

1 Polyunsaturated fatty acids and their endocannabinoid-related metabolites activity at human  
2 TRPV1 and TRPA1 ion channels expressed in HEK-293 cells.

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## 22 ABSTRACT

23 **Background:** Polyunsaturated fatty acids (PUFAs), particularly Omega-3 ( $\omega$ -3) and Omega-6  
24 ( $\omega$ -6) PUFAs, may exert neuroprotective effects via the endocannabinoid system (ECS) and  
25 are promoted as brain health supplements. However, despite their potential role in  
26 endocannabinoid biosynthesis, the impact of PUFAs on ion channels such as TRPV1 and  
27 TRPA1, which are modulated by endocannabinoids, remains incompletely understood.  
28 Furthermore, the potential *in vitro* actions of  $\omega$ -6 to  $\omega$ -3 PUFA combined in the ratios  
29 available in supplements remains uncertain. Therefore, the objective of this study is to  
30 evaluate the functional activity of individual PUFAs, their combination in a specific ratio, and  
31 their endocannabinoid-related derivatives on TRPV1 and TRPA1 ion channels.

32 **Methodology:** We employed a fluorescent Ca-sensitive dye in HEK-293 Flp-In T-REx cells  
33 expressing human TRPV1, TRPA1, or an empty vector to measure changes in intracellular  
34 calcium concentration ( $[Ca^{2+}]_i$ ).

35 **Results:** Capsaicin and certain PUFA derivatives such as DHEA,  $\gamma$ -LEA and AEA stimulate  
36 TRPV1 activity directly, whereas EPA, DHA,  $\gamma$ -LA, and their 9:3:1 ratio triggered TRPV1  
37 response via a mechanism dependent on prior exposure to phorbol ester. Similarly,  
38 cinnamaldehyde and selected PUFA derivatives such as EPEA, DHEA,  $\gamma$ -LEA, 2-AG, 2-AG  
39 ether and AEA triggered TRPA1 response, with EPA, DHA,  $\gamma$ -LA, and the 9:3:1 ratio  
40 showing significant effects at higher concentrations.

41 **Conclusions:** PUFAs alone and their combined form in 9:3:1 ratio stimulate TRPA1 activity,  
42 whereas their metabolites trigger both TRPV1 and TRPA1 response. These findings suggest  
43 new avenues to explore for research into potential mechanisms underlying the neurological  
44 benefits of PUFAs and their metabolites.

45 **Keywords:** *Cannabinoid, Fatty Acid, Inflammation, Omega-3, TRP channel.*

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## 48 **INTRODUCTION**

49

50 Polyunsaturated fatty acids (PUFAs) are crucial for maintaining brain function and has been  
51 used as supplements with claims of improving brain health (Bentsen 2017; Bourre 2009). In  
52 preclinical models, omega-3 ( $\omega$ -3) and omega-6 ( $\omega$ -6) PUFAs have demonstrated  
53 neuroprotective effects via the endocannabinoid system (ECS) (Dyall 2017; Freitas et al.  
54 2018).

55

56 The ECS is an essential part of the central nervous system (CNS) (Skaper & Di Marzo 2012).  
57 Its core components include the lipid derivatives of PUFA's, endocannabinoids (eCB),  
58 enzymes regulating eCB synthesis and breakdown, and cannabinoid receptors CB1 and CB2  
59 (DeMesa et al. 2021; Fezza et al. 2014; Lu & Mackie 2016). Additionally, other G protein-  
60 coupled receptors like GPR55 (Lauckner et al. 2008; Yang et al. 2016), GPR3, GPR119  
61 (Davis 2022) and GPR120 (Im 2009) are the potential member of ECS. Peroxisome-  
62 proliferator activated receptors (PPARs) and transient receptor potential (TRP) ion channels,  
63 are also activated by various cannabinoid ligands, including eCBs (DeMesa et al. 2021; Lu &  
64 Mackie 2016).

65

66 TRP channels are membrane proteins involved in sensing and responding to chemical and  
67 physical stimuli. They are integral to neural signalling processes related to various sensory  
68 perceptions including nociception (Julius 2013; Muller et al. 2019; Sawamura et al. 2017;

69 Zhang et al. 2023). Specific channels within the TRP family, such as TRPV1, TRPV2,  
70 TRPV3, TRPV4, TRPA1, and TRPM8, have been identified as responsive to endogenous,  
71 phyto-, and synthetic cannabinoids (Muller et al. 2019). It is also reported that, these channels  
72 may contribute to eCB signalling, especially within the brain (Nilius & Owsianik 2011).  
73 However, the potential modulation of these ion channels by  $\omega$ -3 and  $\omega$ -6 fatty acids derived  
74 eCBs has not been fully defined (Petermann et al. 2022).

75 Dietary PUFAs activate TRP channels, with eicosapentaenoic acid (EPA) and  
76 docosahexaenoic acid (DHA) shown to activate TRPV1 (Ciardo & Ferrer-Montiel 2017;  
77 Matta et al. 2007) and TRPA1 (Ciardo & Ferrer-Montiel 2017; Motter & Ahern 2012;  
78 Redmond et al. 2014) ion channels. These PUFAs also serve as major precursors for eCB  
79 biosynthesis (Komarnytsky et al. 2021), with resulting eCBs activating TRPV1 and TRPA1  
80 ion channels. It has been reported that 2-arachidonoylglycerol (2-AG), anandamide (AEA)  
81 (Zygmunt et al. 2013; Zygmunt et al. 1999), and N-arachidonoyldopamine (NADA) (Huang  
82 et al. 2002; Raboune et al. 2014) activate TRPV1, while AEA activates TRPA1 (Redmond et  
83 al. 2014). However, there are also other ethanolamides or glycerol conjugates of PUFAs that  
84 may function as eCBs or ligands for related receptors; but their roles and targets in the brain  
85 remain unclear (Bosch-Bouju & Laye 2016; Witkamp 2016). The impact of the  $\omega$ -6 fatty acid  
86 such as  $\gamma$ -linolenic acid ( $\gamma$ -LA) and its ethanolamine derivative on TRPV1 and TRPA1 ion  
87 channels has also not been examined.

88 Dietary intake affects brain PUFA levels (Dyall 2017), and the  $\omega$ -6 to  $\omega$ -3 ratio in the current  
89 Western diet (approximately 20:1) is linked to various health conditions including  
90 autoimmune and inflammatory diseases (Patel et al. 2022; Simopoulos 2002). One possible  
91 reason could be that  $\omega$ -3 long-chain fatty acids such as EPA, DHA as well as some  $\omega$ -6  
92 derived fatty acids, e.g.,  $\gamma$ -LA particularly dihomogamma-linolenic acid, are important for

93 production of anti-inflammatory eicosanoids while  $\omega$ -6 derived fatty acids, mainly AA, are  
94 crucial to produce pro-inflammatory eicosanoids. The presence of higher amounts of pro-  
95 inflammatory eicosanoids such as AA also interferes the synthesis of anti-inflammatory  
96 eicosanoids by competing at the active site of the enzyme, cyclooxygenase (COX) (Calder  
97 2010). Therefore, to mitigate risks linked with excessive  $\omega$ -6 PUFA consumption,  
98 maintaining a balanced  $\omega$ -6 to  $\omega$ -3 ratio of 1:1 to 5:1 is suggested (Patel et al. 2022). Studies  
99 have also indicated that the ratio of  $\omega$ -6 to  $\omega$ -3 fatty acids in tissues is more important for  
100 health benefits than their absolute levels (Ishweki et al. 2015) and combining  $\omega$ -6 and  $\omega$ -3  
101 PUFAs has shown positive health effects, especially in children with Developmental  
102 coordination disorder (DCD) (Richardson & Montgomery 2005). Moreover, it is noted that  
103 maintaining an equilibrium between  $\omega$ -6 and  $\omega$ -3 PUFAs in a healthy diet yields favourable  
104 effects on inflammation and other physiological mechanisms (Gomez-Candela et al. 2011).  
105 Nevertheless, common agreement regarding the ideal ratio remains elusive. Determining the  
106 optimum ratio of  $\omega$ -3 PUFAs, EPA, and DHA for desired health benefits also remains  
107 uncertain (Djuricic & Calder 2021; Gomez-Candela et al. 2011; Mukhametove et al. 2022).  
108  
109 The objective of this study is to evaluate the individual PUFAs (EPA, DHA, and  $\gamma$ -LA), their  
110 combined form in a 9:3:1 ratio, and their endocannabinoid-related derivatives on the  
111 functional activity of human TRPV1 and TRPA1 ion channels, to develop a more complete  
112 picture of the mode of actions of these important dietary molecules.

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116 **METHODOLOGY**

117

118 **Cell culture**

119 Flp-In T-REx HEK-293 cells (Life Technologies, Mulgrave, Victoria, Australia), stably  
120 transfected with human TRPV1 (hTRPV1), TRPA1 (hTRPA1) cDNA (GenScript,  
121 Piscataway, NJ) (Heblinski et al. 2020) or an empty pcDNA5/FR/TO vector, were maintained  
122 in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS)  
123 (Gibco, # 10099-141), 100 U penicillin and 100 µg streptomycin ml<sup>-1</sup> (1% P/S) (Gibco, #  
124 15140-122, Life Technologies, USA), 80 µg.ml<sup>-1</sup> hygromycin B (Invivogen, cat # ant-hg-1),  
125 and 15µg.ml<sup>-1</sup> blasticidin (Invivogen; San Diego, CA, USA, cat # ant-bl-1). The cells were  
126 incubated in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. Upon reaching approximately 90%  
127 confluence in 75cm<sup>2</sup> flasks, they were trypsinized and transferred to poly-D-lysine coated 96-  
128 well plates (Corning, Castle Hill, NSW, Australia) in L-15 medium supplemented with 1%  
129 FBS, 100 U penicillin and 100 µg streptomycin ml<sup>-1</sup> (1% P/S), and 15 mM glucose (80 µL  
130 volume per well). After an overnight incubation (maximum of 16 hours) in humidified room  
131 air at 37°C, TRPV1 and TRPA1 receptor expression was induced 4 hours before  
132 experimentation by adding to each well 10 µL tetracycline solution to a final concentration of  
133 1 µg.ml<sup>-1</sup>.

134

135 **Calcium Assay**

136

137 Intracellular calcium [Ca]<sub>i</sub> levels were assessed using the calcium 5 kit from Molecular  
138 Devices (# R8186, Sunnyvale, CA, USA) on a FlexStation 3 Microplate Reader (Molecular

139 Devices, Sunnyvale, CA, USA). Calcium 5 dye was dissolved in Hank's Balanced Salt  
140 Solution (HBSS) with the composition of (in mM): NaCl 145, CaCl<sub>2</sub> 1.26, MgCl<sub>2</sub> 0.493,  
141 HEPES 22, Na<sub>2</sub>HPO<sub>4</sub> 0.338, NaHCO<sub>3</sub> 4.17, KH<sub>2</sub>PO<sub>4</sub> 0.441, MgSO<sub>4</sub> 0.407 and glucose  
142 1mg/ml (pH adjusted to 7.4, osmolarity = 315 ± 15 mosmol) and used at 50% of the  
143 manufacturer's suggested concentration. Probenecid (Biotium, cat # 50027) which helps to  
144 prevent expulsion of calcium indicator from the cells was added to a final concentration of  
145 1.25 mM. 90µL of the dye were loaded into each well of the plate and incubated for 1 hour  
146 before reading in the FlexStation 3 at 37°C. Fluorescence was recorded every 2 seconds ( $\lambda$   
147 excitation = 485 nm,  $\lambda$  emission = 525 nm) for 5 minutes. After 1 minute of baseline  
148 recording, 20 µL of the drug, dissolved in HBSS with 1% Dimethyl Sulfoxide (DMSO)  
149 (Sigma-Aldrich, Germany, cat # D2650) was added (final DMSO concentration was 0.1% in  
150 well).

151

## 152 **Drugs and reagents**

153

154 All drugs were prepared in DMSO at a concentration of 30 mM and stored at -30°C/-80°C.  
155 Freshly thawed aliquots were used in each experiment and diluted in HBSS containing 0.1%  
156 bovine serum albumin (BSA) (Sigma-Aldrich, US, # A7030). Due to limitations in the  
157 solubility of fatty acids and their derivatives, the highest concentration tested was 30 µM.  
158 EPA, DHA,  $\gamma$ -LA, and their endocannabinoid related derivatives and glycerol conjugates  
159 were procured from Cayman Chemical (Ann Arbor, MI, USA) while cinnamaldehyde (CA)  
160 was obtained from Merck (Castle Hill, NSW, Australia). The 9:3:1 ratio were prepared by  
161 combining 9 % of EPA, 3 % of DHA and 1 % of  $\gamma$ -LA dissolved in DMSO, so that, the  
162 highest concentration tested was 30:10:3 µM. In this study, we explored the effects of a 9:3:1

163 ratio of EPA, DHA, and  $\gamma$ -LA, which reflects the composition found in commercially  
164 available omega-3 supplements. This ratio was chosen because an excess of EPA compared to  
165 GLA is prevalent in these supplements, designed to maximize the anti-inflammatory benefits  
166 associated with omega-3 fatty acids (Baker et al. 2016). All reagents for tissue culture were  
167 sourced from Merck or Life Technologies (Mulgrave, Victoria, Australia). Capsaicin (Caps)  
168 was obtained from Tocris Bioscience, Bristol (# 404-86-4) and capsazepine was from Merck,  
169 USA (# 138977-28-3). PAR-1 agonist peptide (Thr-Phe-Leu-Leu-Arg-NH<sub>2</sub>, # 2660) was  
170 obtained from Auspep (Tullamarine, Victoria, Australia).

171

## 172 **Data analysis**

173

174 The response to agonists was expressed as a percentage change from the average baseline  
175 measurement (1 minute before adding drug). Changes in fluorescence resulting from the  
176 addition of solvent were subtracted before normalization to baseline. Concentration-response  
177 curves, E<sub>max</sub>, EC<sub>50</sub> values and Hill slope were determined using a three-parameter logistic  
178 equation (GraphPad Prism, San Diego, CA). Results are presented as the mean  $\pm$  standard  
179 error of the mean (SEM) from at least six separate experiments conducted in duplicate, unless  
180 otherwise stated. Concentration-response curves (CRC) of the positive controls, capsaicin and  
181 cinnamaldehyde, were performed each day for comparative analysis. Where appropriate,  
182 unpaired Student's t-test were used to compare the responses of individual compounds in  
183 different conditions, while a One-way ANOVA followed by Dunnett's multiple comparisons  
184 test was used to assess potential differences in responses elicited by a range of compounds. P  
185 < 0.05 was considered statistically significant.

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187

188 **RESULTS**

189

190 Application of docosahexaenoyl ethanolamide (DHEA),  $\gamma$ -LEA, AEA, 2- linoleoyl glycerol  
191 (2-LG), NADA, and capsaicin at 10  $\mu$ M produced an elevation  $[Ca]_i$  in HEK-293 TRPV1  
192 cells that was significantly greater than that produced in HEK-293 EV cells (Figure 1, Table  
193 1). However, EPA, DHA,  $\gamma$ -LA, their 9:3:1 ratio, as well as EPEA, 2-AG and 2-AG ether did  
194 not produce a notable change in  $[Ca]_i$  in either cell line, and there was no difference between  
195 the responses in HEK-293 TRPV1 cells and those seen in HEK-293 EV cells (Table 1).

196

197 **Table 1.** Change of Ca5 fluorescence induced by PUFAs and their endocannabinoid related  
198 metabolites in HEK-293 EV, TRPV1 and TRPA1 expressing cells.

DRUG	Mean $\pm$ SEM		
	EV	TRPV1	TRPA1
EPA $\square$	14 $\pm$ 5 $\square$	16 $\pm$ 5 $\square$	58 $\pm$ 42 $\square$
DHA $\square$	11 $\pm$ 2 $\square$	11 $\pm$ 2 $\square$	129 $\pm$ 81 $\square$
$\gamma$ - LA $\square$	9 $\pm$ 2 $\square$	10 $\pm$ 1 $\square$	112 $\pm$ 61 $\square$
9:3:1 $\square$	9 $\pm$ 1	11 $\pm$ 2 $\square$	85 $\pm$ 45 $\square$
EPEA $\square$	3 $\pm$ 1 $\square$	15 $\pm$ 8 $\square$	50 $\pm$ 40 $\square$
DHEA $\square$	10 $\pm$ 3	266 $\pm$ 51* $\square$	107 $\pm$ 50* $\square$
$\gamma$ -LEA $\square$	11 $\pm$ 2	277 $\pm$ 67* $\square$	87 $\pm$ 32* $\square$
AEA $\square$	10 $\pm$ 2	188 $\pm$ 36* $\square$	78 $\pm$ 40 $\square$
2-AG $\square$	4 $\pm$ 2 $\square$	30 $\pm$ 12 $\square$	6 $\pm$ 1 $\square$
2-AG ether $\square$	8 $\pm$ 2 $\square$	39 $\pm$ 24 $\square$	91 $\pm$ 40 $\square$
2-LG $\square$	4 $\pm$ 1	41 $\pm$ 13* $\square$	6 $\pm$ 2 $\square$

NADA□	6 ± 2	262 ± 40 *□	-□
Par-1 (100 μM)□	463 ± 33	-□	-□
Capsaicin□□	12 ± 2	□350 ± 33 *□	-□
Cinnamaldehyde (300 μM)□	9 ± 6	-□	□438 ± 29 *□

199 Drugs were applied at 10 μM. Responses are expressed as a % change in fluorescence from baseline.

200 All experiments represent mean ± SEM of at least 6 independent experiments, except for

201 Cinnamaldehyde in EV cells, which was n=4. An unpaired t-test was conducted to compare the

202 response of PUFAs and their endocannabinoid-related metabolites in HEK-293 EV cell with that of

203 HEK-293 TRPV1 and TRPA1 expressing cells. “\*” Indicates P < 0.05 compared to empty vector cells.

204

205 The effects of the positive control and endocannabinoid-related metabolites of PUFA’s in

206 HEK-293 TRPV1 expressing cells were concentration-dependent. Capsaicin, a prototypic

207 TRPV1 agonist, increased Ca<sup>2+</sup>-dye fluorescence, peaking at a maximum response ( $E_{max} \pm$

208 SEM) of  $340 \pm 19$  % above pre-drug levels. Its potency ( $pEC_{50} \pm SEM$ ) was  $8.0 \pm 0.1$ . The

209 PUFA-derived eCBs DHEA,  $\gamma$ -LEA, AEA, and NADA also triggered TRPV1 activation. In

210 contrast, eicosapentaenoyl ethanolamide (EPEA), 2-AG, 2-AG ether, and 2-LG showed

211 minimal effectiveness (Table 2; Figure 1). Moreover, the response of the endocannabinoid-

212 related metabolites EPEA, 2-AG, 2-AG ether, and 2-LG at 30 μM is significantly lower than

213 the maximal response of capsaicin (10 μM), while the maximal responses of the others did

214 not differ from those produced by the highest concentration of capsaicin (Table S1).

215 However, despite the above-mentioned metabolites of PUFA’s were observed to significantly

216 elevate calcium (Ca) levels, we were unable to report the potency ( $EC_{50}$  values) for these

217 drugs because we could not construct complete concentration-response curves (CRCs) for

218 them, owing to the insolubility of these drugs at higher concentrations.

219

220 **Table 2:** The response of endocannabinoid-related metabolites and their parent compounds in  
 221 HEK-293 TRPV1 and TRPA1 expressing cells.

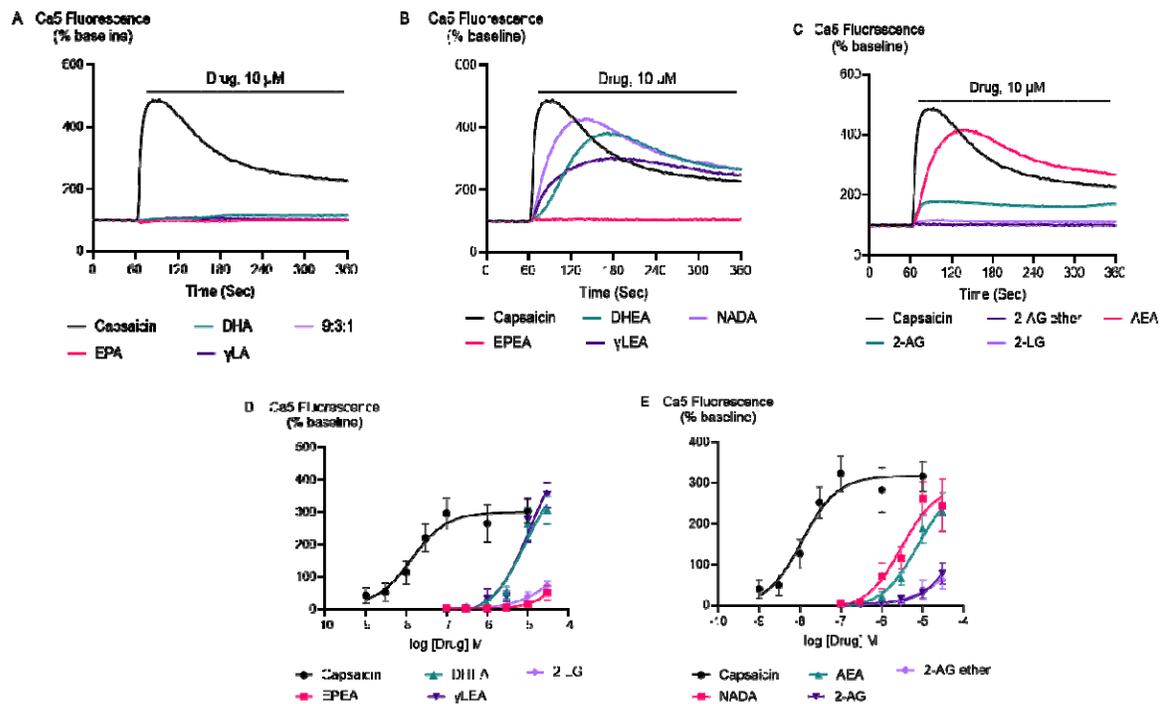
Drugs	TRPV1		TRPA1	
	Change in Ca5 fluorescence	pEC <sub>50</sub> ± SEM	Change in Ca5 fluorescence	pEC <sub>50</sub> ± SEM
	(E <sub>max</sub> ± SEM) %		(E <sub>max</sub> ± SEM) %	
EPA	34±5	n.d	232 ± 67	n.d
DHA	29±0.8	n.d	408 ± 54	n.d
γ-LA	40 ± 11	n.d	255 ± 73	n.d
9:3:1	38±7	n.d	260 ± 61	n.d
EPEA	51 ± 22	n.d	274 ± 27	n.d
DHEA	439±81	5±0.2	335 ± 35	n.d
γ-LEA	354 ± 39	n.d	311 ± 11	n.d
AEA	304±55	5.1± 0.2	206 ± 60	n.d
2-AG	78±26	n.d	109 ± 68	n.d
2-AG ether	63±23	n.d	223 ± 87	n.d
2-LG	75±13	n.d	10 ± 4	n.d
NADA	299±45	5.5±0.2	-	-
Capsaicin	340±19	8.0 ± 0.1	-	-
Cinnamaldehyde	-	-	503±23	4.4±0.1

222 \* "n.d" signifies "not determined," while "-" denotes "not tested." The table displays the  
 223 maximal percentage change in Ca5 fluorescence (E<sub>max</sub>±SEM) for PUFAs and their derivatives  
 224 in HEK-293 TRPA1 and HEK-293 TRPV1 expressing cells, reported either for the highest  
 225 tested concentration (30 μM) or derived from concentration response curves (DHEA, AEA  
 226 and NADA at TRPV1). Capsaicin (TRPV1) and cinnamaldehyde (TRPA1) are included for  
 227 comparison, all values represent the mean + SEM of at least 6 determinations.

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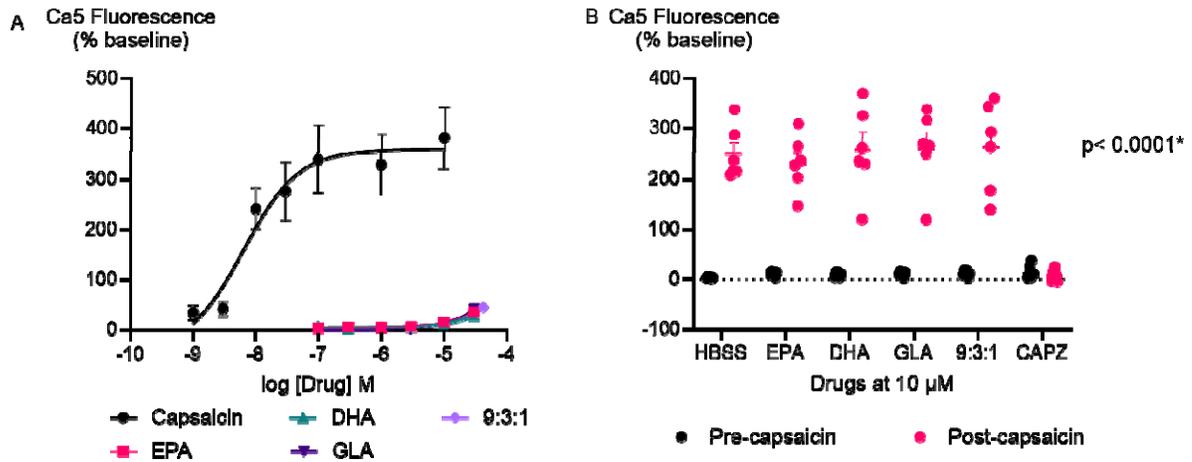
231

232 **Figure 1.** Traces of PUFAs and their endocannabinoid related metabolites at 10 μM (A, B and  
233 C) and concentration-response curves (CRC) for endocannabinoid-related metabolites  
234 capsaicin (D and E) in HEK-293 TRPV1 expressing cells. In A), B) and C) drugs were added  
235 for the duration of the bar. The CRCs were fitted using a three-parameter logistic equation,  
236 each data point represents the mean ± SEM from at least 6 independent experiments  
237 conducted in duplicate.

238

239 The fatty acids, EPA, DHA, γ-LA, or their combination in the ratio of 9:3:1 did not trigger  
240 TRPV1 response (Figure 2A). To test if they inhibited activation of TRPV1 ion channel, we  
241 applied a sub-maximally effective concentration of capsaicin (10 nM) after a 5 minutes  
242 exposure to EPA, DHA, γ-LA or their 9:3:1 ratio. Capsazepine (Bevan et al. 1992; Walpole et  
243 al. 1994) was used as a positive control for inhibition of capsaicin activation of the channel.

244 Pre-incubation with capsazepine (10  $\mu$ M) prevented capsaicin-induced fluorescence changes  
245 ( $P < 0.05$ ) (Figure 2B). However, pre-treatment with EPA, DHA,  $\gamma$ -LA, or their 9:3:1 ratio (10  
246  $\mu$ M) did not affect the capsaicin response in HEK-293 TRPV1 expressing cells.



247

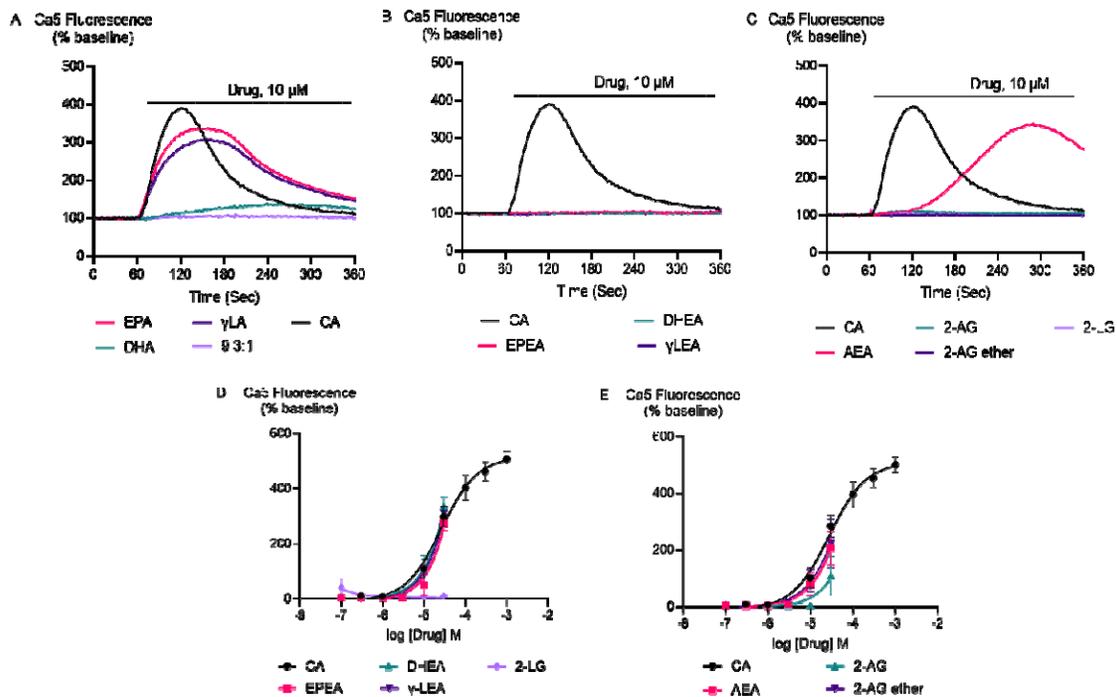
248 **Figure 2.** (A) Concentration response curves of PUFAs in HEK-293 TRPV1 expressing cells;  
249 (B) Effects of PUFAs on responses to capsaicin in HEK-293 TRPV1 expressing cells. PUFAs  
250 or capsazepine (10  $\mu$ M each) were added to the cells for 5 minutes then capsaicin (10 nM)  
251 was added. Changes in  $[Ca]_i$  are expressed as percentage of the pre-drug baseline. The blue  
252 circles represent the response to first drug in individual experiments, while the red circles  
253 correspond to the response to subsequent capsaicin addition. PUFAs (10  $\mu$ M) do not inhibit  
254 the subsequent response to capsaicin (10 nM), whereas preincubation of the cells with the  
255 TRPV1 antagonist capsazepine (CAPZ) (10  $\mu$ M) does ( $P < 0.05$ ).

256

257 Application of DHEA and  $\gamma$ -LEA at 10  $\mu$ M as well as cinnamaldehyde at 300  $\mu$ M produced  
258 an elevation  $[Ca]_i$  in HEK-293 TRPA1 cells significantly greater than that produced in HEK-  
259 293 EV cells. However, at 10  $\mu$ M, EPA, DHA,  $\gamma$ -LA, their 9:3:1 ratio, as well as EPEA, AEA,  
260 2-AG and 2-AG ether did not produce a change in  $[Ca]_i$  different to that seen in HEK-293 EV  
261 cells (Table 1).

262 The effects of the positive control and endocannabinoid-related metabolites of PUFA's in  
263 HEK-293 TRPA1 expressing cells were concentration-dependent. Cinnamaldehyde, a  
264 commonly used activator of TRPA1, increased Ca<sub>v</sub>5 fluorescence, with a maximum effect of  
265 503±23 % above baseline with a pEC<sub>50</sub> ± SEM of 4.4±0.1. EPEA, DHEA, γ-LEA, AEA, 2-  
266 AG, and 2-AG ether, also triggered the TRPA1 ion channel response, while 2-LG had no  
267 effect (Table 2; Figure 3). Among these metabolites, the response of EPEA, AEA, 2-AG, 2-  
268 AG ether, and 2-LG at 30 μM exhibited a significant difference to the response produced by  
269 the highest concentration of cinnamaldehyde (300 μM), while the maximal responses for  
270 DHEA and γ-LEA at the same concentration did not differ from it (*Table S1*).

271

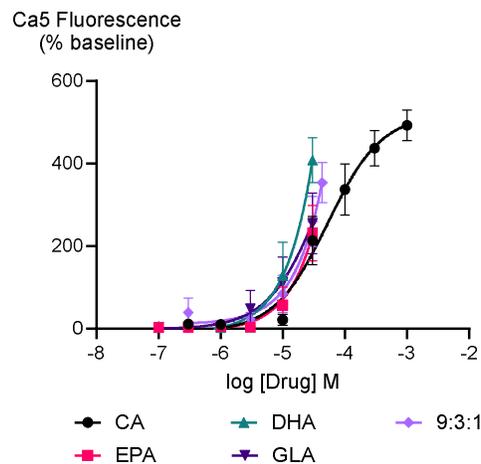


272

273 **Figure 3.** Traces of PUFAs and their endocannabinoid related metabolites at 10 μM (A, B and  
274 C) and concentration-response curves (D and E) for endocannabinoid related metabolites in  
275 HEK-293 TRPA1 expressing cells. In A), B) and C) drugs were added for the duration of the

276 bar. The CRCs were analysed using a three-parameter logistic equation from 6 independent  
277 experiments conducted in duplicate. CA represents Cinnamaldehyde.

278 Furthermore, EPA, DHA,  $\gamma$ -LA, and their combination in a 9:3:1 ratio were found to activate  
279 TRPA1 ion channel. At the highest concentration tested (30  $\mu$ M), their maximum effects were  
280  $232\pm 67\%$ ,  $408\pm 54\%$ ,  $255\pm 73\%$  and  $260\pm 61\%$ , respectively (Table 2; Figure 4).



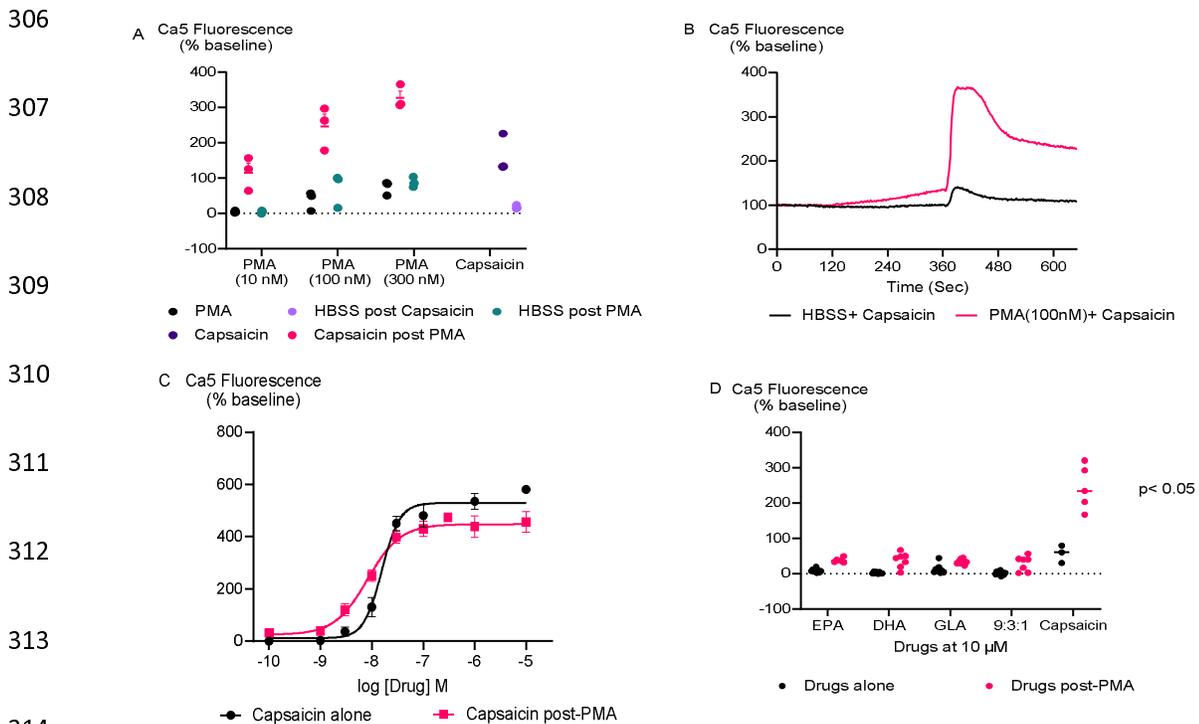
281

282 **Figure 4.** Concentration-response curves of PUFAs in HEK-293 TRPA1 expressing cells. The  
283 CRCs were fitted using a three-parameter logistic equation from 6 separate experiments  
284 conducted in duplicate. CA represents Cinnamaldehyde.

285

286 Matta et al. (2007) reported that some PUFAs, specifically EPA and DHA, activated the rat  
287 TRPV1 ion channel only following incubation with the phorbol ester protein kinase C  
288 activator, Phorbol 12,13-dibutyrate (PDBu). Therefore, we assessed whether phorbol ester-  
289 dependent stimulation of human TRPV1 response by inactive PUFA could also be observed.  
290 We initially determined the effect of phorbol 12-myristate 13 acetate (PMA), another phorbol  
291 ester that is a protein kinase C activator (Jiang & Fleet 2012), on HEK-293 TRPV1  
292 expressing cells. Three concentrations of PMA were tested for 5 minutes followed by

293 challenge with capsaicin at 10 nM (Figure 5A). 10 nM PMA had no effect by itself or on  
294 subsequent capsaicin responses, but 100 nM and 300 nM PMA increased  $[Ca]_i$  by themselves  
295 and potentiated the subsequent response to capsaicin. However, to minimize potentially  
296 confounding effects, a concentration of 100 nM PMA was chosen. For these experiments, the  
297 response to PMA after 5 minutes was subtracted from the subsequent response to capsaicin or  
298 PUFA. PMA (100 nM) increased the effects of low, but not high, concentrations of capsaicin  
299 (Figure 5C), presumably because of saturation of the TRPV1 channels at high agonist  
300 concentrations. The response of the tested PUFAs were also increased after exposure to PMA  
301 ( $P < 0.05$  for each) (Figure 5D). To rule out the possibility that the Ca5 dye was saturated at the  
302 highest concentration of capsaicin tested, we conducted experiments with ionomycin, a  
303 calcium ionophore (Kao et al. 2010) (Figure S2). Ionomycin ( $5 \mu\text{M}$ ) produced a change in  
304 Ca5 fluorescence of  $673 \pm 25 \%$ , significantly greater than that produced by a maximal  
305 effective concentration of capsaicin ( $435 \pm 15 \%$ ).



315 **Figure 5. (A)** Concentration response of PMA's in HEK-293 TRPV1 expressing cells. PMA  
316 were added to the cells at different concentrations for 5 minutes then capsaicin was added at  
317 10 nM. **(B)** Traces of capsaicin and **(C)** Concentration response curves of capsaicin with and  
318 without PMA in HEK-293 TRPV1 expressing cells. **(D)** Effects of PUFAs on responses to  
319 PMA potentiation in HEK-293 TRPV1 expressing cells. The cells were pretreated with PMA  
320 (100 nM) for 5 minutes then each PUFA (10  $\mu$ M) and capsaicin (10 nM) was added. Changes  
321 in  $[Ca]_i$  are expressed as percentage of the pre-drug baseline, with the response to PMA alone  
322 at 5 minutes subtracted. The black circles represent the response of PUFAs without PMA  
323 pretreatment while the pink circles represent the response of PUFAs after PMA potentiation.  
324 The response to PUFAS was potentiated by 5 minutes pre-incubation with PMA, a protein  
325 kinase C activator ( $P < 0.05$  for each).

326

327

## 328 **DISCUSSION**

329

330 The current study demonstrates that some derivatives of PUFAs directly stimulate the TRPV1  
331 and TRPA1. Moreover, EPA, DHA,  $\gamma$ -LA, and their 9:3:1 ratio triggered TRPA1 activity  
332 directly, while stimulation of TRPV1 was only noted after PMA treatment of HEK-293 cells:

333 At 10  $\mu$ M concentration, PUFAs and their endocannabinoid-related metabolites showed  
334 minimal effect on calcium levels in HEK-293 EV cells. However, significant differences were  
335 observed when comparing their effect at the same concentration between EV cells and cells  
336 expressing TRPV1 or TRPA1 receptors. To some PUFAs and their metabolites, when the  
337 concentration was increased to 30  $\mu$ M, there were also notable differences in the responses  
338 between TRPV1, TRPA1-expressing cells and EV cells. These differences could be attributed

339 to the higher concentration needed to activate TRPV1 and TRPA1 channels effectively in the  
340 TRPV1 and TRPA1 expressing cells. It is also possible that the distinct responses are due to  
341 the differential expression of TRPV1 and TRPA1 or other cellular factors in these two cell  
342 types such as differences in signalling pathways, the presence of other receptors, or variations  
343 in the cell membrane properties that affect how the cells interact with or respond to the  
344 metabolites.

345 Transient receptor potential vanilloid subtype 1 (TRPV1), which is widely recognized as the  
346 capsaicin receptor (Caterina et al. 1997), has previously been reported to be responsive to  
347 metabolites of PUFAs such as AEA (Zygmunt et al. 1999) and NADA (Huang et al. 2002)  
348 corroborating our findings. Our research also introduces new evidence indicating that PUFA-  
349 derived endocannabinoids (eCBs) such as DHEA and  $\gamma$ -LEA can trigger TRPV1 activation  
350 while EPEA showed minimal effectiveness. Moreover, consistent with our findings, previous  
351 studies have shown that 2-AG is not very effective at activating these ion channels, but it is  
352 considered a physiologically relevant activator of TRPV1 channels through phospholipase C  
353 (PLC)-mediated mechanisms and potentiation (Petrosino et al. 2016; Zygmunt et al. 2013;  
354 Zygmunt et al. 1999). Additionally, extending the findings for 2-AG, we showed that 2  
355 related molecules, 2-AG ether and 2-LG also failed to modulate TRPV1 in these conditions.

356 Our findings indicate that the fatty acids EPA, DHA,  $\gamma$ -LA, and their combination in a 9:3:1  
357 ratio did not trigger TRPV1 activation or inhibit its activity. As seen in earlier studies with rat  
358 TRPV1, phorbol esters enhance TRPV1 sensitivity to EPA and DHA through PKC-dependent  
359 activation (Matta et al. 2007). In our experiments, this effect was confirmed as a significant  
360 response of TRPV1 to EPA and DHA was only observed when a phorbol ester was present.  
361 Additionally, PMA amplified the TRPV1 response to capsaicin,  $\gamma$ -LA, and a 9:3:1 mixture of  
362 EPA, DHA, and  $\gamma$ -LA.

363 Previous reports have shown that EPA, DHA, and AEA activate TRPA1 (Ciardo & Ferrer-  
364 Montiel 2017; Motter & Ahern 2012; Redmond et al. 2014) which aligns with our findings.  
365 We have extended this work to show that, PUFA-derived endocannabinoids (eCBs) such as  
366 EPEA, DHEA,  $\gamma$ -LEA, and 2-AG ether can stimulate TRPA1 response, whereas 2-LG was  
367 found to be non-effective.

368 Moreover, it has been reported that combining  $\omega$ -6 and  $\omega$ -3 PUFAs is important for achieving  
369 positive health outcomes (LaChance et al. 2016; Puri & Martins 2014). Our result show that  
370 the combination of PUFAs triggers the TRPA1 ion channel activity, which may suggest a  
371 potential mechanism behind some of these observed health benefits. However, it is important  
372 to note that our study does not directly compare the effects of the combined PUFAs with  
373 those of individual components, such as EPA, DHA, and GLA alone. Therefore, it remains  
374 unclear whether the combination of  $\omega$ -6 and  $\omega$ -3 PUFAs in the 9:3:1 ratio is effective in  
375 activating TRPA1 than the individual fatty acids at equivalent concentrations. Therefore,  
376 future studies should aim to investigate whether the combined effects are synergistic,  
377 additive, or simply reflect the sum of their individual actions.

378 Additionally, it is important to consider that consuming a 9:3:1 ratio of  $\omega$ -6 to  $\omega$ -3 PUFAs  
379 does not necessarily translate to the same ratio in the brain or other tissues. The distribution of  
380 these fatty acids in the body may vary due to differences in absorption and metabolism, and  
381 the actual ratio reaching specific tissues such as the CNS is not well understood. Thus,  
382 ongoing research is needed to clarify how these compounds distribute in the brain and other  
383 parts of the body after consumption.

384 Furthermore, while TRPA1 activation plays a role in various physiological processes, its  
385 effects are not universally beneficial. For instance, activation of TRPA1 in sensory neurons is  
386 associated with pain and irritation (McNamara et al. 2007), suggesting that its activation may

387 have different implications depending on the tissue or context. Therefore, given that TRPA1  
388 receptors are expressed in the brain and other tissues (Nilius et al. 2012), it is essential to  
389 consider where these PUFAs and their metabolites are most likely to exert their effects. The  
390 implications of TRPA1 activation in the brain or other tissues also remain unclear and warrant  
391 further investigation to fully understand the potential health impacts.

392 To determine the optimal ratio of  $\omega$ -6 to  $\omega$ -3 PUFAs or between  $\omega$ -3 PUFA's (EPA, and DHA)  
393 for the desired health benefits, our experiment did not explore and compare other possible  
394 combinations and ratios of these compound which is recommended to be completed in future  
395 studies.

396

## 397 **CONCLUSION**

398

399 The studied fatty acids stimulate the TRPA1 ion channel, while their metabolites trigger both  
400 TRPV1 and TRPA1 ion channels activity. Thus, local activation of these channels by PUFAs  
401 and their metabolites may influence neuronal function and provide positive effects through  
402 endocannabinoid-mediated mechanisms (Palazzo et al. 2008). Our findings indicate that these  
403 dietary components could provide neuroprotective effects by modulating these ion channels.  
404 TRPV1 channel activation is beneficial for several neuronal functions, such as regulating  
405 synaptic plasticity, influencing cytoskeleton dynamics, and aiding in cell migration, neuronal  
406 survival, and the regeneration of damaged neurons. It also integrates various stimuli involved  
407 in neurogenesis and network integration (Marsch et al. 2007; Ramírez-Barrantes et al. 2016).  
408 TRPA1 activation is also associated with modulating inflammatory responses and neuropathic  
409 pain, thereby offering protection against neuronal damage and promoting overall brain health  
410 (Nassini et al. 2014). Generally, these results highlight the therapeutic potential of dietary

411 PUFAs in influencing brain function through specific ion channel pathways, which could  
412 support neurological health and aid in preventing neurological diseases. However, while our  
413 study provides important insights into the potential effects of PUFAs on TRPV1 and TRPA1  
414 ion channels, the mechanisms by which orally ingested PUFAs influence brain concentrations  
415 remain unclear. Therefore, future research should focus on elucidating these pathways to  
416 better understand how dietary intake of PUFAs through supplements might alter CNS levels  
417 of these critical compounds.

418

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420

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425

## 426 **List of abbreviations**

<b>Abbreviation</b>	<b>Explanation</b>
2-AG	2-arachidonoylglycerol
2-LG	2-linoleoyl glycerol
AA	Arachidonic Acid
AEA	Anandamide
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
DHA	Docosahexaenoic acid

DHEA	Docosahexaenoyl ethanolamide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
eCB	endocannabinoid
ECS	Endocannabinoid system
EPA	Eicosapentaenoic acid
EPEA	Eicosapentaenoyl Ethanolamide
EV	Empty Vector
FBS	Fetal Bovine Serum
HBSS	Hanks' Balanced Salt Solution
HEK-293	Human Embryonic Kidney cell -293
NADA	N-arachidonoyldopamine
P/S	Penicillin-Streptomycin
PPARs	Peroxisome-Proliferator Activated Receptors
PAR-1	Protease activated receptor 1
PUFA	Polyunsaturated fatty acid
TRP	Transient Receptor Potential
TRPA1	TRP ankyrin
TRPV1	TRP vanilloid
$\gamma$ -LA	$\gamma$ -linolenic acid
$\gamma$ -LEA	$\gamma$ -Linolenoyl ethanolamide
$\omega$ -3	Omega-3
$\omega$ -6	Omega-6

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