# Characterization of novel *N*-Acyl Neurotransmitter/Amino Acid Conjugates (NAAN) on CB1 and TRP family member receptors

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# Abstract

The endocannabinoid family has been extended in recent years from its two first well described members, anandamide and 2-arachidonoyl glycerol, to include a new family of related endogenous lipid-derived compounds composed of a neurotransmitter or amino acid head and a fatty acid tail termed N-acyl amino acid/neurotransmitter conjugate (NAAN). This family of molecules have important effects on G protein-coupled receptors, including the cannabinoid receptors CB1 and CB2; but also have diverse effects on members of the transient receptor potential (TRP) family and other ion channels, thus making them important candidates for the modulation of pain. The aim of this thesis was to investigate the effect of some of these NAAN on heterologously expressed CB1, CB2 and TRPA1 receptors. The effects of NAAN on the signalling pathways and desensitization mechanisms of the CB1 and CB2 receptors were investigated. For the TRPA1 receptor, we investigated how these chemically unreactive compounds might activate a receptor commonly seen as activated by electrophilic agonists, temperature, pressure and pH. Studies were conducted using a 96-wells micro plate reader measuring in real time changes in membrane potential and calcium levels in cell lines expressing recombinant receptors and ion channels.

N-arachidonoyl dopamine (NADA) coupled to Gq-mediated elevations of calcium in CB1 and CB2 expressing cells, but did not act through these receptors to modulate adenylyl cyclase, K channels or ERK activation. NADA did not change the dynamics of CB1 receptor desensitization or affect the allosteric modulators ORG 27569 and PSNCAM-1. Thus, NADA is a highly

biased agonist of CB1 and CB2 toward Gq coupling. Various NAANs activated the TRPA1 receptor, but were less efficacious than the parent molecule, arachidonic acid. Activation of TRPA1 was reduced in a mutant TRPA1 receptor lacking the cysteines necessary for the activation of the receptor by reactive compounds, hinting at possibly another binding site for the receptor. The activation of TRPA1 by arachidonic acid was not blocked by inhibiting enzymes which metabolize it. On the contrary, NDGA, a lipoxygenase inhibitor, activates the TRPA1 receptor and caused cold hyperalgesia in an acetone test in mice .

The findings of this thesis give new insight in the intricacies of the action of NAANs and related compounds on CB1, CB2 as well as the TRPA1 receptor.

# Statement of Candidate

I certify that the work in this thesis entitled "**Characterization of novel *N*-acyl Neurotransmitter/Amino Acid Conjugates (NAAN) on CB1 and TRP family member receptors**" has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University. I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. The research presented in this thesis was approved by Macquarie University Ethics Review Committee, reference number: *09/02/Ex* and *5201001559* approved on 11th of June 2010 and 14th of February 2011 respectively.

_____

William John Redmond

42301203

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# List of abbreviations

| | |
|---|---|
| $[Ca]_I$ | intracellular calcium |
| Δ9-THC | Δ9-tetrahydrocannabinol |
| 2-AG | 2-arachidonoyl glycerol |
| 2-APB | 2-aminoethoxydiphenyl borate |
| AA | arachidonic acid |
| ABN-CBD | abnormal-cannabidiol |
| AC | adenylate cyclase |
| AEA | anandamide, *N*-arachidonoyl ethanolamine |
| ANKTM1 | ankyrin-like with transmembrane domains protein 1 |
| ATP | adenosine triphosphate |
| CA | cinnamaldehyde |
| CB1 | cannabinoid 1 |
| CB2 | cannabinoid 2 |
| cAMP | cyclic adenosine monophosphate |
| CGRP | calcitonin-gene related peptides |
| COOH | carboxyl group |

COX ..... cyclooxygenase

D2 ..... dopamine 2

DAG ..... diacylglycerol

DCP ..... docosapentaeonic

DHA ..... docohexaenoic acid

DRG ..... dorsal root ganglia

DSE ..... depolarization-induced suppression of activation

DSI ..... depolarization-induced suppression of inhibition

ERK1/2 ..... extracellular regulated kinases1/2

FAAH ..... fatty acid amide hydrolase

FFA ..... free fatty acid

GDP ..... guanosine diphosphate

GIRK ..... G-protein gated inwardly rectifying

GLP-1 ..... glucagon-like peptide-1

GPCR ..... G-protein coupled receptor

GPR55 ..... orphan G-protein coupled receptor 55

GRK ..... G-protein coupled kinases

GTP guanosine triphosphate

IP3R inositol 1,4,5-triphosphate channel

LPI 1-α-lysophosphatidylinositol

MAG Monoacyl glycerol lipase

NA-5HT *N*-arachidonoyl serotonin

NAAN *N*-amino acid/neurotransmitter conjugate

NAE *N*-acylethanolamine

NA-GABA *N*-arachidonoyl GABA

NAGly *N*-acyl glycine

NAPE-PLD *N*-acyl-hydrolizing phospholipase D

NG nodose ganglia

NODA *N*-oleoyl dopamine

NPDA *N*-palmitoyl dopamine

NPPB 5-nitro-2-(3-phenylpropylamino)benzoic acid

NSDA *N*-steraoyl dopamine

PA phosphatidic acid

PLA2 phospholipase A2

PLC ..... phospholipase C

PLD ..... phospholipase D

PPAR ..... peroxisome proliferator-activated receptor

$PPP_i$ ..... polyphosphates

PTX ..... *Pertussis* toxin

PUFA ..... poly-unsaturated fatty acid

RhoA ..... ras homolog gene family member A

RFU ..... raw fluorescent unit

RyR ..... ryanodine receptor

TG ..... trigeminal ganglia

TRP ..... transient receptor potential

TRPA ..... transient receptor ankyrin

TRPC ..... transient receptor potential canonical

TRPM ..... transient receptor potential melastatin

TRPML ..... transient receptor potential mucolipin

TRPN ..... TRP no mechanoreceptor potential C

TRPV ..... transient receptor potential vanilloid

TRPP ..... transient receptor polycystic

VGCC ..... voltage-gated calcium channels

# Chapter 1 Introduction

## Chronic Pain

In *Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research*, published in 2011, the Institute of Medicine from the National Academy of Sciences, declared that, in the United States alone, up to $635 billion dollars' worth of resources each year were spent in treatment and loss of productivity due to chronic pain conditions. Of this estimate, 25% of that figure (approximately $150 billion) could be categorized as being spent on inappropriate or unnecessary treatment, tests and procedures. The toll is of importance on productivity as $335 billion loss can be attributed to it, with people in severe pain being half as likely to work full-time compared to the general population (Council., 2011). The magnitude of the aforementioned costs reflects the lack of effective long term pain relief for an important part of this population.

The mainstays of pain treatment remain pharmacotherapy with opioids, such as morphine, cyclooxygenase (COX) inhibitors, such as aspirin and ibuprofen, and the new generation of specific COX-2 inhibitors such as celecoxib. It should be noted that one of the main drugs prescribed and openly sold for the treatment of pain, paracetamol, has a mechanism of action which is still up for debate, as evidence suggests that part of its analgesic effect can come from it being a precursor for an agent

that purportedly acts on the cannabinoid system, AM404 (Hogestatt *et al.*, 2005; Ottani *et al.*, 2006).

In the past decade, drugs with novel mechanisms of action such as the modulators of calcium channels gabapentin and pregabalin have seen a rapid rise in use; and new targets for the treatment of pain have been described. Some drugs acting on these targets include molecules that could modulate transient receptor potential (TRP) channels, the two main G-protein coupled cannabinoid receptors, as well as the enzymatic machinery of the endocannabinoid system, such as fatty acid amide hydrolase (FAAH) and monoacyl glycerol (MAG) lipase (Atwood *et al.*, 2012; Julius, 2013; Pertwee, 2012; Starowicz *et al.*, 2012). Novel insights into the consequences of drug modulation of proteins involved in pain modulation has given us a better understanding of the role of these proteins in physiology and disease. This has led to a renewed interest in how the endogenous ligands of these proteins can act directly on these receptors or through second messengers in order to impact pain modulation.

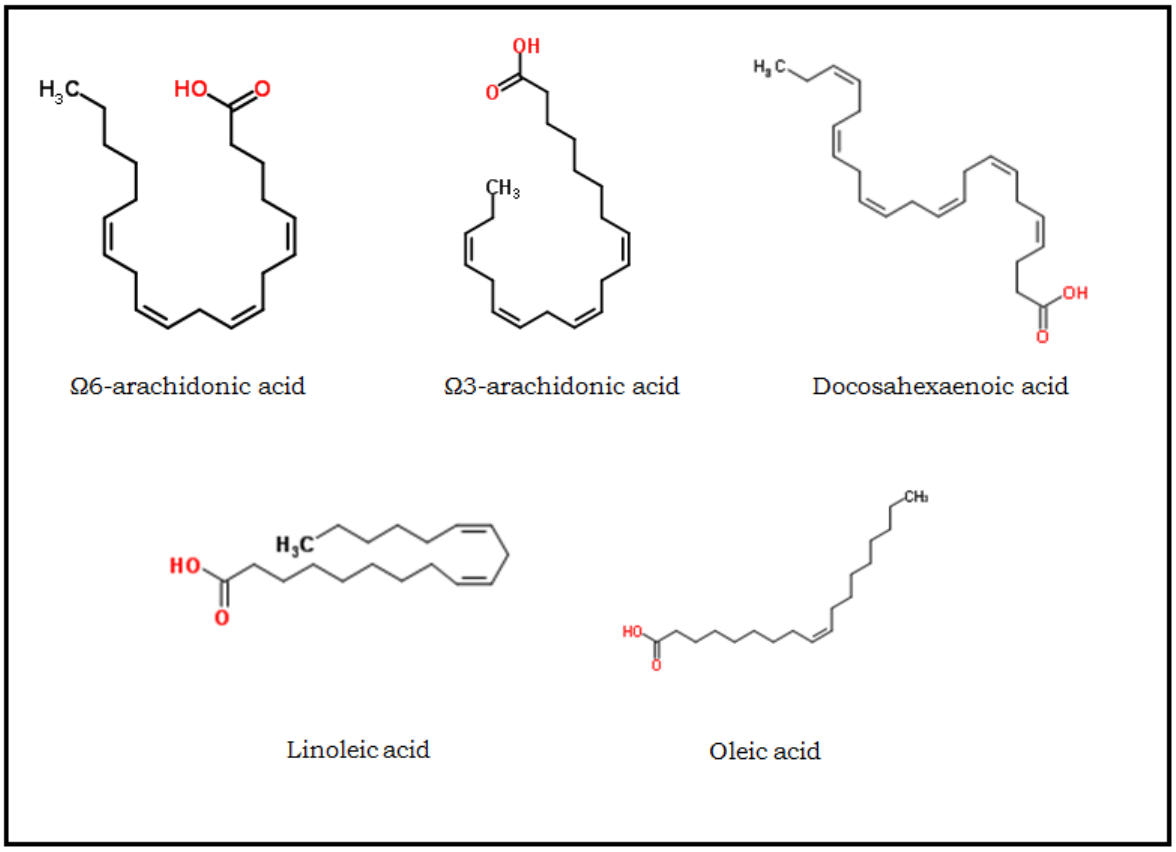
This introduction will summarize and review the current state of literature regarding the pharmacology of endogenous lipids of potential interest as pain modulators. It will also describe the various receptors they activate, with a specific interest toward the two cannabinoid receptors CB1 and CB2, as well as the transient potential receptor channels TRPV1 and TRPA1. Finally, the interaction between these receptors will be discussed.

# Unsaturated fatty acids

## General

Unsaturated fatty acids are lipids that contain at least one double bound in their backbone structure. The human body has a primordial need for poly-unsaturated fatty acids (PUFAs), which can be divided into essential, (meaning that they need to be supplied from the diet in order to be healthy), or not, (meaning that they can be synthesized by the body). A third group, termed, "conditionally essential" represents fatty acids that can become essential under some developmental or disease conditions; and examples include docosahexaenoic acid (an omega-3 fatty acid) and gamma-linolenic acid (an omega-6 fatty acid). Fatty acids possess a carboxyl (COOH) group at one hand and are composed of a straight chain of hydrocarbons. The first carbon next to the carboxyl group is called α, the second β, and so on, with the last position referred to as ω. An ω3-fatty acid, for example, would mean that there is a double-bound found on the third carbon from the $CH_3$ extremity. Endogenous unsaturated fatty acids have usually either an omega-6 or omega-3 unsaturation.

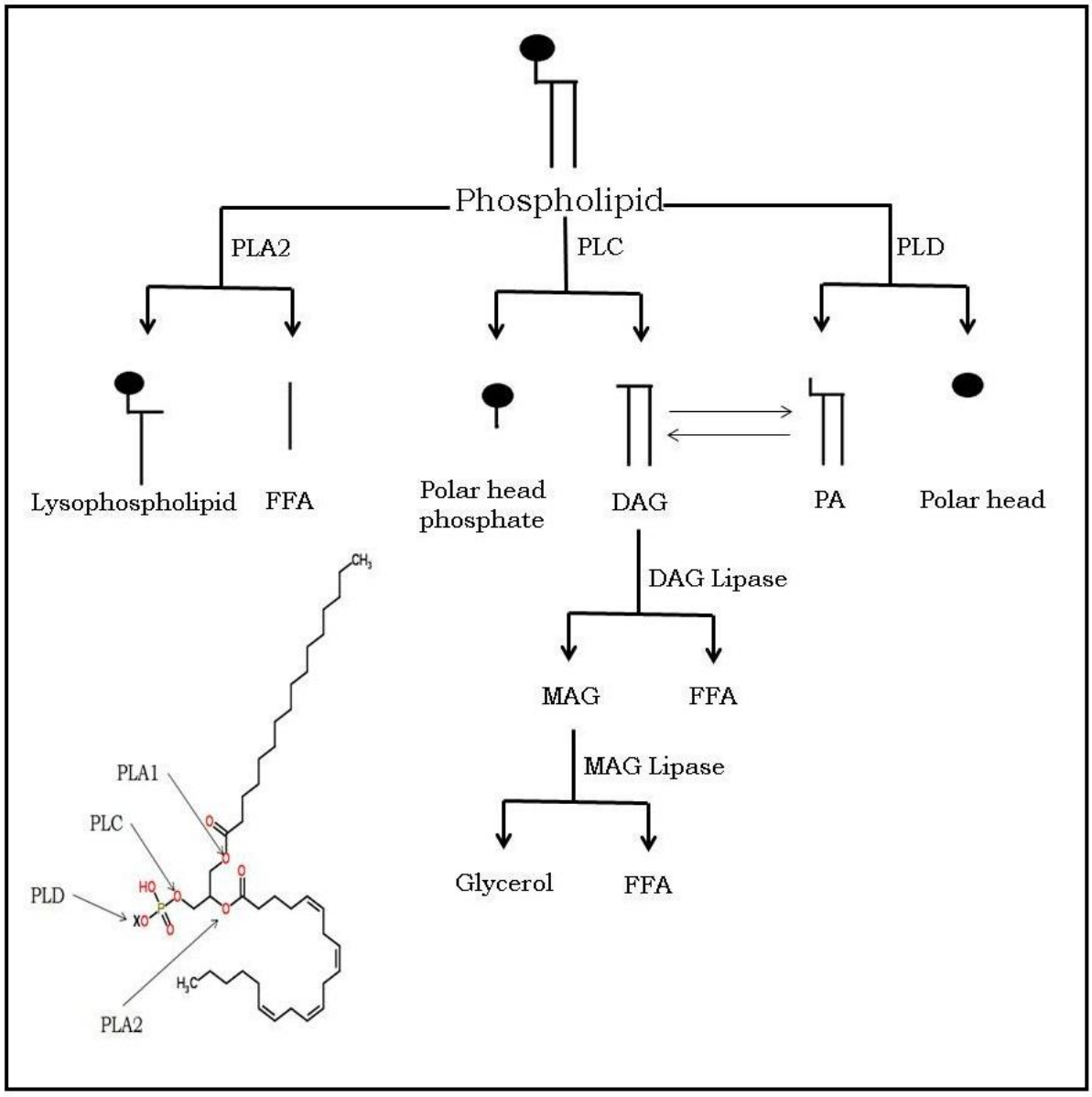
**Figure 1 :** Structure of various PUFAs




In resting cells, PUFAs are stored within the cell membrane, esterified to glycerol in phospholipids. PUFAs are released after a G-protein-dependant receptor event initiates phospholipid hydrolysis and the release of the PUFAs to the intracellular medium. There are three enzymes that can mediate this deacylation mechanism: phospholipase A2 (PLA2), phospholipase C (PLC) and phospholipase D (PLD) which will attack a different site on the phospholipid backbone. PLA2 can release, for example, an arachidonate in a single-step reaction via the hydrolysis of the stereospecific numbering-2 position whereas PLC and PLD do not release free PUFAs directly, rather releasing lipid products containing arachidonate, such as diacylglycerol (DAG) and phosphatidic acid respectively; diacylglycerol lipase and monoacylglycerol can then release PUFAs in the intracellular media (see

figure 1 for schematic) (Piomelli, 2002). For the sake of brevity, only PUFAs of interest for this thesis will be discussed.

**Figure 2:** Synthesis of free PUFAs from cell membranes, adapted from (Piomelli, 2002)



## Arachidonic acid (AA)

Arachidonic acid (AA) (20:4*n*-6) is an endogenous omega-6 PUFA considered non-essential as it can be synthetized by the liver from linoleic acid (Sprecher, 2000). AA comprises approximately 5–15% of total fatty acids in most tissue (Neuringer *et al.*, 1988) and is a major component of cell membranes. It is mostly liberated from phospholipids via the calcium dependent phospholipase $cPLA_2$ (Diez *et al.*, 1994; Kudo *et al.*, 2002; Shikano *et al.*, 1994). It has a plethora of metabolites through its thee main enzymatic pathways (cyclooxygenase, lipoxygenase and peroxidase) leading to the formation of prostaglandins, leukotrienes, thromboxanes and lipoxins. Nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, is studied in **paper 4** of this thesis. It and its metabolites are implicated in a vast array of diverse physiological processes which effects too many pathologies to be reviewed here. Of note for this thesis, AA can be implicated in intracellular signal transduction systems (Shimizu *et al.*, 1990), including neuronal functions such as postsynaptic signalling of excitatory neurons (Kaufmann *et al.*, 1996), modulation of neuronal firing (Scuri *et al.*, 2005) and neurotransmitter release (Ojeda *et al.*, 1989). AA itself is a ligand at intracellular receptors, such as the intracellular calcium release channel inositol 1,4,5-trisphosphate-gated channel (IP3R) and the ryanodine receptor (RyR) (Striggow *et al.*, 1997). AA can also activate various intracellular protein kinase C (PKC) (Schaechter *et al.*, 1993). Action via these receptors and Kinase can all lead to a rise of intracellular calcium. The activation of these receptors by AA will mostly happen following the intracellular release of AA by the activation of various GPCRs. Due to the highly lipophilic nature

of AA, it is unlikely that it could pass the cell membrane without the action of a transporter, or after being firstly metabolized. The passage of arachidonic acid through cell membrane to activate intracellular receptors is a subject that is still debated (Brash, 2001). In vivo, free arachidonic acid levels, and modulation of each of its metabolic pathways, have been shown to influence pain (Smith, 2006).

## Docosahexaenoic acid (DHA)

The most abundant n-3 PUFA in the brain is docosahexaenoic acid (DHA) (22:6*n*-3). Its presence is essential for healthy brain function (Neuringer *et al.*, 1988) although it isn't considered an essential fatty acid as it can be synthesized by the liver from α-lineloic acid (Sprecher, 2000). The calcium-independent phospholipase $iPLA_2$ is the main phospholipid responsible for hydrolizing DHA from its esterified form in membranes (Strokin *et al.*, 2003). DHA is present in high quantity in fish and fish oil and its levels will vary in the brain depending on its availability based on food supplementation, as its biosynthesis from precursors in mammals does not readily occur, even though 60% of the fatty acids that make up neuronal cell membranes consist of DHA (Green *et al.*, 2007). It is particularly concentrated in synaptic membrane (Bazan *et al.*, 1990) and in myelin sheaths (Ansari *et al.*, 1990). Furthermore, its levels also affect the concentration of other PUFA levels in the brain as a diet restricted in DHA and other *n*-3 PUFAs has been linked with higher AA and docosapentaeonic (DCP) acid levels in animal models (Burdge *et al.*, 2002; DeMar *et al.*, 2004; Rapoport *et al.*, 2007).

Interestingly, its metabolites via COX and lipoxygenase metabolic pathways are seen as protective against inflammation, contrary to AA's pro-inflammatory metabolites. Resolvins of the D series and docosatrienes are potent anti-inflammatory and immunoregulatory molecules (Marcheselli *et al.*, 2003), have neuroprotective properties (Okuda *et al.*, 1994) and were termed neuroprotectin (Serhan *et al.*, 2004). These neuroprotectins are now seen as potential novel drugs in pharmacotherapeutic strategies in some neurodegenerative disorders such as Alzheimer's Disease (Lukiw *et al.*, 2010) as well as pathologies where uncontrolled inflammation and redox organ stress occurs (Serhan *et al.*, 2008).

## Oleic acid

Oleic acid, the main component in olive oil and found in high concentration in Mediterranean diets, is a mono-unsaturated fatty acid and it has been showed to be a good substitute to diets rich in saturated fatty acid in order to lower cardiovascular risks according by the World Health Organization (Consultation, 2003). For example, isocaloric replacement of about 5% of energy from saturated fatty acids by oleic acid or other PUFAs has been estimated to reduce coronary heart disease risk by 20–40% mainly via LDL-cholesterol reduction (Kris-Etherton, 1999). Although it is not produced endogenously, oleic acid can be metabolised to form endogenous compounds found in the brain such as oleamide, synthesized by oleoyl coenzyme A in cytochrome (Mueller *et al.*, 2009), and N-oleoyl dopamine (NODA) (Chu *et al.*, 2003; Pokorski *et al.*, 1998).

## Receptors “sensing” UFAs

Fatty acids are important signalling molecules and their detection by nerves and other cells is vital. Taste receptors and enteroendocrine cells of the gastric system, for example, sense dietary fatty acids to regulate proper digestive mechanisms and appetite (Laugerette *et al.*, 2005). Sensing fatty acids derived from phospholipids is also an important signalling pathway. Phospholipase $A_2$ enzymes hydrolyze glycerophospholipids (Murakami *et al.*, 2011) and DAG lipase metabolizes DAG in order to form free PUFAs that can then have important roles in various primordial mechanisms, from inflammation regulation to cell division and wound repair (Funk, 2001; Soberman *et al.*, 2003) both intracellularly or as an intercellular messenger.

PUFAs can regulate taste via inhibition of delayed $K^+$ channels to prolong taste-receptor depolarization (Gilbertson *et al.*, 1997), and bind to peroxisome proliferator-activated receptors (PPARs) to induce gene expression (Hihi *et al.*, 2002). Several orphan GPCRs expressed in enteroendocrine cells are believed to be involved in the sensing of saturated and unsaturated fatty acids (GPR40 (Itoh *et al.*, 2003), GPR41 and GPR43 (Brown *et al.*, 2003; Le Poul *et al.*, 2003), and GPR120 (Hirasawa *et al.*, 2005). Finally, in vertebrates, several members of the transient potential receptor (TRP) channel family appear to be PUFA sensors. Phospholipase C signalling coupled to DAG activate the transient receptor potential canonical (TRPC) 2, 3, 6 and 7 channels (Hardie, 2007). Omega-3 fatty acids inhibit vanilloid-activated TRPV1 receptors (Matta *et al.*, 2007), PUFAs potentiate

TRPV3 and modulate TRPM8 (Andersson *et al.*, 2007) and TRPA1 (Motter *et al.*, 2012). It is although important to note that several of the numerous metabolites of PUFAs are probably more important activators of various TRP channels than PUFAs themselves, for a review on the different effect of arachidonic acid and its metabolites on TRP channel activation, see (Meves, 2008).

## N-amino acid/neurotransmitter conjugates (NAANs)

### General

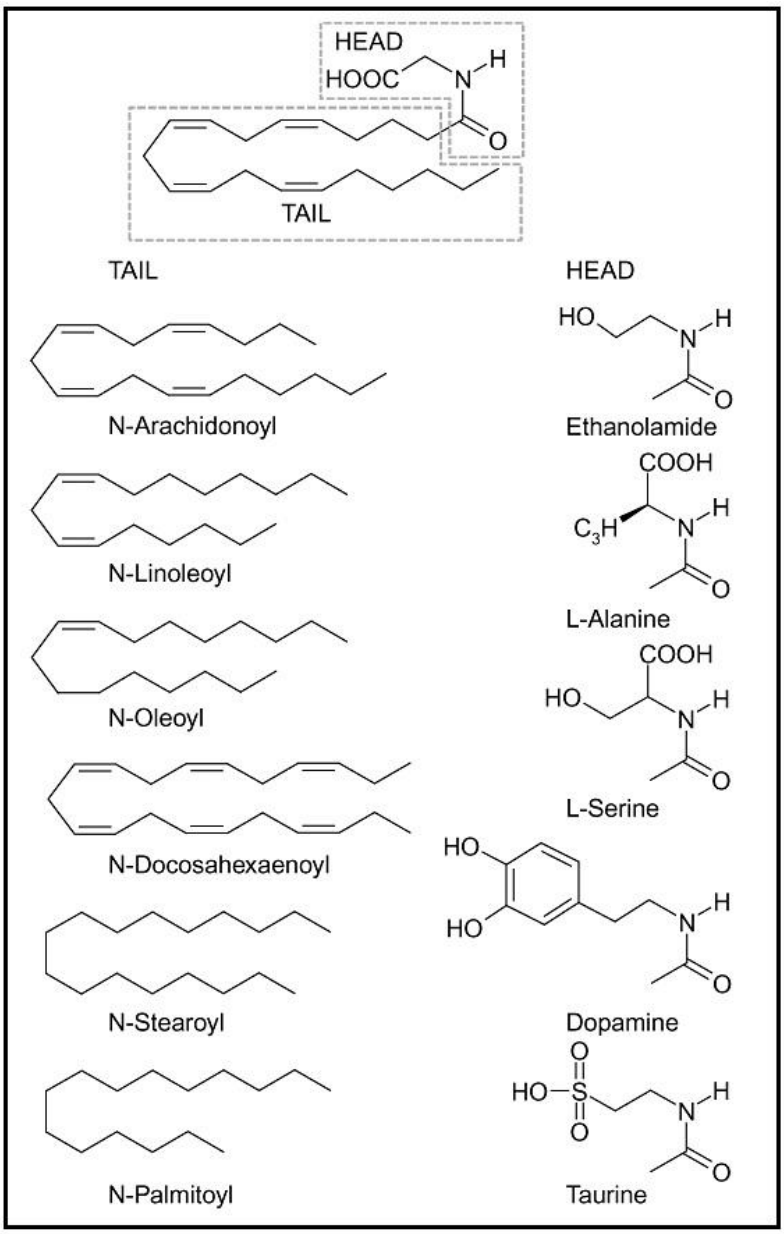
A vast array of lipids are thought to be signalling molecules and some have been reported as mediators of communication within and between cells (Piomelli *et al.*, 2007). One of the most well defined receptor for lipid-mediated receptor activation is the cannabinoid CB1 receptor. Its first endogenous ligand discovered was n-arachidonoyl ethanolamide (anandamide (AEA)) (Devane *et al.*, 1992) and it is seen as the first discovered compound of a family made up of varied ethanolamides with varying fatty acid chain length and unsaturations (Di Marzo *et al.*, 1994). Numerous related endogenous lipid molecules composed of an arachidonic acid or related hydrocarbon tail and an amino acid or neurotransmitter head-group, (such as glycine, GABA or dopamine for example, see figure 2), have been reported in the last decade. There are now more than 70 of these compounds reported in humans (Bradshaw *et al.*, 2009b; Chu *et al.*, 2003; Huang *et al.*, 2001; Huang *et al.*, 2002; Milman *et al.*, 2006; Rimmerman *et al.*, 2008; Saghatelian *et al.*, 2004; Tan *et al.*, 2010).

## Synthesis and degradation

There are currently three main proposed mechanisms for the synthesis of NAANs, though much work is still needed in order to fully understand their production. The first proposed mechanism is through the enzymatic condensation of the amino acid/neurotransmitter head-group with either AA or arachidonoyl coenzyme A. The second is through the sequential modification of a precursor fatty acid conjugate to form the final NAAN. NA-Gly has been showed to be synthesized by both these pathways (Aneetha *et al.*, 2009; Huang *et al.*, 2001; McCue *et al.*, 2008; Merkler *et al.*, 2004). These synthetic pathways have also been proposed for the formation of NADA (Huang *et al.*, 2002) although a third synthesis mechanism, via FAAH, has since been proposed to mediate the condensation of dopamine to AA and is now seen as its prominent synthesis pathway (Hu *et al.*, 2009).

FAAH is also seen as the main degradation pathway, breaking the bond between the head and fatty acid tail of NAANs, as for anandamide (Cravatt *et al.*, 1996). The resulting free AA can then be used for the production of new NAAN molecules (Bradshaw *et al.*, 2009b). The two other proposed mechanisms for degradation either involve modification of the fatty acid backbone by various enzymes, or modification of the amino-acid/neurotransmitter head-group.

**Figure 3:** Structures of *N*-acyl amino acid/neurotransmitter (NAAN). Taken from (Connor *et al.*, 2010).




## *N*-acyl dopamine

The first described compound from this family was N-arachidonoyl dopamine. Before being found endogenously, it was synthetised as a possible fatty acid amide hydrolase (FAAH) inhibitor. A 40-fold affinity preference for

CB1 compared to CB2 was reported (Bisogno *et al.*, 2000). The pharmacological profile of NADA on these receptors was investigated in **paper 2**. NADA was later isolated from bovine brain and has been showed to be a potent agonist at TRPV1, with an $EC_{50}$ at around 50 nM (Huang *et al.*, 2002). Related acyl dopamines oleoyl- (NODA), palmitoyl- (NPDA) and steraoyl- (NSDA) were isolated the following year (Chu *et al.*, 2003). Two synthesis pathways were proposed for NADA, both involving enzyme-mediated conjugation of arachidonic acid with dopamine and is hydrolysed, though more slowly than AEA, by FAAH to form arachidonic acid and dopamine (Hu *et al.*, 2009). Its distribution in the central nervous system is restricted, with its highest density in the striatum, but it is also found in the hippocampus, cerebellum, brain stem, cortex, ventral midbrain and dorsal root ganglia (Bradshaw *et al.*, 2006; Freestone *et al.*, 2013; Huang *et al.*, 2002). It has not been found outside of the CNS but its rate-limiting synthesis enzyme, tyrosine-hydroxylase, is present in the liver, with enhanced concentration in the case of fibrosis (Wojtalla *et al.*, 2012). In the brain, NADA has been showed to tune synaptic transmission on dopaminergic neurons (Marinelli *et al.*, 2007) as well as increase 2-AG production in the midbrain (Freestone *et al.*, 2013). Anandamide and NADA have also both been showed to modulate transmitter release and presynaptic $Ca^{2+}$ levels in the hippocampus.

NADA causes anandamide-like activity when injected systematically, producing increased immobility in the ring test, lowered body temperature, lowered locomotion and delayed response to thermal stimulus (tetrad test for

cannabinoids) (Bezuglov *et al.*, 2001; Bisogno *et al.*, 2000). Systemic administration also reduces opioid-induced emesis and reduces locomotion in ferrets via both TRPV1 and CB1, although its modulation of locomotion appears to involve a non-CB1 and non-TRPV1 mechanism as well (Sharkey *et al.*, 2007). When injected in the periphery, NADA and NODA induce responses in accordance of what one would expect from a TRPV1 such as thermal allodinya in the hindpaw (Chu *et al.*, 2003; Huang *et al.*, 2002), with NODA causing more frequent spontaneous paw lifting and licking. These behavioural effects were described in mice for NODA as well, and sensitization was strongly reduced in KO animals for TRPV1 (Szolcsanyi *et al.*, 2004). Thermal allodynia was reported with topical administration of both NADA and NODA on monkey tails (Butelman *et al.*, 2004). In isolated peripheral organs, NADA causes contraction through a TRPV1-mediated mechanism in guinea pig urinary bladder and bronchi (Harrison *et al.*, 2003). NADA has also been showed to be an anti-fibrogenic factor as it can induce oxidative stress-mediated cell death in hepathic stellate cells, the main fibrogenic cell type in the liver, but not in healthy cells (Wojtalla *et al.*, 2012). It also has neuroprotective properties in the brain after excitotoxic neuronal damage (Grabiec *et al.*, 2012). OLDA is also an endogenous ligand for the GPR119 receptor in the gut and induces the secretion of the incretin and satiety hormone glucagon-like peptide-1 (GLP-1), thus could be of importance in type 2 diabetes (Chu *et al.*, 2008; Lauffer *et al.*, 2009; Overton *et al.*, 2008).

## *N*-acyl Glycine

N-arachidonoyl glycine (NAGly) is probably the most studied NAANwith NADA. It was first synthesized in 1997 (Sheskin *et al.*, 1997). Burstein *et* al. first suggested that NAGly could be a result of a pathway involving oxidation of the hydroxy group of anandamide to form a putative metabolite (Burstein *et al.*, 2000) and this has been confirmed since (Bradshaw *et al.*, 2009a). NAGly was discovered to be present in the mammalian brain, as well as in the spinal cord, the liver, the skin, and in low concentration in the heart (Huang *et al.*, 2001). Cytochrome *c* has also been showed to catalyze the formation of *N*-oleoylglycine from oleoyl-CoA and glycine (Mueller *et al.*, 2007) and N-steraoyl, N-linoleoyl, N-palmitoyl, N-docosahexaenoyl glycine have also all been reported to be endogenous compounds, mostly found in the skin and lungs (Bradshaw *et al.*, 2009b; Rimmerman *et al.*, 2008). NAGly is metabolised by COX-2 to form the prostaglandin $H_2$-Glycine ($PGH_2$) (Burstein *et al.*, 2000; Prusakiewicz *et al.*, 2002) and by FAAH to produce AA and glycine. As the rate of production of these metabolites is as low as 10% the speed at which $PGH_2$ is formed from arachidonic acid and because of its much slower hydrolysis by FAAH when compared to anandamide, NA-Gly can be thought as an inhibitor of both COX-2 and FAAH (Grazia Cascio *et al.*, 2004; Prusakiewicz *et al.*, 2002). Systemic administration of NAGly has thus been reported to increase anandamide levels (Burstein *et al.*, 2002) although its analgesic properties when injected intrathecally do not appear to be mediated by CB1 nor CB2 (Succar *et al.*, 2007; Vuong *et al.*, 2008). Various antinociceptive effects linked with local administration of NA-Gly

have been reported, for review, see (Connor *et al.*, 2010). Its mechanism of action is still not clear, although it appears to involve T-type $I_{Ca}$ in some cases (Barbara *et al.*, 2009; Ross *et al.*, 2009).

## N-arachidonoyl GABA (NA-GABA)

As for NA-Gly, N-arachidonoyl GABA (NA-GABA) was first synthesized as an anandamide congener (Burstein *et al.*, 2000) and isolated in mammals shortly afterward (Huang *et al.*, 2001). It is present in the brain (Bradshaw *et al.*, 2006) and showed modest antinociceptive effect in the hot-plate test in the rat (Burstein *et al.*, 2000) although it did not alleviate pain in an inflammatory model in the rat after being intrathecally administered (Succar *et al.*, 2007). One of its analogs also found endogenously, NA-GABA-OH, shows potent antinociceptive effects in a hot-plate test in the mouse when administered locally. This effect was absent in Cav3.2 knock-out mice (Barbara *et al.*, 2009).

## *N*-arachidonoyl Serotonin (NA-5HT)

N-arachidonoyl serotonin (NA-5HT) is an important modulator of the endocannabinoid system although it is a very poor agonist at CB1 and CB2. It is instead a potent inhibitor of FAAH, the enzyme responsible for the degradation of anandamide as well as the production of arachidonic acid, respectively (Bisogno *et al.*, 1998; Fowler *et al.*, 2003). Its potent inhibition of TRPV1 activation is also considered a part of its endocannabinoid modulating properties (Maione *et*

*al.*, 2007). Finally, NA-5HT is one of the most potent inhibitors described to date of recombinant T type calcium channels (Gilmore *et al.*, 2012), which could potentially yield antinociceptive potential. It was recently revealed to be found, as well as several other *N*-acyl serotonins, *in vivo* in the intestinal cells in the pig (Verhoeckx et al., 2011). Accordingly with its pharmacological profile, NA-5HT has been showed to alleviate pain and inflammation in the brain (de Novellis *et al.*, 2008; de Novellis *et al.*, 2011; Suplita *et al.*, 2005), in the spinal cord (Suplita *et al.*, 2006) and the periphery (Costa *et al.*, 2010; D'Argenio *et al.*, 2006). It has also been showed to elicit anxiogenic properties when injected in the basolateral amygdala via FAAH and TRPV1 modulation (John *et al.*, 2012).

## *N*-acyl Taurine

*N*-acyl taurines were first described in a comparative study of FAAH knock-out mice and their levels went up 10 to 100-fold, on par with increased levels of acyl-ethanolamides (Saghatelian *et al.*, 2006; Saghatelian *et al.*, 2004). In the periphery, polyunsaturated *N*-acyl taurines were found whereas CNS levels were mostly composed of saturated and mono-unsaturated species (Saghatelian *et al.*, 2006). No clear function has been given to *N*-acyl taurines, though they have been found to be weak agonists at various TRP channels (Connor *et al.*, 2010) and inhibitors of T-type $I_{Ca}$ (Barbara *et al.*, 2009).

## Possible unspecific effects

Due to their highly lipophilic nature, NAANs have been proposed as being able to intercalate in the cell membrane, as detergents do, thus theoretically having an effect on membrane fluidity. As well, if its head group is charged, this might potentially influence channels on the cell surface via surface charge screening effects. Even if this happens to be true, these effects have not so far been specifically addressed and cannot account for the full spectrum of activity of these compounds as they can act as *bona fide* ligands at various channels and G-protein coupled receptors (Connor *et al.*, 2010).

# Cannabinoid receptors and the endocannabinoid system

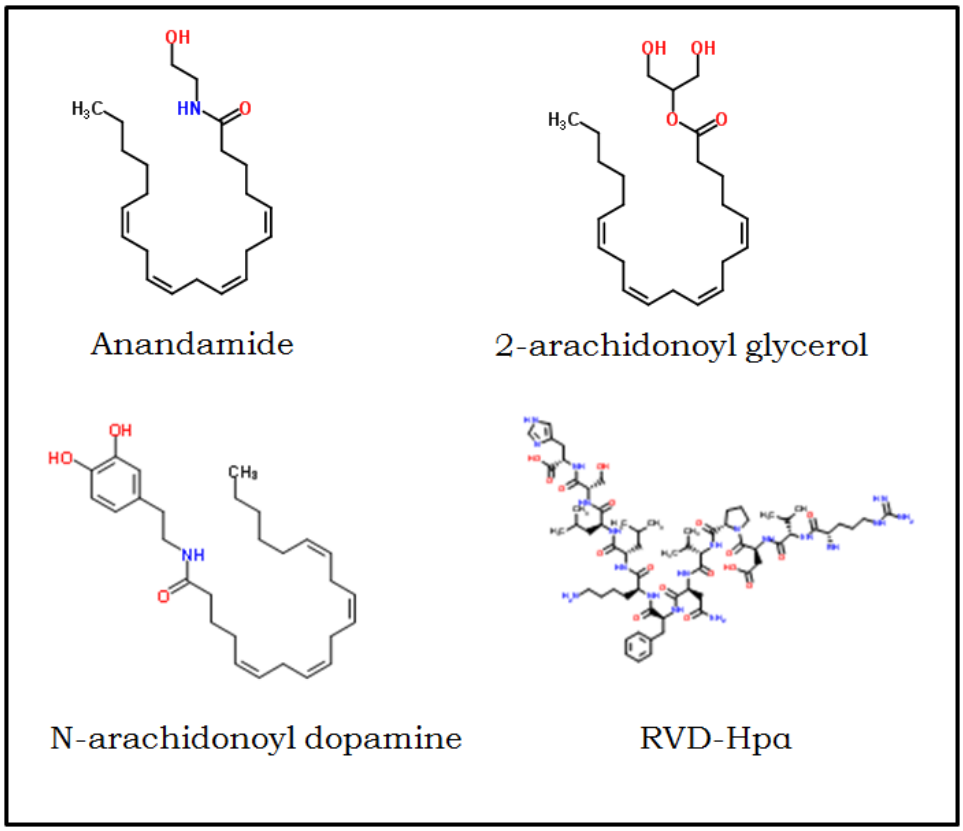
## Cannabinoids

### *Endogenous cannabinoids*

There are currently two well defined endogenous ligands for the endocannabinoid system: arachidonoyl ethanolamide (anandamide) (Devane *et al.*, 1992) and 2-arachidonoyl glycerol (2-AG) (Mechoulam *et al.*, 1995). Anandamide and 2-AG are lipophilic neurotransmitters synthesized by the cleavage of plasma membrane phospholipids and several synthesis pathway have been observed, for review, see (Wang *et al.*, 2009). Anandamide and 2-AG are synthesized on demand at or near the site of action of post-synaptic

neurons (Di Marzo *et al.*, 1994; Piomelli, 2003). Anandamide acts as an agonist for both CB1 and CB2 , but is more efficacious on CB1 (Smita *et al.*, 2007; Sugiura *et al.*, 2002) , while 2-AG acts as a full agonist for both receptors (McAllister *et al.*, 2002; Sugiura *et al.*, 2006). In mammals, anandamide and 2-AG co-exist with ethanolamides of other fatty acids, collectively referred to as *N*-acylethanolamines (NAEs). Anandamide is usually a relatively minor component, while saturated and mono-unsaturated long-chain NAEs being major components and are themselves candidates as potential ligands for these receptors (Yang *et al.*, 1999). Palmithoylethanolamide (Facci *et al.*, 1995), docosoheanoyelethanolamide (Sheskin *et al.*, 1997), oleamide (Leggett *et al.*, 2004), for example, are all agonists, to some extent, at the CB1 and/or CB2 receptor, for review, see (Fonseca *et al.*, 2013; Schmid *et al.*, 2002). Other endogenous molecules possessing a head groups with a fatty acid tail (NAANs) have also been found to bind to the CB1 receptor, like NADA, as well as other endocannabinoids such as arachidonyl glyceryl ether and *O*-arachidonoyl ethanolamine (virodhamine) (Bisogno *et al.*, 2000; Hanus *et al.*, 2001; Porter *et al.*, 2002). Finally, hemopressin, a 9 residue α-hemoglobin-derived peptide, has been described as an inverse agonist at the CB1, and has antinociceptive activity (Heimann *et al.*, 2007). Since its discovery, a search for other α-hemoglobin-derived peptides, resulted in the characterization of two novel CB1 ligands, RVD-Hpα and VD-Hpα, which suggests a novel mode of endocannabinoid regulation (Gomes *et al.*, 2009).

**Figure 4:** Various structures of endocannabinoids




Endocannabinoids are retrograde signalling messengers in GABAergic and glutamatergic synapses as well as modulators of postsynaptic transmission and can interact with other neurotransmitters such as dopamine and norepinephrine (Miller *et al.*, 1995). Retrograde endocannabinoid signalling can mediate forms of short-term synaptic plasticity known as depolarization-induced suppression of inhibition (DSI) (Ohno-Shosaku *et al.*, 2001; Wilson *et al.*, 2001) and depolarisation-induced suppression of excitation (DSE) (Kreitzer *et al.*, 2001), as well as long-term depression at both excitatory (Gerdeman *et al.*, 2002; Robbe *et al.*, 2002) and inhibitory synapses (Chevaleyre *et al.*, 2003; Marsicano *et al.*, 2002), for review, see (Castillo *et al.*, 2012). Postsynaptic depolarization elevating intracellular $Ca^{2+}$ ($[Ca]_I$) via

voltage-gated calcium channels (VGCCs) produces the synthesis of 2-AG in postsynaptic cells, leading to postsynaptic transmission, presumably via $Ca^{2+}$-sensitive enzymes. The elevation of $[Ca]_I$ needed to initiate this synthesis is not limited to channels on the membrane, as activation of $G_q$-coupled GPCRs, such as group I metabotropic glutamate receptors can also generate 2-AG by activating the phospholipase C β (PLCβ) enzyme which leads to an elevation of $Ca^{2+}$ from the endoplasmic reticulum (Katona *et al.*, 2012; Maejima *et al.*, 2001; Varma *et al.*, 2001). Activation of VGCCs and downstream signalling of $G_q$-coupled GPCRs can converge on the same metabolic pathway for the creation of 2-AG, and PLCβ can be seen as a coincidence detector for postsynaptic $Ca^{2+}$ and GPCR signalling (Hashimotodani *et al.*, 2005; Maejima *et al.*, 2005). This interaction might be of importance for the integration of synaptic activity (Brenowitz *et al.*, 2005). The picture is less clear for AEA synthesis. Postsynaptic depolarization and $[Ca]_I$ appear to be of importance for its production, but its exact mechanism is not clearly understood (Di Marzo, 2011). This is in part due to the alternative signalling pathways that can lead to the formation of AEA. Indeed, although the main synthesis pathway for AEA in two successive enzymatic steps, the limiting one being *N*-acyl-hydrolyzing phospholipase-D (NAPE-PLD), a calcium-independent *N*-acyltransferase and other yet unknown NAPE-PLD-independent mechanisms have been proposed (Okamoto *et al.*, 2007).

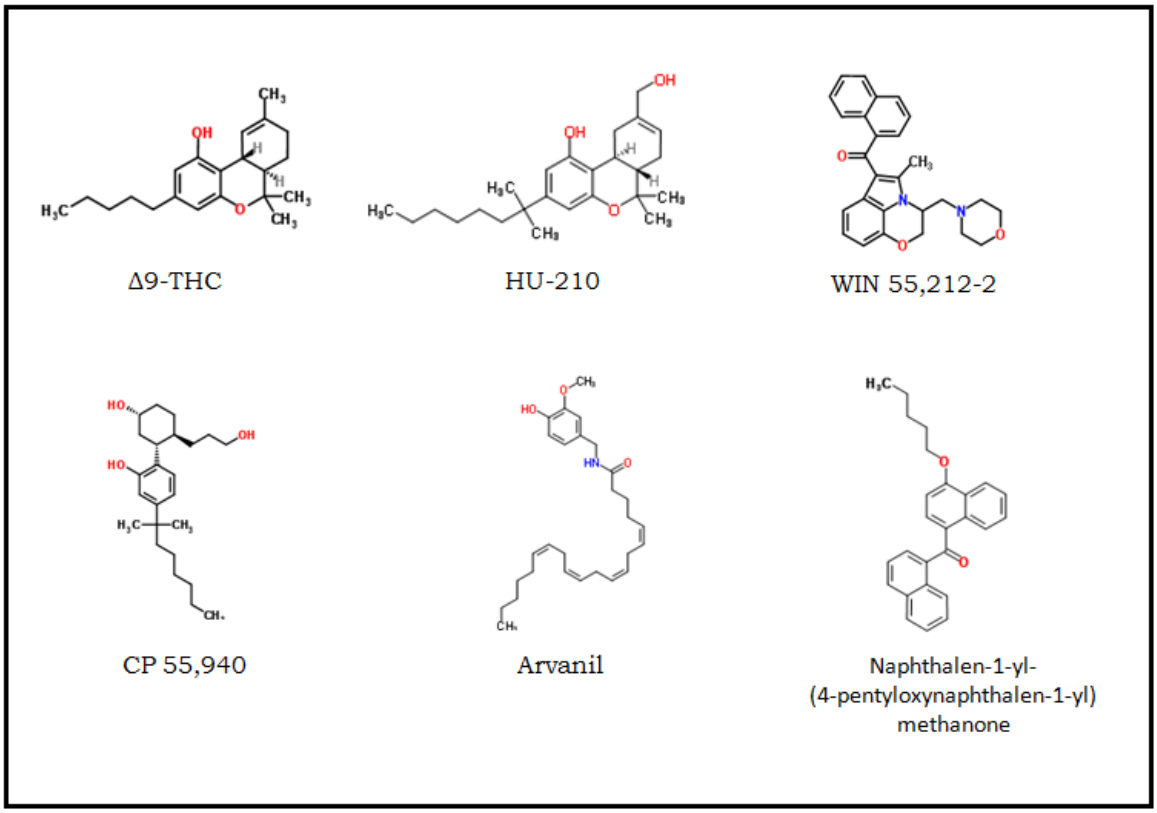
2-AG and AEA are removed rapidly from the intracellular space through diffusion and/or membrane-associated carriers, for review, see (Fowler,

2013). For AEA, one candidate, FLAT, a FAAH-like truncated and catalytically-inactive variant, has been proposed (Fu *et al.*, 2012; Leung *et al.*, 2013). It is likely that different diffusion and/or facilitated diffusion is happening for each endocannabinoid, and it has also been argued that cells may utilize different mechanisms depending upon whether they employ endocannabinoids as signalling molecules or as a source of arachidonic acid (Hillard *et al.*, 2005). FAAH and MAGL are the primary catabolic enzymes of AEA and 2-AG, respectively (Blankman *et al.*, 2007; Cravatt *et al.*, 1996; Di Marzo, 2008; Fegley *et al.*, 2004).

### *Exogenous cannabinoids*

Raphael Mechoulam and collaborators isolated, elucidated the structure and synthesized the first psychoactive cannabinoid compound, Δ9-tetrahydrocannabinol Δ9-THC), from hashish in 1965 (Budzikiewicz *et al.*, 1965; Mechoulam *et al.*, 1965a; Mechoulam *et al.*, 1965b). It has slightly better affinity for CB1 than CB2 but is an agonist for both ($K_i$ of 5 to 80 nM for CB1 and 3 to 75 for CB2) (Pertwee, 2010). There are more than 60 different phytocannabinoids that have been extracted from the plant *cannabis sativa.* Several of these phytocannabinoids show psychoactive and immunomodulatory effects via CB1 and CB2, for review see (Ashton, 2001). These compounds are all highly lipophilic, therefore easily crossing the blood-brain barrier (McGilveray, 2005).

**Figure 5:** Various structures of exogenous cannabinoids.




Through the years, an ever-increasing number of synthetic cannabinoids have been synthesized with varying affinity, potency and molecular structure and a thorough review is out of the scope of this introduction. To summarize, there are four main categories of cannabinoids with completely different molecular structure and there are synthetic ones for each categories, for review see (Pertwee, 2010).

*Classical cannabinoids* are the plant-derived dibenzopyran and its derivatives. HU-210 is one of the main classical synthetic compounds in this category and was synthesized by the group of Mechoulam (Mechoulam *et al.*, 1988). It has similar efficacies as WIN 55,212-2 and CP 55,940 with high

affinities for CB1 and CB2 ($K_i$ of 0.06 to 0.73 nM for CB1 and 0.17 to 0.52 for CB2) and induces long-lasting psychoactive effects *in vivo (Bayewitch et al., 1996).*

*Nonclassical cannabinoids* are mostly the group designed by a Pfizer research team. They are termed nonclassical although they maintain a similar structure to the classical cannabinoids, as they are bicyclic or tricyclic analogues of Δ9-THC that lack a pyran ring. The most widely used in this category is CP 55,940 which shows high efficacy and affinity in the low nanomolar range ($K_i$ of 0.5 to 5.0 nM for CB1 and 0.69 to 2.8 nM for CB2) (Pertwee, 2010).

*Aminoalkylindole cannabinoids* best known member is WIN 55,212-2 as it is widely used in cannabinoid research. It has no resemblance with either the classical, nonclassical or eicosanoid cannabinoids and is thought to bind differently, although on the same orthosteric site of the CB1 receptor as the classical and nonclassical cannabinoids, for review, see (Howlett *et al.*, 2002; Pertwee, 1997) and has a slightly higher affinity for CB2 than CB1 ($K_i$ of 1.89 to 123 nM for CB1 and of 0.28 to 16.2 nM for CB2). It is able to displace both CP 55,940 and HU-210. (Pertwee, 2010).

*Eicosanoids* encompass the endogenous endocannabinoids and related polyunsaturated long-chain fatty acids containing molecules. Arvanil, a nonpungent capsaicin–anandamide hybrid molecule with a capsaicin head and an arachidonic acid tail, has been described as a tool to probe the

vanilloid site on the channel as well as being a CB1/TRPV1 receptors dual activator ($K_i$ of 0.5 and 0.3 µM respectively) (Melck *et al.*, 1999). Agonists that are hydrophilic non-blood-brain barrier permeant agents, such as Naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone and various quinolone-3-carboxamide derivatives (Pasquini *et al.*, 2012) or compounds derived from rimonabant, a synthetic CB1 inverse agonist (Fulp *et al.*, 2012), has been developed to help probe the peripheral cannabinoid system whilst avoiding central side-effects (Thakur *et al.*, 2009). Such a molecule, with both CB1 and CB2 affinity but no central effect, have been showed to reduce pain in rat models of neuropathic pain (Dziadulewicz *et al.*, 2007).

## Cannabinoid receptors CB1 and CB2

### *General*

Cannabinoid receptors CB1 and CB2 are 7-transmembrane G-protein-coupled receptors (GPCR) and are the predominant mediators of cannabinoid effects.

CB1 has been cloned in rat (Matsuda *et al.*, 1990), mouse (Chakrabarti *et al.*, 1995) and human (Gerard *et al.*, 1991) and shows 97-99% amino acid sequence identity between species. This sequence similarity appears to be kept for other non-mammalian species, as the chicken genomic sequence is 98% similar to the zebrafish and 92% similar to the human receptor (McPartland *et al.*, 2003). It is one of the most widely expressed GPCR in the

brain (Herkenham *et al.*, 1991; Herkenham *et al.*, 1990) and can be found at every level of the nervous system, for review of localization in the nervous system see (Mackie, 2005). However, low levels of CB1 have also been described as present in adipose tissue (Cota *et al.*, 2003; Spoto *et al.*, 2006), liver (Osei-Hyiaman *et al.*, 2005), pancreatic islet (Juan-Pico *et al.*, 2006) and the gastro-intestinal tract (Kulkarni-Narla *et al.*, 2000).

CB2 only possess 44% homology with CB1 (Munro *et al.*, 1993). CB2 is principally expressed in blood-borne immune cells and related tissues (Galiegue *et al.*, 1995). Its abundant expression in immune cells can explain most of the cannabinoid-related immunomodulatory activity (Atwood *et al.*, 2010; Berdyshev, 2000; Lynn *et al.*, 1994). Although CB2 was first described as a peripheral receptor, it is also present in the glial and endothelial cells in the brain (Benito *et al.*, 2003; Golech *et al.*, 2004; Nunez *et al.*, 2004). In the CNS, CB2 activation has been shown to alleviate pain (Rivers *et al.*, 2010) and inflammation (Benito *et al.*, 2008) and to be of interest as a therapeutic target in autoimmune diseases (Tanasescu *et al.*, 2010). CB2 can also be directly implicated in inflammation-dependant neurodegeneration pathologies (Ashton *et al.*, 2007). Although most of its more well-defined characteristics are linked with inflammation, CB2 in the CNS could play more than just a role in neuro-immuno activity. Indeed, the activation of CB2 receptors on microglia have been showed to be able to synthesize 2-AG in cultured cells (Carrier *et al.*, 2004; Walter *et al.*, 2003) which could then potentially activate CB1 receptors situated on adjacent neurons. Furthermore, the activation of CB2 receptors on microglia can lead to

glutamatergic-mediated gliotransmission, for review see (Castillo *et al.*, 2012).

### *Signalling*

G-proteins are guanosine diphosphate (GDP)-bound heterotrimers. When a GPCR gets into an active conformation either through the binding of a ligand or through transient constitutive activity, the GDP is exchanged for a guanosine triphosphate (GTP) and the G-protein is cleaved into $G_{\alpha}$ and $G_{\beta\gamma}$ subunits which can then affect various signalling proteins.

CB1 and CB2 preferentially couple to $G\alpha_{i/o}$ G-proteins when the receptor is stabilized in its active state through the binding of a ligand or through constitutive conformational changes favoring an active conformation. Once its subunits are cleaved, its $\alpha_i$ subunit will inhibit adenylate cyclase (AC) activity thus preventing the accumulation of cyclic adenosine monophosphate (cAMP) inside the cell (Caulfield *et al.*, 1992; Howlett, 1984; Howlett *et al.*, 1984; Mackie *et al.*, 1995). Its βγ counterpart is responsible for the activation of G-protein gated inwardly rectifying potassium channels (GIRKs) (Mackie *et al.*, 1995) as well as inhibition of L-, N-, and P/Q-type calcium channels (Caulfield *et al.*, 1992; Mackie *et al.*, 1995). βγ is also proposed to activate mitogen-activated protein kinase (MAP kinase) leading to gene expression, such as Krox 24 (Bouaboula *et al.*, 1995; Graham *et al.*, 2006).

The CB1 receptor is known to be a promiscuous receptor as activation of the receptor with a ligand will usually cause the coupling of more than one G-protein, although $G_{i/o}$ is its most important effector. *Pertussis* toxin (PTX) inhibits the $G_{i/o}$ pathway once GPCR are activated (Burns, 1988). Pre-incubation of PTX before addition of CP55,940 in striatal neurons in primary culture and in CHO cells unmasked an increase in cAMP through adenylyl cyclase activation, showing that the CB1 receptor also couples to Gs G-protein (Glass *et al.*, 1997). Finally, Lauckner *et al.* demonstrated that activation of the CB1 receptor can increase intracellular levels of calcium through Gq coupling and activation of the phospholipase C pathway (Lauckner *et al.*, 2005).

Following the activation of the receptor, as for the majority of GPCRs, the CB1 receptor will become desensitized through the phosphorylation of G-protein coupled kinases (GRK), which facilitates β-arrestin 2 recruitment, causing steric hindrance of G-protein complex interactions, thus preventing further signal cascade from happening through GPCRs (Jin *et al.*, 1999). It is to be noted that for an increasing number of GPCRs, β-arrestin 2 and 3 are now seen as more than adaptor proteins, as they can serve as scaffolds for signalling complexes, for review on β-arrestin roles on GPCR, see (Shenoy *et al.*, 2011). β-arrestin 2 signalling in CB1 has been recently reported after acute and repeated Δ9-THC stimulation, and appears to be region-specific, thus showing multiple overlapping mechanisms of regulation of CB1 (Nguyen *et al.*, 2012).

A mounting body of studies suggests that CB1 receptors can possibly form homo- and/or heterodimers. The first suggestion of CB1 homodimers was reported by Wager-Miller who saw high molecular weight bands suggesting dimerization by western blotting technique (Wager-Miller *et al.*, 2002). As well, immunoprecipitation technique has been used to report a possible heterodimerization between the CB1 and dopamine 2 (D2) receptors (Kearn *et al.*, 2005). Although these two techniques are widely used for detection of GPCR dimers, one needs to keep into consideration that difficulties with membrane solubilisation, formation of GPCR aggregates, inappropriate selection of detergents, remaining membrane patches in supernatant and effects of the receptor glycosylation need to all be taken into consideration (Szidonya *et al.*, 2008). Resonance energy transfer methods has also showed CB1 interacting with D2 and with adenosine $A_{2A}$ (Navarro *et al.*, 2008), as well as CB1 with μ opioid receptor heterodimerization (Rios *et al.*, 2006). These results have all been reported in transfected cells. Different roles for these heterodimerizations have been proposed, with the most likely idea of heterodimerization expanding the repertoire of, as well as making possible region-specific, signalling mechanisms via cannabinoid activation (Hojo *et al.*, 2008; Marcellino *et al.*, 2008; Rozenfeld *et al.*, 2012). The human $CB_1$ gene (*CNR1*) was originally described as consisting of four exons and three introns, with exon 4 containing the entire protein-coding region of the cannabinoid $hCB_1$ receptor (Zhang *et al.*, 2004). It is now known that exon 4 in human and non-human primate can be differentially spliced to produce two coding region splice variants encoding human cannabinoid $hCB_{1a}$ receptors (Shire *et al.*, 1995) and human cannabinoid $hCB_{1b}$ receptors

(Ryberg *et al.*, 2005). Co-expression of the human cannabinoid $hCB_1$, $hCB_{1a}$, and $hCB_{1b}$ receptors have recently been reported to form homodimers, as determined by BRET in transiently transfected HEK 293A cells, and have been showed to increase $G_{i/o}$-dependent extracellular regulated kinases ½ (ERK1/2) phosphorylation (Bagher *et al.*, 2013). Finally, it is important to note that interaction between GPCRs do not necessarily translate to dimerization as co-localization, signalling interactions as well as convergence of downstream signalling all contributing to make dimerization-specific investigation difficult, rendering clear conclusions on the subject more elusive and prone to debate.

These different interactions between various G-proteins, other signalling proteins and the CB1 receptor might come from differential expression of compartmentalisation of G-protein complexes, hetero-oligomerisation with other GPCRs or ligand-selective G-protein association. It is thus possible that various behavioural aspects of CB1 receptor activation can come from these various possible signalling routes (Breivogel *et al.*, 1997; Glass *et al.*, 1997; Glass *et al.*, 1999; Kearn *et al.*, 2005; Lauckner *et al.*, 2005).

In 2005, Price *et al.* described the first three allosteric modulators of the CB1 receptor, hence stating that CB1 possess at least one allosteric site (Price *et al.*, 2005). These organon compounds were shown to increase the binding of radioligand [$^3$H]CP55950 for CB1 receptors on mouse brain membranes; though causing a decrease for the $E_{max}$ of $CB_1$ receptor agonists in stimulation assay of [$^{35}$S]GTPγS binding to mouse brain membranes as well

as a decrease in efficacy of CP55,940 inhibition of electrically evoked contractions of mouse vas deferens. In 2007, Horswill *et al.* proposed a second molecular structure for an allosteric modulator of CB1 in a compound with a similar profile, PSNCBAM-1 (Horswill *et al.*, 2007). We have been able to propose since that what at first appeared to be a paradox between increased affinity and decreased effects of these allosteric modulators come from a change in the dynamics of receptor activation and desensitization, see **paper 2** (Cawston *et al.*, 2013). The allosteric binding site for ORG has been recently described and is situated in the 3-6-7 transmembrane helix (Shore *et al.*, 2013). A second allosteric site, situated on the membrane proximal N-terminus of the receptor has also been proposed (Fay *et al.*, 2013).

### *Desensitization*

The classical model of desensitization for GPCR come from studies of $\beta_2$-adrenergic receptor and has been found to be applicable for many other GPCRs (Inglese *et al.*, 1993; Kovoor *et al.*, 1997; Krupnick *et al.*, 1998). In this model, the agonist-bound GPCR becomes a substrate for kinases called GRK, which phosphorylate serine and/or threonine residues on the GPCR cytoplasmic domains, which pertains them to become in an increased state of affinity for arrestins. This binding of arrestins uncouple GPCRs from their G-protein and prevent further coupling as well as stimulate internalization (Sim *et al.*, 1996). In CB1, mutational studies have shown that a truncation of the receptor at residue 417, but not 438 nor 459, cause a strong attenuation of desensitization without affecting agonist activation. A deletion

mutant with a truncated 417-438 section also fails to elicit desensitization via agonist activation in oocytes (Jin *et al.*, 1999). This lead researchers to study the residues between H8 and H9 helices as potential critical residues for GRK3/βarrestin-2-mediated desensitization. Two putative GRK3 phosphorylation sites exist in the region, S426 and S430 and point-mutation of these residues have shown reduced agonist-induced desensitization as measured by decreased GIRK-mediated hyperpolarization of the cells (Jin *et al.*, 1999) and ERK1/2 phosphorylation via MAP kinase (Daigle *et al.*, 2008a); yet the internalization rates and the concentration of surface receptors as seen through recruitment of βarrestin to the plasma membrane were not significantly different from wild type CB1, tracing a distinction between internalization and desensitization mechanisms (Daigle *et al.*, 2008a; Jin *et al.*, 1999). The effects of ORG27569 and PSNCBAM-1, two allosteric modulators of CB1 on desensitization are shown in **paper 1**.

### *Internalization and trafficking*

The CB1 receptors constitutively internalize in their native state as evidenced by immunocytochemical staining both in cell cultures and in neurons (McIntosh *et al.*, 1998; Pettit *et al.*, 1998). This constitutive activity happens in both transfected cells and neurons (Pertwee, 2005) CB1 have varying rate of internalization as it appears to be faster in transfected cell lines (starting as few as 5 minutes after ligand-mediated activation) (Daigle *et al.*, 2008b; Hsieh *et al.*, 1999; Rinaldi-Carmona *et al.*, 1998) than in primary cultured neurons (Coutts *et al.*, 2001; Leterrier *et al.*, 2006), which could possibly be attributed to different trafficking depending on cell types and

plasma membrane sub-domains (Keren *et al.*, 2003). In hippocampal cells, Leterrier *et al* further reported that constitutive activity and internalization is restricted to the somatodendritic compartment and is mostly absent on the axons, where CB1s accumulate (Leterrier *et al.*, 2006). Cortical neurons showed similar distinction between soma and axons (Mikasova *et al.*, 2008). One possible explanation for this would be that at the synapse but not the soma, expression of regulatory proteins inhibit internalization and/or promote its rapid recycling (Stadel *et al.*, 2011).

For a majority of GPCRs, the carboxyl-terminal of the receptor appears to be of importance for agonist-induced internalization (Trapaidze *et al.*, 1996). This appears to be the case for CB1 as some residues have been proposed to govern endocytosis at the carboxyl-terminal end of H9, and at least 14 residues are implicated (Stadel *et al.*, 2011). For now, the various mutational studies and cell lines used to conduct them prevent us from drawing a clear picture and more in-depth studies are needed.

## Orphan G-protein coupled receptor 55 (GPR55)

The last two decades have seen an exponential interest toward the pharmaceutical potential of the endocannabinoid system, which has lead scientists and pharmaceutical companies in a search for other potential candidate receptor for this family amongst a library of currently orphaned receptors. The first such proposed receptor has been GPR55 for which its sensibility for cannabinoids was first described in two industrial patents from GlaxoSmithKline (Brown AJ, 2001) and from AstraZeneca (Drmota T,

2004) and was further reinforced by a following publication from AstraZeneca relating to cannabinoids' affinity for the receptor (Ryberg *et al.*, 2007). Several reviews have since declared that even if there is little structural relation between GPR55 and the two other cannabinoid receptors (13.5% for CB1 and 14.4% for CB2 in human (Fredriksson *et al.*, 2003; Pertwee, 2010)), GPR55 interact with endocannabinoids (anandamide, 2-AG), phytocannabinoids (Δ9-THC, abnormal-cannabidiol (ABN-CBD)), and synthetic cannabinoid ligands (AM251 rimonabant, CP55,940). The nature and scope of these effects remain unclear, although all parties referring to the complex signalling signature of the receptor and its agonist functional selectivity, thus causing reserve to calling it a *bona fide* CB3 receptor. So far, the only consistent endogenous ligand for the receptor is l-α-lysophosphatidylinositol (LPI) which is not an agonist for CB1 nor CB2 (Oka *et al.*, 2007).

The proposed cannabinoid-mediated signalling pathway for GPR55 is consistent in its inconsistencies with its ligand affinity profile. The first study on cannabinoid signalling pathways in GPR55 used GTP$\gamma$S binding and a FLIPR assay (measuring the changes of fluorescence of a population of cells depicting a correlated change in free $[Ca]_I$) did not see significant changes in calcium but recorded GTP$\gamma$S increased binding to plasma membrane in a PTX insensitive manner. This increased binding was blocked by preincubation with a peptide and an antibody aimed at $G\alpha_{13}$. Consistent with this finding, in another study using HEK cells stably expressing GPR55, LPI caused concentration-dependant GPR55 internalization and stimulated a sustained and oscillatory response in $Ca^{2+}$ release, which is dependent on

$G\alpha_{13}$ coupling and Ras homolog gene family member A (RhoA) GTPase activity. The endogenous cannabinoids anandamide and 2-AG did not cause such responses, but the CB1 receptor antagonist AM251 did (Henstridge *et al.*, 2009). This is in contradiction with the single-cell recorded elevation of $Ca^{2+}$ from intracellular pools responses for AEA and Δ9-THC in HEK293 and mouse DRG cells which were Gq and $G\alpha_{12}$-mediated, PLC activated and PTX-insensitive (Lauckner *et al.*, 2008). The presence of an intact cytoskeleton and a functional RhoA GTPase was suggested as critical for those responses. ERK 1/2 elevation has only been observed for LPI so far (Balenga *et al.*, 2011).

## Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are transcription factors, activated by ligands, which control several physiological and pathological processes. PPARα stimulates oxidation of fatty acids in various organelles, such as mitochondria, peroxisomes and microsomes and stimulates uptake of fatty acids and synthesis of lipoproteins. On the contrary, PPARγ stimulates lipolysis of circulating triglycerides and the subsequent uptake of fatty acids into the adipose cell. It also stimulates binding and activation of fatty acids in the cytosol, which is required for synthesis of triglycerides (Kersten *et al.*, 2000). There are currently 4 isoforms for the receptor ($\alpha$, $\beta, \delta$ *and* $\gamma$). They are heterodimers with the *9-cis* retinoid *X* acid and bind to a specific peroxisome proliferator to regulate elements of transcription of target genes (Gasperi *et al.*, 2013). The ligands for PPARs are eicosanoids, fatty acids and fatty-acid derivatives such as endocannabinoids (Desvergne *et al.*, 1999). AEA and 2-AG can directly

bind and activate PPAR $\alpha$ and $\gamma$ in the micromolar range, resulting in transcriptional activity, preadipocyte activation, interleukin-2 suppression and modulation of glucose uptake (Bouaboula *et al.*, 2005; Gasperi *et al.*, 2007; Rockwell *et al.*, 2006).

## Transient Receptor Channels (TRP)

### General

Transient receptor potential (TRP) channels permits the flux of cations throughout a membrane, thus depolarizing the cell. This change in transmembrane voltage elicits action potential propagation in neurons and can cause muscle contraction (Ramsey *et al.*, 2006). By itself, calcium entry into the cell can be seen as a cellular signalling event and can trigger numeral cellular events such as transcriptional regulation, migration and proliferation (Berridge *et al.*, 2003). TRP channels also play an important role in nonexcitable cells both by directing the calcium entry through plasma membrane channels and controlling the gating of other voltage dependent channels ($Ca^{2+}$, $K^{+}$ and $Cl^{-}$). TRP channel anomalies or absence are linked with various human disorders such as polycystic kidney disease, skeletal dysplasia and familial episodic pain syndrome, to name a few, for review, see Nilius 2007 (Nilius, 2007).

TRP channels all resemble ancestral fly channels in their ability to be activated or modulate PLC-coupled receptors (Venkatachalam *et al.*, 2007) (for more information on the current nomenclature, see (Clapham *et al.*,

2005; Montell *et al.*, 2002). Vertebrate TRP channels are an extended family consisting of more than 30 distinct subtypes which are part of 7 distinct subfamilies: TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPN (no mechanoreceptor potential C), TRPP (polycystic) and TRPV (vanilloid) (Ramsey *et al.*, 2006). Mammals possess 28 members (so far) and have at least a member of all of these subfamilies except for TRPN (Clapham, 2003; Montell, 2005). For the sake of brevity, only mammalian-related TRP information will be discussed in this introduction from this point forward.

TRP proteins are composed of 6 transmembrane domains with a pore located between the fifth and sixth domains, although hydropathy analyses have predicted a seventh N-terminal hydrophobic domain for TRPC, TRPV, TRPM, TRPP and TRPML (Vannier *et al.*, 1998). The study of TRP channels is still in its infancy when compared to other ion channel superfamilies due in part to the current lack of knowledge regarding their structures and structure-related activities (Julius, 2013). It has been suggested that this is for three main reasons, being 1) a dearth of pharmacological agents for manipulating the functions of the channels, 2) no chrystallographic structures except for some intracellular domains as well as 3) low sequence similarity among TRP family members.

There are three mainly consistent aspects linked with this loose family of receptors possessing seemingly disparate functions (Ramsey *et al.*, 2006;

Venkatachalam *et al.*, 2007).The activation of PLC can modulate TRP channel activity via at least three distinct ways which are 1) Hydrolysis of PIP2. 2) production of DAG and 3) production of $IP_3$ and subsequent liberation of $Ca^{2+}$ from intracellular stores. This liberation of $Ca^{2+}$ is believed to then modulate the strength of the subsequent activation of the channel. This PLC pathway can be activated via GPCR tyrosine kinase activation.

1. Direct ligand activation of the channel. Small organic molecules, endogenous lipids, purine nucleotides and inorganic ions and reactive compounds are all possible ligands that can lead to the activation of a TRP channels.
2. Direct activation engaged by changes in temperature and other stimuli such as mechanical stimuli, conformational coupling to $IP_3$ receptors and channel phosphorylation.

## *TRP Channels and Sensing Noxious Stimuli*

Somatosensation encompasses several submodalities that include touch, proprioception, thermosensation, and nociception (Gardner E, 2000). Apart from chemical sensitivity, nociception could simply be considered as an extreme version of touch and temperature sensation. There is still much debate regarding if the distinct aspects between nociception and

somatosensation are merely psychophysiological in nature or if there are also underlying mechanistic distinctions between the two (Julius, 2013).

A noxious stimuli is initially detected by primary afferent sensory nerve fibers that innervate a peripheral target and transmit information to neurons within the dorsal horn of the spinal cord before reaching the brain via ascending neural circuits (Basbaum AI, 2000). There are two main hypotheseses regarding how these primary afferent neurons encode noxious information (Julius, 2013). The *pattern theory* predicts that pain is produced when a stimulus of sufficient intensity elicits a pattern of activity across functionally indistinct sensory nerve fibers, which is then deconstructed in the central nervous system to generate a specific percept representing the noxious stimuli (Melzack *et al.*, 1965). On the other hand, the *specificity theory* proposes that a specific stimulus of specific intensity and/or quality will activate a subset of sensory neurons tuned especially to detect it (Bessou P, 1969). This theory would thus mean that at least part of the information about the modality and the intensity of a stimulus is encoded before the signal reaches the central nervous system. This controversy about how noxious stimuli is encoded brings forward an important issue about if, and to what extent, specific subtypes of afferent nerve fibers might be effective targets for the treatment of pain.

The study of transient receptor channels has helped to elucidate this debate by validating the existence of nociceptors, thus the specificity theory, whilst

providing a framework in order to understand peripheral mechanisms underlying stimulus detection, injury-evoked sensitization and psychophysical coding (Basbaum *et al.*, 2009; Caterina *et al.*, 1999; Patapoutian *et al.*, 2009; Woolf *et al.*, 2007).

## Transient receptor potential vanilloid 1 (TRPV1)

TRPV1 is a member of the larger TRPV channel subfamily which has currently six members divided in two groups: TRPV1-TRPV4 and TRPV5-TRPV6. TRPV1-4 encompasses the so-called thermosensitive TRPV channels activated by heating or cooling stimuli (Moqrich *et al.*, 2005) but as TRPV1-4 channels can be found in regions of the body where drastic temperature changes are unlikely, it has been proposed that temperature might therefore play a permissive role, modifying the activity of these channels, rather than being the sole "purpose" for these channels (Lyall *et al.*, 2004).

TRPV1, also known as the capsaicin receptor, was the first discovered member of this family and the most thoroughly studied. It is a polymodal receptor that can be activated by capsaicin (Caterina *et al.*, 1997) and resiniferatoxin (Seabrook *et al.*, 2002), heat (Cesare *et al.*, 1999), $H^+$ (Tominaga *et al.*, 1998), endocannabinoid lipids such as AEA (Di Marzo *et al.*, 2001; Smart *et al.*, 2000; Zygmunt *et al.*, 1999) and NADA (Bisogno *et al.*, 2000), various eicosanoids (for review, see (Van Der Stelt *et al.*, 2004)) and 2-aminoethoxydiphenyl borate (2-APB), a compound used to inhibit store-

operated Ca2+ channels and IP3 receptors (Chung *et al.*, 2004), amongst others.

TRPV1 receptors are widely expressed but have best been characterized in sensory neurons (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). Knock-out TRPV1 mice exhibit an absence of vanilloid sensibility for nociception, inflammation and thermoregulation tests, showing the importance of TRPV1 in thermal hyperalgesia (Caterina *et al.*, 2000). Some of the inflammatory factors capable of modulating TRPV1 do not act directly on the orthosteric vanilloid site of the receptor and will rather modulate secondary messenger signalling pathways. Various intracellular domains have been described as important loci for the modulation of phosphorylation or the interaction of other proteins such as calmodulin, or with plasma membrane such as $PIP_2$ and other phosphoinositide sites. There are also several proalgesic agents that can sensitize TRPV1 receptors by activating the $G_q$/PLC signalling pathways. These modulations via the cytoplasmic termini loops are widely accepted as explaining the lowered intensity of thermal, agonist-driven or other stimuli, needed to activate TRPV1 in the presence of inflammation (Julius, 2013; McMahon S, 2006).

These nociceptive properties of TRPV1 on afferent neurons cannot explain the role of TRPV1 in the central nervous system, where it has been showed, though with some controversies, to be present (Kauer *et al.*, 2009; Steenland *et al.*, 2006). Using an elegant combination of pharmacology and genetics,

Gibson *et al.* (Gibson et al., 2008) described that capsaicin and 12-(S)-HPETE, an endogenous eicosanoid, can cause long term depression in hippocampal interneurons. Two years later, anandamide was also described to induce long-term depression via TRPV1 activation in the dentate gyrus (Chavez *et al.*, 2010). In 2002, Marinelli et *al.* (Marinelli *et al.*, 2002) showed that the activation of glutamatergic and adrenaline/noradrenaline neurotransmission in the rat locus correleus may be potentiated by TRPV1 activation by capsaicin, and in substantia nigra pars compacta slices, a region where both CB1 and TRPV1 receptors are present, NADA, an agonist for both proteins, has been showed to modulate glutamatergic transmission on DA neurons by direct activation of CB1 and TRPV1 (Marinelli *et al.*, 2007).

## Transient receptor potential ankyrin 1 (TRPA1)

The TRPA1 channel, firstly called ankyrin-like with transmembrane domains protein 1 (ANKTM1), is a 120-130 kDa channel with a similar structure as other TRP channels, containing six putative transmembrane segments with an ion permeable site between S5 and S6. One of the unique characteristics of the protein in term of structure is its 18 ankyrin repeat domains (hence its name) on its long N-terminal moiety (Wu *et al.*, 2010). These repeats are believed to involve protein-protein interactions as well as take part in

channel trafficking to the plasma membrane. Deletion of these repeats has been shown to negatively affect its insertion on the plasma membrane (Nilius *et al.*, 2011). Indeed, TRPA1 activation needs the presence of small intracellular compounds called polyphosphates ($PPP_i$), which binds to the ankyrin repeats, in order for receptor activation to occur (Cavanaugh *et al.*, 2008; Karashima *et al.*, 2008; Kim *et al.*, 2007). The receptor was first described as a cold-sensing channel for temperature lower than its counterpart, the menthol-activated cold-sensing TRPM8 channel (Story *et al.*, 2003). This is still a debated topic (Caspani *et al.*, 2009). Current evidence suggests that TRPA1 is not a major factor in acute cold sensation and that its role might be more of a regulator of injury-evoked cold hypersensitivity (del Camino *et al.*, 2010; Knowlton *et al.*, 2010; Moran *et al.*, 2011).

These days, the main focus of study for this receptor is centred around its agonist-mediated activity and its role as a detector for environmental irritants which can elicit pain and inflammation (Andrade *et al.*, 2012; Basbaum *et al.*, 2009; Bautista *et al.*, 2013). Although numerous cysteines are present throughout the receptor and could therefore be susceptible for electrophilic modifications, three specific cysteine ($Cys^{621}$, $Cys^{641}$ and $Cys^{665}$) residues found on its cytoplasmic N-terminus appear to be essential for the receptor's activation by electrophilic compounds (Hinman *et al.*, 2006). A lysine residue nearby the three main reactive cysteines ($Lys^{708}$) has also showed small but persistent responses in the presence of electrophiles (Hinman *et al.*, 2006). Agonist-mediated covalent modification of cysteine can

occur in several ways such as thiocarbamate forming, Michael adducts, disulfides form protein cysteine-disulfide products or alkylation (Cebi *et al.*, 2007; Nilius *et al.*, 2011). The chemical activators for the receptor include various plant-derived compounds favoured by different cultures for their culinary use such as isothiocyanates (pungent compounds in mustard oil, wasabi and horseradish) (Bandell *et al.*, 2004; Jordt *et al.*, 2004), cynnamaldehyde ((CA) from cinnamon) (Bandell *et al.*, 2004), alicin and diallyl disulphide (from garlic and shallot) (Bautista *et al.*, 2006). Other reactive compounds, which relate more to the threat-sensing theory for the receptor, are acrolein (an irritant used in tear gas) (Bautista *et al.*, 2006), formalin (a highly toxic volatile airpollutant) (McNamara *et al.*, 2007), and 2-pentenal (a metabolic byproduct of chemotherapeutic agents) (Inoue *et al.*, 2010), amongst others. The receptor is also activated by several endogenous products of oxidative or nitrative stress such as 4-hydroxynonenal and $H_2O_2$, hepoxilin (Gregus *et al.*, 2012), the cyclopentenone prostaglandin and the 15-deoxy-δ(12,14)-prostaglandin (Andersson *et al.*, 2008; Trevisani *et al.*, 2007).

However, this mechanism of covalent modification of cysteine cannot take into account the growing number of non-reactive compounds that activate the receptor. Δ9-THC (Jordt *et al.*, 2004), menthol and its analogues (Karashima *et al.*, 2007), nicotine (Talavera *et al.*, 2004), several general anaesthetics (Matta *et al.*, 2008), 5-Nitro-2-(3-phenylpropylamino)benzoic Acid (NPPB) (Liu *et al.*, 2010), all activate the receptor although a direct covalent-modification of cysteine is rather unlikely due to their non-

electrophilic nature. It is to be noted though that the presence of these residues appear to be of a certain importance for the activation of the channel by non-reactive compounds nonetheless, as, for example, a mutagenesis study by Liu (Liu *et al.*, 2010) showed that reactive and non-reactive compounds such as NPPB saw their peak [Cai] response reduced with single cysteine to serine mutations. Xiao *et al.* (Xiao *et al.*, 2008) also described that these residues are important for the activation of the channel mediated by menthol, another non-reactive compound. Endogenous compounds such as anandamide (De Petrocellis *et al.*, 2009) and its fatty acid tail, arachidonic acid (Bandell *et al.*, 2004), have both been described as ligands. Fatty acids of various length and unsaturation have recently been shown to activate the receptor although with lowered activity when compared to arachidonic acid; its second most efficient fatty acid activator being docosahexaeonic acid (DHA) (Motter *et al.*, 2012). In **Paper number 3,** we have investigated the properties of various non-reactive NAANs and related fatty acids on TRPA1 activation.

TRPA1 has also been described as being able to be activated by other receptors. For exemple, bradykinin and adenosine triphosphate (ATP) can both activate the receptor via the activation of Gq-coupled GPCRs through the PLC pathway (Bandell *et al.*, 2004; Jordt *et al.*, 2004). $Ca^{2+}$ released from the endoplasmic reticulum itself can have a dual effect on TRPA1 activation: an initial activation followed by a long-lasting inactivation (Jordt *et al.*, 2004; Nilius *et al.*, 2011; Wang *et al.*, 2008). The exact mechanism through which this activation occurs via signalling pathway modulation remains up for

debate. Either direct activation by calcium of a EF-hand-like motif in the N-terminal domain (Doerner *et al.*, 2007; Zurborg *et al.*, 2007) is in cause, or a calmodulin-independent still unknown mechanism might prevail (Nilius *et al.*, 2011; Wang *et al.*, 2008). Furthermore, TRPA1 might amplify the response of other channels such as TRPV1 when they are coexpressed (Bautista *et al.*, 2006; Bautista *et al.*, 2013).

TRPA1 is a high-conductance cation channel. It consequently has a complex relationship with both voltage and external $Ca^{2+}$. In physiological ionic and divalent-free conditions, a high open probability is seen at negative potentials, whilst positive potentials leads to a voltage-dependant inactivation (Karashima *et al.*, 2008; Karashima *et al.*, 2010; Nilius *et al.*, 2011). More than these single channel recordings, the biophysical properties of the pore of the channel have recently been studied. Pore dilation has been described for TRPA1 with reactive and unreactive compounds (Chen *et al.*, 2009). Using increasing diameter of various organic cations, the diameter of the pore has been estimated to be 11.0 Å (Karashima *et al.*, 2010). The size of this pore appears to be dynamically regulated and depends on the activating agonist and of permeating and non-permeating ions (Banke *et al.*, 2010). This dilation is also heavily controlled by the extracellular $Ca^{2+}$ concentration, as high concentration of extracellular $Ca^{2+}$ restricts pore dilation in its open state whilst its absence results in a longer-lasting dilated open state (Banke *et al.*, 2010). A restricted open state only allows the passage of $Na^{+}$ and $Ca^{2+}$ whereas the dilated state can let through molecules of much greater size, such as the fluorescent dyes Yo-Pro-1 and FM1–43 (Banke *et al.*, 2010; Chen *et al.*, 2009; Karashima *et al.*, 2010). This pore

dilation can be of pharmacological importance since, for example, amiloride, a potassium-sparing diuretic, and some its analogues, have been showed to be more effective inhibitors of TRPA1 in the dilated state compared to the restricted state (Banke, 2011). It is yet unknown if reactive and non-reactive compounds could induce a specific state of pore dilation (Nilius *et al.*, 2011), nor if the three main specific cysteines required for channel activation would have an influence on it. So far, a mutation in a conserved aspartate residue, $Asp^{918}$, has been showed to drastically affect $Ca^{2+}$ sensitivity for pore dilation (Wang *et al.*, 2008) as well as affect conductance (Nilius *et al.*, 2011).

TRPA1 was first described as a nociceptive channel expressed in sensory neurons of the dorsal root ganglia (DRG), trigeminal ganglia (TG) and nodose ganglia (NG) as well as in the organ of corti of the inner ear, hence being involved in nociception and hearing (Bautista *et al.*, 2005; Nagata *et al.*, 2005; Nilius *et al.*, 2012; Story *et al.*, 2003) but has since been described as a widely expressed receptor. It has been reported in the heart, small intestine, lung, skeletal muscle, pancreas and astrocytes, for review see (Nilius *et al.*, 2012; Stokes *et al.*, 2006).

TRPA1 appears to be implicated in pain and inflammation. It is present in the peripheral nervous system in TG, DRG and the spinal cord in unmyelinated C fibers and small myelinated A$\delta$ fibers (Nilius *et al.*, 2012). The channel has been reported as being overexpressed following prolonged inflammation and neuropathy (Obata *et al.*, 2005) and, accordingly, TRPA1 antagonists have been showed to prevent the development and reverse

established mechanical hyperalgesia after inflammation (da Costa *et al.*, 2010; Eid *et al.*, 2008; Petrus *et al.*, 2007). In the lung, activation of TRPA1-expressing nerve fibers promote neurogenic inflammation (Andre *et al.*, 2008; Bessac *et al.*, 2008). According to its oxidative-stress sensing role, its important localization on peptidergic neurons able to release substance P and calcitonin-related peptides (CGRP), and that most TRPA1-positive neurons appear to co-express TRPV1, the receptor is well-suited to contribute to various inflammatory pain syndromes either coming from environmental irritants or endogenous proalgesic agents (Bautista *et al.*, 2006; Bautista *et al.*, 2013; Julius, 2013; Kim *et al.*, 2013; Nilius *et al.*, 2012).

In the central nervous system, TRPA1 has been linked with activation of the cannabinoid CB1 receptor in hippocampal formation (Koch *et al.*, 2011) and is present in the brain stem where it is expressed on the afferent visceral pathway and regulate glutamate release (Sun *et al.*, 2009). Finally, TRPA1, in convergence with TRPV1, is involved in excitatory synaptic inputs to the magnocellular neurosecretory cells (Yokoyama *et al.*, 2011).

# Aims

The main aim of this thesis was to examine the modulatory effects of various NAANs on important cannabinoid effectors, mainly the CB1 and CB2 receptors, and TRP channels, with a specific interest toward pain modulation. We have used recombinant CB1, CB2, TRPV1 and TRPA1 receptors transfected into various cell lines in order to measure, using mainly a 96-well plates fluorescent read, the *flex station*, to measure in real time changes in $[Ca]_I$ and membrane potential.

The aims and hypotheses addressed in this thesis have evolved to some extent over the course of the research. Based on the evidence in the literature, it was intended that the main project for this thesis would focus on examining the effects of a potential NAAN-like drug metabolite, N-arachidonoyl amphetamine, and verify if that novel NAAN-like molecule would have a similar pharmacological profile as NADA. We proposed to investigate if such a compound could be produced endogenously, via a synthesis mechanism similar to that of AM404, following injection of amphetamine. However, the initial experiments we have conducted on the effects of NADA on CB1 receptor activation did not show what was expected of a usual ligand for the receptor, as NADA did not produce the familiar activation patterns seen with other CB1 orthosteric ligands. These intriguing early findings illuminated a possible bias mechanism of action for NADA at the CB1 receptor, which we have decided to pursue.

Similarly, some compounds which were at first intended to be used as controls for the human TRPA1 receptors, such as arachidonic acid and NDGA, turned out to be more efficient at activating the channel than the NAANs we first intended on studying. Because of the current interest for NDGA as a cancer-treatment therapy, and the lack of studies on PUFAs activation of TRPA1 channel, we have decided to investigate them more systematically.

Having come to these realisations, the research in this thesis addresses the following objectives:

*Aim One: Use a quantitative real-time method for studying the effects of CB1 receptor ligands on GIRK-mediated hyperpolarization and desensitization of the cannabinoid receptor CB1 in stably transfected cell lines.* Although NADA has been described as a ligand for the CB1 receptor, no information on the results of NADA's activation on CB1-mediated signalling pathway has so far been described. The first aim of this thesis was to investigate the effects of NADA on the well-established hyperpolarization of cells via the $G_{i/o}$-mediated activation of GIRK channels in AtT20 cells. Similarly, although allosteric modulators of CB1 have been showed not to affect peak-responses of CB1 activation via orthosteric ligands, no information had so far been given regarding the following desensitization of the receptor.

*Aim two: Investigate the $G_q$-mediated activation of the CB1 receptor as well as investigate $G_q$-coupling on the CB2 receptor, using stably transfected cell lines.* As NADA did not show any hyperpolarization via GIRK activation for CB1 receptors, nor any activation nor inhibition of the production of cAMP by AC, thus ruling out $G_{i/o}$ and $G_s$ activity, we have investigated the possible $G_q$-mediated coupling induced by NADA binding to CB1. As $G_q$ coupling leads to increase in $[Ca]_I$, we have used various ways of inhibiting this pathway, from the use of inverse agonist for the receptor to blocking the coupling of the G-protein to the receptor itself. We have also compared G protein involvement in CB1 compared with CB2 receptors for NADA.

*Aim Three: Investigate the increase in $[Ca]_I$ that follows the activation of the human TRPA1 channel in inducible transfected cell lines and assess the implication of reactive cysteines in the activation of the channel by non-electrophilic compounds.* As NAANs are known as ligands able to either activate or inhibit various channels and receptors, we have investigated the effect of several NAANs on the TRPA1 channel as well as the possible effects of PUFAs, from which they are formed. One of the main effects resulting from the activation of these receptors is an increase in $[Ca]_I$, which we have investigated. Three reactive cysteines have been described as being required for the activation of the receptor by electrophilic compounds. We have also investigated the effect of these cysteines on the activation of the receptor by non-reactive ligands for the receptor.

*Aim Four: Investigate the effects on pain modulation of a novel ligand for the TRPA1 receptor.* In order to evaluate if our findings *in vitro* could be translated into *in vivo* physiological responses, we have investigated if the novel binding of NDGA we have discovered could lead to pain modulation in an acetone pain and noxious cold tests when injected in the rat hindpaw.

Aim one is addresed in **paper one**. Aims one and two in **paper two**. Aim three is addressed in **paper three** and aims three and four in **paper four**.

**Chapter 2** **Paper 1**

# Real-time characterization of cannabinoid receptor 1 ($CB_1$) allosteric modulators reveals novel mechanism of action

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**Author Contributions**

MG and EC conceived the study. WJR performed the membrane potential measurements, EC, CB and NG did the BRET and internalization of CB1 receptors assays. EC and MG wrote the paper. MC supervised WJR in his work and helped devise the experimental setting for membrane potential measurements.

**Chapter 3** **Paper 2**

# Identification of *N*-Arachidonoyl Dopamine as a highly biased Gq-preferring ligand at human CB1 and CB2 receptors

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# Abstract

Responses to cannabinoids are primarily mediated by CB1 and CB2 receptors, which couple preferentially to $G_{i/o}$ G-proteins. Restricted coupling to $G_s$ and $G_q$-type G-proteins has been reported for CB1 receptors, although no agonist acts exclusively at one G-protein subtype. Here, we have demonstrated that the CB receptor signalling of *N*-arachidonoyl dopamine (NADA), an endogenous CB1 agonist, has a completely biased signalling pathway signature toward $G_q$ G-protein with no effect via $G_{i/o}$. This $G_q$ activation does not prohibit $G_{i/o}$-coupling by other ligands for the receptor. The presence of NADA prevents the normal internalization mechanisms of the receptor that follows the receptor's activation by other agonist, as well as receptor overexpression when constitutive activity is blocked by an inverse agonist. NADA also appear to activate the CB2 receptor with a similar bias toward $G_q$. Such a signalling profile for CB2 has not yet been presented. Taken together, our results demonstrate a novel completely biased signature profile for NADA on both CB1 and CB2 receptors via $G_q$ G-protein coupling.

# Introduction

Cannabinoid drugs have a wide variety of effects on neuronal and immune system activity whilst endogenous cannabinoid neurotransmitters have a central role in moment to moment control of synaptic transmission in many brain regions via retrograde pre-synaptic mechanisms of action. Responses to cannabinoids are primarily mediated by CB1 and CB2 receptors (Matsuda *et al.*, 1990; Munro *et al.*, 1993). These receptors are G-protein coupled receptors which preferentially couple to $G_{i/o}$ – type G-proteins, leading to an inhibition of adenylyl cyclase (AC) and a modulation of a variety of potassium and voltage–dependent calcium channels (for review see (Howlett, 2005)). Coupling of CB1 receptors to $G_s$ and $G_q$-type G-proteins has also been reported in some circumstances, with resulting stimulation of AC activity (Glass *et al.*, 1997) and mobilization of intracellular calcium ($[Ca]_i$) (Lauckner *et al.*, 2005) respectively. Δ9-THC is the main psychoactive cannabinoid from *Cannabis sativa* (Mechoulam *et al.*, 1965) and a variety of synthetic derivatives of Δ9-THC, as well as structurally unrelated synthetic cannabinoids (e.g. aminoalkylindoles, WIN55212-2 and CP55,940) have been developed (for a complete review, see Pertwee et *al.*, 2010 (Pertwee, 2010)). Cannabinoid receptors, like most GPCRs, are highly dynamic proteins capable of adopting a large number of conformational states. Different suites of conformational state can lead to the coupling of variable sets of downstream effectors. Some evidence suggest that these diverse effects by ligands on conformational changes may be linked with different subsets of residues that form the binding pocket of CB1 (Shim, 2010), leading to

various signature of resulting signalling pathway activations. The CB1 receptor also possesses at least two allosteric sites (Shore *et al.*, 2013; Vallee *et al.*, 2014), capable of modulating specific signalling pathways as well as affecting the time-window of activation for the receptor (Ahn *et al.*, 2012; Cawston *et al.*, 2013; Fay *et al.*, 2013; Price *et al.*, 2005). Despite the diverse chemical entities that can activate CB1 with varying degrees of action on receptor-mediated coupling to various G-proteins, there is no evidence for any ligands causing truly biased activation of any secondary intracellular signalling pathway.

The CB2 receptor only possess 44% homology with CB1 (Munro *et al.*, 1993) and is mostly expressed in blood-borne immune cells and related tissues (Galiegue *et al.*, 1995). Yet, despite their differences in morphology and distribution, a restricted number of ligands act at both receptors, although when they do, the rank order of potency tend to be distinct (Atwood *et al.*, 2012). As well, CB1 and CB2 have been described as favouring slightly different signalling pathways once activated. Although both CB1 and CB2 will mostly mediate their activity via $G_{i/o}$ coupling, CB2-mediated modulation of ion channels is still controversial, with the exception of G-protein-coupled inwardly-rectifying potassium *channels* (GIRKs) activation; and no clear proof has been given for a direct $G_q$ nor $G_s$ coupling (Atwood *et al.*, 2012). Still, some CB2 ligands, like the endogenous endocannabinoid 2-AG, as well as CP55,940, have been shown to activate phospholipase C, leading to the release of calcium in the intracellular medium in CHO hCB2 cells (Shoemaker *et al.*, 2005).

Endocannabinoids are derived from plasma membrane lipids and are characterized by a long carbon chain tail (acyl moiety) and simple head group (Di Marzo *et al.*, 1994). From the two first reported and studied endocannabinoids, n-arachidonoyl ethanolamine (anandamide) and 2-arachidonoyl glycerol (2-AG), a wider group of related lipids have been described as ligands for the receptor. 2-Arachidonyl glyceryl ether (Noladin ether) (Hanus *et al.*, 2001), *O*-arachidonoyl ethanolamine (virhodamine) (Porter *et al.*, 2002) and other compounds with ethanolamide-related head groups or differing fatty acid tails (oleoyl, stearoyl, linoleoyl) have been described through the years, but it appears that fatty acids with more complex head-groups such as dopamine can also serve as ligands for CB1, such as N-arachidonoyl dopamine (NADA) and N-oleoyl dopamine (OLDA) (Bisogno *et al.*, 2000; Huang *et al.*, 2002).

*N*-arachidonoyl dopamine is an endogenous compound that has been found in mammalian brain tissue (Bezuglov *et al.*, 2001; Bisogno *et al.*, 2000; Huang *et al.*, 2002). It has a high affinity for the polymodal TRPV1 channel receptor and a lower affinity for CB1 receptor, although no study has so far depicted a clear picture of this interaction. Despite the fact that a synthesis and degradation pathway have been proposed (Hu *et al.*, 2009; Huang *et al.*, 2002), NADA's physiological roles are still elusive. In the brain, NADA has been showed to tune synaptic transmission on dopaminergic neurons (Marinelli *et al.*, 2007) as well as increase 2-AG production in the midbrain (Freestone *et al.*, 2013). Anandamide and NADA have also both been showed to modulate transmitter release and presynaptic $Ca^{2+}$ levels in the hippocampus. In vivo, NADA causes anandamide-like activity when injected

systematically, acting like cannabinoids on the four branches of the tetrad test for cannabinoids (Bezuglov *et al.*, 2001; Bisogno *et al.*, 2000). Systemic administration also reduces locomotion in ferrets via both TRPV1 and CB1, although its modulation of locomotion appears to involve a non-CB1 and non-TRPV1 mechanism as well (Sharkey *et al.*, 2007). Despite these abundant findings, the signalling pathway signature of NADA for cannabinoid receptors is still absent. So far, NADA has been showed to be more potent than AEA in eliciting an increase in $[Ca]_I$ in non-differentiated N18TG2 neuroblastoma cells via a PTX-sensitive CB1-mediated mechanism (Bisogno *et al.*, 2000). Here, we explored the CB receptor signalling of *N*-arachidonoyl dopamine.

# Results

**NADA binds to CB1 and CB2 receptors.** Binding of NADA to CB1 or CB2 receptors was confirmed by competitive radioligand displacement assays utilising cell membranes generated from HEK-HA-hCB1 or HEK-HA-hCB2. At the CB1 receptor NADA competitively displaced both [$^3$H]-CP55,940 and [$^3$H]-SR141716A with a calculated Ki of 780±240 nM and 230±36 nM (n=4) respectively, similar to what has been previously reported (Bisogno *et al.*, 2000; Huang *et al.*, 2002). At the CB2 receptor NADA competitively displaced [$^3$H]-CP55,940, although with lower affinity than at CB1 with a Ki of 3.4±0.4 μM (n=3).

**NADA does not modify the $G_{i/o}$ signalling at CB1 and CB2 receptors.** NADA (30-100 μM) did not hyperpolarize AtT20-rCB1 cells (Figure 1), nor did it modify the peak hyperpolarization produced by the CB1 agonist CP55,940 (100 pMol – 300 nM). In CHO hCB2 cells, NADA did not cause a GIRK-mediated hyperpolarization by itself nor did it modulate the peak GIRK-mediated change in membrane potential of CP55,940, although it increased the $pEC_{50}$ for CP55,940 on AtT20 hCB2 cells from 8.168±0.162 to 7.336±0.622 for 30 μM NADA (Figure 1).

In HEK 3HA-hCB1 NADA did not modify forskolin-stimulated cAMP production, at concentrations up to 10 μM. At 31.6 μM, NADA did significantly increase cAMP levels above that of forskolin alone ($P$ = 0.002) (Figure 2A). Additionally, both 10 μM and 30 μM NADA significantly

attenuated CP55,940 ($pEC_{90}$ = 10 nM) inhibition of forskolin-stimulated cAMP activity ($P$ = 0.014 and $P$ = 0.009 respectively) (Figure 2B). Incubation with the orthosteric inverse agonist SR141716A 40 nM in the presence of forskolin produces an increase in cAMP production through blockade of constitutive activity via CB1 receptor (Bouaboula *et al.*, 1997; Landsman *et al.*, 1997). The presence of NADA did not change the blockade of constitutive activity for any concentration from 1-31.6 µM ($P$ = 0.111 – 0.743) (Figure 2C). Additionally, as seen with HEK 3HA-hCB1 cells, HEK 3HA-hCB2 cells in the presence of 31.6 µM NADA also showed an increase in forskolin-stimulated cAMP production above the levels cAMP levels of forskolin alone ($P$ = 0.002) (Figure 2D).

**NADA activates $G_q$-mediated signalling pathways in both CB1 and CB2 receptors.** NADA elevated $[Ca]_I$ in CHO-hCB1 cells (nominal $pEC_{50}$ 3.751±2.553, maximum increase in fluorescence at 100 µM 138±21%). The elevations of $[Ca]_I$ by NADA were completely reversed with the pre-incubation for 5 minutes of SR 141716A (1 µM). The increase in $[Ca]_I$ elicited by NADA was independent of $G_{i/o}$ as it was unaffected in pertussis toxin-treated hCB1 cells. N-Oleoyl dopamine, also elevated calcium at 100 µM (76±16%) and 30 µM (32±2%) (Figure 3). NADA caused a greater increase in peak $[Ca]_I$ than CP55,940 and WIN55,212-2, which modestly increased peak $[Ca]_I$ (37±12% and 40±8%).

Pretreating the CHO hCB1 cells with the phospholipase C pathway inhibitor U73122 3 µM inhibited the peak $[Ca]_I$ response for NADA 100 µM. Incubation

with thapsigargin 10 μM, a sarco/endoplasmic reticulum $Ca^{2+}$ pump inhibitor which depletes the intracellular calcium pools, completely prevented any elevation of $[Ca]_I$ with 100 μM NADA, confirming that the $[Ca]_I$ seen after the administration of NADA comes from intracellular pools (Figure 3).

There are currently few options available to directly prevent the coupling of $G_q$ G-proteins to GPCRs. We utilized a palmitoylated peptide (palmitoyl-QLNLKEYNLV) (Strathmann *et al.*, 1990) corresponding to the last 10 amino acids of $G_q$, which has been demonstrated to inhibit GPCR activation of $G_q$, presumably by competing for binding of the receptor to the G-protein (Robbins *et al.*, 2006). The addition of this palpeptide at 10 μM, an hour prior to the administration of NADA 30 μM, caused an inhibition of $[Ca]_I$, from 57±7% to 13±10%. Pre-incubation with a scrambled version of the palpeptide (palmitoyl-NLVLNEKLYQ) did not significantly reduce peak increase in $[Ca]_I$ when compared to NADA 30 μM alone (43±7%), consistent with a $G_q$-mediated pathway (Figure 3).

Surprisingly, as NADA has not so far been reported to activate CB2, NADA produced similar responses in CHO hCB2 cells to those in CHO hCB1 cells for every test relating to $G_q$-mediated calcium mobilization. NADA caused an elevation in $[Ca]_I$ in CHO hCB2 over a similar concentration range as CHO hCB1CB1 to a maximum of 107±8% and a pEC50 of 4.233±1.303. AM630 (300nM) inhibited this increase, reducing NADA-mediated peak response to 30±21% increase in $[Ca]_I$. Overnight incubation with PTX did not reduce the peak increase in $[Ca]_I$ responses of NADA 100 μM. The administration of

U73122 3 μM also caused a diminution of peak $[Ca]_I$ following the administration of NADA 100 μM, from 92±7% to -10±9%. As for CHO hCB1, this increase in calcium came from intracellular pools as depleting intracellular calcium pool using thapsigargin completely prevented any $[Ca]_I$. An hour long incubation with 10 μM of the palpeptide significantly reduced $[Ca]_I$ from NADA 30 μM, from 51±8% to 28±6% but was not significantly different in the presence of a scrambled version of the palpeptide (53±5% peak increase in $[Ca]_I$). OLDA also caused an increase in peak $[Ca]_I$ in CHO hCB2 cells at 100 μM (148±10%) and 30 μM (87±9%). CP55,940 and WIN55,212-2 were both very poor at increasing $[Ca]_I$ in CHO hCB2 cells (17±6% and 6±3%).

**ORG27569 inhibits $G_q$-mediated $[Ca]_I$ in CHO hCB1.** ORG27569 (5-chloro-3-ethyl-N-[2-[4-(1-piperidinyl)phenyl]ethyl]-1H-indole-2-carboxamide), an allosteric modulator that enhances the affinity of CP55,940 for hCB1, prevented peak increase in $[Ca]_I$ for NADA 100 μM from 106±11% to 37±12% in CHO hCB1 when added 5 minutes before NADA, indicating that the presence of this allosteric modulator prevents the coupling of $G_q$ to the receptor. To confirm if this effect is receptor mediated, we have looked at the effects of ORG27569 on the activation of Par 1 agonist 300 nM on the Protease-activated receptor (PAR), a $G_q$-coupled GPCR present in CHO cells. ORG27569 did not prevent PAR-mediated increase in $[Ca]_I$.

**NADA activation of CB1 and CB2 does not cause an increase in pERK.**

In HEK 3HA-hCB1 cells administration of the EC90 for CP55,940 of 15 nM (data no shown) increased ERK 1/2 phosphorylation in the AlphaScreen®

SureFire® assay from 2 minutes until 10 minutes with a peak at 5 minutes (Figure 6A). NADA 30 µM did not cause an increase in ERK 1/2 concentration at the peak time point of 5 minutes post administration as compared to the vehicle response ($P$= 0.429) (Figure 6A). Similarly in HEK 3HA-hCB2 cells, 30 µM NADA did not cause a significant increase in ERK 1/2 phosphorylation at 5 minutes as compared to the vehicle ($P$ = 0.995) (Figure 6B).

**NADA prevents hCB1 internalization in HEK cells.** In internalization assay, CP55,940 caused a concentration dependent rapid internalization of CB1 receptors, with only 37±7% receptors remaining on the cell surface after 30 minute incubation of an $EC_{50}$ concentration of CP55,940, as previously reported (De Petrocellis *et al.*, 2004; Grimsey *et al.*, 2008). NADA, by itself, did not cause CB1 internalization. However, NADA prevented concentration-dependently CP55,940-induced internalization of the receptor, with up to a 90±9% of cell surface receptor remaining after a 30 minutes coincubation with 100 µM NADA. Similarly, 6 hours incubation of an $EC_{90}$ concentration of SR1414716A in HEK hCB1 cells caused a 71±8% increase in cell surface receptor, due to inverse agonism preventing constitutive activity and endocytosis of the receptor (Bouaboula *et al.*, 1997; Grimsey *et al.*, 2010; Landsman *et al.*, 1997; Pertwee, 2003). When applied concurrently, NADA inhibited SR1414716A-induced hCB1 receptor up-regulation in a concentration-dependant manner (figure 7).

**NADA administered to CHO hCB1 cells time-dependently increases 2-AG concentration.** A 100 µM administration of NADA to CHO hCB1 cells

causes a time-dependant increase in the concentration of 2-AG. This increase was significantly higher at 5 (2-fold increase) and 10 minutes (3-fold increase), ($P$ = 0.0474 and P=0.0075 respectively) before returning toward baseline at 15 minutes, as seen using LC/MS/MS analysis (figure 8). This effect was not present following the administration of NADA 100 µM in untransfected cells (data not showed).

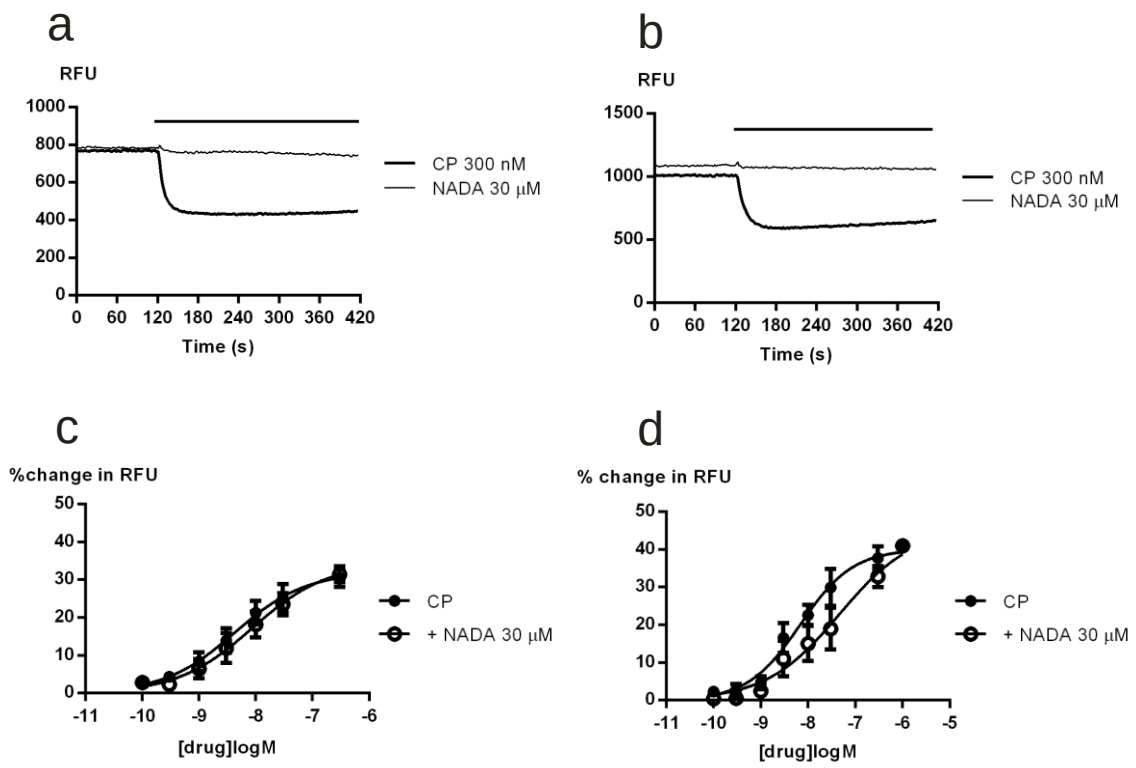
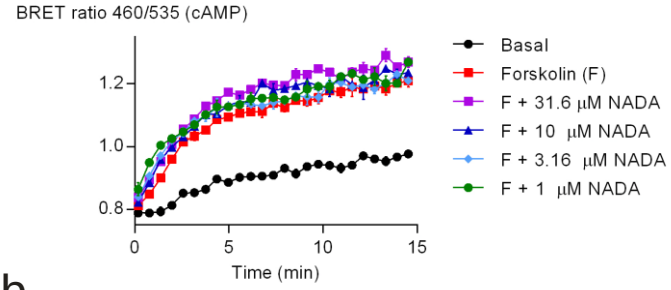


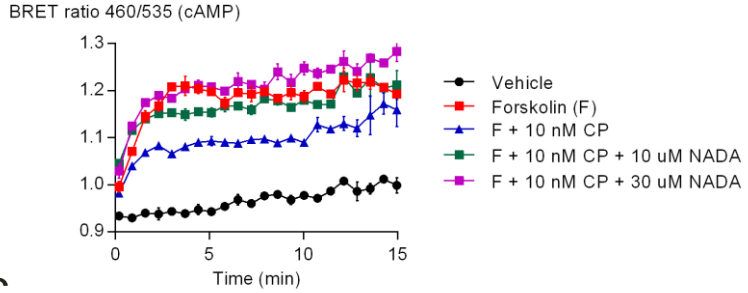

**Figure 1 | NADA does not induce $G_{i/o}$-mediated hyperpolarization via GIRK channels.** **(a)** Raw traces for real-time membrane potential assay in AtT20 cells stably transfected with rCB1 and hCB2 **(b)** Traces are representative of 7 and 6 experiments performed in duplicate. **(c)** Concentration-response curves representing the peak-response in GIRK mediated hyperpolarization caused by CP in the absence or presence of a 5 minutes pre-incubation with NADA 30 μM in AtT20 rCB1 and hCB2 **(d)** cells. Data points represent mean±s.e.m. of 6 and 7 replicates respectively.

**Figure 2 | Cyclic AMP production is not affected by the presence of NADA in hCB1 nor hCB2.** (a) An individual representative real-time cAMP BRET assay for HEK 3HA-hCB1 with 10 μM forskolin (F) in the presence of 31.6 μM -1 μM NADA. Emission data for RLuc and YFP were collected over time and values plotted as raw ratio (± SEM) of emissions 460/535 over time (min). (b) An individual representative real-time cAMP BRET assay for HEK 3HA-hCB1 with 10 μM forskolin (F) in the presence of 10 nM CP55,940 in the presence or absence of either 10 or 30 μM NADA. Emission data for RLuc and YFP were collected over time and values plotted as raw ratio (± SEM) of emissions 460/535 over time (min). (c) An individual representative real-time cAMP BRET assay for HEK 3HA-hCB1 with 10 μM forskolin (F) and 40 nM SR141716A in the presence of 31.6 μM -1 μM NADA. Emission data for RLuc and YFP were collected over time and values plotted as raw ratio (± SEM) of emissions 460/535 over time (min). (d) An individual representative real-time cAMP BRET assay for HEK 3HA-hCB2 with 10 μM forskolin (F) in the presence of 30 μM NADA. Emission data for RLuc and YFP were collected over time and values plotted as raw ratio (± SEM) of emissions 460/535 over time (min).

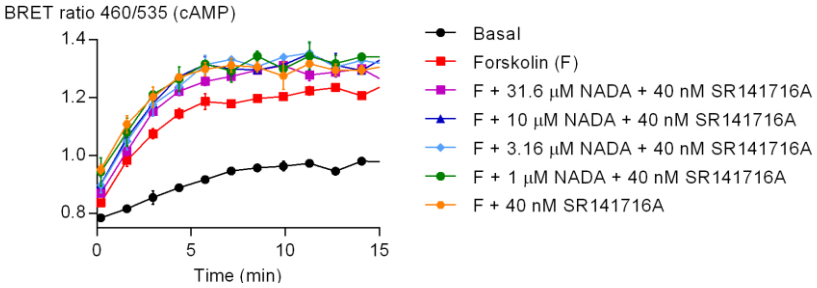
a


BRET ratio 460/535 (cAMP)
1.2
1.0
0.8
0
5
10
15
Time (min)
Basal
Forskolin (F)
F + 31.6 μM NADA
F + 10 μM NADA
F + 3.16 μM NADA
F + 1 μM NADA

b


BRET ratio 460/535 (cAMP)
1.3
1.2
1.1
1.0
0.9
0
5
10
15
Time (min)
Vehicle
Forskolin (F)
F + 10 nM CP
F + 10 nM CP + 10 uM NADA
F + 10 nM CP + 30 uM NADA

c


BRET ratio 460/535 (cAMP)
1.4
1.2
1.0
0.8
0
5
10
15
Time (min)
Basal
Forskolin (F)
F + 31.6 μM NADA + 40 nM SR141716A
F + 10 μM NADA + 40 nM SR141716A
F + 3.16 μM NADA + 40 nM SR141716A
F + 1 μM NADA + 40 nM SR141716A
F + 40 nM SR141716A

d

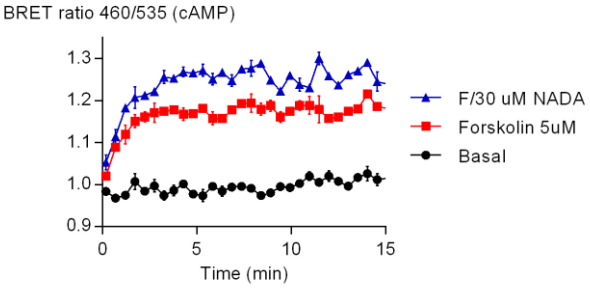
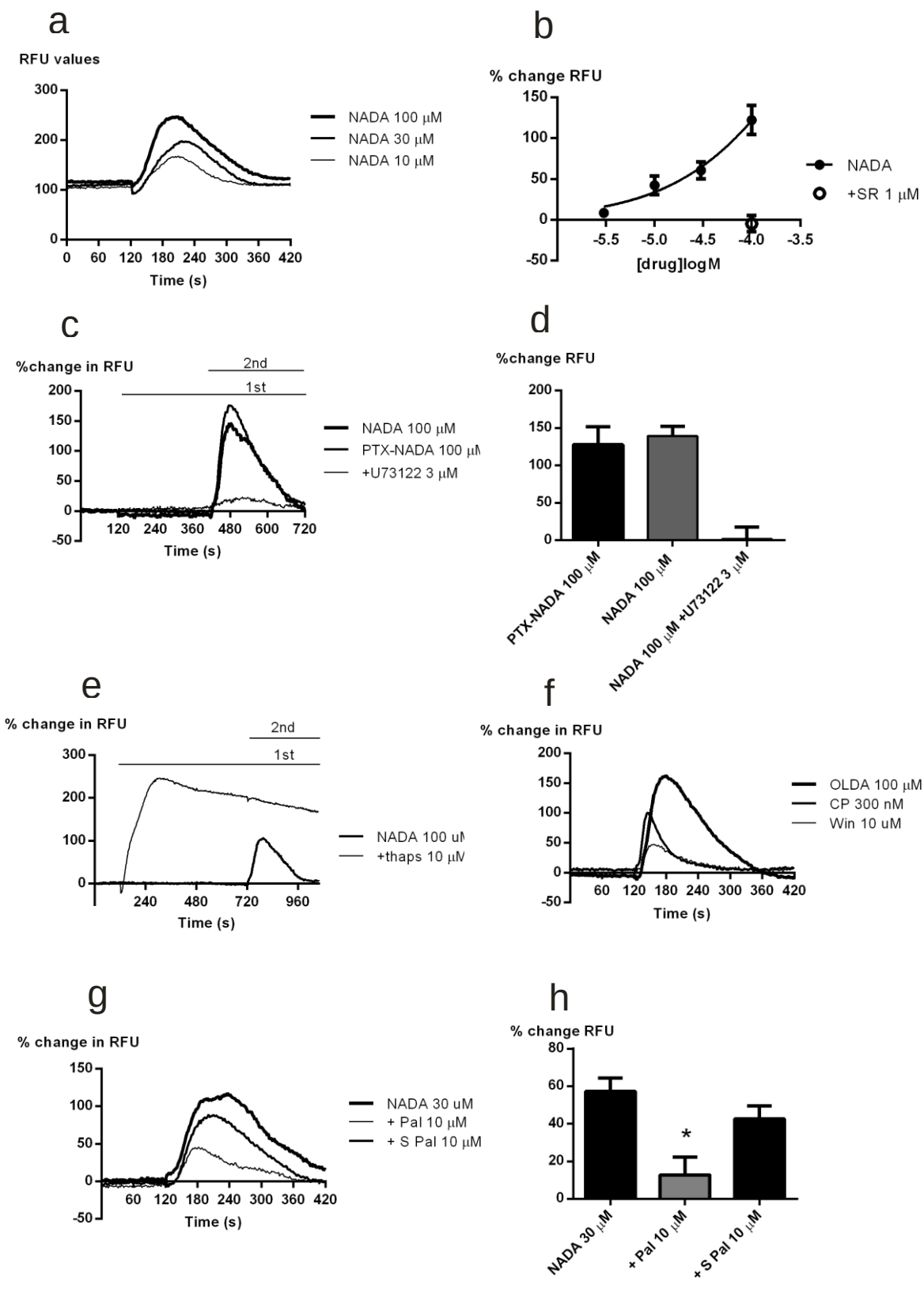

BRET ratio 460/535 (cAMP)
1.3
1.2
1.1
1.0
0.9
0
5
10
15
Time (min)
F/30 uM NADA
Forskolin 5uM
Basal

**Figure 3 | NADA activates the hCB1 receptors which leads to $G_q$-mediated increase in cytosolic $[Ca]_I$ in CHO cells. (a)** Raw traces calcium assay in CHO cells stably transfected with hCB1 receptors, traces are typical of 10 experiments performed in duplicate. **(b)** Concentration-response curves for the peak increase in $[Ca]_I$ in CHO hCB1 cells caused by NADA alone or after 5 minutes pre-incubation with SR (1 μM). Data points represent mean±s.e.m. of 5 experiments made in duplicate. $P<0.05$ for 10 to 100 μM. **(c)** Baseline-corrected traces for Changes in $[Ca]_I$ caused by NADA in the absence or presence of the PLC-pathway inhibitor U73122 3 μM in CHO hCB1, expressed as a % change in fluorescence. **(d)** Normalised peak increase in $[Ca]_I$ to NADA 100 μM for PTX (200 ng/ml) treated and non-treated CHO hCB1 cells as well as in the absence or presence of pre-incubated U73122 3 μM. Data points represent mean±s.e.m. of 5 replicates. *$P<0.05$. **(e)** Baseline-corrected traces representing changes in $[Ca]_I$ for NADA 100 μM in the presence or absence of a 5-minute pre-incubation of the sarco / endoplasmic reticulum Ca2+ ATPase inhibitor thapsigargin 10 μM, which depletes intracellular pools of calcium, in CHO hCB1 cellstraces are typical of 5 experiments performed in duplicate. **(f)** Baseline-corrected traces representing changes in $[Ca]_I$ to OLDA 100 μM, CP 300 nM and WIN 10 μM in CHO hCB1 cells, traces are typical of 5 experiments performed in duplicate. **(g)** Baseline-corrected traces representing changes in $[Ca]_I$ for NADA 30 μM in CHO hCB1 cells in the absence or presence of 60 minutes pre-incubation of a $G_q$-binding palpeptide 10 μM, which prevents $G_q$ coupling to GPCR, or in the presence of a scrambled version of the palpeptide 10 μM, traces are typical of 5 experiments performed in duplicate. **(h)** Normalised peak $[Ca]_I$ caused by NADA in CHO hCB1 cells in the absence or presence of the palpeptide or its scrambled counterpart following 60 minutes incubation. Data points represent mean±s.e.m. of 5 replicates. *$P<0.05$.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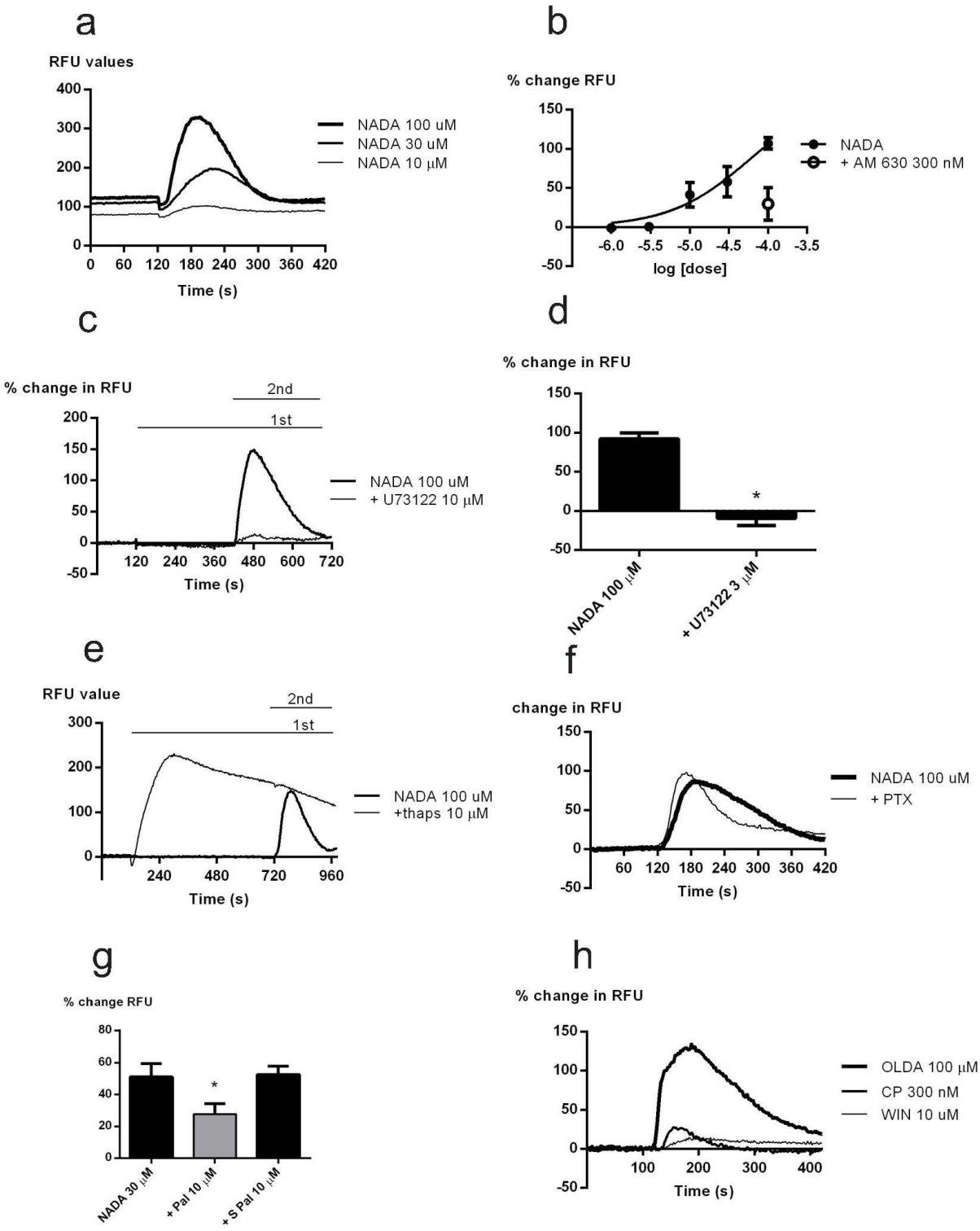
200
100
0
0 60 120 180 240 300 360 420
Time (s)
NADA 100 μM
NADA 30 μM
NADA 10 μM
b
% change RFU
150
100
50
0
-50
-5.5 -5.0 -4.5 -4.0 -3.5
[drug]logM
NADA
+SR 1 μM
c
%change in RFU
2nd
1st
200
150
100
50
0
-50
120 240 360 480 600 720
Time (s)
NADA 100 μM
PTX-NADA 100 μM
+U73122 3 μM
d
%change RFU
200
150
100
50
0
PTX-NADA 100 μM
NADA 100 μM
NADA 100 μM +U73122 3 μM
e
% change in RFU
2nd
1st
300
200
100
0
240 480 720 960
Time (s)
NADA 100 uM
+thaps 10 μM
f
% change in RFU
200
150
100
50
0
-50
60 120 180 240 300 360 420
Time (s)
OLDA 100 μM
CP 300 nM
Win 10 uM
g
% change in RFU
150
100
50
0
-50
60 120 180 240 300 360 420
Time (s)
NADA 30 uM
+ Pal 10 μM
+ S Pal 10 μM
h
% change RFU
80
60
40
20
0
*
NADA 30 μM
+ Pal 10 μM
+ S Pal 10 μM

**Figure 4 | NADA activates the hCB2 receptors which leads to $G_q$-mediated increase in cytosolic $[Ca]_I$ in CHO cells. (a)** Raw traces of calcium assay in CHO cells stably transfected with hCB2 receptors, traces are typical of 5 experiments performed in duplicate. **(b)** Normalised concentration-response curves for the peak increase in $[Ca]_I$ caused by NADA in CHO hCB2 cells in the absence or presence of 5 minutes pre-incubation of AM 630 300 nM. Data points represent mean±s.e.m. of 5 replicates. *$P$<0.05 for 10 to 100 µM. **(c)** Baseline-corrected traces for changes in $[Ca]_I$ caused by NADA in the absence or presence of the PLC-pathway inhibitor U73122 3 µM in CHO hCB2 cells, traces are typical of 7 experiments performed in duplicate. **(d)** Normalised peak increase in $[Ca]_I$ for NADA 100 µM in CHO hCB2 cells in the absence or presence of pre-incubated U73122 3 µM. Data points represent mean±s.e.m. of 7 replicates. *$P$<0.05. **(e)** Baseline-corrected traces representing $[Ca]_I$ for NADA 100 µM in the presence or absence of a 5-minute pre-incubation of the sarco / endoplasmic reticulum Ca2+ ATPase inhibitor thapsigargin 10 µM, which depletes intracellular pools of calcium, in CHO hCB2 cells traces are typical of 5 experiments performed in duplicate. **(f)** Baseline-corrected traces representing changes in $[Ca]_I$ for NADA 100 µM in PTX treated (200ng/ml) and untreated CHO hCB2 cells traces are typical of xx experiments performed in duplicate. **(g)** Normalised peak increase in $[Ca]_I$ caused by NADA in CHO hCB2 cells in the absence or presence of a $G_q$-blocking palpeptide or its scrambled counterpart following 60 minutes incubation. Data points represent mean±s.e.m. of 5 replicates. *$P$<0.05. **(h)** Baseline-corrected traces representing $[Ca]_I$ for OLDA 100 µM, CP 300 nM and WIN 10 µM in CHO hCB2 cells traces are typical of 5 experiments performed in duplicate.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RFU values
400
300
200
100
0
0 60 120 180 240 300 360 420
Time (s)
NADA 100 uM
NADA 30 uM
NADA 10 μM
b
% change RFU
150
100
50
0
-50
-6.0 -5.5 -5.0 -4.5 -4.0 -3.5
log [dose]
NADA
+ AM 630 300 nM
c
% change in RFU
2nd
1st
200
150
100
50
0
-50
120 240 360 480 600 720
Time (s)
NADA 100 uM
+ U73122 10 μM
d
% change in RFU
150
100
50
0
-50
*
NADA 100 μM
+ U73122 3 μM
e
RFU value
2nd
1st
300
200
100
0
240 480 720 960
Time (s)
NADA 100 uM
+thaps 10 μM
f
change in RFU
150
100
50
0
-50
60 120 180 240 300 360 420
Time (s)
NADA 100 uM
+ PTX
g
% change RFU
80
60
40
20
0
*
NADA 30 μM
+ Pal 10 μM
+ S Pal 10 μM
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% change in RFU
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Time (s)
OLDA 100 μM
CP 300 nM
WIN 10 uM

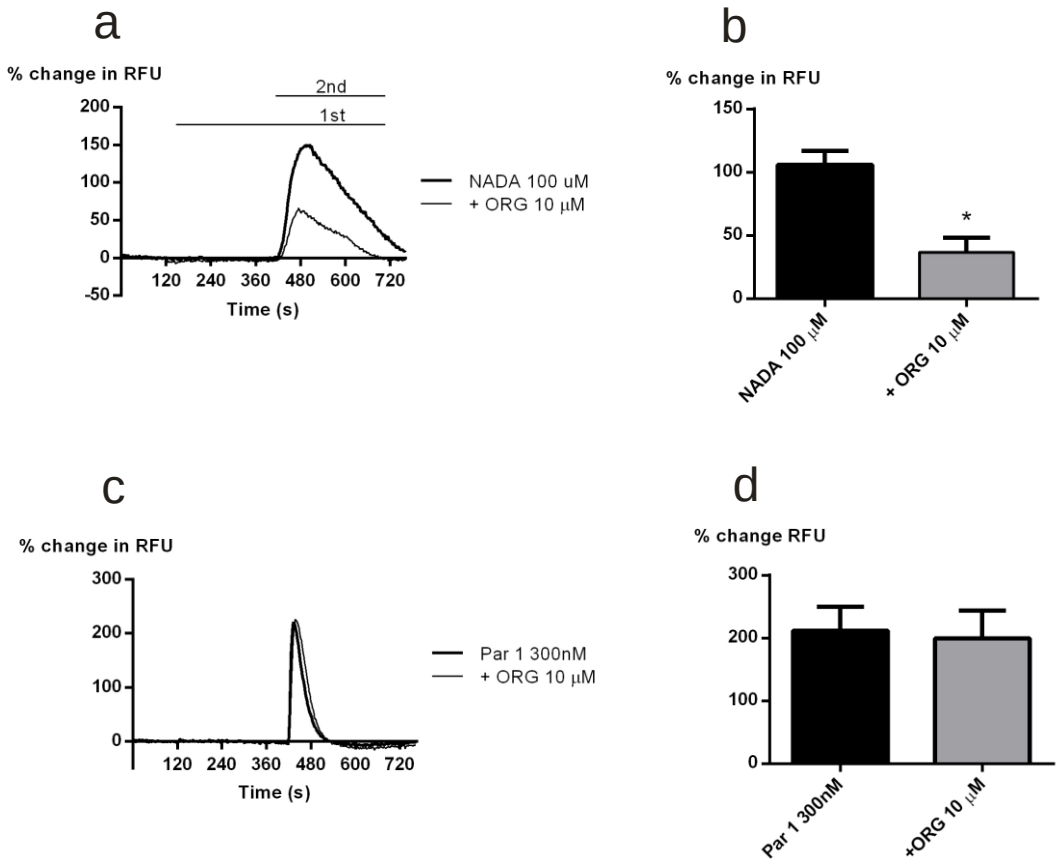



**Figure 5 | The allosteric modulator ORG27569 inhibits NADA $G_q$-mediated $[Ca]_I$ in CHO hCB1 and hCB2 cells. (a)** Baseline-corrected traces representing changes in $[Ca]_I$ for NADA 100 μM or for Par 1 300 nM, a $G_q$-coupled receptor **(c),** in the absence or presence of 10 μM ORG27569 pre-incubated for 5 minutes in CHO hCB1 cells traces are typical of 7 experiments performed in duplicate. **(b)** Normalised peak $[Ca]_I$ for NADA 100 μM or Par 1 300 nM **(d)** in the absence of presence of ORG27569 10 μM following 5 minutes pre-incubation in CHO hCB1. **P*<0.05.

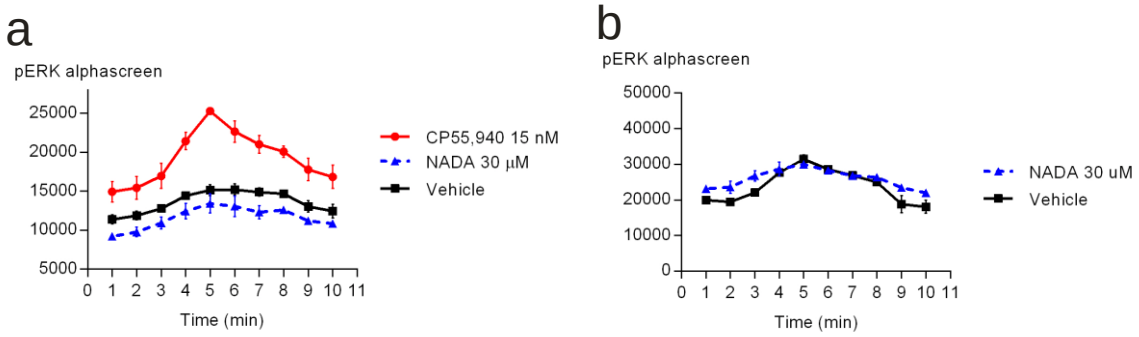
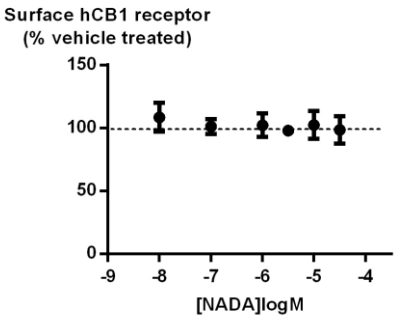



**Figure 6 | NADA does not cause ERK ½ phosphorylation in HEK hCB1 nor hCB2 cells.**

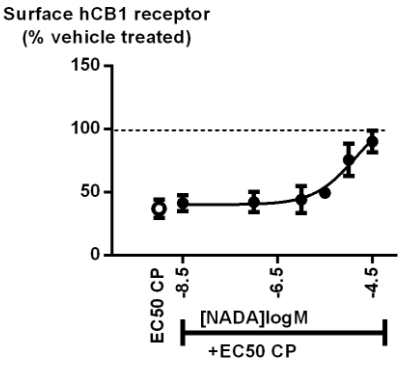
**(a)** An individual representative ERK 1/2 phosphorylation assay over time for HEK 3HA-hCB1 in the presence of vehicle, 15 nM CP55,940 or 30 µM NADA. **(b)** An individual representative ERK 1/2 phosphorylation assay over time for HEK 3HA-hCB2 in the presence of vehicle or 30 µM NADA.

**Figure 7 | NADA does not cause hCB1 receptor internalization but prevents CP induced internalization as well as internalization relating to constitutive activity. (a)** Change in cell surface expression of hCB1 receptors in HEK 293 cells after 30 minutes incubation of various concentration of NADA. Data represents mean±s.e.m., n=3, of triplicates. **(b)** Change in cell surface expression of hCB1 receptors in HEK 293 cells after 30 minutes incubation of an $EC_{50}$ of CP alone or co-incubated with various concentrations of NADA. Data represents mean±s.e.m., n=3, of triplicates. **(c)** Change in cell surface expression of hCB1 receptors in HEK 293 cells after 6 hours incubation of SR141716A, an inverse agonist of CB1, in the absence or presence of various concentrations of NADA. Data represents mean±s.e.m., n=3, of triplicates.

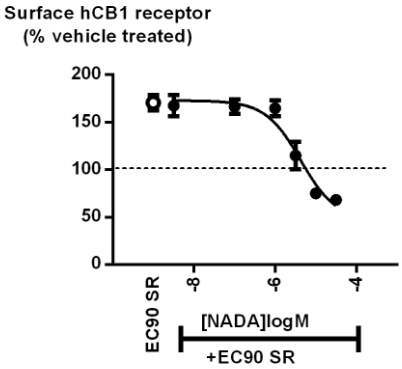
a


Surface hCB1 receptor
(% vehicle treated)
150
100
50
0
-9
-8
-7
-6
-5
-4
[NADA]logM

b


Surface hCB1 receptor
(% vehicle treated)
150
100
50
0
EC50 CP
-8.5
-6.5
-4.5
[NADA]logM
+EC50 CP

c


Surface hCB1 receptor
(% vehicle treated)
200
150
100
50
0
EC90 SR
-8
-6
-4
[NADA]logM
+EC90 SR

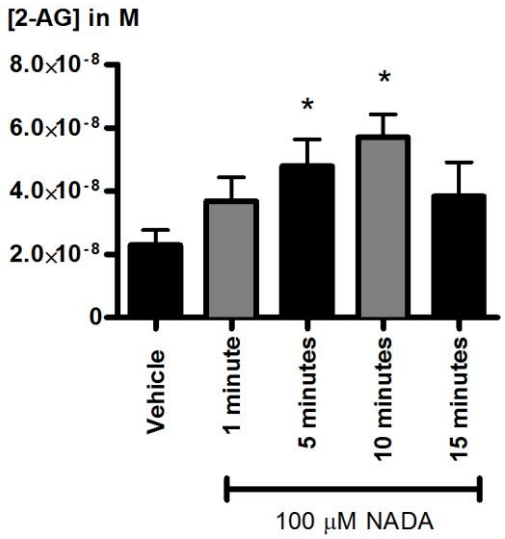



**Figure 8 | NADA administration to CHO hCB1 cells lead to a time-dependant increase in 2-AG levels.** 2-AG concentration levels over time following the administration of vehicle or 100 µM NADA in CHO hCB1 cells. Measurements were conducted every 5 minutes over time and were analysed using MC/LS/LS. Data represents mean±s.e.m., n=5, of duplicates.

# Discussion

The main results of this study are that NADA, an endogenous ligand for the CB1 receptor, has a signal bias toward $G_q$ coupling in CHO hCB1 cells with no clear activation of $G_{i/o}$, the main G-protein transduction pathway for the receptor. A structurally similar molecule, OLDA, produced a similar response. Secondly, we also find that hCB2 couples to $G_q$ G-proteins in the presence of NADA. Although $G_q$ coupling for the CB1 receptor has been described before (Lauckner *et al.*, 2005), $G_q$ coupling to the CB2 receptor has not yet been reported.

Lauckner *et al.* (Lauckner *et al.*, 2005) have first reported that the rat CB1 receptor can couple to the $G_q$ protein. In our assays, we saw increases in calcium for both CP and WIN55292-2 in CHO hCB1 (figure 3) and for CP55,940 in CHO hCB2 (figure 4), which was inhibited by U73122 3 µM and not affected by PTX 200 ng/ml, consistent with coupling to $G_q$. NADA increase in calcium, at maximal concentration, had a higher peak at 100 µM than those seen with maximally effective concentration of CP 55,940 and WIN55292-2 for $[Ca]_I$. NADA produced none of the other usual effects associated with CP55,940 or WIN55,212-2 on GIRK activation, cAMP inhibition in the presence of forskolin nor cAMP production in PTX treated cells, suggesting a transduction bias towards $G_q$. The NADA-induced $[Ca]_I$ came from intracellular pools of calcium as shown by the absence of $[Ca]_I$

once the intracellular pools of calcium from the endoplasmic reticulum were depleted by the application of thapsigargin.

We have further tried to link this $[Ca]_I$ to $G_q$ by specifically aiming to inhibit the binding of $G_q$ to the receptor by using a palpeptide that binds to the tail of the $G_q$ protein. We did see a significantly lowered $[Ca]_I$ in hCB1 and in hCB2 for 30 µM NADA. Although this is consistent with a $G_q$ coupling to the receptor, it is difficult to clearly prove, as $G_q$-specific pharmacological inhibitors are still lacking. We have ensured that our NADA stock was stable over time by doing mass spectrometry analysis in older aliquot of our stock (data not shown), ruling out a possible implication of oxidised or degraded compounds.

There are four main structural classes of ligands that bind to cannabinoid receptors with varying degree of selectivity for the CB1 and the CB2 receptors. Firstly, there is the terpenophenols (phytocannabinoids) and their bicyclic and tricyclic analogues like CP, the aminoalkylindoles like WIN, the naphthoylindole like JWH, the inverse agonists, mainly composed of various SR and AM structures, and finally the endogenous lipids, which contain long hydrophobic alkyl chains and a head group that can vary from ethalonamide (for anandamide) to dopamine in the case of NADA. Some 9-residue alpha-hemoglobin-derived peptides, such as hemopressin, have also been showed to bind to the CB1 receptor (Gomes *et al.*, 2009). Varying degree of receptor stabilisation in an active state favouring the coupling of one or another G-

protein has first been described by Bonhaus *et al.(Bonhaus et al.,* 1998). So far, no ligand for CB1 nor CB2 have been showed to favour a strong bias for a secondary signalling pathway over the preferred pathway via $G_{i/o}$. This finding is specially of interest since hCB2 receptors only share about 44% identity with hCB1 (Munro *et al.*, 1993) and that few molecules show a similar affinity profile for both. Unfortunately, there is no complete crystal structure for CB1 nor CB2, and most studies looking at receptor the conformational changes following activation based on homology modelling of the adenosine $A_{2A}$ receptor template, and much is still required to elucidate the binding properties of endocannabinoids such as anandamide. As OLDA shared a similar $[Ca]_I$ in hCB1 (figure1) and hCB2 (figure 3) as NADA, it appears that the dopamine head group is of importance in the bias we have found between anandamide and NADA. Arachidonic acid itself does not cause changes in $[Ca]_I$ via hCB1 nor hCB2, although it does cause increase in$[Ca]_I$ in WT CHO cells (data not shown).

NADA did not inhibit the responses mediated by CP55,940 in cAMP inhibition nor peak-response of membrane potential changes via GIRK activation in CHO hCB1 cells nor did it affect ERK1/2 phosphorylation. NADA did not affect the peak GIRK-mediated change in membrane potential of CP but changed the EC50 from 5 nM to 50 nM in the presence of 30 µM for CHO hCB2. On the other hand, NADA caused an inhibition of receptor internalization. These effects are all consistent with the low affinity of NADA for these receptors. It is to be noted that the binding affinity presented here does not necessarily translate to functional affinity in functional assays as

the conditions in which these tests were measured differ. It is possible that NADA causes a change in the conformation of the receptor which keeps it in an open conformation for orthosteric ligands but prevents βarrestin recruitment and subsequent internalization. We have encountered such a schism between receptor activation and internalization in the presence of the allosteric modulators of CB1 ORG and PSNCBAM-1 (1-(4-chlorophenyl)-3-3-(6-pyrrolidin-1-ylpyridin-2-yl)phenyl urea) in a previous study (Cawston *et al.*, 2013). Interestingly, the change in conformation caused by ORG when it binds to the receptor, was detrimental to the coupling of hCB1 and hCB2 to $G_q$, seen by reduced peak $[Ca]_I$ following the administration of NADA 100 μM in the presence of ORG 10 μM.

It is to be noted that the concentration of NADA, a low affinity ligand, required to displace CP, a high-affinity ligand, were higher than previously reported in the literature (Bisogno *et al.,* 2000). The concentration of NADA required to increase $[Ca]_I$ in our assay was also higher than the concentration present in the brain (Huang *et al.,* 2002) and out of its known physiological range. However, no study has so far described the concentration of NADA in the periphery, which would be needed to draw a clearer picture. Similarly, NADA levels could hypothetically be higher in certain conditions or at specific points in development although no such mechanism currently known can validate these hypotheses. The colocalization or possible dimerization of CB1 with other calcium channels or $G_q$-mediated GPCRs could potentially lower the needed concentration in order to be efficacious in a physiological setting.

In theory, a strongly biased orthosteric ligand such as NADA should competitively antagonize the signalling mechanisms of an unbiased ligand. This was not completely the case in our findings. Indeed, NADA did not affect GIRK-mediated hyperpolarization caused by CP. However, a small inhibition of $G_{i/o}$-mediated inhibition of cAMP formation following co-administration of CP and forskolin was observed, as well as an inhibition of receptor internalization following CP activation. It is not currently known if a high agonist occupancy ligand might be required for the latter, whereas low affinity occupancy is enough to inhibit GIRK-mediated hyperpolarization. As well, GIRK-mediated hyperpolarization is mediated by the βγ subunits of $G_{i/o}$ whereas cAMP inhibition is mediated by its α counterpart. As for the internalization of the receptor, it requires β-arrestins recruitment. It would thus be possible to think that NADA could influence one but not all of these mechanisms equally. Finally, a change in conformation of the receptor could potentially affect the displacement assays via an action through an allosteric site on the receptor. NADA thus cannot completely be ruled out as a possible ligand for an allosteric site on the receptor or a dual orthosteric/allosteric action. This could potentially explain ORG27569's inhibition of $[Ca]_I$ following NADA administration.

It is of importance to note that this work was done in a purposely restrictive condition system, using cultured cells to test these two main hypothesis with as few confounding factors as possible. In the absence of any information as

to the local concentrations of NADA in native tissue, the physiological implications of this work are unclear, however, we can speculate as to a possible physiological mechanism arising from the elevations of $[Ca]_i$ produced by NADA from this activation. Elevation of calcium via cellular depolarisation or through metabotropic receptor activation (Fukudome *et al.*, 2004; Haj-Dahmane *et al.*, 2005; Kim *et al.*, 2002; Maejima *et al.*, 2001) have been showed to elicit a release of 2-AG from various neuronal cells (for review on $[Ca]_I$ mediated endocannabinoid synthesis, see Hashimotodani *et al.* (Hashimotodani *et al.*, 2007)). $Ca^{2+}$ influx through VGCCs and downstream signalling from Gq-coupled GPCRs, through PLCβ appear to converge into the same metabolic pathway for the synthesis of 2-AG (Castillo *et al.*, 2012). We have thus hypothesized that the activation of the human CB1 receptor by NADA could potentially lead to the synthesis of 2-AG itself. To test this hypothesis, we have measured, using a mass spectrometry reader, the levels of 2-AG post NADA for the next 5 minutes in our CHO hCB1 cells. To test this hypothesis, we have measured, using a mass spectrometry reader, the levels of 2-AG post NADA for the next 15 minutes in our CHO hCB1 cells. We have found significantly higher levels of 2-AG at five and ten minutes following the administration of 100 μM NADA in our assay. Testing this hypothesis in neurons expressing CB1 receptor would be of great interest, but was out of the scope of the present study. We believe our study gives an interesting rationale for further studies on this subject.

Taken together, our results suggest that NADA and related compound OLDA show a novel highly biased signal transduction toward $G_q$ without any clear coupling to $G_{i/o}$ for both the CB1 and CB2 receptors.

# Methods

### Cell culture

HEK cells stably transfected with 3HA-hCB1 (Cawston *et al.*, 2013) and 3-HA hCB2 (Grimsey *et al.*, 2011) were used for cAMP, pERK, internalisation and binding assays. CHO 3HA-hCB1 (Grimsey *et al.*, 2010) and CHO 3HA-hCB2 (Horvath *et al.*, 2012) were used for measurements of changes in $[Ca]_I$. AtT20 rCB1 (Mackie *et al.*, 1995) and hCB2 were used for GIRK-mediated hyperpolarization assays. HEK 293 and AtT20 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U penicillin and 100 µg streptomycin ml-1. HEK 293 cell media also contained hygromycin B 25 µg ml-1 and blasticidin S 5 µg/ml-1. CHO hCB1 and hCB2 cells were incubated in DMEM/F12-HAM media, a 50:50 mixture of DMEM and Ham's F12 media first developed for CHO cells. CHO hCB1 cells were supplemented with [] zeocin and CHO hCB2 cells were supplemented with 500 $\mu g \cdot mL^{-1}$ G-418 . Cells containing either DMEM and DMEM/F12-HAM media were incubated in 5% CO2 at 37˚C in a humidified atmosphere. Cells were grown in 75 $mm^2$ flasks and plated in a volume of 100 µl per well onto clear-bottomed poly-D-lysine coated 96 well plates (Corning, Castle Hill, NSW, Australia) after reaching 90% confluence. Once in the assay plates, cells were grown in L15 medium supplemented with 1%

fetal bovine serum and appropriate antibiotics. The cells were incubated in humidified room air at 37˚C overnight.

**Binding assay**

To generate cell membranes HEK293 cells expressing either hCB1 or hCB2 were grown to 90–100% confluence in 175 $cm^2$ flask and harvested in ice-cold phosphate buffered saline with 5 mM EDTA. Cells were centrifuged at 200×*g* for 10 min and frozen at −80°C until required. Cell pellets were thawed with Tris-sucrose buffer (50mM Tris-HCl, pH 7.4, 200mM sucrose, 5mM $MgCl_2$, 2.5mM EDTA) and homogenized with a glass homogenizer. The homogenate was centrifuged at 1000g for 10 min in a Heraeus multifuge 3SRT for 10 min at 4°C and the pellet discarded. The supernatant was centrifuged in a Sorvall ultracentrifuge 15000 rpm (26 916g) for 30 min (rotor 3335) at 4°C. The final pellet was resuspended in a minimal volume of Tris-sucrose buffer, and aliquoted to avoid repeated freeze-thaw cycles. Protein concentration was determined using the Dc protein assay kit (Bio-Rad, Hercules, CA, USA) following manufacturers protocol. Competition binding assays at 2.5 nM [$^3$H]-CP55,940 or [$^3$H]- SR141716A (both from PerkinElmer) for hCB1, or 2 nM [$^3$H]-CP 55,940 for hCB2 were carried out to determine the $K_i$ values NADA. Membranes (20 µg for hCB1; 7.5µg for hCB2) were incubated with radioligand and a range of concentrations of NADA in binding buffer (50 mM HEPES pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$) with 0.2% (w/v) bovine serum albumin (BSA) (ICP Bio, New Zealand), at 30°C for 60 min. Non-specific binding was determined in the presence of 1 µM non-

radioactive CP55,940 (Tocris Cookson). GF/C Harvest Plates (Perkin Elmer) were pre-soaked in 0.1% polyethylenimine and then washed with 200 µl ice cold wash buffer (50mM HEPES pH 7.4 500mM NaCl and 0.1 % BSA), followed by three 200 µL washes in the same buffer. Harvest plates were then dried overnight at 24 C and then 50 µL of scintillation fluid was added per well. After a 30 minute delay these were scintillation counted in a Microbeta Trilux (Perkin Elmer) for 2 minutes per well. Data was graphed on GraphPad Prism using non-linear regression analysis (one site competition binding, using predetermined Kd values of 2.5nM for CP55,940 at CB1, 1nM for SR141716A at CB1, and 2 nM for CP55,940 at CB2).

**Intracellular calcium measurements**

Intracellular calcium $[Ca]_i$ was measured with the calcium 5 kit from Molecular Devices (Sunnyvale, CA, USA) using a FLEX Station 3 Microplate Reader (Molecular Devices). 100 µl of dye dissolved in HEPES- buffered saline (HBS) containing (in mM): NaCl 140, KCl 5.33, $CaCl_2$ 1.3, $MgCl_2$ 0.5, HEPES 22, $Na_2HPO4$ 0.338, $NaHCO_3$ 4.17, $KH_2PO4$ 0.44, $MgSO_4$ 0.4, glucose 10 (pH to 7.3, osmolarity = 330 ± 5 mosmol) concentration was loaded into each well of the plate for at least 1 hour prior to assay. Fluorescence was measured every 2 seconds (λexcitation = 485nm, λemission = 525 nm for calcium assay and λexcitation = 485nm, λemission = 565 nm for membrane potential assay) for the duration of the experiment. In experiments where one drug addition was made, 50 µL of drug dissolved in HBS was added, for two drug additions, 25 µL was added each time. In all experiments, drugs were added after at least 2 minutes of baseline recording. Assays were carried out at 37˚C.

**Changes in membrane potential measurements**

Changes in membrane potential were recorded using the blue membrane potential dye (Molecular Devices) and a FLEX Station 3 Microplate Reader. A low potassium (no KCl) version of HEPES buffer mentioned above was used for the membrane potential assays. Fluorescence was measured every 2 seconds (λexcitation = 485nm, λemission = 525 nm for calcium assay and λexcitation = 530nm, λemission = 565 nm for membrane potential assay) for the duration of the experiment. The other experimental settings were the same as those mentioned above for intracellular calcium measurements.

**cAMP measurement**

Cellular cAMP was measured as previously described in Cawston et al, 2013. Briefly the pcDNA3L-His-CAMYEL plasmid (ATCC, Manassas, VA, USA), which is a mammalian expression vector that encodes a bioluminescence resonance energy transfer (BRET) cAMP sensor, YFP-Epac-RLuc (CAMYEL) (Jiang *et al.*, 2007) , was transfected into HEK 3HA-hCB1 or HEK 3HA-hCB2 cells using linear polyethyleneimine (PEI, m.w. 25 kDa) (Polysciences, Warrington, PA, USA). Twenty-four hours post transfection cells were re-plated in poly-L-lysine (0.2 mg $mL^{-1}$ in PBS), (Sigma-Aldrich, St Louis, MO, USA) coated white CulturPlate™-96 (PerkinElmer, Waltham, MA, USA) at a density of 55,000 cells per well. After twenty-four hours, cells were serum-starved in Hank's balanced salt solution (HBSS) containing 1 mg/ml Bovine Serum Albumin (BSA) (ICP, Auckland, New Zealand), pH 7.4 for 30 min prior to assay. Five minutes prior to the addition of drugs/vehicle in HBSS plus 1

mg $ml^{-1}$ BSA cells were treated with 5 μM Coelenterazine-h (Promega, Madison, WI, USA) Emission signals were detected simultaneously at 460\25 nM (RLuc) and 560\25nM (YFP), immediately following drug addition with a Victor-Lite plate reader (Perkin Elmer) at 37°C. Raw data is presented as an inverse BRET ratio of emission at 535/460 such that an increase in ratio correlates with an increase in cAMP production.

**Internalisation of cannabinoid receptors**

Surface hCB1 expression and the degree of internalisation was determined utilising a live cell antibody feeding technique and quantified via the Discovery-1 automated fluorescent microscope (Molecular Devices) as previously described (Cao *et al.*, 1999; Grimsey *et al.*, 2008). In brief, HEK 3HA-hCB1 cells were seeded at 30,000 cells/well in poly-L-lysine treated 96-well, flat bottom clear plates (Nunc, Roskilde, Denmark). Approximately twenty four hours later, cells were serum starved in DMEM with 5 mg mL-1 BSA (DMEM-BSA) for 30 minutes at 37°C and subsequently incubated with anti-mouse Monoclonal HA11 antibody (MMS-101P, Covance, Princeton, NJ, USA) diluted 1:500 in DMEM-BSA at 37°C for 30 minutes. After two washes with DMEM-BSA, the agonist CP55940 at EC50-0.5nM (Tocris Bioscience, Bristol, UK) and NADA (Cayman Chemicals, USA) at various concentrations were applied concurrently for 60minutes. Following drug incubation, plates were placed on ice to prevent any further receptor trafficking after which they were incubated with Alexa Fluor® 488-conjugated goat anti-mouse antibody (Life Technologies) diluted 1:300 in DMEM-BSA at room temperature for 30 minutes. Cells were then washed twice in DMEM-BSA,

fixed with 4% paraformaldehyde, and stained with Hoechst 33258 (Life Technologies) diluted 1:500 in Phosphate Buffered Saline with 0.2% Triton-X (PBS-T). Images of the cells were acquired with a Discovery-1 microscope (10x objective, four images per well) and experimental effects quantified using MetaMorph (v.6.2r6, Molecular Devices) by calculating the intensity of fluorescent labelling per cell (Grimsey *et al.*, 2008). Sigmoidal concentration response curves were fitted utilising GraphPad Prism (constrained to top plateau equal or below 100%) to produce *p*EC50 values for independent experiments.

**Receptor Up-regulation**

Up-regulation of surface hCB1 via inhibition of constitutive activity was determined using live cell immunocytochemistry and quantified as per internalisation assay. Cells were plated and treated as above. Following 30 minute serum starve, the inverse agonist SR141716A at EC90-20nM (gift from National Institute of Drug Abuse, Rockville, MD, USA) and NADA at various concentrations were applied concurrently for 6 hours. Following drug incubation, plates were placed on ice to prevent any further receptor trafficking after which they were incubated with anti-mouse Monoclonal HA11 antibody (MMS-101P, Covance, Princeton, NJ, USA) diluted 1:500 in DMEM-BSA at room temperature for 30 minutes. Cells were then washed twice in DMEM-BSA, fixed with 4% paraformaldehyde and incubated with Alexa Fluor® 488-conjugated goat anti-mouse antibody (Life Technologies) diluted 1:400 in goat serum immunobuffer (PBS-T with 1% normal goat serum, Invitrogen; 0.4 mg/mL Merthiolate, Merck, Darmstadt, Germany)

overnight at 4°C. Following this, cells were washed twice with PBS-T and stained with Hoechst 33258 (Life Technologies) diluted 1:500 in (PBS-T). Imaging and quantification were conducted as per the internalisation assay above. Sigmoidal concentration response curves were fitted utilising GraphPad Prism to produce *p*EC50 values for independent experiments.

**ERK1/2 measurements**

AlphaScreen® *SureFire*® Phospho(p)ERK1/2(Thr202/Tyr204) assay kits (PerkinElmer) were utilized following the manufacturers recommended protocol. 40,000 HEK 3HA-hCB1 or HEK 3HA-hCB2 cells per well were seeded in 100 µL culture medium into poly-l-lysine treated 96-well plates (Corning, USA), and incubated at 37°C, 5% $CO_2$ and 95% humidity for 24 h. Cells were serum-starved in 50 µL DMEM plus 5mg/ml BSA overnight prior to drug treatment. All drugs were added at 2x concentration in DMEM + 1mg/ml BSA and incubated for the times indicated. Assay plates were then put onto ice, and media/drug were removed followed by the addition of 30 µl of lysis buffer and plates were agitated for 10 min at room temperature. Cell lysate (5 µL) was transferred into a white 96 well low volume plate (PerkinElmer) and 7 µl detection mix was added. Plates were sealed, wrapped with foil and incubated for 2 - 4 h at RT, and fluorescent signals detected on an EnSpire reader (PerkinElmer).

**Lipid Extraction**

Cells were grown in T-125cm$^2$ flasks to 90% confluency. Drugs were added to 2 flasks separately then combined for each test group. All drugs were

diluted in serum free Dulbecco's Modified Eagle Medium (DMEM). The media the cells were grown in was aspirated off before drug addition. 2.5mL of 1% Dimethyl sulfoxide (DMSO) was added for 5 minutes as a control. 2.5mL of 1uM SR141716A was added for 10 minutes to test the effects of the antagonist alone. 2.5mL of 1uM SR141716A was added for 10 minutes followed by the addition of 100uM NADA for 5 minutes to act as an antagonist control. 100uM N-Arachidonoyl Dopamine (NADA) was then added for 1 minute, 5 minutes, 10 minutes, and 15 minutess. Each flask was diluted with 3:1 volumes of HPLC-grade methanol (eg. 7.5mL of MeOH for 2.5mL of drug) after the fixed time point. Cells were then scraped and the two flasks per each test group were pippetted and combined into a centrifuge tube. 20 µL of 1uM d4AEA was then added to each centrifuge tube. $D_4$AEA was added to act as an internal standard to determine the recovery of the compounds of interest. The tubes were then covered with parafilm and left on ice and in darkness for approximately 2 hours. The samples were then centrifuged at 19,000xG at 24℃ for 20 minutes. The supernatants were then collected and HPLC-grade water was added making the final supernatant/water solution 25% organic in 50mL polypropylene tubes. To isolate the compounds of interest, a Preppy apparatus (Sigma-Aldrich) assembled with 500 mg C18 solid-phase extraction columns (Agilent Technologies, Santa Clara, CA) was used to partially purify the 25% organic solution. The columns were conditioned with 5 mL of HPLC-grade methanol immediately followed by 2.5 mL of HPLC-grade water. The supernatant/water solution was then loaded onto the C18 column, and then washed with 2.5 mL of HPLC grade water followed by 2mL of 40% methanol. Then 1.5mL of 50%, 60%, 70%, and 80% methanol were used for further

purification. Then 1.5mL elutions of 90% and 100% methanol were added to extract the ethanolamides from the column. All elutions were collected in individual autosampler vials and then stored in a -20°C freezer until mass spectrometer analysis.

**LC/MS/MS Analysis and Quantification**

Samples were removed from the -20°C freezer and allowed to warm to room temperature then vortexed for approximately 1 minute before being placed into the autosampler and held at 24°C for LC/MS/MS analysis. 20µL of eluants were injected separately for each sample to be rapidly separated using a C18 Zorbax reversed-phase analytical column (Agilent Technologies, Santa Clara, CA) to scan for individual compounds (mobile phase A: 20% HPLC methanol, 80% HPLC water, 1mM ammonium acetate; mobile phase B: 100% HPLC methanol, 1mM ammonium acetate). Gradient elution (200 µL/min) then occurred under the pressure created by two Shimadzu 10AdVP pumps (Columbia, MD). Next, electrospray ionization was accomplished using an triple quadrupole mass spectrometer. A multiple reaction monitoring (MRM) setting on the LC/MS/MS was then used to analyze levels of each compound present in the sample injection. Synthetic standards were used to generate optimized MRM methods and standard curves for analysis.

**Analysis**

Analysis for all experiments were performed with GraphPad Prism (Version 5.02, GraphPad Software, Inc., La Jolla, CA, USA).

For phosphorylation of ERK 1/2 data, statistical significance was determined by a paired t-test comparing the two groups. Statistical significance was defined as $p < 0.05$. All statistical analyses were performed using SigmaPlot (v.11.0, Systat Software, Chicago, IL, USA).

Data for the cAMP assays were analysed using "Area under the curve" (AUC) analysis in GraphPad Prism (Version 5.02, GraphPad Software, Inc., La Jolla, CA, USA) with data normalised to individual assay basal and forskolin values. Statistical tests were carried out with SigmaPlot (v.12.0, Systat Software, Chicago, IL, USA). Paired t-tests were utilised when comparing two datapoints, 1-way ANOVA for more than two datapoints with one independent variable.

The response to agonists was expressed as a percentage change over the baseline averaged for the 30 seconds immediately prior to drug addition. Changes produced by parallel solvent blanks were subtracted before normalization. Concentration-effect data were fit to a four-parameter logistic Hill equation to derive the $EC_{50}$ values and Hill slope (GraphPad Prism, San Diego, CA). Both NADA and OLDA produced small elevations of [Ca]i in untransfected CHO cells. These changes in [Ca]i were similar to those produced by equivalent concentrations of arachidonic acid in both wt and hCB1 transfected CHO cells, and were not sensitive to inhibitors of PLC. When responses are expressed as % change in RFU, data was corrected by

subtracting the unspecific calcium increases seen in WT CHO cells. These unspecific effects were never above 30% of the maximal response for CB1 nor CB2.

For lipid measurements, the amount of analyte in each sample was calculated by using a combination of calibration curves of the synthetic standards and dueterium-labeled internal standards obtained from the Analyst software. The standards provided a reference for the retention times by which the analytes could be compared. They also helped to identify the specific precursor ion and fragment ion for each analyte which enabled their isolation. These processes provide confidence in the claim that the compounds measured were, in fact, the compound of interest. The amount of each compound was then converted to moles per gram tissue (dry pellet weight post centrifugation), which is how it was statistically analyzed.

***Drugs and reagents***

All drugs unless otherwise specified were made up in ethanol and diluted in saline to give a final concentration of ethanol of 0.05 - 0.1%. NAAN were purchased from Biomol (Plymouth Meeting, PA, USA) or Cayman Chemicals. U73122 and palpeptide were made fresh before each use and were diluted in DMSO and water respectively. Arachidonoyl ethanolamide-d4 (d4-AEA)was purchased from Tocris Bioscience (St. Louis, MO). AEA, PEA, OEA, LEA, and 2-AG were purchased from Cayman Chemical (Ann Arbor, MI). HPLC-grade water and methanol were purchased from VWR International (Plainview, NY). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO).

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**Author Contributions**

MC and WJR conceived the study. WJR performed the calcium and membrane potential measurements, EC, CB and MG did the BRET, ERK1/2 phosphorylation, binding and internalization of CB1 receptors assays. WJR and MC wrote the paper.

# Chapter 4 Paper 3

# Ligand determinants of fatty acid activation of the pronociceptive ion channel TRPA1

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# Ligand determinants of fatty acid activation of the pronociceptive ion channel TRPA1

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## ABSTRACT

**Background and purpose.** Arachidonic acid (AA) and its derivatives are important modulators of cellular signalling. The transient receptor potential cation channel subfamily A, member 1 (TRPA1) is a cation channel with important functions in mediating cellular responses to noxious stimuli and inflammation. There is limited information about the interactions between AA itself and TRPA1, so we investigated the effects of AA and key ethanolamide and amino acid/neurotransmitter derivatives of AA on hTRPA1.

**Experimental approach.** HEK 293 cells expressing hTRPA1 were studied by measuring changes in intracellular calcium ($[Ca]_i$) with a fluorescent dye and by standard whole cell patch clamp recordings.

**Key results.** AA (30 $\mu$M) increased fluorescence in hTRPA1 expressing cells by 370% (notional $EC_{50}$ 13 $\mu$M). The covalent TRPA1 agonist cinnamaldehyde (300 $\mu$M) increased fluorescence by 430% ($EC_{50}$, 11 $\mu$M). Anandamide (230%) and *N*-arachidonoyl tyrosine (170%) substantially activated hTRPA1 at 30 $\mu$M, however, *N*-arachidonoyl conjugates of glycine and taurine were less effective while *N*-acyl conjugates of 5-HT did not affect hTRPA1. Changing the acyl chain length or the number and position of double bonds reduced fatty acid efficacy at hTRPA1. Mutant hTRPA1 (Cys621, Cys641 and Cys665 changed to Ser) could be activated by AA (100 $\mu$M, 40% of wild type) but not by cinnamaldehyde (300 $\mu$M).

**Conclusions and implications.** AA is a more potent activator of TRPA1 than its ethanolamide or amino acid/neurotransmitter derivatives and acts via a mechanism distinct from that of cinnamaldehyde, further underscoring the likelyhood of multiple pharmacologically exploitable sites on hTRPA1.

**Subjects** Biochemistry, Pharmacology

**Keywords** Arachidonic acid, TRP channel, Pain, Inflammation, Arachidonoyl amino acid/neurotransmitter conjugate

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## INTRODUCTION

The transient receptor potential ankyrin 1 channel (TRPA1; *Alexander, Mathie & Peters, 2011*, *Story et al., 2003*) is expressed on primary afferent nociceptors where it detects potentially damaging environmental stimuli such as noxious cold, changes in pH, noxious chemicals and endogenous products of inflammation. Although there is emerging evidence for physiological roles of TRPA1 in cells intrinsic to brain and spinal cord (*Shigetomi et al., 2011*; *Cho et al., 2012*) and TRPA1 is also expressed in the hair cells of the ear (*Corey et al., 2004*), most effects of TRPA1 ligands have been linked with the expression of the channel in the peripheral sensory neurons of the dorsal root, trigeminal and nodose ganglia (*Nagata et al., 2005*; *Story et al., 2003*).

Although a complete description of how TRPA1 is activated by such a wide variety of modulators is yet to be realized, electrophilic agonists such as cinnamaldehyde (CA) and allyl isothiocyanate (AITC) activate TRPA1 via reversible or irreversible covalent modification of cysteine residues located within the intracellular N-terminal domain (*Hinman et al., 2006*; *Macpherson et al., 2007*). The mechanism(s) underlying the activation of TRPA1 by unreactive compounds such as menthol (*Karashima et al., 2007*), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (*Liu et al., 2010*) and $\Delta^9$-tetrahydrocannabinol (*Jordt et al., 2004*) are less well described, although in some cases residues in transmembrane domains appear to be important for channel activation by these ligands (e.g. menthol, *Xiao et al., 2008*). Intriguingly, mutation of cysteine residues which abolish TRPA1 activation by electrophiles appears to also reduce the effectiveness of TRPA1 activation by most unreactive compounds, implying an important general role for these cysteine residues in channel function. For example, a mutagenesis study by (*Liu et al., 2010*) showed that reactive and non-reactive compounds such as NPPB saw their peak [$Ca_i$] response reduced for single cysteine mutations to a serine. Xiao described that these residues are important for the activation of the channel mediated by menthol, another non-reactive compound (*Xiao et al., 2008*). A requirement for formation of disulphide bonds between cysteine residues during channel activation, including activation by non-reactive compounds, might explain why there is lessened activity in the 3x Cys mutants (*Wang et al., 2012*).

TRPA1 is activated by arachidonic acid-derived molecules, including highly reactive isoprostanes, prostaglandins (*Taylor-Clark et al., 2008*), hepoxilins (*Gregus et al., 2012*), epoxyeicosatreinoic acids (*Sisignano et al., 2012*) and the endocannabinoid anandamide (*De Petrocellis & Di Marzo, 2009*). Arachidonic acid (AA) itself has also been reported to activate TRPA1 (*Bandell et al., 2004*; *Motter & Ahern, 2012*). In this study we have examined the activation of recombinant human TRPA1 (hTRPA1) by arachidonic acid and other long chain fatty acids as well as by *N*-arachidonoyl neurotransmitter/amino acid conjugates (NAAN), a large family of endogenous modulators of ion channels and G protein coupled receptors (*Connor, Vaughan & Vandenberg, 2010*). We find that AA itself is the most effective activator of hTRPA1 among these compounds, and modest changes in its structure dramatically alter TRPA1 activity. Mutations in the intracellular Cys

residues that essentially abolish the activity of CA also reduce the effects of AA, suggesting some overlap in the mechanisms through which diverse agonists activate the channel.

## METHODS

### Cell culture

Flp-In TRex HEK 293 (Life Technologies, Mulgrave, Victoria, Australia) were stably transfected with wild type or mutant hTRPA1 or wild type mouse TRPA1 (Genscript, Piscataway, NJ, USA) and cultivated in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U penicillin and 100 $\mu$g streptomycin $ml^{-1}$, hygromycin B 25 $\mu$g $ml^{-1}$ and blasticidin S 5 $\mu$g $ml^{-1}$. Cells were incubated in 5% $CO_2$ at 37°C in a humidified atmosphere. Cells were grown in flasks with a surface area of 75 $mm^2$, once at optimum confluence (approximately 90%), cells were trypsinized and transferred into clear-bottomed poly-D-lysine coated 96 well plates (Corning, Castle Hill, NSW, Australia) in L15 medium supplemented with 1% fetal bovine serum, hygromycin B, and the antibiotics outlined above. The cells were plated in a volume of 100 $\mu$L and were incubated in humidified room air at 37°C overnight. Expression of the TRPA1 receptor or mutants was induced 5–8 h prior to experimentation by addition of with tetracycline, 1 $\mu$g $ml^{-1}$ to each well.

### Calcium assay

Intracellular calcium $[Ca]_i$ was measured with the calcium 5 kit from Molecular Devices (Sunnyvale, CA, USA) using a FLEX Station 3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). 100 $\mu$l of dye dissolved in HEPES- buffered saline (HBS) containing (in mM): NaCl 140, KCl 5.33, $CaCl_2$ 1.3, $MgCl_2$ 0.5, HEPES 22, $Na_2HPO_4$ 0.338, $NaHCO_3$ 4.17, $KH_2PO_4$ 0.44, $MgSO_4$ 0.4, glucose 10 (pH to 7.3, osmolarity = 330 ± 5 mosmol) was loaded into each well of the plate for 1 h prior to testing in the Flexstation at 37°C. Fluorescence was measured every 2 seconds ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 525 nm) for the duration of the experiment. Drugs were added after at least 2 min of baseline recording. In experiments where one drug addition was made, 50 $\mu$L of drug dissolved in HBS was added, for two drug additions, 25 $\mu$L was added each time.

### Electrophysiology

TRPA1 channel currents in HEK293 cells were recorded in the whole-cell configuration of the patch-clamp method (*Hamill et al., 1981*) at room temperature. Dishes were perfused with HEPES buffered saline (HBS) containing (in mM): 140 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 Glucose (pH to 7.3, osmolarity = 330 ± 5 mosmol). Recordings were made with fire-polished borosilicate glass pipettes with resistance ranging from 2–3 M . The internal solution contained (in mM): 130 CsCl, 10 HEPES, 2 $CaCl_2$, 10 EGTA, 5 MgATP (pH to 7.3, osmolarity = 285 ± 5 mosmol). Recordings were made with a HEKA EPC 10 amplifier with Patchmaster acquisition software (HEKA Elektronik, Germany). Data was sampled at 10 kHz, filtered at 3 kHz, and recorded on hard disk for later analysis. Series resistance ranged from 3 to 10 M , and was compensated by at least 80% in all experiments. Leak subtraction was not used. Cells were

exposed to drugs via flow pipes positioned approximately 200 $\mu$m from the cell, drugs were dissolved in HBS immediately before application. All solutions had final ethanol concentration of 0.05%–0.1% v/v.

### Data analysis

The response to agonists was expressed as a percentage change over the baseline averaged for the 30 seconds immediately prior to drug addition. Changes produced by parallel solvent blanks were subtracted before normalization, these changes were never more than 10% of baseline. Concentration-effect data were fit to a four-parameter logistic Hill equation to derive the $EC_{50}$ values and Hill slope (GraphPad Prism, San Diego, CA). Where solubility precluded determining full concentration response curves, the curve maxima were constrained to the maximum increase in $[Ca]_i$ produced by a high concentration of cinnamaldehyde in the same experiment. In these cases drug potency was reported as a notional $EC_{50}$. Comparisons between human and mouse TRPA1 were made after normalising responses to those produced by a maximally effective concentration of CA (300 $\mu$M) included in each experiment. Results are expressed as mean ± s.e.m. of at least 4–5 independent determinations.

### Drugs and reagents

All drugs were made up in ethanol and diluted in HBS to give a final concentration of ethanol of 0.05–0.1%. Because of limits to the solubility of fatty acids and their derivatives, the maximum concentration used was either 30 $\mu$M or 100 $\mu$M, as noted. Arachidonic acid and its derivatives were purchased from Cayman Chemical (Ann Arbor, MI, USA) and NAAN were purchased from Biomol (Plymouth Meeting, PA, USA) or Cayman Chemicals. NPPB was purchased from Tocris Bioscience (Bristol, UK), ruthenium red from Enzo Lifesciences (Farmingdale, NY, USA), HC 030031 and ionomycin were from Ascent Scientific (Avonmouth, UK). Cinnamaldehyde was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All tissue culture reagents were from Sigma-Aldrich, Life Technologies (Mulgrave, Victoria, Australia) or Invivogen, (San Diego, CA, USA).

To independently confirm the activity of adrenic acid and $\omega$3-arachidonic acid, we compared their effects on $Ca_V3.1$ calcium channels with those of $\omega$6-arachidonic acid. Recordings from $Ca_V3.1$ channels were made as described in (*Gilmore et al., 2012*). Cells were stepped repetitively from −86 mV to −26 mV for 20 ms every 10 s. At a concentration of 10 $\mu$M ($n = 3$ each), adrenic acid (64 ± 8%), $\omega$3-arachidonic acid (43 ± 5%) and $\omega$6-arachidonic acid (81 ± 4%) all inhibited $Ca_V3.1$ channels to a degree consistent with previous reports of fatty acid activity on this channel (*Chemin, Nargeot & Lory, 2007*).

## RESULTS

Arachidonic acid has previously been reported to activate mouse and rat TRPA1 expressed in Chinese hamster ovary and HEK 293 cells respectively (*Bandell et al., 2004*; *Motter & Ahern, 2012*). Addition of AA to HEK 293 cells expressing hTRPA1 produced

rapid and sustained elevations of $[Ca]_i$. The increase was concentration-dependent, in our initial series of experiments AA (30 $\mu$M) increased cellular fluorescence by 369 ± 38% over baseline with a notional $EC_{50}$ of 13 ± 4 $\mu$M ($n = 5$). Concentration-response curves were fitted based on the assumption that AA had a similar effect to the highest concentration of CA we used in our experiments. Cinnamaldehyde (*Bandell et al., 2004*) activated hTRPA1 with an $EC_{50}$ of 11 ± 2 $\mu$M, producing a maximum change of fluorescence of 431 ± 29% at 300 $\mu$M ($n = 8$) (Fig. 1). We were reluctant to use higher concentrations of CA because of the possibility of unspecific effects on the cells. Since these studies were completed, it has been reported that at concentration higher than 300 $\mu$M, CA has complex effects on TRPA1 reflecting both activation and inhibition of the channel (*Alpizar et al., 2013*). It is not possible to study this complexity using our experimental design. In our experiments, CA provides a constant reference response between experiments. Addition of the non-selective antagonist of TRPA1, ruthenium red (10 $\mu$M), largely blocked the increase of $[Ca]_i$ caused by AA (30 $\mu$M) and CA (300 $\mu$M) (Fig. 2). The specific antagonist of TRPA1, HC-030031 (30 $\mu$M) abolished the responses to 10 $\mu$M AA and 30 $\mu$M CA (Fig. 2). Cells that were not incubated with tetracycline 4–8 h prior to experimentation showed highly attenuated responses (Fig. 2). In order to test whether saturation of dye responses occurred during experiments, the effects of ionomycin (3 $\mu$M), an ionophore which elevates $[Ca]_i$, were determined. The responses to the highest concentrations of CA (300 $\mu$M) and AA (100 $\mu$M) tested were on average 63 ± 7% and 55 ± 6% of the response to 3 $\mu$M ionomycin, respectively. This indicates that the maximal TRPA1-mediated signal in our cells does not saturate the reporter dye and that we are working within the dynamic range of our experimental system.

To confirm that AA and CA were activating a membrane conductance, whole cell voltage clamp recordings were made from hTRPA1 HEK 293 cells induced overnight with a low concentration of tetracycline (1 $\mu$g mL$^{-1}$). Whole currents were elicited by repeatedly ramping the membrane potential of the cells from −80 mV to +80 mV over 500 ms. The holding potential was 0 mV. AA (10 $\mu$M) produced a rapid increase in membrane current measured at +80 mV (from a baseline of 280 ± 10 pA to a peak of 3.6 ± 1.0 nA, $n = 6$, Fig. 3) that was blocked by co-incubation of the cells with ruthenium red (RR, 10 $\mu$M; control 340 ± 9 pA; in AA and RR 247 ± 6 pA, $n = 6$). Superfusion of the cells with CA (100 $\mu$M) produced a similar current (baseline 306 ± 8 pA, peak 4.6 ± 1.5 nA, $n = 5$, Fig. 3).

Arachidonic acid can be metabolized to a number of molecules that activate TRPA1. To address the possibility that AA metabolites were mediating the observed effects, we preincubated cells with inhibitors of fatty acid amide hydrolase (FAAH), lipoxygenases and cyclooxygenases. *N*-arachidonoyl serotonin (NA-5HT, FAAH, *Maione et al., 2007*), caffeic acid, (lipoxygenases, *Koshihara et al., 1983*) and aspirin (cyclooxygenase, *Vane, 1971*) were used at a concentration of 10 $\mu$M and preincubated with the cells for 10 min before an addition of 10 $\mu$M arachidonic acid. The effect of AA was not altered by application of these enzyme inhibitors, ($P > 0.3$ for each; Fig. 4), leading us to believe

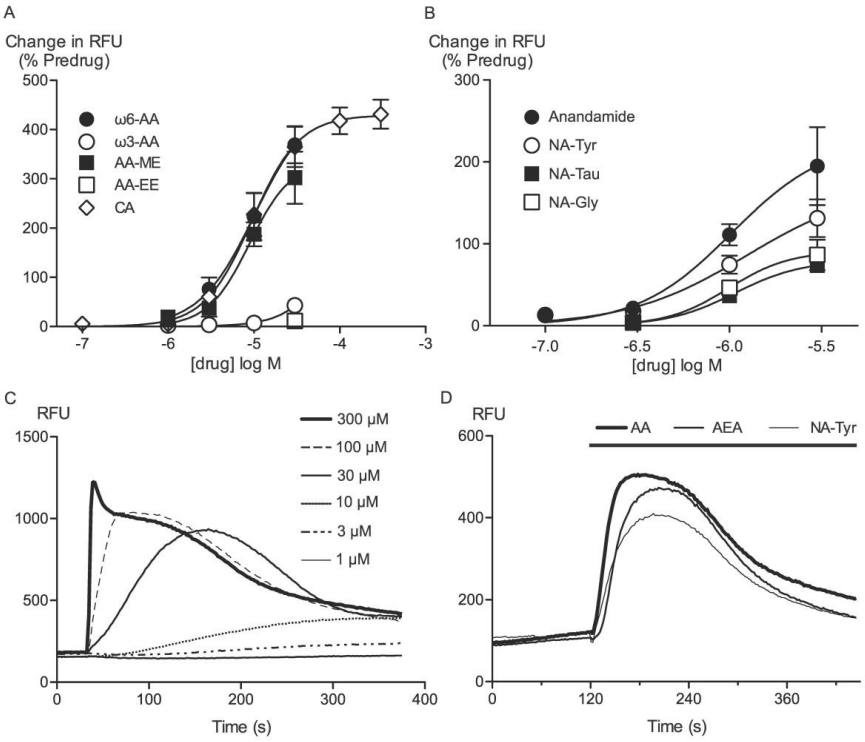


**Figure 1 Arachidonic acid and related molecules elevate calcium in HEK 293 cells expressing hTRPA1.** Changes in intracellular calcium concentration were determined as outlined in the Methods. (A) Concentration response curves for $\omega$6-arachidonic acid ($\omega$6-AA), $\omega$3-arachidonic acid ($\omega$3-AA), arachidonic acid methyl-ester (AA-ME), arachidonic acid ethyl-ester (AA-EE) and cinnamaldehyde (CA) at hTRPA1. Each data point represents the mean ± s.e.m. of 4–5 determinations in triplicate. The curves for AA and CA essentially overlap. (B) Concentration response curves for anandamide, *N*-arachidonoyl tyrosine (NA-Tyr), *N*-arachidonoyl taurine (NA-Tau) and *N*-arachidonoyl glycine (NA-Gly) at hTRPA1. Each data point represents the mean ± s.e.m. of 4–5 determinations in triplicate. (C) Representative traces of change in fluorescence produced by concentrations of CA between 1 $\mu$M and 300 $\mu$M, expressed as raw fluorescence units. CA was applied for the duration of the bar. (D) Representative traces of change in fluorescence produced by 30 $\mu$M anandamide, arachidonic acid and NA-Tyr, expressed as raw fluorescence units. Drugs were applied for the duration of the bar.

that the activation of TRPA1 by AA was direct, and not due to its modification via any of its main metabolic pathways.

We next examined the structural features of arachidonic acid (20:4 $\omega$6) relevant to TRPA1 activation. The relative insolubility of fatty acids meant that determining the maximal possible activation of TRPA1 for most compounds was not possible, and so we chose a fixed concentration of 30 $\mu$M to make comparisons with. Increasing or decreasing the degree of saturation on the fatty acid chain substantially or changing the

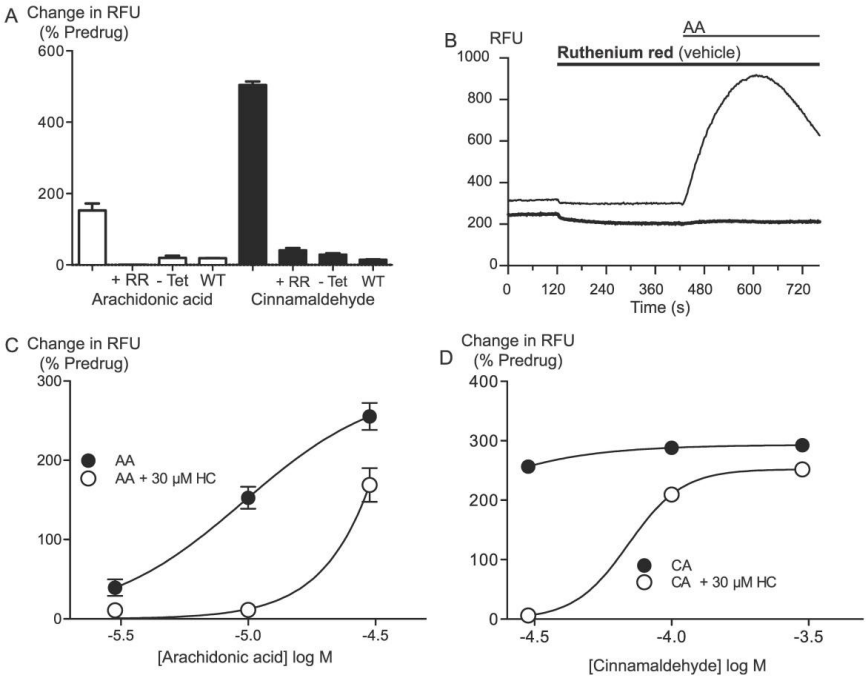


**Figure 2 Arachidonic acid activates hTRPA1.** Changes in intracellular calcium concentration ($[Ca]_i$) was determined as described in the Methods. (A) Elevations of $[Ca]_i$ produced by arachidonic acid (AA, 10 $\mu$M) were absent in Flp-In TRex HEK 293 expressing hTRPA1 not induced with tetracycline, and in untransfected Flp-In TRex HEK 293 cells. The effects of AA (10 $\mu$M) and cinnamaldehyde (CA, 30 $\mu$M) were also strongly reduced by ruthenium red (10 $\mu$M) (representative trace of AA in the presence of RR (B)). HC-030031 (30 $\mu$M), a specific inhibitor of TRPA1, inhibited the elevations of $[Ca]_i$ produced by AA (C) and CA (D) in an apparently competitive manner. Each point represents the mean ± s.e.m of at least 4 determinations. Error bars within the point for (C).

acyl chain length reduced the capacity of the ligand to activate hTRPA1. Docosahexaenoic acid (DHA 22:6 $\omega$3) and linoleic acid (18:2 $\omega$6) produced modest elevations of $[Ca]_i$ in hTRPA1-expressing HEK 293 cells when applied at 30 $\mu$M (Table 1). Adrenic acid (22:4 $\omega$6), oleic acid (18:1 cis-$\omega$9) and elaidic acid (18:1 trans-$\omega$9) produced changes in $[Ca]_i$ of less than 20% at 30 $\mu$M. Arachidonic acid methyl ester (30 $\mu$M) produced similar elevations of $[Ca]_i$ to AA (30 $\mu$M) (Table 1), however, arachidonic acid ethyl ester (30 $\mu$M) was essentially devoid of agonist activity at hTRPA1. Interestingly, $\omega$6-arachidonic acid had a greater agonist activity at hTRPA1 than $\omega$3-arachidonic acid (Table 1).

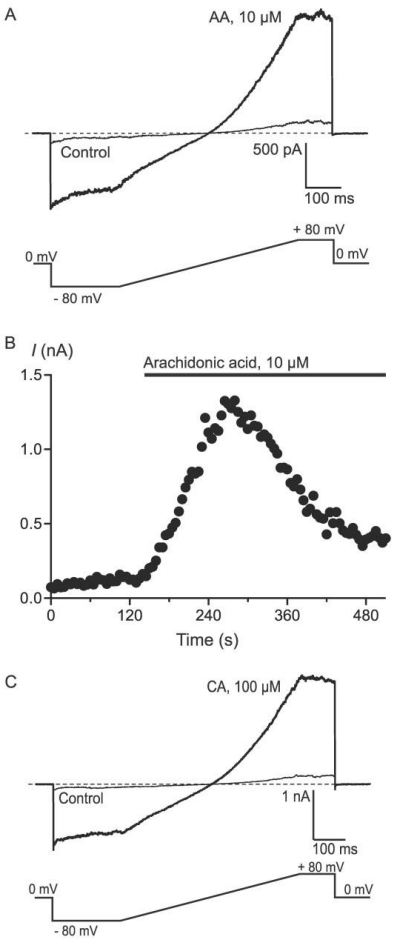


**Figure 3 Arachidonic acid-induced currents in HEK 293 cells expressing hTRPA1.** Whole voltage clamp recordings of membrane currents in HEK 293 cells expressing hTRPA1 were made as outlined in the Methods. (A) Current traces from a hTRPA1-expressing HEK 293 cell in control conditions (thin line) and in the presence of 10 $\mu$M arachidonic acid (AA). Cells were subject to the voltage protocol illustrated beneath the traces. Zero current is designated by the dotted line. (B) A plot of the amplitude of the cell current measured at +80 mV for the same cell, AA was added for the duration of the bar. Typical of 6 similar cells. (C) Current traces from a hTRPA1-expressing HEK 293 cell in control conditions (thin line) and in the presence of 100 $\mu$M cinnamaldehyde (CA). Cells were subject to the voltage protocol illustrated beneath the traces. Zero current is designated by the dotted line. Typical of 5 similar cells.

The first characterization of AA activation of TRPA1 was performed largely with mTRPA1 (*Motter & Ahern, 2012*), and so we compared fatty activation of mTRPA1 with that of hTRPA1 under our experimental conditions. In these experiments the effects of

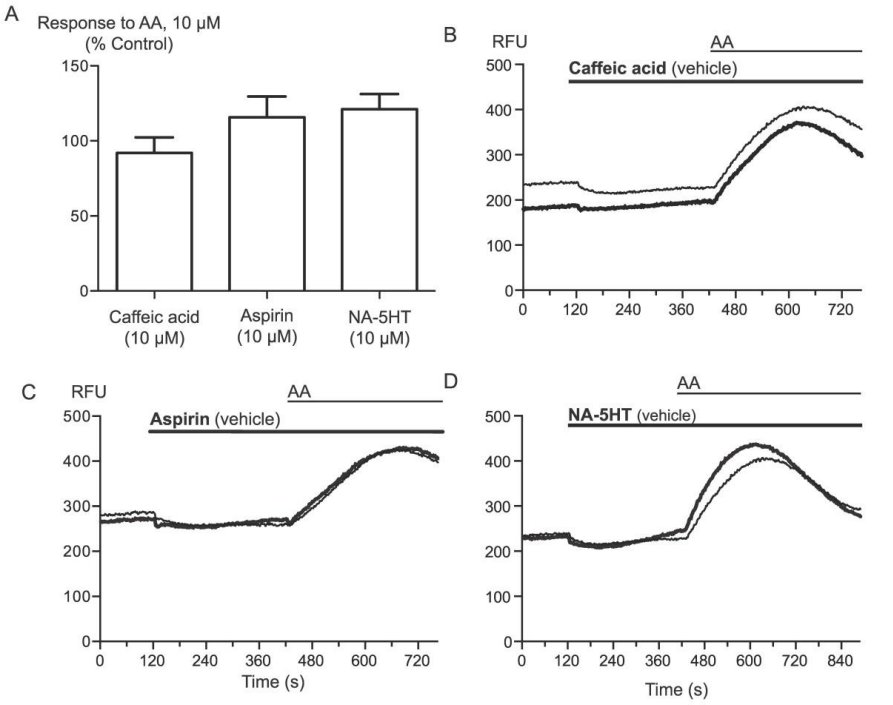


**Figure 4 Inhibitors of arachidonic acid metabolism do not affect arachidonic acid activation of TRPA1.** (A) Changes in intracellular calcium concentration were determined as described in the Methods. Pre-incubation of cells with inhibitors of lipoxygenase (caffeic acid, 10 $\mu$M), fatty acid amide hydrolase (*N*-arachidonoyl 5-HT, 10 $\mu$M) or cyclooxygenase (aspirin, 10 $\mu$M) did not affect elevations of $[Ca]_i$ produced by 10 $\mu$M arachidonic acid through hTRPA1 ($P > 0.35$ for each). Bar graphs represent the mean s.e.m of at least 8 independent determinations per condition. Representative traces for arachidonic acid by itself or in the presence of caffeic acid (B), aspirin (C) and N-arachidonoyl 5-HT (C) provided. They are respectively inhibitors of the lipoxygenase, cyclooxygenase pathways and an inhibitor of fatty acid amid-hydrolase. Each compound was used at 10 $\mu$M.

fatty acids were normalized to the effect produced by a high (300 $\mu$M) concentration of CA, to control for any differences between the number of channels expressed in the mTRPA1 and hTRPA1 cell lines. AA (100 $\mu$M) produced an increase in $[Ca]_i$ that was $74 \pm 12\%$ of that CA at hTRPA1, and $81 \pm 4\%$ at mTRPA1 ($n = 5$ each, $P > 0.6$). Both DHA (100 $\mu$M, $51 \pm 7\%$ and $32 \pm 6\%$ of CA at hTRPA1 and mTRPA1 respectively, $P = 0.125$) and $\omega$3-AA (100 $\mu$M, $16 \pm 5\%$ and $5 \pm 2\%$ of CA at hTRPA1 and mTRPA1 respectively) activated TRPA1 less than the equivalent concentration of AA.

Amino acid/neurotransmitter conjugates of arachidonic acid are a large group of AA derivatives with an incompletely characterized pharmacology. The prototypic NAAN, *N*-arachidonoyl glycine (NA-Gly) produced modest activation of hTRPA1 at the highest

**Table 1 Activation of hTRPA1 by arachidonic acid and derivatives.** Changes in intracellular calcium concentration were determined as outlined in the Methods. Each compound was applied to HEK 293 cells expressing hTRPA1 at a concentration of 30 $\mu$M. Activation of hTRPA1 by cinnamaldehyde was used as a positive control. The values represent the mean ± s.e.m. of the percent changes in raw fluorescence units, $n = 4$–5 determinations per compound.

| Compound | Change in RFU (% Predrug) |
|---|---|
| Cinnamaldehyde (300 $\mu$M) | 426 ± 28 |
| Arachidonic acid C20:4 $\omega$6 | 369 ± 38 |
| Arachidonic acid C20:4 $\omega$3 | 43 ± 10 |
| Arachidonic acid methyl ester | 302 ± 53 |
| Arachidonic acid ethyl ester | 12 ± 4 |
| Docosohexaenoic acid C22:6 $\omega$3 | 121 ± 24 |
| Linoleic acid C18:2 $\omega$6 | 49 ± 15 |
| Arachidonoyl ethanolamide | 195 ± 48 |
| Linoleoyl ethanolamide | 12 ± 5 |
| *N*-arachidonoyl tyrosine | 156 ± 18 |
| *N*-arachidonoyl taurine | 74 ± 26 |
| *N*-arachidonoyl glycine | 87 ± 19 |
| *N*-arachidonoyl dopamine | 32 ± 6 |
| *N*-oleoyl dopamine | 22 ± 3 |
| *N*-arachidonoyl 5-HT | 7 ± 2 |

concentration tested (30 $\mu$M), while the structurally similar endocannabinoid arachidonoyl ethanolamide (anandamide, C20:4 $\omega$6), robustly activated the channel (227 ± 42% increase in $[Ca]_i$, at 30 $\mu$M, Fig. 1, (*De Petrocellis & Di Marzo, 2009*). By contrast, lineoyl ethanolamide (C18:2 $\omega$6) was essentially inactive at hTRPA1. *N*-arachidonoyl tyrosine (NA-Tyr) also activated TRPA1 to a substantial degree (172 ± 20% increase in $[Ca]_i$ at 30 $\mu$M) but other NAAN with aromatic head groups, *N*-arachidonoyl dopamine (NA-DA), *N*-oleoyl dopamine (OL-DA) and NA-5HT, were ineffective at 30 $\mu$M (Table 1). *N*-arachidonoyl taurine (NA-Tau) was also a poor activator of TRPA1 (Table 1). OL-DA is also a potent inhibitor of 5-lipoxygenases (*Tseng et al., 1992*), but it failed to inhibit the effects of AA (30 $\mu$M), the elevation of $[Ca]_i$ was 115 ± 9% by AA alone, and 148 ± 20% in the presence of 30 $\mu$M OL-DA.

AA is unlikely to activate TRPA1 by covalent modification so we sought to determine whether there were differences between AA and CA activation of TRPA1. We first assessed whether AA and CA could activate the channel in a synergistic manner. Prior administration of subthreshold doses of AA (100 nM, 300 nM or 1 $\mu$M) failed to affect the concentration relationship of subsequently applied CA (1 $\mu$M to 300 $\mu$M, Fig. 5).

We next assessed whether activation of TRPA1 by high concentrations of either AA or CA affected the response to a subsequent addition of the other agonist. Application of either drug produced a robust increase in $[Ca]_i$ which declined over the next 15 to 20 min. Addition of CA (300 $\mu$M) after 30 min of AA (100 $\mu$M) produced a very small increase in $[Ca]_i$, as did another addition of AA (100 $\mu$M) at this point. Similarly, application of AA (100 $\mu$M) following CA (300 $\mu$M) also produced only a small increase in $[Ca]_i$. Thus, each agent produced essentially complete cross-desensitization to the

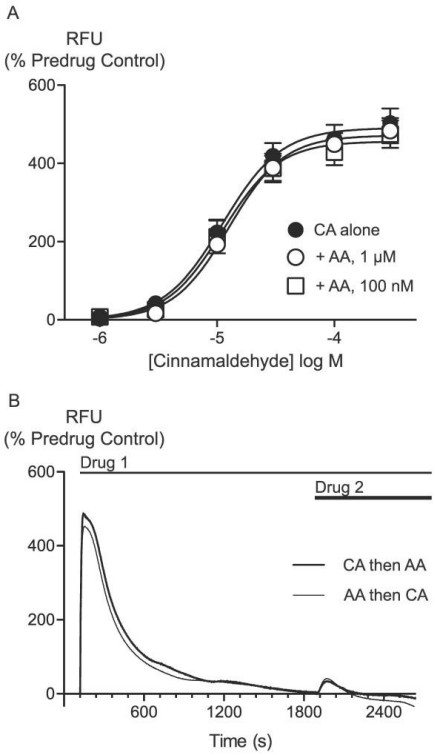


**Figure 5 Lack of interaction between arachidonic acid and cinnamaldehyde in activation of hTRPA1.** Changes in intracellular calcium concentration were determined as outlined in the Methods. (A) Concentration response curves for cinnamaldehyde (CA) in control conditions, and in the presence of arachidonic acid (AA, 100 nM, 1 $\mu$M). Each point represents the mean ± s.e.m. of 4–5 determinations in triplicate. (B) Example traces showing reciprocal cross-desensitization between CA (300 $\mu$M) and AA (100 $\mu$M). Drugs were applied for the duration of the bars. Traces represent typical data from 4-5 independent replicates per condition. RFU = raw fluorescence units.

other (Fig. 5, Table 2). Ionomycin (30 $\mu$M) administered 30 min after the addition of AA (100 $\mu$M) produced an increase in $[Ca]_i$ of 1730 ± 45%, similar to the elevation of $[Ca]_i$ seen when ionomycin 30 $\mu$M is added 30 min after a solvent blank (1390 ± 15%). This indicates that the reduced responses to AA and CA after desensitization were not due to non-specific effects of prolonged elevations of $[Ca]_i$ on the fluorescent dye or cells.

Previous studies have identified 3 intracellular, N-terminal cysteine residues required for hTRPA1 activation by CA (*Hinman et al., 2006*). We examined the effects of mutating all of these residues, Cys 621, Cys 641 and Cys 665 to serine on the activation of hTRPA1 by AA. As previously reported (*Hinman et al., 2006*), application of CA to the 3x Cys mutant hTRPA1 produced essentially no activation of the channel (Fig. 6). By contrast,

**Table 2 Activation of hTRPA1 by cinnamaldehyde or arachidonic acid inhibits subsequent agonist activation of the channel.** Changes in intracellular calcium concentration were determined as outlined in the Methods. Maximally effective concentrations of cinnamaldehyde (CA, 300 $\mu$M) or arachidonic acid (AA, 100 $\mu$M) were applied to HEK 293 cells expressing hTRPA1. Either CA or AA was then applied 30 min later. The first exposure to each agonist essentially abolished the subsequent response. The values represent the mean ± s.e.m. of the percent changes in raw fluorescence units, $n = 3$–5 determinations per condition.

| Drug additions | 1st addition (change in RFU, % predrug) | 2nd addition (change in RFU, % predrug) |
|---|---|---|
| CA then AA | 506 ± 31 | 32 ± 31 |
| AA then Ca | 486 ± 47 | 42 ± 14 |
| CA then CA | 560 ± 20 | −3 ± 6 |
| AA then AA | 486 ± 43 | 57 ± 44 |

the effects of AA were reduced but not abolished in the 3x Cys mutant hTRPA1 (Fig. 6). In these studies we extended the AA concentration response curve to include a concentration of 100 $\mu$M, the resulting $EC_{50}$ in wt hTRPA1 was 13 ± 4 $\mu$M, with a maximum increase in fluorescence of 390 ± 70%. AA (100 $\mu$M) increased fluorescence in 3x Cys mutant hTRPA1 by 155 ± 30%. NPPB is another agonist of TRPA1 which does not bind to the reactive cysteine residues (*Liu et al., 2010*), NPPB-induced elevations of calcium were also reduced but not abolished in the 3x cysteine mutant of hTRPA1 (Fig. 6). These data suggest that mutation of the cysteine residues can reduce but not abolish the activation of TRPA1 by unreactive compounds not structurally related to AA.

## DISCUSSION

The principle finding of our study is that AA and NAANs activate human TRPA1, although NAAN do so less effectively than AA. The activation of TRPA1 by AA and related compounds has a distinct profile from that reported for these compounds at other ion channels such as TRPV1 or $Ca_V3$ calcium channels. We also found that the AA activation of TRPA1 is only partially dependent on the presence of 3 N-terminal intracellular Cys residues that are required for activation of the channel by reactive electrophiles such as cinnamaldehyde. Our data is broadly consistent with studies reporting that AA (10 $\mu$M, (*Bandell et al., 2004*)) and docosohexanoic acid (*Motter & Ahern, 2012*) activate rodent TRPA1, but there appear to be differences in the effects of fatty acids and related compounds at human TRPA1.

We are confident that the effects of AA were being mediated by direct activation of TRPA1, and not by AA metabolites or though unspecific actions of AA on $[Ca]_i$. Under our experimental conditions, AA produced negligible increases in $[Ca]_i$ in untransfected HEK 293 cells, or in HEK 293 cells where TRPA1 expression had not been induced by tetracycline. Further, the effects of AA were blocked by specific (HC-030031, *McNamara et al., 2007*) and non-specific (ruthenium red) TRPA1 antagonists. AA is the parent molecule of a number of reactive compounds that can activate TRPA1 (e.g. *Materazzi et al., 2008*; *Taylor-Clark et al., 2008*; *Gregus et al., 2012*; *Sisignano et al., 2012*), however, the effects of AA were not modified by preincubation of HEK 293 cells with inhibitors of

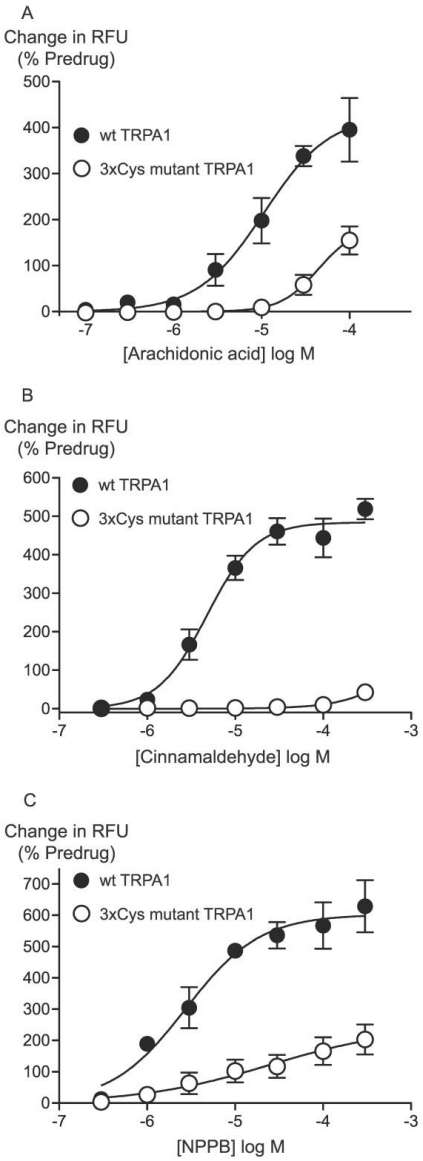


**Figure 6 Distinct amino acid residues in hTRPA1 determine arachidonic acid and cinnamaldehyde activation.** Changes in intracellular calcium concentration were determined as outlined in the Methods. Mutation of Cys621, Cys641 and Cys665 to Ser (3xCys mutant) prevents (A) cinnamaldehyde activation of hTRPA1 but only abrogates (B) arachidonic acid and (C) NPPB activation of the channel. Each point represents the mean ± s.e.m. of at least 5 independent determinations in triplicate. RFU = raw fluorescence units.

cyclo-oxygenase, lipoxygenase and FAAH, making it likely that AA itself was activating TRPA1. It is worth noting that the potency of AA to activate hTRPA1 is very similar to those of prostaglandin-derived TRPA1 agonists identified in previous studies (*Taylor-Clark et al., 2008*; *Materazzi et al., 2008*), but it is significantly less potent than hepoxilin A3 (*Gregus et al., 2012*) or 5,6 epoxyeicosatrienoic acid (*Sisignano et al., 2012*). Thus, metabolism of AA by 12-lipoxygenase or cytochrome P450 epoxygenase may increase the tissue availability of TRPA1 activators, while metabolism via cyclooxygenase is unlikely to, unless the derivatives were substantially more stable than AA, or if they were selectively available in a tissue compartment where AA levels were low.

Arachidonic acid was the most potent activator of hTRPA1 of the fatty acids we examined. In a study published while the present work was in preparation, (*Motter & Ahern, 2012*) used electrophysiological techniques to examine the effects of fatty acids on TRPA1. They focussed on docosahexaenoic acid as their reference compound. DHA activated rTRPA1 with an $EC_{50}$ between 11 and 40 $\mu$M, depending on the membrane potential where activation was measured. At a concentration of 100 $\mu$M, AA produced a similar increase in current to DHA, and this increase was similar to that produced by a high concentration of AITC (1 mM), a covalent TRPA1 agonist. In our experiments, which measured elevations in $[Ca]_i$ produced by activation of hTRPA1, the $EC_{50}$ of AA was about 10 $\mu$M. Interestingly, at 100 $\mu$M, DHA produced significantly less activation of both human and mouse TRPA1 than AA, while adrenic acid (C22:4) was inactive. Fatty acids with shorter acyl chains were also much less active than AA. We also found that $\omega$3-AA was much less effective than $\omega$6-AA at both human and mouse TRPA1. Interestingly, the carboxylic acid moiety of AA appears to be unnecessary for activation of TRPA1, as AA-ME was almost as equally effective as AA, and arachidonoyl ethanolamide and other NAAN retained substantial TRPA1 agonist activity.

*Motter & Ahern (2012)* did not directly compare the potencies of different fatty acids at TRPA1, and our results are largely consistent with theirs, with the exception of the relatively low activity of $\omega$3AA in the present study. It should be emphasized that there are significant differences in the methodology between the two studies. Firstly, our population measurements of TRPA1 activation were conducted at physiological temperature (37°C), a temperatures close to that at which TRPA1 undergoes temperature-dependent inactivation (*Wang et al., 2012*), while the study of Motter and Ahern was done at room temperature, conditions which may favour ligand activation of the channel. Secondly, our work measures both calcium influx through TRPA1 and any subsequent release of intracellular calcium or calcium entry from outside the cell produced by this, which may have an amplifying effect on the signal. Thirdly, our studies were done with the membrane potential of the cells free to vary between the resting potential of CHO cells and the reversal potential of TRPA1 (around 0 mV), the study of (*Motter & Ahern, 2012*) showed some voltage-dependence in the potency of DHA, with the compound being more potent at highly depolarized potentials. Finally, our work is done in intact cells, which may allow distinct mechanisms of channel modulation to those happening in cells subject to whole cell patch-clamp recordings. Nevertheless,

despite the recognized differences in pharmacology between rodent and human TRPA1, arachidonic acid and related compounds appear to act in a qualitatively similar manner.

*N*-acyl amino acids are a large family of lipid mediators that affect a variety of ion channels and receptors important for nociception (*Connor, Vaughan & Vandenberg, 2010*). None of the NAAN tested in the present study were as effective as AA or AEA in activating TRPA1. The most effective was NA-Tyr, with NA-Gly and NA-Tau being less active and NA-DA, NO-DA and NA-5HT being essentially inactive. This profile is quite distinct from that of these compounds at other well characterized effectors, TRPV1 and $Ca_V3$ channels. Notably, NA-DA and NO-DA are agonists at TRPV1 (*De Petrocellis et al., 2004*), while NA-5HT and NA-Tyr are antagonists (*Maione et al., 2007*, Connor, M *et al.* unpublished observations). Neither NO-DA nor NA-5HT inhibited the effects of AA at hTRPA1, suggesting that they interact with TRPA1 very weakly if at all. When considering NAAN modulation of $Ca_V3$ channels, both NA-5HT and NA-DA inhibit these channels with sub-micromolar potencies, as does AEA (*Chemin et al., 2001*; *Ross, Gilmore & Connor, 2009*; *Gilmore et al., 2012*). AA also inhibits $Ca_V3$ channels, but less potently than AEA, NA-5HT or NA-DA (*Chemin, Nargeot & Lory, 2007*; *Ross, Gilmore & Connor, 2009*; *Gilmore et al., 2012*). The rank order of effectiveness for fatty acid inhibition of human $Ca_V3$ channels, C22:6 $\approx$ C22:4 $\approx$ C20:4 > C20:2 > C20:1 > C20:4-methyl ester; (*Chemin, Nargeot & Lory, 2007*)) is quite distinct from that for activation of TRPA1, where C20:4 $\geq$ C20:4-methyl ester > C22:6 $\gg$ C22:4 $\approx$ C20:2 $\approx$ C20:1. The binding site for AA and related compounds has not been identified on either TRPA1 or $Ca_V3$ channels, but the distinct ligand/activity profiles at these channels suggests specific sites of interaction rather than un-specific interactions with the lipid membrane. This idea is reinforced by the very limited effects of the membrane fluidity-modifying detergent Triton-X 100 on TRPA1 (*Motter & Ahern, 2012*).

Several regions of TRPA1 have been shown to interact with ligands. The N-terminal ankyrin repeat domain of hTRPA1 is of major importance for the binding of reactive compounds, with three specific cysteine residues, Cys 621, Cys 641 and Cys 665 identified as crucial for channel activation by AITC and CA (*Hinman et al., 2006*). Mutation of these cysteines and lysine 708 also prevented activation of TRPA1 by 4-hydroxynonenol (*Trevisani et al., 2007*). By contrast, menthol and thymol agonist activity is dependent on specific residues in the fifth transmembrane domain (TM5) of hTRPA1 (*Xiao et al., 2008*) while the channel domains required for hTRPA1 activation by NPPB and farnesyl thiosalicylic acid remain incompletely defined. AA most resembled NPPB in that it retained significant activity in hTRPA1 where Cys621, Cys641 and Cys 665 had been mutated to serine. This channel was largely insensitive to CA. The requirement for intact Cys621/641/665 for full agonist activity of AA and NPPB has not been reported before, but likely reflects the importance of Cys-Cys cross-links involving Cys621 and Cys665 and other N-terminal Cys residues in maintaining the conformation of TRPA1 (*Wang et al., 2012*), rather than indicating that AA or NPPB covalently modify hTRPA1. Our data are consistent with those of (*Motter & Ahern, 2012*), who showed that the presence

of the N-terminal domain of murine TRPA1 was necessary but not sufficient for activation of mouse/drosophila TRPA1 chimeras.

Arachidonic acid is major signalling molecule derived from the actions of phospholipase $A_2$ on membrane phospholipids, and it acts directly on a diverse range of ion channels as well as serving as a precursor for a host of other molecules which activate or inhibit ion channels. Based on the affinity of AA for cyclooxygenase and lipoxygenase enzymes it has been suggested that concentrations of AA up to about 30 $\mu$M may be physiologically relevant (*Attwell, Miller & Sarantis, 1993*). TRPA1 is strongly expressed in subpopulations of sensory neurons and various epithelial cells throughout the body (*Bodkin & Brain, 2011*). There is also evidence for TRPA1-mediated modulation of neurotransmission in brain (*Shigetomi et al., 2011*). Thus, AA actions at TRPA1 could potentially modulate peripheral nociception, central neurotransmission, as well as lung, bladder and cardiovascular function. Our data suggests that there is a specific site where long chain fatty acids or endocannabinoids can interact with and activate TRPA1. Whether this site is the same as that utilized by arachidonic-acid derived molecules is unknown, although it is tempting to speculate that there may be an agonist site utilized by AA that may also provide a binding pocket for AA-derivatives such as 5,6 EET to facilitate their access to the N-terminal Cys residues of TRPA1 required for their activity (*Sisignano et al., 2012*). The definition of the AA binding determinants of TRPA1 may provide insights not only into how this channel is activated, but also how novel antagonists may be developed.

**Abbreviations**

**AA** arachidonic acid (20:4 $\omega$6)

**AEA** arachidonoyl ethanolamide

**AITC** allyl isothiocyanate

**CA** cinnamaldehyde

**$[Ca]_i$** intracellular calcium

**DHA** docosohexaenoic acid

**HBS** HEPES buffered saline

**hTRPA1** human transient receptor potential cation channel subfamily A, member 1

**NAAN** *N*-acyl neurotransmitter/amino acid conjugate

**NA-5HT** *N*-arachidonoyl serotonin

**NA-DA** *N*-arachidonoyl dopamine

**NA-Gly** *N*-arachidonoyl glycine

**NA-Tyr** *N*-arachidonoyl tyrosine

**NA-Tau** *N*-arachidonoyl taurine

**NPPB** 5-Nitro-2-(3phenylpropylamino)benzoic acid

## ADDITIONAL INFORMATION AND DECLARATIONS


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### Competing Interests

The authors declare that they have no competing interests.

### Author Contributions

- William John Redmond performed the experiments, analyzed the data, wrote the paper.
- Liuqiong Gu and Maxime Camo performed the experiments, analyzed the data.
- Peter McIntyre conceived and designed the experiments.
- Mark Connor conceived and designed the experiments, wrote the paper.

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## Author Contributions

MC Sr and WJR conceived the study. WJR performed the calcium measurements, MC Jr did the electrophysiology, LG and PM did the inducible hTRPA1 receptor. WJR and MC Sr wrote the paper.

# Chapter 5 Paper 4

# Nordihydroguaiaretic acid activates hTRPA1

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# Abstract

## Background and Purpose

Nordihydroguaiaretic acid (NDGA) is a major biologically active component of the creosote bush, *larrea tridentate*, and inhibits lipoxygenase enzymes, the SP1 transcription factor and is an antioxidant. NDGA is also used as a "natural" therapeutic agent, and a semisynthetic derivative, meso-tetra-O-methyl nordihydroguaiaretic acid (terameprocol), is being trialled as an anti-cancer agent. Whilst investigating the potential contribution of metabolites of arachidonic acid to activation of the transient receptor potential cation channel subfamily A, member 1 (TRPA1), we found that NDGA alone affected the receptor. This study investigates the activation of TRPA1 by NDGA and terameprocol, and their effects on sensitivity to noxious cold.

## Experimental Approach

hTRPA1 was stably expressed in HEK 293 cells, and channel activations was studied by measuring changes in intracellular calcium ($[Ca]_i$) and whole cell patch clamp recordings. The effects of local NDGA and terameprocol application on acetone-induced paw flinching were examined in mice.

## Key Results

NDGA stimulated hTRPA1 increases in $[Ca]_i$ with a $pEC_{50}$ of 5.4 ± 0.1, and a maximum change in fluorescence of 385 ± 30%. The $[Ca]_i$ and NDGA-induced increase in membrane conductance in hTRPA1 expressing cells was

prevented by the TRPA1 inhibitor HC-030031 30 μM. Terameprocol stimulated hTRPA1 increases in $[Ca]_i$ with a $pEC_{50}$ of 4.5 ± 0.2, and a maximum change in fluorescence of 550 ± 75%. NDGA and terameprocol alone did not produce pain behaviours in mice after hindpaw injection, but did enhance responses to acetone.

**Conclusions and Implications**

NDGA and terameprocol are efficacious activators of TRPA1 and enhance noxious cold stimuli in mice. NDGA should be used with care to probe lipoxygenase involvement in nociception, and further studies are required to assess the safety of NDAG and derivatives as therapeutic agents.

Abbreviations

| | |
|---|---|
| CA | cinnamaldehyde |
| $[Ca]_i$ | intracellular calcium concentration |
| CIPN | chemotherapy-induced peripheral neuropathy |
| HBS | Hepes buffered saline |
| HBSS | modified Hanks balanced salt solution |
| hTRPA1 | human transient receptor potential ankyrin 1 |
| NDGA | nordihydroguaiaretic acid |
| OMeNDGA | tetra-O-methyl nordihydroguaiaretic acid, terameprocol |
| ROS | reactive oxygen species |

# Introduction

Nordihydroguaiaretic acid (NDGA) is a major pharmacologically active component of the creosote bush (*Larrea tridentata*). Creosote extracts ("Chaparral tea") have been traditionally used to treat a wide variety of conditions (Arteaga *et al.*, 2005), and continue to be advertised extensively in unregulated environments, despite well recognized toxicity (Sheikh *et al.*, 1997) and the banning of NDGA from food in the United States more than 40 years ago (http://www.accessdata.fda.gov). NDGA and related molecules inhibit a variety of enzymes and transcription factors and some of these effects are conceivably responsible for anti-inflammatory properties attributed to crude preparations. More recently, the synthetic derivative of NDGA, tetra-O-methyl NDGA (terameprocol, formerly MN4 or EM-1421) has been trialled as an anti-retroviral and anti-cancer agent, largely because of its inhibition of the transcription factor Sp1.

NDGA has been widely used in experimental studies of inflammation (Bhattacherjee *et al.*, 1988; Salari *et al.*, 1984) and its main mechanism of action appears to be through its unspecific inhibition of lipoxygenase (Argentieri *et al.*, 1994; Hope *et al.*, 1983; Lu *et al.*, 2010b) and prevention of oxidization (Floriano-Sanchez *et al.*, 2006; Lu *et al.*, 2010a) by acting as a scavenger of various reactive oxygen species (ROS). It has also been demonstrated, partly via these lipoxygenase and antioxidant properties, as being a tumor growth suppressant (Kubow *et al.*, 2000; Park *et al.*, 2004)

and can lower virus activity, such as the human papilloma virus and HIV (Craigo *et al.*, 2000; Hwu *et al.*, 2008).

While investigating the activation of the pronociceptive ion channel TRPA1 by arachidonic acid and potential metabolites (Redmond WJ, 2014), we unexpectedly found that NDGA itself activated human TRPA1 with a potency similar to that of the widely used agonist cinnamaldehyde (CA). The aims of this study were thus to investigate the pharmacological profile of NDGA as an agonist at the human TRPA1 receptor, as well as to verify if this activation could cause pain-related behaviors in an mice, as other members of this family do. The discovery of TRPA1 agonist activity for NDGA and its derivative teramepracol may provide insight into their potential therapeutic mechanism(s) of action, but also suggest that care should be taken when attributing their biological effects as being solely mediated through enzyme inhibition.

# Methods

*Cell culture*

Flp-In TRex HEK 293 (Life Technologies, Mulgrave, Victoria, Australia) stably transfected with wild type or mutant hTRPA1 (Redmond WJ, 2014) were cultivated in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U penicillin and 100 µg streptomycin $ml^{-1}$, hygromycin B 25 µg $ml^{-1}$ and blasticidin S 5 µg $ml^{-1}$. Cells were incubated in 5% $CO_2$ at 37˚C in a humidified atmosphere. Cells were grown in flasks with a surface area of 75 $mm^2$, once at optimum confluence (approximately 90 %), cells were trypsinized and transferred into clear-bottomed poly-D-lysine coated 96 well plates (Corning, Castle Hill, NSW, Australia) in L15 medium supplemented with 1% fetal bovine serum, hygromycin B, and the antibiotics outlined above. The cells were plated in a volume of 100 µL and were incubated in humidified room air at 37˚C overnight. Expression of the TRPA1 receptor or mutants was induced 5-8 hours prior to experimentation by addition of tetracycline, 1 µg $ml^{-1}$ to each well. Wildtype cells did not express detectable TRPA1 activity. Both wildtype and mutant TRPA1 were expressed under the control of of a tetracycline-sensitive repressor, and expression shortly before experiments.

*Calcium assay*

Intracellular calcium $[Ca]_i$ was measured with the calcium 5 kit from Molecular Devices (Sunnyvale, CA, USA) using a FLEX Station 3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). 100 µl of dye dissolved in

modified Hanks balanced salt solution (HBSS) containing (in mM): NaCl 140, KCl 5.33, $CaCl_2$ 1.3, $MgCl_2$ 0.5, HEPES 22, $Na_2HPO_4$ 0.338, $NaHCO_3$ 4.17, $KH_2PO_4$ 0.44, $MgSO_4$ 0.4, glucose 10 (pH to 7.3, osmolarity = 330 ± 5 mosmol) was loaded into each well of the plate and incubated in room air for 1 hour at 37˚C. All experiments in the Flexstation were carried out at 37 $^oC$. Calcium 5 fluorescence was measured every 2 seconds ($\lambda_{excitation}$ = 485nm, $\lambda_{emission}$ = 525 nm) for the duration of the experiment. Drugs were added after at least 2 minutes of baseline recording. In experiments where one drug addition was made, 50 µL of drug dissolved in HBSS was added, for two drug additions, 25 µL was added each time.

*Electrophysiology*

TRPA1 channel currents were recorded in the whole-cell configuration of the patch-clamp method (Hamill *et al.*, 1981) at room temperature. Dishes were perfused with HEPES buffered saline (HBS) containing (in mM): 140 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$. 10 HEPES, 10 Glucose (pH to 7.3, osmolarity = 330 ± 5 mosmol). Recordings were made with fire-polished borosilicate glass pipettes with resistance ranging from 2-3 MΩ. The internal solution contained (in mM): 130 CsCl, 10 HEPES, 2 $CaCl_2$, 10 EGTA, 5 MgATP (pH to 7.3, osmolarity = 285 ± 5 mosmol). Recordings were made with a HEKA EPC 10 amplifier with Patchmaster acquisition software (HEKA Elektronik, Germany). Data was sampled at 10 kHz, filtered at 3 kHz, and recorded on hard disk for later analysis. Series resistance ranged from 3 to 10 MΩ, and was compensated by at least 80% in all experiments. Leak subtraction was

not used. Cells were exposed to drugs via flow pipes positioned approximately 200 μm from the cell, drugs were dissolved in HBS immediately before application. All solutions had final ethanol concentration of 0.1% v/v.

*Data analysis*

For measurements of drug-induced changes in calcium 5 dye fluorescence, which reflects changes in intracellular concentration ($[Ca]_i$), the response to agonists was expressed as a percentage change over the baseline averaged for the 30 seconds immediately prior to drug addition. Changes produced by parallel solvent blanks were subtracted, these changes were never more than 10 % of baseline. Concentration-effect data from independent experiments, each performed in duplicate or triplicate, was pooled and fit to a four-parameter logistic Hill equation to derive the $EC_{50}$ values and Hill slope (GraphPad Prism, San Diego, CA). Results are expressed as mean ± s.e.m. of at least 4-5 independent experiments unless otherwise stated.

*Behavioural studies*

Experiments were carried out on adult male C57BL/6 mice following the guidelines of the 'NH&MRC Code of Practice for the Care and Use of Animals in Research in Australia' and with the approval of the Royal North Shore Hospital Animal Care and Ethics Committee. Mice initially weighed between 20 – 25 g, and were housed in groups of three in individually ventilated

cages (23 ± 1 °C, humidity 70%) with environmental enrichment and free access to food and water, in a 12:12 hour light-dark cycle.

Animals were allowed to acclimatize to their holding cages and the behavioural testing chambers for 2 - 3 days before any procedures were carried out. All testing was carried out in low level white light (<3 lux). To assess cold sensitivity, the mice were allowed to acclimatise for 20 - 30 minutes prior to testing in elevated perspex cages (15 x 10 x 10 cm) with a wire mesh floor, and 20 µl of acetone was sprayed onto the plantar surface of the left hind paw (Bautista *et al.*, 2006). The number of left hind limb lifts, shakes and licks was then counted over a 2 minute period. Solutions of drugs for intraplantar injection were made up in a vehicle solution which comprised 25 % dimethylsulfoxide (DMSO) and 10 % Tween80 in saline. Intraplantar injections were made in a volume of 15 µl, in under brief isoflurane anaesthesia (2.5 % in saturated $O_2$, 1ml.min$^{-1}$) using a 30-gauge needle. Solutions of drugs for systemic injection were made up in a vehicle solution (15 % dimethylsulfoxide (DMSO) in saline) and were injected intraperitoneally at a volume of 0.12 ml/10g in lightly restrained animals.

In experiments investigating the effect of TRPA1 agonists the protocol was: three predrug behavioural measurements (at 0, 15, 30 minutes), intraplantar agonist or vehicle injection (at 45 minutes), then five behavioural measurements (60, 75, 105, 135 and 165 minutes = 15, 30, 60, 90 and 120 minutes post-agonist). In experiments on the effect of HC-030031 on the TRPA1 agonists the protocol was: three predrug behavioural measurements

(at 0, 15, 30 minutes), systemic injection of HC-030031 (at 45 minutes), two behavioural measurements (at 60, 75 minutes), intraplantar agonist or vehicle injection (at 90 minutes), three behavioural measurements (at 105, 120 and 165 minutes = 15, 30, 60, 90 and 120 minutes post-agonist). Animals were euthanized at the end of the testing period. The experimenter was blinded to the agents being tested.

For the time course experiments, comparisons of drug/vehicle treatment effects over time were made using two-way repeated measures ANOVAs, with time and treatment as a within- and between-subjects factors, respectively (GraphPad Prism). When two-way ANOVAs were significant, post-hoc comparisons between treatment groups at individual time points were made using the Bonferroni adjustment for multiple comparisons. To measure net drug effects, the post-drug measures for acetone induced lifts/shakes/lifts and thermal PWL measures were taken as the average of measurements over 15 – 60 minutes post-drug injection and compared to the pre-injection baseline values (subtracted to give the change relative to baseline). Dose response curves were constructed by fitting data to a four parameter logistic Hill equation (GraphPad Prism). Statistical comparisons of the effect of cinnamaldehyde and NDGA in the presence and absence of antagonists were made using two-way ANOVAs and when significant, post-hoc comparisons were made using the Bonferroni adjustment for multiple comparisons.

*Drugs and reagents*

Drugs for in vitro experiments were dissolved in ethanol and diluted in HBS to give a final concentration of ethanol of 0.05 - 0.1%. NDGA, terameprocol and HC 030031 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Ruthenium red was from Enzo Lifesciences (Farmingdale, NY, USA). Ionomycin was from Ascent Scientific (Avonmouth, UK). CA was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All tissue culture reagents were from Sigma-Aldrich, Life Technologies (Mulgrave, Victoria, Australia) or Invivogen, (San Diego, CA, USA).

# Results

NDGA produced a robust elevation of $[Ca]_i$ in HEK-293 cells expressing hTRPA1, but only a small change in HEK-293-TRPA1 cells where TRPA1 expression had not been induced by tetracycline (Figure 1). The effects of NDGA (30 μM) on $[Ca]_i$ were antagonized by pre-incubation of the cells with the TRPA1 antagonist HC-030031 (30 μM, Figure 1). NDGA increased calcium 5 dye fluorescence with a $pEC_{50}$ of 5.4 ± 0.1, while in parallel experiments, the prototypic TRPA1 agonist cinnamaldehyde (CA) increased fluorescence with a $pEC_{50}$ of 5.3 ± 0.1 (n=5, Figure 2). The maximally effective concentration of NDGA (100 μM) produced a smaller change in fluorescence than a high concentration of CA (300 μM, 385 ± 30% vs 520 ± 25 %, $P < 0.01$, n=5). Since these studies were completed, it has been reported that higher concentration than 300 μM CA can show complex dual activation/inhibition on the TRPA1 channel (Alpizar *et al.*, 2013). Studying this complexity for CA compared to NDGA was out of the scope of this study and was not possible in our experimental design.

To confirm that NDGA was activating a membrane conductance, whole cell voltage clamp recordings were made from hTRPA1 expressing HEK 293 cells induced overnight with a low concentration of tetracycline (1 μg $mL^{-1}$). Whole cell currents were elicited from a holding potential of 0 mV by repeatedly ramping the membrane potential of the cells from -80 mV to + 80 mV over 500 ms, once every 5 s (Redmond *et al.,* 2014). NDGA (10 μM)

produced a rapid increase in membrane current measured at +80 mV (from a baseline of 150 ± 8 pA to a peak of 2.2 ± 0.1 nA, n=6, Figure 3) that was strongly attenuated by co-incubation of the cells with the TRPA1 antagonist HC 030031 (30 μM; control 94 ± 3 pA; after 2 min in HC 030031 110 ± 3 pA; after 3 min in HC 030031 and NDGA, 132 ± 2 pA n=6, Figure 3).

CA and other reactive electrophiles require Cys residues in the intracellular N-terminal of TRPA1 to activate the channel, but this requirement is not shared to the same degree by all TRPA1 agonists {Macpherson, 2007 #41}. NDGA elevated $[Ca]_i$ with an $EC_{50}$ of 4.9 ± 1.7 μM in cells expressing wild type hTRPA1, and 18 ± 3 μM in cells expressing the 3xCys hTRPA1 mutant ($P < 0.01$, n=6). The maximum elevation of $[Ca]_i$ by NDGA was significantly greater in cells expressing wild type hTRPA1 (365 ± 15 %) than in cells expressing the 3xCys mutant hTRPA1 (85 ± 16 %, $P < 0.001$).

Tetra-O-methyl-NDGA (OMeNDGA, terameprocol) is an analog of NDGA being developed as a chemotherapeutic agent. Terameprocol also produced a concentration-dependent increase in calcium 5 fluorescence in cells expressing hTRPA1, with a $pEC_{50}$ of 4.5 ± 0.2 and a maximum change in fluorescence of 550 ± 75 % (300 μM, Figure 5). The effects of terameprocol (30 μM) were antagonized by pre-incubation of cells with HC 030031 (Figure 5, $P < 0.001$, n=6).

In C57BL/6 mice intraplantar injection of NDGA and cinnamaldehyde at doses of up to 300 and 1,000 nmol, respectively, had no effect on hind paw movement and produced no overt behavioural effects. At doses of 3,000 nmol and above, cinnamaldehyde produced whole body responses which were relatively delayed in onset, and included decreased movement and shaking/shivering (Figure 6).

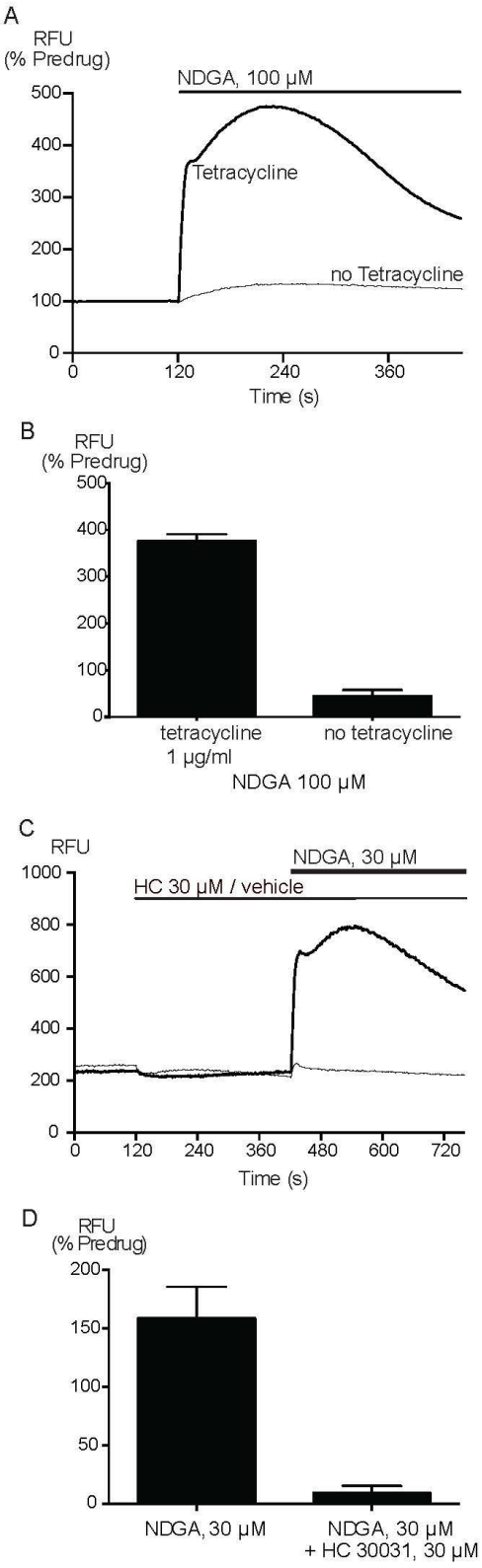
When acetone was sprayed onto the plantar surface of the hind paw before drug injection, it produced on average of 2.0 ± 0.1 localised hind limb responses (hind paw lifts/flinches/licks) which lasted an average of 7.3 ± 0.3 s. Intraplantar injection of cinnamaldehyde produced an increase in the number of localised hind limb responses to acetone which peaked at 15 – 30 min post-injection (Figure 6A). Cinnamaldehyde induced localised hind limb responses at doses from 30 – 1,000 nmol that returned to baseline levels within 60 – 120 minutes after injection (Figure 6A). The increase in the number of localised hind limb responses displayed dose-dependence, with an $EC_{50}$ of 100 ± 5 nmol (Figure 6A, C).

Intraplantar injection of NDGA also produced an increase in the number of localised hind limb responses to acetone which peaked at 15 – 30 min post-injection and gradually returned towards baseline levels (Figure 6B). The increase in the number of NDGA induced localised hind limb responses displayed dose-dependence, with an $EC_{50}$ of 7.0 ± 2.3 nmol (Figure 6B, C). Intraplantar injection of vehicle did not produce a change in the number of

localised hind limb responses to acetone (Figure 6A, B). In addition, intraplantar injection of terameprocol (30 nmol) produced a robust increase in the number of localised hind limb responses to acetone, similar to that observed for NDGA (Figure 6C).

Finally, we examined the effect of systemic injection of the TRPA1 antagonist HC-030031 on the responses to near maximal doses of intraplantar cinnamaldehyde (300 nmol) and NDGA (30 nmol). Systemic injection of HC-030031 (150 $mg.kg^{-1}$) and vehicle did not produce a significant change in the localised hind limb responses to acetone (Figure 6, responses = 0.7 ± 0.3 and 1.1 ± 0.9 for HC-030031 and vehicle, respectively, $p = 0.1, 0.2$). The increase in localised hind limb responses to acetone produced by cinnamaldehyde and NDGA were both significantly less in HC-030031 pretreated animals compared to vehicle pretreated animals (Figure 6D, $p < 0.001$).

**Figure 1. Nordihyroguaiaretic acid (NDGA) activates human TRPA1.** Changes in intracellular calcium ($[Ca]_i$) in HEK293 cells expressing hTRPA1 were measured as outlined as in the Methods. A) Example traces of NDGA actions on HEK293-hTRPA1 cells where expression of hTRPA1 was or was not induced by tetracycline, addition of tetracycline produced a dramatic increase in the effects of NDGA. Traces represent the Raw Fluorescence Units (RFU) normalized to those prior to drug addition. Data from 4 similar experiments is summarized in B), with each bar representing the mean ± s.e.m. of the maximum change in calcium 5 fluorescence. C) Example traces of NDGA effects after pre-incubation of the HEK293-hTRPA1 cells with the TRPA1 antagonist HC 030031 (thin trace) or vehicle (thicker trace). Data from 6 similar experiments are summarized in D) with each bar representing the mean ± s.e.m. of the maximum change in calcium 5 fluorescence. HC 030031 significantly inhibited the effects of NDGA ($P < 0.001$).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+ HC 30031, 30 µM

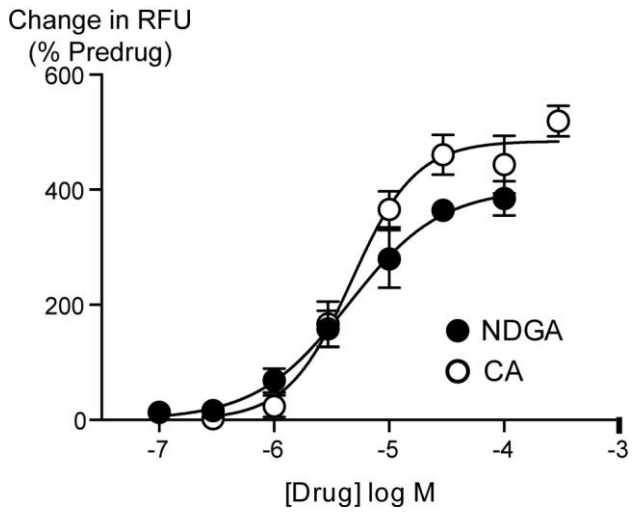
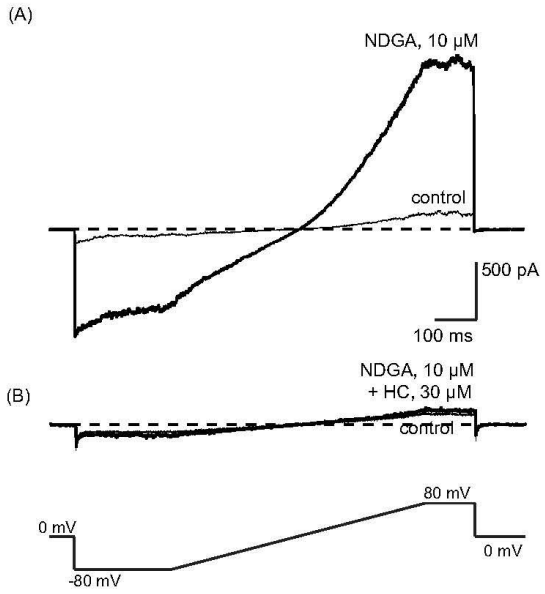



**Figure 2**. **Nordihyroguaiaretic acid (NDGA) activates hTRPA1 with a similar potency to cinnamaldehyde (CA).** Changes in intracellular calcium ($[Ca]_i$) in HEK293 cells expressing hTRPA1 were measured as outlined as in the Methods. Concentration-effect curves for NDGA and cinnamaldehyde were fit with a 4 parameter logistic equation, each point represents the mean ± s.e.m. of the change in fluorescence (RFU) from 5 experiments, each performed in duplicate or triplicate. NDGA elevated $[Ca]_i$ with an $EC_{50}$ of 4.4 μM, cinnamaldehyde elevated $[Ca]_i$ with an $EC_{50}$ of 4.7 μM. The maximum elevation of $[Ca]_i$ by cinnamaldehyde was significantly greater that that produced by NDGA ($P < 0.01$).

**Figure 3. Nordihyroguaiaretic acid (NDGA) activates a membrane conductance in HEK 293 cells expressing hTRPA1.** Whole voltage clamp recordings of membrane currents in HEK 293 cells expressing hTRPA1 were made as outlined in the Methods. A) Current traces from hTRPA1-expressing HEK 293 cell in control conditions (thin line) and in the presence of 10 μM NDGA. The increase in current was prevented by co-application of HC030031 (HC), illustrated in B. These traces are representative of at least 6 cells for each condition. Cells were subject to the voltage protocol illustrated beneath the traces. Zero current is designated by the dotted line.


(A)
NDGA, 10 µM
control
500 pA
100 ms
(B)
NDGA, 10 µM
+ HC, 30 µM
control
80 mV
0 mV
0 mV
-80 mV

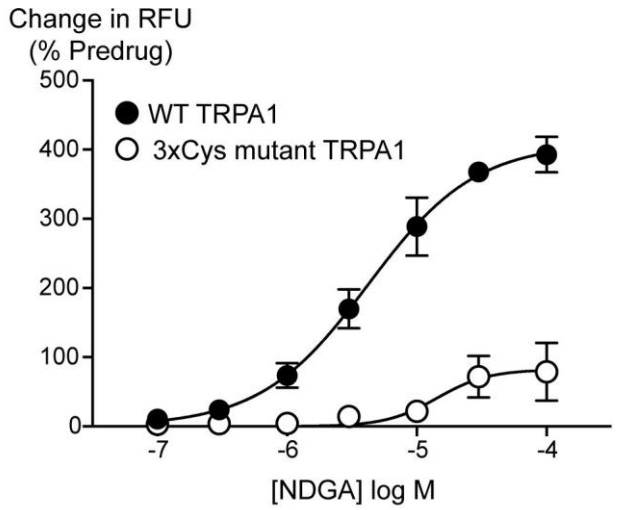
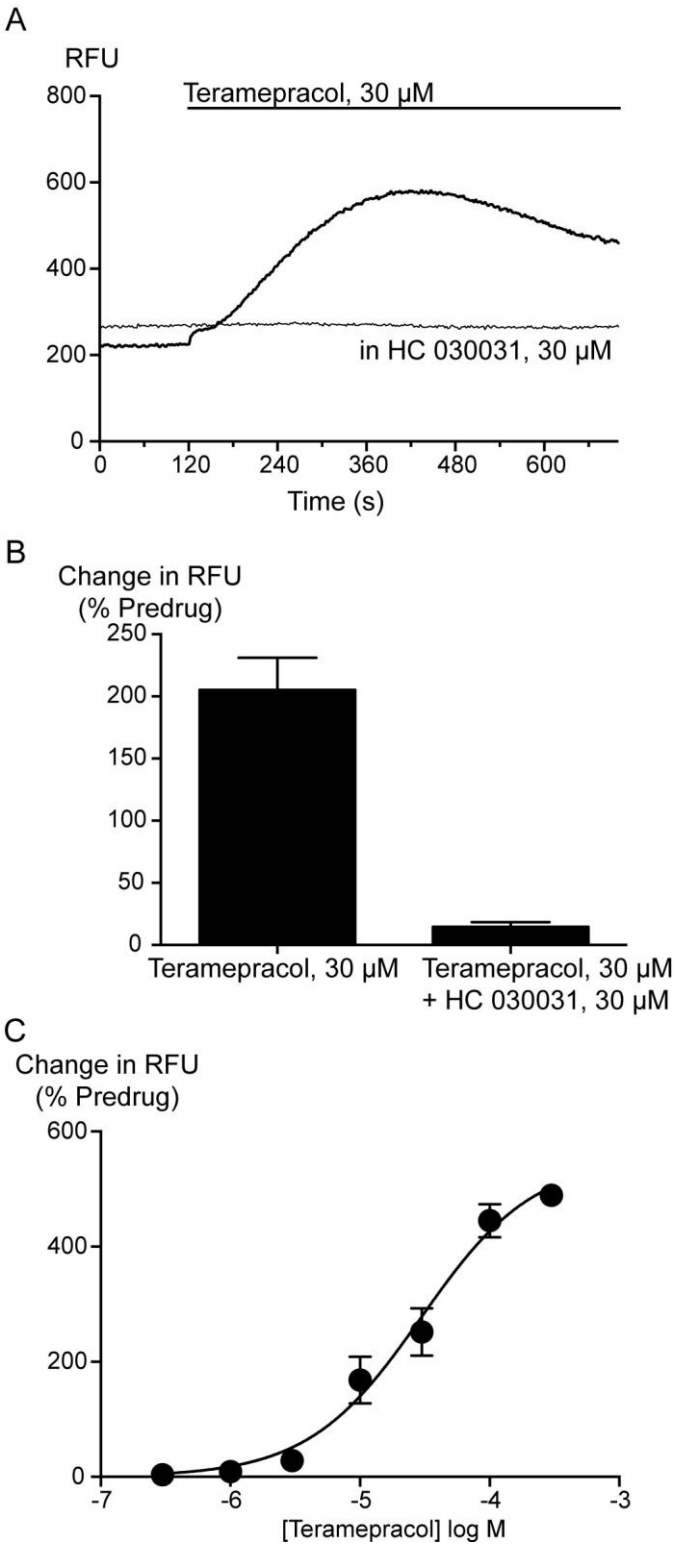



**Figure 4. Nordihyroguaiaretic acid (NDGA) activation of hTRPA1 is strongly dependent on conserved Cys residues in the intracellular N-terminus.** Changes in intracellular calcium ($[Ca]_i$) in HEK293 cells expressing hTRPA1 and mutant hTRPA1 where Cys 621, Cys 641 and Cys 665 were mutated to Ser (3xCys hTRPA1 mutant) were measured as outlined as in the Methods. Concentration-effect curves for NDGA were fit with a 4 parameter logistic equation, each point represents the mean ± s.e.m. of the change in fluorescence (RFU) from 6 experiments, each performed in duplicate or triplicate. In cells expressing wild type hTRPA1, NDGA elevated $[Ca]_i$ with an $EC_{50}$ of 4.9 ± 1.7 μM to a maximum of 365 ± 15 %, while in cells expressing the 3xCys hTRPA1 mutant the NDGA EC50 was 18 ± 3 μM to a maximum of 85 ± 16% ($P < 0.01$ for both $EC_{50}$ and maximum between wild type and 3xCys mutant TRPA1).

**Figure 5. Terameprocol effectively activates hTRPA1.** Changes in intracellular calcium ($[Ca]_i$) in HEK293 cells expressing hTRPA1 were measured as outlined as in the Methods. A) Example traces of terameprocol actions on HEK293-hTRPA1 cells with or without pre-incubation with the TRPA1 antagonist HC 030031. Traces represent the raw Raw Fluorescence Units (RFU). Data from 6 similar experiments is summarized in B), with each bar representing the mean ± s.e.m. of the maximum change in calcium 5 fluorescence, HC 030031 significantly inhibited the effects of terameprocol ($P < 0.001$). C) Concentration-effect curve for terameprocol were fit with a 4 parameter logistic equation, each point represents the mean ± s.e.m. of the change in fluorescence (RFU) from 6 experiments, each performed in duplicate or triplicate. Terameprocol elevated $[Ca]_i$ with an $EC_{50}$ of 30 μM.


A
RFU
Teramepracol, 30 μM
800
600
400
200
0
in HC 030031, 30 μM
0
120
240
360
480
600
Time (s)
B
Change in RFU
(% Predrug)
250
200
150
100
50
0
Teramepracol, 30 μM
Teramepracol, 30 μM
+ HC 030031, 30 μM
C
Change in RFU
(% Predrug)
600
400
200
0
-7
-6
-5
-4
-3
[Teramepracol] log M

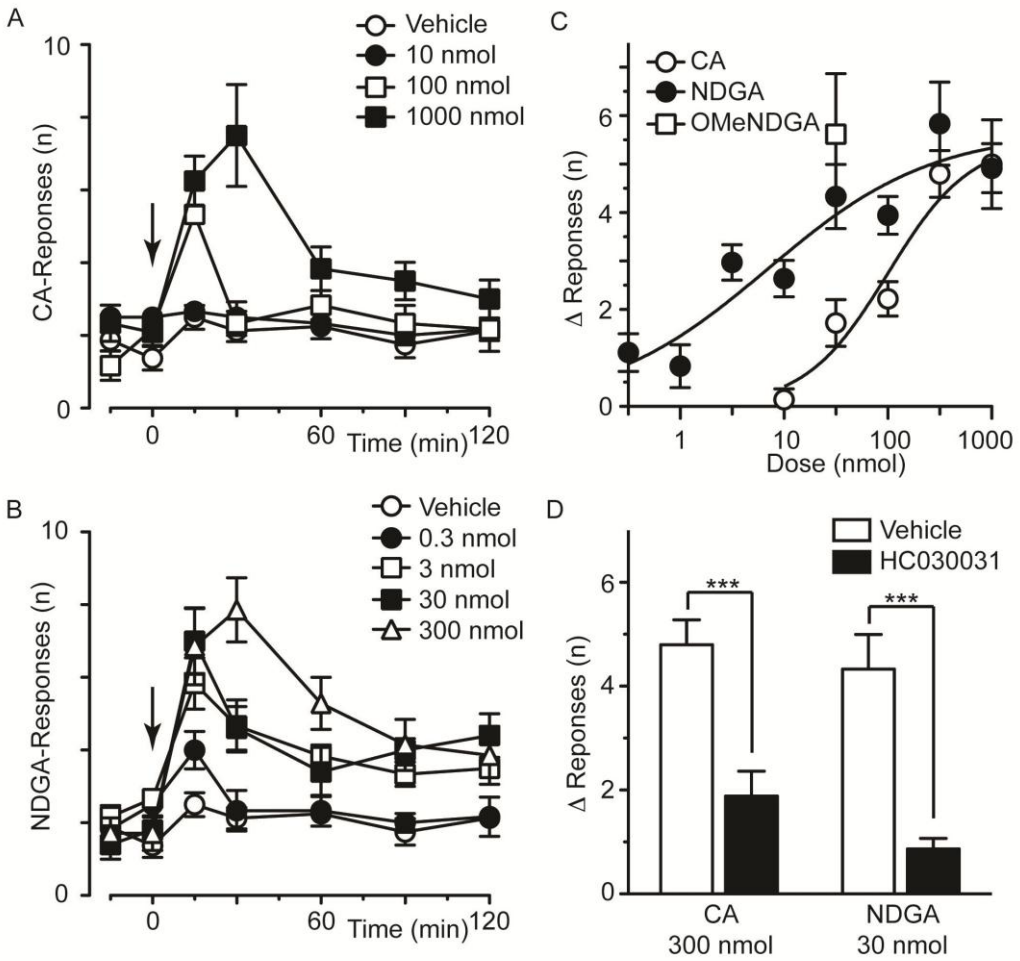



**Figure 6. NDGA enhances responses to cool stimuli.** Time plots of the number of hindpaw lifts/shakes/licks in response to brief topical application of acetone (20 µL) before and after intraplantar injection of a range of doses of A) cinnamaldehyde (CA), B) NDGA, or matched vehicle. C) Dose-effect curve of the average increases in acetone-induced hindpaw lifts/shakes/licks produced by CA, NDGA and terameprocol (OMeNDGA). D) Effect of the TRPA1 antagonist HC-030031 on the increases in acetone-induced hindpaw lifts/shakes/licks produced by CA, NDGA; animals were given an i.p. injection of HC-030031 (150 $mg.kg^{-1}$), or vehicle prior to intraplantar CA/NDGA. In A) and B), CA and NDGA were injected at time 0. In D) *** denotes $P < 0.001$.

# Discussion

The principle finding of this study is that the unspecific lipoxygenase inhibitor NDGA and its analog teramepracol are both efficacious agonists at hTRPA1. Consistent with this, both compounds produced enhanced responses to noxious cold stimuli *in vivo*.

To ascertain that the activation of TRPA1 was not associated with the inhibition of lipoxygenase itself, we have demonstrated that caffeic acid, another another LO inhibitor, did not cause an activation of the channel in a concomitant study (Redmond *et al.,* 2014). No evidence for any "tone" in the production of arachidonic acid metabolites in otherwise unstimulated CHO cells could be detected. The lack of any significant effect of HC-030031 alone suggests a lack of ongoing TRPA1 stimulation.

TRPA1's best defined mechanism of activation, leading to an increase in $[Ca]_I$, comes from the covalent modification of three reactive cysteines situated on the cytosolic N-terminal domain of the channel by reactive molecules such as CA found in cinnamon, allyl isothiocyanate from mustard oil and diallyl disulphide from gralic (Hinman *et al.*, 2006; Macpherson *et al.*, 2007). The mechanism of activation of this channel by the binding of unreactive compounds, such as Δ9-tetrahydrocannabinol (Jordt *et al.*, 2004), menthol (Karashima *et al.*, 2007) and 5-nitro-2-(3-

phenylpropylamino)benzoic acid (Liu *et al.*, 2010), are less well described. We have recently conducted a study in which we have found that, in order for the receptor to be activated by non-reactive fatty acids such as arachidonic acid, the presence of the cysteines on position C621, C641 and C665 were of importance in order to have a full response, but not essential for this activation, which is different from the action of reactive compounds, for which the presence of the cysteines are nearly essential for activation. In order to assess if it was the reactive scavenging coming from the antioxidant effects of NDGA that were the cause for its binding, we have tested NDGA in a mutant hTRPA1 receptor in HEK cells. Similarly to our previous study, NDGA kept part of its increase in $[Ca]_I$ in this mutant human TRPA1 receptor, leading us to believe that its antioxidant properties are not the reason for this activation.

The present results indicate that NDGA enhances behavioural responses to cool stimuli and that this is TRPA1 mediated for a number of reasons. Firstly, intraplantar injection of NDGA and CA alone had no behavioural effects at room temperature, but increased the behavioural nocifensive responses to evaporative cooling during the local topical application of acetone. This is similar to that previously reported for CA and other TRPA1 agonists (del Camino *et al.*, 2010; Gentry *et al.*, 2010) and is indicative of a specific enhancement of responses to cold stimulation. While some studies have observed that TRPA1 agonists produce behavioural responses at room temperature, these have been observed with other agents, or at higher doses of CA (Andrade *et al.*, 2008; Eid *et al.*, 2008; Trevisani *et al.*, 2007; Tsagareli

*et al.*, 2010). Secondly, the NDGA and cinnamaldehyde induced behavioural effects displayed dose dependence with similar potency and efficacy, and this differed to the cellular assay in which NDGA display a relatively higher potency. This may have been due to differences between human and mouse TRPA1, or pharmacokinetic differences following intraplantar injection. Finally, the behavioural effect of both NDGA and cinnamaldehyde were reduced by the TRPA1 antagonist HC-030031, similar to that observed previously (Eid *et al.*, 2008) derivative terameprocol.

NDGA is often used in an unregulated environment as a natural product to treat various ailments and met-NDGA is currently used in clinical trials for cancer. It is thus important to have a complete profile of effects for these two drugs. The implications of the systemic presence of TRPA1 ligands in an organism on physiological mechanisms, such as pain modulation, are still poorly described, and need to be further assessed. There is growing evidence implicating TRPA1 in the transmission of peripheral mechanical stimulation, and peripheral activation could potentially have an important role in noxious mechanoception (McGaraughty *et al.*, 2010) as well as cold hypersensitivity (del Camino *et al.*, 2010). Chemotherapy-induced peripheral neuropathy (CIPN), a severe and painful form of neuropathic pain coming from an increase in oxidative stress, is partly mediated via TRPA1 channels, as various ROS and products of tissue and injury inflammation can activate the receptor (Andersson *et al.*, 2008; Materazzi *et al.*, 2008; Sawada *et al.*, 2008; Trevisani *et al.*, 2007). In a recent study, Trevisani *et al.* have shown that signs of CIPN produced by two chemotherapy agents, bortezomib and

oxaliplatin, were completely abolished in TRPA1 knock-out mice, by the use of HC-030031 or after the co-incubation of the oxidative stress scavenger α-lipoic acid. As NDGA and terameprocol are also scavengers of stress associated free radicals or oxidants, it would be interesting to ascertain their potential effects on noxious mechanoception and cold hypersensitivity in CIPN, as they might have a dual action as scavenger and TRPA1 agonists. The antiviral and anti-tumor growth actions of NDGA and terameprocol do not appear to be mediated via TRPA1 activation, and are likely mediated by inhibition of the SP1 transcription factor. (Chen *et al.*, 1998; Craigo *et al.*, 2000; Huang *et al.*, 2003; Hwu *et al.*, 1998; Park *et al.*, 2003).

TRPA1 also appears to play a role in the peripheral cardiovascular system, although there are few *in vivo* physiological studies that have been conducted using systemic administration of TRPA1 agonists. *In vitro,* some TRPA1 ligands have been showed to cause CGRP-dependent, cyclo-oxygenase-independent relaxation of rat mesenteric arterial rings (Bautista *et al.*, 2005) and rat cerebral arteries via an endothelial-dependant mechanism (Earley *et al.*, 2009). Peripheral TRPA1 activation's influence on blood flow and pressure *in vivo* was studied by Pozsgai *et al.*, and it was reported that TRPA1 activation by trans-CA causes peripheral vasodilatation, which could lead to possibly relevant changes to the autonomic system reflexes and could potentially lead to vasovagal/neurocardiogenic syncope disorders (Pozsgai *et al.*, 2010), which further indicate possible concerns regarding a systematic administration.

In summary, NDGA and its derivative terameprocol, are ligands with a similar efficacy and affinity for the human TRPA1 receptor *in vitro*. As would be expected from a TRPA1 ligand, local administration of NDGA caused enhanced behavioural responses to noxious cold stimuli. Further research on the systemic administration of these compounds and their link with noxious mechanoception and noxious cold sensation, are still needed.

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## Author Contributions

MC Sr and WJR conceived the study. WJR performed the calcium measurements, MC Jr did the electrophysiology, CWV and VM did the behavioural work. WJR and MC Sr wrote the paper.

# Chapter 6 Discussion

*General*

The first modern study in pharmacology that defined efficacy came from Stephenson in 1956. In it, he studied the acetylcholine-like effects of a series of alkyl-trimethyl ammonium salts on the contraction of guinea pig ileum (Stephenson, 1956). The past 50 years have seen an important deepening of knowledge regarding receptor theories, structure and mechanisms of intracellular signalling. Still, most drug efforts, might it be for drug discovery or research, often rely on a single readout, often in a highly artificial system designed for automated screening, in order to classify the effects of ligand-receptor interaction. Within such a system, activity is either measured as a whole cell or tissue response, for example muscle contraction, or a single molecular event, such as influx of cytosolic calcium or depolarization. These interactions are usually measured in two ways, the first one being the dissociation complex of ligand-receptor interaction, called affinity, and the second being the maximal or peak response that can be observed in the system, which is a function of efficacy (Kenakin, 2012; Luttrell *et al.*, 2011). In such a system, affinity and efficacy are highly independent functions; a ligand will be termed a full agonist if it can induce a maximal response on the receptor, partial for a submaximal response and antagonist if it possesses affinity for the receptor but no intrinsic activation.

These principles have provided a framework for the study of ligand-receptor interaction and have brought forward important advances to the field of pharmacology. Nonetheless, the preconception of receptors simply seen as "on" and "off" switches underestimate by far the dynamic and complex mechanisms that are at play in receptor activation, which can lead to the coupling of various downstream effectors for varying periods of time. It would be of interest, in the years to come, for a more complex general model to be devised in order to explain the action of ligands on receptors that encompasses orthosteric ligands, allosteric modulators, other proteins contacting the receptor in the lipid bilayer membrane as well as the cytosolic matrix (Kenakin *et al.*, 2010; Luttrell *et al.*, 2011). This is particularly true for the CB1 receptor, as various ligands have been showed to have different signature for various signalling pathways. The presence of two allosteric site on the receptor further complicates the matter (Bosier *et al.*, 2010).

The need for a better understanding of ligand-receptor interactions is not limited to GPCRs. Indeed, our current knowledge of polymodal ion channels such as TRP channels is still in its infancy; this is in part due to the limited pharmacological tools, mainly specific inhibitors, presently available. This dearth of useful pharmacological tool force the use of genetically-engineered and small interfering RNA strategies, which is a potential cause for the still important disagreements regarding TRP channels assembly, localization and function (Wu *et al.*, 2010). Although one of the main function of TRP channels is their role as chemosensors for exogenous ligands, relatively few endogenous ligands have been thoroughly studied for TRP channel

activation. Therefore, one of the main question regarding TRP channels concerns their "normal" activation in the absence of environmental threats, for the TRPA, TRPV and TRPM families (Wu *et al.*, 2010). These questions about the legitimate activators for these channels (if such a thing exist), would also compel us to consider TRP channels as more than hot or cold sensors. Indeed, even though some TRP channels can be activated by thermal stimuli (mostly at noxious hot or cold temperatures), the localization of some of its thermal-sensing members, such as TRPV1, in the brain and other regions of the body with next to no changes in temperature possible in a viable organism, prevents us from demarking it as the main function of the receptor. It is therefore more likely that changes in temperature can facilitate or be unfavourable to an open state for the receptor, and early reports point that way, as thermodynamic studies tend to show that heat and cold activate the TRP channels via similar conformational changes (Clapham *et al.*, 2011; Julius, 2013). For TRPV1, voltage and temperature have been demonstrated to be important for the gating of the receptor, and higher temperature have been demonstrated as shifting the potential required to activate the receptor, making an open conformation possible at more negative (i.e. physiological) levels (Grandl *et al.*, 2010). In short, a better systematic characterization of the endogenous ligands of these channels is needed.

Finally, some compounds, such as the endocannabinoids AEA and NADA amongst others, can be ligands at both cannabinoid receptors and TRP channels, with varying degrees of affinity and efficacy for each. The peculiar pharmacology of endocannabinoids and their numerous member-related

compounds, such as NAANs, and receptors, such as TRPV1, raise questions regarding the need for such varied endogenous activators (Di Marzo *et al.*, 2012) as well as brings various hypotheses regarding the possible interactions at play in whole organisms. In order to answer such complex questions, more *in vitro* studies are needed in order to understand the pharmacology of endocannabinoids and related NAANs on each of these entities separately in as controlled an environment as possible.

The findings in this thesis relate to these three main topics. Overall, we have put forward the importance of bias signalling in CB1 and CB2 receptors by characterizing the effects of NADA and OLDA, two endogenous compounds, on $G_q$-mediated effects in CHO cells with no measurable coupling to $G_i$ nor $G_s$, a signalling signature that has not been seen before in cannabinoid receptors. Furthermore, we have presented evidences that the presence of an allosteric modulator on the CB1 receptor can modify the rate at which the receptor can de desensitized and internalized without changing the maximal response mediated by the receptor following the binding of various exogenous (CP and WIN), and endogenous (anandamide) orthosteric ligands. For TRP activation, we have shown that several non-electrophilic endogenous NAANs can activate TRPA1 receptor, although to a lesser extent than arachidonic acid itself. We have described that, even though acarichonic acid and other poly-unsaturated fatty acids cannot activate TRPA1 via covalent modification of cysteines, like other electrophilic environmental irritants do, the cysteine residues are of a certain importance, although not completely required, for the activation of the channel. The

cross-desensitization observed between reactive and non-reactive compounds further demonstrated the complex activation mechanism of TRPA1 and prevent us from believing that these two ligand-mediated activations are separate in nature, as they might involve a common protein configuration in order for the receptor to be activated. Finally, we have shown that two novel non-reactive ligands for TRPA1, NDGA and terameprocol, can activate TRPA1 in *in vitro* pharmacology studies, which can be translated to direct pain modulation *in vivo*, where these compounds can produce an enhanced response to noxious cold and acetone stimulation, when injected in the hind paw of mice.

## *Methods optimisation*

Since the aims of the studies we have conducted were wide in range, encompassed various targets, and that multiple measurements were required in order to properly define as much of the whole spectrum of possible effects following the activation of our various receptors as possible; we have based most of our studies on the use of a medium to high-throughput imaging system with real-time readouts capabilities, a machine able to measure both changes in membrane potential and calcium influx, though not at the same time, inside genetically engineered cell lines in an as efficient, fast and non-invasive manner as possible. The *Flexstation* (®), from Molecular Devices, a 96-well plate fluorescence reader, was found to be ideal for this situation. The *Flexstation* has been used in various studies aimed at measuring changes in concentration of intracellular calcium, from T-type

$Ca^{2+}$ channels (Xie *et al.*, 2007) and TRP channels such as TRPA1 (Luo *et al.*, 2011), to $G_q$-mediated elevation of $[Ca]_I$ from intracellular pools for opioid receptor as well as NOP receptor via a chimeric $G_{\alpha\ qi5}$ (Camarda *et al.*, 2013; Camarda *et al.*, 2009), and for various recombinant S1P receptor subtypes (Valentine *et al.*, 2012). Prior studies from our group have also used the *Flexstation* in order to measure GIRK-mediated $G_i$ activated hyperpolarization of cells in order to measure maximum response as well as receptor desensitization with various ligands on the μ-opioid receptor, amongst other (Knapman *et al.*, 2013).

Due to the nature of our projects, it was considered appropriate to utilise immortalised cell lines as opposed to primary cell lines or brain slices. As was outlined in this introduction, endocannabinoids and NAANs possess several targets on GPCRs and various channels, and can also affect enzymatic mechanisms. It was thus best to be able to measure these effects in cell types such as HEK, AtT-20 and CHO cells, which have been used profusely in past receptor trafficking and signalling studies (Lauckner *et al.*, 2005; Liu-Chen, 2004; Tulipano *et al.*, 2007; Wang *et al.*, 2003). Transfection of such cell lines with a receptor of interest makes it possible to have better control over protein construct design and level of expression. In rCB1 HEK cells, the concentration of receptors on the cell surface has been showed to be similar to those in the brain (Glass *et al.*, 1997; Kearn *et al.*, 2005) with levels for CHO and AtT-20 hCB1 believed to be slightly lower. It is also possible, when using more than one cell line for a specific stimulation, to eliminate external factors in order to pinpoint a specific effect that could be

otherwise potentially lost. For example, it was judicious to use AtT-20 stably transfected with rat or human CB1 in studies relating to GIRK-mediated hyperpolarization of the cells. On the contrary, such a cell line, by the presence of GIRK, would be unwanted in order to assess the $G_q$-mediated increase of calcium of agonists, such as WIN and CP, that can activate both $G_i$ and $G_q$ pathways. For TRPA1 studies, we have opted for an inducible expression of the receptors as high concentration of surface receptors were desired in our assays, and the cells would not have been viable for prolonged periods of time.

In order to supplement our findings and verify the veracity of what was discovered during our experiments, our colleagues have used complimentary techniques to our own. Firstly, for TRPA1-relating experiments, whole-cell patch clamp techniques was a good way to verify that what we have seen on multiple cells in our automated assay could be reproduced with more quantitative readings in a single cell. An increase in $[Ca]_I$ seen by changes in the fluorescence of the dye in a *Flexstation* assay does not necessarily translate to channels on the cell surface opening; as this kind of assay cannot easily dissociate the provenance of the $Ca^{2+}$. This information can be provided by patch-clamp techniques. Secondly, although Alisa Knapman from our group has recently developed an assay to look at the inhibition of cAMP by AC when stimulated with forskolin (Knapman *et al.*, 2013), such an assay is more aimed toward rapid and cost-efficient drug screening and is slightly less precise than the readings that one can get from the use of a bioluminescence resonance energy transfer (BRET) cAMP sensor, YFP-Epac-

RLuc (CAMYEL), present in a mammalian vector plasmid transfected into HEK cells. It can also be slightly problematic to use it for drugs that also activate GIRK channels, as further baseline-modification and controls would then be required. BRET techniques were a good fit for the quantitative nature and specificity to cAMP production that was needed in our experiments. Finally, quantification of hCB1 surface receptor was done using a live cell antibody feeding technique and quantified via the Discovery-1 automated fluorescent microscope (Molecular Devices) made it possible to incorporate a time constant to our recording, making it possible to correlate the rates of desensitization and internalization in our assays.

## *Discussion of specific results, perspective and future directions*

**The allosteric modulators ORG27569 and PSNCBAM-1 cause an increased rate of desensitization for the CB1 receptor following the activation of the receptor by orthosteric ligands.** One of the key feature of various experiments conducted in this thesis was the time-based dimension of the measurements conducted, and one of the first result where using time as a variable showed its importance was on the responses associated with the presence of allosteric modulators on the GIRK-mediated hyperpolarization following the activation of the CB1 receptor. Indeed, the increased rate at which the membrane returned to baseline following the activation of cannabinoid ligands, in the presence of ORG27569 or PSNCBAM-1, could be correlated with an increased rate of desensitization of the receptor. As this effect did not affect the peak hyperpolarization response

caused by the activation of the receptor (apart for 10 μM PSNCBAM-1), this effect would have been lost in a single readout. This increased rate of desensitization might help to explain the paradoxical effects of ORG27569 which previously reported that these allosteric ligands caused an increased affinity for the receptor for certain cannabinoid ligands, such as CP55,940, whilst inhibiting the physiological responses on normal cannabinoid-mediated effects *in vivo*, such as *vas deferens* inhibition of contraction (Price *et al.*, 2005). These first observations about time-specific effects of these allosteric modulators were confirmed afterward on the effects of CP55,940 on its $G_{i/o}$-mediated inhibition of cAMP accumulation in the presence of forskolin, as well as modified rate of internalization of the receptors following activation, seen by our colleagues Erin Cawston and Michelle Glass, the co-authors of **paper 1.**

These results help to establish the highly-dynamic nature of cannabinoid-mediated effects, which is useful information for more finely targeted modulation of these receptors for drug-development. They also show the importance of time-dependant analysis of the various signalling pathways that can be modulated once a GPCR is activated. The binding of a ligand, by modifying the energetic balance of the protein and a preference for one conformation, not only modifies the probability for it to achieve each other possible conformational states, but will also lead to the activation of possibly divergent signalling pathway; it will also affect the resulting signalling pathway activation of other ligands, as well as the dynamic inactivation and internalization processes that follow it. In our present case, the presence of

ORG27569 on the CB1 receptor will not only modify the rate at which CP55,940 causes the activation of the $G_{i/o}$-mediated pathways, it will also prevent the increased cAMP response via $G_s$-activation in PTX treated cells, i.e. affecting other possible conformational states, leading to different pathway activation. Similarly, against what one might at first believe, an increased rate of receptor deactivation, via desensitization, does not equate to an increase rate of receptor internalization as a resultant. In this case, ORG27569 limited, rate-dependently, the internalization or the human CB1 receptor, i.e. the conformational change possibly favoured putative GRK-mediated phosphorylation whilst it did not cause a conformational change enhancing β-arrestin-mediated recruitment and following mechanisms of internalization. It was out of the scope of this study to verify the exact dysfunction relating to if it is the β-arrestin recruitment itself, or an underlying mechanism, that was prevented the internalization to occur, although we hope to see this further investigated in the future. Ahn *et al.* have recently shown that a change in β-arrestin recruitment happens in the presence of ORG27569, although these findings were observed in cells expressing constitutively active modified CB1 receptor and would need to be repeated for WT cannabinoid receptors (Ahn *et al.*, 2013).

**NADA activates both CB1 and CB2 receptors, leading to a highly-biased activation of $G_q$-mediated signalling pathways.** The main finding of this thesis is that the endogenous lipid NADA has a completely biased signalling profile of activation for the CB1 receptor via $G_q$ G-protein coupling. This activation leads to an activation of the PLC pathway, which increases $[Ca]_I$

via the release of $Ca^{2+}$ from the endoplasmic reticulum. Oleoyl dopamine, another compound found in the brain, has a similar activation profile for the receptor. By itself, $G_q$-coupling to the CB1 receptor is not novel, it has been reported for both synthetic cannabinoids such as WIN (Lauckner *et al.*, 2005), and hinted at for endocannabinoids, as CB1-mediated increase in $[Ca]_I$ via the PLC pathway has been reported for 2-AG (Sugiura *et al.*, 1996). What is of the most interest in our study is that NADA has a completely different activation profile from these other cannabinoids as it did not activate the two other $G_{i/o}$ nor $G_s$ signalling pathways. Such a level of bias toward a secondary signalling pathway is unheard of for the CB1 receptor, and rare for GPCRs in general (Kenakin, 2012). At the same time, we have been able to confirm that the CB2 receptor can couple to the $G_q$ G-protein in the presence of NADA and OLDA. $G_q$-mediated activation of CB2 has been hinted at in the past, as anandamide has been showed to elevate $[Ca]_I$ via the activation of the PLC in the presence but not the absence of CB2 (Zoratti *et al.*, 2003), and arachidonoyl-chloro-ethanolamide and JWH133, a CB2 agonist, have also been showed to induce PLC-mediated changes in $[Ca]_I$ (De Petrocellis *et al.*, 2007). CB2 has also been showed to be able to couple to chimeric $G_q$ subunit (Malysz *et al.*, 2009). It is thus consistent with the literature that CB2 can couple to $G_q$, so we believe that we have identified the mechanism of action of NADA on hCB2 in CHO cells as being $G_q$-mediated, as blocking the PLC pathway, as well as inhibiting $G_q$ directly with the addition of a specific palpeptide, both significantly prevented the increase in $[Ca]_I$ that followed the addition of NADA.

The implications of these findings are diverse. Even though very high concentration of NADA were needed to activate the receptor, far beyond the expected physiological concentration present *in vivo*, the ability to selectively manipulate physiological functions through the activation of defined signalling pathways could potentially help in the development of safer and more efficacious cannabinoid-based therapeutics aimed at various indications (Bosier *et al.*, 2010). As well, one of the main drawback that currently slows down research on cannabinoid receptors pharmacology is partly due to a lack of a full crystallization structure for the receptor. Having a molecule able to selectively couple the receptor in a "pure" $G_q$-coupled confirmation could thus be of interest in the pursuit of full receptor crystallization.

As an increase in $[Ca]_I$ via 1) the activation of the PLC pathway by the activation of various GPCRs, or 2) via the activation of VGCCs and other $Ca^{2+}$ channels, can lead to an on demand synthesis of 2-AG (Castillo *et al.*, 2012), we have hypothesized that the NADA-induced, cannabinoid-mediated, increase in $[Ca]_I$ that we have described could potentially lead to an increase in 2-AG as well. This happened to be the case in our CHO hCB1 cells, as the addition of 100 µM NADA caused a time-dependant increase in 2-AG up to 5 minutes post-activation. CHO cells are not neurons, and it was out of the scope of this study to verify if such a mechanism can happen *in vivo*, nor define what could be the outcome of such a synthesis pathway on neuronal transmission and plasticity, but some evidences suggest that such a mechanism could theoretically exist. Firstly, the increase in $[Ca]_I$ needed to

initiate 2-AG synthesis appears to be non-discriminative regarding the source of the $[Ca]_I$, as the co-activation of the PLC pathway and VGCCs have been proposed to be able to converge to the same signalling pathway that leads to the production of 2-AG. This simultaneous activation could potentially be used by neurons as a coincidence-detector and has been proposed to be of possible importance for the integration of synaptic activity (Brenowitz *et al.*, 2005). NADA could thus potentially be a good candidate for the dual activation of TRPV1 channels and CB1 receptors when they are co-expressed in the same cells. As well, recent findings regarding the importance of the presence of NADA in 2-AG tone in ventral midbrain neurons has been reported. In these experiments, the endocannabinoid-tone was lost if NADA's synthesis pathway was blocked (Freestone *et al.*, 2013). The production of endocannabinoids following the activation of cannabinoid receptors can sound counterintuitive, as the activation of these receptors usually lead to their desensitization and internalization; but this does not appear to be the case in our findings. NADA did not modulate GIRK-mediated hyperpolarization of the cells nor prevented the inhibition of cAMP formation in the presence of forskolin. Similarly, NADA by itself did not cause the internalization of the CB1 receptors. It could thus be possible that a cannabinoid-mediated loop i.e. activation - causing release - causing activation, could be technically possible. This goes against the pre-synaptic localization usually associated with CB1 receptors, but this could be of interest in developing cells, interneurons of the dorsal horn and neuron-glial cells interactions, to name a few possible candidates.

**Various NAANs and PUFAs activate the TRPA1 receptor by a mechanism that is partly, yet not completely, linked with the presence of three reactive cysteines on its N-terminal tail which are necessary for the activation of the channel by electrophilic agonists.** As various NAANs have been described as agonist and inverse agonist at various TRP channels, we have investigated the potential activation of TRPA1 by a series of NAANs. We have found that several NAANs that we have investigated could activate the channel, although with low peak responses, and at high concentration. What was of more importance was that arachidonic acid itself was a better agonist for the channel. We have thus investigated more systematically this activation. We have found that even for non-electrophilic compounds such as AA, the reactive cysteines required for the activation of the receptor by electrophilic compounds were still somewhat important, although not completely essential, for the opening of the channel to occur. Interestingly, AA and cynnamaldehyde (CA), could both cause the complete desensitization of the channel for one another, hinting at either a similar overall activation or shared desensitization mechanism post-activation. We have hypothesized that AA-mediated activation of the receptor could potentially cause disulphide bond amongst the reactive cysteines when it binds to the receptor. We have also found that the length of the fatty acid was of importance for this activation to occur, as no other PUFA tested could equal the activation recorded for AA, with the second most efficacious being DHA. The location of the unsaturation on the carbon backbone also appear to be of primordial importance, as ω3-AA produced next to no activation of the receptor. Motter *et al.* came to similar conclusion in a paper published whilst we were preparing our manuscript, although they did not see, in mouse

TRPA1, the same differences regarding the positioning of the unsaturations nor any difference between AA and DHA (Motter *et al.*, 2012).

ω6-AA metabolism leads to the formation of a myriad of prostaglandins, leukotrienes and pro-inflammatory compounds that cannot be formed by some other PUFAs, such as DHA. An interesting avenue for these findings would be to correlate the levels of various inflammatory mediators and production of various NAANs in the brain, based on diets rich in ω3-AA, DHA or oleic acid compared to ω6-AA, for example.

**NDGA activates the TRPA1 receptor and modulates pain in noxious cold and acetone tests *in vivo***. The principle finding of this study was that the lipoxygenase inhibitor NDGA and its analog terametpracol (met-NDGA) are both efficacious agonists at hTRPA1. Consistent with this affirmation, both compounds produced enhanced responses to noxious cold stimuli *in vivo*. NDGA and met-NDGA both inhibit a variety of enzymes and transcription factors, and have been proposed as an anti-oxidant, and anti-retroviral and anti-cancer agents, respectively, due to these effects. NDGA is often used in unregulated environment as a natural product to treat various ailments and met-NDGA is currently used in clinical trials. It is thus important to have a complete profile of effects for these two drugs. The implications of the systemic presence of TRPA1 ligands in an organism on physiological mechanisms, such as pain modulation, are still poorly described, and need to be further assessed. There is growing evidence for the

implication of TRPA1 in the neurotransmission of peripheral mechanical stimulation, and peripheral activation could potentially have an important role in noxious mechanoception (McGaraughty *et al.*, 2010) as well as cold hypersensitivity (del Camino *et al.*, 2010).

Taken together, the results in this thesis described the biased signalling pathway of the endogenous lipid NADA on CB1 receptor via $G_q$ and proposed a novel $G_q$-mediated signalling pathway for the human CB2 receptor, showed the novel binding of various NAANs and fatty acids for the TRPA1 via a mechanism that partly require the presence of reactive cysteines as well as described NDGA and met-NDGA as novel ligands for the channel, and sho pain modulatory proprieties via TRPA1 activation in the rat. In the future, it would be of interest to verify the possibility of the proposed loop of CB1 activation – synthesis – activation in more complex system such as neuronal slices. As well, a thorough investigation of the effects of NAANs when cannabinoids and TRP channels are present, and their possible implications on synaptic transmission and possible plastic modification. Finally, I believe that the modulation of fatty acid concentrations in the diet, leading to various levels of NAANs, could potentially have relevant physiological outcomes and could be an interesting avenue for future scientific endeavours.

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Appendix 1 of this thesis has been removed as it may contain sensitive/confidential content