The effects of lysosomal Ca^{2+} release on membrane depolarisation and synaptic plasticity in hippocampus

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Declaration

I, William Foster, declare that the work presented in this thesis is my own unless where explicitly noted.

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Abstract

Intracellular Ca^{2+} signalling is essential for the control of almost every physiological process, from muscular contraction to synaptic transmission. Intracellular Ca^{2+} signals can be generated from both extracellular or intracellular Ca^{2+} stores. Nicotinic acid adenine dinucleotide phosphate (NAADP) is a ubiquitous and important Ca^{2+} mobilising second messenger. NAADP signalling causes Ca^{2+} release from intracellular acidic Ca^{2+} stores. The functional roles of NAADP signalling and acidic store Ca^{2+} release in the central nervous system are relatively unknown Brailoiu et al. (2009b) showed that NAADP signalling enhances membrane excitability in neurons of the medulla, Padamsey and Emptage (2011) (Unpublished) find similar effects in pyramidal neurons of the hippocampus. I used pharmacological manipulations in combination with electrophysiological techniques and dendritic Ca^{2+} imaging to explore the effect of NAADP/acidic store signalling on membrane excitability and synaptic plasticity in pyramidal neurons of the hippocampus. I began by using a membrane permeable form of NAADP (NAADP-AM) to show NAADP/acidic store Ca^{2+} signalling caused membrane depolarisation. I also showed, with intracellular dialysis of Ca^{2+} mobilising second messengers, NAADP is unique in its ability to directly cause membrane depolarisation. I then identified glutamate, acting via metabotropic glutamate receptor 1 (mGluR1), as an endogenous stimulus that causes NAADP-mediated Ca^{2+} release and depolarisation. I next elucidated the signalling pathway responsible for mGluR1/NAADP-mediated depolarisation and showed the requirement of acidic store Ca^{2+} release, and subsequent amplification of this Ca^{2+} via ryanodine receptors by Ca^{2+}-induced Ca^{2+} release. The resulting Ca^{2+} signal caused inhibition of small conductance K^{+} channels (SK channels) and membrane depolarisation. SK channels are described to facilitate the induction of plasticity in hippocampal synapses by modulation of GluN Ca^{2+} entry (Ngo-Anh et al., 2005). Finally, I show that the induction of mGluR1-dependent long-term potentiation requires inhibition of the SK channels via NAADP/acidic store Ca^{2+} signalling. Group 1 mGluRs are implicated in the pathogenesis of neurodevelopmental disorders such as fragile X syndrome. My findings may identify new targets for the treatment of such diseases.

Contents

1.0 Introduction

1.0.1 Stimulus driven intracellular signalling

In order to perform their dedicated roles efficiently and effectively, cells must respond appropriately to both external and internal stimuli. Signals from outside the cell are detected by receptors that subsequently relay, or transduce, the signal across the cell membrane and into the cell, where an appropriate response is generated based upon the stimulus received. Stimuli may also originate from inside the cell itself. This stimulus-response signalling takes a myriad of forms, with many intermediate signalling molecules, however, within this study I focus on the role of calcium ions (Ca^{2+}).

1.0.2 Spatiotemporal dynamics of Ca^{2+} signals

Ca^{2+} is known to regulate a plethora of biological processes including: fertilization (Steinhardt and Epel, 1974), synaptic transmission (Katz and Miledi, 1967) and plasticity, reviewed by Lisman (2001) muscular contraction (Karaki et al., 1997), apoptosis (Giacomello et al., 2007) and transcription (Bading et al., 1997). Tight regulation of intracellular Ca^{2+} is essential to achieve parallel control of multiple and critically important processes within a single cell. Distinct and transient Ca^{2+} signals produced in the cytoplasm and the interaction of this Ca^{2+} with effector proteins, allow physiologically distinct cellular processes to be coordinated in parallel.

Ca^{2+} signals are variable not only in magnitude but also, critically, in their localisation and temporal coding. Furthermore, a Ca^{2+} signal may comprise of several repeated Ca^{2+} signalling events such as a wave or oscillation of Ca^{2+} concentration that the cell may integrate over time. It is the combination of these elements of Ca^{2+} signalling events that produces a unique Ca^{2+} signal and in turn a unique cellular outcome. For example, synaptic transmission requires localised Ca^{2+} release occurring over milliseconds, through voltage-dependent, Ca^{2+} permeable, membrane channels, as reviewed by Takahashi and Momiyama (1993) whereas early development (in oocytes) is dependent on global (throughout the whole cell) Ca^{2+} oscillations occurring over several hours (Whitaker, 2006).

It should be stressed that Ca^{2+} signals can be complex in their localisation, duration, amplitude, and frequency and that these layers of complexity and regulation are essential for producing the correct cellular response to specific stimuli. A regulatory network for Ca^{2+} signalling within the cell is therefore essential. **Figure 1.1** shows the Ca^{2+} signalling network diagrammatically. A great number of individual components constitute each aspect of the Ca^{2+} signalling network, resulting in a myriad of possible combinations and potential cellular outcomes. Collectively, the components of this network are often referred to as the Ca^{2+} signalling 'toolkit'. **Figure 1.4** shows a schematic diagram illustrating a cell with the critical components that comprise the Ca^{2+} signalling toolkit; this diagram may be useful to refer to as the reader progresses through the following chapter.

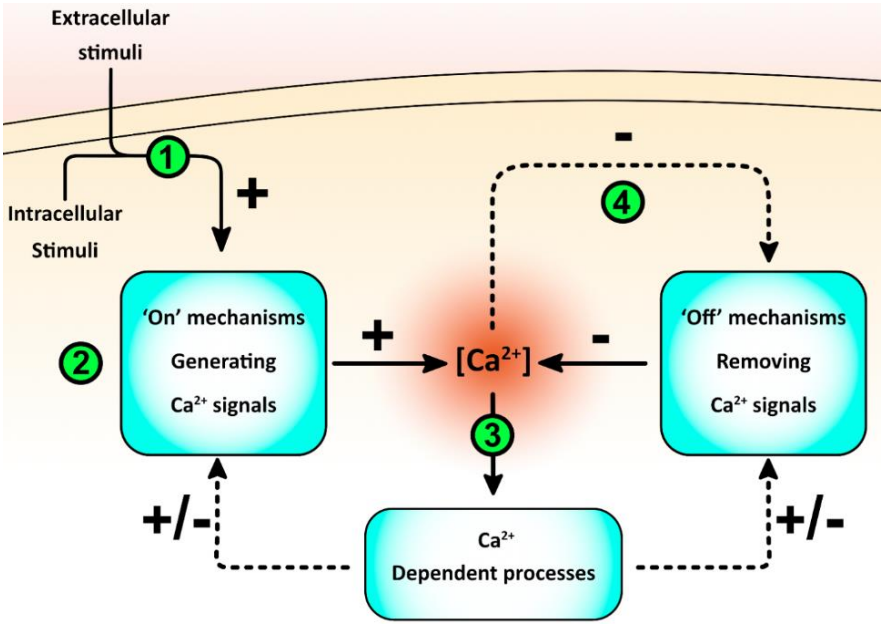

Figure 1.1 The Ca^{2+} signalling network. 1. Ca^{2+} signalling is a stimulus driven event, both extracellular and intracellular stimuli can begin a Ca^{2+} signalling event. **2.** After a given stimulus is detected, cells will initiate a specific and appropriate Ca^{2+} signalling event using 'On mechanisms'. **3.** Specific intracellular Ca^{2+} signals will activate their corresponding Ca^{2+} dependent processes, via Ca^{2+} binding protein 'effectors'. Ca^{2+} signals may further potentiate other 'On' mechanisms or reduce Ca^{2+} signals by initiating 'Off' mechanisms. **4.** After Ca^{2+} signals are generated and have initiated the desired downstream signalling events free Ca^{2+} is removed from the cytoplasm via 'Off' mechanisms and in effect, the Ca^{2+} signal is 'switched off'.

1.0.3 Ca^{2+} signalling On and Off Mechanisms

Ca^{2+} signalling is a stimulus driven process, a diverse range of stimuli exist that cause the production of an intracellular Ca^{2+} signal and therefore turn Ca^{2+} signalling 'On'. These stimuli can originate both intracellularly and extracellularly and the type of stimuli will influence the source and characteristics of the subsequent Ca^{2+} signal. Examples of some stimuli which lead to intracellular Ca^{2+} signalling include, but are not limited to: temperature change, membrane depolarisation, stretch detection, ligand binding to receptors, intracellular Ca^{2+} store depletion and low intracellular Ca^{2+} (Berridge et al., 2000, Berridge et al., 2003).

Ca^{2+} concentration is tightly controlled within the cell, with a cytoplasmic resting concentration of approximately 100 nM, and when activated, Ca^{2+} signals may reach low micromolar concentrations, depending on the localisation (Berridge et al., 2000). Stimuli can recruit a variety of potential Ca^{2+} sources to achieve this increase Ca^{2+} concentration. Firstly, the extracellular fluid, where the Ca^{2+} concentration is suggested to lie between 1-2 mM (Clapham, 2007) and secondly the intracellular stores of Ca^{2+}. Including the: mitochondria, Golgi apparatus, nucleus, endo/sarcoplasmic reticulum (ER/SR), and the acidic stores (endosomes and lysosomes). Intraluminal concentration estimates are suggested for: the ER/SR to be between 1-5 mM (Bygrave and Benedetti, 1996), the mitochondria between 2-20 μM (Pozzan and Rizzuto, 2000, Finkel et al., 2015), and the acidic stores between 400-600 μM (Lloyd-Evans et al., 2008, Christensen et al., 2002). The intracellular stores contain a significant source of Ca^{2+} and each is known to play a major role in intracellular Ca^{2+} signalling. The concentration gradient between cytoplasmic Ca^{2+} and both the intracellular and extracellular stores of Ca^{2+} is substantial. This concentration gradient underlies the rate and effectiveness that Ca^{2+} signals can achieve.

'Off mechanisms' remove Ca^{2+} from the cytoplasm and return intracellular Ca^{2+} concentration to the basal level. They are equally as important as a mechanism for turning Ca^{2+} signals 'On'. It is essential that free Ca^{2+} is removed from the cytoplasm after any desired effects have been achieved, not simply

to prevent undesired activation of other Ca^{2+} dependent mechanisms, but also to prevent toxic precipitations of Ca^{2+} phosphate and disruption to lipid membranes that may occur at a high Ca^{2+} concentration (Case et al., 2007). After the desired effects have been achieved Ca^{2+} is removed from the cytoplasm via one of two routes: 1. Extrusion into intracellular stores of Ca^{2+} or the extracellular space/fluid and 2. Binding to Ca^{2+} sensitive proteins.

1.0.4 Ca^{2+} binding proteins

Generation of specific and appropriate cellular responses to Ca^{2+} signalling events relies on numerous and heterologous Ca^{2+} binding proteins (CBPs). CBPs bind the intracellular Ca^{2+} and are the 'effectors' an initial Ca^{2+} signal. CBPs initiate and/or participate in downstream signalling cascades to produce specific cellular or physiological events. Some CBPs also have the ability to integrate and decode complex Ca^{2+} signals, such as Ca^{2+} oscillations.

CBPs are heterologous in structure and function and are split into three superfamilies. The three superfamilies of CBPs contain hundreds of individual members, each with distinct functionality based upon their Ca^{2+} binding kinetics, distribution, and localisation. Intracellular CBPs comprise two of these superfamilies, with each super-family containing several other families with numerous members. The first of these superfamilies have CBPs containing EF-hand domains, this family contains some of the most ubiquitous and well known CBPs, such as the calmodulin, calcineurin, and neuronal Ca^{2+} sensing (NCS) families. The second super-family contains CBPs which do not present EF-hand domains. This super-family includes proteins essential for intracellular Ca^{2+} homeostasis, such as calreticulin, calsequestrin, the annexins, which have the ability to bind phospholipids and are integral to endo/exocytosis, some kinases such a protein kinase C (PKC) and other Ca^{2+} sensing proteins such as synaptotagmin. As reviewed in Yanez et al. (2012).The third super-family of CBPs possess binding sites to sense extracellular Ca^{2+}. This superfamily contains six other families, which have been grouped based on Ca^{2+} binding domain structure. Their function is generally thought to encompass sensing and

detecting changes in extracellular Ca^{2+} concentration over local microdomains. Local changes in the concentration of extracellular Ca^{2+} are also physiologically important (Hofer, 2005).

The large number of unique CBPs, their pattern of localisation, and differential Ca^{2+} binding properties, coupled with specific spatiotemporal patterns of Ca^{2+} signals ensure that stimuli are appropriately coupled to the relevant cellular response.

1.0.5 Local and Global Ca^{2+} signals

As mentioned in the previous sections, Ca^{2+} signals vary in their spatial distribution. Ca^{2+} entry from the extracellular space or the intracellular stores occurs through membrane channels and produces a brief and local rise in the Ca^{2+} concentration around the channel before it diffuses into the cytoplasm. 'Local' Ca^{2+} signals have been attributed to microdomains between 10-100 nm from the open channel, as reviewed by Bootman et al. (2001). Local Ca^{2+} signals activate effector proteins (CBPs) within the immediate vicinity of the Ca^{2+} permeable channel, they may also recruit further effector proteins and activate downstream signalling cascades. Furthermore, multiple Ca^{2+} permeable membrane channels producing local Ca^{2+} signals in unison allow for summation that can be propagated throughout the cell to cause larger Ca^{2+} signalling events and also global Ca^{2+} signals.

The neuron is a perfect example of how local Ca^{2+} signalling may be utilised to full effect. Neurons possess complex and compartmentalised morphologies with selective distribution of Ca^{2+} permeable channels. Both local and global Ca^{2+} signals are important for generating cellular/physiological responses in neurons, thus allowing for a wider range of downstream signalling outcomes. For example, local Ca^{2+} signals in the presynaptic terminal can result in neurotransmission, whereas local Ca^{2+} signals in the dendrites can affect synaptic strength, spine morphology and protein synthesis. Local Ca^{2+} signalling in neurons may also cause the production of a global Ca^{2+} signalling event, with each type of Ca^{2+} signal producing drastically different effects. Glutamate binding to postsynaptic glutamate receptors can produce markedly different outcomes. Given the correct patterns of synaptic stimulation, glutamatergic neurotransmission can produce a variety of synaptic plasticity events,

which are dependent on specific and local Ca^{2+} signals in the dendritic spine, as reviewed by Lisman (2001) and Shouval et al. (2002). Whereas, overstimulation of glutamate receptors (in the case of ischemic brain injury) produces a global Ca^{2+} signal and results in either apoptosis or necrosis of neurons (Ankarcrona et al., 1995, Orrenius et al., 2003).

Ca^{2+} Sparks and Waves: Spatiotemporal dynamics of intracellular Ca^{2+} store release

Localised Ca^{2+} signalling events can also be produced from the membrane channels on intracellular Ca^{2+} stores. These local Ca^{2+} signalling events are referred to by several names (sparks, puffs, blips and quarks) depending on the cell type, intracellular store and Ca^{2+} release channel. Despite the differences in nomenclature, all of these localised Ca^{2+} signalling events are another important route for producing intracellular Ca^{2+} signals. Like local Ca^{2+} signals produced via plasma membrane Ca^{2+} channels, local Ca^{2+} signals from intracellular stores are spatiotemporally varied. Additionally, local Ca^{2+} signals from intracellular Ca^{2+} stores can also summate to produce larger, regenerative Ca^{2+} signals known as Ca^{2+} waves.

Ca^{2+} sparks/puffs manifest as small and local Ca^{2+} release from the ER/SR due to the coordinated opening of spatially clustered IP_3R or RyRs. Sparks vary in amplitude depending on the number of receptors acting in unison. Sparks were first identified in rodent cardiac cells (Cheng et al., 1993) and have since been described in a variety of other cells types including smooth muscle (Nelson et al., 1995), skeletal muscle (Baylor et al., 2002) and neuronal cells (Koizumi et al., 1999). Sparks may occur spontaneously or be evoked, such as during excitation-contraction coupling in cardiac cells.

Summation of Ca^{2+} sparks/puffs results in larger Ca^{2+} signals known as Ca^{2+} waves. When Ca^{2+} waves are repetitive in nature they are referred to as Ca^{2+} oscillations. Waves and oscillations occur when Ca^{2+} release channels (IP_3R/RyRs) of the ER/SR become sensitised to cytosolic Ca^{2+}. This sensitisation to Ca^{2+} increases their probability of opening and the amount of Ca^{2+} they allow into the cytoplasm in a process referred to as Ca^{2+}-induced Ca^{2+} release (CICR). CICR allows in regenerative and nonlinear Ca^{2+} signals to be produced from the ER/SR and hence high magnitude or global Ca^{2+} signalling events.

The first Ca^{2+} waves to be described in molecular detail are those initiated by membrane receptors which cause the production of the second messenger: inositol 1,4,5-trisphosphate (IP_3) and its action to sensitise the IP_3 receptor to Ca^{2+}. These waves were first noted in Xenopus oocytes (Lechleiter et al., 1991) where Ca^{2+} waves produce a spiral pattern which encodes information required to initiate development of the oocyte. Ca^{2+} waves have since been described in a variety of other cell types including neurons, as reviewed by Ross (2012). RyRs are also known mediate Ca^{2+} waves in a variety of cell types, including smooth muscle (Collier et al., 2000) and neurons (Friel and Tsien, 1992).

A large body of research has now been undertaken on local Ca^{2+} signals from the intracellular Ca^{2+} stores. These signals are implicated in catalysing high threshold Ca^{2+} signalling events, for example, CICR and global Ca^{2+} signalling. Examples of such global Ca^{2+} signalling events include excitation-contraction coupling in cardiac and skeletal muscle, vascular smooth muscle tone, and neuronal secretion, as reviewed by Cheng and Lederer (2008).

The spatial organisation of the proteins involved in the generation, detection and transduction of intracellular Ca^{2+} signals is of key importance. Ca^{2+} signals begin with small localised events. For downstream signalling to occur the correct effector proteins must reside within the 'active zone' of Ca^{2+} permeable membrane channels. This local signalling also underlies how larger scale global Ca^{2+} signalling events can be produced. Local and global Ca^{2+} signals may have drastically different effects even within the same cell type, and therefore the spatial organisation of the members of the Ca^{2+} signalling 'toolkit' is of critical importance for the production of specific and correct cellular responses to stimuli.

1.1 The extracellular Ca^{2+} store

As previously mentioned, cells have a variety of Ca^{2+} stores which they may use to generate an intracellular Ca^{2+} signal. Having multiple sources of Ca^{2+} is beneficial as it allows a greater control and therefore complexity of spatiotemporal Ca^{2+} signalling dynamics. The following section will describe the extracellular Ca^{2+} store and how it may be used to produce Ca^{2+} signals in the cytosol.

The extracellular fluid, where the Ca^{2+} concentration is suggested to lie between 1-2 mM (Clapham, 2007), is the largest Ca^{2+} store which cells make use of. The plasma membrane provides an effective barrier to prevent extracellular Ca^{2+} from entering the cell, therefore, Ca^{2+} entry may only occur through dedicated channels and transporters.

1.1.1 Ca^{2+} permeable plasma membrane channels

Ca^{2+} enters the cell via one of four groups of plasma membrane channels, they consist of: **i.** Voltage-dependent Ca^{2+} channels (VDCCs), **ii.** Transient receptor potential channels **iii.** Ligand gated Ca^{2+} channels **iv.** Store operated Ca^{2+} channels.

i. Voltage-dependent Ca^{2+} Channels

The VDCCs are, as described by their name, gated by changes in the potential difference (voltage) of the plasma membrane. At rest, these channels are predominately closed, and when the cell becomes depolarised they open allowing Ca^{2+} into the cell from the extracellular fluid. There are ten known members of the VDCC family, which is split into three groups: Ca_V1 comprising of L-Type channels ($Ca_V1.1$-1.4), Ca_V2 comprising of P-type ($Ca_V2.1$), N-type ($Ca_V2.2$), and R-type ($Ca_V2.3$) channels, and finally Ca_V3 comprising of T-type Ca^{2+} channels ($Ca_V3.1$-3.3). VDCCs undertake a variety of distinct physiological roles due to distinct voltage activation properties and permeability to Ca^{2+}. For example, Ca_V1 channels cause excitation-contraction/secretion/transcription coupling, Ca_V2 channels cause the initiation of synaptic transmission in neurons and Ca_V3 channels are important for modulation of rhythmic firing activity, such as in the sinoatrial node (Catterall, 2011, Mangoni et al., 2006).

ii. Transient receptor potential channels

Transient receptor potential (TRP) channels are generally non-selective cation channels, there are 28 mammalian members of the TRP channel family. They are split into six subgroups based on structural and functional properties: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and the TRPA (ankyrin). They have both diverse distribution and activation and are responsible for a plethora of functions. Not all TRP channels are found on the plasma membrane,

some are found on the membranes of intracellular compartments. Whilst most but not all (TRPM4 and TRPM5) TRP channels are permeable to Ca^{2+}, the degree of permeability also varies between different members. TRP channels are also regulated by Ca^{2+} itself, allowing both positive and negative feedback loops to exist for their activation (Gees et al., 2010).

iii. Ligand-gated Ca^{2+} Permeable Channels

Various ligand gated Ca^{2+} permeable channels exist on the plasma membrane, they are gated by neurotransmitters such as glutamate, acetylcholine (ACh), serotonin (5-hydroxytryptamine: 5-HT) and adenosine-triphosphate (ATP).

Glutamate receptors

Glutamate gates three subfamilies of ionotropic glutamate receptors: the NMDA (GluN), APMA (GluA) and Kainate (GluK) receptors. The properties and functions of these receptors are discussed in more detail in **1.3.3. Glutamate receptors and postsynaptic Ca^{2+} entry**.

Acetylcholine receptors

Acetylcholine (ACh) can activate both muscarinic/metabotropic (mAChRs) and nicotinic/ionotropic (nAChRs) ACh receptors. The nAChRs are pentameric in structure (comprised of 5 subunits) and these can have both homo and heteromeric composition. There are 17 subunits identified in mammals to date. These receptors are split into four groups depending on the subunits they contain: the 'neuronal type' Group 1 (α9, α10), Group 2 (α7, α8) and Group 3 (α2, α3, α4, α6, β2, β4, β3, α5-3) and the 'muscle type' Group 4 (α1, β1, δ, γ, ε). Many possible combinations of these subunits exist, resulting in a wide range of kinetic, electrophysiological, and pharmacological properties of the nAChRs, and therefore suggesting equally diverse functional properties. The Ca^{2+} permeability is heavily dependent on the subunit composition and a wide range of permeability is observed (Fucile, 2004).

5-HT receptors

5-HT (5-hydroxytryptamine), commonly referred to as serotonin, is another major neurotransmitter in the CNS. 14 members of the 5-HT receptor family have been identified all of which are metabotropic

receptors except 5-HT_3R, which is a non-selective cation channel and highly Ca^{2+} permeable (Ronde and Nichols, 1998). 5-HT_3R is found throughout both the central and peripheral nervous system. In the CNS, 5-HT_3R is localised to presynaptic terminals where it can cause both cellular depolarisation and modulation of neurotransmitter/neuropeptide release via subsequent intracellular Ca^{2+} signalling (Wu et al., 2015).

Purinergic receptors (ATP)

ATP activates both metabotropic purinergic receptors (P2YRs) and ionotropic receptors (P2XRs). The P2XRs are Ca^{2+} permeable, they are widely distributed and found in both excitatory and non-excitatory cells. P2XR consists of three subunits, in either homo or heterotrimeric configuration (Kawate et al., 2009). Seven genes which encode the subunits have been identified thus far. Their functions are also physiologically diverse (Khakh and Henderson, 1998).

iv. Store operated Ca^{2+} entry

Store operated Ca^{2+} channels (SOCs) are found in both excitable and non-excitable cells. They are particularly important in non-electrically excitable cells as these cells contain no VDCCs and reduced routes for Ca^{2+} to enter the cell after intracellular Ca^{2+} stores become depleted. The SOCs are the primary method for allowing Ca^{2+} into the cell to replenish these stores. Several store-operated inward Ca^{2+} currents have been observed (I_{SOCs}). The best understood of these is the Ca^{2+} release-activated Ca^{2+} current or I_{CRAC}, which has been described in a variety of cells, reviewed by Parekh and Putney (2005).

1.1.2 Ca^{2+} extrusion to extracellular fluid

Ca^{2+} may be extruded into the extracellular space via the action of the two transporters: The plasma membrane Ca^{2+} ATPase (**PMCA – Figure 1.6**) and the Na^+/Ca^{2+} exchanger (**NCX – Figure 1.6**). The PMCAs are a ubiquitous and vital regulator of intracellular Ca^{2+}. PMCA are ATPases localised to the plasma membrane. They have a high affinity for Ca^{2+} and operate at low $[Ca^{2+}]_i$, however, they also transport Ca^{2+} at a low capacity, therefore they are suggested to perform a 'maintenance' role,

keeping $[Ca^{2+}]i$ low between 100-200 nM (Brini and Carafoli, 2011). The NCXs are also localised to the plasma membrane in several tissues but are found most abundantly in excitable cells such as in the heart, kidney and neurones. The NCX has a lower affinity for Ca^{2+} than the PMCAs and requires a high $[Ca^{2+}]_i$ to become activated, and therefore is implied to have a role in counteracting high $[Ca^{2+}]_i$ after stimulus driven Ca^{2+} signals are produced (Brini and Carafoli, 2011). The NCX relies on the Na^+ concentration gradient to generate the energy required to extrude one Ca^{2+} for three Na^+. Under certain conditions, the NCX may reverse and allow Ca^{2+} into the cell, though its default operation is for Ca^{2+} extrusion. (Khananshvili, 2014).

1.2 Intracellular Ca^{2+} Stores

Ca^{2+} signals can also be generated via release from the intracellular stores of Ca^{2+}. These stores include the mitochondria, nucleus, Golgi apparatus, ER/SR and the acidic stores (endosomes/lysosomes). Each store has unique, and sometimes multiple routes by which Ca^{2+} release may occur. As several distinct Ca^{2+} stores exist within the cell it is possible to produce Ca^{2+} signals which are spatiotemporally variable. I review the aspects of ER and particularly the acidic Ca^{2+} store signalling here.

1.2.1 Membrane receptor coupling to intracellular Ca^{2+} stores via second messengers

I have discussed the membrane channels which, when activated, allow Ca^{2+} into the cell from the extracellular fluid. However, a great number, of non-channel forming (i.e. metabotropic) membrane receptors also exist and some of these receptors are able to mobilise Ca^{2+} from intracellular stores via second messenger activation. These receptors are an essential component in the detection and transduction of extracellular signals into the cell.

Second messenger molecules act as intracellular transducers, they are responsible for triggering intracellular signalling cascades, and eventually physiological processes, examples of which include: contraction, secretion, transcription, cell differentiation and cell death. It is advantageous to use intermediate or secondary messengers in signalling cascades as they present additional points where

intracellular signals can be controlled. Second messengers may act to amplify or dampen intracellular signals, they also allow greater spatial and temporal control of intracellular signalling cascades. Second messengers are typically only produced/synthesised after receptor activation and their action tends to be transient, as they are often removed/degraded rapidly. There are several classes of second messengers including cyclic nucleotides, arachidonic acid, phosphoinositols, and single unit G-proteins. Ca^{2+} itself is often referred to as a second messenger, and it may act as such, however, in many signalling events it appears further down the cascade, after an initial second messenger.

Three Ca^{2+} mobilising second messengers are well described: inositol 1,4,5-trisphosphate (IP_3), cyclic adenosine diphosphate ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP). A fourth Ca^{2+} mobilising messenger: sphingosine 1-phosphate (S1P) is also described to have Ca^{2+} mobilising action, though our current knowledge on its action is limited. All four of these molecules cause Ca^{2+} release/mobilisation from intracellular stores. I will next detail the major intracellular stores which Ca^{2+} mobilising second messengers act upon.

1.2.2 Endoplasmic reticulum Ca^{2+} signalling

The ER/SR is the largest single intracellular Ca^{2} store, with Ca^{2+} concentrations suggested to reach the millimolar range (Bygrave and Benedetti, 1996), Ca^{2+} loading of the ER is achieved through the action of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). SERCA is uniporter that is membrane bound in the ER/SR, it relies on the hydrolysis of ATP to transport Ca^{2+} into the ER/SR against the concentration gradient. SERCA has multiple regulatory sites, and its function is modulated by a variety of agents including phospholamban and sarcolipin (Periasamy and Kalyanasundaram, 2007). Ca^{2+} release from the ER can be achieved via the action of three Ca^{2+} mobilising second messengers: IP_3, cADPR, and S1P.

IP_3 and ER Ca^{2+} Release

IP_3 is perhaps the best-described Ca^{2+} mobilising second messenger, it is an inositol ring, with phosphate groups at positions 1, 4 and 5, with hydroxyl positions at positions 2, 3 and 6. The structure

is shown in **Figure 1.2A**. Its importance as a Ca^{2+} mobilising second messenger was discovered during the 1970s through seminal work on the blowfly salivary gland by Berridge and colleagues. Berridge noticed that the source of Ca^{2+} required for one route to saliva secretion was from an intracellular store (Prince et al., 1973). He later went on to show that hydrolysis of inositol lipids (phosphatidylinositol 4,5-bisphosphate: PIP_2) resulted in the production of IP_3 (Fain and Berridge, 1979) and caused Ca^{2+} release from the ER (Streb et al., 1983). The Ca^{2+} concentration of the ER is thought to lie between 1-5 mM (Bygrave and Benedetti, 1996) and represents a significant store within the cell (Berridge et al., 2000). IP_3 has since been identified as ubiquitous and conserved second messenger that causes Ca^{2+} release from the ER.

The early studies by Berridge and colleagues presented the first evidence linking extracellular stimulation (through membrane bound metabotropic receptors) and Ca^{2+} mobilisation from intracellular stores. This property is now known to be a fundamental principle for intracellular Ca^{2+} signalling. This major advance caused a key shift in the thinking towards intracellular Ca^{2+} and created a novel field of scientific research.

Over the subsequent years, the pathway linking metabotropic receptor activation to Ca^{2+} mobilisation from to ER was described in further detail. **Figure 1.2B** shows a simplified diagram of such pathway. On activation of G-protein coupled receptors (GPCR), with $G_{q/11}$ α-subunits, the activated $G_{q/11}$ α-subunit dissociates from the GPCR and can 'activate' phospholipase C (PLC). PLC causes hydrolysis of the membrane bound phosphoinositol: PIP_2. The two products of this hydrolysis are diacylglycerol (DAG) and IP_3. DAG is a hydrophobic diglyceride, it remains bound to the plasma membrane where it acts as a signalling molecule for other pathways, DAG is best described for its role as a physiological activator of protein kinase C. IP_3 is hydrophilic and becomes 'freed' from the plasma membrane, and may go on to activate the IP_3R on the ER/SR.

There are three isoforms of the IP_3 receptor (Type 1-3) with varying physiological distribution, though all isoforms are predominantly localised to the ER/SR. They act as ligand gated membrane channels,

with higher selectivity to Ca^{2+} than other cations. The IP_3Rs are modulated by several other molecules, most notably, Ca^{2+} itself, calmodulin and cytosolic ATP (Taylor and Tovey, 2010).

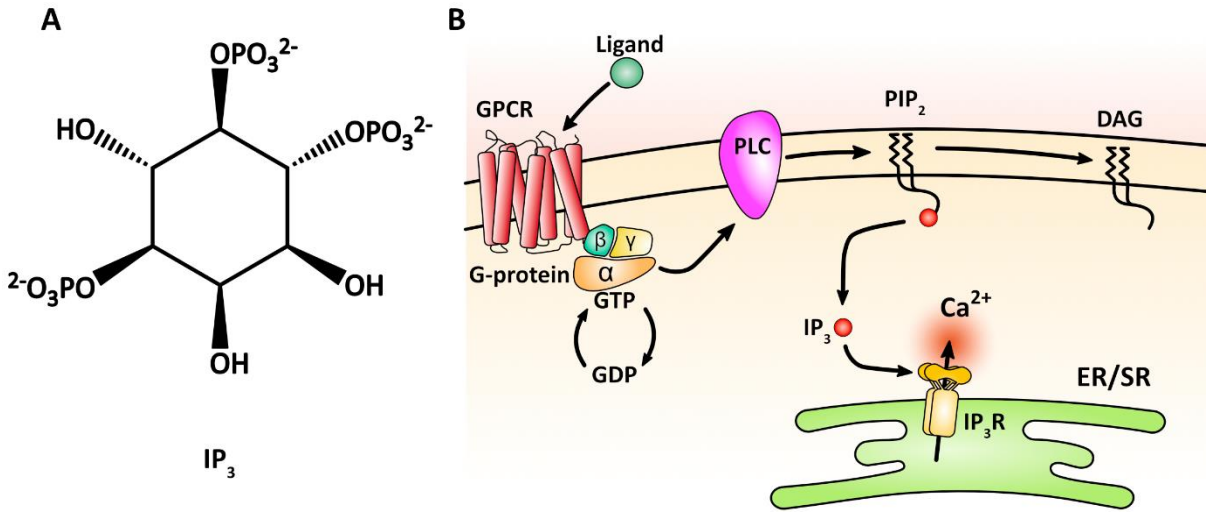

Figure 1.2 The IP_3 signalling pathway. A The chemical structure of IP_3. **B** Ligand activation of GPCRs causes a change in the receptor conformation, this allows the G-protein to exchange GDP for GTP and become 'activated'. The $G_{q/11}$ α-subunit dissociates from the GPCR and 'activates' phospholipase C (PLC), which causes it to hydrolyse PIP_2 into DAG and IP_3. IP_3 is hydrophilic and moves through the cytoplasm, thereby activating the IP_3Rs, causing Ca^{2+} release from the ER/SR. DAG remains bound to the plasma membrane where it activates PKC (not shown).

cADPR and ER Ca^{2+} Release

Cyclic adenosine phosphate-ribose (cADPR) is a cyclic nucleotide based Ca^{2+} mobilising second messenger. Its structure is shown in **Figure 1.3A**. Endogenous cADPR synthesis occurs through the action of a family of enzymes known as the ADP-ribosyl cyclases (ARCs). ARCs have the ability to synthesise cADPR from nicotinamide adenine dinucleotide (NAD+). In mammalian systems, CD38 is considered the main ARC that endogenously synthesises cADPR. However, in CD38-/- knockout mice endogenous cADPR is still detectable, suggesting the presence of alternative ARCs which are able to synthesise cADPR endogenously, such as CD157 (Ceni et al., 2003). Like IP_3, cADPR-induced Ca^{2+} mobilisation has been shown in a variety of experimental preparations, from plants and invertebrates to human cell lines, cADPR is highly effective at causing Ca^{2+} mobilisation from the ER/SR. The cADPR

sensitive channel on the ER/SR is thought to be the ryanodine receptor (RyR). RyRs exist as one of three isoforms and, like the IP_3R, are regulated by multiple intracellular molecules including intracellular Ca^{2+}. However, the manner in which cADPR achieves Ca^{2+} release through RyRs is not entirely clear. Direct binding sites for cADPR on the RyRs are not present and accessory proteins, including calmodulin and FK506-binding protein, are thought to be essential for the correct physiological action of cADPR (Guse, 2015). Therefore, cADPR is suggested to act as a sensitizer of the RyR to Ca^{2+}, and by greatly lowering the threshold for CICR, activates the RyRs (Galione et al., 1991). **Figure 1.3B** shows the hypothesised intracellular signalling pathway for cADPR.

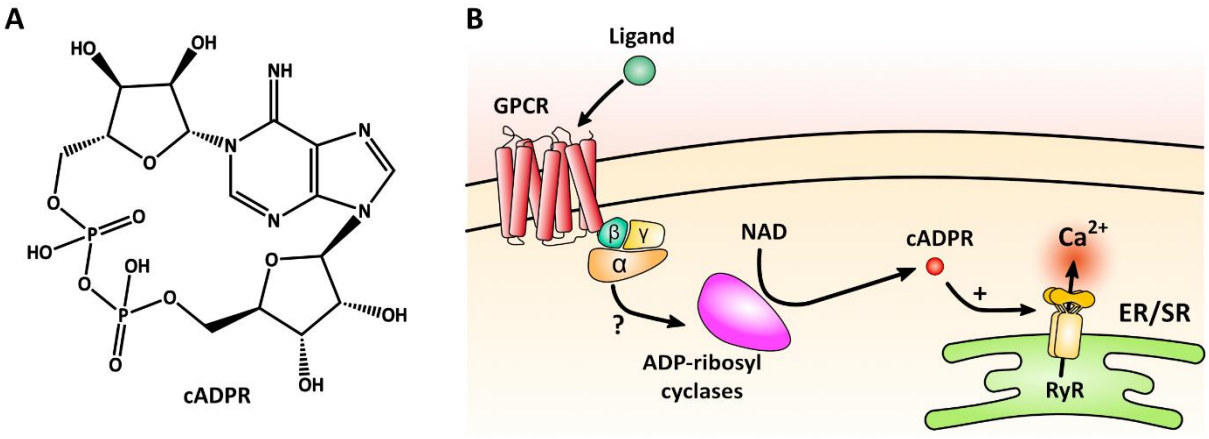

Figure 1.3 The cADPR signalling pathway. A The chemical structure of cADPR, displaying the cyclic nature of the molecule. **B** The activation of ARC enzymes is not well understood but activation of various metabotropic membrane receptors can lead to the synthesis of endogenous cADPR. cADPR is thought to sensitise the RyRs to Ca^{2+}, and therefore initiate CICR from the ER/SR.

S1P and ER Ca^{2+} Release

Perhaps the least well understood of the Ca^{2+} mobilising second messengers is S1P, a sphingolipid that was initially thought to have purely extracellular cell-to-cell signalling properties via GPCRs. However, Ghosh et al. (1990) noted that S1P may also act as an intracellular second messenger with ER Ca^{2+} mobilising properties. S1P is suggested to be synthesised intracellularly via the action of sphingosine kinase; its target receptor is still unidentified, though it is suggested to reside inside the ER (Ghosh et al., 1994).

1.2.3 The acidic Ca^{2+} stores and NAADP

Acidic organelles such as the endosomes or lysosomes have traditionally been thought of as compartments for degradation and waste metabolite recycling. However, more recently they have been identified to have roles in intracellular Ca^{2+} signalling. Nicotinic acid adenine dinucleotide phosphate (NAADP) is a Ca^{2+} mobilising second messenger that has been shown to produce Ca^{2+} release from intracellular acidic stores such as the endosomes and the lysosomes; thus identifying the acidic stores as a significant and physiologically important Ca^{2+} store. The lysosomal/acidic store Ca^{2+} concentration is estimated to be 400-600 μM (Christensen et al., 2002, Lloyd-Evans et al., 2008) Ca^{2+} loading is dependent on the action of a vacuolar H^+ uniporter/ATPase (V-ATPase). The V-ATPase generates a high proton concentration (low pH) inside the lysosome (Christensen et al., 2002) which is then used by H^+/Na^+ exchangers coupled to Na^+/Ca^{2+} or H^+/Ca^{2+} exchangers, to control the Ca^{2+} concentration in the acidic stores (Churchill et al., 2002).

1.2.4 The discovery and characterisation of NAADP as a Ca^{2+} mobilising messenger

NAADP was identified serendipitously by Hon Cheung Lee and colleagues as a contaminant of commercially purchased nicotinamide adenine dinucleotide phosphate (β-$NADP^+$) (Clapper et al., 1987). At this, time Lee and his group were using pyridine nucleotides to study intracellular Ca^{2+} release in sea urchin egg homogenate. Sea urchin eggs have been used in to show that intracellular Ca^{2+} is the key factor for initiating egg metabolism (Steinhardt and Epel, 1974). IP_3 and pyridine nucleotide concentrations were known to become elevated during fertilisation, correlating with raised intracellular Ca^{2+} (Turner et al., 1984). Lee and his group subsequently identified two molecules relating to pyridine nucleotides which could also induce Ca^{2+} release from intracellular stores in sea urchin egg homogenates: cADPR (Lee et al., 1989) and NAADP (Aarhus et al., 1995).

Since its discovery in the sea urchin egg, NAADP and its intracellular Ca^{2+}-mobilising properties have been described in various invertebrate (Aarhus et al., 1995, Aarhus et al., 1996, Churchill et al., 2003, Moccia et al., 2004), mammalian (Bak et al., 1999, Berg et al., 2000, Yamasaki et al., 2005, Cancela et

al., 1999) and plant systems (Navazio et al., 2000). Furthermore, activation of a variety of plasma membrane receptors by physiological stimuli causes the endogenous synthesis of NAADP and subsequent Ca^{2+} release from intracellular stores. Examples of such stimuli include: glucose in clonal pancreatic β-cells (Masgrau et al., 2003), cholecystokinin in mouse acinar cells (Yamasaki et al., 2005), histamine in myometrial cells (Soares et al., 2007), insulin in adipocytes (Song et al., 2012) and isoprenaline (via ß-Adrenergic receptors) in Langendorff-perfused hearts (Lewis et al., 2012). These studies provide strong evidence that NAADP is a bona fide intracellular second messenger with Ca^{2+} mobilising properties. On the basis of such studies, NAADP is now considered to be a ubiquitous and important molecule for mobilisation of intracellular Ca^{2+}.

There are also two features of NAADP signalling which are noteworthy, and are of physiological significance: Firstly, endogenous concentrations of NAADP in mammalian preparations lie within the nanomolar range, approximately 20 nM over a variety of mammalian preparations, as summarised by Guse et al. (2013). In the sea urchin egg, however, basal NAADP concentration is suggested to lie at approximately 300 nM (Churchill et al., 2003). It is suggested NAADP is the most potent of the known Ca^{2+} mobilising second messengers (Galione, 2011).

Secondly, NAADP in unique among second messengers as it has the ability to self-desensitise its receptor. This property also differs slightly between sea urchin egg and mammalian preparations. In the sea urchin egg, subthreshold concentrations (pM) range of NAADP will inactivate the receptor, and prevent further activation and Ca^{2+} release by a usually effective concentration (nM range) of NAADP (Genazzani et al., 1996). The mechanism of this property is not fully understood though it is suggested that it may be due to high affinity/irreversible binding of NAADP to its receptor, which is observed with [32^{P}]NAADP (radioactively labelled NAADP) (Aarhus et al., 1996). Contrarily, in mammalian preparations, subthreshold inactivation of the NAADP receptor does not occur, though suprathreshold inactivation is observed on application of micromolar concentrations of NAADP (Aarhus et al., 1996, Genazzani et al., 1996). Dose response-curves are consequently bell shaped in

mammalian preparations with maximal Ca^{2+} responses observed at around 100 nM NAADP reviewed in Galione (2011) and Guse et al. (2013). This interesting property of self-desensitisation may indicate two (or more) binding sites for NAADP to its receptor, with one site possessing high affinity, and one with low affinity. This theory could explain the difference between the inactivation properties in sea urchin and mammalian systems, where an evolutionary switch in the high/low binding affinity site for activation may have occurred.

A note on methods to detect endogenous NAADP

Intracellular NAADP concentration can be measured using radio-receptor competitive binding assays. The supernatant is extracted from tissue homogenates suspected of containing NAADP. This supernatant is to sea urchin egg homogenate. At low nanomolar concentrations, NAADP is suggested to irreversibly bind to NAADP receptors in the sea urchin egg. Following this [32P]NAADP is added, which binds to remaining receptors and the amount of radioactivity is detected and compared to a standard curve, indicating the amount of occupied NAADP receptors and therefore the concentration of NAADP (Lewis et al., 2007). Alternative methods for detecting NAADP are based upon fluorometric assays which employ the use of coupled enzymatic reactions to produce fluorescent resorufin (Graeff and Lee, 2013, Graeff and Lee, 2002). fluorometric methods are thought to be comparable but do not provide the as greater sensitivity to NAADP detection compared to radio-ligand binding assays.

1.2.5 NAADP endo-synthesis and degradation

Detection of endogenous NAADP has been described in a wide range of experimental preparations, ranging from *E. coli* (Churamani et al., 2004) to invertebrate preparations such as the sea urchin egg and spermatozoa (Churchill et al., 2003), and finally in a variety of mammalian preparations. These include human red blood cells (Churamani et al., 2004), guinea pig hearts (Lewis et al., 2012), mouse uterus, liver, kidney, spleen, and heart (Soares et al., 2007).

In order to be classed as a second messenger NAADP should be shown to be endogenously synthesised and degraded. Agonist induced endo-synthesis of NAADP has been suggested in a number of

experimental preparations, notably in pancreatic acinar cells with the application of cholecystokinin (CCK) (Menteyne et al., 2006) and in pancreatic β-cells with the application of glucose (Arredouani et al., 2010). Ned-19, a specific NAADP antagonist (Naylor et al., 2009), and other pharmacological tools which disrupt lysosomal Ca^{2+} signalling blocked NAADP-mediated Ca^{2+} oscillations that occur in response to these agonists. Other endogenous agonists are shown to cause rises in NAADP concentration, indicating endo-synthesis. These include histamine in myometrial cells (Soares et al., 2007), interleukin-8 and lymphokine activated killer cells (Rah et al., 2010) and thrombin in mouse platelets (Mushtaq et al., 2011). In neurons of the medulla oblongata, Pandey et al. (2009) quantified the amount of NAADP endo-synthesis (using a radio-ligand competitive binding assay) upon application of glutamate. The basal endogenous concentration in mammalian preparations lies in the low nanomolar range (around 10 nM). Upon stimulation, NAADP concentration is suggested to rise to between two to twenty times the basal concentration, summarised by Guse et al. (2013).

Despite reports of NAADP endo-synthesis in a variety of preparations, the enzyme or enzymes responsible for its endo-synthesis are not clearly elucidated. Much of the research effort on NAADP has been focused on identifying NAADP sensitive intracellular Ca^{2+} stores and its receptor. ADP-ribosyl cyclases (ARCs) are favoured as a candidate enzyme family for NAADP endo-synthesis. These enzymes were first characterised as enzymes that could cyclise nicotinamide adenine dinucleotide (NAD) to cADPR. In mammals, two candidate ARCs have been identified thus far: CD38 and CD157. Both are suggested to be multifunctional enzymes that are able to catalyse a base exchange reaction which produces NAADP and nicotinamide from β-NADP and nicotinic acid, *in vitro*, but only under acidic conditions (pH 4) and in the presence of cAMP (Hirata et al., 1994, Aarhus et al., 1995, Itoh et al., 1998). The requirement for acidic conditions is intriguing and has been the basis of the suggestion that mammalian ARCs may reside within the acidic stores of the cell. Furthermore, CD38 and CD157 are typically ectoenzymes (enzymes present on the outside surface of a cell) though some intracellular localisation has been reported (Lee, 2012). Therefore, another possibility is that the ARCs may be bound to intracellular membranes or reside within intracellular compartments, with the products

being transported into the cytoplasm to achieve their effects. There also seems to be tissue specific differences in NAADP synthesising ability of the ARCs. Gene silencing of CD38 in lymphocytes or lymphatic tissue (Schmid et al., 2011) or myometrial cells (Soares et al., 2007) did not reduce the amount of endogenous NAADP detected. Whereas, in other preparations such as pancreatic acinar cells (Cosker et al., 2010) or lymphokine-activated killer cells (Rah et al., 2010), derived from CD38-/- mice show deficient NAADP signalling. It seems that the enzyme or enzymes responsible for endogenous NAADP synthesis are still unidentified. The studies undertaken so far suggest it is likely that multiple ARCs may possess the ability to synthesise NAADP with some ARCs yet to be discovered. Furthermore, it seems there may be tissue specific differences between NAADP synthesising enzymes.

Degradation of NAADP is suggested to occur via the action of a Ca^{2+}-dependent 2'-NAADP-phosphatase based on enzymatic function (Berridge et al., 2002); however, this phosphatase has only been identified in HeLa cells (as the placental isoform of alkaline phosphatase) (Schmid et al., 2012)

1.2.5 Identifying the acidic compartments as NAADP sensitive Ca^{2+} stores

Whilst describing NAADP's action as a Ca^{2+} mobilising messenger in sea urchin egg homogenates, Lee and colleagues also showed that inhibition of IP_3 and cADPR signalling pathways (both of which were known to mobilise Ca^{2+} from the ER/SR), with heparin and 8-amino-cADPR respectively, did not abolish Ca^{2+} release on subsequent application of NAADP. This led to the suggestion by Lee and Aarhus (1995) that NAADP mobilises Ca^{2+} from a previously undescribed store, which was distinct from the ER.

Further evidence of an NAADP sensitive intracellular Ca^{2+} store that is distinct from the ER was obtained in both homogenated (Genazzani and Galione, 1996) and intact (Churchill and Galione, 2001) sea urchin egg preparations. Where preventing ER Ca^{2+} loading with thapsigargin (a SERCA pump inhibitor), in combination with pharmacological antagonism of IP_3 and cADPR signalling pathways failed to inhibit NAADP-evoked Ca^{2+} release.

Lee and Aarhus also undertook a crucial study, where they used gentle centrifugation to create 'stratified' sea urchin eggs, where less dense intracellular compartments (the ER and nucleus) migrate

to the top pole, and denser compartments (the lysosomes and the mitochondria) migrated to the bottom pole. Photolysis of caged IP_3/cADPR caused Ca^{2+} release from top pole, whereas photolysis of caged NAADP caused Ca^{2+} release from the opposite pole to the ER. This study provided more evidence that the NAADP-sensitive Ca^{2+} is distinct from the ER (Lee and Aarhus, 2000).

Further graduated fractionation (using the Percoll media centrifugation method to produce fractions based upon density) of sea urchin egg homogenates was used to purify an NAADP sensitive Ca^{2+} fraction in the stratified sea urchin egg preparation. This fraction did not release Ca^{2+} on application of IP_3 or cADPR, furthermore NAADP sensitivity could be blocked with bafilomycin, a vacuolar proton pump inhibitor, and also protonophores such as nigericin and FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), suggesting the NAADP sensitive compartment is acidic in nature (and therefore ruling out the mitochondria) (Churchill et al., 2002). Binding of [32^{P}]NAADP was also found to be highest in the fraction with compartments enriched with enzymatic markers of the acidic organelles (reserve granules- the sea urchin egg equivalent of the lysosomes). Finally, GPN (glycyl-L-phenylalanine-β-naphthylamide), a substrate for the lysosomal specific cathepsin C that is known to cause hydrolytic perforation of the lysosomes (Berg et al., 1994) selectively abolished Ca^{2+} sensitivity to NAADP in intact and homogenated sea urchin egg preparations. Whilst IP_3 Ca^{2+} signalling remained unaffected (Churchill et al., 2002). In lieu of these results, the NAADP sensitive Ca^{2+} store in sea urchin eggs was suggested to be the reserve granules – and the lysosomes or endosomes in their mammalian counterparts. However, it should be noted that NAADP was not found to mobilise Ca^{2+} from the acidic stores in starfish oocytes (Moccia et al., 2006).

Since describing NAADP's Ca^{2+} mobilising properties in sea urchin egg preparations, a large body of work has now been undertaken in a variety of different mammalian preparations. The first demonstration that NAADP acts as a Ca^{2+} mobilising messenger in a mammalian preparation was described in murine pancreatic acinar cells (Cancela et al., 1999) and this system was used extensively to describe the fundamental properties of NAADP-mediated Ca^{2+} signalling in mammalian cells.

NAADP become established as an endogenous, ubiquitous and physiologically relevant Ca^{2+} mobilising second messenger. The store that NAADP mobilises Ca^{2+} from in mammalian systems is consistently suggested to be the lysosomes/acidic stores, and furthermore, various extracellular ligands have been shown to stimulate endogenous NAADP synthesis upon receptor activation. The last two points will be discussed in further detail throughout the subsequent text.

As mentioned earlier the lysosomes are estimated to contain a Ca^{2+} approximate concentration approximately 400-600 µM (Christensen et al., 2002, Lloyd-Evans et al., 2008), and therefore represent a significant and important intracellular Ca^{2+} store. The lysosomes are not a continuous large vesicle, they are mobile within the cytosol and exist in varying quantities in subcellular compartments. These attributes suggest the ability for highly localised and unique Ca^{2+} mobilising properties.

Furthermore, in the case of some Ca^{2+} signalling events, and especially global Ca^{2+} signalling events, interfering with ER/SR Ca^{2+} signalling can block or reduce NAADP driven Ca^{2+} signals and their associated physiological effects, which can be initiated by NAADP alone. These results suggest interesting features of NAADP's Ca^{2+} mobilising properties. The details of which are stated below.

Firstly, that NAADP may act as a 'trigger', causing a local Ca^{2+}signal, which in turn recruits other Ca^{2+} stores such as the ER to produce larger or even global Ca^{2+} signals. This idea is described as the 'two-pool' theory of intracellular Ca^{2+} signalling by NAADP. The 'two-pool' theory suggests that the acidic stores and the ER are at least functionally, and possibly also physically, coupled to one another. The theory relies on the assumption that the acidic stores and the ER are in close enough proximity to one another that local Ca^{2+} signalling events from the acidic stores would be detected, and sensitise Ca^{2+} release channels on the ER (IP_3R/RyRs) to cytosolic Ca^{2+} and cause CICR. Membrane contact sites (MCSs) between the ER and many organelles, including the mitochondria, endosomes and the Golgi have been described, as reviewed by (Phillips and Voeltz, 2016). These contact sites are stabilised by physical connections in the form of tethering proteins that link the two organelles together. The presence of MCSs is functionally significant, they allow information transfer and signalling events to

occur locally in a tightly controlled manner. This is beneficial in the context of Ca^{2+} signalling, where local Ca^{2+} signals require close proximity to effector proteins in order to initiate further signalling cascades. Following the 'two-pool theory', MCSs between the ER and the acidic stores and their functional significance has been explored in greater detail. The presence of MCSs between the acidic stores and the ER is now well described (Eden, 2016, Helle et al., 2013, Levine and Patel, 2016, Phillips and Voeltz, 2016, Kilpatrick et al., 2013). Functional roles of ER-acidic store MCSs are becoming elucidated, ER-acidic store MCSs are shown to mediate several physiological events including but not limited to: Ca^{2+} signalling between ER and acidic stores (Kinnear et al., 2004, Kinnear et al., 2008), endosomal morphology and function (Kilpatrick et al., 2017), lipid transport (Eden et al., 2016) and endosome fission (Rowland et al., 2014). The presence of these MCSs between the ER and the acidic stores allows for localised signalling and therefore specialised and local physiological events to occur.

Secondly, some specific Ca^{2+} signalling events (and therefore physiological functions) may only be possible if NAADP-induced Ca^{2+} release occurs in combination with the presence and effects of other Ca^{2+} mobilising second messengers (IP_3 and ER Ca^{2+} for example). Two elegant examples which show such specificity are described here: Brailoiu et al. (2006) showed that introduction of NAADP alone to PC12 cells initiates cellular differentiation, whereas introduction of IP_3 and cADPR does not, despite all three second messengers robustly causing Ca^{2+} mobilisation. Yamasaki et al. (2004) demonstrated the importance of specific Ca^{2+} mobilising second messengers for producing agonist induced Ca^{2+} oscillations. This study observed intracellular Ca^{2+} signalling dynamics on the application of neurotransmitters to murine pancreatic acinar cells. Cholecystokinin (CCK) and acetylcholine (ACh) both produced Ca^{2+} oscillations in acinar cells, however, the dynamics were markedly different and furthermore CCK-induced Ca^{2+} oscillations were abolished with agents that block lysosomal Ca^{2+}/NAADP signalling (GPN and bafilomycin) whereas ACh oscillations were not affected by such agents.

Acidic organelles such as the endosomes or lysosomes have traditionally been thought of as compartments for degradation and waste metabolite recycling. However, as a result of the work on the Ca^{2+} mobilising properties of NAADP, the lysosomes are now considered to have critical roles in a variety of important cellular processes. The ability of NAADP to produce local and global Ca^{2+} signalling events, initiated from the acidic stores, has added another layer to our understanding of complexity and control of intracellular Ca^{2+} signals and in turn a deeper understanding of how Ca^{2+}, as a ubiquitous signalling molecule, can initiate so many different physiological processes.

1.2.6 The NAADP receptor

NAADP is now generally accepted to mobilise Ca^{2+} from the lysosome/acidic stores. However, the target to which NAADP binds and the Ca^{2+} release channel on the acidic stores is still uncertain. Several candidates have been proposed and I will discuss the evidence for each in the following section.

Ryanodine receptors

Some groups initially proposed the RyR and the ER in mammalian cells to be the target and Ca^{2+} store associated with NAADP signalling. Mojzisova et al. (2001) used type 2 ryanodine receptors (RyR2) reconstituted into a lipid bilayers and found that NAADP in excess of 1 µM could elicit Ca^{2+} currents. However, this concentration is several orders of magnitude larger than any endogenous concentration of NAADP detected in mammalian preparations thus far. Using a similar approach Hohenegger et al. (2002) found NAADP, in a physiological range (30 nM), could increase the open probability in RyR1s purified from the heart and reconstituted into lipid bilayers. Conversely, the effect was not observed in RyR1s purified from the heart and skeletal muscle (Copello et al., 2001). One has to recognise that studies which rely on the lipid bilayer technique come with the caveat that purification of channel proteins may remove regulatory/modulatory elements that may be critical for physiological function. The RyR is one of the largest intracellular membrane channels with regulation by a variety of additional molecules; their behaviour without the presence of the physiological regulatory elements is, therefore, likely to be markedly different. Interestingly, even in more intact preparations, the effects

of NAADP can also be abolished by blocking the RyRs or interfering with ER Ca^{2+} (Gerasimenko et al., 2003, Steen et al., 2007). These findings can be interpreted one of two ways: either NAADP directly activates the RyRs, or that the NAADP signalling, and therefore acidic store Ca^{2+} release acts in conjunction with ER Ca^{2+} release, perhaps serving as a trigger for CICR. The latter explanation is likely to provide a better explanation. Furthermore, spatiotemporal Ca^{2+} imaging resolution is currently too limited to visualise local and potentially very rapid acidic store Ca^{2+} release. Furthermore, in many of the studies mentioned in the previous section inhibition of the RyR does not always affect NAADP-induced Ca^{2+} release, suggesting NAADP can act independently of RyR in some conditions.

Transient receptor potential channels

Two TRP channels have been suggested to be the target for NAADP: TRPM2 and TRPML-1. TRPM2, a member of the TRP melastatin family, can be activated by NAADP in several preparations (Beck et al., 2006, Toth and Csanady, 2010, Lange et al., 2008), however, in these studies, the EC50s for TRPM2 activation ranged from 1 µM – 730 µM NAADP. TRPM2 is predominantly localised to the plasma membrane, though partial colocalisation with the lysosomal marker LAMP2 is reported (Sumoza-Toledo and Penner, 2011). It seems unlikely that TRPM2 is the target for NAADP. In the studies mentioned, the concentrations of NAADP required for TRPM2 activation are much greater than endogenously detected NAADP concentration, most are far in excess of concentrations shown to desensitise the NAADP receptor in other preparations. Furthermore, thorough pharmacological and genetic manipulations to elements of lysosomal Ca^{2+} pathways and TRPM2 expression, respectively, are yet to be undertaken.

TRP-ML1, a member of the TRP mucolipin family, is also suggested to be the channel responsible for lysosomal Ca^{2+} release. TRP-ML1 purified from lysosomal membranes, in rat liver or bovine smooth muscle cells, and reconstituted into lipid bilayers could be activated by 1 nM - 1 µM NAADP, with higher concentrations causing desensitisation. Furthermore, neither agonists or antagonists of ER Ca^{2+} release channels affected their open probability (Zhang and Li, 2007). Responses to NAADP could also

be blocked by antibodies against TRP-ML1 in the lipid bilayer and by siRNA directed knock-down in coronary arterial myocytes (Zhang et al., 2009). Interestingly, verapamil and nifedipine (L-Type Ca^{2+} channel antagonists) could also block responses to NAADP in purified TRP-ML1 bilayer preparations (Zhang and Li, 2007). These studies provide good evidence that TRP-ML1 could be a target for NAADP. However, in other TRP-ML1 expressing systems, NAADP failed to cause Ca^{2+} release, unless in the presence of a two-pore channel (TPC1 and TPC2). TRP-ML1 is co-localised and can be co-immunoprecipitated with TPC1 and TPC2, suggesting a possible functional or physical cooperation between the two channels (Yamaguchi et al., 2011). Perhaps in the two studies by Zhang et al. (2009), Zhang and Li (2007), contamination of their TRP-ML1 samples with TPCs was possible, which would explain the sensitivity to L-Type Ca^{2+} channel blockers, which are known to block TPCs, owing to their similarity to the voltage dependent Ca^{2+} channels (Patel et al., 2011).

Two-pore channels

The last channels to be implicated as NAADP-sensitive Ca^{2+} release channels on the lysosomes are two-pore channels (TPCs). TPC exists as one of two isoforms in humans and rodents: TPC1 and TPC2, however, most mammals, fish, reptiles and amphibians also possess an additional isoform TPC3 whereas plants and only possess one isoform: TPC1 (Zhu et al., 2010a). Their necessity for NAADP-induced Ca^{2+} release has been shown in a variety of systems, ranging to lipid bilayer preparations to a variety of cell lines, both intact and reconstituted, and primary cell cultures. Furthermore, the number of studies and range of manipulations used in this area has been the most comprehensive; including extensive pharmacological investigation, gene silencing and the use of knock down, knock-out animals. These studies are reviewed in the following section. However, some controversy exists in this area. Initially, the TPCs were suggested to be the target of NAADDP, and also the Ca^{2+} releasing channel of the acidic stores. Three studies during 2009 provided evidence to suggest this hypothesis. The first by Calcraft et al. (2009) showed high-affinity binding of NAADP to the endolysosomal membranes of HEK293 cells overexpressing human TPC2 (TPCN2). Furthermore, pancreatic β-cells from TPC2 KO mice were found to be NAADP insensitive and disruption of the endo-lysosomal proton gradient, with

bafilomycin, abolished NAADP-mediated Ca^{2+} release. Calcraft et al. (2009) also noted that these manipulations did not abolish ER-mediated Ca^{2+} release. Shortly after this, a second study by Brailoiu et al. (2009a) showed overexpression of TPC1 in SKBR3 cells enhanced NAADP-mediated Ca^{2+} signals, whereas silencing of TPC1 with shRNA abolished NAADP-mediated Ca^{2+} signals. Furthermore, this study shows that mutation of a single residue in the suggested pore region of TPC1 also abolished NAADP-mediated Ca^{2+} release. The third study of 2009 by Zong et al. (2009) broadly replicated these findings in HEK293 cells. Studies on the ion selectivity of TPC1 and TPC2 have produced varied results. Lipid bilayer reconstituted human TPC1 (hTPC) suggests permeability to monovalent ions (including protons) as well as Ca^{2+} (Pitt et al., 2014). In the same experimental system, TPC2 was shown to be Ca^{2+} permeable (Pitt et al., 2010). Both studies suggest that TPC1 and TPC2 can be activated by NAADP and is functionally modulated by Ca^{2+}. It is interesting that sensitivity to NAADP is still observed in these lipid bilayer studies despite the lack of regulatory elements.

More recently TPCs have been suggested to be predominantly Na^{+} not Ca^{2+} selective channels regulated by phosphatidylinositol 3,5-bisphosphate (PIP_2). Wang et al. (2012) measured voltage current relationships under a variety of conditions by using voltage clamp of endo-lysosomes treated with vacuolin (cell-permeable triazine that prevents the release of endo-lysosomal contents and causes swelling). Ordinarily, the endo-lysosomal compartments are too small to perform patch clamp on, however, using vacuolin increases their size and allows this to be undertaken. The results produced by Wang et al. (2012) suggest that TPCs are not activated by NAADP, but instead by PIP_2 and form Na^{+} channels, not Ca^{2+} channels. This study also used Ca^{2+} imaging in pancreatic islet cells, their results suggest that TPC1/2 KO animals produce normal Ca^{2+} responses to NAADP. Cang et al. (2013) also used patch clamp electrophysiology of vacuolin swelled endo-lysosomes, and noted activation of TPC1/2 by PIP_2 and modulation by ATP and mTOR and suggested TPC1/2 to be Na^{+} selective.

Structural insights provided by recent crystallisation of TPC1 from *Arabidopsis thaliana* (AtTPC1) (Guo et al., 2016), and functional comparison of human TPC2 (hTPC2) and AtTPC1 (Guo et al., 2017) ion

selectivity has led to the suggestion that hTPC2s are Na^+ selective channels, AtTPC1 has greater selectivity for Na^+ than Ca^{2+} but hTPC1 selectivity is still unclear. Further patch clamp studies were undertaken on vacuolin swollen endo-lysosomes also suggest TPC1 to be Na^+ selective (Cang et al., 2014). Voltage current relationships on vacuoles expressing hTPC1 derived from TPC1 free *A. thaliana* cells suggest that TPC1 is a Na^+ selective channel activated by PIP_2 and regulated by luminal and cytosolic Ca^{2+} (Lagostena et al., 2017).

However, the validity of some of the previously mentioned studies (Cang et al., 2014, Cang et al., 2013, Wang et al., 2012) has been questioned based on the type of TPC KO line used. Except for the N-Terminus entire, TPC protein remained present in these 'TPC-KO'. The N-terminus of TPCs has been suggested to be essential for NAADP sensitivity (Churamani et al., 2013). An extensive re-investigation of the initial findings, with a newly derived KO line, suggested that TPCs are necessary for NAADP-mediated acidic store Ca^{2+} release. This study also included a particularly elegant rescue of NAADP sensitivity, and restoration of acidic store Ca^{2+} signalling, with the viral reintroduction of TPCs to KO animals (Ruas et al., 2015).

It is critical to recognise the challenges presented with undertaking this type of work on the endo-lysosomes. Each technique produces its own experimental confounds which reduce the reliability of the data collected. Reconstitution of membrane channels into non-native membranes without the presence of regulatory elements may produce non-physiological responses. Furthermore, patch clamp of vacuolin swollen endo-lysosomes would change the luminal ionic/proteinaceous composition, as an effect of both the vacuolin itself and dialysis of endo-lysosomal contents into the patch pipette solution. However, the difficulty of producing purified acidic store membrane preparations leaves the investigator with few options.

Finally, NAADP may not directly bind to the any of the channels discussed above. Photoaffinity labelling studies in both sea urchin egg and mammalian preparations suggest NAADP binds to a smaller

molecular weight (22/23kD) protein and not directly to a larger membrane channel (Lin-Moshier et al., 2012, Walseth et al., 2012).

Based on the evidence in the previous section, it seems clear TPCs are essential for NAADP-mediated Ca^{2+} release from the acidic stores. Whether or not they directly bind NAADP seems unlikely based upon photo-affinity labelling studies (Lin-Moshier et al., 2012, Walseth et al., 2012). Moreover, whether TPCs are the channel responsible for releasing Ca^{2+} is unclear, as the discrepancies in the studies on the ion selectivity of TPCs are still present, especially in light of recent crystal structure analysis (Guo et al., 2016, Guo et al., 2017).

In light of the evidence presented above, I suggest that to achieve endogenous acidic store Ca^{2+} release a protein complex is required. This complex would consist of a minimum of three essential components: an NAADP-binding protein and TPC1/TPC2 (perhaps to confer lysosomal excitability via Na^{+} release), and a third component, a Ca^{2+} releasing channel, such as TRP-ML1. Finally, like many protein complexes, it is likely that each component may be subjected to functional modulation by a variety of other molecules; which in turn influence the behaviour of the protein complex as a whole. This may aid explaining the varied results obtained from different experimental systems.

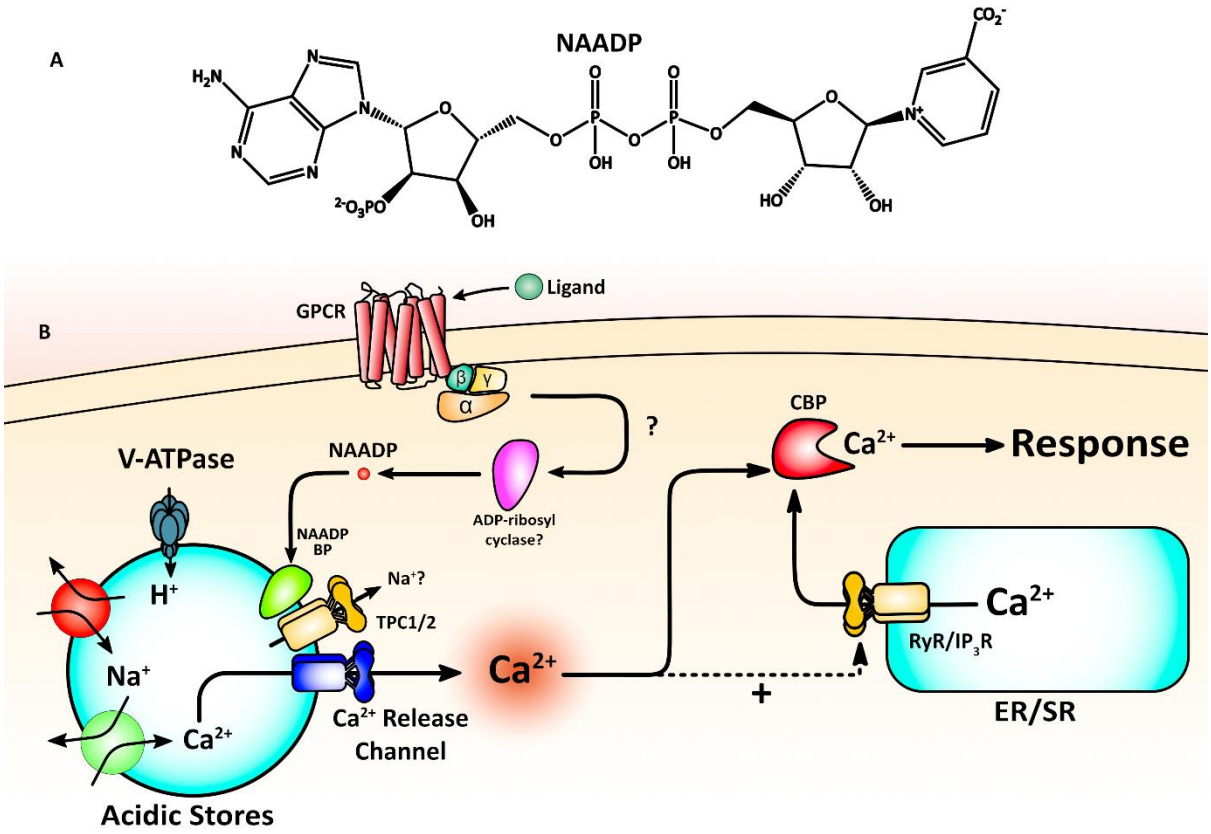

Figure 1.4 The structure of NAADP and a potential intracellular signalling pathway. A The molecular structure of NAADP. **B** Suggested NAADP signalling pathway, where NAADP is synthesised via an ARC after activation of the appropriate cell membrane receptor; this step may occur inside acidic organelles. NAADP binds to a receptor/channel complex on the acidic Ca^{2+} stores either directly or indirectly, which allows Ca^{2+} release from the acidic stores. Acidic store Ca^{2+} acts on its downstream targets and may be further amplified via the RyRs. Abbreviations: GPCR – G-protein coupled receptor, NAADP BP – NAADP binding protein, TPC – two-pore channel, RyR – ryanodine receptor. ER/SR – endo/sarcoplasmic reticulum.

1.2.7 Tools for investigating acidic store/NAADP-mediated Ca^{2+} signalling

In the previous sections, I mentioned a variety of tools which are commonly used to investigate NAADP signalling and lysosomal Ca^{2+} signalling. I will describe their mechanisms of action here.

i. Targeting the NAADP receptor

Several pharmacological tools are available to investigate NAADP signalling. The first class I will describe act upon the NAADP receptor directly. In mammals, the NAADP receptor is activated by nanomolar concentrations of NAADP but also inactivated by desensitising concentrations of NAADP (often in the millimolar range) (Aarhus et al., 1996, Genazzani et al., 1996). This bell shaped dose-response curve has been noted in a wide range of preparations, as reviewed by Guse et al. (2013) and Galione (2011). NAADP is a charged molecule and therefore does not readily pass through the plasma membrane, therefore, NAADP must be either injected or introduced via liposomes. This limits the investigator as both techniques can be technically challenging. Parkesh et al. (2008) described a novel method for synthesising a membrane permeable form of NAADP (NAADP-AM) by attaching acetoxymethyl ester (AM) groups. The AM group negates the charge of NAADP and allows passage through the plasma membrane. Endogenous esterases in the cytosol then cleave the AM groups and allow NAADP to activate its target. As shown with NAADP-AM, it is possible to attach other chemical groups to NAADP. Another example of this method is by attaching a chemical 'cage' group. This allows NAADP to be introduced into cells in an inactive form. When exposed to ultra violet light the cage group is removed and NAADP may act on its target (Lee et al., 1997). The final tool to directly target the NAADP receptor is a pharmacological antagonist known as Ned-19. This compound was identified via virtual screening and is suggested to bind to the NAADP receptor at nanomolar concentrations (Naylor et al., 2009).

ii. Targeting the acidic Ca^{2+} stores

NAADP is well described to mobilise Ca^{2+} from the acidic stores and several pharmacological tools have been shown to specifically disrupt acidic store Ca^{2+} signalling. Bafilomycin A1 is an antibiotic which has been shown to inhibit vacuolar H^+ transporter ATPases, this has a neutralising effect on the acidic stores (Yoshimori et al., 1991), and is suggested to prevent the endogenous Ca^{2+} loading mechanism via H^+/Na^+ and Na^+/Ca^{2+} exchangers (Churchill et al., 2002). GPN (glycyl-L-phenylalanine-β-naphthylamide), a substrate for the lysosomal specific cathepsin C and is known to cause hydrolytic perforation of the lysosomes (Berg et al., 1994, Jadot et al., 1984) and selectively abolishes Ca^{2+} signalling in response to NAADP (Zhu et al., 2010b). Nigericin, a K^+/H^+ exchange ionophore (Steinrauf et al., 1971), has been shown to rapidly dissipate the intraluminal pH of acidic stores, this causes initial Ca^{2+} release, but also a reduction in responses to NAADP after time (Morgan and Galione, 2007, Morgan et al., 2012).

The acidic stores can be visualised with the use of pH sensitive fluorophores such as lysotracker and acidic store Ca^{2+} signalling events can be visualised by a variety of intracellular fluorescent Ca^{2+} indicators. Ca^{2+} chelators such as BAPTA and EGTA have been shown to be effective tools for reducing the downstream effects of acidic store Ca^{2+} release. BAPTA has a high 'on' rate for binding Ca^{2+} and is referred to as a 'fast' chelator, it is capable of inhibiting Ca^{2+} signalling even at nanodomains, whereas EGTA has a similar affinity for Ca^{2+} but a slower 'on' rate and therefore cannot block nano or microdomain Ca^{2+} signalling. EGTA and BAPTA have been useful tools for identifying nano/microdomain coupling between the acidic stores and the ER (Morgan et al., 2013) Both BAPTA and EGTA can be conjugated to AM esters to allow entry across the cell membrane.

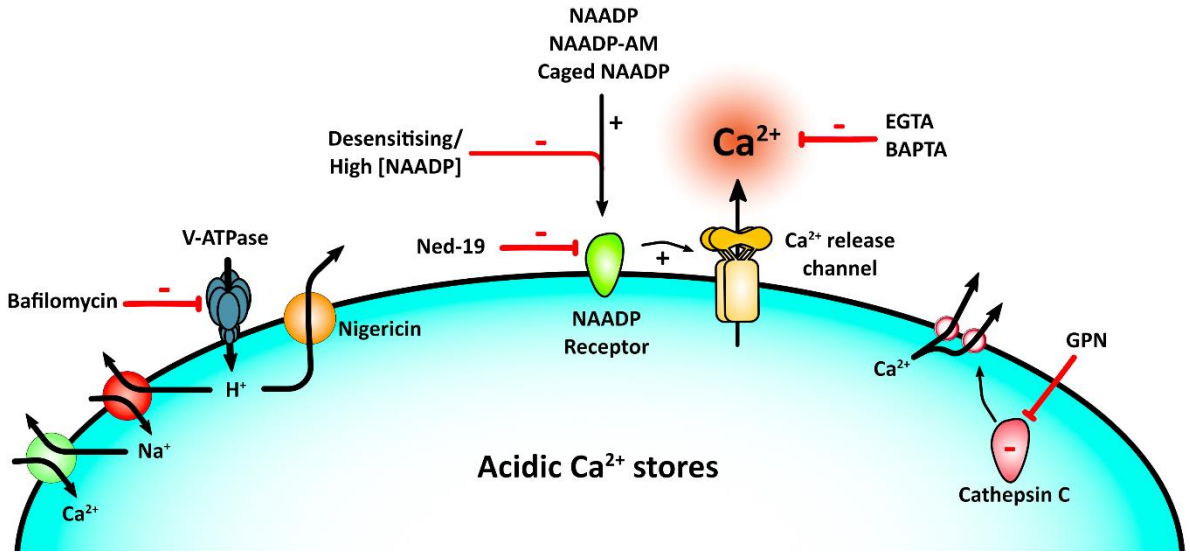

Figure 1.5 Tools to manipulate NAADP and acidic store signalling. Bafilomycin inhibits V-ATPases, preventing proton loading of the acidic stores, this, in turn, prevents Ca^{2+} loading into the acidic stores. The ionophore nigericin removes H^+ from the lysosomes and prevents Ca^{2+} loading in a similar manner as bafilomycin. Ned-19 is an antagonist to NAADP. Desensitising/high concentrations of NAADP will inactivate the NAADP receptor whereas activating concentrations of NAADP will cause acidic store Ca^{2+} release. Membrane permeable NAADP-AM can cross the plasma membrane to activate the NAADP receptors. Photolysis of caged NAADP with UV light can also activate the NAAADP receptor. EGTA and BAPTA chelate intracellular Ca^{2+} and prevent downstream signalling events from the acidic stores. GPN is a substrate for the lysosomal specific cathepsin C and causes hydrolytic perforation of the lysosomes.

1.2.8 Mitochondria, Golgi and nuclear Ca^{2+} signalling

The mitochondria, Golgi apparatus and the nucleus are all known to be significant stores of intracellular Ca^{2+} and are implicated in a wide variety of essential signalling and physiological processes (Berridge et al., 2000). However, I have not explored the role of these Ca^{2+} stores in hippocampal neurons and any potential interaction with NAADP signalling in this thesis. Consequently, I will not discuss their Ca^{2+} signalling properties in detail here as their signalling properties are not relevant to this thesis.

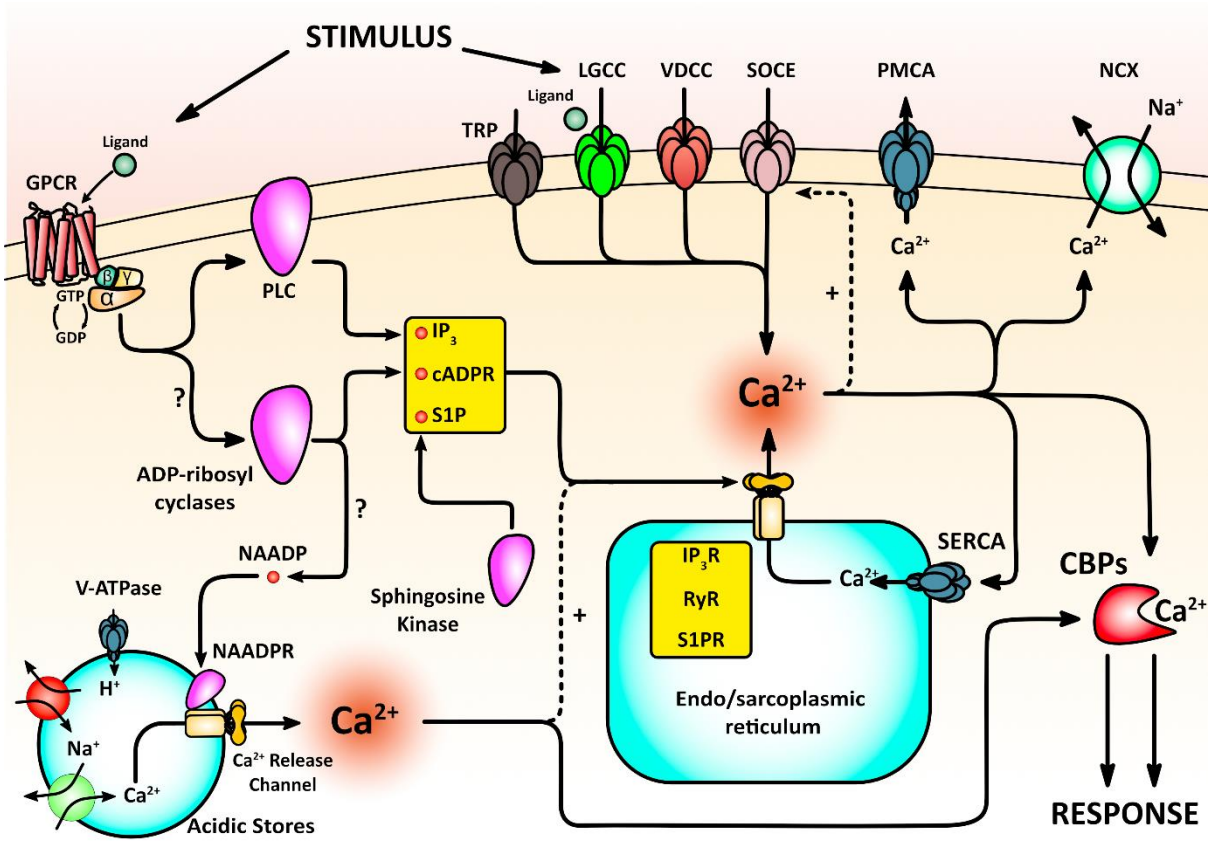

Figure 1.6 The Ca^{2+} Signalling toolkit. This diagram shows the major intracellular Ca^{2+} signalling pathways which I have described. Some elements, such as the mitochondria, Golgi apparatus, and nucleus, are omitted. This diagram highlights the complexity and heterogeneity of the intracellular Ca^{2+} signalling toolkit. Abbreviations. **GPCR**: G-protein coupled receptors, **GTP**: Guanosine-5'-triphosphate, GDP: Guanosine-5'-diphosphate ATP **PLC**: Phospholipase C, **ADP-ribosyl Cyclase**: Adenosine diphosphate ribosyl cyclase. **IP_3**: Inositol trisphosphate, **cADPR**: Cyclic ADP Ribose, **NAADP:** Nicotinic acid adenine dinucleotide phosphate, **SP1**: sphingosine-1-phosphate, **IP_3R:** IP_3 receptor, **RyR**: ryanodine receptor, NAADPR: NAADP receptor, **S1PR**: S1P receptor**, V-ATPase**: Vacuolar ATPase, **SERCA**: sarco/endoplasmic reticulum Ca^{2+} ATPase, **LGCC**: Ligand-Gated Ca^{2+} Channels, **VDCC**: Voltage Dependent Ca^{2+} Channels, **SOCE**: Store operated Ca^{2+} Entry Channels, **PMCA**: Plasma membrane Ca^{2+} ATPase, **NCX:** N^+/Ca^{2+} exchanger, **CBPs**: Ca^{2+} Binding Proteins. **TRP**: transient receptor potential channels.

1.3 Ca^{2+} signalling, glutamate and synaptic plasticity in hippocampus

As demonstrated in the previous section, Ca^{2+} signalling is ubiquitous, complex and versatile, it has roles in practically every cellular process. Here, I focus on the roles of Ca^{2+} at the synapse, with regard to neurotransmission, membrane excitability, and synaptic plasticity.

1.3.1 The hippocampus

The hippocampus has been long since implicated as having critical role in learning and memory. It has a distinct laminar structure and a simple, well defined, network structure consisting of a trisynaptic circuit – **Figure 1.7.** Synaptic responses can be easily identified and recorded over long periods of time (Andersen et al., 1971) using both extracellular/intracellular electrodes in combination with electrical or chemical stimulation. Electrophysiological recordings can also be undertaken in combination with imaging of Ca^{2+} signals. A range of chemical and genetic Ca^{2+} indicators are available for use with a variety of optical methods (Grienberger and Konnerth, 2012). These properties make the hippocampus particularly amenable for studying the synapse and Ca^{2+} signalling events at the synapse. These properties have allowed a great deal of research to be undertaken on synaptic plasticity in the hippocampus. The hippocampus may be used in a variety of preparations including dissociated primary neurons, hippocampal slices, both acute and in culture (organotypic), and finally *in vivo*. Synaptic function is, broadly speaking, functionally similar between different preparations of the hippocampus (Boyer et al., 1998, Bekkers et al., 1990, Brewer, 1997).

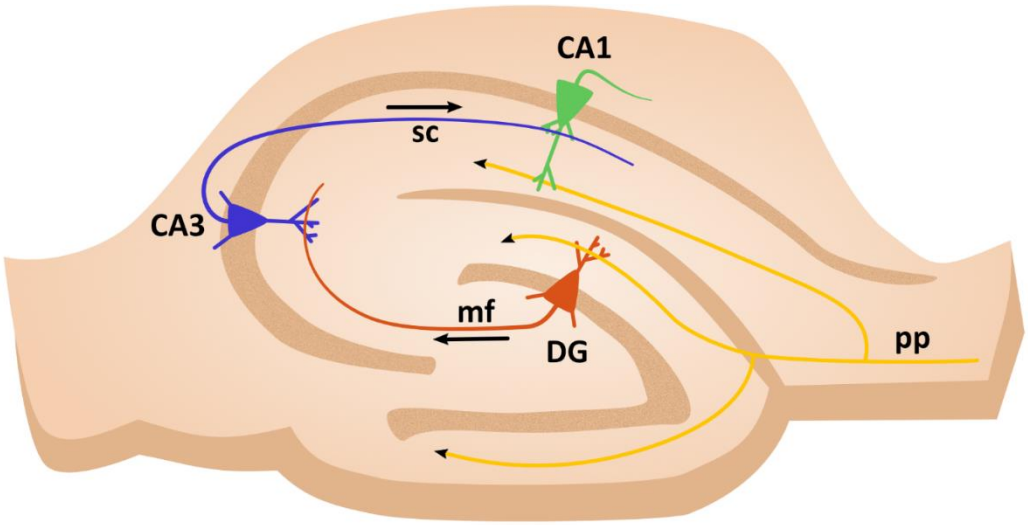

Figure 1.7 Schematic diagram of a hippocampal slice preparation. The structure of the hippocampus is simple and well defined; it consists of a trisynaptic circuit. Projections from the entorhinal cortex synapse with granule cells in the dentate gyrus (DG) via the perforant pathway (pp). Granule cells from the DG project via the mossy fibre pathway (mf) to form synapses with pyramidal neurons in the CA3 region of the hippocampus, which in turn form synapses with CA1 pyramidal neurons via the Schaffer-collateral pathway (sc). Electrical stimulation and both extracellular/intracellular recordings are possible at all three major synapses.

1.3.2 Presynaptic Ca^{2+} and glutamate release

Synapses form electrochemical junctions between neurons, and communication between neurons is generally achieved via chemical neurotransmitters. Glutamate is the major excitatory neurotransmitter in the mammalian brain. It is synthesised at the presynaptic bouton (Palmada and Centelles, 1998) and subsequently packaged into vesicles, which form distinct functional pools within the presynaptic bouton which are: the readily releasable, reserve, and resting pools (Rizzoli and Betz, 2005). Upon cellular depolarisation Ca^{2+} entry via VDCC causes the activation of SNARE proteins resulting in vesicular fusion and glutamate release into the synaptic cleft (Sudhof, 2004).

The intracellular Ca^{2+} stores of the presynaptic bouton are also shown to be important for glutamate release, however, this has not been subject to the same intensity of investigation as Ca^{2+} entry through

VDCCs. Emptage et al. (2001) showed that presynaptic stores of Ca^{2+} mediate spontaneous Ca^{2+} release via CICR, which in turn causes miniature spontaneous glutamate release, and miniature excitatory post synaptic potentials (mEPSPs). This study also showed that these stores contribute to paired pulse facilitation of EPSPs, and therefore short term plasticity. McGuinness et al. (2007) went on to show that the lysosomes (or other acidic Ca^{2+} stores) may contribute to spontaneous glutamate release and mEPSPs. These studies present evidence that the intracellular Ca^{2+} stores are important in regulating presynaptic glutamate transmission and could indicate possible roles in synaptic plasticity.

1.3.3 Glutamate receptors and postsynaptic Ca^{2+} entry

Glutamate release into the synaptic cleft acts upon glutamate receptors located on the postsynaptic dendritic spines. Glutamate receptors can be either ionotropic or metabotropic in nature. Three classes of ionotropic glutamate receptors exist NMDA (GluN), AMPA (GluA) and kainate (GluK). Ionotropic glutamate receptors are comprised of four subunits (Nakanishi and Masu, 1994) which produce pore forming ion channels, and cause direct membrane depolarisation when activated. Several genes encoding receptor subunits are present for each family, and consequently, a variety of possible receptor subunit combinations exist. The receptor subunit composition dictates the activation properties, conductance kinetics, permeability, and localisation of each receptor (Nakanishi and Masu, 1994, Nakanishi et al., 1994).

However, there are general similarities between members of the same family. The GluN receptors are nonselective cation channels, which are permeable to Ca^{2+} (Jahr and Stevens, 1993, Lester et al., 1990). GluNs have slow kinetics of activation but high affinity for glutamate, consequently they remain 'open' for relatively long periods of time upon activation (Lester et al., 1990). Their open channel probability is also regulated by Mg^{2+} in a voltage dependent manner; where at resting membrane potential open probability is low but increases with cellular depolarisation reaching a maximum at around +20 mV (Nowak et al., 1984).

GluA receptors are also non-selective cation channels, however, their permeability to Ca^{2+} is heavily dependent on the subunit composition, where the presence of the GluR2 subunit will effectively render the receptor impermeable to Ca^{2+} (Geiger et al., 1995). They are the primary source of glutamatergic-mediated membrane depolarisation in most neurons, they open and desensitise rapidly, making GluA signalling fast but brief (Gouaux, 2004). GluK receptors have similar channel conductance to GluAs, however, they have slightly slower activation and inactivation properties than GluAs (Lerma et al., 2001) GluKs are thought to only be slightly permeable to Ca^{2+} though this is dependent on receptor subunit composition (Burnashev, 1998, Huettner, 2003).

In addition to the ionotropic glutamate receptors, a family of metabotropic glutamate receptors (mGluRs) are present in neurons. The mGluR family consists of at least of eight G-protein coupled receptors (GPCR), split into three groups based upon pharmacological and functional properties (Niswender and Conn, 2010).

Group 1 contains mGluR1 and mGluR5, both of which are canonically implicated as $G_{\alpha q}$ linked and are suggested to mobilise Ca^{2+} from the ER via IP_3 signalling. Ca^{2+} release from the ER affects local Ca^{2+} dependent processes in the dendrites, including synaptic plasticity and protein synthesis (Raymond et al., 2000). Activation of mGluR1s has also been shown to produce membrane depolarisation and postsynaptic excitability as a result of either inhibition of background K^+ channels (TASK and TREK), or inhibition of short conductance Ca^{2+} activated K^+ Channels (SK) (Tigaret et al., 2016). Both mGluR1/5 are also suggested to cause activation of TRPC channels (Ben-Mabrouk et al., 2012, Kim et al., 2003). Postsynaptic membrane depolarisation and Ca^{2+} signalling are essential components for synaptic plasticity to occur, the Group 1 mGluRs are implicated in both long term potentiation (LTP) and depression of the synapse (LTD) (Anwyl, 1999). Group 1 mGluRs are noted to have perisynaptic localisation on dendritic spines of hippocampal pyramidal neurons (Lujan et al., 1996).

Groups 2 and 3 contain the remaining six members of the mGluR family and are canonically implicated as $G_{\alpha i/o}$ coupled receptors and reduce cyclic adenosine monophosphate (cAMP) synthesis. Their

activation has broad effects, but generally, they reduce neuronal activity by producing hyperpolarisation via activation of K^+ channels (Muly et al., 2007) or inhibition of presynaptic VDCCs (Ferraguti et al., 2008, Ferraguti and Shigemoto, 2006). Activation mGluR Groups 2 and 3 is described to induce LTD at both pre and postsynaptic loci, owing to localisation at both sites (Bellone et al., 2008, Pinheiro and Mulle, 2008).

1.3.4 Ca^{2+} mediated induction of synaptic plasticity

Activity dependent changes to synaptic strength in the neurons of the hippocampus are suggested to form the basis for memory formation and storage. Intracellular Ca^{2+} signalling is well described as essential for the induction of synaptic plasticity in the hippocampus. Various forms of plasticity exist; they are ubiquitously dependent on presynaptic or postsynaptic Ca^{2+} signalling following specific patterns of neural activity.

1.3.5 The current model for GluN receptor dependent plasticity induction

The most prominent and current model for postsynaptic plasticity is focussed upon the role of Ca^{2+} influx through the GluN receptors. Ca^{2+} influx through GluNs may activate both protein kinases and phosphatases, each of which has a differential spatiotemporal sensitivity to Ca^{2+} signals from the GluNs. Transient, high magnitude Ca^{2+} signals result in protein kinase activation, and notably CaMKII activation, as reviewed by Lisman (2001) and Shouval et al. (2002). CaMKII phosphorylates various intracellular proteins which in turn causes exocytosis of GluA1-containing receptors to the extrasynaptic membrane, and subsequent capture of these receptors at the synapse (Hayashi et al., 2000). The increased number of GluA receptors causes a larger postsynaptic response on presynaptic transmitter release, and hence long term potentiation (LTP) of the synapse (Ehlers et al., 2007, Makino and Malinow, 2009, Yang et al., 2010). Conversely, longer lasting Ca^{2+} signals of moderate magnitude in the postsynaptic density (typically a response to weak post synaptic depolarisation) cause the activation of protein phosphatases (notably protein phosphatase 2B and 1) (as reviewed in Lisman (2001) and Shouval et al. (2002)). These phosphatases, dephosphorylate various intracellular proteins

and cause a transition of GluA receptors into the extrasynaptic membrane and their subsequent endocytosis (Malinow and Malenka, 2002). This has the net effect of reducing postsynaptic responses on presynaptic transmitter release, and hence long term depression (LTD) of the synapse (Tardin et al., 2003, Yang et al., 2010). The current model for GluN mediated plasticity is shown diagrammatically in **Figure 1.8.**

1.3.6 mGluR dependent plasticity

Various forms of synaptic plasticity have been attributed to the activation of the mGluRs. Group 1 mGluR-mediated plasticity events are perhaps best described where both LTD and LTP have been noted upon various chemical and electrical plasticity induction protocols (Niswender and Conn, 2010).

Group 1 mGluR-dependent LTD has been described in response to prolonged, low-frequency stimulation with both single and paired pulses (Oliet et al., 1997, Kemp and Bashir, 1999, Kemp et al., 2000, Huber et al., 2000); bath application of Group 1 mGluR agonists such as DHPG (Nosyreva and Huber, 2005, Qian and Noebels, 2006); and co-administration of electrical stimulation with either postsynaptic membrane hyperpolarisation (Stanton and Sejnowski, 1989) or bath application of $GABA_{A/B}$ (Yang et al., 1994) agonists are shown to induce Group 1 mGluR dependent LTD.

mGluR1-dependent LTP has been reported in response to theta burst stimulation coupled with postsynaptic depolarisation in interneurons of the hippocampus (Lapointe et al., 2004) and also described in response to spike timing dependent plasticity protocols in pyramidal neurons of the hippocampus (Tigaret et al., 2016). mGluR1-dependent LTP in pyramidal neurons is dependent on intracellular Ca^{2+} release which is thought to cause inhibition of small conductance Ca^{2+} sensitive K^{+} channels (SK channels).

SK channels are canonically implicated in producing post action potential hyperpolarisation currents (I_{AHP}) in neurons. The I_{AHP} is suggested to rapidly inactivate GluNs by reinstating the Mg^{2+} block and causes reduced Ca^{2+} entry and magnitude of synaptic plasticity (Ngo-Anh et al., 2005). Interestingly, in CA1 pyramidal neurons of hippocampus two isoforms of SK2 are described (long. SK_L and short: SK_s). SK_s are predominantly expressed in at extrasynaptic sites) (Allen et al., 2011), and mGluR1α is also localised to perisynaptic regions (Lujan et al., 1996, Petralia et al., 1997) suggesting close spatial arrangement. Therefore, the proposed inactivation of SK channels by mGluR1 (Tigaret et al., 2016) may indicate differential functions of the SK channel based upon differential sensitivity to the magnitude or source of Ca^{2+} signals. SK channels are reported to be sensitive to Ca^{2+} from both extracellular (Myoga and Regehr, 2011, Marrion and Tavalin, 1998) and intracellular Ca^{2+} stores (Sah and McLachlan, 1991), and are functionally regulated by several other Ca^{2+} sensitive proteins with which they form stable macro-molecular complexes. These include Calmodulin (CaM) (Adelman et al., 2012), phosphatase 2A (PP2A) and Protein kinase CK2 (CK2) (Bildl et al., 2004, Allen et al., 2007). This heterogeneity of SK channel function by Ca^{2+}- reinforces the importance of local Ca^{2+} signalling dynamics.

The plasticity induction protocols of the studies discussed above suggest that Group 1 mGluR-dependent plasticity requires either high magnitude and short synaptic activity or also weaker synaptic activity over a prolonged period. These activation properties are likely to be as a result of their perisynaptic localisation (Lujan et al., 1996), where glutamate must be abundant enough to escape the synaptic cleft into the perisynaptic zones. The current models for Group 1 mGluR mediated plasticity are also shown in **Figure 1.8**.

1.3.7 Intracellular stores and synaptic plasticity

There is a varied body of evidence which implicates an essential role of the intracellular Ca^{2+} stores in synaptic plasticity. Several studies, using GluN receptor mediated plasticity induction protocols, implicate a role of ER Ca^{2+} release via the RyR in both LTP (Wang et al., 1996, Raymond and Redman, 2006, Balschun et al., 1999) and LTD (Nishiyama et al., 2000, Reyes and Stanton, 1996, Futatsugi et al., 1999). The same is noted for Ca^{2+} release via the IP_3R in both LTP (Kwon and Castillo, 2008, Sokolov et al., 2003, Raymond and Redman, 2006, Raymond and Redman, 2002) and LTD (Khodakhah and Armstrong, 1997, Lei et al., 2003, Jo et al., 2010). I should note that the functional aspects of both RyR and IP_3R mediated ER Ca^{2+} release is not clearly elucidated. Suggested functions are inconsistent between these studies, perhaps as a result of different plasticity induction protocols, and experimental preparations used between studies. The functional roles for IP_3R and RyR mediated ER Ca^{2+} release for synaptic plasticity are reviewed extensively by Baker et al. (2013).

Thus far, the evidence for acidic Ca^{2+} store contribution to synaptic plasticity consists of a study by Padamsey et al. (2017), here back-propagating action potentials are shown to Ca^{2+} release from the acidic stores in neuronal dendrites, which initiate a signalling cascade to trigger fusion of lysosomes to the plasma membrane, which when paired with synaptic activity, causes extracellular matrix remodelling and synaptic plasticity

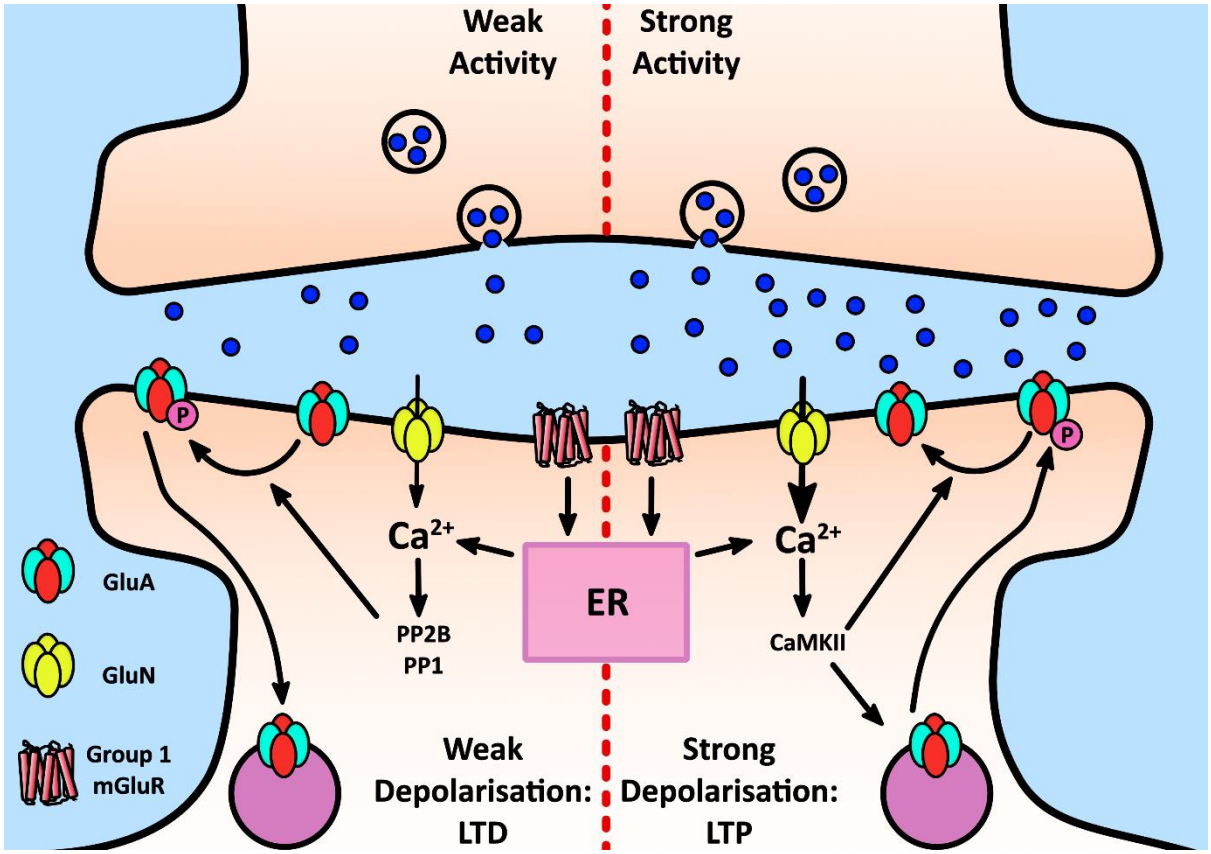

Figure 1.8 The current model for GluN dependent induction of postsynaptic plasticity. LTP occurs in response to synaptic activation of the GluN receptors, which coincides with strong postsynaptic depolarisation. This results in a transient, high magnitude Ca^{2+} signal, which preferentially activates CaMKII (and other Ca^{2+} sensitive protein kinases) and the subsequent exocytosis and synaptic capture of GluA1 containing receptors. Conversely, GluN activation coinciding with weak postsynaptic depolarisation causes modest postsynaptic Ca^{2+} entry. This preferentially activates Ca^{2+} sensitive protein phosphatases and the subsequent removal of GluA receptors from the synapse, followed by their internalisation. Group 1 mGluRs are also included in this diagram to demonstrate a possible route for modulation of plasticity events.

1.4 NAADP and Acidic Ca^{2+} store signalling in neurons

NAADP signalling is implicated in a wide variety of critical processes within the cell and it has been shown to be a ubiquitous and universal Ca^{2+} mobilising messenger. However, specific roles within the central nervous system (CNS) are poorly understood/described thus far.

The first experimental evidence of a role for NAADP in the CNS was shown in brain microsomes by Bak et al. (1999), where NAADP was shown to release Ca^{2+} from stores insensitive to IP_3R and RyR antagonists. A year later (Patel et al., 2000) described widespread and heterogeneous binding sites for NAADP (using radio-ligand binding) in murine brain slices, with the highest affinity in the medulla, hippocampus and the thalamus.

The presently described functions of NAADP/acidic store Ca^{2+} signalling in the CNS seem to be split into four main themes: **1.** Differentiation and growth, **2.** Autophagy, **3.** Effects on neurotransmission and synaptic plasticity, and **4.** Conferring neuronal membrane excitability.

1.4.1 Neuronal differentiation and growth

Brailoiu et al. (2005) demonstrated the presence of NAADP signalling in mammalian neurons for the first time. NAADP, introduced via liposomes, potentiated neurite outgrowth in murine cortical neurons in response to serum and nerve growth factor (NGF). NAADP was shown to cause Ca^{2+} release from acidic intracellular stores and could be blocked by bafilomycin. Ca^{2+} signals were also reduced, but not abolished, by inhibiting IP_3Rs and RyRs. This suggests functional coupling between acidic Ca^{2+} stores and the ER in neurons.

Using the same method of liposomal delivery of NAADP Brailoiu et al. (2006) showed that NAADP triggered differentiation of PC12 cells to a neuronal phenotype by releasing Ca^{2+} from acidic stores. Notably, the introduction of IP_3 and subsequent ER Ca^{2+} release did not cause such differentiation. NAADP produced Ca^{2+} signals over long periods (greater than 25 minutes) whereas IP_3 produced shorter and more transient intracellular Ca^{2+} signals (under 10 minutes). This study underlines the

importance of the different intracellular Ca^{2+} stores in neurons and that different Ca^{2+} mobilising second messengers produce unique Ca^{2+} signals and markedly different cellular responses.

Zhang et al. (2013) also described roles for NAADP, acidic store Ca^{2+} release and TPC2 in neuronal differentiation into neural progenitors produced from mouse embryonic stem (ES) cells. They showed that a membrane permeable form of NAADP (NAADP-AM) caused Ca^{2+} release from the acidic stores. This effect was abolished in ES cells from TPC2 KO animals. They showed TPC2 expression was decreased during differentiation from ES cells into neuronal progenitors, and gradually increased after differentiation was complete. ES cells from TPC2 KO made the differentiation to neuronal progenitors more rapidly but prevented further differentiation into neurons and oligodendrocytes. Conversely, TPC2 overexpression inhibited differentiation from ES cells to neuronal progenitors. These data suggest that acidic store Ca^{2+}, NAADP, and TPC2 signalling inhibits progression from ES cells to neural progenitors, but are required for progression to later neuronal development. These results were broadly replicated by Hao et al. (2016). Finally, Lu et al. (2013) suggest that TPC2 signalling inhibits progression of autophagy during differentiation in ES cells to neurons by alkalizing lysosomal pH.

These studies underline the importance of differential Ca^{2+} signals from intracellular Ca^{2+} stores and provide strong evidence for a role of NAADP signalling in neuronal differentiation. NAADP is also implicated to be important for differentiation and development in a variety of other non-neuronal cell types, as reviewed by Parrington et al. (2015).

1.4.2 Neuronal autophagy

NAADP signalling has been shown to be important for neuronal autophagy. The first indication that NAADP effects autophagy in neural cells was by Pereira et al. (2011). Here they show introduction of NAADP to cultured astrocytes causes Ca^{2+} mobilisation from the acidic stores and an increase vesicular organelle formation and autophagy markers, both of which were reduced in cells expressing a dominant negative TPC2 construct.

The following year Gomez-Suaga et al. (2012a) suggested a functional link between Leucine-rich repeat kinase 2 (LRRK2), endo-lysosomal Ca^{2+}/NAADP signalling and autophagy. Mutations in LRRK2 are implicated through genome wide association studies, in the pathogenesis of both familial (Satake et al., 2009) and sporadic forms (Nalls et al., 2011) of Parkinson's Disease (PD). Therefore, understanding the normal physiological roles for LRRK2 may aid in the identification of novel therapeutic targets for the treatment of PD. Gomez-Suaga et al. (2012b) show that LRRK2 activation causes autophagosome formation which is mediated by a pathway involving Ca^{2+}-dependent protein kinase kinase-β (CaMKK-β) and adenosine monophosphate (AMP)-activated protein kinase (AMPK). They show overexpression of LRRK2 (through viral transfection) causes an increase in autophagosome formation and decreases the number of acidic stores. These effects could be mimicked with the introduction of NAADP-AM, reversed with the NAADP antagonist Ned-19 and blocked with overexpression of dominant-negative TPC channel constructs. They also demonstrated LRRK2 overexpression sensitises cells to stressors associated with abnormal protein degradation (proteasomal inhibitor with MG-13), which was again prevented with Ned-19. Possible mechanisms for the molecular interaction between LRRK2 and TPC/acidic store signalling pathways are explored in Gómez-Suaga and Hilfiker (2012) and Gomez-Suaga et al. (2012a). Pereira et al. (2016) then linked the previously described role of NAADP and acidic store Ca^{2+} signalling in neuronal autophagy to an external stimuli: glutamate. This study showed that external application of glutamate to astrocytes causes autophagy to the same degree as NAADP-AM, which again can be blocked by Ned-19 and siRNA knockdown of TPC1/2. Pandey et al. (2009) had previously shown that glutamate can induce NAADP endosynthesis in astrocytes (and hippocampal neurons). However, the glutamate receptor responsible for NAADP endo-synthesis has not been identified.

These studies give the first evidence for functional roles of NAADP in neurons, furthermore, they implicate NAADP/acidic store signalling to LRRK2, a protein with strong correlation with the pathogenesis of PD, offering functional insights to the disease and also potential therapeutic targets. Finally, they implicate neurotransmission as an external stimulus which can result in NAADP synthesis

which activates acidic store Ca^{2+} signalling and subsequent downstream signalling/physiological events.

1.4.3 Synaptic transmission and plasticity

Aside from the roles in differentiation and autophagy NAADP is also suggested to be linked to synaptic transmission and plasticity via acidic store Ca^{2+} signalling.

The first evidence for an effect of NAADP on neurotransmission was described in *Aplysia californica* by Chameau et al. (2001). In this study, NAADP or IP_3 was microinjected into presynaptic neurons in the buccal ganglia whilst postsynaptic currents or presynaptic Ca^{2+} transients were recorded. Two independent presynaptic stores of Ca^{2+} were identified, one sensitive to NAADP and the other to cADPR/IP_3, with Ca^{2+} mobilisation from either store causing neurotransmitter release and postsynaptic depolarisation.

Having two separate stores of presynaptic Ca^{2+} is extremely beneficial to neurons, as it allows greater control and modulation of presynaptic Ca^{2+}, and therefore neurotransmitter release. The acidic stores and the ER may also be spatially distinct, which increases the spatial control of presynaptic Ca^{2+} signals. Finally, these stores also mobilise Ca^{2+} via independent second messenger pathways which would be likely to have unique activation properties. For example, the NAADP-induced presynaptic Ca^{2+} release may require distinct receptors and patterns of electrical activity compared to IP_3-induced presynaptic Ca^{2+} release.

In the same year, Brailoiu et al. (2001) showed that NAADP, introduced via liposomes, causes intracellular Ca^{2+} release from stores insensitive to IP_3/cADPR. This also caused an increase quantal output and also the probability of neurotransmitter release suggested as a result of increased presynaptic Ca^{2+}. Responses were also enhanced by co-administration of NAADP and IP_3 but inhibited with cADPR, again suggesting that distinct Ca^{2+} mobilising messengers have distinct physiological effects. McGuinness et al. (2007) found similar results in hippocampal slice cultures, where Ca^{2+} release from acidic/lysosomal stores caused an increase in neurotransmitter release, recorded as an

increase in frequency of mini excitatory post-synaptic potentials (mEPSPs) and that disruption of lysosomal Ca^{2+} signalling compromises intracellular Ca^{2+} signals in response to action potentials.

Padamsey et al. (2017) showed that backpropagating action potentials caused Ca^{2+} release from the acidic stores in neuronal dendrites. This initiates a signalling cascade which triggers fusion of lysosomes to the plasma membrane, release of Cathepsin B, and increased activity of matrix metalloproteinase 9, which is an enzyme involved in extracellular matrix remodelling and synaptic plasticity. Inhibition of acidic store Ca^{2+} signalling impaired dendritic spine growth induced by Hebbian plasticity protocols. These data provide evidence for functional roles for NAADP Ca^{2+} signalling in neurotransmission and in synaptic plasticity.

1.4.4 Membrane excitability

NAADP is suggested to have a depolarising effect in neurons. Membrane permeable NAADP (NAADP-AM) causes endolysosomal Ca^{2+} release in neurons of the medulla oblongata and furthermore causes a plasma membrane depolarisation via an unidentified membrane channel (Brailoiu et al., 2009b). Preliminary (unpublished) data produced by (Padamsey and Emptage, 2011) shows that membrane permeable (NAADP-AM) and photolysis of caged NAADP (NAADP-DMNPE) both induce a transient membrane depolarisation in hippocampal slice cultures. NAADP is also suggested to increase Ca^{2+} influx through N-type VDCCs in cultured hippocampal neurons, via exocytotic insertion of N-type VDCCS into the plasma membrane through activity-dependent lysosome fusion (Hui et al., 2015).

The central theme of my thesis was to explore this NAADP-induced membrane depolarisation. I hypothesised that neurotransmitters, such as glutamate, cause endogenous synthesis of NAADP and lysosomal Ca^{2+} release in post-synaptic neurons, which in turn causes cellular membrane depolarisation and that this might impact synaptic plasticity. I initially tested this hypothesis and subsequently explored the possible functions of this depolarisation with regard to synaptic plasticity.

1.5 Concluding remarks

Through this introduction, I have shown that Ca^{2+} signalling is ubiquitous and physiologically essential, in some way, for almost every intracellular signalling event and physiological response. A myriad of possible unique Ca^{2+} signalling events exist, and each is produced in response to a specific stimulus. I have highlighted the importance of the intracellular Ca^{2+} signalling dynamics, showing that different spatiotemporal dynamics and magnitudes of Ca^{2+} signals produce markedly different cellular responses. I went on to discuss the novel Ca^{2+} mobilising second messenger NAADP and its known effects on the central nervous system. I then discussed the importance of Ca^{2+} signalling in neurons of the hippocampus with regard to synaptic plasticity and membrane excitability.

Very little is known about the roles of NAADP within the hippocampus, and even less regarding any possible roles in synaptic plasticity. NAADP has been shown to be synthesised in response to glutamate, via a currently unidentified channel, and has also been able to cause intracellular Ca^{2+} release and membrane depolarisation in neurons (two key features of synaptic plasticity). I propose to examine the functional role of NAADP in synaptic plasticity within the hippocampus in this thesis.

1.6 Aims

1. **To further characterise NAADP-mediated depolarisation in pyramidal neurons of the hippocampus.**

2. **To identify endogenous stimuli and characterise the intracellular signalling pathway which causes NAADP-mediated membrane depolarisation in pyramidal neurons of the hippocampus.**

3. **To identify which membrane channels are responsible for producing NAADP-mediated membrane depolarisation.**

4. **To determine the physiological significance of NAADP-mediated depolarisation, and to identify its role in synaptic plasticity.**

2.0 Methods and statistical analysis

This study was carried out in accordance with the U.K. 1986 Scientific Procedures Act.

2.1 Cell and tissue preparation

Hippocampal slice culture

Slice cultures of the hippocampus were prepared from 6-8 day postnatal (P6) Male Wistar rats were purchased from Harlan Laboratories, Oxford, UK. Rats were sacrificed by cervical dislocation followed by decapitation and removal of the brain. The hippocampi were isolated in ice cold Earle's balanced salt solution (EBSS) with added: HEPES (21 mM), D-(+)-Glucose (27.8 mM) pH adjusted to 7.2 - 7.4 with NaOH, and cut into slices of 350 µm thickness with a McIlwain tissue chopper. The slices were placed into Millicell CM culture plate inserts (PTFE filter, pore size 0.4 µm, diam. 12 mm) in a six well Millicell culture plate (both supplied by Merck Millipore) with 1 mL of culture medium. These cultures were stored at 34.5°C at 5% CO_2. Culture medium composed of 78.8% minimum essential medium (MEM) with GlutaMAX (Gibco), 20% heat inactivated horse serum, 1% B27 with added $CaCl_2$ (1 mM), HEPES (30 mM,) D-(+)-Glucose (26 mM,) $NaHCO_3$ (5.8 mM) and $MgSO_4$ (2 mM). Culture media was renewed every 3-4 days. Experiments were conducted on these slices 6-14 days *in vitro* (D.I.V.).

For experiments, slices were transferred into a recording chamber and perfused with artificial cerebrospinal fluid (aCSF) which comprised of NaCl (145 mM), KCl (2.5 mM), KH_2PO_4 (1.2 mM), $NaHCO_3$ (16.0 mM), glucose (11.0 mM), $CaCl_2$ (3.0 mM) and $MgCl_2$(2.0 mM) aerated with 95% O_2, 5% CO_2. This aCSF was warmed to achieve near-physiological temperatures (31-33°C) an inline heater (built in house).

2.2 Electrophysiology

Whole-cell patch clamp

Either high resistance (16-20 MΩ) or low resistance (5-8 MΩ) patch electrodes were made from borosilicate filament glass capillaries (Harvard Apparatus) with a P-97 Micropipette Puller (Sutter Instrument). For current clamp recordings patch electrodes were filled with internal solution containing 135 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 2 ATP mM, 0.2 mM GTP; pH adjusted to 7.2 with KOH. For voltage clamp recordings a Cs^+ based internal solution was used, in order to reduce the contribution of K^+ to recorded currents and maintain a better clamp, this comprised of the following components: 135 mM CsCl, 2.0 mM $MgCl_2$, 2.0 mM Na_2-ATP, 0.2 mM Na_3-GTP, 10 mM HEPES with pH adjusted to 7.2 with CsOH. Pharmacological agents/second messengers added to the internal solution were conjugated to K^+ salts, and when present, the K-gluconate concentration was reduced by equal molarity in order to maintain osmolarity.

Whole-cell patch clamp recordings were performed on pyramidal neurons from either CA3 or CA1 neurons of hippocampal slice cultures. Voltage/current signals were detected using either an Axoclamp 900A or Axoclamp 2B (Axon instruments/Molecular devices) amplifier, signals were digitised using a Digidata 1440A digitizer then recorded digitally with WinWCP V4.7.9 (Strathclyde Electrophysiology Software). Signals were filtered at 3 kHz and sampled and digitised at 10kHz, 50 Hz noise was eliminated with a Hum Bug (Quest Scientific). Membrane voltages were corrected for liquid junction potentials (LJPs) when relevant, LJPS were calculated to be +15.7 mV for K^+ based internal solutions and +5.0 mV for Cs^- based internal solutions.

2.2.1 Dialysis of Ca^{2+} mobilising messengers

Whole-cell current clamp recordings were performed on pyramidal neurons in the CA1 region of hippocampal slice cultures, 6-14 D.I.V. using low resistance patch electrodes. The Ca^{2+} mobilising second messengers: NAADP, cADPR and IP_3 were included in the internal solution at a range of concentrations (1 nM – 10 mM) whilst the membrane potential of the patch cell was recorded to produce dose-response curves. The access resistance was monitored with a 50 ms hyperpolarising pulse every 15 s. Recordings were rejected if the access resistance changed by more than 20%.

2.2.2 NAADP-AM Experiments

Whole-cell current clamp recordings were performed on pyramidal neurons in the CA3 or CA1 region of hippocampal slice cultures, 6-14 D.I.V. using low resistance patch electrodes. NAADP-AM was introduced directly onto the patch clamped cell via a microelectrode (4-6 MΩ) with a microinjection system (Picospriter) applying ~1-2 nL over 10 seconds. The circulating external solution was maintained at room temperature and included NBQX (10 μM) reduce spontaneous network activity.

2.2.3 Metabotropic glutamate receptor isolation

Whole-cell current clamp recordings were performed on pyramidal neurons in the CA1 region of hippocampal slice cultures, 6-14 D.I.V. High resistance electrodes (16-20 MΩ) were used when isolating metabotropic glutamate receptor responses to prevent dialysis of cytosolic factors. Isolation of various members of the metabotropic glutamate receptor family was achieved with the addition of pharmacological antagonists to the circulating aCSF. Glutamate was either applied via transient bath application (180 s, 300 μM) or glutamate release was evoked via electrical stimulation. Where electrical stimulation was used, an isolated constant current stimulator (Digitimer Ltd) was used to deliver 4 pulses (100 μs, 20-30 μA) at 20Hz to the stratum radiatum via stimulating electrode in a glass pipette (3-5 MΩ) filled with Ca^{2+} free Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.2 mM, Na_2HPO_4, 12 mM $NaHCO_3$ and 5.5 mM D-glucose, pH adjusted to 7.2 with NaOH).

Changes in membrane potential caused by electrical stimulation were determined by subtracting a baseline membrane potential (mean over the 50 ms before electrical stimulation), from the mean membrane potential over 50 ms after electrical stimulation, to give the change in membrane potential (ΔV_m). When bath application of L-Glutamate was used ΔV_m was determined by subtracting a baseline membrane potential (mean of 30 s before glutamate entered the bath), from the mean membrane potential (over 30 s) 180 s post glutamate application.

Kruskal-Wallis and Dunn's Multiple comparisons tests were used to test for statistically significant differences between groups. The Kruskal-Wallis test was selected, to determine statistical significance, as opposed to a one-way ANOVA test as the normality could not be determined from D'Agostino and Pearson omnibus normality tests as the sample size for most groups was too small.

2.2.5 Plasticity experiments

Whole-cell current clamp recordings were performed on pyramidal neurons in the CA1 region of hippocampal slice cultures, 10-14 D.I.V. with low resistance patch electrodes. Excitatory post synaptic potentials (EPSPs) were evoked by delivering current pulses (20-30 µA, 100 µs,) every 15 s to Schaffer collateral axons in stratum radiatum via a monopolar tungsten stimulating electrode with an isolated constant current stimulator (Digitimer Ltd). Synaptic strength was calculated by measuring the initial slope of the EPSP (first 3 ms). A baseline of 20 EPSPs (5 min.) was recorded before a spike time-dependent plasticity (STDP) protocol was delivered. The STDP protocol consisted 1 pre-synaptically evoked EPSP followed by to 2 postsynaptic back propagating action potentials (bAPs) at a 10 ms interval, (bAPs were elicited via somatic current injection between (1-3 nA, 2 ms)). This simulation pattern was repeated 300 times at 5 Hz. After STDP induction, EPSPs were evoked (20-30 µA, 100 µs,) every 15 s for 30 minutes to monitor changes in synaptic strength. STDP induction was delivered within 10 min of establishing whole-cell configuration to avoid dialysis of cytosolic factors necessary for LTP. Access resistance was monitored using hyperpolarising currents (-100 pA), experiments were abandoned if access resistance increased beyond 120 MΩ, or if there was a sudden and considerable

decrease in membrane resistance. EPSP slope analysis was undertaken using pClamp ClampFit electrophysiology software.

2.3 Dendritic Ca^{2+} imaging

Pyramidal neurons in the CA1 region of hippocampal slice cultures, 10-14 D.I.V. were filled with Oregon BAPTA Green 488 (OGB-1) via a patch clamp in whole cell configuration for 1 minute. The patch electrode's internal solution consisted of: 135 mM K-gluconate, 10 mM KCL, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM OGB-1, 10 mM QX314. The patch electrode (3-6 MΩ) was slowly withdrawn in a manner to cause resealing, this prevented dialysis of the neurons contents. The Ca^{2+} dye was allowed to dialyse throughout the cell for 5-10 minutes and then a dendritic branch was located to image. A glass pipette (3-5 MΩ) filled with Ca^{2+} free Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.2 mM, Na_2HPO_4, 12 mM $NaHCO_3$ and 5.5 mM D-glucose) was coated with Albumin from Bovine Serum (BSA) conjugated to Alexa Fluor-488 and placed in the same vertical plane to the dendritic branch in question at a distance of around 20 µm. An isolated constant current stimulator (Digitimer Ltd) was used to deliver 4 current pulses (100 µs, 50-80 µA) at 20Hz to presynaptic neurons and subsequent Ca^{2+} signals in the filled cell were recorded. The circulating external solution was heated to 33°C with an inline heater (built in house) and comprised of aCSF (145 mM NaCl, 2.5 mM KCl, 1.2 mM KH_2PO_4, 16.0 mM $NaHCO_3$, 11.0 mM glucose, 3.0 mM $CaCl_2$ and 2.0 mM $MgCl_2$) aerated with 95% O_2, 5% CO_2. AP5 (50 µM), NBQX (10 µM), LY341495 (100 nM) and MPEP (10 µM) were included in the aCSF to pharmacologically isolate mGluR1 receptors. Anti-oxidants: L-Ascorbic Acid (200 µM) and TROLOX (2 mM) were also included in the aCSF to protect neurons from phototoxicity.

Confocal imaging was undertaken using a Bio-Rad Radiance 2000 laser scan head system attached to an Olympus BX50WI upright microscope equipped with a 60x NA 0.90 water-immersion lens. A 488 nm solid state laser provided the excitation source and galvanometer mirrors enabled rapid XY scanning of tissue. LaserSharp (Bio-Rad) software was used to acquire images, control the area and speed of laser scanning, adjust the laser power, confocal pinhole size, and photomultiplier tube

sensitivity. Images of dendritic branches were acquired over a time series on a single Z-plane (256 x 30 pixels) with a total of 40 images taken over 700 ms. Electrical stimulation began at 70 ms. Changes in fluorescence were quantified as $\Delta F/F = (F - F_{Baseline} / F_{Baseline} - F_{Background})$, where $F_{Baseline}$ reflected the average fluorescence intensity during the first 70 ms of the time series.

2.4 Reagents

NAADP-AM and NAADP were synthesised in house by Professor Grant Churchill using the method described by (Parkesh et al., 2008) and (Aarhus et al., 1995) respectively. Other drugs were purchased from the following suppliers: Abcam: LY341495, CGP55845, D-AP5, Ned-19, JNJ16259685, MPEP, QX314, TTX. Sigma Aldrich: Ryanodine, bafilomycin A1, picrotoxin, NBQX, nicotinic acid adenine dinucleotide (NAAD) and BAPTA, BAPTA-AM, EGTA-AM. Santa Cruz Biotechnology: GPN. Fisher Scientific: OBG-1. Tocris: U73122, U73343.

2.5 Statistical analysis

Statistical significance of comparisons between data sets was assessed using the: Mann-Whitney U test, Kruskal-Wallis/ANOVA with either Dunn's or Dunnett's multiple comparison tests, two-tailed paired t-test, and the Friedman's test for non-parametric paired multiple comparisons. Significance for all tests was assessed as $P<0.05$, averages and standard error of the mean (SEM) are represented in the text as average ± SEM.

2.5.1 Curve fitting

The formula for the equation used to model the bell shaped dose-response curve is shown below. It is essentially an addition of two sigmoidal dose-response curves (stimulation and inhibition curves). Plateau and Dip constants were constrained before curve fitting. *LogEC50$_1$*, *Log EC50$_2$*, and the Hill constants: *nH1* and *nH2* were determined from the data by GraphPad Prism.

$$\boldsymbol{Y = Dip + Section1 + Section2}$$

$$\boldsymbol{where}$$

$$\boldsymbol{Section1 = Span1/(1 + 10\text{^}((LogEC50\_1 - X) * nH1))}$$

$$\boldsymbol{Section2 = Span2/(1 + 10\text{^}((X - LogEC50\_2) * nH2))}$$

$$\boldsymbol{Span1 = Plateau1 - Dip}$$

$$\boldsymbol{Span2 = Plateau2 - Dip}$$

$$\boldsymbol{where}$$

$$\boldsymbol{Plateau1 = 0.0, \quad Dip = 7.000, \quad Plateau2 = 0.0,}$$

$$\boldsymbol{LogEC50_1 = 1.10, \quad LogEC50_2 = 3.845}$$

$$\boldsymbol{nH1 = -0.8053, \quad nH2 = -0.6539}$$

3.0 Introduction of the Ca^{2+} mobilising messenger NAADP causes membrane depolarisation in hippocampal neurons

3.1 Introduction

NAADP, a novel Ca^{2+} mobilising second messenger, causes Ca^{2+} mobilisation from the intracellular acidic stores, such as the lysosomes and endosomes. NAADP-signalling is essential for variety of physiological functions, ranging from stimulus-secretion coupling in endothelial cells (Esposito et al., 2011) and pancreatic β-cells (Arredouani et al., 2015) to neuronal differentiation (Brailoiu et al., 2006) and neuronal autophagy (Gómez-Suaga and Hilfiker, 2012, Pereira et al., 2011). Interestingly, NAADP signalling is also implicated in producing both plasma membrane currents and cellular depolarisation.

Two studies in neurons report an NAADP-mediated membrane depolarisation. The first, by Brailoiu et al., (2009), used bath application of a membrane permeable form of NAADP (NAADP-AM, first described by Parkesh et al. (2008)) whilst using recording membrane potential with current clamp electrophysiology. NAADP is a charged molecule and cannot pass through the plasma membrane. Acetoxymethyl esters (AM) are bonded to NAADP to negate the charge; the same principle is used to introduce a variety of charged molecules into the cytosol. For example, the Ca^{2+} dye: Fura2-AM. Therefore, NAADP-AM can pass across the plasma membrane and endogenous esterases in the cytosol cleave the AM groups, leaving NAADP can act on its receptor. Brailoiu et al. (2009b) showed bath application of NAADP-AM causes membrane depolarisation in dissociated rat medulla oblongata neurons, this depolarisation was dependent on intracellular Ca^{2+}, the ryanodine receptor (RyR), acidic store Ca^{2+}release, and finally an unidentified plasma membrane ion channel.

The second study consists of unpublished work by Padamsey and Emptage (2011) where the effects of NAADP on membrane potential were explored in pyramidal neurons in hippocampal slice culture. 'Caged' NAADP (NAADP-DMNPE) was introduced into the cell via microinjection and 'uncaged' via UV flash photolysis whilst membrane potential was electrophysiologically recorded from pyramidal neurons. Bonding a DMNPE 'cage' group to NAADP prevents activity by NAADP until the chemical

'cage' is removed by photolysis. Padamsey's results show that either photolysis of NAADP-DMNPE or bath application of NAADP-AM caused intracellular Ca^{2+} release, this was expected as NAADP is well described to cause Ca^{2+} release from the acidic stores. However, Padamsey also observed a membrane depolarisation which correlated with the intracellular Ca^{2+} release. This was the first evidence that showed NAADP could influence membrane potential in pyramidal neurons of the hippocampus. However, the membrane channel responsible for this depolarisation was not identified. The findings by Brailoiu et al., (2009) and Padamsey (2011) suggest NAADP can cause a membrane depolarisation in neurons of both the medulla and the hippocampus. This is particularly exciting as this phenomenon has not been previously reported with other intracellular Ca^{2+} mobilising second messengers in neurons.

NAADP signalling has also been shown to affect membrane potential in non-neuronal cell types. In fact, the first evidence that NAADP could affect membrane potential was noted in pancreatic acinar cells. Cancela et al. (1999) showed that introduction of NAADP (via a patch pipette or photolysis of caged NAADP) to pancreatic acinar cells caused Ca^{2+} dependent ionic membrane currents. These currents could be abolished with RyR antagonism, but only reduced with IP_3R antagonism. The channel responsible for the observed membrane channels was not identified. In pancreatic β-cells glucose causes a membrane depolarisation and inward membrane currents. These currents are suggested to occur from the inactivation of ATP sensitive K^+ channels (K_{ATP}) and complexes of sulfonylurea receptor 1 (SUR1) and the inwardly rectifying K^+ channel subunit Kir6.2 (Ashcroft, 2000) and have been found to depend on NAADP and Ca^{2+} release from acidic stores (Kim et al., 2008). Arredouani et al. (2015) suggest the membrane channel responsible for the NAADP mediated depolarisation, in pancreatic β-cells, is a Ca^{2+} sensitive transient receptor potential (TRP) channel, either TRPM4 and/or TRPM5. The synergistic action of NAADP-mediated depolarisation and closure of K^+ channels is suggested to depolarise cells enough to reach the threshold for voltage-dependent Ca^{2+} channel (VDCC) activation and the subsequent secretion of insulin (Arredouani et al., 2015, Arredouani et al., 2010). The studies in pancreatic β-cells present evidence for the presence of a signalling pathway/pathways which couple

extracellular stimuli to intracellular production of NAADP, and in turn activates a signalling cascade whereby acidic store Ca^{2+} release causes cellular depolarisation.

3.2 Aims

1. Confirm application of NAADP can cause membrane depolarisation in pyramidal neurons from the hippocampus.
2. To use pharmacological manipulations to elucidate the signalling pathway underlying NAADP-mediated membrane depolarisation.
3. To determine if NAADP, compared to other Ca^{2+} mobilising messengers, is unique in its ability to produce membrane depolarisation in the hippocampus.

3.3 Results

3.3.1 NAADP-AM causes membrane depolarisation in hippocampal neurons

Pilot experiments indicated that 300 µM NAADP-AM was a concentration which most robustly resulted in membrane depolarisation when applied locally. This concentration was much greater than expected, as NAADP is known to desensitise at higher concentrations (Genazzani et al., 1996, Aarhus et al., 1996). However, due to the nature of delivery, it is likely the concentration reaching the cytosol of the patch clamped cell, was much lower than that applied extracellularly. Fresh NAADP-AM was made into solution daily and was tested on control cells to ensure that it could produce cellular depolarisation before it was used on cells subject to further manipulations.

I began my study by aiming to replicate the findings by Brailoiu et al. (2009b) and Padamsey and Emptage (2011). I first sought to confirm that NAADP-AM caused membrane depolarisation in my experimental preparation; I used a whole cell patch clamp in current camp configuration to record voltage changes in the resting membrane potential of pyramidal neurons in the CA3 and CA1 regions of hippocampal slice cultures. I then used a Picospritzer to introduce 1-2 nL of NAADP-AM over 10 seconds directly onto/above the patched cell. Cells localised to the surface of the slice were chosen in

order to improve the delivery of NAADP-AM. I found the application of NAADP-AM (300 μM) reliably caused transient membrane depolarisation (denoted by ΔV_M) in CA3 and CA1 pyramidal neurons of the hippocampus, this effect could be reproduced in the same cell multiple times. However, the magnitude and length of cellular depolarisation was variable between cells and sometimes between application onto the same cell, this is shown in **Figure 3.1B** where membrane depolarisations after application of NAADP-AM are shown. I suggest the variability of the responses observed is due to the method of NAADP-AM delivery

Figure 3.1C shows representative traces recorded from a single application of NAAADP-AM, NAADP or the Vehicle to CA1 or CA3 pyramidal neurons. The mean amplitude of NAADP-AM induced membrane depolarisations was +9.23 ± 1.34 mV (n=12) relative to the resting membrane potential (**Figure 3.1D**). I hypothesised that the AM groups are essential to allow entry of NAADP into the cell through the plasma membrane and that the charge present on NAADP prevents it from doing so. When NAADP (300 μM), which had not been conjugated to an AM ester, was applied onto patched pyramidal neurons, no significant depolarisation was observed when compared to the depolarisation achieved with NAADP-AM (+9.23 ± 1.34 mV vs. 0.54 ± 0.29 mV, n = 12, 5, $P < 0.0001$) (**Figure 3.1D**). Also, the vehicle (33% Dimethyl sulfoxide (DMSO), 67% Tyrode's solution) did not cause a significant membrane depolarisation when compared to the depolarisation achieved with NAADP-AM (+9.23 ± 1.34 mV vs. -0.92 ± 0.56 mV, n = 12, 6, $P < 0.0001$) (**Figure 3.1D**). These data suggest that NAADP can cause membrane depolarisation, that AM esters allow NAADP-AM to cross the plasma membrane, and neither the vehicle nor that the method of delivery (Picospritzer) caused membrane depolarisation.

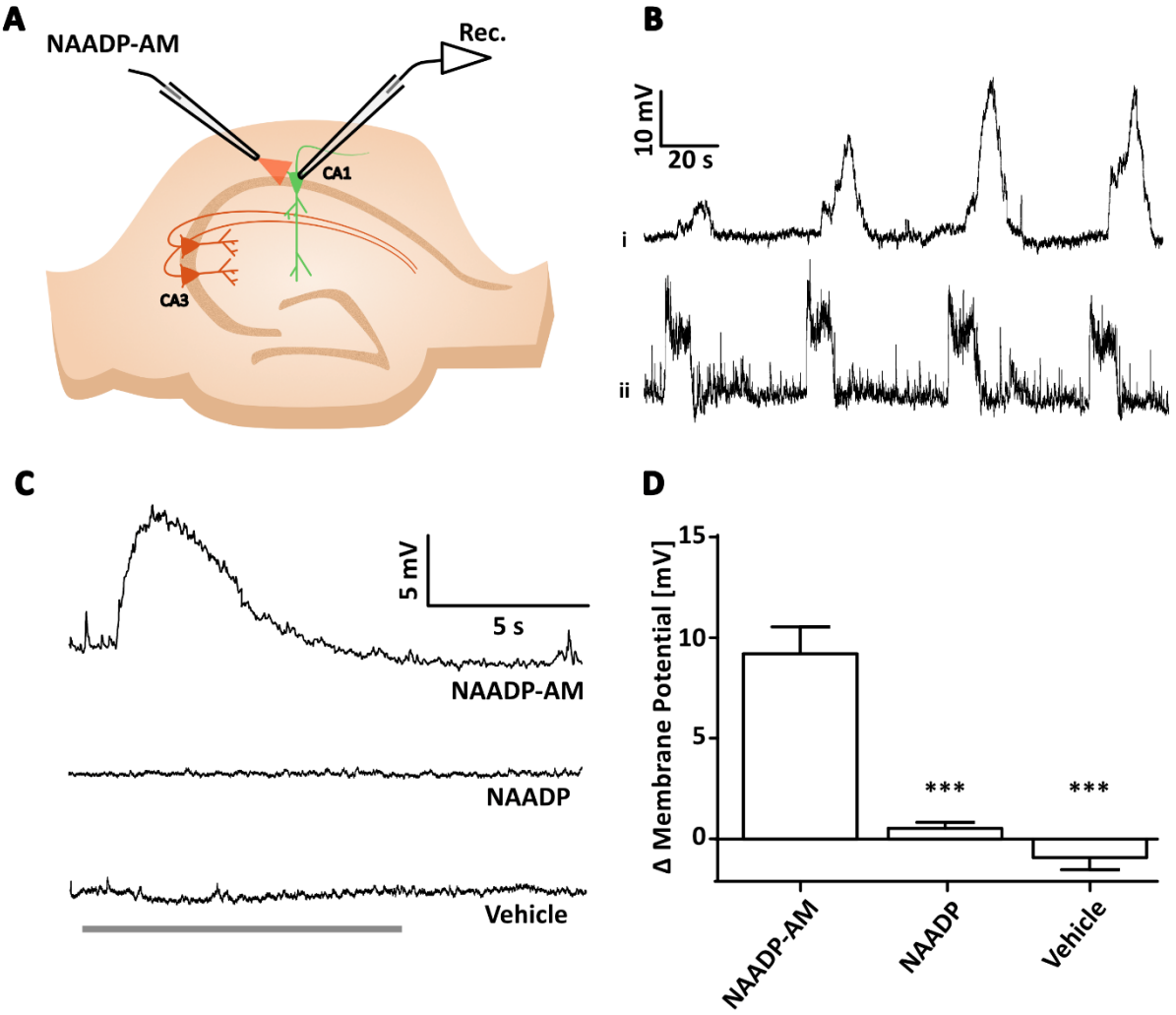

Figure 3.1 The effect of NAADP-AM on resting membrane potential of CA3 and CA1 pyramidal neurons in hippocampal slice culture. A. Diagram showing the experimental configuration. Pyramidal neurons from either the CA1 or CA3 regions of hippocampal slice cultures were patch clamped in current clamp mode, the membrane potential was recorded whilst NAADP-AM was applied onto the patched cell. **B.** Example voltage traces recorded whilst applying NAADP-AM, indicating reproducibility and variability in depolarising responses. **C.** Voltage traces recorded whilst applying either NAADP-AM, NAADP, or Vehicle, for 10 s, the grey bar indicates the time of delivery **D.** Mean ΔV_M with the application of NAADP-AM (300 µM, n=12), NAADP (300 µM, n=5), or Vehicle (n=6). with 30 µM Error bars display SEM. Significant differences in membrane potential between NAADP-AM and other groups are indicated by asterisks where *** = $P < 0.001$.

3.3.2 NAADP-Mediated depolarisation requires Ca^{2+} release from the acidic stores, intracellular Ca^{2+} signalling and the ryanodine receptor

I next examined the mechanism by which NAADP-AM caused this membrane depolarisation. I decided to approach this question by using a series of pharmacological manipulations in combination with the extracellular application of NAADP-AM. Although positive controls were not undertaken to show these pharmacological tools were performing as suggested, each drug was used at a concentration which has been previously reported to be effective in other studies, and at an appropriate concentration according to reported EC50s. Furthermore, each batch of NAADP-AM was tested on control cells to ensure it could reliably cause membrane depolarisation. If NAADP-AM appeared not to cause depolarisation, it was applied to several sites (around 10 µm apart) above patched cell to ensure lack of depolarisation was not due to NAADP-AM not reaching the cell. The pharmacological tools used for these set of experiments did not affect the resting membrane potential of the cells. Typical voltage recordings of the manipulations in combination with the application of NAADP-AM are shown in **Figure 3.2A**. None of the pharmacological tools affected the resting membrane potential of the patched cells. I first sought to determine if NAADP-AM-induced depolarisation was dependent on endogenous NAADP signalling. Two methods were available to achieve this; receptor desensitisation and pharmacological antagonism. The NAADP receptor can be desensitised by high (millimolar) concentrations of NAADP in mammalian cells (Aarhus et al., 1996, Genazzani et al., 1996), after which further addition of NAADP cannot activate the receptor, and downstream events cannot occur. A desensitising concentration of intracellular NAADP (1 mM) was introduced via the patch pipette and allowed to dialyse into the cell for 5 minutes. This manipulation prevented depolarisation on application of additional NAADP-AM (+10.41 ± 1.49 mV vs. +0.04 ± 0.47 mV, n = 11, 6, $P < 0.0001$) (**Figure 3.2B**). NAADP signalling could also be abolished when using the NAADP-receptor antagonist Ned-19 (Naylor et al., 2009). Pre-incubation of slices with Ned-19 (100 µM, 40 min) prevented depolarisation by NAADP-AM (+10.41 ± 1.49 mV vs. +0.18 ± 0.52mV, n = 11, 6, $P < 0.0001$) (**Figure**

3.2B). These data strongly suggest that the observed membrane depolarisation is dependent on NAADP signalling.

NAADP is reported to cause Ca^{2+} release from the endolysosomes (Churchill et al., 2002, Yamasaki et al., 2004). I next examined whether NAADP-mediated depolarisation was dependent on intracellular Ca^{2+} signalling. 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) acts as a Ca^{2+} chelator (Tsien, 1980). BAPTA is fast acting and has a high affinity for Ca^{2+} (binding constant for BAPTA to Ca^{2+} is 500 $\mu m^{-1}s^{-1}$ (Ricci et al., 1998) and can effectively abolish most, including microdomain, intracellular Ca^{2+} signalling events. BAPTA tetra-potassium salt (15 mM) was introduced via the patch pipette, with the aim to buffer the intracellular Ca^{2+} to a low concentration and prevent intracellular Ca^{2+} signalling. I chose to use BAPTA over other Ca^{2+} chelators such as EGTA as other Ca^{2+} chelators are not as rapidly acting or have high enough Ca^{2+} affinity to prevent microdomain Ca^{2+} signalling events. I found that intracellular BAPTA prevented depolarisation by NAADP-AM (+10.41 ± 1.49 mV vs. +0.71 ± 0.13 mV, n = 11, 5, $P < 0.0001$) (**Figure 3.2B**).

I next examined whether NAADP-mediated depolarisation was dependent on Ca^{2+} release from the acidic stores. Lysosomal Ca^{2+} uptake is known to be dependent on high intralysosomal proton concentration (low pH) generated by vacuolar H^+-ATPases (Christensen et al., 2002). Bafilomycin A1 is a vacuolar ATPase inhibitor, it prevents H^+ loading and therefore Ca^{2+} entry into acidic stores (Yoshimori et al., 1991, Christensen et al., 2002). This consequently abolishes acidic store Ca^{2+} signalling. In my experimental system pretreatment with the inhibitor bafilomycin A1 (4 µM, 40 min) also prevented depolarisation by NAADP-AM (+10.41 ± 1.49 mV vs. -0.83 ± 0.54 mV, n = 11, 5, $P < 0.0001$) (**Figure 3.2B**). These data suggest that the depolarisation is dependent on intracellular Ca^{2+} and Ca^{2+} release from the lysosomes.

The ryanodine receptor (RyR) is an endoplasmic reticulum (ER) Ca^{2+} channel; it can be activated and modulated by a variety of messengers including Ca^{2+}. When activated by intracellular Ca^{2+} it causes further Ca^{2+} release from the ER (Zalk et al., 2007), in a process known as Ca^{2+} induced Ca^{2+} release

(CICR) which is shown to be responsible for producing intracellular Ca^{2+} waves in neurons (Friel and Tsien, 1992). In other cell types, Ca^{2+} nanodomains have been suggested to functionally couple acidic stores to ER/SR Ca^{2+} (Morgan et al., 2013, Penny et al., 2015, Penny et al., 2014, Fameli et al., 2014) amplification via such coupling may also be present in hippocampal neurons system. Brailoiu et al. (2009b) showed that NAADP-AM-induced depolarisation could also be abolished with the application of ryanodine. Ryanodine is known to inactivate RyRs in the hippocampus at μM concentrations (Emptage et al., 1999). I found bath application of ryanodine, a ryanodine receptor antagonist, (30 μM, 15 min) prevented membrane depolarisation by NAADP-AM causing in my experimental configuration (+10.41 ± 1.49 mV vs. +0.18 ± 0.14 mV, n = 11, 4, $P < 0.0001$) (**Figure 3.2B**). These data suggest that the depolarisation is dependent on amplification of the initial Ca^{2+} signal (from the endo-lysosomes) by the endoplasmic reticulum via the ryanodine receptor.

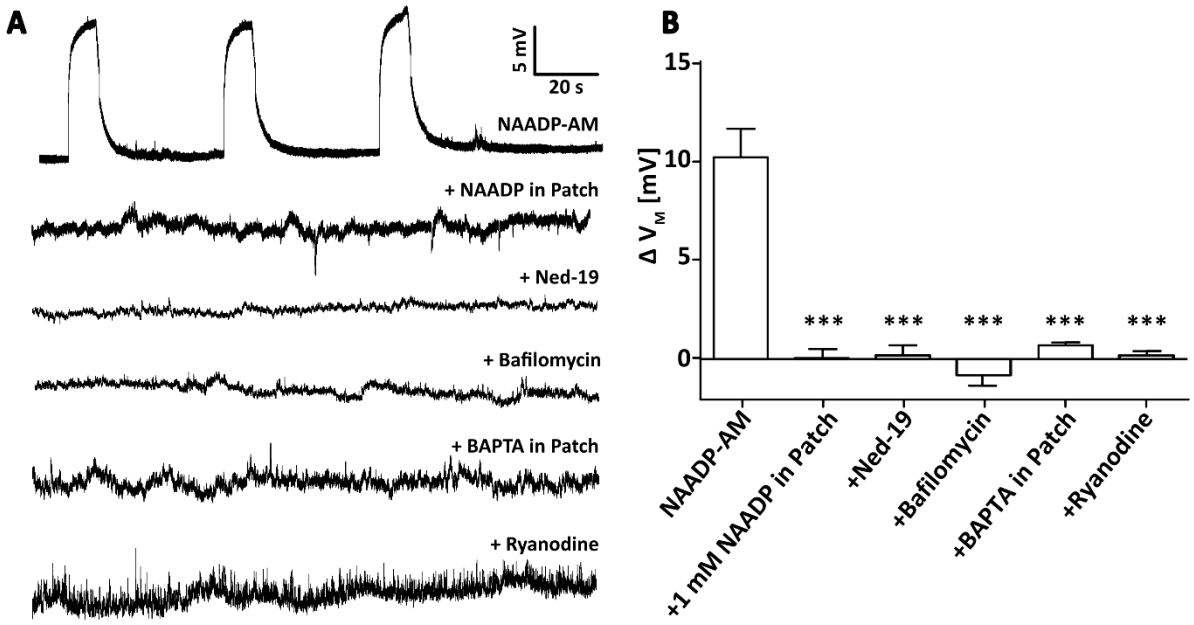

Figure 3.2 The effect of NAADP-AM on resting membrane potential of CA3 and CA1 pyramidal neurons in hippocampal slice culture. A. Typical voltage traces recorded whilst applying either NAADP-AM in combination with either: 1 mM NAADP inside the patch pipette (n=6), preincubation with 100 µM Ned-19 for 40 minutes (n=6), preincubation with 4µM Bafilomycin for 40 minutes (n=5), 15 mM BAPTA inside the patch pipette (n=5) or preincubation with 30 µM Ryanodine for 40 minutes (n=4). **B** Mean ΔV_M when NAADP-AM (300 µM, n=11) was applied in combination with the pharmacological manipulations mentioned above. Error bars display SEM. Significant differences in membrane potential between NAADP-AM and other groups are indicated by asterisks where *** = $P < 0.001$.

3.3.3 NAADP is uniquely able to produce membrane depolarisation among Ca^{2+} mobilising second messengers

The next set of experiments were undertaken in collaboration with a Pharmacology MSc project student (Henry Taylor) while he was undertaking a laboratory rotation with the Emptage group. The project design and mentorship were provided by me.

I wanted to empirically determine whether the ability to produce membrane depolarisation in hippocampal neurons is unique to NAADP, as opposed to the other Ca^{2+} mobilising second messengers. I addressed this question by recording the membrane potential of pyramidal neurons of CA1 in hippocampal slice cultures whilst Ca^{2+} mobilising second messengers were dialysed into the patched cell, **Figure 3.3A** displays representative voltage traces recorded during dialysis of second messenger into the cell. Ca^{2+} mobilising second messengers were included in the intracellular solution at varying concentration and were dialysed into to cell post breakthrough to whole-cell configuration. The three Ca^{2+} mobilising second messengers I used were: NAADP, IP_3 and cADPR. IP_3 and cADPR both are well described to cause Ca^{2+} release from the ER, via the IP_3Rs and RyRs respectively, and neither is reported to mobilise Ca^{2+} from the acidic stores. Whereas, NAADP is well described to mobilise Ca^{2+} from the acidic stores. (Lee and Aarhus, 2000, Churchill et al., 2002, Galione, 2006).

Changes in membrane potential caused by dialysis of second messengers were determined by subtracting a baseline membrane potential (mean over 50 s), after stabilisation of the patch recording post breakthrough to whole cell, from the mean membrane potential (over 50 s, 300 s after the baseline) to give the change in membrane potential (ΔV_M).

I next wanted to identify any statistical differences between the mean ΔV_M observed in control groups and upon the addition of Ca^{2+} mobilising second messengers, at varying concentration.

I show that the addition of NAADP, via patch pipette, to the cytosol of pyramidal neurons in CA1 of the hippocampus, can cause significant membrane depolarisation between the range of 100 nM to 10 µM. I undertook a one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests to

compare each concentration of NAADP, IP_3 and cADPR to their respective control group (no second messenger included in patch). The mean ΔV_M was significantly different to the control group at the following concentrations of NAADP: 100 nM (-0.85 ± 0.37 mV vs +6.11 ± 1.11 mV, P<0.0001), 1 µM (-0.85 ± 0.37 mV vs +4.90 ± 1.19 mV, P < 0.001), and 10 µM (-0.85 ± 0.37 mV vs + 3.39 ± 1.14 mV, P < 0.05). Neither IP_3 or cADPR, at any concentration, produced a significant depolarisation compared to the control group (**Figure 3.3B)**. To ensure IP_3 could cause Ca^{2+} release in pyramidal neurons of the hippocampus, a positive control was undertaken. This control consisted of initially filling a pyramidal neuron with the Ca^{2+} indicator OGB-1 for one minute, allowing the cell to recover and the dye to dialyse through the cell for a further five minutes, then finally re-patching the cell with 10 µM IP_3 included in the internal solution. At 10 µM, IP_3 caused a Ca^{2+} signal to be observed in CA1 pyramidal neurons (data not shown, n=2). A positive control to show cADPR can cause Ca^{2+} release via RyRs was not undertaken, this must be acknowledged as a limitation of the study.

In addition, I undertook two-way ANOVA to compare mean ΔV_M between each concentration of each second messenger used. I found that the both the concentration and type of the second messenger used significantly affected the amount of observed depolarisation ($F_{(8, 110)}$ = 2.142, P = 0.0377 and $F_{(2, 110)}$ = 15.47 P < 0.0001, respectively). I used Dunnett's multiple comparison tests to determine significant differences in ΔV_M between NAADP, IP_3 and cADPR at each concentration of the second messenger used, results are shown (**Figure 3.3C)**. No significant differences in ΔV_M between IP_3 or cADPR are observed at any concentration. However, significant differences ΔV_M between NAADP and IP_3/cADPR were recorded at 100 nM (+6.11 ± 1.11 mV vs -0.03 ±0.32 IP_3 and vs 0.71 ± 0.53 cADPR, where P<0.0001 for both groups) 1 µM (+4.90 ± 1.19 mV vs 0.14 ± 0.37 IP_3, P < 0.001 and vs -0.80 ± 1.14 cADPR: P < 0.0001) and 10 µM (3.39± 1.14 mV vs 0.05 ± 0.48 IP_3, P < 0.05 and -0.47 ± 1.37 cADPR: P < 0.01). Overall these results show clearly that, in the experimental configuration used, NAADP but not IP_3 or cADPR causes membrane depolarisation.

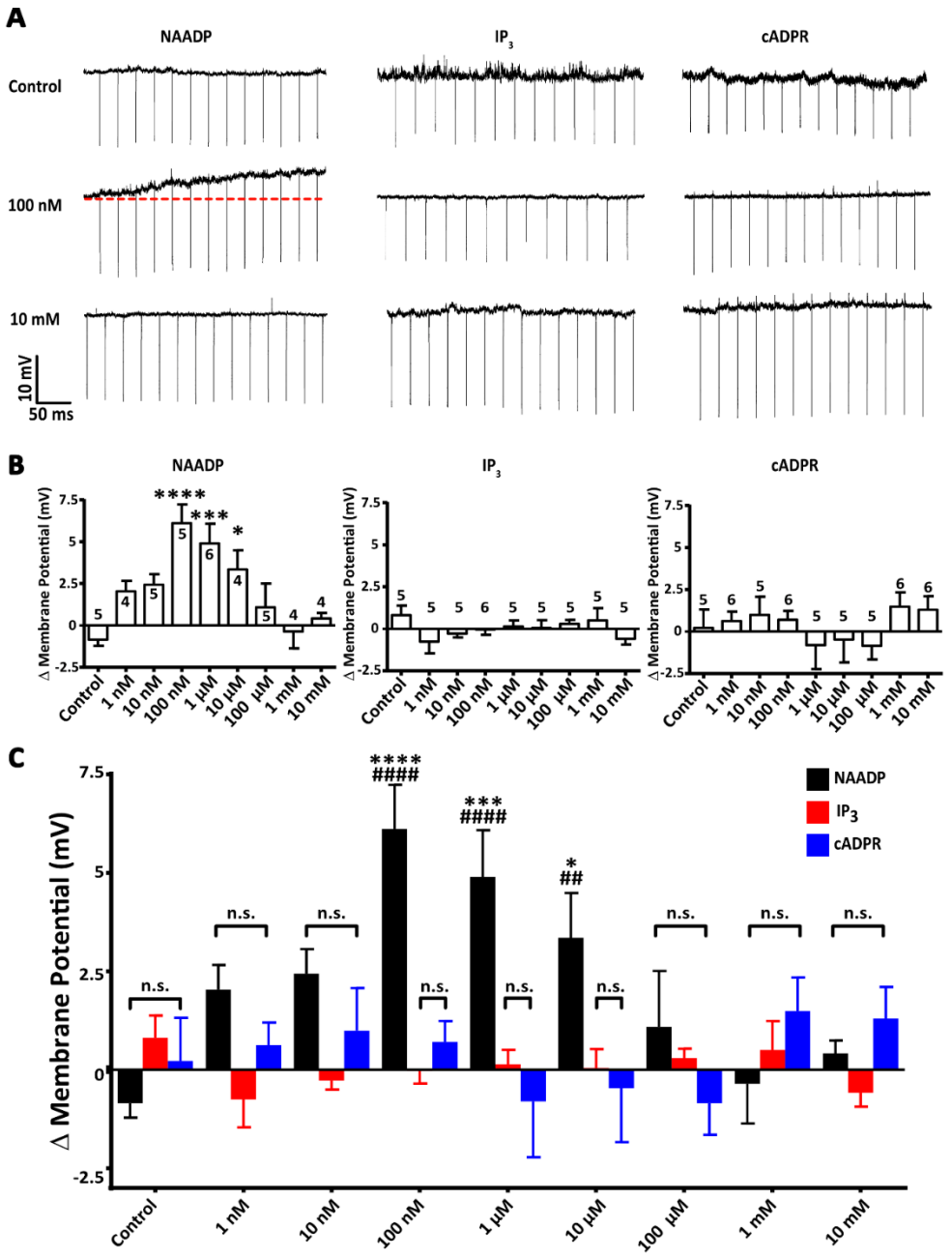

Figure 3.3 Mean ΔV_M on dialysis of Ca^{2+} mobilising second messengers. A. Mean ΔV_M for each concentration of second messenger. Sample size (n) is shown above or inside each column. Error bars display SEM. Significant differences to Control group are shown by asterisks where ****= $P < 0.0001$, ***= $P < 0.001$, *= $P < 0.05$. **B.** Complied Mean ΔV_M from all three groups with two-way analysis of variance. Error bars display SEM. Sample size are the same as in **A**. Significant differences in membrane potential between NAADP and IP_3 are indicated asterisks (*), differences between NAADP and cADPR are indicated by hashes (#). Where ****/#### = $P < 0.0001$, ***/### = $P < 0.001$, **/## = $P < 0.01$, */# = $P < 0.05$.

NAADP has been reported to produce a bell-shaped dose response curve, it is a characteristic feature of NAADP-induced cellular responses (Galione et al., 2011, Guse et al., 2013). The bell-shaped dose-response curve is suggested to be a result of receptor self-desensitisation by a high concentration of NAADP (Genazzani et al., 1996, Aarhus et al., 1996).

I plotted [Second messenger] (nM) vs. ΔV_M on a semi log scale to produce dose-response plots for each second messenger. The dose-response plot produced from NAADP vs ΔV_M generates a clear bell-shaped relationship (**Figure 3.4B**). However, this relationship is not apparent for IP_3 or cADPR (**Figure 3.4C and D**). I used GraphPad Prism to fit a curve based upon a model for a bell shaped dose-response curve (See **2.5.1 Curve fitting**) to the data acquired from intracellular dialysis of NAADP. From this curve, the half maximal effective concentration (EC50) was determined to be 12.5 nM and the half maximal inhibitory concentration (IC50) was determined to be 7.07 µM. The Hill coefficients were reported to be -0.81 stimulation and -0.65 for inhibition, indicating that multiple binding sites for NAADP may exist and that there is a degree of negatively cooperatively binding.

I used GraphPad Prism to compare the goodness of fit between two models applied to the data set produced from each second messenger. I chose to fit a curve based upon either a model for a bell-shaped dose response curve or linear regression where Y=0. To compare the goodness of fit between these two models I employed the Akaike information criterion (AICc) method. Using AICc I found that the model for the bell-shaped dose response curve is more likely to provide a better fit to the NAADP dose-response data than a linear regression model (>99.99% vs. <0.01%, respectively). Conversely, the model for the bell-shaped dose response curve is less likely to provide a better fit than linear regression where Y=0 both IP_3 (2.82% vs. 97.18%, respectively) and cADPR (4.65% vs 95.35%, respectively).

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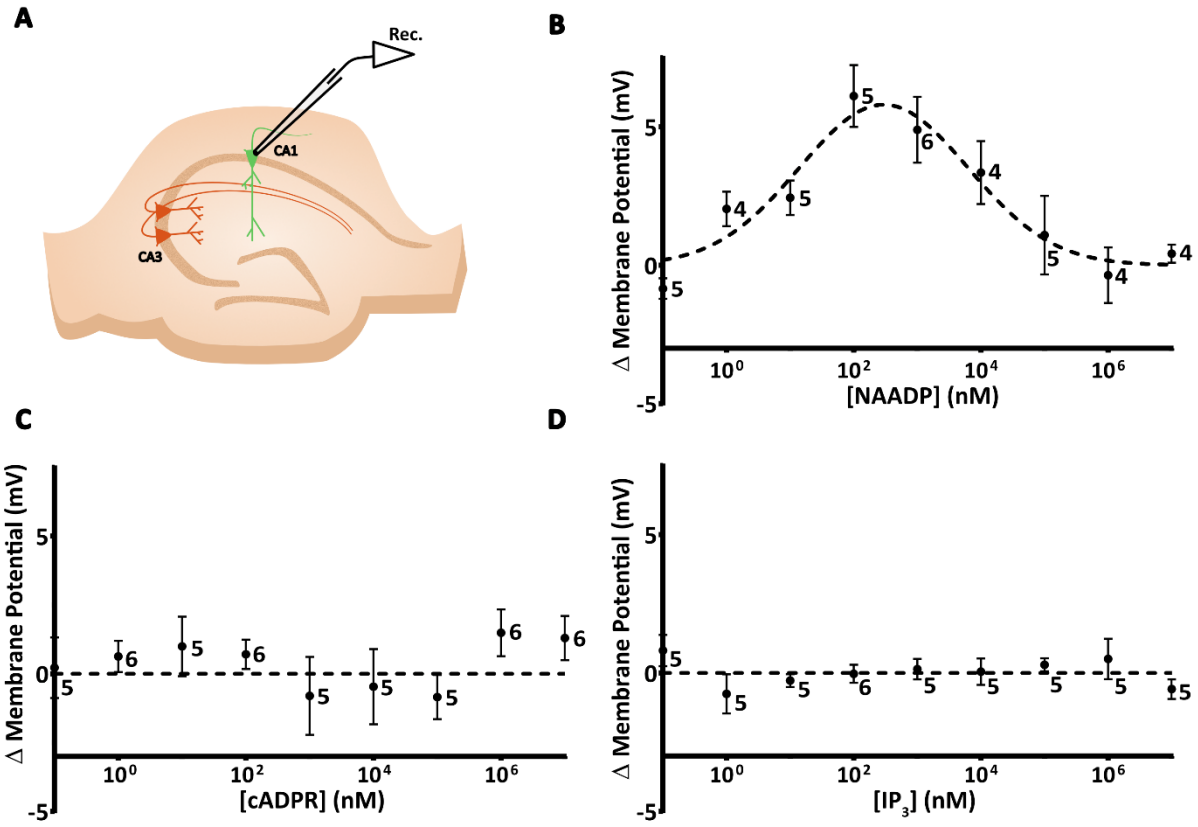

Figure 3.4 Dose response curves for the relationship between membrane potential of pyramidal neurons and concentration of Ca^{2+} mobilising second messengers in patch pipette. **A.** schematic diagram of the experimental configuration. Membrane potential of pyramidal neurons in CA1 of hippocampal slice culture was recorded, in combination with Ca^{2+} mobilising second messengers in the patch pipette. **B.** Dose response curve to NAADP in patch pipette, fitted bell-shaped dose response curve shown by dashed line (R^2=0.5391, d.f.=37, Sy.x = 2.143). **C.** Dose-responses to cADPR in patch pipette, Y=0 shown by the dashed line. **D.** Dose-responses to IP_3 in patch pipette, Y=0 shown by the dashed line. Sample size (n) is shown to the right of each point. Error bars display SEM.

3.4 Discussion

The data I present in this chapter shows that NAADP causes membrane depolarisation in pyramidal neurons in hippocampal slice cultures. I have shown that introduction of a membrane permeant form of NAADP (NAADP-AM) and intracellular dialysis of NAADP via patch pipette both reliably cause

membrane depolarisation. These data are consistent with those observed by Brailoiu et al. (2009b) and Padamsey and Emptage (2011), where NAADP is shown to produce membrane depolarisation in neurons of the medulla and hippocampus respectively. NAADP-mediated membrane depolarisation, with similar dynamics to those observed here and by (Padamsey and Emptage, 2011, Brailoiu et al., 2009b) has also been noted in the pancreatic β-cells (Arredouani et al., 2010, Arredouani et al., 2015). My results provide further evidence that NAADP plays a role in modulating membrane potential in a variety of electrically excitable cells.

The data I present in **Figure 3.1** and **Figure 3.2** broadly replicates those observed by Brailoiu et al. (2009b). I show that for NAADP mediated depolarisation to occur, there is a necessity of an intracellular signalling pathway which requires activation of NAADP signalling, acidic store Ca^{2+}, intracellular Ca^{2+} and the RyR. Both the data I obtained from the extracellular application of NAADP-AM (shown in **Figure 3.1** and **Figure 3.2**) and those presented by Brailoiu et al. (2009b) suggest a role of the RyRs in producing NAADP-mediated cellular depolarisation. I, therefore, suggest that NAADP-mediated acidic store Ca^{2+} is then amplified via the RyR as a result of functional microdomains. Evidence for the existence of these microdomains has been provided from theoretical and experimental data (Aston et al., 2017, Morgan et al., 2013, Penny et al., 2015, Penny et al., 2014).

I show that RyR antagonism prevents NAADP-mediated depolarisation, therefore, it was slightly unexpected that dialysis of cADPR, which is generally accepted to reduce the threshold for activation of the RyRs and can cause ER Ca^{2+} release, as reviewed by Guse (2004), did not produce marked cellular depolarisation at any concentration (**Figure 3.3**). I suggest that a specific spatiotemporal pattern of the Ca^{2+} signal may be required in order to cause cellular depolarisation in pyramidal neurons of the hippocampus. Where Ca^{2+} release must first occur via the acidic Ca^{2+} stores rather than the ER, but the Ca^{2+} signal is required to be amplified via the RyRs/ER to allow for cellular depolarisation. I should note that one of the limitations in this set of experiments is the lack of a positive control for cADPR. To determine whether cADPR produces a Ca^{2+} signal when dialysed into CA1 hippocampal neurons I

suggest an experiment where neurons are filled with a Ca^{2+} sensitive fluorescent indicator and subsequently patched onto with cADPR containing internal solution whilst monitoring fluorescence with confocal imaging. In the same fashion as the positive control undertaken to ensure IP_3 caused a Ca^{2+} release in this experimental preparation. A future experiment which would be interesting and useful to perform comprises of dialysing an agonist for the RyRs, such as caffeine (McPhersonx et al., 1991), into hippocampal neurons whilst recording the membrane potential. If depolarisation was not observed, one could conclude that for NAADP mediated membrane depolarisation to occur, both NAADP and cADPR are required and that NAADP serves as a 'trigger' signal.

The data I present in **Figure 3.3** suggests that NAADP is unique among Ca^{2+} mobilising messengers in its ability to produce cellular depolarisation. A maximum average ΔV_M was recorded at a concentration of 100 nM NAADP (+6.11 ± 1.1 mV, n=6), this is congruent with the maximal dose response recorded in a variety other preparations and systems as reviewed by Guse et al. (2013) and Galione (2011). Interestingly, 1 µM NAADP also produced near maximal membrane depolarisation (+4.89 ± 1.18 mV, n=6), this concentration is greater than those observed for maximal responses in other systems, which are often around closer to 100 nM. This may be a delivery method artefact, with the concentration actually reaching the cytoplasm in order to cause the depolarisation being lower than the 1 µM used in the patch electrode. Additionally, if the depolarisation is dependent on lysosomes located to a specific subcellular compartment, the dendrites, for example, the concentration in subcellular compartments will initially be lower than the soma, as concentration gradient will occur from the site of entry.

The experiments conducted for this chapter have not addressed possible candidates for the depolarising membrane channel. Though I suggest that the channel is regulated by intracellular Ca^{2+}, as the membrane depolarisation is dependent on intracellular Ca^{2+} (blocked by BAPTA – **Figure 3.2**). Brailoiu et al. (2009b) suggest a Ca^{2+} sensitive transient receptor potential (TRP) channel is responsible

for NAADP-mediated depolarisation in neurons, whereas Ashcroft (2000) suggests NAADP-mediated currents occur via the inactivation of K^+ channels in pancreatic β-cells.

I summarise my findings in a schematic diagram (**Figure 3.5**) where I suggest an intracellular signalling pathway which produces NAADP-mediated cellular depolarisation in the hippocampus. I suggest that Introduction of NAADP-AM or NAADP via patch pipette activates the NAADP receptor (NAADPR) and causes Ca^{2+} release from the acidic stores. Ca^{2+} release from the acidic stores is amplified by Ryanodine receptor (RyR) via Ca^{2+} induced Ca^{2+} release (CICR). This causes the activation or inhibition of a Ca^{2+} sensitive membrane channel to cause cellular depolarisation.

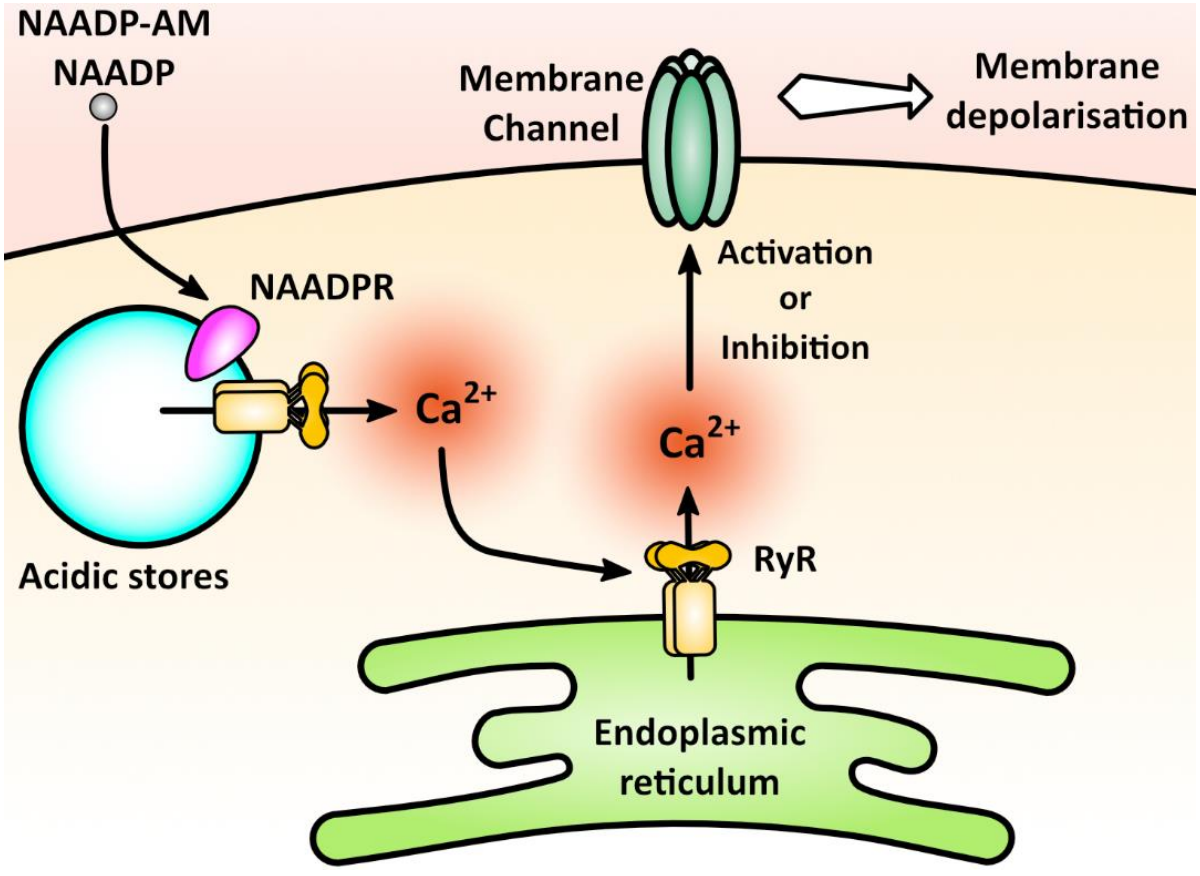

Figure 3.5 Hypothesised pathway for NAADP-mediated depolarisation. The introduction of NAADP-AM or NAADP via patch pipette activates the NAADP receptor (NAADPR) and causes Ca^{2+} release from the acidic stores. Ca^{2+} release from the acidic stores is amplified by Ryanodine receptor (RyR) via Ca^{2+} induced Ca^{2+} release (CICR). This causes the activation or inhibition of a Ca^{2+} sensitive membrane channel to cause cellular depolarisation.

3.5 Conclusion

1. **Confirm application of NAADP can cause membrane depolarisation in pyramidal neurons from the hippocampus.**

I have shown that both extracellular application of membrane permeable NAADP-AM (**Figure 3.1** and **Figure 3.2**) and intracellular dialysis of NAADP (**Figure 3.3**) can robustly cause cellular/membrane depolarisation in pyramidal neurons of the hippocampus.

2. **To use pharmacological manipulations to elucidate the signalling pathway underlying NAADP-mediated membrane depolarisation.**

I have shown, through pharmacological manipulations, that NAADP-mediated depolarisation is dependent on acidic store Ca^{2+} mobilisation, intracellular Ca^{2+}, and the RyR (**Figure 3.2**). I suggest that other intracellular signalling elements may also be present in this hypothesised signalling pathway, however, I show that the above three components are essential for NAADP-mediated depolarisation

3. **To determine if NAADP, compared to other Ca^{2+} mobilising messengers, is unique in its ability to produce membrane depolarisation in the hippocampus.**

I show in **Figures 3.3 and 3.4** that NAADP is unique, among Ca^{2+} mobilising second messengers, in its ability to cause membrane depolarisation, when introduced via patch pipette. Though I recognise that it is possible that dialysis of second messengers via patch pipette may not produce specific spatiotemporal patterns of Ca^{2+} release to cause membrane depolarisation via IP_3 or cADPR.

4.0 Activation of mGluR1 causes membrane depolarisation via NAADP signalling and acidic store Ca^{2+} release

4.1 Introduction

In the previous chapter, I presented experimental evidence that NAADP can produce membrane depolarisation in pyramidal neurons of the hippocampus. I next wanted to determine if NAADP-mediated membrane depolarisation, in CA1 pyramidal neurons of the hippocampus, could be triggered by physiologically relevant stimuli, as opposed to bath/site directed application of NAADP-AM. Pandey et al. (2009) show agonist specific synthesis of NAADP. The addition of glutamate, but not ATP, caused NAADP synthesis, in murine neurons in suspension from whole-brain trituration, with around a 35-fold increase from the basal concentration. Though, the glutamate receptor/receptors responsible for causing NAADP synthesis was not identified. I hypothesised that glutamate, the major transmitter in the hippocampus, could be responsible for inducing NAADP-synthesis leading to NAADP-mediated cellular depolarisation. Many glutamate receptors exist, they are divided into two main families; the ionotropic glutamate receptors, including the GluNs, GluAs and GluKs, and the metabotropic glutamate receptors (mGluRs). mGluRs are G-protein coupled receptors (GPCRs) and act via second messengers (Niswender and Conn, 2010). Though it should be noted that ionotropic glutamate receptors are also reported to have metabotropic functions under certain circumstances (Petrovic et al., 2017, Weilinger et al., 2016). In non-neuronal cell types, several metabotropic (G-protein coupled) membrane receptors are shown to cause intracellular NAADP synthesis and acidic store Ca^{2+} signalling (Soares et al., 2007, Rah et al., 2010, Lewis et al., 2012, Yamasaki et al., 2005).

I proposed that activation of an mGluR could cause intracellular NAADP synthesis. The mGluR family consists of at least of eight members, all of which are GPCRs, split into three groups based upon pharmacological and functional properties (Niswender and Conn, 2010). In addition, Group 1 mGluRs (mGluR1 and mGluR5) are shown to produce membrane depolarisation and affect membrane excitability in neurons of the hippocampus. Congar et al. (1997) show that activation of Group 1

mGluRs in produces modest, transient, inward membrane currents of neurons in the hippocampus, via activation of a Ca^{2+}-dependent non-selective cation channel. Furthermore, other studies have shown activation of mGluR1 can produce intracellular, Ca^{2+}-dependent, postsynaptic membrane depolarisation and or membrane excitability in CA1 pyramidal neurons of the hippocampus. This depolarisation is suggested to be the result of either: inhibition of background K^+ channels (TASK and TREK), inhibition of Ca^{2+} activated K^+ Channels (SK) (Tigaret et al., 2016), inhibition of a K^+ leak channel (Mannaioni et al., 2001) or activation of TRPC channels (Ben-Mabrouk et al., 2012, Kim et al., 2003). mGluR1 and mGluR5, are both canonically implicated as $G_{\alpha q}$ linked and are suggested to mobilise Ca^{2+} from the ER via IP_3 signalling (Raymond et al., 2000). Group 1 mGluRs are noted to have perisynaptic localisation on dendritic spines of hippocampal pyramidal neurons (Lujan et al., 1996). Groups 2 and 3 contain the remaining six members of the mGluR family, they are canonically implicated as $G_{\alpha i/o}$ coupled receptors and reduce cyclic adenosine monophosphate (cAMP) synthesis, their activation has broad effects, but generally they reduce neuronal activity by producing hyperpolarisation a via activation of K^+ channels (Muly et al., 2007) or inhibition of VDCCs (Ferraguti et al., 2008, Ferraguti and Shigemoto, 2006).

4.2 Aims

1. To determine if glutamate can cause NAADP-mediated depolarisation.
2. Were glutamate to cause NAADP-mediated depolarisation, to identify which glutamate receptor produces NAADP-mediated depolarisation.
3. To identify components, of the intracellular signalling pathway responsible for NAADP-mediated depolarisation.

4.3 Results

4.3.1 Metabotropic glutamate receptor 1 activation causes an NAADP mediated depolarisation

As mGluRs act via second messengers, I wanted to determine if mGluRs can produce an NAADP-dependent membrane depolarisation. I addressed this question by patching CA1 pyramidal neurons in hippocampal slice culture. I used current clamp in whole cell configuration with high resistance pipettes (16-20 MΩ) (to prevent dialysis of necessary mGluR co-factors) whilst a modified pattern of electrical stimulation known to activate mGluRs in the hippocampus (Fan et al., 2010) was delivered to *stratum radiatum* (four pulses at 20 Hz). The experimental configuration is shown diagrammatically in **Figure 4.1A**.

I pharmacologically isolated mGluR dependent responses by using AP5 (50 µM) and NBQX (10 µM) to block ionotropic glutamate receptors (GluN, GluA, and GluK), and picrotoxin (100 µM) and CGP 55845 (2 µM) to prevent responses to inhibitory GABAergic interneuron input. The mean ΔV_m was observed to be + 2.09 ± 0.15 mV (**Figure 4.1C**).To demonstrate this depolarisation was indeed the result of mGluR activation I pharmacologically isolated the mGluRs then subsequently bath applied 100 µM LY341495, at such concentration this drug is known to block all members of the mGluR family (Kingston et al., 1998). This significantly reduced/abolished depolarisation after electrical stimulation (+2.09 ± 0.15 mV mGluR isolation vs +0.8 ± 0.15 pan-mGluR blockade, n = 11, 6, $P < 0.05$), shown in **Figure 4.1B and C**. Therefore, I show that an mGluR can cause membrane depolarisation upon synaptic stimulation. I next sought to determine which mGluR/mGluRs were responsible for this depolarisation.

The mGluR family contains 8 members and is split into three groups based upon their pharmacological and function profiles. Group I (mGluR1, mGluR5), Group II (mGluR2, mGluR3) and Group III (mGluR4, mGluR6, mGluR7, mGluR8) (Ferraguti and Shigemoto, 2006, Niswender and Conn, 2010). I systematically isolated members of the mGluR family using series of mGluR subgroup and/or subtype specific pharmacological antagonists with the aim to identify which member/s cause cellular

depolarisation. ΔV_m upon electrical stimulation in the presence of pharmacological antagonists are shown in **Figure 4.1B** and **C**.

LY341495 acts as an mGluR Group II and III antagonist at 100 nM, whereas at 100 µM it acts as a pan-mGluR antagonist (Kingston et al., 1998). The addition of 100 nM LY341493 caused no significant reduction in the depolarisation observed upon synaptic stimulation (+ 2.09 ± 0.15 mV to +2.51 ± 0.46 mV, n = 11, 6, $P < 0.05$), whereas 100 µM LY341495 produced abolished/significantly reduced membrane depolarisation compared to the control group (+ 2.09 ± 0.15 mV to + 0.83 ± 0.15 mV, n = 11, 6, $P < 0.05$) is noted (**Figure 4.1C**). Therefore, I suggest that the observed depolarisation is a Group I dependent effect. I next determined which Group I mGluR subtype (mGluR1/mGluR5) was responsible for the observed depolarisation. MPEP is a selective mGluR5 antagonist at 10 µM (Mannaioni et al., 2001) and JNJ16259685 is a potent and selective mGluR1 antagonist at 300 nM (Lavreysen et al., 2004). MPEP did not cause a reduction in the depolarisation observed (+ 2.09 ± 0.15 mV vs + 1.94 ± 0.22 mV, n = 11, 6, $P < 0.001$), whereas JNJ16259685 significantly reduced the amount of depolarisation (+ 2.09 ± 0.15 mV to + 0.59 ± 0.13 mV, n = 11, 6, $P < 0.001$). Therefore, I suggest that mGluR-mediated depolarisation in hippocampal CA1 pyramidal neurons is dependent on activation of mGluR1.

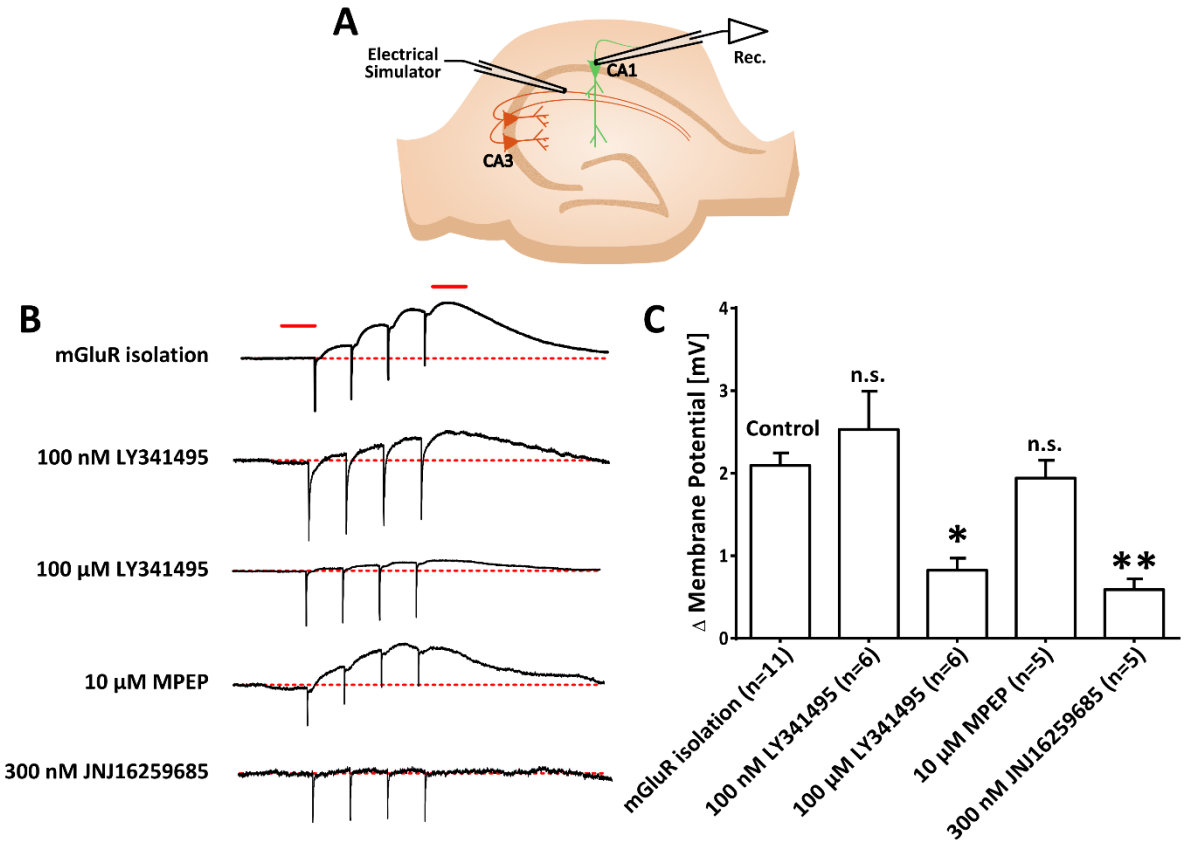

Figure 4.1 Synaptic activation of mGluR1 membrane depolarisation. A. Diagram of experimental configuration. Membrane potential was recorded from pyramidal neurons in the CA1 region of hippocampal slice cultures whilst electrical stimulation 4 pulses at 20 Hz, was delivered to the stratum radiatum. **B.** Representative voltage recordings with: pharmacological isolation of mGluRs with AP5 (50 µM), NBQX (10 µM), picrotoxin (100 µM) and CGP 55845 (2 µM), plus either LY341485 (100 nM), LY341485 (100 µM), MPEP (10 µM) or JNJ16259685 (300 nM). The red lines indicate where membrane potentials were compared before and after stimulation. **C.** Columns show mean ΔV_m of CA1 pyramidal neurons before and after electrical stimulation. Error bars display SEM. Significant differences between membrane potentials are displayed above each column, where ** = $P < 0.01$ and * = $P < 0.05$, n.s. = non-significant.

I next wanted to determine if synaptically evoked mGluR1-mediated membrane depolarisation in CA1 pyramidal neurons is dependent on NAADP signalling and lysosomal Ca^{2+} signalling. I explored this question by isolating the mGluRs (as described previously) and subsequently applying further pharmacological manipulations. ΔV_m upon electrical stimulation in the presence of pharmacological antagonists are shown in **Figure 4.2**

In this set of experiments, pharmacological isolation of the mGluR receptors gave a ΔV_m of +3.60 ± 1.00 mV. LY341495 (100 µM) (pan-mGluR antagonist) was used to ensure responses were indeed mGluR-dependent, and also significantly attenuated mGluR depolarisation in this set of experiments (+3.60 ± 1.00 mV vs. +0.46 ± 0.12 mV, n = 7, 6, $P < 0.001$) (**Figure 4.2**).

A desensitising concentration of intracellular NAADP (1 mM) (Aarhus et al., 1996, Genazzani et al., 1996), introduced via the patch pipette, significantly attenuated responses to mGluR mediated depolarisation (+3.60 ± 1.00 mV vs. +0.38 ± 0.10 mV, n = 7, 6, $P < 0.001$). Whereas nicotinic acid adenosine dinucleotide (NAAD) (1 mM), introduced via the patch pipette, has no effect on synaptically evoked mGluR depolarisation (+3.60 ± 1.00 mV vs. +2.01 ± 0.60, n = 7, 6, $P > 0.9999$) suggesting a specificity to the Ca^{2+} mobilising second messenger NAADP. Pre-incubation with the NAADP receptor antagonist Ned-19 (100 µM, 40 min) (Naylor et al., 2009) also significantly attenuated responses to mGluR depolarisation (+3.60 ± 1.00 mV vs. +0.38 ± 0.10 mV, n = 7, 6, $P < 0.001$). These data suggest mGluR membrane depolarisation is dependent on NAADP signalling (**Figure 4.2**).

I subsequently determined if mGluR-mediated membrane depolarisation is dependent on lysosomal Ca^{2+} signalling. To achieve this, I disrupted lysosomal Ca^{2+} signalling with glycyl-L-phenylalanine 2-naphthylamide (GPN). GPN causes hydrolytic perforation of the lysosomes, without compromising short term cell health (Padamsey et al., 2017) and therefore prevents Ca^{2+} storage and signalling (Berg et al., 1994, Jadot et al., 1984). Acute application of GPN (200 µM) significantly attenuated mGluR-mediated depolarisation by (+3.60 ± 1.00 mV vs. +0.22 ± 0.25 mV, n = 7, 5, $P < 0.001$) (**Figure 4.2**). Significant differences were not observed between LY341495, intracellular NAADP, Ned-19 incubation

or GPN application. From these data, I conclude that mGluR-mediated membrane depolarisation is firstly dependent on NAADP signalling, and secondly on lysosomal Ca^{2+} signalling.

A small residual depolarisation was still observed when mGluRs or mGluR signalling was blocked (**Figures 4.1 and 4.2**). I propose that this may be a result of non-glutamate dependent depolarisation caused by another neurotransmitter, such as ATP.

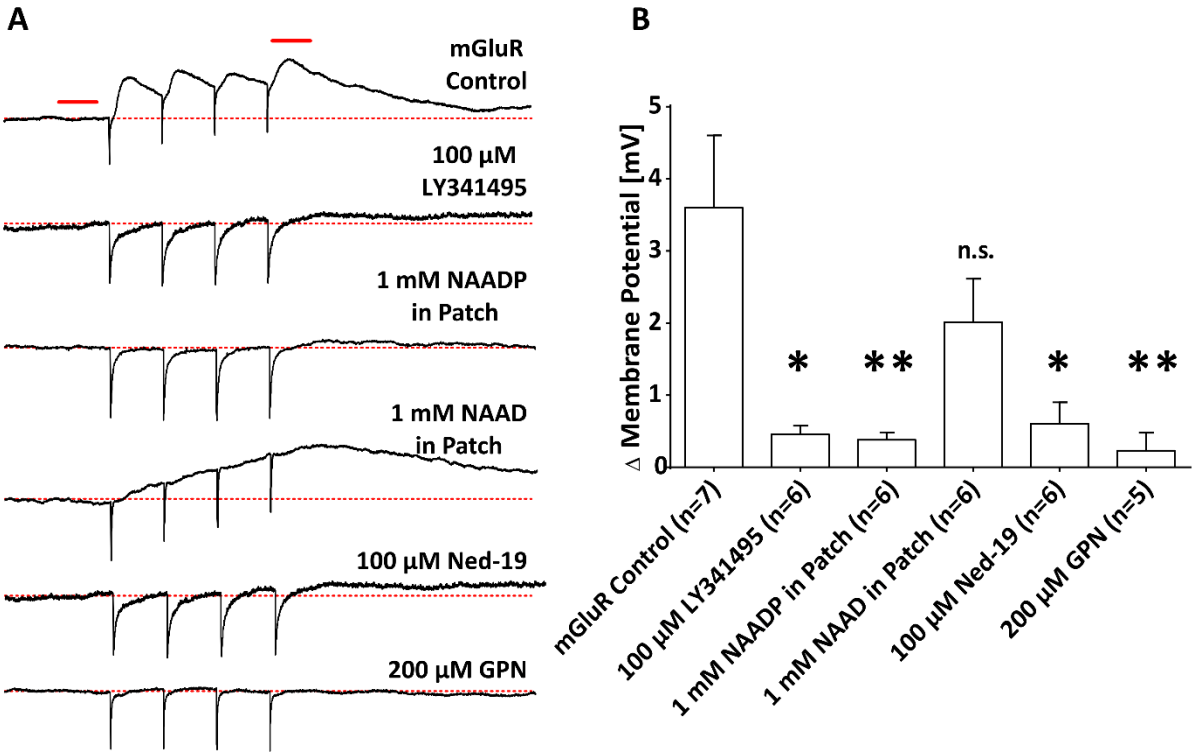

Figure 4.2 Synaptic activation of mGluRs causes an NAADP sensitive membrane depolarisation. A. Representative voltage recordings with: pharmacological isolation of mGluRs with AP5 (50 µM), NBQX (10 µM), picrotoxin (100 µM) and CGP 55845 (2 µM), plus either LY341485 (100 µM), desensitising concentration of NAADP inside the patch pipette (1 mM), NAAD inside the patch pipette (1 mM), preincubation with Ned-19 (60 min., 100 µM) or acute administration of GPN (200 µM). The red lines indicate where membrane potentials were compared before and after stimulation. **B.** Columns show mean ΔV_m of CA1 pyramidal neurons before and after electrical stimulation in each condition. Error bars display SEM. Significant differences between membrane potentials are displayed above each column, where ** = $P < 0.01$ and * = $P < 0.05$, n.s. = non-significant.

4.3.2 mGluR1-mediated depolarisation is a postsynaptic effect

My results from the previous set of experiments suggest that synaptic activation of mGluR1 can cause a membrane depolarisation. In these experiments, I used electrical stimulation to evoke synaptic glutamate release in combination with a series of pharmacological tools to isolate and manipulate the function various cellular targets. Although electrical stimulation is a more physiological method to activate mGluR1s there are possible confounds when used in combination pharmacological manipulations. Off target effects of the drugs used could interfere with presynaptic control mechanisms for glutamate release and occlude any true postsynaptic effects. Therefore, I wanted to ensure the manipulations used to abolish mGluR1/NAADP-mediated depolarisation were acting at the hypothesised postsynaptic site. I also wanted to provide another line of evidence to support my findings for synaptically evoked mGluR1/NAADP mediated depolarisation (**Figure 4.1 and Figure 4.2**).

In order to do this, I pharmacologically isolated mGluR1 (50 µM AP5, 10 µM NBQX, 100 µM picrotoxin, and 2 µM CGP 55845, 100 nM LY341495 and 10 µM MPEP) and transiently (120 s) bath applied extracellular L-glutamate (glutamate), to directly observe post synaptic responses, whilst I recorded membrane potential (current clamp) from CA1 pyramidal neurons in hippocampal slice culture. Furthermore, neurotransmission was also blocked by the presence of TTX (1 µM) to ensure I did not observe any effects of increased presynaptic neurotransmission. I used whole cell configuration with high resistance pipettes (16-20 MΩ) (to prevent dialysis of necessary mGluR co-factors). The experimental configuration is shown in **Figure 4.3A**.

Bath application of L-glutamate (300 µM, 120 s) in combination with mGluR1 isolation also produced modest membrane depolarisation (1.89 ± 0.53 mV), which was also similar in magnitude to the mGluR1-mediated depolarisation observed via electrical stimulation (1.94 ± 0.22 mV, **Figure 4.3C**). To ensure this depolarisation was indeed the result of mGluR1 activation I used either a broad spectrum mGluR antagonist (LY341495, 100 µM) or an mGluR1 specific antagonist (JNJ16259685, 300 nM) to block mGluR signalling. Both LY341495 (100 µM) and JNJ16259685 (300 nM) significantly reduced ΔV_m:

(1.94 ± 0.22 mV vs. +0.16 ± 0.25 mV, P < 0.05) and (1.94 ± 0.22 mV vs. +0.17 ± 0.17 mV, P < 0.05) respectively. Furthermore, the bath application of vehicle (no L-Glutamate) did not cause a significant membrane depolarisation compared to bath application of glutamate (1.94 ± 0.22 mV vs. +0.04 ± 0.23 mV, P < 0.05). These results are shown in **Figure 4.3C** and suggest that the depolarisation observed by application of extracellular glutamate is the result of mGluR1 activation.

Next, I wanted to determine if mGluR1-mediated depolarisation was dependent on NAADP signalling. I used pharmacological isolation of mGluR1 (with the glutamate receptor antagonist cocktail described above) in combination with pharmacological manipulations to disrupt NAADP signalling, the results are shown in **Figure 4.3D**. Firstly, I introduced a desensitising concentration of NAADP via the patch pipette (1 mM) (Aarhus et al., 1996, Genazzani et al., 1996), this prevented mGluR1 depolarisation via application extracellular glutamate (+2.25 ± 0.60 mV vs. -0.10 ± 0.15 mV, P < 0.01). Whereas introduction of NAAD via the patch pipette (1 mM) caused no significant reduction of mGluR1-mediated depolarisation (+2.25 ± 0.60 mV vs. +1.65 ± 0.37 mV, P > 0.99). Pre-incubation (100 µM, 40 minutes) with the NAADP receptor antagonist Ned-19 also prevented mGluR1 depolarisation via application extracellular glutamate (+2.25 ± 0.60 mV vs. +0.25 ± 0.09 mV, P < 0.01). These data suggest that extracellular application of glutamate can activate mGluR1 and cause a membrane depolarisation which is dependent on NAADP signalling.

As mGluR1 is canonically linked to $G_{\alpha q}$ receptor subunits, and IP_3 signalling (Niswender and Conn, 2010); I wanted to determine if an inhibitor of phospholipase C would also block mGluR1-mediated depolarisation. U71322 is a broad spectrum thiol-modifying agent, which is suggested to inhibit PLC, due to the presence of its maleimide group (Bleasdale et al., 1990). Maleimide groups react with sulfhydryl groups and crosslink cysteine groups when introduced to the cytoplasm. Pre-incubation with U71322 (5 µM, 30 minutes) prevented mGluR1-mediated depolarisation (+2.25 ± 0.60 mV vs. +0.16 ± 0.26 mV, P < 0.05).

U73433 is often used as a negative control for U71322, it is structurally the same except it lacks the reactive maleimide group. Pre-incubation with U73433 (5 μM, 30 minutes) had no significant effect on mGluR1-mediated depolarisation (+2.25 ± 0.60 mV vs. +1.75 ± 0.43 mV, $P > 0.99$).

I next attempted to infer how NAADP was produced in CA1 pyramidal neurons. Mechanisms for endogenous NAADP synthesis are poorly understood, however, ADP-ribosyl cyclases (ARCs) are favoured as a candidate enzyme family for NAADP endosynthesis. ARCs are multifunctional enzymes which are able to catalyse a base exchange reaction which produces NAADP and nicotinamide from β-NADP and nicotinic acid (Hirata et al., 1994, Aarhus et al., 1995, Itoh et al., 1998). I introduced a high concentration of nicotinamide (10 mM, included in pipette solution) into the cytosol of the patched cell with the aim to cause end-product inhibition of ARCs (Sauve and Schramm, 2002). I found that intracellular nicotinamide (10 mM) prevented mGluR1 depolarisation from bath application of glutamate (+2.25 ± 0.60 mV vs. -0.04 ± 0.27 mV, $P < 0.001$).

I next sought to determine if intracellular Ca^{2+} signalling components were important in producing mGluR1-mediated cellular depolarisation. I used a series of pharmacological manipulations which targeted the intracellular Ca^{2+} and Ca^{2+} stores, with the aim to determine which Ca^{2+} stores are important for producing mGluR1-mediated cellular depolarisation and the mechanism by which it occurs. These results are shown in **Figure 4.3E**.

Initially, I showed that mGluR1 isolation can produce a membrane depolarisation in these set of experiments where bath application of glutamate produces a mean ΔV_M of +1.94 ± 0.46 mV. I then compared the action of two Ca^{2+} chelators: ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). Both EGTA and BAPTA act as Ca^{2+} chelators, and when introduced into the cytosol, they prevent intracellular Ca^{2+} signalling events.

EGTA has an 'on' rate for binding Ca^{2+} of ≈3-10 $\mu m^{-1} s^{-1}$, whereas BAPTA's 'on' rate for binding Ca^{2+} is several orders of magnitude faster at ≈100–1000 $\mu m^{-1} s^{-1}$ (Naraghi and Neher, 1997, Tsien, 1980).

Consequently, ETGA cannot chelate Ca^{2+} at a rate which is fast enough to prevent nano/microdomain Ca^{2+} signalling events between molecules (Dargan and Parker, 2003), whereas BAPTA can prevent all but elementary Ca^{2+} signalling events (Horne and Meyer, 1997). Comparison between the effects of EGTA and BATPA can be used to make inferences on the spatiotemporal requirements of Ca^{2+} dependent processes. In my experimental system, pre-incubation with a membrane permeable form of EGTA (EGTA-AM, 20 µM, 20 min) did not affect mGluR1-mediated cellular depolarisation (+1.94 ± 0.46 mV vs. +1.71 ± 0.42mV, $P > 0.99$), whereas bath application of a membrane permeable form of BAPTA (BAPTA-AM, 20 µM, 20 min) prevented mGluR1-mediated depolarisation (+1.94 ± 0.46 mV vs. +0.08 ± 0.17mV, $P < 0.05$). These data suggest that mGlurR1-mediated depolarisation is dependent on highly localised or nano/microdomain Ca^{2+} signalling events.

The evidence presented in the previous results chapter (**3.0 Introduction of the Ca^{2+} mobilising messenger NAADP causes membrane depolarisation in hippocampal neurons)**, from the study by Brailoiu et al. (2009b) suggest Ca^{2+} release from both the acidic stores, via NAADP signalling and the ER, via RyRs are essential for producing mGluR1-mediated depolarisation. mGluR1s are suggested to couple to $G_{\alpha q}$ receptor subunits and produce IP_3 and ER Ca^{2+} release via the activation of PLC (Niswender and Conn, 2010). I wanted to ensure that Ca^{2+} release from the IP_3 receptors was also not required for mGluR1-mediated depolarisation. Acute application of Xestospongin C (2 µM, 15 minutes) had no significant effect on mGluR1-mediated depolarisation via stimulation with extracellular glutamate (+1.94 ± 0.46 mV vs. +3.02 ± 1.22 mV, $P > 0.99$), whereas acute application of ryanodine (40 µM, 15 minutes) prevented mGluR1-mediated depolarisation (+1.94 ± 0.46 mV vs. +0.20 ± 0.18 mV, $P > 0.99$).

To ensure Xestospongin C prevented IP_3 mediated Ca^{2+} release in pyramidal neurons of hippocampus a positive control was undertaken. This control consisted of incubating a hippocampal slice with Xestospongin C (2 µM, 15 min.) and subsequently filling a CA1 pyramidal neuron with the Ca^{2+} indicator OGB-1 for one minute. Cells were allowed to recover and the dye to dialyse through the cell for a

further five minutes. Finally, cells were re-patched the cell with 10 µM IP_3 included in the internal solution (a concentration of IP_3 known to cause a Ca^{2+} release in this experimental preparation, see **3.3.3**). When cells were exposed to Xestospongin C (2 µM, 15 min.) 10 µM IP_3 did not cause a Ca^{2+} release in the soma or dendrites of CA1 pyramidal neurons (data not shown, n=2). Indicating that Xestospongin C blocked IP_3Rs in my experimental system.

Finally, I wanted to determine if Ca^{2+} from the acidic stores is essential for mGluR1-mediated depolarisation. I blocked lysosomal Ca^{2+} signalling by acutely applying GPN, this abolished mGluR1-mediated depolarisation (+1.94 ± 0.46 mV vs. +0.05 ± 0.15 mV, $P < 0.05$).

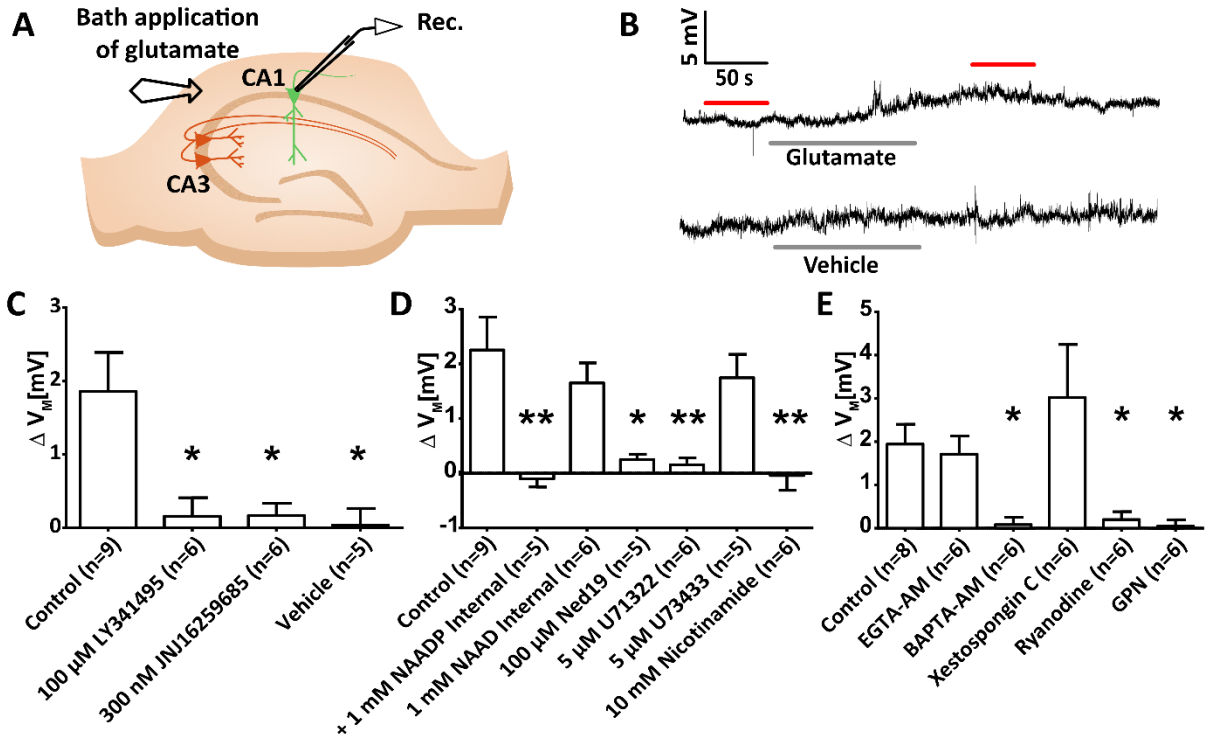

Figure 4.3 mGluR1 activation with extracellular glutamate causes NAADP-sensitive membrane depolarisation in CA1 pyramidal neurons. **A.** Diagram of experimental configuration. Membrane potential was recorded from pyramidal neurons in the CA1 region of hippocampal slice cultures whilst glutamate was transiently perfused in the bath solution (300 µM, 120 s). **B.** Representative voltage recordings during glutamate perfusion and vehicle perfusion. Glutamate perfusion indicated by grey bars. The red lines indicate where membrane potentials were compared before and after glutamate perfusion. **C.** and **D.** columns show mean ΔV_M of CA1 pyramidal neurons before and after glutamate perfusion for each pharmacological manipulation undertaken. Error bars display SEM. Significant differences between membrane potentials are displayed above each column, where ** = $P < 0.01$ and * = $P < 0.05$.

In addition to perfusing hippocampal slices with glutamate, another set of experiments was undertaken (data are not shown) where a Group 1 mGluR agonist ((RS)-3,5-Dihydroxyphenylglycine, DHPG) was transiently (120 s) bath applied whilst I recorded membrane potential (current clamp) from CA1 pyramidal neurons in hippocampal slice culture. During these experiments, mGluR5 was pharmacologically antagonised with MPEP (50 μM) and neurotransmission was prevented with TTX (1 μM). I used whole cell configuration with high resistance pipettes (16-20 MΩ) (to prevent dialysis of necessary mGluR co-factors).

I noted that DHPG could cause a membrane depolarisation, however, it was unaffected by pharmacological tools that interfered with NAADP/acidic store Ca^{2+} signalling including desensitising concentrations of NAADP, Ned-19, and GPN. DHPG is noted to be a biased ligand, where its binding to mGluR1/5 is reported to only cause phosphoinositide hydrolysis (Hathaway et al., 2015). Therefore, I suggest that it is not an effective tool to explore downstream signalling events of mGluR1 involving NAADP signalling. I suggest the methods by which I chose to explore mGluR1 function (synaptic activation and bath applied glutamate in combination with the pharmacological isolation of mGluR1) represent a more appropriate and physiological approach. Hence why I have not attributed much weight to my findings from using DHPG.

4.3.3 mGluR1-mediated Ca^{2+} signalling is dependent on acidic store Ca^{2+}/NAADP signalling and RyRs but not IP_3Rs

The results I present in **Figures 4.1** to **Figure 4.3** provide strong evidence that activation of mGluR1 by glutamate, causes an NAADP-mediated depolarisation which requires acidic store, ER and intracellular Ca^{2+} signalling. I next wanted to visualise intracellular Ca^{2+} signalling events initiated by mGluR1 and determine which intracellular compartments are important for producing mGluR1-mediated Ca^{2+} signals

I began by using confocal microscopy to visualise Ca^{2+} signals in the dendrites, and dendritic spines, of CA1 pyramidal neurons from hippocampal slice cultures. I pharmacologically isolated the mGluR1

receptors (50 µM AP5, 10 µM NBQX, 100 µM picrotoxin, 2 µM CGP 55845, 100 nM LY341495 and 10 µM MPEP). Single CA1 pyramidal neurons were loaded with the Ca^{2+} sensitive dye: Oregon Green 488 BAPTA-1, and 10 mM QX134. I then applied the pattern of electrical stimulation described earlier (4x pulses, 20 Hz) whilst recording Ca^{2+} signals at ~57 frames per second for a total of 700 ms. I repeated these experiments under several pharmacological conditions.

Firstly, I found that electrical stimulation generated dendritic Ca^{2+} signals in the dye-loaded CA1 pyramidal neuron of hippocampal slice cultures. **Figure 4.4Aa** shows a structural image of the dendrite being stimulated, and **Figure 4.4Ad** shows the subtraction of ΔF/F at the baseline from ΔF/F at 300 ms. **Figure 4.4Bi** shows the mean ΔF/F over the imaging window. **Figure 4.4C** shows that the mean ΔF/F at 300 ms was +1.38 ± 0.20 (n=20).

I next ensured that the Ca^{2+} signals I recorded were a result mGluR1 activation, I recorded Ca^{2+} responses with mGluR1 isolation then added 100 µM LY341495 to block mGluR1 responses and repeated the stimulation protocol. This manipulation prevented any significant Ca^{2+} signal being detected (+1.38 ± 0.20 vs 0.18 ± 0.06 mean ΔF/F at 300 ms, n=20, 5, $P < 0.05$) **Figure 4.4 Bii** and **C**.

Pre-incubation (100 µM, 40 min) with the NAADP antagonist Ned-19 also prevented Ca^{2+} events from synaptic activation of mGluR1 **Figure 4.4 Biii** and **C** (+1.38 ± 0.20 vs 0.003 ± 0.06 mean ΔF/F at 300 ms, n=20, 6, $P < 0.001$) suggesting that mGluR1-mediated Ca^{2+} signals depend on NAADP signalling. I also found that acute application of 20 µM ryanodine abolished mGluR1-dependent Ca^{2+} signals **Figure 4.4 Biv** and **C** (+1.38 ± 0.20 vs 0.003 ± 0.06 mean ΔF/F at 300 ms, n=20, 5, $P < 0.05$). Whereas acute application of IP_3 receptor antagonists Xestospongin C (2 µM, 15 min) or 2-APB (50 µM, 15 min) did not significantly reduce mGluR1 mediated Ca^{2+} signalling. Xestospongin C: **Figure 4.4 Bv** and **C** (+1.38 ± 0.20 vs 1.33 ± 0.41 mean ΔF/F at 300 ms, n=20, 5, $P > 0.99$) and 2-APB: **Figure 4.4, Bvi** and **C** (+1.38 ± 0.20 vs 1.64 ± 0.42 mean ΔF/F at 300 ms, n=20, 5, $P > 0.99$). These data suggest that mGluR1-mediated Ca^{2+} signals are dependent on Ca^{2+} release from the acidic Ca^{2+} stores, as they are abolished by Ned-19, and Ca^{2+} release from the ER via the RyR but not the IP_3R.

I had previously found that Xestospongin C prevented IP_3 mediated Ca^{2+} release in pyramidal neurons of the hippocampus through a positive control (described in 4.3.2). I did not undertake a similar positive control for 2-APB, however, it was used at a concentration (50 µM), which is in the range described to inhibit IP_3 mediated Ca^{2+} release in hippocampal neurons (Taufiq et al., 2005, Hu et al., 2015).

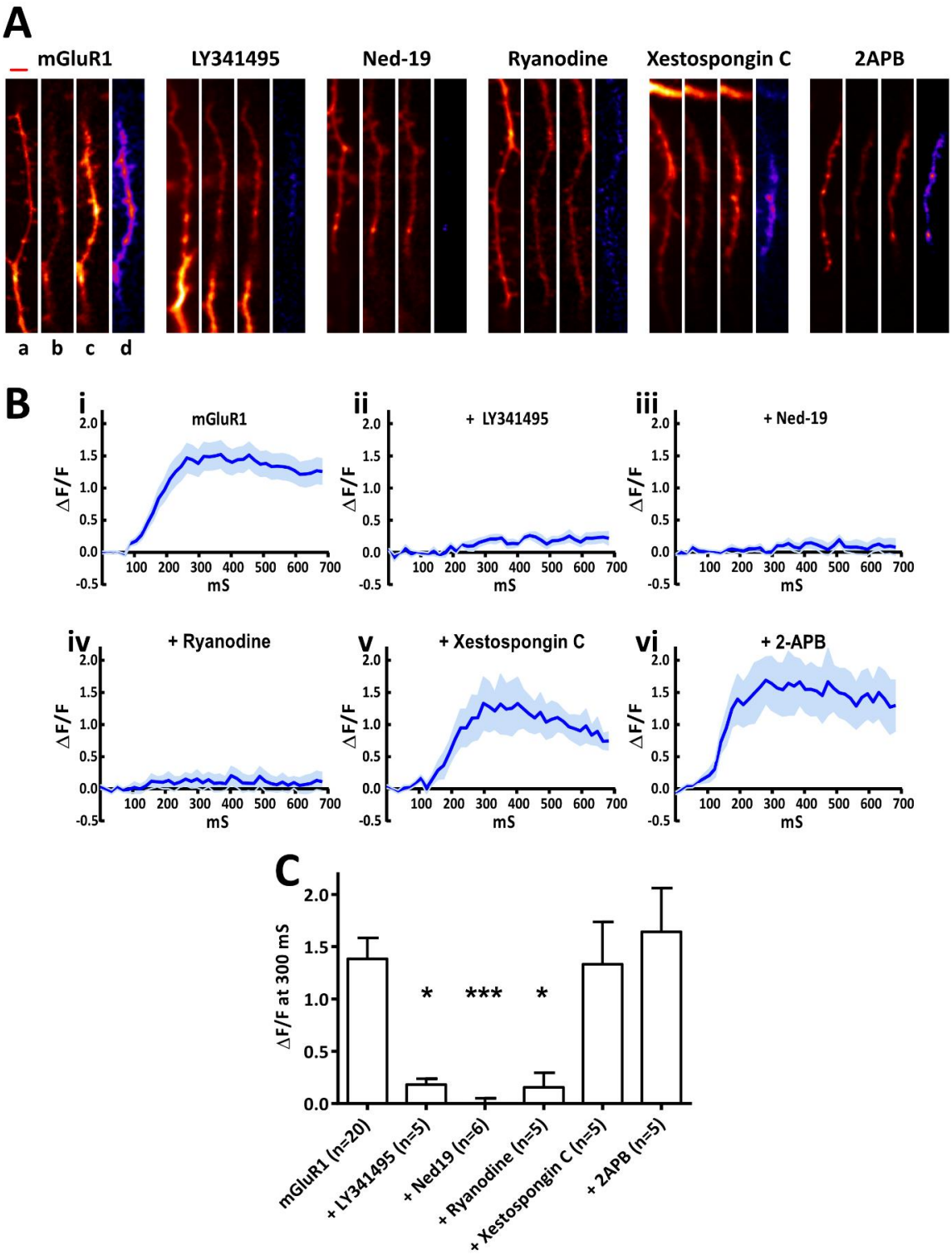

Figure 4.4 mGluR1 dependent Ca^{2+} signalling requires NAADP, acidic store Ca^{2+} release, and microdomain functional coupling with the ryanodine receptor. A. Representative images captured where CA1 pyramidal neurons in hippocampal slice cultures were filled with the Ca^{2+} dye OGB-1 whilst mGluR1 was pharmacologically isolated and electrical stimulation was applied (4 pulses, 20 Hz) **a.** z-

projection shows the structure of the imaged dendritic branch **b.** shows Ca^{2+} signal before electrical stimulation **c.** shows the Ca^{2+} signal at 300 ms **d.** shows subtraction of Ca^{2+} signal before stimulation from Ca^{2+} signal recorded at 300 ms. Red scale bar represents 0.5 µm **B.** ΔF/F over the imaging time course where mGluR1 was pharmacologically isolated in combination with either: acute application of LY341495 (100 µM, 10 min.), preincubation with Ned-19 (100 µM, 1 hr), or acute application of Ryanodine (20 µM, 10 min), Xestospongin C (2 µM, 15 min), or 2-APB (50 µM, 15 min) C. columns show mean ΔF/F before and after electrical stimulation for each pharmacological manipulation undertaken. Error bars show SEM and significant differences between membrane potentials are displayed above each column, where *** = $P < 0.001$ and * = $P < 0.05$.

4.4 Discussion

Through this chapter, I present evidence that the activation of mGluR1s, with synaptic or extracellular glutamate, causes cellular depolarisation and Ca^{2+} signalling events that are dependent on acidic Ca^{2+} stores/NAADP signalling and Ca^{2+} release from the ER via the RyRs but not the IP_3Rs.

The first novel finding I present in this chapter is that activation of mGluR1 causes NAADP-mediated signalling. The central dogma surrounding mGluR1 activation and G-protein coupling is that they couple to $G_{\alpha q}$ receptor subunits and produce IP_3 and ER Ca^{2+} release via the activation of PLC. However, the literature suggests mGluR1 possesses multiple and varied intracellular protein coupling and therefore many possible downstream signalling outcomes upon mGluR1 activation.

There are indeed numerous studies which suggest mGluR1 is intracellularly coupled to $G_{\alpha q}$ subunits (Aramori and Nakanishi, 1992, Hartmann et al., 2004, Houamed et al., 1991, Masu et al., 1991, Thomsen et al., 1993). However, in a variety of systems mGluR1 is shown to enhance cAMP production, a trait normally expected from $G_{\alpha s}$ coupled receptors (Aramori and Nakanishi, 1992, Joly et al., 1995, Pickering et al., 1993, Thomsen, 1996), mGluR1 is also shown to possess residues critical for coupling to adenylate cyclase (Francesconi and Duvoisin, 1998), finally mGluR1 has also been reported to couple to $G_{i/o}$ receptor subunits (Offermanns, 2003). There are three possible mGluR1

receptor subtypes (α,β,γ), produced by alternative splicing, which seem to preferentially couple to different G-protein subunits, depending on isoform and experimental preparation (Hiltscher et al., 1998, Pickering et al., 1993, Thomsen, 1996). Finally, mGluR1 is also suggested to functionally couple with other intracellular signalling molecules, including arachidonic acid, via protein kinase C (PKC) (Aramori and Nakanishi, 1992, Thomsen, 1996), phospholipase D via tyrosine kinases and extracellular Ca^{2+} influx (Kanumilli et al., 2002). It is clear that mGluR1 possess many possible signalling functions, which seems to be dependent on, isoform, cell type and localisation. In CA1 neurons of the hippocampus, only the mGluR1a isoform seems present (Ferraguti and Shigemoto, 2006) and is sub-localised to perisynaptic regions of the dendritic spines (Lujan et al., 1996, Martin et al., 1992). Therefore, I suggest it is entirely feasible that mGluR1 activation could result in NAADP synthesis via interaction of members of its many intracellular signalling pathways.

Several metabotropic receptors have been shown to cause NAADP synthesis upon activation (Soares et al., 2007, Rah et al., 2010, Yamasaki et al., 2005, Lewis et al., 2012), though the transduction pathway between metabotropic receptors, G-protein coupling, and NAADP synthesis was not elucidated during these studies. I used U71322 (**Figure 4.3D**) with the aim to determine whether PLC is essential for the signalling pathway I describe. I found U71322 could abolish mGluR1/NAADP-mediated depolarisation. However, on reflection, I believe U71322 was a poor choice of drug to inhibit PLC. U71322 contains a reactive maleimide group, that causes sulfhydryl cross-linkage of cysteine residues (Bleasdale et al., 1990) and therefore prevents the conformational changes required to produce PLCs enzymatic activity. However, no chemical groups which denote specificity to PLC as opposed to other enzymes are present on U71322, and consequently numerous off target effects have been described for the use of U71322, including but not limited to: block of pertussis toxin-sensitive G proteins ($G_{\alpha s}$), weak activation of PLC or inhibition of lipid kinases in CHO cells (Horowitz et al., 2005) and block of cGMP-induced calcium release in sea urchin eggs (Lee et al., 1998). Finally, U71322 spontaneously forms conjugates with components of cell culture medium including BSA and glutamine

(Wilsher et al., 2007). Therefore, I cannot state with confidence that mGluR1 couples specifically to $G_{\alpha q}$ receptor subunits in my experimental preparation to cause NAADP or IP_3 synthesis.

I also attempted to infer which intracellular enzyme was responsible for synthesising NAADP. ARCs are favoured as a candidate enzyme family for NAADP endo-synthesis and catalyse a base exchange reaction which produces NAADP and nicotinamide from β-NADP and nicotinic acid (Hirata et al., 1994, Aarhus et al., 1995, Itoh et al., 1998). The addition of 10 mM nicotinamide (**Figure 4.3D**) prevented mGluR1 responses to extracellular glutamate. I suggest that nicotinamide, at this concentration, caused end-product inhibition of ARCs. Indicating their possible involvement for NAADP endo-synthesis, though more rigorous exploration of their involvement is required to provide convincing evidence that these enzymes synthesis NAADP in CA1 pyramidal neurons of the hippocampus. From the evidence I present in this chapter, it is not clear which intracellular proteins coupled to mGluR1, are responsible for NAADP synthesis.

The second novel finding I present in this chapter is that, under my experimental conditions, mGluR1 activation causes membrane depolarisation in CA1 pyramidal neurons which require Ca^{2+} release from both the acidic stores and the ER via the RyRs but not the IP_3Rs.

Activation of mGluR1 is ubiquitously shown to cause intracellular Ca^{2+} signalling in neurons, however, the mechanisms and source of Ca^{2+} responsible for creating these Ca^{2+} signals are varied. In the hippocampus, mGluR1 activation in CA1 neurons is shown to produce ER dependent Ca^{2+} release (Rae and Irving, 2004), mGluR1 activation in interneurons of the hippocampus is reported to cause extracellular Ca^{2+} entry through activation of TRP channels (Topolnik et al., 2006). In cerebellar granule cells mGluR1 activation is suggested to mobilise Ca^{2+} from the ER via both RyRs and IP_3Rs (del Rio et al., 1999, Fagni et al., 2000), and also extracellular Ca^{2+} entry through L-Type VDCCs through as a result of functional coupling to RyRs, independent of IP_3R activity (Chavis et al., 1995, Chavis et al., 1996). In CA1 pyramidal neurons, mGluR1s are peri-synaptically localised in dendritic spines (Lujan et al., 1996). Padamsey et al. (2017) confirms the presence of acidic stores in dendritic spines of CA1 pyramidal

neurons, and shows acidic stores are important for the induction and maintenance of long term plasticity (LTP). Due to their proximity in the dendrites, it is plausible that mGluR1 may recruit acidic store Ca^{2+} to produce intracellular Ca^{2+} signalling events.

I suggest that, in my experimental system, activation of mGluR1 causes Ca^{2+} mobilisation from the acidic stores initially which is further amplified via the RyRs. I base this suggestion on the following reasoning. Firstly, the results I obtained from the previous results chapter (**3.0 Figures 3.2** and **3.3**), show that intracellular dialysis of NAADP, but not cADPR, causes membrane depolarisation. I suggest that for this depolarisation to occur NAADP must first be present to produce the correct spatiotemporal Ca^{2+} signal for activation of RyRs. Secondly, acidic store Ca^{2+} signals have been shown to be amplified via the RyR (or IP_3R), in a variety of systems including: SKBR3 cells (Brailoiu et al., 2009a), pulmonary arterial smooth muscle via suggested 'trigger zones' produced by co-localisation of TPCs and RyRs (Kinnear et al., 2008), pancreatic acinar cells (Cancela et al., 1999), primary cultured human fibroblasts, where electron microscopy also shows lysosome-ER membrane contact points (Kilpatrick et al., 2013). These studies provide experimental evidence to justify the 'trigger' hypothesis, suggested by Churchill and Galione (2001), where NAADP causes Ca^{2+} mobilisation from the acidic stores, which serves as a 'trigger' for ER Ca^{2+} release via the RyRs or IP_3Rs. As reviewed by (Galione, 2015, Kilpatrick et al., 2013). Finally, I previously suggested that NAADP-mediated acidic store Ca^{2+} release may be amplified via the RyR as a result of functional microdomains (**3.4**). Evidence for the existence of these microdomains has been provided from theoretical and experimental data (Aston et al., 2017, Morgan et al., 2013, Penny et al., 2015, Penny et al., 2014, Kinnear et al., 2004, Kinnear et al., 2008, Kilpatrick et al., 2013). Furthermore, I present evidence here (**Figure 4.3**) that microdomain Ca^{2+} signalling is required to produce mGluR1-mediated cellular depolarisation, as the 'slow' Ca^{2+} chelator EGTA cannot prevent mGluR1 depolarisation, whereas the 'fast' Ca^{2+} chelator BAPTA can.

Interestingly, in my experimental preparation, IP_3 receptor antagonists did not affect either mGluR1-mediated Ca^{2+} release or membrane depolarisation (**Figure 4.4**). The central dogma suggests mGluR1

couples to $G_{\alpha q}$ receptor subunits to activate PLC and cause Ca^{2+} release via the IP_3 receptors, however, I have already discussed that this is not always the state of affairs and that mGluR1 is quite promiscuous in its intracellular coupling mechanisms. I have also discussed the presence of functional microdomains between the acidic store Ca^{2+} release channels and RyRs specifically, furthermore, I present evidence for the requirements of microdomain Ca^{2+} signalling events in order to produce mGluR1-mediated depolarisation (**Figure 4.3E**). Finally, Fotuhi et al. (1993) shows that there is widespread differential localisation of mGluR1s and IP_3Rs in the brain (including the hippocampus) and with the use of electron microscopy Sharp et al. (1993) shows in CA1 pyramidal neurons the RyRs are predominantly present in axons, dendritic spines, and dendritic shafts near dendritic spines while IP_3Rs are primarily localised in dendritic shafts and cell bodies. Suggesting distinct roles for RyR and IP_3R sensitive Ca^{2+} pools within the ER.

In lieu of these findings, I suggest that activation of mGluR1 by glutamate causes NAADP synthesis, Ca^{2+} release from the acidic stores, which is further amplified via the RyRs due to microdomain coupling. This amplified Ca^{2+} signal acts on a Ca^{2+} sensitive membrane channel to cause membrane depolarisation. This hypothesised signalling pathway is shown diagrammatically in **Figure 4.5**.

I must also note a potential confound during dendritic Ca^{2+} imaging (**Figure 4.4**). Cells were also loaded QX314 (10 mM, included in the patch pipette), QX314 is been shown to block voltage gated Na^{+} channels (VDNCs) from the intracellular domain of CA1 pyramidal neurons and prevents action potentials at this concentration (Perkins and Wong, 1995). VDCCs are activated at a membrane potential threshold which is typically greater than VDNCs, therefore it is unlikely they are able to contribute to any Ca^{2+} signals observed during these experiments. However, I suggest the channel responsible for mGluR1-mediated membrane depolarisation is likely to be distinct from the VDCCs, as the mGluR1/NAADP-mediated depolarisation is far below the threshold of VDCC activation. Though, in the absence of pharmacological antagonists, this membrane depolarisation may contribute to activation of VDCCs which in turn may affect downstream signalling events.

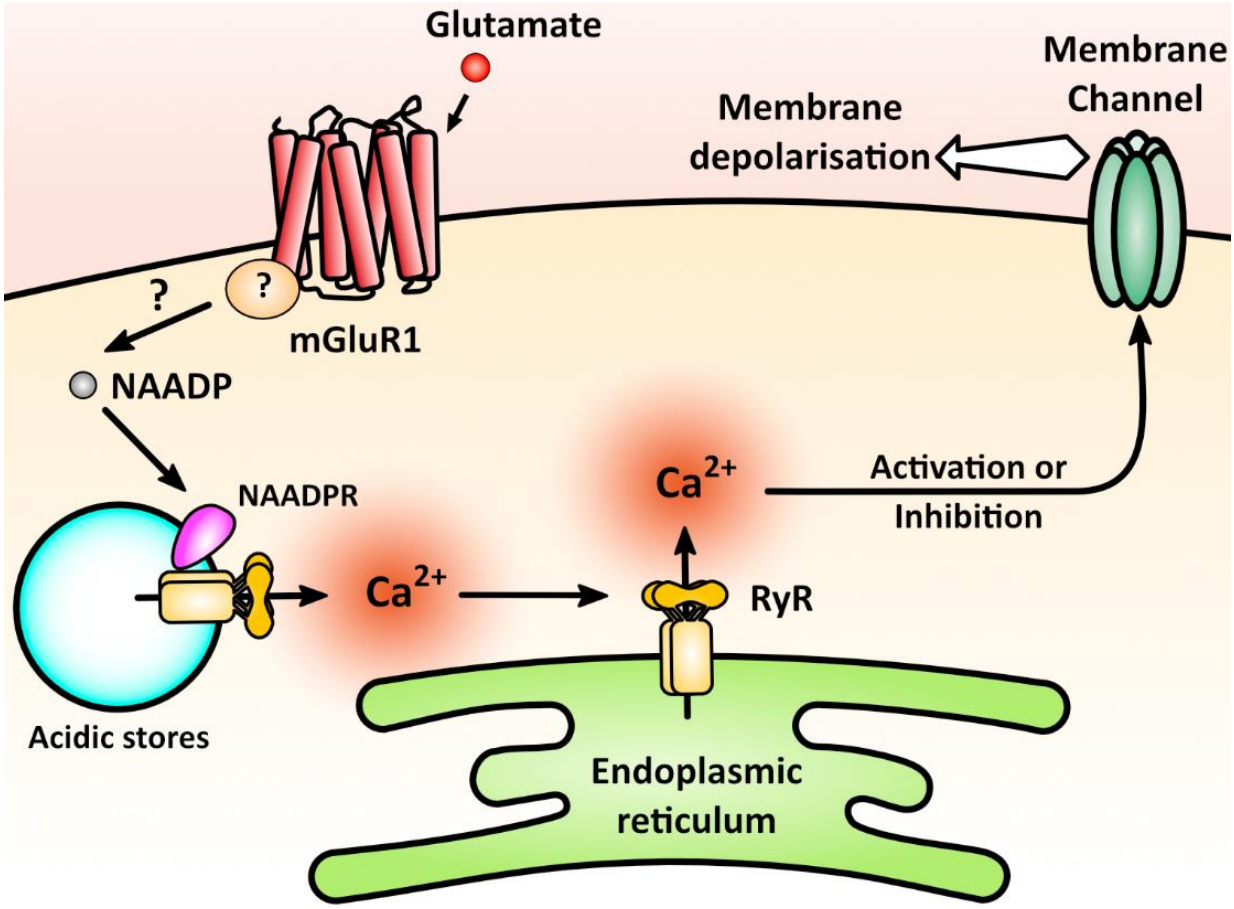

Figure 4.5 Hypothesised pathway for mGluR1-mediated Ca^{2+} release and membrane depolarisation in dendrites of hippocampal neurons. Activation of mGluR1 by glutamate causes NAADP synthesis via an unidentified intracellular enzyme. Intracellular NAADP activates the NAADP receptor (NAADPR) and causes Ca^{2+} release from the acidic stores. Ca^{2+} release from the acidic stores is amplified by Ryanodine receptor (RyR) via Ca^{2+} induced Ca^{2+} release (CICR) and possible microdomain coupling. This causes the activation or inhibition of a Ca^{2+} sensitive membrane channel to cause cellular depolarisation.

4.5 Conclusion

Here I address the aims of this chapter.

1. **To determine if glutamate can cause NAADP-mediated depolarisation.**
2. **Were glutamate to cause NAADP-mediated depolarisation, to identify which glutamate receptor produces NAADP-mediated depolarisation.**

I have shown that glutamate can cause NAADP-mediated membrane depolarisation (**Figures 4.1-4.3**). I show, through pharmacological manipulations, that NAADP-mediated membrane depolarisation is a result of mGluR1 activation specifically and can be achieved via synaptic release, or extracellular application, of glutamate.

3. **To identify components of the intracellular signalling pathway responsible for NAADP-mediated depolarisation.**

I have shown that activation of mGluR1 causes an NAADP-mediated membrane depolarisation, and also an intracellular Ca^{2+} signal. I show that acidic store Ca^{2+} release, amplification of this Ca^{2+} signal via suggested functional coupling to RyRs are required and necessary for mGluR1/NAADP-mediated membrane depolarisation in neurons. Furthermore, I suggest an ARC enzyme could be implicated to synthesis NAADP after mGluR1 activation, as end product inhibition with nicotinamide blocks this depolarisation. I summarise these findings in a proposed signalling pathway (**Figure 4.5**).

The results I have shown in this chapter reveal an entirely novel signalling pathway between mGluR1, NAADP and membrane depolarisation. However, we are left with two important points to address. Firstly, the identity of the membrane channel responsible for mGluR1-mediated depolarisation remains elusive, this must be identified in order to fully characterise this pathway. Secondly, the endogenous function of mGluR1-mediated Ca^{2+} release and depolarisation are also unaddressed. By identifying the membrane channel, I hope to gain insight as to the physiological function of the signalling pathway I describe.

5.0 mGluR1 inhibits SK channels, via acidic store Ca^{2+} signalling, to cause cellular depolarisation

5.1 Introduction

In the previous chapter, I describe a novel signalling pathway which links mGluR1 activation by glutamate to membrane depolarisation, via NAADP synthesis and intracellular Ca^{2+} mobilisation in CA1 pyramidal neurons of the hippocampus. Membrane depolarisation by mGluR1 has been described in a variety of neuronal cell types, reviewed by Anwyl (1999), though the identity of the responsible membrane channel seems to vary between experimental systems. In hippocampal pyramidal cells, Group 1 mGluR-mediated depolarisation (mGluR1/mGluR5) is broadly suggested to occur via either or a combination of inhibition of K^+ leak channels, reduction in Ca^{2+} activated K^+ channels or activation of non-selective ion channels. Here, I discuss evidence for each.

Guerineau et al. (1994) showed broad activation of mGluRs, in CA3 pyramidal neurons, with 1-amino-1,3-dicarboxycyclopentane (ACPD) caused a membrane current consistent to inhibition of K^+ leak channels, these results were replicated, in CA3 pyramidal neurons in hippocampal slices (Luthi et al., 1996) and also in dissociated cultured hippocampal neurons (Wu and Barish, 1999). Takeshita et al. (1996) showed that activation of Group 1 mGluRs (mGluR1/mGluR5) with (S)-3,5-dihydroxyphenylglycine (DHPG) caused both inward and outward currents in neurons from a variety of brain regions, including pyramidal CA3/CA1 neurons. This study also showed, in large cholinergic neurons of rat caudate putamen, mGluR1 specifically caused membrane depolarisation via inhibition of a K^+ leak channel. Chuang et al. (2000) also showed, in CA3 pyramidal neurons, that chemical activation mGluR1/5 also produces membrane depolarisation, and suggested that either a decrease in K^+ conductance and/or activation of an unidentified non-selection cation channel were responsible for this depolarisation.

Reduction in Ca^{2+} activated K^+ channel activity upon chemical activation of mGluRs by ACPD has been reported in CA3 pyramidal neurons (Charpak et al., 1990) and CA1 pyramidal neurons of the

hippocampus (Desai and Conn, 1991). Mannaioni and Marino (2001) suggested distinct physiological roles for Group 1 mGluRs in CA1 pyramidal neurons, where mGluR1 activation caused somatic Ca^{2+} transients and neuronal depolarization, via unidentified channels, whereas mGluR5 activation inhibited Ca^{2+} activated K^+ currents (slow after hyperpolarization potentials). Ireland and Abraham (2002) showed Group 1 mGluR activation in CA1 neurons, caused membrane excitability and inhibition of after hyperpolarisation potentials this study suggested these effects were achieved independently of PLC and IP_3In non-hippocampal preparations mGluR1 is also suggested to cause membrane depolarisation via inhibition, likely via K^+ channels. In the spinal cord mGluR1 is specifically suggested to inhibit K^+ leak channels to cause membrane depolarisation (Kettunen et al., 2003) and in thalamocortical neurons, mGluR1 is shown to cause depolarisation, and a simultaneous increase in input resistance, indicating the closing of a leak channel (Turner and Salt, 2000). Tigaret et al. (2016) showed synaptically evoked mGluR1-dependent plasticity in CA1 pyramidal neurons of the hippocampus is dependent on mGluR1-mediated inactivation of small-conductance K^+ (SK) channels, which is noted to produce enhanced membrane excitability and prolonged excitatory post synaptic potentials (Stocker et al., 1999). Though, Tigaret et al. (2016) does not specifically explore the relationship of mGluR1-mediated inhibition of SK channels with regard to intracellular Ca^{2+} signalling or depolarisation.

Activation of mGluR1 is also implicated in the activation of non-selective cation channels in pyramidal neurons of the hippocampus. Gee et al. (2003) report that chemical activation of either mGluR1 or mGluR5 produced a TRP channel like conductance in CA3 pyramidal neurons. This study shows the current was blocked by broad spectrum TRP channel antagonists including La^{3+}, 2-APB and also intracellular Ca^{2+} chelation. Congar et al. (1997) also report a non-selective cation current, upon activation of Group 1 mGluRs with ACPD in CA1 pyramidal neurons in the presence of K^+ channel inhibitors. Topolnik et al. (2006) also report that in interneurons of CA1 in hippocampus mGluR1 specifically is noted to activate a TRP channel dependent current.

However, much of evidence the suggesting mGluR1/mGluR5 couples to TRP channels comes from studies in other non-hippocampal areas of the brain. Including mGluR1 coupling to: TRPC channels in cerebellar Purkinje cells (Kim et al., 2003, Chang et al., 2012, Charpak et al., 1990, Kang et al., 2014, Shin et al., 2008), unspecified TRP channels in dopaminergic neurons from the midbrain (Bengtson et al., 2004, Tozzi et al., 2003, Cucchiaroni et al., 2010), and TRPV4 in astrocytes (Shibasaki et al., 2014). Finally, I would like to make reference to a study by Brailoiu et al. (2009b) where, in neurons of the medulla oblongata, NAADP-AM was found to activate a cation permeable membrane channel. They suggest the activation of a Ca^{2+} activated, Ca^{2+} impermeable TRP channel could be responsible for this NAADP-mediated depolarisation.

The studies discussed above show clearly that there is broad and diverse coupling between mGluR1 and depolarising membrane channels which seem to be dependent on cell type, experimental preparation, and method of mGluR1 activation.

5.2 Aims

1. To identify the membrane channel responsible for mGluR1-mediated depolarisation in CA1 neurons of the hippocampus.

5.3 Results

5.3.1 TRP Channel Pharmacology

I first explored whether mGluR1 activation caused a membrane depolarisation via a TRP channel. I initially decided to undertake this line of investigation, as Brailoiu et al. (2009b) provided convincing evidence of non-selective ion channel activation upon bath application of NAADP-AM in neurons of the medulla. The TRP channel family contains 28 members, split into six groups (TRPA, TRPC, TRPV, TRPM, TRPML, and TRPP), they are typically non-selective cation channels with diverse distribution and activation and are responsible for a plethora of physiological functions (Gees et al., 2010).

I pharmacologically isolated mGluR1 (1 µM TTX, 50 µM AP5, 10 µM NBQX, 100 µM picrotoxin and 2 µM CGP 55845, 100 nM LY341495 and 10 µM MPEP) and transiently (120 s) bath applied extracellular L-glutamate (glutamate) whilst I recorded membrane potential (current clamp) from CA1 pyramidal neurons in hippocampal slice culture (as described in **4.0**).

I show again that mGluR1 activation by extracellular application of L-Glutamate causes a modest membrane depolarisation (+2.48 ± 0.70 mV) (**Figure 5.1**). I used bath application of two broad spectrum TRP antagonists in an attempt to block mGluR1-mediated depolarisation. I firstly used La^{3+}, which is shown to antagonise most TRP channels at varying concentration (10-100 µM), as reviewed by (Bouron et al., 2015). The addition of 100 µM La^{3+} to the bath solution did not block mGluR1-mediated depolarisation in my experimental preparation (+2.48 ± 0.70 mV vs. +2.98 ± 0.39 mV, n= 7,6, $P > 0.99$) although it should be noted that La^{3+} is reported to activate TRPC1, TRPC4 and TRPC5 channels (Strubing et al., 2001, Schaefer et al., 2000). In order to address this potential confound, I used another broad spectrum TRP channel antagonist (Guinamard et al., 2013): flufenamic acid (FFA) which has also been shown to inhibit TRPC1, TRPC4 and TRPC5 channels (Lee et al., 2003). I found that the addition of 20 µM FFA did not block mGluR1-mediated depolarisation (+2.48 ± 0.70 mV vs. +1.56 ± 0.51 mV, n= 7,6, $P > 0.99$).

Brailoiu et al. (2009b), reports the absence of a Ca^{2+} conductance in the NAADP-AM mediated membrane current. Only two TRP channels are suggested to be Ca^{2+} activated but Ca^{2+} impermeable: TRPM4 and TRPM5 (Hofmann et al., 2003, Launay et al., 2002). I used inhibitors for both TRPM4 and TRPM5, 9-phenanthrol (Grand et al., 2008) and triphenylphosphine oxide (TPPO) (Palmer et al., 2010) respectively, in order to determine if either TRPM4/TRPM5 are responsible for mGluR1-mediated depolarisation. I found TPPO had no effect on mGluR1-mediated depolarisation (+2.48 ± 0.70 mV vs. +3.661 ± 0.58 mV, n= 7,6, $P = 0.95$), whereas 9-phenanthrol abolished mGluR1-mediated membrane depolarisation (+2.48 ± 0.70 mV vs. -0.02 ± 0.52 mV, n= 7,6, $P = 0.047$).

It is interesting that 9-phenanthrol abolished mGluR1 mediated depolarisation, as La^{3+} is well described to block both TRPM4 and TRPM5 channels at concentrations under 100 µM (Bouron et al., 2015, Morita et al., 2007), as is FFA at concentrations between 10-25 µM in neurons (Pace et al., 2007, Partridge and Valenzuela, 2000, Ullrich et al., 2005). Therefore, I would suggest the effect of 9-phenanthrol is not a result of TRPM4 inhibition, but rather, an off target effect. I must acknowledge positive controls were not undertaken to ensure the pharmacological manipulations were causing their desired effects. However, each drug was used at a concentration which has been previously reported to be effective in neurons, and at an appropriate concentration according to reported EC50s.

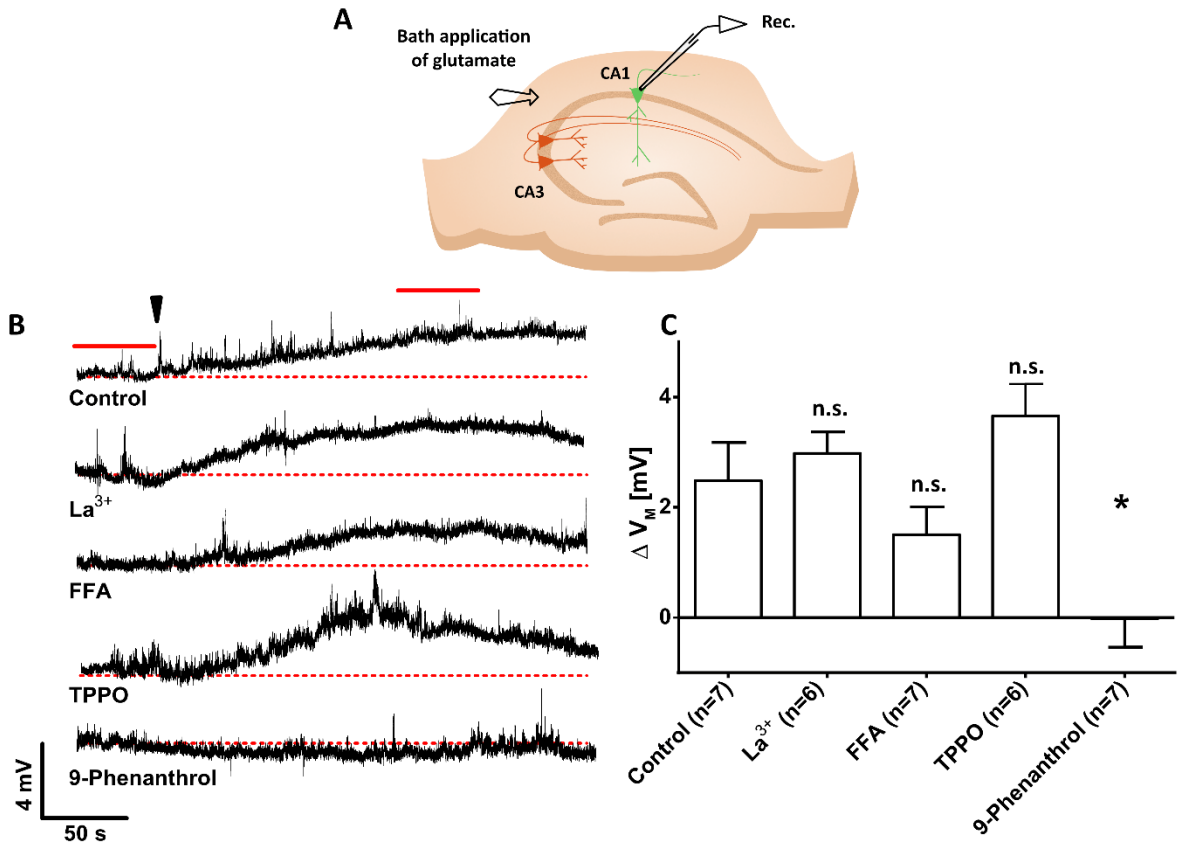

Figure 5.1 mGluR1-mediated depolarisation unlikely to occur via transient receptor potential (TRP) channel activation. A. Diagram showing the experimental configuration, whole cell current clamp recordings were performed on CA1 pyramidal neurons whilst mGluR1 was pharmacologically isolated and L-glutamate was bath applied. **B.** Representative voltage recordings during application of extracellular glutamate (180 s, black arrow indicates where glutamate enters bath) **C**. columns show mean Δ V_M of CA1 pyramidal neurons before and after glutamate perfusion (indicated by red bars in **B**) for each pharmacological manipulation undertaken. All drugs were added to the bath solution at the following concentrations: La^{3+} (100 µM), flufenamic acid (FFA) (20 µM), triphenylphosphine oxide (TPPO) (50 µM) and 9-Phenanthrol (100 µM). Error bars display SEM. Significant differences between membrane potentials are displayed above each column, where ** = $P < 0.01$ and * = $P < 0.05$, n.s. = non-significant.

5.3.2 Inhibition of SK channels is responsible for producing mGluR1-mediated depolarisation in pyramidal neurons

I next decided to explore the possibility that Ca^{2+} regulated K^+ channels (K_{Ca}) could be the channel responsible for mGluR1-mediated depolarisation. As discussed in the introduction, there is a large body of work to suggest that mGluR1 can inhibit various forms of K^+ channels and Tigaret et al. (2016) provides convincing evidence that mGluR1 inhibits SK channels to allow induction of mGluR1-mediated long term plasticity (LTP). Although, Tigaret et al. (2016) does not specifically explore this relationship with regard to depolarisation or intracellular Ca^{2+} signalling dynamics. Therefore, I next explored whether a K_{Ca} channel was responsible for mGluR1-mediated depolarisation.

There are 8 members of the K_{Ca} family, they are grouped based upon conductance: BK (big conductance), IK (intermediate conductance) and SK (small conductance).

As Tigaret et al. (2016) reported inhibition of SK channels by mGluR1, I began by targeting them in my experimental system. Four subtypes of SK channel have been identified in mammals (SK1-4) they are suggested to be voltage insensitive and are modulated by intracellular Ca^{2+} alone. Apamin (bee venom) is noted to selectively block SK channels via allosteric inhibition (Lamy et al., 2010). In neurons, SK2 and SK3, but not SK1 are suggested to be sensitive to apamin (Köhler et al., 1996). SK2 channels are present in the post synaptic density in CA1 pyramidal neurons of the hippocampus (Allen et al., 2011). SK channel activation is traditionally attributed to produce post action potential hyperpolarisation currents (I_{AHP}) in neurons, however, more recently SK channels have become implicated to play roles in dendritic excitability as well as synaptic transmission and plasticity (Faber, 2009, Faber and Sah, 2007, Tigaret et al., 2016).

I, therefore, sought to determine whether apamin could inhibit mGluR1-mediated depolarisation by interfering with SK channel modulation by intracellular Ca^{2+}. I began by pharmacologically isolating mGluR1 (50 µM AP5, 10 µM NBQX, 100 µM picrotoxin and 2 µM CGP 55845, 100 nM LY341495 and 10 µM MPEP) and used whole cell current clamp on CA1 pyramidal neurons in hippocampal slice

culture with high resistance pipettes (16-20 MΩ) in combination with delivering a pattern of electrical stimulation known to activate mGluRs in hippocampus (4 pulses at 20 Hz) (Fan et al., 2010) to stratum radiatum. A diagram of the experimental preparation is shown in **Figure 5.2F** This mGluR1 activation protocol remained consistent to the experiments undertaken in (**4.0**). I found that synaptic activation of mGluR1 produced a modest membrane depolarisation (**Figure 5.2A** and **B**) of (+1.56 ± 0.23 mV, n=6), I then bath applied apamin (200 nM) for 15 minutes. This significantly reduced the amplitude of depolarisation observed (+1.56 ± 0.23 mV vs +0.46 ± 0.20 mV, n=6, $P < 0.05$), subsequently applied GPN (200 µM, 10 minutes) to disrupt acidic Ca^{2+} store signalling, to determine if a further reduction in the depolarisation would be observed, with the aim to show I was targeting the same signalling pathway with both apamin and GPN. I found that GPN had no further effect on reducing the amount of mGluR1-mediated depolarisation after the application of apamin (+0.46 ± 0.20 mV vs +0.37 ± 0.22 mV, n=6, $P > 0.99$). These data suggest that inhibition of the SK channels, by acidic store Ca^{2+}, was responsible for producing mGluR1-mediated depolarisation.

I also repeated this experiment using the same preparation and parameters, except I used transient bath application of L-Glutamate rather than synaptic stimulation. I did this in order to ensure that the addition of apamin and/or GPN was not interfering with presynaptic glutamate release, which would occlude any effect on mGluR1-mediated depolarisation. I bath applied L-Glutamate (300 µM, 120s), whilst pharmacologically isolating mGluR1s (50 µM AP5, 10 µM NBQX, 100 µM picrotoxin and 2 µM CGP 55845, 100 nM LY341495 and 10 µM MPEP) and prevented neurotransmission with TTX (1 µM). These results are shown in **Figure 5.2C** and **D**. I again found modest depolarisation upon mGluR1 activation with extracellular glutamate (+3.42 ± 0.48 mV, n=6), which was significantly reduced by the application of apamin to the bath (200 nM, 15 minutes) (+3.42 ± 0.48 mV vs +0.69 ± 0.48 mV, n=6, $P < 0.05$), subsequent application of GPN I found that GPN had no further effect on reducing the amount of mGluR1 depolarisation after the application of apamin (+0.69 ± 0.48 mV vs +0.81 ± 0.39 mV, n=6, $P > 0.99$). These data concur with the previous experiment and also suggest that SK channels are responsible for producing mGluR1-mediated depolarisation.

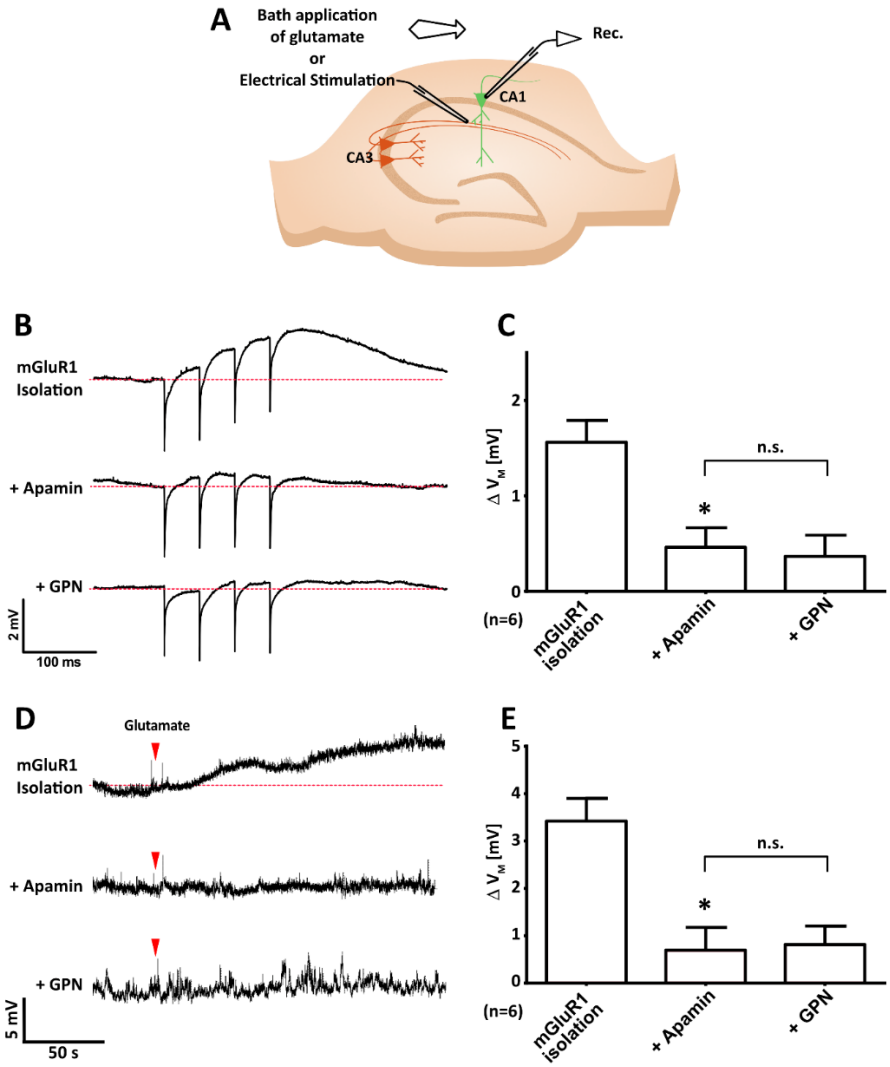

Figure 5.2 mGluR1 inhibits SK channels to cause membrane depolarisation. A. Diagram showing the experimental configuration, whole cell recordings were performed on CA1 pyramidal neurons. mGluR1 was pharmacologically isolated and either glutamate was bath applied or synaptic glutamate release was evoked electrically. **B.** Representative voltage recordings from the same cell (average of 5 consecutive recording sweeps) with: pharmacological mGluR1 isolation, then consecutive addition of 200 nM apamin, then 200 µM GPN. **C.** Mean Δ V_M of CA1 pyramidal neurons before and after electrical stimulation. **D.** Representative voltage recordings from the same cell with mGluR1 isolation, then consecutive addition of 200 nM apamin, then 200 µM GPN, the red arrow indicates where glutamate enters the bath. **E.** Mean Δ V_M of CA1 pyramidal neurons before and after transient application of glutamate (300 µM, 120 s). Error bars display SEM. Significant differences between membrane potentials are displayed above each column, where ** = $P < 0.01$ and * = $P < 0.05$, n.s. = non-significant.

5.4 Discussion

5.4.1 Activation of TRP channels are unlikely to produce mGluR1-mediated membrane depolarisation

The data I present in **Figure 5.1** shows that two broad spectrum TRP antagonists: La^{3+} and FFA do not abolish mGluR1-mediated cellular depolarisation. To address the suggestion that NAADP-AM activates a Ca^{2+} impermeable, non-selective cation channel in neurons (Brailoiu et al., 2009b) I also used inhibitors for Ca^{2+} impermeable Ca^{2+} activated TRP channels TRPM4 and TRPM5. TPPO, an inhibitor for TRPM5, had no effect on mGluR1-mediated depolarisation. Whereas 9-Phenanthtrol, an inhibitor for TRPM4, abolished mGluR1-mediated depolarisation. However, given that both La^{3+} and FFA are shown to inhibit TRPM4 at the concentrations used in this study (Pace et al., 2007, Partridge and Valenzuela, 2000, Ullrich et al., 2005, Bouron et al., 2015, Morita et al., 2007), I suggest that 9-phenanthrol may be affecting another element of the signalling pathway which causes mGluR1/NAADP-mediated depolarisation.

Only two studies have suggested that activation of Group 1 mGluRs (mGluR1/mGluR5) may activate a TRP channel or TRP channel-like conductance in pyramidal neurons of the hippocampus. The first by Gee et al. (2003) which reports chemical activation (DHPG) of either mGluR1 or mGluR5 produces a TRP channel like conductance in CA3 pyramidal neurons. However, this current could be abolished with La^{3+}. The second by Congar et al. (1997) uses ACPD to activate mGluRs and reports a non-selective cation current, but only in the presence of K^+ channel antagonists, and bicuculline, which is also noted to inhibit SK channels (Debarbieux et al., 1998, Johansson et al., 2001, Khawaled et al., 1999).

Whereas a greater body of evidence exists to suggest that mGluR1/Group 1 mGluR activation inhibits of K^+ channels to produce depolarisation in pyramidal hippocampal neurons (Wu and Barish, 1999, Luthi et al., 1996, Guerineau et al., 1994, Takeshita et al., 1996, Chuang et al., 2000, Charpak et al., 1990, Desai and Conn, 1991, Mannaioni et al., 2001, Ireland and Abraham, 2002). It should be noted most of these studies were performed via chemical activation of mGluRs with bath application of ACPD (broad spectrum mGluR agonist) and DHPG (Group 1 mGluR agonist). Whilst these studies present

substantial evidence that mGluRs/mGluR1 can produce a membrane depolarisation via inhibition of K^+ channels, bath application/chemical induction may not best represent physiological activation properties of mGluRs and therefore downstream effects. Bath application of such agonists causes mGluR activation throughout the whole cell, which might produce confounding results based on the differential Group 1 mGluR receptor subtype, and isoform, localisation. In addition, several of these studies were undertaken before Group 1 agonists (DHPG) and specific mGluR antagonists were readily available (Turner and Salt, 2000), therefore it is difficult to determine whether the results observed are a result of mGluR1 or mGluR5 activation.

Finally, DHPG and ACPD are reported to produce binding which is completely biased to phosphoinositide hydrolysis, and produce only a fraction of possible downstream intracellular events, unlike endogenous ligands such as L-Glutamate (Hathaway et al., 2015). Therefore, I suggest the method by which I choose to explore mGluR1 function (synaptic activation/bath applied glutamate in combination with the pharmacological isolation of mGluR1 – **Figure 5.2**) represents a more appropriate and physiological approach.

In conclusion, I suggest there is insufficient evidence from the data I have obtained in my experimental preparation and in the literature to suggest that mGluR1 is activating a TRP channel/non-selective cation channel in CA1 pyramidal neurons of the hippocampus.

5.4.2 mGluR1 inhibits an apamin sensitive K_{Ca} channel to cause depolarisation

The data I present in **Figure 5.2** suggests that mGluR1-mediated depolarisation is produced by transient inactivation of SK channels by Ca^{2+} release from the acidic stores. I have shown that mGluR1-mediated depolarisations are abolished upon the application of apamin, an SK channel antagonist.

Traditionally SK channels are suggested to be voltage insensitive and activated by intracellular Ca^{2+}. They are functionally attributed to produce post action potential hyperpolarisation currents (I_{AHP}) in neurons. SK channels have been shown to become activated by Ca^{2+} entry through R-type (Myoga and Regehr, 2011) and/or L-type VDCCs (Marrion and Tavalin, 1998) but also Ca^{2+} release from the ER via

RyRs (Sah and McLachlan, 1991). In dendritic spines, SK channels are suggested to regulate GluN dependent Ca^{2+} influx in by causing a rapid hyperpolarisation of the postsynaptic dendritic membrane and reinstatement of Mg^{2+} block local intracellular Ca^{2+} signals and therefore modulate LTP (Ngo-Anh et al., 2005).

Therefore, it is slightly counter-intuitive to find mGluR1-mediated Ca^{2+} signals causing SK channels to produce membrane depolarisation rather than hyperpolarisation in my experimental system. This novel finding may be a consequence of intracellular SK channel modulation by other intracellular components. SK channels form stable macro-molecular complexes with several other resident proteins. Calmodulin (CaM) is thought to be the primary Ca^{2+} sensing domain of SK channels, as Ca^{2+} binding sites are absent on the pore-forming unit, and a resident CaM is noted to be present on each subunit of the tetrameric channel, as reviewed by Adelman et al. (2012). Protein kinase CK2 (CK2) and protein phosphatase 2A (PP2A) also are noted to associated with SK channel macromolecular complexes, they are suggested to be resident on the SK2 and SK3 channels. CK2 activity is suggested to decrease SK channel sensitivity to $[Ca]_i$ whereas PP2A is suggested to increase SK channel sensitivity to $[Ca]_i$ (Bildl et al., 2004, Allen et al., 2007). Furthermore, multiple sites for modulation of SK channel activity are predicted, as multiple sites for phosphorylation are reported (Köhler et al., 1996).

Buchanan et al. (2010) show that inhibition of SK channels by M1 muscarinic receptors facilitates long term potentiation in CA1 pyramidal neurons of the hippocampus. They show this effect is dependent on protein kinase C (PKC) activity. PKCα is also shown to positively modulate PP2A activity (Kirchhefer et al., 2014), suggesting a possible intracellular signalling mechanism by which metabotropic receptors can couple to SK channels to regulate their activity. I have not explored this throughout my study but intend to determine if this hypothesised mechanism is responsible for modulating SK channel activity in my experimental system. It seems likely that multiple routes of SK channel regulation are possible furthermore SK channel modulation may not only be dependent on the source of Ca^{2+} but also the spatiotemporal aspects of Ca^{2+} signals, and the downstream CBPs they activate.

In conclusion, I show that I can robustly block mGluR1-mediated membrane depolarisation in CA1 neurons of the hippocampus with apamin (**Figure 5.2**). Apamin is noted to inhibit SK channels and therefore I suggest that temporary inhibition, via acidic store Ca^{2+} signalling, of an SK channel with constitutive K^+ leak activity produces mGluR1-mediated depolarisation. I summarise my findings thus far in a diagrammatic representation shown in **Figure 5.3**.

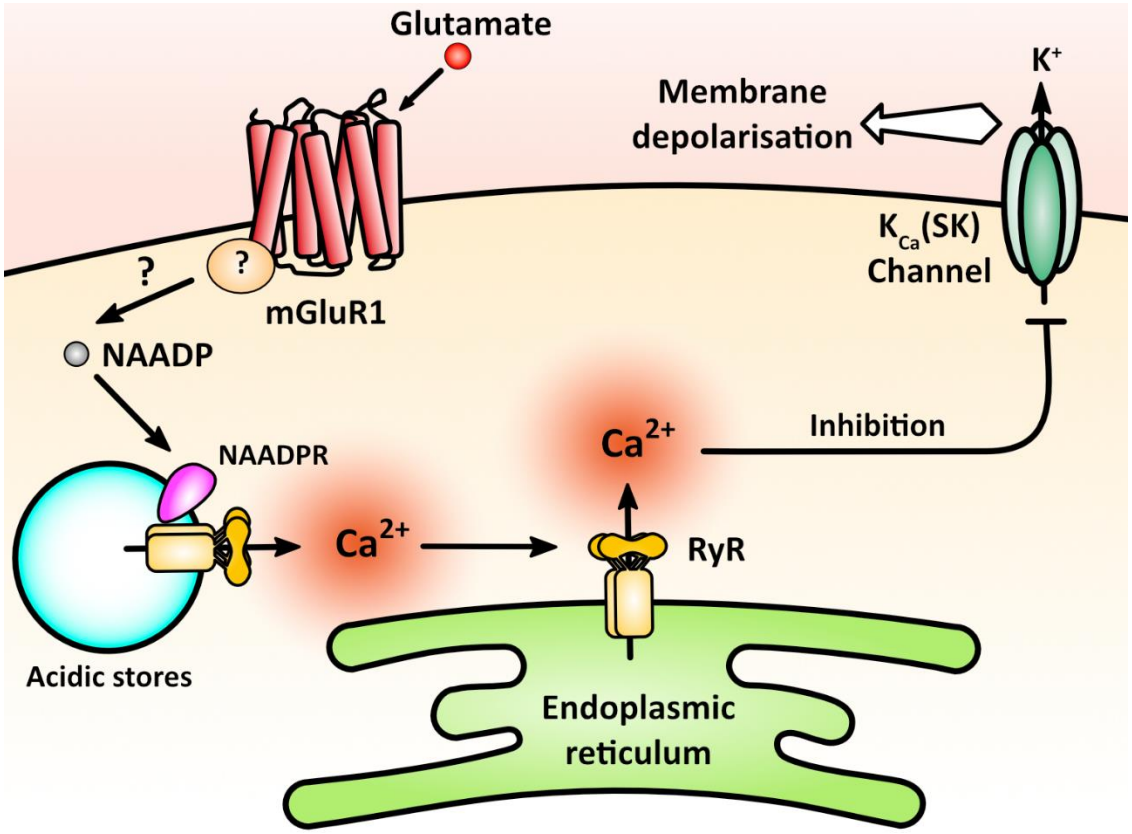

Figure 5.3 Hypothesised pathway for mGluR1-mediated depolarisation. Activation of mGluR1 by glutamate causes NAADP synthesis, intracellular NAADP mobilises Ca^{2+} from acidic stores which are further amplified via the RyRs. This causes temporary inhibition of an SK channel with constitutive K^+ leak activity and therefore results in a net membrane depolarisation.

5.5 Conclusion

1. **To identify the membrane channel responsible for mGluR1-mediated cellular depolarisation in CA1 neurons of the hippocampus.**

From the results in this chapter, I conclude that mGluR1-mediated depolarisation is not the result of activation of a non-selective TRP channel. As both La^{3+} and FFA (broad spectrum TRP antagonists) fail to inhibit mGluR1 mediated depolarisation (**Figure 5.1**).

I suggest that mGluR1 activation causes temporary inhibition of a K_{Ca} channel with constitutive K^+ leak activity and results in a net depolarisation of the cell. I show that this KCa can be blocked with apamin, and therefore I suggest the KCa channel is an SK channel (**Figure 5.2**). Finally, responses post apamin exposure are not reduced further by subsequent application of GPN, suggesting that these two manipulations are acting on the same pathway.

6.0 mGluR1-dependent synaptic plasticity is achieved via inhibition of SK channels by acidic store Ca^{2+} signalling

6.1 Introduction

Chapters **3.0 – 5.0** show experimental evidence for a novel signalling pathway which explains how mGluR1 activation with glutamate causes membrane depolarisation in CA1 neurons of the hippocampus. After mGluR1 is activated with glutamate, NAADP signalling is initiated and causes acidic store Ca^{2+} release, which is further amplified via the ryanodine receptors (RyRs), this Ca^{2+} signal acts to temporarily 'switch off' a constitutively active Ca^{2+}-activated K^+ channels (K_{Ca}) and produce membrane depolarisation. The depolarising K_{Ca} is apamin sensitive and therefore suggested to be a member of the small-conductance K_{Ca} (SK) channel family. This intracellular signalling pathway is shown diagrammatically in (**5.0 Figure 5.3**). I next sought to determine the physiological function of this signalling pathway.

SK channels are thought to be critical regulators of synaptic plasticity in hippocampus, they are localised to dendritic spines in CA1 neurons of hippocampus (Sailer et al., 2004), and are suggested to form functional feedback loops with the GluNs in dendritic spines (Faber and Sah, 2007, Faber, 2009, Adelman et al., 2012). They regulate synaptic plasticity by modulating the amount of Ca^{2+} entry through the GluNs. SK channel activation by extracellular Ca^{2+} through R-type (Myoga and Regehr, 2011) VDCCs or GluNs (Ngo-Anh et al., 2005) is suggested to cause hyperpolarisation and reinstatement of GluN Mg^{2+} block and therefore reduction of Ca^{2+} entry through GluNs (Ngo-Anh et al., 2005, Bloodgood and Sabatini, 2007). Blocking the SK channels with apamin facilitates induction of LTP, and reduces the likelihood of LTD synapses, therefore shifting the point of inflection between LTD and long-term potentiation LTP in CA1 pyramidal neurons of the hippocampus (Stackman et al., 2002, Hammond et al., 2006). SK2 channel plasticity is also shown to contributes to LTP at Schaffer collateral-CA1 synapses (Lin et al., 2008). Inhibition of the SK channels by metabotropic receptor activation, via intracellular Ca^{2+} signalling, is identified as a key contributor for induction of synaptic

plasticity in the hippocampus. Firstly, Buchanan et al. (2010) show that inhibition of SK channels by M_1 acetylcholine receptors, via PKC signalling, facilitates LTP at Schaffer collateral synapses in the hippocampus. Secondly, Tigaret et al. (2016) show mGluR1 activation causes inhibition of SK channels in to produce LTP in CA1 pyramidal neurons, which is dependent on activation of both intra- and extracellular Ca^{2+} stores. The activation or inhibition of SK channels by distinct Ca^{2+} stores allows for differential modulation of Ca^{2+} signal magnitude through GluNs and therefore the magnitude or type of plasticity observed post neural activity.

The current model for postsynaptic plasticity is centered around Ca^{2+} entry through the GluN receptors. Generally, LTP is suggested to occur after strong synaptic activity. This type of neural activity produces large membrane depolarisations in postsynaptic neurons and allows for transient, high magnitude Ca^{2+} entry through GluNs (as their Mg^{2+} block is removed). This type of Ca^{2+} signal initiates downstream signalling cascades resulting in exocytosis, and capture of GluA receptors at the synapse. This long lasting increase number of GluA receptors causes larger postsynaptic responses and hence potentiation of the synapse. (Ehlers et al., 2007, Makino and Malinow, 2009, Yang et al., 2010). Potentiation lasting up to 15 minutes is referred to as short-term potentiation (STP), and the induction of STP is shown to require GluA receptor insertion. Longer lasting changes to synaptic strength (>25 minutes) are referred to as LTP, which requires the induction of STP, but also recruitment of processes to maintain increased synaptic strength, such as *de novo* protein synthesis and extracellular-matrix remodeling. Conversely, longer lasting, moderate magnitude Ca^{2+} signals in the postsynaptic spines (typically a response to weak post synaptic depolarisation) causes a transition of GluA receptors into the extrasynaptic membrane and their subsequent endocytosis (Malinow and Malenka, 2002). This has the net effect of reducing postsynaptic responses and hence long term depression (LTD) of the synapse (Tardin et al., 2003, Yang et al., 2010).

SK channels are shown to influence the induction of LTP, and the intracellular signalling pathway I describe through this study (**2.0** - **5.0**) shows inhibition of SK channels via mGluR1 and acidic store Ca^{2+}

signalling. I wanted to determine if NAADP/acidic store Ca^{2+} signalling and inactivation of SK channels are responsible for the induction of mGluR1-dependent LTP in CA1 pyramidal neurons.

6.2 Aims

1. To determine if induction of mGluR1-dependent synaptic plasticity requires inhibition of SK channels via NAADP/acidic store Ca^{2+} signalling.

6.3 Results

6.3.1 mGluR1-dependent synaptic plasticity at CA3-CA1 synapses is abolished by preventing acidic store Ca^{2+} signalling, and short term plasticity can be rescued via SK channel inhibition with apamin

Tigaret et al. (2016) describe mGluR1-dependent plasticity via inhibition of the SK channels. In this study, a causal spike-timing dependent plasticity (STDP) induction protocol was used to create coincident presynaptic glutamate release and postsynaptic spiking which resulted in mGluR1-dependent potentiation of the synapse. They show that coincident activation of both extracellular Ca^{2+} entry, via VDCCs, and intracellular Ca^{2+} release is required to produce mGluR1-dependent plasticity, suggesting that coordinated activation of distinct sources of Ca^{2+} is required to produce this form of synaptic potentiation.

To determine if mGluR1-dependent plasticity required acidic store Ca^{2+} signalling I began by firstly confirming the STDP protocol used by Tigaret et al. (2016) could induce mGluR1-dependent LTP my experimental configuration. This protocol was shown to produce mGluR1-dependent LTP and consisted of causally pairing one pre-synaptic stimulus (to produce a subthreshold excitatory post synaptic potential (EPSP)) with two back propagating action potentials (bAPs) (100 Hz), elicited in the post synaptic neuron via current injection, at a 10 ms interval. This paired induction protocol was repeated 300 times at 5 Hz and delivered within 10 minutes of whole cell breakthrough to prevent dialysis of factors required for mGluR1-dependent LTP. The experimental configuration and STDP protocol are shown diagrammatically in **Figure 6.1A**. I delivered presynaptic stimulation (1 pulse, 0.5

ms) produce EPSPs every 15 s throughout each experiment. The initial slope (first 3 ms) was used to determine changes in synaptic strength, I compared the mean slope of EPSP during the baseline (5 min) to the mean slope of EPSP between 10-15 min post STDP induction to determine if short term plasticity (STP) had occurred, or between 25-20 min STDP induction to determine if LTP had occurred.

I confirmed that the STDP induction protocol developed by Tigaret et al. (2016) could produce both STP (+153.3 ± 9.39 %, n = 7) and LTP (+153.7 ± 9.58 %, n = 7) in CA1 pyramidal neurons in hippocampal slice culture. To determine the induction of STP/LTP was mGluR1 dependent, I next used the STDP induction protocol in the presence of an mGluR1 specific antagonist (300 nM JNJ16259685) **Figure 6.1C**. Pharmacological antagonism of mGluR1 prevented both STP (**Figure 6.1G:** +153.3 ± 9.39 %, vs +73.08 ± 16.52 %, n = 7,5, $P < 0.01$) and LTP (**Figure 6.1H:** +153.3 ± 9.39 %, vs +78.37 ± 17.14 %, n = 7,5, $P < 0.05$). These data suggest that the STDP induction protocol used requires intact mGluR1 signalling in order to produce either STP or LTP.

I have previously described an intracellular signalling pathway (**Chapters 2.0** and **3.0**) which shows that activation of mGluR1 signalling by synaptic glutamate causes inhibition of SK channels, via acidic store Ca^{2+} signalling. SK channels are described to facilitate the induction of LTP (Stackman et al., 2002, Hammond et al., 2006), I next sought to determine if preventing mGluR1-dependent inhibition of SK channels, by antagonising acidic store Ca^{2+} signalling, prevented mGluR1-dependent plasticity. NAADP mobilises Ca^{2+} from the acidic stores and high concentrations of NAADP also prevent acidic store Ca^{2+} signalling via receptor desensitisation (Aarhus et al., 1996, Genazzani et al., 1996). I included 5 mM NAADP in the intracellular solution of the patch pipette with the intention of selectively inhibiting NAADP signalling, and acidic store Ca^{2+} signalling, in the postsynaptic neuron **Figure 6.1D**. I found that 5 mM NAADP included in the intracellular solution prevented both STP (**Figure 6.1G:** +153.3 ± 9.39 %, vs +81.13 ± 13.17 %, n = 7,5, $P < 0.05$) and LTP (**Figure 6.1H:** +153.3 ± 9.39 %, vs +80.03 ± 16.91 %, n = 7,5, $P < 0.05$). Therefore, I suggest that the induction of mGluR1-dependent STP/LTP requires NAADP/acidic store Ca^{2+} signalling.

Tigaret et al. (2016) show that in CA1 pyramidal neurons of the hippocampus mGluR1-dependent plasticity (LTP) is achieved via inhibition of the SK channels. The intracellular signalling pathway I have described through **Chapters 2.0** and **3.0** shows activation of mGluR1 signalling by synaptic glutamate causes inhibition of SK channels. I also show in **Figure 6.1D, G, H** that inhibition of NAADP/acidic store Ca^{2+} signalling prevents induction of mGluR1-dependent STP/LTP. I hypothesised that the signalling pathway I describe is responsible for the induction of mGluR1-dependent LTP. Therefore, I suggested pharmacological inactivation of the SK channels with apamin (bee venom) would not produce a greater magnitude of STP or LTP upon induction of mGluR1-dependent plasticity with the established STDP protocol, as SK channels would already be inhibited by mGluR1 signalling, and therefore occlude any further effects of apamin. I show in **Figure 6.1 E, G, H** that co-administration of apamin (200 nM) with synaptic induction of mGluR1 dependent plasticity does not increase the magnitude of either STP (**Figure 6.1G:** +153.3 ± 9.39 %, vs +146.5 ± 16.29%, n = 7,7, P > 0.99) or LTP (**Figure 6.1G:** +153.3 ± 9.39 %, vs +139.8± 17.39.29%, n = 7,7, P > 0.99), compared to control experiments.

I show that inhibition of NAADP/acidic store Ca^{2+} signalling with a desensitising concentration of NAADP (5 mM) prevents induction of mGluR1-dependent STP/LTP, **Figure 6.1D, G, H**. I suggest this is because mGluR1 is unable to inhibit SK channels via NAADP/acidic store Ca^{2+} signalling. Therefore, I wanted to test if STP/LTP could be rescued in the presence of 5 mM NAADP by pharmacologically inactivating the SK channels with apamin. I show that with this manipulation, STP can be rescued, where there is no significant difference to either the control group (**Figure 6.1F, G:** +153.3 ± 9.39 %, vs +131.90 ± 21.60 %, n = 7,6, P > 0.99) or mGluR1-dependent plasticity with the presence of apamin (**Figure 1G:** +146.5 ± 16.29%, vs +131.90 ± 21.60 %, n = 7,7, P > 0.99), whereas LTP is not rescued, and is significantly smaller in magnitude than the control group (**Figure 6.1F, H:** +153.3 ± 9.39 %, vs +93.31 ± 24.91%, n = 7,6, P < 0.05).

In conclusion, the results I have obtained in **Figure 6.1** show that the induction of mGluR1-dependent plasticity requires inhibition of the SK channels via NAADP /acidic store Ca^{2+} signalling. I show that STP

(10-15 min), but not LTP (25-30 min), can be rescued by pharmacologically antagonising the SK channels with apamin. This suggests that inhibition of the SK channels is essential for the induction, but not the maintenance of mGluR1-dependent potentiation of synaptic transmission between CA3-CA1 pyramidal neurons of the hippocampus.

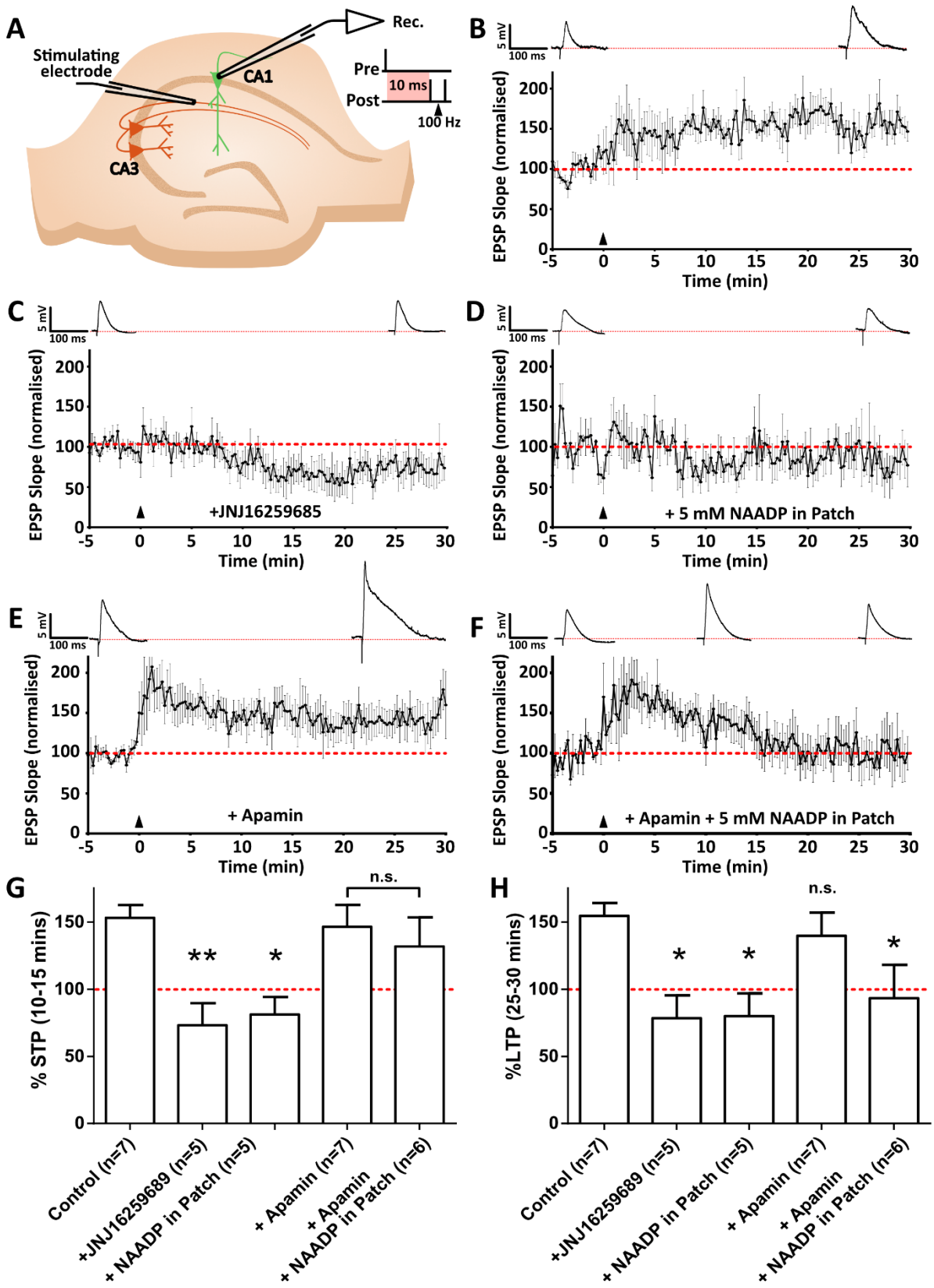

Figure 6.1 Induction mGluR1-dependent LTP requires inhibition of SK channels via acidic store Ca^{2+} signalling. A. Diagram of the experimental configuration, a causal spike time dependent plasticity

protocol was used to induce mGluR1-dependent LTP, where one casual presynaptic stimulation is paired with two back propagating action potentials (100 Hz), at a 10 ms interval. The induction protocol is delivered where t = 0, indicated by the black triangles. **B-F**. Representative EPSP voltage traces displayed at the time they were recorded **B.** This STDP protocol produces LTP lasting at least 30 minutes. **C** The mGluR1 specific antagonist JNJ16259685 (300 nM) prevents LTP with the STDP protocol. **D.** Preventing NAADP/acidic store Ca^{2+} signalling with a desensitising concentration of NAADP (5 mM) also prevents LTP. **E.** Inducing STDP in the presence of SK channel antagonist apamin (200 nM) does not affect the magnitude of STP or LTP. **F.** Apamin rescues STP in the presence of 5 mM NAADP by 're-inhibiting' SK channels. **G.** Mean change in synaptic strength between 10-15 min, indicating STP. **H.** Mean change in synaptic strength between 10-15 min, indicating LTP Error bars display SEM. Significant differences to the control group (unless otherwise stated) are displayed above each column, where ** = $P < 0.01$ and * = $P < 0.05$, n.s. = non-significant.

6.4 Discussion

The current theory of postsynaptic plasticity revolves around Ca^{2+} entry through GluN receptors. Transient, high magnitude rises in intracellular Ca^{2+} through GluNs produces LTP, whereas longer lasting, small magnitude rises in intracellular Ca^{2+} through GluNs produces LTD, as reviewed in Lisman (2001) and Shouval et al. (2002). The SK channels are suggested to form a functional feedback loop with GluNs receptors to regulate the amount of Ca^{2+} entry through GluNs and therefore magnitude and type of synaptic plasticity. Their activation via R-Type VDCC or GluN Ca^{2+} entry (Ngo-Anh et al., 2005, Bloodgood and Sabatini, 2007) is suggested to cause local hyperpolarisation and rapid reinstatement of the GluN Mg^{2+} block, and reducing the amount of LTP observed (Ngo-Anh et al., 2005). However, SK channel inhibition is also noted to occur upon a metabotropic receptor activation and subsequent intracellular Ca^{2+} release. SK channel inhibition is suggested to allow for synaptic potentiation to occur (Buchanan et al., 2010, Tigaret et al., 2016). These studies highlight the

functional importance of distinct Ca^{2+} stores and their ability to produce a variety of downstream events as a result of highly localised and specific Ca^{2+} signalling events.

Throughout this study, I have described an intracellular signalling pathway linking mGluR1 activation to inhibition of the SK channels via NAADP/acidic store Ca^{2+} signalling (**Chapters 3.0 - 5.0**). Pharmacological inhibition of SK channels is shown to facilitate the induction of LTP in the hippocampus (Stackman et al., 2002, Hammond et al., 2006). Additionally, other studies show inhibition of the SK channels by metabotropic receptors, including M_1 acetylcholine receptor (Buchanan et al., 2010), and mGluR1 (Tigaret et al., 2016), is responsible for producing LTP in CA1 pyramidal neurons of the hippocampus. Therefore, I hypothesised that activation of the pathway I have described from specific patterns of neuronal activity allows for the induction of LTP at synapses via inhibition of the SK channels.

I have shown that abolishing NAADP/acidic store Ca^{2+} signalling prevents the induction of an mGluR1-dependent form of synaptic potentiation (**Figure 6.1**). I suggest this because when postsynaptic intracellular NAADP/acidic store Ca^{2+} signalling is removed, synaptic activation of mGluR1 fails to inhibit the SK channels. Leading to insufficient Ca^{2+} entry through the GluNs and a failure to evoke mGluR1-dependent LTP.

I wanted to determine if pharmacologically inactivating the SK channels, whilst blocking NAADP/acidic store Ca^{2+} signalling (thus preventing endogenous inhibition of SK channels), could rescue mGluR1-dependent plasticity. I show that inhibiting NAADP/acidic store Ca^{2+} signalling whilst in the presence of apamin can rescue STP but not LTP. This suggests that the induction of mGluR1-dependent plasticity is a result of inhibition of the SK channels via mGluR1-evoked NAADP/acidic store Ca^{2+} signalling.

The failure to rescue the long-term expression of LTP indicates that the maintenance of synaptic potentiation is also dependent on NAADP/acidic store Ca^{2+} signalling in CA1 pyramidal neurons. Maintenance of LTP in dendritic spines of CA1 pyramidal neurons is shown to be dependent acidic store Ca^{2+} signalling, where activity-dependent exocytosis of Cathepsin B from lysosomes is shown to

regulate long-term plasticity via extracellular matrix remodelling by matrix metallopeptidase 9 (MMP-9) (Padamsey et al., 2017). Therefore, I suggest that the maintenance of mGluR1-dependent LTP (>25 min) could potentially be rescued with the addition of Cathepsin B or MMP9.

A future experiment which will be undertaken in an attempt to understand the mechanism of this form of LTP consists of the following design. The described LTP induction protocol will be applied whilst blocking mGluR1s with JNJ16259685 and in the presence of apamin. As the LTP protocol is spike timing dependent, the post synaptic signalling cascades that are activated by bAPs would, in theory, still allow for LTP to occur despite the removal of mGluR1 signalling if apamin is present as the SK channels would be inactivated further downstream in the signalling cascade.

In conclusion, I suggest the data I have presented through **Chapters 3.0 - 6.0** can be used to present a model for synaptic plasticity via modulation of SK channel function by distinct Ca^{2+} stores. I present this model in **Figure 6.2**. **Figure 6.2A** displays the canonical model for SK channel activation, where **1.** Synaptic glutamate activates GluA receptors to produce **2.** Membrane depolarisation and **3.** Ca^{2+} entry via VDCCS. This causes **4.** Activation of SK channels and local hyperpolarisation, resulting in inhibition of GluNs by reinstating Mg^{2+} block; thereby reducing Ca^{2+} entry through the GluNs and reducing the probability of LTP induction. When synaptic activity is sufficiently strong, the mGluR1 receptors are recruited. **Figure 6.2B** displays my hypothesised model for SK channel modulation via mGluR1. Where **1.** Glutamate activates mGluR1 receptors and causes **2.** NAADP synthesis, which results in **3.** Acidic store Ca^{2+} release, and amplification via the ryanodine receptors (RyR). This causes **4.** inactivation of the SK channels which in turn prevents local hyperpolarisation and **5.** allows greater Ca^{2+} entry through the GluN receptors, and allowing induction of LTP. I suggest that this model explains how distinct routes of intracellular Ca^{2+} signalling allows for distinct types of synaptic plasticity to occur in dendritic spines.

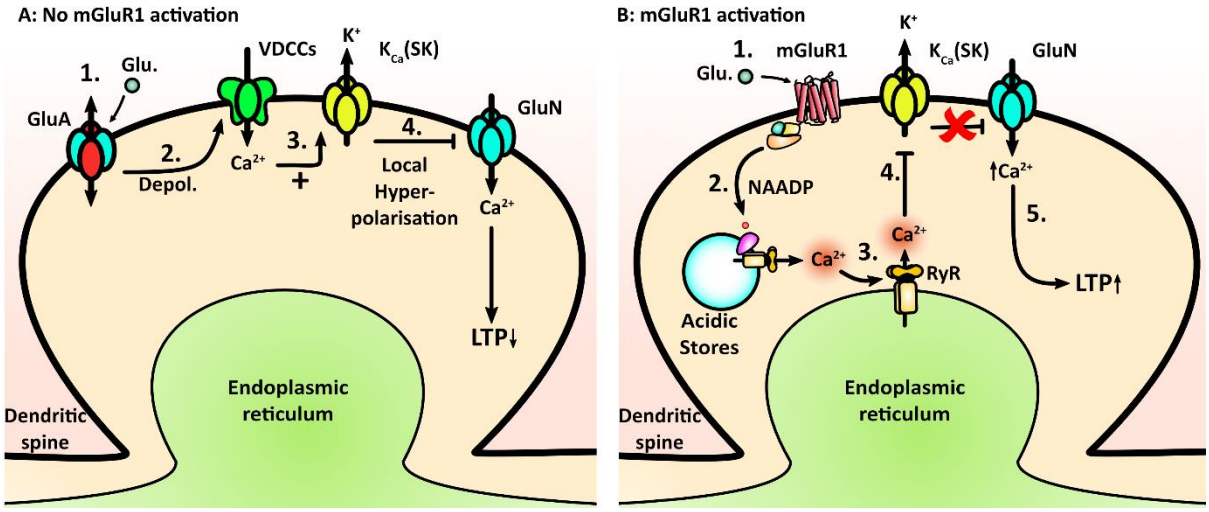

Figure 6.2 Hypothesised model for mGluR1-dependent plasticity. A displays the canonical model for SK channel activation, where **1.** Synaptic glutamate activates GluA receptors to produce **2.** Membrane depolarisation and **3.** Ca^{2+} entry via VDCCS. This causes **4.** Activation of SK channels and local hyperpolarisation, resulting in inhibition of GluNs by reinstating Mg^{2+} block. Thereby reducing Ca^{2+} entry through the GluNs and reducing the probability of LTP induction. Where synaptic activity is sufficiently strong, the mGluR1 receptors are recruited. **B** displays my hypothesised model for SK channel modulation via mGluR1, GluA/VDCC regulation of SK channels is also present here but not shown. Where **1.** Glutamate activates mGluR1 receptors and causes **2.** NAADP synthesis, which results in **3.** Acidic store Ca^{2+} release, and amplification via the ryanodine receptors (RyR). This causes **4.** inactivation of the SK channels which in turn prevents local hyperpolarisation and **5.** allows greater Ca^{2+} entry through the GluN receptors, and allowing induction of LTP.

6.5 Conclusions

Here I address the aims of this chapter.

1. **To determine if induction of mGluR1-dependent synaptic plasticity requires inhibition of SK channels via NAADP/acidic store Ca^{2+} signalling.**

In this chapter I have shown that mGluR1-dependent synaptic plasticity requires intact NAADP/acidic store Ca^{2+} signalling, as the addition of desensitising concentrations of intracellular NAADP prevents mGluR1-dependent plasticity. I have previously described that mGluR1 activation inhibits SK channels via NAADP/acidic store Ca^{2+} signalling (**Chapter 5.0**). Furthermore, I show that the induction and STP can be rescued by pharmacologically inactivating SK channels with apamin, in the presence of desensitising concentrations of intracellular NAADP. In conclusion, I suggest that the induction of mGluR1-dependent plasticity requires inhibition of SK channels via NAADP/acidic store Ca^{2+} signalling.

7.0 Final conclusions

7.1 Key Finding

The induction of mGluR1-dependent LTP is a result of inhibition of SK channels by NAADP/acidic store Ca^{2+} signalling.

7.2 Final conclusion and discussion

Here I summarise the key findings in this study. In **Chapter 3.0** I show that introduction/application of the Ca^{2+} mobilising second messenger NAADP causes membrane depolarisation in pyramidal neurons in hippocampal slice culture. I show that application of a membrane permeant form for NAADP (NAADP-AM) causes membrane depolarisation which is dependent on NAADP signalling, intracellular Ca^{2+}, acidic store Ca^{2+} release and the ryanodine receptor (**Chapter 3.0**. **Figure 3.1**) These results broadly replicate those observed by Brailoiu et al. (2009b). In **Chapter 3.0**. **Figure 3.2** I show that in my experimental system, a bell shaped dose-response curve for NAADP concentration and magnitude of membrane depolarisation is apparent. The bell shaped dose-response curve is a characteristic feature of NAADP-induced cellular responses (Galione et al., 2011, Guse et al., 2013). This provides more evidence that the observed membrane depolarisation is a result of NAADP signalling. Furthermore, I also show that NAADP is unique among direct Ca^{2+} mobilising second messengers in its ability to produce membrane depolarisation in pyramidal neurons of the hippocampus. The introduction of both cADPR or IP_3 failed to produce membrane depolarisation in these neurons. It was surprising that intracellular dialysis of cADPR produced no significant membrane depolarisation, as I show that the ryanodine receptor is required for NAADP mediated membrane depolarisation (**Chapter 3.0**. **Figure 3.1**). This suggests that in order to achieve NAADP mediated depolarisation, Ca^{2+} release from the acidic stores is required initially, which may then be amplified further via the ryanodine receptor by Ca^{2+} induced Ca^{2+} release (CICR).

During **Chapter 4.0** I sought to identify a physiological stimulus which causes NAADP-mediated membrane depolarisation in pyramidal neurons of the hippocampus. Pandey et al. (2009) showed that

the application glutamate to neuronal suspensions causes NAADP synthesis. As glutamate is the major neurotransmitter in the hippocampus, I hypothesised that glutamate could produce an NAADP-mediated membrane depolarisation. I went on to show that synaptic, or extracellularly applied, glutamate can cause NAADP-dependent membrane depolarisation and intracellular Ca^{2+} signalling via metabotropic glutamate receptor 1 (mGluR1) specifically (**Chapter 4.0 Figures 4.1-4.3**), and via the same signalling pathway which I had described in **Chapter 3.0**.

During **Chapter 5.0** I sought to determine the membrane channel responsible for causing mGluR1/NAADP-mediated membrane depolarisation. I first showed that a range of non-specific TRP channel antagonists had no effect on mGluR1-mediated membrane depolarisation (**Chapter 5.0 Figure 5.1**), whereas apamin (bee venom), an SK channel antagonist abolished mGluR1-mediated depolarisation after activation with synaptic or extracellularly applied glutamate (**Chapter 5.0 Figure 5.2**). The final aspect of my thesis was to explore the role of mGluR1 inhibition of SK channels with relation to synaptic plasticity.

In **Chapter 6.0** I show that the induction mGluR1-dependent LTP requires inhibition of the SK channels by NAADP/acidic store Ca^{2+} signalling. When NAADP signalling is abolished, the induction (STP), but not the maintenance (LTP) of mGluR1-mediated synaptic potentiation can be rescued by 're-inhibiting' the SK channels with apamin. The failure to rescue LTP indicates that the maintenance of synaptic potentiation is also dependent on NAADP/acidic store Ca^{2+} signalling in my experimental preparation. Maintenance of LTP in dendritic spines of CA1 pyramidal neurons is shown to be dependent on acidic store Ca^{2+} signalling and extracellular matrix remodelling (Padamsey et al., 2017).

Therefore, throughout my thesis, I have presented an entirely novel intracellular signalling pathway which reveals how mGluR1-dependent plasticity is achieved in neurons of the hippocampus. These findings replicate or unify several aspects of the literature. I have broadly replicated the results of Brailoiu et al. (2009b) and identified mGluR1 as a glutamate receptor which can cause NAADP

signalling, this was not explored by Pandey et al. (2009), where they show the application of glutamate can cause NAADP synthesis.

Inhibition of SK channels by metabotropic receptors is described to be important for certain forms of plasticity. Both M_1 acetylcholine receptors (Buchanan et al., 2010) and mGluR1 (Tigaret et al., 2016) are described to inhibit the SK channels in order to allow for LTP to occur in the hippocampus. Although the manner by which this occurs is not explored extensively in these two studies; I present a model for SK channel inhibition by mGluR1, which helps to explain the findings from these studies.

Group 1 mGluRs (mGluR1/5) are shown to have a functional role in the pathogenesis of fragile X mental retardation (FXS) (Bear et al., 2004). FXS is the most common form of inherited mental retardation, it is caused by transcriptional silencing of the FMR1 gene that encodes the fragile X mental retardation protein (FMRP) (O'Donnell and Warren, 2002). Activation of Group 1 mGluRs is noted to cause a variety of downstream events, including synaptic depression, by initiating translation of pre-existing mRNA near synapses. FMRP normally functions as a repressor of translation of specific mRNAs. In FXS the lack of FMRP is thought to lead to an increase in long term depression (LTD) as Group 1 mGluRs action to translate mRNA at synapses is not counteracted by FMRPs action as a translational repressor. The intracellular signalling pathway I describe could present novel targets to reduce mGluR-mediated LTD, blocking the SK channel is shown to facilitate LTP and change the point of inflection of LTD to LTP in the hippocampus (Stackman et al., 2002, Hammond et al., 2006). Therefore, activation of the signalling pathway I describe could reduce the amount of LTD and reduce the symptoms in FXS.

7.3 Technical limitations

Here I address the technical limitation of the project I have undertaken.

7.3.1 Reliance on pharmacological tools

The experiments in this thesis rely heavily on the use of pharmacological manipulations. Despite demonstrating the effectiveness of these drugs through several lines of evidence (various modes of

electrophysiological recording and Ca^{2+} imaging), I cannot ignore the potential for confounding off-target effects from their use.

A variety of molecular tools have been used in studies investigating acidic store Ca^{2+} release. Both genetic knock-out (Calcraft et al., 2009, Zhu et al., 2010b, Ruas et al., 2015) and knock-in (Ruas et al., 2015) of TPCs have been used to demonstrate their requirement for TPCs in acidic store Ca^{2+} release. Gene silencing or knockdown with short interfering or short hairpin RNA (siRNA/shRNA) have both also been used to try to investigate the channels on the acidic stores that are essential for Ca^{2+} release (Hui et al., 2015, Pandey et al., 2009). Employing these genetic tools may prove useful in future experiments to provide another line of evidence to support the findings with the use of pharmacological tools. Repeating key experiments (mGluR1 mediated membrane depolarisation with electrical stimulation/LTP experiments), with hippocampi derived from animals lacking TPCs, SK channels, or mGluR1 will be undertaken in future work.

7.2.2 Positive controls

I must note the lack of positive controls for some experiments in this study. The following paragraph will reiterate those which are lacking. Positive controls for the following pharmacological tools used were not undertaken: cADPR, 2-APB, La^{3+}, FFA, and TPPO. Positive controls to ensure cADPR that could reliably cause Ca^{2+} release, and that 2-APB could reliably block IP_3R-mediated Ca^{2+} release, will be addressed in future work. These will resemble positive control experiments for verifying IP_3 and Xestospongin C efficacies (See section **4.3.2** for details). Positive controls for the TRP antagonists La^{3+}, FFA, and TPPO, would require isolation of specific TRP channel currents, ideally by genetic means, which is not a practical undertaking in hippocampal slices cultures. It should be noted, however, that the concentration and duration of application used for these antagonists were comparable to those reported to be effective in previous studies.

7.2.3 NAADP and SK channel inhibition

Through this thesis, I have robustly shown that activation of mGluR1 causes NAADP signalling, and membrane depolarisation. I go on to show that the mGluR1-mediated membrane depolarisation can be blocked with apamin, a specific SK channel antagonist derived from bee venom, and that apamin can rescue short term plasticity in the presence of desensitising concentrations of NAADP inside the cell. However, I have not directly shown a link between NAADP and SK channel inhibition. I propose to address this by measuring the sensitivity of currents (using voltage clamp) produced by application of NAADP-AM to SK channel antagonists. I expect SK channel antagonists to inhibit or at least reduce NAADP-AM mediated current. These experiments would also open the opportunity to try different (both pharmacological and non-pharmacological) methods to inhibit SK channel function, and therefore provide additional evidence that acidic store Ca^{2+} release does affect SK channel function.

7.4 Future directions

7.4.1 Restoring both STP and LTP with 'rescue' experiment

The LTP protocol used in this study is spike timing dependent, where by presynaptic stimulation is paired with two postsynaptic bAPs; this pattern of stimulation is repeated 300x at 5 Hz. The presynaptic stimulation causes glutamate release into the synaptic cleft, I propose this glutamate activates mGluR1s and NAADP signalling, which in turn inactivate the SK channels. I show that desensitising concentrations of NAADP prevent LTP, and under these conditions, the addition of apamin can rescue STP but not LTP. The maintenance of LTP is known to require NAADP-dependent lysosomal fusion to the dendritic spines, which can be inhibited by inhibiting NAADP signalling (Padamsey et al., 2017), which explains why both STP and LTP are not rescued in the presence of apamin.

Therefore, another 'rescue' experiment could be undertaken in order to restore both STP and LTP whilst antagonising a component of the signalling pathway. I propose to conduct an experiment where

the LTP induction protocol is delivered whilst blocking mGluR1s with JNJ16259685 and also the SK channels with apamin. The post synaptic signalling cascades that are activated by bAPs would, in theory, still allow for LTP to occur despite the removal of mGluR1 signalling if apamin is present as the SK channels would be inactivated further downstream in the signalling cascade.

7.4.2 SK channel regulation

The intracellular signalling pathway I propose (**Figure 6.2**) relies on the assumption that there is microdomain signalling between mGluR1, the acidic stores, and the SK channels. There is evidence to suggest that the acidic stores are present in dendritic spines (Padamsey et al., 2017) and that it is likely that the mGluR1s and the SK channels lie within close proximity to one another due to their perisynaptic/extrasynaptic localisation (Lujan et al., 1996, Allen et al., 2011). In this study, I have not presented evidence of contact sites between these three proteins. Demonstrating the presence of physical coupling in the form of membrane contact sites between mGluR1s, the acidic stores, and the SK channels would greatly strengthen the findings I have presented.

Exploring the localisation, the co-localisation and physical contact sites between mGluR1s, the acidic stores, and SK channels could be undertaken by employing various techniques. For example, using immunohistochemistry to fluorescently tag mGluR1s, acidic stores, and SK channels would allow for optical detection of their localisation in relation to one another. Furthermore, other methods which rely on immunochemistry (such as Förster resonance energy transfer (FRET)) present the opportunity to detect co-localisation and would present evidence of close physical proximity (within 10 nm of one another). Exploring the proteomic associations of mGluR1s, the acidic stores, and SK channels would be another useful avenue to explore with further experiments. I suggest a pull-down assay and co-immunoprecipitation would be experiments worth undertaking in future experiments to provide evidence of physical interaction between mGluR1s, the acidic stores, and SK channels.

Another element of SK channel regulation which I have not explored in this study is how NAADP-mediated Ca^{2+} release inhibits SK channels. SK channels also are functionally regulated by several Ca^{2+} sensitive proteins with which they form stable macro-molecular complexes with, these include CaM (Adelman et al., 2012), PP2A and CK2 (Bildl et al., 2004, Allen et al., 2007). CK2 activity is suggested to decrease SK channel sensitivity to $[Ca]_i$ whereas PP2A is suggested to increase SK channel sensitivity to $[Ca]_i$. Buchanan et al. (2010) show that inhibition of SK channels by M1 muscarinic receptors facilitates long term potentiation in CA1 pyramidal neurons of the hippocampus. They show this effect is dependent on protein kinase C (PKC) activity. PKCα is also shown to positively modulate PP2A activity (Kirchhefer et al., 2014), suggesting a possible intracellular signalling mechanism by which metabotropic receptors can couple to SK channels to regulate their activity. I propose to use pharmacological inhibitors of protein kinases and phosphatases to explore whether SK channel regulation by NAADP signalling is dependent on PP2A and or CK2.

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