Ca$^{2+}$ Dependent Activation of Extracellular Signal Regulated Kinases 1 and 2: Role of Intrasynaptosomal Ca$^{2+}$ Stores

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Declaration

I, Sohaib Nizami confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Sohaib Nizami
The Best amongst Mankind Is He Who Brings Benefit to Others
Abstract

The aim of the thesis was to examine how Ca\(^{2+}\) activates the ERK pathway and the contribution of Ca\(^{2+}\) released from intracellular stores in physiological and pathophysiological conditions using isolated nerve terminals (synaptosomes) in a presynaptic model. The Ca\(^{2+}\)-dependent phosphorylation/activation of ERK1 and ERK2 stimulated by depolarisation of the plasma membrane or by Ca\(^{2+}\) influx mediated by the ionophore ionomycin was significantly reduced by the removal of external Ca\(^{2+}\). Intrasynaptosomal Ca\(^{2+}\) contribution to the Ca\(^{2+}\)-dependent component of ERK1 and ERK2 phosphorylation/activation was indicated by the depletion of intrasynaptosomal Ca\(^{2+}\) or inhibition of the smooth endoplasmic reticulum Ca\(^{2+}\)-ATPase pump. Two main pathways were found to lead to the release of Ca\(^{2+}\) from intrasynaptosomal stores. Firstly, external Ca\(^{2+}\) influx directly activated ryanodine receptors (RyRs) to mediate Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). Secondly, Ca\(^{2+}\) influx or activation of GPCRs coupled to G\(\text{q/11}\) activated phospholipase C (PLC) to effect PIP\(_2\) metabolism and IP\(_3\) production, with consequent activation of IP\(_3\)-induced Ca\(^{2+}\) release (IPCR). The activation of group I metabotropic glutamate receptor (mGluR1/5) stimulation supported IPCR. Intriguingly, inhibition of Ca\(^{2+}\) influx through voltage-dependent calcium channels (VDCCs) by stimulating GABA\(_B\), group III mGlurS, 5-HT\(_{1A}\) and A\(_1\) receptors was suppressed by prior depletion of the smooth endoplasmic reticulum. Mitochondria and acidic compartments also appear to store Ca\(^{2+}\) intrasynaptosomally, with mitochondrial depolarisation resulting in a transient increase in ERK1 and ERK2 phosphorylation/activation. Finally, a pathophysiological model of nerve terminal ischemia showed that intrasynaptosomal Ca\(^{2+}\) release contributes to the Ca\(^{2+}\)-dependent component of phosphorylation/activation of ERK1 and ERK2 occurring when Na\(^+\)/K\(^+\)-ATPase is inhibited. In conclusion, extracellular Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) store release together support Ca\(^{2+}\) mediated stimulation of the ERK pathway in
synaptosomes. This has important implications in the cross-talk of signalling pathways to ERK1 and ERK2 phosphorylation/activation and neurotransmitter release from nerve terminals in physiological and pathophysiological conditions.
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### Abbreviations

- **2-APB**: 2-aminoethoxydiphenyl borate
- **4-AP**: 4-aminopyridine
- **AMPA**: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
- **BDNF**: Brain derived nerve factor
- **BSA**: Bovine Serum Albumin
- **Ca²⁺**: Calcium
- **CICR**: Ca²⁺-induced Ca²⁺ release
- **cADPR**: Cyclic ADP ribose
- **CalDAG-GEFI**: Ca²⁺ and diacylglycerol-regulated GEFI
- **CalDAG-GEFII**: Ca²⁺ and diacylglycerol-regulated GEFII
- **CaM**: Calmodulin
- **CaMKII**: Ca²⁺/calmodulin-dependent kinase II
- **cAMP**: Cyclic adenosine monophosphate
- **DAG**: Diacylglycerol
- **DHPG**: (S)-3,5-Dihydroxyphenylglycine
- **DMSO**: Dimethyl sulfoxide
- **EGTA**: Ethylene glycolbis(β-aminoethyl ether)-N,N,N,N'-tetraacetic acid
- **ERK1**: Extracellular signal-regulated kinase 1
- **ERK2**: Extracellular signal-regulated kinase 2
- **g**: G force
- **GABA**: γ-Aminobutyric acid
- **GDH**: Glutamate dehydrogenase
- **GEF**: GTP exchange factor
- **GIRK**: G protein-coupled inwardly rectifying K⁺ channel
- **GPCR**: G protein coupled receptor
- **Grb2**: Growth factor receptor-bound protein 2
- **HBM**: Hepes-Buffered medium
- **Hepes**: N-2-hydroxyethylpiperazine-N’-2-ethansulfonic acid
- **HPLC**: High performance liquid chromatography
- **IP₃**: Inositol 1,4,5-trisphosphate
- **IP₃R**: Inositol 1,4,5-trisphosphate receptor
- **IPCR**: IP₃-induced Ca²⁺ release
- **IRBIT**: Inositol 1,4,5-trisphosphate receptor binding protein
- **KCL**: Potassium chloride
- **mGluR1/5**: Metabotropic glutamate receptor 1/5
- **MEK**: MAPK/ERK kinase
- **MgCl₂**: Magnesium chloride
- **Na⁺**: Sodium
- **NAADP**: Nicotinic acid adenine dinucleotide phosphate
- **NaCl**: Sodium chloride
- **NaH₂PO₄**: Sodium dihydrogenphosphate
- **NaHCO₃**: Sodium carbonate
- **NMDA**: N-methyl-D-aspartic acid
- **PI3K**: Phosphatidylinositol-3 kinase
- **PI4K**: Phosphatidylinositol-4 kinase
- **PIP₂**: Phosphatidylinositol 4,5-bisphosphate
- **PIP₃**: Phosphatidylinositol-3,4,5-bisphosphate
- **PKA**: Protein kinase A
- **PKC**: Protein kinase C
- **PLC**: Phospholipase C
Pyk2  Proline-rich tyrosine kinase
RasGRF  Ras-specific guanine nucleotide-releasing factor
RP  Reserve pool
RRP  Ready-releasable pool
RTK  Receptor tyrosine kinase
RyR  Ryanodine receptor
SDS  Sodium dodecyl sulfate
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM  Standard error of the mean
SERCA  Sacro/endoplasmic reticulum Ca\textsuperscript{2+} - ATPase
SNAP-25  25-kDa synaptosome-associated protein
SNAPs  Soluble NSF attachment proteins
SNARE  Soluble N-ethylmaleimide sensitive factor attachment protein receptor
Sos  Son of sevenless
SSV  Small synaptic vesicle
Tris  Tris (hydroxymethyl) aminoethane
Trk  Tropomysin related kinase
VDCC  Voltage-dependent calcium channel
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Chapter 1
1. Introduction

A chemical synapse is structurally a presynaptic nerve terminal, a synaptic cleft and a postsynaptic membrane. Chemical synapses are able to send signals from the presynaptic terminal to the postsynaptic membrane by releasing a neurotransmitter that diffuses across the synaptic cleft and activates the corresponding receptor at the postsynaptic membrane. Accordingly the regulation of neurotransmitter release from the presynaptic nerve terminal is paramount to ensure the postsynaptic membrane is activated to the degree intended. The presynaptic nerve terminal regulates neurotransmitter release by storing the neurotransmitter in small synaptic vesicles (SSVs). Consequently, the release of neurotransmitter into the synaptic cleft can only occur when SSVs fuse with the plasma membrane of the presynaptic nerve terminal. This fusion step of the two plasma membranes of SSVs and presynaptic nerve terminal requires an energy barrier to be overcome.

When an action potential arrives at the presynaptic nerve terminals it opens the Na\(^+\) channels that allow Na\(^+\) entry into the cytosol causing depolarisation. Most importantly depolarisation of the plasma membrane activates voltage-dependent calcium channels (VDCCs) causing influx of calcium (Ca\(^{2+}\)) ions into the cytosol. Depolarisation is terminated by the inactivation of the Na\(^+\) channels followed by efflux of K\(^+\) ions through K\(^+\) channels which returns the plasma membrane to its resting state.

The influx of Ca\(^{2+}\) plays a critical role in the mobilisation, docking, priming and fusion of SSVs, this is a process known as exocytosis. Following exocytosis Ca\(^{2+}\) also plays a critical role in a process of initiation, curvature of the vesicle, scission of the budding vesicle from the plasma membrane and uncoating of adaptor proteins occurs which is known as endocytosis.
The synaptic signal can be boosted by facilitating neurotransmitter release and diminished by inhibiting neurotransmitter release processes known as neuromodulation. There are numerous sites of neuromodulation in the processes of depolarisation, exocytosis and endocytosis. Depolarisation can be modulated by the activation or inhibition of the $K^+$ channels which leads to the increase or decrease of nerve terminal excitability and thereby alteration of $Ca^{2+}$ influx. Furthermore, direct and indirect modulation of VDCCs by presynaptic receptors and signalling cascades regulate the level of $Ca^{2+}$ influx. This is important as it has great effect on the downstream processes of exocytosis and endocytosis. This system allows for modulation at multiple sites and integrates signalling of numerous signalling cascades involving kinases and phosphatases that can regulate the release of neurotransmitter from the presynaptic nerve terminals.

1.1. Neurotransmitter Release

1.1.1. Exocytosis

In the nerve terminals SSVs are compartmentalised into distinct juxtapositioned pools. The ready-releasable pool (RRP) contains SSVs that are docked and primed and ready to undergo the fusion process. The reserve pool (RP) is important in sustaining neurotransmitter release upon repetitive stimulation. Finally the largest pool of SSVs is the resting pool that could be involved in spontaneous release of neurotransmitter but is not involved in the $Ca^{2+}$-dependent release (Fredj & Burrone, 2009). Together the RRP and RP vesicle pools are referred to as the recyclable pools as only the SSVs from these two pools can be mobilised and release neurotransmitter in a $Ca^{2+}$-dependent manner. Upon depolarisation the SSVs in the RRP exocytose but the SSVs in the RP have to be mobilised, docked and primed before they can fuse with the membrane.
The mobilisation of SSVs from the RP to the RRP occurs through the dissociation of SSVs from actin filaments. When the nerve terminals are in the resting state the SSVs in the RP are tethered to actin filaments by synapsins. There are three forms of synapsin I, II and III of which synapsin I and II are the most abundant in the presynaptic nerve terminals. At the presynaptic terminals synapsin I is localised on the cytoplasmic side of the SSVs membranes (De et al., 1983) and it is the ability of synapsin I to interact with actin and SSV membranes that tethers the SSVs and forms the RP (Petrucci & Morrow, 1987). Synapsin I interacts with the SSVs and cytoskeletal elements in a phosphorylation-dependent manner thereby allowing for the modulation of the tethering of the SSVs to actin (Sihra et al., 1989). The importance of synapsin I and II maintaining the RP has been shown in synapsin I or synapsin I and II knockout mice in which the size and mobilisation of the RP was impaired while endocytosis and repriming of SSVs were unaffected (Ryan et al., 1996;Chi et al., 2001;Lonart & Simsek-Duran, 2006). In other studies, it has been shown that Ca\(^{2+}\) influx and brain derived neurotrophic factor (BDNF) are able to enhance neurotransmitter release through increased synapsin I phosphorylation (Nichols et al., 1990;Jovanovic et al., 1996;Jovanovic et al., 2000).

Function of synapsin I can be modulated through seven sites that can be phosphorylated and dephosphorylated. At the N terminus Ser 9 (site 1) is phosphorylated by cAMP-dependent protein kinase (PKA) and Ca\(^{2+}\)/calmodulin-dependent protein kinase I (CaMKI). Furthermore, Ser 62 (site 4) and Ser 67 (site 5) are also localised on the N terminus and are phosphorylated by extracellular signal-regulated kinase 1 & 2 (ERK1 & ERK2). At the C terminus Ser 566 (site 2) and Ser 603 (site 3) are phosphorylated by CaMKII. Ser 551 (site 6) is phosphorylated by ERK1 and ERK2 and cyclin-dependent kinase 5 (CDK5) while Ser 553 (site 7) is phosphorylated by CDK5 (Matsubara et al.,
Sites 1, 2 and 3 are dephosphorylated by protein phosphatase 2A (PP2A) while sites 4, 5 and 6 are dephosphorylated by calcineurin (Jovanovic et al., 2001).

It has generally been observed that phosphorylation of synapsin I reduces its affinity for actin and/or SSVs membrane thus making more SSVs available for exocytosis. While dephosphorylation of synapsin I has the opposing effect and enhances its affinity for actin or SSVs membrane and thereby decreases SSV availability for exocytosis (Jovanovic et al., 2001). Depolarisation of the nerve terminal causes Ca\(^{2+}\) influx that can result in the activation of CaM Kinases, ERK1 and ERK2 and CDK5 which can phosphorylate synapsin I at their target sites. However, it is the strength of the stimulation that determines the phosphorylation state of synapsin I at the phosphorylation sites. It has been shown that CaM Kinases phosphorylate sites 1, 2 and 3 better at weaker stimulations but site 3 can be phosphorylated by stronger stimulations. The ERK kinases phosphorylate sites 4, 5 and 6 at weaker stimulations but at stronger stimulations these sites are dephosphorylated by calcineurin (Chi et al., 2003). Consequently, it seems that only CaM Kinases phosphorylation at sites 1, 2, 3 operates to reduce synapsin I affinity for actin filaments and SSV membranes and thereby increase SSVs availability for exocytosis during normal (weak) depolarisation (Benfenati et al., 1992). Although ERK1 and ERK2 phosphorylate synapsin I at sites 4, 5 (but not at site 6) results in the reduction of synapsin I affinity for actin filaments. During strong depolarisation it is thought calcineurin activation leads to sites 4 and 5 being dephosphorylated and thereby increases synapsin I affinity for actin filaments.

After mobilisation, the SSVs are docked at the active zone by a process that involves bringing SSVs in close proximity to the plasma membrane and VDCCs. The active zone is organised by cytoskeletal multi domain proteins such as piccolo, bassoon and Rab3A
interacting molecule (RIM 1α). Piccolo and bassoon knockout mice show that these proteins are important in the function of maintaining SSV clusters but have no effect on regulating neurotransmitter release.

RIM1α and RIM2α knockout study shows that the RIM proteins are essential for neurotransmitter release and that they compensate each other’s function (Schoch et al., 2006). The reason for this is that RIM1α is able to interact with various proteins that are necessary for neurotransmitter release. RIM1α has been shown to interact directly with the β subunit of VDCCs that mediates Ca^{2+} influx thus anchor vesicles near the VDCCs (Kiyonaka et al., 2007). There are some reports that suggest rabphilin and RIMs are effectors for Rab3A (Deak et al., 2006; Foletti et al., 2001). Rab3A is highly concentrated on the membranes of SSVs and is a key protein (Geppert et al., 1994). The cycling between the Rab3A-GTP form found on the vesicle membrane, and cytosolic Rab3A-GDP form, is a crucial step prior to exocytosis (Stahl et al., 1994). However, other studies argue that rabphilin and RIM are not specific effectors of Rab3A (Fukuda, 2004). Interestingly, synapsin I is another effector for Rab3A (Giovedi et al., 2004), with the two proteins mutually modulating each other’s function (Lonart & Simsek-Duran, 2006).

Furthermore, RIM 1α interacts with Munc 13-1 through the highly conserved zinc finger domains (Koushika et al., 2001). Munc13-1 is a brain-specific presynaptic phorbol ester receptor (Augustin et al., 1999) and is known to be critical in the maturation of SSVs, as neurotransmitter release from Munc13-1 deficient neurons is abolished when triggered by action potentials (Augustin et al., 1999). Furthermore, Munc 13-1 has a C_{2}B domain that is able to bind to two Ca^{2+} ions which facilitates neurotransmitter release (Shin et al., 2010). This interaction between RIM and Munc13
promotes Munc 13-1 interaction with another key protein in the docking of SSVs known as Munc 18-1. Its importance to neurotransmitter release is highlighted by studies which have shown a complete abolition of neurotransmitter release following the deletion of Munc18-1 at the synapse (Verhage et al., 2000).

After docking the SSVs undergo priming which makes them fusion competent and this involves soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins that form a tight four-helix SNARE complex. There are three SNARE proteins thought to be involved in the fusion competence of SSVs which include syntaxin 1, 25kDa synaptosomal-associated protein (SNAP-25) and synaptobrevin (also known as VAMP). Syntaxin 1 and SNAP25 are plasma membrane bound and are referred to as Qa, Qb-Qc – SNAREs respectively while synaptobrevin is bound to the vesicle membrane and is referred to as R-SNARE. This classification is based upon the critical contribution of three glutamines (Q) from syntaxin 1 and SNAP25 and one arginine (R) from synaptobrevin to the formation of a tight four-helix SNARE complex (Rizo & Sudhof, 2002). Studies conducted using tetanus and botulinum-B neurotoxins show that disruption to the formation of the tight four helix SNARE complex results in the inhibition of the neurotransmitter release (Schiavo et al., 1992) (Lawrence & Dolly, 2002).

It is thought that the priming sequence is initiated by the interaction between Munc 13-1 and Munc 18-1. In the resting state Munc 18-1 is bound to syntaxin 1 which conforms syntaxin 1 into a ‘closed’ state. Munc 13-1 interaction with Munc 18-1 leads to the dissociation of Munc18 and syntaxin 1 complex which brings about a conformational change in syntaxin 1 from a ‘closed’ state to an ‘open’ state (Rizo & Sudhof, 2002) thus making it possible for syntaxin 1 to interact with the other SNARE proteins. The
SNARE proteins can interact with other SNARE proteins through the SNARE motif that they possess. Syntaxin 1 which has a single SNARE motif, SNAP25 which has two SNARE motifs and synaptobrevin with a single SNARE motif. It is important in understanding how the SNARE complex forms; syntaxin 1, when it is in its ‘open’ formation has a favourable interaction with SNAP25 to form a binary product. The binary complex allows for the R-SNARE to interact with the Q-SNAREs which forms a loose SNARE complex (Rizo & Sudhof, 2002; Jahn & Scheller, 2006). It is the subsequent regulatory interactions with proteins such as complexins and synaptotagmin that help to form the tight four helix SNARE complex (Rizo & Sudhof, 2002; Jahn & Scheller, 2006; Tang et al., 2006).

After the SSVs are primed, depolarisation of the presynaptic membrane causes an influx of Ca^{2+} through VDCC which leads to the SSVs fusing with the plasma membrane to release neurotransmitter into the synaptic cleft. The exact mechanism as to how fusion occurs is still unclear and it is also debated whether SSVs only undergo full-fusion or also can partially fuse through a ‘kiss-and-run’ mechanism (Gandhi & Stevens, 2003; He et al., 2006).

There are numerous proteins that have been implicated in controlling synaptic fusion including synaptotagmins and complexins. Synaptotagmins have the N-terminus in the SSV membrane with two C-terminal C2 domains that are known as C2A and C2B facing the cytosol (Perin et al., 1991). There are 15 isoforms of synaptotagmins of which only synaptotagmin 1, 2, 3, 5, 6, 7, 9 and 10 are capable of binding to Ca^{2+}. These synaptotagmins are thought to act as Ca^{2+} sensors through their two binding sites for Ca^{2+} at the C2 domains that interact with both SNAREs and acidic phospholipids in a Ca^{2+} dependent manner (Pang et al., 2006). Synaptotagmin 1, 2 and 9 are found on the
SSV membrane and is thought to play the triggering role in fast/synchronous Ca\(^{2+}\) triggered neurotransmitter release (Xu et al., 2007b) and also spontaneous release (Xu et al., 2009).

In order for fusion to occur effectively, synaptotagmin 1 undergoes a conformational change from the cis-conformer to the trans-conformer depending on the Ca\(^{2+}\) concentration. The cis-conformation of synaptotagmin 1 occurs when it is bound to negatively charged phospholipids on the synaptic vesicle membrane and this is the deactivated state of synaptotagmin 1 under resting (1µM) and high (100µM) Ca\(^{2+}\) concentration. The trans-conformation is the converse in which synaptotagmin binds to phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) found in the plasma membrane under physiological Ca\(^{2+}\) concentrations (10µM) and thereby promotes fusion (Lee et al., 2010). Synaptotagmins interact with the C-terminus of SNAP-25 and the disruption of this interaction causes an impairment of fast/synchronous Ca\(^{2+}\) triggered exocytosis (Zhang et al., 2002).

Complexins are small cytosolic proteins that are found to have stimulatory and inhibitory effect on neurotransmitter release depending on the domain of complexins that interact with the SNARE complex (Xue et al., 2007). There is evidence to suggest that the N-termini of the complexins have facilitatory function in fast/synchronous exocytosis (Xue et al., 2010). In addition, the accessory α-helix of complexins has an inhibitory function in fusion by displacing synaptobrevin 2 and thus weakening the SNARE complex (Lu et al., 2010). Furthermore, there is evidence that suggests that complexins and synaptotagmins compete with each other for binding to SNARE complexes and that complexins displacement by synaptotagmins promote SSV fusion (Tang et al., 2006; Maximov et al., 2009).
The last step following exocytosis and before endocytosis is the dissolution of the SNARE complex by N-ethylmaleimide-sensitive factor (NSF) and its cofactor SNAPs. It is still unclear how NSF and SNAP are able to disassemble the SNARE complex but this is clearly an energy dependent step requiring the input of ATP (Jahn & Scheller, 2006) and may in part underlie the ATP-dependence of exocytosis.

1.1.2. Endocytosis

Endocytosis is important in allowing a continuous neurotransmitter release under repetitive stimulation conditions, which would otherwise deplete the RRP. There are several forms of endocytosis that are implicated in the nerve terminal including those described as rapid, bulk and slow. Rapid endocytosis is clathrin-independent and occurs under intense stimulation (Artalejo et al., 1995). Bulk endocytosis is triggered under intense activity and is characterised as a clathrin independent process that internalises a large area of the plasma membrane than that required for SSVs formation (Wu & Wu, 2007; Clayton & Cousin, 2009). The dominant form of endocytosis that is found in the nerve terminals is slow endocytosis which is clathrin-dependent (Granseth et al., 2006). The main process by which the SSV are recycled through the latter mechanism occurs in four stages: clathrin recruitment, curvature of the membrane, scission of the vesicular bud and finally un-coating of clathrin.

Recently, studies have been conducted that indicate that the initiation of endocytosis is by the influx of Ca\(^{2+}\) that activates calmodulin. Furthermore, it has been suggested that at lower stimulation strengths slow endocytosis is observed which gives way to rapid endocytosis with increasing stimulation strengths while even higher stimulation causes bulk endocytosis (Wu et al., 2005; Wu et al., 2009a). The source for the influx of Ca\(^{2+}\) is neither through VDCCs nor does the Ca\(^{2+}\) from the exocytosis contribute to
endocytosis. Interestingly, a protein called Flower is localised on SSVs and the plasma membrane which has Ca\textsuperscript{2+} channel properties. At rest Flower, is localised to the SSVs but is inserted into the plasma membrane at sites for endocytosis after full fusion of SSVs. This concentration of Flower at the site of endocytosis leads to an increase in the influx of Ca\textsuperscript{2+} to initiate endocytosis and is again localised to SSVs after fission (Yao et al., 2009).

The assembly of clathrin on the membrane is achieved by plasma membrane specific adaptors such as the complex AP-2 and AP180. Both of these adaptor proteins interact with clathrin and coordinate the assembly of the clathrin-coated pits with cargo proteins and lipids (Di & De, 2006). Furthermore, SV2A which are localised to the SSVs are important in reconstitution of synaptotagmin 1 to recycled SSVs membranes (Yao et al., 2010). AP-2 is able to interact with synaptotagmin 1 (Zhang et al., 1994b) while synaptobrevin interacts with AP180 and provides another important function in reconstitution of SSVs proteins into the recycling SSVs membranes (Bao et al., 2005). Lack of SSV protein reconstitution can cause disruptions to rapid exocytosis and endocytosis as has been shown in synaptobrevin 2 knockout mice (Deak et al., 2004).

After the clathrin-coated pits have been formed the curvature of the membrane is initiated by epsin (Ford et al., 2002). This curvature recruits BAR domain proteins that include amphiphysin 1 (Wu et al., 2009b), endophilin A1 and syndapin 1 (Andersson et al., 2008). Amphiphysin 1 at the membrane binds to clathrin coated pits and induce further curvature of the SSVs membrane following dephosphorylation by calcineurin (Bauerfeind et al., 1997). The importance of the BAR domain has been highlighted in a report that shows the selective disruption to the endophilin BAR domain inhibits endocytosis (Andersson et al., 2010).
BAR domain proteins, once dephosphorylated recruit dynamin 1 to the neck of the budding vesicle through the interaction of a src-3 homology (SH3) domain and a proline-rich domain (PRD) (Shupliakov et al., 1997). Dynamin 1 is GTPase that requires dephosphorylation by calcineurin to be activated. When dynamin-GTP is hydrolysed, it constricts the size of the neck of the budding SSV and results in the scission of the vesicular bud (Hinshaw, 2000). Dynamin 1 is also regulated by CDK5 which phosphorylates dynamin 1 at Ser774 and Ser778 to reduce endocytosis (Tan et al., 2003; Evans & Cousin, 2007). Furthermore there is evidence that CDK5 is able to also regulate endocytosis by phosphorylating amphiphysin 1 (Tomizawa et al., 2003).

Finally, free clathrin-coated SSVs undergo rapid uncoating supported by ATP-hydrolysing hsc7027 and its chaperones, G-dependent kinase and auxilin. Auxilin knockout mice express impaired clathrin-dependent endocytosis which results in defective synaptic transmission (Yim et al., 2010). The newly formed SSVs can then be filled with the neurotransmitter at a concentration that is dependent on the vesicular neurotransmitter concentration (Wu et al., 2007) and enter the general recycling pool but not the RRP (Wu & Wu, 2009).

1.2. Regulation of neurotransmitter release by modulation of excitability

Neurotransmitter release can be controlled by bringing the membrane potential closer to the depolarisation threshold thus making the plasma membrane more excitable. Conversely, the membrane potential can be moved further from the depolarisation threshold thus making the plasma membrane less excitable.
1.2.1. Sodium channels

There are two types of Na\(^+\) channels, ligand-gated ion channels and voltage-gated sodium channels both of which conduct Na\(^+\) ions across the membrane. The voltage-gated sodium channels have an important role in initiating and propagating the action potential down the axon. The three most important functional features of the voltage-gated sodium channels include voltage-dependent activation, rapid inactivation and selective ion conductance (Catterall et al., 2005). Furthermore, voltage-gated sodium channels are thought to have three states, an open state when they are activated and two closed states that include deactivated and inactivated (Yu & Catterall, 2003).

The voltage-gated sodium channels can also be modulated by different kinases. There is evidence that shows that phosphorylation by protein kinase C (PKC) at site Ser1506 slows inactivation and reduces peak currents of voltage-gated sodium channels (West et al., 1991; Li et al., 1993). Furthermore, activation of the protein kinase A (PKA) pathway is also known to reduce Na\(^+\) currents by accelerating the inactivation state of voltage-gated sodium channels from an open state (d'Alcantara et al., 1999).

1.2.2. Potassium channels

There are four types of potassium channels that include voltage-gated potassium channels, Ca\(^{2+}\)-activated potassium channel, inwardly rectifying potassium channel, tandem pore domain potassium channel and voltage-gated potassium channels. The voltage-gated potassium channels that include the Shaker channels are important in returning the plasma membrane potential to its resting state (Gutman et al., 2005). The Shaker channels also exist in different states when they are activated they are in the ‘open’ conformation but they also have two inactive states. In one inactive state the channel can open if the membrane potential becomes positive inside. The other inactive
state is known as the N-type inactive state occurs by a ‘ball-and-chain’ mechanism and is known to inactive the channel even in conditions that should give a positive effect on the channel (Cuello et al., 2010).

The Shaker channels have phosphorylation sites for PKA. In a study the authors found a phosphate mechanism that slows the kinetics of N-type inactivation but this rate can be reversed by PKA activation (Drain et al., 1994). In addition, PKC activation has been shown to inhibit the Shaker K+ current which could possibly be due to a novel mechanism as PKC does not affect the gating or promote the internalisation of the channel (Boland & Jackson, 1999).

1.2.3. Voltage-dependent Ca2+ channels (VDCCs)

The VDCCs do not regulate the excitability of the plasma membrane but are activated by the depolarisation of the plasma membrane to cause the influx of Ca2+. The importance of Ca2+ entry via VDCCs in neurotransmitter release was first established by the experiments conducted by Bernard Katz. It has now been established there are L-type, P/Q-type, N-type and R-type Ca2+ channels which are activated by high-voltage depolarisation of the plasma membrane. These types are also known as Ca,1.1 to Ca,1.4, Ca,2.1, Ca,2.2 and Ca,2.3, respectively in modern nomenclature. In addition, there are a T-type Ca2+ channels which is activated by low-voltage depolarisation of the plasma membrane, these also being known as Ca,3.1, Ca,3.2 and Ca,3.3 (Catterall et al., 2005).

All the Ca2+ channel subtypes share a common structure in having four or five distinct subunits; α1, α2δ complex, β and γ. The main pore-forming subunit is the α1 also being the voltage sensor and gating apparatus and most of the known sites of channel
regulation by second messengers, drug and toxins (Catterall & Few, 2008). The $\alpha_1$ is composed of four homologous domains and each one of these domain contain six transmembrane helices (conventionally labelled from S1 to S6) and a membrane-associated P-loop between S5 and S6. Through various structure and function studies it has been established that helices S1 to S4 function as voltage sensors while S5 and S6 function as the pore (Catterall, 2000; Catterall & Few, 2008). The other subunits of the Ca$^{2+}$ channels ($\alpha_2\delta$ complex, $\beta$ and $\gamma$) are often referred to as the auxiliary subunits. These auxiliary subunits provide a major contribution to Ca$^{2+}$ channel trafficking and also influence the biophysical properties of the channels (Dolphin, 2009). Furthermore, it has been hypothesised that the antiepileptic and antinociceptive drugs bind to the $\alpha_2\delta$ subunit to perhaps disrupt the trafficking of Ca$^{2+}$ channels to the plasma membrane (Hendrich et al., 2008; Bauer et al., 2010).

Ca$_{2.1}$ and Ca$_{2.2}$ can regulate neurotransmitter release by interacting with exocytotic and endocytotic machinery. There is also suggestion that in neurotransmitter release Ca$_{2.1}$ channels play a more important role than Ca$_{2.2}$ channels (Ambrosio et al., 1997). Neuromodulation by Ca$^{2+}$ channels is capped because the Ca$_{2.1}$ and Ca$_{2.2}$ channels are limited by the binding sites to channel proteins that give them capacity to participate in neurotransmission. Ca$_{2.1}$ could interact with these sites through a synaptic interaction site (synprint) motif in the II-III loop while Ca$_{2.2}$ can interact with its specific sites and compete with Ca$_{2.1}$ channel sites independent of synprint interaction (Cao et al., 2004; Cao & Tsien, 2010). There have been studies which show that using specific antagonists for the Ca$_{2.1}$ and Ca$_{2.2}$ channels ($\omega$-agatoxin IVA and $\omega$-conotoxin GIVA, respectively) block of synaptic currents is more than 100% (Reid et al., 1997). This suggests that there are two VGCCs that are localised in the presynaptic
nerve terminals that include both Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 and that nerve terminals have a non-uniform distribution of these channels (Reid et al., 2003).

It has been shown that Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 channels colocalise with syntaxin-1 and SNAP-25 at the synprint found between the intracellular loop between domains II and III of the Ca\textsuperscript{2+} channel \(\alpha_1\) subunit (Yokoyama et al., 2005; Keith et al., 2007) (Catterall & Few, 2008). In addition, synaptotagmin 1 and 2 have binding sites at the \(\alpha_1\) domain of Ca\textsubscript{v}2.1 channels (Charvin et al., 1997) and the C\textsubscript{2}B domain of synaptotagmin 1 is also able to interact with Ca\textsubscript{v}2.2 channels (Sheng et al., 1997). Furthermore, it has been reported that RIM1 interacts with the \(\beta\) subunit of the Ca\textsuperscript{2+} channels (Kiyonaka et al., 2007). These interactions have led to the suggestions that the Ca\textsuperscript{2+} channels are anchored at the active zone to facilitate SSV fusion. SNARE proteins can regulate the activation properties of Ca\textsuperscript{2+} channels as syntaxin-1 or SNAP-25 binding to synprint is known to cause a negative shift in the activation of Ca\textsuperscript{2+} channels. The inhibitory effect of syntaxin-1 is relieved via SNAP-25 binding and the inhibitory effect of SNAP-25 is relieved via synaptotagmin 1 binding. This regulatory mechanism could ensure that only when the docked SSVs are in place does the Ca\textsuperscript{2+} channel activity increase to initiate neurotransmitter release. There is evidence to suggest the involvement of Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 channels in endocytotic mechanisms. For example, it has been reported that synprint binds to the \(\mu\) subunit of AP-2 and that AP-2 \(\mu\) subunit competes with synaptotagmin 1 is a Ca\textsuperscript{2+}-dependent manner (Watanabe et al., 2010).

Repetitive activation of Ca\textsuperscript{2+} channels can alter neurotransmission in the long term through a process known as short-term synaptic plasticity. This can be in the form of synaptic enhancement through processes termed facilitation, augmentation and posttetanic potentiation (PTP) or synaptic depression (Catterall & Few, 2008; Park &
Kim, 2009). Short-term plasticity in the synaptic terminals is due to the Ca^{2+} accumulation from repetitive stimulations which result in the activation of Ca^{2+} binding proteins that bind to the Ca_{2.1} channel (Mochida et al., 2008). On the other hand, synaptic depression at the presynaptic terminal maybe due to inactivation of Ca_{2.1} by Ca^{2+} binding proteins (Xu et al., 2007a; Mochida et al., 2008).

1.3. Regulation of neurotransmitter release by presynaptic receptors

There are also numerous ionotropic and metabotropic presynaptic receptors that are able to facilitate and inhibit neurotransmitter release. Ionotropic receptors are permeable to ions that determine the membrane potential thus changes in permeability through ionotropic receptor activation can modulate neurotransmitter release. Metabotropic receptors modulate neurotransmitter release by activating second messenger signalling cascades.

1.3.1. Glutamate receptors

The principle excitatory neurotransmitter in the CNS is glutamate; highlighted by the fact that 60% of all neurons are glutamatergic. Glutamate is known to play important roles in learning and memory consolidation in synaptic plasticity processes (Paoletti & Neyton, 2007). There are two families of glutamate receptors: ionotrophic glutamate receptors (iGluRs) which are involved in fast neurotransmission and metabotropic glutamate receptors (mGluRs) that produce their effects through G-protein mediated signalling (Mayer, 2005).

There are three iGluRs that include N-methyl-D-aspartic acid (NMDA), \(\alpha\)-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate receptors. NMDA, AMPA and kainate receptors are found presynaptically and postsynaptically. The
NMDA receptors can allow Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\) to pass into the cell but upon activation the NMDA receptors causes a significant increase in Ca\(^{2+}\) concentration which allows NMDA receptors to play a significant role in mechanisms such as synaptic plasticity. Interestingly, the NMDA are blocked endogenously by Mg\(^{2+}\) and require membrane depolarisation to lift this inhibition (Paoletti & Neyton, 2007).

AMPA receptors are permeable to Na\(^{+}\) and K\(^{+}\) ions but Ca\(^{2+}\) permeability occurs in the absence of the GluR2 or with non-edited GluR2 subunits. Given that native AMPA receptors have the GluR2 thus upon activation they allow Na\(^{+}\) ions to enter which depolarises the cell to lift the Mg\(^{2+}\) inhibition on NMDA receptors (Santos et al., 2009). Furthermore, the AMPA receptors have metabotropic properties and are able to activate G proteins (Wang et al., 1997; Schenk & Matteoli, 2004).

Kainate receptors are permeable to Na\(^{+}\) and K\(^{+}\) ions and have the same conductance for these ions as the AMPA receptors but over a shorter duration (Huettner, 2003). The activation of presynaptic kainate receptors has a facilitatory effect on glutamate release (Perkinton & Sihra, 1999) and GABA release from GABAergic nerve terminals (Mathew et al., 2008; Mathew & Hablitz, 2008). Kainate receptors are also thought to have metabotropic properties and are thought to couple to G\(_{i/o}\) protein in hippocampal interneurons (Rodriguez-Moreno & Sihra, 2007).

There are eight mGluRs receptors including mGluR1 and mGluR5 (group I), mGluR2 and mGluR3 (group II) and mGluR4, mGluR6, mGluR7 and mGluR8 (group III) (Enz, 2007). Group I and group II mGluRs are localised pre- and postsynaptically while group III are localised presynaptically. All mGluRs are seven transmembrane proteins with an external N – terminus that binds agonists and antagonists, a cytosolic C – terminus and
three intracellular loops that together serve to activate cognate G proteins (Enz, 2007). Group I mGluRs are positively linked to PLC through the G$_q$/G$_{11}$ proteins, whereas both group II and III mGluRs are negatively linked to adenylate cyclase through the G$_i$/G$_o$ proteins.

Neurotransmitter release is increased when presynaptic mGluR1/5 receptors are stimulated in glutamatergic nerve terminals (Herrero et al., 1992). These receptors have different affinity for glutamate with mGluR1 having low affinity while mGluR5 has a high affinity in mouse cortical nerve terminals (Musante et al., 2008). However, there is also other evidence that suggests that mGluR1 receptors are not involved in the facilitation of glutamate release (Sistiaga et al., 1998). Interestingly in this regard, the mGluRs can undergo a functional switch in which the facilitatory effect turns to inhibition of glutamate release following receptor desensitisation (Rodriguez-Moreno et al., 1998; Herrero et al., 1998).

The group III glutamate receptors can have a facilitatory or inhibitory effect on evoked glutamate release. Specifically, presynaptic mGluR7 is found to colocalise and crosstalk with other presynaptic receptors (Ladera et al., 2007). The inhibitory effect of mGluR7 can be through Ca$^{2+}$ channel inhibition or decreasing cAMP levels (Millan et al., 2002). Furthermore, mGluR7 is also found to colocalise with Ca$_{2.2}$ channels and activation of mGluR7 inhibits Ca$^{2+}$ influx supporting component glutamate release (Vazquez & Sanchez-Prieto, 1997; Millan et al., 2003). In addition, mGluR7 is also able to inhibit Ca$_{2.1}$ channels through a PKC independent pathway in hippocampal neurons (Martin et al., 2007). The facilitatory effect of mGluR7 on glutamate release is through PLC activation via a pertussis toxin insensitive G-protein after prolonged activation (Martin et al., 2010).
1.3.2. GABA receptors

The major inhibitory neurotransmitter in the central nervous system is $\gamma$-Aminobutyric acid (GABA) that is released from GABAergic nerve terminals. GABA is then able to activate the inhibitory synapses by activating the postsynaptic GABA_A and/or GABA_B receptors but also modulate release of neurotransmitter by presynaptic localisation.

GABA_A receptors are ligand-gated ion channels that are permeable to Cl⁻ anions when GABA molecules bind to two GABA binding sites. GABA_A receptors are pentamers with the subunits deriving from six types of $\alpha$ subunits, three types of $\beta$ subunits, three types of $\gamma$, $\delta$, $\epsilon$, $\theta$ and $\pi$ subunits. Furthermore, there are three $\rho$ subunits that form a receptor and are known as variant of the GABA_A receptor called GABA_A-$\rho$ receptors. The most abundant combination is the $(\alpha_1)_2 (\beta_2) 2\gamma_2$ formation, the minimum requirement for GABA_A receptor function is the inclusion of $\alpha$ and $\beta$ subunits. Different combinations of these subunits forming GABA_A receptors determine the receptor’s agonist affinity, channel conductance and regulation by allosteric modulators.

Benzodiazepines are positive modulators of GABA_A receptors and bind at the interface of $\alpha$ and $\gamma$ subunits. However, benzodiazepine activity only occurs in $\alpha$ subunits that contain a histidine amino acid residue which only includes $\alpha_1$, $\alpha_2$, $\alpha_3$ and $\alpha_5$ subunits (Wafford et al., 2004). Thus, GABA_A receptors that contain $\alpha_4$ or $\alpha_6$ in a combination with $\beta$ and $\gamma_2$ subunits are insensitive to benzodiazepine modulation (Olsen & Sieghart, 2008).

Activation of GABA_A receptors on presynaptic nerve terminals have been shown to be inhibitory or stimulatory. This is likely determined by the differences in the composition of the receptors at the presynaptic nerve terminals. Furthermore, the expression of Cl⁻ transporters such as NKCC1 also has significant effect on the stimulatory or inhibitory
actions of GABA_A receptors. In cortical presynaptic nerve terminals, GABA_A receptor activation decreases depolarisation evoked glutamate release but has no effect on spontaneous glutamate release (Long et al., 2009). In contrast, presynaptic GABA_A receptors in hippocampal neurons facilitate spontaneous glutamate release via depolarisation of the plasma membrane (Jang et al., 2006).

GABA_B receptors are metabotropic and mediate their effects via the activation of G_{i/o} proteins and downstream signalling. However, GABA_B receptors have a different structure from many other seven transmembrane GPCRs in that they exist and function as heterodimers consisting of GABA_B1 and GABA_B2 subunits. This was discovered due to the differences in the affinity of drugs between the native GABA and recombinant GABA_B1 receptors. The GABA_B2 subunit is a seven transmembrane with an N-terminus and it functions to traffic the GABA_B1 subunit to the plasma membrane and link it to the G protein, while GABA_B1 subunit is required for receptor activation. At the plasma membrane these two subunits need to be linked to form a functional GABA_B receptor as GABA_B2 subunit in the absence of GABA_B1 subunit cannot function as an autonomous receptor. The mechanism of GABA_B receptor activation is triggered by agonist binding to the GABA_B1 subunit which causes a conformational change that directs GABA_B2 subunit stimulation leading to G_{i/o} protein activation (Bowery et al., 2002; Bettler et al., 2004). Furthermore, there are also K^+ channel tetramerization domain-containing proteins that act as auxiliary subunits. The assembly of K^+ channel tetramerization domain-containing proteins appear essential for GABA_B G protein signalling and agonist potency of GABA_B receptors (Schwenk et al., 2010).

When GABA_B receptors are activated at the presynaptic nerve terminals they mediate their effects that include inhibition of VDCCs, inhibition of G protein-coupled inwardly
rectifying K\(^+\) (GIRK) channels and modulation of cAMP levels. As GABA\(_B\) receptors couple to G\(_{i/o}\) protein they can inhibit adenylate cyclase thereby decreasing cAMP levels which reduces the activation of protein kinase A (PKA). However, it is unlikely that glutamate release inhibition observed with GABA\(_B\) receptor agonist such as baclofen is due to the down regulation of PKA. Rather a number of studies have shown that GABA\(_B\) reduces glutamate release by inhibiting VDCCs, in particular Ca\(_{2.1}\) channels, and that this inhibition is pertussis toxin sensitive (Wu & Saggau, 1995; Santos et al., 1995; Takahashi et al., 1998; Wang et al., 2004b). While many studies have suggested that inhibition of VDCCs is the primary mechanism by GABA\(_B\) inhibits glutamate release, there other studies that suggest GABA\(_B\) receptors are able to open GIRK channels that would hyperpolarise the neurons and thereby reduce glutamate release. Thus GIRK2 and GIRK3 channels or shown to colocalise with GABA\(_B\) receptors but this inhibition is not pertussis toxin-sensitive (Ladera et al., 2008; Fernandez-Alacid et al., 2009).

1.3.3. Adenosine receptors

Over the years a modulatory role of adenosine receptors in glutamatergic nerve terminals has become increasingly apparent. There are pathways by which adenosine is released into the synaptic cleft to activate the adenosine receptors. Firstly an increase in cAMP levels causes its breakdown by phosphodiesterases to form more 5′-AMP which is further broken down by cytosolic 5′-nucleotides to form adenosine. As the cytosolic concentration of adenosine increases, it diffuses through the nucleotide transporters from the cytosolic to the synaptic cleft. These nucleotide transporters equilibrate the concentration of adenosine across the plasma membrane thus if adenosine concentration in the synaptic cleft is high then adenosine passively diffuses into the cytosol (Wang &
Sihra, 2003). The second pathway that has been suggested is the release of adenosine in an activity dependent manner with the neurotransmitter release (Xia et al., 2009).

All the adenosine receptors are seven transmembrane GPCRs that include $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. Thus, all adenosine receptors are coupled to $G$ proteins, $A_1$ and $A_3$ receptors are coupled to the $G_{i/o}$ protein while $A_{2A}$ and $A_{2B}$ receptors are coupled to the $G_s$ protein (Fredholm et al., 2001; Dunwiddie & Masino, 2001; Fredholm et al., 2005). The $G$ proteins that adenosine receptors are coupled to determine the modulatory effect on neurotransmitter release. The adenosine receptors are distributed to various cells but only the $A_1$ and $A_{2A}$ receptors are thought to be found on presynaptic nerve terminals (Ladera et al., 2007; Sichardt & Nieber, 2007).

Activation of the $A_1$ receptors in cortical nerve terminals results in the inhibition of neurotransmitter release which is likely due to the reduction of $Ca^{2+}$ influx through $Ca_{v2.1}$ and $Ca_{v2.2}$ channels by a membrane-delimited pathway (Ladera et al., 2007; Gundlfinger et al., 2007). This is further supported by evidence which shows $A_1$ receptors negative modulation of $Ca_{v2.2}$ channels in the hippocampal nerve terminals (Manita et al., 2004) where they are abundant, especially the CA1 region (Ambrosio et al., 1997). In addition, in the presynaptic nerve terminals, $A_1$ and $A_{2A}$ receptors can form heteromers which allows the $A_{2A}$ receptors to negatively modulate the $A_1$ receptors thus giving a fine control over neuromodulation (Ciruela et al., 2006).

The $A_{2A}$ receptor has a facilitatory effect on glutamate release but requires a higher concentration of adenosine to be activated over the $A_1$ receptor (Ciruela et al., 2006). Once activated it induces the facilitation by activation of PKA and negative modulation of the $A_1$ receptor. There is evidence that $A_{2A}$ receptor enhances synaptic release in
-aged rats (21-24 months old) that express the receptor more than the $A_1$ receptor (Rebola et al., 2003).

### 1.3.4. Serotonin (5-HT) receptors

There are 14 known types of 5-HT receptors that are divided into seven classes based on their structural and functional characteristics. However, there remain some 5-HT receptors that have yet to have their function classified (distinguished by the lower case notation). The first well defined group contain receptors that are 5-HT$_1$-like that includes 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$ and 5-HT$_{1F}$ receptors. These are GPCRs that activate the $G_{i/o}$ subunit which cause the inhibition of adenylate cyclase. The second group of 5-HT receptors includes 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors which activate $G_{q/11}$ proteins to stimulate PLC activation. 5-HT$_3$ receptors are the only 5-HT receptors that belong to a family of ligand-gated ions channel and its pentameric structure is composed of two subunits, 5-HT$_3$R-A and 5-HT$_3$R-B. The other 5-HT receptor classes include 5-HT$_4$, 5-HT$_6$ and 5-HT$_7$ which also have subtypes and which are thought to activate the $G_s$ subunit to direct the activation of adenylate cyclase. 5-HT$_5$ function has not been characterised but it thought these receptors might interact with the $G_{i/o}$ or $G_s$ subunit. Presynaptically, 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_3$ receptors are localised to glutamatergic neurons in cortex and hippocampus thus are the receptors of interest in the regulation of neurotransmission (Hoyer et al., 1994;Hoyer et al., 2002;Hannon & Hoyer, 2008).

5-HT$_{1A}$ receptors are GPCRs that are coupled to the G protein $G_{i/o}$ which can decrease adenylate cyclase activation and thereby reduce intracellular cAMP levels. However, this does not appear the mechanism of action by which 5-HT$_{1A}$ receptors are able to inhibit glutamate release. Rather these receptors are able to directly inhibit $Ca_{v}2.1$ and
Ca\textsubscript{v}2.2 channels activation thus reducing Ca\textsuperscript{2+} influx during depolarisation (Lin et al., 2001) (Wang et al., 2002). This is supported by reports that show 5-HT\textsubscript{1A} receptor activation causes a reduction in glutamate release hippocampal slices and in an in vivo model that mimics ischemia, therefore highlighting the 5-HT\textsubscript{1A} receptor as a neuroprotective receptor (Mauler et al., 2001). Furthermore, presynaptic 5-HT\textsubscript{1A} receptor activation has been shown to tonically inhibit synaptic GABA release (Koyama et al., 2002).

5-HT\textsubscript{2A} receptors are also GPCRs and found on the presynaptic nerve terminals. 5-HT\textsubscript{2A} receptor activation reduces neurotransmitter release by attenuation of nerve terminal excitability by reduction of Ca\textsuperscript{2+} influx through Ca\textsubscript{v}2.1 channels (Wang et al., 2006). This is supported by another report that shows 5-HT\textsubscript{2A} receptors inhibit glutamate release from rat cerebellar mossy fibers (Marcoli et al., 2001).

5-HT\textsubscript{3} receptor activation has been shown to increase Ca\textsuperscript{2+} concentration in synaptosomes from regions that include the hippocampus, cerebellum, striatum and amygdala. This Ca\textsuperscript{2+} influx is due to the permeability of 5-HT\textsubscript{3} receptors to Ca\textsuperscript{2+} (Nayak et al., 1999). The consequence of Ca\textsuperscript{2+} influx suggests that 5-HT\textsubscript{3} could play a role in neurotransmitter release. Indeed, it has been shown that 5-HT\textsubscript{3} activation causes a transient increase in spontaneous and evoked GABA release (Turner et al., 2004) (Dorostkar & Boehm, 2007). There is also a degree of cross-talk between the 5-HT receptors as has been shown in a report that 5-HT\textsubscript{1A} receptors via G-protein activation are able to inhibit the transient facilitation of GABA release by activation of presynaptic 5-HT\textsubscript{3} receptors (Koyama et al., 2002).
1.4. Regulation of neurotransmitter release by intraterminal signalling cascades

The effect of receptor stimulation can be mediated via two pathways. The first is the membrane-delimited pathway in which the receptor directly interacts with its target and has no cytoplasmic messengers. The second pathway is the downstream activation of signalling cascades. There are numerous signalling cascades that can be themselves regulated thus allowing for cross-talk between different receptors that can lead to amplification or dampening of the signal.

1.4.1. Calmodulin

One of the consequences of Ca\(^{2+}\) entry through VGCCs during depolarisation is the activation of calmodulin (CaM) a Ca\(^{2+}\)-binding protein that is bound to membranes and soluble in the cytoplasm. CaM is sensitive to the increases in cytosolic Ca\(^{2+}\) as it has been shown that CaM in isolation binds to Ca\(^{2+}\) with an \(K_d\) value in the range of 0.5 to 5\(\mu\)M (Saimi & Kung, 2002). CaM is comprised of 4 helix-loop-helix protein folding motifs which are called EF hands. The 4 EF each hands bind to one Ca\(^{2+}\) ion and it has been shown that these sites have different affinities for Ca\(^{2+}\) which increase as Ca\(^{2+}\) ion consecutively bind to one of the EF hand sites. After two Ca\(^{2+}\) ions bind to two their sites this causes the EF hands to pull apart which exposes a hydrophobic pocket within the domain that binds to CaM target proteins (Hook & Means, 2001).

There are numerous target proteins that have been reported for CaM but the main targets for presynaptic terminals are those involved in the regulation of neurotransmitter release. CaM targets Ca\(_{v2.1}\) channels by binding to the IQ motif of the C-terminus domain of the \(\alpha1A\) which direct the inactivation of the channel (Lee et al., 1999;Lee et al., 2000;Lee et al., 2003). Furthermore, this interaction enhances recovery from inactivation and augments further Ca\(^{2+}\) influx by facilitating the Ca\(^{2+}\) current so that it is
greater after full recovery from inactivation (Kim et al., 2008). Conversely, Ca_{V.2.2} channels has IQ motifs that bind to CaM albeit with reduced affinity thus show little Ca^{2+}-dependent inactivation (Hook & Means, 2001). CaM is also shown to interact with the Munc-13 CaM binding motifs which results in the increase of priming activity (Rodriguez-Castaneda et al., 2010). Furthermore, CaM might also play a vital role in endocytosis as it is suggested that CaM initiates all forms of endocytosis which is important in the repletion of RRP (Wu et al., 2009a). CaM in presynaptic function was demonstrated in CaM knockdown study which showed that while neurons were viable, neurotransmitter release was significantly reduced an affect that was rescued via constitutive activation of CaMKII. This suggests CaM boosts synaptic release through the activation of CaMKII (Pang et al., 2010).

1.4.2. **Ca^{2+}/calmodulin-dependent kinase II (CaMKII)**

CaMKII is one of a family of homologous kinases and has broad substrate specificity and it is found in 4 isoforms that include α, β, γ and δ but only α and β isoforms are found in the nervous system (Miller & Kennedy, 1986; Hook & Means, 2001). The major consensus phosphorylation site for CaMKII is RXXS/T though it also phosphorylates Ser or Thr residues in the sequence S/TXD (Yamauchi, 2005).

CaMKII consists of a C-terminus domain that is essential for multimerization and a N-terminal domain that contains the autoinhibitory domain and CaM binding domain (Hook & Means, 2001). Under basal conditions, the autoinhibitory domain and the CaM binding domain bind to each other making the kinase inactive. Binding of Ca^{2+} and CaM to CaMKII displaces the autoinhibitory domain causing the kinase to become activated. CaMKII can then be converted to a Ca^{2+}-independent enzyme by a process of autophosphorylation in which the autoinhibitory domain action is further disrupted by
the phosphorylation of Thr286 found on the α isoforms and Thr287 found on the β isoforms. Autophosphorylation also results in the inactivation of CaMKII through phosphorylation of Thr305 and Thr306 on α and β isoforms respectively after autophosphorylation in a Ca^{2+}-independent manner. This is responsible for the loss of ability of CaM kinase II to bind Ca^{2+} or calmodulin, resulting in a reduction in the kinase activity (Hook & Means, 2001).

CaMKII is able to regulate neurotransmitter release through several effectors. It has been shown that synaptobrevin is a substrate of CaMKII as well as other SNARE proteins thus CaMKII could play an important role in the efficiency of docking and fusion of SSVs (Nielander et al., 1995). However, the effect of α CaMKII deficiency in mice has shown that α CaMKII might not be essential for vesicular cycling including control of the size of the RRP. Nevertheless, α CaMKII might serve an essential function in neurotransmitter release by placing an inhibitory constraint on release during repetitive presynaptic depolarisation (Hinds et al., 2003).

CaMKII activation is known to increase neurotransmitter release through the phosphorylation of synapsin I at sites 2 and 3 which makes more SSVs available for release (Nichols et al., 1990). There is a distribution shift of CaMKII from basal to depolarising stimuli. In basal conditions synapsin I and CaMKII are not associated but after depolarisation they are distributed together. Furthermore, under depolarisation conditions, CaMKII is accumulated at the presynaptic active zone (Tao-Cheng et al., 2006).

CaMKII is also shown to be constitutively bound to Ca_{v}2.1 channels at the α1 subunit even in the absence of Ca^{2+}. It is the binding to Ca_{v}2.1 by which CaMKII exerts its
modulatory effect and not by phosphorylating a specific site. CaMKII modulates the Ca\(_{\text{v}}\)2.1 channels to substantially increase the Ca\(^{2+}\) entry into presynaptic terminals by slowing Ca\(_{\text{v}}\)2.1 channels inactivation and positively shifting the voltage dependence of inactivation (Jiang et al., 2008; Takahashi et al., 2010).

### 1.4.3. Protein kinase A (PKA)

Activation of protein kinase A (PKA) also known as cAMP-dependent protein kinase occurs downstream of second messenger cAMP production stimulation mediated by G protein coupled receptor (GPCR) activation of adenylate cyclase. Intracellular targeting and compartmentation of PKA is determined mainly by association with AKAPs, a family of structurally related proteins.

PKA is a holoenzyme and a heterotetramer that is composed of two regulatory and two catalytic subunits. The regulatory subunit has two cAMP binding sites, A and B which can each bind to two cAMP molecules. All four cAMP molecules need to bind to the two regulatory subunits for PKA to undergo a conformational change. This conformational change dissociates the regulatory and catalytic subunits which in turn become catalytically active (Seino & Shibasaki, 2005).

Activation of PKA can phosphorylate many different substrates that are part of the exocytosis machinery leading to a stimulatory effect in many cases (Leenders & Sheng, 2005). Cysteine string protein which is localized exclusively to vesicle membranes, interacts with syntaxin and synaptotagmin (Evans & Morgan, 2002). Phosphorylation of cysteine string protein by PKA produces an inhibition of the interactions with syntaxin and synaptotagmin (Evans & Morgan, 2003). Snapin is a SNAP-25 associated protein which acts as a SNARE regulator. PKA dependent phosphorylation of Snapin has been
shown to enhance its binding to the SNARE complex and to increase the fusion competence of SSVs. Rabphilin and RIM are also substrates for PKA thus PKA phosphorylation of RIM in mossy fibres is thought to cause an induction of long term potentiation (Leenders & Sheng, 2005). Also both syntaxin 1 and SNAP25 can be phosphorylated by PKA leading to increased neurotransmitter release (Leenders & Sheng, 2005).

During vesicle recruitment synapsin I can be phosphorylated by PKA at site 1 which makes more SSVs available for release leading to a facilitation of neurotransmitter release (Leenders & Sheng, 2005; Menegon et al., 2006). Furthermore, it has been suggested that phosphorylation of synapsin at site 1 is important post-tetanic potentiation (PTP) thereby implying a role for PKA in synaptic plasticity (Fiumara et al., 2007). PKA also plays a stimulatory role in glutamate release by phosphorylating specifically Ca,2.2 channels that produce increased Ca$^{2+}$ influx compared to exocytosis (Wang & Sihra, 2003; Ladera et al., 2007).

1.4.4. Protein kinase C (PKC)

There are 12 isoforms of PKC in mammals which are classified based on their cofactor requirements as classical $\alpha$, $\beta$ and $\gamma$ isoforms that are activated by Ca$^{2+}$, diacylglycerol and lipids. The second category of isoforms is called novel and includes the $\delta$, $\epsilon$, $\eta$ and $\theta$ isoforms, activated by diacylglycerol. There is also a group of atypical isoforms that include $\zeta$ and $\iota/\lambda$ which are activated by neither Ca$^{2+}$ nor diacylglycerol (Morgan et al., 2005; Rosse et al., 2010). The structural studies of PKC have shown that they share a highly conserved carboxyterminal kinase domain that is linked by a hinge region to a more divergent amino-terminal regulatory domain. PKC in the inactive state is auto-inhibited by a pseudo-substrate sequence in the regulatory domain occupying the
substrate binding site in the kinase domain. Binding of the PKC activators to the regulatory domain recruits PKC to the plasma membrane which displaces the bound pseudo-substrate region from the active site, allowing the activated PKC to act on its target substrates (Rosse et al., 2010).

The main presynaptic consequence of PKC activation is the potentiation of neurotransmitter release as has been shown for example in experiments in which phorbol esters have been applied to brain slices (Malenka et al., 1986). The two main exocytotic machinery proteins that are activated by PKC include Munc-18 and SNAP-25. Munc-18 is phosphorylated by PKC at Ser\(^{313}\) causing the disruption of Munc-18 and syntaxin 1 interaction leading to both faster release kinetics and more rapid vesicle recycling (Barclay et al., 2003). More recently it has been hypothesised that phorbol ester potentiation requires the convergence of two pathways, one is the phosphorylation of Munc18-1 by PKC and the second is the activation of Munc13 in a PKC independent manner. The exact molecular mechanism of this interaction is still unclear as to whether Munc18-1 and Munc13 compete for syntaxin 1 binding or Munc18-1 dissociation from syntaxin 1 produces a favourable equilibrium shift towards activation of priming by Munc13 (Wierda et al., 2007).

It has also been shown in various studies that SNAP-25 is phosphorylated at Ser\(^{187}\) by PKC though it has been shown to be a poor substrate in vitro (Morgan et al., 2005). Nevertheless, phosphomimetic mutants of the Ser\(^{187}\) site (Ser to Glu/Asp) has shown a stimulatory effect on the refilling of the RRP after an RRP emptying stimulation, whereas phosphonull mutants (Ser to Ala), or inhibition of PKC, inhibited vesicle pool refilling (Nagy et al., 2002). In hippocampal synapses however it has been reported that
the increase in transmitter release after phorbol esters treatment is not through PKC phosphorylation of SNAP-25 at site Ser\textsuperscript{187} (Finley \textit{et al.}, 2003).

\subsection*{1.4.5. Lipid kinases}

There is a growing interest in the modulatory effect of lipids such as phosphatidylinositol-4,5-bisphosphate (PIP\textsubscript{2}) and phosphatidylinositol-3,4,5-bisphosphate (PIP\textsubscript{3}) have on neurotransmitter release. The Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 channels have been shown to be dually regulated by PIP\textsubscript{2} that maintains but can also inhibit the activity of Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 channels (Wu \textit{et al.}, 2002). Furthermore, there is evidence that decreasing the PIP\textsubscript{2} concentration can lead to postnatal lethality and synaptic defects in mice (Di \textit{et al.}, 2004). The lipid kinases such as phosphatidylinositol 4-kinase (PI4K) and phosphatidylinositol-3 kinase (PI3K) are important in maintaining neurotransmitter release and have been found in presynaptic nerve terminals.

There are different forms of PI4Ks that are grouped as type II which include PI4KI\textsubscript{II}\textalpha and PI4KI\textsubscript{II}\textbeta and type III which include PI4KIII\textalpha and PI4KIII\textbeta (Barylko \textit{et al.}, 2001; Minogue \textit{et al.}, 2001). There is evidence which suggests that the majority of PI4K activity is conducted by the PI4KI\textsubscript{II}\textalpha form (Guo \textit{et al.}, 2003). It is known that the PI4Ks phosphorylate phosphatidylinositol to phosphatidylinositol-4-phosphate which is the first step in the production of phosphatidylinositol-4,5-bisphosphate (PIP\textsubscript{2}) (Gehrmann & Heilmeyer, Jr., 1998; Balla & Balla, 2006). Functional studies have provided evidence that PI4K is localised to SSVs and that inhibition of PI4K can attenuate the Ca\textsuperscript{2+}-dependent component of neurotransmitter release (Wiedemann \textit{et al.}, 1998).

Class-I PI3Ks are heterodimers composed of a catalytic subunit (p110) and an adaptor/regulatory subunit (p85). This class is further divided into the subclass IA and
the subclass IB that are activated by GPCRs (Fresno Vara et al., 2004). The class I PI3K is a member of a lipid kinase family that is characterized by the ability to phosphorylate PIP$_2$ to PIP$_3$. The modulatory effects of PI3K activation include the upstream activation of H-Ras (Hu et al., 1995; Giglione & Parmeggiani, 1998). More recently the lipid kinase PI3K has also been shown to alter neurotransmitter release. Synapsin I is thought to interact with PI3K in intact nerve terminals and that disruption to this interaction decreases the repletion of the RRP which results in a reduction of glutamate release after sustained stimulation (Cousin et al., 2003). In addition, a study conducted showed that PI3K plays a key part in neurotrophin induced synaptic potentiation in presynaptic motoneurons (Yang et al., 2001).

1.5. Regulation of neurotransmitter release by receptor tyrosine kinases

Neurotransmitter release can also be modulated by a class of cell surface receptors that are called receptor tyrosine kinases (RTKs) and that are activated by growth factors known as neurotrophins as well as other extracellular signals. This function adds to diverse key functions of RTKs that include cell survival, morphogenesis, neuronal plasticity and apoptosis.

1.5.1. Trks

There are three different types of tropomysin related kinase (Trk) receptors that include TrkA, TrkB and TrkC. They can be distinguished from other RTKs and have a homologous structure. The extracellular domains of Trks contain two cysteine clusters in between leucine-rich repeats followed by two immunoglobin-like domains and they also have a single cytoplasmic tyrosine kinase domain (Roux & Barker, 2002). These receptors are activated by a wide variety of neurotrophins that bind to the second immunoglobin-like domain of Trks.
Neurotrophins have been divided into four main members that include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophins 3, neurotrophins 4/5 and neurotrophins 6 (Poo, 2001). In addition, they have different affinities for each of the TrK receptors. TrkA receptors have a high affinity for NGF, while TrkB receptors are activated by BDNF and neurotrophins 4/5 and TrkC receptors are stimulated by neurotrophin 3 (Schecterson & Bothwell, 2010).

When the neurotrophins bind to Trk receptors they cause Trks dimerisation and initiate the autophosphorylation of tyrosine residues in their cytoplasmic tyrosine kinase domains. The phosphorylated tyrosine residues function as binding sites for recruiting specific cytoplasmic signalling proteins. A major consequence of TrK receptor activation by neurotrophins is the activation of the extracellular signal regulated kinase (ERK) pathway.

1.5.2. **ERK pathway**

Extracellular signal regulated kinases 1 and 2 (ERK1 and ERK2) are activated through numerous extracellular stimuli that include RTKs, GPCRs and Ca\(^{2+}\)-dependent processes and all these pathways are convergent to the ERK pathway. The ERK pathway is a well characterised cascade that uses the sequential activation of distinct kinases at each level of the cascade. Culminating in the activation of ERK1 and ERK2 which then activate downstream targets found in the nucleus and cytoplasm.

Trk activation leads to the initiation of the ERK pathway through the recruitment of signalling proteins that activate the small GTP binding protein Ras which has three forms that include H-Ras, N-Ras and K-Ras. Ras is a membrane bound protein and undergoes cycles of inactivation and activation depending on the GDP or GTP binding
state, respectively. There is evidence which suggests that the Shc adaptor protein is recruited by phosphorylated tyrosine residues on Trks together with another adaptor protein Grb2. The adaptors then recruit and activate son of sevenless (Sos), a GTP exchange factor (GEF) that is able to exchange the GDP on Ras to GTP and thereby activate Ras (Egan et al., 1993).

The key step that is immediately downstream of Ras is activation of Raf a serine/threonine kinase which is also known as mitogen-activated protein (MAP) kinase kinase. There are three forms of Raf including c-Raf-1, B-Raf and A-Raf that are thought to participate in the ERK pathway. Structural studies looking at Raf show that key features of Raf are that it has a catalytic domain at the C-terminus and regulatory domain at the N-terminus.

In its inactive state c-Raf-1 is in the cytoplasm and forms a complex with Hsp90, Hsp50 and 14-3-3 proteins. It is the 14-3-3 dimer formation at Ser259 and Ser621 that folds Raf into a closed configuration. When Ras is bound to GTP it interacts directly with the N-terminus on c-Raf-1. This does not mutually activate c-Raf-1 but makes it membrane bound. Once at the membrane and bound to the Ras effector loop Raf is able to interact with a second site on Ras through a zinc finger. This interaction with the second site displaces the 14-3-3 half-dimer from the Ser259 to cause a partial activation of c-Raf-1 being still dependent on the presence of Ras. The mechanism of the full activation of c-Raf-1 is debatable but it is thought that c-Raf-1 undergoes phosphorylation at an unidentified site in the catalytic domain. At this site, the binding of 14-3-3 stabilises an open, active configuration of c-Raf-1 which unbinds from Ras-GTP and is translocated to the cytosol where it remains active until deactivated by phosphatase action (Avruch et al., 2001).
Sequentially in the next level of the ERK pathway, the activated form of c-Raf-1 in the cytoplasm acts on MAPK/ERK kinase (MEK) another serine/threonine kinase known as MAP kinase kinase. MEKs are small proteins that have a proline-rich loop which is an important regulatory subdomain found in MEK1 and MEK2 called IX and X subdomains, respectively (Crews et al., 1992; Ohren et al., 2004). This proline-rich loop is not necessary for Raf binding but is important in ensuring the activation of its downstream targets is effective and contains the phosphorylation sites Ser298 and Tyr300 (Dang et al., 1998). Mutational studies have found that Ser218 and Ser222 are important in Raf-1 activation of MEK1 and that phosphorylation of a single site is sufficient for MEK1 activation (Zheng & Guan, 1994). There are seven forms of MEK (MEK1 to MEK7) of which MEK1 and MEK2 are thought to be responsible for ERK1 and ERK2 phosphorylation/activation with MEK2 having higher activity than MEK1 (Zheng & Guan, 1993).

ERK1 and ERK2 kinases are known as central transducers of extracellular signals from growth factors, cytokines, and environmental stresses. The two forms ERK1 and ERK2 are part of the MAPK family and are also known as MAP kinases. These kinases are proline-directed serine/threonine protein kinases that mediate phosphorylation of serine or threonine residues directly upstream of proline. ERK2 knockout mice are not viable and die early in development suggesting that ERK1 is unable to compensate for the function of ERK2. However, knockout studies have shown that ERK1 knockout mice are viable except for the deficits in thymocyte maturation indicating that the functions of ERK1 are largely compensated for by ERK2 (Pages et al., 1999).

Structural studies of ERK1 and ERK2 have shown that they are composed of a small β-strand rich N-terminal lobe and a large α-helix rich C-terminal lobe that are linked by a
hinge region. The N-terminal domain contains a gatekeeper residue that regulates
binding of nucleotide. Furthermore, the gatekeeper residue is part of an N-terminal
structural unit that impedes auto-activation of ERK2 (Emrick et al., 2006). The C-
terminal domain contains the phosphorylation lip and the catalytic loop (Zhang et al.,
1994a; Kinoshita et al., 2008).

Both ERK1 and ERK2 can be in three states, unphosphorylated, mono-phosphorylated
and dual-phosphorylated. In the unphosphorylated state the kinases are inactive with the
phosphorylation lip folded. The mono-phosphorylated states of ERK1 and ERK2 are 2
to 3 fold less active than the fully active form but they may have distinct biological
functions. When the ERKs are in the dual-phosphorylated state the kinases become fully
active and phosphorylate their downstream targets (Zhou & Zhang, 2002). ERK2 is
dually phosphorylated at sites Thr183 and Tyr185 within the phosphorylation lip
(Canagarajah et al., 1997) while Thr202 and Tyr204 sites are phosphorylated in the
phosphorylation lip of ERK1 (Butch & Guan, 1996).

1.5.3. Ca\(^{2+}\) mediated activation of the ERK pathway

The Ca\(^{2+}\)-dependent signalling pathways of the ERK pathway are not well defined but
numerous components have been implicated which includes Src, Pyk2, CaM, CaMKII,
GEFs and GAPs. All of these components are activated directly or indirectly by an
increase in intracellular Ca\(^{2+}\) concentration and act on targets found in the ERK pathway
which can promote or inhibit ERK1 and ERK2 phosphorylation/activation.

Src kinase is a non-receptor tyrosine kinase which consists of three domains, a SH3
domain on the N-terminal, a central SH2 domain and a tyrosine kinase domain and is
enriched in synaptic membrane and vesicles (Ross et al., 1988). The Src kinase has been
implicated in the tyrosine phosphorylation of c-Raf-1 independent of Ras activation (Marais et al., 1995; Stokoe & McCormick, 1997). Furthermore, the A-Raf is also phosphorylated and activated by Src but B-Raf is not activated (Marais et al., 1997). There is evidence that Src can increase influx of Ca\(^{2+}\) through VDCCs when the nerve terminals are stimulated via membrane depolarisation (Wang, 2003). This could be a positive feedback mechanism that further stimulates Src activity.

Another tyrosine kinase proline-rich tyrosine kinase (Pyk2) which contains a protein tyrosine kinase and two proline-rich domains has been suggested to be involved in activation of the ERK pathway. The mechanism of action for ERK pathway activation by Pyk2 is not fully characterised but it is thought an increase in intracellular Ca\(^{2+}\) concentration triggers autophosphorylation of Pyk2 at site Tyr402. This allows Pyk2 to bind to Src through its SH2 domain which causes Src activation. Which can recruit the Grb2/Sos complex via two pathways, firstly Src can phosphorylate Shc and secondly Pyk2 can be phosphorylated at site Tyr881 which allows Pyk2 to recruit the Grb2/Sos complex independent of Shc phosphorylation. The recruitment of the Grb2/SOS complex by either pathway then results in the sequential activation of the ERK pathway (Lev et al., 1995; Dikic et al., 1996; Finkbeiner & Greenberg, 1996; Blaukat et al., 1999).

CaM has been investigated for its role in Ca\(^{2+}\)-dependent activation of the ERK pathway and the results provide a mixture of stimulatory and inhibitory effects which likely occurs due to different conditions (Moreto et al., 2009). All forms of Ras (H-Ras, N-Ras and K-Ras) can bind to CaM and binding of CaM to K-Ras causes an inhibition of its activation (Villalonga et al., 2001). CaM stimulatory effects include the promotion of Raf-1 stimulation from H-Ras activation through PI3K (Moreto et al., 2008). Furthermore, Ras-specific guanine nucleotide-releasing factor (RasGRF) is a GEF that
has IQ motif which allows it to interact with calmodulin in a Ca^{2+}-dependent manner. Studies have shown that CaM binding to RasGRF promotes its activation and thereby the Ras-GTP formation (Farnsworth et al., 1995; Freshney et al., 1997).

CaMKII could also be a downstream target of calmodulin and can be activated to affect the ERK pathway. Ras-GTPase activating protein (SynGAP) is a GAP that hydrolyses the GTP bound to Ras thereby reducing Ras activity and decreases the ERK pathway. CaMKII activation is able to phosphorylate SynGAP and inhibit its activity and thereby enhance the activation of the ERK pathway (Chen et al., 1998). In vascular smooth muscle cells, CaMKII has been shown to inhibit Ca^{2+}-dependent Pyk2 activation of the ERK pathway (Ginnan & Singer, 2002).

An increase in intracellular Ca^{2+} concentration could also directly bind to GEFs that activate Ras and the ERK pathway. Two such GEFs have been described as Ca^{2+} and diacylglycerol-regulated GEFI (CalDAG-GEFI) and CalDAG-GEFII also known as RasGRP. They can be activated by Ca^{2+} and DAG that targets the Rap1 which could lead to B-Raf activation (Kawasaki et al., 1998b; Ebinu et al., 1998). In addition, shoc2 scaffolding protein which has been shown to accelerate the interaction between Ras and c-Raf-1 also requires an increase in intracellular Ca^{2+} concentration (Matsunaga-Udagawa et al., 2010; Yoshiki et al., 2010).

1.5.4. GPCR mediated activation of the ERK pathway

Activation of GPCRs coupled to G_{s/o}, G_{i/o} or G_{q/11} proteins are able to converge and are all able to stimulate the ERK pathway but through different mechanisms (Faure et al., 1994). However, it is thought that these mechanisms work to activate Ras or Raf which
then activates the sequential cascade leading to ERK1 and ERK2 phosphorylation/activation.

Phosphorylation/activation of ERK1 and ERK2 through GPCRs coupled to the Gs protein is thought to involve increases in intracellular cAMP concentration induced by the stimulation of adenylate cyclase. Increases in intracellular cAMP concentration are thought to involve direct action of cAMP and activation of PKA by cAMP on (Ras-proximate-1) Rap1. Rap1 is a small GTP binding protein which is inactive when bound to GDP and is activated by GTP binding. It is thought that Rap1 has inhibitory action on c-Raf-1 but it might also stimulate B-Raf which could result in the activation of the ERK pathway (Ohtsuka et al., 1996). The direct action of cAMP is thought to involve the binding of cAMP to Epac which is a GEF that promotes the GTP binding to Rap1 hence Rap1 activation (Kawasaki et al., 1998a; de et al., 1998). Activation of PKA by cAMP also leads to the phosphorylation of Src at site Ser17 which is thought to activate Rap1 which then consequently activates B-Raf and the ERK pathway (Schmitt & Stork, 2000; Schmitt & Stork, 2002). An example of this is the A2A receptor that can influence the excitatory action of BDNF on hippocampal slices (Diogenes et al., 2004) and the neuromuscular junction (Pousinha et al., 2006). In both of these studies it was shown that it is specifically the A2A receptor that PKA activation is necessary for excitatory actions of BDNF.

The mechanism of ERK1 and ERK2 phosphorylation/activation by GPCRs coupled to the Gq/11 protein is thought to be the stimulation of PLC which metabolises PIP2 to form DAG which can further stimulate PKC activation. It is thought that upon activation PKC can directly phosphorylate c-Raf-1 at sites Ser499 and Ser259 that results in the
activation of the ERK pathway (Kolch et al., 1993; Ueda et al., 1996; Schonwasser et al., 1998).

The GPCRs coupled to $G_{i/o}$ proteins are thought to stimulate the ERK1 and ERK2 phosphorylation through the dissociation of the $\beta\gamma$ subunit. It is not thought the inhibition of adenylate cyclase mediates the stimulatory effects. The dissociation of the $\beta\gamma$ subunit results in an increase in Shc phosphorylation on tyrosine residues which leads to the recruitment of Grb2 and Sos; thus activating the sequential ERK pathway (van et al., 1995). There is also evidence that c-Src might also be activated by the $\beta\gamma$ subunit which results in enhanced Shc and Grb2 complexes (Luttrell et al., 1996; Luttrell et al., 1997).

1.5.5. Regulation of ERK1 and ERK2 phosphorylation/activation

The regulation of ERK1 and ERK2 phosphorylation/activation is important given the diverse range of stimuli for this pathway. The ways in which the ERK1 and ERK2 are regulated is through scaffolding proteins, spatial localisation and MAPK phosphatases (MKPs) activity.

There are several scaffolding proteins including MEK partner 1 (MP1), kinases suppressor of Ras (KSR) and MAPK organizer 1 (MORG1) associated with ERK signalling. These scaffolding proteins act to bring together the different kinases of the ERK pathway and facilitate the activation of ERK1 and ERK2 (Dhanasekaran et al., 2007). MP1 functions as an adaptor that has been shown to specifically bind to MEK1 and ERK1 and facilitate ERK1 phosphorylation/activation. A mutant form of MP1 is unable to bind to MEK1 and is not able stimulate Ras activated ERK pathway (Sharma et al., 2005). Interestingly, MP1 does not interact with Raf, MEK2 or ERK2 and is not involved in ERK2 phosphorylation/activation (Schaeffer et al., 1998). Furthermore, p14
a small protein interacts with MP1 which is required for the activation of the ERK pathway and localises the MP1 and ERK pathway to endosomes and lysosomal compartments (Wunderlich et al., 2001; Teis et al., 2002). MORG1 interacts with MP1 can also associate independently with Raf-1, B-Raf, MEK1, MEK2 ERK1 and ERK2. Interestingly, MORG1 facilitates ERK1 and ERK2 phosphorylation/activation to a specific agonist but not to epidermal growth factor (Vomastek et al., 2004).

KSR1 is also a scaffolding protein that interacts with Raf-1, MEK1, MEK2 and ERK1 and ERK2 as well as other proteins and facilitates the Ras activated ERK pathway. KSR1 binding to MEK1 and MEK2 causes a change in the molecular mass and causes the translocation of MEK from the cytosol to the plasma membrane where it colocalises with Raf-1 and ERK and thereby activates the ERK pathway (Therrien et al., 1996; Stewart et al., 1999). The mechanism of KSR1 binding and translocation of MEK is further regulated by c-TAK1 which constitutively phosphorylates Ser392 on the KSR1 structure. Upon growth factor stimulation, the phosphorylation state of Ser392 is significantly reduced leading to the translocation of KSR1-MEK complex (Muller et al., 2001).

The dephosphorylation of ERK1 and ERK2 by phosphatases is a negative feedback mechanism to regulate their activation. There are two phases of inactivation of ERK1 and ERK2 that are described as rapid and delayed. There is evidence that suggests PP2A dephosphorylates threonine residues and another tyrosine specific phosphatase dephosphorylates tyrosine residues (Alessi et al., 1995). The delayed phase is likely to involve dual specific phosphatases that include MKP 1, 2, 3 and 4 which can dephosphorylate both the phosphoserine/threonine and phosphotyrosine residues in one substrate. In addition, MKPs localise in different compartments; MKP1 and 2 are
expressed in the nucleus, MKP3 is expressed in cytoplasm. MKPs are dual specific phosphatases that are capable of dephosphorylating both phosphorylated tyrosine and threonine residues on ERK1 and ERK2 (Pouyssegur et al., 2002). MKP1 and 2 are activated by the activation of ERK pathway (Brondello et al., 1997) and ERK2 is able to physically associate and activate MKP3 (Camps et al., 1998; Zhou et al., 2001). Furthermore, ERK1 and ERK2 phosphorylation of MKP1 at sites Ser359 and Ser364 reduce the degradation of MKP1 which could thereby prevent prolonged activation of ERK1 and ERK2 (Brondello et al., 1999).

1.5.6. Downstream targets of ERK1 and ERK2

At the presynaptic nerve terminals the major consequence of ERK1 and ERK2 phosphorylation/activation is the modulation of neurotransmitter release by phosphorylation of synapsin 1 at sites 4 and 5 (and possibly 6). BDNF stimulation of TrkB receptors facilitates glutamate release through the activation of ERK1 and ERK2 which phosphorylate synapsin 1 leading to increased recruitment of SSVs (Jovanovic et al., 1996). Furthermore, this facilitation of neurotransmitter release can be attenuated by inhibiting MEK as well as in mice with synapsin deficiency (Jovanovic et al., 2000). There is evidence which suggests that BDNF facilitation of neurotransmitter release in the hippocampal nerve terminals is limited to a subset of nerve terminals (Pereira et al., 2006). ERK involvement in neurotransmitter release is further supported by studies that highlight inhibition of glutamate release by reduced VDCC activity and ERK1 and ERK2 phosphorylation/activation (Chang & Wang, 2009; Chang & Wang, 2010).

Furthermore, there is evidence that ERK1 and ERK2 may also play a role in synaptic plasticity in presynaptic nerve terminals. There is evidence that an ERK-dependent mechanism is responsible for PTP in presynaptic nerve terminals through
phosphorylation of synapsin. In the CA3 region of the hippocampus there is evidence that in these synapses ERK1 and ERK2 phosphorylation/activation modulates presynaptic plasticity through synapsin 1 (Vara et al., 2009). Transgenic mice with constitutively active form of H-ras exhibited enhancements in learning and fear conditionings through the phosphorylation of synapsin 1 by an ERK-dependent pathway (Kushner et al., 2005). The dependence of synapsin 1 in inducing ERK-dependent presynaptic plasticity suggests the mechanism of action is by modulation of SSV mobilisation and neurotransmitter release. Furthermore, down-regulation of the ERK pathway inhibits neurotransmitter release in synapses during development of neuronal circuits (Ghirardi et al., 2004).

1.6. Regulation of neurotransmitter by intracellular Ca\textsuperscript{2+} release

The role of extracellular Ca\textsuperscript{2+} influx on the presynaptic nerve terminal has been well established but the influence of intracellular Ca\textsuperscript{2+} stores on presynaptic nerve terminal functions is less well described. There are three possible stores of intracellular Ca\textsuperscript{2+}, smooth endoplasmic reticulum, mitochondria and NAADP sensitive stores.

1.6.1. Smooth endoplasmic reticulum

Smooth endoplasmic reticulum serves different functions in different cells and can appear different under an electron microscope. In synaptosomes smooth endoplasmic reticulum has been identified as membrane-bound tubules or as isolated vesicles (McGraw et al., 1980a). At the nerve terminals smooth endoplasmic reticulum could function to sequester Ca\textsuperscript{2+} or release Ca\textsuperscript{2+} that contributes to nerve terminal function. The role of the endoplasmic reticulum has been well characterised in other cell types such as smooth muscle cells and at the postsynaptic cell bodies. From these studies certain features and functions of stores have been described that include activity
receptors such as the inositol triphosphate (IP$_3$) receptor and ryanodine receptor. In addition, the Sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps maintain the Ca$^{2+}$ concentration inside smooth endoplasmic reticulum.

There are three forms of IP$_3$ receptors (IP$_3$Rs) that have been cloned including IP$_3$R1, IP$_3$R2 and IP$_3$R3 and through functional studies it has been shown that these forms of receptors differ in IP$_3$ binding affinity and receptor modulation. IP$_3$Rs form tetrameric structures with each subunit having six transmembrane spanning helices with the C-terminus facing the cytosol. The key domains of IP$_3$Rs include the N-terminal suppressor and IP$_3$ binding domain found at the N-terminus, channel forming and coupling domains and gate keeper domain found at the C-terminus (Patterson et al., 2004; Mikoshiba, 2007a; Mikoshiba, 2007b).

IP$_3$Rs can be regulated by phosphorylation sites in the modulatory domain as well as by other proteins. The first form of regulation of IP$_3$Rs is by Ca$^{2+}$ itself. External Ca$^{2+}$ and/or Ca$^{2+}$ release by smooth endoplasmic reticulum causes a positive feedback response releasing more Ca$^{2+}$ though higher Ca$^{2+}$ concentrations have an inhibitory effect on IP$_3$Rs (Bezprozvanny et al., 1991; Boehning et al., 2001). Furthermore, it has been shown that calcium-binding protein 1 in neuroendocrine cells inhibits Ca$^{2+}$ release by IP$_3$Rs from stores (Haynes et al., 2004). Furthermore, PKC is able to phosphorylate IP$_3$Rs at distinct sites which enhances the receptor responsiveness to IP$_3$ (Ferris et al., 1991). At rest, IP$_3$R binding protein (IRBIT) binds to the IP$_3$R at the IP$_3$ binding domain and suppresses the receptor activity. IRBIT also has regulatory domains that can be phosphorylated to regulate IRBIT activity (Ando et al., 2006). IP$_3$ is able to displace IRBIT by competing for the IP$_3$ binding domain thus when IP$_3$ production increases in the cytosol IRBIT is released.
There are three types of ryanodine receptors (RyRs) that including RyR1, RyR2 and RyR3 and they all form homotetrameric complexes. There are numerous modulators that can impact on the gating of the pore that include Ca\(^{2+}\), calmodulin, PKA and CaMKII and these modulatory sites are found on the large N-terminus that faces the cytosol. Modulation of RyRs by Ca\(^{2+}\) is dependent on the concentration as at low concentrations Ca\(^{2+}\) is able to stimulate the RyRs while at higher concentrations the receptors are inhibited. It is thought that EF hand motifs contribute to Ca\(^{2+}\) modulation of RyRs but there are other Ca\(^{2+}\)-binding sites that induce its stimulating effects (Hamilton & Serysheva, 2009). Calmodulin is an inhibitor of RyR when it bound to Ca\(^{2+}\) and when free of Ca\(^{2+}\), calmodulin still inhibits cardiac RyR2 but is an agonist for RyR1. Interestingly, it is thought that calmodulin whether bound or unbound to Ca\(^{2+}\) interacts with the RyRs at the same sites (Zalk et al., 2007). Calstabin 1 and calstabin 2 are enzymes that inhibit RyR and through mutagenesis studies there is evidence that phosphorylation at site Ser2808 by PKA cause the dissociation of the calstabins and an increase in the opening of the channel. Furthermore, CaMKII is able to phosphorylate the RyR2 at ser2814 which also increases channel opening.

The distribution of the receptors types suggests that it is most likely that the RyR2 and RyR3 are localised in cortical nerve terminals. With respect to brain regions RyR1 is highly expressed in the cerebellum, hippocampus and striatum but not in the cortex. RyR2 is expressed in all regions while RyR3 are expressed in the cortex, hippocampus and in low levels in the cerebellum (Giannini et al., 1995). Furthermore, the RyR have been shown to be present in presynaptic nerve terminals using the synaptosomal preparation (Padua et al., 1996).
IP$_3$Rs are stimulated through the PIP$_2$ metabolism pathway. PLC activation results in the metabolism of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) forming two metabolites that are IP$_3$ and diacylglycerol (DAG). This continuous metabolism of PIP$_2$ causes a significant increase in IP$_3$ levels that is then sufficient for IP$_3$R stimulation (Mikoshiba, 2007a). The RyRs on the other hand are largely activated by the influx of Ca$^{2+}$ in a process known as Ca$^{2+}$-induced Ca$^{2+}$ release (CICR). The stimulation of IP$_3$Rs and RyRs results in the efflux of Ca$^{2+}$ from the smooth endoplasmic reticulum into the cytosol.

At the presynaptic nerve terminals studies have focused on the function of intracellular Ca$^{2+}$ stores on neurotransmitter release. There is evidence that at the Purkinje cell synapse RyRs in the presynaptic nerve terminals increase neurotransmitter release to enhance miniature inhibitory postsynaptic currents (Llano et al., 2000; Galante & Marty, 2003). Using hippocampal synapses there is evidence that LTD induction in this system is dependent on Ca$^{2+}$ release mediated by RyRs in the presynaptic nerve terminals (Reyes & Stanton, 1996; Unni et al., 2004). Furthermore, other studies have provided evidence that intracellular Ca$^{2+}$ stores contribute to spontaneous and evoked exocytosis of glutamate from presynaptic nerve terminals (Davletov et al., 1998; Rahman et al., 1999; Capogna et al., 2003). However, it has also been suggested that intracellular Ca$^{2+}$ store release of Ca$^{2+}$ is not sufficient per se for regulating exocytosis but rather functions to modulate the RRP (Khvotchev et al., 2000).

There are numerous studies that have suggested that intracellular Ca$^{2+}$ stores maybe involved in neuronal pathologies. Thus the effect of intracellular Ca$^{2+}$ stores has been investigated in NMDA induced excitotoxicity in cortical neurons. Evidence suggests that endoplasmic reticulum stores release Ca$^{2+}$ through IP$_3$R and RyR activation and
contribute to neuronal death. Moreover, Ca$^{2+}$ release inhibition also attenuated mitochondrial pathways that cause neuronal death (Ruiz et al., 2009). Furthermore, there is evidence that impairment of intracellular Ca$^{2+}$ store function at the presynaptic nerve terminals may possibly be a precursor for Alzheimer’s disease (Zhang et al., 2009). In addition, zonisamide is an antiepileptic drug that is thought to exert its inhibitory effect by enhancing GABA neurotransmitter release through the IP$_3$R activation (Yamamura et al., 2009).

Sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) pumps are able to transport the Ca$^{2+}$ across the membrane of the smooth endoplasmic reticulum via an ATP driven mechanism known as the SERCA catalytic cycle. The SERCA pumps exist in two states in the first (E1) state the SERCA pump has a high affinity for Ca$^{2+}$ and binds to Ca$^{2+}$ on the cytosolic side of the membrane. In the second (E2) state the affinity for Ca$^{2+}$ is reduced leading to release of Ca$^{2+}$ in the lumen of the smooth endoplasmic reticulum. The catalytic cycle is started when the SERCA pump is in the E1 state and binds to two Ca$^{2+}$ ions and ATP. The hydrolysis of ATP increases the formation of a high-energy phosphor-intermediate called the ADP-sensitive form that is phosphorylated and bound to two Ca$^{2+}$ ions. This is followed by a conformational change to the ADP-insensitive form in which the Ca$^{2+}$ binding sites have a lower affinity and are now oriented towards the lumen of the smooth endoplasmic reticulum. Ca$^{2+}$ ions are then released due to the lower affinity which is followed by hydrolysis that forms the E2 state. The SERCA pump then undergoes a conformational change from the E2 state to the E1 state (Brini & Carafoli, 2009).

There are three types of SERCA pumps found in the mammalian cells including SERCA1, SERCA2 and SERCA3. Studies of the structure of SERCA pumps have
identified four domains that are important in the function of the pump. These domains are known as the actuator (A), phosphorylation (P), nucleotide-binding (N) and membrane (M) domains. The M domain is the region of the 10 transmembrane helices and is connected to the A and P domains while the N domain is connected to the P domain. The P domain is important because it contains the amino acid Asp351 which is phosphorylated during the catalytic cycle. The N domain is important because it contains the amino acid Phe487 that is crucial for ATP binding. The A domain is suggested to move significantly during the catalytic cycle. Both these features are thought to be important in the hydrolysis of the phosphorylation site. The two Ca\(^{2+}\) binding sites found in the M domain suggested that this domain is important in the transportation of the Ca\(^{2+}\) (Wuytack et al., 2002).

**1.6.2. Mitochondria**

Mitochondria are an essential component of the cell and are found in presynaptic nerve terminals. They are important in the production of ATP but also studies have pointed to mitochondria playing an important role in Ca\(^{2+}\) homeostasis and dysfunction of the mitochondria is the cause of some pathologies. Mitochondria have an outer and inner mitochondrial membrane. There are several Ca\(^{2+}\) channels and pathways by which Ca\(^{2+}\) is buffered and released by the mitochondria including mCa1 and mCa2, mitochondrial RyR, mitochondrial Na\(^+\)/Ca\(^{2+}\) and H\(^+\)/Ca\(^{2+}\) exchanger.

Ca\(^{2+}\) uptake by mitochondria is mediated by the mitochondrial Ca\(^{2+}\) uniporter (MCU) and non-MCU type that are located in the inner mitochondrial membrane. There is evidence to support that the mitochondrial Ca\(^{2+}\) channels mCa1 and mCa2 are MCU and non-MCU type, respectively (Michels et al., 2009). The mCa1 and mCa2 channels are highly selective for Ca\(^{2+}\) ions and in the presence of Ca\(^{2+}\) are impermeable to Na\(^+\), K\(^+\),
Mg$^{2+}$ and Cl$^-$ ions and that activity is dependent on mitochondrial membrane potential. Through electrophysiological studies the current of mCa1 is found to be inwardly rectifying with a large capacity (Kirichok et al., 2004). The mCa2 current is different from mCa1 because it has smaller single-channel amplitude and a higher open probability. Furthermore, there is a difference in the pharmacology as mCa2 is insensitive to ruthenium 360 (Michels et al., 2009).

Ca$^{2+}$ is able to activate mCa1 which is further supported by the finding that calmodulin is also able to activate mCa1 uptake of Ca$^{2+}$ but CaMKII activation does not have an effect on mCa1 activity (Moreau et al., 2006). The mCa1 channel is regulated by a biphasic mechanism in which the uniporter is inactivated with reduced ability to reuptake Ca$^{2+}$ into the mitochondrial lumen after exposure to high cytosolic Ca$^{2+}$ concentrations. The mechanism by which mCa1 is inactivated is thought to be through the acidification of the inner membrane. Ca$^{2+}$ entry into the inner membrane stimulates ATP synthesis which drives H$^+$ ions into the inner membrane which inactivates mCa1. This can be described as a negative feedback mechanism to prevent Ca$^{2+}$ overload in the mitochondria (Moreau & Parekh, 2008).

The activation of mCa1 and mCa2 receptors is also dependent on the Ca$^{2+}$ microdomains at the mouth of the IP$_3$Rs (Rizzuto et al., 1993; Nassar & Simpson, 2000). There is evidence from Ca$^{2+}$ probes at the outer mitochondrial membrane that shows the Ca$^{2+}$ concentration at several points is 5 to 10 fold higher than the cytosolic Ca$^{2+}$ concentration. Furthermore, these Ca$^{2+}$ microdomains can be formed by IP$_3$Rs on the ER and regions of Ca$^{2+}$ influx through VDCCs (Rizzuto et al., 1998; Giacomello et al., 2010). The close proximity of the ER and mitochondria is thought to also have other functional effects that include the refilling of ER with Ca$^{2+}$ by mitochondrial release of
Ca$_2^+$ (Malli et al., 2005). Furthermore, the IP$_3$R activity of stores that are close to mitochondria is regulated by mitochondria Ca$_2^+$ uptake thus suppressing the positive feedback effect of Ca$_2^+$ release from IP$_3$Rs on IP$_3$Rs (Hajnoczky et al., 1999; Chalmers & McCarron, 2009).

In the outer mitochondrial membrane the voltage-dependent anion channels (VDAC) is thought to play important role in the passage of ions and small molecules into the mitochondria. VDAC are in an open configuration at low potential which it has a high conductance and weak anion-selectivity while at higher potentials they are in the closed state which has a lower conductance and show cation selectivity. As this channel is found on the outer mitochondrial membrane it is an important in the structural coupling between mitochondria and smooth endoplasmic reticulum and has a functional importance in regulating mCa1 and mCa2 exposure to cytosolic Ca$_2^+$ concentration (Shoshan-Barmatz et al., 2004).

Ca$_2^+$ efflux from the mitochondria is by a Na$^+$ dependent pathway through the Na$^+$/Ca$_2^+$ exchanger. The type of Na$^+$/Ca$_2^+$ exchanger enriched at the mitochondria has been identified as NCLX which is not localised in the plasma membrane or the smooth endoplasmic reticulum (Palty et al., 2010). The NCLX is an antiporter in that it uptakes three Na$^+$ ions into the mitochondria in exchange for one Ca$_2^+$ ion efflux into the cytosol. In addition, the exchanger can transport Ca$_2^+$ in either direction under conditions in which Na$^+$ ions are removed the exchanger transports Ca$_2^+$ into the mitochondria. NCLX is regulated through a negative feedback mechanism via Ca$_2^+$ accumulation in the mitochondria, so this could provide a positive contribution of Ca$_2^+$ retention in the mitochondria (Opuni & Reeves, 2000).
There is also a Na\(^+\) independent pathway that has been described to release Ca\(^{2+}\) from the mitochondria. Through RNAi screening Letm1 has been identified as a Ca\(^{2+}\)/H\(^+\) antiporter that is localised in the inner mitochondrial membrane. The antiporter is able to exchange one Ca\(^{2+}\) ion for one H\(^+\) ion in either direction across the inner mitochondrial membrane. In conditions in which the mitochondrial Ca\(^{2+}\) concentration is high and the pH is acidic the antiporter is able to extrude excess Ca\(^{2+}\) ions. At low mitochondrial Ca\(^{2+}\) concentrations and alkaline pH the antiporter uptakes Ca\(^{2+}\) ions at nanomolar concentrations (Jiang et al., 2009).

1.6.3. Acidic stores

There is accumulating evidence that nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP ribose (cADPR) have a prominent role in intracellular Ca\(^{2+}\) signalling. Both of these are formed by ADP ribosyl cyclase that has a synthase and hydrolase activity. It is likely the activation of ADP ribosyl cyclase is due to agonist activation. In support of this hypothesis there is evidence that in hippocampal neurons and glial cells glutamate application results in elevated NAADP cellular levels (Pandey et al., 2009).

However, despite both being generated by ADP ribosyl cyclase, NAADP and cADPR mobilise intracellular Ca\(^{2+}\) through different mechanisms. The cADPR metabolite is thought to act as modulator as it is thought to activate RyRs inducing Ca\(^{2+}\) efflux into the cytosol. However, following RyR activation by cADPR there is a desensitisation of the release mechanism induced by cADPR. The activation of RyR by cADPR requires calmodulin presence and the desensitisation of the release mechanism is due to the dissociation of calmodulin from the RyRs (Lee et al., 1994; Thomas et al., 2002). Furthermore, cADPR has been shown to enhance IP\(_3\) evoked Ca\(^{2+}\) release by increasing
SERCA pump function of sequestering Ca$^{2+}$ (Yamasaki-Mann et al., 2010). In functional studies in the frog motor nerve terminals, it has been shown that cADPR enhances CICR by inhibiting the RyR inactivation and increases the efficiency of activation (Hachisuka et al., 2007). Furthermore, neurotransmitter release has been shown be modulated by cADPR in cholinergic synapses of the buccal ganglion of Aplysia californica (Mothet et al., 1998).

NAADP is thought to act on NAADP receptors found on acidic stores such as lysosomes, release from which has been shown to occur in sea urchin eggs (Churchill et al., 2002). It is thought that NAADP mobilises Ca$^{2+}$ by activating two-pore channels (Calcraft et al., 2009). In lysosomal stores it is thought that TPCN2 a member of the two-pore channel family is activated by NAADP (Zong et al., 2009). The Ca$^{2+}$ released from these acidic stores is independent and does not interact with Ca$^{2+}$ release from the smooth endoplasmic reticulum or the mitochondria. The Ca$^{2+}$ enters the acidic stores via activity of a Ca$^{2+}$-ATPase that pump Ca$^{2+}$ into the store using ATP. Furthermore, the membranes of the acidic stores have a large concentration gradient of protons which can be used by Ca$^{2+}$/H$^+$ exchanger to efflux H$^+$ and uptake Ca$^{2+}$ into the vesicle lumen. In the frog neuromuscular junction it has been shown that NAADP can enhance neurosecretion induced by IP$_3$ and cADPR and that this enhancement activity is distinct from that supported by the smooth endoplasmic reticulum (Brailoiu et al., 2003). Furthermore, there is evidence which suggests that NAADP can also enhance neurotransmitter release in cholinergic synapses of the buccal ganglion of Aplysia californica (Chameau et al., 2001). Interestingly, a study has suggested that NAADP enhances release at the frog neuromuscular junction which could involve NAADP activity of synaptic vesicles in the RRP (Brailoiu et al., 2001).
Ca\(^{2+}\) that is released from acidic stores by NAADP can act on RyRs to activate Ca\(^{2+}\) release from smooth endoplasmic reticulum. However, there is evidence which suggests that Ca\(^{2+}\) released from the acidic stores is transported into the smooth endoplasmic reticulum and so subsequent stimulation of IP\(_3\)Rs or RyRs produce further Ca\(^{2+}\) release (Churchill & Galione, 2001).

1.7. Isolated nerve terminals (Synaptosomes)

In this thesis we need a good model in which the presynaptic effect can be studied in isolation. The paired-recordings method from brain slices is a model that has been developed to understand the functional communication between the neurons. The main advantages of this model are that many different regions of the brains can be studied. Furthermore, this model does not use extracellular stimulations but can use electrical stimulation protocols that ensure the triggering of an action potential at the presynaptic terminal. However, this technique is not appropriate for studying presynaptic nerve terminals in isolation as retrograde signalling can influence the responses at the presynaptic nerve terminal (Debanne et al., 2008).

Isolated nerve terminals (synaptosomes) are encapsulated presynaptic nerve endings that have been sheared off using a homogenisation process. Synaptosomes contain within them the SSVs; some have mitochondria, cytoplasm and smooth endoplasmic reticulum (McGraw et al., 1980a; Whittaker, 1993). In functional terms, synaptosomes can metabolise glucose to make ATP as they have functioning mitochondria. Furthermore, the plasma membrane of synaptosomes contain functional Na\(^+\) and K\(^+\) channels to maintain the membrane potential and ion homeostasis. In addition the SSVs within synaptosomes are competent for storing neurotransmitters and undergoing exocytosis and endocytosis. Synaptosomes have been shown to possess a Ca\(^{2+}\)
dependent exocytotic pool of glutamate (Nicholls & Sihra, 1986) and are sensitive to
tetanus and botulinum A and B toxin (Sanchez-Prieto et al., 1987; McMahon et al.,

The consequences of synaptosomal stimulation can also be studied as they contain
competent and functional signal cascades and downstream kinases and phosphatases.
However, there are some disadvantages of using a synaptosomal model over the cellular
methods. Firstly, synaptosomes cannot make new proteins therefore they have a limited
utility in long term studies. Furthermore, due to their size, single-channel recordings are
not feasible and Ca\textsuperscript{2+} measurement using fluorescent dyes is limited. Lastly, due to the
homogenisation process itself, the synaptosomes that are purified are heterogeneous
containing both glutamatergic and GABAergic nerve terminals. Despite these
shortcomings they are suited to address the questions that are posed in this thesis.

1.8. PhD aims

1. ERK1 and ERK2 phosphorylation/activation could be activated by Ca\textsuperscript{2+}-
dependent mechanisms in presynaptic nerve terminals though the sources of
Ca\textsuperscript{2+} cannot be distinguished. The main objective of the thesis was to investigate
the stimulation paradigms of Ca\textsuperscript{2+}-dependent mechanisms that result in ERK1
and ERK2 phosphorylation/activation. Thus the first aim was to establish a
model that separates the external and internal Ca\textsuperscript{2+}-dependent mechanisms
responsible for ERK1 and ERK2 phosphorylation/activation.

2. In presynaptic nerve terminals there are three potential compartments that store
Ca\textsuperscript{2+} these being the smooth endoplasmic reticulum, mitochondria and acidic
stores. Each store could play a significant role in ERK1 and ERK2
phosphorylation/activation. Thus, the functional role of each of the stores to the phosphorylation/activation of ERK1 and ERK2 was investigated.

3. Intracellular Ca\textsuperscript{2+} stores can be mobilised by different signalling pathways. The two main mechanisms that were targeted included Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) and IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (IPCR).

4. Ca\textsuperscript{2+} homeostasis dysfunction can result in a significant pathophysiological condition. The effect on intracellular Ca\textsuperscript{2+} stores was investigated in a model that mimicked the pathophysiological conditions.
Chapter 2
2. Method

The preparation of synaptosomes has evolved with time with changes in procedure aimed at yielding high concentrations of pure synaptosomes that have good metabolic properties. The key change in the procedure has been the use of type of gradient used during the separation of synaptosomes. Density sucrose gradients were first used but this exposes synaptosomes to very hypertonic conditions thus the metabolic condition of synaptosomes are poor (Whittaker, 1968). The use of polysaccharide polymers with Ficoll gradients yields more metabolic competent synaptosomes as compared to synaptosomes prepared from sucrose gradients. However, synaptosomes were also found to be more heterogeneous (Verity, 1972). Percoll based gradients have been developed which tackles both of these issues. They are non-toxic polymer beads that are iso-osmotic and avoid the effects of dehydration which yields synaptosomes with high metabolic competency. Furthermore, the synaptosomes are purified and the Percoll beads and can be easily removed after centrifugation (Nagy & Delgado-Escueta, 1984; Dunkley et al., 1986; Dunkley et al., 2008). Thus, we have decided to use synaptosomes that are purified using the Percoll gradients.

2.1. Synaptosomal preparation

Synaptosomes were obtained from male Sprague-Dawley rats weighing between 150-200 grams. Animals were sacrificed by decapitation according to the Home Office Animals (Scientific Procedures) Act of 1986. The cerebral cortex was dissected with the hippocampus and any other non-cortical tissues removed. The cortex was then placed into separate centrifuge tubes containing ice-cold sucrose medium (320mM). The cortices were then homogenised using a Telfon pestle and a glass Potter-Elvehjem tissue grinder. The homogenised cortices were then centrifuged in a Beckman J2-21M/E centrifuge at 3,020g for 2 minutes. At the end of the first spin, the first pellet (P1), a
mixture of blood vessels connective tissue and cell body debris, was discarded. The supernatant (S1) was spun at 14,600g for 12 minutes. At the end of this spin, the pellet (P2) contained crude synaptosomes and mitochondria while the supernatant contained various ‘light’ components such as microsomal membranes, ribosomes and myelin.

The supernatant was discarded and the P2 pellets were re-suspended in the sucrose medium (320mM) using a Dounce homogeniser. The re-suspended solution was then gently loaded onto ice-cold Percoll gradients. The Percoll gradients were made of three discontinuous layers of Percoll at concentrations of 3, 10 and 23% (top-bottom). The layered Percoll gradients were then centrifuged at 35,100g for 6 minutes to separate the synaptosomes from mitochondria and myelin. There are several advantages in using Percoll gradients as opposed to other density gradients. Firstly, Percoll gradient separation produces a purer synaptosomal yield and second as the medium is iso-osmotic; it has no dehydration effects on the synaptosomes as is the case for sucrose gradients. Percoll is inert and therefore non-toxic Percoll purification maintains nerve terminal viability.

Following Percoll gradient separation, three bands were observed (from the top): the first band is myelin, the second band contains the synaptosomes and the third band is composed of mitochondria. The first and third bands were discarded but the second band containing the synaptosomes was carefully removed and added to ice-cold HBM: NaCl (140mM), KCl (5mM), NaHCO₃ (5mM), NaH₂PO₄ (1.2mM), MgCl₂ (1mM), glucose (10mM), BSA (1mg/ml) and HEPES (10mM); pH7.4 contained in a 50ml centrifuge tube. These synaptosomes suspended in HBM were then centrifuged at 27,000g for 10 minutes. The resulting pellet contained purified synaptosomes which
were then re-suspended in a small volume of HBM using a Dounce homogeniser and stored on ice.

The concentration of synaptosomes was next determined using the Bradford protein assay (Bradford, 1976). The protein determination allows a reproducible synaptosomal concentration was aliquoted for each preparation and ensures reliability comparability of data between experiments. After determining the synaptosomal concentration from the preparation, an appropriate volume of re-suspended synaptosomes was added to ice-cold test tubes containing 8ml HBM and centrifuged at 3,020g for 10 minutes. The supernatant was discarded and the pellets were stored on ice. The resulting pellet contains the desired amount of purified synaptosomal protein and ready for subsequent use in experiments.

2.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Immunoblotting is a procedure by which specific proteins can be analysed and quantified. There are three stages to the quantification of proteins; the proteins are separated, transferred onto nitrocellulose membranes and immunoblotted with the specific antibodies.

Synaptosomal proteins were separated using SDS-PAGE gels composed of acrylamide (7.5%), bis-acrylamide (3%) and a running buffer consisting of Tris (25mM), glycine (192mM) and SDS (0.1%); pH8.6. The samples contained a STOP solution of Tris (12.5mM), SDS (0.4%), glycerol (2%), 3-mercaptopethanol (1%) and bromophenol blue. The STOP solution was added at the end of each experiment which not only stops the reactions but also denatures the proteins. In addition, SDS in its structure has a hydrophobic chain that bind to the main peptide chain at a ratio of one SDS molecule
for every two amino acid residues. This effectively imparts a negative charge on the protein that is proportional to the mass of that protein and which is greater than the original charge of that protein. Thus proteins migrate down the gel relative to their mass and not to their native charge. The amount of protein that was loaded onto the gels was consistent throughout the experiments at 50µg. The gels were run at room temperature at a constant voltage (40V) therefore increasing temperature does not affect the migration of proteins.

After the proteins have been separated, they were transferred onto a nitrocellulose membrane in electrotransfer buffer containing Tris (23mM), glycine (192mM) and methanol (20%). The transfer was conducted overnight at a constant current of 200mA. After the transfer, the nitrocellulose membrane was stained with Ponceau S solution containing Ponceau Red (0.2%), TCA (3%) and sulfosalicyclic acid (3%); this allowed for the visualisation of the transferred proteins.

The nitrocellulose membrane was then immunoblotted using a standardised procedure for identifying and quantifying ERK1/2 phosphorylation. The membrane was washed three times for 10 minutes each in a Tris buffer saline (TBS)-Tween 20 which contains Tris (20mM); pH 7.6, NaCl (137mM), Tween-20 (0.5%). This ensured that any excess Ponceau S staining was removed and initiated blocking of non-specific binding sites. The membrane was then incubated for an hour in a blocking buffer solution made of dry milk (5%) in TBS-Tween 20. This incubation step reduced non-specific binding of the primary antibody. After the blocking buffer incubation, the membrane was incubated overnight at 4ºC with the primary antibody at a 1 in 1000 dilution in TBS-Tween 20. The primary antibody was purchased from Cell Signalling Technologies and detects endogenous levels of p44 and p42 MAP Kinase (ERK1 and ERK2) when
phosphorylated dually at Thr202 and Tyr204 of ERK1 (Thr185 and Tyr187 of ERK2). The antibody does not cross-react with non-phosphorylated ERK1 and ERK2 and does not cross-react with the corresponding phosphorylated residues of either JNK/SAPK or p38 MAP kinase. Following the overnight incubation, the membrane was washed three times for 15 minutes each in the blocking buffer containing dry milk (5%) to ensure that any unbound primary antibody was removed. After this, the bound primary antibody on the membrane was labelled with the secondary antibody that is conjugated with radioactive $^{125}$I at a 1 in a 1000 dilution for an hour. The membrane was then washed using blocking buffer containing dry milk (5%) for 5 and 10 minutes and TBS-Tween 20 for 5 and 10 minutes to remove any excess secondary antibody. The radioactive membrane was then air-dried and exposed to a phosphoimager screen. The screen was analysed with a Typhoon 9410 phosphoimager screening system. The data was quantified using Image Quant analysis that calculated the volume under the surface of a given area created by a 3-D plot of the pixel locations and pixel intensities.

2.3. Incubation protocols

We have used differing protocols to test the effect various drugs have on ERK1 and ERK2 phosphorylation/activation. This has been done to compensate the preincubation necessity of some drugs to see their effect as the permeability of the drug into the synaptosomes varies from drug to drug.

2.3.1. Standard protocol

In the standard protocol we resuspended the synaptosomes that were in the pellet obtained using the method described in section 2.1. The synaptosomes were resuspended using a HBM buffer solution with BSA (1mg/ml) to give a synaptosomal concentration of 1mg/ml. The resuspended synaptosomes were then incubated at 37°C
for 3 minutes in 1.5ml eppendorf tubes that were kept on ice prior to the incubation. This is necessary as it has been previously shown that in using this protocol temperature can affect the ERK1 and ERK2 phosphorylation/activation. After 3 minutes Ca\(^{2+}\) (1mM) was added to the incubation or in conditions to achieve Ca\(^{2+}\) free incubation EGTA (100\(\mu\)M) was added. In order to cause Ca\(^{2+}\) entry we used 4-AP (1mM) or ionomycin (5\(\mu\)M) after 10 minutes of incubation. We stimulated the synaptosomes with 4-AP or ionomycin for 5 minutes after which we ended the incubation by adding a STOP solution that contains Tris (125mM); pH 6.8, SDS (0.4%), glycerol (2%), 3-mercaptopethanol (1%) and bromophenol blue. In every experiment control incubation was put on ice for the length of time of 37\(^\circ\)C incubation samples which was also ended with the STOP solution.

**Schematic 2-1: Standard protocol timeline**

This schematic shows the standard protocol timeline. Synaptosomes were resuspended in HBM buffer containing BSA (1mg/ml) followed by the Ca\(^{2+}\) addition after 3 minutes.4-AP (1mM) or ionomycin (5\(\mu\)M) was used to stimulate the synaptosomes for 5 minutes and the experiment was ended using the STOP solution.

**2.3.2. Intracellular Ca\(^{2+}\) store repletion protocol**

We devised a protocol that specifically deals with the detecting the effect of Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores on ERK1 and ERK2 phosphorylation/activation. The synaptosomal pellet obtained through the procedure described in section 2.1 was resuspended to give the synaptosomal concentration of 1mg/ml using a HBM buffer solution containing BSA (1mg/ml). Synaptosomes were added to 1.5ml eppendorf tubes that were kept on ice and incubated at 37\(^\circ\)C for 50 minutes. During this preincubation
time various drugs were incubated as required for that specific experiment. Furthermore, if required Ca\(^{2+}\) (1mM) was added during this preincubation period. After 50 minutes the synaptosome suspension was spun down and the supernatant was removed and the synaptosome pellet put on ice. We undertook this step to remove any drug that was added during the preincubation period. The synaptosomes were then resuspended using the HBM buffer containing BSA (1mg/ml) and Ca\(^{2+}\) (1mM) giving a final synaptosomal concentration of 2mg/ml and incubated at 37ºC for 2 minutes. 4-AP (1mM) or ionomycin (5µM) was then used to cause Ca\(^{2+}\) entry into synaptosomes with the stimulation lasting for 5 minutes. The experiment was ended using a STOP solution that contains Tris (125mM); pH 6.8, SDS (0.4%), glycerol (2%), 3-mercaptoethanol (1%) and bromophenol blue. In every experiment control incubation was put on ice for the length of time of 37ºC incubation samples which was also ended with the STOP solution.

Schematic 2-2: Intracellular Ca\(^{2+}\) store repletion protocol timeline
This is the intracellular Ca\(^{2+}\) store repletion protocol timeline. The synaptosomes were resuspended and any drug of interest was added at the beginning of the experiment. After 3 minutes Ca\(^{2+}\) (1mM) was added and synaptosomes were preincubated for 50 minutes and spun down at 14,000rpm for 30 seconds. The synaptosomes were kept on ice and resuspended after 67 minutes with Ca\(^{2+}\) already present in the HBM buffer. 4-AP (1mM) or ionomycin (5µM) was added to the incubation after 70 minutes and this stimulated the synaptosomes which lasted for 5 minutes and the experiment was ended using a STOP solution.

2.3.3. Metabotropic activation protocol

We suspected that the high synaptosomal concentration used in other protocols to show ERK1 and ERK2 phosphorylation/activation is not suitable for metabotropic phosphorylation/activation of ERK1 and ERK2. The high synaptosomal concentration
could lead to high levels of endogenous glutamate in the incubation which could lead to the desensitisation of the glutamate metabotropic receptors. Synaptosomes that were obtained through the procedure described in section 2.1 were first resuspended in synaptosomal concentration of 1mg/ml. The final suspension concentration was then made to 0.067mg/ml using HBM buffer containing BSA (1mg/ml) and incubated at 37°C for 50 minutes. During this preincubation period Ca^{2+} (1mM) was added to the suspension as well as any drugs of interest for the experiment. The synaptosomes were then stimulated by using DHPG (10µM) for 1 minute after which the synaptosomes were spun down and the supernatant removed. The experiment was ended using a STOP solution that contains Tris (125mM); pH 6.8, SDS (0.4%), glycerol (2%), 3-mercaptopethanol (1%) and bromophenol blue. In every experiment control incubation was put on ice for the length of time of 37°C incubation samples which was also ended with the STOP solution.

Schematic 2-3: Metabotropic activation protocol timeline
This is the metabotropic activation protocol timeline. Synaptosomes were resuspended in HBM buffer and any drug of interest for a particular experiment was also added at the beginning of the experiment. After 3 minutes Ca^{2+} (1mM) was added to the incubation. DHPG (10µM) was used to stimulate the synaptosomes for 1 minute after which the synaptosomes were spun down with the supernatant removed. The experiment was ended using a STOP solution.

2.4. Intracellular [Ca^{2+}] Measurement using Fura-2
The measurement of intracellular [Ca^{2+}] is achieved through the use of an ion sensitive fluorescent dye called Fura-2. Fura-2 is a ratiometric dye that fluoresces on binding to Ca^{2+}. Upon Ca^{2+} binding, Fura-2 exhibits a wavelength shift in the peak of its excitation spectrum from 380 nm to 340 nm (Gryniewicz et al., 1985). Fura-2 has four negative
charges which gives Fura-2 its affinity for Ca\(^{2+}\). However, due to the negative charges, Fura-2 is membrane impermeable. To overcome this, a hydrophobic acetomethoxy group is attached to Fura-2 through an ester bond which makes it membrane permeable (Fura-2AM). Once inside the cytosol, the ester bond is hydrolysed leaving Fura-2 trapped in the synaptosomes.

Synaptosomes (0.2mg) were re-suspended in a HBM containing BSA (1mg/ml) and incubated with Fura-2AM at 37°C for 20 minutes. After 3 minutes Ca\(^{2+}\) (0.1mM) was added and at the end of the incubation, synaptosomes were centrifuged for 10,000rpm for 1 minute. The supernatant was removed and the pellet was re-suspended in a HBM containing BSA (1mg/ml). The recording of the trace was started and Ca\(^{2+}\) (1mM) was added after 3 minutes. After 10 minutes, the control or the appropriate drug concentration was added to the synaptosome suspension and the action of the control and drug was measured for 20 minutes (Schematic 2-4).

To ensure that the measurement of intracellular [Ca\(^{2+}\)] is not influenced by varying loads of Fura-2 into synaptosomes, a calibration was conducted at end of each experiment. The maximum binding by intrasynaptosomal loaded Fura-2 to Ca\(^{2+}\) was determined by adding SDS (0.1%). The external Ca\(^{2+}\) is 1mM thus when the synaptosomes are lysed, Fura-2 is saturated by Ca\(^{2+}\) and this produces a maximum fluorescence ratio \(R_{\text{max}}\). The minimum binding by intracellular Fura-2 to Ca\(^{2+}\) was determined with the addition of a mixture of EGTA (10mM) and Tris. EGTA has a greater affinity for Ca\(^{2+}\) than Fura-2 thus EGTA is able to remove any Ca\(^{2+}\) bound to Fura-2. This gives the minimum fluorescence ratio of Fura-2 \(R_{\text{min}}\). The amount of Tris had been previously calibrated to ensure that there is no pH change with the addition of EGTA (10mM).
Schematic 2-4: Intracellular [Ca²⁺] measurement using Fura-2 timeline

Synaptosomes are re-suspended in HBM containing BSA (1mg/ml) and Fura-2AM (5µl) is added to the suspension. During the incubation Ca²⁺ (0.1mM) is added after 3 minutes. After the incubation the synaptosomes were washed and the recording of the experiment conducted. Ca²⁺ (1mM) was added 3 minutes after the start of the recording of the experiment. At 10 minutes HBM or drug of interest was added to the incubation. The calibration was conducted at the 30 minute time point when SDS (0.1%) was added to the incubation and the calibration was completed with the addition of EGTA/Tris (10mM) at the 34 minute time point.

2.5. Glutamate release

At the appropriate time, the synaptosomes were re-suspended for measurement of glutamate release by on-line fluorimetry using a Perkin-Elmer LS-3B spectrofluorimeter. This assay is based on the oxidative deamination of glutamate, catalysed by glutamate dehydrogenase (GDH) and coupled to the reduction of NADP⁺ to NADPH which is measured due to its fluorescence. The settings for the spectrofluorimeter were excitation wavelength 340nm, emission wavelength 460nm. The synaptosomes were re-suspended in 1.5ml HBM containing BSA (1mg/ml) and transferred to the spectrofluorimeter. Constant stirring ensured the synaptosomes were oxygenated. NADP⁺ (1mM) and GDH (50 units/ml) were added to the suspended synaptosomes at time point 0 and Ca²⁺ (1mM) was added after 3 minutes. At 6 minutes, the recording of the release was started to measure fluorescence emission at intervals of 2 seconds. At 10 minutes, one of two secretagogues was added to evoke Ca²⁺-dependent glutamate release; 4-AP (1mM) or ionomycin (5µM). Finally, at 15 minutes, exogenous glutamate (2.5nmol) was added as an internal standard to quantify the glutamate released (Schematic 2-5). Figure 2-1 shows a typical trace in its unanalysed form shown in fluorescence units and the time points at which 4-AP and glutamate were added. The
figure also shows the same trace analysed with the time point of 4-AP addition set at the baseline.

Schematic 2-5: Glutamate release timeline
Synaptosomes were re-suspended in HBM containing BSA (1mg/ml) and NADP⁺ (1mM) and GDH (50 units/ml) was added to the suspension. At 3 minutes Ca²⁺ (1mM) was added and the recording of the trace was started at 6 minutes. Drugs of interest can be added between 7-10 minutes. At 10 minutes 4-AP (1mM) was added and the glutamate (2.5nmol) an internal standard was added at 15 minutes.

2.6. Data Analysis

2.6.1. Immunoblot analysis
Scanning the phosphoimager produced an image of the radioactive bands which were then highlighted using uniform-sized boxes. A similar sized box was used to measure the levels of background radioactivity which was then used to subtract from the sample values. Sample values were then normalised to the 37°C control as shown below:
Normalised Sample Value (%) = \frac{\text{Sample value} - \text{Background value}}{37^\circ\text{C control value} - \text{Background value}} \times 100

Data was then analysed in Microsoft Excel. For data containing more than two sets analysis of variance (ANOVA) was used to assess the statistical significance, followed by Dunnetts post hoc test. The net change in ERK1 and ERK2 phosphorylation/activation was calculated by removing the basal percentage from the stimulated groups for each n. We used Student’s unpaired t-test to assess the statistical significance between two sets of data.

2.6.2. Fura-2 analysis

The intracellular $\text{Ca}^{2+}$ measurement was determined according to the formula:

$$[\text{Ca}^{2+}]_i = \frac{K_d \times (R-R_{\text{min}}) \times S_{f2}}{(R_{\text{max}}-R) \times S_{b2}}$$

$K_d$ is 224nM,
$S_{f2}$ is the minimum fluorescence of the 380nM wavelength
$S_{b2}$ is the maximum fluorescence of the 380nM wavelength
$R$ is the experimental fluorescence ratio
$R_{\text{min}}$ is the minimum fluorescence ratio
$R_{\text{max}}$ is the maximum fluorescence ratio

2.6.3. Glutamate release analysis

The bar graphs of glutamate release in the results section show independent experiments averaged at 300-305 second time points with the mean +/- SEM calculated using these averages. All glutamate release data was analysed using Lotus 1, 2, 3. The glutamate release was calculated using the formula;

$$\text{Glutamate release (nmol/mg)} = \frac{(\text{fluorescence}) \times \text{Glutamate standard}}{F_{\text{std}}}$$

Glutamate standard = 2.5nmol/0.1mg/ml (synaptosomal protein concentration)
$F_{\text{std}}$ = Glutamate standard fluorescence
2.7. Reagents List

Sucrose, HPLC graded water, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), tris(hydroxymethyl)methylamine (tris), glycine and sodium dodecyl sulphate (SDS) were obtained from VWR (UK). Percoll, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(b-aminoehyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), nicotinamide adenine dinucleotide (NADP), 4-aminopyridine (4-AP), baclofen and N⁶-cyclohexyladenosine (CHA) were obtained from Sigma-Aldrich (UK). Ionomycin, thapsigargin, KN93 and PP2 were obtained from Calbiochem (UK). Fura-2acetoxymethyl ester (Fura-2AM) was obtained from Molecular Probes (Cambridge, UK). Acrylamide and bis-acrylamide were purchased as ready-made solutions (30% and 2% solutions, respectively) were obtained from National Diagnostic (UK). W7, 2-aminoethoxydiphenyl borate (2-APB), U-73122, L-AP4, ryanodine, 8-Hydroxy-2-dipropylaminotetralin hydrobromide (8-OH DPAT), (S)-3,5-Dihydroxyphenylglycine (DHPG), FCCP and oligomycin were obtained from Tocris (UK). Rabbit polyclonal phospho-p44/42 MAP kinase (Erk1/2) (Thr202/Tyr204) antibody was obtained from Cell Signaling Technology (UK). Goat ¹²⁵I IgG antibody was obtained from PerkinElmer (UK). High range rainbow marker was obtained from Amersham Bioscience (UK).
Chapter 3
3. Ca$^{2+}$-dependent phosphorylation/activation of ERK1 and ERK2

**Summary:** Ca$^{2+}$-dependent pathways are thought to participate in ERK1 and ERK2 phosphorylation/activation. Using kinase activity as a sensor for an increase Ca$^{2+}$ concentrations, we investigated the role of external and internal sources of Ca$^{2+}$ in the activation of the ERK pathway. We found that there was a significant increase in ERK1 and ERK2 phosphorylation/activation when the synaptosomal membrane was depolarised with 4-AP or by direct Ca$^{2+}$ influx using the Ca$^{2+}$ ionophore, ionomycin. The increase in ERK1 and ERK2 phosphorylation/activation could not be mediated in the absence of external Ca$^{2+}$. Thus this stimulation of ERK1 and ERK2 phosphorylation/activation by 4-AP and ionomycin was found to be completely Ca$^{2+}$-dependent. Furthermore, we found that repletion of intracellular Ca$^{2+}$ stores underpinned 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation. In conclusion, Ca$^{2+}$ is necessary for ERK1 and ERK2 phosphorylation/activation and that intracellular Ca$^{2+}$ stores could also support the Ca$^{2+}$-dependent mechanisms.

3.1. Introduction

In presynaptic nerve terminals, Ca$^{2+}$ influx through VDCCs results in the release of neurotransmitter and the activation of modulatory pathways that increase or decrease the release. One of these modulatory pathways is the ERK pathway that has been shown to enhance neurotransmitter release via brain derived neurotrophic factor (BDNF) activation of the TrkB receptors (Jovanovic et al., 2000). The neurotrophin-mediated ERK1 and ERK2 phosphorylation/activation is described in more detail in section 1.5.2.

In the ‘classical’ pathway involving Trk activation, Grb2 adaptor protein recruitment in turn recruits GTPase – exchange factors (GEFs) such as Sos to the plasma membrane,
resulting in the exchange of GDP for GTP on plasma membrane bound Ras, which is thereby activated. The reverse inactivation is promoted from GTP hydrolysis to GDP by GTPase activating proteins (GAPs). The trigger for the ERK activation cascade is essentially the exchange of GDP to GTP on Ras and mediated by the activity of GEFs and counter balanced by GAPs activity, thus GEFs promote while GAPs reduce ERK1 and ERK2 phosphorylation/activation (Walker et al., 2003). Activation of Ras promotes a conformational change which causes the recruitment/activation of downstream kinases c-Raf-1 and/or B-Raf. Activated c-Raf-1 and/or B-Raf phosphorylate/activate downstream targets, mitogen-activated protein kinase kinase 1 and 2 (MEK1 and MEK2). Phosphorylated/activated MEK1 and MEK2 then cause the dual phosphorylation/activation of their downstream targets ERK1 and ERK2 (Chao, 2003).

Together with the ‘classical’ ERK pathway by Trks, there is evidence to suggest that Ca\(^{2+}\) through multiple mechanisms is able to modulate the ERK activation cascade. One mechanism involves the proline-rich tyrosine kinase (Pyk2) which is activated by increases in Ca\(^{2+}\) concentrations. Activated Pyk2 is thought to phosphorylate Src which then phosphorylates tyrosine sites on Shc, leading to the recruitment of the Grb2/Sos complex. In addition, possibly, activated Src phosphorylation of Pyk2 may allow recruitment of the Grb2/Sos complex which is independent of Shc phosphorylation (Lev et al., 1995). Activated Src may also independently phosphorylate A-Raf and c-Raf-1 directly without the requirement of Ras activation (Marais et al., 1995; Stokoe & McCormick, 1997).

Considering the Ca\(^{2+}\)-dependent mechanism activation of ERK1 and ERK2, increases in Ca\(^{2+}\) could have stimulatory effects on RasGEFs such as Ca\(^{2+}\) and diacylglycerol-regulated GEFI (CalDAG-GEFI) and CalDAG-GEFII that are respectively activated by
Ca\(^{2+}\) and diacylglycerol (DAG), and result in the ERK1 and ERK2 phosphorylation/activation (Kawasaki et al., 1998b). Furthermore, calmodulin (CaM) could also be activated by an influx of Ca\(^{2+}\) which can have stimulatory as well as inhibitory effects on the ERK pathway. Binding of CaM to K-Ras inhibits its activity either through increasing its GTPase activity or inhibiting the transmission of the Ras signals to Raf. Alternatively CaM could also stimulate RasGEFs such as Ras-specific guanine nucleotide-releasing factor (RasGRF) that is directly activated by CaM, thus activating Ras and the consequent downstream ERK1 and ERK2 phosphorylation/activation (Farnsworth et al., 1995). There is evidence therefore multiple pathways that could be activated to initiate and propagate the ERK activation cascade stimulated by Ca\(^{2+}\).

The question remains as to the source(s) of Ca\(^{2+}\) that can mediate the Ca\(^{2+}\)-dependent ERK pathway, requiring a method to differentiate external and internal Ca\(^{2+}\) influences on the mechanism. In order to separate the effects of extracellular and intracellular Ca\(^{2+}\), we established the intracellular Ca\(^{2+}\) store repletion protocol described in section 2.3.2. The protocol is based on the hypothesis that intracellular Ca\(^{2+}\) stores may become partially or completely depleted during the synaptosomal preparation, therefore needing repletion in vitro with preincubation in the presence of external Ca\(^{2+}\).

In presynaptic nerve terminals, there is functional evidence for intracellular Ca\(^{2+}\) stores but these are too small to be detected by imaging fluorimetry widely used with studies with larger compartments. Furthermore, the amount of Ca\(^{2+}\) that is stored and subsequently released upon stimulation falls below the detection capabilities of Ca\(^{2+}\) indicators like Fura-2. However alternatively, it is possible to detect the changes in Ca\(^{2+}\) concentrations by measuring the Ca\(^{2+}\)-dependent activation of kinases through
phosphorylation of their substrates (Long et al., 2009). Thus here we have used Ca\textsuperscript{2+}-dependent ERK1 and ERK2 phosphorylation/activation as sensitive indicator of increases in intrasynaptosomal Ca\textsuperscript{2+} concentrations.

The objective of this chapter is to explore the consequences on ERK1 and ERK2 phosphorylation/activation of Ca\textsuperscript{2+} increases mediated by membrane depolarisation or direct influx through Ca\textsuperscript{2+} ionophore. In addition, we examined the sensitivity of membrane depolarisation and ionophore mediated stimulation to possible mechanism(s) that could be activated by the increase in intracellular Ca\textsuperscript{2+}. Furthermore, we sought to investigate any potential differences or collusion in the contribution of Ca\textsuperscript{2+} between external and internal sources to ERK1 and ERK2 phosphorylation/activation.
3.2. Method

3.2.1. Synaptosomal Preparation

Preparation of synaptosomes has been described in section 2.1.

3.2.2. SDS-PAGE and Immunoblotting

All samples obtained through immunoblotting experiments underwent the procedure described in section 2.2.

3.2.3. Standard protocol

The protocol for the experiments that followed the standard protocol is described in section 2.3.1. The synaptosomes were resuspended in HBM buffer containing BSA (1mg/ml) and incubated at 37°C with drug of interest including PP2 (10µM). After 3 minutes Ca^{2+} (1mM) or EGTA (100µM) was added to the incubation. 4-AP (1mM), ionomycin (5µM) or control (HBM buffer) was added to the incubation at 10 minutes. The experiment was terminated using a STOP solution after 15 minutes of incubation.

3.2.4. Intracellular Ca^{2+} store repletion protocol

Experiments that followed the intracellular Ca^{2+} store protocol are described in section 2.3.2. Synaptosomes were resuspended using the HBM buffer containing BSA (1mg/ml) and incubated at 37°C. After 3 minutes Ca^{2+} (1mM) or EGTA (100µM) was added to the incubation. Synaptosomes were then spun down after 50 minutes of incubation, the supernatant removed and the pellet put on ice. At 67 minute time point from the start of the incubation, synaptosomes were resuspended the second time with HBM containing BSA (1mg/ml) and Ca^{2+} (1mM) or HBM containing BSA (1mg/ml) and EGTA (100µM) and incubated at 37°C. 4-AP (1mM), ionomycin (5µM) or control
(HBM buffer) was then added to the incubation to stimulate synaptosomes for 5 minutes. The experiment was then terminated using the STOP solution.

3.2.5. Statistical analysis

The methods of statistical analysis have been described in section 2.6.1. For data with more than two sets analysis of variance (ANOVA) was used to assess the statistical significance, followed by Dunnetts post hoc test. The net change in ERK1 and ERK2 phosphorylation/activation was calculated by removing the basal percentage from the stimulated groups for each n. We used Student’s unpaired t-test to assess the statistical significance between two sets of data.

3.2.6. Reagents

A stock solution of 4-AP (1mM) was made in water and working solution was further diluted using HBM.

A stock solution of PP2 (10µM) was made using DMSO and further diluted using HBM.

A stock solution of ionomycin (5µM) was made in DMSO and working solution was further diluted using HBM.

A stock solution of EGTA (100µM) was obtained with water and working solution was further diluted using HBM.
3.3. Results

ERK1 and ERK2 phosphorylation/activation is reflected by dual phosphorylation on threonine and tyrosine sites. Using an antibody that specifically detects this dual phosphorylation, we conducted immunoblot analysis to determine the phosphorylation/activation state of ERK1 (p44 ERK) and ERK2 (p42 ERK).

We first assessed the basal activity of ERK1 and ERK2 that is produced once synaptosomes are maintained at physiological temperature (37ºC). Synaptosomes were incubated at different temperatures; one set was maintained at 4ºC while the other set was at 37ºC. There was a significant increase in the basal phosphorylation/activation when synaptosomes incubation is begun by shifting from 4ºC to 37ºC, with ERK1 and ERK2 increasing to 170.5 ± 5.2% and 168.6 ± 6.9% compared to the 4ºC control value, respectively (Figure 3-1D). The phosphorylation/activation of ERK1 and ERK2 at 37ºC suggests that the basal activity of ERK is significant under resting physiological state in synaptosomes.

To investigate the possible role of Ca²⁺ in presynaptic activation of the ERK pathway, we established conditions to obtain depolarisation mediated stimulation of ERK1 and ERK2 phosphorylation/activation. 4-aminopyridine (4-AP) is a K⁺ channel blocker which destabilises the resting plasma membrane and thus increases the likelihood of voltage-gated Na⁺ channels opening and results in depolarisation of the plasma membrane. Previous work in the laboratory has shown that depolarisation of the plasma membrane leads to Ca²⁺ influx through VGCCs, which then leads to the phosphorylation/activation of ERK1 and ERK2. We therefore considered the effect of 4-AP stimulation on ERK1 and ERK2 phosphorylation/activation as compared to 37ºC basal activity. Synaptosomes that were stimulated with 4-AP (1mM) showed significant
Figure 3-1: Basal, 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation using the standard protocol
(A) (B) (C) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively and labels Con = 37ºC control and Iono = Ionomycin shows the condition of each lane. (D) Synaptosomes were incubated at temperatures 4ºC and 37ºC for 15 minutes. Temp = temperature. (E) ERK1 and ERK2 phosphorylation/activation mediated by 4-AP (1mM) stimulation in the presence of Ca²⁺ (1mM). (F) ERK1 and ERK2 phosphorylation/activation mediated by ionomycin (5µM) stimulation in the presence of Ca²⁺ (1mM). All values represent the mean ± SEM. p<0.001(***), p<0.01(**), p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=6).
increase in the phosphorylation/activation of ERK1 (164.1 ± 7.5%) and ERK2 (158.9 ± 5.2%) compared to the 37ºC control (Figure 3-1E).

As an alternative paradigm for increasing Ca²⁺ concentrations without VDCC activation we affected direct increase of Ca²⁺ influx using a Ca²⁺ ionophore, ionomycin. Synaptosomes stimulated with ionomycin (5µM) displayed a significant increase in ERK1 (133.8 ± 3.8%) and ERK2 (135.7 ± 3.7%) phosphorylation/activation when compared to the 37ºC controls (Figure 3-1F). Interestingly, the increase in ERK1 and ERK2 phosphorylation/activation for ionomycin is less when compared to 4-AP stimulation, reflecting the different ‘routes’ of Ca²⁺ entry.

We next wanted to directly confirm that external Ca²⁺ influx is required for ERK1 and ERK2 phosphorylation/activation when the synaptosomes were stimulated with 4-AP. Therefore Ca²⁺ was omitted from the incubation and EGTA (100µM) was added to chelate any residual extracellular Ca²⁺ (Figure 3-2A&B). We found that, in the presence of Ca²⁺ 4-AP mediated stimulation caused a significant increase in ERK1 (145.5 ± 5.1%) and ERK2 (157.5 ± 6.5%) phosphorylation/activation compared to the 37ºC control (Figure 3-2C). In the absence of Ca²⁺, 4-AP mediated stimulation caused no significant increase of ERK1 (102.6 ± 4.4%) and ERK2 (102.4 ± 4.4%) phosphorylation/activation compared to the 37ºC control. In addition, the absence of Ca²⁺ had no significant effect on the basal activity of ERK1 (94.1 ± 8.0%) or ERK2 (91.1 ± 6.5%) compared to the 37ºC control. The net change revealed that there was a significant inhibition of 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation in the absence of Ca²⁺ (Figure 3-2D).
Figure 3-2: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ca^{2+} using the standard protocol

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and E = EGTA shows the condition of each lane.  (B) Timeline of the experiment showing that EGTA (100µM) or Ca^{2+} (1mM) was added at 3 minutes followed HBM or 4-AP (1mM) stimulation after 10 minutes and the experiment ended after 15 minutes of incubation. (C) Basal effect of absence of Ca^{2+} and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of Ca^{2+}. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
To investigate whether ionomycin mediation stimulation of ERK1 and ERK2 phosphorylation/activation was similarly sensitive to external Ca$^{2+}$ we conducted the experiment by omitting external Ca$^{2+}$ with EGTA (100µM) addition (Figure 3-3A&B). While ionomycin mediated stimulation of synaptosomes in the presence of Ca$^{2+}$ resulted in a significant increase in ERK1 (133.8 ± 3.8%) and ERK2 (135.7 ± 3.7%) phosphorylation/activation compared to the 37°C control (Figure 3-3C). We found that in the absence of Ca$^{2+}$ the ionophore did not significantly increase ERK1 (102.6 ± 4.4%) and ERK2 (105.7 ± 3.4%) phosphorylation/activation compared to the 37°C control. In addition, the absence of Ca$^{2+}$ had no significant effect on the basal activity of ERK1 (94.1 ± 8.0%) and ERK2 (91.1 ± 6.5%) compared to the 37°C control. Thus, the net change of ERK1 and ERK2 phosphorylation/activation showed ionomycin mediated stimulation was significantly reduced by the absence of external Ca$^{2+}$ (Figure 3-3D).

Altogether, these results suggest that extracellular Ca$^{2+}$ influx underpins 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation.

Given the demonstrated importance of external Ca$^{2+}$ influx we next investigated the mechanisms activated by the influx of Ca$^{2+}$. One of the Ca$^{2+}$-dependent mechanisms that could be activated includes Src activation through Pyk2. Using the Src inhibitor PP2 (20µM) we investigated how sensitive 4-AP (1mM) mediated stimulation of ERK1 and ERK2 phosphorylation/activation is to Src inhibition (Figure 3-4A&B). ERK1 (162.1 ± 2.5%) and ERK2 (178.6 ± 4.7%) phosphorylation/activation compared to the 37°C control, was significantly increased by 4-AP mediated stimulation in the absence of PP2 (Figure 3-4C). However, there was no significant stimulation of ERK1 (102.3 ± 4.9%) and ERK2 (115.4 ± 8.0%) phosphorylation/activation compared to the 37°C control in the presence of PP2. Interestingly, incubation of PP2 also resulted in a significant decrease of ERK1 (77.6 ± 4.8%) and ERK2 (71.6 ± 0.7%) basal activity.
Figure 3-3: Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ca\(^{2+}\) using the standard protocol

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, Iono = Ionomycin and E = EGTA shows the condition of each lane. (B) Timeline of the experiment showing that EGTA (100µ M) or Ca\(^{2+}\) (1mM) was added at 3 minutes followed HBM or ionomycin (5µ M) stimulation after 10 minutes and the experiment ended after 15 minutes of incubation. (C) Basal effect of absence of Ca\(^{2+}\) and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of Ca\(^{2+}\). (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n shows a significant decrease of ionomycin mediated stimulation in the absence of Ca\(^{2+}\) when compared to the presence of Ca\(^{2+}\). All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
Figure 3-4: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of PP2

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control and P = PP2 shows the condition of each lane. (B) Timeline of the experiment showing that HBM or PP2 (10 µM) was incubated at the start of the experiment with Ca²⁺ (1mM) added at 3 minutes followed by HBM or 4-AP (1mM) stimulation after 10 minutes and the experiment ended after 15 minutes of incubation. (C) Basal effect of PP2 and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of PP2. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
compared to the 37ºC control. Given the basal effect of PP2, the net change showed that ERK1 and ERK2 phosphorylation/activation were still significantly increased in the presence of PP2. However this increase was significantly reduced when compared to ERK1 and ERK2 phosphorylation/activation in the absence of PP2 (Figure 3-4D).

Ionomycin (5µM) mediated stimulation is also Ca²⁺-dependent so we questioned to what degree is this stimulation sensitive to Src inhibition using PP2 (10µM) treatment (Figure 3-5A&B). We found that ERK1 (125.2 ± 8.2%) and ERK2 (130.6 ± 1.6%) phosphorylation/activation compared to the 37ºC control could be significantly increased by ionomycin mediated stimulation in the absence of PP2 (Figure 3-5C). In the presence of PP2, there was a significant inhibition of ERK1 (64.7 ± 4.4%) and ERK2 (67.5 ± 2.1%) phosphorylation/activation compared to the 37ºC control. However, this inhibitory effect might be attributed to the inhibitory effect of PP2 on the ERK1 (71.9 ± 6.7%) and ERK2 (67.3 ± 4.3%) basal activity compared to the 37ºC control. Thus, the net change shows that ERK1 and ERK2 phosphorylation/activation mediated by ionomycin stimulation is completely inhibited in the presence of PP2 (Figure 3-5D). Overall we can conclude that both 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 are sensitive to Src inhibition but to different extents.

We next investigated the role of Ca²⁺ potentially released from intracellular Ca²⁺ stores in the activation of the ERK pathway. We first investigated the basal activity of ERK1 and ERK2 when the synaptosomes are maintained at physiological temperature (37ºC) using the intracellular Ca²⁺ store repletion protocol (Figure 3-6A). We found that there was a significant increase in basal phosphorylation/activation of ERK1 (148.1 ± 6.0%) and ERK2 (158.4 ± 8.4%) compared to the 4ºC controls (Figure 3-6D). The increase in ERK1 and ERK2 phosphorylation/activation using this protocol is comparable to
Figure 3-5: Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of PP2

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, Iono = Ionomycin and P = PP2 shows the condition of each lane. (B) Timeline of the experiment showing that HBM or PP2 (10µ M) was incubated at the start of the experiment with Ca²⁺ (1mM) added at 3 minutes followed by HBM or ionomycin (5µ M) stimulation after 10 minutes and the experiment ended after 15 minutes of incubation. (C) Basal effect of PP2 and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of PP2. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(***), p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
increases using the standard protocol.

We next investigated the effect on ERK1 and ERK2 phosphorylation mediated by 4-AP stimulation of synaptosomes under the intracellular Ca\(^{2+}\) store repletion protocol. Synaptosomes were incubated in the presence of Ca\(^{2+}\) (1mM) during the preincubation stage and stimulated with 4-AP (1mM) in the presence of Ca\(^{2+}\) during the stimulation stage (Figure 3-6B). Under Ca\(^{2+}\)-repletion conditions, there was a significant increase in the phosphorylation/activation of ERK1 (168.3 ± 1.9%) and ERK2 (159.9 ± 3.2%) compared to the 37ºC control (Figure 3-6E).

Naturally, we also wanted to establish the effect on ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation under the intracellular Ca\(^{2+}\) store repletion protocol. Synaptosomes were incubated in the presence of Ca\(^{2+}\) (1mM) during the preincubation stage and stimulated with ionomycin (5 µM) in the presence of Ca\(^{2+}\) during the stimulation stage (Figure 3-6C). We found ionomycin mediated stimulation also caused a significant increase in ERK1 (127.6 ± 3.1%) and ERK2 (125.0 ± 2.1%) phosphorylation/activation compared to the 37ºC control (Figure 3-6F). The 4-AP and ionomycin responses using the intracellular Ca\(^{2+}\) store repletion protocol were comparable to those achieved with the standard protocol suggesting that the preincubation step does not compromise the responsiveness of synaptosomes.

Investigating this further, we sought to establish that 4-AP and ionomycin mediated responses are also completely dependent of Ca\(^{2+}\) using the intracellular Ca\(^{2+}\) store repletion protocol. We first assessed 4-AP mediated stimulation by incubating synaptosomes with Ca\(^{2+}\) (1mM) or in the absence of Ca\(^{2+}\) with EGTA (100µM) addition
Figure 3-6: Basal, 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation using the intracellular Ca\(^{2+}\) store protocol

(A) (B) (C) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively and labels Con = 37\(^\circ\)C control and Iono = Ionomycin shows the condition of each lane. (D) Synaptosomes were incubated for 50 minutes in the presence of Ca\(^{2+}\) (1mM) at 4\(^\circ\)C and 37\(^\circ\)C which is described as the preincubation stage. After spin and removal of the supernatant the pellet were resuspended in the presence of Ca\(^{2+}\) (1mM) and incubated at 37\(^\circ\)C for a further 8 minutes this is described as the stimulation stage. Temp = temperature. (E) Synaptosomes incubated at 37\(^\circ\)C for 50 minutes in the presence of Ca\(^{2+}\) (1mM) were stimulated with 4-AP (1mM) in the presence of Ca\(^{2+}\) (1mM) during the stimulation stage. (F) Synaptosomes incubated at 37\(^\circ\)C for 50 minutes in the presence of Ca\(^{2+}\) (1mM) were stimulated with ionomycin (5\(\mu\)M) in the presence of Ca\(^{2+}\) (1mM) during the stimulation stage. All values represent the mean ± SEM. p<0.001(***) p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=6).
during the preincubation and stimulation stages (Figure 3-7A&B). Similar to previous results, 4-AP mediated stimulation in the presence of Ca\(^{2+}\) in the preincubation and stimulation stage resulted in significant increase in ERK1 (151.4 ± 12.4%) and ERK2 (149.7 ± 5.9%) phosphorylation/activation compared to the 37ºC control (Figure 3-7C). In the absence of Ca\(^{2+}\) during the preincubation and stimulation stages, 4-AP mediated stimulation did not significantly increase ERK1 (70.3 ± 12.4%) but ERK2 (60.7 ± 13.6%) phosphorylation/activation compared to the 37ºC control was significantly reduced. However, ERK2 inhibition could be attributed to the basal activity of Ca\(^{2+}\) absence during the preincubation and stimulation stages. The basal activity of ERK1 (71.7 ± 9.3%) was not affected but ERK2 (52.1 ± 9.79%) was significantly reduced compared to the 37ºC control by the absence of Ca\(^{2+}\) during the incubation. The net change of 4-AP mediated stimulation showed that ERK1 and ERK2 phosphorylation/activation is significantly reduced by the absence of Ca\(^{2+}\) during the preincubation and stimulation stages (Figure 3-7D).

We now assessed the effect absence of Ca\(^{2+}\) would have on ERK1 and ERK2 phosphorylation/activation mediated by ionomycin stimulation. Synaptosomes were incubated in the presence of Ca\(^{2+}\) (1mM) or in the absence of Ca\(^{2+}\) with EGTA (100µM) addition present during the preincubation and stimulation stages (Figure 3-8A&B). Ionomycin mediated stimulation significantly increased ERK1 (122.3 ± 5.3%) and ERK2 (134.4 ± 3.3%) phosphorylation/activation compared to the 37ºC control in the presence of Ca\(^{2+}\) during the preincubation and stimulation stages (Figure 3-8C). While ionomycin mediated stimulation in the absence of Ca\(^{2+}\) caused no significant stimulation of ERK1 (86.9 ± 11.2%) phosphorylation/activation but ERK2 (64.4 ± 0.5%) phosphorylation/activation was significantly reduced compared to the 37ºC control. But this significant reduction could be largely due to the basal activity. There
Figure 3-7: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ca\(^{2+}\) during the preincubation and stimulation stages

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and E = EGTA shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of EGTA (100µM) or Ca\(^{2+}\) (1mM) were incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in EGTA (100µM) or Ca\(^{2+}\) (1mM) and stimulated with 4-AP (1mM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of the absence of Ca\(^{2+}\) during the preincubation and stimulation stages and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of Ca\(^{2+}\). (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
was significant inhibition of basal activity of ERK2 (63.0 ± 4.2%) but not ERK1 (83.6 ± 9.2%) compared to the 37°C control in the absence of Ca$^{2+}$ during the preincubation and stimulation stages. Overall we found that the net change in ERK1 and ERK2 phosphorylation/activation stimulated by ionomycin is significantly reduced by the absence of external Ca$^{2+}$ during the preincubation and stimulation stages (Figure 3-8D). This shows that 4-AP and ionomycin responses are also Ca$^{2+}$-dependent using the intracellular Ca$^{2+}$ store repletion protocol. Interestingly, there is difference in the basal activity between the standard and intracellular Ca$^{2+}$ store repletion protocols. Under the intracellular Ca$^{2+}$ store repletion protocol there is a significant reduction in ERK2 basal activity which does not occur with the standard protocol.

We next sought to delineate the extracellular and intracellular Ca$^{2+}$ contribution to the Ca$^{2+}$-dependent activation of ERK1 and ERK2 phosphorylation/activation using 4-AP and ionomycin mediated stimulation paradigms. According to our hypothesis, intrasynaptosomal Ca$^{2+}$ concentrations are significantly affected during the synaptosomal preparation leading to partial depletion of the intracellular Ca$^{2+}$ stores. The preincubation stage should replete any intracellular Ca$^{2+}$ stores which should enhance their ability to contribute Ca$^{2+}$ to the phosphorylation/activation of ERK1 and ERK2. We thereby reasoned that incubation with EGTA (100µM) instead of Ca$^{2+}$ during the preincubation stage should keep the intracellular Ca$^{2+}$ stores depleted. If intracellular Ca$^{2+}$ stores contribute Ca$^{2+}$ to the stimulation of the ERK pathway, then in these conditions there would be a significant reduction in ERK1 and ERK2 phosphorylation/activation during 4-AP or ionomycin mediated stimulation in the presence of Ca$^{2+}$.

We first tested this hypothesis by looking at the effect of 4-AP stimulation on ERK1
Figure 3-8: Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ca\(^{2+}\) during preincubation and stimulation stages

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, Iono = ionomycin and E = EGTA shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of EGTA (100µM) or Ca\(^{2+}\) (1mM) were incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in EGTA (100µM) or Ca\(^{2+}\) (1mM) and stimulated with ionomycin (5µM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of the absence of Ca\(^{2+}\) during the preincubation and stimulation stages and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of Ca\(^{2+}\). (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
and ERK2 phosphorylation/activation and thus ascertain if intracellular Ca\(^{2+}\) stores are activated when the plasma membrane is depolarised (Figure 3-9A&B). We found that in the presence of Ca\(^{2+}\) during the preincubation and stimulation stages, 4-AP mediated stimulation resulted in significant increase in ERK1 (163.8 \(\pm\) 11.1\%) and ERK2 (149.7 \(\pm\) 5.9\%) phosphorylation/activation compared to the 37\(^\circ\)C control (Figure 3-9C). The absence of Ca\(^{2+}\) during the preincubation stage still resulted in a significant increase of ERK1 (133.0 \(\pm\) 4.9\%) and ERK2 (150.6 \(\pm\) 9.6\%) phosphorylation/activation compared to the 37\(^\circ\)C control when stimulated by 4-AP. The basal activity of ERK1 (99.9 \(\pm\) 4.7\%) and ERK2 (98.8 \(\pm\) 4.0\%) was largely unaffected by the incubation of EGTA during the preincubation stage compared to the 37\(^\circ\)C control. However, the net change shows 4-AP mediated stimulation of ERK1 is significantly reduced but ERK2 phosphorylation/activation was unaffected by the depletion of Ca\(^{2+}\) of intracellular Ca\(^{2+}\) stores (Figure 3-9D).

We next assessed the consequence of direct Ca\(^{2+}\) influx stimulation with ionomycin using this protocol in which the intracellular Ca\(^{2+}\) stores remain depleted (Figure 3-10A&B). We found that incubation of Ca\(^{2+}\) during the preincubation and stimulation stages followed by ionomycin stimulation resulted in significant increase in ERK1 (122.3 \(\pm\) 5.3\%) and ERK2 (133.4 \(\pm\) 3.3\%) phosphorylation/activation compared to the 37\(^\circ\)C control (Figure 3-10C). These responses with ionomycin failed in incubations in which the intracellular Ca\(^{2+}\) stores remain depleted. ERK1 (80.5 \(\pm\) 9.4\%) was unaffected but ERK2 (75.5 \(\pm\) 11.5\%) phosphorylation/activation compared to the 37\(^\circ\)C control was significantly reduced but this could again be attributed to a basal effect. Given that the basal activity of ERK2 (73.0 \(\pm\) 7.3\%) was significantly reduced but ERK1 (85.5 \(\pm\) 9.5\%) which was unaffected compared to the 37\(^\circ\)C control. The net change therefore
shows that depletion of intracellular Ca\textsuperscript{2+} stores significantly inhibits ERK1 and ERK2 phosphorylation/activation mediated by ionomycin stimulation (Figure 3-10D).
Figure 3-9: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ca\(^{2+}\) during the preincubation stage

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control and E = EGTA shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension in the presence of Ca\(^{2+}\) (1mM) or EGTA (100 µM) was used to resuspended synaptosomes and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca\(^{2+}\) (1mM) and stimulated with 4-AP (1mM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of the absence of Ca\(^{2+}\) during the preincubation stage and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of Ca\(^{2+}\) during the preincubation stage. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
Figure 3-10: Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ca\(^{2+}\) during the preincubation stage

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control, Iono = ionomycin and E = EGTA shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension in the presence of Ca\(^{2+}\) (1mM) or EGTA (100µM) was used to resuspended synaptosomes and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca\(^{2+}\) (1mM) and stimulated with ionomycin (5 µM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of the absence of Ca\(^{2+}\) during the preincubation stage and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37 ºC control in the presence and absence of Ca\(^{2+}\) during the preincubation stage. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
3.4. Discussion

The first objective of this chapter was to establish that ERK1 and ERK2 phosphorylation/activation can be mediated by depolarisation of the membrane or Ca\(^{2+}\) ionophore stimulation. Both of these methods of stimulation result in the influx of Ca\(^{2+}\) but though through different pathways. Depolarisation of the membrane results in the activation of VDCCs such as Ca\(_{v2.1}\) and Ca\(_{v2.2}\) channels that leads to the increase in intracellular Ca\(^{2+}\) concentrations. Ca\(^{2+}\) ionophore, ionomycin allows Ca\(^{2+}\) influx without the activation of Ca\(_{v2.1}\) and Ca\(_{v2.2}\) channels thus representing a good way to delineate effects downstream of Ca\(_{v2.1}\) and Ca\(_{v2.2}\) channels. We found that Ca\(^{2+}\) influx either through Ca\(_{v2.1}\) and Ca\(_{v2.2}\) channels mediated by 4-AP stimulation or direct Ca\(^{2+}\) influx mediated by ionomycin stimulation resulted in a significant and reproducible increase in ERK1 and ERK2 phosphorylation/activation.

The second objective of the chapter was to provide supporting evidence that 4-AP and ionomycin mediated stimulation is Ca\(^{2+}\)-dependent and that the underlying mechanisms that could be activated by the influx of Ca\(^{2+}\). We hypothesised that if 4-AP and ionomycin mediated stimulation is Ca\(^{2+}\)-dependent then the absence of external Ca\(^{2+}\) or inhibition of its downstream target(s) should significantly inhibit ERK1 and ERK2 phosphorylation/activation. Indeed, in the absence of external Ca\(^{2+}\) both 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation were significantly inhibited. Given that this resulted in a complete inhibition, we can conclude that stimulation of ERK1 and ERK2 by 4-AP and ionomycin is completely Ca\(^{2+}\)-dependent and no other Ca\(^{2+}\)-independent mechanisms are activated. Thus, we need to consider the Ca\(^{2+}\)-dependent mechanisms potentially involved in ERK1 and ERK2 phosphorylation/activation.
There have been studies conducted that could explain the underlying Ca\textsuperscript{2+}-dependent mechanisms that translate Ca\textsuperscript{2+} influx into ERK1 and ERK2 phosphorylation/activation. Largely, the mechanisms revolve around the hypothesis that promotion of GEFs and inhibition of GAPs stimulate Ras-GTP formation and the subsequent activation of the downstream targets of the ERK activation cascade. One of the underlying mechanisms that we investigated was ERK1 and ERK2 phosphorylation/activation sensitivity to Src inhibition during 4-AP and ionomycin mediated stimulation. Src is a good candidate for mediating the signalling because it has been shown to phosphorylate c-Raf-1 independently of Ras activation, which could thereby activate the ERK pathway (Marais\textit{ et al.}, 1995; Stokoe & McCormick, 1997). Furthermore, Src can be activated by Pyk2 to phosphorylate Shc and Pyk2, both of which subsequently recruit the Grb2/Sos complex again to promote the activation of the ERK pathway (Lev\textit{ et al.}, 1995).

We found that Src inhibition resulted in a complete reduction of ERK1 and ERK2 phosphorylation/activation mediated by ionomycin stimulation. Since this inhibition is absolute, we consider that Ca\textsuperscript{2+} influx through the Ca\textsuperscript{2+} ionophore only converges on the activation of Src. 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation was significantly reduced by Src inhibition but not completely abolished as with ionomycin. This suggests that as well as a Src-dependent mechanism, other Ca\textsuperscript{2+}-dependent mechanisms may be activated by Ca\textsuperscript{2+} influx through Ca\textsubscript{2.1} and Ca\textsubscript{2.2} channels. This could also be a possible explanation for the difference in ERK1 and ERK2 phosphorylation/activation between 4-AP and ionomycin mediated stimulations. The explanation as to why ionomycin mediated stimulation is unable to activate these additional Ca\textsuperscript{2+}-dependent mechanisms require further investigation. These other Ca\textsuperscript{2+}-dependent mechanisms that could be considered might include those involving RasGEFs such as RasGRF that are activated by calmodulin (Farnsworth\textit{ et
In addition, CalDAG-GEFI and CalDAG-GEFII that are activated by Ca\(^{2+}\) could phosphorylate/activate ERK1 and ERK2 through Rap1/B-Raf mechanism. However, Src has been found to tonically phosphorylate and enhance VDCCs thus the Src inhibitory effect could be due to the reduction of Ca\(^{2+}\) influx through Ca\(_{2.1}\) and Ca\(_{2.2}\) channels when stimulated by 4-AP (Wang, 2003). Thus, it could be that 4-AP mediated stimulation activates other Ca\(^{2+}\)-dependent mechanisms but unlike ionomycin stimulation is Src-independent. We could corroborate if 4-AP mediated stimulation is indeed Src-independent by possibly inhibiting the Pyk2 kinase. Under these conditions 4-AP mediated stimulation is Src-independent then Src should still phosphorylate and enhance VDCC, a process not dependent on Pyk2 activation, and the Src inhibitory effect observed here should be occluded. On the other hand if a Pyk2/Src dependent mechanism is activated, then there should be an inhibition of ERK1 and ERK2 phosphorylation/activation by 4-AP mediated stimulation. It is important to clarify the effects of Src as we found that Src inhibition resulted in an inhibition of ERK1 and ERK2 basal activity. This could be due to reduced activity of VDCCs but since these are not activated under basal conditions or it likely shows that basal ERK1 and ERK2 activity is sustained through a Ca\(^{2+}\)-independent Src-dependent mechanism.

The final objective that we wanted to address in this chapter was the source of Ca\(^{2+}\) that contributes to the Ca\(^{2+}\)-dependent mechanisms that phosphorylate/activate ERK1 and ERK2. We considered three possible sources with respect to Ca\(^{2+}\) including the influx of extracellular Ca\(^{2+}\), release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores or possibly a combination extracellular and intracellular Ca\(^{2+}\) sources. We designed an intracellular Ca\(^{2+}\) store repletion protocol in order to delineate the influences of extracellular and intracellular Ca\(^{2+}\) sources which involved preincubation and stimulation stages. The intracellular Ca\(^{2+}\) store repletion protocol does not affect the viability or responsiveness
of the synaptosomes. This is based on the comparison of ERK1 and ERK2 phosphorylation/activation responses with the standard protocol for both 4-AP and ionomycin mediated stimulation. Interestingly, the absence of Ca^{2+} over longer incubation periods during the intracellular Ca^{2+} store repletion protocol significantly reduced ERK1 and ERK2 basal activity. This was not observed using the standard protocol suggesting that the preincubation stage of the intracellular Ca^{2+} store repletion protocol plays a significant role. In addition, this suggests that Ca^{2+}-dependent mechanisms tonically maintain the basal activity of ERK1 and ERK2 in nerve terminals.

The intracellular Ca^{2+} store repletion protocol is based on the hypothesis that after the synaptosomal preparation the intracellular Ca^{2+} stores are somewhat depleted and require incubation Ca^{2+} for repletion. Thus by incubating synaptosomes in the presence (repletion) and absence (depletion) of Ca^{2+} during preincubation stage we could test the effect that depletion of stores would have on Ca^{2+}-dependent stimulation of ERK1 and ERK2 phosphorylation/activation (Schematic 3-1). We found that there was a difference between 4-AP and ionomycin mediated stimulation using this protocol. Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation was completely inhibited by continued depletion of intracellular Ca^{2+} stores. In contrast, 4-AP mediated stimulation of ERK1 phosphorylation/activation was significantly inhibited but ERK2 phosphorylation/activation was unaffected by continued depletion of intracellular Ca^{2+} stores. This suggests that direct Ca^{2+} influx is more sensitive to intracellular Ca^{2+} store depletion than Ca^{2+} influx through Ca_{v}2.1 and Ca_{v}2.2 channels. The reasoning behind this difference could be that the two paradigms activate different mechanisms. Ionomycin mediated stimulation may affect Ca^{2+} release from intracellular which then activates a Ca^{2+}-dependent process perhaps through a Pyk2/Src dependent mechanism.
On the other hand 4-AP mediated stimulation may again instigate Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) store but unlike ionomycin could also cause ERK2 phosphorylation/activation by just the influx of extracellular Ca\(^{2+}\) through the Ca\(_{\text{v}2.1}\) and Ca\(_{\text{v}2.2}\) channels. This might indicate a differential localisation and/or sensitivity of ERK1 and ERK2 to the upstream Ca\(^{2+}\)-dependent mechanisms.

In conclusion, the ERK pathway can be activated in synaptosomes by Ca\(^{2+}\) influx mediated either by membrane depolarisation or by direct Ca\(^{2+}\) entry through the Ca\(^{2+}\) ionophore. In addition, the underlying mechanism may involve the promotion of GEFs through Src-dependent activation that results in Grb2 and Sos recruitment. Finally intracellular Ca\(^{2+}\) stores have been implicated in the phosphorylation/activation of ERK1 and ERK2 by the experiments in this chapter. We therefore proceeded to examine the nature and properties of those Ca\(^{2+}\) stores.
Schematic 3-1: Repletion of possible intracellular Ca\(^{2+}\) stores is a requirement for ERK pathway activation

Influx of extracellular Ca\(^{2+}\) through VDCCs or ionomycin can stimulate the ERK pathway through Src activation which could be stimulated by Pyk2. Increases in [Ca\(^{2+}\)] could also stimulate further release from possible intracellular Ca\(^{2+}\) stores. Incubation with EGTA prior to stimulation could cause the continued depletion of possible intracellular Ca\(^{2+}\) stores which could result in a reduction of intracellular Ca\(^{2+}\) release.
Chapter 4
4. Smooth endoplasmic reticulum

Summary: One of the intrasynaptosomal Ca\(^{2+}\) stores that could potentially contribute Ca\(^{2+}\) to the Ca\(^{2+}\)-dependent process of ERK1 and ERK2 phosphorylation/activation is the smooth endoplasmic reticulum. We targeted three key features of the smooth endoplasmic reticulum including the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump, ryanodine receptors (RyRs) and IP\(_3\) receptors (IP\(_3\)Rs). By inhibiting the SERCA pump we depleted the Ca\(^{2+}\) from the store thus reducing the stimulus capability for the smooth endoplasmic reticulum. We found that this resulted in significant reduction of ERK1 and ERK2 phosphorylation/activation mediated by 4-AP and ionomycin stimulation. Inhibition of RyR using high concentrations of ryanodine showed the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mechanism could be triggered by Ca\(^{2+}\) influx mediated by 4-AP and ionomycin stimulation. Lastly, by inhibiting IP\(_3\)Rs using 2-APB and inhibiting PLC activity using U-73122 we established the presence of an IP\(_3\)-induced Ca\(^{2+}\) release (IPCR) mechanism that can be triggered through Ca\(^{2+}\) influx mediated by 4-AP and ionomycin stimulation. Overall we conclude that smooth endoplasmic reticulum like Ca\(^{2+}\) stores are present in presynaptic nerve terminals and can be mobilised by CICR and IPCR mechanisms which results in ERK1 and ERK2 phosphorylation/activation.

4.1. Introduction

In the previous chapter we produced some evidence indicating that intracellular Ca\(^{2+}\) stores may contribute to ERK1 and ERK2 phosphorylation/activation. The increase in ERK1 and ERK2 phosphorylation/activation is evidently a sensitive assay to indicate intracellular Ca\(^{2+}\) concentrations. According to the literature there are numerous potential compartments that can store Ca\(^{2+}\) which could contribute to cellular processes. One such potential store for intracellular Ca\(^{2+}\) is the smooth endoplasmic reticulum.
which in cells such as the smooth muscle forms the sarcoplasmic reticulum and plays a prominent role in excitation-contraction coupling.

The role that the smooth endoplasmic reticulum plays in presynaptic nerve terminals is less well defined though there have been morphological and functional studies that have identified elements of the smooth endoplasmic reticulum in presynaptic nerve terminals. These morphological studies of synaptosomes have identified membrane-bound tubular or isolated vesicles structures resembling smooth endoplasmic reticulum (McGraw et al., 1980a). Functional studies have identified a facilitatory role of smooth endoplasmic reticulum in neurotransmitter release. One such study has shown that α-latrotoxin secretion of norepinephrine from nerve terminals is enhanced by Ca\(^{2+}\) released from intracellular Ca\(^{2+}\) stores which were proposed to resemble smooth endoplasmic reticulum (Davletov et al., 1998). Furthermore, α-latrotoxin mutant is able to enhance spontaneous and evoked release from CA3 pyramidal neurons by increasing cytosolic Ca\(^{2+}\) concentrations from intracellular Ca\(^{2+}\) stores (Capogna et al., 2003). To identify if a similar facilitatory role of smooth endoplasmic reticulum can be observed on ERK1 and ERK2 phosphorylation/activation using 4-AP and ionomycin mediated stimulation. We targeted three features of smooth endoplasmic reticulum, the SERCA pump, RyR and IP\(_3\)R.

The SERCA pumps establish and maintain the Ca\(^{2+}\) concentration in the lumen of the smooth endoplasmic reticulum. They do this via an ATP driven mechanism known as the SERCA catalytic cycle which pumps Ca\(^{2+}\) from the cytosol into the lumen of the smooth endoplasmic reticulum (Brini & Carafoli, 2009). The functional role of smooth endoplasmic reticulum has been investigated by inhibiting the SERCA pump using the drug thapsigargin. This is based on the hypothesis that the Ca\(^{2+}\) accumulated in the
lumen of the store continuously ‘leaks’ out into the cytosol. This ‘leaked’ Ca$^{2+}$ is recovered by the SERCA pump thus there is no net change in the concentration of Ca$^{2+}$ in the lumen of smooth endoplasmic reticulum under ‘resting’ conditions. Inhibiting the SERCA pump depletes Ca$^{2+}$ from the smooth endoplasmic reticulum as Ca$^{2+}$ still continuously ‘leaks’ out and is now not recoverable by the SERCA pump. This results in a net loss of Ca$^{2+}$ in the lumen of the smooth endoplasmic reticulum. If Ca$^{2+}$ released by the smooth endoplasmic reticulum contributes to a Ca$^{2+}$-dependent process, then the reduction of the store Ca$^{2+}$ concentration should significantly reduce the Ca$^{2+}$-dependent responses.

There are two mechanisms that can be activated that stimulate Ca$^{2+}$ release from smooth endoplasmic reticulum. The first mechanism is known as CICR in which Ca$^{2+}$ is able to stimulate RyR to release store Ca$^{2+}$ and thus further increase the Ca$^{2+}$ concentration in the cytosol (Hamilton & Serysheva, 2009). The trigger for RyR is thought to be Ca$^{2+}$ itself that can be generated by the influx of Ca$^{2+}$ through VDCCs or Ca$^{2+}$ released by other intracellular stores in the proximity of RyR containing compartments. Ca$^{2+}$ stimulation of the RyR has a bimodal nature in that low concentrations (<1µM) have a stimulatory effect while high concentrations (>10 µM) result in the inhibition of the receptors (Nagasaki & Fleischer, 1988).

The second mechanism that can be activated by the release Ca$^{2+}$ from smooth endoplasmic reticulum is IPCR. Here IP$_3$ generation through PIP$_2$ metabolism by phospholipase C (PLC) activation results in the stimulation of IP$_3$Rs found on the smooth endoplasmic reticulum (Mikoshiba, 2007a). But PIP$_2$ metabolism also produces DAG which may activate protein kinase C (PKC) and mediate some independent effects but notably PKC can also phosphorylate IP$_3$Rs to make them more responsive to IP$_3$.
stimulation (Ferris et al., 1991). The IP$_3$Rs also display a bimodal response to concentrations of Ca$^{2+}$ with low concentrations being facilitatory and higher Ca$^{2+}$ concentrations have an inhibitory effect (Bezprozvanny et al., 1991; Boehning et al., 2001).

The objective of this chapter is to investigate the functional role of smooth endoplasmic reticulum during 4-AP and ionomycin mediated stimulation using ERK1 and ERK2 phosphorylation/activation as an indicator for increases in intrasynaptosomal Ca$^{2+}$ concentration. We investigated if 4-AP and ionomycin mediated stimulation results in the activation of a CICR mechanism. Furthermore, we determined whether the IPCR mechanism contributes to Ca$^{2+}$ signalling during 4-AP and ionomycin mediated stimulation of nerve terminals.
4.2. Method

4.2.1. Synaptosomal Preparation

Preparation of synaptosomes has been described in section 2.1.

4.2.2. SDS-PAGE and Immunoblotting

All samples obtained through Immunoblotting experiments underwent the procedure described in section 2.2.

4.2.3. Intracellular Ca\(^{2+}\) store repletion protocol

Experiments that followed the intracellular Ca\(^{2+}\) store protocol are described in section 2.3.2. Synaptosomes were resuspended using the HBM buffer containing BSA (1mg/ml) and incubated at 37\(^{\circ}\)C with any drug of interest such as 2-APB (50\(\mu\)M) or U-73122 (10\(\mu\)M) or thapsigargin (10\(\mu\)M), added to the incubation. After 3 minutes Ca\(^{2+}\) (1mM) or EGTA (100\(\mu\)M) was added to the incubation. Synaptosomes were then spun down after 50 minutes of incubation with the supernatant removed and the pellets put on ice. At the 67 minute time point from the start of the incubation synaptosomes are resuspended a second time with HBM containing BSA (1mg/ml) and Ca\(^{2+}\) (1mM) or HBM containing BSA (1mg/ml) and EGTA (100\(\mu\)M) and incubated at 37\(^{\circ}\)C with the addition of the drug of interest ryanodine (30\(\mu\)M), 2-APB (50\(\mu\)M), U-73122 (10\(\mu\)M) or thapsigargin (10\(\mu\)M) in the resuspension HBM buffer. 4-AP (1mM), ionomycin (5\(\mu\)M) or control (HBM buffer) was then added to the incubation to stimulate synaptosomes for 5 minutes. The experiment was then terminated using the STOP solution.

4.2.4. Statistical analysis

The methods of statistical analysis have been described in section 2.6.1. For data with more than two sets analysis of variance (ANOVA) was used to assess the statistical
significance, followed by Dunnett's post hoc test. The net change in ERK1 and ERK2 phosphorylation/activation was calculated by removing the basal percentage from the stimulated groups for each n. We used Student’s unpaired t-test to assess the statistical significance between two sets of data.

4.2.5. Reagents

A stock solution of 4-AP (1mM) was made in water and working solution was further diluted using HBM.

A stock solution of ionomycin (5µM) was made in DMSO and working solution was further diluted using HBM.

A stock solution of EGTA (100µM) was obtained with water and working solution was further diluted using HBM.

A stock solution of thapsigargin (1µM) was made in DMSO and working solution was further diluted using HBM.

A stock solution of ryanodine (30µM) was obtained with DMSO and working solution was further diluted using HBM.

A stock solution of 2-APB (50µM) was obtained with DMSO and working solution was further diluted using HBM.

A stock solution of U-73122 (10µM) was obtained with DMSO and working solution was further diluted using HBM.
4.3. Results

Smooth endoplasmic reticulum is a potential intracellular Ca\(^{2+}\) store that may be able to release the intracellular Ca\(^{2+}\) to activate Ca\(^{2+}\)-dependent processes such as the ERK pathway. If the Ca\(^{2+}\) stored in smooth endoplasmic reticulum were to be depleted, the hypothesis would be that there should be a significant decrease in ERK1 and ERK2 phosphorylation/activation under 4-AP and ionomycin mediated stimulation.

In order to examine this hypothesis we used thapsigargin which is a well characterised inhibitor of the SERCA pumps found in the smooth endoplasmic reticulum. We conducted the experiment by incubating the synaptosomes with thapsigargin (1µM) in the presence of external Ca\(^{2+}\) (1mM) during the preincubation stage (Figure 4-1A&B). This ensures that any effect observed is due to thapsigargin inhibition of the SERCA pump and not simply a reflection of external Ca\(^{2+}\) absence as demonstrated in chapter 3 (Figure 3-7). Absence of thapsigargin incubation resulted in a significant increase in ERK1 (165.3 ± 1.8%) and ERK2 (159.9 ± 3.2%) phosphorylation/activation compared to the 37ºC control when the synaptosomes were stimulated with 4-AP (Figure 4-1C). This 4-AP mediated stimulation also resulted in significant increase of ERK1 (150.2 ± 2.2%) and ERK2 (133.4 ± 4.5%) phosphorylation/activation compared to the 37ºC control when thapsigargin was included. The basal activity of ERK1 (102.7 ± 5.2%) and ERK2 (91.8 ± 3.4%) compared to the 37ºC control was unaffected in the presence of thapsigargin during preincubation stage. Despite this, the net change of ERK1 and ERK2 phosphorylation/activation showed that thapsigargin incubation significantly reduced 4-AP mediated stimulation (Figure 4-1D).

We next tested this hypothesis on ionomycin mediated stimulation using the same conditions. Thus, we incubated thapsigargin (1µM) in the presence of Ca\(^{2+}\) (1mM)
Figure 4-1: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of thapsigargin during the preincubation stage

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and T = thapsigargin shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without thapsigargin (1µM) and in the presence of Ca²⁺ (1mM) was used to resuspended synaptosomes and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca²⁺ (1mM) and stimulated with 4-AP (1mM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of the presence of thapsigargin during the preincubation stage and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of thapsigargin during the preincubation stage. Thapsi = thapsigargin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=6).
during the preincubation stage (Figure 4-2A&B). Ionomycin mediated stimulation in the absence of thapsigargin during the preincubation stage caused a significant increase of ERK1 (127.6 ± 3.1%) and ERK2 (125.8 ± 1.6%) phosphorylation/activation compared to the 37°C control (Figure 4-2C). Unlike 4-AP mediated stimulation, the incubation of thapsigargin did not significantly stimulate ERK1 (89.6 ± 1.3%) and ERK2 (95.0 ± 3.7%) phosphorylation/activation compared to the 37°C control when stimulated with ionomycin. Interestingly, the ERK1 (87.7 ± 4.4%) basal activity was significantly reduced but ERK2 (99.4 ± 5.1%) basal activity compared to the 37°C control was unaffected by thapsigargin incubation. The net change analysis confirmed that ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation is significantly decreased by thapsigargin incubation (Figure 4-2D). Overall, the results support the hypothesis that both 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation both invoke mobilisation of Ca²⁺ from the smooth endoplasmic reticulum type Ca²⁺ store in nerve terminals.

However, the mechanism for the mobilisation of the aforementioned Ca²⁺ store requires further investigation. It is possible that depletion of smooth endoplasmic reticulum during the preincubation stage subsequently diminishes the ability of 4-AP and ionomycin to stimulate Ca²⁺ release. If this is true then continued depletion of intracellular Ca²⁺ stores during the preincubation and stimulation stages should maintain the inhibitory effect. Therefore, we incubated synaptosomes with Ca²⁺ (1mM) in the presence and absence of thapsigargin (1µM) during the preincubation and stimulation stages (Figure 4-3A&B). We found that the absence of thapsigargin permitted significant increases ERK1 (146.0 ± 2.0%) and ERK2 (160.2 ± 5.0%) phosphorylation/activation compared to the 37°C control during stimulation by 4-AP (Figure 4-3C). 4-AP mediated stimulation of ERK1 (149.8 ± 2.0%) and ERK2 (171.4 ±
Figure 4-2: Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of thapsigargin during the preincubation stage

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, Iono = ionomycin and T = thapsigargin shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without thapsigargin (1µM) and in the presence of Ca²⁺ (1mM) was used to resuspended synaptosomes and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca²⁺ (1mM) and stimulated with ionomycin (5µM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of the presence of thapsigargin during the preincubation stage and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37 ºC control in the presence and absence of thapsigargin during the preincubation stage. Thapsi = thapsigargin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
6.7%) phosphorylation/activation compared to the 37°C control was also significant increase in the presence of thapsigargin. Like previous results the basal activity of ERK1 (91.5 ± 2.3%) and ERK2 (93.4 ± 4.9%) compared to the 37°C control was however unaffected by thapsigargin incubation. Surprisingly, the net change analysis showed that incubation with thapsigargin during the preincubation and stimulation stages actually significantly increased the 4-AP mediated stimulation rather than inhibiting the response (Figure 4-3D). This result may appear to contradict the previous one with 4-AP added following thapsigargin incubation during the preincubation. However, this could reflect the additional role of smooth endoplasmic reticulum in that during 4-AP mediated stimulation it could be sequestering Ca^{2+}.

We next investigated if a CICR mechanism is activated during 4-AP and ionomycin stimulation paradigms. We first wanted examined the CICR pathway by observing the direct consequences of Ca^{2+} influx on ERK1 and ERK2 phosphorylation/activation. Synaptosomes were incubated with Ca^{2+} (1mM) during the preincubation stage to ensure the repletion of intracellular Ca^{2+} stores. This was followed by the stimulation stage in which the synaptosomes were incubated in the presence of EGTA (100µM) and with the absence of Ca^{2+} (Figure 4-4A&B). 4-AP mediated stimulation in the presence of Ca^{2+} caused a significant increase in ERK1 (151.4 ± 9.7%) and ERK2 (164.9 ± 11.3%) phosphorylation/activation compared to the 37°C control (Figure 4-4C). There was a no significant stimulation on ERK1 (125.4 ± 9.7%) and ERK2 (124.2 ± 13.3%) phosphorylation/activation compared to the 37°C control by 4-AP mediated stimulation in the absence of Ca^{2+}. The basal activity of (99.1 ± 2.2%) and ERK2 (94.6 ± 0.9%) compared to the 37°C control was unaffected by the absence of Ca^{2+}. The net change showed that the absence of Ca^{2+} during the stimulation stage significantly reduced 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation (Figure 4-4D).
Figure 4-3: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of thapsigargin during the preincubation and stimulation stages

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and T = thapsigargin shows the condition of each lane. (B) Timeline of the experiment showing that HBM with or without thapsigargin (1µM) and in the presence of Ca²⁺ (1mM) were incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in with or without thapsigargin (1µM) and Ca²⁺ (1mM) and stimulated with 4-AP (1mM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of thapsigargin incubation during the preincubation and stimulation stages and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of thapsigargin. Thapsi = thapsigargin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=7).
Figure 4-4: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ca²⁺ during the stimulation stage

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and E = EGTA shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of Ca²⁺ (1mM) were incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in the presence of EGTA (100µM) or Ca²⁺ (1mM) and stimulated with 4-AP (1mM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of EGTA incubation during the stimulation stages and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of Ca²⁺. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
The consequence of Ca\(^{2+}\) influx of ionomycin was also investigated using the same protocol in which repletion of intracellular Ca\(^{2+}\) stores was followed by stimulation in the absence of Ca\(^{2+}\) during the stimulation stage (Figure 4-5A&B). We found that there was a significant increase in ERK1 (122.3 ± 5.3%) and ERK2 (133.3 ± 2.6%) phosphorylation/activation compared to the 37°C control by ionomycin mediated stimulation in the presence of Ca\(^{2+}\) (Figure 4-5C). As expected the absence of Ca\(^{2+}\) caused ionomycin mediated stimulation to have no significant effect on ERK1 (91.1 ± 7.5%) and ERK2 (87.9 ± 2.9%) phosphorylation/activation compared to the 37°C control. There was also no significant effect on the basal activity of either ERK1 (98.4 ± 4.6%) or ERK2 (92.7 ± 7.1%) compared to the 37°C control by the absence of Ca\(^{2+}\) during the stimulation stage. Overall, the absence of Ca\(^{2+}\) during the stimulation stage significantly reduced ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation as seen from the net change comparison (Figure 4-5D).

From these results, we can conclude that 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation is significantly dependent on Ca\(^{2+}\) influx during stimulation.

We hypothesised that the influx of Ca\(^{2+}\) either through VDCC or direct influx results in the activation of the RyR to activate the CICR mechanism. To investigate the involvement of RyR, we considered inhibiting them with high concentrations of ryanodine. If RyR are involved then 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation should be significantly inhibited. To test RyR involvement, synaptosomes were incubated with Ca\(^{2+}\) (1mM) during the preincubation stage in order to replete the intracellular Ca\(^{2+}\) stores. During the stimulation stage, incubation with ryanodine (30µM) was considered sufficient to inhibit the RyR (Figure 4-6A&B).
Figure 4-5: Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ca\(^{2+}\) during the stimulation stage

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control, Iono = ionomycin and E = EGTA shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of Ca\(^{2+}\) (1mM) were incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in the presence of EGTA (100µM) or Ca\(^{2+}\) (1mM) and stimulated with ionomycin (5µM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of EGTA incubation during the stimulation stages and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of Ca\(^{2+}\). (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
4-AP mediated stimulation resulted in a significant increase in ERK1 (151.6 ± 6.3%) and ERK2 (159.3 ± 6.1%) phosphorylation/activation compared to the 37°C control in the absence of ryanodine incubation (Figure 4-6C). Interestingly, ryanodine incubation reduced the stimulation of ERK1 (106.4 ± 8.7%) and ERK2 (137.8 ± 8.0%) phosphorylation/activation but ERK2 phosphorylation/activation was significantly increased compared to the 37°C control. In addition there was no basal effect of ryanodine incubation during the stimulation stage on ERK1 (95.5 ± 4.8%) or ERK2 (100.0 ± 4.1%) compared to the 37°C control. The net change in ERK1 and ERK2 phosphorylation/activation shows that ryanodine incubation during the stimulation stage significantly reduced 4-AP mediated stimulation (Figure 4-6D).

Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation sensitivity to ryanodine was also examined using the same protocol. After the repletion of intracellular Ca²⁺ stores, ryanodine (30µM) was used to inhibit RyR during the stimulation stage (Figure 4-7A&B). We found that ionomycin mediated stimulation in the absence of ryanodine during the stimulation stage resulted in significant increase in ERK1 (126.7 ± 2.4%) and ERK2 (123.1 ± 2.3%) phosphorylation/activation compared to the 37°C control (Figure 4-7C). There was no significant stimulatory increase of ERK1 (100.7 ± 7.3%) and ERK2 (93.8 ± 8.7%) phosphorylation/activation compared to the 37°C control when stimulated with ionomycin in the presence of ryanodine. Furthermore, ryanodine incubation during the stimulation stage had no significant effect on ERK1 (111.2 ± 1.8%) but the ERK2 (100.8 ± 3.8%) basal activity compared to the 37°C control. From this it clearly evident that ryanodine incubation during the stimulation stage significantly inhibits ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation as shown by the net change (Figure 4-7D). These results indicate that both 4-AP and ionomycin mediated stimulation appear to
Figure 4-6: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of ryanodine during the stimulation stage

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control and R = ryanodine shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of Ca²⁺ (1mM) were incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in the presence of ryanodine (30µM) and Ca²⁺ (1mM) and stimulated with 4-AP (1mM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of ryanodine incubation during the stimulation stages and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of ryanodine. Rya = ryanodine. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
Figure 4-7: Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of ryanodine during the stimulation stage

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and R = ryanodine shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of Ca²⁺ (1mM) were incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in the presence of ryanodine (30 µM) and Ca²⁺ (1mM) and stimulated with ionomycin (5µM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of ryanodine incubation during the stimulation stages and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of ryanodine. Rya = ryanodine. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
activate RyR found on putative smooth endoplasmic reticulum in presynaptic nerve terminals.

To confirm this point we considered the possibility that the inhibitory effects of ryanodine effect on ERK1 and ERK2 phosphorylation/activation could be occluded if the intracellular Ca\(^{2+}\) stores are depleted prior to stimulation. Thus, synaptosomes were incubated in the absence of Ca\(^{2+}\) with EGTA (100µM) during the preincubation stage and then incubated with ryanodine (30µM) during the stimulation stage (Figure 4-8A&B). 4-AP mediated stimulation significantly increased ERK1 (135.8 ± 6.4%) and ERK2 (142.7 ± 8.7%) phosphorylation/activation compared to the 37ºC control with depleted intracellular Ca\(^{2+}\) stores (Figure 4-8C). In the presence of ryanodine 4-AP mediated stimulation still caused a significant increase in ERK1 (140.6 ± 8.5%) and ERK2 (157.0 ± 14.2%) phosphorylation/activation compared to the 37ºC control with depleted of intracellular Ca\(^{2+}\) stores. Incubation with ryanodine and with depleted intracellular Ca\(^{2+}\) stores had no significant effect on the basal activity of ERK1 (95.5 ± 8.7%) and ERK2 (94.3 ± 12.0%) compared to the 37ºC control. The net change analysis shows that 4-AP mediated stimulation in ERK1 and ERK2 phosphorylation/activation was not significantly different (Figure 4-8D). Therefore this indicates that the inhibitory effect of ryanodine that was previously observed in which the intracellular Ca\(^{2+}\) stores were replete is occluded if the stores are prevented from repletion by the absence of Ca\(^{2+}\) during the preincubation stage. We could not apply this protocol to ionomycin mediated stimulation. Previously we had shown that the continued depletion of intracellular Ca\(^{3+}\) stores results in the failure of ionomycin to significantly increase ERK1 and ERK2 phosphorylation/activation (Figure 3-10).
**Figure 4-8: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation with prior absence of Ca²⁺ during the stimulation stage in the presence and absence of ryanodine**

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, ER = EGTA + ryanodine shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of Ca²⁺ (1mM) or EGTA (100 µM) was incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in the presence and absence of ryanodine (30 µM) with Ca²⁺ (1mM) and stimulated with 4-AP (1mM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of absence of Ca²⁺ during preincubation and ryanodine incubation during the stimulation stages and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of ryanodine. Rya = ryanodine. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01 (**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
From the foregoing data, it is evident that there still remains a component of ERK1 and ERK2 phosphorylation/activation that is activated during Ca\(^{2+}\) influx that is ryanodine insensitive. We therefore considered the possibility that IP\(_3\)Rs also being activated by external Ca\(^{2+}\) influx which could act as the trigger for an IPCR mechanism. To target the IP\(_3\)Rs we used the IP\(_3\)R inhibitor 2-APB to elucidate the actions of IP\(_3\)Rs in ERK1 and ERK2 phosphorylation/activation mediated by 4-AP and ionomycin stimulation. Thus, 2-APB (50µM) was included in the presence of Ca\(^{2+}\) (1mM) during the preincubation and stimulation stages (Figure 4-9A&B). There was a significant increase in ERK1 (148.8 ± 8.1%) and ERK2 (133.4 ± 4.1%) phosphorylation/activation compared to the 37°C control mediated by 4-AP stimulation in the absence of 2-APB incubation (Figure 4-9C). Interestingly, 2-APB reduced the 4-AP mediated stimulation of ERK1 (115.7 ± 2.8%) and ERK2 (117.8 ± 3.3%) phosphorylation/activation compared to the 37°C control but ERK2 was still significantly increased. But there is a significant increase of ERK2 and not of ERK1 phosphorylation/. Incubation with 2-APB during the preincubation and stimulation stages did not significantly affect the basal activity of ERK1 (104.7 ± 2.3%) and ERK2 (103.6 ± 4.3%) compared to the 37°C control. The net change in ERK1 and ERK2 phosphorylation/activation shows that there is a significant reduction in 4-AP mediated stimulation of ERK1 and ERK2 in synaptosomes incubated with 2-APB during the preincubation and stimulation stage (Figure 4-9D).

We now investigated the inhibitory effect of 2-APB on ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation. We used the same protocol in which 2-APB (50µM) was included during the preincubation and stimulation stages (Figure 4-10A&B). Ionomycin mediated stimulation of ERK1 (140.1 ± 3.0%) and ERK2 (142.4
Figure 4-9: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of 2-APB

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control, A = 2-APB shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of Ca²⁺ (1mM) with or without 2-APB (50µM) was incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in the presence and absence of 2-APB (50µM) with Ca²⁺ (1mM) and stimulated with 4-AP (1mM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of 2-APB during preincubation and stimulation stages and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of 2-APB. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01 (**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
phosphorylation/activation compared to the 37°C control was significantly increased in the absence of 2-APB (Figure 4-10C). The presence of 2-APB resulted in a decreased stimulation of ERK1 (126.8 ± 5.1%) and ERK2 (127.4 ± 2.7%) phosphorylation/activation compared to the 37°C control mediated by ionomycin. Similar to previous results the basal activity of ERK1 (104.7 ± 2.3%) and ERK2 (103.6 ± 4.3%) compared to the 37°C control were unaffected by the presence of 2-APB during preincubation and stimulation stages. The net change in ERK1 and ERK2 phosphorylation/activation analysis shows that ionomycin mediated stimulation is significantly inhibited by 2-APB incubation during the preincubation and stimulation stages (Figure 4-10D). These results therefore support the hypothesis that Ca²⁺ influx could also be the trigger of mechanisms that lead to the IP₃R activation to affect IPCR and contribute to ERK1 and ERK2 phosphorylation/activation.

Given the indicated involvement of IP₃R in both 4-AP and ionomycin mediated stimulation we sought to target signalling upstream of IP₃R in the generation of the IP₃ signal. The hypothesised IPCR mechanism would suggest IP₃ production is evoked by PLC metabolism of PIP₂, thus inhibition of PLC activity should inhibit IP₃ production and thereby prevent IP₃R mediated stimulation. To investigate if PLC is activated during 4-AP and ionomycin mediated stimulation we used the PLC inhibitor U-73122 and observed it effects on ERK1 and ERK2 phosphorylation/activation. U-73122 (10µM) was incubated in the presence of Ca²⁺ (1mM) during the preincubation and stimulation stages (Figure 4-11A&B). 4-AP mediated stimulation caused a significant increase in ERK1 (148.8 ± 8.1%) and ERK2 (133.4 ± 4.1%) phosphorylation/activation compared to the 37°C control during the absence of U-73122 (Figure 4-11C). Incubation of U-73122 during 4-AP mediated stimulation reduced the responses of ERK1 (117.7 ± 9.7%) and ERK2 (115.4 ± 2.4%) phosphorylation/activation, but ERK2
Figure 4-10: Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of 2-APB

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively. Labels Con = 37°C control, Iono = ionomycin and A = 2-APB shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of Ca²⁺ (1mM) with or without 2-APB (50 µM) was incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in the presence and absence of 2-APB (50 µM) with Ca²⁺ (1mM) and stimulated with ionomycin (5 µM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of 2-APB during preincubation and stimulation stages and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of 2-APB. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. *p<0.01, **p<0.05. (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
Figure 4-11: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of U-73122
(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, U = U-73122 shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of Ca²⁺ (1mM) with or without U-73122 (10µM) was incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in the presence and absence of U-73122 (10µM) with Ca²⁺ (1mM) and stimulated with 4-AP (1mM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of U-73122 during preincubation and stimulation stages and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of U-73122. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
compared to the 37°C control was significantly increased. Incubation of U-73122 during the preincubation and stimulation stage did not have any significant effect on the basal activity of ERK1 (104.5 ± 2.5%) and ERK2 (102.5 ± 4.2%) compared to the 37°C control. The net change therefore reveals that U-73122 incubation significantly reduced ERK1 and ERK2 phosphorylation/activation during 4-AP mediated stimulation (Figure 4-11D).

Since 2-APB incubation had a significant effect on ionomycin stimulation of ERK1 and ERK2 phosphorylation/activation it is reasonable to assume that it might also be sensitive to the PLC inhibition. We investigated the involvement of PLC with this stimulatory paradigm by incubating U-73122 (10µM) during the preincubation and stimulation stages as previously described (Figure 4-12A&B). In the absence of U-73122 incubation ionomycin mediated stimulation significantly increased ERK1 (140.1 ± 3.0%) and ERK2 (142.4 ± 2.3%) phosphorylation/activation compared to the 37°C control (Figure 4-12C). In the presence of U-73122 ionomycin mediated stimulation was able to significantly increase ERK1 (118.8 ± 2.4%) and ERK2 (127.4 ± 2.7%) phosphorylation/activation compared to the 37°C control. Like previous observations the basal effect of ERK1 (104.5 ± 2.5%) and ERK2 (103.6 ± 4.3%) compared to the 37°C control was not significantly affected by U-73122 incubation. Analysis of the net change in ERK1 and ERK2 phosphorylation/activation indicated that ionomycin stimulation is significantly reduced in synaptosome samples incubated with U-73122 during the preincubation and stimulation stages (Figure 4-12D). These results show that 4-AP and ionomycin mediated stimulation are indeed sensitive to PLC inhibition this further supporting the role of IPCR in ERK1 and ERK2 phosphorylation/activation.
Figure 4-12: Ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of U-73122

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control, Iono = ionomycin and U = U-73122 shows the condition of each lane. (B) Timeline of the experiment showing that HBM in the presence of Ca²⁺ (1mM) with or without U-73122 (10 µM) was incubated at the start of the experiment for 50 minutes which was spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in the presence and absence of U-73122 (10µM) with Ca²⁺ (1mM) and stimulated with ionomycin (5µM) and the experiment ended after 75 minutes of incubation. (C) Basal effect of U-73122 during preincubation and stimulation stages and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37º C control in the presence and absence of U-73122. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
4.4. Discussion

The first objective of this chapter was to address the functional role that smooth endoplasmic reticulum can play in nerve terminals during 4-AP and ionomycin mediated stimulation. We examined this by inhibiting the SERCA pumps found on smooth endoplasmic reticulum which should over time result in the depletion of Ca\(^{2+}\) from the store, as it ‘leaks’ out into the cytosol. Using thapsigargin as a store inhibitor, both 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation was significantly reduced. Interestingly, ionomycin mediated stimulation was shown to be more sensitive to Ca\(^{2+}\) depletion from smooth endoplasmic reticulum than 4-AP mediated stimulation.

There are two possible functional roles of smooth endoplasmic reticulum that could be used to explain these results. Firstly, it could be that Ca\(^{2+}\) depletion removes the ability of 4-AP and ionomycin mediated stimulation to stimulate the Ca\(^{2+}\) release from the smooth endoplasmic reticulum. Thus, there is an insufficient Ca\(^{2+}\) concentration to stimulate the Ca\(^{2+}\)-dependent mechanisms of ERK1 and ERK2 phosphorylation/activation. However, in this experiment thapsigargin was not present during the 4-AP and ionomycin mediated stimulation which might suggest that the SERCA pump are functional during the stimulation stage and are able to sequester the influx of Ca\(^{2+}\). Indeed, continued incubation of thapsigargin during the preincubation and stimulation stages indicates that Ca\(^{2+}\) is being sequestered at least for 4-AP mediated stimulation. This is based on the reasoning that as SERCA pumps are unable to sequester the cytosolic Ca\(^{2+}\) during stimulation stage the Ca\(^{2+}\)-dependent mechanisms that phosphorylate/activate ERK1 and ERK2 are further stimulated by the presence of excess Ca\(^{2+}\) in the cytosol. This explanation is further supported by previous studies that show smooth endoplasmic reticulum in synaptosomes is able to sequester Ca\(^{2+}\) from the
cytosol during stimulation (Blaustein et al., 1978; Blaustein et al., 1980; McGraw et al., 1980b; Rasgado-Flores & Blaustein, 1987).

We suspected that ionomycin mediated stimulation sensitivity to thapsigargin could also be due to sequestration, but we need to conduct an experiment in which thapsigargin is incubated continuously. If there is an enhancement of ERK1 and ERK2 phosphorylation/activation by ionomycin mediated stimulation then this points to sequestration by smooth endoplasmic reticulum. The sequestration ability of smooth endoplasmic reticulum could also be important in pathophysiological conditions as failure by smooth endoplasmic reticulum to sequester Ca\(^{2+}\) could result in its excess and cytosolic accumulation in the cytosol.

The second objective of this chapter was to investigate the involvement of CICR mechanism to stimulate Ca\(^{2+}\) release from the smooth endoplasmic reticulum. The CICR mechanism involves an increase in Ca\(^{2+}\) concentration which can be induced by the Ca\(^{2+}\) influx or local intracellular Ca\(^{2+}\) increases. This Ca\(^{2+}\) stimulates RyR which causes Ca\(^{2+}\) release from the smooth endoplasmic reticulum to further increase the Ca\(^{2+}\) concentration and thereby enhance Ca\(^{2+}\)-dependent mechanisms operating in nerve terminals.

By stimulating the synaptosomes with 4-AP and ionomycin in the absence of Ca\(^{2+}\) during stimulation we directly assessed the dependency in Ca\(^{2+}\) influx of ERK1 and ERK2 phosphorylation/activation. The intracellular Ca\(^{2+}\) stores underwent the repletion protocol thus the effects are unlikely to be due to and depletion of intracellular Ca\(^{2+}\) stores. Both 4-AP and ionomycin mediated stimulation had significantly reduced responses in the absence of Ca\(^{2+}\) during stimulation. This indicates that extracellular
Ca$_{\text{2+}}$ influx through VDCCs or Ca$_{\text{2+}}$ ionophore is an essential requirement for ERK1 and ERK2 phosphorylation/activation. In addition, given that the reduction for both 4-AP and ionomycin mediated stimulation was complete; it suggests that no Ca$_{\text{2+}}$-independent mechanisms are involved in ERK1 and ERK2 phosphorylation/activation.

By inhibiting RyR using high concentrations of ryanodine we were able to show that both 4-AP and ionomycin mediated stimulations lead to activation of RyR. RyR have been implicated in the CICR mechanism thus this indicates that Ca$_{\text{2+}}$ influx induced by 4-AP and ionomycin could be triggering Ca$_{\text{2+}}$ release through RyR contain stores (Bouchard et al., 2003). Confirming the inhibitory effect of ryanodine incubation could be occluded by prior depletion of intracellular Ca$_{\text{2+}}$ stores. Although further supports the involvement of ryanodine receptor activation by Ca$_{\text{2+}}$ influx, there appears to be a difference in the responses between 4-AP and ionomycin mediated stimulation. While ionomycin mediated stimulation was completely inhibited by ryanodine inhibition, 4-AP mediated stimulation had a complete inhibitory effect on ERK1 but only partial inhibition on ERK2 phosphorylation/activation. This indicates ionomycin mediated stimulation is able to stimulate Ca$_{\text{2+}}$ release using the CICR mechanism more than 4-AP mediated stimulation and may be indicative of a differential localisation of ERK1 and ERK2.

The final objective of this chapter was to investigate the activation of the IPCR mechanism by 4-AP and ionomycin mediated stimulation. If the mechanism is activated then inhibition of IP$_3$R or decreasing the IP$_3$ production should produce a reduction in ERK1 and ERK2 phosphorylation/activation by 4-AP and ionomycin mediated stimulation. We found that using 2-APB as an IP$_3$R antagonist or inhibiting PLC activity using U-73122, both 4-AP and ionomycin mediated stimulation of ERK1 and
ERK2 phosphorylation/activation were significantly reduced. Interestingly, ryanodine receptor inhibition resulted in a complete reduction of ERK1 and ERK2 phosphorylation/activation mediated by ionomycin stimulation. Targeting the IPCR mechanism with IP3R or PLC inhibition only resulted in a partial reduction. This could highlight a preference of ionomycin mediated stimulation which causes direct Ca$^{2+}$ through ionophore to be more effective in CICR than in invoking IPCR mechanism. The 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation displays the similar pattern for CICR and IPCR mechanisms thus does not appear to have a preference (Schematic 4-1).

In conclusion, in this chapter we show two functional abilities of the smooth endoplasmic reticulum in nerve terminals. Firstly, we provide evidence that the smooth endoplasmic reticulum is able to sequester Ca$^{2+}$ from the cytosol. Secondly, we demonstrated that both CICR and IPCR mechanisms are able to stimulate these Ca$^{2+}$ stores and have a significant effect on Ca$^{2+}$-dependent mechanisms that result in ERK1 and ERK2 phosphorylation/activation.
Schematic 4-1: Influx of Ca$^{2+}$ through VDCCs and ionomycin can stimulate Ca$^{2+}$-dependent mechanisms that activate the ERK pathway

Influx of extracellular Ca$^{2+}$ through VDCC or ionomycin can possibly directly stimulate Pyk2 which activates the ERK pathway and stimulates efflux of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores. (i) Extracellular Ca$^{2+}$ could directly stimulate RyR to efflux Ca$^{2+}$. (ii) Increases in [Ca$^{2+}$] can also stimulate PLC which metabolises PIP$_2$ to form DAG and IP$_3$ which stimulates IP$_3$Rs to release Ca$^{2+}$. (iii) Depletion of intracellular Ca$^{2+}$ stores by inhibiting SERCA pumps diminishes this recruitment decreasing the ERK stimulation response.
Chapter 5
5. Metabotropic activation of IP$_3$-induced Ca$^{2+}$ release

**Summary:** We investigated IP$_3$-induced Ca$^{2+}$ release (IPCR) activation through the stimulation of the metabotropic receptors that increase inositol 1,4,5-trisphosphate (IP$_3$) production. A good candidate in this regard is the mGluR1/5 receptors which are present in nerve terminals and are known to activate phospholipase C (PLC) to facilitate neurotransmitter release. Using the agonist (S)-3,5-dihydroxyphenylglycine (DHPG) to stimulate mGluR1/5 receptors, we found that receptor activation resulted in a significant increase in ERK1 and ERK2 phosphorylation/activation dependent on DHPG concentration and desensitising with time. By targeting components of IPCR mechanism we propose a general mechanism that explains how mGluR1/5 receptor stimulation results in ERK1 and ERK2 phosphorylation/activation. Stimulation of mGluR1/5 with DHPG causes the activation of PLC through the G$_{q/11}$ subunit. PLC activation metabolises PIP$_2$ to form IP$_3$ and DAG; the increase in IP$_3$ production results in the stimulation of IP$_3$ receptors (IP$_3$Rs) found on the smooth endoplasmic reticulum. Stimulation of IP$_3$Rs releases Ca$^{2+}$ from the smooth endoplasmic reticulum which stimulates calmodulin and thereby Ca$^{2+}$/calmodulin-dependent kinase II (CaMKII). The activation of the latter underpins the Ca$^{2+}$-dependent mechanisms that lead to ERK1 and ERK2 phosphorylation/activation.

5.1. Introduction

The IPCR mechanism describes the recruitment of Ca$^{2+}$ from IP$_3$R sensitive stores such as the smooth endoplasmic reticulum to activate Ca$^{2+}$-dependent mechanisms. The Ca$^{2+}$-dependent mechanisms lead to phosphorylation/activation of ERK1 and ERK2 which can be therefore serve as sensitive sensors indicating increases in intrasynaptosomal Ca$^{2+}$ concentrations. We have provided evidence that IPCR mechanism could be activated by the influx of Ca$^{2+}$ through VDCCs mediated by 4-AP.
and ionomycin stimulation (Chapter 4). However, 4-AP and ionomycin mediated stimulation are also able to recruit Ca\(^{2+}\) via other mechanisms such as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). To directly investigate the recruitment of Ca\(^{2+}\) from IP\(_3\)R sensitive stores we considered using metabotropic mediated stimulation. Previous studies have suggested that the IPCR mechanism can be activated through metabotropic receptor mediated stimulation (Herrero et al., 1998).

Metabotropic glutamate receptors (mGluRs) are composed of seven transmembranes, an N-terminal that binds to agonists and antagonist and a C-terminal and intracellular loops which couple to the G protein. There are eight mGluRs that are split into three groups including group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8). Group 1 mGluRs are known to couple to the \(G_{q/11}\) protein, while the group II and III mGluRs are coupled to the \(G_i/G_o\) proteins. The G protein that the mGluR couples to is important in determining the downstream effects of the receptor upon activation. Given that group II and III mGluRs are coupled to \(G_i/G_o\) protein, so they are negatively coupled to adenylate cyclase which results in the decrease of cAMP concentration which generally leads to a reduction in cellular activity. This is in contrast to mGluR1/5 activation which generally have facilitatory actions through its \(G_{q/11}\) protein and PLC activation.

There are two main pathways that can be activated by the stimulation of mGluR1/5 which include an IPCR mechanism and PKC activation. When the mGluR1/5 receptors are stimulated the \(\alpha\) subunit is activated by GTP binding and dissociates from the \(\beta\gamma\) subunit which acts on its own targets. The \(\alpha\) subunit is able to activate PLC bound to the plasma membrane resulting in the metabolism of PIP\(_2\) forming part of the phospholipid content of the inner leaflet of the plasma membrane. The two metabolites that are
formed from PIP$_2$ breakdown include IP$_3$ and diacylglycerol (DAG) which active the IPCR mechanism and PKC, respectively.

DAG remains bound to the membrane and is able to stimulate PKC which has downstream targets on presynaptic channels and the exocytotic machinery but could also directly phosphorylate c-Raf-1 causing its activation and therefore possible downstream activation of the ERK pathway (Kolch et al., 1993; Ueda et al., 1996; Schonwasser et al., 1998). The IP$_3$ metabolite is hydrophilic and therefore is not bound to the membrane and can subsequently be able to diffuse into the cytosol where it interacts with binding sites found on the IP$_3$R. Binding causes the displacement of inositol 1,4,5-trisphosphate receptor binding protein (IRBIT), thus making the IP$_3$R open to effect an efflux of Ca$^{2+}$ from the smooth endoplasmic reticulum (Ando et al., 2006). The Ca$^{2+}$ released could be sufficient for activating Ca$^{2+}$-dependent mechanisms but alternatively it might propagate a Ca$^{2+}$ wave which triggers ryanodine receptors (RyRs) to become active causing further release of Ca$^{2+}$ from the smooth endoplasmic reticulum. Upon removal of IP$_3$ IRBIT is able to bind to the IP$_3$R causing the closing of the channel.

The Ca$^{2+}$-dependent mechanisms that the IPCR mechanism could activate include Src-dependent mechanism inhibition of which has been shown in this thesis to cause suppression of 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation (Chapter 3). The activity of GEFs and GAPs that promote and inhibit, respectively the active form of Ras (Ras-GTP) can regulate the ERK pathway. Calmodulin has been shown to have some inhibitory consequences for the ERK pathway for example it can inhibit K-Ras (Villalonga et al., 2001). However, it can also bind to a RasGEF such as RasGRF to promote the formation of Ras-GTP
(Farnsworth et al., 1995; Freshney et al., 1997). In addition, through PI3K it can stimulate H-Ras activation which promotes c-Raf-1 stimulation and the subsequent downstream activation of the ERK pathway (Moreto et al., 2008). Another stimulatory consequence of calmodulin activation is the activation of CaMKII which has been shown to inhibit SynGAP thus this would again promote Ras activation (Chen et al., 1998).

The objective of this chapter is to investigate whether mGluR1/5 stimulation results in the ERK1 and ERK2 phosphorylation/activation. Moreover, we aimed to test whether any ERK1 and ERK2 phosphorylation/activation is due to the IPCR and the components involved in this mechanism. Furthermore, we investigated the Ca\textsuperscript{2+}-dependent mechanisms that could be activated by the Ca\textsuperscript{2+} release from the smooth endoplasmic reticulum through IP\textsubscript{3}Rs stimulation.
5.2. Method

5.2.1. Synaptosomal Preparation

Preparation of synaptosomes has been described in section 2.1.

5.2.2. SDS-PAGE and Immunoblotting

All samples obtained through immunoblotting experiments underwent the procedure described in section 2.2.

5.2.3. Metabotropic activation protocol

Experiments that followed the metabotropic activation of ERK protocol are described in section 2.3.3. Synaptosomes were resuspended in HBM buffer containing BSA (1mg/ml) with the drug of interest U-73122 (10µM), 2-APB (50µM), thapsigargin (1µM), levetiracetam (100µM), W7 (50µM) or KN-93 (10µM) and incubated at 37ºC. After 3 minutes Ca^{2+} (1mM) was added to the suspension and DHPG (10µM) was added to the incubation after 49 minutes of incubation unless otherwise stated. After 1 minute stimulation with DHPG (10µM) the synaptosomes were spun down and the supernatant removed. The experiment was terminated using the STOP solution.

5.2.4. Glutamate release

Synaptosomes were resuspended in 1.5ml HBM containing BSA (1mg/ml) and transferred to the spectrofluorimeter. Constant stirring ensured that the synaptosomes were oxygenated and NADP^{+} (1mM) and GDH (50 units/ml) were added to the suspended synaptosomes at the start of the experiment which was followed by the addition of Ca^{2+} (1mM) after 3 minutes. When the recording of fluorescence had started we added our drug of interest in this period. Levetiracetam (10 or 100µM) was added at 7 minutes and DHPG (10 and 100µM) was added at 9 minutes. Glutamate release was
then evoked using 4-AP (1mM) at 10 minutes. Finally, at 15 minutes, exogenous glutamate (2.5nmol) was added as an internal standard to quantify the glutamate release.

5.2.5. Statistical analysis

The methods of statistical analysis have been described in section 2.6.1. For data with more than two sets analysis of variance (ANOVA) was used to assess the statistical significance, followed by Dunnetts post hoc test. The net change in ERK1 and ERK2 phosphorylation/activation was calculated by removing the basal percentage from the stimulated groups for each n. We used Student’s unpaired t-test to assess the statistical significance between two sets of data. The bar graphs of glutamate release in the results section show independent experiments averaged at 300-305 second time points.

5.2.6. Reagents

A stock solution of DHPG (10µM) was made in water and working solution was further diluted using HBM.

A stock solution of thapsigargin (1µM) was made in DMSO and working solution was further diluted using HBM.

A stock solution of ryanodine (30µM) was obtained with DMSO and working solution was further diluted using HBM.

A stock solution of 2-APB (50µM) was obtained with DMSO and working solution was further diluted using HBM.
A stock solution of U-73122 (10μM) was obtained with DMSO and working solution was further diluted using HBM.

A stock solution of Levetiracetam (100μM) was made using with water and further diluted using HBM.

A stock solution of W7 (50μM) was made using with water and further diluted using HBM.

A stock solution of KN-93 (10μM) was made using with DMSO and further diluted using HBM.
5.3. Results

We examined the effect of G<sub>q/11</sub> coupled G-protein coupled receptors (GPCRs) stimulation of PLC activation on ERK1 and ERK2 phosphorylation/activation in synaptosomes. We used DHPG to stimulate mGluR1/5 receptors to potentially mobilise the Ca<sup>2+</sup> stores to cause ERK1 and ERK2 phosphorylation/activation. We first conducted a dose-response curve of DHPG at the concentrations of 0.1, 1, 10 and 100µM and quantified the phosphorylation/activation of ERK1 and ERK2. Synaptosomes were stimulated in the presence of Ca<sup>2+</sup> (1mM) for 1 minute by the indicated concentration of DHPG (Figure 5-1A&B). Analysis of immunoblots showed a bell shaped concentration dependency for ERK1 and ERK2 phosphorylation/activation. At 0.1µM DHPG had no significant effect on ERK1 (106.8 ± 6.5%) and ERK2 (108.5 ± 9.4%) phosphorylation/activation compared to the 37°C control (Figure 5-1C). There was a significant increase in ERK1 (129.0 ± 3.4%) and ERK2 (134.9 ± 5.9%) phosphorylation/activation compared to the 37°C control at 1µM DHPG. This was again observed with 10µM DHPG which caused a significant increase of ERK1 (146.7 ± 3.9%) and ERK2 (136.4 ± 5.1%) phosphorylation/activation compared to the 37°C control. However, there appears to be a declining trend at 100µM DHPG, a concentration that caused no significant effect on ERK1 (120.6 ± 8.7%) and ERK2 (123.9 ± 4.6%) phosphorylation/activation compared to the 37°C. For subsequent experiments we employed a DHPG 10µM to elicit a maximum response.

To ensure that 1 minute of DHPG mediated stimulation was sufficient for maximum response, we conducted a time-course of DHPG (10µM) in the presence of Ca<sup>2+</sup> (1mM) with 10, 30, 60, 120, 300 and 600 second(s) stimulations (Figure 5-2A&B). DHPG stimulation for 10 or 30 seconds (10 seconds: ERK1 97.7 ± 6.1% and ERK2 97.6 ±
Figure 5-1: Dose-dependent effect of DHPG on ERK1 and ERK2 phosphorylation/activation

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended with HBM with Ca²⁺ (1mM) added at 3 minutes. Different concentration of DHPG that include 0.1, 1, 10 or 100µM was used at 49 minutes or HBM for control and the experiment ended after 50 minutes of incubation. (C) DHPG effect on ERK1 and ERK2 phosphorylation/activation compared to the 37°C control at concentrations 0.1, 1, 10 and 100µM. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
3.5%; and 30 seconds ERK1 105.9 ± 4.1% and ERK2 102.8 ± 4.4%) had no significant effect on ERK1 and ERK2 phosphorylation/activation compared to the 37°C control (Figure 5-2C). As expected from the previous result DHPG stimulation for a minute caused a significant increase in ERK1 (118.2 ± 4.4%) and ERK2 (116.8 ± 2.1%) phosphorylation/activation compared to the 37°C control. ERK1 and ERK2 phosphorylation/activation was sustained over the 2 and 5 minutes (2 minutes: ERK1 122.4 ± 6.6% and ERK2 118.5 ± 5.5% and 5 minutes: ERK1 121.6 ± 2.2% and ERK2 120.3 ± 2.7%). Subsequently there was no significant effect on ERK1 (113.7 ± 4.8%) and ERK2 (107.9 ± 4.8%) phosphorylation/activation compared to the 37°C control after 10 minutes of DHPG stimulation. The time course shows that initially there is little activity but this is followed by a significant increase which declines with time. For the subsequent experiments we stimulated the synaptosomes for 1 minute with 10µM DHPG.

Having shown that mGluR1/5 mediated stimulation results in ERK1 and ERK2 phosphorylation/activation, we investigated if this stimulation can be attributed to Ca²⁺-release from the smooth endoplasmic reticulum. To do this we incubated synaptosomes with thapsigargin (1µM) to deplete the smooth endoplasmic reticulum in the presence of Ca²⁺ (1mM) and stimulated with DHPG (10µM) for 1 minute (Figure 5-3A&B). We found that in the absence of thapsigargin, DHPG mediated stimulation resulted in a significant increase in ERK1 (133.2 ± 10.6%) and ERK2 (133.0 ± 10.0%) phosphorylation/activation compared to the 37°C control (Figure 5-3C). After thapsigargin incubation we found that the DHPG mediated stimulation of ERK1 (89.5 ± 10.4%) or ERK2 (91.9 ± 7.8%) phosphorylation/activation compared to the 37°C control was diminished. Incubation with thapsigargin had no significant effect on the basal activity of ERK1 (99.2 ± 12.4%) or ERK2 (104.2 ± 9.6%) compared to the 37°C
Figure 5-2: DHPG time course of ERK1 and ERK2 phosphorylation/activation.
(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended with HBM with Ca²⁺ (1mM) added at 3 minutes. DHPG (10µM) was used to stimulate the synaptosomes at 50 minutes or HBM for control and the experiment ended at different time points after DHPG addition that include 10 and 30 seconds, 1, 2, 5 and 10 minutes. (C) DHPG effect on ERK1 and ERK2 phosphorylation/activation compared to the 37°C control over a time course including 10 and 30 seconds, 1, 2, 5 and 10 minutes. All values represent the mean ± SEM. p<0.01(∗∗) p<0.05 (∗). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
Figure 5-3: DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of thapsigargin

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and T = thapsigargin shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without thapsigargin (1µM) and Ca2+ (1mM) addition at 3 minutes. DHPG (10µM) was used to stimulate the synaptosomes at 1 minute and the experiment ended after 50 minutes of incubation. (C) Basal effect of the presence of thapsigargin and DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of thapsigargin. Thapsi = thapsigargin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
control. Overall, from the analysis of the net change, we found that the DHPG mediated stimulation is sensitive to thapsigargin and results in a significant inhibition of ERK1 and ERK2 phosphorylation/activation (Figure 5-3D).

We further investigated the IPCR mechanism by considering the action of PLC. It has been shown that the mGluR1/5 receptor couples through the G protein, $G_{q/11}$ which is capable of activating PLC. In order to directly test if PLC is being activated by mGluR1/5 stimulation, we used the PLC inhibitor U-73122 to examine whether this inhibits the ERK1 and ERK2 phosphorylation/activation in response to DHPG mediated stimulation. Thus, we incubated the synaptosomes with U-73122 (10µM) in the presence of Ca$^{2+}$ (1mM) and stimulated DHPG (10µM) for 1 minute (Figure 5-4A&B). During incubation without U-73122 DHPG mediated stimulation resulted in significant increase in ERK1 (132.4 ± 4.6%) and ERK2 (130.3 ± 4.9%) phosphorylation/activation compared to the 37°C control (Figure 5-4C). However, when U-73122 was present, DHPG mediated stimulation did not increase ERK1 (77.9 ± 6.5%) and ERK2 (79.3 ± 8.7%) phosphorylation/activation compared to the 37°C control. The incubation of U-73122 per se had no significant effect on the basal activity of ERK1 (79.6 ± 10.2%) or ERK2 (80.3 ± 11.9%) compared to the 37°C control. The net change of ERK1 and ERK2 phosphorylation/activation shows that incubation with U-73122 significantly inhibits DHPG mediated stimulation of Ca$^{2+}$ store release (Figure 5-4D).

To ensure that DHPG mediated stimulation is due to the stimulation of IP$_3$Rs found on smooth endoplasmic reticulum we inhibited the latter receptors. Synaptosomes were incubated with 2-APB (50µM) in the presence of Ca$^{2+}$ (1mM) and stimulated with DHPG (10µM) for 1 minute (Figure 5-5A&B). We found that DHPG stimulation in the absence of 2-APB resulted in a significant increase in ERK1 (132.4 ± 4.6%) and ERK2
Figure 5-4: DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of U-73122

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and U = U-73122 shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without U-73122 (10µM) and Ca²⁺ (1mM) addition at 3 minutes. DHPG (10µM) was used to stimulate the synaptosomes at 1 minute and the experiment ended after 50 minutes of incubation. (C) Basal effect of the presence of U-73122 and DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of U-73122. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
Figure 5-5: DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of 2-APB

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and A = 2-APB shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without 2-APB (50 µM) and Ca²⁺ (1mM) addition at 3 minutes. DHPG (10 µM) was used to stimulate the synaptosomes at 1 minute and the experiment ended after 50 minutes of incubation. (C) Basal effect of the presence of 2-APB and DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of 2-APB. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
DHPG mediated stimulation in the presence of 2-APB produced a diminished increase of ERK1 (97.5 ± 10.1%) and ERK2 (94.3 ± 13.6%) phosphorylation/activation compared to the 37ºC control. Incubation with 2-APB by itself had no significant effect on the basal activity of ERK1 (105.0 ± 9.8%) and ERK2 (96.5 ± 12.1%) compared to the 37ºC control. Thus the net change in DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation shows this is a significant inhibition with 2-APB incubation (Figure 5-5D).

To further support the hypothesis that IP₃Rs are indeed activated by mGluR1/5 stimulation, we considered using another inhibitor of IP₃Rs. Levetiracetam is not specific for IP₃Rs like the inhibitor 2-APB because it should also inhibit RyRs but in the context of DHPG mediated stimulation it inhibits IP₃R responses only. Synaptosomes were incubated with levetiracetam (100µM) in the presence of Ca²⁺ (1mM) and stimulated with DHPG (10µM) for 1 minute (Figure 5-6A&B). As expected from previous results DHPG mediated stimulation in the absence of levetiracetam caused a significant increase in ERK1 (133.2 ± 10.6%) and ERK2 (141.2 ± 8.3%) phosphorylation/activation compared to the 37ºC control (Figure 5-6C). However, during incubation with levetiracetam DHPG mediated stimulation did not significantly increase ERK1 (84.4 ± 6.1%) and ERK2 (88.2 ± 8.5%) phosphorylation/activation compared to 37ºC control. We also found that there was no significant effect on the basal activity of ERK1 (88.0 ± 5.0%) and ERK2 (87.0 ± 6.9%) when the synaptosomes were incubated with levetiracetam. The net change in ERK1 and ERK2 phosphorylation/activation shows that DHPG mediated stimulation is significantly reduced in the presence of levetiracetam (Figure 5-6D). Interestingly, the effect of levetiracetam was quite similar to the effect of 2-APB which might be seen to
Figure 5-6: DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of levetiracetam

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and L = levetiracetam shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without levetiracetam (100µM) and Ca²⁺ (1mM) addition at 3 minutes. DHPG (10µM) was used to stimulate the synaptosomes at 1 minute and the experiment ended after 50 minutes of incubation. (C) Basal effect of the presence of levetiracetam and DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37 ºC control in the presence and absence of levetiracetam. Lev = levetiracetam. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
indicate these two drugs are acting on a common target.

We next investigated the Ca\(^{2+}\)-dependent mechanisms that could be activated by the release of Ca\(^{2+}\) from the smooth endoplasmic reticulum. Calmodulin activation occurs following increases in Ca\(^{2+}\) concentration which could result in ERK1 and ERK2 phosphorylation/activation modulation. To test if calmodulin is involved in the mechanism, we incubated synaptosomes with a calmodulin inhibitor W7 (50\(\mu\)M) in the presence of Ca\(^{2+}\) (1mM) and stimulated with DHPG (10\(\mu\)M) for 1 minute (Figure 5-7A&B). We found that in the absence of W7 DHPG mediated stimulation was able to significantly increase ERK1 (133.2 ± 10.6%) and ERK2 (133.0 ± 10.0%) phosphorylation/activation compared to the 37ºC control (Figure 5-7C). In the presence of W7, DHPG mediated stimulation of ERK1 (36.8 ± 3.3%) and ERK2 (23.8 ± 2.5%) phosphorylation/activation compared to the 37ºC control was reduced. This inhibitory effect might however be attributable to the basal effect of W7 incubation which causes a significant inhibition of ERK1 (42.1 ± 5.1%) and ERK2 (23.8 ± 1.9%) compared to the 37ºC control. However, accounting for this, we found from the net change of ERK1 and ERK2 phosphorylation/activation that W7 significantly inhibits DHPG mediated stimulation of the ERK pathway (Figure 5-7D).

Another consequence of Ca\(^{2+}\) release and calmodulin activation in presynaptic nerve terminals is the activation of CaMKII which could also modulate ERK1 and ERK2 phosphorylation/activation. To test the involvement of CaMKII we used an inhibitor of the kinase, KN-93 to examine its effect on DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation. Synaptosomes were incubated with KN-93 (10\(\mu\)M) in the presence of Ca\(^{2+}\) (1mM) and then stimulated with DHPG (10\(\mu\)M) for 1 minute (Figure 5-8A&B). In the absence of KN-93 incubation there was a significant increase
Figure 5-7: DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of W7

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and W = W7 shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without W7 (50µM) and Ca²⁺ (1mM) addition at 3 minutes. DHPG (10µM) was used to stimulate the synaptosomes at 1 minute and the experiment ended after 50 minutes of incubation. (C) Basal effect of the presence of W7 and DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of W7. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
Figure 5-8: DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of KN-93

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and K = KN-93 shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without KN-93 (10µM) and Ca²⁺ (1mM) addition at 3 minutes. DHPG (10µM) was used to stimulate the synaptosomes at 1 minute and the experiment ended after 50 minutes of incubation. (C) Basal effect of the presence of KN-93 and DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence and absence of KN-93. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
in ERK1 (132.4 ± 4.6%) and ERK2 (130.3 ± 4.9%) phosphorylation/activation compared to the 37°C control in response to DHPG stimulation (Figure 5-8C). In the presence of KN-93 however DHPG mediated stimulation of ERK1 (89.2 ± 8.6%) and ERK2 (74.2 ± 5.5%) phosphorylation/activation compared to the 37°C control was suppressed though only the ERK2 was significantly inhibited. Interestingly, KN-93 had a clear significant inhibitory effect on the basal activity of ERK1 (75.1 ± 5.4%) and ERK2 (60.4 ± 6.9%) compared to the 37°C control. The net change analysis shows that both ERK1 and ERK2 phosphorylation/activation mediated by DHPG are reduced in the presence of KN-93 though only ERK1 is significantly inhibited (Figure 5-8D).

Finally, another aspect that we investigated was the effect of intracellular calcium stores on evoked glutamate release. We investigated both the possible stimulatory and inhibitory effects on 4-AP mediated glutamate release by stimulating and inhibiting IP$_3$R, respectively. We used DHPG (10μM) to stimulate the IP$_3$R 1 minute prior to 4-AP (1mM) while levetiracetam (100μM) used to inhibit IP$_3$R was added 3 minutes prior to 4-AP. The initial findings suggest that prior DHPG mediated stimulation might produce a slight facilitation of the 4-AP mediated glutamate release (Figure 5-9A&B). However, levetiracetam incubation per se was unable to effect 4-AP mediated glutamate release (Figure 5-9C&D).
Figure 5-9: Effect of IP₃R stimulation or inhibition on 4-AP mediated glutamate release

Synaptosomes were re-suspended in HBM medium containing BSA (1mg/ml) NADP⁺ (1mM) and GDH (50units/ml). Ca²⁺ (1mM) was added after 3 minutes and levetiracetam (10 or 100µM) was added 3 minutes or DHPG (10 or 100µM) was added 1 minute prior to 4-AP (1mM) addition at 10 minutes and then at 15 minutes glutamate (2.5nmol) is added as an internal standard. (A) A trace of the 10 and 100µM DHPG effect on 4-AP mediated glutamate release. (B) Quantification of the DHPG glutamate release averaged at 300-305 seconds. (C) A trace of the 10 and 100µM levetiracetam effect on 4-AP mediated glutamate release. (D) Quantification of the levetiracetam glutamate release averaged at 300-305 seconds. (n=1).
5.4. Discussion

In this chapter, we determined to investigate the metabotropic activation of intracellular Ca\(^{2+}\) stores, in particular the smooth endoplasmic reticulum. Previous studies have shown a facilitatory effect on glutamate release following mGluR1/5 activation through the mechanisms activated by inositol phospholipid metabolism (Herrero et al., 1992; Schwartz & Alford, 2000). The mechanism that has been proposed to be activated following the mGluR1/5 receptor stimulation is as follows; mGluR1/5 receptors couple to G\(_{q/11}\) proteins which are known to stimulate PLC thus mGluR1/5 stimulation results in PLC activation. PLC activation metabolises PIP\(_2\) to form IP\(_3\) and DAG which subsequently activate signalling pathways that could potentially have facilitatory effects on release. The increased IP\(_3\) production stimulates IP\(_3\)Rs to release Ca\(^{2+}\) from smooth endoplasmic reticulum activating Ca\(^{2+}\)-dependent processes (Schematic 5-1). Increased DAG production stimulates PKC to target its downstream targets such as the facilitation of Ca\(^{2+}\) influx and regulate release (Wang & Sihra, 2004).

DHPG has also been used in many studies as an agonist for the mGluR1/5 receptor though a study has suggested mGluR5 receptors have a higher affinity for DHPG than mGluR1 receptors (Musante et al., 2008). Nevertheless, in most studies DHPG has been used to stimulate mGluR1/5 receptors collectively so the first objective of this chapter was to show any effectiveness of mGluR1/5 stimulation on ERK1 and ERK2 phosphorylation/activation. By conducting a dose-response curve of DHPG, we found that the effect was bimodal with ERK1 and ERK2 phosphorylation/activation peaking at 1\(\mu\)M and proceeding to a decline at the higher concentration (100\(\mu\)M). Furthermore, a time course using DHPG (10\(\mu\)M) also showed ERK1 and EKR2 phosphorylation/activation peaks after 1 minute and is sustained over 5 minutes after which there is towards a decline at the longer time points of stimulation. This suggests
that there is a degree of receptor desensitisation of mGluR1/5 or that there is a mechanism which dephosphorylates ERK1 and ERK2 after prolonged exposure to DHPG. Other studies have pointed to the desensitisation of the mGluR1/5 receptor we suspect this to be the cause for the decline in ERK1 and ERK2 phosphorylation/activation (Herrero et al., 1994).

The second objective of this chapter was to target the individual components of the IPCR mechanism to confirm their significance. We especially targeted the smooth endoplasmic reticulum rather than the other potential intracellular Ca\(^{2+}\) stores because the literature supports IPCR from these stores. In order to test if DHPG mediated stimulation is dependent of Ca\(^{2+}\) release from the smooth endoplasmic reticulum we first depleted the store by inhibiting the SERCA pump thus allowing the ‘leakage’ of Ca\(^{2+}\). We found that by depleting the smooth endoplasmic reticulum, DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation was completely inhibited. This therefore indicates that mGluR1/5 stimulation is sensitive to thapsigargin and also the degree of the inhibition suggests it is unlikely that other potential Ca\(^{2+}\) stores are activated by mGluR1/5 stimulation.

We next investigated the triggers for the release of Ca\(^{2+}\) from the smooth endoplasmic reticulum so we examined the role of PLC activation in ERK1 and ERK2 phosphorylation/activation mediated by DHPG stimulation. According to the mechanism, mGluR1/5 stimulation should active PLC through G\(_{q/11}\) protein thus the inhibition of PLC should result in inhibition of DHPG mediated stimulation. Indeed, inhibiting PLC activity we found that DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation was again completely abrogated implying that PLC activation is obligatory for the DHPG mediated stimulation of the ERK pathway.
To delineate and confirm the downstream pathway from PLC that is responsible for ERK1 and ERK2 phosphorylation/activation we again used the IP$_3$R antagonist 2-APB, to inhibit the receptors resident on smooth endoplasmic reticulum. Inhibiting the IP$_3$Rs we found that ERK1 and ERK2 phosphorylation/activation mediated by DHPG stimulation of mGluR1/5 was again completely abolished. The role of IP$_3$Rs was confirmed using levetiracetam which has been described as an inhibitor of IP$_3$Rs amongst its other targets (Cataldi et al., 2005; Nagarkatti et al., 2008). Known nonspecific actions associated with levetiracetam include the inhibition of RyR at lower concentrations (Nagarkatti et al., 2008). However CICR was not being induced under the condition used here thus any levetiracetam inhibition would likely be a reflection of IP$_3$R inhibition. RyR could be activated by subsequent release of Ca$^{2+}$ due to IP$_3$R activation, but if these are already inhibited by levetiracetam then the subsequent release of Ca$^{2+}$ would not occur and any inhibition of RyR by levetiracetam would be irrelevant. Using levetiracetam to inhibit the IP$_3$R we again found complete abolition of ERK1 and ERK2 phosphorylation/activation mediated by DHPG stimulation.

The combined data from the 2-APB and levetiracetam inhibitory effects on IP$_3$Rs suggest that Ca$^{2+}$ release from the smooth endoplasmic reticulum is essentially responsible for all of the ERK1 and ERK2 phosphorylation/activation mediated by mGluR1/5 stimulation. This further suggests that despite PKC being activated in parallel to the IPCR mechanism the former is not responsible for ERK1 and ERK2 phosphorylation/activation. However, in future experiments we should conduct experiments with PKC inhibition to confirm or refute the role of the kinase in the DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation.
The final objective in this chapter was to investigate the consequence of Ca\(^{2+}\)-dependent mechanisms that are activated by the IPCR mechanism. We targeted the two Ca\(^{2+}\) signal transducers that include calmodulin (CaM) and CaMKII that have been implicated in the regulation of the ERK pathway in our laboratory. We found that the inhibition of CaM resulted in a complete abolition of DHPG mediated stimulation of ERK1 and ERK2 phosphorylation/activation, distinct from the CaM inhibition of the basal activity of both ERK1 and ERK2. In addition, although CaMKII inhibition had a significant effect on the basal activity of ERK1 and ERK2 just like CaM, CaMKII inhibition abrogates ERK1 phosphorylation/activation by DHPG while the effect of ERK2 phosphorylation/activation was reduced but not statistically significantly. This could be expected given that CaM can stimulate ERK1 and ERK2 phosphorylation/activation independently of CaMKII activation.

CaM and CaMKII play a significant role in maintaining the basal activity of ERK1 and ERK2 and both can be further activated by Ca\(^{2+}\) released from the smooth endoplasmic reticulum. There are possible targets that calmodulin and CaMKII could activate which could result in the facilitatory effect on ERK1 and ERK2 phosphorylation/activation that have been described here but this requires further investigation. CaM could for example stimulate RasGRF or could stimulate H-Ras through PI3K so it would be of interest to observe if Ras is activated during DHPG mediated stimulation and whether this is sensitive to calmodulin inhibition. CaMKII could induce its stimulatory effects by inhibiting SynGAP but SynGAP has not been described in presynaptic nerve terminals to date. Furthermore, there is evidence which suggests that SynGAP colocalise with scaffold proteins and NMDA in the postsynaptic cell body (Chen et al., 1998). Thus, an alternative mechanism(s) may need to be invoked to explain how CaMKII regulates the ERK pathway in presynaptic nerve terminals. Finally, given the
inhibitory effect of Src inhibition has had on 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation it would be interesting to see what effect Src inhibition has on DHPG mediated stimulation.

The preliminary examination into the role of smooth endoplasmic reticulum during 4-AP mediated glutamate release was also made. 4-AP mediated stimulation causes influx of Ca\textsuperscript{2+} through VDCCs which acts as the trigger for exocytosis of the neurotransmitter. The role of intracellular Ca\textsuperscript{2+} stores in presynaptic nerve terminals in the exocytotic machinery is unclear though there are studies that have found evidence of a facilitatory role (Davletov et al., 1998; Narita et al., 2000; Ashton et al., 2001). We hypothesised that the intracellular Ca\textsuperscript{2+} stores would also have a facilitatory effect on neurotransmitter release, based on two mechanisms: firstly, the excess Ca\textsuperscript{2+} released by the smooth endoplasmic reticulum supporting exocytosis further and secondly the activation of facilitatory mechanisms by ERK1 and ERK2 mediated phosphorylation/activation leading to SSVs mobilisation. Using DHPG we sought to facilitate 4-AP mediated glutamate release by stimulating IP\textsubscript{3}R. Additionally we also considered that 4-AP mediated glutamate release could be inhibited if IP\textsubscript{3}Rs are inhibited using levetiracetam. Indeed, DHPG incubation enhanced 4-AP glutamate release which could be further stimulatory under submaximal release potentials (Herrero et al., 1992). Levetiracetam had no effect on glutamate release but the lack of effect may indicate that the IPCR mediated by 4-AP does not significantly support release in the absence of mGluR1/5 activation. As these are preliminary experiments further investigation needs to take place before we can draw conclusions.

In conclusion, in this chapter we have shown that mGluR1/5 stimulation results in ERK1 and ERK2 phosphorylation in a transient manner. The mechanism for DHPG
mediated stimulation is the IPCR mechanism which undergoes the following cascade; mGluR1/5 stimulation activates PLC resulting in PIP$_2$ metabolise to significantly increase IP$_3$ concentration which stimulates IP$_3$R. The subsequent release of Ca$^{2+}$ through IP$_3$Rs converges on CaM that independently and through CaMKII activation lead to the phosphorylation/activation of ERK1 and ERK2. The downstream activity of latter supports the facilitation of glutamate release as has been observed with neurotrophin induced activation of the ERK pathway (Jovanovic et al., 1996; Jovanovic et al., 2000).
Schematic 5-1: GPCRs coupled to $G_{\alpha}$ are able to stimulate the efflux of Ca$^{2+}$ from IP$_3$R sensitive stores resulting in the ERK pathway activation

DHPG stimulation of mGluR1/5 receptors results in PLC activation which metabolises PIP$_2$ to form DAG and IP$_3$. (i) Under repletion conditions the increase in [IP$_3$] stimulates IP$_3$Rs to efflux Ca$^{2+}$ which is sufficient for CaM activation. CaM is able to stimulate the ERK pathway and CaMKII which can also activate the ERK pathway. (ii) Depletion of the intracellular Ca$^{2+}$ stores through SERCA pump inhibition using thapsigargin decreases Ca$^{2+}$ efflux from these stores leading to a decrease in the activation of the ERK pathway.
Chapter 6
6. Inhibitory modulation of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release

**Summary:** The Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) mechanism that phosphorylates/activates ERK1 and ERK2 is dependent on the influx of extracellular Ca\textsuperscript{2+} through voltage-dependent Ca\textsuperscript{2+} channels (VDCCs). There is considerable evidence that Ca\textsuperscript{2+} influx through VDCCs is negatively modulated by presynaptic GPCRs that include GABA\textsubscript{B}, group III mGluRs, 5-HT\textsubscript{1A} and A\textsubscript{1} receptors. Thus by stimulating these presynaptic GPCRs we could show that reduced VDCC activity results in a diminished CICR mechanism activation; reported by reduced ERK1 and ERK2 phosphorylation/activation. We show here that stimulation of GABA\textsubscript{B}, group III mGluRs, 5-HT\textsubscript{1A} and A\textsubscript{1} receptors results in a reduction of ERK1 and ERK2 phosphorylation/activation mediated by 4-AP stimulation. This is likely to be due to negative modulation of VDCC activity by these groups of GPCRs. Surprisingly, we also found that prior depletion of the smooth endoplasmic reticulum by inhibiting the SERCA pump resulted in removing the inhibitory effect of these GPCRs on VDCC activity. This perhaps suggests a link between the filling state of the smooth endoplasmic reticulum and the ability of these GPCRs to negatively modulate VDCCs.

6.1. Introduction

When the presynaptic plasma membrane is depolarised it results in the activation of VDCCs such as the Ca\textsubscript{2.1} and Ca\textsubscript{2.2} channels to affect the influx of Ca\textsuperscript{2+} into the nerve terminal. This results in the Ca\textsuperscript{2+}-dependent exocytosis of neurotransmitter release into the synaptic cleft but can also activate other Ca\textsuperscript{2+}-dependent processes including the CICR mechanism. By stimulating ryanodine receptors (RyRs) on the smooth endoplasmic reticulum this mechanism is able to increase the cytosolic Ca\textsuperscript{2+} concentration and thereby possibly enhance the Ca\textsuperscript{2+}-dependent processes. Studies discussed earlier in this thesis have shown that pharmacological inhibition of RyR using
Ryanodine can result in the inhibition of the CICR mechanism. However, physiologically, one way to modulate the CICR mechanism would be to control the influx of Ca\(^{2+}\) through VDCCs upon stimulation. Reduction of Ca\(^{2+}\) influx through VDCCs would reduce the stimulation of RyR thereby decrease Ca\(^{2+}\) release from the smooth endoplasmic reticulum.

In order to decrease the Ca\(^{2+}\) influx during stimulation we considered the presynaptic GPCRs that couple to \(G_{i/o}\) proteins and thereby negatively modulate VDCCs. One of the GPCRs involved in this type of modulation is the GABA\(_B\) receptor. GABA\(_B\) receptors are stimulated by \(\gamma\)-aminobutyric acid (GABA) and are found on presynaptic nerve terminals (Perkinton & Sihra, 1998). Studies have shown that stimulation of GABA\(_B\) receptors using baclofen results in the inhibition of glutamate release because they are able to inhibit both Ca\(_v2.1\) and Ca\(_v2.2\) channels that are involved in exocytic release (Marchetti et al., 1991; Wang et al., 2004b). Furthermore, a study has shown that different GABA\(_B\) receptor subtypes could be involved to affect the various VDCCs to different extents (Guyon & Leresche, 1995). Similarly, after GPCRs that can negatively modulate VDCCs include the group III mGluRs (comprising mGluR4, mGluR6, mGluR7 and mGluR8 subtypes), 5-HT\(_{1A}\) and A\(_1\) receptors all of which are coupled to \(G_{i/o}\) proteins. In addition, they are all found on the presynaptic nerve terminals where they negatively modulate neurotransmitter release (Wang et al., 2002; Wang & Sihra, 2003).

The mechanism by which GABA\(_B\), group III mGluRs, 5-HT\(_{1A}\) and A\(_1\) receptors are able to negatively modulate the VDCCs is thought to be through membrane delimited regulation of the \(\alpha\) subunit of the VDCCs by \(\beta\gamma\) subunit of the G-protein (Kajikawa et al., 1995).
This is based on evidence showing that prevention of the activation of the G proteins results in the occlusion of their inhibitory effects (Takahashi et al., 1998).

The objective of this chapter was to determine whether negative modulation of VDCC activity through GABAergic, group III mGluRs, 5-HT and adenosine receptor stimulation inhibits Ca^{2+}-dependent processes such as ERK1 and ERK2 phosphorylation/activation. Further we asked whether the activation of these GPCRs could also modulate VDCCs following conditions through depletion of the smooth endoplasmic reticulum in which only the external Ca^{2+} influx was able to phosphorylate/activate ERK1 and ERK2.
6.2. Method

6.2.1. Synaptosomal Preparation

Preparation of synaptosomes has been described in section 2.1.

6.2.2. SDS-PAGE and Immunoblotting

All samples obtained through Immunoblotting experiments underwent the procedure described in section 2.2.

6.2.3. Intracellular Ca^{2+} store repletion protocol

Experiments that followed the intracellular Ca^{2+} store protocol are described in section 2.3.2. Synaptosomes were resuspended using the HBM buffer containing BSA (1mg/ml) and Ca^{2+} (1mM) with thapsigargin (1µM) if necessary for the experiment and incubated at 37ºC. Synaptosomes were then spun down after 50 minutes of incubation with the supernatant removed and the pellet was put on ice. At 67 minute time point from the start of the incubation synaptosomes are resuspended the second time with HBM containing BSA (1mg/ml) and Ca^{2+} (1mM) and incubated at 37ºC. The drug of interest adenosine (1µM), baclofen (100µM), 8-OH-DPAT (10µM) or L-AP4 (100µM) was added to the suspension after 2 minutes. The synaptosomes were then stimulated with 4-AP (1mM) at 72 minutes from the start of the incubation for 5 minutes. The experiment was then terminated using the STOP solution.

6.2.4. Statistical analysis

The methods of statistical analysis have been described in section 2.6.1. For data with more than two sets analysis of variance (ANOVA) was used to assess the statistical significance, followed by Dunnetts post hoc test. The net change in ERK1 and ERK2 phosphorylation/activation was calculated by removing the basal percentage from the
stimulated groups for each n. We used Student’s unpaired t-test to assess the statistical significance between two sets of data.

### 6.2.5. Reagents

A stock solution of 4-AP (1mM) was made in water and working solution was further diluted using HBM.

A stock solution of thapsigargin (1µM) was made in DMSO and working solution was further diluted using HBM.

A stock solution of CHA (1µM) was made in water and working solution was further diluted using HBM.

A stock solution of baclofen (100µM) was made in DMSO and working solution was further diluted using HBM.

A stock solution of 8-OH-DPAT (10µM) was made in water and working solution was further diluted using HBM.

A stock solution of L-AP4 (1µM) was made in water and working solution was further diluted using HBM.
6.3. Results

We first investigated the involvement of GABA_B receptors in modulating nerve terminal excitability and Ca^{2+} influx and thereby affect ERK1 and ERK2 phosphorylation/activation mediated by 4-AP stimulation. Using baclofen (100µM) to stimulate GABA_B receptors in the presence of Ca^{2+} (1mM), we assessed its effect on 4-AP (1mM) mediated stimulation (Figure 6-1A&B). In the absence of GABA_B receptor stimulation, we found that 4-AP mediated stimulation resulted in a significant increase in ERK1 (157.5 ± 6.3%) and ERK2 (156.2 ± 4.9%) phosphorylation/activation compared to the 37ºC control (Figure 6-1C). In the presence of baclofen synaptosomes stimulated with 4-AP showed a reduced but significant increase in ERK1 (137.0 ± 6.3%) and ERK2 (131.4 ± 5.2%) phosphorylation/activation compared to the 37ºC control. The basal activity of ERK1 (104.3 ± 3.3%) and ERK2 (95.0 ± 2.4%) compared to the 37ºC control was unaffected by baclofen treatment. The net change analysis revealed a significant inhibition of 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation by GABA_B receptor activation (Figure 6-1D).

We next investigated whether the inhibitory effect of GABA_B receptor activation was affected by the Ca^{2+} depletion of the depleted smooth endoplasmic reticulum Ca^{2+} stores. We used a protocol in which the synaptosomes were incubated with thapsigargin (1µM) in the presence of Ca^{2+} (1mM) during the preincubation stage, after which the synaptosomes were incubated with or without baclofen (100µM) in the presence of Ca^{2+} (Figure 6-2A&B). 4-AP mediated stimulation with thapsigargin incubation, as expected, caused a significant increase in ERK1 (138.2 ± 3.6%) and ERK2 (137.5 ± 9.7%) phosphorylation/activation compared to the 37ºC control in the absence of baclofen treatment (Figure 6-2C). This 4-AP mediated stimulation in the presence of thapsigargin response was still reproducible in the presence of baclofen as
Figure 6-1: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of baclofen

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control and B = baclofen shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension in the presence of Ca²⁺ (1mM) was used to resuspended synaptosomes and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca²⁺ (1mM) and baclofen (100µM) was added at 69 minutes followed by 4-AP (1mM) addition; the experiment ended after 77 minutes of incubation. (C) Basal effect of baclofen during the stimulation stage and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of baclofen. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
ERK1 (148.2 ± 9.2%) and ERK2 (156.0 ± 11.5%) phosphorylation/activation was significantly increased. The preincubation of thapsigargin and baclofen stimulation had no significant effect on the basal activity of ERK1 (106.1 ± 4.5%) and ERK2 (102.1 ± 4.7%) compared to the 37°C control. From the net change of ERK1 and ERK2 phosphorylation/activation we found that, surprisingly there is no inhibitory effect of GABA\(_B\) stimulation under a condition where Ca\(^{2+}\) from smooth endoplasmic reticulum is presumably depleted (Figure 6-2D).

Other presynaptic receptors that could negatively modulate VDCC activity include those constituting group III mGluRs. We hypothesised that if group III mGluRs stimulation results in a reduction in ERK1 and ERK2 phosphorylation/activation it could indicate reduction of VDCC activity. In the presence of Ca\(^{2+}\) (1mM) we used L-AP4 (100µM) an agonist of group III mGluRs to inhibit VDCCs during 4-AP mediated stimulation (Figure 6-3A&B). As expected we found that in the absence of L-AP4 during the stimulation stage there was a significant increase in ERK1 (162.9 ± 9.7%) and ERK2 (155.2 ± 8.5%) phosphorylation/activation compared to the 37°C control mediated by 4-AP stimulation (Figure 6-3C). In the presence of L-AP4, 4-AP mediated stimulation still resulted in a significant increase in ERK1 (148.4 ± 11.9%) and ERK2 (145.4 ± 8.8%) phosphorylation/activation when compared to the 37°C control. The incubation of L-AP4 had no significant effect on the basal activity of ERK1 (119.2 ± 5.4%) and ERK2 (112.7 ± 12.1%) compared to the 37°C control. The net change analysis revealed that there is a significant decrease in ERK1 and ERK2 phosphorylation/activation when the synaptosomes were incubated with L-AP4 mediated by 4-AP stimulation (Figure 6-3D).

Again we next looked at whether the L-AP4 was effected by depletion of Ca\(^{2+}\) from the
Figure 6.2: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation prior incubation with thapsigargin in the presence and absence of baclofen.

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, BT = baclofen and thapsigargin shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without thapsigargin (1µM) in the presence of Ca^{2+} (1mM) incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca^{2+} (1mM) and baclofen (100µM) was added at 69 minutes followed by 4-AP (1mM) addition; the experiment ended after 77 minutes of incubation. (C) Basal effect of thapsigargin and baclofen during the preincubation and stimulation stage, respectively and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control with prior thapsigargin incubation during the preincubation stage in the presence and absence of baclofen. Thapsi = thapsigargin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. *p<0.01(**) *p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
Figure 6-3: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of L-AP4

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control and L = L-AP4 shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension in the presence of Ca²⁺ (1mM) was used to resuspended synaptosomes and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca²⁺ (1mM) and L-AP4 (100µM) was added at 69 minutes followed by 4-AP (1mM) addition; the experiment ended after 77 minutes of incubation. (C) Basal effect of L-AP4 during the stimulation stage and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of L-AP4. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
smooth endoplasmic reticulum. Thus thapsigargin (1µM) in the presence Ca^{2+} (1mM) was added during the preincubation stage and synaptosomes in the presence and absence of L-AP4 (100µM) were stimulated with 4-AP (1mM) during the stimulation stage (Figure 6-4A&B). Preincubation with thapsigargin gives reduced responses but 4-AP mediated stimulation was still able to significantly increase ERK1 (139.9 ± 8.0%) and ERK2 (134.2 ± 9.7%) phosphorylation/activation compared to the 37°C control in the absence of L-AP4 stimulation (Figure 6-4C). Nevertheless when L-AP4 was used for stimulating group III mGluRs we found that there was significant increase in ERK1 (141.9 ± 5.6%) and ERK2 (150.3 ± 6.6%) phosphorylation/activation compared to the 37°C control by 4-AP mediated stimulation. The combination of thapsigargin preincubation and L-AP4 stimulation of group III mGluRs had no effect on the basal activity of ERK1 (110.1 ± 8.1%) and ERK2 (101.5 ± 5.7%) compared to the 37°C control. We compared the net change of ERK1 and ERK2 phosphorylation/activation between 4-AP stimulation in the presence and absence of L-AP4 with prior thapsigargin incubation. We found that there was no significant difference between the two sets of data. Thus it would appear that thapsigargin incubation during the preincubation has occluded the inhibitory effect of L-AP4 (Figure 6-4D).

We continued the investigation into GPCR that negatively modulate VDCCs by targeting the 5-HT_{1A} receptors. We used the agonist 8-OH DPAT to stimulate the 5-HT_{1A} receptors and to see if the reduction in VDCC activity correlated with a reduction in ERK1 and ERK2 phosphorylation/activation. The protocol was similar to one used previously in which after the repletion of intracellular Ca^{2+} stores synaptosomes were stimulated with 4-AP (1mM) in the presence and absence of 8-OH DPAT (10µM) during the stimulation stage (Figure 6-5A&B). 4-AP mediated stimulation caused a significant increase in ERK1 (171.8 ± 5.6%) and ERK2 (162.1 ± 7.0%)
Figure 6-4: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation prior incubation with thapsigargin in the presence and absence of L-AP4

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control, LT = L-AP4 and thapsigargin shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without thapsigargin (1µM) in the presence of Ca2+ (1mM) was used to resuspended synaptosomes and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca2+ (1mM) and L-AP4 (100µM) was added at 69 minutes followed by 4-AP (1mM) addition; the experiment ended after 77 minutes of incubation. (C) Basal effect of thapsigargin and L-AP4 during the preincubation and stimulation stage, respectively and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control with prior thapsigargin incubation during the preincubation stage in the presence and absence of L-AP4. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**), p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
phosphorylation/activation compared to the 37°C control in the absence of 8-OH DPAT (Figure 6-5C). Consistent with previous results with inhibitory GPCR agonists the presence of 8-OH DPAT also resulted in a reduced increase in ERK1 (137.7 ± 7.2%) and ERK2 (124.3 ± 4.8%) phosphorylation/activation compared to 37°C control. There was no significant effect on the basal activity of ERK1 (102.8 ± 4.9%) and ERK2 (96.4 ± 4.9%) compared to 37°C control with 8-OH DPAT incubation. Considering the net change in ERK1 and ERK2 phosphorylation/activation we found that 4-AP mediated stimulation was significantly inhibited in the presence of 8-OH DPAT (Figure 6-5D).

To assess if the 8-OH DPAT inhibitory effect on VDCCs was also abrogated by the depletion of smooth endoplasmic reticulum as seen before, we conducted the experiment under the same protocol. Synaptosomes were incubated with thapsigargin (1µM) in the presence of Ca²⁺ (1mM) and stimulated using 4-AP (1mM) with or without 8-OH DPAT (10µM) during the stimulation stage (Figure 6-6A&B). We found that with prior incubation with thapsigargin and in the absence of 8-OH DPAT there was a significant increase in ERK1 (139.9 ± 8.0%) and ERK2 (134.2 ± 9.7%) phosphorylation/activation compared to the 37°C control with 4-AP mediated stimulation (Figure 6-6C). 4-AP mediated stimulation of ERK1 (135.5 ± 10.1%) and ERK2 (156.3 ± 7.6%) phosphorylation/activation compared to the 37°C control showed a diminished increase in the presence of 8-OH DPAT. 8-OH DPAT incubation during the stimulation stage had no significant effect on the basal activity of ERK1 (106.3 ± 4.0%) and ERK2 (104.4 ± 5.0%) compared to the 37°C control. The net change of ERK1 and ERK2 phosphorylation/activation again shows that with prior thapsigargin incubation 8-OH DPAT mediated inhibition does not affect 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation (Figure 6-6D).
Figure 6-5: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of 8-OH DPAT

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control and D = 8-OH DPAT shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension in the presence of Ca²⁺ (1mM) was used to resuspended synaptosomes and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca²⁺ (1mM) and 8-OH DPAT (10µM) was added at 69 minutes followed by 4-AP (1mM) addition; the experiment ended after 77 minutes of incubation. (C) Basal effect of 8-OH DPAT during the stimulation stage and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of 8- OH DPAT. DPAT = 8-OH DPAT (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
Figure 6-6: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation prior incubation with thapsigargin in the presence and absence of 8-OH DPAT

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control, DT = 8 -OH DPAT and thapsigargin shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without thapsigargin (1µM) in the presence of Ca²⁺ (1mM) for 50 minutes. They were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca²⁺ (1mM) and 8 -OH DPAT (10 µM) was added at 69 minutes followed by 4-AP (1mM) addition; the experiment ended after 77 minutes of incubation. (C) Basal effect of thapsigargin and 8 -OH DPAT during the preincubation and stimulation stage, respectively and 4 -AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37 ºC control with prior thapsigargin incubation during the preincubation stage in the presence and absence of 8-OH DPAT. Thapsi = thapsigargin. DPAT = 8-OH DPAT. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
The last of receptor type that we tested for their ability to negatively modulate VDCCs was the adenosine A₁ receptor. To look at the involvement of A₁ receptors aforementioned we used CHA, a selective agonist for A₁ receptors. Using the same protocol synaptosomes were incubated with Ca²⁺ (1mM) and in the presence and absence of CHA (1µM) and then stimulated with 4-AP (1mM) during the stimulation stage (Figure 6-7A&B). We found that 4-AP mediated stimulation caused a significant increase in ERK1 (151.7 ± 6.0%) and ERK2 (141.9 ± 8.8%) phosphorylation/activation compared to the 37°C control in the absence of CHA (Figure 6-7C). ERK1 (136.2 ± 4.6%) and ERK2 (124.7 ± 7.1%) phosphorylation/activation compared to the 37°C control showed a reduced increase after 4-AP mediated stimulation in the presence of CHA. CHA stimulation had no significant effect on the basal activity of ERK1 (107.0 ± 2.6%) and ERK2 (105.9 ± 0.7%) compared to the 37°C control. The net change in ERK1 and ERK2 phosphorylation/activation confirmed that there is a significant inhibition of 4-AP mediated stimulation in the presence of CHA (Figure 6-7D).

Finally, we investigated if A₁ receptor mediated negative modulation of VDCCs was also mitigated by depleted smooth endoplasmic reticulum. Thapsigargin (1µM) was incubated to deplete the smooth endoplasmic reticulum in the presence of Ca²⁺ (1mM) followed by 4-AP (1mM) mediated stimulation in the presence and absence of CHA (1µM) during the stimulation stage (Figure 6-8A&B). Prior incubation with thapsigargin but absence of CHA resulted in a significant increase of ERK1 (138.5 ± 4.7%) and ERK2 (137.5 ± 9.7%) phosphorylation/activation compared to the 37°C control during 4-AP mediated stimulation (Figure 6-8C). In the presence of CHA there was also a significant but reduced increase in ERK1 (135.6 ± 5.0%) and ERK2 (135.0 ± 6.7%) phosphorylation/activation compared to the 37°C control in incubations with thapsigargin and stimulated. Incubation with thapsigargin and subsequent incubation
Figure 6-7: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of CHA
(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control and C = CHA shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension in the presence of Ca²⁺ (1mM) was used to resuspended synaptosomes and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca²⁺ (1mM) and CHA (1 µM) was added at 69 minutes followed by 4-AP (1mM) addition; the experiment ended after 77 minutes of incubation. (C) Basal effect of CHA during the stimulation stage and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence and absence of CHA. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
Figure 6-8: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation prior incubation with thapsigargin in the presence and absence of CHA

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, CT = CHA and thapsigargin shows the condition of each lane. (B) Timeline of the experiment showing that HBM suspension with or without thapsigargin (1µM) in the presence of Ca²⁺ (1mM) and incubated for 50 minutes. After which they were spun down with the supernatant removed. During the stimulation stage synaptosomes were resuspended in Ca²⁺ (1mM) and CHA (1µM) was added at 69 minutes followed by 4-AP (1mM) addition; the experiment ended after 77 minutes of incubation.

(C) Basal effect of thapsigargin and CHA during the preincubation and stimulation stage, respectively and 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control with prior thapsigargin incubation during the preincubation stage in the presence and absence of CHA. Thapsi = thapsigargin

(D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
with CHA had no significant effect on the basal activity of ERK1 (102.2 ± 4.3%) and ERK2 (97.1 ± 4.2%) compared to the 37°C control. Consistently with the previous observations the net change in ERK1 and ERK2 phosphorylation/activation showed that there was no significant difference with 4-AP mediation stimulation during CHA incubation (Figure 6-8D). Thus, interestingly, when taking into account all four inhibitory receptors treated, we found that there was a consistent effect on ERK1 and ERK2 phosphorylation/activation in both conditions. All the receptors reduced 4-AP mediated stimulation of ERK1 and ERK2 responses presumably through VDCC inhibition but were all unable to modulate Ca^{2+} influx when the smooth endoplasmic reticulum Ca^{2+} stores were depleted.
6.4. Discussion

In this chapter we wanted to negatively modulate the CICR mechanism by reducing the influx of Ca\(^{2+}\) through VDCCs during 4-AP mediated stimulation. We have hypothesised from the results described in Figure 4-1 that there are two sources of Ca\(^{2+}\) that can phosphorylate/activate ERK1 and ERK2. The first source is external Ca\(^{2+}\) entry through VDCCs and the second source are Ca\(^{2+}\) stores in the smooth endoplasmic reticulum. However, the smooth endoplasmic reticulum behaviour is dependent on the concentration of Ca\(^{2+}\) inside the lumen of the store. Repletion of the smooth endoplasmic reticulum causes the efflux of Ca\(^{2+}\) during 4-AP mediated stimulation. Under conditions in which the smooth endoplasmic reticulum is depleted of Ca\(^{2+}\) it may act as sequester of Ca\(^{2+}\) thereby reducing the cytosolic concentration and downstream phosphorylation/activation of ERK1 and ERK2.

In order to negatively modulate the VDCCs we stimulated presynaptic GPCRs that couple to G\(_{i/o}\) proteins, these including GABA\(_B\), group III mGluRs, 5-HT\(_{1A}\) and A\(_1\) receptors. The first objective of the chapter was to investigate the negative modulation of VDCCs by presynaptic GPCRs during intrasynaptosomal Ca\(^{2+}\) repletion conditions. We found that stimulation of GABA\(_B\), group III mGluRs, 5-HT\(_{1A}\) and A\(_1\) receptors resulted in an inhibition of ERK1 and ERK2 phosphorylation/activation. It is likely that this reduction is due to the inhibition of the VDCCs but could also result from a decreased CICR from the smooth endoplasmic reticulum compounding the inhibition of ERK1 and ERK2 phosphorylation/activation (Schematic 6-1).

It is likely that the mechanism for the inhibition of VDCCs is that by the GPCRs is through membrane delimited modulation mediated by the \(\beta\gamma\) subunit of G\(_{i/o}\) proteins. Interestingly, the inhibitory effects of all the receptors were very similar in that they
inhibited the ERK1 and ERK2 phosphorylation/activation to similar extents. Thus, it was not the case that one completely inhibited ERK1 and ERK2 phosphorylation/activation while another only partially inhibited the response. This could perhaps confirm the similarity of these receptors in acting through a similar mechanism.

The final objective of this chapter was to investigate negative modulation of VDCC by GPCRs after depletion of smooth endoplasmic reticulum during preincubation. This would not only eliminate any potential CICR but should also cause the store to sequester Ca\(^{2+}\) from the cytosol. Thus we hypothesised that since there is no Ca\(^{2+}\) release from the smooth endoplasmic reticulum then only the external Ca\(^{2+}\) influx would stimulate the Ca\(^{2+}\)-dependent mechanisms of ERK1 and ERK2 phosphorylation/activation. Therefore, stimulation of the inhibitory GPCRs should then produce an inhibition of Ca\(^{2+}\)-influx mediated by 4-AP and thereby reflect this in reduced ERK1 and ERK2 phosphorylation/activation. However, unexpectedly we found that GABA\(_B\), group III mGluRs, 5-HT\(_{1A}\) and A\(_1\) receptor stimulation did not significantly modulate VDCC activity following depletion of intrasynaptosomal Ca\(^{2+}\) store. This suggests that the depletion of the smooth endoplasmic reticulum through an unknown mechanism is able to occlude or uncouple the inhibitory effects of GABA\(_B\), group III mGluRs, 5-HT\(_{1A}\) and A\(_1\) receptors that were observed in Ca\(^{2+}\) store repletion conditions.

Previous studies have shown that GABA\(_B\) inhibition of exocytosis through reduction of Ca\(^{2+}\) influx through VDCC can be reversed by the stimulation of protein kinase C (PKC) in nerve terminals (Taniyama et al., 1992; Perkinton & Sihra, 1998). It is likely that the depletion of the smooth endoplasmic reticulum Ca\(^{2+}\) stores during the
preincubation stage perhaps initially results in an increase in cytosolic Ca\(^{2+}\) concentration. Over the time period of the preincubation stage in which Ca\(^{2+}\) concentration is high it could possibly result in the stimulation of phospholipase C (PLC) that could metabolise PIP\(_2\) to form IP\(_3\) and diacylglycerol (DAG). The latter could then be sufficient for PKC stimulation and thus uncouple the GPCR inhibitory activity on VDCCs during the stimulation stage. The result in Figure 4-3 supports this mechanism as it was shown that ERK1 and ERK2 phosphorylation/activation mediated by 4-AP stimulation was enhanced by thapsigargin incubation during preincubation and stimulation. However, to better support this mechanism we could directly inhibit PKC under depleted smooth endoplasmic reticulum condition and observe if the GPCR stimulation is now able to once again inhibit ERK1 and ERK2 phosphorylation/activation. If the GPCR are able to inhibit ERK1 and ERK2 phosphorylation/activation under depleted smooth endoplasmic reticulum conditions then this supports a role for PKC as indicated above. Furthermore, we should conduct a time course of thapsigargin incubation over the time period of the preincubation stage and observe if the basal activity of ERK1 and ERK2 is enhanced which might again suggest high Ca\(^{2+}\) concentrations during the thapsigargin incubation.

A more remote but tenable possibility is that smooth endoplasmic reticulum is able to occlude/uncouple GABA\(_B\), group III mGluRs, 5-HT\(_{1A}\) and A\(_1\) receptor activity by an unknown secondary messenger. That is mobilised as a consequence of Ca\(^{2+}\) store depletion and would be akin to the paradigm thought to consist when depletion of Ca\(^{2+}\) stores operate so called store operated Ca\(^{2+}\)-channels. In the current context however such a mechanism remains a matter of speculation.
In conclusion, we found that stimulation of GABA_B, group III mGluRs, 5-HT_1A and A_1 receptors under smooth endoplasmic reticulum repletion results in the inhibition of ERK1 and ERK2 phosphorylation/activation mediated by 4-AP stimulation. The mechanism for this inhibition is most likely through negative modulation of VDCC via the membrane delimited pathway. Curiously, under conditions in which the smooth endoplasmic reticulum is depleted prior to stimulation with 4-AP, the inhibitory effect of GABA_B, 5-HT_1A, group III mGluRs and A_1 receptors on VDCCs is abrogated. Although the mechanism underlying this effect are unclear the implication is that Ca^{2+} loss of intrasynaptosomal stores indicating antagonises the negative modulation of external Ca^{2+}-influx.
Schematic 6-1: Inhibitory GPCRs negative modulation of VDCCs is abrogated by prior depletion of intracellular Ca\(^{2+}\) stores

4-AP mediated depolarisation of the plasma membrane leads to the influx of Ca\(^{2+}\) through VDCC. Influx of Ca\(^{2+}\) can cause further efflux of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores and both sources of stimulate Ca\(^{2+}\)-dependent mechanisms that activate the ERK pathway. (i) Stimulation of inhibitory GPCRs can result in the inhibition of VDCC during 4-AP mediated depolarisation resulting in the reduction of Ca\(^{2+}\) influx and subsequent reduction in ERK pathway activation. (ii) Prior depletion of intracellular Ca\(^{2+}\) store through SERCA pump inhibition oclloes this inhibition perhaps through PKC activation.
Chapter 7
7. Mitochondria and acidic stores

**Summary:** We have investigated the utility of mitochondria and acidic stores as storage compartments of intrasynaptosomal Ca\(^{2+}\). Using the ERK1 and ERK2 phosphorylation/activation as a sensor for increases in intrasynaptosomal Ca\(^{2+}\) concentration, we observed mitochondria and acidic stores contribution to Ca\(^{2+}\)-dependent mechanisms. We found that disruption of mitochondrial function with FCCP and oligomycin caused a transient increase in ERK1 and ERK2 phosphorylation/activation. In addition, inhibition of the SERCA pump enhances the transient effect on ERK1 and ERK2 phosphorylation/activation indicating that the Ca\(^{2+}\) released from the mitochondria in this condition is being sequestered by the smooth endoplasmic reticulum. Furthermore, we also sought to investigate the role of mitochondria during plasma membrane depolarisation mediated by 4-AP. Notably, the disruption of mitochondrial membrane potential resulted in inhibition of ERK1 and ERK2 phosphorylation/activation when the synaptosomes were stimulated with 4-AP. Finally inhibiting Ca\(^{2+}\) accumulation into acidic stores resulted in the decrease of ERK1 and ERK2 phosphorylation/activation indicating their involvement in maintenance of some component of the intrasynaptosomal Ca\(^{2+}\) store. Overall, we found that release of Ca\(^{2+}\) from mitochondria in presynaptic nerve terminals results in ERK1 and ERK2 phosphorylation/activation. In addition, 4-AP mediated stimulation might be sufficient in triggering this release through an unknown mechanism.

7.1. Introduction

We examined the involvement of intracellular Ca\(^{2+}\) stores other than the smooth endoplasmic reticulum in 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation. The two intracellular Ca\(^{2+}\) stores that have been implicated in previous studies include mitochondria and acidic stores.
Mitochondria are found in presynaptic nerve terminals and are described as structures that include the outer mitochondrial membrane, the intermembrane space, the inner mitochondrial membrane, the crista space, and the matrix. There are two important functions that have been identified from studies that have looked at mitochondria. Firstly they are essential in generating mostly all of the ATP that is used in the cell by oxidative phosphorylation. Secondly, there is considerable evidence that mitochondria also have an important function in Ca\(^{2+}\) homeostasis (Malli & Graier, 2010).

The Ca\(^{2+}\) storage function of mitochondria is thought to occur mainly through the MCU and non-MCU that are found in the inner mitochondrial membrane and are also referred to as mCa1 and mCa2, respectively. Both mCa1 and mCa2 are highly selective for Ca\(^{2+}\) over Na\(^{+}\) or K\(^{+}\) and are inwardly rectifying though the mCa2 current is different and has smaller amplitude compared to the mCa1 (Michels et al., 2009). The mCa1 and mCa2 are activated by significant increases in Ca\(^{2+}\) concentrations which are higher than those achieved under normal physiological stimulation conditions. Rather, the mCa1 and mCa2 channels are likely activated by the Ca\(^{2+}\) microdomains created at the mouth of IP\(_3\)Rs found on the smooth endoplasmic reticulum (Rizzuto et al., 1993) (Nassar & Simpson, 2000). The ability of mCa1 and mCa2 to take up Ca\(^{2+}\) can be inhibited by the acidification of the inner membrane which can be achieved through a high exposure to cytosolic Ca\(^{2+}\) or through pharmacological means using protonophore such as FCCP (Moreau & Parekh, 2008).

Efflux of Ca\(^{2+}\) from mitochondria occurs through Na\(^{+}\)-dependent and Na\(^{+}\)-independent mechanisms and is less well characterised. The Na\(^{+}\)-dependent mechanism involves the Na\(^{+}\)/Ca\(^{2+}\) exchanger which are found on the inner mitochondrial membrane and uptakes three Na\(^{+}\) into the lumen in exchange for efflux of one Ca\(^{2+}\) from the mitochondrial
lumen (Palty et al., 2010). Interestingly, in conditions such as low cytosolic Ca\(^{2+}\) concentrations the Na\(^+\)/Ca\(^{2+}\) exchanger can uptake Ca\(^{2+}\). The Na\(^+\)-independent mechanism is thought to involve the H\(^+\)/Ca\(^{2+}\) exchanger which are also found on the inner mitochondrial membrane. The H\(^+\)/Ca\(^{2+}\) exchanger are thought to exchange one H\(^+\) for one Ca\(^{2+}\) in conditions in which Ca\(^{2+}\) concentration is high in the lumen of the mitochondria (Jiang et al., 2009; Santo-Domingo & Demaurex, 2010). In addition, Ca\(^{2+}\) efflux for both exchangers can be sequestered by the smooth endoplasmic reticulum through the SERCA pump activity. This shows the connectivity between two putative intracellular Ca\(^{2+}\) stores.

The other type of potential Ca\(^{2+}\) store are the acidic stores which is based on the role of nicotinic acid adenine dinucleotide phosphate (NAADP) which is thought to be involved in intracellular Ca\(^{2+}\) signalling. NAADP stimulates NAADP receptors found on acidic stores such as lysosomes and other organelles (Zong et al., 2009). There are two pathways by which Ca\(^{2+}\) enters the lumen of the acidic stores. Firstly the Ca\(^{2+}\)-ATPase pump uses ATP to actively accumulate Ca\(^{2+}\) into the store. Secondly, the large concentration gradient of H\(^+\) ions can be used by a Ca\(^{2+}\)/H\(^+\) exchanger to efflux H\(^+\) ions in exchange for Ca\(^{2+}\) into the lumen of the acidic store. The Ca\(^{2+}\) released from acidic stores could then potentially act on ryanodine receptors (RyRs) to promote further Ca\(^{2+}\) release. It may also be sequestered by the smooth endoplasmic reticulum through the SERCA pump thereby increasing the concentration of Ca\(^{2+}\) in the lumen of that store such that subsequent stimulation of smooth endoplasmic reticulum could produce larger than expected responses (Churchill & Galione, 2001).

The objective of this chapter is to identify the effect of release of mitochondrial Ca\(^{2+}\) release on ERK1 and ERK2 phosphorylation/activation. We sought to investigate if the
mitochondrial Ca\(^{2+}\) has a functional role during 4-AP mediated stimulation and identify
the contribution of Ca\(^{2+}\) from acidic stores into the Ca\(^{2+}\)-dependent mechanism of the
ERK1 and ERK2 phosphorylation/stimulation.
7.2. Method

7.2.1. Synaptosomal Preparation

Preparation of synaptosomes has been described in section 2.1.

7.2.2. SDS-PAGE and Immunoblotting

All samples obtained through Immunoblotting experiments underwent the procedure described in section 2.2.

7.2.3. Intracellular Ca\textsuperscript{2+} store repletion protocol

Experiments that followed the intracellular Ca\textsuperscript{2+} store protocol are described in section 2.3.2. Synaptosomes were resuspended in HBM buffer containing BSA (1mg/ml) with thapsigargin (1\mu M) if necessary for the experiment and the suspension was incubated at 37\degree C. After 3 minutes Ca\textsuperscript{2+} (1mM) or EGTA (100\mu M) was to the suspension. Synaptosomes were then spun down at 50 minutes and with the supernatant removed the synaptosomes were put on ice. At 67 minutes from the start of the incubation the synaptosomes were resuspended with HBM buffer containing BSA (1mg/ml) and Ca\textsuperscript{2+} (1mM) with thapsigargin (1\mu M) if it is necessary for the experiment. After 70 minutes bafilomycin A1 (1\mu M), 4-AP (1mM) and/or FCCP (1\mu M) & oligomycin (1\mu g/ml) is added to the suspension at concentrations and at the times stated. The experiment was terminated using a STOP solution.

7.2.4. Statistical analysis

The methods of statistical analysis have been described in section 2.6.1. For data with more than two sets analysis of variance (ANOVA) was used to assess the statistical significance, followed by Dunnetts post hoc test. The net change in ERK1 and ERK2 phosphorylation/activation was calculated by removing the basal percentage from the
stimulated groups for each n. We used Student’s unpaired t-test to assess the statistical significance between two sets of data.

### 7.2.5. Reagents

A stock solution of 4-AP (1mM) was made in water and working solution was further diluted using HBM.

A stock solution of FCCP (1µM) & oligomycin (1µg/ml) was made in DMSO and water, respectively and working solution was further diluted using HBM.

A stock solution of EGTA (100µM) was obtained with water and working solution was further diluted using HBM.

A stock solution of Bafilomycin A1 (1µM) was obtained with DMSO and working solution was further diluted using HBM.

A stock solution of thapsigargin (1µM) was made in DMSO and working solution was further diluted using HBM.
7.3. Results
Ca\(^{2+}\)-stimulated intrasynaptosomal Ca\(^{2+}\) stores evidently support ERK1 and ERK2 phosphorylation/activation. Other than smooth endoplasmic reticulum, there are other Ca\(^{2+}\) stores that may contribute to presynaptic signalling. The first ‘alternative’ Ca\(^{2+}\) stores that could be involved in Ca\(^{2+}\) release are the mitochondria. There is significant evidence to suggest that Ca\(^{2+}\) can accumulate in the lumen of mitochondria driven by oxidative phosphorylation. Combination use of a protonophore (FCCP) and an ATP synthase inhibitor (oligomycin) can be employed to pharmacologically depolarise mitochondria and thereby release mitochondrial Ca\(^{2+}\). Furthermore, the use of FCCP and oligomycin will subsequently prevent mitochondria from accumulating Ca\(^{2+}\).

To observe the effect of FCCP and oligomycin incubation on ERK1 and ERK2 basal activity we first conducted a time course. Synaptosomes were incubated in the presence of Ca\(^{2+}\) (1mM) during the preincubation stage to ensure the repletion of intracellular Ca\(^{2+}\) stores and incubated with FCCP (1µM) & oligomycin (1µg/ml) for 1, 2, 5 and 10 minutes (Figure 7-1A&B). We found that there was a significant increase in ERK1 (121.2 ± 3.8%) and ERK2 (122.8 ± 4.6%) phosphorylation/activation compared to the 37°C control after 1 minute FCCP and oligomycin mediated stimulation (Figure 7-1C). ERK1 (116.1 ± 3.9%) phosphorylation/activation was also significantly increased after 2 minutes of FCCP and oligomycin mediated stimulation but ERK2 (109.5 ± 2.2%) phosphorylation/activation compared to the 37°C control was not significantly different. After 5 minutes of FCCP and oligomycin mediated stimulation phosphorylation/activation of ERK1 (120.8 ± 3.3%) and ERK2 (115.7 ± 4.6%) compared to 37°C control were significantly increased. After 10 minutes FCCP and oligomycin incubation declined to control levels ERK1 (111.1 ± 3.2%) and ERK2 (98.1 ± 4.4%) phosphorylation/activation compared to the 37°C control. Thus, we found that
Figure 7-1: FCCP & oligomycin stimulation effect on ERK1 and ERK2 phosphorylation/activation after 1, 2, 5 and 10 minutes

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of Ca²⁺ (1mM) for 50 minutes. After which they were spun down with supernatant removed and resuspended again in the presence of Ca²⁺ (1mM). FCCP and oligomycin was used to disrupt the mitochondria and the incubation was stopped at various time points. (C) This is the time course of FCCP (1 µM) and oligomycin (1 µg/ml) effect on ERK1 and ERK2 phosphorylation/activation at time points that include 1, 2, 5 and 10 minutes. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=8).
FCCP and oligomycin stimulation of ERK1 and ERK2 phosphorylation/activation was a transient effect with the maximum effect produced at 1 minute and declining thereafter over 10 minutes. In subsequent experiments, we have used the 1 and 10 minutes incubations with FCCP and oligomycin for different paradigms. The 1 minute time point represents the maximum effect of FCCP and oligomycin stimulation while the 10 minutes time point represents the minimum point of the stimulatory effect of FCCP and oligomycin stimulation. While stimulatory activity no longer persists but the mitochondria will be depolarised and therefore presumably unable to sequester Ca^{2+}.

We next investigated if the mitochondrial Ca^{2+} stores are depleted during the synaptosomal preparation and require repletion during the preincubation stage as observed with the smooth endoplasmic reticulum. Thus, we incubated the synaptosomes in the absence of Ca^{2+} with EGTA (100µM) to chelate any residual Ca^{2+} during the preincubation stage and stimulated with FCCP (1µM) and oligomycin (1µg/ml) in the presence of Ca^{2+} (1mM) for 1 minute (Figure 7-2A&B). We found, that in the presence of Ca^{2+} there was a significant increase in ERK1 (122.9 ± 4.3%) and ERK2 (131.3 ± 4.7%) phosphorylation/activation compared to the 37°C control with FCCP and oligomycin mediated stimulation (Figure 7-2C). Unexpectedly, in the absence of Ca^{2+} FCCP and oligomycin mediated stimulation still resulted in a significant increase in ERK1 (125.8 ± 5.7%) and ERK2 (119.6 ± 5.9%) phosphorylation/activation compared to the 37°C control. There was no significant effect on ERK1 (92.7 ± 8.9%) and ERK2 (89.7 ± 5.2%) basal activity compared to the 37°C control in the absence of Ca^{2+} during the preincubation stage. The net change of ERK1 and ERK2 phosphorylation/activation shows that there is no significant difference between the presence and absence of Ca^{2+} during the preincubation stage (Figure 7-2D). Overall, these results suggest that mitochondria are not affected by the presence of Ca^{2+} during the preincubation stage.
Figure 7-2: FCCP and oligomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation for 1 minute in the presence and absence of Ca\textsuperscript{2+} during the preincubation stage

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37\degree C control, E = EGTA and F&O = FCCP & oligomycin show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of Ca\textsuperscript{2+} (1mM) or EGTA (100\mu M) for 50 minutes. After which they were spun down with supernatant removed and resuspended again in the presence of Ca\textsuperscript{2+} (1mM), FCCP (1\mu M) and oligomycin (1\mu g/ml) was used to disrupt the mitochondria for 1 minute and the incubation was stopped at 80 minutes. (C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37\degree C control induced by FCCP and oligomycin in the presence and absence of EGTA during the preincubation stage. F&O = FCCP and oligomycin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean \pm SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
and that the mitochondrial Ca\(^{2+}\) stores are unaffected during the synaptosomal preparation.

The mechanism behind the decline in ERK1 and ERK2 phosphorylation/activation after 10 minutes of stimulation with FCCP and oligomycin was of interest. One possibility is that the smooth endoplasmic reticulum, through the SERCA pump re-uptakes the Ca\(^{2+}\) released by mitochondria thereby reducing the stimulation of the ERK pathway. To examine this possibility, we used thapsigargin to inhibit the SERCA pump which should result in an enhancement of ERK1 and ERK2 phosphorylation/activation if the SERCA pumps are indeed sequestering Ca\(^{2+}\). Thapsigargin (1µM) in the presence of Ca\(^{2+}\) (1mM) was incubated throughout to ensure continued inhibition of the SERCA pumps and stimulated with FCCP (1µM) and oligomycin (1µg/ml) for 1 minute (Figure 7-3A&B). FCCP and oligomycin mediated stimulation for 1 minute resulted in a significant increase in ERK1 (123.0 ± 3.4%) and ERK2 (114.8 ± 2.8%) phosphorylation/activation compared to the 37ºC control in the absence of thapsigargin (Figure 7-3C). In the presence of thapsigargin, FCCP and oligomycin mediated stimulation for 1 minute still resulted in a significant increase in ERK1 (117.0 ± 3.6%) but ERK2 (99.6 ± 4.8%) phosphorylation/activation. This could be due to the unexpected significant inhibitory effect on the basal activity of ERK2 (78.7 ± 2.0%) by thapsigargin incubation, but ERK1 (93.1 ± 5.5%) basal activity compared to the 37ºC control was unaffected. Thus overall, we found that the net change in ERK1 and ERK2 phosphorylation/activation with FCCP and oligomycin stimulation for 1 minute was not significantly different with and without thapsigargin incubation (Figure 7-3D).

Although, the 1 minute FCCP and oligomycin stimulation of synaptosomes was not enhanced by thapsigargin incubation we considered whether an enhancement effect
Figure 7-3: FCCP & oligomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation for 1 minute in the presence and absence of thapsigargin

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control, T = thapsigargin and F&O = FCCP & oligomycin show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of Ca2+ (1mM) with thapsigargin (1µM) for 50 minutes. After which they were spun down with supernatant removed and resuspended again in the presence of Ca2+ (1mM) with thapsigargin (1µM). FCCP (1µM) and oligomycin (1µg/ml) was used to disrupt the mitochondria for 1 minute and the incubation was stopped at 80 minutes. (C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37º C control induced by FCCP and oligomycin in the presence and absence of thapsigargin during the preincubation and stimulation stage. Thapsi = thapsigargin. F&O = FCCP and oligomycin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
could be revealed with 10 minute stimulation. We followed a similar protocol in which thapsigargin (1µM) was incubated continuously in the presence of Ca²⁺ (1mM) but were stimulated with FCCP (1µM) and oligomycin (1µg/ml) for 10 minutes (Figure 7-4A&B). As predicted from previous results, FCCP and oligomycin mediated stimulation for had no significant effect on ERK1 (112.2 ± 4.9%) and ERK2 (97.4 ± 5.4%) phosphorylation/activation compared to the 37°C control in the absence of thapsigargin incubation (Figure 7-4C). But incubation with thapsigargin before FCCP and oligomycin mediated stimulation showed a significant increase of ERK1 (120.7 ± 3.0%) but had no effect on ERK2 (100.4 ± 8.5%) phosphorylation/activation compared to the 37°C control. The presence of thapsigargin did not have a significant effect on the basal activity of ERK1 (94.2 ± 4.3%) but significantly reduced ERK2 (78.7 ± 2.0%) phosphorylation/activation compared to the 37°C control. The net change shows that thapsigargin incubation significantly increases ERK1 and ERK2 phosphorylation/activation stimulated by FCCP and oligomycin for 10 minutes (Figure 7-4D). Overall these results suggest that the SERCA pump found on the smooth endoplasmic reticulum is able to remove the Ca²⁺ released from the mitochondria with time. This underpins the ‘transient’ nature of FCCP and oligomycin stimulation seen in Figure 7-1.

We have shown that the Ca²⁺ released from the mitochondrial stores after FCCP and oligomycin mediated stimulation can phosphorylate/activate ERK1 and ERK2. We next questioned whether this Ca²⁺ release is able to potentiate the 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation which could occur in cases of mitochondrial dysfunction. The protocol we used involved incubation with FCCP (1µM) and oligomycin (1µg/ml) for 1 minute in the presence of Ca²⁺ (1mM) followed by 4-AP (1mM) mediated stimulation (Figure 7-5A&B). We found that in the absence
Figure 7-4: FCCP & oligomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation for 10 minute in the presence and absence of thapsigargin

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control, T = thapsigargin and F&O = FCCP & oligomycin show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of Ca²⁺ (1mM) with thapsigargin (1µM) for 50 minutes. After which they were spun down with supernatant removed and resuspended again in the presence of Ca²⁺ (1mM) with thapsigargin (1µM). FCCP (1µM) and oligomycin (1µg/ml) was used to disrupt the mitochondria for 10 minute and the incubation was stopped at 80 minutes. (C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37 ºC control induced by FCCP and oligomycin in the presence and absence of thapsigargin during the preincubation and stimulation stage. Thapsi = thapsigargin. F&O = FCCP and oligomycin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
of FCCP and oligomycin stimulation there was significant increase in ERK1 (157.3 ± 7.8%) and ERK2 (161.4 ± 6.9%) phosphorylation/activation compared to the 37°C control mediated by 4-AP stimulation (Figure 7-5C). When synaptosomes were incubated with FCCP and oligomycin for 1 minute followed by 4-AP mediated stimulation we found that 4-AP stimulation still resulted in significant increase of ERK1 (152.0 ± 7.7%) and ERK2 (148.8 ± 7.9%) phosphorylation/activation compared to the 37°C control. As expected FCCP and oligomycin mediated stimulation significantly increased ERK1 (126.2 ± 4.3%) and ERK2 (133.2 ± 5.5%) phosphorylation/activation compared to the 37°C control. When this effect of FCCP and oligomycin is taken into account we found in the net change of ERK1 and ERK2 phosphorylation/activation stimulated with 4-AP is significantly inhibited by prior stimulation with FCCP and oligomycin (Figure 7-5D).

We further examined if the 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation could be inhibited by prior stimulation with FCCP and oligomycin stimulation for 10 minutes. We followed the same protocol but synaptosomes were stimulated with FCCP (1µM) and oligomycin (1µg/ml) for 10 minutes in the presence of Ca²⁺ (1mM) prior to 4-AP (1mM) mediated stimulation (Figure 7-6A&B). As previously shown 4-AP mediated stimulation significantly increased ERK1 (141.6 ± 2.5%) and ERK2 (159.0 ± 8.5%) phosphorylation/activation compared to the 37°C control in the absence of FCCP and oligomycin stimulation (Figure 7-6C). Furthermore, 4-AP mediated stimulation was still able to significantly increase ERK1 (128.4 ± 2.7%) and ERK2 (148.8 ± 4.6%) phosphorylation/activation compared to the 37°C control with prior FCCP and oligomycin stimulation. By itself FCCP and oligomycin mediated stimulation for 10 minutes did not significantly affect ERK1 (110.4 ± 6.0%) phosphorylation/activation but surprisingly did significantly
Figure 7-5: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation with or without prior stimulation with FCCP & oligomycin for 1 minute

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control and F&O = FCCP & oligomycin show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of Ca²⁺ (1mM) for 50 minutes. After which they were spun down with supernatant removed and resuspended again in the presence of Ca²⁺ (1mM) and stimulated with or without FCCP (1 µM) and oligomycin (1 µg/ml) for 1 minute after which they were stimulated with 4-AP (1mM) and the incubation was stopped at 85 minutes. (C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37º C control induced by FCCP and oligomycin in the presence and absence of thapsigargin during the preincubation and stimulation stage. F&O = FCCP and oligomycin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(***), p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
increase ERK2 (114.9 ± 3.5%) phosphorylation/activation compared to the 37ºC control. The net change in ERK1 and ERK2 phosphorylation/activation highlighted that 4-AP mediated stimulation is significantly inhibited by prior stimulation with FCCP and oligomycin for 10 minutes (Figure 7-6D). Overall, we found that FCCP and oligomycin incubation with synaptosomes for 1 minute and 10 minutes resulted in a significant reduction of ERK1 and ERK2 phosphorylation/activation mediated by 4-AP stimulation.

Another type of Ca^{2+} containing compartment store that we tested for its presence and capability of stimulating ERK1 and ERK2 phosphorylation/activation was acidic stores constituting either NAADP sensitive stores or indeed secretory vesicles. Acidic stores are able to store Ca^{2+} through the Ca^{2+}/H^{+} exchanger by which Ca^{2+} enters into the lumen of the store in exchange for an H^{+}. The H^{+} transported into the lumen of the store maintains this exchanger by virtue of vacuolar type H^{+-}ATPases. Therefore inhibiting the accumulation of H^{+} should prevent the Ca^{2+}/H^{+} exchanger for exchanging the Ca^{2+} for H^{+} and result in an increase in cytosolic Ca^{2+} and/or depletion of these stores.

To test for the presence of acidic stores we used bafilomycin A1 which inhibits vacuolar H^{+-}ATPases and should prevent Ca^{2+} accumulation into any acidic stores. We conducted a time course of bafilomycin A1 (1µM) incubation for 1, 5, 20 or 40 minutes in the presence of Ca^{2+} (1mM) measuring its effect on ERK1 and ERK2 phosphorylation/activation (Figure 7-7A&B). We found that 1 minute incubation with bafilomycin A1 had no significant effect on ERK1 (100.7 ± 1.1%) and ERK2 (106.1 ± 3.2%) phosphorylation/activation compared to the 37ºC control (Figure 7-7C). But after 5 minutes there was a significant decrease in ERK1 (90.8 ± 1.2%) phosphorylation/activation compared to the 37ºC control but the effect on ERK2 (93.9 ±
Figure 7-6: 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation with or without prior stimulation with FCCP & oligomycin for 10 minutes

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control and F&O = FCCP & oligomycin show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of Ca²⁺ (1mM) for 50 minutes. After which they were spun down with supernatant removed and resuspended again in the presence of Ca²⁺ (1mM) and stimulated with or without FCCP (1 µM) and oligomycin (1µg/ml) for 10 minutes after which they were stimulated with 4-AP (1mM) and the incubation was stopped at 85 minutes. (C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37°C control induced by FCCP and oligomycin in the presence and absence of thapsigargin during the preincubation and stimulation stage. F&O = FCCP and oligomycin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
3.1%) was not statistically significant. We had a similar effect after 20 minutes of bafilomycin A1 incubation at which there was a significant decrease in ERK1 (84.3 ± 1.7%) but not ERK2 (90.0 ± 4.2%) phosphorylation/activation compared to the 37°C control. However, after 40 minutes both ERK1 (83.7 ± 2.5%) and ERK2 (86.5 ± 3.4%) phosphorylation/activation compared to the 37°C control were significantly reduced with bafilomycin A1 incubation. Overall, bafilomycin A1 caused a significant decrease in ERK1 basal activity after 5 minutes and both ERK1 and ERK2 were significantly reduced after 40 minutes. This indicates the presence of acidic Ca^{2+} stores and which could play a role in the maintenance of ERK1 and ERK2 phosphorylation/activation and other Ca^{2+}-dependent mechanisms.
Figure 7-7: Bafilomycin effect on ERK1 and ERK2 phosphorylation/activation after 1, 5, 20 and 40 minutes of stimulation

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control show the condition of each lane. (B) Timeline of the experiment showing that Ca²⁺ (1mM) was added at 3 minutes followed HBM or bafilomycin A1 (1µM) stimulation after which the experiment was ended at various time points that include 51, 55, 70 and 90 minutes. (C) Time course of bafilomycin A1 incubation at 1, 5, 20 and 40 minutes of ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control in the presence of Ca²⁺. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
7.4. Discussion

The first objective of this chapter was to investigate whether the release of Ca\textsuperscript{2+} from mitochondria is able to stimulate Ca\textsuperscript{2+}-dependent mechanisms that result in ERK1 and ERK2 phosphorylation/activation. Using a combination of FCCP (protonophore) and oligomycin (ATP synthesis inhibitor) we depolarised mitochondria in synaptosomes to invoke the release of accumulated Ca\textsuperscript{2+}. The time course following treatment showed that FCCP and oligomycin has a stimulatory effect on ERK1 and ERK2 phosphorylation/activation. However, this effect is transient with the maximum response at 1 minute followed by steadily diminishing response such that at the 10 minute, ERK1 and ERK2 phosphorylation/activation was not significantly different compared to the 37°C control. Together these data show that mitochondrial Ca\textsuperscript{2+} can phosphorylate/activate ERK1 and ERK2 and obviates the suggestion that the inhibition of ATP production leads to a reduction in available substrate for protein phosphorylation (Schematic 7-1).

The Ca\textsuperscript{2+}-dependent mechanisms that could be activated by mitochondrial Ca\textsuperscript{2+} also need to be investigated and could involve the Src-dependent mechanism that has already been shown to be important in 4-AP and ionomycin mediated stimulation. Other Ca\textsuperscript{2+}-dependent mechanisms that could be involved include calmodulin activation. Calmodulin could have stimulatory effects on ERK1 and ERK2 phosphorylation/activation as previously described but some studies have indeed pointed to calmodulin stimulating Ca\textsuperscript{2+} uptake through mCa1 (Moreau et al., 2006). This function is unlikely to occur here as the mCa1 channel is driven by the negative membrane potential which in this case is disrupted by FCCP.
The second objective of this chapter was to investigate the functional role of mitochondria. As we have provided evidence that Ca\(^{2+}\) is indeed stored in mitochondria, it was interesting to examine whether the synaptosomal preparation depletes mitochondria similarly to smooth endoplasmic reticulum stores. If the mitochondria are not depleted than the absence of Ca\(^{2+}\) should not be significantly affect ERK1 and ERK2 phosphorylation/activation mediated by FCCP and oligomycin stimulation. We found that the absence of Ca\(^{2+}\) during the preincubation stage did not affect FCCP and oligomycin mediated stimulation. This indicates that unlike the smooth endoplasmic reticulum which has been shown to require repletion in previous chapters, mitochondria are unaffected by the synaptosomal preparation. The conclusion from this would be that unlike the smooth endoplasmic reticulum Ca\(^{2+}\) does not ‘leak’ out of the mitochondria thus they retain the Ca\(^{2+}\) better than the smooth endoplasmic reticulum.

We also investigated the functional interaction of mitochondria and the smooth endoplasmic reticulum. In previous studies it has been suggested that there is a close functional interactivity between mitochondria and the smooth endoplasmic reticulum which serves, for example, to activate mCa1 channels by the high concentrations of Ca\(^{2+}\) at the mouth of the IP\(_3\)R found on the smooth endoplasmic reticulum. Another possible interaction that could serve to modulate the function of both Ca\(^{2+}\) stores is re-uptake of Ca\(^{2+}\) by SERCA pumps found on the smooth endoplasmic reticulum when Ca\(^{2+}\) is released from the mitochondria. We considered the latter as a possible reason for the mitochondrial release of Ca\(^{2+}\) mediated by FCCP and oligomycin stimulation being transient. This proved to be correct as we found that ERK1 and ERK2 phosphorylation/activation mediated by FCCP and oligomycin stimulation for 10 minutes was enhanced by SERCA pump inhibition. This occurs because by inhibiting the reuptake the released Ca\(^{2+}\) is able to maintain the stimulation of the Ca\(^{2+}\)-dependent
mechanisms of ERK1 and ERK2 phosphorylation/activation. Interestingly, SERCA pump inhibition was unable to enhance ERK1 and ERK2 phosphorylation/activation of FCCP and oligomycin mediated incubated for 1 minute. This may indicate that SERCA pump activity requires some time before it significantly abrogates the release of mitochondrial Ca$^{2+}$.

We also considered whether the Ca$^{2+}$ released by mitochondria could contribute to the Ca$^{2+}$-dependent mechanisms activated by 4-AP mediated stimulation. If 4-AP mediated stimulation induces Ca$^{2+}$ release from mitochondria, then prior release of Ca$^{2+}$ using FCCP and oligomycin mediated stimulation should inhibit ERK1 and ERK2 phosphorylation/activation. However, if mitochondrial Ca$^{2+}$ release is unaffected by 4-AP mediated stimulation, the combined increase in Ca$^{2+}$ concentration might contribute to potentiate ERK1 and ERK2 phosphorylation/activation. We found the former to be the case as 4-AP mediated stimulation was significantly inhibited by prior stimulation of mitochondria with FCCP and oligomycin. Interestingly, the inhibitory effects on ERK1 and ERK2 phosphorylation/activation are observed at both the 1 and 10 minute time points. At the 10 minute time point the Ca$^{2+}$ released by FCCP and oligomycin mediated stimulation should be removed by SERCA pumps as already shown. The inhibition is maintained likely due to the continued depletion as FCCP is preventing Ca$^{2+}$ accumulation in the mitochondria.

The mechanism of Ca$^{2+}$ release by mitochondria mediated by 4-AP stimulation needs further investigation, but it could involve the Na$^+$/Ca$^{2+}$ exchanger found on the inner mitochondrial membrane. 4-AP mediated stimulation causes an increase in Ca$^{2+}$ concentration, but also during stimulation, Na$^+$ channels are opened which significantly increases the intracellular concentration of Na$^+$ (Galvan & Sitges, 2004). This increase
in Na\(^+\) concentration could promote Ca\(^{2+}\) release through the Na\(^+\)/Ca\(^{2+}\) exchanger found on the mitochondria. There are studies that have provided evidence of Ca\(^{2+}\) release via the Na\(^+\)/Ca\(^{2+}\) exchanger in motor nerve terminals which promotes transmitter release (Tsang et al., 2000; Yang et al., 2003). However, the data from the 4-AP mediated stimulation in the absence of extracellular Ca\(^{2+}\) shows lack of significant increase in ERK1 and ERK2 phosphorylation/activation which would dispute this mechanism. In the absence of external Ca\(^{2+}\) the Na\(^+\) concentration should still be significant increased to cause an effect on the Na\(^+\)/Ca\(^{2+}\) exchanger to release Ca\(^{2+}\) from the mitochondria. Nevertheless, this mechanism could be further investigated by observing the effect of Na\(^+\)/Ca\(^{2+}\) exchanger inhibition on 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation. Furthermore, ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation does not increase Na\(^+\) concentration. Thus, ionomycin mediated stimulation should be enhanced by prior stimulation with FCCP and oligomycin using the protocol described here for 4-AP.

The final objective of this chapter was to establish any contribution that acidic stores might make to Ca\(^{2+}\)-dependent mechanisms in the synaptosome. Surprisingly, we found that depletion of acidic stores resulted in the decrease of ERK1 and ERK2 phosphorylation/activation. This suggests that acidic stores in synaptosomes contribute to Ca\(^{2+}\)-dependent mechanisms by tonically activating them. The exact mechanism for this observed inhibition requires further investigation. It is plausible that tonic NAADP activity on acidic stores could be releasing Ca\(^{2+}\) that maintains ERK1 and ERK2 basal activity. The Ca\(^{2+}\) released from acidic stores might also stimulate RyR on the smooth endoplasmic reticulum which also acts to maintain the ERK1 and ERK2 basal activity. It would be very interesting to examine whether the Ca\(^{2+}\) released from acidic stores is sufficient to phosphorylate/activate ERK1 and ERK2 when the acidic stores are
stimulated by NAADP. Furthermore, the stimulatory effect of cADPR in the synaptosomal model would also be of interest as cADPR has been implicated in stimulation of RyR through the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) mechanism. These compartments await the development of cell-permeable analogues of these ligands.

In conclusion, both mitochondria and acidic stores may contribute to intrasynaptosomal Ca\textsuperscript{2+} as indicated by effect on ERK1 and ERK2 phosphorylation/activation. Mitochondrial Ca\textsuperscript{2+} stores are revealed here to be resistant to depletion during the synaptosomal preparation. In addition, the observations that SERCA pumps reuptake Ca\textsuperscript{2+} released from the mitochondria indicates a close proximity of the two Ca\textsuperscript{2+} stores in presynaptic nerve terminals. Most importantly, there is evidence to suggest that mitochondria could be one of the potential Ca\textsuperscript{2+} stores that might contribute to the Ca\textsuperscript{2+}-dependent mechanisms such as ERK1 and ERK2 phosphorylation/activation instigated upon 4-AP mediated stimulation.
Schematic 7-1: Mitochondrial and acidic store effect on the activation of the ERK pathway

4-AP mediated depolarisation results in Ca\(^{2+}\) influx through VDCCs and Na\(^{+}\) influx through voltage-dependent sodium channels. (i) Ca\(^{2+}\) influx can activate the CICR mechanism causing a further release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores and both sources of Ca\(^{2+}\) can stimulate CaM and possibly Pyk2 leading to the activation of the ERK pathway. (ii) Increases in [Na\(^{+}\)] can cause the efflux of Ca\(^{2+}\) from mitochondria which stimulate the ERK pathway through CaM and possibly Pyk2 activation. The efflux of Ca\(^{2+}\) from mitochondria is subsequently sequestered by SERCA pumps. (iii) Acidic stores can sequester cytoplasmic Ca\(^{2+}\) which could be released through unknown stimulus to tonically activate RyR to efflux Ca\(^{2+}\) and maintain the basal activity of ERK1 and ERK2.
Chapter 8
8. Pathophysiological role of intracellular Ca\(^{2+}\) stores

**Summary:** Given that intracellular Ca\(^{2+}\) stores have been shown to be contributing to Ca\(^{2+}\)-dependent processes and maintaining Ca\(^{2+}\) homeostasis, we considered the function of intracellular Ca\(^{2+}\) stores in an ischemia model using nerve terminals. The main consequence of ischemia is a decrease in ATP production which we have mimicked by inhibiting the Na\(^+\)/K\(^+\)-ATPase pump using ouabain. We show that inhibition of the Na\(^+\)/K\(^+\)-ATPase pump results in a significant increase in ERK1 and ERK2 phosphorylation/activation that is a transient and dose-dependent. Further investigation confirmed that this increase in ERK1 and ERK2 phosphorylation/activation is a Ca\(^{2+}\)-dependent process. We also found that the smooth endoplasmic reticulum partially releases Ca\(^{2+}\) which contributes to the Ca\(^{2+}\)-dependent mechanisms. The increase of Ca\(^{2+}\) concentration activates the Ca\(^{2+}\)-dependent mechanisms for ERK1 and ERK2 phosphorylation/activation which have been shown in previous chapters to include calmodulin activation and a Src-dependent mechanism. Ouabain also resulted in the increase in the spontaneous glutamate release but did not facilitate 4-AP evoked glutamate release. Using this pathophysiological model we suggest the intracellular Ca\(^{2+}\) stores especially smooth endoplasmic reticulum contributes to Ca\(^{2+}\)-dependent mechanisms that result in ERK1 and ERK2 phosphorylation/activation.

8.1. Introduction

Under physiological conditions the intracellular Ca\(^{2+}\) concentration is maintained low however in pathophysiological conditions, there is a failure of Ca\(^{2+}\) homeostasis systems which results in high intracellular Ca\(^{2+}\) concentrations and ultimately necrosis. We have so far considered the functional contribution of intracellular Ca\(^{2+}\) stores to Ca\(^{2+}\)-dependent processes such as ERK1 and ERK2 phosphorylation/activation in
physiological conditions. Here we considered the mobilisation of intracellular Ca\(^{2+}\) stores in model pathophysiological condition mimicking the metabolic consequences of ischemia.

Ischemia is a condition that is caused by the restriction in blood supply to an area of the body such as the brain or heart. Restriction of the blood supply causes a drop in the availability of glucose and thus leads to a drop in ATP production which prevents ATP-dependent processes from functioning. One of the major users of ATP in the brain is the Na\(^+/K^+\) -ATPase pump in the plasma membrane and is therefore an obvious casualty during ischemia injury.

Intracellular [Na\(^+\)] is maintained low by the Na\(^+/K^+\) -ATPase pump which transports intracellular Na\(^+\) to the extracellular medium in exchange for K\(^+\) (Lingrel & Kuntzweiler, 1994). It is thus predicted that ATP depletion and thereby reduction of Na\(^+/K^+\) -ATPase activity would lead to the reduction of the Na\(^+\) gradient (Tian et al., 2001) and consequent depolarisation of the plasma membrane. This latter effect can be simulated pharmacologically by Na\(^+/K^+\) -ATPase inhibition using the glycoside ouabain. Interestingly in previous studies it has also been suggested that ouabain interaction with the Na\(^+/K^+\) -ATPase also plays a signalling role by which activation of the ERK pathway leads to the phosphorylation/activation of ERK1 and ERK2 without invoking the bio-energetic effects of Na\(^+/K^+\) -ATPase (Tian et al., 2001; Liu et al., 2003; Wang et al., 2004a; Liang et al., 2006).

There are two mechanisms by which Na\(^+/K^+\) -ATPase inhibition using ouabain could activate the ERK pathway. One of the mechanisms is that the Na\(^+/K^+\) -ATPase acts as a signal transducer which activates Src independently of the changes in Na\(^+\) and K\(^+\).
concentrations (Xie & Askari, 2002). The $\text{Na}^+/\text{K}^+$-ATPase interacts with Src and is thought to inactivate Src by making it less accessible. However ouabain binding to the $\text{Na}^+/\text{K}^+$-ATPase is thought to disrupt this interaction causing the release and activation of Src (Liang et al., 2006). The second potential mechanism does involve the changes in $\text{Na}^+$ and $\text{K}^+$ concentrations. Ouabain has been shown to reverse the concentrations of $\text{Na}^+$ and $\text{K}^+$ in nerve terminals by increasing the $\text{Na}^+$ concentration and decreasing the $\text{K}^+$ concentration, respectively (Archibald & White, 1974). This could disrupt the function of $\text{Na}^+/\text{Ca}^{2+}$ exchanger which efflux $\text{Ca}^{2+}$ in exchange for $\text{Na}^+$ into the cytosol. The increase in $\text{Na}^+$ concentration (decrease in gradient) could therefore inhibit the efflux of $\text{Ca}^{2+}$ leading to an accumulation of $\text{Ca}^{2+}$ concentration in the cytosol. Furthermore, the plasma membrane would be depolarised causing VDCCs to open and mediate further $\text{Ca}^{2+}$ influx.

It has also been reported that inhibition of the $\text{Na}^+/\text{K}^+$-ATPase using ouabain results in the excessive exocytosis of glutamate which could be expected in ischemia. This could be due to the excess $\text{Ca}^{2+}$ available to stimulate glutamate release but also the processes that promote neurotransmitter release are activated. Interestingly, there are studies that point to the recruitment of intracellular $\text{Ca}^{2+}$ stores in the ouabain mediated stimulation of glutamate release (Lomeo et al., 2003; Amaral et al., 2009).

The objective of this chapter is to establish whether inhibition of the $\text{Na}^+/\text{K}^+$-ATPase pump results in significant increases in ERK1 and ERK2 phosphorylation/activation in nerve terminals. Furthermore, we wanted to investigate if any effects on ERK1 and ERK2 is indeed utilise $\text{Ca}^{2+}$-dependent mechanisms. In addition, we asked if intracellular $\text{Ca}^{2+}$ stores contribute to these underlying mechanisms. Finally, we investigated if ouabain mediated stimulation has an effect on neurotransmitter release.
8.2. Method

8.2.1. Synaptosomal Preparation

Preparation of synaptosomes has been described in section 2.1.

8.2.2. SDS-PAGE and Immunoblotting

All samples obtained through Immunoblotting experiments underwent the procedure described in section 2.2.

8.2.3. Standard protocol

The protocol for the experiments that followed the standard protocol is described in section in 2.3.1. The synaptosomes were resuspended in HBM buffer containing BSA (1mg/ml) with drug of interest like PP2 (10µM), EGTA (100µM), Ni^{2+}, Cd^{2+} and Co^{2+} (100µM), W7 (50µM) or thapsigargin (1µM) and incubated at 37ºC. After 5 minutes Ca^{2+} (1mM) was added to the incubation followed by ouabain (100µM) unless stated at 20 minutes. Ouabain stimulated the synaptosomes for 20 minutes and the experiment terminated using a STOP solution after 15 minutes of incubation.

8.2.4. Glutamate release

Synaptosomes were resuspended in 1.5ml HBM containing BSA (1mg/ml) and transferred to the spectrofluorimeter. Constant stirring ensured that the synaptosomes were oxygenated and NADP^{+} (1mM) and GDH (50 units/ml) were added to the suspended synaptosomes at the start of the experiment. After 3 minutes Ca^{2+} (1mM) was added and HBM or ouabain (10 or 100µM) was added at 10 minutes. After incubation for 20 minutes, Ca^{2+}-dependent release was evoked by 4-AP (1mM). Finally, at 15 minutes, exogenous glutamate (2.5nmol) was added as an internal standard to quantify the released glutamate.
8.2.5. Intracellular [Ca\textsuperscript{2+}] measurement using Fura-2A

Synaptosomes (0.2mg) were re-suspended in a HBM containing BSA (1mg/ml) and incubated with Fura-2AM at 37\textdegree C for 20 minutes. After 3 minutes Ca\textsuperscript{2+} (0.1mM) was added and at the end of the incubation, synaptosomes were centrifuged for 10,000rpm for 1 minute. The supernatant was removed and the pellet was re-suspended in a HBM containing BSA (1mg/ml). The recording of the trace was started and Ca\textsuperscript{2+} (1mM) was added after 3 minutes. After 10 minutes, the control or the appropriate drug concentration was added to the synaptosome suspension and the effect of the control vehicle or drug in fluorescence measured over 20 minutes.

8.2.6. Statistical analysis

The methods of statistical analysis have been described in section 2.6.1. For data with more than two sets analysis of variance (ANOVA) was used to assess the statistical significance, followed by Dunnetts post hoc test. The net change in ERK1 and ERK2 phosphorylation/activation was calculated by removing the basal percentage from the stimulated groups for each n. We used Student’s unpaired t-test to assess the statistical significance between two sets of data. The bar graphs of glutamate release in the results section show independent experiments averaged at 300-305 second time points unless stated otherwise.

8.2.7. Reagents

A stock solution of ouabain (100\textmu M) was made using with water and further diluted using HBM.

A stock solution of EGTA (100\textmu M) was obtained with water and working solution was further diluted using HBM.
A stock solution of thapsigargin (1µM) was made in DMSO and working solution was further diluted using HBM.

A stock solution of W7 (50µM) was made using with water and further diluted using HBM.

A stock solution of PP2 (10µM) was made using with DMSO and further diluted using HBM.

A stock solution of Ni^{2+}, Cd^{2+} and Co^{2+} (100µM) was made using with water and further diluted using HBM.
8.3. Results

We investigated a potential role for intrasynaptosomal Ca$^{2+}$ stores in a pathophysiological model by using ouabain to inhibit the Na$^{+}$/K$^{+}$-ATPase pump. We first conducted a dose-response curve with ouabain at 1, 10 and 100µM and quantified the effect on ERK1 and ERK2 phosphorylation/activation (Figure 8-1A&B). We incubated ouabain concentrations (1, 10 or 100µM) for 20 minutes in the presence of Ca$^{2+}$ (1mM). Using this protocol the maximum response of ERK1 (185.4 ± 6.1%) and ERK2 (184.0 ± 6.7%) phosphorylation/activation compared to the 37ºC control was obtained at 100µM ouabain (Figure 8-1C). At 10µM ouabain also caused a significant increase in ERK1 (158.2 ± 13.5%) and ERK2 (147.5 ± 8.4%) phosphorylation/activation compared to the 37ºC control. At 1µM ouabain, we found no significant effect of Na$^{+}$/K$^{+}$-ATPase pump inhibition on ERK1 (109.9 ± 7.0%) and ERK2 (110.9 ± 3.1%) phosphorylation/activation compared to the 37ºC control. These data show that Na$^{+}$/K$^{+}$-ATPase pump inhibition results in the stimulation of ERK1 and ERK2 phosphorylation/activation.

To further elaborate the actions of ouabain on ERK1 and ERK2 phosphorylation/activation, we conducted a time course of ouabain (100 µM) stimulation in the presence of Ca$^{2+}$ (1mM) for 1, 5, 10, 20 or 40 minutes (Figure 8-2A&B). We found that 1 minute ouabain mediated stimulation had no significant effect on ERK1 (111.0 ± 7.5%) and ERK2 (107.9 ± 9.0%) phosphorylation/activation compared to the 37ºC control. However, there was a significant increase in ERK1 (176.3 ± 3.0%) and ERK2 (147.8 ± 4.2%) phosphorylation/activation compared to the 37ºC control after 5 minutes of stimulation mediated by ouabain. The increase in phosphorylation/activation compared to the 37ºC control persisting after 10 minutes (ERK1: 167.1 ± 3.4% and ERK2: 147.4 ± 8.7%) and 20 minutes (ERK1: 150.4 ± 3.4%
Figure 8-1: Ouabain dose-dependent stimulation of ERK1 and ERK2 phosphorylation/activation
(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control show the condition of each lane. (B) Timeline of the experiment shows that Ca²⁺ (1mM) were added at 3 minutes followed HBM or ouabain (1, 10 or 100 µM) stimulation after 10 minutes and the experiment ended after 30 minutes of incubation. (C) Effect of ouabain mediated stimulation at concentrations 1, 10 and 100µM of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence of Ca²⁺. All values represent the mean ± SEM. p<0.01(∗∗) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
and ERK2: 137.5 ± 11.1%) of ouabain mediated stimulation. After 40 minutes of stimulation with ouabain (ERK1 (120.1 ± 11.6%) and ERK2 (103.0 ± 7.7%) phosphorylation/activation) diminished to the 37°C control (Figure 8-2C). Overall we found that ouabain mediated stimulation over the time course resulted in a transient increase in ERK1 and ERK2 phosphorylation/activation which peaked at 5 minutes and reverting to basal levels by 40 minutes.

To investigate the involvement of Ca\textsuperscript{2+} in ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation, we hypothesised that the absence of Ca\textsuperscript{2+} should result in a decrease in ERK1 and ERK2 phosphorylation/activation. We incubated synaptosomes in the absence of Ca\textsuperscript{2+} with EGTA (100 µM) to chelate any residual external Ca\textsuperscript{2+} and stimulated with ouabain (100 µM) for 20 minutes (Figure 8-3A&B). We found that in the presence of Ca\textsuperscript{2+} there was a significant increase in ERK1 (132.3 ± 0.5%) and ERK2 (131.1 ± 1.0%) phosphorylation/activation compared to the 37°C control mediated by ouabain mediated stimulation (Figure 8-3C). However when the external Ca\textsuperscript{2+} was removed, ouabain stimulation of ERK1 (86.1 ± 10.3%) and ERK2 (85.9 ± 14.5%) phosphorylation/activation compared to the 37°C control was abrogated. Notably the basal activity of ERK1 (71.5 ± 8.1%) and ERK2 (71.1 ± 10.5%) compared to 37°C control was also significantly reduced in the absence of external Ca\textsuperscript{2+}. By looking at the net change in ERK1 and ERK2 phosphorylation/activation we found that ouabain mediated stimulation was significantly inhibited in the absence of Ca\textsuperscript{2+} but was not totally abolished (Figure 8-3D). Thus we can conclude that the presence of Ca\textsuperscript{2+} is important for the ouabain stimulation of ERK1 and ERK2 phosphorylation/activation but the inhibition was not complete.
Figure 8-2: Ouabain effect on ERK1 and ERK2 phosphorylation/activation after 1, 5, 10, 20 and 40 minutes of stimulation

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control show the condition of each lane. (B) Timeline of the experiment showing that Ca²⁺ (1mM) was added at 3 minutes followed HBM or ouabain (100µM) stimulation after which the experiment was ended at various time points that include 1 1, 15, 20, 30 and 50 minutes. (C) Time course of ouabain mediated stimulation for 1, 5, 10, 20 or 40 minutes of ERK1 and ERK2 phosphorylation/activation compared to the 37°C control in the presence of Ca²⁺. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=3).
Figure 8-3: Ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ca$^{2+}$.  
(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37ºC control, E = EGTA and Oua = ouabain show the condition of each lane.  
(B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of Ca$^{2+}$ (1mM) or EGTA (100µM) and stimulated with ouabain (100 µM) after 10 minutes and the experiment ended after 30 minutes of incubation.  
(C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37ºC control stimulated by ouabain in the presence and absence of EGTA. 
(D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
The foregoing results show that Na\(^+\)/K\(^-\)-ATPase inhibition results in ERK1 and ERK2 phosphorylation/activation. We next investigated the mechanism underlying this stimulation. One possibility is that ouabain is increasing the intrasynaptosomal Ca\(^{2+}\) concentration, either through the depolarisation of the plasma membrane and/or perhaps through Ca\(^{2+}\) released from smooth endoplasmic reticulum. To look at cytosolic [Ca\(^{2+}\)] directly, synaptosomes were loaded with Fura-2A, a fluorometric Ca\(^{2+}\) indicator in the presence of Ca\(^{2+}\) (1mM) and stimulated with 1µM, 10µM or 100µM ouabain. We show that there was no change in the intracellular Ca\(^{2+}\) concentrations at 1µM ouabain while an increase in Ca\(^{2+}\) concentrations clearly occurs at the 10 and 100µM ouabain (Figure 8-4A). This provides evidence that there are changes in Ca\(^{2+}\) concentrations following ouabain treatment and could be the trigger for the ERK1 and ERK2 phosphorylation/activation. Interestingly, there is no difference in intracellular Ca\(^{2+}\) concentrations between control and ouabain (100µM) after 5 minutes (Figure 8-4B). There is nevertheless a significant increase in ERK1 and ERK2 phosphorylation/activation (Figure 8-4C) suggesting that other (Ca\(^{2+}\)-dependent) mechanisms might also be involved in ERK activation downstream of Na\(^+\)/K\(^-\)-ATPase inhibition.

Given the increase in Ca\(^{2+}\) produced by ouabain the CICR mechanism could be activated to support the stimulation of ERK1 and ERK2 phosphorylation/activation. To confirm whether the Ca\(^{2+}\) influx stimulation of CICR produced by ouabain is triggered by external Ca\(^{2+}\) influx through VDCC, we used Ni\(^{2+}\), Cd\(^{2+}\) and Co\(^{2+}\) to block the VDCCs. Synaptosomes were first incubated with Ni\(^{2+}\), Cd\(^{2+}\) and Co\(^{2+}\) (100µM) in combination, after which they were stimulated with ouabain (100µM) in the presence of external Ca\(^{2+}\) (1mM) for 20 minutes (Figure 8-5A&B). We found that in the absence of Ni\(^{2+}\), Cd\(^{2+}\) and Co\(^{2+}\) incubation there was a significant increase in ERK1 (128.7 ± 3.8%)
Figure 8-4: Intrasyntosomal [Ca\textsuperscript{2+}] measurement with ouabain
(A) The measurement of intrasyntosomal cytosolic [Ca\textsuperscript{2+}] treated with 1, 10 and 100µM ouabain over time. The black line represents the control, the red line represents 1µM ouabain, the green line represents 10µM ouabain and the blue line represents 100µM ouabain. At time point 180 seconds Ca\textsuperscript{2+} (1mM) was added and the control and ouabain concentrations added at 600 seconds. (B) The net change in cytosolic [Ca\textsuperscript{2+}] between ouabain (100 µM) and control. (C) Ouabain (100µM) time course at 1, 5, 10, 20 and 40 minutes of ERK1 and ERK2 phosphorylation/activation. The protocol used conduct this experiment is described in section 8.2.5. Fura data (n=2), ERK1 and ERK2 phosphorylation/activation data (n=5). p<0.001(***) p<0.01(**) p<0.05 (*).
and ERK2 (132.5 ± 3.4%) phosphorylation/activation compared to the 37°C control with ouabain mediated stimulation (Figure 8-5C). In the presence of Ni²⁺, Cd²⁺ and Co²⁺ ouabain mediated stimulation of ERK1 (110.6 ± 5.8%) and ERK2 (109.1 ± 4.5%) phosphorylation/activation compared to the 37º control was reduced significantly. Ni²⁺, Cd²⁺ and Co²⁺ incubation had no significant effect on the basal activity of ERK1 (97.5 ± 6.9%) and ERK2 (87.4 ± 6.2%) compared to the 37ºC control. Thus looking at the net change in ERK1 and ERK2 phosphorylation/activation we found that there was a significant inhibition of ouabain mediated stimulation in the presence of Ni²⁺, Cd²⁺ and Co²⁺ blockade of VDCCs (Figure 8-5D).

To further investigate the nature of the ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation, we looked at the calmodulin dependence. To consider the possible role of calmodulin, we incubated the synaptosomes with W7 (50µM) in the presence of external Ca²⁺ (1mM) and stimulated with ouabain (100µM) for 20 minutes (Figure 8-6A&B). In the absence of W7 we found that there was a significant increase in ERK1 (147.7 ± 5.6%) and ERK2 (172.7 ± 3.1%) phosphorylation/activation compared to the 37°C control with ouabain mediated stimulation. In the presence of W7 ouabain mediated stimulation had a reduced effect on ERK1 (112.5 ± 6.5%) and ERK2 (108.3 ± 5.9%) phosphorylation/activation compared to the 37°C control (Figure 8-6C). Incubation of synaptosomes with W7 also significantly reduced the basal activity of ERK1 (90.9 ± 3.2%) and ERK2 (90.0 ± 5.0%) compared to the 37°C control. However, comparing the net change in ERK1 and ERK2 phosphorylation/activation after ouabain mediated stimulation we found there was a significant inhibition in synaptosomal samples incubated with W7 (Figure 8-6D). These results suggest the involvement of calmodulin in the Ca²⁺-dependent mechanisms activated by the increase in intracellular Ca²⁺ concentration produced by ouabain to
Figure 8-5: Ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of Ni²⁺, Cd²⁺ and Co²⁺

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, B = Ni²⁺, Cd²⁺ and Co²⁺ and Oua = ouabain show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of Ni²⁺, Cd²⁺ and Co²⁺ (100µM) with Ca²⁺ (1mM) added after 3 minutes. They were stimulated with ouabain (100 µM) after 10 minutes and the experiment ended after 30 minutes of incubation. (C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37°C control stimulated by ouabain in the presence and absence of Ni²⁺, Cd²⁺ and Co²⁺ incubation. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=5).
Figure 8-6: Ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of W7

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, W = W7 and Oua = ouabain show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of W7 (50µM) with Ca²⁺ (1mM) added after 3 minutes. They were stimulated with ouabain (100 µM) after 10 minutes and the experiment ended after 30 minutes of incubation. (C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37°C control stimulated by ouabain in the presence and absence of W7 incubation. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=5).
affect ERK1 and ERK2 phosphorylation/activation.

Finally, to further define the Ca\(^{2+}\)-dependent mechanisms involved in ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation looked at the effect of Src inhibitor PP2. Synaptosomes were incubated with PP2 (20\(\mu\)M) in the presence of Ca\(^{2+}\) (1mM) and stimulated with ouabain (100\(\mu\)M) for 20 minutes (Figure 8-7A&B). In the absence of PP2 treatment synaptosomes stimulated with ouabain displayed a significant increase ERK1 (174.3 ± 2.4%) and ERK2 (190.5 ± 8.6%) phosphorylation compared to the 37ºC control (Figure 8-7C). In the presence of PP2, synaptosomes stimulated with ouabain showed a diminished increase in ERK1 (121.3 ± 8.0%) but ERK2 (104.2 ± 6.8%) phosphorylation/activation compared to the 37ºC control. PP2 incubation with synaptosomes also resulted in a significant decrease in the basal activity of ERK1 (74.3 ± 5.6%) and ERK2 (64.8 ± 3.4%) compared to the 37ºC control. Notwithstanding, the analysis of the net change in ERK1 and ERK2 phosphorylation/activation showed that ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence of PP2 incubation was significantly inhibited (Figure 8-7D).

To investigate whether intracellular Ca\(^{2+}\) stores are activated by the Ca\(^{2+}\) influx in the above stimulation of ERK1 and ERK2 phosphorylation/activation we used thapsigargin to deplete the smooth endoplasmic reticulum Ca\(^{2+}\) pools. The synaptosomes were incubated with thapsigargin (1\(\mu\)M) in the presence of Ca\(^{2+}\) (1mM) for 20 minutes then stimulated with 100\(\mu\)M ouabain (Figure 8-8A&B). Ouabain mediated stimulation in the absence of thapsigargin resulted in a significant increase in ERK1 (143.6 ± 3.9%) and ERK2 (140.6 ± 2.5%) phosphorylation/activation compared to the 37ºC control (Figure 8-8C). In the presence of thapsigargin ouabain mediated stimulation resulted in a reduced increase in ERK1 (122.3 ± 5.8%) and ERK2 (123.4 ± 4.9%)
Figure 8-7: Ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of PP2

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, P = PP2 and Oua = ouabain show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of PP2 (10µM) with Ca²⁺ (1mM) added after 3 minutes. They were stimulated with ouabain (100 µM) after 10 minutes and the experiment ended after 30 minutes of incubation. (C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37°C control stimulated by ouabain in the presence and absence of PP2 incubation. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(∗∗) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
phosphorylation/activation compared to the 37ºC control. There was no significant
effect on the basal activity of ERK1 (97.3 ± 1.9%) and ERK2 (96.6 ± 4.0%) compared
to the 37ºC control. Consequently the net change shows that thapsigargin treatment
caused significant inhibition of ERK1 and ERK2 phosphorylation/activation mediated
by ouabain stimulation (Figure 8-8D). These results indicate that the smooth
endoplasmic reticulum contributes to the increase in intracellular Ca²⁺ concentration
which results in ERK1 and ERK2 phosphorylation/activation in response to Na⁷/K⁺-
ATPase inhibition.

There is evidence that ouabain is able to evoke release given the intracellular effects we
have observed with ouabain we investigated the effects on neurotransmitter release from
nerve terminals. There were two effects of ouabain that were investigated; its effect on
basal/spontaneous release and its role in neurotransmitter release evoked by 4-AP.
Synaptosomes were resuspended in HBM containing NADP⁺ (1mM) and GDH (50
units/ml) with Ca²⁺ (1mM) added at 3 minutes. Ouabain (100µM) was added at 10
minutes and 4-AP (1mM) mediated stimulation elicited at 30 minutes. Finally, at 35
minutes, exogenous glutamate (2.5nmol) was added as an internal standard to quantify
the glutamate released. There was an increase in basal/spontaneous glutamate release
when the synaptosomes were stimulated with ouabain at 100µM but not at 10µM
(Figure 8-9A&B). However, 4-AP stimulated release with prior ouabain stimulation
were not affected by the presence of ouabain (Figure 8-9C&D).
Figure 8-8: Ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation in the presence and absence of thapsigargin

(A) Autoradiograph of phosphorylated ERK1 and ERK2 shown as p44 and p42, respectively labels Con = 37°C control, T = thapsigargin and Oua = ouabain show the condition of each lane. (B) Timeline of the experiment showing that synaptosomes were resuspended in the presence of thapsigargin (1µM) with Ca^{2+} (1mM) added after 3 minutes. They were stimulated with ouabain (100 µM) after 20 minutes and the experiment ended after 50 minutes of incubation. (C) The change in ERK1 and ERK2 phosphorylation/activation compared to the 37°C control stimulated by ouabain in the presence and absence of thapsigargin incubation. Thapsi = thapsigargin. (D) The net change of ERK1 and ERK2 phosphorylation/activation which is calculated by removing the basal percentage from the stimulated groups for each n. All values represent the mean ± SEM. p<0.01(**) p<0.05 (*). The procedure in which the ERK1 and ERK2 proteins in the sample were immunoblotted is described in section 2.2. (n=4).
Figure 8-9: The basal/spontaneous and 4-AP stimulated release with ouabain incubation
Synaptosomes were re-suspended in HBM medium containing BSA (1mg/ml) NADP⁺ (1mM) and GDH (50units/ml). After 3 minutes Ca²⁺ (1mM) was added and ouabain (10 or 100µM) was added at 10 minutes followed by 4-AP (1mM) addition at 30 minutes and then at 35 minutes glutamate (2.5nmol) is added as an internal standard. (A) A trace of the basal effect of 10 and 100µM ouabain incubation on glutamate release. (B) Quantification of the basal/spontaneous glutamate release averaged at 1135-1140 seconds. (C) A trace of 4-AP stimulated glutamate release with and without prior incubation of 10 and 100µM ouabain. (D) Quantification of the 4-AP mediated glutamate release averaged at 300-305 seconds. (n=1).
8.4. Discussion

Under physiological conditions we found that intracellular Ca\(^{2+}\) stores in some way contribute to the maintenance and activation of Ca\(^{2+}\)-dependent mechanism leading to the ERK1 and ERK2 phosphorylation/activation. In this chapter we investigated whether the smooth endoplasmic reticulum in pathophysiological model could contribute to rises in Ca\(^{2+}\) that might precipitate excitotoxic consequences.

The pathophysiological mechanism that we wanted to mimic was ischemia which occurs in vivo after restrictions of blood supply to organs including the brain. The restriction of blood supply eventually results in necrosis through a decline in ATP production, rise in intracellular Ca\(^{2+}\) concentration and cell swelling. The pathophysiological model that we formulated does not actually reduce ATP production but rather simulates the consequences of a decline in ATP production that occurs in ischemia. The major user of ATP in the brain is the Na\(^+\)/K\(^+\)-ATPase pump which hydrolyses ATP to transport three intracellular Na\(^+\) into the extracellular medium in exchange for a two K\(^+\). A block of the Na\(^+\)/K\(^+\)-ATPase pump would occur as ATP production declines during ischemia. This can be mimicked by inhibiting the Na\(^+\)/K\(^+\)-ATPase pump pharmacologically using ouabain. This is supported by the reported observation that ouabain inhibition of the Na\(^+\)/K\(^+\)-ATPase pump results in an increase in intracellular Ca\(^{2+}\) concentration which would also occur during ischemia (Tian et al., 2001).

The first objective of the chapter was to investigate if inhibition of the Na\(^+\)/K\(^+\)-ATPase pump by ouabain causes the phosphorylation/activation of ERK1 and ERK2. The dose-dependence curve shows that there was a significant increase in ERK1 and ERK2 phosphorylation/activation at the 10 and 100µM ouabain. We also conducted a time
course of Na\(^+\)/K\(^+\)-ATPase pump inhibition using the 100µM concentration of ouabain. This time course showed a peak of ERK1 and ERK2 phosphorylation/activation at 5 minutes which gradually declines over the time course. Therefore, the effect of Na\(^+\)/K\(^+\)-ATPase pump inhibition on ERK1 and ERK2 phosphorylation/activation appears to be slow and transient. With reference to its transient effect it is unlikely that the inhibition of Na\(^+\)/K\(^+\)-ATPase pump induced by ouabain is reversed in the condition. Rather it is more plausible that despite continuing Na\(^+\)/K\(^+\)-ATPase pump inhibition a secondary mechanism is activated over the time course which reverses ERK1 and ERK2 phosphorylation/activation possibly by dephosphorylation of the kinases.

The next objective of this chapter was to investigate the underlying mechanisms for ERK1 and ERK2 phosphorylation/activation involved by the inhibition of the Na\(^+\)/K\(^+\)-ATPase pump (Schematic 8-1). The primary mechanism we investigated was the effect of Ca\(^{2+}\) and using Fura-2A we showed an increase in the intrasynaptosomal Ca\(^{2+}\) concentration occurring after ouabain treatment. We found that in a slight dose dependent manner Na\(^+\)/K\(^+\)-ATPase pump inhibition resulted in increase in intrasynaptosomal Ca\(^{2+}\) over time. The increase in Ca\(^{2+}\) concentration however does not fully correlate with the increase in ERK1 and ERK2 phosphorylation/activation. They are two observations that highlight this lack of correlation. Firstly, the difference in Ca\(^{2+}\) concentration (control and 100µM ouabain) at the 1 and 5 minute time points were not significantly different, yet ERK1/2 phosphorylation is significantly enhanced between the 1 and 5 minute time points. This shows that at the peak of ERK1/2 phosphorylation/activation is not just due to increases in cytosolic Ca\(^{2+}\) concentration suggesting that another mechanism could be involved at the 5 minute time point. Secondly, although the increase in Ca\(^{2+}\) concentration continues to occur over time, ERK1 and ERK2 phosphorylation/activation tended to decline over the same period of
time. This could suggest the involvement of phosphatase activity as increases in Ca$^{2+}$ concentration occur. In the future, it would be important to test this possibility by pharmacologically inhibiting phosphatases such as PP2A or PP2B and observing if this enhances the ERK1 and ERK2 phosphorylation/activation by allowing persistent activation. Overall, we conclude that Ca$^{2+}$ does influence the ERK1 and ERK2 phosphorylation/activation but there are other mechanisms that have a stimulatory effect on the kinase in a Ca$^{2+}$-independent manner. Those latter affects may relate to the Src dependent effect reported (Tian et al., 2001).

To investigate the mechanisms involved in the phosphorylation/activation of ERK1 and ERK2 induced by the inhibition of the Na$^+$/K$^+$-ATPase pump, we directly addressed the involvement of Ca$^{2+}$ in the ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation. Thus we incubated the synaptosomes in the absence of Ca$^{2+}$ and found that this protocol resulted in a significant inhibition of ERK1 and ERK2 phosphorylation/activation mediated by the Na$^+$/K$^+$-ATPase pump inhibition. Furthermore, the absence of Ca$^{2+}$ resulted in the reduction in the basal activity of ERK1 and ERK2 suggesting that the previous presence of Ca$^{2+}$ in the incubation maintains ERK1 and ERK2 activity.

We considered whether the influx of Ca$^{2+}$ through VDCC activity could be activating the smooth endoplasmic reticulum to release Ca$^{2+}$ or the increase in intrasynaptosomal Ca$^{2+}$ concentration activates the smooth endoplasmic reticulum. So we blocked the VDCC channels using Ni$^{2+}$, Cd$^{2+}$ and Co$^{2+}$ and found in doing so that Na$^+$/K$^+$-ATPase pump inhibition mediated stimulation of ERK1 and ERK2 phosphorylation/activation was significantly inhibited. This indicates that there may be a degree of depolarisation of the membrane by the inhibition of the Na$^+$/K$^+$-ATPase pump which leads to the
influx of Ca\(^{2+}\) which is sufficient for simulating further release of Ca\(^{2+}\) from the smooth endoplasmic reticulum. However, there could also be another possibility given that Ni\(^{2+}\), Cd\(^{2+}\) and Co\(^{2+}\) incubation also inhibits the basal activity of ERK1 and ERK2 indicating that Ca\(^{2+}\) influx occurs through VDCCs under basal conditions. Thus it may be possible that blocking the VDCCs reduces the repletion of the intracellular Ca\(^{2+}\) stores thus limiting the subsequent release of Ca\(^{2+}\) when they are stimulated by other means.

The final objective of this chapter was to investigate the Ca\(^{2+}\)-dependent mechanisms that could be activated by the increase in intracellular Ca\(^{2+}\) concentration. Given that Src activation occurs during Na\(^{+}/K^{+}\)-ATPase pump inhibition we considered whether other Ca\(^{2+}\)-dependent processes are activated by the increase in intracellular Ca\(^{2+}\) concentration. By incubating the synaptosomes with W7, an inhibitor of CaM we found this also resulted in significant inhibition of Na\(^{+}/K^{+}\)-ATPase pump inhibition mediated stimulation of ERK1 and ERK2 phosphorylation/activation. This indicates that in these conditions the Ca\(^{2+}\)-dependent mechanisms are also able to stimulate the ERK1 and ERK2 phosphorylation/activation. This is further supported by the inhibitory effect CaM inhibition had on the activity of ERK1 and ERK2.

We targeted Src because studies have suggested the Na\(^{+}/K^{+}\)-ATPase pump interacts with Src and inhibition of the pump results in release and activation of Src (Tian et al., 2001; Liu et al., 2003; Wang et al., 2004a; Liang et al., 2006). We found that the inhibition of Src significantly reduced ERK1 and ERK2 phosphorylation/activation induced by the Na\(^{+}/K^{+}\)-ATPase pump inhibition. This finding supports the previous studies that have also studied the interaction between Src and the Na\(^{+}/K^{+}\)-ATPase pump. However, the inhibition is not complete suggesting that there are other pathways that could be stimulating ERK1 and ERK2 phosphorylation/activation. Interestingly, we
also found that Src inhibition also significantly reduced the basal activity of ERK1 and ERK2 indicating that Src could be involved in the maintenance of ERK1 and ERK2 activity.

Given the strong evidence that a Ca\(^{2+}\)-dependent process is activated to cause the ERK1 and ERK2 phosphorylation/activation we next investigated if the smooth endoplasmic reticulum contributes to the Ca\(^{2+}\) modulation of this activation. By inhibiting the SERCA pump using thapsigargin we found that ERK1 and ERK2 phosphorylation/activation was significantly inhibited when the Na\(^+\)/K\(^+\)-ATPase pump was inhibited using ouabain. This confirms that the smooth endoplasmic reticulum contributes the Ca\(^{2+}\) by CICR invoking the possibility that this store could play a role in neurodegenerative processes during the pathophysiological inhibition of Na\(^+\)/K\(^+\)-ATPase. We should also consider the possible contributions from the other intracellular Ca\(^{2+}\) stores such as the mitochondria and the acidic stores. Thus in the future it would be interesting to investigate if the dysfunction of the mitochondria further enhances the ERK1 and ERK2 phosphorylation/activation seen in this model.

The increase in Ca\(^{2+}\) concentration and activation of processes such as the ERK pathway should result in the enhancement of the neurotransmitter release. Indeed we found that the basal/spontaneous release was increased by ouabain incubation however evoked release with 4-AP was not enhanced with prior addition of ouabain. Studies have suggested that the intracellular Ca\(^{2+}\) stores that include the smooth endoplasmic reticulum and mitochondria contribute to glutamate release (Lomeo et al., 2003; Amaral et al., 2009). The sensitivity of basal/spontaneous glutamate release induced by ouabain to thapsigargin requires further investigation.
In conclusion, we found that in this chapter, that Na\(^+\)/K\(^+\)-ATPase pump inhibition using ouabain results in ERK1 and ERK2 phosphorylation/activation. This stimulation is dependent on the activation of two pathways, Ca\(^{2+}\)-dependent and Src stimulation of the ERK activation cascade. In addition, we found that the smooth endoplasmic reticulum contributes Ca\(^{2+}\) to the Ca\(^{2+}\)-dependent compartment. This indicates that in conditions that produce an increase in the intrasynaptosomal Ca\(^{2+}\) concentration such as in ischemia, the smooth endoplasmic reticulum further release Ca\(^{2+}\) rather than sequester this excess Ca\(^{2+}\). Therefore, the smooth endoplasmic reticulum in such conditions could contribute neurodegenerative effects from pathophysiological consequent increase in intrasynaptosomal Ca\(^{2+}\).
Schematic 8-1: Ouabain mediated inhibition of Na\(^+\)/K\(^+\)-ATPase through multiple pathways results in ERK1 and ERK2 phosphorylation/activation

Ouabain inhibition of Na\(^+\)/K\(^+\)-ATPase causes the plasma membrane to depolarise causing the activation of the VDCCs that results in Ca\(^{2+}\) influx. (i) Ca\(^{2+}\) influx through VDCCs can stimulate CaM and possibly Pyk2 leading to the stimulation of the ERK pathway. (ii) The CICR mechanism could also be stimulated to release Ca\(^{2+}\) and further support the ERK pathway activation by activating CaM and possibly Pyk2. (iii) Na\(^+\)/K\(^+\)-ATPase inhibition can also cause the release of Src which is able to activate the ERK pathway independent of the Ca\(^{2+}\)-dependent mechanisms. Inhibition of the SERCA pumps using thapsigargin results in decrease in efflux of Ca\(^{2+}\) thus there is a reduction of the ERK pathway stimulation.
Chapter 9
9. Discussion

The regulation of presynaptic nerve terminal activity is important in the maintenance of the central nervous system signalling. Numerous studies have identified key kinases and phosphatases that are important in presynaptic regulation and the role of Ca$^{2+}$ is often paramount. However, most studies to date have largely looked at the influences of external sources of Ca$^{2+}$ that participate in these events. Here we considered the functional role of intracellular Ca$^{2+}$ stores in presynaptic nerve terminals and the contribution these stores might make to the regulation of Ca$^{2+}$-dependent mechanisms.

We sought to study the function of intracellular Ca$^{2+}$ stores solely in presynaptic nerve terminals, without the involvement of the postsynaptic cell body. There are limitations to this model firstly, due to the size of the synaptosomes it is as of yet impractical to directly observe changes in Ca$^{2+}$ concentrations of intracellular Ca$^{2+}$ stores. It would have been a great advantage to directly assess the intracellular Ca$^{2+}$ stores’ ability to raise the intrasynaptosomal Ca$^{2+}$ concentration. In addition, there can be no electrophysiological work conducted due to the size of the synaptosomes. Furthermore, though the experiments conducted are compared to the controls there can be additional experimental variability. For example, in these experiments there are neither controls that ensure that number of actual synaptosomes is comparable between experiments nor can the health of the synaptosomes is assessed during the experiments. In the future, we could conduct a single glutamate release after each preparation. If there is no significant difference between the glutamate releases it would suggest that the number of synaptosomes is similar and that they are healthy.

Using the isolated nerve terminal model does provide numerous advantages that make it a desirable choice for studying Ca$^{2+}$-dependent mechanisms of ERK1 and ERK2
phosphorylation/activation. By using the isolated nerve terminal (synaptosome) model we can achieve the aim aforementioned as it offers metabolically and functionally competent presynaptic nerve terminals that do not have axons or postsynaptic cell body associated. Furthermore, the synaptosomal model has been used extensively to study neurotransmitter release events as well as numerous signalling pathways including the ERK pathway.

The ERK pathway is a sequential process of activation that results from Ras activation to Raf-1 activation which is able to phosphorylate/activate MAPK/ERK kinase 1 (MEK1) and MEK2 which then dually phosphorylates/activates extracellular signal-regulated kinase 1 (ERK1) and ERK2. This cascade can be activated by ‘classical’ route which involves the activation of the tropomysin related kinase (Trks) by neurotrophic factors which activates Ras by the recruitment of growth factor receptor-bound protein 2 (Grb2) and son of sevenless (Sos). Furthermore, increases in intracellular Ca\(^{2+}\) concentration can also activate Ca\(^{2+}\)-dependent mechanisms that promote the activation of the ERK pathway. Thus increases in Ca\(^{2+}\) concentration could be reflected by the increases in the ERK1 and ERK2 phosphorylation/activation in cases in which direct measurement of Ca\(^{2+}\) concentration is less feasible. This case applies to studying intracellular Ca\(^{2+}\) stores in the synaptosomal model in which any potential Ca\(^{2+}\) released from intracellular Ca\(^{2+}\) stores is too small to distinguish from background in such a small compartment.

The first aim that we addressed in this thesis was investigating the Ca\(^{2+}\)-dependent phosphorylation/activation of ERK1 and ERK2 and establishing a model in which the external and internal sources of Ca\(^{2+}\) could be delineated. We have shown that increases in intrasynaptosomal Ca\(^{2+}\) concentration by Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\)
channels (VDCC) or direct Ca\textsuperscript{2+} influx through ionophore result in the significant increase in ERK1 and ERK2 phosphorylation/activation. By removing external Ca\textsuperscript{2+} we have shown that this stimulation of ERK1 and ERK2 is completely Ca\textsuperscript{2+}-dependent. Thus this suggests Ca\textsuperscript{2+}-independent mechanisms are not involved in ERK1 and ERK2 phosphorylation/activation under the conditions used.

There are numerous Ca\textsuperscript{2+}-dependent mechanisms that have been identified which can lead to the phosphorylation/activation of ERK1 and ERK2 but these are not as well characterised as the ‘classical’ TrkB receptor activation. However, Ca\textsuperscript{2+}-dependent mechanisms that have been identified show that these mechanisms interact with the components of the ‘classical’ ERK pathway. The components that Ca\textsuperscript{2+} target induce the promotion of the Ras activation or through direct phosphorylation/activation of c-Raf-1 or B-Raf. The Ca\textsuperscript{2+}-dependent mechanisms that have described include proline-rich tyrosine kinase 2 (Pyk2) activation of Src, calmodulin (CaM), Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII), Ras-GEFs and Ras-GAPs.

The Pyk2/Src mechanism has been shown to be activated by the increases in intracellular Ca\textsuperscript{2+} concentrations and activate Ras by recruiting Grb2 which recruits Sos via two pathways (Lev et al., 1995; Dikic et al., 1996). In the first pathway Pyk2 activation of Src activation results in the phosphorylation of Shc domains by Src and in the second Pyk2 phosphorylation by Src can directly recruit Grb2 which recruits Sos. Although we have not directly assessed the involvement of Pyk2 but using the Src inhibitor we were able to assess if this is type of pathway activated by increasing intracellular Ca\textsuperscript{2+} concentrations. Indeed we find that inhibition of Src does result in the reduction of ERK1 and ERK2 phosphorylation/activation. In addition, we found that
Src inhibition also resulted in significant reduction in the basal activity of ERK1 and ERK2.

We also investigated the effect of CaM inhibition on ERK1 and ERK2 phosphorylation/activation. CaM activation has been shown to have both stimulatory and inhibitory effects on ERK1 and ERK2 phosphorylation/activation. K-Ras interaction with CaM is shown to inhibit its activity which decreases c-Raf-1 activation. The stimulatory effects of CaM could be induced by activation of GEFs such as RasGRF which enhances Ras activation. Furthermore, it has been shown that it can promote PI3K to stimulate H-Ras which leads to c-Raf-1 activation. These differing effects of CaM could be cell/compartment specific. Nevertheless, we found that in the synaptosomal model CaM had stimulatory effect on ERK1 and ERK2 phosphorylation/activation. Thus DHPG and ouabain mediated stimulation of ERK1 and ERK2 phosphorylation/activation was significantly reduced by CaM inhibition. Furthermore, the extent to which the basal activity of ERK1 and ERK2 was affected during CaM inhibition suggests that CaM activation is a key component to the Ca$^{2+}$-dependent mechanisms of ERK1 and ERK2 phosphorylation/activation maintaining the high activity of these kinases. Interestingly, the basal effect of CaM inhibition are enhanced with time suggesting that there is a mechanism by which ERK1 and ERK2 are dephosphorylated which could involve PP2A and/or MAPK phosphatases (MKPs) activity.

Another consequence of CaM activation is the downstream activation of CaMKII which was examined in the DHPG mediated stimulation paradigm. During the stimulation CaMKII only reduced ERK1 phosphorylation/activation but did not affected ERK2 phosphorylation/activation. We found that CaMKII has a stimulatory effect on ERK1
and ERK2 as inhibition of CaMKII resulted in the reduction of basal activity. It has been shown that CaMKII can exert facilitatory effects on ERK1 and ERK2 phosphorylation/activation by inhibiting the SynGAP and thereby reducing the hydrolysis of GTP on Ras. However, SynGAP has been described in the postsynaptic cell body and can colocalise with NMDA receptors and scaffolding proteins found on the postsynaptic cell body. Another possible mechanism for the effect of CaMKII inhibition may be that it reduces Src activity as has been investigated using the vascular smooth muscle cells (Ginnan & Singer, 2002). Therefore, the mechanism by which CaMKII is able to stimulate ERK1 and ERK2 phosphorylation/activation requires further investigation. It would be interesting to observe if indeed CaMKII is able to stimulate Src by possibly directly determining if CaMKII inhibition reduces Src phosphorylation in nerve terminals. Thus, we have identified several Ca\(^{2+}\)-dependent mechanisms in presynaptic nerve terminals that could be stimulated but also appear to maintain the basal activity of ERK1 and ERK2.

Nevertheless the Ca\(^{2+}\)-dependent mechanisms could also have other effects that are independent of intracellular Ca\(^{2+}\) store signalling. There is a study which suggests that Src inhibition results in the reduction of VDCC during 4-AP mediated stimulation (Wang, 2003). This could mean that the inhibition observed of 4-AP mediated stimulation of ERK1 and ERK2 phosphorylation/activation during Src could be due to its inhibitory effects on VDCCs. In addition, ouabain mediated stimulation was shown to be partially inhibited by Src inhibition which again could be due to the VDCC inhibition. However, ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation was completely reduced by Src inhibition. Furthermore, Src inhibition effects on the basal activity of ERK1 and ERK2 could not be due to inhibition of VDCCs as they are not activated at ‘rest’. This suggests that there could be two
potential pathways in which Src is able to enhance ERK1 and ERK2 phosphorylation/activation. Firstly, phosphorylation of Shc and/or Pyk2 by Src could enhance recruitment of Grb2 and subsequent recruitment of Sos could lead to the activation of Ras. Secondly, enhanced Src activity during stimulation could phosphorylate VDCCs to increase the influx of Ca$^{2+}$ which could be a positive feedback mechanism to further enhance its activity or other Ca$^{2+}$-dependent mechanisms.

The main focus of this thesis is to study the functional role of intracellular Ca$^{2+}$ stores in presynaptic nerve terminals. So we constructed a repletion model on the hypothesis that the intracellular calcium stores are depleted by the synaptosomal preparation procedure. Interestingly, we found that a difference in response to 4-AP and ionomycin mediated stimulation under depleted conditions. 4-AP mediated stimulation in the absence of Ca$^{2+}$ only during the preincubation stage affected ERK1 but had no significant effect on ERK2. In contrast, ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation was completely abolished by the absence of Ca$^{2+}$ during the preincubation stage. This shows that the repletion of intracellular Ca$^{2+}$ stores is an important step without which certain stimulation paradigms might not stimulate.

However, this does separate the source of Ca$^{2+}$ that is responsible for this ERK1 and ERK2 phosphorylation/activation. It could be that only extracellular Ca$^{2+}$ influx is responsible or that it is the trigger to activate intracellular Ca$^{2+}$ stores that could contribute to the phosphorylation/activation of ERK1 and ERK2. The third possibility is that it is the combination of Ca$^{2+}$ from extracellular and intracellular Ca$^{2+}$ sources that contribute to ERK1 and ERK2 phosphorylation/activation. Thus in order to delineate the possible internal source of Ca$^{2+}$ from the external source of Ca$^{2+}$ we formed a protocol in which the intracellular Ca$^{2+}$ stores undergo a repletion stage. This is based
on the assumption that intracellular Ca$^{2+}$ stores might be depleted to a certain extent by the synaptosomal preparation and so requires repletion to be fully functional. We found that this repletion step did not affect the viability and responsiveness of the synaptosomes.

The second aim that we addressed in this thesis is the functional role of intracellular calcium store found in nerve terminals. The intracellular Ca$^{2+}$ stores that we have targeted in this thesis include the smooth endoplasmic reticulum, mitochondria and acidic stores.

In order to investigate the presence and mobilisation of intracellular Ca$^{2+}$ stores supporting Ca$^{2+}$-dependent stimulation of ERK1 and ERK2 phosphorylation/activation we took a pharmacological approach to dissect each store individually. The smooth endoplasmic reticulum was the first potential Ca$^{2+}$ store that we examined as it has been shown to be very important in other cell systems such as the smooth muscle cells. Furthermore, smooth endoplasmic reticulum has also been identified in nerve terminals but the data are limited. From the experiments that we have conducted here the presence of smooth endoplasmic reticulum Ca$^{2+}$ stores effective in stimulating ERK1 and ERK2 phosphorylation/activation was confirmed by several experiments.

Smooth endoplasmic reticulum is able to store Ca$^{2+}$ which can be released into the cytosol thus increasing Ca$^{2+}$ concentration and thus provide the facilitatory modulation of Ca$^{2+}$-dependent mechanisms. There are two receptors that are found on the smooth endoplasmic reticulum that able to efflux Ca$^{2+}$ from the store these include the inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) and ryanodine receptors (RyRs).
The second function of the smooth endoplasmic reticulum is to sequester Ca\(^{2+}\) from the cytosol during stimulation. There were two instances which support this conclusion, firstly, thapsigargin incubation throughout the preincubation and stages showed that there was an enhancement of 4-AP mediated stimulation. Yet, the protocol in which thapsigargin was only incubated during the preincubation stage showed that 4-AP mediated stimulation is inhibited. This could suggest that the SERCA pumps have recovered during the stimulation stage and are sequestering the influx of Ca\(^{2+}\) thus reducing the stimulation of Ca\(^{2+}\)-dependent mechanisms. It is also likely that the 4-AP mediated stimulation is reduced because it is unable to stimulate the Ca\(^{2+}\) release from the smooth endoplasmic reticulum. The second piece of evidence that supports the role of smooth endoplasmic reticulum ability to sequester cytosolic Ca\(^{2+}\) comes from the enhancement of ERK1 and ERK2 phosphorylation/activation from Ca\(^{2+}\) released from the mitochondria.

The second store that was investigated as a potential Ca\(^{2+}\) store was the mitochondria. Mitochondria are known to be involved in ATP production and Ca\(^{2+}\) homeostasis but we found that it could also be involved in the release of Ca\(^{2+}\) during 4-AP mediated stimulation. This is supported by another study which points to the function of Na\(^+\)/Ca\(^{2+}\) exchanger on the mitochondria to release Ca\(^{2+}\) as the Na\(^+\) concentration increases by depolarisation of the plasma membrane (Yang et al., 2003). However this requires further investigation as in the absence of Ca\(^{2+}\) there was a complete reduction of the ERK1 and ERK2 phosphorylation/activation. If Ca\(^{2+}\) is released from the mitochondria during depolarisation then ionomycin mediated stimulation should not inhibited but enhanced by prior release of mitochondrial Ca\(^{2+}\).
The third store that was investigated was the acidic stores that include lysosomes and SSVs. The finding that ERK1 and ERK2 basal activity is decreased by depleted acidic stores suggest that the acidic stores are involved in the maintenance of ERK1 and ERK2 phosphorylation/activation. However, areas of the acidic stores need to be explored as it is suspected that it might involve stimulating Ca\textsuperscript{2+} release from RyR on the smooth endoplasmic reticulum. This could also indicate that multiple stores in the nerve terminals are activated during stimulation.

The third aim of this thesis was to investigate the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) and IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (IPCR) mechanisms in nerve terminals. The RyR are stimulated by increases in intracellular Ca\textsuperscript{2+} concentrations that can be brought about Ca\textsuperscript{2+} influx. By using high concentrations of ryanodine we inhibited RyR and showed that 4-AP and ionomycin mediated stimulation of ERK1 and ERK2 phosphorylation/activation is significantly inhibited. We also noted that inhibition of RyR during 4-AP mediated stimulation was only partially inhibited but ionomycin mediated stimulation was completely inhibited. This perhaps indicates that other Ca\textsuperscript{2+}-dependent mechanisms are activated by 4-AP mediated stimulation.

The IP\textsubscript{3}R are stimulated by IP\textsubscript{3} which is produced the metabolism of PIP\textsubscript{2} by PLC was also investigated. We found that 4-AP, ionomycin and DHPG mediated stimulation resulted in the activation of PLC and that inhibition of the IP\textsubscript{3}R resulted in the reduction of the ERK1 and ERK2 phosphorylation/activation. However, there are nonspecific effects of drugs that are used to study IP\textsubscript{3}R. Heparin nonspecific actions include uncoupling of G-protein signalling and activating RyR (Ehrlich et al., 1994) (Taylor & Broad, 1998). Another commonly used IP\textsubscript{3}R inhibitor is xestospongin C which has been used to inhibit Ca\textsuperscript{2+} signalling from IP\textsubscript{3}Rs (Mathew & Hablitz, 2008). However,
xestospongin C has also been described as a potent inhibitor of the SERCA pump (Castonguay & Robitaille, 2002). We considered using 2-aminoethoxydiphenyl borate (2-APB) to inhibit IP$_3$R. While 2-APB is widely used as an IP$_3$R antagonist there have been studies which point to nonspecific actions on the SERCA pump (Bootman et al., 2002; Peppiatt et al., 2003). Although at lower concentrations which we used the nonspecific effects have been shown to be reduced. Thus, we consider that the reduction of ERK1 and ERK2 phosphorylation/activation in the presence of 2-APB to be due to specific inhibition of IP$_3$R.

In addition, to show that PLC activity is involved in the production of IP$_3$ we used a PLC inhibitor called U-73122 which inhibited the ERK1 and ERK2 phosphorylation/activation. This was true for the 4-AP, ionomycin and DHPG mediated stimulation paradigms. Interestingly, ionomycin had a reduced effect on IP$_3$R and PLC activity when compared to its effect on RyR perhaps indicating that it is more favourable towards the CICR mechanism. However, it could also be possible that these effects of PLC inhibition could be due to nonspecific effects. In a study using smooth muscle cells it is reported that U-73122 was able to inhibit direct IP$_3$R and ryanodine receptor activation without involving the activation of PLC suggesting that it has nonspecific actions on the SERCA pumps (Macmillan & McCarron, 2010). However, in our experiments we found that ionomycin mediated stimulation inhibitory effects for thapsigargin was different to the inhibitory effects of U-73122. This could indicate that in the synaptosomal model we are not observing the nonspecific effect of U-73122. Nevertheless, we could clarify if this nonspecific effect is being produced by directly stimulating IP$_3$R and RyR and observing if U-73122 is able to inhibit these responses. If U-73122 is able to inhibit then this could bring into question our previous conclusions about PLC activation but at the very at least the results could further support the
involvement of the smooth endoplasmic reticulum in 4-AP, ionomycin and DHPG mediated stimulation.

The intracellular Ca\(^{2+}\) stores therefore play an important function in presynaptic nerve terminals which could include facilitation of neurotransmitter release. We have done some preliminary experiments that examine the effect of intracellular Ca\(^{2+}\) stores on glutamate release. DHPG mediated stimulation indicates that there is a facilitation of glutamate release. We have shown that intracellular calcium stores release sufficient Ca\(^{2+}\) to stimulate processes that are facilitatory it is hypothesised that 4-AP mediated stimulation should be enhanced by intracellular Ca\(^{2+}\) stores. Previous studies have examined the effect of intracellular Ca\(^{2+}\) stores on glutamate release by using α-Latrotoxin which causes a massive exocytotic release. The study showed that α-Latrotoxin are dependent on Ca\(^{2+}\) released from intracellular sources and require the activation of PLC (Davletov et al., 1998). However, the use of α-Latrotoxin is not very physiological as it also causes Ca\(^{2+}\) independent release by forming pores that allow the neurotransmitter to ‘leak’ out into the cytosol. Thus, later studies have utilised DHPG mediated stimulation to potentiate glutamate release as it does not have non-physiological effects and can directly mobilise the smooth endoplasmic reticulum with minimum side effects. In functional studies there is evidence which suggests that DHPG mediated stimulation can result in neurotransmitter release (Moroni et al., 1998; Reid et al., 1999). The mechanism of action that is proposed to enhance glutamate release through DHPG mediated stimulation has already been described in Chapter 4.

Furthermore, intracellular Ca\(^{2+}\) stores could play a significant role in pathophysiological conditions. As we have shown that inhibition of Na\(^{+}\)/K\(^{+}\)-ATPase pump using ouabain triggers release from the smooth endoplasmic reticulum which could promote the
accumulation of cytosolic Ca\textsuperscript{2+} concentration. We have observed the accumulation of intracellular Ca\textsuperscript{2+} with time and this could also be the trigger for the increase in glutamate release observed with ouabain (100\(\mu\)M). This could therefore cause excitotoxic conditions is the surrounding area of the synapse by excessively activating postsynaptic targets such as ionotropic glutamate receptors. These are important targets in the treatment of ischemia as it is suggested that excessive glutamate presence in the synaptic cleft over stimulates these receptors causing excessive Ca\textsuperscript{2+} influx. In addition, the condition of the presynaptic terminal could be made worse by the stimulation of glutamate receptors found on the presynaptic nerve terminal. We have already shown that mGluR1/5 stimulation can result in the phosphorylation/activation of ERK1 and ERK2 which promotes the mobilisation of SSVs. Therefore, from the initial insult in which cytosolic Ca\textsuperscript{2+} concentrations are continuously rising and the intracellular Ca\textsuperscript{2+} stores contribute to this increase. You can get positive feedback mechanism that continuously promotes the accumulation of Ca\textsuperscript{2+} and ultimately neurosis of the presynaptic nerve terminals and surrounding postsynaptic cell bodies if not controlled.

Another pathophysiological condition that intracellular calcium stores could be significantly contributing to is epilepsy. Levetiracetam is an antiepileptic drug whose mechanism of action is unknown but there have been reports that suggest that it could due to binding of SV2A (Lynch et al., 2004; Gillard et al., 2006). However, a study showed that variation in the SV2A genes does not influence the predisposition towards epilepsy. Furthermore, these variations of SV2A do not influence the levetiracetam response, perhaps indicating that SV2A is not the site for levetiracetam mechanism of action (Lynch et al., 2009). In addition, the expression of SV2A is significantly reduced in several brain areas such as the hippocampus during chronic epilepsy (van Vliet et al.,
2009). This brings into question the effectiveness of levetiracetam as an epileptic drug through binding to SV2A.

In testing the effectiveness of levetiracetam we have found that it is able to significantly inhibit IP3R found on the smooth endoplasmic reticulum which results in the reduction of ERK1 and ERK2 phosphorylation/activation and other possible Ca\(^{2+}\)-dependent mechanisms. The activation of the ERK pathway and Ca\(^{2+}\)-dependent mechanisms can result in the promotion of the neurotransmitter release. However, it is possible in epileptic conditions as with ouabain small insult can result in major changes when the Ca\(^{2+}\) homeostasis is not maintained sufficiently. The preliminary glutamate release data did not show a significant inhibition of the glutamate release with levetiracetam. However, this could be due to the property of levetiracetam in which it only affects epileptic synapses. Thus perhaps the use of mice those that are prone to the epileptic profile could reveal the levetiracetam inhibitory effect on glutamate release. There are studies that have shown that firstly that levetiracetam is able to inhibit both IP3R and RyR on the smooth endoplasmic reticulum (Nagarkatti et al., 2008). Furthermore, there is evidence which shows a novel effect of levetiracetam which is the inhibition of IP3-dependent mechanisms that increase intracellular Ca\(^{2+}\) concentrations in PC12 cells. Levetiracetam was shown to inhibit IP3-dependent increase in Ca\(^{2+}\) by inhibiting the IP3R and reducing the Ca\(^{2+}\) concentration (Cataldi et al., 2005). Another interesting finding is that other antiepileptic drugs can effect on neurotransmitter release through association with IP3R (Yamamura et al., 2009) and RyR (Yoshida et al., 2005).

It would be of interest to observe how the Ca\(^{2+}\)-dependent mechanisms interact with other known stimulatory pathways of ERK1 and ERK2 such as BDNF stimulation of TrkB receptors. BDNF is known to increase ERK1 and ERK2
phosphorylation/activation thus could the release of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores using the stimulatory paradigms described in this thesis result in a further enhancement.

In conclusion we have presented evidence in the thesis which point to the presence of synaptosomal Ca\textsuperscript{2+} stores such as smooth endoplasmic reticulum, mitochondria and acidic stores in presynaptic nerve terminals. All of these stores were capable of modulating Ca\textsuperscript{2+}-dependent mechanisms leading to the ERK1 and ERK2 phosphorylation/activation during basal and stimulatory conditions. The smooth endoplasmic reticulum has been shown to both release Ca\textsuperscript{2+} into the cytosol and sequester Ca\textsuperscript{2+} from the cytosol. Mitochondria could also be a store for Ca\textsuperscript{2+} release during 4-AP mediated stimulation and due to the close proximity to the smooth endoplasmic reticulum might be able to modulate the function of each other. Acidic stores have been shown to maintain the basal activity of ERK1 and ERK2. We also investigated some of the Ca\textsuperscript{2+}-dependent mechanisms that could be activated by Ca\textsuperscript{2+} released from the intracellular Ca\textsuperscript{2+} stores. We found that Src, CaM and CaMKII could be some of the Ca\textsuperscript{2+}-dependent mechanisms that are activated which result in ERK1 and ERK2 phosphorylation/activation. Overall, this thesis points to the complexity of Ca\textsuperscript{2+}-dependent mechanisms in nerve terminals due to the presence of intracellular Ca\textsuperscript{2+} stores.
Schematic 9-1: Presynaptic pathways that phosphorylate/activate ERK1 and ERK2 through Ca\(^{2+}\)-dependent mechanisms.

4-AP mediated depolarisation of plasma membrane can lead to Ca\(^{2+}\) influx through VDCCs and direct Ca\(^{2+}\) influx is mediated by ionomycin. (i) Extracellular Ca\(^{2+}\) influx through VDCCs can activate CaM and possibly Pyk2. (ii) Stimulation of Ca\(^{2+}\) influx through VDCCs and ionomycin can result in direct stimulation of RyR and PLC that result in Ca\(^{2+}\) efflux from RyR and IP\(_3\)Rs. (iii) DHPG stimulation of mGluR1/5 receptors results in PLC activation leading to the breakdown of PIP\(_2\) that increase IP\(_3\).
concentration to stimulate IP$_3$Rs causing Ca$^{2+}$ efflux. (iv) VDCCs can be inhibited by the activation of inhibitory GPCRs which can inhibited by the depletion of the intracellular Ca$^{2+}$ stores possibly through PLC stimulation leading to PKC activation. (v) 4-AP mediated depolarisation can also significantly increase [Na$^+$] which could impact on the mitochondrial Ca$^{2+}$ store to efflux Ca$^{2+}$ that can be taken up by the SERCA pumps found on smooth endoplasmic reticulum but can also be sufficient to stimulate CaM and possibly Pyk2. The acidic store such as SSVs could also contribute Ca$^{2+}$ to Ca$^{2+}$-dependent mechanisms that cause ERK1 and ERK2 phosphorylation/activation. (vi) Na$^+$/K$^+$-exchanger inhibition causes membrane depolarisation resulting in VDCC activation that causes Ca$^{2+}$ influx which causes further Ca$^{2+}$ efflux from intracellular Ca$^{2+}$ stores. CaM can stimulate Ras-GEFs and CaMKII. CaMKII activation can inhibit SynGAP and possibly phosphorylate Src while Pyk2 stimulation results in Grb2 activation and Src phosphorylation. These pathways have a positive contribution to the ERK pathway leading to ERK1 and ERK2 phosphorylation/activation. ERK1 and ERK2 phosphorylation/activation and CaMKII activation can increase the mobilisation of synaptic vesicle from the reserve pool (RP) to the ready-releasable pool (RRP).
References


Cao, YQ & Tsien, RW. (2010). Different relationship of N- and P/Q-type Ca2+ channels to channel-interacting slots in controlling neurotransmission at cultured hippocampal synapses. *J Neurosci*, **30**, 4536-4546.


Martin, R, Durroux, T, Ciruela, F, Torres, M, Pin, JP & Sanchez-Prieto, J. (2010). The metabotropic glutamate receptor mGlu7 activates phospholipase C, translocates munc-


close to IP3-sensitive channels that are sensed by neighboring mitochondria. *Science,* 262, 744-747.


facilitation to inhibition of excitatory synaptic transmission by group I mGluR

Ross, CA, Wright, GE, Resh, MD, Pearson, RC & Snyder, SH. (1988). Brain-specific


Roux, PP & Barker, PA. (2002). Neurotrophin signaling through the p75 neurotrophin

through ryanodine and IP(3) receptors contributes to neuronal excitotoxicity. *Cell Calcium,* 46, 273-281.


64, 289-311.

Botulinum toxin A blocks glutamate exocytosis from guinea-pig cerebral cortical

Santo-Domingo, J & Demaurex, N. (2010). Calcium uptake mechanisms of

Santos, AE, Carvalho, CM, Macedo, TA & Carvalho, AP. (1995). Regulation of
intracellular [Ca2+] and GABA release by presynaptic GABAB receptors in rat
cerebrocortical synaptosomes. *Neurochem Int,* 27, 397-406.

Santos, SD, Carvalho, AL, Caldeira, MV & Duarte, CB. (2009). Regulation of AMPA
receptors and synaptic plasticity. *Neuroscience,* 158, 105-125.


