Investigations into the effects of serine proteases in modulating long lasting depression of synaptic transmission

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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy to the University of Glasgow

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March 2014

Abstract

Long-term depression is a long-lasting decrease in signalling between neurons in the central nervous system. It can be elicited via a number of mechanisms, including NMDA receptors and mGLuR receptors. NMDA-dependent LTD is caused by the endocytosis of AMPA receptors in response to stimuli, whereas mGLuR-dependent LTD acts via the action of kinases. Recent studies have demonstrated a novel form of LTD, which is dependent on serine protease action. Molecular changes in protein expression also accompany the generation of this new type of LTD, although the exact mechanism has not been determined. The aims of this current study are to determine the mechanism by which subtilisin could modulate LTD, and whether this effect was solely dependent on proteolytic action, or other factors. Subtilisin-mediated LTD was elicited in hippocampal slices of mice as an experimental model, and each slice was retained at the end of every experiment to enable protein levels to be analysed via immunoblotting.

Initial experiments were carried out to evaluate the relationship between subtilisin activity and metalloprotease action, based on previous studies that demonstrated subtilisin proteolysis of the synaptic protein VAMP-1, a known target of the metalloprotease tetanus toxin. The putative link between these two proteases was tested by addition of zinc to the perfusing solution, which promotes the activity of zinc-based metalloproteases. Subtilisin however was completely unaffected by this change to extracellular levels of zinc. Experiments were also conducted in the presence of the metal ion chelators EDTA and captopril to determine the contribution of metal ions to subtilisin action. Addition of the general metal chelator EDTA failed to decrease subtilisin action, and the zinc-specific chelator captopril likewise did not display inhibitory action against subtilisin. Attempts were also made to produce a direct comparison between the action of subtilisin and tetanus toxin, however, preliminary experiments were unsuccessful as the tetanus toxin used in the experiment was unable to cause VAMP-1 proteolysis.

The possibility that subtilisin action was solely dependent on their inherent serine protease function was also explored using the general serine protease inhibitor phenylmethanesulfonylfluoride (PMSF). The use of PMSF could provide a better indication of the link between LTD and subtilisin proteolysis, and also shed light on the order of protein degradation. Perfusion of PMSF-inactivated subtilisin prevented the proteolysis of proteins, and also prevented the generation of LTD and abolished the proteolytic activity of subtilisin. These results indicate a close link between the LTD-

inducing and proteolytic effects of subtilisin, and in conjunction with the metalloprotease experiments, suggest a serine protease basis for subtilisin-mediated LTD.

These results suggested that subtilisin action was not based on metalloprotease degradation of VAMP-1 alone, but on a more general proteolytic effect involving other protein targets such as the netrin receptor Unc5H3 and the cytoskeletal protein actin. The specific role of the Unc5H3 and VAMP-1 proteins in the subtilisin process was studied using protein-specific antibodies to prevent proteolysis. Preincubation of hippocampal slices with VAMP-1 proteins did not prevent the proteolytic effects of subtilisin, nor could it prevent the generation of LTD. Preincubation of hippocampal slices with VAMP-1 antibody in the presence of either the cell permeabiliser triton, or the pore-forming chemical streptolysin also had no inhibitory effect on subtilisin action. Hippocampal slices were also preincubated with Unc5H3 antibodies to determine the importance of this protein to the activity of subtilisin; however, this did not change the proteolytic or electrophysiological effects of subtilisin. Addition of either triton or streptolysin to the preincubation solution containing Unc5H3 antibodies failed to prevent the effects of subtilisin on the hippocampal slice, a result identical to those obtained with VAMP-1 antibody preincubations.

The importance of actin proteolysis in mediating subtilisin action was also assessed by addition of the actin stabiliser jasplakinolide to the aCSF used in perfusions to prevent actin degradation. Jasplakinolide was unable to prevent the onset of subtilisin-mediated LTD or its associated degradation of actin.

In order to verify whether the effects of subtilisin represented a novel form of LTD, the LTD elicited by subtilisin was compared to that of more established methods of eliciting LTD, such as the mGluR agonist DHPG. Slices exposed to DHPG failed to elicit LTD, however, the GABA mimetic ethylenediamine (EDA) appeared to be a potent inducer of LTD. EDA produced a significantly smaller LTD effect in comparison to subtilisin, which was not accompanied by degradation of any proteins associated with subtilisin perfusion.

It would be of interest to know whether other serine proteases could cause this LTD effect, and based on previous studies, it is known that members of the S8A subfamily and the S1A subfamily of serine proteases are capable of causing the same effects as subtilisin. To date much work has been performed on subtilisin, and other groups S8A serine proteases such as Proteinase-K. In comparison, very little is known about of the mechanism utilised by group S1A serine proteases in causing LTD. In order to gain a greater understanding of

The possibility that other serine proteases could generate LTD was also investigated as part of this study, with particular emphasis on members of the S8A (to which subtilisin belongs) and S1A subfamily of serine proteases, which are biochemically similar to

members of the S8A subfamily. Previous studies have shown that proteinase-K and cadeprin, both members of the S8A, as well as α-chymotrypsin, a member of the S1A subfamily, can replicate this LTD effect. By studying the impact of α-chymotrypsin on fEPSP and protein degradation, it was hoped that this would provide a much more balanced view on the mechanisms behind the LTD-inducing effects of serine proteases. Previous experiments with subtilisin had ruled out the action of mGluRs or the role of electrically-stimulated LTD as the basis for subtilisin-mediated LTD. For this study these two factors were investigated using α-chymotrypsin in order to discover the different mechanisms utilised by these two serine proteases. Electrically-elicited LTD is known to be sensitive to the action of protein phosphatases, and in the light of this knowledge, experiments were conducted using the phosphatase inhibitors phenylarsine oxide (PAO) and sodium orthovanadate. Sodium orthovanadate did not affect the progression of α-chymotrypsin LTD; however, PAO did significantly decrease the magnitude of this type of LTD. This suggests a possible role for tyrosine kinase in this process, although more experiments would be necessary to provide a definite link as sodium orthovanadate had no effect on the action of chymotrypsin. The involvement of mGLuR in α-chymotrypsin LTD was tested using the p38 MAPK inhibitor SB203580. Perfusion of this chemical did not result in a noticeable decrease in α-chymotrypsin activities, and taken together with previous experiments in subtilisin, suggests that serine protease-mediated LTD is not solely dependent on these two pathways for their neuronal depressive actions.

The results presented here strongly suggest that LTD caused by serine proteases such as subtilisin and α-chymotrypsin do not involve metalloprotease functions. Instead, LTD is closely linked with the proteolysis of a select number of proteins that are cleaved by the endogenous proteolytic functions of these serine proteases. Furthermore, serine protease-mediated LTD is not simply restricted to subtilisin alone, but can involve other members of the S8A subfamily of serine proteases such as α-chymotrypsin. Information gathered over these investigations also suggests the involvement of mechanisms utilised by other forms of LTD, but their overall action depends on categorically different mechanisms.

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DECLARATION

I, Caleb Yan Lik Lui, declare that this thesis was composed by myself, and also that the experiments described therein were performed by myself, except where referenced.

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Caleb Yan Lik Lui

Abbreviations

5-HT4	5-hydroxytryptamine 4
ABP	AMPAR-binding protein
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's Disease
ADAM	a disintegrin and metalloprotease
ADP	Adenosine diphosphate
Ala	alanine
ANOVA	Analysis of Variance
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AP2	Adaptor protein 2
ARP	Actin regulatory protein
Asn	Asparagine
Asp	Aspartate
Bcl2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BHLH	Basic helix-loop-helix
BID	BH3 interacting domain death agonist
BTx	Botulinum Toxin
cAMP	Cyclic adenosine monophosphate
CA1	*Cornuammonis 1*
CA^{2+}	Calcium
CA3	*Cornuammonis 3*
CAP1	Channel-activating protease 1
Cdc42	Cell division control protein 42 homolog
Cdk5	cyclin-dependent kinase 5

CNS	Central nervous system
C-terminal	Carboxyl terminal
DCC	Deleted in Colorectal Cancer
DHPG	Dihydroxylphenylglycine
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
ECL	Enhanced chemiluminescence
E.Coli	*Escherichia Coli*
EDTA	Ethylenediaminetetraaceticacid
ENaC	Epithelial Na^+ channels
(f)EPSP	(field) Excitatory post-synaptic potential
F-actin	Filamentous actin
G-actin	Globular actin
GABA	γ-amino butyric acid
Glyn	Glycine
Glu	Glutamic acid
GluN2B	Glutamate NMDA receptor subunit 2B
GluR2	Glutamate receptor 2
GRIP	glutamate receptor interacting protein
GTPase	Guanosinetriphosphatase
His	Histidine
HIV	Human Immunodeficiency Virus
HtrA	HtrA serine peptidase
Hz	Hertz
IGF	Insulin growth factor
IL-B	Interleukin-B
Ile	Isoleucine

LexA	Lambda excision A
LDL	Low-density lipoprotein
LFS	Low frequency stimulation
LTD	Long term depression
LTP	Long term potentiation
Lys	Lysine
MAP1B	Microtubule-associated protein 1B
MAPK	Microtubule-associated protein kinase
mGLuR	Metabotrophic glutamate receptors
MMP	Matrix metalloprotease
Na^{+}	Sodium
NCS	Neuronal Ca^{2+}sensor
NMDA	*N*-methyl-d Aspartate
NMDAR	*N*-methyl-d Aspartate receptor
NSCL-1	Neuronal stem ell leukemia
N-terminal	Amino terminal
P75NTR	p75 neurotrophin receptor
PAO	Phenylarsine oxide
PC2/PC3	Proproteinconvertase 2/3
PCSK	proproteinconvertase subtilisin/kexin
PD	Parkinson's Disease
PI3K	Phosphoinositide 3-kinase
PICK1	Protein Interacting with PRKCA 1
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulfonylfluoride
PRK1	Protein regulatory kinase 1

PRss8	Protease, serine, 8
RhoA/RhoB	Rho GTPase A/B
RHOGEF	Rho GTPase exchange factor
RIPA	Radioimmuno precipitation assay
RNA	Ribonucleic acid
RNAi	RNA-interference
ROCK	Rho-associated protein kinase
SDS	Sodium dodecyl sulphate
SEM	Standard Error of the mean
Ser	Serine
SNAP-25	Synaptosomal-associated protein 25
SNARE	SNAP (soluble NSF Attachment Protein) Receptor
SOS	Save Overboard Sailors
SO	Sodium Orthovanadate
SP	Signal Peptide
SREBP	Sterol response element-binding protein
TAFI	Thrombin-activatable fibrinolysis inhibitor
TBST	Tris-buffered saline Tween
TeTx	Tetanus Toxin
TIMP/TIMPS	Tissue inhibitors of metalloproteases
TrKB	Tyrosine kinase B
UmuD	UV mutagenesis D
VAMP	Vesicle-associated membrane protein
VASP	vasodilator-stimulated phosphoprotein
VNO	Vomeronasal organ
WASP 2	Wiskott-aldrich syndrome protein homology 2

Acknowledgements

I would like extend my thanks and gratitude to my supervisor, Professor Trevor W. Stone for granting me this opportunity to conduct research at his laboratory, and for his invaluable expertise and advice throughout my time as a PhD student.

I would also like to thank my colleagues in the lab who have been very helpful and supportive over my years of research. In particular, I would like to thank Dr Caroline Forrest for her advice and help in setting up Western blots; Dr Alexandra Ferguson for her invaluable help during my first year of study.

In addition, I would also like to thank my fellow PhD student Khalil Iqbal, for guiding me through the Western blot process, and being available to answer questions whenever. I would also like to thank all Masters students who have been involved with our lab at some point during my time here, for making the lab a better place for all.

I'd like to extend my deepest appreciation to my parents for funding me over the years, as well as their patience in putting up with my often hectic and stressful life as a PhD student. They have always been there for me no matter what, and for that I will always be grateful.

Finally, I'd like to give all glory to God, for giving me this chance to prove myself despite my many failures over the years.

Publications

Stone T.W., Lui C. & Addae J.I. (2011) Effects of ethylenediamine - a putative GABA-releasing agent - on rat hippocampal slices and neocortical activity in vivo. European Journal of Pharmacology, 650(2-3):568-78

Chapter 1 - Introduction

1.1 Protease overview

1.1.1 Protease families

Proteases are involved in numerous physiological functions, and act by cleaving the peptide bonds within proteins. They exist in almost all types of organisms, and in mammalian cells, they have roles in angiogenesis, cell differentiation, matrix remodelling and regulation of the immune system. They are separated into six different families based on their catalytic mechanism, consisting of the threonine, aspartate, cysteine, asparagine, glutamic acid, metalloprotease and serine protease families. Proteases within each family are further divided into clans based on the structure of their cleavage proteolytic and inhibitory sites, which form the basis for their substrate specificity (Rawlings *et al.*, 2005). For the purposes of this investigation, only proteases in the serine, cysteine and metalloprotease are directly relevant.

1.1.2 Cysteine proteases

Cysteine proteases are a family of proteases that utilise a nucleophilic cysteine residue as part of a catalytic triad at their active site for proteolysis. Their members are divided into three clans: calpains, papains (which include cathepsin) and bleomycin hydrolase, and grouped based on their structure (Storer & Ménard, 1994). The catalytic cysteine residue is located at position 25 of the active site of the papain subgroup, which includes cathepsins.

1.1.2.1 Cathepsins

In humans 11 cathepsins are known to exist in humans, where they are expressed ubiquitously in cells due to their role in cell turnover and apoptosis (Berti & Storer, 1995; Turk *et al.*, 2012).

Cathepsins are active in the nervous system, where they regulate cell survival during both development and normal physiological function. During neuronal development, cathepsins B and L work collectively to prevent neuronal apoptosis in the cerebral cortex and cerebellum. In their absence apoptosis occurs, accompanied by an accumulation of lysosomal bodies in neurons and enlargement of axonal processes (Felbor *et al.*, 2002). These proteases have also been implicated in several neurological disorders including schizophrenia, which alters cathepsin K expression levels; and myoclonus epilepsy, which is accompanied by mutations in the cysteine inhibitor cystatin B (Pennacchio *et al.*, 1996; Ko *et al.*, 2006). The action of cathepsin B may also be related to serine proteases as it cleaves urokinase-type plasminogen activator precursor at the same site as kallikrein and plasmin (Kobayashi *et al.*, 1991).

1.1.2.2 Caspases

Another important group of cysteine proteases are the caspases, which regulate the crucial caspase apoptotic pathway in both developing and adult cells. Members of the caspase subgroup are involved in immune system function, such as interleukin-1B-converting enzyme, which converts the inflammation mediator protein IL-B precursor to their active, mature form (Thornberry *et al.*, 1992). Caspases also act within mitochondria, where members such as caspase 8 can trigger apoptosis by cleaving the proapoptotic Bcl2 family member BID (Li *et al.*, 1998).

1.1.3 Metalloproteases

Metalloproteases are proteases that utilise metal ions as part of its catalytic apparatus, and can be separated into groups based on the specific metal ion within the active site. They perform a wide range of physiological roles, and are involved with angiogenesis, the progression of cancer and other disorders. One well-known group of metalloproteases are the zinc metalloprotease family, which utilise zinc ions and are further categorised depending on whether they incorporate single or double zinc ions at the core of their functional site. Zinc metalloproteases are subsequently divided into clans based on the amino acid sequence at their active site, several of which are well known, such as the "a disintegrin and metalloprotease" (ADAM), matrix metalloprotease (MMP) and the tetanus toxin (TeTx)/botulinum toxin (BTx) families (Hooper, 1994)

1.1.3.1 Endogenous metalloproteases

In humans, endogenous metalloproteases include the ADAM family of metalloproteases, which consist of 21 members and regulate the activation of growth factors, receptors and many other signalling molecules by removing the ectodomain of the precursor protein (Edwards *et al.*, 2008). In comparison, matrix metalloproteases (MMPs) are most well known as modulators of long-term synaptic signalling and in particular, the involvement of MMP-9 in long-term potentiation (LTP) is well attested in literature. MMP-9 is intimately involved in the induction of late-phase LTP in hippocampus, where it facilitates actin polymerisation by inactivating cofilin through β1-integrin signalling. Enlargement of dendritic spines is also dependent on the function of MMP-9, which have profound implications for hippocampal-dependent memory due to the dependence of this type of memory on dendritic remodelling (Nagy *et al.*, 2005; Wang *et al.*, 2008). Other MMPs are also involved in hippocampal processes, as evidenced by the effect of MMP inhibitors that reduce LTP magnitude and decreased LTD stability, whereas specific inhibition of MMP-9 had no effect on LTD. (Meighan *et al.*, 2007).

MMPs also expressed outwith the nervous system, where they have roles in processes such as ephrin signalling, where MMPs 1,2,9 and 13 facilitate the release of the active EphrinA1 monomer by cleaving the membrane-attached Ephrin A1 precursor (Beauchamp *et al.*, 2012). Members of both the ADAM and MMP metalloprotease families are noted for their involvement in the formation of proteolytic machinery underlying the progression of Alzheimer's disease. They are also known to actively target adhesion molecules such as N-cadherin on both pre- and postsynaptic membranes, which cause an attenuation of synaptic transmission by reducing the number of synaptic proteins available (Restituito *et al.*, 2011).

1.1.3.2 Clostridial metalloproteases

Other metalloproteases, such as the tetanus/botulinum family of metalloproteases, are also known to have significant effects on the synapse. The tetanus and botulinum toxin groups encompass a number of neurotoxins with potent inhibitory effects on synaptic signalling. This is caused by their selective cleavage of synaptic proteins that prevent the formation of the exocytotic machinery essential for vesicle release. Seven botulinum toxins known to exist, and are denoted as types A, B, C, D, E, F and G, which together target a wide range of synaptic proteins. Botulinum toxin types A and E can cause paralysis by cleaving SNAP-25, a membrane-bound component of the exocytotic SNARE assembly. Another

membrane-SNARE protein, syntaxin, is the target of botulinum toxin C proteolysis, and is triggered in response to stimuli-induced Ca2+ influx (Binz *et al.*, 1994; Binz T. *et al.*, 1994; Mochida *et al.*, 1995). In comparison, types B, D, F and G cleave a set of proteins on the vesicle membrane known as the vesicle-associated membrane proteins (VAMP) or synaptobrevin. The proteolytic site of each subtype varies however, with type G cleaving an ala-ala bond, type F at a Glyn-Lys bond and type D cleaving the following Lys-Ile bond. Botulinum type B has the distinction of sharing the same cleavage site as tetanus toxin when targeting the VAMP protein, with both cleaving the sole Gln-Phe bond in this protein (Schiavo *et al.*, 1994a; Schiavo *et al.*, 1994b; Yamasaki *et al.*, 1994b). Tetanus toxin proteolysis is dependent on the presence of their light chains, as targeted expression of this TeTx domain alone is sufficient to cause VAMP-2 cleavage and loss of Ca^{2+} evoked transmitter release (Gaisano *et al.*, 1994).

1.1.3.3 Inhibition of metalloproteases

Metalloproteases can be inhibited by several different mechanisms, which inactivate their active site via allosteric changes, or disrupt their metalloprotease mechanism by removing the metal ion essential for proteolysis. Examples of inhibitors include the endogenous family of "Tissue inhibitors of metalloproteases" (TIMPs), consisting of four members which act against both ADAMs and MMPs.

TIMPs are involved in the progression of cancer, and the presence of TIMP 1 & 2 in hepatocellular cancer cells is associated with poor disease prognosis, whereas TIMP 3 is located in the extracellular matrix, where it can determine the spread of cancerous cells by acting as a regulator of angiogenesis (Pavloff *et al.*, 1992; Altadilla *et al.*, 2009). TIMPs are also involved in the progression of other disorders, such as Sorsby's fundus dystrophy, which is caused by a mutation in TIMP-3 leading to dysfunctional regulation of angiogenesis (Anand-Apte *et al.*, 1997).

Metalloproteases can also be effectively inhibited by metal ion chelators, which remove the catalytic metal ion required for proteolysis and causing the active site to become inert. One example is captopril, an inhibitor for angiotensin converting enzyme involved in the conversion of angiotensin I to angiotensin II. It inhibits angiogenesis by restricting the movement of endothelial cells, and also possesses anti-tumour properties (Volpert *et al.*, 1996; Smith & Vane, 2003). Captopril is also a capable inhibitor of gelatinous A/MMP 2 activity, and can restrict the growth of lung tumours and metastases (Prontera *et al.*, 1999). Other metal chelators include EDTA, which has been demonstrated to be a very potent inhibitor of zinc proteases such as TeTx and BTx. At a concentration of 1mM, EDTA was

able to inactivate BTx N/D light chain domains responsible for botulinum toxin toxicity (Yamasaki *et al.*, 1994a).

1.1.4 Serine proteases

Serine proteases constitute the largest protease family in existence, and are involved in food processing, cell apoptosis, epidermal homeostasis and neuronal signalling. They are characterised by their use of a serine residue as part of the catalytic mechanism, which is usually accompanied by histidine and aspartate residues forming a catalytic triad. Some serine proteases exhibit atypical catalytic triads that utilise residues other than histidine or aspartate, but are still considered part of the serine protease family. One such example is aspartyl dipeptidase, in which the aspartate residue is replaced by a glutamate residue and is not inhibited by the general serine protease inhibitor PMSF (Conlin *et al.*, 1994). Other examples include the Ser/His/His triad utilised by the cytomegalovirus of the Herpes virus and the catalytic mechanism of sedolisin proteases that rely on a Ser/Glu/Asp triad. In addition to these variations of the serine protease triad, some serine proteases utilise a serine dyad at their active site, an example of which is one comprised of serine and lysine residues. Examples of serine proteases using this dyad active site can be found in E.Coli proteases, such as the Lon protease, type I peptidases and the C-terminal peptidases (Ekici *et al.*, 2008).

1.1.4.1 Structure and mechanism

The key catalytic process of a typical serine protease is dependent on a catalytic triad of Asp-His-Ser residues, and its efficiency can be modified by both endogenous and exogenous factors affecting the active site. The active site of subtilisin BPN' is maintained partly through the presence a hydrogen bond between the histidine and serine residues of the catalytic triad. A network of hydrogen bonds attached to the catalytic aspartic acid residue also contribute to the stability of the active site, with a hydrogen bond establishing a connection between the aspartic acid and histidine residues of the triad (Wright *et al.*, 1969; Kuhn *et al.*, 1998). Amino acids surround the catalytic triad also determine the rate of proteolysis, with the replacement of Ile at position 31 (adjacent to the catalytic Asp residue) with leucine resulting in a 2 to 6-fold increase in enzyme efficiency (Takagi *et al.*, 1988). Additional regulation in some serine proteases such as subtilisin BPN' is provided

by metal ion binding sites. In subtilisin BPN' this consists of 2 cation-binding sites, the first being a site specific for calcium, the second site capable of binding both monovalent and divalent metals with both sites working in unison to provide greater stability for the active site (Alexander *et al.*, 2001). Other examples of serine proteases requiring divalent metal ions include the intracellular subtilisins of gram-positive bacteria, which can be inactivated by addition of chelating agents that disrupt both protease structure and lowers their catalytic rate (Gamble *et al.*, 2012).

A major step in subtilisin-mediated proteolysis is the formation of a tetrahedral intermediate between the protease and the substrate, which facilitates hydrolysis. The structure of this intermediate is established and maintained by an oxyanion hole, which is supported by two hydrogen bonds between the active site residues Ser221 and Asn155. The oxyanion hole also ensures the release of the carbonyl oxygen during the transition from the tetrahedral intermediate into an acyl-enzyme. An additional stabilizing factor during this stage is the presence of a hydrogen bond donated to the target peptide by the enzyme residue Asn155. Replacement of this residue by leucine prevents this bond from being formed, and results in a reduction of the catalytic rate of subtilisin (Robertus *et al.*, 1972; Bryan *et al.*, 1986; Bryan P. *et al.*, 1986). The catalytic rate is also determined by the presence of the serine and histidine residues at the active, as loss or replacement of either residue causes a major decrease in the proteolytic rate. Concurrent loss of both residues results in the most significant decrease in catalysis (Perona & Craik, 2008). Temperature is also a major determinant of serine protease activity, which acts to decrease catalytic activity. This is evident at higher temperatures, as serine proteases that are active at this temperature lack intramolecular interactions, rendering them less stable. For this study subtilisin was chosen, as it is a bacterial protease with optimal function at physiological pH and temperatures, and is therefore suitable for use with in-vitro preparations (Tiberti & Papaleo, 2011).

1.1.4.2 Regulation of serine proteases

Regulation of serine protease action is crucial to normal biological function due to their involvement in many physiological processes. Subtilisin expression is regulated by signaling via inhibitory molecules and autoproteolysis, which is dependent on the autolytic and conformational stability of the enzyme (Wells & Powers, 1986). Changes to the extracellular environment can also be a factor in the suppression several subtilisin-like proteases. For example, fasting decreases the level of proprotein convertase

subtilisin/kexin type 9 (PCSK9) whilst simultaneously increasing cholesterol synthesis, with both regulated by the sterol response element-binding protein (SREBP) 2 (Browning & Horton, 2010). A further regulatory mechanism exists in some serine proteases involving the formation of a complex between the protease and other proteins in a process that increases the removal rate of the serine protease. An example can be found in the removal of subtilisin A from blood, which forms a complex with α-macroglobulin and accelerates the process. (Mitsudo *et al.*, 2003).

1.1.4.3 Inhibition of Serine Proteases

Serine proteases are inhibited by a wide range of both synthetic and biological compounds, reflecting the large number of proteases present in this family. Amongst biological inhibitors, serpins are distinguished as one of the earliest serine protease inhibitors to be discovered, and is also one of the most extensively studied. Serpins are a superfamily of proteins that mainly inhibit serine proteases belonging to the chymotrypsin subfamily, and regulate activities such as blood clotting via anti-thrombin, or neurological processes via neuroserpin. Serpins are also found in the immune system, where they inhibit endogenous enzymes responsible for apoptosis, as well as bacterial proteases such as subtilisin. In the central nervous system, neuroserpin acts as the main regulator for tissue plasminogen, a serine protease with a broad specificity of action involving processes such as neuritogenesis. The neuroserpin-plasminogen interaction is also involved in synaptic plasticity, and also affect learning and memory processes (Miranda & Lomas, 2006).

Likewise, serine protease inhibitors also affect normal physiological function by directly antagonizing the activities of serine proteases and can also determine cell susceptibility to disease. They can prevent cellular damage by endogenous proteases, such as neutrophil-mediated endothelial cell damage. The effects of neutrophils are partly mediated through the release of elastase and other serine proteases, and are inhibited by urinary trypsin inhibitors. These inhibitors are able to inhibit both the extracellular release of elastase by neutrophils as well as decreasing their production and secretion (Nakatani *et al.*, 2001). Serine protease inhibitors are also capable of preventing some autoimmune diseases, such as Netherton syndrome. This disease is caused by mutations in the Spink5 gene which encodes a serine protease inhibitor effective against a range of serine proteases such as subtilisin A and trypsin (Mitsudo *et al.*, 2003). Removal of this serine protease inhibitor also has an adverse impact on the severity of acne rosacea, a disorder caused by an

epidermal serine proteases known as the which is caused by stratum corneum tryptic enzyme (Yamasaki *et al.*, 2007).

In addition, invasion of host cells by pathogenic organisms is also severely restricted by the presence serine protease inhibitors, due to their reliance on serine proteases to facilitate cell penetration. This is demonstrated by the activity of toxoplasma gondii in the presence of two irreversible serine protease inhibitors, which prevents cellular penetration by the parasite in a does-dependent manner (Conseil *et al.*, 1999).

1.1.4.4 Regulation of the cytoskeleton by serine proteases

In addition to adhesion molecules, serine proteases are also capable of targeting other elements of the cytoskeletal structure. A serine protease known as alkaline serine protease exists in the muscle cells of lobsters, where they degrade troponin-C and myosin and help protein turnover within the contractile apparatus (Mykles, 1989). Similar proteases also exist in fish as myofibril-bound serine proteases, although in addition to degrading myosin, actin, tropomyosin and α-actinin are also targets for proteolysis (Cao *et al.*, 2000). Examples of mammalian serine proteases that target cytoskeletal proteins also exist, such as the trypsin-like serine proteases found within intestinal smooth muscle cells of rats. These act as regulators of enzyme catabolism and inactivate native enzymes (Beynon & Kay, 1978). The subtilisin-like proprotein convertase PACE4 is also known to be a regulator of myosin light chain expression, and is a factor controlling myogenic differentiation via the IGF-II pathway (Yuasa *et al.*, 2009).

1.1.4.5 Serine proteases and regulation of Hemostasis

Serine proteases are also intimately involved in haemostasis, with notable roles in platelet formation and control of blood pressure. PCSK-9 is a regulator of epithelial Na^+ channels (ENaC) and can reduce their surface and total cellular expression. This protease induces a change in intracellular trafficking of ENaC subunits by directing more subunits to the proteasomal compartment without impacting on channel endocytosis or cell surface degradation (Sharotri *et al.*, 2012). A serine protease, thrombin, is produced as the final protease of the blood coagulation cascade and as such, it has a crucial role in formation of blood clots by acting on a number of different targets. Amongst these targets are the metalloprotease TAFI, platelet factor XIII and fibrinogen, which undergoes proteolytic activation to produce fibrin. These three proteins are essential to the formation of a stable blood clot. Thrombin also acts as the ligand for protease-activated receptor 1 and 4, which

activate platelets in a process accelerated by the cofactor glycoprotein Ibα (Ramakrishnan *et al.*, 2001; Huntington, 2005).

1.1.4.6 Serine Proteases and the Immune System

Serine proteases form a crucial part of the immune response in mammals, where they have a role in initiating various immune pathways such as the complement system, mediation of the inflammatory response and the elimination of foreign bodies.

1.1.4.6.1 Activators of the Complement Pathway

Members of the serine protease family can be found in the complement system, such as the serine protease complement factor D, which unlike most serine proteases does not require proteolytic activation in order to function. Instead, it is activated by the substrate, complement factor B, a serine protease which represents an important regulatory step for t alternative complement pathway activation (Volanakis & Narayana, 2008). The complement system can also be initiated via the lectin pathway, with activation through either lectinsficolin or mannose-binding lectin (MBL). Both processes involve two MBL-associated serine proteases that are activated in response to lectin signalling and possess proteolytic activity against the complement proteins C2, C3 and C4 (Matsushita *et al.*)

1.1.4.6.2 Mediators of Innate Immune Response

In addition to their role in initiating immunological pathways, some serine proteases can also act directly on invading pathogens, such as neutrophil elastase and granzymes found within white blood cells.

Granzymes are a group of proteases stored within the lytic granules of cytotoxic T cells and natural killer cells, and function as initiators of cell death by utilising the pore-forming protein perforin to trigger cell apoptosis. The role of each member of the granzyme family varies and in humans 5 members are known to exist, denoted as granzymes A, B, H, K and M (Zhang *et al.*, 2001).

Granzyme A activates a DNAse which cause nicks in single-stranded DNA, a feature that characterizes the granzyme A-driven alternative apoptotic pathway (Fan *et al.*, 2003). In addition, granzyme A cleaves laminin proteins A, B and C, which are key elements in maintaining the integrity of the nuclear envelope. Laminin B is also a target for the related granzyme B, although this occurs at a much lower rate, and the protease is completely

ineffective against laminin A and C (Zhang *et al.*, 2001). Granzyme K, which is located on the same chromosome as granzyme A, has similar properties with the latter and act on the same SET complex as granzyme A. However, expression of granzyme K is lower than granzyme A in cytotoxic cells, but both share partial functional redundancy (Zhao *et al.*, 2007). Granzyme H is known for its antiviral properties, which can be traced to their proteolytic action against the adenovirus DNA-binding protein, an essential component necessary for viral DNA replication. This granzyme also has a secondary role as an activator of granzyme B by cleaving the inhibitory 100K assembly protein (Andrade *et al.*, 2007). In comparison to these, granzyme M is mainly expressed in natural killer cells, but is also capable of causing DNA damage in a similar manner to granzyme B by inactivating the inhibitor for caspase-activated DNAse. An additional target of this granzyme is the structural protein α-tubulin, which cause a disorganization of the microtubule network and thereby increasing the susceptibility of the cell to apoptosis (Lu *et al.*, 2006; Bovenschen *et al.*, 2008).

Neutrophil elastase is found within neutrophils, where they co-localise with proteinase 3 and cathepsin G. They belong to the chymotrypsin subfamily of serine proteases, and can mediate inflammation by upregulating interleukin-8 through toll-like receptor 4 signalling. Neutrophil elastases also possess anti-bacterial properties, and are actively involved in processing bacteria for destruction within phagosomes. They restrict the spread of these bacteria by cleaving their virulence factors, lowering both the spread and survival rate of the bacteria (Weinrauch *et al.*, 2001; Devaney *et al.*, 2003). This neutrophil serine protease also promotes blood coagulation in conjunction with the endogenous cathepsin G, although these effects are not systemic, but localized to the thrombus. This mechanism is utilized by the immune system to control bacterial spread during infection, by restricting their presence to a limited number of microvessels (Massberg *et al.*, 2010). During infection serine proteases can also direct the migration of immune cells to the infection site by activating the chemoattractant ligand chemerin, which binds to receptors found on the surface of dendritic cells and macrophages (Zabel *et al.*, 2005).

1.1.4.7 Serine Proteases and Diseases

Serine proteases are commonly found in disease-causing organisms, where they have roles such as assisting cell penetration, facilitating the release infective spores or help regulate the pathogenic maturation process. The activities of viral and bacterial serine proteases are clinically important, as they enable the survival of well-known pathogenic organisms such as the HIV virus and E.Coli. In addition, mutations to endogenous serine proteases in the

body can also increase susceptibility to pathogenic invasion of the body, and underlie a number of neurological disorders.

1.1.4.7.1 Serine Proteases and Pathogenic Disease

Pathogenic organisms such as viruses are highly dependent on serine proteases for multiple stages in their life cycle. The viral serine protease VP4 plays an essential role in processing both avian and fish birnavirus through the use of a serine-lysine catalytic dyad (Lejal *et al.*, 2000). Another example is the hepatitis C virus, which undergoes processing by the NS3 serine protease prior to activation and release. This process is dependent on an induced-fit stabilisation of the catalytic His-Asp hydrogen bond by the substrate, a catalytic step common to many viral proteases (Tomei *et al.*, 1993; Barbato *et al.*, 2000). Furthermore, serine proteases are involved in the activation of HIV envelope proteins by furin, a protease which works in conjunction with proprotein convertase 7 to cleave HIV glycoprotein 160 (Hallenberger *et al.*, 1997).

Serine proteases are also expressed in E.Coli, where they are perform crucial roles to enable the normal function of this bacterium. These include signal peptidase (SP), which removes the N-terminal signal peptide of proteins that have been translocated across the membrane; and the UmuD protease, which is involved in the cellular SOS response to DNA damage. Both proteases utilise the same unusual serine-lysine dyad catalytic mechanism, and share the same arrangement of residues within their active site. Activation of UmuD leads to the inactivation of another serine protease, LexA, which allows SOS genes to become activated (Paetzel & Strynadka, 1999; Luo *et al.*, 2001). Other notable serine proteases in E.Coli include Lon protease, which shares the same catalytic dyad mechanism as SP and UmuD. It regulates the degradation of short-lived regulatory proteins, and also assists in the removal of abnormal or defective proteins (Botos *et al.*, 2003). E.Coli also express the heat shock-inducible protein HtrA, which has serine protease activity and is homologous to the mammalian HtrA2 protein. It promotes cell apoptosis by suppressing the activity of inhibitors against apoptotic proteins. This allows the activation of caspase protease family members, in particular the initiator caspase 3 that triggers the apoptotic pathway (Verhagen *et al.*, 2002).

1.1.4.7.2 Serine Proteases in Neurological Illness

Serine proteases are intimately involved in many physiological processes, and can also be found in the nervous system. Defects in either expression or function of these proteases

underlie several neurological diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis. As an example, the expression of the apoptotic HtrA2 protease is related to the onset of both Alzheimer's and Parkinson's disease, as a disruption to their expression increases susceptibility of striatal neurons to degradation. This is also supported by animal models of the disease, in which mice treated with HtrA2 display symptoms characteristic of the onset of Parkinson's disease. A mutation within the HtrA2 gene can also be found in many patient's suffering from Parkinson's disease, and this compromised mitochondrial function and increased the likelihood of apoptosis (Martins *et al.*, 2004; Strauss *et al.*, 2005). This protease is also linked to amyloid beta activity, a protein that constitutes a major component of amyloid plaques found in the neural tissue of patients suffering from Alzheimer's disease. Intracellular amyloid beta induces neuronal apoptosis via an interaction with HtrA2, and has a potential role in modulating the expression of amyloid processing protein (Park *et al.*, 2004). Other serine proteases affected by AD and PD includes neurosin, which is localized to senile plaques in Alzheimer's disease and to lewy bodies in Parkinson's disease. In both diseases they are severely downregulated in the affected brain regions of patients and are expressed at a lower concentration in the CSF of patients with AD (Ogawa *et al.*, 2000; Mitsui S *et al.*, 2006; Mitsui *et al.*, 2006). Neurosin is also associated with the degradation of myelin proteins such as myelin basic protein and myelin-associated protein, and remyelination of oligodendrocytes after central nervous system insult. This is further supported by a correlation between neurosin and myelin expression levels in a mouse model of multiple sclerosis, with demyelination decreasing and remyelination increasing neurosin expression (Terayama *et al.*, 2005; Bando *et al.*, 2006).

1.1.4.8 Serine Proteases and the Nervous System

Serine proteases also perform important physiological functions in the nervous system. A well-known CNS serine protease is neuropsin, which modulates the turnover of adhesion molecules. Through this mechanism, it regulates the synaptic morphology of neurons and is involved with both the early stage of LTP generation and LTD. Both forms of neuropsin-mediated synaptic plasticity are present postsynaptically, in a manner similar to electrically induced synaptic changes. The action of this protease at a macromolecular level also impacts on physiological function, as inhibition of neuropsin attenuates both the generation of LTP and memory formation in mice (Tamura *et al.*, 2005). Neuropsin is highly expressed in the hippocampus, where it acts as a regulator of LTP and is associated with increased levels of the LTP indicator protein zif268. Its expression is elevated in

response to stimulation, and is one of contributing factors for hippocampal kindling (Chen *et al.*, 1995) The effects of neuropsin are partly mediated through the degradation of fibronectin and collagen, which can disrupt the extracellular environment and affect the stability of neuronal connections (Rajapakse *et al.*, 2005). In hippocampal Schaeffer collateral axons, neuropsin selectively cleaves the synaptic adhesion molecule L1 associated with the development of spatial memory. Neuropsin-dependent LTP relies solely on its proteolytic activity proteins of the extracellular matrix and has no direct effect on NMDA receptors. This is significant as all other forms of LTP are characterised by an NMDA-dependent increase in calcium levels (Komai S *et al.*, 2000; Komai *et al.*, 2000; Matsumoto-Miyai *et al.*, 2003).

1.1.4.9 Subtypes of Serine Proteases

Within each group of serine proteases, members are further classified into distinct clans, and several clans have been particularly well characterised and extensively studied. This includes type II transmembrane protease, tissue kallikrein-related proteases, chymotrypsin and subtilisin groups. Trypsin and chymotrypsin members are two of the most intensively studied members as these were amongst the first serine proteases to be discovered.

1.1.4.9.1 Chymotrypsin subfamily

The trypsin clan of serine proteases are distinguished by an active site containing a His-Asp-Ser catalytic triad, and regulate a number of biological functions such as digestion and synaptic plasticity. They are further subdivided into subfamilies, the most prominent of which are the type II transmembrane proteases, tissue kallikrein-related peptidases, trypsin and chymotrypsin.

1.1.4.9.1.1 Type II Transmembrane Proteases

These membrane-bound serine proteases are found in all vertebrates, where they interact directly with extracellular signalling cues and neighbouring cells (Kitamoto *et al.*, 1995; Hooper *et al.*, 2001) Their main role is to process other digestive proteases, although they also have roles in the regulating cardiac function, auditory function and epithelial homeostasis. An example of an epithelial serine protease is matriptase, which regulates prostatin and controls the initiation of a zymogen cascade crucial to epithelial differentiation (Westler *et al.*, 2002; Szabo & Bugge, 2008). Other members of this clan,

such as CAP1/PRss8, are responsible for maintaining the integrity of the fully formed epithelial barrier. Mutations of this serine protease can result in a loss of occludin and disrupt the function of tight junctions (Leyvraz *et al.*, 2005).

1.1.4.9.1.2 Tissue Kallikreins

These proteases were initially investigated as regulators of blood pressure through bradykinin receptors. Subsequent discovery of kallikrein-related peptidases also demonstrated that this group of serine proteases contribute to epidermal cell desquamation and may also act as possible tumour cell markers (Leeb-Lundberg *et al.*, 2005; Lundwall & Brattsand, 2008).
Notable members of the kallikrein-related peptidase family include kallikrein related protease 8 (neuropsin) which has a major role in regulating brain plasticity, and kallikrein-like protease 6 (neurosin), which is a risk factor for Alzheimer's disease (Mitsui *et al.*, 2006).

1.1.4.9.1.3 Trypsins

Trypsins are well known for their involvement in the digestive process, where they are initially synthesised as inactive trypsinogen molecules. Proteolytic cleavage of this precursor activates the protease by changing the position of active site residues to form a more ordered arrangement. Due to this important physiological role of trypsin, aberrant expression can cause digestive tract disorders such as hereditary pancreatitis, which occurs due to a mutation in trypsinogen. Trypsin also promotes pathological tumour cell growth by activating MMPs, and some members of the trypsin family possess metalloprotease elements are sensitive to changes in metal ion concentrations. One such example is the trypsin-like protease NS3 proteinase, which possesses a zinc-binding domain, supporting the possibility that other serine proteases may not solely rely on serine protease action, but can incorporate mechanisms of other protease families such as metalloproteases (Khosravi-Far *et al.*, 1995; Love *et al.*, 1996; Kukor *et al.*, 2002).

1.1.4.9.1.4 Chymotrypsins

Chymotrypsins are secreted by pancreatic cells in a manner similar to trypsin, but are separated and distinguished as a different clan of serine proteases due to variations in peptide specificity. Both types of serine proteases share a high degree of structural

homology, and trypsin can acquire chymotrypsin-like properties by a combination of S1 site replacement and addition of chymotrypsin surface loops. Trypsin modified in such a manner utilises extended loops in a similar way to chymotrypsin proteolysis, which improves substrate binding. This change converts the normal trypsin specificity for basic side-chains to a preference for hydrophobic side-chains characteristic of chymotrypsin action (Hedstrom *et al.*, 1992). The mechanism of catalysis of both chymotrypsin and trypsin are typical of the serine protease family, involving the formation of an enzyme-substrate complex acting as a tetrahedral intermediate during proteolysis (Bender & Kezdy, 1964).

1.1.4.9.2 Subtilisins

The subtilisin family of serine proteases encompass a number of physiologically important members. Of particular importance are the serine proteases belonging to the proprotein convertase subfamily, and the subtilisin/subtilisin-like proteases.

1.1.4.9.2.1 Proprotein convertases

The proprotein convertase family of subtilisin proteases is involved with a wide range of cellular activities, including regulation of plasma low-density lipoprotein (LDL) levels. This process is regulated by proprotein convertase subtilisin/kexin type 9 (PCSK9), which selectively directs the transport of LDL receptors to lysosomes by interacting with amyloid precursor proteins. These associations are mediated through apolipoprotein B, which prevent autophagic degradation of LDL and increasing the amount available for the vesicular LDL formation (Sun *et al.*, 2012; DeVay *et al.*, 2013; Kosenko *et al.*, 2013). Insulin is also an endocrine target of the subtilisin-like proprotein convertases, with members furin, PCSK2 and PCSK3 responsible for proteolytic activation of proinsulin in islet β cells (Smeekens *et al.*, 1992).

1.1.4.9.2.2 Subtilisin and Subtilisin-like Proteases

The subtilisin subfamily is a group of well-known serine proteases widely used in commercial applications and as a research tool for selective studies on protein modification. They were named after the bacteria, bacilis subtilis, from which these serine proteases were initially extracted. Well-known members include subtilisin BPN' and subtilisin Carlsberg (subtilisin A), and there are over 100 subtilisin-like proteases, known

as subtilases, which share similar active site sequences with established subtilisins (Siezen & Leunissen, 1997). Subtilisin and subtilisin-like proteases are synthesised as precursor proteins consisting of a regulatory signal peptide sequence for secretion connected to the active enzyme domain via an amino acid sequence. This signal peptide directs the initial protease localisation to the cell membrane, undergoing subsequent autolytic cleavage to release the mature enzyme. In the absence of autocatalytic proteolysis, the activation process can be triggered using other subtilisins (Power *et al.*, 1986; Ikemura *et al.*, 1987). Activation of subtilisin BPN' involves dimerization of two precursor subtilisin molecules, which provide mutual assistance to activate each other (Hu *et al.*, 1996). The structure of mature subtilisin enzymes consists of a single polypeptide chain folded into three parts linked by hydrogen bonds, with the active site at its core (Wright *et al.*, 1969).

1.2 Long-term depression

The heterosynaptic form of long-term depression was first characterised in the 1980s, where it was discovered that conjunctive stimulation of climbing fibres could elicit a long-lasting decrease in synaptic signal at parallel fibre-Purkinje cell synapses (Ito & Kano, 1982). This effect had previously been proposed to explain the findings of (Lynch *et al.*, 1977), which suggested that LTP would be accompanied by a concurrent decrease in synaptic signalling in neighbouring cells. Subsequent experiments also demonstrated the existence of homosynaptic LTD, which could be triggered by repetitively stimulating at a frequency of 1-3 Hz (Dudek & Bear, 1992). This represented one of the first experiments related to LTD, and subsequent studies have shown the existence of LTD paradigms in other regions of the brain, such as the hippocampus. LTD generated in the hippocampus has been extensively studied due to its relation to both the formation and storage of memory.

1.2.1 LTD and LTP

Long-term depression (LTD) and long-term potentiation (LTP) represent two opposing types of long lasting alterations to nervous system signalling. Long-term depression describes a process by which a stimulus elicits a sustained decrease in the size of an evoked potential, whereas long-term potentiation has the opposite effect of causing a long-

lasting increase in potential size. The relationship between these effects is most notably demonstrated during the generation of heterosynaptic LTD, and can be interchangeable under certain conditions. Heterosynaptic LTD can be generated in untetanised hippocampal CA1 neurons in response to the expression stimulation-induced LTP in neighbouring cells. This process is dependent on the stimulation-induced release of ATP by astrocytes, which spatially sharpens the initial LTP effect (Chen *et al.*, 2013).

The neuronal expression of either LTD or LTP is regulated by changes to the basal levels of intracellular calcium, with raised levels promoting LTD, and lower levels promoting the formation of LTP. Both processes involve AMPA receptors, but their expression levels are either increased or decreased reflecting the opposing effects of LTP and LTD (Beattie *et al.*, 2000; Xiao *et al.*, 2001).

These two synaptic processes can be regulated by growth factors, such as brain-derived neurotrophic factor (BDNF), which promotes the generation of LTP by activating the tyrosine kinase B (TrKB) receptor. Blockading these receptors using inhibitors prevents LTP and instead facilitates a switch from LTP to LTD (Montalbano *et al.*, 2013). Curiously, the BDNF precursor pro-BDNF has the opposite effect to active BDNF, and promotes the expression of LTD. This is partly explained by pro-BDNF targeting the p75 neurotrophin receptors (p75NTR), in comparison to BDNF, which targets TrKB receptors. Regulation of these two processes by BDNF and pro-BDNF supports the involvement of growth factors in long-term modulation of synaptic signalling (Woo *et al.*, 2005). Types of LTD are categorised based on the mechanism used to elicit these effects, with the most well-known being NMDAR or mGluR-mediated LTD.

1.2.2 NMDA receptor-dependent LTD

LTD elicited by n-methyl-D-aspartate (NMDA) receptors was first discovered as a contributing factor to homosynaptic LTD. Subsequent studies indicated that this type of LTD could also be triggered by low frequency stimulation (LFS). NMDA-dependent LTD involves activation of L-type voltage-gated calcium channels, independent of an increase in intracellular calcium levels. The mechanism by which NMDA receptors trigger LTD is however dependent on a clathrin-based endocytosis of AMPA receptors, a process that occurs after an influx of calcium. This is regulated by the calcium sensing protein hippocalcin that binds to a subunit of the AP2 adaptor complex, a protein acting as a link between clathrin and the proteins to be endocytosed (Palmer *et al.*, 2005). This type of

AMPAR-dependent LTD is stabilised by interaction between the AMPAR-binding protein (ABP) and glutamate receptor interacting protein (GRIP), which is bound to AMPA receptors during LTD. Failure of this association destabilizes the LTD effect, an event that can be mediated through the PICK 1 calcium sensor. The PICK 1 protein also regulates actin turnover at the synaptic site by binding F-actin and promoting the depolymerisation of actin fibers, thereby modifying neuronal morphology (Rocca *et al.*, 2008). The need for F-actin turnover in NMDAR-dependent LTD was also demonstrated by studies where the addition of either the actin stabilizer phalloidin, or a cofilin inhibitory peptide prevented NMDA-mediated LTD. This study also found that changes to actin were regulated by calcium through the calcium-dependent serine-tyrosine phosphatase, calcineurin (Morishita *et al.*, 2005). Additionally, NMDAR-LTD is involved in the lateral transport of these receptors, a process regulated by actin and sensitive to the action of actin stabilizers such as Jasplakinolide, which prevents actin depolymerisation (Ireland & Abraham, 2008)

Other cytoskeletal proteins are also associated with LTD, but mostly through indirect mechanisms by disrupting the cell structure. Silencing the microtubule-associated protein 1B changes synaptic plasticity and impairs LTD generation. This was partly due to the targeting of TIam1, an activator of Rac1 GTPase, which has a downstream effect on actin (Rio *et al.*, 2004). A number of protein kinases, such as Cdk5 (cyclin-dependent kinase 5), are also involved in the progression of NMDA-dependent LTD. Cdk5 activity is linked with that of the nuclear activator p35, and decreasing its expression impairs the generation of both LTD and LTP. This in turn can lead to deficiencies in spatial learning and memory (Ohshima *et al.*, 2005). Glycogen synthase kinase 3 is also a contributor to the induction of LTD, and its expression is further enhanced by LTD via protein phosphatase 1 upon the generation of LTD. Conversely, inhibition of this kinase is also able to inhibit NMDA-dependent LTD (Peineau *et al.*, 2007).

The different subunits of the AMPA receptors are also important to the progression of NMDAR-LTD, with the GluR1 subunit being a particularly important component. This AMPA subunit is normally constitutively phosphorylated by protein kinase A under physiological conditions, however during LTD it undergoes targeted dephosphorylation (Sandkühler J *et al.*, 1997; Lee *et al.*, 1998; Chamberlain *et al.*, 2013).

1.2.3 mGluR-dependent LTD

LTD can also be elicited by metabotropic glutamate receptors (mGluRs), and differ from NMDAR-dependent LTD in several aspects. In the hippocampal CA1 region, they can be triggered through the action of both mGluR1 and mGluR5. In the cerebellum and perihinal cortex, the mechanism for mGLuR-dependent LTD also involves several kinases. Cerebellar mGluR-dependent LTD requires activation of protein kinase C (PKC), as demonstrated by a loss of LTD induction in the presence of PKC inhibitors, and the LTD-mimicking effect of the PKC agonist phorbol-12, 13-diacetate. In the cortex, induction of mGluR-LTD involves a complex formed from the neuronal Ca^{2+}sensor (NCS) protein NCS-1 and PICK 1, proteins that detect changes to the intracellular levels of calcium (Jo *et al.*, 2008). Experiments using hippocampal slice preparations have also demonstrated the necessity of p38 MAP kinase and protein tyrosine phosphatase activation during the induction of synaptically-induced mGluR-LTD (Moult *et al.*, 2008). In addition to kinase, mGLuR-mediated LTD in the mature synapses of the adult brain is accompanied by an increase in protein synthesis. In contrast, mGluR-mediated LTD in the developing nervous system is not associated with any form of protein synthesis, but cause a large decrease in presynaptic function that is absent from the mature synapses. This data indicates within the same type of LTD, different individual mechanisms may be employed under different environmental conditions (Nosyreva & Huber, 2005). At GABAergic synapses, mGluR-dependent LTD cause a decrease in GABA, a process regulated via endocannabinoid signalling (Chevaleyre & Castillo, 2003).

1.2.4 Other forms of LTD

Other forms of LTD are less well-characterised, but several are known to share similarities in mechanism with either NMDAR-dependent or mGLuR-dependent LTD. High frequency stimulation paradigms, previously associated with the formation of LTP alone, are now known to cause of several types of LTD in a number of brain regions. Patterned high frequency stimulation can elicit a type of LTD dependent on AMPA receptor endocytosis, whilst being independent of NMDA receptor signalling. Instead, it requires co-activation of both GABAA and muscarinic acetylcholine receptors that help maintain low levels of postsynaptic depolarisation, a factor which facilitates LTD induction (Zhua *et al.*, 2013). High frequency stimulation is also the basis for long-term depression in kainate receptors,

which relieves slow hyperpolarisation in hippocampal mossy fibre synapses. It is dependent on the activation of adenosine A2A receptors, a process also involved in NMDA-dependent LTP; however, kainate-dependent LTD do not involve NMDA receptor signalling (Chamberlain *et al.*, 2013).

In addition, the endocrine signalling molecule insulin is also capable of generating LTD. This form of LTD is dependent on a concurrent rise in intracellular Ca^{2+} and the activation of protein kinases including phosphate kinase C and phosphatidylinositol 3-kinase (PI3K). It is however independent of either NMDA or mGLuR input, as demonstrated through the use of kynurenic acid, an NMDA receptor blocker, and MCPG, a broad-spectrum metabotropic glutamate receptor antagonist. Both compounds failed to prevent the induction of insulin-LTD, which is mechanistically separate from LTD generated by low frequency stimulation. An interesting feature of this type of LTD is the existence of regional differences in the mechanism used to elicit LTD. In hippocampal neurons, the effects of insulin-LTD are mediated through a clathrin-dependent endocytosis of the GluR2 AMPA receptor subunit present on the postsynaptic membrane, a process shared with LFS-induced LTD. In the neurons of the ventral tegmental region however, insulin induces LTD in excitatory synapses by inhibiting the release of presynaptic glutamate. This is regulated through endocannabinoid signaling, requiring the activation of a receptor tyrosine kinase (Schnabel *et al.*, 1999; Labouèbe *et al.*, 2013).

1.2.5 Serine protease-dependent LTD: A novel form of LTD

A recent study discovered a novel form of LTD, which could be elicited by group S8A serine proteases such as subtilisin. The S8A group of serine proteases encompasses a number of proteins, including subtilisin A, proteinase K, cadeprin and α-chymotrypsin, which are all capable of eliciting LTD (MacGregor *et al.*, 2007). Initial studies suggested that this type of LTD was dependent on the proteolytic action of the serine protease, as LTD induction was sensitive to the action of protease inhibitors. Subsequent studies support the model of a proteolytic basis for subtilisin-mediated LTD, as this long-lasting decrease in synaptic signalling was accompanied by a selective degradation of actin, VAMP-1 and Unc5H3 proteins. This type of LTD was not NMDA or mGluR-dependent, although it shares some similarities in mechanism with other forms of LTD, such as the need for protein synthesis during the establishment of LTD, a process also present in electrically-elicited LTD. Subtilisin-mediated LTD is also sensitive to mGluR antagonists,

indicating a possible indirect role for mGluRs in this type of LTD. Studies have also found that subtilisin-mediated LTD involves proteasome mechanisms, although activation of caspases alone is not the sole cause of LTD (Forrest *et al.*, 2013).

Further evidence of the role of subtilisin in generating LTD was provided by behavioural studies in mice exposed to this protease. Injection of subtilisin into the dorsal hippocampus immediately after an inhibitory avoidance test caused noticeable deficits in memory formation in experimental mice. This impairment was dose-dependent, with effects being most prominent at higher levels of subtilisin infusion. These memory impairments reflect the role of other more established forms of LTD, such as NMDA-LTD, in the regulation of memory formation (Kornisiuk *et al.*, 2011).

1.2.6 Roles of LTD

Physiological roles for LTD have been difficult to ascertain with absolute certainty due to inherent difficulties of eliciting this effect in vivo, as well as the lack of LTD-specific inhibitors. However, over the decades mounting evidence has suggested that LTD is crucial to a number of neuronal processes, and in particular, the formation of memory in several brain regions.

1.2.6.1 Memory and Learning in the Hippocampus

The hippocampal region of the brain has long been thought of as a region heavily involved in the formation of new memories, and the existence of within the neurons of the CA1 neurons have been linked to the memory process. This is supported by evidence from numerous studies in which different aspects of the LTD process were inhibited, leading to disruptions in both morphology and functionality of neuronal structures. An investigation involving the inhibition of the serine/threonine protein phosphatase 2A, a protein necessary for LTD in the forebrain, demonstrated a decrease in NMDAR-dependent LTD. This decrease reduced the abilities of mice when tested in both water maze tests and T maze tests for place recognition (Nicholls *et al.*, 2008). Impairment of dopaminergic neurotransmission by inhibiting the dopaminergic transport protein also caused a decrease in the size of the hippocampal LTD at CA1-CA3 synaptic junctions. In addition, performance in the Morris water maze test was also reduced by an inhibition of the dopamine transporter, which links the effects of LTD to elements of behavioural-related

learning and memory (Morice *et al.*, 2007). Other functions of the hippocampus, such as novelty recognition, are also linked to the expression of LTD. LTD is facilitated by exploration of a novel environment containing either familiar or new objects, with both stimuli triggering effects via dopamine D1/5 receptors and 5-hydroxytryptamine 4 (5-HT4) receptors (Kemp & Manahan-Vaughan, 2004; Lemon & Manahan-Vaughan, 2006). These studies provide evidence of LTD involvement in hippocampal processes and in particular, with regards to learning and memory, that can have behavioural implications.

1.2.6.2 Stress-induced LTD and memory processes

Impairments in spatial memory retrieval within the hippocampus can be attributed to the induction of LTD. LFS-LTD is facilitated by the release of corticosterone, which is dependent on glucocorticosteroid receptor activation and protein synthesis. Antagonists of these processes are therefore capable of blocking the induction of LFS-LTD (Xu *et al.*, 1998). Corticosterone can also elicit mGluR dependent LTD in the hippocampus, as demonstrated by the formation of DHPG-dependent LTD in hippocampal slices of rats subjected to acute stress that caused an elevation of corticosterone levels. In contrast, hippocampal slices from control rats which were not subject to stress, and the resulting spike in corticosterone, did not respond to DHPG (Chaouloff *et al.*, 2007). NMDA-dependent LTD is similarly capable of causing memory impairments, and in particular, the GluN2B subunit is known to be the basis for NMDAR-LTD modulated disruption of memory retrieval. Addition of a GluN2B-selective antagonist promoted memory following acute stress, but produced no effect on controls, highlighting their crucial role in stress-induced memory impairment. General inhibitors of NMDA-dependent LTD is also capable of alleviating stress-induced memory impairments, and these investigations indicate that hippocampal LTD alone is capable of triggering memory impairments (Wong *et al.*, 2007).

1.3 Protein markers

Over the course of this study, the expression levels of several proteins were measured as part of the experiments performed. These encompass a wide range of proteins which are either relevant directly or indirectly to the function of the nervous system.

1.3.1 Netrins

Netrins are a group of signalling molecules that are expressed in several regions of the central nervous system. They act as regulatory factors direct the proper migration of neuronal cells, and provide either chemoattractive or chemorepulsive cues depending on the target receptor.

The balance between these two opposing netrin effects is crucial to the normal development of the nervous system, as demonstrated in animals with disrupted netrin-1 signalling. The loss of netrin can lead to abnormal axonal projections and in the hippocampus cause the failure of commissural axons to cross the midline. In turn, this causes a disorganisation of the neurons travelling to the ipsilateral septum, and the ectopic termination of netrin-1 mutant neurons (Barallobre *et al.*, 2000)

1.3.1.1 Netrins in chemoattraction

In the cerebellum, netrin-1 is released by cells of the lateral ganglionic eminence, acting as a chemoattractant signal to guide the migration of corticofugal neurons to the ganglionic eminence (Métin *et al.*, 1997). Netrin also attracts the neurons of the ventral midbrain, promoting dopaminergic outgrowth to this region and is one of several factors directing thalamorcortical axon projection to the internal capsule. Loss of these signalling cues cause disorganised axon projections through the ventral midbrain and a general misguidance of these neurons (Braisted *et al.*, 2000; Lin *et al.*, 2005). Netrin signalling can be transformed from a chemoattractant signal to a chemorepulsive one by external factors. An example can be found in Xenopus neurons, where the response to netrin is Ca^{2+}-dependent. Blockading Ca^{2+} entry switches cellular response to netrin, from a chemoattractive cue to a chemorepulsive signal (Hong *et al.*, 2000).

1.3.1.2 Netrin in chemorepulsion

In the striatal ventricular zone, netrin-1 is expressed as a chemorepulsive signal that drives the migration of subventricular neurons into the developing striatum (Hamasaki *et al.*, 2001). Netrin-1 is also responsible for directing the termination of dentate gyrus/hilus neurons in the CA3 region of the hippocampus, and inhibition of these signals has implications for memory processes (Steup *et al.*, 2000). Axon projections of spinal commissural neurons are also guided by chemorepulsive cues from netrin signalling. In

their absence, these neurons are unable to project medially and instead continue ventrally where they invade the motor column (Serafini *et al.*, 1996).

1.3.1.3 Mechanism of Action of Netrins

Netrin signalling is regulated by adenosine receptor A2β, and by laminin-1, both of which regulate netrin via cAMP-1 expression. One mechanism of netrin action involves regulating elements of the cytoskeleton within neurons. An example is the phosphorylation of the microtubule-associated protein 1B, which is phosphorylated by glycogen synthase and cyclin-dependent kinases in response to netrin signalling (Höpker *et al.*, 1999; Corset *et al.*, 2000; Rio *et al.*, 2004). Netrin is also able to regulate actin turnover via the Unc5H receptors, which cause an upregulation of actin polymerisation via Rho GTPases (Dent *et al.*, 2004).

1.3.1.4 Netrin Receptors

Netrin receptors are separated into two different groups: Deleted in Colorectal Cancer (DCC), which mediates chemoattraction in response to netrin; and the Unc5H family of receptors, which mediate chemorepulsion in response to netrin. These are often colocalised to the same neuronal pathways, but are generally expressed in different regions. Both types of receptors can be found in Schwann cells after spinal cord injury, where DCC is preferentially expressed in the injury site and the surrounding unmyelinated region, whilst Unc5H receptors are more abundant in the intact nerves (Webber *et al.*, 2011). In some instances both receptors are colocalised together at the same sites, as is the case in retinal ganglion cells, where both are downregulated in response to axotomy of dopamine-containing neurons (Ellezam *et al.*, 2001; Flores *et al.*, 2009). Developmentally, the expression of these two receptors is also linked in the spinal cord, where both receptors are expressed predominantly at different stages of development. During development, DCC receptors constitute the largest portion of netrin receptors being expressed in these neurons. In contrast, Unc5H receptors are the predominant form of netrin receptors in the adult, indicating a change in the role of netrin over this time period (Manitt *et al.*, 2004).

1.3.1.4.1 Deleted in Colorectal Cancer (DCC)

DCC is responsible for mediating the chemoattractant effects of netrin, and are found invarious neuronal processes. Expression of DCC in the locus coeruleus during development is responsible for directing neuronal migration from the dorsal rhombomere to the dorsolateral pontine tegmentum. In the forebrain, DCC guides the migration of luteinizing hormone-releasing hormone neurons from the vomeronasal organ (VNO) to the forebrain. Although both DCC and Unc5H3 are expressed in these cells, only DCC mutations causes abnormal neuronal migration, indicating a more important role for netrin chemoattraction in this process (Schwarting *et al.*, 2001; Schwarting *et al.*, 2004; Shi *et al.*, 2008).

DCC-mediated netrin chemoattraction also involves the BHLH transcription factors NSCL-1 and NSCL-2 as part of neuronal guidance during the formation of the precerebellar nuclei. Reducing the levels of these transcription factors induces an upregulation of Unc5H3 and a subsequent change to netrin chemorepulsion. Both effects act to halt neuronal migration and cause ectopic accumulation of these cells (Schmid *et al.*, 2007).

1.3.1.4.2 Unc5H Receptors

The Unc5H family of receptors consists of four members: Unc5H1, Unc5H2 Unc5H3 and Unc5H4, and are known as dependence receptors due to the presence of death domains which can trigger apoptosis. These apoptotic effects are usually prevented by netrin, which cause the multimerization of Unc5H receptors. The chemorepulsive response mediated through Unc5H receptors however requires a dimerization of Unc5H and DCC receptor cytoplasmic domains (Hong *et al.*, 1999). All members of the Unc5H receptor family mediate netrin chemorepulsion, although variations between members exist both in their expression and their response to netrin binding. These differences are further explored below, with particular emphasis on Unc5H1 and Unc5H3, which are of most relevance to this study.

1.3.1.4.2.1 Unc5H1

The Unc5H1 receptor expression can be found in neurons extending both dorsally and ventrally in the mouse hindbrain during development. They also regulate the dorsal projection of branchiomotor neurons, and are downregulated during initial acquisition of

trace memory in mice, encouraging axonal extension and formation of new nerve terminals (Barrett & Guthrie, 2001; Donahue *et al.*, 2002; Murray *et al.*, 2010). In the spinal cord, Unc5H1 has proapoptotic roles unrelated to netrin signalling, and is also the mediator through which netrin chemorepulsion is effected (Williams *et al.*, 2006). Unc5H1 expression is regulated by the Pax6 transcription factor, c-kinase 1 and protein kinase C, which forms a complex with Unc5H1 receptors to promote their endocytosis. The formation of such a complex can prevent growth cone collapse in response to netrin signalling, indicating the major role of these proteins in modulating neuronal migration and synaptic formation (Williams *et al.*, 2003; Numayama-Tsuruta *et al.*, 2010).

1.3.1.4.2.2 Unc5H3

In contrast to Unc5H1, which has a major role in regulating apoptosis, Unc5H3 is mostly utilised as a chemorepulsive receptor for netrin. Unc5H3 receptor expression is necessary for delineating the rostral cerebellar boundary during development. Mutations of this receptor can cause migrating neuroprogenitor cells to ignore chemorepulsive netrin cues, resulting in the ectopic expression of granule cells. As the position of granule cells are responsible for directing the subsequent migration of Purkinje cells, this also leads to ectopic Purkinje cell expression, leading to an overall reduction in the anatomical size of the cerebellum. These changes also decrease foliation of the cerebellum, producing motor function deficits in mice. Mutations in the gene encoding Unc5H3 receptors also cause ataxia and hyperactivity in experimental mice models (Przyborski *et al.*, 1998; Goldowitz *et al.*, 2000; Choi *et al.*, 2003). Migration of cerebellar interneurons in the first few postnatal weeks is guided by netrin-1 signalling through Unc5H3 receptors at short distances, without the involvement of DCC receptors. Migration within the corticospinal tract is also directed by Unc5H3 receptors. These neuronal movements can be abolished by mutations to the Unc5H3 receptor, causing ectopic migration and reducing the number of decussating neurons at the hindbrain-spinal cord junction (Finger *et al.*, 2002; Guijarro *et al.*, 2006). In dorsal root ganglia, neuronal migration is regulated by netrin-1 signalling through the Unc5H3 receptors expressed in proprioceptive neurons (Guana & Condic, 2003; Masuda *et al.*, 2008).

Both Unc5H3 and Unc5H1 are implicated in the dorsal migration of spinal accessory motor neurons away from the ventral midline region. In this process Unc5H3 signaling has a more prominent role as the loss of Unc5H3 alone can prevent cell migration, whereas loss of Unc5H1 did not interrupt the migration of these neurons (Dillon *et al.*, 2007). The apoptotic role of Unc5H3 is also highlighted in colorectal cancer cells, which express

lowered levels of this receptor. This corresponds to a decreased level of apoptosis, which enhances the spread of these tumour cells (Bernet *et al.*, 2007).

1.3.2 GTPases

Guanine nucleotide binding proteins are a family of enzymes that act by cycling between guanosine-diphosphate bound and guanosine-triphosphate bound states, and are commonly known as Guanosine triphosphatases (GTPases). They are involved in cellular activities such as intracellular vesicle transport, cytoskeletal organisation, cell morphogenesis and protein kinase cascades. The Ras GTPase superfamily is one of the most well known GTPase families, and consists of five groups: Rho/Rac, Ras, ARF, Ran and Rab GTPases. Members of the Rho GTPases in particular are of most interest to this current study (Wennerberg *et al.*, 2005).

1.3.2.1 Rho GTPases

Rho GTPases constitute a major element in the regulation of the actin cytoskeleton and focal adhesions in cells, and are responsible for the organization of actin filaments, specifically the formation of lamellipodia and filopodia (Nobes & Hall, 1999). They are regulated by the guanine nucleotide exchange factors (GEFs), which catalyse the exchange of GDP to GTP and thereby activating the GTPase. Conversely, GTPase activating proteins (GAPS) promote their intrinsic GTPase activity, leading to hydrolytic inactivation (Moon & Zheng, 2003; Rossman *et al.*, 2005). Rho GTPases are separated into different groups depending on their overall structure, the most well known members being Cdc42, RhoA and RhoB. In the context of the current study, RhoA and RhoB levels were measured due to their role as regulators of actin, a protein severely affected by the perfusion of subtilisin and other serine proteases used over the course of this study.

1.3.2.1.1 RhoA GTPase

A major role of RhoA is the regulation of the actin cytoskeleton via ROCK kinase, a process underlying many activities of RhoA such as the loss of cell adherence in response to Ras activation in cell cultures. This is due to either a disruption of acto-cytoskeletal components by RhoA, causing cell integrity to become compromised (Khosravi-Far *et al.*, 1995). RhoA downregulates nuclear migration of precerebellar neurons towards cells expressing netrin by signalling through ROCK, although high levels of netrin-1 are capable

of inhibiting ROCK action an negating the effects of RhoA. This process is caused by the maintenance of cAMP levels via a netrin-dependent mechanism, leading to increased intracellular levels of DCC receptors (Causeret *et al.*, 2004; Moore *et al.*, 2008; Li *et al.*, 2013).

RhoA is also involved in the reduction of growth cone size in response to Semaphorin3A signalling, by decreasing the availability of f-actin patches necessary for growth cone elongation. As a result, actin monomers are formed into intra-axonal F-actin bundles that interact with myosin II to cause axonal contraction. Semaphorin 4D is also capable of causing growth cone collapse via RhoA activation and utilises receptor tyrosine kinase ErB-2 as an intermediate. This actin-myosin response to RhoA expression is also utilised by BDNF to abolish chemoattractant signalling by Cdc42 (Swiercz *et al.*, 2004; Gallo, 2006).

RhoA also assists in axonal guidance by stabilising the region behind the growth cone and preventing the formation of lamellipodia in this area. This restricts growth cone turning by suppressing most protrusions, allowing it to migrate towards a chemoattractive signal (Loudon *et al.*, 2006).

1.3.2.1.2 RhoB GTPase

The gene encoding RhoB is one of a group of rapidly inducible genes activated in response to stimulation or retroviral protein-tyrosine kinases (Jähner & Hunter, 1991). It is a known inducer of cell apoptosis, and has potent inhibitory effects on cell transformation and human tumor growth. RhoB is a target for the tumor suppressing farnesyltranferase inhibitors, which act via RhoB to inhibit cell growth (Du *et al.*, 1999). RhoB itself possesses anti-tumour properties as a trigger for apoptosis, and restricting the activation of Akt kinases by insulin growth factor 1 (Chen *et al.*, 2000). Conversely, inhibition of RhoB via PI3K and Akt kinases represents a crucial step in Ras-mediated oncogenesis (Jiang *et al.*, 2004).

RhoB is also an important regulator of intracellular transport, and is responsible for directing movement of RhoB-containing endosomes to the cell membrane (Sandilands *et al.*, 2004). RhoB also regulates the localization of serine/threonine kinase PRK1 to endosomes and the intracellular trafficking of Cdc42 (Mellor *et al.*, 1998). RhoB also regulates the recruitment of Rac GTPase, allowing changes to the actin cytoskeleton at both central and peripheral sites in preparation for cell migration (Huang *et al.*, 2011).

Recently, RhoB was found to mediate long-term synaptic changes. Its expression is associated with the induction of early-LTP, and by acting through cofilin, operates as a

modulator of spine and dendritic morphology (McNair *et al.*, 2010).

1.3.3 Actin

Actin are cellular proteins that are organised into filaments providing mechanical support to cells and form the primary mechanism for cell motility. They are responsible for transport of vesicles within cells, and assist in the formation of cell-cell junctions necessary for the integrity of multicellular organisms (Pollard & Cooper, 2009). Three different isoforms of actin exist in vertebrates: α, β and ϒ, which are differentiated by changes to the N-terminus. Actin belongs to superfamily of sugar kinases, and fold into two major A/B domains (Bork *et al.*, 1992).

In cells actin either exist as individual G-actin monomers, or are joined together as F-actin filaments. F-actin formation and extension involve addition of ATP-bound G-actin monomers to the positive, or barbed end of the filament. Conversely, decreases in filament length involve the dissociation of ADP-bound G-actin monomers from the negative, or pointed end through the inherent catalytic ATPase mechanism of F-actin. Actin dissociation from the filament is also aided by weakening the bonds binding ADP-actin to the actin filament at the pointed end. The relative balance between the rate of these two activities regulates the overall filament length (Dominguez & Holmes, 2011). F-actin is essential to the formation and regular function of the cell cytoskeleton, and consists of two G-actin actin chains formed into a left-handed helix. Extension and contraction of actin filament length are regulated by the activities of nucleation factors at either end of the filament. Known regulatory factors for actin include cofilin, DNase I, cordon-bleu and profilin. Cofilin and the actin binding protein DNAse I cooperatively bind actin in the cytoplasm and are colocalised with actin in vivo. Cofilin is expressed in both the cytoplasm and the periphery, where it promotes actin depolymerisation, cell protrusions and drives cell migration. In contrast, DNase I expression is limited to the cytoplasm (Chhabra *et al.*, 2002).

Polymerisation of actin is promoted by profilin, which has a high affinity for G-actin monomers that are bound to its C-terminal via an α-helix. Profilin forms a complex that sequesters actin monomers, and stabilises them via a catalytic exchange of actin-bound ADP for ATP in a process that increases the ability of the actin monomer to become polymerised. Profilin activity is more potent at the pointed end of actin filaments and are highly expressed in dynamic cells where there is a need for constant actin turnover, and is

expressed at lower levels in more mature, stable cells (Schluter *et al.*, 1997). Cordon-bleu is another promoter of actin filament length, and act by increasing the assembly of cross-linked actin monomers at the barbed end of actin. It binds actin via three WASP 2 domains (wiskott-aldrich syndrome protein homology 2) and has a key role in increasing neurite outgrowth and branching. Inhibition or loss of cordon-bleu can result in an attenuation of dendritic growth (Ahuja *et al.*, 2007).

Actin filaments, in conjunction with myosin, form part of the acto-myosin mechanism underpinning cell contraction in smooth and striatal muscle cells (Hanson & Lowy, 1963; Graceffa & Dominguez, 2003). Actin-myosin interactions involve a strong association between the DNase-binding loop on actin and the head of myosin, which is stabilised by loops present in both actin and parts of the myosin head (Lorenz & Holmes, 2010).

1.3.3.1 Actin in cell migration and elongation

Cell migration depends on two processes regulating the actin cytoskeleton at the leading edge. Lamellipodium is transient and is present at the extreme front of the leading edge, where it promotes growth cone protrusion and retraction. Further back towards the cell soma, acto-myosin filaments form the lamella that drives the main migratory process (Ponti *et al.*, 2004). Actin involvement in these migratory processes are regulated by growth factors such as platelet-derived growth factor (PDGF), which acts via integrin-linked kinases and P38 MAPK signalling to promote migration. PDGF activation of Rho GTPases represents another form of actin regulation, as Rho GTPase trigger P21-activated kinase phosphorylation of LIM-kinase via P21-activated kinase. This leads to the phosphorylation of cofilin, which is subsequently activated (Edwards *et al.*, 1999; Esfandiarei *et al.*, 2010). Lamellipodia in migrating cells also contain nascent adhesions, where actin binds to the cytoplasmic domains of the integrin adhesion receptors to establish these projections (Choi *et al.*, 2009).

Lamellipodia activity is concurrent with the action of the finger-like cytoplasmic filopodia projections responsible for guiding the growth cone. Filopodia arise from lamellipodia in a process controlled by members of the WASP family of proteins, and the direction of these projections take are partly dependent on the action of Src family kinases, which regulate filopodial motility (Biyasheva *et al.*, 2003; Robles *et al.*, 2005). Filopodial extension and lateral movement depends on a balance between retrograde flow and polymerisation of actin bundles at an angle to the leading edge (Mallavarapu & Mitchison, 1999; Oldenbourg *et al.*, 2000). In the dendritic network, members of the Arp family of proteins regulate actin dynamics by controlling lamellipodia reorganistation. VASP (vasodilator-stimulated

phosphoprotein) molecules also support these activities by preventing the capping of the actin filament at the barbed end and promoting actin filament clustering, which are subsequently stabilised by cross-linkage proteins such as fascin. The importance of these cross-linking proteins is underlined by the use of RNA interference on fascin expression, which results in a decrease in filopodia density and a disruption of their morphology. Conversely, overexpression of fascin promotes extension of existing filopodia and the development of new filopodia (Svitkina *et al.*, 2003; Vignjevic *et al.*, 2006; Applewhite *et al.*, 2007).

The extent of lamellipodia and filopodia growth is dependent on the ratio of G-actin to F-actin within the growth cone, with a higher ratio promoting protrusion (Lee *et al.*, 2013). Filopodia are also important clinically as the driving force behind the invasive behaviour of cancer cells, which utilise actin bundles in conjunction with trafficking proteins such as dynamin to invade and degrade the extracellular matrix (Li *et al.*, 2010).

1.3.3.2 The Actin Cytoskeleton

Another important component of the actin cytoskeleton is the stress fibres, which are found as either dorsal or ventral fibres, or as unattached transverse arcs. Dorsal stress fibers connect to a substrate at one end via focal adhesion points and are assembled by formin-dependent actin polymerisation. In comparison, transverse arcs are unconnected at either end, and are formed by annealing the end of myosin and actin bundles from lamellipodia. However, both types of fibres are capable of becoming ventral fibres and binding focal adhesion points at either end (Hotulainen & Lappalainen, 2006). Interactions between focal adhesion points, dorsal and ventral stress fibres are regulated by talin, vinulin and paxilin, with paxilin expression in particular being elevated prior to actin filament assembly at the focal adhesions (Barry & Critchley, 1994). This dynamic actin assembly is assisted by cortactin, which binds actin and the Arp2/3 complex to act as a link between cadherin adhesions and the actin cytoskeleton. Inhibition of cortactin blocks Arp2/3-dependent actin assembly and the association of actin with cadherin, in addition to attenuating cell growth (Weaver *et al.*, 2002; Helwani *et al.*, 2004).

Actin forms a complex with the cadherin tail and β-catenin in a process requiring the presence of both actin cables and myosin II, which is regulated by α-catenin and vinulin. As cell elongation proceeds, cadherin adhesions are formed sequentially along actin fibres, with fully developed adhesion points found mainly in the lamellipodia. Actin is necessary for the establishment of cadherin adhesions, and as the cell moves cadherin adhesion points are degraded, causing actin depolymerisation (Kobielak & Fuchs, 2004; Lambert *et al.*,

2007). Cadherin adhesions are also involved in Ca^{2+} -driven filopodia penetration of neighbouring cells, which is the first stage in the establishment of cell-cell junctions. These are established by the binding of cadherin to the cytoplasmic end of filopodia, concurrently with the binding of desmosomes to the opposite end in target cells. The process is completed by the reorganisation of actin reorganisation into punctae, a stage crucial to ensuring the complete sealing of the intracellular space (Vasioukhin *et al.*, 2000). At apical junctions, adhesion proteins such as cadherin associate with both actin and other cytoplasmic proteins such as Rac GTPase, which may improve the efficiency of binding.
The integrity of tight junctions are also susceptible to actin depolymerisation, which causes the endocytosis of occludin, a plasma membrane protein integral to the function of tight junctions (Shen & Turner, 2005; Miyoshi & Takai, 2008).
Actin cytoskeletal changes are accompanied by a simultaneous increase in protein biosynthesis, driven by transcription factors activated by actin polymerisation. By acting through several signalling cascade this ultimately leads to the activation of nuclear transcription factors and an increase in protein expression (Olson & Nordheim, 2010).

1.3.3.3 Actin and the endocytotic pathway

F-actin also takes part in clathrin-mediated endocytosis, where it is involved with multiple stages of the clathrin processes, such as their separation and cell surface mobility. Inhibition of actin by either latrunculin A or Jasplakinolide, which both stabilise actin, almost completely inhibits clathrin-related processes (Yarar *et al.*, 2005).
Actin filaments also drive the cell membrane invagination preceding the formation of clathrin-coated endocytotic vesicles. A model of this process involves the polymerisation of actin filaments around the endocytotic site, pushing the invaginated membrane inwards to form a separate vesicle (Galletta *et al.*, 2010).

1.3.4 Synaptic proteins

Synaptic transmission in the nervous system is dependent on exocytotic release of neurotransmitters in response to stimuli. The following steps are observed in this process: synthesis of the neurotransmitter in the Golgi apparatus, followed by vesicular transport to the synaptic site, where they are sequestered by synaptic proteins in preparation for release. In response to stimulation, voltage-gated calcium channels open, elevating intracellular

Ca^{2+} levels and triggering the fusion of vesicles with the cell membrane at the synapse and release their cargo of neurotransmitters. The release mechanism involves a number of proteins, which are collectively known as SNARES (Soluble NSF Attachment Protein receptor). The main proteins belonging to this complex are the synaptobrevins/vesicle-associated membrane proteins (VAMPs), synaptotagmin, SNAP-25, synaptophysin and syntaxin. A disruption to the expression of these proteins is the major cause of many pathological processes, such as botulism, caused by the cleavage of VAMPs by clostridial toxins. As part of this study, the expression levels of several members of the SNARES family were measured. Synaptobrevin/VAMP-1 is a protein highly susceptible to subtilisin cleavage as shown in previous studies, and is therefore a good marker for changes to subtilisin action. Synaptotagmin and synaptophysin both act as synaptic protein controls for VAMP, and are known be more resilient in resisting subtilisin proteolysis based on previous studies.

1.3.4.1 Synaptotagmin

Within the SNARE complex, synaptotagmin selectively binds calcium under physiological conditions, and is responsible for the intracellular response to the calcium influx as a result of stimuli. The essential role of synaptotagmin was demonstrated in a knockout mouse model lacking this protein, which displayed severe disruptions to synaptic transmission and caused the death of homozygous knockout mice within 48 hours of birth (Geppert *et al.*, 1994). In a similar manner, point mutations in synaptotagmin that decrease Ca^{2+} affinity lowers the rate of vesicle release in response to calcium influx (Yoshihara & Littleton, 2002). Synaptotagmin promotes vesicle fusion by binding VAMP in a Ca^{2+}-dependent manner; however, it is also associated with SNAP-25 and syntaxin in a Ca^{2+} independent manner (Stein *et al.*, 2007).

In addition to promoting the exocytosis of vesicles, synaptotagmin is also involved with the endocytosis of spent vesicles from the membrane after neurotransmitter release. A mutation in this protein depleted the amount of synaptic vesicles available for neurotransmitter release by reducing vesicle reuptake, but had no impact on the transport of newly synthesized vesicles to the synaptic site. As membrane fusion proteins are recycled for reuse after neurotransmitter release, inhibition of the endocytotic process decreases the availability of these proteins and prevents the replenishment of synaptic vesicles (Deák *et al.*, 2004). The effectiveness of the synaptotagmin response to Ca^{2+} is also dependent on the composition of the synaptotagmin heterodimers. Some members of the synaptotagmin family such as synaptotagmin IV possess a very low affinity to calcium

in comparison to synaptotagmin I, and can therefore reduce the overall rate of calcium binding at synapses (Littleton *et al.*, 1999). In addition to their roles in endocytosis and exocytosis, synaptotagmin also assists in the recycling process of internalized receptors. Synaptotagmin III is responsible for the formation and delivery of endosomes containing internalized receptors to the pericentriolar endocytic recycling compartment. However, an inhibition of this protein results in a peripheral accumulation of these vesicles, in contrast to their movement under normal physiological conditions (Grimberg *et al.*, 2003).

1.3.4.2 Syntaxins

Syntaxin links the calcium-sensing function of synaptotagmin to the SNARE vesicle fusion apparatus. Syntaxins, SNAP-25 and synaptobrevin form the complex responsible for vesicle docking, determining the state of the vesicle prior to fusion. This complex is also necessary for vesicle fusion to take place, and the loss of syntaxin precludes the formation of a stable SNARE complex (O'Connor *et al.*, 1997; Walter *et al.*, 2010). Syntaxin, and the associated SNAP-25 protein, are both recycled after vesicle fusion and are expressed widely in synaptic vesicles (Walch-Solimena *et al.*, 1995).

1.3.4.3 Synaptosomal-Associated Protein 25 (SNAP-25)

SNAP-25 (Synaptosomal-Associated Protein 25) is a target-membrane SNARE, and is essential for evoked release at neuromuscular junctions and nervous system synapses. They are expressed in the neurons of the mossy fibre region and the dentate gyrus of the hippocampus, and can be found in axonal growth cones during development (Oyler *et al.*, 1989; Osen-Sand *et al.*, 1993).

It is also capable of forming complexes with other SNARE proteins such as the synaptobrevins/VAMPs and syntaxin. SNAP-25 acts as a stabilizing factor for a α-SNAP sensitive complex between VAMP and syntaxin. In their absence, SNAP-25 is able to interact with synaptotagmin, linking the elements of the SNARE complex with calcium-sensing proteins (McMahon & Südhof, 1995; Schiavo *et al.*, 1997; Schiavo G. *et al.*, 1997).

Loss of SNAP-25 also affects synaptic recycling in a similar manner to synaptotagmin, by reducing the readily releasable vesicle pool and abolishing the fast Ca^{2+}-mediated response (Zhang *et al.*, 2002).

1.3.4.4 Synaptophysin

Synaptophysin is a member of the SNARE protein group often associated with VAMP, and consists of two members, synaptophysin and synaptoporin (synaptophysin II). Both are often co-expressed at nerve terminals, and are colocalised within the same vesicles. However, synaptoporin expression is largely restricted to a few selective neuronal populations, whereas synaptophysin expression can be found in most nerve terminals (Fykse *et al.*, 1993).

The synaptophysin protein spans the cell membrane 4 times, with both the amino and carboxyl terminals residing in the cytoplasmic domain. Differences in the structure of synaptophysin between species are due to changes in the intravesicular loops. The cytoplasmic domain however, which contains the Ca^{2+} binding site, is highly conserved between species (Rehm *et al.*, 1986; Johnston *et al.*, 1989).

During postnatal murine brain development, a 80-fold increase in synaptophysin was observed in the cerebellum, correlating with a period of synaptogenesis (Knaus *et al.*, 1986). This relationship between synaptophysin and synaptogenesis is clearly visible during the development of the rat hippocampal dentate gyrus. Changes to synaptophysin expression corresponded to periods of denervation and renervation in this region, and increasing the expression of synaptophysin corresponded to an increase in the size of the inner molecular layer. This suggests that synaptophysin expression can be used as an indication of terminal formation or loss within the region (Masliah *et al.*, 1991).

Synaptophysin expression is also activity-dependent, as cell-cell contact triggers their expression in newly formed puncta, whereas in quiescent cells the expression of these proteins is more dispersed (Chhabra *et al.*, 2002).

Synaptophysin also interacts with VAMP proteins, a process that only occurs in the adult nervous system, as embryonic VAMP is unable to bind the synaptophysin equivalent in the embryo (Becher *et al.*, 1999). Synaptophysin regulates VAMP transport to the synaptic vesicles in a dose-dependent manner, specifically the movement of the VAMP-2 isoform, as the VAMP-1 isoform is unaffected by changes to synaptophysin expression. This targeting mechanism involves the formation of a heterodimer complex between the cytoplasmic domains of both synaptophysin and VAMP-2, which can be further promoted by inhibiting the formation of the VAMP-syntaxin-SNAP 25 complex (Edelmann *et al.*, 1995; Washbourne *et al.*, 1995; Pennuto *et al.*, 2003; Bonanomi *et al.*, 2007).

Synaptophysin is also a regulator of activity-dependent synapse formation, as demonstrated by differences in synaptic formation between cells carrying wild-type and

mutated forms of synaptophysin (Tarsa & Goda, 2002).

Expression of synaptophysin is also clinically relevant due to their role in synaptic signaling. Mice possessing mutated synaptophysin show a reduced capability for object novelty recognition and have spatial learning deficiencies. Synaptophysin expression is also reduced in Alzheimer's disease, where patients display a 50% reduction in the expression of this protein in the parietal, temporal and midfrontal cortex (Masliah *et al.*, 1989; Schmitt *et al.*, 2009).

1.3.4.5 VAMP

The synaptobrevin/VAMP (vesicle-associated membrane protein) family of proteins consist of 10 members in humans, involved in either exocytosis or endocytosis within cells of the nervous system and the neuroendocrine system.

The structure of VAMP-1 consists of three separate domains: a proline-rich domain, an internal domain and a hydrophobic carboxyl-terminal that anchors the protein to the membrane (Trimble *et al.*, 1988). In the central nervous system, VAMP proteins are associated with both synaptic vesicles and the presynaptic plasma membrane, remaining on the membrane of the cytoplasmic domain throughout the vesicle fusion process (Taubenblatt *et al.*, 1999). The most well-known members of the VAMP family, VAMP-1 and VAMP-2, are expressed in the rat central nervous system, but their distribution varies according to location. VAMP-2 is widely distributed and strongly expressed in many major neuronal nuclei and is found in regions including the autonomic motor nuclei, the sensory nuclei and the supraoptic nuclei. In contrast, VAMP-1 expression is present in more specialised areas such as the nerve terminals of thalamic neurons or the zona incerta of the subthalamic nuclei. Both members of VAMP are highly expressed in the locus coeruleus, where they are colocalised with synaptophysin (Trimble, 1993; Raptis *et al.*, 2005).

The synaptobrevin/VAMP proteins can also be found in non-neural tissue, such as the kidneys, spleen or adrenal glands, although variations exist in the type of VAMP proteins expressed by each individual tissue. VAMP-2 is expressed in rat pancreatic islets, where it is involved in the release of secretory granules containing insulin and microvesicles containing GABA (Regazzi *et al.*, 1995; Rossetto *et al.*, 1996). VAMP protein homologues are also associated with exocytosis and reuptake of GluT4-containing small vesicles in rat adipocyte cells, which are released in response to insulin signalling (Cain *et al.*, 1992).

Multimerisation of the VAMP protein leads neurotransmitter release, and this process can be triggered via several mechanisms. Multimerisation can be induced by phosphorylation of VAMP proteins by endogenous kinases, such as the calmodulin-dependent protein kinase II and casein kinase II. Both phosphorylate residues within the cytoplasmic region of VAMP whilst the protein is localised within synaptic vesicles (Nielander *et al.*, 1995).
Application of stimuli can also promote the multimerisation of VAMP and synaptophysin, increasing the proportion of these proteins from 10% to 25% of the total protein expression in synaptosomes via a kinase-independent mechanism (Khvotchev & Südhof, 2004). In addition to synaptophysin complexes, VAMP also forms complexes with syntaxin and SNAP-25, a process which precedes their anterograde axonal transport to nerve endings. These interactions are dependent on the involvement of the VAMP N-terminal, and can be blocked by mutations in this region which prevent subsequent vesicle priming (Shiff & Morel, 1997; Walter *et al.*, 2010).
This complex also has an important association with the Ras GTPase Rab3A, which is necessary for exocytosis in a variety of tissues. Genetic knockout models of VAMP also demonstrate a delay in fast endocytosis after stimulation, a process used for synaptic vesicle recovery. This demonstrates the role of VAMP in replenishing the rapid-release pool of vesicles at the synaptic site (Horikawa *et al.*, 1993; Deák *et al.*, 2004). VAMP action occurs downstream of vesicle docking, as their absence does not prevent vesicle targeting and docking in response to stimuli. Likewise, spontaneous transmitter release is unaffected by the loss of VAMP (Broadie *et al.*, 1995).

In the spinal cord, both VAMP-1 and VAMP-2 are widely expressed, VAMP-1 most prominently in the nerve terminals of the ventral horn, and VAMP-2 found mainly in the dorsal horns. In general VAMP-1 is localised to motor neurones, whereas VAMP type 2 is localised in adrenergic and sensory neurones (Li *et al.*, 1996). The axonal expression in response to nerve damage differs between VAMP-1 and VAMP-2, with VAMP-1 being less severely affected and possessing a faster recovery period than VAMP-2. There were however no differences to the expression of VAMP-1 or VAMP-2 within the cell soma after injury (Jacobsson *et al.*, 1998).

Synaptic proteins also perform crucial roles in the human immune system by facilitating the release of neutrophil granules. The exocytotic release of these granules is dependent on the presence of complexes formed by different VAMP isoforms. Release of both neutrophil-specific and tertiary granules are mediated through complexes containing VAMP-1 and VAMP-2 and SNARE proteins, whereas the release of axurophilic granules

involves formation of a complex containing VAMP-1 and VAMP-7. Both sets of VAMP complexes interact with the plasma membrane by an association with syntaxin (Mollinedo *et al.*, 2006).

1.4 Aims of this project

Based on previous studies, it is known that subtilisin-mediated LTD involves the selective degradation of proteins, although as a relatively new type of LTD, very little is known of its mechanism. The purpose of these investigations was therefore to determine if subtilisin-mediated LTD was dependent proteolytic action alone, and if mechanisms from other types of proteases, such as metalloproteases, were involved. A secondary aim was to determine the relative importance of the proteins most susceptible to subtilisin action to the resultant LTD effect. Finally, the action of subtilisin was compared to those of other types of LTD in order to verify that this was a novel type of LTD. Subtilisin action was also compared to those of another serine protease, to determine whether these effects were restricted to subtilisin alone, or could be replicated by other members of the serine protease family.

1) Potential metalloprotease mechanisms were investigated by altering extracellular conditions to either enhance or inhibit the action of metalloproteases.

2) The importance of proteolytic activity to the generation of LTD by subtilisin was investigated by selectively inhibiting serine protease activity.

3) The importance of the proteins most susceptible to subtilisin action was investigated by targeted blockade using protein-specific antibodies, and for actin, through the use of a polymerising agent.

4) Subtilisin-mediated LTD was also compared to other common types of LTD to establish whether this type of LTD was a novel form of LTD.

5) The ability of other serine proteases to generate effects similar to subtilisin was evaluated by comparing the action of another serine protease, namely α-chymotrypsin, with subtilisin.

Chapter 2 - Methods

2.1 Hippocampal slice preparation

Hippocampal slices were prepared from young adult male mice (ICR strain), weighing between 25-35g. Mice were anaesthetised using an intraperitoneal injection of 25% urethane, at a dosage of 1ml/100g. After a short period, limb and corneal reflexes were checked to determine the effectiveness of anaesthesia. Upon the cessation of these reflexes, the mouse was killed by cervical dislocation. The brain was rapidly removed and placed into ice-cold, oxygenated aCSF containing (mM): KH2PO4 (2.2); KCl (2.0); NaHCO3 (25); NaCl (115); CaCl2 (2.5); MgSO4 (1.2); glucose (10); distilled water. The hemispheres were separated, and the hippocampus dissected from each side. The hippocampi were then sliced transversely using a McIlwain tissue chopper to produce slices with a thickness of 450μm. Slices were transferred to a petri dish containing cold, oxygenated aCSF, for separation into individual slices. The volume of aCSF within the dish was then reduced by removal via a syringe to obtain an optimal level that allowed oxygen diffusion into the slices whilst ensuring they would be sufficiently immersed. The slices were then placed in a closed incubation chamber continuously gassed with 95% O_2/5% CO_2, at room temperature.

2.2 In-Vitro Electrophysiological Recordings

Slices were kept in the incubation chamber for at least 1 hour prior to recording to allow acclimatization and recovery after dissection. Individual slices were retrieved and placed on a wire mesh within a 1ml recording chamber, and held in place using small silver bars placed over the top of the slice. Oxygenated (95% O_2/5% CO_2) aCSF was continuously perfused over the slice at a rate of 3-4ml/min. The temperature of the aCSF was maintained between 28 and 30°C using a water bath. Stimuli were delivered via a concentric, bipolar stimulating electrode, positioned manually using micromanipulators on the stratum radiatum in the CA2 region to elicit orthodromic-evoked responses. Stimuli were produced using a NeuroLog period generator (NL303) and delay-width (NL403), and delivered using a NeuroLog stimulus isolator (NL800) to ensure signal fidelity. The stimulus was

delivered at 330μs square wave pulses at a frequency of 0.1Hz through the bipolar electrode.

Recording electrodes were made using borosilicate glass capillaries, pulled to a fine tip of 1μm using a Kopf vertical pipette puller. Under a microscope, the tip was then broken to a diameter of around 2-8μM to lower the electrical resistance of the electrode, and subsequently filled with 4mM NaCl with a fine needle. The micropipette was then attached onto micromanipulators, and an Ag/AgCl wire electrode, connected to a headstage, was inserted into the top end of the micropipette prior to use. The other end of this electrode was connected to a NeuroLog pre-amplifier (NL104), and the evoked response was filtered using Neurolog filters (NL125) as well as a noise canceller (Hum Bug, Quest Scientific). This response was visualised using a Gould Oscilloscope 1602, and the signal was captured using Cambridge Electronic Design microplus1401 interface onto the computer-based Signal software. Population spikes were recorded by placing the recording electrode on the CA1 pyramidal cell layer; for field excitatory post-synaptic potentials (EPSPs), the electrode was place in the stratum radiatum to record at the synapse junction of CA3 and CA1 pyramidal neurons. (Figure 1.1) A sampling frequency of 10000Hz was used to capture waveforms over the course of the experiment, with stimuli delivered every second during the setup of the experiment, and at 10-second intervals during the course of the experiment. The strength of the stimulus was adjusted using a Neurolog pulse buffer (NL510) to produce a maximum response from the slices; the population spike size being defined by the difference between the positive and negative peak potentials, and the fEPSP size defined as the difference between the negative peak potential and the recovering baseline. For all experiments, after attaining maximal response, the stimuli was reduced to a submaximal response of around 70% prior to the start of recordings to allow for changes in potential size in response drugs perfused over the course of the experiment. For each experiment, a stable baseline was maintained 10 minutes prior to the start to allow an accurate measure of the action of drugs. Percentage values are derived from the average of 10 points prior to addition of the drug. Data from all experiments were analysed and normalised using GraphPad Prism software, with baseline percentage value derived from averaging the 10 points prior to addition of the drug.

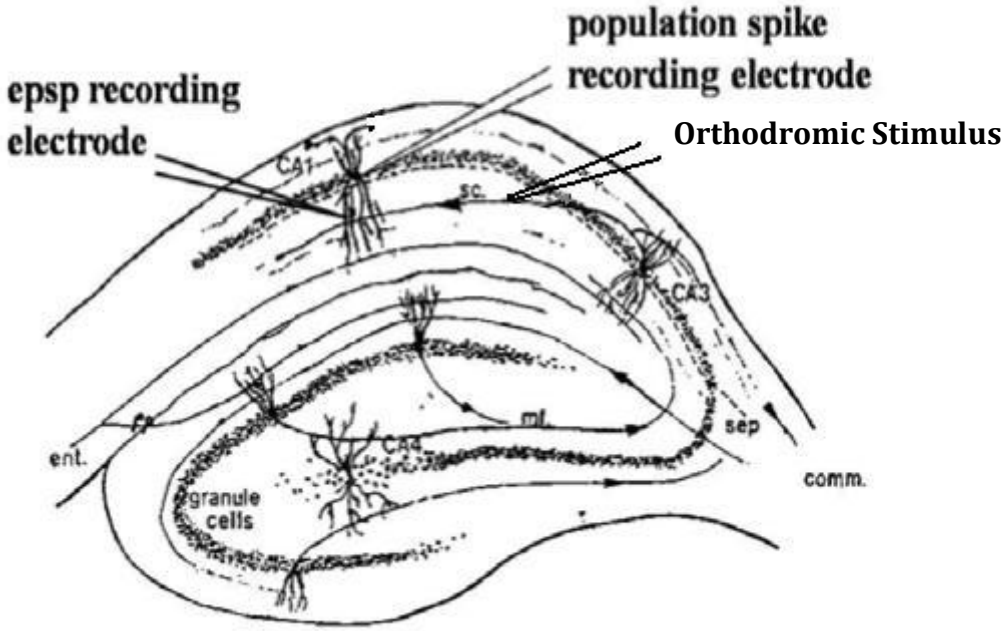


Figure 1.1 Diagram of acute in-vitro mouse hippocampal slice, and locations of stimulating/recording electrodes.

General locations for electrode placement are shown above. Stimulating electrodes were placed within the CA2 region of the hippocampal slice, and recording electrodes in the pyramidal layer (for population spikes) or the stratum radiatum (for fEPSPs). Diagram locations: comm. = commissural; sc. = Schaeffer collaterals; ent. = Entorhinal cortex; mf. = mossy fibres.

2.3 Bradford Assay

After electrophysiology was completed, slices were removed from the recording chamber and placed into Eppendorf tubes kept on dry ice, and subsequently stored at -80°C until further use. Proteins were subsequently extracted from these hippocampal slices for western blotting. 110µl of RIPA buffer (composition: 50mM Tris; 150mM NaCl; 1% Igepoal; 0.5% TritonX 100; 0.1% sodium dodecyl sulphate [SDS]; complete Mini EDTA-free protease inhibitor cocktail tablet {composition: aprotinin; bestatin; calpain inhibitor I; calpain inhibitor II; chymostatin; E-64; leupeptin; α2-macroglobulin; petabloc SC; pepstatin; PMSF; TLCK-HCL; trypsin inhibitor (chicken, egg white); trypsin inhibitor

(soybean)}) was added to the Eppendorf tubes and the mixture of solution and slice tissue was homogenized with a pestle until no visible debris was left. The tubes were then placed in a centrifuge and spun at 13,000 rpm at 4 degrees Celsius for 5 minutes to remove cell debris. Eppendorfs were carefully removed from the centrifuge, and the clear supernatant carefully pipetted into a new Eppendorf tube, taking care not to disturb the pellet at the bottom of the original tube. During this process samples and solutions were kept on ice to minimize protein degradation.

A Bradford Assay was used to measure protein concentration in each sample by comparing the samples to a set standard. The standard concentration curve for this assay was prepared using 0, 0.25, 0.5, 1.0, 1.5 and 2.0µg/ml Bovine Serum Albumin, using a stock solution of 2mg/100ml. Samples for the assay were prepared by adding 4µl sample to 396µl distilled water in an Eppendorf tube. Biorad reagent was mixed with distilled water at a 1:1 dilution, and 200µl was added to each sample and standard solution and mixed thoroughly using a vortex. 200µl from each solution was pipetted into a 96-well plate in duplicates, and measured using fluorometry at 595nm on a plate reader, with values analyzed using Revelation Link software. Once the protein concentration in each sample was established, proteins were normalized using the lowest protein value, by adding distilled water to reach an optimal protein concentration of 12-15µg protein per gel lane.

2.4 Western blotting

After normalisation, samples for the western blots were prepared by mixing 65% sample (32.5µl), 25% 4x sample buffer (12.5µl) and 5% 10x reducing agent (5µl) in Eppendorf tubes. The solution in the tubes were then mixed thoroughly using a vortex before being placed into a water bath and heated to 70°C for 10 minutes, prior to gel loading. A mixture of NuPage MOPS running buffer (50ml) and distilled water (950ml) was prepared, 200µl of which was set aside in a conical flask and 0.5ml NuPage antioxidant added to this solution. NuPage 4-12% Bis-tris gel cassettes was removed from packaging, and rinsed with distilled water. The comb was carefully removed to prevent damage to the loading wells, and rinsed three times with NuPage buffer from the conical flask. After the last rinse, the buffer was left in the wells before being placed in a gel tank for electrophoresis, with the wells on the inside of the central reservoir. The remaining buffer in the conical flask, containing antioxidant, was poured into the central reservoir. The rest of the buffer was added to the outside this reservoir to keep the gel cool and prevent it from drying out.

Each gel was loaded with10µl Amersham Full-Range Rainbow Molecular Weight Marker (RPN800V), and 15µl of prepared samples (for 15-lane gels) or 20µl prepared samples (for 10 or 12-lane gels) into individual wells. The gels were then run for 1 hour and 20 minutes, at 150V, 240 mA. PVDF membranes and transfer buffer were prepared in the interim. Transfer buffer with prepared with the following composition: 50ml NuPageTransfer buffer, 200ml methanol (100ml for one gel), 750ml distilled water and 1ml antioxidant. These were thoroughly mixed before setting aside 200ml of buffer for the central reservoir. The remaining solution was used to soak the filter paper, PVDF membrane, transfer case and sponges for the transfer process. Prior to soaking in transfer buffer, PVDF membranes were soaked in methanol for one minute to allow the membrane to interact with the transfer buffer. Sponges were flattened using a small roller, to ensure they were fully saturated in transfer buffer, before 2 were placed into the case. Once electrophoresis was completed, the gel cassettes were removed from the tank, and placed on a flat surface. The gel cassettes were then cracked around the edges, and the top removed carefully to prevent damage to the gel. Pre-soaked filter paper was placed on top of the gel, which was then turned over, and the gel carefully separated from the other side of the cassette. The PVDF membrane was then placed onto the exposed side with another soaked filter paper on top of the membrane. The filter papers were checked to ensure no bubbles could affect the protein transfer, before being placed onto the sponge stack in the case. Another gel was set up in the same manner as the first, with 3 sponges separating the two gel sets. A final 2 sponges were placed on top of the second gel set before the case cover was added, with all sponges checked for bubbles prior to placement. The complete case was then secured in the running tank. Transfer buffer from the flask was poured into the central reservoir in the case, up to 1cm from the top of the case, to prevent the membrane from drying out. Distilled water was added to the rest of the running tank to keep the case cool during the transfer process. The transfer was run for 1 hour 10 minutes at 30V, 450mA. After the protein transfer was completed, the membrane was separated from the gel, and rinsed several times with distilled water. They were then immersed in ponceau solution over 5 minutes to determine whether the transfer was successful. The membrane was then rinsed with distilled water, before being checked for the presence of protein bands. The membrane was then immersed in 0.1 NaOH for 10-30 seconds to remove the ponceau solution from the membrane. The membranes were again rinsed with distilled water before being stored in TBST. TBST was made by mixing 100ml TBS stock solution (comprising 48.4g TRIS and 160g NaCl in 2l distilled water, pH adjusted to 7.6 using 50% HCl, before addition of 0.5ml Tween), and topped up to 1l with 900ml distilled water. Membranes were subsequently blocked by immersion in a milk-TBST solution (5g milk powder in

100ml TBST) for one hour on a shaker. They were then cut to separate protein bands in order to allow individual primary antibody incubation. Both primary and secondary antibodies were prepared in the milk-TBST solution. Primary antibodies were left in at 4°C on a shaker, before being washed in TBST for three 15-minute intervals. The blots were then incubated using the relevant secondary antibody for 1 hour at room temperature, on a shaker.

The following antibodies were used over the course of these experiments. Unc5H1 (Gt) at 1:1,000 dilution; Unc5H3 (Gt) at 1:1,000 (for EDTA experiments only) and 1:500 dilution; RhoA (Mse) at 1:1,000 dilution; RhoB (Mse) at 1:1,000 dilution; actin (Gt) at 1:10,000 dilution; Synaptotagmin (Mse) at 1:10,000 dilution; Synaptophysin at 1:10,000 dilution and VAMP-1 (Gt) at 1:10,000 dilution. Secondary antibodies used were Donkey anti-goat and Goat anti-mouse, both at a 1:5,000 dilution.

After the secondary incubation was completed, membranes were again washed in TBST for three 15-minute intervals, before being rinsed in water. They were then placed on a flat surface on a plastic folder, and developing ECL solution was added to the membranes. The membranes were then covered for 5 minutes to allow the ECL solution to react with the secondary antibodies, before being placed into the developing cassette. Any excess ECL was blotted off using tissue, before being placed into the developing cassette.

The cassette was then taken into a dark room, and a developing film was placed into the cassette for an initial exposure period of 1 second. This film was then developed, and based on the intensity of the band signal, other film exposures were taken to provide an optimum band for analysis. These films were then scanned, and analysed using ImageJ software developed by National Institutes of Health. A box encompassing the whole band was selected, and three background measurements were made prior to measuring the protein bands in order to take into account any background changes in intensity. Measurements were taken in triplicate, and the average of the three measurements was taken for comparison in each band. These were then analysed by carrying out either t-tests or two-way ANOVAs using GraphPad Prism software. The protein band of each marker was determined by comparing their expected location with the protein ladder, as demonstrated in Figure 1.2.

Results were graphed using excel and Sigmaplot, with each data point represented as mean ± SEM.

All chemicals involved in electrophysiological recordings including aCSF chemicals, subtilisin and chymotrypsin were sourced from Sigma Aldrich. For immunblotting, gel

packs, running buffers and transfer buffers were obtained from Life Technologies. Ponceau solution was obtained from Sigma, ECL solutions from Millipore and Biorad solution (for protein assays) from Bio-Rad Laboratories. Antibodies for immunoblotting were obtained from the following companies: Santa Cruz (Actin, Unc5H1, Unc5H3, RhoA, RhoB goat-anti-mouse, donkey-anti-goat), Millipore (Synaptophysin), R & D Systems (VAMP-1, Synaptotagmin).

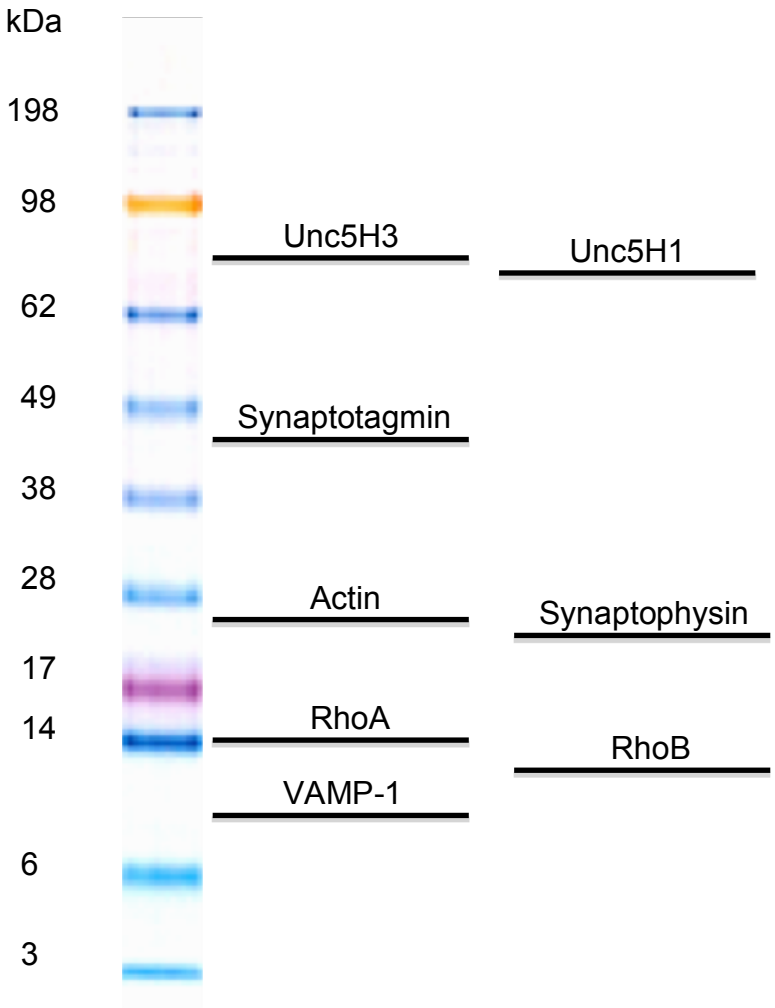


Figure 1.2 Relative size of protein markers

The above diagram indicates the expected size of each protein marker, when compared with the protein ladder on the immunoblot.

Chapter 3 - Contribution of protease mechanisms to subtilisin-mediated LTD

3.1 Metalloprotease mechanisms

3.1.1 Introduction

Based on previous studies, it is known that subtilisin targets a number of proteins, including the synaptic protein VAMP-1 (Forrest *et al.*, 2011). The synaptic proteins comprising the SNARE complex, which include the VAMP proteins, are also established targets of the clostridial metalloproteases TeTx and BTx. These neurotoxins are zinc-dependent metalloproteases, and are therefore susceptible to extracellular changes to metal ion concentrations. This attribute has been demonstrated in multiple studies in which the actions of these metalloproteases have either been enhanced by addition of metal ions, or attenuated using metal chelators (Yamasaki *et al.*, 1994a). Since both subtilisin and the clostridial metalloproteases target VAMP-1, experiments were conducted to evaluate possible contributions of metalloprotease mechanisms to the activities of subtilisin.

3.1.2 Results

3.1.2.1 Extracellular zinc ions do not potentiate subtilisin action

In order to examine whether subtilisin was dependent on metalloprotease action, an experimental protocol was developed in which subtilisin was exposed to exogenously applied zinc ions. Addition of zinc to the perfusate is known to cause a significant increase in TeTx activity, and a similar response with subtilisin would suggest that subtilisin acts via a similar mechanism. To test this hypothesis, the following protocol was used: a fEPSP with amplitude of 0.5-1mV was established for 10 minutes to allow a stable baseline to be measured. An aCSF solution supplemented with 50μM zinc was then perfused over the slice in the recording chamber for 10 minutes, to establish if the addition of zinc alone had any impact on the fEPSP size. If no change to potential size was observed after this period, aCSF containing 4μM subtilisin was perfused onto the slice over

a period of 10 minutes, followed by a 40-minute recovery period in aCSF supplemented with zinc. Control experiments involved perfusion of normal aCSF throughout this period, with the exception of the 10 minutes in which subtilisin was applied to the slice. Addition of zinc did not significantly change the decrease in fEPSP subsequent to subtilisin perfusion ($P = 0.7767$, Figure 3.1a). No significant changes were observed in protein expression as measured by immunoblotting between slices exposed to subtilisin in aCSF, or in aCSF supplemented with zinc ($P > 0.05$, Figure 3.1b) for all proteins.

3.1.2.2 The metal ion chelator EDTA does not inhibit subtilisin activity

The action of metalloproteases can also be attenuated through the use of chelators that remove the catalytic metal ion at the active site. One chelator commonly used to inhibit metalloproteases is ethylenediaminetetraacetic acid, or EDTA. Previous studies have suggested that this chelator can act as an effective inhibitor against the metalloprotease action of both BTx and TeTx (Tetx and BTx B). If EDTA had a similar inhibitory effect on subtilisin, this could indicate a role for metal ions in the action subtilisin. To test this hypothesis, a protocol similar to previous experiments with zinc was adopted. After the formation of a stable fEPSP baseline of 0.5-1.0 mV was obtained over 10 minutes, aCSF supplemented with 20μM EDTA was applied to the slice over a period of 10 minutes, again to check for any differences in fEPSP size. Following this period the slice was perfused with 4μM subtilisin dissolved in aCSF, before a 40-minute recovery period was observed. In control experiments normal aCSF was perfused throughout, with the exception of 10 minutes during which subtilisin was applied to the slice. Addition of EDTA did not significantly change the decrease in fEPSP subsequent to subtilisin perfusion (Figure 3.2a). No significant changes were observed in the expression of most proteins measured via immunoblotting (Figure 3.2b). Expression of VAMP-1 however did differ significantly between slices perfused with EDTA + aCSF and slices perfused with aCSF alone. $N = 4$ for both treatment groups.

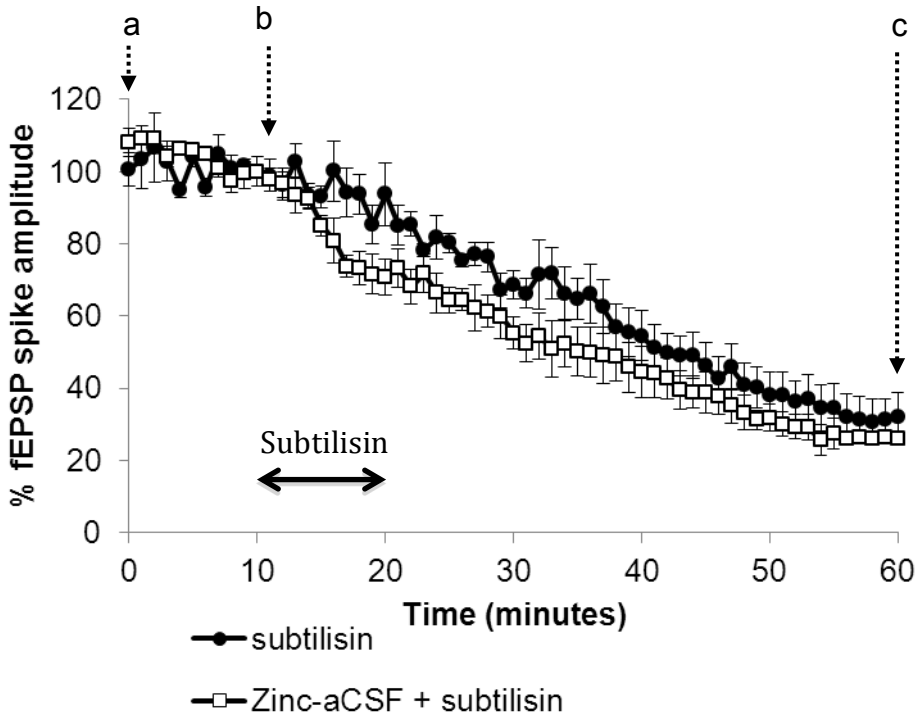
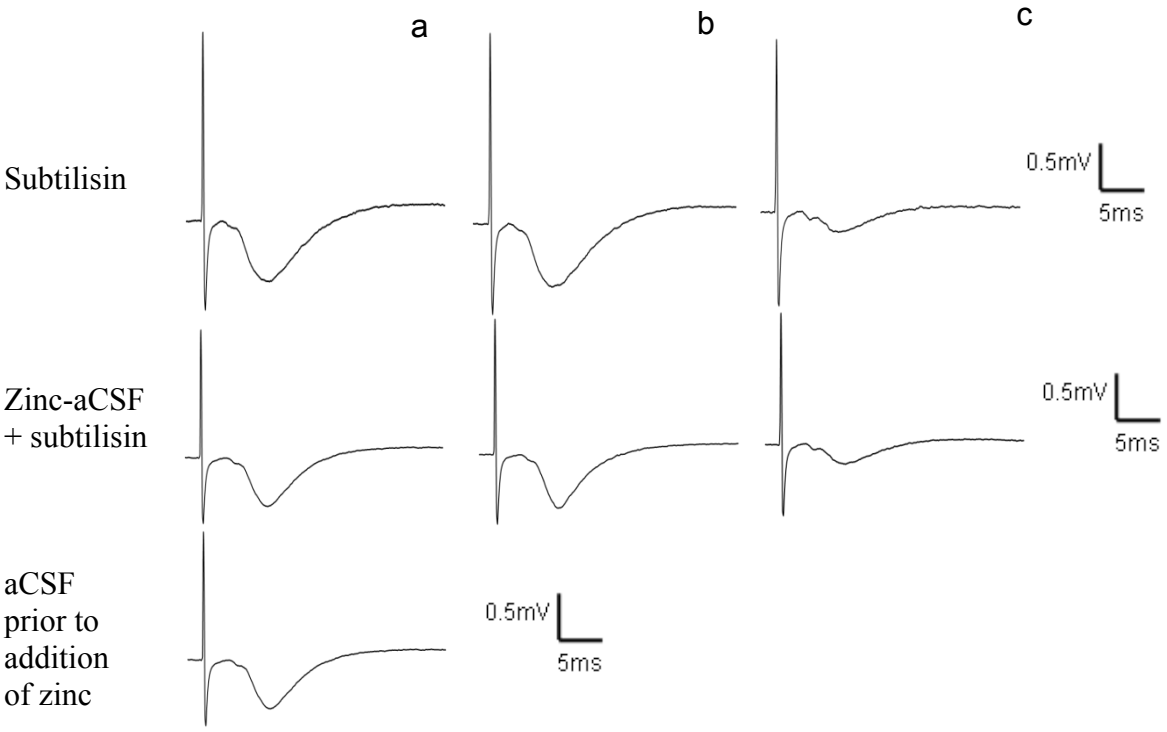


Figure 3.1a Effect of aCSF supplemented with zinc on hippocampal fEPSP size after subtilisin perfusion.

The subtilisin-mediated decrease in fEPSP size was not affected by addition of aCSF supplemented with 50µM zinc to aCSF (27.07 ± 2.22%, N=4) when compared to controls (31.92 ± 7.03%, N=4) (Students t-test, P = 0.6585). Arrows a, b and c indicate time points at which traces were taken.



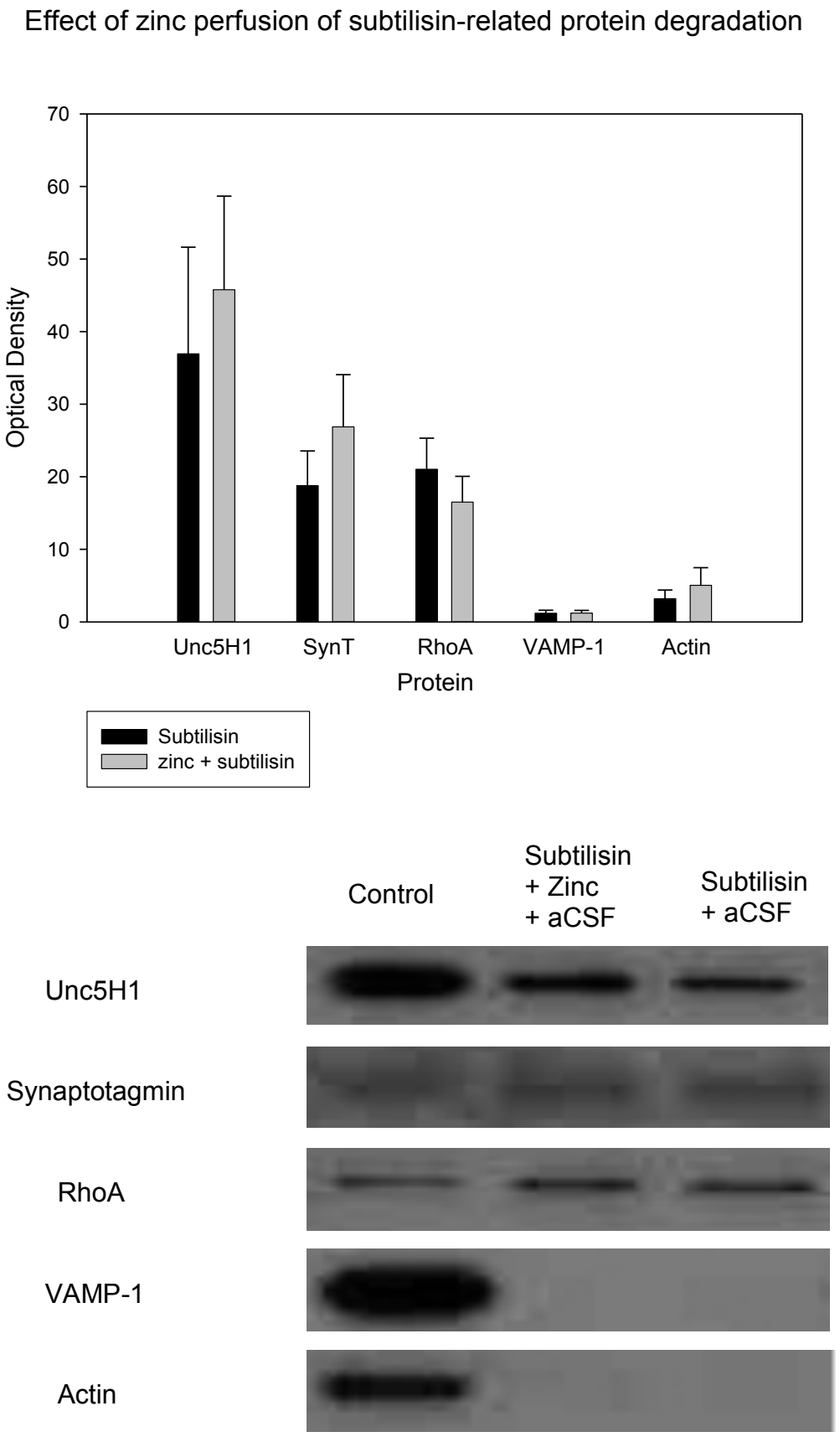


Figure 3.1b Effect of 50µM zinc on protein degradation in response to subtilisin.

No significant changes in protein expression were observed across all markers between slices treated with zinc and with aCSF alone. ($P > 0.05$, $N = 4$)

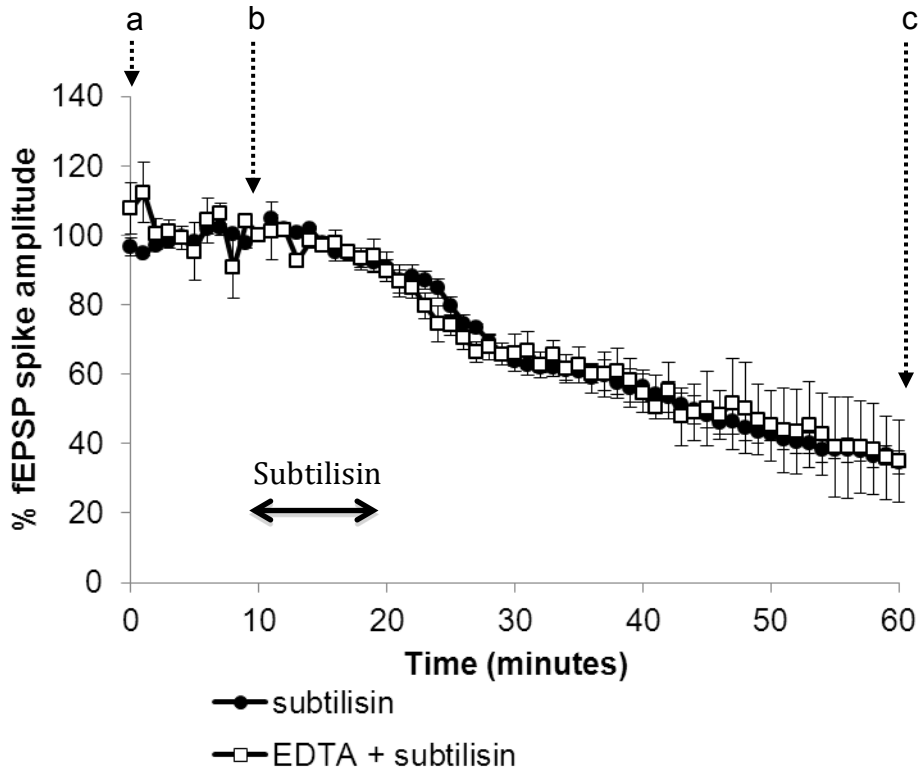
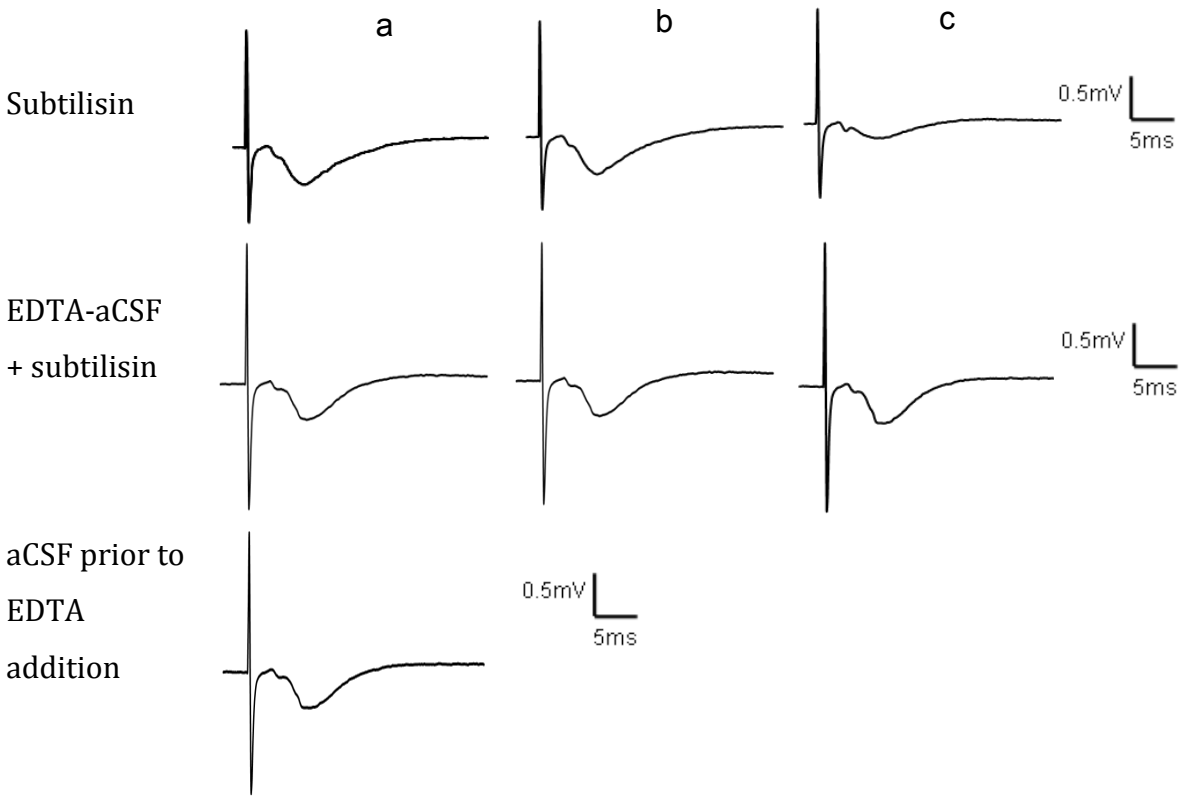


Figure 3.2a Effect of aCSF supplemented with EDTA on hippocampal fEPSP size after subtilisin perfusion.

The subtilisin-mediated decrease in fEPSP size was not affected by addition of aCSF supplemented with 20μM EDTA to aCSF (34.96 ± 11.85, N=4) when compared to controls (31.06 ± 3.823, N=4) (Students t-test, P = 0.3132). Arrows a, b and c indicate time points at which traces were taken.



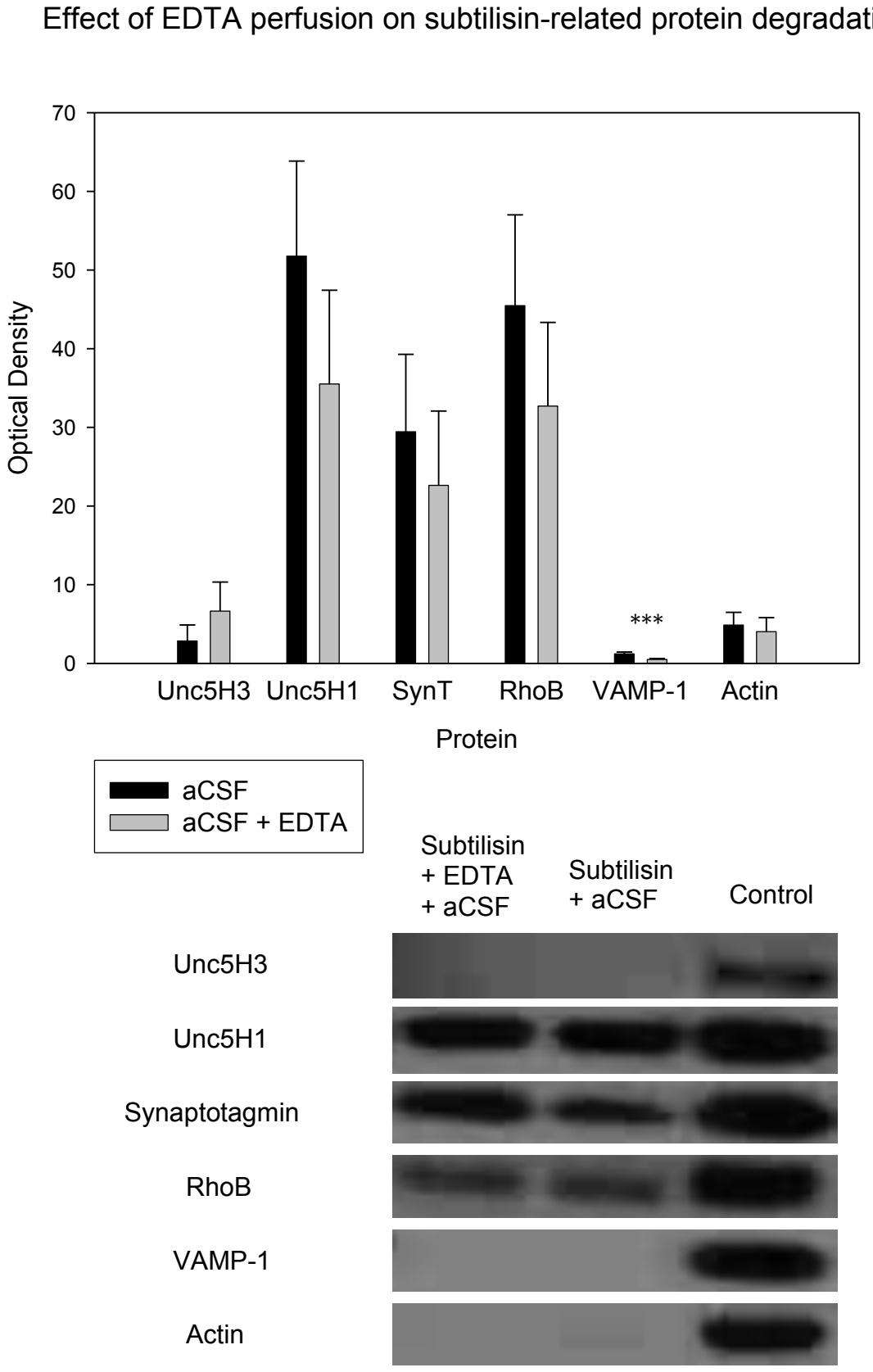


Figure 3.2b Effect of 20µM EDTA on protein degradation in response to subtilisin.

No significant changes in protein expression were observed across all markers between slices treated with EDTA and with aCSF alone. ($P > 0.05$, $N = 4$), with the exception of VAMP-1, which was extremely significant ($P < 0.0001$).

3.1.2.3 Captopril is ineffective as an inhibitor of subtilisin

Captopril is an ACE inhibitor that can be used as an effective chelator of zinc metalloproteases. Unlike EDTA however, it is specific for zinc endopeptidases such as the clostridial toxins (Schiavo *et al.*, 1993). The protocol used during this experiment was identical to the one used for both EDTA and zinc, however, in this experiment aCSF was supplemented with 100μM captopril. Baseline levels in normal aCSF were established over a 10-minute period, followed by another 10-minute slice exposure to aCSF containing captopril. This was followed by 10 minutes of subtilisin perfusion, before a 40-minute washout/recovery period in aCSF containing captopril.

No significant changes (Figure 3.3a, $P = 0.515$) were observed in the progression of subtilisin-mediated LTD between hippocampal slice treated with captopril (63.98 ± 20.84%, N=3) and slices perfused with normal aCSF (39.39 ± 27.47%, N=3). No significant changes were observed in protein expression as measured by immunoblotting between slices exposed to subtilisin in the presence of zinc (Figure 3.3b, $P > 0.05$) for all proteins.

3.1.2.4 Comparison of Subtilisin and Tetanus Toxin effects

The clearest indication of whether subtilisin action was a result of metalloprotease activity would be to compare the action of subtilisin alongside a metalloprotease. To this end, an experiment was planned for comparing the effects of tetanus toxin with subtilisin. Preliminary experiments were carried out to measure the effects of TeTx on hippocampal slice recordings, which demonstrated no difference between the two groups (Figure 3.4, $P = 0.549$). Immunoblotting of these hippocampal slices however indicated no change in the expression of any proteins, including VAMP-1, the target for tetanus toxin (data not shown).

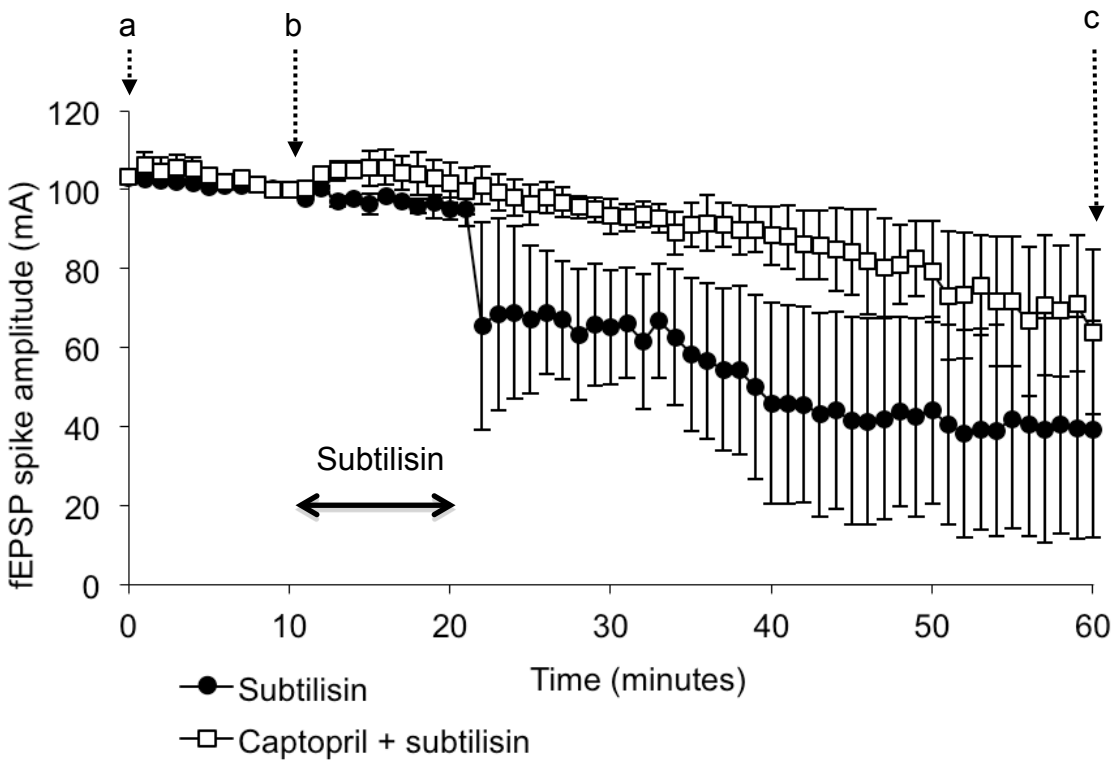
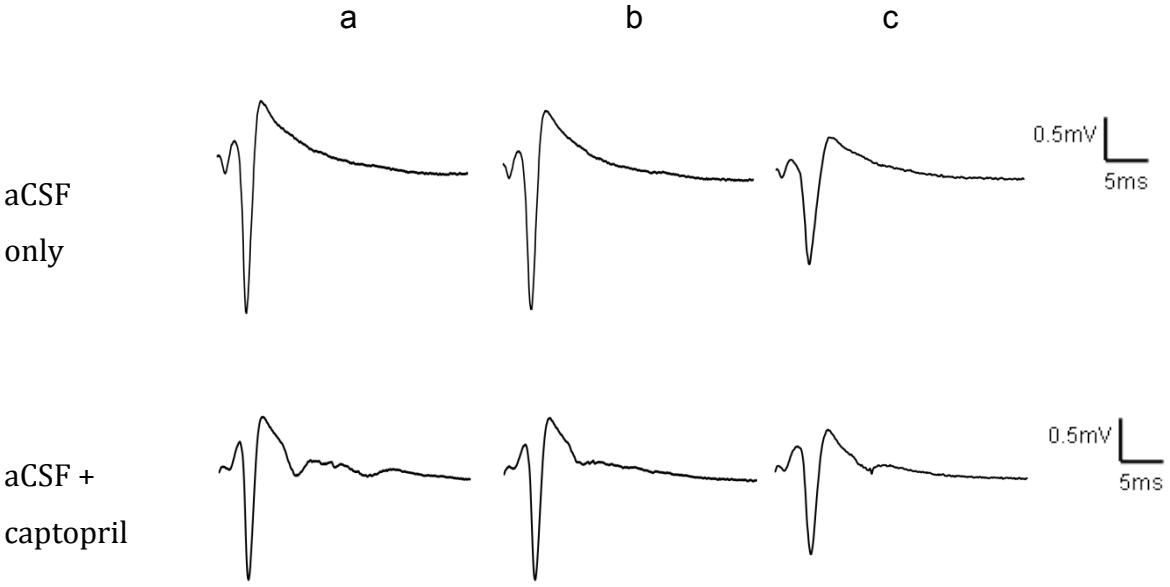


Figure 3.3a Effect of aCSF supplemented with captopril on hippocampal fEPSP size after subtilisin perfusion.

The subtilisin-mediated decrease in fEPSP size was not affected by addition of aCSF supplemented with 100µM captopril to aCSF (39.39 ± 27.47, N=3) when compared to controls (63.98 ± 20.84, N=3) (Students t-test, P =). Arrows a, b and c indicate time points at which traces were taken.



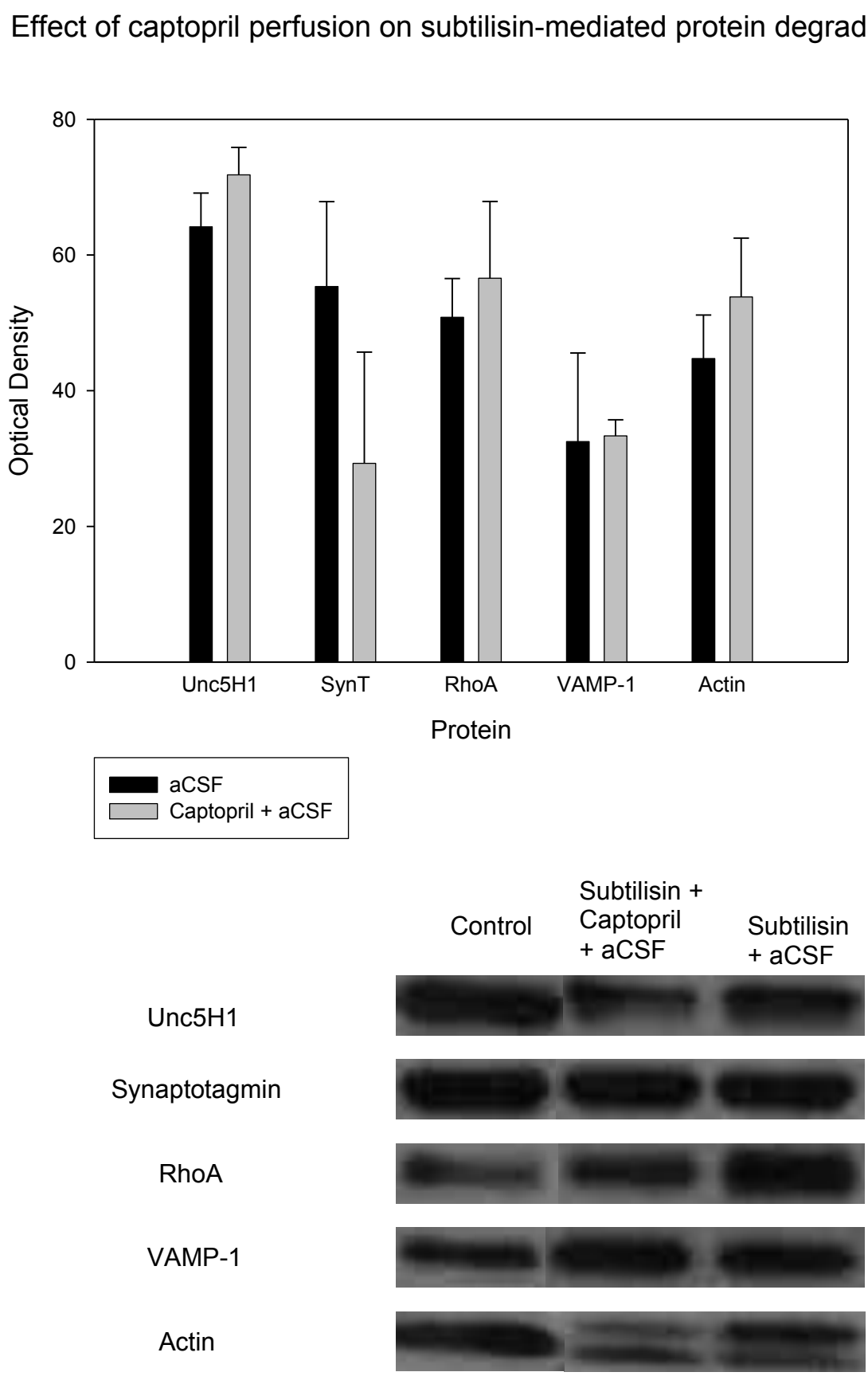


Figure 3.3b Effect of 100µM captopril perfusion on protein degradation in response to subtilisin.

No significant changes were observed between slices perfused with aCSF + captopril and slices perfused with aCSF alone. N =3 for both treatment groups.

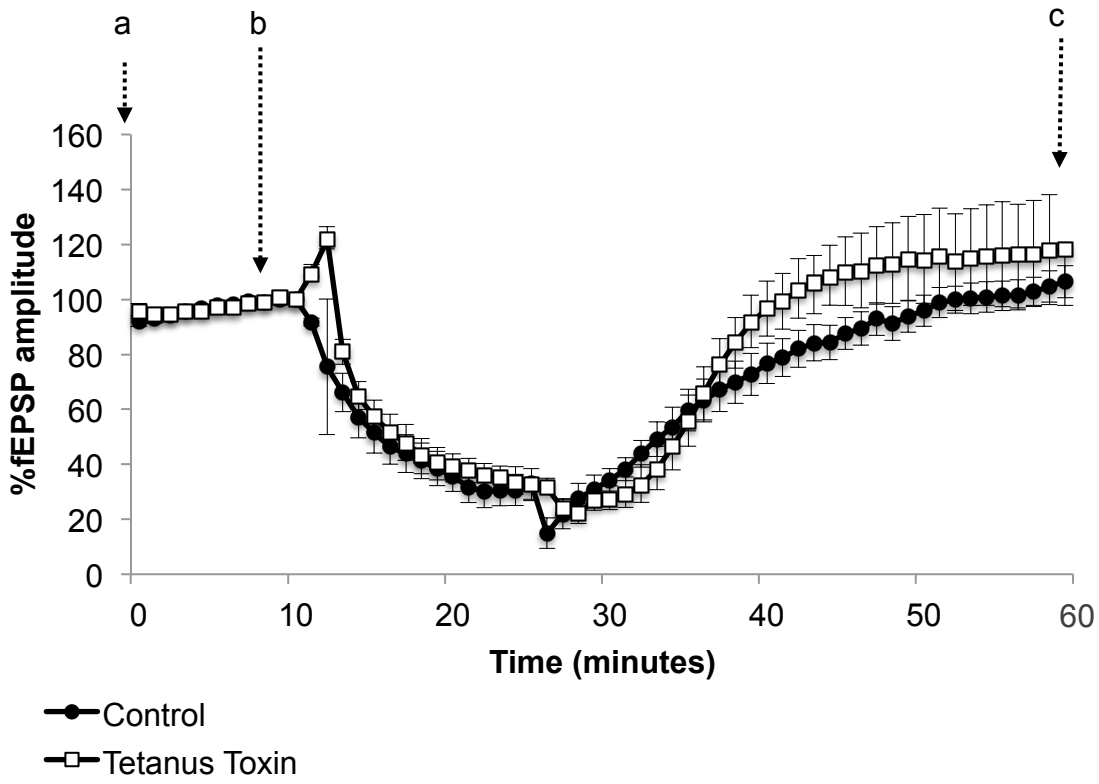
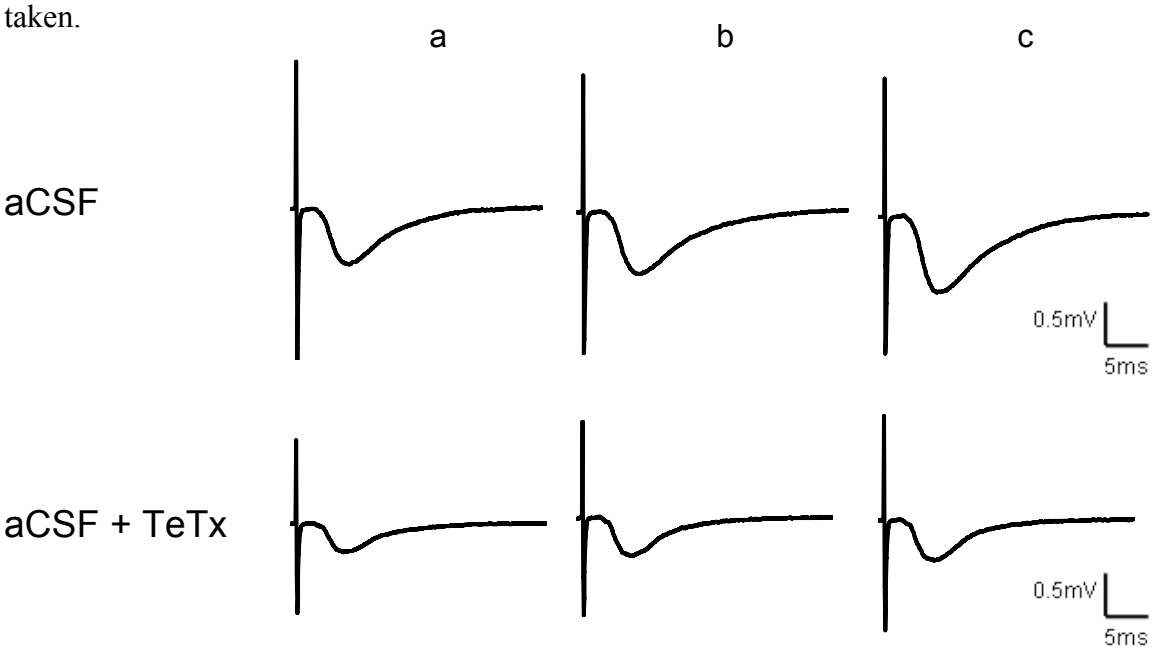


Figure 3.4 Comparison of hippocampal fEPSP response between a 15-minute exposure to TeTx and a 15-minute stoppage period in aCSF.

No differences were observed in the end fEPSP size between slices exposed to 2μM TeTx over a period of 15 minutes, and slices left in normal aCSF over this period. Slices exposed to TeTx (118.22 ± 5.57, N=7), slice exposed to normal aCSF (104.97 ± 20.78, N=7), P = (), no significant. Arrows a, b and c indicate time points at which traces were taken.



3.1.3 Discussion

These experiments were conducted as a result of the similar pattern of VAMP cleavage by both subtilisin and tetanus toxin. This suggested two possibilities; firstly, that subtilisin and tetanus toxin could share a metalloprotease mechanism. Another possibility was that subtilisin action depended on the activation of a metalloprotease possessing similar properties to tetanus toxin, which could explain the degradation of VAMP-1 by both proteases. However, none of the previous literature regarding clostridial toxins has demonstrated that they are capable of generating long-term depression. Similarly, all previous studies regarding subtilisin-mediated LTD have been focused on its characteristics as a serine protease and have not examined the possibility of a role for metalloproteases. If subtilisin action was dependent on the action of metalloproteases, this would represent a new function for the metalloprotease family. Initial experiments were based on the assumption that subtilisin possessed a zinc-dependent mechanism, as VAMP-1 was similarly targeted by tetanus toxin, a zinc-dependent metalloprotease (Yamasaki *et al.,* 1994a). Tetanus toxin is known to be sensitive to the application of chelators such as EDTA, and the exogenous application of zinc ions (Simpson *et al.,* 1993) However, the addition of zinc ions did not increase the potency of subtilisin, as would be expected of a zinc dependent metalloprotease. Furthermore, addition of the zinc-metalloprotease inhibitor captopril to aCSF failed to inhibit subtilisin, further supporting the view that zinc did not have an important role in subtilisin action. Experiments were also carried out to determine whether subtilisin could utilise, or possess, general metalloprotease functions involving other metal ions. As a general chelator of metal ions, EDTA represents an effective inhibitor of many metalloproteases. However, application of EDTA did not have a significant effect on subtilisin-mediated LTD and proteolysis, unlike their effects on metalloproteases such as botulinum toxins (Yamasaki *et al.,* 1994b), therefore discounting the influence of metal ions in subtilisin action. The failure of tetanus toxin to cause VAMP-1 degradation, as demonstrated by immunoblotting, prevented a definitive comparison between the mechanisms of subtilisin and an established metalloprotease. Further optimisation of the application of tetanus toxin would be necessary to rectify this anomaly. However, based on negative results of all other experiments regarding the role of metalloproteases in subtilisin action, it would appear likely that metalloprotease mechanisms do not contribute to subtilisin action.

There were several limitations to the experimental protocol, would could have an impact on the results presented here. The addition of zinc could lead to a disruption of the ionic

balance within the cell; however, electrophysiological data did not demonstrate a noticeable impact in potential when zinc-supplemented aCSF was applied. The use of zinc at a concentration of 50μM might also be too low to generate a measurable change in potential due to the action of buffering agents within the aCSF. Furthermore, the use of 20μM of EDTA may be insufficient to buffer endogenous zinc, as previous experiments were conducted with EDTA at millimolar concentrations (Schiavo *et al.,* 1993). In addition, the presence of other metal ions found within the aCSF could also affect the efficiency of these chelators in binding zinc.

3.2 - Serine protease mechanisms

3.2.1 Introduction

Previous studies have demonstrated the existence of a potential link between the LTD effect caused by subtilisin, and selective degradation of proteins presumably based on its status as a serine protease. However, to date the relationship between these two processes is unclear, and it is not known whether perfusion of subtilisin was capable of eliciting LTD outwith its serine protease action. In order to obtain a better understanding of this relationship, experiments were conducted using the general serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). PMSF possesses inhibitory action against a range of serine proteases, including the chymotrypsin subfamily that shares a similar catalytic active site as subtilisin (MacGregor *et al.,* 2007). Initial experiments were conducted using PMSF at a concentration of 200μM; a second experiment was also conducted using a much lower PMSF concentration of 5μM.

3.2.2 Results

3.2.2.1 Effect of 200μM PMSF on subtilisin-mediated activities

The first experiment was conducted by preparing a solution containing 200μM PMSF and 4μM subtilisin dissolved in aCSF. This solution was gassed with a mixture of 95% O_2 and 5% CO2 over a period of 30 minutes to allow interactions to take place between the protease inhibitor and subtilisin molecules. In this intervening period, a fEPSP with an

amplitude of 0.5-1mV was again elicited in a hippocampal slice, and maintained over a period of 10 minutes to allow formation of a stable baseline. The PMSF-subtilisin perfusate was then perfused over the slice for a 10-minute period, followed by a 40-minute recovery period in which the slice was perfused with normal aCSF. In control slices, subtilisin dissolved in aCSF was perfused in place of a PMSF-subtilisin solution over a 10-minute exposure period. Normal aCSF was perfused throughout the whole experiment, except for a 10-minute period in which aCSF supplemented with either subtilisin alone or PMSF + subtilisin was perfused over the slice. Perfusion of hippocampal slices with PMSF + subtilisin caused a major attenuation of subtilisin activity in comparison to perfusion with subtilisin alone, with the difference being statistically significant ($P =<0.0001$, $N=4$, Figure 3.5a). Furthermore, results from immunoblotting between the two treatment groups reflected the differences found in electrophysiological recordings ($P < 0.05$ for all proteins, $N =4$, Figure 3.5b). A small depressive effect in fEPSP size was also observed immediately after perfusion of the PMSF-subtilisin solution began, and although the potential recovered during the washout period, this could unexpected effect could have an impact on the results obtained.

3.2.2.2 Effect of 5μM PMSF on subtilisin-mediated activities

In order to determine whether the decrease in fEPSP size was due to an interaction of PMSF with subtilisin, or due to a high PMSF concentration, experiments were conducted with a lower concentration of 5μM PMSF. A secondary aim of these experiments was to determine whether the degradation of a specific protein could underlie the generation of LTD. By comparing the effects of PMSF at two different concentrations, it could be possible to determine the protein responsible for the LTD effect. The protocol used for this experiment was the similar to the one used for testing 200μM PMSF, but with the use of a 5μM concentration of PMSF. Normal aCSF was again perfused throughout the whole experiment, with the notable exception of a 10-minute period in which the mixture of PMSF and subtilisin, dissolved in aCSF, was perfused.

At a concentration of 5μM, PMSF was able to completely block the onset of LTD in response to subtilisin perfusion ($P < 0.0001$, $N = 6$, Figure 3.6a), and preventing the degradation of proteins ($P < 0.05$ for all protein markers, $N = 6$, Figure 3.6b)

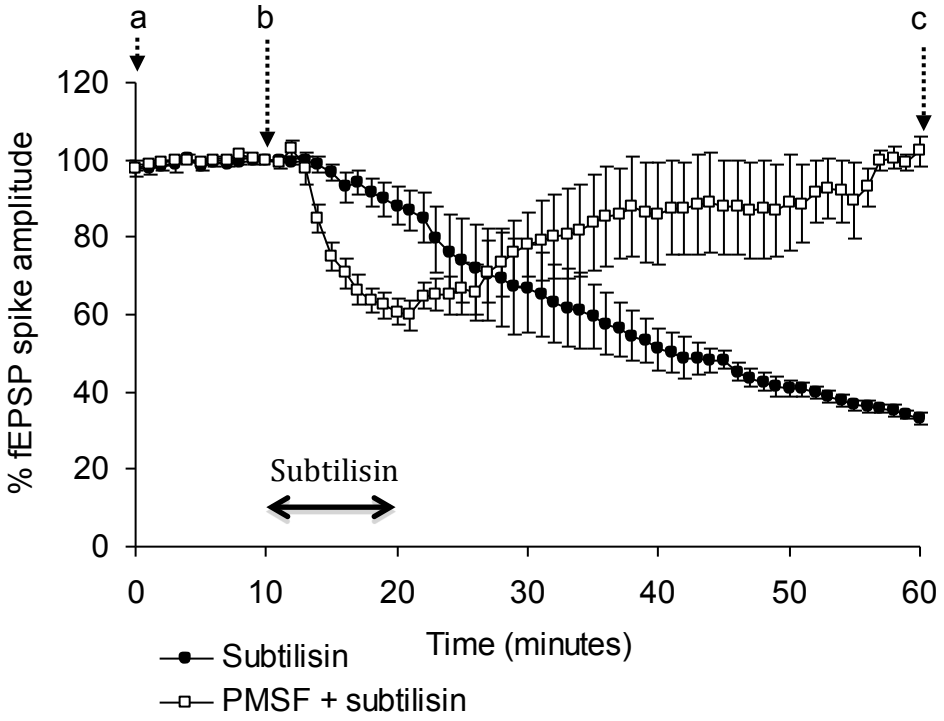
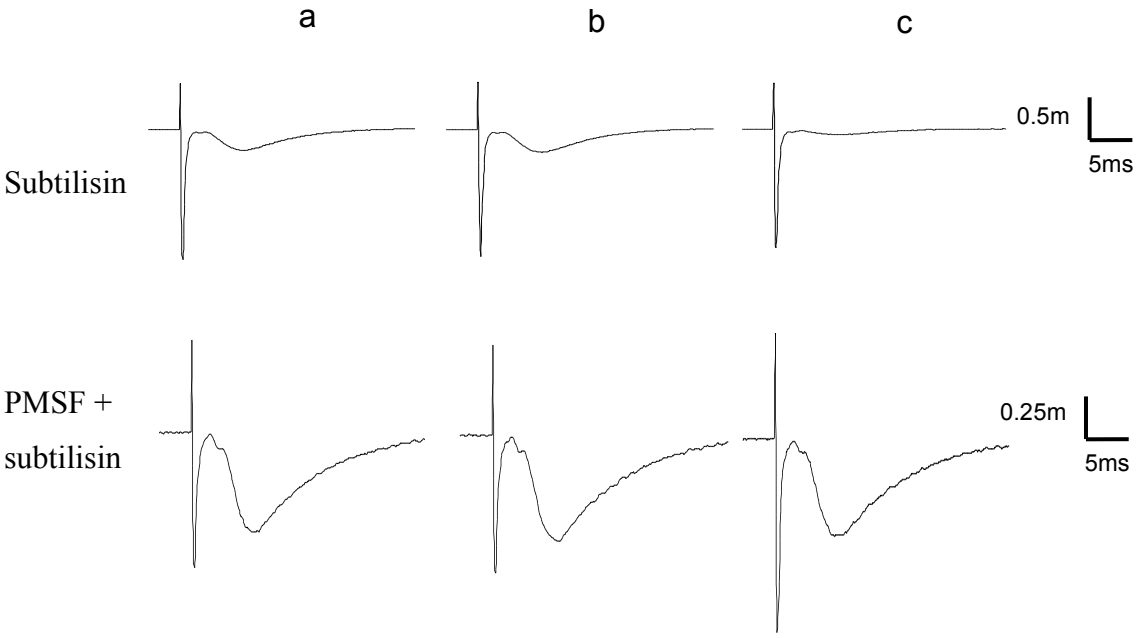


Figure 3.5a Effect of co-perfusion of 200µM PMSF on fEPSP response to 4µM subtilisin perfusion.

Slices were treated with either 4µM subtilisin alone or a mixture of 200µM PMSF and 4µM subtilisin. Pretreatment of subtilisin with PMSF significantly attenuated the fEPSP response to subtilisin (102.16 ± 3.63%, N = 4) in comparison to subtilisin alone (alone 32.95 ± 1.55%, N = 4) ($P<0.0001$). Of note was the initial decrease in PMSF-treated slices, reached a maximum value of 59.78 ± 3.38% (N =4) baseline. Arrows a, b and c indicate time points at which traces were taken.



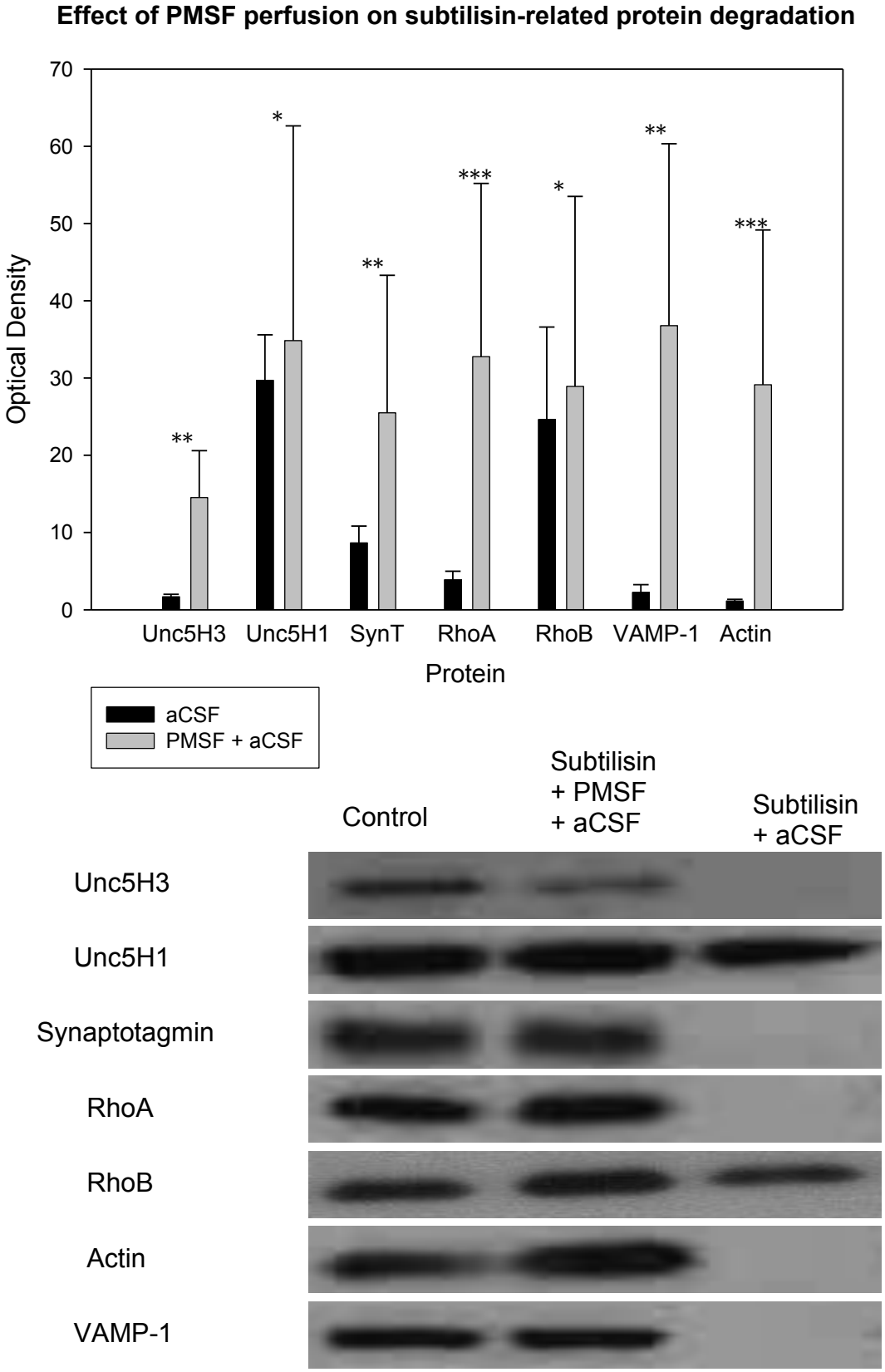


Figure 3.5b Effect of co-perfusing 200µM PMSF on protein degradation response to 4µM subtilisin.

Significant differences between the two treatments were observed in all protein markers: Unc5H1, RhoB ($P<0.05$, *), Unc5H3, SynT and VAMP-1 ($P<0.001$, **); RhoA, Actin ($P<0.0001$, ***). N = 4 for both subtilisin and PMSF + subtilisin.

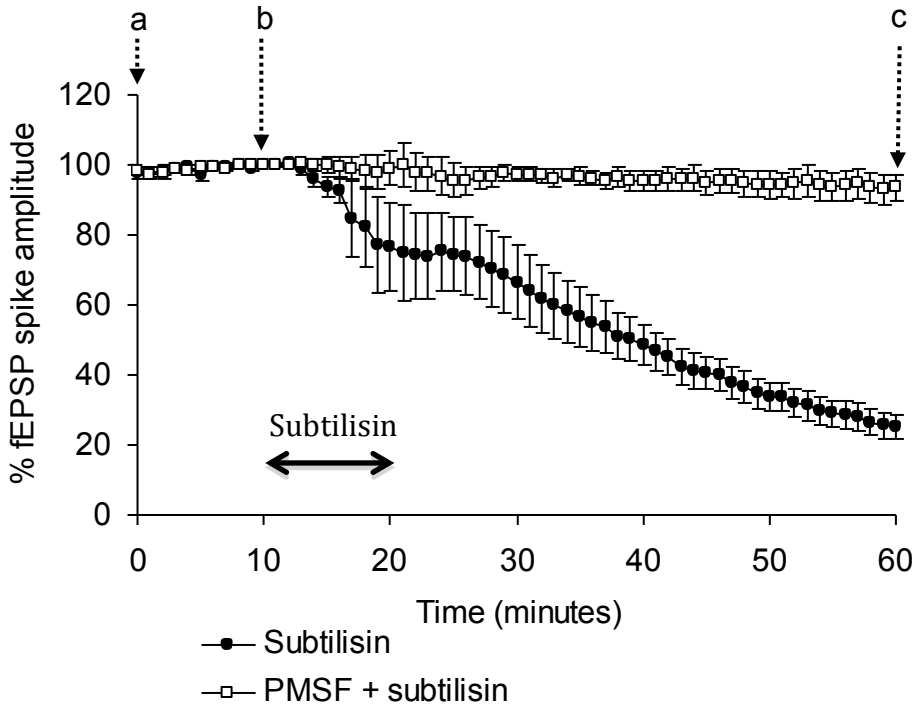
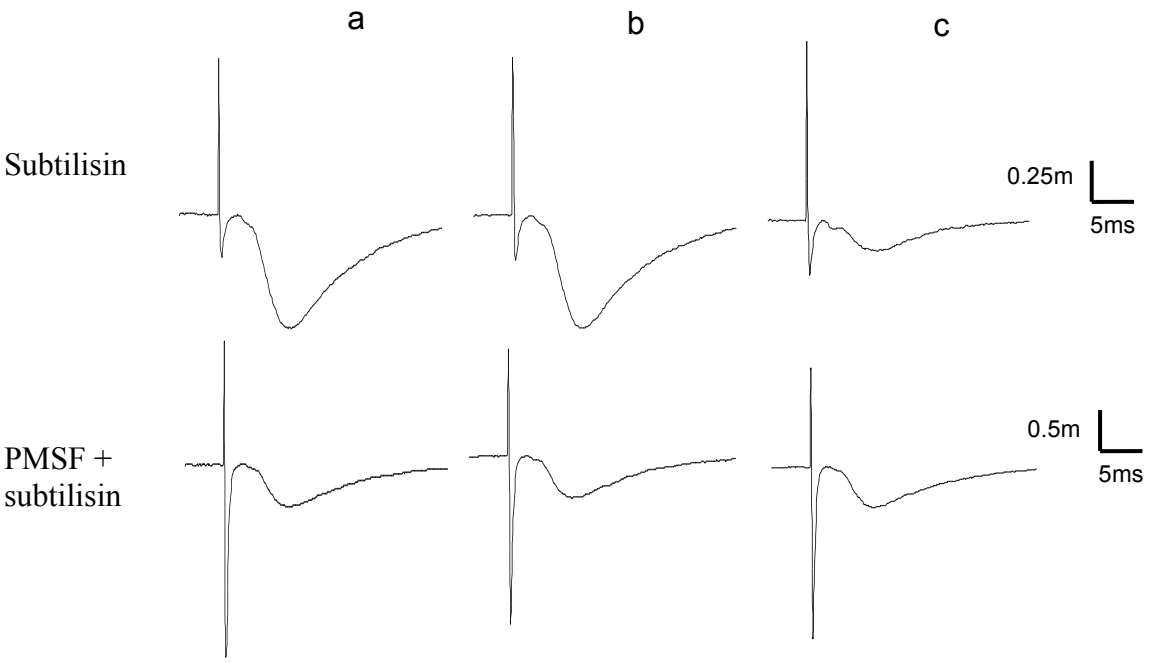


Figure 3.6a Effect of co-perfusion of 5µM PMSF the on fEPSP response to 4µM subtilisin perfusion

Pretreatment of subtilisin with PMSF significantly attenuated the fEPSP response to subtilisin (93.51 ± 3.8%, N = 6) in comparison to subtilisin alone (25.2 ± 3.63%, N = 6) ($P < 0.0001$). Arrows a, b and c denote time points at which traces were taken as shown below.



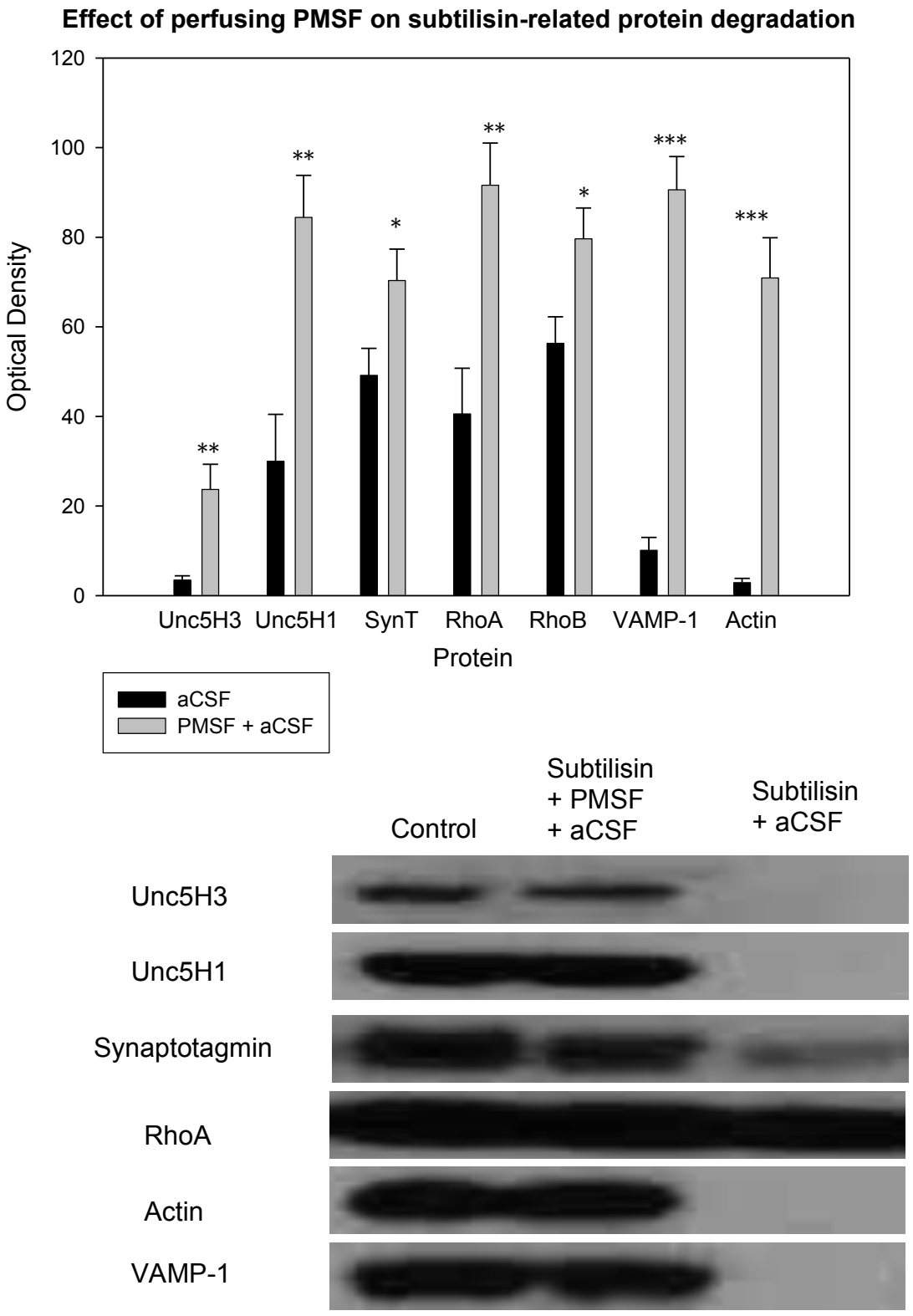


Figure 3.6b Effect of co-perfusing 5µM PMSF on protein degradation response to 4µM subtilisin

Significant differences between the two treatments were observed in all protein markers: SynT, RhoB ($P<0.05$,*), Unc5H3, Unc5H1 and RhoA ($P<0.001$, **); VAMP-1, Actin ($P<0.0001$, ***). N = 11 for both subtilisin and PMSF + subtilisin.

3.2.3 Discussion

Based on the negative results of experiments regarding the contribution of metalloprotease mechanisms to the action of subtilisin, it was suggested that subtilisin-mediated LTD was instead dependent on their endogenous serine protease mechanism. In order to confirm this theory, the serine protease action of subtilisin was inhibited through the use of the general serine protease inhibitor PMSF. An additional aim was to confirm whether the proteolysis of proteins in response to subtilisin perfusion was related to the generation of LTD. Using two concentrations of PMSF, the possibility of a sequential breakdown of proteins was also explored, which could result in the identification of the precise mechanism of subtilisin action. However, both concentrations of PMSF produced a similar inhibition LTD generation usually found after perfusion of subtilisin. PMSF at both these concentrations were also equally effective in preventing protein degradation in response to subtilisin. These results reinforce the view that subtilisin action is based solely on its serine protease function, in agreement with a previous study demonstrating the effectiveness of PMSF as an inhibitor of subtilisin and related serine proteases (MacGregor *et al.,* 2007). These experiments did not however provide any new data on its mechanism of action. Further experiments into the role of serine protease mechanisms could be conducted using lower concentrations of PMSF, as subtilisin is completely inhibited by 5μM PMSF. At a concentration of 200μM, PMSF also generated an initial decrease in potential size, which could be the result of an inhibition of endogenous serine proteases. Reducing the concentration of PMSF to 5μM abolished this effect. A more specific serine protease inhibitor, such as eglin (McPhalen *et al.,* 1985) could be more efficient in determining the susceptibility of individual proteins to subtilisin degradation, therefore highlighting any potential proteolytic cascades in response to the induction and onset of LTD.

Chapter 4 – Investigations into the role of specific protein targets in subtilisin-mediated LTD.

4.1 Importance of VAMP-1 proteins to the action of subtilisin

4.1.1 Introduction

The action of proteases can be effectively inhibited by preventing interfering with the binding of the substrate to the active site. This can be effected through a change in the structure of the active site by substituting key residues at this site, or by altering the recognition site on the substrate via methylation or phosphorylation.

The shape of the substrate recognition site can also be changed using antibodies targeted to this site, and is known to be effective in preventing the action of proteases against substrates. An example of this method is the use of antibodies against malarial merozoites, which prevent the activation of a key surface protein by proteolytic processing (Blackman *et al.*, 1994). Of particular interest is a study that demonstrated that antibodies against VAMP proteins could prevent its degradation by tetanus toxin (Poulain *et al.,* 1993) It is therefore plausible that antibodies could also prevent the degradation of VAMP-1 by subtilisin. The use of antibodies would also allow an evaluation into whether structural changes to these proteins themselves could trigger LTD.

For the purposes of this investigation, protein-specific antibodies against Unc5H3 and VAMP-1 were used, as these were the proteins most affected by the action of subtilisin. The following protocol was used for all antibody experiments; with minor changes between each separate set of experiments. After a 1-hour recovery period following dissection, hippocampal slices were separated into two groups into petri dishes, each containing approximately the same number of slices. One dish was set aside as a control, and slices within were exposed to aCSF alone throughout the incubation period. Slices in the second dish was incubated in an aCSF solution containing a set concentration of either VAMP-1 or Unc5H3 antibodies, and both control and antibody dishes were subsequently left in the incubation chamber gassed with a mixture of 95% O_2 and 5% CO_2 for 20 minutes at room temperature. At the end of this period, aCSF in both dishes was removed via a syringe, and the slices were carefully washed 3 times with fresh, oxygenated aCSF.

After these washes were completed, normal aCSF was added to both dishes and slices placed back into the incubation chamber for a recovery period of 30 minutes prior to use. For experiments involving triton, slices were exposed to a more concentrated antibody solution for an initial period of 5 minutes, before aCSF containing the 0.01% triton was added to produce the desired antibody concentration, and left to incubate for a further 15 minutes. Thereafter they were washed 3 times as before, and left to incubate for 30 minutes at room temperature prior to use. Experiments with streptolysin involved incubation of slices with 500ng streptolysin over a period of 5 minutes, before the addition of antibody-containing aCSF for the remaining 15-minute period of incubation. Subsequent washes and recovery incubation period was identical to the other experiments.

4.1.2 Results

4.1.2.1 Effect of 1:5000 VAMP-1 antibody preincubation on subtilisin

VAMP-1 antibodies were initially applied to hippocampal slices at a concentration of 1:5000. A solution containing this concentration of antibodies was prepared by addition of 0.4µl antibody to 20ml of aCSF, a volume that represents the total volume of the petri dish. Preincubation of hippocampal slices with VAMP-1 antibodies at this concentration did not result in any significant changes ($P > 0.05$, $N = 4$, Figure 4.1a) to the extent of subtilisin-mediated fEPSP depression. This result was also mirrored by the findings from immunoblotting, which demonstrated no change ($P > 0.05$ for all markers, apart from Unc5H1 ($P = 0.0063$), $N = 4$, Figure 4.1b) in the levels protein expression, including the levels of VAMP-1.

4.1.2.2 Effect of hippocampal slice preincubation with 0.01% triton and 1:5000 VAMP-1 antibody on the effects of subtilisin

As the use of 1:5000 VAMP-1 antibodies failed to cause any changes to either fEPSP response or protein expression levels, it was possible that this was due to the inability of the antibodies to penetrate the cells within the hippocampal slice. In an effort to eliminate this variable, the permeabilising agent triton was be used to facilitate antibody entry into the cells. A 5ml aCSF solution containing 0.4µl VAMP-1 antibody was prepared and incubated with the hippocampal slices over a period of 5 minutes. A 15ml aCSF solution

containing 2µl triton was prepared, and subsequently added to the slices to yield a total triton concentration of 0.01% and incubated for a further 15 minutes. Preincubation of hippocampal slices in with the mixture of 0.01% triton and 1:5000 VAMP-1 antibodies did not cause any significant changes to the size of the LTD effect in response to subtilisin perfusion ($P > 0.05$, $N = 4$, Figure 4.2a). Likewise, no significant differences were observed in the pattern of protein degradation between slices preincubated with the triton-VAMP-1 solution, and those preincubated in aCSF alone ($P > 0.05$, $N = 3$, Figure 4.2b).

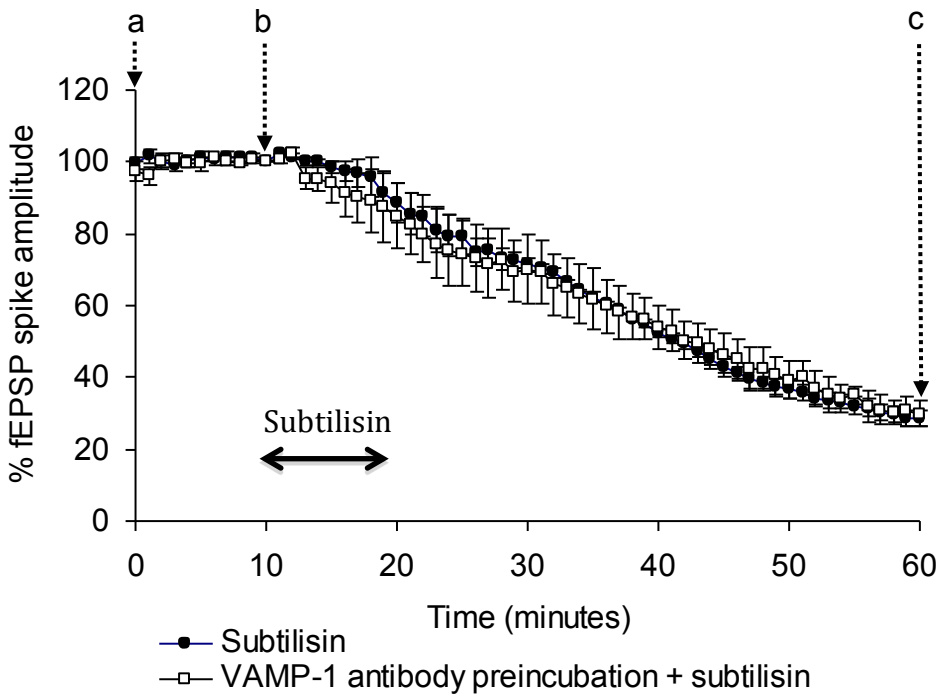
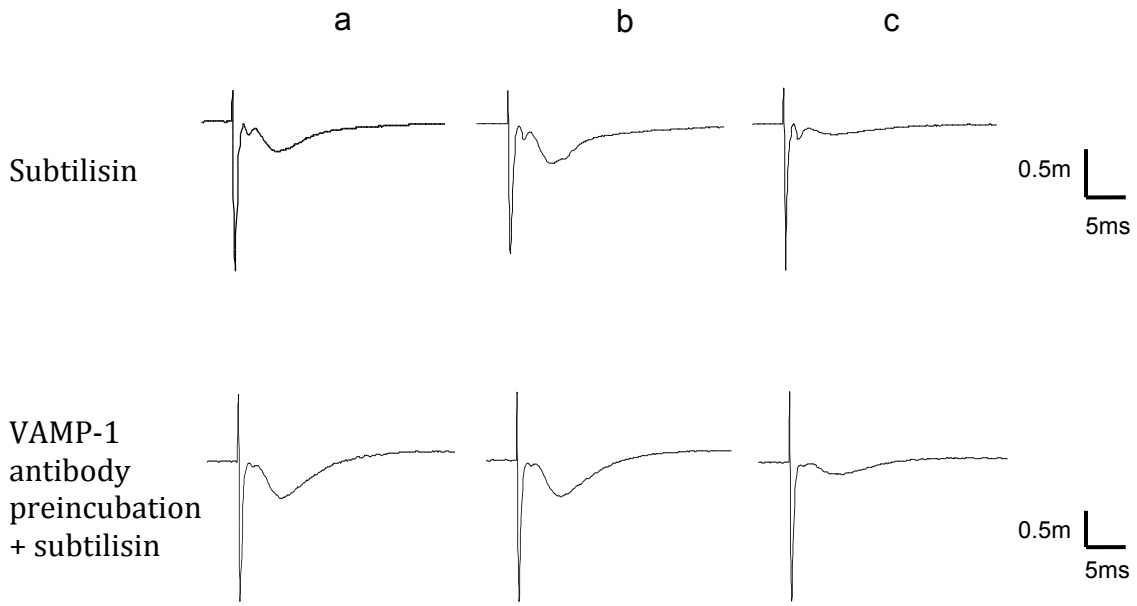


Figure 4.1a Effect of preincubating hippocampal slices with VAMP-1 antibodies on fEPSP response to subtilisin perfusion.

Preincubation with VAMP-1 antibodies did not change the fEPSP response to subtilisin (29.99 ± 3.45%, N=3) when compared with slices incubated in aCSF alone (28.51 ± 2.21%, N=4)(P = 0.0968). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



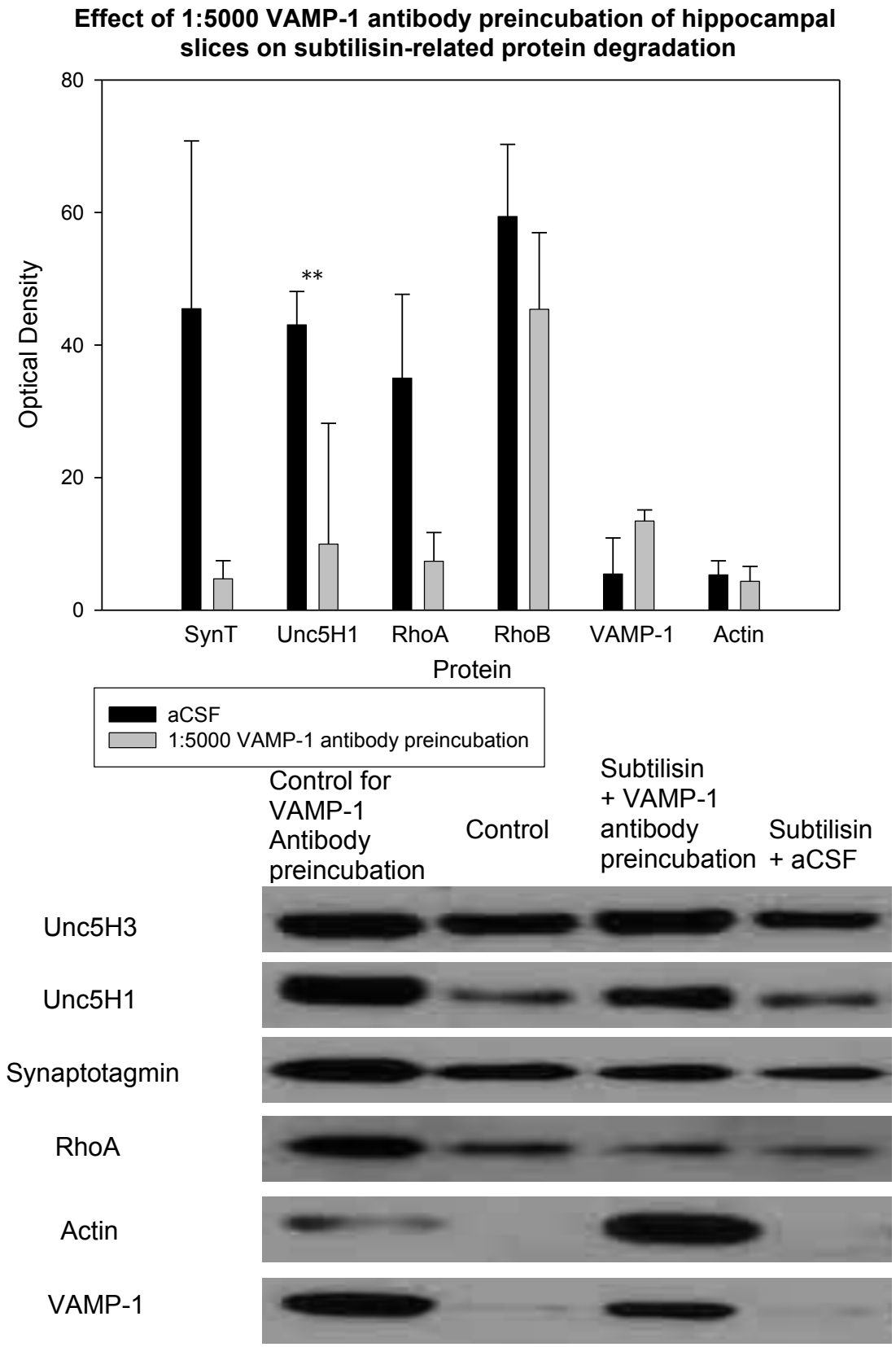


Figure 4.1b Effect of preincubating hippocampal slices with 1:5000 VAMP-1 antibodies on protein expression in response to subtilisin.

There was a very significant difference in protein expression for Unc5H1 (P = 0.0063, **) between the two groups; all other proteins were not significantly different. N = 3 for both antibody preincubation and untreated groups.

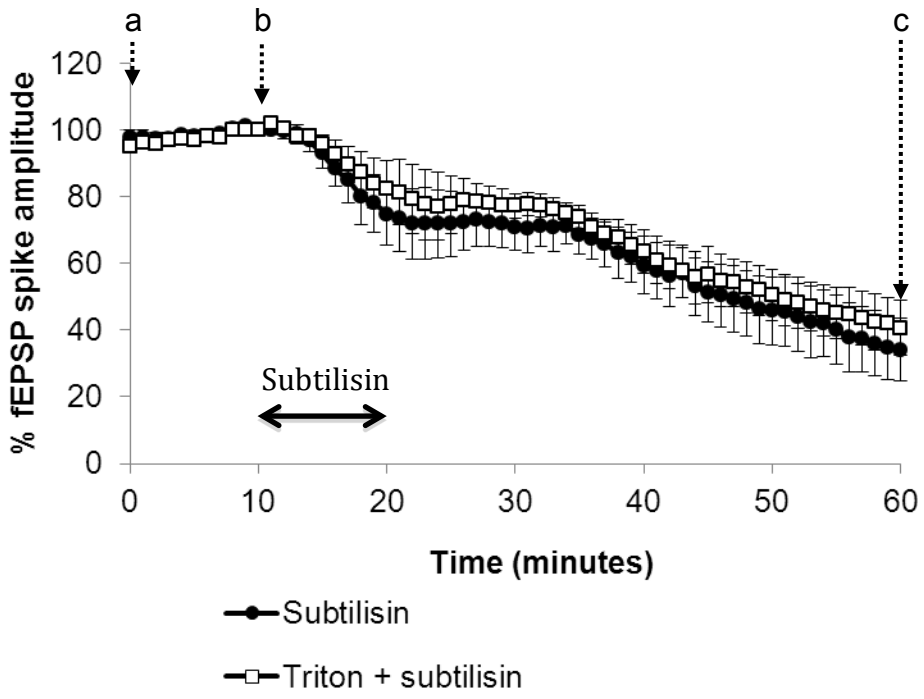
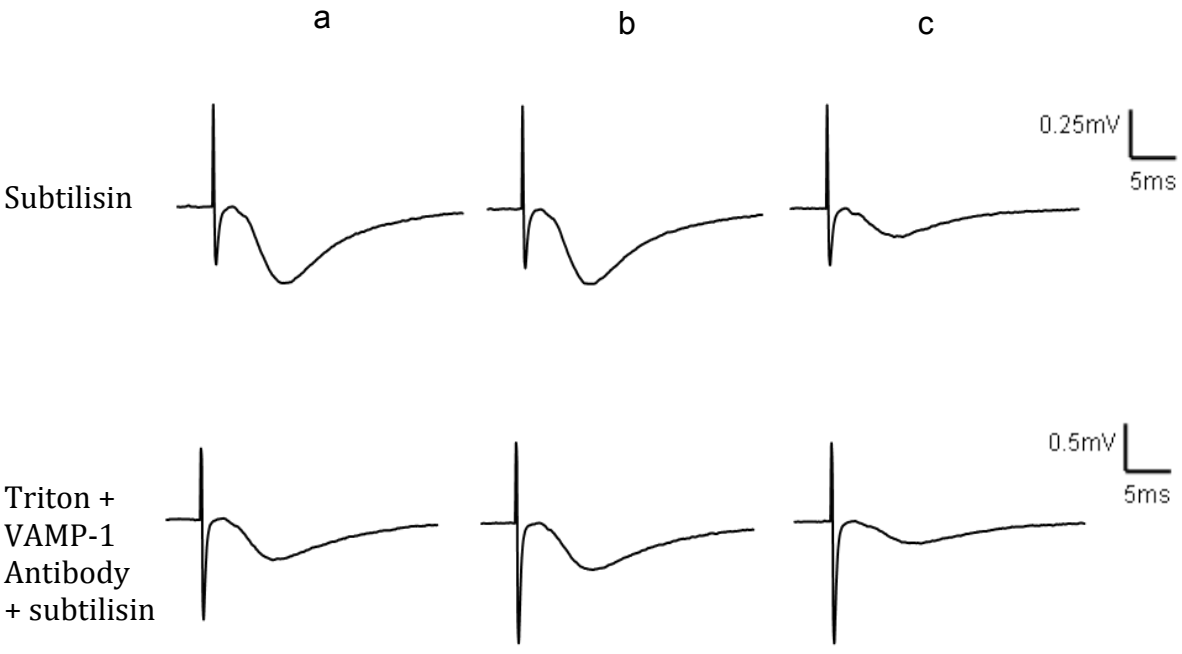


Figure 4.2a Effect of preincubating hippocampal slices with 0.01% triton and 1:5000 VAMP-1 antibodies on fEPSP response to subtilisin perfusion.

Preincubation with triton and VAMP-1 antibodies did not change the fEPSP response to subtilisin (40.69 ± 8.39%, N=4) when compared with slices incubated in aCSF alone (34.18 ± 9.41%, N=4)(P = 0.631). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



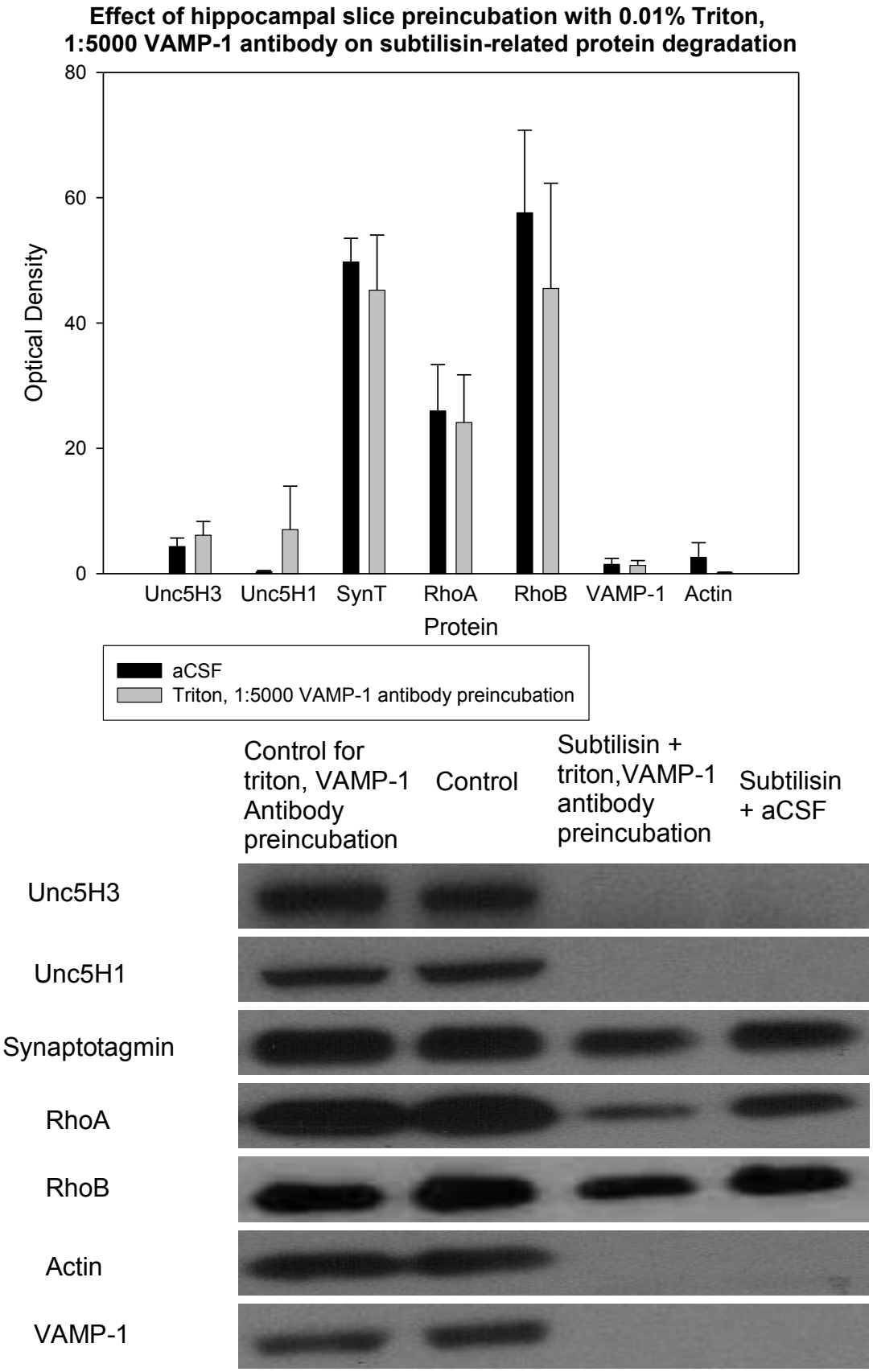


Figure 4.2b Effect of preincubating hippocampal slices with 0.01% triton and 1:5000 VAMP-1 antibodies on protein expression in response to subtilisin.

No significant differences were observed in protein expression between the two treatment groups. N = 4, P > 0.05 for all proteins in antibody preincubation and untreated groups.

4.1.2.3 Effect of 1:500 VAMP-1 antibody preincubation on subtilisin

Preincubation of slices with 1:5000 VAMP-1 antibodies had no effect on subtilisin-mediated LTD, or protein degradation in response to subtilisin perfusion. The presence of triton in the preincubation solution likewise did not produce any noticeable change to either process, suggesting that the concentration of VAMP-1 antibody could be insufficient to make an impact on the action of subtilisin. To test this hypothesis, further experiments were be conducted using a higher concentration of 1:500 VAMP-1 antibody in order to increase the rate of antibody-protein interactions. The antibody solution was prepared by addition of 4µl antibody to 20ml of aCSF, which represented the total volume of the petri dish. Preincubation of hippocampal slices with VAMP-1 antibodies at a concentration of 1:500 did not result in any significant changes (Figure 4.3a; $P > 0.05$, $N = 4$) to the extent of subtilisin-mediated LTD when compared to control slices. This result was also mirrored by data obtained immunoblotting on the same hippocampal slices, which demonstrated no change (Figure 4.3b) in the levels of VAMP protein expression.

4.1.2.4 Effect of hippocampal slice preincubation with 100ng/ml streptolysin and 1:5000 VAMP-1 antibody on the effects of subtilisin

VAMP-1 antibodies, even at an increased concentration of 1:500, failed to elicit any changes to the progression of subtilisin-mediated LTD or the associated protein degradation. The use of permeabilising agents was again considered, and since the permeabilising chemical triton was ineffective in preventing subtilisin action, an alternative was considered. The pore-forming compound streptolysin is capable of forming pores large enough to allow the entry of antibodies into cells, and could therefore be an effective method of facilitating cellular entry of VAMP-1 antibodies for this experiment. (Walev *et al.*, 2001) For this experiment, a 5ml aCSF solution containing 200ng streptolysin was formulated and incubated with the hippocampal slices over a period of 5 minutes. A 15ml aCSF solution containing 4µl of VAMP-1 antibody was prepared with 15ml aCSF, and subsequently added to the slices to produce an antibody 1:500 which was incubated for a further 15 minutes. Results of this preincubation protocol involving a combination of streptolysin and VAMP-1 antibody yielded no significant changes to the extent of the LTD response to subtilisin (Figure 4.4a; $P > 0.05$, $N = 4$). Protein degradation was also not inhibited by the addition o or in protein degradation (Figure 4.4b) as a result of subtilisin perfusion.

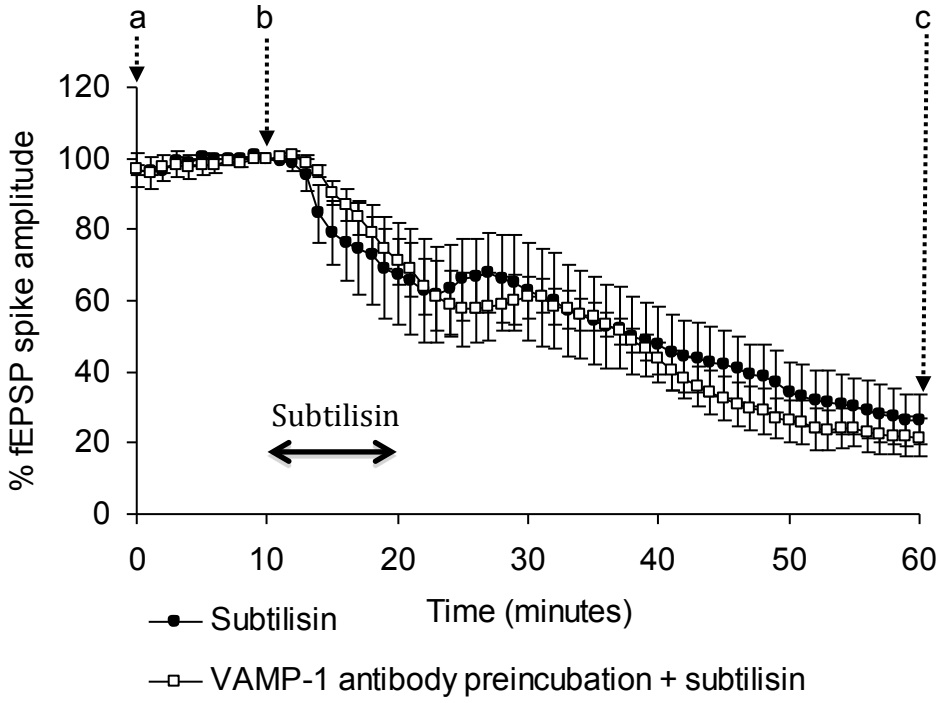
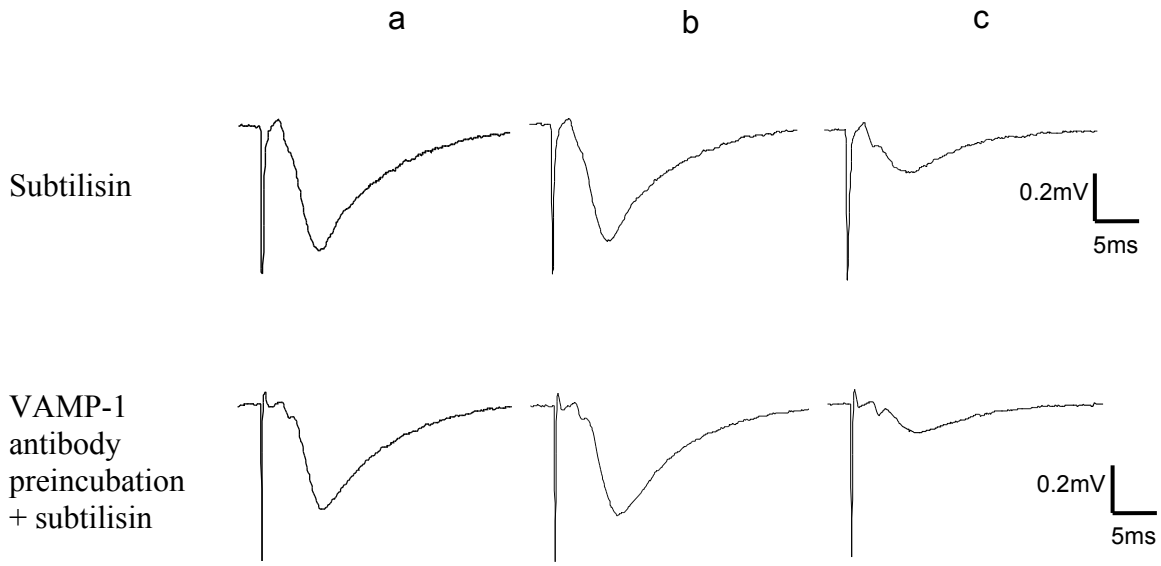


Figure 4.3a Effect of preincubating hippocampal slices with 1:100 VAMP-1 antibodies on fEPSP response to subtilisin perfusion.

Preincubation with VAMP-1 antibodies did not change the fEPSP response to subtilisin (21.49 ± 5.5%, N=4) when compared with slices incubated in aCSF alone (26.54 ± 6.91%, N=4)(P = 0.5883). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



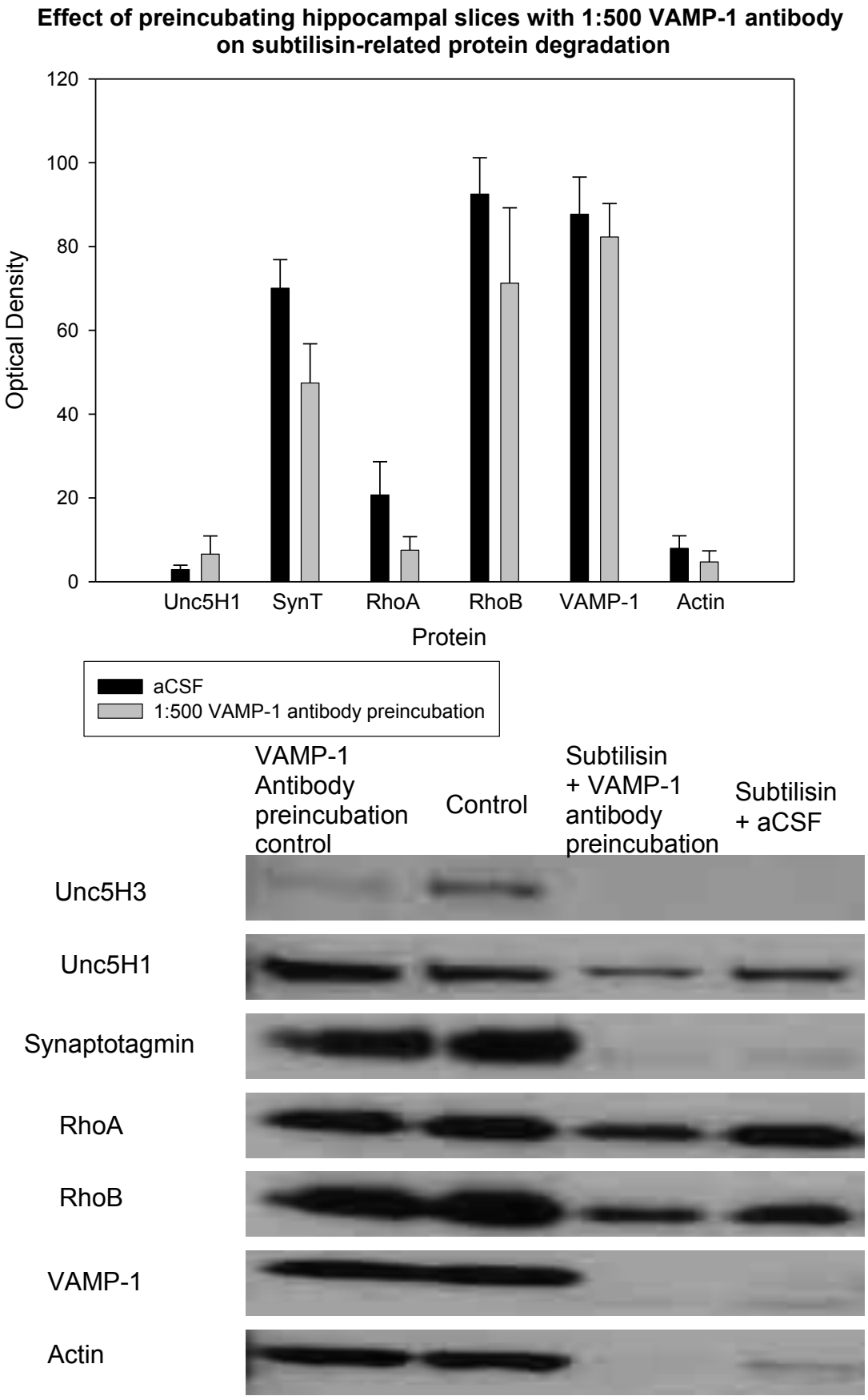


Figure 4.3b Effect of preincubating hippocampal slices with 1:500 VAMP-1 antibodies on protein expression in response to subtilisin.

No significant differences in protein expression were observed between the antibody preincubation and untreated groups. N = 4, P > 0.05 for all proteins

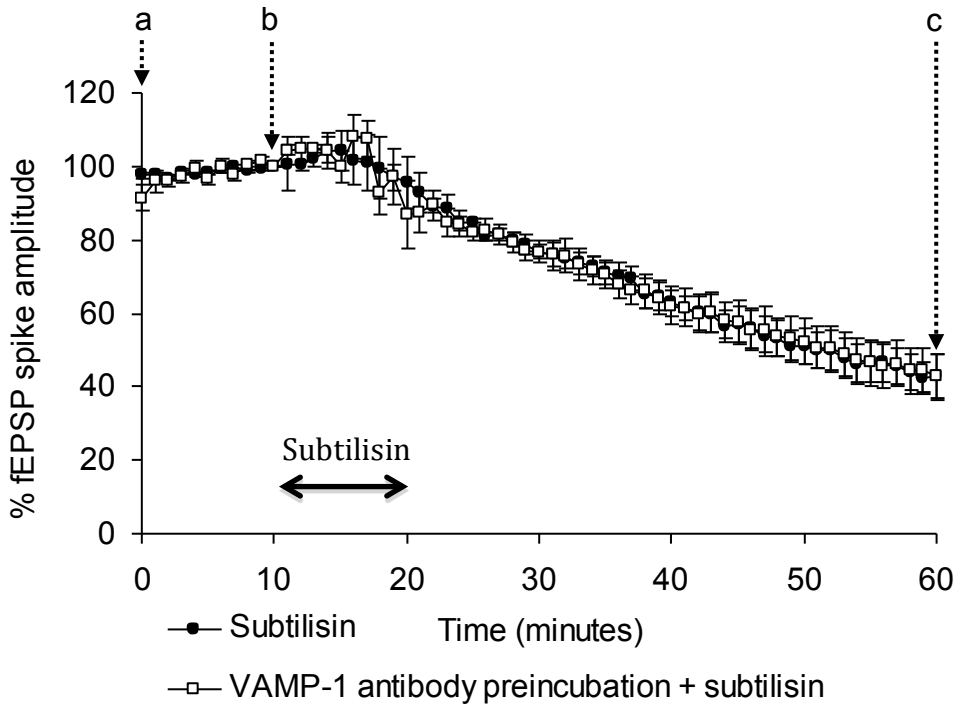
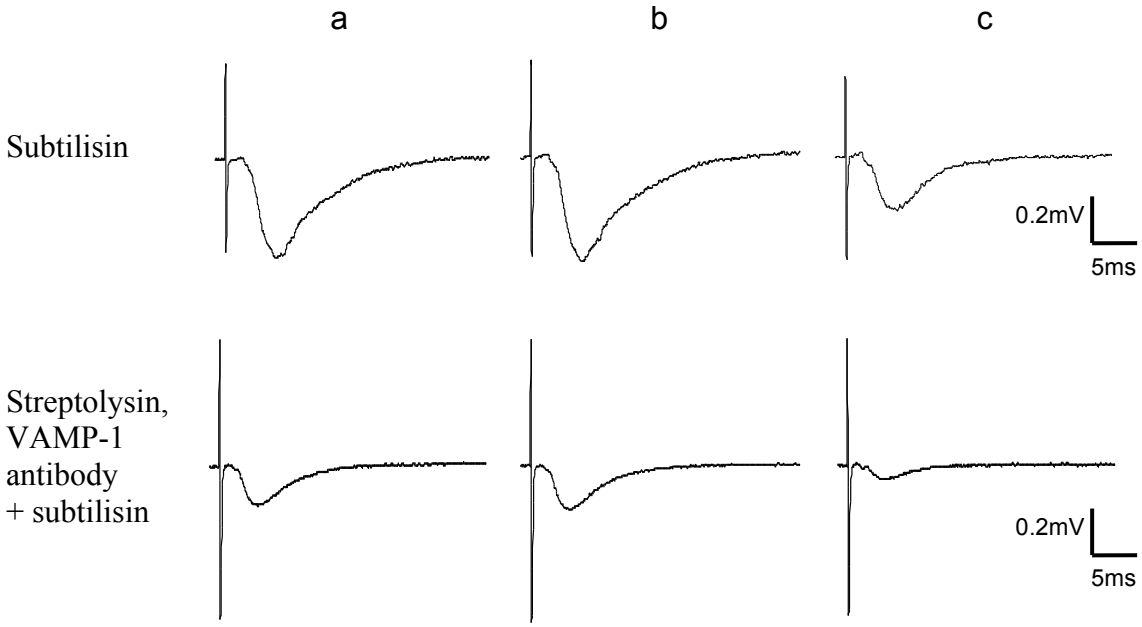


Figure 4.4a Effect of preincubating hippocampal slices with 100ng/ml streptolysin and VAMP-1 antibodies on fEPSP size.

Subtilisin-mediated fEPSP response to slice preincubation with 100µg streptolysin, 1:500 VAMP-1 antibody. Preincubation with streptolysin and VAMP-1 antibodies did not change the fEPSP response to subtilisin (42.65 ± 6.38%, N=4) when compared with slices incubated in aCSF alone (42.85 ± 5.77%, N=4)(P = 0.8512). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



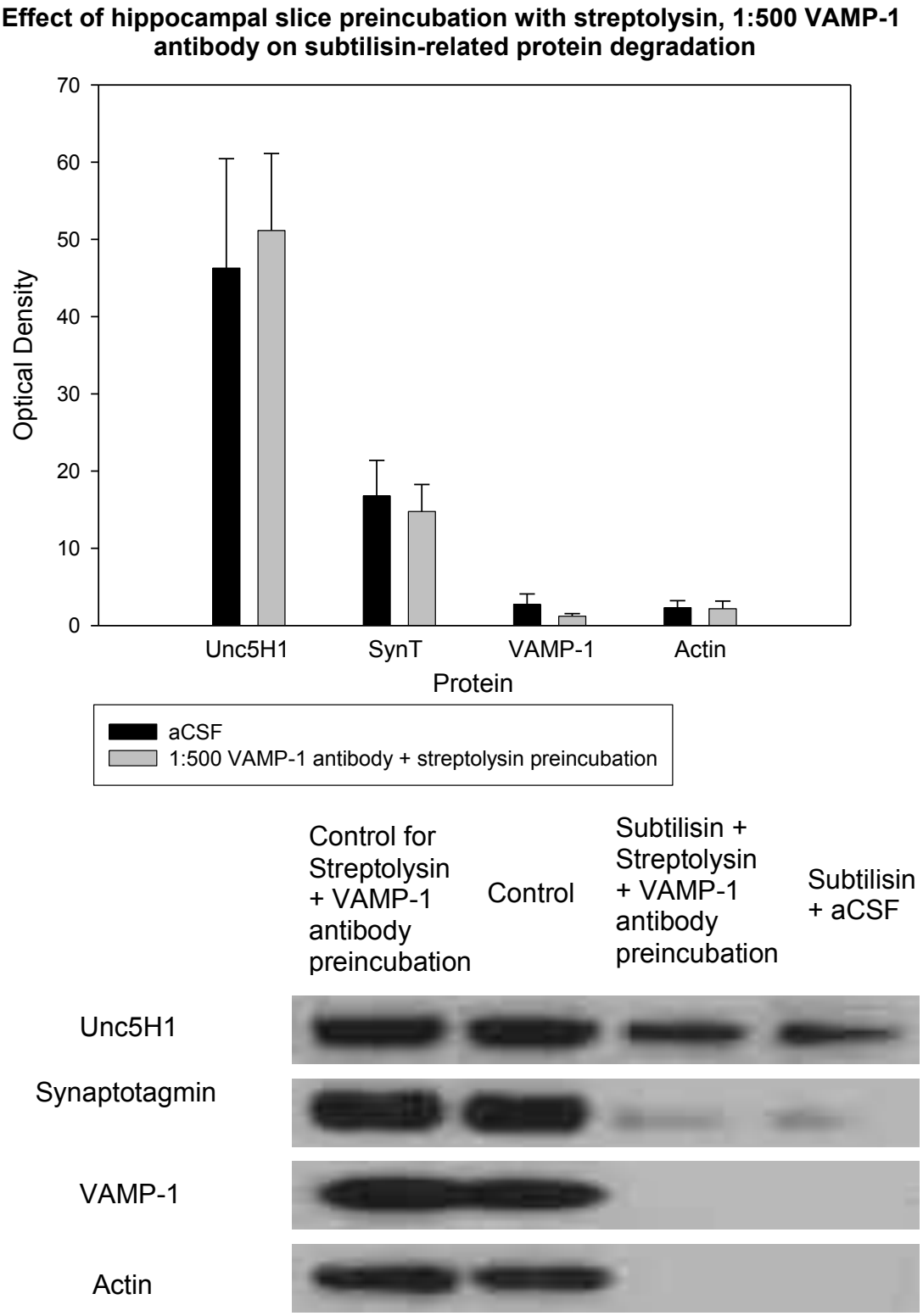


Figure 4.4b Effect of hippocampal slice preincubation with 100ng/ml streptolysin and 1:500 VAMP-1 antibodies on protein expression in response to subtilisin.

There were no significant differences in the pattern of protein degradation between slices preincubated with a mixture of streptolysin and VAMP-1 antibody and control slices exposed to aCSF alone. N=4, P >0.05 for all protein markers.

4.2 Effect of Unc5H3 antibody preincubation on the action of 4μM subtilisin

4.2.1 Introduction

The Unc5H3 is an established target for subtilisin action, and also acts as a regulator for actin via the Rho GTPases. Since actin relatively susceptible to subtilisin degradation, interfering with the proteolysis of Unc5H3 could represent an effective way of preventing subtilisin activity.

4.2.2 Results

4.2.2.1 Effect of Unc5H3 antibodies at a concentration of 1:1000 on the action of subtilisin

The first experiments into the effects of hippocampal slice preincubation with Unc5H3 antibodies were conducted using an antibody concentration of 1:1000. The antibody-aCSF solution was prepared by addition of 2μl Unc5H3 antibody to 20ml of aCSF, held in a petri dish. Slices underwent three washes in normal aCSF before they were left to recover at room temperature. Hippocampal slice preincubations using Unc5H3 antibodies at a concentration of 1:1000 did not result in any significant changes ($P > 0.05$, $N = 4$ (aCSF), $N = 5$ (triton + Unc5H3 antibody), Figure 4.5a) to the extent of subtilisin-mediated LTD. Similar results were also obtained from immunoblots of the hippocampal slices used for recordings, demonstrating no significant differences in protein degradation between slices preincubated in Unc5H3 antibodies, and those preincubated with aCSF alone ($P > 0.05$, Figure 4.5b).

4.2.2.2 Effect of hippocampal slice preincubation with 0.01% triton and 1:1000 Unc5H3 antibody on the effects of subtilisin

Hippocampal slices were also preincubated with a mixture of 0.01% triton and 1:1000 Unc5H3 antibodies, in an attempt to increase the intracellular concentration of antibody to allow greater interaction with the Unc5H3 protein. For this study, a 5ml aCSF solution containing 2µl VAMP-1 antibody was incubated with the hippocampal slices over a period of 5 minutes. A 15ml aCSF solution containing 2µl triton was prepared, and subsequently added to the slices to yield a total triton concentration of 0.01% and incubated for a further 15 minutes. Hippocampal slice preincubation with triton + Unc5H3 antibodies did not result in any significant changes to the progression of subtilisin-mediated LTD ($P > 0.05$, $N = 4$, Figure 4.6a). The protein expression levels of Unc5H3 differed very significantly ($P = 0.0063$) in slices preincubated with triton and Unc5H3, however, no other significant changes were found in the other protein markers measured ($P > 0.05$, $N = 3$, Figure 4.6b)

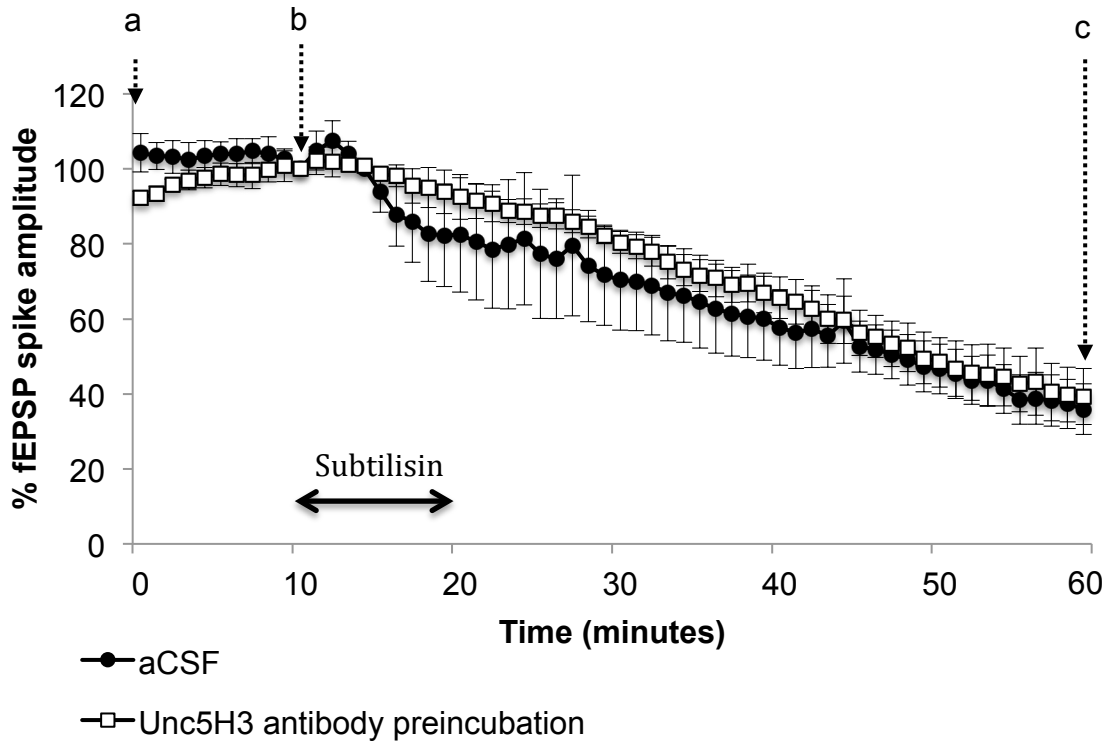
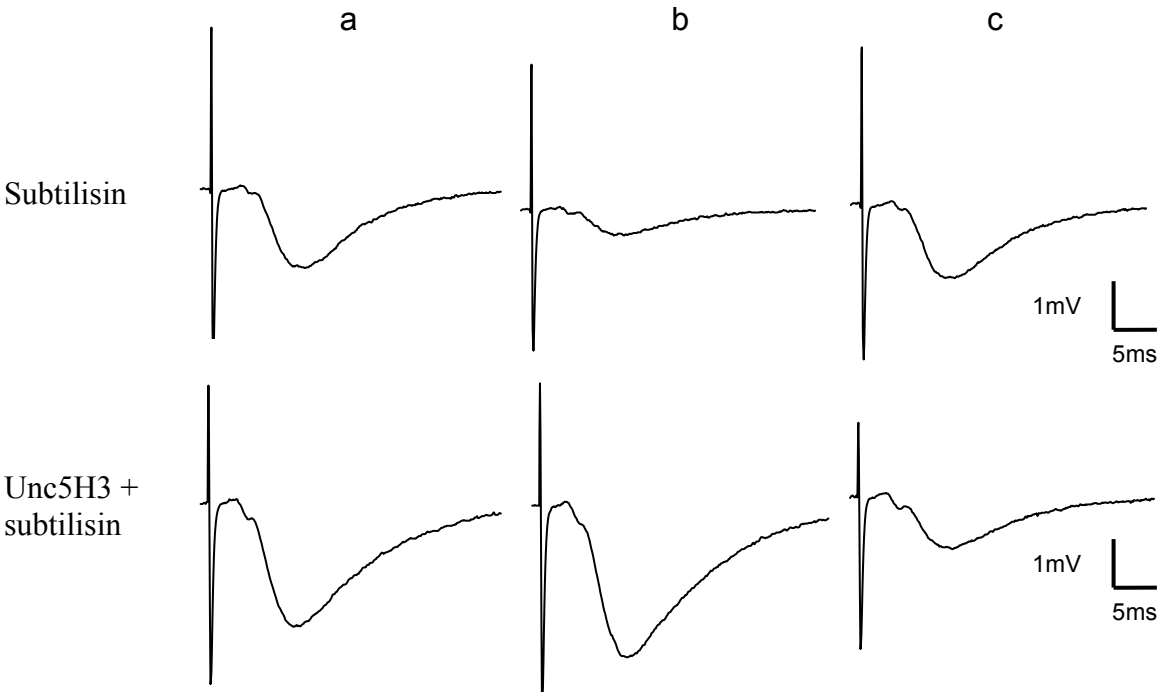


Figure 4.5a Effect of preincubating hippocampal slices with 1:1000 Unc5H3 antibodies on fEPSP response to subtilisin perfusion.

Subtilisin-mediated fEPSP response to slice preincubation with 1:1000 Unc5H3 antibodies. Preincubation with streptolysin and Unc5H3 antibodies did not change the fEPSP response to subtilisin (38.44 ± 5.91%, N=4) when compared with slices incubated in aCSF alone (33.44 ± 6.54%, N=4)(P = 0.5907). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



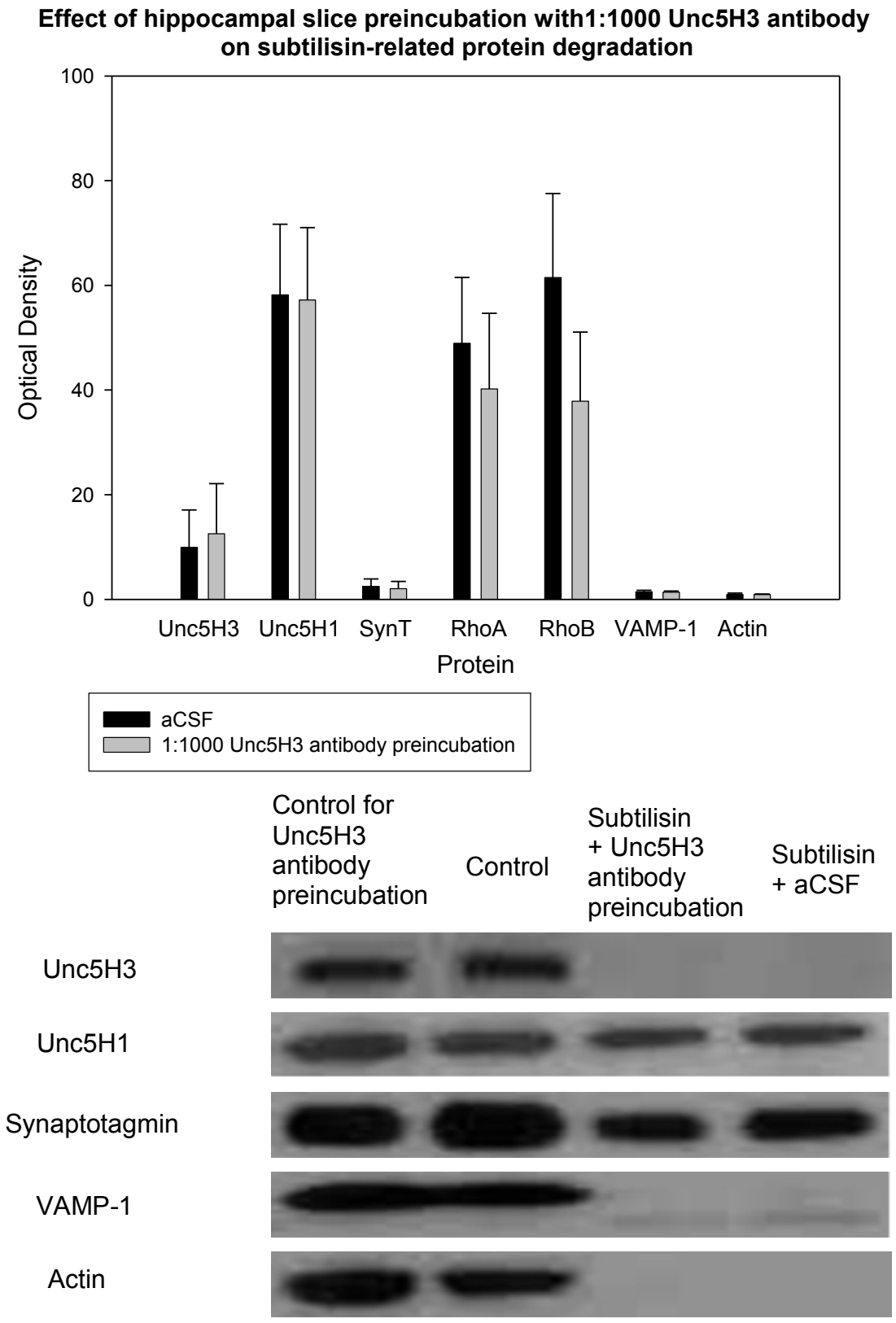


Figure 4.5b Effect of preincubating hippocampal slices with 1:1000 Unc5H3 antibodies on protein expression response to subtilisin perfusion.

No significant differences in the pattern of protein degradation was observed between slices preincubated with Unc5H3 antibody and control slices exposed to aCSF alone N=3, $P > 0.05$ for all proteins

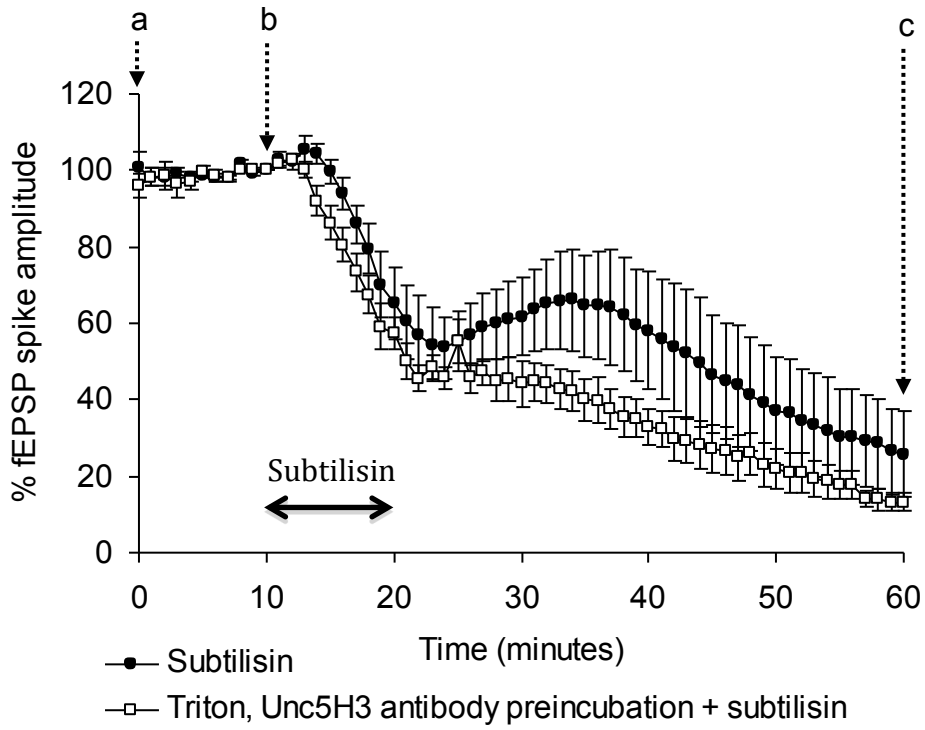
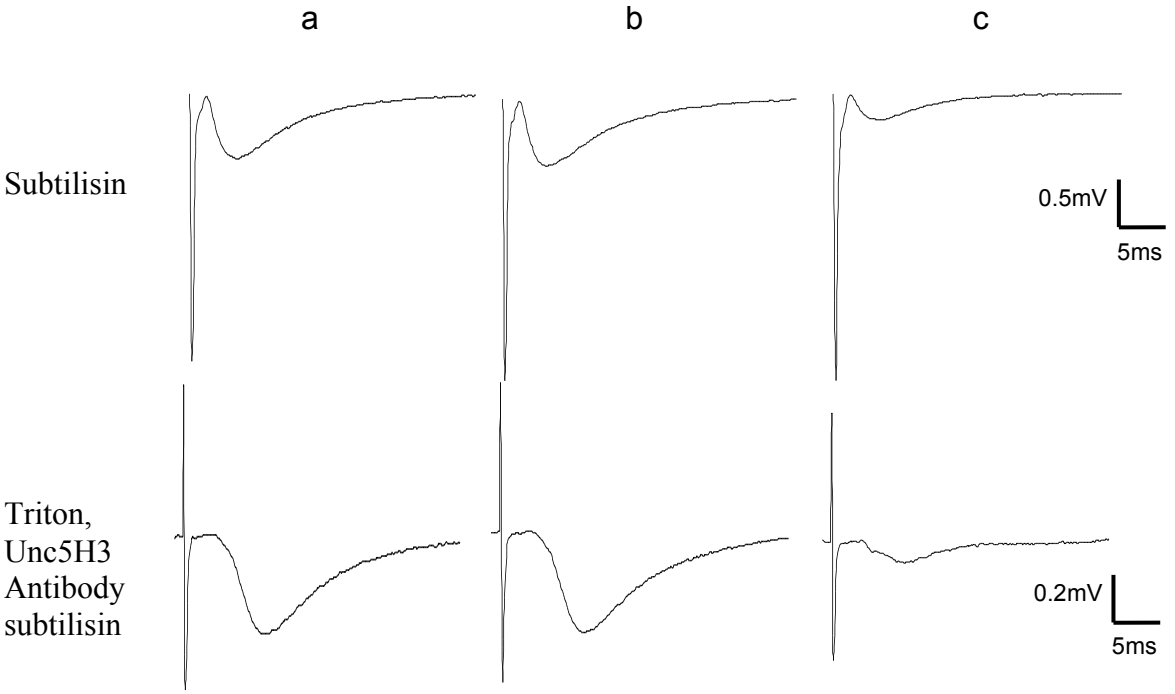


Figure 4.6a Effect of preincubating hippocampal slices with 0.01% triton, 1:1000 Unc5H3 antibodies on fEPSP response to subtilisin perfusion.

Preincubation with triton and Unc5H3 antibodies did not change the fEPSP response to subtilisin (13.13 ± 2.33%, N=5) when compared with slices incubated in aCSF alone (25.78 ± 11.01%, N=4)(P = 0.2476). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



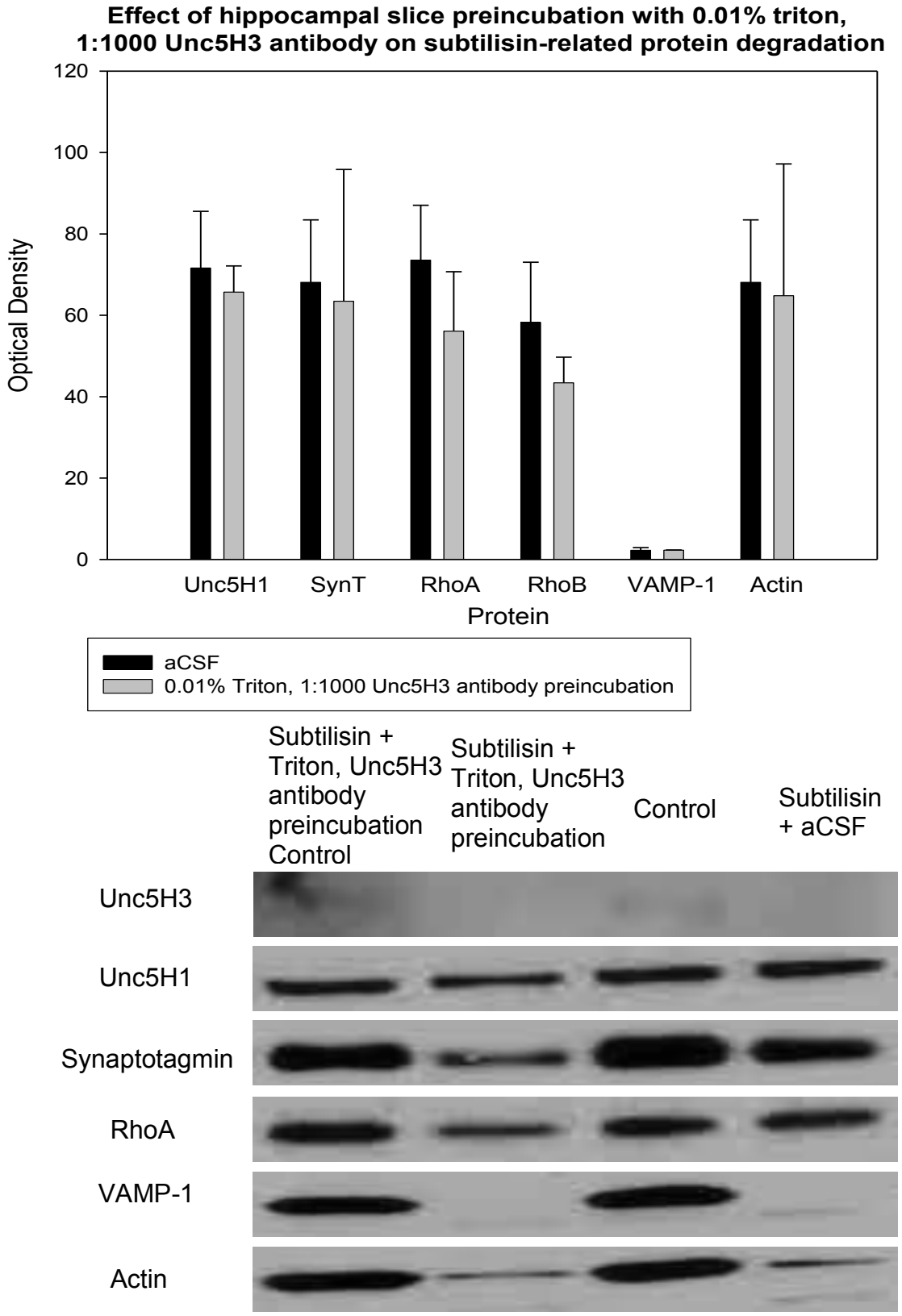


Figure 4.6b Effect of preincubating hippocampal slices with 0.01% triton and 1:1000 Unc5H3 antibodies on protein expression response to subtilisin.

There were no significant differences in the pattern of protein degradation between slices preincubated with a mixture of streptolysin and Unc5H3 antibody and control slices exposed to aCSF alone. N=3, $P > 0.05$ for all protein markers.

4.2.2.3 Effect of 1:100 Unc5H3 antibody preincubation on subtilisin

Preincubation with 1:1000 Unc5H3 antibodies was ineffective in preventing subtilisin action, and the addition of triton to the preincubation solution also failed to elicit any changes in the hippocampal response to subtilisin. In an attempt to increase the number of antibody-protein interactions, further experiments were conducted using a higher concentration of 1:100 Unc5H3 antibody. Preincubation of hippocampal slices with Unc5H3 antibodies at a concentration of 1:100 did not produce significant changes ($P > 0.05$, $N = 3$, Figure 5.7a) to the LTD effect in response to subtilisin perfusion. The ineffectiveness of triton + Unc5H3 antibody preincubation in preventing subtilisin action was also reflected in the pattern of protein degradation, which did not differ significantly between slices preincubated with triton and Unc5H3, and slices preincubated with aCSF alone ($P > 0.05$, Figure 4.7b).

4.2.2.4 Effect of hippocampal slice preincubation with 100ng/ml streptolysin and 1:100 Unc5H3 antibodies on the effects of subtilisin

Preincubation of hippocampal slices with 1:100 Unc5H3 antibodies alone did not cause any noticeable change to the effects of subtilisin on the hippocampal slice. Because of the lack of change in either LTD effect or the pattern of proteolysis with the preincubation of Unc5H3 antibodies alone, an experiment was attempted using a combination of 100ng streptolysin and 1:100 Unc5H3 antibody. This would likely increase the amount of antibodies penetrating into the neurons by facilitating transport via membrane pores. For this experiment, the protocol followed was identical to the one used during experiments with streptolysin and VAMP-1 antibodies, with the major exception being the use of antibodies against Unc5H3 rather than VAMP-1. Preincubation of hippocampal slices using a mixture of streptolysin and Unc5H3 antibodies failed to prevent LTD in response to subtilisin perfusion ($P > 0.05$, $N = 4$, Figure 4.8a) and the associated protein degradation ($P > 0.05$, Figure 4.8b)

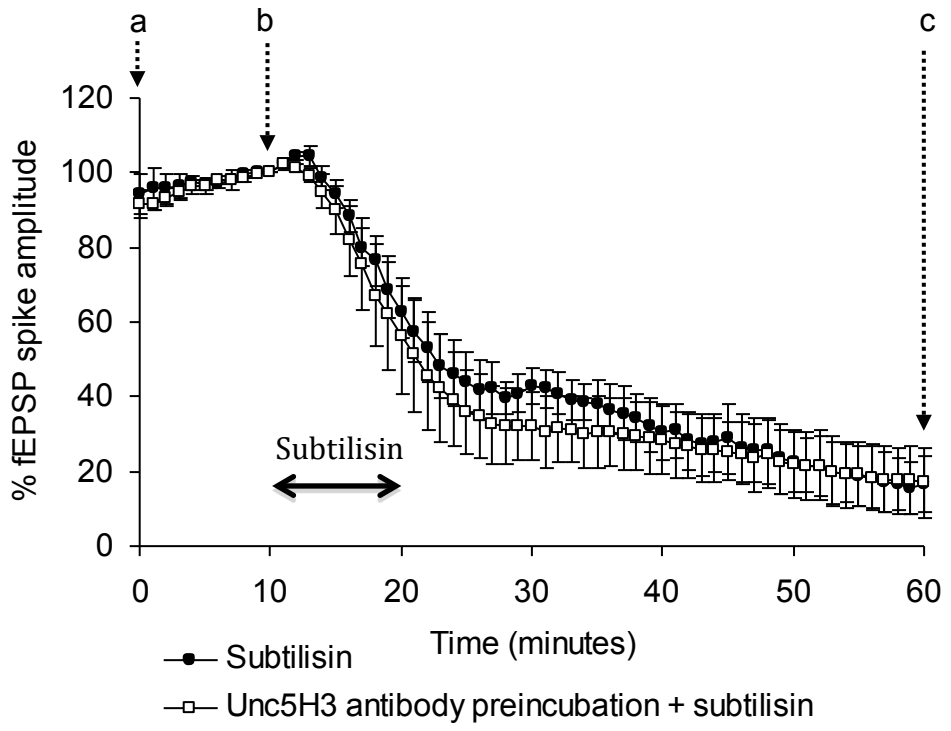
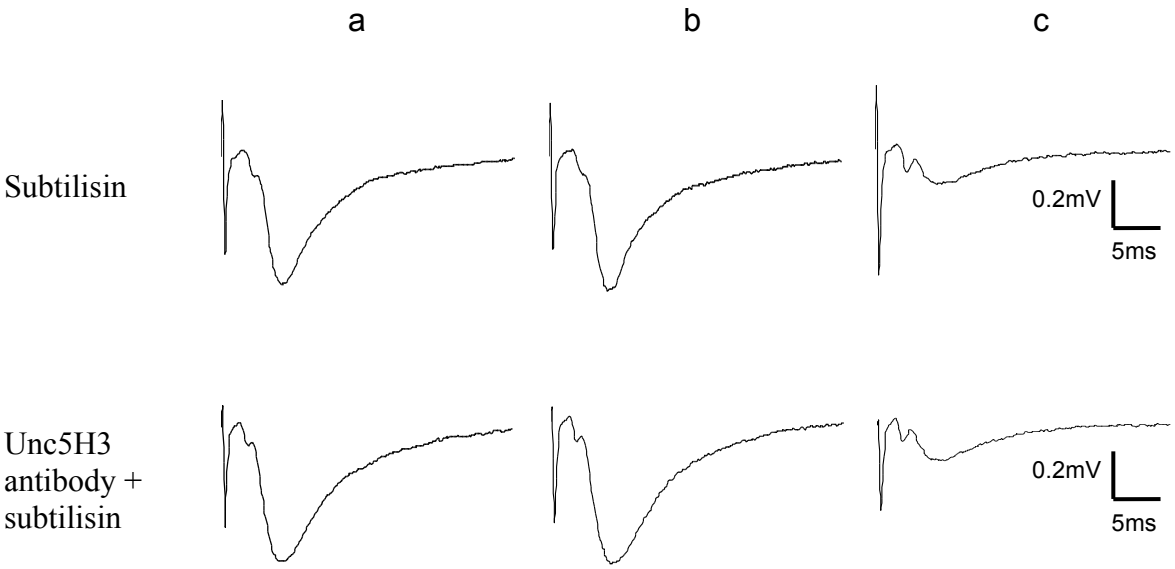


Figure 4.7a Effect of preincubating hippocampal slices with 1:100 Unc5H3 antibodies on fEPSP response to subtilisin perfusion.

No significant differences were detected in the fEPSP response to subtilisin perfusion between slices preincubated with Unc5H3 antibody (16.92 ± 9.18%, N=3), and control slices exposed to aCSF alone (16.39 ± 7.54%, N=3; P = 0.9667). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



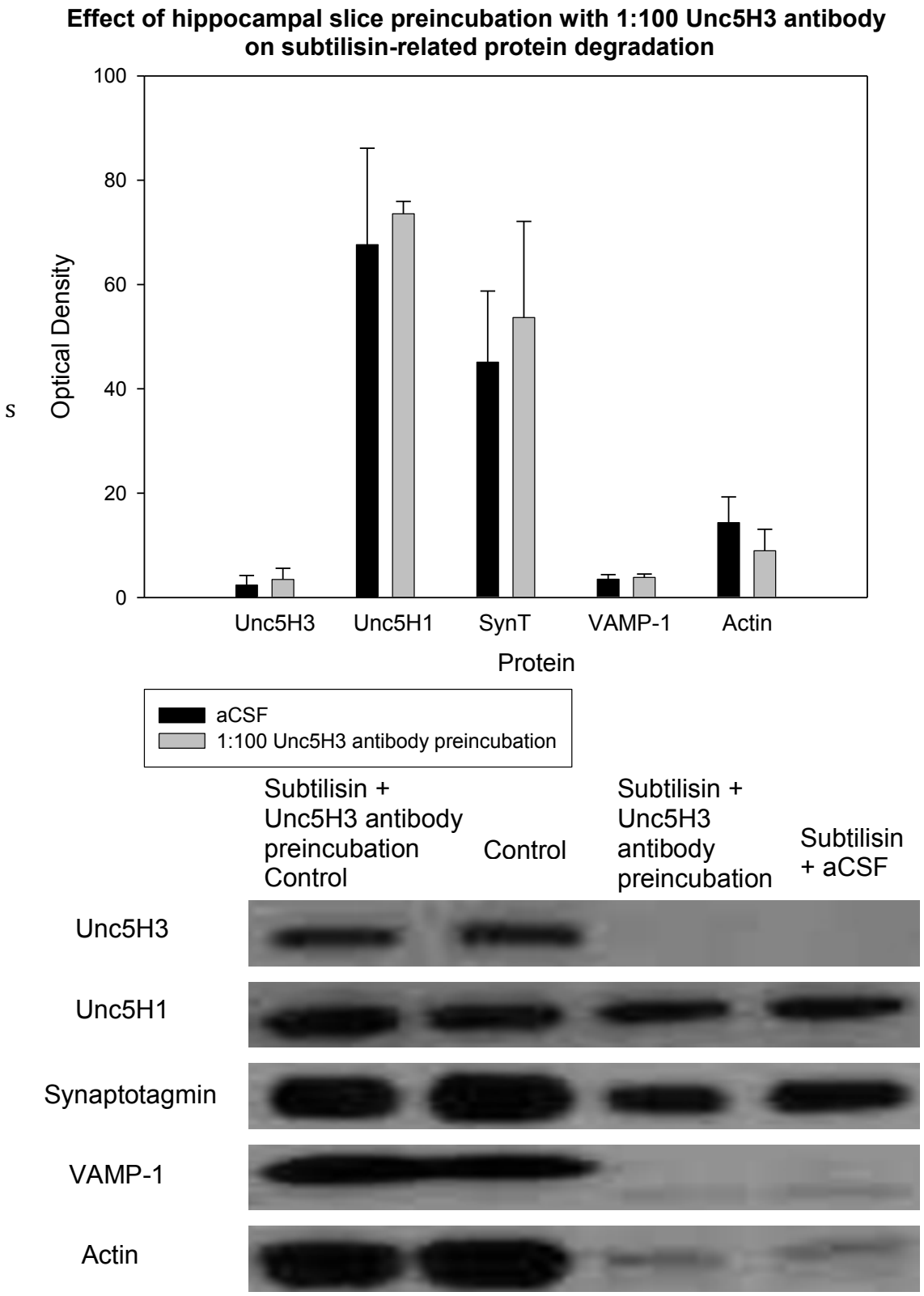


Figure 4.7b Effect of preincubating hippocampal slices with 1:100 Unc5H3 antibodies on protein expression response to perfusion of subtilisin.

The pattern of protein degradation did not differ between slices preincubated with Unc5H3 antibody and control slices exposed to aCSF alone. N=3, $P > 0.05$ for all proteins measured

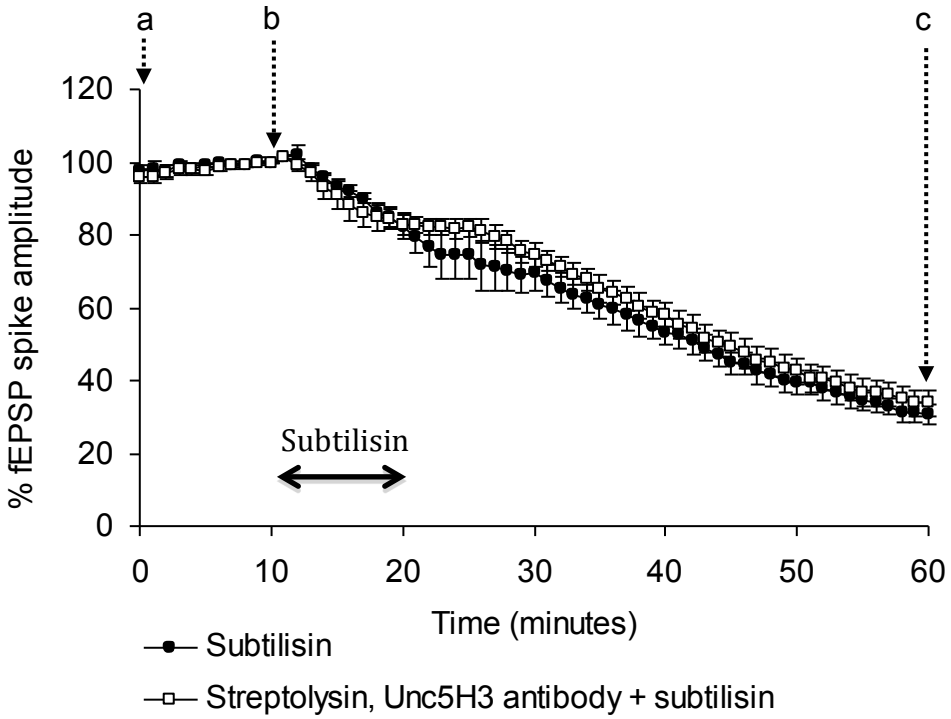
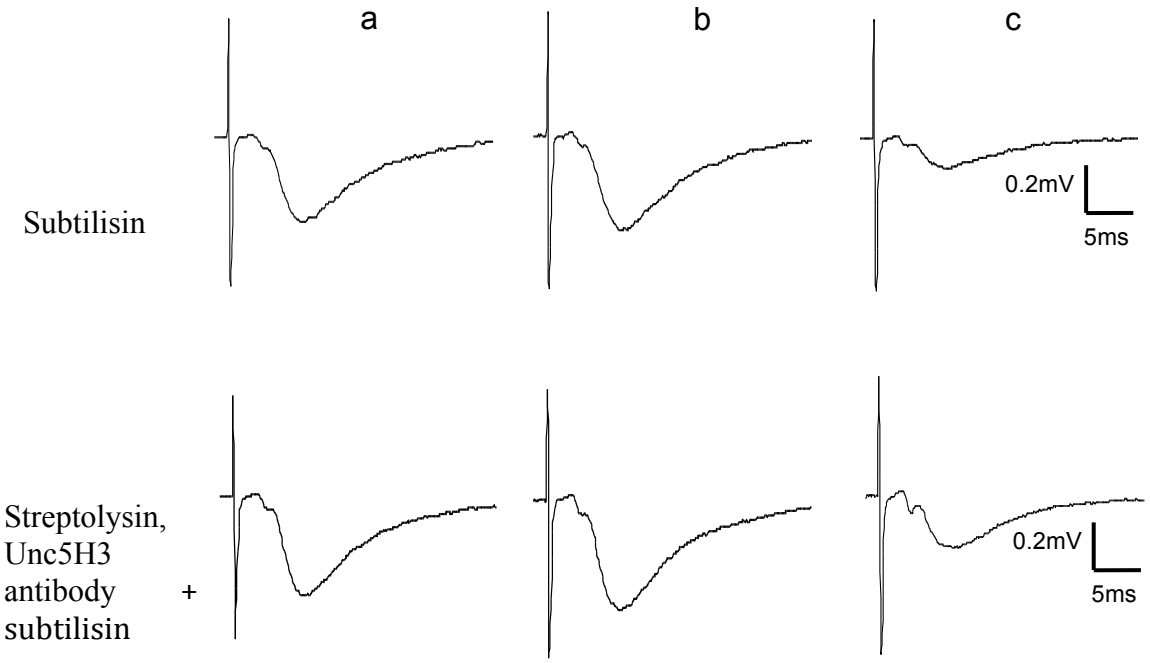


Figure 4.8a Effect of hippocampal slice preincubation with 100ng/ml streptolysin, 1:100 Unc5H3 antibodies on fEPSP response to subtilisin perfusion.

The fEPSP response to subtilisin perfusion was not significantly different between slices preincubated with streptolysin and Unc5H3 antibody (35.06 ± 4.38%, N=4), and control slices exposed to aCSF alone (27.78 ± 2.80%, N=4)(P = 0.211). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



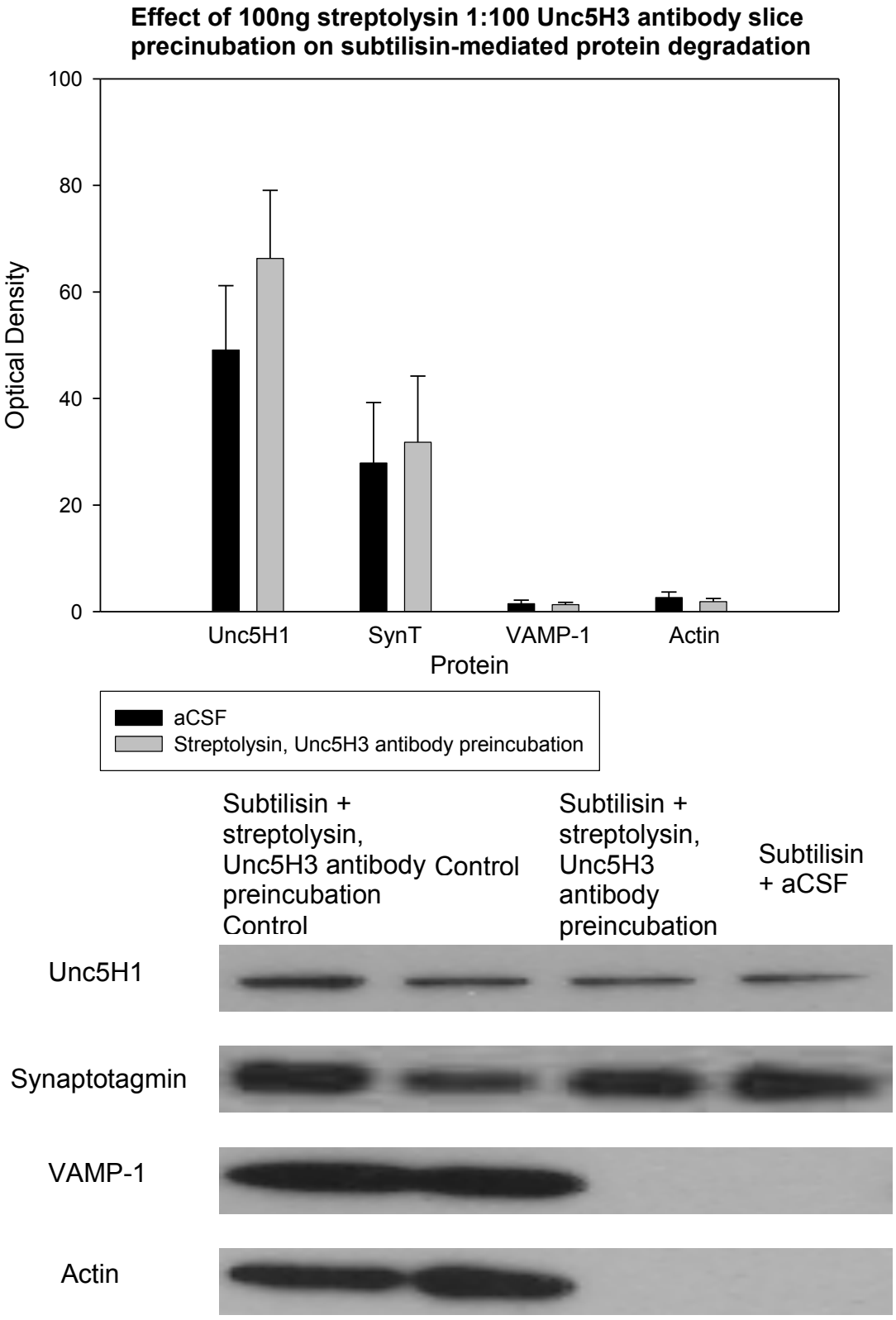


Figure 4.8b Effect of hippocampal slice preincubation with 100ng/ml streptolysin and 1:100 Unc5H3 antibodies on protein expression response to subtilisin.

No significant differences in the pattern of protein degradation were observed between slices preincubated with a mixture of streptolysin and Unc5H3 antibody and control slices exposed to aCSF alone. N=6, P >0.05 for all protein markers.

4.3 Validation of the effects of triton and streptolysin

4.3.1 Introduction

As permeabilising and pore-forming compounds respectively, triton and streptolysin alone have the potential to influence the action of subtilisin. In order to eliminate this possibility, experiments were carried out with each compound alone to determine they could affect the results obtained with antibody preincubations.

4.3.2 Results

4.3.2.1 Effect of 0.01% triton on subtilisin response

This investigation was carried out to determine whether the permeabilising effects of triton alone would cause a change in the way the hippocampal slices responded to subtilisin. For this experiment a modified version of the previous antibody preincubation protocol was used. Hippocampal slices were exposed to 0.01% triton over a period of 20 minutes, followed by 3 washes in aCSF and a 30-minute recovery period. Preincubation of triton did not have any adverse effects on progression of subtilisin-mediated LTD, and values for triton preincubation did not differ significantly from those attained with aCSF alone ($P > 0.05$, $N = 4$, Figure 4.9a). The decrease in expression of selected proteins were likewise unaffected by slice preincubation in triton ($P > 0.05$ for all proteins, Figure 4.9b)

4.3.2.2 Effect of 100ng/ml streptolysin on subtilisin response

Investigations were also carried out to determine whether the use of streptolysin would impact on the actions of subtilisin. Hippocampal slices were preincubated with 100µg streptolysin over a 20-minute period, followed by 3 washes in aCSF and a 30-minute recovery period prior to use. Streptolysin preincubation alone did not alter the decrease in fEPSP after subtilisin perfusion ($P > 0.05$, $N = 4$, Figure 4.10a); in addition, it did not change the pattern of protein degradation after the addition of subtilisin ($P > 0.05$ for all proteins, Figure 4.10b).

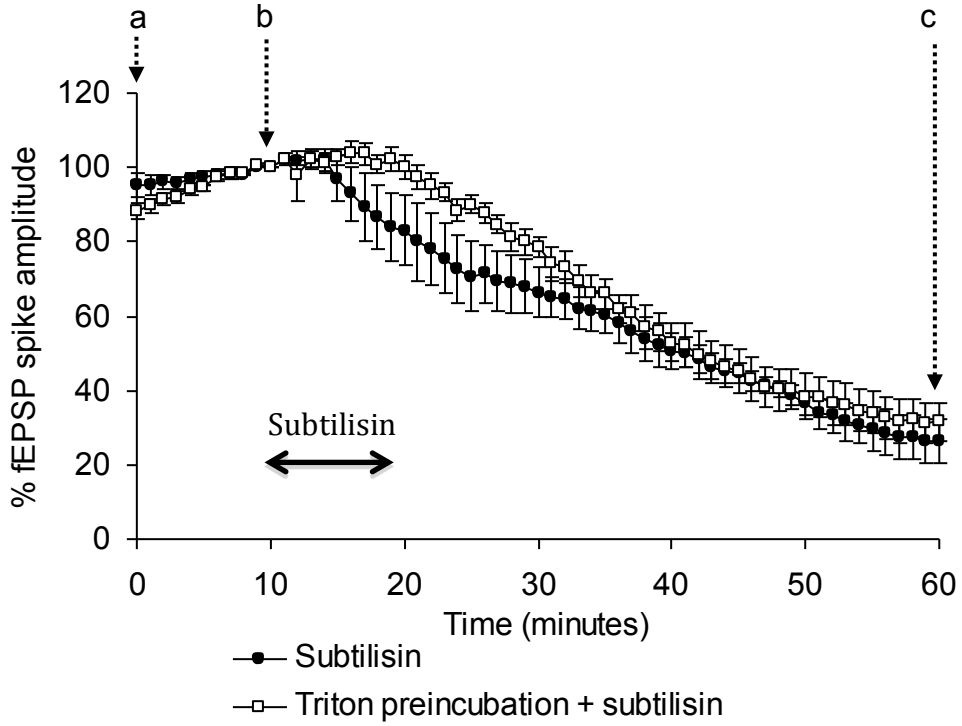
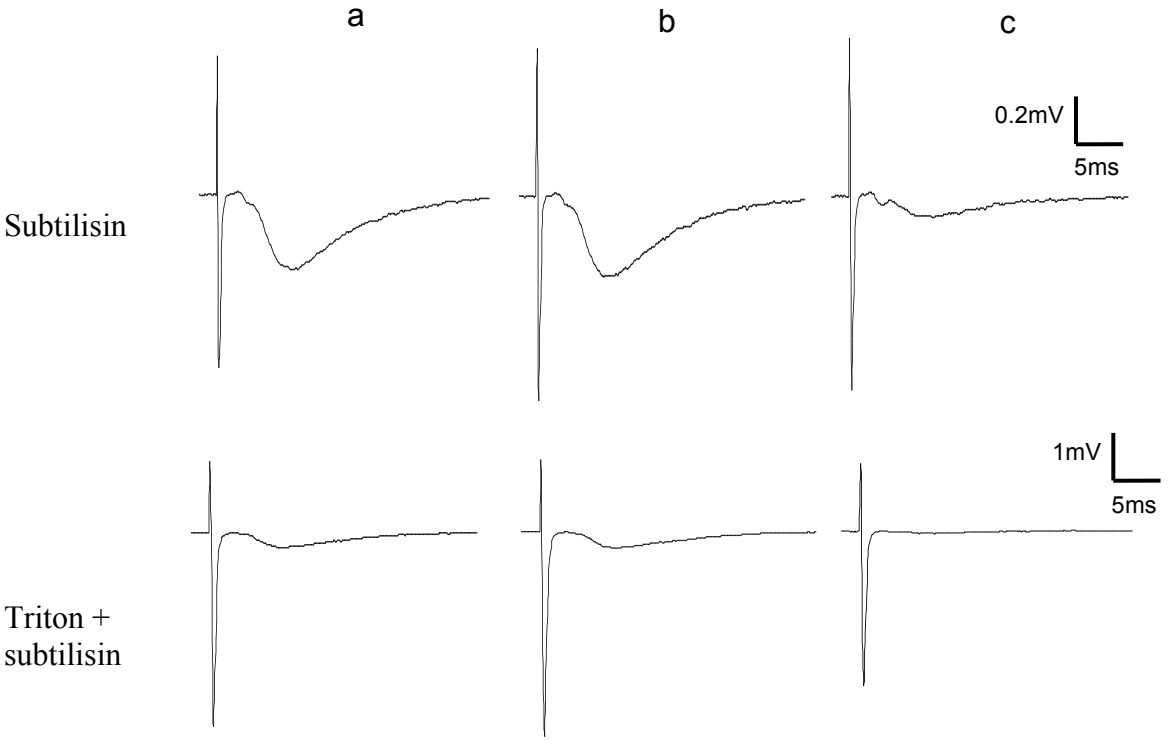


Figure 4.9a Effect of preincubating hippocampal slices with 0.01% triton alone on fEPSP response to subtilisin.

Preincubation with triton antibodies alone did not demonstrate a change to the fEPSP response to subtilisin (31.54 ± 5.07%, N=4) compared with slices incubated in aCSF alone (26.38 ± 6.09%, N=4; P = 0.5388). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



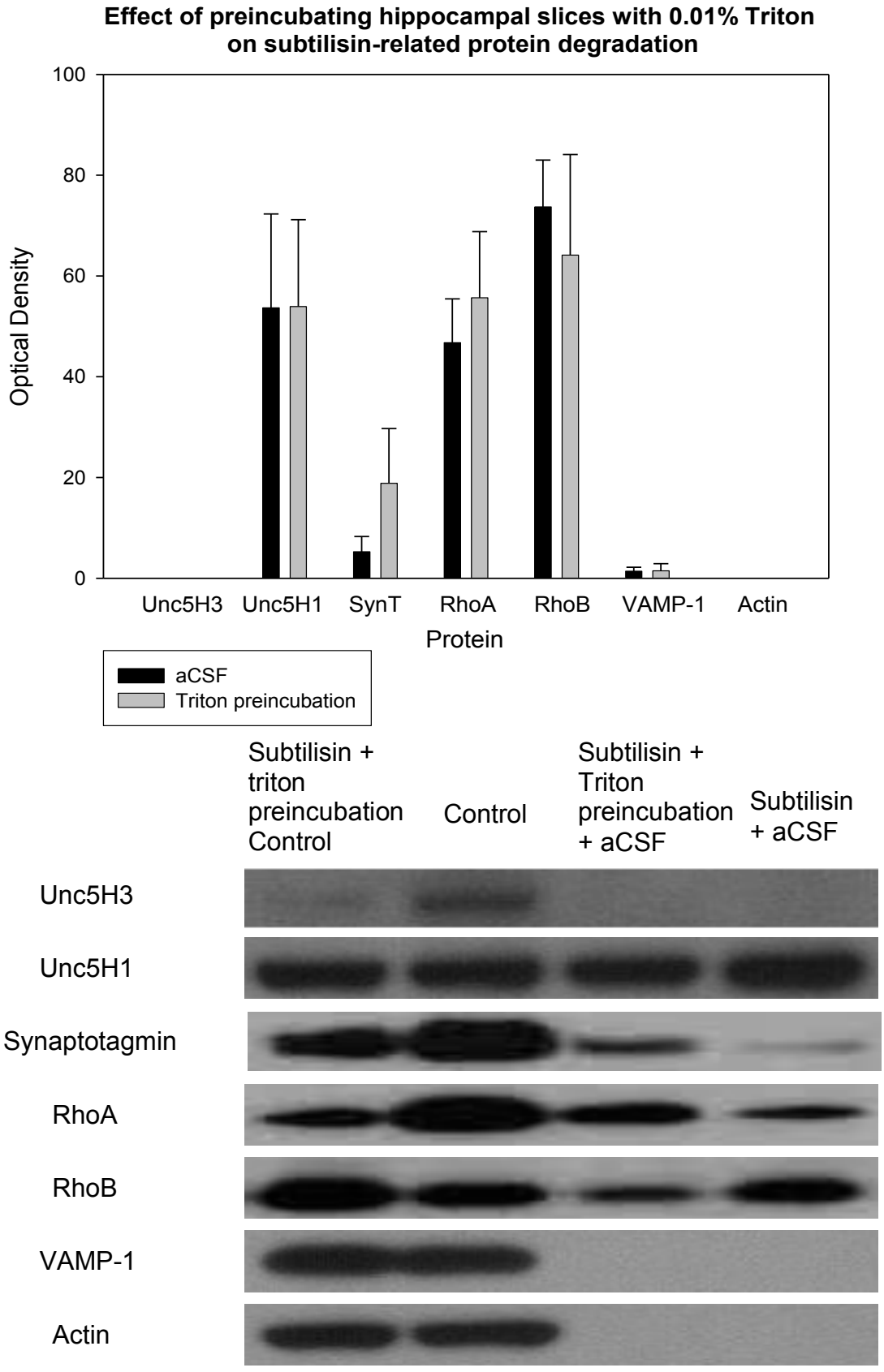


Figure 4.9b Effect of preincubating hippocampal slices with triton alone on protein expression in response to subtilisin.

No significant differences in the pattern of protein degradation were observed between slices preincubated with triton and control slices exposed to aCSF alone. N = 4, P >0.05 for all protein markers.

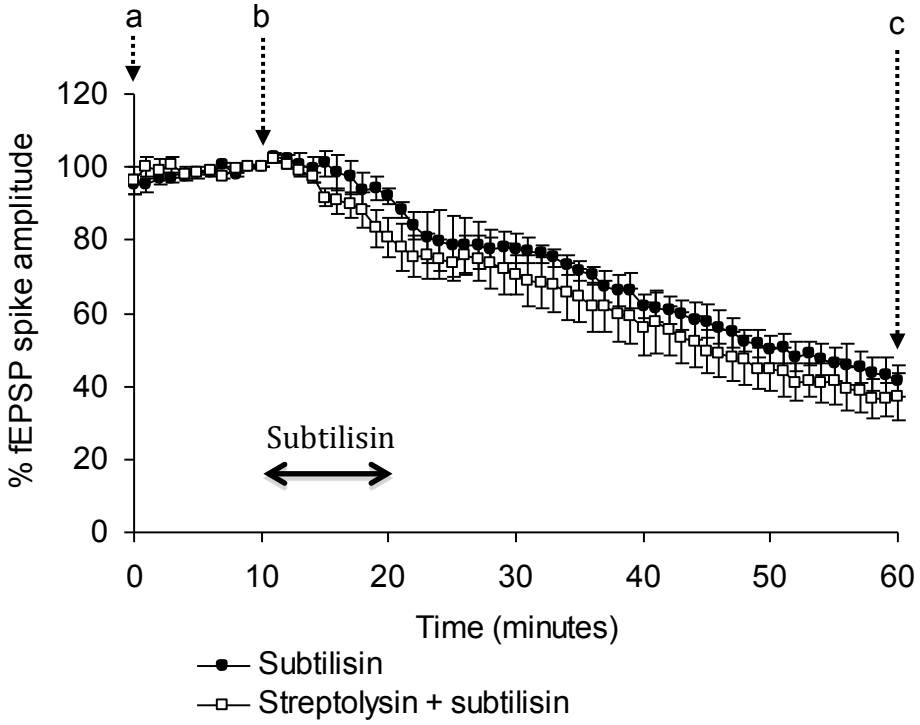
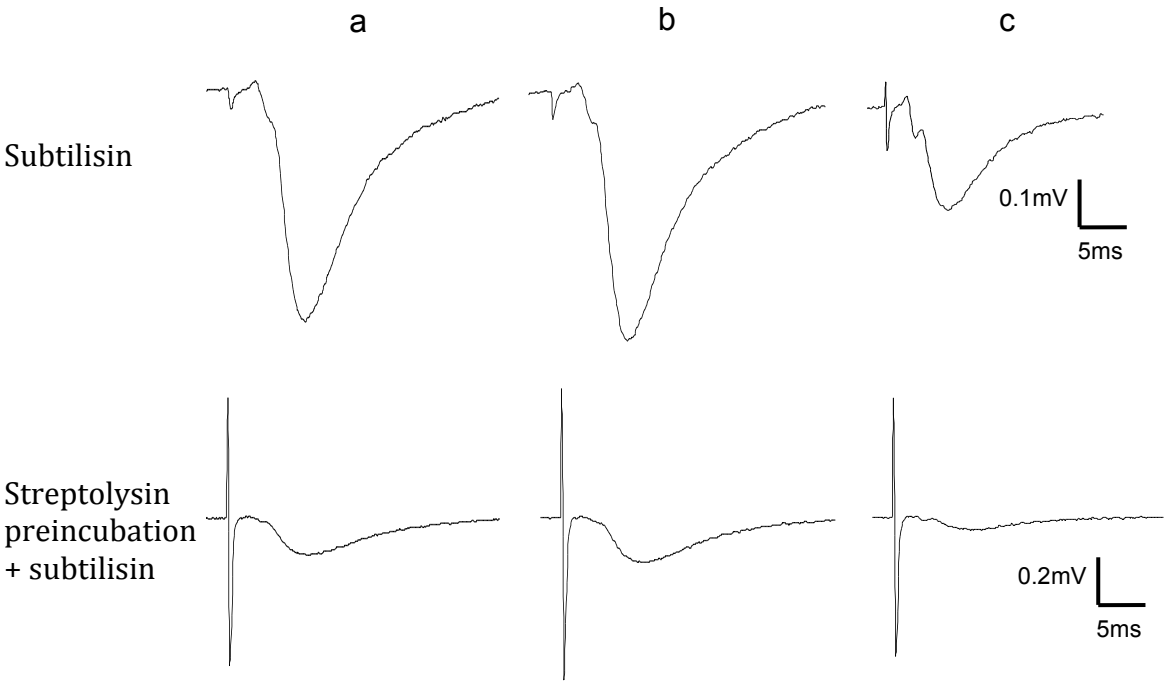


Figure 4.10a Effect of preincubating hippocampal slices with 100ng streptolysin alone on fEPSP response to subtilisin.

Preincubation with streptolysin antibodies did not change the fEPSP response to subtilisin (37.28 ± 6.54%, N=4) compared with slices incubated in aCSF alone (41.53 ± 4.47%, N=4)(P = 0.222). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



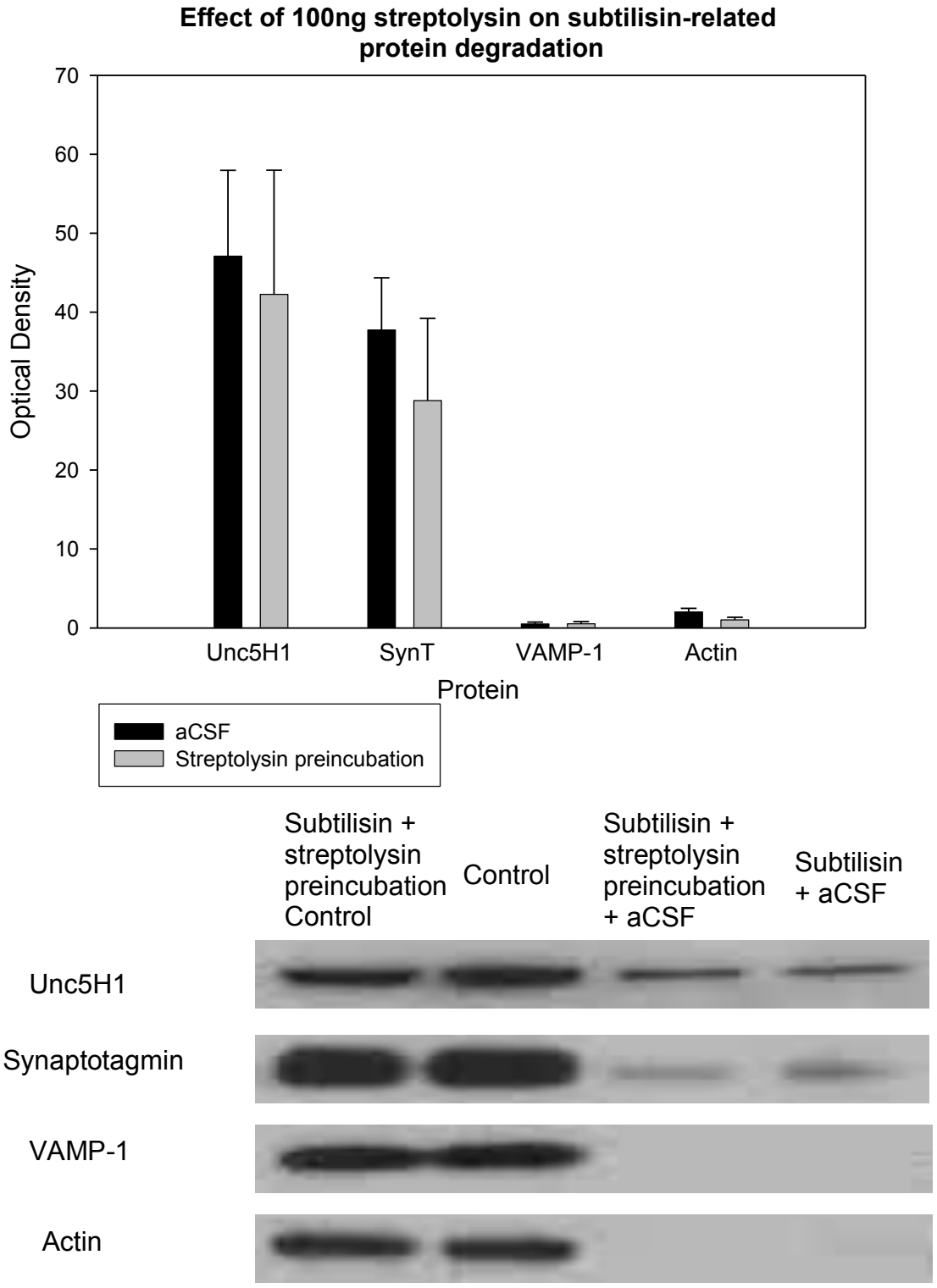


Figure 4.10b Effect of preincubating hippocampal slices with 100ng/ml streptolysin on protein expression in response to subtilisin.

No significant differences in the pattern of protein degradation were observed between slices preincubated with 100µg streptolysin and control slices exposed to aCSF alone. N = 4, P >0.05 for all protein markers.

4.4 Discussion

Protein-specific antibodies represent an accurate method of identifying a protein of interest amongst a heterogeneous group of other proteins. In addition, recent studies have also shown that antibodies provide an effective way of altering the protein structure, without changing the original amino acids present within the protein. This mechanism can also be used to block the cleavage sites on a protein and interfering with the function of several proteases.

In this study, antibodies against VAMP-1 and Unc5H3 proteins were used in an attempt to block their proteolysis by subtilisin. Initial experiments were conducted on the synaptic protein VAMP-1, as a disruption of the synaptic site would produce the most obvious change in synaptic signalling. The first experiments utilised VAMP-1 antibodies at a concentration of 1:5000, but were unsuccessful in producing an inhibitory effect on subtilisin action. Addition of 0.01% triton to the VAMP-1 antibody solution used for hippocampal slice preincubation was also ineffective in modulating the subtilisin response. VAMP-1 antibodies at a higher concentration of 1:500 were used in the next set of experiments in an attempt to increase the potential for antibody-protein interactions. However, even at this concentration VAMP-1 antibodies were still unable to elicit any noticeable changes to the activity of subtilisin. The presence of 100ng/ml streptolysin, which caused the formation of pores large enough to allow antibody entry, was unable to facilitate the entry of VAMP-2 antibodies into the cells.

Experiments were also conducted using antibodies against the Unc5H3 proteins, which was also one of the proteins most susceptible to subtilisin degradation. There was a possibility that VAMP-1, being an intracellular protein, was less sensitive to the antibodies used in vitro. In comparison, the Unc5H family of receptors, including Unc5H3, are transmembrane receptors, and therefore could be more readily bound by antibodies. However, use of Unc5H3 antibodies at concentrations of 1:5000 and 1:500 alone failed to have any impact on the progression of LTD or proteolysis in response to subtilisin. Addition of either triton or streptolysin to the antibody solution also failed to produce any changes to the decrease in fEPSP and protein degradation after perfusion of subtilisin.

These experiments suggest that antibodies may be an ineffective method of inhibiting subtilisin action, unlike the activity of tetanus toxin, which was decreased in the presence of antibodies against VAMP-1 (Poulain *et al.*, 1993). However, several variables may have influenced the results obtained in these experiments. The hippocampal slices were only incubated with the antibody solution over a period of 20 minutes, even in the presence

of pore-forming chemicals. The length of this incubation period may be insufficient for allowing successful antibody penetration into the cells, or the formation of stable antibody-protein complexes. Furthermore, during the incubation period hippocampal slices were kept within a static incubation chamber, which could limit the interaction of antibodies with the target protein. Additionally, this could lead to high levels of antibodies localised at several different locations, which might not correspond to the regions from which fEPSPs were elicited during electrophysiological recordings.

The use of antibodies more suited to immunoblotting may also have a role in the low impact of these antibodies in the hippocampal slices, as this could decrease the efficiency of their protein binding and reduce the likelihood of antibody penetration. Use of the permeabilising agent triton-X may not have been effective due to the relatively low concentration (0.01%) utilised for these experiments. Previous experiments in fixed cells utilised triton at a concentration of 0.1% to cause membrane permeabilisation and allow antibody entry (Appleby *et al.,* 2011), although these experiments require the use of live tissue for electrophysiology, and therefore may require a lower triton concentration. In a similar manner, a concentration of 100ng/ml streptolysin may have been insufficient for forming enough pores to allow effective antibody entry. This initial concentration was based on results of cell studies in literature, which utilised 20-40ng/ml streptolysin to permeabilise cells (Ogino *et al.,* 2009). However, as hippocampal slices are much thicker and require a greater diffusion time, therefore a streptolysin concentration of many magnitudes higher may be necessary. Experiments involving hippocampal slice preincubation in either triton or streptolysin alone did not result in significant changes to subtilisin-mediated LTD, suggesting that these chemicals did not have an impact on LTD or protein degradation mediated through subtilisin. Use of these permeabilising agents is also likely to have non-specific effects on the hippocampal slice, or the neurons within. Triton-X, as a detergent, is capable of causing irreversible cell damage due to the cell membrane, leading to a high level of cell death. However, at the concentration used during the current experiments, this is unlikely to occur as cell membranes would be capable of recovering after a wash-out period (Koley & Bard, 2010). Of greater concern is the potential for uncontrolled entry and exit of molecules, which may change the intracellular balance of ions. Similarly, previous literature on the use of streptolysin-O indicates that these toxins are capable of inhibiting the uptake of certain amino acids, namely phenylalanine and alanine, affecting the rate of protein synthesis, and have causing a change to the progression of long-term synaptic changes. In addition, streptolysin also causes a clustering of membrane cholesterol, and reducing the fluidity of the lipid membrane. This can in turn lead to a decrease in receptor turnover on the neuronal

surface, reducing synaptic signalling and potentially causing a reduction in the number of active synaptic connections.

Future experiments into the importance of these proteins might involve the use of antibodies raised specifically to detect proteins in hippocampal slices, which should provide a much more accurate result. Alterations to the protocol used in these experiments could involve the use of a shaker to produce a better distribution of antibodies within the preincubation solution. This could however damage the neuronal network by disrupting synaptic connections, and therefore has to be closely monitored. The incubation time for hippocampal slices may also need to be increased, although the use of acute hippocampal slices will limit the amount of time available. The use of fluorescent tags on the antibodies may also allow better visualisation and optimisation of antibody entry into neurons.

4.5 Investigations into the effects of an actin stabiliser, Jasplakinolide, on subtilisin-mediated neuronal effects and protein expression.

4.5.1 Introduction

In addition to the degradation of Unc5H3 and VAMP-1, subtilisin also selectively targets actin, a structural protein essential for maintaining both cytoskeletal integrity and preserving the functionality of neurons. Loss of this protein has been proven to cause a disruptive effect to neuronal signalling by through the loss of synaptic function. Therefore, it is plausible that the long-term depression caused by subtilisin is due to a selective degradation of the actin cytoskeleton, leading to a prolonged period of disrupted synaptic signalling. In addition, actin is a downstream effector protein for Unc5H3 signalling, and these investigations could shed new light into the role of Unc5H3 proteins in subtilisin action. In order to determine the relative importance of actin to the actions of subtilisin, an attempt was made to prevent the depolymerisation of actin using the actin stabiliser jasplakinolide.

Jasplakinolide is a compound that promotes actin stabilisation in vitro by increasing the rate of actin nucleation, and preventing their depolymerisation. It is ideally suited to use with in vitro preparations as their use with in vivo preparations can result in cytotoxic effects due to uncontrolled actin polymerisation (Bubb *et al.,* 1994; Bubb *et al.,* 2000) In the nervous system, jasplakinolide can act to increase the duration of long-term potentiation in rat hippocampal slices when perfused throughout the stimulus period and for a period following LTP induction (Messaoudi *et al.*, 2007).

4.5.2 Results

In this experiment 100μM Jasplakinolide was perfused over the hippocampal slice for a 10-minute period before subtilisin perfusion, throughout the perfusion period and 10 minutes after subtilisin perfusion ceased.

Addition of jasplakinolide did not decrease the extent subtilisin-mediated LTD (Figure 4.11a), also did not prevent the degradation of proteins after subtilisin perfusion (Figure 4.11b).

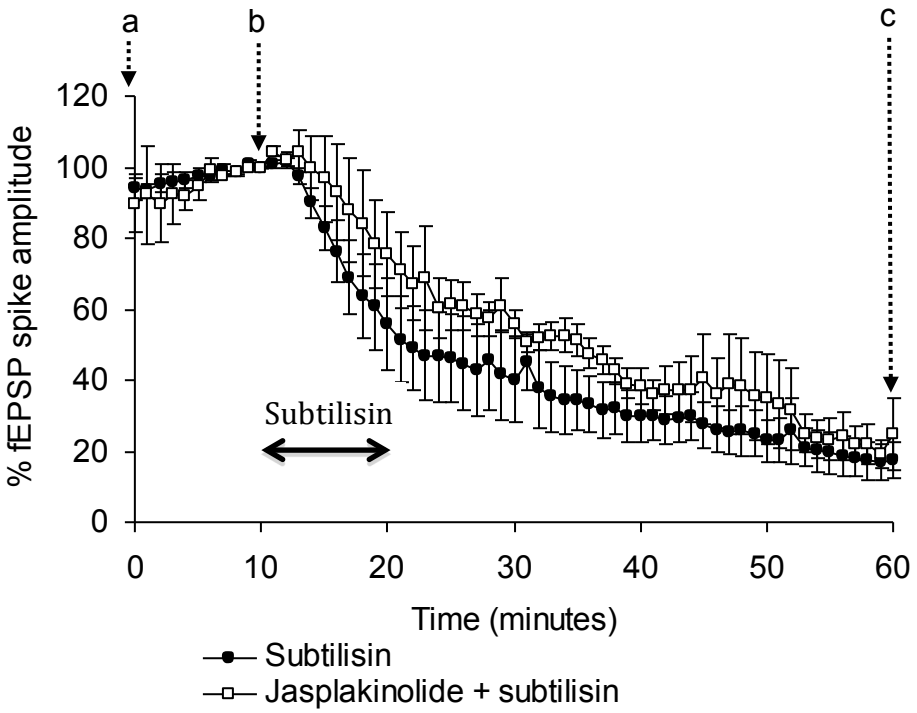
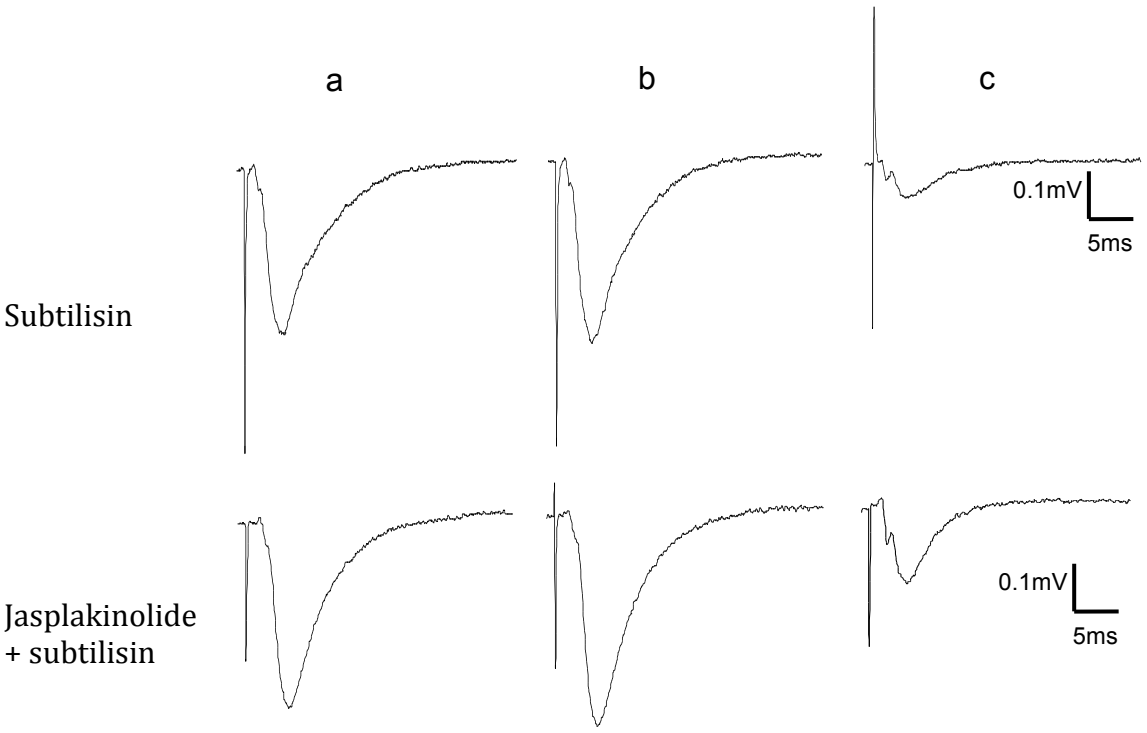


Figure 4.11a Effect of 100µM Jasplakinolide on fEPSP response to subtilisin action. Addition of Jasplakinolide did not change the fEPSP response to subtilisin (17.90 ± 4.22%, N=4) when compared with slices incubated in aCSF alone (17.36 ± 5.12%, N=4)(P = 0.9375). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



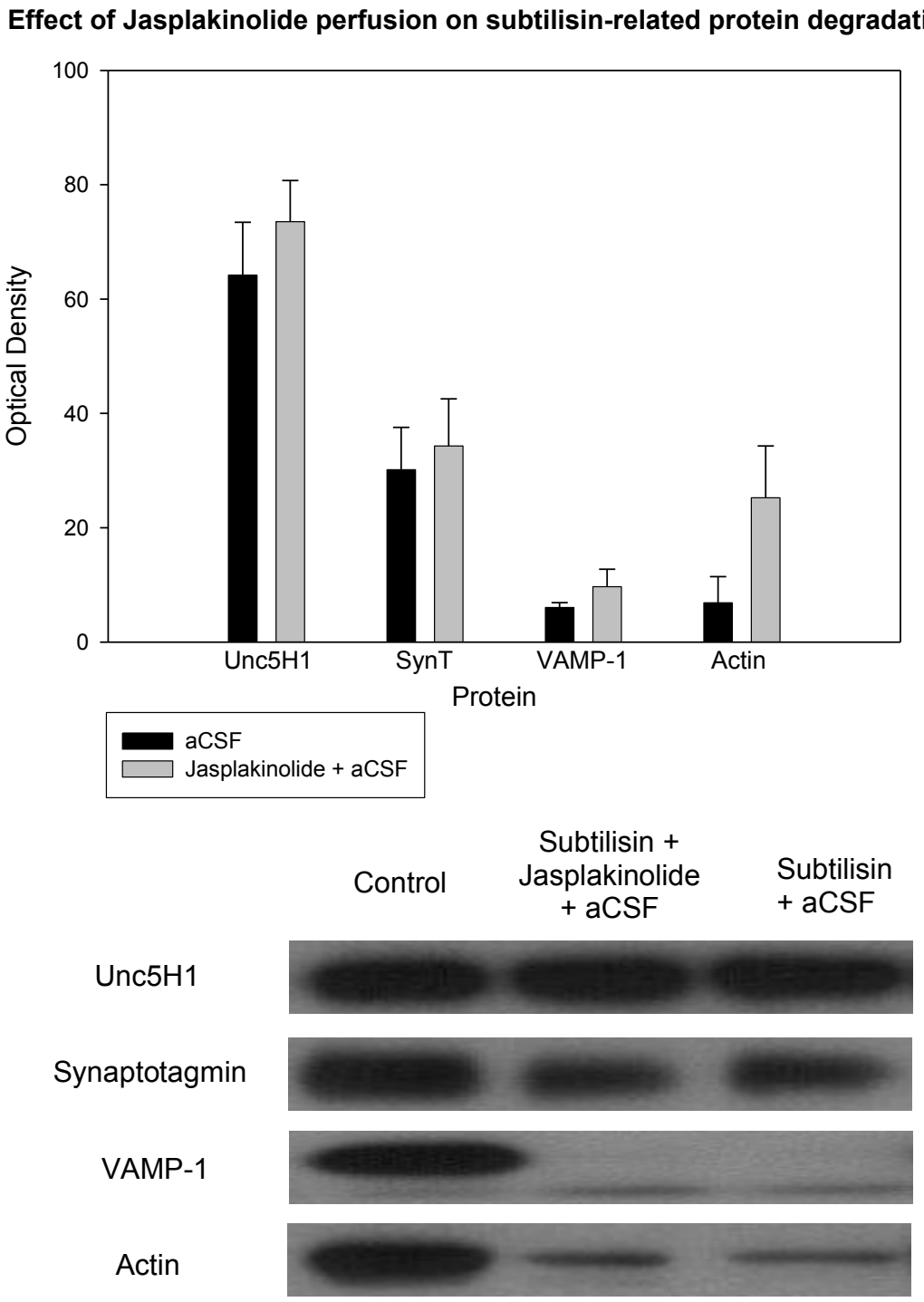


Figure 4.11b Effect Jasplakinolide perfusion on protein expression in response to subtilisin.

No significant differences in the pattern of protein degradation were observed between slices perfused with jasplakinolide and control slices perfused with aCSF alone during the administration of subtilisin. N = 4, P >0.05 for all protein markers.

4.5.3 Discussion

The degradation of actin by subtilisin represents a potentially significant mechanism of inhibition, as actin is involved in a wide range of cellular activities. The main function of this protein is to maintain the cell structure via the actin cytoskeleton and to facilitate the intracellular trafficking of vesicles (Pollard & Cooper, 2009) Disruption of either functions has major implications for cell-cell signalling, including at synaptic junctions. A disruption of actin could potentially affect the integrity of synapses by producing alterations to the surrounding cytoskeleton; preventing or interfering with vesicle re-uptake due to the loss of f-actin fibres, and interfere with the transport of neurotransmitter vesicles to the synapse (Yarar *et al*, 2005). Stabilising or even reversing the effects of actin degradation could represent an effective way of preventing decreases to synaptic signalling.

For this experiment, the actin stabiliser Jasplakinolide was used to promote actin polymerisation and ensure the stability of existing actin fibres. However, this did not appear to have the desired effect in preventing subtilisin-mediated changes to fEPSP, and analysis of protein degradation indicated that Jasplakinolide was unable to prevent the degradation of actin. Other proteins were similarly degraded by subtilisin even in the presence of jasplakinolide, suggesting that actin may not have such an essential role in the action of subtilisin as was initially believed. One possible explanation is that the jasplakinolide was unable to penetrate the cells of the hippocampal slices, and therefore could not cause a change to the intracellular actin molecules within the neurons. An alternative explanation is that jasplakinolide molecules were not given sufficient time to bind to actin due to the high perfusion rate used during the experiment. The concentration of jasplakinolide used for these experiments were scaled up from the concentrations used for neurons, although an even higher concentration may be required to in order to have an effect on hippocampal slices (Jaworski *et al.,* 2009). Despite these results, further experiments could explore the use of other factors regulating actin, due to the importance of actin to the function of many cellular processes.

Chapter 5 - Comparison of the effects of subtilisin and other forms of LTD

5.1 Introduction

In order to establish whether subtilisin-mediated LTD was a novel form of LTD, the characteristics of this form of LTD was compared to other more established methods of eliciting LTD. Of particular interest was whether protein degradation had a role in other types of LTD, as selective proteolytic degradation of proteins appears to be the process that characterised subtilisin-mediated LTD. One well-studied type of LTD is dependent on the action of mGluRs, and can be elicited chemically using the mGluR agonist DHPG (Huber *et al.*, 2000). For these experiments, the effect of 17μM DHPG was compared against the effects of subtilisin-mediated LTD. This was tested by a substitution of subtilisin-containing aCSF for one that contained DHPG, which was perfused over a 10-minute period in a similar manner to subtilisin.

5.2 Results

5.2.1 Comparison of the effects of 17μM DHPG against 4μM subtilisin

At a concentration of 17μM, DHPG was expected to generate an mGLuR-mediated LTD. However, over the course the study, none of the hippocampal slices perfused with DHPG produced an LTD, and therefore could not be compared to the LTD generated by subtilisin (Figure 5.1a). Immunoblotting of hippocampal slices was also carried out to determine whether DHPG alone could cause protein degradation, but due to the failure of DHPG to produce LTD, this hypothesis remained unproven (Figure 5.1b). Perfusion of DHPG alone, without the generation of LTD, did not result in any notable protein degradation.

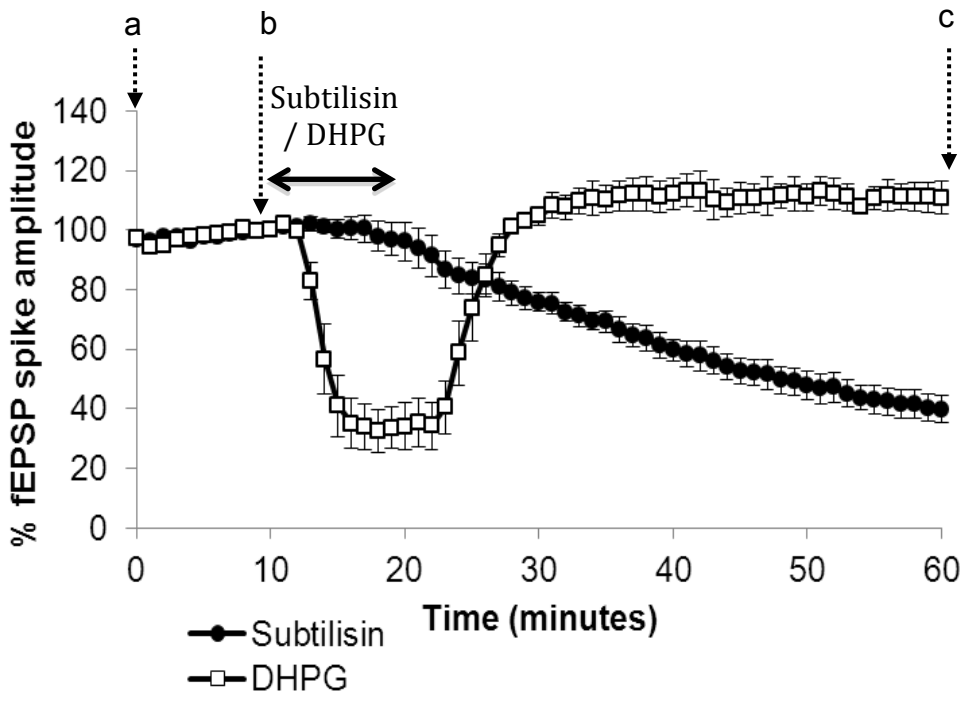
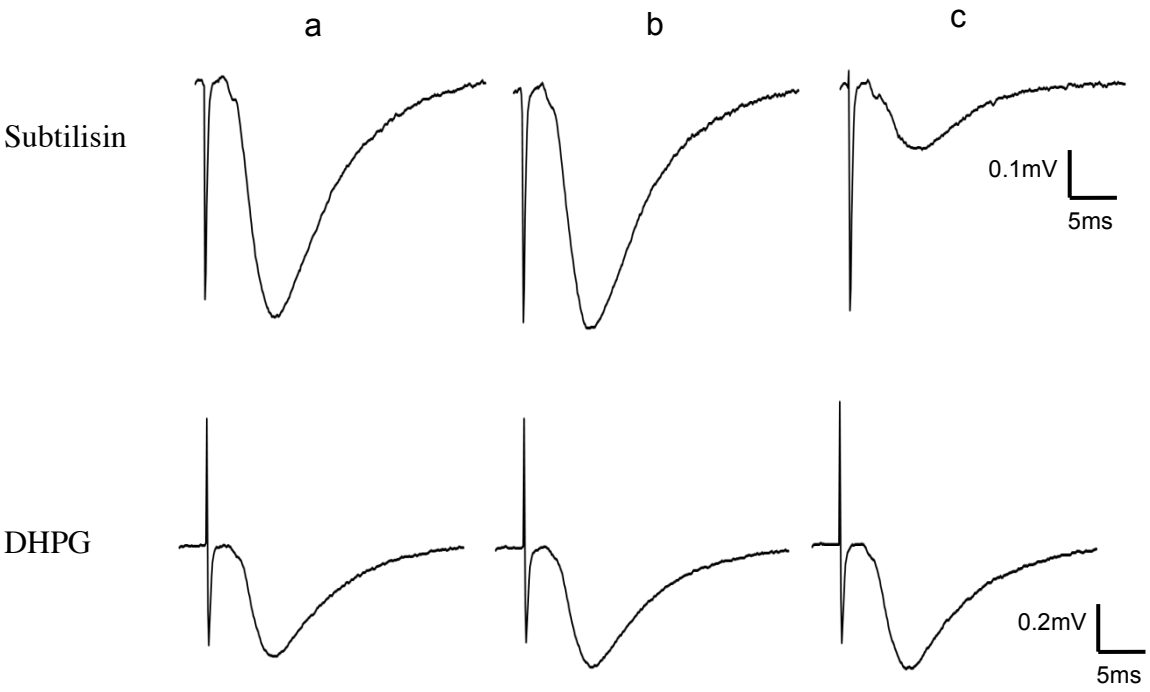


Figure 5.1a Comparison of DHPG and subtilisin effects on fEPSP size.

Perfusion of DHPG at a concentration of 17μM did not elicit LTD in comparison to the perfusion of 4μM subtilisin. Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



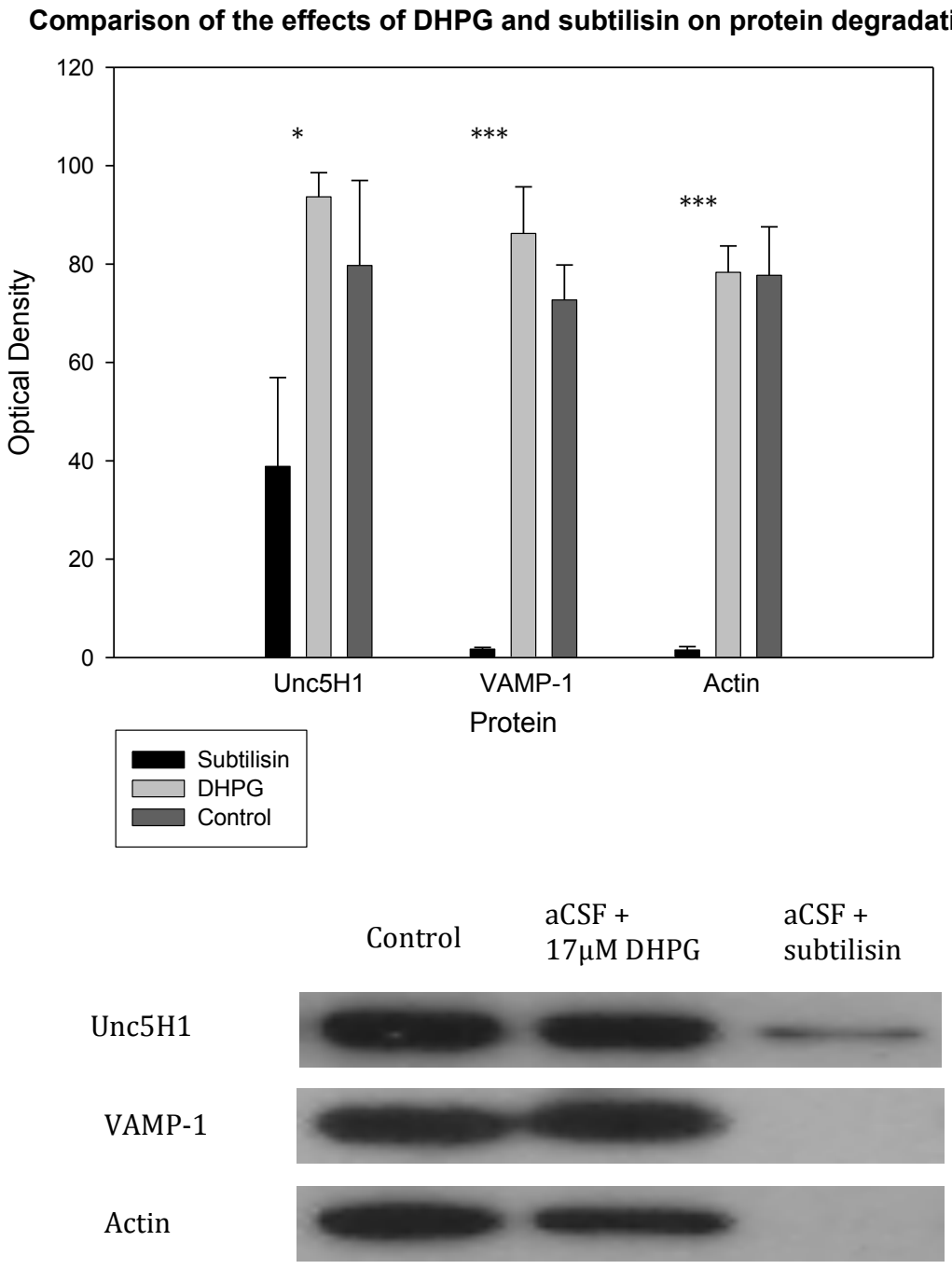


Figure 5.1b Comparison of 17µM DHPG and 4µM subtilisin perfusions on protein expression.

Perfusion of subtilisin caused a significant decrease in protein expression compared with controls. In comparison, slices perfused with DHPG exhibited protein levels comparable with control slices. A significant difference in Unc5H1 expression was observed between slices perfused with subtilisin and DHPG ($P = 0.0263$, *). In addition, perfusion of subtilisin caused extremely significant decreases in expression of both VAMP-1 ($P < 0.0001$, ***) and actin ($P = 0.0001$, ***) in comparison to slices treated with DHPG.

5.2.2 Comparison of the neuronal effects of EDA and subtilisin

Another compound capable of causing LTD is the GABA mimetic ethylenediamine (EDA). The ability of EDA to generate LTD was accidentally discovered in a study to investigate the pharmacological attributes of EDA, and to assess their impact in vivo. Perfusion of EDA in the study resulted in the generation of an LTD effect in a portion of the hippocampal slices. The mechanisms involved however are unclear, as no other GABA mimetic compounds are known to generate such an effect. It was suggested that the LTD-inducing effects of EDA was a result of modifications to receptors such as mGluRs, resulting in an mGluR-dependent LTD effect (Stone *et al.*, 2011). The aim of the following experiment was therefore to provide a comparison between subtilisin, a bacterial protease, and the inhibitory compound EDA, in its role as an inducer of LTD.

Experiments were conducted using 1mM EDA as a replacement for subtilisin during the 10-minute perfusion period. Any LTD effects generated by EDA perfusion were then compared with those caused by subtilisin. Perfusion of EDA elicited a much weaker LTD, as reflected by a decrease in fEPSP amplitude of around 20%. In comparison, subtilisin-mediated LTD typically reduced the amplitude to around 70-80% of the original fEPSP size (Figure 5.2a, $P > 0.0001$). EDA-LTD did not appear to depend on proteolytic action, as none of the proteins measured were significantly degraded in the presence of EDA, in sharp contrast to subtilisin-mediated LTD (Figure 5.2b)

5.3 Discussion

These experiments were conducted primarily to compare the characteristics of subtilisin-mediated LTD with LTD mediated through other mechanisms. In particular, subtilisin-mediated LTD is characterised by the selective degradation of several proteins, and it would be of great interest to determine whether this also occurs in other types of LTD. Comparison of the effects of subtilisin with DHPG, an mGLuR agonist was not successful, as DHPG did not generate an LTD effect. There is a possibility that the failure of DHPG to elicit LTD was a result of the concentration of DHPG being too low to elicit such an effect, as many previous experiments have utilised DHPG at a concentration of 20μM or higher (Rush *et al.,* 2002). A test concentration of 100μM DHPG was also applied to a small number of slices, however, this was also ineffective in generating LTD. The failure of DHPG to elicit LTD at this concentration suggests that the protocol used in these experiments may have been at fault, and a longer period of DHPG perfusion may be necessary to elicit an LTD effect. A second attempt at comparing the effects of LTD utilising MGluR-mechanisms was made using the GABA mimetic compound EDA. Previous experiments have indicated that perfusion of EDA is capable of generating LTD (Stone *et al.*, 2011), and this was proven during the experiment, where EDA almost always caused LTD induction. Furthermore, the generation of LTD by EDA was not accompanied by protein degradation, indicating that protein degradation was likely restricted to LTD generated by subtilisin alone.

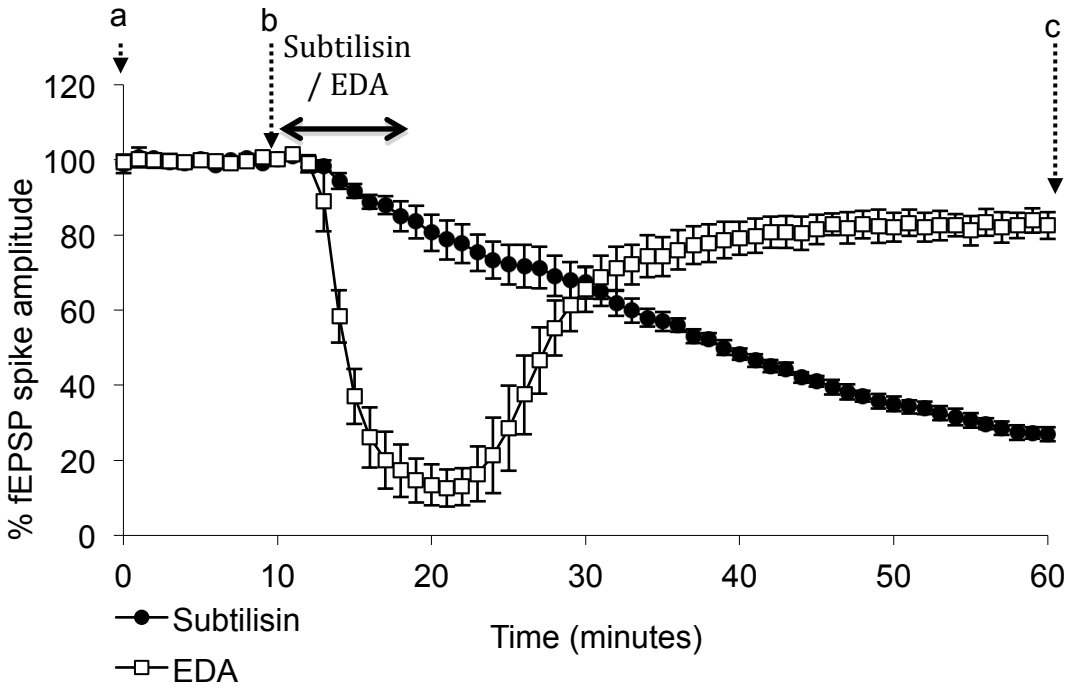
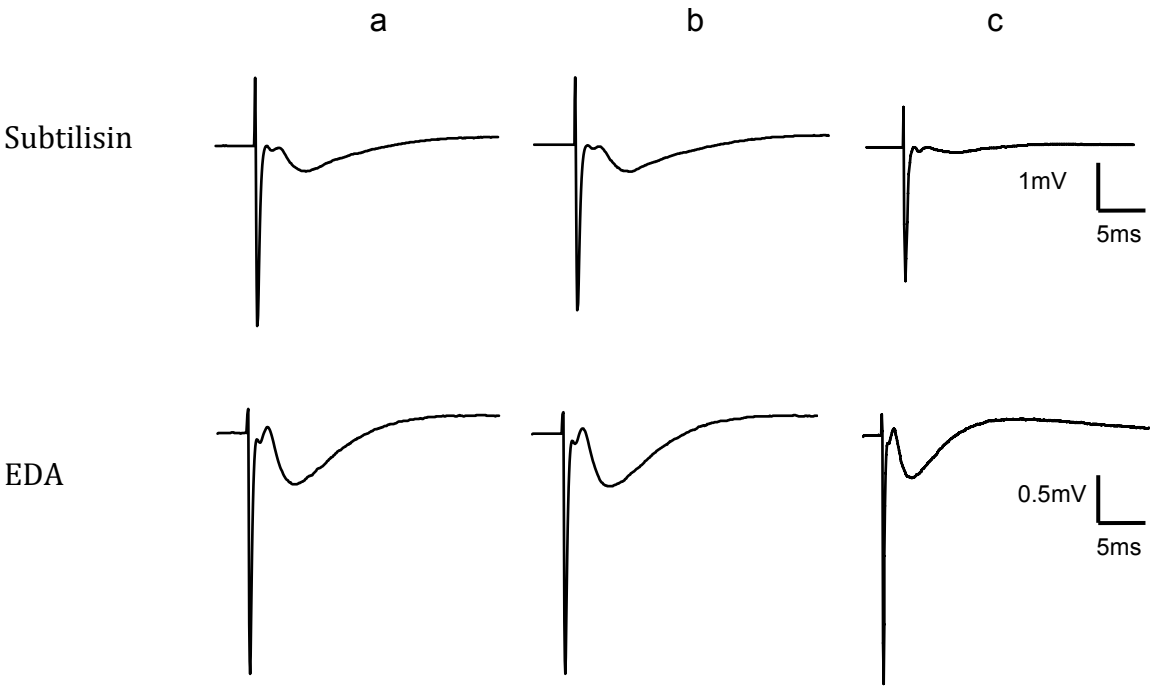


Figure 5.2a Comparison of the effects of EDA and subtilisin perfusion on end fEPSP size.

Perfusion of Ethylenediamine (EDA) at a concentration of 1mM produced a LTD effect of (82.49 ± 3.53% baseline, N = 4), which was substantially less than the LTD effect caused by perfusion of 4μM subtilisin (26.97 ± 1.87% baseline, N = 4, P < 0.0001). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



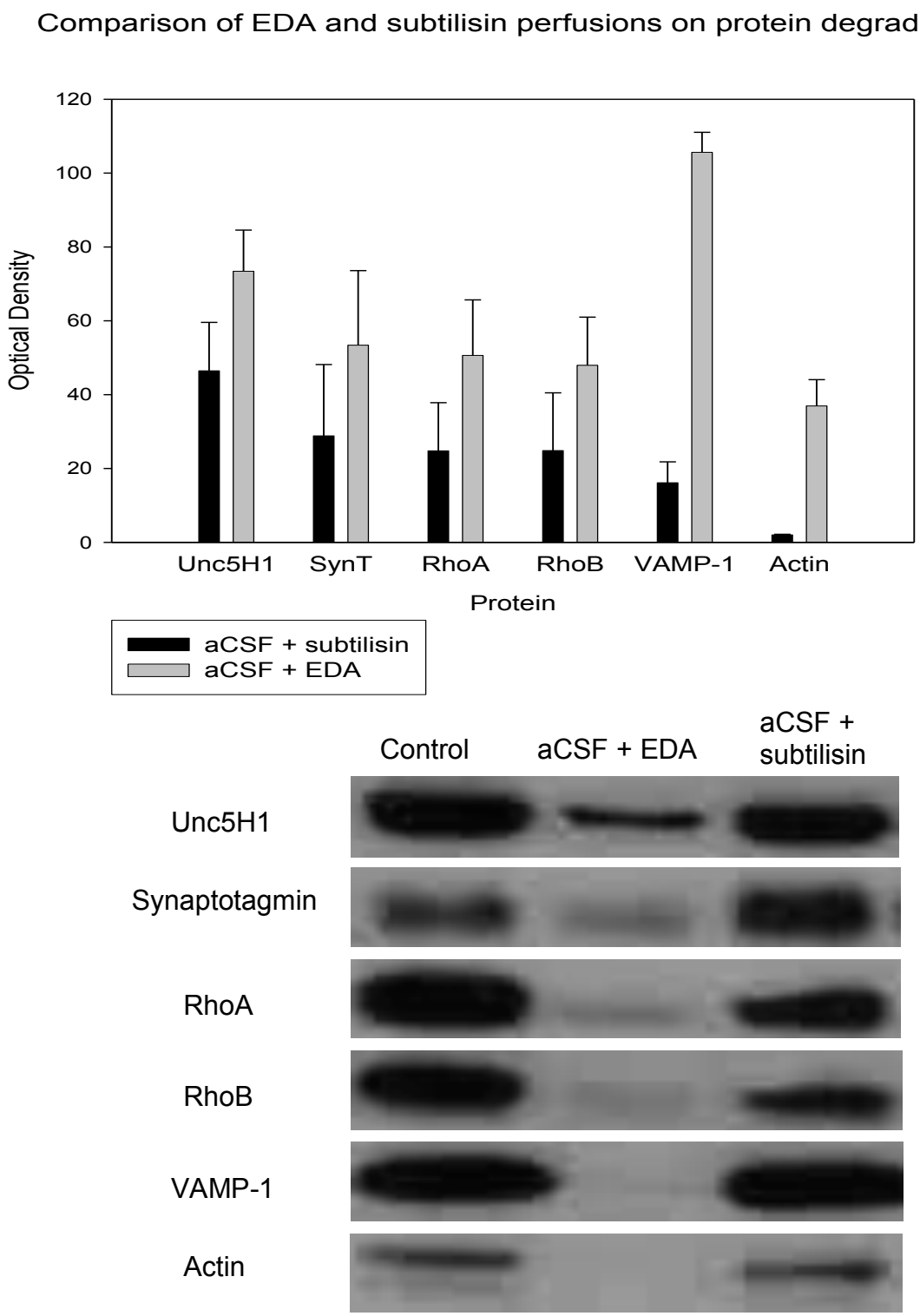


Figure 5.2b Comparison of 1mM EDA and 4µM subtilisin perfusions on protein expression.

Perfusion of subtilisin caused a significant decrease in protein expression compared with controls. In comparison, slices perfused with EDA exhibited protein levels comparable with control slices. Significant differences in protein expression were observed between slices treated with subtilisin alone and those perfused with EDA, however, when compared to controls only those exposed to subtilisin differed significantly (Subtilisin vs. EDA $P > 0.05$; $N = 4$).

Chapter 6 - Investigations into the activities of the subtilisin-like serine protease, chymotrypsin

6.1 Comparison of chymotrypsin and subtilisin potency

6.1.1 Introduction

Most studies on serine protease mediated LTD to date have been performed using subtilisin as the inducing agent. However, past studies have demonstrated other members of the S8A group of serine proteases can also elicit LTD, including the serine protease α-chymotrypsin (MacGregor *et al.*, 2007). However, the potency of chymotrypsin is several magnitudes less than subtilisin, and therefore higher concentrations of chymotrypsin were needed to produce the same effect. The optimal concentration of chymotrypsin for use in these experiments was determined through an evaluation of chymotrypsin activity at different concentrations.

6.1.2 Results

6.1.2.1 Comparison of 4μM chymotrypsin and 4μM subtilisin action

All previous experiments in this study involved the use of subtilisin at a concentration of 4μM. Chymotrypsin was therefore perfused at a concentration of 4μM to provide an accurate comparison between the activities of these two serine proteases within the current experimental protocol.

Perfusion of chymotrypsin produced a decrease in fEPSP size which was significantly lower than those produced by subtilisin (Figure 6.1a, $P = 0.0032$). The higher potency of subtilisin in comparison to chymotrypsin was also reflected in the assessment of protein expression levels. Hippocampal slices treated with subtilisin expressed significantly lower levels of proteins in all markers when compared to slices treated with chymotrypsin. (Figure 6.1b, $P < 0.05$ for all proteins)

6.1.2.2 Comparison of the effects of 6μM chymotrypsin and 4μM subtilisin

Chymotrypsin at a concentration of 4μM was less potent than 4μM subtilisin, and as a result, a higher concentration of chymotrypsin was required to produce a comparable LTD effect. Chymotrypsin was therefore tested at the higher concentration of 6μM chymotrypsin in an attempt to produce a similar LTD size.

Despite this increase in concentration, there was still a significant difference ($P = 0.0073$, Figure 6.2a) in the end LTD size between slices perfused with chymotrypsin and those perfused with subtilisin. Perfusion of 6μM chymotrypsin was still insufficient to cause the same level of LTD as 4μM subtilisin perfusion. However, the difference in end fEPSP between chymotrypsin and subtilisin was much reduced at this concentration. For the experiment comparing 4μM chymotrypsin against 4μM subtilisin, a difference in end fEPSP size of 37.68 ± 6.13% was observed. In the current experiment, the difference in end fEPSP size between 6μM chymotrypsin and 4μM subtilisin perfusions was 21.37 ± 5.97%.

Analysis of protein degradation in response to either chymotrypsin or subtilisin also demonstrated the presence of significant differences in the expression levels of both synaptophysin and VAMP-1. However, expression of both Unc5H1 and actin were similar between chymotrypsin and subtilisin (Figure 6.2b).

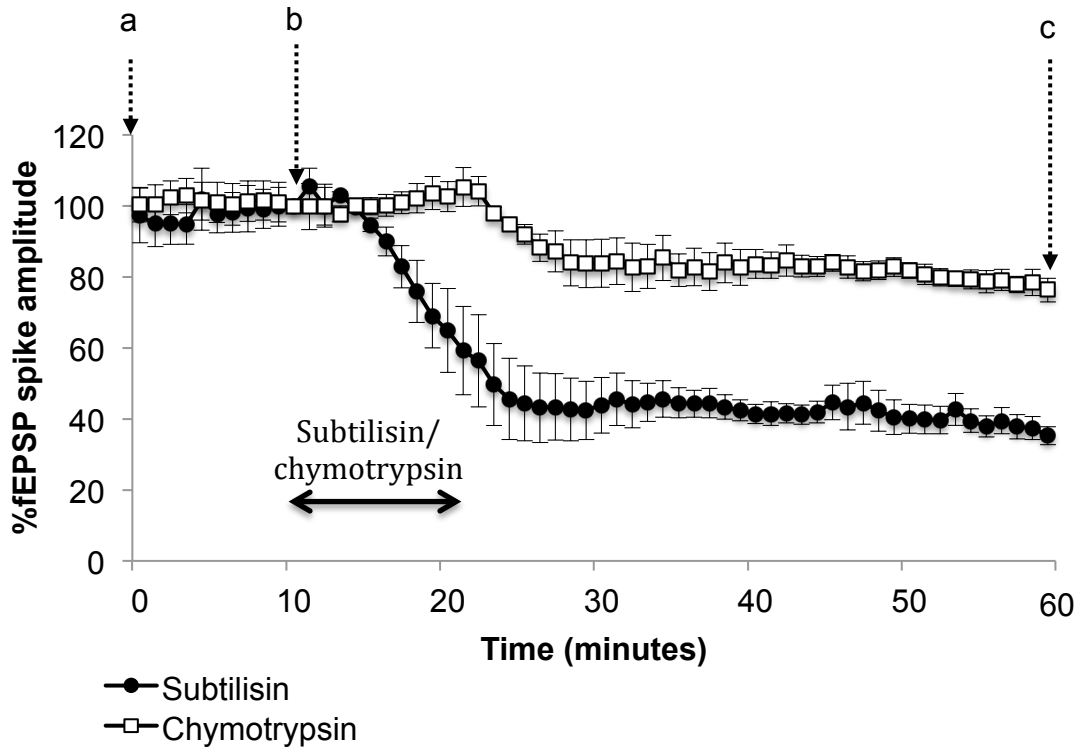
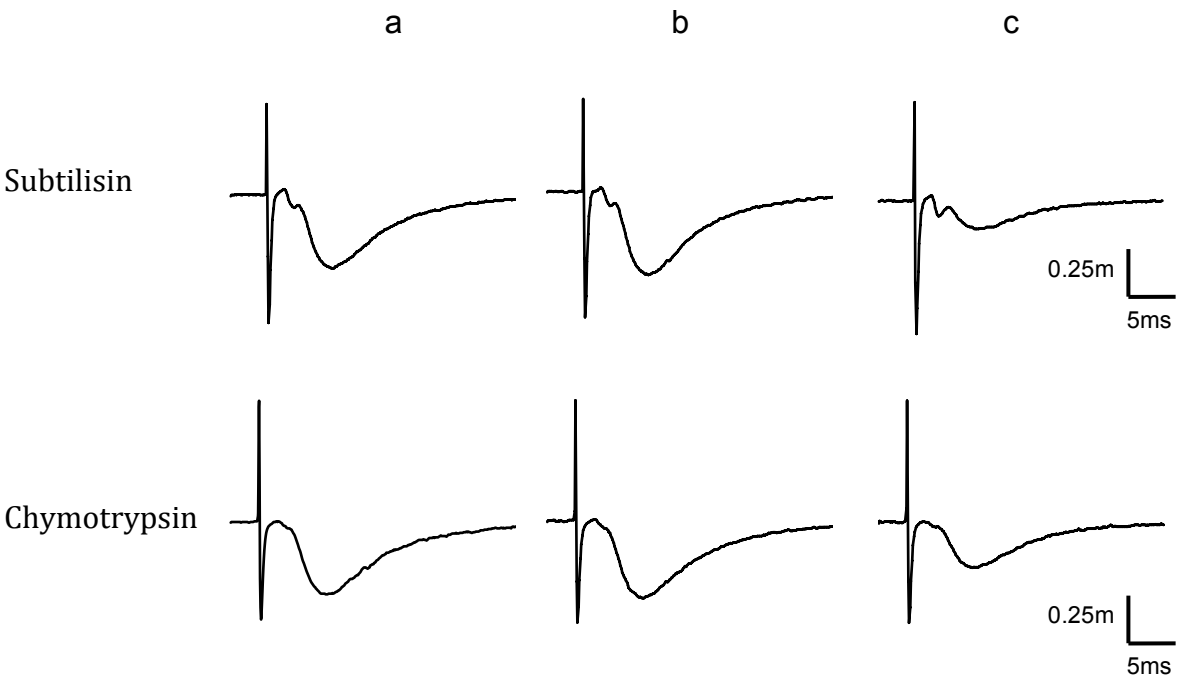


Figure 6.1a Comparison of the fEPSP effects of 4µM Chymotrypsin and 4µM subtilisin perfusion.

The reduction in fEPSP after chymotrypsin perfusion (73.38 ± 6.08%, N =4) was significantly lower than the rate of fEPSP reduction after subtilisin perfusion (37.05 ± 4.69%, P = 0.0032). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



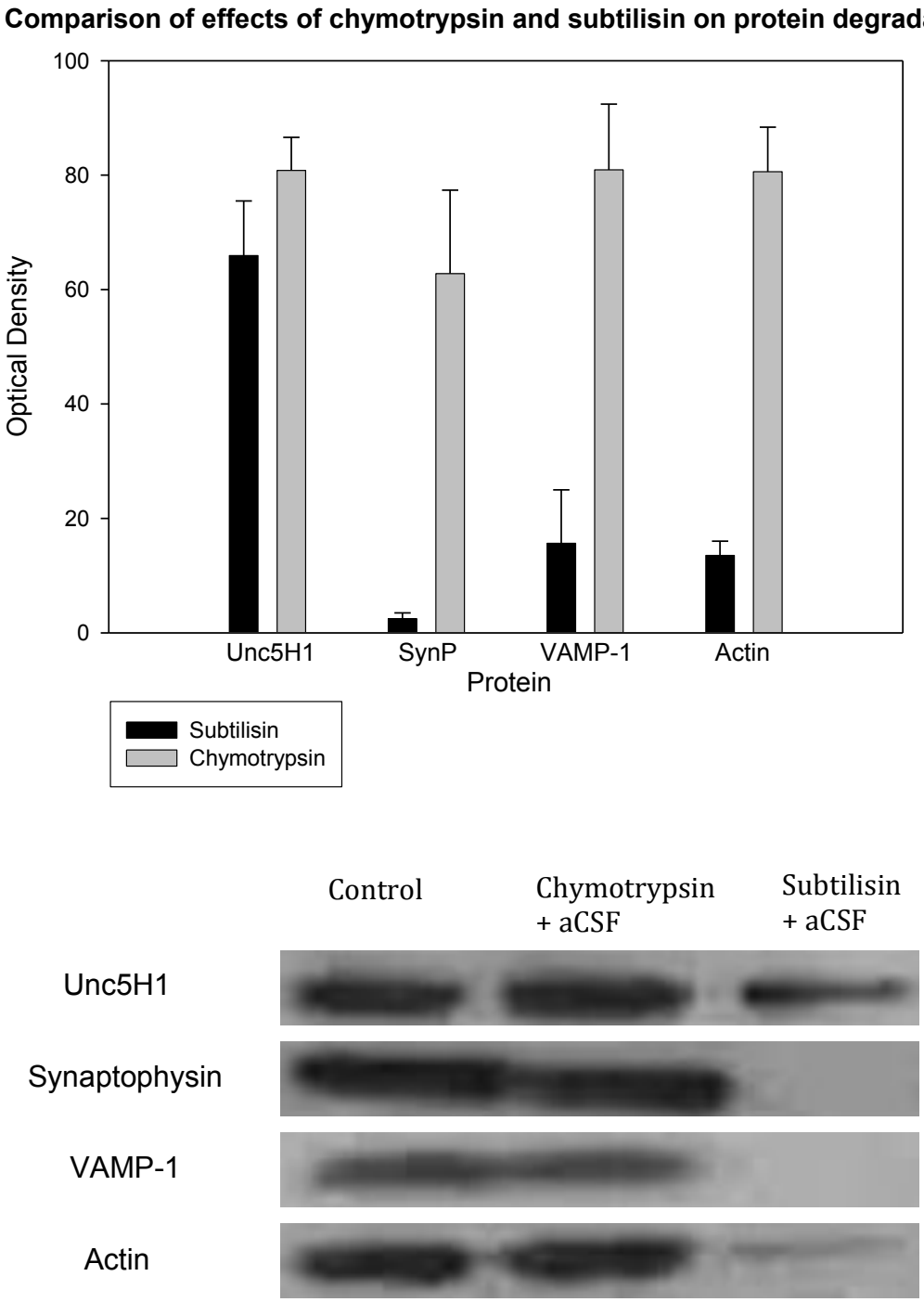


Figure 8.1b Comparison of the effects of 4µM chymotrypsin and 4µM subtilisin on protein degradation.

Protein degradation after perfusion of 4µM chymotrypsin was significantly lower than after subtilisin perfusion. $P < 0.05$ for all proteins, N =4)

N = 4 for both chymotrypsin treatment and subtilisin treatment.

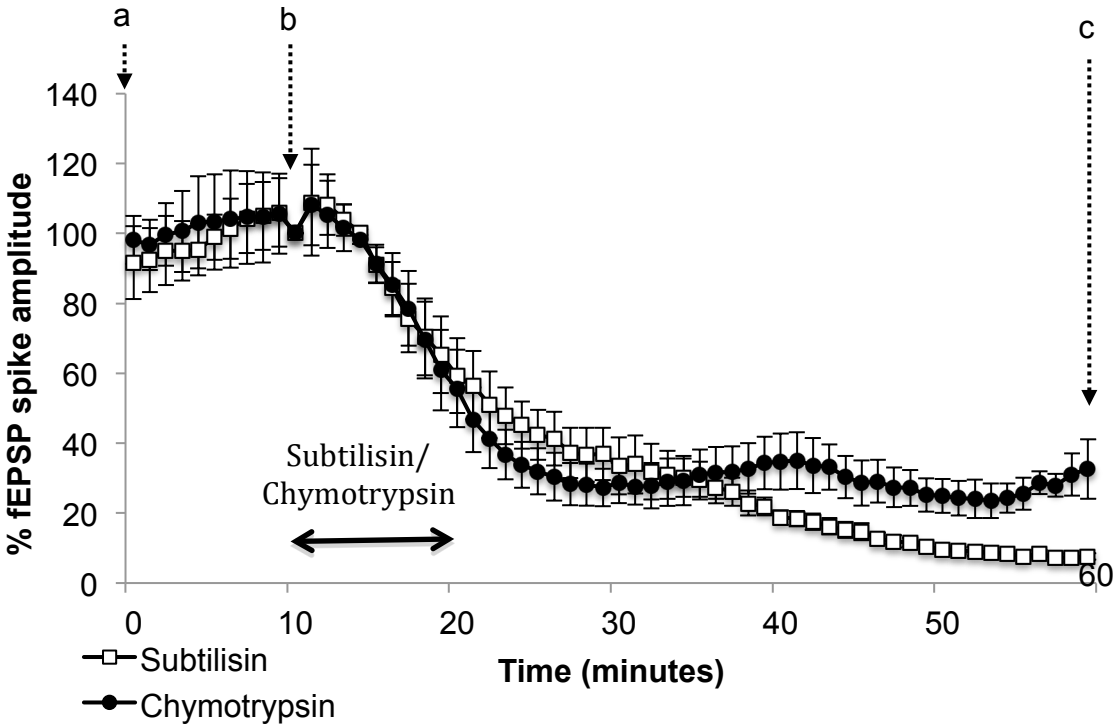
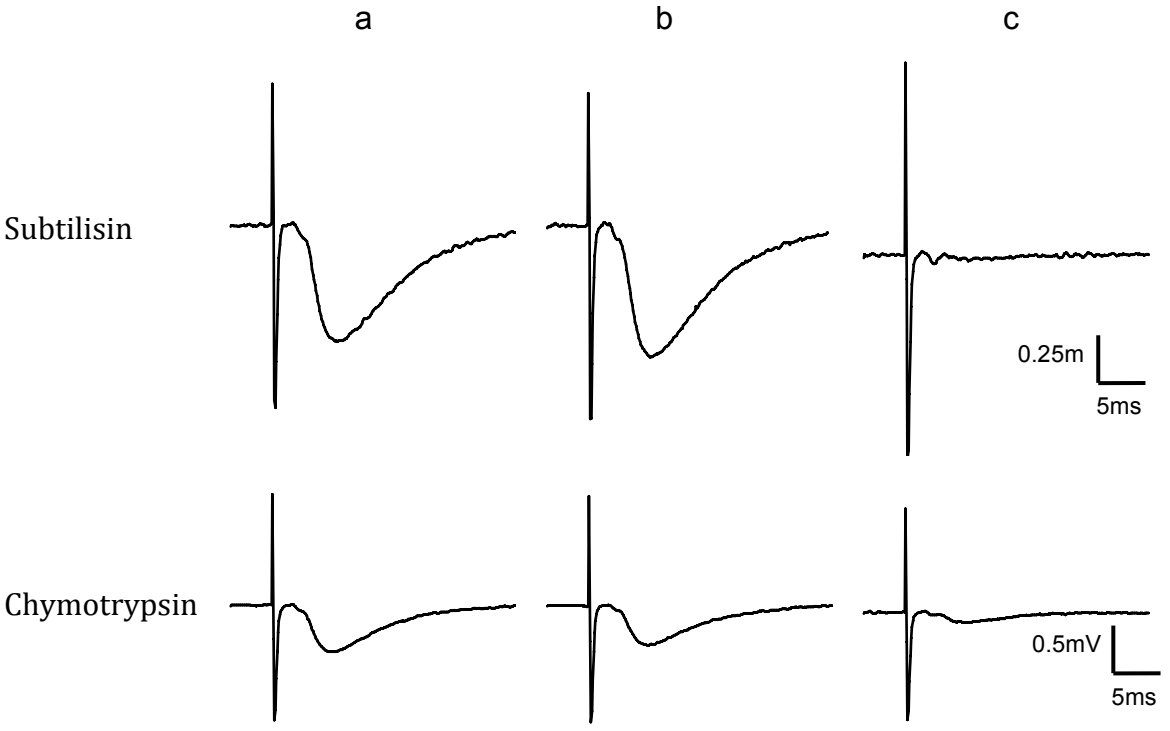


Figure 6.2a Comparison of the effects of 6µM Chymotrypsin and 4µM subtilisin on fEPSP size.

The reduction in fEPSP after chymotrypsin perfusion (30.11 ± 5.79%, N =4) was significantly lower than the rate of fEPSP reduction after subtilisin perfusion (7 ± 0.65%, P = 0.0073). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



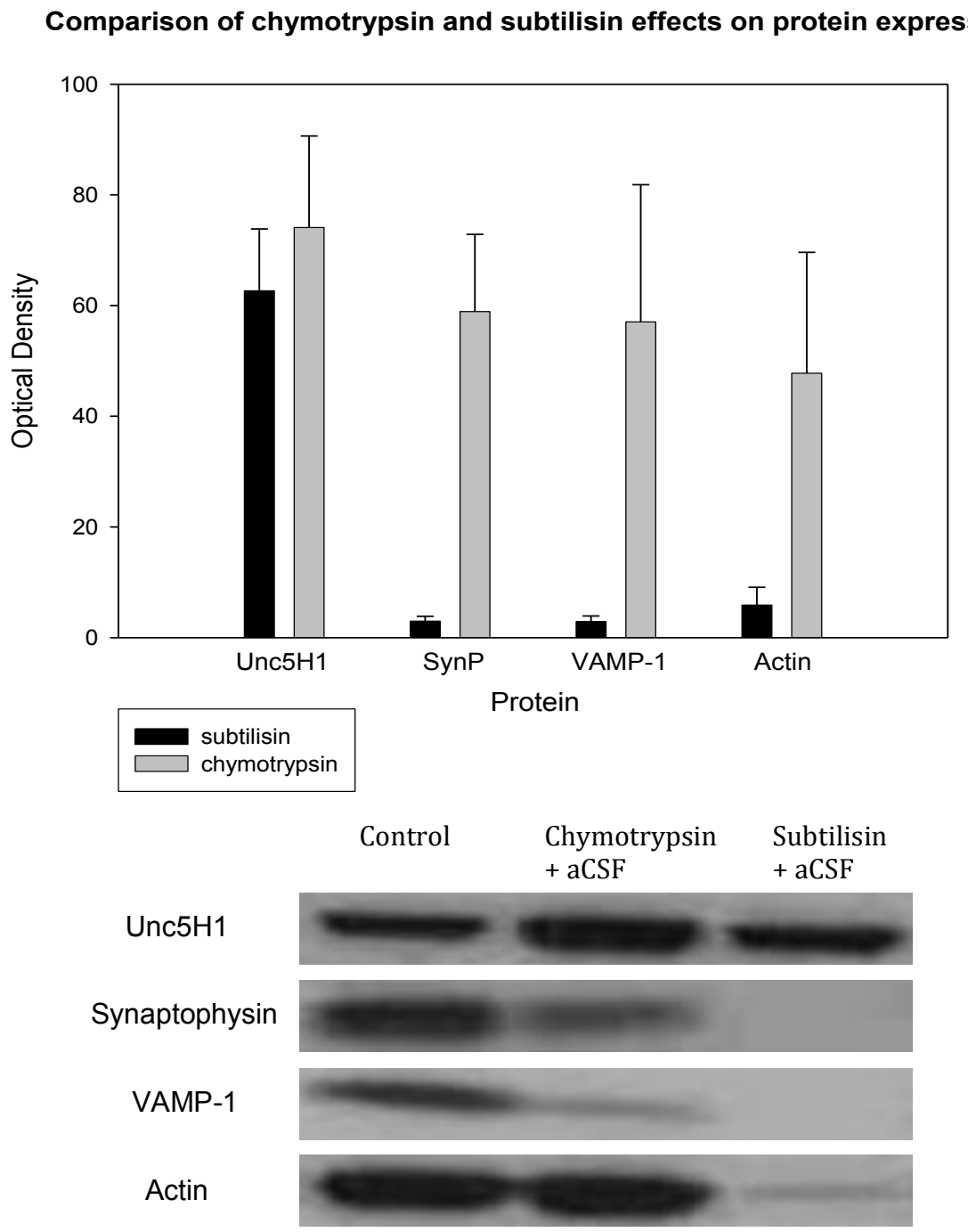


Figure 6.2b Comparison of 6µM chymotrypsin and 4µM subtilisin perfusion effects on protein degradation.

Perfusion of 4µM chymotrypsin resulted in a significant difference in levels of synaptophysin (P = 0.007) and VAMP-1 (P = 0.036) expression. Expression of Unc5H1 and actin however did not show any significant variations. N =4

6.1.3 Discussion

Previous studies have shown that chymotrypsin and subtilisin differ in their relative potencies in the generation of LTD (MacGregor *et al.,* 2007), and this was further supported by the results obtained over these experiments. At a concentration of 4μM, chymotrypsin generated an LTD of approximately 75% baseline fEPSP, in comparison to the LTD generated by subtilisin, which decreased fEPSP to around 35% baseline. This difference was also reflected in the pattern of protein degradation caused by these serine proteases, with chymotrypsin perfusion having minimal effect on protein expression, even in proteins normally most susceptible to subtilisin degradation.

The effect of an increased chymotrypsin concentration of 6μM was compared with the effects of 4μM subtilisin. The LTD effect generated by chymotrypsin was still weaker than subtilisin, with chymotrypsin producing a decrease of around 30%, and subtilisin decreasing fEPSP size to around 7.5% baseline. At this concentration, chymotrypsin was still unable to cause major changes to protein expression, in contrast to subtilisin perfusions, which produced a significant decrease in the expression of all protein markers. Despite the difference in potency between these two serine proteases, chymotrypsin at a concentration of 6μM was able to generate an LTD effect of a similar magnitude to those induced by subtilisin. However, this effect on fEPSP was not accompanied by a similar level of protein degradation, leading to the possibility that mechanisms other than protein degradation may be responsible for this LTD effect.

Based on these results, a chymotrypsin concentration of 6μM was selected for use in subsequent experiments, as this concentration produced an LTD effect of comparable size to 4μM subtilisin.

6.2 Investigations into the mechanism of chymotrypsin-mediated LTD.

6.2.1 Introduction

Initial experiments confirmed that chymotrypsin could cause a similar LTD effect as subtilisin, which was also accompanied by selective protein degradation. However, the potency of chymotrypsin was far lower than subtilisin, suggesting potential differences in their mechanisms. Chymotrypsin-mediated LTD was therefore evaluated against other common types of LTD using inhibitory compounds previously tested on subtilisin. The role of phosphatases was examined using the inhibitors phenylarsine oxide (PAO) and sodium orthovanadate, which block the activation of phosphatases essential to electrically-stimulated LTD. Both electrically-stimulated and mGluR-dependent LTD requires p38 MAP kinase activity, and the contribution of this kinase to chymotrypsin-induced LTD was investigated using the selective inhibitor SB203580.

6.2.2 Results

6.2.1 Effect of 25μM PAO on the action of 6μM chymotrypsin

For this set of experiments 25μM PAO was added to the aCSF and perfused over the hippocampal slices throughout the experimental period, up to 30 minutes of the recovery period after chymotrypsin perfusion. Perfusion of PAO significantly decreased the effects of chymotrypsin, causing a LTD effect of 73.81 ± 3.96% of baseline values (N = 3). In comparison, perfusion of chymotrypsin in aCSF alone caused an LTD decrease of 10.41 ± 2.15% (N =4), which was extremely significant ($P < 0.0001$, Figure 6.3).

6.2.2 Effect of 1mM sodium orthovanadate on 6μM chymotrypsin

The effect of sodium orthovanadate on the action of chymotrypsin was evaluated by dissolving this chemical in the aCSF used for perfusing the hippocampal slices during electrophysiological recordings. Sodium orthovanadate was used at a concentration of 1mM, and slices were perfused with this modified aCSF for the duration of the experiment,

up to 30 minutes into the recovery period, when perfusion was switched back to ordinary aCSF.

Perfusion of sodium orthovanadate did not significantly alter the LTD response in the hippocampal slice when compared with chymotrypsin perfusion in aCSF alone. LTD generated by chymotrypsin in the presence of sodium orthovanadate caused a 77.76 ± 3.47% (N = 4) reduction in fEPSP size; chymotrypsin in normal aCSF caused a fEPSP decrease of 71.82 ± 3.23 (N =5). Therefore the perfusion of sodium orthovanadate did not produce a significant change to the chymotrypsin-mediated effects on fEPSP (P = 0.2525, Figure 6.4).

6.2.3 Effect of SB203580 on 6μM chymotrypsin

A final experiment was conducted to determine the effects of the p38 MAP kinase inhibitor SB203580 on the activity of chymotrypsin. Over the course of the electrophysiological recording period, hippocampal slices were exposed to 5μM SB203580 dissolved in aCSF for 50 minutes, before being perfused with normal aCSF for the last 10 minutes of the recovery period. Perfusion of this inhibitor did not produce a significant change to the size of the LTD in response to chymotrypsin perfusion (P = 0.2398, Figure 6.5).

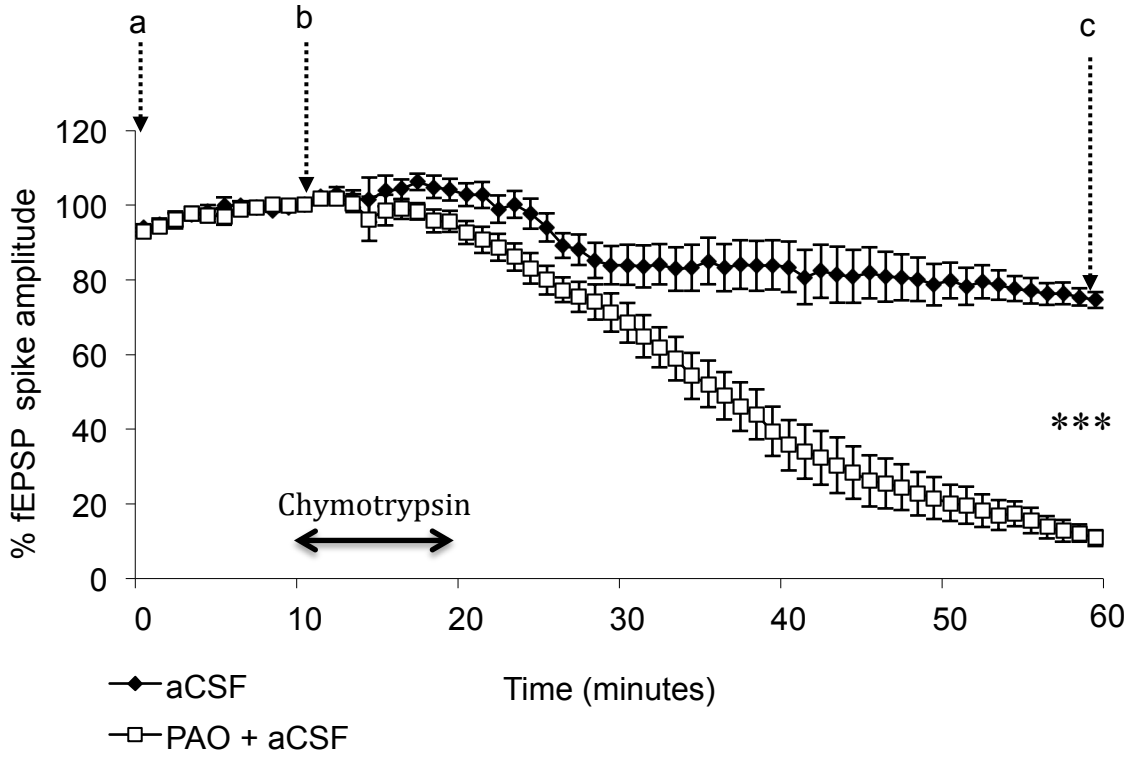
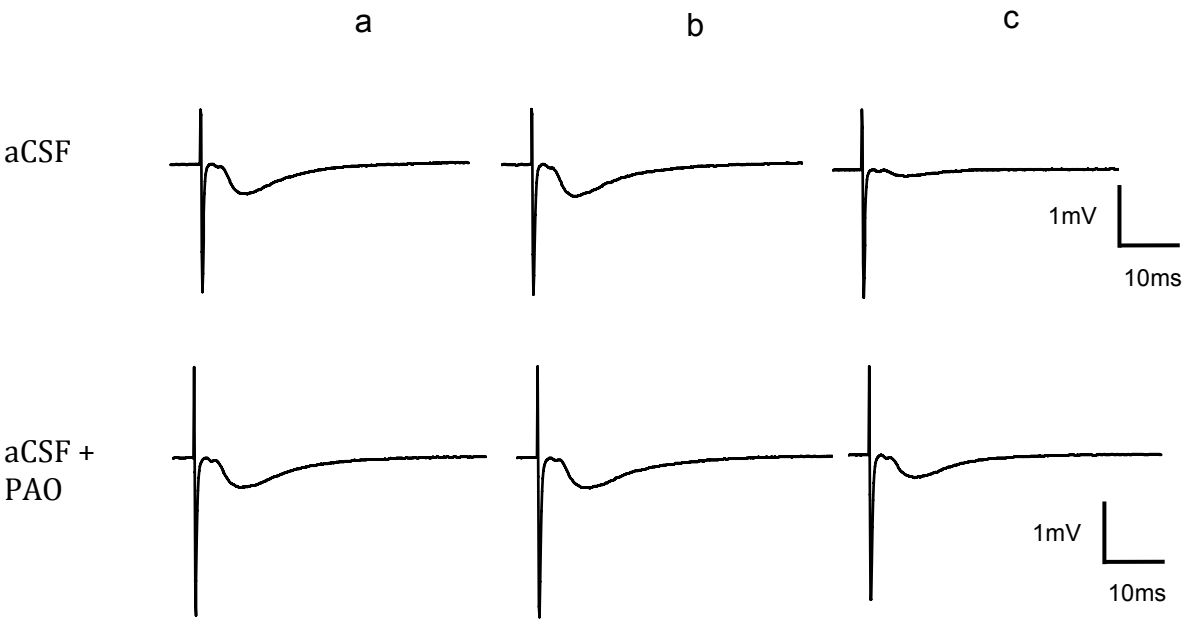


Figure 6.3a Effect of perfusing 25µM phenylarsine oxide (PAO) on fEPSP response to 6µM chymotrypsin.

The reduction in fEPSP size in response to chymotrypsin perfusion was more prominent in slices perfused with aCSF supplemented with PAO (10.41% ± 2.15%, N = 4) than slices perfused with aCSF alone (73.81 ± 3.95%, N =3, P<0.0001). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



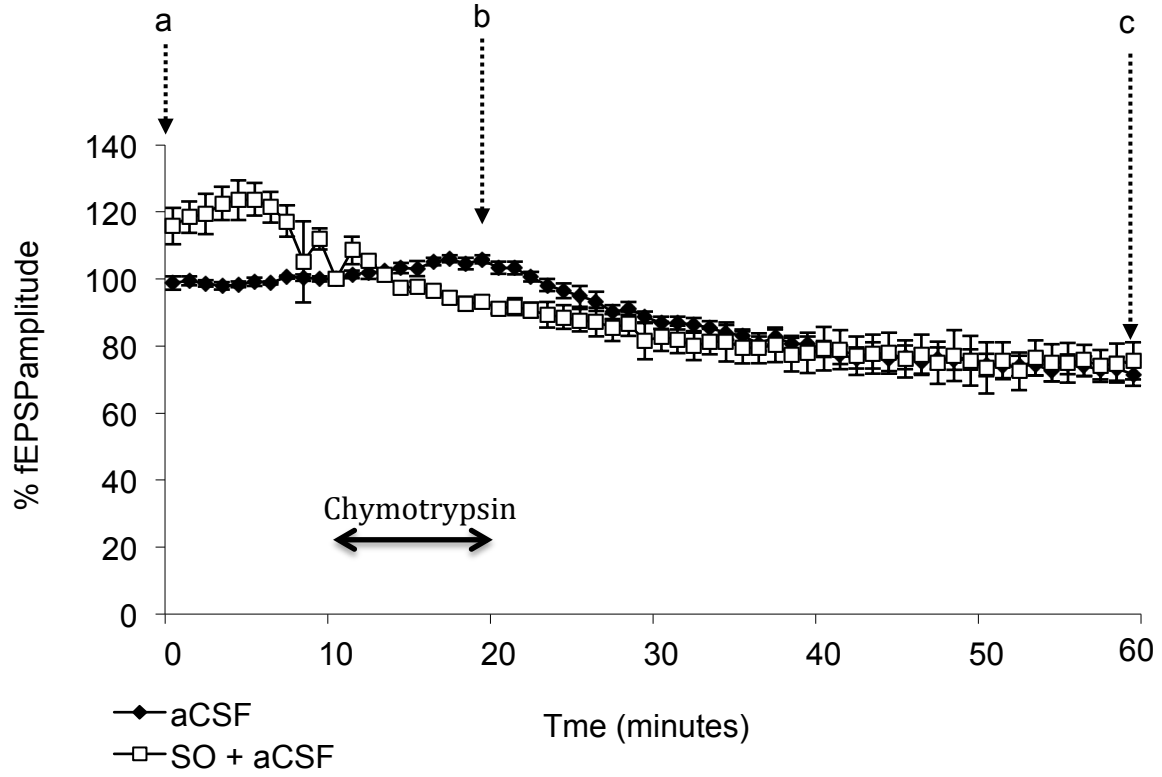
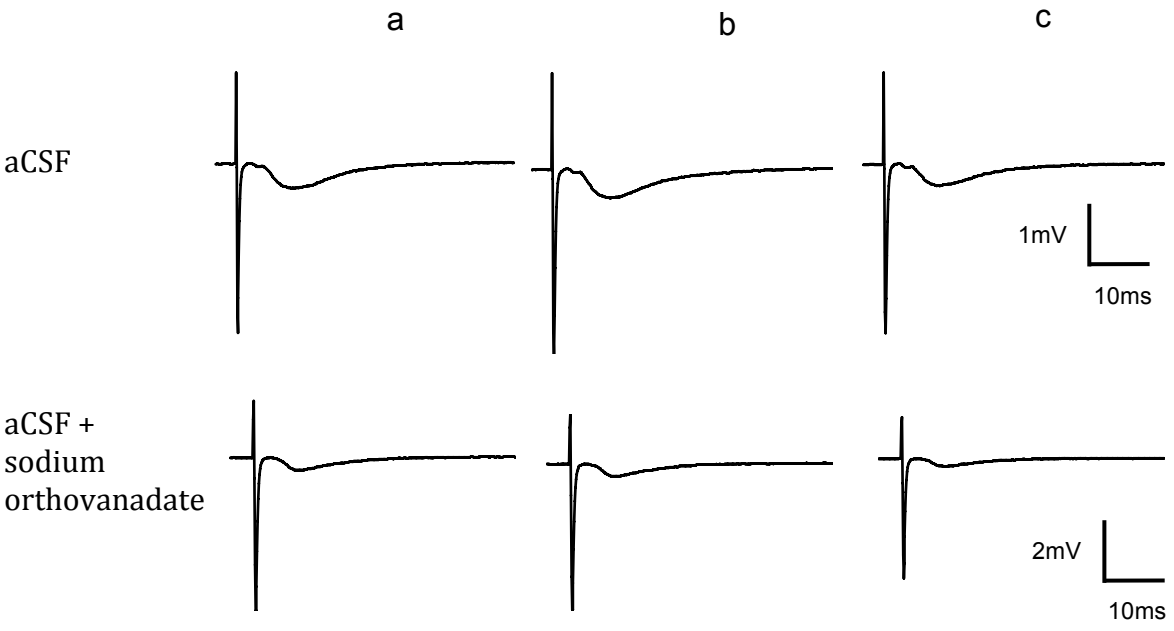


Figure 6.4 Effect of perfusing 1mM sodium orthovanadate (SO) on fEPSP response to 6µM chymotrypsin.

The reduction in fEPSP size in response to chymotrypsin perfusion did not differ significantly between slices perfused with aCSF alone (71.82 ± 3.23%, N =5) than slices perfused with aCSF supplemented with SO (77.76% ± 3.47%, N =4; P = 0.2525). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



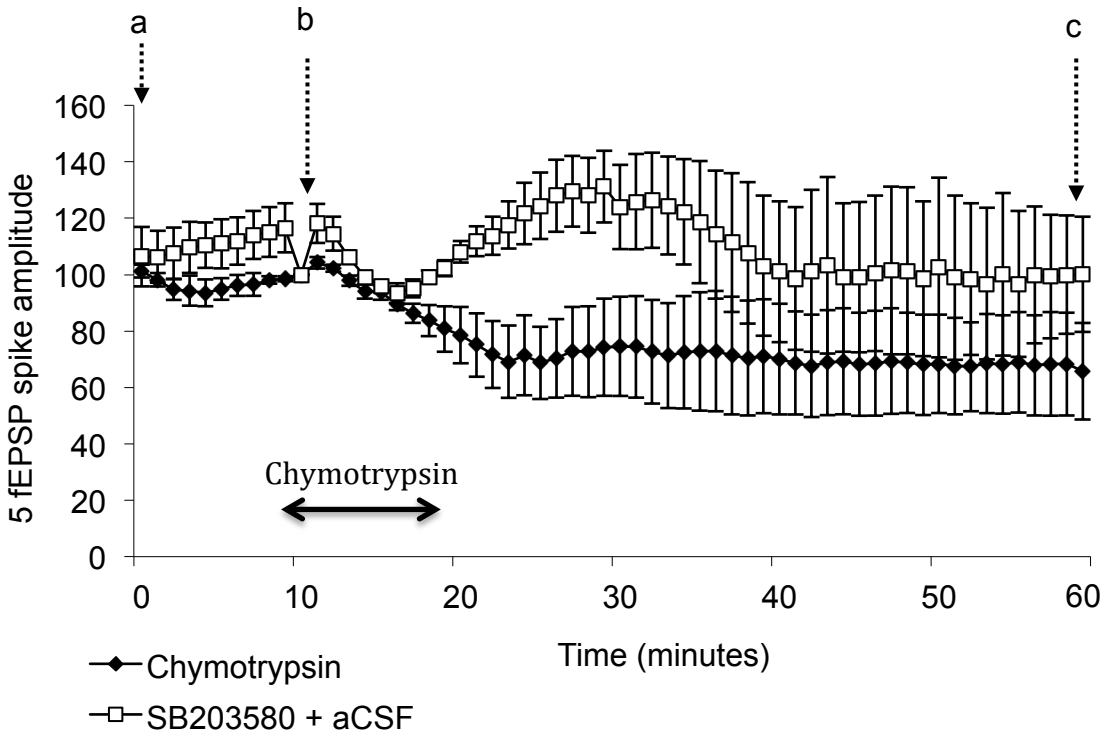
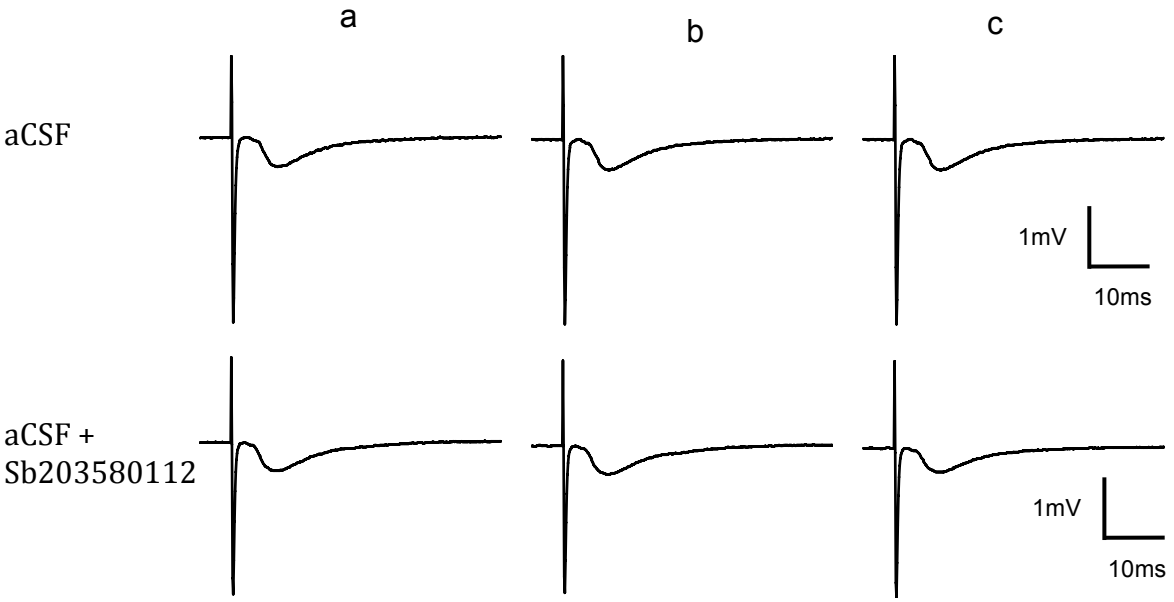


Figure 6.5 Effect of perfusing 5µM SB203580 on fEPSP response to 6µM chymotrypsin

The reduction in fEPSP size in response to chymotrypsin perfusion was not significantly different in slices perfused with aCSF supplemented with SB203580 (99.01 ± 19.27%, N = 4) than slices perfused with aCSF alone (65.65 ± 16.81%, N =4, P = 0.2398). Arrows a, b & c indicate time points at which sample fEPSPs were taken, as shown below.



6.3 Discussion

Previous experiments on serine protease-mediated LTD was mainly focussed on the action of subtilisin. However, it is known that other members of the S8A serine protease subfamily, to which subtilisin belongs, also possess LTD-inducing properties (MacGregor *et al.,* 2007). These other serine proteases vary in their potency and most are less effective in generating LTD when compared to subtilisin. However, at a sufficiently high concentration, these other serine proteases can produce an LTD of similar amplitude to those generated by subtilisin. In order to investigate the action of these other serine proteases, experiments were conducted on α-chymotrypsin, a serine protease that possesses a similar structure as subtilisin. In addition, its role as an endogenous serine protease can also make these experiments more relevant to a physiological context. Initial experiments involved optimising the concentration of chymotrypsin to produce an LTD of a similar size to subtilisin by testing the effect of 4μM and 6μM chymotrypsin. Results suggested that chymotrypsin at concentration of 6μM was able to produce an LTD of around 70% baseline fEPSP, which was comparable to LTD generated by subtilisin. This chymotrypsin concentration was therefore used for all subsequent experiments to enable a comparison to be made with subtilisin action.

Past studies on subtilisin-mediated LTD have demonstrated that new form of LTD may utilise some of the mechanisms involved with electrically-induced or mGLuR-induced LTD. Evaluating of the contribution these mechanisms make in the action of chymotrypsin would provide a clearer understanding of processes underlying serine protease induced LTD. The contribution of protein phosphatases involved in electrically-induced LTD was assessed using the protein phosphatase inhibitors PAO and sodium orthovanadate. The involvement of p38 MAP kinases, which contribute to mGluR-mediated LTD, was assessed using the inhibitory compound SB203580 which prevents the activity of this kinase. The addition of SB203580 had no effect on chymotrypsin action, suggesting that mGLuR activity does not have a major role in this new LTD mechanism. In contrast, experiments with sodium orthovanadate and PAO were less conclusive. The size of the chymotrypsin-mediated LTD was reduced in the presence of PAO, but sodium orthovanadate had no measurable effect on chymotrypsin activity. As a consequence of these contradictory results, it is unclear if protein phosphatases are a factor in the generation of chymotrypsin-dependent LTD.

Comparison of these results with those attained with subtilisin suggests that chymotrypsin-mediated LTD is dependent on slightly different mechanisms.

Further experiments using different inhibitors may provide a better understanding of whether these mechanisms are utilised by chymotrypsin, and help determine the exact mechanisms used by this serine protease to generate LTD. Immunoblotting could also be performed on these hippocampal slices to determine whether the protein phosphatase or MAP kinase inhibitors could have an impact on chymotrypsin-mediated protein degradation.

Chapter 7 - General Discussion

7.1 Contribution of serine and metalloprotease mechanisms to subtilisin-mediated LTD

7.1.1 Metalloprotease mechanisms and subtilisin action

The possibility that subtilisin could act via metalloprotease mechanisms was based on the observation that subtilisin selectively cleaved the synaptic protein VAMP-1. The VAMP proteins are known to be major targets for botulinum and tetanus toxins, both of which are zinc-dependent metalloproteases. The possibility that subtilisin could utilise these mechanisms was explored using three different experimental protocols. Initial experiments were conducted by addition of 50μM zinc to the aCSF perfused throughout the experiment in order to enhance the activity of zinc-dependent metalloproteases. These experiments failed to produce any changes to the progression of subtilisin-mediated LTD, and did not decrease protein degradation in response to subtilisin perfusion. Furthermore, the use of 100μM captopril, a potent inhibitor of zinc metalloproteases, failed to elicit any change to the action of subtilisin. The contribution of other metalloproteases was also examined by addition of the general metal chelator 20μM EDTA to aCSF in order to inhibit metalloprotease activity. Experiments were also conducted to compare the action of subtilisin directly with tetanus toxin; however, the use of tetanus toxin in the experimental protocol did not produce any protein degradation, even on their target protein, VAMP-1. These experiments suggest that subtilisin action is not dependent on metalloproteases; however, there were also several shortcomings in the protocol which could have influenced these results. The concentration of zinc was in the range of several micromolar, but the aCSF itself contains buffer chemicals in molar concentrations. There is therefore a possibility that these buffer chemicals could chelate most exogenous zinc ions added to the aCSF, leaving a very small number of ions to interact with the hippocampal slice. Similarly, the use of EDTA at a micromolar concentration may not be sufficient to chelate zinc ions, as other metal ions are present at molar concentrations. These concentrations would therefore need to be increased to provide a more reliable measurement of the contribution of metal ion to subtilisin action. This could potentially in major changes to

aCSF composition, leading the further difficulties in comparisons between slices in normal aCSF and altered aCSF. The experimental protocol used for comparing tetanus toxin and subtilisin action in this study was not effective, as the lack of protein degradation by tetanus toxin suggests that it was inactive during the administration period. As such, another protocol will be necessary to provide a proper comparison between these proteases, possibly involving a higher concentration of tetanus toxin, as concentrations of up to 100μM are known to cause changes to evoked potentials in hippocampal slices (Calabresi *et al.,* 1989).

7.1.2 Role of serine protease mechanisms in subtilisin-mediated LTD

Subtilisin itself possesses inherent proteolytic capabilities as a serine protease, and the contribution of these mechanisms were analysed through the use of the general serine protease inhibitor PMSF. These experiments were conducted in an attempt to separate the proteolytic and LTD-inducing effects of subtilisin, and explore the relative importance of each proteolytic target of subtilisin. The use of PMSF would prevent the proteolytic action of subtilisin, and could determine whether LTD was closely-related to protein degradation, as suggested by previous data. The results of the current experiments support this view, as addition of PMSF was able to prevent both protein degradation, and the generation of LTD. However, these experiments failed to distinguish between each protein, and was therefore of limited value. Further experiments with other serine protease inhibitors may be more selective and produce a partial inhibition of subtilisin activity, which would allow better quantification of their effects on both neuronal signalling and protein expression.

7.2 Importance of VAMP-1, Unc5H3 and actin degradation for subtilisin-mediated LTD

These three proteins are most susceptible to subtilisin proteolysis, and their degradation may have an important part in the progression of subtilisin-mediated LTD.

7.2.1 Effect of Unc5H3 and VAMP-1 antibodies on subtilisin action

The importance of either VAMP-1 or Unc5H3 was tested using protein-specific antibodies to target these particular proteins. Preincubation of hippocampal slices with antibodies failed to prevent the action of subtilisin, even in the presence of pore-forming chemicals such as triton or streptolysin. This indicates that VAMP-1 and Unc5H3 may not cause subtilisin-mediated LTD, but could be an indication of subtilisin activity. However, other explanations for these results are also possible, as there are a number of variables in the protocol used for these experiments. The antibodies used for these experiments were optimised for molecular studies, and not for use with in-vitro preparations of hippocampal slices. There is therefore a possibility that the antibodies themselves could not penetrate the hippocampal slices sufficiently to produce an effect. In addition, there was no method of visualising or measuring the amount of antibody that had penetrated the hippocampal slice, which could potentially be remediated through the use of fluorescent-tagged antibodies. The location of subtilisin action is also unclear, and it is possible that the antibodies binding site was sufficiently distant from the cleavage site that they had no impact on proteolytic cleavage. The duration of the preincubation period could also be a cause for these negative results, as antibody penetration of cells requires a lengthy period of time, and an extended preincubation period may help facilitate an increased number of antibody-protein interactions. However, a balance had to be struck between preincubation time, and slice viability, due to the use of acute hippocampal slices which decrease in function over time. A preincubation period several hours long may not provide sufficient time for accurate electrophysiological recordings to be made from these slices.

The aim of these experiments using antibodies against either Unc5H3 or VAMP-1 was to determine the relative importance of these proteins to subtilisin-mediated LTD. Other methods should be explored to specifically target these proteins, such as the use of RNAi to silence the appropriate genes encoding these proteins.

7.2.2 Impact of the actin stabiliser Jasplakinolide on subtilisin action

Addition of jasplakinolide to the aCSF perfused over the hippocampal slice during recordings did not have an impact on subtilisin-mediated LTD or protein degradation. In particular, it did not affect the levels of actin within the hippocampal slice, leading to the possibility that it did not interact with actin, or at such a low level that it could not have an

overall impact on actin. A higher concentration of jasplakinolide might provide a better indication of the role of actin in subtilisin-mediated LTD and provide a measurable change to actin polymerisation. Alternatively, addition of exogenous actin at a sufficiently high concentration may compensate for the degradation by subtilisin and give a better understanding of the actin dynamics underlying subtilisin action.

7.3 Comparison of DHPG and EDA-mediated LTD with subtilisin-mediated LTD

A comparison was made between subtilisin-mediated LTD and LTD generated by other means, in order to establish whether differences existed between these types. One of the major forms of LTD is dependent on the action of mGluRs, which can be elicited by the mGluR agonist DHPG (Palmer *et al.,* 1997). A comparison was made between the effects of perfusing subtilisin and DHPG on hippocampal slices, with the aim of providing a direct comparison between these two types. However, perfusion of DHPG did not result in the generation of an LTD effect, preventing a comparison using this compound. A second experiment was conducted using ethylenediamine, a GABA mimetic previously known to be capable of generating LTD (Stone *et al.,* 2011). Perfusion of EDA produced an LTD effect that was smaller in magnitude than the LTD generated by subtilisin. Furthermore, it was not accompanied by protein degradation, unlike subtilisin-mediated LTD, supporting the previous view that this was a new form of LTD closely associated with protein degradation. Additional confirmation of these results will require a repeat of the DHPG experiments, as EDA is not well-studied as an inducer of LTD in comparison to DHPG or MCPG, which are well-attested as LTD inducers in literature.

7.4 Comparison of subtilisin and chymotrypsin-mediated LTD

Previous studies have shown that serine proteases related to subtilisin, such as α-chymotrypsin, are also capable of producing LTD effects. This serine protease is

structurally similar to subtilisin, but is less potent in causing LTD. Initial experiments to optimise the use of chymotrypsin supports this view, as 6μM chymotrypsin was required to produce a similar LTD effect as 4μM subtilisin.

Experiments were also conducted to evaluate the contribution of protein phosphatases and protein kinases to chymotrypsin action, which were previously tested on subtilisin-mediated actions (Forrest *et al.,* 2011). These results suggest that chymotrypsin action involve elements of other LTD mechanisms, in a similar manner to subtilisin, which also utilises mechanisms from other types of LTD. An interesting finding from these experiments is that chymotrypsin-LTD is less dependent on protein degradation than subtilisin. Chymotrypsin-mediated LTD at 6μM was similar in to those generated by subtilisin at 4μM; however, protein degradation at this chymotrypsin concentration occurred at a much lower rate. This suggests that chymotrypsin may be a better model for studying the relationship between serine protease-induced LTD and protein degradation.

7.5 Issues on the interpretation of western blots

Western blotting is a useful tool for measuring the presence of proteins, however, it is also semi-quantitative, as the final measurements can be influenced by a number of different variables. For these experiments, calculations were made to load 10-12μg of protein into each gel lane, but without the presence of an accurate loading control, this could not be definitively verified. An effective housekeeping protein would increase the reliability of the current results, but due to the proteolytic nature of subtilisin action against a wide range of proteins, this was not feasible.

The effectiveness of the protein transfer process may also vary between each blot, as there was no protocol in place to measure the amount of proteins being transferred. The Ponceau staining used for these experiments only provided a qualitative measurement of the protein transfer, which if successful would allow the next stage of the protocol to be performed.

In experiments carried out over a number of blots, these variables may be even more prominent, as the response of each blot to blocking, antibody preincubation and chemiluminescence processes will be different. The strength of each protein band is also very subjective, and is dependent on the exposure time of each film, with longer periods providing a stronger signal. If samples are spread over several blots, this may prevent the

detection of small changes to protein expression. An additional issue is that some proteins respond too strongly to the ECL solution, causing a high chemiluminescence value that causes individual protein bands to merge. This complicates the quantification process, as measurements may no longer reflect the size of a single protein band, but instead incorporate signals from neighbouring lanes.

The reliability of the results obtained using immunoblotting could also be improved by the use of additional controls. Protein levels in experiments involving the addition of chemicals to aCSF, such as EDTA, or antibody preincubations agents were not measured in the absence of subtilisin. Therefore the current results may be influenced by the treatments themselves, and not accurately reflect the impact of subtilisin on these slices.

Chapter 8 - Summary

8.1 Main Findings

1) The action of subtilisin was unaffected by either the addition of zinc, which potentiates the action of zinc-dependent metalloproteases, or the addition of the zinc-specific chelator captopril. These results indicate that although subtilisin and the zinc metalloprotease share the same proteolytic target in VAMP-1, their mechanism of action is likely different.

2) Addition of EDTA to the perfusion medium did not attenuate the action of subtilisin. This result suggests that not only is subtilisin not associated with zinc metalloproteases, other metalloproteases do not participate in the mechanisms utilised by subtilisin.

3) Inhibition of the serine protease action of subtilisin via PMSF also abolished the LTD-inducing action of subtilisin. This suggests that these two properties of subtilisin may be linked in some way.

4) VAMP-1 and Unc5H3 antibodies were intended to selectively block subtilisin action on their respective proteins. This did not occur and the presence of antibody alone did not make an impact on the action of subtilisin.

5) Jasplakinolide was unable to prevent actin degradation by subtilisin, although this may have been due to a general failure of action, as there was no sign of actin polymerisation in the slices treated with this actin stabiliser.

6) Comparing LTD between the mGLuR agonist and subtilisin was not possible as DHPG did not elicit an LTD in all slices tested. However, subtilisin-mediated LTD was compared to the LTD generated by a GABA-mimetic compound, ethylenediamine. The LTD generated by subtilisin was significantly bigger in magnitude than the one generated by EDA; furthermore, EDA-mediated LTD did not demonstrate any significant proteolytic activities against the proteins analysed, further supporting the view that subtilisin-mediated LTD represents a novel form of LTD.

7) Comparing the effects of subtilisin and α-chymotrypsin, subtilisin appeared to be more potent, requiring only a concentration of 4μM to cause a decrease in fEPSP size of around 70-80%. Similar values were only attained by α-chymotrypsin at a concentration of 6μM.

8) Subtilisin and chymotrypsin appear to share a similar mechanism of action, which is not dependent on the mechanisms used to generate electrical LTD or mGluR-dependent LTD. However, whilst subtilisin was completely unaffected by the activity of both sodium orthovanadate and PAO, LTD induced by α-chymotrypsin was significantly reduced by the presence of PAO in the perfusion medium.

8.2 Future work

Due to the failure of several of these experiments to work as intended, some, such as the experiments conducted with VAMP-1 and Unc5H3 antibodies may be worth revisiting, but using antibodies raised specifically for the task. In addition, the effect of subtilisin on actin has not yet been elucidated despite the attempts made during this investigation. As actin is such a crucial protein to the normal function of cells and neurons, it would be advisable to repeat these experiments, but with optimised concentrations of jasplakinolide. The research presented here is only an overview of some of the molecular elements of subtilisin action, and indeed effects of serine proteases on the aspects of neuronal signalling. The similarity in action between subtilisin and chymotrypsin also lends itself to the possibility that other unknown serine proteases may also possess these same attributes. The ability of α-chymotrypsin to induce LTD is of great interest, as unlike subtilisin, a bacterial protease, α-chymotrypsin is an endogenous serine protease, which could be a more attractive option considering their natural presence in the body. The finding that the effects of subtilisin could be duplicated with chymotrypsin, albeit at a slightly higher concentration, also make this an attractive model for supporting evidence gathered from experiments on subtilisin, and may even represent a good replacement for subtilisin. In light of the results obtained both in this investigation and in previous studies, it may be worth examining other endogenous serine proteases such as neuropsin to determine whether they have the potential to cause the same LTD effects or disruption to memory as subtilisin. Subtilisin alone is also known to impair the consolidation of memory formation in mice, when injected into their dorsal hippocampi. Other behavioural tests would help to expand on the

current knowledge of subtilisin's physiological roles. Finally, serine proteases have a role in a number of neurological disorders such as Parkinson's disease and dementia, and subtilisin, or its associated proteases such as α-chymotrypsin, may become important models for studying these diseases.

8.3 Conclusions

The initial discovery of serine protease-mediated LTD stemmed from a study which discovered a new serine protease, cadeprin, which belonged to the S8A group of small serine proteases. As part of an evaluation into its activities, cadeprin was found to cause a novel form of LTD, which was associated with degradation of proteins, a characteristic not found in other forms of LTD. Comparison of cadeprin with other members of the S8A group and the related S1A group of serine proteases indicated that other serine proteases, such as subtilisin and chymotrypsin, could also generate a similar LTD effect (MacGregor *et al.,* 2007). Further studies into this new type of LTD, using subtilisin as a model, indicates that subtilisin-mediated LTD is accompanied by a selective degradation of proteins, with the most affected being the synaptic protein VAMP-1, the netrin receptor Unc5H3 and the cytoskeletal protein actin (Forrest *et al.,*2011). Based on these previous studies, the current experiments were conducted to determine the relative importance of these proteins to the action of subtilisin, and also to learn more about the mechanisms underlying this LTD effect. Results presented in this study indicate that the proteolytic action of subtilisin does not utilise metalloprotease mechanisms, but is dependent on its inherent serine protease mechanism. Furthermore, these results confirmed the close association of protein degradation with the LTD effect generated by subtilisin, although this effect is not dependent on the proteolysis of VAMP-1, Unc5H3 or actin alone. These results also confirm the differences between subtilisin-mediated LTD and LTD induced by mGluR mechanisms, although more experiments would be needed to improve the reliability of this result. Comparison of the effects of chymotrypsin and subtilisin also indicate that these two serine proteases utilise similar mechanisms for producing LTD, as evidenced by the susceptibility of Unc5H3, VAMP-1 and actin to degradation by both proteases. Both the proteolytic and LTD-inducing effects of chymotrypsin were weaker than subtilisin, in keeping with previous data on the relative potencies of these two proteases (MacGregor *et al.,* 2007). However, unlike subtilisin, chymotrypsin action was

affected by the presence of PAO, suggesting a possible explanation for the differences in activity between these two serine proteases.

Long-term depression is known to have major roles in the acquisition and retention of memories, and interference with this process is the cause of several major degenerative diseases. In both Alzheimer's disease and Parkinson's disease, pathological progression involves an initial loss of short-term memory deficits, followed by more severe symptoms such as bradykinesia (for Parkinson's disease) or speech impairment and the loss of long term memory (in Alzheimer's). Subtilisin, or serine protease-mediated LTD, have previously been shown to affect the consolidation of short-term memories in mice. Direct injection of subtilisin into the mouse hippocampus after a training session, resulted in an impairment of their response to foot-shock stimuli (Kornisiuk *et al.,* 2010). It is therefore possible for serine protease-mediated LTD to be used as a model for the early stage symptoms of both AD and PD. In addition, a trypsin-like serine protease neurosin, is known to be localised within amyloid plaques of patients suffering from Alzheimer's disease (Ogawa *et al.,* 2000). This suggests that serine proteases can be an important factor in the progression of this disease, and the possibility that subtilisin or related serine proteases such as chymotrypsin may become useful tools for investigating these disorders.

The results obtained in this study have helped to provide a more detailed view of the LTD mediated through subtilisin, as they have indicated that the actions of this serine protease is unlikely to occur via metalloprotease mechanisms. These experiments also prove that other serine proteases such as chymotrypsin are capable of generating LTD with similar characteristics, opening the possibility that endogenous serine proteases with important roles in physiology and pathological conditions may also display this synaptic effect.

Chapter 10 - References

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