Generation of recombinant toxin molecules from
Clostridium tetani and Clostridium botulinum

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Natalie Kikidopoulos


School of Applied Sciences

College of Science, Engineering and Health

RMIT University

April 2013
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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

_____________________________
Natalie Kikidopoulos

19 April 2013
Acknowledgements

I would like to first thank my supervisor Prof. Peter Smooker for taking me on as a PhD student after I completed my Honours degree under his supervision. He has been an invaluable resource and friend during my time as a PhD candidate, and I am thankful for his guidance and support. I would also like to thank my second supervisor Prof. Peter J. Coloe for his guidance and support throughout the project.

Without Tony and Russell this project would not have been possible, so I would like to thank them for the opportunity to work on a fantastic research project which has the potential to help many people suffering from this disorder.

Thank you to Dr Andrew Hung who used his magical protein modelling powers to help me with some modelling work, and to Tom who has recently joined the project and has already done some great work for the project.

I would like to thank all of the people in the Biotech lab and in the Biotechnology and Environmental Biology department for their help over the years. Specific thanks must go to those who have offered insight and technical knowledge like old-school PhD students who were finishing as I was getting started: Manvendra, Xenia and Luke, and to those whom I spent most of my time in the lab with: Aya, Fiona, Amber and Monica (and Tim!) who were not just lab-mates, but also great friends.

Thank you to all my morning tea ladies (and gents) who have provided good times and good laughs during times of stress and success: Danka, Aya, Shannon, Helen, Olivia and Leanne, and those who were part of the morning tea crew over the years: Fiona and Lisa. Without these people, coffee-time would not have been as much fun!

And finally, thank you to my closest friends and family for their supports over the years. To those who have been around forever and to those who have recently come into my life and have helped make this journey unforgettable.

Natalie
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<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>Cam</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Co&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Cobalt</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
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<td>Deoxyribonuclease</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
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<td>g</td>
<td>Gram</td>
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<td>g</td>
<td>Relative centrifugal force</td>
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<td>h</td>
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<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Water</td>
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<tr>
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<tr>
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<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>One thousand Daltons (kilodalton)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal dose causing death in 50% of test group</td>
</tr>
</tbody>
</table>
M Molarity
MCS Multiple cloning site
μF Microfarad
mg Milligram
MG H₂O Molecular grade water
min Minutes
mL Millilitre
μL Microlitre
 mM Millimolar
μM Micromolar
MQ H₂O Milli-Q® water
MW Molecular weight
NaCl Sodium chloride
NaOH Sodium hydroxide
ng Nanogram
Ni²⁺ Nickel
nM Nanomolar
NMJ Neuromuscular junction
OD Optical density
OSA Obstructive sleep apnoea
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
PCR Polymerase chain reaction
pH Negative algorithm of hydrogen ion concentration
R Resistance to antibiotic
RNase Ribonuclease
rpm Revolutions per minute
s Seconds
SDS Sodium dodecyl sulphate
SNAP-25 Synaptosome-associated protein of 25 kDa
SNARE Soluble N-ethylmaleimide-sensitive-factor (NSF) attachment protein receptors
TEMED N,N,N',N'-tetramethylethylenediamine
Tm°C Melting temperature
Tris-HCl Tris (hydroxymethyl) amino methane
U Units
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>V</td>
<td>Voltage</td>
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<tr>
<td>VAMP-2</td>
<td>Vesicle-associated membrane protein-2 (Synaptobrevin-2)</td>
</tr>
<tr>
<td>λ</td>
<td>Lambda phage DNA</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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Summary

Obstructive sleep apnoea is a muscular disorder that disrupts breathing during sleep and is caused by a loss of muscle tone in the muscles of the upper airway which become flaccid and physically block the airway. Sleep is disrupted due to the restriction of air intake caused by upper airway resistance and is characterised by pauses in breathing which may last for up to 60 seconds. Individuals are forced to gasp for air in order for breathing to resume, and the disorder is characterised by daytime sleepiness, waking with a headache and disturbances in concentration and memory. If left untreated, obstructive sleep apnoea can lead to increased blood pressure and risk of stroke, brain damage or heart attack. Current treatment options include mechanical and non-mechanical devices to increase upper airway patency, and surgery to remove excess soft tissue which blocks the airway during sleep. These treatments have low adherence rates due to their impact on lifestyle, or are not suitable for all individuals. This project focussed on developing a novel treatment for obstructive sleep apnoea to correct the loss of muscle tone. Tetanus toxin produced by Clostridium tetani, causes spastic paralysis by preventing the release of inhibitory neurotransmitters within the central nervous system which normally signal muscles to relax. The result is an increase in muscle tone leading to rigid paralysis, which can be exploited for the treatment of obstructive sleep apnoea. Localised injection of tetanus toxin has been demonstrated to induce tetany in the upper airway of British Bulldogs, who naturally have sleep apnoea-like events during sleep, without systemic effects. Due to the widespread immunisation against tetanus toxin through vaccination with tetanus toxoid, this project aims to develop a chimeric holotoxin consisting of the heavy chain of tetanus toxin and the botulinum type A light chain to circumvent pre-existing immunity to tetanus toxin. Tetanus and botulinum toxins have structural similarities, and while they elicit opposing effects, their mode of action is similar. The individual domains, which retain function upon separation, have previously been substituted among domains of other Clostridial neurotoxins. In conclusion, individual toxin domains from tetanus and botulinum type A toxin will be developed and recombined to produce a novel chimeric holotoxin for the treatment of obstructive sleep apnoea.
Chapter 1
Introduction
1.1 Sleep apnoea

Sleep apnoea (SA) is a breathing disorder characterised by irregular breathing patterns during sleep. The condition is a form of sleep disordered breathing (SDB), with sufferers experiencing recurrent arousals during sleep caused by the brief cessation of breathing. There are three types of SA, (i) central sleep apnoea (CSA), caused by a neurological disorder; (ii) obstructive sleep apnoea (OSA), where sleep is interrupted as a result of a physical blockage of the upper airway; and (iii) complex sleep apnoea (CompSA), which is caused by a combination of both. The effects of SDB range from collapse of the upper airway, increased upper airway resistance, pauses in breathing, and decreased saturated oxygen levels in the blood which lead to hypoxia. Irregular breathing patterns continue throughout the night, and result in the individual feeling fatigued the following day (Wolk et al., 2003).

In all types, the apnoea-hypopnoea index (AHI) is used as a quantitative measure of disease severity, and it refers to the number of apnoeas occurring per hour of sleep. An apnoea refers to the cessation of breathing, whilst a hypopnoea is a reduction in the volume of air passing through the upper airway, or a period of very shallow breathing (Young et al., 1993). SA is classified as obstructive or central, based on the whether or not respiratory effort is employed to reinstate breathing during sleep, respectively. The physical obstruction of the upper airway observed in OSA, results from the collapse of the surrounding tissue or muscles in the upper airway. Due to the narrowing of the airway, there is an increase in airway resistance, and sufferers are forced to gasp for air so that breathing may resume. CSA is a neurological disorder which does not allow for breathing to be restored through physical effort. Causes of CSA are of a central origin, and not due to a physical blockage of the airway. CompSA is seen when an individual with OSA continues to experience brief cessations of breathing and interrupted sleep, even once the obstruction has been removed. In such cases, the residual effects of SDB are attributed to CSA-like events, and are generally treated as such. Despite varying causes, all three types of SA display an overlap in symptoms and in all instances, treatment options are aimed at reducing the AHI (Young et al., 1993).

Treatment options vary, depending on the type of SA, however mechanical devices to assist breathing during sleep are the most commonly prescribed. These forms of treatment are very effective in treating symptoms of SA and reducing the risk of comorbidities, however compliance is generally low due to the impact that these devices have on the patient’s lifestyle. In view of the necessity for more convenient treatment options, the focus of this
review will be on the options currently available, and the proposal of a novel therapeutic approach to the treatment of OSA.

1.2 Central sleep apnoea

Central sleep apnoea (CSA) is characterised by impaired central respiratory drive. An interruption of nerve impulses from the brain to the thoracic and abdominal muscles involved in respiration causes muscles to cease movement, and breathing pauses for at least 10 seconds during sleep (Yumino and Bradley, 2008). Disturbances in electrical signals may result from damage to the region of the brainstem which controls breathing and may be from acute brainstem injury or central nervous system disease such as encephalitis, or from neurological disorders such as Parkinson’s disease (Aurora et al., 2012). CSA may also be seen in association with Cheyne-Stokes respiration or other medical conditions such as congestive heart failure, high altitude environments, illicit drug use, obesity, or in normal healthy subjects (Aurora et al., 2012; Eckert et al., 2007).

CSA occurs because the central nervous system cannot react quickly enough to changes in CO₂ levels in the blood. Upon expiration, the level of oxygen in the blood decreases and the level of CO₂ increases, prompting the body to draw in more oxygen. The inhaled oxygen replaces the built up CO₂ in the blood, and the cycle continues. These normal breathing patterns are controlled by chemoreceptors which respond to changes in CO₂ levels through a shift in the concentration of hydrogen ions (Eckert et al., 2007). Over-responsive chemoresponses which are sensitive to small changes to CO₂ levels, cause unstable ventilatory control and may result in irregular breathing patterns such as Cheyne-Stokes respiration. This irregular form of breathing occurs in CSA sufferers whereby individuals exhibit heavy, deep, often quick breathing, gradually becoming shallow, and then stopping temporarily. This pattern of breathing repeats to form an abnormal cycle of hyperventilation and apnoeas. The restricted air supply throughout the night results in inadequate ventilation and compromised gas exchange, inducing symptoms of restless sleep, waking with a headache and daytime sleepiness (Eckert et al., 2007).

1.2.1 Symptoms of CSA

Due to fragmented sleep caused by CSA, sufferers wake often during the night and experience nocturia, daytime sleepiness or fatigue, waking with a headache, and disturbances in concentration and memory (Eckert et al., 2007). Sympathetic activity is increased which has been shown to cause elevated left ventricular pressure and is a major contributor to the development of heart failure (Yumino et al., 2009).
1.2.2 Treatment of CSA

Treatment to reduce the AHI in CSA sufferers includes continuous positive airway pressure (CPAP), or bi-level positive airway pressure (BPAP). Although generally used as the treatment of choice for OSA, CPAP has improved breathing during sleep in CSA sufferers, however the mechanisms are unclear (Eckert et al., 2007). Adaptive servo-ventilation (ASV) is used in patients with Cheyne-Stokes breathing and congestive heart failure, and may be used successfully in CSA patients (Aurora et al., 2012; Ramar et al., 2012). ASV works by precisely adjusting the air pressure delivered according to changes in breathing dynamic. Once the pressurised oxygen normalises breathing, the system reduces delivery to a level which maintains a patent airway, increasing again when breathing becomes unstable. ASV has been effective in improving breathing and AHI in CSA sufferers (Aurora et al., 2012; Morgenthaler et al., 2007).

Pharmacotherapies for CSA act centrally to stimulate respiration. Metabolic alkalosis occurs in CSA patients from the loss of hydrogen ions and may cause unstable respiration. The use of medications such as Acetazolamide, a carbonic anhydrase inhibitor, has been shown to stabilise breathing in CSA patients by creating a mild metabolic acidosis, which increases hydrogen ion concentration and thus the drive to breathe (Selim et al., 2012; Yumino and Bradley, 2008). Side effects of high dose Acetazolamide include tingling sensations or shortness of breath, which may result from drug-induced increased ventilation, however low-dose administration may reduce such effects (Javaheri, 2006). Theophylline, a bronchodilator and heart muscle stimulant, has also shown improvements in daytime sleepiness in CSA patients, but has been shown to cause arrhythmias and nausea (Aurora et al., 2012; Selim et al., 2012). It is not surprising that pharmacotherapy has limited supporting evidence for success as the sole source of treatment, since CSA is a systemic disorder, however, it may be suitable for use in conjunction with other therapies for the treatment of CSA (Selim et al., 2012).

In cases of Cheyne-Stokes related CSA, where hypocapnia (reduced levels of CO₂ in the blood) results, the administration of carbon dioxide during sleep increases the drive to breathe and may reduce hypopnoea (Aurora et al., 2012; Yumino and Bradley, 2008). Hypoxia (reduced dissolved O₂ in the blood), may cause an over-response to subsequent increased levels of CO₂ in the blood, and may result in hyperventilation after an apnoeic event. This condition may be treated by the delivery of oxygen (Yumino and Bradley, 2008). Effects of oxygen delivery may be patient-specific and there is still no definitive treatment for CSA or CSA associated with heart failure.
1.3 Obstructive sleep apnoea

Obstructive sleep apnoea (OSA) is a muscular disorder that disrupts breathing during sleep. OSA is characterised by pauses in breathing, known as apnoeas, which occur a number of times per hour sleep. Unlike CSA, where thoraco-abdominal movement ceases due to an interruption in central signalling, these muscles continue to work in patients with OSA. However, a loss of muscle tone or rigidity causes the muscles to become flaccid or collapse, thereby obstructing the airway when the individual is recumbent (Ankichetty et al., 2011). Breathing pauses for at least 10 seconds as little air is able to pass through the blocked airway, and patients are forced to gasp for air in order for breathing to resume (Eckert et al., 2007). The loss of a patent airway increases upper airway resistance and leads to loud snoring noises, as attempts to inhale air through the occluded airway are made. Increased upper airway resistance leads to a hypopnoea (period of shallow breathing), followed by an apnoea (pause in breathing), which may last for up to 60 seconds. This breathing-pause-breathing cycle continues throughout sleep, with apnoea-hypopnoea events occurring between 5 and 30 times per hour sleep, depending on severity (Ankichetty et al., 2011). Although individuals are not always roused during sleep, additional respiratory drive is required to reinstate breathing. This results in restless sleep, accompanied by classical symptoms such as waking with a headache and drowsiness throughout the day.

1.3.1 Pathophysiology of OSA

OSA is caused by a collapse of the muscles in the upper airway causing increased upper airway resistance during sleep. The upper airway is separated into three regions: the nasopharynx (nasal passage); the oropharynx (hard palate on the roof of the mouth to the base of the epiglottis); and the hypopharynx or laryngopharynx (base of the tongue to the larynx (throat)) (Schwab, 2005). More than 20 muscles make up the pharyngeal region and are involved in breathing and swallowing. The position of the soft palate is coordinated by 11 different muscles and both respiratory and non-respiratory functions such as swallowing, are controlled by the coordinated activity of all muscles (Series, 2002). The airway or pharynx is maintained in an open state by the action of the pharyngeal dilator muscle. The movement of air over the surface of the pharynx and increases in lung volume also aid in maintaining a patent airway (Schwab, 2005; Series, 2002). As the pharynx does not contain any bony structures for support, the activity of these muscles is crucial for the maintenance of a patent airway.

A loss of tone of the dilator muscles, and soft tissue of the pharyngeal wall including the uvula (soft tissue which hangs from the posterior edge of the soft palate at the back of the
throat), causes the muscles to collapse and block the airway when the individual is supine (Figure 1.1). The airway, however, is not completely blocked, but its size is reduced and a much smaller volume of air passes at a much higher resistance during inspiration and expiration (Young et al., 1993). Enlargement of the adipose tissue surrounding the upper airway muscles, known as pharyngeal fat pads, are also a predisposing factor of OSA (Al Lawati et al., 2009; Schwab, 2005). Changes in craniofacial structure occur during sleep when the mandible relaxes and becomes shorter, reducing the space for the surrounding soft tissue structures, such as the tongue, to rest. The tongue and lateral pharyngeal walls become enlarged in the limited space, and the walls of the pharynx repeatedly collapse, making breathing during sleep difficult (Schwab, 2005). Factors predisposing individuals to OSA may be a combination of genetic and behavioural, however, the basis of disease is an abnormal physiology of the upper airway.

### 1.3.2 Risk factors of OSA

Risk factors associated with OSA range from physical (reversible), genetic and other irreversible factors. Obesity is the only major reversible risk factor associated with OSA. A large neck circumference and skin folds present due to excess body fat, puts pressure on the upper airway and reduces the size of the pharynx during sleep. Individuals predisposed to seasonal rhinitis are at a higher risk of developing OSA and the AHI is higher in patients with rhinitis, suggesting that symptoms such as nasal congestion may play a role in OSA (Al Lawati et al., 2009). Smoking also increases the risk of OSA, possibly due to inflammation of the upper airway and the instability caused to sleep (Al Lawati et al., 2009). Alcohol may also increase upper airway resistance during sleep and exacerbate OSA symptoms (Young et al., 1993).

#### 1.3.2.1 Age and sex

Advanced age and the male sex are also risk factors with 4% of males aged 30 – 60, and 2% of women of the same age group affected by OSA (Young et al., 1993). Males tend to carry excess body fat in the upper body and have larger upper airway soft tissue structures than females. The incidence of OSA is thus increased in males, compared to females who have a predominantly lower body fat distribution (Al Lawati et al., 2009). With fat loss, neck circumference may be reduced, placing less pressure on the upper airway and allowing air to pass through the pharynx unobstructed. There is, however, an increased risk of OSA seen in post-menopausal women, irrespective of age or other risk factors. It may be that female
Figure 1.1: Muscles of the upper airway involved in OSA. Reproduced from Victor (1999).

Normal airway (left) and abnormal airway showing obstruction during sleep (right). A loss of tone in the muscles of the soft palate may obstruct the airway when the individual is supine.
hormones play a role in protecting woman against OSA (Al Lawati et al., 2009; Launois et al., 2007). It is difficult to estimate the effects of ethnicity on predisposition to OSA due to differences in environmental risk factors such as diet and lifestyle (Young et al., 1993).

1.3.2.2 Genetic factors

Although there is no direct association between genetic factors and OSA, these factors do determine the physiology of the upper airway. Deposits of adipose tissue in specific areas of the body is determined by genetic factors, and enlarged pharyngeal fat pads may increase the risk of OSA (Schwab, 2005). Differences in craniofacial structure between various ethnicities and/or people, such as the size and shape of the mandible and surrounding soft tissue structures, can reduce the size and therefore quality of the airway (Al Lawati et al., 2009; Schwab, 2005). The thickness of the tongue and pharyngeal walls can also reduce airway quality and predispose individuals to OSA (Schwab, 2005). OSA has been observed at a higher incidence in African Americans and Asians at a given body mass index (BMI) (Al Lawati et al., 2009).

1.3.3 Incidence of OSA

OSA occurs in 2 – 4% of the developed world, and even with a wide, albeit general, range of symptoms, 1 out of 5 adults will show minimal symptoms or be asymptomatic (Young et al., 1993). These cases cause a large burden of morbidity. Childhood OSA occurs in 2 – 3% of healthy children (Friedman and Goldman, 2011), whilst 5% of adults in the developed world are expected to have undiagnosed OSA (Young et al., 1993). With the increasing prevalence of obesity in the general population, it is likely that there will be an increased incidence of OSA with subsequent public health burdens also increasing over time (Young et al., 1993).

1.3.4 Symptoms and consequences of OSA

OSA is described as strenuous breathing due to the gasping or choking sounds which occur as attempts are made for breathing to resume after a pause in breath. Snoring is common in OSA, and sufferers may be roused during sleep when gasping for breath. Daytime sleepiness or fatigue results from interrupted sleep, and may be responsible for workplace and/or motor vehicle accidents (Akerstedt et al., 2002). A recent review of the literature showed that depression is seen in 5 – 63% of OSA sufferers and that the incidence of depression is higher in OSA sufferers than within the general population (Ejaz et al., 2011).
Alterations in mood may result in a decreased sex drive and overall poor quality of life, thus exacerbating depression (Ejaz et al., 2011; Malhotra and White, 2002).

The sympathetic nervous system is activated by repeated arousals from sleep, which may cause irreversible neuronal damage when prolonged periods of hypoxia are repeatedly experienced during sleep (Ejaz et al., 2011). Activation of the sympathetic nervous system also results in sleep fragmentation, and hypoxia of the brain and heart (Malhotra and White, 2002; Young et al., 1993). Hypoxia of the brain may result in decreased neurocognitive function and impairment of memory and attention (Bawden et al., 2011), and clinical manifestations include loss of grey matter in areas of the brain responsible for cognitive function, memory processing and learning (Morrell et al., 2003).

Loss of sleep caused by OSA can alter monocyte proinflammatory cytokine responses and result in autoimmune disorders such as diabetes mellitus, arthritis and cardiovascular disease through the effects on the production of Interleukin-6, TNF-alpha and C-reactive protein, respectively (Irwin et al., 2010). OSA is an independent risk factor for enhanced platelet activation and aggregation (Kondo et al., 2011), hypertension, and therefore cardiovascular morbidity (Maruyama et al., 2012; Wolk et al., 2003; Young et al., 1993; Young et al., 2002). OSA is also an independent risk factor for atherosclerosis, which increases the risk of myocardial infarction or stroke (Drager et al., 2007). Cardiovascular events may result due to hypoxia of the heart and an increase in C-reactive protein, a biomarker for systemic inflammation, and atherosclerosis which may occur independently of other risk factors such as smoking, diabetes or hypertension. Other clinical manifestations of OSA include hypertension due to decreased circulating oxygen (hypoxaemia) and increased CO$_2$ circulating in the blood (hypercapnia) (Young et al., 1993).

Symptoms of OSA are exacerbated with the use of sedatives, anaesthetics and pain relief medication. Under general anaesthesia, arousal responses may be slowed (Ankichetty et al., 2011), and snoring during intubation may cause uvular oedema and result in postoperative complications (Ankichetty et al., 2011; Ejaz et al., 2011). OSA sufferers may also be at an increased risk of type II diabetes and stroke, likely to be associated with obesity and hypertension (Fredheim et al., 2011; Malhotra and White, 2002; Olsen et al., 2008). It is possible to reverse the clinical consequences of OSA with adequate treatment. The first step, however, is to establish the cause of symptoms.
1.3.5 Diagnosis of OSA

Diagnosis of OSA is initially based on observed symptoms, usually by the bed partner. A history of snoring, restless sleep and choking sounds may only be apparent to the bed partner, as the patient may be unaware of such events occurring throughout the night. Physical examination to measure neck circumference and BMI are typically performed, however definitive diagnosis is usually made via overnight polysomnography (PSG), or CT or MRI whilst the patient is awake, to examine the size of the upper airway. PSG serves as the standard diagnostic test for OSA and measures the number of apnoeas and hypopnoeas per hour of sleep, providing diagnosis based on an apnoea-hypopnoea index (AHI) (Olsen et al., 2008; Punjabi, 2008). Diagnosis of OSA is usually considered if a patient displays >5 respiratory events per hour sleep with concurrent symptoms of excessive daytime sleepiness, snoring, and arousals from sleep due to choking. An AHI>15 per hour sleep in the absence of other symptoms, is also considered as a positive OSA diagnosis (Olsen et al., 2008).

PSG is a test typically performed overnight in a sleep clinic for the diagnosis of sleep apnoea. It uses electroencephalogram, electromyogram, electrooculogram, oronasal airflow, and oxyhaemaglobin to measure physiological signals during sleep and determine the AHI, and snoring is recorded with a microphone (Shiomi et al., 2011). An apnoea occurs when breathing ceases for at least 10 seconds and it is at this point when the apnoea is classified as obstructive or central, based on whether effort is employed for breathing to resume. A hypopnoea is measured when a 25 – 50% reduction in oronasal airflow is observed, in addition to a reduction in oxyhaemoglobin saturation or with arousal from sleep (Punjabi, 2008). A patient displaying AHI<5 per hour is considered normal, an AHI of 5 – 14 is considered to be mild OSA, an AHI of 15 – 29 is moderate OSA, and >30 events per hour is classified as severe OSA (Ejaz et al., 2011). An AHI>5 with concurrent excessive daytime sleepiness were once the only symptoms used for diagnosing OSA. However, due to the increased risk of life-threatening cardiovascular events, some sufferers who record an AHI>5 with the absence of daytime sleepiness are also classified as having OSA (Ejaz et al., 2011).

Overnight PSG is quite labour intensive and requires patients to stay overnight in a sleep clinic. Qualified personnel are required to carry out testing and interpret results, with some symptoms such as snoring counted manually by a sleep technician. Such observations may be subjective, especially in instances when severity of snoring is reported by sleep technicians (Shiomi et al., 2011). Inconsistencies between different sleep labs with regard to data collection and interpretation, may make it difficult to study trends amongst various populations (Punjabi, 2008).
1.3.6 Treatment of OSA

It is well established that OSA has severe and potentially life threatening clinical consequences if left untreated. There are a number of treatment options which have been proven to be effective in reversing symptoms and clinical manifestations of OSA. Treatments range from mechanical devices which are used to regulate the pressure within the upper airway during sleep, oral devices to maintain an open airway, surgery, medications, and behavioural modifications which may aid in the reduction of OSA symptoms. Despite studies which have shown promising success rates for some treatments, patient compliance is usually the determining factor of individual success. Many patients will initially accept and adhere to the use of daily treatments used during sleep, but after a period of time, patients cease use and symptoms return. Some patients may not be good candidates for other methods of treatment such as surgery, or may be unwilling to adopt certain behavioural modifications. Treatment options currently available will be discussed.

1.3.6.1 Continuous positive airway pressure (CPAP)

Once OSA is confirmed by PSG, the treatment of choice by sleep specialists to reduce the AHI to below 5 and control OSA symptoms, is the continuous positive airway pressure (CPAP) device (Kushida et al., 2005). The device consists of a mask worn over the nose or face, depending on patient requirements, and connected to a pump which regulates pressure within the nasopharynx (Figure 1.2) (Bakker et al., 2012). A fixed air pressure, determined during a titration PSG, is applied and acts as a pneumatic splint to prevent the collapse of the muscles of the upper airway (Malhotra et al., 2000; Olsen et al., 2008). The patient is able to breathe normally while the CPAP device is in use, and the symptoms of snoring and choking arousals are eliminated. It is well documented that consistent CPAP use is able to decrease the AHI to less than 5 events per hour, decreases daytime sleepiness, improves oxygenation and cognitive performance and clinical manifestations may also be reversed (Aloia et al., 2003; Cheng et al., 2011; Ejaz et al., 2011). Improvements in hypertension are also seen with the reduction of autonomic activity during sleep (Thomas et al., 2005).

1.3.6.2 CPAP compliance

Although CPAP is non-invasive and its use has been shown to improve symptoms of OSA, it is estimated that 50% of patients who are prescribed this therapy, are non-compliant after 1 year (Ballard et al., 2007). The treatment is not widely accepted due to the uncomfortable nature of the device. The mask may restrict mobility during sleep, while the volume of the
Figure 1.2: CPAP device showing mask secured over the nose and mouth. Reproduced from Ejaz et al. (2011).
pump during operation may keep the individual, as well as other(s) sleeping in the same area, awake. There may also be problems with spontaneous intimacy with the bed partner while the device is in use (Zozula and Rosen, 2001). The mask can cause irritation to the face, stiffness of the nose, and/or claustrophobia, and air leaks around the mask may reduce its effectiveness (La Piana et al., 2011; Zozula and Rosen, 2001). Unfavourable effects from the device have been reported in 15 – 45% of patients (Zozula and Rosen, 2001). Modifications may be made to the device to assist in comfort, such as the use of humidifiers, sponge pads placed on the bridge of the nose to cushion the face, and pumps with reduced noise output (Malhotra et al., 2000). However, adherence rates have not increased at the same rate as the improvements made to these newer machines, possibly indicating that compliance is not solely based on mechanical issues with the CPAP device.

Compliance is defined as use of the CPAP device for at least 4 h per night on at least 70% of nights over 3 months (Aloia et al., 2003). Of the patients who are prescribed CPAP treatment and take it home, only 50 – 75% actually use the treatment effectively (Zozula and Rosen, 2001), and the suboptimal rate of CPAP adherence is a major cause of OSA treatment failure (La Piana et al., 2011; Olsen et al., 2008). Since CPAP compliance does not seem to be affected by the side effects from the mechanical parts alone, there may be psychological and motivational issues involved in adherence (Olsen et al., 2008).

Social support seems to be important in patient adherence to CPAP use (Olsen et al., 2008). Training sessions to educate patients and their bed or living partners on the benefits of adherence, and familiarisation with the device has been effective in improving compliance rates. However, re-training may be required after 1 year in order to ensure compliance long-term (La Piana et al., 2011; Smith, S. et al., 2004). It has been shown that CPAP compliance is high in married couples, and low in those who live alone or are working, thus indicating the importance of support in the form of encouragement of CPAP use from a living partner (Gagnadoux et al., 2011). Involvement of friends or co-workers may provide a strategy to improve compliance in unmarried patients. It is especially important for sufferers who are working to have social support, as excessive daytime sleepiness may result in occupational or motor vehicle accidents (Akerstedt et al., 2002). Often, those who travel for work or have competing interests will prioritise work over CPAP adherence (Gagnadoux et al., 2011). As compliance of CPAP use is low, and consistent use of treatment is critical in reversing OSA symptoms, there are other therapeutic options for patients who are not willing to use CPAP.
1.3.7 Other positive airway pressure devices

Bi-level positive airway pressure (BPAP) devices can be used to deliver different inspiratory and expiratory pressures, which allows for less effort for expiration compared to CPAP. However, these devices are expensive and studies have indicated that CPAP is more effective in the treatment of OSA (Ballard et al., 2007; Reeves-Hoche et al., 1995). With adherence rates being generally similar between the two devices, CPAP is the superior option, therefore BPAP is rarely prescribed (Malhotra and White, 2002).

Autotitrating devices deliver variable pressure in a similar manner to BPAP, but is determined by factors such as snoring and flow limitation (Malhotra and White, 2002). Autotitrating devices have been used for more hours per night than CPAP, but overall, long term adherence rates were similar, as were the effects of treatment on OSA symptoms (Hudgel and Fung, 2000).

Expiratory positive airway pressure (EPAP) nasal devices are a disposable stand-alone device in the form of an adhesive patch worn over the nares. A valve, placed over the centre of the nares allows minimal resistance of air through the nose during inspiration and increases air flow resistance during expiration. Expiration pressure is created and time of expiration is longer, two factors which may stop the airway from collapsing, however the precise mechanism of this is unclear (Walsh et al., 2011). EPAP is not recommended as a stand-alone treatment for individuals who are non-adherent to CPAP, however it may be used in addition to other treatments for OSA.

1.3.8 Alternative treatment options

Other treatment approaches to reducing the symptoms of OSA include weight loss to reduce neck circumference, restriction of alcohol consumption and use of sedatives to prevent supine sleep (Ankichetty et al., 2011; Malhotra and White, 2002). Airway size may be enlarged through tonsillectomy or nasal steroids may be used to decrease inflammation of the airway in patients who suffer from chronic rhinitis (Friedman and Goldman, 2011). Long term outcomes of steroid use are still unknown.

1.3.8.1 Oral appliances

Oral appliances are recommended for patients who have mild OSA or primary snoring, and who do not meet the criteria or do not respond to treatment with behavioural modifications
such as weight loss. They can be used in patients with moderate to severe OSA who cannot use CPAP either because of non-compliance or intolerance (Sari and Menillo, 2011). Oral appliances can be used to maintain an open pharyngeal airway by preventing the tongue and surrounding tissue from collapsing and obstructing the airway.

The mandibular advancement splint (MAS), is a non-adjustable block which rests on the mandible and retains it in an extended position during sleep. Titratable oral appliances perform as a splint and also create a space between the back of the tongue and the posterior pharyngeal wall, effectively inhibiting the tongue from blocking the airway when the patient is supine (Figure 1.3) (Sari and Menillo, 2011). Compliance of such devices is usually poor as they cause significant discomfort (Malhotra and White, 2002).

1.3.8.2 Surgery

If patients refuse to adhere to, or fail with CPAP and/or oral devices, another option for treatment of OSA is surgery of the upper airway. Surgical procedures involve removal of redundant soft tissue, which is causing blockage of the upper airway. The most common procedure is uvulopalatopharyngoplasty (UPPP), in which the soft tissue of the lateral pharyngeal walls and the uvula (fleshy part which hangs from the roof of the mouth at the back of the throat), are resected to increase the size of the airway. Aside from the invasive nature of this procedure and possible postoperative complications, about 41% of patients only see a reduction of the AHI to less than 20 events per hour sleep (Malhotra and White, 2002), which is still classified as moderate OSA. Symptoms of snoring may cease, however, sleep disordered breathing continues in many cases, leading to silent sleep apnoea (Malhotra and White, 2002). There are instances where modified UPPP surgery in small test groups (N=<50) has been effective at improving patient AHI (Mackay et al., 2013). Laser-assisted uvulopalatoplasty (LAUP) is usually performed to relieve symptoms of snoring, but has been used in the treatment of OSA to increase the size of the pharyngeal airway (Olszewska et al., 2012). Under local anaesthesia, the laser causes targeted thermal tissue damage to create vertical channels in the soft palette, thus widening the airway. This treatment may be required prior to UPPP surgery. Although the AHI may be reduced by as much as 50% after surgery, this may still not completely reverse symptoms in patients with severe OSA (Ejaz et al., 2011; Olszewska et al., 2012).

Maxillomandibular advancement (MMA) is used to lengthen the upper (maxilla) and lower (mandible) jaw as much as 9 mm and 12 mm, respectively, and increase the calibre of the upper airway (Hsieh and Liao, 2012). The procedure may create cosmetic changes, and little
Figure 1.3: Oral appliances for the treatment of OSA. Reproduced from Sari and Menillo (2011).

Titratable oral appliance (top) and the mandibular advancement splint (MAS) (bottom).
attention has been paid to the effects on the surrounding structures such as the skeleton and upper airway post-surgery. It is thus difficult to determine whether improvements to AHI are due to changes in the upper airway tissues or structure of the jaw (Hsieh and Liao, 2012). It has been shown to improve the AHI to a greater extent than UPPP, although scores remain above the threshold of AHI<5 (Blumen et al., 2009; Boyd et al., 2012). MMA in conjunction with UPPP may alleviate OSA symptoms, however patients of one study reported post-operative complications such as difficulty swallowing and numbness of the lower lip for two years post-procedure (Liu et al., 2012).

Surgery is an invasive procedure for which some patients may not be suitable candidates, or may be reluctant to undergo due to associated health risks. In obese patients with OSA there is a risk of perioperative morbidity associated with surgery (Gali et al., 2009). There is a significant risk associated with extubation after general anaesthesia which may result in oedema and collapse of the soft tissue or damage to the uvula, as well as risks associated with pharyngeal tone and arousal responses, which may be diminished after use of anaesthesia (Cavallone and Vannucci, 2013; Kaw et al., 2006).

1.3.8.3 Pharmacotherapy

Even with CPAP use, some sufferers may continue to experience daytime sleepiness. Whilst there is no effective drug treatment currently available for the treatment of OSA, drugs are available to reduce symptoms of daytime sleepiness with concurrent CPAP use. Madofinil, a wakefulness promoting drug has been approved by the U.S. Federal Drug Administration (FDA) for the treatment of narcolepsy, and has also been used to relieve residual daytime sleepiness in patients using CPAP (Darwish et al., 2010; Weaver et al., 2009). Armodafinil is the R-isomer of racemic Madofinil, and has a longer half-life. Both drugs have been used for the treatment of CPAP-treated OSA, however symptoms of increased blood pressure and chest pains have been noted for both medications (Black et al., 2010; Darwish et al., 2010).

1.3.9 Treatment of OSA in children and the elderly

OSA in children is usually caused by enlarged tonsils which obstruct the airway and may cause the airway to collapse during sleep. The resulting symptoms are similar to those seen in adults, with neurocognitive and behavioural dysfunction predominating, and may include failure to thrive (Friedman and Goldman, 2011). Surgical removal of the tonsils and adenoids is used in the treatment approach; however 20% of children who undergo adenotonsillectomy suffer residual OSA after surgery. It is also possible that tonsils may
regrow after surgical removal, thus anti-inflammatory treatment or the use of steroids delivered orally or intranasally, may be a better option to reduce adenotonsillar size and relieve the symptoms of OSA in children (Friedman and Goldman, 2011).

OSA in the elderly (beyond age 60) may show signs of involuntary urination, nocturia, ophthalmic conditions and recurring falls due to disturbances in neurocognitive function. CPAP is the treatment of choice in the elderly (Launois et al., 2007).

1.4 Complex sleep apnoea

Complex sleep apnoea (CompSA) is described as the presence of apnoea-hypopnoea events in OSA patients, even once a patent airway is restored with CPAP treatment. Patients usually present as having OSA, however treatment with CPAP may not resolve SDB. The persistent events are central, rather than obstructive and are due to highly sensitive chemoresponses leading to unstable ventilatory control, as in the case of CSA (Kuzniar and Morgenthaler, 2008). Symptoms may improve over time with CPAP use; however the mechanism for this remains unclear. There are conflicting reports on the success of CPAP treatment in CompSA with some cases reporting no change or worsening of symptoms with CPAP use, therefore other methods of treatment are generally recommended (Gilmartin et al., 2005). Adaptive servo-ventilation (ASV) used in the treatment of CSA, and non-invasive positive pressure ventilation (NPPV) treatments have been shown to improve nocturnal breathing in CompSA patients with Cheyne-Stokes respiration, with ASV being a promising first candidate for treatment of CompSA (Allam et al., 2007; Morgenthaler et al., 2007; Ramar et al., 2012; Willson et al., 2001).

1.4.1 Costs associated with diagnosis and treatment

Costs associated with PSG for the diagnosis of OSA have increased over time in Australia. From 1995 – 2004, the Australian Medicare system has reimbursed patients AUD$162.8 million for 417,533 PSG, with the annual cost of PSG increasing from AUD$9.2 million in 1995 to AUD$26.7 million in 2004, and the demand for PSG per 100,000 between 1995 and 2004 rising 150% above population growth (Marshall et al., 2007). Despite the increase in cost of PSG over time, it only accounted for 0.29% of the total Medicare budget in 2004, and it remains the gold standard diagnostic test for SA today.
The costs to the Australian government associated with surgery between 1995 and 2007 was AUD$8.2 million for almost 20,000 UPPP procedures, and AUD$1.1 million on just over 3000 LAUP (laser-assisted) (Marshall et al., 2010).

With the increasing costs to the public health system and the rising trend of OSA associated within the population and subsequent increase in cases of OSA, a more widely accepted and cost-effective treatment option is required.

1.5 Proposal of a novel therapeutic for OSA

Currently available treatment options for OSA have poor compliance rates due to significant impact on lifestyle or comfort, in the case of CPAP and oral devices, or increased risks associated with side effects in the case of surgery and pharmacotherapy. In obese patients, weight loss may improve SA symptoms, however not all patients are able to achieve significant weight loss in order to reverse symptoms of OSA. It is clear from the evidence presented, that a novel therapeutic approach to the treatment of OSA that is likely to be accepted by sufferers, has minimal impact on lifestyle and comfort, and is cost effective to patients and the government, is required.

The underlying cause of OSA is the loss of muscle tone in the upper airway. A rational approach to correcting this problem is to increase the tone of these muscles, thus opening the airway and allowing the un-obstructed passage of air during nocturnal breathing. This is the rationale behind surgery, however due to the invasive nature of the procedure and the difficulties of performing it on obese individuals, a less invasive procedure that will be more widely accepted and elicit the same results, is necessary. A novel approach to the treatment of OSA to increase muscle tone in the upper airway involves the targeted delivery of bacterial toxins which cause muscle contraction.

An agent known to increase muscle tone is tetanus neurotoxin (TeNT). This neurotoxin, formed in nature by the bacterium Clostridium tetani, acts to inhibit muscle relaxation, effectively paralysing the muscle in a rigid state. This event is termed tetany. Controlled administration of tetanus toxin to induce localised tetany in the area of the upper airway is a feasible approach to the treatment of OSA. Targeted delivery of the toxin in appropriate doses may increase muscle tone in the immediate area of administration without causing any adverse or widespread reaction to the individual. In the section to follow, the current use of tetanus toxin and the related botulinum type A neurotoxin (BoNT/A) will be discussed, and their potential to treat the cause and symptoms of OSA will be explored.
1.5.1 Genus Clostridium

Clostridia are gram positive, spore-forming, obligate anaerobic bacilli found widespread in the environment, and are responsible for causing significant infection in humans and animals. Clostridia have played a significant role in both human and animal infections with some members of the genus responsible for colitis in humans (C. difficile), food poisoning in humans and necrotic enteritis in poultry (C. perfringens), wound infections in humans (C. sordellii) and gas gangrene (C. novyi, C. septicum and C. perfringens). In all cases, organisms cause disease through the production of toxins. Two species of great interest to the scientific community are Clostridium tetani and Clostridium botulinum, the toxins of which cause tetanus and botulism, respectively. These organisms are able to survive harsh environments by laying in a dormant state within spores which are resistant to extreme conditions. Due to their broad distribution, it is not uncommon for these organisms to contaminate foods and enter the body through abrasions and wounds. Once a spore enters the body, the host conditions allow the spores to germinate and produce live vegetative cells which are then free to produce toxin. The group of toxins produced by these bacteria are known as the family of Clostridial neurotoxins (CNTs) (Hatheway, 1990).

1.5.2 Disease caused by C. tetani and C. botulinum

1.5.2.1 Tetanus caused by C. tetani

Tetanus was first reported by the Greeks more than 20 centuries ago and was initially thought to be a condition of the nervous system. In 1891, the bacterium was isolated, characterised and named Clostridium tetani, from the Greek word tetanos, meaning “contraction” (Kitasato, 1891). The bacterium itself is predominantly found in soil and is widespread in the environment and the intestines of mammals. Spore formation allows C. tetani to survive harsh environmental conditions such as dry heat and high moisture, and also gives C. tetani cells their drumstick appearance. Disease occurs when the spores enter a wound, gaining entry from an infected article, most commonly a rusty nail, or through contact with soil or manure. Spores may also gain entry through burns, ulcers, fractures, operative wounds or during intravenous drug use. The low oxygen environment of a wound promotes the germination of the spores to form active bacterial cells which then produce a toxin called tetanospasmin, producing the effects of tetanus. The toxin may lay dormant for a period of a few days to weeks after exposure and generally, the shorter the incubation period, the more severe disease which results (Hatheway, 1990).
A lesion formed in the infected area usually goes unnoticed, as the wound from which the spores gained entry is usually still evident, with the first symptoms being sporadic contractions of the muscles surrounding the wound site. The toxin then travels in a retrograde fashion from the axon terminals to the CNS, where it acts on nerves to continuously activate the contraction of muscles. Disease progression is demonstrated by spasms in facial and neck muscles, leading to trismus or lockjaw, where the muscles of the jaw become rigid due to the constant firing of nerve impulses which contract the muscles. Eventually, these muscle spasms spread down the torso and then very quickly throughout the body, giving way to generalised tetanus. If left untreated, death results from the paralysis of the muscles required for breathing (Hatheway, 1990).

Neonatal tetanus mainly occurs from infection of the umbilical stump and usually occurs in third world countries where an infected instrument is used to cut the umbilical cord, or infected material is used to cover the stump (Hatheway, 1990). Initially, neonates are able to feed and cry normally in the first few days after birth, but then lose these abilities after a few days, and become rigid and suffer from spasms (WHO, 2006).

1.5.2.2 Botulism caused by C. botulinum

Botulism has been documented since the 18th century and was first referred to as “sausage poisoning”. In 1882 Justinus Kerner published the first correctly documented cases of foodborne botulism with associated neurological symptoms (Hatheway, 1990), however, it wasn’t until 1949 when Burgen and colleagues discovered that BoNT blocked neurotransmitter release (Burgen et al., 1949).

There are various ways in which an individual may develop botulism. Disease may occur from infection with preformed toxin, or infection with the organism itself which produces toxin after growing in the gastrointestinal tract or a wound. In all cases of botulism, the toxin travels from the small intestine to the circulation via the lymphatic system, where it can then act locally on the peripheral nervous system to inhibit the neuronal signals that activate muscles. A flaccid muscular paralysis results within 24 h of toxin entry, with paralysis initiating at the head and descending symmetrically, affecting the muscles involved in breathing. Flaccid paralysis caused by BoNTs occurs by blocking the release of synaptic vesicles containing acetylcholine from motorneurons.

Foodborne botulism is the most common form of botulism and occurs by the ingestion of food containing either preformed toxin, or the organism which then produces toxin in the gut,
with symptoms including nausea and vomiting (Hatheway, 1990). Foodborne botulism is most commonly contracted from home-preserved vegetables or fish which have been inadequately prepared, with the food rarely appearing spoiled (Davletov et al., 2005; Hatheway, 1990). The cooking process kills vegetative cells, however botulinal spores which are resistant to high temperatures survive the cooking process, and germinate if kept at the optimal temperature (Hatheway, 1990).

Foodborne botulism occurs via toxin entry through the gut, and in order to avoid degradation within the acidic environment of the stomach, BoNTs are produced along with a complex of proteins which protect the toxin from the harsh environment. When present in a high dose, ingestion of pure toxin is able to cause disease, however most frequently the toxin is ingested as a progenitor toxin complex (Maksymowych et al., 1999). The toxin complex consists of the BoNT toxin, haemagglutinin (HA) proteins and non-toxic non-haemagglutinin (NTNH) proteins which are non-covalently associated with the toxin. These proteins ensure that BoNT is not degraded by the acidic environment of the stomach, which would otherwise degrade the toxin after direct ingestion from food or after toxin production within the gut (Hatheway, 1990; Simpson, 2004). For certain strains of *C. botulinum*, however, the toxin is released in its single-chain inactive form and the proteases present in the stomach convert it into the active di-chain form (Simpson, 2004).

Wound botulism although rare, may occur in intravenous drug users or after bone fracture when the organism gains entry to a wound and produces toxin. The resulting disease is the same as foodborne botulism and the method of intoxication of the wound is similar to that observed for *C. tetani* (Hatheway, 1990; Schiavo et al., 2000).

Inhalational botulism results from the aerosolisation of BoNT and is a risk to workers handling the toxin and as an agent of bioterrorism (Simpson, 2004). Both the progenitor toxin complex and pure BoNT are able to bind to airway epithelial cells and cross into the circulation via transcytosis to cause disease (Park and Simpson, 2003). Although the toxin is unstable in aerosols, there have been attempts to use BoNT as a bioweapon in Japan in the early 1990's (Arnon et al., 2001), and in Iraq during the Gulf War (Patocka et al., 2005).

Infant botulism occurs after the ingestion of spores. In adults, even if spores are ingested and are able to germinate, it is difficult for *C. botulinum* to compete for an adequate niche and colonise the gastrointestinal tract. In infants <1 year of age however, where the gut flora has not yet been established, *C. botulinum* may colonise the intestinal tract. Toxin produced in the gut is then absorbed into the bloodstream and causes botulism. Initial signs of infant botulism are infrequent bowel movements and constipation, followed by loss of head control,
lethargy, difficulty swallowing and feeding, and an altered cry. Most commonly, botulinal spores are ingested from honey, thus it is advised that honey should not be fed to children younger than 1 year of age (Hatheway, 1990; Simpson, 2004).

1.6 Diagnosis and treatment

1.6.1 Tetanus

Due to the unique nature of clinical symptoms of tetanus, diagnosis of tetanus is usually based purely on observation. The presence of *C. tetani* may be confirmed by isolation of the organism from the wound, however this is usually not required as it takes 48 h for initial growth to be seen in culture and most standard biochemical tests are negative (Hatheway, 1990).

Initial treatment of tetanus is immunotherapy with human tetanus immunoglobulin which neutralises the tetanospasmin toxin. If the vaccination status for the individual is unknown or incomplete, patients are treated as non-immune and are given the tetanus vaccine immediately (Miyagi and Shah, 2011). A 114 kDa collagenase (ColT) protease is expressed by *C. tetani* along with TeNT and catalyses the cleavage of collagen to aid in tissue damage during infection (Bruggemann and Gottschalk, 2004). Surgical debridement may therefore be required to remove infected tissue in an attempt to limit the spread of infection (Trujillo *et al*., 1987). Muscle relaxants and sedatives may also be used in the treatment of tetanus, and antibiotic therapy may be used to limit further progression of disease. Without hospitalisation to manage the symptoms of disease, mortality from tetanus is usually 100% (WHO, 2006).

1.6.2 Botulism

Culture methods are rarely used today due to time limitations, with *C. botulinum* requiring 5 days growth under strict anaerobic conditions. Botulism can be diagnosed by the detection of toxin from the faces, serum, gastric contents or by the isolation of *C. botulinum* bacteria from the faeces or wound (Hatheway, 1990). The standard method of detection of BoNT in food was once the mouse bioassay, with diluted samples injected into mice to observe symptoms of disease. However, in an attempt to move away from *in vivo* diagnosis, *in vitro* assays have been developed for toxin detection (Stanker *et al*., 2008). Neutralisation bioassays employing ELISA are generally not as sensitive as the mouse bioassay, but may be ideal for same-day screening of samples (Kegel *et al*., 2007; Stanker *et al*., 2008). PCR methods may be used, however sample enrichment is usually required prior to performing PCR (Dahlenborg *et al*.,
2001; Lindstrom et al., 2001). In cases where in vivo assays are required to observe systemic effects and/or for high sensitivity, assays involving animals may be required. Such assays may be lethal (observation of systemic paralysis), or non-lethal (local paralysis), with the former being the most sensitive (Lindstrom and Korkeala, 2006).

Treatment of botulinum poisoning can be performed before or after exposure, by targeting any one of the three key steps involved in intoxication (Swaminathan, 2011). Despite its therapeutic benefits, botulinum toxins are still considered potential biological warfare agents, thus treatment options remain an important area of research.

1.6.2.1 Antitoxin treatment of botulism

Botulism is still considered a candidate for bioterrorism, thus appropriate measures for treatment are required (Middlebrook, 2005). A heptavalent antitoxin produced by the CDC in the U.S.A. contains equine-derived, neutralising monoclonal antibodies against all seven types of botulinum toxins (BoNT/A – /G). This antitoxin therapy has recently replaced the bivalent AB antitoxin and monovalent BoNT/E antitoxin (Mayers et al., 2001). Horses are used for the production of antitoxin antibodies as they are able to produce a large volume of blood in a short amount of time (Mayers et al., 2001). The efficacy of antitoxin production is limited by the risks associated with the use of heterologous preparations. Heterologous immunoglobulins, which are recognised as foreign by the human immune system may cause undesirable side effects such as serum sickness, which may lead to anaphylaxis. However, antitoxin therapy shortens the time of illness and reduces the risk of mortality from botulism and is thus still a standard method of treatment (Mayers et al., 2001).

Treatment for infant botulism continues to use a human-derived bivalent antitoxin, BabyBIG (botulism immunoglobulin), for disease caused by BoNT/A and /B (CDC, 2010; Chalk et al., 2011).

1.6.2.2 Peptide inhibitors

There is no pharmacotherapy currently available for the prevention or treatment of botulism, but attempts have been made to create peptide inhibitors of BoNT/A to inhibit the activity of the light chain (LC) which is responsible for the paralytic effects seen in botulism (Li, B. et al., 2011; Silvaggi et al., 2008). Phage display has been used for the in vivo screening of peptide inhibitors against BoNT (Zdanovsky et al., 2001), as well as in vitro mRNA display, which identified peptide inhibitors that could inhibit the activity of the LC at a much lower
concentration than those identified by the former method (Yiadom et al., 2005). Peptide inhibitors which are able to inactivate circulating BoNT/A after poisoning may replace heterologous antitoxin therapy as the standard method of treatment for botulism.

1.6.2.3 Neutralising antibodies

The identification of neutralising antibodies is an important step in the development of monoclonal antibody/antitoxin treatment against BoNTs, and may also be useful for vaccine development. Phage display comparing human immune and non-immune sera (Amersdorfer et al., 2002), and a yeast display library (Levy et al., 2007), have been used to identify previously unknown neutralising epitopes of BoNT/A. These epitopes may be used in the development of human antibodies to replace the equine-derived antibodies currently used in the treatment of BoNT/A infection (Mayers et al., 2001).

1.7 Prevention

Clostridia typically have a high AT content which can impact expression of genes in a heterologous host, and so native toxin is used in the preparation of toxoid vaccines (Hatheway, 1990). Such methods pose a health risk to workers involved in preparing these vaccines, thus new strategies for vaccination against CNTs are under investigation. Although tetanus and botulinum toxins have therapeutic potential, vaccines are still required as they continue to be a cause of life-threatening disease.

1.7.1 Tetanus toxoid vaccine

Tetanus is successfully controlled through administration of a tetanus toxoid vaccine which induces humoral immunity. Formaldehyde treatment of the native toxin renders it non-toxic, however the conformation of the TeNT holotoxin remains intact, preserving its immunogenicity (Behrensdorf-Nicol et al., 2008). Three doses of tetanus toxoid are required to achieve protective levels of circulating antibodies in adults, and five doses are recommended for children, with a booster injection required every 10 years in order to maintain adequate levels of antitoxin antibodies (WHO, 2006). In immunised females, maternal antibodies to tetanus toxin are transmitted to the foetus via the placenta, preventing neonatal tetanus. In third world countries where immunisation rates are low, neonatal tetanus is still quite prevalent, with the World Health Organization (WHO) estimating 180,000 neonatal deaths from tetanus in 2002 (WHO, 2006). The minimum amount of circulating
antitoxin antibodies required to maintain immunity to tetanus is 0.01 IU/mL, and is
determined by ELISA, however, cases of tetanus in individuals with antibody titres above this
threshold have been reported (Crone and Reder, 1992; Livorsi et al., 2010).

The production of tetanus toxoid from native toxin poses a significant risk to workers due to
the possibility of residual tetanus toxin activity after inactivation with formaldehyde
(Behrensdorf-Nicol et al., 2008). Due to the dangerous nature of this method of vaccine
preparation, novel methods to develop tetanus vaccines are being investigated. The C-
terminal 451 amino acid fragment of tetanus toxin (H_C), termed fragment-C, is involved in the
initial binding step of the toxin to its target cell (Francis, 2000). This domain is the most
immunogenic region of the toxin, and can be prepared by recombinant methods in a
heterologous host (Qazi et al., 2006). Such methods pose minimal risks to workers, thus
fragment-C is a promising candidate for a novel, safer tetanus vaccine. Although fragment-C
has been shown to induce protective levels of antibodies to tetanus neurotoxin, it did not
produce antitoxin antibodies equivalent to the tetanus toxoid vaccine (Qazi et al., 2006).
Attempts at vaccine production involving fragment-C have been made with this domain
delivered alone (Qazi et al., 2006), or as part of a fusion molecule for the co-delivery of
another immunoprotective peptide (Boucher et al., 1994). It has also been assessed as part
of a DNA vaccine (Anderson et al., 1996), as an oral vaccine with fragment-C delivered in an
attenuated Salmonella enterica (Terry et al., 2005), and as a nasal or epicutaneous vaccine
with fragment-C delivered by an adenovirus vector (Shi et al., 2001). Efforts are ongoing,
however tetanus toxoid prepared by formaldehyde treatment of native tetanus toxin is still the
vaccine given today.

1.7.2 Botulinum vaccines

Aside from the threat of bioterrorism, botulism occurs rarely; therefore it is unnecessary to
vaccinate entire populations against the disease. BoNT types A and B have cosmetic
applications and are also used in the treatment of neuromuscular disorders, adding to the
reasons it is impractical to vaccinate against C. botulinum.

Two vaccines against botulism are licensed for use, a pentavalent vaccine which protects
against BoNT/A – /E, which is no longer available from the Centres for Disease Control
(CDC), due to issues with protective levels across all serotypes being maintained equally,
and local reactions to booster injections (CDC, 2011; Henkel et al., 2011); and a heptavalent
vaccine protecting against BoNT/A – /G, both of which are produced by chemically
inactivated BoNT purified from C. botulinum. These vaccines are reserved for workers at risk
of occupational exposure such as those who handle pure toxin during research or antitoxin
production, and military personnel who may be at risk of biological warfare (Keller, 2008; Patocka et al., 2005; Smith, T. J. et al., 2005).

Due to the complex nature of toxoid vaccine preparation, alternative approaches to vaccine production against botulism have been investigated. A recombinant heptavalent subunit vaccine has been produced which comprises fragment-C (Hc) from the seven BoNT serotypes (Baldwin et al., 2008), due to the highly immunogenic nature of this domain. A vaccine using fragment-C of BoNT/A (Clayton et al., 1995; Villaflores et al., 2013), and a trivalent vaccine encompassing fragment-C of BoNT/A, /B and /E has been shown to induce effective levels of antitoxin immunity in mice trials (Zichel et al., 2010), however, another study has shown that immunisation with a vaccine targeting one subtype of BoNT/A does not necessarily protect against other BoNT/A subtypes (Henkel et al., 2011). Sequence variability present within subtypes of the seven BoNT serotypes makes it difficult to create a vaccine using inactivated native toxin (Smith, T. J. et al., 2005). Thus, studies are underway to create a botulinum toxoid by targeted mutagenesis using recombinantly expressed BoNT sequences, thus negating the need to isolate BoNT from its native reservoir (Agarwal et al., 2005). There is evidence to suggest that BoNTs can enter the circulation via inhalation, indicating that BoNT Hc may also be a candidate for an inhalation vaccine (Park and Simpson, 2003).

1.8 Clostridial family of neurotoxins

The Clostridial neurotoxin family is comprised of eight immunologically distinct neurotoxins, tetanus neurotoxin (TeNT) produced by C. tetani, and seven botulinum neurotoxins (BoNTs), designated types A – G, produced by C. botulinum, which are the most potent toxins known to man. Botulinum toxin types E and F are also produced by Clostridium butyricum and Clostridium baratti, respectively, and Clostridium argentinense was previously known as C. botulinum type G (Ghanem et al., 1991; Hatheway, 1990). However, it is the toxins of C. botulinum which cause significant disease in humans and animals. The LD50 in humans is 1 ng/kg body weight for BoNT and 2.5 ng/kg for TeNT, and the LD50 in mice is 0.5 ng/kg for BoNT and 1 ng/kg for TeNT (Gill, D. M., 1982). The toxins of C. tetani and C. botulinum are the causative agents of the diseases tetanus and botulism, respectively.

CNTs interfere with the movement of skeletal and smooth muscle by interfering with the secretion of vesicles containing neurotransmitters at the neuromuscular junction (NMJ). All CNTs poison nerve terminals and have a similar mode of action; however TeNT and BoNT illicit opposing effects as their intracellular activity take place in different parts of the nervous
system. Tetanus is defined as spastic paralysis and occurs as a result of TeNT-mediated blockade of the release of inhibitory neurotransmitters from inhibitory interneurons in the CNS. Without a signal released from inhibitory interneurons to cease muscle contraction, skeletal muscle is continuously activated, resulting in spastic paralysis. Botulism has the opposite effect by inhibiting the release of the neurotransmitter acetylcholine (ACh) which is released from the NMJ of motoneurons in the peripheral nervous system to activate skeletal muscle. With the release of ACh blocked, the motoneuron is unable to activate the muscle and flaccid paralysis results (Rossetto et al., 2001).

1.8.1 Toxin types

The seven serologically distinct BoNT toxin serotypes are found in various reservoirs. BoNT/A, /B, /E and /F have been found in human outbreaks of botulism, whilst BoNT/C is found in birds such as ducks and poultry. Humans lack the neuronal receptor to which BoNT/D bind, rendering it ineffective in humans, but it can be found in cattle. BoNT/G is found in soil, but has also been linked to infection in humans (Davletov et al., 2005). Interestingly, rats and chickens are resistant to TeNT intoxication due to a single amino acid substitution at the TeNT cleavage site within the target substrate VAMP-2 (Kegel et al., 2007). The following section will explore the genetic characteristics of TeNT and BoNT/A.

1.8.1.1 Tetanus neurotoxin

A single serotype of tetanus toxin occurs and it is able to infect both humans and animals in a similar manner (Driemeier et al., 2007; Langner et al., 2011). The gene for tetanospasmin (tetX) is situated on a 75 kb extra-chromosomal plasmid within C. tetani, designated pE88 (Eisel et al., 1986; Finn et al., 1984), and non-toxigenic strains which lack this plasmid also exist in nature (Hatheway, 1990). The plasmid containing tetX has a 75.5% AT content and a high gene density, with 61 coding regions making up 67.1% of the plasmid (Bruggemann and Gottschalk, 2004).

The tetX gene has an AT content of 72.1% and encodes a 1315 amino acid protein with a molecular weight of 150.7 kDa, which has sequence similarity to BoNT/A, /B and /E (Eisel et al., 1986), with both TeNT and BoNT/A translated from monocistronic mRNA (Binz et al., 1990; Eisel et al., 1986). Toxin synthesis is regulated by the tetR gene which acts as an alternative RNA polymerase sigma factor. The TetR protein has a 67% similarity to the corresponding BotR/A protein expressed by BoNT/A (Bruggemann and Gottschalk, 2004; Couesnon et al., 2006). The TeNT toxin is produced as a single-chain protein after which the
N-terminal methionine is removed, converting the toxin to its mature form. The toxin is then released from the bacterium following cell lysis, however, there is no evidence of a signal peptide involved in the secretion of the toxin from the cell (Eisel et al., 1986). Upon release of the single-chain mature protein from the bacterial cell, the toxin is proteolytically nicked which converts the protein to its active di-chain form. The active holotoxin is a 151 kDa di-chain protein consisting of a 52.2 kDa LC comprising residues P2 – K446, linked by a disulfide bridge to the 98.3 kDa heavy chain (HC), residues S458 – D1315 (DiMari et al., 1982; Eisel et al., 1986; Kriegstein et al., 1990). The conversion of the single-chain protein to the di-chain form is required for TeNT intoxication of neuronal cells.

1.8.1.2 Botulinum neurotoxins

Botulinum neurotoxins are chromosomally expressed and the \textit{botA} gene encodes a 1,296 amino acid single-chain BoNT/A protein. BoNT/A has a molecular weight of 149.4 kDa and displays an overall 33.8\% sequence similarity to TeNT, with the translocation domain \textit{H\textsubscript{n}} having a similarity of 47.9\% (Binz et al., 1990). Like TeNT, mature BoNTs consist of an N-terminal 50 kDa LC, residues P2 – K488, linked by a disulfide bridge to a C-terminal 100 kDa HC, residues A499 – L1296. BoNTs are also proteolytically nicked to create the active di-chain form. Nicking occurs as a result of endogenous or exogenous proteases, depending on whether they are produced by proteolytic or non-proteolytic strains, respectively (Gordon and Leppla, 1994).

Unlike TeNT which is produced as a pure toxin, BoNTs are expressed along with a complex of proteins which aid in the intoxication process. BoNTs entering the body through the gut are able to survive the harsh environment of the stomach due to the presence of non-toxic proteins including non-toxic non-haemagglutinin (NTNH/A), and haemagglutinin (HA) proteins, which are expressed simultaneously with BoNT/A to form complexes ranging from 300 – 900 kDa (Couesnon et al., 2006). Together, these proteins form the progenitor BoNT/A toxin. These additional proteins remain non-covalently associated with BoNT and do not play a role in BoNT toxicity, but protect the toxins from the low pH environment and proteolytic enzymes present within the stomach until the toxin crosses the epithelial layer of the gut and enters the circulation (Hatheway, 1990; Kukreja and Singh, 2007; Maksymowych and Simpson, 1998).

BoNT nomenclature is based on the order of discovery and does not reflect the duration of action of each of the BoNTs. BoNT/A is the longest acting toxin, with a duration of action of approximately 6 months in humans, followed by BoNT/C, /B, /F and /E (Davletov et al., 2005). Various subtypes of BoNT/A exist, with BoNT/A1 being the subtype used in the
production of anti-toxin therapy. The various subtypes show differences in amino acid sequence, with the greatest variability seen in regions of the LC domain required for substrate recognition (Arndt et al., 2006b). Sequence variation between toxin types and within subtypes results in little cross-reactivity in monoclonal antibody binding and neutralisation, which has significant implications on the development of diagnostics, anti-toxin therapy and vaccine production (Smith, T. J. et al., 2005).

1.8.2 Toxin structure

All CNTs undergo three post-translational modifications after expression as a single-chain polypeptide, to produce the active di-chain molecule. First, the N-terminal methionine residue is removed, then specific cysteine residues interact to form disulfide linkages, and finally proteolytic nicking of the molecule by endogenous (or exogenous) proteases converts the single-chain peptide into the active di-chain form (Helting et al., 1979; Krieglstein et al., 1990). Nicking of CNTs to their active di-chain form may also be performed by treatment with trypsin (Bergey et al., 1989; Dekleva and DasGupta, 1989). Cleavage of BoNT/A by endogenous or exogenous proteases, or by trypsin to create the active di-chain molecule, occurs at Lys439 and Lys449, thus removing a 10 amino acid linker sequence from the mature protein (DasGupta and Dekleva, 1990; Jensen et al., 2003), whilst cleavage of TeNT by endogenous and exogenous proteases occurs within residues 445 – 461 (Krieglstein et al., 1990). Treatment of the holotoxin with papain liberates fragment-C, which consists of the C-terminal binding domain of the heavy chain (Hc). This fragment is used to investigate the binding, internalisation and trafficking of CNTs (Fairweather et al., 1986; Halpern et al., 1990).

CNTs share significant secondary structure homology, which is a function of their high sequence similarity (Lebeda and Olson, 1994). The ~150 kDa holotoxin consists of a 50 kDa LC and a 100 kDa HC, connected by a disulfide bond to give a classical A – B toxin type structure. All eight CNTs have a similar overall function and cleave soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins at presynaptic nerve terminals, however they target different substrates at different the peptide bonds and elicit paralysis of varying durations.

BoNTs target vesicle fusion machinery at the neuromuscular junction of neurons in the peripheral nervous system, while TeNT blocks the release of inhibitory neurotransmitters from inhibitory interneurons in the CNS. Although they act on different parts of the nervous
system and illicit opposing effects, all CNTs share a similar intracellular function which is attributed to the conserved structure amongst the proteins (Figure 1.4).

The LC is the catalytic portion of the toxin and acts as a zinc metalloprotease, with a zinc binding motif HexxH + E forming the active site of the LC (Simpson et al., 1993). The zinc atom plays a catalytic role and is essential for the inhibition of neurotransmitter release (Schiavo et al., 1992a; Schiavo et al., 1992b). The zinc ion is organised around residues His222, His226, Glu261 and a water-coordinated molecule through the first Glu in the zinc-binding motif (Glu223) (Lacy et al., 1998), the arrangement of which is consistent with zinc proteins (Coleman, 1992).

The heavy chain is divided into the 50 kDa N-terminal (HN) domain, involved in translocating the LC into the cellular cytosol, and the 50 kDa C-terminal (HC) domain, which contains residues involved in receptor binding. The C-terminal portion of the heavy chain (HC), can be further divided into an N-terminal (HCN) domain displaying a lectin-like jelly roll, and C-terminal (HCC) domain, with a β-trefoil region (Emsley et al., 2000). The HCC domain contains residues critical for receptor binding, however the function of the HCN domain remains unclear (Swaminathan, 2011).

The LC domains of CNTs share a high amino acid sequence similarity with a 36.5% similarity amongst the 8 CNT types, and the highest similarity is seen between TeNT and BoNT/B which share 51.6% identity (Kurazono et al., 1992). These two CNTs cleave the same SNARE substrate, albeit at different peptide bonds (Chen, S. et al., 2008b). The crystal structures of BoNT/A (Lacy et al., 1998), and TeNT (Umland et al., 1997) have been determined and show structural similarities between the catalytic and binding domains of both CNTs. The TeNT LC has a similar overall secondary structure to the LC of BoNT/A, /B and /E (Breidenbach and Brunger, 2005a), and the HC domains of TeNT and BoNT/A contain an N-terminal β-barrel and a C-terminal β-trefoil domain linked by an alpha helix (Lacy and Stevens, 1999). Whilst BoNT/A and TeNT share a considerable degree of primary sequence similarity, the highest similarity observed for the heavy chain domain is seen within the HN translocation domain, whilst the HC binding domain of TeNT and BoNT/A show the least degree of similarity. This is consistent with this region being highly immunogenic amongst the eight serologically distinct CNTs. It is also expected that the HC region would show some degree of difference due to the unique binding and intracellular trafficking routes amongst CNTs, a function which is determined by this domain (Lacy and Stevens, 1999).

There are 6 half-cysteine residues in sulfhydryl form, and two disulfide bonds forming an intrachain disulfide bond linking Cys869 with Cys1093 within the HC, and an interchain bond


Figure 1.4: General structure of CNT domains. Reproduced from Brunger and Rummel (2009).

General structure of CNT domains in the active di-chain form (bottom), and the crystal structure of BoNT/B (top).
joining Cys439 of the LC with Cys467 of the HC of TeNT (Figure 1.5) (Krieglstein et al., 1990). The corresponding residues forming the interchain disulphide bond in BoNT/A are Cys429 and Cys453 (Lacy et al., 1998). The position of the interchain disulfide bridge is conserved in all CNTs and is necessary for toxicity, however the remaining cysteines are not conserved amongst CNTs (Krieglstein et al., 1990; Qazi et al., 2007).

Proximal to the H<sub>N</sub> domain of CNTs lies the “belt” region which is composed of ~50 amino acid residues encoded by the translocation domain, but is associated with the catalytic light chain domain in the tertiary structure (Swaminathan, 2011). The belt has been shown to encircle the active site of the LC and is connected by a disulfide bond and other non-covalent interactions (Li, L. and Singh, 2000; Swaminathan, 2011), with the active site buried about 20 Å within the protein beneath the belt region in the unreduced holotoxin (Brunger et al., 2007). Although not directly involved in the activities dictated by the H<sub>N</sub> domain such as translocation of the LC and pore-formation, the belt may occlude the active site and protect it from solvent until the LC is able to reach its catalytic substrate (Fischer et al., 2012). BoNT/A LC (LC/A) is most catalytically active in the absence of the belt (Gul et al., 2010; Lacy et al., 1998), which is consistent with the hypothesis that the belt acts only as a chaperone, assisting in the translocation of the toxin from the endosome to the cytosol (Brunger et al., 2007).

In order for CNT toxins to poison neurons, they must be present in the active di-chain form, and toxins are rendered non-toxic after the separation of the light and heavy chains (Eisel et al., 1986). The section to follow will explore the mode of action of CNTs by looking at the function of the individual domains.

### 1.8.3 Toxin mode of action

The presynaptic terminals of motorneurons where CNTs enter neuronal cells, is located in the periphery, while the cell body is located in the CNS. Once the toxin has bound to membrane receptors on the surface of neurons, it is internalised into the motor neuron and TeNT is transported in a retrograde fashion along the axon and across the blood-brain barrier to the cell body. Following secretion, TeNT is taken up by adjacent inhibitory interneurons via trans-synaptic spread, while BoNTs remain at the NMJ of motorneurons in the periphery. CNTs then cleave one of the SNARE proteins involved in synaptic vesicle fusion (Caleo and Schiavo, 2009; Lalli and Schiavo, 2002).
Figure 1.5: Location of disulfide bonds in tetanus toxin.
Graphical representation of the location of sulfhydryl residues and the interchain and intrachain disulfide bonds in the tetanus toxin sequence. Adapted from Qazi et al. (2007) and Kriegstein et al. (1990).
There are four main steps involved in CNT toxicity, binding, internalisation, translocation and cleavage (summarised in Figure 1.6). Both TeNT and BoNTs act on the presynaptic terminals of NMJs, but within different types of neurons. The key difference between TeNT and BoNTs is that TeNT undergoes retrograde axonal transport which transmits the toxin toward the CNS, whilst BoNTs travels in an anterograde direction to act on motorneurons in the peripheral nervous system. The different cellular trafficking of TeNT and BoNT explains the distinct target sites and therefore the different clinical symptoms of the two diseases.

1.8.3.1 Binding to neuronal cells is mediated by the HCs domain

CNTs enter the circulatory system from a point of entry via transcytosis (Maksymowych and Simpson, 1998), and enter neurons via receptor-mediated endocytosis (BoNTs) (Simpson, 2004), or a clathrin-dependent pathway (TeNT) (Deinhardt et al., 2006). The key to the differential trafficking of TeNT and BoNTs is attributed to the cell surface receptors initially bound on target neuronal cells. It is hypothesised that after initial binding to gangliosides, TeNT and BoNTs bind a secondary protein receptor which results in differential sorting of CNTs after internalisation (Montecucco, 1986).

Cell binding is a function of the HCs domain which targets CNTs specifically to neuronal cells (Lalli et al., 1999). A dual receptor model was hypothesised in 1986 to explain how (i) CNTs undergo differential trafficking, and (ii) bind preferentially to gangliosides present on neuronal cells, as they are found in high concentrations on cells throughout the nervous system and within the spleen (Montecucco, 1986; Sinha et al., 2000). It is thought that gangliosides act as low affinity binding receptors and their presence in high concentrations accumulates CNTs on the surface of neurons, allowing them to then bind to a secondary high affinity low-density protein receptor (Swaminathan, 2011). The hypothesis that the secondary receptor is a protein was confirmed when binding of TeNT to rat brain membranes was decreased following pre-treatment with proteases (Lazarovici and Yavin, 1986; Yavin and Nathan, 1986). It is proposed that since TeNT and BoNTs bind to gangliosides, it is this secondary protein receptor which determines the internal trafficking of CNTs (Rummel et al., 2003).

In the case of TeNT, targeted mutation of the HCs domain affected both binding to gangliosides and retrograde axonal transport. Mutations which did not affect ganglioside binding, still decreased binding of the HCs to motorneurons and also inhibited retrograde transport, indicating that this region contained a separate receptor site which was essential to retrograde trafficking of TeNT (Sinha et al., 2000). There is also a lack of competition between TeNT and BoNTs with regard to binding, suggesting that they bind to different cellular receptors on the surface of neuronal cells (Habermann and Dreyer, 1986).
Figure 1.6: CNT mode of action. Reproduced from Turton et al. (2002).

Mode of action of botulinum toxin. 1) Binding to neuronal cell via toxin-specific receptors. 2) Internalisation into an intracellular vesicle. 3) Translocation of toxin from the endosome to the cytosol. 4) Cleavage of toxin-specific SNARE proteins prevents SNARE complex formation and inhibits fusion of secretory vesicles with the synaptic membrane.
1.8.3.2 Gangliosides

Gangliosides are a class of glycosphingolipids comprised of a sialic acid oligosaccharide, and are found predominantly in the membranes of neuronal cells. They contain a common core to which one to four sialic acids are bound, with these regions exposed to the extracellular space, while the nonpolar regions are embedded in the plasma membrane (Mocchetti, 2005). The various types of gangliosides are composed of different combinations of sugar residues such as glucose, galactose, fructose, N-acetylg glucosamine and N-acetylgalactosamine, linked to a ceramide base. The total number of sialic acid residues contained within the gangliosides is used to classify the different species as monosialogangliosides (GM), disialogangliosides (GD), trisialogangliosides (GT), and tetrasialogangliosides (GQ) (Mocchetti, 2005). The nomenclature is further classified based on the order of migration of the gangliosides via thin layer chromatography, indicated by the numbers 1, 2, 3, etc., and finally, gangliosides are assigned to the series “asialo-”, “a”, “b”, or “c” to indicate 0, 1, 2, or 3 sialic acid residues linked to the inner galactose unit (Svennerholm, 1964). TeNT and BoNT bind to the GD₁b and GT₁b series of gangliosides (Rummel et al., 2003; Rummel et al., 2004), with TeNT showing a preference for ganglioside GT₁b (Winter et al., 1996).

Two ganglioside molecules can bind simultaneously to TeNT Hₐ, while BoNTs only bind a single ganglioside with low affinity (Chen, C. et al., 2009; Dong et al., 2006; Rummel et al., 2004). TeNT Hₐ contains two carbohydrate binding pockets which bind to gangliosides, a sialic acid binding pocket and a lactose binding pocket (Chen, C. et al., 2008a; Chen, C. et al., 2009; Fotinou et al., 2001). The sialic acid binding site is crucial for the binding of the Hₐ to gangliosides and for neurotoxicity while the lactose binding site is highly conserved in TeNT, and all BoNTs (except BoNT/D), indicating that it is essential for the binding of Hₐ to gangliosides (Rummel et al., 2003).

The position of binding site 1 is common to both TeNT and BoNTs and contains a lactose-binding site and a sialic acid binding site, respectively. The second binding site in TeNT is a sialic acid binding site; however the corresponding site in BoNTs does not bind any part of gangliosides. This site may instead interact with a protein receptor and also indicates that the sialic acid-binding site (site 2), in TeNT may also be involved in the selective trafficking of TeNT to the CNS (Jayaraman et al., 2005).

Oligomerisation is demonstrated by TeNT as seen by cross-linking of Hₐ molecules upon binding to gangliosides (Fotinou et al., 2001). This is a concentration-dependent process thought to be used to accumulate TeNT (specifically Hₐ), in lipid rafts in order to increase the
concentration of the toxin and bring it in close proximity to the secondary protein receptor to which it binds prior to endocytosis (Qazi et al., 2007). TeNT H2 has been observed to bind cell membranes in a punctuate manner, with binding corresponding to the concentration of toxin in microdomains called lipid rafts (Herreros et al., 2001; Lalli et al., 1999). The C-terminal domain of the TeNT heavy chain can undergo retrograde axonal transport in the absence of the remainder of the molecule with the 34 C-terminal residues of the tetanus heavy chain (residues 1282 – 1315), and particularly the region surrounding His1293, having been found crucial for binding to gangliosides (Emsley et al., 2000; Shapiro et al., 1997).

TeNT may interact with multiple carbohydrate binding sites (Bizzini et al., 1977; Emsley et al., 2000; Halpern and Loftus, 1993). As well as binding to gangliosides GD1b and GT1b on the neuronal membrane, TeNT also binds glycosylphosphatidylinositol (GPI)-anchored proteins (Deinhardt et al., 2006). GPI-anchored proteins and gangliosides are present on lipid rafts, along with cholesterol and other sphingolipids and concentrate TeNT molecules at the cell membrane, which is thought to enhance uptake of the toxin into neuronal cells (Francis et al., 2004a; Herreros et al., 2001). A 15 – 20 kDa N-glycosylated protein has been shown to be a GPI-anchored protein and proposed to specifically bind TeNT on the surface of spinal cord cells and motorneurons, acting as a co-receptor for TeNT binding to neurones (Herreros et al., 2000; Herreros et al., 2001).

BoNTs bind gangliosides and synaptic vesicle protein SV2, an integral membrane protein found on synaptic and endocrine secretory vesicles (Dong et al., 2006; Mahrhold et al., 2006). It is hypothesised that once BoNT/A enters a cell and deactivates the neuron by inhibiting synaptic vesicle release, there is a decrease in the amount of SV2 receptors exposed on the surface of the cell thereafter. This phenomenon allows for BoNT present in the circulation to then bind active cells which have yet to be poisoned and still have a greater amount of SV2 receptors on their surface (Dong et al., 2006).

### 1.8.4 Internalisation into neuronal cells

Following attachment to neuronal cells, BoNTs are internalised through the plasma membrane via receptor-mediated endocytosis (Simpson, 2004), while TeNT employs a clathrin-dependent pathway after toxin bound to receptors has sorted through lipid rafts (Deinhardt et al., 2006). TeNT travels to the CNS within round vesicles and tubular structures to avoid acidification which would otherwise lead to degradation of the toxin (Lalli and Schiavo, 2002), and internalisation is enhanced by neurotrophic factors (Roux et al., 2006).
Once CNTs have entered neuronal cells, the di-chain molecule is localised in an acidic endosomal compartment and requires translocation into the cytosol to perform its catalytic function.

1.8.5 Translocation of the LC into the cytosol

Following internalisation of CNTs in their di-chain form into endosomes, the acidic environment of the endosome causes a conformational change in the holotoxin which results in the separation of the LC from the HC and the translocation of the LC into the cytosol, where it is free to cleave its substrate (Matteoli et al., 1996). This process occurs much like diphtheria toxin which is transported to the cytosol from acidic vesicles (Sandvig and Olsnes, 1980). Translocation of the LC from the endosome into the cytosolic compartment involves six steps, summarised below.

(i) pH-induced conformational changes expose hydrophobic regions
(ii) HN domain creates a channel to the cytosolic compartment
(iii) LC is translocated through the pore and into the cytosol
(iv) Reduction of disulfide bond which links the light and heavy chains
(v) non-covalent linkages holding the heavy and light chain together dissociate
(vi) the light chain refolds into its active form

The pH within the endosome which is lowered by the action of a protein pump, induces changes in structural conformation of the holotoxin. The structure-altering effects of the low pH environment within the endosome are completely reversible, as shown by the maintenance of enzymatic activity of LC/A before and after exposure to low pH (Li, L. and Singh, 2000). Acidification of the LC by the early endosome occurs from pH 4.55 to pH 4.40 (Puhar et al., 2004). Concanamycin A, which inhibits the acidification of intracellular compartments has been shown to inhibit the activity of BoNT/A, indicating that exposure to low pH within the endosome is crucial for toxicity (Keller et al., 2004).

The di-chain molecule is partially unfolded, exposing hydrophobic regions, and the mostly alpha-helical HN domain inserts itself into the endosomal membrane, creating a channel (Blaustein et al., 1987; Boquet and Duflot, 1982; Hoch et al., 1985; Sheridan, 1998).

The pore-forming ability of the HN domain facilitates transport of the toxin from the endocytic vesicle to the cytosol of the cell in an N- to C-terminus direction (Fischer and Montal, 2007; Koriazova and Montal, 2003). It is critical that the disulfide bond connecting the LC and HC remains intact during translocation, as premature reduction of the bridge prior to channel
formation prevents translocation from the endosome (Fischer and Montal, 2007; Schiavo et al., 1990; Wey et al., 2006). The LC is also partially unfolded as it moves into the cytosol, a step which is critical for the efficient translocation of the LC across the hydrophobic endosomal membrane (Bade et al., 2004).

Upon entry to the cytosol, reducing conditions dissociate covalent and non-covalent interactions such as the disulfide bridge holding the LC and HC together, and the “belt” which covers the catalytic zinc endopeptidase domain of the LC (Li, L. and Singh, 2000; Puhar et al., 2004). Finally, the light chain structure refolds as it leaves the acidic environment of the endosome and enters the more neutral environment of the cytosol where it is then able to bind to a SNARE substrate on the presynaptic membrane and catalyse its target peptide bond (Fisher and Montal, 2006; Hoch et al., 1985; Simpson, 2004).

1.8.6 Cleavage of SNARE proteins

The selectivity of CNTs to cleave one of four proteins which make up the SNARE complex in neuronal cells is mediated by the LC. Substrates targeted by CNTs are the soluble NSF attachment protein receptors (SNAREs), which include the 25 kDa synaptosomal-associated protein (SNAP-25), synaptobrevin II, also known as vesicle associated membrane protein-2 (VAMP-2), and syntaxin (Sutton et al., 1998). The SNARE complex is essential for the secretion of chemical signals in eukaryotic cells, and is not specific to neuronal cells. VAMP proteins such as synaptobrevin occur on the surface of synaptic vesicles, whilst SNAP-25 and syntaxin are bound to the plasma membrane. The SNARE complex (Figure 1.7), is formed when synaptic vesicles come close to the pre-synaptic plasma membrane in order to release vesicular contents into the post-synaptic junction (Davletov et al., 2005).

The light chains of CNTs are zinc-dependent endopeptidases which hydrolyse members of the SNARE family at a specific peptide bond, unique to each CNT (Schiavo et al., 1992a; Schiavo et al., 1992b), and zinc has been shown to be essential for the catalytic activity of CNTs (Simpson et al., 1993). Cleavage of SNARE proteins inhibits fusion of synaptic vesicles with the pre-synaptic membrane of the neuron, preventing exocytosis and release of neurotransmitters from the pre-synaptic membrane (McMahon et al., 1993). The LC has been shown to also cleave SNARE proteins in non-neuronal cells, indicating that neuronal specificity is solely a function of the heavy chain (Chaddock et al., 2000; Penner et al., 1986).
Figure 1.7: Formation of the SNARE complex. Reproduced from Humeau et al. (2000).

Exocytosis occurs when a synaptic vesicle docks and fuses to the plasma membrane, through the formation of the SNARE complex. In its free state (A), the synaptic vesicle is sensitive to proteolytic attack by CNTs prior to formation of the SNARE complex (B). Once the SNARE complex is formed and the synaptic vesicle has docked (C), it is able to fuse with the plasma after an inflow of calcium ions (D1). D2 shows a cross section of the normal formation of the SNARE complex.
When the SNARE complex is fully assembled, it is resistant to proteolytic attack by CNTs as target cleavage sites may not be exposed in this configuration, thus CNTs must cleave individual SNARE proteins when present in their intermediate state, prior to SNARE assembly (Sutton et al., 1998).

1.9 Toxin specificity and duration of action

Unlike most proteases, CNTs require extended regions of substrate in order to bind and cleave efficiently, but do not require a consensus region for recognition of the cleavage site (Breidenbach and Brunger, 2005b).

TeNT and BoNT/B both cleave VAMP-2 at the same peptide bond however they have unique recognition sites, with TeNT requiring an additional 20 amino acids to form a stable interaction with the substrate and cleave VAMP-2 (Figure 1.8) (Chen, S. et al., 2008b). These differences can be attributed to the different location of the amino acid residues responsible for cleavage in the active site of the LC (Rao et al., 2005). TeNT requires 51 amino acid residues of VAMP-2 for cleavage, and hydrolyses the Gln76 – Phe77 peptide bond in the substrate (Sikorra et al., 2008).

BoNT/D, /F, and /G also cleave VAMP, but at different peptide bonds to TeNT and BoNT/B, while BoNT/A, /E, and /C cleave SNAP-25. BoNT/C also cleaves syntaxin and is the only CNT able to cleave two different substrates (Baldwin and Barbieri, 2007). LC/A binds directly to residues 80 – 110 of SNAP-25 on the plasma membrane in order to cleave the SNARE protein (Chen, S. and Barbieri, 2011). The deletion of 10 N-terminal residues or 68 C-terminal residues from TeNT LC renders the domain nontoxic (Kurazono et al., 1992).

BoNT/A initially binds to SNAP-25 along the belt region at residues 156 – 202, and cleavage occurs at the Gln197 – Arg198 peptide bond of SNAP-25 (Breidenbach and Brunger, 2004; Chen, S. et al., 2007). Nine amino acid residues are removed from the C-terminus of SNAP-25, allowing it to maintain its association with syntaxin (Chen, S. and Barbieri, 2006). The 10 N-terminal and 57 C-terminal residues from LC/A are required for catalytic activity (Kurazono et al., 1992).
Figure 1.8: Target SNARE cleavage sites of CNTs. Reproduced from Humeau et al. (2000).

Cleavage sites of TeNT and BoNT/A – /G are indicated by arrows and the open boxes indicate the binding sites of the CNT light chains.
BoNT/E also cleaves SNAP-25, but requires a much shorter recognition region (residues 167 – 186), than BoNT/A, and cleaves at the peptide bond between residues 180 – 181. Cleavage by BoNT/E removes 26 amino acids from SNAP-25, which leads to the dissociation of SNAP-25 from synatxin (Chen, S. and Barbieri, 2006). The difference in length of SNAP-25 removed between the toxin types does not however, translate to an increased duration of action. In fact, paralysis by BoNT/A persists the longest of all BoNTs, while BoNT/E displays the shortest paralytic time of less than 4 weeks and is thus generally not clinically useful (Eleopra et al., 1998). BoNT/E is able to poison neurons at a faster rate than BoNT/A, possibly due to the translocation step occurring more quickly in BoNT/E (Wang et al., 2008). It has been demonstrated that simultaneous injection of BoNT/A and BoNT/E saw muscle activity reinstated in the shorter time after BoNT/E paralysis, and not of the longer acting BoNT/A (Eleopra et al., 1998).

The speed of recovery of vesicle fusion machinery is also affected by the rate at which damaged SNARE proteins are replaced with newly synthesised proteins (Foran et al., 2003). For example, SNAP-25 cleaved by LC/E is quickly removed and replaced, thus permitting the SNARE complex to reform and reinitiate synaptic vesicle fusion in a shorter period of time than when cleaved with BoNT/A (Meunier et al., 2003). Reasons for such differences may be due to the sub-cellular localisation of the LCs. LC/A has been found to localise at the plasma membrane in a punctuate manner, with localisation directed by a di-leucine motif present within the first eight amino acids of the LC/A sequence. LC/E is found mainly within the cytosol where proteases may degrade the LC protein over time (Fernandez-Salas et al., 2004).

Further supporting evidence on the effects of the di-leucine motif come from a study which showed that fusing active LC/E to catalytically inactive full-length BoNT/A delivered LC/E to cholinergic neurons and extended the duration of action of LC/E to that of LC/A. It was revealed that C-terminal di-leucine motif present in BoNT/A was responsible for its long duration of action (Wang et al., 2011).

1.10 Therapeutic utility of CNTs

A striking feature of CNTs, much like other toxins with an A – B type toxin structure, is that individual domains maintain their function in the absence of the other domains. This trait opens a door for the use of individual toxin domains from CNTs as tools in the treatment of disorders involving secretory abnormalities and the nervous system. CNTs may be delivered
as whole toxins for a targeted, low-dose treatment, or as individual domains fused to other CNT or non-CNT proteins to exploit the function of individual domains.

In the absence of the light chain, the heavy chain is a sophisticated delivery vehicle and fragment-C of TeNT has been used to deliver cargo proteins attached in place of the LC to the CNS, without systemic TeNT poisoning. The LCs of TeNT and BoNT can be targeted to a desired cell type by attachment to a specific cell-binding receptor to disrupt SNARE assembly, even in cells outside of the nervous system. It was first realised that the muscle relaxing effects of BoNTs could be useful as a therapeutic in the 1970’s, 150 years after Justinus Kerner’s first observations of botulism in a patient (Chaddock and Marks, 2006).

CNTs can be used for the delivery of cargo proteins to the cytosol of neuronal cells. If CNT toxicity is not desired, the cargo protein may be coupled to a deactivated LC after mutation of the active site (Bade et al., 2004; Li, Y. et al., 2001), or the cargo protein may completely replace the LC region (Goodnough et al., 2002). Such molecules are termed targeted secretion inhibitors and they may be engineered to target cell types outside the nervous system, with cell targeting determined by the binding domain included in the toxin molecule (Stancombe et al., 2012). Targeted delivery of controlled amounts of toxin has been shown to assist in the treatment of muscular disorders. However, the effects of both CNTs are reversible, so therapeutic use requires recurrent application, determined by the length of time the toxin remains active at the target site.

1.10.1 Therapeutic use of tetanus toxin

In cases where the effects of the native TeNT are desired, delivery of low-dose TeNT to a targeted area will illicit the effects of the native toxin in a controlled manner. TeNT fragment-C was first suggested as a non-toxic carrier of cargo proteins to neurons within the CNS by Bizzini and colleagues in 1977 (Bizzini et al., 1977). Fragment-C of TeNT is generally included in the production of therapeutic molecules as it retains the binding and trafficking properties of TeNT, but lacks the catalytic activity of the light chain. A key feature of TeNT fragment-C is that it is able to avoid degradation during retrograde axonal transport, ensuring the attached cargo protein is delivered to the CNS intact (Lalli and Schiavo, 2002).

Drugs cannot usually penetrate the blood-brain barrier because of specialised capillary endothelial cells which prohibit entry of large molecules such as peptides, proteins and viruses (Pardridge, 2002). However, the ability of TeNT to travel in a retrograde fashion via the motorneuron allows it to act as a non-viral delivery system to transport small molecules to the CNS. TeNT has been suggested for use in the treatment of Alzheimer’s disease and
spinal cord injury as a non-viral carrier for the delivery of therapeutic molecules (Singh et al., 2010).

Chemical conjugation of molecules to fragment-C or expression as a recombinant protein with the cell-binding domain, has been shown to deliver the cargo protein to the CNS in vivo and in vitro (Schiavo et al., 2000). Cargo proteins delivered by fragment-C include therapeutic proteins and growth factors, with some examples listed below.

A glial cell line-derived neurotrophic factor (GDNF) chemically conjugated to TeNT fragment-C showed increased neuronal survival and repair in vitro and in vivo, which has the potential for use in the treatment of neurodegenerative disorders such as Parkinson’s disease and amyotrophic lateral sclerosis (Larsen et al., 2006).

Superoxide dismutase-1 (SOD-1) has been shown to be protective in cases of stroke and trauma and in Parkinson’s disease. Delivery of SOD-1 to the CNS may be therapeutic to disease, however it is rapidly cleared from circulation when delivered orally (Francis et al., 1995). To overcome this, the 19 kDa subunit of SOD-1 was expressed as a recombinant protein with TeNT fragment-C in E. coli, and delivered by both intramuscular injection and directly into the CNS through injection into the CSF. The amount of SOD-1-TTC delivered was enhanced and it was also retained for longer in the brain matter than when SOD-1 was injected alone (Benn et al., 2005; Francis et al., 2004a; Francis et al., 1995).

TeNT fragment-C has been fused to the diphtheria catalytic and transmembrane domains as a novel approach to deliver a cargo molecule linked to the CNS (Francis et al., 2004b).

One study of particular interest used native tetanus toxin for the treatment of obstructed breathing in British Bulldogs. British bulldogs are at risk of obstructed breathing caused by laryngeal collapse in the upper airway, which is usually treated with surgery (Torrez and Hunt, 2006). Localised injections of TeNT in the upper airway increased muscle tone compared to the placebo and this study is an excellent indicator for the use of TeNT to increase muscle tone in the upper airway (Sasse et al., 2005).

These examples provide unequivocal support for the potential of tetanus neurotoxin, or fragments thereof, for use in the delivery of therapeutic molecules to the CNS in the treatment of neuromuscular disorders.
1.10.2 Therapeutic use of botulinum toxin

BoNTs have been used in controlled toxicity since the 1970's, when American scientist Alan Scott first showed that small doses of BoNT injected into over-stimulated muscles could cause muscle relaxation without any adverse systemic effects in primates, and later used BoNT/A for the treatment of strabismus in humans (Scott, 1981). Well known for its cosmetic uses and for the treatment of muscular disorders involving excessive muscle contraction, BoNTs have also been used in the treatment of other muscle spasticity disorders due to their ability to reversibly block the innervation of both striated and smooth muscles.

BoNT/A is the most commonly used serotype due to its longevity of action, and was approved for the treatment of eye conditions and hemifacial spasms in 1989 by the U.S. FDA, under the commercial name Oculinum, and after its acquisition by Allergan in 1989, BoNT/A was marketed as BOTOX®. BoNT/A is also available as Dysport which is distributed by Ipsen and has been approved for the treatment of blepharospasm (eyelid twitch), cervical dystonia (neck and shoulder muscle spasms), hemifacial spasm (spasms on one side of the face), muscle spasms in children and ophthalmological disorders, and BoNT/B (Myobloc, approved in 2000), has also been used in treatment of dystonia (Goldman and Wollina, 2010; Wortzman and Pickett, 2009). BoNTs have also been used in the treatment of overactive smooth muscles and constipation by inhibiting the contraction muscle in the gastrointestinal system, and also sphincters, in the case of anal fissure (Brisinda et al., 2004).

BoNT/A has been used in the treatment of a range of dystonias (overactive muscle contractions of a neurological origin), such as oromandibular dystonia, writer’s and other occupational cramps, foot dystonia, axial dystonia (affecting the midline of the body), Tourette’s syndrome, tremor and spasticity (Truong and Jost, 2006). BoNT/A has also been used in the treatment of secretory disorders such as hyperhydrosis (excessive sweating), and sialorrhea (excessive salivation) (Keith and John, 2010).

BoNTs have been used as a muscle relaxant for headaches (Jabbari, 2008) and BOTOX® is commonly used for cosmetic purposes to soften lines and wrinkles around the eyes, forehead and mouth (Goldman and Wollina, 2010). The local paralysis caused by targeted injection of BoNT/A relaxes the muscles and flattens the skin, preventing the appearance of deep lines in the face.

A range of medical disorders are associated with chronic pain, such as multiple sclerosis, chronic migraines, arthritis, and back and shoulder pain, and these afflictions are associated with a decreased quality of life. In cases where sufferers do not respond to commonly available medications for the alleviation of pain or fail to respond due to overuse, botulinum
toxin serves as an alternative treatment option (reviewed in Dolly and O’Connell (2012) and Mahant et al. (2000)). The 100 kDa LC and H$_{N}$ fragment of BoNT/A (LH$_{N}$/A) has been coupled to a lectin in order to cleave SNAP-25 within neurons responsible for transmitting pain signals to the CNS, for the treatment of pain in rats. The lectin targeting moiety from *Erythrina cristagalli* delivers LH$_{N}$/A to nociceptive afferent neurons located in the periphery, where it is then able to inhibit SNARE complex formation and prevent the release of synaptic vesicles containing substance P, involved in transmission of signals to the CNS which are then perceived as pain (Duggan et al., 2002).

The LH$_{N}$/A has also been chemically conjugated to wheat germ agglutinin (WGA), targeting pancreatic B-cells to inhibit the release of insulin. This WGA binding domain permitted binding to the cell line which is normally resistant to the effects of BoNT/A, and the LH$_{N}$/A was able to translocate the LC to the cell cytosol and inhibit neurotransmitter release in a manner analogous to the native toxin (Chaddock et al., 2000). Another study fused the LH$_{N}$/C to epidermal growth factor to prevent hypersecretion of mucus in asthma and chronic obstructive pulmonary disease patients (Foster et al., 2006).

The aim of BoNT use is to deliver a targeted and controlled dose of toxin to reduce the level of neuronal activity, without completely inhibiting it. In cases where excessive and uncontrolled peripheral cholinergic neuronal activity occurs, therapeutic doses of BoNT will reduce the level to near normal. These characteristics of BoNT action have been exploited successfully for use in cosmetic applications and therapeutic treatment of muscular disorders.

### 1.11 Rationale of the project

This review has shown that a novel approach to the treatment of OSA which is more widely accepted to sufferers will not only show improvements in the quality of life, but also have an impact on the financial burden associated with treatment of this disease. It has been well established that a loss of tone in muscles of the upper airway obstruct breathing during sleep, and that current treatment options seek to increase muscle tone with surgery, or maintain a patent airway through the use of mechanical and/or non-mechanical devices. A novel approach to the treatment of OSA is proposed whereby CNT toxins may be used to increase muscle tone in the upper airway. It has been demonstrated in the literature that botulinum toxin is able to cause localised paralysis and it is commonly accepted for use in cosmetic purposes. It has also been demonstrated that tetanus toxin is able to elicit muscle contracting effects in a dose-dependent manner.
It is proposed that tetanus toxin is used to cause targeted, localised muscle contraction in muscles of the upper airway to increase muscle tone, and remove the obstruction to the airway in OSA sufferers.

The use of native tetanus toxin is limited due to widespread immunity in the population from routine vaccination. It is also important that individuals maintain a protective level of circulating antibodies, and continue to receive booster vaccinations for the remainder of their lives. For this reason, the wild-type tetanus toxin is not practical for therapeutic use. To address this issue, it is proposed that a chimeric neurotoxin comprised of the cell-binding and translocation domains of tetanus neurotoxin fused to the catalytic light chain domain of botulinum type A toxin will create a novel chimeric CNT which is able to illicit the effects of native tetanus after localised injection. The use of the BoNT/A light chain in place of the TeNT LC is for two reasons: (i) BoNT/A LC has been shown to maintain paralysis for the longest duration of time of all CNTs (Davletov et al., 2005; Eleopra et al., 1998); and (ii) its use will assist in evading host immune responses to the chimeric toxin, as the majority of the population should not display immunity to this domain. Although the vast majority of the developed world has been vaccinated against tetanus toxin, the use of the TeNT heavy chain may still be applicable (Fishman et al., 2006).

Antibody responses to TeNT occur prior to the toxin binding to neuronal cells. When delivered intramuscularly, THC may evade circulating antibodies and bypass the immune response. The injected toxin may have enough time to bind to neuronal cells due to an insufficient level of circulating antibodies to neutralise the entire injected toxin before it is able to bind, even in a newly vaccinated animal. In that time, TeNT may bind neuronal cells and be internalised (Fezza et al., 2000; Fishman et al., 2006).

The use of the tetanus toxin heavy chain will ensure that LC/A is delivered to the CNS where it can effectively cause tetany in the muscles surrounding the injection site. It is anticipated that over time, much like the case with the use of BOTOX®, that injection of the novel chimeric toxin will induce neutralising antibodies. Thus, the future aims of this project are to alter the immunogenic regions of the toxins in order to evade the host immune system, but still maintain native activity.

The scope of this thesis is to use recombinant DNA technology to express the LC/A and TeNT HC in a heterologous host and reconstitute these toxin domains to produce a novel chimeric CNT holotoxin. The chimeric toxin should be able to bind to motorneurons and display retrograde axonal transport to inhibitory interneurons within the CNS and cleave the
SNAP-25 substrate. A potential novel treatment for OSA involving a simple set of injections given up to twice per year would be less invasive and far more reliable than surgery, which may improve compliance rates of treatment. Thus, the proposed novel toxin molecule could be the future of OSA treatment, a disorder impacting millions of people worldwide.
Chapter 2

General Materials and Methods
2.1 General procedures

General materials and methods are listed below, with specific details for methods listed in the respective chapters. Chemicals used were of analytical or molecular grade and were stored at ambient temperature unless stated otherwise.

All reagents were of analytical or molecular grade and were prepared with deionised water from the Millipore Milli-Q® water system (MQ water) (Millipore, USA); whilst media was prepared using distilled water (dH₂O).

All glassware, media and solutions were sterilised by autoclaving at 121°C for 20 min. Media containing supplements and/or antibiotics were autoclaved and cooled to 55°C before the addition of any supplement(s) or antibiotic(s). All media was prepared using aseptic technique and stored at 4°C until required.

Solutions were delivered using Finnpipette® digital pipettes (ThermoFisher Scientific, U.S.A.) which included ranges of 0.5 – 10 μL, 5 – 50 μL, 20 – 200 μL, 200 – 1000 μL, 1 – 5 mL, 2 – 10 mL, and a 50 – 300 μL multichannel pipette. Volumes above 10 mL measured with a measuring cylinder.

Volumes of 1.5 mL or less were separated by centrifugation with the Eppendorf microcentrifuge 5415D and volumes up to 50 mL were separated by centrifugation using either the Beckman Allegra™ 21R centrifuge or the Heraeus Multifuge 1S-R centrifuge. High speed centrifugation was performed using a Beckman J2-21 M/E Super Centrifuge.

Weighing of solids less than 2 g was performed using the Mettler Toledo XS105 Dual Range top loading analytical balance, and weighing of solids greater than 2 g was performed using the ISSCO Model 300 top loading balance.
2.2 General equipment and suppliers

96-well, colourless, flat bottom plates
Nunc, Denmark

96-Well Microplate Polystyrene Black (350µl well volume)
Chromacol, United Kingdom

Acrodisc® (0.2 µm and 0.45 µm)
Pall, U.S.A.

Balances:
- XS105 Dual Range analytical balance
  Mettler Toledo, Australia
- ISSCO Model 300 balance
  ISSCO, Australia

Biological safety cabinet class II, BH2000 series
Clyde-Apac, Australia

BioPhotometer
Eppendorf, Germany

Centrifuge tubes:
- 1.5 mL microfuge tubes
  Sarstedt, Germany
- 10 mL centrifuge tubes
  Sarstedt, Germany
- 15 mL centrifuge tubes
  Sarstedt, Germany
- 50 mL centrifuge tubes
  Greiner Bio-One, Germany

Centrifuges:
- Eppendorf 5414D bench top centrifuge
  (maximum speed 16,000 x g)
  Eppendorf, Germany
- Heraeus Multifuge 1S-R centrifuge
  (maximum speed 5000 x g)
  Thermo Electron Corporation, U.S.A.
- Allegra™ 21R centrifuge
  (maximum speed 5000 x g)
  Beckman Coulter, U.S.A.
- Beckman J2-21 M/E Super centrifuge
  (maximum speed 50,400 x g)
  Beckman Coulter, U.S.A.

Cryovials (1.8 mL)
Iwaki, Japan

Dry block heater
Ratek, Australia

Electrophoresis power supply:
- EPS 3000xi
  (BioRad Laboratories, U.S.A.)
- PowerPac 300
  (BioRad Laboratories, U.S.A.)

Electrophoresis units and components:

DNA
- Mini gel (Mini-sub cell GT cell)
  BioRad Laboratories, U.S.A.
- Midi gel (Wide mini-sub cell GT cell)
  BioRad Laboratories, U.S.A.

Protein
- Mini-PROTEAN® Tetra Cell
  BioRad Laboratories, U.S.A.

Electroporation cuvettes (0.2 cm)
Molecular Bio Products, U.S.A.
GelDoc imaging system: BioRad Laboratories, U.S.A.
FLUOstar Optima microplate reader: BMG Labtech, Germany
iBlot dry gel transfer device: Invitrogen Corporation, U.S.A.
iMark microplate absorbance reader: BioRad Laboratories, U.S.A.
Milli-Q® water filtration system: Millipore, U.S.A.
Petri dishes: BioLab, Australia
pH meter: Metrohm AG, Switzerland
Platform orbital shaker: Ratek, Australia
Polypropylene gravity flow column (1 mL): Qiagen, Germany
Pulse controller & Gene pulser apparatus: BioRad Laboratories, U.S.A.
Px2 Thermal Cycler: Thermo Electron Corporation, U.S.A.

Syringes:
1, 3, 5, 10, 20, and 60 mL
SGE 100 μL glass syringe: SGE Analytical Science, Australia

Transilluminator (UV): Novex, Australia
UVette® (10 mm): Eppendorf, Germany

Vivaspin 20
30,000 Da MWCO
50,000 Da MWCO
500 Da MWCO
Diafiltration cup: Sartorius-Stedim, Germany

Water bath: Ratek, Australia

2.3 General materials and suppliers

2.3.1 General solutions

1,4-Ditiothreitol: (Roche, Germany), stored at 4°C.
2-(N-morpholine)-ethanesulfonic acid (MES): (Sigma-Aldrich, Germany).
β-mercaptoethanol: electrophoresis purity (BioRad Laboratories, U.S.A.).
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES): 1 M (Invitrogen Corporation, U.S.A.), stored at 4°C.
Acetic acid (glacial): (Merck, Germany).
Acrylamide/bisacrylamide solution: 40% acryl/bis (Amresco, U.S.A.), stored at 4°C.
Acqua Stain: (Bulldog Bio Inc., U.S.A.), stored at 4°C.
Adenosine triphosphate (ATP): 20 mM ATP (Sigma-Aldrich, Germany), stored at -20°C.
**Agarose:** 1.5-2.5 % (w/v) agarose, DNA grade (Bioline, England).

**Ammonium persulphate (APS):** 10% (w/v) ammonium persulphate (Sigma-Aldrich, Germany), stored at 4°C.

**Ampicillin:** 100 mg/mL ampicillin stock solution (CSL, Australia), filtered through a 0.2 μm filter. Working concentration of 100 μg/mL, aliquots stored at -20°C.

**Bacteriological agar:** (Oxoid, England).

**BCIP/NBT substrate solution:** BCIP/NBT (Amresco, U.S.A.), stored at 4°C. Mixed 1:1 with detection buffer before use.

**Blocking buffer:** 5% skim milk (Diploma, Australia) prepared in TBS before use.

**Blocking solution:** PBS containing 3% BSA prepared before use.

**Bovine serum albumin (BSA):** 1 – 10 mg/mL BSA (Promega, U.S.A.), aliquots stored at -20°C.

**Bradford reagent:** 100 mg Coomassie Brilliant G-250 (Sigma-Aldrich, Germany) dye dissolved in 50 mL 95% (v/v) ethanol (Merck, Germany), then mixed with 100 mL 85% (v/v) phosphoric acid (Chem Supply, Australia), and brought up to 1 L with dH2O. Stored at 4°C and filtered through a 0.45 μm filter before use.

**Bromophenol blue:** (BDH, U.S.A.).

**Chelating sepharose™ fast flow:** (GE healthcare, Sweden), charged with 0.2 M nickel sulphate (NiSO₄) (BDH, U.S.A.) or 0.2 M cobalt chloride (Co₂Cl) (BDH, U.S.A.)

**Chloramphenicol:** 34 mg/mL Chloramphenicol stock solution (Sigma-Aldrich, Germany) in 96% ethanol (Merck, Germany), filtered through a 0.45 μm filter. Working concentration of 34 μg/mL, aliquots stored at -20°C.

**ClearPAGE Instant Blue Stain:** (C.B.S. Scientific Company, U.S.A.), stored at 4°C.

**Cobalt chloride (Co₂Cl):** 0.2 M Co₂Cl (BDH, U.S.A.), filtered through a 0.45 μm filter.

**Coomassie Brilliant G-250:** (Sigma-Aldrich, Germany).

**Deoxynucleoside triphosphates (dNTP):** 10 mM of each dATP, dTTP, dGTP, and dCTP (Bioline, England), stored at -20°C.

**Detection buffer:** 100 mM Tris-HCl (Merck, Germany), 100 mM NaCl (BDH, U.S.A.), pH 9.5.

**Dextran sulfate:** 10 mg/mL dextran sulfate (Sigma-Aldrich, Germany), stored at -20°C.

**DNA loading buffer (11x):** 10% (w/v) ficoll®-400 (BDH, U.S.A.), 50% (v/v) glycerol (BDH, U.S.A.), 0.5% (w/v) Orange G (Sigma-Aldrich, Germany), 1% (w/v) SDS (Merck, Germany), 10 mM EDTA (BDH, U.S.A.), 50 mM Tris-HCl (pH 8.0) (Merck, Germany).

**Ethanol:** 70% (v/v), 96% (v/v), 100% (v/v) (Merck, Germany).

**Ethidium bromide (EtBr):** 6 mg EtBr (Sigma-Aldrich, Germany) in 2 L MQ water.

**Ethylenediaminetetra-acetate (EDTA):** 0.5 M EDTA (Merck, Germany).

**Formaldehyde:** 37% (Sigma-Aldrich, Germany).

**Glycerol:** 10% (v/v) sterile glycerol (BDH, U.S.A.).

**HisPur Cobalt 1 mL spin columns:** (ThermoFisher Scientific, U.S.A.), stored at 4°C.
Hydrochloric acid (HCl): 32% (Merck, Germany).

**iBlot Transfer stacks:** iBlot gel transfer stacks (nitrocellulose), Regular (2 gels) or Mini (1 gel) (Invitrogen Corporation, U.S.A.).

**IMAC stripping solution:** 0.5 M NaCl (BDH, U.S.A.), 50 mM EDTA (Merck, Germany).

**Imidazole:** 5 M Imidazole (Sigma-Aldrich, Germany), filtered through a 0.45 μm filter, stored at 4°C in a light-proof container.

**Isopropyl-β-D-thiogalactopyranoside (IPTG):** 1 M IPTG (Progen, Australia) filtered through a 0.2 μm filter, aliquots stored at -20°C.

**Lambda (λ) DNA:** stored at -20°C (Promega, U.S.A.).

**MES/NaCl buffer:** 20 mM 2-(N-morpholine)-ethanesulfonic acid (Sigma-Aldrich, Germany), 0.1 M NaCl (BDH, U.S.A.), pH 5.0.

**Methanol:** 100% (Merck, Germany).

**Mini-PROTEAN TGX Precast gels:** 4 – 15% (BioRad Laboratories, U.S.A.), stored at 4°C.

**Molecular grade water (MGH2O):** Distilled water, DNAse and RNase free (Invitrogen Corporation, U.S.A.).

**Nickel sulfate (NiSO4):** 0.2 M NiSO4 (BDH, U.S.A.), filtered through a 0.45 μm filter.

**PBST:** PBS (Oxoid, England) with 0.05% (v/v) Tween 20 (Promega, U.S.A.).

**Phenylmethanesulfonyl fluoride (PMSF):** 200 mM (Sigma-Aldrich, Germany) in anhydrous (100%) ethanol (Merck, Germany), stored at 4°C.

**Phosphate buffered saline (PBS):** 1 tablet Dulbecco’s A PBS (Oxoid, England) in 100 mL mH2O (sodium chloride 0.8%, potassium chloride 0.02%, disodium hydrogen phosphate 0.115% potassium dihydrogen phosphate 0.02%).

**Phosphoric acid:** 85% (Chem-Supply, Australia).

**Precision Plus Protein™ Dual Colour Standard:** 10 μL per well (BioRad Laboratories, U.S.A.), stored at -20°C.

**Precision Plus Protein™ Kaleidoscope™ prestanted standard:** 10 μL per well (BioRad Laboratories, U.S.A.), stored at -20°C.

**Precision Plus Protein™ standard:** 10 μL per well (BioRad Laboratories, U.S.A.), stored at -20°C.

**Protein binding buffer:** 50 mM Tris-HCl (pH 8.0) (Merck, Germany), 150 mM NaCl (BDH, U.S.A.), 10 mM imidazole (Sigma-Aldrich, Germany), prepared before use.

**Protein elution buffer:** 50 mM Tris-HCl (pH 8.0) (Merck, Germany), 150 mM NaCl (BDH, U.S.A.), 100 mM – 200 mM Imidazole (Sigma Aldrich, Germany), prepared before use.

**Protein lysis buffer:** 50 mM Tris-HCl (pH 8.0) (Merck, Germany), 150 mM NaCl (BDH, U.S.A.), 20 mM imidazole (Sigma-Aldrich, Germany), 1 mg/mL lysozyme (Amresco, U.S.A.), prepared before use.

**Protein storage buffer:** 50 mM Tris-HCl (pH 8.0) (Merck, Germany), 150 mM NaCl (BDH, U.S.A.), stored at 4°C.
**Protein wash buffer**: 50 mM Tris-HCl (pH 8.0) (Merck, Germany), 150 mM NaCl (BDH, U.S.A.), with increasing concentrations of imidazole (20, 50, 100, and 150 mM), prepared before use.

**Resolving SDS-PAGE gel**: For 10 mL of 10% separating gel: 4.8 mL dH₂O, 2.5 mL 40% (v/v) acryl/bis (Amresco, U.S.A.), 2.5 mL 1.5 M Tris-HCl (pH 8.8) (Merck, Germany), 0.1 mL 10% (w/v) SDS (Merck, Germany), 0.1 mL 10% (w/v) ammonium persulfate (Sigma-Aldrich, Germany), 0.004 mL Tetra-methyl-ethylenediamine (TEMED) (BioRad Laboratories, U.S.A.).

**Ribonuclease A (RNaseA)**: 0.1 µg/µL (Sigma-Aldrich, Germany), stored at -20°C.

**S30 T7 High-Yield Protein Expression System**: stored at -80°C (Promega, U.S.A.).

**SDS sample loading buffer (5 x)**: 60 mM Tris-HCl (Merck, Germany), 25% (v/v) glycerol (BDH, U.S.A.), 2% (w/v) SDS (Merck, Germany), 1.4 mM 2-mercaptoethanol (BioRad Laboratories, U.S.A.), 0.1% (w/v) bromophenol blue (Sigma-Aldrich, Germany), stored at -20°C.

**SDS-PAGE running buffer (1 x)**: 3% (w/v) Tris-HCl (Merck, Germany), 14.2% (w/v) glycine (Amresco, U.S.A.), 1% (w/v) SDS (Merck, Germany).

**Skim milk**: powder (Diploma, Australia).

**SNAPtide® hydrolysis buffer**: 50 mM HEPES (pH 7.4) (Invitrogen Corporation, U.S.A.), 0.05% Tween 20 (Promega, U.S.A.).

**Sodium acetate (NaOAc)**: 3 M NaOAc (pH 5.2) (BDH, U.S.A.).

**Sodium Chloride (NaCl)**: 0.15 M to 5 M NaCl (BDH, U.S.A.).

**Sodium dodecyl sulphate (SDS)**: 10% (w/v) (Merck, Germany)

**Sodium Hydroxide (NaOH)**: 0.1 M NaOH (Merck, Germany).

**Stacking SDS-PAGE gel**: For 5 mL of 5% stacking gel: 3.4 mL dH₂O, 0.83 mL 40% (v/v) acryl/bis (Amresco, U.S.A.), 0.63 mL 1.0 M Tris-HCl (pH 6.8) (Merck, Germany), 0.05 mL 10% (w/v) SDS (Merck, Germany), 0.05 mL 10% (w/v) ammonium persulfate (Sigma-Aldrich, Germany), 0.005 mL TEMED (BioRad Laboratories, U.S.A.).

**Sulphuric acid (H₂SO₄)**: 1 M sulphuric acid (BDH, USA).

**TBST**: TBS with 0.05% (v/v) Tween 20 (Promega, U.S.A.).

**TEMED (N,N,N',N'-tetramethylethylenediamine)**: Electrophoresis purity (BioRad Laboratories, U.S.A.).

**Tetramethylbenzidine (TMB)**: TMB substrate reagent A and substrate reagent B (BD Biosciences, U.S.A.) were mixed in a ratio of 1:1 and used within 10 min, stored at 4°C.

**Tris (hydroxymethyl) amino methane (Tris-HCl)**: 1 M stock solution (Merck, Germany), adjusted to pH 8.0 with HCl.

**Tris-Acetate-EDTA (TAE) buffer (1x)**: 40 mM Tris-HCl (pH 8.0) (Merck, Germany), 20 mM acetic acid (Merck, Germany), 2 mM EDTA (Merck, Germany).

**Tris Buffered Saline (TBS)**: 25 mM Tris-HCl (pH 7.4) (Merck, Germany), 0.18 M NaCl (BDH, U.S.A.).
**Tryptone:** (Oxoid, England).

**Tween 20:** polyoxethylene sorbitan monolaurate (Promega, U.S.A.).

**Urea:** 2 to 8 M urea (BDH, U.S.A.) in 0.1 M Tris-HCl (pH 8.0).

**VAMP-2 Cleavage buffer:** 20 mM Tris-HCl (pH 8.0) (Merck, Germany), 50 mM NaCl (BDH, U.S.A.).

**Whole cell lysis buffer:** 0.1 M Tris-HCl (pH 8.0) (Merck, Germany), 2% (w/v) SDS (Merck, Germany), 15% (v/v) glycerol (BDH, U.S.A.).

**Yeast extract:** (Oxoid, England).

### 2.3.2 General media

All media was prepared using distilled water and sterilised by autoclaving at 121°C for 15 min. For agar media, bacteriological agar was added prior to autoclaving.

**EnPresso™ tablet cultivation set:** EnBase® medium tablets (white bag), booster tablet (black bag), EnzI'm (600 U/L) stored at 4°C, AirOtop seal flask closures (BioSilta, Finland).

**Luria-Bertani (LB) broth:** 1% (w/v) tryptone (Oxoid, England), 0.5% (w/v) yeast extract (Oxoid, England), 1% (w/v) NaCl (BDH, U.S.A.). For LB agar 1% (w/v) bacteriological agar (Oxoid, England) was added.

### 2.3.3 Enzymes

All enzymes were used in the supplied buffer according to the manufacturer’s instructions, and were stored at -20°C unless otherwise stated.

**Deoxyribonuclease I (DNAse I):** 10 U/μL DNAse A (Sigma-Aldrich, Germany).

**EKMax™ Enterokinase:** 1.0 U/μL (Invitrogen Corporation, U.S.A.).

**Expand polymerase:** Expand long template enzyme (ELT), system 1 (Roche, Germany).

**Lysozyme solution:** 10 mg/mL lysozyme (Boehringer Mannheim, Germany) in dH₂O, filtered through a 0.45 μm filter, stored at -20°C.

**T4 DNA ligase:** 3 U/μL T4 DNA ligase (Promega, U.S.A.).

### 2.3.3.1 Restriction enzymes

Restriction enzymes were used in the supplied buffer according to the manufacturer’s instructions and stored at -20°C. The recognition sequences are listed in Table 2.1.
**EcoRI**: 12 U/μL (Promega, U.S.A).
**PstI**: 10 U/μL (Promega, U.S.A).
**PvuII**: 12 U/μL (Promega, U.S.A).
**Xhol**: 10 U/μL (Roche, Germany).

### 2.3.4 Antibodies

- **Goat anti-rabbit IgG – AP**: stored at 4°C (Abcam, England).
- **Goat anti-rabbit IgG – HRP**: stored at 4°C (Abcam, England).
- **Mouse anti-His IgG**: stored at -20°C (GE Healthcare, Sweden).
- **Rabbit anti-** *Clostridium botulinum A toxoid IgG**: stored at -20°C (Abcam, England).
- **Rabbit anti-tetanus toxin IgG**: stored at 4°C (Abcam, England).

### 2.3.5 Commercial kits

- **DNA sequencing kit (ABI version 3.1)**: Big Dye, 5x dilution buffer, stored at -20°C (Monash University, Australia).
- **Enterokinase removal kit**: stored at 4°C (Invitrogen Corporation, U.S.A.).
- **PureYield™ plasmid miniprep system**: (Promega, U.S.A.).
- **QIAquick gel extraction kit**: (Qiagen, Germany).
- **QIAprep spin miniprep kit**: (Qiagen, Germany).

### 2.3.6 Toxins

- **Botulinum type A light chain, recombinant protein**: 10 µg, stored at -20°C (List Biological Laboratories, U.S.A.).
- **Tetanus toxin light chain**: 10 µg, stored at -20°C (List Biological Laboratories, U.S.A.).
- **Tetanus toxin C-fragment**: 10 µg, stored at 4°C (List Biological Laboratories, U.S.A.).
- **Tetanus toxin C-fragment**: 10 µg, stored at 4°C (Sigma-Aldrich, Germany).
- **Tetanus toxin from *C. tetani***: 25 µg, stored at 4°C (Sigma-Aldrich, Germany).
Table 2.1: Restriction enzymes used in this study and their recognition sequence.

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Recognition sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>…G↓AATTC…</td>
</tr>
<tr>
<td>KpnI</td>
<td>…GGTAC↓C…</td>
</tr>
<tr>
<td>PstI</td>
<td>…CTGCA↓G…</td>
</tr>
<tr>
<td>PvuII</td>
<td>…CAG↓CTG…</td>
</tr>
<tr>
<td>XhoI</td>
<td>…C↓TCGAG…</td>
</tr>
</tbody>
</table>

Where ▼ represents the point of cleavage.
2.3.7 Substrates

GST-Synaptobrevin-2 (GST-VAMP-2), recombinant protein: 100 µg, stored at -20°C (List Biological Laboratories, U.S.A.).

SNAPtide® (FITC/DABCYL) FRET substrate: 200 nmoles, stored at -20°C (List Biological Laboratories, U.S.A.).

2.4 Microbiological methods

2.4.1 Bacterial strains, plasmids and recombinant proteins

The description of all the bacterial strains used throughout this study is listed in Table 2.2. A description of all plasmid vectors used and those developed throughout the study is listed in Table 2.3 and Table 2.4, respectively.

2.4.2 Bacterial storage conditions

*Escherichia coli* strains were stored at -80°C in 15% glycerol.

2.4.3 Bacterial Culture Conditions

All *E. coli* bacteria containing plasmids were grown in LB broth or on LB agar containing 100 µg/mL ampicillin and 34 µg/mL Chloramphenicol at 37°C unless stated otherwise. *Escherichia coli* BL21(DE3)pLysS not containing any plasmid construct was grown in LB broth with 34 µg/mL chloramphenicol for maintenance of the pLysS plasmid at 37°C unless stated otherwise. All media was sterilised by autoclaving at 121°C for 15 min. All broths inoculated with *E. coli* were shaken on a Ratek orbital shaker at 200 revolutions per min (rpm).
Table 2.2: Bacterial strains used throughout this study.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Genotype/description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> BL21(DE3)pLysS</td>
<td><em>E. coli</em> B F&lt;sup&gt;-&lt;/sup&gt; dcm ompT hsdS (r&lt;sub&gt;B&lt;/sub&gt;− m&lt;sub&gt;B&lt;/sub&gt;−) gal λ(DE3) [pLysS Cam&lt;sup&gt;R&lt;/sup&gt;]</td>
<td>Invitrogen Corporation, U.S.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F'/&lt;em&gt;end&lt;/em&gt;A1 hsdR17&lt;sup&gt;(rK-, mK+)&lt;/sup&gt; supE44 thi-1 recA1 gyrA96 (NalR) relA1 Δ(lacZYAargF) U169 Φ80d lacZ_M15</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td></td>
<td>deoR phoA λ&lt;sup&gt;−&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> Origami&lt;sup&gt;TM&lt;/sup&gt;2(DE3)pLysS</td>
<td>Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F&lt;sup&gt;+&lt;/sup&gt; [lac+ laci&lt;sub&gt;l&lt;/sub&gt;] pro] (DE3) gor522::Tn10 trxB pLysS (Cam&lt;sup&gt;R&lt;/sup&gt;, Str&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Novagen, Germany</td>
</tr>
</tbody>
</table>
**Table 2.3: Plasmids used throughout this study.**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype/description</th>
<th>Diagram</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC57</td>
<td>2.7 kb cloning plasmid</td>
<td>Figure 3.1</td>
<td>Fermentas, Canada</td>
</tr>
<tr>
<td>pRSET-A</td>
<td>2.9 kb protein expression plasmid, N-terminal His₆-tag, T7 promoter, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Figure 3.2</td>
<td>Invitrogen Corporation, U.S.A.</td>
</tr>
</tbody>
</table>

Vector maps in Chapter 3.
Table 2.4: Expression plasmids and description of the proteins used and developed throughout this study.

<table>
<thead>
<tr>
<th>Expression plasmid</th>
<th>Protein</th>
<th>Description/Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTLC</td>
<td>TLC</td>
<td>Recombinant codon optimised tetanus toxin light chain</td>
<td>GenScript, U.S.A.</td>
</tr>
<tr>
<td>pTHC</td>
<td>THC</td>
<td>Recombinant codon optimised tetanus toxin heavy chain</td>
<td>GenScript, U.S.A.</td>
</tr>
<tr>
<td>pB/ALC</td>
<td>B/ALC</td>
<td>Recombinant codon optimised botulinum toxin type A light chain</td>
<td>GenScript, U.S.A.</td>
</tr>
</tbody>
</table>

Vector maps in cloning chapter
2.4.4 Bacterial manipulation

2.4.4.1 Preparation of electrocompetent cells

Two millilitres of an overnight *E. coli* culture was used to inoculate 200 mL LB broth and the cells were grown at 37°C with vigorous shaking until absorbance at 600 nm was 0.3 – 0.4 (early mid log phase). When cells reached mid log phase, the flask was chilled on ice for 15 – 30 min. All centrifugation steps were carried out in a 4°C centrifuge for 15 min at 4,000 x g. Aliquots of 50 mL were transferred to 50 mL sterile tubes and pelleted by centrifugation. The cells were resuspended in 50 mL ice-cold sterile MQ water and pelleted by centrifugation. The cells were then resuspended in 25 mL ice-cold sterile MQ water and pelleted again. Cells were resuspended in a total of 5 mL of ice-cold 10% sterile glycerol, and cells were then combined and pelleted a final time. Ten per cent ice-cold sterile glycerol was added to a final volume of 50% of the cell pellet and divided into 40 μL aliquots. Electrocompetent cells were stored at -80°C.

2.4.4.2 Electrotransformation

This method was obtained from the manual supplied with the BioRad Gene Pulser apparatus. One microliter of purified ligation mixture was added to 40 μL of electrocompetent *E. coli* cells prepared above. The cells and ligation mixture were transferred to an ice-cold sterile electroporation cuvette (Section 2.2) and tapped lightly to settle the mixture to the bottom and remove bubbles. The cuvette was placed in the apparatus and the Gene Pulser (Section 2.2) was set at 25 μF, 200 Ω, and 2.50 kV. The sample was pulsed once and 1 mL of LB broth was immediately added to the cuvette to resuspend the cells. After transferring the medium to a 1.5 mL microfuge tube, samples were incubated on a shaker at 37°C for 1 h. One hundred microliters of sample was plated onto LB agar containing the appropriate antibiotics and the remaining sample pelleted by centrifugation and resuspended in 150 μL of supernatant, then spread onto LB agar containing antibiotics, and incubated at 37°C overnight.
2.5 DNA methods

2.5.1 Nucleic acid isolation

2.5.1.1 Plasmid DNA extraction (miniprep)

Plasmids were grown in *E. coli* DH5α and purified using the QIAprep Spin Miniprep kit (Qiagen, Germany) as per the manufacturer’s protocol.

2.5.1.2 Plasmid DNA extraction for cell-free expression (miniprep)

The PureYield™ plasmid miniprep system (Promega, U.S.A.) was used to achieve highly pure and endotoxin-free plasmid DNA for use in cell-free expression. Plasmids were grown in *E. coli* DH5α and purified according to the manufacturer’s protocol.

2.5.1.3 DNA extraction from agarose

Digested inserts were separated by electrophoresis on a 1.5% - 2.5% agarose gel. The DNA was excised from the gel and then purified using the QIAquick gel extraction kit (Qiagen, Germany), as per the manufacturer’s protocol.

2.5.2 DNA analysis

2.5.2.1 Lambda DNA marker

A solution of 50 ng/μL digested λ-DNA was prepared by incubating 50 μg λ-DNA (Promega, U.S.A.) with 180 U of *Pst*I, 90 μL of 10x Buffer H, 1 mg/mL BSA and MQ water made up to 900 μL, at 37°C overnight. Two microliters of 11x DNA loading buffer was added to 18 μL of the digested λ-DNA and run on a 1.5% DNA agarose gel to check for complete digestion. Eighty eight microlitres of 11x DNA loading buffer was then added to the remaining digestion to make up the standard marker. Twenty microliters was run on each DNA agarose gel. The marker was stored at 4°C. A diagram of *Pst*I digested λ-DNA is shown in Appendix 1.
2.5.2.2 Agarose gel electrophoresis

Agarose gels ranging from 1.5% – 2.5% were used to separate and visualise DNA samples. DNA samples mixed with 2 μL of 11x DNA loading buffer, and 20 μL of λ-PstI marker containing loading dye were loaded onto the gel. After electrophoresis at voltages ranging from 60 V to 100 V, gels were stained in a 3 mg/mL EtBr water bath for 5 min, and then destained under running water for 30 min.

DNA products were visualised with a BioRad GelDoc transilluminator (Section 2.2) and photographed using the GelDoc imaging system (Section 2.2). The relative sizes of DNA products were estimated by comparison to the λ-PstI marker (Appendix 1).

2.5.3 Quantification of nucleic acids

Quantitation of nucleic acids was performed using one of two methods. For precise quantitation of DNA, a spectrophotometer was used, and for approximate quantitation of DNA, DNA was separated on an agarose gel and compared to known standards of the λ-PstI marker (Appendix 1).

2.5.3.1 Quantitation of DNA by gel electrophoresis

Lambda DNA marker (Section 2.5.2.1) was separated on an agarose gel along with sample DNA fragments. The quantity of each digested sample fragment was compared with intensities of known quantities of DNA within the DNA marker.

2.5.3.2 Spectrophotometric quantification of DNA

The Eppendorf BioPhotometer was used to determine the optical density (OD) of a DNA solution at a wavelength of 260 nm. Protein contamination was indicated by reading at 280 nm. An OD of 1.0 was equal to 50 µg/mL of double-stranded DNA.
2.5.4 DNA manipulation

2.5.4.1 Restriction enzyme digestion of DNA

The restriction enzymes used in this study are listed in Table 2.1. Five units of each restriction enzyme was used in the supplied buffer for restriction enzyme digestion of 50 – 100 ng of plasmid DNA, with 2 µg BSA in a final volume of 20 µL. Reactions were incubated in a 37°C water bath for 4 h. After digestion, the entire volume was separated on a 1.5% – 2.5% agarose gel.

2.5.4.2 Sequencing PCR

Forward or reverse T7 promoter primers were used for sequence analysis of constructs. This analysis was performed to confirm the presence and orientation of inserts and to confirm all inserts were in frame with the start codon of the plasmid vector. Sequencing components (Table 2.5), cycling conditions (Table 2.6) and precipitation of PCR products (Section 2.5.4.4) were performed as per the protocol provided by Micromon DNA Sequencing Facility, Monash University, Melbourne, Australia.

2.5.4.3 Primers

Primers were obtained as lyophilised samples from Geneworks, Australia. The description of the sequencing primers used in this study is shown in Table 2.7.

2.5.4.4 Precipitation of DNA from solution

For 20 µL of DNA, 2 µL of 3 M sodium acetate pH 5.2, and 50 µL of absolute ethanol (96%) were added, mixed and left at room temperature for 10 min. Samples were pelleted by centrifugation for 15 min and the DNA recovered by ethanol precipitation. Samples were centrifuged for 2 min and the DNA pellet was left to dry at room temperature under sterile conditions. DNA was resuspended in 10 µL of MG H₂O.
Table 2.5: Sequencing reaction components.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 Forward OR Reverse Primer (50 ng/μL)</td>
<td>1</td>
</tr>
<tr>
<td>Big Dye</td>
<td>2</td>
</tr>
<tr>
<td>5x Dilution buffer</td>
<td>3</td>
</tr>
<tr>
<td>Template DNA</td>
<td>300 ng</td>
</tr>
<tr>
<td>MQ water</td>
<td>To 20 μL</td>
</tr>
<tr>
<td><strong>Total reaction volume</strong></td>
<td><strong>20 μL</strong></td>
</tr>
</tbody>
</table>
Table 2.6: Sequencing reaction cycling conditions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (ºC)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STAGE 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>96</td>
<td>1 min</td>
</tr>
<tr>
<td><strong>STAGE 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>96</td>
<td>10 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>50</td>
<td>5 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>60</td>
<td>4 sec</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
</tr>
<tr>
<td>Name</td>
<td>Sequence 5’-3’</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>T7 Forward</td>
<td>TAATACGACTCACTATAGGG</td>
<td>Sequencing primer for pRSET-A</td>
</tr>
<tr>
<td>T7 Reverse</td>
<td>GCTAGTTATTGCTCAGCGG</td>
<td>Sequencing primer for pRSET-A</td>
</tr>
</tbody>
</table>
2.5.4.5 Ligation

Ligations were performed using digested and purified DNA at a ratio of 1:3 (vector:insert), as calculated using the formula below from Sambrook (2001).

\[
\text{ng of vector required} \times \frac{\text{size of insert (kb)}}{\text{Size of vector (kb)}} \times \text{molar ratio of insert to vector (1:3)} = \text{ng of insert required for ligation}
\]

Typically, ligations were performed with the addition of 2 Weiss Units of T4 DNA ligase in the supplied buffer, and 1 mM ATP in a final volume of 10 μL. Reactions were incubated in a 16°C water bath overnight. The ligated DNA was then purified prior to electrotransformation.

2.5.4.6 Purification of ligation DNA for electroporation

In preparation for electroporation, ligation products were precipitated from the buffers used in the reaction, to prevent arcing during electrotransformation. For a 10 μL ligation reaction, 0.5 μL of dextran sulfate (10 mg/mL stock) and 1 μL of 3 M sodium acetate were added and mixed. Twenty five microliters of absolute ethanol was added to the mix and precipitated DNA was pelleted by centrifugation for 15 min at 16,000 x g. The pellet was resuspended in 100 μL 70% (v/v) ethanol and centrifugation was repeated for 5 min. The supernatant was removed and the pellet left to dry at room temperature under sterile conditions. The DNA was resuspended in 5 μL of molecular grade water and 1 μL was used for electrotransformation. The remaining DNA was stored at -20°C.
2.6 Protein methods

2.6.1 Protein quantitation

2.6.1.1 Bradford Assay

The protein concentration of purified samples was determined by the Bradford method. Standard albumin solution (1 mg/mL) was used to prepare protein standards of 0 to 20 µg. Ten microlitres of sample was made up to 100 µL with 0.15 M NaCl to which 1 mL of Bradford reagent (Section 2.3.1) was added, mixed well and allowed to stand for 2 min at room temperature. Two hundred microlitre aliquots of each sample were transferred to a 96-well microtitre tray in duplicate and absorbance was read at 600 nm.

2.6.1.2 Spectrophotometric quantification of protein

The Eppendorf BioPhotometer was used to determine the optical density (OD) of a protein solution at a wavelength of 280 nm. The concentration of the protein sample (mg/mL) was equal to the absorbance at 280 nm (with a path length of 1 cm).

2.6.2 Protein visualisation

2.6.2.1 SDS-PAGE

Proteins were separated by one-dimensional 10% reducing SDS-PAGE. After pouring the resolving gel, distilled water was poured over the gel to ensure the gel interface was level after it set. After the gel had set and the water removed, the 5% stacking gel was then layered over the top of the resolving gel and a cast inserted between the glass slides to form the wells. The protein gel system was assembled as per the manufacturer’s protocol (BioRad Laboratories), and then placed within the gel tank containing 1 x SDS-PAGE running buffer. Protein samples were added to 5 x SDS sample loading buffer, boiled for 3 min and then loaded onto the gel together with a Precision Plus Protein™ Standard (Appendix 2). The prestained Kaleidoscope™ protein standard (Appendix 2), or Precision Plus Protein™ Dual Colour Standard (Appendix 2), was used if the gel was to be used for immunoblotting, while the unstained Precision Plus Protein™ standard was used for gels that were to be stained by Coomassie. Electrophoresis was performed at 60 V for 30 min, and then 180 V for 50 min.
2.6.2.2 Coomassie instant stain

Instant stain was used in place of Coomassie staining for some gels. Thirty millilitres of ClearPAGE Instant Blue Stain (C.B.S. Scientific Company, U.S.A.), or Acqua Stain (Geneworks, Australia) (Section 2.3.1), (or enough to cover gel(s)) was used for staining SDS-PAGE gels after electrophoresis. Bands were visible within 10 – 15 min, however Coomassie instant Stain was allowed to resolve bands for up to 1 h. A destaining step was not required with the use of ClearPage Instant Stain and Acqua Stain.

2.6.2.3 Immunotransfer

Western blotting was used to transfer proteins from an SDS-PAGE gel to a nitrocellulose membrane for further analysis. After SDS-PAGE, the gel was removed from the gel system and the stacking gel removed. The iBlot dry blotting system was used according to the manufacturer’s instructions. The order of materials set up for the iBlot system is shown below built from the anode stack, bottom:

1. Anode stack, bottom
2. Protein gel
3. iBlot filter paper (pre-soaked in deionised water)
4. Cathode stack, top
5. Disposable sponge (attached to the lid of the apparatus)

The cassette was closed after removing any air bubbles from the sandwich with the blotting roller and the transfer was performed (Program 3; 7 min). After transfer was complete, the nitrocellulose membrane was used for immunoblotting and all other components were discarded.

2.6.2.4 Immunoblotting

The nitrocellulose membrane was removed from the iBlot system and blocked in blocking buffer (Section 2.3.1), for 1 h. All incubations and washes were performed on a rotating shaker. The membrane was washed twice in TBS for 2 min, and then the primary antibody diluted in blocking buffer was added to the blot and left for 2 h at room temperature (or overnight at 4ºC). The blot was washed twice in TBS for 5 min, and the secondary antibody conjugated with AP was added and incubated at room temperature for up to 2 h. The blot was washed three times in TBST for 5 min and then incubated in detection buffer for 5-10
min. To develop the AP conjugate, the blot was immersed in BCIP/NBT substrate solution until the desired resolution was achieved. The blot was washed in dH2O to stop the reaction.

2.6.3 Protein expression and purification

2.6.3.1 Protein expression

For protein expression, constructs were electrotransformed into *E. coli* BL21(DE3)pLysS or *E. coli* Origami™2(DE3)pLysS for each expression (Section 2.4.4.2). The next step varied for the growth medium used for protein expression; however, in all cases *E. coli* BL21(DE3)pLysS or *E. coli* Origami™2(DE3)pLysS containing the expression vector was grown in the presence of 100 mg/mL ampicillin and 34 mg/mL chloramphenicol.

2.6.3.2 Pilot expression study

Following electrotransformation of constructs into *E. coli* BL21(DE3)pLysS or *E. coli* Origami™2(DE3)pLysS and overnight grown on LB agar containing antibiotics, a single colony was inoculated into 2 mL LB broth containing antibiotics and grown at 37°C until the absorbance at 600 nm reached 0.6 – 1.0, after which time the culture was left at 4°C overnight. The next day, the 2 mL pre-culture was pelleted by centrifugation and resuspended in 2 mL fresh LB broth. Fifty millilitres of LB broth was inoculated with 1% pre-culture and antibiotics, and grown at 37°C until the absorbance at 600 nm reached 0.3 – 0.6. A 1 mL aliquot was taken before a final concentration of 1 mM IPTG was added to the remaining culture. One millilitre aliquots were taken at 1 – 5 h after IPTG induction. Cell pellets were washed twice in 1 mL of 10 mM Tris-HCl, pH 8.0, resuspended in 0.5 mL whole cell lysis buffer (Section 2.3.1), and then boiled for 3 min. Samples were separated by SDS-PAGE gel (Section 2.6.2.1).

2.6.3.3 Protein expression in LB broth

The next day, a single colony was inoculated into 2 mL LB broth containing antibiotics and grown at 37°C until the absorbance at 600 nm reached 0.6 – 1.0, after which time the culture was left at 4°C overnight. The next day, the 2 mL pre-culture was pelleted by centrifugation and resuspended in 2 mL fresh LB broth. Two hundred millilitres of LB broth in a 1 L flask was inoculated with 1% pre-culture and antibiotics and grown at the desired temperature (25°C, 30°C, 37°C, 42°C), until the absorbance at 600 nm reached 0.3 – 0.6. A final
concentration of 1 mM IPTG was added and protein expression was induced at the required
temperature for the length of time found to be optimal through the pilot study.

2.6.3.4 Protein expression with EnPresso™ tablet media set (BioSilta)

Protein expression was conducted following the manufacturer’s protocol. Briefly, following
electrotransformation of the construct into *E. coli* BL21(DE3)pLysS or *E. coli*
Origami™2(DE3)pLysS, the next day a single colony was inoculated into 2 mL LB broth
containing antibiotics and grown at 37°C for 7 h. Five hundred microlitres of the pre-culture
was added to 50 mL sterile dH₂O in a 500 mL flask along with the antibiotics, the white bag
containing the media in tablet form, and 25 µL of EnzI’m, both of which were supplied in the
kit from BioSilta (Section 2.3.2). The flask was sealed with an AirOtop seal (BioSilta). The
culture was grown overnight at 30°C and the next day a final concentration of 1 mM IPTG
was added along with the black bag containing the glucose tablet and 50 µL of EnzI’m
(BioSilta) to the culture and induction of protein expression was allowed to proceed for 8 h.

2.6.3.5 Preparation of cell lysate

Following induction of expression, cells were harvested by centrifugation at 4,700 x g for
15 min at 4°C. The cell pellet was resuspended in 5 mL of protein lysis buffer per gram
weight of pellet and left at -80°C overnight (first freeze). The next day, a total of four
freeze/thaw cycles was performed using a -80°C freezer and a 37°C water bath. Thirty units
of DNase I was added after the second cycle and incubated at 37°C for 10 min. For some
samples, a final concentration of 1 mM PMSF was added at each thaw step for a total of four
times. Cell debris was pelleted by centrifugation at 4,700 x g for 15 min at 4°C. Clarification
was repeated if necessary. The cell lysate was then applied to nickel or cobalt charged
sepharose resin pre-equilibrated with binding buffer containing the same concentration of
imidazole as was in the protein lysis buffer.

2.6.3.6 Cell-free protein expression

Expression vectors used in cell-free expression were first purified using the PureYield™
plasmid miniprep system (Promega, U.S.A.), following the manufacturer’s instructions.

Cell-free protein synthesis was performed using the S30 T7 High-yield protein expression
system (Promega, U.S.A.), as per the manufacturer’s protocol. Briefly, T7 S30 extract
(circular), S30 Premix plus and the plasmid DNA template with a T7 promoter were
combined and incubated at 37°C with shaking at 300 rpm. Reactions were performed in 50 µL volumes. Reaction components are listed in Table 2.8.

Reactions were stopped by incubation in an ice water bath for 5 min. Five microliters of the cell-free reaction was combined with 14 µL of TBS and 1 µL of 0.1 µg/µL RNaseA and incubated at room temperature for 5 min. Samples representing 10% of the total cell-free reaction were analysed by SDS-PAGE (Section 2.6.2.1).

2.6.4 Detection and solubilisation of inclusion bodies

2.6.4.1 Detection of inclusion bodies

Following induction of protein expression in *E. coli* BL21(DE3)pLysS or *E. coli* Origami™2(DE3)pLysS, 1 mL of culture was pelleted by centrifugation at 10,000 x g for 1 min. The cell pellet was resuspended in 100 µL protein lysis buffer (Section 2.3.1). Cells were lysed by a total of four freeze/thaw cycles using a -80°C freezer and a 37°C water bath, and 30 U DNase I was added after the second cycle and incubated at 37°C for 10 min. Cell debris was pelleted by centrifugation at 16,000 x g for 5 min. The supernatant was transferred to a new tube and the remaining pellet was resuspended in 100 µL of MQ water. Ten microliters of the pellet and supernatant samples were analysed by SDS-PAGE (Section 2.6.2.1) and visualised using with Coomassie instant stain (Section 2.6.2.2) and immunoblotting with appropriate antisera (Section 2.6.2.3 – 2.6.2.4).

2.6.4.2 Pilot solubilisation of inclusion bodies

Following induction of protein expression in *E. coli* BL21(DE3)pLysS or *E. coli* Origami™2(DE3)pLysS, the cell pellet was resuspended in 5 mL of protein lysis buffer per gram weight of pellet. Cells were lysed by a total of four freeze/thaw cycles using a -80°C freezer and a 37°C water bath, and 30 U DNase I was added after the second cycle and
Table 2.8: Reaction components for cell-free transcription/translation procedure.

<table>
<thead>
<tr>
<th>Component</th>
<th>Per 50 µL reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 S30 extract, circular</td>
<td>18 µL</td>
</tr>
<tr>
<td>S30 Premix plus</td>
<td>20 µL</td>
</tr>
<tr>
<td>DNA template</td>
<td>1 µg</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>Up to 50 µL</td>
</tr>
</tbody>
</table>
incubated at 37°C for 10 min. Cell debris was pelleted by centrifugation at 1,000 x g for 5 min at 4°C. Inclusion bodies remaining in the supernatant were pelleted by centrifugation at 27,000 x g for 15 min at 4°C. The pellet, containing inclusion bodies was resuspended in 1.5 mL MQ water and 100 µL aliquots were separated into 1.5 mL microfuge tubes. Aliquots were pelleted by centrifugation at 1,000 x g for 5 min and the supernatant was discarded. Inclusion bodies were washed in 100 µL of 0.1 M Tris-HCl of varying pH (pH 8, 9, 10, 11.5, 12 and 12.5), or 100 µL of 0.1 M Tris-HCl of varying pH and 2 M urea. Inclusion bodies were pelleted by centrifugation at 1,000 x g for 5 min and the supernatant was transferred to a new 1.5 mL microfuge tube, and pellets were resuspended in 100 µL of MQ water. Ten microliters of the pellet and supernatant samples were analysed by SDS-PAGE and visualised using with Coomassie instant stain (Section 2.6.2.2) and immunoblotting with appropriate antisera (Section 2.6.2.3 – 2.6.2.4).

2.7 Protein purification

2.7.1 Immobilised metal affinity chromatography (IMAC)

2.7.1.1 IMAC column preparation

A 1 mL gravity flow column was charged before use by passing 0.5 CV of 0.2 M nickel sulphate (NiSO₄) (Ni²⁺), or 0.2 M cobalt chloride (Co₂Cl) (Co²⁺) through the column. The column was then washed with at least 5 CV of distilled water to remove unbound metal ions and equilibrated with 5 CV of binding buffer.

2.7.1.2 IMAC purification

Cell lysate containing expressed recombinant protein was applied to a pre-equilibrated IMAC column. The column was washed with 5 CV of wash buffer containing increasing concentrations of imidazole and the flow through was collected in 1 mL fractions and analysed for unbound protein via the Bradford method (Section 2.6.1.1). No colour change indicated the absence of protein in the wash. Protein was eluted with 5 CV of elution buffer and collected in 1 mL aliquots. Samples were analysed by SDS-PAGE (Section 2.6.2.1).
2.7.1.3 IMAC column cleaning and regeneration

Gravity flow columns were re-used up to 4 times, and stripped and cleaned between purifications. The column was cleaned by stripping the nickel from the column with 5 CV of IMAC stripping solution. Residual EDTA from the stripping solution was removed by washing the column with 5 - 10 CV of 2 M NaCl, followed by washing with 5 - 10 CV of MQ water, 5 CV of 1 M NaOH and a final wash with 5 - 10 CV of MQ water. Columns were stored in 20% ethanol at 4°C and recharged with nickel or cobalt before re-use.

2.7.1.4 IMAC purification – HisPur Cobalt spin columns

HisPur Cobalt 1 mL spin columns (ThermoFisher Scientific, U.S.A.) were purchased pre-charged with cobalt and were used following the manufacturer’s instructions. All centrifugation steps were performed at 700 x g for 2 min at 4°C. Prior to use, the resin was rinsed twice with 2 CV MQ water and pre-equilibrated with binding buffer. Cell lysate was applied to the column and incubated on a rotating platform shaker for 30 min on ice, to allow binding of the His$_6$-tagged proteins to the cobalt-charged resin. The flow through was collected by centrifugation and the column was then washed 3 times with 2 CV wash buffer. The His$_6$-tagged proteins were eluted 3 times in 1 CV elution buffer. Samples were analysed by SDS-PAGE (Section 2.6.2.1).

2.7.1.5 HisPur Cobalt spin column cleaning and regeneration

After use, the HisPur Cobalt spin column was washed with 10 CV of MES/NaCl buffer (Section 2.3.1) and then 10 CV dH$_2$O. Resin was stored as 50% slurry in 20% ethanol at 4°C.

2.7.1.6 Batch purification (protein miniprep)

For small scale protein purification, His$_6$-tagged proteins were purified using a batch purification method. One hundred microlitres of sepharose resin in a 1.5 mL centrifuge tube was charged with 0.5 CV of 0.2 M NiSO$_4$. All centrifugation steps were performed at 1000 x g for 2 min. Excess nickel was removed by rinsing 3 times with 5 CV dH$_2$O and resin was pre-equilibrated with 5CV binding buffer. His$_6$-tagged protein samples were applied to the resin and incubated for 10 min on ice before washing with 5 CV wash buffer and eluting in 5 CV protein elution buffer. Samples were analysed by SDS-PAGE (Section 2.6.2.1).
### 2.7.2 Protein processing

#### 2.7.2.1 Protein concentration and buffer exchange

Proteins purified by IMAC underwent ultrafiltration using a centrifugal device to concentrate the sample and to remove any small molecular weight proteins still present after IMAC purification. Buffer exchange was performed to replace the protein elution buffer with a buffer required for downstream protein assays and for protein storage at -20°C. After pre-rinsing the Vivaspin 20 (30 kDa or 50 kDa MWCO) centrifugal device twice with 10 mL MQ water, the protein in elution buffer was added and ultracentrifugation was performed at 5,000 x g for 10 min at 4°C. After the protein elution buffer filtered through the device, and <2 mL of protein, 10 mL of protein storage buffer (PSB) (Section 2.3.1) was added to the diafiltration cup (Section 2.2) inserted into the device, and centrifugation was repeated in 10 min intervals until the volume of PSB was reduced to less than 1 mL. The retentate was then recovered and transferred from the device to a 1.5 mL microfuge tube. Typically, 250 – 500 μL of retentate was recovered. If required, concentration was adjusted so that it was no higher than 1.5 mg/mL with protein storage buffer.

#### 2.7.2.2 Storage

Proteins were stored at -20°C for up to 2 months in protein storage buffer (Section 2.3.1).

#### 2.7.2.3 His<sub>6</sub>-tag removal

Removal of the N-terminal affinity tag was performed by treatment with EKMax™ Enterokinase (Invitrogen Corporation, U.S.A.). A pilot study was performed whereby 20 µg of His<sub>6</sub>-tagged protein was incubated with various amounts of enterokinase (0, 0.001, 0.01, 0.1, 1, 4 U/reaction), for various lengths of time (1 h, 2 h, 16 h), at room temperature. Reactions were analysed by SDS-PAGE (Section 2.6.2.1), and a Western blot (Section 2.6.2.3 – 2.6.2.4), probing with antisera to the His<sub>6</sub>-tag (mouse anti-His IgG) (Section 2.3.4).

Once enterokinase treatment was optimised, the enzyme was removed from the reaction using the Enterokinase removal kit (Invitrogen Corporation, U.S.A.) (Section 2.3.5), following the manufacturer’s instructions. Following enterokinase removal from the reaction, the cleaved His<sub>6</sub>-tag was then removed by batch purification (protein miniprep), (Section 2.7.1.6). Enterokinase-treated samples were applied to sepharose resin pre-charged with 0.2 M NiSO<sub>4</sub> and equilibrated with protein wash buffer without imidazole. The enterokinase-free sample was added to the charged resin and the His<sub>6</sub>-tag was allowed to bind for 30 min on
ice. After centrifugation at 1000 x g for 2 min, the His$_6$-tag-free supernatant was recovered and analysed by SDS-PAGE (Section 2.6.2.1).
Chapter 3

Construction of neurotoxin chains from *Clostridium tetani* and *Clostridium botulinum*
3.1 Introduction

The preceding section outlined the literature relating to the problem of OSA and the requirement of a novel, less-invasive and acceptable treatment option to surgery and mechanical and non-mechanical devices. The potential of exploiting the function of CNTs to create a novel chimeric CNT neurotoxin for the treatment of OSA was also presented. In this chapter, individual toxin domains, namely botulinum type A light chain (B/ALC), and the light and heavy chains of tetanus toxin (TLC and THC), are engineered using recombinant DNA technology for expression in an *E. coli* heterologous host.

In order for the novel chimera to be useful in the treatment of OSA, the toxin is required to (i) retain the specific cell-binding activity of native TeNT and undergo retrograde axonal transport to inhibitory interneurons in the CNS, mediated by the HC domain; (ii) translocate the LC from the endosome into the cytosolic compartment of the inhibitory interneuron, mediated by the HN domain; and (iii) cleave one of the SNARE proteins to block neurotransmitter release from the inhibitory interneuron, mediated by the LC (Turton *et al.* 2002).

After recombinant protein expression of the toxin domains in *E. coli*, the purified proteins will joined via conjugation of the free thiol groups present at the C- and N-terminus of the LC and HC chains, respectively, creating the di-chain molecule which mimics the structure of native CNTs. This final chimeric toxin will comprise the catalytically active B/ALC, and the heavy chain cell-binding and translocation domains of tetanus toxin (THC). The native tetanus toxin will also be re-created by conjugating the recombinantly expressed TLC and THC, to serve as an experimental control. This recombinant tetanus toxin will be used to assess the efficiency of the expression and conjugation procedure by comparing its activity with that of native tetanus toxin.

TeNT and BoNT domains will be expressed individually and later reconstituted to create the di-chain toxins, as opposed to expression as full-length single-chain holotoxins. Safety concerns over the potential toxicity of recombinant single-chain toxins which have been shown to retain catalytic function in the un-nicked form, underpins the basis of this approach (Li, Y. *et al.*, 2001). Although it has been documented that recombinantly expressed individual toxin domains may display a reduced biological activity to the native toxin (Fairweather *et al.*, 1993), it has also been demonstrated that CNT toxin domains can be expressed individually and later reconstituted to create an active di-chain molecule with activity comparable to that of the native holotoxin (Li, Y. *et al.*, 1999; Li, Y. *et al.*, 1994).
The recombination of CNT domains has previously been performed to create chimeric holotoxins comprising recombinant fragments from both BoNTs and TeNT (Poulain et al., 1991; Wang et al., 2008), and full-length native TeNT (Li, Y. et al., 1999; Li, Y. et al., 2001), and BoNTs (Band et al., 2010; Pellett et al., 2011), have also been recombinantly expressed. TeNT HC fragments have been joined to non-CNT proteins for the delivery of therapeutic molecules to the CNS (Barati et al., 2002; Benn et al., 2005; Francis et al., 2000; Francis et al., 2004b; Francis et al., 1995; Larsen et al., 2006; Roux et al., 2006), and BoNT fragments have also been joined to non-CNT proteins to exploit their cell-targeting and catalytic functions (Bade et al., 2004; Duggan et al., 2002).

It is well known that *C. tetani* and *C. botulinum* genomes have a low GC content (Hatheway, 1990). Due to the codon bias displayed by *E. coli* for certain transfer RNA (tRNA), recombinant expression of genes with a low GC content (or high AT content), in *E. coli* has been reported to affect translation efficiency, and may lead to a reduction in the yield of protein expression (Andersson and Kurland, 1990). Due to the differences in codon usage within Clostridia, the three toxin sequences were codon optimised to suit the expression host; a method which has previously been used to increase recombinant protein expression of CNTs in *E. coli* (Makoff et al., 1989).

The objectives of this part of the study are to obtain codon optimised sequences of TLC, THC and B/ALC. The sequences will be cloned into the expression vector, pRSET-A. *Escherichia coli* will be used for the heterologous expression of the recombinant toxin domains from pRSET-A, and the recombinant proteins will contain an N-terminal His<sub>6</sub>-tag. The affinity tag will be used to aid in the purification and identification of the recombinant proteins in the next part of the study. A pilot expression study is performed on each of the proteins to assess the time and temperature required to obtain an optimal yield of recombinant protein expression. The results from the pilot study will determine the conditions used for the expression of protein in the following section.
3.2 Materials and Methods

3.2.1 Construct design

3.2.1.1 CNT genes

The gene sequences of tetanus toxin (Accession no. X06214) and botulinum type A toxin strain Hall 183 (Accession no. EF033126), were obtained from the NCBI nucleotide database.

The tetanus toxin coding sequence (CDS) consists of 4,338 nucleotides, with the TLC encoded by nucleotides 1 – 1,371, and the heavy chain by nucleotides 1,372 – 2,577. The mature TeNT protein lacks the N-terminal methionine in the LC, therefore the recombinant TeNT proteins express residues P2 – A457 of the LC, and S458 – D1315 of the HC (Eisel et al., 1986).

The botulinum type A LC is encoded by nucleotides 1 – 1,344. The mature B/ALC also lacks the N-terminal methionine, and the recombinant protein expresses residues P2 – K448 (Zhang et al., 2003).

Sequences were manipulated using the SE Central suite of analysis tools (Clone Manager), and construct maps for TLC, THC and B/ALC in pRSET-A were created using Clone Manager.

3.2.2 Codon optimisation and gene synthesis

The services of GenScript Corporation (NJ, USA), were employed to synthesise the toxin chains. Codon optimisation of the three nucleotide sequences was performed using the OptimumGene™ codon optimisation tool (GenScript Corp., U.S.A.), and sequences were optimised for expression in *E. coli*.

The synthesised toxin domains were cloned by EcoRV into pUC57 (Fermentas) plasmid cloning vector (Figure 3.1), and supplied as a 4 μg plasmid miniprep by GenScript. Following receipt, sequences were sub-cloned into pRSET-A (Figure 3.2), and further investigated for protein expression. Pilot expression was performed in *E. coli* BL21(DE3)pLysS (Invitrogen), and THC was also expressed in *E. coli* Origami™2(DE3)pLysS (Merck Millipore), to observe
Figure 3.1: pUC57 vector (Fermentas, U.S.A.).

Reproduced from pUC57 product specifications sheet (Fermentas, Canada).
Figure 3.2: pRSET vector map (top) and multiple cloning site of pRSET-A (bottom) (Invitrogen, U.S.A.).
Reproduced from pRSET-A user manual (Invitrogen, U.S.A.).
the optimal temperature for expression and duration of induction following addition of the inducer, IPTG.

### 3.2.2.1 Analysis of codon optimisation

The codon adaptation index (Sharp and Li, 1987), GC content and codon frequency distribution (Grosjean and Fiers, 1982), analysis was performed using online resources from GenScript (GenScript, 2013).

### 3.2.3 In silico protein characterisation

The SE Central suite (Clone Manager) was used to estimate the molecular weight and isoelectric point (pI) of the recombinant His$_6$-tagged proteins, and PeptideCutter (ExPASy) (Gasteiger E., 2005) was used to predict protease cleavage sites within the codon optimised sequence of the toxin chains. Hydropathicity profiles were constructed using the Hopp-Woods (Hopp and Woods, 1981) and Kyte-Doolittle algorithms (Kyte and Doolittle, 1982), and plots were created using a generator from Colorado State University (Toolkit, 2013). Protein templates for modelling were obtained from the Protein Data Bank (PDB) (Berman et al., 2000). The crystal structure of tetanus toxin light chain (PDB ID. 1Z7H) (Breidenbach and Brunger, 2005a), heavy chain (PDB ID. 1FV3) (Zozula and Rosen, 2001), and botulinum type A toxin (PDB ID. 3BTA) (Lacy et al., 1998), were used as templates to create a model of the proposed novel chimeric holotoxin consisting of B/ALC and THC. Models were constructed using Modeller 9.10 (Sali and Blundell, 1993), and viewed using Swiss-PDB Viewer version 4.0.1 (Guex and Peitsch, 1997).

### 3.2.4 Cloning of toxin chains into pRSET-A

#### 3.2.4.1 Cloning of TeNT light chain into pRSET-A

To facilitate cloning of TLC into the multiple cloning site (MCS) of pRSET-A, 5’ XhoI and 3’ PstI restriction enzyme sites were added to the nucleotide sequence during synthesis. Sequence analysis performed using the SE Central suite (Clone Manager), indicated that no additional nucleotide bases were required to ensure the TLC coding sequence remained in frame with the ATG start codon of pRSET-A.
Codon optimised TLC containing the above-mentioned 5’ and 3’ restriction sites was supplied by GenScript in the pUC57 vector (Figure 3.3). Restriction enzyme digestion of TLC from pUC57 and pRSET-A containing no insert, was performed using XhoI and PstI (Section 2.5.4.1). Following digestion, reactions were separated on a 1.5% DNA agarose gel (Section 2.5.2.2). Excision of the double digested TLC and pRSET-A from the agarose gel was performed and the DNA was purified using the QIAquick gel extraction kit (Section 2.3.5), then concentrated to a smaller volume (Section 2.5.4.4) to facilitate ligation. After overnight ligation at 16°C (Section 2.5.4.5), the DNA was purified (Section 2.5.4.6), and 1 μL was used for electrotransformation (Section 2.4.4.2) into electrocompetent *E. coli* DH5α cells. Colonies that grew on LB agar containing 100 mg/mL ampicillin were screened for the presence of the TLC insert.

### 3.2.4.2 Cloning of TeNT heavy chain into pRSET-A

The restriction sites used to facilitate cloning of THC into the MCS of pRSET-A were 5’ XhoI and 3’ *Pvu*II which were added to the nucleotide sequence during synthesis. Sequence analysis performed using the SE Central suite (Clone Manager), indicated that the addition of these restriction enzyme sites was sufficient for the THC coding sequence to remain in frame with the ATG start codon of pRSET-A.

Codon optimised THC was supplied by GenScript in the pUC57 vector with 5’ XhoI and 3’ *Pvu*II restriction sites (Figure 3.4). Restriction enzyme digestion of THC from pUC57 and pRSET-A containing no insert, was performed using XhoI and *Pvu*II (Section 2.5.4.1). Following digestion, reactions were combined and the DNA was concentrated to a smaller volume (Section 2.5.4.4) to facilitate ligation. After overnight ligation at 16°C (Section 2.5.4.5), the DNA was purified (Section 2.5.4.6), and 1 μL was used for electrotransformation (Section 2.4.4.2) into electrocompetent *E. coli* DH5α cells. Colonies that grew on LB agar containing 100 mg/mL ampicillin were screened for the presence of the THC insert.

### 3.2.4.3 Cloning of BoNT/A light chain into pRSET-A

The restriction sites used to facilitate cloning into the MCS of pRSET-A were 5’ *Pst*I and 3’ *Eco*RI, which were added to the nucleotide sequence during synthesis. Sequence analysis using the SE Central suite (Clone Manager) indicated that the B/ALC coding sequence was not in frame with the start codon of pRSET-A, so a single C nucleotide was added between the restriction enzyme recognition sequence at the 5’ end of B/ALC to allow the sequence to be translated in frame with the ATG start codon of pRSET-A.
Figure 3.3: Codon optimised gene encoding TLC in pUC57 (Fermentas), supplied by GenScript, U.S.A.
Figure 3.4: Codon optimised gene encoding THC in pUC57 (Fermentas), supplied by GenScript, U.S.A.
Codon optimised B/ALC was supplied by GenScript in the pUC57 vector with 5’ EcoRI and 3’ PstI restriction sites (Figure 3.5). Restriction enzyme digestion of B/ALC from pUC57 and pRSET-A containing no insert, was performed using EcoRI and PstI (Section 2.5.4.1). Following digestion, reactions were separated on a 1.5% DNA agarose gel (Section 2.5.2.2). Excision of the double digested B/ALC and pRSET-A was performed and the DNA was purified using the QIAquick gel extraction kit (Section 2.3.5), then concentrated to a smaller volume (Section 2.5.4.4) to facilitate ligation. After overnight ligation at 16°C (Section 2.5.4.5), the DNA was purified (Section 2.5.4.6), and 1 μL was used for electroporation (Section 2.4.4.2) into electrocompetent E. coli DH5α cells. Colonies that grew on LB agar containing 100 mg/mL ampicillin were screened for the presence of the B/ALC insert.

### 3.2.4.4 Colony screening

All clones were analysed for the presence and orientation of inserts and to confirm that all inserts were in frame with the start codon of the pRSET-A expression vector by sequencing PCR (Section 2.5.4.2) (results not shown), and by restriction digestion using the restriction enzymes which corresponded to the 5’ and 3’ restriction sites engineered into the synthesised sequences (Section 2.5.4.1).

### 3.2.5 Pilot protein expression study

Following successful cloning of inserts into pRSET-A and confirmation by sequencing PCR, a pilot expression study (Section 2.6.3.2), was performed to assess the optimal temperature and time required for induction of protein expression. One millilitre aliquots were collected at 0 – 5 h after induction of protein expression with IPTG, and 10 – 15 µg of protein was analysed by SDS-PAGE (Section 2.6.2.1) and staining with Coomassie instant stain (Section 2.6.2.2). Western blot (Section 2.6.2.3 – 2.6.2.4) was also performed, probing with antibodies directed at the His$_6$-tag (mouse anti-His IgG), and to the toxin domains (rabbit anti-tetanus toxin IgG, and rabbit anti-C. botulinum A toxoid IgG) (Section 2.3.4).
Figure 3.5: Codon optimised gene encoding B/ALC in pUC57 (Fermentas), supplied by GenScript, U.S.A.
3.3 Results

3.3.1 Codon optimisation

Codon optimisation of the wild-type Clostridial nucleotide sequences showed improvements in percentage GC content of the toxin domains, codon adaptation index (CAI) scores, and codon frequency distribution (CFD) (Figures 3.6A and B, 3.7A and B, and 3.8A and B). The GC content of all three toxin chains was increased from below 30% in the wild-type to above 40% in the codon optimised sequences, with increases in GC content of between 12 – 17% across the three sequences. CAI scores increased from ~0.6 to above 0.8 in all sequences after codon optimisation, with increases of between 0.2 – 0.3. Finally, all CFD results showed an increase in the number of codons present in the pool of codons most frequently used by *E. coli* (91 – 100%) after codon optimisation, with increases of between 20 – 32 codons now present in the most frequently used pool.

Alignments of the wild-type and codon optimised nucleotide sequences of TLC, THC and B/ALC is shown in Figures 3.9, 3.10 and 3.11, respectively. After codon optimisation, sequences displayed differences in primary sequence identity of 22% for TLC, 25% for THC and 26% for B/ALC (data not shown).

3.3.2 Cloning of toxin chains into pRSET-A

The pRSET-A expression vector (Figure 3.2) contains an ATG start codon upstream of the MCS, from where initiation of the His6-tag, enterokinase cleavage site and ribosomal binding site (along with other elements) begins. Transcription of the sequence inserted into the MCS is also initiated from this site. For this reason, the synthesised toxin sequences lack the initial methionine at the 5’ position, with the exception of THC which does not contain an N-terminal start codon in the native amino acid sequence. A 3’ stop codon was added to both light chain sequences to terminate transcription. Insertion of a stop codon was not required for THC, which retained the stop codon at the 3’ end of the native amino acid sequence.

The N-terminal affinity tag encoded by the pRSET-A expression vector added 39 residues to the N-terminus of the recombinant TLC and THC, and 42 residues to the N-terminus of the recombinant B/ALC (due to the position of the MCS) (data not shown).
Figure 3.6A: Wild-type TLC Codon Adaptation Index, GC content and Codon Frequency Distribution.

Figure 3.6B: TLC Codon Adaptation Index, GC content and Codon Frequency Distribution after codon optimisation by GenScript.
Figure 3.7A: Wild-type THC Codon Adaptation Index, GC content and Codon Frequency Distribution.

Figure 3.7B: THC Codon Adaptation Index, GC content and Codon Frequency Distribution after codon optimisation by GenScript.
Figure 3.8A: Wild-type B/ALC Codon Adaptation Index, GC content and Codon Frequency Distribution.

Figure 3.8B: B/ALC Codon Adaptation Index, GC content and Codon Frequency Distribution after codon optimisation by GenScript.
Figure 3.9: Alignment of wild-type (TLC) and codon optimised (TLC CO) tetanus toxin light chain nucleotide sequences.
Figure 3.10: Alignment of wild-type (THC) and codon optimised (THC CO) tetanus toxin heavy chain nucleotide sequences.
Figure 3.11: Alignment of wild-type (B/ALC) and codon optimised (B/ALC CO) botulinum type A toxin light chain nucleotide sequences.
3.3.2.1 Tetanus toxin light chain (TLC)

The codon optimised TLC nucleotide sequence consists of 1,371 bp encoding the 456 amino acid native tetanus toxin light chain. The final construct pTLC is 4,266 bp in length, and consists of the 1,371 bp TLC and the ~2,900 bp pRSET-A vector (Figure 3.12A). TLC (~1.3 kb) was digested from the pUC57 vector (~2.7 kb) using *Xho*I and *Pst*I (Figure 3.12B), and following ligation, screening of clones with *Xho*I and *Pst*I (Figure 3.12C) and sequencing PCR (data not shown) confirmed the presence of TLC in pRSET-A (pTLC).

3.3.2.2 Tetanus toxin heavy chain (THC)

The codon optimised THC nucleotide sequence consists of 2,577 bp encoding the 858 amino acid native tetanus toxin heavy chain. The final construct pTHC is 5,469 bp in length, and consists of the 2,577 bp THC and the ~2,900 bp pRSET-A vector (Figure 3.13A). THC (~2.5 kb) was digested from the pUC57 vector (~2.7 kb) using *Xho*I and *Pvu*II (Figure 3.13B), and following ligation, screening of clones with *Xho*I and *Pvu*II (Figure 3.13C) and sequencing PCR (data not shown) confirmed the presence of THC in pRSET-A (pTHC).

3.3.2.3 Botulinum type A toxin light chain (B/ALC)

The codon optimised B/ALC nucleotide sequence consists of 1,344 bp encoding the 447 amino acid native botulinum type A toxin light chain. The final construct pB/ALC is 4,231 bp in length, and consists of the 1,344 bp B/ALC and the ~2,900 bp pRSET-A vector (Figure 3.14A). B/ALC (~1.3 kb) was digested from the pUC57 vector (~2.7 kb) using *Pst*I and *Eco*RI (Figure 3.14B), and following ligation, screening of clones with *Pst*I and *Eco*RI (Figure 3.14C) and screening PCR (data not shown) confirmed the presence of B/ALC in pRSET-A (pB/ALC).

3.3.3 Pilot expression study

All three constructs expressed recombinant protein at the predicted molecular weight, (estimated by Clone Manager, data not shown), when visualised using SDS-PAGE and Western blotting.
Figure 3.12: Construction of pTLC.

(A) Diagrammatic representation of pTLC. The pTLC construct encodes the codon optimised sequence for the mature TLC, spanning residues P2 – A457 of the wild-type amino acid sequence, with an N-terminal His6-tag and C-terminal stop codon. The gene is inserted within the MCS of the pRSET-A vector and is expressed under the control of a T7 promoter. (B) Double digest of TLC in pUC57. Lane 1, λ-PstI; Lane 2, pUC57/TLC digested with XhoI and PstI. (C) Cloning into pRSET-A. Lane 1, λ-PstI; Lane 2, TLC insert in pRSET-A (pTLC) digested with XhoI and PstI.
Figure 3.13: Construction of pTHC.

(A) Diagrammatic representation of pTHC. The pTHC construct encodes the codon optimised sequence for the mature THC, spanning residues S458 – D1315 of the wild-type amino acid sequence, with an N-terminal His6-tag and C-terminal stop codon. The gene is inserted within the MCS of the pRSET-A vector and is expressed under the control of a T7 promoter. (B) Double digest of THC in pUC57. Lane 1, λ-PstI; Lane 2, pUC57/THC digested with Xhol and PvuII. (C) Cloning into pRSET-A. Lane 1, λ-PstI; Lane 2, THC insert in pRSET-A (pTHC) digested with Xhol and PvuII.
Figure 3.14: Construction of pB/ALC.

(A) Diagrammatic representation of pB/ALC. The pB/ALC construct encodes the codon optimised sequence for the mature B/ALC, spanning residues P2 – K488 of the wild-type amino acid sequence, with an N-terminal His_{6}-tag and C-terminal stop codon. The gene is inserted within the MCS of the pRSET-A vector and is expressed under the control of a T7 promoter. (B) Double digest of B/ALC in pUC57. Lane 1, λ-PstI; Lane 2, pUC57/B/ALC digested with PstI and EcoRI. (C) Cloning into pRSET-A. Lane 1, λ-PstI; Lane 2, B/ALC insert in pRSET-A (pB/ALC) digested with PstI and EcoRI.
3.3.3.1  pTLC pilot expression study

The recombinant His$_6$-tagged TLC, encodes amino acids P2 - A457 of native tetanus toxin and consists of 495 amino acids with a predicted molecular weight of 56.7 kDa. Expression vector pTLC was expressed in *E. coli* BL21(DE3)pLysS and the pilot expression study was performed in LB broth at 30°C (Figure 3.15), and 37°C (Figure 3.16). TLC expression was optimal at 30°C for 3 h after induction with IPTG.

3.3.3.2  pTHC pilot expression study

The recombinant His$_6$-tagged THC, encodes the C-terminal 858 amino acids of the native tetanus toxin (S458 – D1315), and consists of 897 amino acids with a predicted molecular weight of 102.7 kDa. Expression vector pTHC was expressed in *E. coli* BL21(DE3)pLysS and the pilot study was performed in LB broth at 30°C (Figure 3.17), and 37°C (Figure 3.18). THC expression was optimal at 30°C for 3 h after induction with IPTG.

Expression vector pTHC was also expressed in *E. coli* Origami™ 2(DE3)pLysS and the pilot study was performed in LB broth at 30°C (Figure 3.19), and 37°C (Figure 3.20). THC expression in this strain was optimal at 30°C for 3 h after induction with IPTG.

3.3.3.3  pB/ALC pilot expression study

The recombinant His$_6$-tagged B/ALC encodes amino acids P2 – K448 of native botulinum type A toxin and consists of 489 amino acids with a predicted molecular weight of 56 kDa. Expression vector pB/ALC was expressed in *E. coli* BL21(DE3)pLysS and the pilot expression study was performed in LB broth at 30°C (Figure 3.21), and 37°C (Figure 3.22). B/ALC expression was optimal at 30°C for 3 h after induction with IPTG.
Figure 3.15: pTLC 30°C pilot expression in *E. coli* BL21(DE3)pLysS.
Lane 1, Marker; Lane 2 - 7, time-point samples collected 0 – 5 h pre- and post-induction with 1 mM IPTG, 10 µg/well. (A) Pilot expression of TLC at 30°C visualised by SDS-PAGE and Coomassie instant stain. (B) Pilot expression of TLC at 30°C as detected by immunoblotting with mouse anti-His IgG. (C) Pilot expression of TLC at 30°C as detected by immunoblotting with rabbit anti-tetanus toxin IgG.
Figure 3.16: pTLC 37°C pilot expression in *E. coli* BL21(DE3)pLysS.
Lane 1, Marker; Lane 2 - 7, time-point samples collected 0 – 5 h pre- and post-induction with 1 mM IPTG, 10 µg/well. (A) Pilot expression of TLC at 37°C visualised by SDS-PAGE and Coomassie instant stain. (B) Pilot expression of TLC at 37°C as detected by immunoblotting with mouse anti-His IgG. (C) Pilot expression of TLC at 37°C as detected by immunoblotting with rabbit anti-tetanus toxin IgG.
Figure 3.17: pTHC 30°C pilot expression in *E. coli* BL21(DE3)pLysS.
Lane 1, Marker; Lane 2 - 7, time-point samples collected 0 – 5 h pre- and post-induction with 1 mM IPTG, 10 µg/well. (A) Pilot expression of THC at 30°C visualised by SDS-PAGE and Coomassie instant stain. (B) Pilot expression of THC at 30°C as detected by immunoblotting with mouse anti-His IgG. (C) Pilot expression of THC at 30°C as detected by immunoblotting with rabbit anti-tetanus toxin IgG.
Figure 3.18: pTHC 37°C pilot expression in *E. coli* BL21(DE3)pLysS.
Lane 1, Marker; Lane 2 - 7, time-point samples collected 0 – 5 h pre- and post-induction with 1 mM IPTG, 10 µg/well. (A) Pilot expression of THC at 37°C visualised by SDS-PAGE and Coomassie instant stain. (B) Pilot expression of THC at 37°C as detected by immunoblotting with mouse anti-His IgG. (C) Pilot expression of THC at 37°C as detected by immunoblotting with rabbit anti-tetanus toxin IgG.
Figure 3.19: pTHC 30°C pilot expression in *E. coli* Origami™ 2(DE3)pLysS. Lane 1, Marker; Lane 2 - 7, time-point samples collected 0 – 5 h pre- and post-induction with 1 mM IPTG, 10 µg/well. (A) Pilot expression of THC at 30°C visualised by SDS-PAGE and Coomassie instant stain. (B) Pilot expression of THC at 30°C as detected by immunoblotting with mouse anti-His IgG. (C) Pilot expression of THC at 30°C as detected by immunoblotting with rabbit anti-tetanus toxin IgG.
Figure 3.20: pTHC 37°C pilot expression in *E. coli* Origami™ 2(DE3)pLysS.
Lane 1, Marker; Lane 2 - 7, time-point samples collected 0 – 5 h pre- and post-induction with 1 mM IPTG, 10 µg/well. (A) Pilot expression of THC at 37°C visualised by SDS-PAGE and Coomassie instant stain. (B) Pilot expression of THC at 37°C as detected by immunoblotting with mouse anti-His IgG. (C) Pilot expression of THC at 37°C as detected by immunoblotting with rabbit anti-tetanus toxin IgG.
Figure 3.21: pB/ALC 30°C pilot expression in *E. coli* BL21(DE3)pLysS.
Lane 1, Marker; Lane 2 - 7, time-point samples collected 0 – 5 h pre- and post-induction with 1 mM IPTG, 15 µg/well. (A) Pilot expression of B/ALC at 30°C visualised by SDS-PAGE and Coomassie instant stain. (B) Pilot expression of B/ALC at 30°C as detected by immunoblotting with mouse anti-His IgG. (C) Pilot expression of B/ALC at 30°C as detected by immunoblotting with rabbit anti-*C. botulinum* A toxoid IgG.
Figure 3.22: pB/ALC 37°C pilot expression in *E. coli* BL21(DE3)pLysS.
Lane 1, Marker; Lane 2 - 7, time-point samples collected 0 – 5 h pre- and post-induction with 1 mM IPTG, 15 µg/well. (A) Pilot expression of B/ALC at 37°C visualised by SDS-PAGE and Coomassie instant stain. (B) Pilot expression of B/ALC at 37°C as detected by immunoblotting with mouse anti-His IgG. (C) Pilot expression of B/ALC at 37°C as detected by immunoblotting with rabbit anti-*C. botulinum* A toxoid IgG.
3.3.4 Characterisation of recombinant His$_6$-tagged proteins

3.3.4.1 Hydropathicity profiles

Hydropathicity profiles of the His$_6$-tagged protein sequences were performed in silico using the Kyte-Doolittle and Hopp-Woods algorithms. All plots showed peaks for all three His$_6$-tagged protein sequences were above 0 using the Hopp-Woods algorithm for hydrophilicity (Figures 3.23A, B and C). Hydrophobicity plots showed that peaks were below 1.6 overall, when using the Kyte-Doolittle algorithm (Figures 3.24A, B and C). The exception was THC which has one peak around residue 255 which approaches 1.6 on the Kyte-Doolittle scale (Figure 3.24B).

3.3.4.2 Isoelectric point (pI)

The theoretical pI estimated using Clone Manager are shown in Table 3.1.

3.3.4.3 Protease cleavage sites

The His$_6$-tagged protein sequences were screened for potential protease cleavage sites using PeptideCutter (ExPASy), and the proteases found to cleave the recombinant proteins are listed in Table 3.2. Numbers correspond to the number of times the recombinant protein is cleaved by the listed protease, and proteases listed in bold are inhibited by the protease inhibitor phenylmethanesulfonyl fluoride (PMSF).

3.3.4.4 Protein modelling

A model of the proposed novel chimeric holotoxin was constructed by joining the B/ALC and THC sequences in Modeller 9.10 (Sali and Blundell, 1993), and viewed using Swiss-PDB Viewer version 4.0.1 (Guex and Peitsch, 1997). The BoNT/A holotoxin template (3BTA) (Figure 3.25A), and the chimeric holotoxin superimposed on the BoNT/A holotoxin (Figure 3.25B), show that the proposed chimeric holotoxin is structurally similar to the native BoNT/A structure, with differences mainly seen in the heavy chain domain.
Figure 3.23: Hydrophilicity plots of (A) TLC, (B) THC and (C) B/ALC performed using the Hopp-Woods algorithm.
Figure 3.24: Hydrophobicity plots of (A) TLC, (B) THC and (C) B/ALC performed using the Hopp-Woods algorithm.
Table 3.1: Theoretical pl of the recombinant His$_6$-tagged proteins.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Isoelectric point (pl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC</td>
<td>5.38</td>
</tr>
<tr>
<td>THC</td>
<td>6.14</td>
</tr>
<tr>
<td>B/ALC</td>
<td>6.35</td>
</tr>
</tbody>
</table>
Table 3.2: Protease cleavage sites of the recombinant His$_6$-tagged proteins as predicted by PetideCutter (ExPASy).

<table>
<thead>
<tr>
<th>Protease</th>
<th>THC</th>
<th>B/ALC</th>
<th>TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg-C proteinase</td>
<td>27</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Asp-N endopeptidase</td>
<td>61</td>
<td>31</td>
<td>39</td>
</tr>
<tr>
<td>Asp-N endopeptidase + N-terminal Glu</td>
<td>109</td>
<td>60</td>
<td>65</td>
</tr>
<tr>
<td>BNPS-Skatole</td>
<td>13</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>CNBr</td>
<td>-</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Chymotrypsin-high specificity (C-term to [FYW], not before P)</td>
<td>95</td>
<td>62</td>
<td>52</td>
</tr>
<tr>
<td>Chymotrypsin-low specificity (C-term to [FYWML], not before P)</td>
<td>198</td>
<td>123</td>
<td>118</td>
</tr>
<tr>
<td>Clostripain</td>
<td>27</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Enterokinase</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Formic acid</td>
<td>61</td>
<td>31</td>
<td>39</td>
</tr>
<tr>
<td>Glutamyl endopeptidase</td>
<td>48</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Iodosobenzoic acid</td>
<td>13</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>LysC</td>
<td>75</td>
<td>41</td>
<td>34</td>
</tr>
<tr>
<td>LysN</td>
<td>75</td>
<td>-</td>
<td>34</td>
</tr>
<tr>
<td>NTCB (2-nitro-5-thiocyanobenzoic acid)</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Pepsin (pH1.3)</td>
<td>281</td>
<td>152</td>
<td>139</td>
</tr>
<tr>
<td>Pepsin (pH&gt;2)</td>
<td>181</td>
<td>111</td>
<td>97</td>
</tr>
<tr>
<td>Proline-endopeptidase [*]</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>398</td>
<td>214</td>
<td>198</td>
</tr>
<tr>
<td>Staphylococcal peptidase I</td>
<td>45</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>245</td>
<td>133</td>
<td>132</td>
</tr>
<tr>
<td>Trypsin</td>
<td>100</td>
<td>57</td>
<td>47</td>
</tr>
<tr>
<td>Caspase1</td>
<td>-</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Caspase4</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.25: (A) The BoNT/A (3BTA) holotoxin. (B) Superimposition of BoNT/A (3BTA) holotoxin (blue), and the proposed novel chimeric CNT neurotoxin (red) comprised of B/ALC and THC.
3.4 Discussion

This chapter described the development of three expression vectors pTLC, pTHC and pB/ALC, which contain the codon optimised sequences of the tetanus toxin light chain (TLC) and heavy chain (THC), and the botulinum type A light chain (B/ALC), respectively. Codon optimised gene sequences were synthesised by GenScript, U.S.A. and provided in a pUC57 vector. Following sub-cloning into pRSET-A, clones expressed a recombinant His₆-tagged protein which migrated at the predicted molecular weight when visualised by SDS-PAGE after a pilot expression study. All three proteins were reactive with anti-His₆ antisera, the TLC and THC were also reactive with anti-tetanus toxin antisera, and B/ALC was reactive with anti-BoNT/A antisera.

Only a single toxin type exists amongst the various strains of *C. tetani* (Accession no. X06214) (Eisel *et al.*, 1986), while the sequence of botulinum type A toxin strain Hall 183 (Accession no. EF033126) (Zhang *et al.*, 2003), was used in chain construction.

The initial methionine present in the light chain sequences was omitted from the synthesised sequences, as it is not present in the native mature toxin. This residue was not required in the recombinant sequence as translation was initiated from the start codon in pRSET-A. It has also been demonstrated that inclusion of the initial methionine may negatively impact on protein stability and biological activity and may induce an immunogenic response when included in therapeutic proteins (Liao *et al.*, 2004).

3.4.1 Codon optimisation

The genomes *C. tetani* and *C. botulinum*, like many Clostridium spp., are rich in A-T nucleotides and therefore employ tRNA codons that do not occur frequently in non-AT-rich genomes. *Escherichia coli*, used as the heterologous host to express the recombinant proteins, is known to have an alternate codon usage to that of AT-rich genomes such as Clostridia (Grantham *et al.*, 1980).

Genes which are highly expressed within an organism, such as the tetanus and botulinum neurotoxins, generally employ a large number of codons which are rarely used in heterologous organisms such as *E. coli*. These rare codons are present in low quantities in *E. coli* and are usually only required for weakly expressed genes (Grantham *et al.*, 1981; Grosjean and Fiers, 1982). During expression in an *E. coli* host, the low availability of tRNA codons employed by Clostridial genes may have detrimental effects on heterologous translation efficiency (Zdanovsky and Zdanovskaia, 2000). The outcomes may be a low yield
of full-length recombinant protein expression and/or premature termination of the polypeptide chain (Andersson and Kurland, 1990).

It has been demonstrated that the yield of heterologous expression of Clostridial proteins increased after codon optimisation of sequences (Makoff et al., 1989; Stratford et al., 2000; Tregoning et al., 2003; Zdanovsky and Zdanovskaia, 2000). In order to enhance recombinant protein expression yield in \textit{E. coli}, the toxin chains used in this study were codon optimised.

Codon optimisation was performed by GenScript Corporation (NJ, USA), using the OptimumGene™ codon optimisation tool with requirements for codon bias indicated for expression in an \textit{E. coli} host. Rare codons were removed from the nucleotide sequence and replaced with codons which have a higher frequency of use in \textit{E. coli}, while amino acid sequences remained unchanged. The CAI, GC content and CFD of the codon optimised sequences were improved compared to that of the wild-type sequence for each TLC, THC and B/ALC, as shown in Figures 3.6 – 3.8, respectively.

\subsection*{3.4.1.1 Codon adaptation index (CAI)}

Sequence analyses included the use of a Codon Adaptation Index (Sharp and Li, 1987) to measure the relative codon usage frequency within the target nucleotide sequence. The CAI tool functions on the hypothesis that codons used throughout highly expressed proteins will be available in larger quantities than those only used in weakly expressed proteins, and that codons contained in this pool may differ from those used in highly expressed genes of other organisms (Ermolaeva, 2001; Grantham et al., 1981). A score is calculated by comparing the target sequence with a set of reference codons known to be used in highly expressed genes in the target \textit{E. coli} host. Through this index, a CAI of 1.0 is ideal, and the lower the score, the higher the likelihood that protein expression yield will be low. Using GenScript’s OptimumGene™ codon optimisation tool the CAI score was increased from approximately 0.6 to >0.8 in all three toxin sequences.

\subsection*{3.4.1.2 GC content adjustment}

The optimal percentage range of GC content for heterologous expression in \textit{E. coli} ranges from 30 – 70\% (Han et al., 2010). The wild-type sequences contained a very low GC content (<20\%), which may have had a negative effect during expression in \textit{E. coli}. Codon optimisation increased the GC content to an average of 43\% across the three sequences.
3.4.1.3 Codon frequency distribution (CFD)

The CFD tool calculates frequently used codons present in *E. coli* and groups them based on availability (GenScript, 2013). Sequences were analysed for the types of codons they require and from which computed group those codons belong. The ideal sequence would require codons from the highest group based on availability, and contain no codons from the lower groups which are present in small quantities in *E. coli* or not at all. The CFD scale computed groups from 0 – 100 based on their usage frequency. Codons with values below 30 were altered to enhance expression efficiency by the OptimumGene™ codon optimisation tool.

3.4.2 Expression host and vector choice

3.4.2.1 Bacterial expression host – *E. coli*

*Escherichia coli* is a commonly used bacterium for the expression of recombinant proteins. Its attractive features include fast growth using minimal growth medium, and high growth yield from relatively small volumes of culture. The smaller volumes of culture used allow for greater ease of handling and purification and induction of expression is specific and can be controlled by the addition of a chemical inducer. Using this heterologous host provided a quick and safe method for expression of the individual toxin domains.

3.4.2.2 Bacterial expression strain – *E. coli* BL21(DE3)pLysS

The host strain used for recombinant expression of neurotoxin chains was *E. coli* BL21(DE3)pLysS. This strain carries the DE3 bacteriophage lambda lysogen which contains the T7 RNA polymerase gene, responsible for driving transcription of the target gene cloned into the pRSET-A expression vector. The pLysS plasmid contains a chloramphenicol resistance gene (Cam^R^) and encodes a T7 phage lysozyme to restrict basal level expression of T7 polymerase, and subsequently of the target gene. The T7 lysozyme binds to T7 polymerase and inhibits initiation of transcription of the target gene from the T7 promoter. When an inducer such as the synthetic lactose analogue isopropyl-1-thio-β-D-galactopyranoside (IPTG) is added to the culture, the repression of T7 polymerase is removed and the T7 promoter can initiate expression of the target recombinant protein (Dubendorff and Studier, 1991). Generally, basal expression is restricted when over-expression of recombinant protein is toxic to the host cells, leading to cell death. Although the neurotoxin chains are non-toxic when individually expressed, stringent control is beneficial to prevent any difficulties with proliferation and differentiation of the host cell which may be caused by the introduction of a heterologous protein. Like all B strains of *E. coli*, this
strain is deficient in the *lon* protease and the *ompT* outer membrane protease which may degrade proteins during purification (Grodberg and Dunn, 1988).

### 3.4.2.3 Bacterial expression strain – *E. coli* Origami™ 2(DE3)pLysS

The *E. coli* Origami™2 strain is a K-12 derivative and contains mutations in the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes to create an oxidising environment within the *E. coli* cytoplasm, a condition which aids in the formation of disulfide bonds. The presence of an intrachain disulfide bond in the THC may indicate the requirement of such conditions to ensure correctly folded THC during recombinant expression in *E. coli*. This strain also contains the DE3 bacteriophage lambda lysogen and pLysS plasmid, which contain the T7 RNA polymerase gene and T7 phage lysozyme, respectively. Although the full-length tetanus toxin heavy chain has previously been expressed in a soluble form in *E. coli* (Li, Y. *et al.*, 1999), it is possible that reducing environment of the *E. coli* cytoplasm may cause THC to misfold during cytoplasmic expression (Gill, R. T. *et al.*, 1998).

### 3.4.2.4 Expression vector – pRSET-A

Sequences were cloned into the pRSET-A expression vector (Invitrogen, U.S.A.) by restriction enzyme digestion and the resulting recombinant protein is expressed with an N-terminal His<sub>6</sub>-tag under the control of a T7 promoter. Translation is initiated via an ATG start codon present upstream of the His<sub>6</sub>-tag and stopped by the inclusion of a TAA stop codon at the 3’ end of the inserted sequence. An enterokinase recognition site is present between the His<sub>6</sub>-tag and the coding sequence of the target gene to facilitate removal of the His<sub>6</sub>-tag from purified recombinant protein. The pRSET-A vector is maintained in the expression host through ampicillin resistance and His<sub>6</sub>-tagged proteins were purified by a simple chromatographic method, immobilised metal affinity chromatography (IMAC).

### 3.4.3 Pilot expression study

The pilot expression study was performed in *E. coli* BL21(DE3)pLysS for pTLC, pTHC and pB/ALC, and also in *E. coli* Origami™ 2(DE3)pLysS for pTHC. Results indicate that all three recombinant proteins express well at both 30°C and 37°C, however TLC (Figure 3.15 – 3.16) and THC (Figure 3.17 – 3.20), which expressed equally well at both temperatures, expressed fewer non-specific proteins at 30°C. B/ALC expressed a higher yield of protein at 30°C (Figure 3.21 – 3.22). Expression at 30°C has been used previously for the expression of Clostridial genes in *E. coli* in order to decrease degradation of the recombinant protein within
the *E. coli* cytoplasm and to decrease the production of inclusion bodies (Baldwin *et al.*, 2004; Zhou *et al.*, 1995).

All of the recombinant constructs expressed successfully from the pRSET-A vector in *E. coli* and were reactive with anti-His and toxin-specific antisera. However, reactivity with proteins which migrated at both a higher and lower molecular weight than the target protein was evident. The origin of these proteins is still unclear, but may be elucidated in later sections of this study. Expression and IMAC purification methods will be explored in the subsequent chapters, along with *in vitro* assays to assess the activity of the individual recombinant toxin domains.
Chapter 4

Protein expression, purification and functional assays of tetanus and botulinum type A toxin light chains
4.1 Introduction

The preceding section outlined the development of the tetanus toxin and botulinum type A light chains and cloning into the pRSET-A expression vector. After evaluation of the optimal conditions required for expression of the recombinant proteins, this section describes the optimisation of small-scale expression and purification of the recombinant proteins.

CNTs are the most potent toxins known to mankind, and there is a risk of toxicity associated with handling full-length toxins (Francis et al., 2000). For this reason, the toxin domains will be expressed individually and later conjugated to create the holotoxin. This will also facilitate the subsequent creation of various holotoxin “serotypes”. The light chains of CNTs make up the C-terminal catalytic domain which cleaves one of the SNARE proteins involved in synaptic vesicle fusion at pre-synaptic junctions. These domains, which retain their catalytic function independent of the heavy chain domains of CNTs, will be used to create the recombinant holotoxin TLC + THC, and the novel chimeric holotoxin B/ALC + THC. The proposed recombinant holotoxins are being created via the conjugation of individual light and heavy chains.

The first section of this part of the study is to express the recombinant light chains in *E. coli*. Two types of growth media and various temperatures will be evaluated to determine the optimal conditions required to obtain a high yield of soluble protein. The second part of the study is to purify the proteins from the bacterial host using immobilised metal affinity chromatography (IMAC). IMAC utilises the N-terminal His$_6$-tag expressed as part of the recombinant protein to isolate the proteins from *E. coli*. Multiple purification configurations will be evaluated to find the optimal conditions for recombinant protein purification. Following purification of the light chains, their activity will be assessed in functional assays which monitor the toxin domains for cleavage of their target substrate.

Tetanus toxin light chain naturally cleaves synaptobrevin-2 (VAMP-2) at the pre-synaptic membrane, and a recombinant substrate will be used to assess the cleavage ability of purified TLC. Botulinum type A toxin light chain naturally cleaves SNAP-25, and a fluorogenic substrate will be used for the assessment of SNAP-25 cleavage by purified B/ALC.
4.2 Materials and methods

4.2.1 Expression in LB broth

Expression vectors pTLC and pB/ALC were electrotransformed into *E. coli* BL21(DE3)pLysS for each expression (Section 2.4.4.2). After overnight growth of transformants on LB agar containing the appropriate antibiotics, a single colony was sub-cultured into 2 mL LB broth containing antibiotics and grown at 37°C until the absorbance at 600 nm reached 0.6 – 1.0. The next stage of the protocol, growth to mid-log phase (absorbance at 600 nm of 0.3 – 0.6), was also performed in LB broth at 37°C (Section 2.6.3.3). Induction of protein expression with IPTG was performed in LB broth for 3 h at the required temperature. After 3 h induction, cell lysates were prepared for IMAC purification (Section 2.6.3.5).

TLC was expressed for 3 h after addition of IPTG at 25°C, 30°C, 37°C and 42°C in LB broth, and B/ALC was expressed for 3 h after addition of IPTG at 30°C and 37°C in LB broth.

4.2.2 Expression in EnPresso™

Expression vectors pTLC and pB/ALC were electrotransformed into *E. coli* BL21(DE3)pLysS for each expression (Section 2.4.4.2). After overnight growth of transformants on LB agar containing the appropriate antibiotics, a single colony was sub-cultured into 2 mL LB broth containing antibiotics and grown at 37°C until the absorbance at 600 nm reached 0.6 – 1.0. The next stage of the protocol, growth to mid-log phase (absorbance at 600 nm of 0.3 – 0.6), was performed in EnPresso™ tablet medium (Section 2.3.2) at 30°C. Induction of protein expression with IPTG was performed in EnPresso™ tablet medium for 8 h at 30°C (Section 2.6.3.4). After 8 h induction, cell lysates were prepared for IMAC purification (Section 2.6.3.5). TLC and B/ALC expression in EnPresso™ was performed with and without the addition of 1 mM PMSF protease inhibitor (Section 2.3.1) during preparation of the cell lysate (Section 2.6.3.5).

4.2.3 IMAC purification

Following preparation of the cell lysate (Section 2.6.3.5), samples were purified by IMAC (Section 2.7.1.2). IMAC purification was performed using Chelating Sepharose™ fast flow (Section 2.3.1), charged with 0.2 M nickel sulphate (Ni²⁺) (Section 2.7.1.1), or HisPur Cobalt 1 mL spin columns (ThermoFisher Scientific, U.S.A.), (Section 2.7.1.4). His₉-tagged proteins
in the cell lysate were allowed to bind to the pre-charged resin, which was pre-equilibrated with protein binding buffer (Section 2.3.1). The column was then washed in 5 column volumes (CV) of protein wash buffer (Section 2.3.1) containing 20 mM and 50 mM imidazole and then eluted in protein elution buffer (Section 2.3.1).

4.2.3.1 TLC purification

Purification of TLC expressed in LB broth was performed by IMAC pre-charged with 0.2 M nickel sulphate, or 0.2 M cobalt chloride (Co²⁺) (Section 2.7.1.2). TLC was eluted in protein elution buffer (Section 2.3.1) containing 100 mM imidazole.

A double purification procedure using nickel IMAC was also performed for TLC expressed in LB broth at 30°C. Following preparation of the cell lysate, TLC was purified by IMAC (Section 2.7.1.2), and the eluted protein was concentrated and buffer exchanged (Section 2.7.2.1), into protein binding buffer containing 10 mM imidazole (Section 2.3.1) to facilitate binding to the IMAC column during the second purification step. The concentrated protein sample was then purified a second time in a newly prepared nickel IMAC column using the same protocol (Section 2.7.2.1). TLC was eluted in protein elution buffer (Section 2.3.1) containing 100 mM imidazole, and the concentration and buffer exchange protocol (Section 2.7.2.1) was repeated. Double-purified TLC was buffer exchanged to protein storage buffer (Section 2.3.1).

Purification of TLC expressed in EnPresso™ tablet media was performed by IMAC pre-charged with 0.2 M nickel sulphate (Section 2.7.1.2), or HisPur Cobalt 1 mL spin columns (ThermoFisher Scientific, U.S.A.), (Section 2.7.1.4). TLC was eluted in protein elution buffer (Section 2.3.1) containing 100 mM imidazole.

Ten to twenty micrograms of protein was separated by SDS-PAGE (Section 2.6.2.1) and visualised by staining with Coomassie instant stain (Section 2.6.2.2) and by Western blot (Section 2.6.2.3 – 2.6.2.4), probing with antibodies directed at the His₆-tag (mouse anti-His IgG), and to the toxin domain (rabbit anti-tetanus toxin IgG) (Section 2.3.4).

4.2.3.2 B/ALC purification

Purification of B/ALC expressed in LB broth was performed by IMAC pre-charged with 0.2 M nickel sulphate (Section 2.7.1.2). Purification of B/ALC expressed in EnPresso™ tablet
media was performed by IMAC pre-charged with 0.2 M nickel sulphate (Section 2.7.1.2), or HisPur Cobalt 1 mL spin columns (ThermoFisher Scientific, U.S.A.), (Section 2.7.1.4).

B/ALC was eluted in protein elution buffer (Section 2.3.1) containing 100 mM imidazole. Five to ten micrograms of protein was separated by SDS-PAGE (Section 2.6.2.1) and visualised by staining with Coomassie instant stain (Section 2.6.2.2) and by Western blot (Section 2.6.2.3 – 2.6.2.4), probing with antibodies directed at the His$_6$-tag (mouse anti-His IgG), and to the toxin domain (rabbit anti-\(C.\ botulinum\) A toxoid IgG) (Section 2.3.4).

4.2.4 Concentration and buffer exchange

Following elution of both TLC and B/ALC from the IMAC column, the proteins present in the protein elution buffer were applied to a pre-rinsed 30 kDa molecular weight cut-off (MWCO) Vivaspin 20 centrifugal device (Section 2.2), and samples were concentrated to a small volume (typically <1 mL) before performing buffer exchange (Section 2.7.2.1) to protein storage buffer (Section 2.3.1). Proteins were stored in aliquots at -20°C for up to 2 months, or until required.

4.2.5 Enterokinase treatment

Cleavage of the affinity tag from the recombinant proteins was performed using treatment with EKMax™ enterokinase (Invitrogen, U.S.A.), (Section 2.3.3). A pilot assay (Section 2.7.2.3) was performed to assess the optimal amount of enzyme, temperature and time of incubation required to remove the His$_6$-tag from recombinant TLC and B/ALC.

4.2.5.1 His$_6$-tag removal

Once the optimal amount of enzyme and time of incubation was determined, enterokinase was removed from the reaction using the Enterokinase removal kit (Invitrogen Corporation, U.S.A.) (Section 2.3.5), and the cleaved His$_6$-tag was removed using a modified method of batch purification (protein miniprep) (Section 2.7.1.6). Samples were analysed by SDS-PAGE (Section 2.6.2.1) and visualised by staining with Coomassie instant stain (Section 2.6.2.2) and by Western blot (Section 2.6.2.3 – 2.6.2.4), probing with an antibody directed at the His$_6$-tag (Section 2.3.4) to detect the presence of the tag.
4.2.6 Functional assays

Cleavage assays were used to assess the activity of the recombinant light chains. The native substrate cleaved by TLC is VAMP-2 (also known as synaptobrevin-2), while B/ALC cleaves SANP-25. A recombinant GST-VAMP-2 substrate and a SNAP-25 FRET substrate (Section 2.3.7), was used to assess the cleavage ability of TLC and B/ALC, respectively.

4.2.6.1 VAMP-2 cleavage assay

A GST-Synaptobrevin-2 (GST-VAMP-2) recombinant protein substrate (38 kDa) (List Biological Laboratories, U.S.A.), (Section 2.3.7), was used to assess the cleavage ability of recombinant TLC. Reactions were performed in 1.5 mL microfuge tubes and consisted of 5 µM GST-VAMP-2 and 200 nM or 500 nM toxin in VAMP-2 cleavage buffer (Section 2.3.1) in a 20 µL reaction volume. Reactions were incubated for 1 h at 37°C, then visualised using SDS-PAGE (Section 2.6.2.1). Reactions were performed with recombinant TLC and His-free TLC (His₆-tag removed) as test samples, a recombinant tetanus toxin light chain (List Biological Laboratories, U.S.A.), (Section 2.3.6) as the positive control, and recombinant B/ALC and His-free B/ALC (His₆-tag removed) (developed in this study), as negative controls. Cleavage of GST-VAMP-2 by TLC reduces the size of the recombinant substrate from 38 kDa to 36 kDa.

4.2.6.2 SNAP-25 FRET assay

A SNAP-25 FRET substrate, SNAPtide® (FITC/DABCYL) (List Biological Laboratories, U.S.A.), (Section 2.3.7), was used to assess the cleavage ability of recombinant B/ALC. Reactions were performed in a 96-Well black polystyrene microplate (Section 2.2), and consisted of 5 µM SNAPtide® (FITC/DABCYL), and 5 nM toxin in SNAPtide® hydrolysis buffer (Section 2.3.1). Reactions were performed in a 250 µL volume and assays were performed in triplicate using a FLUOstar Optima microplate reader (BMG Labetech, Germany) (Section 2.2). The fluorogenic substrate and SNAPtide® hydrolysis buffer were pre-warmed at 37°C for 15 min prior to addition of toxins to ensure equilibrium. Reactions were initiated after the addition of the toxins and the assay was performed for 100 min at 37°C, and relative fluorescence units were measured as an indication of SNAPtide® (FITC/DABCYL) cleavage over time. Increases in fluorescence upon cleavage of the SNAPtide® substrate were measured by excitation and emission wavelengths 490 nm and 523 nm, respectively, with a cutoff of 495 nm. Reactions were performed with recombinant B/ALC and His-free B/ALC (His₆-tag removed) as test samples, a recombinant botulinum type A toxin light chain (List Biological Laboratories, U.S.A.), (Section 2.3.6) as the positive
control, and recombinant TLC (developed in this study), as the negative control. Results were analysed using Microsoft Excel 2007.

4.3 Results

4.3.1 Protein expression

4.3.1.1 TLC expression and purification from LB broth

TLC was expressed with a final concentration of 1 mM IPTG for 3 h in LB broth at 25°C, 30°C, 37°C and 42°C to investigate the effect of temperature on TLC expression. TLC expressed at 25°C (Figure 4.1), 30°C (Figure 4.2), 37°C (Figure 4.3), and 42°C (Figure 4.4) in LB broth was purified by nickel IMAC and the recombinant protein was eluted in protein elution buffer containing 100 mM imidazole. Following purification with IMAC, concentration and buffer exchange, 10 – 20 µg of protein was separated using SDS-PAGE and visualised by staining with Coomassie instant stain (Figures 4.1A – 4.4A). Following immunotransfer, membranes were probed by immunoblotting, with antisera to the His₆-tag (mouse anti-His IgG) (Figures 4.1B – 4.4B), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figures 4.1C – 4.4C), for the detection of the His₆-tag and tetanus toxin domain, respectively. Cross-reactivity is evident with both antisera which detect proteins at both a larger and smaller molecular weights to TLC.

A significant amount of purified protein (10 – 20 µg) was separated by SDS-PAGE, and this should be taken into account when estimating the purity of TLC. TLC reacted with both His₆-tag and anti-toxin antisera, and cross-reactivity is evident with both antisera which detected proteins at both a larger and smaller molecular weights to TLC. TLC migrated to the predicted molecular weight of 56.7 kDa. Induction of protein expression at 30°C was determined to be optimal for TLC expression (Figure 4.2), with expression yields typically ranging from 0.7 – 0.9 mg/mL.

Double-purification of TLC after expression at 30°C in LB broth (Figure 4.5A, Lane 2) was performed to remove reactive species present at both lower and higher molecular weights to TLC, which remained after concentrating the eluted sample in a 30 kDa MWCO Vivaspin 20 centrifugal device. Figure 4.5A Lane 2 shows TLC after the first purification and buffer exchange step and Figure 4.5A, Lane 4 shows TLC after the second purification and buffer exchange step. Figures 4.5B and 4.5C show Western blot results after probing with anti-His₆
Figure 4.1: TLC expressed at 25°C in LB broth and purified by Ni²⁺ IMAC.
Lane 1, Marker; Lane 2, 10 µg purified TLC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of TLC probed with mouse anti-His IgG. (C) Immunoblot detection of TLC probed with rabbit anti-tetanus toxin IgG.
Figure 4.2: TLC expressed at 30°C in LB broth and purified by Ni²⁺ IMAC.
Lane 1, Marker; Lane 2, 20 µg purified TLC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of TLC probed with mouse anti-His IgG. (C) Immunoblot detection of TLC probed with rabbit anti-tetanus toxin IgG.
Figure 4.3: TLC expressed at 37°C in LB broth and purified by Ni²⁺ IMAC.
Lane 1, Marker; Lane 2, 10 µg purified TLC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of TLC probed with mouse anti-His IgG. (C) Immunoblot detection of TLC probed with rabbit anti-tetanus toxin IgG.
Figure 4.4: TLC expressed at 42°C in LB broth and purified by Ni²⁺ IMAC.
Lane 1, 10 µg purified TLC; Lane 2, Marker. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of TLC probed with mouse anti-His IgG. (C) Immunoblot detection of TLC probed with rabbit anti-tetanus toxin IgG.
Figure 4.5: TLC expressed at 30°C in LB broth and purified twice by Ni²⁺ IMAC.

(A) SDS-PAGE stained with Coomassie instant stain. Lane 1 and 3, Marker; Lane 2, 20 µg purified TLC (first purification); Lane 4, 20 µg purified TLC (second purification). (B) and (C) Lane 1, Marker; Lane 2, 20 µg purified TLC (first purification); Lane 3, 20 µg purified TLC (second purification). (B) Immunoblot detection of TLC probed with mouse anti-His IgG. (C) Immunoblot detection of TLC probed with rabbit anti-tetanus toxin IgG.
IgG and anti-tetanus toxin IgG antisera, respectively.

Cross-reactivity is evident with both antisera which detected proteins at both higher and lower molecular weights to TLC. Results indicate that the double-purification procedure did not remove these proteins.

### 4.3.1.2 TLC expression and purification from EnPresso™ tablet media

TLC purification after expression in EnPresso™ tablet media was performed using both nickel and cobalt IMAC. Induction of protein expression was performed for 8 h at 30°C in EnPresso™, however all subsequent steps leading to purification were identical to those in previous section for LB broth (Section 4.3.1.1).

TLC expressed in EnPresso™ tablet media was purified using nickel IMAC and 5 – 10 µg of purified, concentrated and buffer exchanged protein was separated by SDS-PAGE and visualised by staining with Coomassie instant stain (Figure 4.6A). Following immunotransfer membranes were probed by immunoblotting, with antisera to the His<sub>6</sub>-tag (mouse anti-His IgG) (Figure 4.6B), and tetanus toxin (rabbit anti-tetanus toxin IgG), (Figure 4.6C) for the detection of the His<sub>6</sub>-tag and tetanus toxin domain, respectively.

Protein yields of ~1.5 mg/mL of purified TLC were obtained from expression in EnPresso™. When considering that a significant amount of purified protein (5 – 10 µg) has been analysed by SDS-PAGE, TLC appears to be 80 – 90% pure. TLC expression in EnPresso™ media (Figure 4.6 – 4.7) produced a higher yield of recombinant TLC with fewer lower molecular weight proteins, compared to expression at various temperatures in LB broth (Figures 4.1 – 4.4). However, it must be noted that TLC often migrated to just below the 50 kDa band in the protein standard marker when expressed in EnPresso™.

To compare the influence of the metal ion used in IMAC purification, TLC expressed in EnPresso™ was also purified using cobalt IMAC. Five micrograms of purified, concentrated and buffer exchanged protein was separated by SDS-PAGE and visualised by staining with Coomassie instant stain (Figure 4.7A) and Western blot, with membranes probed by immunoblotting with antisera to the His<sub>6</sub>-tag (mouse anti-His IgG) (Figures 4.7B), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figure 4.7C). The use of cobalt IMAC did not show a reduction in the number of reactive species present at either higher or lower molecular weights to TLC (Figure 4.6 – 4.7).
Figure 4.6: TLC expressed at 30°C in EnPresso™ and purified by Ni²⁺ IMAC.
Lane 1, Marker; Lane 2, 10 µg purified TLC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of TLC probed with mouse anti-His IgG. (C) Immunoblot detection of TLC probed with rabbit anti-tetanus toxin IgG.
Figure 4.7: TLC expressed at 30°C in EnPresso™, and purified by Co²⁺ IMAC.
Lane 1, Marker. Lane 2, 5 µg purified TLC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of TLC probed with mouse anti-His IgG. (C) Immunoblot detection of TLC probed with rabbit anti-tetanus toxin IgG.
TLC expressed in EnPresso™ was also purified after treatment with protease inhibitor PMSF during preparation of the cell lysate. Figures 4.8A, B and C show 10 µg of purified, concentrated and buffer exchanged protein separated by SDS-PAGE and visualised by staining with Coomassie instant stain and Western blot, probing with antisera to the His₆-tag (mouse anti-His IgG), and tetanus toxin (rabbit anti-tetanus toxin IgG), respectively. The use of PMSF did not demonstrate a reduction in the number of reactive species present at either higher or lower molecular weights to TLC detected after immunoblotting (Figure 4.8).

### 4.3.1.3 B/ALC expression and purification from LB broth

B/ALC expression was performed with a final concentration of 1 mM IPTG for 3 h in LB broth at 30°C and 37°C to investigate the effect of temperature on B/ALC expression. B/ALC expressed at 30°C (Figure 4.9) and 37°C (Figure 4.10) in LB broth was purified by nickel IMAC and the recombinant protein was eluted in protein elution buffer containing 100 mM imidazole. Following purification by IMAC, concentration and buffer exchange, 5 – 10 µg of protein was separated using SDS-PAGE and visualised by staining with Coomassie instant stain (Figures 4.9A – 4.10A). Following immunotransfer, membranes were probed by immunoblotting, with antisera to the His₆-tag (mouse anti-His IgG) (Figures 4.9B – 4.10B), and *C. botulinum* type A toxin (rabbit anti-*C. botulinum* A toxoid IgG) (Figure 4.9C – 4.10C), for the detection of the His₆-tag and *C. botulinum* toxin domain, respectively. His₆-tagged B/ALC migrated to the predicted molecular weight of 56 kDa and the yield of B/ALC expressed at 30°C in LB broth (Figure 4.9) was higher and showed fewer proteins at a lower molecular weight to B/ALC when compared to expression at 37°C (Figure 4.10).

### 4.3.1.4 B/ALC expression and purification from EnPresso™ tablet media

The purification of B/ALC after expression in EnPresso™ tablet media was performed using both nickel and cobalt IMAC. Induction of protein expression was performed for 8 h at 30°C in EnPresso™, however all subsequent steps leading to purification were identical to those in previous section for LB broth (Section 4.3.1.3).

B/ALC expressed in EnPresso™ tablet media was purified using nickel IMAC and 10 µg of purified, concentrated and buffer exchanged protein was separated by SDS-PAGE and visualised by staining with Coomassie instant stain (Figure 4.11A). Following immunotransfer, membranes were probed by immunoblotting with antisera to the His₆-tag (mouse anti-His IgG) (Figure 4.11B), and *C. botulinum* type A toxin (rabbit anti-*C. botulinum*
Figure 4.8: TLC expressed at 30°C in EnPresso™ with PMSF and purified by Ni²⁺ IMAC.
Lane 1, Marker; Lane 2, 10 µg purified TLC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of TLC probed with mouse anti-His IgG. (C) Immunoblot detection of TLC probed with rabbit anti-tetanus toxin IgG.
Figure 4.9: B/ALC expressed at 30°C in LB broth and purified by Ni²⁺ IMAC.
Lane 1, Marker; Lane 2, 10 µg purified B/ALC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of B/ALC probed with mouse anti-His IgG. (C) Immunoblot detection of B/ALC probed with rabbit anti-*C. botulinum* A toxoid IgG.
Figure 4.10: B/ALC expressed at 37°C in LB broth and purified by Ni\textsuperscript{2+} IMAC.
Lane 1, Marker; Lane 2, 5 µg purified B/ALC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of B/ALC probed with mouse anti-His IgG. (C) Immunoblot detection of B/ALC probed with rabbit anti-\textit{C. botulinum} A toxoid IgG.
Figure 4.11: B/ALC expressed at 30°C in EnPresso™ and purified by Ni²⁺ IMAC.
Lane 1, Marker; Lane 2, 10 µg purified B/ALC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of B/ALC probed with mouse anti-His IgG. (C) Immunoblot detection of B/ALC probed with rabbit anti-\textit{C. botulinum} A toxoid IgG.
A toxoid IgG) (Figure 4.11C), for the detection of the His6-tag and C. botulinum toxin domain, respectively. B/ALC expression in EnPresso™ media (Figure 4.11) produced a higher yield of recombinant B/ALC with fewer proteins present at a lower molecular weight to B/ALC when compared to expression at 30°C (Figure 4.9) and 37°C (Figure 4.10) in LB broth.

B/ALC expressed in EnPresso™ was also purified after treatment with protease inhibitor PMSF during preparation of the cell lysate (Section 2.6.1.4). Figures 4.12A, B and C show 10 µg of purified, concentrated and buffer exchanged protein separated by SDS-PAGE and stained with Coomassie instant stain. Following immunotransfer, membranes were probed with antisera to the His6-tag (mouse anti-His IgG), and C. botulinum type A toxin (rabbit anti-C. botulinum A toxoid IgG), respectively. The use of PMSF showed some improvements in the reduction of reactive species present at a lower molecular weight to B/ALC, detected after immunoblotting (Figure 4.12).

To compare the influence on the metal ion used for IMAC purification of B/ALC, B/ALC expressed in EnPresso™ was also purified using cobalt IMAC. Ten micrograms of purified, concentrated and buffer exchanged protein was separated by SDS-PAGE and visualised by staining with Coomassie instant stain (Figure 4.13A) and Western blot, probing with antisera to the His6-tag (mouse anti-His IgG) (Figures 4.13B), and C. botulinum type A toxin (rabbit anti-C. botulinum A toxoid IgG) (Figure 4.13C). The use of cobalt IMAC did not remove reactive species present at both higher and lower molecular weights to B/ALC.

4.3.2 His6-tag cleavage and removal

4.3.2.1 Enterokinase treatment

Cleavage of the affinity tag from the recombinant proteins was performed by treatment with enterokinase. A pilot assay was performed to assess the optimal amount of enzyme, temperature and time of incubation required to remove the His6-tag from TLC and B/ALC. Figures 4.14A – D and figures 4.15A – D show the enterokinase pilot assay for TLC and B/ALC, respectively.

For TLC, His6-tag removal was achieved after incubation with 1 U enterokinase for 2 h at room temperature, indicated by the absence of a band on the anti-His Western blot for this sample (Figure 4.14B). The inability of the anti-His antibody to produce a colorimetric reaction for this sample indicates the absence of a detectable limit of His6-tag within the sample.
Figure 4.12: B/ALC expressed at 30°C in EnPresso™ with PMSF and purified by Ni²⁺ IMAC.

Lane 1, Marker; Lane 2, 10 µg purified B/ALC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of B/ALC probed with mouse anti-His IgG. (C) Immunoblot detection of B/ALC probed with rabbit anti-"C. botulinum" A toxoid IgG.
Figure 4.13: B/ALC expressed at 30°C in EnPresso™ and purified by Co²⁺ IMAC.
Lane 1, Marker; Lane 2, 10 µg purified B/ALC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of B/ALC probed with mouse anti-His IgG. (C) Immunoblot detection of B/ALC probed with rabbit anti-C. botulinum A toxoid IgG.
Figure 4.14: TLC enterokinase pilot assay.

(A) – (C) SDS-PAGE stained with Coomassie instant stain. Lane 1, Marker; Lane 2 – 7, 20 µg TLC treated with 0, 0.001, 0.01, 0.1, 1 and 4 U enterokinase, respectively. (A) TLC enterokinase pilot assay incubated for 1 h at room temperature. (B) TLC enterokinase pilot assay incubated for 2 h at room temperature. (C) TLC enterokinase pilot assay incubated for 16 h at room temperature; Lane 8, Marker. (D) Immunoblot detection of TLC enterokinase pilot assay probed with mouse anti-His IgG. Lane 1 – 3, 20 µg TLC treated with 0, 0.1, 1 U enterokinase for 2 h at room temperature; Lane 4, Marker.
Figure 4.15: B/ALC enterokinase pilot assay.

(A) and (C) SDS-PAGE stained with Coomassie instant stain. Lane 1, Marker; Lane 2 – 7, 20 µg B/ALC treated with 0, 0.001, 0.01, 0.1, 1 and 4 U enterokinase, respectively. (A) B/ALC enterokinase pilot assay incubated for 1 h at room temperature. (B) B/ALC enterokinase pilot assay incubated for 2 h at room temperature. Lane 1 and 6, Marker; Lane 2, 3, 4, 5, 7 and 8, 20 µg B/ALC treated with 0, 0.001, 0.01, 0.1, 1 and 4 U enterokinase, respectively. (C) B/ALC enterokinase pilot assay incubated for 16 h at room temperature. (D) Immunoblot detection of B/ALC enterokinase pilot assay probed with mouse anti-His IgG. Lane 1, Marker; Lane 2 – 4, 20 µg B/ALC treated with 0, 0.1, 1 U enterokinase for 1 h at room temperature; Lane 5 – 7, 20 µg B/ALC treated with 0, 0.1, 1 U enterokinase for 2 h at room temperature.
For B/ALC, His$_6$-tag removal was achieved after incubation with 1 U enterokinase for 2 h at room temperature, indicated by the absence of a band on the anti-His Western blot for this sample (Figure 4.15B).

### 4.3.2.2 His$_6$-tag removal

Prior to the removal of the free His$_6$-tag cleaved from the recombinant proteins during enterokinase treatment, the enzyme was first removed from the reaction using the Enterokinase removal kit (Invitrogen Corporation, U.S.A.). Following enterokinase removal, cleaved His$_6$-tag was removed using a modified method of batch purification (protein miniprep). Samples were analysed by SDS-PAGE and a Western blot, probing with an antibody directed at the His$_6$-tag to detect the presence of the affinity tag. Protein migrated to the predicted molecular weight for both His-free TLC (data not shown) and B/ALC (Figure 4.16). No bands were present on anti-His Western blots (data not shown), indicating that His$_6$-tag was completely removed from the enterokinase-treated protein.

### 4.3.3 Functional assays

Functional assays were performed for the detection of cleavage activity of recombinant TLC and B/ALC. A cleavage assay for the detection of TLC activity was analysed by observing a shift in molecular weight of the GST-VAMP-2 substrate by SDS-PAGE, and cleavage of a fluorogenic SNAP-25 substrate by B/ALC was analysed by measuring fluorescence emitted by FRET.

#### 4.3.3.1 VAMP-2 cleavage assay

Samples containing recombinant positive control TLC, and the recombinant TLC (Figure 4.17A), and His-free TLC (Figure 4.17B) developed in this study show a shift in the size of GST-VAMP-2 from 38 kDa to 36 kDa, indicating cleavage of GST-VAMP-2 at the target peptide bond of native TLC. Incubation of GST-VAMP-2 with recombinant B/ALC (Figure 4.17A), His-free B/ALC (Figure 4.17C), and without the addition of toxin (Figure 4.17A, B, C), does not display the 36 kDa cleavage product of GST-VAMP-2.
Figure 4.16: His$_6$-tag removal from B/ALC.
SDS-PAGE stained with Coomassie instant stain. B/ALC treated with enterokinase and removal of cleaved His$_6$-tag by batch purification. Lane 1, Marker; Lane 2, 200 mM imidazole wash (contains cleaved His$_6$-tag, ~6 kDa); Lane 3, His-free B/ALC collected after His$_6$-tag was removed from the sample.
Figure 4.17: VAMP-2 cleavage assay.

(A) to (C) SDS-PAGE stained with Coomassie instant stain. (A) Lane 1, Marker; Lane 2, 200 nM recombinant TLC (positive control); Lane 3, 500 nM recombinant TLC (positive control); Lane 4, 200 nM TLC (this study); Lane 5, 500 nM TLC; Lane 6, 200 nM B/ALC (this study, negative control); Lane 7, 500 nM B/ALC; Lane 8, GST-VAMP-2 only. (B) Lane 1, Marker; Lane 2, GST-VAMP-2 only; Lane 3, 200 nM His-free TLC; Lane 4, 500 nM His-free TLC. (C) Lane 1, Marker; Lane 2, GST-VAMP-2 only; Lane 3, 200 nM His-free B/ALC (negative control); Lane 4, 500 nM His-free B/ALC. NB. GST-VAMP-2 substrate used in (B) and (C) was provided as a 50% pure preparation from List Biological Laboratories, U.S.A., which explains the presence of two major bands at ~26 – 30 kDa in figures (B) and (C).
4.3.3.2 SNAP-25 FRET assay

A fluorogenic SNAP-25 substrate, SNAPtide® was used to assess the cleavage ability of B/ALC FRET analysis. The positive control BoNT/A LC showed the highest increase in fluorescence over time, following by B/ALC, His-free B/ALC, and the negative control TLC showed the lowest fluorescence over the course of the assay (Figure 4.18).
Figure 4.18: SNAP-25 FRET assay.
LC/A (dark blue), recombinant B/ALC positive control (List Biological Laboratories, U.S.A); B/ALC (purple), B/ALC developed in this study; His-free B/ALC (aqua), His-free B/ALC developed in this study; TLC (red), TLC developed in this study (negative control).
4.4 Discussion

This section of the study reports the recombinant expression of soluble, catalytically active tetanus toxin light chain and botulinum type A toxin light chain. The individual catalytic light chain domains of CNTs have previously been expressed in *E. coli* for use in the characterisation of residues involved in catalytic activity and mode of action (Agarwal *et al.*, 2005; Baldwin *et al.*, 2004; Chen, S. and Barbieri, 2006; 2011; Chen, S. *et al.*, 2008b; Chen, S. *et al.*, 2007; Fernandez-Salas *et al.*, 2004; Gul *et al.*, 2010; Kurazono *et al.*, 1992; Sikorra *et al.*, 2008). The LCs have been used in structural characterisation studies (Arndt *et al.*, 2006a; Breidenbach and Brunger, 2004; 2005a; Gul *et al.*, 2010; Li, L. and Singh, 2000; Rao *et al.*, 2005; Silvaggi *et al.*, 2008), and to investigate light chain inhibitors for the prevention of CNT toxicity (Li, B. *et al.*, 2011; Silvaggi *et al.*, 2008; Yiadom *et al.*, 2005). Light chains have also been produced by recombinant expression with the aim of creating a CNT holotoxin either expressed as a single-chain holotoxin or via conjugation with a CNT heavy chain domain (Li, Y. *et al.*, 1994; Wang *et al.*, 2008), or other cell-targeting moiety (Duggan *et al.*, 2002; Hopp and Woods, 1981; Stancombe *et al.*, 2012).

The use of *E. coli* for heterologous expression of LCs has proved to be a convenient host for the expression of recombinant CNT light chains in a soluble form (Agarwal *et al.*, 2004; Baldwin *et al.*, 2004; Chen, S. and Barbieri, 2006; 2011; Chen, S. *et al.*, 2008b; Chen, S. *et al.*, 2007; Fernandez-Salas *et al.*, 2004; Gilsdorf *et al.*, 2006; Jensen *et al.*, 2003; Li, L. and Singh, 2000; Rawat *et al.*, 2008; Sikorra *et al.*, 2008; Yiadom *et al.*, 2005). Yields of TLC and B/ALC recombinant proteins from EnPresso™ were typically ~1.5 mg/mL, and the TLC and B/ALC light chain domains developed in this study were expressed in a soluble form and shown to be catalytically active both in the presence and absence of the N-terminal affinity tag.

4.4.1 TLC expression and purification

TLC expression in LB broth at both 30°C and 37°C produced a high yield of soluble protein in a relatively pure form. TLC expressed at the predicted molecular weight of ~56.7 kDa. The presence of proteins at both higher and lower molecular weights to TLC were initially thought to be non-specific proteins which had co-purified with the His$_6$-tagged TLC. These proteins were present when TLC was expressed between 25°C to 37°C, but not when expressed at 42°C. Taking into consideration the significant decrease in yield of TLC expression at this temperature, this temperature was not deemed ideal for recombinant TLC production. A significant amount of purified protein (10 – 20 µg) was separated by SDS-PAGE for TLC
expressed at 25°C, 30°C and 37°C (Figures 4.1 – 4.3), and when considering this, the purity of TLC can be estimated at between 80 – 90%. However, further attempts to remove these “non-specific” proteins by means of double-purification by IMAC, by expression in EnPresso™ tablet media, with the use of ions with different affinities for binding the His₆-tag during IMAC, and the use of PMSF during the preparation of the cell lysate were performed.

The second step of the double-purification protocol was performed to remove non-specific proteins, and the second purification step was performed to purify TLC from a sample with fewer non-specific proteins, which may have allowed for more specific binding of TLC to the IMAC column. Results indicate that the “non-specific” proteins remained after the second purification step, even after the sample concentration following the first purification. The use of a 30 kDa MWCO centrifugal device should have removed the smaller molecular weight proteins, however proteins as small as 10 kDa which reacted with the His₆-tag and anti-toxin antisera, remained following concentration with the device.

Of the ions commonly used in IMAC, nickel has the least affinity to the His₆-tag and it may therefore allow the binding of endogenous proteins which have a minimum of at least two consecutive histidine residues (Stewart et al., 1998). In an attempt to increase the affinity for His₆-tagged proteins during IMAC purification, divalent cobalt was tested. Cobalt is able to bind His₆-tags with a higher affinity than nickel; however the “non-specific” proteins continued to co-purify with TLC and reacted with the His₆-tag and anti-toxin antisera (Stewart et al., 1998).

The use of the protease inhibitor PMSF during preparation of the cell lysate to inhibit cleavage of TLC by proteases produced by E. coli, did not have an effect on the amount of “non-specific” proteins present after TLC purification.

These results taken together indicate that these “non-specific” proteins may in fact be C-terminal degradation products of TLC. These truncated products would retain the N-terminal His₆-tag and therefore bind to the column with high affinity during purification and co-elute with full-length TLC. It has yet to be determined whether these truncated products are as result of premature termination of translation or due to proteolytic degradation of a small percentage of TLC species which may have misfolded after expression. The presence of these truncated TLC proteins in the purified sample constituted only about 10 – 20% of the total protein content, and was not deemed to be of concern due to the presence of a high yield of soluble TLC.
A protein which migrates between 100 – 150 kDa was present after purification and also detected during immunoblotting within some TLC samples (Figures 4.2 – 4.8, with the exception of Figure 4.4). It is unknown what this reactive species migrating at ~100 kDa is, but given its reaction with antisera, it may be a doublet of TLC which is present due to incomplete reduction of the sample.

TLC expressed in EnPresso™ produced a higher yield of protein than expression in LB broth, which was expected due to the nature of this growth medium. EnPresso™ is designed as a fed-batch expression system and includes an enzymatically controlled slow release glucose delivery which allows slow bacterial growth at 30°C (Baneyx and Mujacic, 2004). Slow growth results in slower recombinant protein production, which allows more time for the proteins to fold correctly in the E. coli host. Recombinant expression using EnPresso™ was performed for 8 h (6 – 24 h is recommended) and slow-release glucose delivery during induction of protein expression and addition of proprietary enzymes (EnZI’m, BioSilta), resulted in a high yield of recombinant TLC protein expression.

TLC was successfully expressed and purified from E. coli BL21 in a high yield at a relatively high purity and was assessed for activity in the VAMP-2 cleavage assay in the presence and absence of the His6-tag.

### 4.4.1.1 His6-tag removal

His6-tag removal was performed to assess whether the affinity tag and/or the method used for its removal would impact on the activity of TLC in the functional assay. The final conjugated holotoxin would not contain the affinity tags, thus it was important to assess if catalytic activity was maintained after its removal. Optimal conditions of His6-tag cleavage were treatment with 1 U enterokinase for 2 h at room temperature (Figure 4.14). The absence of reactive species in the enterokinase-treated sample after Western blot with anti-His antisera, provided further evidence to suggest that the majority of proteins present in the sample were C-terminal degradation products of TLC (present due to either premature termination of translation or degradation of misfolded protein after expression) and retained the N-terminal His6-tag.

Following enterokinase removal with a commercial kit, batch purification (small-scale IMAC) was performed to remove the free and uncleaved His6-tag from TLC. Removal of the His6-tag was confirmed by SDS-PAGE and immunoblotting with antisera targeting the His6-tag. The absence of a visible colorimetric detection on the Western blot confirmed the absence of the His6-tag (data not shown).
4.4.1.2 VAMP-2 functional assay

The GST-VAMP-2 substrate (List Biological Laboratories, U.S.A.), is a 38 kDa recombinant protein consisting of the 97 residue GST-tag, and 116 residues of native VAMP-2 (lacking the final 19 residues of the native VAMP-2), and is usually provided as a 90% pure protein from the manufacturer (List Biological Laboratories, U.S.A.). Upon cleavage of GST-VAMP-2 at the Gln76 – Phe77 peptide bond of VAMP-2, the size of the recombinant substrate displays a shift in molecular weight from 38 kDa to 36 kDa.

TLC expressed in EnPresso™ for 8 h at 30°C was used for the assessment of catalytic activity in the VAMP-2 cleavage assay. Test samples showed that the TLC developed in this study displayed catalytic activity comparable to that of the recombinant TLC positive control (51.2 kDa) (List Biological Laboratories, U.S.A.). TLC both with and without the His6-tag cleaves GST-VAMP-2 in a concentration-dependent manner, as seen by an increase in the amount of cleavage product, and concomitant decrease in the amount of uncleaved GST-VAMP-2 between the 200 nM and 500 nM toxin reactions (Figure 4.17). B/ALC and His-free B/ALC developed in this study, served as a negative control for the VAMP-2 assay, and did not demonstrate cleavage ability for VAMP-2 (Figure 4.16). As this is not a natural substrate for B/ALC, this was expected.

4.4.2 B/ALC expression and purification

B/ALC expression in LB broth produced a high yield of protein at the predicted molecular weight of B/ALC (~56 kDa). B/ALC expression at 30°C produced a higher yield of protein than expression at 37°C; however expression in EnPresso™ for 8 h at 30°C gave the highest yield of B/ALC expression. There were fewer non-specific proteins present at a smaller molecular weight to B/ALC in the preparation compared to that of TLC; however despite the use of a 30 kDa MWCO centrifugal device, these bands remained in the sample after concentration. The origin of these smaller molecular weight proteins were assumed to be C-terminal degradation products, as seen during purification of TLC (Section 4.4.1), since they reacted with both anti-His and anti-C. botulinum A toxoid antisera. The use of PMSF was therefore investigated to inhibit proteolytic degradation of B/ALC; however it did not reduce the number of truncated proteins which remained after purification. The use of cobalt IMAC purification did not alter the purification profile of B/ALC, which was anticipated once the proteins were determined to be C-terminal degradation products which retained the N-terminal His6-tag.
A protein which migrates between 100 – 150 kDa was present after purification and was reactive with anti-His and anti-toxin antisera within B/ALC samples (Figures 4.9A, 4.11, 4.12, 4.13). Much like the case with TLC (Section 4.4.1), it is likely that this reactive species migrating at ~100 kDa is a doublet of the B/ALC which may be present due to incomplete reduction of the sample. The presence of truncated forms of B/ALC in the sample (albeit very few products when compared to those observed in the TLC preparation), was not deemed to be of concern due to the presence of a high yield of soluble B/ALC.

B/ALC was successfully expressed and purified from *E. coli* BL21 in a high yield at a relatively high purity and was assessed for activity in the SNAP-25 FRET assay in the presence and absence of the His$_6$-tag.

### 4.4.2.1 His$_6$-tag removal

Optimal conditions of His$_6$-tag cleavage were treatment with 1 U enterokinase for 2 h at room temperature (Figure 4.15). Although a faint band is present within the Western blot probed with antisera to the His$_6$-tag in this sample (Figure 4.15D, Lane 7), any uncleaved His$_6$-tag still associated with B/ALC would have been removed during batch purification, and would therefore not be present in the final His$_6$-free sample.

Successful removal of the His$_6$-tag was confirmed by SDS-PAGE and immunoblotting with antisera targeting the His$_6$-tag. The absence of a visible colorimetric detection on the Western blot confirmed the absence of the His$_6$-tag (data not shown).

### 4.4.2.2 SNAP-25 functional assay

The SNAP-25 FRET substrate, SNAPtide® (FITC/DABCYL) (List Biological Laboratories, U.S.A.) was used to measure catalytic activity of toxins with the amount of substrate cleaved proportional to the increase in fluorescence over time. B/ALC expressed in EnPresso™ for 8 h at 30°C was used for the assessment of catalytic activity in the SNAP-25 FRET assay. Test samples showed that the B/ALC developed in this study displayed catalytic activity. The TLC developed in this study served as a negative control for the SNAP-25 assay, and did not demonstrate cleavage ability for SNAP-25 (Figure 4.18). As this is not a natural substrate for TLC, this was expected.

B/ALC both with and without the His$_6$-tag demonstrated a higher fluorescence than the negative control TLC (this study); however neither B/ALC preparation displayed fluorescence
as high as that of the recombinant full-length B/ALC positive control (50 kDa) (List Biological Laboratories, U.S.A.). The fluorescence of B/ALC over time was higher than that of His-free B/ALC, indicating that the method of His<sub>6</sub>-tag removal may have reduced the catalytic activity of the protein, however the causes of the reduced activity need to be further investigated.

4.5 Conclusion

This section of the study has described the recombinant expression of the tetanus toxin and botulinum toxin type A light chains in *E. coli*. Recombinant proteins were purified at a high yield in soluble form and displayed catalytic activity comparable to that displayed by the positive control proteins. TLC was demonstrated to be equally as active as the positive control protein when assayed for VAMP-2 substrate cleavage, however B/ALC, although clearly active compared to the negative control TLC, was not as active as the positive control B/ALC.

One study has demonstrated the importance of the C-terminus of LCs in solubility, stability and catalytic activity of the LC domain due to the presence of hydrogen bonds which stabilise the structure of the domain and maintain the catalytic active site (Baldwin *et al.*, 2004). It is possible that the difference in catalytic activity seen between B/ALC and its positive control may be due to the misfolding of the B/ALC protein or alterations to the C-terminal residues. Western blot analysis of both TLC and B/ALC demonstrated C-terminal degradation products (many more in TLC than B/ALC), which were not significantly inhibited with the use of the protease inhibitor, PMSF. It is unclear as to whether degradation is caused by endogenous proteases, or perhaps by zinc-metalloprotease activity of the LCs themselves, which has been reported by Baldwin (Baldwin *et al.*, 2004).

Further investigation to optimise the expression and purification of both TLC and B/ALC is required and may also include different methods of purification, the use of a protease inhibitor cocktail to inhibit proteolytic attack by other proteases to which TLC and B/ALC are predicted to be susceptible (Table 3.2). Although B/LC activity is currently not optimal, it may still be useful for the conjugation to THC to create the chimeric Clostridial holotoxin.
Chapter 5
Protein expression and purification of tetanus toxin heavy chain
5.1 Introduction

Following the expression of the tetanus toxin (TLC) and botulinum type A (B/ALC) toxin light chains in the previous section; this final section of the study outlines the small-scale expression and purification of the recombinant tetanus toxin heavy chain (THC) domain. In order for the novel chimeric holotoxin to perform its catalytic function within inhibitory interneurons in the CNS, the resulting molecule must be able to bind to the native receptors on the neuronal target cells and following internalisation, have the ability to translocate the catalytic light chain into the cytosol of the cell where it can then cleave its target SNARE protein.

Expression of the heavy chain domain of tetanus toxin normally includes the C-terminal binding domain, H\textsubscript{C} (or fragment-C), which is sufficient for cell-binding and retrograde trafficking, but does not retain the translocation function of the H\textsubscript{N} domain. However, expression of full-length tetanus toxin heavy chain in \textit{E. coli} has also been reported (Li, Y. \textit{et al.}, 1999).

The recombinant heavy chain protein expressed in this section of the study contains an N-terminal His\textsubscript{6}-tag and the native ~50 kDa N-terminal (H\textsubscript{N}) domain (residues S458 – V879), which is mostly alpha-helical and displays pore-forming ability to translocate the LC domain from the endosome and into the cytosol of the cell after internalisation, a step which is critical for the catalytic activity of the LC (Boquet and Duflot, 1982). It also contains the ~50 kDa C-terminal (H\textsubscript{C}) receptor binding domain (residues I880 – D1315) of native tetanus toxin. The C-terminal portion of the heavy chain (H\textsubscript{C}), can further be divided into the N-terminal (H\textsubscript{CN}) domain (residues 865 – 1110), displaying a lectin-like jelly roll domain, and C-terminal (H\textsubscript{CC}) (residues 1110 – 1315), with a β-trefoil domain (Emsley \textit{et al.}, 2000). The H\textsubscript{CC} domain contains residues critical for receptor binding, however, it is still unclear as to the function of the H\textsubscript{CN} domain (Swaminathan, 2011). THC binds to gangliosides GT\textsubscript{1b} and a secondary protein receptor present on the surface on neuronal cells through binding sites present in the C-terminal domain of the H\textsubscript{C} (H\textsubscript{CC}) (Montecucco, 1986).

The first section of this part of the study is to express the recombinant heavy chain in \textit{E. coli}. Two types of growth media and two strains of \textit{E. coli} will be evaluated, as well as a cell-free culture method to determine the optimal conditions required to obtain a high yield of soluble protein. The second section of the study aims to purify the heavy chain using IMAC. IMAC utilises the N-terminal His\textsubscript{6}-tag expressed as part of THC for isolation from \textit{E. coli}. Multiple
purification configurations will be evaluated to find the optimal conditions for purification of the recombinant proteins.
5.2 Materials and methods

5.2.1 Expression in LB broth

Expression vector pTHC was electrotransformed into *E. coli* BL21(DE3)pLysS or *E. coli* Origami™2(DE3)pLysS for each expression (Section 2.4.4.2). After overnight growth of transformants on LB agar containing the appropriate antibiotics, a single colony was subcultured into 2 mL LB broth containing antibiotics and grown at 37°C until the absorbance at 600 nm reached 0.6 – 1.0. The next stage of the protocol, growth to mid-log phase (absorbance at 600 nm of 0.3 – 0.6), was also performed in LB broth at 37°C (Section 2.6.3.3). Induction of protein expression with IPTG was performed in LB broth for 3 h at the required temperature. After 3 h induction, cell lysates were prepared for IMAC purification (Section 2.6.3.5). Induction of protein expression with IPTG was performed in LB broth for 3 h at 25°C, 30°C, 37°C and 42°C in *E. coli* BL21(DE3)pLysS, and for 3 h at 37°C in *E. coli* Origami™2(DE3)pLysS.

5.2.2 Expression in EnPresso™

Expression vector pTHC was electrotransformed into *E. coli* BL21(DE3)pLysS or *E. coli* Origami™2(DE3)pLysS for each expression (Section 2.4.4.2). After overnight growth of transformants on LB agar containing the appropriate antibiotics, a single colony was subcultured into 2 mL LB broth containing antibiotics and grown at 37°C until the absorbance at 600 nm reached 0.6 – 1.0. The next stage of the protocol, growth to mid-log phase (absorbance at 600 nm of 0.3 – 0.6), was performed in EnPresso™ tablet medium (Section 2.3.2) at 30°C. Induction of protein expression with IPTG was performed in EnPresso™ tablet medium for 8 h at 30°C (Section 2.6.3.4). After 8 h induction, cell lysates were prepared for IMAC purification (Section 2.6.3.5). THC expressed in EnPresso™ was performed with and without the addition of 1 mM PMSF protease inhibitor (Section 2.3.1) during preparation of the cell lysate.

5.2.3 Detection of inclusion bodies

THC expressed in *E. coli* BL21(DE3)pLysS in LB broth at 37°C and in EnPresso™ at 30°C was assessed for inclusion body formation (Section 2.6.4.1). THC expressed in *E. coli* Origami™2(DE3)pLysS in LB broth at 37°C was also assessed for inclusion body formation (Section 2.6.2.1). Following expression of THC and preparation of cell lysate, samples were
assessed for the presence of inclusion bodies by analysing the cell debris which remained after preparation of the cell lysate. After addition of 5x SDS sample loading buffer and heating at 100°C for 3 min, the presence of THC in the “pellet” sample was indicative of inclusion bodies.

5.2.3.1 Pilot solubilisation of inclusion bodies

Following the determination of inclusion body formation, a pilot study was performed to assess conditions required to solubilise inclusion bodies present in the cell lysate (Section 2.6.4.2). Inclusion bodies were solubilised in 0.1 M Tris-HCl of varying pH (pH 8 – 12.5), with and without 2 M urea. Supernatant released from solubilised inclusion bodies and the remaining cell debris were analysed by SDS-PAGE (Section 2.6.2.1).

5.2.4 Cell-free expression

Expression vector pTHC was purified using the PureYield™ plasmid miniprep system (Promega, U.S.A.), (Section 2.3.5) following the manufacturer’s instructions for expression in the S30 T7 High-yield protein expression system (Promega, U.S.A.), (Section 2.3.1). Due to the small volume of cell-free expression, THC was purified by batch purification (Section 2.7.1.6) using nickel IMAC. The IMAC resin was washed in 5 column volumes (CV) of protein wash buffer (Section 2.3.1) containing 50 mM imidazole and THC was eluted in protein elution buffer (Section 2.3.1) containing 100 mM imidazole and samples were analysed by SDS-PAGE (Section 2.6.2.1).

5.2.5 IMAC purification

Following preparation of the cell lysate (Section 2.6.3.5), samples were purified using IMAC (Section 2.7.1.2). IMAC purification was performed using Chelating Sepharose™ fast flow (Section 2.3.1), charged with 0.2 M nickel sulphate (Ni²⁺) or 0.2 M cobalt chloride (Co²⁺) (Section 2.7.1.1). After the His₆-tagged proteins in the cell lysate were allowed to bind to the pre-charged resin, which was pre-equilibrated with protein binding buffer (Section 2.3.1). The column was then washed in 5 CV of protein wash buffers (Section 2.3.1) containing 20 mM and 50 mM imidazole. THC was eluted in protein elution buffer (Section 2.3.1) containing 100 mM imidazole (Section 2.3.1).
5.2.5.1 THC purification

A double purification procedure using Ni$^{2+}$ IMAC was performed for THC expressed in LB broth at 30°C. Following preparation of the cell lysate, THC was purified using IMAC (Section 2.7.1.2), and the eluted protein was concentrated and buffer exchanged (Section 2.7.2.1), into protein binding buffer containing 10 mM imidazole (Section 2.3.1) to facilitate binding to the IMAC column during the second purification step. The concentrated protein sample was then purified a second time in a newly prepared nickel IMAC column, using the same protocol (Section 2.7.2.1). The THC protein was eluted in protein elution buffer (Section 2.3.1) containing 100 mM imidazole, and the concentration and buffer exchange protocol (Section 2.7.2.1) was repeated. The double-purified THC was buffer exchanged to protein storage buffer (Section 2.3.1).

Purification of THC expressed in EnPresso™ tablet media was performed using IMAC pre-charged with 0.2 M nickel sulphate or 0.2 M cobalt chloride (Section 2.7.1.2), and THC was eluted in protein elution buffer (Section 2.3.1) containing 100 mM imidazole.

THC expressed in cell-free culture and THC expressed in EnPresso™ following the solubilisation of inclusion bodies were purified using nickel batch purification (protein miniprep) (Section 2.7.1.6). Samples were analysed by SDS-PAGE (Section 2.6.2.1) and visualised by staining with Coomassie instant stain (Section 2.6.2.2) and by Western blot (Section 2.6.2.3 – 2.6.2.4), probing with antisera directed at the His$_6$-tag (mouse anti-His IgG), and to the toxin domain (rabbit anti-tetanus toxin IgG) (Section 2.3.4).

5.2.6 Concentration and buffer exchange

Following the elution of THC from the IMAC column, protein present in protein elution buffer was applied to a pre-rinsed 50 kDa molecular weight cut-off (MWCO) Vivaspin 20 centrifugal device (Section 2.2), and samples were concentrated to a small volume (typically <1 mL) before performing buffer exchange (Section 2.7.2.1) to protein storage buffer (Section 2.3.1). Protein was stored in aliquots at -20°C for up to 2 months, or until required.

5.2.7 Enterokinase treatment

Cleavage of the affinity tag from the recombinant proteins was performed using treatment with EKMax™ enterokinase (Invitrogen, U.S.A.), (Section 2.3.3). A pilot assay (Section
2.7.2.3) was performed to assess the optimal amount of enzyme, temperature and time of incubation required to remove the His$_6$-tag from recombinant THC.

5.2.7.1 His$_6$-tag removal

Once the optimal amount of enzyme and time of incubation was determined, enterokinase was removed from the reaction using the Enterokinase removal kit (Invitrogen Corporation, U.S.A.) (Section 2.3.5), and the cleaved His$_6$-tag was removed using a modified method of batch purification (protein miniprep) (Section 2.7.1.6). Samples were analysed by SDS-PAGE (Section 2.6.2.1) and visualised by staining with Coomassie instant stain (Section 2.6.2.2) and by Western blot (Section 2.6.2.3 – 2.6.2.4), probing with an antibody directed at the His$_6$-tag (Section 2.3.4) to detect the presence of the tag.

5.3 Results

5.3.1 Protein expression

5.3.1.1 THC expression and purification from LB broth in E. coli BL21

THC was expressed in E. coli BL21(DE3)pLysS with a final concentration of 1 mM IPTG for 3 h in LB broth at 25°C, 30°C, 37°C and 42°C to investigate the effect of temperature on THC expression. THC expressed at 25°C (Figure 5.1), 30°C (Figure 5.2), 37°C (Figure 5.3), and 42°C (Figure 5.4) in LB broth was purified by nickel IMAC and the recombinant protein was eluted in protein elution buffer containing 100 mM imidazole. Following purification with IMAC, concentration and buffer exchange, 5 – 15 µg of protein was separated using SDS-PAGE and visualised by staining with Coomassie instant stain (Figures 5.1A – 5.4A). Following immunotransfer, membranes were probed by immunoblotting, with antisera to the His$_6$-tag (mouse anti-His IgG) (Figures 5.1B – 5.4B), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figures 5.1C – 5.4C), for the detection of the His$_6$-tag and tetanus toxin domain, respectively. Cross-reactivity is evident with both antisera which detect proteins at both a larger and smaller molecular weights to THC. His$_6$-tagged THC migrated to the predicted molecular weight of 102.7 kDa and induction of protein expression was optimal between 25°C (Figure 5.1) and 30°C (Figure 5.2). However, due to the extended length of time required for growth at 25°C, THC expression at 30°C was determined to be optimal for high yield production of THC.
Figure 5.1: THC expressed in *E. coli* BL21 at 25°C in LB broth and purified by Ni$^{2+}$ IMAC.

Lane 1, Marker; Lane 2, 15 µg purified THC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.2: THC expressed in *E. coli* BL21 at 30°C in LB broth and purified by Ni^{2+} IMAC.

Lane 1, Marker; Lane 2, 10 µg purified THC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.3: THC expressed in *E. coli* BL21 at 37°C in LB broth and purified by Ni$^{2+}$ IMAC.

Lane 1, Marker; Lane 2, 10 µg purified THC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.4: THC expressed in *E. coli* BL21 at 42°C in LB broth and purified by Ni²⁺ IMAC.

Lane 1, Marker; Lane 2, 5 µg purified THC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Double-purification of THC expressed in *E. coli* BL21(DE3)pLysS at 30°C (Figure 5.5A) was performed to remove “non-specific” proteins which remained after concentrating the eluted sample in a 50 kDa MWCO Vivaspin 20 centrifugal device. Figure 5.5A, Lane 2 shows THC after the first purification and buffer exchange step, and Figure 5.5A, Lane 4 shows THC after the second purification and buffer exchange step. Figures 5.5B and 5.5C show Western blot results after probing with anti-His IgG and anti-tetanus toxin IgG antisera, respectively.

Cross-reactivity is evident with both antisera which detected proteins at lower molecular weights to THC. Results indicate that the double-purification procedure did not remove the non-specific proteins present at both higher and lower molecular weight to the THC.

### 5.3.1.2 THC expression and purification from EnPresso™ tablet media in *E. coli* BL21

THC expressed in *E. coli* BL21(DE3)pLysS in EnPresso™ tablet media was purified using both nickel and cobalt IMAC. Induction of protein expression was performed for 8 h at 30°C in EnPresso™, however all subsequent steps leading to purification were identical to those in previous section (Section 5.3.1.1).

THC expressed in EnPresso™ tablet media was purified using nickel IMAC and 10 µg of purified, concentrated and buffer exchanged protein was separated by SDS-PAGE and stained with Coomassie instant stain (Figure 5.6A). Following immunotransfer, membranes were probed by immunoblotting with antisera to the His6-tag (mouse anti-His IgG) (Figure 5.6B), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figure 5.6C), for the detection of the His6-tag and tetanus toxin domain, respectively.

THC expressed in EnPresso™ (Figure 5.6) did not significantly increase the yield of recombinant THC when compared to THC expressed at 25°C – 37°C in LB broth (Figures 5.1 – 5.3). However, due to the more simple method of expression with EnPresso™ than with LB broth, THC expressed at 30°C in EnPresso™ was determined to be optimal for this study. It must be noted that noted that THC often migrated to just below the 100 kDa band in the protein standard marker when expressed in EnPresso™.

To compare the influence of the metal ion used for IMAC purification of THC, THC expressed in EnPresso™ was also purified using cobalt IMAC. Ten micrograms of purified, concentrated
Figure 5.5: THC expressed in *E. coli* BL21 at 30°C in LB broth and purified twice by Ni²⁺ IMAC. 

(A) SDS-PAGE stained with Coomassie instant stain. Lane 1 and 3, Marker; Lane 2, 10 µg purified THC (first purification); Lane 4, 10 µg purified THC (second purification). (B) and (C) Lane 1, Marker; Lane 2, 10 µg purified THC (first purification); Lane 3, 10 µg purified THC (second purification). (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.6: THC expressed in *E. coli* BL21 at 30°C in EnPresso™ and purified by Ni²⁺ IMAC.
Lane 1, Marker; Lane 2, 10 µg purified THC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
and buffer exchanged protein was separated by SDS-PAGE and visualised by staining with Coomassie instant stain (Figure 5.7A). Western blotting was performed and membranes were probed by immunoblotting with antisera to the His$_6$-tag (mouse anti-His IgG) (Figures 5.7B), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figure 5.7C). The use of cobalt IMAC did not result in a reduction in the number of proteins at a lower molecular weight to THC when the results of Figure 5.6 and Figure 5.7 are compared.

THC expressed in EnPresso™ was also purified after treatment with protease inhibitor PMSF during preparation of the cell lysate. Figures 5.8A, B and C show 10 µg of purified, concentrated and buffer exchanged protein separated by SDS-PAGE and visualised by staining with Coomassie instant stain, and Western blot probed with antisera to the His$_6$-tag (mouse anti-His IgG), and tetanus toxin (rabbit anti-tetanus toxin IgG), respectively. The use of PMSF showed some improvements in reducing the amount of non-specific protein detected after immunoblotting.

### 5.3.1.3 THC expression and purification from LB broth in *E. coli* Origami™2

THC expressed in *E. coli* Origami™2(DE3)pLysS was performed with a final concentration of 1 mM IPTG for 3 h in LB broth at 30°C (Figure 5.9), and was purified by cobalt IMAC. THC was eluted in protein elution buffer containing 100 mM imidazole. Following purification by IMAC, concentration and buffer exchange, 5 µg of protein was separated using SDS-PAGE and visualised by staining with Coomassie instant stain and by Western blot, probing with antisera to the His$_6$-tag (mouse anti-His IgG) (Figure 5.9B), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figure 5.9C), for the detection of the His$_6$-tag and tetanus toxin domain, respectively. THC was expressed at a lower yield from *E. coli* Origami™2(DE3)pLysS in LB broth compared to expression at the same temperature in *E. coli* BL21(DE3)pLysS (Figure 5.2), however there were fewer reactive species of a lower molecular weight to THC present after expression in *E. coli* Origami™2(DE3)pLysS.

### 5.3.1.4 THC expression and purification from EnPresso™ tablet media in *E. coli* Origami™2

THC expressed in *E. coli* Origami™2(DE3)pLysS for 8 h at 30°C in EnPresso™ tablet media was purified using cobalt IMAC (Figure 5.10). Three micrograms of purified, concentrated
Figure 5.7: THC expressed in *E. coli* BL21 at 30°C in EnPresso™ and purified by Co²⁺ IMAC.

Lane 1, Marker; Lane 2, 10 µg purified THC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.8: THC expressed in *E. coli* BL21 at 30°C in EnPresso™ with PMSF and purified by Co²⁺ IMAC.

Lane 1, Marker; Lane 2, 10 µg purified THC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.9: THC expressed in *E. coli* Origami™2 at 30°C in LB broth and purified by Co²⁺ IMAC.

Lane 1, Marker; Lane 2, 5 µg purified THC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.10: THC expressed in *E. coli* Origami™2 at 30°C in EnPresso™ and purified by Co²⁺ IMAC.

Lane 1, Marker; Lane 2, 3 µg purified THC. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
and buffer exchanged protein was separated by SDS-PAGE and visualised by staining with Coomassie instant stain (Figure 5.10A). Following immunotransfer, membranes were probed by immunoblotting with antisera to the His$_6$-tag (mouse anti-His IgG) (Figure 5.10B), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figure 5.10C), for the detection of the His$_6$-tag and tetanus toxin domain, respectively. THC was expressed from *E. coli* Origami™2(DE3)pLysS in EnPresso™ at a yield comparable to that after expression in *E. coli* BL21(DE3)pLysS in EnPresso™ with PMSF (Figure 5.8). There was a reduction in the number of reactive species present at a lower molecular weight compared to THC expressed in *E. coli* BL21(DE3)pLysS at 30°C in EnPresso™ (Figure 5.7).

### 5.3.2 Detection of inclusion bodies

#### 5.3.2.1 Detection of inclusion bodies from *E. coli* BL21

THC expressed in *E. coli* BL21(DE3)pLysS at 37°C in LB broth (Figure 5.11) and at 30°C in EnPresso™ (Figure 5.12) was analysed for the presence of inclusion bodies. Ten microlitres of cell lysate and cell debris or “pellet” sample were analysed by SDS-PAGE and visualised by staining with Coomassie instant stain (Figure 5.11A and 5.12A), and by Western blot, probing with antisera to the His$_6$-tag (mouse anti-His IgG) (Figure 5.11B and 5.12B), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figure 5.11C and 5.12C), for the detection of the His$_6$-tag and tetanus toxin domain, respectively. THC was detected in the pellet sample indicating the presence of inclusion bodies after expression in at 37°C in LB broth (Figure 5.11) and at 30°C in EnPresso™ (Figure 5.12).

#### 5.3.2.2 Detection of inclusion bodies from *E. coli* Origami™2

THC expressed in *E. coli* Origami™2(DE3)pLysS at 37°C in LB broth and at 30°C in EnPresso™ was analysed for the presence of inclusion bodies. Ten microlitres of cell lysate and cell debris or “pellet” sample were analysed by SDS-PAGE and stained with Coomassie instant stain (Figure 5.13A and 5.14A), and probing with antisera to the His$_6$-tag (mouse anti-His IgG) (Figure 5.13B and 5.14B), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figure 5.13C and 5.14C) for the detection of the His$_6$-tag and tetanus toxin domain, respectively, following immunotransfer. THC was detected in the pellet sample indicating the presence of inclusion bodies after expression at 37°C in LB broth (Figure 5.13) and at 30°C in EnPresso™ (Figure 5.14).
Figure 5.11: Detection of inclusion bodies for THC expressed in *E. coli* BL21 at 37°C in LB broth.
Lane 1, Marker; Lane 2, 10 µL soluble fraction; Lane 3, 10 µL insoluble fraction. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.12: Detection of inclusion bodies for THC expressed in *E. coli* BL21 at 30°C in EnPresso™.

Lane 1, Marker; Lane 2, 10 µL insoluble fraction; Lane 3, 10 µL soluble fraction. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.13: Detection of inclusion bodies for THC expressed in *E. coli* Origami™2 at 37°C in LB broth.

Lane 1, Marker; Lane 2, 10 µL soluble fraction; Lane 3, 10 µL insoluble fraction. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.14: Detection of inclusion bodies for THC expressed in *E. coli* Origami™2 at 30°C in EnPresso™.

Lane 1, Marker; Lane 2, 10 µL soluble fraction; Lane 3, 10 µL insoluble fraction. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
5.3.3 Solubilisation of inclusion bodies

Following the detection of inclusion body formation after THC was expressed in *E. coli* BL21(DE3)pLysS) at 37°C in LB broth (Figure 5.11) and at 30°C in EnPresso™ (Figure 5.12), a pilot study was performed to determine the optimal conditions required to solubilise the inclusion bodies. Inclusion bodies were isolated following THC expression at 37°C in LB broth (Figure 5.15) and at 30°C in EnPresso™ (Figure 5.16), and were treated with buffer containing 0.1 M Tris-HCl at varying pH with and without 2 M urea. The pellet samples and the supernatant collected following cell lysis were analysed by SDS-PAGE and stained with Coomassie instant stain (Figure 5.15A – C and 5.16A – C) and by Western blot, probing with antisera to the His₆-tag (mouse anti-His IgG) (Figure 5.15D and 5.16D), and tetanus toxin (rabbit anti-tetanus toxin IgG) (Figure 5.15E and 5.16E), for the detection of the His₆-tag and tetanus toxin domain, respectively. The presence of THC at ~102.7 kDa in supernatant samples was indicative of solubilised THC, whilst the presence of THC in pellet samples indicated THC had not been completely solubilised in the buffer.

THC expressed in inclusion bodies at 37°C in LB broth was completely solubilised by treatment with buffer containing 0.1 M Tris-HCl pH 12.5 and with buffer containing 0.1 M Tris-HCl pH 12 and 2M urea as indicated by the absence of protein in the pellet sample after treatment with these buffers (Figure 5.15B, Lane 4; Figure 5.15C, Lane 6; Figure 5.15D, Lane 5 and 9; and Figure 5.15E, Lane 4 and 8).

THC expressed in inclusion bodies at 30°C in EnPresso™ was not completely solubilised after treatment with the buffers assessed, however buffer containing 0.1 M Tris-HCl pH 12.5 and 2 M urea solubilised the majority of THC as indicated by a reduction in the amount of THC present in the pellet sample and a larger amount of THC present in the corresponding supernatant sample (Figure 5.16C, Lane 6 and 7; and Figure 5.16D – 5.16E, Lane 4 and 5).
Figure 5.15: Solubilisation of inclusion bodies for THC expressed in *E. coli* BL21 at 37°C in LB broth.

(A) SDS-PAGE stained with Coomassie instant stain. Lane 1 and 4, Marker; Lane 2 and 3, 0.1 M Tris-HCl pH 12.5, 10 µL insoluble and soluble fraction; Lane 5 and 6, 0.1 M Tris-HCl pH 12 and 2 M urea, 10 µL insoluble and soluble fraction. (C) and (D) Lane 1 and 6, Marker; Lane 2 and 3, 0.1 M Tris-HCl pH 12.5, 10 µL insoluble and soluble fraction; Lane 4 and 5, 0.1 M Tris-HCl pH 12.5 and 2 M urea, 10 µL insoluble and soluble fraction. (C) Immunoblot detection of THC probed with mouse anti-His IgG. (D) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.16: Solubilisation of inclusion bodies for THC expressed in *E. coli* BL21 at 30°C in EnPresso™.

(A) SDS-PAGE stained with Coomassie instant stain. Lane 1 and 4, Marker; Lane 2 and 3, 0.1 M Tris-HCl pH 12.5, 10 µL insoluble and soluble fraction; Lane 5 and 6, 0.1 M Tris-HCl pH 12.5 and 2 M urea, 10 µL insoluble and soluble fraction. (C) and (D) Lane 1 and 6, Marker; Lane 2 and 3, 0.1 M Tris-HCl pH 12.5, 10 µL insoluble and soluble fraction; Lane 4 and 5, 0.1 M Tris-HCl pH 12.5 and 2 M urea, 10 µL insoluble and soluble fraction. (C) Immunoblot detection of THC probed with mouse anti-His IgG. (D) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
5.3.3.1 Purification of solubilised inclusion bodies

THC was purified from inclusion bodies following expression in *E. coli* BL21(DE3)pLysS at 30°C in EnPresso™ and solubilisation in 0.1 M Tris-HCl pH 12.5 and 2 M urea (determined in Section 5.3.3), using batch purification (Figure 5.17). There is a high degree of cross-reactivity evident with antisera directed at the His<sub>6</sub>-tag (Figure 5.17B) which detected proteins at both a smaller and larger molecular weights to THC, however the immunoblot probed with antisera to tetanus toxin (Figure 5.17C) shows a dominant protein at the approximate molecular weight of THC. THC is present in the flow-through collected after the cell lysate was bound to the column, indicating that not all of the THC bound to the nickel resin.

5.3.4 Cell-free expression

THC was expressed in the S30 T7 High-Yield protein expression system using *E. coli*-extracted factors for transcription and translation in a cell-free system. Following batch purification using nickel IMAC, 8 µL of the cell-free reaction (representing approximately 20% of the 50 µL reaction) was separated using SDS-PAGE and visualised by staining with Coomassie instant stain (Figure 5.18A) and by Western blot, probing with antisera to the His<sub>6</sub>-tag (mouse anti-His IgG) (Figures 5.18B). During batch purification, the resin was washed with protein wash buffer containing 50 mM imidazole (Figure 5.18A and B, Lane 2), and eluted in protein elution buffer containing 100 mM imidazole (Figure 5.18A and B, Lane 3). THC was detected at ~102.7 kDa in both wash and elution samples of the Western blot (Figure 5.18B), and there was no cross-reactivity demonstrated with the anti-His antisera.

5.3.5 His<sub>6</sub>-tag cleavage and removal

5.3.5.1 Enterokinase treatment

Cleavage of the affinity tag from recombinant THC was performed using treatment with enterokinase. A pilot assay was performed to assess the optimal amount of enzyme, temperature and time of incubation required to remove the His<sub>6</sub>-tag from THC. Figures 5.19A – D show the enterokinase pilot assay for THC.
Figure 5.17: Solubilised inclusion bodies for THC expressed in *E. coli* BL21 at 30°C in EnPresso™ purified by Ni²⁺ IMAC.

Lane 1, Marker; Lane 2, 20 µL of column flow through; Lane 3, 20 µL of 100 mM imidazole elution. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG. (C) Immunoblot detection of THC probed with rabbit anti-tetanus toxin IgG.
Figure 5.18: THC expressed by cell-free expression and purified by Ni\textsuperscript{2+} IMAC.
Lane 1, Marker; Lane 2, 20 µL of 50 mM imidazole wash; Lane 3, 20 µL of 100 mM imidazole elution. (A) SDS-PAGE stained with Coomassie instant stain. (B) Immunoblot detection of THC probed with mouse anti-His IgG.
Figure 5.19: THC enterokinase pilot assay.

(A) – (C) SDS-PAGE stained with Coomassie instant stain. Lane 1, Marker; Lane 2 – 7, 20 µg THC treated with 0, 0.001, 0.01, 0.1, 1 and 4 U enterokinase, respectively. (A) THC enterokinase pilot assay incubated for 1 h at room temperature. (B) THC enterokinase pilot assay incubated for 2 h at room temperature. Lane 8, Marker. (C) THC enterokinase pilot assay incubated for 16 h at room temperature. (D) Immunoblot detection of THC enterokinase pilot assay probed with mouse anti-His IgG. Lane 1, Marker; Lane 2 – 4, 20 µg THC treated with 0, 0.1, 1 U enterokinase for 2 h at room temperature.
Complete His<sub>6</sub>-tag removal was achieved after incubation with 1 U enterokinase for 2 h at room temperature, indicated by the absence of a band on the anti-His Western blot for this sample (Figure 5.19D). The inability of the anti-His antibody to produce a colorimetric reaction for this sample indicates the absence of a detectable limit of His<sub>6</sub>-tag within the sample.

### 5.3.5.2 His<sub>6</sub>-tag removal

Prior to the removal of the free His<sub>6</sub>-tag cleaved from recombinant THC during enterokinase treatment, the enterokinase enzyme was first removed from the reaction using the Enterokinase removal kit (Invitrogen Corporation, U.S.A.). Following enterokinase removal, cleaved His<sub>6</sub>-tag was removed using a modified method of batch purification (protein miniprep). Samples were analysed by SDS-PAGE and a Western blot probing with an antibody directed at the His<sub>6</sub>-tag to detect the presence of the affinity tag. Protein migrated to the predicted molecular weight for His-free THC (Figure 5.20) and no bands were present on anti-His Western blots (data not shown), indicating that His<sub>6</sub>-tag was completely removed from the enterokinase-treated protein.
Figure 5.20: His$_6$-tag removal from THC.
SDS-PAGE stained with Coomassie instant stain. THC treated with enterokinase and removal of cleaved His$_6$-tag by batch purification. Lane 1, Marker; Lane 2, 200 mM imidazole wash (contains cleaved His$_6$-tag, ~6 kDa); Lane 3, His-free THC collected after His$_6$-tag was removed from the sample.
5.4 Discussion

This section of the study reports the recombinant expression of tetanus toxin heavy chain. Fragment-C of tetanus toxin, which contains the C-terminal domains involved in cell-binding and retrograde trafficking of TeNT is generally used in the development of CNT-based therapeutic molecules (Charles et al., 1991; Francis et al., 2004a). However, in order to create a chimeric holotoxin which is able to deliver the light chain domain to inhibitory interneurons in the CNS and translocate the LC into the cytosol, the cell-binding and trafficking function of fragment-C and the translocation function of the Hₙ domain needed to be retained. Therefore, full-length recombinant THC was expressed in this study. A previous report has demonstrated the successful production of recombinant full-length tetanus toxin heavy chain from E. coli for the purpose of creating a recombinant tetanus holotoxin by reconstitution of the heavy chain with recombinant tetanus toxin light chain (Li, Y. et al., 1999). Based on the success of this report, this study attempted to produce recombinant THC from E. coli BL21 and E. coli Origami™2.

5.4.1 THC expression and purification from E. coli BL21

There was some difficulty in obtaining THC in a highly pure form after purification by IMAC. C-terminal degradation of THC was indicated by the presence of proteins of a smaller molecular weight which reacted with both anti-His and anti-tetanus toxin antisera. The use of PMSF showed a slight reduction in the amount of non-specific protein, providing further evidence that these proteins may be degradation products of recombinant THC. Degradation has possibly occurred at both N- and C-terminal ends of THC, however only C-terminal degradation products have co-purified with full-length THC since they retain the N-terminal His₆-tag required for purification by IMAC.

Due to the reducing environment in the E. coli cytoplasm disulfide bond formation will be prevented, which may cause misfolding of the protein (Baneyx and Mujacic, 2004; Stewart et al., 1998). The detection of THC in both soluble and insoluble fractions indicated the possible misfolding of THC in the cytoplasm of E. coli BL21 and subsequent partial deposition into insoluble aggregates in the form of inclusion bodies. Protein misfolding may cause exposure of protease-sensitive regions which undergo proteolytic attack by endogenous proteases present in the E. coli cytoplasm. Inclusion bodies are resistant to proteolysis and should be comprised solely of full-length misfolded recombinant THC, however, solubilisation of inclusion bodies still failed to achieve THC in a pure form.
5.4.2 THC expression and purification from \textit{E. coli} Origami\textsuperscript{TM}2

In an attempt to produce an optimal environment for the formation of the intrachain disulfide bond and thus correctly folded recombinant THC, THC was expressed in the \textit{E. coli} Origami\textsuperscript{TM}2 strain. This strain has been designed to enhance disulfide bond formation in the \textit{E. coli} cytoplasm by the introduction of mutations in the thioredoxin reductase (\textit{trxB}) and glutathione reductase (\textit{gor}) genes. Analysis of THC expressed in \textit{E. coli} Origami\textsuperscript{TM}2 indicated that there were fewer reactive proteins present at a lower molecular weight to THC after IMAC purification; however these proteins were similar to those which reacted from THC expressed in \textit{E. coli} BL21. THC was detected in the insoluble fraction after expression in \textit{E. coli} Origami\textsuperscript{TM}2, indicating that THC was not correctly folded and continued to form insoluble aggregates.

5.4.3 Cell-free expression

The S30 T7 High-Yield protein expression system (Promega, U.S.A.) allows for transcription and translation of DNA sequences with a T7 promoter by using T7 RNA polymerase for transcription and factors required for translation extracted from \textit{E. coli} for cell-free protein synthesis. The use of a cell-free expression system for recombinant THC production did not achieve completely pure THC, but demonstrated a single reactive protein at \(\sim 102.7\) kDa in the anti-His Western blot. The presence of only a single reactive protein indicated that THC was not degraded during cell-free expression, the intrachain disulfide bond was potentially formed and the protein was most likely present in its correctly folded form. Difficulties in obtaining pure THC from cell-free expression indicated that this method of recombinant THC production is still not optimal for this study.

Despite the inability to obtain pure, correctly folded recombinant THC, the function of THC was assessed using an ELISA. THC was assessed for its ability to bind to its native receptor, ganglioside GT\textsubscript{1b}; however difficulties with the assay prevented any conclusions being drawn about the binding activity of recombinant THC (data not shown).

In summary, full-length THC was successfully expressed in \textit{E. coli}, however as many others have found it is difficult to obtain this in a pure, homogenous form. In the next chapter, future studies that may be performed to overcome the limitations of this expression system will be discussed.
Chapter 6

Conclusion and future direction
Obstructive sleep apnoea affects 2 – 4% of the population (Young et al., 1993) and causes significant comorbidities which impact on the quality of life. Current treatment options such as oral appliances cause significant discomfort (Malhotra and White, 2002), while pharmacotherapy has resulted in adverse side effects (Black et al., 2010; Darwish et al., 2010). Surgery is a significantly invasive procedure that may not be suitable for some individuals or may not completely reverse symptoms in patients with severe OSA (Ejaz et al., 2011; Olszewska et al., 2012). Although the most successful treatment for reversing OSA symptoms, the non-invasive CPAP device still displays a 50% non-compliance rate 1 year after prescription (Ballard et al., 2007). It is evident that a treatment for OSA which is convenient and more widely accepted by sufferers is necessary.

Tetanus toxin, which causes muscle spasticity by increasing muscle tone, may be used in the treatment of OSA. Controlled administration of tetanus toxin to the muscles of the upper airway involved in obstruction during sleep may increase muscle tone by inducing localised tetany. The use of tetanus toxin in the treatment of airway obstruction which occurs during sleep and under anaesthesia in British Bulldogs, has demonstrated that controlled injection of the toxin into muscles of the upper airway, does in fact relieve OSA symptoms without adverse effects from toxin administration (Sasse et al., 2005).

The tetanus toxin heavy chain domain has been used as a carrier for the transport of cargo proteins to the central nervous system (Bizzini et al., 1977; Francis et al., 2004a) indicating that although the majority of the population are vaccinated against tetanus, the toxin domains are still feasible for use in therapeutic molecules (Fishman et al., 2006). Nevertheless, a method to reduce the potential immunogenicity of the toxin molecule is to replace the tetanus toxin light chain domain with that of the botulinum type A light chain. This chimeric CNT holotoxin will employ the tetanus toxin heavy chain to deliver the botulinum type A light chain to the CNS where it can act on inhibitory interneurons to cause muscle spasticity in the area of injection. The botulinum toxin light chain domain has been used in targeted secretion inhibitors (Stancombe et al., 2012), and the use of the botulinum type A holotoxin in the treatment of muscle spasticity disorders (Truong and Jost, 2006) demonstrates that controlled toxicity with CNTs has therapeutic potential. It may also be possible to replace regions of the heavy chain not involved in neuronal targeting with botulinum sequences.

Recombinant toxin molecules comprising domains from various CNTs have previously been reported (Poulain et al., 1991; Wang et al., 2008), and recombinant CNT domains have been expressed individually in E. coli and later reconstituted to create an active chimeric holotoxin (Li, Y. et al., 1999; Li, Y. et al., 1994). The aims of this study were to express recombinant CNT toxin domains of tetanus toxin light (TLC) and heavy (THC) chains and the botulinum
type A toxin light chain (B/ALC) in order to create a novel chimeric CNT holotoxin comprising B/ALC + THC for the treatment of OSA. The native tetanus toxin would be re-created using recombinantly expressed TLC + THC to assess the method of expression and conjugation to create the chimeric holotoxin by comparing its activity to the native tetanus toxin.

The strategy used in this study was to express the heavy and light chains as individual proteins, with a view to conjugation to form active toxin. This was done for both safety concerns (the individual chains are non-toxic) and to facilitate easy domain swapping when making the different "serotypes". This study demonstrated the expression of the tetanus toxin and botulinum type A light chain domains in a relatively pure and active form. Recombinant light chains were purified to 80 – 90% purity via IMAC after expression in *E. coli*, and were shown to be active in functional assays. The tetanus toxin light chain was at least as active as the commercially obtained positive control tetanus toxin light chain as demonstrated by the successful cleavage of its native substrate, VAMP-2. The BoNT/A light chain developed in this study was catalytically active as demonstrated by the cleavage of its native substrate, SNAP-25 in a FRET assay; although activity was slightly reduced from that of the commercially obtained positive control BoNT/A light chain.

Proteins which migrated at both a higher and lower molecular weight to the light chains observed in both preparations after purification were thought to be C-terminal degradation products which retain the N-terminal His-tag and thus co-purified with the full-length light chain proteins. These proteins were reactive with both anti-His and anti-toxin-specific antisera, indicating that they are truncated forms of the full-length protein. Given the amount of protein loaded onto SDS-PAGE gels, these impurities comprise only 10 – 20% of the total protein obtained after purification of the light chain toxin domains, and both light chains display catalytic activity even in the presence of these impurities. In preparation for animal experiments, a final clean-up of the light chain proteins may be performed using methods such as size-exclusion chromatography or ion exchange chromatography for the removal of truncated forms of the proteins. The tetanus and botulinum type A toxin light chain domains developed in this study were catalytically active and are satisfactory for use in the reconstitution with the tetanus toxin heavy chain to create the final chimeric holotoxin.

Difficulty in the expression of the tetanus toxin heavy chain domain meant that it could not be obtained in a pure, soluble form. The majority of the literature reports the soluble expression of the C-terminal heavy chain domain (H<sub>CC</sub>) also known as fragment-C (Bizzini et al., 1977; Francis et al., 2004a; Lalli and Schiavo, 2002; Larsen et al., 2006). This 50 kDa domain has been expressed successfully in *E. coli* and retains the cell-binding and trafficking activity of the tetanus toxin, but lacks the translocation activity of the H<sub>N</sub> domain. The full-length heavy
The tetanus toxin heavy chain contains four sulfhydryl groups, one involved in forming the interchain disulfide bridge with the LC, two free sulfhydryl groups, and two which form an intramolecular disulfide bond (Krieglstein et al., 1990). The presence of this intramolecular disulfide bond may have an impact on the correct folding of the protein in the *E. coli* cytoplasm during expression. The reducing environment of the *E. coli* cytoplasm is typically not favourable for the formation of disulfide bonds (de Marco, 1999), however since THC has previously been expressed in a soluble form from *E. coli*, it was expected that the same would be possible in this study. Despite the unfavourable conditions of the *E. coli* cytoplasm, it was expected that even if recombinant THC was expressed in a misfolded form, the conditions required to reconstitute the LC and HC to create the final chimeric holotoxin which involves reducing and then oxidising the proteins, would allow formation of the intrachain and interchain disulfide bonds (Li et al., 1999).

The detection of THC in inclusion bodies after expression in *E. coli* BL21 indicated that THC was possibly misfolding during expression and forming protein aggregates within the *E. coli* cytosol. THC expressed using the EnPresso™ tablet media at 30°C was expected to both increase the yield of recombinant protein, and prevent the formation of protein aggregates due to the nature of the expression system. The EnPresso™ tablet media uses a slow release glucose delivery providing a fed-batch system for the slow growth of *E. coli*. The rate at which recombinant protein is produced is reduced increasing the time for synthesis and folding to occur (Krause et al., 2010). It has been demonstrated that over-expression or expression at a fast rate of recombinant proteins may result in the aggregation of folding intermediates within the cell cytoplasm (Vasina and Baneyx, 1997). Initially, the reducing environment of the *E. coli* cytoplasm was not deemed an obstacle, since proteins would be exposed to a reduced environment followed by an oxidised environment during the reconstitution process (Li et al., 1999). However, since inclusion bodies were formed both after expression in *E. coli* BL21 in LB broth at 37°C and in EnPresso™ at 30°C, it was evident that the reducing conditions within the *E. coli* cytoplasm may have been hampering the correct folding of recombinant THC.
Expression of THC in *E. coli* Origami™ gave promising results. This strain contains a double mutation (*trx*B and *gor*) to create an oxidising environment within the *E. coli* cytoplasm to enhance expression of recombinant proteins containing disulfide bonds (de Marco, 1999). Although THC was still present in inclusion bodies after expression in this strain, the protein was purified in a highly pure form from the soluble fraction of the cell lysate, as indicated by fewer non-specific or truncated THC proteins present after purification. These results indicate that THC expression in *E. coli* Origami™ may provide optimal conditions for the formation of the intrachain disulfide bond within THC and therefore create correctly folded recombinant protein. This strain has previously been used for the expression of proteins (Seras-Franzoso *et al*., 2012) and antibodies (Subedi *et al*., 2012) which have proved to be difficult to express in a soluble form due to the presence of one or more disulfide bonds, and it has also been used for the expression of the light chain and translocation domain of BoNT/A (LHn/A) which retains the interchain disulfide bond (Stancombe *et al*., 2012). The misfolding of THC and accumulation of the protein in inclusion bodies may be used to an advantage. Catalytically active BoNT/A, B/ and E LC has successfully been purified in an active form from inclusion bodies (Ahmed and Smith, 2000; Zdanovsky and Zdanovskaia, 2000), indicating that it may be possible to optimise conditions for the purification of THC from inclusion bodies. However, purification of full-length THC from inclusion bodies has not been reported in the literature.

Further optimisation of THC expression is required to elucidate the mechanism behind protein misfolding. It is possible that proteases present within the *E. coli* cytoplasm are contributing to the degradation of the C-terminal region of THC. Protease inhibitor PMSF showed some improvements in the reduction of C-terminal degradation products when used during purification to protect the recombinant protein from proteolytic attack during cell lysis. However, a protease inhibitor cocktail containing multiple protease inhibitors may show further improvements be inhibiting the activity of a wider range of proteases. Another strategy to limit proteolytic attack during protein expression is to use an expression vector that targets the recombinant THC to the periplasm of *E. coli*. Secretion of recombinant proteins to the oxidising environment of the periplasm following expression has been shown to enhance folding of proteins containing disulfide bonds (de Marco, 1999). The pMAL expression vectors (NEB, U.S.A.) are able to express recombinant protein fused to a maltose-binding protein (MBP), which has been shown to increase solubility of recombinant proteins (Kapust and Waugh, 1999).

For safety reasons, the toxin domains created in this study were expressed individually with the aim to the reconstitute the light and heavy chains to create the active di-chain molecule. However, expression of full-length single-chain toxins has been performed and is a feasible option for the future development of chimeric CNT holotoxins (Li *et al*., 2001; Wang *et al*.,
2008). The ultimate aims of the project will include the production of sequence variants of chimeric holotoxins in an attempt to create tetanus subtypes that maintain the native function of THC, but are less immunogenic than the native toxin. Expression of single chain sequence variants will not facilitate easy domain swapping for the creation of different “serotypes”, but will allow for rapid production of active molecules to assess activity of the novel toxins.

The work undertaken in this study demonstrates that it is possible to create catalytically active recombinant CNT toxin molecules by expression in *E. coli*. Functional assays for the detection of catalytic activity of CNT light chains has also been established and this study provides a firm basis for the expression of CNT toxins as therapeutic molecules. However, further work to optimise the soluble expression of THC is required before the novel chimeric CNT holotoxin can be created for the treatment of OSA.
References


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Appendices
Appendix 1 – DNA standard

Figure A1. λ-DNA ladder

(A) Lambda DNA digested with PstI. PstI digested λ-DNA separated by agarose gel electrophoresis with size and quantity of individual DNA fragments specified. Reproduced from Lambda DNA/PstI marker certificate of analysis (Fermentas).
Appendix 2 – Protein standards

Figure A2. Protein standards