

Imperial College
OF SCIENCE, TECHNOLOGY AND MEDICINE

**α -LATROTOXIN AND ITS
RECEPTOR, LATROPHILIN:
STRUCTURE-FUNCTION
RELATIONSHIPS**

A Thesis Submitted for Ph.D. in Biochemistry
and Diploma of Imperial College

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For my parents

Abstract

α -Latrotoxin (LTX) is a potent stimulator of neuroexocytosis. Biochemical analysis shows that LTX is a dimer under native conditions. In the presence of divalent cations these dimers assemble into tetramers. Cryo-electron microscopy and angular reconstitution have been used to determine the three-dimensional structure of these oligomers. These structure-function studies reveal that the tetramer (dimensions 100 x 180 x 180 Å) possesses a central 10 Å-diameter pore large enough to allow the passage of divalent cations and small molecules. As a tetramer, the normally hydrophilic LTX gains a hydrophobic base, which can insert into liposomes permeabilising their membrane. Thus, a model for the pore-forming ability of LTX is presented. Structure-functional analysis has also been applied to the novel LTX receptor, latrophilin (LPH). Latrophilin is an unusual G protein-coupled receptor in that it is cleaved just before the first transmembrane domain to form two fragments: NTF (~ 120 kDa, containing the N-terminus) and CTF (~ 65 kDa, containing seven transmembrane regions and the C-terminus). After cleavage the NTF surprisingly remains anchored to synaptic membranes (SPM). This is unlikely due only to interactions with the CTF as firstly, chemical treatments of SPM designed to disrupt protein-protein interactions cannot release the NTF into solution and secondly, a LPH mutant containing just the NTF can anchor to the surface of COS-7 cells, as shown by confocal microscopy. The amount of CTF that co-purifies with the NTF on a LTX affinity column depends on the physiological state of the brain membrane used for extraction, suggesting that the two fragments can behave independently in the membrane. It is also shown in this study that LPH interacts physically and functionally with the specific heterotrimeric G proteins and that the receptor is presynaptic. These findings strengthen the idea that LPH is the physiological receptor involved in the second, pore-independent mode of LTX action.

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Author publications

Some of the results contained in this thesis have been published in the following journals:

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Abbreviations

APS	Ammonium persulphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid
CTF	C-terminal fragment of latrophilin (65 kDa)
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GPI	Glycosphosphatidyl-inositol
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HRP	Horse radish peroxidase
LTX	α -Latrotoxin
NAGA	N-acetylglucoseamine
NMDA R	N-methyl-D-aspartate receptor
NTF	N-terminal fragment of latrophilin (120 kDa)
PAGE	Polyacrylamide gel electrophoresis
PSD-95	Postsynaptic density protein (95 kDa)
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulphate
SNAP-25	Synaptic vesicle associated protein (25 kDa)
TEMED	N, N, N', N'-Tetra-methyl-ethylenediamine
TRITC	Tetramethylrhodamine isothiocyanate
WGA	Wheat germ agglutinin

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CHAPTER ONE



GENERAL INTRODUCTION

1.1 Neurotransmission and the black widow spider

The nervous system

The nervous system may be defined anatomically as an organised group of cells specialised for the conduction of a stimulus impulse from a sensory receptor through a nerve cell network to the site at which the response occurs. Neurones are abundant cells in the nervous system and by far the most important. Their fundamental task is signalling and their every aspect (size, shape, development and organisation within the brain) is geared towards receiving and transmitting information. The majority of neurones consist of three distinct regions: (1) a cell body or soma containing the nucleus; (2) a nerve fibre or axon (transmitting process); and (3) the dendrites (receiving processes). The axon arises from the soma at a region called the axon hillock or initial segment. It is here that the plasma membrane generates nerve impulses (action potentials). The axon conducts these impulses away from the soma or dendrites towards other neurones. At the axon terminal, and sometimes along its length, are specialised nerve structures that form junctions (synapses) with other neurones and with muscle cells. Each chemical synapse has a bulbous presynaptic terminal specialised for rapid secretion of neurotransmitters (chemical messengers) and a postsynaptic cell containing receptors, separated by a gap (of about 0.02 microns) called the synaptic cleft (Figure 1.1a). Neurotransmitters diffuse across this cleft when released from vesicles and bind to their specific receptors present on the postsynaptic cell triggering a postsynaptic potential, which can depolarise the next cell. Neurones thus transmit information through the nervous system by an alternating chain of electrical signals (within cells) and chemical signals (between cells) as illustrated in Figure 1.1b.

Generation of action potentials

The plasma membrane of the neurone is semipermeable to K^+ and slightly permeable to Cl^- and Na^+ . In the extracellular fluid electroneutrality is maintained by high concentration of Na^+ balanced by high concentrations of Cl^- , together with trace quantities of impermeant anions such as bicarbonate, phosphate and sulphate. In the cytoplasm the concentration of K^+ is high but the concentration of Cl^- is insufficient to balance the sum of positive forces. Instead electroneutrality inside is maintained by impermeant proteins and by phosphates.

This ordered arrangement of electroneutrality on either side of the neurone's plasma membrane and semipermeability of the membrane produces a difference in electrical potential across the cell's surface. The inside of the membrane is more negatively charged than the outside and this membrane potential, in most neurones, is between -60 and -75 mV. This potential is also known as the resting potential (i.e. when the membrane is not generating a nerve impulse it is at rest). The neurone is thus described as being polarised and any changes that make the inside more negative is known as hyperpolarisation and anything that makes it less negative is known as depolarisation.

Whenever a physical stimulus, e.g. taste or touch, is sensed by a specific sensory receptor cell, the energy of the stimulus (e.g. mechanical, chemical) is transformed into an electrical response. Sensory receptors are linked to ion channels in the membrane and upon stimulation of these receptors the ion channels open, permitting the influx of Na^+ (greater positive charge) into the cell. A local potential is created, which, if sufficiently large, will generate an action potential. The threshold for

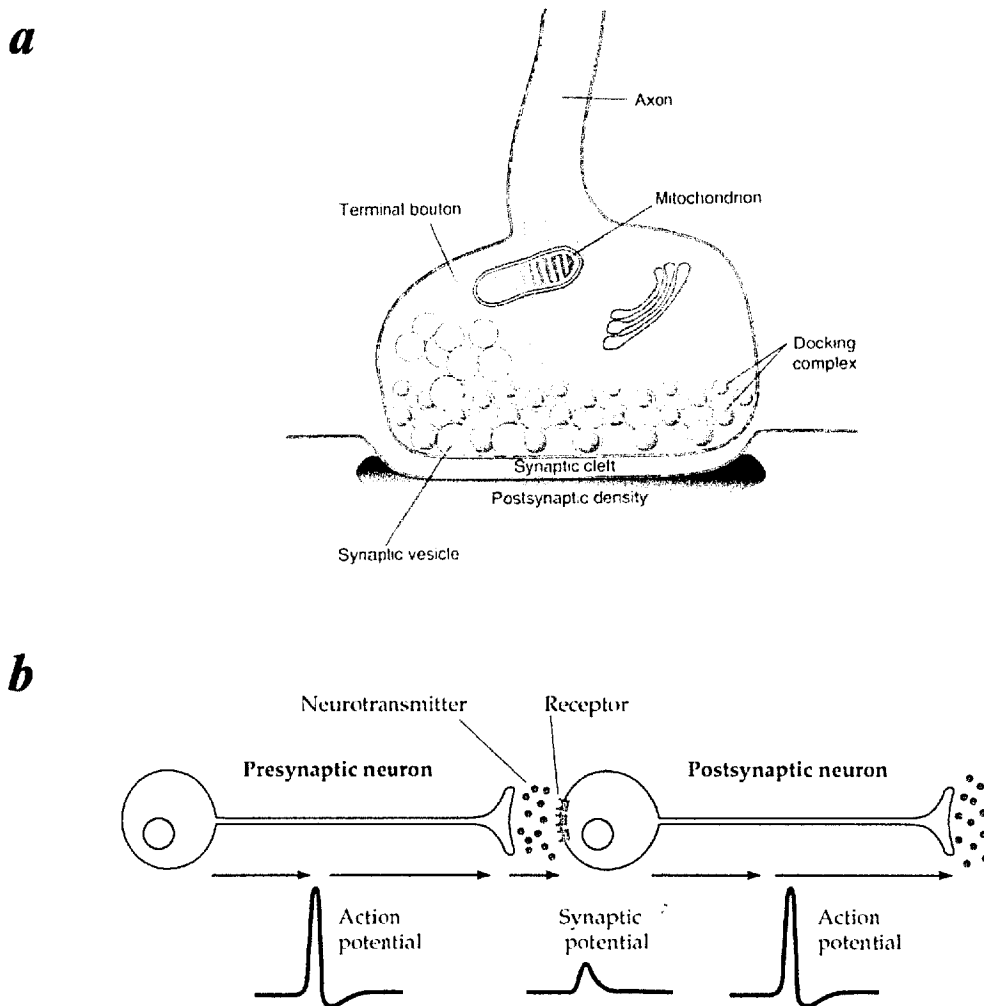


Figure 1.1 Neurotransmission¹. *a*, The synapse. Axons branch and terminate in many bulb-like structures called boutons. In the presynaptic cell, a docking complex at the active zone tethers synaptic vesicles (filled with neurotransmitters) in close apposition to the postsynaptic cell. *b*, Simple diagrammatic representation of how neurones transmit information. An action potential is generated at the initial segment of a neurone. This is transmitted down the axon towards the nerve terminal. Neurotransmitters released from the presynaptic membrane into the synaptic cleft are recognised by their specific receptors on the postsynaptic membrane (at the postsynaptic density). This triggers a local synaptic potential, which is capable of depolarising the next neurone and so the process repeats.

¹Sources of illustrations: *a*, Robert E. Kingsley, Concise Text of Neuroscience, 1996; *b*, Zach W. Hall, An Introduction to Molecular Neurobiology, 1992

the generation of an action potential is about -58 mV, that is, when the neurone is depolarised to this level, the voltage-dependent sodium channels become fully activated allowing Na^+ to flood the cell. Such is the extent of Na^+ entry that the membrane potential reaches a maximal level of about $+35$ mV.

At the peak of the action potential the sodium channels become inactivated. At the same time voltage-dependent potassium channels open and K^+ diffuses out of the neurone [for a recent review of the structure and function of voltage dependent sodium and potassium channels see Catterall, 1999]. These two processes permit the faster return of the cell to the polarised state (inside of the cell negative to the outside). This is called repolarisation and after this there is a period in which a second action potential cannot be generated no matter how large the electrical stimulus. This is known as the absolute refractory period, which is then followed by a relative refractory period where a nerve impulse can be generated but only if the stimulus is greater than the current originally required. Shortly after this, the neurone returns to its normal resting state, which requires reinstating the original intracellular concentrations of Na^+ and K^+ achieved through the work of Na^+K^+ ATPase and ion pumps.

The nerve impulse is transmitted down the nerve fibre like a wave, the membrane depolarising in front of the spike of potential and repolarising behind. The action potential does not decrease in amplitude as it is propagated along the axon. To aid conduction, long axons acquire an insulating myelin sheath, which provides a high resistance, low capacitance electrical insulator. The sheath is interrupted at regular intervals by gaps called nodes of Ranvier, where the nerve membrane is exposed to the external environment and where there is a high concentration of voltage-dependent sodium channels. In this fashion, the nerve impulse jumps in a saltatory-like fashion

from node to node, and electrical stimulation can proceed very long distances in a matter of milliseconds. The nerve impulse continues along the plasma membrane until it reaches the nerve ending. There it is turned into chemical events, which will now be discussed.

At the nerve terminal

The release of neurotransmitters from nerve terminals occurs through regulated exocytosis – the fusion of vesicles with membranes. The synaptic vesicles containing neurotransmitters ready for release are tethered at a special region of the presynaptic cell called the active zone, which is in close apposition to the postsynaptic cell. A nerve impulse depolarises the nerve terminal membrane, and this stimulates the opening of voltage-dependent calcium channels (VDCCs) present in the presynaptic membrane. As a result, calcium enters into the cell from the extracellular fluid down its concentration gradient. The increase of cytosolic calcium levels serves as a stimulus for exocytosis, which occurs in less than a millisecond after the entry of Ca^{2+} . Data obtained from imaging the distribution of Ca^{2+} , and theoretical considerations suggests that fast transmitter release is triggered by elevations of intracellular calcium to at least 100 μM near the sites of vesicle fusion [Augustine *et al.*, 1991], however, recently, it has been shown that $[\text{Ca}^{2+}]_i$ four times as less ($\sim 25 \mu\text{M}$) can induce fast release and 80% depletion of the readily releasable pool of vesicles from central synapses of the calyx of Held [Scheneggenburger & Neher, 2000].

The VDCCs [for review see Catterall, 1998] are highly concentrated at the active zone. This has three advantages when it comes to triggering neurotransmitter release: (1) as the calcium channels are near to the vesicles only the $[\text{Ca}^{2+}]$ at the active zone is increased and not in the whole nerve terminal. As a consequence, this also

reduces the time taken for the $[Ca^{2+}]$ within the cell to reach the threshold value necessary to stimulate vesicular release; (2) the same number of calcium channels are better suited to increasing the local concentration of Ca^{2+} if clustered than if spread all over the presynaptic terminal and finally, (3) release can be terminated quickly as the local concentration of Ca^{2+} at the active zone quickly decreases by diffusion. Together these features allow rapid, high frequency release of neurotransmitters in response to action potentials.

What happens between the period of Ca^{2+} entry and the fusion of synaptic vesicles with the presynaptic membrane is still unknown and a wide area of study in the field of neuroscience. Exocytosis is a multi-step process and requires several protein complexes to permit the vesicle to fuse with the plasma membrane and thus release neurotransmitter into the synaptic cleft. SNARE proteins (short for SNAP REceptors) are thought to play a pivotal role in the fusion event. SNAREs can be classed into two categories: those localised on the target membrane (t-SNAREs), i.e. syntaxin and SNAP-25 families, and those localised on the membrane of the vesicle (v-SNAREs) i.e. synaptobrevin and its relatives. Many SNAREs, in addition to these three neuronal proteins, have been found over the last few years [for review see Jahn & Südhof, 1999]. A feature of all SNAREs is a common domain of ~60 residues, which is referred to as the SNARE motif [Weimbs *et al.*, 1997].

The three major neuronal SNAREs, SNAP-25, syntaxin 1A/B and synaptobrevin 2 interact in a 1:1:1 stoichiometry *in vitro* to produce a stable core complex. The trimeric core complex is actually formed by the interaction of the SNARE motifs [Hao *et al.*, 1998; Fasshauer *et al.*, 1998] of the three proteins (one domain each from syntaxin 1A/B and synaptobrevin 2, encompassing residues 199-243

and 27-96, respectively, and two domains from SNAP-25, spanning 1-84 and 143-206). The crystal structure of the core complex revealed that this tight complex is made up of four twisted coiled-coiled α -helical domains [Sutton *et al.*, 1998], aligned in parallel with an overall length of 12 nm [Poirier *et al.*, 1998; Katz *et al.*, 1998]. It is thought that the interaction of t-SNAREs with the v-SNARE creates this intertwined 'trans' complex, which promotes the fusion of vesicles with the presynaptic membrane by bringing the membranes within 4 nanometres of each other [Jahn & Südhof, 1999]. Indeed, purified SNAREs alone have been shown to cause fusion when reconstituted into artificial membranes [Nickel *et al.*, 1999].

ATP hydrolysis by N-ethylmaleimide-sensitive factor (NSF) is required at some stages either preceeding the priming of vesicles just before fusion, or after endocytosis. Upon hydrolysing ATP, NSF causes the disassembly of the complex (probably in its cis-form) and the recycling of the SNAREs. Although membrane fusion requires the trans-SNARE complexes to form, these may actually disassemble before the actual fusion event occurs as shown with yeast vacuoles [Ungermann *et al.*, 1998]. This suggests that yet another macromolecule may be involved in the membrane fusion event. In accordance with this hypothesis exocytosis in sea urchin eggs occurs apparently in the absence of SNARE complexes [Coorsen *et al.*, 1998]. A recent publication by Peters *et al.* [2001] working on yeast vacuoles, has suggested that the terminal event of fusion is the formation of a proteolipid channel complex after the trans-SNARE complex has formed. They propose that this channel forms by the dimerisation of V0 (the integral membrane part of the vacuolar H⁺-ATPase) from opposing membranes. It remains to be tested whether proteolipid channels are involved in the fusion of vesicles in the plasma membrane.

Another component crucial to exocytosis is the “Ca²⁺ sensor”. Several Ca²⁺-binding proteins have putatively been suggested as the target for Ca²⁺ after its influx. These include calmodulin, CaM Kinase II (Ca²⁺/calmodulin-dependent protein kinase II), annexin and CAPS (Ca²⁺-dependent activator protein for secretion) [see review by Lang, 1999 and references within]. The candidate protein that has been most extensively studied is synaptotagmin I found on the membrane of synaptic vesicles. This protein possesses two Ca²⁺-binding repeats (termed C₂ domains). One of these domains (C₂A) binds to the SNARE protein syntaxin 1A/B at concentrations of Ca²⁺ necessary to stimulate fast exocytosis. However, synaptotagmin I is probably one of a number of Ca²⁺ sensor proteins as exocytosis still occurs in its absence [Geppert *et al.*, 1994].

Toxin tools in neuroscience

Neurotoxins are venom components from bacteria, plants and animals that are directed against the nervous system [for reviews of all commonly used neurotoxins and their effects see Conn, 1992; Hucho, 1995]. Normally each neurotoxin will act upon a specific function of the nervous system. Due to this specificity they are ideal tools for neuroscientists to use in the elucidation of the basic mechanisms of neurotransmission. For example, tetrodotoxin and saxitoxin are specific blockers of the voltage-dependent Na⁺ channels while ω -conotoxin is specific for a certain type of Ca²⁺ channel. Tetanus and botulinum neurotoxins act by cleaving specific regions of synaptobrevin, SNAP-25 or syntaxin 1A/B resulting in a complete blockade of vesicular transmitter release [see Montecucco & Schiavo, 1995; Pellizzari *et al.*, 1999; Schiavo *et al.*, 2000]. The use of these clostridial neurotoxins led to the original identification of the SNARE proteins and their role in vesicle docking and fusion. The focus of this thesis is a natural tool that comes from the infamous black widow spider (*Latrodectus mactans* genus).

Black widow spider toxin (α -latrotoxin)

The bite of the black widow spider can cause muscle spasms, breathing difficulties and temporary paralysis in humans (latrotoxicism) but is rarely fatal. These effects are caused entirely by α -latrotoxin (LTX), a component of the spider's venom, which acts specifically on the vertebrate nervous system, causing massive exocytosis from peripheral synapses [reviewed by Rosenthal & Meldolesi, 1989]. The resulting neurotransmitter exhaustion causes the symptoms of latrotoxicism while the over-released transmitters themselves can cause a wide range of detrimental pharmacological changes in organ systems. LTX is unique as a molecular tool for a number of reasons:

1. Unlike other known neurotoxins, LTX does not initially inhibit the nervous system but stimulates it by inducing the release of synaptic vesicles. Exhaustive stimulation may lead to depletion of presynaptic transmitter stores and result in a blockade of further neurotransmission.
2. It is the most potent stimulator of exocytosis known. Although delayed in action compared to depolarising agents such as high K^+ , the toxin's overall effect is far more extensive [McMahon *et al.*, 1990];
3. It apparently possesses no enzymatic activity and therefore, functions by a specialised mechanism of action.

The study of LTX (in order to elucidate its molecular mechanism) and its molecular targets will, in turn, improve our understanding of the presynaptic mechanisms of the human nervous system. For the remainder of this Chapter, the knowledge that has been obtained about LTX and its neuronal receptors over the past 30 years is reviewed.



Figure 1.2 The black widow spider². The black widow is just one of over 30,000 recognised members of the carnivorous spider order (Araneida). About six species exist whose distribution covers Africa, Australasia, North and South America, southwestern Asia and southern Europe. The poisonous females, distinguished by a red hourglass marking on their underside, occasionally ensnare and eat the males after mating – hence the name black widow. Its venom is 15 times more potent than an equal weight of rattlesnake venom (β -bungarotoxin) but its 1.7 mm fangs (chelicera) inject a much smaller amount.

²**References:** Alfred Knopf, National Audubon Society Field Guide to North American Insects and Spiders, New York (1995); Lawrence Stack, New England Journal of Medicine (1997) Volume 336, page 1649

1.2 Purification of LTX from the venom

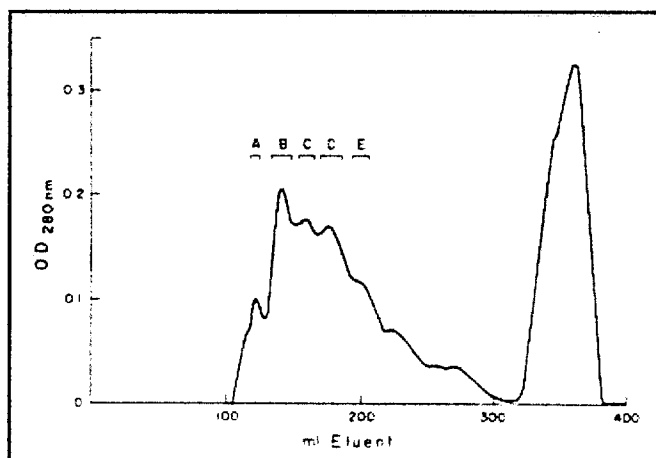
Purified LTX is usually isolated from the spider venom using several rounds of high-performance liquid chromatography (HPLC) [Frontali *et al.*, 1976; Grasso, 1976; Ushkarav & Grishin, 1986; Cavalieri *et al.*, 1987; Grebinozhko & Nikolaenko, 1987; Krasnoperov *et al.*, 1990; Grasso & Mastrogiacomo, 1992]. In the first published purification method, aqueous extracts of the venom glands were chromatographed on a Sephadex G-200 gel resulting in the elution of several neurotoxin protein peaks [Frontali *et al.*, 1976]. Only fraction B (containing LTX) was active on vertebrate synaptosomes (see Figure 1.3a). Further purification of fraction B on DEAE Sephadex A-50 yielded LTX in protein fraction B₅ (Figure 1.3b).

In another approach venom extract was applied to a Sephadex G-100 SF gel (filtration) column, then subjected to ion-exchange on DEAE-Sephadex A-50 [Grasso, 1976]. The peak eluting at about 0.3 M salt was concentrated by freeze-drying and purified to homogeneity by filtration on a Bio-Gel A 0.5 m column. These authors observed a single band of LTX on SDS-PAGE but the precise conditions of electrophoresis were not revealed. Although Frontali and coworkers in 1976 also initially observed a single band relating to LTX on SDS-gels, when lower acrylamide concentrations were used for electrophoresis, fraction B₅ (Figure 1.3b) could be resolved into two bands. This indicates that although LTX can be purified 10-12 fold after two steps of chromatography [Grasso, 1976], at least one major contaminant remains. This was later identified as δ -latroinsectotoxin [see Ashton *et al.*, 2000].

Alternatively, immunoaffinity chromatography may be used to isolate LTX directly from the venom [Pashkov *et al.*, 1989]. This is a more rapid purification method but requires very specific monoclonal antibodies. However, it still produces a toxin contaminated with at least one low molecular weight peptide [Volkova *et al.*, 1995]. This 70 amino acid residue protein (~ 8 kDa) has been shown to co-purify with LTX using all methods, probably due to stable complex formation [Kiyatkin *et al.*, 1992]. The peptide, originally named low molecular weight protein (LMWP), is a non-toxic acidic protein (pI 4.3) [Gasparini *et al.*, 1994; Kiyatkin *et al.*, 1995]. Attempts to separate LTX and LMWP in native conditions by different chromatographic methods have been unsuccessful [Grishin *et al.*, 1993].

The small peptide's primary structure shows similarity to erabutoxin A, a snake venom toxin [Kiyatkin *et al.*, 1992], but possesses even more homology to crustacean hyperglycemic hormones, suggesting a possible structural relationship between these proteins [Gasparini *et al.*, 1994]. After cloning, LMWP was renamed latroductin [Pescatori *et al.*, 1995]. Despite co-purifying in some LTX preparations latroductin is unlikely to play a functional role in toxin action. Indeed, recently, recombinant α -latrotoxins have been produced in baculovirus expression systems and shown to stimulate neurotransmitter release similar to native LTX despite being devoid of latroductin [Ichtchenko *et al.*, 1998; Volynski *et al.*, 1999a].

a



b

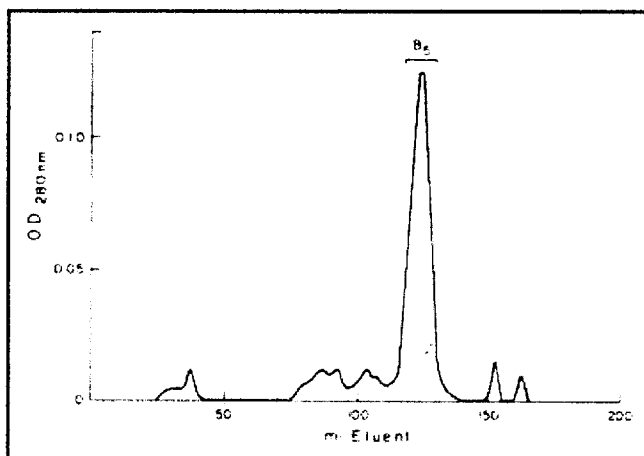


Figure 1.3 Purification of α -latrotoxin by HPLC. *a*, Aqueous extracts of venom glands were first chromatographed on a size exclusion column (Sephadex G-200) to yield peaks A-E that caused depolarisation and release of neurotransmitters in different phyla. Only fraction B was toxic for vertebrates. *b*, When fraction B was subsequently run through an anion-exchange column (DEAE Sephadex A-50) the major peak eluted (fraction B₅) contained all the LTX. This two-step chromatographic procedure is most commonly used to isolate the toxin in the literature [after Frontali *et al.*, 1976].

1.3 The biochemical properties of LTX

The molecular mass of LTX was previously determined to be in the range of 125-130 kDa by SDS-electrophoresis [Frontali *et al.*, 1976; Grasso & Mastrogiacomo, 1992] and 110 kDa by sieve gel chromatography [Frontali *et al.*, 1976]. Others have proposed that the toxin may be a dimer of two 118 kDa subunits [Ushkarev & Grishin, 1986]. This observation was made by gel filtration and native gel electrophoresis on a LTX preparation obtained by conventional techniques.

Purified LTX is a slightly acidic protein, which can be resolved into four bands by isoelectric focusing with isoelectric points distributed between pH 5.2 and 5.5 [Frontali *et al.*, 1976]. It must be noted, however, that these estimations were done on a preparation contaminated by δ -latroinsectotoxin (refer to section 1.2). Black widow spider toxin is unglycosylated and possesses no detectable enzymatic action (in particular proteolytic or phospholipase activity) [reviewed by Grasso, 1988]. LTX's functional activity can be prevented through proteolysis by a variety of enzymes and reducing agents such as 5 mM β -mercaptoethanol [Hurlbut & Ceccarelli, 1979; Meldolesi, 1982; Scheer *et al.*, 1984].

Other properties include an LD₅₀ of about 0.02 μ g pure protein/g body weight for the toxin, which was obtained by subcutaneous injection of mice [Grasso, 1976]. Its ED₅₀ (concentration of LTX that causes 50% of its maximum measurable release of neurotransmitter from synaptosomes or cell systems) was found in the range 10^{-10} to 10^{-9} M.

1.4 The effects of LTX

At the neuromuscular junction (also termed endplate), even without electrical stimulation, there is always a small amount of spontaneous neurotransmitter release. This gives rise to the detection of miniature endplate potentials (MEPPs) at the postsynaptic membrane (caused by released transmitter binding to its postsynaptic receptor). These endplate potentials typically have an amplitude of 1 mV (far below the 70 mV threshold for depolarisation) and typically occur once a second. When LTX is applied to the neuromuscular junction (NMJ) it causes a massive increase in the frequency of MEPPs and depolarisation [Longenecker *et al.*, 1970]. The confirmation that these observations reflect the result of massive exocytosis came by way of several electron microscopy studies on the NMJ. Upon application of the toxin, extensive vesicle release could be seen from the active zone of the presynapse followed by their fusion with the presynaptic membrane, thus increasing the surface membrane area [Ceccarelli *et al.*, 1979]. In addition, “dimples” or “pits” appeared beside active zones that represented vesicles joined to the membrane by exocytosis [Hurlbut & Ceccarelli, 1979].

Electron microscopy of PC12 cells also reveals two distinct components to LTX action: (a) partially Ca^{2+} - independent stimulation of exocytosis (“early effect”) and (b) strictly Ca^{2+} -dependent cell lysis (“late toxic effect”) [Watanabe *et al.*, 1983]. During the early effect (~ 1 minute after toxin addition), many secretory granules are observed in various stages of exocytosis. Later (after 8-10 minutes), swelling of various organelles are observed characteristically caused by Ca^{2+} influx [Grasso *et al.*, 1980]

that ultimately lead to cell death. Such morphological changes have also been seen in the mammalian central nervous system, either in slices [Tzeng *et al.*, 1978b] or in synaptosomes [Watanabe & Meldolesi, 1983] and may reflect apoptosis (programmed cell suicide). When Ca^{2+} was absent from the incubation media exocytosis in PC12 cells was delayed by about 2-7 minutes.

In fact LTX does not require extracellular Ca^{2+} to cause vesicle release provided another divalent cation such as Mg^{2+} , Ba^{2+} , or Sr^{2+} is present [Meldolesi *et al.*, 1983]. Moreover, divalent cations may not be essential as LTX causes release in the presence of EDTA at the frog neuromuscular junction in hypertonic solutions [Misler & Hurlbut, 1979]. This distinguishes its mode of action from that of depolarising agents and ionophores that function solely by inducing Ca^{2+} entry into the presynapse.

LTX does not appear to be selective for different types of vertebrate synapse but acts upon them all irrespective of the neurotransmitter involved. Examples include acetylcholine [Tzeng *et al.*, 1978a and Gorio *et al.*, 1978], γ -aminobutyric acid (GABA) [Tzeng *et al.*, 1978a; Storchak *et al.*, 1994], noradrenaline [Grasso & Senni, 1979], enkephalin [Janicki & Habermann, 1983], dopamine [Cull-Candy *et al.*, 1973] and glutamate [McMahon *et al.*, 1990]. Therefore, α -latrotoxin is a general synaptic stimulatory agent, which adds to its effectiveness as a tool in molecular neurobiology [see Grasso, 1988; Schiavo *et al.*, 2000]. It cannot stimulate release from most non-neuronal cell types even at high concentrations [see Hurlbut & Ceccarelli, 1979; Scheer *et al.*, 1984; Rosenthal & Meldolesi, 1989] although neuroendocrine cells are also targets for the toxin and have been widely used to study LTX mechanism of action [Bittner, 2000].

In addition to acting on exocytosis of small synaptic vesicles (SSVs), α -latrotoxin is capable of stimulating release from large dense cored vesicles (LDCVs), e.g., peptides from chromaffin cells [Kobayashi *et al.*, 1986; Barnett *et al.*, 1996], sensory and enteric neurons [De Potter *et al.*, 1997; Waterman & Maggi, 1995], and catecholamines from PC12 cells [Grasso *et al.*, 1982]. These cell types have been widely used to study LTX's mechanism of action as they all contain endogenous LTX receptors [Saito *et al.*, 1985; Liu & Misler, 1998b]. Only at the neuromuscular junction does LTX apparently selectively stimulate the release of SSVs (containing acetylcholine) but not LDCVs containing peptides [Matteoli *et al.*, 1988]. However, this study did not measure the actual release of peptides. Rather, the residual immunoreactivity of the terminals was used to suggest that there was no release from LDCVs; no positive control or quantitation was provided to demonstrate that immunoreactivity could be a good measure of release. Although the low number of LDCVs did not apparently change after the toxin application, it remains possible that release, in fact, occurred, but unlike SSVs, LDCVs did not fuse with the membrane completely. The fact that LTX can cause the release of peptides from chromaffin cells and neurons suggests multiple or universal mechanisms of toxin action.

It is accepted that LTX causes two types of neurotransmitter release: true vesicular exocytosis (a process requiring energy in the form of ATP) and an energy-independent escape of neurotransmitters from the cytoplasm [McMahon *et al.*, 1990]. It was first demonstrated by these authors that although LTX causes a complete depletion of ATP levels in synaptosomes within 3 minutes, the toxin still releases a significant amount of glutamate after 6 minutes suggesting that an alternative energy-independent process may be responsible. This was confirmed by release experiments using synaptosomes first exhausted of ATP and then stimulated with LTX. Under these

conditions two amino acids that are largely excluded from synaptic vesicles but are abundant in the cytoplasm (D- and L-aspartate) were also released with glutamate. This occurred under conditions where the reversal of uptakers for these amino acids was prevented. Consistent with these findings, neurotransmitter release by LTX is even seen from PC12 cells and synaptosomes that have been poisoned with clostridial neurotoxins [see Davletov *et al.*, 1998 and references within]. The fact that LTX still stimulates release after the use of these neurotoxins confirms that there is a component of toxin-induced neurotransmitter release that is non-vesicular.

The ATP-dependent vesicular release is, at least partially, due to divalent cation influx and can further be broken down into two components: Na^+ -dependent and Na^+ -independent [Deri *et al.*, 1993]. In the presence of Na^+ in the extracellular buffer, LTX causes massive acetylcholine release from synaptosomes (accompanied by a rise in intracellular Ca^{2+} levels). In the absence of Na^+ , less neurotransmitter release is observed although intracellular Ca^{2+} levels still increase. The explanation for this difference is that Na^+ influx into the nerve terminals down its concentration gradient will induce depolarisation and opening of endogenous Ca^{2+} channels that will add to the stimulus for exocytosis [see Nicholls *et al.*, 1982].

There are no absolute pharmacological inhibitors of LTX action but non-specific inhibition can occur using concanavalin A and wheat germ agglutinin. These lectins work by reducing the level of toxin binding to its receptor [Rubin *et al.*, 1978; Meldolesi, 1982; Ushkarev & Grishin, 1986]. Certain trivalent cations, for example, La^{3+} and Gd^{3+} can completely block the effects of LTX in rat striatal synaptosomal preparations probably by preventing Ca^{2+} -influx [Scheer, 1989].

1.5 Structural studies of LTX

The primary structure of LTX (Figure 1.4) was deduced by screening a cDNA library of venom glands from the black widow spider [Kiyatkin *et al.*, 1990]. Structural analysis of overlapping cDNA clones revealed a continuous sequence of 5408 base pairs encoding a polypeptide of ~1401 amino acid residues (~ 157 kDa). This is much larger than the 130 kDa protein observed by purification of the venom. The N-terminus of LTX is preceded by a recognition site of furin (instead of a defined signal peptide) and there is another potential furin site in the C-terminal region of the precursor [Volynski *et al.*, 1999a]. It is possible that during maturation, a furin-like protease hydrolyses LTX at these sites, producing an active toxin. Indeed, molecular engineering of a protein terminated at both places produced a toxin of exactly 131.5 kDa, consistent with the size isolated from the venom, as estimated by SDS-PAGE, and which displayed full activity of native LTX [Ichchenko *et al.*, 1998; Volynski *et al.*, 1999a].

Hydrophobicity profile analysis of LTX shows that it possesses insufficient hydrophobic segments to form a membrane spanning α -helix [see Grishin, 1996]. However, its ankyrin-like repeats may facilitate toxin-membrane interactions. Recent studies indicate that this repetitive ankyrin motif is responsible for high affinity binding to membrane proteins and cytoskeletal elements [for review see Sedgwick & Smerdon, 1999].

α -Latrotoxin has three invariant cysteines in the N-terminal region that are conserved in all latrotoxins [Dulubova *et al.*, 1996]. Recombinant LTXs, designed with substitutions of these cysteines are no longer able to bind to LTX receptors [Ichtchenko *et al.*, 1998]. This implies that the N-terminus of LTX is important in toxin-receptor interactions. Interestingly, another mutant (LTXN4C) made by the same authors where a four amino acid insertion was placed between the N-terminus and the first ankyrin repeat, bound to receptors but could not stimulate norepinephrine or glutamate release from synaptosomes. This suggests that the receptor binding site and functionally active region of LTX are different.

A set of monoclonal antibodies raised against LTX [Pashkov *et al.*, 1993] indirectly indicates the presence of four separate functional domains in the toxin structure. Although it is not known which epitopes of LTX these antibodies recognise they have been shown to interfere with (1) receptor binding, (2) Ca^{2+} influx, (3) the rate of neurotransmitter release and lastly, (4) the ability of LTX to form pores in artificial membranes. Another monoclonal antibody (named 4C4.1) has been reported to abolish the ability of the toxin to stimulate $^{45}\text{Ca}^{2+}$ influx in PC12 cells [Cattaneo & Grasso, 1986]. Ca^{2+} -dependent catecholamine release was still evoked, albeit decreased, if the toxin was allowed to bind PC12 cells first and then challenged with antibodies. This again suggests that the receptor binding site and the functional region of the LTX structure involved in Ca^{2+} -influx are separate.

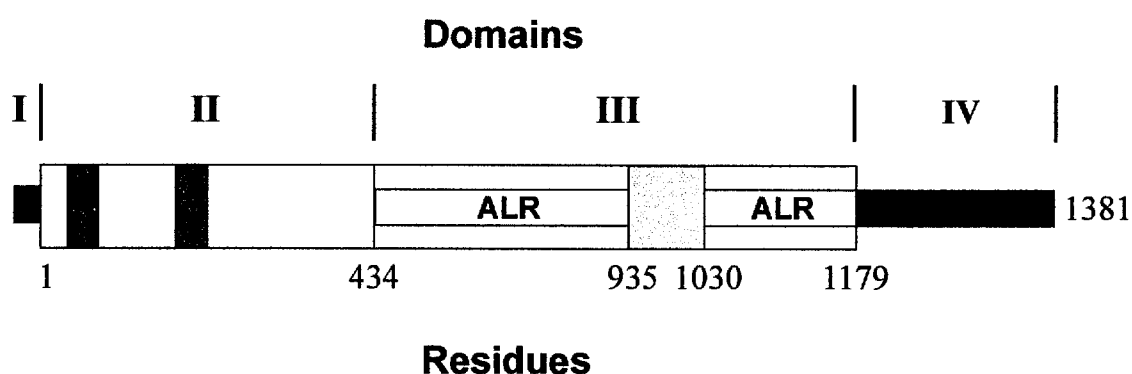


Figure 1.4 The primary structure of α -latrotoxin. Putatively, LTX can be subdivided into four domains (I-IV). *Domain I* – an N-terminal leader sequence that possesses a recognition site for furin (R-M-R-R). *Domain II* – an N-terminal region of ~ 434 residues. The three invariant cysteines located within this domain (common to all α -latrotoxins) may be important for receptor binding as their substitution prevents LTX-receptor interactions. This region also contains 2 putative membrane-binding segments (red boxes), which are thought to be involved in LTX ion channel formation. *Domain III* – consisting of 22 ankyrin-like repeats (ALR) and covering ~ 745 amino acid residues. This segment contains a putative receptor-binding region (grey box). *Domain IV* – a C-terminal domain (~ 200 residues) that possesses another possible furin site (K-F-R-R). Cleavage by a furin-like protease here and at the furin site in domain I would yield a protein of 131.5 kDa [based on Grishin, 1996; Dulubova *et al.*, 1996 and Volynski *et al.*, 1999a].

1.6 Other Black Widow Spider toxins

LTX is a major protein of a number of spider toxins present in the venom of the black widow, collectively described as the “latrotoxins”. Other members of this family include α -latrocrustotoxin (α -LCT) and five latroinsectotoxins (α -, β -, γ -, δ - and ϵ -LIT), which act upon crustacean and insect nerve terminals, respectively [reviewed by Rosenthal & Meldolesi, 1989; Grishin, 1998]. Although LTX has been the most studied toxin due to its vertebrate specificity, some research has also been carried out on α -LCT, α -LIT and δ -LIT, which will now be discussed.

The primary structures for α - and δ -latroinsectotoxins were determined from a spider venom gland cDNA library using a similar method as described for LTX [see Kiyatkin *et al.*, 1990]. The putative α -LIT precursor is a 1411-amino-acid protein with a calculated molecular mass of ~ 158 kDa and pI 6.38 [Kiyatkin *et al.*, 1993]. The calculated molecular mass differs from that determined for native α -LIT (~ 130 kDa) on SDS-PAGE. Like LTX, mature α -LIT is probably derived from double processing in the N-terminal and C-terminal regions of the primary translation product [Volynski *et al.*, 1999a].

The same is probably also true for δ -LIT, which was cloned as 1214 residues encoding for a 130 kDa protein, whereas native δ -LIT, isolated from the venom, is 110 kDa [Dulubova *et al.*, 1996]. Mass spectrometry of native δ -LIT reveals the absence of residues for the C-terminal portion. In addition, full-length recombinant δ -

latroinsectotoxin appears to be inactive but a mutant lacking the C-terminal portion triggers neurotransmitter release from insect synapses [Dulubova *et al.*, 1996].

Comparisons of the primary sequences of α -LIT, δ -LIT and LTX show that all three toxins are structurally similar [Dulubova *et al.*, 1996; Magazanik *et al.*, 1992; Grishin *et al.*, 1992]. They all possess the same four-domain structure as described for LTX in Figure 1.3. δ -Insectotoxin is 190 residues shorter than the other two toxins but possesses 37% homology with LTX and 38% similarity with α -LIT. About 16% of total residues are conserved in all three toxins [Dulubova *et al.*, 1996]. Alignment of the α -LIT and α -LTX amino acid sequences shows an overall similarity of 34% [Kiyatkin *et al.*, 1993]. Recent cloning and sequencing of α -LCT (a protein of ~120 kDa) has revealed 68% and 31% homology with α -LIT and LTX, respectively [Volynski *et al.*, 1999b].

High-affinity latroinsectotoxin receptors have been found in the membranes of insect nerve tissue. Binding of α -LIT to these receptors is independent of divalent cations but Mg^{2+} and Ca^{2+} are essential for α -LIT induced neurotransmitter release [Magazanik *et al.*, 1992]. Both α -LIT and δ -LIT can form channels permeable to Ca^{2+} in artificial bilayers (conductances of ~5 pS and ~20 pS, respectively have been reported) [Shatursky *et al.*, 1995; Dulubova *et al.*, 1996].

α -Latrocrustotoxin is specific for crustacean synapses. Binding of this toxin to its receptor is also independent of Ca^{2+} but the neurotransmitter release elicited from the crayfish neuromuscular junction is dependent on this divalent cation [Burmistrov *et*

al., 1997]. α -LCT causes influx of Ca^{2+} into the crayfish NMJ, an effect that is unaltered by blockers of endogenous Ca^{2+} -channels [Elrick & Charlton, 1999].

The binding features of insect-specific and crustacean-specific latrotoxins to their respective nerve terminals and the formation of Ca^{2+} -permeable pores are two characteristics they share with LTX (discussed later). Interestingly, the binding of α -LIT and α -LCT in the absence of divalent cations suggest that they bind to phylum-specific homologues of latrophilin (Ca^{2+} -independent receptor for LTX, see section 1.10) rather than homologues of neurexin I α (Ca^{2+} -dependent receptor for LTX, see section 1.8).

Thus, all latrotoxins seem to undergo the same processing from the primary translation product and stimulate exocytosis at synapses, albeit in different phyla. As they have similar primary structures they are likely to share the same mechanism of action. Hence, the complete elucidation of the mechanism of action of one toxin should reveal valuable clues to the function of the other toxins.

1.7 Mechanisms of LTX action

The mechanism of α -latrotoxin action remains to be fully elucidated but two hypotheses have been proposed [reviewed by Henkel & Sankaranarayanan, 1999]. Firstly, upon being recruited by LTX receptors, the toxin penetrates the plasma membrane to form a pore/channel that allows influx of Ca^{2+} and other ions. The increase of cytosolic calcium levels is well known to trigger exocytosis [see Matthews, 1996]. Indeed, LTX causes a massive Ca^{2+} -influx in PC12 and chromaffin cells [Grasso *et al.*, 1980; Watanabe *et al.*, 1983; Grasso & Mastrogiacomo, 1992; Krasnoperov *et al.*, 1999]. However, LTX can stimulate neurotransmitter release in the absence of extracellular Ca^{2+} [Misler & Hurlbut, 1979] suggesting that there is a second, Ca^{2+} -independent mechanism distinct from the pore. It is therefore possible that LTX, upon binding to its receptor, triggers an intracellular signalling cascade leading to vesicle release.

Supporting the latter observation, LTX can increase the frequency of miniature postsynaptic currents in hippocampal cells without Ca^{2+} -influx [Capogna *et al.*, 1996] and can stimulate insulin secretion from pancreatic β -cells in the absence of any ion fluxes [Lang *et al.*, 1998]. Moreover, even when Ca^{2+} is present in the medium, it does not need to enter the LTX-sensitive cells to stimulate secretion [Capogna *et al.*, 1996; Michelena *et al.*, 1997; Davletov *et al.*, 1998]. It appears therefore, that the toxin possesses a signalling function in addition to its channel-forming ability. These two mechanisms of LTX action need not be mutually exclusive and there is a wealth of data

supporting both cases, which will now be discussed in the following sections, beginning with the possible role of LTX's known receptors in intracellular signalling.

1.8 The Neurexins

Neurexin I α (NRX), a protein varying in size from 160-220 kDa, was identified and isolated by affinity chromatography of detergent-solubilised rat or bovine membranes on a LTX column [Scheer & Meldolesi, 1985; Petrenko *et al.*, 1988; 1990]. The presence of Ca²⁺ is essential for this toxin-receptor interaction [Davletov *et al.*, 1995] and has given rise to the alternative name for NRX, the Ca²⁺-dependent receptor for α -latrotoxin.

The structure of the neurexins

Neurexin I α is a member of a large family of neuronal cell surface proteins collectively known as “the neurexins”. There are three related vertebrate neurexin genes termed I, II and III [see Missler & Südhof, 1998c]. A fourth neurexin gene (IV) has been found in *Drosophila* that has a human homologue called Caspr2 [Baumgartner *et al.*, 1996; Poliak *et al.*, 1999]. Although neurexin IV is closely related, it possesses subtle differences from the other neurexins in its domain structure and arrangement. Hence it is not considered to be a true neurexin [Missler *et al.*, 1998a]. Each vertebrate NRX gene has two independent promoters, which encode a longer (neurexin α) and a shorter (neurexin β) form of the protein. The difference in size between the two mRNAs is about 3 kilobases. Thus, there are six predominant neurexin proteins in mammals: I α , I β , II α , II β , III α and III β , but due to extensive alternative splicing over 1000 different neurexin isoforms may be generated [Ullrich *et al.*, 1995; Missler & Südhof, 1998c]. All α - and β -neurexins share a similar domain structure (Figure 1.5).

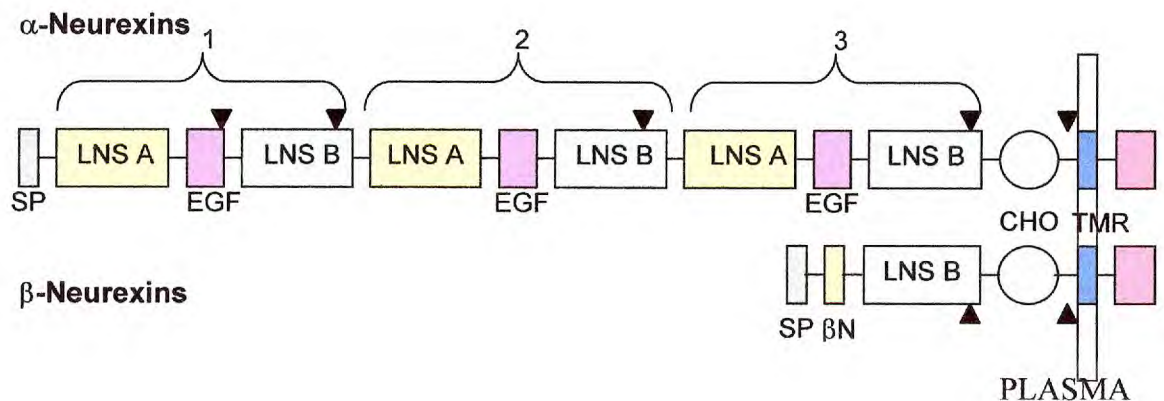


Figure 1.5 Domain structure of the neurexins. α-Neurexins commence with a signal peptide (SP) followed by three repeats (shown in the figure as 1, 2 and 3). Each repeat consists of a central epidermal growth factor (EGF)-like domain between two LNS domains (sequence motifs found in laminin A, neurexin and sex hormone-binding globulin) [Missler & Südhof, 1998b]. After the repeats, there is an O-linked sugar (CHO) sequence followed by a short single-pass transmembrane region (TMR) that anchors the neurexins to the membrane and finally, a cytoplasmic tail (shown in pink). β-Neurexins possess a signal peptide region, a unique N-terminus (βN) and a single LNS region before the O-linked sugar, TMR and cytoplasmic tail domains [Ushkaryov *et al.*, 1994]. Being transcribed from the same genes with the respective α-forms, β-neurexins are completely identical to α-neurexins in their C-terminal two-thirds. Splice sites are indicated by black triangles. Alternative splicing can lead to the generation of some soluble neurexin isoforms [Ushkaryov & Südhof, 1993].

Neurexin I α is the Ca²⁺-dependent LTX receptor

The only member of the neurexin family that binds to LTX is neurexin I α (NRX). Most splice variants of neurexin I α bind to LTX strongly (K_d of 4 nM) in Ca²⁺. Neurexin I β did not bind LTX [Davletov *et al.*, 1995]. Recently, it was suggested that β -neurexins transfected into PC12 cells act as functional LTX receptors [Sugita *et al.*, 1999]. However, these experiments were done under conditions milder than physiological; in fact, physiological salt concentrations prevent any interaction of LTX with β -NRX. In addition, these studies were conducted in a cell line that possesses endogenous LTX receptors [Rosenthal *et al.*, 1990], so these results must be viewed with some caution. Also, in the work of Sugita and coworkers it was shown that recombinant neurexin I α lacking the intracellular C-terminal region and therefore, the ability to signal, did not prevent LTX action in the presence of Ca²⁺. Thus, in Ca²⁺, LTX only requires neurexin I α for recruitment to the membrane but stimulates secretion by another mechanism in this cell line.

The ligands and probable functions of the neurexins

The neurexins share homology with the extracellular matrix proteins agrin, laminin and slit, all involved in axon guidance and synaptogenesis, which suggests a role for the receptors in cell-cell interactions [Ushkaryov *et al.*, 1992]. Ligands for α and β -neurexins have been identified that also indicate roles for these receptors in cell adhesion and in addition, intracellular signalling.

Via its cytoplasmic tail both α - and β -neurexins interact with a possible intracellular signalling protein called CASK that contains calcium-calmodulin-kinase-like, SH3 and guanylate kinase-like domains [Hata *et al.*, 1996]. It is possible that the

activation of neurexin I α by LTX in the presence of Ca²⁺ may stimulate CASK to modify neurosecretion. In addition to CASK, β -neurexins bind to a family of extracellular Ca²⁺-dependent ligands called the neuroligins [Ichtchenko *et al.*, 1995; 1996]. The cytoplasmic tail of the neuroligins can bind to the abundant postsynaptic membrane protein PSD-95, which in turn can bind to CASK on cytoplasmic tail of β -neurexins. This brings about the formation of a neuroligin-neurexin junction. Thus, one predicted that the function of β -neurexins is in the formation of intracellular junctions [Nguyen & Südhof, 1997]. A recent paper by Scheiffele *et al.* [2000] elegantly showed that when neuroligin was expressed on non-neuronal cells, neurons in the same co-culture formed synapses with these non-neuronal cells, and the interaction was β -neurexin dependent.

α -Neurexins, in contrast to β -neurexins, have putative extracellular ligands termed neuroexophilins (~29 kDa), first identified by their co-purification with neurexin I α [Petrenko *et al.*, 1993b]. These *N*-glycosylated ligands have five domains but no transmembrane region. As they resemble peptide hormones this provides further evidence of a possible role for α -neurexins in extracellular cell signalling [Petrenko *et al.*, 1996; Missler & Südhof, 1998a].

1.9 Is neurexin the physiological receptor for LTX?

NRX is thought to be the physiological LTX receptor involved in intracellular signalling leading to neurosecretion based on two criteria: it is brain specific and interacts with synaptotagmin I. However, there are several pieces of evidence that imply that although NRX is a functional LTX receptor, it is not the physiological receptor for LTX as will now be discussed.

To be selective for presynaptic membranes, LTX must be targeted there by specific presynaptic receptors. This idea was initially supported by immunofluorescence studies using LTX and anti-LTX antibodies [Valtorta *et al.*, 1984], which showed that the toxin bound exclusively at presynaptic nerve terminals but was lacking at nerve fibres. Although immunocytochemical labeling has revealed that the neurexins are mainly neuronal proteins [Ushkaryov *et al.*, 1992; Ullrich *et al.*, 1995] they have also been found on myelinated nerves and non-neuronal perineurial cells in the electric organ of the elasmobranch fish [Russell & Carlson, 1997], as well as in non-neuronal neuroendocrine cells [Bittner, 2000]. As NRX is not nerve terminal-specific there is a question mark over the receptor's role in LTX action.

Synaptotagmin I is a synaptic vesicle protein that binds Ca^{2+} and may play the role of Ca^{2+} -sensor in exocytosis [Littleton & Bellen, 1995]. Therefore, when neurexin I α was found to co-purify with this protein from the brain on a LTX affinity column [Petrenko *et al.*, 1991], it was believed that a direct link to the neurosecretion machinery had been found. Indeed, it has been shown that the cytoplasmic tail of

neurexin I α binds to synaptotagmin [Hata *et al.*, 1993; Perin, 1994]. However, these interactions only occur at low salt concentrations (< 0.6 M NaCl) and may be non-specific and non-functional. Significantly, neurotransmitter release by LTX in the presence of Ca²⁺ is not impaired in synaptotagmin I-knockout mice [Geppert *et al.*, 1994]. Also, PC12 cells deficient in synaptotagmin I showed no LTX-induced release of norepinephrine in Ca²⁺-independent conditions [Shoji-Kasai *et al.*, 1994]. However, this effect was apparently reversed by transfection of the synaptotagmin I gene into this cell line. This work illustrates that although synaptotagmin I is important in exocytosis, a receptor, that only binds to LTX in the presence of Ca²⁺, and therefore is not neurexin, plays a key role in LTX induced neurotransmitter release.

Studies with neurexin I α -knockout mice were even more conclusive in proving that the receptor is not essential for LTX action [Geppert *et al.*, 1998]. LTX-induced neurotransmitter release occurs to the same extent in the knockout mice as in wildtype mice, with or without Ca²⁺. α -Latrotoxin binding to membranes, however, decreases by 50% in mutant mice compared to wildtype mice and this may be due to the loss of Ca²⁺-dependent binding sites, although the authors did not reveal whether the number of Ca²⁺-independent binding sites also decreased in mutant mice. This is consistent with observations in neuronal cells, PC12 cells and synaptosomes [Tzeng *et al.*, 1979; Meldolesi, 1982; Meldolesi *et al.*, 1983; Rosenthal *et al.*, 1990], which show that Ca²⁺ is not necessary for LTX binding to membranes although binding increases in the presence of this divalent cation.

As Ca²⁺ is not necessary for LTX to mediate its function there must be a second Ca²⁺-independent receptor involved in LTX action. In fact two populations of high affinity ($K_d \sim 0.3 \times 10^{-10}$ M) presynaptic LTX binding sites have been demonstrated in

bovine synaptosomal membranes and PC12 cells, one being Ca^{2+} -dependent, the other Ca^{2+} -independent [Rosenthal *et al.*, 1990]. Autoradiography has been used to study the relative distribution and importance of these two types of LTX binding sites in rat brain sections [Davletov *et al.*, 1998]. Apart from a heavy concentration of Ca^{2+} -specific LTX binding sites in the thalamus, most LTX binding sites are Ca^{2+} -independent, indicating that the latter is crucial for LTX binding and hence, function. It has even been estimated that brain membranes contain ~ 10 - 15 fold more Ca^{2+} -independent receptor than NRX [Davletov *et al.*, 1998].

1.10 Latrophilin – Ca²⁺-independent receptor for LTX

Repetition of the affinity purification of LTX-binding proteins using immobilized toxin in the absence of Ca²⁺ revealed a single specific band of 120 kDa eluting with high salt [Davletov *et al.*, 1996; Krasnoperov *et al.*, 1996]. This protein had in fact been present in previous purifications where Ca²⁺ was present [Petrenko *et al.*, 1990; 1993] but, being of similar molecular weight to LTX, was considered to be toxin leaching from the column and was not investigated. This protein could be concentrated and further purified using a wheat germ agglutinin column without loss of LTX-binding activity [Ushkarev & Grishin, 1986; Davletov *et al.*, 1996].

In view of this protein's high affinity for LTX (dissociation constant 0.5-0.7 nM) with or without Ca²⁺ it was named latrophilin (LPH) [Davletov *et al.*, 1996]. In the literature, this receptor is also known as CIRL, calcium-independent receptor for α -latrotoxin [Krasnoperov *et al.*, 1997] or CL, short for CIRL/LPH [Sugita *et al.*, 1998]. The number "1" now commonly follows this particular protein, e.g., LPH1 or CL1 as other homologues have been found (see below).

Latrophilin is a brain specific, *N*-glycosylated protein that is more abundant in the cerebrum than in the cerebellum [Davletov *et al.*, 1996; Krasnoperov *et al.*, 1996]. Latrophilin's content in the bovine brain is estimated as 0.33 pmol/mg protein [Davletov *et al.*, 1996] which compares favorably to the total number of estimated LTX-binding sites (~ 0.25 pmol/mg protein) [see Rosenthal & Meldolesi, 1989; Scheer & Meldolesi, 1985]. LPH is apparently a membrane-bound protein as the receptor

could only be isolated using detergent-solubilised brain membranes but could not be extracted using a 1 M NaCl wash of unsolubilised membranes. In addition, phase-separation using Triton X-114 separates LPH into the detergent phase indicating its hydrophobic nature [Davletov *et al.*, 1996].

Latrophilin homologues

Molecular cloning has shown that LPH1 (Figure 1.6) is a novel protein of 1471 amino acids giving it a molecular mass of about 180 kDa inclusive of glycosylation [Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997]. The apparent discrepancy between its predicted size and that purified from the LTX column (~ 120 kDa) has not been solved and may be explained by the protein travelling abnormally on SDS-PAGE or alternatively, by part of the receptor not being isolated. Sequence comparisons show that LPH1 belongs to the superfamily of G protein-coupled receptors (GPCRs).

GPCRs are the largest superfamily of receptors involved in cellular signal transduction. Approximately 1-2% of all cDNA in mammals encode receptor proteins that belong to this family [reviewed by Watson & Arkinstall, 1994; Wess, 1997]. These receptors are characteristically all (a) integral membrane proteins, (b) possess an extracellular N-terminus and an intracellular C-terminus and (c) structurally composed of 7 α -helical cylinders that form transmembrane regions (TMRs) connected by extracellular and cytoplasmic loops.

Two homologues of LPH1 have been discovered in rat and bovine cDNA libraries called LPH2 and LPH3 [Sugita *et al.*, 1998; Ichchenko *et al.*, 1999; Matsushita *et al.*, 1999]. Hydrophobicity plots reveal that they are 165-175 kDa serpentine receptors like LPH1 with 7 TMRs homologous to the secretin/calcitonin

receptor (SecR) subgroup (see section 1.11). In the rat overall sequence similarity between the three is 54-63% with extracellular and TMRs being most conserved and the intracellular region being more variable. LPH2 and 3 possess alternative splice sites, as does LPH1 [Sugita *et al.*, 1998].

Bovine LPH1 is homologous to its rat counterpart, the extracellular domains showing highest identity (99.1%) while the C-termini share 96% between both species. Northern blotting of RNA has revealed that LPH1 and 3 are virtually brain-specific whereas LPH2 is ubiquitous and is most abundant in the liver, although it cannot be isolated by LTX column affinity chromatography, suggesting that it is not a true LTX receptor [Matsushita *et al.*, 1999].

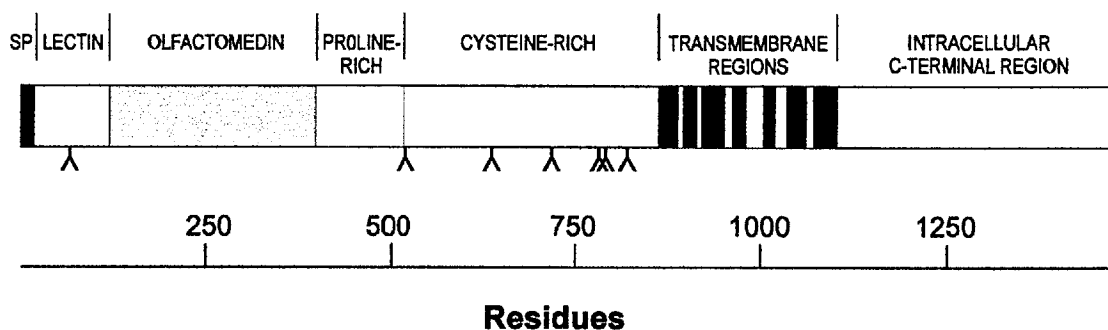


Figure 1.6 Structure of latrophilin. LPH1 has an extremely long extracellular domain for a GPCR consisting of ~ 849 residues. This is broken down into the following: a hydrophobic signal peptide (SP) followed by a 92 residue cysteine-rich region homologous to galactose-binding lectin. This is succeeded by a 290 residue region related to olfactomedins and glaucoma-gene myocilin. The extracellular domain is completed by a proline- and threonine-rich linker sequence, another long region of about 280 residues with homology to BAI1, 2 and 3 (large serpentine receptors of unknown function) and finally, a 90 residue cysteine-rich region. Branched structures indicate hypothetical *N*-linked glycosylation sites. The remaining 372 residues of LPH1 comprise the transmembrane segment that contains seven putative α -helices, characteristic for GPCRs, which is followed by an intracellular C-terminal portion. There are several proline rich domains at the C-terminus of latrophilin, which may be implicated in the interaction with SH3 domains of signalling proteins. There are also numerous phosphorylation sites over the whole cytoplasmic tail that may be involved in receptor desensitisation. The high-affinity LTX-binding site is predicted to be located within residues 467-891 [based on Lelianova *et al.*, 1997; Sugita *et al.*, 1998 and Krasnoperov *et al.*, 1999].

1.11 The secretin/calcitonin family of GPCRs

Over the past 10 years a distinct subgroup of G protein-coupled receptors has been described and collectively named the Secretin receptor family (SecR). The SecR family is characterised by a long N-terminus containing several highly conserved residues, including six cysteines that may form disulphide bonds and may be important for ligand binding. The ligands for these GPCRs are peptide hormones such as secretin [Ishihara *et al.*, 1991], gastric inhibitory protein (GIP) [Usdin *et al.*, 1993] and vasoactive intestinal peptide (VIP) [Ishihara *et al.*, 1992].

Members within the SecR family share 25-30% overall sequence similarity, particularly in the transmembrane region but similarity to other GPCR families is very small [Donnelly, 1997]. Latrophilin shares up to 45% sequence identity with the TMRs of the SecR family [Lelianova *et al.*, 1997]. It is, therefore, quite possible that the endogenous ligand for LPH may be a peptide hormone. Interestingly, LTX has been reported to have areas of structural homology with secretagogic hormones of the SecR family such as VIP and secretin [Holz & Habener, 1998]. On this basis the authors synthesised a chimeric peptide containing long LTX sequences that stimulates Ca^{2+} signalling and insulin secretion in human β -cells and MIN6 insulinoma cells.

Early experiments in the search for endogenous ligands for LTX receptors showed that certain bovine brain cytoplasmic fractions could compete with LTX for binding to bovine membranes [Zhukareva *et al.* 1992]. From such fractions a 90 kDa protein was isolated that was “ α -latrotoxin-like” in its ability to form Ca^{2+} -permeable

channels in lipid bilayers like LTX (discussed later) with a conductance in the range 20-200 pS and thus, named LL-protein. However, work on this isolated protein has never been completed. It has not been cloned and its ability to bind LTX receptors (whether NRX or LPH) has not been shown.

1.12 How may latrophilin function in neurosecretion?

As latrophilin is a G protein-coupled receptor, it must interact with a specific G protein (so called because they bind GTP). G proteins are heterotrimeric (composed of α , β and γ subunits) but are classified according to the identity of their unique G_α subunit [see Stryer & Bourne, 1986]. Based on amino acid similarity, G proteins have been classified into four broad subgroupings: G_i , G_s , $G_{12/13}$ and G_q . Within these groups there are multiple members [Hamm & Gilchrist, 1996]. Generally, G_i inhibits activity of adenylate cyclase, G_s stimulates activity of adenylate cyclase and phosphodiesterase, $G_{12/13}$ is thought to be involved in the stimulation of cell growth and G_q in the increase in phospholipase C activity [see Fields & Casey, 1997 and references within].

It has long been known that GTP-binding proteins are important in the calcium-regulated exocytosis of vesicles or granules [see Lang, 1999; Vitale *et al.*, 2000]. Certainly, heterotrimeric G proteins are present on the plasma membranes and, in addition, they have been discovered on the membranes of LDCVs and small synaptic vesicles in neuronal cells as well as on the granules of other secretory cells [Ahnert-Hilger *et al.*, 1994]. Thus, G protein α subunits are thought to play key roles in calcium-stimulated exocytosis and this has been demonstrated in a number of systems, for example, in insulin-secreting cells [Lang *et al.*, 1995], pancreatic zymogen granules [Ohnishi *et al.*, 1997] and in chromaffin cells [Gasman *et al.*, 1997].

There are two stages of Ca^{2+} -dependent exocytosis, an ATP-dependent priming of the exocytotic machinery followed by the Ca^{2+} -triggered fusion event (ATP-

independent) [Bittner & Holz, 1992]. Studies on G protein α subunits using patch clamp techniques [Kreft *et al.*, 1999] and permeabilised cell systems [see review by Gomperts, 1990] implicate their action in the late ATP-independent stage of exocytosis after vesicle priming [Komatsu *et al.*, 1993].

Thus, the concept that LTX causes massive synaptic vesicle release via a G protein linked to latrophilin is highly plausible. Even before latrophilin was identified as a high affinity LTX receptor, the toxin was seen to cause phosphatidyl inositol 4,5-bisphosphate (PIP₂) hydrolysis and the production of 1,4,5-inositol triphosphate (IP₃) in PC12 cells [Vicentini & Meldolesi, 1984; Rosenthal *et al.*, 1990]. This effect was only seen in the presence of extracellular Ca²⁺ and was attributed to Ca²⁺-sensitive phospholipase C (PLC). Consistent with these observations, LTX can stimulate IP₃ production in COS cells transfected with latrophilin [Lelianova *et al.*, 1997]. Similar activation of PIP₂ hydrolysis was seen with LTX on synaptosomes in the presence of Ca²⁺ [Ichtchenko *et al.*, 1998].

In cells, after PLC-catalysed hydrolysis of PIP₂, the IP₃ produced can bind to specific receptor proteins located on intracellular membrane organelles, stimulating the opening calcium channels that release Ca²⁺ from intracellular, non-mitochondrial stores into the cytosol [Eberhard & Holz, 1988]. The rise of calcium levels in the cytosol would stimulate synaptic vesicle release, and this is how LPH may be linked to the neurosecretion pathway.

In theory, these Ca²⁺ stores would need to be located near the sites of release, i.e. the active zone, in order to raise the local concentration of Ca²⁺ to $\sim 25\text{-}100\text{ }\mu\text{M}$

required for exocytosis. Interestingly, in many non-excitable cells it has been found that the depletion of Ca^{2+} stores triggers the influx of extracellular Ca^{2+} into the cytosol through a predicted store-operated calcium channel (SOC) or capacitance channel in the plasma membrane [Putney & McKay, 1999]. The principal function of this is to provide Ca^{2+} to replenish the intracellular stores, however, it is conceivable that this may also prolong the increased cytosolic calcium concentration at the active zone and thus stimulate synaptic vesicle release. Little is known about the nature of the capacitance channel or the signalling pathway that activates it although several transient receptor potential (TRP) proteins have been proposed as candidate SOCs [Dutta, 2000].

1.13 LTX channel/pore formation

One of LTX's major effects is the stimulation of Ca^{2+} -influx in PC12 cells and synaptosomes, which could trigger exocytosis [Grasso *et al.*, 1980; Meldolesi *et al.*, 1984; Nicholls *et al.*, 1982]. These effects are not due to the activation of endogenous channels, as the toxin effect is not inhibited by Na^{+} -channel blockers (e.g., tetrodotoxin), K^{+} -channel blockers (e.g., 4-aminopyridine) and organic Ca^{2+} -channel blockers (e.g., ω -conotoxin, verapamil and nifedipine). Additionally, divalent cations that are known blockers of Ca^{2+} channels (e.g., Co^{2+}) do not prevent LTX-induced release of neurotransmitter in Ca^{2+} -containing media and actually support toxin-induced release in the absence of extracellular Ca^{2+} [see Meldolesi *et al.*, 1986 and references within]. All of this data suggests that the toxin itself forms the channel.

There have been numerous demonstrations of this ability. In artificial phospholipid-cholesterol membranes, channels with a large conductance (100-400 pS) were formed presumably by LTX inserting across the lipid bilayer [Finkelstein *et al.*, 1976; Robello *et al.*, 1984]. These channels were similar to calcium channels of excitable membranes [Sokolov *et al.*, 1983] and were permeable to Ca^{2+} but could not be inhibited by organic calcium channel inhibitors [Mironov *et al.*, 1986]. These results, therefore, imply that LTX can open channels in membranes devoid of LTX receptors. However, another study in artificial membranes disputes this suggestion. Scheer and colleagues in 1986 reconstituted LTX receptors into liposomes. The preparation apparently contained both neurexin Ia and latrophilin as toxin binding was observed with different divalent cations and in EDTA. Under these conditions LTX

caused $^{45}\text{Ca}^{2+}$ efflux from liposomes containing the reconstituted receptors but not from liposomes absent of them. Typical conductances of these channels recorded (in the presence of Ca^{2+}) were 65 pS.

The idea that receptors aid the formation of LTX channels is strengthened by the fact that the toxin only opens pores in cell systems expressing LTX receptors. In PC12 cells LTX formed channels permeable to Na^+ , K^+ and Ca^{2+} [Wanke *et al.*, 1986]. These channels were much smaller in conductance (about 15 pS) but like those seen by Finkelstein *et al.*, 1976 in artificial systems, could not be prevented or reversed by endogenous channel blockers. Furthermore, in PC12 cells, toxin induced intake of radiolabelled Ca^{2+} was completely blocked if cells were pre-exposed to micromolar concentrations of concanavalin A. This was in all probability caused by inhibition of a concanavalin-sensitive 'receptor'. Thus, LTX receptors play an integral role in the formation of the LTX-induced Ca^{2+} pores [Meldolesi *et al.*, 1986].

Another study of receptor involvement in LTX-induced channel formation came by way of patch-clamp studies on *Xenopus* oocytes expressing both LTX receptors (injection of rat brain messenger RNA) [Filippov *et al.*, 1994]. Channels formed in oocytes varied widely in conductance from 13 to 200 pS but were not observed in oocytes that had not been injected with rat mRNA suggesting that the receptor is essential for channel formation.

High affinity receptors for LTX have been found in a neuroblastoma cell line, NG108-15, [Hurlbut *et al.*, 1994]. The affinity constant for LTX interaction with receptors is similar to that of PC12 cells (2×10^{-9} M) but NG108-15 cells have six times more binding sites. In this cell system, LTX was able to form nonselective channels

that had a conductance comparable to that observed in artificial lipid bilayers (300 pS). These channels were permeable to monovalent and divalent cations and could be blocked by 1 mM La^{3+} . Interestingly, in Ca^{2+} -containing solutions, not all LTX formed channels. Only when Ca^{2+} was removed or Mg^{2+} was supplemented, were more channels observed. Upon removal of Ca^{2+} , LTX would seemingly no longer remain bound to neurexin $\text{I}\alpha$, which strengthens the idea that latrophilin may be more important than neurexin in mediating the various functions of LTX whether channel formation or receptor-mediated signalling.

Most recently, LTX channels have been demonstrated to form in bovine chromaffin cells and HEK293 cells transfected with LPH1 [Hlubek *et al.*, 2000]. The conductance of the channels in both cell types is 400 pS in the absence of extracellular calcium. The presence of Ca^{2+} reduced channel currents. LPH mutants (full-size receptor minus the C-terminal region or the extracellular portion plus a single TMR) or neurexin $\text{I}\alpha$ expressed in these cells also produced LTX induced channels indicating that extracellular binding sites, irrespective of type, are important in tethering the toxin to the membrane but not latrophilin's TMRs.

The literature describing LTX pore formation, as can be seen, remains rather confusing. On one hand, the majority of LTX channels require the presence of receptors to form but this is not the case in every system. Also of some concern is the lack of consistency between the size of the conductances measured. Even in the same system (e.g., oocytes) conductances can vary widely from 13 to 200 pS. This problem has undoubtedly risen from the use of different LTX preparations, purified to varying degrees, by the various experimenters. As will be shown in Chapter 3 (our own experiments to characterise the channel-forming properties of LTX) impure toxin

preparations (containing the equivalent of 10 pM LTX) can form channels in planar bilayers whereas 10 pM of a highly purified LTX cannot. Moreover, the involvement of the LTX pore in the toxin's effect may, therefore, be contested. Thus, in the absence of Ca^{2+} , LTX still stimulates release in some systems, and this ability has been interpreted as an indication of the direct involvement of the receptor in stimulation of secretion, or of the sensitivity of the release apparatus to other cations (Na^+) flowing through the pore [Adam-Vizi *et al.*, 1993].

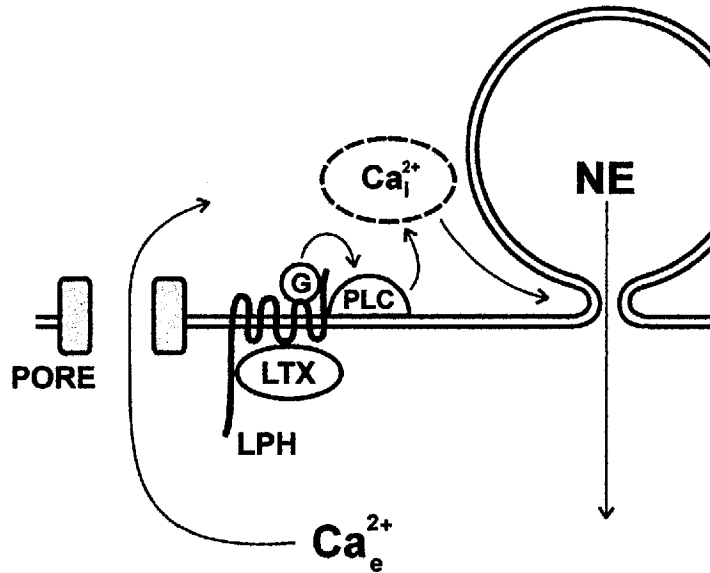
1.14 Current model for LTX action

To summarise the last few sections, LTX mediates two types of neurotransmitter release: Ca^{2+} -dependent and Ca^{2+} -independent. Both of these could occur through its interaction with a single receptor, latrophilin, which is more abundant than neurexin I α in the brain and can bind LTX under all conditions. Furthermore, LTX causes PIP_2 hydrolysis and the production of IP_3 . Structural studies reveal that this effect of LTX is likely to be mediated through the toxin's interaction with latrophilin as this receptor belongs to the superfamily of G protein-coupled receptors. Moreover, there are multiple components to LTX-induced release:

- vesicular release dependent on divalent cation (e.g., Ba^{2+} , Ca^{2+} , Mg^{2+} and Sr^{2+}) influx, ATP and Na^+ ;
- vesicular release dependent on divalent cations and ATP but Na^+ -independent;
- non-vesicular release (non-specific leakage through the plasma membrane), which is independent of ATP or Na^+ or divalent cations.

In an attempt to explain and consolidate these many observations, Davletov *et al.* [1998] proposed the latest model for mechanisms of α -latrotoxin action (see Figure 1.7). These authors noticed that intracellular Ca^{2+} chelators reduced the degree of Ca^{2+} -dependent norepinephrine (NE) release from synaptosomes stimulated by LTX, confirming previous hypotheses that the toxin's mode of action is through increasing intracellular Ca^{2+} concentrations. Interestingly, the omission of Mg^{2+} from their incubation buffers abolished the radiolabelled influx of extracellular Ca^{2+} into

a



b

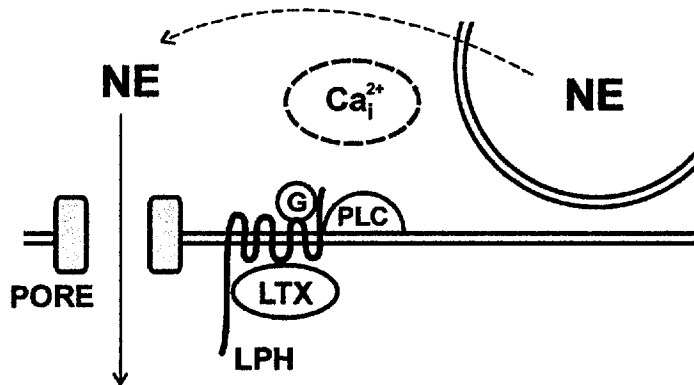


Figure 1.7 Proposed mechanism of α -latrotoxin action. ***a***, LTX is targeted to the presynaptic membrane by its recognition of latrophilin. Once there LTX causes two effects (i) the opening of a pore in the cell membrane allowing extracellular Ca^{2+} influx (Ca^{2+}_e) and (ii) the activation of G protein-coupled phospholipase C (PLC), which leads to the mobilization of Ca^{2+} from endogenous stores (Ca^{2+}_i). Both mechanisms synergistically lead to neurotransmitter release. ***b***, Once open, the pore will allow the escape of neurotransmitters from the cytoplasm such as norepinephrine (NE), as shown in the figure [after Davletov *et al.*, 1998].

synaptosomes, yet the extent of NE release was not altered. It is plausible that firstly, extracellular calcium, without entering the cell, acts as a co-factor for LTX-induced activation of the receptor(s). Alternatively, a very small local influx of Ca^{2+} near the release sites may go undetected by radioactive Ca^{2+} measurements, but may be crucial in stimulating exocytosis. Secondly, internal Ca^{2+} stores might be mobilised to raise Ca^{2+} concentrations in the cytoplasm to levels necessary to stimulate exocytosis. Indeed, when drugs that deplete intracellular calcium stores (thapsigargin) or inhibit the coupling of G proteins to phospholipase C (aminosteroid U73122) were used, the effect of LTX-induced NE release decreased even in the presence of high extracellular Ca^{2+} ($[\text{Ca}^{2+}]_e$).

This suggested a possible role for latrophilin in a synergistic mechanism of action where Ca^{2+} -dependent vesicular neurotransmitter release actually consists of two components: (a) direct Ca^{2+} influx through a LTX-mediated pore, and (b) activation of latrophilin leading to the mobilisation of intracellular Ca^{2+} stores, an effect particularly important when $[\text{Ca}^{2+}]_e$ is low. These suggestions, however, did not explain the Ca^{2+} -independent effect observed, but it was found that this portion of release was not blocked by the cleavage of the SNAREs by clostridial neurotoxins as shown earlier by McMahon *et al.* [1990]. They concluded that the Ca^{2+} -independent exocytosis was due to the leakage of neurotransmitter from the cytoplasm through LTX-induced pores.

Therefore, the mechanism of LTX action is rather complex and still unresolved. How LTX opens Ca^{2+} channels and whether latrophilin is the molecular target for the toxin are still unknown. In this thesis, structure-function studies on LTX and latrophilin have gone some way towards answering these questions. First, as discussed in Chapter 3, a novel procedure of toxin purification is suggested that allows the isolation of LTX,

free from any contaminants. The structure of this toxin was studied biochemically and using cryo-electron microscopy (Chapter 4). LTX was found to exist as a stable homodimer capable of forming cyclical tetramers. Such tetramers were able to insert into lipid bilayers and permeabilise the membrane to cations and small molecules, thus, explaining the pore-forming ability of LTX.

Second, latrophilin is studied in detail (Chapters 5-7) and shown to consist of two fragments, termed the NTF (N-terminal fragment) and the CTF (C-terminal fragment), which originate from one precursor. These fragments may interact but also behave as separate proteins anchored in the membrane. Both the NTF and the CTF are found in the presynaptic plasma membrane where the CTF is able to interact with specific heterotrimeric G proteins, $G\alpha_q$ and $G\alpha_o$. This work has laid the first basis of understanding multiple LTX actions and suggested new methods and approaches to elucidating the structure and physiological function of LPH.

CHAPTER TWO



MATERIALS & METHODS

2.1 Purification and characterisation of LTX

2.1-1 Gel filtration and anion exchange

100 μ l of lyophilised *Latrodectus mactans lugubris* venom (Fauna Ltd., Institute of Zoology, Kazakhstan) was dissolved in 1 ml of 50 mM Tris-HCl pH 8.3, 0.15 M NaCl (buffer A), in the presence of 20 mM EDTA. The sample was clarified by centrifugation (5 minutes, 13,000 g) and the resulting pellet was re-extracted with 0.1 ml of buffer A to decrease loss of soluble protein. The combined extract was applied to a Showdex KW-803 gel filtration column and eluted with buffer A at a flow rate of 0.25 ml/min using a Waters 626 LC system. Fractions containing LTX (collectively termed GF1), as determined by Western blotting, were then applied onto a Millipore Protein-Pak Q AP-1 column (equilibrated in buffer A) and eluted at a flow rate of 1 ml/min using a gradient of [NaCl]: 20 min, 0.15 M; 15 min, linear gradient 0.15-0.33 M; 20 min, 0.33 M; 30 min, linear gradient 0.33-1 M.

2.1-2 Native preparative electrophoresis

Fractions containing LTX after anion exchange (collectively termed IE3), as identified by SDS-PAGE, were pooled and concentrated to 3 ml by ultrafiltration using a Centriprep-30 concentrator (Amicon). During the concentration step salt-free buffer was added to reduce the [NaCl] to $\sim$ 0.15 M. For preparative native PAGE, a 3.5 cm-long gel (6% polyacrylamide) was prepared for a BioRad Model 491 Prep Cell (see Table 3 at the back). The toxin sample (to which glycerol and bromophenol blue were added to final concentrations of 10% and 0.005%, respectively) was applied to the top of this gel under running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine), and

electrophoresis was carried out overnight at 200 V. Proteins (continuously monitored at 280 nm) were eluted from the Prep Cell at a flow rate of 0.4 ml/min and collected in 6 ml fractions. Fractions containing α -latrotoxin were combined, concentrated to ~5 ml using another Centriprep 30 unit and stored in aliquots at -70°C .

2.1-3 Channel formation in lipid bilayers

These experiments were performed in association with the laboratory of Richard Ashley (Physiology Department, University of Edinburgh) using samples of venom, GF1, IE3 and LTX obtained by the purification method described above. Planar bilayers were formed from purified synthetic lipids (Avanti), suspended at a concentration of 30 mg/ml in *n*-decane (Aldrich). The bilayers, which comprised pure palmitoyl-oleoyl phosphatidylethanolamine (POPE) or an equimolar mixture of POPE with palmitoyl-oleoyl phosphatidylserine (POPS), were cast across a 0.3 mm diameter hole in a polystyrene partition separating two solution-filled chambers, as described elsewhere [Williams, 1995]. The contents of these chambers were changed by perfusion (≥ 10 volumes), as required. The membranes were voltage-clamped using a Biologic RK-300 patch clamp amplifier (Intracel, UK), and transmembrane currents were low-pass filtered at 10 kHz and recorded. The LTX was added to the *cis* side of the bilayers, which was voltage-clamped relative to the grounded *trans* side. Data was post-filtered and digitally sampled for analysis using pClamp 6 (Axon). Relative ion permeabilities were calculated from reversal potentials measured under biionic conditions, using appropriate forms of the Goldman-Hodgkin-Katz and Fatt-Ginsborg equations.

2.1-4 Synaptosomal release experiments

Synaptosomes were prepared from one rat brain using the Ficoll gradient approach (full description in 2.4-1) and resuspended in 5 ml of a physiological buffer (132 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM D-Glucose, 0.1 mM Na₂EGTA, 20 mM HEPES, pH 7.2). Where necessary, MgCl₂ and Na₂EGTA were replaced with 1.2 mM CaCl₂. 1 ml of this suspension was incubated with 2.5 µCi (6 nmol) of [³H]norepinephrine ([³H]NE, New England Products) for 30 min at 37°C, before being washed three times with 1 ml of the physiological buffer. 150 µl of these pre-loaded synaptosomes were then mixed with 5 nM of LTX, incubated for 10 min at 37°C, and pelleted in a microcentrifuge. Aliquots of the supernatant were combined with 6 ml of scintillation liquid (Sigma) and measured in a β-spectrometer. Experiments were performed in triplicate.

2.1-5 Synaptosomal binding experiments

For binding, LTX was iodinated by the chloramine-T method (see below). Different nM concentrations of this radiolabelled toxin were mixed with synaptosomes (1 mg protein/ml, prepared by Ficoll gradients) in 50 mM Tris-HCl pH 8.3, 5 mM EDTA. After incubating at room temperature for 10 min, the samples were diluted into 3 ml of 50 mM Tris-HCl pH 8.3, 0.15 M NaCl and filtered through a glass-fibre filter (GF/F, Whatman). The filters were immediately washed with 6 ml of the TBS buffer (completed within 10 seconds) and their radioactivity was determined in a γ-spectrometer.

2.1-6 Molecular weight determination by gel filtration

The gel filtration of preparative α -latrotoxin was carried out on a Superose 6 column (Pharmacia) connected to a BioLogic system (BioRad), which facilitated accurate data analysis. The retention time of the toxin on the column (eluted using 50 mM Tris-HCl pH 8.3 at 0.4 ml/min) was measured against the following protein standards (from Sigma): carbonic anhydrase (29 kDa), ovalbumin (43 kDa), alcohol dehydrogenase (150 kDa), β -amylase (200 kDa), apoferritin (443 kDa), thyroglobulin (669 kDa) and bovine serum albumin (BSA) oligomers (66, 132, 199, 265 kDa). The toxin and standards were prepared to 1 mg/ml concentration and 200 μ l of each sample was used for chromatography.

2.1-7 Molecular weight estimation by dynamic light scattering (DLS)

200 μ l of preparative LTX (1 mg/ml) was injected into a DynaPro-801 instrument (Protein Solutions Inc.) and its molecular mass automatically calculated by Dynamics™ software. Fifteen measurements were taken from five separate LTX preparations. To verify the internal calibration of the instrument the protein standards of known molecular weight (see above) were also injected (200 μ l of 1 mg/ml preparations).

2.1-8 Native gels

Samples were loaded onto Novex pre-made gradient 4-12% polyacrylamide gels under native conditions (refer to Table 3 at the back for sample buffer formulation). Electrophoresis was performed in 50 mM Tris-HCl pH 8.3, 192 mM glycine buffer at 130 V constant for 7 hours (gel pre-run for 30 minutes) to achieve

suitable separation. The protein bands were then visualised by staining with Coomassie R-250.

2.1-9 Iodination of LTX

LTX was iodinated using the chloramine-T method [Ushkarev & Grishin, 1986]. Briefly, a column containing 10 ml of P4 Bio-Gel coarse resin (BioRad) was swelled in warm dH₂O and degassed under vacuum. A 100 µl aliquot of LTX (~ 5 µM concentration) was mixed with 50 µl of 25 mM Tris-HCl (pH 7.6), 12 µl of Na¹²⁵I (1 mCi, ICN Radiochemicals) and 15 µl of chloramine-T (1 mg/ml, Sigma). This preparation was pipetted up and down at room temperature for 1 minute before 15 µl of sodium metabisulphite (1 mg/ml, Sigma) and 30 µl of sodium iodide (3 mg/ml, Sigma) were added to quench the reaction. The mixture was then carefully applied onto the P4 column. 1 ml fractions were eluted using PBS or TBS (as required) and counted using a γ-spectrometer in order to estimate specific radioactivity. Samples of the radiolabelled toxin fractions were run on SDS-PAGE to check for degradation before being used within a week for various studies.

2.1-10 Cross-linking of LTX

The cross-linker, bis[sulfosuccinimidyl]suberate (BS³, Pierce) was prepared as a 25 mM stock solution in phosphate buffered saline (PBS, pH 7.2) just before use. For the reaction, 1 nM of ¹²⁵I-LTX (iodinated in PBS) was incubated with 30 µM of BS³ for 30 minutes at room temperature in PBS supplemented with 5 mM EDTA. The reaction was then quenched for 15 minutes using 1 M Tris-HCl, pH 7.5 (added to a final concentration of 50 mM).

2.1-11 Affinity between LTX dimers

1 mg of unlabelled LTX was coupled to 1 ml CNBr-activated Sepharose 4B to produce an "LTX column" (for full details see 2.3-1). A "BSA column" was synthesised by coupling 1 mg of BSA to 1 ml of CNBr-Sepharose. 10 µl aliquots of each column were blocked for 45 minutes using 1% BSA in 50 mM Tris-HCl pH 8.3 buffer, and then incubated with 2-50 nM ^{125}I -LTX for 30 minutes, at room temperature, in the Tris buffer (containing 0.1% BSA). Divalent cations or EDTA were supplemented into the buffer where necessary. After incubation, the columns were washed and counted in a γ -counter.

2.2 Three-dimensional structural studies of LTX

2.2-1 Cryo-electron microscopy and image processing

A 4 μ l drop of a 1 mg/ml LTX preparation (supplemented with divalent cations or detergent as required) was applied onto an electron microscope grid covered by a holey carbon film. The excess liquid was blotted away with filter paper and the grid immediately plunged into liquid nitrogen-cooled ethane in order to vitrify the sample [Dubochet *et al.*, 1988]. Images were taken in a CM200 cryo-microscope (Philips) equipped with a field-emission gun (FEG), at 50,000 x magnification and 3.1 ± 0.2 μ M defocus.

The micrographs were digitised using an EmiL linear CCD densiometer at a 14 μ M step size (equivalent to 2.8 Å on the specimen scale). Molecular images were interactively selected using the IMAGIC-5 software [van Heel *et al.*, 1996] on DEC workstations running under UNIX. The contrast transfer function (CTF) of the microscope was determined by finding zero positions of Thon rings. As images were taken within a narrow range of defocus values, correction of the CTF was applied to the final reconstruction. The images were processed by multi-reference alignment technique [Dube *et al.*, 1993] and multivariate statistical classification [van Heel, 1989] in order to find the characteristic molecular views in the sample. Characteristic views, or class averages, are averages of several images of a molecule in the same orientation. This procedure suppresses noise inherent in low-dose electron microscopy and enhances the structural signal. Relative orientations of the characteristic views were defined by the angular reconstitution approach [van Heel *et al.*, 1987].

After finding the orientations, the reconstruction of the 3-D distribution of molecular densities was calculated using the exact filter back projection algorithm [Harausz *et al.*, 1986]. The obtained 3-D map was then refined iteratively as described [Serysheva *et al.*, 1995; Schatz *et al.*, 1995]. To assess the resolution of the resulting 3-D structure a set of class averages was split into two halves and two different 3-D maps were calculated. Comparison of the two structures using Fourier Shell Correlation (FSC) [van Heel *et al.*, 1986] revealed that a resolution of ~ 14 Å was achieved for the tetramer and ~ 18 Å for the dimer.

2.2-2 Preparation of the N-terminal antibody to LTX

A mouse was injected with four doses of ~ 20 µg of SDS-denatured LTX (obtained as described above) to produce LTX antiserum. To isolate the antibody specific to the N-terminus, a fusion protein was constructed by subcloning residues 81-218 of LTX into pET23 vector in frame with six His residues (Novagen). The fusion protein expressed in *E. coli* was isolated using a nickel column (Invitrogen) and transferred onto PVDF membranes (Millipore). The membranes were blocked using 5% non-fat milk, 0.1% Tween in TBS and incubated with the LTX antiserum overnight. After several washes in TBS, specific antibodies were eluted with 0.1 M glycine-HCl pH 2.5 and neutralised using unbuffered Tris base. For electron microscopy, LTX was incubated with 2 mM MgCl₂ to produce tetramers before being mixed with the N-terminal specific antibodies in a 1:1 molar ratio at room temperature. This preparation was then applied onto holey carbon grids and prepared for cryo-microscopy as described above.

2.2-3 Preparation of liposomes

Liposomes were prepared by hydration of 1 mg of pre-liposomes (L- α -phosphatidylcholine, β -oleoyl- γ -palmitoyl; Sigma) in 0.5 ml of 25 mM Tris-HCl pH 8.2, 2 mM MgCl₂. This suspension was mixed with LTX at a molar ratio of 2000:1 (lipid:toxin). After a 15-minute incubation at room temperature, the specimens were chilled on ice and prepared for electron microscopy as described earlier. Side views of LTX attached to the membrane were selected for alignment and classification. No symmetry was applied to the class averages.

2.3 Isolation of latrophilin

2.3-1 Preparation of the LTX and BSA columns

1 mg of purified LTX and 1 mg of pure BSA (Pierce) were each coupled to 1 ml of CNBr-activated Sepharose-4B (Pharmacia) using a modified version of the manufacturer's method. Briefly, after the reactive groups of Sepharose-4B had been activated with a quick 1 mM HCl wash, they were partially blocked by incubation with coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3), for 30 minutes. Following this, the Sepharose beads were incubated with the appropriate ligand (dialysed into the coupling buffer), overnight, at 4°C. After washing, all remaining active groups were blocked using 0.1 M Tris-HCl, pH 8.0, for 2 hr. The column preparation was completed with three cycles of washing with alternate pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.3 and pH 6.8). This pH range ensured that LTX would not be denatured.

2.3-2 Preparation of the brain for chromatography

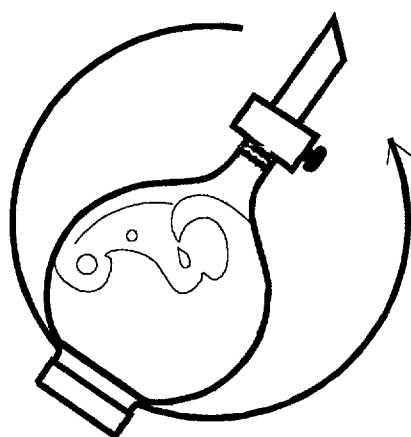
Twenty rat brains (Harlan Sera Labs.) were homogenised in 110 ml of 50 mM Tris-HCl pH 7.6, 0.2 M NaCl, 5 mM EDTA until a uniform suspension was formed using a polytron homogeniser (Janke & Kunkel Ultra-Turrax T25). Immediately after this, 330 ml of 50 mM Tris-HCl (pH 7.6), 0.2 M NaCl, 2% Thesit buffer was added and the mixture permitted to solubilise for 1-2 hr. The preparation was subsequently centrifuged for 4 hr at 30,500 g in a T-19 rotor (Beckman). The supernatant was recovered and typically yielded 5 x 80 ml total brain aliquots for chromatography. All steps of this procedure were carried out at 4°C or on ice, and a mammalian protease inhibitor cocktail³ (Sigma # P8340) was also present throughout. Divalent cations were added during homogenisation and solubilisation, as required.

2.3-3 LTX affinity chromatography

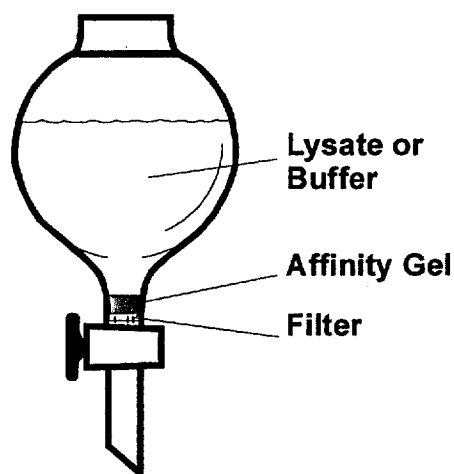
Affinity chromatography was performed by the batch method using a 100 ml Teflon separation funnel (Sigma) with a filter inserted into the stopcock assembly (as in Figure 2.1). This allowed the use of the same vessel for both incubation and elution of proteins bound to the adsorbant. The 80 ml aliquots of solubilised rat brain (in the presence of 2 mM Mg^{2+} or EDTA) were incubated with the LTX column for at least 2 hr or overnight. After washing with 100 column volumes of wash buffer (50 mM Tris-HCl pH 7.6, 0.2 M NaCl, 0.2% Thesit, 2 mM Mg^{2+} or EDTA), specific proteins were eluted from the column with 10-30 ml of elution buffer (50 mM Tris-HCl pH 7.6, 1 M NaCl, 0.2% Thesit, 2 mM Mg^{2+} or EDTA), as required. All steps of the procedure were performed at 4°C with protease inhibitors present.

2.3-4 Wheat germ agglutinin (WGA) chromatography

1 ml of WGA-Sepharose-4B (Pharmacia) was placed into a 100 ml Teflon separation funnel with a filter inserted into the stopcock assembly to make a “WGA column”. 1 M salt eluates from the LTX column were diluted with a regular Tris buffer in order to reduce [NaCl] to ~ 0.5 M and then loaded onto this second column. After a 2 hr incubation, the lectin was washed with 100 column volumes of 50 mM Tris-HCl (pH 7.6), 0.5M NaCl, 0.2% Thesit, and then all specifically bound proteins were eluted with 8 x 0.5 ml of 100 mM *N*-acetylglucosamine (NAGA) in the same buffer. For latrophilin preparations enriched in heterotrimeric G proteins, 20-100 μM Mg^{2+} and 2 mM GDP were included during purification.



**Incubation
with rotation**



**Washing or
Elution**

Figure 2.1 Affinity chromatography by the batch method. A Teflon separation funnel with a filter inserted into the stopcock assembly makes a perfect vessel for carrying out LTX, BSA and WGA affinity chromatographies. The affinity gel (Sephacrose) can be incubated with brain proteins, washed and eluted in this setup without any appreciable loss of the toxin, BSA or lectin.

³**Sigma mammalian protease inhibitor (# P8340) contains:** 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), bestatin, leupeptin and aprotinin.

2.4 Preparation of brain fractions and other tissues

2.4-1 Synaptosomes

Purification of synaptosomes was performed using the Ficoll gradient method. Brains were removed from two rats within one minute of their death (Schedule 1). To the dissected cerebral cortices, 40 ml of 0.32 M sucrose, 10 mM HEPES pH 7.2 (buffer A) was added and the brain material crushed with 10 up and down strokes of a Potter-Elvehjem homogeniser (clearance 0.2 mm). This homogenate was briefly spun down in a Heraeus table-top centrifuge (2 minutes, 2,200 *g*) to yield a pellet "P1" and a supernatant "S1". The supernatant was further spun down for 20 minutes at 12,000 *g* using a Sorvall SS34 rotor to yield another pellet and supernatant ("P2" and "S2"). This second pellet was resuspended in 3 ml of buffer A.

Two 7.5%/12% (w/v) Ficoll (Pharmacia) gradients were prepared in buffer A in tubes suitable for an SW40Ti rotor (Beckman). The resuspended "P2" was layered equally onto the gradients and centrifuged at 64,000 *g* for 1 hour. The synaptosomes were recovered at the 7.5%/12% Ficoll interface with a pipette. To remove the Ficoll, the synaptosomes were washed in a physiological buffer e.g., 132 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1 mM MgCl₂, 10 mM D-Glucose, 0.1 mM Na₂EGTA, 20 mM HEPES, 1.2 mM Na₂HPO₄ (pH 7.2). The composition of this buffer was changed according to requirements.

2.4-2 Synaptic plasma membranes (SPM)

After preparing synaptosomes, as described above, they were incubated for 10 min at 37°C before being quickly pelleted in a bench-top centrifuge (Heraeus, 4000 *g*,

3 min). The resulting pellet was osmotically lysed in 30 ml of ice-cold 10 mM Tris-HCl pH 7.6, 50 μ M Ca^{2+} or Mg^{2+} buffer containing protease inhibitors (Sigma # P8340). The lysate was subjected to three freeze/thaw cycles at -70°C after which it was centrifuged at 100,000 g for 30 min, at 4°C , using the SW40Ti rotor (Beckman), to yield pellet "P3" (crude SPM) and supernatant "S3". The S3 supernatant was used for synaptic vesicle purification (see 2.4-4).

2.4-3 Enrichment of pre- and postsynaptic membranes

Crude SPM from the brains of five freshly killed rats were resuspended in 5 ml of 15 mM Tris-HCl (pH 7.6), 0.4 M NaCl, 0.15 M DTT and homogenised briefly (10 sec) with a polytron homogeniser (Janke & Kunkel Ultra-Turrax T25) set to 8,000 rpm on ice. The material was layered onto a single three-step sucrose density gradient {0.8 M, 1.0 M and 1.2 M (w/v) in the Tris/salt/DTT buffer} that had been prepared in a tube for an SW28 rotor (Beckman). The gradient was centrifuged at 100,000 g for 3 hr after which the "presynaptic" membranes were removed from the 0.8 M/1.0 M sucrose interface and the "postsynaptic" membranes from the 1.0 M/1.2 M sucrose interface. All steps of this procedure were carried out at 4°C .

2.4-4 Preparation of synaptic vesicles

The supernatant "S3" (obtained from 5 rat brains) was centrifuged at 165,000 g for 2 hours, at 4°C , using the SW40Ti rotor (Beckman). This yielded a small pellet "P4" rich in synaptic vesicles and a clear supernatant "S4". The pellet was resuspended in 1.5 ml of 40 mM sucrose, 4 mM HEPES and loaded on a sucrose gradient (100 mM to 800 mM in 100 mM steps, each 2.75 ml) prepared in HEPES. This was centrifuged in a SW28 rotor (Beckman) at 65,000 g , for 5 hours, at 4°C . After this, 2 ml fractions were collected using a capillary tube and peristaltic pump. Fractions that contained

synaptic vesicles were identified by specific antibodies (synaptophysin and synaptobrevin) after Western blotting (see 2.7-1). These were subsequently washed to remove sucrose and concentrated, as needed.

2.4-5 Other Tissues

Crude postnuclear membranes (i.e. "P2") were prepared from ~ 2 g of liver, small intestine, heart and kidney as described for synaptosomes above.

2.5 Biochemical studies of the NTF and CTF

2.5-1 Detergent solubilisation of latrophilin

50 μ l of synaptic plasma membranes (2 mg protein/ml) were resuspended in 0.5 ml of 50 mM Tris-HCl (pH 7.6), 0.2 M NaCl buffer and various detergents: Thesit (0 to 2% w/v); SDS (0 to 0.1%); Triton X-100 (0.5% w/v); and CHAPS (0.5% w/v). These were incubated for 10 minutes at 4°C after which pellets and supernatants were separated by a microcentrifuge spin (13,000 g, 5 minutes). Proportionate amounts of each pellet and supernatant fraction were loaded onto SDS-electrophoresis, transferred onto PVDF membrane and probed with appropriate antibodies (refer to section 2.7).

2.5-2 Chemical treatments

A fresh SPM preparation was first treated with 100 mM DTT in 50 mM Tris-HCl (pH 7.6) for 30 minutes at 37°C in order to reduce all disulphide bonds. A 20 μ l aliquot of these SPM (1 mg protein/ml) was dispensed into tubes where they were subjected to the following chemical treatments: 50 mM glycine-HCl (pH 2.5), 0.1 M NaOH (pH 14), 2 M NaI, 4 M MgCl₂, 2 M KCl, 1 M NaCl, 8 M urea and, finally, 50 mM Tris-HCl, pH 7.6, (as a control). Treatment of membranes was carried out in a volume of 300 μ l at room temperature for 30 minutes (urea for 5 minutes). After this period, the samples were diluted 12-fold using regular buffer (acid and alkali treatments were titrated to neutral pH after dilution using Tris base and HEPES, respectively). The membranes and supernatants were separated by a 10 min spin in an ultracentrifuge (350,000 g) and then loaded onto SDS-PAGE, as described (see 2.7-1).

2.5-3 PI-PLC treatment of membranes

SPM were incubated with 0.1 M NaOH, as described immediately above. The pellet obtained after ultracentrifugation was resuspended in 50 mM HEPES (pH 7.4) buffer and treated with 1 unit of phosphatidylinositol-specific phospholipase C (PI-PLC, Boehringer-Mannheim) for 2 hr, at 37°C. Following this, the membranes were pelleted in a microcentrifuge (13,000 g, 5 minutes).

2.5-4 Sucrose density gradients

Sucrose density gradients containing ten 1 ml layers, encompassing stepwise from 8% to 17% (w/v) sucrose, were prepared in 50 mM Tris-HCl (pH 6.8), 0.2 M NaCl, 0.2% Thesit and 2 mM EDTA (for SW40Ti rotor tubes). SDS (0.5%) and DTT (25 mM) were added to the gradients where necessary. These were left for 2 hr to permit diffusion of the layer interfaces before samples of solubilised rat brain or purified native latrophilin (in final total volumes of 400 µl) were added. The gradients were centrifuged at 164,000 g for 16 hours, at 4°C, after which they were fractionated from the bottom using a glass capillary tube and peristaltic pump. 0.5 ml fractions were collected, which were subsequently analysed on SDS-PAGE.

2.5-5 Depolymerisation of tubulin

Synaptosomes, prepared as described in 2.4-1, were incubated with 1 mM colchicine (Sigma) that had been dissolved in DMSO (1% final concentration in the buffer). After 1 hr at 37°C, the synaptosome pellet was washed twice with physiological buffer (2.4-1) and then solubilised in 2.5 ml of 50 mM Tris-HCl pH 7.6, 0.2 M NaCl, 1% EDTA and 2% Thesit (containing protease inhibitors). The mixture was diluted 4-fold using the same buffer (absent of Thesit) in order to reduce the

detergent concentration to ~ 0.5% and then centrifuged (SW40Ti, 164,000 g for 45 min) to remove any undissolved debris. The supernatant was loaded onto a fresh LTX affinity column (~ 50 µl) overnight. After washing, bound proteins were eluted with 500 µl of 1x SDS-loading buffer (see Table 1 at the back). The experiment was repeated with synaptosomes incubated in (1) physiological buffer and (2) in the presence of 1% DMSO to act as the controls.

2.5-6 Dephosphorylation of the CTF

Purified native CTF was treated with 1 unit of alkaline phosphatase (Sigma) in the presence of 1 mM MgCl₂ and 0.1 mM ZnCl₂. The reaction was performed at pH 10 for 2 hours at 37°C to optimise enzymatic activity. Samples were collected at 30 min intervals, titrated to pH 6.8, and prepared for SDS-PAGE analysis (see later). As a control, the experiment was duplicated without the enzyme being present.

2.5-7 Deglycosylation of the NTF

Native latrophilin obtained after LTX and WGA column chromatographies was resuspended in 50 mM Tris pH 7.6, 0.2 M NaCl, 0.2% Thesit and 2 mM CaCl₂, and treated with 10 mU Neuraminidase (Boehringer Mannheim) for 1 hr followed by 1 unit of PNGase (*N*-glycosidase F, Boehringer Mannheim) for a further hour. All incubations were performed at 37°C, after which samples were precipitated with chloroform/methanol and loaded on SDS-gels (2.7-1).

2.6 Cell culture studies

2.6-1 Cell System

COS-7 cells (a gift from Claire Isacc, Department of Biology, Imperial College) were cultured in “complete medium,” which comprised of Dulbecco’s modified Eagle’s medium (DMEM, GIBCO-BRL) supplemented with 10% fetal bovine serum (heat inactivated, GIBCO-BRL), 1% L-glutamine and 1% antibiotic (penicillin and streptomycin), at 37°C in a humidified atmosphere containing 5% CO₂. COS-7 cells, grown to about 50% confluency in 100-mm dishes (Triple Red), were transiently transfected with the appropriate construct (kindly provided by Dr. Vera Lelianova, see Figure 2.2) or vector alone.

2.6-2 Transfection protocol

5 µg of DNA was mixed with 150 µl of DMEM and 25 µl of SuperFect reagent (Qiagen). After 5-10 min incubation at room temperature, 1.5 ml of complete medium was added to the transfection mix. This mixture was then transferred to plates containing COS-7 cells that had been washed once in PBS. The cells were then incubated at 37°C for 2.5 hr before the transfection mix was removed and the cells washed again with PBS. Finally, 4 ml of complete medium was added and the cells left to grow in a 5% CO₂ atmosphere at 37°C.

2.6-3 Biotinylation of LPH-12

Biotinylation was performed on COS-7 cells expressing LPH12 (see Figure 2.2), 24 hr after transfection, using sulphosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin, Pierce), essentially as specified elsewhere [Prince & Welsh, 1998].

All procedures were carried out on ice or at 4°C, and in the presence of protease inhibitors (Sigma # P8340). Trypan blue assay was used to verify that 95% of the COS cells were intact before starting the surface labelling procedure. On plates, COS cells ($\sim 3 \times 10^6$) were washed twice with ice-cold PBS containing 1 mM MgCl₂. Biotinylating reagent was then added (0.5 mg/ml in 5 ml of PBS) to each plate, for 30 minutes, with gentle rocking every 5 minutes. The cells were again washed with ice-cold PBS before being gently scraped off their plates in 4 ml of the same buffer.

After pelleting in a Hereaus centrifuge (2000 g), the cells were solubilised in 600 µl of 50 mM Tris-HCl (pH 7.6), 0.2 M NaCl, 2% CHAPS buffer for 2 hr, assisted by homogenisation (15 up and down strokes of a disposable 19.5 G syringe needle). The samples were diluted four-fold by adding 50 mM Tris-HCl (pH 7.6), 0.2 M NaCl, in order to reduce [CHAPS] to 0.5%. They were then centrifuged at 13,000 g (microcentrifuge) for 30 minutes to remove unsolubilised debris. To the supernatant, 50 µl of avidin-agarose beads (Sigma) were added for one hour. After washing with the incubation buffer, specific proteins were eluted with 300 µl of SDS-urea sample loading buffer (see Table 2 at the back). As control, COS cells expressing LPH12 were treated in the same way without biotinylation.

2.6-4 Immunofluorescence procedures and confocal microscopy

COS-7 cells were grown on multi-well chamber slides (Lab-tek Chamber Slide System, Nunc) and transfected as described above. After 24 hr, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 minutes at room temperature, and finally, washed with PBS again. Where required, cells were permeabilised with 0.05% Triton X-100, which was kept throughout after fixation. Non-specific binding sites were blocked by a 30 min incubation with 3 % goat serum in PBS (Gibco-BRL). For

detection of the N-terminal fragment of latrophilin, anti-NTF was used as the primary antibody and FITC-conjugated (green) goat anti-rabbit (Sigma) as the secondary antibody. For CTF staining, anti-Myc antibody was utilised, which was then visualised with TRITC-conjugated (red) goat anti-mouse antibody (Sigma). The primary and secondary antibodies were each applied for 1 hr and separated by 6 x 5 min washes with PBS. After incubation with the secondary antibody the COS cells were washed, mounted using Vectashield Mounting Medium (Vector Laboratories) and analysed by confocal laser-scanning microscopy. Specimens were imaged with a LSM 510 system (Zeiss) mounted on an upright microscope (Axioskop-2, Zeiss) using dual excitation at 488 and 633 nm. Light emitted by doubly stained preparations was analysed using a combination of an FITC-type narrow band-pass filter block (505-530 nm) and a long pass filter block (≥ 650 nm). Images were collected using oil-immersion objective (Plan-Neofluar, 40x/1.3, Zeiss).

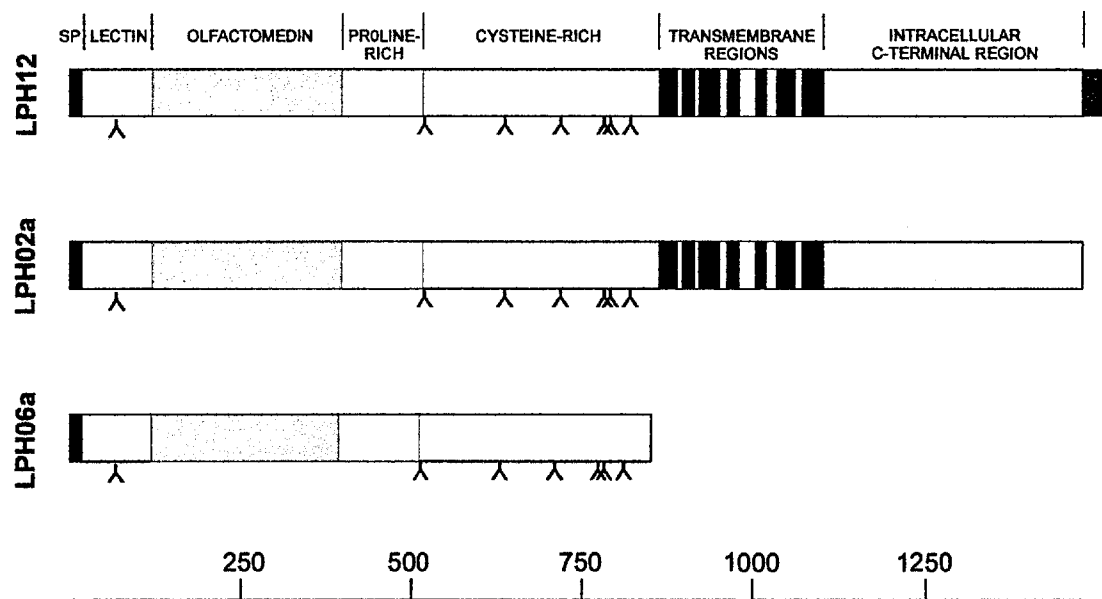


Figure 2.2 Latrophilin constructs. LPH02a and LPH12 are full-size latrophilin constructs. LPH12 possesses a Myc epitope region at its C-terminal half (shown as a red box), which permits the recognition of the full-size receptor and the CTF with a specific, commercially available antibody. LPH06a contains only the extracellular portion of latrophilin, terminating right after the sequence TNFAVLMAHRE.

2.7 Other techniques

2.7-1 SDS-PAGE

Discontinuous SDS-PAGE was performed according to Laemmli [1970] using BioRad Mini-Protean II apparatus (see Table 1 for gel formulae). 5 cm-high resolving gels were prepared onto which a 2 cm-high stacking gel was placed. The thickness of the gels varied (0.75 mm, 1.0 mm or 1.5 mm) depending on the sample volume to be loaded. Electrophoresis was carried out at 100-140 volts with 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% SDS (available in stock solution from BioRad) as the anode and cathode buffer.

Proteins were either dissolved in a 1:1 ratio with 2x sample buffer or in a 1:5 ratio of 6x buffer to sample (see Table 1 at the back). Before loading onto gels, samples were boiled for 3 minutes to allow disulphide bond reduction by β -mercaptoethanol. Alternatively, proteins were completely precipitated by adding 2 sample volumes of a chloroform/methanol (1:1) mixture followed by brief centrifugation. The precipitate recovered from the solvent-water interface was washed once with methanol to remove any remaining chloroform and then dried in air. Following this, the pellet was dissolved in 1x sample buffer and boiled as above.

2.7-2 SDS-urea PAGE

This was performed under the same running conditions as regular SDS-PAGE using a resolving gel and a stacking gel containing 8 M urea (Sigma electrophoresis grade). Refer to Table 3 at the back for gel formulae and sample loading buffer.

2.7-3 Coomassie Staining

The gels were stained for at least 30 minutes in Coomassie brilliant blue R250 (0.025 g of Coomassie in 70% distilled water, 20% methanol and 10% acetic acid). Excess dye was then removed by extensive washes with destaining solution (70% distilled water, 20% methanol and 10% acetic acid). The gels were then equilibrated with distilled water, before being dried using the Gel-air system (BioRad).

2.7-4 Western blotting

For immunostaining, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore Immobilon-P), using a wet blotting mini-electrotransfer unit (Sigma-Aldrich). Transfers of LTX and δ -LIT were carried out for 4 hr (rat tissue and purified LPH for 8 hr) at constant current (150 mA) in 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% methanol. The electrotransfer unit was kept cool (4°C) using a re-circulating pump.

2.7-5 Immunostaining

The Western blot was blocked using 5% non-fat dried milk in TBS-Tween (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.2% Tween-20) for 1-2 hr. The membrane was then stained with primary antibody (in an appropriate dilution) for 1 hr. Following this, it was washed in TBS-Tween (4 x 5 min) before secondary antibodies conjugated to HRP (Amersham or Sigma) were added for a further 30 minutes. After another 4 x 5 min washes with TBS-Tween, the bound antibody was visualised by the Luminol reaction as specified by the manufacturer (Pierce). The membranes were exposed to Luminol sensitive film (Kodak BioMax).

2.7-6 Source of antibodies

Antibodies to G-protein α subunits (G_{common} , G_i , G_o , G_s and G_z) were from Santa Cruz Biotechnology; anti-LTX and anti- δ -LIT were made by Catherine Manser by injecting the purified protein into mice or rabbits; anti-syntaxin, anti-synaptobrevin, anti-SNAP-25 and anti-Kv1.2 were kindly provided by Professor J.O. Dolly; anti-tubulin and anti-synaptotagmin were acquired from Affiniti; anti-NMDA receptor, anti-PSD-95 and anti-synaptophysin were purchased from Boehringer Ingelheim. An antibody to the N-terminus of latrophilin (anti-NTF or anti-120) was made by injecting rabbits with the protein isolated from a LTX column. Rabbit and chicken antibodies to the C-terminal portion of latrophilin (anti-CTF or anti-CT) were prepared using a GST construct conjugated with the LPH cDNA encompassing amino acid residues 1175-1466. Both the anti-NTF and anti-CTF antibodies were affinity-purified, as described below.

2.7-7 Peptide antibody to latrophilin

A peptide to the third cytoplasmic loop of LPH (CSVLPDSSRRLDNIK) was synthesised by Genosys Biotechnologies. This synthetic peptide (P.10) was coupled to the carrier protein KLH (keyhole limpet hemacyanin) and used to immunize rabbits and chickens four times at two-week intervals. The eggs laid by the chickens were collected over a one-month period post-immunisation. From the yolks, IgGs (IgY) were purified with a protein concentration of 28 mg/ml. Rabbits were bled shortly after the last injection; the immune serum was either used as such or anti-peptide antibodies were purified on a column with conjugated cognate peptide.

2.7-8 Affinity purification of anti-NTF and anti-CTF antibodies

Antibodies specific to the N-terminal fragment (NTF) and C-terminal fragment (CTF) were affinity purified from rabbit or chicken serum by a method similar to that described for anti-LTX antibody previously (2.2-2). Fusion proteins containing 1) glutathione-S-reductase (GST) and NTF; 2) dihydrofolate reductase (DHFR) and CTF were expressed in *E. coli*, isolated by affinity chromatography on glutathione (GSH) or nickel-column, respectively, and then transferred onto PVDF membranes (Millipore). The membranes were blocked using 5% non-fat milk, 0.1% Tween in PBS containing 0.2% sodium azide and incubated with the appropriate antiserum overnight. After several washes in PBS, specific antibodies were eluted from the membranes with 0.5M glycine pH 2.4, 0.5M NaCl and neutralised using unbuffered Tris base.

2.7-9 Concentration assay

All protein concentrations were determined using the Bicinchoninic acid (BCA) Protein Assay (Pierce) using the room temperature protocol (range 20 – 2000 µg/ml).

2.8 Sequencing of protein bands

2.8-1 The p55 band (tubulin)

For sequencing of the 55 kDa doublet, the 1 M eluates from 5 LTX column chromatographies were combined and completely precipitated using chloroform/methanol. The resulting pellet was dissolved in 240 μ l of 2x SDS-loading buffer and loaded onto SDS-PAGE. After electrophoresis, the doublet was identified, without fixing, by Coomassie dissolved in water. It was sequenced from trypsin-digested gel pieces (after modifying arginines) by Dr. Nick Totty at the Ludwig Institute for Cancer Research, London, as described elsewhere [Leliana *et al.*, 1997].

2.8-2 The C-terminal fragment of latrophilin

COS-7 cells were transfected with LPH02a (see Figure 2.2). After harvesting these cells (3×10^6), the cell pellet was solubilised in CHAPS buffer as described in 2.6-3. The resulting supernatant was applied to a fresh LTX column (~ 0.4 ml) overnight. Following several washes with 25 mM Tris-HCl (pH 8.2), 0.15 M NaCl, 0.2% CHAPS, 1 mM $MgCl_2$, latrophilin was eluted off the column in 1 ml fractions using the same buffer but containing 2 M NaCl. The flow-through was reloaded onto the LTX column another 3 times to exhaust the receptor. Fractions enriched with CTF (identified by SDS-PAGE and Western blotting) were combined and concentrated using a Centricon-10 unit (Amicon). DTT sample loading buffer (60 mM Tris-HCl pH 6.8, 100 mM DTT, 5% glycerol) was added to the CTF preparation in a 1:1 ratio, which was then run on a special sequencing gel (refer to Table 4 at the back). The gel was transferred onto PVDF membrane using the regular method. The CTF bands were

identified by water-dissolved Coomassie stain. These were excised from the membrane and sent for sequencing to Graham Duncan Kemp at the University of St. Andrews.

CHAPTER THREE



ISOLATION AND CHARACTERISATION OF α -LATROTOXIN

3.1 Overview

The majority of research carried out on LTX has focussed on measuring and/or observing its effects in different neuronal and non-neuronal systems. The results of these studies have provided plausible arguments for both of its proposed mechanisms of action (“channel” and “receptor-mediated” theories, as reviewed in Chapter 1). Although much information has been acquired about the function of LTX, little research effort has been devoted towards understanding how the toxin could open a pore/channel or how it could activate and interact with its receptors. To address these questions, one of the major aims of this thesis has been to investigate the biochemistry and three-dimensional (3-D) structure of LTX to see what clues this could reveal about the toxin’s mechanism of action.

Admittedly, previous biochemical and high-resolution structural studies on LTX were complicated by the unavailability of a truly pure α -latrotoxin preparation. Conventional methods of LTX isolation (e.g., HPLC or antibody column) produced a good preparation for functional studies but still contaminated with at least one other ~110 kDa protein [Frontali *et al.*, 1976] or sometimes small molecular weight latroectin [Grishin *et al.*, 1993]. Therefore, the starting point for this section of work has been the removal of these artifacts from the final LTX preparation.

In this chapter, a novel method for LTX purification is presented that produces perhaps the purest toxin isolated to date. This preparation is suitable for cryo-electron microscopy studies of LTX (Chapter 4). Moreover, biochemical analysis of the toxin

has revealed that it exists as a dimer (~ 260 kDa) in its native state. The discovery that LTX is able to oligomerise may have significant implications in its mode of action.

3.2 Results

3.2-1 Gel filtration and anion exchange chromatographies

After two chromatographic steps, the major peak of the anion exchange chromatography (IE3), eluting with 0.33 M NaCl, contained all of the LTX loaded from gel filtration (Figures 3.1 and 3.2). However, SDS-PAGE on an 8% gel followed by Coomassie staining revealed the presence of two proteins of similar molecular weight in this fraction: LTX (upper band) and δ -latroinsectotoxin (lower band), as identified by specific antibodies (see Figure 3.2b). If more dense gels were used (e.g., 10% polyacrylamide or higher), then the two bands merged into one (data not shown).

Attempts to use HPLC to further separate LTX and δ -LIT were limited in success. Although it was possible to obtain some fractions containing pure LTX and pure δ -LIT the yield was low, as the majority of these proteins always co-eluted together (data not shown). This is not surprising because the intrinsic charge of the two toxins is very similar over a wide pH range e.g., -29 and -26, respectively, for LTX and δ -LIT at pH 8.3 [calculated using the Protean database]. This makes separation based on ionic charge unsuitable.

Interestingly, it was discovered that the two toxins could be separated when fraction IE3 (from anion-exchange) was run on non-denaturing PAGE gels (Figure 3.2c). By using a variety of polyacrylamide gel concentrations it was determined that a 6% concentration, as shown in the figure, was optimal for the separation. More dense polyacrylamide gels required longer electrophoresis times for the same degree of

separation and too little polyacrylamide (below 6%) reduced the degree of resolution of proteins (results not shown).

3.2-2 Native preparative electrophoresis

The knowledge provided by these observations was used to create a novel third purification step – preparative electrophoresis (Figure 3.3a). Preparative electrophoresis is a technique that permits the separation of proteins based on their size by continuous elution from a polyacrylamide gel. The eluted proteins can be collected in fractions in a manner very much resembling a chromatographic step. A 6% non-denaturing gel was used, which permitted the complete isolation of LTX from the mixture of the two toxins, as confirmed by SDS-PAGE with Coomassie staining (Figure 3.3b). The time interval between the insect-specific toxin (the first to elute out) and the vertebrate toxin was about 3.5 hours when using a gel 3.5 cm in length.

The original method of preparative electrophoresis was performed using a much longer gel (8 cm). This was also effective at separating the proteins but, as expected, took much longer to finish (~ 15 hours from the start for the final LTX fractions to elute out). Progressively the length of the gel was shortened in order to obtain the two purified toxins more efficiently (data not shown). A 3.5 cm gel was found to be an ideal height for loading a sample of ~ 5 ml volume and the subsequent separation of proteins. Later it was found that the concentrating and desalting of anion exchange fraction IE3 could be omitted, as it did not affect LTX yields, but the presence of the salt increased the time taken for the sample to enter the gel. It may be possible to further reduce the height of the gel to obtain faster separation but the resolution may diminish.

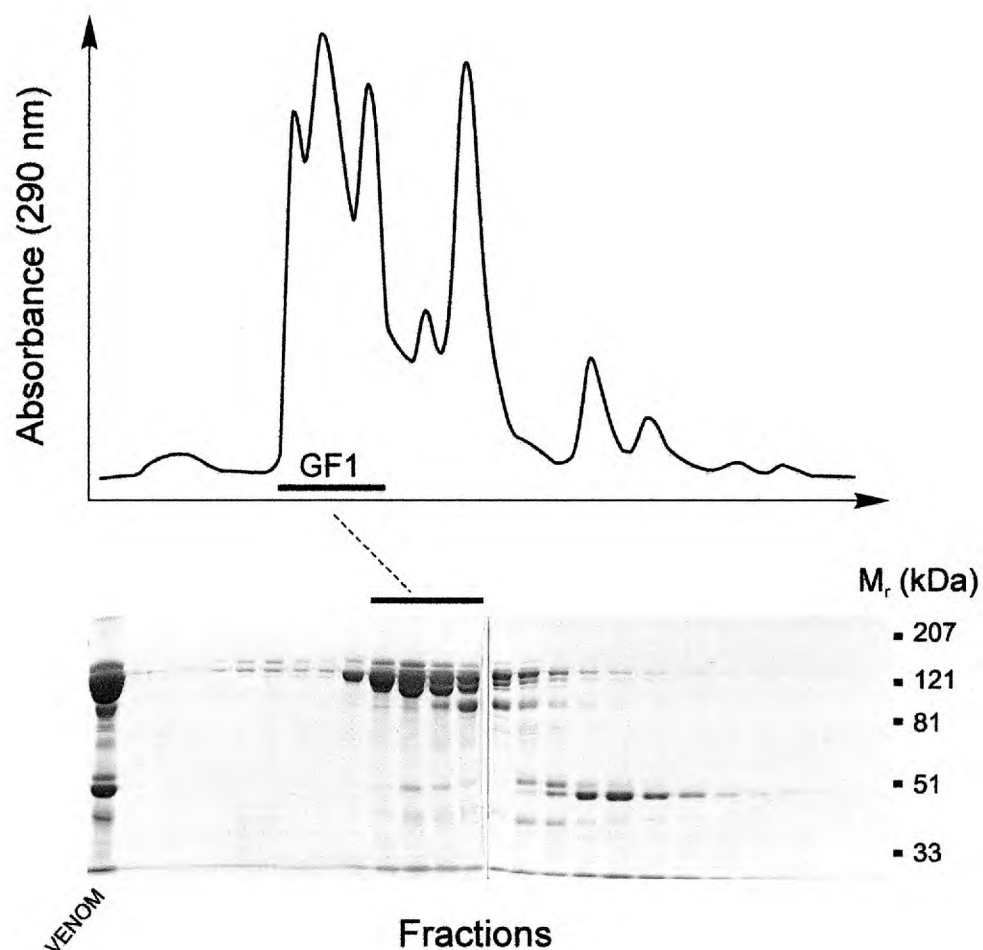


Figure 3.1 Gel filtration of black widow spider venom. *Top*, Elution profile. Lyophilised *Latrodectus mactans lugubris* venom was dissolved in 50 mM Tris-HCl pH 8.3, 0.2 M NaCl containing 20 mM EDTA and separated on a Showdex KW-803 column at 0.25 ml/min as described in Chapter 2. *Bottom*, SDS-PAGE analysis of fractions. Samples were run on an 8% SDS-gel and stained with Coomassie R250. The position of molecular weight markers is given on the right. Fractions collectively termed GF1 contained most of the LTX, as detected with specific antibodies to the toxin (see Figure 3.2b).

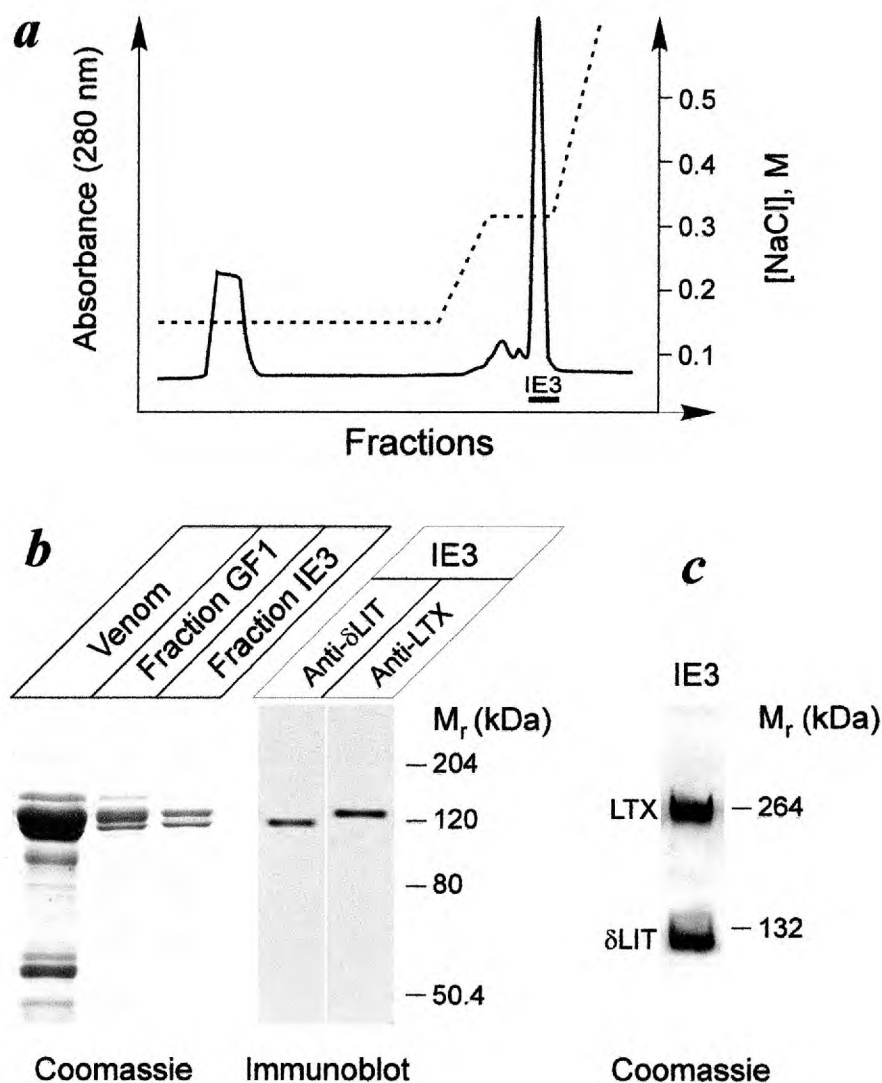


Figure 3.2 Anion exchange of fraction GF1. *a*, Profile of protein elution from loading fraction GF1 (containing LTX) onto a Millipore ProteinPak Q column (AP-1) and eluting by a gradient of [NaCl] (refer to Chapter 2). *b*, SDS-PAGE analysis of fractions from anion exchange. Samples were loaded on an 8% SDS gel, which was subsequently stained with Coomassie (as indicated). Alternatively, protein bands from fraction IE3 were transferred onto PVDF membrane and stained with antibodies specific to LTX or δ -LIT. Notice the differential staining. The position of molecular mass markers is shown on the right. *c*, Analysis of fraction IE3 by native PAGE in a 6% gel. Note the considerably faster migration of δ -LIT compared to LTX, a finding that led to the use of preparative electrophoresis as a new purification step. Oligomers of BSA were used as molecular mass markers.

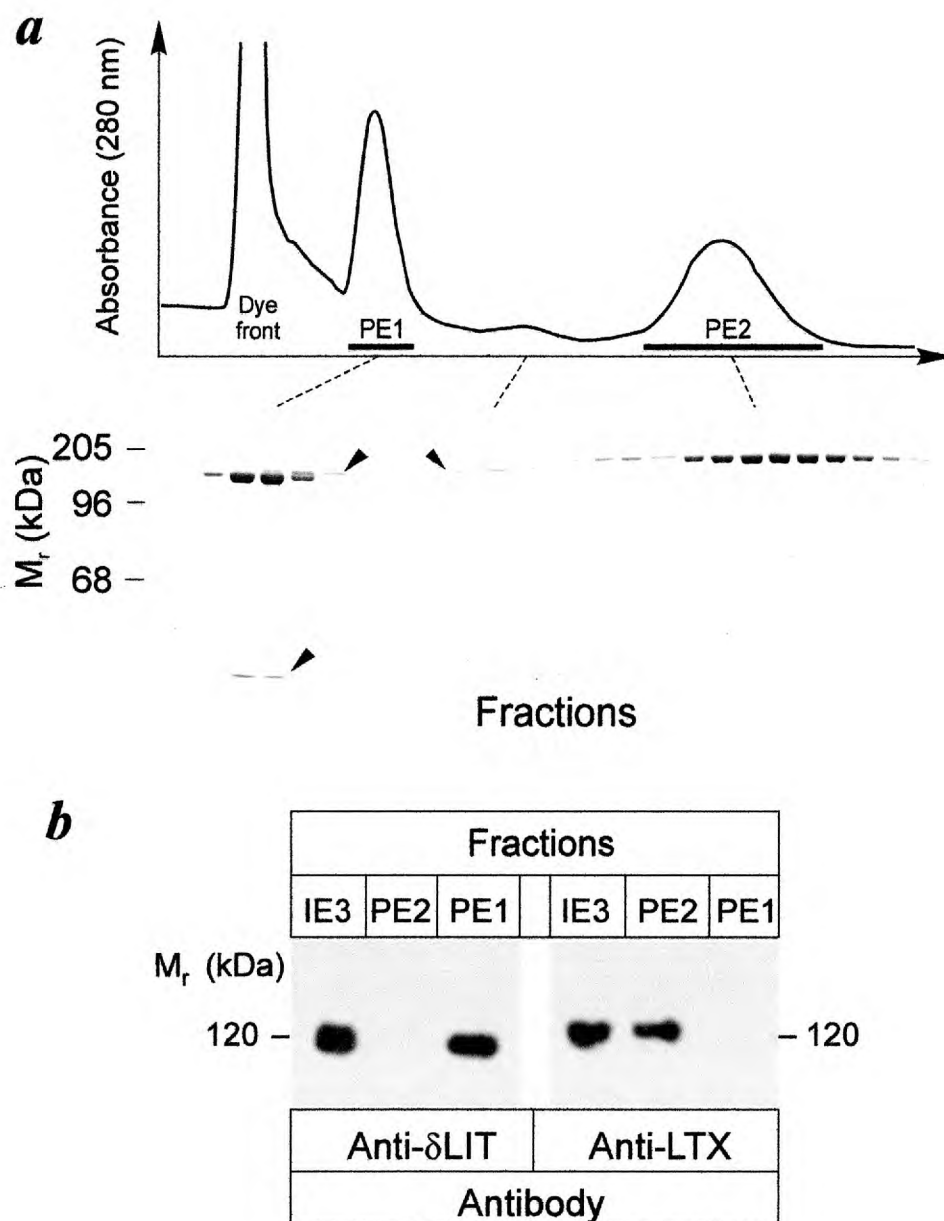


Figure 3.3 Preparative electrophoresis yields highly pure LTX and δ -LIT. Fraction IE3 from anion exchange chromatography was concentrated, desalted and run on a non-denaturing preparative 6% gel. **a**, Top, profile of protein elution from the preparative gel. Bottom, analysis of fractions by SDS-PAGE with Coomassie staining; molecular size marker positions are shown. Note the complete separation of δ -LIT (fraction PE1) from LTX (fraction PE2) and the removal of minor protein contaminants (arrowheads). **b**, Western blotting of pooled fractions PE1 and PE2 from preparative PAGE. Position of a molecular size marker is shown on both blots. Immunostaining confirms the presence of pure δ -LIT and LTX in fractions PE1 and PE2, respectively.

3.2-3 Purity of the preparation

SDS-PAGE analysis of preparative LTX (fraction PE2) shows a single band at 130 kDa with the total absence of any other proteins (Figure 3.3a) including the 7 kDa latroductin (LMWP) that has been found to co-purify with LTX in most preparations [Kiyatkin *et al.*, 1992; Gasparini *et al.*, 1994; Pescatori *et al.*, 1995]. LTX lacking latroductin retains its full activity as shown by multiple physiological experiments, as well as by the synthesis of recombinant LTXs absent of latroductin that are fully functional [Ichtchenko *et al.*, 1998; Volynski *et al.*, 1999a].

3.2-4 Properties of fraction PE2

LTX isolated by preparative PAGE is at least as functional as previously reported preparations. First, the ability of iodinated PE2 LTX to bind latrophilin in synaptosomes was examined. Experiments were performed in Mg^{2+} or EDTA to preclude the binding of the toxin to neurexin I α (Figure 3.4a). In order to more accurately determine the maximal binding (B_{max}) and the dissociation constant (K_d) of LTX to its receptor, the binding data was linearised into a Scatchard plot (Figure 3.4b). In such a graph, the ratio of bound to free toxin is plotted as a function of the bound toxin. If receptor sites are independent of each other and sufficiently diluted, this function is linear. Any co-operativity of binding (negative or positive) leads to deviations of the function from the straight line. In the case shown in Figure 3.4b, no co-operativity is observed. Therefore, the K_d may be directly determined from the slope of this line. For both conditions it appears to be ~ 0.45 nM.

Furthermore, the Scatchard plot allows one to determine the theoretical maximal concentration of binding sites (B_{max}). This is done by extrapolating the graph to the x-axis, i.e. to the condition where free toxin concentration vastly exceeds the

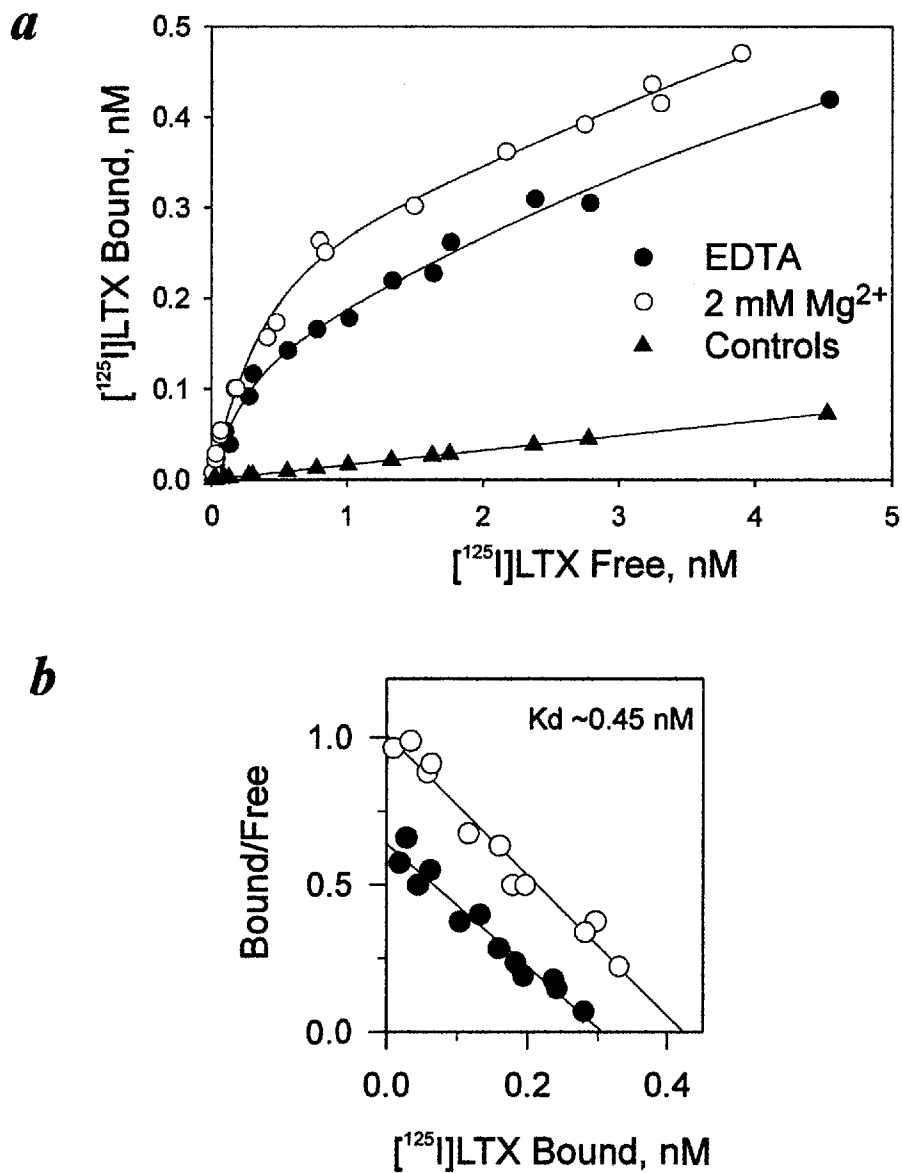


Figure 3.4 Preparative-prepared LTX binds to receptors. *a*, Binding of the toxin to synaptosomes measured under various conditions. Non-specific toxin binding was determined in the presence of 100-fold excess of unlabelled LTX (controls). ***b***, Scatchard plot of the data from *a*. Note that LTX has the same affinity to its receptor under both conditions ($K_d \sim 0.45$ nM), although the maximal binding is higher in the presence of Mg^{2+} .

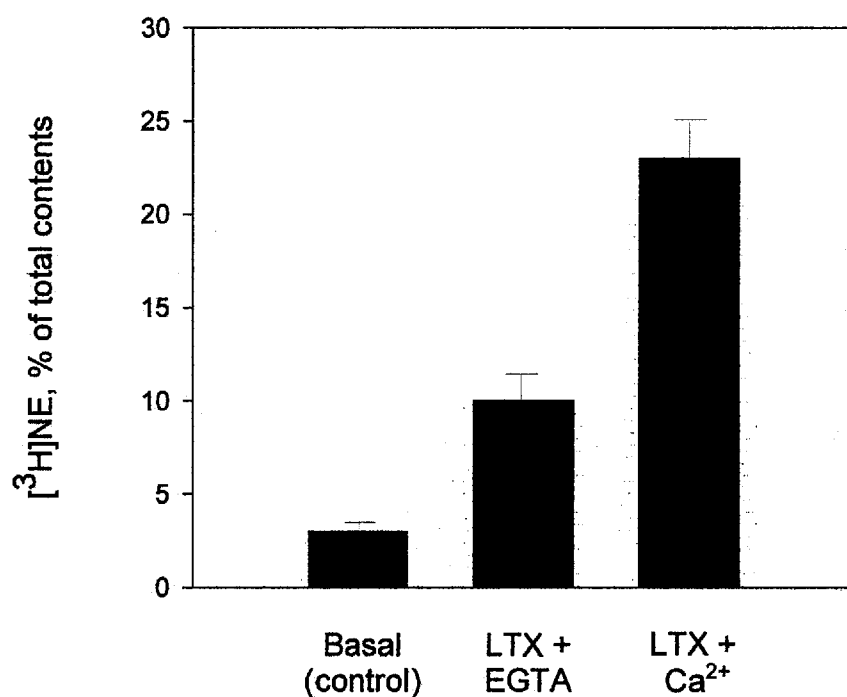


Figure 3.5 Preparative-prepared LTX induces norepinephrine (NE) release. Rat cerebral cortical synaptosomes were loaded with [³H]NE as described in Chapter 2. Samples were stimulated for exactly 10 min with LTX in the presence of 1.2 mM Ca²⁺ or 0.1 mM EGTA. Respective controls were treated the same but without the addition of LTX. As the two controls produced similar results they are grouped into a single bar for basal release.

bound toxin concentration and, therefore, where no more binding can occur. The graph clearly shows that the maximal binding of radiolabelled toxin to latrophilin is higher in Mg^{2+} than in EDTA. Second, the PE2 fraction stimulates the release of norepinephrine from synaptosomes in the presence or absence of extracellular Ca^{2+} as shown in Figure 3.5. In addition, PE2 was found to be toxic to mice when injected into their blood stream. A dose of 70 μg caused death by asphyxiation within 10-15 minutes. A similar dose of PE1, in contrast, was not fatal to mice (personal observations).

3.2-5 LTX channels in lipid bilayers

The ability of LTX to form channels in pure lipid bilayers was next investigated (Figure 3.6). The toxin within the crude spider venom and in the various steps of purification was tested. Venom, equivalent to 10 pM pure LTX, induced channels with currents of about 20 pA within 2 min. The channels were permeable to both Ca^{2+} and K^+ , and had conductances ranging from 60 pS to 200 pS, suggesting that the venom contained several different channel-forming components. Fractions GF1 and IE3 also induced channels with currents of ~ 30 pA although only 1-2 channels were observed despite prolonged exposure. However, when a 1000-fold excess of preparative LTX was used (10 nM), toxin channels were not seen to form even after several hours, either when added to pre-formed bilayers or to membranes that were broken and reformed in the continued presence of LTX (Figure 3.6). These findings suggest that many pore-forming constituents of the venom are gradually removed from LTX by the purification procedure, resulting in a pure toxin that is unable to form pores in pure lipid membranes even at concentrations of 10 nM.

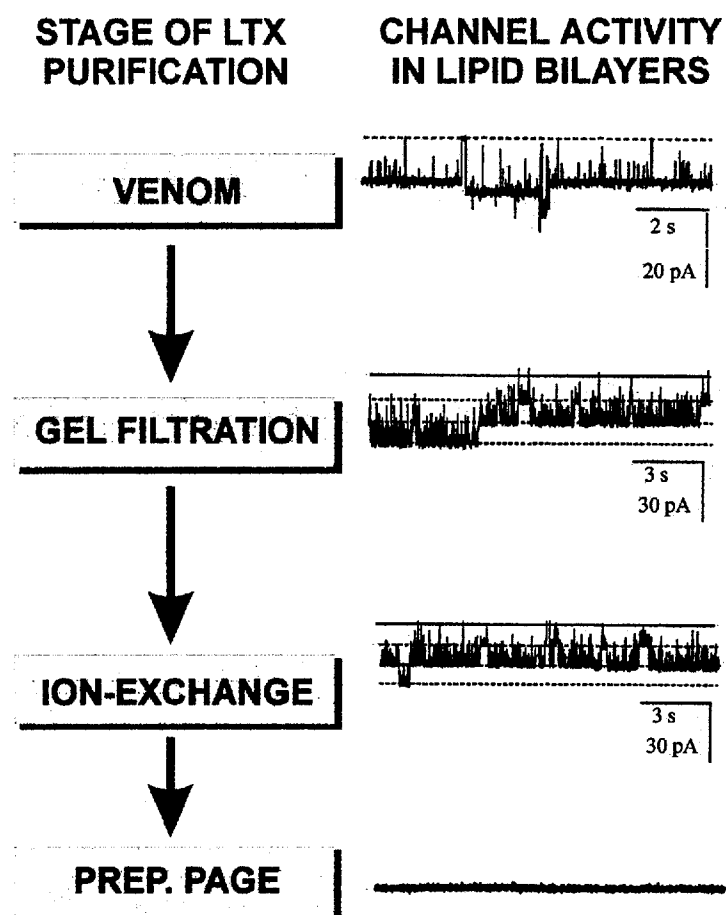


Figure 3.6 Channel-forming properties of different LTX-containing fractions. The ability of pure LTX, crude spider venom, and fractions GF1 (from gel filtration) and IE3 (from anion exchange) to form channels in pure lipid bilayers was tested as described in the Materials and Methods. Notice how the venom, GF1 and IE3 give rise to channels with similar range but preparative-purified LTX does not produce any channel activity (refer to text).

3.2-6 Molecular weight characterisation of LTX

It is remarkable that the procedure developed to purify LTX exploits two physical properties of proteins: size exclusion (gel filtration chromatography), charge separation (anion exchange chromatography) and size exclusion again (native preparative electrophoresis). LTX has always been believed to be a protein of about 110-130 kDa based on estimations by SDS-PAGE [see review by Rosenthal & Meldolesi, 1989], yet its complete separation from δ -LIT by preparative electrophoresis suggested that its molecular weight was larger than that of the insect toxin. Initial estimates of LTX's size were compared against bovine serum albumin (BSA) oligomers (a monomer is ~ 66 kDa) on a native gel (Figure 3.2c). LTX had the same mass as BSA tetramers (~ 264 kDa). This suggested that LTX was a dimer of two 130 kDa subunits, a hypothesis proposed by Ushkarev and Grishin [1986]. Determining the molecular weight of native LTX was crucial to our understanding of the toxin's mechanism of action as its oligomerisation into a dimer might have a functional relevance. Therefore, the next set of experiments was designed to conclusively and accurately determine the molecular weight of LTX.

The first technique used to estimate the molecular mass of purified LTX was gel filtration using a Superose 6 column (Pharmacia) connected to a BioRad Biologic system allowing the automated calculation of the data obtained. Commercial protein standards (Sigma) were run against LTX (Figure 3.7). The retention time of LTX on this column was consistent with its mass being about 260 kDa, i.e. dimeric. The experiment was repeated with other gel filtration columns such as the Showdex KW-803 with reproducible results (data not shown).

To verify this molecular weight, dynamic light scattering (DLS) was used, a method independent of molecule's charge and interaction with chromatographic media (Figure 3.8). This measures the time scale of intensity fluctuation of the light scattered by irradiated molecules and, under the assumption of Brownian motion of a molecule, determines a hydrodynamic radius, R_H . From R_H , the Dynamics™ software can calculate the molecular weight automatically. Fifteen samples from five different preparations of LTX were tested and the average hydrodynamic radius obtained was ~ 65.5 Å, which corresponded to a molecular mass of 255 kDa. The internal calibration of the machine was also checked using the same protein standards used in gel filtration (Figure 3.8). Therefore, two independent techniques concluded that LTX is not 130 kDa in size, as widely believed, but is a dimer of 2 x 130 kDa subunits.

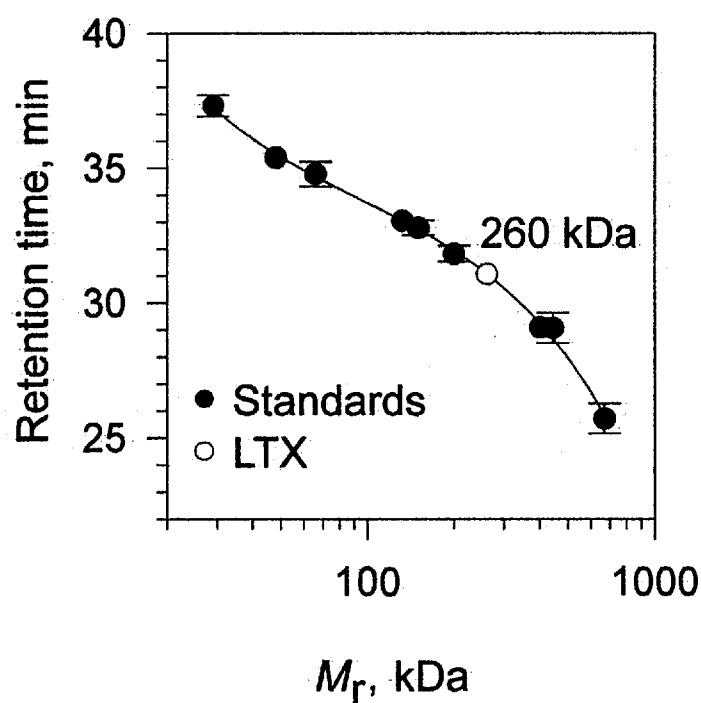


Figure 3.7 Estimation of the molecular mass of native α -latrotoxin. Gel filtration of LTX was performed on a Superose 6 column eluted with 50 mM Tris-HCl, pH 8.3, at 0.4 ml/min. Peak retention times were plotted against known M_r values for standard proteins (see Materials and Methods) and revealed the native toxin to be a dimer.

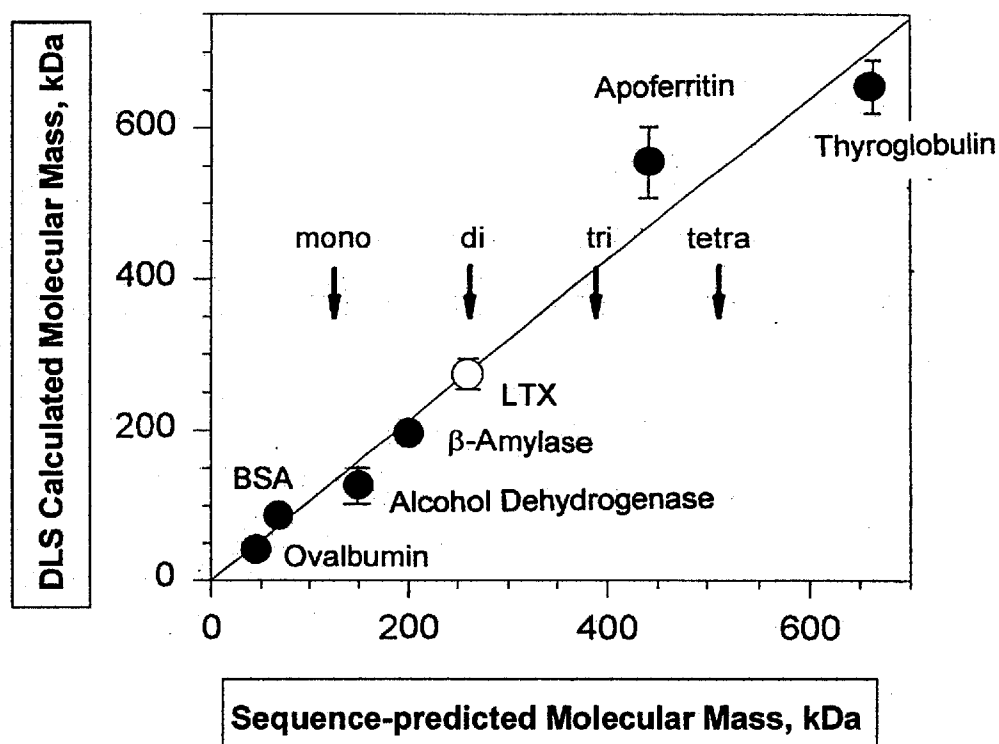


Figure 3.8 The molecular mass of LTX estimated by dynamic light scattering (DLS). 15 samples of α -latrotoxin (1 mg/ml) from 5 separate preparations were injected into a DynaPro-801 instrument, and their molecular weight automatically calculated by the DynamicsTM software to be ~ 265 kDa. To check the internal calibration of the machine, the protein standards used to calibrate the Superose column (Figure 3.7) were also injected at the same concentration. Their mass, as calculated by DLS (vertical axis) was compared to their known molecular weight (horizontal axis). Notice the good correlation of determined and known molecular weights, validating the accuracy of this technique. Note that when the calculated molecular weight of LTX is plotted on the calibration curve it corresponds to the dimer value (255 kDa) on the predicted molecular mass axis.

3.2-7 The stability of LTX dimers

Low concentrations (0.2% w/v) of non-ionic detergents such as Triton X-100 or zwitterionic detergents such as CHAPS as well as 8 M urea were unable to break LTX dimers into monomers on native gels demonstrating their relative stability (gels not shown). However, preparative LTX always appeared monomeric in SDS-denaturing electrophoresis. This suggested that LTX dimers could be disrupted by sodium dodecyl sulphate, an anionic detergent, and by the conditions employed during SDS-PAGE. To test this hypothesis, purified LTX was treated with 0.1 or 1% SDS together with 5% β -mercaptoethanol (β ME), boiled briefly and then run on a non-denaturing 4-12% gradient gel. The denatured LTX samples showed the presence of LTX monomers that travelled like BSA dimers (~ 132 kDa) in addition to LTX dimers formed by the re-association of monomers when SDS and β ME were being removed during electrophoresis (Figure 3.9). The toxin also appeared to be aggregating into trimers and tetramers as the two reagents were being removed indicating that LTX is capable of further oligomerisation.

3.2-8 Cross-linking experiments

In a further experiment, iodinated LTX was subjected to treatment with Bis[Sulfosuccinimidyl]suberate (BS³), a homobifunctional cross-linking reagent that links together ϵ -lysines of active amino groups. After incubation, the toxin sample was denatured using SDS-gel loading buffer and run on a Novex 4-12% gradient gel under SDS-PAGE conditions (Figure 3.10b). Although cross-linking is never 100% efficient, exposure of the dried gel onto film revealed the presence of a large number of dimers demonstrating that the monomers within the LTX dimer are close enough to each other to be irreversibly linked together.

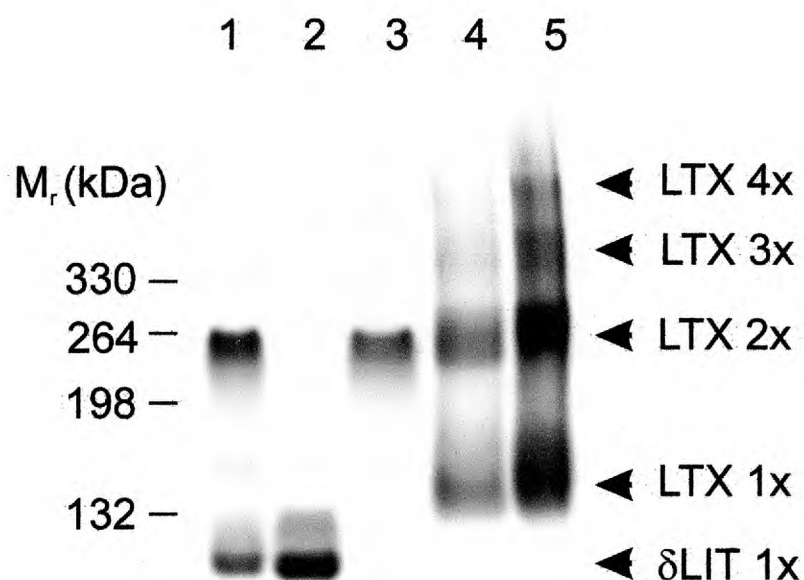


Figure 3.9 Pure LTX runs as a dimer on native PAGE. Purified LTX was run in a native 4-12% polyacrylamide gel subsequently stained with Coomassie. δ -Latroinsectotoxin (δ -LIT) was used as an internal reference because it has a unitary M_r (110 kDa) and charge similar to those of LTX but does not form dimers under the conditions employed. Lanes: (1), mixture of δ -LIT and LTX; (2) pure δ -LIT; (3) pure LTX; (4) pure LTX denatured with 5% β -mercaptoethanol and 1% SDS; (5) the same with 0.2% SDS. Molecular size markers (BSA oligomers) are shown on the left.

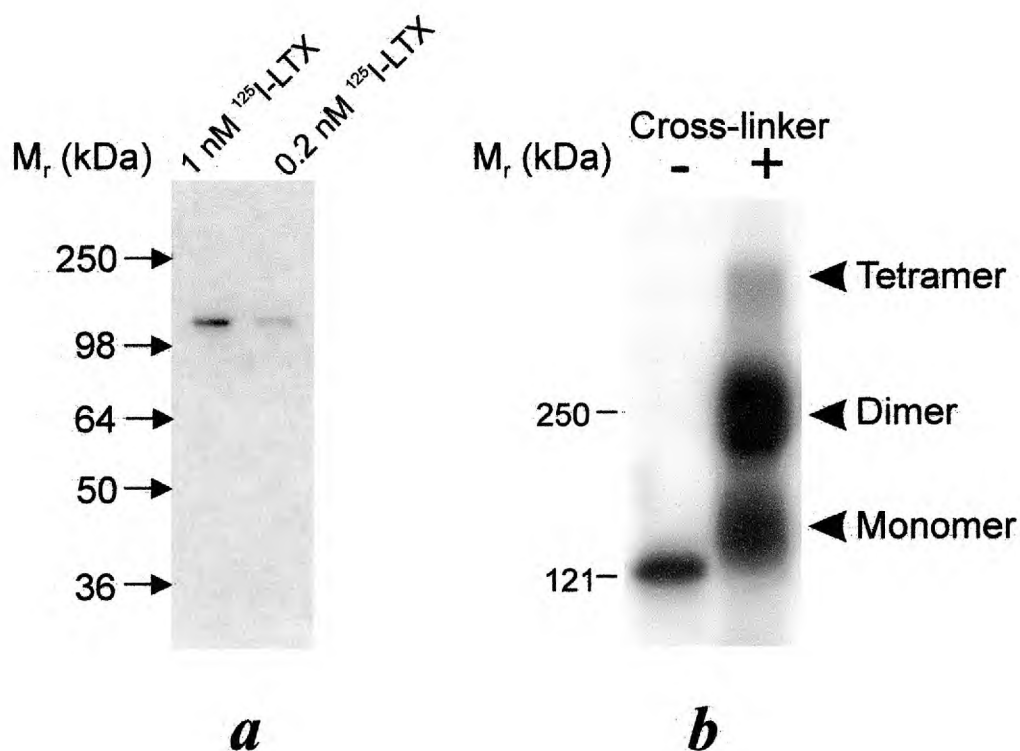


Figure 3.10 Cross-linking of preparative LTX shows dimers and tetramers. *a*, Preparative LTX iodinated by the chloramine-T method (refer to text) was run on SDS-PAGE. The gel was dried between two cellophane sheets and exposed to film. Note that there are no LTX degradation products. *b*, 1 nM of this iodinated toxin was subsequently incubated in the presence or absence of a crosslinker (BS³) as described in Chapter 2, then separated in a gradient 4-12% polyacrylamide gel containing SDS and exposed to film. Under these denaturing conditions a large amount of LTX dimers was visible. Notice the higher molecular weight band, which is in the position of LTX tetramers.

3.3 Discussion

The original aim of this work was to produce a pure LTX preparation for functional and structural studies, and at great yields. One possibility was to prepare an affinity column containing anti-LTX antibody and purify LTX by this method. This technique is used by some researchers [e.g., Pashkov *et al.*, 1993] but is, however, limiting due to the low capacity of the antibody column, i.e. the LTX-containing fraction must be reloaded several times to exhaust it. In addition, bound toxin must be eluted with 4 M MgCl_2 and then dialysed into a suitable buffer. Not only does this make the procedure almost as time-consuming as conventional HPLC but it is not unusual for a little of the antibody to co-elute from the column and thus, contaminate the final preparation. For high-resolution structural studies such eventualities are undesirable.

It was observed that in native conditions e.g., native-PAGE, gel permeation chromatography and DLS, α -latrotoxin behaves as a dimer. These dimers are relatively stable hence this property of LTX was exploited to separate the vertebrate-specific toxin from δ -latroinsectotoxin (which is not a dimer under the conditions used) by native preparative electrophoresis.

The most important criteria for any modification of the conventional LTX purification procedure is whether the new step will (a) improve the purity of LTX and (b) retain the high yields seen after anion-exchange chromatography. It has been found that this three-step procedure routinely yields about 1 mg of both LTX and δ -LIT from

100 μ l of lyophilised *Latrodectus mactans lugubris* venom with virtually no loss of protein between the second and third phases. Preparative electrophoresis also removes at least three other impurities from the final LTX preparation as indicated by the arrows in Figure 3.3. These impurities do not stain with antibodies to either δ -LIT or LTX and have not been characterised yet.

The oligomerisation of LTX may have a functional significance. The toxin is widely believed to form a pore or open a pore in neuronal and neuroendocrine cells (reviewed in Chapter 1). It has been difficult to envisage how (after recruitment by specific receptors) LTX, which is soluble and possesses a limited number of hydrophobic residues, can interact with plasma membranes in such a way as to carry out this effect. The ability of LTX to oligomerise may result in increased hydrophobicity that would permit better interactions between the toxin and the membrane. Although LTX is dimeric, it is conceivable that the toxin can oligomerise further as a small amount of tetramer was seen, in addition to dimers, during the cross-linking procedure with BS³ (Figure 3.10b).

Interestingly, at physiological concentrations, pure LTX cannot form channels in lipid bilayers (Figure 3.6). The literature concerning LTX channel formation has been somewhat confusing (refer to section 1.13 of Chapter 1). On one hand, picomolar concentrations of toxin have been shown to open Ca²⁺-permeable channels in artificial membranes for example, the experiments of Finkelstein *et al.*, 1976. However, other published work reveals that LTX can only open such channels in cell systems or artificial membranes expressing LTX receptors e.g., Scheer *et al.*, 1986. Equally as puzzling has been the observation of a variety of different channel conductances in the various systems tested.

It is possible that previous observations of LTX pores in artificial lipid membranes may be attributed to residual contamination by other pore-forming venom components. The large variety of conductances obtained with the crude venom and IE3 fraction in our experiments supports this idea. When such contaminants are removed after preparative electrophoresis the ultra pure toxin can no longer form channels even when concentrations of ~ 10 nM are used.

In fact further studies revealed that LTX could form channels in lipid bilayers devoid of LTX receptors only if extremely high concentrations of the pure toxin (equivalent to 100 nM) were used [Volynski *et al.*, 2000]. This is consistent with the data to be presented in Chapter 4, which will show that such concentrations of LTX can form pores in pure liposomes as a result of the toxin's tetramerisation. LTX receptors can facilitate high local concentrations of the toxin, thereby promoting oligomerisation and consequently, membrane insertion, which may explain why channels are only seen in receptor-expressing cells. Unfortunately, previous works did not compare the same toxin preparation on pure lipid bilayers and receptor-deficient cells as well as on bilayers and cells with receptors present. Such studies can now be performed reliably in the future using the extremely pure LTX isolated by the novel three-step purification procedure.

3.4 Summary



A novel three-step method for isolating large yields of highly pure LTX and δ -LIT has been developed.



The new purification procedure removes other contaminants from the final LTX and δ -LIT preparations.



Preparative LTX is fully functional.



Native LTX is an oligomer of 2 x 130 kDa subunits and is capable of further oligomerisation.



The LTX dimer is stable but can be reversibly disrupted by SDS and β -mercaptoethanol.

CHAPTER FOUR



FREE-DIMENSIONAL STRUCTURAL STUDIES OF α -LATROTOXIN

4.1 Overview

The finding described in the last chapter that LTX is capable of oligomerisation presents a plausible explanation for how the toxin could form channels. To explore this possibility, this section of my thesis is devoted to the study of the three-dimensional (3-D) structure of LTX. This was feasible due to the availability of the protein in a highly purified form after preparative electrophoresis (Chapter 3). Cryo-electron microscopy (cryo-EM) and angular reconstitution were used to obtain the 3-D reconstructions of the toxin. A recently developed method of vitrified sample preparation made it possible to observe molecules in their native environment; therefore, LTX was studied in the presence or absence of divalent cations, which are necessary for its biological activity [Misler & Hurlbut, 1979]. Recently, the 3-D structure of another toxin, pneumolysin, has been solved using a similar approach [Gilbert *et al.*, 1999].

The structural work presented here was a joint project between our laboratory (Ushkaryov Group) and the Structural Biology Group at Imperial College. I was involved in the preparation of samples for microscopy and preliminary processing of micrographs. Dr. E. Orlova performed the statistical and structural analysis. Therefore, the chapter is focused on the biochemical interpretation of the determined structure and its possible mode of action.

It is shown, for the first time, that LTX exists in two oligomeric forms: in EDTA it is largely dimeric but in the presence of divalent cations these homodimers

can assemble into tetramers. The tetramer has dimensions of 100 x 180 x 180 Å, with a hole of ~ 10 Å in diameter at its centre, large enough to allow divalent cations or small molecules to pass through. Its base is hydrophobic, which allows the tetramer to insert into lipid bilayers completely permeabilising them. Thus, LTX belongs to the group of pore-forming toxins.

4.2 Results

4.2-1 Image analysis and characteristic views of LTX oligomers

Images of LTX in the presence or absence of divalent cations in vitreous ice were taken by Brent Gowen in a cryo-microscope as described in Materials and Methods. Toxin molecules observed in micrographs had different appearances. Some of them had a square shape formed by twelve maximums of electron density. Other views did not show any symmetry and resembled one-half of the square-shaped molecule. Multivariate statistical analysis of these images revealed several characteristic views of the toxin (Figure 4.1). The square-shaped molecules had a 4-fold (C_4) symmetry and represented LTX tetramers. They were the predominant form of LTX seen in micrographs, in particular the bottom view (Figure 4.1, b1). Classes appearing like “clenched-fists” (e.g., Figure 4.1, b5) represented tetramer side views. Other characteristic views that resembled halves of the square-shapes were asymmetric and represented LTX dimers (Figure 4.1, d1-6).

4.2-2 The 3-D reconstruction of the tetramer

The 3-D distribution of the tetramer densities was reconstructed using 128 class averages. The structure was determined with a 14 Å resolution assessed by Fourier Shell Correlation (FSC). The 3-D map (Figure 4.2) shows that the tetramer resembles a 4-bladed propeller. The toxin has a side of 180 Å, a height of 100 Å and a volume of $\sim 608,000 \text{ Å}^3$. Based on average protein density (0.84 Da/Å^3) this corresponds to a molecular mass of $\sim 520 \text{ kDa}$, which is consistent with the presence of four 130 kDa LTX monomers, confirming the structure to be a tetramer. The lower portion of the

propeller-like structure is approximately 155 x 155 Å (Figure 4.2b). The most significant feature of the tetramer is an opening that pierces its centre. This channel has an average diameter of 25 Å but is constricted at the top to 10 Å and widens at the bottom into a 36 Å diameter vestibule. Holes of up to 13 x 13 Å appear in the channel walls.

Within the tetramer structure three characteristic domains belonging to each LTX monomer can be distinguished: wing, body and head (Figure 4.2, see also Figure 4.5). The bodies are “L-shaped” and form the backbone of the tetramer, while the heads come together to form the central channel. The wings extend horizontally from the bulk of the structure when viewed from the side (Figure 4.2c).

4.2-3 The 3-D reconstruction of the dimer

The addition of detergent (0.2% CHAPS) to the toxin before cryo-freezing was found to completely disrupt tetramers and facilitated dimer formation. The 3-D map of the dimer was calculated using 120 class averages (~ 2000 molecular images) without imposing symmetry to achieve 18 Å resolution as determined by FSC. The dimer resembles half the tetramer (Figure 4.3) and the wing, body and heads of its two monomeric units are easily recognisable. The relative orientation of monomers in the dimer is slightly different to that in the tetramer (refer to Figure 4.3), suggesting that conformational changes occur during the transition of one of two LTX dimers into the tetramer.

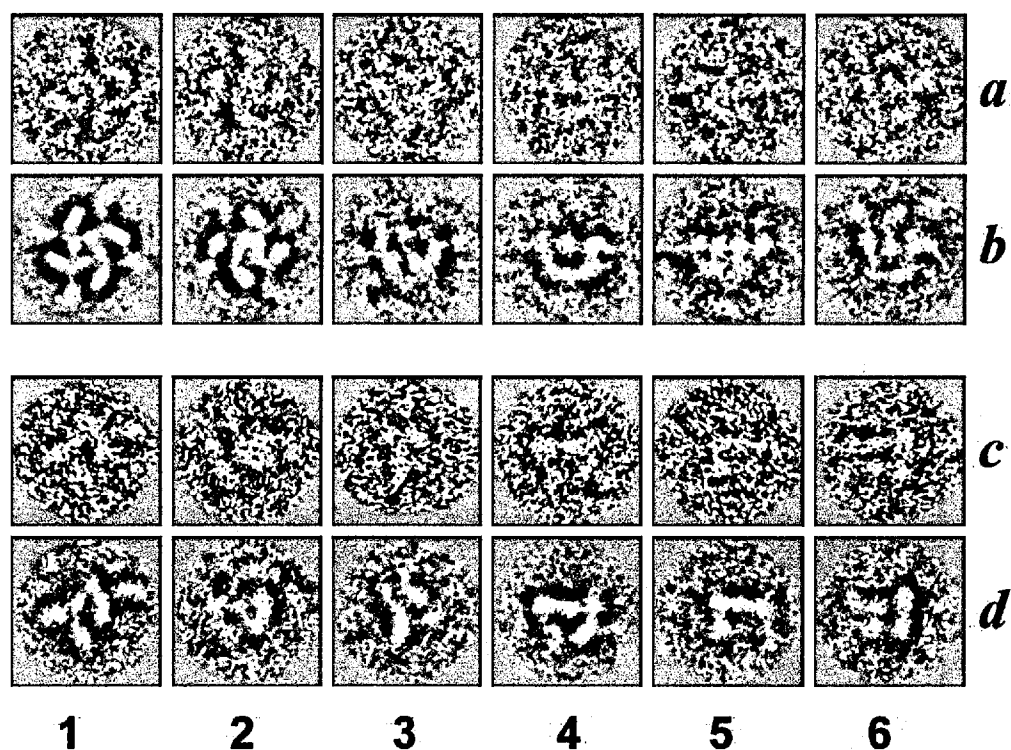


Figure 4.1 Raw images and characteristic views of LTX oligomers. From 60 digitised micrographs a set of ~ 7000 images of LTX oligomers was extracted and subjected to multivariate statistical analysis. *a*, Representative raw images and *b*, corresponding characteristic views of LTX tetramers. The bottom view of the tetramer (b1) was predominant, while both the top view (b2) and the side views (b3-b6) were much rarer. *c*, Raw images and *d*, characteristic views of LTX dimers.

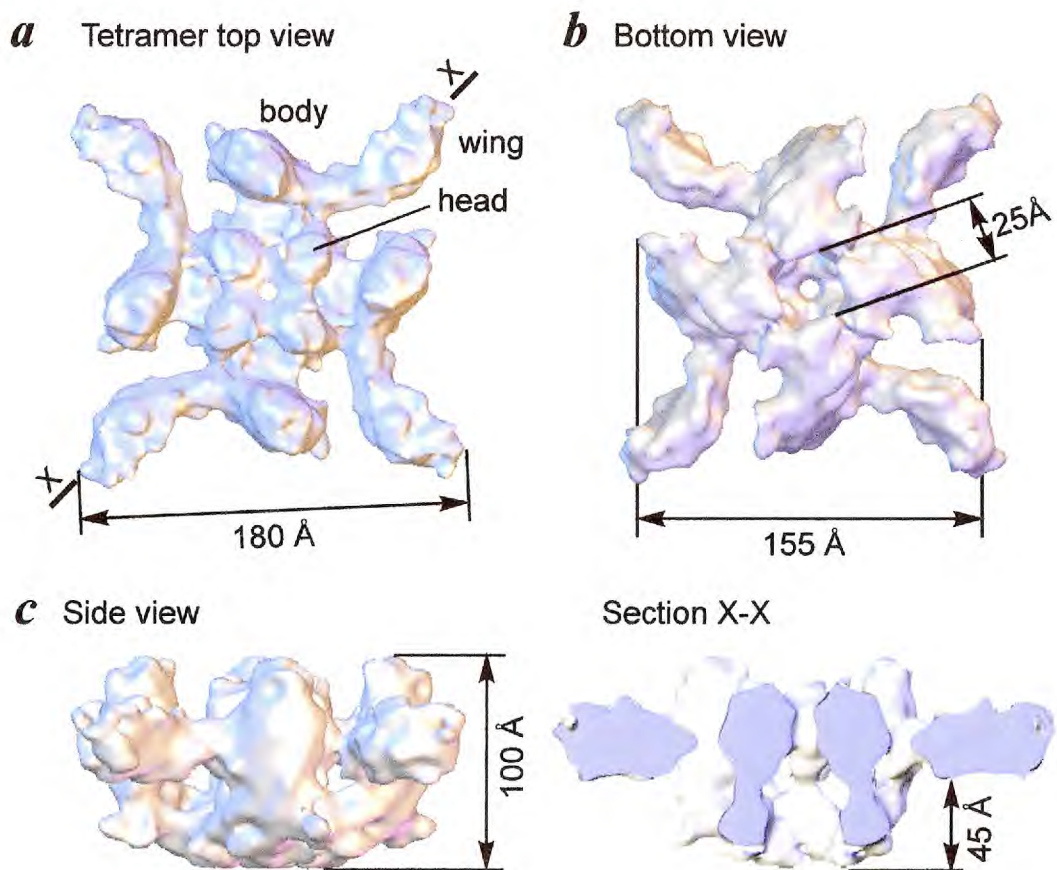


Figure 4.2 The 3-D reconstruction of the LTX tetramer. *a*, The top and *b*, bottom views of the tetramer. *c*, The side and cut-open views of the tetramer. The final structure was calculated from 128 class averages that included ~1900 original molecular images. Notice the distinct “propeller-like” shape of the tetramer when seen from above or below and the central hole of ~25 Å in diameter. Note also how the “wings” extend from the main bulk of the structure (refer to Figure 4.4 for nomenclature), which may provide sites for interaction with LTX receptors (see text).

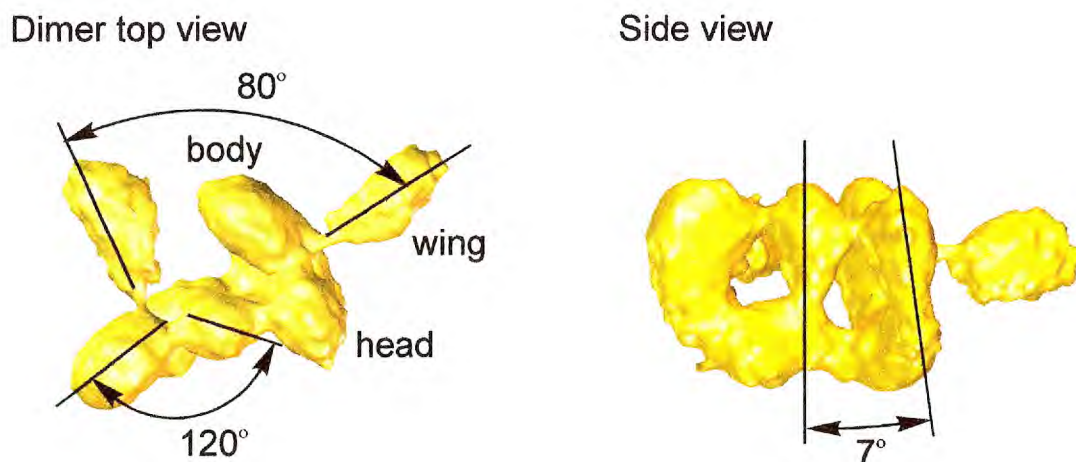


Figure 4.3 The 3-D reconstruction of the LTX dimer. The image of the LTX dimer was reconstructed from 120 characteristic views (~ 2000 molecular images). The top and side views are shown. The “wings”, “bodies” and “heads” (refer to text) are easily distinguishable although the dimer is asymmetric unlike the tetramer. The monomers in the dimer lean towards each other by 7° (right) while they are parallel and vertical in the tetramer. In addition, the in-plane body-head angles are wider in the dimer (120°) than in the tetramer (100°). Together, these suggest that substantial conformational changes occur when dimers assemble into tetramers.

4.2-4 The LTX monomer and localisation of the N-terminus

Images of the LTX monomer were computationally extracted from the LTX dimer and tetramer, and all of its possible projections calculated. The monomer consists of the three domains described above (Figure 4.4a). Volumetric analysis reveals that of the ~130 kDa that makes up the LTX monomer, the wing accounts for ~ 36 kDa, the body ~ 76 kDa and the head ~ 18 kDa.

In order to locate the N-terminus of the toxin an antibody (Ab) prepared to an 18 kDa fragment of LTX's N-terminus (see Materials and Methods) was used. In raw images of the Ab-LTX complexes (in the presence of Mg^{2+}), two or three antibody molecules were always seen attached to the tetramer's wing-tips (Figure 4.5). This represented the Ab recognising different epitopes on the N-terminus. As the antibody was never observed to bind to any other domain of LTX in 600 raw images, it can be concluded that the wing contains the toxin's N-terminus.

The domains of the LTX monomer are linked in the order wing-body-head, separated by two narrow junctions. As the wing contains the N-terminus of the toxin, one can expect that the head possesses its C-terminus. In support of this hypothesis, the LTX head shows primary and 3-D structural homology with the C-terminus of other short ankyrin-like proteins e.g., GABP β [see within Orlova *et al.*, 2000]. This permits the putative alignment of the domain structure of the LTX monomer with the toxin's primary structure (Figure 4.4b), which predicts that the head domain consists of 5 ankyrin-like repeats (ALRs) and the body domain is mainly composed of LTX's other ALRs.

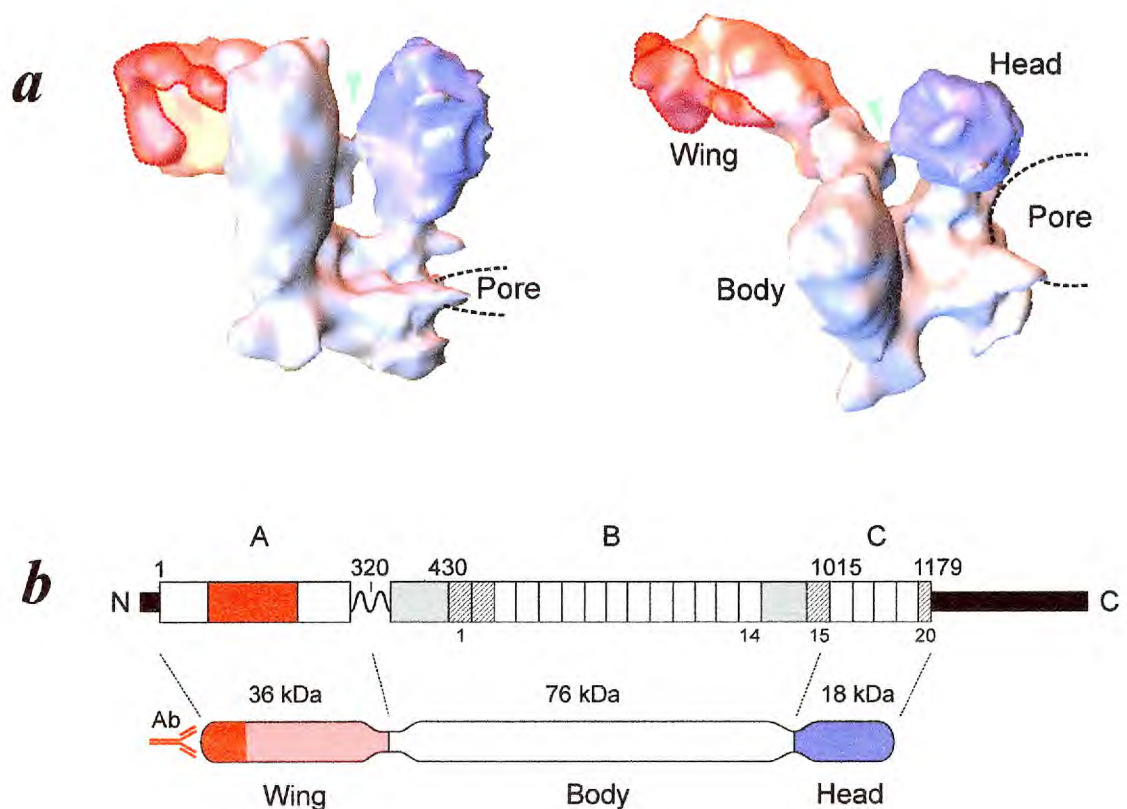


Figure 4.4 The domain structure of the LTX monomer. *a*, Two views of the LTX monomer illustrating the three structural domains of the molecule: wing (pink), body (silver), and head (blue). The position of the tetramer's central pore is shown by the dashed line; binding sites for the N-terminal antibody are shown in dark red. The green arrowheads point at the connection between the head and the body, possibly a disulphide bond *b*, The sequence of LTX (top) is compared to a linear representation of the domain structure of LTX (below). The numbers above the sequence show amino acid positions of region boundaries. Narrow boxes numbered 1-20 correspond to ankyrin repeats (imperfect or incomplete repeats are hatched); grey, sequence regions lacking homology to other proteins; wavy line, coiled coil region; red, the fragment used for N-terminal antibody isolation; thick black lines, fragments proteolytically cleaved during LTX maturation (see General Introduction).

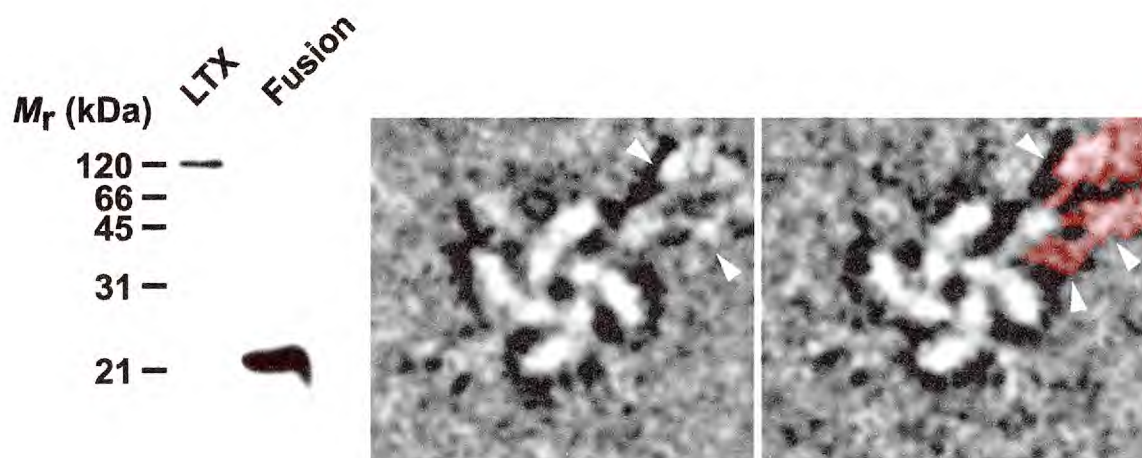


Figure 4.5 Localisation of LTX's N-terminus in the tetramer. *Left*, The affinity-purified antibody (Ab) to LTX's N-terminus recognises native LTX and the 18 kDa fragment (containing a 6 His tag) used for its isolation from rabbit serum (see also Figure 4.4). *Right*, Complexes of LTX with the N-terminal antibody were studied by cryo-EM in the presence of Mg^{2+} . Approximately 600 raw images were collected from four micrographs and ~ 350 best bottom views were selected for analysis. Averaged positions of Ab molecules associated with LTX tetramers are indicated by arrowheads (left) and by the pink coloration (right) on the resulting averaged images (each consisting of ~ 90 original images).

4.2-5 Analysis of LTX in liposomes

The structure of the LTX tetramer suggested that it could make a channel. The next question asked was whether this tetramer could interact with biological membranes. As a preliminary experiment, cryo-EM was used to examine the interaction of LTX tetramers with liposomes (Figures 4.6 and 4.7), as performed recently for the pore-forming toxin, pneumolysin [Gilbert *et al.*, 1999]. The interaction of LTX with liposomes devoid of LTX receptors was much slower than with cells expressing neurexin or latrophilin [Volynski *et al.*, 2000, paper in press] and required high toxin concentrations (~100 nM).

To verify that LTX was actually incorporated into the membrane rather than just adsorbed onto the liposome's surface, only tangential views were taken for image analysis (Figure 4.6b). Due to the vast difference in contrast between the lipid membrane and toxin particles, the LTX side views could only be aligned but reconstruction could not be performed. Cryo-EM of 22 averaged side views revealed that the toxin tetramer inserted into the membrane with its lower half, termed base (Figure 4.7a). This averaged characteristic view was compared to the side view of the tetramer rotated around its C4 axis (Figure 4.7b) and the rotated tetramer overlaid with an image of the lipid bilayer (Figure 4.7c). The characteristic view and projected images of the tetramer were almost identical, which suggested that the central pore could completely permeabilise the lipid bilayer.

4.2-6 Role of divalent cations in oligomerisation

Of the two oligomeric forms of LTX seen in electron micrographs only the tetramer possessed the features of a channel. This implied that the LTX tetramer represented the active state of the toxin. LTX usually requires the presence of divalent

cations to show biological activity (Chapter 1); therefore, the effect of such cations on the LTX oligomerisation state was examined. Cryo-EM was performed on LTX treated with EDTA or divalent cations before cryo-freezing (Figure 4.8). In EDTA, 90% of all viewed images in the micrographs were of the LTX dimer. The presence of divalent cations changed the majority of the molecules (95%) into LTX tetramers. Thus, divalent cations facilitate tetramer formation.

To further study the role of divalent cations in LTX oligomerisation, the affinity of LTX dimers to each other was investigated. Native (dimeric) LTX was iodinated as described in Materials and Methods. This was incubated with native LTX immobilized on CNBr-activated Sepharose in the presence of EDTA or divalent cations. The affinity of the dimers increased 50-fold in the presence of the divalent cations compared to EDTA (Figure 4.9). This supported the theory that divalent cations promote LTX oligomerisation.

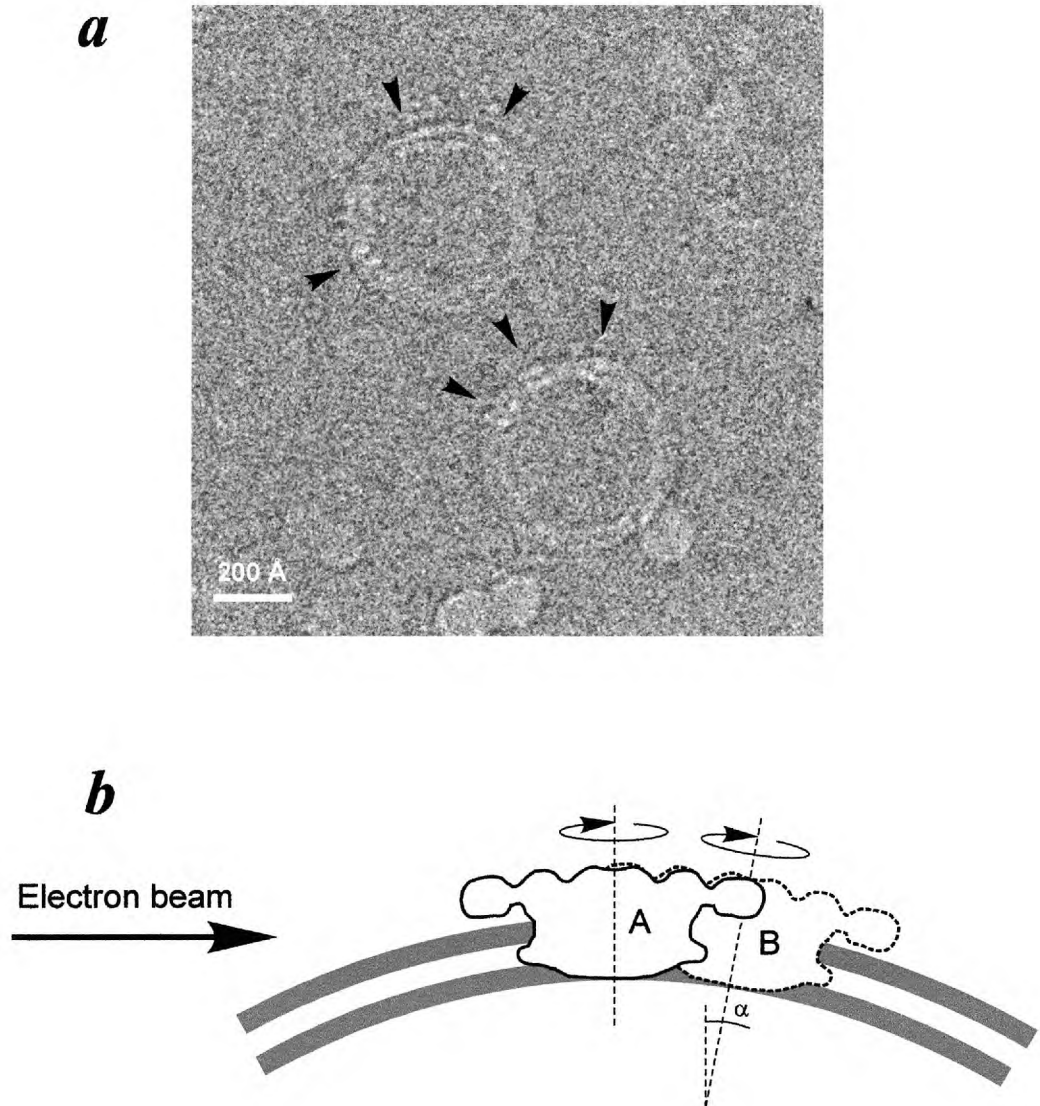


Figure 4.6 Insertion of LTX into membranes. *a*, Raw images of liposomes with inserted LTX tetramers (indicated by arrowheads). Electron-dense material (membrane and toxin) appears white against the grey background. Where LTX has incorporated into the lipid bilayers, electron density increases slightly both within and outside the membrane but not inside the liposome, demonstrating that the toxin does not internalise. *b*, To verify that LTX was actually incorporated into the membrane rather than just adsorbed onto the liposome's surface, only tangential views were taken for image analysis.

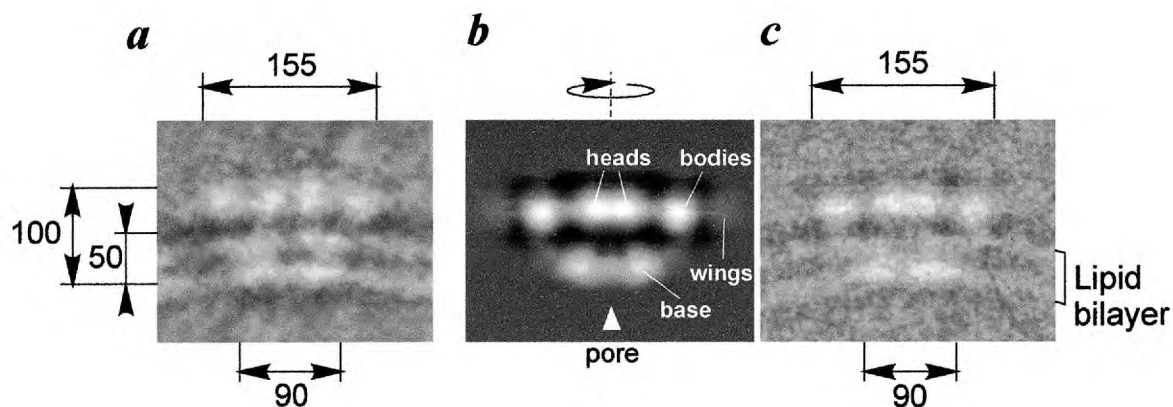


Figure 4.7 Cryo-EM of membrane-incorporated LTX. *a*, Average of 22 side views of the toxin inserted into the lipid bilayers. *b*, Side projection of the 3-D map of the tetramer rotationally averaged around the C4 axis. *c*, Same as in *b*, but overlaid with the image of the lipid bilayer. Note the similarity of images *a* and *c*. All figures are given in Ångströms. Notice that the LTX tetramer has a height of 100 Å but only half of this structure inserts into the bilayers.

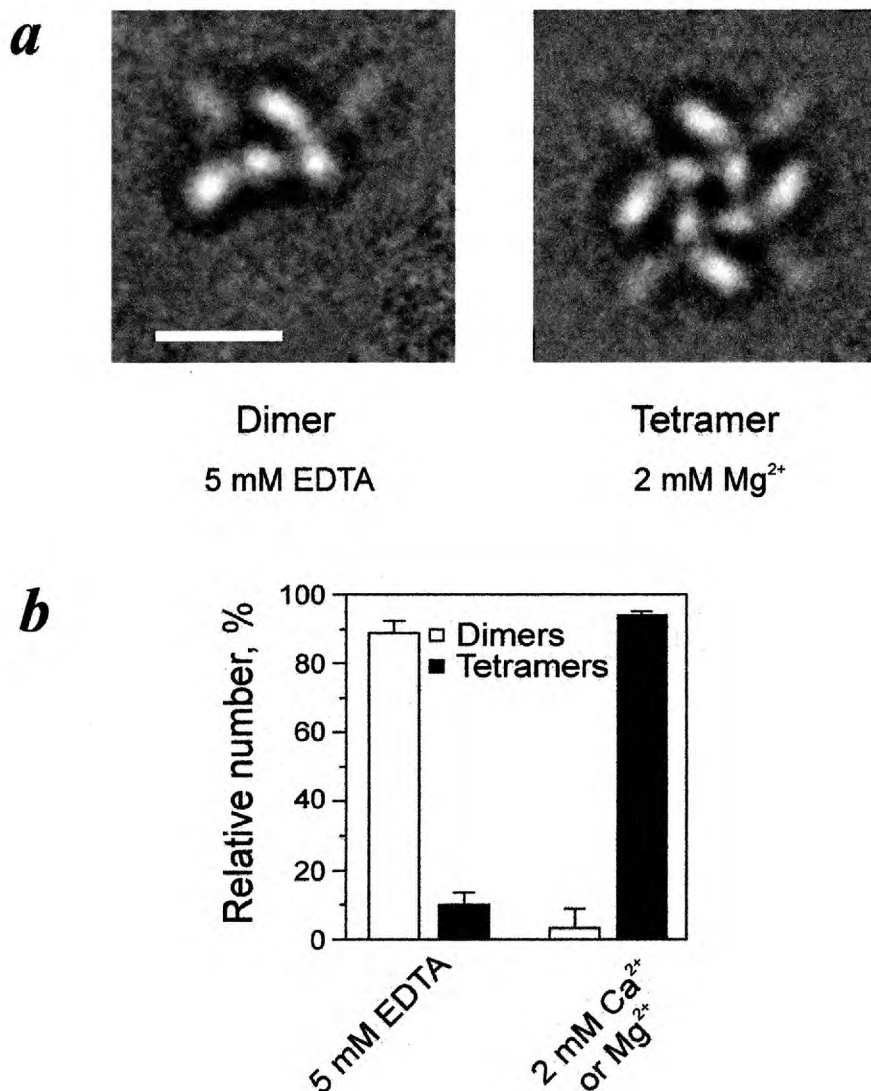


Figure 4.8 Divalent cations promote assembly of LTX dimers into tetramers. *a*, LTX dimers and tetramers visualised by cryo-EM. Raw images were selected from six digitised micrographs of LTX specimens prepared in the presence of 5 mM EDTA or 2 mM Mg²⁺ (as indicated). Obtained images were aligned and classified as described in Chapter 2. The resulting characteristic (average) views are shown. Scale bar, 100 Å. *b*, Relative abundance of the dimers and the tetramers in the presence or absence of divalent cations (as indicated). These were measured from the same micrographs as described above. Note that even in 5 mM EDTA about 10% of LTX is in the tetrameric form.

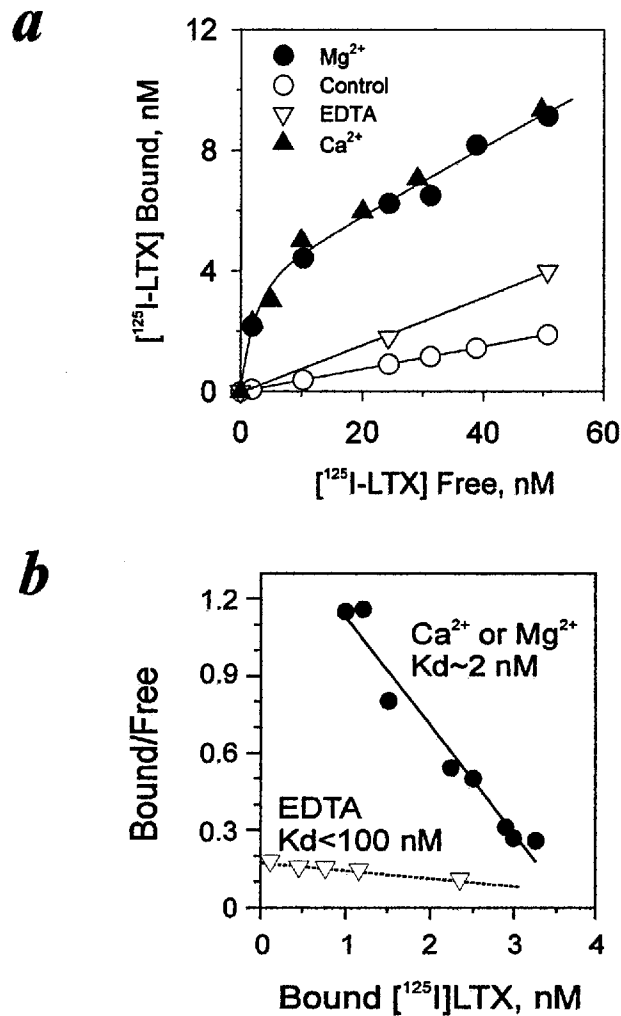


Figure 4.9 Affinity of LTX dimers increases in divalent cations. *a*, Aliquots of preparative-purified toxin immobilized on Sepharose were incubated with different concentrations of $[^{125}\text{I}]\text{LTX}$ in the presence of 2 mM MgCl_2 , CaCl_2 or EDTA. An identical column with BSA was used to calculate the non-specific toxin interaction with the matrix (control). Notice the affinity of dimers increases 50-fold in the presence of the divalent cations. *b*, Scatchard plot of the results shown in *a*. From this plot the K_d of the LTX tetramers was determined as ~ 2 nM.

4.3 Discussion

In this chapter, the three-dimensional structure of LTX is reported. Knowing the structure can help in understanding more about the toxin's mechanism of action. Although structural studies alone will not solve the phenomenon of how LTX stimulates neurotransmitter release, they can give us valuable clues. Previously, it could not be comprehended how a soluble toxin like LTX with very short, although multiply repeated, hydrophobic regions (within each ALR) could interact with the presynaptic membrane, and mediate the opening of a pore seen in various systems. The biochemical (chapter 3) and cryo-EM studies have shown that LTX can oligomerise. LTX is a dimer in its native state but in the presence of divalent cations, which are necessary for its biological activity, LTX forms tetramers. It can, therefore, be concluded that the LTX tetramer is the functionally active form of the toxin. In EDTA, 10% of all LTX seen in micrographs remains in the active tetrameric form. This small amount of tetramer may be responsible for the release facilitated by LTX in EDTA [Misler & Hurlbut, 1979; Ashton *et al.*, 2000].

The LTX tetramer is reminiscent of a channel. From the tetramer's orientation after insertion into the lipid bilayers, it appears that (i) its base is hydrophobic; (ii) the base can fully penetrate the membrane; (iii) the heads and wings remain on the outer surface of the membrane; (iv) the pore at the centre of the structure can fully permeabilise the bilayers. This suggests that LTX itself forms the pore that has been described in the literature, rather than stimulating the opening of an endogenous pore in the membrane, and that LTX receptors are not involved in the pore structure. In support

of this, firstly, LTX tetramers insert into artificial biological membranes (liposomes) that possess no proteins with which the toxin can interact. The insertion of the toxin into liposomes was inefficient in our system due to the lack of receptors and, therefore, concentrations of 100 nM, well above physiological levels, were required. This explains the results presented in Chapter 3 where toxin concentrations of 10 nM (10-fold less than used on liposomes) could not form channels in artificial lipid bilayers (it has recently been confirmed that 100 nM LTX can indeed make channels in planar lipid bilayers [Volynski *et al.*, 2000]).

Secondly, pure lipid bilayers are typically ~ 50 Å thick. The LTX tetramer is 100 Å in height but only half of this structure was observed to penetrate the liposomal membranes. Thus, oligomerised LTX is indeed capable of spanning the presynaptic plasma membrane. In this case, the central channel will completely permeabilise the membrane. Lastly, LTX most probably binds to its receptors via its wings i.e. the N-terminus, as has been hypothesised elsewhere [Itchtchenko *et al.*, 1998]. The wings of the LTX tetramer do not insert into the membrane and therefore, receptors are unlikely to be physically involved in the LTX channel. Instead, LTX receptors have a different role in pore formation (i) they recruit LTX to the presynaptic plasma membrane; (ii) they increase the local concentration of the toxin at the membrane, thus, facilitating tetramerisation; (iii) they possibly orientate the tetramers correctly for insertion into membranes.

The three-dimensional structure of the LTX tetramer and its insertion into bilayers is consistent with previous hypotheses made, and data obtained, from studies of LTX primary structure. It has been suggested that LTX is not biologically active unless a furin-like endoprotease cleaves it at the C-terminus (in addition to another

fragment cleaved at its N-terminus) [Volynski *et al.*, 1999a]. The spider venom is produced by “holocrine secretion”, i.e. disruption of the cells of the secretory epithelium, which literally burst and pour their contents into the lumen of the venom gland. As a result, the venom contains not only toxins but also all other cell constituents, including proteases. The presence of trypsin-like proteases in the spider venom has been shown in an early work of Huidobro-Toro *et al.*, [1982]. In the LTX tetramer, the four heads (C-terminal domains) are in contact with each other forming the external mouth of the pore. It is possible that unless cleavage occurs at the furin site, the LTX heads are not able to tetramerise and/or form the channel, thus, resulting in a functionally inactive toxin.

The L-shaped bodies of the LTX monomer each contain about 17 of LTX's 22 ankyrin-like repeats (the remainder being in the heads). Ankyrin repeats are found in many different proteins and are thought to mediate interactions between and within proteins [for reviews see Bork, 1993; Sedgwick & Smerdon, 1999]. Therefore, the ankyrin repeats in the bodies may also be responsible for LTX oligomerisation as well as providing the LTX tetramer with a hydrophobic base to interact with presynaptic membranes. This may also explain why a recombinant LTX, that had a four amino acid insertion placed between the wing and body domains, bound to LTX receptors but could not stimulate transmitter release, as it was unable to oligomerise [Ichtchenko *et al.*, 1998].

In conclusion, a 3-D structure of LTX has been described that provides the first model for α -latrotoxin pore formation. LTX is one of the smallest proteins whose structure has been solved to date using single particle cryo-EM method; therefore, reaching 14 Å resolution has been a remarkable achievement in itself [refer to Saibil,

2000]. However, the ultimate goal is to view LTX's structure at atomic resolution and to view the foldings of the peptide backbone (for which two-dimensional crystals will be required), and to observe the toxin in complex with its receptor in the membrane. These studies will be able to help us to comprehend the conformational changes involved in toxin-receptor interactions and to elucidate further LTX's pore-forming mechanism of action.

4.4 Summary



LTX exists in two oligomeric states: dimers and tetramers. The 3-D structures of these oligomers have been determined at 18 Å and 14 Å resolution, respectively, by cryo-EM and angular reconstitution.



“Two halves make a hole” – two LTX dimers come together and form a tetramer. The LTX tetramer resembles a pore and is probably the biologically active toxin species as divalent cations promote its tetramerisation.



The base of the tetramer can insert into liposomes completely permeabilising their membrane.



Therefore, LTX itself forms the pore involved in the pore-dependent mechanisms of α -latrotoxin action.

CHAPTER FIVE



IDENTIFICATION OF PROTEINS INTERACTING WITH LPH

5.1 Overview

Chapters 3 and 4 have presented a plausible explanation for the pore-dependent mechanism of α -latrotoxin action. The next three parts of this thesis will focus on elucidating the structure and physiological function of latrophilin in order to determine its involvement in the pore-independent mode of LTX action.

To put the timescale of this particular chapter into context, latrophilin was first identified as a novel 120 kDa high-affinity receptor for LTX by my co-workers in 1996 [refer to Davletov *et al.*, 1996]. Shortly after that paper was published our laboratory cloned LPH1, which revealed the receptor to be a member of the family of G protein-coupled receptors (GPCRs) [subsequently published in Lelianova *et al.*, 1997]. This discovery was immediately followed by the experimental work described herein.

Being a GPCR, latrophilin is likely to have specific heterotrimeric G proteins associated with it, which couple the receptor to its appropriate effectors. Affinity chromatography of solubilised brain membranes on immobilized LTX was used to investigate the specific G proteins (and other proteins) associating with LPH, as the procedure affords a 25,000-fold enrichment of the receptor [Davletov *et al.*, 1996]. Standard SDS-PAGE analysis [according to Laemmli, 1970] of the LTX column eluate shows that only the 120 kDa band of LPH is specific for the toxin when chromatographies are performed in Mg^{2+} or in EDTA, although tubulin is also enriched. In addition, it is shown that latrophilin interacts physically and functionally with the G proteins, $G\alpha_q$ and $G\alpha_o$.

5.2 Results

5.2-1 α -Latrotoxin affinity chromatography of solubilised rat membranes

Rat total brain was solubilised in Thesit as described in the Materials and Methods and then passed through a Sepharose column containing 1 mg of covalently attached LTX, purified by the native preparative electrophoresis approach. As a control, 1 mg of BSA (an acidic protein like LTX and also capable of oligomerisation) was irreversibly attached to Sepharose beads to make another column. To investigate proteins that were co-purifying specifically with LPH on the LTX affinity column, all chromatographies were performed either in 2 mM Mg^{2+} or 2 mM EDTA, as this removed contamination by neurexin I α (Figure 5.1b).

In these chromatographies only the band corresponding to latrophilin was observed to be wholly specific for the LTX column (Figure 5.1a). However, when the eluates from the LTX column were compared to those from the BSA column, there seemed to be an enrichment of a protein of approximately 55 kDa on the toxin column (Figure 5.1a). To investigate whether this second protein was specifically binding to the LTX column, several toxin column eluates were combined, dialysed to remove high salt, concentrated, and run through a new LTX column. The second pass of this material through an LTX column removed some of the other bands seen after the first affinity chromatography (Figure 5.2a), suggesting that this step could be used to improve the purity of isolated latrophilin. However, the 55 kDa band (often appearing as a doublet of 55 and 57 kDa) was not removed and its presence was even more apparent (Figure 5.2).

a

NaCl	0.2 M		1 M	
BSA-Column	+		+	
LTX-Column		+		+

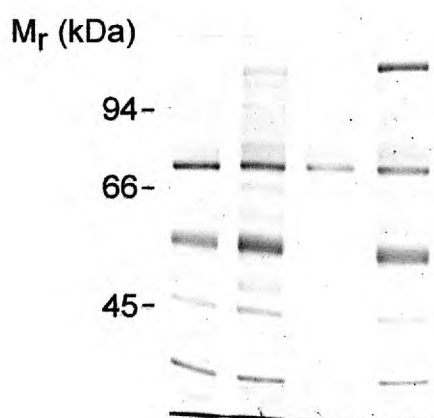
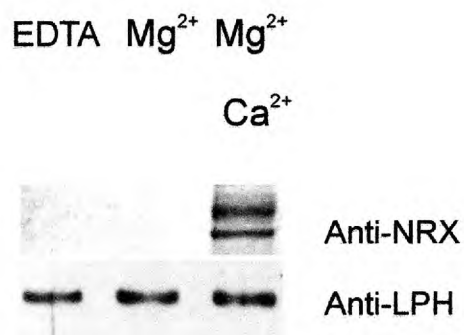
*b*

Figure 5.1 Only the 120 kDa protein is specific for the LTX column. *a*, 1 mg of purified LTX was coupled to Sepharose as described in Chapter 2 to make an LTX affinity column. The procedure was repeated with 1 mg of BSA to produce a control column. Total solubilised rat brain membranes were incubated with each column in 2 mM Mg^{2+} and after several washes with 0.2 M NaCl, specific proteins were eluted with 1.0 M NaCl. Comparison of the eluates on SDS-PAGE from both columns reveals that only LPH (120 kDa) is specific for LTX, however, a protein of 55 kDa is also enriched on the toxin column. The same results were seen in experiments with 2 mM EDTA. *b*, All LTX column chromatographies were performed in 2 mM Mg^{2+} or EDTA as this excludes the purification of neurexin I α (NRX), which only binds to the toxin when Ca^{2+} is present (here shown by immunostaining with specific antibodies).

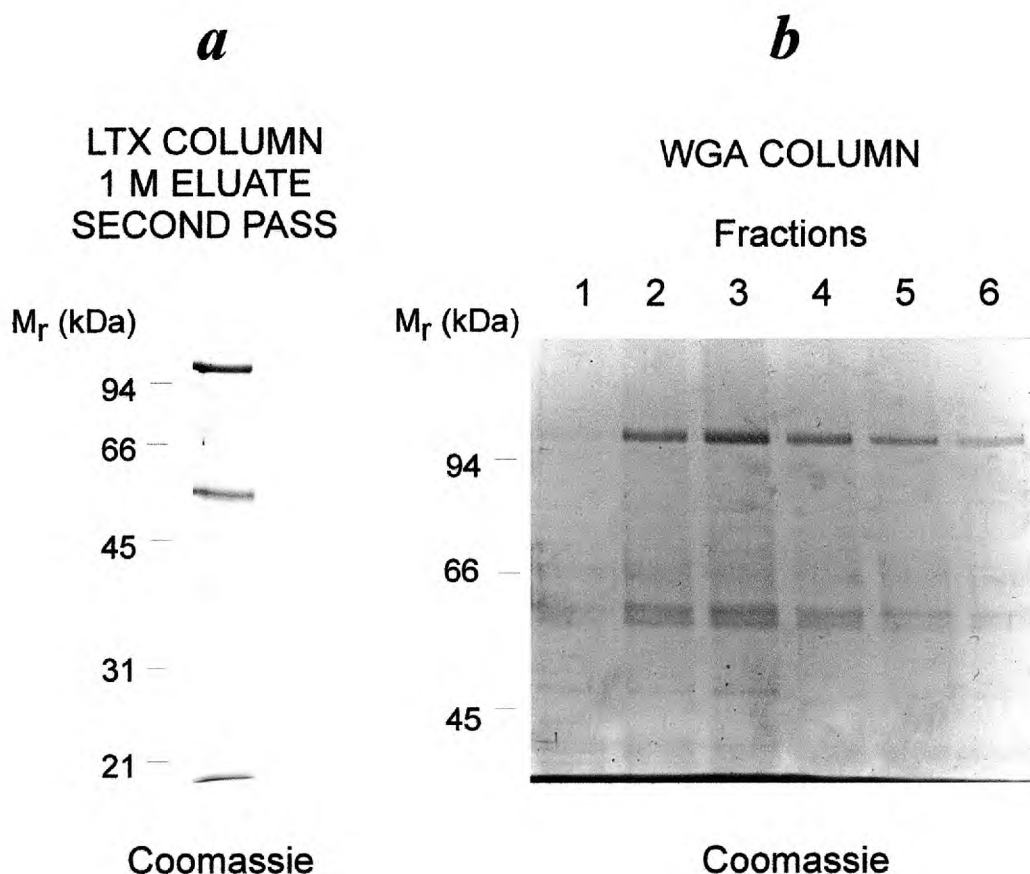


Figure 5.2 The 55 kDa protein co-purifies with latrophilin. *a*, To further test the interaction of the 55 kDa protein with latrophilin, several 1.0 M eluates from the LTX column were combined, dialysed to remove high salt, concentrated, and passed through a fresh LTX column. The interaction of the two proteins survived this second chromatography. Notice, however, the removal of some other non-specific protein bands after a second pass through the LTX column. *b*, To check whether the 55 kDa protein was interacting with LTX or latrophilin specifically, the eluate from the LTX column was passed through WGA-Sepharose. After washing with 0.5 M NaCl (*fraction 1*) proteins were eluted with 100 mM *N*-acetylglucosamine (*fractions 2-6*). Note that latrophilin (120 kDa) and the 55 kDa band are the major proteins eluting from the column and that their elution profiles coincide. The relative position of molecular weight markers is indicated on both gels.

This implied that either the 55 kDa protein was co-purifying with latrophilin or was binding tightly to LTX. The latter could occur if the protein possessed the same affinity constant as the receptor for the toxin. To distinguish between the two possibilities a second affinity chromatography step was introduced using a completely different system, wheat germ agglutinin (WGA). This lectin was previously shown to bind latrophilin through the receptor's *N*-glycosylation but not LTX [Ushkarev and Grishin, 1986; Davletov *et al.*, 1996]. Upon passing the LTX column eluate through the lectin column, latrophilin and the 55 kDa protein co-purified (Figure 5.2b). Both proteins were clearly visible on SDS-PAGE with Coomassie staining, their elution profiles coinciding. The sequencing of this doublet (as described in Materials and Methods) gave peptides for β and γ tubulin (MREIVHIQAG and MPREITLQL, respectively).

5.2-2 G proteins co-purify with latrophilin

When chromatography of solubilised rat brain on the LTX column was conducted in the presence of low micromolar Mg^{2+} and 2 mM GDP, a 40 kDa protein could be detected by Coomassie staining of the eluate fraction run on an SDS-PAGE gel. This protein was shown to be a G protein α -subunit by Western blotting using an antibody that recognised all α subunits of heterotrimeric G proteins. The G protein band had been absent from the receptor preparations made in millimolar Mg^{2+} or in EDTA (Fig. 4.2), pointing at the physiological relevance of this interaction. This band could also be stained with specific anti-G α_o and anti-G $\alpha_{q/11}$ antibodies but not with antibodies directed to other G protein α subunits (Figure 5.3).

To verify the specificity of the co-elution of latrophilin and the G proteins from the affinity column, solubilised brain membranes were also passed through a new

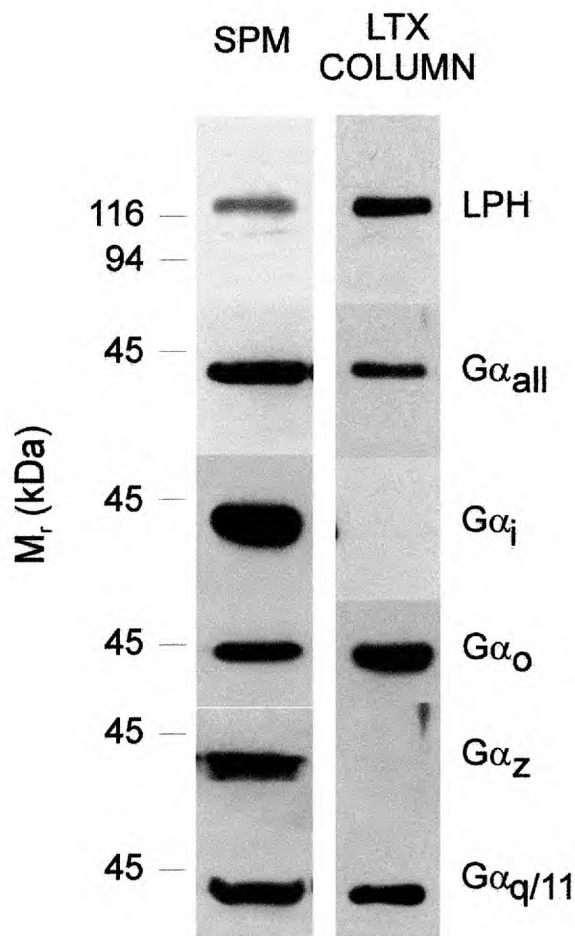


Figure 5.3 Specific G proteins elute from the LTX affinity column. Synaptosomal plasma membranes (SPM) were solubilised in 20-100 μM Mg^{2+} and 2 mM GDP and loaded onto the LTX affinity column, which was then extensively washed and eluted with 1 M NaCl. Equal amounts of SPM and the LTX column eluate were separated in an SDS-gel, transferred onto PVDF membrane and immunostained with antibodies to latrophilin (LPH) or G protein α -subunits (as shown on the right). The two lanes are not meant for quantitative comparison with each other. Numbers on the left denote the position of molecular weight standards. Notice the absence of association between latrophilin and $G\alpha_i$ and $G\alpha_z$.

control column containing immobilised BSA under the same conditions. In the BSA eluate no $G\alpha_{q/11}$ or $G\alpha_o$ could be detected (not shown). Thus, these $G\alpha$ -subunits seemed to specifically interact with latrophilin bound to immobilised LTX.

To demonstrate further the specificity of this interaction, a second round of affinity chromatography was introduced. The eluate from the toxin column was loaded onto a WGA column and eluted with *N*-acetylglucosamine (NAGA). Since G proteins are not glycosylated, they do not bind the lectin column (not shown); however, if a G protein interacts with the glycosylated toxin receptor, it should be eluted off the WGA column in parallel with latrophilin. Indeed, as Figure 5.4 shows, elution of G protein from the lectin column perfectly matched that of latrophilin. Furthermore, during the chromatography of brain proteins, if the LTX column was washed with GTP instead of GDP and then eluted with 1 M NaCl, much less G protein remained in the latrophilin fraction (Figure 5.5, lower panel). Thus, an excess of GTP is able to reverse the interaction of latrophilin with its requisite G protein(s), suggesting that this association is not only physical but also functional.

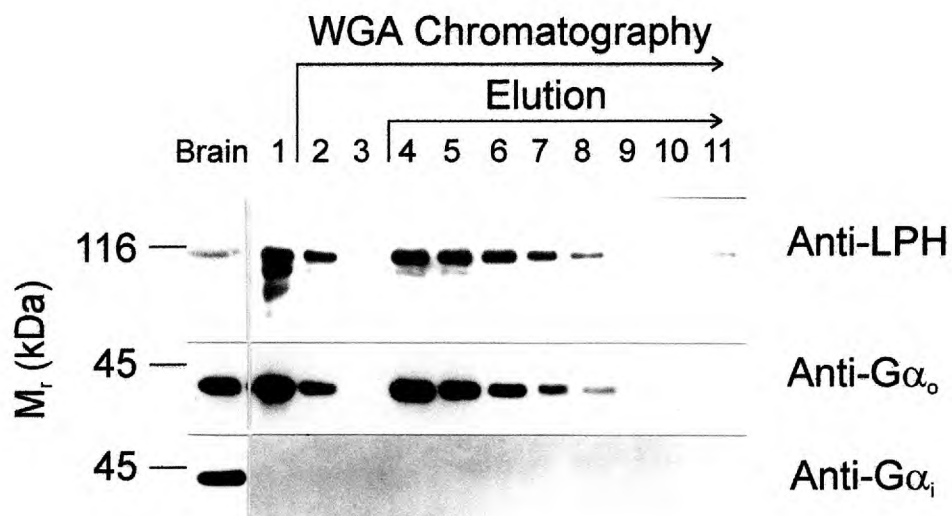


Figure 5.4 G proteins co-purify with latrophilin. The eluate from the LTX column (*lane 1*) containing both latrophilin and G α subunits was subsequently loaded onto a WGA column (*lane 2*, flow through), washed (*lane 3*), and eluted with 100 mM *N*-acetylglucosamine (*lanes 4-11*). Fractions were analysed by SDS-PAGE and immunoblotting with respective antibodies. Notice how the elution of G proteins matches the profile of latrophilin during the second chromatographic step.

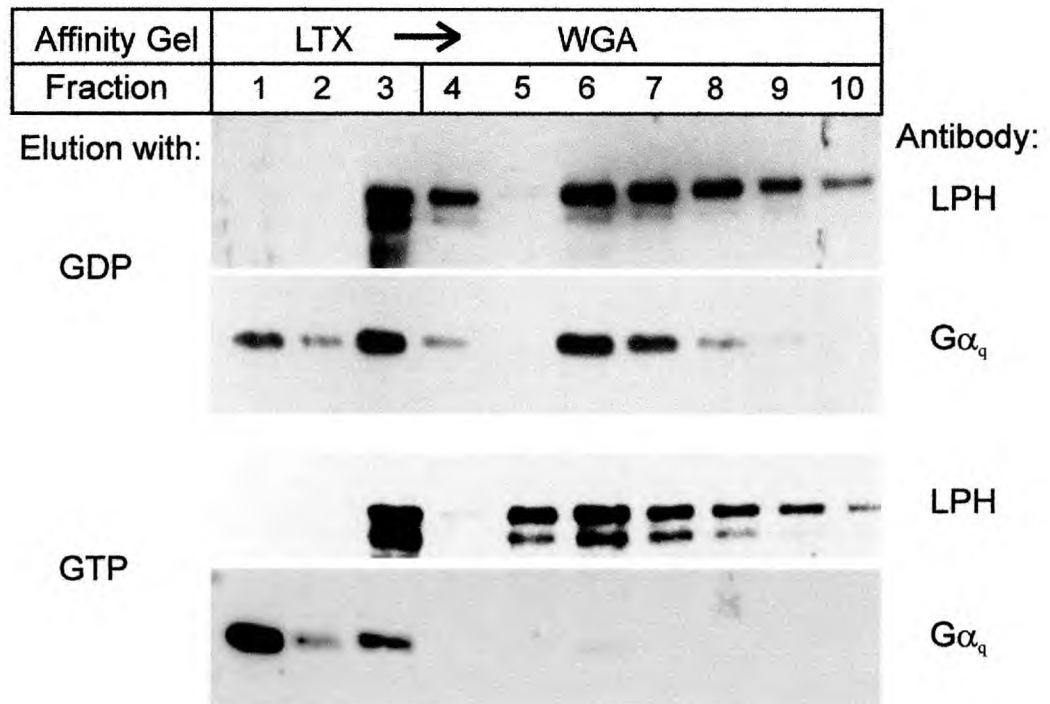


Figure 5.5 GDP stabilises the G protein-latrophilin complex whereas GTP reverses this interaction. The LTX affinity column was loaded with solubilised synaptosomal plasma membranes, washed and first eluted with 2 mM GDP or GTP (lanes 1 and 2, upper or lower panels respectively) and then with 1 M NaCl (lane 3). This eluate was loaded onto a second affinity column containing immobilized WGA, which was then washed and eluted with 100 mM *N*-acetylglucosamine (lanes: 4, flow through; 5, buffer wash; 6-10, consecutively eluted fractions). After electrophoresis and Western blotting, the protein bands were stained with antibodies against latrophilin (LPH) or $G\alpha_q$. Note that when the LTX column is eluted with GTP, much less G protein remains in the complex with latrophilin.

5.3 Discussion

A two-step affinity chromatography procedure was used to detect proteins specifically co-purifying with latrophilin. In this study it was found that latrophilin interacted specifically with two G protein α subunits. When latrophilin purification is performed on a LTX column in the presence of GDP and low micromolar Mg^{2+} , a protein of 42 kDa co-purifies with the receptor, which reacts with antibodies against $G\alpha_o$ and $G\alpha_q$ but not any others tested. This interaction survived the second chromatography on a lectin column, indicating that their purification is stable. It was also found that whereas GDP promoted the association of G proteins to latrophilin, an excess of GTP reversed it. This indicates a functional interaction because GPCRs are known to bind G proteins in the GDP-bound (but not GTP-bound) state.

It is possible, however, that latrophilin binds G proteins indirectly, via a protein that has not been detected on the gels, even though the only other bands observed by Coomassie stain are those for latrophilin and tubulin (in 2 mM EDTA or Mg^{2+}). Recent work strongly suggests a functional link between latrophilin and phospholipase C (PLC) [Davletov *et al.*, 1998; Rahman *et al.*, 1999]. The coupling of a GPCR with PLC is usually provided by a $G\alpha_q$ [Selbie & Hill, 1998] and, therefore, it may not be a coincidence that such a G protein α subunit was found to co-purify with latrophilin.

Although latrophilin binds to G protein α subunits, the effects of the receptor may be mediated alternatively, or in addition, by G protein $\beta\gamma$ subunits. Only in the last few years has the $G\beta\gamma$ subunit been recognised as a signal transduction molecule in its

own right and is now known to directly regulate as many different protein targets as the $G\alpha$ subunit. For example, $G\beta\gamma$ has been shown to positively stimulate muscarinic acetylcholine receptors associated with cardiac K^+ channels [Logothetis *et al.*, 1987], phospholipases, β -adrenergic receptor kinase and certain types of adenylate cyclase [reviewed by Clapham & Neer, 1993]. Different types of Ca^{2+} channels including N-type and P/Q-type channels involved in neurotransmitter release are also influenced by $G\beta\gamma$ [Herlitze *et al.*, 1996; Ikeda *et al.*, 1996]. As $G\beta\gamma$ does not bind GTP, its functional lifetime depends on the rate of hydrolysis of GTP by its associated $G\alpha$ subunit [see Stryer & Bourne, 1986]. At present, there is no data to implicate the $G\beta\gamma$ subunits, or exclude them from, the LTX-evoked effects.

In contrast to G proteins that required the presence of GDP and Mg^{2+} for isolation, tubulin was found to equally bind to latrophilin in the presence or absence of divalent cations (personal observation). This interaction could be non-functional as tubulin is an abundant cytoskeletal protein of synaptosomes. Alternatively, tubulin may interact with other cytoskeletal proteins to influence exocytosis. The cytoskeleton forms a cortex of filaments underlying the plasma membrane and prevents an uncontrolled fusion of vesicles with the membrane. Actin is known to play an important role in the process of exocytosis [Gasman *et al.*, 1997]. The actin filaments may bind to membrane proteins via tubulin, thus, providing a direct and fast control mechanism of recruiting vesicles to release sites.

Also, it has been speculated that the interaction of heterotrimeric G proteins with tubulin may influence cellular signalling mechanisms. Tubulin, like G proteins is a guanine nucleotide-binding protein. Studies have shown that tubulin can donate its exchangeable GTP to certain subtypes of G proteins and thereby facilitate G protein

function. The most widely studied example of this is the activation of adenylyl cyclase through $G\alpha_s$ [Popova *et al.*, 1994]. Conversely, G proteins can also influence the polymerisation and depolymerisation of tubulin [see review by Ravindra, 1997]. Changes to the conformational state of tubulin at one locus may be transmitted through the cytoskeleton network to a region some distance away. This in turn may affect other receptors and/or G proteins, and represents another way that tubulin can be involved in signalling, i.e. communication through physical forces. Thus, the co-purification of tubulin with latrophilin may yet turn out to have a functional significance.

5.4 Summary



The 120 kDa latrophilin band is the only specific protein for the LTX column under the chromatography and electrophoresis conditions used.



Tubulin co-purifies with the latrophilin.



Two G proteins, $G\alpha_o$ and $G\alpha_q$ specifically and functionally co-purify with LPH when chromatographies are performed in the presence of GDP and micromolar Mg^{2+} .

CHAPTER SIX



TRANSLATIONAL PROCESSING OF LATROPHILIN

6.1 Overview

The full sequence of latrophilin predicted its molecular mass to be 159 kDa, or, inclusive of the cleavable glycosylation, 185 kDa [Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997]. However, only a 120 kDa band had been seen on SDS-PAGE gels [Davletov *et al.*, 1996; Krasnoperov *et al.*, 1996]. A possible explanation for this was the abnormal mobility of latrophilin caused by intramolecular interactions that made the receptor more compact and, therefore, resulted in its faster migration on gels. Alternatively, it meant that a part of the receptor (60-65 kDa) had not been isolated. The second idea seemed unlikely at the time, as there was no protein of 60-65 kDa isolating specifically off the LTX column (Chapter 5).

Sequences of tryptic peptides from the 120 kDa protein confirmed that it contained latrophilin's N-terminus; no peptides from the C-terminal half of the molecule were obtained. To address the uncertainty of whether the 120 kDa band contained the C-terminus of latrophilin, antibodies to a peptide from this region were used to stain isolated native receptor preparations. As this chapter will reveal, these antibodies do not stain the 120 kDa band but recognise a protein of ~ 65 kDa. N-terminal sequencing of this 65 kDa band reveals that it must contain all seven transmembrane regions of latrophilin in addition to the C-terminus. Therefore, latrophilin is cleaved to produce two receptor fragments, which have been named the NTF (N-terminal fragment, or 120 kDa) and the CTF (C-terminal fragment, or 65 kDa).

6.2 Results

6.2-1 N- and C-terminal antibodies against LPH stain different proteins

Two sera (rabbit and chicken) containing antibodies to the intracellular C-terminal region of latrophilin (generated against amino acid residues 1175-1466) were affinity-purified as described in Materials and Methods. Together with an affinity-purified antibody against the 120 kDa protein (anti-120), they were tested on immunoblots containing LTX column eluates prepared for electrophoresis by precipitation with chloroform/methanol. The C-terminal antibodies and the anti-120 antibody showed differential staining (Figure 6.1). The two C-terminal antibodies did not recognise the 120 kDa protein but, instead, stained the top of the transferred resolving gel. Conversely, anti-120 recognised the ~ 120 kDa protein specifically. Since both C-terminal antibodies behaved similarly this initially suggested that the 120 kDa fragment did not possess the C-terminus.

The staining at the top of gels indicated that the C-terminal half of latrophilin could be SDS-insoluble, and this prevented its resolution by SDS-PAGE. The next strategy was therefore to find conditions that would dissolve the C-terminus. The approach that succeeded first was to warm eluates at 37°C in the SDS-urea loading buffer before loading onto gels containing urea. This abolished the staining at the top of the blots and, instead, both C-terminal antibodies specifically stained a series of four bands varying from about 65-68 kDa (Figure 6.1a). However, when urea was removed from both the sample buffer and the gel, the same series of bands was stained by the antibodies (Figure 6.1b). This result indicated that urea was unnecessary for

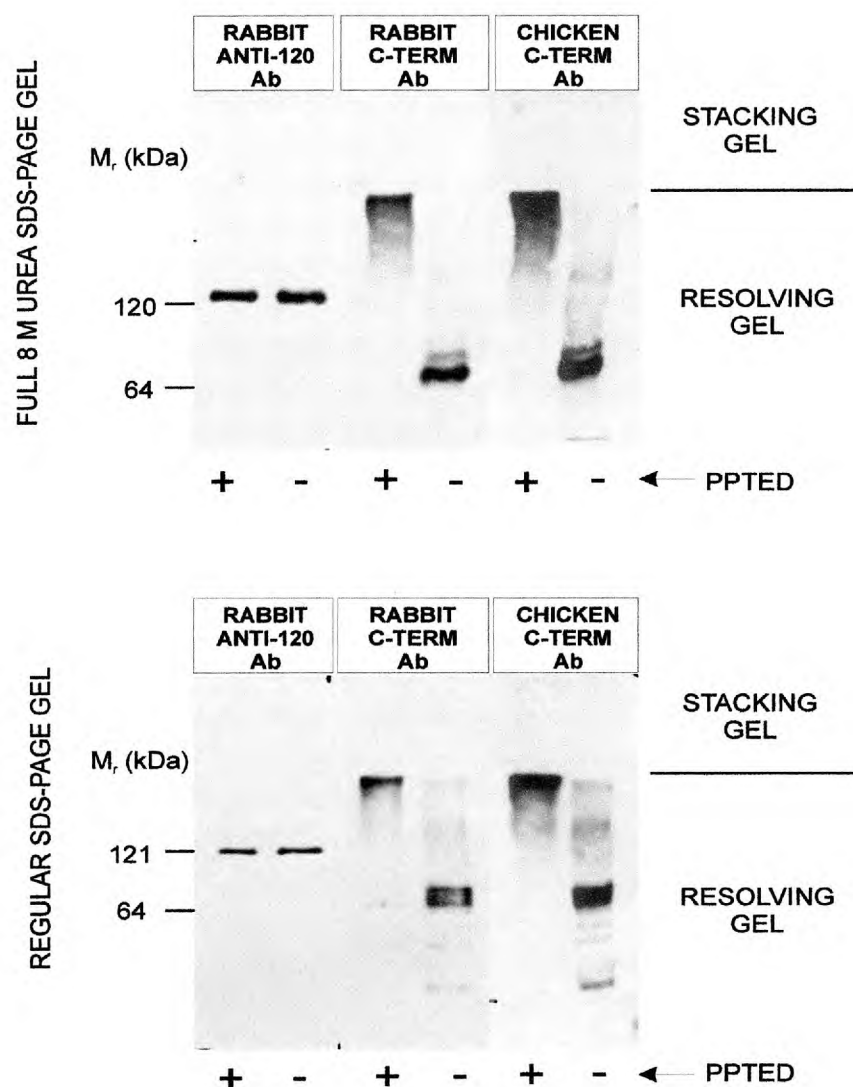


Figure 6.1 Antibodies to the N- and C-termini of LPH show differential staining. A rabbit affinity-purified antibody (Ab) to the 120 kDa latrophilin band that contains the receptor's N-terminus (anti-120) and a rabbit and a chicken antibody specific to a region of LPH's C-terminus (C-term antibody) were used to stain LPH isolated from a LTX column. The LTX column eluate was either precipitated (ppted) in chloroform/methanol or left untouched before being dissolved in an appropriate sample loading buffer, as described in Chapter 2. These preparations were run on a full 8 M urea gel (upper figure) or on regular SDS-PAGE (lower figure) and then transferred onto PVDF membrane. Notice that the two C-terminal antibodies stain the same protein band of approximately 65-68 kDa whereas the N-terminal antibody is specific for the 120 kDa band. Note also that if samples are precipitated, the 65 kDa band remains at the top of the resolving gel unlike the 120 kDa band, which is unaffected. Position of molecular mass markers is indicated on the left.

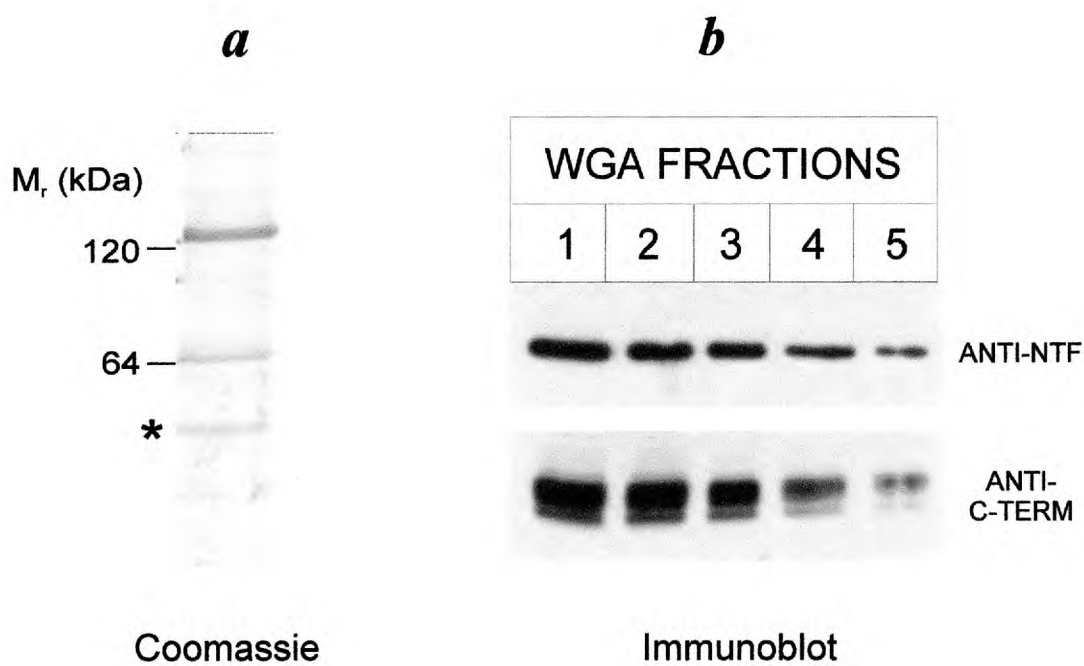


Figure 6.2 The 65 kDa protein co-purifies with the NTF. *a*, Latrophilin was isolated from total solubilised rat brain using two-step affinity chromatography (LTX and WGA columns, refer to Chapter 2). 2 mM EDTA was present throughout. The eluate from the lectin column was dissolved in regular SDS-PAGE buffer (without boiling the sample) and run on an 8% SDS-gel. The gel was subsequently stained with Coomassie R250. The position of tubulin is marked with an asterisk. *b*, Consecutive fractions (1-5) eluted off the WGA column were run on SDS-PAGE as described above, transferred onto PVDF membrane, and then stained with antibodies specific for the NTF and the C-terminal portion of LPH. Notice how the elution profile of the 65-68 kDa bands matches that of the NTF. Positions of molecular mass markers are indicated on the figure.

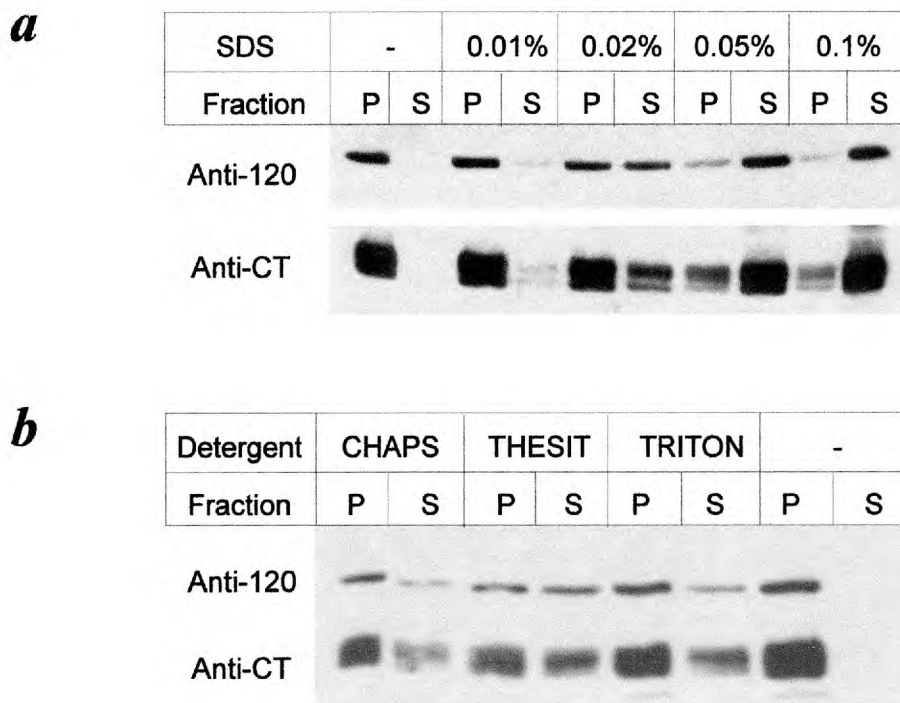


Figure 6.3 The 120 kDa and 65 kDa latrophilin fragments show similar patterns of solubilisation in different detergents. *a*, Synaptic plasma membranes (SPM) were incubated for 10 min in different concentrations of SDS. After this period, pellets (P) and supernatants (S) were collected by a centrifuge spin (refer to Materials and Methods). Equivalent amounts of each pellet and supernatant were dissolved in SDS-loading buffer (without boiling), run on SDS-PAGE and Western blotted. The membrane was then stained with antibodies against the N-terminus (anti-120) and C-terminal fragment (anti-CT) of LPH. *b*, SPM were incubated with 0.5% (w/v) CHAPS, Thesit or Triton X-100 for 10 min after which pellets and supernatants were collected and analysed as described in *a*.

solubilisation of the C-terminal portion of LPH and that its irreversible precipitation could be avoided by neither precipitating with chloroform/methanol nor boiling the sample. As the 120 kDa protein contains latrophilin's N-terminus but not its C-terminus it has been named "NTF" (N-terminal fragment).

With this new knowledge, all subsequent LTX and WGA affinity column eluates were loaded on SDS-PAGE without precipitating or boiling the samples. Under these conditions, a second specific band for the LTX column of ~ 65 kDa was visible with Coomassie stain. This band survived a second chromatography on the WGA column resulting in an eluate containing three major proteins (NTF, 65 kDa and tubulin), when chromatographies of total brain were performed in 2 mM EDTA throughout (Figure 6.2a). The NTF and 65 kDa protein had a similar pattern of elution from the lectin column as shown by respective antibodies, suggesting an interaction (Figure 6.2b). The two proteins also demonstrated the same degree of solubilisation in different detergents (Figure 6.3), which again implied that the N-terminal and C-terminal portions of latrophilin remained together.

6.2-2 Calibration curves for the N-terminal and C-terminal latrophilin antibodies

We attempted to calibrate the intensity of staining of the two proteins with their respective antibodies. Initially we examined how the anti-NTF and anti-65 kDa antibodies stained the full-size receptor. This was achieved by expressing the construct LPH02a in COS-7 cells, which produced a receptor of ~ 180 kDa (refer to Figure 6.8). As this protein, being full-size, contained an equimolar amount of the N-terminal and C-terminal portions of latrophilin it was suitable for use in comparing the relative staining capabilities of the two antibodies at different exposure times (Figure 6.4). The anti-120 kDa and anti-65 kDa antibodies, each at the same dilution, stained latrophilin

with virtually a 1:1 intensity (when measured within the linear segments of the concentration dependence curves). This relationship held over a wide range of exposure times (20 seconds to 60 seconds) but was not as reliable after 1.5 minutes. As there was linearity of staining between 30 seconds to 50 seconds we chose this period as the optimal exposure time for our experiments.

We next examined how the two antibodies stained the latrophilin fragments isolated from the total brain on the LTX affinity column. The NTF stains very well with Coomassie dye on gels. As it is roughly the same molecular mass as purified LTX (run on SDS-PAGE), by comparing the intensity of the Coomassie-stained LPH N-terminus with that of known concentrations of purified LTX it is possible to approximately quantify the amount of NTF present in a LTX column eluate run on an electrophoretic gel. Using this method, we ran 5-50 ng of the NTF (contained within the total LTX column eluate) on a gel, transferred it onto PVDF and then stained the membrane with antibodies to the NTF and the 65 kDa protein at different exposure times. This permitted us to quantitatively assess the staining of the N-terminal fragment by the anti-NTF antibody and compare this with staining of the C-terminal fragment by the anti-65 kDa antibody. Computational analysis showed that the relative intensity of the NTF to the 65 kDa protein was about 1.5 to 1 (refer to Figure 6.7) over a wide range of protein concentration and exposure times. This suggested that the LTX column eluates from the brain contained, on average, 1.5 times less C-terminal fragment than the NTF.

6.2-3 The N-terminal and C-terminal halves of LPH are not strongly linked

The finding that more of the N-terminal fragment was present in latrophilin isolated from total brain was interesting as this implied that at least part of the NTF was

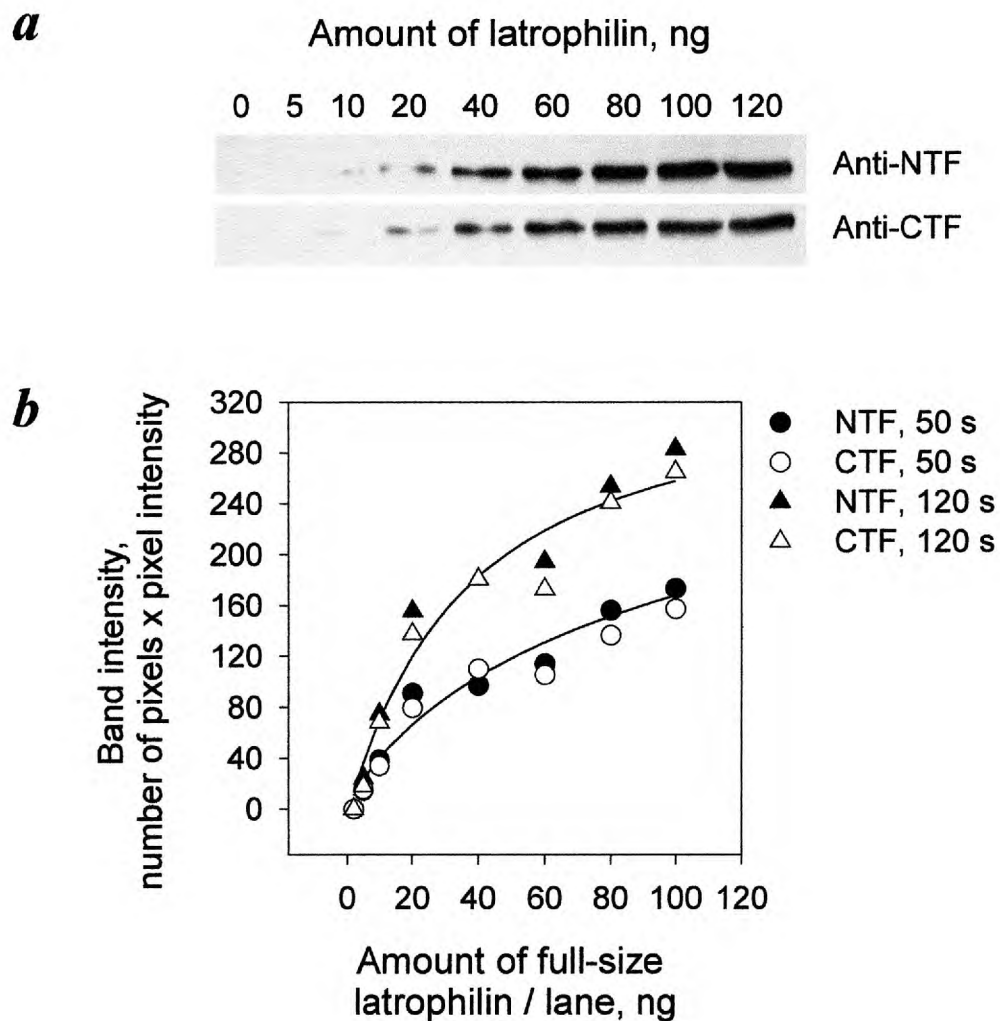


Figure 6.4 Calibration of antibodies. *a*, Staining of the same full-size latrophilin (LPH02a) expressed in COS-7 cells with affinity purified antibodies to the NTF and 65 kDa protein (CTF). Exposure 50 sec. *b*, Example of computer-assisted densitometry of blots as in *a* exposed for 50 sec or 120 sec. Note the almost identical staining of the full-size protein with both antibodies.

not connected to the C-terminal portion of the receptor. Indeed, if the two fragments co-purified in equimolar amounts off the LTX column, then their staining intensity would be the same, as shown using full-size receptor expressed in COS-7 cells. To explore this further, we studied the effects of changing the conditions of LTX affinity chromatography on the co-purification of the two proteins.

First, chromatographies of total brain on a LTX column were compared in EDTA and in different divalent cations (Ca^{2+} , Mg^{2+} and Ba^{2+}) known to stimulate or support exocytosis. These chromatographies were performed on mini-LTX columns (~ 100 μl of immobilised LTX on Sepharose) in triplicate. After several washes with 0.2 M NaCl, the columns were eluted directly with 1x SDS-loading buffer, thus, ensuring removal of all proteins from the LTX column as denaturation of LTX prevents toxin-receptor interactions [Davletov *et al.*, 1996]. Interestingly, staining with respective antibodies on immunoblots revealed that in the presence of 2 mM Mg^{2+} or Ca^{2+} , the 65-68 kDa bands stained with 40% less intensity compared to 2 mM EDTA or Ba^{2+} incubations (Figure 6.5). The relative staining of the NTF isolated, on the other hand, was unaffected by changing the conditions of chromatography. According to the antibody calibration curves these results suggested that the same relative amount of NTF had been purified in all chromatographies, however, the reduced intensity of the 65-68 kDa bands in Mg^{2+} or Ca^{2+} was a reflection of less of this protein co-purifying with the N-terminal fragment in the presence of these divalent cations compared to in Ba^{2+} and in EDTA. This trend was consistently seen in our experiments and implies that any interaction between the 120 kDa and 65 kDa proteins is not always strong but possibly depends on specific physiological conditions.

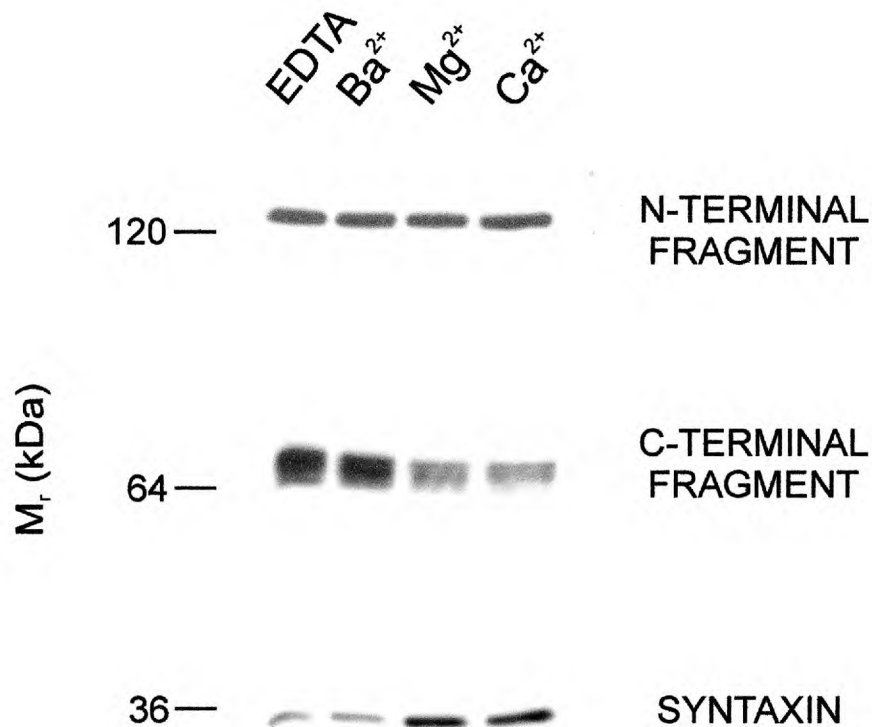


Figure 6.5 Less of the 65 kDa protein co-purifies with the NTF in Ca²⁺ and Mg²⁺. Latrophilin was isolated from total rat membranes on miniature LTX columns in the presence of 2 mM EDTA, Ba²⁺, Mg²⁺ or Ca²⁺ as described in the text. After elution, samples were run on SDS-PAGE, transferred onto Immobilon and stained with various antibodies, as indicated. Note that the relative amount of NTF isolated is similar under all conditions. However, in Mg²⁺ and Ca²⁺, the CTF stains with less intensity than in EDTA or in Ba²⁺. Syntaxin was also detected in LTX column eluates. Positions of molecular weight markers are given on the left.

Second, LTX affinity chromatography was performed using different membrane preparations. Total brain membranes, synaptosomes and SPM were solubilised in Thesit and incubated with mini-LTX columns in 2 mM EDTA. Due to differences in total protein concentration, all samples eluted with 1x SDS-loading buffer were equalised for their NTF content (determined by antibody staining on blots and assessed by computer-aided densitometry). The relative staining of 65 kDa protein co-purifying with the NTF was then visualised using the anti-C-terminal antibodies on immunoblots (Figure 6.6a). We observed that the intensity of staining of the 65 kDa fragment was substantially higher in the total brain preparation compared with latrophilin isolated from synaptosomes and SPM. Considering the relative ratio of NTF:65 kDa bands in the total brain membranes as 1:1 (Figure 6.6c), this ratio in synaptosomal and SPM receptor isolates was estimated as 5:1. Thus, it could be reasoned that comparatively, more 65 kDa protein was eluting with the NTF from the total brain than from the other membrane preparations.

Furthermore, we quantitatively assessed the staining of the N-terminal fragment by the anti-NTF antibody from the SPM preparation as we had done for the total brain earlier (Section 6.2-3) and compared this with staining of the C-terminal fragment by the anti-65 kDa antibody. Computer-aided densitometry showed that the relative intensity of the NTF to the 65 kDa protein was about 5 to 1 in the SPM preparations (Figure 6.7) at different protein concentrations and exposure times. Whereas the brain preparation contained approximately 1.5 times less C-terminal fragment than the NTF, in SPM preparations, there was five times as little 65 kDa protein co-purifying with the NTF. A possible reason for the non-equimolar isolation of the 65 kDa protein from synaptosomes and SPM could potentially be smaller amounts of this protein in these

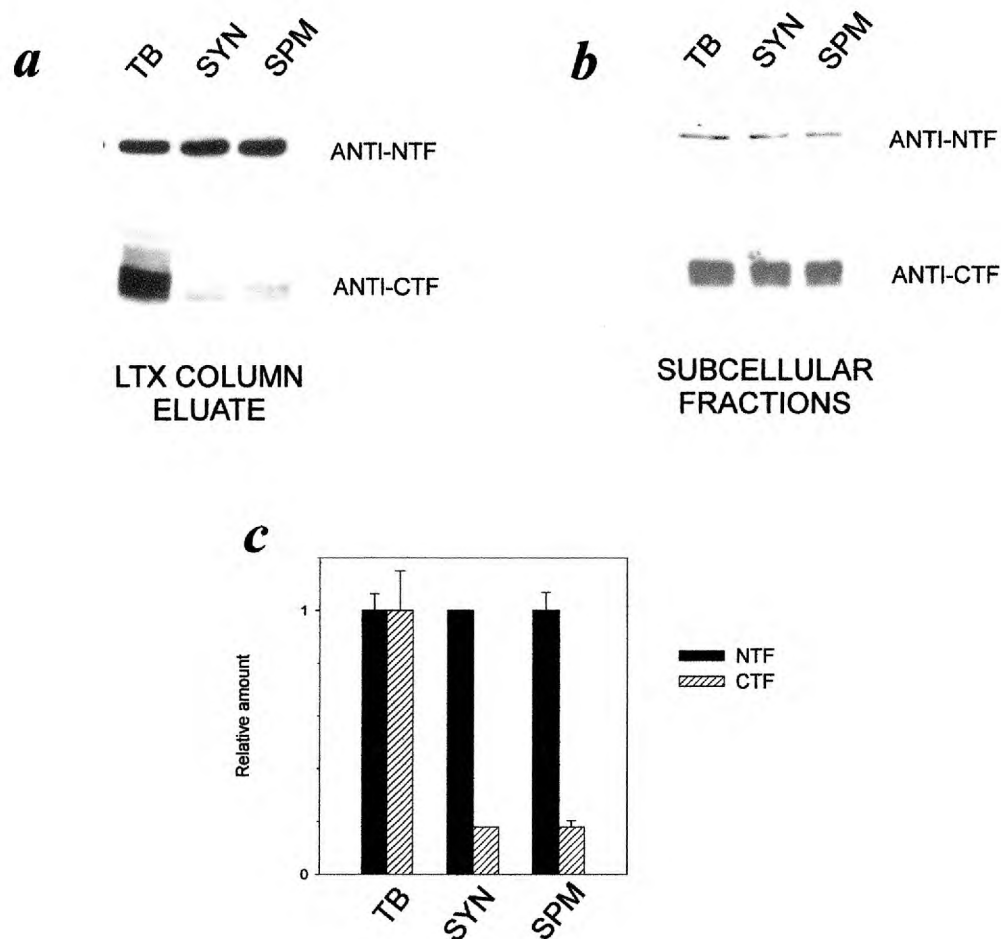


Figure 6.6 Less of the 65 kDa protein (CTF) co-purifies with the NTF in synaptosomes and SPM. Total brain membranes (TB), synaptosomes (SYN) and synaptic plasma membranes (SPM) were solubilised in Thesit and used for LTX affinity chromatography as described in the text. *a*, The eluates as analysed on Western blots by immunostaining with respective antibodies. Notice how little the CTF stains with the NTF from synaptosomes and SPM compared to total brain membranes. *b*, Immunostaining of subcellular brain fractions. Note the similar staining of NTF and CTF, respectively, in all three types of membrane. *c*, Bar chart illustrating the relative amounts of NTF and CTF from different brain fractions, isolated on LTX columns.

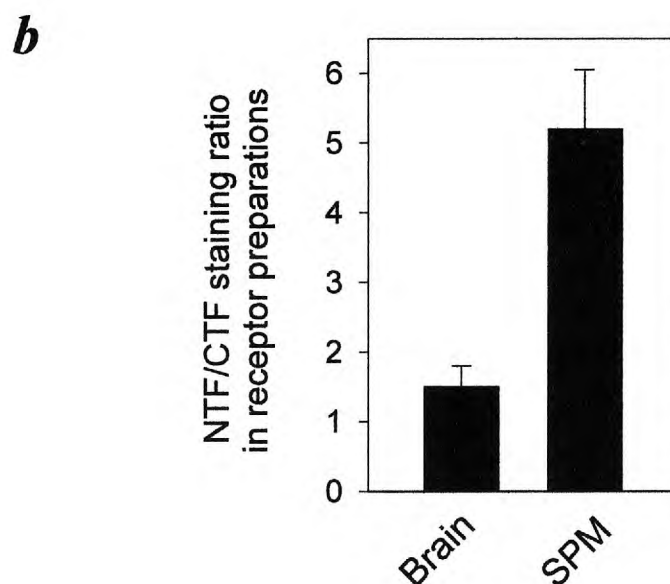
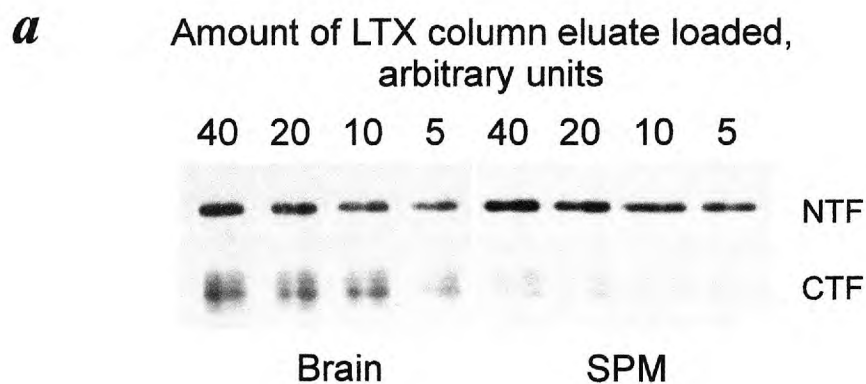


Figure 6.7 The 120 kDa (NTF) and 65 kDa (CTF) latrophilin fragments do not co-purify in equimolar amounts off the LTX column. Preparations of total brain membranes or synaptic plasma membranes (SPM) were passed through fresh LTX affinity columns. **a**, Different amounts of the LTX column eluates loaded and stained on the same blot with anti-NTF and anti-CTF antibodies. **b**, The ratio of NTF/CTF staining measured for several amounts of LTX column eluate and different film exposures (20 to 60 sec). Note there is approximately 1.5 times less CTF than NTF isolating from the brain off the column. In SPM eluates, > 5 times as little CTF co-purifies with the NTF.

membrane preparations. Therefore, samples of total brain membranes, synaptosomes and SPM were directly (i.e. before passing through the LTX column) separated by SDS-PAGE and stained with anti-NTF and anti-C-terminal antibodies on immunoblots. This revealed that the relative amounts of 120 kDa and 65 kDa protein, respectively, were equal in all three samples (Figure 6.6b). This suggests that relatively lower amounts of the C-terminal fragment isolated on the LTX column from processed membranes are due to some functional changes that this protein may undergo during membrane preparation, and are not due to degradation or to initially low quantity of this fragment in such membranes. Hence, it can be concluded that the interaction of the NTF and 65 kDa protein is actually dependent on the state of the membrane from which it is isolated.

6.2-4 The 65 kDa receptor fragment is phosphorylated

It was noticed that the anti-CTF antibody usually stained four bands between 65-68 kDa. The close proximity of these bands suggested that they might differ by a small modification e.g. phosphorylation. To test this theory, a WGA latrophilin preparation (from total brain) was treated with alkaline phosphatase, an enzyme that dephosphorylates proteins modified by all types of phosphorylation. After incubation with this enzyme, the four bands were gradually reduced to the 65 kDa one similar in its mobility to the C-terminal fragment from COS cells expressing latrophilin (Figures 6.8a). The 65 kDa protein was most likely unphosphorylated in these cells lacking the system of latrophilin signalling and/or desensitisation. This suggests that the 65 kDa protein has three phosphorylation states.

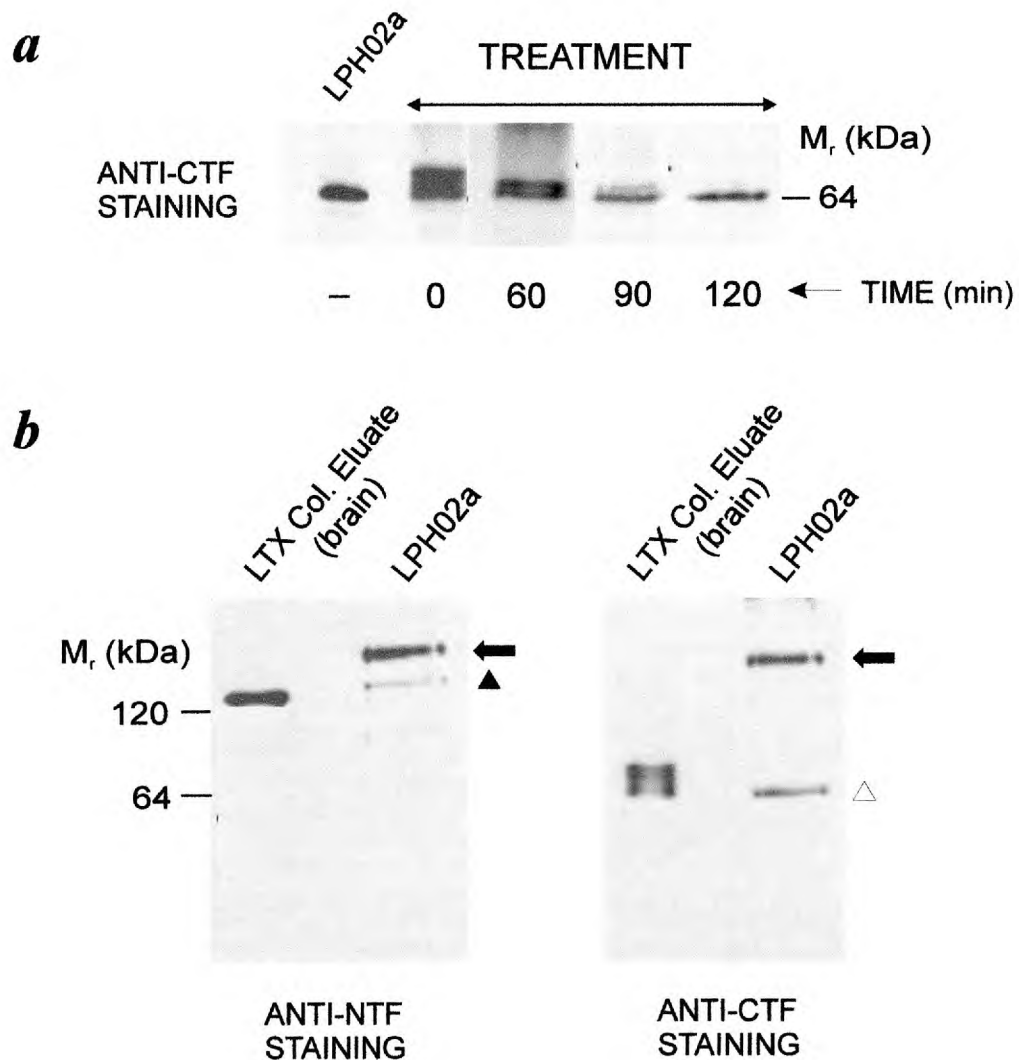


Figure 6.8 The CTF is phosphorylated. *a*, The CTF of latrophilin obtained from rat brain after LTX and WGA column chromatographies consists of four close bands (time zero). This preparation was treated with alkaline phosphatase over a 2 hour period as described in Chapter 2. Immunostaining of this blot with anti-CTF antibody shows that the four bands are reduced to a single band of ~ 65 kDa with time, indicating that the three uppermost bands of the CTF represent different phosphorylated forms of the receptor fragment. *b*, When construct LPH02a is expressed in COS-7 cells the majority of latrophilin is full size (~ 180 kDa, ←), as demonstrated by the specific staining of anti-NTF and anti-CTF antibodies. However, the NTF (▲) and CTF (△) are also present. The latter appears as a sharp band of ~ 65 kDa because it is presumably not phosphorylated unlike in the brain.

6.2-5 Sequencing of the 65 kDa band

To confirm that the 65 kDa band contained the C-terminal portion of latrophilin it was sequenced from receptor expressed in COS-7 cells as it was possible to obtain large amounts of the unphosphorylated 65 kDa protein from these cells (Figure 6.8b). The N-terminus of the 65 kDa band began with the sequence TNFAVL, which indicated a point of cleavage between L⁸⁰⁹ and T⁸¹⁰ residues just upstream of the first transmembrane region of LPH (Figure 6.9). As the protein also contains the very C-terminal peptide (as demonstrated by immunostaining) the whole fragment between T⁸¹⁰ and the C-terminus of latrophilin has been named the “CTF” (C-terminal fragment).

In addition, another N-terminal sequence has been determined in some CTF preparations (ISLVC), indicating that a second cleavage site is present in latrophilin within the first transmembrane region (Figure 6.9). The significance of this site is not known as the size of the CTF produced in such a case is ~ 60 kDa, which has rarely been detected on gels. It is most likely that this cleavage is caused by a protease co-purifying with the CTF, as it can be blocked by protease inhibitors employed during protein isolation procedures.

6.2-6 The NTF and CTF are both brain specific

As the CTF contained the transmembrane domains and C-terminus of latrophilin it was logical to suggest its brain specificity, as had been demonstrated for the NTF previously [Davletov *et al.*, 1996; Krasnoperov *et al.*, 1996]. To further prove the latrophilin localisation using also the anti-C-terminal antibodies, membrane fractions were prepared from the following rat tissue: brain, kidney, liver, heart and small intestine. Equal amounts of each preparation were loaded on SDS-PAGE and

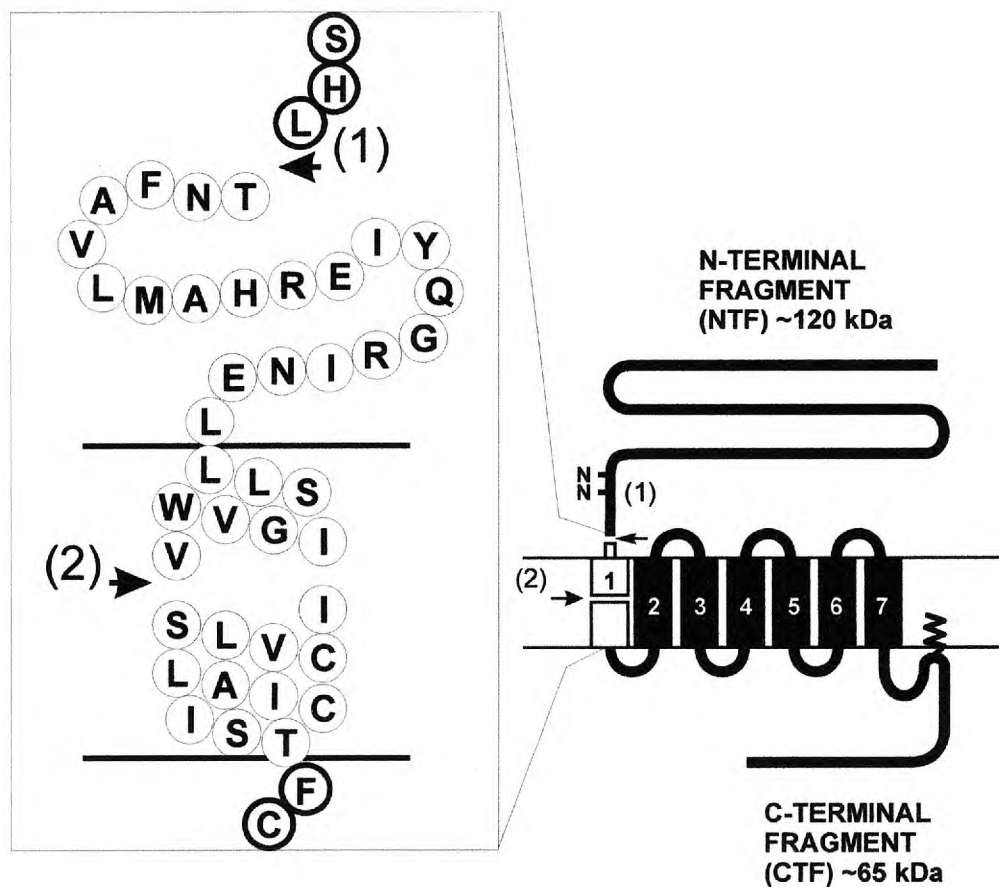


Figure 6.9 Latrophilin is cleaved in two positions. The principle cleavage site (1) is at a position 20 amino acids upstream of the first transmembrane domain resulting in the production of the NTF and CTF products described in the figure. Therefore, the CTF, in addition to the latrophilin's C-terminus, contains all seven of its transmembrane regions. Also, a second cleavage site (2) was identified within the first transmembrane region, as indicated. The size of the product formed in this case is ~ 60 kDa, which is sometimes seen on SDS-gels.

stained directly with an affinity-purified antibody to the CTF after blotting. The C-terminal antibody recognised the four bands of 65-68 kDa only in the brain preparation (Figure 6.10, lower panel).

The immunoblot was also stained with the anti-NTF antibody for comparison (Figure 6.10, upper panel). The 120 kDa fragment of latrophilin was detected in the brain fraction as expected but nowhere else. The anti-120 also stained a band of ~ 85 kDa band extremely well in the brain. This protein is, however, not related to latrophilin as it does not purify on the LTX column and other batches of anti-NTF antibody recently tested do not stain the 85 kDa band (personal observations).

6.2-7 Presynaptic localisation of latrophilin

To be the physiological receptor for LTX, latrophilin must not only be brain-specific but it must be distinctively presynaptic. To demonstrate this, we next attempted to isolate presynaptic membranes by chemically dissociating the pre- and postsynaptic membranes at the synaptic cleft. Using a combination of mild homogenisation in a DTT-containing buffer (see Materials and Methods) followed by separation on sucrose density gradients, it was possible to obtain fractions enriched in pre- or postsynaptic membranes from a preparation of total synaptic membranes (SPM), as shown by the use of specific antibody markers (Figure 6.11). The method was a modification of that applied by Ratner & Mahler [1983] to isolate an enriched preparation of postsynaptic membranes.

When these preparations were stained with anti-NTF and a peptide antibody specific to the third cytoplasmic loop of latrophilin (LPH-3CL), both antibodies explicitly recognised the presynaptic membrane fraction (Figure 6.11). The specificity

of the peptide antibody was confirmed by its successful exhaustion against the cognate peptide used to generate it (Figure 6.12). Interestingly, an antibody specific to the neurexins also preferentially stained the enriched presynaptic membrane fraction (Figure 6.13) although a large proportion of both α - and, to a lesser extent, β -NRX still resided in the “postsynaptic membranes”.

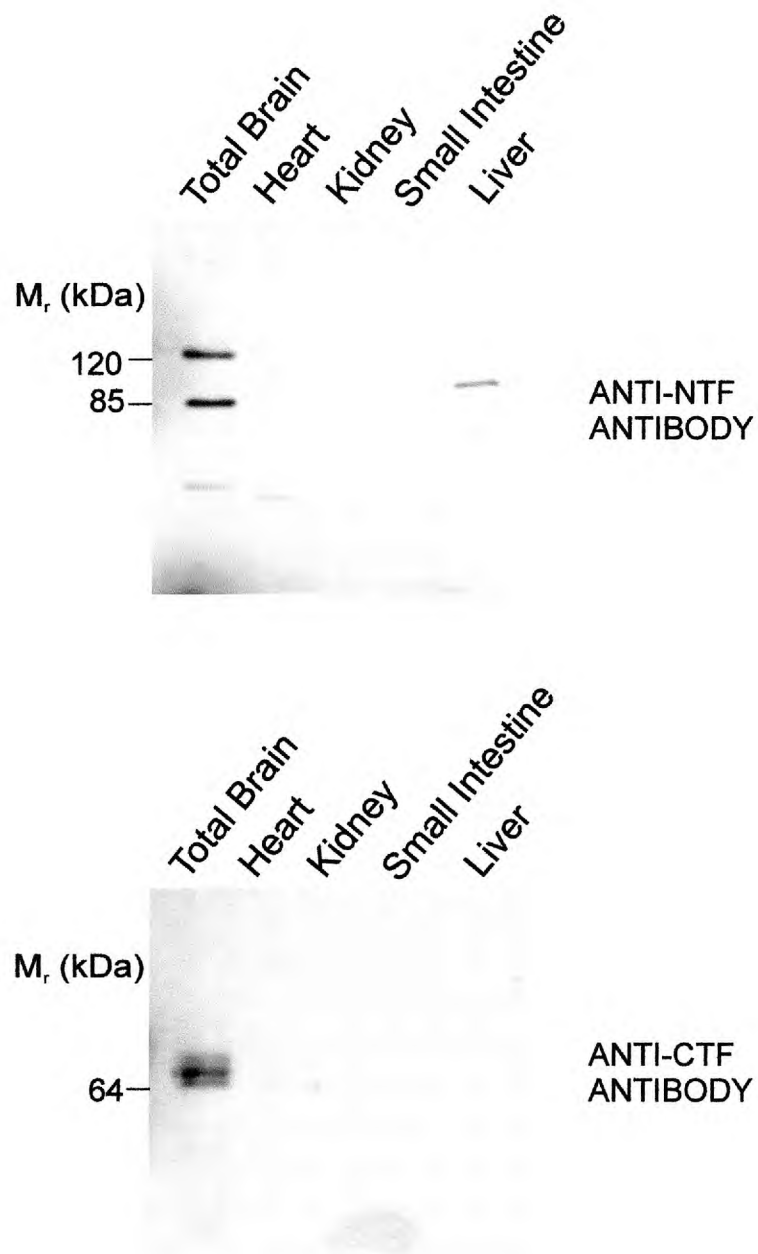


Figure 6.10 The NTF and CTF are brain specific. 50 μ g of each tissue was loaded on SDS-PAGE without boiling the samples. After Western blotting onto PVDF, the membranes were stained with affinity-purified antibodies to the NTF and CTF. Both the NTF (120 kDa) and CTF (65 kDa) are specific to the brain. The anti-NTF antibody also recognises well a band of $\sim$ 85 kDa in the brain, which, however, is unrelated to LPH (see text).

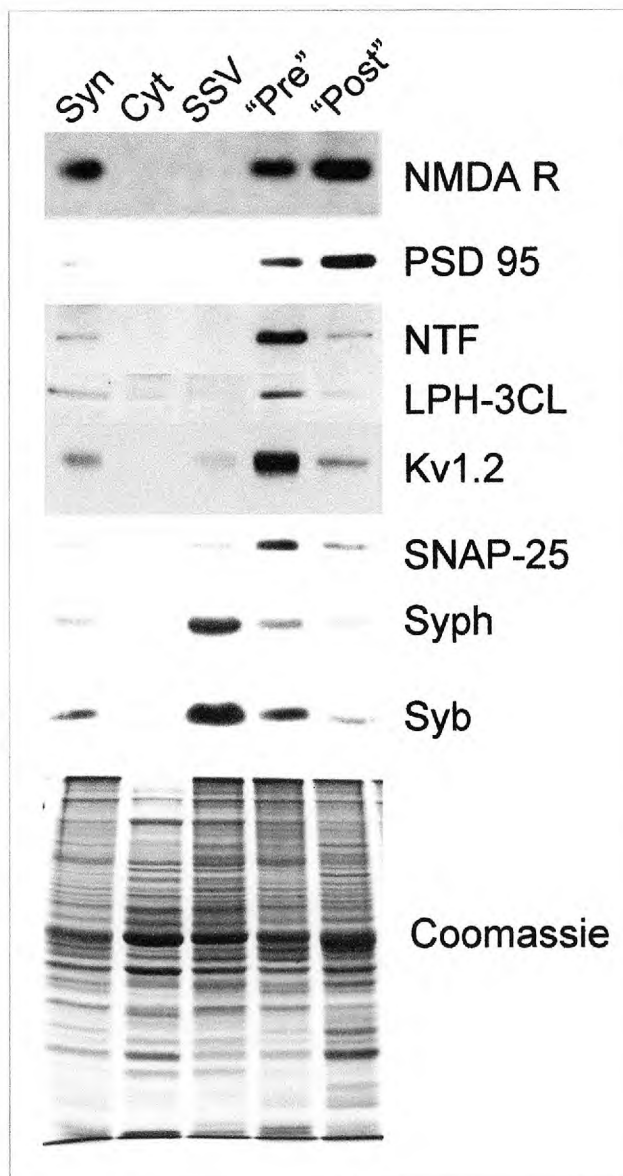


Figure 6.11 Latrophilin is a presynaptic receptor. Subcellular brain fractions, synaptosomes (Syn), cytosol (Cyt), synaptosomal synaptic vesicles (SSV), “presynaptic” and “postsynaptic” membranes (“Pre” and “Post,” respectively) were prepared as described in Materials and Methods. After equalising the preparations for protein content these were Western blotted and then stained using antibodies specific for marker proteins of presynaptic membranes (Kv 1.2 and SNAP-25), postsynaptic membranes (NMDA R and PSD 95) and synaptic vesicles (synaptophysin, Syph and synaptobrevin, Syb). An affinity-purified antibody to the N-terminus (NTF) and a peptide antibody to the third cytoplasmic loop of latrophilin (LPH-3CL) were also used to stain the preparation. The two latrophilin antibodies preferentially stained presynaptic membranes.

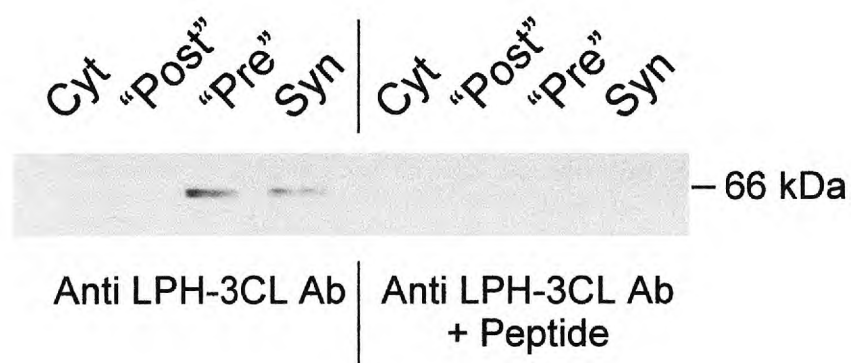


Figure 6.12 Testing the specificity of LPH-3CL. An aliquot of anti-LPH-3CL antibody was exhausted against the cognate peptide (P.10, see Materials and Methods) and then used to stain a blot containing synaptosome (Syn), cytosol (Cyt), "pre-" and postsynaptic membranes preparations ("pre" and "post," respectively). Compared to the control (left) the antibody staining was completely abolished after exhaustion proving it to be specific.

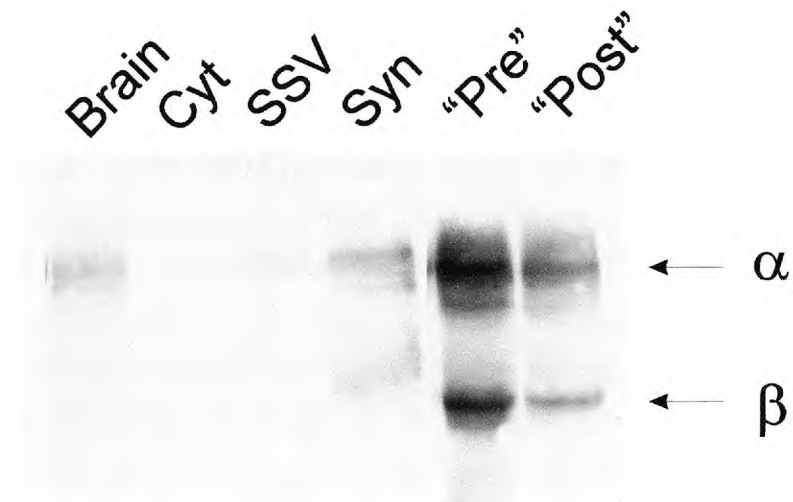


Figure 6.13 Neurexin is also presynaptic. Total brain (Brain), synaptosomes (Syn), cytosol (Cyt), synaptosomal synaptic vesicles (SSV), presynaptic ("Pre") and postsynaptic ("Post") membrane preparations (all equalised for total protein content) were also stained with antibodies against the neurexins. Like latrophilin, the staining of both α - and β -neurexins was preferentially presynaptic.

6.3 Discussion

Previously, all latrophilin preparations obtained from the LTX affinity column had been analysed after their precipitation by chloroform/methanol [Davletov *et al.*, 1996; Krasnoperov *et al.*, 1996 and Chapter 5]. Under these conditions only a 120 kDa band was seen wholly specific for the column, and the CTF, a protein of ~ 65 kDa, was missed. Sample precipitation or boiling appears to induce irreversible precipitation of the CTF, after which only small amounts can be detected in the resolving gel after SDS-PAGE. The size of the CTF fragment when added to the molecular mass of the NTF gives a product of ~ 185 kDa, consistent with that expected for the receptor from its amino acid sequence and the determined extent of glycosylation [Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997]. However, the existence of two distinct fragments is indicative of receptor cleavage. N-terminal sequencing of the CTF reveals that the position of this cleavage is just before the first transmembrane domain hence the CTF contains not only the C-terminus of latrophilin but also all seven of its transmembrane regions.

The principle position of cleavage determined in our work is in agreement with the work of Krasnoperov *et al.*, 1997. These authors sequenced the whole eluate from the LTX column and found the sequence TNFAVLMAHREYI, beginning at amino acid residue 810 of latrophilin (just upstream of the first transmembrane region). However, they saw the CTF as a band of ~ 85 kDa on SDS-urea PAGE. In addition, when sequencing a protein preparation containing many proteins, it is possible to obtain sequences from products of degradation that can arise during purification. By

sequencing an isolated 65 kDa band from LPH-expressing cells (refer to 6.2-5), we found that the N-terminus of this protein indeed contains TNFAVL. It is possible, therefore, that the large size of the CTF (~ 85 kDa) seen in the work of Krasnoperov and colleagues was due to its abnormal mobility in urea-containing gels.

A second site of cleavage was also determined within the first transmembrane domain of latrophilin. Cleavage at both positions could be prevented by the use of the protease inhibitor, MG-132, on latrophilin-expressing cells. It is of interest to note that MG-132 is a non-specific inhibitor of γ -secretases, which are aspartyl proteases responsible for the cleavage of receptors (such as amyloid precursor protein and Notch) within the plasma membrane [see review by Hooper *et al.*, 1997]. The physiological significance of an intramembrane cleavage site within latrophilin is unknown at present but it would result in the physical separation of the NTF and CTF whilst retaining both subunits on the membrane. Perhaps this occurs to prevent further signalling by this G protein-coupled receptor after over-stimulation by its endogenous ligand or is part of latrophilin's mechanism for turnover.

After cleavage the NTF and CTF can interact as the two fragments demonstrate a similar pattern of elution from affinity columns and solubilisation from membranes in different detergents when stained with specific antibodies. In addition, an anti-CTF antibody was able to co-immunoprecipitate the NTF with the CTF [Krasnoperov *et al.*, 1997]. This explains the co-purification of G proteins, which normally bind to the third cytoplasmic loops of GPCRs, with the NTF; their solubilisation in sample buffer was unaffected by precipitation and boiling (Chapter 5). However, our results (Figures 6.5, 6.6 and 6.7) illustrate that the interactions between the NTF and CTF are not always maintained and are subject to precise conditions. First, Ca^{2+} or Mg^{2+} apparently

decreased the relative amount of CTF eluting from the LTX column compared to EDTA but the relative amount of NTF never altered. Second, the relative proportion of CTF isolated from synaptosomes or SPM was much lower than in latrophilin preparations from the total brain (by about 5-fold).

In the presence of Ca^{2+} or Mg^{2+} during affinity chromatographies, the CTF may lose some affinity to the NTF. It is equally plausible that under these conditions the CTF has reduced affinity for LTX itself. In either case the results indicate that the NTF and CTF interactions are not stable. They also imply that the NTF is the more important fragment involved in recruiting LTX to the presynaptic membrane. This is consistent with its extracellular nature. The use of different membrane preparations for LTX affinity chromatography also alters the amount of CTF isolating with the NTF. This most likely reflects the greater separation of the two latrophilin fragments caused by the extensive manipulation of the brain membranes. Certainly, to prepare synaptosomes or SPM (refer to Materials and Methods), latrophilin as well as all other brain proteins must undergo several rounds of homogenisation and centrifugation, all of which could probably disrupt forces holding together the NTF and CTF.

The CTF is phosphorylated, which is consistent with the predicted possible phosphorylation sites for cAMP-dependent protein kinase, protein kinase C and casein kinase II within this fragment [Leliana *et al.*, 1997]. The state of phosphorylation of this fragment may influence its interaction with the NTF and will need to be investigated in future experiments. In receptor preparations syntaxin, one of the SNARE proteins involved in synaptic vesicle release [reviewed by Schiavo *et al.*, 2000] was detected in LTX column eluates. Indeed, the CTF has been shown to interact with syntaxin using immunoprecipitation experiments with anti-syntaxin and anti-CTF

antibodies [Krasnoperov *et al.*, 1997]. Therefore, latrophilin may have a direct link to the neurosecretion machinery through its interaction with this protein.

The NTF and CTF are both brain-specific and, more importantly, presynaptic; thus, both protein fragments are delivered to the same functional compartment of the neuronal cell. This strengthens the idea that latrophilin is the physiological receptor for LTX, as the toxin is known to act presynaptically [Valtorta *et al.*, 1984]. Neurexin Ia is also present in the presynapse, and could function in LTX action along with latrophilin. However, its separation into the presynaptic membrane fraction is less dramatic as that of LPH. This may reflect NRX's localisation also in myelinated nerves and astrocytes [Russell & Carlson, 1997], which are not easily removed from either the pre- or postsynaptic membrane fractions by the chemical treatment used on SPM.

The cleavage of latrophilin into the NTF and the CTF occurs within the cell, probably in the endoplasmic reticulum, soon after its synthesis while the NTF remains somehow anchored in the membrane [Volynski and Ushkaryov, unpublished observations]. What could be the functional significance of this complicated post-translational processing of this G protein-coupled receptor? As the two fragments interact but can be separated under certain conditions, it is tempting to speculate that the NTF could be an endogenous ligand of the CTF. Such a relationship is known to occur in protease receptors (e.g., thrombin receptor) which are cleaved upon ligand binding; the small peptide product remains bound to the extracellular domain of the seven transmembrane region fragment and is responsible for the activation of the receptor [reviewed in Vergnolle, 2000].

Alternatively, the NTF and CTF could be co-receptors binding another ligand. The separation of the two proteins could induce intracellular signalling, while phosphorylation of the CTF may be the result of its desensitisation by the cell's recovery mechanism. In fact latrophilin may represent just one receptor in a new subfamily of GPCRs. For example, CD97 (a member of the SecR subfamily of GPCRs like LPH) is also proteolytically cleaved before the first membrane-spanning domain [Gray *et al.*, 1996]. Based on sequence comparisons, other G protein-coupled receptors such as polycystin 1 are reported to do the same [see Ponting *et al.*, 1999]. Therefore, the continued study of latrophilin will not just reveal new pathways of exocytosis but help to understand the mode of action of several other GPCRs.

6.4 Summary



Latrophilin is cleaved just upstream of the first transmembrane region to produce two fragments: the NTF (N-terminal fragment, or 120 kDa) and the CTF (C-terminal fragment, or 65 kDa).



N-terminal sequencing and immunostaining of the CTF reveals that it must contain all seven of latrophilin's transmembrane regions and its C-terminus.



The CTF is not resolved on SDS-gels if latrophilin preparations are concentrated by precipitation before sample preparation or the samples boiled before loading.



The CTF has three phosphorylated forms.



A method to enrich pre- and postsynaptic membranes using sucrose gradients reveals that both latrophilin and neurexin I α are presynaptic

CHAPTER SEVEN



FRAGMENTS GENERATED
BY CLEAVAGE BEHAVE AS
SEPARATE PROTEINS

7.1 Overview

Latrophilin is cleaved into two fragments, the NTF and the CTF, shortly after synthesis. Both are transported to the same functional subcellular compartment (presynaptic membranes) but may only be linked conditionally. The work reported in this section continues the investigation into the behaviour of the two latrophilin fragments after proteolytic modification, using molecular biology and biochemical approaches.

It is shown by immunofluorescent confocal microscopy that the NTF and CTF are expressed on the surface of COS-7 cells but are practically not co-localised. Using the same technique it is demonstrated that the NTF itself can exist independently of the CTF at the cell surface. Together with data obtained from the biochemical analysis of the N-terminal fragment, this suggests that the 120 kDa protein is strongly anchored to the plasma membrane after being transported there. Thus the NTF and CTF may behave as separate proteins after cleavage, which, however, are capable of interaction under certain conditions. LTX may bring about this functional interaction as the toxin can bind both receptor fragments (shown by sucrose density gradients).

7.2 Results

7.2-1 Expression of LPH constructs in COS-7 cells.

In all, three latrophilin constructs have been expressed in COS cells for the work described in chapters 6 and 7: LPH02a, LPH06a and LPH12 (see Figure 2.2). Before any of these mutant latrophilins were used, they were characterised for their expression of protein and binding to LTX. Maximal binding of LTX to expressed latrophilins was seen about 24 hr post-transfection (Figure 7.1a). This trend was observed for all three constructs. After this period, the binding decreased rapidly, although the overall amount of latrophilin expressed in cells continued to increase up to 40 hr before stabilising, as determined by immunostaining (Figure 7.1b and 7.1c). The degree of radiolabelled-LTX binding to cells did not correlate with the expression of full-size latrophilin, but instead, with the appearance of the LPH N-terminal cleavage products (termed α and β), as shown in the lower panel of Figure 7.1c. The peak of α and β expression occurred at 24 hr consistent with measurement of maximal LTX binding. It, therefore, appears that the NTF binds LTX and, in order to do so, the N-terminal fragment must be transported to the cell surface and reside there after latrophilin is cleaved. Since a high level of expressed receptor together with good binding to α -latrotoxin was observed 12 to 24 hours after transfection, all COS cell experiments were carried out within this period.

7.2-2 Are the NTF and CTF co-localised at the cell surface?

Latrophilin is cleaved within the endoplasmic reticulum (ER) lumen just after synthesis and then transported to the cell surface [Kirill Volynski, personal

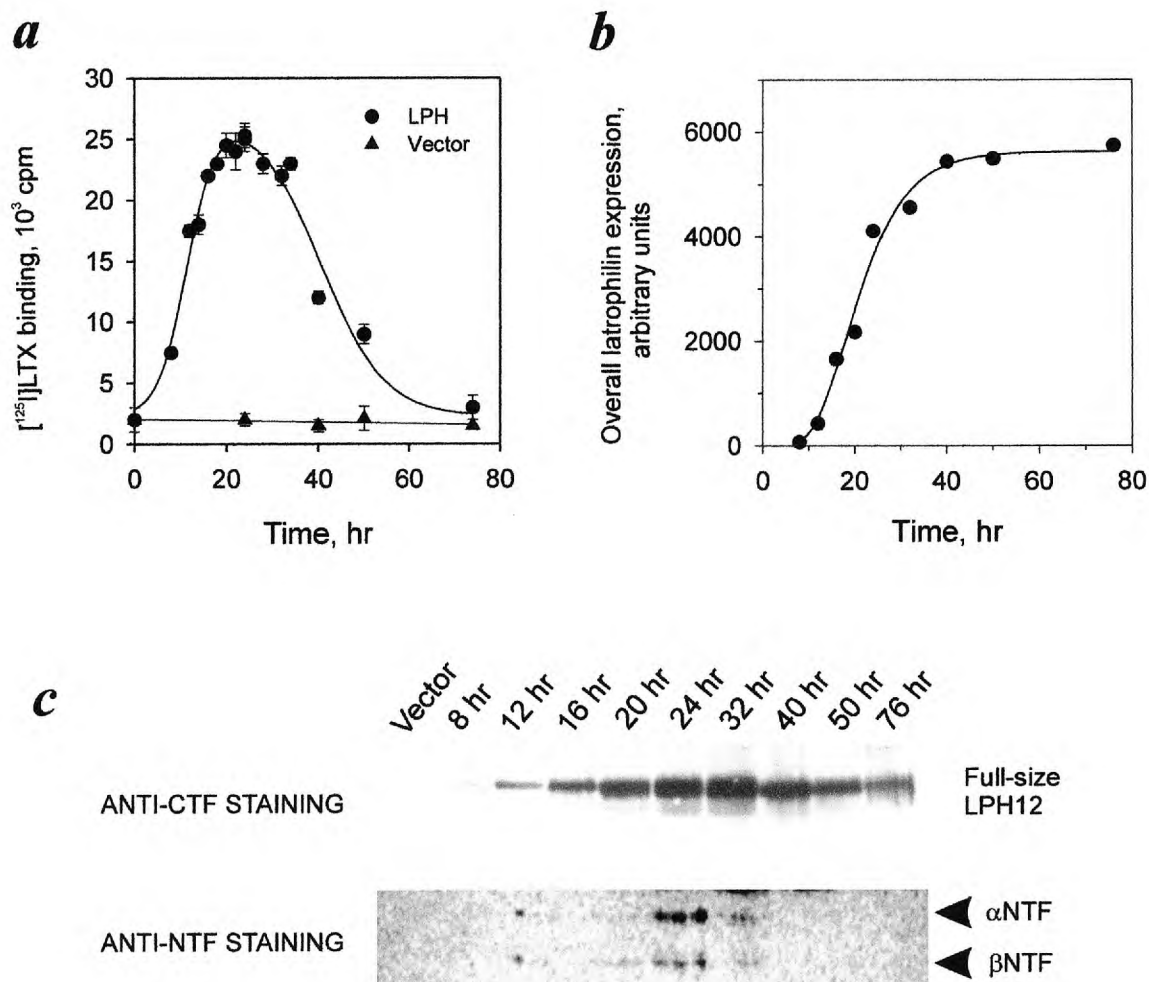


Figure 7.1 Binding and expression of LPH12. *a*, The binding of $[^{125}\text{I}]\text{LTX}$ to COS cells expressing LPH12 was examined over a 76 hr period. As a control, this was compared to COS cells transfected with vector alone (see Materials and Methods). Maximal binding occurs at about 24 hours post-transfection. *b*, The overall amount of latrophilin synthesised by COS cells. This increases until about 40 hr post-transfection. *c*, The expression of LPH12 protein checked using immunostaining. The vector control loaded on the blot was taken after 24 hr. The upper panel shows the expression of the full-size receptor, which peaks at 32 hr, and the lower panel shows the appearance of LPH cleavage products, α and β (both N-terminal fragments). Notice that the maximal binding of iodinated-LTX to COS cells correlates with the peak of αNTF and βNTF .

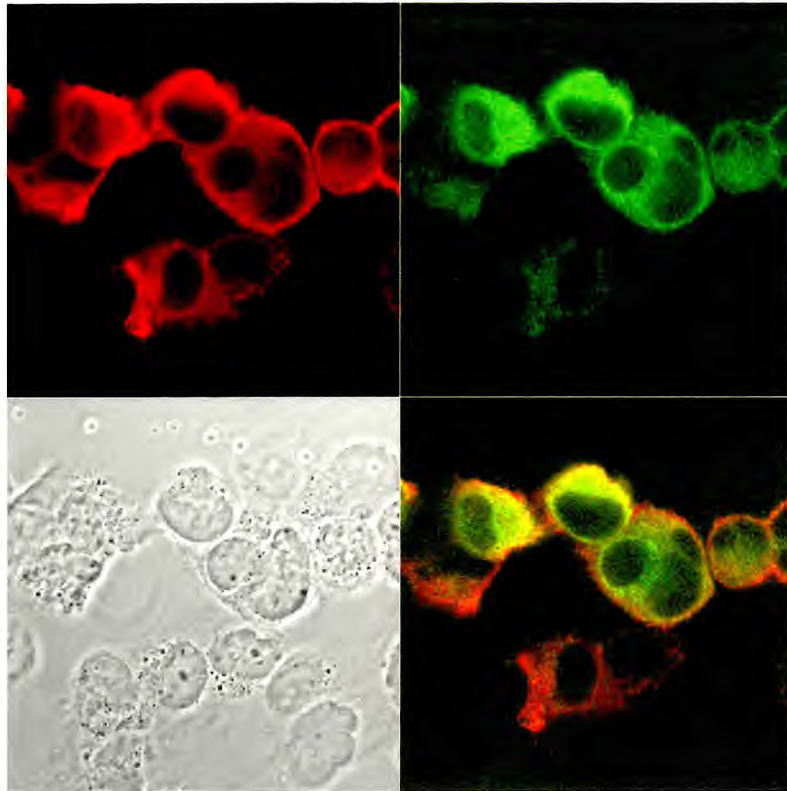


Figure 7.2 The NTF and CTF are mostly not co-localised. LPH12 was expressed in COS-7 cells. After 24 hr, confocal microscopy and immunostaining was used to visualise the distribution of the two latrophilin fragments. Cells were permeabilised after fixing in order to visualise the intracellular CTF. *Top left*, Red fluorescence indicates position of the CTF. *Top Right*, Green fluorescence indicates position of the NTF. *Bottom left*, Phase contrast image of the COS cells used for this experiment. *Bottom right*, The overlay of the NTF and CTF fluorescence shows where the two fragments co-localise (visible as yellow). The complete co-localisation is not abundant and can be attributed to the full-size LPH, which is known to be present in the cells. Note that NTF is mostly localised in some vesicular compartment inside the cell, while the CTF is present in other vesicles as well as on the plasma membrane.

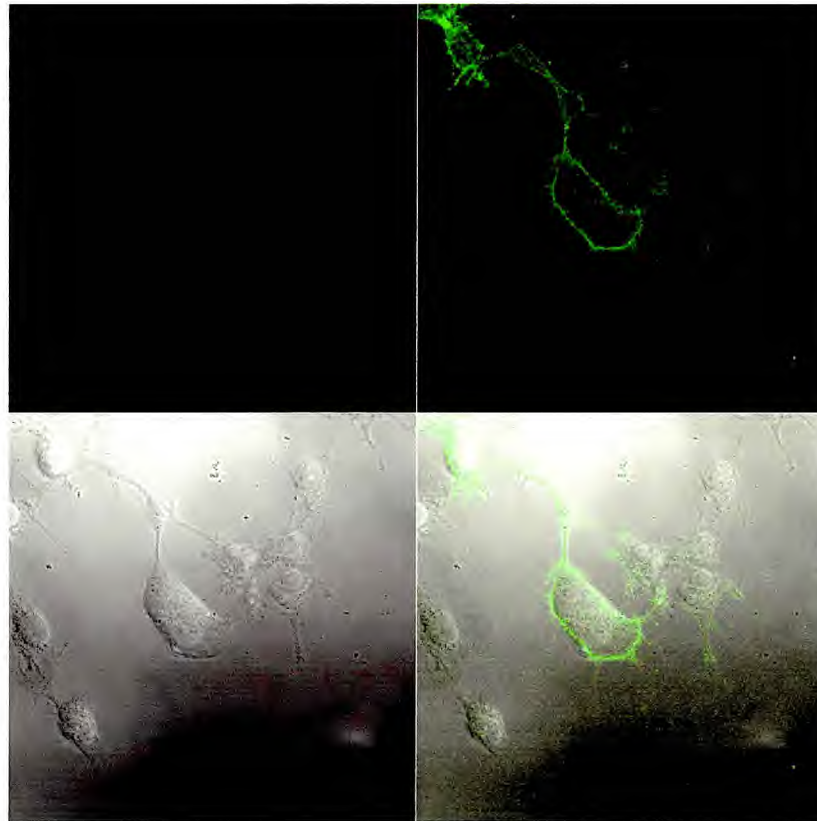


Figure 7.3 The NTF is expressed on the COS cell surface. COS-7 cells expressing LPH12 were immunostained with anti-NTF and anti-CTF antibodies without permeabilising cells, 24 hr post-transfection. Confocal microscopy was used to visualise the specific fluorescence. *Top Left*, Red fluorescence, indicative of the CTF, is absent when cells are not permeabilised. *Top Right*, The green fluorescence, indicative of the NTF, is found on the cell surface. *Bottom Left*, Phase contrast image of the COS cells used for this experiment. *Bottom Right*, Overlay of the anti-NTF staining onto the phase contrast image of the COS cells. Note that not all cells are transfected with latrophilin, but only those that are fluoresce demonstrating the specificity of the anti-NTF antibody.

communication]. In order to determine whether the NTF and CTF remain together, we studied the localisation of the two latrophilin fragments on the surface of COS-7 cells expressing LPH-12, using immunocytochemistry and confocal microscopy. To obtain staining for the intracellular CTF, the COS cells were permeabilised with 0.05% Triton X-100. The staining of the NTF (green fluorescence) and the Myc epitope on the CTF (red fluorescence) were clearly visible inside COS cells (in the ER and Golgi complex) but also at the cell surface (Figure 7.2). At the cell surface, the red CTF staining appeared on the outer perimeter of the cells whilst the green NTF staining was more evident in the inner perimeter, suggesting that the NTF and CTF are physically located in different compartments at the plasma membrane and are, therefore, not co-localised. This was confirmed when the two fluorescent markers were overlayed to reveal that only a small proportion of the NTF and CTF co-localises (as shown by the yellow colour in Figure 7.2).

Since a substantial amount of the uncleaved, full-size latrophilin is detectable on Western blots, some co-localisation of the N-terminal and the C-terminal staining (yellow colour) can be attributed to this uncleaved protein, suggesting that the cleaved NTF and CTF practically do not co-localise. Thus, after cleavage, the two latrophilin fragments are transported to the membrane surface but the majority of them are not together. This result also presents the first evidence that the NTF can anchor to the cell surface independently of the CTF (see later). Interestingly, most of the NTF staining was found within the cells when studies were performed using permeabilised cells. To prove that the NTF is indeed on the cell surface, experiments were repeated without permeabilisation. Under these conditions, the NTF staining was clearly observed on the membrane surface (Figure 7.3), indicating that only a small proportion of this fragment

is either transported to the surface or remains there after the majority of the NTF is endocytosed.

7.2-3 Surface labelling of COS cells expressing LPH-12

Although very little yellow colour was seen at the cell surface in the overlaid NTF- and CTF-stained images, it was not clear whether this was due to co-localisation of the two fragments or to the presence of the full-size protein on the plasma membrane. To address this question, surface labelling of COS cells expressing LPH12 was performed using biotinylation. The potential sites for biotinylation were the NTF and the extracellular loops of the CTF. To isolate biotinylated proteins, they were pulled down using avidin-agarose beads. These were then subsequently stained with anti-NTF and anti-CTF antibodies on immunoblots. Although the cleaved latrophilin products were predominantly stained by the two antibodies a trace of full-size latrophilin was also visible (Figure 7.4). Consistent with the findings using immunocytochemical labelling of COS cell membranes, these results suggest that (i) low amounts of full-size, uncleaved latrophilin can be transported to the surface of COS cells and (ii) there is almost no co-localisation of the NTF and the CTF even on the cell surface (as the yellow colour indicating co-localisation can be attributed to the full-size protein).

7.2-4 The NTF is membrane bound

Because the cleavage site in latrophilin is localised upstream of the first transmembrane domain, the proteolytic processing of this protein in the ER should theoretically result in a soluble NTF fragment. If the NTF is bound reversibly to the surface via some protein-protein interactions (e.g., with the CTF), some of the N-terminal fragment may be expected to dissociate from the membranes with time.

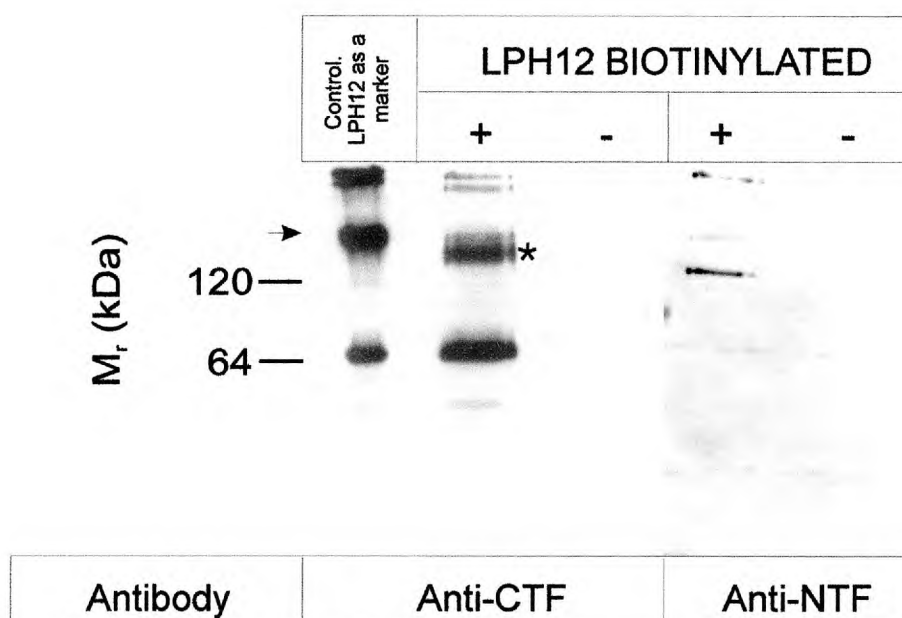
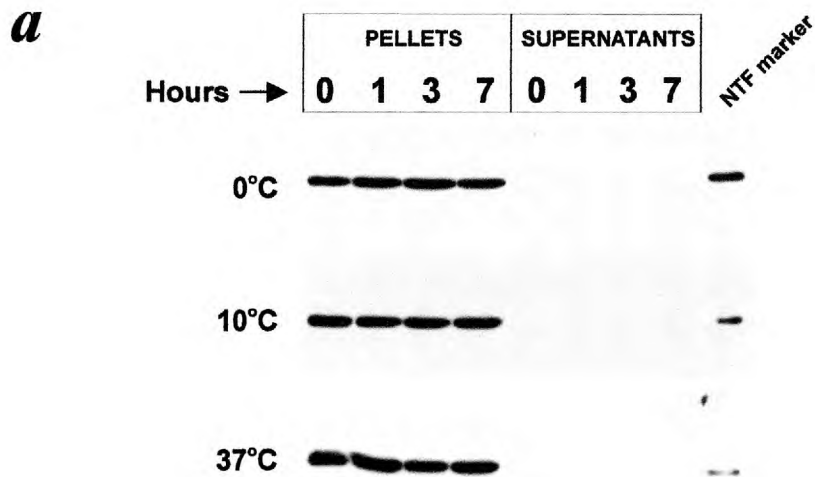


Figure 7.4 Surface labelling of COS cells using biotinylation. LPH12-expressing COS cells were subjected to biotinylation one day after transfection, as described in the Materials and Methods. Labelled proteins were precipitated with avidin-agarose beads after solubilising the cells, and stained with specific antibodies, as shown. For control, COS cells expressing LPH12 were treated in the same way but without the addition of the reagent. The arrow marks the position of the full-size receptor of which a trace can be seen labelled by either antibody after biotinylation. The band heavily stained by the anti-CTF antibody below the full-size receptor is in the position of the CTF dimer (marked by an asterisk). Relative positions of other molecular mass markers are indicated on the figure.

We, therefore, examined whether there was a gradual increase in the release of the NTF from synaptic membranes over time by incubating a fresh preparation of SPM for seven hours at 3 different temperatures (0°C, 10°C and 37°C). At hourly time-points pellet and supernatant fractions were prepared (as described in Materials and Methods). These were examined on an immunoblot with an affinity-purified antibody to the NTF (Figure 7.5a). Over seven hours, no soluble NTF was detected in the supernatants and the amount of NTF stained in the pellet fractions remained constant. In a further experiment, the solubility of the NTF from SPM was compared to the α -subunit of potassium channel, Kv1.2. This protein was chosen because it is presynaptic, as is LPH (Chapter 6) and possesses six transmembrane regions making it an integral membrane protein [see Harvey, 1997]. Remarkably, the solubilisation of NTF in increasing amounts of Thesit exactly matched that of Kv1.2 (Figure 7.5b). Taken together, these studies suggest that the extracellular portion of latrophilin is strongly membrane-bound.

7.2-5 Does the CTF anchor the NTF to the membrane?

The NTF could behave as a membrane-bound protein due to some extremely strong protein-protein interactions, probably with the CTF. To investigate this possibility, chemical treatments were performed on SPM to disrupt protein-protein interactions. Synaptic membranes were first treated with DTT to reduce any disulphide bonds before incubation with the various reagents, which included high and low pH, high molarity chaotropic salts and even urea (for a short period; otherwise, urea dissolved the membranes) (Figure 7.6). Although many proteins were released into the solution after such harsh treatments (as shown by the Coomassie staining), one hundred percent of the NTF, as could be detected by affinity-purified antibodies, remained bound to the membranes.



b

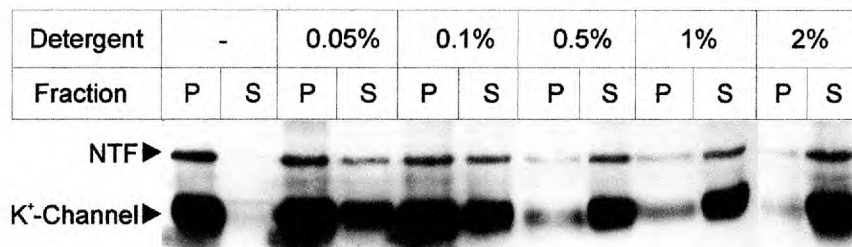


Figure. 7.5 The NTF is strongly bound to the plasma membrane. *a*, Synaptic plasma membranes (SPM) were incubated in 50 mM Tris-HCl pH 7.6, 0.2 M NaCl buffer for 7 hr at three different temperatures: 0°C, 10°C and 37°C. At regular time-points an aliquot of the suspension was removed and pelleted in a microcentrifuge. The resulting pellet and supernatant were examined for their content of the NTF by immunoblotting. Purified native LPH is loaded as a positional marker in the far right lane. *b*, The solubilisation of the NTF from SPM was compared to a large integral membrane protein, K⁺ channel Kv1.2. Pellets (P) and supernatants (S) obtained from a 10 min incubation of membranes with increasing amounts of Thesit are shown.

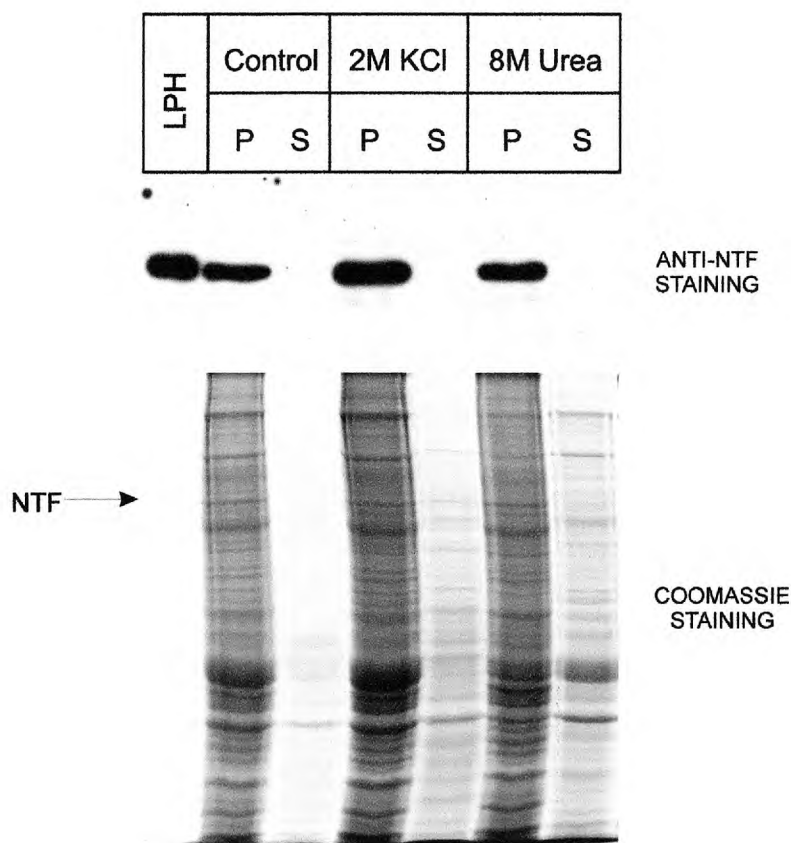
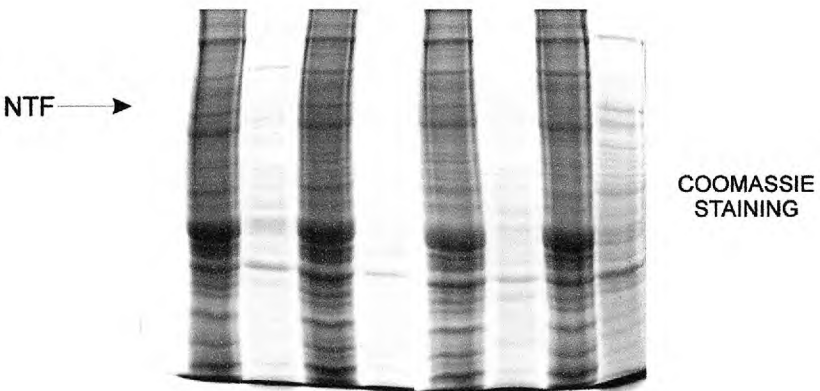
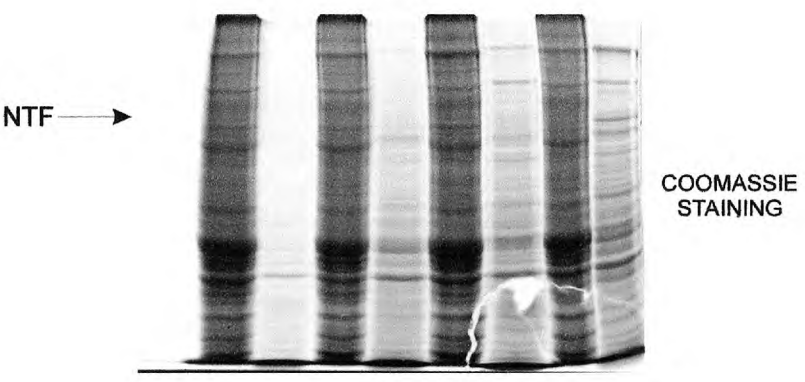


Figure 7.6 Does the CTF anchor the NTF to the membrane through protein-protein interactions? Here and on the following page synaptic plasma membranes (SPM) were incubated with a variety of chemical treatments that should disrupt potential non-covalent interactions between the NTF and CTF. On the SDS-gels, a control (SPM incubated with a Tris/salt buffer) was loaded in parallel with a marker for the NTF (LPH isolated from the LTX column). The arrows on the left of the gels indicate the position of the affinity-isolated N-terminal fragment.

LPH	Control		DTT		1M NaCl		4M MgCl ₂	
	P	S	P	S	P	S	P	S



LPH	Control		pH2.5		pH14		2M NaI	
	P	S	P	S	P	S	P	S



A second approach was used to further study the potential influence of the CTF in anchoring the NTF to the membrane. A latrophilin construct, LPH06a, was prepared in Dr. Ushkaryov's laboratory that consisted of the extracellular portion of latrophilin (inclusive of the extracellular cleavage site) but stopped just before the first transmembrane region (refer to Figure 2.2). When transfected into COS-7 cells, this construct produced two LTX-binding proteins: a ~ 135 kDa protein that was secreted into the medium and a ~ 121 kDa protein that remained in the COS cell pellet (Figure 7.7). As the 121 kDa latrophilin mutant was not secreted and lacked the CTF, it was interesting to see whether it was expressed on the surface of COS-7 cells. Therefore, immunostaining and confocal microscopy were performed on non-permeabilised cells expressing LPH06a, as described earlier. Indeed, specific green fluorescence pertaining to the NTF was observed on the surface but not inside the cells (Figure 7.8). This demonstrated that latrophilin, in the absence of the CTF, could still be transported to the cell surface where it anchors by an unknown mechanism, further supporting the lack of co-localisation reported above (Figures 7.2 and 7.3).

7.2-6 Is the NTF anchored by GPI modification?

As the NTF appeared to be anchoring to membranes on its own after cleavage, we next examined whether this was through glycosphosphatidyl-inositol (GPI) addition. A GPI anchor is the most common form of posttranslational modification that attaches secreted proteins to plasma membranes [Brown, 1992]. Therefore, membranes were treated enzymatically with phosphatidylinositol specific-phospholipase C (PI-PLC) to find out if the NTF could be released into the medium. Synaptic plasma membranes were first incubated in mild alkali (100 mM NaOH), to remove any palmitate residues that might protect the GPI anchor from cleavage by the enzyme, and

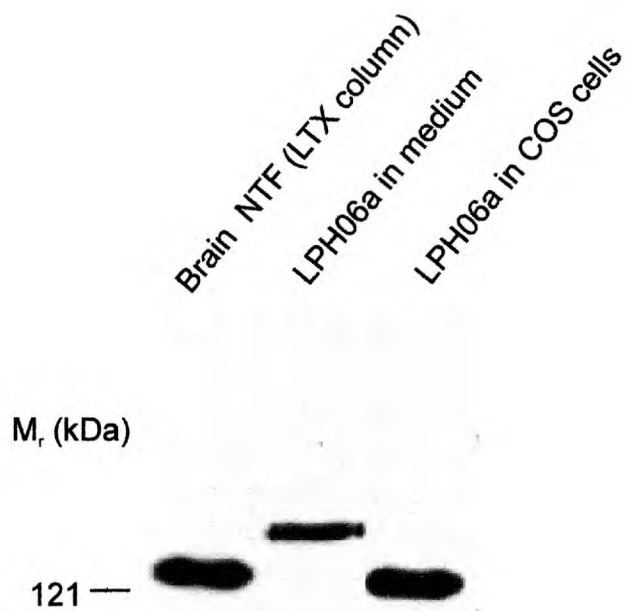


Figure 7.7 LPH06a construct expressed in COS-7 cells. COS-7 cells transfected with LPH06a were harvested 24 hr after transfection and dissolved in the SDS-urea sample buffer in parallel with a sample of the medium above these cells. After SDS-electrophoresis, proteins were transferred onto Immobilon and immunoblotted with the the N-terminal antibody, with chemiluminescent detection. Brain NTF by LTX affinity chromatography was used as a control.



Figure 7.8 The NTF can be expressed at the cell surface independently of the CTF. Immunofluorescence and confocal microscopy were used to visualise the specific staining of LPH06a expressed in COS cells. The construct, LPH06a, which is effectively the NTF, was labelled with an anti-NTF antibody 24 hr after transfection after fixation without permeabilising the cells. *Left*, the green fluorescence labels LPH06a at the surface of the COS cell. *Right*, Phase contrast image of the same cell.

then treated with PI-PLC at 37°C. However, even after a two-hour treatment, no NTF was released into solution (Figure 7.9).

7.2-7 Deglycosylation of the NTF

To estimate the amount of *N*-linked glycosylation in the NTF, the fragment isolated from rat brain by two steps of affinity purification (see Materials and Methods) was deglycosylated by *N*-glycosidases (Figure 7.10). The molecular weight of the NTF was reduced from ~ 121 kDa to ~ 118 kDa by neuraminidase alone and even further to ~ 110 kDa after PNGase (*N*-glycosidase F). The resulting protein still had a larger molecular weight (by ~ 15 kDa) than that calculated on the basis of its cleavage upstream of the first transmembrane domain. *O*-Glycosidase failed to remove this remaining extra molecular weight (not shown), suggesting that the remaining modification is either a carbohydrate resistant to all glycosidases or another undefined moiety.

7.2-8 How may the NTF and CTF interact?

In Chapter 5, tubulin, the abundant cytoskeletal protein, was identified as a major protein co-purifying with latrophilin. As the NTF and CTF may be located close to each other on the membrane surface after cleavage, it is possible that this protein that underlies the plasma membrane, upon solubilisation, may independently bind to both the NTF and the CTF and, thus, explain the co-purification of these fragments on a LTX column. To test this possibility an SPM preparation was treated with colchicine in order to depolymerise tubulin. When the solubilised colchicine-treated SPM were passed through a LTX affinity column, tubulin no longer eluted as shown by a specific antibody on blots (Figure 7.11). The relative amounts of NTF and CTF, however, did not alter from the control. Therefore, tubulin plays no role in the co-purification of the

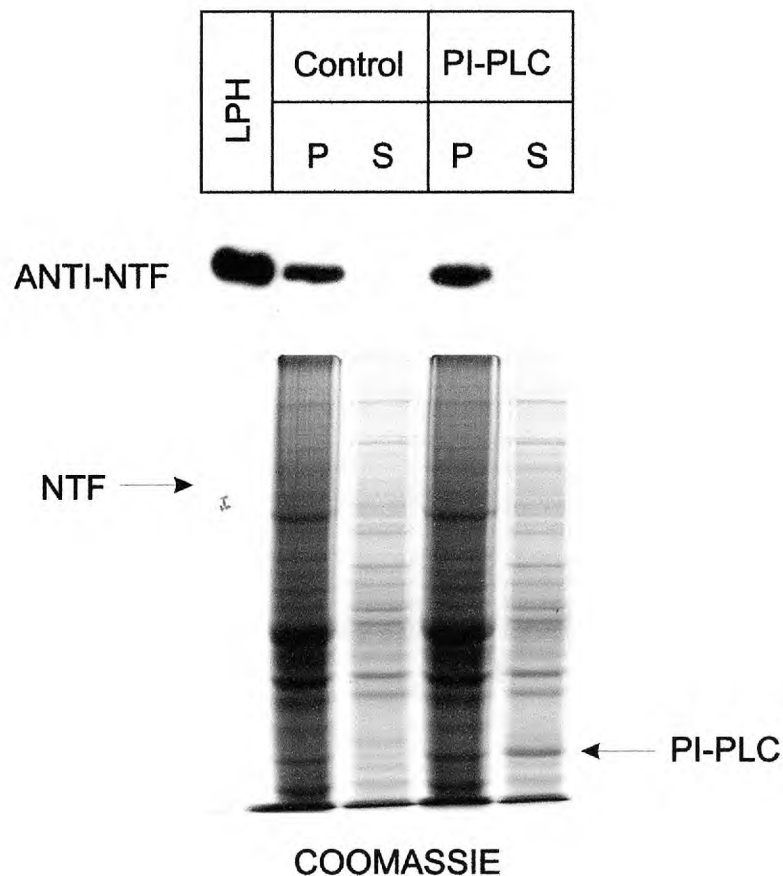


Figure 7.9 The NTF is not anchored to membranes via regular GPI. SPM were treated with phosphatidyl inositol specific-phospholipase C (PI-PLC) as described in Materials and Methods. After incubation with the enzyme, the membranes were pelleted in a microcentrifuge. Equivalent amounts of pellet (P) and supernatant (S) were loaded on SDS-PAGE after precipitation with chloroform/methanol. Following transfer onto PVDF membrane the blot was probed with an affinity-purified antibody to the NTF. The control membranes were treated in the same way without the addition of PI-PLC.

Rat NTF			G-L	Treatment
-	+	+	-	Neuraminidase
-	-	+	-	PNGase



Figure 7.10 Deglycosylation of latrophilin. Purified rat latrophilin was incubated with neuraminidase and PNGase (*N*-glycosidase F), as indicated. Treated samples were precipitated with chloroform/methanol and then resolved on a 5% polyacrylamide gel. After transfer onto PVDF, the blot was stained with an antibody specific for the NTF. A GST-construct of LPH (G-L) expressed in bacteria of known molecular mass (100 kDa) was loaded on the gel as a marker. Positions of other molecular weight markers are indicated by the arrows.

NTF and CTF. At the same time, this interesting result suggests that one of the fragments (most likely the CTF) specifically binds the polymerised form of tubulin.

The interaction of the NTF and CTF and the effect of LTX on them were also investigated using sucrose density gradients. In normal total brain, the NTF produces a defined peak between fractions 9 to 18, whereas the CTF is spread over the whole length of the gradient and is also found in fractions 1 to 8 as well as 19-23 (Figures 7.12a, 7.13a). In other words, there is a large proportion of CTF that does not interact with the NTF, reminiscent of the observations on the surface of COS-7 cells (refer to Figure 7.2). The detection of the CTF alone at the bottom of the gradient suggests that it can form large complexes with itself or other unidentified proteins. Certainly, as we have seen above, the CTF can form dimers, hence it could form higher complexes.

When SDS is included in the gradient, this detergent disrupts the complexes formed by both latrophilin fragments. The NTF and CTF shift positions to lighter molecular weights (Figures 7.12b and 7.13b). However, there is a proportion of the CTF devoid of any NTF (fractions 10-15). Remarkably, when excess LTX is added to the total brain extract before running the gradient, the CTF dramatically ceases to spread out and travels in the same fractions with the NTF as though the toxin stimulates the association of the two latrophilin fragments with each other (Figures 7.12c and 7.13c). These observations may mimic what occurs *in vivo* on the plasma membrane. It is interesting to observe that the NTF and CTF obtained from latrophilin isolated from the brain by a LTX column travels together much like the experiment where excess LTX was added to the total brain, again suggesting that interaction of the latrophilin

fragments with LTX on the column stimulates the association of the two fragments (Figures 7.12d and 7.13d).

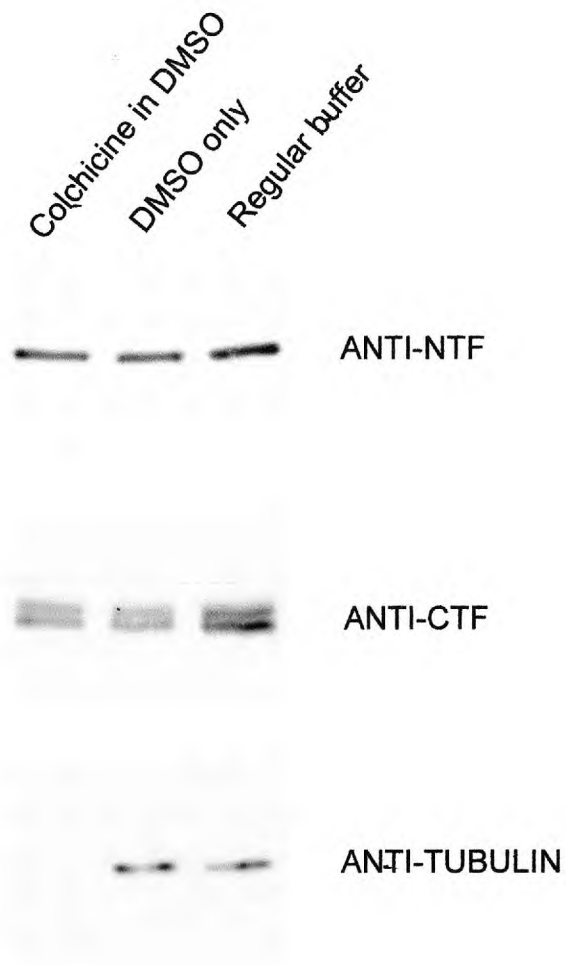


Figure 7.11 Tubulin co-purifies with the receptor but does not mediate the interaction of the NTF and CTF. Rat cerebral cortical synaptosomes were incubated with 1mM colchicine (in 1% DMSO) to depolymerise tubulin. Control synaptosomes were either incubated with 1% DMSO or regular buffer (see Materials and Methods). The synaptosomes were then solubilised in Thesit and passed through fresh LTX columns. After SDS-electrophoresis and Western blotting the preparations were stained with specific antibodies, as indicated. Notice that tubulin is not isolated off the affinity column when synaptosomes have been pre-treated with colchicine, however, the amount of the latrophilin fragments co-purifying does not differ from the controls.

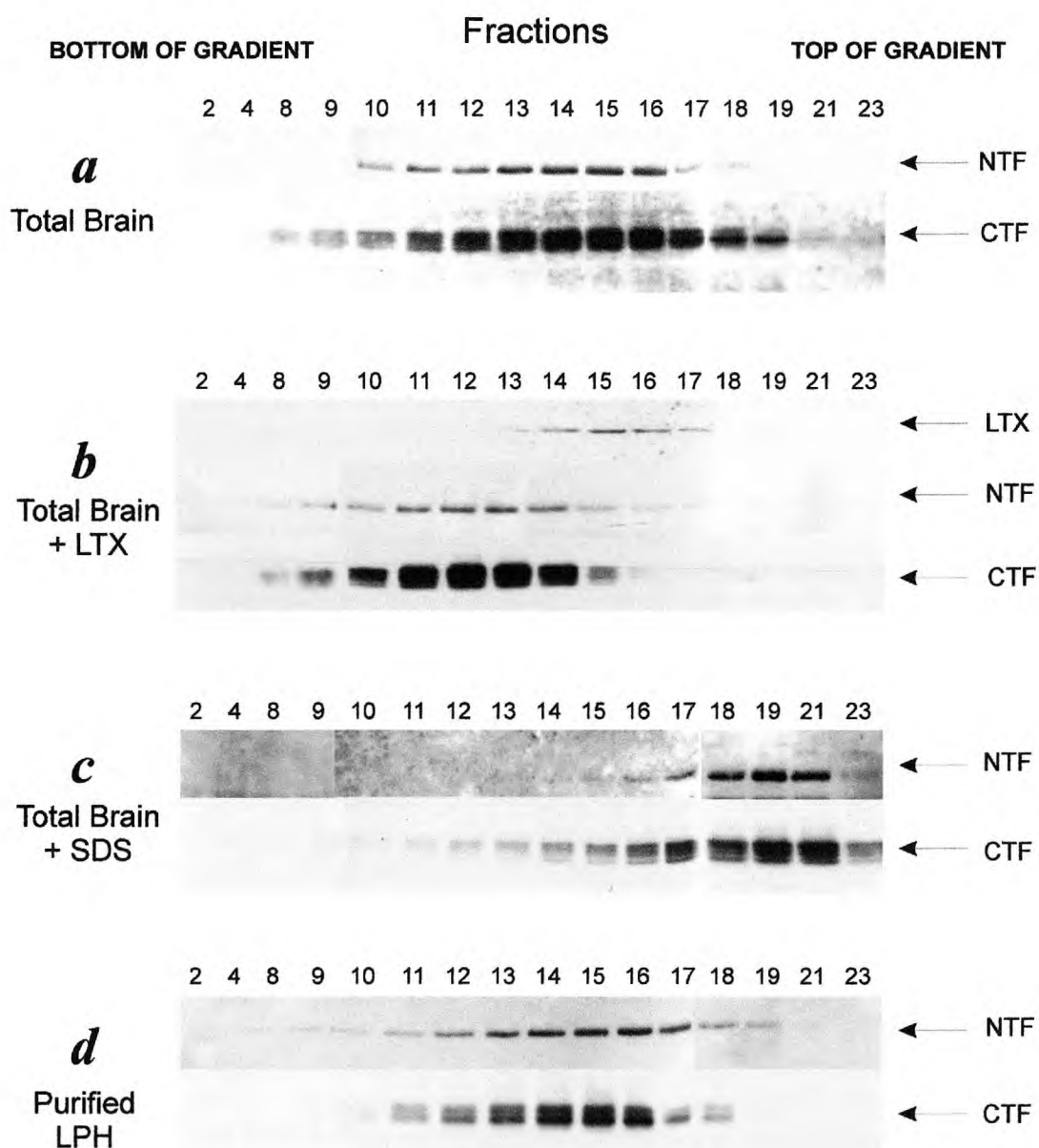


Figure 7.12 LTX may bind both the NTF and the CTF. Sucrose density gradients were run of total solubilised rat brain membranes in different conditions, as indicated (*a-c*). Purified receptor from the LTX affinity column was also run (*d*). Positions of the NTF, CTF and LTX have been identified by immunostaining of the fractions on Western blots with specific antibodies. These results have been plotted on Figure 7.13.

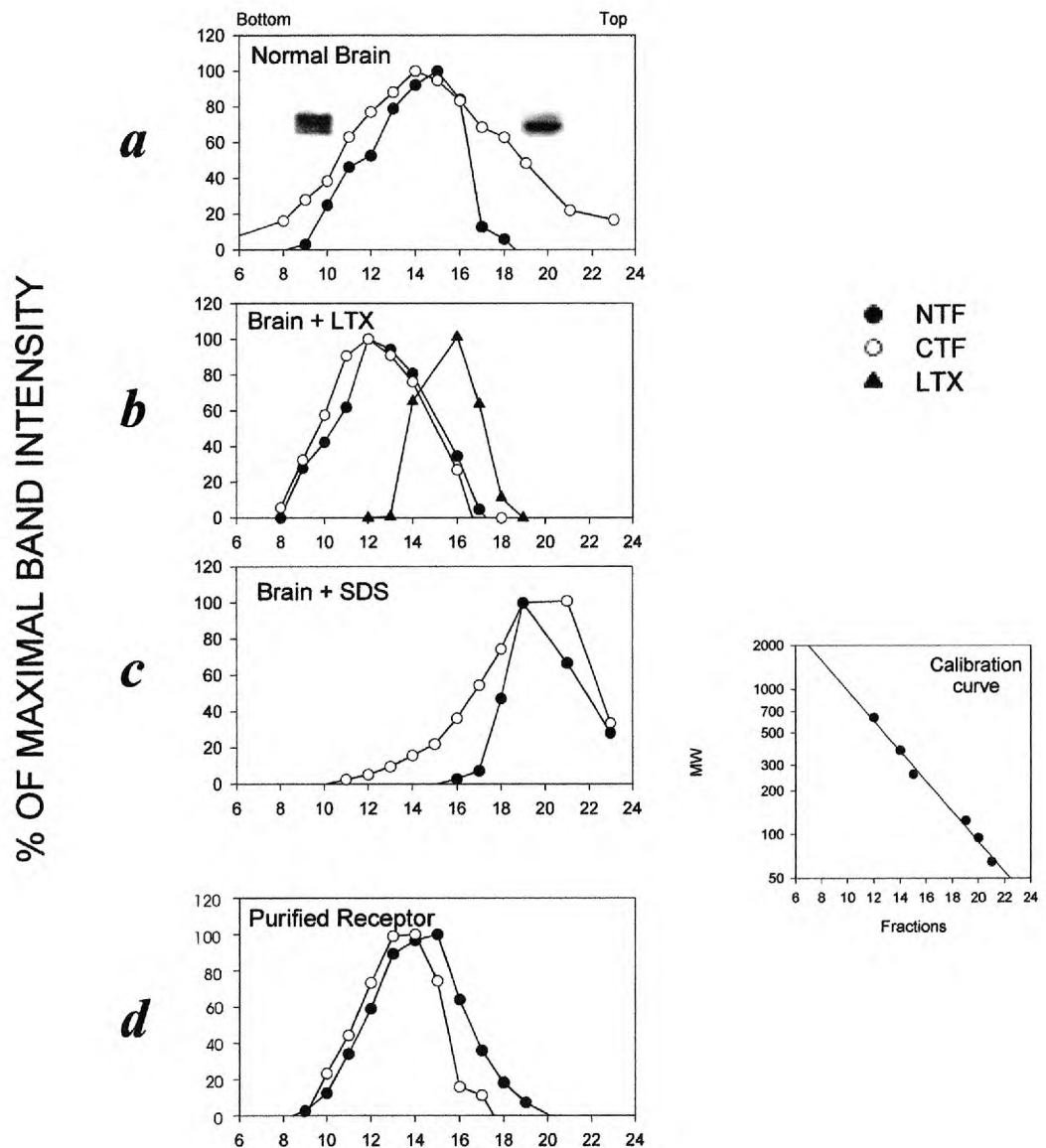


Figure 7.13 LTX may bind both the NTF and the CTF. Analysis of the sucrose density gradients (from Figure 7.12) according to % of maximal band intensity reveals that much of the CTF does not travel together with the NTF but this changes when excess LTX is added (*a-c*). The fragments of the receptor isolated from the LTX column travel together (*d*). These results suggest that LTX either facilitates the interaction of the NTF and CTF or binds each fragment separately. The calibration curve is based on the position of the peak of LTX in fraction 16 as a 255 kDa protein.

7.3 Discussion

The work reported in this section was aimed at attaining an important basic knowledge about the behaviour of the NTF and CTF after latrophilin is proteolytically modified. The finding in the last chapter that apparently less CTF co-purifies with the NTF on a LTX column from a synaptosome or SPM preparation prompted the study of the localisation of the two fragments on the surface of COS cell membranes. It was found that the majority of the NTF and CTF exist independently at the cell surface as well as in most intracellular compartments; therefore, the two fragments of latrophilin may behave as separate proteins after cleavage.

The minor coincidence of co-localisation of the two fragments on the membrane surface may be attributed to the presence of a small amount of the full-size receptor, rather than interacting NTF and CTF, as confirmed by the biotinylation experiment. This was probably a result of incorrect processing at the ER and/or Golgi complex, possibly due to overexpression of the protein. Certainly, in isolated rat nerve terminals, no full-size receptor was ever seen.

After latrophilin is cleaved at the principal site just before the first transmembrane region, it could be expected that the NTF would be released from the plasma membrane after being transported there. However, the data presented in this chapter disputes this assumption. Undeniably, the NTF solubilises in detergent like a truly membrane-bound protein (Kv1.2) and is not released from SPM with time. Both observations imply that after cleavage, this fragment somehow strongly anchors to the

membrane. One mechanism that could explain this is strong non-covalent protein-protein interactions between the NTF and CTF, the latter containing seven transmembrane α -helices. For this reason, chaotropic chemical treatments were performed on SPM but none was successful in removing the 120 kDa protein from the membranes. It is unlikely that the NTF is bound to the CTF by disulphide bonds, as the SPM used for these experiments were pretreated with 100 mM DTT for 30 minutes at 37°C to reduce such bonds.

Nevertheless, there was still a chance that potential NTF-CTF interactions were resistant to the variety of treatments used. To eliminate any doubt, a second molecular biology approach was utilised to investigate the role of the 65 kDa protein in anchoring the NTF. A construct, that was essentially the NTF alone, was transfected in COS-7 cells. Immunostaining and confocal microscopy demonstrated that this 121 kDa protein could be expressed on the cell surface, where it was able to bind LTX (refer to Figure 7.1). Together with the LPH12 confocal microscopy data, demonstrating the lack of co-localisation of the NTF and CTF, this result indicates that after latrophilin's cleavage, the NTF can exist as an independent membrane-bound receptor. Incidentally, the other protein produced in LPH06a expressing cells (~ 135 kDa) was probably secreted into the medium as a result of abnormal glycosylation. Clearly, its molecular weight is too large to represent the true NTF of 121 kDa, which was not soluble.

These findings contrast with the NTF-CTF protein-protein interaction theory proposed by Krasnoperov and coworkers [1997], which was used to explain why the N-terminal fragment is not secreted. Their assumption was based on two observations: (a) the CTF co-purified with the NTF during affinity chromatography and (b) an anti-CTF antibody could co-immunoprecipitate the NTF, both of which suggested an association

between the two fragments. However, these observations were only based on the staining of immunoblots. Indeed, we also saw the NTF and CTF apparently co-purify from a LTX column when blots were stained with specific antibodies. However, our calibration curves for the antibodies illustrated that the amount of CTF purified on a LTX column was much lower than that of the NTF, especially from enriched membrane preparations (Chapter 6). This does not support the hypothesis that all of the NTF is bound to the membrane by strong protein-protein interactions with the CTF. Instead, it is proposed that after the cleavage of latrophilin, the NTF can behave as an independent integral membrane receptor for LTX, which under certain conditions may bind the CTF.

If the CTF is not involved in tethering the extracellular domain, then by what mechanism does the NTF attach to the cell surface? One possible explanation considered was through GPI addition, the most common form of posttranslational modification that anchors secreted proteins to the plasma membrane. However, the attempts to remove the 120 kDa protein from SPM using PI-PLC were unsuccessful. In a similar experiment, purified latrophilin, immobilised on PVDF membrane, was treated with alkali and PI-PLC. A specific antibody was then used to stain for the cross-reactive determinant (CRD) epitope, as described elsewhere [Guther *et al.*, 1994]. The CRD is common to all GPI anchored proteins and is exposed upon PI-PLC cleavage. However, the CRD was not detected in the NTF (results not shown).

Although the identity of the hydrophobic anchor on the NTF has not been determined in this work, there are two pieces of evidence of its existence. Firstly, when the NTF (directly from the LTX column) was subjected to a phase separation experiment in Triton X-114 it completely partitioned into the detergent phase indicating

its hydrophobic nature [Davletov *et al.*, 1996]. As argued previously, this is unlikely to be due to an interaction with the CTF as the two latrophilin fragments do not co-purify in equimolar amounts off the affinity column. It is more likely that the NTF possesses an inherent hydrophobic anchor.

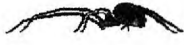
Secondly, the use of *N*-glycosidases could only reduce the NTF from a starting molecular mass of ~ 121 kDa to a final molecular weight of ~ 110 kDa (see Figure 7.10). Latrophilin's extracellular domain is predicted to be ~ 95 kDa without glycosylation but the NTF could not be reduced to this molecular weight even after long incubations with the enzymes. The observed difference between the expected and obtained molecular masses (~ 15 kDa) may be explained by a posttranslational modification or an interaction with another protein that anchors the NTF to the membrane. It may be argued that the 15 kDa unaccounted for is *O*-linked glycosylation, which is apparently resistant to *O*-glycosidases [see within Davletov *et al.*, 1996]. Indeed, the NTF-GST fusion protein expressed in a bacterial cells produces a protein of ~ 100 kDa, which exactly equals its predicted molecular weight [Vera Lelianova, personal communication]. This bacterial NTF cannot possess either *N*- or *O*-linked glycosylation by virtue of the system used to express it, which supports the hypothesis that the NTF expressed in mammalian cells possesses an additional modification that may serve as an anchor. Future experiments will need to address the nature of the membrane anchor present in the N-terminal fragment.

Although the NTF and CTF are not co-localised, the fact there is some co-purification suggests that they can be linked. The relationship of the two latrophilin fragments and the role of α -latrotoxin in their interaction were studied using sucrose density gradients. Experiments using total solubilised brain show that a significant

proportion of the CTF does not interact with the NTF. However, when an excess of α -latrotoxin is present, the two fragments come together in the same fractions. These findings reflect the idea that NTF and CTF may be separate but in close vicinity with each other such that LTX can bring them together to form a functional receptor. This may be due to the proposed ability of LTX to bind either fragment. This explains how latrophilin could be linked to the stimulation of neurosecretion through a G protein-stimulated signaling cascade upon the addition of LTX.

The endogenous mechanisms that may induce an interaction of the two LPH fragments remain to be discovered but in this chapter we eliminate tubulin as having an active role. Perhaps, the NTF acts as an endogenous ligand for the CTF and there may be specific conditions that bring them together. For instance, it was noticed in a previous chapter that Mg^{2+} or Ca^{2+} could weaken the interaction between the two fragments, which appears to be stronger in the presence of EDTA. It is important to know the precise physiological conditions that would induce an equimolar interaction of the two cleaved fragments; we could then hope to understand better how this amazing and unusual G protein-coupled receptor may be linked to novel neuroexocytotic pathways.

7.4 Summary



The NTF and CTF do not necessarily co-localise on the surface COS cells.



The NTF is strongly anchored to the membrane independently of protein-protein interactions with the CTF.



A regular glycosphosphatidyl-inositol (GPI) anchor is not involved in attaching the NTF to the membrane.



The NTF and CTF may behave as separate proteins after cleavage.



LTX may bring about the functional interaction of the NTF and CTF.

Conclusions and future work

α -Latrotoxin has two proposed mechanisms of action:

1. The opening of an LTX-induced pore on the plasma membrane permitting the influx of divalent cations and Na^+ , and possible efflux of neurotransmitters (pore-forming mechanism);
2. The stimulation of LTX receptors leading to the activation of a receptor-mediated signalling cascade causing neurosecretion (pore-independent mechanism);

It is now possible to update the current theory of LTX's mode of action by considering the results of this research work and some other recent publications.

(a) Pore-forming mechanism of α -latrotoxin

The ability of LTX to form pores or channels in a variety of model systems has long been recognised (see Chapter 1). However, it was previously not known whether LTX itself formed the pore or whether it stimulated the opening of an endogenous channel by interacting with another, yet uncharacterised protein as hypothesised by some [Ichchenko *et al.*, 1998]. Perhaps the latter of the two theories was favoured as it was difficult to envisage how a hydrophilic toxin could insert into the membrane. The key to understanding the pore-forming ability of LTX has been the discovery that the toxin can oligomerise (Chapter 3). Oligomerisation is an energy-efficient mechanism that can bury the hydrophilic domains within the oligomer, while exposing

hydrophobic regions. While the toxin normally exists in the dimeric form, the addition of divalent cations such as Ca^{2+} or Mg^{2+} drives its conformational change into a tetramer. LTX acquires a hydrophobic base, which improves interactions between the toxin and membrane and allows the toxin to insert into membranes, completely permeabilising them. This is illustrated in the scheme below:

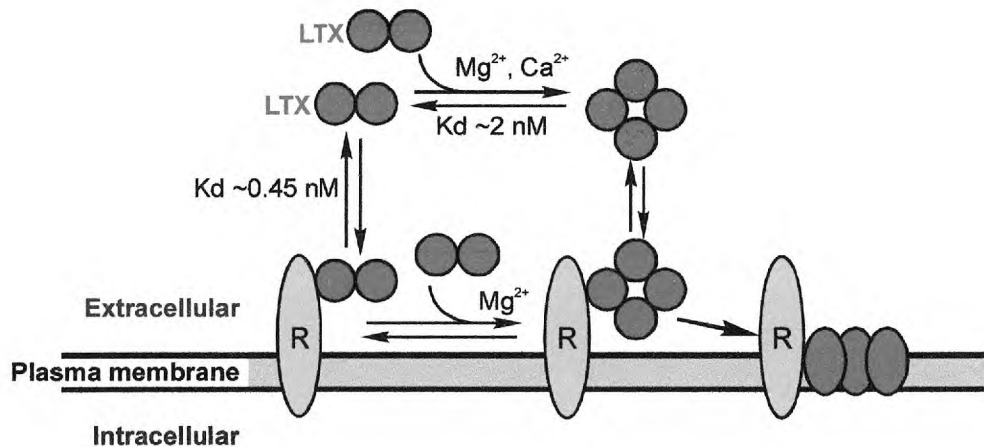


Figure 8.1 Toxin oligomerisation and pore formation. The LTX dimers and tetramers are in equilibrium that depends on the toxin concentration and the presence of divalent cations. Both oligomers interact with receptors (R) but only the tetramers form membrane pores. The dissociation constant between toxin dimers is estimated to be $\sim 2 \text{ nM}$ in the presence of divalent cations (Chapter 4) and $\sim 0.45 \text{ nM}$ between the toxin and receptors (Chapter 3).

Therefore, LTX can be considered to belong to the group of pore-forming toxins such as aerolysin, anthrax protective antigen and δ -endotoxin CytB [reviewed by Gouaux *et al.*, 1997]. These toxins are hydrophilic in the monomeric state but gain amphipathicity and, therefore, the ability to interact with membranes upon oligomerisation facilitated by divalent cations. The influx of divalent cations and Na^+ through the pore would trigger exocytosis. Meanwhile the open pore would also permit the non-vesicular release of transmitters from the cytoplasm.

Although divalent cations are necessary for LTX pore formation, as they promote tetramerisation, LTX receptors are even more crucial. Receptors are firstly necessary to target the toxin to the presynapse but their second function is to increase the local concentration of the toxin and probably orientate it correctly, which would also promote tetramerisation and, as a consequence, membrane permeabilisation. Indeed, whereas toxin insertion into pure lipid membranes was inefficient and required [LTX] of ~ 100 nM (Chapter 4), concentrations as little as 0.5 nM LTX have been demonstrated recently to insert into synaptosomes incubated in physiological buffers [Khvotchev & Südhof, 2000]. As a signalling function of the receptor is not required for the pore-effect, either latrophilin or neurexin (both present in synaptosomes) may facilitate this process. This is supported by observations that a wild type latrophilin mutant, a latrophilin mutant devoid of most of the CTF and the structurally unrelated neurexin I α produced identical high conductance LTX channels when independently transfected into HEK293 cells [Hlubek *et al.*, 2000].

(b) Pore-independent mechanism of α -latrotoxin

In conditions of high extracellular Ca^{2+} and/or Mg^{2+} , the LTX pore is probably the major mechanism of LTX action [Davletov *et al.*, 1998]. However, the pore alone cannot explain the release of neurotransmitters that occurs in the absence of divalent cations when pore formation is minimal, as demonstrated by the absence of ionic fluxes [Misler & Hurlbut, 1979; Capogna *et al.*, 1996; Lang *et al.*, 1998]. This suggests that a receptor-mediated mechanism is also occurring. In the process of studying the Ca^{2+} -independent LTX-evoked secretion, a novel high affinity LTX receptor has been discovered (latrophilin), which binds to the toxin even in the absence of divalent cations [Davletov *et al.*, 1996]. This receptor is proposed to be linked to the

phospholipase C-catalysed production of IP₃ [Vicentini & Meldolesi, 1984; Rosenthal *et al.*, 1990; Lelianaova *et al.*, 1997]. This secondary messenger may stimulate the release of Ca²⁺ from intracellular stores [Simpson *et al.*, 1995] that could act as an additional stimulus for neurotransmitter release. The other known high affinity receptor for LTX, neurexin I α , only binds to the toxin in the presence of Ca²⁺ and is structurally distinct from G protein-coupled receptors. This has warranted a greater interest in the study of latrophilin.

Consistent with the receptor-mediated theory of α -latrotoxin action, LPH has been found to physically and functionally couple to G proteins (Chapter 5). One of those in particular, G α_q , has a known linkage to phospholipase C-activation in other systems [Selbie & Hill, 1998]. This receptor is also presynaptic (Chapter 6). Although only Western blotting of membrane fractions was used to illustrate its presynaptic localisation in this work, this has been confirmed by immunofluorescent confocal microscopy of frog neuromuscular junctions using our highly specific anti-NTF and anti-CTF antibodies [Meunier *et al.*, 2001, paper in preparation]. The two results strengthen the claim that latrophilin is the true physiological receptor involved in the pore-independent effects of LTX action.

A surprising finding, however, has been the discovery that latrophilin possesses an unusual, for a GPCR, structure in that it is cleaved between its extracellular long N-terminus domain and the transmembrane signalling domains (Chapter 6). Although the two latrophilin fragments are close to each other on the membrane surface they are not co-localised (Chapter 7). Since the NTF binds LTX, how could this activate the CTF,

the signalling fragment of this protein? The two fragments, however, can interact. There are probably some physiological conditions that bring about the functional interaction of the two fragments allowing them to act as a functional receptor. Such conditions have not been determined yet. The interaction, or its disruption, may indeed be brought about by LTX itself, as analysis of latrophilin deletion mutants has pinpointed the high-affinity LTX binding site to be between residues 467-891, that is, the C-terminal half of the NTF and the first transmembrane region of the CTF [Krasnoperov *et al.*, 1999]. Alternatively, it could be speculated that LTX can directly stimulate the CTF upon its insertion into the membrane. However, it is clear from physiological and biochemical data that latrophilin still plays a key role in LTX action. The current theory for the mechanism of α -latrotoxin action is illustrated in Figure 8.2 below.

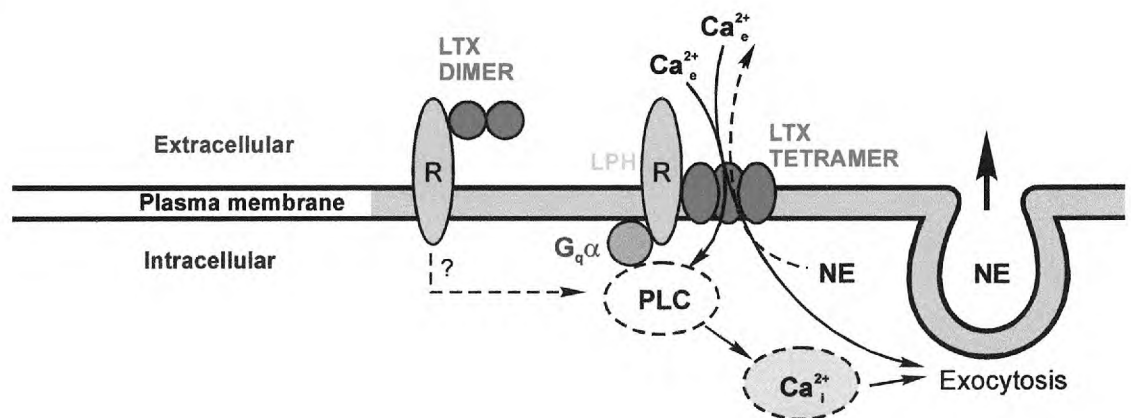


Figure 8.2 Current mechanism of LTX action. Latrophilin (LPH) targets LTX to the plasma membrane where, in its tetrameric form, it can insert and permeabilise the presynapse. The entry of Ca^{2+} through the pore will stimulate the vesicular release of neurotransmitters such as norepinephrine (NE). The pore also permits the escape of cytoplasmic neurotransmitters (the Ca^{2+} -independent, non-vesicular, effect of the toxin). Additionally, LTX dimers and tetramers activate latrophilin directly, which through $\text{G}_q\alpha$ is linked to the phospholipase C (PLC)-catalysed mobilisation of Ca^{2+} from intracellular Ca^{2+} stores. This contributes to the stimulus for vesicular release. Neurexin 1 α also plays a role in LTX pore formation but is not involved in the intracellular signalling mechanism.

The influx of Ca^{2+} through a LTX channel and intracellular Ca^{2+} release has also been reported in peptide-secreting gonadotropes [Tse & Tse, 1999] thus suggesting that this theory of LTX action is universal. Although the work presented in this thesis has gone some way towards improving our understanding of how LTX functions it has not solved the mechanism of LTX action. Many questions remain unanswered, particularly over the mode of action of latrophilin. The cleavage of the receptor must have a function and this remains to be determined. The endogenous ligands for LPH need to be found, which may ultimately prove the importance of latrophilin in neurosecretion. Alternatively, it could be speculated that the NTF can act as a ligand for the CTF after latrophilin cleavage in a similar manner to that seen in protease receptors such as the thrombin receptor [Vergnolle, 2000]. Therefore, conditions that bring together a strong interaction or cause the complete separation of the two protein fragments will also need to be addressed in future experiments.

Although the LTX tetramer has been visualised inserting into lipid bilayers by cryo-EM, thus proposing a mechanism for LTX pore formation, these experiments will need to be repeated using reconstituted receptors to mimic conditions *in vivo*. In preliminary studies of this nature, brain-isolated receptors were found to be unsuitable as they can only be obtained in the presence of detergents, which disrupt LTX tetramers into dimers during sample preparation for cryo-EM. The approach must use recombinant neurexin or latrophilin expressed and isolated in detergent-free conditions. One suggestion would be to express receptors, tagged with histidine, in an insect cell line. The soluble receptor would be secreted into the medium where a nickel column would permit easy isolation of the tagged receptor. This is similar to the method used to produce recombinant LTX recently [Volynski *et al.*, 1999a]. Therefore, there are many exciting times ahead in the study of α -latrotoxin and its receptor, latrophilin.

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Table 1 – SDS-PAGE

The gel formulae given are for the BioRad Mini-Protean II system. The 6x loading buffer can be diluted to 2x or 1x with dH₂O. The anode and cathode buffer are 25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS (available in stock solution from BioRad).

Resolving Gel	4%	6%	8%	10%	12%
Distilled water	3.25 ml	2.88 ml	2.50 ml	2.10 ml	1.75 ml
50% Glycerol	1.50 ml	1.50 ml	1.50 ml	1.50 ml	1.50 ml
1.5 M Tris-HCl pH 8.8	1.87 ml	1.87 ml	1.87 ml	1.87 ml	1.87 ml
40% Acrylamide/Bis 37.5:1	0.75 ml	1.13 ml	1.50 ml	1.90 ml	2.25 ml
10% SDS	75 µl	75 µl	75 µl	75 µl	75 µl
10% APS	50 µl	50 µl	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl	5 µl	5 µl

1% Stacking Gel	6x Loading Buffer		
Distilled water	2.50 ml	1 M Tris-HCl pH 6.8	3.5 ml
0.5 M Tris-HCl pH 6.8	0.94 ml	SDS (solid)	1.2 g
40% Acrylamide/Bis 37.5:1	0.28 ml	100% Glycerol	3.6 ml
10% SDS	38 µl	β-mercaptoethanol	2.0 ml
10% APS	50 µl	Bromophenol blue	0.015 g
TEMED	5 µl		

Table 2 – SDS-urea PAGE

Apart from the gel composition and the sample loading buffer, all procedures are the same as described for regular SDS-PAGE (after Bollag, Rozycki & Edelstein, 1996).

<u>4% Separating Gel (10 ml)</u>		<u>1% Stacking Gel (4 ml)</u>
30% Acrylamide, 0.8% Bis-acrylamide	1.33 ml	0.13 ml
1.5 M Tris-HCl (pH 8.8), 0.4% SDS	2.5 ml	-
0.5 M Tris-HCl (pH 6.8), 0.4% SDS	-	1.0 ml
Urea	4.8 g	1.9 g
H ₂ O	2.57 ml	1.47 ml
10% Ammonium Persulphate	50 µl	30 µl
TEMED	5 µl	5 µl

8 M SDS-urea sample loading buffer (10 ml)

Electrophoresis grade urea (Sigma)	3.6 g
SDS (solid)	0.4 g
DTT	0.154 g
1 M Tris-HCl pH 6.8	600 µl
Bromophenol blue	0.015 g

Make up to 10 ml with H₂O

Table 3 – Native electrophoresis

The formula below can be used to make a gel of up to 4.5 cm in height in a BioRad Model 491 Prep assembly tube. Preparative gels are typically left to polymerise for 12-24 hr at 4°C before use. For purifying LTX, the upper and lower buffer composition in the Prep Cell is 25 mM Tris-HCl, 192 mM glycine (pH 8.3).

6% Native Preparative Gel

0.75 M Tris-HCl pH 8.8/10% glycerol	20 ml
40% Acrylamide/Bis Solution 37.5:1	6 ml
Distilled water	14 ml
10% APS	175 µl
10% TEMED	175 µl

The following buffer composition was used to prepare samples for loading onto Novex pre-made gradient gels under native conditions.

Novex Native-PAGE sample buffer (2x)

1 M Tris-HCl pH 6.8	3.5 ml
100% Glycerol	3.6 ml
Bromophenol blue	0.015 g
dH ₂ O	2.2 ml

Table 4 – Gel for sequencing the CTF

The following method has been adapted for the BioRad Mini-Protean II system (after Dunbar & Wilson, 1994).

<u>Resolving Gel</u>		<u>Stacking Gel</u>
1.5 M Tris-HCl pH 7.5	3.75 ml	-
1 M Tris-HCl pH 8.8	-	1.5 ml
30% Acrylamide/Piperazine diacrylamide (37.5:1)	4 ml	0.99 ml
50% Glycerol	3 ml	-
10% SDS	150 µl	60 µl
H ₂ O	4 ml	3.51 ml
10% Ammonium Persulphate	75 µl	45 µl
TEMED	7.5 µl	6 µl

<u>Lower Electrode Buffer (4x)</u>	<u>Upper Electrode Buffer (4x)</u>
36.34 g Tris base	12.11 g Tris base
0.8 g SDS	0.8 g SDS
150 ml H ₂ O	150 ml H ₂ O
adjust to pH 8.8 and make up to 200 ml with H ₂ O	adjust to pH 6.8 and make up to 200 ml with H ₂ O

Note: 750 µl of 10 mM glutathione was added per 150 ml of 1x upper electrode buffer before running.