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α-Latrotoxin and Its Receptors

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Abstract

α-Latrotoxin (α-LTX) from black widow spider venom induces exhaustive release of neurotransmitters from vertebrate nerve terminals and endocrine cells. This 130-kDa protein has been employed for many years as a molecular tool to study exocytosis. However, its action is complex: in neurons, α-LTX induces massive secretion both in the presence of extracellular Ca^{2+} (Ca^{2+}_e) and in its absence; in endocrine cells, it usually requires Ca^{2+}_e. To use this toxin for further dissection of secretory mechanisms, one needs an in-depth understanding of its functions. One such function that explains some α-LTX effects is its ability to form cation-permeable channels in artificial lipid bilayers. The mechanism of α-LTX pore formation, revealed by cryo-electron microscopy, involves toxin assembly into homotetrameric complexes which harbour a central channel and can insert into lipid membranes. However, in biological membranes, α-LTX cannot exert its actions without binding to specific receptors of the plasma membrane. Three proteins with distinct structures have been found to bind α-LTX: neurexin Iα, latrophilin 1 and receptor-like protein tyrosine phosphatase σ. Upon binding a receptor, α-LTX forms channels permeable to cations and small molecules; the toxin may also activate the receptor. To distinguish between the pore- and receptor-mediated effects, and to study structure-function relationships in the toxin, α-LTX mutants have been used. At least one non-pore-forming α-LTX mutant can activate latrophilin, a G protein-coupled receptor, causing release of Ca^{2+} from intracellular stores. Latrophilin action still requires Ca^{2+}_e and may trigger transmitter secretion either by itself or by activating Ca^{2+}-channels and/or inducing Ca^{2+} waves. These results reveal two Ca^{2+}_e-dependent mechanisms of α-LTX action (membrane pore formation and signalling via latrophilin), but how α-LTX triggers Ca^{2+}_e-independent neurotransmitter release still remains unexplained. Hypotheses for this action include direct interaction with intracellular components involved in exocytosis or the effects of α-LTX pores.

Keywords

α-latrotoxin; exocytosis; neurexin; latrophilin; protein tyrosine phosphatase σ

1. α-LTX and release of neurotransmitters

The notorious black widow spider (*Latrodectus* genus) and its venom, first studied by ancient Greeks (Aristotle 350 B.C.), entered the era of modern science in the 1930's with the discovery of its proteinacious active principles (D'Amour et al. 1936) and, following the

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demonstration that it affects synaptic neurotransmitter release (Longenecker et al. 1970) and the isolation of toxic components (Frontali et al. 1976), gripped the attention of neurobiologists. It played a role in the debate leading to the Ca^{2+} (Augustine et al. 1987) and vesicular quantum (Ceccarelli and Hurlbut 1980) paradigms of neurotransmission. However, some details of α-LTX actions still remain mysterious, despite its widespread use as a stimulant of neurosecretion.

The venom contains at least 86 unique proteins (Duan et al. 2006), including several homologous LTXs which play a role in its toxicity to insects and crustaceans (Grishin 1998), with only one, α-LTX, targeting vertebrates specifically; reviewed by Rosenthal and Meldolesi (1989). α-LTX is usually isolated from spider venom by conventional chromatography (Frontali et al. 1976; Tzeng et al. 1978), but to achieve homogeneity and remove contaminants (Volkova et al. 1995; Pescatori et al. 1995) that may endow the preparation with uncharacteristic properties (Umbach et al. 1998), preparative native electrophoresis should ideally be used (Ashton et al. 2000).

1.1. Target cells

The toxin induces strong and sustained release of neurotransmitters and hormones from secretory cells capable of regulated exocytosis (Rosenthal et al. 1990). The effects of α-LTX on neurosecretion were first described on a cellular level in the 1970's at frog neuromuscular junctions (NMJ) (Longenecker et al. 1970; Frontali et al. 1976), and later in mouse brain slices (Tzeng et al. 1978), in synaptosomes (isolated brain nerve terminals) from rat (Grasso et al. 1978), dog (Tzeng and Siekevitz 1979) and guinea pig (Nicholls et al. 1982), primary cerebellar granule cell cultures (Grasso and Mercanti-Ciotti 1993) and in hippocampal organotypic cultures (Capogna et al. 1996). In addition, catecholamine-secreting chromaffin cells (Picotti et al. 1982) and PC12 cell line (Robello et al. 1987), insulin-secreting pancreatic β-cells in primary or derived cell cultures (Lang et al. 1998), oxytocin- and vasopressin-secreting neurohypophysis cells (Hlubek et al. 2003) and luteinising hormone-secreting rat gonadotropes (Tse and Tse 1999) were also used to characterise the toxin's effects on secretion from non-neuronal excitable cells. A secretory cell not sensitive to α-LTX still remains to be found.

1.2. Site and mode of action

From the earliest description of toxin's actions on neuronal systems, it emerged that α-LTX affects specifically the presynaptic element, from which it causes massive neurotransmitter release (e.g. Longenecker et al. 1970). The toxin has no major enzymatic activities (Frontali et al. 1976). Crucially, α-LTX has been discovered to create Ca^{2+}-permeable channels in lipid bilayers (Finkelstein et al. 1976), and a large body of evidence shows that Ca^{2+} influx through membrane channels induced by α-LTX in the presynaptic membrane accounts for a major part of its effect. Pore formation occurs in all the biological systems mentioned above, but the features of α-LTX-triggered release cannot be fully explained by the toxin pore.

The effect of α-LTX at NMJs is usually delayed and develops fully after ~10 min, although synaptosomes react much faster. It is detected electrophysiologically as an increase in the frequency of spontaneous miniature postsynaptic potentials at NMJs (Longenecker et al. 1970; Misler and Hurlbut 1979; Ceccarelli and Hurlbut 1980; Tsang et al. 2000) and excitatory or inhibitory postsynaptic currents at central synapses (Capogna et al. 1996). In addition, α-LTX affects action potential-evoked, synchronous release, and does this in a time-dependent manner: initially, it enhances evoked potentials, but eventually inhibits (Capogna et al. 1996) or blocks them (Longenecker et al. 1970; Hurlbut and Ceccarelli 1979; Liu and Misler 1998). Finally, when used in higher concentrations, α-LTX can cause morphological deformation and cell death, as reviewed by Sudhof (2001).

However, the most surprising feature of the α-LTX-evoked secretion is that it can occur both in the presence and absence of Ca^{2+}_e.

1.3. Ca^{2+}-independent release

The ability of α-LTX to trigger neurotransmitter exocytosis in the absence of extracellular Ca^{2+} remains particularly interesting and inexplicable to the field (Longenecker et al. 1970; Ceccarelli et al. 1979; see also Sudhof (2001) and Ushkaryov et al. (2004) for review). This is clearly different from depolarisation-induced exocytosis, which is Ca^{2+}-dependent, but not unlike the effect of hypertonic sucrose. The possibility that α-LTX-induced release involves an unknown, Ca^{2+}-independent mechanism which may also occur during normal synaptic activity has provided the *casus belli* for many a quest for α-LTX structure and receptors that could trigger neurotransmission *via* intracellular mechanisms.

In neurones, the Ca^{2+}-independent secretion is restricted to small synaptic vesicles, as demonstrated by synaptosomal and NMJ experiments, where glutamate, GABA and acetylcholine are released in the absence of Ca^{2+}, while catecholamines or peptides are not (Matteoli et al. 1988; Davletov et al. 1998; Khvotchev et al. 2000). Ca^{2+}-independent release does not normally occur in endocrine cells (Grasso et al. 1980; Michelena et al. 1997; Silva et al. 2005), although in some cultured cells it does (Meldolesi et al. 1983; Lang et al. 1998; Tse and Tse 1999).

The characteristics of Ca^{2+}-independent release are peculiar: it requires the presence of divalent cations, such as Mg^{2+}, which can be added or removed in succession, causing respective bouts of secretion or its cessation (Misler and Hurlbut 1979). In the absence of Mg^{2+}, this release can be supported by slightly hypertonic sucrose, by itself insufficient to cause secretion (ibid.). The Ca^{2+}-independent release can be blocked by millimolar La^{3+} (Rosenthal et al. 1990) or concanavalin A (Grasso et al. 1978; Boehm and Huck 1998). It may involve release of Ca^{2+} from mitochondria, as observed in peripheral (Tsang et al. 2000) and not central synapses (Adam-Vizi et al. 1993), but it is unclear if stored Ca^{2+}_i itself can trigger release.

As Ca^{2+} is important for endocytosis, the sustained secretory activity in its absence eventually depletes NMJ terminals (Longenecker et al. 1970; Ceccarelli and Hurlbut 1980), but not synaptosomes (Watanabe and Meldolesi 1983), of all vesicles. Electrophysiological recordings at NMJs show that in the absence of Ca^{2+} α-LTX causes a large but slow rise in the frequency of spontaneous exocytotic events, which then slow down and cease altogether; bursts of miniatures are never observed.

1.4. Ca^{2+}-dependent release

In the presence of Ca^{2+}_e, α-LTX also causes a slow and large increase in the frequency of miniature events, overlaid by bursts of release (Ceccarelli et al. 1979). As in the absence of Ca^{2+}, depletion of vesicles and block of exocytosis also occurs, but because vesicles are able to recycle for some time, the total number of quanta released is twice higher in Ca^{2+} than in its absence (Fesce et al. 1986; Auger and Marty 1997). It appears that the gradual rise and fall of the frequency of vesicle fusion events is a common feature of α-LTX-induced release both with and without Ca^{2+}_e, and that Ca^{2+} adds an extra component, increasing the total release.

Influx of Ca^{2+} through α-LTX pores might explain Ca^{2+}-dependent secretion, but not the bursts of miniatures. These may be due to Ca^{2+} waves caused by activation of phospholipase C (PLC) (Vicentini and Meldolesi 1984; Davletov et al. 1998) and release of intracellular Ca^{2+} (Ca^{2+}_i). In fact, U73122 (blocks PLC activation by G proteins), thapsigargin (depletes Ca^{2+} stores) and 2-APB (inhibits activation of Ca^{2+} stores) block α-LTX action (Davletov et

al. 1998; Ashton et al. 2001; Capogna et al. 2003), implicating a G protein cascade in Ca^{2+}-dependent toxin-induced release.

2. The structure of α-LTX

To understanding and explaining the complex activities of α-LTX, its primary and three-dimensional (3D) structure has been thoroughly studied.

2.1. Sequence analysis

α-LTX is synthesised as a 157 kDa polypeptide (Kiyatkin et al. 1990) (Figure 1A) by free ribosomes in the cytosol of secretory epithelial cells of the venom glands (Cavalieri et al. 1990). These cells disintegrate (Smith and Russell 1966) and expel toxin into the gland lumen together with various proteases (Duan et al. 2006). Here, it is cleaved at both termini by a furin-like protease (Volynski et al. 1999), producing an active α-LTX of ~131 kDa (Kiyatkin et al. 1990; Ichtchenko et al. 1998).

The most striking feature of the α-LTX primary structure is a series of 22 ankyrin repeats that account for about two thirds of the sequence (Figure 1B) (Kiyatkin et al. 1995). Ankyrin repeats are found in a wide variety of proteins and generally mediate protein-protein contacts but, unlike other protein binding motifs, they take part in a wide range of interactions and do not have a specific target (Sedgwick and Smerdon 1999). The N-terminal third of α-LTX shows no significant homology to other proteins. This region contains three conserved cysteines important for structural stability and activity of all LTXs (Kiyatkin et al. 1995; Ichtchenko et al. 1998).

α-LTX is a mostly hydrophilic protein that does not have a classical hydrophobic signal peptide or clear transmembrane regions (TMRs). The hydrophilic nature of α-LTX seemed for a long time at odds with its membrane insertion capabilities, but a study of its 3D structure (Orlova et al. 2000) shed light on this issue (Section 2.2).

2.2. Higher-order structures

So far, crystallisation of α-LTX has not been reported. However, the toxin has been successfully studied by cryo-electron microscopy (cryo-EM) (Orlova et al. 2000), a method that allows visualisation of proteins instantly frozen in their native conformations. The size of the toxin tetramer (~520 kDa) proved ideal for this approach to reveal medium-resolution (14 Å) information, and even the dimer (~260 kDa) was reconstructed to below 20 Å resolution, revealing detailed information on the domain organisation of the toxin in its two oligomeric states (Orlova et al. 2000).

2.2.1. Domain structure of the monomer—The 3D cryo-EM reconstructions of the α-LTX tetramer and dimer (Orlova et al. 2000) show that the monomer consists of three domains (Figure 1C): (1) The wing, at the N-terminus, has an estimated molecular mass of 36 kDa. Thought to comprise mainly α-helices, it has no close homologues among proteins of known 3D structure. The wing links to the rest of the toxin molecule through a narrow rigid connection. (2) The body comprises the first 17 ankyrin repeats of the α-LTX sequence. Its estimated molecular mass is 76 kDa. The ankyrin repeats forming the "vertical" part of the body are tightly packed into a characteristic structure, reminiscent of ankyrin. However, the lower part of the body is bent and has a more complex arrangement. (3) At the C-terminus is the head, the smallest domain, with an estimated 18.5-kDa mass. It consists of 4.5 ankyrin repeats and demonstrates high spatial homology to ankyrin-repeat-containing proteins with known 3D structures (Orlova et al. 2000).

Despite having a large number of ankyrin repeats (superseded only by the 24 repeats in ankyrin itself), α-LTX does not assume the monotonous arch-like shape so characteristic of proteins with multiple ankyrin repeats, such as the 12-repeat ankyrin fragment (Michaely et al. 2002), the human protein phosphatase HEAT (15 repeats), or the porcine ribonuclease inhibitor LRR (16 repeats) (Andrade et al. 2001). Instead, the ankyrin repeat-containing part of α-LTX is broken in several places and forms clearly delimited domains and sub-domains probably reflecting sequence divergence of some repeats and the functional specialisation of the sub-domains.

2.2.2. Quaternary structures—Although some monomers have been observed by cryo-EM in EDTA-treated α-LTX, the toxin almost always exists as a stable dimer (Orlova et al. 2000; Ashton et al. 2001). In the asymmetric dimer, the two monomers are associated "head to tail" by tight multipoint contacts mediated by the "horizontal" parts of the bodies and by the sides of the head domains (arrowhead in Figure 1C).

Association of dimers, strongly catalysed by Mg^{2+}, produces a cyclical structure (Figure 1C) that can contain four monomers only. The tetramer has C4 rotational symmetry and resembles a bowl, in which the bottom is formed by the "horizontal" parts of the bodies. This part is important for penetration into lipid bilayers, and it is likely that structural rearrangements required for tetramerisation expose the surface regions favourable to interaction with lipid bilayers. In addition, this part represents the intracellular mouth of the channel, with a large (30 Å) central hole in its center.

Above this, in the centre of the "bowl", the four heads form a cylindrical assembly surrounding the channel (Figure 1C), which is restricted at one point to 10 Å (Figure 2; see also Section 3.4.7). This constriction most probably corresponds to the cation binding site (selectivity filter) of the α-LTX channel (Section 3.4.1).

The wings extend sideways from the body domains perpendicular to the central symmetry axis of the tetramer and could participate in the binding to some receptors (see Sections 2.3 and 4). They also seem to mediate homotypic interactions, causing tetramers to assemble into flat 2D crystals, often containing large numbers of tetramers (Lunev et al. 1991). These lattices could underlie the frequently described phenomenon of α-LTX channel clusterisation (Robello et al. 1987; Krasilnikov and Sabirov 1992; Filippov et al. 1994; Van Renterghem et al. 2000).

2.3. Recombinant α-LTX

Detailed structure-function analysis of α-LTX is impossible without generating mutants. The difficulty inherent to this approach is to ensure proper folding of this large protein. Two groups relied on baculovirus expression and successfully purified active recombinant toxins. Ichtchenko et al. (1998) used two 8-histidine tags for purification, whereas Volynski et al. (1999, 2003) utilised a monoclonal antibody.

Recently, active recombinant α-LTX has been generated using bacteria in which both thioredoxin reductase and glutathione reductase are inactivated to improve the formation of disulphide bonds in expressed proteins (Li et al. 2005). The toxin is expressed as a fusion with glutathione-S-transferase (GST), which is used for affinity purification of the recombinant toxin and can be subsequently removed by selective proteolysis. Considering the relative ease of generating recombinant proteins in bacteria, this approach will facilitate structure-function studies of α-LTX.

Using these methods, several useful observations regarding the functions of the α-LTX domains have been made by analysing various mutants of recombinant toxin. These results are discussed in relevant sections below.

3. Membrane pore

How the hydrophilic α-LTX inserts into lipid membranes and makes cation-permeable pores is not fully known, but an in-depth insight into the mechanisms of channel formation has been gained by combining cryo-EM, biochemical and biophysical studies with toxin mutagenesis. α-LTX pore formation consists of at least three steps: toxin tetramerisation, interaction with a specific cell-surface receptor and, finally, membrane insertion. Many experimental procedures can affect some of these steps and thereby prevent or assist channel formation.

3.1. Tetramerisation

The tetrameric state of α-LTX is divalent cation-dependent. Accordingly, treatment with EDTA renders purified α-LTX mostly dimeric, while subsequent addition of millimolar Ca^{2+} or Mg^{2+} promptly restores tetramerisation (Ashton et al. 2000). This transition, requiring conformation changes (Orlova et al. 2000) and catalysed by divalent cations, may explain the dependence of α-LTX pore-mediated actions on Mg^{2+} (e.g. Misler and Hurlbut 1979). Tetramerisation can also be triggered by amphipathic molecules (Ashton et al. 2000), possibly membrane lipids.

Interestingly, La^{3+} at a concentration higher than 100 μM also greatly reduces the number of tetramers (Ashton et al. 2001). In addition, this trivalent cation is able to block the channels of membrane-inserted tetramers (see Section 3.4.6), making La^{3+} useful in studying pore-independent toxin effects.

The role of tetramerisation in toxin pore formation has been vividly illustrated by mutagenesis of α-LTX. In particular, Ichtchenko et al. (1998) have generated an interesting mutant (α-LTX^{N4C}) that contains a four-amino acid insert between the N-terminal domain and the ankyrin repeats (Figure 1B). This insert apparently causes a conformational change enabling α-LTX^{N4C} to form dimers but not cyclical tetramers (Volynski et al. 2003). Accordingly, although α-LTX^{N4C} still binds its receptors (Ichtchenko et al. 1998), it fails to incorporate into the membrane and form pores (Ashton et al. 2001; Volynski et al. 2003), providing a powerful argument that the tetramer is the molecular species that inserts into membranes.

3.2. Receptor interaction

Although α-LTX is able to insert into pure lipid membranes (Finkelstein et al. 1976), reconstituted receptors greatly enhance the rate of insertion (Scheer et al. 1986). Biological membranes seem even more refractive to the toxin: when cells do not possess α-LTX receptors, no pore formation can be detected (Hlubek et al. 2000; Van Renterghem et al. 2000; Volynski et al. 2000), whereas expression of exogenous receptors allows abundant α-LTX insertion and concomitant channel formation. Receptors, thus, confer specificity to the pore-mediated effects of α-LTX. It is not clear whether receptors are directly involved in membrane insertion, simply concentrate toxin near the membrane or organise membrane lipid domains to make them accessible to α-LTX. Importantly for some aspects of toxin-evoked secretion, interaction with one of the receptors, neurexin (NRX), is Ca^{2+}-dependent, while interaction with the other two, latrophilin 1 (LPH1) and protein tyrosine phosphatase σ (PTPσ), is completely Ca^{2+}-independent (see Section 4 for more details).

The role of the structural domains of α-LTX in receptor interaction has been studied by mutagenesis. Ichtchenko et al. (1998) have generated three point-mutants by replacing conserved cysteines in the N-terminal domain (Figure 1B) with serines. These mutants (C14S, C71S, C393S) are inactive in release, probably because they do not bind α-LTX receptors. Accordingly, chemical reduction of disulphide bonds in α-LTX abolishes receptor binding (ibid.). Thus, proper folding of the N-terminal domain, aided by disulphide bonds, is essential for toxin-receptor interactions.

In another work, Li et al. (2005) have deleted the C-terminal ankyrin repeats 15 to 22, comprising the head and part of the body (Figure 1B), and found that this mutant fails to bind LPH1 but still interacts with NRX Iα. This suggests that α-LTX complex with LPH1 requires an intact C-terminal quarter of the toxin molecule. Whether this sequence binds LPH1 directly or affects the conformation of other α-LTX regions that actually interact with LPH remains to be addressed. Clearly, the C-terminal region is not necessary for binding of NRX Iα.

3.3. Membrane insertion

The insertion of the α-LTX channel into black lipid membranes is unidirectional (Robello et al. 1984), suggesting that the toxin has evolved specialised domains to help it enter lipid bilayers. The direct observation of tetramers inserted into artificial lipid membrane (Orlova et al. 2000) indicates that the “bowl” formed by the body domains is immersed deeply in the lipid, while the wing domains remain splayed on, and slightly buried into, the extracellular surface of the bilayer, while the heads are exposed to the extracellular space (Figure 2). In this model, some regions of the body domains are exposed to the cytosol, consistent with the observation that protease treatment from that side of the lipid bilayer modifies the inserted channel (Robello et al. 1984; Chanturia and Lishko 1992) and supportive of the hypotheses that α-LTX could interact with intracellular release machinery (Khvotchev et al. 2000) or act as a fusogen catalysing vesicle fusion (Sokolov et al. 1987; Lishko et al. 1990).

In addition to the presence of receptors, the ionic composition of buffer (Finkelstein et al. 1976) and the makeup of lipid bilayer (Robello et al. 1984) can influence the rate of α-LTX insertion into lipid membranes. It is possible that membrane microenvironment affects the insertion of a receptor-bound tetramer also in a physiological context. For example, at 0 °C, α-LTX binds to receptor-containing membranes, but it is not inserted (Khvotchev et al. 2000; Volynski et al. 2000).

Another feature of α-LTX is that it co-purifies with a small protein, called LMWP (Volkova et al. 1995) or latrodectin (Pescatori et al. 1995). It does not affect the toxicity of α-LTX nor has secretory activity of its own (Volkova et al. 1995). However, when LMWP is removed (Ashton et al. 2000), the toxin becomes unable to form channels in artificial lipid bilayers, although its receptor-mediated permeation of biological membranes is unhindered (Volynski et al. 2000). It is tempting to speculate that LMWP helps wild-type α-LTX to penetrate lipid bilayers. To test this, recombinant toxins lacking LMWP need to be studied in pure lipid membranes.

The receptor-aided insertion of α-LTX into biological membranes has been recently investigated using recombinant toxin N-terminally fused to GST and expressed in bacteria (Li et al. 2005). When, after purification, the GST moiety is removed, the resulting wild type recombinant α-LTX inserts into the plasma membrane and induces strong cation currents in tsA cells (HEK-293-derived) transfected with NRX Iα, whereas the GST-fused toxin is much less effective. As both the GST-free and GST-fused toxins bind well to the receptors (Li et al. 2005), it is clear that GST attached to the α-LTX N-terminus decreases the efficiency of toxin incorporation into membranes. However, despite its large size and

hydrophilicity, GST does not block channel formation completely, indicating that when α-LTX inserts into the membrane, the wing does not need to penetrate the bilayer.

This idea is further supported by the analysis of a series of α-LTX truncation mutants, lacking 1, 2, 3 or 8 C-terminal ankyrin repeats (Li et al. 2005). When tested in NRX Iα-transfected tsA cells, some of these constructs induce cation conductance, others completely lack this ability. As all these mutants possess unaltered N-terminal wing domains, the wing apparently does not participate in pore formation, while the structure of the C-terminal head is crucial for membrane insertion. This is consistent with the model of the membrane-inserted α-LTX tetramer in Figure 2.

3.4. Features of the pore

3.4.1. Ion selectivity—Once formed, the α-LTX channel only mediates cationic currents, probably because negatively charged acidic side chains line the channel (Finkelstein et al. 1976). Most significantly, the α-LTX channel is permeable to Ca^{2+} (Finkelstein et al. 1976; Krasil'nikov et al. 1982; Mironov et al. 1986), and it is this aspect of the α-LTX channel that is most often considered. However, this channel is not very selective, and Ba^{2+}, Sr^{2+}, Mg^{2+} as well as Li^{+}, Cs^{+} and, importantly, Na^{+} and K^{+} currents are also carried by the channel (Krasil'nikov et al. 1982; Mironov et al. 1986).

A detailed analysis of the permeability of α-LTX channels to cations and non-electrolytes (Mironov et al. 1986; Robello 1989; Krasilnikov and Sabirov 1992) postulates that the negatively charged selectivity filter of the channel is located close to the extracellular side of the membrane, fitting in with the hypothesis that the C-terminal head domains form the selectivity filter (see Section 2.2.2).

This hypothesis finds additional confirmation in the features of the α-LTX truncation mutants described above (Section 3.3) (Li et al. 2005). These mutants have 1 to 8 ankyrin repeats removed from their C-termini and form cation channels of dramatically different conductivities. For example, α-LTxΔ1 mutant mediates an enormous conductance, by far exceeding that of wild type toxin. It is possible that the removal of the last ankyrin repeat lifts an obstruction for cation movements in the extracellular mouth of the channel. In contrast, α-LTxΔ2 and α-LTxΔ3 make very inefficient channels, probably due to perturbations of the channel lining. Finally, α-LTxΔ8 does not induce any cation currents, as it seemingly cannot form tetramers.

3.4.2. Ca^{2+} currents—Although Ca^{2+} only carries a small proportion of currents through cell membrane-inserted α-LTX channels (Hurlbut et al. 1994; Tse and Tse 1999), the influx of Ca^{2+} through presynaptically-targeted α-LTX channels is most often referred to, because of the well-established link between presynaptic $[Ca^{2+}]$ and neurotransmitter release. There is a wealth of evidence indicating that in conditions favourable to channel formation (e.g. in the presence of divalent cations), influx of extracellular Ca^{2+} through α-LTX channels is an important aspect of α-LTX action.

Increases in $[Ca^{2+}]_i$ upon α-LTX addition in the presence of $Ca^{2+}{}_e$ have been detected in synaptosomes (Nicholls et al. 1982; Meldolesi et al. 1984), native PC12 (Grasso et al. 1980; Meldolesi et al. 1984) and chromaffin (Barnett et al. 1996) cells as well as in receptor-transfected COS (Volynski et al. 2000; Ashton et al. 2001), BHK (Krasnoperov et al. 1997) and neuroblastoma cells (Volynski et al. 2004).

While the bulk of this effect is due to calcium influx through the toxin channel, it is possible that the rise in $[Ca^{2+}]_i$ is contributes to by Ca^{2+} release from intracellular stores (Tse and Tse 1999; Tsang et al. 2000; Liu et al. 2005) or influx through endogenous Ca^{2+} channels

(Nicholls et al. 1982; Lajus et al. 2006). In LPH-transfected neuroblastoma cells, Ca^{2+}_i waves caused by LPH signalling are observed in response to non-pore forming α-LTX (Volynski et al. 2004). Nevertheless, influx of Ca^{2+} through the channel formed by wild type toxin appears more dramatic.

3.4.3. Na^+ currents—α-LTX channels inserted in artificial membranes are permeable to Na^+ (Krasil'nikov et al. 1982; Robello et al. 1984), which is modulated by Ca^{2+} added to the topologically extracellular side of the bilayer (Mironov et al. 1986). Inward Na^+ currents are observed electrophysiologically in *Xenopus* oocytes, PC12 and neuroblastoma cells (Wanke et al. 1986; Filippov et al. 1994; Hurlbut et al. 1994), and α-LTX elevates $[Na^+_i]$ in synaptosomes detectable with a fluorescent dye (Deri and Adam-Vizi 1993), although not with radioactive Na^+ (Storchak et al. 1994).

Such currents probably account for at least one of α-LTX actions: tetrodotoxin-insensitive depolarisation of artificial and biological membranes (Grasso and Senni 1979; Nicholls et al. 1982; Scheer et al. 1986). Although depolarisation could cause neurotransmitter release by activating voltage-gated Ca^{2+} channels, Ca^{2+} flow through the α-LTX channel probably overwhelms this effect (Nicholls et al. 1982), so the role of depolarisation in α-LTX action is unclear.

In addition, Na^+ currents through α-LTX channels can underlie some of the α-LTX-induced non-vesicular neurotransmitter release by causing, for example, the collapse of the cross-membrane Na^+ gradient and the reversal of some (McMahon et al. 1990) but not all (Deri and Adam-Vizi 1993) transmitter uptake pumps.

α-LTX-mediated Na^+ influx can also induce mitochondria of frog NMJ synapses (Tsang et al. 2000) and neurohypophysis terminals (Hlubek et al. 2003) to release large amounts of Ca^{2+} into the cytosol because of the reversal of their Na^+/Ca^{2+} exchangers, although this seems to be inconsequential for secretion in the absence of Ca^{2+}_e. In contrast, increases in $[Ca^{2+}]_i$, partially dependent on Na^+_e, correlate with acetylcholine release in synaptosomes (Deri and Adam-Vizi 1993). Generally, a rise $[Na^+]_i$ in nerve terminals correlates with enhanced secretion (Meiri et al. 1981). Thus, Na^+ currents represent yet another facet of the α-LTX channel actions at the synapse and could account for some of the toxin-evoked Ca^{2+}_e-independent vesicular release. However, α-LTX may be able to induced release even in Ca^{2+}-free and Na^+-free medium in synaptosomes (Storchak et al. 1994) and frog NMJs (Tsang et al. 2000).

3.4.4. Factors affecting conductance—A perplexing feature of the literature that explores α-LTX channels is the extremely wide range of single-channel conductances, ranging from 15 pS (Wanke et al. 1986; Krasilnikov and Sabirov 1992) to 100-300 pS (Finkelstein et al. 1976; Van Renterghem et al. 2000) or even 1100 pS (Krasilnikov and Sabirov 1992). To put these figures in perspective, the voltage-gated K^+ channel Kv1.2 and the ryanodine-sensitive Ca^{2+}-release channels have conductances of 14-18 pS and ~700 pS, respectively (Lindsay et al. 1994; Gutman et al. 2005) (Figure 2).

Several plausible hypotheses can explain this variability. Firstly, the venom contains a range of pore-forming toxins with varying conductances. Secondly, there is evidence that the make-up of the permeated membrane (Robello et al. 1984; Scheer et al. 1986; Krasilnikov and Sabirov 1992) and variations in lipid packing and order (Chanturia and Lishko 1992), but not the type of receptor present (Hlubek et al. 2000; Van Renterghem et al. 2000), can affect the properties of α-LTX channels.

Interestingly, Ca^{2+} inhibits the conductance of α-LTX channels to monovalent cations, causing a "flickery block", in artificial membranes (Mironov et al. 1986; Krasil'nikov et al. 1988), neuroblastoma cells (Hurlbut et al. 1994) and embryonic kidney cells (Hlubek et al. 2000), although this is not apparent in receptor-expressing oocytes (Filippov et al. 1994). Mg^{2+} positively modulates the conductivity of toxin channel for Ca^{2+} (Davletov et al. 1998; Van Renterghem et al. 2000).

Finally, the frequent observation of bursts of conductance, likely corresponding to synchronised opening and closing events, has led many authors to suggest that α-LTX channels form clusters in the membrane (Robello et al. 1987; Krasilnikov and Sabirov 1992; Filippov et al. 1994; Van Renterghem et al. 2000). Such clustering is plausible from a structural point of view, as crystalline arrays of α-LTX tetramers are observed by EM (Lunev et al. 1991) (see Section 2.2.2), and it may account for some of the variability in conductances reported in the literature.

3.4.5. Gating—The channel is open most of the time, with an open probability of 0.8 (Filippov et al. 1994). Closure events, albeit rare, have been observed in many systems (Finkelstein et al. 1976; Wanke et al. 1986; Hurlbut et al. 1994), and several channels can open and close in concert (Krasilnikov and Sabirov 1992; Filippov et al. 1994; Tse and Tse 1999). Membrane depolarisation may increase the likelihood of closure events (Filippov et al. 1994; Van Renterghem et al. 2000), and Ca^{2+} and Mg^{2+} modulate gating (Filippov et al. 1994; Hurlbut et al. 1994). The molecular basis of gating is currently unknown, but is likely to involve head domains.

3.4.6. Divalent and trivalent cations—The α-LTX pore is permeable to alkaline earth cations, whose affinities for the channel decrease in the following sequence: $Mg^{2+} > Ca^{2+} > Sr^{2+} \geq Ba^{2+}$ (Mironov et al. 1986). Transition metal cations ($Cd^{2+} > Co^{2+} \geq Ni^{2+} \geq Zn^{2+} > Mn^{2+}$) strongly block Ca^{2+} and K^{+} currents through α-LTX channels in artificial membranes (Mironov et al. 1986). This block is only effective when the cation is applied from the *cis*-side (equivalent to the extracellular side) of the membrane.

Millimolar concentrations of trivalent cations, such as Yb^{3+}, Gd^{3+}, Y^{3+}, La^{3+} and Al^{3+}, block the pore-mediated effects of α-LTX in synaptosomes (Scheer 1989). The nature of this effect is complex. For example, Al^{3+} blocks the binding of α-LTX to synaptosomes (Scheer 1989), while La^{3+} prevents tetramerisation (Ashton et al. 2000) and thereby channel insertion and formation.

Most trivalent cations, at 50-100 μM, block previously inserted channels (Scheer 1989; Hurlbut et al. 1994; Van Renterghem et al. 2000) and inhibit α-LTX-mediated Ca^{2+}-uptake, while La^{3+} blocks depolarisation as well (Scheer 1989). Channel inhibition by trivalent cations is very important because La^{3+} is essentially the only reagent that blocks the Ca^{2+}-independent α-LTX-evoked neurotransmitter secretion in neurons (Scheer 1989; Capogna et al. 2003).

The use of trivalent cations for the purpose of dissecting the mechanisms of α-LTX action, however, is complicated by their well-described ability to trigger neurotransmitter release in the absence of other stimuli (Scheer 1989). Furthermore, at low concentrations, both La^{3+} (at 15 μM) and Cd^{2+} (at 50 μM) support, rather than block, the secretagogue activity of the toxin (Misler and Falke 1987).

3.4.7. Permeation of molecules/leakage—It is possible that the water-filled α-LTX channel, which is relatively wide (~10 Å at its narrowest (Krasilnikov and Sabirov 1992; Orlova et al. 2000), can pass small molecules. Indeed, α-LTX channels inserted in the

membranes of synaptosomes, NMJ nerve terminals and receptor-transfected COS7 cells appear to pass fluorescein (Stokes-Einstein radius, R_e = 4.5 Å) and norepinephrine ($R_e \leq 4$ Å) (Davletov et al. 1998; Rahman et al. 1999; Volynski et al. 2000), shown in Figure 2 for comparison with 8-hydrated calcium ion (R_e = 4.2 Å) and the toxin channel. Analysis of "impermeant" cations commonly used in channel studies reveals that α-LTX channels are poorly permeable (Hurlbut et al. 1994) to glucosamine H^+ (R_e = 4.6 Å) and not significantly permeable (Tse and Tse 1999) to N-methyl-D-glucamine (R_e = 5.2 Å), thus limiting the pore diameter by ~10 Å.

These observations are particularly relevant to the experimental use of α-LTX in neurotransmission studies, since α-LTX has been shown in several systems to cause non-vesicular release by allowing leakage of cytoplasmic neurotransmitters (McMahon et al. 1990; Deri et al. 1993; Davletov et al. 1998). This flux could be mediated by the α-LTX channel itself, by local disruptions of cellular membranes, or by reversal of transmitter uptake pumps driven by Na^+ gradient (see Section 3.4.3). Synaptosomes seem to be particularly sensitive to an increase in hydrostatic pressure, which may occur when influx of Na^+ or Ca^{2+} leads to a concomitant influx of water.

3.5. Can the pore explain everything?

It is now universally accepted that α-LTX can insert into, and permeabilise, artificial and biological membranes. Cation currents can explain some, but not all, of the toxin's effects. For example, it is not clear how the α-LTX channel could mediate Ca^{2+}_e-independent exocytosis in neurons. Although it would be tempting to assign some of α-LTX actions in the absence of Ca^{2+}_e to Na^+ currents, lack of Na^+ does not prevent Ca^{2+}-independent secretion (Tsang et al. 2000). Cation flux-associated incursion of terminals by water could be involved, but α-LTX effect on intracellular osmotic pressure has not been characterised yet.

Based on the results of α-LTX mutagenesis, strong correlation exists between pore formation and stimulation of Ca^{2+}-dependent exocytosis from neuroendocrine cells. However, in some experiments with chromaffin cells, α-LTX action does not involve Ca^{2+} entry (Michelena et al. 1997). In addition, α-LTX sensitises chromaffin cells to Ca^{2+} even when the cells are permeabilised and toxin pores should have no effect; this involves protein kinase C (PKC) activation (Bittner and Holz 2000). Furthermore, the ability of $\alpha\text{-LTX}^{N4C}$ to induce Ca^{2+}-dependent exocytosis without forming pores implicates a stimulating mechanism other than pore formation.

Therefore, α-LTX receptors, which are so crucial for the toxin's action in all biological systems, have been comprehensively studied.

4. Receptors

The three currently known receptors for α-LTX - NRX, LPH (or CIRL) and PTPσ - were all isolated from brain extracts by affinity chromatography on immobilised α-LTX (Petrenko et al. 1990; Davletov et al. 1996; Krasnoperov et al. 1996; Krasnoperov et al. 2002a). Surprisingly, they were purified only as a result of three separate efforts several years apart. In part, this piecemeal discovery of α-LTX receptors reflects differences in purification procedures; in part, it is due to the researchers' inability to explain all the actions of the toxin by the features of each newly found receptor, warranting further attempts at isolating the "ultimate" target of α-LTX. While new proteins interacting weakly/transiently with the toxin and mediating some of its activities may still be found, there is no doubt that NRX Iα, LPH1 and PTPσ exhaust the repertoire of major α-LTX receptors. As these proteins are discussed in detail below, it is important to remember that, with any receptor, wild-type α-

LTX can insert into the membrane and form pores (or engage in other interactions), causing strong direct effects that make definitive conclusions regarding receptor signalling, or lack of it, difficult. Only when membrane insertion and pore formation are blocked (as, for example, in α-LTX^{N4C} mutant, see 2.3), can the observed effects be attributed to the action of one or the other receptor.

4.1. Neurexin

The first α-LTX receptor to be identified was isolated from solubilised bovine brain by toxin-affinity chromatography in the presence of Ca^{2+} (Petrenko et al. 1990) and termed neurexin (NRX) (Ushkaryov et al. 1992) (Figure 3).

4.1.1. Structure—NRX is a member of a family of polymorphous neuronal cell-surface receptors (Ushkaryov et al. 1992; Ushkaryov and Sudhof 1993; Ullrich et al. 1995) (Figure 3A). Three homologous genes encode NRXs I, II and III. Each gene, in turn, is transcribed from two independent promoters (α and β), leading to a long (α) and a short (β) forms of each NRX (Missler et al. 1998a). The resulting six main NRXs are further diversified by extensive alternative splicing at five splice sites. As a result of independent combinations of variable-size inserts, hundreds of NRX isoforms may be produced (Ullrich et al. 1995; Missler and Sudhof 1998). Given the differential expression of the three NRX genes in brain regions (Ullrich et al. 1995), developmental regulation of the two promoters and cell-specific alternative splicing (Ushkaryov and Sudhof 1993; Ullrich et al. 1995; Occhi et al. 2002), NRXs could play the role of neuronal recognition tags (Missler and Sudhof 1998).

All NRXs have a single transmembrane region (TMR) and a short cytoplasmic tail. In α-NRXs, the large ectodomains are composed of six LNS domains (Figure 3A) characteristic of many adhesion molecules. Pairs of LNS(A) and LNS(B) domains, with EGF-like sequences in the middle, form three “major repeats” (Figure 3A). In contrast, β-NRXs have one LNS(B) domain and a unique N-terminal sequence. All NRXs contain an extensively O-glycosylated Thr- and Ser-rich region upstream of the TMR (Ushkaryov et al. 1992; Ushkaryov et al. 1994).

4.1.2. Distribution—NRXs are present mostly in brain (Ushkaryov et al. 1992) but also in pancreas and lung (Occhi et al. 2002). They are differentially expressed in brain regions, showing partially overlapping patterns of distribution of their six main forms and region-specific splice variants (Ullrich et al. 1995). It is difficult to localise NRX proteins at the cellular level, partly because the respective α- and β-forms are almost identical immunologically. As demonstrated by light microscopy, NRXs are expressed at synapses (Ushkaryov et al. 1992; Dean et al. 2003), but in the absence of EM data, their distribution between the pre- and postsynaptic membranes is not clear. Based on the presynaptic action of α-LTX, NRX Iα is implicitly presynaptic; β-NRXs are assigned to presynaptic membranes due to their interaction with neuroligins, indeed localised in the postsynaptic membrane by EM (Song et al. 1999). It remains to be seen whether any NRXs may be expressed postsynaptically.

4.1.3. Binding of α-LTX—Analysis of NRX isolated from brain by α-LTX-affinity chromatography (Petrenko et al. 1990; Petrenko et al. 1993) shows that out of the six main isoforms only NRX Iα binds toxin with sufficient affinity (Davletov et al. 1995), and this interaction strictly requires Ca^{2+} (Petrenko et al. 1990; Davletov et al. 1995).

The α-LTX binding site has not been mapped in a systematic effort, but incidental evidence implicates the third major repeat, LNS(A3/B3). In particular, α-LTX binds neither a NRX Iα mutant lacking the LNS-domains B2, A3 and B3, nor the full-size NRX Iα with an insert

in LNS(B3) (Davletov et al. 1995). Although LNS(B3) domains of all three β-NRXs are able to interact with α-LTX in a splicing-dependent manner under mild conditions (Sugita et al. 1999), they are not sufficient for strong toxin binding: NRX Iβ does not pull down α-LTX (Davletov et al. 1995), nor are β-NRXs isolated from brain by α-LTX chromatography (Petrenko et al. 1990). Finally, a NRX Iα mutant, containing only LNS(A3/B3), binds the toxin well (Li et al. 2005). Thus, the strong interaction of α-LTX with NRX Iα is mediated by both LNS(A3) and LNS(B3), while splicing in LNS(B3) regulates this binding.

4.1.4. Proposed functions—The structure of NRXs is consistent with a function in cell adhesion and/or ligand binding. Indeed, the extracellular domains of α-NRXs interact specifically and strongly with neurexophilins (Petrenko et al. 1993; Petrenko et al. 1996; Missler et al. 1998b), a family of glycosylated, proteolytically processed hormone-like 30-kDa proteins with species-specific differential expression in mammalian tissues (Petrenko et al. 1996; Missler and Sudhof 1998). The biological role of neurexophilins is unknown (Missler et al. 1998b), but their tight binding to the LNS(B1) domain of α-NRXs implies long-term/structural, rather than short-term/signalling, interactions.

In addition, both α- and β-NRXs can interact with neuroligins, a family of neuronal proteins that contain a region of homology to esterases in their extracellular domains and a single TMR (Ichtchenko et al. 1995; Ichtchenko et al. 1996) (Nguyen and Sudhof 1997; Boucard et al. 2005). Neuroligin-1 has been identified owing to its ability to bind all three β-NRXs - only if they lack an insert at splice site 4 (Ichtchenko et al. 1995) (Figure 3). Neuroligins are also differentially spliced, and the absence of an insert at splice site B enables neuroligins to bind also α-NRXs (Boucard et al. 2005). These proteins are localised on the postsynaptic membrane of excitatory (Song et al. 1999) and inhibitory (Varoqueaux et al. 2004) synapses. Their Ca^{2+}-dependent, splicing-controlled interaction with β-NRXs (Ichtchenko et al. 1996; Nguyen and Sudhof 1997; Comoletti et al. 2006) is important for synapse formation (Scheiffele et al. 2000; Dean et al. 2003; Chih et al. 2006), differentiation (Levinson et al. 2005) and maturation (Dresbach et al. 2004; Varoqueaux et al. 2006).

In the cytoplasmic tail of NRXs, there is a short C-terminal site that binds to PDZ domain-containing scaffold proteins, CASK (Hata et al. 1996) and Mint (Biederer and Sudhof 2000). These proteins interact with each other and with Ca^{2+} channels; Mint also makes complexes munc18, a protein involved in vesicle exocytosis (Verhage et al. 2000). Therefore, NRXs may participate in arranging transmitter release mechanisms. Indeed, knockout of all three α-NRXs has demonstrated that they play a role in organising N- and P/Q-type Ca^{2+} channels (Zhang et al. 2005) in nerve terminals (Missler et al. 2003) and endocrine cells (Dudanova et al. 2006). Even though β-NRXs have the same C-terminal sequences as the respective α-NRXs, the two isoforms participate differently in synaptogenesis and transmitter secretion. In particular, α-NRXs are apparently not involved in synapse formation (Missler et al. 2003) but may influence the expression of postsynaptic NMDA (but not AMPA) glutamate receptors (Kattenstroth et al. 2004).

4.2. Latrophilin

Because NRX Iα requires Ca^{2+} to bind α-LTX, it cannot mediate the toxin's effects in the absence of Ca^{2+}. The quest for a Ca^{2+}-independent receptor continued, and a major protein was eventually isolated by α-LTX-chromatography of solubilised brain proteins and termed latrophilin (LPH) (Figure 4) (Davletov et al. 1996; Lelianova et al. 1997), or Ca^{2+}-independent receptor of α-LTX (CIRL) (Krasnoperov et al. 1996; Krasnoperov et al. 1997). LPH binds toxin in the presence or absence of divalent cations (Davletov et al. 1996). It was not discovered earlier because its 120-kDa toxin-binding fragment was confused on SDS

gels with α-LTX, while the second (65 kDa) fragment was lost due to its quantitative precipitation upon boiling with SDS.

4.2.1. Structure—LPH is a large (185 kDa) heptahelical receptor. It comprises three major domains (Figure 4A): (1) a long, glycosylated N-terminal extracellular domain; (2) seven hydrophobic TMRs; and (3) a long cytoplasmic tail. The ectodomain contains regions of homology to: galactose-binding lectin; the surface-attached extracellular matrix protein olfactomedin (Snyder et al. 1991; Loria et al. 2004); a hormone receptor motif (HRM) found also in other G-protein-coupled receptors (GPCRs) and probably involved in ligand binding; a "Stalk" domain important for proteolytic cleavage (Chang et al., 2003); and the G-protein-coupled receptor proteolysis site (GPS). The seven TMRs are similar to the corresponding regions of the secretin/calcitonin GPCRs that bind peptide hormones and induce release of various substances (Harmar 2001).

Based on this homology and on the length of ectodomains, LPH and similar receptors have been initially classified as "Long N-terminus group B" GPCRs (Hayflick 2000; Stacey et al. 2000). However, it is recognised now (Fredriksson and Schioth 2005) that they form a separate group, termed "adhesion GPCRs". This nomenclature stems from the fact that these receptors contain various cell adhesion modules in their ectodomains. The group includes 33 proteins identified by their function/activity or through genome searches (Fredriksson and Schioth 2005).

LPH has two very similar homologues, LPH2 and LPH3 (Matsushita et al. 1999). The family is also called CIRL1-3 (Ichtchenko et al. 1999) and CL1-3 (an abbreviation combining the two other names of the protein, CIRL and LPH) (Sugita et al. 1998). The LPHs have seven sites of alternative splicing (Sugita et al. 1998; Matsushita et al. 1999). Some splice variants encode receptors with an altered third cytoplasmic loop or a truncated cytoplasmic tail (Matsushita et al. 1999), modifications that are likely to affect coupling to G proteins.

4.2.2. Unusual architecture—"Adhesion GPCRs" have an unusual architecture (Gray et al. 1996; Krasnoperov et al. 1997; Volynski et al. 2004). They contain two functional parts: an N-terminal cell adhesion receptor-like extracellular domain and a C-terminal domain with seven TMRs and a signalling potential (Hayflick 2000; Stacey et al. 2000). Intriguingly, all these receptors are post-translationally cleaved at the GPS domain (Krasnoperov et al. 1997) (Figure 4A, B) into N- and C-terminal fragments (NTF and CTF), which correspond to the two functional parts. This constitutive proteolysis, occurring in the endoplasmic reticulum, is prerequisite for LPH cell surface delivery (Krasnoperov et al. 2002a; Volynski et al. 2004). The GPS is localised upstream of the first TMR, yet the ectodomain is not released into the medium, apparently because it is attached to the membrane by a hydrophobic anchor (Volynski et al. 2004).

The peculiar biology of LPH has been studied in neuroblastoma cells stably expressing recombinant LPH1 (Volynski et al. 2004). Following delivery to the plasma membrane, the two receptor fragments can dissociate and behave as independent cell-surface proteins. However, under certain conditions, the fragments re-associate. This re-assembly, mediated only by seven most N-terminal amino acids of the CTF, is induced by detergents and also by α-LTX binding to the NTF (ibid.). Ternary α-LTX-NTF-CTF complexes form large patches in the plasma membrane, triggering formation of molecular dimers of the CTF. This process temporally and spatially correlates with signal transduction, which can be visualised using the non-pore-forming mutant $\alpha\text{-LTX}^{N4C}$ (see Section 2.3). This signalling requires the full-size CTF and involves activation of PLC and release of Ca^{2+} from intracellular stores (ibid.). It is interesting that both in this model system and in central synapses (Ashton et al. 2001;

Capogna et al. 2003), α-LTX^{N4C} induces Ca^{2+} signals only in the presence of Ca^{2+}_e. Calcium ions may serve here as a co-factor for LPH activation or they may enter the cytosol through an LPH-activated Ca^{2+} channel(s).

4.2.3. Distribution—LPHs are differentially distributed in mammalian tissues: while LPH1 and LPH3 are enriched in brain, LPH2 features more prominently in other tissues (Sugita et al. 1998; Ichtchenko et al. 1999; Matsushita et al. 1999). Similar to NRX Iα (Ushkaryov et al. 1992; Occhi et al. 2002), very small levels of LPH1 mRNA can be detected outside brain, especially in kidneys and pancreas, and both receptors reside in endocrine cells, such as pancreatic β-cells (Lang et al. 1998). Based on α-LTX binding and action, which are dramatically inhibited by the LPH gene knockout, this receptor is localised in presynaptic terminals. In a yeast two-hybrid system, the C-terminal cytoplasmic tail of LPH can bind Shank (Tobaben et al. 2000; Kreienkamp et al. 2000), an ankyrin repeat-containing protein of postsynaptic density, although the specificity and significance of this interaction is unclear.

4.2.4. Binding of α-LTX—LPH1 has a very high affinity for α-LTX (Kd ~0.1-1.5 nM) (Volynski et al. 2000; Ichtchenko et al. 1999), while the interaction of LPH 2 with toxin is more than 10-fold weaker (Ichtchenko et al. 1999). α-LTX binds the Stalk/GPS domains of the NTF (Krasnoperov et al. 1999), while the lectin and olfactomedin regions upstream could participate in adhesion interactions with the extracellular matrix or adjacent cells (Figure 4B). Hence, the NTF is thought to remain on the cell surface at specialised cell-cell or cell-matrix junctions (Volynski et al. 2004) and, thus, define the sites where the LPH fragments can interact with each other. If endogenous CTF-mediated signalling requires NTF-CTF complex formation (as with α-LTX), then LPH signals may be restricted to adhesion foci.

4.2.5. Proposed functions—The functions of LPH remain to be elucidated. As follows from the results of LPH1 gene knockout (Tobaben et al. 2002), this receptor is not crucial for basic neuronal survival and activity, although LPH2 and 3 may compensate for the lack of LPH1. The structure of all adhesion GPCRs indicates that these receptors can transform extracellular interactions into intracellular signalling. Being involved in some pathways leading to release of stored Ca^{2+}, LPH may participate in the cell contact-dependent fine tuning of Ca^{2+}_i levels and modulation of transmitter secretion.

4.3. Protein tyrosine phosphatase σ

In the search for a Ca^{2+} independent α-LTX receptor, affinity chromatography in the absence of Ca^{2+} was used to isolate brain proteins with any affinity for α-LTX.

Sequencing of all proteins in the α-LTX column eluate (Krasnoperov et al. 1996; Krasnoperov et al. 2002b) has revealed that, in addition to LPH1, PTPσ can also bind to the column as a set of two minor bands.

4.3.1. Structure—PTPσ is a member of the family of receptor-like PTPs that contain cell adhesion molecule-like extracellular domains, a single TMR and cytoplasmic phosphatase domains (Tonks 2006). In PTPσ, the extracellular domain comprises three immunoglobulin-like modules and, depending on alternative splicing, from four to eight fibronectin type III-like domains (Figure 5A). The cytosolic portion of the protein contains a catalytically active phosphatase domain and a pseudo-phosphatase.

Reminiscent of LPH, PTPσ is proteolytically cleaved 125 residues upstream of the TMR into two fragments, which remain non-covalently associated (Figure 5A) (Yan et al. 1993;

Aicher et al. 1997). The proteolysis occurs inside the cell but, unlike in LPH, is not necessary for cell surface delivery of PTPs (Serra-Pages et al. 1994). On the cell surface, the P-subunit of PTPσ can be further cleaved by the metalloprotease TACE (Ruhe et al. 2006) six residues upstream of the TMR (Aicher et al. 1997). This process, stimulated by treating cells with Ca^{2+} ionophores or phorbol esters (ibid.), or by interaction of PTPσ with the epidermal growth factor receptor (EGFR) (Ruhe et al. 2006), leads to the shedding of the E-subunit and sequestration of the P-subunit (Figure 5B), thus downregulating the PTPσ function.

4.3.2. Distribution—The large splice variant of PTPσ is found in most tissues, while the shorter variant is mostly brain-specific (Yan et al. 1993; Pulido et al. 1995) but, similar to NRX Iα and LPH1, is also found in small amounts in some other tissues (Yan et al. 1993), which may be due to autonomic innervation.

4.3.3. Binding of α-LTX—Despite the very low binding of this protein to toxin columns, the α-LTX-PTPσ interaction is specific and maps onto the fibronectin domains 2 and 3 (Krasnoperov et al. 2002b). These are present in both PTPσ1 and PTPσ2, but on over-expression of these splice variants in pancreatic β-cells, only PTPσ1 mediates the secretory action of α-LTX (Lajus and Lang 2006). This may be because the toxin binds PTPσ2 too far from the membrane and is then unable to insert into the plasma membrane.

4.3.4. Proposed functions—As phosphorylation of proteins localised in focal adhesions controls cell-cell and cell-matrix interactions, dephosphorylation is also important for the regulation of cell adhesion. Indeed, PTPσ is involved in negative regulation of axonal growth (McLean et al. 2002; Thompson et al. 2003) and growth cone pathfinding (Meathrel et al. 2002). Apart from EGFR (see 4.3.1), PTPσ interacts with N-cadherin and dephosphorylates it, augmenting its catenin-mediated attachment to the cytoskeleton and inhibiting axonal growth (Figure 5B). In PTPσ knockout mice, N-cadherin becomes over-phosphorylated (Siu et al. 2006) and unable to interact with catenins and the cytoskeleton, leading to stimulated axonal growth (McLean et al. 2002).

The structure of PTPσ is consistent with its role in binding ligands on the surface of other cells or in the extracellular matrix. It interacts with heparin sulphate proteoglycan in the basement membrane (Sajnani-Perez et al. 2003). It may also bind to the C-terminal domain of cell surface-exposed nucleolin, a normally nuclear protein that is presented on the surface of developing muscle cells (Alete et al. 2006). However, these complexes seem to mediate structural or long-term regulatory interactions rather than short-term signalling to the neuronal secretory machinery.

4.4. α-LTX receptors and signalling

4.4.1. NRX Iα—Can NRXs transduce intracellular an exocytotic signal? All currently known cytoplasmic interactions of α- and β-NRXs appear to be stable, non-enzymatic, structural connections that are essential for neuronal development, synapse organisation and long-term regulation but are unlikely to underlie a massive and relatively fast response induced by α-LTX. However, toxin binding to NRXs might cause conformational changes in Mint, which, by interacting with munc18, might regulate the availability of docking sites for synaptic vesicles. On the other hand, even a truncated NRX Iα that lacks the cytoplasmic tail can mediate the sensitivity of PC12 cells to wild-type α-LTX (Sugita et al. 1999). The most likely explanation of this is that toxin inserts into the membrane on binding any membrane-attached form of NRX Iα (Volynski et al. 2000; Hlubek et al. 2000), and because NRX experiments require Ca^{2+}_e, influx of this cation through the toxin pore can trigger maximal exocytosis irrespective of any NRX Iα-mediated signalling. With this in mind,

there is no doubt that NRXs (full-size or C-terminally truncated) constitute functional receptors for α-LTX (Sugita et al. 1999), but only in the sense that they provide binding sites for the toxin, which subsequently exerts its own actions.

4.4.2. LPH1—LPH1 is the only receptor capable of G-protein-mediated signalling induced by α-LTX in central synapses (Davletov et al. 1998; Ashton et al. 2001; Section 1.4). Furthermore, LPH1 is directly involved in α-LTX^{N4C}-induced signalling in LPH-transfected neuroblastoma cells (Volynski et al. 2004). This mutant activates PLC also in neurons, causing release of $Ca^{2+}{}_i$ (Ashton et al. 2001; Volynski et al. 2003; Volynski et al. 2004) and leading to neurotransmitter release, which is blocked when the Gq cascade is inhibited by various drugs (Davletov et al. 1998; Ashton et al. 2001; Capogna et al. 2003). LPH1 also co-purifies with Gq (Rahman et al. 1999).

The endogenous ligand of LPH1 is currently unknown, and it is interesting to consider an invertebrate LPH orthologue from *C. elegans* (Mee et al. 2004; Willson et al. 2004). The nematode protein LAT-1 (B0457.1) lacks the olfactomedin region but contains the domains required for α-LTX binding (HRM, Stalk and GPS). Although its identity to vertebrate LPH1 is not too high (18-45 % in the area of toxin binding), it mediates the lethal effect of an α-LTX homologue, ε-latroinsectotoxin (Mee et al. 2004). *C. elegans* also expresses NRX and PTPσ homologues, but only the knockdown of LPH renders the worms resistant to ε-latroinsectotoxin (Mee et al. 2004). Interestingly, the worm LPH mediates the action of the anthelmintic octadepsipeptide emodepside, which causes paralysis in nematodes (Willson et al. 2004). By the coupling to LAT-1, emodepside activates Gαq and PLC-β (ibid.). The product of the PLC activity, IP_3 stimulates release of Ca^{2+} from intracellular stores, known to be important for α-LTX activity in mammals (Davletov et al. 1998; Capogna et al. 2003). In addition, the effect of emodepside involves UNC-13, a protein implicated in control of vesicular exocytosis and activated by diacyl glycerol, another product of PLC. It is possible that such different agonists as emodepside and LTX^{N4C} can stimulate similar signalling from LPH orthologues, suggesting that at least part of the α-LTX effect on exocytosis is due to LPH signal transduction.

Is LPH involved only in one type of signalling, to Ca^{2+} stores? In fact, α-LTX^{N4C}, acting via LPH, can activate Ca^{2+} and inhibit K^+ channels in pancreatic β-cells (Lajus and Lang 2006), raising the possibility that, similar to many other GPCRs, LPH may be linked to ion channels. In addition, in endocrine cells, α-LTX^{N4C} triggers $Ca^{2+}{}_e$-independent release of stored calcium and also activates PKC (Liu et al. 2005; Bittner et al. 2005). Thus, α-LTX can both provide an exocytotic stimulus (by releasing stored Ca^{2+}) and sensitise the secretory apparatus to it (by activating PKC).

4.4.3. PTPσ—Only LTX binding to PTPσ1, but not to PTPσ2, leads to secretory response in β-cells (Lajus and Lang 2006). This indicates that LTX apparently does not evoke any signalling through PTPσ, which probably simply aids toxin pore formation. Indeed, the multimeric toxin must induce the dimerisation of PTPσ, leading to reciprocal inhibition of the catalytic domains (Tonks 2006). Furthermore, while all three receptors are found naturally occurring in pancreatic β-cells, the distribution of PTPσ does not correlate with the endogenous sensitivity of these cells to α-LTX (Lang et al. 1998; Lajus and Lang 2006).

4.4.4. α-LTX mutants—As α-LTX^{N4C} (see 2.3) does not form pores, it probably stimulates exocytosis by receptor mediated signalling. Therefore, the activity of α-LTX^{N4C} has been investigated in Ca^{2+}-dependent and Ca^{2+}-independent exocytosis from neurons and Ca^{2+}-dependent exocytosis from neuroendocrine cells.

In Ca^{2+}-free media, the mutant toxin does not stimulate substantial exocytosis in neurons (Ashton et al. 2001; Volynski et al. 2003; Capogna et al. 2003). This strongly indicates that Ca^{2+}-independent exocytosis, so characteristic of wild type α-LTX, requires more than receptor activation. However, α-LTX^{N4C}, strongly stimulates transmitter release from neurons in the presence of Ca^{2+}_e, presumably due to its ability to activate LPH1 (Ashton et al. 2001; Capogna et al. 2003; Volynski et al. 2003). The specific details of how receptor activation triggers exocytosis remain to be established, but it is clear that thapsigargin-sensitive release of Ca^{2+}_i plays a pivotal role (Ashton et al. 2001; Capogna et al. 2003).

α-LTX^{N4C} is also able to stimulate Ca^{2+}-dependent exocytosis from endocrine cells: it is active in chromaffin cells in the presence of Ca^{2+} or Sr^{2+} (Volynski et al. 2003) and in pancreatic β-cells (Liu et al. 2005; Lajus and Lang 2006). On the other hand, bacterially expressed α-LTX^{N4C} appears unable to stimulate Ca^{2+}-dependent exocytosis from PC12 cells (Li et al. 2005), but this may be due to mutant's misfolding or by specific characteristics of the PC12 cell line used.

4.4.5. Receptor knockouts—The genes for all three α-LTX receptors have been knocked out in mice, and these mutations are not lethal. NRX Iα knockout mice show no obvious behavioural phenotype (Geppert et al. 1998). LPH1 knockout causes social problems in mice (Tobaben et al. 2002), consistent with LPH1 involvement in schizophrenia (Chen and Chen 2005). PTPσ knockout leads to developmental abnormalities in the nervous system and outside it (Meathrel et al. 2002; Batt et al. 2002).

In mice lacking the NRX Iα gene, Ca^{2+}-dependent α-LTX binding to brain membranes is inhibited by 60 % (Geppert et al. 1998), although the Ca^{2+}-independent binding seems to be affected as well (ibid.). LPH1 knockout leads to the loss of 75% of Ca^{2+}-independent binding of α-LTX but also affects the Ca^{2+}-dependent binding (Tobaben et al. 2002). The NRX-LPH1 double knockout still binds about 25% of α-LTX compared to wild type mice, both in the presence and absence of Ca^{2+} (ibid.). Ca^{2+}-dependent release of glutamate induced by wild-type α-LTX in synaptosomes from the NRX Iα, LPH1 and NRX Iα -LPH1 knockout mice is similarly inhibited, while Ca^{2+}-independent secretion, is most grossly affected in the LPH1 single knockout, but not abolished. Surprisingly, the inactivation of both LPH1 and NRX Iα is less detrimental for the toxin-stimulated release than the LPH1 knockout alone (ibid.). These data suggest that the two α-LTX receptors, NRX Iα and LPH1, may functionally interact (Sudhof 2001), e.g. by reciprocally influencing each other's expression or synaptic delivery and, thus, altering the actions of α-LTX. The removal of both NRX Iα and LPH1 from synaptic cleft may allow the pore-forming toxin to access PTPσ more freely, with subsequent membrane insertion and Ca^{2+}-independent effects. It would be interesting to test non-pore-forming α-LTX mutants on neuronal preparations from NRX Iα, LPH1 and PTPσ knockout mice.

5. Overview of mechanisms

α-LTX causes Ca^{2+}_e-dependent and -independent release of neurotransmitters (Figure 6). Dense core vesicles require Ca^{2+}_e, while synaptic vesicles, containing amino acids or acetylcholine, can be stimulated in its absence. However, in the presence of Ca^{2+}, transmitter is stronger overall due to initially unperturbed vesicle recycling.

All α-LTX actions in biological systems require receptors, which provide binding sites for the toxin on the cell surface. Once the toxin is bound, part of its Ca^{2+}-dependent action is due to pore formation and influx of Ca^{2+}. This mechanism triggers the release of both readily releasable and reserve pools of vesicles (Ashton et al. 2001). Another action is based on receptor-mediated signalling, which involves stimulation of PLC, production of IP_3 and

diacyl glycerol, with respective release of stored Ca^{2+} and activation of PKC. This mechanism, most likely mediated by LPH1, affects readily releasable vesicles only. Both the pore- and receptor-mediated signals can be amplified by the release of Ca^{2+}_i and influx of Ca^{2+}_e, producing Ca^{2+} waves. Mitochondria can contribute to the increase in $[Ca^{2+}]_i$. The pore-mediated component of the Ca^{2+}-dependent action can be blocked by La^{3+}, whereas the receptor-mediated action cannot (Ashton et al. 2001; Capogna et al. 2003).

In the absence of Ca^{2+}_e, α-LTX only binds to LPH1 and PTPσ. Ca^{2+}-independent exocytosis requires the presence of Mg^{2+} and toxin insertion into the plasma membrane, but these conditions also induce formation of α-LTX channels. Influx of Na^+ and efflux of K^+ through these channels and associated efflux of small molecules and influx/efflux of water may cause secretion. In addition, transmitter release can be caused by membrane perturbation or direct interaction with secretory machinery. Some secretion may be non-vesicular. Receptor-mediated signalling can cause the activation of PKC in some cells. However, Ca^{2+}-independent release is blocked by La^{3+}, indicating that toxin pores play a crucial role in this release.

Finally, although α-LTX receptors (at least LPH1) can transduce a signal, it is obvious that this signalling cannot explain all the effects of α-LTX. So, while the use of the toxin has brought about the discovery of neuronal cell adhesion protein families (NRX and LPH) and new knowledge about PTPσ, the toxin's effect in the absence of Ca^{2+}_e remains unclear, and its study may throw light onto the very basic mechanisms of exocytosis and different levels of its regulation.

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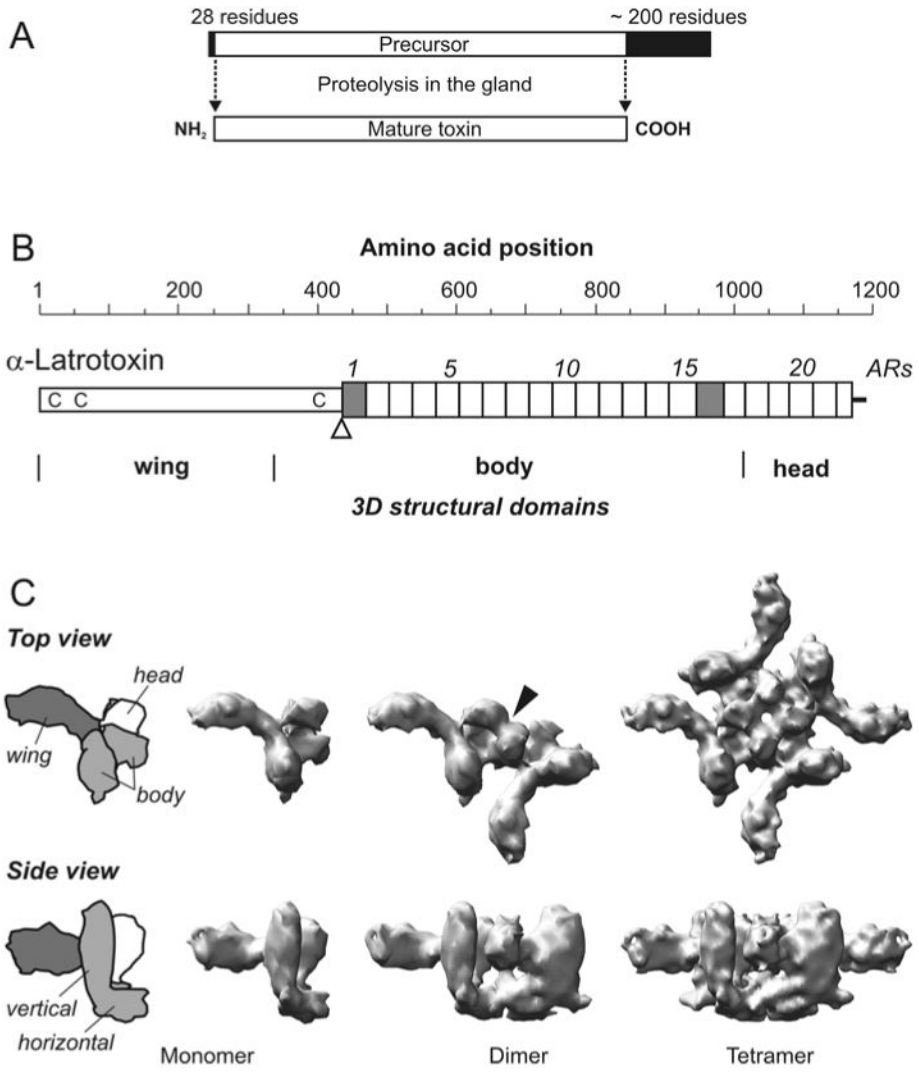

Figure 1.

The structure of α-LTX. A, Schematic of α-LTX processing in the venom gland. B, Primary and domain structure. The numbered boxes, ankyrin repeats (ARs). Grey, imperfect repeats; C, conserved cysteines residues in the N-terminal domain; open arrowhead, insert in the mutant α-LTX^{N4C}. Protein domains identified from the 3D structure (Orlova et al. 2000) are delimited below. C, 3D reconstructions of the α-LTX monomer, dimer and tetramer, viewed from the top and side. The monomer has been computationally extracted from the experimentally determined tetramer structure. Leftmost image, a scheme of the monomer, with the domains designated by different shades of grey. Filled arrowhead, strong association of the head domains in the dimer.

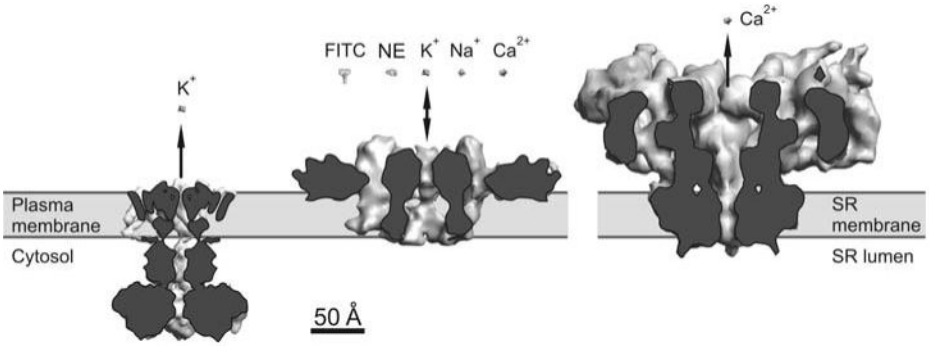

Figure 2.

Membrane topography of the α-LTX pore. Cross-section of the α-LTX tetramer embedded in a membrane (as observed in liposomes) (Orlova et al. 2000) is shown alongside the cut-open voltage-dependent K^+ channel (Kv1.2) (Long et al. 2005) and Ca^{2+} release channel (ryanodine receptor) (Serysheva et al. 2005). Fully hydrated cations and molecules known to permeate through the respective channels are shown next to each reconstruction (FITC, fluoresceine isothiocyanate; NE, norepinephrine). The narrowest part of the α-LTX channel is 10 Å. Molecular images were produced using the UCSF Chimera package (Pettersen et al. 2004).

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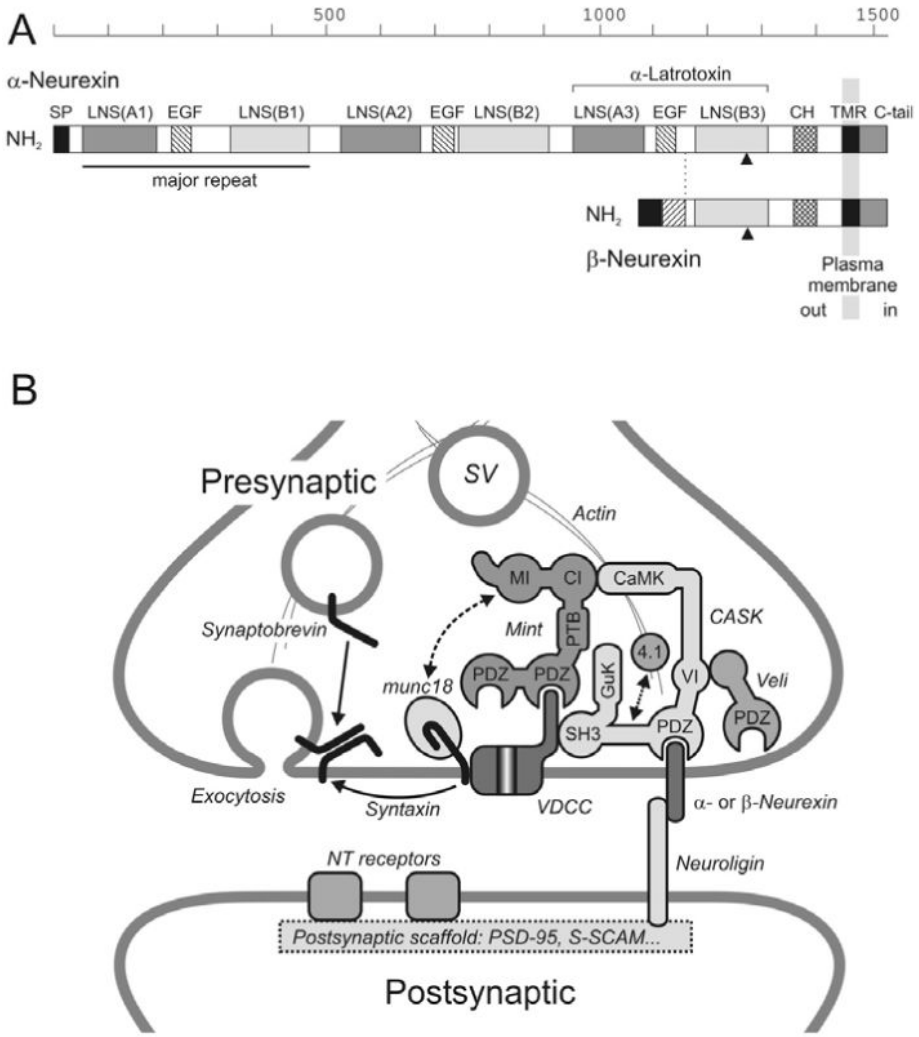

Figure 3.

Generalised structure and functions of neurexins. A, Domain structure of α- and β-neurexins. Scale above shows amino acid positions. Arrowheads, splice site 4; SP, signal peptide; LNS, laminin G/NRX/sex hormone-binding globulin-like domains; EGF, epidermal growth factor-like repeats; CH, O-linked carbohydrate attachment domain; bracket, α-LTX binding site. α- and β-NRXs are identical to the right of the dotted line. B, Scheme of NRX interactions. Domain names: CaMK, Ca^{2+}, calmodulin-dependent protein kinase homology; CI, CaMK-interacting; GuK, membrane-associated pseudo-guanylate kinase; MI, munc18-interacting; VI, Veli (vertebrate Lin7)-interacting. Other abbreviations: 4.1, protein 4.1 bound to actin; Mint, munc18-interacting protein; NT, neurotransmitter; PTB, phosphotyrosine binding; SH3, src homology; S-SCAM, synaptic scaffolding molecule; SV, synaptic vesicle; VDCC, voltage-dependent Ca^{2+}-channel. Dotted arrows, protein interactions; solid arrows, formation of the SNARE complex involved in exocytosis of synaptic vesicles.

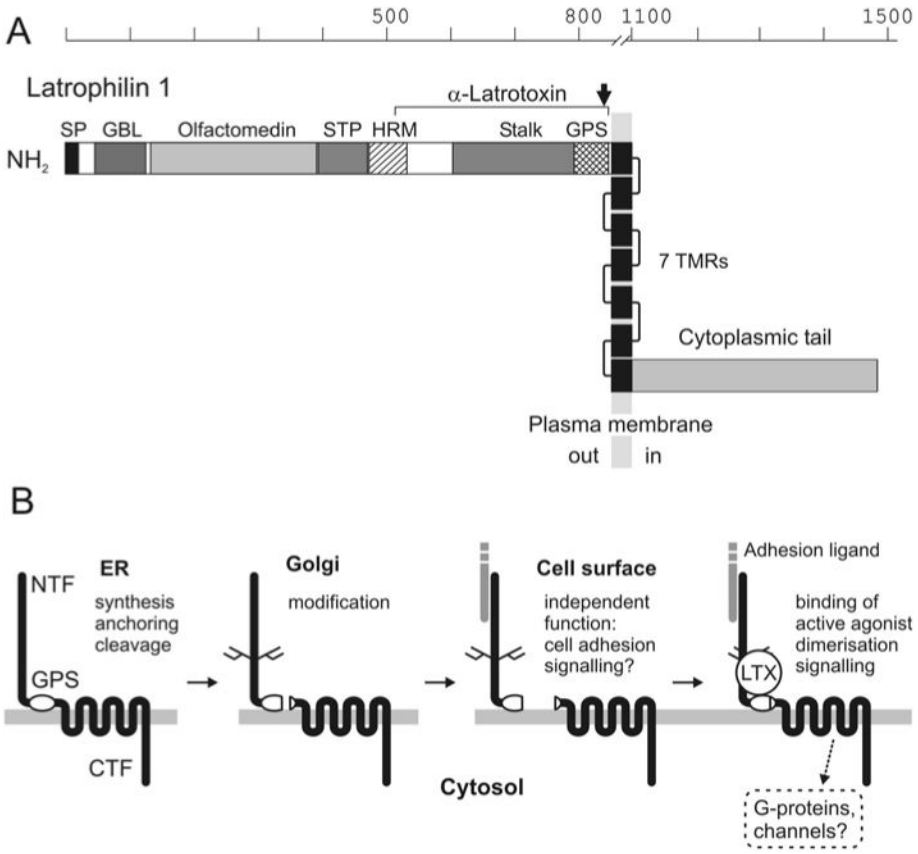

Figure 4.

LPH structure and processing. A, Generalised domain structure of latrophilins. 7TMRs, seven transmembrane regions and corresponding loops; arrow, cleavage point; bracket, the site of α-LTX binding in LPH1; GBL, galactose-binding lectin; GPS, GPCR-proteolysis site domain; HRM, hormone receptor motif; STP, Ser-, Thr- and Pro-rich domain. B. Scheme of LPH processing and functioning (Volynski et al. 2004). See text for details. ER, endoplasmic reticulum.

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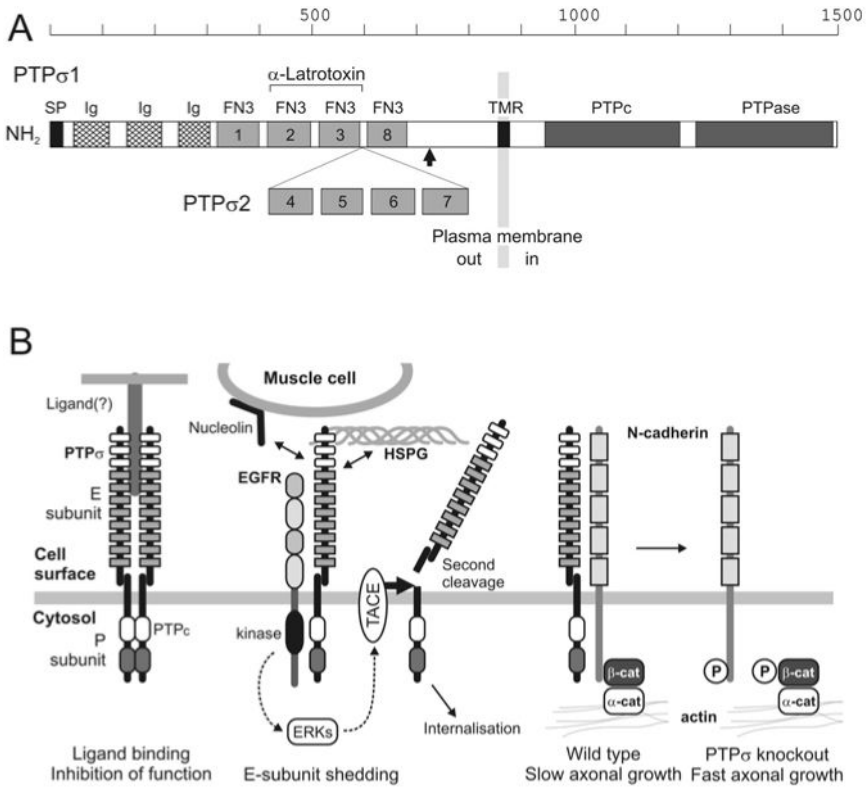

Figure 5.

PTPσ structure and functions. A. Generalised domain structure of receptor-like PTPσ. FN3, fibronectin type III-homology domains; Ig, immunoglobulin-like domains; PTPc, catalytic PTP domain; PTPase, pseudo-phosphatase domain. Arrow, proteolytic cleavage site; bracket, α-LTX binding site. B, Cellular functions and interactions of PTPσ. Ligand binding leads to dimerisation and inhibition of the intracellular domains. PTPσ interaction with EGFR activates the ERK-mediated signalling and upregulates the TACE metalloprotease which cleaves the P subunit and releases E subunit. PTPσ may interact with HSPG of the basement membrane and nucleolin on muscle cells. PTPσ binds N-cadherin and dephosphorylates it, causing increased adhesion and inhibition of axonal growth. In mice lacking PTPσ, N-cadherin is hyper-phosphorylated; this reduces adhesion and increases neuronal growth. EGFR, epidermic growth factor receptor; ERKs, MAP kinases ERK1 and ERK2; HSPG, heparin sulphate proteoglycan; cat, catenin.

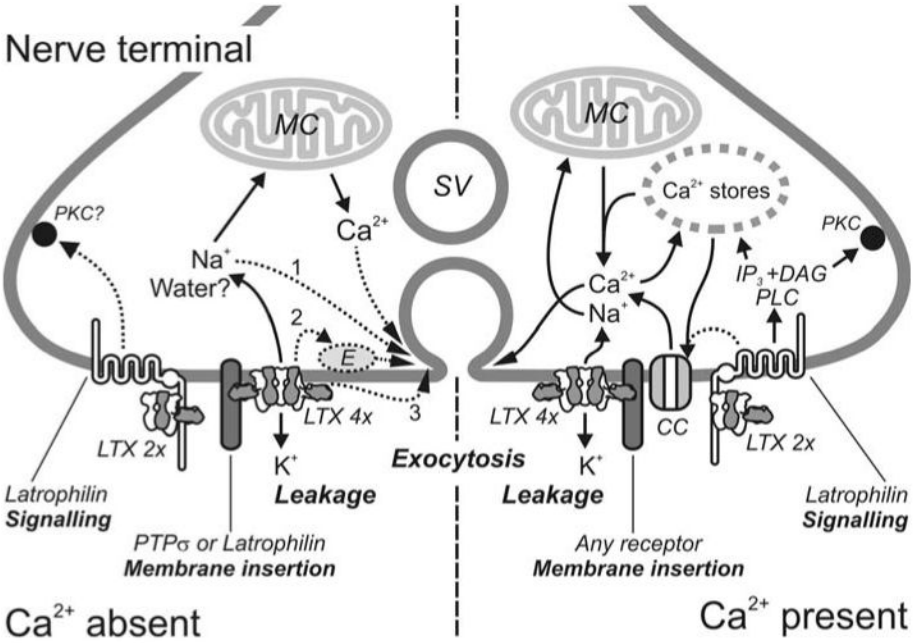

Figure 6.

Diverse mechanisms of α-LTX action. *Right*, Ca^{2+} is present in the medium. The pathways shown are described in the text. CC; Ca^{2+} channels; DAG, diacyl glycerol; LTX 4x, α-LTX tetramers; MC, mitochondria. *Left*, Ca^{2+}-free conditions. For main comments, see text. The possible pathways for Ca^{2+}-independent exocytosis shown include: (1) high concentrations of Na^{+} mimicking Ca^{2+}; (2) the internalised domains of α-LTX interacting with components of the exocytotic machinery (E); (3) α-LTX exerting direct fusogenic action.

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