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Penelope's web: using α-latrotoxin to untangle the mysteries of exocytosis

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Abstract

For more than three decades, the venom of the black widow spider and its principal active components, latrotoxins, have been used to induce release of neurotransmitters and hormones and to study the mechanisms of exocytosis. Given the complex nature of α-latrotoxin actions, this research has been continuously overshadowed by many enigmas, misconceptions and perpetual changes of the underlying hypotheses. Some of the toxin's mechanisms of action are still not completely understood. Despite all these difficulties, the extensive work of several generations of neurobiologists has brought about a great deal of fascinating insights into presynaptic processes and has led to the discovery of several novel proteins and synaptic systems. For example, α-latrotoxin studies have contributed to the widespread acceptance of the vesicular theory of transmitter release. Presynaptic receptors for α-latrotoxin – neurexins, latrophilins and protein tyrosine phosphatase σ – and their endogenous ligands have now become centerpieces of their own areas of research, with a potential of uncovering new mechanisms of synapse formation and regulation that may have medical implications. However, any future success of α-latrotoxin research will require a better understanding of this unusual natural tool and a more precise dissection of its multiple mechanisms.

Keywords

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

Neurons communicate with each other and with target cells at synapses. Given the very small size of most synapses, their direct analysis is difficult. Fortunately for scientists, synapses are a target of multiple natural neurotoxins that can inhibit (or activate) synaptic mechanisms. By using these poisons as molecular tools, scientists have been able to scrutinize many molecules and processes involved in synapse formation, functioning and regulation. However, the conversion of nature's powerful weapons into specific research tools has proved difficult because most neurotoxins have evolved to cause multiple effects, ensuring universal and robust responses in targeted neurons. Therefore, the use of unmodified natural toxins has often led to contradictory results and conflicting conclusions.

This problem has been vividly illustrated by the history of research based on the application of α-latrotoxin (α-LTX) from black widow spider venom (BWSV). α-LTX, a potent stimulant of secretion in neurons and endocrine cells, has been used to stimulate nerves since the 1930's (D'Amour *et al*. 1936). The effect of α-LTX is very strong and apparently straightforward. However, like so many other toxins, α-LTX acts through multiple

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mechanisms, which have been obscuring its molecular targets. The deeper researchers delved into the mode of action of α-LTX, the more complex it appeared, with every new finding leading to a thorough revision of the previous, so painfully built and seemingly so fitting, theory. In this sense, α-LTX studies resemble the myth of Penelope, the wife of Odysseus, who weaved a robe (web) by day, only to undo the work by night. Despite the clear connotations with this proverbial description of something being perpetually done but never finished, α-LTX research has actually brought about a great deal of important new molecules and concepts.

This review will examine how, in a long series of rigorous studies conducted by various laboratories, α-LTX and other black widow spider latrotoxins (LTXs) have not only improved our understanding of the complex mechanisms of neurotransmitter release, but also led to the discovery of a number of novel proteins, which, in turn, have since commanded their own lines of investigation.

Spider venom

The black widow spider (genus *Lactrodectus*) has horrified humans for millennia – mostly by its very painful (sometimes fatal; Bogen and Loomis 1936) bite, but not least by its striking coloration and enigmatic behavior. A more scientific study of the venom, and its exploitation in research, began in the 1930s, when it was first discovered that the active ingredients of the venom were proteins (D'Amour *et al.* 1936), acting upon neurons (Sampayo 1944). However, it was not until its direct effects on presynaptic neurotransmitter release were clearly demonstrated (Longenecker *et al.* 1970), that several generations of neurobiologists – with Penelope's fidelity – dedicated their research to untangling the web of intrigue surrounding this infamous spider and the cellular effects of its potent venom.

Black widow spider venom contains a plethora of unique proteins (Duan *et al.* 2006), of which the large LTXs are the principal toxic components. There are at least seven highly homologous LTXs. The best characterized of these is the vertebrate-specific toxin, α-LTX (Grasso 1976; Frontali *et al.* 1976). The venom also contains five insect-specific toxins, called latroinsectotoxins α, β, γ, δ and ε (Grishin 1998), and one crustacean-specific protein, α-latrocrustatoxin (Krasnoperov *et al.* 1990; Volynskii *et al.* 1999). Although these toxins have been employed in research much less, their application has largely confirmed, in respective animal taxa, the data obtained with α-LTX.

Actions in secretory cells

From the pioneering study of the toxin's action at the frog neuromuscular junction (NMJ) (Longenecker *et al.* 1970), it emerged that the venom causes exhaustive neurotransmitter release by acting specifically at the presynaptic nerve terminal. Later, in numerous experiments, this effect was unequivocally attributed to α-LTX. At low, subnanomolar concentrations, the toxin has no morphological effect on the nerve terminal; however, its action can be detected electrophysiologically as an increase in the frequency of miniature end-plate potentials (mepps) (Longenecker *et al.* 1970). At high, nanomolar concentrations, the toxin causes massive neurotransmitter release (Ceccarelli *et al.* 1979), which is sustained for a considerable amount of time and followed by substantial morphological changes in nerve terminals and even neuronal cell death. There is a concomitant dramatic drop in ATP levels and disintegration of the plasma membrane (McMahon *et al.* 1990), which is most strongly manifested in central synapses (Davletov *et al.* 1998; Ashton *et al.* 2001).

The effects of α-LTX on release of various neurotransmitters and peptide hormones have also been studied in synaptosomes from rat (Grasso *et al.* 1978), dog (Tzeng and Siekevitz 1979) and guinea pig (Nicholls *et al.* 1982) brain, rat and mouse brain slices (Frontali *et al.*

1972), primary cerebellar granule cell cultures (Grasso and Mercanti-Ciotti 1993), organotypic hippocampal cultures (Capogna *et al.* 1996) and neurohypophysis (Hlubek *et al.* 2003). The toxin has also been shown to stimulate exocytosis form non-neuronal excitable cells, such as PC12 cells (Grasso *et al.* 1982), chromaffin cells (Picotti *et al.* 1982), pancreatic β-cells and cell lines (Lang *et al.* 1998) and mast cells (Zhou and Misler 1995). However, α-LTX-induced release of glutamate in cultured astrocytes has also been reported (Parpura *et al.* 1995).

The potency of α-LTX as a secretagogue is quite remarkable: it causes complete depletion of millions of synaptic vesicles in frog NMJs (Ceccarelli *et al.* 1979; Fesce *et al.* 1986) and can even stimulate exocytosis at central synapses when the vesicle fusion machinery has been perturbed by knockout of one or another SNARE protein (Deák *et al.* 2009).

In general, LTXs from the spider venom have similar mechanisms of action, which are, however, limited to specific organisms. The reason for such a specialization of these highly homologous toxins is that, in order to act, they must bind to cell surface receptors, which appear to be distinct in different animal taxa (reviewed in Rohou *et al.* 2007).

Transmitter release and Ca^{2+}

One of the most intriguing aspects of α-LTX action, discovered early on at the frog NMJ (Clark *et al.* 1970; Longenecker *et al.* 1970; Ceccarelli *et al.* 1979), was the toxin's ability to cause transmitter release in both the presence and absence of extracellular calcium ions (Ca^{2+}_e). This distinguished α-LTX action from Ca^{2+}-dependent depolarization-induced exocytosis (Gorio *et al.* 1978a) and made the toxin's effect extremely interesting.

It was established by the initial work (Ceccarelli *et al.* 1979) and many subsequent experiments that the Ca^{2+}_e-dependent and -independent mechanisms of α-LTX action are distinct by many criteria:

1. The characteristics of exocytosis evoked by α-LTX with and without Ca^{2+}_e are very different (Ceccarelli and Hurlbut 1980; Tsang *et al.* 2000). In the absence of Ca^{2+}_e, secretion is tonic: the frequency of spontaneous exocytotic events rises slowly and, on reaching a broad peak, decreases gradually (Fig. 1a), consistent with quasi-stationary tonic exocytosis (Fesce *et al.* 1986). By contrast, in the presence of 1-2 mM Ca^{2+}_e, α-LTX causes clonic release, appearing as fast bursts of acetylcholine exocytosis. These are later overlaid by a continual tonic rise in mepps frequency, which subsequently also declines (Fig. 1b) (Longenecker *et al.* 1970; Ceccarelli *et al.* 1979; Fesce *et al.* 1986; Volynski *et al.* 1999; Lelianova *et al.* 2009).

2. When α-LTX-evoked stimulation is submaximal, the Ca^{2+}-dependent and -independent actions are additive and both occur in the presence of Ca^{2+}_e. Therefore, the frequency of transmitter exocytosis and the total number of quanta released are much higher in the presence of Ca^{2+}_e than in its absence (Fesce *et al.* 1986; Valtorta *et al.* 1988; Auger and Marty 1997; Davletov *et al.* 1998; Ashton *et al.* 2001). This is most vivdly illustrated in mouse NMJs (Fig. 1b-d): when Ca^{2+}_e is added to terminals already responding to wild-type toxin, the frequency of exocytosis increases 2-20 times, depending on the underlying frequency of Ca^{2+}_e-independent release.

It must be pointed out that because both LTX actions have a common target – the vesicles present in a synapse, the size of the vesicular pool can affect this comparison. Thus, the toxin-evoked exocytosis in excitatory central synapses is often similar with or without Ca^{2+} (Capogna *et al.* 1996; Deák *et al.* 2009). Yet, the mechanisms of the Ca^{2+}-dependent and -independent components of α-LTX action are very different (Deák *et al.* 2009).

3. The Ca^{2+}-independent toxin action also depends on divalent cations and can be fully supported by Mg^{2+} (Misler and Hurlbut 1979). As will be discussed below, Mg^{2+} promotes α-LTX tetramerization and pore formation, suggesting that tonic exocytosis is caused by α-LTX forming cation pores in the membrane.

Thus, α-LTX appears to induce two forms of secretion: clonic exocytosis that occurs only in the presence of Ca^{2+}_e and tonic exocytosis that can be recorded both in the presence and absence of Ca^{2+}_e. Given the additivity of tonic and clonic actions (Fig. 1b-d), the toxin is thought to trigger both types of transmitter release in the presence of Ca^{2+}_e (Davletov *et al.* 1998; Ashton *et al.* 2001).

Therefore, one of the major problems encountered when α-LTX is used to study synaptic transmission has been the difficulty of measuring the Ca^{2+}-dependent α-LTX action in isolation. This conundrum was addressed by subtracting the secretion in the absence of Ca^{2+}_e from the total secretion in its presence (Fesce *et al.* 1986; Davletov *et al.* 1998). Although this brought about some important insights into the Ca^{2+}-dependent toxin's actions (Davletov *et al.* 1998; Rahman *et al.* 1999; Ashton *et al.* 2000; Ashton *et al.* 2001), it was clearly inadequate. An alternative approach to selectively inhibiting the Ca^{2+}-independent component, was based on the application of micromolar La^{3+} together with α-LTX (Capogna *et al.* 2003; see also below).

However, because La^{3+} can stimulate transmitter release in its own right (Angaut-Petit *et al.* 1998), by far the best results have been obtained with mutant α-LTX, LTX^{N4C} (Ichtchenko *et al.* 1998) (see below for details). This mutant does not form pores (Ashton *et al.* 2001; Volynski *et al.* 2003; Capogna *et al.* 2003) and consequently only causes high-frequency clonic secretion and no tonic release (Fig. 1e). Similar to the clonic exocytosis caused by wild-type α-LTX, the effect of LTX^{N4C} critically requires Ca^{2+}.

A characteristic feature of α-LTX action in the absence of Ca^{2+}_e is that it causes eventual cessation of all exocytosis. This has been attributed to the Ca^{2+}_e requirement for endocytosis and recycling of synaptic vesicles, meaning that in Ca^{2+}_e-free media vesicles fusing with the plasma membrane become stranded in it (Ceccarelli and Hurlbut 1980; Valtorta *et al.* 1988). As vesicle recycling is not impeded in the presence of Ca^{2+}, multiple rounds of exocytosis can occur and toxin's effect continues for much longer, as shown in the frog NMJ (Ceccarelli and Hurlbut 1980; Fesce *et al.* 1986). However, exocytosis induced by wild-type toxin eventually stops even in the presence of Ca^{2+}_e (Fig. 1a-d) (Longenecker *et al.* 1970; Ceccarelli and Hurlbut 1980; Lelianova *et al.* 2009). The most likely reason for this is that the tonic, high-frequency exocytosis gradually overwhelms vesicle recycling (J. Suckling, K. Volynski, V. Lelianova, R. Ribchester, Y. Ushkaryov, paper in preparation).

It would follow then that if the Ca^{2+}-independent α-LTX mechanism is blocked, the Ca^{2+}-dependent toxin action might continue indefinitely. Indeed, it does when nerve terminals are stimulated by LTX^{N4C} (Fig. 1e). Interestingly, a similar Ca^{2+}-dependent clonic release (without the tonic component) has been described at the frog NMJ stimulated by brown widow spider venom in saline containing Ca^{2+} but not Mg^{2+} (del Castillo and Pumplin 1975). Using focal extracellular recordings, these bursts were attributed to independent activation of single active zones (del Castillo and Pumplin 1975). The addition of Mg^{2+} in these experiments led to the appearance of both clonic and tonic phases. Therefore, true dissection of the Ca^{2+}-dependent and independent mechanisms of α-LTX action can only be accomplished using α-LTX mutants similar to LTX^{N4C}.

Finally, the two α-LTX actions target distinct types of synapses. Experiments in different neuronal preparations have demonstrated that α-LTX stimulates Ca^{2+}-independent exocytosis of glutamate, γ-aminobutyric acid or acetylcholine, but not catecholamines or

peptides (Matteoli *et al.* 1988; Davletov *et al.* 1998; Khvotchev *et al.* 2000). Endocrine cells, containing large dense-cored granules, are also insensitive to α-LTX in the absence of Ca^{2+} (Grasso *et al.* 1982; Picotti *et al.* 1982). On the other hand, all neurotransmitters can be released by the Ca^{2+}_e-dependent action, which is specifically induced by LTX^{N4C} (Ashton *et al.* 2001; Volynski *et al.* 2003; Capogna *et al.* 2003; Lelianova *et al.* 2009). As we shall see later in this review, LTX^{N4C} has proved to be very useful and has helped to change many prevailing concepts about the actions of wild-type α-LTX and physiological synaptic mechanisms. The possible contribution of the toxin pore to the Ca^{2+}-independent α-LTX action will also be discussed below.

The data described above establish the following important facts: (1) α-LTX evokes exocytosis by two mechanisms, Ca^{2+}-dependent and -independent; (2) these two mechanisms are distinct; (3) the tonic Ca^{2+}-independent action develops slowly but ultimately leads to a total block of neurotransmitter release; (4) the Ca^{2+}-dependent bursts of high frequency exocytosis appear abruptly and periodically but occur over a long time; (5) the Ca^{2+}-independent exocytosis requires the presence of Mg^{2+}; (6) synaptic vesicles carrying distinct neurotransmitters have different sensitivities to the two toxin actions.

Contribution to the exocytosis debate

One extremely important outcome of the first phase of toxin studies was its contribution to the debate regarding the nature of neurotransmitter release. At the time, transmitter exocytosis (as opposed to transporter-mediated outflow) was still a controversial idea, and the unique ability of α-LTX to cause sustained release of transmitters in the absence of Ca^{2+}_e played a decisive role in this argument. As mentioned above, synaptic vesicles, fused with the plasma membrane, require Ca^{2+}_e for their endocytosis (Ceccarelli and Hurlbut 1980). If α-LTX is applied in a Ca^{2+}-free medium, fused vesicles become stranded in the membrane and eventually depleted from the terminal. This elegant paradigm, based on the peculiar features of α-LTX, was successfully employed in a series of cornerstone works (Ceccarelli *et al.* 1973; Gorio *et al.* 1978a). Finally, using electron microscopy and electrophysiological recordings, the authors concluded that quantal release at the NMJ occured as long as vesicles were present in the nerve terminal (Ceccarelli and Hurlbut 1980). This lent enormous support to the exocytotic theory of transmitter secretion (Hurlbut *et al.* 1990).

On the other hand, α-LTX never seizes to bring surprises, and the toxin has been also shown to cause non-vesicular release of neurotransmitters (McMahon *et al.* 1990; Deri and Adam-Vizi 1993; Davletov *et al.* 1998; Volynski *et al.* 2000). This Ca^{2+}-independent action has been attributed to the large pore made by the toxin in the plasma membrane (as described below), and the relative amount of this non-vesicular leakage depends on the particular experimental approach used (see Ashton *et al.* 2000).

It is uncontestable, however, that α-LTX causes mostly vesicular secretion. This is clearly demonstrated by the wealth of electrophysiological recordings of individual quantal events (mepps). In addition, as a truly vesicular process, the toxin's action requires SNARE proteins and is blocked by clostridial neurotoxins which cleave different SNAREs (Dreyer *et al.* 1987; Janicki and Habermann 1983; Stahl *et al.* 1994; Capogna *et al.* 1996; Aguado *et al.* 1997; Capogna *et al.* 1997; Davletov *et al.* 1998; Rahman *et al.* 1999; Ashton *et al.* 2001). Only botulinum toxin A (BoNT/A) may not fully inhibit α-LTX-induced secretion because Ca^{2+} influx via the toxin pore rescues SNAP-25 cleaved by BoNT/A (Cull-Candy *et al.* 1976; Dreyer *et al.* 1987; Angaut-Petit *et al.* 1998).

Both Ca^{2+}-dependent and -independent effects of α-LTX were found to need intact synaptobrevin, syntaxin and SNAP-25 (Capogna *et al.* 1996; Capogna *et al.* 1997; Davletov

et al. 1998; Rahman *et al.* 1999; Ashton *et al.* 2001). The requirement of SNARE proteins and unperturbed exocytotic machinery for the Ca^{2+}-independent α-LTX-induced exocytosis at central synapses has been recently confirmed using mice with knocked out synaptobrevin, SNAP-25 or Munc13-1 (Deák *et al.* 2009).

However, in a new twist of Penelope's tale, the Ca^{2+}-dependent α-LTX action still occurred in neurons lacking synaptobrevin 2, SNAP-25 or Munc13-1 (Deák *et al.* 2009). This effect was mimicked by the Ca^{2+} ionophore ionomycin and was fully attributed to the pore-forming activity of α-LTX, suggesting that a novel pathway of vesicular fusion exists that does not require the presence of all SNARE proteins and that can be activated by a large ingress of Ca^{2+} into the terminal (Deák *et al.* 2009). Given the well-documented sensitivity of the Ca^{2+}-dependent α-LTX action in wild-type nerve terminals to acute removal of SNAREs by cleavage with clostridial toxins, the striking results in SNARE knockout mice probably reflect an unusual assembly of the exocytotic machinery. It is tempting to speculate that in the continuous absence of synaptobrevin or SNAP-25, different synaptotagmins might take over the role of the missing SNARE, giving rise to this atypical Ca^{2+}-dependent, but action potential-insensitive, pathway of exocytosis.

Membrane pore formation

The initial findings using α-LTX suggested that it could be a perfect molecular tool to study intrinsic presynaptic processes underlying neurotransmitter exocytosis. However, early into α-LTX research, a fascinating discovery appeared to solve the mystery of α-LTX action (and made it suddenly less attractive to many researchers): α-LTX was shown to spontaneously insert itself into artificial lipid bilayers and form cation-selective channels/pores (Finkelstein *et al.* 1976). Clearly, influx of Ca^{2+} through such pores could induce vesicular exocytosis, making the toxin receptors mere passive acceptors and, therefore, not worth in-depth studies. It was perhaps only due to the Ca^{2+}-independent action of α-LTX, which could not be adequately explained by the effect of the pore and might arguably be mediated by the receptors, that the α-LTX research continued.

Inevitably, much interest also focused on the nature of α-LTX-induced cation channels. In particular, it was unclear how this large hydrophilic protein could penetrate the lipid bilayer and what the role of receptors in pore formation was.

These channels are permeable to cations (especially divalent), but not to anions (Finkelstein *et al.* 1976; Mironov *et al.* 1986). The toxin pores are insensitive to specific blockers of Na^+, K^+ and Ca^{2+} channels (Wanke *et al.* 1986; Scheer 1990). Only trivalent cations (e.g. La^{3+}) and Cd^{2+} block the toxin pore (or its formation) (Scheer 1989; Rosenthal *et al.* 1990; Ashton *et al.* 2001; Van Renterghem *et al.* 2000). When forming the pore, the toxin inserts itself into the membrane, at least partially, as it becomes protected against trypsinization at 37°C but not at 0°C, when the membrane is impervious to protein insertion (Khvotchev *et al.* 2000). The involvement of any proteins/peptides, usually contaminating α-LTX preparations (Volkova *et al.* 1995; Pescatori *et al.* 1995), has been ruled out, as even highly purified α-LTX (Ashton *et al.* 2000) still forms cation permeable pores (albeit inefficiently) in lipid bilayers devoid of any receptors (Volynski *et al.* 2000). The α-LTX pores can be blocked by anti-toxin antibodies applied extracellularly (Cattaneo and Grasso 1986; Chanturiya *et al.* 1996), indicating that the toxin does not cross the lipid bilayer entirely.

Some researchers dismissed the relevance of toxin insertion into lipid membranes because α-LTX could not penetrate the plasma membrane of cells lacking its receptors. Therefore, it was proposed that biological membranes are generally refractory to toxin and that receptors allow α-LTX to reach the lipid bilayer and insert itself into it. Indeed, when α-LTX receptors were cloned (see later), their expression on the surface of non-neuronal cells made

the latter a target for efficient α-LTX pore formation (Volynski *et al.* 2000). Moreover, the features of the toxin channels in such receptor-expressing cells were similar to those in pure lipid bilayers and did not depend on the nature of the receptor (Hlubek *et al.* 2000; Van Renterghem *et al.* 2000).

The major question was (and still remains) what role do toxin pores play in α-LTX-induced exocytosis? Superficially, Ca^{2+}_e influx through the pores induced by the toxin in the plasma membrane can account for Ca^{2+}-dependent neurotransmitter release and/or membrane depolarization (Hurlbut *et al.* 1994). Indeed, the effect of α-LTX in neurons lacking SNARE proteins is fully explained by the toxin pore (Deák *et al.* 2009).

However, the toxin does not always cause depolarization (Lang *et al.* 1998), and the channel formation cannot easily explain the Ca^{2+}-independent release. Many different mechanisms of how the toxin pore could mediate secretion in the absence of Ca^{2+} have been proposed. For example, the pore passes Na^+, which might replace Ca^{2+} in some of its reactions (Adam-Vizi *et al.* 1993). In addition, influx-efflux of other cations and influx of water (Krasilnikov and Sabirov 1992) could tip the cell's homeostasis.

To complicate the story further, the pores were found to also pass large substances, such as fluorescent dyes and neurotransmitters and possibly ATP (McMahon *et al.* 1990; Deri and Adam-Vizi 1993; Davletov *et al.* 1998; Volynski *et al.* 2000). This suggested (Ashton *et al.* 2000) that biochemically measured neurotransmitter discharge triggered by α-LTX could often represent an outflow of cytosolic neurotransmitter through the toxin pore.

Some of the hypothetical mechanisms of Ca^{2+}-dependent exocytosis have been dismissed by direct experiments. For instance, the influx of Na^+ has been found to be inconsequential because, when extracellular Na^+ was replaced with glucosamine or choline-Cl, the Ca^{2+}-independent effect of α-LTX persisted (Gorio *et al.* 1978b; Tsang *et al.* 2000). However, other mechanisms were more difficult to test. From this point of view, it is interesting to recall that Ca^{2+}-independent α-LTX-induced transmitter release is specifically blocked by La^{3+} (Scheer 1989; Ashton *et al.* 2001), while some (but not all) Ca^{2+}-dependent secretion is resistant to trivalent cations (Ashton *et al.* 2001; Capogna *et al.* 2003). This is remarkably consistent with the α-LTX pore (but not the receptor-mediated action) being blocked (or disrupted) by La^{3+} (Scheer 1989; Hurlbut *et al.* 1994; Ashton *et al.* 2001). It is tempting to suggest that the Ca^{2+}-independent toxin's action and some Ca^{2+}-dependent mechanism are mediated by the toxin pore (or at least coincide with it), while a certain part of the specific Ca^{2+}-dependent action is actually pore-independent and must be mediated by toxin's interaction with receptors or associated proteins (Ashton *et al.* 2001).

Perhaps the most plausible hypothesis as to how the toxin pore can mediate its Ca^{2+}-independent action is that an inflow of water through the toxin pores could cause swelling of nerve terminals, exerting some mechanical effect on the presynaptic plasma membrane. In this sense, the action of α-LTX pore in the absence of Ca^{2+} is similar to the effect of hypertonic sucrose: hypertonicity also acts by creating a tension in the presynaptic membrane (Rosenmund and Stevens, 1996), while the lack of Ca^{2+}_e prevents recycling of synaptic vesicles during application of hypertonic sucrose solutions (Ashton and Ushkaryov, 2005).

It can be concluded from the toxin pore studies, which resulted in nearly a hundred publications from many different laboratories, that (1) α-LTX indeed has an ability to form membrane pores; (2) these pores are permeable to cations (especially Ca^{2+}) but are blocked by trivalent cations; (3) the toxin makes such pores in the membrane of any cells as long as they expresses, naturally or artificially, any of its receptors; (4) when inserted into the lipid

membrane, the α-LTX molecule faces both the cytosolic and the extracellular side of the membrane; (5) the role of the pore in the toxin's action remains controversial.

α-LTX structure

Given the toxin's complex action, much hope was initially placed in the cloning and sequencing of α-LTX. However, the structural studies, too, have not been without controversies.

Sequence analysis (Kiyatkin *et al.* 1990) and immunocytochemistry (Cavalieri *et al.* 1990) shows that α-LTX is synthesized on free cytosolic ribosomes in the spider's venom glands as a large pro-toxin, which is proteolytically processed in the lumen of the venom gland to produce the mature toxin with a molecular mass of ~130 kDa (see Ushkaryov 2002). The latter contains two domains: the N-terminal one-third of the molecule has no significant sequence homology to known proteins, while the C-terminal two-thirds contain 22 ankyrin-like repeats (ALRs), the most prominent feature of α-LTX (Kiyatkin *et al.* 1995). ALRs are found in a wide variety of unrelated proteins with diverse functions, and are thought to mediate intra- and/or inter-molecular interactions (Li *et al.* 2006). Consistent with its obligatory processing in the venom gland, the pro-toxin is inactive (Kiyatkin *et al.* 1995). However, it becomes fully active if a stop codon is placed after the ALR-containing domain. This recombinant construct has been subsequently used in many studies.

Unfortunately, the primary structure of α-LTX did not provide any clear clues as to how it could stimulate transmitter release or form membrane pores, and researchers became interested in the spatial organization of the toxin. Several attempts at crystallization have so far been unsuccessful. However, cryo-electron microscopy, a method that allows one to observe individual molecules frozen in their native conformation, has proved extremely useful (Orlova *et al.* 2000). As can be seen from the three-dimensional (3D) reconstructions (Orlova *et al.* 2000), α-LTX in solution is not a monomer, but rather a stable dimer, which can undergo further oligomerization into tetrameric complexes. The four toxin monomers create a central channel that fully pierces the structure. Intriguingly, this tetramerization is facilitated by physiological concentrations of divalent cations (Mg^{2+} and Ca^{2+}) (Ashton *et al.* 2000), and the presence of at least one of these cations is absolutely required for α-LTX action (Misler and Hurlbut 1979; Rosenthal *et al.* 1990). It follows then that when the toxin is active (i.e. in the presence of Mg^{2+} or Ca^{2+}), it exists almost exclusively in its tetrameric form.

The 3D structure of α-LTX has provided perhaps the best evidence so far to support the ability of α-LTX to make membrane pores. The toxin tetramer has been shown to spontaneously insert itself into the lipid bilayer of liposomes (Orlova *et al.* 2000), with the central hole connecting the outside and inside of the vesicle. This insertion is thought to be possible due to the hydrophobic nature of the tetramer's base. The hole in the middle of the tetramer has a sufficiently large internal diameter of (10 – 25 Å) (similar to that estimated by Krasilnikov and Sabirov 1992) to permit the influx of Na^{+} and Ca^{2+}, but also the efflux of neurotransmitters and cytosolic components in a Ca^{2+}-independent non-quantal fashion (as proposed by (McMahon *et al.* 1990; Deri and Adam-Vizi 1993; Davletov *et al.* 1998).

Electron microscopy has also uncovered the remarkable ability of α-LTX tetramers to form two-dimensional crystals (Lunev *et al.* 1991); A. Rohou, M. van Heel and Y. Ushkaryov, paper in preparation). If the toxin arranged into such lattices on the cell surface, this could explain the synchronous opening of multiple channels demonstrated in several systems (Robello *et al.* 1987; Krasilnikov and Sabirov 1992; Filippov *et al.* 1994) and the detrimental effects of large toxin concentrations on neuronal cells mentioned above.

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These studies provided a spectacular insight into the overall structure of α-LTX and the likely mechanism of its pore formation, but the significance of the toxin pore is still disputed. The main reasons for that is the ability of toxin to induce Ca^{2+}_e-independent transmitter release (not easily explained by the pore) and the importance of its presynaptic receptors.

Non-pore forming α-LTX mutant

Even before any receptors were isolated and studied, some experiments suggested that α-LTX was able to trigger intracellular signaling. In particular, it was shown that the toxin led to the activation of phospholipase C (PLC) and production of inositol-1,4,5-trisphosphate (IP3), causing subsequent release of Ca^{2+} from intracellular Ca^{2+} stores (Vicentini and Meldolesi 1984). However, persistent pore formation by the cell-bound toxin has obviously complicated studies of the receptors' functions (Volynski *et al.* 2000). For this reason, a toxin mutant retaining the high affinity for the receptors and lacking the ability to form pores would be an ideal tool to study the effect of receptor activation only.

Serendipitously, a mutant recombinant toxin has been produced (termed LTX^{N4C}) (Ichtchenko *et al.* 1998), containing a thrombin recognition site inserted upstream of the ALR-containing domain. This insert perturbs the molecule folding but in a very peculiar way: LTX^{N4C} retains the high affinity for α-LTX receptors and the capacity to activate hydrolysis of phosphoinositides, but lacks the natural ability of α-LTX to form membrane pores (Ashton *et al.* 2001). This is because LTX^{N4C} can assemble into dimers but not tetramers (Volynski *et al.* 2003) which are required for pore formation (Orlova *et al.* 2000). In full agreement with this theory, the mutant toxin does not support ion fluxes in cell membrane, but it does, in fact, elicit neurotransmitter release (Ashton *et al.* 2001; Volynski *et al.* 2003; Capogna *et al.* 2003). Therefore, LTX^{N4C} has become the first tool that allows one to study neurotransmitter release induced exclusively by the activation of α-LTX receptors.

To summarize the actions and molecular mechanisms of α-LTX, we believe that the Ca^{2+}-independent effect of LTX (1) is based on its tetramerization, membrane insertion and pore formation. The pore may pass water, leading to a mechanical perturbation of the presynaptic plasma membrane, which causes vesicular exocytosis in a manner similar to that of sucrose. The pore also mediates non-vesicular leakage of neurotransmitters and other molecules from the cytosol, leading to rundown of terminals. The Ca^{2+}-dependent effect (2) consists of at least two actions: (a) Ca^{2+} influx through the toxin pore, which is induced by wild-type α-LTX and leads to vesicular exocytosis (in some cases even in the absence of SNARE proteins, and (b) receptor stimulation, which is induced by both wild-type α-LTX and the mutant LTX^{N4C} and requires both Ca^{2+}_e and stored Ca^{2+}. When wild-type α-LTX is used in the presence of Ca^{2+}_e, all these actions appear to take place.

α-LTX receptors

From all the evidence provided by massive research into α-LTX action, it followed that toxin receptors could hold the key to presynaptic regulatory mechanisms hijacked by α-LTX, and so several laboratories began a hot pursuit of the receptors. However, a long period of "web weaving and undoing" lay ahead.

Essentially the same method was applied in several studies aimed at isolating α-LTX receptors: affinity chromatography of solubilized brain extracts on a column with immobilized toxin. In hindsight, the outcome of these studies was very surprising: to date, three structurally unrelated receptors have been isolated by this procedure. In chronological order these are: (1) neurexin (Petrenko *et al.* 1990); (2) latrophilin, or CIRL (Davletov *et al.*

1996; Krasnoperov *et al.* 1996); and (3) receptor-like protein tyrosine phosphatase σ (PTPσ) (Krasnoperov *et al.* 2002b). The significance of this diversity of toxin's targets is still unclear. Perhaps, the evolutionary pressure on α-LTX has ensured its universal ability to attack all secretory cells that could be reached by the toxin but differed in receptor composition. Otherwise, α-LTX could evolve in parallel with evolutionary changes in the repertoire of receptors in the vertebrate nervous and endocrine systems. Whatever the reason for this versatility is, it means that the toxin can cause distinct effects on binding (1) the same type of receptor in different cell types, or (2) different types of receptors in the same cell type. This, obviously, has important implications for any attempts at deciphering the mechanism of toxin's action.

Neurexin

Neurexin Iα was initially purified together with a set of other proteins, considered to be subunits of the receptor complex (Petrenko *et al.* 1990). Through cloning and sequencing of the genes for the largest constituents of this "receptor complex", a novel family of highly polymorphic neuronal cell-surface proteins was discovered and called neurexins (Ushkaryov *et al.* 1992). Neurexin Iα, a neuronal cell-surface protein, binds α-LTX with high affinity (Kd ~ 4 nM) (Petrenko 1993), in a strictly Ca^{2+}-dependent manner (Davletov *et al.* 1995).

Neurexins contain large extracellular domains (composed of epidermal growth factor-like repeats and LNS repeats that are also present in laminin and sex hormone-binding globulin), one transmembrane region (TMR) and a very short cytoplasmic tail. They exhibit high but regimented heterogeneity based on the existence of three homologous genes, each of which can be transcribed from two independent promoters and alternatively spliced (Ushkaryov *et al.* 1992; Ushkaryov and Sudhof 1993). The two promoters direct the synthesis of the longer α-neurexins and the shorter β-neurexins (Ushkaryov *et al.* 1994) (Fig. 2). This generates six principle neurexins: Iα, Iβ, IIα, IIβ, IIIα and IIIβ. In addition, α-neurexins are alternatively spliced at five canonical positions and β-neurexins at two, with the splice sites used independently and containing variable inserts. These combinations can potentially create more than 1000 distinct neurexins in the brain (Ullrich *et al.* 1995).

Neurexins and synapse formation—The polymorphic structure of the neurexins, their specific differential neural distribution (Ullrich *et al.* 1995; Missler *et al.* 1998), and their sequence similarity to extracellular matrix proteins (laminin, slit, and agrin) suggest a function in cell recognition and cell-adhesion at the nerve terminal (Fig. 2). Indeed, neurexins are expressed almost entirely presynaptically in the brain (Berninghausen *et al.* 2007). The role for neurexins as presynaptic cell-adhesion molecules is supported by the discovery of their endogenous ligands, neurexophilin and neuroligins (Nguyen and Sudhof 1997; Missler *et al.* 1998). Neurexophilin is a small (29 kDa) secreted peptide which binds to the extracellular domain of neurexin Iα (Petrenko *et al.* 1996). Neuroligin is a post-synaptic transmembrane protein that (in a splicing-dependent manner) binds β- or α-neurexins and facilitates synapse formation (Scheiffele *et al.* 2000) (Fig. 2).

Neuroligins are involved in the maturation of synapses by validating excitatory versus inhibitory synapses (Chubykin *et al.* 2007). Mice lacking neuroligin or neurexin genes show improper synapse function and are not viable, although synapse formation itself is not affected (Missler *et al.* 2003; Varoqueaux *et al.* 2006).

An intriguing link to neurodevelopmental disorders was made when genetic screens revealed that mutations in the neurexin and neuroligin genes, although infrequent and not prerequisite, are among the numerous genetic causes that may contribute to susceptibility towards autism spectrum disorders and mental retardation (Szatmari *et al.* 2007; Glessner *et al.* 2009; Maestrini *et al.* 2009). Studies on one particular NL3 mutation associated with

autism shows enhanced inhibition and impaired social interactions in mice (Tabuchi *et al.* 2007). Therefore, gaining a better understanding of the molecular mechanism of these proteins may prove useful in treating or even preventing certain disorders of the central nervous system.

Intracellularly, neurexins interact with scaffolding proteins (Biederer and Sudhof 2000), the synaptic vesicle protein synaptotagmin (Petrenko *et al.* 1991) and mediate functional coupling of voltage-gated Ca^{2+} channels to the presynaptic machinery (Missler *et al.* 2003). These interactions constitute a link from neurexins to both synaptic vesicles and the vesicle fusion apparatus. Thus, neurexins participate in the structural organization, maturation and functional maintenance of presynaptic terminals.

Latrophilin

After α-neurexin was shown to strictly require Ca^{2+} for α-LTX binding (Davletov *et al.* 1995), it became obvious that the Ca^{2+}-independent α-LTX actions must be mediated by a different receptor. Investigators started searching for neuronal receptors able to bind α-LTX in the absence of Ca^{2+}. After a number of attempts, a brain-specific protein was isolated that bound α-LTX specifically. Based on its high affinity for α-LTX (0.5 – 0.7 nM) the new receptor was termed latrophilin (Davletov *et al.* 1996). The same protein was independently purified by another laboratory and named CIRL (calcium-independent receptor of α-LTX) (Krasnoperov *et al.* 1996). In the literature, this protein is sometimes referred to as CL, which stands for CIRL/latrophilin (Sugita *et al.* 1998).

Unusual structure/function of latrophilin—Latrophilin comprises a long extracellular domain, seven hydrophobic TMRs and a long cytoplasmic tail (Lelianova *et al.* 1997; Krasnoperov *et al.* 1997) (Fig. 2). The extracellular domain is connected to the first TMR via a short GPS motif (for G protein-coupled receptor proteolysis site; Krasnoperov *et al.* 1997). This motif contains four conserved cysteine residues and a cleavage site that divides the protein into non-covalently bound N- and C-terminal fragments (NTF and CTF, respectively).

This constitutive cleavage, occurring in the endoplasmic reticulum, is required for latrophilin delivery to the cell surface (Krasnoperov *et al.* 2002a; Volynski *et al.* 2004). Here, the two subunits can dissociate and behave as independent cell-surface proteins that are delivered to distinct parts of the cell membrane and are recycled separately. To explain this, NTF has been proposed to anchor in the membrane independently of the CTF (Volynski *et al.* 2004).

Under certain conditions (e.g. the binding of agonist, α-LTX), the latrophilin fragments are able to reassociate. Treatment of latrophilin expressing cells with the non-pore-forming LTX^{N4C}, results in the formation of large ternary α-LTX-NTF-CTF complexes on the plasma membrane which are able to mediate signal transduction.

Latrophilin has two very similar homologues, latrophilin 2 and latrophilin 3 (Sugita *et al.* 1998; Matsushita *et al.* 1999; Ichtchenko *et al.* 1999). Despite their high sequence homology, latrophilins are differentially distributed in mammalian tissues: latrophilin 1 and latrophilin 3 are brain-specific, while latrophilin2 is ubiquitously expressed. Similar to neurexin Iα (Ushkaryov *et al.* 1992; Occhi *et al.* 2002), very small levels of latrophilin 1 mRNA can be detected outside brain, in the kidneys and lung, and both receptors reside in endocrine cells, such as pancreatic β-cells (Lang *et al.* 1998). Although all three latrophilins are present in the brain, at least latrophilin 1 is strictly expressed in neurons but not glial cells (Kreienkamp *et al.* 2000). There have been suggestions that latrophilin 1 may be postsynaptic because its CTF can interact in a yeast two-hybrid system with the PDZ

domain of Shank 1 (Tobaben *et al.* 2000; Kreienkamp *et al.* 2000), a postsynaptic scaffolding protein (Sheng and Kim 2000). However, this result may reflect the normal interaction of the ubiquitous latrophilin 2 rather than the neuronal latrophilin 1 because this binding occurred at the C-terminus (Kreienkamp *et al.* 2000), which has the same sequence in both latrophilin isoforms. In contrast, the dramatic inhibition of α-LTX binding and action in the latrophilin knockout mouse (Tobaben *et al.* 2002), clearly indicate that latrophilin 1 is localized in presynaptic terminals. Moreover, a series of comprehensive studies (H. Matsushita, O. Berninghausen, M. A. Rahman, J.-P. Silva, A. Tonevitsky, Y. Ushkaryov, submitted for publication) has demonstrated unequivocally that latrophilin 1 is not only presynaptic but also enriched at the active zones.

Recent experiments have shown an interaction of CTF of latrophilin with TRIP8b, a cytosolic protein that binds clathrin and subunits of the AP2 complex, suggesting a role for latrophilin and TRIP8b in receptor-mediated endocytosis (Popova *et al.* 2007; Popova *et al.* 2008).

Latrophilin as a GPCR—Latrophilin has become one of the founding members of a new family of GPCRs, known as “adhesion GPCRs” (Fredriksson *et al.* 2003). These receptors have relatively conserved CTFs and entirely unrelated NTFs. The NTFs are unusually large and complex and contain various motifs likely to participate in cell adhesion. CTFs comprise seven TMRs and are expected to function as GPCRs. Thus, this group of receptors represents natural chimeras of adhesion and signaling receptors (Krasnoperov *et al.* 2002a). Latrophilin is the first receptor of this group that has been shown to bind specifically to G proteins, namely, Gαo and Gαq/11 (Rahman *et al.* 1999; Serova *et al.* 2008). This interaction is strong, but also functional because it is disrupted by conditions that allow G-protein activation and dissociation from the receptor (Rahman *et al.* 1999). Coupling to Gαq/11 can activate PLC, with subsequent signaling to intracellular Ca^{2+} stores and Ca^{2+}i-mediated Ca^{2+}_e influx, ultimately resulting in neurotransmitter release.

Many questions still remain in the latrophilin story. The identification of its endogenous ligand(s) would greatly aid in understanding its function. In an effort to find such a candidate, we have recently identified a postsynaptic protein, called Lasso (for latrophilin associated synaptic surface protein), that binds latrophilin with high affinity (J.-P. Silva, V. Lelianova, P. Hitchin, M. A. Rahman, A. Dell and Y. Ushkaryov; paper in preparation). The structure of Lasso and the role of its interaction with latrophilin (Fig. 2) are currently being investigated.

PTPσ

When α-LTX affinity chromatography of brain extract was conducted in the absence of Ca^{2+}, in addition to latrophilin, a small amount of receptor-like protein tyrosine phosphatase σ (PTPσ) was also identified (Krasnoperov *et al.* 2002b). In contrast to neurexin and latrophilin which were discovered as a result of toxin chromatography, PTPσ had been known before this work and in this respect is not, strictly speaking, an outcome of α-LTX research.

PTPσ is a member of the family of receptor-like PTPs that contain cell adhesion molecule-like extracellular domains, a single TMR and one or two cytoplasmic phosphatase domains (reviewed by Tonks 2006). The extracellular domain contains N-terminal immunoglobulin-like modules and from four to eight fibronectin type III-like domains (Fig. 2). The cytosolic portion of the protein contains a catalytically active PTP phosphatase domain and a pseudo-phosphatase. Similar to latrophilin, PTPσ is proteolytically cleaved into two fragments, but the resulting subunits remain non-covalently associated.

PTPσ has two splice variants: the large variant is found in most tissues, while the shorter one, which binds α-LTX, is mostly brain-specific (Yan *et al.* 1993; Pulido *et al.* 1995) but, similar to neurexin Iα and latrophilin 1, is also found in small amounts in some other tissues (Yan *et al.* 1993). There have been no suggestions that PTPσ is involved in regulation of exocytosis, but and its primary function may be the regulation of axonal growth and synapse formation. PTPσ interacts with extracellular matrix, EGF receptors and N-cadherin. By dephosphorylating N-cadherin, PTPσ regulates its interaction with the cytoskeleton and inhibits growth of axons (McLean *et al.* 2002; Siu *et al.* 2006) (Fig. 2). In addition, deposphorylation of proteins localized in focal adhesions may also be important for the regulation of cell-cell contacts.

Which receptor transduces an α-LTX signal?

Thus, in defiance of all expectations, the search for α-LTX receptors has only complicated the α-LTX field. The toxin has been found to interact with three structurally and functionally unrelated receptors, making its mechanism of action very hard to understand, unless one assumes that toxin's effect is limited to pore formation. However, do the three receptors contribute equally to the toxin's effect at the synapse?

α-Neurexin can only bind to α-LTX in the presence of Ca^{2+}_e (Davletov *et al.* 1995), while the toxin can act in Ca^{2+}-free buffers, indicating that one or both of its Ca^{2+}-independent receptors (latrophilin and PTPσ) must be involved in this action of α-LTX. Knockout of α-neurexin in mice has demonstrated that it definitely contributes to α-LTX binding in the presence of Ca^{2+} (Geppert *et al.* 1998). However, the secretory effect of α-LTX – both in the presence and absence of Ca^{2+} – is only moderately affected in the knockout mice. Thus, α-neurexin, is non-critical for both Ca^{2+}-dependent and -independent mechanisms of α-LTX.

There is no doubt, however, that neurexins can serve as functional α-LTX receptors, by providing toxin binding sites. This, for instance, explains the increase in sensitivity of PC12 cells to α-LTX when such cells are transfected neurexin (Sugita *et al.* 1999). Pore formation by the wild-type toxin used in these experiments, explains this effect. In addition, neurexins apparently play a structural (rather than signaling) role in the synapse and are unlikely to mediate the fast exocytotic signal induced by α-LTX.

More compelling evidence that rules out the necessity of neurexins in α-LTX action has perhaps come from the observation based on the use of LTX^{N4C} (Volynski *et al.* 2003). LTX^{N4C}-evoked neurotransmitter release is strictly Ca^{2+}-dependent (Ashton *et al.* 2001; Capogna *et al.* 2003). However, Sr^{2+}, which does not aid toxin binding to α-neurexin, fully supports the stimulatory effect of LTX^{N4C} (Volynski *et al.* 2003).

These considerations make it difficult to assign neurexins a role in the α-LTX action other than that of passive toxin acceptors. Of course, this does not in the least diminish their role in synaptic physiology.

As shown above, latrophilin binds α-LTX with the highest affinity, compared to the other two receptors. The effect of α-LTX at nerve terminals has been associated with an increased breakdown of phosphoinositides and cytosolic concentration of Ca^{2+} (reviewed in Scheer *et al.* 1984). Moreover, the toxin's effect on release of norepinephrine and amino acid neurotransmitters is inhibited by thapsigargin (which depletes intracellular Ca^{2+} stores), 2-aminoethoxydiphenyl borate (which blocks IP3-induced Ca^{2+} release), or U73122 (which inhibits PLC) (Davletov *et al.* 1998; Rahman *et al.* 1999; Ashton *et al.* 2001; Capogna *et al.* 2003). It is not surprising then that latrophilin is not only structurally capable of mediating these effects, but has also been shown to interact with G proteins (see above). In fact, even

in receptor-transfected non-neuronal cells, LTX^{N4C} was able to stimulate release of intracellular Ca^{2+} and this effect was fully dependent on the presence of functional latrophilin (Volynski *et al.* 2004; Silva *et al.* 2009). This indicates that coupling of latrophilin to Ca^{2+} stores is responsible for at least some transmitter exocytosis induced by α-LTX. Finally, knockout of latrophilin in mice (Tobaben *et al.* 2002) leads to a 50-70 % decrease in toxin binding but, at the same time, to a much greater inhibition of toxin's effect.

The structure of PTPσ is consistent with its role in binding ligands on the cell surface. Similar to latrophilin, it is also capable of signaling. PTPσ has been shown to interact with heparin sulphate proteoglycan in the basement membrane (Sajnani-Perez *et al.* 2003) and some cell adhesion molecules. However, the binding of the toxin dimer (or tetramer) to PTP, should lead to its dimerization and inhibition (Tonks 2006), rather than activation, of phosphatase activity. In addition, α-LTX binds PTPσ within the fibronectin-like domains 2 and 3 (Krasnoperov *et al.* 2002b). These two domains are present in both splice variants of PTPσ, but only the shorter variant mediates the secretory action of wild-type α-LTX in pancreatic β-cells (Lajus and Lang 2006), suggesting that it simply facilitates toxin pore formation by bringing α-LTX closer to the plasma membrane.

Taken together, these considerations establish latrophilin as the most likely receptor that mediates an exocytotic signal from the toxin. However, α-LTX causes many different effects, and it is quite possible that some of the long-term signals are mediated specifically by neurexin or PTPσ. It is also likely that some toxin's actions – in particular its pore formation in the cell membrane – bypass any signaling that can be triggered by the receptors.

What are the results of α-LTX research?

So, after about forty years of modern research using α-LTX, what do we know about mechanisms of action of this fascinating neurotoxin? An overview of α-LTX knowledge base, with all its uncertainties and complex logical links, is provided in Fig. 3.

Even though the effect of toxin application in various systems is obvious and well-documented, the molecular machinery underlying its mode of action are still highly debated and controversial. α-LTX is a naturally occurring highly poisonous agent which is used by black widow spiders to inflict excruciating pain and scare away its enemy (a vertebrate animal). It is, therefore, not surprising that the toxin's effects are so powerful and diverse, while its mechanisms (including its targeting of multiple receptors and several modes of actions) are so complex. Scientists have tried to utilize this natural toxin to answer their questions regarding exocytosis, but perhaps often interpreted the results in a rather simplistic manner.

Nevertheless, the α-LTX studies have led to the formulation of extremely important scientific concepts. It now appears obvious that neurotransmitter release occurs via exocytosis, but in the early days of α-LTX research, it was a hotly debated topic. Toxin studies have also led to the discovery of several presynaptic receptors and their ligands (in particular neurexins/neuroligins and latrophilin/Lasso) (Fig. 2). The heterophilic trans-synaptic interactions involving these receptor systems have been shown to control synapse formation, maturation and/or activity.

There is also a much greater understanding of the toxin itself. It is now generally accepted that the wild-type α-LTX can form non-selective cation channels and that this ability stems from the tetramerization of toxin molecules. Based on these insights, researchers have tried to move away from using this rather blunt tool and, instead, have engineered various recombinant toxin mutants which could be used with more precision and less uncertainty.

For example, the toxin mutant LTX^{N4C} has become a new tool enabling researchers to specifically study α-LTX-induced receptor-mediated exocytosis. While extremely beneficial, LTX^{N4C} is still not an ideal tool, leaving many questions unanswered. Indeed, although LTX^{N4C} does not form membrane pores and acts via receptors (Volynski *et al.* 2003), it still requires Ca^{2+} to evoke neurotransmitter release (Capogna *et al.* 2003). So it remains to be investigated whether Ca^{2+}_e enters via latrophilin-activated Ca^{2+} channels (Lajus and Lang 2006), or whether this cation serves as a co-factor for latrophilin. In addition, LTX^{N4C} binds both latrophilin and neurexin (and possibly PTPσ), making it impossible to unequivocally assign its signaling pathways.

For these reasons, a series of remarkable deletion mutants of α-LTX (Li *et al.* 2005) seem to offer a great potential. These authors showed that the removal of just one C-terminal ALR makes the toxin pore enormously conductive, while further C-terminal truncations lead to the total loss of pore forming ability. It would be very interesting to compare the ability of these truncation mutants to tetramerize and induce transmitter exocytosis in neurons.

Finally, α-LTX has also been hypothesized to penetrate partially through the cell membrane and interact with exocytotic proteins on the cytosolic side and thus stimulate receptor-independent effects (Khvotchev *et al.* 2000). This promises to open yet other novel aspects of toxin's action and – more importantly – tell us more about the intricate mechanisms of synaptic function.

Even though much controversy still exists, it is clear that the "web" (Fig. 3) covering α-LTX will be eventually completely removed.

Remaining questions and new avenues

Surprisingly, after all the years of α-LTX use in synaptic research, the mechanism/s of its Ca^{2+}-independent action are still unknown. Novel toxin mutants and unconventional approaches will have to be employed to address this question.

α-LTX receptors are now being studied in-depth. Several questions in this field that require more work: What are the physiological functions of these receptors? What are the consequences of their interactions with their respective endogenous ligands? How do the different toxin receptors interact with each other? Why is the structure of latrophilin so unusual and why can this receptor exchange fragments with other aGPCRs? Finally, what are the signaling mechanisms of each α-LTX receptor?

There is no doubt that studies of α-LTX and its receptors should continue, but at a new, modern level.

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Abbreviations used

3D	three-dimensional
ALR	ankyrin-like repeat
BWSV	black widow spider venom
Ca^{2+}_e	extracellular calcium ions

CIRL	Ca^{2+}-independent receptor of α-latrotoxin
CTF	C-terminal fragment
GPCR	G-protein-coupled receptor
IP3	inositol-1,4,5-trisphosphate
α-LTX	α-latrotoxin
LTXs	latrotoxins
mepps	miniature end-plate potentials
NMJ	neuromuscular junction
NTF	N-terminal fragment
PLC	phospholipase C
PTPσ	receptor-like protein tyrosine phosphatase σ
TMR	transmembrane region

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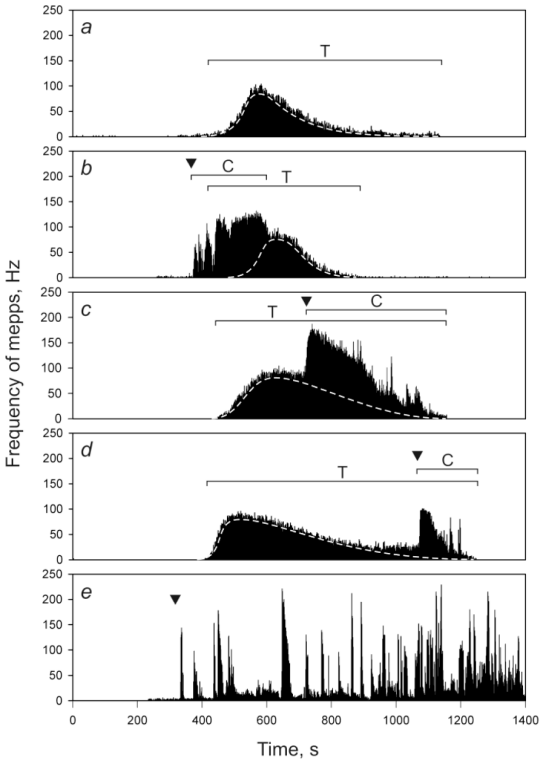

Fig. 1.

Dissection of the two distinct mechanisms of α-LTX action in the presence and absence of 2 mM Ca^{2+}_e. (a) A representative recording at the mouse NMJ of mepps induced by 1 nM wild-type α-LTX in the absence of Ca^{2+}_e. (b-d) Similar recordings using wild-type α-LTX, with Ca^{2+}_e added at different times (arrowheads). Two phases are clearly visible: clonic (C) and tonic (T). Only the tonic phase occurs in the absence of Ca^{2+}; it is characterized by a smooth, slowly rising and falling pattern (dotted lines) and always results in the cessation of spontaneous release events, even after the subsequent addition of Ca^{2+}. Clonic exocytosis only occurs in the presence of Ca^{2+} and is additive with tonic release. (e) In a similar recording, on addition of Ca^{2+} (arrowhead), 1 nM LTX^{N4C} only triggers the clonic phase. This burst-like spontaneous exocytosis continued for 5 hr. (From (Lelianova *et al.* 2009); modified).

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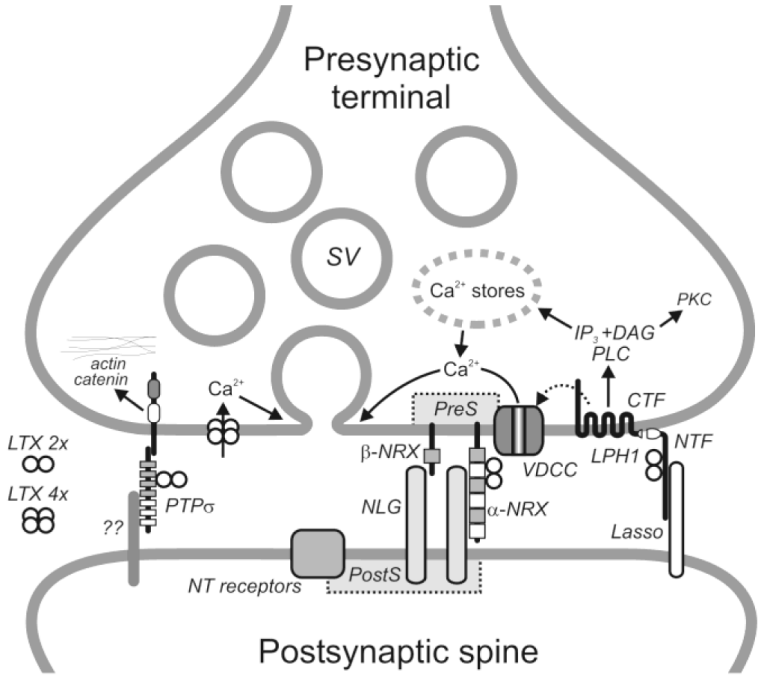

Fig. 2.

Generalized scheme of the α-LTX receptors and some of its mechanisms of action at the synapse. The wild-type toxin is dimeric, but it assembles into tetramers in the presence of millimolar Mg^{2+}. After binding to its receptors, tetrameric α-LTX inserts itself into the plasma membrane and forms non-selective cation channels. The subsequent influx of Ca^{2+} is able to mediate Ca^{2+}-dependent α-LTX evoked neurotransmitter release. Mutant LTX^{N4C} (dimeric only) is able to stimulate receptor-mediated neurotransmitter release which requires both intracellular and extracellular Ca^{2+}. This mode of action may involve voltage-dependent Ca^{2+} channels and is probably mediated by latrophilin 1, which, upon binding the toxin, signals via the PLC cascade. Both neurexin and latrophilin 1 appear to organize VDCCs. Abbreviations used here are: DAG, diacyl glycerol; LPH1, latrophilin 1; LTX 4x, α-LTX tetramers; LTX 2x, α-LTX dimers; NLG, neuroligin; NRX, neurexin; NT, neurotransmitter; PKC, protein kinase C; PreS, presynaptic scaffolding; PostS, postsynaptic scaffolding; SV, synaptic vesicles; VDCC; voltage-dependent Ca^{2+} channels.

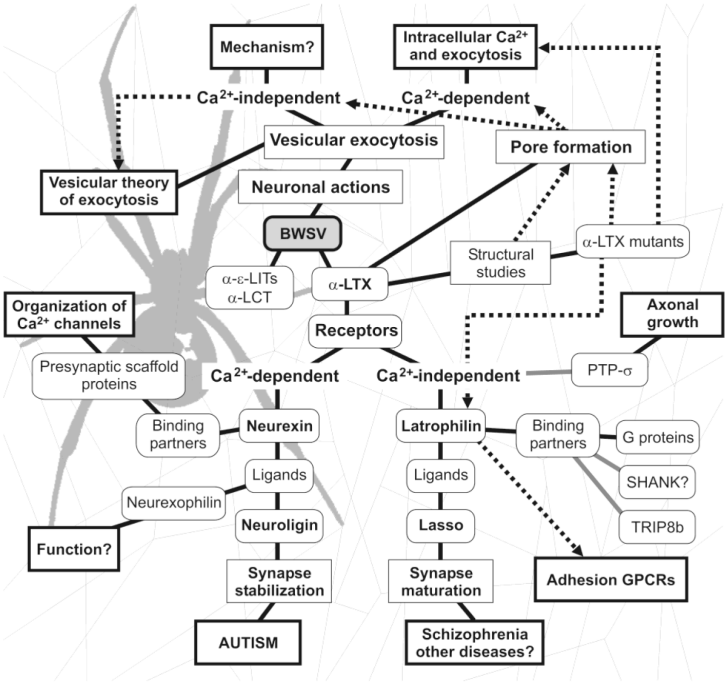

Fig. 3.

A web of intrigue. A summary of processes (rectangles) and molecules (rounded rectangles) revealed using BWSV and α-LTX. Key proteins, concepts and actions are shown in bold. Solid black lines indicate causal links between findings; solid gray lines show interactions of unknown significance; dotted arrows signify contributions to the understanding of respective phenomena. Other toxins from BWSV, α-ε-latroinsectotoxins (α-ε-LITs) and α-LCT, have largely confirmed the findings made using α-LTX (Rohou *et al.* 2007).