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Lynx1 prototoxins: critical accessory proteins of neuronal nicotinic acetylcholine receptors

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Abstract

Nicotinic receptors of the cholinergic system are ligand-gated ion channels, responding to the excitatory neurotransmitter, acetylcholine, and the addictive component of tobacco, nicotine. They help to transduce salient information in the environment by activating specific neural circuitry in normal and disease states. While nicotinic receptors are promising neurological and neuropsychiatric disorder targets, they have fallen out of favor after several late-stage clinical failures. Targeting the complex of the nicotinic receptor, including lynx1 accessory proteins, could be the key to unlocking the intractable nAChR for therapeutic development. Lynx1 binds to the extracellular face of the nAChR and acts as a critical modulator, suppressing memory, learning, and plasticity. Lynx1 removal in animal models leads to memory and plasticity enhancements, some of which have therapeutic relevance for neuropsychiatric and neurological disease. A review of the lynx1 accessory modulator and its role in modulating neuronal nAChRs will be discussed.

The promise and challenge of targeting nicotinic receptors

The cholinergic system is an excitatory neurotransmitter system involved in the modulation of circuits associated with a range of complex functions and there has been considerable interest in developing cholinergic drugs for a range of neurological and neuropsychiatric indications [1•]. Three of the four major therapeutics prescribed to Alzheimer's patients target the cholinergic system [2]. Raised acetylcholine levels in the brain can be achieved by inhibiting the enzyme acetylcholinesterase, which breaks down acetylcholine. Clinical improvements associated with this treatment, however, are limited [3]. This is due partly to loss of efficacy due to tachyphylaxis by agonist-based compensatory mechanisms such as receptor desensitization, downregulation, *etc.* Several therapeutic programs to develop agents specifically targeting nicotinic receptors have been actively pursued, with a number of pharmacological classes (e.g. agonists, partial agonists, allosteric modulators, inverse agonists and blockers), of multiple nicotinic receptor subtypes for multiple indications [1•,4]. After much effort, however, clinical failures have blunted enthusiasm for targeting

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nicotinic receptors, with only a couple of successful approvals. The reasons are manifold: nAChRs are part of a large family of highly related genes (α1–10, β2–4) which exist in a number of combinations of heteropentameric or homopentameric subtypes. This makes the select targeting of individual subtypes difficult and prone to off-target effects. The most abundant subtypes, α4β2 and α7 nAChRs, are distributed widely throughout the CNS, leading to on-target side effects from actions outside the intended brain regions [1•]. Some of the biophysical properties of nAChRs also present a challenge to therapeutic development: desensitization after channel opening is a feature of most nAChR subtypes [1•]. An agonist-based therapeutic targeting orthosteric binding will be working against these factors.

Complexes of nAChRs with lynx1 – an allosteric approach

Inclusion of accessory molecules within the nAChR complex could circumvent some of these issues because it more faithfully recapitulates the nAChR as they exist in the brain. Lynx1 is part of a large gene family, the Ly6/uPAR/neurotoxin superfamily, that codes for proteins exhibiting a highly successful receptor binding fold, the three-finger toxin fold, many of which bind tightly to nAChRs and modulate their function (Refs. [5–7], reviewed in Refs. [8,9•]; and Ref. [10]). Lynx1 has been shown to bind tightly to nAChRs *in vitro* [11] *in vivo* [12•], and to the invertebrate nAChR homolog, AChBP, at nM affinity [13]. As the most well-characterized member of the toxin-like prototoxins, lynx1 is emerging as a significant factor in complex and disease brain states, and will be the focus of this review.

Lynx1 exerts allosteric effects by promoting receptor desensitization and slowing recovery from desensitization of α4β2 nAChRs [11]. Removal of lynx1 leads to nondesensitizing and hypersensitive nAChRs so presumably a lynx1 inhibitor would be less subject to tachyphylaxis.

There is a 10-fold higher EC_{50} between brain nicotinic responses [14] versus heterologously expressed nAChR ones in cells without lynx1, whereas addition of lynx1 in heterologous systems resembles brain nAChR function [11]. Cell-based screening assays including the entire receptor complex are likely to yield better leads, as compared to screening nAChRs alone, with potentially few drop-outs during pre-clinical development.

Functional modulation of circuits by lynx1

The ability of lynx1 to influence circuit function has implications for the cognitive aspects of neurological and neurodevelopmental diseases [15•,16••]. Removal of the lynx1 brake leads to enhanced cognitive effects, such as augmented associative learning ability [14], and reopening of the critical period of plasticity in the visual cortex [17–19], although subcortical effects have also been reported [12•,16••,20]. The cell type-specific expression of lynx1 [21], for instance, PV-positive GABAergic neurons in the visual cortex [17], and 5HT-3 positive neurons in the auditory cortex [22••], influences the relative weighting of the nAChR response to acetylcholine to emphasize some nodes in a circuit among others. The inhibitory influence of lynx1 within circuits may amplify gain modulation, which is associated with cortical nicotinic activation [23], and even modify the plasticity window of experience-based circuit modification, which has implications for brain repair [17,22••,24].

Lynx1 influences on receptor stoichiometry, specificity and number

The influences of lynx1 on nAChRs are long-term and numerous. In addition to functional effects on gating, lynx1 can also influence receptor stoichiometry. At a single channel level, the co-expression of lynx1 on α4β2 results in an increase in the larger amplitude, faster inactivating openings of agonist-induced responses, associated with the low sensitivity, LS, $(\alpha4)_3(\beta2)_2$ stoichiometry [11]. Lynx1 has been shown to interact in the endoplasmic reticulum (ER) in cells only expressing α4 nAChRs, or at α4:α4 dimers, an interface which exists in only the LS, $(\alpha4)_3(\beta2)_2$ stoichiometry [25]. Removal of the GPI-anchor in this latter case did not influence nAChR responses suggesting that some of the effects occur during receptor maturation [25]. Such long-term interactions can influence a number of features of nAChR biology, including maturation, receptor number and stoichiometry. Gating effects of lynx1 can be revealed more clearly if the stoichiometry can be fixed to a single population, which has been accomplished by concatemerization, synthesizing the entire pentamer through fusion of nAChR subunits into a single polypeptide.

Single channel studies using concatemeric α3β4 nAChRs indicate nAChRs are more often in the closed state when cells are co-transfected with lynx1 [26]. There are different effects depending on which cDNA concatemer, representing the different stoichiometry of the receptor, was used. When coexpressed with lynx1, there was a reduction in the number of receptors of the $(\alpha3)_2(\beta4)_3$–nAChR stoichiometry [26]. For the $(\alpha3)_3(\beta4)$-nAChR stoichiometry, on the other hand, the effects were largely functional, reducing unitary conductance, and enhancing closed dwell times. In heterologous expression systems, lynx1 has also been shown to influence α5α3β4 nAChRs [26] and α6β2 nAChRs [20]. *In vivo*, lynx1 has been shown through genetic studies to influence α6* nAChRs in the striatum, α6β2 nAChR in the colliculus [20], and β2* and α7 nAChRs [14].

Three dimensional structure of lynx1

The NMR solution structure of lynx1 has indicated that it exhibits a three-fingered fold common to α-neurotoxins [27]. The disulfide bridges between the cysteine residues stabilize the head of the protein, from which three beta-sheet rich loops emerge. The secondary structure of ws-lynx1 represents two antiparallel β-sheets, one consisting of two strands in loop I, and four strands going across loops I–III. The second part of loop III is relatively disordered. While the 3D-topology of elapid neurotoxins and lynx1 is evident, lynx1 and α-neurotoxins share distinct features as well. Within the Ly6/uPAR/toxin superfamily, structural data of toxin/nAChR complexes support interfacial binding for three-finger fold proteins, exemplified in αneurotoxins (Ref. [28], reviewed in Ref. [8]). Functional studies demonstrate binding of lynx1 in the receptor extracellular domain, globally similar to toxin binding, although mutagenesis studies with a synthetic, secreted, version of lynx1, ws-lynx1, indicate more subtle features which are distinct from those of neurotoxins [29].

The nAChRs twist: clues from molecular dynamics simulations

nAChRs transition through several functional states, open, closed and desensitized, *etc*. [30–32]. Global changes in the pentameric receptor are thought to involve a coordinated twist in

the five subunits leading to an increase in the channel diameter, thus allowing for ion flow through this enlarged pore [30]. Molecular dynamics simulations of the GPI-anchored lynx1 bound to α4:α4 interfaces of α4β2 nAChRs embedded in the membrane (Figure 1) indicate prolonged interaction of lynx1 with the receptor C-loop [33••]. The C-loop is involved in the transition from the closed (agonist unbound) state to the open (agonist bound) state [30] by capping the agonist binding site. In molecular dynamics simulations with lynx1, the C-loop is restricted in either the open or bound state, as compared to the nAChR alone, which transitions freely between open, closed, and desensitized states [33••]. Unimpeded, the C-loop may not influence channel opening greatly, per se [34], but binding to an accessory protein could impede the twist. This supports a 'lock and key' notion for lynx1 function, and the idea that brain nAChR are restricted in these state transitions by lynx1 binding.

Functional studies using soluble lynx1

Our current understanding of lynx1 function is that it is a membrane-bound, GPI-linked protein. Variations in effects have been reported when lynx1 is co-expressed with nAChRs in its GPI-anchored form, as opposed to when applied externally to the nAChR-containing cell in a soluble form [11,27,29,35]. Studies of the secreted variant of lynx1, ws-lynx1, have been illuminating for isolating the gating capability of lynx1 independent of receptor stoichiometry. Removal of the GPI-attachment site allows for straightforward purification of lynx1, ws-lynx1 [27,35], with subtle differences in function as compared to the native GPI-anchored lynx1 [11,27,35,36]. The application of ws-lynx1 can influence peak currents of α4β2 [27,35] and α7 nAChRs in a dose-dependent manner [27,29]. It has been shown to inhibit proliferative activity in non-neuronal cells [37]. Ws-lynx1 has been shown to bind to multiple nAChRs from brain extracts, α3–7, β2,and β4 nAChR, subunits [38] as well as a bacterial homolog of the nAChR, GLIC [13] and mAChRs [27]. *In vivo* demonstration of native lynx1: nAChR complexes have only been shown as yet with β2*-nAChRs [12•], and α7 nAChRs [39••] so the specificity of lynx1 binding needs to be considered in the context of expression in the brain. A model that encompasses the experimental differences between ws-lynx1 versus native, GPI-lynx1, is that the tethering of the nAChR to the membrane slows the rate of transitions of the nAChR (Figure 2).

Therapeutic potential of lynx1

There have been suggestions that lynx1 inhibition would raise cholinergic tone by releasing the molecular brake on nAChRs [40], indicating its potential as a therapeutic target. Titration of lynx1 dosage will be important, as lynx1 has a neuroprotective effect in aged brains and hence complete lynx1 inhibition would not be warranted as a long-term therapeutic solution [14,41]. A link between Alzheimer's disease and lynx1 is emerging. Ws-lynx1 can influence synaptic plasticity in the hippocampus, a memory structure associated with early Alzheimer's pathology [42•] and lynx expression [35]. Lynx1 is subject to transcriptional fluctuations [40] and demonstrates a modest (10%) downregulation in animal models of Alzheimer's disease [38]. Alzheimer's disease is associated with an increase in a toxic peptide, soluble β-amyloid (Aβ) [43], which has been shown to enter into neurons via nAChRs [38]. A demonstration of the potential utility of a lynx-based therapeutic, comes from studies on ws-lynx1 which have shown that ws-lynx1 can compete for binding of Aβ

to nAChRs, and as such to lower Aβ toxicity [38], and abolish the negative effect of Aβ on synaptic plasticity [42•]. This is suggestive of anti-toxic activity of peptides or proteins derived from lynx-like proteins by binding to nAChRs.

Disruptions of lynx1 and nAChRs present a challenge since protein–protein interactions can be difficult to disrupt by a small molecule. The water-soluble variant of lynx1, ws-lynx1 has potential as a therapeutic because it has been shown to partially displace endogenous lynx1 for binding at nAChRs potentially leading to a partial loss-of-function of lynx1 [39••]. Further, it can breach the blood–brain barrier with intranasal delivery [39••]. Even more promising, synthetic circularized peptides derived from lynx1 and lynx1-like family members have been found to bind to α7 and the ectodomains of α9 nAChR subtypes [44••]. If it too can penetrate the blood–brain barrier, its relatively small size increases its potential as a biologic therapeutic. Other α7 nAChR-binding proteins have been shown to induce transfer of macromolecules across the blood–brain barrier and into cells through α7 nAChR mediated transport [45] or direct binding in the case of viruses [46], indicating a potential for the development of carrier transport peptides-based on nicotinic receptor binding protein sequences.

Lynx1 removal has been associated with schizophrenia [15•] and biological signatures in an autism model [16••], indicating an involvement in neurodevelopmental disorders. The emerging understanding of lynx1 in neurological, neuropsychiatric and neurodevelopmental disorders underscores the immense potential of lynx1 accessory proteins to revive nicotinic receptor therapeutic development.

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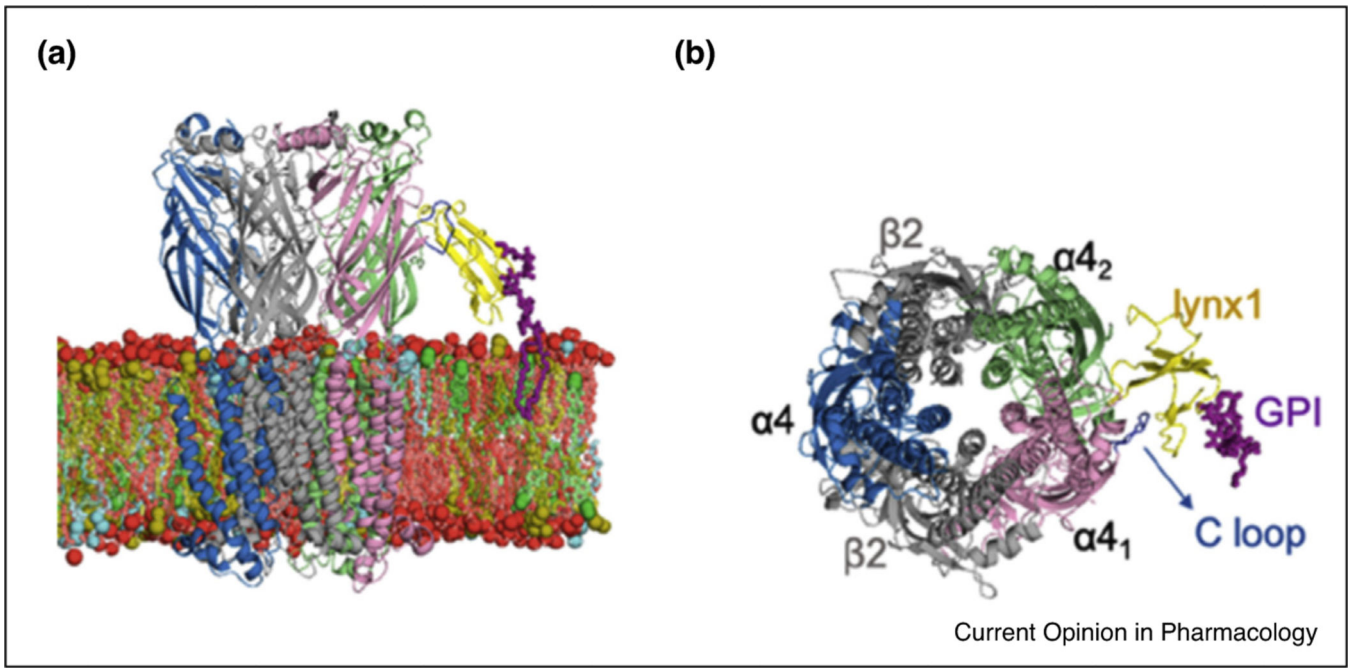

Figure 1.

Model of lynx1 and α4β2 nAChRs embedded in the membrane.

Lynx1 (yellow), GPI anchor of lynx1 (purple), α4 nAChR C loop (blue).

(a) Side view.

(b) En face view.

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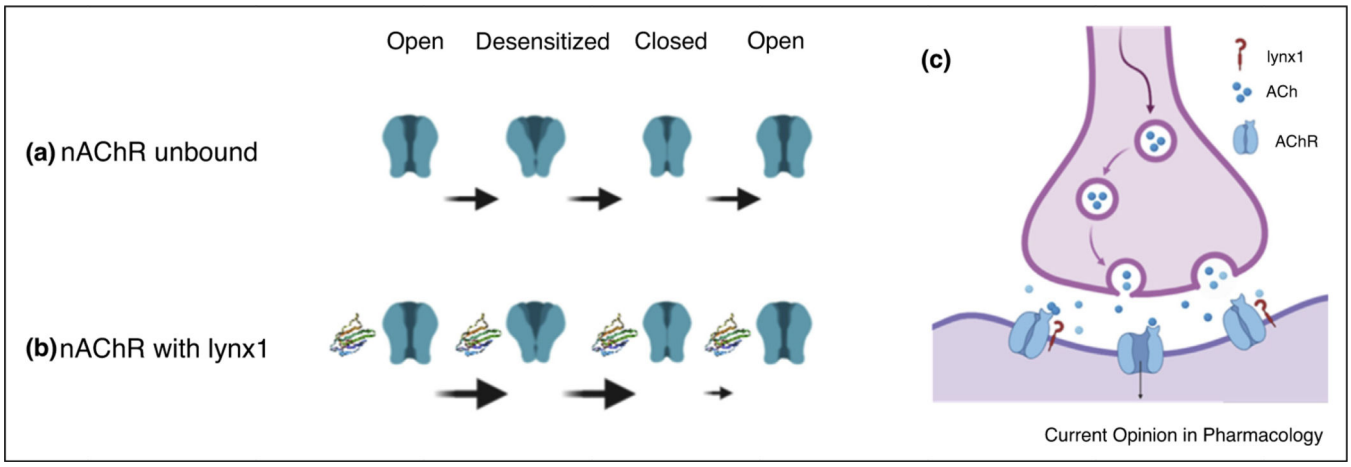

Figure 2.

Schematic of possible lynx1 action on transition states of nAChRs.

(a) nAChR (green) states.

(b) nAChR with lynx1 present (2L03, ribbon).

(c) Reduced cholinergic drive at synapses with lynx1-bound nAChR complexes.