

# Three-Finger Proteins from the Ly6/uPAR Family: Functional Diversity within One Structural Motif

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**Abstract**—The discovery in higher animals of proteins from the Ly6/uPAR family, which have structural homology with snake “three-finger” neurotoxins, has generated great interest in these molecules and their role in the functioning of the organism. These proteins have been found in the nervous, immune, endocrine, and reproductive systems of mammals. There are two types of the Ly6/uPAR proteins: those associated with the cell membrane by GPI-anchor and secreted ones. For some of them (Lynx1, SLURP-1, SLURP-2, Lypd6), as well as for snake  $\alpha$ -neurotoxins, the target of action is nicotinic acetylcholine receptors, which are widely represented in the central and peripheral nervous systems, and in many other tissues, including epithelial cells and the immune system. However, the targets of most proteins from the Ly6/uPAR family and the mechanism of their action remain unknown. This review presents data on the structural and functional properties of the Ly6/uPAR proteins, which reveal a variety of functions within a single structural motif.

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The Ly6/uPAR family got its name from the names of two representatives: lymphocyte antigen-6 (Ly6) and urokinase-type plasminogen activator receptor (uPAR). Proteins from this family are characterized by presence of one or several LU domains consisting of 60-90 amino acids (a.a.) (Fig. 1). The LU domain includes a  $\beta$ -structural core, stabilized by a system of four invariant disulfide bonds, and three extended loops (Fig. 2). Thereby, the Ly6/uPAR proteins are often called three-finger proteins. The functional diversity of the Ly6/uPAR proteins is connected with variability of the loop regions. The loop regions can also contain additional disulfide bonds (Fig. 2). To date, the Ly6/uPAR proteins have been found in insects [1], fish [2], amphibians [3], reptiles [4], birds [5], and mammals [6]. The best-known three-finger proteins are snake neurotoxins, which act on numerous targets

such as nicotinic acetylcholine receptors (nAChRs), muscarinic acetylcholine receptors (mAChRs),  $\alpha/\beta$ -adrenergic receptors, gamma-aminobutyric acid (GABA) receptors, acid-sensitive ion channels (ASIC), etc. [7]. Conservation of the three-finger structural motif points on the great functional significance of the mammalian Ly6/uPAR proteins. In the human genome, there are 35 genes encoding three-finger proteins [8], most of which remain poorly studied. Ly6/uPAR proteins can be associated with the cell membrane by glycosylphosphatidylinositol anchor (GPI anchor), and they can be secreted. In this review, the Ly6/uPAR proteins of insects and chordates are considered, and information about the function of mammalian three-finger proteins is discussed.

## SNAKE TOXINS

### $\alpha$ -Neurotoxins and nicotinic acetylcholine receptors.

Toxins are major protein components of snake venoms.  $\alpha$ -Neurotoxins, the best-studied snake toxins, are highly specific inhibitors of nAChRs, which are ligand-gated ion channels.  $\alpha$ -Neurotoxins are considered as tools for studying nAChRs properties, and as prototypes of drugs for the treatment of diseases of the nervous system [9].

*Abbreviations:*  $\alpha$ -Bgtx,  $\alpha$ -bungarotoxin; ACh, acetylcholine; GABA, gamma-aminobutyric acid; GPI-anchor, glycosylphosphatidylinositol anchor; Ly6, lymphocyte antigen 6; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; tPA, tissue plasminogen activator; uPAR, urokinase plasminogen activator receptor; WTX, nonconventional toxin from *Naja kaouthia*.

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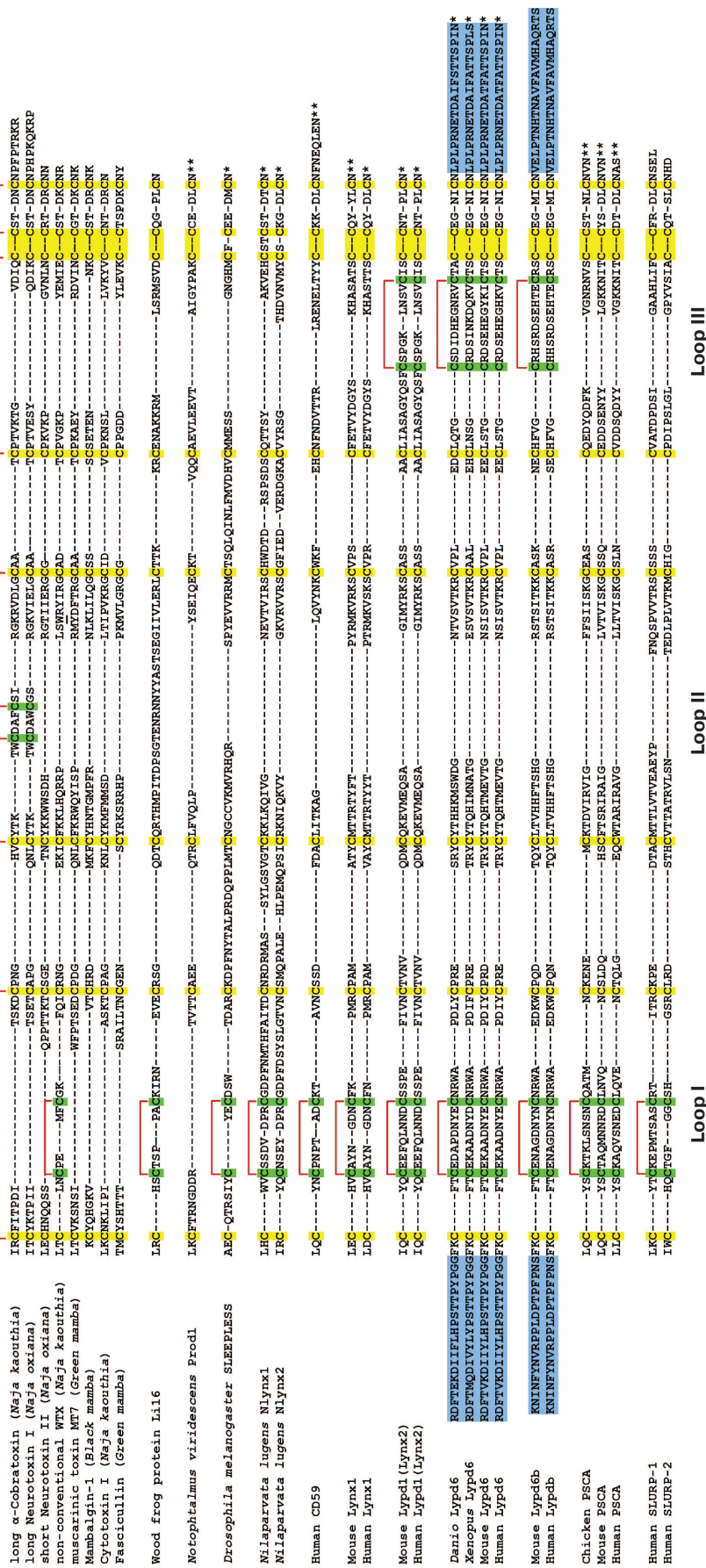


Fig. 1. Comparison of amino acid sequences of three-finger proteins of different origin. Conservative Cys residues are shown in yellow. Cys residues participating in the formation of additional disulfide bonds are shown in green. Additional N- and C-terminal sequences of Ly6d6 and Ly6d6b are shown in blue. A single asterisk indicates protein sequences for which presence of a GPI-anchored form is shown, and two asterisks show proteins for which presence of both GPI-anchored and secreted form is reported.

Snake  $\alpha$ -neurotoxins contain 60-75 a.a. and 4-5 disulfide bonds. There are short-chain  $\alpha$ -neurotoxins (60-62 a.a., 4 disulfide bonds) and long-chain  $\alpha$ -neurotoxins (66-75 a.a., having a fifth disulfide bond in the central loop; Fig. 2). Both types of  $\alpha$ -neurotoxins efficiently interact with muscle type nAChRs, but only the long-chain  $\alpha$ -neurotoxins act on neuronal  $\alpha$ 7-nAChRs [10]. It was revealed that the main structural motif of the  $\alpha$ -neurotoxins interacting with nAChRs is the tip of the central loop. The fifth disulfide bond in the central loop is essential for the interaction with  $\alpha$ 7-nAChRs (Fig. 3) [10, 11].

#### Neurotoxins and muscarinic acetylcholine receptors.

Neurotoxins isolated from snake venom can also target mAChRs related to G-protein coupled receptors (GPCR) [12]. There are muscarinic neurotoxins isolated from mamba venom (MT1-MT7, four disulfide bonds) that interact with different types of mAChRs as agonists, antagonists, and allosteric modulators [12]. Allosteric antagonists MT3 and MT7 isolated from *Dendroaspis angusticeps* demonstrate high specificity for M1 and M4 mAChRs, whereas other muscarinic toxins are less specific [12]. It was shown that the central loop of MT7 is the main structural determinant of high-affinity interaction with M1 mAChRs [13].

**Non-conventional toxins.** There are about 30 non-conventional toxins isolated from Elapidae venoms having an additional fifth disulfide in the loop I. The function and targets of these toxins have been poorly studied [14]. In general, these non-conventional toxins are characterized by lower toxicity ( $LD_{50} = 5-80$  mg/kg) than  $\alpha$ -neurotoxins ( $LD_{50} = 0.04-0.30$  mg/kg) [14]. However, there are also highly toxic non-conventional toxins, for example,  $\gamma$ -bungarotoxin isolated from *B. multicinctus* ( $LD_{50} \sim 0.15$  mg/kg). Phylogenetic analysis revealed that the family of non-conventional toxins is not homogeneous. Thus, some (mainly those from *Naja* spp.) have a close relationship with muscarinic mamba toxins, while toxins isolated from *Bungarus* spp. have a close relationship with long-chain  $\alpha$ -neurotoxins [4]. Non-conventional (weak) toxin WTX isolated from *Naja kaouthia* ( $LD_{50} > 2$  mg/kg) combines the properties of  $\alpha$ -neurotoxins and muscarinic toxins. WTX irreversibly blocks with low affinity ( $IC_{50} \sim 10$   $\mu$ M) muscle type and  $\alpha$ 7-nAChRs, but can also interact with different types of mAChRs as allosteric modulator [15]. The central loop of WTX, which demonstrates high conformational plasticity, is important for interaction with both nAChRs and mAChRs (Fig. 4, [16, 17]).

**Neurotoxins and GABA<sub>A</sub>-receptors.** Recently, the three-finger toxins MmTX1 and MmTX2 increasing the sensitivity of  $\gamma$ -aminobutyric acid receptors (GABA<sub>A</sub> receptors) to agonists were isolated from *Micrurus mipartitus* snake venom [18].  $\alpha$ -Bgtx, previously known as a highly specific nAChR inhibitor, was found to selectively inhibit GABA<sub>A</sub>-evoked currents through the channel of the  $\alpha_2\beta_2\gamma_2$  receptor, although with significantly less efficacy [19]. The ability of three-finger neurotoxins to inhibit

GABA<sub>A</sub> receptors has also been demonstrated in the case of  $\alpha$ -cobratoxin, neurotoxin I, and toxin WTX [20]. It turned out that an important structural determinant of neurotoxin I for interaction with GABA<sub>A</sub> receptors is the central loop, as in the case with nAChRs and mAChRs [20].

#### Neurotoxins and acid-sensitive ion channels (ASIC).

Two three-finger proteins called mambalgins-1 and mambalgins-2 demonstrating analgesic effect comparable with the effect of morphine were isolated from black mamba venom [21]. Mambalgins target the acid-sensitive channels (ASIC) responsible for pain in mammals (ASIC1a, ASIC2a, and ASIC1b) [21]. Recently, the crystal structure of mambalgins-1 was determined. It has been shown that the binding site of the peptide to the ASIC1a channel is located in the central loop (amino acid residues Phe27, Leu32, and Leu34; Fig. 5) [22].

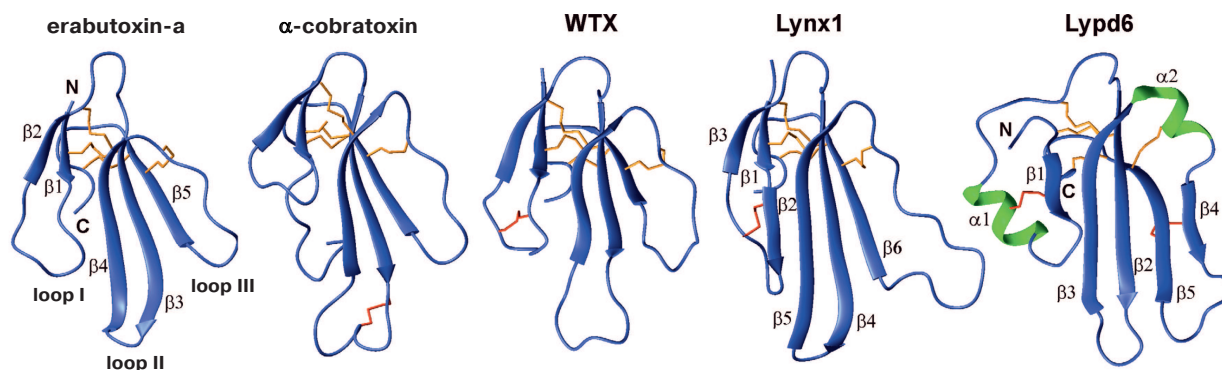
**Cytotoxins.** The target of three-finger cytotoxins is the cell membrane. The tips of the cytotoxin loops are rich in hydrophobic amino acid residues forming a membrane-binding motif [23]. The cytotoxins interact with anionic lipids of the membrane of various cells, for example of myocytes, and they induce the disintegration of the cell membrane [24]. An antitumor activity of cytotoxins associated with their accumulation in lysosomes was shown on lung carcinoma A549 and leukemia HL60 cells. When the concentration of cytotoxins in lysosomes exceeds some threshold value, the lysosomal membrane is disrupted, thus initiating apoptosis or necrosis of cancer cells [25].

**Inhibitors of acetylcholinesterase.** Fasciculins from mamba venom prevent neuromuscular transmission by inhibiting acetylcholinesterase, which hydrolyzes ACh in the synaptic cleft [26]. The fasciculin structure is similar to that of short  $\alpha$ -neurotoxins (Fig. 2). Fasciculins interact with acetylcholinesterase by the first and second toxin loops (Fig. 6). The first loop provides a larger interaction area with the enzyme and the second loop rich in hydrophobic residues blocks the access of the substrate (ACh) to the acetylcholinesterase catalytic site [27].

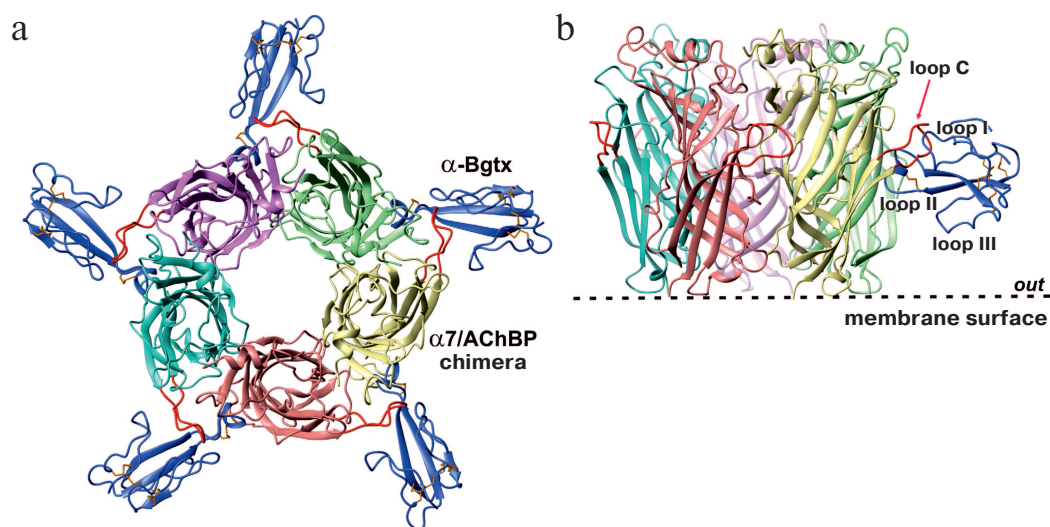
## INSECT NEUROMODULATORS

**Sleepless, a regulator of sleep in *Drosophila*.** The *sleepless* gene has been identified in *Drosophila*. It encodes a three-finger protein responsible for sleep. The SLEEPLESS protein is anchored to cell membrane of neurons by a GPI anchor and is highly expressed in the brain [1]. The loss of SLEEPLESS causes a significant decrease in sleep duration. A moderate decrease in SLEEPLESS expression weakly affects the baseline sleep, but significantly reduces the recovery sleep following sleep deprivation. The known mutation *quiver*, which affects the current through the K<sup>+</sup> channel Shaker, is located at the *sleepless* gene. The expression of Shaker in

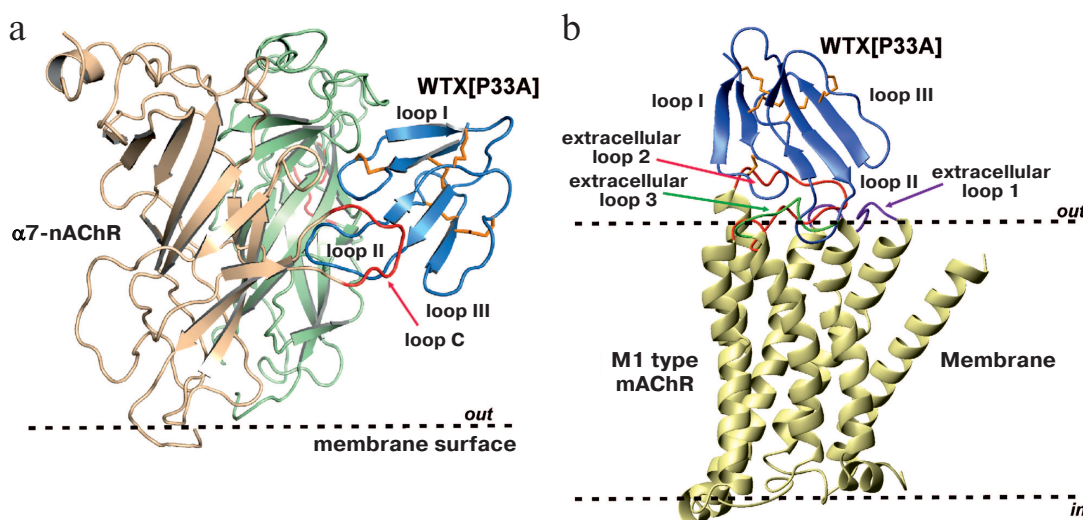




**Fig. 2.** Structure of the LU domains of the three-finger erabutoxin-a from *Laticauda semifasciata* (PDB code 1QKD),  $\alpha$ -cobratoxin from *Naja kaouthia* (PDB code 2CTX), WTX[P33A] from *Naja kaouthia* (PDB code 2MJO), human Lynx1 (PDB code 2LO3) and human Lypd6 (Shenkarev et al., in preparation for publication). Conservative and additional disulfide bonds are shown in orange and red, respectively.



**Fig. 3.** Structure of the  $\alpha$ -bungarotoxin ( $\alpha$ -Bgtx) complex with a chimeric protein homologous to the extracellular domain of  $\alpha 7$ -nAChR (PDB 4HQP) [11]. The top view (a) and the side view (b) are shown. Different subunits of the receptor are shown in different colors. Red color shows the C-loop of the receptor closing the entrance to the orthosteric ligand-binding site.



**Fig. 4.** Models of complexes of non-conventional toxin WTX with the extracellular domain of  $\alpha 7$ -nAChR (a) and M1 type mAChR (b). In the case of nAChR, the central loop of the toxin interacts with the orthosteric binding site of the receptor [16], and in the case of mAChR it interacts with the allosteric binding site [17].



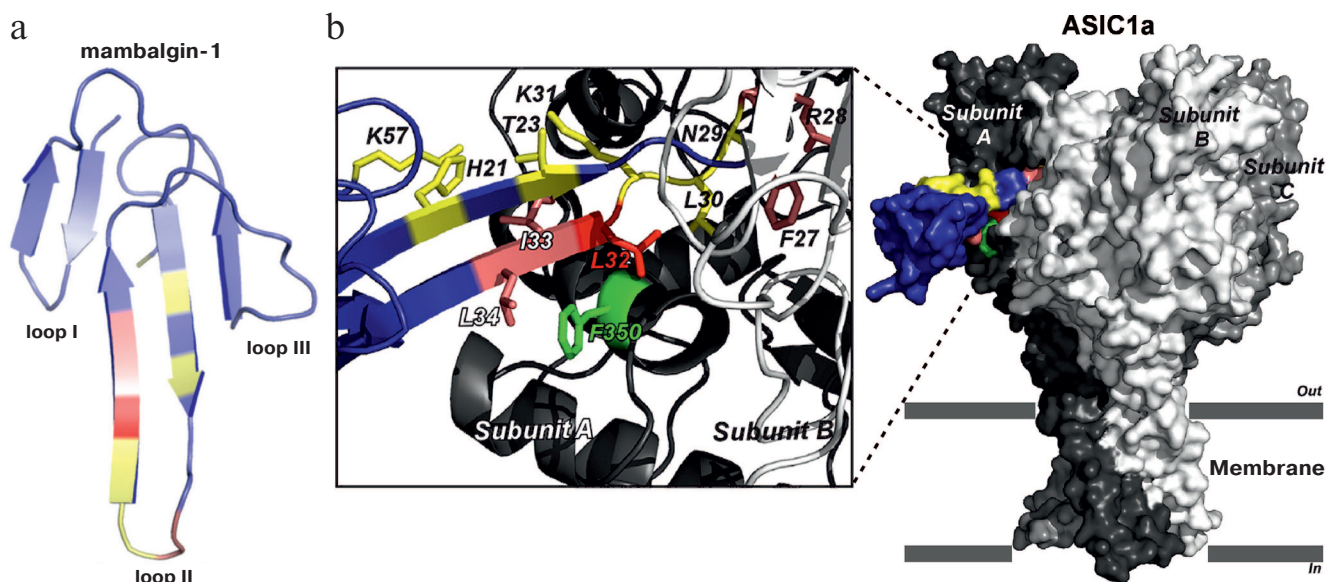


Fig. 5. Model of the complex of mambalgin-1 with the ASIC1a channel. The crystal structure of mambalgin-1 (a) and contacts of the toxin central loop with the channel (b) are shown. The figure was adapted from [22].

*sleepless* mutants is decreased [1], while SLEEPLESS enhances Shaker expression and directly interacts with the ion channel, increasing the open probability [1, 28]. In addition, SLEEPLESS is an antagonist of nAChRs

[28]. Both factors lead to reduced excitability of nerve cells as well as to a decrease in synaptic transmission, which in turn results in a transition from wakefulness to sleep [28]. The key role of the SLEEPLESS central loop in the interaction with both nAChRs and Shaker has been revealed [29].

**Lynx1 and lynx2 from the brown planthopper *Nilaparvata lugens*.** The nAChRs, target for neonicotinoid insecticides, mediate rapid cholinergic synaptic transmission in the insect brains. Two three-finger proteins NI-lynx1 and NI-lynx2 from the brown planthopper *Nilaparvata lugens* modulate the nAChR activity, increasing agonist-induced macroscopic currents through the  $N\alpha 1/\beta 2$  heteromeric receptors, but do not change receptor sensitivity to agonists and desensitization properties [30]. The Y151S mutation of the  $\alpha 1$  nAChR subunit increases insect resistance to neonicotinoid insecticides (imidacloprid). It has been shown that NI-lynx1 and NI-lynx2 proteins significantly increase the amplitude of imidacloprid-evoked current at mutant  $N\alpha 1/\beta 2$  receptors, but do not affect the ACh-evoked current [30]. Thus, NI-lynx1 and NI-lynx2 can act as factors affecting the sensitivity of insects to neonicotinoid insecticides [30].

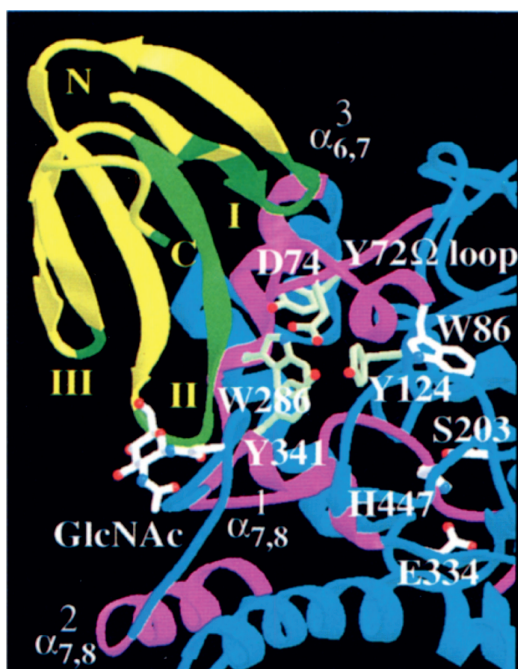


Fig. 6. Structure of the complex of fasciculin-2 with acetylcholinesterase. The second loop of fasciculin-2 interacts with the peripheral anionic site of the enzyme, whereas the loop I binds to the acetylcholinesterase  $\Omega$ -loop. The sites of fasciculin and the enzyme involved in the complex formation are shown in green and purple colors, respectively. The figure was adapted from [27].

### THREE-FINGER PROTEINS FROM FISH AND AMPHIBIANS

**Li16 protein of the tree frog *Rana sylvatica*.** The tree frog *Rana sylvatica* experiences a many-week winter hibernation with freezing of the body up to 65%. Freezing involves adaptation mechanisms that prevent long-term ischemia and protect macromolecules from disruption.

The gene *li16* encoding 89-a.a. protein with five disulfide bonds was found in the tree frog (Fig. 1, [31]). The level of Li16 expression significantly increases during the first day of freezing. Hypoxia and dehydration also lead to increase of Li16 expression. Defrosting and restoration of the normal oxygen level lead to decline in *li16* gene expression to the control level, that possibly points to the important role of Li16 in the development of resistance to ischemia during freezing [31].

**Prod1 protein, a regulator of limb regeneration in salamandra.** Prod1 is a membrane-tethered three-finger protein that regulates regeneration in salamanders, determining the direction of limb growth [3]. Impaired *prod1* expression blocks the formation of the radius and ulna and outgrowth of the anterior digits [32]. Recently, it was shown that Prod1 plays an important role in the regulation of cell adhesion. Membrane-bound Prod1 molecules aggregate in the cell membrane and interact with Prod1 molecules on the membrane of neighboring cells, triggering cell adhesion during limb regeneration [33].

**Lypd6 protein, a regulator of fish and frog embryogenesis.** During the development of zebrafish *Danio rerio*, *lypd6* expression was found at the stages of blastula, gastrula, segmentation, and organogenesis [2]. It was shown that the three-finger protein Lypd6 enhances the Wnt/ $\beta$ -catenin signaling, which regulates embryogenesis and cell differentiation [2]. The overexpression of the Wnt/ $\beta$ -catenin signaling inhibitors Axin1 and Dkk1 suppresses the *lypd6* expression at the gastrula and somitogenesis embryonal stages, and the overexpression of the signal activator Wnt8 in contrast increases the *lypd6* expression at the somitogenesis stage. Blocking of *lypd6* expression using morpholine oligonucleotides leads to morphological changes in embryos of *Danio rerio*, but the co-injection of Lypd6 mRNA restores normal development of the embryos (Fig. 7). Lypd6 tethering in membrane rafts by GPI anchor was shown to be necessary for interaction with the Lrp6/Frizzled8 receptor complex and activation of Lrp6 phosphorylation in the membrane rafts [2]. A Lypd6 protein homolog was also found in *Xenopus* frogs (Xlypd6; Fig. 1). Expression profile of Xlypd6 during embryogenesis resembles the expression profile of Lypd6 in *Danio rerio* [2].

Recently, a cluster of genes encoding three secreted three-finger proteins was found in chromosome 2 of *Danio rerio*. The function of these proteins is currently unknown, but expression in the endoderm indicates their involvement in the development of internal organs [34].

### THREE-FINGER PROTEINS OF MAMMALS

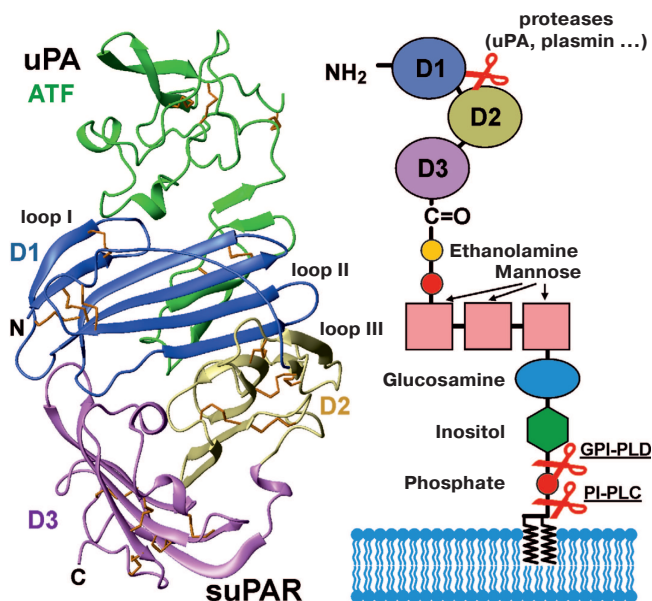
**Glycoprotein CD59 – an inhibitor of membrane attack complex or protectin.** CD59 is a membrane-bound regulatory protein of the complement system. This protein is found in various blood, epithelial, endothelial, and placenta cells. Soluble forms of CD59 have been found in saliva, amniotic fluid, and urine [35]. Mutations of the *cd59* gene or genes of enzymes responsible for the synthesis of glycosylphosphatidylinositol lead to partial or complete absence of CD59 protein. As a result, red blood cells undergo complement-mediated lysis, which can lead to the development of hemolytic anemia [36]. The spatial structure of human CD59, determined by NMR spectroscopy in 1994, was the first proof that three-finger proteins are expressed in mammals [35]. The protein has a three-finger structure characteristic for snake neurotoxins, but unlike  $\beta$ -structural toxins, the elongated CD59 third loop contains an  $\alpha$ -helical region.

**Urokinase-type plasminogen activator receptor (uPAR).** The urokinase uPA receptor is synthesized by normal and tumor cells and presents on monocytes, fibroblasts, platelets, and endothelium. The uPA/uPAR system plays an important role in cell proliferation, differentiation, adhesion, migration, plasminogen activation, and remodeling of extracellular matrix and basal membrane [37]. uPAR is bound to the membrane by GPI anchor, but there is also a soluble form of the receptor – suPAR (Fig. 8). A high level of suPAR is a negative prognosis of tumor disease, and uPAR inhibition may be a strategy for cancer treatment [38].

The uPA receptor consists of three LU domains – D1, D2, and D3 (Fig. 8) [39]. These domains participate in the interaction with the N-terminal domain of uPA (ATF), which activates plasminogen. Domains D2 and D3 can be cleaved out and interact with the lipoxin A4



**Fig. 7.** Blocking of *lypd6* expression using morpholine oligonucleotides (*lypd6*<sup>-</sup>) leads to morphological changes in embryos of *Danio rerio*. Co-injection of mRNA encoding *lypd6* restores the morphology of the embryo. The figure was adapted from [2].



**Fig. 8.** Structure of the complex of the N-terminal fragment of uPA (ATF) and water-soluble uPAR (suPAR, PDB 2FD6) [39]. The three LU domains of the uPAR molecule (D1, D2, D3) are shown in different colors. GPI anchor linked to the C-terminal sequence of uPAR attaches the receptor to the cell membrane. On the right, a schematic structure of uPAR containing the GPI anchor is shown. The sites of possible uPAR molecule cleavage that could lead to the formation of active soluble signal forms of the receptor are shown.

receptor (LXA4R) transmitting chemotaxis signals [37]. In addition, uPAR can bind to integrins that promotes intercellular interactions and adhesion [37].

#### Lynx1 is a factor of neuronal plasticity in mammals.

Lynx1 was the first three-finger protein found in the CNS of mammals [6]. Lynx1 is expressed in the Purkinje cells, cerebellum nuclei, cerebral cortex, and hippocampus [6]. Moreover, co-localization of Lynx1 attached to the neuronal membrane by GPI anchor and nAChRs was detect-

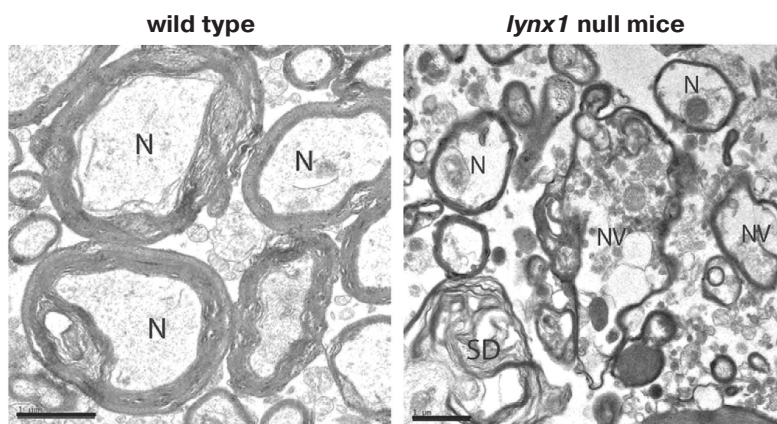
ed in the cerebral cortex, thalamus, substantia nigra, cerebellum, hippocampus, and amygdala [40]. In addition, Lynx1 is expressed by GABA-ergic parvalbumin interneurons of the visual cortex in mouse brain [41].

Co-expression of membrane-anchored Lynx1 with  $\alpha 4\beta 2$ -nAChRs in mammalian cell culture leads to an increase in the time and degree of receptor desensitization, as well as channel conductivity [40]. In the endoplasmic reticulum, Lynx1 affects the assembly of  $\alpha 4\beta 2$ -nAChRs by stabilizing  $\alpha 4/\alpha 4$ , but not  $\beta 2/\beta 2$  dimers. This results in a shift of the receptor stoichiometry to  $(\alpha 4)_3(\beta 2)_2$ -nAChRs. They have reduced sensitivity to ACh compared with  $(\alpha 4)_2(\beta 2)_3$ -nAChRs [42].

The neurons of *lynx1* knockout mice are more sensitive to nicotine. Knockout mice receiving nicotine demonstrated an increased sensitivity in the fear conditioning test and an enhanced motor activity in the rotarod test [43]. Transgenic mice with overexpression of the secreted form of Lynx1 (without the GPI anchor) also showed increased motor learning ability, whereas mice with overexpression of GPI-anchored Lynx1 did not. Thus, the soluble form of Lynx1 can act as a potential tool for modulating of ACh-dependent brain plasticity and learning mechanisms [44].

Knockout mice have displayed the destruction of nerve bundles, the loss of nerve fibers and neuronal nuclei, as well as vacuolization and destruction of myelin sheath during aging in the dorsal striatum (Fig. 9). In contrast, these morphological features were not observed in young transgenic mice and in heterozygous mice of any age. This suggests the possibility of maintaining neuronal health with increasing concentration of Lynx1 [45].

It is known if a juvenile animal has one eye closed for several weeks (monocular deprivation) and then it is opened after the end of a critical period, the vision in this eye is not restored. In the case of adult *lynx1* knockout mice, complete restoration of vision was observed after the eye was opened [46]. Monocular deprivation leads to



**Fig. 9.** Breakdown in nerve fiber (N) bundles in 13-month-old *lynx1* null mice. In *lynx1* null mice an increase in the distance between the fibers, disordered myelin sheaths (denoted as SD), and intracellular vacuolization of the axon (designated as NV) are observed. The bar scale is 1  $\mu$ m. The figure was adapted from [45].



increased activity of tissue plasminogen activator (tPA) in the primary visual cortex. Normally, tPA activity does not change in adult animals [47]. Adult *lynx1* knockout mice demonstrated increased activity of tPA accompanied by a decrease in dendrite spine density and a change in the formation of ocular dominance [47]. Deletion of *lynx1* was shown to increase the rate of appearance and disappearance of dendrite spines in the visual cortex of adult animal brains [48]. Thus, Lynx1 is one of the key factors regulating neuronal plasticity.

**Soluble analog of Lynx1.** The development of the bacterial expression system for the water-soluble domain of Lynx1 without a GPI anchor [49] made possible detailed study of its structural and functional properties. For example, previously unknown targets of Lynx1 were found ( $\alpha 3\beta 2$ -nAChRs, muscular nAChRs and M3 mAChRs). It was shown that Lynx1 at low concentration (1  $\mu$ M) activates and at high concentration (10  $\mu$ M) inhibits  $\alpha 7$ -nAChRs, while only inhibitory effect of Lynx1 was demonstrated at  $\alpha 3\beta 2$  and  $\alpha 4\beta 2$  nAChRs (Fig. 10). It was found that Lynx1 interacts with nAChRs in an allosteric mode [50].

Using NMR spectroscopy, it was shown that Lynx1, like  $\alpha$ -neurotoxins, has a three-finger  $\beta$ -structural spatial organization [50]. However, in contrast to snake neurotoxins, the extended third loop of Lynx1, possibly interacting with the receptor, demonstrates great conformational mobility in the ps-ns time range and does not have an ordered structure in solution (Fig. 11). Similar dynamic properties have been described for the central loop of the non-conventional toxin WTX, which can interact with low affinity with both nAChRs and mAChRs [17]. Perhaps, the high plasticity of the loops is one of the factors that determines simultaneously the ability to interact with different molecular targets and to have a low affinity for them. For comparison, snake  $\alpha$ -neurotoxins with more ordered loops (Fig. 11) inhibit nAChRs with significantly greater affinity [9]. Based on the Lynx1 structure, models of the complexes of the neuromodulator with nAChR have been proposed. According to the models of these complexes, Lynx1 interacts with the outside of the receptor loop C without penetration into the orthosteric binding site and does not prevent the interaction between the receptor and agonists [50, 51].

**Role of Lynx1 in pathophysiology of Alzheimer's disease.** One of the reasons for cognitive impairment in the brain of patients with Alzheimer's disease is nAChRs dysfunction [52]. The co-expression of Lynx1 and nAChRs in the brain regions responsible for memory and learning [53] suggests the involvement of Lynx1 in the development of Alzheimer's disease [54]. It was shown that water-soluble Lynx1 competes with oligomeric  $\beta$ -amyloid peptide (1-42) for binding to nAChR subunits isolated from the human brain homogenate. Moreover, the preincubation of cultured cortical neurons with the water-soluble Lynx1 significantly reduced the cytotoxic effect of  $\beta$ -amyloid peptide

(1-42) [54]. On the other hand, it was found that the expression level of Lynx1 in the cerebral cortex of transgenic mice with  $\beta$ -amyloid and tau pathology is significantly lower compared with healthy mice [54]. Perhaps, the cognitive function impairment in Alzheimer's disease is associated with a decreased Lynx1 expression level.

**Lynx1 modulates cholinergic activity in healthy and tumor cells of bronchial epithelium.** Besides the brain, Lynx1 expression was also detected, although in much lower amounts, in lung tissue [53]. Lynx1 has been shown to act as a negative modulator of nAChRs in healthy and tumor lung tissues [55]. In healthy lung, Lynx1 downregulates the increased expression of nAChRs and GABA receptors observed upon chronic nicotine treatment, and it controls mucin synthesis [55]. A decreased Lynx1 expression is observed in lung cancer. Blocking of *lynx1* gene expression by interfering RNA stimulates cancer cell growth, while *lynx1* overexpression reduces cell proliferation [55]. Thus, Lynx1 can be considered as a prototype for drugs for asthma, chronic obstructive pulmonary disease, and lung cancer treatment [55]. Interestingly, in addition to Lynx1, expression of the neuromodulator Lynx2 was also observed in lung tissues [55].

**Lynx2 neuromodulator.** The expression of *lynx2* was first detected in neurons of the peripheral and central nervous systems in mouse embryos [56]. A high level of *lynx2* expression was observed in the hippocampal CA1 region, dentate gyrus neurons, deep layers of cerebral cortex, and spinal cord neurons [56]. The *lynx2* mRNA was found in the visual cortex [46], hippocampus, and frontal cortex [57]. During the first two weeks of the postnatal period, the *lynx2* mRNA level in the frontal cortex and hippocampus increases, followed by a gradual decline that emerges on a plateau at 60- and 26-day age, respectively [53].

It was shown that Lynx2 reduces ACh-induced current through  $\alpha 4\beta 2$ -nAChRs expressed in *Xenopus* oocytes and enhances their desensitization [57]. On the other hand, Lynx2 reduces  $\alpha 4\beta 2$ -nAChRs expression on the cell membrane by preferentially forming a complex with  $\alpha 4$ , rather than the  $\beta 2$  receptor subunit [58]. Perhaps, Lynx2 affects the  $\alpha 4\beta 2$ -nAChR stoichiometry during the receptor assembly in the endoplasmic reticulum, as shown previously for Lynx1 [42].

Despite having similar pharmacological properties, *lynx1* and *lynx2* expression profiles in the brain differ significantly. This probably indicates the involvement of these neuromodulators in different processes. This hypothesis is supported by experiments with *lynx2* knockout mice demonstrating normal motor and sensorimotor activity compared to the wild type [57] in contrast to *lynx1* knockout mice [43].

**Neuromodulator *lypd6*.** The expression of the *lypd6* gene encoding a membrane-tethered three-finger protein was found in the cerebral cortex and the spinal cord in mice [59]. In rats, *lypd6* expression was found in the brain, lung, kidneys, heart, liver, and prostate [60]. In humans, *lypd6*

expression was demonstrated in different tissues, especially in the brain and heart [61]. Expression of *lypd6*, as well as *lynx1*, was shown in the GABA-ergic interneurons of the mouse visual cortex, although the *lynx1* expression was observed only in the parvalbumin interneurons, while *lypd6* expression was revealed only in the somatostatin interneurons [41]. In the serotonin-expressed interneurons, neither *lynx1*, nor *lypd6* mRNA was detected [41]. Differentiation of the expression patterns of various neuromodulators in the brain regions can be of great importance for directed and specific modulation of individual populations of interneurons in the treatment of mental disorders [41].

It has been shown that *Lypd6* increases the amplitude of the  $Ca^{2+}$  current in response to nicotine in the neurons of the mice trigeminal ganglion [59]. In line with this, transgenic mice with *Lypd6* overexpression demonstrate increased locomotor activity and visceral hyperalgesia, indicating an increase in cholinergic tone [59]. Using inhibitors of  $\alpha 7$ -nAChR, it was found that the target of *Lypd6* is not homopentameric  $\alpha 7$ -nAChRs, but other types of nAChRs [59]. In contrast, the water-soluble recombinant analog of *Lypd6* fused with glutathione-S-transferase inhibits the nicotine-induced current in the CA1 region in the hippocampal slices [60]. The discrepancy in the functional activity of endogenous and recombinant neuromodulators may be due to presence of glutathione-S-transferase in the recombinant protein. A similar effect of additional sequences on neuromodulator activity against nAChRs has also been observed for human SLURP-1 [62].

Nicotine administration to rats in the prenatal and early postnatal period resulted in an increased *Lypd6* expression level in the hippocampus, which was not observed in adult animals [60]. At the same time, significant changes in the *Lynx1* and nAChR  $\beta 2$  subunit expression profiles were not detected [60]. Attempts to obtain mice with blocked *lypd6* expression led either to death or to infertile animals [59]. This, taken together with the high homology of the amino acid sequences of mouse and *Danio rerio* *Lypd6* (~87%; Fig. 1), possibly indicates the special role of *Lypd6* and associated with it cholinergic activation in embryonic development.

**Neuromodulator *lypd6b*.** The *lypd6b* gene expression was found in testes, lungs, stomach, prostate, brain, and other human organs [63]. *Lypd6b* is expressed in glutamate-ergic and GABA-ergic neurons in the visual cortex of adult mice [41]. The primary structure of *Lypd6b* is characterized by a high degree of homology with *Lypd6* (~54%; Fig. 1). Similarly to *Lypd6*, *Lypd6b* has in its structure a C-terminal amino acid sequence to which a GPI anchor can potentially attach, but presence of a GPI anchor has been experimentally confirmed only for *Lypd6* [2]. Unlike other Ly6/uPAR proteins, *Lypd6* and *Lypd6b* besides classical three-finger domain [64] have unusual additional extended N- and C-terminal sequences adjacent to the LU domain (Fig. 1). The role of these extended sequences is still unknown. Co-expression

of *Lypd6b* with  $\alpha 3\beta 4$ -nAChR increases the desensitization rate and the sensitivity of  $(\alpha 3)_3(\beta 4)_2$  nAChRs to ACh in *Xenopus* oocytes. Moreover, *Lypd6b* inhibits  $\alpha 3\alpha 5\beta 4$ -nAChRs, but it does not affect  $\alpha 3\alpha 5\beta 4$  receptors, which are distinguished by the replacement of D398N in the  $\alpha 5$  subunit associated with the development of nicotine addiction [65].

**PSCA, a prostate stem cell antigen.** PSCA exists in membrane-associated and soluble forms [66, 67]. PSCA is expressed in various organs and tissues, such as bladder, kidney, esophagus, stomach, skin, prostate basal cells, and placenta tissues [68]. PSCA is a marker for some tumors – prostate, stomach, and bladder [69]. PSCA demonstrates an oncogenic activity in prostate cancer and glioma, but PSCA suppresses cell growth in gastric and gallbladder cancers [68].

PSCA expression was found in the chicken ciliary ganglion neurons on the 14th day of embryonic development. In the late stages of development of chicken embryos, low *pzca* expression was also observed in the pectoral muscles, liver, ovaries, testicles, heart, and cerebellum. Significantly higher levels of *pzca* were found in the telencephalon and peripheral nervous system [5]. Neuronal tissue of adult mice, as in the case of chicken embryos, contains much more *pzca* than nonneuronal tissues. Moreover, correlation of *pzca* expression with expression of  $\alpha 7$ -nAChRs was found [5].

PSCA blocks activation of  $\alpha 7$ -nAChRs in the ciliary ganglion neurons and rescues neurons from cell death, and overexpression of *pzca* in chicken embryos leads to a decrease in the death of the choroid neurons, but not ciliary ganglion neurons [5]. PSCA inhibits nicotine-induced phosphorylation of MAP kinase ERK1/2 in PC12 cells, possibly thus regulating synaptic plasticity [67]. The levels of PSCA were increased by approximately 70% in the frontal cortex of patients with Alzheimer's disease compared with healthy people. This probably indicates involvement of PSCA in the pathogenesis of Alzheimer's disease [67].

**Secreted proteins SLURP-1 and SLURP-2.** SLURP-1 and SLURP-2 are found in epithelial and immune cells of mammals and are considered as auto/paracrine regulators of epithelial homeostasis [70]. SLURP proteins control the growth, migration, and differentiation of epithelial cells, as well as the development of inflammation and tumors [70]. SLURP-1 has antiproliferative activity and promotes apoptosis of human keratinocytes [71], while SLURP-2 accelerates cell growth, decreasing their differentiation and depressing response to proapoptotic signals [70]. It has been shown that SLURP proteins regulate skin and mucous wound healing [72] and participate in protecting skin cells from oncogenic transformation caused by nitrosamines [70, 71]. Point mutations in the *slurp-1* gene cause an autosomal inflammatory disease of the skin and nails called Mal de Meleda [73]. Inhibition of the *slurp-2* gene results in

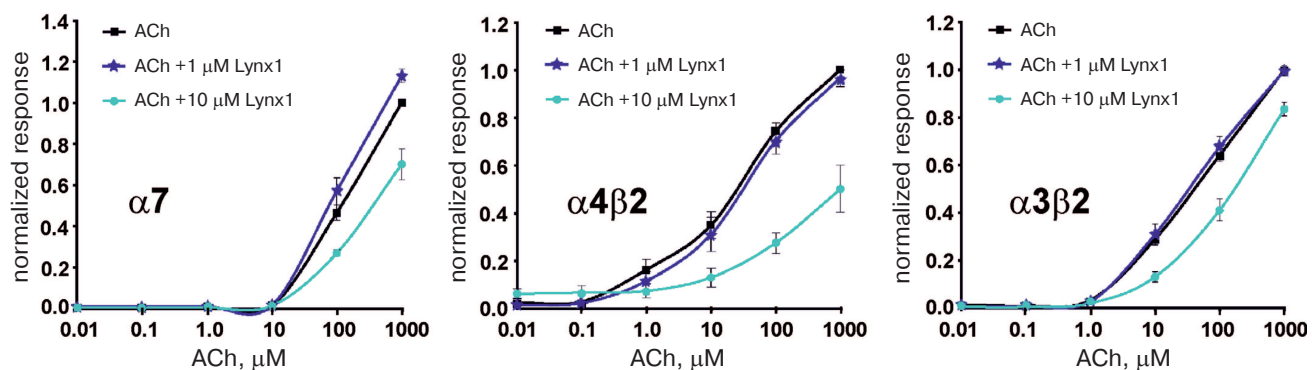


Fig. 10. Effect of the water-soluble LU-domain of Lynx1 on ACh-induced currents on  $\alpha 3\beta 2$ ,  $\alpha 4\beta 2$ , and  $\alpha 7$ -nAChR expressed in *Xenopus* oocytes. The figure was adapted from [50].

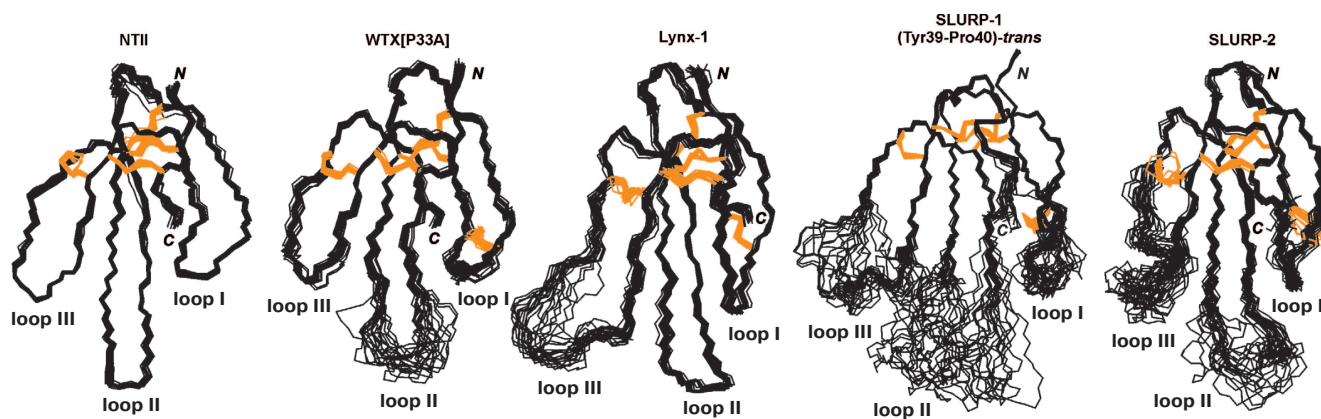


Fig. 11. Superposition of spatial structures determined by NMR spectroscopy for neurotoxin II from *Naja oxiana* (NTII), WTX[P33A], Lynx1, SLURP-1, and SLURP-2 (codes in the PDB database 1NOR, 2MJO, 2LO3, 2MUO, and 2N99, respectively). Disulfide bonds are shown in orange color.

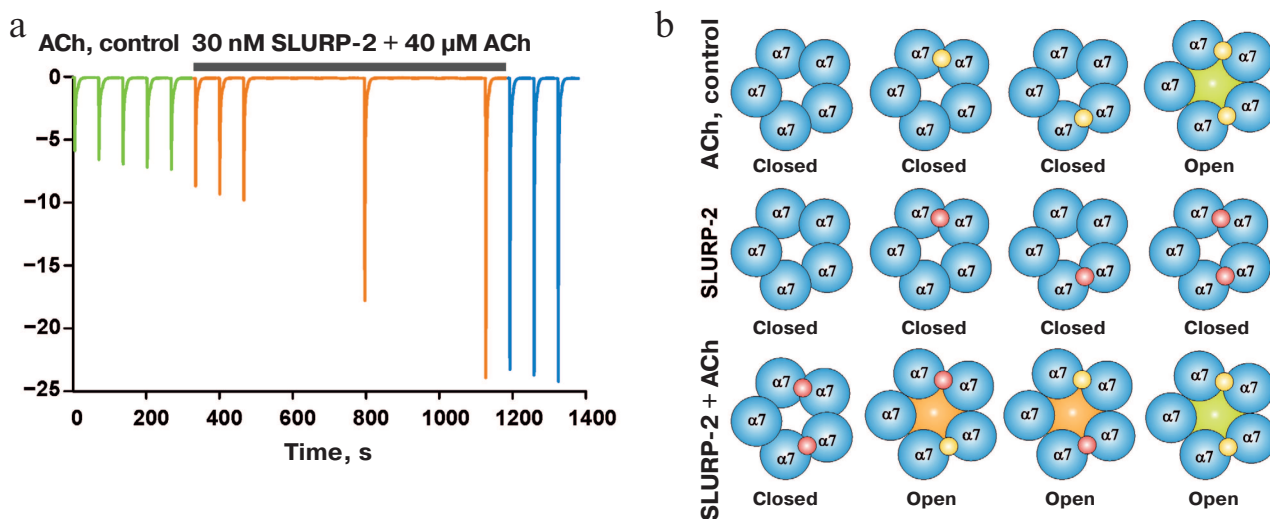


Fig. 12. “Priming” effect of SLURP-2 on  $\alpha 7$ -nAChR expressed in *X. laevis* oocytes. a) Electrophysiological records of ACh-induced current in absence and presence of 30 nM SLURP-2. Currents were elicited by 5 s pulses of 40  $\mu$ M ACh. A representative trace from nine independent experiments is shown. Green traces represent the responses evoked by ACh in absence of the compound, orange traces are the responses evoked by the same ACh test pulse in presence of SLURP-2, and blue traces are the responses evoked by ACh after terminating the SLURP-2 application. b) A diagram explaining the “priming” effect of SLURP-2 on  $\alpha 7$ -nAChR. The ACh and SLURP-2 molecules are indicated by yellow and red balls, respectively. The figure was adapted from [82].



development of palmar-plantar keratoderma [74], and the *slurp-2* expression level is increased in psoriasis patients [75]. SLURP-1 expression was detected in human HT-29 colorectal adenocarcinoma cells, and the level of SLURP-1 expression in these cells is significantly reduced by nicotine treatment [76]. Incubation of HT-29 cells with recombinant proteins SLURP-1 and SLURP-2 significantly inhibits cell growth [77]. SLURP proteins may also be involved in the work of the immune system [78] and are expressed in sensory neurons [79].

SLURP-1 selectively interacts with  $\alpha 7$ -nAChRs and inhibits the ACh-induced current on  $\alpha 7$ -nAChR expressed in *Xenopus* oocytes, with  $IC_{50} \sim 1 \mu M$ . Besides, SLURP-1 does not compete with  $^{125}I$ -labeled  $\alpha$ -Bgtx for interaction with  $\alpha 7$ -nAChR, indicating that SLURP-1 binds not to the orthosteric binding site of the receptor [62]. The antiproliferative activity of SLURP-1 is probably associated with its interaction with  $\alpha 7$ -nAChR. It is assumed that the interaction of SLURP-1 with  $\alpha 7$ -nAChR triggers intracellular processes associated with both  $Ca^{2+}$  current through the receptor channel and activation of kinases by a metabotropic pathway [80]. This is confirmed by the lack of influence of  $\alpha$ -Bgtx on the antiproliferative activity of SLURP-1 [62].

The structure of SLURP-1 is unusual for three-finger proteins: there is a well-structured core, but none of the three loops of the molecule forms an ordered structure (Fig. 11). This, together with the *cis-trans* isomerization of the Tyr39–Pro40 peptide bond in the central loop, causes the unusual conformational plasticity of the loops in solution. Such flexible structure indicates the possibility that SLURP-1 binds to different targets associated with signal transmission by ionotropic and metabotropic pathways.

Until recently, it was believed that the auto/paracrine activity of SLURP-2 was mediated by its selective interaction with  $\alpha 3\beta 2$ - and  $\alpha 9$ -nAChR [81]. However, in 2016 it was shown that SLURP-2 could extract from the cerebral cortex the  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$  nAChR subunits, indicating its wider pharmacological profile [82]. It was shown that SLURP-2 inhibits ACh-induced currents through  $\alpha 4\beta 2$ - and  $\alpha 3\beta 2$ -nAChRs expressed in *Xenopus* oocytes [82]. In the case of  $\alpha 7$ -nAChRs, SLURP-2, like Lynx1, at concentrations of  $\leq 1 \mu M$  significantly increases the ACh-evoked current, but inhibits the receptor at higher concentrations [82]. Under certain conditions, SLURP-2 could increase the current amplitude through  $\alpha 7$ -nAChR more than 5-fold (Fig. 12). The observed effect of receptor activation at low ligand concentrations recalls the “priming effect” described for other nAChR ligands, such as tubocurarine [83]. In addition, it was reported that SLURP-2 could allosterically modulate human M1 and M3 mAChRs [82]. The interaction of SLURP-2 with  $\alpha 3\beta 2$ -nAChR and M3-mAChR increases human keratinocyte proliferation and interaction with  $\alpha 7$ -nAChR leads to inhibition of cell growth [82]. It is known that keratinocytes express various types of acetylcholine

receptors at different stages of maturation, so the effects of SLURP-2 could depend on the stage of cell development.

As in the case of WTX, Lynx1, and SLURP-1, the SLURP-2 loops exhibit significant conformational plasticity due to motions in the ps-ns time range (Fig. 11) [82]. Interestingly, Lynx1 and SLURP-2 are the products of one gene, but the proteins have a low degree of homology due to an alternative splicing ( $\sim 30\%$ ). Nevertheless, analysis of the pharmacological and structural properties of the Lynx1 and SLURP-2 molecules indicates great functional similarity of these proteins.

More than 40 years ago, three-finger proteins, having structural homology with snake  $\alpha$ -neurotoxins and often similar molecular targets, for example, nicotinic acetylcholine receptors, were found in mammals. This raised many questions regarding evolutionary relationships in the Ly6/uPAR family. Today, many of these questions remain open. The detection of three-finger proteins in insects, fish, and amphibians indicates that snake neurotoxins have later evolutionary origin and probably have been “developed” by nature based on endogenous regulators of the vital receptors [4]. Apparently, in the course of evolution, neurotoxins accumulated their unique properties, namely, high selectivity, affinity, and the ability to block target receptors irreversibly.

All known to date endogenous members of the Ly6/uPAR family play crucial roles in the functioning of the organism. Violation of their expression leads to the development of various diseases or is lethal. Recent advances in genome sequencing and proteome analysis give a hope for the discovery of new representatives of the three-finger protein family in various organisms. Therefore, the creation of effective recombinant systems for the Ly6/uPAR protein production making it possible to obtain milligram quantities of the proteins for structural and functional studies is especially important [84].

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