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# Scorpion toxins interact with nicotinic acetylcholine receptors

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**Neurotoxins are among the main components of scorpion and snake venoms. Scorpion neurotoxins affect voltage-gated ion channels, while most snake neurotoxins target ligand-gated ion channels, mainly nicotinic acetylcholine receptors (nAChRs). We report that scorpion venoms inhibit α-bungarotoxin binding to both muscle-type nAChR from *Torpedo californica* and neuronal human α7 nAChR. Toxins inhibiting nAChRs were identified as OSK-1 (α-KTx family) from *Orthochirus scrobiculosus* and HelaTx1 (κ-KTx family) from *Heterometrus laoticus*, both being blockers of voltage-gated potassium channels. With an $IC_{50}$ of 1.6 μM, OSK1 inhibits acetylcholine-induced current through mouse muscle-type nAChR heterologously expressed in *Xenopus* oocytes. Other well-characterized scorpion toxins from these families also bind to *Torpedo* nAChR with micromolar affinities. Our results indicate that scorpion neurotoxins present target promiscuity.**

**Keywords:** neurotoxin; nicotinic acetylcholine receptor; potassium channels; scorpion; snake; venom

Animal venoms, such as those of scorpions and snakes, are complex mixtures containing peptide and protein components and a single venom may contain several hundred individual toxins. Scorpion and snake venoms comprise toxins affecting different systems in the prey organism, including cardiovascular, nerve, muscle, and some other systems. Peptide toxins from scorpion venoms are well known to target voltage-gated sodium (Navs) and potassium channels (Kvs), while some of them possess antimicrobial activity [1,2]. Data about other biological targets for scorpion toxins are in the process of accumulation; thus, substances possessing anticoagulant [3] and antiviral activity [4] were found in scorpion venoms or produced from venom gland cDNA libraries. Scorpion toxins interacting with Navs are the most potent in killing prey. Many of them bind to Navs quite efficiently manifesting nanomolar-range affinity [5]. Some toxins targeting Kvs possess even higher affinities for their targets; for instance, agitoxins inhibit Kv1.1, Kv1.3, and Kv1.6 channels with picomolar affinity [6]. There are several families and subfamilies of toxins interacting with

**Abbreviations**
[$^{125}$I]α-Bgt, mono-iodinated (3-[$^{125}$I]iodotyrosyl$^{54}$)-α-bungarotoxin; Kv, voltage-gated potassium channel; MBP, maltose-binding protein; nAChR, nicotinic acetylcholine receptor; Nav, voltage-gated sodium channel; Trx, thioredoxin; α-Bgt, α-bungarotoxin.

Navs or Kvs. The channel specificities and potencies are varied between families and subfamilies. If we consider, for example, scorpion toxins acting on Kvs, they are grouped into seven families: α-, β-, γ-, δ-, ε-, κ-, and λ-KTx based on homology, 3D folding pattern, and activity, as systemically represented in the Kalium database [7–9]. Each family consists of several subfamilies, which include individual toxins grouped by homology.

On the contrary, snakes of Elapidae family are famous to contain α-neurotoxins efficiently inhibiting some types of nicotinic acetylcholine receptors (nAChRs) [10]. So-called long-chain α-neurotoxins such as α-bungarotoxin (α-Bgt) from the Taiwanese banded krait *Bungarus multicinctus* [11] or α-cobratoxin from the monocled cobra *Naja kaouthia* [12] block with high affinity both neuronal α7, α8, α9α10 and muscle-type nAChRs. Recently we and other laboratories have shown that some α-neurotoxins can block ionotropic receptors of γ-aminobutyric acid [13,14].

There have been no data about scorpion toxins of known structure interacting with nAChRs in the literature; and in our search for new ligands of nAChRs, we have studied scorpion venoms and found that some toxins isolated from these venoms show the capacity to block nAChRs. We report here the inhibitory activity of representatives of two families of scorpion toxins known as Kv blockers.

## Materials and methods

Mono-iodinated (3-[$^{125}$I]iodotyrosyl$^{54}$)-α-bungarotoxin ([$^{125}$I]α-Bgt; 500 Ci·mmol$^{-1}$) was prepared as described in [15]. nAChR-enriched membranes from the electric organs of *Torpedo californica* ray were kindly provided by Professor Ferdinand Hucho (Free University of Berlin, Germany), and $GH_4C_1$ cells transfected with the human α7 nAChR gene were a gift from Eli-Lilly (Indianapolis, IN, USA).

### Scorpions and scorpion venom

Specimens of the black Central Asian scorpion *Orthochirus scrobiculosus* (family Buthidae) were collected in the deserts of Kazakhstan and Uzbekistan, while specimens of the Vietnam forest scorpion *Heterometrus laoticus* (family Scorpionidae) were collected in the An Giang province of Vietnam and bred at the Institute of Applied Materials Science VAST, Ho Chi Minh City; they were fed with crickets and locusts. Scorpions were milked by electric stimulation, and the obtained venom of *O. scrobiculosus* was lyophilized and kept at −80 °C, while that of *H. laoticus* was dried over anhydrous $CaCl_2$ and kept at −20 °C until use.

### Scorpion toxin isolation and purification

Our general approach to venom separation has been published previously [16,17]. Crude lyophilized venom of *O. scrobiculosus* was dissolved in 10% $CH_3CN$ with addition of 0.1% trifluoroacetic acid (TFA) to the final concentration of 10 mg·mL$^{-1}$. It was then subjected to centrifugation (5 min, 15 400 ***g***) and the supernatant was separated by size-exclusion chromatography on a TSK 2000SW column (7.5 × 600 mm, 12.5 nm pore size, 10 μm particle size; Tosoh Corporation, Tokyo, Japan) in 10% $CH_3CN$ and 0.1% TFA at 25 °C and a flow rate of 0.5 mL·min$^{-1}$; detection was performed at 210 nm. The most active fraction in radioligand analysis was then separated into subfractions using reversed-phase HPLC on a Jupiter $C_5$ column (4.6 × 250 mm, 30 nm pore size, 5 μm particle size; Phenomenex, Torrance, CA, USA) in a 60-min linear gradient of $CH_3CN$ (0–60%) in 0.1% TFA at 25 °C. Detection was performed at 210 and 280 nm. The purity of the isolated active component was confirmed using reversed-phase HPLC on a Vydac 218TP54 $C_{18}$ column (4.6 × 250 mm, 30 nm pore size, 5 μm particle size; Separations Group, Hesperia, CA, USA) in otherwise the same separation conditions as above.

The toxin from *H. laoticus* venom was purified using an approach described earlier [18]. In brief, crude *H. laoticus* venom was first fractionated by gel-filtration on Sephadex G-50 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) column (Fig. 2 in [18]), followed by reversed-phase HPLC of fraction 4, which was lethal to mice (Fig. 5 in [18]). Final purification was achieved by one additional step of reversed-phase HPLC using a shallower gradient of acetonitrile (Fig. 5, left inset in [18]). Fraction 4.6.3 inhibited [$^{125}$I]α-Bgt binding to *T. californica* membranes and was used for further study.

### Recombinant toxin production

Charybdotoxin from the deathstalker *Leiurus quinquestriatus* (Chtx; Kalium name α-KTx1.1; UniProt ID P13487) was produced recombinantly in *Escherichia coli* BL21(DE3) according to [19]. Briefly, Chtx-encoding DNA sequence was assembled from synthetic oligonucleotides by PCR and cloned into pET-32b vector (Novagen, Merck KGaA, Darmstadt, Germany) at Kpn1 and BamH1 restriction sites. Controlled gene expression was induced by IPTG, and Chtx was produced as a fusion protein with thioredoxin (Trx), which was purified by metal-chelate affinity chromatography on a cobalt resin (HisPur Cobalt Resin; Thermo Fisher Scientific, Waltham, MA, USA). The carrier protein Trx was cleaved off by enteropeptidase light chain [20] (the DDDDK proteolysis motif immediately precedes Chtx in the fusion amino acid sequence), and the resulting recombinant toxin was purified by reversed-phase HPLC. Similar to some other toxins [21], Chtx contains an N-terminal pyroglutamate residue, which was spontaneously formed from glutamine in solution as followed by MS.

Recombinant hongotoxin-1 from the bark scorpion *Centruroides limbatus* (Hgtx; Kalium name α-KTx 2.5; UniProt ID P59847), kaliotoxin-1 from the fat-tailed scorpion *Androctonus mauritanicus* (Ktx; Kalium name α-KTx 3.1; UniProt ID P24662), and agitoxin-2 from *L. quinquestriatus* (AgTx-2; Kalium name α-KTx 3.2; UniProt ID P46111) were produced in *E. coli* Rosetta-gami(DE3)pLysS (Novagen, Merck KGaA, Darmstadt, Germany) according to [22]. Briefly, in this case the peptide-encoding sequences were cloned at Kpn1 and HindIII restriction sites into the vector pET-23d (Novagen) also containing the *MalE* gene and a linker sequence from pET-32b as described [22]. Expression was induced with IPTG, the peptides were produced as fusion proteins with maltose-binding protein (MBP), and in this case a TEV protease cleavage site immediately preceded the target sequences. The fusion proteins were purified on Ni-NTA agarose (Qiagen, Venlo, The Netherlands). TEV protease was produced as described [23] and used to cleave off the carrier protein MBP. Recombinant toxins were purified by reversed-phase HPLC.

## MALDI mass spectrometry

For analysis, peptides were dissolved at a concentration of 2 mg·mL$^{-1}$ in 20 mM $NH_4HCO_3$ (pH 8). Mass spectra were obtained for intact full-length peptides before and after disulfide bond reduction with 5 mM dithiothreitol, as well as for their fragments after limited proteolysis with trypsin. Trypsinolysis was carried out for 5–20 min at room temperature with a peptide to trypsin ratio of 200 : 1 (w/w). For a number of tryptic fragments, MS/MS analysis was perfomed.

MALDI–TOF MS analysis was performed on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, GmbH, Bremen, Germany) equipped with an Nd laser. Aliquots of 0.2 μL of the samples were mixed on a steel target with 0.5 μL of 2,5-dihydroxybenzoic acid (Sigma-Aldrich, St. Louis, MO, USA) solution (30 mg·mL$^{-1}$ in 30% acetonitrile, 0.5% TFA). Monoisotopic $MH^+$ molecular ions were measured in the reflectron mode; the accuracy of mass peak measurement was within 50 p.p.m. Fragmentation spectra were obtained in the LIFT mode, the accuracy of daughter ion measurement was within 0.5 Da. Peptide identification was carried out on the combined MS+MS/MS data using the Mascot search service (www.matrixsciene.com) in the SwissProt database.

## Peptide microsequencing

Reduction, pyridylethylation, and N-terminal amino acid sequence determination of the purified peptides were performed as described [24].

## Peptide synthesis

HelaTx1 (Kalium name κ-KTx 5.1; UniProt ID P0DJ41) from *H. laoticus* and spinoxin (Kalium name α-KTx 6.13; UniProt ID P84094) from the Asian giant forest scorpion *Heterometrus spinifer* were prepared by peptide synthesis. Solid-phase peptide synthesis was carried out using an automatic peptide synthesizer Myltisyntech Syro II as in [25]. Briefly, peptides were prepared utilizing solid-phase synthesis with Fmoc-methodology using diisopylcabodiimide activation. The C-terminal amino acid was attached to a polystyrene resin with chlorotrityl chloride handle and peptide assembly was performed. After the synthesis, the protected peptidyl-polymer was subjected to a total deprotection/cleavage using a mixture of TFA, ethanedithiol, m-cresole, and dimethylsulfide (9 : 0.3 : 0.3 : 0.3, v/v/v/v) for 40 min at 25 °C and the cleaved peptide was purified by reversed-phase HPLC. A pure linear peptide was dissolved in 50 mM $NH_4HCO_3$ in water/acetonitrile (90 : 10, v/v) to a final concentration of 0.5 mg·mL$^{-1}$. The resulting solution was stirred on air overnight, then acetonitrile was evaporated under vacuum, and residual solution was acidified by 1% (v/v) acetic acid and subjected to HPLC.

## Radioligand analysis

Concentrations of peptides were determined by spectrophotometry using molar extinction coefficients at 280 nm calculated using GPMAW (Lighthouse data) or at 214 nm according to [26].

Analysis of competition between scorpion toxins and [$^{125}$I]α-Bgt for receptor binding was carried out on two targets: muscle-type nAChRs in membrane preparation of *T. californica* ray electric organ and human neuronal α7 nAChR expressed in $GH_4C_1$ cell line. For competition binding assays, suspensions of *T. californica* nAChR-rich membranes (1.25 nM α-Bgt binding sites) in 20 mM Tris-HCl (pH 8.0) containing 1 mg·mL$^{-1}$ BSA (binding buffer) or human α7 nAChR expressing cells (0.9 nM α-Bgt binding sites) in binding buffer were incubated for 3 h at room temperature with various amounts of scorpion toxins, followed by an additional 5 min incubation with 0.8 nM [$^{125}$I]α-Bgt. Nonspecific binding was determined by preliminary incubation of the preparations with 20 μM α-cobratoxin. The membrane and cell suspensions were applied to glass GF/C filters (Whatman, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) presoaked in 0.25% polyethylenimine, and unbound radioactivity was removed from the filter by washing three times with 3 mL of 20 mM Tris-HCl (pH 8.0) containing 0.1 mg·mL$^{-1}$ BSA (washing buffer). The bound radioactivity was determined using a Wizard 1470 automatic gamma counter (Wallac Oy, Turku, Finland). The results were analyzed using ORIGIN 7.5 (OriginLab, Northampton, MA, USA) fitting to one-site dose–response competition curves.

## Electrophysiology measurements

Oocytes were gathered *via* surgery of *Xenopus laevis* frogs according to a procedure approved by a local ethics

committee (protocol number 251/2018 26.02.18; Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry). In brief, incision of about 10 mm was made on a benzocaine-anesthetized frog and part of the ovarium was removed and placed in 2 mg·mL$^{-1}$ type 1 collagenase solution (Gibco, Thermo Fisher Scientific). After 2 h of collagenase treatment, oocytes were transferred to the ND96 buffer solution (5 mM HEPES/NaOH at pH 7.6, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$) and injected with pRBG plasmids mixture encoding adult murine muscle nAChR ($\alpha1_2\beta1\varepsilon\delta$). After 2–3 days of incubation at 18 °C, the measurements were performed using a turbo TEC-03X amplifier (npi electronic GmbH, Tamm, Germany). Acetylcholine (100 μM) alone or mixed with OSK1 was applied at 5-min intervals. OSK1 was applied for 5 min before the application of acetylcholine/OSK1 mixture. Wash out was performed with a constant flow of the ND96 buffer for 5 min after each drug application. Current amplitudes were measured, normalized and expressed as mean ± SEM. The results were fitted to the dose–response model in ORIGIN 7.5.

## Results

### Testing scorpion venoms for binding to nicotinic acetylcholine receptors

The venoms of several scorpion species, that is *L. quinquestriatus*, *O. scrobiculosus*, and *H. laoticus* were tested for ability to affect nAChRs. Radioligand analysis with radioactive α-bungarotoxin ([$^{125}$I]α-Bgt) was used to determine if scorpion venom components interact with the classic ligand-binding site of nAChRs. The membranes of the electric organ from *T. californica* containing muscle-type nAChRs, as well as $GH_4C_1$ cells heterologously expressing human neuronal α7 nAChRs were utilized as receptor sources. It was found that all three venoms competed with [$^{125}$I]α-Bgt for binding to both receptor types (Fig. 1). The venoms of *O. scrobiculosus* and *H. laoticus* were chosen for further studies since we had investigated these venoms previously [18,27] and elaborated fractionation conditions. Active compounds were isolated by chromatographic separation of the venoms.

### Scorpion toxin isolation and purification

To purify the active compounds, several steps of liquid chromatography were applied. In the process of purification, all fractions were checked for inhibitory activity toward nAChR from *T. californica* electric organ and human α7 nAChR. First the venoms were separated by gel-filtration as demonstrated in Fig. 2A for *O. scrobiculosus* venom. Fraction 3 was chosen for further investigation as the most active one (Fig. 2C). After its separation using reversed-phase HPLC (Fig. 2B), all collected subfractions were tested against *T. californica* nAChR in the same system. The greatest activity was detected in subfraction 4 (Fig. 2D). This subfraction was shown to contain a pure peptide with a measured molecular mass of 4205.3 Da, which was used for further studies.

An analogous approach was used for the separation of *H. laoticus* venom (data not shown). As a result, a peptide with a molecular mass of 2915.4 Da possessing inhibitory activity against *T. californica* and human α7 nAChRs was isolated. We isolated a toxin with the same mass earlier from *H. laoticus* venom [18], and here we determined its primary structure.

### Toxin primary structure determination

The toxin isolated from *O. scrobiculosus* venom was easily identified as the previously described potassium channel blocker OSK1 (Kalium name α-KTx 3.7;

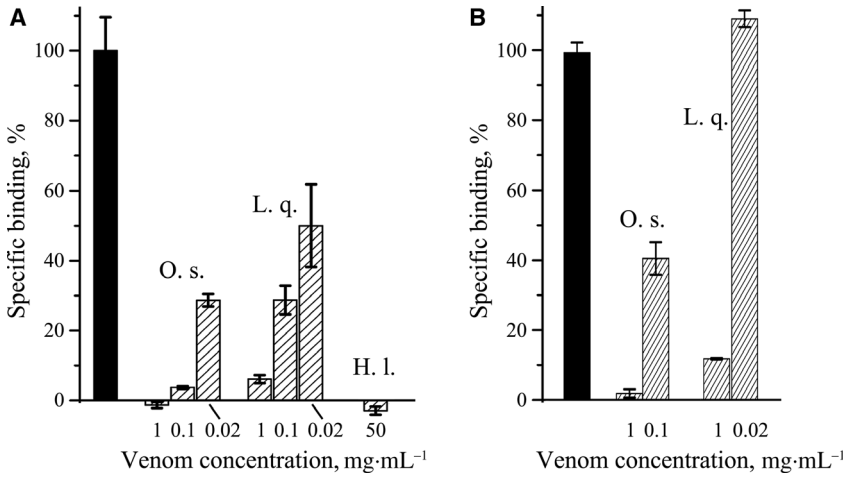


**Fig. 1.** Competition of crude scorpion venoms of *Orthochirus scrobiculosus* (O. s.), *Leiurus quinquestriatus* (L. q.), and *Heterometrus laoticus* (H. l.) at different concentrations with radiolabeled α-Bgt for binding to *Torpedo californica* (A) and human α7 (B) nAChRs. Each bar is the mean ± SEM value of two measurements for each venom concentration in single experiment. Black bars correspond to control.

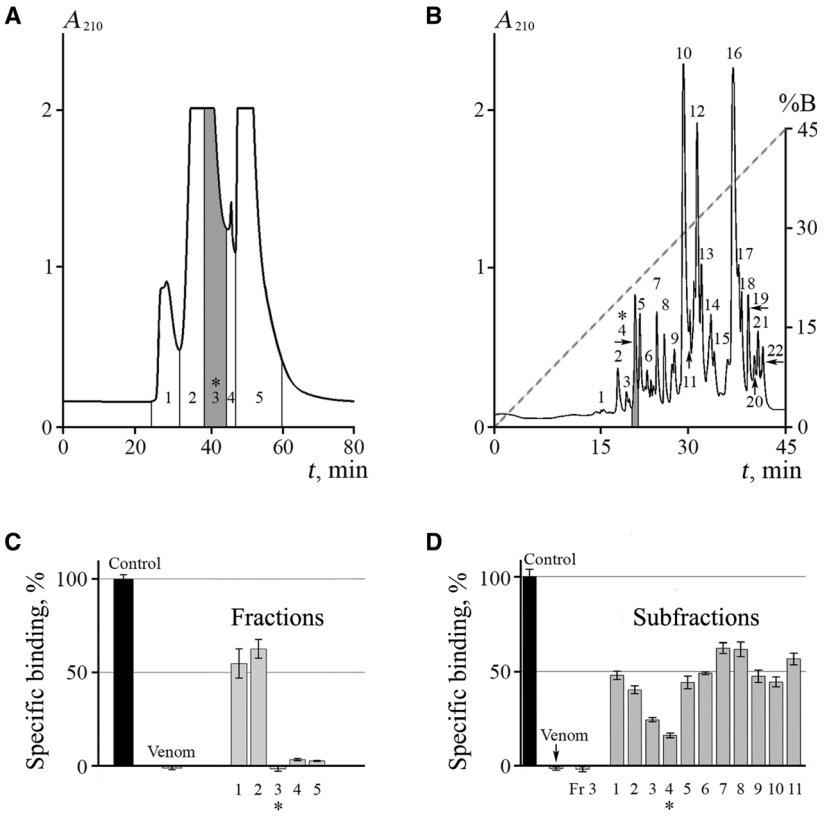


**Fig. 2.** Isolation and purification of nAChR ligands from the venom of *Orthochirus scrobiculosus*. (A) Venom separation by size-exclusion chromatography. Collected fractions are numbered and the most active fraction is shaded. (B) Separation of fraction 3 from (A) by reversed-phase HPLC. Acetonitrile gradient is shown by a dashed line. Collected subfractions are numbered and the most active subfraction is shaded. (C, D) Specific binding assay of fractions obtained from size-exclusion chromatography (C) and subfractions from reversed-phase HPLC (D) to nAChR. The bar height corresponds to the fraction of labeled α-Bgt remaining bound to *Torpedo californica* nAChR. The most active fraction and subfraction are marked with asterisks.

UniProt ID P55896) from the α-KTx 3 subfamily [28,29] based on the molecular mass and retention time on reversed-phase HPLC column. We routinely use this purification step for isolation of OSK1 from *O. scrobiculosus* venom.

The structure of *H. laoticus* toxin was established by MS using a peptide mass fingerprinting approach (Table 1). MS/MS analyses of both the complete polypeptide chain and peptide fragments achieved by trypsin digestion were performed. Based on the obtained data, we deduced the full amino acid sequence of *H. laoticus* toxin. MS data indicated that its C-terminal residue is amidated. The established sequence was identical to that of HelaTx1 toxin (Kalium name κ-KTx 5.1; UniProt ID P0DJ41) from the κ-KTx 5 subfamily characterized previously in *H. laoticus* venom [30]. Interestingly, a toxin derivative with an oxidized methionine was found in *H. laoticus* venom along with the nonoxidized peptide. Sufficient amounts of this toxin for biological activity studies were prepared by solid-phase peptide synthesis.

## Interaction of scorpion toxins with nAChRs

The activity of identified scorpion toxins was determined by radioligand analysis using [$^{125}$I]α-Bgt as a ligand and membranes of *T. californica* electric organ as the source of the muscle-type nAChRs. It was found that both toxins inhibited [$^{125}$I]α-Bgt binding to the membranes with $IC_{50}$ values of 0.505 ± 0.040 μM and 1.4 ± 0.1 μM for OSK1 and HelaTx1, respectively (Fig. 3A,B, Table 2). The $GH_4C_1$ cells transfected with the human α7 nAChR gene were used as a source of neuronal receptor type. Both toxins demonstrated a 30- to 50-fold decreased affinity toward this receptor. Analysis of the HelaTx1 inhibition curves revealed significant differences in Hill coefficients between the *Torpedo* and α7 nAChRs (Table 2). Interestingly, HelaTx1 derivative with an oxidized methionine was almost as active as the native toxin on both receptor types (Fig. 3B).

These results inspired us to investigate the receptor inhibitory activity of a number of well-known scorpion toxins blocking potassium channels. We selected the classic Chtx, Ktx, and AgTx2 as well as Hgtx and spinoxin representing α-KTx and κ-KTx families and four different subfamilies. All of them presented activity at concentrations in the same order of magnitude as OSK1 and HelaTx1 in relation to both *Torpedo* (Fig. 4A) and α7 (Fig. 4B) nAChRs. The most effective inhibitor of the *Torpedo* receptor was spinoxin, which showed the same affinity ($IC_{50}$ of 0.490 ± 0.030 μM) as OSK1 (Fig. 3A).

**Table 1.** Comparison of theoretical digestion fragments of toxin HelaTx1[a] with those found in tryptic digest of toxin isolated in this work.

| Molecular masses (Da) | | | Position in amino acid sequence of HelaTx1 |
|---|---|---|---|
| Determined | Calculated | Sequence[a] | |
| 1855.0 | 1854.9 | RTKKCMQKCNREHGH* | 11–25 |
| 1698.9 | 1698.8 | TKKCMQKCNREHGH* | 12–25 |
| 1597.9 | 1597.8 | SCKKECSGSRRTKK | 1–14 |
| 1469.8 | 1469.7 | SCKKECSGSRRTK | 1–13 |
| 1341.6 | 1341.6 | CMQKCNREHGH* | 15–25 |
| 1240.6 | 1240.7 | SCKKECSGSRR | 1–11 |
| 1084.5 | 1084.5 | SCKKECSGSR | 1–10 |
| 1010.5 | 1010.5 | KCMQKCNR | 14–21 |
| 882.4 | 882.4 | CMQKCNR | 15–21 |
| 851.4 | 851.4 | CNREHGH* | 19–25 |
| 794.4 | 794.4 | ECSGSRR | 5–11 |
| 637.4 | 637.3 | KCMQK | 14–18 |
| 532.4 | 532.4 | RRTK | 11–14 |
| 509.3 | 509.2 | CMQK | 15–18 |

[a]Amino acid sequence of HelaTx1 SCKKECSGSRRTKKCMQKCNREHGH*. Here and in the table the asterisk indicates amidated C-terminal residue. Fragments sequenced by *de novo* MS/MS are underlined.

### Electrophysiological assays

To check if the identified scorpion toxins are capable of not only binding to but also blocking the functional activity of nAChRs, electrophysiological experiments were carried out on adult mouse muscle-type nAChR heterologously expressed in *Xenopus* oocytes. It was found that OSK1 manifesting fairly high activity in radioligand assay on the *Torpedo* receptor inhibited reversibly the currents induced by 100 μM acetylcholine in such nAChR (Fig. 5). The block was dose-dependent and an $IC_{50}$ of 1.6 μM was calculated with the 95% confidence interval (1.5–1.8 μM).

## Discussion

Animal toxins are widely used as molecular tools to study diverse receptors and ion channels. These applications are based on highly effective and selective binding of toxins to their biological targets. The first neurotoxin applied for receptor research was α-Bgt, which continues to be used as a selective marker of muscle-type and α7 nAChRs [31]. α-Bgt belongs to the family of three-finger toxins, which are found in the venoms of snakes mostly from Elapidae family and include so-called α-neurotoxins [10]. These toxins are effective inhibitors of certain nAChR types and until recently have been regarded as selective nAChR ligands. It should be noted that there are several structural types of snake toxins interacting with nAChRs and differing in receptor selectivity [32]. These are so-called short-chain neurotoxins selectively inhibiting muscle-type nAChRs, long-chain neurotoxins blocking both muscle type and α7 nAChRs as well as nonconventional toxins which in addition to inhibition of muscle-type and α7 nAChRs interact with muscarinic acetylcholine receptors. Also noncovalent and disulfide-bound dimers of these toxins exist and the dimers are efficient inhibitors of several neuronal type nAChRs [33]. However, a few years ago, several laboratories found that α-Bgt and some other α-neurotoxins inhibit ionotropic GABA receptors [13,14]. This finding suggests that snake α-neurotoxins may exert a wider array of biological effects than it has been previously thought.

Scorpion and spider venoms are traditionally regarded as the major reservoir of toxins affecting the voltage-gated ion channels. However, some snake venoms contain toxins interacting with these channels as well. Thus, dendrotoxins from mamba venoms are well-known blockers of Kvs [34]. These toxins belong to the Kunitz inhibitor structural type. Recently, a new toxin called calliotoxin has been identified in the venom of the blue coral snake *Calliophis bivirgatus*, which enhances the peak inward current and delays the inactivation of Nav1.4 channel [35]. It is the first identified Nav activator from snake venom belonging to the large family of three-finger toxins.

Scorpion venoms contain toxins acting mostly on voltage-gated ion channels and classified into several families and subfamilies generally based on the following four criteria: the ion channel affected (sodium, potassium, calcium, or chloride), specific receptor to which the toxin binds, three-dimensional structure of the toxin, and the type of response induced (activation/inactivation of the receptor) [1,7]. In general, most of these toxins modify the gating mechanism of Nav channels or block Kv channels. However, there are no data in the available literature about scorpion toxins with established structure affecting ligand-gated ion channels.

In this work, we have tested several scorpion venoms and found that they are able to inhibit nAChRs of the α7 and muscle types. Activity-driven isolation of active compounds has resulted in purification of scorpion toxins interacting with nAChRs. Their structure elucidation has shown that they are known toxins OSK1 and HelaTx1 belonging to α-KTx 3 and κ-KTx 5 scorpion toxin subfamilies, respectively. Radioligand assay has shown that both toxins interact more efficiently with the muscle-type nAChRs manifesting an $IC_{50}$ around 1 μM (Fig. 3A,B, Table 2). Their affinities

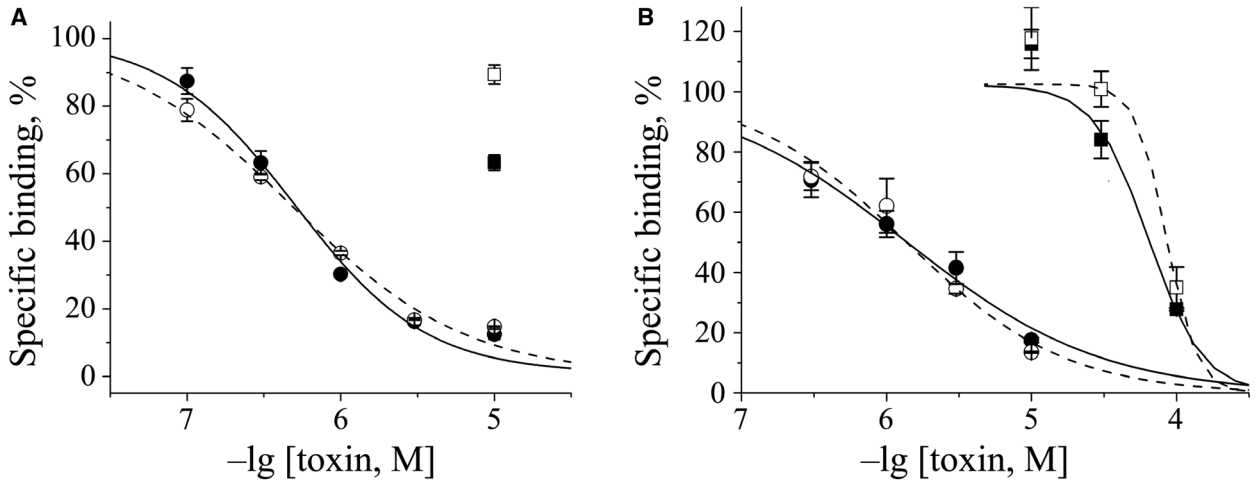


**Fig. 3.** Interaction of the most active anticholinergic components of *Orthochirus scrobiculosus* and *Heterometrus laoticus* venoms with *Torpedo californica* and human α7 nAChRs. (A) Inhibition curves for OSK1 (filled symbols, solid line) and spinoxin (open symbols, dashed line) toward *Torpedo* (circles) and α7 (squares) receptors. (B) Inhibition curves for synthetic analogs of the HelaTx1 with intact (filled symbols, solid lines) and oxidized Met residue (open symbols, dashed lines) toward *Torpedo* (circles) and α7 (squares) receptors. Each point is the mean ± SEM value of two measurements for each concentration in two independent experiments. The respective $IC_{50}$ values (mean ± SEM) are collected in Table 2.

**Table 2.** $IC_{50}$ values and Hill coefficients ($n_H$) for the inhibition of [$^{125}$I] α-Bgt binding to nAChRs by scorpion and snake toxins.

| Toxin | *Torpedo californica* nAChR $IC_{50}$, μM ($n_H$) | Human α7 nAChR $IC_{50}$, μM ($n_H$) | Reference |
|---|---|---|---|
| HelaTx1 | 1.4 ± 0.1 (0.65 ± 0.04) | 64 ± 7 (2.3 ± 0.4) | This work |
| OSK1 | 0.505 ± 0.040 (1.02 ± 0.07) | ~ 20 | This work |
| Spinoxin | 0.490 ± 0.030 (0.77 ± 0.04) | ≫20 | This work |
| α-Bgt | 0.000270 ± 0.000013 (3.4 ± 0.5) | 0.000240 ± 0.000031 (1.13 ± 0.13) | [37] |
| α-Cobratoxin | 0.0045 ± 0.0003 | 0.105 ± 0.010 | [42] |
| Azemiopsin | 0.18 ± 0.03 (one site); 0.030 ± 0.015 and 0.8 ± 0.4 (two sites) | 22 ± 2 | [42] |
| Vurtoxin | 0.26 ± 0.02 | 14 ± 5 | [43] |
| WTX | 3.0 ± 0.5 | 14.8 ± 1.3 | [53] |

to human neuronal α7 nAChRs are much lower with the $IC_{50}$ values around 20–60 μM. Interestingly, a HelaTx1 analog with an oxidized methionine residue was isolated from *H. laoticus* venom, and it was obtained during peptide synthesis with a yield almost equal to that of the nonoxidized product. The affinities of both peptides to nAChRs were almost the same (Fig. 3B).

Noteworthy is the significant difference in Hill coefficients obtained for HelaTx1, their value being 0.65 and 2.3 for the *Torpedo* and neuronal α7 receptors, respectively (Table 2). Although there are some limitations of the Hill equation, for example, the assumption that ligand molecules bind to a receptor simultaneously, it is widely used for ligand–receptor interaction studies. A Hill coefficient of less than unity may indicate negative cooperativity or two independent binding sites with different affinities [36]. Indeed, recently we have discovered a new toxin named αδ-bungarotoxin that binds with different affinities to two sites on the muscle-type nAChR with a Hill coefficient of 0.65 [37]. The low coefficient of HelaTx1 may also indicate its binding with different affinities to two sites on *Torpedo* nAChR. The limitations of the Hill equation were analyzed in detail by Weiss [38]; it was shown that sequential or independent ligand binding to several binding sites even without cooperativity resulted in a Hill coefficient of higher than unity. Neuronal α7 nAChR can bind α-Bgt at five identical binding sites [39], and sequential or independent toxin binding to these sites may result in a Hill coefficient of higher than unity. This is exactly

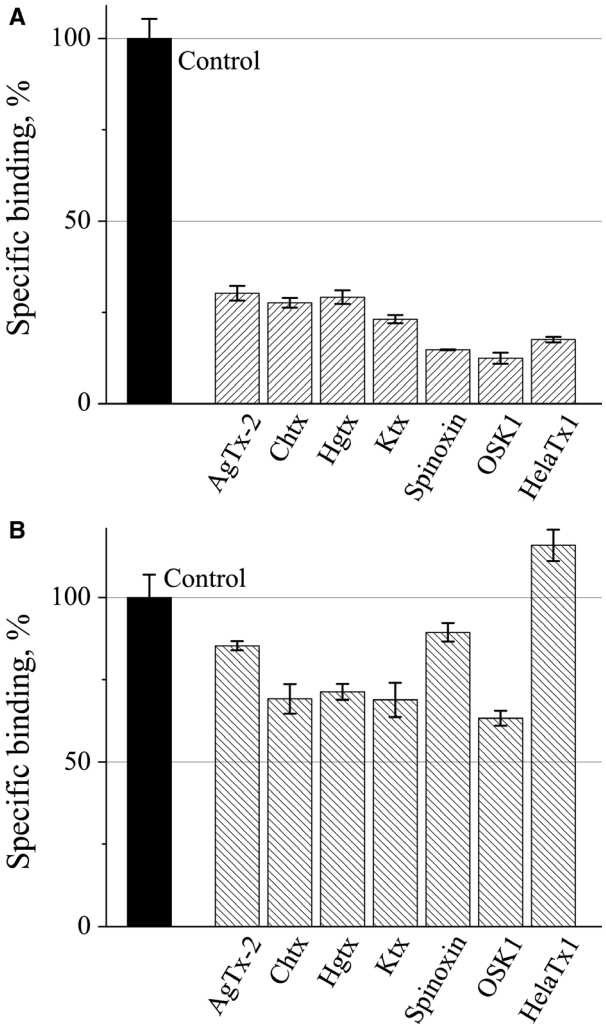


**Fig. 4.** Competition of different scorpion toxins at 10 μM with radiolabeled α-Bgt for binding to *Torpedo californica* (A) and human α7 (B) nAChRs. Each bar is the mean ± SEM value of two measurements in single experiment.

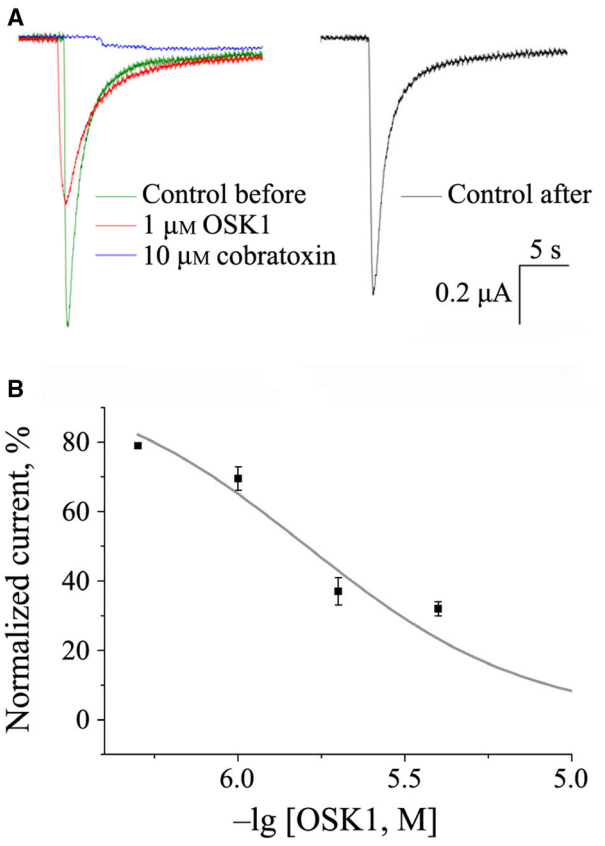


**Fig. 5.** OSK1 action on mouse muscle-type nAChR studied by electrophysiology. (A) Representative traces of acetylcholine-evoked currents in control (green line, before addition of OSK1, and black line, after toxin wash out) and in the presence of 1 μM OSK1 (red line) or α-cobratoxin (blue line). (B) Dose–response curve for the inhibition of mouse muscle-type nAChR by OSK1.

what we have obtained for HelaTx1 interaction with α7 nAChR, the Hill coefficient being equal to 2.3 (Table 2). Interestingly, some scorpion toxins showed Hill coefficients of higher than unity in the interaction with potassium channels. Thus, BmSKTx1 (α-KTx family) from the Chinese scorpion *Mesobuthus martensii* manifested a coefficient of 2.2 in the interaction with small conductance calcium-activated potassium channels [40], and BmP09 from the same scorpion presented a coefficient of 1.8 in large conductance calcium-activated and Kvs [41].

A number of other well-characterized scorpion toxins in our hands behaved in a similar way toward muscle-type and neuronal α7 nAChRs (Figs 3A and 4). It is worthwhile to compare the potency of scorpion toxins acting on nAChRs with that of neurotoxins from snake venoms (Table 2). Although $IC_{50}$ values for scorpion toxins determined in this study are much higher than those for three-finger α-neurotoxins or the linear peptide neurotoxin azemiopsin from the Fea's viper *Azemiops feae* [42], they are only a little bit higher than those for different phospholipases $A_2$ including vurtoxin from the Steppe viper *Vipera renardi* [43] and of the same order as for the three-finger weak toxin WTX from *N. kaouthia* [44].

In electrophysiology experiments, one of the toxins (OSK1) blocked the functional activity of adult mouse muscle-type nAChR expressed in *Xenopus* oocytes in the micromolar concentration range (Fig. 5). At this time, we can only speculate about the physiologically relevant concentrations of scorpion toxins injected into prey. In insects, representing the main scorpion prey, nAChRs play an important role in mediating fast

cholinergic synaptic transmission and are targeted by many insecticides. Because insects are small animals, we assume that after scorpion sting toxins may well reach concentrations sufficient to block nAChRs.

It should be mentioned that OSK1, Chtx, AgTx2, Hgtx, and Ktx are very efficient blockers of Kvs manifesting activity in the low nanomolar range [6,28,45,46]. Thus, the interaction with nAChRs may hardly be considered as their main biological effect. Spinoxin is not such an efficient Kv blocker, yet its activity against certain isoforms is also in the nanomolar range [47]. As concerns HelaTx1, it is a very weak blocker of Kv1.1 ($EC_{50}$ of 9.9 μM) and Kv1.6 (~ 60% block at 30 μM) [30]. Considering its higher affinity to muscle-type nAChRs ($IC_{50}$ of 1.4 μM) one may suggest that the biological target of HelaTx1 could be a ligand-gated ion channel.

HelaTx1 belongs to the κ-KTx family of potassium channel toxins from scorpion venom and most probably structurally resembles κ-hefutoxin-1 (κ-KTx 1.1) conforming to the CSα/α fold and containing two short antiparallel α-helices stabilized by two disulfide bonds [48]. A family of plant defense peptides called α-hairpinins shares these structural features with κ-KTx [49]. It has been shown that both κ-KTx and α-hairpinins may serve as scaffolds for rational functional design [50,51], and HelaTx1 can accommodate considerable structural changes [52]. In addition, the activity of the well-known peptide neurotoxins α-conotoxins demonstrating high selectivity to diverse nAChR subtypes [10] and showing structural similarity to κ-KTx (being short α-helical peptides with two disulfide bridges) can also be rationally manipulated [15]. We therefore believe that HelaTx1 may serve as an attractive scaffold for the design of new nAChR ligands.

## Conclusions

We have found that some scorpion toxins inhibit α-Bgt binding to both *Torpedo* and α7 nAChRs. The affinities of scorpion toxins depend on their structure; members of α-KTx family such as OSK1, Chtx, AgTx2, Hgtx, and Ktx interact 3–4 orders of magnitude better with Kvs than with nAChRs, while the affinity of HelaTx1, a representative of κ-KTx family, to muscle-type nAChRs is one order of magnitude higher than to Kvs. Electrophysiological experiments on the mouse muscle-type nAChR heterologously expressed in *Xenopus* oocytes showed that OSK1 inhibited acetylcholine-induced current in this receptor, demonstrating the functional blocking effect. Thus, our results indicate that scorpion neurotoxins can interact with multiple biological targets.

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## Author contributions

VIT, AAV, and YNU conceived and supervised the study; IEK, PBO, DSK, and MNZ designed experiments; ANH provided key reagents; IEK, PBO, MNZ, NSE, IAI, AMG, OVN, DSK, NAP, and MVS performed experiments; IEK, PBO, MVS, AAV, DSK, and YNU analyzed the data; IEK, PBO, VIT, AAV, and YNU wrote the manuscript.

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