Tetrodotoxins Secretion and Voltage-Gated Sodium Channel Adaptation in Ribbon Worm *Kulikovia alborostrata* (Takakura, 1898) (Nemertea)

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Nemertea is a phylum of marine worms, counting more than 1300 species, most of which are active predators. Many species of these worms bear various toxins, including tetrodotoxin (TTX) – a potent low molecular weight neurotoxin of bacterial origin.

Despite the more than 30 years of studying TTX in nemerteans, many questions regarding its functions, mechanisms ensuring its accumulation and usage remain unclear. For many TTX-secreting animals, the function of the toxin as the predators' deterrent was suggested. The realization of this function in ribbon worms supposes the recovery of TTX in secreting cells through migration from the tissues of the internal environment. In the current research, using 17 specimens of the ribbon worm Kulikovia alborostrata, we studied the dynamics of TTXs concentration in the secretion produced at different time intervals and toxins localization at different stages of the excretion process. To accumulate TTXs and specifically use them as antipredator defense or for prey immobilizing during hunting, animals should have molecular mechanisms ensuring resistance to the toxin. The resistance mechanisms known for some TTX-bearing animals represent mutations in TTX targets – voltage-gated sodium (NaV) channels in the region of the selective filter. In the current research, for the first time, a search for the amino acid substitutions, leading to a decrease of the affinity of the NaV1 channel to TTX in nemerteans, was performed.

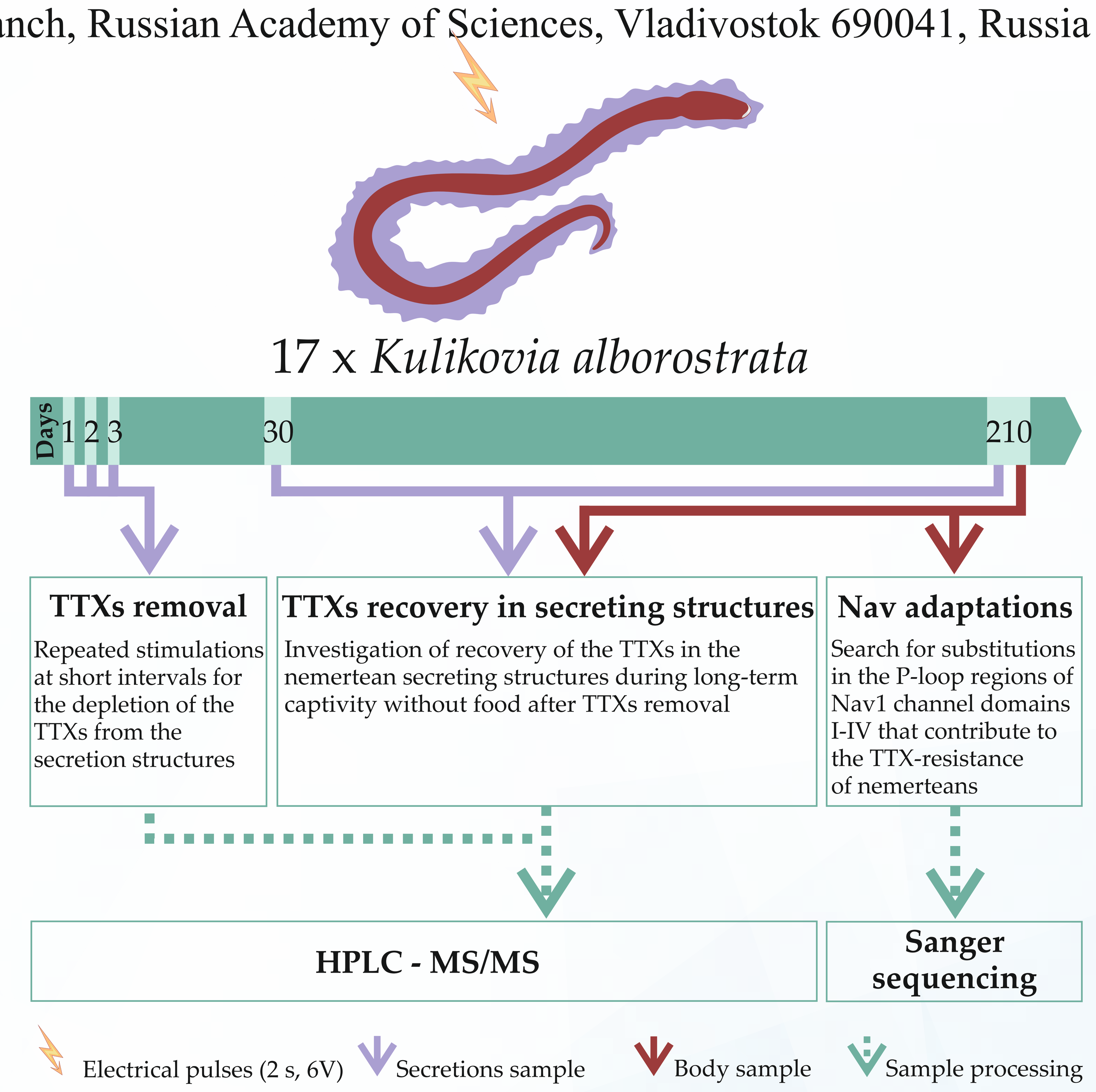

Figure 1. Scheme of the experimental design for studies of tetrodotoxins (TTXs) secretion and Nav1 adaptation in the ribbon worm *Kulikovia alborostrata*

TTXs removal and recovery in secreting structures

№. of specimen			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Secretion (TTXs, ng/g)	Day 1	TTX	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
		5,6,11-trideoxyTTX	0.675	0.264	0.229	-	-	-	-	-	-	-	-	-	0.144	-	-	-	-
	Day 2	TTX	0.340	0.357	+	0.556	+	+	+	+	+	-	+	+	-	-	-	-	+
		5,6,11-trideoxyTTX	1.905	3.171	0.156	6.244	0.399	-	1.690	0.774	-	0.440	-	0.835	-	-	-	-	0.199
	Day 3	TTX	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-
		5,6,11-trideoxyTTX	-	-	-	-	-	-	-	-	-	-	-	0.287	0.115	-	0.093	-	-
	Day 30	TTX	+	+	-	0.306	-	-	-	-	-	+	-	+	+	-	+	-	-
		5,6,11-trideoxyTTX	-	+	+	-	0.338	+	0.862	0.413	+	0.253	0.178	0.547	0.140	+	0.068	-	+
	Day 21	TTX	+	+	+	+	+	+	+	+	0.305	+	+	+	+	+	+	+	+
		5,6,11-trideoxyTTX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Body (TTXs, ng/		TTX	+	+	+	1.166	0.492	0.146	+	+	1.286	+	0.306	0.441	+	+	+	+	+
		5,6,11-trideoxyTTX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 1. Tetrodotoxin (TTX) and 5,6,11-trideoxyTTX in extracts of nemertean Kulikovia alborostrata. +: <limit of quantification (0.6 ng/mL of extract); -: not detected.

TTXs secretion

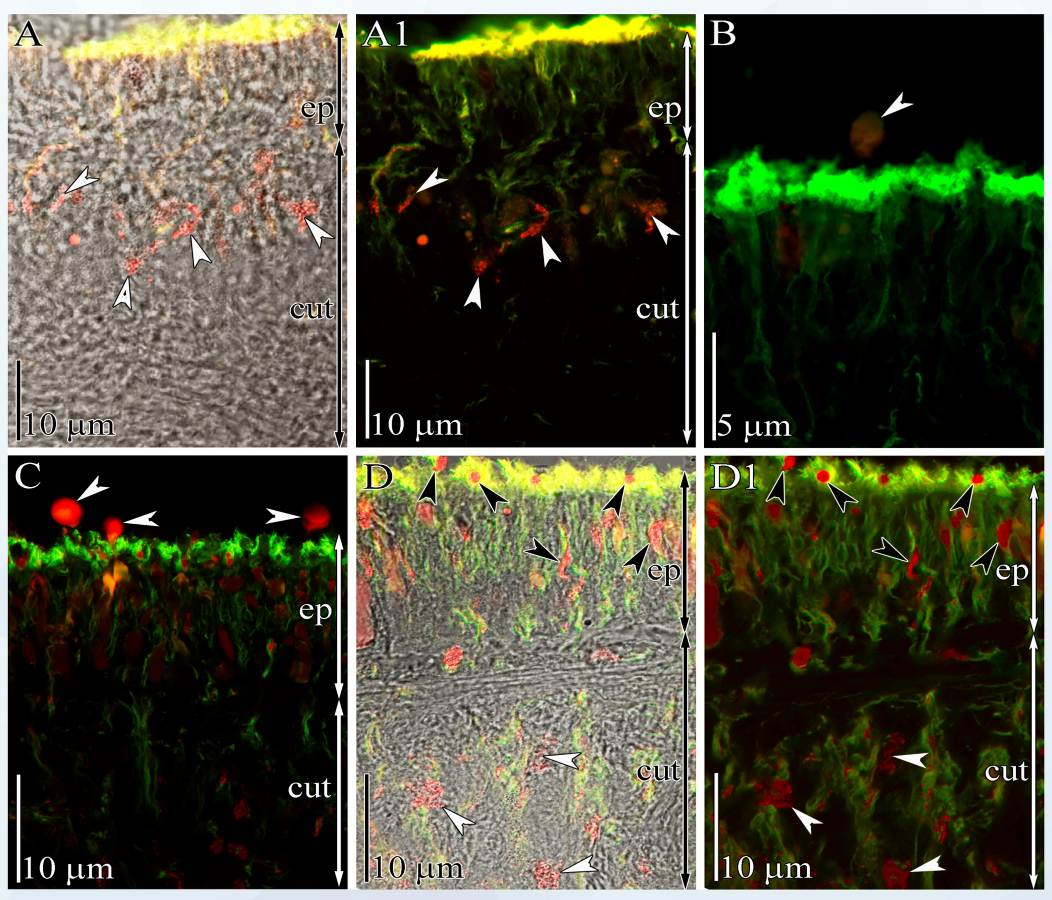

Figure 2. Confocal laser scanning micrographs (Z-projections) of transverse sections of the body wall of the intact (A,B) and stimulated (C,D) specimens of *Kulikovia alborostrata*. Red: tetrodotoxin-like immunoreactivity; green: cilia and cytoskeleton, acetylated tubulin immunoreactivity. (A,A1) Panoramic view showing subepidermal gland cells with toxin-positive granules (arrowheads). (B) TTX-positive spherical secretion (arrowhead) above the apical surface of the epidermis. Panoramic view showing TTX-positive spherical secretions (arrowheads) above the apical surface of the epidermis. (D,D1) Panoramic view of integument showing subepidermal gland cells with TTX-positive granules (white arrowheads) and their epidermal extensions with TTX-positive granules (black arrowheads). Legends: cut, cutis; ep, epithelium.

TTXs adaptation

	Domain I (404)	Domain II (758)	Domain III (1239)	Domain IV (1534)
Homo sapiens NaV1.4	TQDYWENLFQ	CGEWIET	VATFKGWMDI	TSAGWDGLLN
Takifugu rubripes NaV1.4b	TQDFWENLFQ	CGEWIES	VATFKGWTDI	TSAGWDGLLS
Takifugu rubripes NaV1.6b	??DYWEG???	??EWIET	????KGWMD?	??AGWDG???
Thamnophis sirtalis NaV1.4	TQDYWENLFQ	CGEWIET	VATFKGWMDI	TSAGWNVLLN
Taricha granulosa NaV1.3	TQDAWENLYQ	CGEWIES	VATFKGWMDI	TSAGWDGLLA
Notospermus geniculatus NaV1	TQDYWENLYQ	CGEWIES	VATFKGWTEI	TSAGWNYVLN
Kulikovia alborostrata NaV1	TQDYWEGVYH	CGEWIES	VATFKGWTEI	TSAGWNSVLD

Figure 3. Sequence alignment of P-loop regions of Nav channels domains I–IV of *Homo sapiens*(TTX-sensitive channel), TTX-resistant animals (*Takifugu rubripes, Thamnophis sirtalis, Taricha granulosa*), and *Kulikovia alborostrata*, studied herein. The four key amino acids constituting the selectivity region—aspartate-glutamate-lysine-alanine (DEKA) motif are marked in blue. Amino acid substitutions associated with TTX channel resistance are marked in red. Amino acids involved in TTX binding and coordination are marked in yellow. Accession numbers or references: *H. sapiens* Nav1.4: P35499; *T. rubripes* Nav1.4b: Q2XVR6; *T. rubripes* Nav1.6b; *T. granulosa* Nav1.3: A0A6G9W273; *T. sirtalis* Nav1.4: A0A1W5T2B2.

The studies have shown:

- A low rate of TTX recovery in the nemertean secreting structures; after 30 days, TTX was recovered in only one-third of the studied ribbon worms. This leads us to suggest a low probability of targeted usage of TTX by the toxic nemertean *K. alborostrata* as a repellent through its secretion in mucus.
- The TTX analogue 5,6,11-trideoxyTTX, on the contrary, fully migrated from the body wall into the secreting cells and was completely lost through the release of secretion after 30 days of the experiment, which may indicate the specificity of its transfer in response to stimulation and possible targeted usage.
- The sequences of the P-loop regions of Nav1 channel domains I–IV of all 17 studied specimens are identical and have amino acid substitutions, which were shown for TTX-resistant organisms, and, according to the literature data, can contribute to TTX resistance.