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Animal Neurotoxins in Neurobiological Research

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Animal venoms and poisons are in many cases complex mixtures[1], containing biogenic amines, phospholipases, hyaluronidases, proteolytic enzymes and various types of toxins. Some of them have specific effects on the nervous system and are collectively designated 'neurotoxins'. Their extremely specific action on different stages in the complex mechanism of signal conductance between nerve cells and their target organs have made them valuable tools in the hands of the neurobiologist. In view of the restricted space, only a selection of neurotoxins can be discussed here. Several extensive reviews and monographs on the biology of animal poisons and neurotoxins have recently been published and can be referred to for more details[2–6]. In the present article, the selected neurotoxins will be discussed in the sequence of their action on signal transmission: nerve fibre, presynaptic nerve terminal and postsynaptic membrane. Finally, two toxins with apparently central excitatory action will be mentioned.

Table I gives a summary of some of the animal neurotoxins used in neurobiological research. Not all of these are pure substances, which complicates the interpretation of their action. For instance, the venoms of the Australian tiger snake[7] and of the Black Widow spider[8,9] have been used without purification of their neurotoxins, but have provided valuable information because of the specificity of their action. While all snake and arthropod neurotoxins isolated so far have proved to be polypeptides[3,4,10–12], others like the pufferfish poison Tetrodotoxin and the functionally closely related Saxitoxin (the poison of Pacific shellfish) have unique and complex chemical structures[6,13,14].

In Table II the neurotoxins to be discussed in this article are classified according to their site of action. Two general conclusions can be drawn: 1. The pure neurotoxins have extremely specific actions and block neural transmission at only one distinct and selective site. With unpurified venoms or only partially purified toxins, various sites of action are simultaneously affected, complicating the interpretation of their action. Only purified toxins should thus be used as tools in neurobiology.

2. The neurotoxins acting pre- or postsynaptically at the nerve terminal have been mostly used for experiments on the neuromuscular junction, since the nerve-muscle preparation allows a clear separation of pre- and postsynaptic sites. So far, very little work appears to have been done on their action on synapses in the central nervous system or peripheral ganglia. The finding that the polypeptide toxins cross the blood-brain barrier only very slowly and in very small amounts[3,15–17], has also cast some doubt on a physiologically significant central function. This aspect has only very recently received renewed attention[18–21].

(A) *Toxins with action on the nerve fibre: Tetrodotoxin (TTX) and Saxitoxin (STX)*

Tetrodotoxin, the poison from the pufferfish (Gen. *Fugu*), also found in the Californian newt, *Taricha torosa* (Salamandridae), has been isolated and its unusual and unique structure identified[22–25]. A poison with similar action, but as yet unknown chemical structure is Saxitoxin, which occurs in Pacific shellfish and is produced by a dinoflagellate which grows

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Table I. Some animal neurotoxins used in neurobiological research

Reptilia	Elapidae (snakes)	Gen. *Naja* (Cobras)	Cobra α-toxins Cobra toxins T_3
		Bungarus cinctatus (Banded krait)	α-Bungarotoxin α-BuTX β-Bungarotoxin β-BuTX
		Notechis scutatus (Austral. tiger snake)	'Tiger snake venom' TSV
	Crotalidae (snakes)	*Crotalus durissus terrificus* (S. Amer. rattlesnake)	Crotoxin, Crotamin 'neurotoxin'
Amphibia	Anura	*Physalaemus fuscumaculatus* (Argentine frog)	Physalaemin
		Dendrobates histrionicus (Columbian frog)	Histrionicotoxin HTX
	Urodela	*Taricha torosa* (California newt)	Tetrodotoxin TTX
Pisces		Gen. *Fugu* (Pufferfish)	Tetrodotoxin TTX
Mollusca	Lamellibranchiata	*Saxidomas giganteus* (Alaska butter clam) *Mytilus california* (California mussel)	Saxitoxin STX (produced by dinoflagellate Gonyaulex catenella)
Arthropoda	Arachnoidea	*Latrodectus mactans tredecimguttatus* (Black widow spider)	'Black widow spider venom' BWSV
		Tityus serrulatus (Scorpion)	Tityustoxin TsTX
	Tracheata	*Apis mellifera* (Honey bee)	Apamin

periodically in the water around the shellfish beds[26]. In contrast to snake neurotoxins, TTX and STX do not act on the neuromuscular junction, but on the nerve fibres. At 10^{-9}–10^{-7} *M*, TTX quickly (within sec) abolishes the action potential of single myelinated fibres[27] and blocks conduction in frog sciatic nerve[28] and lobster giant axon[29,30], without affecting the resting potential[29,30]. At the motor endplate, a block of conductance without depolarization is observed, with no change in the sensitivity of the endplate to acetylcholine[31]. Externally applied acetylcholine produces normal depolarization in the presence of TTX and STX. Since it was found that the endplate potentials (EPP) disappear before the miniature endplate potentials (MEPP)[31], it is deduced that the axon is affected rather than the nerve terminal. Voltage-clamp studies show that the action of TTX and STX is to block, rapidly and specifically, the initial peak transient inward Na^+-current, without affecting the late steady-state K^+-conductance[23,27,29,30]. Studies on structural derivatives of TTX demonstrate that the entire molecule is required for its action, and that the presence of the guanidinium group alone is not sufficient without the hydroxyl-group at C_4 and the hemilactal *O*-bridges[29]. The blocking action is also specific for the outside of the membrane: TTX does not block the action potential or the Na^+-current when internally perfused through the squid giant axon at high concentrations[32]. The effect of TTX on Ca^{++}-dependent electrogenic systems has not yet been thoroughly studied, but it has been found that the toxin does not seem to have a blocking action[33–35].

TTX and STX have become widely-used tools for the elucidation of the properties of the early transient channel and of the connection between the action potential and transmitter release[5,24]. Thus, it was found that TTX acts on the early transient channel itself, not on the ions, and acts independently of the direction of ion movement through the channel[36,37]. Use of TTX allowed an estimation of the density of early transient channels on the lobster giant axon. About 13 channels per μm^2 were found. The blocking of the Na^+-channels by TTX does not affect transmitter release caused by muscular depolarization by K^+ at the squid giant synapse[38]. Such experiments have suggested

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Table II. Site of action of some neurotoxins of animal origin

Action on nerve fibre	Tetrodotoxin	Block channels for initial peak transient inward Na^+-current. Total block of conduction in nerve fibres.
	Saxitoxin	
Presynaptic action	β-Bungarotoxin	Depletes synaptic vesicles in cholinergic synapses.
	Black widow spider venom	Disrupts synaptic vesicles in motor nerve terminals and destroys synaptic membrane. Acts equally on ACh and Glu motor synapses (insects). Not a pure toxin.
	Tiger snake venom	Disrupts the process linking membrane depolarization to transmitter secretion. Not a pure toxin.
	Tityustoxin	Increases release of ACh from rat cerebral cortex slices.
Postsynaptic action	Cobra toxins	Selectively bind to ACh receptors. Curare-like action, anti-depolarizing. Reversible by washing, except toxin T_3 from *Naja naja siamensis*.
	α-Bungarotoxin	Same action as cobra toxins. Acetylated derivatives used. Irreversible by washing.
	Tiger snake venom	Same action as cobra toxins. Irreversible by washing. Not a pure toxin.
	Crotoxin	Motor endplate desensitized to action of ACh. Not a pure toxin.
	Perhydro-Histrionicotoxin	Reversible block of excitatory ionic transduction sites at motor endplates (topologically distinct from ACh receptors).
Central excitatory action	Physalaemin	Strong depolarizing (excitatory) action on spinal motoneurones.
	Apamin	Augments excitatory monosynaptic and polysynaptic pathways in spinal cord.

that neither a change in Na^+-conductance nor influx of Na^+-ions is necessary for K^+-stimulated transmitter release. The activation of release appears to be dependent only on the level of membrane potential at the presynaptic terminal[24]. Recent experiments with TTX have provided support for the hypothesis that permeability changes are brought about by the movement of charged particles or dipoles within the membrane, in response to changes in the membrane potential[39]. While the action of TTX on the nerve fibre is now well understood, few studies have been made on its effect in the central nervous system. Iontophoretically applied TTX depressed the excitability of cortical neurones, prevented the generation of synaptically evoked responses, and abolished the excitant effects of glutamate and acetylcholine[20]. This central effect seems to be caused by reduction in the excitability of the postsynaptic membranes in neurones[21]. The latter authors, however, did not find any interference of TTX with the depolarizing action of glutamate.

(B) *Toxins with presynaptic action*

1. *β-Bungarotoxin (β-BuTX).* This component of the venom of the banded krait, *Bungarus cinctatus*, a polypeptide, acts exclusively at the presynaptic side of the neuromuscular junction. It thus belongs to the 'Group 2' toxins (LEE[3]). Its effect is associated with a loss of synaptic vesicles from the nerve terminals[40,41]. The postsynaptic acetylcholine response is not reduced. The reduction of acetylcholine release is preceded by a facilitated release of the transmitter, shown by an increased frequency of postsynaptically recorded MEPPs. The size (= quantum content) of EPPs is progressively reduced to the level of MEPPs, and finally complete failure of the action potential-induced release of transmitter occurs[40,42]. The amplitude of EPPs on repetitive stimulation is retained, suggesting failure of the release mechanism. Either high Mg^{++} or low Ca^{++} delays, whereas a higher rate of stimulation accelerates the action of β-BuTX[40]. Electronmicroscopy has shown that β-BuTX induces an increase in the number of opened synaptic vesicles, a decrease in the total number of vesicles, and finally an almost total depletion of vesicles in the nerve terminals at the neuromuscular junction[41]. Central cholinergic synapses are apparently not blocked by β-BuTX[42]. There appears to be a mutual antagonism between β-BuTX and botulinum toxin (which also inhibits the release mechanism at the neuromuscular junction), although it is suggested that they act at different but closely related sites[33].

2. *Black Widow Spider Venom (BWSV).* The venom extracted from the homogenized poison glands of the Black Widow spider (*Latrodectus mactans tredecimguttatus*) by cold saline selectively blocks motor nerve endings in amphibians[43,44]. In the cat, a progressive and irreversible failure of neuromuscular transmission occurs[9]. Electronmicroscopy shows a progressive disruption of vesicles[45] and membranes in the presynaptic terminal, ending in complete destruction and

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loss of all subcellular organelles. There is no damage to the postsynaptic membrane[9]. Electophysiological studies on the glutaminergic neuromuscular junction of the locust *Schistocerca gregaria*[8] showed qualitatively similar effects to those on cholinergic synapses. As at the frog myoneural junction, BWSV transiently increases the frequency of MEPPs before totally abolishing them.

3. *Australian Tiger Snake Venom (TSV)*. The venom of the elapid snake *Notechis scutatus scutatus* contains toxins both with post- and with presynaptic action (see below)[46]. On the sciatic-sartorius nerve-muscle preparation of the toad *Bufo marinus*, the presynaptic-acting toxin causes a complete block of neuromuscular transmission without affecting the action potential in the axon[7]. The muscle failed to respond to indirect stimulation, and no EPPs or MEPPs could be recorded from the muscle fibres. The mechanism postulated is a selective disruption of the process linking membrane depolarization to transmitter release[7].

4. *Tityustoxin (TsTX)*. The purified toxin from the venom of the scorpion *Tityus serrulatus*, Tityustoxin[47] increases the release of acetylcholine when incubated with rat cerebral cortex slices[48]. This effect is dependent on the presence of Na^+ or Ca^{++} in the incubation medium, but independent of K^+-concentration. TTX completely blocks the stimulation by TsTX. It has been suggested[48] that TsTX might be a useful tool for studying acetylcholine metabolism at the presynaptic nerve terminal.

(C) *Toxins with postsynaptic action*

1. *α-Bungarotoxin (α-BuTX)*. One of the two major neurotoxins from the venom of the banded krait (*Bungarus cinctatus*, Elapidae), α-BuTX produces an irreversible anti-depolarizing neuromuscular block at the postsynaptic membrane of the motor endplate[3,4,15]. The purified toxin, unlike the crude venom, does not inhibit the release of acetylcholine from nerve endings[42]. The mode of action of α-BuTX is identical to that of D-tubocurarine, in that endplate potentials (EPP) and miniature endplate potentials (MEPP) are depressed without affecting conduction in the nerve terminal, without changing the resting electrical properties of the muscle membrane, and without affecting the K^+ response[15,40,49,50]. α-BuTX accumulates on the motor endplate zone of mouse or rat diaphragm[16,17,51], and binds specifically and irreversibly to the acetylcholine receptors in mammalian muscle and in electroplax of *Electrophorus* and *Torpedo*[52,53]. This specificity is demonstrated by the protection against binding of α-BuTX obtained by pre-treatment with acetylcholine, D-tubocurarine, or carbachol[40,52,53].

This specificity of action has made α-BuTX an extremely valuable tool for the isolation and quantitative determination of acetylcholine receptor sites in the motor endplate. Using J^{125}-labeled α-BuTX, FAMBROUGH and HARTZELL[54] found that rat diaphragm contains 1.4 to 4.0×10^7 receptor sites per endplate, roughly proportional to bodyweight. BARNARD, WIECKOWSKY and CHIU[55] studied the binding of H^3-α-BuTX to mouse and chicken skeletal muscle endplates and found very similar values, 3 to 5×10^7 receptor sites per endplate. The N, *O*-diacetylated and N, N, *O*-triacetylated derivatives of α-BuTX act exactly like the parent compound as stable, specific and irreversible binding agents of acetylcholine receptors[56], and have been used to estimate the number of receptor sites per endplate in rat diaphragm, giving a value of $1.9–2.2 \times 10^7$. Quite recently PORTER et al.[57], using electronmicroscopic autoradiography with H^3-α-BuTX, found two classes of receptor sites in vertebrate (frog and rat) muscle: both are irreversibly blocked by α-BuTX, but only one class (in the endplate region) is reversibly blocked by D-tubocurarine. They also found a significant number of α-BuTX-binding sites dispersed throughout the muscle fibres. There is no evidence as yet that these extra-synaptic components are a form of acetylcholine receptor. It has also been shown, using α-BuTX, that although the number of specific receptor sites in the motor endplates is about equal to the number of acetylcholinesterase active centers, these sites are topologically distinct and clearly not identical[19,55,58,59,61]. In contrast to its irreversible binding to acetylcholine receptors, α-BuTX binds reversibly to the postsynaptic ion conductance modulator sites also blocked by Histrionicotoxin[62], thus distinguishing the modulator from the receptor sites. Recently, α-BuTX has been

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used as a probe for acetylcholine receptors in cultured neurones from chick embryo sympathetic ganglia[18], demonstrating that its action is not exclusively confined to the cholinergic neuromuscular junction.

2. *Cobra toxins.* Like α-BuTX, the cobra (Gen. *Naja*, Elapidae) toxins so far isolated (α-toxins, toxin T_3) belong to 'Group 1'[3], with exclusively postsynaptic action. Their action is like that of D-tubocurarine, anti-depolarizing, prevented by treatment with acetylcholine, and not affecting the K^+ response[40, 50, 63–65]. The cobra toxins have been shown to bind specifically to the cholinergic receptors in the electric tissues of *Electrophorus* and *Torpedo*[52, 53, 66, 67]. In contrast to α-BuTX, the neuromuscular block by cobra toxins can in general be reversed either by neostygmine or by repeated washing[40, 50, 63]. Cobra toxin T_3 from *Naja naja siamensis* (*kaouthia*) seems to be an exception in this case, by blocking irreversibly[40, 50, 63]. Pretreatment with D-tubocurarine can protect the muscle from the action of cobra toxins, suggesting a competition of the two substances for the same post-synaptic receptor site[15]. The identity of action of cobra toxins and D-tubocurarine is further suggested by electrophysiological studies, which show that cobra toxin depresses EPPs without affecting the terminal spike, the resting membrane potential or the action potential of the muscle[64]. Like α-BuTX, cobra toxins have little or no effect on muscle, peripheral nerve or on the central nervous system[50, 64, 68, 69].

The H^3-α-toxin from *Naja nigricollis* has been used by BOURGEOIS et al.[70] to localize the cholinergic receptor sites in *Electrophorus* electroplax by means of high-resolution autoradiography. They found that the toxin binds exclusively to the innervated side of the electroplax, and that about 100 times more toxin per unit area is bound under the nerve terminals than between the synapses. The values found were about 400 sites per μm^2 in the extrasynaptic area, 33,000 per μm^2 in the subsynaptic membrane, and 39 per μm^2 on the non-innervated face of the electroplax. KARLSON et al.[71] isolated the nicotinic acetylcholine receptor by biospecific chromatography on insolubilized neurotoxin from *Naja naja siamensis* (*kaouthia*). The receptor molecules in homogenized electric organ of *Torpedo* were adsorbed onto the column-bound toxin, and subsequently desorbed by carbachol. The T_3-toxin from the same Thailand cobra was used by LESTER[72, 73] for electrophysiological studies on the mechanism of transmission at the frog myoneural junction. The results support the finding that cobra toxins bind specifically to the acetylcholine receptors, causing an irreversible exponential decline in the amplitude of the endplate potential as a result of a decrease in the sensitivity of the receptors for acetylcholine.

The unpurified venom of the Australian tiger snake (*Notechis scutatus scutatus*), also a member of the Elapid family, has recently been shown to interact with acetylcholine receptors in the sciatic-sartorius nerve-muscle preparation of the toad *Bufo marinus*[7]. This venom also shows distinct presynaptic effects (see above).

3. *Crotoxin.* The venoms of the Crotalid snakes do not all contain neurotoxins[3]. From the venom of the South American rattlesnake, *Crotalus durissus terrificus*, a neurotoxic polypeptide fraction called Crotoxin has been isolated, which produces a neuromuscular block of non-depolarizing type, caused by a decrease in sensitivity of the endplate to the depolarizing action of acetylcholine[74]. Crotoxin can be separated into various fractions, called 'Crotamine', 'Neurotoxin', and 'Proteolytic Enzyme'[3, 11].

4. *Histrionicotoxin (HTX).* The perhydroderivative (H_{12}-HTX) of this neurotoxin from the Columbian frog, *Dendrobates histrionicus*[75], blocks reversibly the excitatory ionic transduction system in the subsynaptic and sarcolemmal membranes of mammalian skeletal muscle[62]. This site is also competitively and reversibly blocked by α-BuTX. It has been suggested[62] that at least two types of sites participate in the excitation of synapses by acetylcholine: the receptor site, irreversibly blocked by α-BuTX, also blocked competitively by curare, and a second site, part of the cholinergic ion conductance modulator, reversibly blocked by α-BuTX and H_{12}-HTX. Evidence that H_{12}-HTX affects Na^+ and K^+ permeability rather than acetylcholine binding is afforded by its effect upon the action potential generation. The results indicate that both acetylcholine receptor sites and a nearly equal number of receptor-associated sites exist at vertebrate neuromuscular junctions[62].

(D) *Toxins with central excitatory action: Physalaemin and Apamin*

The neurotoxic polypeptide from the skin of the South American frog, *Physalaemus fuscumaculatus*, has been isolated and its structure identified as an

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endecaptide, confirmed by synthesis[76,77]. This peptide, Physalaemin, exerts a powerful hypotensive action and stimulates smooth muscle in dog and rabbit[76,78]. It has a strong depolarizing action on spinal motoneurones, about 500 times as strong as glutamate on a molar basis. This depolarizing action is not changed when action potentials are blocked by 10^{-7} *M* TTX, or when the Ca^{++}-concentration is lowered. Physalaemin may thus be related to an excitatory transmitter in the spinal cord.

The neurotoxic polypeptide from the venom of the honey bee, Apamin, produces uncoordinated, uninterrupted movements in mice culminating in generalized convulsions[10,79]. Apamin increases the amplitudes of the monosynaptic extensor reflex potentials and of the polysynaptic flexor reflex potentials in spinally transsected cats[80]. It thus resembles Physalaemin in its central excitatory action, which is quite unlike that of all other known polypeptide neurotoxins.

Conclusions

It is evident from a review of the literature that animal neurotoxins have so far been used mostly for the investigation of the function of the neuromuscular junction. This is because many neurotoxins act selectively in vivo on the motor endplate and, as mentioned before, because the nerve-muscle preparation is a particularly suitable experimental system for studies in this field. In consequence, much more is known about the structure and function of the neuromuscular junction than about other types of synapses. As this review shows, animal neurotoxins have only quite recently been used on material from the CNS, and the first results reveal that different types of central synapses are also sensitive to these toxins. Here is, therefore, a new and wide-open field of study, in which animal neurotoxins could prove to be valuable tools for the neurobiologist. For example, studies on transmitter release and reuptake in tissue slices or tissue cultures, and studies on the function of Ca^{++}-ions in transmitter release mechanisms could benefit greatly from the use of selectively acting neurotoxins. They could also assist in the investigation of neuropathological conditions. In particular, the use of combinations of pure neurotoxins acting at distinct sites should provide more detailed information on those steps in signal transmission at the synapse which are still obscure: for instance, the connection between the action potential and transmitter release, the role of calcium, ion transport channels in the postsynaptic membrane, and the molecular nature of receptor sites for transmitters other than acetylcholine.

Addendum. Early work on the biochemistry of snake venoms came from the laboratory of E.A. ZELLER in Basel. In 1944, he first reported the existence of a new and special L-aminoacid oxidase in the venom of *Vipera aspis*[81]. In the following years, he identified the same enzyme, called 'ophiooxidase', in the venoms of other snakes (*Naja, Bothrops atrox, Vipera latastei, V. libetina*)[82] and studied its substrate specificity[83]. In 1949 and 1950, he investigated the substrate specificity, kinetics and inhibitors of snake venom cholinesterase[84,85] and ATPase[86].

Zusammenfassung

Die tierischen Neurotoxine sind Substanzen unterschiedlicher chemischer Struktur, denen gemeinsam die ausserordentlich spezifische Wirkung auf verschiedene Stufen der Signalübermittlung im Nervensystem ist. Dadurch werden sie zu wertvollen Hilfsmitteln für die Neurobiologie bei der Untersuchung der komplexen Mechanismen der elektrischen und chemischen Signalübermittlung. Nach ihrem Wirkungsort unterscheidet man folgende Typen von Neurotoxinen: a) auf die Nervenfaser wirkend: Tetrodotoxin (Pufferfisch) und Saxitoxin (Muscheln). Diese Toxine mit komplexer chemischer Struktur blockieren selektiv die Kanäle des Na^{+}-Transportes an der Nervenmembran. b) Präsynaptisch wirkend: Beispiele sind β-Bungarotoxin, «Tiger Snake Venom» (Schlangen), «Black Widow Spider Venom» (Spinne) und Tityustoxin (Skorpion). Diese Toxine mit Peptidstruktur zerstören Vesikel und andere Strukturen an der präsynaptischen Nervenendigung. c) Postsynaptisch wirkend: Beispiele sind die Kobratoxine, α-Bungarotoxin, Crotoxin (Schlangen) und Histrionicotoxin (Frosch). Diese Gruppe von Toxinen mit Peptidstruktur (ausser Histrionicotoxin) bindet an verschiedene Arten von Rezeptoren und Ionentransportkanälen an der postsynaptischen Membran und hat sich besonders wertvoll zur Isolierung und Präparation der Acetylcholin-Rezeptoren an der motorischen Endplatte erwiesen. d) Zentral excitatorisch, also wie erregende Transmitter, wirken Physalaemin (Frosch) und Apamin (Biene).

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