Accepted Manuscript

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Please cite this article as: Mathilde R.Israel, Michael Morgan, Bryan Tay, Jennifer R.Deuis, Toxins as tools: Fingerprinting neuronal pharmacology, Neuroscience Letters https://doi.org/10.1016/j.neulet.2018.02.001

Toxins as tools: Fingerprinting neuronal pharmacology

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Highlights

- Toxins that target the nervous system are structurally and functionally diverse.
- Many toxins display exquisite selectivity for neuronal ion channels and receptors.
- These toxins have proved to be useful tools to probe neuronal function.

Abstract

Toxins have been used as tools for decades to study the structure and function of neuronal ion channels and receptors. The biological origin of these toxins varies from single cell organisms, including bacteria and algae, to complex multicellular organisms, including a wide variety of plants and venomous animals. Toxins are a structurally and functional diverse group of compounds that often modulate neuronal function by interacting with an ion channel or receptor. Many of these toxins display high affinity and exquisite selectivity, making them valuable tools to probe the structure and function of neuronal ion channels and receptors. This review article provides an overview of the experimental techniques used to assess the effects

that toxins have on neuronal function, as well as discussion on toxins that have been used as tools, with a focus on toxins that target voltage-gated and ligand-gated ion channels.

1. Introduction

In this review, we use the term 'toxin' broadly to include any small molecule or peptide of biological origin that has activity on neuronal ion channels or receptors, regardless of the route of exposure. This includes 'poisons', which are toxins that are ingested or absorbed, and 'venoms', which are toxins that are injected. It should be noted that all compounds can be considered toxic, and that it is the dose that determines toxicity, a concept first devised by physician Paracelsus in the 16th century. Therefore, neurotoxins are generally distinguished by their ability to bind to a neuronal ion channel or receptor with relatively high affinity (in the nanomolar to micromolar range).

The biological origins of toxins discussed in this review are diverse, ranging from single cell organisms, such as bacteria and algae, to multicellular organisms, which includes plants and animals. These toxins may be produced to deter predators, such as capsaicin produced by chilli peppers, or used to capture prey, as is the case for most venom-producing animals such as spiders, snakes, scorpions and cone snails. It is therefore not surprising that toxins that target the nervous system have independently evolved many times over in numerous plant and animal species. They modulate neuronal functions in many ways, generally leading to the disruption of normal electrical activity or chemical messenger signalling. Many of these toxins display high affinity and selectivity for a particular molecular target, making them useful pharmacological tools to probe neuronal ion channel and receptor function. However, it should be noted that even toxins lacking exquisite selectivity are still useful pharmacological tools, with numerous examples of toxins in the literature used to discover novel ion channel subtypes, improve our understanding of ion channel structure-function relationships, and identify novel allosteric binding sites, all of which provide essential insights for the rational design of more selective probes in the future. In addition, toxins can be used to identify novel therapeutic targets, with the most notable example being the ω-conotoxins establishing $Ca_V2.2$ as a novel therapeutic target for the treatment of neuropathic pain.

The aim of this review is to provide an overview of the techniques commonly used to assess the effects that toxins have on neuronal function (Figure 1), to provide notable examples of toxins that have been used in the literature as tools to fingerprint neuronal pharmacology, and to discuss the limitations of using toxins as tools.

2. Techniques for assessing neuronal function

2.1 Patch-clamp electrophysiology

One of the most common experimental techniques for assessing neuronal function is electrophysiology. In these experiments the electrical properties of neurons, associated with the flow of ions, are probed using micropipettes [125]. The principles of electrophysiology are

used to study both intracellular and extracellular properties of cells. Furthermore, emerging technology such as planar patch clamp and multi-electrode array platforms continue to enhance our understanding of neuronal function in a high-throughput manner [130, 165]. In practice, neurons isolated in culture, in *ex vivo* slices (eg. brain, spinal cord) or *in vivo* are probed with a small pipette filled with a solution similar to the intracellular contents [55, 188]. In general, for whole-cell patch clamping after generation of a "gigaseal" between the cell membrane and the patch pipette, the cell membrane is breached allowing access between the intracellular electrolyte and the recording electrode. Resulting information is then compared to a reference electrode in the extracellular solution. In this configuration, neurons can be "clamped" for voltage or current, measuring the trans-membrane ionic current or membrane voltage, respectively. Many variations on this protocol have been devised including perforated patch, inside-out, and outside-in, however the principles of micropipette, reference and recording electrode set-up remain largely the same [81]. The use of different intracellular and extracellular solution combinations allows the experimenter to isolate particular membrane-spanning channels, and the use of temperature control devices whilst recording increases the scope of electrophysiology, contributing to the discovery of temperature sensitive channels [69]. Electrophysiology has and continues to be the gold standard for assessing the pharmacological effects of toxins on ion channels [137, 142]. Toxins are perfused or added to the intracellular or extracellular recording solutions and the subsequent effect on ion channel biophysical properties are studied.

2.2 Calcium imaging

Calcium imaging remains a robust tool for assessing neuronal function. The influx of calcium via a number of different membrane bound channels tightly controls many intracellular processes [124]. Calcium imaging requires calcium indicators, including Fura-2 and Fluo-4, which exhibit changes in fluorescence upon the binding of calcium ions [174]. Neurons, *in vitro*, *ex vivo* or *in vivo*, with an indicator present are excited at appropriate wavelengths and captured by fluorescence microscopy using a charge coupled device (CCD) camera in real time

4

[76]. Using this technique, toxin molecules can be perfused directly onto neurons to assess effects on Ca^{2+} permeability. Toxins may act directly on Ca^{2+} permeable channels or cause perturbations in intracellular Ca^{2+} levels via second messenger systems. Crucially, calcium imaging gives the user the ability to visualise more than one neuron at a time, and thus neuronal subpopulations can be identified based on functional response. Indeed toxins have been used in this manner to assess subpopulation-specific responses in a high-content approach known as "constellation pharmacology" [169]. This technique uses the selective nature of toxins to identify constellations of receptor and channel types functionally expressed on single or populations of neurons [43].

2.3 Single-Fiber Recordings

The rodent saphenous skin-nerve preparation is a valuable technique used to study responses of individual primary sensory neurons to mechanical, thermal and/or chemical stimuli [50, 148]. In this method, the saphenous nerve along with the skin of the dorsal hind paw and lower leg is removed and placed in an organ bath chamber. The proximal end of the saphenous nerve is placed in a separate recording chamber and immersed in paraffin oil. To obtain a single-fiber recording, the nerve is desheathed and teased apart until mechanical probing identifies a single receptive field on the skin. The fiber is then classified based on conduction velocity (C-fiber < 1 m/s, A-fiber 1.6-12 m/s), mechanical von Frey threshold (low threshold 1-5.7 mN, high threshold 5.7-128 mN), and responsiveness to cold and heat [195]. The technique can be used to study the role of specific ion channels or receptors on neuronal excitability using transgenic mice or selective compounds, including toxins. To test the effect of a toxin, the receptive field on the skin is isolated with a ring to allow continual perfusion of the toxin to the peripheral nerve terminal. Spontaneous activity and responses to mechanical, cold and/or heat stimuli can then be compared before and after the addition of a toxin. A similar methodology is employed in transgenic mice, however due to the heterogeneity of primary sensory neurons; a much larger sample size is needed.

2.4 Compound Action Potential Recordings

Compound action potential (CAP) recordings are used to study the electrical conduction properties of a whole nerve, with the sciatic nerve commonly used in studies assessing cutaneous primary sensory neurons. In this method, a nerve segment is isolated and mounted between two suction electrodes: one that delivers electrical stimuli and one that records the signal [96]. An electrical stimulus is required to synchronize action potentials, as asynchronous action potentials cannot be recorded from a whole nerve [80]. The CAP waveform shape, amplitude and latency of A- or C-fibers can then be compared before and after perfusion of a selective compound to the axon. When testing toxins, it may be necessary to desheath the nerve, as some toxins cannot cross the perineurium [78].

2.5 Neurotransmitter Release

The release of neurotransmitters is an important neurological mechanism for communication between neurons. Changes in release of neurotransmitters can be indicative of a pharmacological response to a drug or toxin, of pathology such as with Parkinson's disease or addiction or can be a sign of neuronal plasticity. Measuring the neurotransmitter release is an important technique and some of the commonly used methods are provided below.

Microdialysis is a minimally invasive method to measure neurotransmitter and other mediator release *in vivo* [187]. The microdialysis equipment generally comprises of a tubular semipermeable dialysis membrane that is inserted into the anatomical region to be sampled. An exogenous physiological solution is perfused along the membrane and the analyte of interest is able to cross from the tissue through the membrane by following the concentration gradient. A slow flow rate of the exogenous physiological fluid allows for the analyte to cross and come to equilibrium with the sampled tissue. Samples of perfusate are collected over periods of time and the concentrations of different neurotransmitters determined by using compound-optimized chromatography [35, 46]. This technique can analyse a wide array of neurotransmitters with high sensitivity. However due to relatively large volumes of sample required for quantification, measures of neurotransmitters are provided in blocks of time ranging from minutes to hours.

6

Electrochemical detection of neurotransmitters has been the primary method used *in vivo*. Fast scanning cyclic voltammetry is a technique that is commonly used to measure dopamine, but also other monoamines (5-HT, adrenaline and noradrenaline) release from neurons *in situ* tissue preparations and *in vivo* [88, 150, 167]. The technique consists of placing a carbon fiber microelectrode at the brain region of interest and a current sufficient to oxidise monoamines is passed through it. The oxidation of the monoamine neurotransmitters allows for current flow at the electrode, which is converted to a concentration by prior equilibration [88]. Fast scanning cyclic voltammetry provides exact measurements of neurotransmitter release in real time, from precise areas of the brain. The technique provides nanomolar to micromolar concentrations and each voltogramm cycles every 100 milliseconds providing resolution of tonic and phasic neurotransmitter release patterns [150].

2.6 Neuron ablation

Plant-derived toxins known as ribosome-inactivating proteins (RIPs), such as saporin from the seeds of *Saponaria officinalis* (soapwort), can be used as tools to selectively kill specific cell populations. They cause irreversible damage to ribosomes, which leads to the inhibition of protein synthesis and ultimately cell death [166]. The specificity comes by conjugation of RIPs to targeting proteins, such as antibodies, hormones, or ligands, enabling the RIP to internalise into the cell where it is active [138, 166]. This technique has been used extensively to target cancer cells by conjugation of an RIP to a monoclonal antibody that selectively binds to a cancer cell surface-specific antigen, with many 'immunotoxins' undergoing clinical trials [6, 139]. These axonally transported RIP toxins can also be used to ablate specific neuronal populations *in vivo*. This approach was used to ablate Neuropeptide Y (NPY) receptor expressing neurons, by conjugating NPY to saporin, which identified an important role for NPY receptor-expressing neurons in mediating anxiety behaviors [114]. Similarly, isolectin B_4 (IB_4), used to distinguish small-diameter sensory neurons into peptidergic (IB_4-negative) and non-peptidergic (IB_4-positive), is routinely conjugated with saporin to assess the role of non-peptidergic neurons in nociception by targeted chemical removal [179, 183]. One intriguing possibility is RIPs

conjugated to venom-derived peptides, particularly those that display high level of specificity for their target (many of which described in detail below). Hypothetically, this would lead to highly specific ablation of certain neuronal subpopulations.

2.7 Behavioral studies

While the methods discussed above provide crucial insights into the molecular mechanisms underlying neuronal excitability, no method currently exists that can model an intact nervous system without using a whole animal. Therefore, *in vivo* experiments using electrophysiological recordings in anaesthetized animals or behavioral studies in awake, non-anaethetized animals have been developed.

Animal models play a crucial role in defining animal behaviors and determining the role of receptors and neurological pathways in those behaviors. Animal models of cognition [123], memory, reward [113], aversion [47] and decision making [131], along with models of pathology such as depression [133], addiction [113], dementia [123] amongst others have been essential in developing an understanding of the molecular mechanisms and neurological pathways that mediate them. Determining the importance of specific brain regions and receptor subtypes in physiological and pathological conditions has been made possible through microinjection of specific pharmacological agents (often selective toxins) into regions of interest and measuring behavioral changes. Stereotaxic surgery allows the accurate insertion of cannulas into specific brain regions by the use of landmarks bregma and lambda on the skull in reference to the brain atlas [72, 136]. Once animals recover from their surgery, pharmacological tools can then be infused slowly into the brain region of conscious animals and their behavior determined.

Animal models have also played a crucial role in our understanding of pain pathways, with mice and rats the most common species used in pain behavioral studies [120]. As pain cannot directly be measured in rodents, withdrawal of the hind paw or tail from a noxious mechanical, thermal, chemical or electrical stimulus is routinely used to quantify 'pain-like' behaviors [49].

Increased or decreased nociception is then inferred based on the level of stimulus required to elicit a withdrawal response. This can be performed in naïve rodents or in rodents that have had a pain state induced by administration of a compound, tissue damage, inflammation or nerve injury. The role of specific ion channels or receptors in the sensation of noxious mechanical, thermal, chemical or (less commonly) electrical stimuli can then be assessed using transgenic mice or following administration of a selective compound, including toxins. For peripheral administration, toxins are routinely delivered by shallow subcutaneous injection into the hind paw (intraplantar injection), as this allows a high local concentration to be reached at sensory nerve endings and reduces the risk of systemic adverse effects [52]. For delivery to the central nervous system (CNS), toxins (particularly peptides) often require administration by intrathecal injection, intracerebroventricular injection, or microinjection into specific brain regions, as they generally cannot cross the blood-brain-barrier [23].

3. Toxins used as probes

While there are likely hundreds of examples of toxins that have been used as tools to probe neuronal function in the literature, the aim of this review is not to provide an exhaustive list, but rather to provide the reader with examples of toxins that have made significant contributions to field of neuronal pharmacology. This review will focus on toxins that act on voltage-gated and ligand-gated ion channels; however there have also been a number of toxins identified that act at G-protein-coupled receptors [31, 51, 154].

3.1 Voltage-gated ion channels

3.1.1 Voltage-gated sodium channels

Voltage-gated sodium channels (Na_V) are pore-forming membrane proteins that permit the influx of sodium ions across cell membranes, a process that is essential for the generation and propagation of action potentials in electrically excitable cells. Because of this, numerous plants and animals have evolved toxins that target Na_V channels for prey capture and/or predator defence, including cone snails, spiders, snakes, scorpions and sea anemone [21, 62, 97, 189].

In mammals, nine different Na_V channel subtypes (Na_V1.1-1.9) have been described, with five subtypes (Na_V1.1, 1.6, 1.7, 1.8, 1.9) expressed on adult peripheral sensory neurons [15, 70, 71]. Despite high sequence homology (>50%) between Na_V subtypes [175], several toxins have been isolated that are selective for one or more Na_V subtypes (see [85] for review), making them useful tools to probe Na_V channel function.

The most notable example is tetrodotoxin (TTX), produced by marine bacteria and bioaccumulated in puffer fish, which is widely used to differentiate Na_V channels as either TTX sensitive ($IC_{50} < 10$ nM; Na_V1.1, 1.2, 1.3, 1.4, 1.6, 1.7) or TTX resistant ($IC_{50} > 1$ μM; Na_V1.5, 1.8, 1.9) [85]. TTX is routinely used in patch-clamp electrophysiology on dorsal root ganglion neurons to isolate TTX resistant current, attributable to Na_V1.8 and Na_V1.9 [153]. TTX is also used in combination with Na_V1.8 or Na_V1.9 knockout mice to elucidate the specific contribution of these channels to neuronal excitability. This approach has identified Na_V1.8 as the primary mediator of action potential propagation in the distal axonal segment of C-fibers using CAP recordings [96]. Application of TTX to the receptive fields of Na_V1.8$^{+/+}$ and Na_V1.8$^{-/-}$ mice in the saphenous skin-nerve preparation has also identified Na_V1.8 as the primary mediator of action potential generation at peripheral nerve terminals at low temperatures (10 °C) in C-fibers [196].

The use of toxins has also been pivotal in elucidating the role of Na_V1.6 in pain, as loss of functional Na_V1.6 in Scn8a$^{med/med}$ mice results in the development of hind limb paralysis and premature death 21 days post partum, making the strain unsuitable for *in vivo* behavioral studies [26]. Intraplantar injection of μ-conotoxin GIIIA (Na_V1.1/Na_V1.6 inhibitor), but not TIIIA (Na_V1.1/Na_V1.2 inhibitor) [185], significantly reverses oxaliplatin- and ciguatoxin-induced cold allodynia in mice, suggesting a role for Na_V1.6-expressing neurons as mediators of pathological cold pain *in vivo* [53, 194]. In line with this, intraplantar administration of the β-scorpion toxin Cn2 in mice, a selective and potent Na_V1.6 activator (EC_{50} 39 nM) [156], causes spontaneous flinching, mechanical allodynia, and enhances 4-aminopyridine (K_V channel

blocker) cold allodynia, providing further evidence for the involvement of $Na_V1.6$ in peripheral pain pathways [53].

A similar approach has also been used to identify a role for $Na_V1.1$ in peripheral pain pathways. Application of δ-theraphotoxin-Hm1a, a selective and potent $Na_V1.1$ activator (EC_{50} 38 nM), to the receptive fields of A-fibers in the saphenous skin-nerve preparation leads to an increase in firing frequency in response to mechanical stimuli [132]. Consistent with an increase in mechanical excitability, intraplantar injection of δ-theraphotoxin-Hm1a also causes spontaneous pain behaviors and mechanical allodynia in mice, which are partially reversed in peripherin-Cre $Na_V1.1$ conditional knockout mice [132].

3.1.2 Voltage-gated potassium channels

Voltage-gated potassium channels (K_V) are pore-forming membrane proteins that permit the efflux of potassium ions across cell membranes, influencing action potential threshold, waveform and frequency. As such, numerous species have evolved toxins that target K_V channels, including scorpions, spiders, sea anemones, snakes and cone snails [129]. In mammals, forty different K_V α-subunits have been described to date, which are grouped into twelve subfamilies ($K_V1.x$-$K_V12.x$) that combine to form either homo- or heterotetrameric channels, the composition of which can alter pharmacology [4]. Due to the number of K_V α-subunits, which is further complicated by the number of possible tetrameric combinations, determining the activity of toxins across all K_V channels is arduous and typically not performed. Despite this, several toxins that target K_V channels have been used as tools to study the role of K_V channels in neuronal excitability.

Dendrotoxins, from the venom of both the green and black mamba snakes, are potent inhibitors of $K_V1.1$, $K_V1.2$ and $K_V1.6$ [79], and have been used as pharmacological tools for decades to inhibit slowly inactivating or delayed rectifier potassium currents (I_{DR}) in both the central and peripheral nervous system. The important role of $K_V1.1$ and $K_V1.2$ in regulating CNS excitability was first revealed by administration of α-dendrotoxin in the brain of rats, which

11

caused spontaneous seizures [11], and was later confirmed by the development of spontaneous seizures in $K_V1.1$ and $K_V1.2$ knockout mice [25, 163]. In addition, α-dendrotoxin is commonly used with 4-aminopyridine to assess the contribution of I_{DR} currents in trigeminal and dorsal root ganglion neurons [116, 190].

BDS-I, isolated from the venom of the sea anemone *Anemonia sulcata*, is an inhibitor of $K_V3.1$, $K_V3.2$ and $K_V3.4$ [59, 192]. BDS-I was recently used in combination with α-dendrotoxin and 4-aminopyridine to identify K_V3 channels as key contributors to spike repolarization in small capsaicin-sensitive dorsal root ganglion neurons [106]. While useful to probe K_V3 channel function under patch-clamp conditions that isolate potassium current, caution should be used in interpreting the results obtained using BDS-I in other methods, as BDS-I was recently found to potently activate $Na_V1.7$ [107].

Ts8, isolated from the venom of the Brazilian yellow scorpion *Tityus serrulatus*, is an inhibitor of $K_V4.2$ channels (IC_{50} 313-652 nM), with no effect on $Na_V1.2$, $Na_V1.4$, $Na_V1.6$, $K_V1.1$-1.6, $K_V2.1$, $K_V3.1$, $K_V7.1$, $K_V7.2$, $K_V7.4$, $K_V7.5$ or $K_V10.1$ at 1 μM [143]. Intraplantar injection of Ts8 (60 μM) in mice causes spontaneous pain behaviors and mechanical allodynia, revealing a role for peripheral $K_V4.2$ in nociception. While mechanical allodynia is consistent with the phenotype present in $K_V4.2^{-/-}$ mice, $K_V4.2$ is also expressed in the brain and spinal cord [82]. Therefore, Ts8 is a useful tool to assess specific block of $K_V4.2$ in periphery sensory neurons, as large peptide-based toxins are unlikely to cross the blood brain barrier, however Ts8 may have activity at other K_V channels at the concentrations used to elicit pain behaviors *in vivo*.

3.1.3 Voltage-gated calcium channels

Voltage-gated calcium channels (Ca_V) are pore-forming membrane proteins that open in response to membrane depolarisation to permit the influx of calcium ions, which is essential for the regulation of many intracellular processes, including neurotransmitter release. Toxins that target Ca_V channels have been isolated from the venom of many species, including cone snails, spiders, scorpions and snakes [22]. In mammals, ten different Ca_V α-subunits have been

described, which are divided into three families (Ca$_V$1, Ca$_V$2 and Ca$_V$3), producing five distinct types of calcium currents in neuronal and non-neuronal tissues, known as L-type (Ca$_V$1.1-1.4), P/Q-type (Ca$_V$2.1), N-type (Ca$_V$2.2), R-type (Ca$_V$2.3) and T-type (Ca$_V$3.1-3.3) [33]. In combination with the dihydropyridines that selectively block L-type current, the venom-derived peptides ω-agatoxin IVA, ω-conotoxin GVIA, and SNX-482 (spider toxin), which selectively block P/Q-type current, N-type current, and R-type current, respectively, have proved to be useful tools in the isolation of Ca$_V$ currents [3, 44]. Most noteworthy, the ω-conotoxins have been pivotal in establishing Ca$_V$2.2 on the central terminals of primary sensory neurons as a therapeutic target for neuropathic pain, with the ω-conotoxin MVIIA (zinconotide, Prialt) currently approved by the FDA for the treatment of severe refractory pain by intrathecal injection [2].

3.2 Ligand-gated ion channels

3.2.1 Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand gated ion channels that are activated by the endogenous ligand acetylcholine and the exogenous ligand nicotine. The membrane ion channel is comprised of a combination of 5 subunits (subunits α1-10, β1-4 and γ, δ and ε), arranged around a pore, consisting of either a homomeric or heteromeric arrangement of subunits [5, 73, 94]. The arrangement of these subunits confers selectivity to various exogenous ligands. Acetylcholine and nicotine bind directly to the boundary between subunits (5 binding sites), stabilising the opened state of the channel allowing the passage of cations (Na$^+$, K$^+$ and sometimes Ca^{2+}) through the pore [5]. Nicotinic receptors are found in the peripheral nervous system, at the neuromuscular junction and in the autonomic nervous system, as well as the CNS [5]. In the brain, activation of nAChRs commonly results in the release of neurotransmitters dopamine, serotonin, glutamate and γ-hydroxybutyric acid [5]. Neuronal nAChRs have a role in a number of pathological conditions including addiction [64], depression [119], Alzheimer's disease, Parkinson's disease [109] and various forms of epilepsy and schizophrenia [102].

13

3.2.1.1 Neuronal nAChR agonists

A number of plants and animals have evolved selective toxins and venoms that can be utilised against predators or against their prey. Due to high levels of nAChR expression and their physiological importance within the nervous system, a number of defensive and predatory mechanisms have evolved in plants, animals and bacteria to both activate and inhibit nAChRs. These plants and animals have provided a diverse resource of pharmacological tools to study nAChRs. These tools have become particularly important, as antibodies for neuronal nicotinic receptors have been shown to lack specificity in knockout mice [122]. There are a number of toxins that have been identified that act on the nicotinic receptors at the neuromuscular junction, however this review will focus on the neuronal nAChR.

Toxins that act on nAChRs have been historically used for medicinal, recreational and for execution purposes. Poison-Hemlock, an extract from *Conium maculatum* was famously described by Plato as the poisoned tea used to execute Socrates [149]. Poison hemlock contains a number of piperidine alkaloids with coniine and *γ-coniceïne* the most prevalent nAChR actives responsible for the plant's toxicity [100, 101]. The most well-known nAChR toxin is nicotine from the nightshade family of plants, a toxin commercially produced from the *Nicontania tobacum* plant. In the late 16th century, the tobacco plant was used to treat various disorders from pain to parasites, while in the early 19th century extracted nicotine became one of the earliest toxins to be used as an experimental tool to provide evidence of the autonomic nervous system. Nicotine has greatest affinity for the α4β2 nAChRs (low nanomolar), which is the predominant nAChR expressed in the brain, but also acts at α3β4 and α7 nAChR (high nanomolar to low micromolar) [87]. Radiolabelled nicotine is used as an agonist to determine receptor densities in healthy brains and in cases of pathology, however other agonists have been identified in more recent years that provide greater utility [14, 27]. There has been great interest in developing and identifying selective and non-selective nAChRs agonists for the treatment of various neurological conditions and as research tools to elucidate neurological mechanisms. Cytisine is an alkaloid partial agonist at β2 containing nAChRs and is found in plants from the

Leguminosae family. It has been shown to be effective in clinical trials for the treatment of nicotine addiction [182], and has also shown efficacy in animal models of other types of addiction and compulsive behavior [13, 159].

While plants have provided an abundant source of nAChR agonists, a number of ligands have been found from other sources. Anatoxin-A is a bicyclic amine alkaloid neurotoxin produced by cyanobacteria of the *Anabaena, Oscillatoria* and *Aphanizomenon* (blue green algae) species amongst others. Anatoxin-A exerts its toxic effects primarily by binding irreversibly to and activating α4β2 nAChR [170]. Anatoxin-A is a potent agonist at the nicotinic receptors and has been used as a tool to elucidate the pharmacology of nAChRs [8, 67, 115] and to measure neurotransmitter release *in vitro* [29, 39] and *in vivo* [28]. Epibatidine is an alkaloid secreted on the skin of the Ecuadorian poison arrow frog (*Epipedobates tricolor*), and potently activates α4β2 and α3β4 nAChRs [68, 144]. Epibatidine binds to nAChRs with very high affinity and radiolabelled epibatine has been used to study the binding and conformational changes of nAChRs as well as changes to receptor subunit expression in disease states and brain regions [95, 145, 159]. The toxin has also been used to elucidate neurotransmitter release mechanisms [184] and has been used in animal models to demonstrate the role of nAChRs in analgesia [144].

3.2.1.2 Neuronal nAChR antagonists

Cone snails have developed a diverse array of short peptides for both hunting prey and defense against predators. Within their venom are a family of 12-19 amino acid disulphide bond stabilised peptides that are highly selective for nAChRs known as the α-conotoxins. To date all cone snail venoms studied have been found to contain a nAChR inhibitor. One of the first neuronal nAChR antagonists identified was the α-conotoxin ImI, an α7 nAChR selective antagonist (with some activity at α9) [89]. Subsequently a range of other selective neuronal α-conotoxins have been identified, including BuIA an α6 subunit nAChR selective antagonist [9], AuIB an α3β4 nAChR antagonist [112], PIA an α6 subunit-containing inhibitor [61], MII and PnIA α3β2 nAChR inhibitors [30], PnIB and EpI α7 nAChR antagonists [128], AnIB an α3β2

and α7 nAChR inhibitor [111], GIC an α3β2 and α6β2β3 nAChR antagonist, RgIa and Vc1.1 potent nAChR α9α10 inhibitors [63, 177], and GID an α3β2, α7 and α4β2 nAChR inhibitor [127]. The α-conotoxins have played a pivotal role in nAChR pharmacology and have been used to elucidate nAChR subtype roles in different diseases. Some examples of how α-conotoxins have been used as tools, include determining which subtypes are expressed within certain brain regions and how they change in animal models of addiction by radioligand binding [159], determining that inhibition of α9α10 nAChR with Vc1.1 reduces neuropathic pain in animal models [155, 177], determining brain regions involved in α6 mediated addiction pathways by electrophysiology [64] or in animal models by microinjection into a reward centre of the brain [99].

While cone snails have produced a vast array of selective antagonists, a number of neuronal nAChR inhibitors have been identified from other plant and animal sources. A number of snakes (cobras, elapids) produce α-neurotoxins that act at neuronal nAChRs. α-Bungarotoxin is a 74 amino acid peptide, with 5 disulfide bridges identified in the venom of the *Bungarus multicinctus* snakes [34]. It was first found to block the neuromuscular junction, however subsequent studies found it also was also an α7 nAChR inhibitor. α-Bungarotoxin has provided an invaluable tool for examining neuronal function. The toxin has been used to identify α7 nAChR binding sites within the brain and changes to expression in response to various neurological pathologies by radioligand binding in brain slices [40, 77], to visualise α7 nAChR expression by tagging the toxin with a green fluorescent protein [158], to affinity isolate proteins using a biotinylated α-bungarotoxin [158], and to determine α7 nAChR meditated behavioral changes through microinjection into certain brain regions [1].

Methyllycaconitine is a deterpinoid alkaloid toxin produced by the Delphinium plant genus that acts as an antagonist at α7 nAChRs. It has been used extensively as a pharmacological tool to elucidate α7 nAChR mediated roles in memory, cognition, and depression as well as determining nAChR agonist mediated neurological effects [48, 66, 119, 180]. The toxin has

been used to elucidate α7 nAChR roles in animal models of Parkinson's disease [109] and to determine mechanism of beta-amyloid neurotoxicity in *in vitro* models of Alzheimer's [108].

3.2.2 Acid-sensing ion channels

Acid-sensing ion channels (ASICs) are pore-forming membrane proteins activated by extracellular protons, which are thought to be important in the detection of extracellular acidic pH during inflammation and ischemia. In mammals, six different ASIC subunits have been described (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, ASIC4) that combine to form either homo- or hetero-trimers. Toxins that target ASICs have been isolated from the venom of spiders, snakes and sea anemones, and have been pivotal in establishing the role of ASICs in ischemic stroke, neuronal inflammation, and pain [41].

The Texas coral snake toxin MitTx was the first toxin used to elucidate the role of ASIC1a and ASIC1b activation in pain. MitTx is a potent activator of ASIC1a (EC_{50} 9 nM) and ASIC1b (EC_{50} 23 nM), with at least 100-fold selectivity over the other ASIC subunits [18]. Intraplantar injection of MitTx (5 μM) causes spontaneous pain behaviors in mice, which are significantly attenuated in ASIC1$^{-/-}$ mice, confirming a role for ASIC1 in pain [18]. In addition, calcium imaging of mouse dorsal root ganglion (DRG) neurons with MitTx identified that functional ASIC1 is mainly co-expressed with TRPV1 (33%) and NF200 (53%), markers of small nociceptive and myelinated neurons, respectively [18]. MitTx was also used to determine the crystal structure of the chicken ASIC1a in the open state [10].

Mambalgin-1, from the venom of the black mamba, is a potent inhibitor of ASIC1a and ASIC1b homomers and heteromers [58]. Administration of mambalgin-1 is antinociceptive in multiple mouse models of pain, revealing ASIC1 subunits as novel therapeutic targets for pain [56, 58]. Interestingly, the antinociceptive effects of mambalgin-1 are absent in ASIC1a$^{-/-}$ mice following intrathecal administration, but not peripheral administration, suggesting ASIC1a mediates the central effects and ASIC1b mediates the peripheral effects [56, 58].

APETx2, discovered from the venom of the sea anemone *Anthopleura elegantissima*, is a potent inhibitor of ASIC3 homomers (IC_{50} 63 nM) and heteromers (IC_{50} 100-2000 nM) [57]. APETx2 has been used as a tool to elucidate the role of peripheral ASIC3 channels in acid-induced and inflammatory pain in rodents [54, 93]. However, APETx2 was later found to inhibit $Na_V1.8$ (IC_{50} 2.6 μM), which may contribute to its antinociceptive effects *in vivo* [17].

3.2.3 Transient receptor potential channels

Transient Receptor Potential (TRP) channels are a large family of cation permeable channels that are activated by a variety of stimuli. Structurally, most TRP channels share six transmembrane spanning regions along with some degree of sequence homology [105]. TRP channels are further classed into seven sub-families; TRPC, TRPV, TRPA, TRPM, TRPN, TRPP and TRPPML [147]. While TRP channels are expressed in both excitable and non-excitable cells, they play a distinct role in sensory neuro-signalling. Functionally, TRP channels are transducers that "sense" external stimuli, including mechanical, thermal and chemical stimuli [37, 38, 105, 147]. The result of TRP channel activation is cation influx leading to generator potentials, which can depolarise the membrane to trigger an action potential.

Toxins have been utilised to delineate the function of TRP channels in neurons. TRPV1, one of the best studied TRP channel subtypes was in fact discovered, in part, due to its ability to bind capsaicin from chilli peppers [32]. TRPV1 is also activated by heat, thus the burning sensation associated with members of the *Capsicum* genus is caused by direct activation of TRPV1 by capsaicin. Indeed, many TRP channels are modulated by plant derived toxins including menthol (TRPM8; cold sensitive) and mustard oil (TRPA1; chemosensitive) (For review see; [178]). Recently, venom-derived toxins have shed light on TRP channels *in vitro* and *in vivo* [161]. This includes the double knot toxin DkTx from *Ornithoctonus huwena* (Chinese bird spider), which has provided insight into TRPV1 structure and gating [19]. Furthermore, investigation of centipede derived RhTx using electrophysiology, calcium imaging and *in vivo* pain behavior assays proffered new evidence on the mechanism of TRPV1 activation [191]. Although capsaicin remains a simple way to identify TRPV1, which is a marker commonly used to

identify nociceptive neurons, toxins continue to be critical for defining the difference between ligand-gated and stimulus-gated (ie. temperature) activation.

Crotalphine, a venom-derived peptide from *Crotalus durissus terrificus* (South American rattlesnake) is a potent activator of TRPA1 (EC_{50} 46 nM) [24]. Unlike other TRPA1 activators, such as allyl isothiocyanate from mustard oil that cause pain following topical application [91], intraplantar injection of crotalphine (10 μM) causes no pain behaviors in mice. Instead, crotalphine is analgesic in multiple inflammatory models of pain due to desensitization of the TRPA1 *in vivo* [24]. Crotalphine therefore provides a novel mechanism of targeting TRPA1 that may be used for the development of new TRPA1 therapeutics.

Toxins derived from the dinoflagellate *Gambierdiscus toxicus*, known as ciguatoxins (CTX), have been utilised to study the contribution of TRP channels in cold sensing [176]. P-CTX-1 causes cold allodynia in patients suffering from ciguatera, a symptom that is recapitulated in rodents following intraplantar injection of P-CTX-1 (3-10 nM) [194]. It is therefore ideally placed to assess the contribution of ion channels and neuronal populations in cold sensing and pathological cold pain [12, 103]. Indeed, ciguatoxin-induced cold allodynia is mediated by TRPA1-expressing neurons, however P-CTX-1 itself does not directly activate TRPA1, but is a non-selective activator of Na_V channels [83, 176]. This provides an example of how toxins can be used to provide unique insight into gross neuronal function.

3.2.4 Ionotropic glutamate receptors

Ionotropic glutamate receptors (iGluRs) are ligand-gated cation channels that open upon binding of the neurotransmitter glutamate, a key excitatory neurotransmitter in the CNS, to generate excitatory postsynaptic current. In humans, 18 iGluR subunits have been described that assemble into tetramers to form three receptor families, known as NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid) and kainate receptors, originally named based on their sensitivity to agonists. In fact, toxins were pivotal in the discovery of AMPA receptors (formally known as quisqualic acid receptors) and kainate

receptors. Each iGluR subunit has a similar structural architecture, consisting of an extracellular N-terminal domain, an extracellular ligand-binding domain that binds glutamate, three membrane-spanning segments (M1, M3 and M4), and finally an intracellular C terminal domain [92]. Despite playing a key role in excitatory neurotransmission, relatively few toxins that selectivity act on iGluRs are reported in the literature.

3.2.4.1 NMDA receptors

NMDA receptors form tetramers from up to seven different subunits (GluN1, GluN2A-D, GluN3A-B). Unlike AMPA and kainate receptors, NMDA receptors require binding of both glycine (to GluN1 or GluN3) and glutamate (to GluN2) at distinct binding sites to open [135, 171]. At the very least, functional NDMA receptors express one GluN1 and either GluN2 subunits alone or a combination of GluN2 and GluN3 [42, 134]. The heterogeneity of the NMDA receptors based on this subunit array underpins its diverse role in neuronal function [135]. Intriguingly, while ligand binding is essential for channel opening, ion flux through NMDA receptors is also modulated by voltage-dependent binding of extracellular Mg^{2+} and Zn^{2+}, which impede the passage of cations and in a sense, block the channel [117].

Argiotoxin-636 (ArgTX-636), from the orb-weaver spider *Argiope lobata*, is a potent inhibitor of NMDA receptors containing the GluN2A or GluN2B subunits (IC_{50} 5-9 nM), and has been used to probe the channel structure and mechanism of channel block [140, 146]. ArgTx-636 interacts with the pore-loop M2 and transmembrane M3 sections, which form the core of the NMDA receptor, leading to steric block of cation flow [140]. The use of ArgTx-636 as a tool to study NMDA receptor function is limited by activity at the AMPA receptor subunit GluA1 (IC_{50} 77 nM), however analogues with improved selectivity have been developed [141].

Conantokins are small linear peptides (17-24 amino acids) isolated from several different *Conus* species that inhibit NMDA receptors, with varying selectivity for the GluN2 subunits [104]. For example, the well-studied conantokin-G (Con-G; from *Conus geographus*) is selective for NMDA receptors containing the GluN2B subunit, inhibiting the receptor by competitively

binding with glutamate at the extracellular ligand binding domain of GluN2B [60, 186]. Differences in the selectivity of conantokins for GluN2B over GluN2A, GluN2C and Glu2D gives scope to rationally design selective inhibitors of these different NMDA receptor subunits to tease apart their role in neuronal function by pharmacological inhibition [173].

Aberrant NMDA receptor function has been implicated in a variety of different neuronal abnormalities including Alzheimer's disease, chronic pain, and ischemia/reperfusion injury [42, 135, 193]. Thus, the prospect of selective pharmacological inhibition of NMDA receptor subunits by toxins is an exciting therapeutic possibility.

3.2.4.2 AMPA receptors

AMPA receptors are the primary channel associated with fast excitatory neurotransmission in the CNS [74, 171]. They are heterotetramers formed by up to four subunits (GluA1-4), with GluA1/GluA2 heterotetramers most abundant in the CNS [20, 75]. The presence of the GluA2 subunit renders AMPA receptors impermeable to Ca^{2+} due to the presence of an additional positive charge in the pore (R607) [84]. Quisaqualic acid, from the seeds of *Quisqualis indica*, was originally used to identify the AMPA receptor, but was superseded by the agonist AMPA, due to poor selectivity over kainate and metabotropic glutamate receptors [160].

Con-ikot-ikot, a large 86 amino acid peptide isolated from the venom of *Conus striatus*, selectively enhances AMPA receptor currents by blocking desensitization, and is active at all four subunits (GluA1-4) [181]. Con-ikot-ikot has no detectable activity on kainate receptors containing GluK2 or NMDA receptors containing GluN1/GluN2A, suggesting it is selective for AMPA receptors, although activity at other iGluR subunits remains to be confirmed. Con-ikot-ikot recently proved useful for resolving the crystal structure of the GluA2 AMPA receptor in the activated state, interacting with the extracellular ligand binding domain [36].

3.2.4.3 Kainate receptors

Like the AMPA receptor, the kainate receptor was discovered due to activation by kainate, a naturally occurring neurotoxin isolated from the red algae *Digenea simplex*. Kainate receptors

are tetramers formed by one or more combinations of five subunits (GluK1-5), with GluK1-3 subunits capable of forming functional homo- or hetero-tetrameric channels, while GluK4 and GluK5 require heterotetrameric assembly with one of the GluK1-3 subunits [86, 121]. Compared to NMDA and AMPA receptors, less is known about kainate receptors due to a lack of selective pharmacological probes. Kainate and the structural analogue domoate, also isolated from marine algae, interact with the glutamate-binding site to activate kainate receptors (and less potently AMPA receptors), with subtle differences in activity and selectivity at GluK1, GluK2 and GluK3 [86, 157]. The agonist 5-iodowillardiine, isolated from the seeds of *Acacia* and *Mimosa* species, is a selective agonist of the GluK1 subunit (EC50 0.21 μM), with no effect at GluK2 and GluK3 at concentrations > 300 μM [7, 168].

3.2.5 GABA$_A$ receptors

The endogenous ligand of the GABA$_A$ receptor is γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the CNS. Upon GABA binding, GABA$_A$ receptors open to allow the influx of chloride ions, ultimately causing hyperpolarisation of the neuron and rapidly inhibiting synaptic transmission in the brain. Structurally, GABA$_A$ receptors are pentamers arranged to form a central pore, made up of 19 subunits α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3, with the majority of heteromers consisting of two α subunits, two β subunits, and one other subunit [118, 162]. GABA$_A$ receptors are the target of the barbiturate and benzodiazepine classes of drugs, thus they have clinical potential for the treatment of many neurological disorders.

Initially, bicuculline and picrotoxin, alkaloid toxins isolated from plants known to cause convulsions, were discovered to be antagonists of the GABA$_A$ receptor and were used to the study its function [90, 126]. Many venom-based toxins with activity at GABA$_A$ receptors have also been reported, including α-bungarotoxin, α-cobratoxin and the α-conotoxin ImI, however these toxins also act at nAChRs, as both receptors belong to the pentameric Cys-loop superfamily of ligand-gated ion channel receptors [98]. Recently, two toxins named MmTX1 and MmTX2, isolated from the venom of the Costa Rican coral snake, were found to potently

22

modulate GABA$_A$ receptors, without displacing the binding of several nAChR ligands to rat brain synaptosomes, suggesting selectivity for GABA$_A$ receptors [152]. Both toxins allosterically increased the GABA$_A$ receptor affinity for agonists, in a similar manner to benzodiazepines, making them useful drug leads to develop novel therapeutics that modulate GABA$_A$ receptors.

4. Limitations of using toxins as probes

Despite being highly potent for a molecular target of interest, the main limitation of toxins is the potential for off-target activity. Off-target activity may occur within channels or receptors of the same class due to high sequence homology or it may occur within different ion channel or receptor classes due to structural homology. Promiscuous activity is particularly evident amongst venom-derived peptides that target the voltage-sensing domain of voltage-gated ion channels, with venom-derived peptides from spiders often reported to have activity at Na$_V$, K$_V$ and Ca$_V$ channels [16, 65, 132, 164]. However, it should be noted that compared to most other probes, including synthetic small molecules and endogenous ligands, toxins in general remain to be the most selective modulators available for voltage-gated and ligand-gated ion channels.

Often the use of a toxin as a tool may be limited by the amount of material available to researchers. This is because many toxins are difficult to produce synthetically or recombinantly in large quantities and purification from the original biological source may not be possible. An example of this is the alkaloid Na$_V$ channel activator batrachotoxin, which was originally purified from the skin glands of the now endangered poison dart frog [45]. Difficulties in chemical synthesis of batrachotoxin, which were only recently overcome [110], have so far limited the widespread use of this toxin as a pharmacological tool.

Knowing the effective concentration of a toxin at the target of interest can be problematic, particularly in *ex vivo* and *in vivo* studies. Sometimes, the concentration required to produce a functional response far exceeds the potency of the toxin determined by *in vitro* functional assays. Examples of this include the ASIC1 activator MitTx (EC$_{50}$ 9-23 nM), the Na$_V$1.1

23

activator δ-theraphotoxin-Hm1a (EC_{50} 38 nM) and the $K_V1.1$, $K_V1.2$ and $K_V1.6$ inhibitor α-dendrotoxin (EC50 1-25 nM), which need to be administered by intraplantar injection at concentrations of 5 μM, 5 μM, and 100 μM, respectively, to elicit pain behaviors in mice [18, 116, 132]. This therefore can make it difficult to determine if the administered concentration is indeed selective for the target of interest based on *in vitro* selectivity data, and may require the use of knockout animals (when available) to confirm target specificity.

5. Conclusion

Toxins are a structurally and functionally diverse group of compounds that interact with many different neuronal ion channels and receptors. From the discovery of novel molecular targets, to the determination of ion channel and receptor structure and function, there is no doubt that toxins have and will continue to be essential tools to probe neuronal function. Advances in high-throughput electrophysiology and fluorescence-based assays, in combination with improved proteomics, transcriptomics and mass spectrometry, will undoubtedly lead to the discovery of novel toxins with activity at neuronal ion channels and receptors currently lacking selective pharmacological probes [142, 151]. In addition, improvements in chemical synthesis and recombinant expression technology of peptide-based toxins will also increase the quantity of toxins available to be used in functional studies in the future [172].

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Figure Legends

Figure 1. Overview of the experimental techniques commonly used to assess toxin effects on neuronal function.