Neurotoxins: From Frogs and Fish to Spiders and BOTOX

Neuroscience Teaching Unit

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ISCI 2A18

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INSTRUCTOR'S MANUAL

PART A - INSTRUCTOR'S OVERVIEW

(1012 words)

The purpose of this twenty-five hour unit is to illustrate concepts in neuroscience using the mechanism action of various neurotoxins on different parts of the nervous system. In addition, parts of this unit will act as an introduction into research methods and experimental design in biology and neuroscience.

This unit consists of five sections. The latter four sections each focus on a single neurotoxin illustrating a different aspect of nervous system function. These are meant to act as a vectors to introduce detailed descriptions of the nervous system, as well as important concepts in biochemistry and cell biology. Each section should be evaluated separately via a short quiz and/or a cumulative final unit test.

Section 1: General Knowledge (5 hours)

Lesson (Topic)	Main Concepts/Points
Overview of Nervous System (1 hour)	● Basic components and importance of the nervous system ● The nervous system in animals o Distinguishing between the central and peripheral nervous system
Neurons and Synapses (1.5 hours)	● Neuron cell structure (structures involved in signaling) o Discussion: why do neurons need to communicate? ● Electrical vs. chemical synapses, basic neurotransmitters
Membrane Potential (1.5 hours)	● Definition of membrane potential ● Comparison of passive transport and active transport (facilitated diffusion vs. active transport) ● Similarities and differences between transmembrane proteins, ion channels, transporters, and pumps
Introduction to Neurotoxins (1 hour)	● Definition of a neurotoxin with an emphasis on diversity of compounds that can exist (e.g. defense against predation)

Section 2: Tetrodotoxin (5 hours)

Lesson (Topic)	Main Concepts/Points
Background (0.5 hours)	● Cultural significance in Japan ● Tetrodotoxin-producing animals ● Theories regarding tetrodotoxin production o Bacterial symbiosis o Bioaccumulation from tetrodotoxin-producing bacteria at the lowest trophic level
The Action Potential (0.75 hours)	● Recap of membrane potential ● Purpose of the action potential ● Events of the action potential o Depolarization

	○ Hyperpolarization
The Voltage-Gated Sodium Channel (0.75 hours)	● Domains and segments (briefly) ○ Noting amino acid helix formation, which form segments, which form a domain in the protein, then subunits, etc. ● Change in conformation due to depolarization ○ Noting how structure/properties of certain segments are related to function ● Role of the sodium channel in the sequence of events in the action potential
Interaction of Tetrodotoxin and the Sodium Channel (1 hour)	● Sodium channel is not perfectly selective for sodium ions ● Presence of a guanidinium group allows tetrodotoxin to enter the pore ● Amino acid substitutions (i.e. mutations) in the sodium channel can decrease binding affinity for tetrodotoxin and allows for tetrodotoxin resistance in certain tissues
Key Experiments involving Tetrodotoxin (1 hour)	● Primary principle: the selectivity of tetrodotoxin for the sodium channel allows for simple, deliberate manipulation of the action potential
Wave Summation Activity (1 hour)	● Illustration of how turning off the current of one ion illustrates its properties

Section 3: α-Latrotoxin (5 Hours)

Lesson (Topic)	**Main Concepts/Points**
Introduction (1 Hour)	● Brief introduction to black widow spiders and α-latrotoxin ● Protein conformational changes ○ Discuss types of protein conformational changes (i.e. ligand-dependent, voltage-dependent) ○ What a conformational change is and examples ○ Protein activation cascades (i.e. one protein is activated by a ligand, which causes it to interact with another protein, etc.)
Synaptic Vesicle Release (1.5 Hours)	● Voltage-gated calcium channels - structure and function ● Function of synaptotagmin and complexin ● Binding to SNARE complex and phospholipids in the vesicle membrane - mechanism ● Opening of the fusion pore ● Diffusion of neurotransmitters across the synaptic cleft
α-Latrotoxin Mechanism of Action (1.5 Hours)	● High-concentration ○ Incorporation into the presynaptic membrane ● Low-concentration ○ Extracellular calcium-dependent: binding to receptors in the presynaptic membrane, pore formation ○ Extracellular calcium-independent: binding to receptors in the presynaptic membrane, release of cellular calcium stores ● Cell death and paralysis
Pore Formation Activity (1 Hour)	● Demonstration of calcium influx via pore formation versus calcium influx via voltage-gated channels

Section 4: Botulinum (5 Hours)

Lesson (Topic)	**Main Concepts/Points**
General Introduction (1 Hour)	● Introduction to the *Clostridium botulinum* bacteria ● Toxin production in the bacteria ● Overlying biology of the cell and how it functions in anaerobic conditions ● Review of antigenics
Physiology of the Neuromuscular Junction (1 Hour)	● Key components of the neuromuscular junction ○ The motor neuron and innervated muscle fiber ● Specific morphological changes ○ How morphological changes are conducive to neuromuscular transmission ● Introduction to neurotransmitters
Botulinum in the Neurotoxin (1 Hour)	● The agonistic effects of botulinum on regular nerve signal transduction ○ Basic chemical overview of the molecule ● Introduction to microbiological processes of proteolysis and endocytosis ● Focus on how botulinum uses these processes to act ○ Interactions with SNAP-25
Clinical Applications of Botulinum (1 Hour)	● How can botulinum be used safely and effectively despite its potency? ● History of discovery of treatments and how they have helped treat various muscular disorders ● Cosmetic applications of Botox
Neuromuscular Response rate activity (1 Hour)	● Measuring the response rate of muscles ○ Response time from visual observation of stimulus to corresponding muscle activation ● Comparisons of response rates between individuals and demographics ● Hypothesize reasons for discrepancies between response rates

Section 5: Epibatidine (5 hours)

Lesson (Topic)	**Main Concepts/Points**
Background about epibatidine and the Scientific Method (1 Hour)	● Discovery of epibatidine o Illustrate scientific method, noting the many studies and trials needed to elucidate and synthesize the compound ● Epibatidine-associated side effects ● Use for visualization of retinal waves
nAChRs (1 Hour)	● Introduction to receptors and subcomponents ○ Review of neurotransmitter release from presynaptic cell including diffusion across synaptic cleft ● Overview and significance of nAChRs
Agonists vs. Antagonists (1 Hour)	● Contrast definitions of agonists and antagonists ● Definition of nicotinic agonists ● High affinity of epibatidine in comparison to nicotine and acetylcholine (to be linked to by next lesson)
Cation-π Interactions and Hydrogen Bonding (1 Hour)	● Review of intermolecular forces (particularly hydrogen bonding). ● Introduction to π bonds (within the context of aromatic rings); cation-π interactions and their importance for binding of epibatidine
Modelling Intermolecular Forces using Magnets (1 Hour)	● Students will construct a simple model exploring how acetylcholine and its agonists nicotine and epibatidine bind to nAChRs using simple materials (magnets, a paper plate, and rulers) ● Encourage students to explore different options and structures, using the two magnets in the centre of the plate and ruler to model cation-π interactions ○ Side magnets on plate and rulers are used to illustrate hydrogen bonding between epibatidine/nicotine and nAChRs

PART B - LOGISTICS WORKSHEET

Group identifier D

Running title Neurotoxins: From Fish and Frogs to Spiders and BOTOX

1) Appropriate grade level: Grade 12

2) Recommended background concepts in:

Biology: Cell structure, protein function, evolution, bioaccumulation, antigens, antagonism vs, agonism

Chemistry: Intermolecular forces, ions and charges, covalent bonds, molecular concentrations

Math: Basic arithmetic, effective graph analysis

Physics: Voltage and electricity, wave superposition

Other

3) Major concepts students should learn or master in this project

- Intra- and inter-neuronal communication
- Clinical applications of neurotoxins
- Potential of using neurotoxins for research

4) Time required to: a) set up: 3 hrs**, b) deliver**: 25 hrs**, c) clean up:** 1 hrs**.**

5) Space and equipment requirements:

- Average-sized classroom able to hold a class of approximately 25 students
- A teacher with a minimum of a B.Sc. in Biology, preferably with a biochemistry background
- A projector with a screen

6) Supplies required (provide quantities):

- 15 meter sticks
- 12 paper plates
- 100 small (student) magnets
- 25 printed worksheets for activity 1 (see part C in instructor's manual)
- 5 buckets
- 5 water balloons
- 5 pairs of scissors
- 15 plastic water bottles
- 3 rolls of duct tape
- 5 wooden skewers

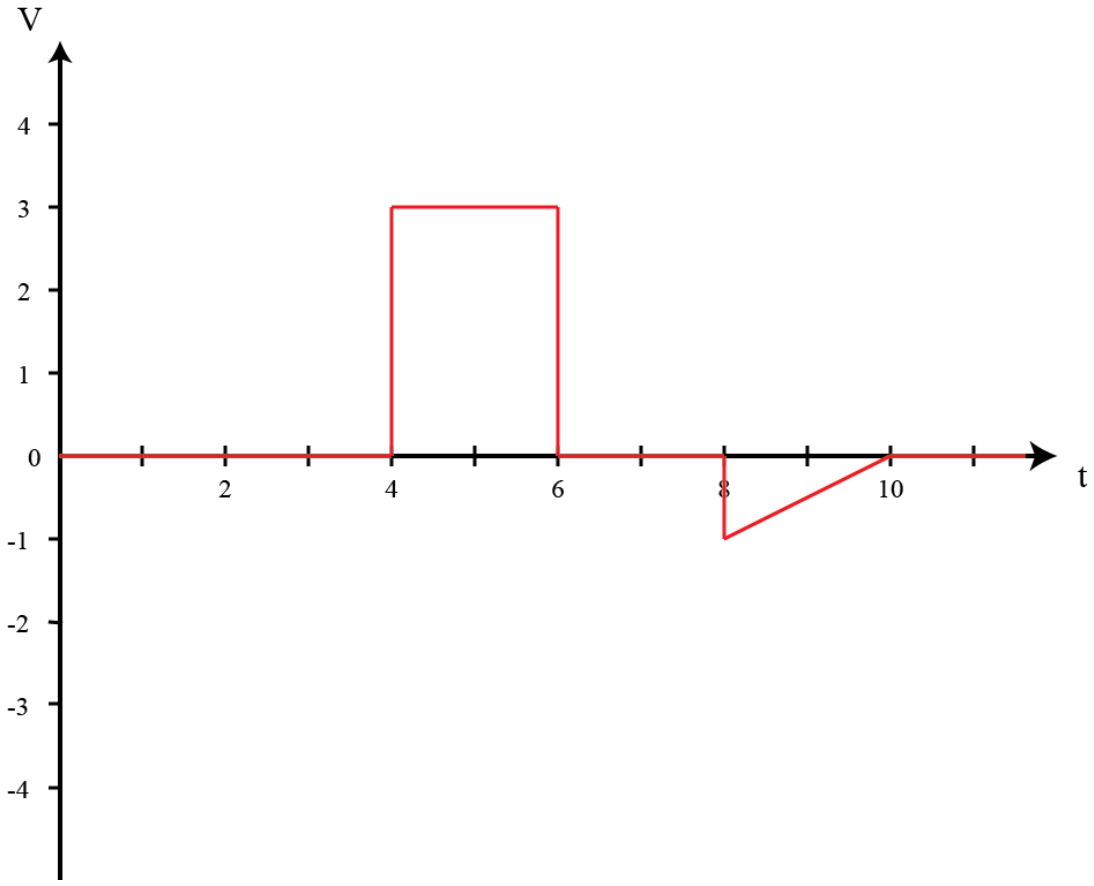
PART C - ADDITIONAL WORKSHEETS

Activity 1: Student Worksheet – Tetrodotoxin

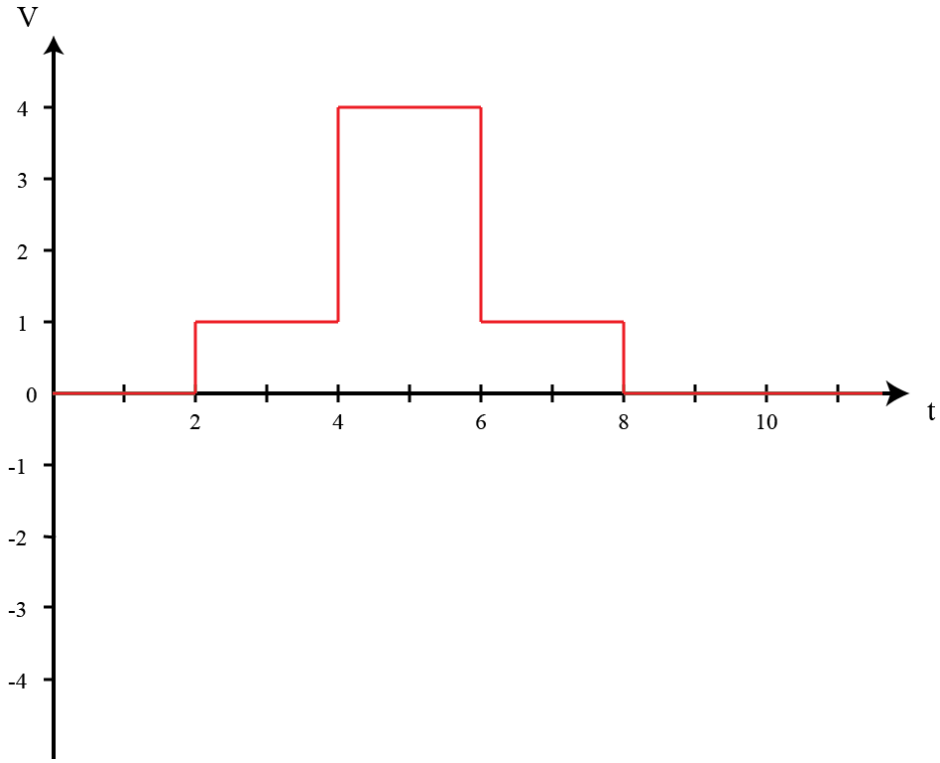
Tetrodotoxin, due to its selectivity for the sodium channels, is useful for isolating currents in the action potential. By turning off the sodium current, an experimenter can observe the change in membrane potential due to potassium only, and can therefore easily determine what ions are flowing in a given direction at a certain time.

Instructions
The following four graphs are made from three superimposed pulses (A, B, and C). From these graphs, draw the shape of the individual pulses. Three sets of axes are provided for you to draw the individual pulses on page 3 and 4.

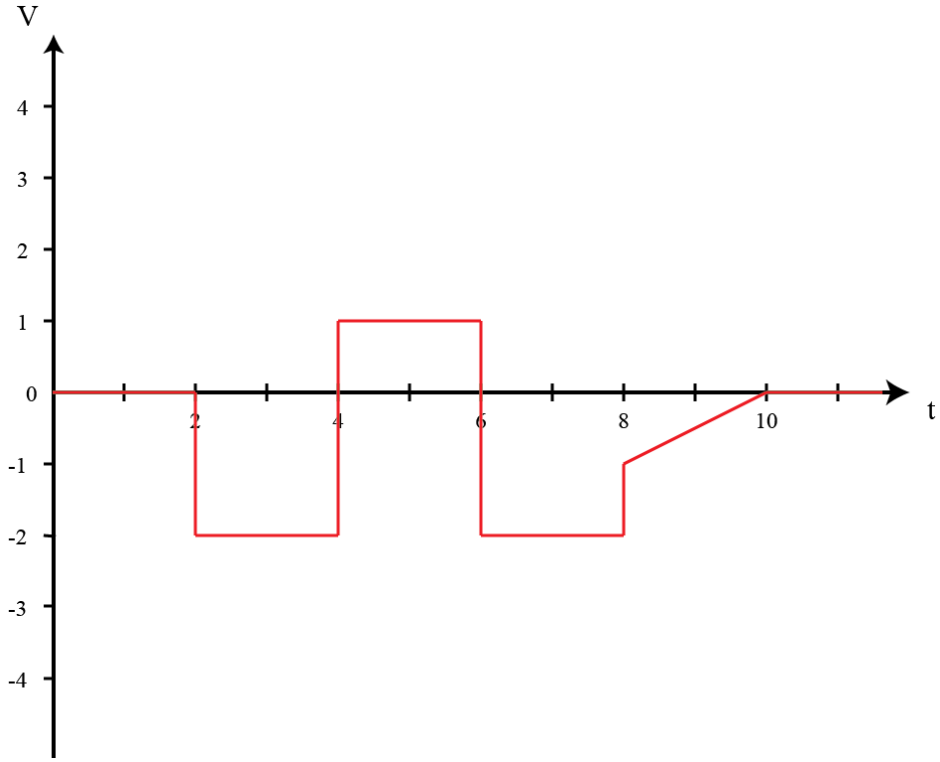
A + B + C



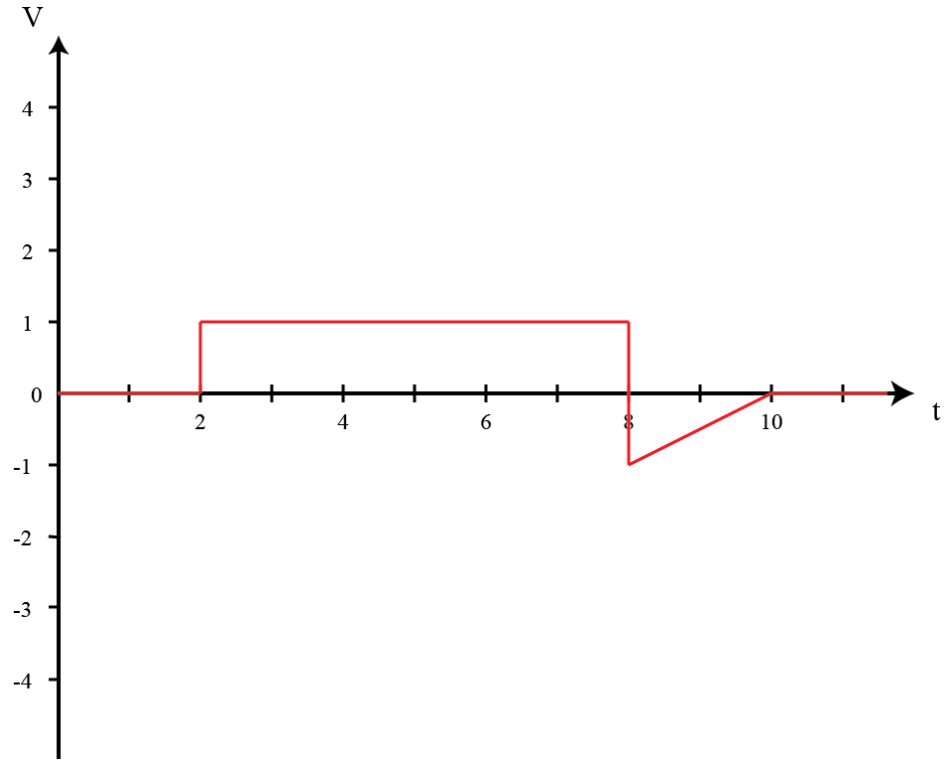
A + B



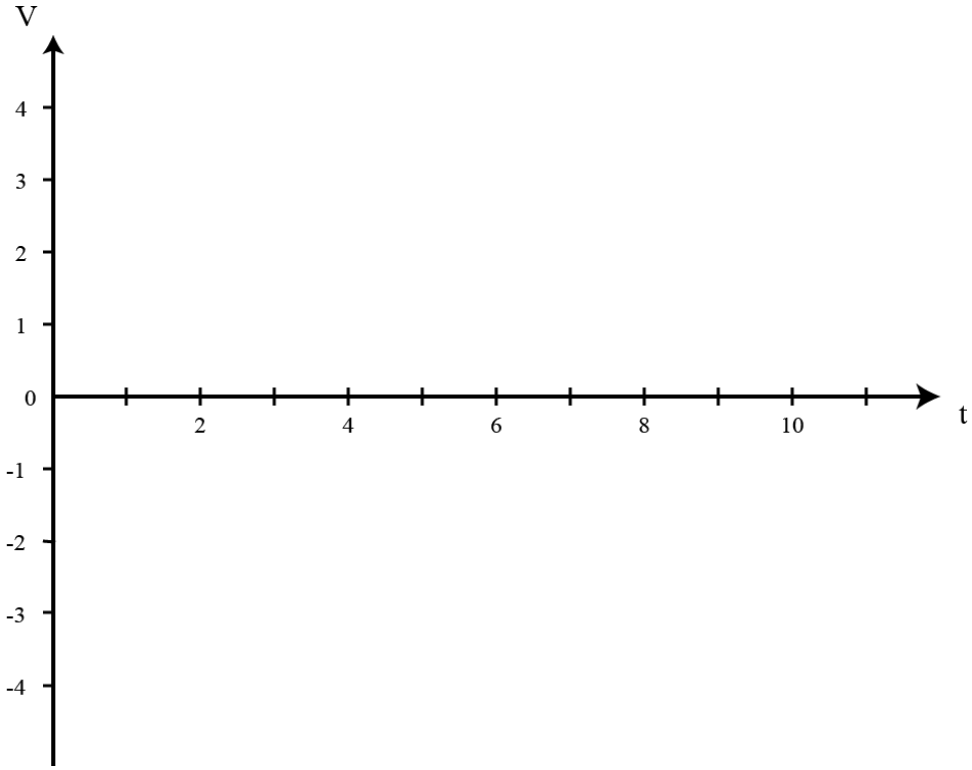
B + C



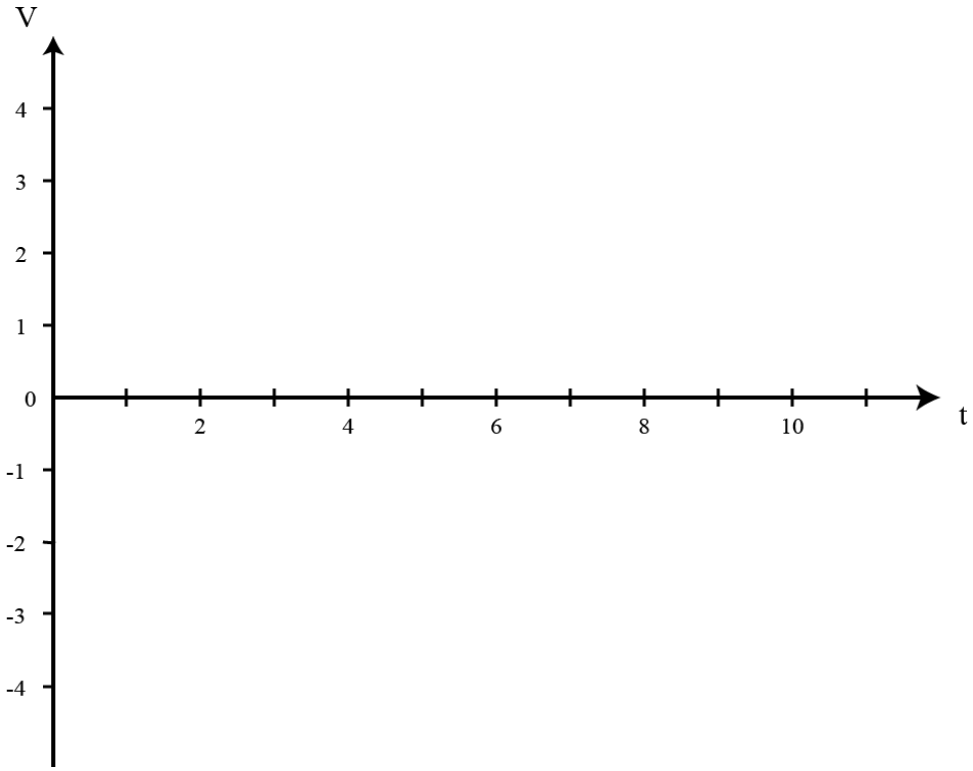
A + C



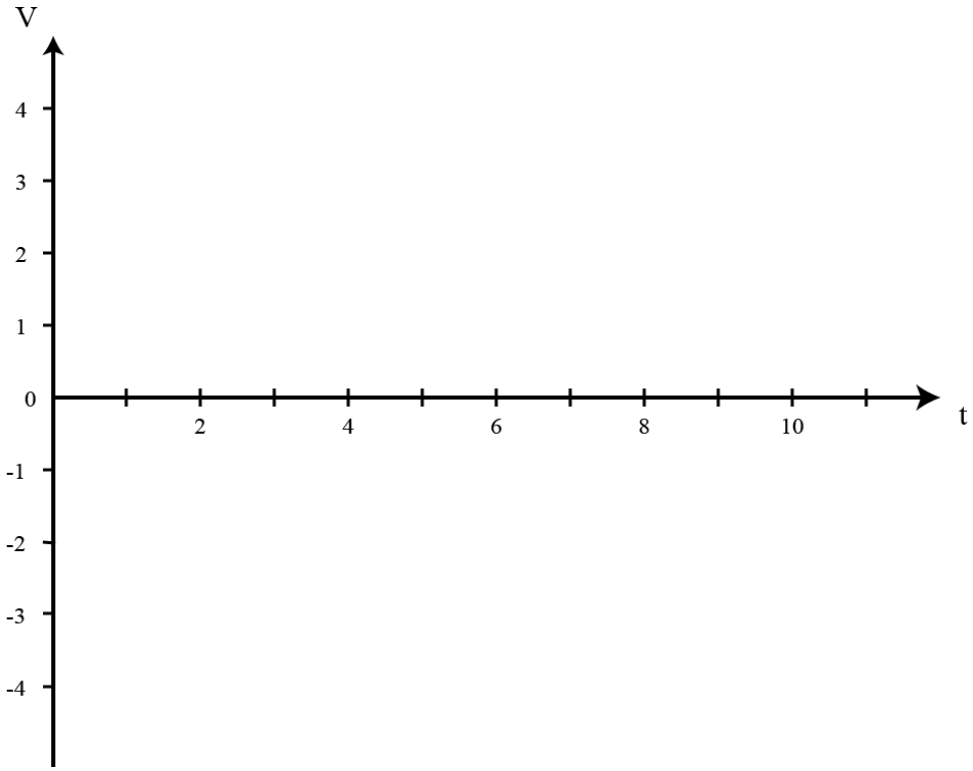
Pulse A



Pulse B

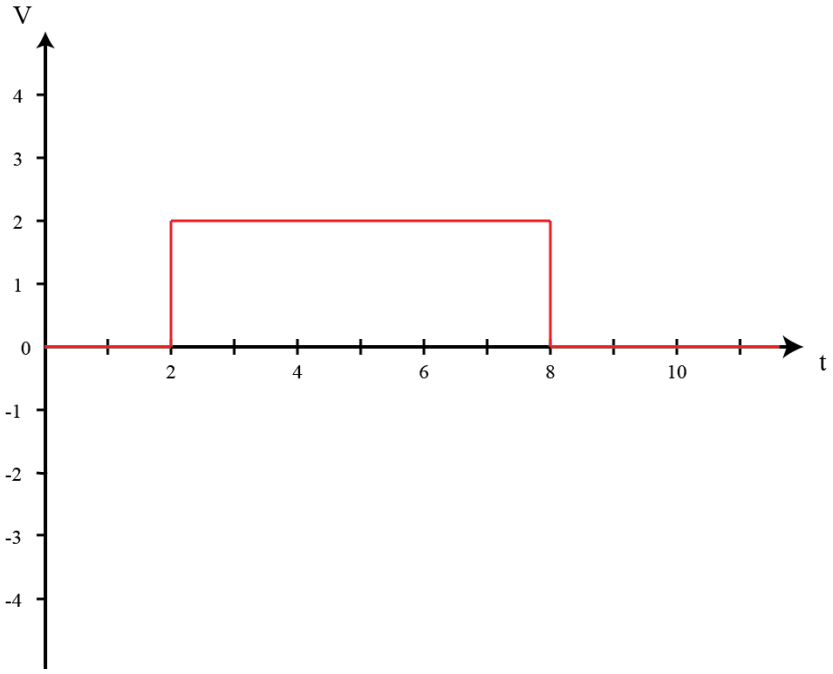


Pulse C



Activity 1: Answer Key – Tetrodotoxin

Pulse A



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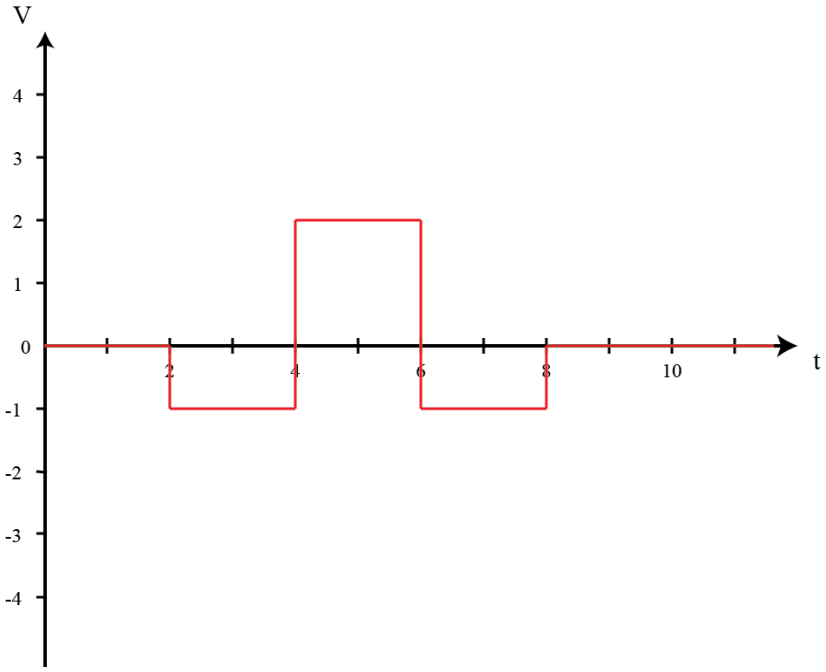
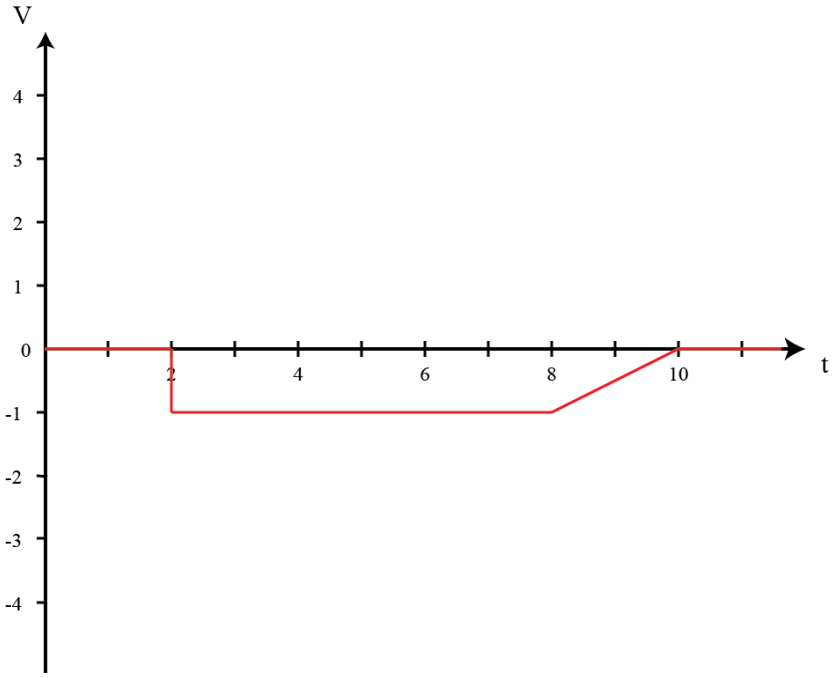


Pulse C



Activity 4: Background Knowledge – Epibatidine

Receptors along the membranes of neurons are made up of proteins, assembled from the folding of monomers called amino acids (Figure 1). "R" represents a substituent group that varies depending on the specific amino acid.

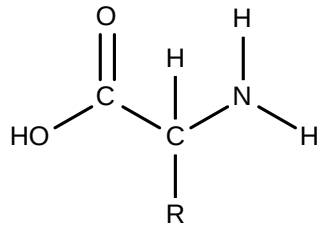


Figure 1. A typical amino acid

An important amino acid found in the Nicotinic Acetylcholine Receptor (nAChR) is tryptophan. As can be seen in Figure 2, this amino acid's R group is quite large, containing a fused ring structure. Aromatic rings are created from alternating single and double bonds in what is known as a conjugated π bond system. As a result, electrons in this system are delocalized (spread out within the system), producing a net negative charge on the overall "face" of the ring.

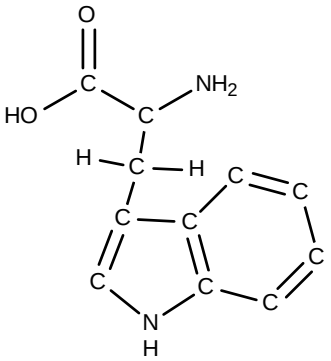


Figure 2: The structure of tryptophan. Note that for simplicity, the hydrogen atoms attached to carbon atoms on each of the rings are not shown.

As you have learned, epibatidine, nicotine, and acetylcholine are all able to bind to nAChRs. Each of these three molecules has a positive formal charge on a nitrogen atom (Figure 3). As a result, the nitrogen atom can be thought of as similar to a cation (positively charged ion). In turn, each compound can bind to tryptophan's aromatic rings via interactions known as **cation-π bonds**. In the three structures shown below, focus on the charged nitrogen atom, rather than the entire compound as a whole. Note the presence of hydrogen atoms in epibatidine and nicotine in polar N-H bonds that make it possible for hydrogen bonds to form at receptor sites.

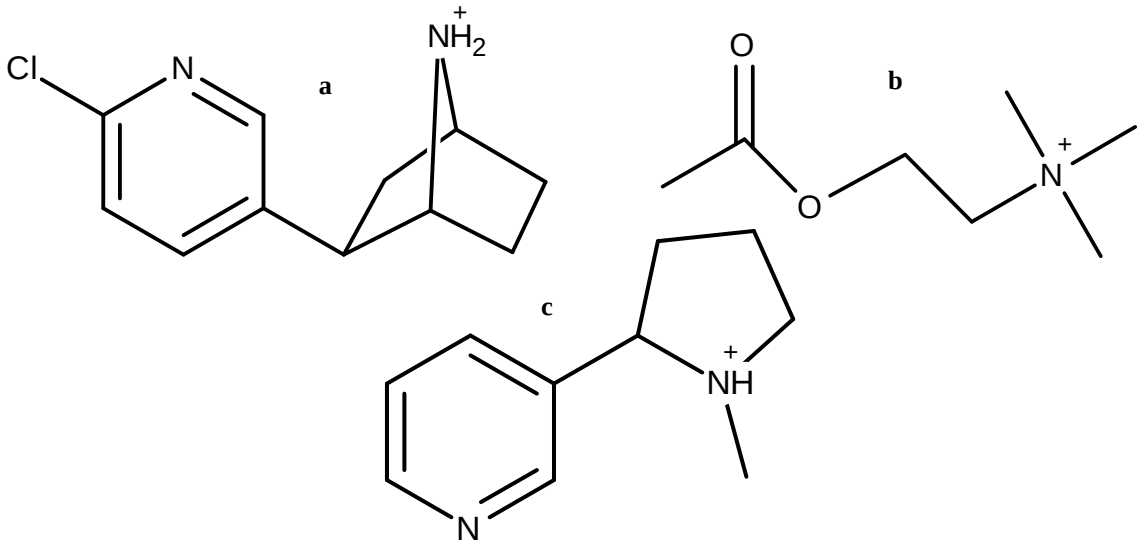


Figure 3. Structures of a) epibatidine, b) acetylcholine, and d) nicotine.

Together, these interactions can be modelled using the analogue in Figure 4. Here nicotine binds with tryptophan (labelled as "Trp B") via a cation-π interaction from the positive nitrogen ion towards the negative face of an aromatic ring, seen in Figure 1. As well, a hydrogen bond forms between nicotine and tryptophan, at the site of the positive formal charge in nicotine.

This analogue takes place in an aqueous environment, in the presence of other amino acids (e.g. Asn, and Leu). Hydrogen bonds therefore form between these amino acids and water molecules.

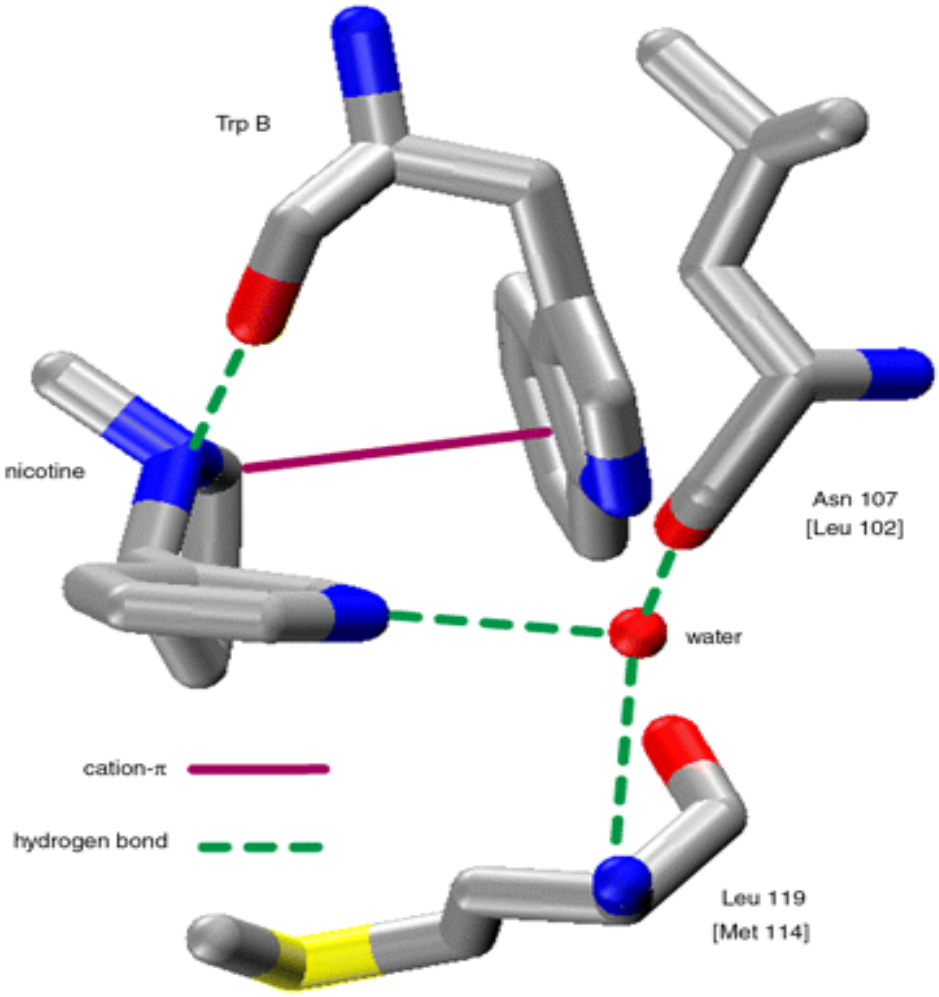


Figure 4. A visualization of cation-π interactions and hydrogen bonds. (Blum et al., 2010).

In this activity, you will be modeling the **cation-π interactions** and **hydrogen bonding** using simple tools: magnets, a plate, and rulers. Think carefully about each step of the procedure as you perform each step and how each part of the procedure demonstrates the bonds that are may be present between the possible ligands (epibatidine, acetylcholine, and nicotine) and nAChRs.

Activity 4: Post-Activity Questions – Epibatidine

1. Why did you place the two magnets in the centre of the plate and ruler? What type of interaction are they used to represent and why did you not move them when reconfiguring the system?

2. What do the single magnets on the outside of the plate and ruler represent? What type of interactions are the single magnets used to represent in comparison to the magnets in the centre of the ruler and ring?

3. What trends did you see when you were trying to separate the magnets? What was the purpose of applying this force on the magnet?

4. Describe the optimal conditions that you designed in Part 3 to ensure the most stable bond formed. How does this relate to the importance of specific ligand-receptor interactions?

Activity 4: Post-Activity Questions Answer Key – Epibatidine

1. Why did you place the two magnets in the centre of the plate and ruler? What type of interaction are they used to represent and why did you not move them in the ring when reconfiguring the system?

Sample Answer: Two magnets were placed in the centre of the plate to represent strong **cation-π interactions.** They were not moved within the ring when reconfiguring the system as the charge within an aromatic ring should remain only within the entire "face" of the ring (i.e. moving the magnet within the ring could extend the "negative face" to outside of the closed, conjugated π system).

2. What do the single magnets on the outside of the plate and ruler represent? What type of interactions are the single magnets used to represent in comparison to the magnets in the centre of the ruler and ring?

Sample Answer: The single magnets on the outside of the plate and ruler represent possible locations for hydrogen bonding. Hydrogen bonds tend to be weaker intermolecular forces than **cation-π** interactions. Students were able to move the hydrogen bonds in order to observe the importance of position of atoms in intermolecular bonding.

3. What trends did you see when you were trying to separate the magnets? What was the purpose of applying this force on the magnet?

Sample Answer: The more connections the magnets had, the more difficult it tended to be to separate the magnets. Applying the force on the magnet represents different intermolecular forces acting on the ligand and receptor; the more tightly bound the ligand to the receptor, the more stable the complex becomes (typically described in terms of great affinity that the ligand has for the receptor)

4. Describe the optimal conditions that you designed in Part 3 to ensure the most stable bond formed. How does this relate to the importance of specific ligand-receptor interactions?

Sample Answer: The most stable conformation produced had all magnets on the ruler lined up with the magnets on the plate. This idea suggests that the ability of ligands such as epibatidine to bind so strongly with the nAChR receptor depends on specific positions of atoms that are able to exploit intermolecular forces (cation-π bonds, hydrogen bonding, etc.) effectively.

PART D - BACKGROUND KNOWLEDGE

(4833 words)

General Knowledge

The nervous system

All animals have a nervous system that allows them to respond efficiently to stimuli. This system serves as a mode of communication in the body, and uses electrical and chemical signaling to respond to sensory information. Such signals are relayed by neurons, the basic units of the nervous system. While neurons can vary greatly in morphology, depending on the type of nervous tissue within which they are found, all neurons have common specialized features.

Neurons and Synapses

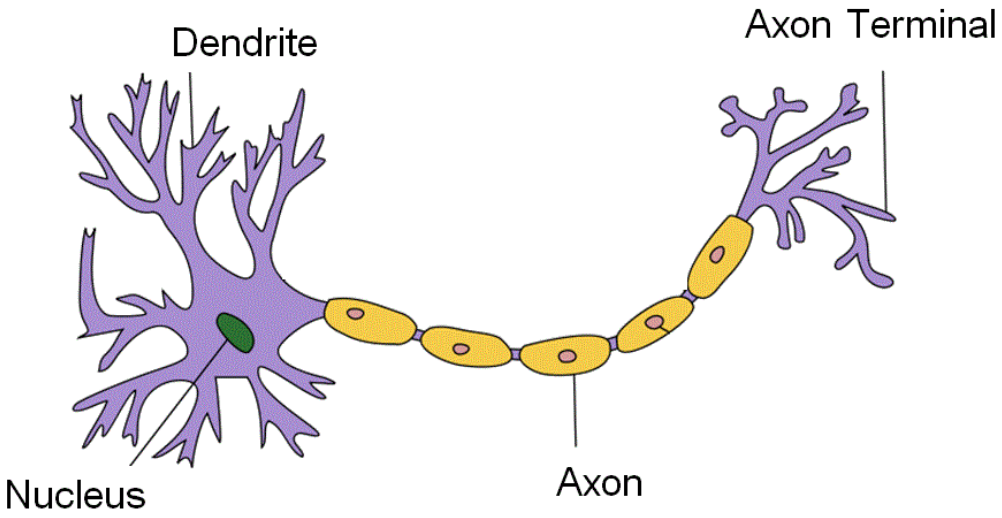


Figure 1. Diagram of a neuron. It has extensive branching in the axon terminal and the dendrites can receive inputs from many sources (Nottingham Science Blog, 2012).

The neuron (Figure 1) is a specialized cell that can receive input from one or more sources and transmit the signal to neurons and other cells. Each neuron has dendrites, which receive incoming signals, an axon, which sends outgoing signals, as well as a cell body, or soma, which performs other cell functions like maintenance. Different types of neurons have different degrees of branching in both the axon and the dendrite.

The synapse is the space where two cells in the nervous system meet, and is the site of interneuron communication. There are two types of synapses in the nervous system: electrical and chemical. Electrical synapses exist where the electrical signal passes directly from one neuron to another, and are known as gap junctions. In chemical synapses, an electrical impulse down the axon, or action potential, triggers the release of chemicals from the transmitting, or presynaptic neuron into the synaptic cleft. This causes a response in the receiving, or postsynaptic neuron. The arrival of an action potential at the axon terminal causes neurotransmitter release into the synaptic cleft via synaptic vesicle exocytosis. The neurotransmitters then diffuse across the cleft and induce a response in the postsynaptic neuron.

Active and passive transport

The membrane potential of the neuron is defined as the difference in electric potential between the interior and exterior of the neuron membrane. The membrane potential of a neuron is a consequence of differing ion concentrations on either side of the membrane. This is a result of unequal, selective permeability of the membrane to ions. The movement of ions across the membrane, and consequently the membrane potential, is maintained by transmembrane proteins. These transport proteins are classified as either passive or active. Passive transport occurs through membrane channels and occurs along the electrochemical gradient of the substance. In contrast, active transport proteins, such as pumps and transporters, move various substances against the electrochemical gradient.

In neurons, the resting membrane potential is primarily maintained by active sodium-potassium-ATPase (or Na-K-ATPase) ion pumps. Na-K-ATPase uses ATP to pump out three sodium ions for every two potassium ions it pumps in, creating a net difference in charge between the outside and inside of the cell and establishing an overall negative membrane potential.

Passive transport channels can be open and closed at different times, depending on changes in the cellular environment. For example, many ion channels are voltage-gated, meaning that they open when a certain membrane potential is reached. Other channels are ligand-gated, opened by a specific molecule binding to the channel or associated proteins. In some cases, the binding of the ligand to a channel directly causes a conformational change in the channel, meaning that the physical properties of the channel are modified such that specific molecules are allowed to pass through. In some other cases, a ligand-receptor interaction may transduce a pathway that indirectly causes activation of a channel. Channels can also be active or inactive based on other stimuli, such as temperature and stretching.

Overview of neurotoxins

Neurotoxins are historically defined as substances capable of damaging or weakening nervous tissue function after their introduction into the body (Kellaway, 1939). Neurotoxins come from a variety of different biotic and abiotic sources. Many notorious deadly poisons and venoms in nature are neurotoxins, for example the venoms of poison dart frogs and black widow spiders. However, despite their deadliness, neurotoxins have also been essential for the purposes of the discovery of many components of the nervous system.

In this unit, students will explore four different neurotoxins (tetrodotoxin, botulinum, α-latrotoxin, and epibatidine) designed to outline aspects of the nervous system like action potentials, neural signalling, neurotransmitter release and binding, and receptor agonists. The sections below will provide an overview of the effects, mechanisms and importance of each neurotoxin on the nervous system.

Tetrodotoxin and the discovery of the action potential

History

Tetrodotoxin (TTX) is a potent neurotoxin that is most notably found in pufferfish. Poisoning most commonly occurs in Japan, where ingestion of the puffer as sashimi is considered a delicacy. From 1987 to 1996, there were 488 cases of tetrodotoxin poisoning in Japan, resulting in 32 deaths (Noguchi and Ebesu, 2001). Symptoms of poisoning include paresthesias, weakness, and paralysis, followed by hypotension and central nervous system dysfunction. Death is usually caused by respiratory muscle paralysis, which eventually leads to respiratory failure. There is currently no known antidote or antitoxin. Treatment, therefore, generally attempts to alleviate the toxic effects of the neurotoxin, such as the administration of atropine to increase heart rate and raise blood pressure (Noguchi and Ebesu, 2001; Sims and Ostman, 1986).

This neurotoxin has historically been of particular importance to neuroscientists. In the 1960s, there was abundant research into the mode of action of tetrodotoxin, and how it affects the propagation of the action potential down nerves (Narahashi et al., 1964). Tetrodotoxin is among the most studied neurotoxins, and its effects have helped scientists understand how the nervous system works. Tetrodotoxin is highly specific in its mode of action, which makes it an extremely powerful tool for determining certain properties of the nervous system.

Origin and occurrence in nature

In several species of pufferfish, tetrodotoxin is found in multiple areas of the body, including the skin, intestine, and muscle, but is most concentrated in the liver and ovary (Narahashi, 2008). However, while tetrodotoxin is best known for being found in pufferfish, it occurs in several other marine species, including, but not limited to, salamanders, starfish, several worms, goby fish, horseshoe crabs, and blue-ringed octopi (Miyazawa and Noguchi, 2001). Its main purpose is chemical defense against predators.

Several mechanisms have been proposed to explain the occurrence of tetrodotoxin in animals. Since tetrodotoxin has been found in such a wide variety of species, it has been suggested that tetrodotoxin could be produced by bacterial symbionts and not by the animals themselves. Tetrodotoxin is generally found within a specific host organ, and it is hypothesized that symbiotic bacteria may benefit from the **secondary metabolites** produced in these organs. This is further supported by the existence of tetrodotoxin-producing bacteria, which have been isolated from many animals, including pufferfish (Chau et al., 2011). However, these experiments have not been performed in all animals containing tetrodotoxin, and thus there is still some uncertainty as to the accuracy of bacterial symbiosis as the origin of the toxin.

It has been proposed, instead, that bioaccumulation is the primary reason for occurrence in some animals. At the lowest level, there are tetrodotoxin-producing bacteria, and the toxin is magnified as it is consumed along higher trophic levels. This is supported by the possibility of non-toxic pufferfish, when grown in captivity with a non-toxic diet (Noguchi et al., 2006).

The action potential

The toxicity of tetrodotoxin comes from its interference with the **action potential**, which is a sequence of events that results in the **depolarization** of the membrane, and is the mechanism by which a signal is sent down the axon of a neuron. The action potential happens in several distinct stages:

1. The membrane is depolarized to a “threshold potential” which triggers the rest of the action potential.
2. At the **threshold potential**, voltage-gated sodium channels open to allow an influx of sodium ions into the cell. Membrane potential increases.

3. Within milliseconds, the sodium channels close, and voltage gated potassium channels open to facilitate an efflux of potassium. Membrane potential decreases.
4. Membrane potential **hyperpolarizes** past resting potential, and potassium channels close. Active transport mechanisms re-establish resting potential.

The voltage-gated sodium channel

The sodium channel, like most **transmembrane proteins**, is made up of several different subunits. The α-subunit is the primary functional unit (Marban et al., 1998), and consists of four homologous domains, each with six transmembrane segments (figure 2). The channel opens by changing conformation in response to depolarization of the membrane and allows the flow of ions through the pore. A "hinged lid" mechanism inactivates the channel milliseconds after opening (Yu and Caterall, 2003).

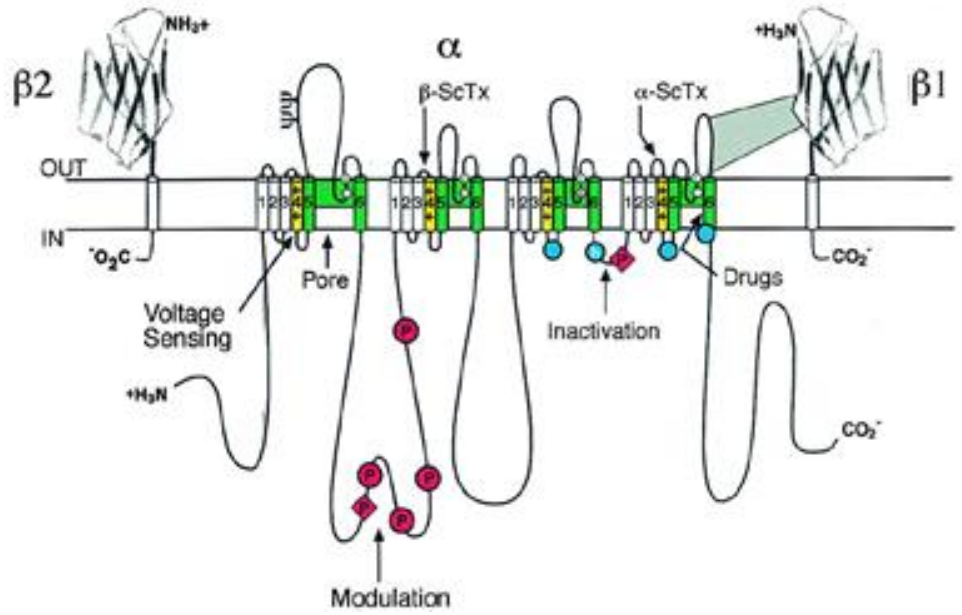


Figure 2. A schematic of the α-subunit of the voltage-gated sodium channel. The S4 segments (yellow) in each domain contain charged amino acid residues which move in response to depolarization, causing a conformational change in the S5-S6 segment pore region (green) to allow the flow of ions (Caterall, 2000). The intracellular loop between domains III and IV folds under the pore to close the channel (Yu and Caterall, 2003).

Interaction of tetrodotoxin with the sodium channel

Tetrodotoxin (Figure 3) acts from the extracellular side of the membrane, and binds to the region between the S5 and S6 segments, which constitute the ion selectivity filter and pore lining (Cestèle and Caterall, 2001; Caterall, 2000). When bound to the channel, it effectively acts as a cork in a bottle and restricts the passing of other ions.

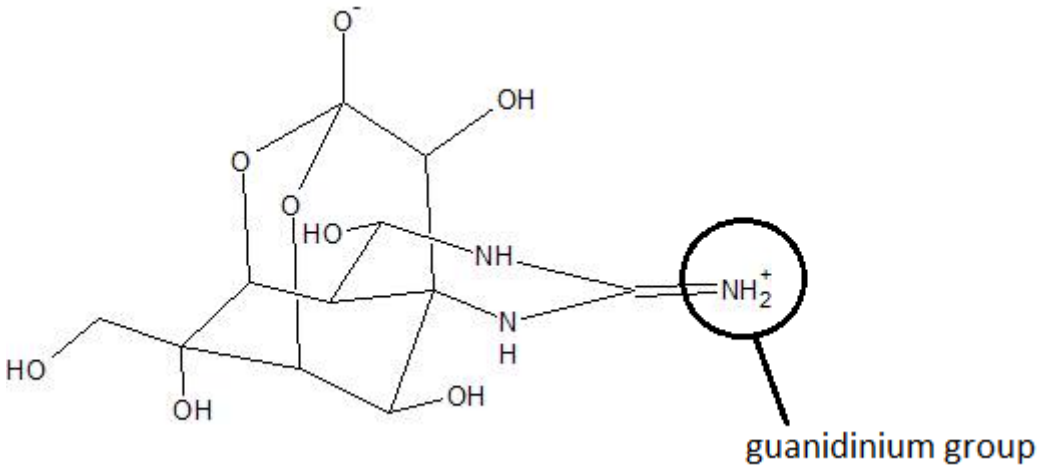


Figure 3. The chemical structure of tetrodotoxin. It has been shown that the guanidinium ion can act as a substitute for sodium ions. Therefore, the presence of a guanidinium group allows the toxin to "infiltrate" the pore and bind to the selectivity filter (Larramende et al., 1956; Hille, 1971; Kao and Nishiyama, 1965; Fozzard and Lipkind, 2010).

The subtypes of sodium channels that are present in tetrodotoxin-containing species are more resistant to tetrodotoxin. Depending on the animal and tissue, several amino acid substitutions are made in several locations to decrease **binding affinity** to tetrodotoxin. For example, in pufferfish, a non-aromatic amino acid substitutes an aromatic amino acid in the channel's binding domain for tetrodotoxin, while in newt-eating garter snakes, there are different, novel amino acid substitutions that increase tetrodotoxin resistance, which are proposed to have been coevolved with the production of tetrodotoxin as a defense mechanism in newts (Noguchi and Arakawa, 2008; Geffeney et al., 2005). Subtypes of sodium channels expressed in certain mammalian tissues, such as cardiac muscles and parts of the **peripheral nervous system**, are also tetrodotoxin-resistant (Goldin, 2001).

Key experiments using tetrodotoxin

In an experiment by Sretavan, Shatz, and Stryker (1988), tetrodotoxin was used to inhibit action potential activity in the visual pathway of developing cats in the fetal stage. It was found that in these cats, there was abnormal neuron **arborization** branching in the **thalamus,** which is part of the pathway for visual processing. This study demonstrated that proper development in this area depends on action potential activity.

A landmark study conducted by Narahashi, Moore, and Scott (1964) used tetrodotoxin in lobster axons to selectively block the sodium-carrying current while artificially inducing depolarization. This left the potassium-carrying current intact, while blocking the sodium current, thereby revealing the ions responsible for different parts of the action potential.

Finally, in another classical experiment conducted by Katz and Miledi (1967), tetrodotoxin and **tetraethylammonium** (which blocks potassium channels) were used to block the flow of sodium and potassium ions in the presynaptic membrane. The presynaptic membrane was then depolarized artificially, and a response was seen in the postsynaptic membrane. This experiment showed that the release of neurotransmitters was related to the membrane potential, but independent of sodium and potassium currents.

α-Latrotoxin: an illustrator of calcium's role at the synapse

Background

Latrotoxins are potent neurotoxins that are produced by spiders of the genus *Lactrodectus*. Most *Lactrodectus* venoms are effective against invertebrates, such as crustaceans and insects, but the venom of some species can also paralyse vertebrates (Deák et al., 2009). α-latrotoxin, often referred to as α-LTx, is found in the venom of the black widow spider (*Lactrodectus mactans*) and is one such vertebrate-effective neurotoxin (Sudhöf, 2001). Black widow spiders are highly venomous and easily recognizable by their distinct black and red colouring (Heller, 2013). Their bites are seldom fatal to healthy humans, but can kill children and older adults. They are found in southern and western regions of the United States, typically in barns and other outdoor structures in the south-western United States. Intoxication of the spider's toxin is commonly characterized by extreme muscle pains, and death is usually preceded by seizures (Heller, 2013).

α-Latrotoxin was first seen to have blocking effects in frog neuromuscular junction transmission by Longenecker *et al.* in 1970. Once isolated, α-latrotoxin became an important tool for studying the role of calcium ions in **synaptic vesicle** release at the **synapse** (Meldolesi, 1984).

Synaptic vesicle release

α-Latrotoxin acts by disrupting the natural process of interneuron chemical signal transmission. In a normal neuron, a signal is transmitted from the axon terminal of one neuron to the dendrite of another through the **exocytosis** of a synaptic vesicle. These vesicles, containing **neurotransmitters**, fuse with the presynaptic membrane. This causes neurotransmitters to diffuse into the synapse and be received by the postsynaptic dendrite. Usually, neurotransmitter release is caused by an action potential flooding the axon terminal of an axon, causing a short, synchronous burst of neurotransmitter release through exocytosis, which is then followed by neurotransmitter releases that are smaller in magnitude and less frequent (Neher and Sakaba, 2008).

This action potential-evoked neurotransmitter release is caused by the rush of calcium ions (Ca^{2+}) into the axon terminal of the presynaptic neuron (Südhof, 2012). When the action potential **depolarizes** the membrane, **voltage-gated** Ca^{2+} channels in the neuron membrane open (Catterall, 2011). This occurs because the generated electric field causes a segment of the **protein domain** to move outward and rotate, which initiates a conformational change, opening a pore in the protein. A loop of the pore outside of the membrane contains Ca^{2+}-selective glutamate residues, allowing the ion to enter when the channel is open (Catterall, 2011). The influx of Ca^{2+} is detected and mediated by the protein sensor synaptotagmin (Syt) and its **cofactor** complexin (Südhof, 2012).

Syt is found on the presynaptic axon membrane and binds calcium-dependently to phospholipids and SNARE proteins on synaptic vesicles (Südhof, 2012). Ca^{2+} binding to Syt1 (the most common type of synaptotagmin, found in most neurons) triggers fast neurotransmitter release by inducing Syt1 to interact with SNARE complexes and with phospholipids on the synaptic vesicle membrane simultaneously. This event is facilitated by the cofactor complexin, which binds to a **SNARE complex** on the synaptic vesicle membrane and activates the protein complex. Syt1 and complexin, once bound to the synaptic vesicle's proteins and phospholipids, open a fusion pore to the phospholipid bilayer of the presynaptic axon membrane. This causes the vesicle to bind to the membrane and exocytosis follows, diffusing neurotransmitters into the synaptic cleft (Südhof, 2012).

Mechanism of action

α-Latrotoxin can trigger synaptic vesicle exocytosis in the absence of an action potential, and in fact, independently of extracellular calcium (Henkel and Sankaranarayanan, 1999). At high concentrations of toxin in the cell, α-latrotoxin inserts itself directly into the presynaptic membrane (Ushkaryov, Volynski and Ashton, 2004). Though **hydrophilic** overall, the neurotoxin is amphipathic, meaning that a part of the peptide can bind to **hydrophobic** surfaces. It is a **tetramer** and has a central channel, which, when incorporated into the presynaptic axon membrane, allows substances to pass through it, including Ca^{2+}. An unregulated influx of Ca^{2+} into the axon causes massive exocytosis of synaptic vesicles through the Syt1-mediated mechanism described above (Figure 4). However, direct insertion of the toxin protein into the membrane, without receptor assistance, can only occur when α-latrotoxin is present at a concentration that exceeds its normal **active concentration** by at least two orders of magnitude (Ushkaryov, Volynski and Ashton, 2004).

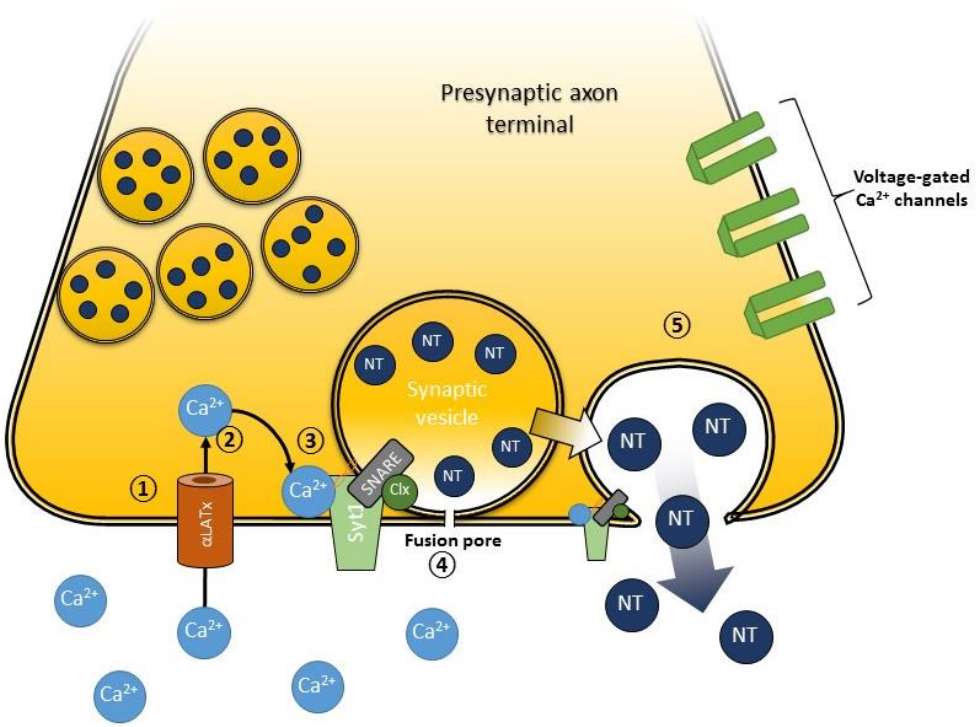


Figure 4. The calcium-dependent, high [α-LTx] mechanism of action. 1) α-LTx inserts itself into the presynaptic axon membrane, allowing 2) Ca^{2+} ions to diffuse into the axon. 3) Syt1 can then bind Ca^{2+}- and complexin-dependently to SNARE proteins and phospholipids in the synaptic vesicle membrane. 4) This complex then forms a fusion pore between the axon membrane and the vesicle membrane, resulting in the 5) exocytosis of the synaptic vesicle and diffusion of neurotransmitters into the synaptic cleft.

At lower concentrations of α-LTx, insertion into the membrane is mediated by **integral** neural membrane proteins, and can occur whether extracellular calcium ions are present (calcium-dependent insertion) or not (calcium-independent insertion). **Classical synapses**, which have pre-docked vesicles containing neurotransmitters such as glutamate, GABA, and acetylcholine, do not require a calcium ion influx for massive exocytosis via α-LTx (Sudhöf, 2001). However, non-classical synapses, which do not have pre-docked vesicles, require calcium ions for α-LTx insertion to occur (Sudhöf, 2001). In this case, α-LTx binds to neurexin-1α, a protein receptor for the toxin, and forms a pore, in the presence of Ca^{2+} (Ushkaryov, Volynski and Ashton, 2004). α-LTx-activated neurexin-1α modulates exocytosis by binding to Syt1, which then causes vesicle exocytosis (Henkel and Sankaranarayanan, 1999). Furthermore,

activated neurexin-1α also interacts with neurexophilins and CASK proteins, both of which are proteins involved in the neurotransmitter **secretory pathway**.

In the extracellular calcium-independent pathway, α-LTx has been shown to bind to latrophilin/CIRL (LPH), a latrotoxin-specific protein receptor, which has phospholipase C (PLC) as a downstream effector (Deák et al., 2009). PLC activates a **phosphorylation cascade** which releases Ca^{2+} from intracellular stores, which triggers vesicle exocytosis through the regular neurotransmitter release mechanism (Henkel and Sankaranarayanan, 1999). This mechanism has been studied through a mutated form of α-LTx, α-LTx^{N4C}, which is unable to form pores, but still causes neurotransmitter release (Deák et al., 2009).

The mass exocytosis of synaptic vesicles caused by α-latrotoxin eventually results in extensive **morphological changes** in the axon terminal, including disintegration of the plasma membrane and significant drops in ATP levels, which often results in neuronal cell death (Silva, Suckling and Ushkaryov, 2009).

Neurotoxic effects of botulinum on the neuromuscular system

Background

Botulinum is a naturally occurring neurotoxin which is secreted by the bacteria ***Clostridium botulinum***. The bacteria produces eight known antigenetically unique variations of the toxin (A, B, C1, C2, D, E, F and G) (Shukla and Sharma, 2005). Which toxin variant is produced depends on the strain of bacteria producing it. Different strains of *C. botulinum* are related to different environmental settings and their associated toxins have varying effects on human health (Peck, 2009). For example, botulinum toxins A, B and E are found in foodborne bacterial strains and can cause **botulism**. The toxin is one of the deadliest substances ever studied, with one gram being enough to kill one million adult humans (Rao, Starr, Morris and Lin, 2007). Death typically occurs due to the paralytic effects of the neurotoxin on respiratory organs. All types of the botulinum toxin act through signal interference at the neuromuscular junction. This causes paralysis, as muscles are unable to receive signals from the brain.

Physiology of the Neuromuscular Junction

The neuromuscular junction contains two principal components: the **motor neuron** and the **muscular fiber**, both of which are highly specialized cells (Sanes, 1999). Neuromuscular junctions begin as the connection of a neuron with a muscular fiber. As the connection is established, the neuron and muscular fiber undergo morphological changes so as facilitate a dynamic and functional synapse (Hall, 1993). Within the neuromuscular junction, the muscular tissue (also known as the sarcolemma) is repeatedly folded in order to maximize the amount of surface area exposed to the synaptic cleft (Figure 5) (Sine, 2012). The sarcolemma also contains an extremely high density of **acetylcholine receptors** (AChRs), which maximize neurotransmitter receptivity (Dennis, 1981).

In the motor neuron, production of **acetylcholine** is rapidly increased through enzyme-catalyzed synthesis from choline and acetyl coenzyme A (acetyl-CoA) within the mitochondria of the cell (Taylor and Brown, 1999). Furthermore, the synthesis and storage of acetylcholine is highly localised to the distal **axonal terminal**, which lies directly above the synaptic cleft. High concentrations of acetylcholine in the presynaptic motor neuron and high density of acetylcholine receptors in the muscle fiber ensure a highly sensitive and sustainable connection (Sanes and Lichtman, 1999).

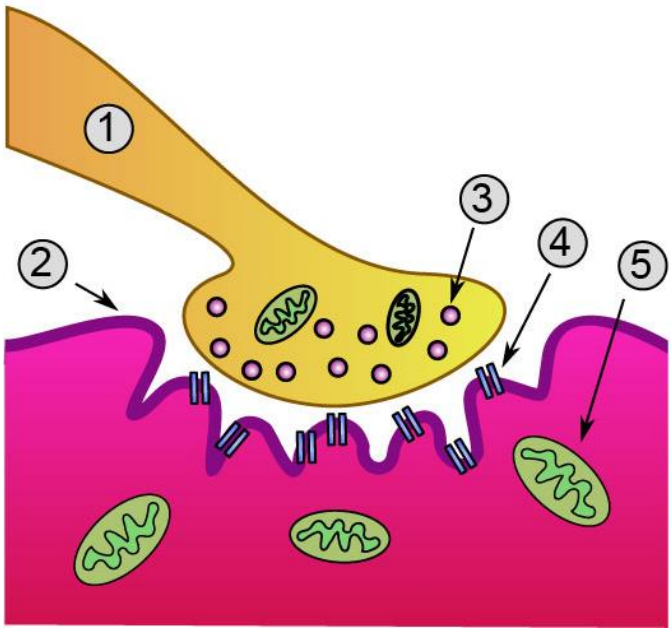


Figure 5. A visual representation of the neuromuscular junction. 1) Motor neuron; 2) Sarcolemma of muscle fiber; 3) Acetylcholine vesicles in the axonal terminal; 4) Acetylcholine receptors; 5) Muscular mitochondria (Dake, 2005).

Signal transduction in the neuromuscular junction

As previously explained, the influx of Ca^{2+} from ions allows for **exocytosis**, where the acetylcholine vesicle fuses to the presynaptic membrane and diffuses into the synaptic cleft. The vesicle is able to fuse with the membrane partially as a result of Synaptosomal-Associated Protein 25 (**SNAP-25)**, which is found in the presynaptic membrane. SNAP-25 acts as a target-SNARE component in the trans-SNARE complex responsible for vesicle-membrane fusion (Hall and Sanes, 1993). After having diffused into the synaptic cleft, acetylcholine is taken up by its respective ligand-gated receptors. The acetylcholine binds to a subunit of the receptor and causes it to undergo a conformational change. This conformational change allows for a second subunit to increase its affinity for acetylcholine (Sine, 2012). This cooperative bonding of two acetylcholine molecules for every receptor allow for a sustained postsynaptic response, which is essential for effective dexterity within the muscular system. As such, botulinum acts primarily through interference with the synaptosomal associated protein 25 (SNAP-25) (Blasi et al., 1993).

Botulinum (Type A) in the Neuromuscular Junction

Botulinum's mechanism of action is explained in part by its basic chemistry. The toxin is a relatively heavy molecule, weighing approximately 150 kDa. The molecule is comprised of one "heavy" and one "light" polypeptide chain connected by a disulfide bond. (Nigam and Nigam, 2010). These two components work in tandem to infiltrate the presynaptic cell. The heavy chain binds to high-affinity receptors at the presynaptic site, after which the disulphide bond between the chains is cleaved and the light chain enters the cytoplasm by **endocytosis.** After entering the cell, the light chain cleaves SNAP-25 (Nigam and Nigam, 2010). The destruction of the protein is carried out through **proteolysis**. This effectively disrupts the ability of the cell to release acetylcholine, due to the fact that acetylcholine-containing vesicles can no longer fuse with the presynaptic membrane to undergo exocytosis. After having been introduced to the neuromuscular junction, maximum paralysis typically occurs within 4-6 days and can last up to 10-12 weeks (Sakaguchi, 1982).

The distinct types of botulinum toxin (A, B, C1, C2, D, E, F and G) differ primarily in the sequencing of their amino acids which form the peptides in the chain, which determines its selectivity for different proteins in the presynaptic cell (Satterfield et al., 2010). For example types B, D, and F will affect the vesicle associated membrane protein (VAMP) which is found on the vesicle membrane, instead of the presynaptic neuron membrane. However, botulinum toxin type A is the most pervasive and well-studied type of botulinum toxin, as well as used most frequently in clinical settings.

Clinical Applications

Although the botulinum toxin is highly deadly, it also has valuable clinical applications. Most notably, botulinum is the active ingredient in the **BOTOX** drug. The use of botulinum in a clinical setting originated in the late 1970s, when scientists saw its potential for treating disorders caused by muscle overactivity, such as strabismus (a disorder which causes over activity in the eye muscles) (Scott, 1980). It was discovered that the highly localised injection of a few picograms of the toxin effectively prevented spastic muscle movement for extended periods of time with little to no side effects. Soon, other muscular disorders such as dystonia (sustained muscle spasms) and spasmodic torticollis (involuntary spasms of the neck) were treated with the type A botulinum toxin (Hallett, 1999). Furthermore, cosmetic applications of the botulinum toxin target wrinkles by creating partial paralysis in small muscle fibers in the face.

Most clinical applications of botulinum only result in partial paralysis. Since every muscle fiber in the body is controlled by multiple neuromuscular junctions (i.e. multiple motor neurons), a small dose of the toxin inhibits enough of the junctions to weaken the muscles' ability to respond to a stimulus, but not completely inhibit voluntary control (Pellizzari, Rossetto, Schiavo and Montecucco, 1999). The effects of inhibition are only temporary, however, since new junctions will form to replace the affected ones.

Epibatidine

History and discovery

In 1974, biologists John Daly and Charley W. Myers visited Southern Ecuador to study toxic compounds present within dendrobatid (poison dart) frogs (Daly et al., 2000). Daly and Myers analyzed frog skin extracts for the presence of alkaloids, a diverse group of nitrogen-containing compounds often used for defense against predation (Daly et al., 1978). After removing the skin tissue, epibatidine was isolated in the species now known as *E. anthonyi* (Daly et al., 1978; Tasso et al., 2013). Epibatidine is a powerful analgesic (pain reliever) as well as capable of producing severe rigidity in white mice tails (Daly et al., 1978), known as a **Straub Tail Response**. However, it is only present in small quantities and is therefore difficult to extract. As a result, nearly two decades passed before epibatidine's structure was elucidated via spectroscopic techniques (Figure x) (Spande et al., 1992).

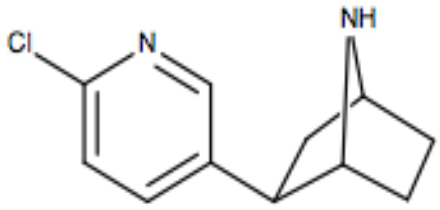


Figure 6. The chemical structure of epibatidine, determined by Spande et al. in 1992.

A new structure and proposed mechanism of action for epibatidine

It was first proposed that epibatidine acted similarly to opioids, since symptoms of intoxication such as delayed pain response and Straub Tail were similar to the responses of mice treated with morphine (Daly et al., 1978; Bilbey, Salem and Grossman, 1960). However, opioid antagonists were found to be ineffective in blocking the action of epibatidine, and a much lower dose of epibatidine was required to induce a similar response to morphine (Li et al., 1993; Spande et al. 1992; Sullivan et al. 1994). Further studies therefore suggested that epibatidine binds to and activates the nicotinic acetylcholine receptor (nAChR) (Sullivan et al., 1994; Badio and Daly, 1994).

Receptors and the nAChR

Normally, after a neurotransmitter is released from the presynaptic membrane and diffuses across the synaptic cleft, it binds to either of two different types of receptors lining the postsynaptic membrane: an **ionotropic receptor,** which is also an ion channel and therefore allows the passage of ions directly following the binding of a ligand; or a **metabotropic receptor** which, in response to a ligand, triggers a series of events that indirectly activates or inactivates a nearby ion channel (Nicholls et al., 2011).

nAChRs are pentameric ionotropic receptors that can be found at neuromuscular junctions as muscle-type receptors and throughout the rest of the nervous system as neuronal-type receptors (Millar and Harkness, 2008). These receptors are activated by the binding of the neurotransmitter acetylcholine, which causes conformational changes in the receptor, consequently allowing the inflow of sodium and potassium and depolarizing the postsynaptic membrane (Nicholls et al., 2012; Dani, 2001). nAChRs can also sometimes allow the inflow of calcium ions, and as they are sometimes present on some presynaptic membranes, the flow of calcium ions through open nAChRs can cause additional release of neurotransmitters (Shen and Yakel, 2009; Kalamida et al., 2007).

Agonists mimic the activity of acetylcholine by binding at the active site or at an allosteric site. Notably, nAChR's name stems from nicotine's ability to act as an agonist by binding to an allosteric site on the

receptor, producing an equivalent effect (Dani, 2001; Hansen et al., 2006). Confirming epibatidine's status as a nicotinic agonist was the observation that the nicotinic **antagonist** mecamylamine hindered epibatidine's ability to bind to nAChRs (Badio and Daly, 1994).

Binding to nAChRs

A comparison of acetylcholine, nicotine, and epibatidine in their protonated forms reveals that each compound contains a positive formal charge at a nitrogen atom (Figure 7).

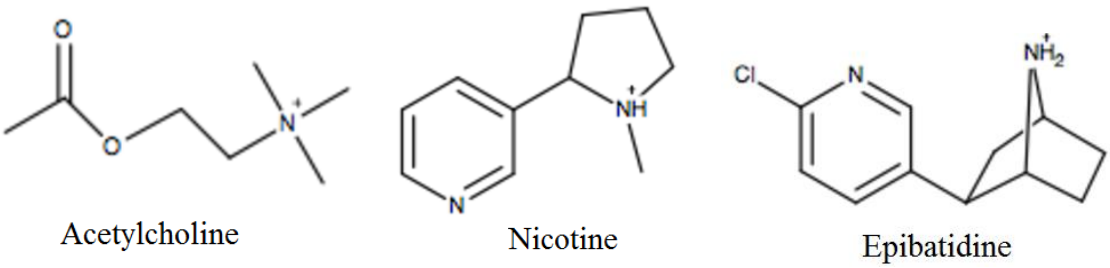


Figure 7. Protonated forms of acetylcholine, nicotine, and epibatidine. Notice the presence of a positive formal charge at a nitrogen atom, known as the cationic centre.

Acetylcholine, nicotine, and epibatidine are able to bind to activation sites on nAChRs as a result of **cation-π interactions**, which occur when cations are attracted to delocalized electrons in a conjugated π system (Xiu et al., 2009; Cashin et al., 2005). In acetylcholine, nicotine, and epibatidine, a quaternary ammonium compound acts as a cationic centre (Cashin et al., 2005). nAChRs are composed of several amino acids that contain substituent aromatic groups (e.g. tryptophan), which allow the positively charged ligand to interact with the negative "face" of the aromatic ring (Cashin et al., 2005). In nicotine and epibatidine, the presence of a hydrogen atom attached at the cationic centre (polar N-H bond) allows the formation of hydrogen bonds to the backbone carbonyl atom in amino acids (Xiu et al., 2009; Cashin et al., 2005). A summary of these interactions is shown in Figure 8.

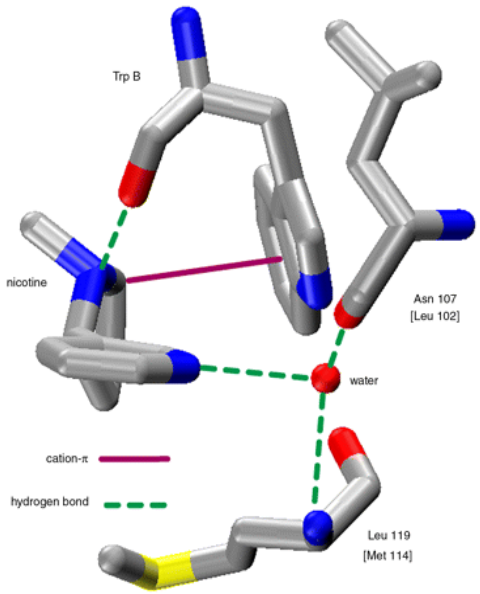


Figure 8. A simplification of the interactions taking place at the nAChR receptor by isolating a nicotine molecule and various amino acids in an aqueous environment. Here, nicotine is seen interacting with Trp B (the amino acid tryptophan) via a cation-π interaction (purple) and a hydrogen bond (green). Note that the hydrogen bond extends from the hydrogen atom attached to the cationic centre. As there are other amino acids and water molecules present, additional hydrogen bonds are present in this visualization (Blum, Lester, and Dougherty, 2010).

Epibatidine has a greater affinity for nAChRs than nicotine, yielding a high response even at low concentrations (Gerzanich et al., 1995). In muscular nAChRs, nicotine is unable to form cation-π interactions and strong hydrogen bonds, as its molecular shape prevents it from being able to approach the receptors as close as epibatidine (Cashin et al., 2005; Xiu et al., 2009). As cation-π interactions are present between nicotine and neuronal nAChRs, the difference in affinity at these nAChRs remains ongoing areas of research (Cashin et al., 2005).

Toxicity and physiological effects

The analgesic properties of epibatidine are believed to come from its strong affinity to two types of nAChRs found in the central nervous system (CNS), the α4β2 and α7 receptors (Abdrakhmanova et al., 2008; Cucchiaro et al., 2008). When activated, these receptors are known to induce pathways associated with pain relief, such as anti-inflammatory mechanisms (Wang, 2003; Hosur and Loring, 2011). However, epibatidine is toxic even in small concentrations, as it tends to be non-selective when binding to receptors. Instead of binding only to α4β2 and α7 receptors in the CNS in order to produce pain relief, epibatidine can also bind to peripheral nAChRs, such as the α3β4 receptor (Cucchiaro et al., 2008). This can result in harmful side effects, such as seizures, hypertension, and respiratory paralysis (Cucchiaro et al., 2008). Synthesizing analogues of epibatidine that can mimic the analgesic properties and prevent deadly side effects continue to be important areas of research (Abdrakhmanova et al., 2008; Cucchiaro et al., 2008).

Similarly to how tetrodotoxin was used to help determine the location of action potentials, epibatidine has also been used to analyze early visual development in mammals by blocking short bursts of action potentials, known as spontaneous retinal activity or retinal waves (Cang et al., 2005; Huberman, 2007). Normally, these waves are important for the creation of coherent circuitry in the retina in mammals prior to the development of sight (Mooney et al., 1996; Wong 1999). The waves are activated by different receptors during three distinct stages. The importance of epibatidine in visualizing these waves occurs during stage II (1-2 postnatal weeks in mice), when acetylcholine binds to nAChR receptors to induce action potentials. As a result, exposure to the potent agonist epibatidine desensitizes the receptors and makes it more difficult for acetylcholine to induce further action potentials (Huberman, 2007).

PART E - ANNOTATED READING LIST

The following are recommended readings for the instructor, organized by neurotoxin.

Tetrodotoxin

Geffeney, S.L., Fujioto, E., Brodie E.D., Brodie, E.D. Jr., and Ruben, P.C., 2005. Evolutionary diversification of TTX-resistant sodium channels in a predator-prey interaction. *Nature*, 434, pp.759-763.

- In this paper, the authors present findings of tetrodotoxin resistant sodium channels in garter snakes that prey on tetrodotoxin-producing newts. Perhaps the most interesting finding is that these are novel (and therefore recently evolved) mutations, rather than the previously known mutations found in tetrodotoxin-containing animals and mammalian cardiac tissue. This is a paradigm of natural selection, and elegantly brings together evolutionary, ecological and biochemical ideas of neurotoxins.

Narahashi, T., Moore, J.W., Scott, W.R., 1964. Tetrodotoxin Blockage of Sodium Conductance Increase in Lobster Giant Axons. *The Journal of General Physiology* 47, pp.965-974.

- This study by Narahashi et al. was highly influential and extremely important to our understanding of the action potential and the mechanism of action of tetrodotoxin. Using the voltage-clamp technique, which essentially artificially depolarizes the membrane, they measured the current across the membrane with and without tetrodotoxin, which conclusively confirmed that tetrodotoxin blocked the early sodium current without affecting the resting membrane potential and the late potassium current.

Yu, F.H., and Caterall, W.A., 2003. Overview of the voltage-gated sodium channel family. *Genome Biology*. 4(3), pp.207.1 – 207.7.

- Caterall is an influential researcher in the area of voltage-gated channels. This relatively concise review goes in depth on the structure of the sodium channel, and the understanding of the function and dynamics of the sodium channel. In addition, Yu and Caterall discuss the subtypes of sodium channels and briefly describes those subtypes resistant to tetrodotoxin.

α-latrotoxin

Longenecker, H.E., Hurlbut, W.P., Mauro, A., and Clark, A.W., 1970. Effects of black widow spider venom on the frog neuromuscular junction. Effects on end-plate potential, miniature end-plate potential and nerve terminal spike. *Nature*, 225(5234), pp.701–703.

- A landmark, classical study, Longenecker and colleagues were the first to describe the effects of black widow spider venom in the synapse. They discovered the revolutionary fact that black widow spider venom acts on the nerve terminal, even independently of the presence of extracellular calcium ions.

Silva, J.-P., Suckling, J., and Ushkaryov, Y., 2009. Penelope's web: using alpha-latrotoxin to untangle the mysteries of exocytosis. *Journal of neurochemistry*, 111(2), pp.275–90.

- This massive review outlines the role that α-latrotoxin has played in discovering the mechanisms of vesicle release at the synapse, including interactions with latrophilins, mechanisms of pore formation, and possible malfunctions in this machinery as a link to autism.

Südhof, T.C., 2012. Calcium control of neurotransmitter release. *Cold Spring Harbor perspectives in biology*, 4(1), p.a011353.

- This review summarizes current knowledge of neurotransmitter release exhaustively and succinctly. Südhof, a 2013 Nobel Prize winner in physiology and medicine for his work on synaptic signalling, clearly explains vesicle exocytosis as a result of calcium ion influx, in terms

of the protein pathways involved. An excellent example of impeccable scientific writing, it is an important read for those wishing to better understand signalling at the synapse.

Botulinum

Dennis, M.J., 1981. Development of the neuromuscular junction: inductive interactions between cells. *Annual Review of Neuroscience*, 4, pp.43–68.

- This journal entry in the Annual Review of Neuroscience provides a comprehensive overview of the physiology and basic chemistry which is involved in the neuromuscular junction. The article also looks at how neuromuscular junctions develop as we age and how neuromuscular junctions in various parts of the body will differ. Furthermore, the article brings in an evolutionary perspective by looking at nuances in neuromuscular junctions from an interspecies and intraspecies point of view.

Hallett, M., M.D. 1999, "Editorial: One man's poison--clinical applications of botulinum toxin", *The New England Journal of Medicine*, vol. 341, no. 2, pp. 118-120.

- Although focusing mostly on clinical applications of the drug, this journal article does an excellent job of detailing the biochemical mechanisms of action observed in the botulinum neurotoxin. Furthermore the article brings excellent relevance to the topic as it discusses at length the past and current methods by which botulinum is used and various safety measures which are taken to ensure that the drug is not potent. While slightly dated, the article still provides relevancy as many of the procedures which are discussed are still used today.

Taylor, P., and Brown, J.H., 1999. *Synthesis, Storage and Release of Acetylcholine.* Available at: <http://www.ncbi.nlm.nih.gov/books/NBK28051/> [Accessed 19 Nov. 2015].

- This entry focuses on the neurotransmitter acetylcholine and its role in the neuromuscular junction. Without a comprehensive understanding of acetylcholine's biochemical properties and biophysical behaviours within the synapse, the understanding of the effect of botulinum on the neuromuscular system will be very difficult. Of particular interest within this article are the sections discussing the transportation of acetylcholine through the presynaptic membrane as this is the underlying mechanism with which botulinum interferes.

Epibatidine

Cashin, A.L., Petersson, E.J., Lester, H.A., Dougherty, D.A.., 2005. Using physical chemistry to differentiate nicotinic from cholinergic agonists at the nicotinic acetylcholine receptor. *Journal of the American Chemical Society*, 127(1), pp.350–6.

- Provides an excellent overview and insight into the intermolecular forces present between acetylcholine, nicotine, and epibatidine and the nAChR receptor in both neuronal and muscle cell type nAChR receptors. For the purposes of this study and many other experiments looking into cation-π bond interceptions, the protein AChBP secreted from the glial cell of snails is used as an appropriate analogue for muscle-type receptors. Well-written with an effective introduction and conclusion that highlights ideas behind the different affinities of each molecule at particular nAChR receptors.

Daly, J.W., Brown, G.B., Mensah-Dwumah, M.M., Myers, C.W., 1978. Classification of skin alkaloids from neotropical poison-dart frogs (dendrobatidae). *Toxicon*, 16(2), pp.163–188.

- A landmark paper in which Daly et al. describe the first observation of epibatidine as an “extremely potent analgesic agent that causes Straub tail in mice” (p. 186). Although brief in its description of the neurotoxin, this paper marks the start of a decades-long attempt to elucidate the structure of epibatidine and synthesize more epibatidine for further study. An important read for instructors intending to get insight into the historical context of this alkaloid and other compounds within poison dart frogs.

Spande, T.F., Garraffo, H.M., Edwards, M.W., Yeh, H.J.C., Pannell, L., and Daly, J.W., 1992. Epibatidine: a novel (chloropyridyl)azabicycloheptane with potent analgesic activity from an Ecuadoran poison frog. *Journal of the American Chemical Society*, 114(9), pp.3475–3478.

- An important article that was the first to correctly identify the complete structure of epibatidine. For the interested reader, detailed spectroscopic outputs are presented on pages 3476-3477. A natural continuation of early work performed by John Daly, this paper serves as further evidence that epibatidine and morphine act as analgesic agents via different mechanisms.

Sullivan, J.P., Decker, M.W., Brioni, J.D., Donnelly-Roberts, D., Anderson, D.J., Bannon, A.W., Kang, C.-H., Adams, P., Piattoni-Kaplan, M., and Buckley, M.J., 1994. (+/-)-Epibatidine elicits a diversity of in vitro and in vivo effects mediated by nicotinic acetylcholine receptors. *Journal of Pharmacology and Experimental Therapeutics*, 271(2), pp.624–631.

- Building off past evidence by John Daly and his colleagues, Sullivan et al. (1994) are one of the first teams to identify the nicotinic properties of epibatidine. The paper compares and contrasts nicotine and epibatidine, finding that the later has a much greater affinity for nAChRs. Results of this study helped pave the way for increased interest into the role of epibatidine's and its potential to be synthesized as a derivative for use as an alternative analgesic agent for morphine.

PART F - GLOSSARY OF TERMS

ACETYLCHOLINE – the primary "messenger" for neuronal signals between the nervous system and the muscular system. Acetylcholine is responsible for the "activation" of muscles as dictated by the brain. Substances which inhibit the effects of Acetylcholine are known as anticholinergics.

ACETYLCHOLINE RECEPTOR – a ligand gated channel which responds to the binding of acetylcholine. In addition to acting as a receptor, acetylcholine receptors act as ion channels which induce an action potential in the muscle fiber when they bind with acetylcholine.

ACTION POTENTIAL – an electrical signal propagated through changing membrane potential in the neuron.

ACTIVE CONCENTRATION – the concentration at which a toxin has a measurable effect on the cells it affects.

AGONIST – an exogenous chemical species that can mimic the activity of certain neurotransmitters and bind to the same or allosteric sites on a receptors in order to produce the same or enhanced results.

ANTAGONIST – an exogenous chemical species that can blocks the activity of certain neurotransmitters by bind to the same or allosteric sites on a receptors, producing a diminished or non-existent response

ARBORIZATION – a branch-like structure at the end of neurons.

AXONAL TERMINAL – the terminal end of a presynaptic neuron which acts as the "hub" of various processes associated with neuronal communication.

BINDING AFFINITY – the strength of attractive forces between two chemical structures.

BOTOX – a cosmetic drug used to reduce the appearance of wrinkles and creases within the skin by partially paralysing the facial muscles used to distort the face. The active ingredient in BOTOX is extremely small amounts of the botulinum neurotoxin.

BOTULISM – a disease caused by the introduction of the botulinum neurotoxin to the body. Foodborne botulism is the most common type of the disease within humans as improperly stored/handled food provides ideal conditions for the *Clostridium Botulinum* bacteria to grow. Symptoms of the disease include double vision, drooping eyelids, slurred speech difficulty swallowing and muscle weakness.

CATION-π BOND INTERACTION – the interaction and subsequent bond formed by the attraction between an electron-dense, π-bond system (e.g. benzene) and a positively-charged cation.

CLASSICAL SYNAPSE – a synapse in which a single presynaptic neuron affects a single postsynaptic neuron.

CLOSTRIDIUM BOTULINUM – an anaerobic bacteria which produces the botulinum toxin as a natural defence against predators. The neurotoxin forms within spores produced by the bacteria. The spores can only grow in anaerobic conditions.

COFACTOR – a molecule whose presence is essential for the function of a protein.

DEPOLARIZATION – an increase in membrane potential, usually in a neuron.

ENDOCYTOSIS – a form of active cellular transport where external agents (such as a neurotoxin) are internalized by the cell membrane.

EXOCYTOSIS – an active form of cellular transportation where a vesicle membrane is fused with the cell membrane and the contents of the vesicle are expelled from the cell. In the context of a neuron, the neurotransmitter vesicle will fuse with the neuron membrane and will be expelled into the synaptic cleft.

HYDROPHILIC – of a molecule; able to form molecular attractions with water. Usually polar.

HYDROPHOBIC – of a molecule; incapable of forming molecular attractions with water. Usually non-polar and fat-soluble.

HYPERPOLARIZATION – an increase in the magnitude of the membrane potential; usually refers to a change that makes a neuron's membrane potential more negative and reducing the likelihood of an action potential.

IONOTROPIC RECEPTORS – structures that allow for the direct flow of ions after ligands have bound to them.

INTEGRAL – of a protein; embedded in the cell membrane.

METABOTROPIC RECEPTORS – structures that allow for the indirect flow of ions after ligands have bound to them by acting nearby ion channels.

MORPHOLOGICAL CHANGES – changes to the physical structure of some aspect of an organism. Can be on a system, cellular, or molecular level.

MOTOR NEURON – specialized neurons which are responsible for relaying signals to muscle fibers.

MUSCULAR FIBER – a cylindrical subunit of a muscle made of myocytes (muscle cells). Muscle fibers are the portions of the muscle which are in contact with the motor neurons. Synchronous contraction or relaxation of fibers is what allows for movement.

NEUROTRANSMITTER – chemicals able to transmit a signal across a synapse. Have a variety of mechanisms of signal transmission.

PERIPHERAL NERVOUS SYSTEM – all nervous system tissue that does not include the brain and spinal cord.

PROTEIN DOMAIN – a section of a protein that has a specific function other than structure. Can function separately from the rest of the protein.

PROTEOLYSIS – the hydrolysis of proteins into their amino acid constituents. The process is almost always catalysed by enzymes known as proteases.

SECONDARY METABOLITE – a substance produced by an organism that is generally not directly involved in growth or reproduction.

SECRETORY PATHWAY – the mechanism of the movement of proteins and other molecules out of the cell.

SNAP-25 – a protein responsible for mediating the fusion of the acetylcholine vesicle membrane to the neuron membrane. The protein acts within the trans-SNARE complex which is responsible for matching the vesicle membrane to the neuron membrane.

SNARE COMPLEX – the complex formed by a bound SNARE protein and a vesicle, where SNARE proteins are a family of proteins responsible for vesicle fusion and exocytosis.

STRAUB TAIL RESPONSE – the presence of a stiff "S-shaped" tail akin to local paralysis; historically seen after exposure to opioids, such as morphine

SYNAPSE – the space between two neurons that allows the transmission of a chemical or electrical signal.

SYNAPTIC VESICLE – a vesicle, containing neurotransmitters, which are released into the synapse.

TETRAETHYLAMMONIUM – a neurotoxin that inhibits the action of the potassium channel.

TETRAMERIC PROTEIN – a protein made up of four protein subunits.

THALAMUS – a structure of the brain responsible for transmitting sensory and motor signals, among other functions.

THRESHOLD POTENTIAL – the membrane potential that must be achieved in order to initiate an action potential in a neuron.

TRANSMEMBRANE PROTEINS – a protein embedded in a biological membrane that has components on both sides of the membrane.

VOLTAGE-GATED CHANNEL – a protein channel which opens when a specific membrane potential is reached.

STUDENT MATERIAL

PART A - INTRODUCTION

Figure 1. A few of the many sources of neurotoxins present in the world around you.

In past years, you have likely studied the importance of the human nervous system and discussed its role as the control centre of the body. At the core of this system lies a vast communication network, which sends signals from the brain in the central nervous system down the spinal cord to peripheral nerves all over the body. Over time, scientists have carefully determined many of the underlying mechanisms at play in this system. But what happens when something goes wrong?

A variety of chemical compounds known as neurotoxins have both plagued and fascinated humanity for millennia. While they are known by many names, including “venoms”, “toxins”, and “poisons”, all neurotoxins share a single characteristic: the ability to harm the nervous system in some way. Neurotoxins are created from a number of different sources. Often, they tend to arise in the form of defense mechanisms developed by organisms to ward off predation. As a result, they can produce a number of deadly side effects in organisms when they come into contact with these highly potent chemicals.

Despite the many risks associated with neurotoxins, some cultures have incorporated organisms into their cuisine that naturally contain some of these deadly compounds. For instance, the puffer fish is considered a local Japanese delicacy. Improper preparation of the dish can result in accidental consumption of the neurotoxin tetrodotoxin, which can lead to symptoms as serious as paralysis or even death. However, tetrodotoxin has been of significant importance in scientific research, as it is able to inhibit the transmission of electrical signals, or action potentials, from one end of a neuron to another. Decades ago, experiments involving tetrodotoxin allowed scientists to discover action potentials, forever transforming their understanding of the propagation of signals along a neuron.

Neurotoxins have also been essential in exploring the mechanisms at play in interneuron communication. During chemical signaling, molecules called neurotransmitters leave one neuron (presynaptic cell) and head to receptors on an adjacent neuron (postsynaptic cell). Binding to these receptors on the postsynaptic cell activates chemical properties that initiate the process of creating action potentials. Certain neurotoxins have been found to enhance the rate of neurotransmitter release, such as α-latrotoxin, potent chemicals secreted by the black widow spider. While exposure to α-latrotoxin can have toxic effects on the health of organisms, it has become invaluable in neuroscience research for modelling interneuron communication.

Despite the many harmful aspects associated with neurotoxins, researchers envision using these very effects to enhance quality of life for individuals. For instance, the neurotoxin botulinum comes from the bacteria *Clostridium botulinum*. The compound can attack neuromuscular junctions, locations where neurons are connected to muscles and regulate movement, resulting in paralysis. However, local paralysis of certain neuromuscular junctions can be used safely in BOTOX therapy, a form of cosmetic facial treatment. Medical applications include the possibility of using this neurotoxin to treat muscle disorders such dystonia (sustained muscle spasms) and spasmodic torticollis (involuntary spasms of the neck).

Epibatidine, derived from skin extracts of the poison dart frog *E. anthonyi*, is another example of a neurotoxin that has fascinated scientists. Although part of an important defense mechanism, the neurotoxin itself comes from the frog's diet! Epibatidine is able to bind to a postsynaptic receptor called the nAChR. Normally, the neurotransmitter acetylcholine binds to nAChR as a means of initiating an action potential in the postsynaptic cell; however, nicotine as well as epibatidine are able to act as agonists, compounds that mimic the chemical activity of acetylcholine and by binding to the nAChR. Epibatidine has a very high affinity for nAChR receptors even when present in small concentrations. In addition to the normal responses seen with acetylcholine, epibatidine can also producing analgesic effects (pain relief) when it binds to particular type of nAChR receptors. However, it also produces harmful side effects in the form of seizures and respiratory failure by binding to a different form of nAChR receptors simultaneously. Taking advantage of these properties, scientists have also used epibatidine to visualize early retinal pathways developing in mammals by blocking retinal waves, quick bursts of propagating action potentials.

Throughout this unit, each of the above neurotoxins will be described in more depth in order to provide you with real-world applications that describe various components of past and current research in neuroscience. As you read through these chapters, remember to ask yourself the following questions: 1) How does each neurotoxin explore a different aspect of the nervous system? 2) What are the potential risks associated with each neurotoxin? 3) Can these downsides be overcome or used in a positive light? 4) How has each neurotoxin changed the face of neuroscience research? Enjoy!

PART B - ACTIVITY INSTRUCTIONS

Activity 1: Tetrodotoxin as a research tool

Due to its selectivity for the sodium channels, tetrodotoxin is useful for isolating currents in the action potential. By turning off the sodium current, an experimenter can observe the change in membrane potential due to potassium only, and can therefore easily determine what ions are flowing in a given direction at a certain time. This activity mimics an experiment done by Narahashi et al. in 1964. In this experiment, by using tetrodotoxin to "turn off" the sodium current in the action potential, they were able to isolate the late potassium current. Instructions for this activity are included in the given worksheet.

Activity 2: α-Latrotoxin pore formation

The purpose of this activity is to model pore formation and calcium ion influx due to high α-latrotoxin concentrations in the neuron axon.

You will need a bucket, water, a water balloon, scissors, one or more plastic water bottles, some duct tape, and some kind of tool that can poke a hole in a balloon.

To set up this demonstration (Figure 1), first take your water balloon and cut as many small (~1 cm radius) circles in it as you have water bottles. Cut off the necks of the water bottles and insert the resulting bottlenecks into the holes in the balloon; seal them with duct tape. This is your extracellular environment.

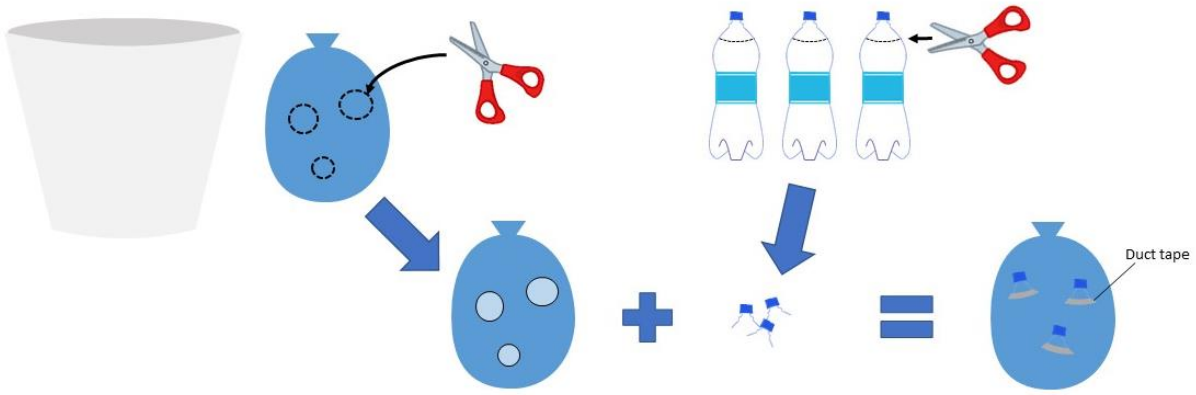


Figure 1. How to set up your "extracellular environment".

Next, fill your water balloon with water (Figure 2A). If you notice water leaking, use more tape to seal the holes further. Open the bottlenecks by unscrewing the plastic cap. What happens? **Given your knowledge of synaptic vesicle exocytosis and that the bottlenecks represent voltage-gated calcium ion channels and the interior of the bucket represents the interior of the axon, what does your hand represent? What about the water?**

Finally, poke a hole in the water balloon (Figure 2B). What happens? **Why would an uncontrolled influx of water (i.e. calcium ions) be bad for the cell?**

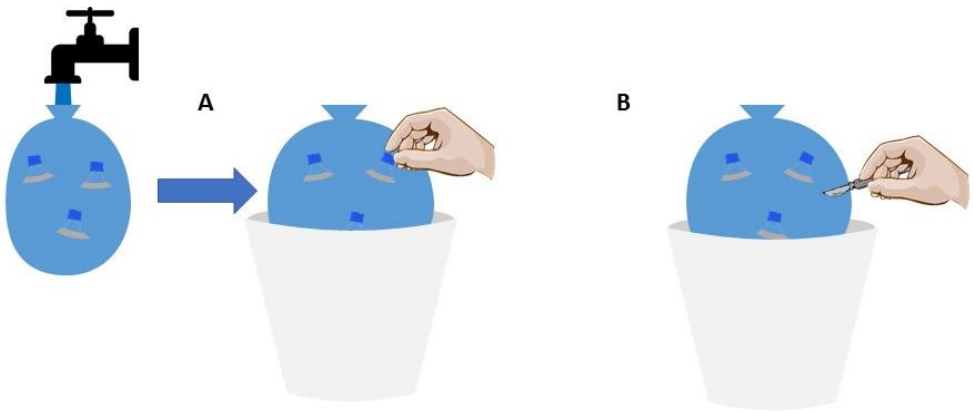


Figure 2. Simulating calcium influx into the axon terminal.

Activity 3: Botulinum and neuromuscular response rates

The purpose of this activity is to look at response rates within the neuromuscular system.

To complete this activity we only need one meter stick. Have one individual hold the meter stick in the air and have another person with their fingers outstretched at the base of the ruler. The individual holding the ruler will release the stick at an unspecified time while the individual with their fingers outstretched will try to catch the ruler as quickly as possible.

The response rate can be determined using the formula

$$t = \sqrt{\frac{2d}{g}}$$

Where t represents the response rate (in seconds), d represents the distance the ruler fell before being caught (in meters), and g represents the acceleration due to gravity (9.81 m/s^2).

With this activity, there will be different response times throughout the class. **Discuss possible reasons for discrepancies in response times between individuals. Consider factors that could affect reaction times (e.g. older vs. younger students).** Complete multiple trials of the experiment. **Can response time be improved with practice?**

Activity 4: Epibatidine interactions with nAChRs

In pairs, you will use simple material to model the presence of cation-π interactions and hydrogen bonds between receptors and ligands. As you go through this activity, think about why you are using each of these materials and what each step of the procedure represents.

Part 1 - Creating the aromatic ring (Figure 3)

1. Take the provided paper plate and flip it over.

2. Using a black marker, draw the structure of benzene, a typical aromatic structure.

3. Place and tape two magnets with the N (North) side face up in the centre of your molecule. Tape two single magnets (with the N side face up) on opposite sides, approximately equidistant from the centre magnet:

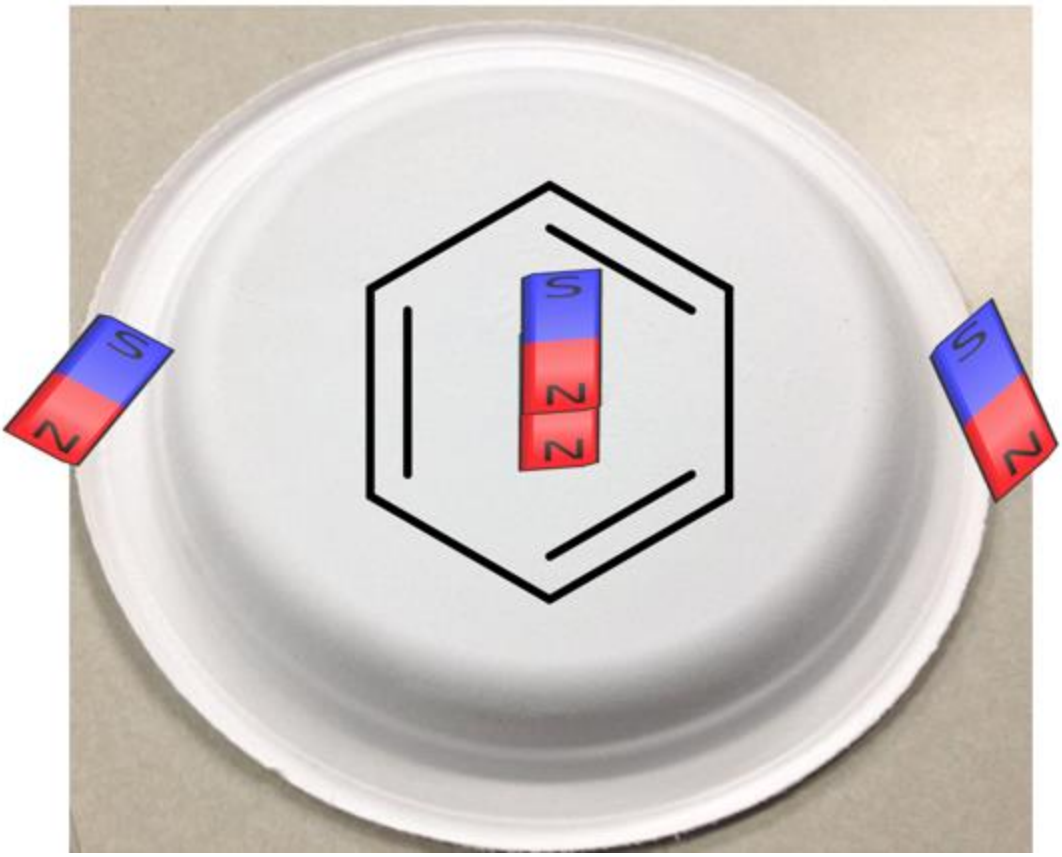


Figure 3. Setup of magnets on a plate as described in part 1.

Part 2 - Creating the ligands

1. Using tape and making sure that the S (South) Side is facing up, prepare three different rulers, such that:

a) One ruler has two magnets only in the centre.

b) One ruler has two magnets in the centre and a single magnet on one of the ends

c) One ruler has two magnets in the centre and a single magnets on each of the ends (Figure 4).

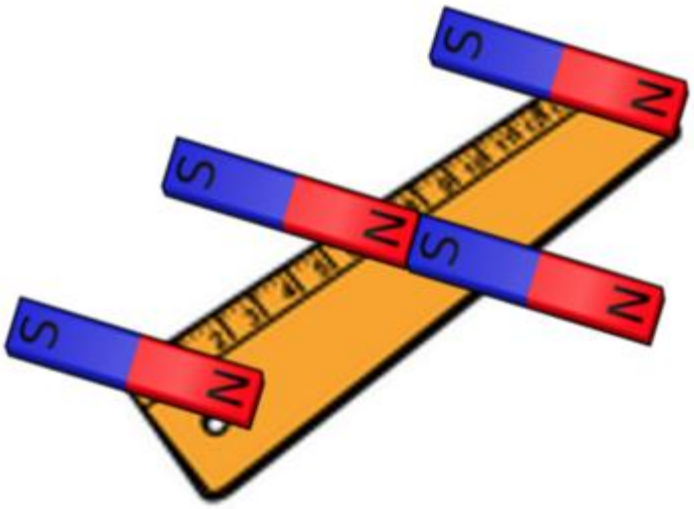


Figure 4. Setup of magnets on ruler c) as described in part 2.

Part 3 - Time to bind!

1. Bring each ruler one at a time towards your plate. After the ruler is attracted to and binds to the plate, try to pull it off and comment on the difficulty associated with doing this task. Above your desk, you may also turn the plate upside down and observe the effects of gravity.

2. Modify the experimental design to improve binding. Consider changing the position of the magnets on the outside of the ring and those on the ruler. However, you should not move the magnet in the centre of the ring. Why?

3. Complete the attached student worksheet.

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