Veterinary Bioscience: Cells to Systems

Practical class 8: Neuromuscular transmission

Dr Graham Mackay (Dept. Biochemistry and Pharmacology) *gmackay@unimelb.edu.au*

Learning Outcomes

Upon completion of this CAL and studying its content, students should be able to:

- Describe the process of chemical transmission that occurs at the neuromuscular junction.
- Describe how the activity of the neuromuscular junction can be modulated by pharmacological agents.
- Appreciate that drugs with selective activity can be used to reveal cellular mechanisms.

Using Drugs as Tools to Understand Neurotransmission

On reaching the nerve terminal of a motor neurone, an action potential will cause the release of the neurotransmitter **acetylcholine** (**ACh**). ACh is able to diffuse across the synaptic cleft where it can interact with **nicotinic ACh receptors** on the plasma membrane of the skeletal muscle cells. Nicotinic ACh receptors are ligand-gated ion-channel receptors and their activation by agonist binding leads to channel gating and the influx of Na^+ into the cell. This causes the depolarisation of muscle cells which, if it reaches threshold, triggers the opening of voltage gated Na^+ channels and initiates an action potential which spreads throughout the muscle. This then causes the release of intracellular Ca^{2+} from the sarcoplasmic reticulum initiating muscle contraction (figure 1).

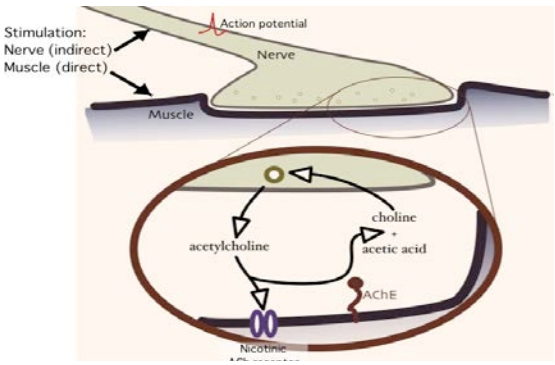


Figure 1. Control of muscle contraction at the neuromuscular junction.

The ability of ACh to interact with ACh nicotinic receptors is inhibited by the activity of the enzyme **acetylcholinesterase** (**AChE**). This enzyme hydrolyses the neurotransmitter, rendering it inactive (*Figure 1*). Thus, the extent of muscle contraction will depend upon the amount of ACh released by the neural action potential, the expression of nicotinic receptors, and the activity of AChE.

In today's computer-aided learning session we will investigate the pharmacological manipulation of electrically-induced muscle responses at the neuromuscular junction.

The Preparation

The experimental procedure involves a commonly used preparation in studies investigating the neuromuscular junction, the **rat phrenic nerve-hemidiaphragm**. The diaphragm is composed of skeletal muscle and the phrenic nerve is a conventional motor neurone. The preparation is maintained in an appropriate buffer/nutrient solution (Krebs solution) and is arranged so that the phrenic nerve can be electrically stimulated and that contraction of the skeletal muscle can be measured via a tension transducer (figure 2). The stimulator can also be switched so that it **directly** stimulates the skeletal muscle, causing muscle contraction without phrenic nerve activity. This can be a useful tool for investigating the site of action of a particular drug (i.e. is the drug affecting muscle or nerve function?).

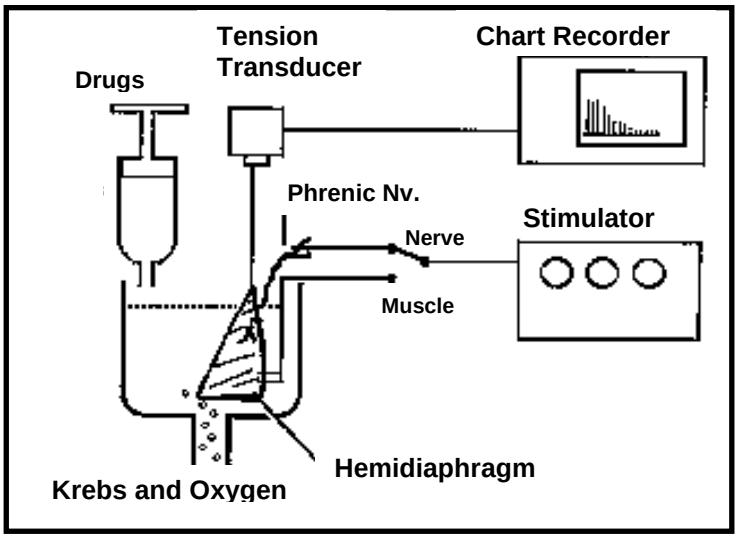


Figure 2. The Rat Phrenic Nerve-Hemidiaphragm Preparation

Drugs of known concentrations can be added directly to the organ bath and, as the organ bath volume is known, the final drug concentration can be calculated. Today we will investigate the effects of FIVE different drugs on the hemidiaphragm's contractile response to phrenic nerve stimulation. These are:

- **Tubocurarine**
  - a nicotinic cholinoceptor antagonist (a component of a South American Indian arrow poison) used as a skeletal muscle relaxant
  - competitively prevents activation of the nicotinic ACh receptor
- **Atropine**
  - a muscarinic cholinoceptor antagonist

- **Neostigmine**
  - an acetylcholinesterase inhibitor
  - increases the effectiveness of ACh released from the nerve
- **Tetrodotoxin**
  - a voltage-operated Na^+-channel blocker derived from a marine bacterium
- **Suxamethonium**
  - an agonist at nicotinic cholinoceptors but acts as a skeletal muscle relaxant
  - *discovering how this is possible is one of the learning outcomes of the prac…*

Experimental Protocols

The software package “Virtual Twitch” (developed by The University of Strathclyde, Glasgow, Scotland) will be made available in the Practical class session via MyUniApps (see below for details), or can be downloaded onto your own PC (or cross-platform Mac) from the following site:

http://spider.science.strath.ac.uk/sipbs/page.php?show=software_sims

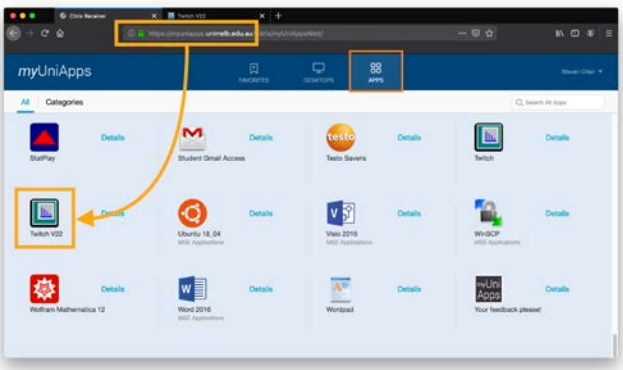
Load a web browser and type 'myuniapps.unimelb.edu.au'

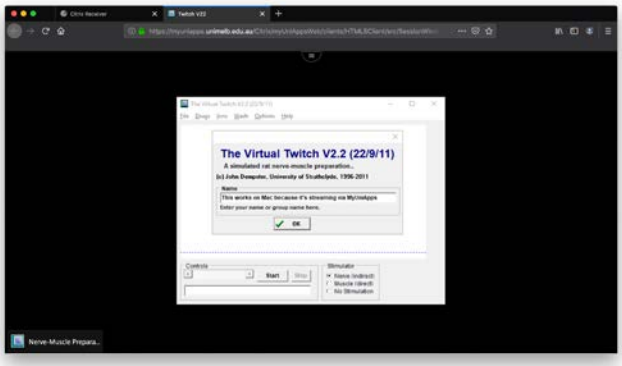
When starting the myUniApps for the first time, you will be asked to install Citrix Receiver if not already present on your computer. Just follow the instructions on screen. Visit the myUniApps FAQ for more detailed setup instructions.

To install Virtual Twitch:

Log in with your University account and choose either Citrix Receiver (this option needs Citrix Receiver installed, which is also available - see above) OR Light version (stream within the browser). Locate 'Virtual Twitch' after clicking 'Apps'.
This will take a couple of minutes to load as it is streaming down Virtual Twitch to the endpoint. Once loaded the software can be used within the web browser if 'Light version' was selected or via the Citrix Receiver on your device.

A Zoom link will be provided so that you can join the class.





Getting Started

- Select the **Twitch folder** and open the programme **Twitch**.
- You will be prompted to enter your name/group – you do not have to do this.
- The **file menu** is used to start a new preparation in the case of the tissue showing signs of loss of activity due to prolonged stimulation or the addition of a drug with a mechanism of action that is slow to diminish even after washing.
- The **drugs menu** contains a list of 6 pharmacological agents - we will study 4-5 of these but you are welcome to explore the activities of the other agents if you have time today (or in your own time). Some information on what these drugs do can be found in the **help menu** and a more detailed description can be found in any good Pharmacology textbook (*e.g.* Rang & Dale's Pharmacology*.*, 9th edition).
- We **will not** explore the **ions menu** today - again this can be done in your own time if desired.
- There are 2 options in the **wash menu** - today we will use **Normal Krebs** washes only.
- The **options menu** controls the trace and background colours.
- The **help menu** has information on the drugs used in the experiments as well as information on the preparation etc.
- At the bottom right hand corner of the box you will see the **stimulator controls**- ensure that the stimulator is set on **Nerve (indirect)**. The stimulator will electrically excite the phrenic nerve at a set frequency and impulse strength - this **can not** be varied in the experiment.
- **Click on start** - immediately you will see diaphragm contractions (twitches) of around 60 g tension occurring. You can **move the mouse cursor around to measure the magnitude of the muscle-twitch responses** (tension values and time (seconds) are shown at the bottom left hand side of the screen).
- In real life experiments of this kind, obtaining a twitch response to phrenic nerve stimulation is an important event that confirms that the sometimes tricky dissection has been a success.

You are now ready to start experimentation!

<u>Experiment 1</u> The effect of tubocurarine on neurally-induced skeletal muscle contraction.

After starting stimulation of the phrenic nerve, watch the level of skeletal muscle contraction to ensure it is relatively consistent. Sometimes tissues take some time to equilibrate to their new *in vitro* conditions. Take an average measurement of the basal muscle contractions you observe (enter the values obtained in the table below).

You are going to construct a **concentration-inhibition curve**, where the concentration of tubocurarine will be altered and its effects on muscle contraction induced by nerve stimulation measured.

- From the **drugs menu** select **tubocurarine**. Then select the concentration of $1x10^{-7}$ M **(NB: if any concentration is not immediately available it can be directly typed into the concentration window)** and select "inject drug". Monitor the contractions you observe and when they have reached a consistent level take a few readings (using the cursor), enter them into the table on the next page and then average the contractions.
- Select **Normal Wash** from the wash menu and wash the tissue 5 times - you can do this one after the other. You will notice that the response returns to the basal readings.
- From the drug menu select tubocurarine and select $3x10^{-7}$ M. Allow the effect of the drug to reach a plateau, take a contraction reading and repeat the washing steps.
- Repeat this process for tubocurarine concentrations of $5x10^{-7}$M, $1x10^{-6}$M, $3x10^{-6}$ M and $5x10^{-6}$ M (NB: **do not wash out** after the last concentration has reached is maximum effect).
- After the last concentration has produced is maximum effect, switch the stimulator to **muscle (direct)** setting.

What do you observe?

- **Return the stimulator to nerve (indirect) and wash the preparation 4 times with normal Krebs.**

Tubocurarine (M)	Tension (g) (take a few readings when the response has plateaued)	Average Tension (g) (take an average of the readings in the previous column)	Inhibition (%)
0 (basal readings)			
$1x10^{-7}$			
$3x10^{-7}$			
$5x10^{-7}$			
$1x10^{-6}$			
$3x10^{-6}$			
$5x10^{-6}$			
Direct Muscle Stimulation			

Often we express inhibition data as the % inhibition achieved. This can be calculated directly as:

((Basal Tension –Tension in presence of drug)/Basal tension) x 100

For example: Basal tension = 60 g

Tension in presence of tubocurarine = 40 g

% inhibition = ((60-40)/60) x 100 = 33 %

✍ **Sketch a log concentration - inhibition (%) curve for tubocurarine**. *(NB: You sketch does not need to be highly accurate- but take care if you decide to use Excel.. are the spacings on the X-axis appropriate?)*

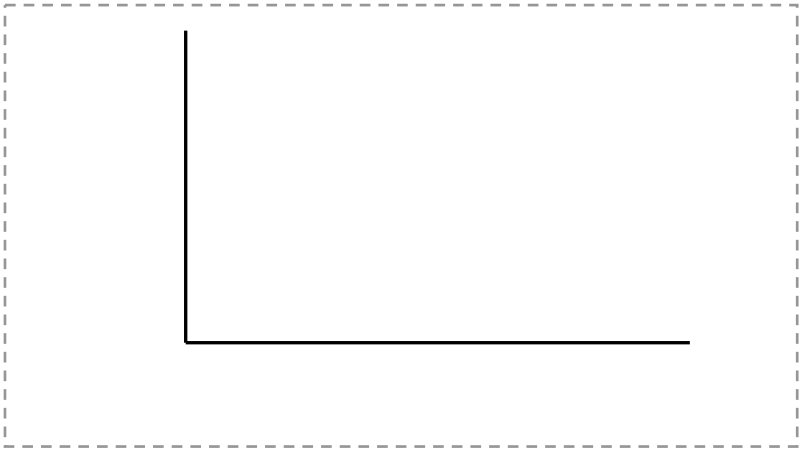
From this curve we can calculate a value called the inhibitory concentration 50%, otherwise known as the **IC_{50}**. This corresponds to the concentration of drug required to reduce the tissue response by 50% of the maximum. Using the same methodology that you have used to estimate EC_{50} values, estimate the IC_{50} value of tubocurarine from the graph. *Validate your answer by examination of your raw data.*

✍ **Estimated IC_{50} value tubocurarine** =

Questions

1. What happened to the response after the tubocurarine was washed out of the organ bath? What might this suggest about the type of antagonist that tubocurarine is?

2. In another experiment we add varying concentrations of ACh directly to the muscle preparation, the contractions are measured, and a log concentration-response curve generated for ACh. **i)** What shape of curve might arise from conducting this experiment? Sketch it on the exes below- make sure you label the axes appropriately. **ii)** The ACh is removed and tubocurarine (10^{-6} M) is added to the muscle preparation and the log concentration-response curve to ACh is repeated. What effect would you expect tubocurarine to have on the ACh concentration-response curve? Draw this on the axes below and consider why the two curves are different.



3. Despite the continued presence of tubocurarine and the almost complete blockade of neurally-mediated skeletal muscle contraction, the diaphragm was still able to contract maximally when the tissue was stimulated directly. Why is this?

Experiment 2 **The effect of atropine on neurally-induced skeletal muscle contraction**

- Look at the basal contraction of the diaphragm to nerve stimulation - it should be around 60 g of tension.
- Using the same protocol as described above obtain a concentration-response relationship to the drug atropine. This time select the concentrations $1x10^{-7}$, $1x10^{-6}$, $1x10^{-5}$, $1x10^{-4}$, $1x10^{-3}$ M. Use the table on the next page to compile your data. **NB:** as you do this, consider the concentration of atropine you are using and how fast you might expect to see an effect of the drug- *i.e.* if you have a hypothesis, skip ahead in the concentration-response to test this.

Atropine (M)	Tension (g)	Average Tension (g)	Inhibition (%)
0			
$1x10^{-7}$			
$1x10^{-6}$			
$1x10^{-5}$			
$1x10^{-4}$			
$1x10^{-3}$			
Direct Muscle Stimulation			

Questions

1. Are you able to determine an IC_{50} value for atropine? Are you able to determine if atropine is an agonist or an antagonist from this experiment?

2. Both tubocurarine and atropine are antagonists of ACh receptors. Why is only tubocurarine effective in the present experiment?

3. Would you expect atropine to be able to inhibit carbachol-induced contraction of gastrointestinal smooth muscle? Why is this?

Experiment 3 **The effect of neostigmine on neurally-induced skeletal muscle contraction**

Neostigmine is an acetylcholinesterase (AChE) inhibitor. It is able to bind to AChE and thereby inhibits the enzymes ability to hydrolyse ACh.

✍ From your knowledge of the cholinergic system what do you predict will be the effect of neostigmine on the diaphragm contractile response to phrenic nerve stimulation?

- Add neostigmine ($2x10^{-7}$ M) to the organ bath and monitor the response. Does the effect match your prediction?

✍ What do you notice after washing out the neostigmine from the organ bath? What does this suggest about the effects of the drug on AChE?

Questions

- A commonly used class of insecticides are the organophosphates which can cause toxic effects in humans and animals. These toxins covalently modify and inactivate AChE. How are the effects of the organophosphates likely to compare to neostigmine in the present model?

Experiment 4 **Reversal of tubocurarine-induced blockade by neostigmine**

For this experiment it is best to select a **new rat** from the **file menu**.

- Again, start nerve stimulation and after a suitable equilibration period add tubocurarine ($1x10^{-6}$ M).
- Allow the inhibition to take maximal effect **- do not wash away the drug**.
- Whilst still in the presence of tubocurarine add neostigmine ($2x10^{-7}$ M). What do you observe?

Questions

1. Describe the mechanism that underlies neostigmine's ability to reverse tubocurarine blockade of the neuromuscular junction.

2. The autoimmune disease **myasthenia gravis**, which can produce skeletal muscle weakness and paralysis, results from a loss of nicotinic ACh receptors. Do you think that AChE-inhibitors may have a role in therapy of this disease? Describe why you think this is the case?

<u>Experiment 5</u> Effect of neostigmine on suxamethonium-induced neuromuscular blockade

For this experiment it is best to select a **new rat** from the **file menu**.

- Again start nerve stimulation and, after a suitable equilibration period, find a concentration of **suxamethonium** that produces the same amount of muscle blockade as tubocurarine ($1x10^{-6}$ M).
- Allow the inhibition to take maximal effect **- do not wash away the drug**.
- Whilst still in the presence of suxamethonium add **neostigmine** ($2x10^{-7}$ M).

✍ What do you observe? How does this compare to the effect of neostigmine on tubocurarine-induced blockade?

✍ How does a nicotinic receptor agonist (suxamethonium) produce skeletal muscle relaxation? *(you will most likely have to consult a pharmacology textbook, or other trusted online sources, to work this out).*

✍ Describe why neostigmine has different effects on the muscle relaxation induced by tubocurarine and suxamethonium. *(you will most likely have to consult a pharmacology textbook, or other trusted online sources, to work this out).*

Experiment 6 Identification of ambiguously labelled compounds

Experiment 6

You have been provided with TWO tubes that have been poorly labelled.

One tube contains the potentially deadly **tetrodotoxin**, which is a Na^+ channel blocker. The toxin is produced by a marine bacterium that accumulates in certain tissues of the puffer fish. Besides being VERY dangerous (minimal lethal dose in the mouse 8 μg/kg) this reagent is hard to isolate and is expensive and hence discarding both tubes is not an option.

The other tube contains the muscle relaxant drug **tubocurarine**. YOU DO NOT KNOW THE CONCENTRATIONS OF THESE SUBSTANCES.

What experiments could you conduct using the rat phrenic nerve–hemidiaphragm preparation in order to identify which tube contains which agent. Use the space below to work out your strategy and then conduct the experiment.

Use this page for your notes and sketches