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**WORKSHOP ON THE USE OF RECEPTOR BINDING ASSAY (RBA)**

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# *Detection of Saxitoxin and its Relatives from the Molecular to the Mouse*

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# Detection of saxitoxin and its relatives, from the molecular to the mouse

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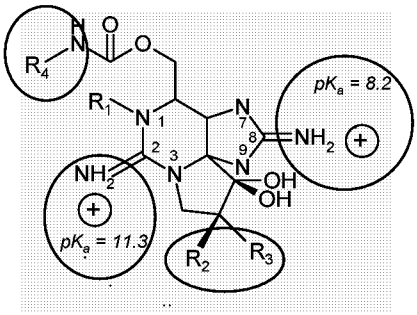
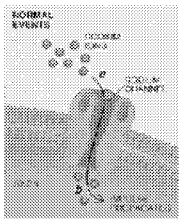
## The paralytic shellfish poisons

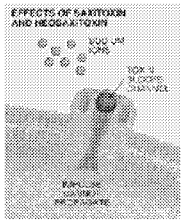
Family of toxins based upon saxitoxin:




## What do PSP's do?

- PSPs block the extracellular pore of the sodium channel
- The sodium channel spans the cell membrane and selectively allows $Na^+$ ions to pass during an action potential
- PSP's therefore render excitable tissue such as nerve and muscle functionless
- Can lead to respiratory paralysis of PSP victims
- Not all sodium channel isoforms bind saxitoxin





## Mouse bioassay for PSPs

- Mouse intra-peritoneal injection
- "official" method (AOAC, APHA, FDA)
- Measures mammalian toxicity and relevant to monitoring for human safety
- First used in 1930's
- McFarren (1959) published collaborative study (11 labs)
- Used spiked and naturally toxic extracts
- Average standard error was 17%
- Underestimated toxicity by 40% at lowest toxicity level
- Endorsed as official method in 1965
- Intraperitoneal route irrelevant to oral toxicity
- Yessotoxins and other new toxins potent via this route but not orally available so not a public health issue

## Mouse assay protocol

- Official Methods of Analysis, 1990
- Toxin absorbed through blood vessels lining the peritoneal cavity
- Proper compliance requires a stock colony of healthy mice within a certain weight range (19-21 g)
- Prior to conducting assay, must standardise mouse colony sensitivity to an STX standard
- Standardise by repeated injections of a known amount of STX until death time of 5-7 minutes is regularly recorded (n >= 10)
- Assay then repeated a day or two later with the same solutions of STX and then the entire experiment is repeated again with a new batch of STX to ensure reproducibility
- Similar to standard STX solution, test samples are concentrated or diluted until mouse death is 5-7 minutes
- PSTs are quantitated by comparing death time, recorded as the last gasp, to a standard series of mouse units

## Mouse bioassay for PSPs

- Many decades of use since Sommer & Meyer (1937)
- Enormous amount of data enabled continual validation of assay
- Expensive due to maintenance of mouse colonies
- Animal experimentation becoming increasingly unpopular
- Prone to human error, with end point being last gasp of the mouse, a subjective judgment by the experimenter
- Other sources of error arise form poor injection technique
- If toxic extract not injected exclusively into the peritoneal cavity, some ends up in subcutaneous compartments or in the digestive tract itself, so toxicity may be underestimated
- Error can arise from PST entering testicular cavity of male mice leading to sexual differences in toxin sensitivity
- Discrepancies observed due to presence of metals or common salts found in marine samples

## Tissue culture detection

- Receptor based ($Na^+$ channel in cell membrane)
- Non-radioactive and usually colourometric
- Cells containing Na channels swell in the presence of veratridine, a Na channel activator
- Swelling is enhanced with ouabain, a $Na^+$ / $K^+$ ATPase inhibitor which prevents removal of excessive Na ions allowed in by veratridine
- Addition of both drugs at appropriate concentration results in cell lysis or severe morphological changes that affect cell viability
- Na channel blocker such as STX, TTX or an active analogue is added to the cell culture media prior to the addition of these drugs, the influx of Na ions is prevented & cells remain viable
- Will detect compound(s) that affect veratridine and ouabain binding to Na channel
- Initially, cells were manually counted as alive or dead and prone to experimenter error
- Assay later modified to spectrophotometric measurement of the product of cellular metabolism of tetrazolium salts

## Tissue culture detection

- Inexpensive, requiring only basic laboratory equipment such as tissue culture facilities and a plate reader
- Mimics whole organism activity and has a high correlation with the mouse bioassay
- Sensitivity governed by duration of assay with best results achieved after 24 hours
- Non-specific agents in marine extracts may cause cellular toxicity and confound assay results
- In addition to detecting Na channel blocking agents, has also been employed to detect Na channel enhancing compounds such as brevetoxins and ciguatoxins

## Sodium channel receptor bioassays

- Basic method changed little from beginnings in the early '70's
- Relies on membrane preparations from tissue containing Na channels, typically brain tissue, and binding of $^3$H-STX
- Can also use cell lines highly expressing $Na^+$ channels (cloned or native)
- Has equal affinity for tetrodotin and its analogues
- Uses tritiated saxitoxin but could also use tritiated tetrodotoxin
- Formatted into microtitre filtration plates (rapid throughput)
- Detection of PSTs achieved by incubation of receptor and radioligand in presence of test sample, salt and buffer
- Salt used must be unable to pass through the open Na channel to prevent alteration of membrane potential and hence conformation of the Na channel
- Usually incorporate choline chloride to maintain osmotic balance

## Sodium channel receptor bioassays

- After appropriate length of time, incubation mixture filtered through glass fiber type C filter with the Na channel / $^{3}$H-STX complex trapped on filter
- Filter washed with cold buffer solution and radioactivity bound on the filter quantitated by scintillation counting
- Detection of PSTs in a sample is evident by a reduction in the amount of $^{3}$H-STX bound to the synaptosomes and hence a reduction in the radioactive signal from scintillation counting
- Alternatively, free $^{3}$H-STX can be separated from bound radioligand by passage of assay mixture through a cation exchange chromatography resin which retains charged toxin and allows receptor bound $^{3}$H-STX to pass through into a vessel for scintillation counting
- Na channel affinity for STX decreases with increased temperature and in a technical sense, is best to maintain experiments as close to 0 °C as possible.
- Toxin binding is diminished at pH's below 6 due to an effect upon essential amino acids within the Na channel

## Sodium channel receptor bioassays

- Experiments must be well buffered to prevent test samples affecting assay, especially those produced using the AOAC procedure where solvent is 0.1N HCl
- Binding of STX by Na channel depends on both guanidino groups borne by STX being in charged state
- With pKa's of 8.2 and 11.3, the guanidino groups deprotonate as the pH moves to these pKa's and unable to bind to the channel
- This therefore constrains this assay to a narrow pH range.
- Monovalent and divalent cations affect binding of STX, thus the potential of false negatives increases if test samples contain significant quantities of salt
- Binding of PSTs to the Na channel determines mammalian toxicity
- Toxicity observed by this method correlates well with mouse lethality bioassay
- Cheaper assay than mouse lethality and tissue culture in terms of consumables and reagents but requires expensive equipment such as a scintillation counter and generates radioactive waste

## Saxiphilin receptor bioassays

- Saxiphilin is an STX receptor protein unrelated in structure to the Na channel
- Member of the transferrin family which binds and circulates iron throughout the body of many animals
- Differs from Na channel in that it selectively binds STX with a sub-nanomolar affinity but is insensitive to TTX
- Saxiphilin found in wide range of vertebrates and invertebrates including species that have no demonstrated contact with potential sources of PSTs such as terrestrial invertebrates and vertebrates
- In essence, saxiphilin assay is similar to Na channel assay in that the protein is incubated with $^{3}$H-STX, test sample and salt in a buffered solution
- Filtration of reaction mixture with polyethylimine treated glass fibre type B filters which enables glass fibres to bind soluble proteins
- PSTs in a test sample reduces the radioactive signal from scintillation counting of filter bearing saxiphilin / $^{3}$H-STX complex

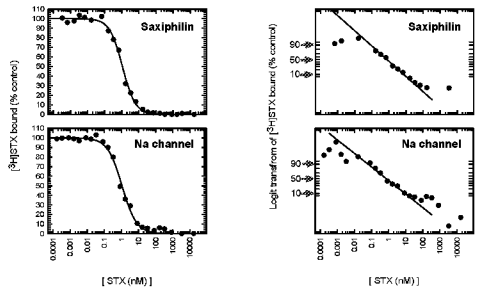
## Saxiphilin receptor bioassays

- Like Na channel, can also be conducted using cation exchange chromatography resin for separation of bound and free $^3$H-STX
- Able to detect several PST analogues with equal affinity with the exception of the sulfated C class toxins.
- Assay has recently been used for the detection of PSTs in a variety of samples including an extensive study using AOAC prepared extracts and correlated favourably with Na channel receptor, mouse lethality bioassay and HPLC analysis
- Also tolerates complex matrices, high salt and gives reproducible results over a wide range of pH's
- Is subject to the same problems as the Na channel assay in that it requires radioactively labelled STX and the dilemmas associated with radioactive handling and waste management:
  - Safe radioisotope handling by lab personnel
  - Radioactive waste management
  - Scintillation fluid waste
    - Microtitre plate assays dramatically reduce scintillation fluid usage

## Quantification from competition curves

- Method 1
  - Competition curves [$^3$H]STX bound = 100*([STX]$^n$/([STX]$^n$ + $IC_{50}{}^n$))
    - $IC_{50}$ is volume of crude extract to cause 50% inhibition
    - n is the slope of the curve
  - When concentration of the $^3$H-STX in experiment is much greater than $K_D$ of the receptors $^3$H-STX, the $IC_{50}$ of unlabelled STX versus $^3$H-STX equivalent to concentration of $^3$H-STX used
  - Above equation converts to [STXeq] = $((100-F)/F)^{1/n} * IC_{50}$
    - F = % bound [$^3$H]STX relative to controls in extract presence
    - n & $IC_{50}$ as above
  - [STXeq] then calculated taking into account concentration of [$^3$H]STX, assay sample volume, volume of test extract
- Method 2
  - Competition curves can be linearised by logit transformation of y-axis of standard competition curve
  - Most reliable linearity between 30-70% inhibition

## Quantification from competition curves



## Immunological approaches

- Antibody production requires STX to be transformed into an antigenic entity
- Different antibody batches may give varied results
- Colourometric
- Rapid-throughput (microtitre plate format)
- Enzyme-linked immunofiltration assay, radioimmunoassays and indirect ELISA
- Main problem is inherent specificity for a particular PST
- Both monoclonal and polyclonal antibodies reported to date possess little crossreactivity with other PSTs apart from the one against which the antibody was raised, presenting difficulties for samples containing a variety of PSTs
- Likely need to mix antibodies
- Time from inoculation to antibody harvest can be several months
- Multiple animals required raising spectre of animal use
- Immunological approaches have ability to be formatted into qualitative techniques to be taken into the field (eg MIST Alert

## RIDASCREEN Saxitoxin assay

- A direct competitive enzyme linked immunosorbent assay (dc-ELISA) used for detection of STX and neoSTX using antibodies generated to toxin linked to a carrier protein to manufacture an antigenic epitope
- Basis for a commercially available kit (Ridascreen ® Saxitoxin Test)
- dc-ELISA technique uses microtitre plate wells coated with antibody to which test sample and a conjugate of STX with the enzyme, horse radish peroxidase (STX-HRP), are added simultaneously to then compete for the STX antibody
- If test sample contains STX in sufficient quantities, then will outcompete STX-HRP for the binding sites
- If no STX is present in the test sample, the STX-HRP will saturate the STX antibody
- A HRP chromogenic substrate is added to the plate after washing to remove unbound test sample and STX-HRP and a colour forms if HRP is present as the STX-HRP
- No colour formation reveals the presence of STX in the test sample that prevented the STX-HRP binding to the antibody coated microtitre plate

## RIDASCREEN Saxitoxin assay

- Correlation with the mouse bioassay is ~ 60%
- Underestimated toxicity relative to mouse lethality by a factor of ~2
- Simple to perform but requires a photometric plate reader
- Limitations attributed to the specificity by the antibodies towards individual PSTs
- Shown would underestimate total toxicity if the sample were to contain high levels of neoSTX, GTX 1 or GTX 4
- Partly reflects that antibodies are raised to one PST only, usually STX
- RidaScreen soon to release new version based on a new monoclonal Ab

| Toxin | Ridascreen polyclonal | Ridascreen monoclonal |
|---|---|---|
| Saxitoxin | 100 % | 100 % |
| dcSTX | 27.8 % | 18.2 % |
| GTX 2/3 | 12.1 % | 86.3 % |
| C 1 / 2 | 0.5 % | 3.0 % |
| neoSTX | 3.1 % | 8.5 % |
| GTX 1/4 | 0.3 % | 15.1 % |

## Tissue biosensor

- Na ion electrode used to detect fluctuations in [$Na^+$] across a frog bladder membrane which is rich in Na channels
- Na channel blocking agents such as STX or TTX alter the membrane permeability to $Na^+$ detected as potential change
- Average measure takes ~ 5 minutes making it a comparatively fast
- Will not distinguish between STX and TTX
- Very good correlation with mouse lethality bioassay
- Depends on intact Na channels present in the frog bladder membrane and as such has differing sensitivities for various PSTs
- Frog bladder Na channels may have different toxin sensitivities than mammalian Na channels
- Little work done on the characterisation of the assay with regard to physiocochemical properties such as pH variation, salts or other chemical factors that may affect the integrity of the method

## Whole organism assays (not mouse)

- Chick embryo's, brine shrimp, desert locust and even some bacteria have been examined for their suitability to detect PST's
- Same basic principle of observing an effect on the organism, usually whether it is alive or dead, upon application of a PST or a PST containing sample
- As an example, the house fly bioassay involves injection of samples into the common house fly *Musca domestica*
- Responses based on fly movement are scored as zero for no movement or one for movement
- Rapid and cost effective method with a single fly able to be processed every minute
- Does not consume much sample as it only requires an injection volume of only 1 to 1.5 microlitres
- Claimed that the 'salt effect' that plagues the mouse bioassay is not observed with this system
- Tedious and technically challenging method

## Fluorescent probes

- Toxin activities reflected in changes in cellular properties beyond the direct action by the toxin on the Na channel
- One measure looks at variations in intracellular $Ca^{++}$ resulting from action of PSTs on Na channels in rat cortical primary cultures
- Blockade of the Na channel by PSTs reduces peaks of intracellular calcium induced by electrical stimulus, as measured by a calcium sensitive fluorescent dye.
- Another fluorescent method of PST detection relies on the membrane potential dependent distribution of a fluorescent dye bis (1,3-diethylthiobarbituric acid) trimethine oxonol (bis-oxonol).
- Neuroblastoma cells equilibrated with the dye followed by addition of the Na channel activator, veratridine, to depolarise the membrane
- STX contained in a test sample reduces the effect of veratridine and subsequently change the distribution of bis-oxonol across the cell membrane which is then measured fluorometrically

## Molecule, cell, organism

- Molecular assays obey the law of mass action
  - Receptor + ligand $\underset{k_d}{\overset{k_a*[L]}{\rightleftharpoons}}$ Receptor.ligand
- Cellular assays:
  - Law of mass action
  - Cell metabolisem
    - Active and inactive metabolites
- Organism
  - Absorption
    - Epithelia of blood, gut, skin
  - Distribution
    - Blood, lymphatics
  - Metabolism
    - Toxic and non-toxic metabolites
  - Excretion
    - Urine, defecation

## New paralytic shellfish poisons

- More lipophilic so potentially better able to be taken up through gut
- As potent as saxitoxin
- Common sample pretreatment may remove these toxins and so not detected
- More commonly occurring than first realised
- Confounding current assay results??