NEURAL RECEPTORS OF NICOTINE ADDICTION

OXFORD BROOKES UNIVERSITY

Functional Analysis of the Human Alpha4Beta2 receptor expressed in *Xenopus laevis* oocytes for the Molecular Binding of Smoking Cessation Drugs

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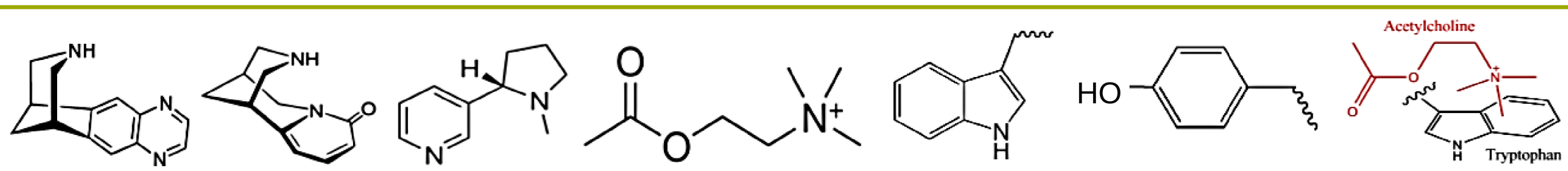
Nicotine addiction is a familiar problem for many people with clear consequences in society, especially after the First World War where cigarettes were distributed free of charge to troops as a convenient source of nicotine, and advertising offered them as the diet alternative to sweets.

Two drugs currently used to aid smoking cessation are varenicline and cytisine. They are known to mimic the effects of nicotine on the alpha4beta2 nicotinic acetylcholine receptors in the brain, which give it the addictive qualities, however their side-effects vary and can be very serious(1). These nicotinic receptors are responsible for the much of mechanism of nicotine addiction(2), and investigating the binding mechanisms of smoking cessation drugs will help form a better understanding of their interactions and provide targets for improved pharmaceuticals.

By introducing six separate single point mutations into the cloned human cDNA for these receptors, the structure of the binding site in the extracellular region of the receptor was altered specifically to provide a measure of how strongly each point that is already known to interact with nicotine will also interact with cytisine and varenicline. The differences in binding on a molecular level could account for the variation in side-effects. To measure the effect of the mutation, the mutant cDNA was injected into *Xenopus laevis* oocytes (frog eggs), which were large enough to be probed with electrodes(3). Sufficient cation-π interactions between the agonist to aromatic residues in the ligand binding domain induce a conformational change in the 3D structure of the receptor, opening the channel pore and causing a voltage change across the membrane(4). This change can be measured and gives an indication the functionality of the mutant receptors on exposure of the drug, and comparing them with the response of non-mutated cDNA will reveal molecular binding differences. Any mutation which prevents a response is assumed to be involved in the binding of that agonist. The aim is to provide a starting point for further research by establishing whether or not any differences in molecular binding actually exist.



1. METHODS

Oligonucleotide primer design:

Primers were designed to introduce mutations into six selected sites on the α and β chain genes for the extracellular binding region of the receptor (mutations named W88A, Y126A, W182A, Y223A and Y230A on the alpha chain, and W82A on the beta chain). These mutations were point mutations designed to convert the original aromatic tyrosine or tryptophan residues into alanine, removing the binding abilities of the phenol group. Alanine was the chosen substitute due to the less reactive nature of the -CH3 R group and the relatively smaller size, which reduces interference.

Mismatch and possible hairpins were also considered in the design, with hairpin avoidance given greater importance over %mismatch.

PCR Procedure:

The polymerase chain reaction technique was used to clone and amplify the mutant cDNA, along with the wild type of each gene. High fidelity polymerase from Promega was used to reduce transcription error during cycling. After cycling the parental cDNA was digested and the success of the procedure was checked using Gel Electrophoresis.

E. Coli transformation:

Supercompetent *E. coli* cells were transformed with the cDNA for amplification before selection of colonies for culture in ampicillin treated LB broth.

QIAprep Spin Miniprep (QIAGEN):

The cultures were pelleted by centrifugation and the pellet was resuspended in buffer before the cDNA was purified using the QIAGEN QIAprep Spin Miniprep Kit. The concentration of cDNA eluted was measured with a Nanovue machine (Table below, left).

Oocyte injection:

The purified linear DNA was injected into selected stage V-VI oocytes with a polished sterile glass nanoliter pipette. The oocytes are then incubated for 48-72 hours.

Functional Analysis:

The function of the mutant and wildtype receptors was measured in response to cytisine or varenicline using the HiClamp Machine (Multi Channel Systems). The concentration response curves produced (Graphs, right) were fitted with the Hill equation and statistically analysed with GraphPad Software (GraphPad Prism 7.04)

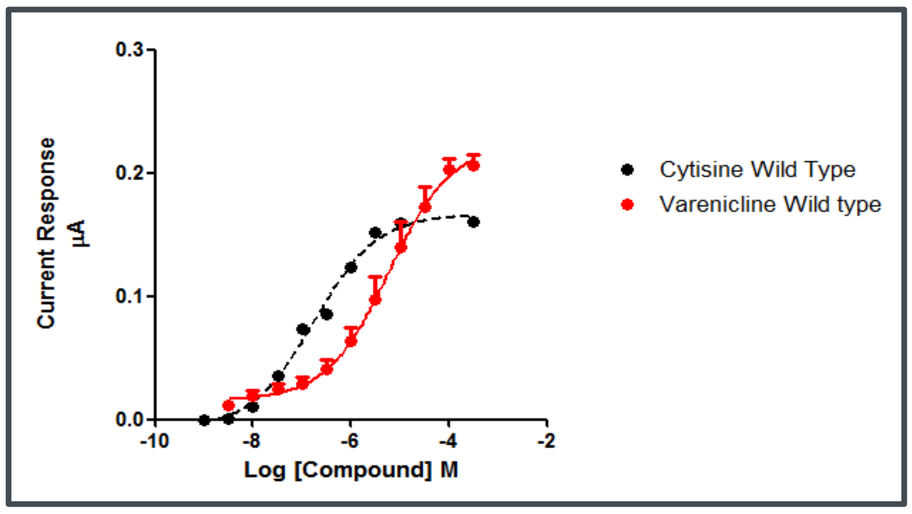
Mutation	concentration (µg/ml)	A260/A280	EC_{50}	SEM (µM)
αW88A	294.5	1.852	0.17	0.001
αY126A	300.5	1.832	0.21	0.001
αW182A	198.0	1.776	5.60	0.100
αY223A	367.0	1.826	5.10	1.000
αY230A	271.0	1.856	13.50	0.500
βW82A	560.0	1.848	13.90	2.000

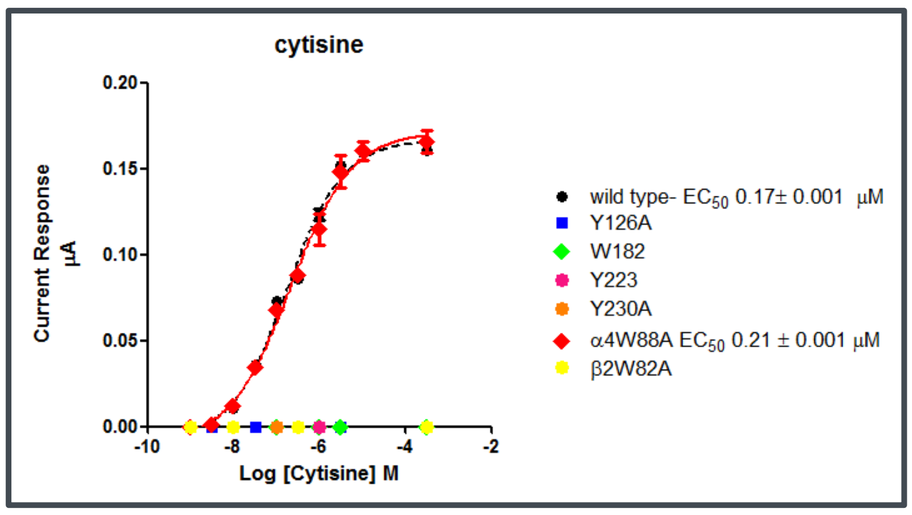
Drug	Comparison (EC_{50}s)	p value	t value	Significant
Cytisine	wt & W88A	<0.0001	28.28	yes
Varenicline	wt & W88A	0.6365	0.4975	no
	wt & Y126A	<0.0001	15.49	yes
	wt & Y230A	0.006	4.145	yes

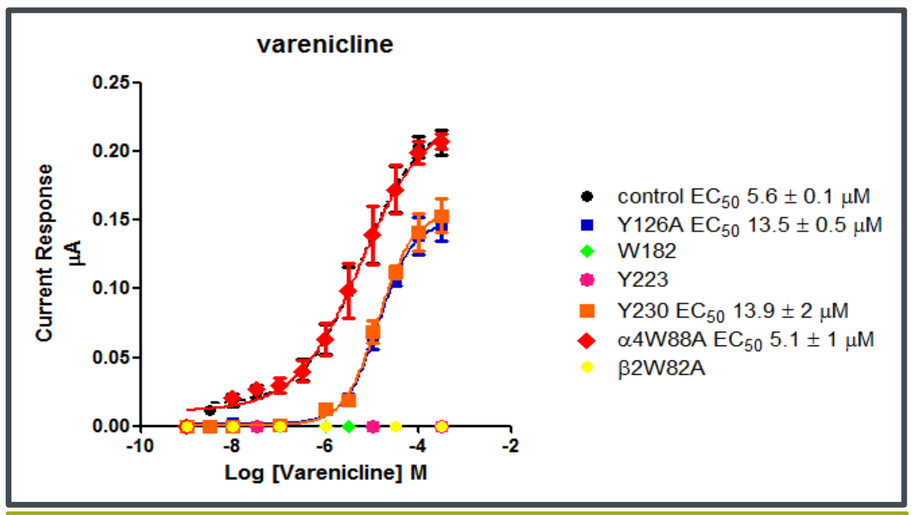
2. RESULTS

Summary:

- Three of the mutations abolished all functionality of receptors for both cytisine and varenicline (W182A, Y223A and W82A)
- Two sites also showed diminished responses for varenicline (Y126A and Y230A) but abolished responses all to cytisine
- W88A showed no significant effect on binding of varenicline compared to wild type, but the response to cytisine was shown by statistical analysis of EC_{50}s to be significantly different from the wild type (see Tables and Graphs)







3. CONCLUSIONS

- There are differences in the molecular binding of varenicline and cytisine
- The differences in binding suggest different molecular interactions between amino acids of the ligand binding domain and the agonist
- Surprisingly, some mutations caused diminished results but still elicited responses, indicating other molecular interactions are present besides cation-π
- Further investigation of other residues could reveal a better image of binding mechanisms
- Removing the function properties of aromatic amino acids known to be involved in agonist binding is a sufficient method to indicate these initial differences and fulfil the specified aims
- The neural pathways of nicotine addiction are extremely complex and no concrete conclusions about the impact of molecular binding on side effects can be drawn from this data alone

The results from this project show that differences do exist between the binding of the two drugs, as well as highlight a few key areas for further investigation, mainly other interactions (besides the cation-π interaction interfered with here) between the ligand binding domain and the agonist and eventually establishing a link between binding interactions and clinical side effects.

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Graphs were produced by the project supervisor using GraphPad and the tables were produced by myself using both GraphPad and Excel.
For more detail, please see my Dissertation: Masureik, R. (2018) *Functional Analysis of the Human Alpha4Beta2 receptor Expressed in Xenopus laevis oocytes for the Molecular Binding of Smoking Cessation Drugs.* Oxford Brookes University.