α7 Nicotinic Acetylcholine Receptor-Mediated Calcium Signalling in Neuronal Cells

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

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Abstract

α7 nicotinic acetylcholine receptors (nAChR) are highly permeable to Ca\textsuperscript{2+} and are clinical targets for Alzheimer's disease and schizophrenia. The aim of this work was to examine α7 nAChR-mediated Ca\textsuperscript{2+} signalling in neuronal cells using three different methods, and to evaluate the effects of the desensitizing agonist and prototypical smoking-cessation drug sazetidine-A on α7 nAChRs.

Initial studies used 96-well plate assays with SH-SY5Y cells to characterize responses evoked by the α7 nAChR-selective agonist PNU-282987 and positive allosteric modulator PNU-120596. This was complemented by live-imaging of cortical cultures, where the compounds evoked robust Ca\textsuperscript{2+} responses from 12 % of cells. Co-application with Cd\textsuperscript{2+}, ryanodine and xestospongin-C significantly inhibited these responses, suggesting the involvement of voltage-gated Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release. CNQX and MK801 also significantly inhibited α7 nAChR mediated Ca\textsuperscript{2+} elevations, indicating a role for glutamate release.

A high-content screening assay was developed to further examine these phenomena. Exploratory experiments using KCl, AMPA and NMDA validated a protocol that could be used to image Ca\textsuperscript{2+} elevations in large cell populations. Inconsistent responses to PNU-120596 and PNU2-282987 were also observed, reflecting the scarcity of α7 nAChRs in cortical cultures and the need for assay optimization. Combination with immunofluorescent labelling revealed α7 nAChR mediated Ca\textsuperscript{2+} elevations in a subpopulation of astrocytes and neurons, some of which were GABAergic.

PNU-120596 potentiated the effects of sazetidine-A in SH-SY5Y cells (EC\textsubscript{50} 0.4 µM) eliciting responses in 14 % of cells in cortical cultures in a methyllycaconitine-sensitive manner, consistent with α7 nAChR activation. Pre-incubation with sazetidine-A concentration-dependently attenuated subsequent α7 nAChR-mediated responses in SH-SY5Y cells (IC\textsubscript{50} 476 nM) and cortical cultures, suggesting that α7 nAChRs could play a role in the behavioural effects of sazetidine-A.

These comparative experiments enhance our understanding of α7 nAChR signalling and provide a new method to study them further.
For my Mother and Father
“What do I know about God and the purpose of life? I know that this world exists.”

– Wittgenstein
# Table of Contents

Acknowledgements ........................................................................................................... xiv

Publications.......................................................................................................................... xv

Abbreviations ...................................................................................................................... xvi

Chapter 1: Introduction........................................................................................................ 1

1.1 ................................................................................................................................. Structure and Diversity of nAChRs

................................................................................................................................. 2

1.1.1 Structural overview ................................................................................................. 2

1.1.2 The ACh binding site ............................................................................................... 4

1.1.3 nAChR diversity and stoichiometry ......................................................................... 7

1.1.4 Gating ..................................................................................................................... 9

  1.1.4.1 From ligand binding to pore opening – Allosteric transitions ................................ 9

  1.1.4.2 The nAChR ion channel – Cation selectivity ......................................................... 10

  1.1.4.3 nAChR desensitization ........................................................................................ 11

1.1.5 Allosteric modulation of nAChRs ........................................................................... 13

  1.1.5.1 Positive allosteric modulators of nAChRs – Type I ................................................ 14

  1.1.5.2 Positive allosteric modulators of nAChRs – Type II .............................................. 18

  1.1.5.3 Negative allosteric modulators of nAChRs .......................................................... 21

1.2 ................................................................................................................................. Physiological roles of nAChRs

................................................................................................................................. 23

1.2.1 nAChR distribution ................................................................................................. 23

1.2.2 nAChR mediated Ca\(^{2+}\) signalling .................................................................... 26

  1.2.2.4 Voltage gated Ca\(^{2+}\) channels .............................................................................. 27

  1.2.2.5 Ionotropic glutamate receptors ........................................................................... 29

  1.2.2.6 Ca\(^{2+}\) permeability of nAChRs .......................................................................... 29

  1.2.2.7 nAChR mediated Ca\(^{2+}\) signaling pathways ......................................................... 31

  1.2.2.8 Ca\(^{2+}\) dependent nAChR modulation of neurotransmitter release ................. 34

  1.2.2.9 Long term Ca\(^{2+}\) dependent effects mediated by nAChRs .................................. 40

1.3 ................................................................................................................................. nAChRs as drug targets

................................................................................................................................. 43

1.3.3 Cholinergic deficits .............................................................................................. 43
1.3.4 Neuroprotection

1.3.5 Addiction

1.4 Summary

1.5 Aims of the thesis

Chapter 2: Materials & Methods

2.1 Materials

2.1.1 Compounds

2.1.2 Antibodies

2.2 Methods

2.2.1 SH-SY5Y cell culture

2.2.2 Primary cell culture

2.2.3 96-well plate Ca^{2+} fluorescence assays

2.2.4 Live cell imaging of Ca^{2+} fluorescence

2.2.5 High content microscopy: Ca^{2+} assays with immunofluorescence

2.2.6 Statistics

Chapter 3: Mechanisms of \alpha_7 nAChR Mediated Calcium Signalling in Primary Cortical Cultures

3.1 Introduction

3.1.1 Measuring intracellular Ca^{2+} signals

3.1.2 SH-SY5Y cells

3.1.3 Mouse primary cerebral cortical cultures

3.1.4 Aims

3.2 Results

3.2.1 PNU1 reveals \alpha_7 nAChR mediated Ca^{2+} elevations SH-SY5Y cells

3.2.2 Preliminary experiments and method development in mouse primary cortical cultures
3.2.3 \( \alpha_7 \) nAChR mediated Ca\(^{2+} \) elevations in mouse cortical cultures are dependent on VGCC activation ................................................................. 78

3.2.4 \( \alpha_7 \) nAChR mediated Ca\(^{2+} \) elevations in mouse cortical cultures are dependent on ER mediated Ca\(^{2+} \) release ................................................................. 79

3.2.5 Contribution of glutamatergic neurotransmission to \( \alpha_7 \) nAChR mediated Ca\(^{2+} \) elevations in mouse cortical cultures ................................................................. 84
  3.2.5.1 Dependence on AMPA receptors ................................................................. 84
  3.2.5.2 Dependence on NMDA receptors ................................................................. 88
  3.2.5.3 Dependence on metabotropic glutamate receptors ................................. 92

3.3 ........................................................................................................... Discussion
  ........................................................................................................... 94

  3.3.1 Summary ................................................................................................. 94
  3.3.2 PNU1 reveals \( \alpha_7 \) nAChR mediated Ca\(^{2+} \) elevations in SH-SY5Y cells .... 95
  3.3.3 Live imaging of \( \alpha_7 \) nAChR mediated Ca\(^{2+} \) elevations in mouse primary cortical cultures .................................................................................. 97
  3.3.4 VGCCs and ER contribute to \( \alpha_7 \) nAChR mediated Ca\(^{2+} \) elevations in mouse primary cortical cultures ................................................................................. 98
  3.3.5 \( \alpha_7 \) nAChR-mediated Ca\(^{2+} \) elevations in mouse primary cortical cultures: evidence of excitatory transmission ......................................................... 102
  3.3.6 Conclusions ......................................................................................... 104

Chapter 4: Development of a High-Content Screening Assay to Investigate Calcium Signalling in Primary Cortical Cultures ........................................ 105

4.1 ........................................................................................................... Introduction
  ........................................................................................................... 106

  4.1.1 High content screening ........................................................................... 106
  4.1.2 Aims .................................................................................................. 107

4.2 ........................................................................................................... Results
  ........................................................................................................... 108

  4.2.1 Validation of a high-content screening Ca\(^{2+} \) imaging assay ................. 108
  4.2.2 Measuring ionotrophic glutamate receptor induced Ca\(^{2+} \) elevations using HCS 112
  4.2.3 \( \alpha_7 \) nAChRs mediated Ca\(^{2+} \) elevations from subpopulations in primary cortical cultures .......................................................................................................... 116
  4.2.4 Immunofluorescent characterization of primary cortical cultures ........ 122
Chapter 5: Characterization of Sazetidine-A at $\alpha_7$ and non-$\alpha_7$ nAChRs

5.1 Introduction

5.2 Results

5.3 Discussion
5.3.4 Desensitization of α7 and non-α7 nAChRs by sazetidine-A in SH-SY5Y cells 173
5.3.5 Desensitization of α7 nAChRs in primary cortical cultures by sazetidine-A 174
5.3.6 Reactivation of desensitized α7 nAChRs by PNU1 ................................. 175
5.3.7 Characterization of novel azetidine/pyrrolidine containing compounds 176
5.3.8 Conclusions ..................................................................................... 177

Chapter 6: General Discussion & Conclusions ............................................. 179

6.1 .............................................................................................................. Conclusions
................................................................................................................. 180
6.1.1 Summary of α7 nAChR-mediated Ca\textsuperscript{2+} signalling mechanisms in primary
cortical cultures ...................................................................................... 180
6.1.2 Comparison of methods used to measure α7 nAChR mediated Ca\textsuperscript{2+} signalling
in this thesis ............................................................................................... 183

6.2 .............................................................................................................. Future Perspectives
................................................................................................................... 185
6.2.1 Further investigations of α7 nAChR-mediated Ca\textsuperscript{2+} signalling in cortical
cultures ........................................................................................................ 186
6.2.2 Higher content ................................................................................... 188

Bibliography .............................................................................................. 191
Table of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.1</td>
<td>Basic structure of nAChRs.</td>
<td>3</td>
</tr>
<tr>
<td>1.1.2</td>
<td>ACh binding site of nAChRs.</td>
<td>5</td>
</tr>
<tr>
<td>1.1.3</td>
<td>Ligand binding induced conformational changes.</td>
<td>6</td>
</tr>
<tr>
<td>1.1.4</td>
<td>Subunit composition of nAChR subunits.</td>
<td>8</td>
</tr>
<tr>
<td>1.1.5</td>
<td>Distinct conformational states of nAChRs in equilibrium.</td>
<td>9</td>
</tr>
<tr>
<td>1.1.6</td>
<td>Amino acid residues lining the nAChR ion channel in the open and closed states.</td>
<td>11</td>
</tr>
<tr>
<td>1.1.7</td>
<td>Type I and II positive allosteric modulators.</td>
<td>14</td>
</tr>
<tr>
<td>1.1.8</td>
<td>Computer docking simulation of proposed PNU-120596 binding cavity.</td>
<td>20</td>
</tr>
<tr>
<td>1.2.1</td>
<td>Distribution of nAChR subtypes in the rodent brain.</td>
<td>24</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Subcellular nAChR distribution.</td>
<td>25</td>
</tr>
<tr>
<td>1.2.3</td>
<td>Possible routes of Ca$^{2+}$ entry into cytosol following nAChR activation.</td>
<td>32</td>
</tr>
<tr>
<td>1.2.4</td>
<td>Presynaptic nAChRs can influence Ca$^{2+}$ signaling events in postsynaptic cells via glutamate release.</td>
<td>35</td>
</tr>
<tr>
<td>3.1.1</td>
<td>Chemical structures of Ca$^{2+}$ chelators EGTA, BAPTA, fura-2 and fluo-2.</td>
<td>60</td>
</tr>
<tr>
<td>3.2.1</td>
<td>PNU1 strongly potentiates αbgt sensitive intracellular calcium elevations in SH-SY5Y cells</td>
<td>65</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Concentration response profile of PNU1 potentiation of choline and PNU2 evoked intracellular calcium elevations in SH-SY5Y cells</td>
<td>67</td>
</tr>
<tr>
<td>3.2.3</td>
<td>PNU1 potentiates low concentrations of choline and PNU2.</td>
<td>68</td>
</tr>
<tr>
<td>3.2.4</td>
<td>KCl evokes robust F$^{340}$:F$^{380}$ increases in mouse primary cortical cultures</td>
<td>69</td>
</tr>
<tr>
<td>3.2.5</td>
<td>PNU2 alone does not elicit a detectable response.</td>
<td>70</td>
</tr>
<tr>
<td>3.2.6</td>
<td>PNU1 alone does not affect F$^{340}$:F$^{380}$, but enhances PNU2 stimulation.</td>
<td>71</td>
</tr>
<tr>
<td>3.2.7</td>
<td>Spontaneous F$^{340}$:F$^{380}$ oscillations are silenced by TTX.</td>
<td>72</td>
</tr>
<tr>
<td>3.2.8</td>
<td>Rising basal F$^{340}$:F$^{380}$ during long periods of recording.</td>
<td>73</td>
</tr>
<tr>
<td>3.2.9</td>
<td>MLA fully blocks responses evoked by PNU1 in combination with PNU2 in mouse primary cortical cultures</td>
<td>75</td>
</tr>
<tr>
<td>3.2.10</td>
<td>Repetitive stimulation of mouse primary cortical cultures with PNU2 in combination with PNU1</td>
<td>76</td>
</tr>
<tr>
<td>3.2.11</td>
<td>α7 mediated calcium elevations are inhibited by voltage gated calcium channel blockade by Cd$^{2+}$ in mouse primary cortical cultures.</td>
<td>79</td>
</tr>
<tr>
<td>3.2.12</td>
<td>α7 mediated calcium elevations are not significantly inhibited by L-type voltage gated calcium channel blockade by nifedipine in mouse primary cortical cultures.</td>
<td>80</td>
</tr>
<tr>
<td>3.2.13</td>
<td>α7 nAChR mediated calcium elevations are inhibited by ryanodine receptor blockade in mouse primary cortical cultures.</td>
<td>81</td>
</tr>
</tbody>
</table>
Figure 3.2.14: α7 nAChR mediated calcium elevations are inhibited by xestospongin C in mouse primary cortical neurons .......................................................... 82
Figure 3.2.15: AMPA evoked calcium responses are blocked by CNQX in mouse primary cortical cultures .................................................................................. 85
Figure 3.2.16 α7 nAChR mediated calcium elevations are partially blocked by CNQX in mouse primary cortical cultures ............................................................... 86
Figure 3.2.17: MK801 blocks NMDA evoked Ca^{2+} elevations in mouse primary cortical cultures ................................................................................................. 87
Figure 3.2.18 α7 nAChR mediated calcium elevations are partially blocked by MK801 in mouse primary cortical cultures ............................................................... 89
Figure 3.2.19 α7 nAChR mediated calcium elevations are partially blocked by CNQX and MK801 in mouse primary cortical cultures .............................................. 90
Figure 3.2.20: Effects of mGluR1 blockade on intracellular calcium elevation elicited by PNU2 in combination with PNU1 ................................................................. 91
Figure 3.2.21: Contributions to α7 mediated [Ca^{2+}]_e elevations in mouse primary cortical cultures ................................................................................................. 92
Figure 4.2.1: KCl evokes robust F_{525} increases in large populations of mouse primary cortical cultures .......................................................................................... 108
Figure 4.2.2: Segmentation of fluorescently labelled primary cortical cultures .................. 109
Figure 4.2.3 KCl evokes robust F_{525} increases in large populations of mouse primary cortical cultures .......................................................................................... 111
Figure 4.2.4: AMPA evoked Ca^{2+} elevations in mouse primary cortical cultures are partially blocked by CNQX ................................................................. 113
Figure 4.2.5: NMDA evoked Ca^{2+} elevations in mouse primary cortical cultures are blocked by D-AP5 ......................................................................................... 114
Figure 4.2.6: Responses to PNU2 in the presence of PNU1 are difficult to detect in large populations of primary cultured cortical cells .............................................. 115
Figure 4.2.7: α7 evoked Ca^{2+} elevations in large populations of mouse primary cortical cultures ................................................................................................. 116
Figure 4.2.8: PNU2 in combination with PNU1 evoked Ca^{2+} elevations in large populations of mouse primary cortical cultures in the presence of bicuculline .............. 117
Figure 4.2.9: Stimulation evoked F_{525} increases in large populations of mouse primary cortical cultured cells .................................................................................. 118
Figure 4.2.10: Percentage of cells responding to various stimuli as measured by changes in F_{525} ........................................................................................................ 119
Figure 4.2.11: Effects of various antagonists on α7 mediated F_{525} increases in large populations of mouse primary cortical cultured cells ........................................ 120
Figure 4.2.12: Immunofluorescent labelling of mouse primary cortical cultures ................. 121
Figure 4.2.13: Attempted segmentation of GFAP positive fluorescent images ....................... 122
Figure 5.2.14: Novel azetidine/pyrrolidine compounds A-E do not compete for binding with epibatidine .................................................................................................................................................. 165

Figure 5.2.15: Effects of novel azetidine/pyrrolidine containing compounds A-E on intracellular calcium elevations in SH-SY5Y cells............................................................................................................................................... 166

Figure 6.1.1 Summary of α7 nAChR-mediated Ca²⁺ elevation mechanisms in mouse primary cortical cultures............................................................................................................................................... 180

Figure 6.1.2: Summary of possible consequences following activation of presynaptic α7 nAChRs in primary cortical cultures............................................................................................................................................... 181

Table 6.1.1: Comparison of methods used to measure intracellular Ca²⁺ elevations in this thesis. ........................................................................................................................................................................ 182
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Publications

Refereed articles


Poster communications


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ca$^{2+}$]$_{ec}$</td>
<td>Extracellular calcium concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$_{ic}$</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>5-HI</td>
<td>5-hydroxyindole</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine; serotonin</td>
</tr>
<tr>
<td>αbgt</td>
<td>α-bungarotoxin</td>
</tr>
<tr>
<td>αCtxMII</td>
<td>α-conotoxin MII</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxymethyl ester</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>AMPAR</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Arc</td>
<td>Activity regulated cytoskeletal associated protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Aβ</td>
<td>β-amyloid</td>
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<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>Bcl</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA1-3</td>
<td>Cornu Ammonis 1-3</td>
</tr>
<tr>
<td>CaBP</td>
<td>Calcium binding proteins</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calcium/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CHRNA7</td>
<td>α7 nAChR gene</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium induced calcium release</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
</tbody>
</table>
CREB | Cyclic adenosine monophosphate response element binding protein  
---|---  
ddH₂O | Distilled, deionised water  
DHβE | Dihyro-β-erythroidine  
DIV | Days in vitro  
DMEM | Dulbecco’s modified eagles media  
DMSO | Dimethylsulphoxide  
EC₅₀ | Concentration of agonist capable of evoking half-maximal response  
ECD | Extracellular domain  
EDTA | Ethylenediaminetetracetic acid  
EGTA | Ethylenglycol-bis-(β-aminoethylether)-N,N,N′,N′'-tetracetic acid  
ER | Endoplasmic reticulum  
ERK | Extracellular signal regulated kinase  
FBS | Foetal bovine serum  
g | gram  
GABA | γ-aminobutyric acid  
GAD | Glutamic acid decarboxylase  
GPCR | G-protein coupled receptor  
GSK3β | Glycogen synthase kinase 3β  
h | Hour  
HCS | High-content screening  
HEK-293 | Human embryonic kidney-293 cells  
HEPES | N-[2-hydroxyethyl]piperazine-N’-[2-ethane sulfonic acid]  
IC₅₀ | Concentration of antagonist capable of reducing maximal response by half  
ICD | Intracellular domain  
IEG | Immediate early gene  
iGluR | Ionotropic glutamate receptor  
IP₃ | Inositol 1,4,5-trisphosphate  
IP₃R | Inositol 1,4,5-trisphosphate receptor  
JAK | Janus Kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
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<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>KYNA</td>
<td>Kynurenic acid</td>
</tr>
<tr>
<td>LDT</td>
<td>Laterodorsal tegmental nucleus</td>
</tr>
<tr>
<td>LGIC</td>
<td>Ligand gated ion channel</td>
</tr>
<tr>
<td>LTD</td>
<td>Long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>Ly6/uPAR</td>
<td>Ly6/urokinase receptor like gene family</td>
</tr>
<tr>
<td>m</td>
<td>Metre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>mec</td>
<td>Mecamylamine</td>
</tr>
<tr>
<td>mEPSC</td>
<td>Miniature excitatory post-synaptic current</td>
</tr>
<tr>
<td>mEPSP</td>
<td>Miniature excitatory post-synaptic potential</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MLA</td>
<td>Methyllycaconitine</td>
</tr>
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<td>NAc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NAM</td>
<td>Negative allosteric modulator</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium/calcium exchanger</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-d-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-d-aspartate receptor</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PAM</td>
<td>Positive allosteric modulator</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PC12</td>
<td>Pheochromocytoma Cells</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>PCSA</td>
<td>Prostate stem cell antigen</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PFC</td>
<td>Pre-frontal cortex</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PNU1</td>
<td>PNU-120596</td>
</tr>
<tr>
<td>PNU2</td>
<td>PNU-282987</td>
</tr>
<tr>
<td>PPN</td>
<td>Pedunculopontine nucleus</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RP</td>
<td>Reserve pool</td>
</tr>
<tr>
<td>RRP</td>
<td>Readily releasable pool</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco-endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SLURP-1/2</td>
<td>Ly6/urokinase receptor like gene family related protein</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SPCA</td>
<td>Secretory pathway calcium ATPase</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TSS</td>
<td>Tyrode’s salt solution</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>VGSCs</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>XeC</td>
<td>Xestospongin-C</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
The animal nervous system, with its unparalleled connectivity, epitomizes the integration of divergent elements forming complex entities in nature. At the heart of this connectivity lies what now constitute the targets of drug development, first theorized by physiologist John Langley in 1905 as a “receptive substance” (Langley, 1905), membrane receptor molecules are the interfaces that transduce specific biological signals between cells. The particular subject of Langley’s insights was the same effector underlying the actions of the poison curare speculated by Claude Bernard to be localized at the junction between nerve and muscle almost 50 years earlier, now known as the nicotinic acetylcholine receptor (nAChR). It was not until 1970 that the nAChR was characterized biochemically (Changeux et al., 1970) as the first ligand gated ion channel (LGIC) ever to be identified. The nAChR is now known to encompass a large family of receptor subtypes with diverse functional properties. They fulfil a range of physiological roles, many of which follow from a receptor mediated increase in intracellular calcium ions (Ca\(^{2+}\)), central to a tremendously diverse range of biological signalling events in a multitude of different cells and tissues. As such, nAChRs are implicated in a spectrum of diseases and it intuitively follows that nAChRs present worthy targets for pharmaceutical intervention in medicine. This introduction will discuss the current state of knowledge about nAChRs as drug targets that modulate cellular Ca\(^{2+}\) signalling, covering their basic structure and function, physiological roles, clinical significance, and relevance to this thesis.

### 1.1 Structure and Diversity of nAChRs

At the centre of complex interactions in nature that give rise to biology are proteins, for which form and function are inextricably linked. Knowledge and understanding of the structure of protein molecules like nAChRs can help us to pharmacologically manipulate their function. This section will describe the structure of nAChRs, their diversity, their function as LGICs and their modulation by a variety of endogenous and exogenous ligands.

#### 1.1.1 Structural overview

The study of some of nature’s most exotic species has provided enlightening and important discoveries regarding nAChRs. The electric predatory fish, *Torpedo marmorata* and *Electrophorus electricus* possess organs that enable the generation of
an electric discharge to stun their prey which were found to express an extremely high density of nAChRs (Kistler and Stroud, 1981), making them ideal sources for the purification, isolation and eventual cloning of nAChRs. This was enabled by the use of another predatory weapon, the venom of the krait snake *Bungarus multicinctus*, α-bungarotoxin (abgt), which binds strongly to muscle nAChRs resulting in their inactivation at the neuromuscular junction, causing paralysis (Changeux et al., 1970).

Early studies resolved the nAChR subunits α, β, γ and δ and their pentameric arrangement in cell membranes (Raftery et al., 1980). Subsequent cloning studies further illuminated the complexity of nAChR diversity (Ballivet et al., 1982; Noda et al., 1982, 1983; Claudio et al., 1983; Seguela et al., 1993; Groot Kormelink and Luyten, 1997; Kormeinka and Luyten, 1997). So far, 17 different nAChR subunits have been identified in vertebrates and labelled α1-10 (α8 only found in birds), β1-4, γ, δ, and ε on the basis of structural similarities. Pentameric assembly of prescribed subunit combinations yields an abundance of nAChR subtypes with distinct functional

![Figure 1.1](image.png)

**Figure 1.1.1: Basic structure of nAChRs.**

- **a.** nAChR subunit topology: a complex folded β-sheet rich extracellular N-terminal domain contains a pair of disulphide bonded cysteine residues that characterizes the cys-loop LGIC receptor superfamily. Four amphipathic transmembrane α-helical domains (M1-M4) are joined by intra- or extracellular loops, forming a variable intracellular domain and a short extracellular C-terminal domain. **b.** Five nAChR subunits arrange in a ring spanning the membrane forming a central pore. ACh binding sites are formed at subunit interfaces. Other sites for alternative ligands exist close to the ACh binding site and within the transmembrane domain. Residues of the M2 transmembrane domain line the lumen of the pore, which selectively allow the passage of mono- and divalent cations. The central pore is also the site of action for channel blocking, non-competitive antagonists.
properties and diverse distribution throughout the nervous system and periphery (Dani and Bertrand, 2007; Albuquerque et al., 2009; Taly et al., 2009; Changeux, 2012).

nAChRs are pentameric LGICs, allosteric proteins whose structure allows the transduction of ligand binding to opening of the intrinsic ion channel leading to cation flux. Figure 1.1.1a shows the basic topology of a single nAChR subunit, comprised of a large complex, folded β-sheet rich extracellular N-terminal domain, 4 transmembrane α-helical domains (M1-M4) joined by intracellular or extracellular loops, and a short extracellular C-terminal end. The N-terminal region of each subunit contains a pair of disulphide-bonded cysteine residues separated by 13 amino acids, known as the ‘cys-loop’, a signature polypeptide motif which defines the ‘cys-loop’ LGIC receptor superfamily, to which GABA_A, glycine and 5-HT_3 receptors also belong (Dani and Bertrand, 2007; Albuquerque et al., 2009). In some subunits, part of the extracellular N-terminal region also forms half of an ACh binding site with another subunit. Upon pentameric arrangement, as shown in Figure 1.1.1b, the membrane spanning regions of nAChR subunits form a central pore, which is lined by the hydrophobic residues of the M2 segments in the closed conformation, whilst the M4 segments bare the outward facing, membrane-lipid interface.

1.1.2 The ACh binding site

The primary endogenous ligand of the nAChR, the neurotransmitter ACh binds to a region on the extracellular domain referred to as the orthosteric or canonical binding site, as do many other non-endogenous agonists such as nicotine. As shown in Figure 1.1.1b, other sites also exist on nAChRs, which accommodate a wealth of alternative ligands that affect nAChR function in various ways expanding their complexity further still (see section 1.1.5).
Much of our knowledge of the orthosteric nAChR binding site with which ACh interacts comes from x-ray crystallographic structures of the ACh binding protein (AChBP) expressed by the aquatic mollusks *Lymnaea stagnalis* (Brejc et al., 2001) and *Bulinus truncatus* (Celie et al., 2005), and the marine mollusk *Aplysia californica* (Hansen et al., 2005). These proteins share ~20% sequence homology with the N-terminal domain of nAChRs, and more importantly an identical ACh binding site to muscle type nAChRs, represented in Figure 1.1.2. Select combinations of nAChR subunit interfaces form ACh binding sites. Subunits α1, α2, α3, α4 and α6 form the principle (+) face, with polypeptide loops A-C, which generally determine ligand selectivity, whereas subunits δ, ε, γ, β2 and β4 can form the complementary (-) face with polypeptide loops D-F, and generally determine ligand affinity (Albuquerque et al., 2009). A minimum of two binding sites is required for full receptor function. α7, α8 (in avian species; not found in mammals), α9 and α10 subunits are capable of forming ACh binding sites with both (+) and (-) faces, and can therefore arrange to form homomeric nAChRs, which contain five binding sites. The remaining subunits α5, β1 and β3 do not form ACh binding sites, but instead are classified as accessory subunits (Kuryatov et al., 2008); they are not classically thought to contribute to
ligand binding but still affect receptor function, and as described in section 1.1.5, it could be possible that these subunits harbour alternative ligand binding sites.

α-subunits are defined by a pair of adjacent cysteine residues (Cys-192, Cys-193; *Torpedo* numbering) on polypeptide loop C of the N-terminal extracellular domain, this loop also contains two tyrosine residues (Tyr-190, Tyr-198, *Torpedo* numbering), which along with a tryptophan-tyrosine pair on both loops A (Trp-86, Tyr-93, *Torpedo* numbering) and B (Trp-149, Tyr-151, *Torpedo* numbering) are responsible for binding ligands via π-cation interactions (Celie et al., 2004; Changeux and Taly, 2008). When an agonist ligand binds, loop C extends round the ligand-binding pocket, moving 7.3 Å towards loop F of the adjacent (-) subunit face and trapping the ligand inside (Figure 1.1.3), resulting in conformational changes that open the receptor ion channel. The extent of this conformational movement varies depending on the efficacy of the agonist; partial agonists with weaker efficacy would cause a more modest rotation. Competitive antagonist binding on the contrary extends loop C in the other direction by 4.3 Å, preventing receptor ion channel opening (Figure 1.1.3; Hansen et al., 2005).

![Figure 1.1.3: Ligand binding induced conformational changes.](image-url)
1.1.3 nAChR diversity and stoichiometry

As described above, the various nAChR subunits form either hetero- or homomeric pentamers of defined composition on the basis of the formation of at least two ACh binding sites. In heterologous expression systems, many more nAChR subunit combinations are expressed than have been evidenced \textit{in vivo}, implying the existence of strict regulatory mechanisms on nAChR expression at the post-translational level (Albuquerque et al., 2009). Figure 1.1.4 represents the most common subunit arrangements in mammalian cells, ranging from the simple homomeric \( \alpha7 \) and \( \alpha9 \) nAChRs to the more complex heteromeric pentamers, such as the \( \alpha4(\beta2)_2\alpha6\beta3 \) and \( (\alpha6\beta2)_2\beta3 \) nAChRs. Additional confusion results from heteromeric nAChRs which contain different ratios of the same subunits, often with significant implications for their function. For example \( \alpha4\beta2^*, \alpha6\beta2^*, \alpha3\beta4^* \) and \( \alpha3\beta2^* \) (\( \ast \) denotes undisclosed subunits; Lukas et al., 1999; see Figure 1.1.4) nAChRs, which can exist with either three \( \alpha \) and two \( \beta \) subunits, or two \( \alpha \) and three \( \beta \) subunits. The most well studied example are the \( (\alpha4)_2(\beta2)_3 \) and \( (\alpha4)_3(\beta2)_2 \) nAChR stoichiometries, which are distinguished by their differential sensitivities to ACh as high-sensitivity (HS) and low-sensitivity (LS) stoichiometries respectively (Nelson et al., 2003; Moroni et al., 2006; Carbone et al., 2009). These arrangements also differ in their conductance (Nelson et al., 2003), \( \text{Ca}^{2+} \) permeability (Tapia et al., 2007; see 1.2.2.6) and sensitivity to various agonists and modulators (see 1.1.5). It has now been reported that a functionally distinct agonist binding site at the \( \alpha4(+) / (\text{--})\alpha4 \) interface of the LS-\( \alpha4\beta2 \) confers these differences in sensitivity, and its occupation by agonists is required for the full activation of this receptor subtype (Harpsøe et al., 2011; Mazzaferro et al., 2011; Benallegue et al., 2013). \( \alpha4\beta2^*, \alpha3\beta4^* \) and \( \alpha3\beta2^* \) nAChRs can also accommodate the accessory \( \alpha5 \) subunit, which confers a range of functional differences, such as increased receptor open kinetics (Nelson and Lindstrom, 1999), increased \( \text{Ca}^{2+} \) permeability (Gerzanich et al., 1998; Tapia et al., 2007; see 1.2.3.3),
Figure 1.1.4: Subunit composition of nAChR subunits.

A diverse repertoire of heteropentamers are formed from various combinations of two subunits of α1, α2, α3, α4, and/or α6 with two subunits of δ, ε, γ, β2 and/or β4, which form the ACh binding site interfaces represented by yellow segments. Subunits α5 and β3 do not form ACh binding sites but can act as accessory subunits in the fifth position denoted by an asterisk. Subunits α7 and α9 can form homopentamers, whilst α10 assembles with α9 to give five ACh binding sites in both cases.
altered desensitization kinetics (Gerzanich et al., 1998; Kuryatov et al., 2008; Wageman et al., 2013; see 1.1.4.3), increased sensitivity to ACh and nicotine (Wang et al., 1996), and resistance to nicotine induced upregulation (Wageman et al., 2013). Recently the possibility of an α4/α5 interface binding site was negated in a site-directed mutagenesis study (Marotta et al., 2013), but interestingly Jin et al. (2013) reported that α5 could take the place of a binding site forming β2 subunit with no effect on receptor function. Together, the assortment of nAChR subunits creates a range of unique pentameric blends whose characteristics differ on many levels from conformational kinetics and ion permeability to ligand sensitivity.

1.1.4 Gating

1.1.4.1 From ligand binding to pore opening – Allosteric transitions

nAChRs are allosteric proteins that exist in an equilibrium between three main conformational states that are described by the Monod-Wyman-Changeux model as

![Figure 1.1.5: Distinct conformational states of nAChRs in equilibrium](image)

nAChRs exist in equilibrium between resting (closed channel), active (open channel) and desensitized (closed channel) conformational states. Transitions between these states are influenced by the binding of ligands. The desensitized state can be further divided into fast and slow onset.

resting, active and desensitized (Figure 1.1.5; Monod, Wyman, & Changeux, 1965). As such, nAChRs exhibit constitutive activity even in the absence of agonists, but binding
of agonists lowers the energy barrier for the transition from resting to open states considerably, as it does also for transition to the desensitized state, in which ligands are tightly bound, but the channel remains closed (see 1.1.4.3). The desensitized state can be further subdivided between fast- and slow-onset. Antagonist binding on the other hand favours the resting state, raising the energy barrier for activation even higher. The nAChR therefore can be viewed as a dynamic process for which the probability of transition between distinct conformational states is strongly influenced by the binding of various ligands.

1.1.4.2 The nAChR ion channel – Cation selectivity

Figure 1.1.6 shows the amino acid residues lining the pore lumen in the Torpedo nAChR in the open and closed states. The coupling of ligand binding to ion channel opening occurs rapidly via conformational changes in the quaternary structure of the nAChR protein. Rotation of loop C upon agonist binding (Figure 1.1.3) generates torque on the α-subunits, which drives the rotation of M2 domains lining the pore resulting in a widening of the channel from ~3 Å to ~8 Å and moving the hydrophobic residues Val-255 and Leu-251 (Torpedo numbering) away from the channel, replacing them with hydrophilic residues Ser-252 and Ser-248 (Torpedo numbering) that allow the passage of hydrated Na\(^+\), K\(^+\) and Ca\(^{2+}\) ions (Albuquerque et al., 2009; Taly et al., 2009). These crucial residues that form a ring lining the pore are referred to as the gate, and they are highly conserved throughout α-subunits (Jensen et al., 2005). Ion selectivity of the channel has been demonstrated by mutagenesis studies to be dependent on multiple residues situated in the ECD and ICD. Mutating Glu-237 on the cytoplasmic side of M2 to alanine (Glu-241 Torpedo numbering) for example abolishes Ca\(^{2+}\) permeability in α7 nAChRs (Galzi et al., 1992; Bertrand et al., 1993). Insertion of a proline residue between positions 236 and 237, as well as replacing Val-251 with a threonine residue converts the α7 nAChR ion selectivity from cationic to anionic (Galzi et al., 1992). In the extracellular region, Li et al. (2011) identified a ring of aspartate residues (Asp-44; α7 numbering), which was confirmed to be essential for Ca\(^{2+}\) permeability by Colón-Sáez and Yakel (2013), who replaced these residues with alanine, abolishing Ca\(^{2+}\) currents measured by fluorescent imaging of a genetically encoded Ca\(^{2+}\) indicator. It can be concluded that some aspects of ion channel selectivity are governed by the effects of individual residues, whereas others
appear to be more dependent on alterations in tertiary and/or quaternary structure of the protein.

![Figure 1.1.6: Amino acid residues lining the nAChR ion channel in the open and closed states.](image)

The M2 transmembrane domains constitute the lumen of the nAChR ion channel pore. In the closed state (a), a ring of non-polar residues facing outwards form a gate (residues indicated by red lines) that prevents the transmission of hydrated cations. Following the binding of agonist molecules, conformational changes in the quaternary structure of the protein cause the M2 α-helices to rotate, replacing the non-polar valine and leucine residues with negatively charged serine residues, which allow the passage of hydrated cations (b; adapted from Jensen et al., 2005; Albuquerque et al., 2009).

### 1.1.4.3 nAChR desensitization

A prominent feature of nAChR behaviour is the intrinsic tendency to succumb to desensitization in the presence of continued agonist stimulation (Quick and Lester, 2002). In this conformation nAChRs can no longer induce a physiological response, although it should be noted that the lack of a physiological response in a certain state does not attribute biological redundancy, which is easily forgotten when it presents itself as an experimental nuisance. Indeed, it has been proposed that the desensitized state could serve as a mechanism for fine tuning a response, protecting cells from over-excitation (from Ca^{2+} especially, see 1.2.2), providing a memory of recent stimuli, and even as a way of sequestering ligands or “trapping” (Quick and Lester, 2002; Giniatullin et al., 2005).

The process of nAChR desensitization, although intrinsic to the receptor, can be altered by many factors such as agonist structure, concentration and duration of exposure; nAChR subunit composition and stoichiometry; and the external
environment, which includes experimental expression systems (Giniatullin et al., 2005). For example, the rate at which \( \alpha 7 \) nAChRs desensitize in the presence of high agonist concentrations is notoriously rapid, on the scale of milliseconds; whereas \( \alpha 4\beta 2^* \) and \( \alpha 3\beta 4^* \) nAChRs desensitize more slowly, within seconds (Fenster et al., 1997). Distinct forms of desensitization have been reported to exist, classically divided between fast- and slow-onset (Figure 1.1.5), although more recently with the aid of high-resolution electrophysiological recordings and the use of allosteric ligands and receptor mutations that modulate desensitization, extensions have been made to the original categories to accommodate more exotic states (Williams et al., 2011c, 2012; see 1.1.5.2).

Fast-onset desensitization occurs rapidly in the presence of higher agonist concentrations (\( \mu \text{M-}\text{mM} \)), whereas slow-onset desensitization as its name suggests occurs more gradually on the scale of seconds to minutes and in response to low concentrations of agonists (nM), because of this it is also termed "high-affinity desensitization" (Giniatullin et al., 2005). The molecular determinants of nAChR desensitization have been alluded to by site directed mutagenesis studies, and are proposed to exist in the M2 domain and the extracellular N-terminal domain. For example, mutation of S284L in the \( \alpha 4 \) nAChR subunit M2 region increases the speed of fast-onset desensitization (Matsushima et al., 2002), whereas Val-287 has been reported to underlie the faster desensitization kinetics conveyed by the \( \beta 2 \) nAChR subunit when combined with \( \alpha 4 \) (De Fusco et al., 2000), and the M2 region residue Lys-247 is crucial to the characteristic rapid desensitization of \( \alpha 7 \) nAChRs (Revah et al., 1991). The inclusion of some nAChRs subunits into the accessory position of heteromeric pentamers has been shown to yield consistent effects on desensitization such as the desensitization-enhancing effect of an \( \alpha 5 \) subunit on \( \alpha 3\beta 2 \) and \( \alpha 3\beta 4 \) (Wang et al., 1996; Gerzanich et al., 1998; Groot-Kormelink et al., 2001), although a recent study reported \( \alpha 5 \) to confer HS-\( \alpha 4\beta 2\beta 3 \) nAChRs greater resistance to desensitization (Wageman et al., 2013). The inclusion of \( \beta 2 \) subunits has been shown to have complimentary effects at different nAChR subtypes, increasing the rate of desensitization of \( \alpha 4\beta 2 \) nAChRs (Nelson et al., 2003), but slowing that of \( \alpha 7 \) nAChRs expressed in \textit{X. laevis} oocytes (Khiroug et al., 2002).

The diversity of nAChR subtypes as governed by their subunit composition conveys distinct ion permeability and desensitization characteristics due to
differences in amino acid sequence in various regions. These properties directly influence the downstream cellular and physiological effects of nAChR activation, which will be the concern of section 1.2, before that however there remains an additional layer of modulatory possibilities to be described that continues the theme of allosteric transitions in nAChRs.

### 1.1.5 Allosteric modulation of nAChRs

Additional sites at which ligand binding also affects the conformational equilibria of nAChRs, known as allosteric or non-competitive binding sites exist at diverse locations such as the N-terminal extracellular region, the non-canonical subunit interface binding sites, sites within the TM domains and regions at the protein-lipid interface (Bertrand and Gopalakrishnan, 2007; Arias, 2010; Thomsen and Mikkelsen, 2012a). Ligands acting in this way are categorized according to their overall effect on nAChR function, although it should be noted that for many compounds acting allosterically at nAChRs, these effects are dependent on multiple factors such as concentration of the ligand, duration of exposure to ligand and nAChR subunit composition and stoichiometry. Compounds that positively affect nAChR function are termed non-competitive agonists or positive allosteric modulators (PAMs – previously known as allosteric potentiating ligands; APLs), again by the way in which their effects are achieved. Non-competitive agonists promote activation via allosteric binding sites, whereas PAMs are defined by their lack of intrinsic agonist activity, but their ability to enhance agonist induced receptor activation. PAMs can be further subdivided by the way in which they bring about enhanced receptor function: those that increase the peak current of a response (type I PAMs) and those that affect the time course of a response (type II PAMs; Figure 1.1.7; Grønlien et al., 2007). Both types increase the potencies of agonists and increase their maximal efficacies. Conversely, compounds that negatively affect nAChR function include non-competitive antagonists and negative allosteric modulators (NAMs), also differentiated by the manner in which their effects are realized: non-competitive antagonists act by interacting with intraluminal sites, blocking the ion channel, a phenomenon that also becomes prevalent at high concentrations of non-specific compounds. NAMs are generally considered to stabilize inactive/non-conducting receptor conformations (Arias, 2010).
1.1.5.1 Positive allosteric modulators of nAChRs – Type I

Non-selective nAChR ligands that have been reported to enhance the peak current response induced by agonist stimulation include the acetylcholinesterase (AChE) inhibitors physostigmine and galantamine, and the morphine derivative codeine, (Storch et al., 1995; Samochocki et al., 2003), the neurotransmitter 5-hydroxytryptamine (5-HT) (Schrattenholz et al., 1996), the plant tropane alkaloids atropine and scopolamine (Zwart and Vijverberg, 1997; Smulders et al., 2005), the C-terminus of AChE (Greenfield et al., 2004), the 2-amino-5-keto thiazole analogue LY2087101 (Broad et al., 2006) and the GABA<sub>A</sub> receptor PAM CCM1 (Ng et al., 2007). α7 nAChR selective type I PAMs include the tryptophan metabolite 5-hydroxyindole (5-HI), (Zwart et al., 2002; Arnaiz-Cot et al., 2008; López-Hernández et al., 2009; Pérez-Alvarez et al., 2012), albumin derivatives (Conroy et al., 2003), the naturally occurring isoflavone and tyrosine kinase inhibitor genistein (Bertrand et al., 2008; Grønlien et al., 2010), the synthetic anthelmintic drugs ivermectin (Krause et al., 2007).
1998; Collins et al., 2011), morantel (Seo et al., 2009; Cesa et al., 2012; Chrisman et al., 2013), pyrantel and oxantel (Cesa et al., 2012), the 5-HT_{2B/C} receptor antagonist SB-206553 (Dunlop et al., 2009), and the urea derivative NS-1738 (Timmermann et al., 2007; Bertrand et al., 2008). The natural bryozoan product desformulflustrabromine (Kim et al., 2007; Liu, 2013), the endogenous estrogenic steroid 17-β-estradiol (Paradiso et al., 2001), Zn^{2+} (Hsiao et al., 2001, 2006, 2008; Moroni et al., 2008), and the synthetic compounds NS-9283 (Timmermann et al., 2012; Olsen et al., 2013) and NS-206 (Olsen et al., 2013) are examples of type I PAMs that show selectivity for α4β2* nAChRs. The endogenous metabolite choline, produced by the hydrolysis of ACh by AChE, which was reported to act as a full agonist at α7 nAChRs and weak partial agonist at α3β4* nAChRs (Alkondon et al., 1997b; Alkondon and Albuquerque, 2006), was also found to potentiate ACh-induced responses at α4β4 nAChRs in *X. laevis* oocytes at concentrations under 10 mM (Zwart and Vijverberg, 2000), at higher concentrations however, choline was found to inhibit ACh responses at α4β2* nAChRs, suggesting a complex, sub-type specific regulatory role for choline at nAChRs (Alkondon and Albuquerque, 2006). Members of the Ly6/urokinase receptor like gene family (Ly6/uPAR) have also been reported to exhibit type I PAM activity (and NAM activity; see section 1.1.5.3) at nAChRs, namely the secreted Ly6/uPAR related protein-1 (SLURP-1) (Chimienti et al., 2003), and Lypd-6 (Darvas et al., 2009).

The first compounds to be observed as nAChR PAMs were the naturally occurring acetylcholinesterase (AChE) inhibitors physostigmine and galantamine, isolated from the calabar bean and the common snowdrop respectively. Both are currently prescribed as symptomatic therapeutics for patients suffering from Alzheimer’s disease, where it is now proposed that in addition to AChE inhibition and subsequent elevation of ACh availability at cholinergic synapses, direct interaction with nAChRs also play a role (Maelicke et al., 2000). Physostigmine was originally reported to act as an open channel blocker, and at lower concentrations, a weak agonist at muscle nAChRs – independent from AChE inhibition. Sensitivity to blockade by competitive nAChR antagonists was at first conflicting (Shaw et al., 1985; Pereira et al., 1993), but later studies provided more evidence that physostigmine, as well as galantamine and the morphine derivative codeine, were acting independently of the ACh binding site (Okonjo et al., 1991; Storch et al., 1995; Akk and Steinbach,
Channel activation by physostigmine and galantamine were inhibited by the monoclonal antibody FK1 and benzoquinonium, implying action at a binding site close to but distinct from that of ACh (Okonjo et al., 1991; Pereira et al., 1993, 1994). Photoaffinity labelling of \[^3^H\]physostigmine at Torpedo nAChR, subsequent proteolytic cleavage and sequencing indicated a binding site within the \(\alpha\) subunit, in which the residue Lys-125 was strongly labelled and therefore implicated in binding (Schrattenholz et al., 1993). Although single-channel activation events have been observed in response to these compounds alone, they did not amount to whole-cell currents as observed in response to ACh. It was therefore suggested that they may act primarily by sensitizing nAChRs to submaximal orthosteric activation (Storch et al., 1995), a concept that was later confirmed (Samochcki et al., 2003). More recent studies using a mutational approach and photoaffinity labelling with protein sequencing methods have provided evidence for multiple binding sites for galantamine and physostigmine at the canonical and non-canonical subunit interfaces, and an intraluminal site, perhaps with different consequences for nAChR activity for each (Hansen and Taylor, 2007; Hamouda et al., 2013).

Discovery of a site that binds exogenous compounds distinct from the ACh binding site raised the possibility of an endogenous ligand existing for this site. Indeed, evidence has been provided for competitive actions of the astrocytic metabolite kynurenic acid (KYNA; see section 1.1.5.3) with galantamine at \(\alpha_7\) nAChRs (Lopes et al., 2007), and interestingly, the neurotransmitter 5-hydroxytryptamine (5-HT) has been reported act in a similar way to galantamine, with potentiating effects that are also sensitive to the FK1 antibody, suggesting actions at the same binding site. As with galantamine, higher, micromolar concentrations inhibited responses to ACh rather than potentiating them (Schrattenholz et al., 1996). These findings may imply cross-neurotransmitter effects as a further modulatory phenomenon in neural networks.

By creating a range of \(\alpha_7/5HT_3\) receptor chimeras, Bertrand et al. (2008) revealed a novel allosteric site in the extracellular M2-M3 loop of \(\alpha_7\) nAChRs which was crucial for the potentiating actions of the synthetic type I PAM NS-1738. Using a similar approach, Grønløen et al. (2010) reported that the same M2-M3 region is important for the actions of the naturally occurring isoflavone genistein, which also acts as a type I \(\alpha_7\) nAChR PAM. Substitution of this region with a 5-HT\(_{3\alpha}\) receptor
sequence however did not affect potentiation elicited by 5-HI, indicating a distinct binding site for genistein and NS-1738.

Interestingly, the α4+/(-)α4 subunit interface at the LS-α4β2 (see 1.1.3) nAChR has recently been characterized as a third, low-sensitivity ACh binding site, whose occupancy is required for full activity, corresponding to the low sensitivity component of the biphasic response to ACh seen with this receptor (Harpsoe et al., 2011; Mazzaferro et al., 2011). Most αβ2 nAChR PAMs so far reported are selective only for the LS-α4β2, but Olsen et al. (2013) have recently reported that the novel αβ selective PAM NS-206 binds to a site distinct to that of NS-9283. NS-206 potentiated ACh evoked responses at both HS- α4β2 and LS-α4β2 nAChRs, whereas NS-9283 only potentiated the LS-α4β2 stoichiometry. When applied together, additive effects were seen, indicating distinct binding sites. This was confirmed by using chimeric nAChRs and site directed mutagenesis approaches, revealing that NS-206 bound to a transmembrane site on the α4 subunit, whereas NS-9283 exerted its actions at the α4+/(-)α4 subunit interface.

As expected for most proteins, ionic content of the surrounding solution is important for function, but more specific interactions of certain ions have been shown to affect nAChR function such as Ca²⁺ and Zn²⁺. Ca²⁺ has been shown to interact with Glu-172 in the (-) face of the ACh binding site producing allosteric potentiation of peak responses and increased agonist affinity at α7 nAChRs (Mulle et al., 1992b; Galzi et al., 1996). Also, Zn²⁺ ions have been reported to confer complementary allosteric actions at different nAChR subtypes. At concentrations of 1-100 μM, Zn²⁺ potentiated ACh current responses at rat α2β2, α2β4, α3β4, α4β2 and α4β4 nAChR heteromers expressed in X. Laevis oocytes, whereas it inhibited ACh responses at α3β2 nAChRs (Hsiao et al., 2001). As with most of the allosteric modulators discussed so far, Zn²⁺ had no effect on the rate of nAChR desensitization, but in contrast to modulation by physostigmine, galantamine, and 5-HI, Zn²⁺ potentiation was seen in the presence of saturating concentrations of ACh (Hsiao et al., 2001). At both high sensitivity (HS)-α4β2 and low sensitivity (LS)-α4β2 nAChRs expressed in X. laevis oocytes, the two non-canonical α4(-)/(+β2 subunit interfaces were found to accommodate Zn²⁺ ions which resulted in inhibition of ACh induced responses. At LS-α4β2 nAChRs however, Zn²⁺ also resulted in a potentiation of ACh induced responses at concentrations of up to 100 μM, above which inhibition was caused.
Potentiation in this case was found to require the α4(+)/-α4 interface (Moroni et al., 2008). Zn$^{2+}$ potentiation was blocked by dyethylpyrocarbonate (DEPC), which carbethoxylates imidazole rings, implicating histidine residues in its coordination with nAChRs. Site directed mutagenesis confirmed that His-195 of the α4(+) face and Glu-224 of the α4(−) face were important for Zn$^{2+}$ potentiation (Hsiao et al., 2006; Moroni et al., 2008).

So far this section has focused on PAMs that increase peak responses but do not affect desensitization rates, widely referred to as type I PAMs. A large proportion of the compounds discussed so far have also been selective for the α7 nAChR subtype, which is the most well studied nAChR subtype with respect to allosteric modulation (Bertrand and Gopalakrishnan, 2007; Thomsen and Mikkelsen, 2012c). It is also the prototypical nAChR subtype for the study of type II PAMs; compounds that affect the time course of receptor activation.

### 1.1.5.2 Positive allosteric modulators of nAChRs – Type II

Due to the rapid desensitization of α7 nAChRs in the continued presence of agonist molecules, measurement of their responses requires high-resolution electrophysiological techniques combined with rapid solution exchange methods (Papke and Porter Papke, 2002; Fedorov et al., 2012). Type II PAMs have therefore become extremely useful for studying α7 nAChRs, as they massively prolong receptor activation, boosting the overall signal. The urea derivative PNU-120596 (PNU1; Hurst et al., 2005), used extensively in this thesis to reveal α7 nAChR responses is certainly the most well studied compound of this class, others include TQS (Grønlien et al., 2007; Gill et al., 2011), A-867744 (Faghih et al., 2009), A-549291 (Gopalakrishnan et al., 2011), JNJ-1930942 (Dinklo et al., 2011) and the intriguing allosteric agonist 4BP-TQS (Gill et al., 2011; Papke et al., 2013).

As measured by two-electrode voltage-clamp electrophysiology in *X. laevis* oocytes expressing α7 nAChRs, the character of ACh-induced currents in the presence of PNU1 and TQS are radically different from responses to ACh alone, or in the presence of the peak current enhancing type I PAMs such as 5-HI, genistein or NS-1738 (figure 1.1.7; Grønlien et al., 2007). The typical rapidly decaying currents evoked by ACh at α7 nAChRs are greatly prolonged. Further scrutiny of these responses indicates a distinct secondary component, more evident at lower PAM
concentrations, as a weakly decaying, slower onset and higher peak current response (Figure 1.1.7). It has been hypothesized that type II PAMs destabilize the agonist induced desensitized state of α7 nAChRs, driving the receptor back to the open conformation. As the response decays however, the receptor appears to exhibit a distinct form of desensitization that is insensitive to further activation by agonist in the presence of PAM, and also depends on the concentration of agonist (Williams et al., 2011b).

A parallel between the effects of agonist stimulation observed in the presence of type II PAMs at wild type α7 nAChRs can be drawn with the behaviour of the L247T (the ion channel gate; see Figure 1.1.6) mutant α7 in response to agonist stimulation (Revah et al., 1991). The mutation affects the pore region, and renders what would usually be a desensitized non-conducting state a conducting one. In support of this, compounds that usually stabilize the desensitized state such as DHβE, hexamethonium and tubocurarine were found to elicit large responses, whereas αbgt blocked them, indicating that it instead stabilizes the resting state (Bertrand et al., 1992).

Computer docking studies conducted by Young et al. (2008) proposed PNU1 to bind within a cavity inside the transmembrane domains of α7 nAChRs, surrounded by the residues Ser-222 and Ala-225 from the M1 domain, Met-253 from the M2 domain, and Phe-455 and Cys-459 from the M4 domain (Figure 1.1.8). Interestingly, the same study also reported this to be the site of action of LY-2087101, which in contrast to PNU1 does not affect the time course of agonist induced responses, highlighting the importance of a reciprocal relationship between ligands and binding sites for the overall effect of receptor function. It was also recently published that potentiation by PNU1 is highly cooperative and requires at least 4 binding sites to be occupied to confer potentiation, and even here, potentiation is roughly half that of a fully saturated pentamer (Dacosta and Sine, 2013).
Perhaps the most striking effects of a compound acting at an allosteric site so far reported are those of the TQS derivative 4BP-TQS, again thought to act within the TMDs. Like PNU1 and TQS, 4BP-TQS greatly enhances agonist-induced responses and reduces desensitization, but also has highly potent and efficacious intrinsic agonist activity and does not compete for binding at the orthosteric site. The selective α7 nAChR competitive antagonist methyllycaconitine (MLA) blocked 4BP-TQS in a non-competitive manner, and the mutation M253L in the proposed type II PAM binding site in the TMDs (Young et al., 2008) abolished 4BP-TQS agonist activity, whereas mutating the orthosteric ACh binding site W148F had no effect on 4BP-TQS activity but denied ACh activity (Gill et al., 2011). In a more recent study, Papke et al., (2013) proposed a separate binding site for the agonist actions of 4BP-TQS distinct from the orthosteric site and the TMD site on the basis that its agonist activity is rapidly reversible upon washout, as opposed to its potentiating activity, and the activation is MLA sensitive. The residue Trp-55 was found to be essential for coupling the orthosteric site to the transmembrane site, as mutating this residue removed the necessity for simultaneous orthosteric agonist binding with PAM binding to elicit maximum responses from 4BP-TQS, and conveying intrinsic agonist activity to PNU1 and TQS, it also rendered 4BP-TQS induced activation insensitive to blockade by MLA.
1.1.5.3 Negative allosteric modulators of nAChRs

A variety of compounds have been reported to negatively affect nAChR function, by either channel blocking or stabilizing the non-conducting resting or desensitized conformational states. Compounds that act as non-specific NAMs at nAChRs include channel blockers like MK801, amantadine, memantine (Buisson and Bertrand, 1998) and mecamylamine (Varanda et al., 1985); steroid hormones such as progesterone (Valera et al., 1992); fatty acids (Dalziel et al., 1980; Fernández-Carvajal et al., 2006; Colón-Sáez and Yakel, 2011); members of the Ly-6/uPAR gene family lynx-1 (Miwa et al., 1999; Ibañez-Tallon et al., 2002), lynx-2 (Tekinay et al., 2009), and the prostate stem cell antigen (PSCA; Hruska et al., 2009); the synthetic compounds UCI 30002 (Yoshimura et al., 2007) and COB-3 (González-Cestari et al., 2009); the west African psychedelic plant drug ibogaine (Arias et al., 2010); the dissociative drug of abuse phencyclidine (PCP; Arias et al., 2010); the antidepressant bupropion (Alkondon and Albuquerque, 2005; Radhakrishnan et al., 2013); the flavouring and cigarette additive menthol (Hans et al., 2012; Ashoor et al., 2013; Kabbani, 2013); and various general anaesthetics (Bondarenko et al., 2013; Mowrey et al., 2013). Negative regulators of α7 nAChRs have been reported to include the endogenous metabolite KYNA (Hilmas et al., 2001; Albuquerque and Scwarz, 2013), the cannabinoid receptor agonist anandamide (Oz et al., 2003), the Alzheimer’s Disease associated amyloid-β (Aβ) protein (Parri et al., 2011), and the synthetic compound HDMP (Abdrakhmanova et al., 2010). For α4β2 nAChRs, Zn$^{2+}$ (Moroni et al., 2008) and the synthetic COB-3 analogue KAB-18 (Henderson et al., 2010) have been described to exhibit NAM effects.

Aside from channel blocking sites, NAMs have been proposed to bind to diverse sites on nAChRs. KYNA is thought to exert its actions at the FK1 antibody binding site originally proposed for physostigmine, galantamine and codeine (see 1.1.5.1) on the ECD of α-subunits a short distance from the orthosteric binding site. NAMs acting at subunit interfaces include Zn$^{2+}$ and KAB-18. Zn$^{2+}$ is thought to exert inhibitory effects via a site at the non-canonical α4(-)/(+)/β2 subunit interface involving His-195 on the α4 subunit and Asp-218 on the β4 subunit (Moroni et al., 2008; see 1.1.5.4), whilst KAB-18 was hypothesized to bind 10 Å from the canonical α4(+) /(-)β2 binding site with residues Phe-118, Glu-60 and Thr-58 on the β2 subunit, resulting in a proposed obstruction of C-loop closure around the orthosteric agonist binding site (Pavlovicz et al., 2011; Henderson et al., 2012). Elsewhere, a
transmembrane binding site was recently proposed to mediate the inhibitory actions of menthol at α7 nAChRs, involving residues Thr-292 and Leu-250 based on computer modelling of muscle type nAChRs (Ashoor et al., 2013).

The Ly-6/uPAR gene family products, lynx-1 and -2, and the analogous prostate stem cell antigen (PSCA) are structurally homologous to αbgt and lynx-1 has been shown to compete with [125I]αbgt for binding at α7 nAChRs (Lyukmanova et al., 2011). It is anchored to the membrane via a glycophasmatidylinositol (GPI) anchor (Miwa et al., 1999), and was found to enhance α7 nAChR mediated responses to ACh at 1 μM, but inhibit α7, α4β2* and α3β2* nAChRs at 10 μM (Lyukmanova et al., 2011). Lynx-2 was found to decrease ACh potency at α4β2 nAChRs expressed in X. laevis oocytes (Tekinay et al., 2009), and PSCA was reported to antagonize α7 nAChR mediated responses (Hruska et al., 2009). Together the Ly-6/uPAR gene family products (also see 1.1.5.1) represent a class of endogenous allosteric modulators that regulate nAChR function.

Perhaps less conventionally, the natural environment surrounding nAChRs can be described as having properties that allosterically affect receptor function, such as the composition of phospholipids and cholesterol content in the cell membrane, and the ionic content of the aqueous phases that encapsulate the intracellular and extracellular domains (ICD; ECD; described above; 1.1.5.1). As well as providing a selective barrier to cells, the phospholipid bilayer constituting the cell membrane provides a fluid environment that permits a degree of motility to allow conformational changes of transmembrane proteins to occur. Cell membranes are heterogeneous entities composed of a range of different phospholipids whose physical properties contribute to the overall fluid character of the membrane and affect transmembrane proteins more directly at the protein-lipid interface. This occurs via electrostatic interactions or hydrogen bonds between positive charges on the protein and phosphate carbonyl of the hydrophilic layer of the membrane, and also by van der Waals interactions between hydrophobic residues on the protein and fatty-acid chains of the membrane (Poveda et al., 2013). Cholesterol presents another important constituent of cell membranes, and was found in early studies to be required for proper functioning of purified nAChRs (Dalziel et al., 1980). It has also been demonstrated to contribute to the speed of α7 nAChRs desensitization and agonist affinity (Colón-Sáez and Yakel, 2011). Here, when cholesterol and
sphingomyelin were depleted by sequestering agents and enzymatic breakdown, α7 nAChRs desensitized more slowly, an effect that was reversed upon replacement of cholesterol and sphingomylin, implicating these molecules as α7 nAChR NAMs.

Together, the various compounds described above and their diverse actions at nAChRs form fragments of a bewilderingly complex puzzle that can only be expected to deepen as high throughput drug discovery programs are utilized and greater resolution structural details of receptors are elucidated. Observations of this wealth of modulatory phenomena have shifted our understanding of nAChRs from the simple ligand-binding, open/closed binary view of LGICs to that of a dynamic equilibrium of interconvertible conformational states permitted by the three-dimensional protein structure and its surrounding environment.

Having focused on the nAChR structure and their behaviour and modulation by endogenous and exogenous factors, it is now appropriate to discuss their implications in a physiological setting. The scope of the next section will expand beyond the horizon of the nAChR receptor protein and into the processes it influences in cells, and how they are integrated into biological systems such as neural networks. A major determinant of these effects is the high relative permeability of nAChRs to Ca$^{2+}$, a ubiquitous and fundamental biological actor integral to a vast and diverse range of biochemical processes from reproduction to programmed cell death, memory formation to muscle contraction. The mechanisms of nAChR mediated Ca$^{2+}$ signalling are the focus of this thesis, and will be discussed in the following section.

1.2 Physiological roles of nAChRs

nAChRs are expressed throughout the body and respond primarily to ACh, this section will strive to communicate the diversity of events mediated by nAChRs, which project from their fundamental properties as LGICs. As much of this diversity is shaped by the surrounding environment, it is of great relevance to first summarize the distribution of nAChRs, with a focus on the mammalian CNS.

1.2.1 nAChR distribution

nAChRs are expressed in a wide range of neuronal and non-neuronal cells, and within distinct sub-cellular locations. Various techniques have been used to ascertain this including mRNA analysis, electron microscopy, radioligand binding,
immunoprecipitation, functional studies, positron emission tomography (PET) and single-photon emission computed tomography (SPECT). Although these methods each has its limitations, such as lack of specificity or spatial resolution, together they have contributed towards a substantial understanding of the macro- and microscopic distribution of nAChRs in the body (Gotti and Clementi, 2004; Gotti et al., 2006; Hurst et al., 2013).

In peripheral, non-neuronal regions, nAChRs are expressed in cells as diverse as those of the immune system, skin, muscle, kidneys, bladder, lungs, cardiovascular system, and auditoryvestibular system (Wessler and Kirkpatrick, 2008; D’hoedt and Bertrand, 2009; Hurst et al., 2013). nAChRs are also extensively expressed in the autonomic nervous system where their main function is to mediate fast excitatory synaptic transmission. Here, subunits α3, α5, α7, β2 and β4 combine to form nAChRs subtypes comprised of α3β4(α5/β2) and α7 pentamers (Rust et al., 1994). The main focus of experiments within this thesis however is on the role of nAChRs in cells of the CNS, where nAChRs are expressed on neuronal cells at pre-, post- and peri-
synaptic subcellular locations (Figure 1.2.2), and on glial cells (Albuquerque et al., 2009).

Figure 1.2.1 shows the broad and overlapping expression of nAChR subtypes in the various regions of the rodent brain, reflecting widespread cholinergic innervation originating from the basal forebrain nuclei, pedunculopontine (PPN) and laterodorsal tegmental nuclei (LDT), and also from cholinergic interneurons in various brain regions such as the cortex and hippocampus. It is also thought however, that a significant proportion of nAChRs in the CNS respond to diffuse, paracrine levels of ACh and perhaps choline in a process known as volume transmission (Sarter et al., 2009). The α7 and α4β2* subtypes are the most widely and abundantly expressed, especially in regions such as the cortex and hippocampus, associated with learning and declarative memory processes (Marks et al., 1986; Gotti et al., 2006). It is important to note that due to the far-reaching connectivity of the brain, various nAChR subtypes that are not expressed by cells originating in a particular region of the brain may exist on pre-synaptic terminals of neurons projecting to that region and hence will exert local influence. This is of particular relevance to experiments that comprise this thesis, which were conducted on primary cultured cells from the mouse cortex, which receives innervation from diverse subcortical regions in the intact brain. Culturing the cortex alone will omit any of this innervation and the respective nAChRs expressed at terminal regions.

Some nAChR subtypes are much more restricted in their expression pattern to various discreet brain regions such as the α6* nAChRs.
The experimental systems in this thesis however, comprise SH-SY5Y neuroblastoma cells and mouse primary cortical cultures (see section 3.1). Neurons of the cortex have been found to express α7 and α4β2(α5) nAChRs, with α7 nAChRs also expressed by astrocytes (Gotti et al., 2006; Mao et al., 2008; Pirttimaki et al., 2013).

1.2.2 nAChR mediated Ca^{2+} signalling

The coupling of ligand binding to ion channel opening in nAChRs results in a selective alteration of the physiological ionic gradient usually maintained by the cell membrane. By virtue of the nAChR ion channel selectivity filter (see 1.1.4.2), intracellular effects of nAChR activation begin with the influx of Na\(^+\) and Ca\(^{2+}\) ions. Ca\(^{2+}\) is well suited to its role in biological signalling, as a divalent cation it forms more stable complexes than its monovalent counterparts, and due to the geometry, flexibility and dimensions of protein structures, Ca\(^{2+}\)'s larger radius provides a better fit than that of Mg\(^{2+}\) within the various binding pockets presented by proteins (Bading, 2013). Binding and coordination reactions also occur ~1000 times faster with Ca\(^{2+}\) than with Mg\(^{2+}\) (Williams, 2006). The concentration of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{ic}\)) is strictly controlled; several reasons can be ascribed to this: (i) the environment in which primitive organisms evolved on Earth is thought to have had relatively similar [Ca\(^{2+}\)] to resting cytoplasmic [Ca\(^{2+}\)] (~100 nM) of today's organisms, which gradually increased with the geological evolution of Earth (Kazmierczak et al., 2013); (ii) high [Ca\(^{2+}\)] results in the disruption of key biochemicals such as phosphate groups, proteins, nucleic acids and lipid membranes (Kazmierczak et al., 2013); and (iii) the heterogeneity of Ca\(^{2+}\) dependent biological processes and the ubiquity of Ca\(^{2+}\) as a signalling entity. Spatial and temporal control of biological Ca\(^{2+}\) signals is achieved by specialized cell morphologies that convey cellular microdomains such as neuronal dendritic spines or astrocytic processes, membrane-bound organelles to provide a barrier as in the case of the endoplasmic reticulum and mitochondria, and various calcium binding proteins (CaBPs) or active ion pumps that strongly buffer the intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{ic}\)) (Grienberger and Konnerth, 2012; Bading, 2013). The maintenance of a steep concentration gradient for Ca\(^{2+}\) provides a driving force for rapid influx, and several amplification mechanisms exist to boost Ca\(^{2+}\) signals above the threshold needed to overcome Ca\(^{2+}\) buffering or extrusion mechanisms (Berridge et al., 2003).
LGICs such as nAChRs; purinergic P2X receptors; transient receptor type C (TRPC) channels; and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) type ionotropic glutamate receptors (iGluRs; see 1.2.2.5) comprise the starting point for Ca\(^{2+}\) entry into cells either directly or by a two step process requiring Na\(^+\) influx which results in membrane depolarization, and subsequent activation of voltage gated Ca\(^{2+}\) channels (VGCCs; see 1.2.2.4) (Berridge, 1998; Berridge et al., 2003; Grienberger and Konnerth, 2012; Bading, 2013). Elevated [Ca\(^{2+}\)]\(_c\) from either of these sources may then activate Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR) from the endoplasmic reticulum (ER) or Golgi apparatus via stimulation of ryanodine receptors (RyRs) or inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs) (Berridge, 1998; Berridge et al., 2003; Grienberger and Konnerth, 2012; Bading, 2013). G-protein coupled receptor activation can also lead to IP\(_3\)R mediated Ca\(^{2+}\) release from the ER via G\(_q\)-mediated activation of phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), releasing IP\(_3\) (Berridge, 1998; Berridge et al., 2003; Grienberger and Konnerth, 2012; Bading, 2013). Various Ca\(^{2+}\)-dependent protein signalling cascades proceed from any of these points depending on location within the cell. Intracellular free Ca\(^{2+}\) is also rapidly sequestered by (i) CaBPs such calbindin D-28, calretinin and parvalbumin in the cytoplasm, and calnexin calreticulin and calsequestrin in the ER; (ii) active pumps such as the plasma membrane Ca\(^{2+}\) ATPases (PMCAs), sarco(endo)plasmic reticulum Ca\(^{2+}\) ATPases (SERCAs) or secretory pathway (Golgi) pumps (SPCAs); or (iii) membrane ion exchanger proteins such as the Na\(^+\)/Ca\(^{2+}\) exchangers (NCXs) found on plasma, ER and mitochondrial membranes, as well as mitochondrial H\(^+\)/Ca\(^{2+}\) exchangers and the mitochondrial Ca\(^{2+}\) uniporter and permeability transition pores (Berridge, 1998; Berridge et al., 2003; Bading, 2013).

The aim of this section is to describe the details of nAChR mediated events with a particular focus on intracellular Ca\(^{2+}\) signalling, introducing concepts that are vital to the majority of experiments that comprise this thesis, and beginning with a summary of the various cellular contributors of Ca\(^{2+}\) signals.

**1.2.2.4 Voltage gated Ca\(^{2+}\) channels**

Expressed at the cell membrane, VGCCs are pivotal molecular threshold detectors that play a role in the transduction of electrical stimuli into the elevation of [Ca\(^{2+}\)]\(_c\) to
initiate diverse physiological events in a broad range of cell types (Catterall, 2011). The presence of a voltage sensor, thought to be conferred by a single, arginine-rich transmembrane α-helix in each of the four pore forming six-transmembrane repeats of the protein allows the coupling of electrical excitation to Ca\textsuperscript{2+} flux (Bezanilla, 2000). An extracellular pore loop containing glutamate residues has been shown to confer Ca\textsuperscript{2+} selectivity (Heinemann et al., 1992), similar to that seen in nAChRs (see 1.2.2.6). Subtypes are distinguished according to their pore-forming subunits, biophysical characteristics and pharmacological distinctions, which are expressed in different cell types and subcellular domains, summarized in Table 1.2.1.

L-type VGCCs (Ca\textsubscript{v}1.1-1.4) are characterized by their long-lasting Ca\textsuperscript{2+} currents and sensitivity to dihydropyridine drugs such as nifedipine, they mediate excitation-contraction coupling in skeletal, smooth and cardiac muscle; endocrine secretion; gene transcription; and in neurons are found predominantly on cell bodies and dendrites (Catterall, 2000). P/Q- (Ca\textsubscript{v}2.1) and N-type (Ca\textsubscript{v}2.2) channels are often associated with the release of neurotransmitters at nerve terminals and are sensitive to blockade by ω-conotoxin GVIA and ω-agatoxin respectively (Catterall and Few, 2008). R-type (Ca\textsubscript{v}2.3) channels can be blocked by the tarantula toxin SNX-492, and are found on dendrites, whilst the novel drug ML218 inhibits T-type channels, which are characterized by their transient activation and are involved in pacemaker activity and repetitive firing (Catterall, 2000; Xiang et al., 2011).

<table>
<thead>
<tr>
<th>Channel</th>
<th>Current</th>
<th>Pharmacology</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca\textsubscript{v}1.1-1.4</td>
<td>L (&quot;Long-lasting&quot;/&quot;DHP sensitive&quot;)</td>
<td>Dihydropyridine sensitive</td>
<td>Muscle contraction; endocrine secretion; transcription; neuronal Ca\textsuperscript{2+} transients in cell bodies, dendrites; audition; neurotransmitter release from sensory cells</td>
</tr>
<tr>
<td>Ca\textsubscript{v}2.1</td>
<td>P/Q (&quot;Purkinje&quot;)</td>
<td>ω-agatoxin sensitive</td>
<td>Neurotransmitter release; dendritic Ca\textsuperscript{2+} transients</td>
</tr>
<tr>
<td>Ca\textsubscript{v}2.2</td>
<td>N (&quot;Neuronal&quot;)</td>
<td>ω-conotoxin-GVIA sensitive</td>
<td>Neurotransmitter release; dendritic Ca\textsuperscript{2+} transients</td>
</tr>
<tr>
<td>Ca\textsubscript{v}2.3</td>
<td>R (&quot;Residual&quot;)</td>
<td>SNX-482 sensitive</td>
<td>Neurotransmitter release; dendritic Ca\textsuperscript{2+} transients</td>
</tr>
<tr>
<td>Ca\textsubscript{v}3.1-3.3</td>
<td>T (&quot;Transient&quot;)</td>
<td>-</td>
<td>Pacemaking and repetitive firing</td>
</tr>
</tbody>
</table>
1.2.2.5 Ionotropic glutamate receptors

The primary mediators of fast excitatory transmission in the CNS, iGluRs comprise tetrameric glutamate-gated cation channels split into three groups on the basis of agonist pharmacology as AMPA, kainate, and NMDA receptors (Traynelis et al., 2010). With respect to Ca$^{2+}$ permeability, NMDARs dominate this class of receptors and are crucial initiators of Ca$^{2+}$ signalling events that underlie long-term synaptic changes. Ca$^{2+}$ permeability of NMDARs is affected by subunit composition and phosphorylation, and more importantly their activation requires co-agonism of glutamate with either D-serine or glycine, and is voltage-dependent due to Mg$^{2+}$ blockade at resting membrane potentials. These characteristics add an important level of signal control considering the gravity of NMDAR effects on cell behaviour, and qualify NMDARs as coincidence detectors. Indeed, over-activation of NMDARs, especially in non-synaptic regions is known to result in excitotoxicity (Hardingham et al., 2002; Lynch and Guttmann, 2002).

NMDARs work in concert with AMPA receptors, which primarily flux Na$^{+}$ in response to glutamate-induced activation, providing the necessary membrane depolarization to initiate excitatory postsynaptic potentials (EPSCs) and remove the Mg$^{2+}$ block on NMDARs (Traynelis et al., 2010). AMPAR tetramers lacking the GluA2 subunit have a higher relative permeability to Ca$^{2+}$ and can also initiate cellular Ca$^{2+}$ signalling events independently of NMDARs (Jonas et al., 1994; Perkinton et al., 1999).

1.2.2.6 Ca$^{2+}$ permeability of nAChRs

nAChRs have a high relative Ca$^{2+}$ permeability, which makes them important initiators of cellular Ca$^{2+}$ signalling pathways. Determination of Ca$^{2+}$ permeability of ion channels was initially ascertained by whole-cell electrophysiological measurements of ion channel currents at different holding potentials, allowing the ionic reversal potential to be estimated (potential difference at which current equals zero). When repeated with different concentrations of extracellular Ca$^{2+}$ ($[\text{Ca}^{2+}]_{\text{ec}}$), a shift in the reversal potential occurs, which allows the theoretical estimation of the relative permeability of Ca$^{2+}$ to Na$^{+}$ ($P_{\text{Ca}}/P_{\text{Na}}$) by using the Goldmann-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949; Lewis, 1979). As described in section 1.1.5 the environment surrounding an ion channel receptor, including $[\text{Ca}^{2+}]_{\text{ec}}$
can significantly affect its function, presenting a major drawback in this method when the necessary whole cell recordings are made in the presence of very high $[\text{Ca}^{2+}]_{\text{ec}}$ (Mulle et al., 1992b). A more direct method was pioneered by Neher and colleagues (Schneggenburger et al., 1993), which allows an estimate of fractional $\text{Ca}^{2+}$ current ($P_f$) to be made by combining fluorimetric measurements of cells loaded with the ratiometric $\text{Ca}^{2+}$ indicator fura-2 (Grynkiewicz et al., 1985; see section 3.1.1) with the recording of whole-cell currents. Caveats also exist with this technique, as the calculation of $[\text{Ca}^{2+}]_{\text{ec}}$ from ratiometric fluorescence values relies on a sophisticated calibration protocol, and an assumption that the sole source of $\text{Ca}^{2+}$ influx is through the ion channels in question. This can be controlled for with respect to VGCCs by maintaining a constant voltage clamp, but not for CICR from the ER. Finally, it must be assumed that any $\text{Ca}^{2+}$ influx will be completely bound by fura-2 so that it can be measured (Fucile, 2004; Pankratov and Lalo, 2013). Despite these imperfections, the above techniques have been used extensively to provide estimates of ion channel $\text{Ca}^{2+}$ permeability, which are useful for comparison (Reviewed by Fucile, 2004; Pankratov and Lalo, 2013).

$\text{Ca}^{2+}$ permeability of nAChRs is dependent on subunit composition and stoichiometry (see 1.1.3), the most notable being that of the homomeric nAChRs such as the $\alpha_7$ subtype, which was reported to exhibit a $P_f$ of 11.4 % and 8.8 % for human and rat $\alpha_7$ nAChRs transiently expressed in GH4C1 cells respectively (Fucile et al., 2003). This rivals that of NMDARs which was reported to command $P_f$ of 8.2 % to 14.1 % depending on subunit composition and expression system (Burnashev et al., 1995; Garaschuk et al., 1996; Egan and Khakh, 2004). In contrast, $P_f$ determined for both human and chick $\alpha_4\beta_2$ nAChRs expressed in human embryonic kidney-293 (HEK293) cells was much lower at 3.1 % (Egan and Khakh, 2004). Using the reversal potential method on $X.\ laevis$ oocytes heterologously expressing various nAChR subtypes, a study by Tapia et al. (2007) reported that $\text{Ca}^{2+}$ permeability of heteromeric nAChRs is increased by the incorporation of subunits $\alpha_4$, $\alpha_5$ and $\beta_3$ due to a negatively charged glutamate residue (Glu-261; $\alpha_4$ numbering) on the extracellular edge of the M2 domain, which in $\alpha_7$ nAChR pentamers forms a ring that is crucial for cationic selectivity (Galzi et al., 1992; see 1.1.4.2). At $\beta_2$ subunits however, a positively charged lysine residue in the same position restricts $\text{Ca}^{2+}$
permeability. In line with this, Galzi et al., (1992) also reported higher relative Ca\(^{2+}\) permeability for LS-\(\alpha4\beta2\) than HS-\(\alpha4\beta2\).

### 1.2.2.7 nAChR mediated Ca\(^{2+}\) signalling pathways

In Figure 1.2.3, a simplified diagram of a cell shows the basic mechanisms of Ca\(^{2+}\) signal amplification following nAChR activation which can be summarized as: (i) Ca\(^{2+}\) influx through nAChR, (ii) depolarization of the cell membrane mediated by Na\(^+\) influx through nAChRs causing activation of VGCCs, (iii) CICR from ER via RyRs and/or IP\(_3\)Rs, and (iv) IP\(_3\) mediated Ca\(^{2+}\) release from ER. Interestingly, the first two routes are thought to be mutually exclusive as nAChR mediated Ca\(^{2+}\) influx occurs at resting or hyperpolarized membrane potentials, whereas VGCCs as their name suggests are only activated under conditions of depolarization, illustrating divergent mechanisms for different physiological situations (Mulle et al., 1992a). Further confounding this simplistic categorization, local LGIC or VGCC mediated [Ca\(^{2+}\)]\(_{ic}\) elevations can cause subsequent CICR from the ER. More specifically, the ER has been shown to act as a kind of Ca\(^{2+}\) capacitor: as Ca\(^{2+}\) enters the cytosol via LGICs or VGCCs, SERCAs actively pump it into the ER, loading it with Ca\(^{2+}\) (Garaschuk et al., 1997; Berridge, 1998). Periods of more intense neuronal stimulation can therefore lead to a cumulative increase in ER [Ca\(^{2+}\)], sensitizing them to further stimulation. The buildup of Ca\(^{2+}\) slowly decreases over time however, as Ca\(^{2+}\) leaks back into the cytoplasm where it is rapidly buffered by CaBPs, or diffuses within the ER. This system provides a memory of recent excitatory events in the same cellular location, and serves as a threshold mechanism by which larger global Ca\(^{2+}\) signals can be generated that initiate long term changes in cell behaviour by being propagated to the nucleus (Berridge, 1998; Bading, 2013).

Another synergistic effect exists involving the IP\(_3\)R, which can be activated by either Ca\(^{2+}\) or IP\(_3\), but to a stronger degree when both are present, acting as a coincidence detector for multiple inputs (Berridge, 1993; Simpson et al., 1995). As well as being a possible preventative measure to protect against uncontrolled excitation, activity dependent desensitization of nAChRs and other LGICs in the presence of continued agonist stimulation presents an additional memory of previous events that at its most basic level is intrinsic to the receptors themselves, but is also influenced by intracellular Ca\(^{2+}\) dependent metabolic processes, specifically the Ca\(^{2+}\)
dependent phosphorylation of the intracellular regions of nAChRs by protein kinase A (PKA) and protein kinase C (PKC), which enhance recovery from desensitization.

Calcineurin has the opposite effect of Ca\(^{2+}\) dependently dephosphorylating nAChRs, delaying their recovery from the desensitized state (Quick and Lester, 2002; Giniatullin et al., 2005).

At the neuromuscular junction where the effects of nAChRs were first studied, synaptically released ACh activates postsynaptic nAChRs on skeletal muscle cells leading to an influx of Na\(^+\) ions that results in membrane depolarization. This initiates action potentials that are channelled to voltage-sensitive dihydropyridine receptors on the cell membrane, which are physically coupled to RyRs on the sarcoplasmic reticulum\([\text{Ca}^{2+}]_{\text{io}}\), which in turn release Ca\(^{2+}\) into the cytosol, eventually leading to muscle contraction (Fleischer and Inui, 1989). nAChRs also mediate excitatory postsynaptic potentials (EPSPs) in neurons of the autonomic ganglia (Wang et al., 2002), but in the CNS nAChRs are thought to play a more modulatory role in neurotransmission, and in the generation of Ca\(^{2+}\) signals that have more long-term

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**Figure 1.2.3: Possible routes of Ca\(^{2+}\) entry into cytosol following nAChR activation.**

Upon nAChR activation, Na\(^+\) and Ca\(^{2+}\) ions enter the cell via the nAChR ion channel. Na\(^+\) influx results in depolarization of the cell membrane, triggering voltage-gated Ca\(^{2+}\) channels (VGCCs) on the cell membrane. Ca\(^{2+}\) entering directly through nAChRs can stimulate Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR) from the endoplasmic reticulum (ER) via both ryanodine receptors (RyRs) and/or inositol-1,4,5-trisphosphate receptors (IP\(_3\)Rs).
effects. Examples of fast cholinergic transmission in the CNS have however been reported (Alkondon et al., 1998; Frazier et al., 1998a, 1998b).

In the last two decades, there have been numerous reports of different nAChR subtypes coupling to distinct Ca\(^{2+}\) signalling pathways in different cell types, cell locations and brain regions, reflecting the segregation of Ca\(^{2+}\) signalling to achieve diverse physiological actions (Dajas-Bailador and Wonnacott, 2004; Shen and Yakel, 2009). nAChRs are capable of elevating \([\text{Ca}^{2+}]_c\) alone (Trouslard et al., 1993), and it has been reported that local \(\alpha7\) nAChR-mediated \([\text{Ca}^{2+}]_c\) elevations in filopodia of SH-SY5Y human neuroblastoma cells (overexpressing \(\alpha7\) nAChRs) were not sensitive to either the RyR antagonist ryanodine (Ry) or hyperpolarization using the K\(^+\) ionophore valinomycin, ruling out contributions from VGCCs and RyR mediated CICR (Gilbert et al., 2009). Another study reported that both \(\alpha3^*\) and \(\alpha7\) nAChR mediated Ca\(^{2+}\) transients in IMR-32 human neuroblastoma cells were not coupled to VGCCs or RyR mediated CICR either (Gilbert et al., 2009; Ween et al., 2010). Fayuk and Yakel (2005) have also demonstrated \(\alpha7\) nAChR-mediated \([\text{Ca}^{2+}]_c\) elevations in hippocampal CA1 interneurons from rat brain slices that were insensitive to the general VGCC blocker Cd\(^{2+}\) and Ry. Evidence for nAChR mediated \([\text{Ca}^{2+}]_c\) elevations recruiting secondary Ca\(^{2+}\) amplification mechanisms involving VGCCs and ER mediated release is also well documented however, highlighting the diversity of physiological processes influenced by nAChRs. For example, in chick ciliary ganglion neurons, stimulation of \(\alpha7\) nAChRs (Vijayaraghavan et al., 1992; Rathouz and Berg, 1994) and non-\(\alpha7\) nAChRs (Rathouz and Berg, 1994) resulted in \([\text{Ca}^{2+}]_c\) elevations that were VGCC-dependent, as were nicotine-induced \([\text{Ca}^{2+}]_c\) elevations in rat medial habenula neurons (Mulle et al., 1992b), and \(\alpha7\) nAChR-mediated \([\text{Ca}^{2+}]_c\) elevations in rat hippocampal neurons (Barrantes et al., 1995a). Chick sympathetic neuronal cell body \([\text{Ca}^{2+}]_c\) elevations evoked by the ganglionic nAChR agonist dimethylphenylpiperizinium (DMPP) were reported to involve L- and N-type VGCCs because as reported of their inhibition by the selective VGCC antagonists \(\omega\)-conotoxin and (+)Bay k 8644 respectively (Dolezal et al., 1996). In other studies, Khiroug et al., (1997) reported that non-\(\alpha7\) nAChRs coupled to VGCCs in rat chromaffin cells, whilst in rat intercardiac ganglion neurons it was shown that muscarinic AChRs (mAChRs) acted through IP\(_3\)Rs to raise \([\text{Ca}^{2+}]_c\) whereas nAChRs were coupled to CICR from RyRs.
(Beker et al., 2003), as were α9 nAChRs in guinea pig outer cochlear hair cells (Evans et al., 2000).

Investigations have begun to build an increasingly detailed picture by providing evidence linking distinct Ca²⁺ amplification pathways to particular nAChR subtypes in the same cellular systems. Among them, Tsuneki et al. (2000) reported that in dopaminergic neurons of the substantia nigra pars compacta (SNc) from mouse brain slices, both α7 and β2* nAChRs were coupled to RyR-mediated ER Ca²⁺ release, but only β2* nAChRs were coupled to L-type VGCCs. Sharma and Vijayaraghavan (2001) reported that in rat hippocampal astrocytes, α7 nAChR [Ca²⁺]ic elevations were coupled to ER-mediated release. Another study by Shoop et al. (2001) using chick ciliary ganglion cells distinguished the local Ca²⁺ transients mediated by α7 nAChRs alone that were confined to dendritic spines with α3* nAChR-mediated global Ca²⁺ events that were dependent on both L-type VGCCs and RyR-mediated ER release. Having observed α7 nAChR-mediated Ca²⁺ transients that were isolated by the morphological characteristic of dendritic spines, the same group later discovered that in hippocampal aspiny interneurons, α7 nAChR-mediated Ca²⁺ transients were instead constrained by the close association and physical coupling to PMCA2 pumps via postsynaptic density protein 95 (PSD-95) (Gomez-Varela et al., 2012). Furthermore, two studies from Wonnacott’s group detailed the segregation of α7 and α3* nAChR mediated [Ca²⁺]ic elevations to ER mediated Ca²⁺ release (both RyR- and IP₃R-mediated) and L-type VGCCs respectively in both SH-SY5Y cells (Dajas-Bailador et al., 2002a) and rat adrenomedullary pheochromocytoma (PC12) cells (Dickinson et al., 2007). Here, in SH-SY5Y cells it was also found that the ER-mediated release could be divided into short- and long-lasting components by their differential sensitivities to Ry and the IP₃R blocker xestospongin-C respectively. Similar studies have finely dissected the nAChR subtype dependent Ca²⁺ signalling pathways in bovine chromaffin cells revealing that non-α7 nAChRs coupled to L-type VGCCs and CICR whilst α7 nAChRs coupled to non-L-type VGCCs and CICR (del Barrio et al., 2011).

1.2.2.8 Ca²⁺ dependent nAChR modulation of neurotransmitter release

Numerous cases have also been described in which particular physiological roles for the distinct Ca²⁺ signalling pathways are initiated by different nAChR subtypes. A
A significant proportion of nAChRs in the CNS are proposed to be located on presynaptic terminals, where their activation can stimulate or modulate the release of neurotransmitters such as glutamate, glycine, dopamine, noradrenaline, serotonin, and γ-aminobutyric acid (GABA) (Wonnacott, 1997; Vizi and Lendvai, 1999; Zappettini et al., 2011b; Garduño et al., 2012). This can be generalized to occur in two main ways: (i) occurring on a scale of microseconds, nAChR mediated depolarization of presynaptic terminals or boutons, resulting in the activation of VGCCs that provide the Ca\(^{2+}\) influx necessary to stimulate the rapid process of Ca\(^{2+}\) dependent exocytosis of neurotransmitter containing vesicles by SNARE complexes, and (ii) presynaptic potentiation of neurotransmitter release, also known as presynaptic plasticity, a longer and more complex process involving Ca\(^{2+}\) dependent metabolic pathways that
increase the probability and magnitude of release, producing effects that can last over timescales of seconds to minutes (Shen and Yakel, 2009).

Following on from Figure 1.2.3, Figure 1.2.4 shows the next level of possible Ca²⁺ signalling routes that exist in synaptically connected systems. Here, [Ca²⁺]_{ic} elevations initiated by presynaptic nAChRs can indirectly lead to [Ca²⁺]_{ic} elevations in the postsynaptic cells via the facilitation of glutamate release, which activates postsynaptic AMPA and NMDA receptors, allowing Ca²⁺ influx. Na⁺ influx through AMPA receptors can also activate Ca²⁺ influx through postsynaptic VGCCs, and as in the single-cell model (Figure 1.2.3), free Ca²⁺ can stimulate CICR from RyR or IP₃Rs on the ER. Glutamate release can also activate mGluR₁, leading to the PLC catalysed hydrolysis of PIP₂ to generate IP₃, which acts at IP₃Rs to release Ca²⁺ from the ER (see

![Diagram of calcium signalling](image)

**Figure 1.2.4:** Presynaptic nAChRs can influence Ca²⁺ signaling events in postsynaptic cells via glutamate release.

Presynaptic nAChRs raise Ca²⁺ in terminals, either activating or potentiating the release of neurotransmitters such as glutamate. Glutamate diffuses across the synaptic cleft, activating postsynaptic AMPA and NMDA type ionotropic glutamate receptors (iGlRs), which flux Na⁺/Ca²⁺, leading to activation of VGCCs and possibly CICR via RyRs and IP₃Rs on the ER. Activation of metabotropic glutamate receptors type I (mGluR₁) can also raise postsynaptic Ca²⁺ via G-protein catalysed conversion of PIP₂ to IP₃, which activates IP₃Rs on the ER to release Ca²⁺.
Earlier studies reported stimulation of neurotransmitter release by presynaptic nAChRs on ganglionic neurons, where it was found that DMPP evoked [³H]Ach release from neurons of the guinea pig myenteric plexus were sensitive to ω-conotoxin, implicating N-type VGCCs (Takahashi et al., 1992). In contrast to nicotine’s actions at cell bodies, VGCCs were not implicated in the mediation of nicotine evoked noradrenaline release from cultured chick sympathetic neurons, implying that nAChRs could elevate [Ca²⁺]ᵋ sufficiently alone (Dolezal et al., 1996). This was also the case for the facilitation of GABA release by β²* nAChRs from slices of thalamus sensory nuclei (Léna and Changeux, 1997), and in anatoxin-A evoked glycine release from rat spinal dorsal horn substantia gelatinosa neurons (Kiyosawa et al., 2001). In rat sympathetic axon terminals however, nicotine was found to evoke Ry-sensitive Ca²⁺ spikes (Brain et al., 2001). A report by Soliakov and Wonnacott, (1996) provided evidence for N-type VGCCs mediating anatoxin evoked [³H]dopamine release from rat striatal synaptosomes, which was confirmed by Turner (2004), although here it was found that P/Q-type VGCCs also mediate this effect. In the latter study, αβgt sensitive Ca²⁺ dependent enhancement of [³H]dopamine release by nicotine or choline was reported to be independent of VGCCs in contrast to most other studies, but acted via a calmodulin dependent pathway to increase the readily releasable pool of neurotransmitter vesicles. Indeed, numerous reports have now presented a dichotomy between the effects of presynaptic α7 and non-α7 nAChR-mediated neurotransmitter release, with accompanying differences in Ca²⁺ signalling mechanisms. The more Ca²⁺ permeable α7 nAChR is well established to play a role in the enhancement of transmitter release rather than its direct mediation, which is ascribed to the less Ca²⁺ permeable, depolarization-inducing heteromeric nAChRs.

To measure presynaptic potentiation in intact neurons forming synaptic connections, the frequency of low-level, constitutive neurotransmitter release can be measured from the postsynaptic neuron (miniature EPSPs; mEPSCs) in the presence of tetrodotoxin (TTX), which blocks voltage gated Na⁺ channels (VGSCs) and therefore prevents action potential mediated neurotransmitter release on the presynaptic neuron, and blocks suprathreshold excitatory events on the postsynaptic neuron. In the event of presynaptic potentiation, an increase in the frequency of mPSCs is expected, as the probability and magnitude of release is enhanced. Gray et al.
(1996) used this method to measure nicotine-mediated presynaptic potentiation of hippocampal mossy fibre terminals in rat brain slices. The increased mEPSC frequency was sensitive to αbgt and MLA, implicating α7 nAChRs, and was dependent on [Ca^{2+}]_{ec}. Fura-2 was then used to image nicotine-evoked Ca^{2+} elevations in the mossy fibre terminals, which were unaffected by AMPA and NMDA receptor blockade. Intraterminal Ca^{2+} elevations evoked by nicotine were additive with VGCC-dependent Ca^{2+} elevations evoked by electrical stimulation, suggesting that α7 nAChRs could elevate Ca^{2+} without the need for VGCCs, or acted synergistically. Similar studies have also reported VGCC-independence of α7 nAChR-mediated glutamatergic potentiation in medial habenula and interpeduncular synapses in co-culture (Girod et al., 2000), and in neurons of the nucleus of the solitary tract (Kalappa et al., 2011), where presynaptic α7 and non-α7 nAChR-mediated enhancement of mEPSCs frequency was also independent of ER-mediated Ca^{2+} elevations. Furthermore, a variety of reports have provided evidence that links the potentiation of glutamate release by presynaptic α7 nAChRs to RyR-mediated CICR (Sharma and Vijayaraghavan, 2003; Le Magueresse and Cherubini, 2007; Sharma et al., 2008). Studies measuring the release of neurotransmitters from synaptosomes found that whereas α7 nAChR-mediated neurotransmitter release was dependent on CICR, β2* nAChR-mediated release was VGCC dependent. This was reported for [3H]D-aspartate (glutamate surrogate) release from rat PFC synaptosomes (Dickinson et al., 2008) and GABA release from rat hippocampal synaptosomes (Zappettini et al., 2011a). Wang et al. (2006) however reported that α7 nAChR-mediated glutamate release from rat PFC synaptosomes was coupled to N- and P/Q-type VGCCs, and in a separate study, non-α7 nAChRs on sympathetic terminals of the mouse vas deferens were coupled to RyR-mediated CICR (Williams et al., 2011a). Lastly, glutamatergic terminals synapsing with 5-HT neurons in the dorsal raphe nuclei (DRN) were found to couple α4β2 but not α7 nAChRs to both VGCCs and CICR, as measured by increased frequency of mEPSCs (Garduño et al., 2012).

The modulatory aspect of Ca^{2+} dependent presynaptic nAChR-mediated potentiation of neurotransmitter release has been hypothesized to reflect dynamic alterations in the availability of readily releasable neurotransmitter vesicles that affects the probability and magnitude of release. In support of this, electron microscopy studies discovered two distinct pools of neurotransmitter containing
vesicles designated: (i) the readily releasable pool (RRP) proximal to the presynaptic membrane, and (ii) the reserve pool (RP) located distal to the presynaptic membrane, containing vesicles whose association with the actin cytoskeleton is mediated by a neuron specific phosphoprotein called synapsin I (Pieribone et al., 1995). Metabolic cascades that result in the phosphorylation of synapsin I by CaMKII and extracellular signal regulated kinase (ERK)/mitogen activated kinase (MAPK) at specific sites trigger vesicle dissociation from actin whereas Src kinase phosphorylation mediates re-aggregation of synaptic vesicles to actin (Kushner et al., 2005; Cesca et al., 2010; Messa et al., 2010). Association and dissociation of vesicles to actin is thought to occur in response to different modes of neuronal stimulation, where high-frequency stimulation as opposed to low-frequency stimulation triggers recruitment of RP vesicles to the RRP (Pieribone et al., 1995), and also in response to neurotrophins such as brain derived neurotrophic factor (BDNF) (Jovanovic et al., 2000). Interestingly, the CICR-dependent facilitation of glutamate release from PFC synaptosomes by α7 but not β2* nAChR stimulation was found to be dependent on ERK/MAPK phosphorylation, and correlated with an increase in synapsin I phosphorylation at ERK/MAPK specific sites (Dickinson et al., 2008), whereas a study probing the post-receptor effects of acute nicotine treatment in mice in vivo reported a β2* but not α7 nAChR-dependent effect of increased CaMKII activity which was correlated with an increase in synapsin I phosphorylation at CaMKII specific sites in the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Jackson et al., 2009). Using a slightly different approach, Gomez-Varela and Berg (2013) supplemented evidence for the important role played by α7 nAChRs in modulating presynaptic potentiation of glutamate release by showing that lateral mobility of α7 nAChRs on glutamatergic terminals in cultured rat hippocampal neurons affected the extent of the enhancement of glutamate release. By restricting mobility of α7 nAChRs with cross-linking antibodies, the authors observed an increase in the frequency of mEPSCs that was accompanied by an enlargement of the RRP of synaptic vesicles. α7 nAChRs expressed by astrocytes were also found to release glutamate following elevations of [Ca²⁺]ᵣ, although amplification mechanisms were not defined (Pirttimaki et al., 2013), although as noted, previous studies have linked α7 nAChRs on astrocytes to RyR-dependent Ca²⁺ release from the ER. Other studies have implied the dependence of α7 nAChR-mediated presynaptic potentiation on protein kinase A.
(PKA) in hippocampal mossy fibre terminals (Cheng and Yakel, 2014), and in mouse ventral hippocampal neurons long lasting (30 min) $[\text{Ca}^{2+}]_\text{c}$ elevations in axons and terminals that underlie presynaptic facilitation of glutamate release were identified to arise from $\alpha 7$ nAChR stimulation requiring subsequent CICR from IP$_3$Rs, activation of PLC and CaMKII (Zhong et al., 2013).

When taken together these reports illustrate the need for further study to reconcile some seemingly contradictory results. Indeed, many of the publications outlined above have only concentrated on certain aspects nAChR mediated Ca$^{2+}$ signalling, leaving others open to speculation. Whereas it may be that different methods of assessment of Ca$^{2+}$ signals can affect bias towards a particular mechanism, differences in brain regions or cell type probably account for much of the diversity.

1.2.2.9 Long term Ca$^{2+}$ dependent effects mediated by nAChRs

Some targets of Ca$^{2+}$ signalling occur long distances from initial Ca$^{2+}$ flux, and require sustained or global $[\text{Ca}^{2+}]_\text{c}$ elevation, such as those at the nucleus, where their effects influence gene expression, producing long-lasting changes in cell behavior (Bading, 2013). In an environment that strongly buffers [Ca$^{2+}$], this requires mechanisms that amplify or transduce the initial signal (see 1.2.2). The principle mechanisms by which these goals are achieved are: (i) the activation of somatic VGCCs proximal to the nucleus occurring after backpropagation of action potentials, and (ii) via regenerative Ca$^{2+}$ waves propagated along the ER towards the nucleus, mediated by successive instances of IP$_3$R mediated CICR. The ER membrane is continuous with the nuclear envelope, and Ca$^{2+}$ can diffuse passively through the nuclear pore complex (Wang et al., 2002). Once in the nucleus, Ca$^{2+}$ can initiate signalling events that influence gene transcription, such as the activation of transcription factors, histone modification, DNA methylation, and the inhibition of cell death signals (Dajas-Bailador and Wonnacott, 2004; Shen and Yakel, 2009; Bading, 2013).

As discussed in section 1.2.2.8, nAChR mediated Ca$^{2+}$ signalling at presynaptic locations modulates neurotransmitter release in short- and long-term processes. With respect to long-term signalling that involves the modulation of gene transcription, it has been reported that nAChR-dependent signalling events can lead to the upregulation of enzymes required for the synthesis of neurotransmitters such
as tyrosine hydroxylase (TH). The mechanism was found to be dependent on CICR from the ER, PKA and the ERK/MAPK pathway in response to α7 nAChR stimulation (Gueorguiev et al., 2000).

Of course, the effects of modulated neurotransmitter release and presynaptic potentiation directly affect postsynaptic cells (Figure 1.2.4), as the rate and extent of postsynaptic stimulation is increased. At glutamatergic synapses, increased synaptic input activity underlies the process of synaptic plasticity known as long term potentiation (LTP), widely regarded to underlie learning and memory (Bliss and Lomo, 1973; Lynch, 2004). At its most basic, LTP involves the activity-dependent potentiation of synaptic efficacy in response to increased stimulation that results in postsynaptic \([\text{Ca}^{2+}]_c\) elevations that trigger signalling pathways leading to, in chronological order: (i) increased iGluR and mGluR receptor expression at the postsynaptic density, (ii) transcription of genes and protein synthesis, and (iii) morphological and structural changes in postsynaptic regions such as dendritic spines. Nicotinic agents have long been known to have positive effects on cognition (Levin and Simon, 1998), and in addition to their facilitatory effects at presynaptic locations, nAChRs at postsynaptic locations have been shown to exert complementary influences on LTP in the hippocampus which are crucially dependent on the timing of stimulation, and present a mechanism by which pre- and postsynaptic cholinergic stimuli can be integrated across a synapse (Ji et al., 2001; Gu et al., 2012).

Various downstream actors involved in the later stages of LTP have been reported to show activation following nAChR stimulation, such as PKA, CaMKII/IV, ERK/MAPK and cyclic AMP (cAMP) response element binding protein (CREB), which mediate signal transduction pathways that converge at the nucleus, resulting in the induction of immediate early genes (IEGs) such as c-Fos and activity regulated cytoskeletal associated protein (Arc) (Sweatt, 2001; Bading, 2013). These IEGs are strongly associated with LTP and the pro-cognitive effects of α7 nAChR activation (Thomsen et al., 2010). c-Fos encodes a range of nuclear transcription factors, responsible for the induction of various genes in response to neuronal stimulation (Curran and Morgan, 1995) whilst Arc plays a more local synaptic role; its mRNA is translated at dendrites where it regulates processes integral to LTP such as AMPA receptor trafficking and cytoskeletal expansion (Bramham et al., 2008).
Early studies reported that nicotine induced c-Fos via a mechanism independent of extracellular Ca\(^{2+}\) in PC12 cells (Greenberg et al., 1986), and it was found that \(\alpha_7\) nAChR-mediated Ca\(^{2+}\) signals that were partially dependent on VGCCs lead to phosphorylation of ERK1/2 via PKA and MEK in SH-SY5Y cells and rat hippocampal neurons (Dajas-Bailador et al., 2002b). In chick ciliary ganglion neurons, Chang and Berg (2001) reported that nicotine activated CREB, but only when L-type VGCCs were silent, as they were found to activate the protein phosphatase calcineurin, that dephosphorylates CREB. The same lab then provided evidence suggesting that CREB activation in hippocampal neurons is independent of VGCC activation, but involves CICR, CaMKII/IV and ERK/MAPK (Hu et al., 2002). More recently, a study by Bitner et al. (2007) correlated the \(\alpha_7\) nAChR agonist A-582941 mediated enhanced performance in cognitive tasks on rodents and primates with increased ERK1/2 phosphorylation in PC12 cells, and ERK1/2 and CREB phosphorylation in mouse cingulate cortex and hippocampus. Furthermore, A-582941 and another \(\alpha_7\) nAChR agonist SSR180711 have been found to elevate c-Fos and Arc in the PFC and nucleus accumbens shell (Hansen et al., 2007; Kristensen et al., 2007; Thomsen et al., 2008). Drawing together elements of nAChR-mediated gene transcription with CICR, Ziviani et al. (2011) reported nicotine-induced, CREB phosphorylation-dependent upregulation of RyR2 in areas of the brain involved in addiction and cognition, which drove further CREB phosphorylation in a positive feedback mechanism. By affecting long-term downstream signalling pathways, it has also been widely reported that nAChRs mediate neuroprotection (covered later in the context of disease and therapeutics; 1.3.4) and promote survival in a variety of cell types and models (Belluardo et al., 2000; Dajas-Bailador and Wonnacott, 2004; Mudo et al., 2007). From these varied reports, it can be seen that nAChRs play vital roles in physiological events that are coordinated by distinct and controlled intracellular Ca\(^{2+}\) pathways whose effects range from the near instantaneous to long-term events that shape neuronal activity.

The overall picture built up by this fragmentary jigsaw of experimental evidence betrays the startling complexity, segregation and integration of nAChR mediated Ca\(^{2+}\) signalling in neuronal cells. Like the shadows on the walls of Plato’s Cave, experimental techniques, however powerful, can only lend a mere silhouette or trace of nature’s inner workings. But armed with scientific rigor and a humble
approach to the interpretation of observations, these sightings can be of great utility, as in the pursuit of pharmacological agents to treat disease.

1.3 nAChRs as drug targets

Sections 1.1 and 1.2, have described nAChRs as dynamic biological signal transducers that influence a diverse range of important cellular events, and can be modulated by a kaleidoscopic palette of endogenous and exogenous compounds. This section will focus on a major driving force behind research aiming to better understand nAChRs and their place within the labyrinth of physiology: the quest for drug development to treat disease.

The pervasive expression of nAChRs throughout the body, and their potential to affect diverse and important biological events naturally implicates them in a range of diseases, and their apparent susceptibility to modulation entitles them as worthy drug targets. Indeed, nAChRs have been reported to play a role in the pathology and/or potential alleviation of a variety of diseases such as Alzheimer's disease (AD), schizophrenia, Parkinson's disease (PD), addiction, pain, depression, epilepsy, cancer and immune disorders. This section will summarize the links between nAChRs and disease and potential treatment.

1.3.3 Cholinergic deficits

ACh is a significant neurotransmitter in the brain, acting at nAChRs and mAChRs, thought to play a central role in cognitive functions such learning, attention and working memory (Klinkenberg et al., 2011). Cholinergic influence in the brain is particularly widespread, owing to extensive projections from neurons originating in the basal forebrain, PPN and LDT and also from cholinergic interneurons in the striatum, which are thought to act both locally and in a more diffuse manner by volume transmission (Sarter et al., 2009). Deficits in cholinergic function have been linked to diseases that manifest an impaired cognitive component, prime examples being AD and schizophrenia. Whilst ostensibly describing two very different illnesses, schizophrenia and AD share an overlapping etiological element and entail massive socioeconomic burden. Here, the reasons for nAChRs presenting possible therapeutic targets will be discussed.
Schizophrenia is an umbrella term given to a spectrum of mental illness that encompasses a broad aetiology of genetic and environmental factors, thought to affect 1% of the world’s population. Symptoms are traditionally categorized as positive (gain of function), which include hallucinations and delusions; negative (loss of function) comprising social withdrawal, lack of affect and anhedonia; and cognitive, which manifests as learning, attentional and working memory deficits (Young and Geyer, 2013). Patients also exhibit a high incidence of comorbidity with other psychiatric illnesses such as depression, substance misuse and anxiety-related disorders; symptomatic expression is highly unique (Buckley et al., 2009). Although many factors are thought to be involved such as misregulation of the dopaminergic and glutamatergic systems, there are several lines of evidence implicating a role for nAChRs in the aetiology and/or treatment of schizophrenia. Firstly, nicotine has been shown to exert positive effects on both schizophrenics and healthy controls in tests of attentional performance (Rezvani and Levin, 2001). Furthermore, the α7 nAChR subtype in particular has been implicated in schizophrenia, as it was found that a sensory gating deficit to the P50 evoked potential (Adler et al., 1982), a characteristic endophenotype of schizophrenia was linked to polymorphisms at chromosome location 15q13-14, harbouring the α7 nAChR gene (CHRNA7) (Freedman et al., 1997; Severance and Yolken, 2008; Finlay-Schultz et al., 2011), although this has not been shown in all studies (Cabranes et al., 2012). Adding to this, decreased expression of α7 nAChRs has been found in post-mortem schizophrenic brains (Freedman et al., 1995; Court et al., 1999; Marutle et al., 2001), and it has recently been suggested that a partial duplication of the α7 nAChR gene, CHRFAM7A, has a negative effect on α7 nAChR function and is only found in humans (Araud et al., 2011). When co-expressed in X. laevis oocytes, CHRFAM7A inhibited ACh induced α7 nAChR currents, and had no functional activity when expressed alone, which was hypothesized to result from a truncated protein product that lacks an ACh binding site. Another theory proposed that due to the high incidence and intensity of tobacco smoking (up to 80% for schizophrenics compared to 25% in the general population), schizophrenic patients were possibly self-medicating with nicotine in an attempt to mitigate cognitive symptoms, or antipsychotic drug side-effects (Kumari and Postma, 2005). Although the link between schizophrenia and smoking is well reported in the literature, there may be other factors influencing its prevalence, such as the need for a structured
routine, alleviation of boredom and pleasurable effects (Keltner and Grant, 2006). Importantly, it has been reported that the tobacco industry has played a major role in the funding and propagation of research and ideas that suggest schizophrenics need to smoke and even that they are somehow immune to adverse health effects associated with smoking such as cancer, as well as suppressing research that contradicted these claims (Prochaska et al., 2008).

Notwithstanding these debates, there has been considerable research into developing drugs targeting the α7 nAChR for the possible alleviation of cognitive deficits in schizophrenia, with promising findings in preclinical studies with α7 nAChR agonists and PAMs (Reviewed in Hajós and Rogers, 2010; Hurst et al., 2013; Wallace and Bertrand, 2013; Young and Geyer, 2013). As schizophrenia is uniquely human disease however, drug development in the preclinical stages must rely on animal models of schizophrenic-like phenotypes and often results in poor translation to the clinic (Jones et al., 2011; Hurst et al., 2013), and as mentioned above, the heterogeneous symptomatic presentation of schizophrenia poses further hurdles for rigorously controlled clinical trials. Of note, the α7 nAChR partial agonist GTS-21 was found to improve P50 auditory gating deficits but did not significantly improve cognitive symptoms in schizophrenics, although some improvement in negative symptoms was reported (Olincy et al., 2006; Freedman et al., 2008). The α7 nAChR agonist and 5-HT3 antagonist EVP-6124 was recently reported to improve cognitive symptoms in schizophrenics in a proof of concept trial (Preskorn et al., 2014), and the full α7 nAChR agonist TC-5619 was found to have beneficial effects on cognitive and negative symptoms in an exploratory trial (Lieberman et al., 2013). In conclusion, both preclinical and clinical evidence supports the further development of drugs targeting nAChRs for schizophrenia, although numerous factors such as the broad aetiology of the disease, substance abuse (including tobacco smoking) and medical history of patients make this a significantly challenging endeavour.

Sharing the cognitive, cholinergic counterpart, the role of nAChRs in AD is somewhat more conspicuous than in schizophrenia. AD is the most prevalent form of dementia; a devastating disease and a growing socioeconomic burden exacerbated by an aging population. The first and until recently only drugs to be prescribed for AD were AChE inhibitors such as galantamine (see 1.1.5.1), rivastigmine and donepezil, that primarily act to increase the half-life of ACh in the brain to compensate for the
progressive loss of cholinergic neurons characteristic of the disease (Schliebs and Arendt, 2011). As mentioned in section 1.1.5.1, these drugs have also been recognized to directly modulate nAChRs, and it has long been known that nAChR density in the brain decreases as AD progresses (Perry et al., 1987). β-amyloid (Aβ) plaques and neurofibrillary tangles, the widely reported "pathological hallmarks" of AD have also been observed to interact with nAChRs (Wang et al., 2000, 2003), revealing a promising avenue for potential therapeutic intervention which reiterates a facet of nAChR mediated signalling alluded to in section 1.2.2.9, nAChR mediated neuroprotection.

### 1.3.4 Neuroprotection

nAChR mediated signalling pathways have been proposed to confer cell survival and anti-inflammatory effects and are relevant to other diseases such as PD, inflammatory diseases and cancer as well as AD. A variety of *in vitro* and *in vivo* experiments have shown nAChR agonists to be neuroprotective against toxicity induced by glutamate, kainate, Aβ, MPTP, paraquat, ethanol, nerve growth factor (NGF) deprivation, glucose and oxygen deprivation, oxidative stress, brain injury and cerebral ischemia (Mudo et al., 2007; Bencherif, 2009; Shimohama, 2009; Quik et al., 2012). nAChR mediated neuroprotection is thought to result from the overlapping effects of downstream cellular signalling cascades that result in the inhibition of apoptosis and inflammation, and the stimulation of plasticity (see section 1.2.2.9) and cell survival, most of which but not all are Ca\(^{2+}\) dependent. Among them are the PKA mediated ERK/MAPK activation of CREB; the CaMK and phosphatidylinositol 3-kinase (PI3K)/Akt pathway; the Janus kinase 2 (JAK2)/PI3K and/or signal transducer and activator of transcription 3 (STAT3) cascades; and the inhibition of nitric oxide synthase (NOS), which inhibits NO induced necrosis. Together, these pathways enhance cell survival and plasticity via increased TH, CREB and B-cell lymphoma 2 (Bcl-2) and Bcl-x activity; whilst reducing caspase 3, 8 and 9 and nuclear factor-κB (NFκB) activity, inhibiting apoptosis (Dajas-Bailador and Wonnacott, 2004; Mudo et al., 2007; Bencherif, 2009; Shimohama, 2009; Parri and Dineley, 2010; Quik et al., 2012).

In AD, the prevailing hypothesis of neurotoxicity relies on increased production and oligomerization of Aβ peptides, products of amyloid precursor
protein (APP) cleavage, which lead to excitotoxic insult (Esposito et al., 2013). Aβ has been reported to bind with high affinity to α7 and α4β2 nAChRs in the low picomolar range (Wang, 2000; Wang et al., 2000), where it is thought to play a role in synaptic plasticity in a physiological setting (Parri and Dineley, 2010). In AD however, the prolonged exposure, increased concentration and oligomerization of Aβ blocks α7 nAChR function and causes internalization of toxic α7-Aβ complexes (Nagele et al., 2002). The interaction of Aβ with α7 nAChRs was also found to lead to phosphorylation of the cytoskeletal protein tau via ERK and JNK-1, providing a possible link between the two biochemical markers of AD (Wang et al., 2003), and it was later found that α7 nAChR activation decreased tau phosphorylation via reduced activity of GSK3β (Bitner et al., 2009). The reduction of α7 nAChR-mediated pro-survival and pro-LTP effects are compounded by the effects of Aβ on glutamate homeostasis at excitatory synapses. At healthy glutamatergic synapses, activation of postsynaptic NR2A containing NMDARs favours the Ca²⁺ dependent activation of CaMKII, ERK and CREB, promoting LTP and cell survival, whilst inhibiting p38-MAPK and GSK3β pathways that lead to LTD (Esposito et al., 2013). Higher concentrations and oligomerization of Aβ block astrocytic glutamate uptake, allowing it to accumulate, leading to desensitization of iGluRs and the activation of non-synaptic, NR2B containing NMDARs via glutamate spill over, blocking CREB activation and triggering cell death signals (Hardingham et al., 2002). Overall, these findings legitimize the development of α4β2 and α7 nAChR selective agonists for the treatment of AD. As such, nicotine has widely been reported to exert protective effects in animal models of Alzheimer-like neurodegeneration (Shimohama, 2009; Inestrosa et al., 2013), leading some to suggest a decreased incidence of AD in smokers, whilst other studies have disputed this claim. A meta-analysis of 43 studies controlling for tobacco industry affiliation however found that tobacco smoking was not protective, but was indeed a significant risk factor for AD (Cataldo et al., 2010). Possible reasons for this include the difference between the effects of tobacco smoking, which entails a profusion of adverse health effects, and nicotine alone; not to mention the chronic exposure to nicotine in the smokers’ brain compared to animal and in vitro studies.

nAChR mediated neuroprotection is also being pursued for the treatment of PD, a highly debilitating disease characterized by the progressive degeneration of
dopaminergic neurons in the nigro-striatal pathway, leading to persistent tremor, rigidity and bradykinesia (Quik and Wonnacott, 2011; Quik et al., 2012). Current treatments such as L-dopa increase the amount of dopamine in the brain similar to the AChE approach used in AD, providing symptomatic relief but failing to halt disease progression, whilst also producing an array of side effects such as dyskinesia and psychiatric symptoms (Olanow and Schapira, 2013). The causes of PD are uncertain, although various environmental and genetic risk factors have been described. Strikingly, it has been consistently demonstrated that tobacco smoking is negatively associated with incidence of PD (Noyce et al., 2012), which has driven research to show that nicotine protects against nigro-striatal damage and enhances dopaminergic transmission in animals, most likely via α4β2* and α6β2* nAChRs (Quik et al., 2006a, 2006b). Currently, the outcome of small scale clinical trials using nicotine to treat motor symptoms are disappointing, although there is promising evidence of nicotine reducing L-dopa induced dyskinesia (Quik and Wonnacott, 2011; Quik et al., 2012; Hurst et al., 2013).

1.3.5 Addiction

Continuing the theme of nAChR mediated dopaminergic modulation, and recalling the effects of nicotine on memory and the involvement of nAChRs in synaptic plasticity (section 1.2.2.9), this final section will be concerned with addiction, which is of relevance to chapter 5. Addiction arises when long-term changes occur in certain brain regions in response to rewarding stimuli such as drug taking, gambling, eating, etc. Clinically, the symptoms of addiction are defined by the loss of control and compulsive repetition of said behaviours despite obvious negative consequences, and involve the building of tolerance, which precipitates a withdrawal syndrome upon removal of the rewarding stimulus (Nestler, 2013). Addiction is a complex behaviour affected by many genetic and environmental factors, and is driven by cellular mechanisms that underlie memory formation in various brain regions, the most important being the mesolimbic system dopaminergic neurons that originate in the VTA and project to the NAc. Several other brain regions such as the hippocampus, amygdala and PFC are also integral to addiction, which are central to memory and decision making behaviours (Nestler, 2005, 2013). This section will focus on nicotine addiction and therapeutic strategies targeting nAChRs for possible treatments.
Tobacco smoking is currently the leading preventable cause of death in the world and a major economic and public health burden (World Health Organization, 2008). The primary addictive ingredient of tobacco smoke is thought to be nicotine, and it is the most well studied. It has however long been known that tobacco smoke contains >4000 chemicals, many of which are harmful and carcinogenic, and some pharmacologically active, such as β-carbolines which are potent inhibitors of monoamine oxidase (MAO) enzymes, responsible for the degradation of dopamine, noradrenaline and serotonin (Essman, 1977; van Amsterdam et al., 2006). Added to this, there are reports of at least 599 additives in cigarettes that have been proposed to enhance the addictive properties of nicotine and facilitate its delivery (Rabinoff et al., 2007), leading some to suggest that the problem of tobacco addiction involves more than nicotine alone. Indeed, it has been demonstrated that nicotine alone is weakly reinforcing compared to nicotine in combination with MAO inhibitors in rodents (Guillem et al., 2005; Kapelewski et al., 2011), and this may present another potential therapeutic strategy.

Nicotine acts on nAChRs in the brain to promote the release of a range of neurotransmitters (see section 1.2.2.8). The role of nAChRs in the mesolimbic pathway is thought to be critical to the rewarding effects of nicotine, which result from increased dopamine release in the NAc, and has been shown to require β2* nAChRs (Picciotto et al., 1998; Benowitz, 2008), and although not necessary for developing nicotine addiction, some evidence also suggests a role for α7 nAChRs in enhancing drug-seeking behaviour (Brunzell and McIntosh, 2012). Elevated dopamine release in the NAc occurs directly via nAChRs on dopamine cell bodies in the VTA and their terminals, and also indirectly via nAChR mediated facilitation of glutamate release and the inhibition of GABA release following nAChR desensitization (Benowitz, 2008; Leslie et al., 2013). Following repeated exposure to nicotine, several long-term changes have been observed in brain reward circuitry, which contribute towards the establishment of dependence. This includes the nicotine-mediated desensitization, cell-surface upregulation and subunit substitution of nAChRs, and the strengthening of neural connections in brain regions critical to addiction via LTP (Buisson and Bertrand, 2002; Benowitz, 2008).

Current cessation strategies for nicotine addiction include nicotine-replacement therapy (NRT), in the form of chewing gum, transdermal patches and
vaporizers, which aim to prevent craving experienced by quitters by maintaining a level of nicotine in the patient’s body to prevent withdrawal symptoms, whilst removing the negative health risks of smoking tobacco. In a similar vein, smoking cessation drugs that have activity at nAChRs are currently available; mecamylamine, bupropion and varenicline have been used in such a way (Hurst et al., 2013). The subject of chapter 5, the desensitizing nAChR agonist sazetidine-A has also shown promising findings in pre-clinical studies for reducing nicotine-seeking behaviour. These compounds are thought to work by blocking access of nicotine to nAChRs in the brain, whilst providing a nicotine-like effect to placate withdrawal symptoms (Polosa and Benowitz, 2011).

To conclude, this section presents a worthy case for the development of nAChR ligands as therapeutics for a range of diseases, that are not exhaustively described here (for recent review see Hurst et al., 2013). Some drugs that target nAChRs are already available and prescribed such as AChEs and varenicline, with more currently in the clinical trial pipeline and a great abundance of candidate drugs constituting the base of the drug development pyramid, reflecting the need for further and continued research.

1.4 Summary

This introduction has described nAChRs as a group of allosteric transmembrane proteins that gate Na\(^+\) and Ca\(^{2+}\), thereby influencing a diverse spectrum of physiological processes from muscle contraction to cognition. We have also outlined an abundance of ligands that differentially influence the conformational states of nAChRs, both endogenous and exogenous, via diverse binding sites which could potentially be used to treat diseases such as schizophrenia, AD, PD and addiction.

1.5 Aims of the thesis

This work is divided into three results chapters, unified by the underlying theme of Ca\(^{2+}\) signalling, which is used to measure nAChR function in each case. The main focus of these studies is the highly Ca\(^{2+}\)-permeable \(\alpha7\) nAChR, which is expressed in diverse brain regions, and associated with cognitive processes. Explicitly, the aims of these chapters are:
1. Investigation of the mechanisms of α7 nAChR mediated Ca^{2+} signalling in cells of the mouse cerebral cortex in culture, using live imaging.

2. Development of a high-throughput microscopy and analysis protocol to combine functional Ca^{2+} measurements with immunofluorescent cellular characterization in large cell populations, further detailing the mechanisms of α7 nAChR-mediated Ca^{2+} elevations in mouse primary cortical cultures.

3. Characterization of the desensitizing nAChR agonist and prototypical smoking cessation drug, sazetidine-A at α7 nAChRs, using functional Ca^{2+} fluorimetry and live imaging.
Chapter 2: Materials & Methods
2.1 Materials

2.1.1 Compounds
NaCl, MgCl₂, MnCl₂, Triton X-100, (-)-nicotine hydrogen tartrate, nifedipine, mecamylamine hydrochloride, choline bitartrate, DHβE, CdCl₂, ionomycin, Hoechst-33342 and NMDA were purchased from Sigma-Aldrich (UK); KCl, CaCl₂, NaH₂PO₄, NaHCO₃, glucose and HEPES were purchased from Fisher Scientific (UK); fluo-3 AM, fura-2 AM, α-bungarotoxin and pluronic f-127 were purchased from Life Technologies (UK); sazetidine-A dihydrochloride, tetrodotoxin citrate, CGP54626 hydrochloride, GABA, methyllycaconitine citrate, (S)-AMPA, (+)-MK801 maleate, 5-iodo-A85380 dihydrochloride, (-)-bicuculline methochloride were purchased from Tocris Bioscience (UK); ryanodine, LY456236 hydrochloride, D-AP5 were purchased from Abcam (UK); Xestospong-C was purchased from Calbiochem (UK). Novel azetidine/pyrrolidine containing compounds were a gift from Dr. John Fossey (School of Chemistry, University of Birmingham). PNU-120596 and PNU-282987 were provided by Pfizer Inc.

2.1.2 Antibodies
Rabbit anti-glial fibrillary acidic protein (GFAP), rabbit anti-microtubule associated protein type 2 (MAP2), rabbit anti-neurofilament, mouse anti-glutamic acid decarboxylase (GAD) and mouse anti-synaptophysin were purchased from Millipore (UK). Anti-rabbit Alexa Fluor-546 and anti-mouse Alexa Fluor-488 conjugated secondary antibodies were obtained from Life Technologies (UK).

2.2 Methods

2.2.1 SH-SY5Y cell culture
Human neuroblastoma SH-SY5Y cells (ECACC, Salisbury, UK; passages 16-27) were cultured as previously described (Ridley et al., 2001). In brief, cultures were maintained in Advanced Dulbecco’s modified Eagle’s media (DMEM/F12), supplemented with 2% foetal bovine serum (FBS), 2 mM L-glutamine, 190 U/ml penicillin and 0.2 mg/ml of streptomycin in 94 x 16 mm tissue culture dishes in a
humidified chamber at 37 °C with 5 % CO2. Cells were seeded 1:2 into 96-well plates; experiments were performed 72 h later with confluent cultures.

2.2.2 Primary cell culture

Mouse primary cortical cultures were prepared as previously described (Hoey et al., 2009). Briefly, a pregnant female CD1 mouse (University of Bath, UK) was sacrificed by cervical dislocation and E18 embryos were harvested. Cortices were dissected in PBS with 30 % glucose (Ca\(^{2+}\) and Mg\(^{2+}\) free) under a dissection microscope before being dissociated with a fire-polished glass Pasteur pipette. Tissue was then centrifuged at 500 g for 5 min, resuspended in neurobasal medium (w/out phenol red) supplemented with B27, 2mM L-glutamine and 60μg/ml penicillin and 100 μg/ml streptomycin (Life Technologies, UK). Cortices from a single brain were suspended in 12 ml media. For live imaging experiments (section 2.2.4), cells were grown on 25 mm round glass coverslips (thickness no. 1) coated with 20 μg/ml poly-D-lysine (Sigma-Aldrich, UK) and placed in 6-well tissue culture plates (Corning, USA). For high content microscopy experiments, cells were grown in 24-well visiplates (Perkin-Elmer, UK). Cells were allowed to grow for 10-14 d in vitro (DIV) at 37 °C in a humidified atmosphere of 95 % air and 5 % CO2.

2.2.3 96-well plate Ca\(^{2+}\) fluorescence assays

Increases in [Ca\(^{2+}\)]\(_c\) were measured as described previously (Dajas-Bailador et al., 2002a). Briefly, cells were washed twice with Tyrode’s salt solution (TSS: 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 0.2 mM NaH\(_2\)PO\(_4\), 12 mM NaHCO\(_3\), 5.5 mM glucose; pH 7.4) and incubated with the membrane-permeable, Ca\(^{2+}\) sensitive dye fluo-3 AM (10 μM) and 0.02 % pluronic F127 for 1 h at room temperature in darkness. Cells were then washed twice with TSS before pre-incubation with 80 μl antagonists, modulators or TSS (20 min for αbgt, 10 min for all others; 1.25 times final concentration). Changes in fluorescence (excitation 485 nm, emission 538 nm) were measured using a Fluoroskan Ascent fluorescent plate reader (Thermo Scientific, UK). Basal fluorescence was measured for 5 s before drugs were automatically dispensed (20 μl; 5 times final concentration) and fluorescence was monitored for a further 20 s.
Normalisation was achieved by determining the maximum and minimum fluorescence values of each fluo-3 AM signal using 0.2 % Triton X-100 (F<sub>max</sub>) followed by 40 mM MnCl<sub>2</sub> (F<sub>min</sub>). Data were calculated as a percentage of F<sub>max</sub>-F<sub>min</sub>.

**2.2.4 Live cell imaging of Ca<sup>2+</sup> fluorescence**

Changes in [Ca<sup>2+</sup>]<sub>i</sub> in individual cells were monitored using live cell imaging (Concord System, Perkin Elmer, UK). Mouse primary cortical cultures (10-14 DIV) were washed twice with Ca<sup>2+</sup> buffer (140 mM NaCl, 5.0 mM KCl, 1.0 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 10 mM glucose, 5.0 mM HEPES; pH 7.4) and incubated with the Ca<sup>2+</sup> sensitive dye fura-2 AM (5 μM) and 0.02 % pluronic F127 for 1.5 h at room temperature in darkness. After another two washes with buffer, coverslips were assembled into a temperature controlled (37 °C) perfusion chamber (Series 20 PH2 platform with a RC-21BR chamber, Harvard Apparatus, MA, USA) and mounted on an inverted fluorescence microscope. Buffer and drug solutions were pre-heated to 37 °C and perfused at a rate of 5 ml/min under gravity feed. Fura-2 AM was excited at 340 and 380 nm using a SpectroMaster I and emissions at 510 nm were detected with an intensified Ultrapix PDCI low light level CCD camera. Experiments were carried out in the presence of 1 μM tetrodotoxin (TTX) pre-incubated for at least 1 min prior to recording unless otherwise stated. During long drug pre-incubations, perfusion was switched off to conserve solutions, and recording was turned off to prevent photobleaching.

Data were analysed with Ultraview software (Perkin Elmer, UK) and expressed as a ratio of F<sub>340</sub>:F<sub>380</sub> following subtraction of background fluorescence using a region of interest (ROI) in which no cells could be seen. For successive drug treatments on the same cells, initial peak F<sub>340</sub>:F<sub>380</sub> for each selected ROI was normalized to 100 % following subtraction of mean basal F<sub>340</sub>:F<sub>380</sub>. Subsequent responses in the presence of antagonists/modulators or after washout were calculated as a percentage of the original response from the same ROI. These values were then averaged within experiments, such that n values reflect the number of independent cultures examined.

[Ca<sup>2+</sup>]<sub>i</sub> was calculated by using the Gryniewicz equation:

\[
[Ca^{2+}]_{ic} = \frac{K_d \times \beta \times (R - R_{mix})}{(R_{max} - R)}
\]
where \( R_{\text{max}} \) is given by \( F_{340}:F_{380} \) following perfusion of cultures with 10 mM Ca\(^{2+}\) buffer containing 2 \( \mu \)M ionomycin, and \( R_{\text{min}} \) is given by \( F_{340}:F_{380} \) following perfusion of cultures with Ca\(^{2+}\)-free buffer containing 2 \( \mu \)M ionomycin and 1 mM EGTA. Parameters were entered in the Ultraview software (Perkin Elmer, UK) for calculation, assuming \( K_d = 244 \) nM of Ca\(^{2+}\) for fura-2 (Gryniewicz et al., 1985).

### 2.2.5 High content microscopy: Ca\(^{2+}\) assays with immunofluorescence

Optimized method, devised following preliminary experiments described in chapter 4. [Ca\(^{2+}\)]\(_{\text{ic}}\) changes and immunofluorescence in large cell populations were imaged using high content microscopy (INCell Analyzer 2000, GE healthcare, UK). Primary cultures (10-14 DIV) were washed twice with Ca\(^{2+}\) buffer (140 mM NaCl, 5.0 mM KCl, 1.0 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 10 mM glucose, 5.0 mM HEPES; pH 7.4) and incubated with the Ca\(^{2+}\) sensitive dye fluo-3 AM (10 \( \mu \)M), the nucleic acid dye Hoechst-33342 (10 \( \mu \)M) and 0.02 \% pluronic F127 for 1.5 h at room temperature in darkness (200 \( \mu \)l per well). Cells were then washed twice with buffer before pre-incubation with 400 \( \mu \)l antagonists, modulators or buffer (10 min; 1.25 times final concentration). Two basal images were then acquired at 455 and 525 nm following excitation at 350 and 490 nm 6.4 s apart, followed by automatic dispensation of drugs (100 \( \mu \)l; 5 times final concentration). Then three more images were captured at both wavelengths at 10 s intervals.

After completion of the Ca\(^{2+}\) imaging assay, cells were washed twice with PBS and fixed in 4 \% PFA for 20 min. Cell were washed again three times with PBS, and incubated in PBS containing 3 \% BSA and 0.1 \% Triton X-100 to block non specific antibody binding. Primary antibodies (1:1000) in PBS were then added to cells in the presence of 1 \% BSA and 0.1 \% Triton X-100 and left overnight at 4 \(^\circ\)C. Cells were then washed three times for 5 min before fluorescently tagged secondary antibodies in PBS with 1 \% BSA and 0.1 \% Triton X-100 were added for 1 h at room temperature. Cells were again washed three times with PBS, and left in PBS containing 10 \( \mu \)M Hoechst-33342. Fixed and immunostained cells were then imaged a second time using high content microscopy at appropriate wavelengths.

Image processing and analysis is covered extensively in chapter 4, section 4.2. HCS Ca\(^{2+}\) imaging assays were conducted separately in triplicate due to the need for drug pre-incubation between different conditions. Imaging data from each triplicate
was then saved automatically as an image stack. In order to analyse data from an entire 24-well plate simultaneously, image stacks were combined using INCell Translator software (GE Healthcare, UK) according to a customized filename metadata expression, which could extract parameters such as date, column, row, time-point and wavelength from image filenames and directories. Image stacks could then be analysed with customized algorithms using INCell Developer Toolbox software (GE Healthcare, UK), and visualized using a file-link to Spotfire (TIBCO, Boston, USA) for inspection.

The final algorithm used to identify, track and measure the fluorescence intensity of cell bodies linked to a nucleus constructed in INCell Developer Toolbox software is as follows: Nuclear objects were assigned to Hoescht-33342 stained images using object segmentation with kernel size 5, sensitivity 20. A post-processing sieve was added to exclude objects smaller than 8 μm². Cell objects were assigned to the fluo-3 stained image using object segmentation, kernel size 25, sensitivity 30. Post-processing modules were then added: sieve 10 μm², clump breaking including secondary targets with radius 2, and border object removal. To link cells through the time series, track and block was defined to use an extended blocking method according to time-point. Target linking was then used to combine cell and nuclear objects at each time-point. Output measures were selected to report fluorescence intensity for each individual cell object, which were then manipulated to express the change in F₅₂₅ between basal and stimulated time-points (ΔF₅₂₅).

2.2.6 Statistics
Statistical tests were carried out in Prism 6 (GraphPad Software, Inc; CA, USA) as indicated in figure legends. Statistical significance was defined by P < 0.05 for all tests; independent experiments constituting a single n were defined by those conducted on cultures from separate animals or separate cell-line passages.

One-way analysis of variance (ANOVA) tests assumed normal distribution of data, and were used in conjunction with post-hoc multiple comparisons tests to detect statistically significant differences between treatment groups. Comparisons were taken from pre-selected treatment group pairs (indicated by connecting lines in figures) and corrected using Bonferroni’s multiple comparisons test.
One sample t-tests were used to assess statistically significant differences between treated conditions and the hypothetical value of 100 % representing the control response (see 2.2.4).

Prism 6 was also used to fit concentration response curves to the Hill equation:

\[ y = \frac{a}{1 + \left(\frac{k}{x}\right)^n} \]

where \( y \) is the change in \( F_{538} \) (\( F_{max} - F_{min} \)) evoked by the concentration \( x \) of a drug. \( a \) is the asymptotic maximum, and the EC\textsubscript{50} or IC\textsubscript{50} value is represented by \( k \), whilst \( n \) is the Hill coefficient. Concentration response curves of stimulation (Figure 5.2.2) were generated using the non-linear regression log[agonist] vs. response (three parameters) function. Concentration response curves of inhibition (Figure 5.2.7) were generated using the non-linear regression log[inhibitor] vs. normalised response function, which constrains the range of \( y \) values to between 0-100 %, and uses a Hill slope of -1.
Chapter 3: Mechanisms of α7 nAChR Mediated Calcium Signalling in Primary Cortical Cultures
3.1 Introduction

As discussed in section 1.2.2, the intrinsic capacity of nAChRs to flux Ca\textsuperscript{2+} places them in a pivotal position to influence an extremely diverse spectrum of physiological events. Incidentally, Ca\textsuperscript{2+} also presents an effective means of measuring functional nAChR activity in an experimental scenario, owing to its inherent chemical properties. With the aid of a modest pharmacological toolkit, the dark tunnels that comprise the labyrinth of intracellular Ca\textsuperscript{2+} signalling can be partially illuminated to provide insight into the role played by nAChRs in health and disease.

This chapter will detail experiments that aim to disentangle the various Ca\textsuperscript{2+} signalling pathways that either directly or indirectly result from the stimulation of α7 nAChRs in primary cultured cells from mouse cerebral cortex with the aid of fluorescent Ca\textsuperscript{2+} indicators and live cell imaging. Initial experiments used SH-SY5Y cells in high throughput Ca\textsuperscript{2+} fluorescence assays to evaluate the pharmacological profile of selective compounds used to stimulate α7 nAChRs.

As the majority of background information relevant to these experiments was discussed in the general introduction (chapter 1), this section will be concerned with describing the experimental models and methods used to evaluate nAChR mediated Ca\textsuperscript{2+} signalling in neuronal cells.

3.1.1 Measuring intracellular Ca\textsuperscript{2+} signals

Intracellular Ca\textsuperscript{2+} has historically been measured using either Ca\textsuperscript{2+} binding photoproteins or chemical Ca\textsuperscript{2+} indicators as reporter molecules in combination with microscopy. The earliest Ca\textsuperscript{2+} indicator to be used was the photoprotein aequorin, isolated from the luminescent jellyfish *Aequorea* (Shimomura et al., 1962), since then numerous Ca\textsuperscript{2+} chelating fluorophores and genetically encoded Ca\textsuperscript{2+} indicators have been developed, alongside ever increasing imaging capabilities (Grienberger and Konnerth, 2012).

In the experiments that comprise this thesis, extensive use is made of the chemical Ca\textsuperscript{2+} indicators fluo-3 and fura-2, which will be the focus of this section (Figure 3.1.1). These indicators belong to a family of highly selective Ca\textsuperscript{2+} chelators derived from the EGTA analogue 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA), which binds two Ca\textsuperscript{2+} ions via its four carboxylic acid
groups. The addition of fluorescent chromophore groups makes fluo-3 and fura-2 capable of reporting changes in [Ca\textsuperscript{2+}] (Tsien, 1980; Gryniewicz et al., 1985; Minta et al., 1989). Important differences between fluo-3 and fura-2 exist and have implications for their use when measuring [Ca\textsuperscript{2+}]. When chelating Ca\textsuperscript{2+}, the fluorescence of fluo-3 is increased up to 40-fold, whereas fura-2 undergoes a spectral shift. This feature of fura-2 arises from its dual spectroscopic excitation peaks at 340 and 380 nm, in contrast to the single excitation peak of fluo-3 at 488 nm. The excitation of fura-2 at either 340 or 380 nm is dependent on [Ca\textsuperscript{2+}], and therefore the ratio of fluorescence emitted at 510 nm resulting from excitation at either 340 or 380 nm is directly correlated to [Ca\textsuperscript{2+}] and can be calibrated using minimum and maximum Ca\textsuperscript{2+} buffer solutions to calculate [Ca\textsuperscript{2+}] using the Gryniewicz equation (Gryniewicz et al., 1985).

![Figure 3.1.1: Chemical structures of Ca\textsuperscript{2+} chelators EGTA, BAPTA, fura-2 and fluo-2.](image)

Fura-2 and fluo-3 chelate Ca\textsuperscript{2+} in the same way as EGTA and BAPTA, but the addition of chromophore groups makes them useful as Ca\textsuperscript{2+} indicators. Fura-2 is excited by light at 340 and 380 nm depending on [Ca\textsuperscript{2+}], and emits light at 510 nm. Fluo-3 is excited by light at 488 nm and emits at 526 nm.

The ratiometric fluorescence measurement confers several advantages, including the cancelling out of confounding variables such as sample thickness and uneven dye
distribution (Gryniewicz et al., 1985), but limits temporal imaging resolution to the speed of excitation wavelength switching between 340 and 380 nm.

To facilitate loading, both fluo-3 and fura-2 are made cell permeable by the addition of an acetoxymethyl ester group, which is cleaved by intracellular esterases, once again rendering them cell impermeable and locking them in the cytosol (Tsien, 1981). It should be remembered that as Ca²⁺ indicators chelate Ca²⁺, they act as exogenous Ca²⁺ buffers, altering the natural Ca²⁺ buffering capacity of the cell (Neher, 1995), slightly distorting the free cytosolic [Ca²⁺] dynamics from that in the absence of indicator. Using these compounds, we aim to measure Ca²⁺ signalling in SH-SY5Y cells and mouse primary cortical cultures.

3.1.2 SH-SY5Y cells

The systematic screening of pharmacological agents to assess their functional properties in vitro requires a relatively uniform biological model, which is satisfied by the use of immortalized cell lines; a relatively cheap, low maintenance and easily accessible source of experimental biological material that will continue to divide indefinitely. SH-SY5Y cells are a sub-clone from the SK-N-SH cell line, originally derived from a metastatic human bone tumour biopsy, first sub-cloned into SH-SY, SH-SY5 and finally SH-SY5Y cells (Biedler et al., 1978; Kovalevich and Langford, 2013). They comprise both adherent and non-adherent cells, and have been described to present two distinct morphologies: “neuroblast-like” and “epithelial-like” (Ross et al., 1983).

SH-SY5Y cells have been reported to express α3, α5, β2 and β4 nAChR subunit mRNA as evidenced by northern blot analysis (Lukas et al., 1993). Radioligand binding studies using [¹²⁵I]αbgt also provided evidence for surface expression of α7 nAChRs, whereas a two site model could be fitted for high affinity [³H]ACh binding experiments, suggesting two distinct non-α7 nAChR subtypes (Lukas et al., 1993; Ridley et al., 2001). Functional ⁸⁶Rb⁺ efflux assays suggested that the majority of nAChRs expressed by SH-SY5Y cells were non-α7 due to αbgt insensitivity (Lukas et al., 1993). Peng et al. (1994) also cloned the α7 nAChR subunit cDNA from SH-SY5Y cells, confirming that when expressed in X. laevis oocytes, the resulting receptors exhibited rapidly desensitizing, inwardly rectifying currents that triggered Ca²⁺ sensitive Cl⁻ channels, characteristic of functional α7 nAChRs expressed in oocytes.
Furthermore, expression of cloned human α3, α5, β2 and β4 cDNA in X. laevis oocytes suggested assembly of functional surface α3β2, α3β4, α3β2α5 and α3β4α5 nAChRs and it was also found that native SH-SY5Y α3 subunits were immuno-associated with α5 subunits, and that half of these were associated with β2 subunits (Wang et al., 1996; Gerzanich et al., 1998). Surface expression of α7 and α3β2* nAChRs in SH-SY5Y cells was reported to be upregulated by nicotine as shown by $[^{125}\text{I}]\text{abg}$ and $[^{3}\text{H}]\text{epibatidine}$ binding studies (Ridley et al., 2001), and there have been reports of nAChR mediated $[\text{Ca}^{2+}]_c$ elevations recruiting VGCCs and CICR (Dajas-Bailador et al., 2002a). Together, these studies endorse SH-SY5Y cells as an appropriate experimental model for studying nAChR mediated $[\text{Ca}^{2+}]_c$ signals in a high throughput fashion.

### 3.1.3 Mouse primary cerebral cortical cultures

The majority of experiments comprising this thesis were carried out on primary cultured cells from embryonic day 18 CD1 mouse cerebral cortex, which at this level of development has been reported to exhibit pharmacological properties of nAChR binding sites similar to those of the adult rodent brain (Dávila-García et al., 1999). Due to the fact that neurons are post-mitotic cells, they do not divide and cannot therefore be used continuously like immortalized cell lines (e.g. SH-SY5Y cells; above), and must be freshly prepared. Primary cultures from rodent brain tissue have been extensively used for pharmacological, neurochemical and toxicological studies, and provide a convenient system to investigate the properties of neuronal and/or glial cells from certain brain regions in isolation (for review, see Suñol et al., 2008). Following ultrastructural characterization, Robert et al., (2012) concluded that functional characteristics of primary cultured rat neurons faithfully represent those of neurons in vivo, forming processes, synaptic connections and exhibiting spontaneous electrical activity, although abnormally shaped organelles were found.

Cortical cultures have been described as containing mainly glutamatergic and ~40 % GABAergic neurons (Suñol et al., 2008). Numerous studies have measured release of glutamate (Bianchi et al., 2007; Lin et al., 2010) and GABA from primary cortical cultures (Hertz et al., 1984; Cai and Erdö, 1992; Schaffhauser et al., 1998; Bianchi et al., 2007; García et al., 2008), and expression of vesicular neurotransmitter transporters is reported to be similar to that in vivo (De Gois et al., 2005; Fattorini et...
al, 2009; Fiederling et al., 2011). The expression of nAChRs in cortical cultures is evidenced to be the same as that in vivo from numerous pharmacological and ligand binding studies, confined to α7 and α4β2 subtypes. Among them, Barrantes et al. (1995b) reported α7 nAChRs in rat cortical cultures labelled with [125I]αbglt in 36 % of cells, compared to 95 % of cells in hippocampal cultures. Numerous other studies also provide evidence for α7 expression in cortical neurons (Jensen et al., 1997; Hu et al., 2009; Lin et al., 2010; Hammond et al., 2013), and also astrocytes (Wang et al., 2013) and microglia (Morioka et al., 2014). Competition binding studies using [3H]cytisine and [3H]epibatidine provided evidence for α4β2 nAChR subtypes (Dávila-García et al., 1999; Hilmas et al., 2001), and nicotine mediated neuroprotection of hypoxic and glutamatergic insult was found to be blocked by αbglt, DHβE and β2 KO (Hejmadi et al., 2003; Stevens et al., 2003); whereas 5-iodo-A85380 (5-IA) mediated neuroprotection and nicotine mediated increases in phosphor-ERK were blocked by DHβE and β2 KO respectively (Steiner et al., 2007; Ueda et al., 2008).

Evidence also exists for the expression of mAChRs (Hammond et al., 2013), group I, II and III mGluRs (Prézeau et al., 1994; Schaffhauser et al., 1998; Koga et al., 2010; Ayala et al., 2012), GABA_A (Hu and Ticku, 1994; Pomés et al., 1994; García et al., 2006), GABA_B (New et al., 2006), AMPA (Cai and Erdö, 1992; Fischer et al., 2002; Rainey-Smith et al., 2010; Hoey et al., 2013), NMDA (Hilmas et al., 2001; Hoey et al., 2009; Lin et al., 2010), 5-HT (Bianchi et al., 2007), dopamine (Kurokawa et al., 2011, 2012) and adrenergic receptors in cortical cultures (Hansson et al., 1984; Suñol et al., 2008). Relevant to experiments of this chapter which concern intracellular Ca^{2+} signalling mechanisms, RyR-1 and -2 have been reported in cortical cultures (Kurokawa et al., 2011, 2012), as have L-type VGCCs (Stevens et al., 2003; Hoey et al., 2009). Together, evidence favours the use of primary cortical cultures as a convenient neuronal model, which can be easily imaged and pharmacologically manipulated, and contains the relevant receptors for the work comprising this thesis.

3.1.4 Aims

This chapter aims to evaluate the mechanisms of α7 mediated [Ca^{2+}]_i elevations in cells of the mouse cortex using the α7 selective PAM PNU1 and selective agonist PNU2. Numerous possible routes of Ca^{2+} entry to cells are possible in this system (see section 1.2.2), which can be interrogated by the use of selective antagonists.
Initially, we aim to assess the concentration response profiles of PNU1 and PNU2 in a high throughput SH-SY5Y cell based fluorimetry assay. With the information gained from these experiments, we will progress to using mouse primary cortical cultures, which exhibit synaptic connectivity and bear a closer resemblance to neural networks found in the brain. Having established robust \([\mathrm{Ca}^{2+}]_{i}\) elevations in response to \(\alpha 7\) stimulation with PNU1 and PNU2, we aim to test their dependence on the various sources of \(\mathrm{Ca}^{2+}\):

1. VGCCs, blocked by \(\mathrm{Cd}^{2+}\), L-type VGCCs blocked by nifedipine.
2. RyRs, blocked by Ry.
3. IP\(_3\)Rs, blocked by XeC.
4. AMPA and NMDA receptors, blocked by CNQX and MK801 respectively.
5. mGluR\(_1\), blocked by LY456236.
3.2 Results

3.2.1 PNU1 reveals α7 nAChR mediated Ca^{2+} elevations in SH-SY5Y cells

SH-SY5Y cells were loaded with fluo-3 AM and a high throughput 96-well plate assay was used to monitor [Ca^{2+}]_i to evaluate the conditions for observing α7 nAChR responses. To activate α7 nAChR, we employed the endogenous agonist choline (Alkondon et al., 1997b), the synthetic α7 nAChR selective agonist PNU-282987 (PNU2; Hajós et al., 2005) and selective α7 nAChR positive allosteric modulator PNU1 (Hurst et al., 2005).

Following pre-incubation of SH-SY5Y cells with fluo-3 AM, KCl (100 mM) was applied to give a positive control response, yielding increases of 17.68 ± 2.64 % (n =

![Figure 3.2.1: PNU1 strongly potentiates αbgt sensitive intracellular calcium elevations in SH-SY5Y cells](image)

SH-SY5Y cells loaded with fluo-3 AM were stimulated with nicotine (30 μM; red bars), choline (1.5 mM; blue bars) and PNU2 (1 μM; green bars) in the presence or absence of PNU1 (10 μM; dashed bars). Responses observed in the presence of PNU1 were challenged with the α7 nAChR selective antagonist α-bungarotoxin (αbgt; 100 nM; hatched bars). Fluorescence at 538 nm was measured for 20 s following stimulation with nicotinic agonists or TSS (white bar) alone or following 5 min pre-incubation and co-application of PNU1 or 20 min pre-incubation of αbgt. Data are presented as per cent maximum fluorescence determined by addition of 0.2 % Triton X-100 minus minimum fluorescence quenched by 350 mM MnCl_2. Bars represent mean ± SEM of at least 4 independent experiments; * P < 0.05, *** P < 0.001, **** P < 0.0001, significantly different from nicotine, choline or PNU2 or in combination with PNU1, non-paired one-way ANOVA, Bonferroni’s multiple comparisons test.
18; not shown). In order to observe nAChR-mediated responses, we applied 30 μM nicotine, previously reported to be the approximate EC₅₀ for nicotine in this assay (Ridley et al., 2002; Dajas-Bailador et al., 2003; Figure 3.2.1). This resulted in fluorescence increases of 12.69 ± 3.25 %. Cells were then exposed to the α7 nAChR selective agonists choline (1.5 mM; EC₅₀ reported in electrophysiological studies by Alkondon et al., 1997) and PNU2 (1 μM; EC₅₀ in electrophysiological studies Hajós et al., 2005). Alone these compounds had very little effect on fluorescence, comparable to that of TSS alone. However, when cells were pre-incubated with PNU1 (10 μM; maximally effective concentration for potentiation Hurst et al., 2005; Dickinson et al., 2007) for 5 min, co-application of choline (1.5 mM) or PNU2 (1 μM) resulted in large and rapid fluorescence increases of 48.95 ± 12.21 % and 43.81 ± 11.04 % respectively, that were significantly different from choline (1.5 mM) and PNU2 (1 μM) alone. PNU1 (10 μM) was also co-applied with nicotine (30 μM) for comparison, yielding fluorescence increases of 34.57 ± 7.43 %, a 2.72 ± 0.22 fold potentiation.

To confirm α7 nAChR specificity of these responses, the α7 nAChR selective antagonist αβgt (100 nM) was pre-incubated with SH-SY5Y cells for 20 min prior to agonist stimulation. Under these conditions, responses to choline (1.5 mM) or PNU2 (1 μM) in the presence of PNU1 (10 μM) were completely blocked (fluorescence changes of 2.70 ± 0.46 % and 1.56 ± 0.19 % respectively).

We then examined the concentration response profile of PNU1 in combination with choline (1.5 mM) or PNU2 (1 μM; Figure 3.2.2). Following two reports from Uteshev's lab (Gusev and Uteshev, 2010; Kalappa et al., 2010), that showed that PNU1 could enhance "sub-threshold, physiological (~10 μM)" concentrations of choline at α7 nAChRs by using patch-clamp electrophysiology in tuberomammillary neurons in hypothalamic slices, and hippocampal CA1 pyramidal neurons, we assessed the ability of PNU1 to potentiate the responses to lower concentrations of choline and PNU2, as well as the concentrations used in Figure 3.2.1. For this we chose to use PNU2 at 100 nM, roughly equivalent to the difference in concentration between 1.5 mM and 10 μM used for choline.
SH-SY5Y cells loaded with fluo-3 AM were pre-incubated with varying concentrations of PNU1 (0.3 µM – 100 µM) for 5 min before stimulation with choline (10 µM, 1.5 mM; a) or PNU2 (30 nM, 100 nM, 1 µM; b). Fluorescence at 538 nm was then measured for 20 s. Data are presented as per cent maximum fluorescence determined by addition of 0.2 % Triton X-100 minus minimum fluorescence quenched by 350 mM MnCl₂. Points represent mean ± SEM or range of at least 2 independent experiments. Data were fitted to the Hill equation.

SH-SY5Y cells loaded with fluo-3 AM were pre-incubated with increasing concentrations of PNU1 (0.3 µM – 100 µM), and stimulated with choline (1.5 mM). Here there was concentration-dependent potentiation by PNU1 with EC₅₀ estimated at 4.49 µM, which reached maximal levels of ~50 % at ~100 µM. Upon stimulation of cells with physiological concentrations of choline (10 µM), PNU1 (1 µM – 100 µM) concentration-dependently potentiated responses with estimated EC₅₀ of 16.33 µM, also approaching maximal levels of ~12 % at ~100 µM.

Cells pre-incubated with varying concentrations of PNU1 (0.3 µM – 100 µM) were then stimulated with PNU2 (1 µM). A concentration-dependent relationship was observed for PNU1 potentiation with estimated EC₅₀ of 6.80 µM, tending to maximum of ~64 % at ~100 µM. To evaluate the effects of lower agonist concentrations, we chose a concentration 100 times lower (100 nM), as with choline above. Here, PNU1 potentiated responses with a similar EC₅₀ of 13.60 µM, approaching maximum of ~33 % at ~100 µM. Finally, we used a lower concentration of PNU2 (30 nM). Here, pre-incubation of PNU1 (0.3 µM – 100 µM) was relatively ineffective at boosting PNU2 induced responses, with maximum fluorescence increase of ~4 %.
Figure 3.2.3: PNU1 potentiates low concentrations of choline and PNU2.

SH-SY5Y cells loaded with fluo-3 AM were stimulated with choline (10 μM; blue bars) and PNU2 (100 nM; green bars) in the presence or absence of PNU1 (30 μM; dashed bars). Responses observed in the presence of PNU1 were challenged with the α7 nAChR selective antagonist α-bungarotoxin (αbgt; 100 nM; hatched bars). Fluorescence at 538 nm was measured for 20 s following stimulation with choline or PNU2 alone or following 5 min pre-incubation and co-application of PNU1 or 20 min pre-incubation of αbgt. Data are presented as percent maximum fluorescence determined by addition of 0.2 % Triton X-100 minus minimum fluorescence quenched by 350 mM MnCl₂. Bars represent mean ± SEM of at least 4 independent experiments; *P < 0.05, **P < 0.01 significantly different from choline or PNU2 or in combination with PNU1, non-paired one-way ANOVA, Bonferroni's multiple comparisons test.

Figure 3.2.3 shows that the low concentrations of choline (10 μM) and PNU2 (100 nM) are potentiated by PNU1 (30 μM) by 5.67 ± 1.32 and 9.70 ± 2.13 fold respectively. Pre-incubation and co-application of αbgt (100 nM; 20 min) blocked these effects.

In summary, so far we have demonstrated the ability of PNU1 to reveal responses evoked by α7 nAChR selective agonists choline and PNU2. Concentration-response dependence of potentiation by PNU1 was explored, and it was found that potentiation by PNU1 occurred even at lower “sub-threshold” concentrations. Potentiated responses were completely blocked by the homomeric nAChR antagonist αbgt. Equipped with the information gained in these high throughput assays using the SH-SY5Y cell line, we used this knowledge to inform live imaging experiments on mouse primary cortical cultures, which exhibit a high degree of intercellular connectivity and a heterogeneous cell population. Here, sequential drug perfusions could be applied, and their effects on individual cells imaged in real time.
3.2.2 Preliminary experiments and method development in mouse primary cortical cultures

In contrast to the SH-SY5Y cell experiments, monitoring Ca\textsuperscript{2+} flux in cortical neurons was a new departure for the laboratory and required some preliminary experiments to optimize the system, based on that used by Innocent et al. (2008) for PC12 cells. Mouse primary cortical cultures (10-14 DIV) were loaded with the ratiometric Ca\textsuperscript{2+} sensitive dye fura-2 AM and perfused with 1.8 mM Ca\textsuperscript{2+} buffer at 37 °C. Fluorescence was measured at 510 nm after dual excitation at 340 and 380 nm to give the fluorescence ratio \textit{F}_{340}\textit{F}_{380}. Initially, basal fluorescence was measured for 30 s before switching perfusion to KCl (40 mM) for 20 s as a positive control, followed again by buffer (Figure 3.2.4). Drugs were applied by gravity feed at a rate of \textasciitilde{}5 ml/min and

![Image](image.png)

**Figure 3.2.4:** KCl evokes robust \textit{F}_{340}\textit{F}_{380} increases in mouse primary cortical cultures

Mouse primary cortical cultures (10-14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Basal fluorescence (a) was monitored for 60 s before 20 s application of KCl (40 mM; b). Still images show fluorescence in pseudocolour, (black/blue = low \textit{F}_{340}\textit{F}_{380}, red/white = high \textit{F}_{340}\textit{F}_{380}) taken during buffer perfusion and KCl stimulation. Time trace (c) shows coloured lines corresponding to 42 regions of interest selected as responding cells, circled in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm (\textit{F}_{340}\textit{F}_{380}).
typically took ~20 s to reach cells through perfusion lines and provoke a response. In one experiment, KCl evoked responses from 35/42 regions of interest (ROIs; Figure 3.2.4b) in a densely populated field of view. The average peak F₃₄₀:F₃₈₀ increase was 0.87 (SD = 0.21), and was slightly variable between ROIs, ranging from 1.01-1.91 above basal levels (0.59; SD = 0.02). Following calibration, average basal [Ca²⁺]ᵢ was determined by the Grynkiewicz equation (see methods; Grynkiewicz et al., 1985) to be 278.91 ± 86.74 nM, which is higher than others report (<100 nM; Dajas-Bailador et al., 2000; Beker et al., 2003; Evans and Cousin, 2007), furthermore basal [Ca²⁺]ᵢ of some cells was calculated to be “0 nM”. Three repeats of calibration were carried out, but did not give repeatable results, therefore here we present data as F₃₄₀:F₃₈₀.

![Figure 3.2.5: PNU2 alone does not elicit a detectable response.](image)

Cultured cells (10-14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Basal fluorescence was monitored for 30 s before 20 s application of PNU2 (3 μM). Buffer was perfused for another 80 s before a 20 s application of KCl (40 mM). Coloured lines represent individual regions of interest – all cells responding to KCl. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm (F₃₄₀:F₃₈₀).

Having observed positive responses to KCl, we then aimed to verify the presence of functional α7 nAChRs in mouse primary cortical cultures by stimulating them with α7 nAChR selective drugs whilst monitoring changes in the fluorescence ratio F₃₄₀:F₃₈₀.
Alone, 20 s perfusion with PNU2 did not elicit a detectable increase in $F_{340}:F_{380}$ (repeated 3 times; Figure 3.2.5). In this experiment, subsequent perfusion of 40 mM KCl for 20 s to the same cells resulted in large and rapid increases in $F_{340}:F_{380}$ of 0.33 ± 0.03 (5 cells) above basal levels (0.44 ± 0.17), confirming that the cells were responsive. We therefore used the α7 nAChR PAM PNU1 to enhance α7 nAChR activation to detectable levels. Pre-incubation of PNU1 (10 μM) for 3 min had no measurable effect on basal $F_{340}:F_{380}$ (Figure 3.2.6). However, co-application of PNU2 (3 μM) with PNU1 (10 μM) resulted in large $F_{340}:F_{380}$ increases of 0.42 ± 0.08 above basal (0.30 ± 0.03), averaged from 35 experiments on 14 independent cultures. These responses occurred in a subpopulation of cells counted at 12 % of all visible cells from a sample of 20 measurements from 14 independent cultures, a much lower proportion than that responding to KCl (40 mM; 82 %). Individual responses to PNU2 (3 μM) in the presence of PNU1 (10 μM) were highly variable; peak $F_{340}:F_{380}$ increases measured of 455 cells from 14 independent cultures ranged from 0.00 to 2.26 with an average of 0.46 ± 0.04 above basal levels (0.32 ± 0.01) with a standard deviation of 0.92. For this reason when comparing different drug treatments to control responses,

![Figure 3.2.6: PNU1 alone does not affect $F_{340}:F_{380}$, but enhances PNU2 stimulation.](image)

Cultures (10-14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Basal fluorescence was monitored for 30 s before 3 min pre-incubation of PNU1 (10 μM) prior to 20 s co-application of PNU2 (3 μM). Coloured lines represent five regions of interest – all cells responding to PNU2 (3 μM) in the presence of PNU1 (10 μM). Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm ($F_{340}:F_{380}$).
measurements were always sequential and taken from the same cell, control responses are normalized to 100%.

During preliminary experiments, it was noticed that F_{340}:F_{380} measured from cells would sometimes oscillate spontaneously (Figure 3.2.7a), in the absence of drugs. We therefore examined including the voltage gated Na^+ channel blocker tetrodotoxin (1 μM; Figure 3.2.7b). This reduced the noise that these oscillations caused in the F_{340}:F_{380} signal. TTX also served to prevent axonal transmission of action potentials, thus isolating cell bodies from their nerve terminals. TTX was therefore routinely included in all subsequent experiments (Murphy et al., 1992).

In some experiments, F_{340}:F_{380} was recorded for over 10 min to observe sequential drug treatments that required long pre-incubation times, it was noticed that basal F_{340}:F_{380} levels began to increase after long periods of time, consistent with a photobleaching effect (Figure 3.2.8). During long drug pre-incubations therefore, F_{340}:F_{380} was switched off, until 30 s before stimulation, to limit recording time to 2-3 min.

![Figure 3.2.7: Spontaneous F_{340}:F_{380} oscillations are silenced by TTX](image)

Cultures (10-14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Basal fluorescence was monitored for 30 s in the absence (a) or presence (b) of TTX (1 μM; same cells). Coloured lines represent individual regions of interest. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm (F_{340}:F_{380}).
To confirm the α7 nAChR selectivity, responses to PNU2 with PNU1 were challenged with the α7 nAChR selective competitive antagonist methyllycaconitine (MLA 100 nM; as opposed to using abgt, which requires lengthy pre-incubation, ~20 min). Control responses to PNU2 (20 s; 3 μM) in the presence of PNU1 (10 μM; 3 min) gave average peak $F_{340}:F_{380}$ increases of $0.20 \pm 0.13$ above basal levels ($0.25 \pm 0.06$). MLA pre-incubated for 10 min fully blocked subsequent responses elicited by PNU2 (3 μM) in combination with PNU1 (10 μM; 93.86 ± 7.26 %; Figure 3.2.9a, d) in all previously responding cells, (39 cells pooled from 4 experiments conducted in 3 independent cultures; SD = 11.00 %; Figure 3.2.21). Following 10 min washout, 3 min pre-incubation and co-application of PNU1 with PNU2 for 20 s resulted in a variable recovery of the original response (89.68 ± 22.73 %; Figure 3.2.9c, d).

![Graph](image)

Figure 3.2.8: Rising basal $F_{340}:F_{380}$ during long periods of recording.

Cultures (10-14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm fluorescence was monitored for 25 min. Coloured lines represent individual regions of interest. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm ($F_{340}:F_{380}$).

In one experiment, cultures were exposed to PNU1 (10 μM) in combination with PNU2 (3 μM) multiple times to evaluate whether responses would remain consistent (Figure 3.2.10). Initially, cells were pre-incubated with PNU1 (10 μM) for 3 min, and then exposed to three consecutive 20 s PNU2 (3 μM) co-applications, 50 s
apart (Figure 3.2.10a). Here a gradual downward trend in the average peak response from 8 cells was observed, from a $F_{340}:F_{380}$ ratio of 0.82 ± 0.16 in the initial response to 0.77 ± 0.14 in the second response, and 0.57 ± 0.08 in the third response. Cells were also subjected to consecutive stimulations separated by 3 min washout periods (Figure 3.2.10b). This protocol did not result in an obvious decrease in the responses, although different cells exhibited a variable response to successive stimulations over time (standard deviation between responses from individual cells ranged from 0.05 – 0.34). We therefore chose to allow cells to recover with a 3 min washout following stimulation in subsequent experiments.

Having confirmed the existence of functional α7 nAChRs in mouse primary cortical cultures, and a reliable method of activating them to produce increases in $F_{340}:F_{380}$ with the selective compounds PNU1 and PNU2, we sought to examine the responses for contributions from other sources of Ca$^{2+}$. To do this we would use compounds that selectively blocked the various routes of Ca$^{2+}$ entry into the cytosol.
Chapter 3: Mechanisms of α7 nAChR-Mediated Calcium Signalling in Primary Cortical Cultures

Figure 3.2.9: MLA fully blocks responses evoked by PNU1 in combination with PNU2 in mouse primary cortical cultures.

Cultures (10–14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal \( \frac{F_{340}}{F_{380}} \); after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with MLA (100 nM) and PNU1 (10 μM) prior to recording \( \frac{F_{340}}{F_{380}} \); finally, following 10 min wash out, the protocol was repeated in the absence of MLA (c). Still images show fluorescence in pseudocolour: (black/blue = low \( \frac{F_{340}}{F_{380}} \), red/white = high \( \frac{F_{340}}{F_{380}} \)) taken during PNU2 stimulation. Traces show coloured lines corresponding to 20 regions of interest selected as responding cells, circled in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak \( \Delta \frac{F_{340}}{F_{380}} \) increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 3 independent experiments; ** \( P < 0.01 \) significantly different from initial response to PNU2 in combination with PNU1 (100 %), one sample t-test.
Figure 3.2.10: Repetitive stimulation of mouse primary cortical cultures with PNU2 in combination with PNU1

Cultures (10-14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C, and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal $F_{340}/F_{380}$ for 30 s, after which PNU2 (3 μM) was co-applied for 20 s. (a i) Two further PNU2 applications (3 μM; 20 s) were given after 50 s intervals, perfusion of PNU1 (10 μM) was left on the whole time. (a ii) Cells were exposed to three more consecutive PNU2 (3 μM; 20 s) co-applications in the presence of PNU1 (10 μM; 30 s) followed by 3 min washouts. Traces show coloured lines corresponding to individual regions of interest selected as responding cells. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm. Bars (a ii, b ii) represent mean peak $F_{340}/F_{380}$ of consecutive responses to PNU2 in the presence of PNU1 from traces a i and b i respectively.
3.2.3 α7 nAChR mediated Ca$^{2+}$ elevations in mouse cortical cultures are dependent on VGCC activation.

If α7 nAChR mediated [Ca$^{2+}$]$_{ic}$ elevations in mouse primary cortical cultures were augmented by VGCC activation following depolarization of the cell membrane due to α7 nAChR mediated Na$^+$ influx, we hypothesized that increases in F$_{340}$:F$_{380}$ in response to PNU2 in the presence of PNU1 would be diminished by non-selective blockade of VGCCs with Cd$^{2+}$. As discussed in section 1.2.2.4, various VGCC subtypes exist performing specialized roles in different cells and tissues. Here we also attempted to evaluate the dependence of α7 nAChR mediated [Ca$^{2+}$]$_{ic}$ on L-type VGCC by using the dihydropyridine drug nifedipine.

Initial application of PNU2 (3 μM) in combination with PNU1 (10 μM) yielded average peak F$_{340}$:F$_{380}$ increases of 0.23 ± 0.10 above basal levels (0.29 ± 0.08). Pre-incubation and co-application of Cd$^{2+}$ (10 min; 50 μM; Figure 3.2.11b, d) with PNU2 (3 μM) in combination with PNU1 (10 μM) resulted in a 70.07 ± 5.07 % inhibition of the original response elicited by PNU2 (3 μM; Figure 3.2.11a, d) and PNU1 (10 μM) in all cells examined, although to a more variable degree than blockade observed for MLA, with standard deviation of 21.27 % from 34 cells in 3 independent cultures (Figure 3.2.21). Following 10 min washout, a third stimulation with PNU2 (3 μM; Figure 3.2.11c, d) in combination with PNU1 (10 μM) elicited a response that had recovered to 76.56 ± 3.99 %.

In experiments where nifedipine was used, we observed average peak F$_{340}$:F$_{380}$ increases above basal levels (0.35 ± 0.04) of 0.70 ± 0.34 in response to PNU2 (3 μM; 20 s; Figure 3.2.12a, d) in the presence of PNU1 (10 μM; 3 min) from 23 cells in 3 cultures. Pre-incubation and co-application of nifedipine (3 μM; 10 min) had no significant effect on responses elicited by PNU2 (3 μM; 20 s) in combination with PNU1 (10 μM; 3 min) but there was a downward trend of 31.89 ± 15.61 %. This effect was highly variable, with standard deviation of 42.00 % (Figure 3.2.21). After 10 min washout, responses to further stimulation with PNU2 (3 μM; 20 s) with PNU1 (10 μM; 3 min) did not recover but declined further to 48.33 ± 9.99 % of the original response (Figure 3.2.12c, d).
3.2.4 α7 nAChR mediated Ca\(^{2+}\) elevations in mouse cortical cultures are dependent on ER mediated Ca\(^{2+}\) release

To determine whether α7 nAChR mediated \([Ca^{2+}]_c\) elevations in mouse primary cortical cultures depend on amplification by ER mediated Ca\(^{2+}\) release, we challenged responses to PNU2 in the presence of PNU1 with ryanodine and xestospongin-C (XeC), which block ryanodine receptors and IP\(_3\) receptors respectively, mediators of Ca\(^{2+}\) induced Ca\(^{2+}\) release from the ER (see section 1.2.2).

The initial control response to PNU2 (3 μM) in combination with PNU1 (10 μM) resulted in average peak F\(_{340}\):F\(_{380}\) increases of 0.46 ± 0.13 above basal levels (0.27 ± 0.02; Figure 3.2.13a, d). Following 3 min washout, 10 min pre-incubation and co-application of Ry (30 μM) variably impeded the response to PNU2 (3 μM) with PNU1 (10 μM) by an average of 58.75 ± 2.42 % (Figure 3.2.13b, d), with standard deviation of 24.18 % from 75 cells in 3 independent cultures (Figure 3.2.21). The response then recovered to 78.84 ± 9.74 % of control after 10 min washout (Figure 3.2.13c, d).

Similarly, when cells were subjected to 10 min pre-treatment with XeC (5 μM), co-application of PNU2 (3 μM) and PNU1 (10 μM) resulted in a 60.84 ± 11.70 % block of the original response (0.31 ± 0.18 above basal 0.26 ± 0.10; SD = 26.15; 42 cells; 3 cultures; Figure 3.2.21), which then recovered to 60.00 ± 12.35 % following 10 min washout. Figure 3.2.14 shows a striking example of an experiment where XeC (5 μM) almost completely blocked the initial response to PNU2 (3 μM) in the presence of PNU1 (10 μM).

To summarize, so far we have demonstrated that mouse primary cortical cultures loaded with fura-2 AM respond to the α7 nAChR selective agonist PNU2 in combination with the α7 nAChR selective positive allosteric modulator PNU1 in an MLA sensitive manner. These responses are also significantly blocked by the general VGCC blocker Cd\(^{2+}\), the ryanodine receptor antagonist ryanodine, and the IP\(_3\) receptor antagonist XeC to variable degrees. Blockade of L-type VGCCs however did not have a significant effect. The following experiments aimed to investigate whether the α7 nAChR mediated \([Ca^{2+}]_c\) elevations extend beyond single cells by means of neurotransmission.
Figure 3.2.11: α7 mediated calcium elevations are inhibited by voltage gated calcium channel blockade by Cd2+ in mouse primary cortical cultures.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal F340:F380 for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with Cd2+ (50 μM) and PNU1 (10 μM) prior to recording F340:F380, before, during and after co-stimulation with PNU2 (3 μM; b). Finally, following 10 min wash out, the protocol was repeated in the absence of Cd2+ (c). Stills images show fluorescence in pseudocolour, (black/blue = low F340:F380, red/white = high F340:F380) taken during PNU2 stimulation. Traces show coloured lines corresponding to 9 regions of interest selected as responding cells, indicated with coloured arrow heads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak F340:F380 increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 3 independent experiments; ** P < 0.01 significantly different from initial response to PNU2 in combination with PNU1 (100 %), one sample t-test.
Figure 3.2.12: α7 mediated calcium elevations are not significantly inhibited by L-type voltage gated calcium channel blockade by nifedipine in mouse primary cortical cultures. Cultures (10-14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM, 3 min) before recording basal \( F_{340:F_{380}} \) for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with nifedipine (3 μM) and PNU1 (10 μM) prior to recording \( F_{340:F_{380}} \) before, during and after co-stimulation with PNU2 (3 μM; 20 s; b). Finally, following 10 min wash out, the protocol was repeated in the absence of nifedipine (c). Still images show fluorescence in pseudocolour, (black/blue = low \( F_{340:F_{380}} \); red/white = high \( F_{340:F_{380}} \)) taken during PNU2 stimulation. Traces show coloured lines corresponding to 8 regions of interest selected as responding cells, indicated with coloured arrow heads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak \( F_{340:F_{380}} \) increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 3 independent experiments.
Figure 3.2.13: α7 nAChR mediated calcium elevations are inhibited by ryanodine receptor blockade in mouse primary cortical cultures.

Cultures (10-14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal $F_{340}/F_{380}$ for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with ryanodine (30 μM) and PNU1 (10 μM) prior to recording $F_{340}/F_{380}$ before, during and after co-stimulation with PNU2 (3 μM; 20 s; b). Finally, following 10 min wash out, the protocol was repeated in the absence of ryanodine (c). Still images show fluorescence in pseudocolour, (black/blue = low $F_{340}/F_{380}$, red/white = high $F_{340}/F_{380}$) taken during PNU2 stimulation. Traces show coloured lines corresponding to 7 regions of interest selected as responding cells, indicated with coloured arrow heads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak $F_{340}/F_{380}$ increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 3 independent experiments; ** $P < 0.01$ significantly different from initial response to PNU2 in combination with PNU1 (100 %), one sample t-test.
Chapter 3: Mechanisms of α7 nAChR-Mediated Calcium Signalling in Primary Cortical Cultures

Figure 3.2.14: α7 nAChR mediated calcium elevations are inhibited by xestospongin C in mouse primary cortical neurons.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (30 μM, 3 min) before recording F340:F380 for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with XeC (5 μM) and PNU1 (10 μM) prior to recording F340:F380 before, during and after co-stimulation with PNU2 (3 μM; 20 s; b). Finally, following 10 min wash out, the protocol was repeated in the absence of XeC (c). Still images show fluorescence in pseudocolour; (black/blue = low F340:F380, red/white = high F340:F380) taken during PNU2 stimulation. Traces show coloured lines corresponding to 7 regions of interest selected as responding cells, indicated with coloured arrow heads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from an example of almost full blockade of PNU2 and PNU1 evoked responses by XeC. Bars (d) represent mean peak F340:F380 increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 3 independent experiments; ** P < 0.01 significantly different from initial response to PNU2 in combination with PNU1 (100 %), one sample t-test.
3.2.5 Contribution of glutamatergic neurotransmission to α7 nAChR mediated Ca\(^{2+}\) elevations in mouse cortical cultures

α7 nAChRs are well known to reside on presynaptic terminals in neurons of the CNS where activation enhances the probability of neurotransmitter release, a Ca\(^{2+}\) dependent process (Wonnacott, 1997). We hypothesized that if α7 nAChRs existed on glutamatergic terminals in mouse primary cortical cultures, application of PNU2 in combination with PNU1 would indirectly lead to [Ca\(^{2+}\)]\(_{i}\) elevations in postsynaptic cells, via facilitation of glutamate release and subsequent activation of postsynaptic ionotropic and/or metabotropic glutamate receptors (iGluRs and/or mGluRs; see section 1.2.2.5). To test this, we used a range of antagonists to block the various GluRs: CNQX for AMPA type GluRs, MK801 for NMDA type GluRs, and LY456236 (LY4) for group I mGluRs.

3.2.5.1 Dependence on AMPA receptors

Firstly, we assessed the ability of AMPA to elicit [Ca\(^{2+}\)]\(_{i}\) elevations in mouse primary cortical neurons. Cells were loaded with fura-2 and perfused with buffer at 37 °C as above, and stimulated with AMPA (20 s; 10 μM) which resulted in average F\(_{340}:F\(_{380}\) increases of 1.14 ± 0.70 (45 cells pooled from 6 experiments on 2 independent cultures) above basal levels (0.38 ± 0.02). In 3 of these experiments (from 1 culture), cells were also exposed to PNU2 (3 μM) in the presence of PNU1 (3 min pre-incubation; 10 μM) to compare the response amplitude and number of responding cells to that induced by AMPA (10 μM). We observed F\(_{340}:F\(_{380}\) increases of 0.76 ± 0.07 above basal levels (0.37 ± 0.03), 92.18 ± 8.45% of the response evoked by AMPA (10 μM), but this occurred in only 5/31 cells that responded to AMPA (10 μM). In these experiments, pre-incubation and co-application of the competitive AMPAR antagonist CNQX (10 min; 10 μM; Honoré et al., 1988) blocked further responses to AMPA (20 s; 10 μM) by 50.31 ± 1.41% of the original response, recovering to 76.48 ± 23.32% when stimulated with AMPA (20 s; 10 μM) again after 10 min washout.

Having demonstrated robust responses to AMPA and their blockade by CNQX, we sought to evaluate the dependence of [Ca\(^{2+}\)]\(_{i}\) elevations evoked by α7 nAChR stimulation in primary cortical cultures. Following control responses to PNU2 (20 s; 3 μM) in the presence of PNU1 (3 min; 10 μM), which gave F\(_{340}:F\(_{380}\) increases of 0.30 ± 0.16 above basal levels (0.28 ± 0.65), blockade by CNQX (10 min; 10 μM) variably
attenuated further responses by $37.49 \pm 4.75 \%$. Notably, the standard deviation of the % block of responses to PNU2 in the presence of PNU1 by CNQX measured in 34 cells was 56.27 %, more variable than any of the conditions tested so far (Figure 3.2.21). Following 10 min washout, responses to PNU2 (20 s; 3 μM) in the presence of PNU1 (10 μM) recovered to $73.78 \pm 6.33 \%$ of control.
Chapter 3: Mechanisms of α7 nAChR-Mediated Calcium Signalling in Primary Cortical Cultures

Figure 3.2.15: AMPA evoked calcium responses are blocked by CNQX in mouse primary cortical cultures.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal $F_{340}:F_{380}$ for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. Following 3 min washout, basal fluorescence was again recorded for 30 s before AMPA (10 μM) was applied to cells for 20 s (b). After 3 min wash out, cells were then pre-incubated for 10 min with CNQX (10 μM) prior to recording $F_{340}:F_{380}$ before, during and after co-stimulation with AMPA (10 μM; 20 s; c). Finally, following 10 min wash out, cells were stimulated a third time with AMPA (10 μM; 20 s; d). Still images show fluorescence in pseudocolour, (black/blue = low $F_{340}:F_{380}$, red/white = high $F_{340}:F_{380}$) taken during agonist stimulation. Traces show coloured lines corresponding to 6 regions of interest selected as AMPA responding cells, indicated with coloured arrow heads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (e) represent mean peak $F_{340}:F_{380}$ increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 2 independent experiments.
Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37°C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal $F_{340}/F_{380}$ for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with CNQX (10 μM) and PNU1 (10 μM) prior to recording $F_{340}/F_{380}$ before, during and after co-stimulation with PNU2 (3 μM; 20 s; b). Finally, following 10 min wash out, the protocol was repeated in the absence of CNQX (c). Still images show fluorescence in pseudocolour, (black/blue = low $F_{340}/F_{380}$, red/white = high $F_{340}/F_{380}$) taken during PNU2 stimulation. Traces show coloured lines corresponding to 4 regions of interest selected as responding cells, indicated with coloured arrowheads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak $F_{340}/F_{380}$ increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 4 independent experiments; ** $P < 0.01$ significantly different from initial response to PNU2 in combination with PNU1 (100 %), one sample t-test.
3.2.5.2 Dependence on NMDA receptors

In a single experiment, we stimulated fura-2 AM loaded primary mouse cortical cultures with NMDA (50 μm), which elicited average $F_{340}:F_{380}$ peak increases of 0.30 ± 0.05 from 9 cells. Pre-incubation and co-application of the non-competitive NMDA receptor channel blocker MK801 (2.5 μM; 10 min; Wong et al., 1986) completely blocked these responses (98.90 ± 0.42 %). After 30 s washout, a further NMDA (50 μM) application for 20 s gave an average peak $F_{340}:F_{380}$ increase of 37.75 ± 10.47 %.

Figure 3.2.17: MK801 blocks NMDA evoked Ca$^{2+}$ elevations in mouse primary cortical cultures

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Basal $F_{340}:F_{380}$ was recorded for 30 s, after which NMDA (50 μM; a) was applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with MK801 (2.5 μM) prior to recording $F_{340}:F_{380}$ before, during and after co-stimulation with NMDA (50 μM; 20 s; b). Cells were then stimulated again with NMDA (50 μM; 20 s) after 30 s washout (b). Still images show fluorescence in pseudocolour, (black/blue = low $F_{340}:F_{380}$, red/white = high $F_{340}:F_{380}$) taken during PNU2 stimulation. Traces show coloured lines corresponding to 9 regions of interest selected as responding cells. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm.

Having observed a inhibition of NMDA mediated [Ca$^{2+}$]$_{ic}$ elevations with MK801, we now tested the hypothesis that [Ca$^{2+}$]$_{ic}$ elevations in mouse primary cortical cultures were contributed to by NMDA receptor activation following presynaptic α7 nAChR mediated glutamate release. After stimulating fura-2 AM loaded cultures with to PNU2 (3 μM; 2 s) in the presence of PNU1 (10 μM; 3 min), we
observed average peak increases in $F_{340}:F_{380}$ of 1.80 ± 0.43 over basal levels (0.38 ± 0.05) from 45 responding cells from 4 separate cultures. Pre-incubation and co-application of MK801 (2.5 μM) variably blocked these responses by an average of 57.44 ± 15.86 %. The variability of % block by MK801 is described by a standard deviation of 35.01 % (46 cells; 3 cultures; Figure 3.2.21). Responses then recovered to 48.05 ± 10.14 % after a further application of PNU2 (3 μM; 20 s) with PNU1 (10 μM; 3 min) following 10 min washout.

Experiments that followed combined CNQX and MK801 in an attempt to block both AMPA and NMDA receptor contributions to α7 nAChR mediated $[Ca^{2+}]_c$ elevations. Initial responses to PNU2 (3 μM; 20 s) in the presence of PNU1 (10 μM; 3 min) yielded average peak increases in $F_{340}:F_{380}$ of 0.43 ± 0.05 above basal levels (0.38 ± 0.01), which were variably blocked 56.07 ± 16.57 % by 10 min pre-incubation and co-application of CNQX (10 μM) and MK801 (2.5 μM). Here, the variability of the % block is described by the standard deviation of 34.64 % (19 cells; 2 cultures; Figure 3.2.21). Following 10 min washout, responses recovered to 68.76 ± 4.98 %. There was no sign of additivity in the effect of CNQX and MK801, although more experiments would be needed to confirm this.
Figure 3.2.18: α7 nAChR mediated calcium elevations are partially blocked by MK801 in mouse primary cortical cultures.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37°C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal F$_{340}$:F$_{380}$ for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with MK801 (2.5 μM) and PNU1 (10 μM) prior to recording F$_{340}$:F$_{380}$ before, during and after co-stimulation with PNU2 (3 μM; 20 s; b). Finally, following 10 min wash out, the protocol was repeated in the absence of MK801 (c). Still images show fluorescence in pseudocolour, (black/blue = low F$_{340}$:F$_{380}$, red/white = high F$_{340}$:F$_{380}$) taken during PNU2 stimulation. Traces show coloured lines corresponding to 4 regions of interest selected as responding cells, indicated with coloured arrow heads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak F$_{340}$:F$_{380}$ increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 4 independent experiments; * P < 0.05 significantly different from initial response to PNU2 in combination with PNU1 (100 %), one sample t-test.
Chapter 3: Mechanisms of α7 nAChR-Mediated Calcium Signalling in Primary Cortical Cultures

Figure 3.2.19: α7 nAChR mediated calcium elevations are partially blocked by CNQX and MK801 in mouse primary cortical cultures.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal $F_{340}/F_{380}$ for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with CNQX (10 μM), MK801 (2.5 μM) and PNU1 (10 μM) prior to recording $F_{340}/F_{380}$ before, during and after co-stimulation with PNU2 (3 μM; 20 s; b). Finally, following 10 min wash out, the protocol was repeated in the absence of CNQX and MK801 (c). Still images show fluorescence in pseudocolour, (black/blue = low $F_{340}/F_{380}$, red/white = high $F_{340}/F_{380}$) taken during PNU2 stimulation. Traces show coloured lines corresponding to 4 regions of interest selected as responding cells, indicated with coloured arrow heads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak $F_{340}/F_{380}$ increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± range from 2 independent experiments.
Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37°C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal $F_{340:380}$ for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with MK801 (2.5 μM) and PNU1 (10 μM) prior to recording $F_{340:380}$ before, during and after co-stimulation with PNU2 (3 μM; 20 s; b). Finally, following 10 min wash out, the protocol was repeated in the absence of MK801 (c). Still images show fluorescence in pseudocolour, (black/blue = low $F_{340:380}$, red/white = high $F_{340:380}$) taken during PNU2 stimulation. Traces show coloured lines corresponding to 4 regions of interest selected as responding cells, indicated with coloured arrow heads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak $F_{340:380}$ increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 4 independent experiments; * $P < 0.05$ significantly different from initial response to PNU2 in combination with PNU1 (100 %), one sample t-test.

Figure 3.2.18: α7 nAChR mediated calcium elevations are partially blocked by MK801 in mouse primary cortical cultures.
α7 nAChR-mediated calcium elevations are partially blocked by CNQX and MK801 in mouse primary cortical cultures.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal $F_{340}/F_{380}$ for 30 s, after which PNU2 (3 μM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with CNQX (10 μM), MK801 (2.5 μM) and PNU1 (10 μM) prior to recording $F_{340}/F_{380}$ before, during and after co-stimulation with PNU2 (3 μM; 20 s; b). Finally, following 10 min wash out, the protocol was repeated in the absence of CNQX and MK801 (c). Still images show fluorescence in pseudocolour, (black/blue = low $F_{340}/F_{380}$, red/white = high $F_{340}/F_{380}$) taken during PNU2 stimulation. Traces show coloured lines corresponding to 4 regions of interest selected as responding cells, indicated with coloured arrow heads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak $F_{340}/F_{380}$ increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± range from 2 independent experiments.
3.2.5.3 Dependence on metabotropic glutamate receptors
As well as the ionotropic AMPA and NMDA type GluRs, mGluR1 also play a role in elevating $[Ca^{2+}]_c$ postsynaptically following synaptic glutamate transmission, via IP$_3$ receptor mediated release from the ER (Niswender and Conn, 2010). To test the possibility that group mGluR1 is involved in $[Ca^{2+}]_c$ elevations that occur following stimulation of α7 nAChRs in mouse primary cortical neurons, we attempted to challenge responses to PNU2 in combination with PNU1 to the selective mGluR1 antagonist LY456236 (LY4).

PNU2 (3 μM) in the presence of PNU1 (10 μM; 3 min) provoked average peak $F_{340}:F_{380}$ increases of 0.33 ± 0.03 above basal levels (0.38 ± 0.00). In the presence of LY4 (10 μM; Shannon et al., 2005; Varty et al., 2005) there was a downward trend of 28.75 ± 12.95 % (Figure 3.2.20). The overall effect was highly variable, giving a standard deviation of 53.89 % (24 cells; 2 cultures; Figure 3.2.21). In 4 of these cells, responses were actually enhanced by 58.76 ± 4.18 %. After 10 min washout, average peak increases of $F_{340}:F_{380}$ following stimulation with PNU2 (3 μM; 20 s) in the presence of PNU1 (10 μM; 3 min) remained at a similar level of 54.25 ± 23.80 %.

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**Figure 3.2.20: Effects of mGluR1 blockade on intracellular calcium elevation elicited by PNU2 in combination with PNU1.**

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal $F_{340}:F_{380}$ for 30 s, after which PNU2 (3 μM) was co-applied for 20 s. After 3 min washout, cells were then pre-incubated for 10 min with LY4 (10 μM) and PNU1 (10 μM) prior to recording $F_{340}:F_{380}$ before, during and after co-stimulation with PNU2 (3 μM; 20 s). Finally, following 10 min washout, the protocol was repeated in the absence of LY4. Bars represent mean peak $F_{340}:F_{380}$ increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 2 independent experiments.
Figure 3.2.21: Contributions to α7 mediated [Ca^{2+}]_{e} elevations in mouse primary cortical cultures.

Summary of experiments presented in previous figures. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording basal F_{340:F380} for 30 s, after which PNU2 (3 μM) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with MLA (100 nM), Cd^{2+} (50 μM), nifedipine (Nif; 3 μM), ryanodine (Ry; 30 μM), xestospongin C (XeC; 5 μM), CNQX (10 μM), MK801 (2.5 μM), or LY4 (10 μM) in the presence of PNU1 (10 μM) prior to recording F_{340:F380} before, during and after co-stimulation with PNU2 (3 μM; 20 s). Data points represent individual ROIs selected as responding cells pooled from repeat experiments, presented as percentage of initial response to PNU2 in combination with PNU1. Black lines represent mean ± SD.
3.3 Discussion

In this chapter we have investigated mechanisms of α7 nAChR mediated Ca\(^{2+}\) elevations in mouse primary cortical cultures using fura-2 AM live cell imaging, following initial experiments that aimed to optimize conditions for α7 nAChR stimulation with the selective PAM PNU1 and agonist PNU2 in SH-SY5Y cells.

3.3.1 Summary

In SH-SY5Y cells loaded with fluo-3 AM exposure to the α7 nAChR selective agonists choline and PNU2 had no measurable effect on F\(_{538}\). In the presence of PNU1 however, large fluorescence increases were observed that were dependent on the concentrations of agonists and PNU1. These responses were blocked by αbgt, confirming their α7 nAChR selectivity.

In primary cortical cultures, a protocol was developed to image changes in [Ca\(^{2+}\)]\(_{c}\) with the aid of fura-2 AM and live cell imaging. Here, drugs were applied via a perfusion system, so that individual cells could be subjected to repeated stimulation in the presence of antagonists. Initially, cultures were stimulated with KCl, which elicited large responses from the majority of cells imaged. TTX was used to prevent spontaneous Ca\(^{2+}\) oscillations, and recording time was limited to prevent photobleaching and focus drift. No responses were observed following application of PNU2, however in the presence of PNU1, a sub-population of cells responded to PNU2 with considerably variable magnitude. These responses were consistently blocked by MLA, confirming α7 selectivity.

To assess the contribution to these responses of other sources of Ca\(^{2+}\) we used selective inhibitors. The general VGCC blocker Cd\(^{2+}\), the RyR blocker Ry, and the IP\(_3\)R blocker XeC all significantly attenuated responses induced by PNU2 in combination with PNU1, but the degree of inhibition was variable. The L-type VGCC blocker nifedipine also produced a downward trend in responses to PNU1 and PNU2. Blocking AMPA and NMDA receptors with CNQX and NMDA was also shown to variably inhibit responses to PNU2 in the presence of PNU1, whilst there was no significant reduction when blocking mGluR\(_1\) with LY4.
3.3.2 PNU1 reveals α7 nAChR mediated Ca\(^{2+}\) elevations in SH-SY5Y cells

Application of nicotine (30 µM) to SH-SY5Y cells loaded with fluo-3 AM evoked modest and sustained increases in F\(_{538}\) indicative of [Ca\(^{2+}\)]\(_c\) elevations similar to that reported previously in this assay (Dajas-Bailador et al., 2002), which are attributed to heteromeric α3β2(α5) and α3β4(α5) nAChRs (see section 3.1.2). In contrast, application of the selective α7 nAChR agonists choline (1.5 mM) and PNU2 (1 µM) failed to evoke detectable responses, consistent with the widely reported rapid desensitization kinetics that characterize the α7 nAChR subtype (Giniatullin et al., 2005). It is well established that to observe activation events mediated by rapidly desensitizing receptors such as α7 nAChRs requires fast solution exchange methods of agonist application, as the peak response of the receptor occurs so rapidly that by the time peak agonist concentration in the extracellular environment is achieved, the receptor desensitizes (Papke and Porter Papke, 2002; Fedorov et al., 2012; Pesti et al., 2014). The kinetic properties of α7 nAChRs make them notoriously difficult to study, and the dependence of receptor activation events on solution exchange speed confounds pharmacological observations such as agonist concentration response relationships (Pesti et al., 2014). However, when the α7 nAChR selective type II PAM PNU1 (10 µM) was pre-incubated for 5 min prior to stimulation, a significant potentiation was observed for nicotine, choline and PNU2. This effect has been widely reported, for example in PC12 cells using the same assay with choline, nicotine and the α7 nAChR selective agonist Compound A (Dickinson et al., 2007; Innocent et al., 2008); in α7 nAChR expressing SH-EP1 cells with Ca\(^{2+}\) fluorimetry with ACh (Hurst et al., 2005); and in electrophysiological studies in X. laevis oocytes and rat hippocampal neurons with ACh and PNU2 (Hurst et al., 2005; Grønlien et al., 2007; Williams et al., 2011c, 2012). Potentiation by PNU1 is thought to occur via destabilization of the α7 nAChR desensitized state without affecting ion selectivity (Hurst et al., 2005; Grønlien et al., 2007; Williams et al., 2011b; Szabo et al., 2014). The effects of PNU1 on the desensitized state of α7 nAChRs are analogous to the effects of the α7 nAChR L247T mutant, which modifies the pore region, transforming what would be a non-conducting desensitized state in a wild-type receptor into a conducting one (Revah et al., 1991; Bertrand et al., 1992), and it was also recently reported that PNU1 binds to the desensitized state with 100-fold higher affinity than the resting state (Szabo et al., 2014). Pre-incubation with the α7 nAChR antagonist αbgt (100 nM) completely
blocked the effects of PNU1, supporting the α7 nAChR selectivity of this approach and emphasizing its advantages in studying α7 nAChR responses.

Examination of the concentration dependence of PNU1 potentiation of choline (1.5 mM) and PNU2 (1 μM) yielded EC$_{50}$ estimates of 15.89 μM and 4.17 μM respectively (Figure 3.2.2), which are slightly higher than the EC$_{50}$ value of PNU1’s potentiating effects reported by Grønlien et al. (~1.5 μM; 2007). In electrophysiological studies that are able to detect α7 nAChR responses to agonist alone, co-application of PNU1 is consistently reported to shift the EC$_{50}$ to the left, and increase E$_{\text{max}}$ of agonists such as ACh, PNU2 and GTS-21 (Hurst et al., 2005; Grønlien et al., 2007). Here we also assessed the potentiating effects of PNU1 when combined with lower concentrations of agonists, following reports from Uteshev’s lab (Gusev and Uteshev, 2010; Kalappa et al., 2010), who showed that physiological concentrations of choline found in cerebrospinal fluid (CSF; ~10 μM) were significantly potentiated by PNU1, activating native α7 nAChRs in hypothalamic tuberomammillary neurons and hippocampal CA1 pyramidal neurons. We also observed significant potentiation of 10 μM choline and a lower concentration of PNU2 (100 nM) by PNU1 (10 μM), which were both sensitive to \textalpha bgt (100 nM). The effects of PNU1 were not observed in the presence of 30 nM PNU2, highlighting the agonist concentration dependence of PNU1. Assessment of the concentration dependence of PNU1 potentiation yielded rightward shifted EC$_{50}$ values and decreased maximum stimulation values.

These data suggest that α7 PAMs like PNU1 could be used as therapeutics aimed at enhancing endogenous α7 nAChR function, retaining spatio-temporal specificity of endogenous stimulation, which could be a safer approach than using nicotinic agonists, as was suggested by Uteshev’s group (Gusev and Uteshev, 2010; Kalappa et al., 2010). This hypothesis raises many concerns however, as the intended therapeutic use of any pharmacological agent entails unwanted side effects. By dramatically reducing desensitization such PAMs greatly increase total ion flux and could allow cytotoxic levels of Ca$^{2+}$ to accumulate in cells, and undermine the physiological memory of recent events and protective mechanisms that receptor desensitization is thought to provide. Changing the kinetics of a receptor so much could severely disrupt synchrony in neural networks (Reviewed in Williams et al., 2011; Uteshev, 2012).
3.3.3 Live imaging of α7 nAChR mediated Ca\(^{2+}\) elevations in mouse primary cortical cultures

Using primary cultured mouse cortex allows the interrogation of Ca\(^{2+}\) signalling mechanisms in a more physiological setting, albeit still far removed from the native environment of the mammalian brain. Here, as opposed to in SH-SY5Y cell cultures, a diverse population of brain cells including neurons and glia (see chapter 4 for immunofluorescent characterization studies) that exhibit network connectivity is available for study (see 3.1.3). The elevated complexity of this system may in some ways be considered an experimental drawback, requiring a greater degree of control and certainty of any conclusions. Certainly, the use of higher resolution techniques of observation like live imaging used here dramatically reduce experimental throughput, which highlights the importance of the SH-SY5Y cell experiments in informing more basic pharmacological factors. The paramount advantage of this system lies in the ability to image individual cells in real time over multiple drug applications, and evaluate the effects of α7 nAChR mediated Ca\(^{2+}\) elevations on synaptic activity.

As above, application of PNU2 alone failed to elicit detectable responses in mouse cortical cultures. When PNU1 was pre-incubated and co-applied with PNU2 however, we were able to observe sizable, MLA sensitive increases in F\(_{340}\):F\(_{380}\) indicative of [Ca\(^{2+}\)]\(_{c}\) elevations from a subpopulation of cells that numbered fewer than those responding to stimulation with KCl. This suggests that the number of excitable cells exceeds the number of cells responding to α7 nAChR stimulation, possibly reflecting a more sporadic expression of α7 nAChRs in the entire cell population. This agrees with earlier studies by Barrantes et al., (1995b), who reported \([^{125}\text{I}]\alpha\beta\text{gt}\ binding in 36 % of cortical neurons and Hammond et al. (2013), who recently reported that nAChR influence of spontaneous network activity was limited to a subset of circuits in rat primary cortical neurons.

Average peak F\(_{340}\):F\(_{380}\) increases in response to PNU2 in combination with PNU1 were highly variable, which could reflect the diversity of [Ca\(^{2+}\)]\(_{c}\) elevation events mediated by α7 nAChRs in these cultures. α7 nAChRs are known to be expressed in different neuronal locations such as pre-synaptic terminals, cell bodies and dendrites (Fabian-Fine et al., 2001; Jones and Wonnacott, 2004; Dickinson et al., 2008; Hammond et al., 2013), as well as on astrocytes (Shen and Yakel, 2012;
Chapter 3: Mechanisms of α7 nAChR-Mediated Calcium Signalling in Primary Cortical Cultures

Pirttimaki et al., 2013; Talantova et al., 2013) and microglia (Morioka et al., 2014), and are known to mediate a range of Ca\(^{2+}\) dependent cellular events including neurotransmitter release (reviewed in section 1.2.2.8). Indeed, the aim of this chapter is to define the mechanisms that contribute to such events, which have not yet been reported for mouse primary cortical cultures.

### 3.3.4 VGCCs and ER contribute to α7 nAChR mediated Ca\(^{2+}\) elevations in mouse primary cortical cultures

Responses to PNU2 in combination with PNU1 were sensitive to blockade by the general VGCC blocker Cd\(^{2+}\), the RyR blocker Ry and the IP\(_3\)R blocker XeC, indicating that VGCCs and ER mediated Ca\(^{2+}\) release follow from α7 nAChR stimulation in mouse primary cortical cultures. The L-type VGCC antagonist nifedipine affected responses to a lesser extent, and was non-significant, further experiments would therefore be needed to confirm their involvement, but this suggests that non-L-type VGCCs possibly play a more substantial role. This could be investigated by using VGCC selective antagonists such as ω-conotoxin GVIA for N-type, ω-agatoxin for P/Q-type, and SNX-482 for R-type VGCCs. The greater degree of variation seen from blockade of VGCCs, RyRs and IP\(_3\)Rs compared to blockade of α7 nAChRs with MLA (see Figure 3.2.21 for summary) suggests significant heterogeneity of nAChR-Ca\(^{2+}\) coupling mechanisms. It is likely that multiple mechanisms play a simultaneous role in α7 nAChR-mediated Ca\(^{2+}\) elevations. To determine the relative influence of each mechanism, further study would be needed in which blockade is attempted with various combinations of the antagonists used here.

As described in section 1.2.2, nAChR mediated Ca\(^{2+}\) elevations have been reported to couple to VGCCs and/or ER mediated release, seemingly dependent on nAChR subtypes and experimental preparations. It is important to reiterate here that α7 nAChRs are highly permeable to Ca\(^{2+}\) (see section 1.2.2.6), and it therefore follows that a proportion of the Ca\(^{2+}\) elevations observed in these experiments are due to direct flux through α7 nAChR channels. Indeed, there are numerous reports of α7 nAChRs mediating Ca\(^{2+}\) elevations, or Ca\(^{2+}\) dependent events directly, for example in rat hippocampal mossy fibre terminals (Gray et al., 1996), glutamatergic terminals in MHb-IPN co-cultures (Girod et al., 2000), dendritic spines of chick ciliary ganglion neurons (Shoop et al., 2001), stratum radiatum interneurons of the rat hippocampus
(Fayuk and Yakel, 2005), filopodia of SH-SY5Y cells overexpressing a7 nAChRs (Gilbert et al., 2009), IMR-32 neuroblastoma cells (Ween et al., 2010), and glutamatergic terminals of neurons of the solitary tract nucleus (Kalappa et al., 2011).

This is even more relevant with the inclusion of PNU1, which as described above, dramatically increases channel open time by destabilizing the desensitized state, therefore allowing a greater total Ca\(^{2+}\) flux directly through a7 nAChRs. As no Ca\(^{2+}\) increases were detected in response to agonists alone (Figure 3.2.5), it can be concluded that Ca\(^{2+}\) or Na\(^{+}\) flux is so brief (see above) that it also fails to recruit secondary mechanisms such as VGCCs and ER mediated Ca\(^{2+}\) release. Conversely, the inclusion of PNU1 could cause such a large ion flux that cellular mechanisms that usually constrain Ca\(^{2+}\) signals would be overwhelmed, leading to indiscriminate activation of all secondary Ca\(^{2+}\) amplification mechanisms that would usually be more tightly regulated in a physiological setting. Such is the difficulty in studying these receptors; experimental limitations must be balanced with decreased physiological relevance. Despite these concerns, there have been reports of a7 nAChR mediated Ca\(^{2+}\) elevations evoked by agonists in the presence of PNU1 that did not activate VGCCs or CICR (Gilbert et al., 2009; Ween et al., 2010). To evaluate the proportion of \([\text{Ca}^{2+}]_i\) elevations in mouse primary cortical cultures resulting from direct a7 nAChR mediated ion flux, all other possible routes would have to be completely blocked simultaneously.

It is reasonable to suggest that in many situations in mouse primary cortical cultures, a7 nAChRs mediate local transient \([\text{Ca}^{2+}]_i\) elevations as described elsewhere (Shoop et al., 2001; Gilbert et al., 2009) which are not resolved by the imaging equipment used here. In a physiological setting, the coincidence of cholinergic inputs may determine the summation of local into global Ca\(^{2+}\) signals, and the proximity of a7 nAChRs to VGCCs, RyRs and/or IP\(_3\)Rs would also influence this.

Also worthy of note are the effects highly augmented levels of \([\text{Ca}^{2+}]_i\) (such as that made possible by PNU1) could have on a7 nAChR function and the implications for repeated stimulation, as it has been reported that Ca\(^{2+}\) dependent mechanisms can modulate nAChR desensitization via phosphorylation by PKA and PKC, which promotes recovery of desensitization, and dephosphorylation by calcineurin, which delays recovery (Khiroug et al., 1998; Giniatullin et al., 2005).
Chapter 3: Mechanisms of \( \alpha_7 \) nAChR-Mediated Calcium Signalling in Primary Cortical Cultures

The involvement of VGCCs in \( \alpha_7 \) nAChR mediated \( Ca^{2+} \) elevations and \( Ca^{2+} \) dependent events have been widely reported with conflicting results depending on the experimental preparation. \( Cd^{2+} \) blocked \( \alpha \) bgt sensitive \( Ca^{2+} \) elevations evoked by nicotine in chick ciliary ganglion neurons (Vijayaraghavan et al., 1992; Rathouz and Berg, 1994) and hippocampal neurons (Barrantes et al., 1995a), indicating coupling of \( \alpha_7 \) nACHRs to VGCCs. VGCC subtype specific information was also revealed by using the L-type VGCC blocker isradipine, which blocked \( \alpha_7 \)nAChR mediated \( Ca^{2+} \) elevations in rat GH3 pituitary cells (Feuerbach et al., 2005), whereas the use of \( \omega \)-conotoxin MVIIIC implied non-L-type VGCCs were involved in \( \alpha_7 \) nAChR mediated \( Ca^{2+} \) increases evoked by PNU2 in combination with PNU1 in bovine chromaffin cells (del Barrio et al., 2011). A study on rat PFC synaptosomes by Wang et al. (2006) also reported 4-aminopyridine (4AP) evoked glutamate release that was enhanced by nicotine in an MLA but not DH\( \beta \)E sensitive manner, and mimicked by choline, suggesting involvement of \( \alpha_7 \) but not \( \beta_2^* \) nAChRs. This effect was blocked by \( \omega \)-conotoxin GVIA and \( \omega \)-agatoxin IVA, implying that \( \alpha_7 \) nAChR mediated potentiation of glutamate release is reliant on N- and P/Q-type VGCCs. Studies conducted in the Wonnacott lab directly contradict these findings (Dickinson et al., 2008), here enhanced release of \( ^{[3]}H \)D-aspartate (used as a surrogate for glutamate) mediated by \( \beta_2^* \) nAChR activation was coupled to VGCCs whereas \( \alpha_7 \) nAChRs were coupled to ER release via RyRs and IP\(_3\)Rs. Several reasons can be ascribed to these conflicting results: (i) Wang et al. (2006) observed the effects of nicotinic agents on 4AP evoked release, whereas Dickinson et al. (2008) were studying the effects of nicotinic agents on basal release; (ii) Wang et al. (2006) were measuring basal endogenous glutamate release via on-line fluorimetry of NADPH produced by oxidative deamination of released glutamate by glutamate dehydrogenase (GDH), whereas Dickinson et al. (2008) were measuring the release of \( ^{[3]}H \)D-aspartate in place of glutamate; and most importantly (iii) the \( \alpha_7 \) nAChR selectivity of nicotine’s glutamate release enhancing effects reported by Wang et al. (2006) are inferred from blockade by MLA at 10 \( \mu \)M, a concentration that is high enough to block all nACHRs, including \( \alpha_4\beta_2^* \) nAChRs (IC\(_{50}\) of MLA for \( \alpha_4\beta_2^* \) nAChRs = 0.2 \( \mu \)M; Ward et al., 1990; Absalom et al., 2013), although the authors reported no effect of the \( \beta_2^* \) nAChR selective antagonist DH\( \beta \)E. The authors also reported similar enhancement of 4AP evoked glutamate release by choline at 10 \( \mu \)M, which is well below the concentration able to elicit measurable
effects from α7 nAChRs (EC_{50} = 1.5 mM; Alkondon et al., 1997; and see above 3.2.1). In contrast, Dickinson et al. (2008) used the α7 nAChR selective agonist compound A, and confirmed selectivity by blocking with the selective antagonist αbgt. Furthermore, similar nAChR-Ca^{2+} coupling mechanisms to those reported by Dickinson et al. (2008) were reported for hippocampal synaptosomes (Zappettini et al., 2010, 2011a), with ER mediated CICR being mostly responsible for α7 nAChR mediated potentiation of glutamate, aspartate and GABA release. Indeed, α7 nAChR coupling to CICR from the ER has been extensively described elsewhere, for example in SNc neurons in mouse brain slices (Tsuneki et al., 2000), hippocampal astrocytes (Sharma and Vijayaraghavan, 2001), SH-SY5Y cells (Dajas-Bailador et al., 2002a), hippocampal mossy fibre terminals from rat brain slices (Sharma and Vijayaraghavan, 2003; Sharma et al., 2008), rat dentate gyrus (Welsby et al., 2006), hippocampal Schaffer collateral synapses (Le Magueresse and Cherubini, 2007), bovine chromaffin cells (del Barrio et al., 2011), and ventral hippocampal axons (Zhong et al., 2013). Evidence suggests that both RyRs and IP_{3}Rs contribute to this effect, but with differing temporal dynamics. Previously in SH-SY5Y cells, Dajas-Bailador et al. (2002) reported that long term (10 min), αbgt sensitive [Ca^{2+}]_{i} elevations evoked by nicotine were significantly blocked by XeC, whereas Ry more significantly blocked the early stages (20 s) of the response. More recently, it was reported that in glutamatergic axons of the ventral hippocampus, IP_{3}Rs were responsible for the sustained (up to 30 min) increase in [Ca^{2+}]_{i} evoked by nicotine in an αbgt, but not DhβE sensitive manner (Zhong et al., 2013). To test this in mouse primary cortical cultures, one could give long (20-30 min) drug applications, and investigate the effect of Ry vs. XeC on sustained Ca^{2+} signals.

Another important factor to note when observing CICR is the tendency of ER to accumulate Ca^{2+} via active pumps following periods of increased activity as a sort of “Ca^{2+} capacitor” (see section 1.2.2). To investigate this effect, we would need to conduct experiments where sequential α7 nAChR stimulation was given, followed by blockade with Ry and/or XeC to confirm ER mediated CICR dependence. Alternatively, thapsigargin could be applied, which blocks SERCA pumps, preventing uptake of Ca^{2+} into the ER (Sharma and Vijayaraghavan, 2003).

In our experiments, VGCC or CICR activation following α7 nAChR stimulation could be occurring either pre- or post-synaptically. VGCC or CICR could also be a
result of the widely reported effect of α7 nAChR stimulation promoting glutamate release, discussed in the following section. In fact, as greater direct nAChR mediated Ca$^{2+}$ flux occurs at resting or hyperpolarized potentials, whereas VGCCs flux Ca$^{2+}$ at depolarized potentials, this may be the case (Mulle et al., 1992a).

### 3.3.5 α7 nAChR-mediated Ca$^{2+}$ elevations in mouse primary cortical cultures: evidence of excitatory transmission

The inference that in mouse primary cortical cultures, α7 nAChR activation results in glutamate release, causing postsynaptic [Ca$^{2+}$]$_{ic}$ elevations via AMPA and NMDA receptors is evidenced by the variable blockade of responses to PNU2 in combination with PNU1 by CNQX and/or MK801. A wealth of studies have previously reported potentiation of glutamate release via α7 nAChR activation in different preparations and brain regions such as frontal and pre-frontal cortex (Rousseau et al., 2005; Dickinson et al., 2008; Konradsson-Geuken et al., 2009, 2010; Livingstone et al., 2010; Bortz et al., 2013), hippocampus (Gray et al., 1996; Sharma and Vijayaraghavan, 2003; Welsby et al., 2006; Sharma et al., 2008; Zappettini et al., 2010; Gomez-Varela and Berg, 2013; Halff et al., 2014), striatum (Kaiser and Wonnacott, 2000; Marchi et al., 2002; Livingstone et al., 2009; Northrop et al., 2010), cerebellum (Zwart et al., 2002), VTA (Mao et al., 2011), and as gliotransmitter release by hippocampal (Pirttimaki et al., 2013) and cortical (Talantova et al., 2013) astrocytes.

The variability of the block observed here (Figure 3.2.21) indicates the presence of both pre- and postsynaptic α7 nAChR. Although, as CNQX did not fully block control responses to AMPA (~50%; Figure 3.2.15), it cannot be assumed that the inhibition of PNU2 in combination with PNU1 by CNQX (Figure 3.2.16) reveals the component of the response that is not dependent on glutamate release, i.e. responses evoked by solely by somatodendritic α7 nAChRs. Furthermore, residual AMPAR activation may still sufficiently depolarize postsynaptic neurons to relieve the Mg$^{2+}$ block on NMDARs, resulting in a much larger response than if AMPARs were fully blocked. Also, NMDA was seen to elicit large Ca$^{2+}$ responses in the presence of Mg$^{2+}$ (in buffer; Figure 3.2.17), which suggests the presence of endogenous glutamate release activating AMPARs, relieving the Mg$^{2+}$ block.

As both CNQX and MK801 both significantly blocked α7 nAChR responses, indicating involvement of AMPA and NMDARs, which exist on the same postsynaptic
cells and work in concert (Traynelis et al., 2010), one would expect their effects to be additive when applied in combination (Figure 3.2.19). This has been described elsewhere, for example in rat striatum and PFC where α7 nAChRs activated by choline in the presence of PNU1 enhanced the release of [3H]dopamine, which is dependent on glutamate release (Livingstone et al., 2009). Our results however fail to show additivity when CNQX and MK801 were combined, which could possibly be due to the heterogeneity of Ca^{2+} signalling mechanisms activated following stimulation with PNU2 in the presence of PNU1 in these cultures as shown above (Figure 3.2.21), which would obscure such an effect. It could also be the case that antagonist concentrations are not maximal. Further studies and a larger sample size, or repeated applications of PNU1 in the presence of PNU2 with and without CNQX or MK801 followed by both antagonists in combination to the same cells may also give a better chance of observing this effect. Here, responses showing dependence on iGluRs could be examined again for the additive effect.

As mentioned in the previous section, post-synaptic [Ca^{2+}]_i elevations could be partly due to the activation of VGCCs or CICR following initial Na^+/Ca^{2+} influx through iGluRs, and it would be difficult to ascertain the proportion of [Ca^{2+}]_i increase mediated solely by iGluRs, as these secondary mechanisms exist on both sides of the synaptic cleft, and are reputed to be necessary for α7 nAChR mediated glutamate release (see above). α7 nAChRs are also thought to be present in somatodendritic locations in primary cortical cultures (Hammond et al., 2013), further confounding the dissection of pre- vs. post-synaptic Ca^{2+} signals. An attempt to block VGCCs and CICR on the postsynaptic cell would therefore also block glutamate release. However, total blockade of iGluRs would reveal the component that comprises [Ca^{2+}]_i elevations due to activation of postsynaptic α7 nAChRs, and any subsequent amplification by VGCCs and/or CICR. As well as glutamate release, α7 nAChR activation is also known to facilitate the release of GABA, for example in the hippocampus (Zappettini et al., 2011a). If this is the case in primary cortical neurons, which have been reported to contain a substantial proportion of GABAergic cells (Suñol et al., 2008), it would be expected that blockade of GABA_A and/or GABA_B receptors with would potentiate α7 nAChR mediated Ca^{2+} signals. This situation is investigated in the following chapter.
Our experiments did not yield significant evidence for the involvement of mGluR₁ in α7 nAChR mediated $[\text{Ca}^{2+}]$ elevations, which could occur via the $G_q$ mediated generation of IP₃ (see section 1.2.2.7). This was investigated following reports mGluR₁ association with α7 nAChRs in hippocampal LTP (Welsby et al., 2006), but the lack of effect may be due to low expression levels reported by others for this mGluR subtype in primary cortical cultures (Ayala et al., 2012). To confirm the functional presence of mGluR₁ and their ability to raise $[\text{Ca}^{2+}]_c$ following α7 nAChR activation, experiments would need to conducted using selective mGluR₁ agonists, to which the inhibitory effects of LY4 could be tested. It is possible that other mGluR subtypes play a role here, as evidence suggests higher expression of mGluR₂/₃ (Prézeau et al., 1994; Schaffhauser et al., 1998), which are coupled to inhibitory $G_i$-proteins and are mainly expressed presynaptically (Niswender and Conn, 2010). As such, it could be envisioned that blockade of these mGluRs could have a potentiating effect on α7 nAChR mediated $\text{Ca}^{2+}$, by disinhibition as described for GABARs above.

### 3.3.6 Conclusions

The experiments presented in this chapter provide evidence of α7 nAChRs mediating $[\text{Ca}^{2+}]$ elevations via multiple routes that include VGCCs, RyR and IP₃R mediated CICR from the ER, and also AMPA and NMDARs via glutamate release. From this we can conclude that α7 nAChRs are expressed pre- and postsynaptically in mouse primary cortical cultures. Further study would be needed to completely define these mechanisms, by using combinations of antagonists. The role of α7 nAChRs in the release of GABA could also be investigated, as could the inhibitory influence of type II and III mGluRs.
Chapter 4: Development of a High-Content Screening Assay to Investigate Calcium Signalling in Primary Cortical Cultures
4.1 Introduction

The development of automated analysis capable of extracting multiparametric observations from biological images that can be acquired in high-throughput fashion has major implications for experimental output and discovery in biomedical research. High-content screening (HCS) has enabled the generation of extremely large data sets from large cell populations in relatively short periods of time (~10^6 cells per experiment), making it an effective solution for assay optimization, repetitive investigations, subtle variable changes, and increasing statistical power. This chapter details exploratory work aimed at developing a HCS protocol to further investigate nAChR mediated Ca^{2+} signalling mechanisms described in chapter 3.

4.1.1 High content screening

Over the last decade, large-scale biological microscopy has become increasingly sophisticated owing to the development of HCS protocols that acquire, process, analyse and archive vast amounts of imaging data (Abraham et al., 2004). These methods have also impacted neuroscience research, and have been extensively used in the field of drug discovery (Dragunow, 2008). At a basic level, HCS can be used to greatly increase the speed, accuracy and objectivity of simple but laborious tasks such as cell counting. Due to the flexibility of currently available HCS software however – and with the correct markers – these simple tasks can be extended to include multiparametric observations such as organelle counting and morphological analysis that exceed even the capabilities of human visual inspection. The inclusion of liquid handling and environmental control modules inflates the possibilities further still, enabling live cell assays to be conducted in an array of different conditions. In fact, simultaneous multiparametric measurements can be applied to cell assays to improve the validity of findings, as has been described for cell toxicity assays (Abraham et al., 2004; Götte et al., 2010).

The complexity of neuronal and glial cell morphology increases the necessity for more sophisticated and stringent analysis algorithms to segment objects based on bright-field or fluorescent images. This is a challenging endeavour, but has been pioneered and reported in the literature with some success (Narayan et al., 2007). Probably the best example is the automated neurite outgrowth analysis (Ramm et al,
2003; Radio et al., 2010), which tracks changes in the length of cellular processes, and has been used to report Aβ toxicity in primary cortical and hippocampal cultures (Hu et al., 2007; Evans et al., 2008). Other potential applications of HCS for neuroscience include tracking receptor internalization, protein aggregation, cell migration and transcription factor translocation (Dragunow, 2008). The advantages of HCS are clearly vast, but it should be stressed that it cannot completely surpass some aspects of smaller scale imaging experiments, such as real-time manual manipulation that allows for greater creativity in exploratory investigations, and greater image resolution. In conclusion, HCS is an exciting field that promises to greatly accelerate biological research and reduce the time and resources spent on simple laborious procedures.

4.1.2 Aims
This chapter aims to develop a high content imaging protocol that can detect Ca²⁺ elevations in primary cortical cultures using fluorescent indicators in a multi-well plate format that could potentially be used to further investigate α7 nAChR mediated Ca²⁺ signalling. An integral part of this is the development of an accurate and reliable automated image segmentation and analysis algorithm, capable of recognizing individual cells in large populations, tracking them over a time series, and extracting the desired parameters of fluorescence intensity.

The second aim was to develop a more conventional immunofluorescent characterization of the cortical cultures with the ultimate aspiration of combining this with Ca²⁺ measurements. This would significantly enhance the analytic value of the assay and add greater insight to Ca²⁺ signaling mechanisms in these cultures.
4.2 Results

4.2.1 Validation of a high-content screening Ca\(^{2+}\) imaging assay

Live imaging experiments described in chapter 3 required primary cortical cultures to be grown on glass coverslips and assembled into a perfusion chamber, which was a lengthy procedure. To enhance experimental throughput, the INCell Analyzer 2000 (GE Healthcare, UK) high-content microscope was chosen to develop an assay with a 24-well plate format that could screen multiple conditions in a shorter time. Imaging large cell populations was central to the aims of these experiments; as such, the 10X objective was used, giving a field of view of 2048 X 2048 pixels, corresponding to a size of 1.52 mm\(^2\). The INCell Analyzer 2000 contains an integrated liquid handling system, allowing solutions to be aspirated from a separate “compound” plate, and dispensed onto cells in the “sample” plate. As with SH-SY5Y cell experiments described in chapter 3, the single-wavelength indicator flou-3 AM was used to report changes in \([\text{Ca}^{2+}]_{\text{ic}}\) as the microscope lacked a dual excitation filter wheel required to image fura-2 ratiometric fluorescence. In contrast to the fluorescent plate reader assay used for SH-SY5Y cell experiments however, flou-3 intensity was used for imaging, allowing individual cells to be examined. Cells were also stained with Hoechst-33342 (Hoechst) to reveal cell nuclei to aid analysis. Due to limitations in the temporal resolution of image acquisition in this system, cells could not be imaged in real-time as in live-cell imaging experiments. Instead, a protocol was devised to measure the changes in flou-3 mediated fluorescence at 525 nm \((F_{525})\) in individual cells following drug application from Hoechst and flou-3 positive regions from five time-points allocated \(t_1\), \(t_5\), \(t_1\) \((0\ s)\) and \(t_2\) \((6.4\ s)\) were acquired in the absence of drug; and \(t_3\), \(t_4\), and \(t_5\) acquired in sequential 10 s intervals following automated drug application.
Chapter 4: Development of a High-Content Screening Assay to Investigate Calcium Signalling in Primary Cortical Cultures

Figure 4.2.1: KCl evokes robust $F_{525}$ increases in large populations of mouse primary cortical cultures.

Cultures (10-14 DIV) loaded with fluo-3 AM and stained with Hoechst-33342 were stimulated with buffer (a) or KCl (40 mM; b). Images show fluorescence at 525 (fluo-3; excitation 490 nm; green) and 455 nm (Hoechst-33342; excitation 350 nm; blue) before (i) and after (ii) stimulation, zoom inset shows responding cells (location indicated by white arrow).

Upon visual inspection of images acquired at “basal” time-points (t1-2), low levels of spontaneous changes in fluorescence measured at 525 nm ($F_{525}$) were apparent in a small proportion of cells (Figure 4.2.1ai, bi). Application of buffer alone directly to the liquid layer resulted in large $F_{525}$ increases at “stimulation” time-points (t3-5), evident upon visual inspection of images. This was rectified by dispensing drug “off-center” down the side of the well, minimizing liquid disturbance that previously resulted in false positive responses (Figure 4.2.1aii). As in previous experiments (chapter 3), KCl (40 mM) was applied to evoke a positive response, resulting in clear $F_{525}$ increases seen at t3-5 in a large proportion of cells (Figure 4.2.1bii), indicating the suitability of this protocol for detecting drug evoked $[Ca^{2+}]_{ic}$ in primary cortical neurons.

As discussed in section 4.1.1, an integral part of HCS is the powerful automated image processing and data analysis that can be developed using specialist software.
Here we have used the INCell Developer Toolbox software (GE Healthcare), which allows the creation of highly flexible and specific algorithms to process image data and segment fluorescent objects. The complex morphology of cells in primary cortical cultures made this a challenging endeavour. The first step was to generate a nuclear mask from the Hoechst stained images, from which would be defined the central region of an individual cell. Here, following optimization by trial and error and manual inspection of segmented images, the object segmentation function was set to encircle cell nuclei with a kernel size (minimum object size) of 5X5 pixels and grey level sensitivity (minimum brightness of an object relative to background; 0-100) set to 20. A post-processing module, “sieve” was then added to remove small objects of less than 8 μm², giving a nuclear segmentation mask that appeared to reliably determine individual cell nuclei (Figure 4.2.2a). The second step was to use flou-3 fluorescence images to generate a cell mask (Figure 4.2.2b), which would then be
linked to the nuclear mask so that each cell had to contain a nucleus to be included in the analysis (Figure 4.2.2c). Following optimization, kernel size was set to 25X25 pixels, and grey level sensitivity at 30. Objects smaller than 10 μm² were removed by the “sieve” function. In order to distinguish individual cells clustered together, the “clump break” module was used with a radius of 2 pixels, and any objects that touched the image border were removed from analysis.

Images acquired from time-points t₁-t₅ were then linked in an image stack so that F₅₂₅ intensity of individual cells defined by segmented objects could be tracked through the time-series. To quantify drug-evoked changes in F₅₂₅, the difference between the mean F₅₂₅ of t₃-t₅ and t₁-t₂ for each single cell data point was defined as ΔF₅₂₅. The average number of fluo-3 and Hoechst positive cells per field of view was 3573 (SD = 1056; n = 380 wells from 16 cultures) giving an average of 2358 cells/mm².

Mean F₅₂₅ for t₁-t₂ was calculated to be 208.53 (arbitrary fluorescence intensity units) with SD of 15.87 (380 wells; 16 independent cultures). This SD value was then defined as the ΔF₅₂₅ threshold for a response to stimulation, allowing an estimate of the percentage of cells responding to drug in each condition. After applying the algorithm described above, changes in basal F₅₂₅ between t₁ and t₂ were found to be minimal, averaging at 0.49 (SD = 0.54; n = 16 cultures). The average ΔF₅₂₅ following application of buffer alone was -4.63 ± 1.17 (see Figure 4.2.9). On average, 1.38 % of cells could be classed as responders following application of buffer alone (see Figure 4.2.10), with mean ΔF₅₂₅ of 41.66 ± 6.35 from this small subpopulation.

Analysis of images shown in Figure 4.2.1 gave an average ΔF₅₂₅ of -7.65 (SD = 18.08; n = 6436 cells) in response to buffer alone, compared to 127.70 (SD = 145.6; n = 4138 cells) in response to KCl (40 mM; Figure 4.2.3). Data collected from experiments on 7 independent cultures gave an average F₅₂₅ increase of 116.45 ± 17.68 that was significantly different from responses to buffer alone (Figure 4.2.9). Here, 85.57 % of cells were classed as responders (ΔF₅₂₅ > 15.87; see above), with a mean ΔF₅₂₅ of 129.41 ± 16.82 in the responding population (Figure 4.2.10). These results support the validity of the acquisition protocol and analysis algorithm to measure changes in [Ca²⁺]ᵢₑ in primary cortical cultures.
Chapter 4: Development of a High-Content Screening Assay to Investigate Calcium Signalling in Primary Cortical Cultures

4.2.2 Measuring ionotropic glutamate receptor induced Ca$^{2+}$ elevations using HCS

Having observed clear responses to KCl, the assay was then used to assess pharmacological stimulation of ionotropic glutamate receptors using AMPA (10 μM) and NMDA (50 μM), which gave robust responses in live imaging experiments (chapter 3). Upon exposure to AMPA (10 μM), cultures loaded with fluo-3 AM and Hoechst showed average $F_{S25}$ increases of 68.32 ± 9.78, an elevation that was significantly different to that observed following application of buffer alone (see Figure 4.2.9). The subpopulation of responding cells (46.39 %; Figure 4.2.10) showed $F_{S25}$ increases of 146.78 ± 8.69. Pre-incubation and co-application of CNQX attenuated these responses by 57.98 ± 14.43 % (Figure 4.2.9), also reducing the percentage responding cells to 27.73 % (Figure 4.2.10). These values agree with live cell imaging experiments in which CNQX blocked AMPA induced responses by 50.31 ± 1.41 % (chapter 3; section 3.2.5.1). An example of responses to AMPA (10 μM) in the presence or absence of CNQX (10 μM) is shown in Figure 4.2.4. Images and cell data plots show clear $F_{S25}$ increases following application of AMPA (10 μM), attenuated by CNQX (10 μM).

![Figure 4.2.3 KCl evokes robust $F_{S25}$ increases in large populations of mouse primary cortical cultures.](image)

In a single experiment, cultures (10-14 DIV) loaded with fluo-3 AM and stained with Hoechst-33342 were stimulated with buffer (a) or KCl (40 mM; b). Five images in total were acquired at both wavelengths: two before (t1, t2; 6.4 s interval), and three after (t3, t4, t5; 10 s intervals) stimulation. Data are presented as the difference between mean $F_{S25}$ at t3-t5 and t1-t2 ($\Delta F_{S25}$). Each point represents a single cell defined by co-localization of Hoechst-33342 and fluo-3 segmentation masks, tracked from t1-t5. Dotted black line shows mean $\Delta F_{S25}$ with SD (grey shading).
NMDA (50 μM) also significantly increased $F_{525}$ by $51.95 \pm 9.47$ compared to buffer alone (Figure 4.2.9). Responding cells were determined to comprise 33.65 % of total cells imaged (Figure 4.2.10), with $F_{525}$ increases of $112.05 \pm 14.22$ from within this sub-population. Pre-incubation and co-application of MK801 (2.5 μM; 10 min) resulted in responses that were reduced by $82.76 \pm 23.76$ % compared to NMDA alone (Figure 4.2.9), which is comparable to that observed in live cell imaging experiments in which MK801 blocked NMDA induced responses by $98.90 \pm 0.42$ % (see section 3.2.5.2). Here the presence of MK801 reduced the number of responding cells to 16.27 % (Figure 4.2.10). In a single experiment, 10 min pre-incubation of another antagonist, D-APV (100 μM), completely blocked the response to a subsequent application of NMDA by $93.76 \pm 0.59$ % (Figure 4.2.5).

Together, these data support a HCS approach to measuring Ca$^{2+}$ elevations in primary cortical cultures. Having observed positive responses to KCl, AMPA and NMDA, which were affected by appropriate antagonists, we then attempted to measure $\alpha7$ nAChR mediated Ca$^{2+}$ elevations using the method described above.
Figure 4.2.4: AMPA evoked Ca\textsuperscript{2+} elevations in mouse primary cortical cultures are partially blocked by CNQX.

Cultures (10-14 DIV) loaded with fluo-3 AM and stained with Hoechst-33342 were stimulated with AMPA (10 μM) alone (a) or in the presence of CNQX (b: 10 μM; 10 min pre-incubation). Images show fluorescence at 525 nm (fluor-3; excitation 490 nm; green) and 455 nm (Hoechst-33342; excitation 350 nm; blue) before (I) and after (II) application of AMPA, zoom inset shows responding cells (location indicated by white arrow). Five images in total were acquired at both wavelengths; two before (t1, t2: 6.4 s interval) and three after (t3, t4, t5: 10 s interval) stimulation. Data (III) are presented as the difference between mean F\textsuperscript{525} at t1-t5 and t2-t5 (ΔF\textsuperscript{525}). Each point represents a single cell defined by co-localization of Hoechst-33342 and fluo-3 segmentation masks, tracked from t1-t5. Dotted black line shows mean ΔF\textsuperscript{525} with SD (grey shading).
Cultures (10–14 DIV) loaded with fluo-3 AM and stained with Hoechst-33342 were stimulated with NMDA (10 μM) alone (a) or in the presence of D-APS (b; 100 μM; 10 min pre-incubation). Images show fluorescence at 525 nm (fluorescein, excitation 490 nm; green) and 455 nm (Hoechst-33342; excitation 350 nm; blue) before (i) and after (ii) application of NMDA. Zoom inset shows responding cells (location indicated by white arrow). Five images in total were acquired at both wavelengths: two before (t1, t2; 6.4 s interval), and three after (t3–t5; 10 s intervals) stimulation. Data (iii) are presented as the difference between mean F525 at t1, t2 and t3–t5 (ΔF525). Each point represents a single cell defined by co-localization of Hoechst-33342 and fluo-3 segmentation masks, tracked from t1–t2. Dotted black line shows mean ΔF525 with SD (grey shading).
4.2.3 α7 nAChRs mediated Ca\(^{2+}\) elevations from subpopulations in primary cortical cultures

Following pre-incubation with PNU1 (10 μM; 3 min), cells loaded with fluo-3 AM and stained with Hoechst were stimulated with PNU2 (3 μM) resulting in variable responses between cultures, with an average ΔF\(_{525}\) of 2.66 ± 6.41 (Figure 4.2.9). The mean percentage of responding cells – although highly variable (SD = 25.80) – was 13.94 % (Figure 4.2.10); in agreement with live cell imaging experiments (12 %; chapter 3). Pre-incubation and co-application of MLA (100 nM) resulted in an average ΔF\(_{525}\) of -4.30 ± 1.46 (Figure 4.2.9), and also reduced the number of responding cells to 4.65 % (Figure 4.2.10), the effect of MLA on ΔF\(_{525}\) induced by PNU2 in the presence of PNU1 was not significantly different.

Figure 4.2.7 shows an example of an experiment in which a noticeable F\(_{525}\) increase of 53.89 ± 46.69 was observed following stimulation with PNU2 (3 μM) in the presence of PNU1 (10 μM). Within the 81.85 % of responding cells, the average F\(_{525}\) increase was 65.08 ± 43. Here, MLA reduced ΔF\(_{525}\) to 1.32 ± 9.00, confirming α7 nAChR specificity. In other experiments however, changes in F\(_{525}\) following application of PNU2 in combination with PNU1 were much less obvious, and blockade by MLA had little effect (Figure 4.2.6).

![Figure 4.2.6: Responses to PNU2 in the presence of PNU1 are difficult to detect in large populations of primary cultured cortical cells.](image)

Cultures (10-14 DIV) loaded with fluo-3 AM and stained with Hoechst-33342 were stimulated with PNU2 (3 μM) in the presence of PNU1 (10 μM; 3 min pre-incubation; a) or in the presence of MLA (b; 100 μM; 10 min pre-incubation. Data are presented as the difference between mean F\(_{525}\) at t\(_1\)-t\(_5\) and t\(_1\)-t\(_5\) following excitation at 490 nm (ΔF\(_{525}\)). Each point represents a single cell defined by co-localization of Hoechst-33342 and fluo-3 segmentation masks, tracked from t\(_1\)-t\(_5\). Dotted black line shows mean ΔF\(_{525}\) with SD (grey shading).
Figure 4.2.7: \( \alpha \text{T} \) evoked \( \text{Ca}^{2+} \) elevations in large populations of mouse primary cortical cultures.

Cultures (10-14 DIV) loaded with fluo-3 AM and stained with Hoechst-33342 were stimulated with PNU2 (3 \( \mu \)M) in the presence of PNU1 (10 \( \mu \)M; 3 min pre-incubation; a) or in the presence of MLA (b; 100 \( \mu \)M; 10 min pre-incubation). Images show fluorescence at 525 nm (fluo-3; excitation 490 nm; green) and 455 nm (Hoechst-33342; excitation 350 nm; blue) before (i) and after (ii) application of PNU2, zoom inset shows responding cells (location indicated by white arrow). Five images in total were acquired at both wavelengths: two before (\( t_1, t_2 \); 6.4 s interval), and three after (\( t_3, t_4, t_5 \); 10 s intervals) stimulation. Data (iii) are presented as the difference between mean \( F_{525} \) at \( t_1-t_5 \) and \( t_1-t_5 \) following excitation at 490 nm (\( \Delta F_{525} \)). Each point represents a single cell defined by co-localization of Hoechst-33342 and fluo-3 segmentation masks, tracked from \( t_1-t_5 \). Dotted black line shows mean \( \Delta F_{525} \) with SD (grey shading).
Chapter 4: Development of a High-Content Screening Assay to Investigate Calcium Signalling in Primary Cortical Cultures

Figure 4.2.8: PNU2 in combination with PNU1 evoked Ca\textsuperscript{2+} elevations in large populations of mouse primary cortical cultures in the presence of bicuculline.

Cultures (10-14 DIV) loaded with fluo-3 AM and stained with Hoechst-33342 were stimulated with PNU2 (3 μM) in the presence of PNU1 (10 μM) and bicuculline (Bic; 1 μM; 10 min pre-incubation). Images show fluorescence at 525 nm (fluor-3; green) and 455 nm (Hoechst-33342; blue) before (i) and after (ii) application of PNU2, zoom inset shows responding cells (location indicated by white arrow). Five images in total were acquired at both wavelengths: two before (t\textsubscript{1}, t\textsubscript{2}; 6.4 s interval), and three after (t\textsubscript{3}, t\textsubscript{4}, t\textsubscript{5}; 10 s intervals) stimulation. Data (iii) are presented as the difference between mean F\textsubscript{525} at t\textsubscript{1}-t\textsubscript{5} and t\textsubscript{2}-t\textsubscript{5} following excitation at 490 nm (ΔF\textsubscript{525}). Each point represents a single cell defined by co-localization of Hoechst-33342 and fluo-3 segmentation masks, tracked from t\textsubscript{1}-t\textsubscript{5}. Dotted black line shows mean ΔF\textsubscript{525} with SD (grey shading).
Figure 4.2.9: Stimulation evoked $F_{525}$ increases in large populations of mouse primary cortical cultured cells.

Cultures (10-14 DIV) loaded with fluo-3 AM and stained with Hoechst-33342 were stimulated with buffer, KCl (40 mM), AMPA (10 μM) in the presence or absence of CNQX (10 μM), NMDA (50 μM) in the presence of MK801 (2.5 μM), or PNU2 (3 μM) in combination with PNU1 (10 μM) with or without MLA (100 nM). Antagonists were pre-incubated for 10 min prior to stimulation. Data are presented as the difference between mean $F_{525}$ following excitation at 490 nm before and after stimulation ($\Delta F_{525}$), averaged from 2-9 independent experiments ± SEM; *** $P < 0.001$, **** $P < 0.0001$ significantly different from buffer, non-paired one-way ANOVA, Bonferroni’s multiple comparisons test; each point represents the mean $\Delta F_{525}$ of a triplicate from a single experiment.
Cultures (10-14 DIV) loaded with flou-3 AM and stained with Hoechst-33342 were stimulated with buffer, KCl (40 mM), AMPA (10 µM) in the presence or absence of CNQX (10 µM), NMDA (50 µM) in the presence or absence of MK801 (2.5 µM), or PNU2 (3 µM) in combination with PNU1 (10 µM), with or without MLA (100 nM). Antagonists were pre-incubated for 10 min prior to stimulation. Data are presented as the percentage of responding cells (defined as $\Delta F_{525} > 15.87$ following excitation at 490 nm), averaged from 2-9 independent experiments ± SEM; * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ significantly different from buffer, non-paired one-way ANOVA, Bonferroni’s multiple comparisons test; each point represents the percentage of responding cells per triplicate from a single experiment.

Figure 4.2.10: Percentage of cells responding to various stimuli as measured by changes in F<sub>525</sub>.
As in chapter 3, responses to PNU2 (3 μM) in combination with PNU1 (10 μM) were examined in the presence of various antagonists that block cellular Ca\(^{2+}\) elevation mechanisms. In contrast to live cell imaging experiments, no significant effects could be resolved here following pre-incubation and co-application of these antagonists with PNU1 and PNU2 (Figure 4.2.11). Notably, pre-incubation of the GABA\(_A\) receptor antagonist bicuculline (1 μM) potentiated the effects of PNU2 (3 μM) in combination with PNU1 (10 μM), elevating F\(_{525}\) to an average of 5.02 ± 5.47. This effect was inconsistent, but an example of a clear response to PNU2 (3 μM) in the presence of PNU1 (10 μM) and bicuculline (1 μM) is shown in Figure 4.2.8. In conclusion, these experiments provide some evidence that this HCS method could be used to investigate α7 nAChR Ca\(^{2+}\) signalling, although further optimization may be necessary.

![Figure 4.2.11: Effects of various antagonists on α7 mediated F\(_{525}\) increases in large populations of mouse primary cortical cultured cells.](image-url)

Cultures (10-14 DIV) loaded with fluo-3 AM and stained with Hoechst-33342 were stimulated with PNU2 (3 μM) in combination with PNU1 (10 μM) in the presence or absence of either MLA (100 nM), Cd\(^{2+}\) (50 μM), ryanodine (Ry; 30 μM), CNQX (10 μM), MK801 (2.5 μM) CNQX (10 μM) in combination with MK801 (2.5 μM), bicuculline (Bic; 1 μM) or CGP55845 (CGP 1 μM). Antagonists were pre-incubated for 10 min prior to stimulation. Data are presented as the difference between mean F\(_{525}\) before and after stimulation following excitation at 490 nm (ΔF\(_{525}\)), averaged from 2-9 independent experiments ± SEM; \(*^* \ P < 0.001, \***^* \ P < 0.0001\) significantly different from buffer, non-paired one-way ANOVA, Bonferroni’s multiple comparisons test; each point represents the mean ΔF\(_{525}\) of a triplicate from a single experiment.
4.2.4 Immunofluorescent characterization of primary cortical cultures

Primary cultures prepared from mouse cerebral cortex have been previously described as a heterogeneous population of cell types (see section 3.1.3). Here we used immunofluorescent labelling to characterize cultures for different cell types. Cultures were fixed in 4% paraformaldehyde (PFA) and fluorescently tagged secondary antibodies were used to detect binding of primary antibodies against microtubule associated protein type 2 (MAP2) revealing extensive dendritic structures characteristic of neuronal cells (Figure 4.2.12). Dendritic processes could

![Figure 4.2.12: Immunofluorescent labelling of mouse primary cortical cultures.](image)

Cultures (10-14 DIV) were fixed and stained with Hoechst-33342 (blue) and incubated with primary antibodies (green) specific to glial fibrillary acidic protein (GFAP), microtubule associated protein type 2 (MAP2), glutamic acid decarboxylase (GAD) or synaptophysin (SP), followed by incubation with Alexa Fluor-488 tagged secondary antibodies. Cells were then imaged at 455 (Hoechst-33342) and 525 nm (Alexa Fluor-488) following excitation at 350 and 490 nm respectively. Scale shown applies to all images.
be seen to extend over 200 μm in length, and were extensively interconnected. Antibodies specific to glial fibrillary acidic protein (GFAP) revealed cells displaying multiple extensive processes characteristic of astrocytes. These processes appeared more variable and restricted in length, and exhibited a degree of overlap. The complex and extensive morphologies shown in these images present a serious challenge for segmentation, as it is difficult to ascertain the boundaries of individual cells even by eye. An attempt to segment GFAP positive structures is shown in (Figure 4.2.13).

To reveal the presence of GABAergic cells, fixed cultures were probed with antibodies specific to glutamic acid decarboxylase, revealing comparatively sparse, cell body sized regions of high fluorescent intensity as well as extensive punctate regions that appeared to follow the dendritic-like patterns shown by MAP2 staining (Figure 4.2.12). Lastly, following incubation with antibodies for the pre-synaptic terminal protein synaptophysin, a much more dense punctate pattern of fluorescence

![Figure 4.2.13: Attempted segmentation of GFAP positive fluorescent images.](attachment:image.png)

Cultures (10-14 DIV) were fixed and stained with Hoechst-33342 (blue) and incubated with primary antibodies specific to glial fibrillary acidic protein (GFAP), followed by incubation with Alexa Fluor-488 tagged secondary antibodies. Cells were then imaged at 455 (Hoechst-33342) and 525 nm (a, Alexa Fluor-488) following excitation at 350 and 490 nm respectively. CellProfiler software was used to segment nuclei based on Hoechst-33342 staining (b, green outlines), followed by the attempted segmentation of GFAP positive regions (b, pink outlines). Segmented objects based on GFAP positive regions linked to a segmented nuclei are shown in (c).
was revealed (Figure 4.2.12). By using primary and secondary antibody pairs, three colour images could be generated, as shown in (Figure 4.2.14). Here, cells were stained with Hoechst and probed with anti-GAD primary antibodies, were co-labelled with either anti-GFAP, -MAP2 or -neurofilament specific primary antibodies from a different species and two different fluorescent secondary antibodies were used for visualization. From these images, neuronal cells appear to outnumber astrocytes, whilst GAD positive cells could be judged at around 20 % of total neuronal cells. GAD positive regions co-localized more with neuronal markers compared to astrocytic markers.

![Figure 4.2.14: Three-colour immunofluorescent labelling of mouse primary cortical cultures.](image)

Cultures (10-14 DIV) were fixed and stained with Hoechst-33342 (blue) and incubated with primary antibodies specific to glutamic acid decarboxylase (a-c: GAD; green) and either glial fibrillary acidic protein (a: GFAP, red), microtubule associated protein type 2 (b: MAP2; red), or neurofilament (c: NF; red), followed by incubation with Alexa Fluor-488 or -546 conjugated secondary antibodies. Cells were then imaged at 455 (Hoechst-33342), 525 nm (Alexa Fluor-488) and 605 nm (Alexa Fluor-546) following excitation at 390, 490 and 543 nm respectively.

Together, these images show predominantly neuronal cultures with a substantial presence of astrocytes. GABAergic neurons and synapses are also evident. These data raised the possibility of gaining further information that could inform Ca$^{2+}$ measurements shown in sections 4.2.1-3, which will be explored in the following section.

### 4.2.5 Combining the high content Ca$^{2+}$ imaging assay with immunofluorescent labelling

The aim of HCS is to maximize throughput and data output from an assay. Following the success of exploratory experiments aimed at detecting Ca$^{2+}$ elevations in primary cortical cultures, we aimed to add insight by combining these experiments with
immunofluorescent labelling. To do this, cells were fixed after the Ca^{2+} imaging assay, stained with fluorescent antibodies and re-imaged. Images had to be re-aligned according to the nuclear (Hoechst) stain due to movement of the cell culture plate from its original position following replacement into the microscope plate holder. This is a common problem in imaging, and various methods exist for correcting it. Here we have used the normalized cross correlation method from the align function in CellProfiler image analysis software (Broad Institute; Figure 4.2.15). Multiple fluorescent images could then be combined to an image stack for viewing in ImageJ (NIH). In this section, a selection of images compiled in this manner will be described.

**Figure 4.2.15: Re-alignment of fluorescent images based on nuclear stain.**

Following Ca^{2+} imaging (see 4.2.1), primary cortical cultures (10-14 DIV) were probed for various markers using immunofluorescence. Upon returning cell culture plates to the microscope, the field of view imaged was not properly aligned with the original image as shown by Hoechst staining (a; red, green; both represent nuclei). To rectify this, Hoechst-33342 images from before and after immunofluorescent labelling were used to realign images in CellProfiler, using the normalized cross correlation method (b). X- and Y- offset coordinates could then be applied to immunofluorescence images to align them with fluo-3 positive images of the same cells.
The alignment of multiple fluorescent images enables the possibility of further detail being extracted from the HCS assay. The following examples show the potential of this method. Figure 4.2.16 shows KCl (40 mM) evoked $F_{525}$ increases, as described previously in Figure 4.2.1, but with added GFAP (Figure 4.2.16a) or NF (Figure 4.2.16b) immunofluorescent labelling. Upon visual inspection, co-localization of fluo-3 and NF exceeds that of fluo-3 and GFAP (yellow regions), mostly seen in large, cell body-like structures. These images also show the pre-dominance of neuronal compared to astrocytic cell types, which in Figure 4.2.16 can be estimated by eye to be in the ratio ~9:1.

Figure 4.2.17 shows increases in $F_{525}$ stimulated by PNU2 (3 μM) in the presence of PNU1 (10 μM) and bicuculline (1 μM), combined with GFAP (Figure 4.2.17a), NF (Figure 4.2.17b) or GAD (Figure 4.2.17c) immunolabelling. Co-localized increases in $F_{525}$ with immunolabelling are indicated by white arrows in each image. Responses to PNU2 (3 μM) in the presence of PNU1 (10 μM) and bicuculline (1 μM) are relatively sparse compared to responses evoked by KCl (40 mM), as described by results in sections 4.2.1 and 4.2.3. Upon visual inspection, these responses appear to occur more in non-GABAergic neuronal cell types, but some co-localization is evident.

Finally, experiments were conducted to combine fluo-3 $F_{525}$ changes with double immunolabelling. Figure 4.2.18 shows an example of this where NMDA was applied to cultures, followed by immunolabelling with anti-GFAP and -GAD antibodies. Here, a larger proportion of cells can be seen responding than in Figure 4.2.17, consistent with data presented in sections 4.2.2 and 4.2.3. Some GFAP positive regions can be seen responding to NMDA.
Figure 4.2.16: KCl evoked [Ca\textsuperscript{2+}]\textsubscript{e} elevations in primary cortical cultures stained for GFAP and NF.

Cultures (10-14 DIV) loaded with fluo-3 AM (green) and stained with Hoechst-33342 (blue) were imaged at 455 and 525 nm following excitation at 350 and 488 nm respectively before and after stimulation with KCl (40 mM). Cultures were then fixed in 4 % PFA and probed with primary antibodies to GFAP (a; red) and NF (b; red), before detection with Alexa Fluor-546 conjugated secondary antibodies, in combination with Hoechst-33342. Cultures were imaged again at 605 and 455 nm following excitation at 543 and 350 nm respectively, and aligned using the normalized cross correlation coefficient method in CellProfiler software according to the Hoechst-33342 staining pattern of both images.
Figure 4.2.17: α7 nAChR mediated [Ca\textsuperscript{2+}]\textsubscript{ic} elevations in primary cortical cultures stained for GFAP, NF and GAD.

Cultures (10-14 DIV) loaded with fluo-3 AM (green) and stained with Hoechst-33342 (blue) were imaged at 455 and 525 nm following excitation at 350 and 488 nm respectively before and after stimulation with PNU2 (3 μM) in combination with PNU1 (10 μM) and bicuculline (Bic; 1 μM). Cultures were then fixed in 4 % PFA and probed with primary antibodies to GFAP (a; red) and NF (b; red), and GAD (c; red), before detection with Alexa Fluor-546 conjugated secondary antibodies, in combination with Hoechst-33342. Cultures were imaged again at 605 and 455 nm following excitation at 543 and 350 nm respectively, and aligned using the normalized cross correlation coefficient method in CellProfiler software according to the Hoechst-33342 staining pattern of both images. White arrows indicate co-localization of increased fluo-3 mediated F\textsubscript{525} before and after stimulation, with immunolabelled GFAP (a), NF (b) or GAD (c).
Figure 4.2.18: NMDA evoked Ca$^{2+}$ elevations in primary cortical neurons showing GFAP and GAD positive cells.

Cultures (10-14 DIV) loaded with fluo-3 AM (green) and stained with Hoechst-33342 (blue) were imaged at 455 and 525 nm following excitation at 350 and 488 nm respectively before and after stimulation with NMDA (50 μM). Cultures were then fixed in 4 % PFA and probed with primary antibodies to GFAP (red) and GAD (yellow), before detection with Alexa Fluor-488 and -546 conjugated secondary antibodies, in combination with Hoechst-33342. Cultures were imaged again at 605, 525 and 455 nm following excitation at 543, 490 and 350 nm respectively, and aligned using the normalized cross correlation coefficient method in CellProfiler software according to the Hoechst-33342 staining pattern of both images.
4.3 Discussion
The experiments presented in this chapter aimed to develop a HCS method that could potentially be used to further investigate nAChR mediated Ca$^{2+}$ signalling mechanisms in primary cortical cultures.

4.3.1 Summary
Using the INCell Analyser 2000 high content microscope and the INCell Developer Toolbox software (GE Healthcare), an algorithm was constructed to automatically segment cells in fluorescent Hoechst and fluo-3 labelled images, and report fluorescence intensity over a time series, which included automated drug application. Using this protocol, we have shown clear and significant fluo-3 mediated fluorescence intensity increases to stimulation with KCl, AMPA and NMDA on an individual cell basis from populations of ~3000 cells. Attenuation of these responses by antagonists CNQX, MK801 and D-APV was also reported.

Some evidence for α7 nAChR mediated [Ca$^{2+}$]$_e$ elevations in response to PNU2 in the presence of PNU1 was shown, although this was too inconsistent to discern any significant quantitative effects of blocking the various routes of Ca$^{2+}$ amplification that were investigated in chapter 3.

The multi-well plate format used here also allowed further analysis to be conducted by immunofluorescent labelling following the Ca$^{2+}$ imaging assay. We provide examples of fluorescent images that show co-localization of Ca$^{2+}$ elevations evoked by various drugs with markers for astrocytic, neuronal and GABAergic cells.

4.3.2 High content Ca$^{2+}$ imaging assay and analysis
Investigations utilizing HCS to observe primary cultured neuronal cells have been reported in the literature, mostly for the purposes of neurotoxicity assays using neurite outgrowth analysis protocols (Ramm et al., 2003; Hu et al., 2007; Evans et al., 2008; Anderl et al., 2009; Götte et al., 2010; Radio et al., 2010). There have been fewer attempts however to monitor live cells from primary neuronal cultures using HCS, and to our knowledge none so far that have combined live cell Ca$^{2+}$ imaging assays with phenotypic labelling. Cornelissen et al., (2013) recently described HCS-like analysis of Ca$^{2+}$ imaging data of primary cultured hippocampal neurons, although acquisition was performed on a standard confocal microscope rather than an
automated HCS system, allowing much greater temporal resolution than our experiments. Here the authors imaged 25-35 cells per field of view, analogous to experiments presented in chapter 3. Although probably not high throughput enough to qualify as a HCS protocol, this study highlights the advantages of automated analysis techniques, which can be applied to smaller-scale, higher-resolution experiments to increase efficiency and reduce bias. Limited temporal resolution in our assay was due to excitation/emission switching at each time-point and liquid-handling operations. The possibility of environmental control in the assay was available, but it would have further increased time-delays between measurements, so was not used. These factors highlight the compromise between high-content and high-resolution that must be met with currently available technology.

The assay developed here was determined to be a valid and reliable method for measuring Ca\textsuperscript{2+} elevations in large populations of primary cortical cultures, as evidenced by significant changes in fluo-3 mediated fluorescence in response to stimulation with KCl (40 mM), AMPA (10 μM) and NMDA (50 μM; see sections 4.2.1-2). These changes were clear from visual inspection of images before and after stimulation, and by plotting cell-by-cell fluorescence intensity data on a scatter graph. In these plots, a large data spread was seen for ΔF\textsubscript{525} measured for each cell, making the mean value less representative of the data set. For this reason, a threshold was set to also determine the percentage of responding cells as any ΔF\textsubscript{525} greater than one standard deviation of the average F\textsubscript{525} recorded at resting conditions, and a mean ΔF\textsubscript{525} of these “responding” cells was also presented. A much greater proportion of cells were seen to respond to KCl, AMPA and NMDA than to PNU2 in the presence of PNU1, which is consistent with live-imaging experiments in chapter 3, and reflects reports in previous studies that α7 nAChRs are expressed in a smaller subpopulation of cortical neurons as assessed by αbgt binding (33 %; Barrantes et al., 1995).

In these experiments, blockade of AMPA (10 μM) with CNQX (10 μM) and MK801 (2.5 μM) was evident, although not statistically significant in ANOVA post-hoc comparisons. Due to the lack of a perfusion system in this assay, antagonism experiments could not be conducted sequentially on the same cells as in chapter 3, and therefore relied on comparing the mean ΔF\textsubscript{525} values from different populations of cells. Large cell populations with a large data spread reduces the chances of observing a statistically significant difference. The decreased percentage of
responding cells supports evidence of a block, but here it was inappropriate to report
the mean ΔF₅₂₅ of responding cells, as this would highlight the smaller percentage of
cells that were not blocked below the threshold, whilst ignoring those that had been
blocked to below threshold levels.

4.3.3 α7 nAChR mediated Ca²⁺ elevations

The HCS Ca²⁺ imaging assay has provided some evidence of α7 nAChR mediated Ca²⁺
elevations, although these were inconsistent between experiments. When these
responses were clearly visible however, they were blocked by MLA, adding to their
validity as α7 nAChR mediated responses. The percentage of supra-threshold
responses from the total population also support this conclusion. In some cases, F₅₂₅
increases from t₁-₂ to t₅-₅ following application of PNU2 (3 μM) in the presence of
PNU1 (10 μM) were not discernable by visual inspection of images, as a degree of
constitutive activity was seen and could possibly have obscured responses from a
small subpopulation of cells. This further justifies the use of a difference between
average F₅₂₅ at stimulated and basal time-points, as it increases the chances of a
change in F₅₂₅ being the result of stimulation rather than spontaneous activity. In
some cases however, there was very little difference between basal and stimulated
F₅₂₅ following stimulation with PNU2 in the presence of PNU1, which indicates that
either the responses were too low, too short in duration, or did not occur possibly
due to a much lower level of α7 nAChR expression. A lack of temporal resolution
could also make responses from subpopulations of cells harder to detect. With a small
collection of time-points, it is more difficult to assess the duration of spontaneous
events, which in live cell imaging experiments would be more easily disregarded
compared to longer, stimulation-induced events.

Due to the inconsistency of the responses, it was difficult to discern any
significant effects of the antagonists investigated in chapter 3. Again, it may be that
trying to detect a partial blockade of a response that occurs within a small
subpopulation of cells is more difficult with lower spatio-temporal resolution. The
inclusion of bicuculline (1 μM) to block GABA_A receptors had a potentiating effect in
some experiments. This is to be expected if α7 nAChRs are located on GABAergic
nerve terminals, promoting the release of GABA, a predominantly inhibitory
neurotransmitter (Obata, 2013). Positive immunofluorescent labelling for GAD (see
sections 4.2.4-5) also supports the existence of GABAergic cell bodies and terminals in primary cortical cultures. Blocking postsynaptic GABA<sub>A</sub> receptors would push the equilibrium of GABA mediated inhibition vs. glutamatergic excitation towards the glutamatergic excitatory side, manifesting in an increased $\Delta F_{525}$ and percentage of responding cells. Further experiments would be needed to confirm this phenomenon. In agreement with this, $\alpha 7$ nAChR mediated GABA release has been reported in the rat hippocampus as examined by electrophysiology and synaptosomal neurotransmitter release assays (Alkondon et al., 1997a, 1999; Zappettini et al., 2011a). $\alpha 7$ nAChRs on GABAergic interneurons have been found to modulate inhibition of pyramidal cells in the pre-frontal cortex (Couey et al., 2007; Poorthuis et al., 2012).

### 4.3.4 Enhancing the assay with immunofluorescent labelling

The results of immunofluorescent labelling experiments indicate the presence of astrocytes and neurons, a smaller proportion of which (~ 20 %) appear to be GABAergic, which agree with reports in the literature (discussed in 3.1.3). The lower density of punctate fluorescent objects revealed by GAD immunolabelling compared to synaptophysin immunolabelling also supports this (Figure 4.2.12). The heterogeneity of cell types in these cultures warrants the use of a sophisticated HCS protocol that can report these differences between cells, and this would greatly enhance our knowledge about nAChR mediated Ca$^{2+}$ signalling in primary cortical cultures. We have shown that such an approach is possible in principle, by combining Ca$^{2+}$ imaging assay data with immunofluorescent labelling. Segmentation of complex cell morphologies such as astrocytes makes this a daunting task, but could be made easier by optimizing culture density for imaging. Optimization of immunofluorescent labelling conditions would also help to resolve individual cells. The wealth of software tools for image processing and analysis currently available makes this even more likely to bear fruit in the future.

Astrocytes were found to be present in primary cortical cultures as assessed by GFAP labelling, but were outnumbered by neuronal cells by roughly 9:1, although this was variable (compare Figure 4.2.12 to Figure 4.2.16). The difference may be due to the age of the cultures, as it has been reported that astrocytes continue to divide in contrast to neurons (Horner and Palmer, 2003). The presence of astrocytes in these
cultures could be regarded as an experimental drawback if one desires to examine a purely neuronal population. It should be noted however that these cultures are used to model the mammalian brain, which albeit orders of magnitude more complex than its flat representation used here, contains an abundance of non-neuronal cell types. In fact, the intact brain glia are known to outnumber neurons substantially (Azevedo et al., 2009). Glial cells are well known to play a supportive role to neurons both physically and biochemically (Vernadakis, 1996), and omitting them completely could compromise cell viability and physiological relevance (Rita et al., 2012). For these reasons the development of an assay that can reliably distinguish differences between cell types in culture makes the presence of these cells more of an advantage than a limitation.

Qualitative analysis of combined images revealed Ca^{2+} elevations in astrocytes and neurons following α7 nAChR stimulation (Figure 4.2.17). In line with these observations, α7 nAChR-mediated Ca^{2+} elevations in astrocytes have been reported in cells of the rat hippocampus (Sharma and Vijayaraghavan, 2001; Pirttimaki et al., 2013) and cortex (Talantova et al., 2013). The images also showed that a small proportion of GAD-positive neurons exhibited increased fluorescence following α7 nAChR stimulation. Again, evidence for α7 nAChR-mediated Ca^{2+} elevations in GABAergic neurons has been reported in the hippocampus (Gomez-Varela et al., 2012). The scarcity of these responses in GAD-positive cells supports the notion that a greater proportion of α7 nAChRs are present on glutamatergic cells or terminals, which has been reported elsewhere for the hippocampus (Gray et al., 1996; Zappettini et al., 2010; Cheng and Yakel, 2014), nucleus of the solitary tract (Kalappa et al., 2011) and PFC (Dickinson et al., 2008; Livingstone et al., 2010).

**4.3.5 Conclusions**

The experiments conducted in this chapter provide a powerful method for measuring Ca^{2+} signalling in large populations of heterogeneous cells. The ability to combine this data with immunofluorescent labelling from the same individual cells greatly enhances the possibilities for gaining further insight into Ca^{2+} signalling and perhaps other markers. However, the low temporal resolution makes it challenging to quantitatively evaluate low proportions of weak responses such as those derived from α7 nAChRs accurately, and further optimization may be required.
Chapter 5: Characterization of Sazetidine-A at α7 and non-α7 nAChRs
5.1 Introduction

The plethora of candidate compounds screened in the process of modern drug discovery inevitably yields structures which convey intriguing properties that as well as being potentially clinically useful, can greatly enhance our understanding of disease pathology and receptor function. Prime examples discussed in section 1.1.5 and used extensively in the experiments that comprise this thesis are the positive allosteric modulators (PAMs), of which benzodiazepines, which target GABA<sub>A</sub> receptors, have a long history of use in medicine.

This chapter will focus on the characterization of sazetidine-A, a compound that exhibits a potentially useful behaviour at nAChRs, that of receptor desensitization, a property proposed to convey therapeutic benefits to a range of diseases. The experiments that are described in this chapter are aimed at characterizing the effects of sazetidine-A at α7 and non-α7 nAChRs in SH-SY5Y cell populations, and in mouse primary cortical cultures, both with the aid of fluorescent calcium indicators to report nAChR activation. In this section I will describe the chemical, pharmacological and behavioural aspects of sazetidine-A that have been reported in the literature.

5.1.1 Sazetidine-A: Chemistry & pharmacology

Sazetidine-A (6-[5-((2S)-2-azetidinylmethoxy]-3-pyridinyl]-5-hexyn-1-ol; fig. 5.1.1) is a derivative of A-85380 (3-[(2S)-2-azetidinylmethoxy]pyridine; fig. 5.1.1), a 3-pyridyl ether compound originally synthesized as a structural analogue of the archetypal nAChR ligand epibatidine (Figure 5.1.1), exhibiting picomolar affinity for nAChRs and a more clinically desirable selectivity profile (Abreo et al., 1996). The drawback of nAChR ligands epibatidine and nicotine for clinical use lies in their tendency to affect both the ganglionic (α3β4*) and neuronal (α4β2*) type nAChRs, the former thought to mediate cardiovascular and gastrointestinal side-effects (Cucchiaro et al., 2008).
In an effort to produce nAChR ligands with a similar selectivity profile that could be used as radiolabelled probes for identifying nAChR populations both in vivo and in vitro, Koren et al. (1998) synthesized a range of halogenated derivatives of A-85380. It was found that iodination at position 5 of the pyridine nucleus of A-85380 not only yielded a compound capable of fulfilling this need (5-iodo-A-85380; fig. 5.1.1), but also conferred even greater selectivity for α4β2* nAChRs (Mukhin et al., 2000; Xiao and Kellar, 2004). In a similar vein, the development of sazetidine-A emerged from attempts to modify A-85380 with long side-chain groups in the hope of generating nAChR ligands that could be valuable for affinity purification and/or fluorescent labeling (Xiao et al., 2006). Although successful in pre-clinical studies (section 5.1.2), it should be cautioned that the acetylene group present on sazetidine-A pre-disposes it to metabolic instability and could therefore discourage its use in clinical research (Liu et al., 2011), but despite this sazetidine-A remains an interesting lead compound.

Table 5.1.1 presents K_i values of various nAChR subtypes for sazetidine-A reported in the literature. The initial study conducted by Xiao et al. (2006)
demonstrated high selectivity of sazetidine-A for human and rat α4β2 over rat α3β4 nAChRs in [3H]epibatidine competition binding assays, with binding affinity for rat α4β2 nAChRs 24,000 times higher than that for rat α3β4. Later binding studies reported binding affinities for the same receptor subtypes in a similar range, although species and expression systems differed somewhat from the original publication (table 5.1.1; Zhang et al., 2012; Liu et al., 2013; Wageman et al., 2013). Among these studies, binding affinities of sazetidine-A at α7 nAChRs were also reported, with affinities of 670 nM for rat, and 3300 ± 745 nM for human receptors, over 10,000 times less than the affinities for α4β2 nAChRs of their respective species (Zhang et al., 2012; Liu et al., 2013). It is also interesting to note the large differences between the α3β4/α4β2 Kᵢ ratios reported for rat (60,000) and human (100) nAChRs, as this could have detrimental consequences for translating the behavioral effects of sazetidine-A (see section 5.1.2) to humans.

The first measurements of sazetidine-A's functional activity (all in vitro activity to date summarized in Table 5.1.2) were made using the ⁸⁶Rb⁺ efflux assay in human embryonic kidney (HEK) 293 cells stably expressing rat α4β2 and α3β4 nAChRs and SH-EP1 cells expressing human α4β2 nAChRs (Xiao et al., 2006). Here, no receptor activation was observed in response to the application of sazetidine-A. Antagonist effects were also absent when sazetidine-A was co-applied with other agonists such as nicotine. Pre-incubation of sazetidine-A for 10 min however potently inhibited α4β2 nAChR activation by nicotine with an IC₅₀ value of ~30 nM. Furthermore, the effect was long lasting: after wash off, α4β2 nAChR function had only recovered by 35 % after 1 h, compared to almost full recovery from nicotine induced desensitization; it was these observations that led the authors to coin the term “silent desensitizer” to describe the activity of sazetidine-A for nAChRs.
Table 5.1.1: Comparison of binding affinities of sazetidine-A at nAChR subtypes from the literature

Competition binding assays from four publications carried out using [3H]epibatidine. K_i values are mean ± SEM where given; K_i ratios are given to the nearest hundred. Experimental preparation in which nAChRs were expressed is shown in brackets; s.t.: stably transfected; r: rat; h: human; m: mouse.

<table>
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<tr>
<th>Authors (Year)</th>
<th>r.α4β2</th>
<th>m.α4β2</th>
<th>r.α3β4</th>
<th>h.α4β2</th>
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<th>h.α7</th>
<th>a3β4/a4β2</th>
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<tr>
<td>Xiao et al. (2006)</td>
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<td>10,000 ± 3000</td>
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<td>0.64 ± 0.32</td>
<td>—</td>
<td>24,000</td>
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<tr>
<td></td>
<td>0.16</td>
<td>(s.t.HEK)</td>
<td></td>
<td>(s.t.SH-EP1)</td>
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<td>Zhang et al. (2012)</td>
<td>0.05 ± 0.0</td>
<td>3000 ± 630</td>
<td>—</td>
<td>0.26 ± 0.2</td>
<td>30 ± 2</td>
<td>3300 ± 745</td>
<td>r: 60,000</td>
<td>h: 13,000</td>
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<tr>
<td></td>
<td>0.062</td>
<td>(PC-12)</td>
<td></td>
<td>(s.t.SH-EP1)</td>
<td>(SH-SY5Y)</td>
<td>(s.t.HEK/RIC3)</td>
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<td></td>
<td>1900</td>
<td>670</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>11,000</td>
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<td></td>
<td></td>
<td>(s.t.HEK)</td>
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<td>(s.t.HEK)</td>
<td></td>
<td>(s.t.HEK)</td>
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<tr>
<td>Wageman et al. (2013)</td>
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<td>0.06 ± 0.01(m. cortex)</td>
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<td>—</td>
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Table 5.1.2: Comparison of functional activity reported in the literature for sazetidine-A

Data from 10 separate publications

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<td>Non-linked Concatameric h.β2-α4-β2-α4-α4</td>
<td>Concatameric h.β3-α6-β2-α6-β2</td>
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<td>Kuryatov and Lindstrom (2011)</td>
<td>Concatameric h.β3-α6-β2-α6-β2</td>
<td>Concatameric h.β3-α6-β2-α4-β2</td>
<td>Concatameric h.β3-α6-β2-α6-β2</td>
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<td>Zhang et al. (2012)</td>
<td>h.(α4)β2(B2)</td>
<td>h.α4β2α5</td>
<td>h.(α4)β2(B2)</td>
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<td>Liu et al. (2013)</td>
<td>h.α4β2</td>
<td>r.α4β4</td>
<td>Yxaα4β2H1</td>
<td>s.t. HEK</td>
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**Notes:**
- EC_{50} (nM) represents the concentration of the agonist that produces 50% of the maximum effect.
- E_{max} (% ACh) represents the maximum effect in percentage of the maximum response.
- IC_{50} (nM) represents the concentration of the antagonist that reduces the maximum response to 50%.

**References:**
- Kozikowski et al. (2009)
- Liu et al. (2013)
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<tr>
<th></th>
<th>$E_{\text{max}}$ (% ACh)</th>
<th>$I_C_{50}$ (nM)</th>
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<th>$m.\alpha_4\beta_2^*$</th>
<th>$m.\alpha_4\beta_2$</th>
<th>$m.\alpha_4\beta_2 + m.(\alpha_4\beta_2)\alpha_5$</th>
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<td>$D_{\text{max}}$ (% ACh)</td>
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<td>$77 \pm 7$</td>
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<td>0.12 ± 0.05</td>
<td>0.63 ± 0.1</td>
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<td>$D_{\text{IC}_{50}}$ (nM)</td>
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<td>90 ± 5</td>
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<th>$E_{\text{max}}$ (% ACh)</th>
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<tr>
<td></td>
<td>55 ± 0.8</td>
<td>99 ± 2</td>
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<td>12 ± 2</td>
<td>4.6</td>
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<td>44</td>
<td>100</td>
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<td>55 ± 0.8</td>
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<th>$E_{\text{max}}$ (%)</th>
<th>s.t. tsA20; membrane potential assay</th>
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<td></td>
<td>44</td>
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<td>0.48 ± 0.02</td>
<td>0.28 ± 0.2</td>
<td>330 ± 100</td>
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<td>3500 ± 400</td>
<td>330 ± 100</td>
<td>3500 ± 400</td>
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Chapter 5: Characterization of Sazetidine-A at $\alpha_7$ and non-$\alpha_7$ nAChRs
Paradoxically, a subsequent study by Zwart et al. (2008) reported potent agonist effects of sazetidine-A eliciting \[^{3}H\]dopamine release from rat striatal slices with an EC\(_{50}\) of \(~1\) nM. Addition of 100 nM \(\alpha\)-conotoxin MII (\(\alpha\)CtxMII) did not affect the EC\(_{50}\) value but reduced the E\(_{\text{max}}\) by roughly half, indicating – as seen before with the structural analogue 5-iodo-A-85380 – substantial contribution from \(\alpha_6\beta_2^*\) nAChRs which partly mediate dopamine release in the striatum (Mogg et al., 2004). In the same report, electrophysiological recordings from \(X.\ laevis\) oocytes expressing human \(\alpha_4\beta_2\) nAChRs were made to establish a possible reason for the lack of agonist activity reported previously by Xiao et al. (2006). By varying the ratio of \(\alpha_4\) to \(\beta_2\) nAChR subunit cDNAs injected into oocytes, the effect of sazetidine-A on different \(\alpha_4\beta_2\) nAChR stoichiometries could be studied. It was found that whilst full agonist activity was observed for sazetidine-A at high sensitivity \(\alpha_4\beta_2\) (HS-\(\alpha_4\beta_2\)) nAChRs, only 6\% efficacy relative to acetylcholine was found for the lower sensitivity \(\alpha_4\beta_2\) (LS-\(\alpha_4\beta_2\)) nAChRs, implying that sazetidine-A is a stoichiometry selective, as well as subtype selective nAChR agonist. This compelling finding was later confirmed by Carbone et al. (2009), who also used concatenated receptor subunit constructs to express different \(\alpha_4\beta_2\) nAChR stoichiometries separately in \(X.\ laevis\) oocytes. No difference was reported for concatameric and non-linked \(\alpha_4\beta_2\) nAChRs. Zhang et al., (2012) also reported full efficacy of sazetidine-A for stably transfected human HS-\(\alpha_4\beta_2\) in HEK-293 cells, whereas at LS-\(\alpha_4\beta_2\) and \(\alpha_4\beta_2\alpha_5\) nAChRs it was much lower (98 ± 1 \%, 0.8 ± 4 \%, 0.02 ± 2\% respectively, relative to ACh). This group also reported similar potency and efficacy for desensitization of HS-\(\alpha_4\beta_2\), LS-\(\alpha_4\beta_2\) and \(\alpha_4\beta_2\alpha_5\) nAChR subtypes (see Table 5.1.2).

Sazetidine-A’s functional attributes were again studied using the \(^{86}\text{Rb}^+\) efflux assay in \(SH\)-EP1 cells expressing human nAChRs during an investigation into the chemistry and pharmacology of nine of its structural analogues (Kozikowski et al., 2009). EC\(_{50}\) values of 5.8, 25, 340 and 2200 nM; and efficacy relative to nicotine of 55, 98, 97 and 51 \% were reported for \(\alpha_4\beta_2\), \(\alpha_4\beta_4\), \(\alpha_3\beta_4\) and \(\alpha_1\) nAChRs respectively. Here sazetidine-A acted as a partial agonist at \(\alpha_4\beta_2\) nAChRs in disagreement with the two previous studies described above, again possibly due to differences in nAChR stoichiometry in the experimental system used. This again highlights the importance of nAChR subunit stoichiometry in determining the functional effects of sazetidine-A.
Elaborating on the findings published by Zwart and colleagues (2008) with respect to sazetidine-A’s actions at α6β2* nAChRs, Kuryatov and Lindstrom (2011) generated and expressed concatameric constructs of (α6β2)β3 nAChRs and different (α6β2)(α4β2)β3 nAChR stoichiometries in X. laevis oocytes. They reported high potency and almost full efficacy for sazetidine-A at (α6β2)(α4β2)β3 (EC₅₀ = 20, 45 nM and Eₘₐₓ = 86, 73 % for β3-α6-β2-α4-β2 and β3-α4-β2-α6-β2 concatamers respectively). At (α6β2)β3 nAChRs, sazetidine-A was less potent and less efficacious, with EC₅₀ = 157 nM and Eₘₐₓ = 22 %. A recent study by Wageman et al. (2013) probed the selectivity of sazetidine-A for α4β2* nAChR subtypes even further by employing αCtxMII or α6 KO mice in combination with α5 KO mice, and found that the presence of the α5 subunit in α4β2* nAChRs decreased the potency of sazetidine-A acting as a desensitizer against ACh in mouse striatal synaptosomes.

Recently it was reported that similar to its preference for activating HS-α4β2α5 over LS-α4β2α5 nAChRs, sazetidine-A also selectively desensitizes HS-α4β2α5 nAChRs over LS-α4β2α5 (Campling et al., 2013; Eaton et al., 2013), in contrast to earlier findings (Zhang et al., 2012). Whiteaker’s group resolved this phenomenon further still, by heeding the work of Harpsøe et al., (2011) and Mazzaferrro et al., (2011) who demonstrated that LS-α4β2α5 nAChRs contain a third, low-sensitivity agonist binding site between the α4(+)/(-)α4 subunit interface. It was found that following 10 min desensitization pre-treatment with sazetidine-A, concatameric LS-α4β2α5 in contrast to HS-α4β2α5 nAChRs still responded to acute ACh application, which was attributed to allosteric modulator-like activity at the unbound α4(+)/(-)α4 binding site, reactivating the receptor. The authors therefore interpreted sazetidine-A to be selective for the α4(+)/(-)β2 over the α4(+)/(-)α4 binding site (Eaton et al., 2013). Considering this, it is very interesting to note that Zhang et al., (2012) found that sazetidine-A showed similarly low potency and efficacy at α4β2α5 compared to LS-α4β2α5 nAChRs, suggesting that the α4β2α5 subtype also contains a non-canonical binding site. However, recent studies employing a mutation approach similar to that used to establish the α4(+)/(-)α4 binding site dispute an analogous α5(+)/(-)α4 binding site in the α4β2α5 nAChR (Marotta et al., 2013). Nevertheless, expression of these subunits as linked, concatameric constructs has provided evidence that as well as occupying the 5th, accessory position, the α5 subunit can replace a β2 subunit that forms a canonical binding site with α4, with no apparent functional impact (Jin et al.,
2013). The reasons for sazetidine-A’s low activity at α4β2α5 remain obscure and warrant further study.

Owing to previous efforts, which used a combination of transfected RIC-3 and the chemical chaperones valproic acid and 4-phenylbutyric acid to allow high expression levels of functional α7 nAChRs in cell lines (Kuryatov et al., 2013), Lindstrom’s group were also able to measure the functional effects of sazetidine-A at separately expressed α7 nAChRs for the first time, using a fluorescent membrane potential assay. They report it to have full agonist and desensitizer activity at α7 nAChRs with EC$_{50}$ and IC$_{50}$ values of 1.2 and 3.5 μM respectively, approximately 10 fold less potent than at α3β4 nAChRs, and 100 fold less than at α4β2 nAChRs, which Campling et al., (2013) declare is not within the clinically achievable range.

Another striking and potentially clinically useful property of sazetidine-A came to light in a report from Hussmann et al. (2012), who found that in contrast to nicotine and another nAChR ligand varenicline, currently prescribed as a smoking cessation aid, 2 week chronic administration of sazetidine-A did not upregulate nAChRs in rodent brain in vivo measured using saturating concentrations of [³H]epibatidine. This effect occurred at two to six times the behaviorally active dose of sazetidine-A in a nicotine self-administration test. Also contrary to nicotine and varenicline, the concentration of sazetidine-A measured in the brains of chronically treated animals was only 10 % of that found in blood, yet with a K$_i$ of 0.05 nM for nAChRs in rat cortex (Zhang et al., 2012) this was still theoretically sufficient to occupy all α4β2 nAChRs in the brain (~30 nM). Sazetidine-A was however found to significantly upregulate nAChRs in rat primary cortical cultures, in agreement with previous findings in HEK-293 cells (Xiao et al., 2006). Hussmann et al., (2012) suggested that the difference between in vivo and in vitro nAChR upregulation by sazetidine-A reflects the poorer ability of sazetidine-A relative to nicotine and varenicline to penetrate the blood brain barrier and subsequent cell- and organelle-membranes in vivo. It would therefore be able to reach the brain at sufficient concentrations to occupy cell surface nAChRs, but perhaps fail to reach the ER in sufficient concentrations to influence nAChR upregulation. This could be a highly valuable attribute for clinical uses that rely on occupation of nAChRs but would otherwise be limited by nAChRs upregulation.
To summarize, the 3-pyridyl ether derivative sazetidine-A has been found to exhibit high selectivity for β2* nAChRs over β4* and α7 nAChRs in binding studies comparable to its forerunner analogues, whilst displaying three intriguing and quite possibly valuable attributes: the ability to desensitize nAChRs for extended periods of time, differential selectivity for α4β2 nAChR stoichiometries, and failure to upregulate nAChRs in the brain *in vivo* at behaviourally active doses. The next section will explore the implications of these characteristics by evaluating the findings garnered from pre-clinical research involving sazetidine-A.

### 5.1.2 Evaluation of sazetidine-A’s effects in pre-clinical models

A steady stream of animal studies has begun to provide evidence for the potentially beneficial effects of sazetidine-A in a range of clinical disorders including addiction, depression, pain, and attention deficits. Whilst offering therapeutic potential, sazetidine-A’s unique pharmacological properties described above are also adding to our understanding of the underlying pathology and neurobiology of such clinical conditions in a reciprocal fashion. A recurrent theme in nAChR related disorders is the at-first paradoxical notion of the ability of both agonists and antagonists to provide the same beneficial effects. These observations are however reconciled when one takes into account the intrinsic propensity of nAChRs to desensitize in the presence of continued agonist stimulation, presenting a physiological antagonistic effect. The remainder of this section will be concerned with evidence of such effects produced by sazetidine-A.

Cucchiaro and colleagues, (2008) reported significant analgesic effects of sazetidine-A in the formalin test of nociception in mice at doses above 0.5 mg/kg. These results were later confirmed by Zhang et al. (2012) and Alsharari et al. (2012). The former screened a variety of α4β2*-selective agonists with varying activation/desensitization properties and reported that stronger desensitizers of α4β2* nAChRs elicit better analgesia. Interestingly, this study also concluded that desensitization of α4β2α5 nAChRs; at which sazetidine-A’s effects were similar to those observed for LS-α4β2 nAChRs was more important in determining antinociceptive effects of nicotinic compounds. Alsharari et al., (2012) also used the tail flick and hot plate models of acute nociception in which sazetidine-A and varenicline, another α4β2 nAChR partial agonist, failed to induce significant
analgesia. Together these studies demonstrate the potential therapeutic benefit of
sazetidine-A for chronic, but not acute pain conditions.

Sazetidine-A, like other compounds affecting α4β2 nAChRs such as
camylamine, A-85380 (Buckley et al., 2004), SIB-1508Y (Ferguson et al., 2000),
arenicline (Rollem et al., 2009), cytisine (Mineur et al., 2007) and TC-1734 (Gatto et
al., 2004), was found to increase time spent swimming in the mouse forced swim test
(Kozikowski et al., 2009; Turner et al., 2010; Caldarone et al., 2011), designed to
measure antidepressant drug efficacy in mice. Typical antidepressant drugs such as
sertraline and desipramine reduce time spent immobile by a mouse during an
allocated time left in a water filled container, a measure of behavioural helplessness
(Porsolt et al., 1977). These pre-clinical findings provide incentive to further study
the effects of nAChR agents like sazetidine-A on depressive symptoms.

Promising findings in the field of addiction have also been reported for
sazetidine-A. It reduced nicotine self-administration in rats (Levin et al., 2010;
Rezvani et al., 2010), with no attenuation in effectiveness even after a 4-week period
of chronic treatment (Johnson et al., 2012). These effects are due to partial agonism of
sazetidine-A for nAChRs, and long lasting desensitization, which would block the
effects of nicotine. Alcohol self-administration was also significantly reduced in
selectively bred alcohol preferring rats by both chronic and acute treatment with
sazetidine-A (Rezvani et al., 2010). Here, in contrast to the nicotine self-
administration studies, tolerance began to develop after the seventh day of treatment.
Furthermore, as measured by the novelty-induced hypophagia and marble burying
tests, acute sazetidine-A mitigated anxiogenic effects of nicotine withdrawal in mice
(Turner et al., 2013). Paterson et al. (2010) reported that sazetidine-A, like
arenicline and nicotine exhibited reinforcing effects in rats, although to a lesser
degree. As described above (section 5.1.1), another important aspect to note with
respect to sazetidine-A’s potential use for addiction therapy is the lack of nAChR
upregulation reported in the brains of rodents following chronic treatment with
doses that reduced nicotine self-administration, as this could contribute to the
reduction of nicotine withdrawal symptoms, by reducing the number of available
nAChRs (Hussmann et al., 2012).

Sazetidine-A, which is reputedly able to desensitize α4β2 nAChRs for hours
after brief activation (Xiao et al., 2006) was seen to enhance and restore MK801 and
scopolamine impaired performance in the visual signal task (Rezvani et al., 2011, 2012a), a rodent model of sustained attention (Bushnell, 1995). To address the question of whether activation or inactivation via desensitization is the dominant mechanism of sazetidine-α7’s attention enhancing effects, Levin et al. (2013a) used antagonists of α4β2 and α7 nAChRs, dihydro-β-erithroidine (DHβE) and methyllycaconitine (MLA) respectively in the same behavioural paradigm, achieving similar results. This group also investigated hypothermic responses in rodents, a well-known effect of nicotinic agonists, caused by sazetidine-α7. In two separate studies it was found that DHβE and β2 subunit knockout but not MLA or α7 subunit knockout reduced sazetidine-α7 induced hypothermic responses in rodents (Rezvani et al., 2012b; Levin et al., 2013b). This suggests that the beneficial effects of nicotinic agonists are indeed due to a physiological antagonist effect, which can result from nAChR desensitization. This would also be expected from receptor exposure to drug in vivo, which would be over relatively long timescales.

5.1.3 Summary
In consideration of its qualities described thus far, sazetidine-α7 represents a promising lead compound for the development of treatments for a range of clinical disorders in which nAChRs are implicated, specifically pain, depression, addiction, and attention deficits. To reiterate its pharmacological profile, sazetidine-α7 is a compound which displays full agonist activity for HS-α4β2, partial agonist activity for α6β2* and almost no agonist activity for LS-α4β2 nAChRs, making it subtype as well as stoichiometry selective. It also potently desensitizes nAChRs for long periods of time, and fails to upregulate nAChRs in vivo. Relatively little attention so far has been paid to the actions of sazetidine-α7 nAChRs, which have been reported to modulate motivation to self-administer nicotine in rodents (Brunzell and McIntosh, 2012). Widespread α7 nAChR expression in peripheral systems also raises the possibility of them mediating of side-effects to nicotinic drugs (Filippini et al., 2012). This receptor subtype therefore deserves further study with respect to the effects of sazetidine-α7.

5.1.4 Aims
The aim is to evaluate the functional activity of sazetidine-α7 at α7 and non-α7 nAChRs. Sazetidine-α7 has been reported to activate and strongly desensitize nAChRs,
attributes that are potentially relevant for clinical treatment of addiction and depression. It has been shown to be highly selective for \( \alpha_4\beta_2^* \) compared to \( \alpha_3\beta_4^* \) nAChRs (Xiao et al., 2006; Zwart et al., 2008; Kozikowski et al., 2009; Zhang et al., 2012; Campling et al., 2013; Liu et al., 2013), with evidence for substantial activity at \( \alpha_6\beta_2^* \) nAChRs (Zwart et al., 2008; Kuryatov and Lindstrom, 2011), although at the time of conducting these experiments little has so far been published for its functional activity at \( \alpha_7 \) nAChRs. Therefore the aim was to examine the ability of sazetidine-A to both activate and desensitize \( \alpha_7 \) and non-\( \alpha_7 \) nAChRs, as these effects could be relevant to its clinical profile.

Using the SH-SY5Y human neuroblastoma cell line, which express \( \alpha_3, \alpha_5, \alpha_7, \beta_2, \beta_4 \) nAChR subunits (Lukas et al., 1993), the effects of sazetidine-A on nAChRs, and their concentration dependence will be assessed in a high throughput 96-well plate calcium assay. Sazetidine-A’s effects will then be examined on an individual cell basis in mouse primary cortical cultures, using live cell imaging. Finally, in this chapter we also aim to characterize the effects of five novel compounds on \( \alpha_7 \) and non-\( \alpha_7 \) nAChRs.
5.2 Results

5.2.1 PNU-120596 potentiates intracellular calcium elevations elicited by sazetidine-A in SH-SY5Y cells: contributions from α7 and non-α7 nAChRs.

Previously, Zwart et al. (2008) reported that sazetidine-A evoked [³H]noradrenaline ([³H]NA) release in rat hippocampal slices with an EC50 = 4.5 ± 2.5 μM, an effect attributed solely to α3β4* nAChRs (Luo et al., 1998; Anderson, 2000; Kennett et al., 2012). We therefore used high concentrations of sazetidine-A in preliminary experiments using SH-SY5Y cells that express these nAChR subunits in order to evoke robust responses.

![Graphs showing intracellular calcium elevations](image)

**Figure 5.2.1: Intracellular calcium elevations evoked by sazetidine-A in SH-SY5Y cell populations.**

SH-SY5Y cells loaded with fluo3-AM were stimulated with sazetidine-A (10 μM, a, b; 100 μM, c, d) in the presence (dark blue bars) or absence (light blue bars) of PNU1 (10 μM). Responses were challenged with the general nAChR antagonist mecamylamine (30 μM; checkered bars) and the α7 nAChR selective antagonist MLA (100 nM; dashed bars). Fluorescence at 538 nm was measured for 20 s following stimulation with sazetidine-A (b) and (d). Data are presented as percent maximum fluorescence determined by addition of 0.2 % Triton X-100 minus minimum fluorescence quenched by 350 mM MnCl2 (a) and (c). Bars represent mean ± SEM of at least 4 independent experiments; * P < 0.05, ** P < 0.01, *** P < 0.001 significantly different from sazetidine-A or in combination with PNU1, non-paired one-way ANOVA, Bonferroni's multiple comparisons test.
Initially, sazetidine-A alone was applied to cells loaded with fluo-3AM and fluorescence at 538 nm ($F_{538}$) was monitored for 20 s as shown in representative traces (Figure 5.2.1b, d). At concentrations of 10 (Figure 5.2.1a, b) and 100 μM (Figure 5.2.1c, d), sazetidine-A elicited changes in $F_{538}$ indicative of $[\text{Ca}^{2+}]_{ic}$ elevations of 27.97 ± 4.35 % and 30.3 ± 4.4 % respectively. Responses to 100 μM sazetidine-A appeared to reach maximal levels faster than those evoked by 10 μM sazetidine-A (Figure 5.2.1b, d). These responses were sensitive to blockade by the general nAChR channel blocker mecamylamine (30 μM), but unaffected by the α7 nAChR selective antagonist MLA (100 nM). Significant potentiation of these responses were observed following pre-incubation and co-application of 10 μM PNU1 (2.33 ± 0.72 and 2.24 ± 0.48 fold respectively) potentiation was abolished in the presence of mecamylamine (30 μM) or MLA (100 nM; Figure 5.2.1).

![Concentration-response profile of sazetidine-A, intracellular calcium elevations in SH-SY5Y cell populations.](image)

SH-SY5Y cells loaded with fluo3-AM were stimulated with sazetidine-A (0.1 – 100 μM) in the presence (grey squares) or absence (black circles) of PNU1 (10 μM). Fluorescence at 538 nm was measured for 20 s following stimulation with sazetidine-A. Data are presented as percent maximum fluorescence determined by addition of 0.2 % Triton X-100 minus minimum fluorescence quenched by 350 mM MnCl$_2$. Points represent mean ± SEM of at least 8 independent experiments. Data were fitted to the Hill equation following exclusion of data point representing the response to 100 μM sazetidine-A in the presence of PNU1.

We then examined the concentration-response profile of sazetidine-A in SH-SY5Y cells with and without PNU1 (Figure 5.2.2). Cells loaded with fluo-3 AM were stimulated with sazetidine-A alone at concentrations of 0.1-100 μM, giving a sigmoidal concentration-response relationship with $EC_{50} = 4.17$ μM that began to saturate at 100 μM. In the presence of PNU1, responses were much more variable (data point for response at 100 μM sazetidine-A in the presence of PNU1 has been
excluded as an outlier to allow points to fit a curve described by the Hill equation, with EC$_{50}$ = 0.42, and saturation beginning at around 10 µM.

In a single experiment, the potentiation of sazetidine-A by PNU1 was compared to that of nicotine and another highly selective α4β2 nAChR agonist, 5-Iodo-A85380 (5-Iodo; Figure 5.2.3). Nicotine (30 µM) and 5-Iodo (1 µM) evoked $F_{538}$ elevations of 14.79 ± 2.12 % and 28.86 ± 4.73 % respectively, which were potentiated 4.00 ± 0.21 and 2.35 ± 0.28 fold by pre-incubation and co-application of PNU1 (10 µM). These levels of potentiation by PNU1 are in the same range as observed for the potentiation of 10 µM sazetidine-A (2.33 ± 0.72 fold; Figure 5.2.1).

Having observed the agonist effects and concentration response relationship of sazetidine-A in the presence or absence of PNU1 in SH-SY5Y populations, we sought to examine the responses in greater detail with the aid of live cell imaging. Here, individual cell responses can be imaged in real time, and by employing a perfusion system cells can be subjected to sequential drug applications, allowing imaging of a response and its subsequent antagonism. For these experiments, we used primary cells cultured from mouse cortex, which express α4, α5, α7 and β2 nAChR subunits (Gotti et al., 2006; Mao et al., 2008). The cultures comprise neuronal and glial cells that exhibit synaptic connections and a higher degree of complexity that is more physiologically relevant to the brain.
5.2.2 PNU-120596 reveals sazetidine-A evoked calcium elevations in mouse primary cortical cultures

Mouse primary cortical cultures were loaded with the ratiometric calcium sensitive dye fura-2 AM and continuously perfused with buffer at 37 °C. Stimulation with sazetidine-A at 10 nM, 100 nM and 1 μM for 20 s was not found to elicit changes in fluorescence ratio (F₃₄₀:F₃₈₀) in any cells examined (n = 1 culture, 2 replicates x 2 measurements, 23 regions of interest; Figure 5.2.4a-c). At 10 μM however, sazetidine-A inconsistently provoked short bursts of elevated F₃₄₀:F₃₈₀ indicative of [Ca²⁺]ᵢ changes in a subpopulation of cells from 4/8 measurements (mean F₃₄₀:F₃₈₀ increase

Figure 5.2.4: Live cell calcium imaging of mouse primary cortical cultures perfused with sazetidine-A.

Cultures (10-14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Basal fluorescence was monitored for 30 s before 20 s application of sazetidine-A (0.01 - 10 μM). KCl (40 mM) was added for 20 s following a null response to sazetidine-A (d). Coloured lines represent individual regions of interest: (a), (b), (c) – all visible cells, (d) & (e) – all responding cells. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm (F₃₄₀:F₃₈₀).
Chapter 5: Characterization of Sazetidine-A at α7 and non-α7 nAChRs

= 0.22 ± 0.13; Figure 5.2.4d-e). KCl (40 mM) was applied to non-responsive cells as a positive control; resulting in rapid F340:F380 increases of 0.28 ± 0.01 that returned to basal levels upon washout (33 cells from two measurements; n = 1; Figure 5.2.4d).

In one experiment, nine cells gave short, burst like elevations in F340:F380 (0.04 ± 0.01) in response to a 20 s application of 10 μM sazetidine-A (Figure 5.2.5a). After 3 min washout, mecamylamine (30 μM) was applied to the same cells for 10 min, before co-applying 10 μM sazetidine-A again for 20 s, which failed to produce a repeated response in all cells (Figure 5.2.5b). Following 10 min washout however, a third application of 10 μM sazetidine-A for 20 s in the absence of mecamylamine resulted in a 45.22 ± 0.00 % recovery of the original response (Figure 5.2.5c). Spontaneous elevations in basal F340:F380 were observed at the start of recording (Figure 5.2.5a,c), artifacts similar to this have been observed previously at the start of F340:F380 recordings in this experimental system (see section 3.2.2).

To examine the potential agonist effects of sazetidine-A at α7 nAChRs in mouse primary cortical cultures, we employed the α7 selective PAM PNU1 to amplify any responses that may be obscured by rapid desensitization typical of this receptor. A representative experiment is shown in figure 5.2.6a-c. Following 3 min pre-incubation of PNU1 (10 μM), 20 s co-application of sazetidine-A (10 μM; Figure 5.2.6a), gave more consistent and prolonged increases in F340:F380 of 0.12 ± 0.04 above basal levels (0.15 ± 0.02) from 14 % of visible cells imaged in six experiments from three separate cultures, similar to the percentage of cells responding to α7 nAChR stimulation with PNU1 (10 μM) in combination with PNU2 (3 μM; 12 %; 3.2.2). These responses were also completely blocked by 10 min pre-incubation and co-application of the α7 selective antagonist MLA (100 nM; Figure 5.2.6b, d). On average, responses recovered to 32.43 ± 9.42 % of the initial stimulation following 10 min wash out (Figure 5.2.6c, d), notably lower than the recovery of responses to PNU1 in combination with PNU2 following blockade with MLA, where 89.68 ± 22.73 % % recovery was observed (3.2.2).

So far, we have assessed the agonist effects of sazetidine-A in fluo-3 AM loaded SH-SY5Y cells in a population based calcium fluorescence assay, and in fura-2 AM loaded mouse primary cortical cultures using live cell imaging. The next section will concern a behavior attributed to sazetidine-A that can be distinguished from the effects described above, namely that of receptor desensitization. Here we will use
similar methods of enquiry to observe this phenomenon in SH-SY5Y cells and mouse primary cortical cultures. Desensitization of a receptor can only be indirectly reported, to do so we must measure the ability of the desensitizing agent, in this case sazetidine-A, to impede the response elicited by an agonist compound. We will pre-incubate cells with sazetidine-A for 10 min prior to agonist stimulation, as reported by Xiao et al. (2006). To activate non-α7 nAChRs we will use nicotine (100 μM), and to activate α7 nAChRs we will use PNU1 (10 μM) in combination with PNU2 (3 μM).
Chapter 5: Characterization of Sazetidine-A at α7 and non-α7 nAChRs

Figure 5.2.5: Mecamylamine sensitive calcium transients evoked by sazetidine-A in mouse primary cortical cultures.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Basal fluorescence $F_{340}/F_{380}$ was monitored for 30 s before 20 s application of sazetidine-A (10μM; a) followed by 3 min washout. Cells were then pre-incubated with mecamylamine (30 μM, 10 min) and imaged again during co-application of sazetidine-A (10 μM; b). After 10 min washout, cells were imaged a third time whilst being perfused with sazetidine-A (10 μM; 20 s; c). Images (a), (b) and (c) show $F_{340}/F_{380}$ in pseudocolour (black/blue = low $F_{340}/F_{380}$ red/white = high $F_{340}/F_{380}$) taken during application of sazetidine-A (10 μM) in the presence or absence of mecamylamine (30 μM). Traces show coloured lines corresponding to nine individual regions of interest that responded to sazetidine-A, circled in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a single experiment.
Figure 5.2.6: Sazetidine-A elicits intracellular calcium elevations in the presence of PNU1 in mouse primary cortical cultures.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 µM; 3 min) before recording basal F_{340}/F_{380} for 30 s, after which sazetidine-A (10 µM; a) was co-applied for 20 s. After 3 min wash out, cells were then pre-incubated for 10 min with MLA (100 nM) and PNU1 (10 µM) prior to recording F_{340}/F_{380} before, during and after co-stimulation with sazetidine-A (10 µM; 20 s; b). Finally, following 10 min wash out, the protocol was repeated in the absence of MLA (c). Still images show fluorescence in pseudocolour; (black/blue = low F_{340}/F_{380}, red/white = high F_{340}/F_{380}) taken during agonist stimulation. Traces show coloured lines corresponding to two regions of interest selected as responding cells indicated with coloured arrowheads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (d) represent mean peak F_{340}/F_{380} increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 3 independent experiments; **** P < 0.0001 significantly different from initial response to sazetidine-A in combination with PNU1 (100 %), one sample t-test.
5.2.3 Desensitization of nAChRs by sazetidine-A in SH-SY5Y cells

To assess the ability of sazetidine-A to desensitize α3β4* nAChRs, cells loaded with fluo-3 AM were pre-incubated with sazetidine-A for 10 min before addition of nicotine (fig. 5.2.7a). In the absence of sazetidine-A, 100 μM nicotine evoked changes in fluorescence of 33.89 ± 7.21 %. This was concentration-dependently attenuated by sazetidine-A (0.01 – 100 μM) with IC$_{50}$ = 0.52 μM (Figure 5.2.7a).

![Figure 5.2.7](image)

**Figure 5.2.7**: Sazetidine-A concentration-dependently inhibits nicotine and PNU1+PNU2 evoked intracellular calcium elevations in SH-SY5Y cell populations.

SH-SY5Y cells loaded with fluo-3 AM were stimulated with nicotine (100 μM; a) or PNU2 (3 μM; b) following 10 min pre-incubation of PNU1 (10 μM) in the presence or absence of sazetidine-A (0.01 μM – 100 μM). Fluorescence at 538 nm was measured for 20 s following stimulation with nicotinic agonists. Data were calculated as percent maximum fluorescence determined by addition of 0.2 % Triton X-100 minus minimum fluorescence quenched by 350 mM MnCl$_2$, expressed as a percentage of responses measured in the absence of sazetidine-A. Points are fitted to the Hill equation and represent mean ± SEM of at least 10 independent experiments.

Sazetidine-A’s ability to desensitize α7 nAChRs was then evaluated by stimulating cells with PNU2 (3 μM) in the presence of PNU1 (10 μM), which elicited increases in fluorescence of 44.47 ± 9.70 %. As above, 10 min pre-incubation of sazetidine-A (0.01 – 100 μM) resulted in a concentration-dependent diminution of this response with a similar IC$_{50}$ = 0.48 μM (Figure 5.2.7b).

After the pre-incubation phase, in which sazetidine-A was added in combination with PNU1, it was noticed that measurements of basal F$_{538}$ were rising with increasing concentrations of sazetidine-A (Figure 5.2.8). After pre-incubation of 1 μM sazetidine-A and 10 μM PNU1, basal F$_{538}$ increased 171.51 % from basal F$_{538}$ in the absence of drugs. In the absence of PNU1 however, 1 μM sazetidine-A only increased F$_{538}$ by 7.12 %. This raises a concern that the ability of PNU1 to overcome
α7 nAChR receptor desensitization could compromise subsequent measurements of responses to agonist stimulation. However, the comparability of sazetidine-A’s inhibition of nicotine and PNU2 in combination with PNU1-evoked responses argues against this.

5.2.4 Desensitization of nAChRs by sazetidine-A in mouse primary cortical cultures

Mouse primary cortical cultures loaded with fura-2 AM were perfused with buffer at 37 °C. After 10 min pre-incubation with 500 nM sazetidine-A in combination with 10 μM PNU1 as above, stable baseline $F_{340}:F_{380}$ was observed prior to the addition of PNU2 (3 μM). The baseline was not significantly different from that observed in the absence of drugs (mean $F_{340}:F_{380}$ in absence of drugs = 0.30 ± 0.06, mean pre-incubation $F_{340}:F_{380} = 0.26 ± 0.04$; Figure 5.2.9).
Figure 5.2.9: Pre-incubation of sazetidine-A in combination with PNU1 in cortical cultures does not significantly alter pre-stimulation F<sub>340</sub>:F<sub>380</sub>.

Mouse primary cortical cultures (10-14 DIV) were loaded with fura-2 AM and perfused with 1.8 mM calcium buffer at 37 °C. Cultures were pre-incubated with buffer or sazetidine-A (500 nM) in combination with PNU1 (10 μM) for 10 min. Fluorescence at 510 nm following excitation at 340 and 380 nm was measured for 10 s. Data are presented as mean F<sub>340</sub>:F<sub>380</sub> ± SEM from individual ROIs from 3 independent experiments.

In the absence of sazetidine-A, PNU2 (3 μM) in the presence of PNU1 (10 μM) evoked F<sub>340</sub>:F<sub>380</sub> responses of 0.46 ± 0.23 above basal levels, a 3.68 ± 0.02 fold increase above responses evoked by sazetidine-A (10 μM) used as an agonist in combination with PNU1 (10 μM; Figure 5.2.6). Although the magnitude of these responses observed between cells was highly variable (Figure 5.2.10a-c), any inhibition observed was consistent, as has been observed previously for α7 nAChR responses in this system (see section 3.2.2). In light of our previous experiments in SH-SY5Y cells (fig. 5.2.7b), we chose to use 500 nM sazetidine-A, roughly equivalent to its IC<sub>50</sub> (0.48 μM) for blocking an α7 nAChR response.

Pre-incubation and co-application of sazetidine-A (500 nM) for 10 min in combination with PNU1 (10 μM) significantly reduced subsequent responses to PNU2 (3 μM) by 59.22 ± 12.40 % (Figure 5.2.10d), in agreement with the justification for using 500 nM sazetidine-A. After 10 min washout, responses to PNU2 (3 μM) in the presence of PNU1 (10 μM) only recovered to 57.38 ± 17.83 % of the initial response (fig. 5.2.10d), markedly lower than recovery from antagonism by MLA (89.68 ± 22.73 %; see Figure 3.2.9).

To examine the effect further, we repeated this experiment with 10 μM sazetidine-A (Figure 5.2.11). Here, desensitization of the response elicited by PNU2 (3
µM) in combination with PNU1 (10 µM) was stronger than that observed with 500 nM sazetidine-A, producing a more substantial block of 85.76 ± 4.91 %. After 10 min washout, the response recovered to 59.62 ± 11.41 %, again lower than that observed for recovery from an MLA (100 nM; Figure 3.2.9) block.

Having observed the desensitization effects of 10 min sazetidine-A pre-incubation on subsequent agonist stimulation (Figure 5.2.10, Figure 5.2.11), we hypothesized that such a desensitized state could be reversible by PNU1, as reported for other α7 nAChR agonists (Hurst et al., 2005; Grønlien et al., 2007). To test this, we planned to pre-incubate sazetidine-A for 10 min at the higher concentration of 10 µM as above, in order to achieve a higher level of desensitization (Figure 5.2.11), before applying PNU1 (10 µM) in an attempt to re-open desensitized α7 nAChRs.
Figure 5.2.10: Pre-incubation of 500 nM sazetidine-A reduces α7 nAChR mediated intracellular calcium elevations in mouse primary cortical cultures. Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording F_{340}/F_{380} for 30 s prior to addition of PNU2 (3 μM; 20 s; a), followed by 3 min wash out. Sazetidine-A (500 nM) in combination with PNU1 (10 μM) were then pre-incubated for 10 min, after which F_{340}/F_{380} was again recorded for 30 s before addition of PNU2 (3 μM; 20s; b). Finally, after 10 min wash out and pre-incubation of PNU1 (10 μM; 3 min), F_{340}/F_{380} was recorded for 30 s before a third co-application of PNU2 (3 μM; 20 s; c). Still images show fluorescence in pseudocolour; (black/blue = low F_{340}/F_{380}, red/white = high F_{340}/F_{380}) taken at peak response. Traces show coloured lines corresponding to nine regions of interest indicated with coloured arrowheads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm (F_{340}/F_{380}) from a representative experiment. Bars (d) represent mean F_{340}/F_{380} increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from 4 independent experiments; * P < 0.05 significantly different from initial response to PNU2 in combination with PNU1 (100 %), one sample t-test.
Chapter 5: Characterization of Sazetidine-A at α7 and non-α7 nAChRs

Figure 5.2.11: Pre-incubation of 10 μM sazetidine-A reduces α7 nAChR mediated intracellular calcium elevations in mouse primary cortical cultures.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU1 (10 μM; 3 min) before recording F340:F380 for 30 s prior to addition of PNU2 (3 μM; 20 s; a), followed by 3 min wash out. Sazetidine-A (10 μM) in combination with PNU1 (10 μM) were then pre-incubated for 10 min, after which F340:F380 was again recorded for 30 s before addition of PNU2 (3 μM; 20 s; b). Finally, after 10 min wash out and pre-incubation of PNU1 (10 μM; 3 min), F340:F380 was recorded for 30 s before a third co-application of PNU2 (3 μM; 20 s; c). Still images show fluorescence in pseudocolour, (black/blue = low F340:F380, red/white = high F340:F380) taken at peak response. Traces show coloured lines corresponding to 2 regions of interest indicated with coloured arrowheads in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm (F340:F380) from a representative experiment. Bars (d) represent mean peak F340:F380 increase from basal expressed as a percentage of the initial stimulation response from the same region of interest ± SEM from a single experiment.

162
5.2.5 Reactivation of desensitized nAChRs with PNU1 in mouse primary cortical cultures

Initially, cells were pre-incubated with PNU2 (3 μM) for 10 min, before co-applying PNU1 (10 μM) for 20 s, as was done above with agonist applications. We observed a

Figure 5.2.12: Positive allosteric modulator PNU1 re-activates α7 nAChRs desensitized by agonists PNU2 and sazetidine-A.

Cultures (10-14DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cells were pre-incubated with PNU2 (3 μM; a i) or sazetidine-A (10 μM; b i) for 10 min prior to addition of PNU1 (3 μM; 20 s; a ii; b ii). Still images show fluorescence in pseudocolour, (black/blue = low F\textsubscript{340:F380}, red/white = high F\textsubscript{340:F380}). Traces (a iii; b iii) show coloured lines corresponding to individual regions of interest indicated with coloured triangles in each image. Data are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm from a representative experiment. Bars (c) represent mean basal or peak F\textsubscript{340:F380} from 1 (PNU2; green bars) and 2 (sazetidine-A; blue bars; ± range) independent experiments.
slow increase in $F_{340}:F_{380}$ to 0.17 ± 0.60 above basal levels from a small proportion of cells that occurred after a ~20 s delay (Figure 5.2.12ai-iii, c) after PNU1 co-application. Pre-incubation of sazetidine-A (10 μM) followed by addition of PNU1 (10 μM) induced similar increases in $F_{340}:F_{380}$ in a subpopulation of cells (0.19 ± 0.12 above basal; Figure 5.2.12bi-iii, c) that were also delayed by approximately 20 s when compared with responses elicited by co-applying PNU2 (3 μM; Figure 5.2.10, Figure 5.2.11) or sazetidine-A (10 μM; Figure 5.2.6) after pre-incubation of PNU1 (10 μM; 3 min).

To summarize, we have examined two distinct facets of sazetidine-A’s activity with respect to α7 and non-α7 nAChRs, in fluo-3 AM loaded SH-SY5Y cell populations, and on an individual cell basis in mouse primary cortical cultures loaded with fura-2 AM. The final section of this chapter will describe experiments in which five novel compounds (Figure 5.2.13A-E) sharing structural similarities to sazetidine-A were characterized in the SH-SY5Y cell populations as above. Here, we aimed to evaluate any potential agonist or desensitizing activity of the compounds and their specificity to nAChRs.
5.2.6 Characterization of novel azetidine/pyrrolidine containing compounds

Sazetidine-A and related nAChR ligands such as A-85380 and its 5-iodo derivative share a common structural motif comprising a four membered, nitrogen-containing ring known as an azetidine group (Figure 5.1.1; Figure 5.2.13). Five membered nitrogen-containing rings or pyrrolidine groups (Figure 5.2.13) also feature prominently in nAChR ligands, examples including nicotine and the clinical candidate ABT-089 (Lin et al., 1997). Figure 5.2.13 displays the structures of sazetidine-A, four azetidine containing compounds (Figure 5.2.13 A-D) and a single pyrrolidine

![Chemical structures of azetidine, pyrrolidine, sazetidine-A and novel compounds A-E.](image)

Bn: Benzene; Ph: Phenol
derivative (Figure 5.2.13 E) that were examined with respect to their activity at nAChRs (Feula et al., 2010).

Firstly, competition-binding assays were undertaken using 200 nM [³H]epibatidine to label heteromeric nAChRs in rat brain membranes to evaluate the affinity of compounds A-E for the orthosteric ACh binding site of nAChRs (Figure 5.2.14). At 100 μM, none of the compounds affected the binding of [³H]epibatidine as measured by scintillation counting.

![Figure 5.2.14: Novel azetidine/pyrrolidine compounds A-E do not compete for binding with epibatidine](image)

nAChRs in rat brain membranes were labelled with 200 nM [³H]epibatidine in the presence or absence of increasing concentrations of nicotine of sazetidine-A, or compounds A-E (100 μM). These experiments were conducted by Sarah Foale using the method of Houghtling et al., (1995) as described in Whiteaker et al., (1998). Data were fitted to the Hill equation.

Compounds A-E (100 μM) were then applied to fluo-3 AM loaded SH-SY5Y cells to assess their potential agonist activity at nAChRs (Figure 5.2.15a). In stark contrast to nicotine (100 μM; 32.82 ± 6.00 %), PNU2 (3 μM) in combination with PNU1 (10 μM; 41.54 ± 9.31 %) and sazetidine-A (100 μM; 17.97 ± 14.02), little change in F<sub>538</sub> was observed in response to application of compounds A-E (2.73 ± 0.63 %, 3.26 ± 0.24 %, 2.98 ± 0.42 %, 4.29 ± 0.40 %, 3.01 ± 0.70 %). There was no significant difference between F<sub>538</sub> observed in response to compounds A-E, nor did they give significantly different responses from buffer (3.01 ± 0.71 %).
Chapter 5: Characterization of Sazetidine-A at α7 and non-α7 nAChRs

As described above in similar experiments with sazetidine-A, compounds A-E were then examined for their potential ability to antagonize nAChR responses (Figure 5.2.15b, c). Again we have used nicotine (100 μM) and PNU2 (3 μM) in combination with PNU1 (10 μM) to evoke α7 and non-α7 nAChR responses respectively. Pre-incubation with each of the compounds A-E (100 μM) for 10 min significantly inhibited nicotine (100 μM; fig. 5.2.14b) induced increases in F538 (88.43 ± 2.44 %, 60.37 ± 9.51 %, 87.30 ± 3.31 %, 80.12 ± 11.31 %, 89.29 ± 0.64 %), as did sazetidine-A (100 μM) and mecamylamine (100 μM).

Figure 5.2.15: Effects of novel azetidine/pyrrolidine containing compounds A-E on intracellular calcium elevations in SH-SY5Y cells.

SH-SY5Y cells were loaded with fluo-3 AM and stimulated with nicotine (100 μM; pink bars; a, b), PNU2 (3 μM; green bars; a, c) in the presence of PNU1 (10 μM), sazetidine-A (100 μM; dark blue bars; a) or compounds A-E (100 μM; light blue bars; a) for 20 s whilst monitoring fluorescence at 538 nm. Dashed bars (b, c, d) indicate 10 min pre-incubation of compounds A-E (100 μM), sazetidine-A (Saz; 100 μM) or mecamylamine (Mec; 100 μM) before stimulation. Data are presented as percent maximum fluorescence determined by addition of 0.2 % Triton X-100 minus minimum fluorescence quenched by 350 mM MnCl₂. Bars represent mean ± SEM of at least 3 independent experiments; * P < 0.05, ** P < 0.01, *** P < 0.001 significantly different from nicotine, PNU2 in combination with PNU1 or KCl alone, non-paired one-way ANOVA, Dunnett’s multiple comparisons test.
Increases in F$_{538}$ in response to application of PNU2 (3 μM) in combination with PNU1 (10 μM; Figure 5.2.15c) however were only significantly inhibited by 10 min pre-incubation of compounds C and D ($71.92 \pm 7.03\%$ and $83.66 \pm 0.96\%$), although a downward trend was also observed for compounds A, B and E ($22.33 \pm 11.10\%, 47.82 \pm 21.39\%, 18.54 \pm 5.70\%$).

As shown by our lab previously (Dajas-Bailador et al., 2002a), nAChR mediated $[\text{Ca}^{2+}]_{ic}$ elevations in SH-SY5Y cells are dependent on multiple secondary mechanisms such as the release of calcium from intracellular stores and via depolarization induced opening of voltage gated calcium channels (VGCCs). We hypothesized that compounds A-E could be interfering with such mechanisms rather than directly acting at nAChRs to inhibit $[\text{Ca}^{2+}]_{ic}$ elevations in SH-SY5Y cells. In order to evaluate the specificity of these effects with respect to nAChRs, we repeated the 10 min pre-incubation of compounds A-E, but this time stimulated cells with KCl (100 mM; Figure 5.2.15d), which depolarizes cells resulting in activation of VGCCs, raising $[\text{Ca}^{2+}]_{ic}$. Application of KCl (100 mM) alone to fluo-3 AM loaded SH-SY5Y cells evoked F$_{538}$ increases of $27.32 \pm 5.36\%$. Following 10 min pre-incubation, these responses were significantly inhibited by compounds D and E ($75.10 \pm 3.15\%, 83.74 \pm 3.17\%$), and a downward trend was observed for compound C ($46.33 \pm 26.35\%$). Compounds A, B and sazetidine-A had no significant effect on KCl evoked changes in F$_{538}$.

In this section, we have described experiments that aimed to characterize the novel azetidine/pyrrolidine containing compounds A-E. We extended these methods to examine potential agonist or antagonist effects of novel compounds, and finally to assess their specificity for nAChRs.
5.3 Discussion
In this chapter we have investigated the effects of the highly selective, stoichiometry dependent, nAChR desensitizing agonist sazetidine-A at α7 and non-α7 nAChRs using a high throughput fluo-3 AM based calcium fluorimetry assay in SH-SY5Y cell populations, and subsequently in finer detail with the aid of fura-2 AM live cell imaging in mouse primary cortical cultures to observe these effects in real time on an individual cell basis.

5.3.1 Summary
In SH-SY5Y cells, sazetidine-A alone produced concentration dependent increases in F$_{538}$ indicative of [Ca$^{2+}$]$_c$ elevations that were sensitive to blockade by the general nAChR antagonist mecamylamine, but unaffected by the presence of the α7 selective antagonist MLA, consistent with activation of heteromeric nAChRs. Pre-incubation of the α7 selective PAM PNU1 was seen to potentiate these responses in an MLA sensitive manner. Mouse primary cortical cultures inconsistently responded to sazetidine-A alone, an observation that contrasted with sazetidine-A’s effects in the presence PNU1 where it elicited consistent MLA-dependent responses in a small subpopulation of cells.

Using a 10 min sazetidine-A pre-incubation protocol we observed a concentration dependent reduction in F$_{538}$ increases evoked by nicotine or PNU2 in combination with PNU1, consistent with reports of sazetidine-A mediated nAChR desensitization (Xiao et al., 2006). This effect was replicated in mouse primary cortical cultures for responses evoked by PNU2 in the presence of PNU1. Application of PNU1 following PNU2 or sazetidine-A pre-incubation was seen to elevate F$_{340}$:F$_{380}$ in a manner consistent with a reversal of agonist driven α7 nAChR desensitization.

Finally, the effects of the azetidine or pyrrolidine containing compounds A-E were evaluated in SH-SY5Y cells. Here, no competition for the orthosteric nAChR binding site was observed, and no agonist activity was observed for any of the compounds, but they were all seen to negatively affect nicotine-evoked F$_{538}$ elevations following 10 min pre-incubation. Compounds C and D also seen to diminished F$_{538}$ increases evoked by PNU2 in the presence of PNU1. Lastly, in an
attempt to clarify nAChR specificity of these effects, we observed inhibition of KCl evoked $F_{538}$ elevations only by compounds D and E.

### 5.3.2 Sazetidine-A activates nAChRs in SH-SY5Y cells

Application of sazetidine-A alone to SH-SY5Y cells loaded with fluo-3 AM provoked rapid and prolonged increases in $F_{538}$, indicative of [$Ca^{2+}]_{lc}$ elevations that were sensitive to blockade by mecamylamine, implying activation of nAChRs. Examination of the concentration dependence of this effect yielded an $EC_{50}$ of 4.17 μM, which agrees with the values reported by Kozikowski et al. (3.4 μM; 2009), in the same cell line using the $^{86}Rb^+$ efflux assay, and by Zwart et al. (4.5 μM; 2008) in rat hippocampal slices measured by $[^3H]NA$ release, which is reputed to rely on activation of $\alpha3\beta4^*$ nAChRs (Luo et al., 1998; Azam and McIntosh, 2006; Kennett et al., 2012). Both groups ascribed the activating effects of sazetidine-A to $\alpha3\beta4^*$ nAChRs, although SH-SY5Y cells also express $\alpha3\beta2^*$ nAChRs which could well be contributing to these responses (Wang et al., 1996; Gerzanich et al., 1998; Ridley et al., 2001). Considering the greater affinity of sazetidine-A at $\beta2^*$ compared to $\beta4^*$ nAChRs reported in the literature (Xiao et al., 2006; Zwart et al., 2008; Kozikowski et al., 2009; Kuryatov and Lindstrom, 2011; Zhang et al., 2012; Liu et al., 2013), this seems highly probable, and could be confirmed by testing the sensitivity of these responses to $\alpha$CtxMII or $\alpha$CtxPnIA that preferentially block $\alpha3\beta2$ nAChRs (Cartier et al., 1996; Luo et al., 1999). The presence of $\alpha5$ nAChR subunits in SH-SY5Y cells could also influence the results of experiments, as their presence as an accessory subunit in nAChRs has been shown to enhance calcium permeability of $\alpha3^*$ nAChRs to levels comparable to that of $\alpha7$ nAChRs, but also increases the rate of their desensitization (Wang et al., 1996; Gerzanich et al., 1998).

Responses to sazetidine-A in SH-SY5Y cells were significantly potentiated by pre-incubation and co-application of the $\alpha7$-selective PAM PNU1 (10 μM) in an MLA (100 nM) sensitive manner, suggesting recruitment of $\alpha7$ nAChRs. Since conducting these experiments, sazetidine-A was reported to have full agonist efficacy at $\alpha7$ nAChRs and $EC_{50}$ and $IC_{50}$ values of 1.2 and 3.5 μM respectively (Campling et al., 2013). The inclusion of PNU1 in our experiments increased the potency for sazetidine-A to elicit [$Ca^{2+}]_{lc}$ elevations by a factor of ~10 (from 4.17 to 0.42 μM), similar to the level of potentiation seen with ACh (Hurst et al., 2005), and raised the
maximum response by ~48%. It should be noted that responses in the presence of PNU1 were highly variable (Figure 5.2.2) compared to sazetidine-A alone. There are reports of α7 nAChR expression in SH-SY5Y cells decreasing after larger passage numbers, which could be a possible reason for this. Nevertheless, replicates within experiments also exhibited a high degree of variability, which suggests that passage number may not be the cause, as these replicates would be from the same passage. We propose another reason: that α7 nAChRs are expressed by a subpopulation of SH-SY5Y cells, and the variability is an effect of measuring fluorescence changes in populations of cells in which α7 nAChRs are non-homogenously expressed in the region of measurement between replicates. To confirm this, immunofluorescence experiments with fluorescently tagged αBgt could be used to evaluate the uniformity of α7 nAChR expression in SH-SY5Y populations. Indeed, poor expression of α7 nAChRs has limited their study in cell lines until very recently, owing to the work of (Kuryatov et al., 2013), who were able to enhance expression by the use of chemical chaperones and RIC-3.

Combining PNU1 with other nAChR agonists such as nicotine or 5-iodo resulted in potentiation in the same range as that of sazetidine-A (4.00 ± 0.21, 2.35 ± 0.28 and 2.33 ± 0.72 fold respectively), which indicates similar levels of activity for these compounds at α7 nAChRs, albeit only in the presence of PNU1. The functional activity of sazetidine-A at α7 nAChRs without PNU1 would require higher resolution techniques such as patch-clamp electrophysiology or the fluorescent membrane potential assay employed by Campling et al., (2013) in cells in which α7 nAChRs were highly expressed.

5.3.3 PNU-120596 reveals sazetidine-A evoked calcium elevations in mouse primary cortical cultures

In mouse primary cortical cultures, application of sazetidine-A alone at concentrations of 10 nM, 100 nM and 1 μM had no effect on $F_{340}/F_{380}$, whereas 10 μM inconsistently elicited short bursts of activity following perfusion, which were blocked by pre-incubation and co-application of mecamylamine (30 μM) in one experiment. This could indicate activation of α4β2* nAChRs, although further repeated observations of this effect would be needed to make a reliable judgment. Neurons of the mouse cortex are known to express α4β2(α5) nAChRs (Gotti et al., 2006; Mao et al., 2008), so given the reported selectivity of sazetidine-A for α4β2
nAChRs (Xiao et al., 2006; Zwart et al., 2008; Kozikowski et al., 2009; Kuryatov and Lindstrom, 2011; Zhang et al., 2012; Liu et al., 2013), we would have expected it to elicit responses at much lower concentrations than were used in SH-SY5Y experiments above. It is possible however, that the LS-(α4)3(β2)2 stoichiometry and/or the α4β2α5 nAChR predominates in these cultures, at which sazetidine-A was reported to act as a very poor partial agonist (Zwart et al., 2008; Carbone et al., 2009; Zhang et al., 2012). This would explain the lack of responses to sazetidine-A alone, although as previously mentioned, further experiments would be needed to confirm that this is a reliable effect. A way to examine this hypothesis would be to evaluate the effects of α4β2 nAChR stoichiometry selective agonists in the cultures. The compounds TC-2559 and ABT-894 were reported to be more efficacious at LS-(α4)3(β2)2 than HS-(α4)2(β2)3 nAChRs (HS/LS: 357 %/28 % ; 120 %/50 % respectively; relative to ACh), which would make them suitable for addressing this question (Zwart et al., 2006; Zhang et al., 2012). Another possibility for the difference in responses to sazetidine-A alone between SH-SY5Y cells and mouse primary cortical cultures is the fact, mentioned above, that in contrast to mouse cortex, SH-SY5Y cells also express α3β2(α5) nAChRs, which are highly permeable to calcium and perhaps more sensitive to activation by sazetidine-A than LS-(α4)3(β2)2 nAChRs. Conversely, in the presence of PNU1 (10 μM), sazetidine-A consistently evoked comparatively robust and prolonged responses from a subpopulation of cells. These responses were completely blocked by 10 min pre-incubation and co-application of MLA (100 nM).

An interesting finding here was that after being blocked by MLA (100 nM), the individual cell responses to sazetidine-A (10 μM; 32.43 ± 9.42 % recovery) in combination with PNU1 (10 μM) did not recover as well as responses elicited by PNU2 (3 μM; 89.68 ± 22.73 % recovery; Figure 3.2.9) in combination with PNU1 (10 μM) This suggests that after 10 min washout, sazetidine-A is still bound to α7 nAChRs leaving them desensitized. This effect was first reported in the initial study by Xiao et al. (2006) for α4β2 nAChRs, which remained significantly desensitized after 1 h incubation in fresh media. Desensitization of nAChRs by sazetidine-A will be the subject of following sections.
5.3.4 Desensitization of α7 and non-α7 nAChRs by sazetidine-A in SH-SY5Y cells

Consistent with the reports of others (Xiao et al., 2006; Kozikowski et al., 2009; Zhang et al., 2012; Campling et al., 2013; Eaton et al., 2013; Wageman et al., 2013), our experiments have provided evidence for the desensitization effects of sazetidine-A at nAChRs. In SH-SY5Y cells, pre-incubation of sazetidine-A for 10 min concentration-dependently inhibited responses to nicotine (100 μM) and PNU2 (3 μM) in combination with PNU1 (10 μM), with IC50 values of 0.52 and 0.48 μM respectively. We attribute this to desensitization of α3β4(α5) and α3β2(α5) nAChRs, and α7 nAChRs respectively. These IC50 values are approximately 10 fold lower than the EC50 values discussed above, which confirms previous findings that reported sazetidine-A to be a more potent desensitizer than an agonist at nAChRs (Zhang et al., 2012; Campling et al., 2013; Eaton et al., 2013).

It was noticed in these experiments that during the pre-incubation phase, the combination of sazetidine-A and PNU1 would result in elevated basal F538 before agonist application had commenced, compared to sazetidine-A pre-incubation in the absence of PNU1 (Figure 5.2.8). We attribute this effect to the ability of PNU1 to reverse α7 nAChR desensitization and reveal agonist induced channel activation effects, therefore during the pre-incubation phase these receptors were being activated for prolonged periods of time, raising [Ca2+]c levels, reflected in the increased basal F538 values measured. This could have affected the ability of cells to respond again to subsequent agonist stimulation, either through depletion of internal Ca2+ stores or further receptor desensitization, possibly to a state that is not reversible by PNU1 (Williams et al., 2011b). This raises concerns about the reliability of measuring sazetidine-A’s desensitization effects at α7 nAChRs. Since α7 nAChRs desensitize so readily upon agonist stimulation, it is not possible to test its true effect without the use of higher resolution techniques such as electrophysiology. Here we use PNU1 to overcome this desensitization in order to amplify the effects of α7 nAChR stimulation to detectable levels in our assay. Thus, in reality the effect being examined here is more appropriately described as the propensity for sazetidine-A to desensitize α7 nAChRs in the presence of PNU1. Williams et al., (2011) described two differing states of α7 nAChR desensitization, which are distinguished by their reversibility by type II PAMs such as PNU1. The difficulty here is that sazetidine-A also appears to activate α7 nAChRs in the presence of PNU1, but perhaps with much
longer pre-incubation times α7 nAChRs would inactivate to the PAM-resistant state, which would then allow subsequent measurement of desensitization to further agonist stimulation. Such an effect is interesting and deserves further study. The next section will discuss our attempts to measure this effect in individual cells in primary cortical neurons.

5.3.5 Desensitization of α7 nAChRs in primary cortical cultures by sazetidine-A

It is interesting that the effects described in section 5.2.3 (Figure 5.2.8) for the elevated basal fluorescence in SH-SY5Y cells following 10 min pre-incubation of sazetidine-A in combination with PNU1 were not observed in primary cortical cultures. This could be due to differences in the model system being used. Here, pre-incubation of 500 nM sazetidine-A in the presence of 10 μM PNU1 for 10 min inhibited responses to subsequent perfusion of 3 μM PNU2 by 59.22 ± 12.40 %, which validates the IC50 value found for sazetidine-A as a desensitizer for α7 nAChRs in SH-SY5Y cells above (0.48 μM), and reassures us that our observations of rising basal F538 in these experiments did not hugely affect the examination of sazetidine-A’s potency as a desensitizer.

As described above for responses to sazetidine-A in the presence of PNU1 in cortical cultures (Figure 5.2.6), the recovery of the response to PNU2 (3 μM) in combination with PNU1 (10 μM), after blockade by sazetidine-A and following 10 min washout (57.38 ± 17.83 % recovery; Figure 5.2.11) was lower than that seen for antagonism by MLA (100 nM; 89.68 ± 22.73 %; Figure 3.2.9), again supporting the notion that sazetidine-A desensitizes α7 nAChRs for long periods of time. It would be interesting to test this effect for longer washout periods to ascertain a measure of its ultimate duration. This finding agrees with the study by of Xiao et al., (2006), who reported long lasting desensitization of α4β2 nAChRs by sazetidine-A hours after washout in HEK-293 cells. The long lasting effects of sazetidine-A are highly relevant to pre-clinical research, as it would imply that sazetidine-A’s effects could continue long after the drug had been cleared from the system in vivo, due to it being bound to and desensitizing nAChRs for long periods of time. Hussmann et al., (2012) reported that low micromolar concentrations of sazetidine-A are achievable in the rat brain following subcutaneous injection of 2.3 mg/kg. It has also been noted that α7 nAChR receptor populations exhibit a balance of desensitization and low-level activation.
dubbed “smoldering activation” in the presence of sazetidine-A (Campling et al., 2013), which could be achieved by low micromolar concentrations reported to be effective in our experiments. There is also a great deal of evidence for α7 nAChRs in the periphery, especially in cells of the immune system (Razani-Boroujerdi et al., 2007; Skok, 2009; Filippini et al., 2012). It could be envisaged that a compound like sazetidine-A, which remains bound to receptors for long periods of time, could affect their signaling.

5.3.6 Reactivation of desensitized α7 nAChRs by PNU1

We compared the ability of PNU1 to reverse desensitization by agonists PNU2 and sazetidine-A in mouse primary cortical neurons. This effect has been well documented for ACh and nicotine in electrophysiological studies (Hurst et al., 2005; Grønlien et al., 2007; Williams et al., 2011b). However, considering the reputation of sazetidine-A as a strongly desensitizing compound, we thought it an intriguing prospect to examine its reversibility relative to other agonists by PNU1. The live imaging system with primary cortical cultures allowed this to be done. We discovered that application of PNU1 following 10-min pre-treatment of both PNU2 and sazetidine-A resulted in responses of similar magnitude and duration, suggesting similar reactivation of α7 nAChRs desensitized by either PNU2 or sazetidine-A. These responses occurred after a ~20 s delay compared to responses evoked by PNU2 or sazetidine-A application following pre-incubation of PNU1, suggesting slower kinetics of reactivation by PNU1 compared to activation by PNU2 or sazetidine-A in the presence of PNU1. Further studies would be needed to evaluate the propensity of sazetidine-A to drive these receptors into the PAM resistant desensitized state described by Williams et al. (2011a, 2011b) in X. laevis oocytes.

This alludes to an interesting prospect that was touched upon in recent publications (Harpsøe et al., 2011; Mazzaferro et al., 2011; Benallegue et al., 2013; Eaton et al., 2013) describing the α4(+)/(-)β2 binding sites in both the LS-α4β2 and HS-α4β4 nAChR stoichiometries contributed to HS phase responses that were identical in magnitude per-receptor, but the α4(+)/(-)α4 binding site in the LS-α4β2 stoichiometry could act to boost this effect. Furthermore, following desensitization pre-treatment with sazetidine-A, acute agonist application could reactivate
desensitized LS-α4β22, similar to the effect we describe for desensitized α7 nAChRs with PNU1 (Eaton et al., 2013).

5.3.7 Characterization of novel azetidine/pyrrolidine containing compounds

We evaluated the potential activities of several novel compounds (Figure 5.2.13; A-E) containing structurally similar motifs to sazetidine-A. None of the compounds affected the binding of [3H]epibatidine to rat brain membranes, suggesting that they do not bind to the orthosteric ACh binding site of nAChRs, whereas sazetidine-A dose dependently competed for binding. At first glance the chemical structures of these compounds possess two essential components of the nAChR pharmacophore that are shared by compounds like nicotine, epibatidine and sazetidine-A – a charged group, i.e. a protonatable nitrogen atom that forms a π-cation interaction with a tryptophan residue on the nAChR; and a hydrogen bond acceptor, i.e. the lone electron pair present on the pyridine nitrogen atom which is reputed to co-ordinate a water molecule, forming a bridge to the complimentary nAChR subunit (Abreo et al., 1996; Celie et al., 2004; Dallanoce et al., 2013). Closer scrutiny of the geometric arrangement of these ingredients in compounds A-E however, allows further insights to emerge that may help to explain their very low affinity observed for the nAChR binding site. The distance between the two nitrogen atoms, or internitrogen (N-N) distance for optimum affinity is espoused to be 4.6 Å (Abreo et al., 1996). Compared to sazetidine-A, nicotine or epibatidine, the protonatable azetidine nitrogen is two chemical bonds closer to the pyridine nitrogen on compounds A-E, which could put it out of the optimum N-N distance, and also restrict bond rotation. Another factor that could further obstruct binding for compounds A-E is the benzene ring bound to the protonatable nitrogen atom, which adds considerable steric hindrance effects, further constraining positioning of the pharmacophore.

Interaction with α7 binding sites was not carried out due to lack of availability of radioligand, moreover, it could be possible that compounds A-E affect different sites on nAChRs, or somehow interfere with nAChR function. We therefore used functional Ca²⁺ assays as above to evaluate the potential activities of compounds A-E in SH-SY5Y cells. The failure of compounds A-E is consistent with their inability to compete for binding to heteromeric nAChRs and indicate no activity from other mediators of Ca²⁺ elevations such as GPCRs.
Following pre-incubation as for sazetidine-A above, compounds A-E (100 μM) significantly inhibited subsequent responses to nicotine, whereas only compounds D and E significantly blocked responses to PNU2 in the presence of PNU1. This could suggest that compounds A-E non-competitively block α3β2(α5) or α3β4(α5) nAChRs, and compounds D and E also block α7 nAChRs. There is however a possibility that these effects are not due to specific interactions with nAChRs, but are achieved by a non-specific mechanism that inhibits [Ca^{2+}]_c elevations. At the high concentrations tested (100 μM), this could be due to open channel block of nAChRs, or VGCCs which have been shown to mediate nAChR induced [Ca^{2+}]_c elevations in SH-SY5Y cells (Dajas-Bailador et al., 2002a). We evaluated the latter by challenging responses to acutely applied KCl (100 mM), which elevates [Ca^{2+}]_c via recruitment of VGCCs, and found evidence that the actions observed for compounds D and E were indeed dependent on this mechanism, whereas compounds A-C had no significant effect. Further studies using lower concentrations of compounds A-C were not deemed useful. Taken together, these results do not suggest any obvious potential for compounds A-E with respect to nAChRs.

### 5.3.8 Conclusions

The experiments described in this chapter aimed to characterize the activity of sazetidine-A at α7 and non-α7 nAChRs. We assessed functional activation of nAChRs by the use of fluorescent calcium binding dyes, and conclude that sazetidine-A both activates and desensitizes nAChRs in SH-SY5Y cells and mouse primary cortical cultures. We attribute these effects to α3β2(α5) and α3β4(α5) nAChRs in the former. In both preparations, PNU1 either potentiated or revealed agonist effects of sazetidine-A, indicating activity at α7 nAChRs. Desensitization of α7 nAChR mediated responses was also seen, and appeared to be long lasting. This desensitization was analogous to that produced by the α7 agonist PNU2, as it was reversed by subsequent application of PNU1.

The clinical implications of these findings may be minor, but it is possible that sazetidine-A could enter the brain at low micromolar concentrations and therefore affect central α7 nAChRs (Hussmann et al., 2012). There is also the possibility of affecting peripheral α7 nAChRs, such as those present on immune cells that mediate signal-transduction pathways involved in the anti-inflammatory or oncogenic effects.
of α7 nAChR ligands possibly independently of ion channel function (Razani-Boroujerdi et al., 2007; Skok, 2009; Thomsen and Mikkelsen, 2012b).

This work adds to the growing body of literature that has seen divergence of the originally reported claims of sazetidine-A as an α4β2 nAChR selective “silent desensitizer”. However it may be that in vivo, during prolonged exposure, the net activity of sazetidine-A is one of physiological antagonism of nAChRs resulting from its long lasting desensitization effects, as has been suggested (Levin et al., 2013a, 2013b).
Chapter 6: General Discussion & Conclusions
6.1 Conclusions

This thesis presents investigations that aimed to define the mechanisms of α7 nAChR-mediated Ca\(^{2+}\) elevations in primary cultured cells from the mouse cerebral cortex using selective pharmacological agents. α7 nAChRs are characterized by their high relative Ca\(^{2+}\) permeability (Fucile et al., 2003), sensitivity to blockade by αbgt (Zhang et al., 1994), and rapid activation-desensitization kinetics (Fenster et al., 1997). They are widely expressed in the mammalian brain, where they mediate Ca\(^{2+}\) dependent signalling pathways that modulate neurotransmitter release, synaptic plasticity, gene transcription, and neuroprotection (Dajas-Bailador and Wonnacott, 2004). As such, they are thought to play a role in cognitive processes such as working memory and attention, and are implicated in diseases that manifest cognitive deficits such as schizophrenia and Alzheimer’s disease (Bencherif and Lippiello, 2010; Hurst et al., 2013; Wallace and Bertrand, 2013b).

Fluorimetric Ca\(^{2+}\) assays and live imaging were employed to study α7 nAChR-mediated Ca\(^{2+}\) signalling processes, and also to characterize the actions of the desensitizing agonist and prototypical smoking-cessation drug, sazetidine-A at native α7 nAChRs, showing that the sensitivity of this subtype to sazetidine-A could contribute to its behavioural effects or possibly mediate side-effects. In addition, a novel HCS Ca\(^{2+}\) imaging and analysis assay that can be combined with immunofluorescent labelling to add further insight to Ca\(^{2+}\) signalling mechanisms in different cell types from large populations was optimized.

6.1.1 Summary of α7 nAChR-mediated Ca\(^{2+}\) signalling mechanisms in primary cortical cultures

These experiments provide evidence for the involvement of multiple secondary Ca\(^{2+}\) amplification mechanisms including the activation of VGCCs and CICR via RyRs and/or IP\(_3\)Rs, summarized in Figure 6.1.1a. They also demonstrate that α7 nAChRs in primary cortical cultures raise Ca\(^{2+}\) by promoting glutamatergic transmission, implying the existence of both pre- and post-synaptic α7 nAChRs. As shown in Figure 6.1.1b, these
Figure 6.1.1 Summary of α7 nAChR-mediated Ca²⁺ elevation mechanisms in mouse primary cortical cultures.

(a) α7 nAChR activation with selective compounds PNU1 and PNU2 resulted in increased fura-2 mediated F₃₄₀/F₃₈₀ increases indicative of [Ca²⁺]ᵢ elevations that were blocked by MLA. Treatment with Cd²⁺, Ry and XeC significantly inhibited these responses, indicating coupling of α7 nAChRs to VGCCs, RyRs and IP₃Rs respectively. Sazetidine-A in the presence of PNU1 also evoked [Ca²⁺]ᵢ elevations that were blocked by MLA. (b) Blocking AMPA and NMDA receptors with CNQX and MK801 respectively also diminished α7 nAChR-mediated Ca²⁺ elevations, indicating the involvement glutamatergic neurotransmission.
processes should be interdependent, and further studies could evaluate the effects of blocking multiple routes of Ca\(^{2+}\) elevation simultaneously (see section 6.2).

Exploratory experiments using a HCS Ca\(^{2+}\) imaging assay combined with immunofluorescent labelling provide evidence for α7 nAChR-mediated Ca\(^{2+}\) elevations occurring in astrocytes and neuronal cells, a subpopulation of which appear to be GABAergic. Figure 6.1.2 shows the possible consequences of presynaptic α7 nAChR activation in synaptically connected cells in a population consisting of glutamatergic and GABAergic neurons. In this simplified two-cell model,

![Diagram](image)

**Figure 6.1.2: Summary of possible consequences following activation of presynaptic α7 nAChRs in primary cortical cultures.**

Activation of presynaptic α7 nAChRs expressed on glutamatergic (i, ii) and GABAergic (iii, iv) neurons in primary cortical cultures could facilitate release of glutamate (green) or GABA (blue), having excitatory (+) effects via iGluRs, or inhibitory effects (−) via GABA\(_R\)s on postsynaptic cells respectively. Dotted lines indicate action potential inhibition by TTX, isolating synapses from axons. Depending on the combination of cells types at a synapse, the net effects possible in a two-cell model are (i) Activation of excitation – net activation, (ii) Activation of inhibition – net inhibition, (iii) inhibition of inhibition – net activation (in the presence of endogenous excitatory activity), and (iv) inhibition of excitation – net inhibition.
four outcomes are possible, resulting in either net excitation or inhibition depending on the types of cells synaptically connected. In the intact brain, there is evidence for inhibitory GABAergic interneurons in the PFC, where nAChRs modulate activity in a layer-specific manner (Couey et al., 2007; Poorthuis et al., 2012).

### 6.1.2 Comparison of methods used to measure α7 nAChR mediated Ca\(^{2+}\) signalling in this thesis

Three different methods were used to measure \([\mathrm{Ca}^{2+}]_{\mathrm{ic}}\) elevations: (i) fluorescent plate reader based \(\mathrm{Ca}^{2+}\) assays, (ii) live cell \(\mathrm{Ca}^{2+}\) imaging, and (iii) HCS \(\mathrm{Ca}^{2+}\) imaging assays. Table 6.1.1 summarizes and compares the different attributes of these methods.

**Table 6.1.1: Comparison of methods used to measure intracellular \(\mathrm{Ca}^{2+}\) elevations in this thesis.**

<table>
<thead>
<tr>
<th></th>
<th>Fluorescent plate-reader</th>
<th>Live imaging</th>
<th>High content screening</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluorophore</strong></td>
<td></td>
<td></td>
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<tr>
<td>Fluo-3; only single excitation recording available.</td>
<td></td>
<td></td>
<td>Fluo-3; only single excitation recording available.</td>
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<tr>
<td><strong>Cells</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cells must be confluent for reliable readings; limited to cell lines.</td>
<td>Can image primary cultures or cell lines.</td>
<td>Can image primary cultures or cell lines.</td>
<td></td>
</tr>
<tr>
<td><strong>Throughput</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>High – 96-well plate completed in ~ 1 h.</td>
<td>Low – one coverslip per hour.</td>
<td>High – 24-well plate format imaged in ~30 min.</td>
<td></td>
</tr>
<tr>
<td><strong>Imaging</strong></td>
<td></td>
<td></td>
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<tr>
<td>No imaging capabilities, records fluorescence from centre of well in real-time.</td>
<td>Single cell imaging in real-time from 40X objective, manual stage control.</td>
<td>Single cell imaging from prescribed fields of view; 10X, 20X and 40X objectives available, time-points limited by ex/em switching and liquid handing operations.</td>
<td></td>
</tr>
<tr>
<td><strong>Drug application</strong></td>
<td>Automatic drug dispensing, no perfusion system. ~100 μl/well</td>
<td>Perfusion system enables sequential drug application and imaging of same cells. Large volumes (&gt;20 ml) needed.</td>
<td>Automatic drug dispensing, no perfusion system. ~2ml/well.</td>
</tr>
<tr>
<td><strong>Environmental control</strong></td>
<td>Temperature control available, not used in</td>
<td>Temperature control integrated into perfusion</td>
<td>Environmental control possible but reduces image</td>
</tr>
</tbody>
</table>
### Chapter 6: General Discussion & Conclusions

<table>
<thead>
<tr>
<th>Feature</th>
<th>Assay due to time constraints.</th>
<th>System and cell chamber.</th>
<th>Acquisition speed considerably.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segmentation</td>
<td>Not applicable.</td>
<td>Manual selection of ROIs – Intuitive and flexible, but user biased.</td>
<td>Fully automated image segmentation, processing and analysis. Reduced bias, increased throughput. Complex morphologies present a challenge for segmentation algorithms.</td>
</tr>
</tbody>
</table>

| Data analysis and manipulation | Data output easily manipulated in spreadsheet format. | Manageable data set sizes, requires some advanced manipulation. | Vast data sets, requires more specialist skills and more powerful computer processor to manipulate. |

| Data storage                   | Very little data storage capacity needed, <100 kb per experiment | Large data storage capacity needed for movies. <1 Gb per experiment | Very large data storage capacity essential. >5 Gb per experiment |

| Miscellaneous                  | Can be combined with immunofluorescent labelling. |

| Findings, with relevance to α7 nAChRs | Sazetidine-A activates and desensitizes native α7 nAChRs at low μM concentrations in SH-SY5Y cells. | α7 nAChR-mediated Ca\textsuperscript{2+} elevations in primary cortical cultures are coupled to multiple intracellular mechanisms, and promote glutamatergic neurotransmission. | α7 nAChR mediated Ca\textsuperscript{2+} elevations occur in astrocytes, and glutamatergic and GABAergic neurons. |

It is important to note the difference between fluorophores used to report changes in [Ca\textsuperscript{2+}]\textsubscript{c} in these experiments, and to highlight their strengths and weaknesses that make them appropriate for certain applications. Fura-2 is well suited to measuring kinetic properties of Ca\textsuperscript{2+} waves as in live imaging experiments due to its relatively fast $K_{\text{off}}$ for Ca\textsuperscript{2+} (Dissociation rate; 23 s\textsuperscript{-1}; Paredes et al., 2008) compared to single-wavelength indicators like fluo-3 ($K_{\text{off}} =$ 90 s\textsuperscript{-1}; Zou et al., 2004). Fura-2 is also more well-suited for measuring small changes in resting [Ca\textsuperscript{2+}]\textsubscript{c} because of its relatively low $K_{d}$ for Ca\textsuperscript{2+} (140 nM; Gryniewicz et al., 1985) compared to fluo-3 ($K_{d} = 400$ nM; Minta et al., 1989). For our experiments, it was more important to measure
[Ca\(^{2+}\)]_c changes to sequential drug applications in individual cells, and analysis of the kinetic properties of these [Ca\(^{2+}\)]_c elevations was not an important experimental aim per se. We could therefore have used single-wavelength indicators in place of fura-2 in these experiments without changing the experimental design significantly. There are other advantages of using ratiometric Ca\(^{2+}\) indicators like fura-2 however, such as the minimization of technical issues including uneven loading or leakage of the indicator and photobleaching (Paredes et al., 2008). The main reasons for choosing these different fluorophores here were based on the imaging equipment available. Improved single-wavelength indicators are available such as fluo-4, which has increased brightness and faster loading compared to fluo-3 (Paredes et al., 2008), and could have been used in the plate reader or HCS experiments.

In conclusion, the complimentary attributes of these methods allow different scientific questions to be investigated, and together they can each provide an interlocking piece of the larger puzzle. By considering the advantages and limitations of these methods when formulating experimental aims, they can be used to their potential whilst minimizing their hindrances. Higher-throughput techniques are advantageous when screening multiple conditions in a short time, but lack the flexibility of more labour-intensive techniques that permit higher-resolution. By combining the two approaches, a complementarity can be achieved, like using a course- and fine-toothed comb. Certain aspects of more advanced HCS technology are an exception to the compromise between resolution and throughput, as illustrated by the combination of Ca\(^{2+}\) imaging with immunofluorescent labelling explored in chapter 4, which gives more detail to each experiment. As HCS technology advances, it can be envisaged that this dichotomy is blurred even further.

**6.2 Future Perspectives**

This section will describe further studies and alternative approaches that could be used to investigate \(\alpha7\) nAChR-mediated Ca\(^{2+}\) signalling mechanisms. A long list of potential experiments to follow nature down the rabbit-hole can be imagined; in practice however, these ideas are limited by the experimental techniques available.
6.2.1 Further investigations of α7 nAChR-mediated Ca\textsuperscript{2+} signalling in cortical cultures

The results of experiments described in chapter 3 support the premise that multiple mechanisms play a role in α7 nAChR-mediated Ca\textsuperscript{2+} elevations. However, in a complex physiological system such as primary cortical cultures it may not be the case that all of these mechanisms occur simultaneously in the same location or for the same function. This is reflected by our results, in which a variable degree of antagonism of α7 nAChR-mediated Ca\textsuperscript{2+} elevations is seen after blocking these mechanisms with selective inhibitors (Figure 6.1.1). By combining antagonists for each of the possible Ca\textsuperscript{2+} amplification mechanisms, the contribution of individual mechanisms could be evaluated alone.

Contribution of different VGCC-subtypes merits further investigation, as although Cd\textsuperscript{2+} significantly blocked α7 nAChR-mediated Ca\textsuperscript{2+} elevations, using nifedipine to block L-type VGCCs did not achieve a clear effect. This suggests involvement of other subtypes such as N-, P/Q-, R-, and T- type VGCCs, which could be investigated by using selective toxins (see section 1.2.2.4). The large drug volumes required in live-imaging experiments precluded this possibility due to the expense of these toxins, but by using the HCS Ca\textsuperscript{2+} imaging assay described in chapter 4 this would be much more feasible. An alternative to the pharmacological approach used in this thesis to investigate the involvement of various cellular actors in α7 nAChR-mediated Ca\textsuperscript{2+} elevations would be to use RNA interference (RNAi) to inhibit transcription of candidate genes or “gene knockdown” in primary cortical cultures (Davidson et al., 2004). It should be noted however, that this approach does not remove candidate proteins 100 %, but could serve as a useful – and in some cases more specific – comparison.

To expand the findings of experiments that provided evidence for the involvement of glutamatergic neurotransmission in α7 nAChR-mediated Ca\textsuperscript{2+} elevations (Figure 6.1.1b), the postsynaptic excitatory effects of glutamate release could be evaluated by the use of 4-aminopyridine to stimulate synaptic vesicle exocytosis in the presence of bicuculline to block postsynaptic inhibitory effects of GABA. This would allow quantification of excitatory synaptic effects in the cultures, and also allow the comparison of secondary Ca\textsuperscript{2+} amplification mechanisms recruited by iGluRs to those recruited by α7 nAChRs. Conversely, neurons could be injected
with tetanus toxin to block glutamate release (Gomez-Varela and Berg, 2013), allowing postsynaptic α7 nAChR-mediated Ca$^{2+}$ signalling mechanisms to be observed without the interference from iGluR-mediated mechanisms. This approach is technically challenging, labour intensive and low-throughput to the point of a few cells per experiment. Following on with glutamatergic mechanisms, our experiments did not provide significant evidence for the involvement of mGluR$_1$ in α7 nAChR-mediated Ca$^{2+}$ elevations in primary cortical cultures. It would be helpful to ascertain whether activation of these receptors by selective agonists results in any measurable Ca$^{2+}$ responses, as it may be that these receptors are expressed at a low level, as reported by Ayala et al. (2012).

The influence of GABARs on α7 nAChR-mediated Ca$^{2+}$ elevations deserves more attention, as α7 nAChRs are known to reside on GABAergic terminals, acting to facilitate the release of GABA (Alkondon et al., 1997a, 1999; Zappettini et al., 2011a; Poorthuis and Mansvelder, 2013). The majority of research in this area is focused on the hippocampus, probably due to the higher expression of α7 nAChRs in this brain region (Barrantes et al., 1995b), and therefore the greater likelihood of achieving robust and reliable observations. In our experiments, immunofluorescent staining for GAD revealed the presence of GABAergic cell bodies and punctate objects suggestive of pre-synaptic terminals in primary cortical cultures. Co-labelling of GAD with Alexa Fluor-conjugated abgt could strengthen evidence for α7 nAChRs existing at GABAergic nerve terminals. Our experiments provided some evidence for this, as the pre-incubation and co-application of bicuculline potentiated α7 nAChR responses in some HCS experiments.

Looking slightly more broadly at the possibilities for future work in this area, different methodological approaches should be considered to measure [Ca$^{2+}$]$_c$. As HCS experiments indicate the presence of different cell types, another approach to measuring cellular Ca$^{2+}$ more specifically in certain cell types could be appropriate. A way of doing this would be use genetically encoded Ca$^{2+}$ indicators (GECIs) to target particular cell types. This has been achieved using a specific promoter to amplify expression of the GECI G-CaMP2 in interneurons, but not Purkinje cells in mouse cerebellum (Kuhn et al., 2012). This alternative may reduce the ambiguities present in fluorescent co-localization methods used in chapter 4, as the Ca$^{2+}$ signal would only be coming from cells expressing the GECI, and therefore more specific. These
indicators do however have inherent difficulties, such as the need for viral transfection of cells, compared to the straightforward passive loading of AM tagged chemical Ca\textsuperscript{2+} indicators.

6.2.2 Higher content

HCS analysis raises the possibilities of data extraction astronomically, and experiments described in chapter 4 have only scratched the surface. As well as automating the analysis of cytosolic Ca\textsuperscript{2+} imaging assays as described in chapter 4, this technology enables many other avenues to be explored. Higher magnification microscopy could enable imaging and automated segmentation of more local Ca\textsuperscript{2+} events such as those occurring in neurites and subcellular compartments, aided by the ability to combine immunofluorescently labelled images. Distinguishing the effects of pre- and post-synaptic \textalpha 7 nAChRs is a difficult obstacle to overcome, but with the HCS Ca\textsuperscript{2+} imaging assay this could be achieved using higher magnification imaging in combination with post-assay immunfluorescent labelling for pre-synaptic markers such as synaptophysin, or post-synaptic markers such as PSD-95.

Further analysis of the glial composition of these cultures also warrants investigation, as does their effect on \textalpha 7 nAChR-mediated signalling. The presence of other glial cells such as microglia was not evaluated here, but could be observed by immunofluorescent labelling with antibodies for cluster of differentiation molecule 11b (CD11b; Perego et al., 2013). Another interesting approach would be to grow pure astrocyte or microglial cultures to examine \textalpha 7 nAChR-mediated Ca\textsuperscript{2+} signalling mechanisms in these cell types.

Other variables to be considered in these cultures include the developmental state of cells in culture, and their plating density. A method for culturing hippocampal neurons at low density was described by Kaech and Banker, (2006), which relies on the use of coverslips to sandwich low density neurons on to an astrocyte feeder layer, which is more appropriate for live cell imaging experiments which utilizes a coverslip chamber. This approach would allow more reliable quantitation of single cell observations, although it should be noted the level of cell density in a fundamentally interconnected physiological system could change its behaviour.

An obvious partner to the experiments covered in this thesis would be to also assess the mechanisms by which other nAChRs such as \textalpha 4\beta 2* subtypes raise Ca\textsuperscript{2+} in
primary cortical cultures as a comparison. Previous studies in this lab suggest distinct Ca\(^{2+}\) coupling mechanisms for α7 and β2* nAChRs in PFC synaptosomes in the context of facilitated [\(^{3}H\)]D-aspartate release (Dickinson et al., 2008). Within the timeframe here it was only possible to concentrate on α7 nAChRs, but given the progression seen in HCS Ca\(^{2+}\) assay development this could be assessed in parallel in future studies.

Despite intense research for the past half-century, very few drugs targeting nAChRs are currently marketed, the exception being the smoking cessation agent varenicline. Although recent years have seen an ever-growing portfolio of nAChR ligands with diverse effects on receptor function and more desirable selectivity profiles, the tight therapeutic window required for these ligands to exert significant effects in the CNS without causing unacceptable side effects remains the main difficulty. Other limiting factors such as the complexity and contradictory nature of ligand-receptor interactions (i.e. desensitisation), diverse subunit composition giving possible undefined targets, and spatio-temporal considerations are often the reasons for poor performance in the clinic (Hurst et al., 2013). As this thesis shows however, ligands that fail in the clinic can often provide useful research tools such as PNU1 and sazetidine-A, whose insights may at some point aid future drug discovery efforts. The obstacles to developing nAChR drugs may be daunting in their numbers, but an optimistic perspective would allow that further research might eventually grant the necessary foundations for effective therapeutics.

In the quest to treat some of the most peculiar and debilitating illnesses that warp and destroy the very essence of what it is to be human, any advances in our understanding of the enigmatic workings of the brain are welcome. The findings presented here are relevant to understanding the pathology and potential treatment of Alzheimer’s disease (Bencherif and Lippiello, 2010), schizophrenia (Young and Geyer, 2013) and addiction (Levin et al., 2010) to name a few. In addition, the analytical power of the HCS assay developed here may well contribute to these endeavours.

Drawing to a close, the almost fractal expansion of concentric levels of interaction emanating from the activation of a relatively scarce and extraordinary receptor leaves one awestruck by the complexity and convoluted structure of nature. The α7
nAChR is a spectacular structure in itself, but is a mere speck within a vast interconnected web of interaction in the microscopic world.

The End
Bibliography


Eaton, J.B., Lucero, L.M., Stratton, H., Chang, Y., Cooper, J.F., Lindstrom, J.M., et al. (2013). The unique α4(+)/(-)α4 agonist binding site in (α4)3(β)2 subtype nicotinic acetylcholine receptors permits differential agonist desensitization pharmacology vs. the (α4)2(β2)3 subtype. J. Pharmacol. Exp. Ther. 4:


with antidepressant, neuroprotective and long-lasting cognitive effects. CNS Drug Rev. 10: 147–66.


Henderson, B.J., González-Cestari, T.F., Yi, B., Pavlovicz, R.E., Boyd, R.T., Li, C., et al. (2012). Defining the putative inhibitory site for a selective negative allosteric


Kurokawa, K., Mizuno, K., and Ohkuma, S. (2012). Increase of ryanodine receptors by dopamine D1 receptors is negatively regulated by γ-aminobutyric acid type B


Langley, J.N. (1905). On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curari. J. Physiol. 33: 374–413.


metabotropic glutamate receptors, in primary rat cortical cultures. Brain Res. 782: 91–104.


Sazetidine-A Activates and Desensitizes Native \( \alpha 7 \) Nicotinic Acetylcholine Receptors

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Abstract The aim of this study was to investigate the ability of sazetidine-A, a novel partial agonist at \( \alpha 4\beta 2 \) nicotinic acetylcholine receptors (nAChRs), to affect the function of native \( \alpha 7 \) nAChRs in SH-SY5Y cells and primary cortical cultures. The \( \alpha 7\)-selective positive allosteric modulator PNU-120596 was used to reveal receptor activation, measured as an increase in intracellular calcium using fluorescent indicators. In the absence of PNU-120596, sazetidine-A elicited mecamylamine-sensitive increases in fluorescence in SH-SY5Y cells (EC\(_{50}\) 4.2 \( \mu M \)) but no responses from primary cortical neurons. In the presence on PNU-120596, an additional response to sazetidine-A was observed in SH-SY5Y cells (EC\(_{50}\) 0.4 \( \mu M \)) and robust responses were recorded in 14 % of cortical neurons. These PNU-120596-dependent responses were blocked by methyllycaconitine, consistent with the activation of \( \alpha 7 \) nAChRs. Preincubation with sazetidine-A concentration-dependently attenuated subsequent responses to the \( \alpha 7\)-selective agonist PNU-282987 in SH-SY5Y cells (IC\(_{50}\) 476 nM) and cortical cultures. These findings support the ability of sazetidine-A to interact with \( \alpha 7 \) nAChRs, which may contribute to sazetidine-A’s actions in complex physiological systems.

Keywords Live cell calcium imaging · SH-SY5Y cells · Primary cortical neurons · PNU-120596

Sazetidine-A (6-[5-[(2S)-2-azetidinylmethoxy]-3-pyridinyl]-5-hexyn-1-ol) has the profile of a potent partial agonist at \( \alpha 4\beta 2 \) nicotinic acetylcholine receptors (nAChRs) and has attracted interest as a lead compound for several therapeutic targets, making detailed knowledge of its wider activity an important consideration. Sazetidine-A is a derivative of the nicotinic agonist A-85380, developed to bear a long side chain for potential attachment of fluorescent or photoaffinity probes [1]. In binding assays it showed improved selectivity for \( \alpha 4\beta 2 \) nAChRs, compared with A-85380, with \( K_i \) values of 0.4 and 0.6 nM for rat and human \( \alpha 4\beta 2 \) nAChRs respectively.

In contrast to the parent molecule, sazetidine-A appeared to be devoid of agonist activity at recombinant human \( \alpha 4\beta 2 \) nAChRs and was described as a ‘silent desensitizer’ [1]. Subsequent studies revealed sazetidine-A to be a stoichiometry-dependent agonist capable of fully activating high sensitivity human \( \alpha 4\beta 2 \) (HS-\( \alpha 4\beta 2 \)) nAChRs, whereas it had negligible efficacy, relative to acetylcholine, at lower sensitivity \( \alpha 4\beta 2 \) (LS-\( \alpha 4\beta 2 \)) nAChRs [2, 3]. Presumably the stable cell lines employed in the original study predominantly expressed the low sensitivity form. The ‘accessory’ subunit occupying the fifth position in the pentameric nAChR, a position that does not directly contribute to either of the two high affinity, orthosteric agonist binding sites, nevertheless can influence receptor properties [4]. In this case the subunit occupying this position appears to determine the ability of sazetidine-A to interact with \( \alpha 7 \) nAChRs, which may contribute to sazetidine-A’s actions in complex physiological systems.

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value ~ 30 nM), compared with the rapid recovery following preincubation with nicotine [1]. More recent studies that have taken into account its differential interaction with the two stoichiometries of α4β2 nAChRs have shown that sazetidine-A selectively desensitizes HS-α4β2,3 nAChRs over LS-α4β2 [7, 8]. Its partial agonist profile has raised interest in sazetidine-A as a therapeutic lead for numerous indications, including drug dependence [9, 10], cognitive and attentional deficits [11], pain [12] and depression [13]. Given the complex contributions of multiple nAChRs in the CNS to brain function and behaviours, it is important to understand the specificity of agents such as sazetidine-A. It binds with much lower affinity but acts as a full or sub-stantial agonist at recombinant α4β4, α3β4, and α6β2αnAChRs [1, 6, 7]. Sazetidine-A’s activation of α7 nAChRs has only recently been documented, with very different EC_{50} values and efficacies at human (1.2 μM; 100 %) and rat (60 μM; 6 %) α7 nAChRs [7, 14]. The contribution of α7 nAChRs to the clinical targets mentioned above [15, 16], as well as α7 nAChRs credited with functions in peripheral systems that could mediate side-effects [17] suggest that this nAChR subtype, in particular, merits further attention. Hitherto, sazetidine-A’s activation of native α7 nAChRs has not been reported. In this study we examined the ability of sazetidine-A to activate and desensitize α7 nAChRs in SH-SY5Y cells and primary cortical neurons.

Materials and Methods

Materials

Triton X-100, (-)-nicotine hydrogen tartrate and mecanylamine hydrochloride, were purchased from Sigma-Aldrich (Poole, Dorset, UK); B27, l-glutamine, antibiotics, fluo-3 AM, fura-2 AM, and pluronic f127 were obtained from Life Technologies (Paisley, UK); sazetidine-A dihydrochloride, tetrodotoxin citrate, methyllycaconitine citrate and 5-iodo-A85380 dihydrochloride were purchased from Tocris Bioscience (Avonmouth, UK); PNU-120596 and 5-iodo-A85380 dihydrochloride were purchased from Aldrich (Poole, Dorset, UK); B27, Triton X-100, (-)-nicotine hydrogen tartrate and mecalazine, were obtained from Sigma-Aldrich (Poole, Dorset, UK); B27, L-glutamine, antibiotics, fluo-3 AM, fura-2 AM, and pluronic F127 were purchased by Pfizer Inc. USA; general reagents were purchased from Fisher Scientific (Loughborough, UK).

Methods

SH-SY5Y Cell Culture

Human neuroblastoma SH-SY5Y cells (ECACC, Salisbury, UK; passages 16–27) were cultured as previously described [18]. In brief, cultures were maintained in Advanced Dulbecco’s modified Eagle’s media (DMEM/F12), supplemented with 2 % fetal bovine serum (FBS), 2 mM l-glutamine, 190 U/ml penicillin and 0.2 mg/ml of streptomycin in 94 × 16 mm tissue culture dishes in a humidified chamber at 37 °C with 5 % CO₂. Cells were seeded 1:2 into 96-well plates, experiments were performed 72 h later with confluent cultures.

Mouse Primary Cortical Cultures

Primary cultures were prepared from embryonic mouse cortices as previously described [19]. Briefly, time-mated pregnant female CD1 mice were killed by cervical dislocation and E18 embryos were harvested. Cortices were dissected in PBS with 30 % glucose (Ca^{2+}- and Mg^{2+}-free) and dissociated with a fire polished glass Pasteur pipette. Tissue was centrifuged at 500 g for 5 min, resuspended in neurobasal medium supplemented with B27, 2 mM l-glutamine and 60 μg/ml penicillin and 100 μg/ml streptomycin (12 ml medium per brain). For live imaging experiments, cells were plated on 25 mm round glass coverslips (thickness no. 1) coated with 20 μg/ml poly-(d-)lysine, in 6-well tissue culture plates (Corning, USA). Cells were allowed to grow for 10–14 days in vitro (DIV) at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂.

Ca^{2+} Fluorimetry

SH-SY5Y Cells

Increases in [Ca^{2+}]_{ic} were measured as described previously [20]. Briefly, cells were washed twice with Tyrode’s salt solution (TSS: 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 1.8 mM CaCl₂, 0.2 mM NaH₂PO₄, 12 mM NaHCO₃, 5.5 mM glucose; pH 7.4) and incubated with the membrane-permeable, Ca^{2+} sensitive dye fluo-3 AM (10 μM) and 0.02 % pluronic F127 for 1 h at room temperature in darkness. Cells were then washed twice with TSS before pre-incubation (10 min) with 80 μM antagonists, modulators or TSS. Changes in fluorescence (excitation 485 nm, emission 538 nm) were measured using a Fluoroscan Ascent fluorescent plate reader (Thermo Scientific, UK). Basal fluorescence was measured for 5 s before agonist (20 μl) was added and fluorescence was monitored for a further 20 s. Calibration of responses was achieved by determining the maximum and minimum fluorescence values of each fluo-3 AM signal, by application of 0.2 % Triton X-100 (F_{max}) followed by 40 mM MnCl₂ (F_{min}). Data were calculated as a percentage of F_{max} – F_{min}. Concentration response data were fitted to the Hill equation and half maximal effective concentrations determined.
**Cortical Cultures**

Changes in $[\text{Ca}^{2+}]_i$ in individual cells of mouse E18 cortical cultures grown on glass coverslips were monitored using live cell imaging (Concord System, Perkin Elmer, UK). Cortical cultures (10–14 DIV) were washed twice with calcium buffer (140 mM NaCl, 5.0 mM KCl, 1.0 mM MgCl$_2$, 1.8 mM CaCl$_2$, 10 mM glucose, 5.0 mM HEPES; pH 7.4) and incubated with the ratiometric Ca$^{2+}$-sensitive dye fura-2 AM (5 μM) and 0.02 % pluronic F127 for 1.5 h at room temperature in darkness. After another two washes with buffer, coverslips were assembled into a temperature controlled (37 °C) perfusion chamber (Series 20 PH2 platform with a RC-21BR chamber, Harvard Apparatus, MA, USA) and mounted on an inverted fluorescence microscope. Buffer and drug solutions were pre-heated to 37 °C and perfused at a rate of 5 ml/min. Fura-2 AM was excited at 340 and 380 nm using a SpectroMaster I and emissions at 510 nm were detected with an intensified Ultrapix PDCI low light level CCD camera. All experiments were carried out in the presence of 1 μM tetrodotoxin (TTX) pre-incubated for at least 1 min prior to recording. During long drug pre-incubations perfusion was switched off to reduce drug use, and recording was turned off to prevent photobleaching.

Data were analysed with Ultraview software (Perkin Elmer, UK) and expressed as a ratio of $F_{340}/F_{380}$ following subtraction of background fluorescence taken from a region in which no cells could be seen. For successive drug treatments on the same cells, initial peak $F_{340}/F_{380}$ ratio for each individual responding region of interest (ROI) was normalized to 100% following subtraction of mean basal $F_{340}/F_{380}$ ratio recorded immediately before drug application. Subsequent responses in the presence of antagonists/modulators or after washout were calculated as a percentage of the original response from the same ROI. These values were then averaged within experiments, such that n values reflect the number of independent cultures examined.

**Statistical Analysis**

Statistical significance was evaluated by ANOVA with post-hoc test, or t-test as appropriate, with details given in figure legends.

**Results**

**Effects of Sazetidine-A on Ca$^{2+}$ Responses Initiated by Native Human nAChRs Expressed in SH-SY5Y Cells**

SH-SY5Y cells express $\alpha3$, $\alpha5$, $\alpha7$, $\beta2$, and $\beta4$ nAChR subunits [21–23] consistent with the formation of functional $\alpha3^*$ and $\alpha7$ nAChRs [20]. Based on the sensitivities of recombinant non-$\alpha4\beta2$ nAChRs, sazetidine-A was examined at 10 and 100 μM in SH-SY5Y cells loaded with the Ca$^{2+}$ indicator fluo-3 AM. Both concentrations of sazetidine-A produced a similar increase in fluorescence and this response was abolished in the presence of 30 μM mecamylamine (Fig. 1). The $\alpha7$-selective antagonist methyllycaconitine (MLA; 100 nM) was without effect. This suggests that under the conditions of the assay, sazetidine-A activates $\alpha3$-containing nAChRs but not $\alpha7$ nAChRs, consistent with previous findings for other agonists [24]. However, in the presence of the $\alpha7$-selective positive allosteric modulator (PAM) PNU-120596 (10 μM) [25], sazetidine-A evoked significantly larger increases in fluorescence that were partially blocked by both mecamylamine and by MLA (Fig. 1). This suggests that PNU-120596 reveals an $\alpha7$ nAChR-mediated increase in intracellular Ca$^{2+}$.

The response elicited by 10 μM sazetidine-A in the presence of PNU-120596 (2.3 ± 0.7 fold increase in fluorescence, Fig. 1) is comparable to that observed with the structurally related agonist 5-iodo-A85380 (1 μM; 2.3 ± 0.2 fold increase) and with nicotine (30 μM; 4.0 ± 0.2 fold increase), both tested in the presence of the PAM (data not shown). Increases in fluorescence in response to sazetidine-A were concentration dependent (Fig. 2). The concentration response curve was shifted to the left in the presence of PNU-120596. EC$_{50}$ values of 4.2 and 0.4 μM were derived for sazetidine-A in the absence and presence of PNU-120596, respectively.

The propensity of sazetidine-A to antagonize nAChRs in SH-SY5Y cells was assessed by preincubating cultures with increasing concentrations of sazetidine-A for 10 min, followed by stimulation with 100 μM nicotine (to activate $\alpha3$-containing nAChRs) or the $\alpha7$-selective agonist PNU-282987 (10 μM), in the presence of the PAM PNU-120596. Maximally effective agonist concentrations were deployed to elicit the optimum signal for quantitating inhibition. In both cases sazetidine-A produced a concentration-dependent inhibition of agonist-evoked responses, with IC$_{50}$ values of 522 and 476 nM respectively (Fig. 3).

**Effects of Sazetidine-A on $\alpha7$ nAChR-Mediated Ca$^{2+}$ Signals in Mouse Cortical Neurons**

Experiments were carried out on mouse E18 primary cortical cultures to assess the effects of sazetidine-A on native $\alpha7$ nAChRs in cells with a more neuronal phenotype. Cortical cultures were loaded with fura-2 AM and changes in fluorescence indicative of changes in intracellular Ca$^{2+}$ were monitored by live cell imaging. Sazetidine-A alone (10 nM–10 μM; 20 s application) failed to evoke any change in fluorescence, except for occasional, inconsistent...
increases at the highest concentration tested. In contrast, 40 mM KCl consistently produced robust responses from a majority of cells (data not shown). Following preincubation with PNU-120596, co-application of 10 μM sazetidine-A with the PAM evoked sustained responses from 14% of cells (average from 6 experiments from 3 independent cultures). Responses were completely blocked by 100 nM MLA, with partial recovery (32.4 ± 9.4% of initial response) following 10 min washout (Fig. 4).

Sazetidine-A was examined for its ability to attenuate responses from α7 nAChRs in cortical neurons by sequential application of the α7 nAChR agonist PNU-282987 alone (in the presence of PNU-120596) and following exposure to sazetidine-A for 10 min (Fig. 5). Sazetidine-A applied at 500 nM, a concentration approximating the IC50 value derived from SH-SY5Y cells (Fig. 3), decreased the response to PNU-282987 by 59%, whereas preincubation with 10 μM sazetidine-A resulted in a stronger block of 86%. This effect was not due to run-down of responses or exhaustion of the Ca2+ indicator as responses recovered to 57 and 60% of control, respectively, after 10 min washout of sazetidine-A (Fig. 5).

Discussion

In this study we have exploited the PAM PNU-120596 to reveal activity of native α7 nAChRs [27], in order to examine the actions of sazetidine-A on α7 nAChRs expressed in SH-SY5Y cells and mouse cortical cultures. In the absence of the PAM, sazetidine-A evoked mecamylamine-sensitive increases in fluorescence in SH-SY5Y cells that were insensitive to MLA. The EC50 value of

Fig. 1 Intracellular calcium elevations evoked by sazetidine-A in SH-SY5Y cell populations. SH-SY5Y cells loaded with fluo-3 AM were preincubated for 10 min with or without antagonist (mecamylamine (Mec), 30 μM, chequered bars, or MLA, 100 nM, cross-hatched bars) and/or PNU-120596 (PNU1, 10 μM, grey bars) before addition of sazetidine-A (Saz, 10 μM, a, b; 100 μM, c, d) in the presence (grey bars) or absence (white bars) of PNU-120596 (10 μM). Fluorescence at 538 nm was monitored for 20 s; representative traces are shown (b, d; some antagonist curves omitted for clarity). The increase in fluorescence at 20 s is presented as a percentage of the maximum fluorescence determined by addition of 0.2% Triton X-100 minus the minimum fluorescence quenched by 350 mM MnCl2 (a, c). Bars represent the mean ± SEM of at least 4 independent experiments; *P < 0.05, **P < 0.01, significantly different from sazetidine-A alone or in combination with PNU-120596, non-paired one-way ANOVA, Bonferroni’s multiple comparisons test.
4 μM is consistent with the activation of human α3β4* nAChRs in SH-SY5Y cells; at heterologously expressed α3β4 nAChRs sazetidine-A is a relatively weak agonist, with efficacy ranging from ~0 to 100 % in different studies, presumably reflecting differences in stoichiometry, species and methodology [1, 7, 9]. The lack of α7 nAChR responses in the absence of the PAM is likely to reflect the rapid kinetics of the receptor, as other agonists were previously found to be without effect in this assay [24]. However, sazetidine-A was recently reported to activate rat α7 nAChRs with very low efficacy [14] although another study using a different assay and overexpressed human α7 nAChRs, reported 100 % efficacy [7]. The MLA-sensitive enhancement of responses to sazetidine-A in the presence of the PAM PNU-120596 is indicative of the recruitment of α7 nAChRs. The lower EC50 determined in the presence of PNU-120596 is likely to underestimate the true EC50 for sazetidine-A at α7 nAChRs as this PAM shifts the agonist concentration–response relationship to the left by approximately 0.8 of a log unit [25]. This suggests that the EC50 value for activation of α7 nAChRs in SH-SY5Y cells by sazetidine-A would be in the low μM range, comparable with the recent report that sazetidine-A activated recombinant α7 nAChRs in the absence of a PAM with an EC50 value of 1.2 μM, using a sensitive fluorescence assay to measure changes in membrane potential [7]. A higher EC50 value of 60 μM was found using two-electrode voltage clamp recording from Xenopus oocytes expressing rat α7 nAChRs [14]. The ability of a brief (10 min) incubation with sazetidine-A to ameliorate responses to subsequent stimulation of α3* or α7 nAChRs is consistent with its propensity to desensitize nAChRs. There was a concern that this experiment would be compromised by the requirement for preincubation with the PAM, alongside sazetidine-A, in order to reveal α7 nAChR-evoked responses. Although PNU-120596 prolongs the activation of α7 nAChRs [25], the duration of this effect is relatively short-lived with return to baseline within

![Fig. 2](image_url) **Fig. 2** Concentration dependence of sazetidine-A-evoked responses in SH-SY5Y cells. SH-SY5Y cells loaded with fluo-3 AM were stimulated with sazetidine-A (0.1–100 μM) in the presence (solid black circles) or absence (open black circles) of PNU-120596 (PNU1; 10 μM). Fluorescence at 538 nm was measured for 20 s following stimulation with sazetidine-A. The increase in fluorescence at 20 s is presented as a percentage of the maximum fluorescence determined by addition of 0.2 % triton X-100 minus the minimum fluorescence quenched by 350 mM MnCl2. Points represent the mean ± SEM from 8 independent experiments. Data were fitted to the Hill equation and EC50 values for sazetidine-A in the absence and presence of PNU-120596 were calculated to be 4.2 and 0.4 μM respectively.

![Fig. 3](image_url) **Fig. 3** Sazetidine-A inhibits responses evoked by nicotinic agonists in SH-SY5Y cells. SH-SY5Y cells loaded with fluo-3 AM and preincubated with sazetidine-A (0.01–100 μM) for 10 min before stimulation with nicotine (100 μM; solid circles, a) or PNU-282987 (3 μM) together with PNU-120596 (10 μM; open circles, b). The PAM was also present during the preincubation period in b. Fluorescence at 538 nm was measured for 20 s following stimulation. Normalised responses at 20 s are expressed as a percentage of the response to agonist in the absence of sazetidine-A. Points represent mean ± SEM of at least 4 independent experiments and are fitted to the Hill equation, yielding IC50 values of 522 nM and 476 nM for sazetidine-A versus nicotine and versus PNU-282987, respectively.
evoked responses in the absence of the PAM, attributed to
measured in both cases and that 120596 argues that an inhibitory effect of sazetidine-A is
selective agonist PNU-282987 in the presence of PNU-
5 min \[\text{perfused with 1.8 mM calcium buffer at } 37 \, ^\circ \text{C and imaged under a fluorescence microscope at 510 nm. Cultures were pre-incubated with PNU-120596 (PNU1; 10 \, \mu M; 3 min). Basal fluorescence (F}_{340:F}_{380} was recorded for 30 s before stimulation with sazetidine-A (Saz; 10 \, \mu M; 20 s). Following 3 min washout, cells were pre-incubated with MLA (100 nM; 10 min) and PNU-120596 (10 \, \mu M; 3 min) prior to recording F}_{340:F}_{380} before, during and after co-stimulation with sazetidine-A (10 \, \mu M; 20 s). Following 10 min washout, the protocol was repeated in the absence of MLA. Panels a, b, c show representative images of the same field of cells taken during the 3 successive stimulations with sazetidine-A before, during and after exposure to MLA; scale bar 150 \, \mu M. Fluorescence is shown in pseudocolour, (black/blue = low F}_{340:F}_{380}, red/white = high F}_{340:F}_{380}). Two cells that responded to stimulation in a representative experiment are indicated by the arrowheads and their individual fluorescence profiles are shown below; fluorescence changes are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm. d Averaged data from 3 independent cultures are presented graphically. Bars represent the mean ± SEM peak F}_{340:F}_{380} increase above basal, expressed as a percentage of the response to the initial stimulation with sazetidine-A in the presence of PNU-120596, from the same region of interest. ****P < 0.0001 significantly different from initial response to sazetidine-A in combination with PNU-120596, one sample t-test

Fig. 4 Sazetidine-A elicits intracellular calcium elevations in the presence of PNU-120596 in mouse cortical cultures. Mouse E18 primary cortical cultures (10–14 DIV) were loaded with fura-2 AM, perfused with 1.8 mM calcium buffer at 37 °C and imaged under a fluorescence microscope at 510 nm. Cultures were pre-incubated with PNU-120596 (PNU1; 10 \, \mu M; 3 min). Basal fluorescence (F}_{340:F}_{380} was recorded for 30 s before stimulation with sazetidine-A (Saz; 10 \, \mu M; 20 s). Following 3 min washout, cells were pre-incubated with MLA (100 nM; 10 min) and PNU-120596 (10 \, \mu M; 3 min) prior to recording F}_{340:F}_{380} before, during and after co-stimulation with sazetidine-A (10 \, \mu M; 20 s). Following 10 min washout, the protocol was repeated in the absence of MLA. Panels a, b, c show representative images of the same field of cells taken during the 3 successive stimulations with sazetidine-A before, during and after exposure to MLA; scale bar 150 \, \mu M. Fluorescence is shown in pseudocolour, (black/blue = low F}_{340:F}_{380}, red/white = high F}_{340:F}_{380}). Two cells that responded to stimulation in a representative experiment are indicated by the arrowheads and their individual fluorescence profiles are shown below; fluorescence changes are presented as a ratio of fluorescence emitted at 510 nm following excitation at 340 and 380 nm. d Averaged data from 3 independent cultures are presented graphically. Bars represent the mean ± SEM peak F}_{340:F}_{380} increase above basal, expressed as a percentage of the response to the initial stimulation with sazetidine-A in the presence of PNU-120596, from the same region of interest. ****P < 0.0001 significantly different from initial response to sazetidine-A in combination with PNU-120596, one sample t-test

The IC_{50} values for this effect were ~0.5 \, \mu M (Fig. 3). This could be relevant to clinical applications of sazetidine-A when therapeutic concentrations may approach these levels [28] (see below). Moreover, Campling et al. [7] recently highlighted ‘smouldering activation’ of nAChRs resulting from the balance within a population of receptors of sustained desensitization versus activation, such that the impact of chronic agonist concentrations will be complex.

The sensitivity of \(\alpha7\) nAChRs to sazetidine-A was reinforced by studies in primary cortical neurons. Interestingly, no changes in fluorescence were detected in response to sazetidine-A in the absence of the PAM. This was surprising as functional \(\alpha4\beta2\) nAChRs might have been anticipated to be present in cortical neurons. Possible explanations are that they are only present in the LS-

\(\alpha4\beta2\) stoichiometry, or that they are absent at this developmental stage. Although \(\alpha4\beta2\) nAChRs have been documented on thalamocortical afferents [29], projection neurons would not be present in the cortical cultures. However \(\alpha4\beta2\) nAChRs may also occur on intrinsic cortical neurons [30, 31]. Alternatively, \(\alpha4\beta2\) nAChRs might not initiate detectable changes in intracellular \(\text{Ca}^{2+}\), due to the presence of TTX in the perfusing buffer.

In contrast, in the presence of PNU-120596 sazetidine-A elicited robust responses from a minority of cells, estimated as 14 % of the total population. This proportion is consistent with measurements using a selective \(\alpha7\) nAChR agonist together with the PAM (Brown and Wonnacott, unpublished observation). The almost total blockade of these responses by 100 nM MLA confirmed that they arise from activation of \(\alpha7\) nAChRs. Recovery following 3 min washout was partial and somewhat variable, possibly reflecting sazetidine-A’s propensity to desensitize nAChRs. This was supported by the ability of sazetidine-A to produce a concentration-dependent-decrease in responses to PNU-282987, with sensitivity similar to that observed in SH-SY5Y cells.
The manuscript does not contain clinical studies or patient data. Standards of animal care were in accordance with the ARRIVE guidelines and UK law.

**Conflicts of interest** The authors declare that they have no conflict of interest.

**References**


