Investigating PYY3-36 and PYY3-36 analogues in the development of an obesity therapy

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For my parents, and Barry
Abstract

Obesity, defined as a body mass index greater than 30kg/m², has reached the scale of a pandemic. The increasing prevalence of obesity, and its associated morbidity and mortality, together with limited treatment options, underscores an urgent need for effective therapeutic interventions. Gut hormones have been identified as integral factors in the regulation of appetite. One such gut hormone is Peptide YY (PYY), a postprandial satiety hormone that communicates nutritional status to the central nervous system. PYY is processed to generate the principle circulating form PYY3-36, which acts on Y2 receptors in the brainstem and hypothalamus to reduce appetite. Chronic intravenous infusion of PYY3-36 induces weight loss in rodents and acute intravenous infusion to obese humans reduces food intake. Furthermore, obese humans have been reported to display a blunted postprandial rise in PYY3-36, suggesting PYY3-36 is a potential anti-obesity drug target. However, exogenous PYY3-36 is rapidly cleared and has a short circulating half-life. Additionally, at supraphysiological levels PYY3-36 can produce nausea in humans. The administration of long-acting PYY3-36 analogues to the obese may overcome these limitations. This thesis investigates the design and development of PYY3-36 analogues, and their potential in the treatment of obesity. I have investigated modifications to different domains within the primary structure of PYY3-36 in order to elucidate Y2R affinity, susceptibility to proteolytic degradation and biological activity. I demonstrated that a combination of substitutions in different domains can create a long acting analogue. I have examined sites at which PYY3-36 is susceptible to degradation by specific proteases and identified that inhibition of meprin metalloendopeptidases can prolong the plasma longevity and anorectic actions of PYY3-36. I also found that substitution of a section of the α-helix of PYY3-36 with a conserved α-helical epitope creates a long-acting analogue, that is more efficacious than PYY3-36 in chronically reducing food intake and body weight in a diet-induced obese rodent model. Finally, in investigating a slow-release delivery system for PYY3-36 analogues, I have shown that substituting His residues into the α-helix-substituted analogue promotes chelation with Zn in vitro, and facilitates a slow-release pharmacokinetic profile in vivo, that may circumvent the side effects associated with administering high levels of PYY3-36.
Declaration of contributors

The majority of work described in this thesis was carried out by the author. All collaboration and assistance is described below.

Chapter 2
Feeding studies were carried out with assistance from members of the PYY group.

Chapter 3
The feeding studies and plasma levels studies were carried out with assistance from Dr James Minnion, Dr Keisuke Suzuki and Joy Shillito. The nephrectomy studies were carried out with assistance from Samar Ghourab and Klara Hostomska.

Chapter 4
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Contents

Abstract ........................................................................................................................................... 3
Declaration of contributors ................................................................................................................ 4
Acknowledgements ........................................................................................................................... 5
Index of Figures .................................................................................................................................. 9
Index of Tables ................................................................................................................................... 15
Abbreviations used in thesis .............................................................................................................. 19

Chapter 1: General Introduction ...................................................................................................... 21
  1.1 Introduction ................................................................................................................................. 22
    1.1.1 Obesity ................................................................................................................................ 22
    1.1.2 Cause and origin of obesity ................................................................................................. 22
  1.2 The Physiology of Appetite and Metabolism .............................................................................. 23
    1.2.1 Appetite control centres: overview ...................................................................................... 23
    1.2.2 The hypothalamus ............................................................................................................... 26
    1.2.3 Hypothalamic neuropeptides regulating appetite control .................................................. 33
    1.2.4 Peripheral adiposity signals ............................................................................................... 46
    1.2.5 Hormones secreted from the GI-tract regulating energy balance ....................................... 48
  1.3 Design and development of PYY3-36 anti-obesity therapies .................................................. 65
    1.3.1 Current licensed obesity treatments ..................................................................................... 65
    1.3.2 Use of PYY-36 as a potential antiobesity therapy .............................................................. 67
    1.3.3 Structural design of PYY analogues .................................................................................... 68
  1.4 Overview ..................................................................................................................................... 70

Chapter Two: Alterations to the primary sequence of PYY3-36 in elucidating structure/activity and for the design of long acting analogues ...................................................... 72
  2.1 Introduction and aims .................................................................................................................. 73
    2.1.1 Use of analogues to investigate peptide properties and structure activity relations .............. 74
    2.1.2 Structural properties of PYY and PYY3-36 ........................................................................ 75
  2.2 Aim of studies .............................................................................................................................. 85
  2.3 Materials and Methods .............................................................................................................. 85
    2.3.2 Peptide design: investigating the importance of specific regions of PYY3-36 in receptor binding and bioactivity .......................................................................................... 87
    2.3.3 Production of hY2R overexpressing cell line ...................................................................... 91
    2.3.4 Cell Culture ....................................................................................................................... 98
    2.3.5 Receptor binding studies .................................................................................................... 101
    2.3.6 Degradation assay using KBB membrane ......................................................................... 103
    2.3.7 In vivo studies .................................................................................................................... 105
    2.3.8 Statistics ............................................................................................................................ 106
  2.4 Results ..................................................................................................................................... 107
    2.4.1 Investigations into the role of the C terminus of PYY3-36 in structure activity relations at the Y2R and in its in vivo bioactivity ................................................................. 107
2.4.2 Investigations into the role of the mid-section of PYY3-36 in structure activity relations at the Y2R and in vivo bioactivity ...................................................... 117
2.4.3 Investigations into the role of the N-terminal of PYY3-36 in structure activity relations at the Y2R and in vivo bioactivity ...................................................... 124
2.4.4 Effect of modifications to improve longevity on receptor binding, degradation and in vivo bioactivity ................................................................. 130
2.4.5 Effect of combining longevity modifications with simple amino acid substitutions upon food intake ................................................................. 138
2.4.6 The effects of combining amino acid changes within the entire PYY sequence on receptor binding, susceptibility to degradation and in vivo bioactivity ........... 144

2.5 Discussion ........................................................................................................... 156

Chapter Three: Investigating the metabolic clearance and degradation of PYY3-36 as a tool in developing a long-lasting PYY3-36 therapy ............................................. 170

3.1 Introduction ........................................................................................................ 171
3.2 Aim of studies .................................................................................................... 175
3.3 Materials and Methods .................................................................................... 178
  3.3.1 Materials .................................................................................................... 178
  3.3.2 Purified protease degradation studies ......................................................... 178
  3.3.3 Quantitative HPLC .................................................................................. 178
  3.3.4 MALDI-ToF Mass Spectrometry ................................................................. 179
  3.3.5 KBB degradation studies ......................................................................... 179
  3.3.6 In vivo studies .......................................................................................... 180
  3.3.7 Statistics .................................................................................................. 185

3.4 Results ................................................................................................................. 186
  3.4.1 The effect of nephrectomy in rats on PYY3-36 pharmacokinetics ............... 186
  3.4.2 Effect of the Zn metalloendopeptidases NEP and meprin β on PYY3-36 .... 188
  3.4.3 Effect of KBB membranes on PYY3-36 ...................................................... 193
  3.4.4 Characterisation of PYY3-36 peptide fragments ....................................... 195
  3.4.5 Characterisation of a PYY3-36 analogue with an Asp substitution ............ 197
  3.4.6 Effect of NEP and meprin β inhibition on PYY3-36 degradation in vitro .... 201
  3.4.7 Effect of NEP and meprin β inhibition on PYY3-36 bioactivity ................. 204

3.5 Discussion ............................................................................................................ 210

4 Chapter Four: Development of PYY3-36 analogues: modifications to 3D structure and use of a metal ion delivery system to promote slow release .......................... 217

4.1 Introduction ........................................................................................................ 218
  4.1.1 Evidence for therapeutic potential of PYY3-36 ........................................ 218
  4.1.2 Development of a PYY3-36 analogue with an altered α-helix ................. 221
  4.1.3 Use of a metal ion delivery system in developing a slow-release administration regime for PYY3-36 analogues ......................................................... 222

4.2 Hypotheses & Aims .......................................................................................... 224

4.3 Methods .............................................................................................................. 226
  4.3.1 Peptides .................................................................................................... 226
  4.3.2 PYY3-36 analogue design ........................................................................ 226
  4.3.3 PYY3-36 receptor binding assays .............................................................. 228
  4.3.4 Animals .................................................................................................... 228
4.3.5 Acute PYY3-36 analogue feeding studies in mice ........................................... 229
4.3.6 Acute PYY3-36-αLT behavioural study in mice ............................................ 229
4.3.7 PYY3-36, PYY3-36-αLT and PYY3-36-NPro-αLT pharmacokinetic studies in rats .......................................................................................................................... 230
4.3.8 Investigations into the substitution of His residues in PYY3-36 NPro αLT in the development of a Zn delivery system .................................................. 232
4.3.9 Radioimmunoassays for PYY3-36 and PYY3-36 analogues .......................... 233
4.3.10 Assessment of the chronic effects of PYY3-36 analogues administered with Zn to diet-induced obese mice ................................................................. 234
4.3.11 Measurement of total body fat in animals treated with PYY3-36-αLT using whole body proton magnetic resonance spectroscopy (1H-MRS) ..................... 234
4.3.12 Statistics ....................................................................................................... 235

4.4 Results ............................................................................................................. 236
4.4.1 Investigations into substituting αLT into the α-helix (residues 16-23) of PYY3-36 or NPro-PYY3-36 .................................................................................. 236
4.4.2 Investigations into the substitution of His residues in PYY3-36 NPro αLT in the development of a Zn delivery system ................................................. 247
4.4.3 Assessment of the use of a Zn delivery system to prolong the chronic bioactivity of PYY3-36 analogues in vivo ...................................................................... 254

4.5 Discussion ..................................................................................................... 260
4.5.1 The effect of substituting the LT/LIT/Exendin-4 conserved region into the α-helical region (residues 16-23) of PYY3-36 .................................................................. 261
4.5.2 The effect of the addition of N-Pro to PYY3-36-αLT ..................................... 263
4.5.3 The effect of chronic administration of PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H ........................................................................... 267

Chapter 5: Final Discussion ..................................................................................... 271

5.1 Final Conclusions and Future Prospects ......................................................... 273
5.1.1 Structure activity relationships and design of long-acting analogues .......... 273
5.1.2 Insight into the metabolic clearance and degradation of PYY3-36 for design of analogues resistant to specific proteases and/or the use of protease inhibitor therapies ................................................... 276
5.1.3 Use of αLT substitution to modify the 3D structure of PYY3-36 and Zn-precipitation systems to allow a slow release pharmacokinetic profile ................. 277

Appendices .......................................................................................................... 317

6.1 Appendix I: Amino Acids .............................................................................. 317
6.2 Appendix II: Solutions used in Thesis ............................................................ 318
6.3 Appendix III: Vectors ................................................................................... 320
6.4 Appendix iv: Radioimmunoassay .................................................................. 321

Manuscripts in Submission ................................................................................... 325

Presentations ...................................................................................................... 325
Index of Figures

Figure 1.1. Schematic diagram showing hypothalamic nuclei in the rodent brain, which are involved in energy homeostasis in mammals................................................................. 27
Figure 1.2. Three dimensional view of the right hemisphere of the rodent hypothalamus.. 32
Figure 1.3. A schematic diagram showing the POMC precursor molecule and major cleavage products. ............................................................................................................. 34
Figure 1.4. Illustrative representation of putative model for regulation of appetite by the arcuate nucleus........................................................................................................... 45
Figure 1.5. Summary of the mechanisms of action of the key hormones involved in energy homeostasis.............................................................................................................. 54
Figure 1.6. The human amino acid sequences of the members of the PP-fold family. ........ 57
Figure 1.7. Illustrative representation of putative model for mechanism of action of PYY and PYY3-36............................................................................................................. 64

Figure 2.1. Conserved ‘PP-fold’ structure of PYY.............................................................. 77
Figure 2.2. Structure of PYY as determined by NMR (left) and as representative conformers.. ................................................................. 78
Figure 2.3. Image showing 3D solution structures of A) PYY and B) PYY3-36 showing a stable and destabilized PP-fold respectively ................................................................. 79
Figure 2.4. Structure of human PYY and PYY3-36 as determined by NMR, and putative models for PYY and PYY3-36 binding conformations at the A) Y1 and B) Y2 receptors..80
Figure 2.5. Chemical structure of stereoisomers of proline (blue box) and isoleucine (green box) used in PYY3-36 analogue design................................................................. 90
Figure 2.6. Representative binding affinity curve of C-terminal-hexapeptide substituted analogues at the Y2 receptor ................................................................. 109
Figure 2.7. The effect of subcutaneous administration of 20, 60, 200 or 600nmol/kg PYY3-36 (n=8-9) on food intake in fasted mice ........................................................................ 110
Figure 2.8. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 30-substituted PYY3-36 analogues. ............ 113
Figure 2.9. Representative binding affinity curve of C-terminal-hexapeptide substituted analogues at the Y2 receptor................................................................. 115
Figure 2.10. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with C-terminal hexapeptide substitutions.

Figure 2.11. Representative binding affinity curve of position 19 substituted analogues at the Y2 receptor.

Figure 2.12. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 19-substituted PYY3-36 analogues.

Figure 2.13. Representative binding affinity curve of position 23 substituted analogues at the Y2 receptor.

Figure 2.14. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 23-substituted PYY3-36 analogues.

Figure 2.15. Representative binding affinity curve of position 4-substituted analogues at the Y2 receptor.

Figure 2.16. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 4-substituted PYY3-36 analogues.

Figure 2.17. Representative binding affinity curve of position 6-substituted analogues at the Y2 receptor.

Figure 2.18. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 6-substituted PYY3-36 analogue.

Figure 2.19. Representative binding affinity curve of analogues with the addition of a Cys, Pro or D-Pro at the N-terminal at the Y2 receptor.

Figure 2.20. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with N-terminal addition of Pro, D-Pro or Cys.

Figure 2.21. Representative binding affinity curve of analogues with a stereoisomer of Ile at position 3 at the Y2 receptor.

Figure 2.22. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with a stereoisomer at position 3.

Figure 2.23. Representative binding affinity curve at the Y2R of PYY3-36 analogues with a substitution of Lys at Ala12 or the addition of a 12 carbon fatty acid (lauroyl) to Lys12.
Figure 2.24. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with a Lys only or acylated Lys at position 12 ................................................................. 140

Figure 2.25. Representative binding affinity curve at the Y2R of PYY3-36 analogues with a substitution of Ile3 for D-allo-Ile and Ala12 for Lys with the addition of lauroyl to Lys12............................................................... 142

Figure 2.26. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with combined stereochemical and acyl group substitutions at position 3, 6 and 12 ................................................................. 144

Figure 2.27. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with combined substitutions at positions 19, 23 and 30 and with or without the addition of an N-terminal Pro. ................................................................. 147

Figure 2.28. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with combined substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro ........................................................................................................ 152

Figure 2.29. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with combined substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro. ................................................................. 155

Figure 3.1. NEP specificity matrix using the nomenclature for the interaction of proteases with their substrates ................................................................. 176

Figure 3.2. Dimeric structure of meprin A and B ................................................................. 176

Figure 3.3. Meprin-β specificity matrix using the nomenclature for the interaction of proteases with their substrates ................................................................. 177

Figure 3.4. Effect of unilateral or bilateral nephrectomy on PYY3-36 plasma levels in rats at 2 min, 5 min, 10 min, 15min, 30min and 60min post-injection. ................................................................. 187

Figure 3.5. Effect of unilateral or bilateral nephrectomy on PYY3-36 plasma levels in rats at 2 min, 5 min, 10 min, 15min, 30min and 60min post-injection ................................................................. 187

Figure 3.6. Representative reverse-phase HPLC absorption profile following in vitro digestion of: A) 2nmol GnRH with 200ng NEP for 60 min at 37°C PYY3-36 and B) 2nmol PYY3-36 with 200ng NEP for 60 min at 37°C ................................................................. 190

Figure 3.7. Representative reverse-phase HPLC absorption profile following in vitro digestion of 2nmol PP or PYY3-36 with 200ng NEP for 60 min at 37°C ................................................................. 191
Figure 3.8. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of PYY3-36 with 200ng NEP over 60 min, 120 min and 240 min at 37°C. ... 192
Figure 3.9. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of PYY3-36 with 200ng meprin β over 5, 15, 30 and 60 min at 37°C. .......... 192
Figure 3.10. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with A) 0.62mg/ml, 1.25mg/ml and 2.50mg/ml KBB for 30 min at 37°C and B) 0.25mg/ml KBB for 5 min, 15 min and 30 min at 37°C. .......................... 194
Figure 3.11. Schematic showing cleavage sites for meprin β and KBB on A) PYY3-36 and B) PYY3-36 Asp10......................................................... 200
Figure 3.12. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with 200ng NEP or 200ng NEP & 10nmol phosphoramidon for 30 min at 37°C. ........................................................................ 202
Figure 3.13. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with 1.25mg/ml KBB or 1.25mg/ml KBB & 100nmol phosphoramidon for 30 min at 37°C. .......................................................... 202
Figure 3.14. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with 200ng meprin β or 200ng meprin β & 10nmol actinonin for 30 min at 37°C.............................................................. 203
Figure 3.15. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with 1.25mg/ml KBB or 1.25mg/mlKBB with 50/100nmol actinonin for 30 min at 37°C .......................................................... 203
Figure 3.16. Effect of phosphoramidon (20mg/kg; sc) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and phosphoramidon alone (20mg/kg, sc) on food intake at 0-1h, 1-2h, 2-3h, 4-8h and 8-24h post-injection .......................... 204
Figure 3.17. Effect of phosphoramidon (20mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and phosphoramidon (20mg/kg; ip) alone on cumulative food intake over 8h...................................................... 205
Figure 3.18. Effect of phosphoramidon (20mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and phosphoramidon (20mg/kg; ip) alone on cumulative food intake over 8h...................................................... 206
Figure 3.19. Effect of actinonin (20mg/kg, ip) on plasma PYY3-36 IR at 20, 60 and 90 min after PYY3-36 injection (100nmol/kg, sc) in mice.......................................................... 207
Figure 3.20. Effect of actinonin (20 or 40mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and actinonin (20 or 40mg/kg; ip) on food intake at A: 0-1h, B: 1-2h, C: 2-4h, and D: 4-6h post-injection. ................................................................. 208

Figure 3.21. Effect of actinonin (20 or 40mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and actinonin (20 or 40mg/kg; ip) alone on cumulative food intake over 6h .......................... 209

Figure 3.22. Effect of actinonin (20 or 40mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and actinonin (20 or 40mg/kg; ip) alone on cumulative food intake over 6h .......................................................... 210

Figure 4.1. Computer generated image of the insulin hexamer........................................ 224

Figure 4.2. Homologous amino acid sequences of Ex4, αLTX and αLIT are shown with residues that are either identical or conserved in bold. .................................................. 227

Figure 4.3. Effect of PYY3-36(50nmol/kg; sc) and PYY3-36-αLT (20nmol/kg / 50nmol/kg; sc) on food intake at 0-1h, 1-2h, 2-4h, 4-8h and 8-24h post-injection, and on cumulative food intake over 24h. ................................................................. 236

Figure 4.4. Dose response effect of PYY3-36(500nmol/kg or 10,000nmol/kg; sc) and PYY3-36-αLT(500,1000,5000 or 10000nmol/kg; sc) on food intake at 0-1h, 1-2h, 2-4h and 4-8h post-injection. ......................................................... 238

Figure 4.5. Dose response effect of PYY3-36(500nmol/kg or 10,000nmol/kg; sc) and PYY3-36-αLT(500,1000,5000 or 10000nmol/kg; sc) on food intake at 8-24h, 0-24h, 24-48h and 0-48h post-injection........................................................................... 238

Figure 4.6. Pie charts depicting the proportion of observations of each of the predefined behaviours a) 0-1h and b)1-2h post-injection of saline, LiCl (127mg/kg) or PYY3-36-αLT (500nmol/kg). .................................................................................. 241

Figure 4.7. Binding affinity curves comparing PYY3-36 and PYY3-36-αLT binding at the A) human or B) mouse Y2R................................................................. 242

Figure 4.8. Binding affinity curves comparing A)PYY3-36 and PYY3-36-NPro binding and B) PYY3-36 and PYY3-36-NPro-αLT binding at the human Y2R.......................... 243

Figure 4.9. Effect of 1000nmol/kg (sc) of PYY3-36-NPro-αLT and PYY3-36-αLT on food intake at 0-1h, 1-2h, 2-4h, 4-8h, 8-24h, 24-48h post-injection, and on cumulative food intake over 0-24h and 0-48h post-injection .................................................................................. 245

13
Figure 4.10. Plasma concentration/time curves of PYY3-36 (grey), PYY3-36-αLT (blue) and PYY3-36-NPro-αLT (purple) following administration of a 100nmol/kg dose of PYY3-36 or analogue. .................................................................................................................. 246

Figure 4.11. Graphs showing the amount of PYY3-36 or PYY3-36 analogue that dissolves into solution and the amount that precipitates out of solution at physiological pH........... 248

Figure 4.12. Graphs showing the amount of PYY3-36-NPro-αLT-4H that dissolves into solution and the amount that precipitates out of solution at physiological pH........... 249

Figure 4.13. Plasma concentration/time curves of PYY3-36-NPro-αLT-4H (light green) and PYY3-36-NPro-αLT-4H with Zn (dark green) following subcutaneous administration of a 300nmol/kg dose of PYY3-36-NPro-αLT-4H in saline solution (pH4.5) or in a saline/Zn solution containing a molar ratio of 1:1 Zn:peptide (pH 4.5)........................................... 251

Figure 4.14. Effect of 1000nmol/kg (sc) of PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H on food intake at 0-1h, 1-2h, 2-4h and 4-8h post-injection............. 252

Figure 4.15. Effect of 1000nmol/kg (sc) of PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H on food intake at 8-24h and 24-48h post-injection, and on cumulative food intake over 24 and 48h. .................................................................................................................. 253

Figure 4.16. Binding affinity curves comparing PYY3-36 and PYY3-36-NPro-αLT-4H binding at the human Y2R.......................................................... 254

Figure 4.17. Effect of daily administration of 300nmol/kg of PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H (sc, all with Zn) on food intake over 49 days. .................................................................................................................. 256

Figure 4.18. Effect of daily administration of 300nmol/kg of PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H (sc, all with Zn) on body weight change (g) over 49 days. .................................................................................................................. 256

Figure 4.19. Effect of daily administration of 300nmol/kg of PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H (sc, all with Zn) on percentage change from initial body weight over 49 days (n =6-8)......................................................... 257

Figure 4.20. Effect of daily administration of 300nmol/kg of PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H (sc, all with Zn) on acute food intake over 4h post-injection on day 1 (a) and over 0-4h (b) and 4-8h (c) post-injection on day 26... 258

Figure 4.21. Effect of daily administration of 300nmol/kg PYY3-36-αLT with Zn (sc) over 49 days on adiposity......................................................................................... 259
Index of Tables

Table 2.1. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at positions 30 within the binding hexapeptide of the C terminus................................................................. 108

Table 2.2. Statistical significance of the effects on food intake of PYY3-36 controls or position 30-substituted PYY3-36 analogue derived from food intake data from each study day................................................................. 112

Table 2.3. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at positions 31, 34 or 36 within the binding hexapeptide of the C terminus................................................................. 114

Table 2.4. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions in the binding hexapeptide derived from food intake data for each study day................................................................. 116

Table 2.5. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 19 within the mid-section................................................................. 118

Table 2.6. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions at position 19 derived from food intake data for each study day................................................................. 120

Table 2.7. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 23 in the mid-section................................................................. 121

Table 2.8. Statistical significance for PYY3-36 controls or PYY3-36 analogues with substitutions at position 23 derived from food intake data for each study day................................................................. 123

Table 2.9. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 4 in the N-terminal................................................................. 124
Table 2.10. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions at position 4 derived from food intake data for each study day............................................................ 126

Table 2.11. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 6 in the N-terminal ........................................ 127

Table 2.12. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions at position 6 derived from food intake data for each study day............................................................................................................ 129

Table 2.13. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with the addition of Cys, Pro or D-Pro at the N-terminal.. .................. 131

Table 2.14. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with amino acid additions at the N-terminal of PYY3-36 derived from food intake data for each study day............................................................................................................ 134

Table 2.15. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 3 in the N-terminal.............................. 135

Table 2.16. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with a stereoisomer of Ile at position 3 derived from food intake data for each study day............................................................................................................ 137

Table 2.17. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with different substitutions at positions 12 in the N-terminal........ 138

Table 2.18. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with a stereoisomer of Ile at position 23 derived from food intake data for each study day............................................................................................................ 140

Table 2.19. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with combined substitutions at positions 3 and/or 12 within the N-terminal............................................................................................................ 141

Table 2.20. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with a stereochemical substitutions at position 3 with either an amino acid substitution at position 6 or 12 and/or the addition of an acyl group derived from food intake data for each study day............................................................................................................ 143

Table 2.21. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with combined substitutions at positions 19, 23 and/or 30 of PYY3-36.. ............................................................................................................................................... 145
Table 2.22. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with combined substitutions at positions 19, 23 and 30 and with or without the addition of an N-terminal Pro derived from food intake data for each study day. ................................................................. 147

Table 2.23. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with combined substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro. ................................................................. 149

Table 2.24. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro derived from food intake data for each study day. .......... 151

Table 2.25. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with combined substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro. ................................................................. 153

Table 2.26. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with combined substitutions at position 23 and 30 with or without the addition of Pro derived from food intake data for each study day. ........................................... 155

Table 3.1. Summary of each treatment group, designated 1-6, for studies described in sections 1.3.6.3 and 1.3.6.4.......................................................................................................................... 184

Table 3.2. Half-disappearance rate For PYY3-36 in sham, UNx and BNx rats (mean ±SEM, n=5-6) calculated using equation k=ln2/t1/2. .................................................................................................................. 188

Table 3.3. Creatinine levels in sham, UNx and BNx rats at 60 min (mean ±SEM, n=5-6). 188

Table 3.4. Summary of mean percentage degradation after PYY3-36 (2nmol) was incubated with NEP (200ng; n=6) or meprin β (200ng; n=7), and after increasing doses or incubation times. ........................................................................................................................................................................... 193

Table 3.5. MW of peptide fragments following incubation of PYY3-36 (2nmol) with KBB (1.25mg/ml), meprin β (200ng) or NEP (200ng) over 5, 15 or 30 min. ........................................... 196

Table 3.6. MW of peptide fragments after PYY3-36-Asp10 (2nmol) was incubated with KBB (1.25mg/ml), meprin β (200ng) or NEP (200ng) over 5, 15 or 30 min. ........................................... 199

Table 4.1. Summary PYY3-36 levels (pmol/L) in either fasted (12h overnight fast) or postprandial (30 min after a buffet meal for Batterham et. al., and 90 min after a 420 calorie meal for Le Roux et. al.) obese and lean patients, and in obese patients that had undergone Roux-En-Y gastric banding (RYGB) surgery 6-36 months before the study. ................. 221
Table 4.2. Table showing median frequency and inter-quartile range (in square brackets) of each of the predefined behaviours observed over 0-1h and 1-2h post-injection of saline, LiCl (127mg/kg) or PYY3-36-αLT (500nmol/kg).
Abbreviations used in thesis

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>AcN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti related protein</td>
</tr>
<tr>
<td>AP</td>
<td>Area postrema</td>
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<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine- and amphetamine-regulated transcript</td>
</tr>
<tr>
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</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
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<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
</tr>
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</tr>
<tr>
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<td>Conditioned taste aversion</td>
</tr>
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<td>Dorsomedial hypothalamus</td>
</tr>
<tr>
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<tr>
<td>Acronym</td>
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<td>Neprilysin</td>
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<td>Polymerase chain reaction</td>
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<td>Preoptic area</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Paraventricular nucleus</td>
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<tr>
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<td>Peptide YY</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
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<tr>
<td>RQ</td>
<td>Respiratory Quotient</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-Activity Relationship</td>
</tr>
<tr>
<td>sc</td>
<td>Subcutaneous</td>
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<tr>
<td>SNS</td>
<td>Sympthetic nervous system</td>
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</tr>
<tr>
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<td>WAT</td>
<td>White adipose tissue</td>
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Chapter 1: General Introduction
1.1 Introduction

1.1.1 Obesity

Obesity is now at the scale of a pandemic in the modern developed world (World Health Organization, 2006). Obesity (Body mass index $>30\text{kg/m}^2$) is associated with an increased risk of developing a range of debilitating conditions including type II diabetes, hypertension, stroke, cardiovascular disease, osteoarthritis, gallbladder disease and a number of cancers (Kopelman, 2000; Calle et al., 1999). This presents a substantial economic and societal burden. Worldwide, there are 300 million obese humans, and prospects suggest that more than half of the world’s population will be overweight or obese by 2015 (World Health Organization, 2006). Lifestyle changes to reduce energy intake and increase energy output can promote weight loss, but there is resistance to broad adoption by many populations and promotion of such lifestyle changes has had little impact on the rising levels of obesity (Rennie and Jebb, 2005). Thus there is an urgent requirement for the scientific community to elucidate the physiology of appetite and energy homeostasis, in order to facilitate the development of antiobesity therapies. Such therapies would ideally be able to restore appetite control and prevent co-morbidities through the use of the body’s own satiety infrastructure, rather than by acting on the ubiquitous neurotransmitter systems that previous anti-obesity drugs acted upon. In recent years a number of scientific advances in our understanding of the regulation of appetite and energy expenditure have been made. Identifying physiological drug targets and conducting physiological and pharmacological investigations will aid the design and development of effective therapeutic interventions for obesity.

1.1.2 Cause and origin of obesity
Interestingly, despite short-term mismatches in energy balance, humans have historically been able to equalize energy intake with energy output resulting in minimal change in body weight over a period that spans many meals. This ‘thrifty’ genetic constitution resulted in the ability to store calories as fat during periods of abundance, and is thought to have been fuelled by famine and favoured by natural selection (Prentice, 2005). Such a genotype has become detrimental in the modern ‘obesogenic’ environment, as this is characterized by readily available, high-fat food sources and a reduced requirement for energy expenditure. This in turn may lead to dysregulation of modulators controlling energy balance and in the development of obesity, especially amongst genetically susceptible individuals (Bloom et al., 2008). Obesity results from a state of positive energy balance, in which energy intake exceeds energy expenditure (Berridge, 2004). Maintenance of an overweight phenotype is associated with compensatory changes in energy expenditure and feeding, which counteracts the maintenance of a body weight that is different from the usual weight (Leibel et al., 1995). This suggests that obesity is characterized by defence of an elevated body weight, or metabolic ‘set-point’ (Morton et al., 2006)

1.2 The Physiology of Appetite and Metabolism

1.2.1 Appetite control centres: overview

Energy homeostasis is controlled by the CNS, which monitors metabolic status using a number of neural and humoral signalling mechanisms. Peripheral signals originating from adipose tissue and the gut encode information regarding adiposity and acute energy status respectively. This input is received by the brainstem, often via the vagal nerve, and the hypothalamus in the forebrain, and is interpreted in order to allow homeostatic regulation of food intake and energy expenditure. The brainstem and hypothalamus relay to and receive information from the limbic system and midbrain, which mediate emotion, memory and hedonic reward. Interaction of these homeostatic pathways with higher centres allows an organism to adapt to food source availability and location in an unpredictable environment, ultimately determining survival (Morton et al., 2006). Energy homeostasis appears to be governed by three broad signalling mechanisms: neurotransmitters signalling within feeding centres of the central nervous system (CNS), hormonal signals secreted in proportion to body fat and satiety signals released postprandially from the gastrointestinal (GI) tract (Woods et
The work presented in this thesis focuses on utilising the latter signalling mechanism as a potential therapeutic target for obesity.

### 1.2.1.1 Hypothalamic regulation of energy balance

The hypothalamus is an evolutionarily ancient structure found in the forebrain, above the brainstem and pituitary gland and below the thalamus, occupying the ventral portion of the diencephalon and interfacing with both sides of the third ventricle (Swanson et al., 1987; Simerley and Paxinos, 1998). Its structure, which is highly evolutionarily conserved, is complex, with a number of interconnected nuclei containing neuronal populations that can have opposing or synergistic functions. It is a key homeostatic regulator of functions that are necessary for survival, including thermal regulation, circadian rhythms, osmotic balance, sexual behaviour and energy metabolism. The hypothalamus is divided into nuclei that contain distinct neuronal cell types and perform specific functions, and will be discussed in further detail in section 1.2.2.

Within the arcuate nucleus (ARC) of the hypothalamus, termed the infundibular nucleus in man, there exist two populations of neurones that exert opposing effects on appetite. The anorexigenic neuronal population express the anorectic transcripts proopio-melanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), while the orexigenic population express the orexigenic neuropeptides neuropeptide Y (NPY) and agouti-related peptide (AgRP) (Broberger et al., 1998a; Broberger et al., 1998b; Elias et al., 1998). The close proximity of the hypothalamus to the pituitary gland allows it to act as a direct link between the nervous and endocrine systems. Factors secreted from the hypothalamus act on the anterior pituitary, which is regulated by hypothalamic releasing factors released from the median eminence and transported to the pituitary in the hyophyseal portal circulation, allowing the release of hormones from endocrine cells into the systemic circulation. For instance corticotrophin-releasing-hormone (CRH) is released from the hypothalamus and stimulates adrenocorticotropic hormone (ACTH) release from the anterior pituitary, which mediates the stress response and can modulate food intake (Tsigos and Chrousos, 2002). The posterior pituitary is neuronal in origin and is composed of the neural fibres and terminals of...
magnocellular neurones originating in the supraoptic and paraventricular nuclei of the hypothalamus. Hormones including oxytocin and vasopressin are synthesised in cell bodies of the hypothalamus and transported to the posterior pituitary for release into the general circulation (Kombian et al., 2002).

1.2.1.2 Extra-hypothalamic regulation of energy balance

1.2.1.2.1 The brainstem

The hypothalamus has connections with other brain regions implicated in the control of appetite and energy homeostasis, including the brainstem. The brainstem receives peripheral stimuli from the gastrointestinal tract regarding energy availability and food ingestion, thus providing a neuroanatomical link between the gut and CNS. The afferent vagal nerve, which is a conduit through which mechanoreceptors and chemoreceptors in the gastrointestinal tract can signal to the brainstem, has cell bodies in the nodose ganglia and projects to the dorsal vagal complex (DVC) in the brainstem. The DVC contains key areas involved in appetite regulation: the nucleus tractus solitarius (NTS) and the area postrema (AP), which interface with the hypothalamus (Schwartz, 2000; Abbott et al., 2005a). A number of gastrointestinal (GI) hormones signal to the hypothalamus via vagal-brainstem circuitry, and vagal afferent fibres are also stimulated by gastric distension, nutrient composition, pH and osmolarity, resulting in subsequent neuronal activation within the NTS (Schwartz, 2006). The NTS also contains receptors for the adiposity hormone, leptin, and leptin administration into the NTS reduces food intake (Morton et al., 2006). POMC and NPY neuronal populations within the brainstem extend to the hypothalamus and other brain areas (Ter Horst et al., 1989; Mountjoy et al., 1994). Interestingly, decerebrate rats, in which connections between the brain stem and forebrain are surgically severed, are able to compensate for changes in the composition of individual meals offered to them, but when challenged with a reduction in meal number are unable to adjust their meal size (Grill and Smith, 1988). These data suggest that the brainstem does not just act as a conduit to the hypothalamus, but itself plays a role in homeostatic, short-term adjustments in individual meal consumption. The hypothalamus, in contrast, is thought to control longer term changes in energy homeostasis (Morton et al., 2006).
1.2.1.2.2 Brain areas involved in the hedonic aspects of appetite

Eating is a rewarding experience and the reward value of food is modulated by energy status. Fasted rats display increased hedonic reactivity to food compared to satiated rats, while reward value is reduced after central injection of appetite-modulating hormones, such as leptin (Berridge, 1991). Thus energy restriction is thought to increase the sensitivity of reward-related circuits (Morton et al., 2006). Taste stimuli also interact with brain areas controlling reward and emotion, which is thought to influence appetite. Oral taste receptor cells transmit information regarding taste via afferent sensory fibres to the NTS. The NTS relays this information to the parabrachial nucleus in the hindbrain, the ventral tegmental area (VTA) in the midbrain, and multiple sites in the forebrain, including the nucleus accumbens (NAc), striatum, thalamus and cerebral cortex (Schwartz, 2006; Norgren and Leonard, 1973; Norgren, 1976). Hedonic responses to food intake involve dopamine release from neurones projecting from the VTA to the NAc, striatum and cortex. The lateral hypothalamus, a hypothalamic nucleus that is thought to partly regulate orexigenic behaviour, receives projections from the VTA and NAc, and contains neurones that have a potent orexigenic effect upon stimulation. These neurones are under tonic inhibition that is relieved by the activation of reward pathways (Kelley et al., 2005; Schwartz, 2006; Morton et al., 2006). Single neurone recordings from non-human primates demonstrates that orbitofrontal cortex neurones, which receive projections from the VTA and NAc, will only respond to taste or olfactory stimuli if the primate is hungry. Functional magnetic resonance imaging in humans has demonstrated that taste neurones in the orbitofrontal cortex respond to taste stimuli in a ‘hunger dependent’ manner. Neuronal activity observed in the orbitofrontal cortex in response to a liquid food stimulus has been found to correlate with the decrease in subjective pleasantness of food as it is eaten to satiety. This demonstrates that neurones that respond to the rewarding value of food ingestion in the orbitofrontal cortex are regulated by satiety-related inputs (Kringelbach et al., 2003).

1.2.2 The hypothalamus

The hypothalamus is an evolutionarily conserved structure within the brain which controls energy homeostasis (see fig. 1.1 & 1.2). It surrounds the 3rd ventricle and lies above the pituitary (Simerley and Paxinos, 1998). Specific hypothalamic nuclei were first implicated in
appetite regulation in the 1940s, when Hetherington and Ranson lesioned discrete hypothalamic nuclei in rodents. They found that surgical transection of the ventromedial nucleus, arcuate nucleus, paraventricular nucleus and dorsomedial nucleus disrupted daily food intake, enhancing appetite (Hetherington and Ranson, 1983; Brobeck, 1946). Conversely, it was discovered that destruction of the lateral hypothalamic area in rodents resulted in the cessation of feeding, leading to starvation and death (Anand and Brobeck, 1951). These studies suggested a ‘dual-centre’ model, whereby distinct feeding and satiety centres within the hypothalamus govern appetite. Over the years, this has been expanded using more refined techniques. We now know that feeding is regulated by a complex array of CNS circuits.

![Diagram of hypothalamic nuclei](image)

**Figure 1.1. Schematic diagram showing hypothalamic nuclei in the rodent brain, which are involved in energy homeostasis in mammals.** ARC - Arcuate nucleus, PVN - paraventricular nucleus; VMH - ventromedial hypothalamus; DMN - dorsomedial hypothalamus; LH - lateral hypothalamic area; OC - optic chiasm. Adapted from (Neary et al., 2004).

### 1.2.2.1 The arcuate nucleus

The ARC is thought to contain neurones responsible for mediating food intake. Situated at the base of the hypothalamus next to the floor of the third ventricle, the ARC contains a number of neuronal cell bodies that together form an ‘arc’ or bow shape (Schwartz et al., 2000). The ARC has an incomplete blood-brain barrier and is thus thought to be able to directly respond to circulating peripheral signals such as leptin, ghrelin and insulin (Migrenne et al., 2006; Kalra et al., 1999).
The ARC contains anorectic and orexigenic neurones which synthesize effector molecules that translate peripheral signals into a neuronal response. There are two major populations of appetite-regulating neurones in the ARC which have opposing effects on food intake. Within the ventromedial ARC, there is an orexigenic (appetite-stimulating) neuronal population that expresses neuropeptide Y (NPY), which has high affinity for Y1 and Y5 receptors located on POMC neurones, which express the NPY Y1 receptor and are inhibited by NPY. NPY-expressing neurones co-express agouti-related peptide (AgRP), which is an antagonist at MC3/MC4 receptors on second order neurons regulating food intake in the PVN and LHA (Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993; Bagnol et al., 1999). A neuronal subset adjacent to, but distinct from, the NPY/AgRP neurones express POMC, which is post-translationally processed to generate peptides, including alpha-melanocyte-stimulating hormone (αMSH) (Schwartz, 2006). These POMC neurones co-express cocaine- and amphetamine-related transcript (CART). Both neuronal populations express leptin receptors, which implies that leptin is able to modulate neurones that regulate both anorexigenic and orexigenic output (Cheung et al., 1997; Baskin et al., 1999). NPY/AgRP neurones are inhibited by peripheral satiety signals, such as leptin, insulin and PYY, and stimulated by the peripheral orexigenic hormone ghrelin. Inhibition of NPY/AgRP neurones allows the activation POMC neurones, which are under tonic inhibition from NPY/AgRP neurones (Baskin et al., 1999; Cowley et al., 2001; Cowley et al., 2003). The putative model for appetite regulation by the ARC is that satiety and adiposity-related peripheral signals are sensed by POMC/CART and NPY/AgRP neurones, allowing both anorectic and orexigenic output (see fig. 1.4). This information is then transmitted to second order neurones in other hypothalamic areas, including the PVN, VMH and LH, as well as higher order centres, to facilitate homeostatic control of energy balance (Lenard and Berthoud, 2008; Kalra et al., 1999).

1.2.2.1.1 Regulation of neuronal populations within the arcuate nucleus by neurotransmitters

ARC neurones do not only project to regions outside of the ARC. They also signal within the ARC to regulate neurones. The inhibitory neurotransmitter GABA is released from NPY neurones in the ARC (Horvath et al., 1999). GABA-ergic nerve terminals form synaptic contacts with POMC cell bodies, on which GABAA receptors are found (Meister, 2007;
Ovesjo et al., 2001). Electron microscopy has shown co-localisation of NPY and GABA in synaptic termini that associate with POMC neurones. NPY increases the frequency of GABA-mediated inhibitory post-synaptic potentials in ARC POMC neurones, suggesting there is tonic GABA inhibitory input onto POMC neurones from NPY/AgRP neurones (Cowley et al., 2001). This may represent a fail-safe mechanism to ensure food intake behaviour is possible in the absence of NPY release or ‘hunger’ per se. In support of this, mice lacking the GABA_A transporter in NPY/AgRP neurones in the ARC have a lean phenotype (Tong et al., 2008). The autoinhibitory NPY Y2R is expressed on both NPY and POMC neurones, and it is therefore possible that locally released NPY may inhibit both NPY neurones and POMC neurones, allowing auto-regulation of NPYergic activity and the inhibition of anorectic POMC neurones (Broberger et al., 1997). As both NPY and POMC neurones express the MC3R, a short feedback loop may exist between these neurones. An MC3R agonist has been shown to increase GABA-mediated inhibitory postsynaptic potentials in POMC neurones. This suggests that the expression of MC3R by POMC neurones may allow autoreceptor regulation of POMC neuronal activity in response to α-MSH release, thus providing an extra control mechanism for the effects of anorectic hormones, such as leptin, on POMC neurones (Cowley et al., 2001).

1.2.2.2 The paraventricular nucleus

The PVN is adjacent to the superior side of the 3rd ventricle in the anterior hypothalamus. It is highly vascularised, and PVN neurones project to regions including the posterior pituitary and median eminence. The PVN contains well-characterised magnocellular and parvocellular neurosecretory neurones, which play a role in a variety of behaviours. The PVN sends parvocellular neuronal projections to the median eminence, from where peptides are released into the hypothalamo-pituitary portal system and travel to the anterior pituitary where they regulate the release of pituitary hormones into the general circulation.

The PVN is an important area for integrating and relaying signals mediating energy homeostasis, and is innervated by axons projecting from the ARC NPY/AgRP and POMC/CART neurones, from NPY neurones in the brainstem and from orexin neurones of the LH, which release the corresponding neuropeptides at terminals within the PVN (Baker and Herkenham, 1995; Sawchenko et al., 1985). Microinjection into the PVN of major
orexigenic peptides increases food intake, and in situ hybridisation studies have revealed the presence of orexigenic receptors, including the Y1R and the Y5R within this nucleus (Gerald et al., 1996; Parker and Herzog, 1999; Trivedi et al., 1998). NPY release increases in the PVN in response to fasting, but not in other areas of the hypothalamus, suggesting it is one of the major sites of physiological NPY release that relates to food intake (Kalra, 1991). ARC NPY/AgRP and POMC/CART neurones project extensively to second order CRH and Thyrotrophin-releasing hormone (TRH) neurones in the PVN (Bai et al., 1985; Schwartz, 2001). Alpha-MSH reduces food intake, and NPY increases food intake, when injected directly into the PVN (Kim et al., 2000a; Stanley and Leibowitz, 1985). As injection of CRH or TRH directly into the PVN decreases food intake; these neurones may be stimulated by POMC/CART neurones and/or inhibited by NPY/AgRP neurones (Krahn et al., 1988; Vogel et al., 1979). Destruction of the PVN causes hyperphagia and body weight gain, suggesting that the overall tone in the PVN is anorectic (Leibowitz et al., 1981) However, this phenotype may be due to the lack of anorectic POMC/CART neuronal input from the ARC.

1.2.2.3 The ventromedial hypothalamus

The VMH is located directly above the ARC. Anand and Brobeck discovered that lesions to the VMH resulted in hyperphagia and obesity. The VMH was suggested to be a centre for satiety and to control the hypothalamic ‘set point’ for body weight homeostasis (Brobeck, 1946). The size of the VMH and its neural activity (measured by glucose turnover) is reduced in obesity-prone rats compared to normal rats. This is not the case for the ARC (Levin and Sullivan, 1989; Levin, 1995). Although most peptides involved in the regulation of food intake have not been detected in the VMH, this region does contain receptors for appetite-regulating peptides, including NPY, galanin and leptin (Schick et al., 1993; Stanley et al., 1985; Satoh et al., 1997a). Microinjection of the orexigenic peptides NPY and galanin into the VMH increase food intake. Interestingly, diet-induced obese mice display increased levels of NPY mRNA in the VMH (Guan et al., 1998). Leptin microinjection into the VMH reduces food intake and rats with VMH lesions are unable to respond to leptin (Satoh et al., 1997b). There are POMC projections to the VMH, and the VMH expresses MC3 and MC4 receptors. Alpha-MSH inhibits glutamatergic neurones in the VMH (King, 2006; Fu and van den Pol, 2008). Furthermore, the VMH expresses the anorectic peptide, brain-derived-neurotrophic factor (BDNF), a neurotrophin important in neuronal plasticity and development
as well as energy homeostasis. BDNF expression in the VMH declines during fasting in rodents, an effect prevented by ICV administration of an MC4R agonist (Castren et al., 1995; Xu et al., 2003). BDNF neurones in the VMH may therefore be a downstream mediator of the effects of α-MSH on energy balance. The VMH is also suggested to be a glucose monitoring centre (Borg et al., 1994; King, 2006).

### 1.2.2.4 The dorsomedial nucleus

The DMN is located immediately dorsal to the VMH. Lesioning of the DMN results in hypophagia, hypodipsia and growth retardation in rodents. Like the PVN, the DMN is responsive to most orexigenic peptides, including NPY, ghrelin, galanin and the orexins (Kalra et al., 1999). The DMN is innervated by NPY efferents from the ARC and also extends projections to other hypothalamic nuclei involved in food intake, including the PVN (Bai et al., 1985). The DMN contains CCK cell bodies and CCK-A receptors, and rats with lesions in the DMN show an attenuated response to the effects of peripheral CCK-8 (Bellinger and Bernardis, 1984). Microinjection of CCK-8 into the DMN results in a potent reduction in feeding, and down-regulates NPY gene expression (Chen et al., 2008). Rats lacking CCK receptors are obese and hyperphagic and exhibit an increase in NPY expression in the DMN (Bellinger and Bernardis, 2002). A role for CCK in regulating NPY levels in the DMN has therefore been postulated. Recently, adenoassociated-virus (AAV) was used to overexpress NPY in the rat DMN, resulting in an increase in food intake and body weight, and an exacerbation of diet-induced obesity (Yang, 2009). Furthermore, mice fed on a high fat diet show an increase in c-fos, which is an early gene and marker of neuronal activation, expression in the DMN and rats with lesions in the DMN do not become as obese as sham-operated rats when fed a high fat diet (Bellinger and Bernardis, 2002). This suggests that the DMN may play a physiological role in body weight regulation.

### 1.2.2.5 The lateral hypothalamus

The LH is more vaguely defined than hypothalamic nuclei, and is laterally adjacent to the PVN, VMH and DMN. It comprises a large, diffuse neuronal population that contains a number of melanin concentrating hormone (MCH) and orexin neurones. Lesions of the LH in the rat result in aphagia, adipsia and a reduction in body weight, while electrical stimulation
of the LH causes hyperphagia. These findings led to the ‘dual centre’ hypothesis that the LH is a ‘hunger centre’, interacting closely with the ‘satiety centre’ in the VMH (Steinbaum, 1965; Brobeck, 1946). However, the LH is heavily innervated by serotonergic and catecholaminergic circuitry, and is involved in a wide variety of behaviours that can affect feeding, including circadian rhythms and spontaneous movement (Bernardis and Bellinger, 1996). The LH receives direct input from the ARC and is innervated by extensive NPY, AgRP and α-MSH containing nerve terminals that are in contact with orexigenic MCH and orexin neurones (Broberger et al., 1998a)

Figure 1.2. Three dimensional view of the right hemisphere of the rodent hypothalamus. 3V: third ventricle; AHA: anterior hypothalamic area; ARC: arcuate nucleus; AV3V: anteroventral area of third ventricle; CI: capsula interna; DP: dorsal parvocellular subnucleus of paraventricular nucleus (PVN); DMN: dorsomedial nucleus; F: fornix; LHA: lateral hypothalamic area; LM: lateral magnocellular subnucleus of paraventricular nucleus; LPOA: lateral preoptic area; ME: median eminence; MP: medial parvocellular PVN; MPO: medial preoptic area; OT: optic tract; Pe: periventricular nucleus; ScH: suprachiasmatic nucleus also (SCN); SON: supraoptic nucleus; SI: substantia inomminata; ST:
subthalamic nucleus; VMH: ventromedial hypothalamus; VP: ventral parvocellular subnucleus of paraventricular nucleus (Berthoud, 2002).

1.2.3 Hypothalamic neuropeptides regulating appetite control

1.2.3.1 The melanocortin system

Melanocortins are products of the POMC gene and the POMC precursor polypeptide. The four melanocortins are ACTH, α-MSH, β-MSH and γ-MSH, which mediate their effects through five G-protein-coupled receptors (GPCR) classified MC1R- MC5R. The opioid receptor ligand, β-endorphin, is another class of peptide encoded by the POMC gene (see fig. 1.3). The POMC gene is expressed in the pituitary, where it is processed to produce ACTH, which is released into the general circulation to act on MC2R in the adrenal gland to regulate glucocorticoid production. POMC is also expressed in the skin and hair follicle, where melanocortins act in a paracrine manner to regulate pigment production through the MC1R. The MC5R is also expressed in the periphery, where it appears to regulate secretions from exocrine glands (Cone, 2005). In the brain, POMC is expressed in neurones within the ARC of the hypothalamus and the NTS in the brainstem. Melanocortins then act as agonists at downstream sites expressing the MC3R and MC4R. As previously described, POMC is co-expressed with CART in most POMC-positive neurones in the rostro-caudal ARC (see section 1.2.2.1) (Jacobowitz and O'Donohue, 1978; Haskell-Luevano et al., 1999; Williams, 2005). AgRP is another neuropeptide that is a component of the melanocortin system. AgRP acts as an endogenous antagonist at the MC3R and MC4R, which will be further discussed later in this section (Ollmann et al., 1997).
Melanocortins are involved in mediating the effects of metabolic signals upon food intake and energy expenditure. ICV administration of α-MSH reduces food intake (Poggioli et al., 1986). Alpha-MSH also stimulates the release of TRH from hypothalamic explants and increases plasma TSH levels. These effects on the HPT axis may account for the stimulatory effects of α-MSH in rodents on energy expenditure (Pierroz et al., 2002; Kim et al., 2000b).

POMC levels in the ARC are sensitive to energy status; fasting reduces levels of POMC mRNA in the ARC. In contrast, the administration of leptin increases levels of POMC mRNA (Mizuno et al., 1998; Schwartz et al., 2000). Genetic defects in the POMC gene result in early-onset obesity, adrenal insufficiency and red hair pigmentation in humans (Krude et al., 1998). This phenotype has been recapitulated in rodent models. POMC knockout mice display obesity, hyperphagia, altered pigmentation, adrenal insufficiency, and, interestingly, a decrease in metabolic rate (Coll and Loraine Tung, 2009).

The MC3R and MC4R are expressed predominantly in brain regions implicated in energy homeostasis (Mountjoy et al., 1994). MC3R or MC4R agonists reduce food intake, increase oxygen consumption and decrease respiratory quotient in rodents (Fan et al., 1997; Hwa et
ICV administration of MC3R/MC4R antagonists increases food intake in rodents and MC4R knockout mice are obese, hyperinsulinaemic and hyperphagic (Huszar et al., 1997). These data suggest a role for the melanocortin receptors in tonic regulation of feeding and energy expenditure. A frameshift mutation in the MC4R locus was the first heritable mutation discovered to cause obesity in humans (Yeo et al., 1998). A number of MC4R mutations have subsequently been recognised as heritable causes of morbid obesity in humans (Vaisse et al., 2000). The role of the MC3R in obesity is less well-defined, as the high-affinity MC3R agonist γ-MSH has not been reported to affect food intake when administered ICV (Abbott et al., 2000). However, MC3R knockout mice exhibit increased fat mass and an increase in the ratio of weight gain to food intake, though they are not hyperphagic (Chen et al., 2000). These data suggest that the MC3R does play a role in energy homeostasis. Further experiments are required to characterise this role.

AgRP is a 40 amino acid neuropeptide, which acts as a functional antagonist of melanocortin signalling by binding the MC3R and MC4R (Ollmann et al., 1997). In mice, AgRP overexpression under the β-actin promoter, which allows ubiquitous overexpression, results in an obese phenotype similar to that of MC4R knockout mice (Graham et al., 1997; Huszar et al., 1997). Furthermore, ICV administration of AgRP at picomolar doses results in hyperphagia that lasts over 24h (Hagan et al., 2000). This is initially mediated by functional antagonism of the MC3R and MC4R, as ICV administration of AgRP blocks the anorectic effect of a MC3R/MC4R agonist administered simultaneously. However, when AgRP is administered 24h prior to an MC3/MC4R agonist, it does not block the anorectic effect of a MC3R/MC4R agonist, suggesting that the orexigenic effect of AgRP is maintained by an a mechanism other than ameliorating melanocortin signalling at later time points (Hagan et al., 2000). AgRP is found primarily in the ARC, where 95% of AGRP-immunoreactive neurones co-express NPY. These AgRP/NPY neurones project to other hypothalamic nuclei important in the regulation of appetite, including the PVN and DMN (Broberger et al., 1998b). AgRP expression is dependent on nutritional state, as AgRP levels increase 18-fold following a 48h fast in mice, whereas POMC levels exhibit only a moderate change, falling 25% after a 96 hour fast (Hahn et al., 1998; Brady et al., 1990). This suggests that the hypothalamus responds to a negative energy state by increasing the AgRP expression, allowing the down-regulation of anorectic melanocortin tone by increasing antagonistic activity at the
melanocortin receptors. Conversely, a state of positive energy balance results in a reduction in AgRP expression, allowing an increase in anorectic melanocortin tone (see fig. 1.4).

1.2.3.2 CART

Cocaine- and amphetamine- regulated transcript (CART) is a transcript that is so named because its expression in the brain (Douglass et al., 1995) was found to be upregulated by acute administration of cocaine or amphetamine (Douglass and Daoud, 1996). Alternative splicing of the transcript, followed by removal of a propeptide signal sequence gives rise to a number of peptides, including CART (55-102), which is the putative active CART peptide (Kristensen et al., 1998). CART mRNA is found in the ARC (where it is co-expressed with POMC mRNA), PVN, SON and rostral VMH. CART is thought to play a role in addictive and reward related behaviours; it has a variety of effects on dopaminergic systems in the brain and is increased in the nucleus accumbens of cocaine users (Murphy, 2005). Though a receptor has not yet been identified, the finding that CART activates neurones in nuclei related to appetite, the ARC, PVN and DMN, suggests that CART receptors are expressed in these sites (Vrang et al., 1999b). In the PVN, CART is co-localised with TRH and with the orexigenic peptide galanin, while in the LH and DMN CART is co-localised with the orexigenic peptide MCH (Vrang et al., 1999a; Broberger, 1999). ICV administration of CART (55-102) reduces dark phase and fasting-induced feeding in rodents (Kristensen et al., 1998; Kalra et al., 1999). ICV administration of a CART antiserum to rats increased feeding, suggesting a physiological role for CART in appetite regulation (Kristensen et al., 1998). Furthermore, CART mRNA was found to be downregulated in the ARC of obese animal models and upregulated after peripheral administration of leptin to obese mice (Lambert et al., 1998). However, CART has been reported to have an orexigenic effect when administered into discrete hypothalamic nuclei, including the ARC and VMH (Abbott et al., 2001). These data suggest that CART may play a role in transmitting information between both anorectic and orexigenic appetite circuits in the hypothalamus. Further studies are required to elucidate the role of CART in energy homeostasis.
1.2.3.3 CRH

CRH-expressing neurones are localized in the PVN (Dallman et al., 1993). CRH is a 41 amino acid peptide, and is the main hypothalamic hormone stimulating the production of ACTH from the pituitary, which in turn stimulates glucocorticoid release from the adrenal gland. Glucocorticoids are the major peripheral hormones mediating the stress response in mammals. CRH also signals with the CNS. CRH mediates its effects via the GPCRs CRH1 and CRH2, which are expressed throughout the brain (Wong et al., 1994). Intracerebroventricular administration of CRH reduces dark-phase and fasting induced feeding in rats, an effect that is thought to be mediated via the CRH-1 and CRH-2 receptors in the PVN (Morley and Levine, 1982; Kalra et al., 1999). Peripheral administration of CRH increases energy expenditure and fat oxidation in humans (Smith et al., 2001). A CRH antagonist has also been shown to prevent stress-induced anorexia using a tail pinch stress paradigm (Heinrichs et al., 1992). Recently, ICV administration of BDNF has been found to increase CRH expression in the PVN and that the effect of ICV BDNF on feeding is CRHR-2 dependent, suggesting CRH may regulate anorectic BDNF signalling in the PVN (Toriya et al., 2010). However, the role of CRH in physiological appetite regulation is controversial (Kalra et al., 1999).

1.2.3.4 NPY

NPY is a 36 amino acid member of the PP-fold family of peptides that includes the gut hormone PYY and the pancreatic hormone, pancreatic polypeptide (PP) (Tatemoto et al., 1982). The PP-fold family will be discussed in further detail in section 1.2.6.1. There are 5 distinct types of receptor in mammals that bind the PP-fold peptides, denoted Y1R-Y5R. However, although the Y3R has been designated an NPY-preferring subtype after pharmacological studies using a variety of different tissues, it has not been cloned despite several attempts. The existence of the Y3R is thus a source of contention due to the possibility that the Y3R pharmacological profile represents a mix of those of the cloned receptors (Berglund et al., 2003).

Each Y receptor has a unique distribution pattern and function according to the nature and location of its ligand and the affinity with which it binds. All known PP-fold peptide receptors are found in neuronal cells of the peripheral and central nervous systems, as well as
non-neuronal tissues, and are able to bind two out of the three PP-fold ligands (Berglund et al., 2003). The Y receptor/PP-fold family system is unique in that it provides an example of a multireceptor/multiligand system in which three full-length peptides share four receptors for which they have different affinities. At endogenous levels these peptides may share functions or at least modulate those of each other. In short, NPY and PYY have high affinity for Y1R, Y2R and Y5R, while PP has high affinity for Y4R (Lindner et al., 2008). Y1R and Y4R require the entire N terminus of their ligands for binding, while Y5R is able to bind peptides that lack the first amino acid and Y2R is able to bind much shorter ligands (NPY13-36) or analogues with central deletions (Lindner et al., 2008). However the C-terminal region has been found to be essential for Y receptor binding for all natural ligands (Merten et al., 2007). A number of acidic residues, including Asp$^{6.59}$ located in the extracellular loops of the Y receptors have been proposed to form electrostatic interactions with basic residues in the ligand, allowing binding of the ligand to its Y receptor. The effects of NPY on energy homeostasis are thought to be mediated by the Y1R and Y5R (see fig. 1.4).

NPY is abundant in the peripheral and central nervous systems, where it has a variety of established and putative functions (Chronwall and Zukowska, 2004). In the hypothalamus NPY-containing cell bodies are found primarily in the ARC and DMN, and dense fibre networks are found in other areas of the hypothalamus, including the PVN (Pelletier, 1990). The AgRP/NPY co-expressing neurones in the ARC are the best characterised NPY-expressing neuronal population (Hahn et al., 1998). NPY neurones from the ARC innervate the PVN, DMN and LH, as NPY neurone destruction prevents the formation of NPY-immunoreactive fibres innervating these areas (Bai et al., 1985). Y1R and Y5R mRNA is found in the supraoptic nucleus (SON) and the ARC, where Y1R is prominent in the lateral POMC neurone-containing portion (Parker and Herzog, 1999). Pre-treatment of rats with a Y1R antagonist or leptin prevents the effect of NPY on feeding and prevents the expression of c-fos in the DMN and the magnocellular region of the PVN, showing these areas are activated in response to centrally-administered NPY, and are possible targets for physiological NPY signalling (Yokosuka et al., 1998). NPY is a potent orexigen. ICV administration or microinjections of NPY into hypothalamic nuclei including the PVN, LH and VMH stimulates appetite. Chronic ICV administration of NPY causes obesity in rodents (Clark et al., 1984; Stanley et al., 1985; Zarjevski et al., 1993). Evidence also suggests that NPY plays a role in modulating energy expenditure. Intra-PVN administration of NPY causes changes in markers of thermogenesis and lipogenesis, including a decrease in uncoupling
protein-1 (UCP-1) in brown adipose tissue (BAT) and an increase in lipoprotein lipase activity in white adipose tissue (WAT) (Billington et al., 1994). This suggests NPY may promote a reduction in energy expenditure and the deposition of fat during a state of negative energy balance. There is also evidence for a physiological role of NPY in the regulation of appetite and energy expenditure. In rodents, NPY immunoreactivity exhibits diurnal variation, with the highest NPY concentration in the ARC and PVN evident at the onset of the dark phase, the natural feeding period for nocturnal animals (Jhanwar-Uniyal et al., 1990). NPY immunoreactivity in the PVN is increased following a fast. Blockade of endogenous NPY signalling by ICV administration of an NPY-antibody attenuates fasting-induced feeding by 30% in rats (Lambert et al., 1993; Calza et al., 1989). Unexpectedly, NPY knockout mice display normal feeding behaviour and body weight, and display normal fasting-induced hyperphagic behaviour (Erickson et al., 1996). A variety of explanations have been offered for this counter-intuitive finding. The most likely appears to be that developmental compensation allows alternative orexigenic circuits to fulfil the role of NPY when it is absent (Chronwall and Zukowska, 2004). Post natal ablation of NPY/AgRP neurones results in starvation, whereas neonatal ablation has no effect on feeding behaviour. These authors proposed developmental compensatory mechanisms in NPY/AgRP-lacking neonates allowed normal feeding regulation (Luquet et al., 2005). The adaptive ability of hypothalamic neuronal circuits regulating behaviours critical for species survival, such as feeding, for instance through neuronal and synaptic plasticity, may underlie the lack of phenotype of neonatal NPY and NPY/AgRP knockout mice.

1.2.3.5 Orexin A & B

Orexin A (33 amino acids) and Orexin B (28 amino acids), also known as hypocretins 1 and 2 respectively, were discovered by two groups concomitantly (De et al., 1998; Sakurai et al., 1998). The orexins are synthesized in neurones of the dorsal and lateral hypothalamus that innervate brain areas involved in appetite regulation, including the ARC, PVN and brainstem (De et al., 1998; Sakurai et al., 1998). Orexin A and B stimulate food intake when administered centrally, and prepro-orexin mRNA, which encodes both neuropeptides, is upregulated in fasted animals. The orexins mediate their effects via the G-protein coupled orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R). Orexin A has a ten-fold greater binding affinity for OX1R over OX2R, whereas both orexin A and B bind with similar
affinity to OX2R. Though both OX1R and OX2R are expressed widely in the brain, including in the hypothalamus, the receptors do have distinct expression patterns: OX1R is found primarily in the locus coeruleus, while OX2R is found in the tuberomamillary nucleus and other regions associated with wakefulness (Trivedi et al., 1998; Marcus et al., 2001). Orexin A stimulates food intake more potently than orexin B, which may be due to activation of both OX1R and OX2R (Sakurai et al., 1998). Microinjection of orexin A into the PVN and LH, the sites of OX2R immunoreactivity, also stimulates feeding. Orexin efferents from the LH make direct synaptic contact with NPY/leptin receptor co-expressing neurones in the ARC, suggesting that orexin neurones may mediate appetite through a direct excitatory effect at leptin-regulated NPY neurones in the ARC (Horvath et al., 1999). In support of this, orexin-induced feeding is suppressed by pre-treatment with Y1R or Y5R antagonist administered ICV in rodents (Dube et al., 2000; Yamanaka et al., 2000). Interestingly, orexin neurones have recently been found to be sensitive to a variety of peripheral metabolic cues. Orexin neurones in the LH are activated by hypoglycaemia via the vagal-brainstem-hypothalamic pathway, and the hypothalami of hypoglycaemic rats exhibit a ten-fold increase in orexin B immunoreactivity (Cai et al., 2001). Orexin also appears to be involved with ghrelin-induced actions, since pre-treatment of rodents with anti-orexin antibodies prevents the orexigenic effects of ghrelin (Toshinai et al., 2003).

The data supporting a role for orexin in energy homeostasis must be interpreted with caution, since orexins are highly involved in promoting wakefulness. Analysis of post-mortem brains from patients with the sleep disorder narcolepsy has revealed low levels of central orexin, and a reduced number of orexin neurones in the hypothalamus (Thannickal et al., 2000). A well-characterised canine model of narcolepsy, which is very similar to human narcolepsy, is also associated with a loss of orexin neurones and orexin in cerebrospinal fluid (Ripley et al., 2001). Dogs with familial narcolepsy have a mutation in the OX2R gene, but normal orexin levels (Lin et al., 1999). Mice with ablated orexin neurones exhibit a narcolepsy-like phenotype, as well as late-onset obesity despite hypophagia, which suggests a role for orexin in the link between energy balance and wakefulness (Hara et al., 2001). It has been proposed that orexin neurones may promote wakefulness during periods of negative energy balance (Tsujino and Sakurai, 2009).
1.2.3.6 MCH

MCH is synthesized in the LH in a population of neurones which projects to a number of other hypothalamic areas involved in appetite regulation. MCH is so named because it was originally discovered as a pituitary peptide with a pigment aggregation property in the salmon form (Kawauchi et al., 1983). In mammals, MCH is a 17 amino acid peptide that retains the conserved ring structure seen in salmon (Vaughan et al., 1989). MCH binds to the receptors MCHR1 and MCHR2, which are both GPCRs. MCH-R1 mRNA is found in the brain regions to which MCH-immunoreactive neurones project, and in the hypothalamus is most highly expressed in the DMN, VMH, PVN and ARC (Kokkotou et al., 2001). MCHR2 has 38% sequence identity to MCHR1 and is not found in rodents. For this reason studying the MCHR2 has proven more difficult and less is known regarding the role of MCHR2 in mediating the effects of MCH in other species such as humans (Nahon, 2006). ICV administration of MCH stimulates food intake in rodents. A putative physiological role for MCH in the regulation of energy balance is supported by the finding that MCH mRNA is upregulated by fasting in rodents and MCH knockout mice have reduced body weight due to hypophagia and increased metabolic rate (Qu et al., 1996; Shimada et al., 1998). Chronic oral administration of an MCH1 receptor antagonist reduces body weight and increases energy expenditure in diet-induced obese mice (Nahon, 2006; Ito et al., 2009; Ito et al., 2010). MCH1 receptors may therefore be useful pharmacological targets for obesity, though the role of MCH in other physiological factors, including reproduction, mood, growth and water intake, may lead to side effects (Rivera, 2008).

1.2.3.7 Opioids

Most mammals will eat beyond their metabolic requirements when given access to highly palatable foods. There are thought to be in-built systems identifying the rewarding nature of certain foods beyond their nutritional content. The rewarding aspects of food consumption are commonly associated with the hedonic aspects of addiction. Opioids are thought to be key mediators of palatability and the hedonic aspects of feeding, as well as reward (Kelley et al., 2002). There are three endogenous, biologically active opioids in the hypothalamus. β-endorphin is a 31 amino acid peptide encoded by the POMC gene, dynorphin A is an 8 amino acid peptide encoded by the prodynorphin (PDYN) gene and the enkephalins, in the form of
either Met-or Leu-enkephalin, are 5 amino acid peptides encoded by the proenkephalin (PENK) gene. The POMC, PDYN and PENK genes share a similar overall structure, and their respective propeptides have conserved cysteines in the N-terminal region and an opioid ‘core’ region consisting of a Tyr/Phe-Gly-Gly-Phe motif. Endogenous opioids bind the mu, delta and kappa opioid receptors (Sundstrom et al., 2010; Kalra et al., 1999).

β-endorphin is expressed in the ARC and brainstem, and activates the mu receptor to increase food intake. Central administration of β-endorphin increases food intake in rodents (Kalra et al., 1999). Enkephalin and dynorphin-producing neurones are more widely distributed in the hypothalamus, and are thought to mediate their orexigenic effects via the delta and kappa receptor subtypes. Opioids may have a physiological role in food ingestion mediating both metabolic need and palatability. NPY neurones make synaptic contact with β-endorphin dendrites and ICV NPY increases β-endorphin release in the PVN (Horvath et al., 1999). Furthermore, the non-specific opioid receptor antagonist naloxone attenuates feeding in response to NPY administered into the PVN. Naloxone was found to reduce NPY-induced neuronal activation in the brainstem and medulla, but not in the hypothalamus. In contrast, naloxone increased neuronal activation following palatable food intake. These data suggest that opioid receptors in the brainstem may play a role in post-ingestive feedback in the brainstem and PVN, and in mediating palatable food intake (Glass et al., 1999). Unexpectedly, β-endorphin null mice that retain normal melanocortin signalling are hyperphagic and obese, which was unexpected in light of the orexigenic effect of β-endorphin (Appleyard et al., 2003). The physiological importance of opioid signalling in energy homeostasis is still unknown, but it is widely accepted that their receptors are involved in mediating the reward-based aspects of appetite.

1.2.3.8 Galanin

Galanin is a 29 amino acid, C-terminal amidated peptide originally discovered in the intestine (Tatemoto et al., 1983). Galanin activates three GPCRs: GALR1 and GALR2, which have similar affinity for galanin, and GALR3, which has a lower affinity for galanin compared to GALR1 and GALR2. These three receptors exhibit distinct but overlapping expression throughout the periphery and the CNS, including the hypothalamus (Lang et al., 2007). Populations of galanin-producing neurones exist within the ARC, DMN and PVN, and
innervate most areas of the hypothalamus. Administration of galanin into the PVN, LH and VMH increases food intake in satiated animals, though to a lesser extent than NPY (Schick et al., 1993). Galanin neurones make direct contact with NPY neurones in the ARC and in the PVN, which suggests some cross-talk between these neurones. Opioids appear to mediate part of the effect of galanin on feeding, as pre-treatment with naloxone attenuates the orexigenic effect of galanin, and galanin synapses are also found in close proximity to NPY neurones in the ARC. Total hypothalamic galanin levels do not change in response to fasting and changes in leptin, suggesting that galanin may not be a physiological orexigen during periods of negative energy balance (Kalra and Horvath, 1998). Chronic ICV infusion of galanin does not increase food intake and body weight gain (Smith et al., 1994). Whether galanin plays a physiologically significant role in energy balance and appetite remains to be determined (Leibowitz, 2005).

1.2.3.9 Endocannabinoids

The endocannabinoids are lipids derived from arachidonic acid that are synthesized enzymically from precursors in cell membranes. Anandamide and 2-arachidonoylglycerol (2-AG) are the two most widely studied endocannabinoids, and are ligands for the cannabinoid receptors CB1R and CB2R, GPCRs that share 44% sequence identity. CB1R is found ubiquitously, but is most highly expressed in the brain and spinal cord, while CB2R is expressed in the periphery, most highly in immune and adipose cells (Onaivi, 2009). However, a second CB2R isoform has recently been identified in the brain (Liu et al., 2009). The study of endocannabinoids has revealed a system which may interlink hedonistic and homeostatic aspects of appetite within the brain and periphery.

Anandamide and 2-AG act on CB1 receptors in the CNS to increase food intake. CB1 receptor blockade results in hypophagia and weight loss, and CB1 receptor null mice exhibit a reduction in fasting-induced feeding. Administration of 2-AG into the nucleus accumbens shell, an area that processes the rewarding/addictive value of food, potently increases food intake, an effect attenuated by administration of a CB1R antagonist (Kirkham et al., 2002). Anandamide and 2-AG levels increase in the limbic forebrain and 2-AG levels increase in the hypothalamus in response to fasting. These results suggest endocannabinoids physiologically regulate the response to fasting in the limbic system, which contains areas that process
emotion and reward, as well as in the hypothalamus. Therefore, the endocannabinoids may mediate appetite motivation. Defective leptin signalling results in elevated hypothalamic levels of endocannabinoids in rodent models, and acute leptin treatment reduces elevated levels of cannabinoids in ob/ob mice (Di, V et al., 2001). This suggests there is tonic activation of CB-1 receptors by endocannabinoids in a leptin-dependent manner. Endocannabinoid levels in plasma and adipose tissue are elevated in diet-induced obese rodents and obese humans, and a CB1R knockout mouse is resistant to diet-induced obesity (De Kloet and Woods, 2009). A CB-1 receptor antagonist, rimonabant, has been used to treat obesity in humans. Rimonabant has now been withdrawn from the market due to an increase in the incidence of mood-related disorders (Christensen et al., 2007). The psychiatric side effects associated with CB-1 receptor antagonism are likely to be due to the widespread expression of CB-1 receptors in the brain and their involvement in a number of cognitive and reward systems (De Kloet and Woods, 2009).
Figure 1.4. Illustrative representation of putative model for regulation of appetite by the arcuate nucleus. The functionally opposing Neuropeptide Y (NPY)/Agouti-related peptide (AGRP) and proopio-melanocortin (POMC)/Cocaine-and-amphetamine-related transcript (CART) neurones within the ARC form a regulated network due to interconnecting fibres. Both sets of neurones project to the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA), which are sites of second-order neurones involved in the regulation of food intake. Circulating factors such as the hormones leptin and insulin inhibit NPY/AgRP and stimulate POMC/CART neurones via the leptin receptor (Lepr) and insulin receptor respectively. Leptin is able to suppress orexigenic NPY/AgRP neuronal activity and stimulate anorectic POMC/CART neuronal activity via the Lepr. Ghrelin is secreted from the stomach and is able to stimulate NPY/AgRP neurones via the ghrelin receptor (Ghsr). Y1r: Y1 receptor, Mc3r: melanocortin 3 receptor, Mc4r: melanocortin 4 receptor. Adapted from (Barsh and Schwartz, 2002).
1.2.4 Peripheral adiposity signals

Over 50 years ago Kennedy introduced an “adiposity negative-feedback” model for energy homeostasis. This model suggests that the brain detects changes in body fat mass through circulating signals from the periphery. The brain is then able to implement homeostatic measures to adjust energy input/output to achieve the long term stability of fat stores observed in most humans (Kennedy, 1953).

The criteria for a putative negative feedback signal include; 1) the signal must circulate at levels proportional to body fat content and must enter the brain, 2) the signal must promote weight loss by acting upon neuronal circuits involved in energy balance and 3) inhibition of the these neuronal actions must result in hyperphagia and increased body weight. Many nutrients (e.g. free fatty acids, glucose), cytokines (e.g. IL-6, TNF-α) and hormones (e.g. glucocorticoids) fulfil several of these criteria, but currently only insulin and leptin fulfil all of them (Morton et al., 2006).

1.2.4.1 Leptin

Leptin is a 167 amino acid protein synthesized primarily in adipocytes. Leptin binds six receptors (Ob Ra-Ob Rf), which are widely expressed. The long form of the leptin receptor, Obese Receptor B (ObRb), has an extended intracellular domain that is necessary for leptin’s effects on appetite. Ob-Rb belongs to the gp 130 family of cytokine receptors. ObRb is coupled to the JAK-STAT signal transduction pathway (Margetic et al., 2002), activation of which in leptin-responsive neurones results in the inhibition of the release of orexigenic peptides, such as NPY, MCH and AgRP, and stimulation the release of anorexigenic factors, including α-MSH, CART and CRH (Schwartz et al., 2000; Morton et al., 2006).

In the 1970s, Coleman reported mutations in the ob and db genes that caused obesity and diabetes. Coleman expanded on Kennedy’s hypothesis by performing parabiosis experiments. Parabiosis of the ob/ob mouse to a WT mouse rescued diabetes in the ob/ob mouse. However, parabiosis of the db/db mouse with a WT mouse did not rescue the obesity and diabetes observed (Coleman and Hummel, 1969; Coleman, 1973). These results suggested the ob gene codes for a putative factor regulating energy homeostasis. It was not until 1994 that the ob gene was cloned, allowing the discovery that a mutation in the ob gene resulted in absence of
its protein product (later named leptin), resulting in the ob/ob phenotype (Zhang et al., 1994). The ob protein was then shown to be undetectable in ob/ob mice and high in db/db mice. Chronic administration of leptin to ob/ob mice reduced body weight, food intake, circulating glucose and insulin, and restored metabolic parameters and energy expenditure to wildtype levels (Pellemounter et al., 1995). An inactivating mutation in ObRb explains the leptin-resistant phenotype of the db/db mouse (Chen et al., 1996).

In the ARC, ObRb is expressed primarily on NPY and POMC neurones (Cheung et al., 1997). ObRb mRNA is increased in NPY/AgRP neurones during fasting and leptin inhibits NPY mRNA expression in the ARC and NPY neurones in vitro, suggesting leptin inhibits NPY/AgRP neurones (see fig. 1.4) to produce its anorectic effect (Spanswick et al., 1997; Kalra et al., 1999). Leptin is most effective at reducing food intake when administered into the ARC, but the VMH and LH are also responsive (Satoh et al., 1997a). In line with this, restoration of leptin signalling exclusively in the ARC attenuates, but does not prevent, hyperphagia and obesity in ObRb-null rats (Schwartz et al., 1996). Leptin activates POMC neurones in the ARC and increases the expression of POMC and CART mRNA, suggesting differential regulation of NPY/AgRP and POMC/CART neurones in the ARC (Cowley et al., 2001; Kristensen et al., 1998). When leptin levels are reduced during fasting, the HPA axis is activated, whilst the reproductive and thyroid axes, as well as the immune system, are suppressed, indicating leptin can regulate a number of physiological systems (Ahima et al., 1996; Lord et al., 1998).

Humans with inactivating mutations in the ob gene exhibit congenital obesity, which is responsive to leptin treatment (Farooqi et al., 1999). However, the majority of obese humans have high circulating leptin and are resistant to the anorectic effects of leptin as observed in ob/ob mice (Considine et al., 1996). Furthermore, while peripheral or ICV administration of leptin reduces food intake in lean animals, these anorectic effects are attenuated in obese rodents (Cusin et al., 1996; Halaas et al., 1995). The prevalence towards leptin resistance in the obese may reflect the fact that inability to store fat when food is easily accessible is evolutionarily disadvantageous in the long term.
1.2.4.2 Insulin

Insulin is a 51 amino acid peptide secreted in proportion to adiposity by pancreatic β cells. In addition to its role in glucose homeostasis, insulin also affects fat metabolism and regulates the release of fat stores from the liver (Bhathena, 2006). Insulin binds to the insulin receptor, a ubiquitous tyrosine kinase transmembrane receptor, resulting in an intracellular cascade allowing influx of glucose, through recruitment of the Glut-4 transporter. Obesity is characterized by hyperinsulinaemia, insulin resistance and hyperglycaemia, all features of the metabolic syndrome and type II diabetes. Insulin receptors are widely expressed in the CNS. In the hypothalamus, they are most highly expressed in the ARC (Havrankova et al., 1978; Werther G.A. et al., 1987). Studies suggest that central insulin signalling plays a key role in regulating energy homeostasis. Insulin enters the brain in proportion to its circulating levels, and central administration of insulin to rodents reduces food intake and body weight (Baura et al., 1993; Woods et al., 2004). In support of a physiological role for insulin in energy balance, chronic central infusion of insulin antibodies increases food intake and body weight in rats (McGowan et al., 1992). Mice deficient in neuronal insulin receptors develop obesity and impaired fertility, suggesting that insulin physiologically regulates energy homeostasis and reproduction via central pathways (Bruning et al., 2000).

Together this evidence suggests that leptin and insulin may both act as signals of body fat stores and modulate energy balance through cross-talk at the level of the ARC.

1.2.5 Hormones secreted from the GI-tract regulating energy balance

The gastrointestinal (GI) tract is the largest endocrine organ in mammals, and releases over 20 peptide hormones that regulate endocrine processes. Most of these hormones are released in response to gut nutrient load, and are able to modulate the digestive process, by acting on tissues including smooth muscle, paracrine glands and the peripheral nervous system. Concomitantly, these hormones signal a change in metabolic status, which in turn influences physiological and behavioural adaptation (Fried et al., 1988). Acute feelings of hunger and satiety are thought to be mediated by changes in circulating gut hormones. The primary sites upon which these hormones are thought to act in mediating appetite are the vagus nerve, brainstem and hypothalamus (Murphy, 2006).
1.2.5.1 Pancreatic polypeptide

A PYY gene duplication event is thought to have given rise to the gene coding for PP, a 36 amino acid peptide structurally similar to NPY and PYY, which is also a member of the PP-fold family (Hort et al., 1995). PP has a high affinity for the Y4R, which is widely expressed peripherally and centrally. In the brain it is expressed in the brainstem and in hypothalamic nuclei involved in appetite regulation, including the PVN, DMN, VMH and ARC (Parker and Herzog, 1999; Small et al., 1997). In contrast to other Y receptors, the Y4R is the least conserved receptor; there is only 75% identity between the human and rat sequences (Larhammar, 1996). PP is secreted in proportion to the calorific content of food. Levels of circulating PP remain elevated for up to 6 hours postprandially (Adrian et al., 1976). Central and peripheral administration of PP dose-dependently reduces food intake in rodents (Asakawa et al., 1999). Peripheral administration in normal-weight humans has also been shown to reduce appetite but the effect of PP in obese humans has yet to be reported (Batterham et al., 2003b; Jesudason et al., 2007). Transgenic mice overexpressing PP exhibit reduced food intake and body weight (Ueno et al., 1999). The reduction in food intake by PP is abolished by vagotomy in rodents, suggesting PP acts via the vagal-brainstem-hypothalamic route to mediate food intake. Peripheral administration of PP reduces hypothalamic NPY expression and orexin expression in the LH, and increases POMC expression in the ARC and BDNF expression in the VMH (Asakawa, 2003; Sainsbury et al., 2010). Peripheral administration of PP activates early gene expression in POMC neurones in the ARC, and has no effect in mice lacking hypothalamic MC4R, which supports a role for the melanocortin system in mediating the effects of PP on appetite (Lin et al., 2009). PP administration also increases energy expenditure; increasing oxygen consumption and the discharge of sympathetic efferent nerves innervating the adrenal gland and BAT (Asakawa, 2003). As the vagus has been shown to be essential for its effects, PP is hypothesized to have an indirect effect on the hypothalamus to reduce food intake. At present the physiological role of PP in appetite remains unknown.
1.2.5.2 The preproglucagon derived peptide hormones

The endocrine L-cells in the GI tract are responsible for the release of a number of gut hormones. The L-cells release a large precursor protein known as preproglucagon. Post-translational processing of preproglucagon results in a number of biologically-active peptides, including glucagon, GLP-1 and glucagon-like peptide 2 (GLP-2) and oxyntomodulin (OXM). Of these, GLP-1 and OXM play a role in appetite, and are discussed in detail below.

1.2.5.2.1 GLP-1

GLP-1 is secreted from intestinal L cells in two major forms: GLP-1\textsubscript{1-37} and GLP-1\textsubscript{1-36 amide}. Further processing of these forms results in GLP-1\textsubscript{7-37} and GLP-1\textsubscript{7-36amide}; the latter is the major circulating form, and will be referred to as GLP-1 from now on. GLP-1 is a powerful incretin that at physiological concentrations stimulates biosynthesis and secretion of insulin (Gutniak et al., 1992). The serine protease DPP-iv inactivates GLP-1 (Mentlein, 1999). Both GLP-1\textsubscript{7-37} and GLP-1\textsubscript{7-36amide} are equally potent in activating the GLP-1 receptor, which is widely expressed throughout the periphery and in the hypothalamus, where it is found in the PVN and ARC (Bullock et al., 1996; Thorens and Waeber, 1993; Shughrue et al., 1996). Peripheral administration of GLP-1 increases c-fos expression in the PVN, NTS and area postrema, but not in GLP-1R knockout mice, suggesting that the actions of GLP-1 are mediated by central GLP-1R in these sites (Baggio et al., 2004). GLP-1 is also synthesized within the CNS, and is found in neurones in the area postrema and the NTS. These neurones send projections to the DMN and PVN (Larsen et al., 1997). Central and peripheral administration of GLP-1 reduces food intake in rodents (Tang-Christensen et al., 2001; Turton et al., 1996). The saliva of the Gila monster lizard, Heloderma suspectum, contains a peptide known as exendin-4, which is a potent agonist at the GLP-1 receptor. Exendin9-39, a truncated form of this peptide, acts as a competitive antagonist at the same receptor. Central administration of exendin9-39 for 3 days increased food intake in satiated rats, suggesting that GLP-1 plays a physiological role in appetite regulation (Tang-Christensen et al., 2001; Turton et al., 1996). Peripheral GLP-1 administration in humans dose-dependently reduces food intake (Verdich et al., 2001). However, mice with targeted disruptions in the GLP-1R display no abnormalities in feeding behaviour or body weight but are hyperglycaemic, which
suggests that the primary endogenous role of GLP-1 is in mediating glycaemia in response to food ingestion (Scrocchi et al., 1996). Even if peripheral GLP-1 is not a physiological appetite regulator, evidence from clinical trials of exendin-4 in patients with diabetes suggests the GLP-1 system may be a useful pharmacological target for weight loss (Kendall et al., 2005).

1.2.5.2.2 Oxyntomodulin

Like PYY and GLP-1, OXM is released postprandially from L cells of the distal GI tract, in levels proportional to calorific content of a meal. Central or peripheral administration of OXM reduces food intake in rodents. OXM appears to signal through the GLP-1 receptor. OXM is ineffective in GLP-1 receptor null mice, and its anorectic effect is blocked by the GLP-1R antagonist exendin9-39 (Dakin et al., 2001). Like GLP-1, peripheral OXM increases c-fos expression in the NTS, area postrema and PVN (Baggio et al., 2004). Intra-ARC injection of OXM reduced food intake in rats. However, the anorectic effect of peripherally-administered OXM, but not GLP-1, is blocked by prior administration of exendin9-39 into the ARC. Furthermore, OXM has a 50-fold lower affinity for the GLP-1 receptor, but appears to reduce food intake with similar potency (Dakin et al., 2001). This suggests OXM and GLP-1 may mediate their pharmacological effects on food intake via different pathways. Further studies are required to determine whether OXM mediates its anorectic effects exclusively via the GLP-1R.

OXM may also stimulate energy expenditure. Chronic central administration of OXM results in reduced weight gain in rats compared to pair-fed controls, and chronic peripheral administration of OXM to humans over 4 days significantly increased activity-related energy expenditure, assessed by continuous electronic movement monitoring (Dakin et al., 2002). In addition chronic administration of OXM to obese humans results in weight loss, which is promising regarding the utility of the GLP-1 system as an anti-obesity therapeutic target (Wynne et al., 2005; Wynne et al., 2006).
1.2.5.3 **Cholecystokinin**

Cholecystokinin (CCK) is a peptide hormone secreted by K cells in the duodenum, jejunum and proximal ileum, as well as by specialised neurones in the myenteric plexus and brain (Larsson and Rehfeld, 1978; Moran and Schwartz, 1994). CCK is released from the small intestine in response to the ingestion of protein and fat. CCK is present in multiple bioactive forms, which are all derived from the precursor preprocholecystokinin (Moran and Schwartz, 1994). CCK exerts its actions via binding to the G-protein coupled receptors, CCK1 and CCK2. CCK1 receptors are found throughout the brain, notably in the NTS, DMN and AP as well as in the pancreas and on vagal efferents. CCK2 receptors are also distributed throughout the brain, the afferent vagus nerve and the stomach (Moran, 2000; Moran and Kinzig, 2004). Both CCK1 and CCK2 receptors mediate the peripheral functions of CCK, including stimulation of pancreatic enzyme secretions and gallbladder contraction (Moran, 2000). Central and peripheral administration of CCK-8 (the predominant form of CCK in the brain) reduces food intake in rats and was the first gut hormone to be shown to influence food intake (Moran and Kinzig, 2004). Peripheral CCK is thought to signal via the vagal nerve to the brainstem, as vagotomised rats do not respond to peripherally-administered CCK (Crawley et al., 1984). CCK1 receptor antagonists increase food intake in rodents and humans, and the Otsuka Long-Evans Tokushima Fatty rat, which lacks the CCK-1 receptor, is hyperphagic and obese, and exhibits increased NPY mRNA in the DMN. This suggests that CCK plays a physiological role in energy homeostasis (Bi and Moran, 2002). However, prolonged administration of CCK does not affect food intake, due to a compensatory increase in meal number, despite a decrease in meal size (Crawley and Beinfeld, 1983). The administration of long-acting CCK analogues does not cause weight loss in obese and normal-weight humans (Jordan et al., 2008). Therefore, although CCK is a physiological signal regulating meal size, it is probably not useful as a treatment for obesity.

1.2.5.4 **Ghrelin**

Ghrelin is a 28 amino acid peptide agonist of the growth hormone secretagogue receptor (GHSR), produced by the oxyntic cells of the stomach (Kojima et al., 1999). Ghrelin was named for its potent effect in stimulating growth hormone secretion (Kojima et al., 1999). Fasting increases circulating ghrelin levels, which are reduced upon food intake (Cummings
et al., 2001). In obese individuals circulating ghrelin is low, but increases to a normal level following weight loss. Further, the postprandial reduction in circulating ghrelin is poorly regulated in the obese, which may be a contributing factor towards difficulty in weight loss implementation (Cummings et al., 2002). Ghrelin is currently the only endogenous orexigenic signal known to originate from the GI tract. Peripheral and central administration of ghrelin potently induces feeding in rodents, and chronic administration to rats causes hyperphagia and obesity. Peripheral administration of ghrelin to humans also increases food intake (Wren et al., 2001a). Peripherally, ghrelin is thought to mediate glucose homeostasis; reducing glucose sensitivity by inhibiting insulin production and secretion in the pancreatic β cell (Sun et al., 2006; Sun et al., 2007). The ARC was found to be the most sensitive to the orexigenic effects of ghrelin compared to other hypothalamic nuclei (Wren et al., 2001b). Blockade of the endogenous action of ghrelin by central infusion of ghrelin antibodies results in a decrease in fasting-induced refeeding, suggesting that ghrelin is a physiological orexigenic signal from the periphery to the brain (Nakazato et al., 2001).

Transgenic rats with attenuated GHSR signalling in catecholaminergic neurones in the ARC have a hypophagic, lean phenotype and are unresponsive to the orexigenic effects of growth hormone secretagogues. This suggests that ARC GHSR-ghrelin signalling may mediate the orexigenic and adipogenic effects of ghrelin (Shuto et al., 2002). Further, central administration of ghrelin increases the expression of hypothalamic AgRP and GHSR is expressed on NPY/AgRP neurones (Kamegai et al., 2000; Gnanapavan et al., 2002). Peripheral administration of ghrelin increases c-fos expression in the ARC AgRP/NPY neurones (Wang et al., 2002). The orexigenic effect of peripheral ghrelin administration is completely abolished in mice lacking NPY and AgRP (Chen et al., 2004). These data suggest that NPY/AgRP neurones mediate the orexigenic effect of ghrelin. An indirect mechanism of action of ghrelin via the vagal-brainstem-hypothalamic pathway has also been postulated as vagotomy abolishes the orexigenic effect of peripheral ghrelin administration (Date et al., 2002). The absence of a lean phenotype of global ghrelin knockout mice and GHSR deficient mice is surprising. However, young ghrelin knockout mice exposed to a high fat diet gained less weight than wildtype littermates. Furthermore, ablation of GHSR specifically in the ARC results in a lean hypophagic phenotype, suggesting that ghrelin does play a role in regulating food intake (Kirchner et al., 2010).
Figure 1.5. Summary of the mechanisms of action of the key hormones involved in energy homeostasis. PYY3-36, GLP-1 and OXM can directly stimulate anorectic pathways in the hypothalamus and indirectly via the vagal-brainstem afferents. CCK is thought to affect anorectic appetite pathways via the NTS in the brainstem, while ghrelin acts via the vagus and signals directly to the hypothalamus to stimulate food intake. Adapted from (Wynne et al., 2005). CCK- cholecystokinin, GLP-1- glucagon-like peptide 1, NTS- nucleus tractus solitaries, OXM- oxyntomodulin, PYY- peptide YY.
1.2.5.5 Peptide YY

PYY was isolated from the porcine intestine in 1980 (Tatemoto, 1982). PYY is a 36 amino acid peptide hormone postprandially secreted from L cells of the gastrointestinal tract in proportion to food intake. PYY belongs to the PP-fold family, which also includes NPY and PP (see fig. 1.6 for amino acid sequences). Peptides of the PP-fold family act on the Y receptors, which are a part of the GPCR superfamily (see section 1.2.3.4). The human NPY gene is localised on chromosome 7p15.1, while human PYY and PP genes are located on chromosome 17q21.1 (Conlon, 2002). C-terminal amidation of these proteins is required for biological activity. The PP-fold peptide family is characterised in solution by a classic ‘U’ shaped structure, which is formed by a polyproline helix between residues 1-8 and an α-helix at residues 15-32. These two helices are connected by a β-turn. The conformation of this structure determines the selectivity of these peptides for the Y receptors (Conlon, 2002).

The Y2R is a 381 amino acid protein found predominantly in the brain and in the peripheral nervous system. Antagonist and knockout studies have implicated Y2R in delayed gastric emptying, bone formation, angiogenesis, vasoconstriction and behavioural effects such as memory enhancement, circadian rhythms, analgesia, anorexia and anxiety (Berglund et al., 2003; Parker and Balasubramaniam, 2008; Lindner et al., 2008). All Y receptors are coupled to the G inhibitory protein (Gi). Activation of the Y receptors thus results in inhibition of intracellular adenylate cyclase activity and therefore cAMP accumulation. Activation of the Y2R also reduces the peak amplitude of calcium currents via selective N-type calcium channels (Wiley et al., 1993; Toth et al., 1993). The Y2R is thought to be located presynaptically in the CNS, acting as an autoreceptor which prevents further neurotransmitter release. In situ hybridization in the rat brain has revealed Y2R mRNA in the hippocampus, hypothalamus, amygdala and brainstem, whilst in the periphery Y2R is found in sympathetic, parasympathetic and sensory neurones of the peripheral nervous system (Gustafson et al., 1997). Brainstem regions that express the Y2R include the nucleus of the solitary tract and the lateral reticular nucleus; areas which relay information between the hypothalamus and spinal cord. In the hypothalamus, Y2R is found in the ARC, preoptic nucleus (PON) and dorsomedial nucleus (DMN). More than 80% of arcuate NPY neurones co-express the Y2R (Broberger et al., 1997; Wolak et al., 2003; Gustafson et al., 1997). Y2R is confined to the medial portion of the ARC, which contain the NPY neurones while Y1R is prominent only in
the lateral, POMC neurone-containing portion (Parker and Herzog, 1999). In the human brain, the Y2R predominates over other Y receptor subtypes, and is additionally found in the dentate gyrus and cerebral cortex (Caberlotto et al., 1998). Furthermore, the Y2R is the only Y receptor expressed in the lateral septum, an area involved in the integration of efferent and afferent neuronal signalling within the limbic system, and which may be an important downstream effector in the homeostatic regulation of appetite.

1.2.5.5.1 The distribution of PYY and PYY3-36

PYY and PYY3-36 can be detected by radioimmunoassay in human plasma, with PYY3-36 forming the majority of circulating PYY in the fed state (Grandt et al., 1992). Unlike NPY, which is only found in neurones, PP and PYY are found mostly within the endocrine cells of the pancreas and gastrointestinal tract respectively. In mammals, PYY is found in the ileum, colon and in the rectum (Ekblad and Sundler, 2002). During endocrine cell differentiation, PYY appears to be the earliest hormone released by colonic endocrine cells. This highlights a speculative role for PYY in the development of the gastrointestinal tract (Upchurch et al., 1996). PYY is also found in the CNS, where it is expressed in the medulla oblongata, nucleus reticularis, dorsal medulla, NTS, hypothalamus, pons and spinal cord, and in the enteric neurones of the gastric mucosa (Ekblad and Sundler, 2002; Pieribone et al., 1992).
| residue position | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
|-----------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| DPP-IV cleavage |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| N-terminus      | Tyr| Pro| Ile| Lys| Pro| Glu| Ala| Pro| Gly| Glu| Asp| Ala| Ser| Pro| Glu| Glu| Leu| Asn| Arg| Tyr| Tyr| Ala| Ser| Leu| Arg| His| Tyr| Leu| Asn| Leu| Val| Thr| Arg| Gln| Arg| Tyr|
| Mid-section     |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| C-terminus      |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| PP              | Ala| Pro| Leu| Glu| Pro| Val| Tyr| Pro| Gly| Asp| Asn| Ala| Thr| Pro| Glu| Gln| Met| Ala| Gln| Tyr| Ala| Ala| Asp| Leu| Arg| Arg| Tyr| Ile| Asn| Met| Leu| Thr| Arg| Pro| Arg| Tyr|
| NPY             | Tyr| Pro| Ser| Lys| Pro| Asp| Asn| Pro| Gly| Glu| Asp| Ala| Pro| Ala| Glu| Asp| Met| Ala| Arg| Tyr| Tyr| Ser| Ala| Leu| Arg| His| Tyr| Ile| Asn| Leu| Ile| Thr| Arg| Gln| Arg| Tyr|

**Figure 1.6. The human amino acid sequences of the members of the PP-fold family.** Amino acids on PYY cleaved by dipeptidyl-peptidase-iv (DPP-iv) to generate PYY3-36 are shaded in grey. The N terminus is shaded in red, the mid-section is shaded in blue and the C terminus is shaded in green for the PYY sequence. PYY- peptide YY, PP- pancreatic polypeptide, NPY- neuropeptide Y.
1.2.5.5.2 The release of PYY

Circulating levels of PYY increase after the ingestion of a meal. Endogenous PYY plasma levels peak in proportion to food intake 1-2 hours after a meal, and remain at a plateau level for around 6 hours (Adrian et al., 1985). The amount of PYY secreted by L-cells has been shown to rise in proportion to an increased ratio of fat intake to carbohydrate and protein intake in a meal (Greeley, Jr. et al., 1989; Lin and Chey, 2003). The L cells of the colon and ileum are thought to be the main source of circulating PYY (Greeley, Jr. et al., 1989; Adrian et al., 1987; Ekblad and Sundler, 2002). Unlike other gut hormones, PYY release is not affected by gastric distension (Pedersen-Bjergaard et al., 1996). Ileocoloctomy has been found to abolish the release of PYY in response to the administration of a fatty acid into the duodenum, and basal PYY levels are low in patients who have undergone colonic resection and ileostomy. Triggers for PYY release include the intraluminal presence of certain markers of ingestions of a meal, such as glucose, lipids, short-chain fatty acids, amino acids, gastric acid and bile acids. L-cells are thus thought to monitor intake of substances into the small bowel and colon and to release PYY in response to the presence of these markers (Onaga et al., 2002; Ballantyne, 2006).

Evidence suggests that the majority of circulating PYY is released from the distal small intestine and/or large intestine. However, since a meal given intraduodenally increases circulating PYY before nutrients are able to reach the L cells of the distal gastrointestinal tract, a neural and/or hormonal response has been proposed to underlie its immediate release. The vagus nerve complex may mediate this putative neural regulation, while regulatory gut peptides such as vasoactive intestinal peptide (VIP), CCK, gastrin and GLP-1 have been suggested as humoral mediators of release (Greeley, Jr. et al., 1989; Ballantyne, 2006; Naslund et al., 1999; McGowan and Bloom, 2004). A considerable number of gastrointestinal diseases associated with appetite loss are characterized by elevated PYY levels, which may indicate a protective role for this peptide in reducing food intake during diseased states (Liu et al., 1996).
Obese patients have been reported to exhibit significantly lower levels of PYY compared to people with a normal body mass index (BMI), though this has not been the case in other studies (Batterham et al., 2003a). However, another group found that PYY plasma levels were similar between normal-weight and obese patients using the same assay. Significantly, in this study obese patients did show blunted postprandial PYY3-36 plasma levels (Stock et al., 2005). Interestingly post-prandial PYY levels are elevated in obese patients that have undergone Roux-en-Y gastric bypass surgery (Le Roux et al., 2006a). It has been proposed that abnormal delivery of undigested fats to the small bowel, common in Roux-en-Y and gastrointestinal disease, may be responsible for the increase in PYY, thus providing heightened anorectic signalling during diseased states, when this may provide a selective advantage in promoting survival (Korner et al., 2005; Vincent and Le Roux, 2008).

1.2.5.5.3 The in vivo effects and mechanism of action of PYY

Since the 1980s, endogenous PYY has been known to play a fundamental role in gastric motility and secretion (Pappas et al., 1985). PYY is a mediator of the ‘ileal brake’, which involves the slowing of gastric emptying in response to nutrients in the distal small intestine (Spiller et al., 1988). PYY also inhibits glucose-induced insulin secretion, gastric and pancreatic secretion, reduces gallbladder emptying, enhances vasoconstriction in the GI tract and pancreas, and augments salt and water absorption in the colon (Adrian et al., 1985; Bottcher et al., 1989). At supra-physiological doses, PYY3-36 was recently found to inhibit the diarrhoea induced by prostaglandin-E2 through the reduction of intestinal fluid secretion and colonic transit (Moriya et al., 2010). PYY3-36 has been shown to acutely reduce food intake when administered peripherally at physiological concentrations in both normal and obese rodents (Chelikani et al., 2006a; Batterham et al., 2002; Chelikani et al., 2005). Initially, other laboratories were unable to reproduce these results (Tschop et al., 2004). Subsequently, the effects of PYY3-36 on food intake have been shown to be susceptible to stress; careful acclimatisation of animals to the injection and handling procedure is necessary before feeding studies are performed (Halatchev et al., 2004; Chaudhri et al., 2008; Abbott et al., 2006).
Chronic peripheral administration of PYY3-36 has been shown to reduce food intake and body weight gain or adiposity in normal-weight and obese rodents (Chelikani et al., 2007; Batterham et al., 2002; Abbott et al., 2005b). However, PYY3-36 has not produced a chronic anorectic effect in all studies using different administration and dosing regimens. Intravenous (iv) infusion has proven the most reproducible method for PYY3-36 administration (Chelikani et al., 2006a; Scott et al., 2005). Iv administration of PYY3-36 significantly reduced 24h caloric intake in normal-weight and obese humans (Batterham et al., 2003a). Supraphysiological doses of PYY3-36 produce conditioned taste aversion in rats and nausea in humans (Chelikani et al., 2006b; Le Roux et al., 2008). PYY null mice exhibit hyperphagia and increased adiposity, which is reversed by treatment with PYY3-36 (Batterham et al., 2002). Interestingly, PYY is potently and dose-dependently orexigenic when administered centrally into the 3rd, 4th or lateral ventricles, the PVN or the hippocampus. This effect is thought to be due to PYY activating Y1R and Y5R, as it is abolished in Y1R and Y5R knockout mice. The most potent orexigenic effect is observed after administration into the 4th ventricle suggesting that the hindbrain may be important (Morley et al., 1985; Corp et al., 1990; Hagan, 2002; Batterham et al., 2002). In physiological terms it seems unlikely that postprandial concentrations of PYY released from L-cells would diffuse to activate central Y1/Y5 receptors, though there is recent evidence that central neurones may synthesize PYY (Morimoto et al., 2008). PYY3-36 from the periphery accesses the ARC, which is thought to be incompletely isolated from the general circulation by the blood brain barrier (Migrenne et al., 2006; Peruzzo et al., 2000). The striking contrast between central and peripheral administration highlights the intricate and sensitive nature of the PP-fold family multi-receptor/multi-ligand system.

PYY3-36 has been found to be a potent agonist of Y2R in pharmacological studies (Grandt et al., 1992). Evidence suggests that the Y2R is responsible for the anorectic effects of PYY3-36 as Y2R antagonists attenuate the effects of PYY3-36 on food intake, and Y2R-null mice are insensitive to peripheral PYY3-36 administration (Batterham et al., 2002; Abbott et al., 2005a). Y2R is thought to be a presynaptic autoinhibitory receptor and is expressed on most NPY-positive neurones in the ventromedial ARC (Broberger et al.,
However it is not expressed on POMC neurones in the ventrolateral ARC, which express postsynaptic Y1R in close apposition to NPY terminals (Colmers et al., 1991; Broberger et al., 1997). Administration of PYY3-36 into the ARC potently and dose-dependently reduces food intake during the dark phase and during re-feeding of fasted rodents. Furthermore, administration of a Y2R antagonist into the ARC prevented the effects of exogenous PYY3-36 on food intake in rats and increased food intake in satiated rats, implying that endogenous PYY3-36 regulates food intake (Batterham et al., 2002; Abbott et al., 2005b). The specificity of this effect is supported by the fact that the Y2R antagonist has no effect when administered into the PVN, which is thought to be less accessible to circulating peptides (Batterham et al., 2002). The brainstem and ARC exhibit an increase in c-fos immunoreactivity after peripheral administration of PYY3-36 at physiological doses, whereas other sites involved in energy homeostasis, namely the anterior hypothalamus, the DMN, the LH, the PVN, the VMH and the central nucleus of the amygdala, did not exhibit an increase in c-fos immunoreactivity after PYY3-36 administration (Blevins et al., 2008).

Whether the effect of PYY3-36 on Y2R-expressing neurones in the ARC is directly and/or indirectly mediated is unknown. The ARC is relatively permeable; and the circulation and transportation of PYY3-36 across the blood brain barrier via a non-saturable mechanism has been discovered. A direct action of PYY3-36 on the ARC has therefore been postulated (Peruzzo et al., 2000; Nonaka et al., 2003). iv infusion of PYY3-36 to rats increases c-fos in the NTS and AP in the hindbrain, which may transmit anorectic signals to the ARC. The NTS and AP are directly innervated by vagal sensory neurones from the gut, which express Y2R and are stimulated by PYY3-36 (see fig. 1.7) (Koda et al., 2005). Meal related satiety signals may therefore be transmitted via the vagal neurones to increase activity within neurone populations of the NTS and AP, which project to the hypothalamus. Since the Y2R is expressed in the NTS and AP, PYY3-36 may also directly activate these neurone populations. Furthermore, it is unclear whether neuronal activation in the ARC precedes or follows the activation of the NTS. The ARC is linked to the NTS via descending pathways, and the NTS is linked to the ARC via an ascending noradrenergic pathway, through which for example, the orexigenic gastric hormone ghrelin has been suggested to exert its effects (Blevins et al., 2008; Date et al., 2006). Some studies have shown abrogation of the effects
of PYY3-36 in rodents following vagotomy, whereas others failed to demonstrate any differences in feeding after PYY3-36 administration to vagotomised or capsaicin-treated animals (Koda et al., 2005; Abbott et al., 2005a; Talsania, 2005; Halatchev and Cone, 2005). The ablation of the vagal-brainstem-hypothalamic pathway may have an indirect effect on the ability of the ARC to respond to circulating hormones. These results suggest that the effects of PYY3-36 may be mediated in part via vagal afferents (see fig. 1.7).

Recently, other areas of the brain involved in hedonic aspects of feeding behaviour have been suggested to play a role in mediating the effects of PYY on appetite. Functional magnetic resonance imaging carried out in humans during infusions of postprandial levels of PYY3-36 revealed changes in neural activity in the hypothalamus as well as in the orbitofrontal cortex, which is involved in hedonic behaviour. During PYY3-36 infusion, mimicking the fed state, the amount of neural activity in the orbitofrontal cortex was correlated with the amount of food eaten during a subsequent buffet meal. The amount of neural activity in the hypothalamus during the control infusion was correlated with the amount of food eaten during the subsequent meal (Batterham et al., 2007). This indicates a potential role for PYY3-36 in the hedonic control of appetite. PYY3-36 may inhibit NPY neurones and/or excite POMC neurones in the ARC (Batterham et al., 2002). In a study investigating the time-course of the effects of PYY3-36 on POMC and NPY expression, ARC POMC expression was elevated by 6- and 2.4-fold at 6h and 24h after PYY3-36 administration respectively, whereas NPY mRNA levels were suppressed 2.3 fold at 6h but were not significantly altered at 24h (Challis et al., 2003). Electrophysiological studies show contrasting results. One study found that PYY3-36 exerts a robust inhibitory effect on both NPY cells and POMC cells identified by selective GFP expression in transgenic mice, contradicting previous findings in which PYY3-36 was found to stimulate POMC neurones and inhibit NPY neurones (Challis et al., 2003; Koda et al., 2005; Batterham et al., 2002). Y2R-selective agonists have been found to reduce the release of NPY from hypothalamic explants in vitro, while a Y2R antagonist increases NPY release (King et al., 2000). Since POMC neurones primarily express Y1R, in vitro electrophysiological inhibition by PYY3-36 may be mediated by this receptor. However, this may not reflect the mechanism through which endogenous PYY3-36 acts in the brain.
GAD, the neuronal GABA marker, is also co-localized with NPY in the ARC. These GABA-ergic nerve terminals form synaptic contacts with POMC cell bodies, on which GABAA receptors are found (Meister, 2007; Ovesjo et al., 2001). Thus it is feasible that PYY3-36 may attenuate release of the inhibitory neurotransmitter GABA from presynaptic NPY-terminals onto neighbouring POMC neurones, resulting in their disinhibition. In support of this hypothesis, PYY3-36 has been shown to inhibit GABA transmission through mechanisms including the reduction of glutamatergic excitatory synaptic input to GABA neurones (Acuna-Goycolea et al., 2005; Acuna-Goycolea and van den Pol, 2005). The anorectic effect of PYY3-36 is still evident in MC4R knockout mice and POMC knockout mice (Marsh et al., 1999; Challis et al., 2004; Halatchev et al., 2004), suggesting that any stimulatory effect of PYY3-36 on POMC neurones is not critical for its anorectic effects.

In summary, the evidence indicates three, possibly redundant mechanisms through which PYY3-36 may exert an anorexigenic effect within the hypothalamus: 1) Y2R-mediated inhibition of NPY neurones, 2) Y2R-mediated GABA disinhibition of POMC and 3) input into ARC neuronal populations from vagal-brainstem afferents (see fig. 1.7). Overall, this evidence highlights a putative physiological role for PYY3-36 in the control of satiety through activation of the Y2R on NPY neurones in the ARC.
Figure 1.7. **Illustrative representation of putative model for mechanism of action of PYY.**
NPY/AgRP neurons and POMC neurons signal to the PVN and other hypothalamic nuclei to increase or decrease appetite respectively. PYY3-36 may cause meal termination by direct action involving presynaptic inhibition of NPY neurones, which reduces orexigenic NPY and AgRP tone, and/or via disinhibition of POMC neurones. PYY3-36 may also have an indirect effect on food intake via the vagal-brainstem pathway, which projects to the ARC. ARC- arcuate nucleus, AgRP- Agouti-related peptide, DPP-iv-dipeptidyl peptidase iv, NPY- neuropeptide Y, POMC- proopio-melanocortin, PVN- paraventricular nucleus, PYY- peptide YY, Y2R- Y2 receptor.
1.3 Design and development of PYY3-36 anti-obesity therapies

1.3.1 Current licensed obesity treatments

The development of effective pharmacotherapies for obesity is receiving urgent attention due to side effects associated with current treatments. A modest weight loss (<5kg/year) in obese patients has been found to improve a variety of parameters, including insulin sensitivity, glycaemic control, dyslipidaemia and hypertension (Goldstein, 1992; Van Gaal et al., 1997). Drugs that can be used to treat obesity are normally classified into three groups: drugs that elicit hypophagia by acting on homeostatic appetite systems or hedonic reward systems, drugs that interfere with fat absorption and drugs that increase energy expenditure and thermogenesis. Sibutramine reduces food intake by blocking presynaptic serotonin and norepinephrine reuptake in the central and peripheral nervous systems. The hypophagic effect is thought to result from differential regulation of POMC and NPY/AgRP neurones in the ARC via 5-HT occupancy of the 5-HT$_{2C}$ on POMC neurones and 5-HT$_{1B}$ receptors on NPY/AgRP neurones (Heisler et al., 2006). Sibutramine treatment results in a modest reduction in body weight, and is also associated with modest increases in heart rate and blood pressure due to peripheral sympathetic effects (Nisoli and Carruba, 2000). Sibutramine is no longer licensed for management of obesity, as it was associated with cardiovascular side effects, as well as headache, dry mouth, insomnia and constipation. Rimonabant is now a previously licensed obesity drug, which reduced food intake by acting on the endocannabinoid system, affecting hedonic reward pathways. Rimonabant was developed to reverse the overactive endocannabinoid system seen in obesity by antagonising CB-1 receptors. Rimonabant was removed from the market due to adverse psychological effects, likely due to effects on reward-associated behaviours (Bloom et al., 2008; Heal et al., 2009). The only FDA-approved longterm anti-obesity drug is orlistat, which works by interfering with fat absorption. Orlistat inhibits gastrointestinal lipases that hydrolyze triglycerides into free fatty acids in the intestine, resulting in the failure to absorb around 30% of energy ingested as fat. This results in a modest reduction in body weight but also provokes a number of unpleasant side effects, including diarrhoea,
bloating, abdominal pain, dyspepsia and deficiencies in fat-soluble vitamins (Li and Cheung, 2009).

As discussed, treatment options for obesity are currently limited due to unwanted side effects by their actions on central neurotransmitter systems or limited efficacy. Drugs that elicit hypophagia by affecting homeostatic appetite systems might be expected to be associated with nausea, which is the most extreme manifestation of satiety, and those that affect hedonic reward systems to reduce the hedonic value of pleasurable experiences, which may result in adverse mood-related disorders (Bloom et al., 2008). Drugs that act to reduce nutrient absorbance might be expected to cause gastrointestinal problems and vitamin deficiencies (McCarthy, 2000). It is therefore desirable to develop treatments that act in accord with the body’s own satiety infrastructure, which lower body weight to a level that reduces morbidity and mortality. As mentioned above, bariatric surgery is currently the most effective, permanent treatment for obesity, though it is associated with considerable cost and is therefore not viable for the obese population as a whole. Previous surgical strategies included reducing stomach volume or reducing absorption by jejunoileal anastomosis. Gastric banding and Roux-En-Y gastric bypass (RYGB) are the two most commonly performed surgeries. RYBG combines the restrictive effects of gastric banding (which reduces the volume of the stomach) with diversion of nutrients away from the gastric fundus and proximal small bowel through the construction of a small pouch to which the jejunum is attached. RYGB is associated with greater weight loss than gastric banding, which may be explained by altered patterns of secretion of PYY, OXM and GLP-1 (Field et al., 2009). Several studies have found sustained and increased circulating PYY after gastric bypass surgery in humans and in rodent models, which may explain the maintained weight loss observed (Pournaras et al., 2010). Blockade of endogenous PYY by administering a PYY antibody increases food intake in rodents that have undergone bariatric surgery, and post-bariatric surgery weight gain correlates with lower plasma PYY levels, suggesting that PYY plays a role in reducing food intake and weight gain post-surgery (Le Roux et al., 2006a; Neary and Batterham, 2009b; Meguid et al., 2008). These observations have encouraged research into gut hormones as potential new targets for obesity pharmacotherapy. The development of a gut hormone therapy may allow a less-
invasive, more cost-effective and more physiological alternative to bariatric surgery, orlistat and sibutramine.

### 1.3.2 Use of PYY3-36 as a potential antiobesity therapy

A change in lifestyle to reduce energy intake and increase energy expenditure is theoretically the most ideal treatment for obesity, but has proven unsuccessful to tackle the obesity crisis. Current licensed obesity therapies such as orlistat, which acts in the gut to prevent fat absorption, and previously-licensed obesity therapies including sibutramine and rimonabant, which act in the CNS to impair appetite, cause only modest weight loss and are associated with side effects. The only effective long-term obesity treatment is gut bypass surgery, which is expensive and incurs significant risk of surgical or post-surgical morbidity and mortality. Rather than acting on ubiquitous neurotransmitter systems, the development of an anti-obesity therapy should ideally work with the body’s own satiety infrastructure to reduce body weight (Cottrell et. al. 2007).

Understanding the role of gut hormones, in particular PYY, in appetite regulation may aid the development of antiobesity therapies which act on endogenous systems to restore body weight regulation. Obese patients have been reported to have low basal and postprandial circulating levels of PYY3-36, but still retain sensitivity to the anorexigenic effects of exogenous PYY3-36 (Batterham et al., 2003a). This finding is promising in terms of the utilisation of PYY as a therapeutic agent, as it implies that the obese are not resistant to PYY3-36, as they are to leptin and insulin. Simple replacement of the peptide in vivo presents a challenge as PYY is rapidly metabolised in the circulation and supraphysiological doses are thus required to maintain effects on food intake. Physiological doses of PYY3-36 result in a reduction in calorie intake in humans, but higher doses of PYY3-36 have been reported to produce transient nausea in humans (Le Roux et al., 2008; Degen et al., 2005). Furthermore, recent studies have found that rodents given acute PYY3-36 injections show an increase in food intake after treatment with PYY3-36 is ceased (Parkinson et al., 2008). This ‘overswing response’ may indicate a compensatory homeostatic drive to increase food intake after chronic PYY-induced anorexia.
PYY3-36 reduces food intake for 2-4 hours after an iv infusion and has a plasma half-life of only 8 min in rats (Lluis et al., 1989). The chronic effects of PYY3-36 on food intake and body weight are dependent on route of administration and dosage pattern. Chronic subcutaneous administration of PYY3-36 via osmotic minipump has been found to reduce food intake in rodents but only for the first 3-4 days of administration. An hour long intravenous infusion every other hour for ten days produced a 20% reduction in food intake, a 7% reduction in body weight and a 35% reduction in adiposity in rats (Chelikani et al., 2005). This suggests that the efficacy of PYY3-36 is extremely sensitive to route and pattern of administration. If PYY3-36 analogues are to be used in the treatment of obesity they will need to counteract escape and overswing effects while allowing a sensible route of administration and dosage pattern to humans, e.g. daily subcutaneous administration. PYY analogues that last longer in the circulation and at the site of injection due to protease-resistance, which exhibit a more potent interaction with the Y2 receptor, and which show better penetration may have a more durable anorectic effect, allowing for the development of a practical delivery system for human pharmacotherapy.

1.3.3 Structural design of PYY analogues

Determination of the functional and structural properties of PYY3-36 may provide further insight into its mechanism of action. Furthermore, for gut peptides with potential therapeutic applications, clinical utility is often limited by their short duration of action and the consequent need for repeated administration. Repeated administration or utilising supraphysiological dosage regimes often leads to tachyphylaxis and side effects respectively (Reidelberger et al., 2008). Rational modifications of the peptide sequence or its secondary structure may enhance the bioactivity and/or the half-life of the peptide, allowing less frequent, lower doses to be used (Druce et al., 2009). As a pharmacological drug candidate, native PYY3-36 is rapidly cleared from the circulation, with a reported half-life of 8 min in the rat, 13 min in the mouse, 19 min in the rabbit and >30 min in the pig (Nonaka et al., 2003; Sileno, 2005; Ito, 2006; Lluis et al., 1989). In humans, PYY levels return to baseline around 30 min after a steady state infusion (Sloth et al., 2007b). PYY3-36
therefore has limited therapeutic efficacy, which makes metabolically stable and long-acting PYY3-36 analogues highly desirable. Attachment of polyethylene glycol polymers to PYY3-36 creates PEGylated PYY3-36 analogues. These analogues have prolonged half-lives of up to 24h because of their increased hydrodynamic size and reduced renal clearance. However the receptor potency of these analogues is sacrificed due to PEGylation and the in vivo efficacy of these analogues is only moderately better than that of PYY3-36 (Ortiz et. al. 2007, Lumb et. al. 2007, Shechter et.al. 2005). Design and development of long-acting analogues that exhibit increased Y2 receptor selectivity is therefore required.

### 1.3.3.1 Design of PYY analogues based on receptor efficacy and peripheral clearance

Design of effective and long-lasting PYY analogues must take into account two important factors: a) the affinity of PYY analogues for the Y2 receptor and b) the resistance of PYY analogues to degradation in the circulation (and in the subcutaneous space if administration is to be via the subcutaneous route). Rational amino acid substitutions can enhance the binding affinity of a peptide for its receptor. This is dependent on the 3D structure of the peptide, which allows it to fit inside and interact with the active site of the receptor. Binding affinity is most commonly measured using receptor binding assays, which have been used for other gut hormone analogue development programmes (Druce et al., 2009; Liu et al., 2010). In practice gut hormone analogue development has normally relied on finding a balance between good receptor efficacy and longevity in vivo, through protease resistance and/or resistance to kidney filtration. In order to make rational amino acid substitutions it is necessary to determine the amino acids in native PYY3-36 that are susceptible to degradation. This may aid the development of long-acting analogues. In addition, determining the enzymes responsible for breaking down PYY3-36 in vivo may give rise to the utility of specific enzyme inhibitors in the treatment of obesity. Finally, other peptide analogue development programmes have focussed on modifying the delivery solution in which the peptide is administered, in order to encourage precipitation of peptides at physiological pH, and therefore a subcutaneous depot of peptide that allows slow steady release into the circulation (Brange and Langkjær, 1997; Owens, 2002).
1.4 Overview

The work contained in this thesis aims to elucidate the structure-function relationship of PYY at the Y2R in order to design and develop more potent and longer-acting analogues of PYY3-36. The first chapter establishes the effects of modifications to the amino and carboxy termini of PYY on susceptibility to proteolysis, binding affinity at the Y2R and in vivo efficacy. As this ‘screening’ approach did not identify long-acting PYY3-36 analogues, the second chapter investigates the physiological degradation profile of PYY3-36. This aimed to elucidate amino acid sites at which substitution may prevent endogenous degradation and prolong the in vivo efficacy of native peptide. The final chapter focuses on modification of the secondary structure of PYY3-36 and on combining these long-acting analogues with a slow-release delivery solution that allows prolonged efficacy in animals and humans.

Hypothesis and aims

I hypothesized that the studies described in the following chapters would help to elucidate the structure activity relationship of PYY3-36, the way in which PYY3-36 is metabolized and degraded and, by altering the primary and/or secondary structure of PYY3-36, would create a longer acting analogue. I also hypothesized that the use of a slow-release delivery system for long-acting analogues may be efficacious in the chronic administration of PYY3-36 analogues to rodent models of diet-induced obesity. My studies aimed to determine whether:

- rational changes to the PYY primary sequence increase resistance to proteolytic degradation, binding affinity at the Y2R and efficacy in reducing food intake in vivo
- the use of kidney brush border-induced degradation as a tool in the analysis of amino acid sites that confer resistance to degradation facilitates the development of analogues with a longer action in vivo and which retain potency at the Y2R receptor
• knowledge of the physiological enzymatic degradation of PYY3-36 facilitates design of longer acting analogues and/or the use of an enzyme inhibitor/PYY3-36 co-administration regime
• substitution of the α-helix of PYY3-36 will prolong its bioactivity
• use of a metal ion precipitation system allows slow release from subcutaneous depots and prolong the biological effects of PYY3-36
Chapter Two: Alterations to the primary sequence of PYY3-36 in elucidating structure/activity and for the design of long acting analogues
2.1 Introduction and aims

As described in the previous chapter, evidence suggests that increasing circulating PYY3-36 levels may be beneficial in the obese. Obese patients remain sensitive to the anorectic effects of PYY3-36 (Batterham et al., 2003a), and bariatric surgery, currently the only effective therapeutic strategy for obesity, may mediate its effects partly through raised levels of PYY (Korner et al., 2005; Le Roux et al., 2006a). Due to the costs and associated complications of bariatric surgery, a logical therapeutic approach would be to mimic the changes in endogenous gut hormones that occur after bariatric surgery. Knowledge of the structure/activity relationships of the PYY3-36 molecule, particularly those involving its interaction with the Y2R, through which PYY3-36 exerts its anorectic effect, and the regions of PYY3-36 susceptible to proteolytic cleavage, may allow rational modifications to be made to the molecule in order to develop a potent and/or long-acting PYY3-36 analogue.

Understanding the structure-activity relationship (SAR) between a peptide and its receptor or receptors is a major focus of drug design. Nearly half of all drugs currently in clinical use act on GPCRs (Christopoulos, 2002). Characterizing the functional groups and structural properties of the peptide ligand responsible for a specific biological activity is important in understanding the mechanism of action of the peptide and the way in which its receptor mediates its bioactivity. For peptides with potential therapeutic applications, such as PYY3-36, clinical use is often limited by short duration of action and the need for repeated administration or higher doses to obtain the required biological effect (Le Roux et al., 2008). This provides impetus to create analogues that are longer acting because of their increased resistance to proteolytic degradation, but which retain or have improved Y2R affinity. This should lead to a more sustained and therapeutically useful biological effect. Knowledge regarding the structural features of PYY susceptible to proteolytic cleavage and which participate directly or indirectly in Y2R binding is thus helpful when making rational modifications to the amino acid sequence of PYY in order to design an analogue with
clinical utility. Information regarding the secondary and tertiary/3D structure may assist in predicting the conformational outcomes of changes to the primary structure.

2.1.1 Use of analogues to investigate peptide properties and structure activity relations

In order for a ligand to interact with and bind its receptor it must contain a specific combination of amino acids that presents the correct steric, electrostatic and hydrophobic complement. This set of features is known as the pharmacophore, which was first defined by Paul Ehrlich as ‘a molecular framework that carried the essential features responsible for a drug’s pharmacokinetic activity’ and is now defined as ‘an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger (or block) its biological response’ (Wermuth C.G et al., 1998). Peptide analogues can have a variety of altered characteristics compared to the natural ligand, such as receptor selectivity and affinity, intracellular signal transduction or physiological response, susceptibility to proteolytic degradation and bioavailability. All of these characteristics can be modified by the ability of the molecule to cross barrier membranes, which allow access of peripherally administered substances to different compartments in the body (Hruby, 2002).

A number of strategies have been employed in the rational development of peptide analogues. Structure affinity/activity studies can be used to elucidate the essential and non-essential components of the ligand. Sequential truncation from the N- or C- terminal of the analogue can be used to determine the minimal structural requirements for receptor binding and bioactivity. A classic method to define residues that are involved in the ligand-receptor interaction is the use of amino acid scans. This involves sequential substitution of each residue in the ligand with a small, uncharged amino acid such as Ala (see appendix I for full list of amino acids and abbreviations), which has minimal effects on the secondary structure of the peptide. This technique can be used to investigate the importance of direct interactions of specific ligand side chains with the receptor (Lindner et al., 2008). Similarly, scanning with D-amino acids provides information about the orientation and the steric
requirements of amino acids in the ligand. For example, D-amino acids can stabilize β-turn/hairpin secondary structure, so the finding that a D-amino acid substitution confers an increase in receptor affinity and bioactivity may suggest that a β-turn conformation is important for the SAR of PYY3-36. It is also useful to modify the chiral, hydrophobic, hydrophilic and acid/base properties of key amino acids, in order to obtain further insight into the requirements of the binding pocket of the receptor. Once the structural elements in the peptide that are responsible for receptor affinity and bioactivity have been established it is then possible to determine the three-dimensional relationships of these pharmacophore properties using techniques such as nuclear magnetic resonance (NMR) and X-ray crystallography to predict 3D peptide structure and circular dichroism to predict helicity and folding. One drawback to three-dimensional modelling is that peptides do not hold a well-defined structure in aqueous solution and therefore artificial simulation of a peptide’s tertiary structure may not reflect its endogenous conformations (Hruby, 2002).

In essence, knowledge of the SAR between a peptide and its receptor acts as a framework to facilitate the design of analogues that retain or show improved receptor binding affinity and a sustained or improved biological effect. It may then be possible to further modify the peptide to include structural features that enhance resistance to proteolytic degradation and increase the half-life of the peptide in the circulation and/or improve blood brain barrier permeability.

2.1.2 Structural properties of PYY and PYY3-36

2.1.2.1 PYY as part of a complex multireceptor/multiligand system

The PP-fold/Y receptor system is an example of a complex multireceptor/multiligand system, and comprises three ligands that bind four receptors with varying affinity and selectivity. NPY and PYY bind the Y1, Y2 and Y5 receptors, whereas PP preferentially binds the Y4R but is still able to bind the Y5R with high affinity. N-terminal Tyr-Pro degradation of PYY by the enzyme dipeptidyl peptidase-iv (DPP-iv) reduces its ability to
bind the Y1, Y4 and Y5 subtypes, transforming it into a specific Y2 receptor agonist. Position 34 is crucial for Y2R recognition, as position 34-substituted analogues do not bind Y2R but do bind Y1R. A change in tertiary conformation of Pro34-PYY has been shown by nuclear magnetic resonance (NMR), which may explain the inability of this molecule to bind the Y2R (Keire et al., 2002). Y1R, Y4R and Y5R require both the C and N-terminal of their ligands for activation whereas Y2R receptor activation only requires an intact C terminus. Since ligands for Y2R mainly interact with the C-terminus, ligands shortened at the N-terminal will readily bind the Y2R. Y1R, Y2R and Y5R are highly conserved between species. For example, the mouse and human Y2R coding sequences are 94% homologous and the transmembrane region sequences are 98% homologous (Larhammar, 1996). This evolutionary conservation may reflect the physiological importance of these receptors in the homeostatic regulation of energy homeostasis.

2.1.2.2 The three-dimensional structure of PYY and PYY3-36

The crystal structure of avian PP was first described using X-ray analysis in the 1980s, leading to computational modelling of potential structures of mammalian PP, NPY and PYY (Glover et al., 1984). Further studies using circular dichroism and nuclear magnetic resonance (NMR) consistently found evidence for a folded structure, known as the ‘PP-fold’, characterized by a polyproline-like N-terminal (Pro2-Pro5-Pro8), which is folded back onto a long amphipathic \( \alpha \)-helix at residues 15-30 and stabilized by hydrophobic contacts with Leu17, Tyr20, Leu24 and Tyr27 and a dipole moment (see fig. 2.1) (Bjornholm et al., 1993). The polyproline helix and \( \alpha \)-helix are connected by a \( \beta \) turn, brought about by Gly9, which is highly conserved in the PP-fold peptide family and between species. The C-terminal hexapeptide of PYY is required for receptor recognition, and is also highly conserved (Nygaard et al., 2006). The PP-fold may function to protect the molecule from proteolytic degradation, which is in accord with studies showing that proteolytic cleavage at the N-terminal can be enhanced by removing residues from the C terminus (Schwartz et al., 1990). Although the overall homology between peptides of the PP-fold family varies considerably, the conserved residues are thought to be important in
stabilization of PP-fold and the C-terminal. The conserved residues in the PP-fold family of peptides include Pro2, Pro5, Pro8, Gly9, Tyr20 and Tyr 27, which are important in maintaining the PP-fold, and Val31, Thr32, Arg33, Gln34, Arg35 and Tyr36, which are located in the C-terminal and are important for receptor binding (Nygaard et al., 2006; Conlon, 2002; Ahn et al., 2009).

Figure 2.1. Conserved ‘PP-fold’ structure of PYY. The interacting hydrophobic amino acid residues, including prolines (red), tyrosines (green) and leucines (yellow) in the hydrophobic core of human PYY influence the PP-fold structure. Figure adapted from (Nygaard et al., 2006).

An amphipathic $\alpha$-helix is thought to anchor or ‘dock’ the ligand onto the membrane where it can then be recognised by its receptor in a particular 3D conformation (Moroder et al., 1993). This concept has been supported by studies showing that PYY undergoes a conformational change upon binding to dodecylphosphocholine micelles, which are used as a membrane mimetic in NMR studies. Interestingly, it has been shown that members of the PP-fold family with similar binding profiles display similar conformations when micelle-bound, in particular the orientation of the C-terminal segment. In its micelle-bound state, the N-terminal and mid-section of PYY are released from the folded form and the hydrophobic residues within the $\alpha$-helix interact with the hydrophobic interior of the membrane (see fig. 2.2) (Lerch et al., 2004).
Figure 2.2. Structure of PYY as determined by NMR (left) and as representative conformers showing the N and C termini (right) A) in solution and B) bound to micelle membrane mimetics. This model proposes that PP-fold hormones are docked to the membrane by hydrophobic interactions and destabilization of the PP-fold, allowing diffusion along the membrane in order to undergo receptor recognition. In both PYY and NPY, the residues Arg33 and Arg35, which are involved in receptor binding, are similarly positioned when bound to the membrane mimetic, and the residue Tyr36, which is essential for receptor binding, is positioned within the water-membrane interface. Adapted from (Lerch et al., 2004).

In solution, PYY, PYY3-36 and the specific Y1R agonist PYY-Pro34 have clearly different conformations, showing that minor changes in primary structure can have large conformational repercussions and consequently major effects on receptor specificity. Whether PYY is recognised by the Y receptors in the conformation it adopts in solution or after docking to membranes is a source of contention. Some authors argue that the less well-defined, micelle-bound form is essential for receptor-recognition and therefore
predicts affinity (Lerch et al., 2004). Others argue that the amphipathic nature of the peptide allows accumulation of ligand at the membrane surface, making receptor binding more likely to occur, but that the peptide will re-adopt the PP-fold form for receptor recognition, particularly of the Y1R, which requires both the N and C terminus for recognition (Nygaard et al., 2006). The Y2R only requires the ligand C terminus for binding and has a higher affinity for PYY3-36 over PYY. PYY3-36 has a destabilized PP-fold in solution, possibly due to the lack of interaction between Pro2-Tyr27 that occurs in the more stable, well-defined form PYY (see fig. 2.3). Therefore, it is possible that PYY3-36 and/or PYY exhibit the ‘micelle bound’ form upon Y2R binding, and adopt the PP-fold form for Y1R binding (see fig. 2.4). Authors have also speculated that destabilization of the PP-fold may render the peptide more susceptible to proteolytic degradation, resulting in increased susceptibility of PYY3-36 to proteolytic attack (Nygaard et al., 2006). Clarification of the pharmacological importance of the 3D structure of PP-fold peptides in conferring Y receptor recognition and selectivity may aid the design of PYY analogues stabilized in a state favoured by the receptor.

![Image showing 3D solution structures of A) PYY and B) PYY3-36 showing a stable and destabilized PP-fold respectively.](image)

*Figure 2.3. Image showing 3D solution structures of A) PYY and B) PYY3-36 showing a stable and destabilized PP-fold respectively.* Taken from the MMDB database (Wang, 2007). Adapted from (Nygaard et al., 2006).
Figure 2.4. Structure of human PYY and PYY3-36 as determined by NMR, and putative models for PYY and PYY3-36 binding conformations at the A) Y1 and B) Y2 receptors. This model proposes that the classical PP-fold solution structure of PYY is necessary to bind to the Y1 receptor but not the Y2 receptor. This is supported by the indication that PYY3-36 has a destabilized PP-fold, low affinity for the Y1R and increased affinity for the Y2R compared to PYY. In PYY, the conserved residues Pro2, Pro5 and Pro8 interact with the conserved hydrophobic residues Leu17, Tyr20, Leu24 and Tyr27, forming the PP-fold. This structure is proposed to be destabilized due to the loss of the Pro2-Tyr27 interaction following the removal of Tyr-Pro by DPP-iv to form PYY3-36, thus increasing the affinity for the Y2R. Adapted from (Nygaard et al., 2006).

2.1.2.3 The role of the N-terminal of PYY and PYY3-36 in receptor affinity and proteolytic degradation

PYY has been reported to be susceptible to cleavage at its N-terminal by DPP-iv and Aminopeptidase P, which cleave the N-terminal Tyr-Pro or Tyr respectively. The actions of these enzymes are thought to be most prevalent in the gut, as these cleavage products were evident after incubation of PYY with jejunal brush border membranes and were prevented by pre-incubation with the relevant inhibitor (Medeiros and Turner, 1994). Removal of Tyr-Pro from the N-terminal of PYY by DDP-iv appears to be the most physiologically relevant
finding, as PYY3-36 is the predominant circulating form of PYY. This cleavage also reduces the ability of the peptide to bind the Y1, Y4 and Y5 subtypes, transforming it into a selective Y2 receptor agonist (Cabrele and Beck-Sickinger, 2000; DeCarr et al., 2007). The N-terminal (residues 1-12) of PYY is therefore important in generating Y2 receptor selectivity. Recently, it has also been suggested that the N-terminal binds to extracellular Y receptor sections, anchoring the ligand as it enters the binding pocket of the receptor. Since ligands for Y2R mainly interact with the C-terminal, ligands truncated at the N-terminal, including PYY3-36 and PYY13-36, are able to bind the Y2R. Shorter fragments, including PYY18-36, PYY22-36, PYY25-36 and centrally truncated [Ahx5-24]NPY also exhibit selective binding for the Y2R over the Y1R and Y5R (Cabrele and Beck-Sickinger, 2000). Further truncation of PYY beyond Arg25 results in a significant loss of affinity at the Y2R (DeCarr et al., 2007). This indicates that only part of the α-helix and C-terminal of PYY is essential to bind to the Y2R. While truncation of N-terminal residues from PYY3-36 creates Y2R-selective agonists, it also leads to attenuated affinity for the Y2R (Lindner et al., 2008). In support of the importance of the N-terminal in binding affinity, an Ala scan of NPY indicated that Pro5 plays an important role in Y2R binding, as [Ala5]NPY and [Ala8]NPY exhibited the largest decrease in affinity at the Y2R. The Ala scan also indicated that residues within the proline helix at the N-terminal of NPY interact with hydrophobic side chains in the C-terminal regions. Interactions between Pro5:Tyr27 and Pro8:Tyr20 are thought to be essential in stabilizing secondary structure and allowing optimal Y2 receptor-binding affinity. As these residues are conserved between NPY and PYY, these findings are also likely to apply to the PYY sequence.

Several N-terminally modified PYY analogues have been described. N-terminal truncated forms of PYY, such as PYY13-36, have reduced receptor binding affinity and bioactivity compared to PYY, and it has thus been suggested that residues 1-12 stabilize the peptide. This is supported by study of the tertiary structure of PYY, which suggests the N-terminal contributes to the amphiphilicity of the peptide and binding to the extracellular domains of the receptor (Zou et al., 2009). A PYY analogue in which the native Ala residues were replaced with the charged residues Glu and Lys, (Glu3 Lys7[PYY]), was designed to increase the amphiphilicity of the polyproline helix and thus stabilise the secondary
structure of PYY. This analogue showed increased *in vitro* bioactivity in the rat vas deferens assay compared to PYY, and a 10-fold greater increase compared to PYY13-36, suggesting that modifications to the N-terminal of PYY can influence its bioactivity (Minakata and Iwashita, 1990).

### 2.1.2.4 The role of the midsection of PYY and PYY3-36 in receptor affinity and proteolytic degradation

As discussed, 3D analysis has suggested that the amphiphilic nature of the α-helix, (residues 17-31) appears to stabilize the tertiary structure of PP fold peptides, which may contribute towards Y receptor affinity and selectivity. Circular dichroism studies comparing the helicity of Ala-substituted analogues of NPY have revealed considerable correlation between helicity and receptor binding, underlining the importance of the α-helix in receptor binding. As the members of the PP-fold peptide family show almost identical tertiary structure when membrane-bound, Zerbe and colleagues hypothesized that the ligands are recognized in their membrane-associated form, resulting in receptor binding following diffusion along the membrane. The amphipathic α-helix has been proposed to direct peptides to the membrane-water interface and to act as an anchor in guiding the C-terminal, which is flexible in solution, into the correct conformation for receptor binding (Zerbe et al., 2006). An Ala scan of PYY3-36 revealed that Ala substitutions in the α-helix at positions 20, 25 and 27-29, which are required for hydrophobic interactions in forming the PP-fold, reduces bioactivity (Ahn et al., 2009). The importance of the α-helical region of NPY in receptor binding is also demonstrated by large decreases in the affinity to the Y1R, Y2R and Y5Rs when the 19-23 amino acid segment of the NPY α-helix is exchanged for the PP 19-23 segment (Lerch et al., 2005).
2.1.2.5 The role of the carboxy terminal of PYY and PYY3-36 in receptor affinity and proteolytic degradation

There is considerable evidence that the C-terminal of PYY is essential for Y2R binding and bioactivity. The acidic residue Asp at position 6.59 within the Y2R is thought to interact ionically with the Arg33 residue at the C terminus. Furthermore, all Ala substitutions within the C-terminal hexapeptide (residues 31-36) of either PYY3-36 or NPY lead to a reduction or loss of binding affinity and bioactivity in vivo (Beck-Sickinger et al., 1994; Ahn et al., 2009). In particular, Arg35 and Tyr36 play crucial roles in the recognition of PYY by the Y2R, as individual Ala substitutions result in >1000-fold decreases in affinity. Interestingly, substitution of Gln for Pro at position 34 transforms PYY into a specific Y1R agonist. Substitution of the Tyr36 by other aromatic residues such as Phe does not alter binding (Lindner et al., 2008). The C terminus fragment of NPY is unable to bind the Y2R, and requires the α-helical mid-section of NPY for successful binding, suggesting that this region may stabilise the C terminus in its interaction with the Y2R (Fuhlendorff et al., 1990). Little information is available on the amino acids in the primary sequence of PYY and PYY3-36 that are susceptible to proteolytic cleavage in vivo. Studies testing the effect of kidney brush border (KBB) membranes on PYY in vitro have suggested that position 29-30 may be cleaved by the Zn metalloendopeptidase neprilysin EC34.11 (NEP). Substitutions at position 30 may therefore prolong the bioactivity of PYY3-36. However, it is unknown whether the proteolytic effects of NEP also occur in vivo (Medeiros and Turner, 1994).

2.1.2.6 Peptide modifications to enhance longevity in vivo

There are a number of peptide modifications that have been used to prolong the half-life of peptides in therapeutic drug development. Longer chains are less susceptible to proteolytic degradation than shorter chains. However, using complex amino acids with bulky side chains may alter secondary structure and thus affect ligand-receptor interaction and
bioactivity. Small neutral amino acids, such as Ala, Gly and Pro, are more useful in the elongation of peptides, as they are less likely to impact on secondary structure.

There is also evidence that the addition of acyl chains can prolong the half-life of peptides in vivo (Peters, Jr., 1985). Acylation of peptides promotes binding to albumin, a large 67kDa extracellular protein abundant in plasma and the subcutaneous space, where exogenous peptides are likely to be delivered. Albumin has eight fatty-acid binding sites and is known to bind reversibly to endogenous non-esterified fatty acids, as well as to several pharmacological agents. In humans, the normal loading of albumin is about 1 mole of fatty acid: 1 mole albumin, which leaves a surplus of binding sites available to bind acylated peptide (Peters, Jr., 1985). Binding of peptides to albumin may protect the N-terminal from proteolytic cleavage and renal filtration. This approach has been successfully applied to insulin and proglucagon-derived peptides. For example, the fatty acid acylated GLP-1 analogue liraglutide has a longer plasma half-life than native GLP-1 and potently reduces glycaemic excursions in diabetic patients (Madsen et al., 2007). Another study found that acylation at the α-amino group of N-terminally-truncated PYY analogue PYY24-36 markedly improved Y2R affinity, while maintaining Y2R selectivity over Y1R and Y5R (Potter et al., 1994). As steric conformation of this analogue remained consistent with the published structure of the C-terminal of PYY, it was suggested that acylation of this analogue stabilised the molecule and thus altered preference for the Y2R.

Other methods of reducing susceptibility to degradation include conjugation to biotin or polyethylene glycol (PEG)-ylation, which also reduce proteolysis and kidney filtration (Peleg-Shulman et al., 2004; Shechter et al., 2005). However, despite increasing the half-life of a peptide, biotinylation and PEGylation can interfere with the steric conformation and surface availability of the residues of small peptides, resulting in reduced receptor binding affinity and low bioactivity of the peptide in vivo. Conventional PEGylation of PYY3-36, which results in a stable PEGylated PYY3-36, has been found to abolish the anorectic effect of PYY3-36. This can be overcome by introducing PEG chains via a chemical bond that allows the release of peptide in its active conformation at a steady and reliable rate in vivo. For this, a slow, consistent and homogenous rate of hydrolysis under
physiological conditions is crucial to permit the desired pharmacokinetic profile of the peptide (Peleg-Shulman et al., 2004). Reversible PEGylation of PYY3-36 using (2-sulfo)-9-fluorenylmethoxycarbonyl (FMS)-PEG moieties, allows the release of PYY3-36 from PEG in just this manner, resulting in a consistent concentration of circulating peptide, and a substantial increase in half-life and bioactivity of PYY3-36 (Shechter et al., 2005).

2.2 Aim of studies

- To investigate the contribution of the different domains of PYY3-36 to its bioactivity and susceptibility to degradation.
- To examine the effect of rational changes to individual domains of PYY3-36 on receptor binding affinity and its effect on food intake.
- To use modifications to prolong the half-life of PYY3-36 to generate analogues with potential therapeutic application

2.3 Materials and Methods

2.3.1.1 Peptides

PYY1-36 and PYY3-36 were purchased from Bachem Ltd. Analogues were synthesized by Advanced Biotechnology Services, Imperial College London (London, UK) and Biomol International LP (Exeter, UK). Peptides were synthesised and purified using the approach described below.
2.3.1.2 Peptide Synthesis

Peptides were synthesized on resins derivatised with one of a number of cleavable linkers, using an Fmoc/t-butyl-based solid-phase synthesis strategy (Wellings and Atherton, 1997). Temporary Nα-amino group protection was afforded by the Fmoc-group, with t-butyl ethers being utilised for the protection of Tyr, Ser and Glu residues. His and Lys side chains were protected as their Nτ and Nε-tertbutyloxy carbonyl (Boc) derivatives respectively, cysteine as its S-trityl derivative, and arginine guanidine moiety as its Pbf derivative. Where Nε-acylation of Lys was required, orthogonal protection was afforded by the incorporation of 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)3-methyl-butyl Lys (Lys(ivDde)), which was deprotected on solid phase using 2% hydrazine in DMF.

2.3.1.3 Peptide cleavage

Upon completion of synthesis peptides were cleaved from solid support, with removal of side chain protecting groups, by treatment with aqueous trifluoroacetic acid (TFA) (95%) containing triisobutylsilane (TIS) (5%) as a scavenger. TFA and scavengers were removed by evaporation and trituration.

2.3.1.4 Purification of peptides and quality control

Peptides were purified by reverse phase preparative high performance liquid chromatography (HPLC) followed by lyophilisation. The purified product was subsequently analysed by reverse-phase HPLC and matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-ToF MS).
2.3.2 Peptide design: investigating the importance of specific regions of PYY3-36 in receptor binding and bioactivity

2.3.2.1 PYY3-36 analogues with C-terminal substitutions

The C terminus hexapeptide section of PYY3-36 is essential for binding the Y2R (Larhammar, 1996; Lindner et al., 2008). Amino acids at key positions in this sequence are highly conserved across species, and therefore are believed important for biological function (Conlon, 2002). Analogues with substitutions at positions 31, 33 and 36 were designed in order to determine the importance of these particular amino acids in Y2R binding and subsequent bioactivity and whether these alterations may enhance bioactivity (refer to fig. 1.6 for PYY sequence). Previously, Ala-scanning at position 30 showed that Ala substitution had only a minor detrimental effect on the anorectic effect of PYY3-36, suggesting this residue is not critical for its bioactivity and is therefore a candidate for substitution in drug design. Furthermore, previous work showed that substituting Leu30 with the hydrophobic residue Trp increased the affinity of PYY22-36 at the Y2R. Hydrophobic residues at position 30 may therefore be important for receptor affinity (Balasubramaniam et al., 2000). Analogues were designed with both hydrophobic and positively charged, hydrophilic amino acid substitutions at position 30 and were screened for Y2R binding affinity and susceptibility to proteolytic degradation in vitro, and ability to inhibit food intake in vivo.

2.3.2.2 PYY3-36 analogues with mid-section substitutions

Previous Ala-scanning at position 19 or 23 showed that Ala substitutions at these sites had little effect on the anorectic effect of PYY3-36. In addition, neither Arg19 or Ser23 are well-conserved, suggesting these residues are not critical for the bioactivity of PYY3-36 and may therefore be candidates for substitution in drug design (Ahn et al., 2009). The mid-section, which contains the α-helix of PYY3-36, is not essential for binding, but is involved in promoting stability of the peptide and of the C-terminal binding portion of PYY3-36.
(Zerbe et al., 2006). In order to alter the charge within the midsection and putatively promote stability, peptides were synthesized with a positively charged Lys or His instead of a positively charged Arg at position 19. Previous work showed that Ser23 is important in the bioactivity of PYY22-36 in the intestine (Balasubramaniam et al., 2000). An analogue was therefore synthesized with Ala instead of Ser at position 23 to test whether Ser23 is important for Y2R binding and ability to inhibit food intake. A peptide was also synthesized with a Glu substitution at position 23 in order to determine whether altering the charge at this site alters the bioactivity of PYY3-36.

2.3.2.3 PYY3-36 analogues with N-terminal substitutions

PYY1-36 and PYY3-36 are the endogenous circulating forms of PYY (Grandt et al., 1993), while PYY2-36 has not been reported to be a circulating form of PYY. I hypothesised that that addition of an amino acid residue at the N-terminal would protect the peptide from proteases that are able to cleave PYY1-36 and PYY3-36 endogenously. The addition of an amino acid at the N-terminal of PYY3-36 may also alter its helicity and 3D conformation, since significant differences have been shown between the structures of PYY1-36 and PYY3-36 (Nygaard et al., 2006). PYY3-36 was therefore synthesized with an N-terminal Pro, the amino acid that occurs at position 2 in the endogenous PYY1-36 sequence and is the first residue that forms part of the N-terminal proline helix of PYY (Nygaard et al., 2006). Another PYY3-36 analogue was synthesized with an N-terminal Cys addition, in order to determine whether a mildly hydrophobic amino acid at this position protects the molecule against proteolytic attack while having minimal effects on structure/activity at the Y2R. The effects of these analogues on Y2R affinity, susceptibility to proteolytic degradation and ability to inhibit food intake was then assessed. Previous Ala-scan work revealed that substitution of Lys for Ala at position 4 resulted in an analogue with greater bioactivity than PYY3-36, which decreased food intake by 36% relative to saline (Ahn et al. 2009). This was unexpected, as Lys4 is highly conserved between species. Therefore, two further amino acid substitutions at this site were explored: one similar to the native Lys and one with the opposite charge moiety. His was substituted for Lys at this position in
order to determine whether a slight change in amino acid side chain may protect the molecule from proteolytic attack whilst retaining binding affinity (Conlon, 2002). PYY3-36 Glu4 was also synthesized in order to determine whether a negatively charged amino acid at position 4 affects the stability of the peptide and therefore its bioactivity. Ala-scan experiments also showed that substitution of Ala for Glu at position 6 had little effect on the bioactivity of PYY3-36. Considering that Glu6 is very well conserved across species, the fact that substituting it for Ala had little effect was surprising and suggested that position 6 may be a potential candidate for drug design. In order to investigate this, three different substitutions were made at position 6, including a positively charged Lys, a neutral/polar Ser and a hydrophobic/aromatic Val.

2.3.2.4 PYY3-36 analogues with substitutions intended to promote longevity

Addition of an acyl side chain to a peptide can increase its binding to plasma proteins and thus reduce its breakdown by peptidases and impede metabolic clearance by the kidney (Knudsen et al., 2000). Analogues of PYY3-36 were produced with a lauroyl acyl side chain, which is an 18 carbon fatty acid chain, attached to a Lys at position 12. An acyl side chain require a Lys for attachment, and a location separate from the α-helix and C-terminal, and on the β-turn, was used in an attempt to minimise effects on secondary structure and prevent interference in receptor binding (Knudsen et al., 2000). An analogue containing the Lys12 substitution in an unacylated form was also synthesized and tested in order to separate out the effects of the addition of the lauroyl acyl group from the effects of Lys12. Stereochemical changes are often introduced into peptides in an effort to reduce the conformational fit of the substrate into the active sites of peptidases, and have been utilised in GLP-1 analogue design (Hinke et al., 2002). A PYY3-36 analogue with a stereochemical change at Ile3 (PYY3-36 D-allo-Ile3) was designed and has been previously reported (Parkinson et al., 2008). An enantiomer of D-allo-Ile, D-Ile was substituted for Ile3 in order to investigate the effects of stereochemical reorientation of side chains at position 3 on proteolytic degradation at the N-terminal. In the same vein, analogues with a D-isomer of Pro at the N-terminal were designed (see fig. 2.5 for structures of stereoisomers). Following
on from these results, the most effective ‘longevity’ substitutions were combined with simple substitutions, including Ser6, in a single analogue, in order to determine whether the effects on bioactivity are summative.

2.3.2.5 PYY3-36 analogues with combined substitutions

The results of the studies described above demonstrated the effects of altering the structure of PYY3-36 in specific regions of the peptide. The rationale behind these modifications was to establish the importance of certain regions in Y2R binding and susceptibility to proteolytic degradation, and to determine whether these alterations can reduce clearance in
vivo, thus prolonging activity, and/or increase Y2 receptor binding affinity. The endpoint for all of these studies was the effect of the changes on the anorexigenic activity of peripherally administered PYY3-36. Different combinations of these simple substitutions were then generated in order to design single analogues with improved receptor binding and/or reduced susceptibility to proteolytic degradation.

2.3.3 Production of hY2R overexpressing cell line

2.3.3.1 Production of competent bacteria

To allow entry of DNA-containing plasmids into gram negative bacteria, they are transformed to become competent to take up DNA. This is achieved by incubating the bacteria with cations (Hanahan, 1983).

Materials
LB (appendix II)
Tetracycline (Sigma) 10 mg/ml in absolute ethanol
TFB I (appendix II)
TFB II (appendix II)
XL1-Blue E.coli (Stratagene Ltd.)

Method
One hundred millilitres of LB supplemented with 5µg/ml tetracycline (LB_{tet}) was inoculated with a colony of XL1-Blue and incubated overnight, with vigorous shaking, at 37°C. One millilitre of this culture was inoculated into 100ml of fresh LB_{tet} (prewarmed to 37°C) and incubated at 37°C, with vigorous shaking, until the bacteria were in log phase growth (OD_{550} = 0.4-0.5). The bacteria were recovered by centrifugation at 800g for 15min. The bacteria were resuspended in 40ml ice cold TFBI and incubated on ice for 10min. The bacteria were recovered as above and resuspended in 4ml TFBII and incubated...
on ice for 15min. Following incubation 50µl aliquots of cell suspension were frozen in a dry ice/ethanol bath and stored at -70°C.

2.3.3.2 Electrophoresis of DNA fragments

Additional steps to promote efficient transformation of the bacteria are required. Incubating the bacteria on ice facilitates attachment of the plasmid to the bacterial cell membrane, while the heat shock step facilitates transport of the plasmid containing the antibiotic resistance genes into the bacteria. The subsequent incubation allows expression of the ampicillin resistance gene before exposure to the antibiotic.

Materials
LB (appendix II)
LB agar plates (appendix II)
Competent bacteria
pCMV6-XL5-Y2 receptor (Origene; Appendix III)

Method
A 50µl aliquot of frozen competent bacteria was thawed on ice, 10ng of plasmid added and the mixture incubated on ice for 20min. The reaction was then incubated at 42°C for 2min followed by incubation on ice for 2min. Two hundred microlitres of LB was then added and the reaction incubated at 37°C for 30 min. Simultaneously, agar plates (supplemented with ampicillin) were dried and the transformed bacteria added to the plate. The bacteria were spread over the surface of the agar, the plates inverted and incubated at 37°C overnight.

2.3.3.3 Small scale preparation of plasmid

Initially, plasmids were isolated on a small scale culture to allow several clones to be analysed simultaneously (Sambrook and Russell, 2001). To isolate plasmids from bacteria
it is necessary to disrupt the cell wall to release the plasmid and at the same time remove contaminating proteins, genomic DNA and RNA. The method used involves disruption of the cell wall by treatment with alkaline SDS followed by precipitation of bacterial debris with sodium acetate. This step also removes most of the genomic DNA, since in bacteria this is anchored to the cell wall. The RNA is removed at a later stage by either treatment with RNase A or alkali.

Materials

- LB
- SDS (appendix II)
- 3M Sodium acetate pH 4.6 (appendix II)
- Phenol/chloroform/isoamyl alcohol (25:24:1 dilution) (VWR International)
- GTE (appendix II)
  - 25mM Tris-HCl pH 8.0
  - 10mM EDTA
  - 50mM Glucose
- Isopropanol
- 0.3M sodium chloride (appendix II)
- Absolute ethanol

Method

Two millilitres LB supplemented with 0.05mg/ml ampicillin (LB (amp)) were inoculated with a single bacteria colony and incubated overnight at 37°C with vigorous shaking. One and a half millilitres of the culture were centrifuged for two min at 12300g to pellet the bacteria. The supernatant was discarded and the pellet resuspended in 100µl GTE. Two hundred microlitres alkaline SDS was added and the sample incubated on ice for 5min followed by the addition of 150µl 3M sodium acetate and a further incubation on ice for 5min. The precipitated bacterial debris was removed by centrifugation for 5min at 12300g at room temperature and 350µl supernatant was transferred to a clean tube. An equal volume of phenol/chloroform/isoamyl alcohol was added and the sample mixed. The phases were separated by centrifugation for 3min at 12300g and the aqueous phase
transferred to a fresh tube. The DNA was precipitated by the addition of 0.6 volumes of isopropanol and incubated at room temperature for 10 min. DNA was dissolved in 100 µl 0.3 M sodium chloride, 250 µl ice cold absolute ethanol added, and the solution incubated at -20°C for at least 1 h. The DNA was recovered by centrifugation at 12300 g at room temperature and dissolved in 10 µl GDW.

2.3.3.4 Restriction endonuclease digestion of plasmid DNA

In order to select which clones contained the correct plasmid, DNA was digested with restriction endonuclease enzymes analysed by gel electrophoresis. Clones containing the correct size inserts were chosen for large scale preparation.

Materials
Plasmid DNA
Restriction endonucleases: EcoR I and Xho I (New England Biolabs)
10x Restriction buffer (as supplied)
10x BSA (Sigma)

Method
In separate reactions, the products of small scale preparation of plasmid were diluted in autoclaved GDW, BSA and restriction buffer, to give a final concentration of 1x BSA and 1x restriction buffer. Restriction endonucleases were added to five times excess and the volume of enzyme added was kept below 10% of the final volume. The reaction was incubated for a minimum of 1 h at 37°C.

2.3.3.5 Electrophoresis of DNA fragments

After restriction digestion, the DNA was size fractionated by electrophoresis on an agarose gel to confirm whether an insert was present.
Materials
Agarose, type II-A medium EEO (Sigma)
50x TAE (appendix II)
    2 M Tris-Acetate pH 8.5
    0.5 M EDTA pH 8.0
Ethidium bromide (10mg/ml) (Sigma)
DNA marker (1KB ladder, Invitrogen)
Gel loading buffer (appendix II)

Method
A 0.7% (w/v) agarose gel was prepared by dissolving the agarose in 1x TAE using a microwave oven. The gel was cooled to 45°C and ethidium bromide added to a final concentration of 0.5µg/ml. Three microlitres of loading buffer were added to 10µl of restriction enzyme digest. One microlitre of DNA marker was added to 9µl GDW and treated the same way. The samples were loaded onto the gel and electrophoresed at 1.5V/cm. The DNA was visualised by illumination with UV light (300 nm).

2.3.3.6 Large scale plasmid purification

This method is used to produce the plasmid on a larger scale, and is followed by a caesium chloride gradient to further purify the plasmid.

Materials
LB
MP1 (appendix II)
    25mM Tris-HCl pH 8.0
    10mM EDTA
15% Sucrose
0.3M Sodium chloride
Lysozyme (Sigma)
SDS (appendix II)
3M Potassium acetate pH 4.6 (appendix II)
DNase free RNase A (Sigma)
Phenol/chloroform/isoamyl alcohol (25:24:1 dilution)
1x TE (appendix II)

**Method**

Following small scale preparation of the plasmid the size of the insert was confirmed by restriction digest as described. A large scale preparation of the plasmid was carried out prior to purification using a caesium chloride gradient. A small quantity of bacteria containing the plasmid with the correct size was inoculated into 500ml LB and incubated at 37°C overnight with vigorous shaking. The bacteria were recovered by centrifugation for 8min at 3000g (HS-4 rota in RC-5B super speed centrifuge, Du Pont) at 4°C. The pellet was resuspended in 25ml MP1 supplemented with 2mg/ml of lysozyme and the samples incubated at room temperature for 5min. Fifty millilitres of alkaline SDS were added, the sample mixed until clear and incubated on ice for 5min. Then 37.5ml of 3M potassium acetate were added, the samples mixed and the bacterial debris removed by centrifugation for 15min 9000g (HS-4 rota in RC-5B super speed centrifuge) at 4°C. The supernatant was transferred to a clean tube and 0.6 volumes of isopropanol added and samples incubated on ice for 15min. As before the DNA was recovered by centrifugation (9000g, 4°C). The pellet was dissolved in 10ml 1xTE, RNase A added to a concentration of 0.1mg/ml and the reaction incubated at 37°C for 30min. Addition of an equal volume of phenol/chloroform/isoamyl alcohol was used to terminate the reaction. The solution was then mixed and phases separated by centrifugation for 20min at 10000g and 4°C. The aqueous phase was transferred to a new microtube and 0.1 volumes 2M sodium acetate, pH 5.2 and one volume isopropanol added, and the reaction incubated at -20°C for at least 1h.
2.3.3.7 **Caesium chloride gradient purification**

This technique was used to purify large quantities of plasmid. The purification method depends on the decrease in density of nucleic acids when they bind ethidium bromide. Ethidium bromide binds by intercalation into the DNA, causing the DNA helix to partially unwind. Binding occurs to a higher degree in linear genomic DNA or nicked plasmid DNA than the closed circular supercoiled plasmid DNA, so the different states of DNA form separate bands in the caesium gradient.

**Materials**

TES (appendix II)
- 50 mM Tris-HCl, pH 8.0
- 50 mM Sodium chloride
- 5 mM EDTA
Caesium chloride (Sigma)
Ethidium bromide (10mg/ml)
Isopropanol, caesium chloride saturated (appendix II)
1x TE (appendix II)
Mineral oil

**Method**

The DNA obtained from section 2.3.3.6 was recovered by centrifugation for 20 min at 24000g (HB4 rotor in RC5B super speed centrifuge, Du Pont) and 4°C and dissolved in 7.85ml TES. Eight grams of caesium chloride were dissolved in the DNA solution, 150µl ethidium bromide added and the solution mixed. The sample was loaded into a polyallomer tube (Ultracrimp, Du Pont), overlaid with mineral oil, the tube sealed and centrifuged for 16h at 20°C and 185500g (A1256 rotor, OTD-55B centrifuge, Du Pont). After centrifugation, the DNA bands were visualised by UV illumination and the band containing the closed circular DNA removed using a 20G needle and a 2ml syringe. The ethidium bromide was removed from the plasmid by repeated extraction with an equal volume of caesium chloride saturated isopropanolol, until both phases were colourless. The
DNA was precipitated by addition of two volumes of GDW and six volumes of room temperature absolute ethanol. The DNA was recovered by centrifugation for 15 min at room temperature and 24000g, the supernatant removed and the pellet dissolved in 0.4 ml GDW. The DNA was ethanol precipitated, recovered by centrifugation for 10 min at 12300g, dissolved in 1 ml GDW and quantified spectrophotometrically.

2.3.3.8 Quantification of DNA by spectrophotometer

The DNA was diluted 1:100 in GDW and 1 ml placed into a quartz cuvette. The absorbance was read at 260 and 280 nm, to check for contamination of DNA by RNA and protein (UV0160 spectrophotometer, Kyoto, Japan). DNA samples with an optical density of 1.8-2.0 (optical density = 260 nm/280 nm) were considered clean enough for further use. The concentration of DNA was calculated using the following formula: concentration (µg/ml) = (A\textsubscript{260} x dilution factor x 50)/1000.

2.3.4 Cell Culture

2.3.4.1 Maintenance of cells

Materials
HEK 293T cells (LGC Promochem)
Dulbecco’s modified medium (DMEM) without sodium pyruvate containing 4.5 g/l Glucose (Invitrogen)
Foetal bovine serum (FBS) (Invitrogen)
100x Antibiotic (Invitrogen)
    100 IU/ml Penicillin
    100 µg/ml Streptomycin
Non-enzyme cell dissociation buffer (Sigma)
Method

HEK 293T cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic. Medium was changed every 2-3 days and the cells sub-cultured when 80-90% confluent using a non-enzymatic cell dissociation buffer. Briefly, the medium was aspirated from the flask and 2ml/50 cm\(^2\) flask surface area of room temperature cell dissociation buffer added. Cells were incubated at room temperature until they detached from the flask. Five millilitres of fresh medium was then added to the flask and the cells recovered by centrifugation for 5min at 100g. The cells were resuspended in fresh medium and transferred to a new flask at a dilution of 1:5.

2.3.4.2 Cell passage

Once cells had reached 80-90% confluence, DMEM media was removed using a 10ml pipette, 10ml of cell-dissociation buffer (0.5M EDTA, pH8.0; Invitrogen) added and the flask incubated at room temperature for 15min. Cells were agitated to dislodge them from the bottom of the flask, and 5ml of the cell suspension was removed and added to a new flask containing 35ml media. This process was repeated using the remaining 5ml cell suspension. DMEM media was removed and replaced with fresh media 24h after passage.

2.3.4.3 Transfection of cells with the Y2R DNA construct

2.3.4.3.1 Polyethylenimine-mediated gene transfer

In order to create a readily available source of the human Y2R, the plasmid containing the DNA construct for this receptor was transfected into HEK 293T cells. PEI is a cationic polymer in which every third atom is an amine nitrogen residue. In the presence of 5% glucose, PEI and DNA interact to form stable complexes which are taken up by pinocytosis across the cellular plasma membrane.
Materials
0.1M 25kD PEI (Aldrich Chemical Company; appendix II)
stereile water
10% Glucose
Plasmid DNA
Geneticin (Invitrogen, UK)

Method
Twenty four h prior to transfection, cells were sub-cultured and plated at a density of 20000 cells/60 mm plate (Invitrogen) in standard media. Transfections were carried out when the cells were 70% confluent.

PEI/DNA complexes were made with PEI (average $M_W \sim 25000$) according to the method of Abdallah et al (Abdallah et al., 1996). A 0.1M stock solution of 25kD PEI pH 7.0 was prepared and filtered through a 0.2µm filter prior to use. The plasmid DNA was prepared such that each plate was transfected with 6µg DNA in 5% (w/v) glucose (the 10% glucose solution was also filtered through a 0.2µm filter). The cells were co-transfected with pCMV6-XL5-Y2 receptor and pcDNA3.1+ (plasmid containing geneticin resistance gene; Invitrogen) and 9 nitrogen equivalents of PEI. The amount of PEI required was calculated according to the ratio of PEI amine nitrogen equivalents to DNA phosphate where 1µm of 0.1M PEI = 100nmol amine nitrogen and 10µg DNA = 30nmol DNA phosphate. Thus for 9 nitrogen equivalents 45µg DNA (135nmol DNA phosphate) requires 810nmol amine nitrogen i.e. 8.1µl of 0.1M PEI.

Four plates were transfected with each receptor plasmid, and pcDNA3.1, and two plates with receptor plasmid only (controls). PEI solution was slowly added to the DNA solution and glucose, vortexed for 30 seconds and allowed to stand at room temperature for 10min before use. The PEI/DNA/glucose mix was added slowly to the cells and incubated for 3h at 37°C, after which the media was removed and replaced with standard media (DMEM supplemented with 10% FBS and 1% antibiotic and cells maintained as described in 2.3.9.1). Forty eight hours later, media was supplemented with 800µg/ml geneticin, and the
media replaced every 48h and re-supplemented with fresh geneticin until all control cells were killed (observed as displaced cells). Remaining cells from each plate were then transferred to T-10 flask (1 plate to 1 flask), and maintained as previously described.

2.3.5 Receptor binding studies

2.3.5.1 Preparation of Y2R-expressing membranes

In order to investigate the affinity of PYY3-36 analogues for the hY2R, membranes were prepared from cultured hY2-expressing cells. Cell media was removed and cells were removed from approximately 30 T 175cm2 flasks by scraping in ice-cold 0.02M phosphate buffered saline (PBS). Cells were centrifuged for 5min at 800rpm (Sigma Laboratory centrifuges 3, K18, rotor No. 19777-H). Cell pellets were then homogenized for 15 sec at 3000rpm (Beckman J2-21 Rotor) in ice cold homogenization buffer (1mM HEPES buffer pH 7.4 containing 10µg/ml soybean trypsin inhibitor (Sigma), 0.5µg/ml pepstatin (Sigma), 0.5µg/ml antipain (Sigma), 0.5µg/ml leupeptin, 0.1mg/ml benzamidine (Sigma) and 1ml 100KIU/ml aprotinin (Sigma) at 4°C (Beckman J2-21, rotor JS-13.1)). The pellet was resuspended in 50mM HEPES buffer pH 7.4 containing 10µg/ml soybean trypsin inhibitor (Sigma), 0.5µg/ml pepstatin (Sigma), 0.5µg/ml antipain (Sigma), 0.5µg/ml leupeptin, 0.1mg/ml benzamidine (Sigma) and 1ml 100KIU/ml aprotinin (Sigma) using a motorised homogeniser (IKA, Germany), for 2 min. The mixture was then centrifuged as before except at 2800rpm in order to remove nuclei and cell debris (Beckman J2-21 Rotor), and the resulting supernatant transferred to the ultracentrifuge (Sorvall Ltd, Stevenage, UK) and spun at 38000rpm at 4°C for 1 h. The resulting membrane-containing pellet was resuspended in approximately 15-20ml 50mM HEPES buffer using a hand-held Teflon homogeniser (Jencons) and stored at -70°C in 100µl aliquots.
2.3.5.2 **Biuret assay**

The membrane was then tested in order to determine protein concentration. A standard curve was made up using 10mg/ml bovine serum albumin (Sigma), which was used to construct a standard curve (0, 1, 2, 3, 4 and 5mg of protein/500µl) with dH20. Membrane was assayed at 20µl and 200µl and volumes were adjusted to 500µl with dH20. Three millilitres of biuret reagent was then added to all tubes. Tubes were incubated at room temperature for 30 min, after which the absorbance at 540nm was measured in a spectrophotometer (UV-160, Shimadzu) with a zero reference using the 0mg protein standard. The absorbance values of the sample of membrane were converted to mg protein by linear regression analysis.

2.3.5.3 **Y2 Receptor binding assay**

The receptor binding assay was optimised for pH, temperature, incubation time and buffer. Iodinated PYY1-36 was used as the competitive ligand as it is known to bind with high affinity to hY2R and is easily iodinated (Grandt et al., 1992). All tubes were assayed in triplicate except for those containing the highest and lowest concentrations of PYY3-36 analogue, which were assayed in duplicate. Cell membranes (10ng protein) from a CHO hY2R-overexpressing cell line were used for these assays as described in section 1.3.5.1. Cell membrane was incubated for 90 min at room temperature in siliconized polypropylene tubes (Sigma) with $^{125}$I-PYY1-36 (500Bq:100pM) in binding buffer (2mM MgCl2, 6.5mM CaCl2, 20mM HEPES pH 7.4, 1% BSA (MP Biomedicals), 0.25%Tween20 (Sigma), 4mM phenylmethanesulphonylfluoride (PMSF) (Sigma), 0.5mM diprotin A (Sigma), 0.5mM phosphoramidon (Sigma)) in a final assay volume of 0.5ml. The Y2R-$^{125}$I-PYY1-36-unlabelled peptide (analogue or PYY3-36) complex was separated from free tracer by centrifugation at 15600g (Sigma Laboratory Centrifuges 3, K18) for 3 min. The supernatant was removed and discarded and the pellet resuspended and washed with 0.5ml binding buffer and a re-centrifugation step at 15600g (as above) in order to recover the pellet after the wash. The supernatant was again removed and the resulting pellets counted in a $\gamma$ counter (model NE1600, Thermo Electron Corporation) for 240 seconds. Specific binding
was calculated as the difference between the amount of $^{125}$I-PYY1-36 peptide bound in the absence and presence of a specific concentration of unlabelled peptide which was then used to calculate the % specific binding at different concentrations of peptide. IC50 values (the concentration at which 50% of receptors are bound) were calculated using Graphpad Prism version 5.00 for Windows (GraphPad Software, San Diego, California).

2.3.6 Degradation assay using KBB membrane

2.3.6.1 Preparation of rat KBB membranes

Rat KBB membranes were prepared by the method of homogenisation and differential centrifugation (Booth and Kenny, 1974). Rats were killed by decapitation and kidneys dissected and snap frozen in liquid nitrogen and stored at -70°C. To prepare the membranes, kidneys were placed in cold 0.5M sucrose and the renal cortices dissected out and weighed. The cortices were finely sliced and minced, and then gently homogenised at a ratio of 1g tissue to 6ml 0.5M sucrose using a 15ml hand-homogeniser (Jencons). Tissue was then further homogenized using three strokes of a motorized 15ml homogenizer with a Teflon pestle at 1000rpm. Following this, 1M MgCl2 was added to a final concentration of 10mM in the homogenate, and the homogenate incubated for 15 min in the ice bath to ensure aggregation of other subcellular structures by bivalent metal ions. The suspension was then centrifuged at 3000 rpm (Beckman J2-21, rotor JS-13.1) for 20min at 4°C, the supernatant resuspended and then centrifuged at 10000 rpm (Beckman J2-21, rotor JS-13.1) for 12 min at 4°C. The supernatant was discarded and the pellet resuspended in 100µl of 300mM mannitol in 12mM Tris-Cl (pH7.4), with 10mM MgCl2. This was then centrifuged at 3700 rpm at 4°C (Sigma Laboratory Centrifuge 3 K18). The supernatant was resuspended and centrifuged for 12min at 10000rpm at 4°C. The pellet was then resuspended in 10ml of 300mM mannitol in 12mM HEPES (pH7.4) and the Biuret assay used to determine protein concentration (see section 2.3.5.2).
2.3.6.2 **PYY3-36/PYY3-36 analogue degradation assay using KBB**

PYY3-36 (2nmol) was incubated with or without 12.5µg, 25µg or 50µg, KBB in digest buffer (300mM mannitol in 12mM HEPES, pH7.4) at 37°C in a total volume of 140µl. For the inhibitor studies, 100nmol phosphoramidon or actinonin was included in the digests, either with or without peptide. The reaction was terminated at 5, 15, 30 or 60 min time-points by the addition of 10µl 30% trifluoroacetic acid (TFA). Samples were then centrifuged at 12500xg for 5 min at room temperature in order to separate enzyme from peptide solution. A dose response experiment using different concentrations of KBB was carried out in order to determine a concentration at which 1) >50% of peptide was degraded and 2) break down products could be visualized. This concentration was used in the remainder of peptide/KBB incubations. Incubation samples were then analysed by HPLC.

2.3.6.3 **High Performance Liquid Chromatography**

Incubation samples were then analysed by HPLC (Jasco HPLC system: solvent delivery system PU-2080 plus, autosampler AS-2057 plus, degasser DG2080-53, dynamic mixer 2080-32, UV detector uv-2075) using a Gemini C\textsubscript{18} (5µm particles, 250mm x 4.6 mm) column. Samples (100µl of supernatant from the terminated incubation) were injected onto the C\textsubscript{18} column and eluted using a linear ACN/water gradient running from 15-60% ACN over 35 min. As the retention time for actinonin was similar to that of PYY3-36, a shallower gradient of 20-35% ACN was used for the actinonin experiments to avoid co-elution of these compounds. The eluted peptides and peptide fragments were detected spectrophotometrically at a wavelength of 214nm. Percentage degradation of peptide was calculated by comparing area under the peak (AUP) on HPLC chromatograms of incubations with and without enzyme (Graphpad Prism version 5.00 for Windows, GraphPad Software, San Diego, California).
2.3.7 In vivo studies

2.3.7.1 Animals

All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project Licence 70/5516). Adult male C57/BL6 mice (Harlan, UK) weighing 20-25g or adult male Wistar rats (Charles River, UK) weighing 250-300g were maintained in individual cages under controlled temperature (21-23°C) and lights (12:12 h light:dark cycle, lights on at 0700h). Animals had ad libitum access to water and RM1 diet (Special Diet Services). To minimise stress animals were regularly handled and acclimatised to subcutaneous (sc) injections according to the exact protocol on the experimental study day.

2.3.7.2 Acute PYY3-36 or PYY3-36 analogue feeding studies in mice

Studies were carried out during the early light phase (0900h). Mice were overnight-fasted in order to reduce circulating levels of endogenous PYY (Adrian et al., 1985). Mice were randomly divided into treatment groups with the same average body weight and administered a sc injection of either saline, PYY3-36 (50nmol/kg) or PYY3-36 analogue (50nmol/kg). Peptides were dissolved in a maximum volume of 100µl saline. Body weight was recorded before injection. Mice were returned to their cages after injection with access to a pre-weighed amount of food. Food was then weighed at 1, 2, 4, 8 and 24h intervals post-injection and the body weight recorded at 24h post-injection. Subcutaneous administration of 50nmol/kg PYY3-36 resulted in a significant or a trend towards a reduction in food intake for the first 2 h post-injection in most analogue studies compared to saline-treated controls. The food intake of animals injected with PYY3-36 and PYY3-36 analogues at 1 h, 2 h, 4 h and 8 h intervals was compared to the food intake of those injected with saline and analysed for significance. The p values were then compared
between PYY3-36 and PYY3-36 analogues. Analogues that resulted in a more prolonged anorexigenic effect than PYY3-36 were considered long acting.

### 2.3.8 Statistics

All data are presented as means ± SEM. For the PYY3-36 dose response study, food intake data was analyzed using one-way ANOVA with Dunnet’s *post hoc* test (GraphPad Prism version 5.00 for Windows; GraphPad Software, San Diego, California). For PYY3-36 analogue studies, food intake data was compared using one-way ANOVA with Tukey’s *post hoc* multiple comparison test (GraphPad Prism version 5.00 for Windows; GraphPad Software, San Diego, California). In all cases, p<0.05 was considered to be statistically significant. The large-scale nature of the analogue studies made it unfeasible to administer all analogues in the same study. Taking this into account, PYY3-36 analogues were compared using p values derived from comparison of PYY3-36 analogue vs saline control and vs PYY3-36 control from the study day on which the analogue was tested. For clarity, in an additional set of figures, the effect of PYY3-36 analogues representing alterations to specific regions of PYY3-36 on food intake are displayed in the same figure by expressing the data as a percentage of the saline control from the particular study day on which the analogue was tested. In these figures, saline and PYY3-36 control values are shown as the mean of the values for all study days for that category. These figures are intended to aid qualitative interpretation of the results, and statistical analyses were not carried out on these data.
2.4 Results

2.4.1 Investigations into the role of the C terminus of PYY3-36 in structure activity relations at the Y2R and in its in vivo bioactivity

Substitutions at various residue positions (see table 2.1) within the C terminus of PYY3-36 were carried out in order to investigate a) the structural importance of these amino acids in binding the Y2R and b) whether alterations at this site could alter the peptide’s susceptibility to proteolytic degradation and c) whether these alterations have effects on in vivo bioactivity of PYY3-36 (Fauchere JL and Thurieau, 1992).

2.4.1.1 Effect of substitutions at position 30 of PYY3-36 on affinity at the Y2R and resistance to proteolytic degradation

Replacing Leu30 with a positively charged Lys or Arg residue increased the affinity of the peptide for the Y2R by approximately 1.5-fold compared to PYY3-36. Substitution of His or Ile at position 30 reduced the affinity for the Y2R by 2-fold compared to PYY3-36. Substituting Leu30 with the hydrophobic residues Phe or Val worsened the binding affinity at the Y2R by 3-fold (see fig. 2.6 for binding curves and table 2.1 for IC50 values). PYY3-36 and PYY3-36 analogues were incubated with KBB membranes to investigate susceptibility to proteolytic degradation. Degradation of PYY3-36 was increased by 63 ±6% and 17 ±14% when Leu30 was substituted for Lys or Phe respectively. All other substitutions at position 30 resulted in a decrease in degradation of PYY3-36; by 30 ±3% for His30, 1 ±4% for Arg30, 13 ±2% for Ile30 and 8 ±12% for Val30 (see table 2.1: degradation shown as mean percentage of PYY3-36 degradation).
<table>
<thead>
<tr>
<th>Peptide/analogue</th>
<th>IC50 at Y2 receptor</th>
<th>% of PYY3-36 degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (nM)</td>
<td>SEM</td>
</tr>
<tr>
<td>PYY3-36</td>
<td>0.17</td>
<td>0.002</td>
</tr>
<tr>
<td>PYY3-36 Lys 30</td>
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<td>0.02</td>
</tr>
<tr>
<td>PYY3-36 His 30</td>
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<td>0.05</td>
</tr>
<tr>
<td>PYY3-36 Arg 30</td>
<td>0.10</td>
<td>0.005</td>
</tr>
<tr>
<td>PYY3-36 Ile 30</td>
<td>0.30</td>
<td>0.02</td>
</tr>
<tr>
<td>PYY3-36 Phe 30</td>
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<td>0.11</td>
</tr>
<tr>
<td>PYY3-36 Val 30</td>
<td>0.61</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 2.1. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at positions 30 within the binding hexapeptide of the C terminus. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue. The mean IC50 of PYY3-36 derived from assays carried out on the same day is shown for comparison. For the degradation studies, PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. The percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation carried out on the same day.
Figure 2.6. Representative displacement binding curves for C-terminal-hexapeptide substituted analogues at the Y2 receptor A) PYY3-36 Lys30 (mean IC50: 0.13nM), B) PYY3-36 His30 (mean IC50: 0.31nM), C) PYY3-36 Arg30 (mean IC50: 0.1nM), D) PYY3-36 Ile30 (mean IC50: 0.3nM), E: PYY3-36 Phe30 (mean IC50: 0.56nM) and F) PYY3-36 Val30 (mean IC50: 0.61). A control PYY3-36 binding assay carried out at the same time is shown as a reference on each binding affinity plot. Assays were carried out in triplicate and the IC50 is derived from a minimum of 3 assays. Increasing concentrations of PYY3-36 analogue were incubated with a fixed concentration of $^{125}$I PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I PYY1-36 bound with and without unlabelled competing peptide.
2.4.1.2 Effect of PYY3-36 on food intake in mice (dose finding study)

The effect of sc administration of PYY3-36 in C57/BL6 mice was examined to establish the dose required to produce a robust reduction in food intake (n=8-9 /group) (see fig. 2.7). Administration of PYY3-36 sc at all doses tested significantly reduced cumulative food intake for the first four hours post-injection when compared to saline controls. A significant reduction in food intake was observed over 8h following administration of 60nmol/kg, 200nmol/kg and 600nmol/kg PYY3-36 when compared to saline controls. At 8-24h post-injection PYY3-36 resulted in a dose-dependent trend towards an increase in food intake, a finding that has been previously defined as an ‘overswing’ response to the acute anorectic effect of PYY3-36 (Fauchere JL and Thurieau, 1992).

Figure 2.7. The effect of subcutaneous administration of 20, 60, 200 or 600nmol/kg PYY3-36 (n=8-9) on food intake in fasted mice. Results are means ± SEM. **p<0.01, ***p<0.001 vs saline (one-way ANOVA with Dunnett’s post-hoc adjustment).
2.4.1.3 Effect of substitutions at position 30 of PYY3-36 on food intake

All position 30-substituted analogues significantly reduced food intake 0-1h post-injection. At 1-2h post-injection, analogues substituted with Ile at position 30 had no significant effect on food intake compared to saline or PYY3-36 controls. Analogues substituted with His, Arg, Phe or Val reduced food intake to a similar extent as PYY3-36 at the same time points (see table 2.2). At the 2-4h interval, PYY3-36 Lys30 significantly reduced food intake whereas PYY3-36 had no effect on food intake at this time point. By the 4-8 h interval, no position 30 substituted analogues were more effective than PYY3-36 in reducing food intake (see table 2.2). All position-30 substituted analogues significantly reduced cumulative food intake up to 8h post-injection when compared to saline controls. However, mice treated with PYY3-36 Val30 ate significantly more than mice treated with PYY3-36 over 8h. PYY3-36 Val30 had no effect on food intake by 24h post-injection as opposed to PYY3-36, which resulted in a significant reduction in cumulative food intake over 24h compared to saline controls. Examining the percentage reduction in food intake for position 30 substituted analogues suggested that a Lys30 substitution most effectively reduced food intake compared to other position 30-substituted analogues. This is likely due to the fact that PYY3-36 Lys30 was the only analogue able to significantly inhibit food intake at the 2-4h interval and/or that it resulted in less of an overswing response at 4-8h post-injection compared to the other analogues tested (see fig. 2.8).
<table>
<thead>
<tr>
<th>Group</th>
<th>0-1h</th>
<th>1-2h</th>
<th>2-4h</th>
<th>4-8h</th>
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</table>

Table 2.2. Statistical significance of the effects on food intake of PYY3-36 controls or position 30-substituted PYY3-36 analogue derived from food intake data from each study day. All food intake (g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc adjustment ( *p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.01, $$$p<0.001$ vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.
Figure 2.8. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 30-substituted PYY3-36 analogues. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.

2.4.1.4 Effect of substitutions within the C-terminal hexapeptide of PYY3-36 on Y2R binding and resistance to proteolytic degradation

Substituting Gln34 with Asn was detrimental to Y2R binding. Substituting Val 31 with Lys had little effect on the Y2R binding, while substituting Tyr36 with Phe resulted in a slight increase in Y2R binding affinity (see fig. 2.9 for binding curves and table 2.3 for IC50 values). PYY3-36 and PYY3-36 analogues were incubated with KBB to investigate susceptibility to proteolytic degradation. Degradation of PYY3-36 was decreased by 5 ±1% when Val31 was substituted for Lys, by 31 ±6% when Gln34 was substituted for Asn and by 36 ±2 % when Tyr36 was substituted for Phe (see table 2.3: degradation of analogues shown as mean percentage of PYY3-36 degradation).
<table>
<thead>
<tr>
<th>Peptide/analogue</th>
<th>IC50 at Y2 receptor</th>
<th>% of PYY3-36 degradation</th>
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<td>Mean (nM)</td>
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<td>PYY3-36</td>
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Table 2.3. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at positions 31, 34 or 36 within the binding hexapeptide of the C terminus.

A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue. The mean IC50 of PYY3-36 derived from assays carried out on the same day is shown for comparison. For the degradation studies, PYY3-36 or analogue (2nmol) was incubated with KBB (50μg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. The percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation carried out on the same day.
Figure 2.9. Representative displacement binding curves for C-terminal-hexapeptide substituted analogues at the Y2 receptor A) PYY3-36 Lys31 (mean IC50: 0.19nM), B) PYY3-36 Asn34 (mean IC50: 0.64nM) and C) PYY3-36 Phe36 (mean IC50: 0.13nM). A control PYY3-36 binding assay carried out at the same time is shown as a reference on each binding affinity plot. Assays were carried out in triplicate and the IC50 is derived from a minimum of 3 assays. Increasing concentrations of PYY3-36 analogue were incubated with a fixed concentration of $^{125}\text{I}$ PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}\text{I}$ PYY1-36 bound with and without unlabelled competing peptide.
**2.4.1.4.1 Effect of substitutions within the C-terminal hexapeptide of PYY3-36 on food intake**

PYY3-36 Lys 31 significantly reduced food intake at 0-1h post-injection compared to saline and to a similar extent to PYY3-36 controls (see table 2.4). However, PYY3-36 Lys31 was less effective in reducing food intake at 1-2h compared to PYY3-36 controls. PYY3-36 Asn34 had no effect on food intake at any time point post-injection when compared to saline controls, and was less effective in reducing 0-1h food intake and cumulative food intake over 8h compared to PYY3-36 controls. PYY3-36 Phe36 significantly reduced 0-1h and 1-2h food intake compared to saline controls and to a similar extent as PYY3-36 controls (see table 2.4). PYY3-36 Phe36 significantly reduced cumulative food intake over 4h and 8h compared to saline. Comparison of p values between these two groups suggested that PYY3-36 Phe36 was less effective than PYY3-36 at reducing cumulative food intake. Examining the percentage reduction in food intake for analogues with substitutions at positions 31, 34 and 36 suggested that all substitutions tested were detrimental to the bioactivity of PYY3-36 when compared to PYY3-36 controls (see fig. 2.10).

<table>
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Table 2.4. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions in the binding hexapeptide derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test (*p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.05, $$ p<0.01 vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.
Figure 2.10. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with C-terminal hexapeptide substitutions. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.

2.4.2 Investigations into the role of the mid-section of PYY3-36 in structure activity relations at the Y2R and in vivo bioactivity

Substitutions at various residue positions (see tables 2.5 and 2.7) within the mid-section of PYY3-36 were carried out in order to investigate a) the structural importance of these amino acids in binding the Y2R and b) whether alterations at this site could alter the peptide’s susceptibility to proteolytic degradation and c) whether these alterations have effects on in vivo bioactivity of PYY3-36 (Fauchere JL and Thurieau, 1992).
2.4.2.1 Effect of substitutions at position 19 of PYY3-36 on receptor binding at the Y2R and susceptibility to proteolytic degradation

Replacing Arg19 with a positively charged Lys increased the affinity of the peptide for the Y2R compared to PYY3-36. Substitution of His at position 19 had no effect on affinity for the Y2R compared to PYY3-36 (see fig. 2.11 for binding curves and table 2.5 for IC50 values). Degradation of PYY3-36 was decreased by 32 ±0.2% and by 8 ±4% when Arg19 was substituted for Lys or His respectively (see table 2.5: degradation of analogues shown as percentage of PYY3-36 degradation).

<table>
<thead>
<tr>
<th>Peptide/analogue</th>
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<th>% of PYY3-36 degradation</th>
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Table 2.5. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 19 within the mid-section. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.
Figure 2.11. Representative displacement binding curves for position 19 substituted analogues at the Y2 receptor A) PYY3-36 Lys19 (mean IC50: 0.12nM) and B) PYY3-36 His19 (mean IC50: 0.18nM). A control PYY3-36 binding assay carried out at the same time is shown as a reference on each binding affinity plot. Assays were carried out in triplicate and the IC50 is derived from a minimum of 3 assays. Increasing concentrations of PYY3-36 analogue were incubated with a fixed concentration of $^{125}$I PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I PYY1-36 bound with and without unlabelled competing peptide.

2.4.2.2 Effect of substitutions at position 19 of PYY3-36 on food intake

PYY3-36 Lys 19 significantly reduced food intake at 0-1h post-injection and cumulative food intake over 8h compared to saline and to a similar extent as PYY3-36 controls at the same time point (see table 2.6). However, over 24h, PYY3-36 significantly reduced food intake compared to saline, whereas PYY3-36 Lys19 had no effect on food intake compared to saline and resulted in significantly higher food intake than PYY3-. PYY3-36 His19 significantly reduced food intake at 0-1h compared to saline, but to less of an extent than PYY3-36 at this time point (see table 2.6). PYY3-36 His19 was significantly less effective than PYY3-36 controls at reducing cumulative food intake over 8h.

Examining the percentage reduction in food intake for analogues with substitutions at position 19 suggested that all substitutions were detrimental to the bioactivity of PYY3-36
when compared to PYY3-36 controls and that animals treated with PYY3-36 Asn34 exhibited a slight increase in % food intake compared to saline controls (see fig. 2.12).

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Table 2.6. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions at position 19 derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test (*p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.05 vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.

Figure 2.12. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 19-substituted PYY3-36 analogues. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.
2.4.2.3 Effect of substitutions at position 23 of PYY3-36 on receptor binding and susceptibility to proteolytic degradation

Replacing Ser23 with Ala increased the affinity for the Y2R compared to PYY3-36 by approximately 1.5 fold, while substitution of Ser23 with a negatively charged Glu had no effect on the affinity of the peptide for the Y2R compared to PYY3-36 (see fig. 2.13 for binding curves and table 2.7 for IC50 values). PYY3-36 and PYY3-36 analogues were incubated with KBB to investigate susceptibility to proteolytic degradation. Degradation of PYY3-36 was increased by 21 ±4% and decreased by 49 ±9% when Ser23 was substituted for Ala or Glu respectively (see table 2.7: degradation of analogues shown as percentage of PYY3-36 degradation).

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<th>Peptide/analogue</th>
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<th>% of PYY3-36 degradation</th>
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Table 2.7. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 23 in the mid-section. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37ºC and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.
Figure 2.13. Representative displacement binding curves for position 23 substituted analogues at the Y2 receptor A) PYY3-36 Ala23 (mean IC50: 0.12nM) and B) PYY3-36 Glu23 (mean IC50: 0.21nM). A control PYY3-36 binding assay carried out at the same time is shown as a reference on each binding affinity plot. Assays were carried out in triplicate and the IC50 is derived from a minimum of 3 assays. Increasing concentrations of PYY3-36 analogue were incubated with a fixed concentration of $^{125}$I PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I PYY1-36 bound with and without unlabelled competing peptide.

**2.4.2.4 Effect of substitutions at position 23 of PYY3-36 on food intake**

PYY3-36 Ala23 significantly reduced food intake at 0-1h and 2-4h intervals post-injection to a similar extent as PYY3-36 (see table 2.8). PYY3-36 Ala23 significantly reduced cumulative food intake over 24h compared to saline, and to a similar extent as PYY3-36 compared to saline at the same time point. PYY3-36 Glu23 significantly reduced food intake at 0-1h, 1-2h and 2-4h intervals and on cumulative food intake over 8h post-injection compared to saline, and to a similar extent as PYY3-36 (see table 2.8).

Examining the percentage reduction in food intake for analogues with substitutions at position 23 suggests that Glu23 slightly decreased % food intake compared to PYY3-36 controls, whereas an Ala23 substitution had no effect on the bioactivity of PYY3-36 when compared to PYY3-36 controls (see fig. 2.14).
Table 2.8. Statistical significance for PYY3-36 controls or PYY3-36 analogues with substitutions at position 23 derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test (*p<0.05, **p<0.01, ***p<0.001 vs saline). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.

Figure 2.14. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 23-substituted PYY3-36 analogues. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.
2.4.3 Investigations into the role of the N-terminal of PYY3-36 in structure activity relations at the Y2R and in vivo bioactivity

Substitutions at various residue positions (see table 2.9) within the mid-section of PYY3-36 were carried out in order to investigate a) the structural importance of these amino acids in binding Y2R and b) whether alterations at this site could alter the peptide’s susceptibility to proteolytic degradation and c) whether these alterations have effects on in vivo bioactivity of PYY3-36 (Fauchere JL and Thurieau, 1992).

2.4.3.1 Effect of substitutions at position 4 of PYY3-36 on receptor binding and susceptibility to proteolytic degradation

Replacing Lys4 with a positively charged His reduced the affinity of PYY3-36 at the Y2R by 3-fold, while substitution of a negatively charged Glu resulted in a 9-fold decrease in affinity at the Y2R (see fig 2.15 for binding curves and table 2.9 for IC50 values). Degradation of PYY3-36 was decreased by 7 ±13% and by 20 ±10% when Lys4 was substituted for His or Glu respectively (see table 2.9: degradation of analogues shown as percentage of PYY3-36 degradation).

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<th>Peptide/analogue</th>
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Table 2.9. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 4 in the N-terminal. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37ºC and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.
Figure 2.15. Representative displacement binding curves for position 4-substituted analogues at the Y2 receptor A) PYY3-36 His4 (mean IC50: 0.51nM) and B) PYY3-36 Glu4 (mean IC50: 0.90nM). A control PYY3-36 binding assay carried out at the same time is shown as a reference on each binding affinity plot. Assays were carried out in triplicate and the IC50 is derived from a minimum of 3 assays. Increasing concentrations of PYY3-36 analogue were incubated with a fixed concentration of $^{125}$I PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I PYY1-36 bound with and without unlabelled competing peptide.

2.4.3.2 Effect of substitutions at position 4 of PYY3-36 on food intake

PYY3-36 His4 significantly reduced food intake at 0-1h and 1-2h intervals post-injection compared to saline and to a similar extent as PYY3-36. PYY3-36 significantly reduced food intake at the 2-4h interval, and significantly increased food intake at the 4-8h interval compared to saline controls. In contrast, PYY3-36 His4 had no significant effect on food intake at both the 2-4h and 4-8h intervals (see table 2.10). PYY3-36 His4 significantly decreased cumulative food intake over 4h compared to saline, but this was significantly higher than PYY3-36 controls at the same time point. Furthermore, PYY3-36 His4 had no effect on food intake over 8h, whereas PYY3-36 significantly decreased food intake compared to saline. PYY3-36 Glu4 significantly reduced food intake compared to saline but was significantly less effective at reducing food intake compared to PYY3-36 at the 0-1h interval. PYY3-36 Glu4 resulted in a significant reduction in cumulative food intake over 8h, but this was to less of an extent than PYY3-36 controls (see table 2.10). Examining the percentage reduction in food intake suggested that substitution of either a
His or Glu for Lys4 were detrimental to the bioactivity of PYY3-36 when compared to PYY3-36 controls (see fig. 2.16).

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Table 2.10. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions at position 4 derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test (*p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.05$ vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.

Figure 2.16. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 4-substituted PYY3-36 analogues. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.
2.4.3.3 Effect of substitutions at position 6 of PYY3-36 on receptor binding at the Y2R and susceptibility to proteolytic degradation

Replacing Glu6 with a polar Ser, a hydrophobic Val or a positively charged Lys increased the affinity of PYY3-36 at the Y2R by approximately 1-fold (see fig 2.17 for binding curves and table 2.11 for IC50 values). Degradation of PYY3-36 was increased by 17 ±2% and decreased by 21 ±11% and 18 ±5% when Glu6 was substituted for Ser, Val or Lys respectively (see table 2.11: degradation of analogues shown as percentage of PYY3-36 degradation).

<table>
<thead>
<tr>
<th>Peptide/analogue</th>
<th>IC50 at Y2 receptor</th>
<th>% of PYY3-36 degradation</th>
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<tr>
<td>Lys 6</td>
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<td>0.06</td>
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</table>

Table 2.11. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 6 in the N-terminal. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.
Figure 2.17. Representative displacement binding curves for position 6-substituted analogues at the Y2 receptor A) PYY3-36 Ser6 (mean IC50: 0.12nM), B) PYY3-36 Val6 (mean IC50: 0.12nM) and C) PYY3-36 Lys6 (mean IC50:0.11nM). A control PYY3-36 binding assay carried out at the same time is shown as a reference on each binding affinity plot. Assays were carried out in triplicate and the IC50 is derived from a minimum of 3 assays. Increasing concentrations of PYY3-36 analogue were incubated with a fixed concentration of ¹²⁵I PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of ¹²⁵I PYY1-36 bound with and without unlabelled competing peptide.

2.4.3.4 Effect of substitutions at position 6 of PYY3-36 on food intake

PYY3-36 Ser6 significantly reduced food intake compared to saline at the 0-1h and1-2h interval compared to saline controls, and to a similar extent as PYY3-36 controls. PYY3-36 Ser6 significantly increased food intake compared to PYY3-36 controls at the 2-4h interval. At the 4-8h interval, PYY3-36 Ser6 significantly decreased food intake compared to PYY3-36, which resulted in significantly higher food intake compared to saline at this time point.
PYY3-36 Ser6 significantly reduced cumulative food intake over 4h compared to saline and resulted in significantly higher food intake compared to PYY3-36, but had no effect on food intake by 8h. PYY3-36 Val6 significantly reduced food intake at 0-1h post-injection compared to saline controls, and to a similar extent as PYY3-36 controls, but failed to reduce food intake at 2-4h post-injection as observed for PYY3-36 controls (see table 2.12). PYY3-36 Val6 significantly reduced cumulative food intake over 4h compared to saline, and to a similar extent as PYY3-36 controls, but failed to reduce 8h cumulative food intake as observed for PYY3-36 controls. PYY3-36 Lys6 significantly reduced food intake 0-1h post-injection compared to saline controls to a similar extent as PYY3-36, and resulted in significantly higher food intake at the 2-4h interval compared to PYY3-36 controls (see table 2.12). PYY3-36 Lys6 significantly reduced cumulative food intake over 8h, but to a less of an extent than PYY3-36. Examining the percentage reduction in food intake suggested that substitution of Glu6 for either a polar Ser, a positively charged Lys or a hydrophobic Val was detrimental to the bioactivity of PYY3-36 when compared to PYY3-36 controls (see fig. 2.18).

<table>
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Table 2.12. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions at position 6 derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test (*p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.05, $$ p<0.01 vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.
2.4.4 Effect of modifications to improve longevity on receptor binding, degradation and in vivo bioactivity

2.4.4.1 Effect of the addition of a Cys or Pro tail at the N-terminal of PYY3-36 on receptor binding at the Y2R and susceptibility to proteolytic degradation

The addition of Cys or the stereoisomer D-Pro to the N-terminal of PYY3-36 decreased the affinity for the Y2R by approximately 2-fold compared to PYY3-36, while the addition of Pro to the N-terminal of PYY3-36 did not affect the affinity of the peptide for the Y2R compared to PYY3-36 (see fig. 2.19 for binding curves and table 2.13 for IC50 values). Degradation of PYY3-36 was decreased by 53 ±2% and 50 ±13% when Cys or Pro respectively was added to the N-terminal, and increased by 29 ±6% when D-Pro was added.

Figure 2.18. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of position 6-substituted PYY3-36 analogue. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.
to the N-terminal (see table 2.13: degradation of analogues shown as percentage of PYY3-36 degradation).

<table>
<thead>
<tr>
<th>Peptide/analogue</th>
<th>IC50 at Y2 receptor</th>
<th>% of PYY3-36 degradation</th>
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<td>N-D-Pro</td>
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Table 2.13. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with the addition of Cys, Pro or D-Pro at the N-terminal. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.
Figure 2.19. Representative displacement binding curves for analogues with the addition of a Cys, Pro or D-Pro at the N-terminal at the Y2 receptor A) PYY3-36 N-Cys (mean IC50: 0.42nM), B) PYY3-36 N-Pro (mean IC50: 0.15nM) and C) PYY3-36 N-DPro (mean IC50: 0.43nM). A control PYY3-36 binding assay carried out at the same time is shown as a reference on each binding affinity plot. Assays were carried out in triplicate and the IC50 is derived from a minimum of 3 assays. Increasing concentrations of PYY3-36 analogue were incubated with a fixed concentration of $^{125}\text{I}$ PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}\text{I}$ PYY1-36 bound with and without unlabelled competing peptide.
2.4.4.2 Effect of the addition of a Cys, Pro or D-Pro tail at the N-terminal of PYY3-36 on food intake

N-Cys PYY3-36 significantly reduced food intake at 0-1h and 1-2h post-injection compared to saline controls and to a similar extent as PYY3-36 controls (see table 2.14). At the 2-4h interval, N-Cys PYY3-36 significantly reduced food intake compared to saline and PYY3-36 controls, whereas PYY3-36 had no significant effect on food intake. Both N-Cys PYY3-36 and PYY3-36 controls significantly reduced food intake to a similar extent over 24h compared to saline (see table 2.14). N-Pro-PYY3-36 significantly reduced food intake at 0-1h post-injection compared to saline controls and to the same extent as PYY3-36 (see table 2.14). At the 2-4h interval, N-Pro-PYY3-36 significantly reduced food intake compared to saline and PYY3-36 controls, whereas PYY3-36 had no effect on food intake. N-Pro PYY3-36 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls and over 24h compared to saline. N-D-Pro-PYY3-36 significantly reduced food intake 0-1h post-injection compared to saline controls and to a similar extent as PYY3-36 at the same time point (see table 2.14). At the 2-4h interval, N-DPro-PYY3-36 significantly reduced food intake compared to saline and PYY3-36 controls, while at the 4-8h interval N-D-Pro PYY3-36 resulted in significantly higher food intake compared to saline. N-Pro PYY3-36 significantly reduced cumulative food intake over 24h compared to saline, but to less of an extent than PYY3-36. Examining the percentage reduction in food intake suggested that the addition of Pro at the N-terminal of PYY3-36 is more effective than addition of Cys at the N-terminal of PYY3-36 in reducing food intake (see fig. 2.20).
Table 2.14. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with amino acid additions at the N-terminal of PYY3-36 derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test ( *p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.05 vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.

<table>
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Figure 2.20. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with N-terminal addition of Pro, D-Pro or Cys. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.
2.4.4.2.1  

Effect of stereochemical changes at Ile-3 of PYY3-36 on receptor binding and susceptibility to proteolytic degradation

Substitution of Ile3 for the stereoisomers D-Ile or D-allo-Ile increased the affinity of the peptide for the Y2R by approximately 1-fold compared to PYY3-36 (see fig. 2.21 for binding curves and table 2.15 for IC50 values). Degradation of PYY3-36 was increased by 8 ±18% and decreased by 9 ±3%, when Ile3 was substituted for its stereoisomers D-Ile or D-allo-Ile respectively (see table 2.15: degradation of analogues shown as percentage of PYY3-36 degradation).

<table>
<thead>
<tr>
<th>Peptide/analogue</th>
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<th>% of PYY3-36 degradation</th>
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</tr>
<tr>
<td>D-allo-Ile 3</td>
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Table 2.15. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with substitutions at position 3 in the N-terminal. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.
Figure 2.21. Representative displacement binding curves for analogues with a stereoisomer of Ile at position 3 at the Y2 receptor A) PYY3-36 D-Ile3 (mean IC50: 0.12nM), B) PYY3-36 D-allo-Ile3 (mean IC50: 0.11nM). A control PYY3-36 binding assay carried out at the same time is shown as a reference on each binding affinity plot. Assays were carried out in triplicate and the IC50 is derived from a minimum of 3 assays. Increasing concentrations of PYY3-36 analogue were incubated with a fixed concentration of $^{125}$I PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I PYY1-36 bound with and without unlabelled competing peptide.

2.4.4.2.2 Effect of stereochemical changes at Ile3 of PYY3-36 on food intake

PYY3-36 D-Ile3 significantly reduced food intake 0-1h and 1-2h post-injection compared to saline controls and to a similar extent as PYY3-36 controls (see table 2.16). PYY3-36 D-Ile3 significantly reduced food intake over 8h but had no significant effect on food intake over 24h compared to saline controls and in contrast to PYY3-36, which significantly reduced food intake over 24h. PYY3-36 D-allo-Ile3 significantly reduced food intake 0-1h and 1-2h post-injection compared to saline controls and to a similar extent to PYY3-36 (see table 2.16). PYY3-36 D-allo-Ile3 significantly reduced cumulative food intake over 24h compared to saline controls and to a similar extent as PYY3-36. Examining the percentage reduction in food intake analysis suggested that substituting Ile3 for the stereoisomer D-allo-Ile3 is more effective than D-Ile3 PYY3-36 or PYY3-36 in reducing food intake (see fig. 2.22).
Table 2.16. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with a stereoisomer of Ile at position 3 derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test ( *p<0.05, **p<0.01, ***p<0.001 vs saline). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.

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<th>Group</th>
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<th>1-2h</th>
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<tr>
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Figure 2.22. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with a stereoisomer at position 3. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.
2.4.5 Effect of combining longevity modifications with simple amino acid substitutions upon food intake

2.4.5.1.1 Effect of the addition of a fatty acid to the charged group of Lys-substituted PYY3-36 on receptor binding

Substitution of Ala12 for Lys12 had no significant effect on the affinity of PYY3-36 for the Y2R, while substitution of Lys12-lauroyl decreased the affinity of the peptide for the Y2R by 9-fold compared to PYY3-36 (see fig. 2.23 for binding curves and table 2.17 for IC50 values). Degradation of PYY3-36 was increased by 24 ±2% and decreased by 99 ±0.9% when Ala12 was substituted for Lys or Lys-lauroyl respectively (see table 2.17: degradation of analogues shown as percentage of PYY3-36 degradation).

<table>
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<tr>
<th>Peptide/analogue</th>
<th>IC50 at Y2 receptor</th>
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<tr>
<td>Lys 12-Lauroyl</td>
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Table 2.17. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with different substitutions at positions 12 in the N-terminal. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.
2.4.5.1.2  Effect of the addition of a fatty acid to PYY3-36 Lys12 on food intake

PYY3-36 Lys12 significantly reduced food intake 0-1h post-injection, but had no effect on food intake at any later interval and had no significant effect on cumulative food intake. In contrast, PYY3-36 significantly reduced food intake at 0-1h, 1-2h and 2-4h post-injection as well as cumulative food intake over 8h. PYY3-36 Lys12-Lauroyl significantly reduced food intake 0-1h post-injection compared to saline controls, 1-2h post-injection compared to saline and 2-4h post-injection compared to saline and PYY3-36 controls. PYY3-36 Lys12-Lauroyl significantly reduced cumulative food intake over 8h post-injection compared to saline controls and to a similar extent as PYY3-36. Examining the percentage reduction in food intake analysis suggested that substitution of Ala12 with Lys has a
detrimental effect upon PYY3-36 bioactivity, while the addition of a lauroyl fatty acid to Lys12 results in an analogue that has similar bioactivity to PYY3-36 (see fig. 2.24).

<table>
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Table 2.18. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with a stereoisomer of Ile at position 23 derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test ( *p<0.05, **p<0.01, ***p<0.001 vs saline). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.

Figure 2.24. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with a Lys only or acylated Lys at position 12. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.
2.4.5.2 **Effect of combining acylation at Lys 12 and stereochemical modifications at the N-terminal in a single analogue**

2.4.5.2.1 **Effect of combining acylation at Lys 12 and stereochemical modifications at the N-terminal on receptor binding and susceptibility to proteolytic degradation**

Combining D-allo-Ile3 with Lys12 has minimal effect on the affinity of PYY3-36 for the Y2R, while adding a lauroyl to Lys12 of PYY3-36 D-allo-Ile3 Lys12 decreased affinity for the Y2R by 13 fold compared to PYY3-36 and PYY3-36 D-allo-Ile3 Lys12 (see fig. 2.25 for binding curves and table 2.17 for IC50 values). Degradation of PYY3-36 was decreased by 62 ±0.3% when Ile3 and Ala12 were substituted for D-allo-Ile and Lys respectively and 99 ±0.5% when Ile3 and Ala12 were substituted for D-allo-Ile and Lys-lauroyl respectively (see table 2.17: degradation of analogues shown as percentage of PYY3-36 degradation).

<table>
<thead>
<tr>
<th>Peptide/analogue</th>
<th>IC50 at Y2 receptor</th>
<th>% of PYY3-36 degradation</th>
</tr>
</thead>
<tbody>
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<td>PYY3-36</td>
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<td>100 (0)</td>
</tr>
<tr>
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<td>D-allo-Ile 3 Lys 12-lauroyl</td>
<td>2.4 (0.4)</td>
<td>1.5 (0.5)</td>
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</table>

Table 2.19. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with combined substitutions at positions 3 and/or 12 within the N-terminal. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.
Figure 2.25. Representative displacement binding curves for PYY3-36 analogues with a substitution of Ile3 for D-allo-Ile and Ala12 for Lys with the addition of lauroyl to Lys12 at the Y2 receptor. A) PYY3-36 D-allo-Ile3 Lys12 (mean IC50: 0.16nM), B) PYY3-36 D-allo-Ile3 Lys12-Lauroyl (mean IC50: 2.4nM). A control PYY3-36 binding assay carried out at the same time is shown as a reference on each binding affinity plot. Assays were carried out in triplicate and the IC50 is derived from a minimum of 3 assays. Increasing concentrations of PYY3-36 analogue were incubated with a fixed concentration of $^{125}$I PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I PYY1-36 bound with and without unlabelled competing peptide.

2.4.5.2.2 Effect of combining acylation at Lys 12 and stereochemical modifications at the N-terminal on food intake

PYY3-36 D-allo-Ile3 Lys12 significantly reduced intake at 0-1h post-injection compared to saline and PYY3-36 controls, and at 1-2h post-injection compared to saline controls. PYY3-36 D-allo-Ile3 Lys12 resulted in significantly higher food intake at 4-8h post-injection (see table 2.18). PYY3-36 D-allo-Ile3 Lys12 significantly decreased cumulative food intake over 4h compared to saline, but resulted in significantly higher food intake compared to PYY3-36 over 4h and 8h. PYY3-36 D-allo-Ile3 Lys12-Lauroyl significantly reduced food intake compared to saline controls, but resulted in significantly higher food intake compared to PYY3-36 controls at 0-1h post-injection, and significantly decreased food intake 2-4h post-injection compared to saline and PYY3-36 controls (see table 2.18), whereas PYY3-36 had no significant effect in food intake at 2-4h post-injection. PYY3-36
D-allo-Ile3 Lys12-Lauroyl significantly reduced cumulative food intake over 8h compared to saline controls, and to a higher extent than PYY3-36. Examining the percentage reduction in food intake suggested that combining D-allo-Ile at position 3 with Lys12-Lauroyl at position 6 is more effective than combining D-allo-Ile3 with Lys at position 12 in enhancing the bioactivity of PYY3-36 (see fig. 2.26).

<table>
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</tbody>
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Table 2.20. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with a stereochemical substitutions at position 3 with either an amino acid substitution at position 6 or 12 and/or the addition of an acyl group derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test ( *p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.05,$$p<0.01, $$$ p<0.001 vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.
2.4.6 The effects of combining amino acid changes within the entire PYY sequence on receptor binding, susceptibility to degradation and in vivo bioactivity

2.4.6.1 Investigations into combining changes at positions 19, 23 and 30, with and without the addition of a proline tail

2.4.6.1.1 Effect on receptor binding and susceptibility to proteolytic degradation

All combinations of substitutions in the analogues investigated resulted in increased binding affinity at the Y2R compared to PYY3-36. Combining all substitutions into a single analogue, to create N-Pro Lys19 Ala23 Lys30 increased Y2R binding affinity by approximately 2 fold (see table 2.19 for IC50 values). Degradation of PYY3-36 was
decreased by 24 ±3% when Ser23 and Leu30 were substituted for Ala and Lys respectively, 21 ±0.5 % when Arg19 and Ser23 were substituted for Lys and Ala respectively, 11 ±2 % when Arg19 and Leu30 were both substituted for Lys, 10 ±6 % when Arg19 and Leu30 were both substituted for Lys and Pro was added to the N-terminal and by 26 ±0.6% when Arg19, Ser23 and Leu30 were substituted for Lys, Ala and Lys respectively and with the addition of Pro at the N-terminal (see table 2.19: degradation of analogues shown as percentage of PYY3-36 degradation).

<table>
<thead>
<tr>
<th>Peptide/analogue</th>
<th>IC50 at Y2 receptor</th>
<th>% of PYY3-36 degradation</th>
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<td>N-Pro Lys19 Ala23 Lys30</td>
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Table 2.21. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with combined substitutions at positions 19, 23 and/or 30 of PYY3-36. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37ºC and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.

2.4.6.1.1 Effect of combined substitutions at positions 19, 23 and 30 on food intake

All analogues significantly reduced food intake 0-1h post-injection compared to saline (see table 2.20). PYY3-36 Ala23 Lys30 significantly reduced food intake 2-4h post-injection compared to saline, whereas PYY3-36 had no significant effect on food intake at this time.
point. PYY3-36 Ala23 Lys30 also significantly reduced food intake 4-8h post-injection compared to PYY3-36. PYY3-36 Ala23 Lys30 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls. PYY3-36 Lys19 Ala23 significantly reduced food intake 1-2h and 2-4h post-injection compared to saline controls, and to a similar extent as PYY3-36 (see table 2.20). PYY3-36 Lys19 Ala23 also significantly reduced food intake at 4-8h compared to PYY3-36 controls. PYY3-36 Lys19 Ala23 significantly reduced cumulative food intake over 8h compared to saline and to a similar extent as PYY3-36 controls (see table 2.20). PYY3-36 Lys19 Lys30 significantly reduced food intake 2-4h post-injection compared to saline controls (see table 2.20). PYY3-36 Lys19 Lys30 also significantly reduced cumulative food intake over 24h compared to saline controls, whereas PYY3-36 did not significantly reduce cumulative food intake over 24h. PYY3-36 N-Pro Lys19 Lys30 significantly reduced food intake 1-2h and 2-4h post-injection compared to saline controls. PYY3-36 N-Pro Lys19 Lys30 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls, and over 24h compared to saline controls. PYY3-36 N-Pro Lys19 Ala23 Lys30 significantly reduced food intake at 1-2h post-injection compared to saline and PYY3-36 controls; 2-4h post-injection compared to saline and PYY3-36 controls and at 4-8h post-injection compared to PYY3-36 controls (see table 2.20). PYY3-36 N-Pro Lys19 Ala23 Lys30 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls, and over 24h compared to saline controls. Examining the percentage reduction in food intake data suggested that all analogues tested were more effective than PYY3-36 in reducing cumulative food intake compared to saline controls. The combination of Lys19 Lys30 was more effective than the combination of Ala23 Lys30 or Lys19 Ala23 in reducing food intake. Lys 19 Lys30 and N-ProLys19 Ala23 Lys30 appeared to be most effective in reducing percentage food intake compared to PYY-36 controls over 8 or 24h (see fig 2.27).
Table 2.22. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with combined substitutions at positions 19, 23 and 30 and with or without the addition of an N-terminal Pro derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test ( *p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.05, $$ p<0.01 vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.

<table>
<thead>
<tr>
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<tr>
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<tr>
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</table>

Figure 2.27. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with combined substitutions at positions 19, 23 and 30 and with or with or without the addition of an N-terminal Pro. All peptides were administered at a low dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.
2.4.6.2  Investigations into combining substitutions at positions 6, 23 and 30 and the addition of Pro at the N-terminal

2.4.6.2.1  Effect of combining substitutions at position 6, 23 and 30 with the addition of an N-terminal Pro on receptor binding and susceptibility to proteolytic degradation

All combinations of substitutions in the analogues investigated resulted in a similar or an increased binding affinity at the Y2R compared to PYY3-36 apart from PYY3-36 Ser6 Glu23 Lys30, which decreased Y2R affinity by approximately 1.5 fold compared to PYY3-36. Combining substitutions at position 6, 23 and 30 into a single analogue with the addition of Pro to create N-Pro Val6 Ala23 Lys30 or N-Pro Ser6 Ala23 Lys30 increased Y2R affinity by approximately 2 fold (see table 2.21 for IC50 values). Degradation of PYY3-36 was decreased by 20 ±3% when Ser23 and Leu30 were substituted for Glu and Lys respectively, by 34 ±3% when Glu6 Ser23 Leu30 were substituted with Ser, Glu and Lys respectively by 23 ±1 % when Glu6, Ser23 and Leu30 were substituted for Ser, Ala and Lys respectively, by 23 ±9% when Glu6, Ser23 and Leu30 were substituted for Ser, Ala and Lys respectively with the addition of Pro at the N-terminal, by 32 ±1% when Glu6, Ser23 and Leu30 were substituted for Val, Ala and Lys respectively with the addition of Pro at the N-terminal and by 65 ±3% when Glu6, Ser23 and Leu30 were substituted for Lys, Ala and Lys respectively with the addition of Pro at the N-terminal (see table 2.21: degradation of analogues shown as percentage of PYY3-36 degradation).
<table>
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<tr>
<th>Peptide/analogue</th>
<th>IC50 at Y2 receptor</th>
<th>% of PYY3-36 degradation</th>
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</thead>
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Table 2.23. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with combined substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2Y receptor was used to determine the IC50 of each analogue vs PYY3-36.

2.4.6.2.2 Effect of combining substitutions at position 6, 23 and 30 with the addition of an N-terminal Pro on food intake

All analogues tested significantly reduced food intake 0-1h post-injection to the same or to a higher extent as PYY3-36. PYY3-36 Glu23 Lys30 significantly reduced food intake 2-4h post-injection compared to saline and PYY3-36 controls and at 4-8h post-injection compared to PYY3-36 controls (see table 2.22). PYY3-36 Glu23 Lys30 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls and over 24h compared to saline controls. PYY3-36 Ser6 Glu23 Lys30 significantly reduced cumulative food intake over 8h compared to saline and to a similar extent as PYY3-36 controls (see table 2.22). PYY3-36 Ser6 Ala23 Lys30 significantly reduced food intake 1-2h post-injection compared to saline controls, but had no effect on food intake at 2-4h post-injection, whereas PYY3-36 significantly reduced food intake at this time point (see table 2.22). PYY3-36 Ser6 Ala23 Lys30 significantly reduced cumulative food intake over 8h.
compared to saline and to a similar extent as PYY3-36 controls and over 24h compared to saline controls. PYY3-36 N-Pro Ser6 Ala23 Lys30 significantly reduced food intake 1-2h and 2-4h compared to saline controls and at 4-8h compared to PYY3-36 controls (see table 2.22). PYY3-36 N-Pro Ser6 Ala23 Lys30 significantly reduced cumulative food intake over 8h and 24h compared to saline and PYY3-36 controls. PYY3-36 Val6 Ala23 Lys30 significantly reduced food intake 2-4h post-injection compared to saline controls, whereas PYY3-36 had no effect on food intake at the time-point (see table 2.22). PYY3-36 N-Pro Val6 Ala23 Lys30 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls. PYY3-36 N-Pro Lys6 Ala23 Lys30 significantly reduced food intake 1-2h and 2-4h post-injection compared to saline controls and to a similar extent as PYY3-36 (see table 2.22). PYY3-36 N-Pro Lys6 Ala23 Lys30 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls and over 24h compared to saline controls. Examining the percentage reduction in food intake suggests that all analogues tested were more effective than PYY3-36 in reducing cumulative food intake compared to saline controls up until 8h post-injection. PYY3-36 N-Pro Ser6 Ala23 Lys30 appeared to be most effective in reducing percentage food intake over 8h, with no major differences between groups by 24h (see fig. 2.28).
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<th>0-8h</th>
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</tr>
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</tr>
</tbody>
</table>

Table 2.24. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test ( *p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.05, $$ p<0.01, $$$p<0.001 vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.
Figure 2.28. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with combined substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.

2.4.6.3 Investigations into combining substitutions at positions 23 and 30 with the addition of Pro at the N-terminal

2.4.6.3.1 Effect of combining substitutions at positions 23 and 30 with or without the addition of Pro on receptor binding and susceptibility to proteolytic degradation

All combinations of substitutions in the analogues investigated increased Y2R affinity compared to PYY3-36 apart from PYY3-36 N-Pro Glu23, which decreased Y2R affinity by approximately 1.5 fold. PYY3-36 N-Pro Ala23 increased Y2R affinity by approximately 2-fold. Combining substitutions at position 23 and 30 into a single analogue with the addition of Pro, in PYY3-36 N-Pro Ala23 Arg30 or N-Pro Ala23 His30, increased Y2R affinity by approximately 1 fold, and PYY3-36 N-Pro-Ala23 Lys30, increased Y2R affinity by approximately 6 fold (see table 2.23 for IC50 values). Degradation of PYY3-36 was
decreased by 53 ±2% when Ser23 was substituted for Glu23 with the addition of Pro at the N-terminal, by 82 ±6% when Ser23 was substituted for Ala with the addition of Pro at the N-terminal, by 16 ±4% when Ser23 and Leu30 were substituted for Ala and Arg respectively with the addition of Pro at the N-terminal and by 53 ±1% when Ser23 and Leu30 were substituted for Ala and Lys respectively with the addition of Pro at the N-terminal. Degradation of PYY3-36 was increased by 1 ±2% when Ser23 and Leu30 were substituted for Ala and His respectively, with the addition of Pro at the N-terminal (see table 2.23: degradation of analogues shown as percentage of PYY3-36 degradation).

<table>
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<tr>
<th>Peptide/analogue</th>
<th>IC50 at Y2 receptor</th>
<th>% of PYY3-36 degradation</th>
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<td>Mean (nM)</td>
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<tr>
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Table 2.25. Summary of Y2R binding affinity and resistance to proteolytic degradation of PYY3-36 analogues with combined substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro. PYY3-36 or analogue (2nmol) was incubated with KBB (50µg) over 30 min at 37°C and the parent peptide and peptide fragments separated by HPLC. For the degradation studies, the percentage degradation of analogues was calculated by comparing AUC of parent peptide peaks and corrected to the percentage degradation of the control PYY3-36 incubation run at the same time. A receptor binding assay measuring analogue binding at the Y2R was used to determine the IC50 of each analogue vs PYY3-36.

2.4.6.3.2 Effect of combining substitutions at positions 23 and 30 with or without the addition of Pro on food intake

All analogues significantly reduced food intake 0-1h post-injection (see table 2.24). PYY3-36 N-Pro Glu23 significantly reduced food intake 1-2h post-injection compared to saline.
and to a similar extent as PYY3-36 controls (see table 2.24). PYY3-36 N-Pro Glu23 significantly reduced cumulative food intake over 24h compared to saline controls and to a similar extent as PYY3-36 controls. PYY3-36 N-Pro Ala23 significantly reduced food intake 2-4h post-injection compared to saline and PYY3-36 controls. PYY3-36 N-Pro Ala23 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls and over 24h compared to saline controls. PYY3-36 N-Pro Ala23 Arg30 significantly reduced food intake 2-4h post-injection compared to saline and PYY3-36 controls and at 4-8h compared to PYY3-36 controls. PYY3-36 N-Pro Ala23 Arg30 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls but had no effect on 24h food intake, as observed for PYY3-36 controls. PYY3-36 N-Pro Ala23 His30 significantly reduced intake 2-4h post-injection compared to saline and PYY3-36 and at 4-8h compared to PYY3-36 controls. PYY3-36 N-Pro Ala23 His30 significantly reduced cumulative food intake over 8h compared to saline and PYY3-36 controls but had no effect on 24h food intake, as observed for PYY3-36 controls. PYY3-36 N-Pro Ala23 Lys30 significantly reduced food intake 1-2h and 2-4h post-injection compared to saline and PYY3-36 and 4-8h compared to PYY3-36 controls. PYY3-36 N-Pro Ala23 Lys30 significantly reduced cumulative food intake over 8h and 24h compared to saline and PYY3-36. Examining the percentage reduction in food intake suggests that all analogues tested were more effective than PYY3-36 in reducing cumulative food intake compared to saline controls. PYY3-36 N-Pro Ala23 Lys30 appeared to be most effective in reducing percentage food intake over 8h and PYY3-36 N-Pro Ala23 most effective in reducing % food intake over 24h (see fig. 2.29).
Table 2.26. Statistical significance of the effects on food intake of PYY3-36 controls or PYY3-36 analogues with combined substitutions at position 23 and 30 with or without the addition of Pro derived from food intake data for each study day. Food intake(g) data for each study group was compared to saline controls and food intake data for PYY3-36 analogues compared to saline controls and PYY3-36 and analysed using one-way ANOVA with Tukey’s post-hoc test ( *p<0.05, **p<0.01, ***p<0.001 vs saline; $p<0.05, $$ p<0.01 vs PYY3-36). Arrows pointing downwards or upwards indicate a reduction or increase in food intake respectively compared to saline or PYY3-36.

Figure 2.29. Cumulative food intake (expressed as percentage of saline controls on each study day) following administration of PYY3-36 analogues with combined substitutions at positions 6, 23 and 30 and with or without the addition of an N-terminal Pro. All peptides were administered at a dose of 50nmol/kg. Results are means ± SEM. PYY3-36 and vehicle are shown as an average of all study days for the purpose of clarity, n=7-12.
2.5 Discussion

The first set of studies described in this chapter aimed to investigate the effects of changes to different sections of the PYY3-36 molecule in order to demonstrate elements that are important for receptor binding and sites susceptible to degradation, shedding light on the structure/function relationships of PYY3-36 in vitro and in vivo. Obese humans exhibit a reduction in food intake after iv infusions of PYY3-36 at doses that mimic postprandial levels (Batterham et al., 2003a; Le Roux et al., 2008). PYY3-36 is therefore a potential anti-obesity treatment. However, the therapeutic window of PYY3-36 is extremely narrow: high doses induce nausea, while low doses have no effect on food intake in humans (Halatchev and Cone, 2005; Le Roux et al., 2008; Reidelberger et al., 2008). A PYY3-36 administration regime is likely to require a long-acting analogue of native peptide in order to retain potency while circumventing side effects associated with higher doses, such as nausea. This would ideally involve subcutaneous injection, in order to avoid the costs and inconvenience associated with continuous iv delivery. A successful example of such an administration protocol for a peptide hormone is the GLP-1 analogue liraglutide, which has an Arg34 substitution and a palmitoyl fatty acid addition at Lys26 and is administered by daily subcutaneous injection. The alterations to the peptide structure facilitate binding to circulating albumin, self-oligomerization and resistance to DPP-iv-mediated degradation. The resulting increased half-life and 24h blood glucose control means that liraglutide can be delivered once daily via the subcutaneous route (Barnett, 2009; Montanya and Sesti, 2009; Lovshin and Drucker, 2009). Therefore, analogues of PYY3-36 may have improved biological properties compared to the native peptide, including greater potency and longer duration of action, and a greater therapeutic potential in the treatment of obesity.

Peptides generally have limited clinical utility due to rapid clearance and/or degradation in the circulation. Therefore, analogues need to be designed that circumvent this problem. The goal in analogue design is to prolong or enhance the activity of a peptide at its receptor, and requires an interdisciplinary approach, in which the use of conformational modifications must be weighed against peptide stability, receptor affinity and/or selectivity, membrane permeability, elimination from the circulation, and physiological aspects relating to the
subjects receiving the analogue (eg. normal-weight or obese, etc) (Witt et al., 2001; Egleton and Davis, 1997; Fauchere JL and Thurieau, 1992; Deacon and Holst, 2006). This goal can be achieved by:

1) Reducing degradation: endogenous peptides are rapidly degraded by proteolytic enzymes, according to the body compartment to which the analogue is administered.

2) Reducing elimination: peptides are normally eliminated via renal and hepatic routes, according to their size and lipophilicity.

3) Increasing selectivity/affinity for the receptor: peptides can be altered to enhance their ability to recognize and interact with receptors.

4) Increasing bioavailability: peptides acting on receptors expressed in the CNS must transverse the blood-brain barrier. This process is affected by size, flexibility, conformation and biochemical properties and sequence structure of the amino acids of a peptide.

5) Increasing the time at binding sites within the receptor compartment: efflux mechanisms can affect the ‘on-time’ between a peptide and its receptor.

The common methodology in the design of gut hormone analogues, has been used for GLP-1 and OXM analogues, involves reducing degradation (1) and clearance (2) and increasing selectivity and/or affinity for the receptor (3) (Druce et al., 2009; Green et al., 2004; Lee et al., 2006). The methodologies 1) and 3) are used in the experiments described in this chapter to elucidate the importance of certain regions of PYY3-36 in the structure/function relations at the Y2R and develop PYY3-36 analogues for use as an obesity treatment.

Importantly, I did not alter any conserved positions known to be important in maintaining the PP-fold structure of PYY3-36, which constitutes a polyproline-like N-terminal (Pro5-Pro8), folded back onto an amphipathic α-helix that is stabilized by hydrophobic contacts with Leu17, Tyr20 and Leu24. This was to be consistent with my aim to explore the role of specific positions within different regions of the peptide, and minimise the confounding effects of severely altering the ability of the peptide to fold in solution. However, NMR
structure analysis would be required to confirm that substitutions at the sites I altered did not markedly affect tertiary structure. Since a number of the amino acid residues I used were charged or hydrophobic, it is likely that the overall amphiphilicity of the peptide is affected in some analogues. The amphiphilicity of the peptide influences its lipophilicity and ability to interact with the membrane. Since PYY3-36 exhibits higher Y2R affinity than PYY and a less stable tertiary structure that is thought more conducive to binding the C-terminal-requiring Y2R, it seems possible that PYY3-36 adopts its membrane-bound conformation upon Y2R binding (Nygaard et al., 2006; Lerch et al., 2004; Keire et al., 2000). Therefore, it is possible that the substitutions that alter amphiphilicity may stabilise the conformation of PYY3-36 required for Y2R binding. Carrying out tertiary/3D structure analysis will be beneficial in determining mechanism of action of analogues that show potential as anti-obesity therapies, but was not feasible in these preliminary structure/activity studies.

Though the C terminus hexapeptide is required for binding, an intact mid-section and N-terminal is thought to stabilise the conformation of the peptide in the state required for binding, since N-terminally truncated analogues have poor affinity, but high selectivity at the Y2R. Furthermore, the PP-fold-forming amino acid sites in the polyproline and α helices likely function to protect the binding portion of the molecule from proteolytic cleavage (Nygaard et al., 2006). Therefore alterations at sites in the N-terminal, mid-section, and at position 30 (which is at the C terminus but is not involved in binding the Y2R), may enhance or reduce binding affinity and resistance to proteolytic degradation. The activity of each peptide in vivo was often, but not always, related to the effect of the modification on both Y2 receptor affinity and resistance to proteolytic degradation by KBB membranes. Use of a cell line that over expresses the Y2R is a commonly used approach to test binding affinity for PYY3-36 (Pedersen et al., 2009; Pedersen et al., 2010b). A number of ubiquitous endopeptidases known to be important in the degradation of regulatory hormones are concentrated in the KBB, which is as a widely used tool to study proteolytic degradation of hormones (Medeiros and Turner, 1994; Pankow et al., 2007). Therefore these parameters were investigated in the design of PYY3-36 analogues.
The first set of experiments explored the effects of alterations to the amino acid sequence of PYY3-36 within the C terminus, mid-section and N-terminal of the peptide. Though position 30 is not essential for binding and bioactivity, it is adjacent to the 6 highly conserved amino acids within the C terminus binding hexapeptide, which are crucial for Y2R recognition and binding. Leu30 is also the last residue within the amphipathic α-helix. Previous studies have reported significant correlation between Y2R affinity and helicity, which has led to the hypothesis that the α-helix stabilizes the interaction between Y2R and the C terminus, which is normally flexible in solution (Zerbe et al., 2006; Lerch et al., 2004; Ahn et al., 2009). Replacing Leu with the hydrophobic residue Trp at position 30 on PYY22-36 increases its affinity at the Y2R (Balasubramaniam et al., 2000). In my studies, substituting Leu30 with a hydrophobic Phe, Val or Ile (an isomer of Leu), worsened Y2R binding affinity, and had a less potent effect on food intake than PYY3-36. In contrast, replacing Leu30 with the positively charged amino acids Lys or Arg improved Y2R affinity. The Lys30 substitution improved bioactivity in vivo, significantly reducing food intake at the 2-4h interval compared to saline, and thus having a more prolonged effect than other position 30-substituted analogues. This was surprising, considering a Lys at position 30 renders PYY3-36 around 60% more susceptible to degradation in vitro. The specific properties of the charged side chain of Lys may impart changes to the amphiphilicity of this analogue that improve Y2R affinity and/or activity. In contrast, His worsened Y2R binding affinity but did not alter bioactivity compared to PYY3-36. Here the maintained effect on food intake may be explained by reduced proteolytic degradation in vivo, since in vitro a His30 substitution reduces degradation by 30%. The imidazole side chain of His may abolish the beneficial effects of other positively charged side chains at this position. My data provides impetus to further investigate whether a positive charge at position 30 stabilizes the interaction of the C terminus at the Y2R, perhaps by studying changes in receptor activity, by measuring its effects on cAMP, and assessing the 3D structure of these analogues in solution and whilst membrane bound using nuclear magnetic resonance imaging.

Within the binding hexapeptide, substitution of Val31 for Lys did not alter Y2R affinity or resistance to proteolytic degradation, but significantly worsened bioactivity compared to
PYY3-36. Substitution of Gln34 for Asn had a detrimental effect on binding and prevented degradation of PYY3-36 by around 30%. The fact that Asn34 reduced bioactivity in vivo may be explained by an almost 4-fold reduction in Y2R affinity. Since PYY Pro34 also binds to the Y1R, another possible explanation is that PYY3-36 Asn34 activates the orexigenic Y1R (Keire et al., 2000). In Y2R agonist design, precaution must be taken to prevent increases in affinity for the Y1R and Y5R, since the activation of these receptors elicits orexigenic effects which may negate any anorectic activity resulting from Y2R activation. Indeed, one focus of PYY analogue development has been the design of Y2R selective analogues, which have very low affinity for the Y1R and Y5R but retain affinity at the Y2R (Pedersen et al., 2010b). However, attempting to selectively reduce the affinity for the Y1R and Y5R is difficult and can compromise Y2R affinity, reducing potency in vivo. Although PYY3-36 is only orexigenic when administered ICV, peripherally-administered PYY3-36 analogues that are selective for Y1R and/or Y5R may activate these receptors in the ARC, resulting in orexigenic effects. Substituting Tyr36 with an aromatic Phe slightly increased Y2R affinity, which is in agreement with a previous study in which modifications at Tyr36 were well tolerated (Pedersen et al., 2009). Though the Phe36 substitution did not alter binding affinity and prevented degradation by around 30%, it was slightly less effective than PYY3-36 in reducing food intake, though this difference did not reach statistical significance. These results are consistent with Ala scan studies, which found that any individual substitution at positions 31-36 had a detrimental effect on PYY3-36 bioactivity, highlighting the importance of the C terminus hexapeptide in PYY3-36 bioactivity (Ahn et al., 2009).

In situations where PYY3-36 analogues show maintained Y2R affinity, but poor bioactivity, the analogue may be acting as an antagonist or inverse agonist, which can only be determined by analysis of the intracellular signalling pathways mediated by Y2R upon analogue binding. To further investigate this, a sensitive, whole cell assay would be required to measure the EC50 of PYY3-36 analogues for decreasing cAMP, since the Y2R is negatively coupled to adenylate cyclase via Gi (Toth et al., 1993). This would require prior stimulation of adenylate cyclase using a compound such as forskolin. Another possibility for discrepancies between Y2R affinity and in vivo bioactivity is that there are
differences between Y2R expressed in vivo and Y2R expressed on solubilised cell membranes, as the in vitro preparation may lack the physiological intracellular machinery that would be present in neurones expressing Y2R and mediating the effects of PYY3-36 analogues in vivo. There may also be differences in affinity of analogues for the human Y2R and murine Y2R; the human Y2R was used in the binding affinity studies due to the relevance of analogues in treating human obesity. However, this discrepancy is unlikely, as the human and mouse Y2 receptors are 94% homologous (Rose et al., 1995; Nakamura et al., 1996). Lack of correlation between bioactivity and resistance to proteolytic degradation may be due to a number of factors that were previously outlined in this discussion, including bioavailability to Y2 receptors located on the vagus, brainstem and/or hypothalamus.

The mid-section of PYY3-36 contains the amphipathic α-helix (residues 15-30), which may play a role in guiding the C terminus to the membrane/water interface, a concept that has previously been exploited in PYY3-36 analogue design (Pedersen et al., 2010a). Therefore, substitutions using charged amino acids were made to determine whether altering the charge ratio may be beneficial for Y2R binding and PYY3-36 bioactivity. Replacing Arg19 with His or Lys had no effect or resulted in a minor increase in Y2R affinity respectively. Lys19 increased the resistance of PYY3-36 to proteolytic degradation by around 20%, while Arg19 had little effect. The fact that Lys19 significantly worsened the bioactivity of PYY3-36 was surprising considering this substitution slightly increased Y2R affinity and resistance to degradation. His19 significantly reduced bioactivity compared to PYY3-36, which may be due to the fact that this analogue was more susceptible to degradation than PYY3-36. Considering that substitution of position 19 using very similar amino acids had a detrimental effect on bioactivity without affecting Y2R affinity, and that it is conserved amongst vertebrates, this position may play a role in PYY3-36 bioactivity, that is dependent on other factors such as metabolic clearance or bioavailability.

Ser23 is important in the antisecretory effects of PYY3-36 in the intestine (Balasubramaniam et al., 2000). This residue is not well-conserved, and occurs as Ala in the chicken and various amphibian species, and Asp in the python (Conlon, 2002).
Replacing a polar Ser at position 23 with a simple, aliphatic Ala increased Y2R affinity and slightly decreased resistance to proteolytic degradation compared to PYY-36. The bioactivity of PYY3-36 Ala23 was unaltered compared to PYY3-36, which is in agreement with previous PYY3-36 Ala scanning experiments (Ahn et al., 2009). A Glu substitution at position 23 was also made, to investigate whether the addition of a negatively charged side chain, which is likely to affect the amphiphilicity of the peptide, would improve Y2R affinity and/or resistance to proteolytic degradation. Replacing Ser23 with a negatively charged Glu maintained Y2R affinity and reduced degradation by around 50%. This analogue was more effective than PYY3-36 in reducing food intake acutely, though this difference did not reach statistical significance. This data implies that position 23 is a potential candidate for further screening in PYY3-36 drug design.

Substituting a positively charged Lys4 for a positively charged His had a considerable detrimental impact on Y2R affinity, which may be due to the imidazole ring present in the side chain of His that is not present in Lys. Altering the charge at position 4 by substituting Lys for Glu was even more detrimental to Y2R affinity, suggesting that this highly conserved site is important in the structure/activity relationship at the Y2R. Considering that both substitutions had little effect on degradation, the significant loss of bioactivity compared to PYY3-36 is probably due to the loss of Y2R binding affinity. Since a neutral Ala substitution at this position has previously been shown to result in a substantial increase in bioactivity (Ahn et al., 2009), it is likely that the detrimental impact on receptor binding after His or Glu substitution at position 4 is due to altering the charge of the peptide; it is more likely to impact on the shape of the peptide and its dipole moment, which is thought to be helix-stabilizing in PYY (Nygaard et al., 2006).

All substitutions for Glu6, including an aliphatic Val, a neutral, polar Ser and a positively charged Lys improved Y2R binding affinity, suggesting that this position is a potential candidate for drug design. A Ser at position 6 maintained the acute effect of PYY3-36 upon food intake, but was significantly less effective than PYY3-36 at later time points. Here, the detrimental effect may be explained by an increase in proteolytic degradation in vivo, as Ser6 increased in vitro degradation by around 17%. Similar to Ser6, both Lys6 and Val6
maintained the acute effect of PYY3-36 on food intake, but were less effective than PYY3-36 at later time points, while these substitutions only slightly increased proteolytic resistance \textit{in vitro}. These data suggest that these substitutions improve Y2R affinity, but may be more useful combined with other substitutions that prolong or maintain the effect of PYY3-36 at later time points.

The addition of Pro to the N-terminal of PYY3-36 enhanced Y2R affinity; whereas D-Pro did not. Furthermore, N-Pro markedly reduced proteolytic degradation of PYY3-36 by around 50%, while D-Pro increased the susceptibility of PYY3-36 to degradation by around 30%. In line with these results, an N-Pro substitution significantly enhanced the effect of PYY3-36 upon cumulative food intake over 8 hours post-injection, suggesting both receptor binding and resistance to degradation were improved \textit{in vivo}. Furthermore, a D-Pro substitution was less effective than PYY3-36 in its bioactivity, though this difference did not reach statistical significance. These results suggest that steric conformation is an important factor that can have a large effect on the structure-activity relationships of PYY3-36 analogues. In PYY1-36, Pro2 interacts with Tyr27, a bond which plays a role in maintaining stable conformation in solution (Glover et al., 1984). NMR analysis showed that the PP-fold in PYY3-36 is less well-defined compared to PYY1-36, which may mean that PYY3-36 is more susceptible to degradation. D-Pro may be unable to interact with Tyr27 due to steric hindrance, while interaction of N-Pro with Tyr27 may stabilise the 3D structure, reducing flexibility in solution and thereby preventing degradation (Nygaard et al., 2006; Schwartz et al., 1990). The enzyme DPP-iv is able to cleave peptides when Pro is at position 2 in PYY, resulting in PYY3-36. However, DPP-iv is only able to cleave Pro when it is the first amino acid on the amino side of the scissile bond (P1 position), and in this analogue Pro is on the carboxy side of the scissile bond (P1’ position; see \url{www.merops.sanger.ac.uk}). Enzymes that normally encounter and cleave PYY or PYY3-36 may therefore be unable to cleave PYY2-36 (Grandt et al., 1992). Furthermore, since PYY and PYY3-36 have highly different 3D conformations, PYY2-36 may have an equally different tertiary structure, which may shield the remaining sequence from proteolytic attack. Therefore, an N-terminal Pro may prove useful in PYY3-36 drug design. The addition of Cys at the N-terminal had a detrimental effect upon Y2R affinity, but prevented
proteolytic degradation to a similar degree as substitution of Pro at this site. Interestingly, the addition of Cys at the N-terminal was less effective than PYY3-36 at reducing food intake acutely, but at later intervals (between 2-4 h post-injection) was significantly more effective than PYY3-36. This suggests that in some cases enhanced Y2R affinity may be important for acute effects on food intake, but that chronic bioactivity cannot be maintained to the same extent as PYY3-36 if the peptide is more susceptible to proteolytic degradation.

Substituting Ile3 for its stereoisomers D-Ile or D-allo-Ile slightly improved binding, and had little effect on resistance to proteolytic degradation. Interestingly, D-allo-Ile3 was more potent in reducing food intake over 24h compared to saline, though this difference did not reach statistical significance compared to PYY3-36. In contrast, D-Ile3 was less effective in reducing food intake over 8h compared to PYY3-36, though again, this difference did not reach statistical significance. As neither binding nor resistance to proteolytic degradation could account for these differences in bioactivity it is likely that position 3 may be structurally important in another factor influencing bioactivity, such as differential Y receptor selectivity. This would need to be further explored by testing the binding affinity of these analogues at the Y1R and Y5R receptors.

The next set of experiments aimed to evaluate the effects of modifications that can improve longevity in peptide analogues on PYY3-36 bioactivity. Some of these modifications can improve the half-life of a peptide by reducing kidney filtration and/or proteolytic degradation (Markussen et al., 1996; Knudsen et al., 2000). Lauroyl, an 8 carbon fatty acid, was attached at position 12, a region on the β turn; after the proline helix and before the α-helix, to minimise the risk of altering the 3D PP-fold structure or the ‘micelle-bound’ structure. As hoped, the ‘control’ analogue, which only contained the Lys substitution (for attachment to lauroyl), did not alter Y2R affinity. Substitution of Ala12 for Lys12-lauroyl had a detrimental effect on Y2R affinity, reducing it by almost 10 fold, but prevented KBB-induced degradation of PYY3-36 by 99%. Though fatty acid moieties are useful in increasing peptide half-life by promoting binding to plasma proteins and increasing lipophilicity (thus allowing penetration of the peptide through cell membranes), they often reduce pharmacological activity at the receptor (Muranishi et al., 1991; Knudsen et al.,
The Lys12 substitution alone had a detrimental effect on PYY3-36 bioactivity, though this did not reach statistical significance compared to PYY3-36. Interestingly, Lys12Lauroyl had a detrimental effect on bioactivity in the first hour post-injection compared to PYY3-36, but at later time points significantly improved bioactivity compared to PYY3-36, though its effect on cumulative food intake was not significantly different to PYY3-36. This data warrants further investigation into using acylated Lys substitutions to prolong PYY3-36 bioactivity, for instance by acylating the naturally-occurring lysines in native PYY3-36 to prevent any interference associated with alterations of other positions, as has been successfully accomplished in liraglutide (Degn et al., 2004).

Combining D-allo-Ile 3 with Lys12 maintained Y2R affinity, which may suggest that the individual substitutions conferred a summative improvement in Y2R affinity. PYY3-36 D-allo-Ile3 Lys12 exhibited a substantial reduction in degradation, by around 60%, which was surprising considering neither substitution on its own had an appreciable effect on degradation. On this basis, D-allo-Ile3 was combined with Lys12-Laur. Surprisingly, the control analogue, PYY3-36 D-allo-Ile3 Lys12, was initially more effective than PYY3-36, but was significantly less bioactive than PYY3-36 between 4-8h and cumulatively over 8h post-injection, which may be due to the detrimental effects of Lys12 outweighing the beneficial effects of D-allo-Ile3 on bioactivity. Similar to the Lys12Laur analogue, the D-allo-Ile3 Lys12Lauroyl combination proved to be significantly less effective than PYY3-36 at acutely reducing food intake, but by the 2-4h interval was significantly more potent than PYY3-36. The acute effect is likely due to reduced Y2R affinity, reflected in a reduction in receptor ‘on-time’ when peptide concentrations are at their peak, while the late effect may be due to reduced proteolytic degradation, since in vitro KBB-induced degradation was prevented by nearly 100%. The Lys-Laur substitution may also allow binding of PYY3-36 to plasma proteins, such as albumin, thereby reducing renal filtration. This data suggests that combining a Lys-fatty acid conjugate substitution with a D-allo-Ile3 stereoisomeric substitution in a single analogue is beneficial in prolonging the effect of PYY3-36 on food intake.
The next set of experiments aimed to evaluate whether combining substitutions within different regions of PYY3-36 in a single analogue might be summative in improving PYY3-36 bioactivity. The analogues are grouped into different categories for clarity, though because of the large number of analogues and the limited animal numbers, they could not all be tested within the same feeding study. I found that the effect of single substitutions on Y2R affinity, resistance to proteolytic degradation and food intake did not necessarily predict these effects when combined. In the first set of experiments, I investigated the effects of combining substitutions at positions 19 and 23 in the N-terminal with position 30 in the C terminus, along with the addition of Pro to the N-terminus. All combined analogues improved Y2R affinity, with combined N-Pro Lys19 Ala23 Lys30 substitutions increasing Y2R binding affinity by around 2 fold. The addition of N-Pro to the N-terminal of Lys19 Lys30 did not enhance resistance to proteolytic degradation, which was unexpected considering the addition of Pro to the N-terminal of PYY3-36 reduced degradation by 50%. However, this may be due to unfavourable structural effects of Lys19 Lys30 substitutions on PYY3-36, as this analogue also exhibited an increase in degradation compared to its individual substitutions. Despite this, combined Lys19 Lys30 substitutions significantly reduced cumulative 24h food intake compared to saline, whereas PYY3-36 did not. The N-Pro Lys19 Lys30 combination was equally effective in reducing food intake over 24h. All combined substitutions investigated were significantly more effective than PYY3-36 in reducing food intake, though only three analogues, PYY3-36 Lys19 Lys30, PYY3-36 N-Pro Lys19 Lys30 and PYY3-36 N-Pro Lys19 Ala23 Lys30, significantly reduced food intake over 24h. Out of these analogues, N-Pro Lys19 Ala23 Lys30 appeared to be most effective in reducing food intake, as it was the only analogue that significantly reduced food intake compared to PYY3-36 at 2-4h and 4-8h post-injection, and potently reduced food intake over 24h compared to saline, though the 24h food intake was not significantly different to PYY3-36. This improvement appears to be due to the addition of an Ala23 substitution, which is in line with the slight, non-significant improvement in bioactivity following the individual Ala23 substitution and previous work showing that position 23 is important in the Y2R-mediated anti-secretory activity of PYY3-36 (Balasubramaniam et al., 2000; Moriya et al., 2010).
The next set of analogues were designed in order to investigate the effects of combining substitutions at positions 6, 23 and 30 with or without the addition of Pro at the N-terminal. The improved affinity of all but one of the peptides, and the improved bioactivity of all the peptides implies that amino acids within the N-terminal, mid section and C terminus play a role in Y2R affinity and bioactivity. However, there were no significant correlations between resistance to proteolytic degradation and the degree of enhanced bioactivity, though all analogues showed improved resistance to proteolytic degradation by KBB.

Combining Glu at position 23 with Lys at position 30 was significantly more effective than PYY3-36 in reducing food intake at 4-8h, and significantly reduced cumulative food intake over 24h. This shows that Glu at position 23 is more effective in increasing PYY3-36 bioactivity than Ala at position 23 when combined with Lys30. However, the addition of Ser at position 6 to the combined Glu23 Lys30 substitutions did not significantly improve PYY3-36 bioactivity, which may be due to the slight decrease in Y2R affinity. Combining the Ser6 substitution with Ala23 and Lys30 improved Y2R affinity by 2 fold and reduced 24h food intake, whereas PYY3-36 did not, suggesting that Ala at position 23 is more beneficial than Glu when combined with Ser6 and Lys30. Interestingly, the addition of Pro at the N-terminal to create PYY3-36 N-Pro Ser6 Ala23 Lys30 significantly reduced food intake at 4-8h and cumulative food intake over 24h compared to PYY3-36, and is therefore the only analogue thus far to exhibit a significantly improved 24h food intake profile compared to both saline and PYY3-36. Although this analogue exhibited a 2-fold increase in Y2R affinity and around a 20% reduction in degradation, these initial properties were also observed for PYY3-36 Ser6 Ala23 Lys30, suggesting that they are not solely responsible for the improved bioactivity. The effect of the Pro tail may improve resistance to degradation of PYY3-36 by circulating proteases that are not expressed in KBB, or may alter the bioavailability of the analogue at central Y2 receptors. Peptides with individual Ser6, Ala23 or Lys30 substitutions substantially increased the susceptibility of PYY3-36 to proteolytic degradation. However, combining these substitutions reduces proteolytic degradation, suggesting that other factors, such as secondary and 3D structure, influence a peptide’s susceptibility to KBB proteases. Interestingly, combining the substitutions N-Pro Lys6 Ala23 Lys30 reduced KBB-mediated degradation of PYY3-36 by 65%, and though
this peptide was more effective than PYY3-36 in reducing cumulative food intake over 8 hours, it had no significant effect on 24h food intake.

The next set of experiments evaluated the effects of combining substitutions at positions 23 and 30 with the addition of Pro at the N-terminal. Combining N-Pro with Glu at position 23 had the most detrimental effect on Y2R affinity out of all analogues with combined substitutions, which is reflected in the fact that N-Pro Glu23 did not significantly improve bioactivity compared to PYY3-36. However, substituting an Ala instead of a Glu at position 23 in the same analogue improved Y2R affinity and resulted in an analogue significantly more effective than PYY3-36 in reducing cumulative food intake over 8 hours, and was the only analogue to significantly inhibit food intake at 4-8h compared to saline. The prolonged effect on food intake may be explained by the fact that combining N-Pro and Ala23 substitutions reduced KBB-mediated degradation by around 80%. As substituting a hydrophobic Leu for a positively charged amino acid at position 30 appeared to be beneficial, Lys, His or Arg were then combined into the N-Pro Ala 23-substituted analogue. All three analogues, PYY3-36 N-Pro Ala23 Lys/His/Arg30 exhibited an enhanced Y2R affinity. PYY3-36 N-Pro Ala23 Lys30 was most effective in reducing food intake, as it significantly reduced 24h food intake to a level similar to the analogue PYY3-36 N-Pro Ser6 Ala23 Lys30. However, this may suggest that Ser6 does not play a role in enhancing the bioactivity of this analogue. Combining N-Pro, Ala23 and Lys30 substitutions reduced the IC50 at the Y2R to 0.03nM and improved resistance to KBB-mediated degradation by 50%, whereas the same analogue with either His or Arg at position 30 had small effects on these parameters. The KBB has therefore served as a useful tool in detecting which analogues may be more resistant to physiological degradation. However, due to the multitude of other factors that play a role in determining the bioactivity of a peptide, the in vitro Y2R affinity and resistance to proteolytic degradation does not always predict the longevity or potency of the peptides in vivo.

In conclusion, individual positions have been shown to be important in the bioactivity of PYY3-36 in vivo. These included positions 3 and 4 in the N-terminal, position 19 in the mid-section, and positions 31 and 34 in the C terminus. These investigations helped
elucidate several sites that had potential in drug design that exhibited an unaltered or improved Y2R affinity and/or bioactivity in vivo. A combination of several substitutions in different domains of PYY3-36 were subsequently discovered which significantly increase the longevity of the anorectic effect of PYY from 2 hours to between 4 and 8 hours. Two analogues with changes in the N-terminal and α-helix of PYY3-36, PYY3-36 N-Pro Ser6 Ala23 Lys30 and PYY3-36 N-Pro Ala23 Lys30, , showed a significantly more potent effect between 4 and 8 hours and over 24 hours post-administration compared to PYY3-36. These substitutions also allow a more potent interaction with the human Y2 receptor, which implies that they may be beneficial in the development of PYY3-36 analogues for use in the treatment of human obesity. Furthermore, the substitution of an acylated Lys residue into position 12 has been shown to almost completely reduce the susceptibility of PYY3-36 to degradation by enzymes of the kidney brush border, and significantly prolong the effect of PYY3-36 on food intake. This demonstrates that acylation of PYY3-36 can prolong its bioactivity, as observed in other peptide analogue programs, such as for liraglutide. My work has also shown that steric alterations at position 3 can also be utilised to enhance the Y2R affinity and potency of PYY3-36. As these studies have indicated that proteolytic degradation can be an important determinant in bioactivity, the design of further long-acting analogues may require study of the physiological degradation profile of native PYY3-36. Knowledge regarding the cleavage sites of specific KBB proteases, together with the knowledge gleaned regarding the domains and amino acid sites at which substitutions can be made that enhance the affinity of PYY3-36 at the Y2R, may enhance the design of PYY3-36 analogues.
Chapter Three: Investigating the metabolic clearance and degradation of PYY3-36 as a tool in developing a long-lasting PYY3-36 therapy
3.1 Introduction

There is debate as to whether PYY plays a role in the pathogenesis of obesity. The first report on PYY plasma levels in the obese suggested that obesity is a PYY-deficient state, as patients exhibit a blunted postprandial response and decreased basal levels (Batterham et al., 2003a). However, another group using the same assay as used in the Batterham et al study found that fasting PYY plasma levels were similar between normal-weight and obese volunteers levels, and another group has since reported that fasting PYY concentrations are not correlated with BMI (Kim et al., 2005). Significantly, Stock et al did report that obese patients showed blunted postprandial PYY plasma levels (Stock et al., 2005) and Le Roux and colleagues subsequently confirmed a reduced postprandial response in obese patients (Le Roux et al., 2006b). Guo and colleagues reported a positive correlation between postprandial release of PYY and changes in ratings of satiety after a meal. In another prospective study, the authors found that peak PYY concentrations were negatively associated with adiposity markers and body weight change and positively associated with postprandial satiety ratings (Guo et al., 2006). In rodent studies, mice exposed to a high fat diet became obese and exhibited a reduction in circulating PYY. Furthermore, the phenotype of PYY knock-out mice, which display hyperphagia and increased adiposity, was reversed by exogenous replacement with PYY (Batterham et al., 2006). Taken together, these results suggest that a blunted postprandial rise in PYY3-36 may be associated with obesity, but that the ability of obese individuals to respond to PYY is not homogenous.

Obese patients retain sensitivity to the anorectic effects of PYY3-36, which is in contrast to the resistance seen in the obese to leptin administration (Zelissen et al., 2005; Schwartz et al., 2000). Thus PYY3-36 may be useful as an anti-obesity therapeutic (Batterham et al., 2003a). However, simple replacement of PYY3-36 to the obese presents a challenge as the peptide is rapidly metabolised in the circulation, and moderately high levels have
unpleasant side effects, and chronic administration can lead to tachyphylaxis (Adrian et al., 1986). Exogenous PYY undergoes rapid enzymatic clearance resulting in a short half-life of around 8 min during an iv infusion in rats (Lluis et al., 1989). Administration of PYY3-36 at supraphysiological levels induces conditioned taste aversion in rodents and nausea in humans (Halatchev and Cone, 2005; Le Roux et al., 2008; Reidelberger et al., 2008). Dosing strategy and route of administration is critical to ensuring sustained chronic effects of PYY3-36, (Reidelberger et al. 2008). The development of long-acting PYY3-36 analogues should therefore aim to develop peptides which have an extended half-life, but which do not result in the activation of nausea pathways or result in tachyphylaxis.

Investigating the physiological metabolic degradation profile of PYY3-36 will suggest the enzymes able to cleave PYY3-36 and the sites at which this degradation occurs. Characterization of the amino acid sites susceptible to proteolysis may aid design of long-acting PYY3-36 analogues, by determining amino acid changes that may reduce the susceptibility of the molecule to breakdown. Alternatively, the use of specific enzyme inhibitors may prolong the biological effect of endogenous PYY3-36. These approaches have been successfully utilised in the therapeutic development of drugs based upon the incretin hormone GLP-1. GLP-1 is subject to N-terminal degradation by the enzyme dipeptidyl peptidase-iv (DPP-iv). Exendin-4 is a DPP-iv resistant analogue of GLP-1 that has a longer half-life than native peptide and an improved insulinotropic effect. Exendin-4 is now a licensed treatment for type II diabetes, which improves glycaemic control and reduces body weight (Bloom et al., 2008). Furthermore, DPP-iv inhibitors have insulinotropic effects and are useful anti-diabetic drugs in their own right (Deacon and Holst, 2006). However, the mechanisms by which PYY3-36 is physiologically degraded are currently poorly characterized (Neary and Batterham, 2009a).

Meprin B, DPP-iv and neprilysin (NEP) are widely expressed on epithelial cell surfaces, and are highly concentrated in the brush border of the kidney. Meprin B and NEP have previously been found to degrade PYY (Medeiros and Turner, 1994; Bertenshaw et al., 2001). Elevated levels of PYY and a number of other gut-derived peptides have been found in chronic renal failure patients, presumably due to a reduction in the degradation
and/or filtration of circulating peptide (Hegbrant et al., 1991). Cell surface enzymes are of interest as they initiate peptide hydrolysis at the extracellular face of the plasma membrane, where circulating endogenous regulatory peptides, as well as exogenously administered peptides are most likely to exist in vivo (Fauchere JL and Thurieau, 1992).

NEP is a widely-expressed plasma membrane of the M13 family of Zn metallopeptidases, which is involved in the inactivation of a number of regulatory peptidase (Erdos and Skidgel, 1989). NEP is most highly expressed in the brush border of the kidney cortex, and is also expressed in intestinal enterocytes, endothelial cells (though only at a low concentration on vascular endothelial cells), fibroblasts and neutrophils, and is known to regulate a large number of signalling peptides both peripherally and in the brain as a synaptic ectoenzyme (Erdos and Skidgel, 1989; Turner et al., 2001). Recently the gene for human NEP-2 was cloned from a human foetal-brain library, and the predicted protein was found to be 54% identical to human NEP (Bonvouloir et al., 2001). Unlike NEP, which is abundantly expressed in the kidney and brain, NEP-2 is expressed widely in distinct neuronal populations of the CNS and most highly in the testis (Turner et al., 2001). NEP has broad specificity and cleaves on the N-terminal side of hydrophobic amino acid, with a preference for leucine, followed by phenylalanine (denoted L>F) (see fig. 3.1). Medeiros and Turner found that NEP cleaved PYY at residues Asp29 and Leu30 of the PYY3-36 α-helix, generating the inactive metabolites PYY3-29 and PYY31-36 (Medeiros and Turner, 1994). As the C-terminal of PP-fold family peptides is essential for Y receptor binding, NEP was proposed to inactivate PYY, though this has not been proven, suggesting that NEP, and perhaps related enzymes, may be important in the physiological degradation of PYY3-36. PYY3-36 analogues with NEP-resistant substitutions may demonstrate an increased functional half-life in vivo.

DPP-iv is a protease that has a small active site that preferentially cleaves following the two amino acid sequences Xaa-Pro or Xaa-Ala, where Xaa stands for any amino acid. Elucidation of its crystal structure revealed that only amino acids with small side chains are able to fit into the active site of DPP-iv, which restricts possible amino acids in the P1 position to proline, alanine or glycine (Rasmussen et al., 2003). DPP-iv is primarily
expressed in the brush borders and microvilli of the kidney cortex, intestinal enterocytes, hepatocytes in the liver and epithelial cells in the pancreas. DPP-iv is also located on endothelial cells of blood vessel walls and is found as a soluble enzyme in blood plasma (Mentlein, 1999). Endogenously released PYY is therefore likely to encounter DPP-iv upon its release from L cells of the gastrointestinal tract.

The meprins are Zn metalloendopeptidases of the astacin family. Meprin A is a heterodimer of meprin α and β subunits, while meprin B is a homodimer of meprin β subunits. The meprin α subunit is secreted from the membrane, while meprin β is membrane bound and has a cleavage domain which allows the enzyme to be secreted (see fig. 3.2) (Becker et al., 2003). Meprin A has been found to preferentially cleave peptides with small and aromatic residues in position P1’ (S>F>A>T>M>Y>G) and with Pro at position P2’ (see fig. 3.3). A previous study found that PYY is a substrate for meprin B, but not meprin A, which implicates the β subunit in PYY degradation. Incubating PYY (100µM) with meprin B (2nM) was found to result in 32% degradation of PYY as demonstrated by quantitative HPLC analysis. The authors proposed the cleavage site of Meprin B to be Asp10-Glu11 after MALDI-TOF analysis of the breakdown products (Bertenshaw et al., 2001). The cleavage sites for NEP and meprins on PYY appear to be downstream of position 3. However, the effects of these enzymes on PYY3-36, the more selective Y2R agonist, have not been investigated.

NEP and Meprin B are expressed ubiquitously, but are particularly highly expressed in the kidney brush border (KBB) of the proximal convoluted tubule (Sterchi et al., 2008; Erdos and Skidgel, 1989). The meprins constitute 5% of total protein in the KBB, second only to actin in quantity (Bond et.al. 2005). The KBB contains a number of microvillar peptidases thought to be responsible for degradation of peptides within the glomerular filtrate in the proximal tubule (Carone et al., 1984). Chronic renal failure patients exhibit high levels of PYY, which suggests that the kidney may be an important site for clearing and/or degrading PYY (Hegbrant et al., 1991). These patients also have reduced appetite, though the mechanism behind this cachexia has not been identified. In addition, membranes of KBB have previously been utilised as a tool to study peptide degradation as they possess
peptidolytic activity reflective of enzymes expressed throughout the periphery (Medeiros and Turner, 1994; Yamaguchi et al., 1994).

This work is aimed at the design and discovery of PYY analogues that display potent binding and activation at the Y2R and are resistant to degradation by circulating and membrane-bound peptidases. Substitutions of amino acids on key sites of the PYY molecule will alter the 3D structure of the protein thereby altering its efficacy at the Y2 receptor and its susceptibility to proteolytic degradation and/or renal clearance.

### 3.2 Aim of studies

1) To clarify whether the kidney is a major anatomical site for PYY3-36 filtration/inactivation by measuring plasma levels after exogenous administration of PYY3-36 to nephrectomised and sham-operated rats.

2) To determine the role of the metalloendopeptidases NEP and meprin B in the physiological clearance of PYY3-36 and the amino acid sites at which PYY3-36 is susceptible to hydrolysis by these enzymes *in vitro* and *in vivo*.

3) To ascertain whether the cleavage sites of these enzymes may be utilised as targets for analogue development and to investigate whether use of specific enzyme inhibitors, such as neprilysin and actinonin, prolongs the bioactivity of PYY3-36.
### Table: Amino Acid Preference for NEP

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Figure 3.1. NEP specificity matrix using the nomenclature for the interaction of proteases with their substrates as designated by Schecter and Berger (taken from Merops database: [www.merops.sanger.ac.uk](http://www.merops.sanger.ac.uk)). Residues on the amino side of the scissile bond are numbered P1 through P4 counting outward while residues on the carboxy side are numbered P1’ through P4’ counting outward. Numbers are arbitrary units and represent relative preference for certain residues, with higher numbers representing higher preference. NEP is thought to cleave when hydrophobic amino acids Arg and Met are in P1’ (Rawlings, 1999).

Figure 3.2. Dimeric structure of meprin A and B. Meprin A is a heterodimer of meprin α and β subunits, and meprin B exists as a homodimer of meprin β subunits. The meprin α subunit is secreted from the membrane, while meprin β is membrane bound and has a cleavage domain which allows the enzyme to be secreted. Taken from: (Sterchi et al., 2008).
Figure 3.3. Meprin-β specificity matrix using the nomenclature for the interaction of proteases with their substrates designed by Schecter and Berger. Residues on the amino side of the scissile bond are numbered P1 through P4 counting outward while residues on the carboxy side are numbered P1’ through P4’ counting outward. Numbers are arbitrary units and represent relative preference for certain residues, with higher numbers representing higher preference. Meprin-β is thought to cleave when negatively charged amino acids Asp and Glu are in P1’, Pro in P2’ and Glu in P1 (adapted from the Merops database: www.merops.sanger.ac.uk) (Rawlings, 1999).
3.3 Materials and Methods

3.3.1 Materials

PYY3-36 was purchased from Bachem Ltd (UK). Human recombinant meprin β (EC 3.4.24.18) was a gift from Dr Christoph Becker-Pauly of Munster University (Becker-Pauly et al., 2007). Human recombinant NEP (EC3.4.24.11) was purchased from R&D Systems. Phosphoramidon and actinonin were purchased from Sigma-Aldrich (UK).

3.3.2 Purified protease degradation studies

PYY3-36 (2nmol) was incubated with or without 200ng recombinant human NEP or 200ng recombinant human meprin β subunit in digest buffer (50mM Tris-HCL, 50mM NaCl pH7.5) at 37°C in a total volume of 140µl (Druce et al., 2009). For the inhibitor studies, 10nmol phosphoramidon or actinonin was included in the digests, either with peptide and enzyme or with peptide only as a control step. The reaction was terminated at the stated time-point by adding 10µl 30% trifluoroacetic acid (TFA). Samples were then centrifuged at 12500xg for 5 min at room temperature.

3.3.3 Quantitative HPLC

Incubation samples were then analysed by reverse phase HPLC (Jasco HPLC system: solvent delivery system PU-2080 plus, autosampler AS-2057 plus, degasser DG2080-53, dynamic mixer 2080-32, UV detector uv-2075) using a Gemini C18 (Phenomenex, Macclesfield UK; 5µm particles, 250mm x 4.6 mm) column. Sample (100µl of supernatant from the terminated incubation) was eluted from the C18 column using a linear acetonitrile (AcN)/water gradient. A gradient of 15-60% AcN over 35 min was used for most experiments. Under these conditions PYY3-36 eluted at 20 min. For the inhibitor studies, inhibitors in digest buffer were run through the HPLC in order to determine their retention
times. As the retention time for actinonin was similar to that of PYY3-36, a shallower gradient of 20-35% AcN was used for the actinonin experiments to avoid co-elution of these compounds. Under these conditions PYY3-36 eluted at 30 min. The eluted peptide and peptide fragments were detected spectrophotometrically at a wavelength of 214nm. Percentage degradation of peptide was calculated by comparing area under the curve (AUC) on HPLC chromatograms of incubations with and without enzyme or membrane (Graphpad version 5.00 for Windows, GraphPad Software, San Diego, California).

3.3.4 MALDI-ToF Mass Spectrometry

Selected samples were also analysed using matrix assisted laser desorption / ionisation-time of flight (MALDI-TOF) mass spectrometry. Samples were dissolved using 100µl Solution A (60% AcN/40% water/0.1%TFA). The matrix (10mg/ml alpha-cyano-4-hydrocinnamic acid in Solution A) was applied to the sample plate (0.5µL) followed immediately by the sample (0.5µL) and the plate air dried. The mass spectrometer (Shimadzu Axima-CFR) was set to positive linear mode. Data was acquired for samples after near point calibration using a mixture of five peptides across the mass range 1,000-6,000. Peptide fragments resulting from the cleavage of PYY3-36 were identified (taking into account the monoisoptic mass allowance) using the program FindPept (http://www.expasy.ch/tools/findpept.html).

3.3.5 KBB degradation studies

3.3.5.1 PYY3-36 degradation assay using KBB

For the preparation of KBB membranes and protein concentration assay see Chapter 2, section 2.3.5.2 and 2.3.6.1. PYY3-36 (2nmol) was incubated with or without 2.5mg/ml, 1.25mg/ml or 0.625mg/ml KBB in digest buffer (300mM mannitol in 12mM HEPES, pH7.4) at 37°C in a maximal volume of 140µl. For the inhibitor studies, 100nmol phosphoramidon or actinonin was included in the digests, either with or without peptide. The reaction was terminated at 5, 15, 30 or 60 min time-points by the addition of 10µl 30%
trifluoroacetic acid (TFA). Samples were then centrifuged at 12500xg for 5 min at room temperature in order to separate membrane from peptide solution. KBB was found to have a potent dose and time dependent effect on PYY3-36 breakdown. The KBB incubation protocol was therefore optimised to a concentration and incubation time that reliably resulted in >50% degradation of PYY3-36, so that both parent peptide and peptide fragments could be observed on the chromatogram. A dose response experiment over a fixed incubation time of 30 min using increasing concentrations of KBB (0.625, 1.25 and 2.5mg/ml) was carried out in order to determine a concentration at which 1) >50% of peptide was degraded and 2) break down products could be detected. Subsequently, a concentration of 1.25mg/ml was used in the remainder of peptide/KBB incubations. Incubation samples were then analysed by HPLC and MALDI-ToF mass spectrometry as previously described.

### 3.3.6 In vivo studies

#### 3.3.6.1 Animals

All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project Licence 70/5516). Adult male C57/BL6 mice (Harlan, UK) weighing 20-25g were maintained in individual cages under controlled temperature (21-23°C) and lights (12:12 hour light:dark cycle, lights on at 0700h). Animals had *ad libitum* access to water and normal chow RM1 diet (Special Diet Services). To minimise stress animals were regularly handled and acclimatised with two subcutaneous (sc) and intraperitoneal (ip) injections. All feeding studies were carried out during the early light phase (0800h-1000h). Mice were overnight fasted before each study morning 1) in order to reduce endogenous PYY and PYY3-36 and 2) because anorectic effects are more easily detected in the fasted state.
3.3.6.2 PYY3-36 pharmacokinetics study in nephrectomised rats

Rats were randomized according to body weight and divided into 3 groups: unilateral nephrectomy, bilateral nephrectomy and sham. On study day rats were anaesthetized with 67mg/kg hypnorm (1:30 ratio of fentanyl: fluanisone; Vetapharma) mixed with 3mg/kg hypnovel (midazolam; Pfizer) given ip. Prior to the first incision rats were administered 0.5mg of hypnovel sc and placed in dorsal recumbence on a heated surgical table at 37°C.

A midline abdominal incision was made to expose the viscera. The abdominal contents were displaced and a single suture was tied in a double knot around either one or both renal pedicles to allow unilateral or bilateral nephrectomy respectively. The displaced abdominal contents were then restored and wound clips used to close the skin. A 2 cm ventral incision was made in the skin about 1 cm from the midline, starting from the pectoralis muscle and extending 2 cm toward the head. The jugular vein, which is directly beneath the subcutaneous tissue, was exposed by blunt dissection using fine point curved forceps and haemostats (Fine Science Tools, Heidelberg, Germany) to hold the skin. Using surgical sutures (VICRYL PLUS, Ethicon, USA) a ligature was placed with the aid of the fine point curved forceps around the exposed part of the jugular vein towards the head. This served to occlude blood flow from the head, minimising blood loss after the vein is nicked to insert cannula. A loose ligature is also placed near the distal end of the exposed vein. A small nick was then made in the vein between the 2 ligatures using spring style micro-scissors (Fine Science Tools, Germany). A bevelled cannula was then inserted through the nick, extending about 0.5 cm into the vein, and the loose ligature was then tied firmly around the cannulated vein. The cannula used was a polyethylene tubing (inner diameter: 0.40 mm, outer diameter: 0.80 mm; Smiths Medical International, UK) attached to a needle and a 1ml syringe pre-filled with a 5% heparin saline solution to inhibit blood coagulation. PYY3-36 (100nmol/kg) was then injected iv into the penile vein. A 300µl blood sample from the jugular vein was then taken to represent time 0. Blood sampling from the jugular vein was then carried out at 2, 5, 10, 15, 30 and 60 min post injection and collected into eppendorfs containing aprotinin (Trasylol, Bayer, Germany) to inhibit blood protease action and blood
coagulation. After each sample was taken the vein was flushed with 100µl of the heparin saline solution. After the final time point, rats were killed using an overdose of sodium pentobarbitone (Merial Animal Health Ltd, UK). Plasma was separated from whole blood by centrifugation at 6000rpm for 10 min, and stored at -20°C until use.

3.3.6.2.1 PYY3-36 radioimmunoassay

An established in-house PYY radioimmunoassay was used to detect PYY3-36 immunoreactivity (IR) in plasma samples from various time points (Adrian et al., 1985). This technique relies upon competition between a radiolabelled antigen and an unlabelled antigen for antibody binding sites. A fixed amount of 125I-labelled PYY3-36 antigen and a fixed amount of antibody is added to the sample (see Appendix iv). The ratio of bound to unbound radiolabelled antigen provides a measure of the quantity of unlabelled antigen present in the sample. The unbound antigens are separated from the antigen:antibody complex and the amount of label is measured using a gamma counter. Separation of bound and unbound antigen was carried out using a secondary antibody binding method. The secondary antibody binds the primary antibody, creating a secondary antibody:primary antibody:antigen complex. Centrifugation then allows separation of the smaller unbound antigen into the supernatant, while the bound secondary antibody is located in the pellet. A standard curve is constructed using known amounts of labelled peptide. In this way, it is possible to quantify the absolute values of unlabelled peptide in each sample. The PYY3-36 assay was performed in 0.06M phosphate buffer (see appendix II) containing 1% BSA. Samples and standards were assayed in duplicate in a total volume of 700µl. 125I-Labelled PYY3-36 was prepared by the iodogen method and purified by HPLC. 125I-Labelled PYY3-36 was prepared at 200 counts per 10 seconds per 100µl and 100µl was added to each tube. The assay measured the biologically active components, both the full-length PYY1–36 and the fragment PYY3–36. The antiserum, Y21, was produced in a rabbit against synthetic porcine PYY (Bachem) coupled to BSA glutaraldehyde and used at a final dilution of 1:50,000. Similar to all current PYY assays, the antibody cross-reacts fully with
PYY1–36 and PYY3–36 but not with PP, NPY, or any other gastrointestinal hormone. The samples were incubated for 3 days at 4°C before separation of free and antibody-bound label by sheep antirabbit antibody. Two hundred microliters of unextracted plasma were assayed, whereas 200 µl of PYY-free colloid fluid, Hemacel, was added to standards and other reference tubes to negate any effects of nonspecific assay interference. The assay detected changes of 2 pmol/liter, with intra- and interassay coefficients of variation of 5.8 and 9.8%, respectively.

3.3.6.2.2 Creatinine ELISA

Creatinine was measured as it is a known marker of kidney failure. The 60 min time point plasma samples were assayed for creatinine using a commercially-available creatinine ELISA (Abbott Diagnostics UK) and was performed by Hammermsith Hospital Clinical Chemistry service.

3.3.6.3 Phosphoramidon/PYY3-36 co-administration feeding study in mice

Mice were divided into four treatment groups and administered a sc injection (maximum volume 100µl) of either: saline, phosphoramidon (10mg/kg), PYY3-36 (50nmol/kg) or PYY3-36 (50nmol/kg) & phosphoramidon (10mg/kg) (n=10; see table 3.1). The dose of phosphoramidon used was based on a previous study, in which NEP was inhibited over 4 hours using this dose (Day et al., 2005). Food intake was measured at 1, 2, 3, 4 and 8 hour intervals post-injection.

3.3.6.4 Actinonin/PYY3-36 co-administration feeding study in mice

The study was carried out as described above. Mice were randomly divided into six treatment groups and administered an intraperitoneal injection followed by a subcutaneous injection 15 min later (maximum volume 100µl) of: vehicle (20% ethanol 80% saline) &
saline, actinonin (20mg/kg) & saline, actinonin (40mg/kg) & saline, vehicle & PYY3-36 (50nmol/kg), actinonin (20mg/kg) & PYY3-36 (50nmol/kg) and actinonin (40mg/kg) & PYY3-36 (50nmol/kg) (n=7-10; see table 3.1). A dose of 20mg/kg actinonin was used due to its ability to inhibit meprin activity over 4h, and this dose was increased in order to investigate whether the effects of actinonin were dose-responsive (Carmago et al., 2002). Based on our finding that PYY3-36 alone did not reduce food intake at the 4-8h interval, food intake was measured at 1, 2, 3, 4 and 6 hour intervals post-injection so that any anorectic effect between 4 and 6 h post-injection could be detected.

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<td>2</td>
<td>PYY3-36 (50nmol/kg)</td>
<td>vehicle</td>
</tr>
<tr>
<td>3</td>
<td>PYY3-36 (50nmol/kg)</td>
<td>actinonin (20mg/kg)</td>
</tr>
<tr>
<td>4</td>
<td>PYY3-36 (50nmol/kg)</td>
<td>actinonin (40mg/kg)</td>
</tr>
<tr>
<td>5</td>
<td>saline</td>
<td>actinonin (20mg/kg)</td>
</tr>
<tr>
<td>6</td>
<td>saline</td>
<td>actinonin (40mg/kg)</td>
</tr>
</tbody>
</table>

Table 3.1. Summary of each treatment group, designated 1-6, for studies described in sections 1.3.6.3 and 1.3.6.4.

3.3.6.5 Actinonin/PYY3-36 plasma levels study in mice

This study was carried out in order to assess whether actinonin has the ability to enhance pharmacological levels of exogenously-administered PYY3-36. Mice fasted for 4h prior to the study were randomly divided into four treatment groups and administered an intraperitoneal injection followed by a subcutaneous injection 15 min later (maximum volume 100µl) of: vehicle (20% ethanol 80% saline) & saline, actinonin (20mg/kg) & saline, vehicle (20% ethanol 80% saline) & PYY3-36 (50nmol/kg) and actinonin (20nmolg/kg) & PYY3-36 (50nmol/kg) (n=8-13: all groups except actinonin only: n=2-5). Time zero was recorded from the second injection and mice were killed by CO₂ asphyxiation at 0, 30, 60, 90 and 120 min post-injection. Blood was immediately removed
via cardiac puncture using heparinised syringes and placed in eppendorfs containing 20µl protease inhibitor (aprotinin) on ice. Plasma was then separated, stored and assayed for PYY3-36 as described in section 1.3.3.2.

3.3.7 Statistics

All data are presented as means ± SEM. The interval food intake data and plasma levels study data was analyzed using one-way ANOVA with Tukey’s post hoc test (GraphPad Prism version 5.00 for Windows; GraphPad Software, San Diego, California). Cumulative food intake data was compared using the generalized estimating equation with the Mann-Whitney U adjustment (Stata 9, StataCorp LP, College Station, TX). In all cases, p<0.05 was considered to be statistically significant.
3.4 Results

3.4.1 The effect of nephrectomy in rats on PYY3-36 pharmacokinetics

This study was carried out in order to analyse the contribution of the kidney to the removal of exogenously-administered PYY3-36 from the circulation. As discussed, PYY has a short half-life in mammals. It was therefore assumed that PYY3-36 would be cleared in a similar manner, and so plasma sampling was carried out at 0, 2, 5, 10, 15, 30 and 60 min post-injection of PYY3-36 (100nmol) (n=5-6 per group). Time zero was recorded at 10 min post-nephrectomy surgery. At 0 min post administration of PYY3-36 (and 10 min post-surgery), there were no differences in plasma PYY3-36 immunoreactivity (IR) between sham-operated controls and those that underwent unilateral (UNx) or bilateral (BNx). After 2, 5, 10 and 15 min there were no significant differences between groups. At 30 min post administration BNx animals had significantly higher PYY3-36 plasma levels (see fig. 3.4 and 3.5). At 60 min post administration there were no significant differences between groups. The half-disappearance rate for PYY3-36 in sham rats was 25.9 min, which increased to 30.6 min and 45.4 min with the loss of function of one (UNx) or both (BNx) kidneys respectively (see table 3.2). Loss of kidney function was confirmed by assaying the plasma samples for creatinine, which is a by-product of muscle metabolism and accumulates in kidney failure. As expected creatinine levels were increased in rats that had undergone both unilateral and bilateral nephrectomy, though for the UNx group this did not reach statistical significance (see table 3.3).
Figure 3.4. Effect of unilateral or bilateral nephrectomy on PYY plasma levels in rats at 2 min, 5 min, 10 min, 15min, 30min and 60min post-injection. Results are means ± SEM. ***p < 0.001 vs. sham (ANOVA with post-hoc Dunnett’s adjustment), n = 6.

Figure 3.5. Effect of unilateral or bilateral nephrectomy on PYY3-36 plasma levels in rats at 2 min, 5 min, 10 min, 15min, 30min and 60min post-injection. Results are means ± SEM (n=6). ***p< 0.001 vs. sham (ANOVA with post-hoc Dunnett’s adjustment), n = 6.
Half-disappearance rate of PYY3-36

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (min):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>25.9 ± 2.9</td>
</tr>
<tr>
<td>UNx</td>
<td>30.6 ± 5.8</td>
</tr>
<tr>
<td>BNx</td>
<td>45.4 ± 0.75</td>
</tr>
</tbody>
</table>

Table 3.2. Half-disappearance rate for PYY3-36 in sham, UNx and BNx rats (mean ±SEM, n=5-6) calculated using equation $k=\ln2/t_{1/2}$. Rats administered 100nmol/kg PYY3-36 iv

Creatinine levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma concentration (μmol/L):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>46 ± 2.1</td>
</tr>
<tr>
<td>UNx</td>
<td>56.4 ± 3.4</td>
</tr>
<tr>
<td>BNx</td>
<td>64.8** ± 3.3</td>
</tr>
</tbody>
</table>

Table 3.3. Creatinine levels in sham, UNx and BNx rats at 60 min (mean ±SEM, n=5-6). **p < 0.01 vs. sham (ANOVA with post-hoc Dunnett’s adjustment), n = 6.

3.4.2 Effect of the Zn metalloendopeptidases NEP and meprin β on PYY3-36

3.4.2.1 Dose-response effect of NEP and meprin β on PYY3-36

Following incubation of PYY3-36 with 200ng NEP over 60 min, HPLC analysis revealed 14 ±3 % degradation (n=6). Due to the unexpected low potency of the effect of NEP on PYY3-36, gonadotrophin-releasing hormone (GnRH), a known substrate for NEP, was then utilised as a positive control in an experiment testing the effect of NEP on PYY3-36.
Following incubation of GnRH or PYY3-36 with 200ng NEP, HPLC analysis revealed 60% degradation of GnRH and 9% degradation of PYY3-36 (see fig. 3.6). A further control experiment was carried out in which the effect of NEP on pancreatic polypeptide (PP), a member of the PP-fold family closely related to PYY3-36, was investigated. Following incubation of 2nmol PP or PYY3-36 with 200ng NEP, HPLC analysis revealed 40% degradation of PP and 13% degradation of PYY3-36 (see fig. 3.7). A dose response experiment in which PYY3-36 was incubated with 200ng, 400ng and 800ng NEP over 60 min resulted in 5 ±1%, 24 ±6% and 25 ±0.2% degradation of PYY3-36 respectively (n=3). As a 60 min incubation with meprin β at the same concentration resulted in 100% degradation of PYY3-36, a shorter incubation of 30 min was carried out. Following incubation of PYY3-36 with 200ng meprin β over 30 min, HPLC analysis revealed 57 ± 4% degradation of parent peptide (n=5). A dose-response experiment in which PYY3-36 was incubated with 200ng, 400ng and 800ng meprin β over 30 min resulted in 43 ±9%, 53 ±12% and 74 ±8% degradation of PYY3-36 respectively (n=3). A summary of the dose response study results is shown in table 3.4.
Figure 3.6. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of: A) 2nmol GnRH with 200ng NEP for 60 min at 37°C PYY3-36 and B) 2nmol PYY3-36 with 200ng NEP for 60 min at 37°C. Digests were analysed by HPLC on a 15-60% water/acetonitrile (AcN) gradient over 35 min. Peptide fragments were detected by spectrophotometry at a wavelength of 214nm.
3.4.2.2 Time-dependent effect of NEP and meprin β on PYY3-36

Incubation of PYY3-36 with 200ng NEP resulted in 12 ±2%, 40 ±3% and 50 ±10% degradation following a 60, 120 or 240 min incubation respectively (n=3, see fig. 3.8 for representative example). Incubation of PYY3-36 with 200ng meprin β over 5, 15 or 30 min resulted in 39 ±5%, 41 ±6% and 52 ±10% degradation respectively (n=3; see fig. 3.9 for representative example). A summary of time-course study results is shown in table 3.4.
Figure 3.8. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of PYY3-36 with 200ng NEP over 60 min, 120 min and 240 min at 37°C. Digests were analysed by HPLC on a 15-60% water/acetonitrile gradient over 35 min. Peptide fragments were detected by spectrophotometry at a wavelength of 214nm.

Figure 3.9. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of PYY3-36 with 200ng meprin β over 5, 15, 30 and 60 min at 37°C. Digests were analysed by HPLC on a 20-35% water/acetonitrile gradient over 35 min. Peptide fragments were detected by spectrophotometry at a wavelength of 214nm.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Dose</th>
<th>Incubation time</th>
<th>% degradation</th>
<th>Dose</th>
<th>Incubation time</th>
<th>% degradation</th>
<th>Dose</th>
<th>Incubation time</th>
<th>% degradation</th>
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<tbody>
<tr>
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<td>60</td>
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<tr>
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<td>400ng</td>
<td>120</td>
<td>40 ± 3</td>
<td>400ng</td>
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<td>50 ± 10</td>
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<td>800ng</td>
<td>240</td>
<td>50 ± 10</td>
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<td>5</td>
<td>39 ± 5</td>
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<td>15</td>
<td>41 ± 6</td>
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<td>400ng</td>
<td>15</td>
<td>41 ± 6</td>
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<td>800ng</td>
<td>30</td>
<td>52 ± 10</td>
<td>800ng</td>
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</tr>
</tbody>
</table>

Table 3.4. Summary of mean percentage degradation after PYY3-36 (2nmol) was incubated with NEP (200ng; n=6) or meprin β (200ng; n=7), and after increasing doses or incubation times (dose used: 200ng meprin β/NEP) (n=2-3). Percentage degradation is shown as means ± SEM. Digests were analysed by HPLC on a 20-35% water/AcN gradient over 35 min and percentage degradation calculated using AUC.

3.4.3 Effect of KBB membranes on PYY3-36

KBB was found to have a potent dose and time dependent effect on PYY3-36 breakdown (see fig. 3.10). The KBB incubation protocol was therefore optimised to a concentration and incubation time that reliably resulted in >50% degradation of PYY3-36, so that both parent peptide and peptide fragments could be observed on the chromatogram. A dose response experiment over a fixed incubation time of 30 min using increasing concentrations of KBB (0.625, 1.25 and 2.5mg/ml) was carried out in order to determine a concentration at which 1) >50% of peptide was degraded and 2) break down products could be detected. Subsequently, a concentration of 1.25mg/ml was used in the remainder of peptide/KBB incubations. Incubation samples were then analysed by HPLC as previously described.
Figure 3.10. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with A) 0.62mg/ml, 1.25mg/ml and 2.50mg/ml KBB for 30 min at 37°C and B) .25mg/ml KBB for 5 min, 15 min and 30 min at 37°C. Digests were analysed by HPLC on a 15-60% water/acetonitrile gradient over 35 min. Peptide fragments were detected by spectrophotometry at a wavelength of 214nm.
3.4.4 Characterisation of PYY3-36 peptide fragments

3.4.4.1 NEP and meprin β-induced peptide fragments

After incubation of PYY3-36 with NEP, MALDI-ToF mass spectrometry was utilised to determine the mass of peptide fragments derived from NEP. Putative sequences for peptide fragments were calculated according to the sequence of PYY3-36. Short incubations were utilised in order to determine the first cleavage that occurs upon exposure of the peptide to the enzyme. After a 30 min incubation with NEP, the major peak detected was PYY3-36, which is consistent with the HPLC findings. In addition, a peptide fragment with a MW of 2529 was detected. However, the 2529 fragment did not match a MW that was in accord with the sequence of PYY3-36 and was therefore considered an impurity. Furthermore, this fragment was not observed after PYY3-36 incubation with KBB, in which NEP is highly expressed (Turner et al., 2001). A number of PYY3-36 fragments were observed after incubation of PYY3-36 with meprin β, and appeared in a time-dependent manner. A 5 min incubation with meprin β resulted in molecules with molecular weights corresponding to PYY11-36, while 15 and 30 min incubations both resulted in molecules with molecular weights corresponding to PYY11-36, PYY12-36 and PYY13-36. These fragments suggested the presence of meprin β-cleavage sites at Glu10-Asp11, Asp11-Ala12 and Ala12-Ser13.

3.4.4.2 KBB-induced peptide fragments

A time-course of KBB-induced PYY3-36 degradation was analysed by MALDI-TOF to determine whether the same peptide fragments generated by incubation with meprin β or NEP are evident following incubation of PYY3-36 with KBB. Both 5 and 15 min incubations of PYY3-36 with KBB resulted in molecules with molecular weights corresponding to PYY11-36 and PYY15-36; while a 30 min incubation resulted in PYY11-
36, PYY15-36 and PYY13-36. This was consistent with the cleavage sites Glu10-Asp11 and Ala12-Ser13 as suggested by incubation with meprin β, though a putative cleavage site at Pro14-Glu15 was also observed (see table 3.5 & fig. 3.11).

<table>
<thead>
<tr>
<th>Enzyme/membrane</th>
<th>Incubation time (min)</th>
<th>MALDI-ToF MW</th>
<th>Putative sequence of peptide products</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sequence</td>
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<td></td>
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<td>Glu15-Tyr36</td>
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</table>

Table 3.5. MW of peptide fragments following incubation of PYY3-36 (2nmol) with KBB (1.25mg/ml), meprin β (200ng) over 5, 15 or 30 min. Samples were analyzed by MALDI-TOF mass spectrometry. Putative sequences for peptide fragments were calculated according to the sequence of PYY3-36. Putative cleavage sites consistent for both meprin β and KBB are shown in bold.
3.4.5 Characterisation of a PYY3-36 analogue with an Asp substitution

A PYY3-36 analogue was designed with an Asp at position 10 in order to investigate whether an extra putative cleavage site for meprin-β would increase the susceptibility of PYY3-36 to breakdown by meprin β.

3.4.5.1 Meprin β-induced peptide fragments

A 5 min incubation of PYY3-36 Asp10 with meprin β resulted in molecules with molecular weights corresponding to PYY10-36, PYY11-36, PYY12-36 and PYY13-36; a 15 min incubation resulted in molecules with molecular weights corresponding to PYY10-36, PYY11-36, PYY12-36, PYY13-36 and PYY15-36 and a 30 min incubation resulted in molecules with molecular weights corresponding to PYY10-36, PYY11-36, PYY12-36 and PYY13-36 (see table 3.5). Incubation of PYY3-36 Asp10 resulted in peptide fragments that were not found after meprin β was incubated with PYY3-36. These included molecules with molecular weights corresponding to PYY10-36 and PYY15-36. Furthermore, molecules with molecular weights corresponding to PYY12-36 and PYY13-36 appeared at 5 min when PYY3-36 Asp10 was the substrate for meprin β; whereas these were only detected at 15 min when PYY3-36 was the substrate for meprin β (see table 3.6 & fig. 3.11).

3.4.5.2 KBB-induced peptide fragments

A 5 min incubation of PYY3-36 Asp10 with KBB resulted in molecules with molecular weights corresponding to PYY10-36, PYY11-36, PYY12-36, PYY13-36 and PYY15-36; while a 15 and 30 min incubation resulted in molecules with molecular weights corresponding to PYY10-36, PYY11-36, PYY12-36, PYY13-36 (see table 3.5). These peptide fragments are consistent with all meprin β-induced cleavage sites on PYY3-36 Asp10 at Gly9-Asp10, Asp10-Asp11, Asp11-Ala12, Ala12-Ser13 and Pro14-Glu15, though
the corresponding peptide fragments appeared at different times during incubation with meprin β (see table 3.6 & fig. 3.11). Incubation of PYY3-36 Asp10 resulted in peptide fragments that were not found after KBB was incubated with PYY3-36. These included molecules with molecular weights corresponding to PYY10-36 and PYY12-36. Furthermore, molecules with molecular weights corresponding to PYY11-36 and PYY13-36 appeared at 5 min when PYY3-36 Asp10 was the substrate for meprin β, whereas this only found after 15 min and 30 min respectively when PYY3-36 was the substrate for meprin β (see table 3.6 & fig. 3.11).
Table 3.6. MW of peptide fragments after PYY3-36-Asp10 (2nmol) was incubated with KBB (1.25mg/ml), meprin β (200ng) or NEP (200ng) over 5, 15 or 30 min. Samples were analysed by MALDI-TOF mass spectrometry. Putative sequences for peptide fragments were calculated according to the sequence of PYY3-36. Putative cleavage sites consistent for both meprin β and KBB are shown in bold.

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<th>Enzyme/membrane</th>
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Figure 3.11. Schematic showing cleavage sites for meprin β and KBB on A) PYY3-36 and B) PYY3-36 Asp10. Blue lines indicate cleavage sites found during PYY3-36 breakdown, while red lines indicate cleavage sites when an Asp substitution is made at position 10.
3.4.6 Effect of NEP and meprin β inhibition on PYY3-36 degradation in vitro

3.4.6.1 Effect of NEP and meprin β inhibition on KBB-induced PYY3-36 degradation

Subsequently the effect of the NEP inhibitor phosphoramidon on PYY3-36 degradation by KBB membranes was tested. KBB membranes express NEP at high concentrations, and were used as in situ model for meprin β activity (Sterchi et al., 2008). The NEP inhibitor phosphoramidon (10nmol) prevented NEP-induced degradation of PYY3-36 by 95 ±5% and the accumulation of peptide fragments in vitro (n=2, see fig 3.12). Pre-treatment with phosphoramidon (100nmol) prevented KBB-induced PYY3-36 degradation by approximately 36 ±13% (n=2, see fig. 3.13). In this experiment, a second peak was observed corresponding to phosphoramidon. Since this peak was observed in a control run in which phosphoramidon was incubated with KBB (but not PYY3-36) at the same doses, it is likely that phosphoramidon itself is hydrolysed by KBB. The effect of the meprin β inhibitor actinonin on PYY3-36 degradation by KBB membrane was investigated. The meprin inhibitor actinonin prevented meprin β-induced degradation of PYY3-36 by 96 ±2% degradation in vitro (n=3; see fig. 3.14) and the subsequent accumulation of PYY11-36, PYY12-36 and PYY13-36 as determined by mass spectrometry. Pre-treatment with actinonin (100nmol) prevented KBB-induced PYY3-36 degradation by approximately 100% (n=3, see table 3.7). We also investigated whether the inhibition of meprin B within KBB prevents the accumulation of the peptide fragments observed using mass spectrometry. Incubation of actinonin (100nmol) with KBB and PYY3-36 over 15 min prevented cleavage at Glu10-Asp11 and the subsequent appearance of PYY11-36, but did not prevent the appearance of PYY15-36. However, as MALDI-ToF is not a quantitative technique, this result did not confirm that inhibition of meprin β-induced degradation results in a reduction in the amount of a specific peptide fragment.
Figure 3.12. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with 200ng NEP or 200ng NEP & 10nmol phosphoramidon for 30 min at 37°C. 200ng NEP only and 10nmol phosphoramidon alone were run as controls. Digests were analysed by HPLC on a 15-60% water/acetonitrile gradient over 35 min. Peptide fragments were detected by spectrophotometry at a wavelength of 214nm.

Figure 3.13. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with 1.25mg/ml KBB or 1.25mg/ml KBB & 100nmol phosphoramidon for 30 min at 37°C. 1.25mg/ml KBB only and 100nmol phosphoramidon alone were run as controls. Digests were analysed by HPLC on a 15-60% water/acetonitrile gradient over 35 min. Peptide fragments were detected by spectrophotometry at a wavelength of 214nm.
Figure 3.14. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with 200ng meprin β or 200ng meprin β & 10nmol actinonin for 30 min at 37°C. 200ng meprin β only and 10nmol actinonin only were run as controls. Digests were analysed by HPLC on a 20-35% water/acetonitrile gradient over 35 min (5-40 min). Peptide fragments were detected by spectrophotometry at a wavelength of 214nm.

Figure 3.15. Representative reverse-phase HPLC absorption profile following *in vitro* digestion of 2nmol PYY3-36 with 1.25mg/ml KBB or 1.25mg/ml KBB with 50/100nmol actinonin for 30 min at 37°C. 1.25mg/ml KBB only, 50nmol and 100nmol actinonin only were run as controls. Digests were analysed by HPLC on a 20-35% water/acetonitrile gradient over 35 min (5-40 min). Peptide fragments were detected by spectrophotometry at a wavelength of 214nm.
3.4.7 Effect of NEP and meprin β inhibition on PYY3-36 bioactivity

3.4.7.1 Effect of phosphoramidon on PYY3-36 bioactivity in vivo

In order to determine the role of NEP in the clearance and subsequent bioactivity of PYY3-36 in vivo phosphoramidon (20mg/kg) was subcutaneously co-administered with PYY3-36 (50nmol/kg) to fasted mice and the effect on food intake measured at 1h, 2h, 3h, 4h and 8h (n=10, see fig. 3.16). PYY3-36 significantly reduced food intake at 0-1h and 1-2h post-injection. PYY3-36 did not alter food intake significantly at any other time point observed. PYY3-36 co-administered with phosphoramidon significantly reduced food intake 0-1h, 1-2h, 2-3h and 2-4h post-injection. At 4-8h there were no significant differences between groups. Phosphoramidon alone significantly reduced food intake at 1-2h post-injection (see fig. 3.16).

Figure 3.16. Effect of phosphoramidon (20mg/kg; sc) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and phosphoramidon alone (20mg/kg, sc) on food intake at 0-1h, 1-2h, 2-3h, 4-8h and 8-24h post-injection. Results are means ± SEM. ***p < 0.001 vs. saline, **p < 0.01 vs. saline, *p < 0.05 vs. saline (ANOVA with post-hoc Tukey’s multiple comparison adjustment), n = 10.
PYY3-36 significantly reduced cumulative food intake over 8h post-administration. PYY3-36 co-administered with phosphoramidon significantly reduced cumulative food intake over 8h post-administration. Phosphoramidon significantly reduced cumulative food intake over 2 h post-administration [0-2 h food intake (g) 1 ±0.07 (saline), 0.8 ±0.06 (phosphoramidon) (p<0.05 vs saline), data not shown].

Figure 3.17. Effect of phosphoramidon (20mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and phosphoramidon (20mg/kg; ip) alone on cumulative food intake over 8h. Results are means ± SEM. **p<0.01 PYY3-36 & phosphoramidon vs saline, ***p<0.001 PYY3-36 and PYY3-36 & phosphoramidon vs. saline (General Estimating equation (GEE) using Mann-Whitney U test for equal variances), n = 10.
Figure 3.18. Effect of phosphoramidon (20mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and phosphoramidon (20mg/kg; ip) alone on cumulative food intake over 8h. Results are means ± SEM. **p<0.01 PYY3-36 or PYY3-36 & phosphoramidon vs saline (GEE using Mann-Whitney U test for equal variances), n = 10.

3.4.7.2 Effect of actinonin on PYY3-36 plasma levels in vivo

The effect of actinonin on PYY3-36 plasma levels was tested in mice that were fasted for four hours prior to the study. At 0, 20, 60 and 90 min post-administration, 50nmol/kg actinonin had no effect on PYY3-36 IR, with basal circulating levels (<100pmol/L) found at all time points observed (see fig. 3.19). At 0 min, actinonin administered with PYY3-36 resulted in a trend towards an increase in PYY3-36 IR compared to controls administered PYY3-36 only. At 20 and 60 min, mice administered actinonin with PYY3-36 had significantly higher circulating levels of PYY3-36 IR. At 90 min post-administration there were no significant differences between PYY3-36 and PYY3-36 & actinonin treatment groups.
Figure 3.19. Effect of actinonin (20mg/kg, ip) on plasma PYY1-36/PYY3-36 IR at 20, 60 and 90 min after PYY3-36 injection (100nmol/kg, sc) in mice. Actinonin (20mg/kg) administered as a control. Results are means ± SEM. **p < 0.001 vs PYY3-36 (ANOVA with post-hoc Dunnett’s adjustment), n = 8-12.

3.4.7.3 Effect of actinonin on PYY3-36 induced bioactivity in vivo

To determine the role of meprin β in the clearance and subsequent bioactivity of PYY3-36 in vivo, actinonin (20mg/kg or 40mg/kg) was subcutaneously co-administered with PYY3-36 (50nmol/kg) to fasted mice and the effect on food intake measured at 1h, 2h, 3h, 4h and 6h post-injection (n=8-12, see fig. 3.20). PYY3-36 significantly reduced food intake at 0-1h and 1-2 h post-injection. PYY3-36 co-administered with 20mg/kg actinonin significantly reduced food intake at 0-1h, 1-2h and 2-4h post-injection. PYY3-36 administered with 40mg/kg actinonin significantly reduced food intake at 0-1h, 1-2h, 2-3h and 2-4h post-injection. There were no significant differences between groups at 4-6h post-injection.
Figure 3.20. Effect of actinonin (20 or 40mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and actinonin (20 or 40mg/kg; ip) on food intake at A: 0-1h, B: 1-2h, C: 2-4h, and D: 4-6h post-injection. Results are means ± SEM. *p < 0.05 vs. saline, **p < 0.01 vs. saline, ***p < 0.001 vs. saline (ANOVA with post-hoc Tukey’s multiple comparison adjustment), n =10.

PYY3-36 administered alone and PYY3-36 administered with actinonin at both doses significantly reduced cumulative food intake over 6h compared to saline. Interestingly, PYY3-36 administered with 40mg/kg actinonin significantly reduced food intake compared to PYY3-36 administered alone. Actinonin administered at 20mg/kg or 40mg/kg had no effect on food intake at any interval, but significantly reduced cumulative 0-6h food intake (see fig. 3.21 & 3.22).
0-6h food intake: ***p<0.001 PYY3-36 & actinonin (both doses) vs saline, $p<0.05$ PYY3-36 & 40mg/kg actinonin vs PYY3-36, **p<0.01 20mg/kg actinonin vs saline, *p<0.05 40mg/kg actinonin vs saline.

Figure 3.21. Effect of actinonin (20 or 40mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and actinonin (20 or 40mg/kg; ip) alone on cumulative food intake over 6h. Results are means ± SEM. *p<0.05 40mg/kg actinonin vs saline, **p<0.01 20mg/kg actinonin vs saline, ***p<0.001 PYY3-36, PYY3-36 & 20mg/kg actinonin, PYY3-36 & 40mg/kg actinonin vs saline, #p<0.05 PYY3-36 & 40mg/kg actinonin vs PYY3-36 (GEE with Mann-Whitney U test for equal variances), n = 10.
Figure 3.22. Effect of actinonin (20 or 40mg/kg; ip) co-administered with PYY3-36 (50nmol/kg; sc), PYY3-36 alone (50nmol/kg; sc) and actinonin (20 or 40mg/kg; ip) alone on cumulative food intake over 6h. Results are means ± SEM. *p<0.05 40mg/kg actinonin vs saline, **p<0.01 20mg/kg actinonin vs saline, ***p<0.001 vs saline, #p<0.05 vs PYY3-36 (GEE with Mann-Whitney U test for equal variances), n = 10.

3.5 Discussion

PYY3-36 is thought to be a key hormone in the regulation of satiety, and its role in appetite and obesity has been widely studied (Batterham et al., 2002; Chaudhri et al., 2008; Boggiano et al., 2005; Small and Bloom, 2005; Chandarana and Batterham, 2008). These studies aimed to exploit its anorectic effects through the development of long-acting protease-resistant PYY analogues and/or the use of specific enzyme inhibitors to prolong the half-disappearance time of native PYY3-36. In order to develop this strategy it is first necessary to understand the biological mechanisms of the inactivation and processing of PYY3-36. Identifying the enzymes which catalyse the physiological breakdown of PYY3-36 will aid the future development of analogues resistant to these peptidases. The kidney is thought to be a major proteolytic centre for other regulatory peptides such as atrial natriuretic peptides and endothelins (Medeiros and Turner, 1994). Study of the kidney is also relevant to PYY analogue development since subcutaneously administered peptide is likely to bypass hepatic ‘first pass’ metabolism and will therefore be subject to renal metabolism (Fauchere JL and Thurieau, 1992). Zn metalloendopeptidases that degrade regulatory peptides, such as NEP and the meprins are concentrated on the KBB within the proximal convoluted tubule. These enzymes are thought to allow sequestration of amino
acids from the glomerular filtrate and also the inactivation of peptides before reabsorption (Carone et al., 1984). As these enzymes are mostly ubiquitously expressed plasma membrane peptidases, but are concentrated in microvilli within the glomerular tubule, the kidney brush border membrane is a useful model to study protease degradation for drug development (Fauchere JL and Thurieau, 1992). Therefore knowledge of the renal enzymes important in the inactivation of PYY3-36 might help to identify specific cleavage sites which can then be altered allowing development of protease-resistant analogues. This work aimed to study the effects of cell-surface metalloendopeptidases expressed in the KBB on PYY3-36 breakdown.

Rat models of acute unilateral and bilateral kidney failure, which exhibited the expected rise in creatinine levels (a marker of kidney failure), showed a reduction in the removal of intact PYY3-36 from the circulation. PYY/PYY3-36 radioimmunoassay revealed a significant increase in plasma PYY3-36 IR at 30 min post-injection of PYY3-36. The radioimmunoassay used detects both PYY and PYY3-36. In order to ensure that the quantification of PYY3-36 was not confounded by differences in endogenous circulating PYY, the animals were fasted prior to the study, which reduces circulating PYY3-36 (Adrian et al., 1985). Since a bolus dose of PYY3-36 was given iv, it is extremely likely that PYY3-36 is the form of PYY-immunoreactivity detected by the radioimmunoassay in these plasma samples. Bilateral nephrectomy resulted in a significant percentage increase in PYY3-36 plasma levels at 15 and 30 min post-injection. However, by 60 min, PYY3-36 levels were reduced in bilaterally-nephrectomised rats to levels observed in sham-operated rats. This suggests that the kidney is not the sole anatomical site for PYY3-36 removal. As a relatively small peptide, PYY3-36 is likely to be subject to glomerular filtration and processing by peptidases within the brush borders of the proximal tubule for sequestration of amino acids (Carone et al., 1984). It is likely that iv-administered peptide is also subject to degradation by ubiquitous plasma membrane peptidases expressed in subcutaneous tissue, and in the epithelial cells of the blood vessel walls (Fauchere JL and Thurieau, 1992).
To study this further, I investigated the effects of purified plasma membrane Zn metalloendopeptidases previously implicated as potential candidates in PYY degradation. NEP was not as effective at degrading PYY3-36 as it was for the positive controls GnRH and PP, suggesting it may not be a key player in degradation of intact PYY3-36. Increasing the dose of NEP resulted in slight, but not marked increases in the degradation of PYY3-36, with 2-fold and 4-fold increases in enzyme concentration only resulting in a less than 10% increase in degradation. NEP appeared to be more effective in degrading PYY3-36 after longer incubation times. A time-course of NEP incubation with PYY3-36 revealed a marked increase in degradation between 2 and 4 hours, as at 2 hours NEP resulted in 15% degradation of PYY3-36 whereas at 4 hours 62% degradation was observed. After 4 hours a concomitant increase in the number of peaks that eluted at earlier time points could also be observed, suggesting an increase in the number of breakdown products. My findings contrast with those of Medeiros & Turner, who found a similar concentration of NEP resulted in an almost complete (88%) hydrolysis of PYY1-36 in two hours. This may be explained by differences in the enzyme; my studies used a commercially-available recombinant human NEP and those of Medeiros & Turner used NEP isolated from pig cortices (Medeiros and Turner, 1994). Additionally, PYY1-36 and PYY3-36 exhibit markedly different 3D conformations, and the tertiary structure of PYY1-36 may be more prone to NEP-induced catabolism, though this remains to be investigated.

In order to clarify the validity of these findings, the role of NEP in PYY3-36 degradation in situ and in vivo was investigated using the specific NEP inhibitor, phosphoramidon (Ki=2nM) (Bertenshaw et al., 2001). The fact that phosphoramidon partially prevents degradation of PYY3-36 by KBB membrane suggested a potential physiological role for NEP in the degradation of PYY3-36. However, mass-spectrometry analysis did not reveal any viable peptide fragments after incubation with NEP, which provided a similar result to incubation of PYY3-36 with NEP incubation, ie. only very few breakdown products could be detected. Phosphoramidon partially prevented degradation of PYY3-36 by KBB membranes, which led me to investigate whether phosphoramidon is able to prolong the anorectic effect of PYY3-36 in vivo. Both PYY3-36 and PYY3-36/phosphoramidon-treated groups exhibited a reduction in food intake over 8 hours. Interestingly, mice co-
administered phosphoramidon with PYY3-36 ate significantly less in the 2-3 hour and 2-4 hour intervals post-injection compared to saline-treated mice, while mice treated with PYY3-36 did not exhibit a reduction in food intake at these intervals. However, animals treated with phosphoramidon alone also exhibited a reduction in food intake, though only during the 2nd hour post injection. During the refeeding period, phosphoramidon may prevent the degradation of other endogenous anorectic gut hormones such as GLP-1 (Plamboeck et al., 2005) or PP (Minnion et. al., unpublished findings). Taken together, these results suggest that NEP does not readily breakdown PYY3-36 *in vitro*, yet inactivation of NEP slightly prolongs the effect of PYY3-36 on food intake. Considering the fact that NEP has a predominantly time-dependent effect on PYY3-36 *in vitro* and minimal effects on PYY3-36 degradation *in vivo*, it is possible that NEP may be important in sequential cleavage of bioactive fragments of PYY3-36. In accord with a role for NEP in such sequential cleavage reactions, a recent study showed that NEP will only cleave the natriuretic hormone, B-type natriuretic peptide (BNP) after it has been metabolised by meprin A (Pankow et al., 2007).

Unlike NEP, human recombinant meprin β has a potent dose- and time-dependent effect on PYY3-36. A previous study suggested that meprin B cleaves PYY at Glu10-Asp11 (Bertenshaw et al., 2001). Since meprin B is a homologue of meprin β subunits, it is likely that these subunits would behave identically *in vivo*. I undertook further investigations using different time points, which revealed that PYY3-36 is susceptible to meprin β cleavage at a number of sites. The first site at which meprin β cleaves PYY3-36 was Glu10-Asp11, followed by Asp11-Ala12 and Ala12-Ser13. This is consistent with the specificity of the active site of meprin B, which prefers acidic amino acids in positions near and adjacent to its cleavage site (Bertenshaw et al., 2001). Peptide fragments found after incubation of KBB with PYY3-36 were consistent with those found after incubation with meprin β, but not with the breakdown product found after incubation with NEP. PYY11-36 and PYY13-36 were the common products for all time points observed, while PYY13-36 was found at the 30 min incubation time point. An analogue with an Asp substitution at position 10 was used to determine whether the introduction of another Asp would increase the number of meprin β specific cleavage sites on PYY3-36, since Asp is highly preferred.
by the active site of meprin β (Bertenshaw et al., 2001). Furthermore, since Glu is the native amino acid at position 10, it could be assumed that an Asp substitution would not severely alter the tertiary structure of PYY3-36, and therefore its general ability to interact with an enzyme. Meprin β cleaved PYY3-36 Asp10 at Gly9-Asp10 which was also observed after incubation of PYY3-36 Asp10 with KBB. All peptide fragments found after meprin β incubation were also found after KBB incubation, suggesting that endogenous meprin β behaves similarly to recombinant meprin β in vitro. Meprin β also cleaves PYY3-36 Asp10 at the sites on which it cleaves PYY3-36, but the associated peptide fragments, PYY12-36 and PYY13-36 were observed more readily, suggesting that Asp is also beneficial for the steric requirements of meprin β at positions upstream and downstream of the P1’ position. This data supports further investigation into whether substitution of negatively charged amino acids at key sites within PYY3-36 may protect against meprin β-induced degradation.

The meprin β inhibitor actinonin (Ki 1.7µM) completely prevented PYY3-36 degradation by KBB in vitro, which implies that inhibition of this enzyme within KBB membranes prevents the ability of KBB to breakdown PYY3-36. This data is unsurprising considering that meprin expression in the kidney brush borders is second only to actin in abundance (Sterchi et al., 2008). The degradation of PYY3-36 by KBB can therefore be viewed as a model for meprin β-induced degradation. These findings are in contrast to the fact that phosphoramidon only resulted in partial prevention of PYY3-36 degradation by KBB and are in line with the results of the dose-response and time course studies, which showed that meprin β was more effective in degrading PYY3-36 in vitro. The inhibition of meprin α by actinonin is unlikely to play a role in KBB-induced degradation of PYY3-36 as the enzyme meprin A, a homooligomer of meprin α subunits, has not been reported to cleave PYY in vitro (Bertenshaw et al., 2001). In vivo, meprin α is secreted as a homooligomer but is also found as a heterooligomer in association with meprin β in the plasma membrane of the apical brush border of the renal epithelium. The role of meprin α heterooligomers in PYY3-36 degradation remains to be fully clarified, and it is feasible that meprin α/β heterooligomers could also account for KBB-induced degradation of PYY3-36. Until analogues of actinonin that are able to differentially target α and β subunits are developed, this
objective remains unfeasible (Kruse et al., 2004). Nevertheless, since the use of actinonin in characterizing the role of meprins in vivo is recognized by other laboratories and actinonin does not inhibit any known enzymes able to cleave PYY3-36, the role of meprin β in PYY3-36 degradation was further investigated (Sterchi et al., 2008).

The fact that KBB-mediated PYY3-36 degradation is highly sensitive to the actions of actinonin prompted further investigation into the role of meprin β in the in vivo bioactivity of PYY3-36. The effect of actinonin on PYY3-36 plasma levels was investigated in order to determine whether meprin β may play a role in PYY3-36 degradation in vivo. Mice administered actinonin with PYY3-36 had significantly higher plasma PYY levels at 20 and 60 min compared to mice administered PYY3-36 alone. Since dual administration of actinonin and PYY3-36 resulted in an increase in PYY-like immunoreactivity in mice, we hypothesized that meprin β inhibition could prolong the effect of PYY3-36 on food intake. Subsequently, we found that dual actinonin/PYY3-36 administration prolonged the anorectic effect of PYY3-36 between 2-4 hours post-injection, as PYY3-36 alone was no longer effective at reducing food intake after 2 hours and actinonin administered alone had no effect on feeding at this time point. Interestingly, mice treated with PYY3-36 and 40mg/kg actinonin showed a significant reduction in cumulative food intake over 6 hours compared to mice administered PYY3-36 only, which suggests that actinonin may have potential in alleviating the degradation of PYY3-36 and enhancing its bioactivity. Actinonin administered on its own resulted in a trend towards a reduction in food intake at the later time point (4-6h) and a significant decrease in cumulative food intake over 6 h compared to saline, which may reflect the elevation of endogenous postprandial anorectic gut hormones by actinonin, which would peak shortly after refeeding. The fact that actinonin allows PYY3-36 levels to remain elevated for longer may explain the dual effect of actinonin and PYY3-36 on food intake. However, the effect of actinonin on re-feeding induced release of PYY3-36, as well as other anorectic gut hormones, including GLP-1, OXM and PP, needs to be further investigated to determine whether actinonin may be useful in manipulating circulating levels of gut hormones.
Taken together, these findings support a potential role for meprin β in the degradation of PYY3-36, and provide impetus for further investigation into using meprin inhibitors as a therapeutic intervention to prolong the half-life of PYY3-36 and/or designing meprin-resistant PYY3-36 analogues.
Chapter Four: Development of PYY3-36 analogues: modifications to 3D structure and use of a metal ion delivery system to promote slow release
4.1 Introduction

4.1.1 Evidence for therapeutic potential of PYY3-36

As discussed in Chapter 2, mimicking the chronic elevation of circulating PYY achieved by bariatric surgery is a potential therapeutic approach for obesity (Korner et al., 2005; Le Roux et al., 2006a). A number of studies in humans have explored the therapeutic potential of PYY3-36 administration in reducing appetite, and have drawn a number of conclusions. Batterham and co-workers reported that iv infusion of PYY3-36 in normal-weight human volunteers reduced caloric intake by 36% during a buffet meal presented 2 hours post-infusion, compared to saline-infused volunteers. Food diaries indicated that PYY3-36 significantly reduced cumulative food intake by 33% compared to saline controls over 24h post-infusion (Batterham et al., 2002). The dose used in this study, 0.8pmol/kg/min over 90 min (eg. 5040pmol in total for a 70kg man), resulted in plasma levels of around 43.5pmol/L over 30 min post-infusion, which is similar to the postprandial PYY3-36 levels of approximately 50pmol/L as reported by Grandt et al (Grandt et al., 1992). These results therefore suggested that physiological levels of PYY3-36 achieved during an infusion can inhibit cumulative food intake over 24h. However, PYY3-36 is quickly degraded when administered iv, with plasma levels returning to basal after 30 min post-infusion to humans. This is in line with the reported half-life of PYY3-36, which is 11 min in the dog and 8 min in the rat (Lluis et al., 1989; Pappas et al., 1985). A study by Le Roux and co-workers examining the effects of a 90 min infusion of graded doses of PYY3-36 (0.2, 0.4, 0.5, 0.6, 0.7 and 0.8 pmol/kg/min), found a significant correlation between dose and plasma levels of PYY3-36, and a dose-dependent reduction in caloric intake in normal weight subjects (Le Roux et al., 2006b). A similar study was carried out in normal weight volunteers, assessing the effects of 0.2, 0.4 and 0.8pmol/kg/min infused over 120 min on appetite and food intake. This study suggested that the low dose produced plasma levels (approximately 50pmol/L) within the physiological range and had little effect on food intake, but the higher doses resulted in increased, pharmacological plasma levels (around 136pmol/L for the 0.8pmol/kg/min dose) of PYY3-36, which is in contrast to what was previously reported in the two previous studies. The authors found that 0.8pmol/kg/min PYY3-36 resulted in a
32% reduction in caloric intake. However, this dose also resulted in an increase in adverse events, including abdominal discomfort, nausea and vomiting (Degen et al., 2005). Similarly, Le Roux and co-workers showed that iv infusion of PYY3-36 (1pmol/kg/min over 90 min), which resulted in supraphysiological plasma concentrations of PYY3-36 (up to 140pmol/L), resulted in nausea in 5 out of 6 normal-weight subjects, without any further reduction in caloric intake over that observed using lower doses (Le Roux et al., 2008). In agreement with the other PYY3-36 human infusion studies, Sloth and colleagues found that a 0.2 pmol/kg/min infusion of PYY3-36 had no effect on food intake and no adverse effects in normal weight and obese volunteers. A dose of 0.8 pmol/kg/min PYY3-36 resulted in peak plasma levels of around 160pmol/L and significantly reduced caloric intake by around 19% compared to controls. However, this infusion also resulted in adverse effects including nausea and abdominal discomfort, though these were not reported when the test meal was received. The similar doses of PYY3-36 appear to result in variable plasma levels achieved by different laboratories, which may be due to differences in study protocol, subjects or plasma hormone assays. However, the fact that both studies achieving higher plasma levels of PYY3-36 report adverse effects in their volunteers suggests that supraphysiological PYY3-36 plasma levels should be avoided in the development of PYY3-36 as a therapeutic agent (Sloth et al., 2007b).

Batterham and co-workers were first to report that PYY significantly reduced food intake in obese subjects. They found that a dose of 2nmol/kg mean surface area reduced food intake by around 30% after a 90min infusion (which produced plasma levels of around 57pmol/L) and significantly reduced 24h cumulative food intake (Batterham et al., 2003a). Whether PYY circulates at lower levels in the obese, and whether obese patients exhibit a blunted postprandial rise in PYY is controversial. A summary of observed fasting and postprandial PYY plasma levels in the lean and obese is summarised in table 4.1. Interestingly, in obese male volunteers, graded doses of PYY3-36 (25-100pmol/kg lean body mass) injected daily via the subcutaneous route over 5 days significantly decreased satiety and hunger scores without causing nausea, but did not significantly reduce food intake. The protocol followed in this study, which was to gradually increase the dose of PYY3-36 over 5 days, may promote tolerance to PYY3-36 (Sloth et al., 2007a). It is also possible that the doses used in
this study were insufficiently high to elicit a significant reduction in food intake. Merck Research Laboratories investigated the effects of intranasal administered PYY3-36 to obese patients 3 times daily over 12 weeks, at doses of 200µg or 600µg dose, achieving maximal plasma levels of around 43pmol/L and 106pmol/L respectively. Although the low dose group exhibited a mean weight loss of around 2kg over 12 weeks, 29.5% and 9.1% of the patients reported at least one episode of nausea and vomiting respectively, while 70% of the patients in the 600µg group discontinued the study due to adverse effects (Gantz et al., 2007). In general, low doses of PYY3-36 do not cause nausea or substantially inhibit food intake, whereas doses producing higher levels of PYY3-36 inhibit food intake and result in acute adverse effects. Therefore, although PYY3-36 is promising in terms of its ability to reduce appetite in obese humans, it is evident that its therapeutic window is extremely narrow, and is likely to be sensitive to a variety of factors.

I therefore hypothesized that developing an analogue with a more long-lasting effect on food intake, and that could be administered in a way that would allow slower, more sustained release, may prevent the side effects associated with PYY3-36 administration. Developing an analogue in such a way may allow lower doses to be used that circumvent the nausea threshold, but still achieve a pharmacological effect at Y2R. Activation of biological pathways that elicit nausea may decline when PYY3-36 is administered chronically, particularly if its appearance in the circulation is slow and sustained. For example, in a 20 week drug trial, although sc administration of the GLP-1 analogue liraglutide resulted in nausea, 80% of nausea events occurred in the first 4 weeks of the study, and declined over the remaining 16 weeks. This behavioural tolerance to nausea may be aided by the stable, steady levels of circulating liraglutide achieved due to its markedly increased half-life of about 13h (compared to the 1-2 min half-life of GLP-1) (Astrup et al., 2009).
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<th>Author &amp; Publication date</th>
<th>Obesity Fasted</th>
<th>Obesity Post-prandial</th>
<th>Lean Fasted</th>
<th>Lean Post-prandial</th>
<th>Bariatric surgery Fasted</th>
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<td>(Batterham et al., 2003a)</td>
<td>10.2 ±0.7</td>
<td>14.4 ±1.2</td>
<td>16.9 ±0.8</td>
<td>23.5 ±0.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Le Roux et al., 2006b)</td>
<td>7.1 ±1.3</td>
<td>-</td>
<td>9.3 ± 0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Pfluger et al., 2007)</td>
<td>10.8 ±0.9</td>
<td>-</td>
<td>12.9 ±1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Le Roux et al., 2006a)</td>
<td>13.8 ±7.1</td>
<td>16 ±8.1</td>
<td>15.4 ±8.3</td>
<td>23.7 ±9.5</td>
<td>18.5 ±5.2</td>
<td>40.4 ±7.8</td>
</tr>
</tbody>
</table>

Table 4.1. Summary PYY3-36 levels (pmol/L) in either fasted (12h overnight fast) or postprandial (30 min after a buffet meal for Batterham et al 2003., and 90 min after a 420 calorie meal for Le Roux et al. 2006) obese and lean patients, and in obese patients that had undergone Roux-En-Y gastric banding (RYGB) surgery 6-36 months before the study. Data is included from four separate studies. Only data from studies which quoted values for plasma levels are included. Conversion from metric units (pg/ml) to Systeme International units (pmol/L) was performed using the conversion factor 0.25 for PYY.

4.1.2 Development of a PYY3-36 analogue with an altered α-helix

The α-helix is the most common motif in protein secondary structures and is involved in the folding and stability of peptide/protein structures, and thus bioactivity. For example, the α-helical C-terminal region of GLP-1 binds and activates GLP-1R (Adelhorst et al., 1994; Runge et al., 2007). Exendin-4, a hormone found in the saliva of the Gila monster binds and activates GLP1-R with higher affinity and potency respectively. The central region of
exendin-4 (residues 11-27), which shares four identical residues with GLP-1, adopts an α-helical secondary structure which is important in the enhanced GLP1-R bioactivity of exendin-4 (Goke et al., 1993; Runge et al., 2008; Runge et al., 2007). Interestingly, these α-helical regions in exendin-4 and GLP-1 share homology with an α-helical region in latrotoxin (LT), which is a protein isolated from the venom of the black widow spider, *Latroductus tredecimguttatus*. LT is a toxin that causes spontaneous discharge of neurotransmitters from motor and sensory neurones, resulting in latrotoxism, which involves neuromuscular pain and cardiovascular symptoms in humans following *Latroductus* spider bites (Ushkaryov, 2002). LT also has ‘GLP-1-like’ effects on the endocrine system, which include increasing intracellular calcium (Ca\(^{2+}\)), and initiating insulin exocytosis (Holz and Habener, 1998; Davletov et al., 1998; Lang et al., 1998). Residues 970-981 of LT, which are found in the hypothesized GLP-1R binding domain, share considerable homology with residues 17-28 of the α-helical region of Exendin-4 (Holz and Habener, 1998). This region of LT is also found in the latroinsectotoxin (LIT) protein, which is another protein derived from Latroductus, but which is able to stimulate neurotransmitter release at insect neuromuscular junctions (Holz et al., 2000). A chimeric GLP-1 peptide, in which residues 17-28 were substituted with the α-helical region of residues 970-981 of LT, maintained the ability of endogenous GLP-1 to stimulate an increase in intracellular Ca\(^{2+}\) concentration and insulin secretion from human β-cells and insulinoma cells. This suggests that the homologous α-helical epitopes within Exendin-4, GLP-1 and LT are important in their bioactivity, possible through the stabilising effects of this α-helical region. Subsequently, I chose to investigate whether substitution of residues 16-23 of the α-helix of PYY3-36 with a combination of residues from LT (970-981) and LIT (952-962), to create the analogue PYY3-36-αLT, would affect the bioactivity of PYY3-36 in vivo. The affinity of PYY3-36αLT at the Y2R, the effects on food intake and the clearance of this peptide were therefore examined.

4.1.3 Use of a metal ion delivery system in developing a slow-release administration regime for PYY3-36 analogues

PYY3-36 is soluble at neutral physiological pH (around pH7), which is the pH found in the subcutaneous space and blood, both potential sites for PYY3-36 and PYY3-36 analogue
The isoelectric point of a peptide is the pH at which the peptide has no net charge and, as a result, is more likely to precipitate out of solution. Altering the solubility of a peptide by modifying its isoelectric point, for instance through increasing the number of charged residues in its amino acid sequence, can allow the peptide to precipitate in vivo. This precipitation out of solution may increase the duration of action of the peptide, by increasing the time it takes for a peptide to fully dissolve and enter the circulation. For instance, a peptide administered sc may exhibit slow, sustained release into the circulation if it has an isoelectric point near physiological pH, due to the formation of a subcutaneous depot of precipitated peptide. This method has been used in the development of the long-acting insulin analogue glargine, which has two positively charged Arg amino acids introduced to the β chain causing a shift in the isoelectric point from 5.4 to 6.7. This results in precipitation at the site of injection, and a steady, sustained, ‘peakless’ release of insulin glargine in humans (Lepore et al., 2000).

Another method that utilizes a similar concept is the administration of the peptide in combination with a metal salt solution. Zn (Zn) salts are most commonly used and are able to prolong the duration of bioactivity of several peptides (HOMAN et al., 1954; Gietz et al., 2000; Brange and Langkjaer, 1997). Zn ions are able to bind to charged His residues to form a Zn-peptide precipitate complex. Zn, and the other transition metals, copper and nickel have also been shown to complex with luteinizing hormone-releasing hormone (LHRH) at physiological pH mainly by binding to its His residue (Bal et al., 1989; Gerega et al., 1988). Zn has been used in the development of a number of pharmaceutical insulin formulations. Interestingly, Zn may play a physiological role in insulin storage. Within β-cells of the pancreas, the insulin precursor molecule, proinsulin, readily dimerizes. These proinsulin dimers form hexamers through interactions between peptide His residues and Zn (see fig. 4.1). Proinsulin hexamers undergo proteolysis, resulting in insulin hexamers, which exhibit crystallisation and reduced solubility. This Zn-insulin hexamerization is a well-known insulin storage mechanism (Brange, 1994; Ashcroft and Ashcroft, 1992). Hexameric insulin suspensions are used for diabetes treatment. These formulations are administered sc, and, due to the decreased solubility at physiological pH, are longer acting.
and are absorbed more slowly than native insulin (Brange and Langkjaer, 1997; Owens, 2002). In the presence of excess Zn at physiological pH, insulin hexamers can also form complex rhombohedral crystal structures that further reduce insulin solubility, and which have been used in the formulation of long-acting insulin treatments (Brange and Langkjaer, 1997; Owens et al., 2001). On these grounds, I investigated whether administering PYY3-36 or PYY3-36 analogues substituted with His with Zn enhances their ability to precipitate at physiological pH, and whether this reduces the clearance of these peptides in vivo, prolonging their bioactivity.

Figure 4.1. Computer generated image of the insulin hexamer. Three insulin dimers are hexamerized through interactions between His residues of insulin peptides and Zn ions (Dunn, 2005).

4.2 Hypotheses & Aims

I hypothesized that substitution of part of the α-helical domain of PYY3-36 with the αLT sequence would enhance the bioactivity of PYY3-36, as the literature suggested that stabilizing the α-helix and 3D conformation of PYY3-36 may be beneficial in analogue design. Following from these results, I hypothesized that the addition of Pro would prolong
the bioactivity of PYY3-36-αLT, due to the enhanced resistance to proteolytic degradation and enhanced bioactivity of PYY3-36 N-Pro (see Ch. 2). I subsequently hypothesized that the substitution of His residues in PYY3-36-αLT at sites implicated by my previous PYY3-36 analogue structure/affinity studies would enhance the precipitation of PYY3-36 and reduce its clearance from the circulation.

Finally, I hypothesized that the chronic administration of PYY3-36 analogues with Zn to a diet-induced obese mouse model would reduce body weight gain without causing tolerance, due to a slow-release, steady state pharmacokinetic profile.

Therefore my aims were to investigate the effects of:

- α-LT-substituted PYY3-36 analogues on acute food intake
- α-LT substitutions on the Y2R affinity and pharmacokinetics of PYY3-36
- Zn on the solubility of PYY3-36 analogues at physiological pH
- His-substitutions on the ability of Zn to modulate that solubility and clearance of PYY3-36 analogues
- chronic administration of PYY3-36 analogues with Zn on food intake and body weight gain in a diet-induced obese mouse model
4.3 Methods

4.3.1 Peptides

PYY1-36 and PYY3-36 were purchased from Bachem Ltd (St Helen’s, UK). Other peptides were purchased from Bachem Ltd (UK) or Biomol International LP (Exeter, UK). Peptides were synthesized, cleaved and purified as previously described (see Ch. 2, section 2.3.1.1-4).

4.3.2 PYY3-36 analogue design

A common feature of design for all PYY3-36 analogues investigated in this chapter is the substitution of an epitope homologous to LTX (970-981), LIT (952-962) and Ex4 (17-28) alpha helices at residues 16-23 of PYY3-36. PYY3-36-αLT was therefore the framework for the design of the other two analogues investigated (see fig. 4.2). The addition of Pro at the N terminus of PYY3-36-αLT, creating PYY3-36-NPro-αLT was carried out due to the enhanced bioactivity of PYY3-36-NPro observed in previous studies described in chapter 2 (see section 2.4.4). The substitution of His at various sites in PYY3-36-αLT was carried out at positions in the N terminus, mid-section and C terminus, in order to maximise the putative interaction between His and Zn. The selection of amino acid residues for His substitution was also carried out according to previous studies described in chapter 2. Although His did not improve bioactivity at these positions, analogues substituted with His or other positively charged amino acids retained the ability to bind the Y2R and reduce food intake. The primary sequences of the analogues investigated are shown in fig 4.2.
Figure 4.2. Homologous amino acid sequences of Ex4, αLTX and αLIT are shown with residues that are either identical or conserved in bold. The amino acid sequences of PYY3-36 and the analogues designed are shown, with pink shading for the residues substituted with the αLT/LIT sequences, and red shading for the residues substituted with His residues. See Appendix I for amino acid codes.
4.3.3 PYY3-36 receptor binding assays

The procedure for the preparation of the human Y2R plasmid, Y2R transfection into cells, production of Y2R-expressing cell membrane preparation and the human Y2R binding assay was described in chapter 2 and is identical to the procedures used for the mouse Y2R. The mouse Y2R plasmid was a kind gift from Professor Herbert Herzog (University of New South Wales, Australia). The method for the mouse Y2R binding assay did not differ from the human Y2R binding assay (see Ch.2, section 2.3.5.3).

4.3.4 Animals

Male C57BL/6 mice weighing 20 - 25 g (Charles River, Kent, UK) were used for feeding and behavioural studies. Animals were maintained in individual cages under controlled temperature (21-23°C) and lights (12:12 h light:dark cycle, lights on at 0700h). Animals had *ad libitum* access to water and RM1 diet (Special Diet Services). To minimise stress animals were regularly handled and acclimatised to subcutaneous (sc) injections. Diet induced obese (DIO) mice weighing 33-40 g were used for the chronic feeding studies. This phenotype was generated by allowing C57/BL6 mice *ad libitum* access to high fat diet (energy content: 60% kCal fat, 20% kCal protein, 20% kCal carbohydrate; Research Diets Inc., NJ, USA) for 18 weeks prior to the start of the study. The mice remained on this diet throughout the chronic studies (Liu 2010). Male Wistar rats weighing 150 - 200 g (Charles River) were used for pharmacokinetic studies. These animals were and allowed a minimum acclimatisation period of 72 hours before terminal studies. All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project Licence 70/6402).
4.3.5 Acute PYY3-36 analogue feeding studies in mice

Acute feeding studies in mice were carried out as previously described (see Ch.2, section 3.4.4). For studies investigating higher doses of PYY3-36 or PYY3-36 analogues on food intake, food was weighed at 48h post-injection, in addition to being weighed at 1h, 2h, 4h, 8h and 24h intervals.

For the low dose PYY3-36-αLT study, mice were administered 20/50nmol/kg PYY3-36-αLT, saline or 50nmol/kg PYY3-36 (sc, n = 10-12 per group). For the high dose PYY3-36-αLT study, mice were administered saline, 500/1000/10000nmol/kg PYY3-36-αLT or 500/1000nmol/kg PYY3-36 (n=10-12/group). For the study investigating the effects of the addition of N-Pro to PYY3-36-α-LT on food intake, mice were administered 1000nmol/kg PYY3-36-α-LT or 1000nmol/kg PYY3-36-α-LT. For the study investigating the effects of the addition of His residues to PYY3-36-NPro-α-LT, mice were administered 1000nmol/kg PYY3-36-α-LT, 1000nmol/kg PYY3-36-NPro-α-LT or 1000nmol/kg PYY3-36-NPro-α-LT-4H.

4.3.6 Acute PYY3-36-αLT behavioural study in mice

In line with the procedures for acute feeding studies, mice were overnight fasted. In the early light phase mice were administered saline, 500nmol/kg PYY3-36-αLT or 127mg/kg LiCl (sc). LiCl was used as a positive control due to its ability to induce an aversive behavioural response (Yamamoto et. al. 1992). Behavioural patterns were monitored for 2h immediately after injection by observers blinded to the treatments. Behaviour was classified into 6 categories:

1. Feeding (including drinking)
2. Locomotion (including rearing, climbing and burrowing)
3. Grooming
4. Resting
5. Head Down (animal in an abnormal posture: hunched appearance, with piloerection
6. Pica (consuming a substance without nutritional value, such as faeces or bedding)

Each animal was observed for 12 seconds, every 5 minutes. Each 12 second period was subdivided into three four second periods, in which the behaviour of each mouse was recorded. In this way, each animal had a total of 36 behaviours recorded per hour. This method of behavioural analysis is a modified version of a well characterized method established by Fray and co-workers (Fray et al., 1980; Wren et al., 2002).

4.3.7 PYY3-36, PYY3-36-αLT and PYY3-36-NPro-αLT pharmacokinetic studies in rats

4.3.7.1 Effect of αLT and NPro-αLT modifications on PYY3-36 pharmacokinetics in the anaesthetized and conscious rat

This study was carried out in order to assess the effects of α-LT and NPro-αLT modifications to PYY3-36 on the clearance of PYY3-36 in vivo. There are a number of methods to take plasma samples for pharmacokinetics studies. Serial sampling from the jugular vein allows plasma samples to be easily withdrawn at multiple time points and the observation of a pharmacokinetic profile for each animal over a period of time, unlike terminal blood sampling studies. As PYY has been reported to have a short half-life of around 8 min in the rat (Lluis et al., 1989), serial sampling was utilised to assess plasma levels of PYY3-36 and PYY3-36 analogues at time points over 1h immediately after injection (Waynforth and Fleckmell, 1992). In order to measure plasma levels of PYY3-36 and PYY3-36 analogues at later time points post-injection, blood samples were collected from conscious animals at 30min and 60min post-injection (in order to assess whether the results from the two different methods utilised for collecting bloods were comparable), and also at 2h, 6h and 24h post-injection. In the pharmacokinetic analyses subsequently carried out, Tmax is the time for the exogenously administered peptide to reach its maximum plasma concentration, while Cmax is its maximum plasma concentration (Graphpad Prism version 5.00 for Windows, GraphPad Software, San Diego, California). Percentage relative bioavailability is a measure of the amount reaching the circulation of two different formulations of a drug. This was calculated for
peptide administered sc either with or without Zn using the equation: Relative bioavailability = AUC of peptide with Zn/AUC of peptide without Zn * 100 (Hoag and Hussain, 2001).

4.3.7.1.1 Serial Sampling from the jugular vein of anaesthetized rats injected with PYY3-36-αLT or PYY3-36-NPro-αLT

Rats were randomized according to body weight and divided into treatment groups (100nmol/kg PYY3-36, PYY3-36-αLT or PYY3-36-NPro-αLT; n=4-6/group). On study day rats were anaesthetized with 67mg/kg hypnorm (1:30 ratio of fentanyl:fluanisone; Vetapharma) mixed with 3mg/kg hypnovel (midazolam; Pfizer) given intraperitoneally (ip). The isolation and cannulation of the jugular vein as was described in chapter 3 (section 1.3.6.2). Rats were then injected with 100nmol/kg (sc, final volume 50µl) PYY3-36, PYY3-36-αLT or PYY3-36-NPro-αLT. Blood sampling from the jugular vein was then carried out at 0min, 5min, 10min, 15min, 30min, 45min and 60 min post injection and collected into eppendorfs containing aprotinin (Trasylol, Bayer, Germany) to inhibit blood protease action and blood coagulation. After the final time point, rats were killed using an overdose of sodium pentobarbitone (Meridial Animal Health Ltd, UK). Plasma was separated from whole blood by centrifugation (3-18K centrifuge, rotor 12348; Sigma, UK) at 6000rpm for 10min, and stored at -20°C until use.

4.3.7.1.2 Blood collection from conscious rats injected with PYY3-36-αLT or PYY3-36-NPro-αLT

Rats were randomized according to body weight and divided into treatment and time point groups (100nmol/kg PYY3-36, PYY3-36-αLT or PYY3-36-NPro-αLT; n=4-6/time point/group). At time 0, rats were injected with 100nmol/kg PYY3-36, PYY3-36-αLT or PYY3-36-NPro-αLT. Rats were then decapitated at 30min or 60min, and at 1h, 2h, 6h or 24h post-injection. The early (30min or 60min) time points were taken in order to compare the two methods utilised for taking plasma and to compare plasma levels of PYY3-36 between conscious and anaesthetized rats. Trunk blood was collected into eppendorfs containing aprotinin (Trasylol; Bayer, Germany) to inhibit blood protease action and blood coagulation. Plasma was separated from whole blood
by centrifugation (3-18K centrifuge, rotor 12348; Sigma, UK) at 6000rpm for 10min, and stored at -20°C until analysis by RIA.

4.3.8 Investigations into the substitution of His residues in PYY3-36 NPro αLT in the development of a Zn delivery system

4.3.8.1 Investigation into the solubility of PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H when in a Zn solution

PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H were investigated for the ability to precipitate in vitro when in Zn solution at physiological pH (pH 7.4). ZnCl₂⁺ (Sigma, UK; referred to as Zn in this thesis) is the commercially-available anhydrous form of Zn that was purchased and used in all studies.

Peptides (10mg/ml) were dissolved in saline or in a saline solution containing a molar ratio of 2:1 Zn:peptide at pH 4.5 (final volume 1 ml). Bovine serum albumin (BSA, Sigma, UK) (0.5%) was added and the pH was increased to 7.4 by the addition of 0.2M NaOH. At this pH some peptides precipitated. The precipitate was then centrifuged for 2min at 8000 rpm. The supernatant was removed and stored at -20°C to measure for the concentration of dissolved peptide. The pellet was resuspended in 1ml saline (pH 7.4) and either a) stored at -20°C if little or no peptide initially precipitated or b) incubated on a shaking tray at 37°C for 1h if substantial precipitate was observed. After the wash, any remaining precipitate was centrifuged as before, the supernatant removed and stored and the pellet resuspended in saline (pH 7.4). This process was repeated up to five times or until no precipitate was seen following centrifugation. The pellet from the fifth (or final) wash was dissolved in 1ml saline at (pH 4.5). All samples were stored at -20°C until analysis by RIA.

4.3.8.2 Effect of Zn and the introduction of His residues in PYY3-36 NPro-αLT-4H on pharmacokinetics in the conscious rat

The pharmacokinetics studies described in section 4.1.4.8 showed that anaesthetics may be associated with an increase in peptide plasma levels at 30min and 60min post-
administration compared to peptide plasma levels from conscious rats. This may be due to decreased clearance or degradation of peptide, since anaesthetics reduce perfusion of blood to tissues and reduce renal glomerular filtration (Burchardi and Kaczmarczyk, 1994). Therefore, for this subsequent study, blood was taken from conscious rats only via the decapitation method as previously described. Rats were administered 300nmol/kg PYY3-36-αLT, PYY3-36-NPro-αLT or 2P-PYYα-H4 (final volume 20 µl-10mg/ml as used in the solubility studies) at a final molar concentration of 1:1 Zn:peptide. A lower ratio of Zn:peptide was used because the solubility studies implied full precipitation at 2:1 Zn:peptide, even after five 1h washes. It is necessary for the peptide to deprecipitate to cause its biological effects, and therefore a lower Zn concentration was used in the hope that a favourable amount of precipitation could be attained, translating to a slow increase in circulating peptide levels. Saline at a pH of 4.5 was used as a vehicle to ensure initial peptide solubility. Rats (n=4-6/time-point/group) were decapitated at 15min and 30min and 1h, 2h, 4h, 8h and 24h post-injection. Trunk blood was collected into lithium-heparin tubes containing aprotinin and stored on ice immediately after collection. Plasma was separated from whole blood by centrifugation at 6000rpm for 10min, and stored at -20°C until analysis by RIA.

4.3.9 Radioimmunoassays for PYY3-36 and PYY3-36 analogues

Plasma PYY-like immunoreactivity was measured using a specific and sensitive RIA, as described in Chapter 3 (section 3.3.6.2.1 and appendix iv). The PYY3-36 analogues had the following cross-reactivity with the Y21 antibody: PYY3-36-αLT and PYY3-36-NPro-αLT: 20-25% and PYY3-36-NPro-αLT-4H: 11%. As a specific antibody was not yet raised in the time that these assays were carried out concentrations of peptide analogues were increased within the standard curve, in order to allow detection of the respective peptides in accord with their respective cross reactivity with Y21. A standard concentration of 1pmol/ml was used for PYY3-36, 5pmol/ml was used for PYY3-36-αLT and PYY3-36-NPro-αLT and 10pmol/ml was used for PYY3-36-NPro-αLT-4H. The intra- and inter-assay coefficients of variation were 6% and 10% respectively. All analogue assays were incubated for 3 days at 4°C before separation of free and antibody-bound label by secondary antibody (sheep anti-rabbit antibody).
4.3.10 Assessment of the chronic effects of PYY3-36 analogues administered with Zn to diet-induced obese mice

Male C57BL/6 DIO mice (33-40g) were randomised according to body weight into treatment groups of 8 mice, each with an equal average body weight. Mice were administered saline with 2.4mM Zn (pH 4.5) or 300nmol/kg PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT or PYY3-36-NPro-αLT at a 1:1 Zn:peptide molar ratio (pH4.5; sc). One hour post-injection mice were given a pre-weighed amount of high fat diet. During the early light phase (07:00h) of the following day, food was weighed and removed. Therefore, mice had access to high fat diet during the dark phase from 17:00h until 07:00h and were fasted during the light phase from 07:00h until 17:00h over 48 days. This protocol was followed so that the Zn:peptide solution is given time to precipitate during the 1h between injections and re-feeding, allowing release during the dark phase, in which rodents are most active. Acute food intake was measured at 0-4h post-injection on day 1 and around half-way through the study (day 26), in order to investigate whether the mice develop tolerance to the effects of PYY3-36 or PYY3-36 analogues administered with Zn over time. Food intake was also measured at 4-8h post-injection in order to investigate whether PYY3-36 or PYY3-36 analogues administered with Zn have a delayed effect on food intake.

4.3.11 Measurement of total body fat in animals treated with PYY3-36-αLT using whole body proton magnetic resonance spectroscopy (1H-MRS)

Whole body 1H-MRS spectroscopy for analysis of body composition was carried out on mice after they had been administered saline or PYY3-36-αLT over 49 days (see section 1.3.10). Animals were anaesthetised with a 2-4% isoflurane-oxygen mix maintained throughout the scan via a face-mask, and were scanned on a 4.7T Unity Inova MR Scanner (Varian Inc, CA, USA) using a Quad 72 linear whole body coil. Temperature and respiration was monitored throughout the scan using monitoring equipment (SA Instruments Inc, NY, USA) and animals were maintained at room temperature. 1H MR spectroscopy was performed using a single pulse sequence with a repetition time (TR)
of 10 seconds, a pulse angle of 45°, four averages and a spectral width of 20,000 Hz. The spectra were analyzed using MestReC software (Santiago de Compostela, Spain). An exponential line broadening was applied, prior to phasing and baseline correction and peak integration of the water (reference at 4.7 ppm) and lipid (reference at 1.2 ppm) were obtained. The whole body percentage adiposity was calculated using the following equation: %adiposity = 100x lipid integral/(lipid integral + water integral + (0.38xwater integral)). This equation takes into account the ratio of water to lean mass in the body of 0.38 (Mystkowski et al., 2000).

**4.3.12 Statistics**

Acute food intake expressed as mean ± standard error of the mean (SEM) was analysed by one way ANOVA using post-hoc Tukey’s multiple comparison adjustment to determine the p value for significant differences in food intake between PYY3-36-treated groups, PYY3-36 analogue-treated groups and saline. As the data from the behavioural study exhibited abnormal distribution it was analysed using Kruskal-Wallis one-way ANOVA on ranks (Systat 11, San Jose, CA, USA). Cumulative data from the chronic feeding studies was analysed using the generalized estimating equation and the Mann-Whitney U test (Systat 11, San Jose, CA, USA). Plasma levels of PYY3-36-NPro-αLT-4H expressed as mean ± SEM from the pharmacokinetic study in which PYY3-36-NPro-αLT-4H was administered with or without Zn was analysed using an unpaired Students t-test. In all cases, p<0.05 was considered to be statistically significant.
4.4 Results

4.4.1 Investigations into substituting αLT into the α-helix (residues 16-23) of PYY3-36 or NPro-PYY3-36

4.4.1.1 Effect of PYY3-36-NPro-αLT on food intake in mice

4.4.1.1.1 Effect of PYY3-36-αLT at low doses on food intake

PYY3-36 and both doses of PYY3-36-αLT significantly reduced food intake at 0-1h and 1-2 h post-injection (p <0.01 vs saline). PYY3-36-αLT (50nmol/kg) resulted in a more potent reduction in food intake than PYY3-36 (50nmol/kg), though this effect did not reach statistical significance compared to PYY3-36 (50nmol/kg) at 2-4h post-injection, while the lower dose of PYY3-36-αLT (20nmol/kg) had no effect on food intake by 2-4h. PYY3-36-αLT (50nmol/kg) significantly increased food intake at 8-24h compared to saline controls. There were no significant differences between groups at any other time point observed (see fig. 4.3).

Figure 4.3. Effect of PYY3-36 (50nmol/kg; sc) and PYY3-36-αLT (20nmol/kg / 50nmol/kg; sc) on food intake at 0-1h, 1-2h, 2-4h, 4-8h and 8-24h post-injection, and on cumulative food intake over 24h. Results are means ± SEM. *p<0.05, **p<0.001 vs. saline (ANOVA with post-hoc Tukey’s multiple comparison adjustment), n = 8-10.
4.4.1.1.2 Effect of PYY3-36-αLT at supraphysiological doses on food intake

The dose response effect of PYY3-36 and PYY3-36-αLT on food intake was then investigated. All doses of PYY3-36 and PYY3-36-αLT significantly reduced food intake at 0-1h, 1-2 h and 2-4h post-injection compared to saline. Animals administered PYY3-36-αLT at all doses ate significantly less than animals administered the high dose of PYY3-36 (10,000nmol/kg) at 0-1h post-injection (see fig. 4.4). Animals administered the high dose of PYY3-36 (10,000nmol/kg) or all doses of PYY3-36-αLT (500/ 1000/ 5000/ 10,000nmol/kg) ate significantly less than animals administered the lower dose of PYY3-36 (500nmol/kg) at 2-4h and 4-8h post-injection (see fig 4.4). PYY3-36 (10,000nmol/kg) and all doses of PYY3-36-αLT significantly reduced food intake at 4-8h post-injection, while PYY3-36 (500nmol/kg) had no effect on food intake compared to saline controls (500nmol/kg PYY3-36: p>0.05 vs saline; 500 /1000 /5000 /10,000nmol/kg PYY3-36-αLT: p<0.001 vs saline). PYY3-36(10,000nmol/kg) and PYY3-36-αLT (500 /1000 /5000 /10,000nmol/kg) significantly reduced food intake at 8-24h compared to saline controls (10,000nmol/kg PYY3-36: p<0.05 vs saline; 500 /1000 /500 /10,000 nmol/kg PYY3-36-αLT: p<0.001 vs saline). Animals administered all doses of PYY3-36-αLT (500 /1000/ 5000/ 10,000nmol/kg) ate significantly less than animals administered PYY3-36 (500nmol/kg). Animals administered PYY3-36-αLT (1000 /5000/10,000nmol/kg) ate significantly less than animals treated with PYY3-36 (10,000nmol/kg) at 8-24h in a dose-dependent manner (see fig. 4.5). At 24-48h, only the two highest doses of PYY3-36-αLT (5000nmol/kg/ 10,000nmol/kg) significantly reduced food intake compared to saline controls, PYY3-36 (500nmol/kg) and PYY3-36 (10,000nmol/kg). PYY3-36 (10,000nmol/kg) significantly reduced cumulative food intake over 24h and 48h compared to saline controls, whereas PYY3-36 (500nmol/kg) did not. All doses of PYY3-36-αLT significantly and dose-dependently reduced cumulative food intake over 24h and 48h compared to saline controls and PYY3-36 (500nmol/kg). Over 48h, the three highest doses of PYY3-36-αLT (1000/5000/10,000nmol/kg) significantly reduced cumulative food intake compared to the highest dose of PYY3-36 (10,000nmol/kg) (see fig 4.5). Although PYY3-36 (10,000nmol/kg) significantly reduced cumulative food intake over 48h, animals treated with PYY3-36 (10,000nmol/kg) ate more than animals administered the lowest dose of PYY3-36-αLT.
Figure 4.4. Dose response effect of PYY3-36 (500nmol/kg or 10,000nmol/kg; sc) and PYY3-36-αLT (500, 1000, 5000 or 10000nmol/kg; sc) on food intake at 0-1h, 1-2h, 2-4h and 4-8h post-injection. Results are means ± SEM. **p<0.01, ***p<0.001 vs. saline, ###p<0.001 vs. PYY3-36 (500nmol/kg); $p<0.05 vs. PYY3-36 (10,000nmol/kg) (ANOVA with post-hoc Tukey’s multiple comparison adjustment), n = 8-10.

Figure 4.5. Dose response effect of PYY3-36(500nmol/kg or 10,000nmol/kg; sc) and PYY3-36-αLT (500, 1000, 5000 or 10000nmol/kg; sc) on food intake at 8-24h, 0-24h, 24-48h and 0-48h post-injection. Results are means ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. saline; ###p<0.001 vs. PYY3-36 (500nmol/kg); $p<0.05, $$$p<0.001 vs. PYY3-36 (10,000nmol/kg) (ANOVA with post-hoc Tukey’s multiple comparison adjustment), n = 8-10.
4.4.1.2 Effect of PYY3-36-NPro-αLT on behaviour in mice

To determine whether the potent effect on feeding may be due to nausea, the behavioural effects of PYY3-36-αLT (500nmol/kg) were assessed. LiCl (127mg/kg, ip) was used as a positive control, as it is a known aversive stimulus, producing internal malaise (Yamamoto et al., 1992). PYY3-36-αLT significantly decreased feeding behaviour at 0-1h and 1-2h post-injection compared to saline controls (0-1h feeding saline vs. PYY3-36-αLT: p<0.05; 1-2h food intake: saline vs. PYY3-36-αLT: p<0.01; see table 4.2). PYY3-36-αLT significantly increased grooming at 0-1h post-injection compared to saline controls, but had no affect on grooming by 1-2h post-injection (0-1h grooming: saline vs. PYY3-36-αLT: p<0.01; see table 4.2). LiCl (127mg/kg) significantly reduced food intake and increased head down behaviour at 0-1h post-injection compared to saline controls, but had no effect on any behaviour by 1-2h post-injection (0-1h feeding: saline vs. LiCl: p<0.01; 0-1h head down: saline vs. LiCl: p<0.0; see table 4.2). PYY3-36-αLT had no effect on head down behaviour at any time point studied. A pie-chart depicting the proportion of behaviours observed in each time period is also shown in fig. 4.6.
<table>
<thead>
<tr>
<th></th>
<th>Feeding</th>
<th>Locomotion</th>
<th>Grooming</th>
<th>Resting</th>
<th>Head Down</th>
<th>Pica</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) 0-1h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>22 [19-24]</td>
<td>4.5 [3.8-6.2]</td>
<td>1 [1-2]</td>
<td>1 [0-1.5]</td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
</tr>
<tr>
<td>LiCl</td>
<td>12.5 [6.8-18.8]**</td>
<td>2 [1-3]</td>
<td>0.5 [0-2]</td>
<td>2 [1-3.75]</td>
<td>9 [7:14.2]**</td>
<td>0 [0-0]</td>
</tr>
<tr>
<td>PYY3-36-αLT</td>
<td>13 [11.5-16.5]*</td>
<td>8 [5.5-11]</td>
<td>4 [3-7.5]**</td>
<td>1 [0-2]</td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
</tr>
<tr>
<td><strong>B) 1-2h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5.5 [0.75-0.95]</td>
<td>1.5 [0-4.25]</td>
<td>1.5 [0-4.5]</td>
<td>19 [12.8-22.5]</td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
</tr>
<tr>
<td>LiCl</td>
<td>12 [4-12]</td>
<td>4 [3-6]</td>
<td>4 [2-6]</td>
<td>12 [7-18]</td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
</tr>
<tr>
<td>PYY3-36-αLT</td>
<td>0 [0-0]**</td>
<td>0 [0-5]</td>
<td>1 [0-6]</td>
<td>25 [21.5-30]</td>
<td>0 [0-0]</td>
<td>0 [0-0]</td>
</tr>
</tbody>
</table>

Table 4.2. Table showing median frequency and inter-quartile range (in square brackets) of each of the predefined behaviours observed over 0-1h and 1-2h post-injection of saline, LiCl (127mg/kg) or PYY3-36-αLT (500nmol/kg). Each mouse was observed for 12 sec every 5 min during each 1h time period. This 12 sec period was further divided into three 4 sec periods, and the behaviour of each mouse was recorded in each section of the time period. Each mouse had a total of 36 behaviours recorded per h. *p<0.05, **p<0.01 vs. saline (Kruskal-Wallis One-Way Analysis of Variance), n = 8-10 per treatment, per behaviour.
Figure 4.6. Pie charts depicting the proportion of observations of each of the predefined behaviours a) 0-1h and b) 1-2h post-injection of saline, LiCl (127mg/kg) or PYY3-36-αLT (500nmol/kg). Results are shown as percentage of total behaviours observed over the 1h time period stated (n=8-10 per treatment, per behaviour).
4.4.1.3 Effect of PYY3-36-αLT on affinity at the human and mouse Y2R

A Y2R binding assay was used to investigate whether increased receptor affinity may account for the observed increase in bioactivity of PYY3-36-α-LT. Substitution of residues 16-23 of PYY3-36 with α-LT resulted in a 2-fold lower affinity for the human Y2R (PYY3-36 IC50: 0.17 ±0.03nM, PYY3-36-αLT IC50: 0.34 ±0.03nM (see fig. 4.7a). In order to investigate whether this lower Y2R affinity observed for PYY3-36-α-LT is specific to the human Y2R, a mouse Y2R binding assay was also carried out. PYY3-36 α-LT also had a 2-fold lower affinity for the mouse Y2R (PYY3-36 IC50: 0.16 ±0.03nM, PYY3-36-αLT IC50: 0.34 ±0.05nM (see fig. 4.7b).

Figure 4.7. Binding affinity curves comparing PYY3-36 and PYY3-36-αLT binding at the A) human or B) mouse Y2R. The curves are means ±SEM of 3-4 assays. Increasing concentrations of PYY3-36 or PYY3-36-αLT were incubated with a fixed concentration of ¹²⁵I PYY and Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of ¹²⁵I-PYY bound with and without unlabelled competing peptide.
4.4.1.4 Effect of PYY3-36-NPro-αLT on Y2R affinity

The remaining binding studies were performed using the human and mouse Y2R, but only data for the human Y2R is shown, as there was little or no difference between binding of PYY3-36 analogues to the mouse and human Y2R. The addition of Pro to the N terminus of PYY3-36 slightly increased Y2R affinity (PYY3-36 IC50: 0.17 ±0.009nM, PYY3-36-NPro IC50: 0.15 ±0.05nM (mean from 3 assays; see fig. 4.8a). The addition of Pro to the N terminus of PYY3-36-αLT reduced Y2R affinity by 2.6 fold (PYY3-36 IC50: 0.16 ±0.01nM, PYY3-36-NPro-αLT IC50: 0.42 ±0.12nM (see fig. 4.8b).

![Graph A](image1)

![Graph B](image2)

**Figure 4.8.** Binding affinity curves comparing a) PYY3-36 and PYY3-36-NPro binding and b) PYY3-36 and PYY3-36-NPro-αLT binding at the human Y2R. The curves are shown as means of 3-4 assays. Increasing concentrations of PYY3-36 or PYY3-36-NPro-αLT were incubated with a fixed concentration of $^{125}$I-PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I PYY bound with and without unlabelled competing peptide.
**4.4.1.5 Comparison of the effect of PYY3-36-αLT and PYY3-36-NPro-αLT on food intake**

As the effect of the addition of Pro at the N-terminus of PYY3-36 significantly prolongs the effect of PYY3-36 on food intake (see Chapter 2; section 2.4.4), the effect of PYY3-36-αLT on food intake was then compared that of PYY3-36-NPro-αLT. A dose of 1000nmol/kg was used as it was the lowest dose of PYY3-36-αLT that significantly reduced food intake over 0-48 compared to saline and compared to a 10x higher dose of PYY3-36 (10,000nmol/kg, see section 1.4.1.1.2). PYY3-36-αLT and PYY3-36-NPro-αLT significantly reduced food intake over 0-1h, 1-2h, 2-4h, 4-8h and 8-24h post-injection and cumulative food intake over 24h compared to saline controls and to a similar level of potency, apart from 0-1h post-injection, when animals administered PYY3-36-αLT ate significantly less than animals administered PYY3-36-NPro (see fig. 4.9). By 24-48h post-injection PYY3-36-αLT had no significant effect on food intake, while PYY3-36-NPro-αLT significantly reduced food intake compared to saline controls, though this effect was not statistically different from that of PYY3-36-αLT (see fig. 4.9). Over 48h, both PYY3-36-αLT and PYY3-36-NPro-αLT significantly reduced cumulative food intake compared to saline controls (see fig. 4.9).
Figure 4.9. Effect of 1000nmol/kg (sc) of PYY3-36-NPro-αLT and PYY3-36-αLT on food intake at 0-1h, 1-2h, 2-4h, 4-8h, 8-24h, 24-48h post-injection, and on cumulative food intake over 0-24h and 0-48h post-injection. Results are means ± SEM. *p<0.05, ***p<0.001 vs. saline; $p<0.01$ vs. PYY3-36-NPro-αLT (1000nmol/kg) (ANOVA with post-hoc Tukey’s multiple comparison adjustment), n = 7-10.

### 4.4.1.6 Effect of αLT and NPro-αLT modifications on PYY3-36 pharmacokinetics in the anaesthetized and conscious rat

The plasma levels of PYY3-36 and PYY3-36 analogues following sc injection were then investigated in anaesthetized fasted male rats. In the anaesthetized rat, the maximum plasma concentrations ($C_{max}$) following subcutaneous administration of PYY3-36(100nmol/kg), PYY3-36-αLT (100nmol/kg) or PYY3-36-NPro-αLT (100nmol/kg) PYY3-36 were 5806 ±1119pmol/L ($T_{max}$: 15min), 3744 ±615pmol/L ($T_{max}$: 10min) and 8263 ±1490pmol/L ($T_{max}$: 30min) respectively (n=4-6 per group, see fig. 4.10a). In order to measure plasma levels of PYY3-36 and PYY3-36 analogues at later time points post-injection, blood samples were collected from conscious animals at 30min, 60min, 2h, 6h and 24h post-injection (n=4-6 per group, see fig. 4.10b). As these peptides were assayed using different concentrations of standard, due to different cross-reactivity with the Y21 PYY antibody, statistical analysis was not carried out, and these plasma concentration/time curves serve only to
compare the Cmax and Tmax of the peptides, and the time at which the peptide plasma levels return to baseline.

Figure 4.10. Plasma concentration/time curves of PYY3-36 (grey), PYY3-36-αLT (blue) and PYY3-36-NPro-αLT (purple) following administration of a 100nmol/kg dose of PYY3-36 or analogue. Plasma was collected from a) anaesthetized rats via the jugular vein or b) conscious rats from trunk blood after decapitation. Results are means ± SEM.
4.4.2 Investigations into the substitution of His residues in PYY3-36 NPro αLT in the development of a Zn delivery system

4.4.2.1 Effect of α-LT or NPro-αLT substitutions and Zn on peptide solubility at physiological pH

PYY3-36 and PYY3-36 analogues were investigated for their ability to precipitate in a solution containing Zn at physiological pH (i.e. the pH encountered by peptides upon entering the subcutaneous space). These studies were done in order to assess whether it is possible for PYY3-36 analogues to form a subcutaneous bolus through chelating with Zn ions, allowing slow release into the circulation. Around 92% of total PYY3-36 (10mg/ml) initially dissolved in a saline solution (pH 4.5) remained in solution when the pH was increased to 7.4, with approximately 8% precipitation. Washes were not carried out for PYY3-36 due to the low precipitation observed (see fig. 4.11a). However, of PYY3-36 dissolved in a saline/Zn solution (2:1 Zn:peptide ratio; pH 4.5), only 6% of total PYY3-36 remained in solution when the pH was increased to 7.4. Over the next five washes (pH 7.5), 66% of total PYY3-36 dissolved, leaving 28% of total PYY3-36 in the final precipitate (see fig 4.11b).

Approximately 67% of PYY3-36-αLT initially dissolved in saline remained in solution when the pH was increased to 7.4. After the first wash another 15% of PYY3-36-αLT dissolved, leaving 18% of total PYY3-36-αLT in the final precipitate (see fig. 4.11c). When PYY3-36-αLT was dissolved in a saline/Zn solution (2:1 Zn:peptide ratio; pH 4.5), 6% of total PYY3-36-αLT remained in solution. Over the following 5 washes 24% of total PYY3-36-αLT dissolved, leaving 70% in the final precipitate (see fig. 4.11d).

Around 82% of PYY3-36-N-Pro-αLT initially dissolved in saline remained in solution when the pH was increased to 7.5, leaving 18% of total PYY3-36-αLT in the precipitate (see fig. 4.11e). In contrast, when PYY3-36-NPro-αLT was dissolved in saline/Zn (2:1 Zn:peptide ratio; pH 4.5), no PYY3-36-NPro-αLT remained in solution, with 100% precipitating. Over the following 5 washes 12% of the total peptide dissolved, leaving 88% in the final precipitate (see fig 4.11f).
Figure 4.11. Graphs showing the amount of PYY3-36 or PYY3-36 analogue that dissolves into solution and the amount that precipitates out of solution at physiological pH. Percentage peptide (all 10mg/ml; a&b: PYY3-36, b&c: PYY3-36-αLT, d&e: PYY3-36-NPro-αLT) initially dissolved (Initial D) in a solution of saline at pH 7.4, dissolved following 1h incubations in a solution of saline at pH7.4 at 37ºC (washes) and in the final precipitate (Final P) is shown. The solutions contained either peptide only or peptide with Zn at a molar ratio of 2(Zn):1(peptide) in a final volume of 1ml. Washes took place only if a precipitate was observed when the solution was brought to pH 7.4. Y axis is displayed as a logarithmic scale.
Almost none of the total PYY3-36-NPro-αLT-4H (10mg/ml) initially dissolved in a saline solution (pH 4.5) remained in solution when the pH was increased to 7.4, with around 99.8% precipitating. During each of the following 5 washes, around 5-12% of total PYY3-36-NPro-αLT-4H dissolved, leaving over 72% in the final precipitate (see fig. 4.12a).

No PYY3-36-NPro-αLT-4H dissolved in a saline/Zn solution (2:1 Zn:peptide ratio; pH 4.5) could be detected in solution when the pH was increased to 7.4. The following 5 washes (pH7.5) had no effect on peptide solubility, as no PYY3-36-NPro-αLT-4H could be detected in the supernatant of each wash. Subsequently, 100% of total PYY3-36-NPro-αLT-4H remained in the final precipitate (see fig 4.12b).

Figure 4.12. Graphs showing the amount of PYY3-36-NPro-αLT-4H that dissolves into solution and the amount that precipitates out of solution at physiological pH. Percentage PYY3-36-NPro-αLT-4H (10mg/ml) initially dissolved (Initial D) in saline at pH 7.4, dissolved following 1h incubations in saline at pH7.4 at 37°C (washes) and in the final precipitate (Final P) is shown. The solutions contained either a) PYY3-36-NPro-αLT-4H only or b) PYY3-36-NPro-αLT-4H with Zn at a molar ratio of 2(Zn):1(peptide) in a final volume of 1ml. Y axis is displayed as a logarithmic scale.
4.4.2.3  Effect of Zn and the introduction of His residues in PYY3-36 NPro-αLT-4H on pharmacokinetics in the conscious rat

The plasma levels of PYY3-36-NPro-αLT-4H with or without Zn following sc injection were then investigated in male rats by collecting plasma from trunk blood following decapitation. Administration of PYY3-36-NPro–αLT-5H (300nmol/kg, sc) without Zn resulted in a C\text{max} of 8750 ±689, and a T\text{max} of 15min. Plasma levels of PYY3-36-NPro–αLT-5H administered without Zn were significantly higher than those following administration of PYY3-36-NPro–αLT-5H with Zn at 15min and 45min (see fig 4.13). Administration of PYY3-36-NPro–αLT-5H (300nmol/kg, sc) with Zn (1:1 peptide/Zn ratio) resulted in a C\text{max} of 4950 ±686, and a T\text{max} of 24h. Plasma levels of PYY3-36-NPro–αLT-5H administered with 1:1 Zn were significantly higher than those following administration of PYY3-36-NPro–αLT-5H without Zn at 24h (see fig 4.13). The areas under the curve for PYY3-36-NPro–αLT-5H and PYY3-36-NPro–αLT-5H with 1:1 Zn were 2,246000 and 4,188000 pmol/L/min respectively. The relative bioavailability of PYY3-36-NPro–αLT-5H with 1:1 Zn was 186% of that for PYY3-36-NPro–αLT-5H without Zn. As the standard concentration in the standard curve of the PYY3-36-NPro-αLT-4H assay was increased ten-fold to allow for the fact that PYY3-36-NPro-αLT-4H only has 11% cross-reactivity with the PYY antibody used in this assay, the baseline level of PYY3-36 is not a true representation of endogenous PYY3-36 levels (>50pmol/L) (Grandt et al., 1994). Since the purpose of these assays was to compare pharmacokinetics with and without Zn, rather than to detect small changes in endogenous peptide, the reduced cross-reactivity of Y21 with PYY3-36-NPro-αLT-4H is unlikely to significantly affect the reliability of these results.
Figure 4.13. Plasma concentration/time curves of PYY3-36-NPro-αLT-4H (light green) and PYY3-36-NPro-αLT-4H with Zn (dark green) following subcutaneous administration of a 300nmol/kg dose of PYY3-36-NPro-αLT-4H in saline solution (pH4.5) or in a saline/Zn solution containing a molar ratio of 1:1 Zn:peptide (pH 4.5). Plasma was collected from rat trunk blood after decapitation at 15min, 30min, 60min, 2h, 4h, 8h and 24h post-administration, and at 0 min, 8h and 24h from rats that were injected with saline/Zn solution only, in order to measure basal PYY3-36. Results are means ± SEM. *p<0.05, ***p<0.001 vs PYY3-36-NPro-αLT-4H with Zn; *p<0.05 vs PYY3-36-NPro-αLT-4H (Unpaired students t-test; n=3-4).

4.4.2.4 Comparison of effect of PYY3-36-αLT, PYY3-36 NPro-αLT and PYY3-36 NPro-4H-αLT, on acute food intake in mice

PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H significantly reduced food intake over 0-1h, 1-2h, 2-4h and 4-8h post-injection compared to saline controls and to a similar level of potency, apart from 0-1h post-injection, when animals administered PYY3-36-αLT or PYY3-36-NPro-αLT-4H ate significantly less than animals administered PYY3-36-NPro-αLT (see fig. 4.14).

At 8-24h post-injection, PYY3-36-αLT and PYY3-36- NPro-αLT significantly reduced food intake to a similar extent compared to saline and PYY3-36- NPro-4H-αLT (p<0.001 vs. saline; p<0.001 vs. PYY3-36- NPro-4H-αLT), which had no effect
on food intake compared to saline. By 24-48h none of the analogues tested reduced food intake compared to saline, though PYY3-36-αLT and PYY3-36-NPro-αLT significantly reduced food intake compared to PYY3-36- NPro-4H-αLT (see fig. 4.15). Over 48h, both PYY3-36-αLT and PYY3-36-NPro-αLT significantly reduced cumulative food intake to a similar extent compared to saline controls and compared to PYY3-36- NPro-4H-αLT, while PYY3-36-NPro-αLT-4H significantly reduced food intake compared to saline (see fig. 4.15).

![Figure 4.14](image-url)

**Figure 4.14.** Effect of 1000nmol/kg (sc) of PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H on food intake at 0-1h, 1-2h, 2-4h and 4-8h post-injection. Results are means ± SEM. **p<0.01 vs. saline, ***p<0.001; $p<0.05, $$$p<0.01$ vs. PYY3-36-NPro-αLT (1000nmol/kg) (ANOVA with post-hoc Tukey’s multiple comparison adjustment), n = 8-11.
4.4.2.5  Effect of PYY3-36 NPro-4H-αLT on Y2R affinity

Four His substitutions at positions 4, 6, 19 and 30, which are positions that were previously investigated in Ch. 2, were made in the analogue PYY3-36-NPro-αLT, creating PYY3-36-NPro-αLT-4H. PYY3-36-NPro-αLT-4H had approximately 4.6 fold reduced Y2R affinity compared to PYY3-36 (PYY3-36 IC50: 0.16 ±0.03, PYY3-36-NPro-αLT-4H IC50: 0.79 ±0.12 (mean from 3 assays; see fig. 4.16).
Figure 4.16. Binding affinity curves comparing PYY3-36 and PYY3-36-NPro-αLT-4H binding at the human Y2R. The curves are shown as means of 3-4 individual experiments. Increasing concentrations of PYY3-36 or PYY3-36-NPro-αLT-4H were incubated with a fixed concentration of $^{125}$I PYY and human Y2R-expressing membrane. Specific binding was calculated as the difference between the amount of $^{125}$I PYY bound with and without unlabelled competing peptide.

4.4.3 Assessment of the use of a Zn delivery system to prolong the chronic bioactivity of PYY3-36 analogues in vivo

As Zn appears to promote precipitation of PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H at physiological pH in vitro, and appears to promote a slow release profile when co-administered with PYY3-36-NPro-αLT-4H in vivo, the chronic effects of the co-administration of Zn with PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT or PYY3-36-NPro-αLT-4H in vivo were investigated.

Mice were randomized according to body weight so that each group had an equal starting average body weight. Each group was administered 300nmol/kg PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT or PYY3-36-NPro-αLT-4H in a 1:1 Zn:peptide ratio (pH4.5) via the sc route at 07:00h daily, for a period of 49 days (7 weeks). PYY3-36 administered with Zn had no effect on cumulative food intake or body weight over the duration of the study (see fig.4.17, 4.18 & 4.19).

PYY3-36-αLT administered with Zn significantly reduced cumulative food intake and body weight gain over the 49 day study period compared to vehicle and PYY3-36 controls (see fig. 4.17). By the 49th day, mice administered vehicle or PYY3-36 with
Zn weighed 103.6 ±1.4% or 100.8 ±2.1 % of their initial average body weights respectively, while mice administered PYY3-36-αLT with Zn weighed 89 ±1.1% of their initial average body weight (see fig. 4.19). PYY3-36-NPro-αLT administered with Zn significantly reduced cumulative food intake throughout the 49 day study period compared to saline controls, though from day 18 onwards this effect was less potent than it was until day 17 compared to vehicle controls (see fig. 4.17). PYY3-36-NPro-αLT also significantly reduced cumulative food intake compared to PYY3-36 controls, though only up until day 8 (see fig. 4.17). PYY3-36-NPro-αLT administered with Zn significantly reduced body weight gain throughout the 49 day study period, though between day 8 and day 31 this effect was less potent compared to saline than its initial (1-7 day) and late (31-49 day) effect (see fig. 4.18). PYY3-36-NPro-αLT also significantly reduced body weight gain compared to PYY3-36 controls, from days 1-9 and days 31-46 (see fig. 4.18). By the 49th day, mice administered vehicle or PYY3-36 with Zn weighed 103.6 ±1.4% or 100.8 ±2.1 % of their initial average body weights respectively, while mice administered PYY3-36-NPro-αLT with Zn weighed 88.1 ±1.2% of their initial average body weight (see fig.4.19). PYY3-36-NPro-αLT-4H administered with Zn significantly reduced cumulative food intake throughout the 49 day study period, though from day 11 onwards this was at a reduced magnitude compared to its initial effect (day 1-10). PYY3-36-NPro-αLT-4H also significantly reduced cumulative food intake compared to PYY3-36 controls, though only up until day 5 (see fig. 4.17). PYY3-36-NPro-αLT-4H administered with Zn significantly reduced body weight gain throughout the 49 day study period, though between day 11 and day 32 this was effect was less potent than for its initial (day 1-10) and late (day 32-49) effect compared to vehicle controls (see fig. 4.18). PYY3-36-NPro-αLT-4H also significantly reduced body weight gain compared to PYY3-36 controls, though only up until day 6 (see fig. 4.18). By the 49th day, mice administered vehicle or PYY3-36 with Zn weighed 103.6 ±1.4% or 100.8 ±2.1 % of their initial average body weights respectively, while mice administered PYY3-36-NPro-αLT with Zn weighed 94.4 ±1.6% of their initial average body weight (see fig.4.19).
Figure 4.17. Effect of daily administration of 300nmol/kg of PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H (sc, all with Zn) on food intake over 49 days. Results are means ± SEM. **p<0.01, *p<0.05 vs. saline; $$p<0.01, $p<0.05 vs. PYY3-36 (GEE with Mann-Whitney U test), n =6-8.

Figure 4.18. Effect of daily administration of 300nmol/kg of PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H (sc, all with Zn) on body weight change (g) over 49 days. Results are means ± SEM. **p<0.01, *p<0.05 vs. saline; $$p<0.01, $p<0.05 vs. PYY3-36 (GEE with Mann-Whitney U test), n =6-8.
Figure 4.19. Effect of daily administration of 300nmol/kg of PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H (sc, all with Zn) on percentage change from initial body weight over 49 days (n =6-8).

The acute food intake over 4h post-injection of all groups was measured on day 1 and day 26 at 0-4h post-injection, (16:00h-20:00h) and on day 26 at 4-8h post-injection (20:00h-00:00h). PYY3-36 significantly reduced food intake compared to vehicle controls over 0-4h post-injection on day 1 and day 26 of the study. PYY3-36-αLT significantly reduced food intake on day 1 of the study, but resulted in a non-significant reduction in food intake on day 26. This lack of statistical significance may be due to the fact that the vehicle group at around 10% less on day 1 than on day 26 at 0-4h post-injection. Animals administered PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H ate significantly more than animals administered PYY3-36 at 0-4h post-injection on both day 1 and day 26 (see fig. 4.20a & b). Food intake was measured at 4-8h post-injection on day 26. PYY3-36 and PYY3-36-αLT had no effect on food intake at 4-8h, whereas PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H significantly reduced food intake compared to vehicle and PYY3-36 controls (see fig. 4.20c).
Figure 4.20. Effect of daily administration of 300nmol/kg of PYY3-36, PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H (sc, all with Zn) on acute food intake over 4h post-injection on day 1 (a) and over 0-4h (b) and 4-8h (c) post-injection on day 26. Animals had access to high fat diet from 1h post-injection. Results are means ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. saline; $p<0.05, $$$p<0.001 vs. PYY3-36 (one-way ANOVA using Tukey’s post-hoc adjustment), n =6-8.
4.4.3.1 Effect of chronic administration of PYY3-36-aLT on total body fat content

As measured by 1H MR spectroscopy, the percentage adiposity of mice administered PYY3-36-aLT with Zn over 49 days was 24.4 ±1.7% (n=6) of total body fat, which was significantly lower than that of animals administered vehicle over 49 days, which was 37.2 ±3.2 % (n=8).

Figure 4.21. Effect of daily administration of 300nmol/kg PYY3-36-aLT with Zn (sc) or saline with Zn (sc) over 49 days on adiposity. Data are means ± SEM. *p<0.05 vs. vehicle (students paired two-tailed t-test).
4.5 Discussion

The studies described in this chapter aimed to create a longer acting PYY3-36 analogue by modifying the two and three dimensional structure of PYY3-36, by modifying the solution in which the analogue is administered and by modifying the charged amino acids of PYY3-36 to optimise it for administering in the delivery solution. PYY3-36-αLT was designed by substituting part of the α-helix, which in PP-fold peptides is important for receptor affinity and peptide stability, with a conserved α-helical motif derived from the venom of *Latrodectus* spiders and used in the long-acting GLP-1 analogue Exendin-4 (Fuhlendorff et al., 1990; Holz and Habener, 1998; Runge et al., 2008). The following studies investigated the mechanistic basis for the improved bioactivity observed for this analogue, and whether the addition of Pro at the N-terminus improves bioactivity and pharmacokinetic profile further, as previously observed for PYY3-36 in Chapter 2. The final studies aimed to utilize Zn ions in developing a delivery system for PYY3-36 or PYY3-36 analogues that prolongs the circulating time of PYY3-36 through enhancing the ability of peptides to form a subcutaneous depot. This may allow the administration of lower doses of PYY3-36 or PYY3-36 analogues in order to avoid side effects such as nausea or abdominal discomfort associated with high iv doses of PYY3-36 in humans. I also hypothesized that the substitution of His residues at sites characterised in my structure/affinity studies described in chapter 2 would create a more favourable, ‘peakless’ pharmacokinetic profile when administered with Zn, due to chelation between Zn and His ions. I hypothesized that the subcutaneous depot formed by such an analogue might translate into an effect on food intake that is less potent than other PYY3-36 analogues, but is sufficient to drive a sustained reduction in body weight due to its slow release profile. In this chapter, the anorectic properties, receptor binding affinity, behavioural effects, solubility and plasma clearance of PYY3-36-αLT were investigated. Studies investigating the effects of N-Pro and His modifications to the PYY3-36-αLT sequence on solubility, pharmacokinetics and chronic food intake were also carried out.
4.5.1 The effect of substituting the LT/LIT/Exendin-4 conserved region into the α-helical region (residues 16-23) of PYY3-36

At low doses of 20 and 50 nmol/kg, PYY3-36-αLT did not have a strikingly improved bioactivity compared to PYY3-36. PYY3-36-αLT at 50nmol/kg had a more potent effect on food intake than PYY3-36 (50nmol/kg) at 2-4h compared to saline. Furthermore, the fact that the ‘overswing’ response appeared to be greater at 8-24h for PYY3-36-αLT may reflect that this peptide was initially more potent than PYY3-36, resulting in a greater homeostatic drive to eat once the effects of low dose PYY3-36-αLT had worn off (Parkinson et al., 2008). The acute anorectic effects of PYY3-36-αLT were also tested at higher doses (500, 1000, 5000 and 10,000nmol/kg). The lowest PYY3-36 dose (500nmol/kg) reduced food intake until the 2-4h interval, but did not significantly reduce cumulative food intake over 24h. In contrast, the same PYY3-36-αLT dose (500nmol/kg) significantly reduced food intake up to the 8-24h interval and reduced cumulative food intake over 48h post-injection. These animals ate significantly less than animals administered PYY3-36 (500nmol/kg) at these time points. PYY3-36-αLT dose dependently reduced food intake over 48h compared to saline and PYY3-36 controls. Interestingly, animals administered PYY3-36-αLT (1000nmol/kg) ate significantly less than animals treated with a ten-fold higher dose of PYY3-36 at the 8-24h interval and over 48h post-injection. This suggests that the αLT substitution at positions 16-23 markedly improves the long term bioactivity of PYY3-36. In order to investigate whether the more potent and longer-lasting effect of PYY3-36-αLT was due to activating pathways that produce aversion, the effects of PYY3-36-αLT on behaviour were examined. LiCl, an emetic agent that causes anorexia after peripheral administration in rodents by increasing behavioural aversion to food, was used as a positive control (McCann et al., 1989; Nachman and Ashe, 1973). In rodents, LiCl produces a learned aversion to palatable substances, such as sucrose solution (15%), when administered at the same time (Eckel and Ossenkopp, 1996; Nachman and Ashe, 1973; Ossenkopp and Eckel, 1995). PYY3-36-αLT at 500nmol/kg significantly decreased feeding and increased grooming behaviour compared to saline controls. Excess grooming has been hypothesized to occur during the induction of a stress response in rodents, suggesting that PYY3-36-αLT may activate stress circuits in the brain, though this requires further investigation (Rodriguez Echandia et al., 1983; Kametani, 1988). However, injection of PYY3-36-
αLT may also cause skin irritation at the injection site, which would explain its acute effect on grooming. Between 1-2h post-injection PYY3-36-αLT had no effects on grooming behaviour, but still reduced food intake compared to saline controls. This suggests that the anorectic effects of PYY3-36-αLT are independent of any stress response induced by PYY3-36-αLT. LiCl significantly reduced food intake with more potency than PYY3-36 in the first hour post-injection, and increased head-down behaviour, which is characterised by an abnormal, hunched posture and pilo-erection. Importantly head-down behaviour was not observed in mice administered PYY3-36-αLT. It is possible that PYY3-36-αLT shifts the rodent behavioural satiety sequence (BSS), shortening the period in which feeding occurs and advancing grooming and resting periods usually observed following feeding (Halford et al., 1998). It has been hypothesized that agents that induce illness disrupt the BSS, and that natural satiety factors shift the BSS, without disrupting it (Scott et al., 2005; Antin et al., 1975). An iv injection of PYY3-36 after an overnight fast has been shown to shift the rodent behavioural satiety sequence to the left, shortening feeding time and therefore reducing the latency to resting behaviour. PYY3-36 did not result in aversive effects in this study, and its effects on behaviour were inhibited by pre-treatment with a Y2R antagonist, indicating that the effect of PYY3-36 on behaviour is specific to the Y2R (Scott et al., 2005). This study is in line with my results, as out of the total behaviours observed PYY3-36-αLT reduced the proportion of feeding in the first and second hours post-injection, and increased the percentage of resting by 10% compared to saline in the 2nd hour post-injection. LiCl appeared to have a disruptive effect on the BSS, as it increased head-down behaviour and reduced feeding in the 1st h, while in the 2nd h resting was approximately 27% lower than observed for saline-treated animals, and feeding behaviours returned to the levels observed for saline controls. These data suggest that the anorectic effects of PYY3-36-αLT in mice at this dose are not due to illness, but due to a shifted BSS. Further experiments testing the length of time spent feeding and the latency to rest for PYY3-36-αLT compared to PYY3-36 would be useful in clarifying any differences in potency of behavioural effects between PYY3-36 and PYY3-35-αLT.

PYY3-36-αLT was not any more susceptible to degradation by KBB membranes (see ch. 2) or the metalloproteases meprin β or NEP (see ch.3) in vitro compared to PYY3-36 (data not shown). This is in agreement with the fact that the cleavage sites of KBB
or meprin β on PYY3-36 were not altered in the sequence of PYY3-36-αLT. The fact that the affinity of PYY3-36-αLT is reduced by around 2-fold is unexpected considering the enhanced bioactivity of this analogue, and that most analogues with enhanced bioactivity investigated previously in this thesis exhibit either a maintained or increased Y2R binding affinity compared to PYY3-36. Further work investigating the intracellular response to PYY3-36-αLT using cAMP Y2R activity assays is required to validate this finding. Furthermore, binding studies using Y1R and Y5R overexpressing cell lines may help to identify whether PYY3-36-αLT is a more selective Y2R agonist, and cAMP assays using Y1R/Y5R overexpressing cells may clarify whether PYY3-36-αLT acts as an antagonist at either of these receptors, which might explain the increased longevity of this analogue in reducing food intake compared to PYY3-36 (Kanatani et al. 1996). As Y receptors that have effects on food intake are expressed in the brain, the ability of PYY3-36-αLT to cross the blood-brain-barrier may influence its bioactivity. PYY3-36 is thought to crosses the blood-brain-barrier by non-saturable transmembrane diffusion (Nonaka et al., 2003). The structure or charge of PYY3-36-αLT may enhance its ability to diffuse across the blood-brain barrier compared to PYY3-36, which might explain the enhanced bioactivity of PYY3-36-αLT (Banks, 2009). This could be examined by comparing the percentage difference in radiolabelled PYY3-36-αLT and PYY3-36 in the brain following peripheral administration.

4.5.2 The effect of the addition of N-Pro to PYY3-36-αLT

The addition of N-Pro to PYY3-36-αLT slightly reduced the binding affinity of PYY3-36-αLT. Previous studies showed that PYY3-36 N-Pro exhibits a more prolonged anorectic effect compared to PYY3-36 (see chapter 2). In full length PYY, the Pro residue at position 2 forms hydrophobic interactions with the conserved Tyr residue at position 27, which confers a more stable and well-defined conformation in solution when compared to PYY3-36 (Glover et al., 1984; Nygaard et al., 2006). Although the PP-fold is still observed in PYY3-36, it is less well-defined than in PYY1-36, which may render PYY3-36 more unstable and susceptible to proteolytic degradation (Nygaard et al., 2006). This is in agreement with my studies showing that PYY3-36 N-Pro is less susceptible to degradation by KBB when compared to PYY3-
36 (see chapter 2). PYY3-36-NPro-αLT (1000nmol/kg) had a significantly less potent effect on food intake compared to PYY3-36-αLT (1000nmol/kg) in the first hour, but exhibited a more prolonged anorectic effect, significantly reducing food intake at 8-24h post-injection compared to saline controls, when PYY3-36-αLT had no effect. This is in agreement with the hypothesis that the addition of Pro at the N-terminal of PYY3-36 and PYY3-36 analogues prolongs the bioactivity of PYY3-36 \textit{in vivo}, possibly due to a reduction in proteolytic degradation.

I next investigated whether the α-LT and/or N-Pro-αLT modifications increase the longevity of circulating PYY3-36 after administration to rats. Two methods were used for blood sampling. The first study carried out utilized a method involving serial sampling from the jugular vein of anaesthetized rats, a technique which allows withdrawal of blood from the same animal over consecutive time points. In the second study, plasma was acquired from trunk blood of rats decapitated at later time points post-injection, but including 30 and 60 min time points, in order to compare the plasma levels achieved using the two different techniques. Interestingly, PYY3-36-αLT (100nmol/kg) reached peak concentrations 5 min earlier than PYY3-36. Analysis of plasma levels from trunk blood of decapitated rats at later time points revealed that PYY3-36-αLT (100nmol/kg) plasma levels remained elevated compared to baseline at 2h, 6h and 24h post-injection, whereas PYY3-36 levels returned to baseline levels by 2h post-injection. PYY3-36-NPro-αLT reached its $C_{\text{max}}$ 15 min later than PYY3-36. Like PYY3-36-αLT, PYY3-36-NPro-αLT (100nmol/kg) plasma levels remained elevated compared to baseline at 2h, 6h and 24h post-injection. This suggests that PYY3-36-αLT and PYY3-36-NPro-αLT may be less prone to clearance or proteolytic degradation than PYY3-36 \textit{in vivo}, which may explain the more potent and prolonged effect of PYY3-36-αLT and PYY3-36-NPro-αLT on food intake.

I also investigated possible methods to increase the duration of action of the PYY3-36 and PYY3-36 analogues \textit{in vivo}. A longer plasma half-life is a favourable property of a peptide drug as it increases the time between each injection, improving the practicality of the drug administration regimen. Pharmaceutical insulin formulations have been developed with altered pharmacodynamic properties. Several insulin formulations consist of Zn solutions and result in the formation of an insulin-Zn precipitate complex at neutral pH. This allows a slow sustained release of insulin
when sc administered (Brange and Langkjaer, 1997; Owens et al., 2001). The GLP-1 analogue, BMS-686117, forms a poorly water soluble adduct when added to a Zn solution. A 1.5:1 Zn/BMS-686117 suspension has a lower $C_{\text{max}}$ and a greatly extended duration of release, with peptide detectable in plasma over 24h from time of injection following sc administration in dogs when compared to the administration of BMS-686117 without Zn(II), which lasts up to 8h post-injection (Qian et al., 2009).

The ability of PYY3-36, PYY3-36-αLT and PYY3-36-NPro-αLT to precipitate at physiological pH (the pH encountered after administration in vivo) was examined in vitro. This was tested either in a saline solution or in a saline solution with Zn. All four peptides completely dissolved in a saline solution at a concentration of 10 mg/ml at pH 7.4. In the presence of Zn at a molar concentration of 2:1 Zn: peptide, a minor percentage of PYY3-36 precipitated out of solution. Interestingly, a much larger percentage of peptide precipitated out of solution for both PYY3-36-αLT and PYY3-36-NPro-αLT in the presence of Zn, and after a 5 hour wash in pH7.4 saline, about 10-30% of the original peptide concentration still remained in precipitate form. This suggests that the PYY3-36 analogues with the altered α-helical regions have a greater tendency to form a Zn-peptide complex than PYY3-36 and are more likely to form a precipitate when injected into subcutaneous tissue, which is at neutral pH. The fact that plasma levels of PYY3-36-αLT and PYY3-36-NPro-αLT took longer to return to baseline than PYY3-36 may suggest that these peptides may have a small natural tendency to precipitate in the subcutaneous tissue. Therefore, the effect of the addition of His residues to PYY3-36-NPro-αLT was investigated, in the hope that the precipitation profile might be even further improved by the ability of Zn to interact with His residues (Brange and Langkjaer, 1997; Owens et al., 2001). His residues were substituted at positions 4, 6, 19 and 30, since in the structure/affinity studies described in chapter 2, although His did not improve bioactivity at these positions, analogues substituted with His or other positively charged amino acids retained the ability to bind the Y2R and reduce food intake. PYY3-36 also has a naturally occurring His residue at position 26. PYY3-36-NPro-αLT-4H (10mg/ml) had precipitated in vitro when in a saline solution at pH 7.4 without Zn, probably due to the fact that the increase in the number of His residues altered the charge and isoelectric point of this peptide. After five 1 hour incubations at 37°C, over 72% of the original peptide remained in precipitate form. When in a saline solution at a 2:1
Zn ratio, PYY3-36-NPro-αLT-4H completely precipitated and no peptide was detected in solution after the 5 washes, leaving 100% in the final precipitate. These results prompted further investigation into the effects of Zn on the pharmacokinetic profile of PYY3-36-NPro-αLT-4H in vivo. A similar concentration as used in vitro was used to administer PYY3-36-NPro-αLT-4H in vivo (10mg/ml in solution ~ 300nmol/kg in 20µl). When administered without Zn, a peak in PYY3-36-NPro-αLT-4H levels was detected at 15min, followed by a gradual decrease in peptide levels until 24h post-administration, when peptide levels dropped to around PYY3-36 basal levels. Interestingly, the opposite effect was observed when PYY3-36-NPro-αLT-4H was co-administered with a 1:1 Zn/peptide molar ratio. A lower molar concentration of Zn was used as the precipitation studies indicated that all PYY3-36-NPro-αLT-4H remained precipitated even after 5 washes, and this is not entirely favorable in vivo, as some peptide needs to dissolve in order to observe an anorectic effect. Peptide plasma levels slowly and gradually increased, until significantly higher levels were observed at 24h compared to basal and PYY3-36-NPro-αLT-4H without Zn peptide levels. This suggests that the increased proportion of charged His residues promote interaction between PYY3-36-NPro-αLT-4H and Zn in the sc depot, allowing slow release into the circulation. The effect of PYY3-36-NPro-αLT-4H on food intake was then compared to PYY3-36-αLT and PYY3-36-NPro-αLT in order to determine whether the increase in charge brought about by the His residues had a detrimental effect on bioactivity. PYY3-36-NPro-αLT-4H (1000nmol/kg) only reduced food intake up until 4-8h post-injection, whereas the other analogues were effective for significantly longer than PYY3-36-NPro-αLT-4H, reducing food intake up until 8-24h compared to saline and PYY3-36-NPro-αLT-4H and 24-48h compared to PYY3-36-NPro-αLT-4H. This reduction in potency of PYY3-36-NPro-αLT-4H may be attributable to the fact that its Y2R affinity was reduced by 4.6 fold compared to PYY3-36. I hypothesized that the improved pharmacokinetic profile after PYY3-36-NPro-αLT-4H administration with Zn may prevent a reduced bioactivity associated with decreased Y2R affinity. I therefore decided to investigate the effects of all three analogues administered with Zn on chronic food intake and body weight in a mouse model of diet-induced obesity.
4.5.3 The effect of chronic administration of PYY3-36-αLT, PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H

The male DIO C57BL/6 model was used for the mouse chronic study. When ad lib fed on a high fat diet this strain of mice is highly susceptible to developing obesity, showing a marked increase in body weight gain and adiposity compared to other much more obesity resistant strains such as the A/J mouse (Surwit et al., 1995). In addition to the obese phenotype, the DIO C57BL/6 mouse model develops other disease phenotypes commonly associated with human obesity, including insulin resistance, glucose intolerance, dyslipidaemia, leptin resistance and hypertension, making this phenotypical strain a suitable model for assessing novel anti-obesity therapies (Parekh et al., 1998; Winzell and Ahren, 2004).

PYY3-36 did not significantly reduce food intake or body weight over the 49 day study period; although towards the end of the study a trend towards a reduction in body weight was observed. This may be due to the acclimatization of the saline group to the study procedure with time, resulting in this group gaining weight towards the end of the study. The reduced potency for the PYY3-36 group may be due to a compensatory increase in food intake between the daily injections or changes in circulating levels of other neuroendocrine signals or the expression levels of their receptors. Parkinson and co-workers have demonstrated that PYY3-36 administered ip during the early light phase to ad lib fed mice acutely decreases food intake, but increases food intake during the dark phase (Parkinson et al., 2008).

PYY3-36-αLT administered daily with Zn significantly reduced food intake over the 49 day study period, an effect which did not appear to wane with time when compared to saline. This analogue also significantly reduced body weight gain over the entire study period compared to saline and PYY3-36. This resulted in a reduction of about 10% body weight compared to PYY3-36 and saline and a significant reduction in the percentage of body fat compared to saline by the end of the study. In contrast, the animals administered PYY3-36-NPro-αLT exhibited a less potent effect on food intake during the study compared to the effect that was observed for PYY3-36-αLT. However, by the end of the study these mice exhibited a similar % weight loss to mice treated with PYY3-36-αLT.
PYY3-36-NPro-αLT-4H has a lower affinity to the Y2R and a less potent anorectic effect compared to PYY3-36-αLT. Daily administration of PYY3-36-NPro-αLT-4H at a 1:1 Zn/peptide molar ratio may mimic peptide release from an osmotic minipump in that a continuous low release of peptide from a sc depot leads to a slow, but gradual increase in peptide plasma levels over 24 hours. Continuous sc administration of PYY3-36 using osmotic minipumps produces anorectic effects that are effective for only 3-4 days in lean and obese mice and rats (Pittner et al., 2004; Tschop et al., 2004) while intermittent infusion of PYY3-36 over a 21 day study period produces a sustained reduction in daily food intake and reduces body weight gain and fat deposition (Chelikani et al., 2006a). In a seven day chronic PYY3-36 osmotic minipump infusion study, a reduction in plasma leptin levels was observed after the first day of infusion, which may contribute to a greater drive to eat (Unniappan and Kieffer, 2008). In fact, the continuous administration of several other anorexigenic peptides including CCK and GLP-1 also results in transient anorectic effects which are not sustained longer than a few days (Al-Barazanji et al., 2000; Arnelo et al., 1996; Crawley and Beinfeld, 1983; Donahey et al., 1998; Lukaszewski and Praissman, 1988). PYY3-36, as well as CCK and GLP-1, all bind to GPCRs and excess exposure of agonists to GPCRs often results in receptor downregulation and tolerance (Grady et al., 1997; Eison and Mullins, 1996; Barnes, Jr., 1996). In order to investigate whether PYY3-36 analogues that more readily form a sc depot in vitro may result in tolerance and escape due to a similar plasma release profile as observed for chronic PYY3-36 administration by osmotic minipumps, acute feeding was monitored at the beginning and after 26 days of the chronic feeding study. Interestingly, over 0-4h following injection on both day 1 and day 26, PYY3-36 and PYY3-36-αLT significantly reduced food intake, PYY-36-NPro-αLT and PYY3-36-NPro-αLT-4H had no effect on food intake compared to vehicle controls. The maintained acute effect of PYY3-36 after 26 days suggests that the lack of effect on chronic food intake and body weight is not due to acute receptor downregulation, but may be due to a compensatory increase in feeding at later time points post-injection. The fact that PYY3-36-αLT reduced food intake with a similar potency to PYY3-36 suggests that its enhanced potency may be due to a lack of compensatory orexigenic drive, though this requires further investigation. Results from the in vitro precipitation studies suggested that PYY3-36-NPro-αLT and PYY3-36-NPro-αLT-4H have a
natural tendency to precipitate at physiological pH in contrast to PYY-36 and PYY3-36-αLT, which did not. Furthermore, the ability of these two analogues to precipitate was enhanced in the presence of Zn, with 100% of peptide initially precipitating at physiological pH, which suggested that these analogues were likely to form a sc slow-release depot in vivo. Therefore, the lack of effect of these analogues on acute 0-4h food intake, and their delayed anorectic effect occurring over 4-8h post-injection were expected. The fact that chronic treatment of a diet-induced obese mouse model with PYY3-36-NPro-αLT-4H did not result in reduced body weight gain may be due to a high amount of precipitation in the presence of Zn, coupled with its reduced Y2R affinity. Altering the ratio of Zn to peptide may therefore improve the chronic bioactivity of this analogue. Current ongoing studies in dogs have shown that a single dose of PYY3-36-NPro-αLT-4H can achieve sustained, steadily rising plasma levels over a week without inducing nausea, in contrast to PYY3-36, which results in a sharp rise in plasma concentrations associated with vomiting, that is then followed by a return to basal plasma levels by 24h. Whether or not this ‘slow-release’ profile is sufficient to achieve weight loss is currently under investigation in our laboratory.

In conclusion, the work described in this chapter shows that substitution of positions 16-23 of the α-helix of PYY3-36 with the conserved epitope αLT enhances the bioactivity and plasma longevity of PYY3-36 without inducing behavioural aversion, and despite a reduced Y2R affinity. As found in previous studies described in this thesis, the addition of N-Pro to PYY3-36-αLT did not alter Y2R affinity, but enhanced the longevity of PYY3-36-αLT after acute administration, though this was less effective than PYY3-36-αLT in reducing body weight gain during chronic administration. Finally, a Zn precipitation method was developed that significantly enhanced the precipitation of PYY3-36, PYY3-36-αLT and most notably, PYY3-36-NPro-αLT at physiological pH. An analogue based on the structure of PYY3-36-NPro-αLT, PYY3-36-NPro-αLT-4H, was found to completely precipitate at physiological pH in the presence of Zn, and remain insoluble over five hour long washes at physiological pH in vitro, and plasma levels remaining elevated by 24h post-injection in vivo, suggesting a slow-release profile. Further investigations are required to elucidate the position and number of histidines in the PYY3-36-αLT sequence required to give a suitable pharmacokinetic profile when co-administered with Zn while minimally attenuating the anorectic effects. However, due to the
markedly increased duration of anorectic action and the enhanced plasma longevity of the PYY3-36-αLT-based analogues, these peptides may provide a framework for the design of further PYY3-36 analogues for the development of anti-obesity therapeutic.
Chapter 5: Final Discussion
The increasing prevalence of obesity is due to interplay between current environmental factors (such as cheap, calorie-dense food sources and the reduced requirement for physical exercise) and genetic factors that predispose to weight gain (Weinsier et al., 1998; Bloom et al., 2008). Understanding appetite regulatory systems may facilitate the design and development of novel anti-obesity therapies. Targeting endogenous gut hormones for their use in such therapies may be more promising than previously investigated appetite suppressing agents, such as rimonabant and sibutramine, which have ubiquitously-expressed receptors that regulate multi-functional neurotransmitter systems. Such drugs often have a multitude of side effects; both rimonabant and sibutramine have been withdrawn from the market because of their side effect profile (Bloom et al., 2008; Heal et al., 2009; Kulkarni, 2008; Kulkarni, 2010). The most effective treatment for obesity is bariatric surgery, but it is impractical to apply to the general obese population due to its cost and risk of mortality. Recent research has suggested that increased circulating PYY contributes to the reduced appetite and body weight gain observed after bariatric surgery, suggesting mimicking the post-bariatric state by peripherally administering PYY to the obese may be a logical, less costly therapeutic approach (Korner et al., 2005; Le Roux et al., 2006a). However, the exogenous administration of gut hormones to promote weight loss in the obese has its own challenges. One major disadvantage is that gut hormones are extremely short-lived in the circulation, which limits their efficacy and ease of administration. The development of long-acting analogues of gut hormones is therefore an approach used by an increasing number of labs. GLP-1, for example, was used as the basis of the development of liraglutide, which is now used as a once-daily drug to treat diabetes type II (Croom and McCormack, 2009). Another disadvantage to using PYY and PYY3-36 as drugs is that the therapeutic window for dosage appears narrow, with supraphysiological plasma levels resulting in nausea and/or vomiting, and low plasma levels resulting in poor efficacy (Degen et al., 2005; Le Roux et al., 2008; Sloth et al., 2007b; Gantz et al., 2007).

My studies are divided into three sections. In the first section, I aimed to investigate the effects of altering different domains of the primary sequence of PYY3-36 to shed light on its structure-activity relationships, and to attempt to create more potent and/or longer acting analogues. In the second section I aimed to investigate how the native
peptide, PYY3-36, is cleared and degraded in the circulation, for the purpose of rational analogue design and/or the use of an enzyme inhibitor therapy to prolong the half-life of the native peptide. In the third section I aimed to alter the secondary structure of PYY3-36 by substituting part of its $\alpha$-helix. As this analogue had a more sustained anorectic effect than PYY3-36, it was then used as a framework to design further analogues implementing the findings of the structure activity studies of PYY3-36 described in the first section. In the third section, I also aimed to investigate altering the delivery solution for PYY3-36 analogues using a Zn precipitation system to allow analogues to form a subcutaneous, slow-release depot, in order to render them longer-acting and less likely to induce side effects when administered chronically to rodent models of obesity.

5.1 Final Conclusions and Future Prospects

5.1.1 Structure activity relationships and design of long-acting analogues

PYY3-36 has been shown to reduce food intake in both rodents and humans. This effect is preserved in obese humans, making PYY3-36 an attractive target in the development of long-acting analogues for the treatment of obesity. A series of studies were carried out to investigate whether alterations to PYY3-36 would provide insight into the sites important for receptor binding and susceptibility to proteolytic degradation. In line with the known importance of the C terminus binding hexapeptide, substitution of residues here markedly reduced the bioactivity of PYY3-36 (Ahn et al., 2009). Interestingly, only PYY3-36 Asn34 exhibited markedly reduced Y2R affinity. The worsened bioactivity but retained Y2R affinity observed for substitution of Val31 and Tyr36 suggests that their substitution with Lys and Phe respectively may reduce Y2R selectivity rather than affinity (Lindner et al., 2008). The binding hexapeptide is therefore an unlikely candidate domain for amino acid substitution for drug design. As the helicity of PYY3-36 is known to correlate with Y2R affinity, and residues in the $\alpha$-helix adjacent to the binding hexapeptide may guide the C terminus to the membrane water interface, position 30 was investigated as a potential site for drug design (Zerbe et al., 2006). Unexpectedly, the substitution of
Leu30 with other hydrophobic amino acids was detrimental to Y2R affinity and bioactivity, while substitution with positively charged amino acids improved bioactivity, warranting further investigation into position 30 as a potential target for PYY3-36 analogue design. In the α-helix of PYY3-36, replacing Arg19 with Lys or Ser23 with Ala had little effect on PYY3-36 bioactivity and Y2R affinity, suggesting that these sites have minor involvement in the structure activity relationships of PYY3-36, and are candidates for alteration to aid drug design. Residues within the N terminus may bind to the extracellular domains of the Y receptors, and may also be important in determining the amphipilicity and 3D folding of the molecule, protecting the mid-section and C terminus from proteolytic attack (Nygaard et al., 2006). The importance of the N terminus in Y2R affinity has been demonstrated by studies showing that truncated analogues bind the Y2R with high selectivity over the Y1R and Y5R, but with poor affinity (DeCarr et al., 2007).

Generally, substitutions that increased or maintained Y2R affinity, but that also increased susceptibility to proteolytic degradation resulted in an initial improvement, but a delayed reduction, in bioactivity. This suggests that Y2R binding affinity is important for the acute effects of PYY3-36 upon food intake, but that at later time points, increased susceptibility to degradation may reduce bioactivity of these analogues compared to PYY3-36. Within the N terminus, Lys4 appears to be critical to maintain Y2R affinity, while substitutions at Glu6 were able to enhance Y2R affinity. Interestingly, adding Pro to the N terminus of PYY3-36 vastly reduced the susceptibility of the molecule to proteolytic degradation and maintained Y2R affinity, an effect that appeared to be dependent on steric conformation. This suggests that the N terminus is important in Y2R affinity, possibly due to alteration of the 3D structure of PYY3-36, as Pro2 and Tyr27 interact in PYY, stabilising its conformation (Nygaard et al., 2006; Schwartz et al., 1990; Glover et al., 1984). Furthermore, N-Pro was the only simple modification able to significantly improve the effect of PYY3-36 on bioactivity at later time points. The fact that when added to the N terminus of PYY3-36, Cys reduced proteolytic degradation to a similar degree as Pro, but was detrimental to Y2R affinity, suggests that elongating the N terminus can prevent it from proteolytic attack, but that specific residues must be used to maintain Y2R affinity. In summary, all domains of PYY3-36 appear to be important to Y2R affinity and/or the anorectic effects of PYY3-36. Although there was a weaker correlation
between susceptibility to proteolytic degradation and the anorectic effects of PYY3-36, there were clear cases in which resistance to *in vitro* proteolytic degradation could explain enhanced *in vivo* bioactivity. In general, the effect of single substitutions on Y2R affinity, resistance to proteolytic degradation and food intake did not necessarily predict these effects when combined. For example, all combined analogues tested had improved Y2R affinity. Using a systematic approach, a combination of several substitutions in different domains of PYY3-36 were subsequently discovered which significantly increase the longevity of the anorectic effect of PYY from 2 hours to between 4 and 8 hours. Two analogues with changes to the N-terminal and α-helix of PYY3-36, PYY3-36 N-Pro Ala23 Lys30 and PYY3-36 N-Pro Ser6 Ala23 Lys30, showed a significantly more potent effect over 24 hours post-administration compared to PYY3-36. These substitutions also increased affinity for the human Y2 receptor, which implies that they may be beneficial in the development of PYY3-36 analogues for use in the treatment of human obesity.

Additional modifications to PYY3-36 were investigated for their ability to improve the longevity of the molecule, in particular the addition of an acyl side chain. Previous work has indicated that intermittent, twice-daily peripheral administration of PYY3-36 can reduce food intake and adiposity in rodents (Chelikani et al., 2006a). However, such frequent administration to humans is impractical and costly. Such regimens may be circumvented by a long-acting analogue of PYY3-36 that requires less frequent administration. The addition of an acyl group to the N terminus of PYY3-36 completely prevents proteolytic degradation *in vitro*. This translated to a detrimental effect on acute bioactivity, perhaps due to reduced Y2R affinity, but significantly enhanced bioactivity at later time points, consistent with reduced *in vitro* proteolytic degradation. Combining the Lys12-lauroyl substitution with a steric change at position 3 also enhanced PYY3-36 bioactivity. This data warrants further investigation into using acylated Lys substitutions to prolong PYY3-36 bioactivity. Careful positioning of the acyl group, and/or combining acyl group additions with substitutions known to enhance Y2R affinity is a feasible aim for future investigations. Acylation of the naturally-occurring lysines in native GLP-1 to generate a longer acting peptide has been successfully accomplished for liraglutide (Degn et al., 2004). Apart from receptor binding and susceptibility to degradation, there are a number of parameters that can be investigated in structure activity...
relationships, such as Y2R selectivity compared to Y1R and Y5R, bioavailability of the peptide to the receptors expressed in the CNS, and efflux mechanisms affecting the ‘on-time’ of a peptide at its receptor. Therefore, future work may aim to investigate these parameters in investigating structure activity relationships where the altered bioactivity of an analogue is not explained by changes in receptor affinity or susceptibility to proteolytic degradation.

5.1.2 Insight into the metabolic clearance and degradation of PYY3-36 for design of analogues resistant to specific proteases and/or the use of protease inhibitor therapies

A series of studies were carried out to investigate whether the kidney is an important site for PYY3-36 clearance, as observed for other gut hormones such as OXM (Kervran et al., 1990). As chronic renal failure patients exhibit high levels of PYY and are cachectic, the kidney was hypothesized to be an important anatomical site for the inactivation of PYY (Hegbrant et al., 1991). The results suggested that the kidney is initially important for removal of PYY3-36 from the circulation, but it is not the sole location where this occurs. Further studies aimed to examine whether the metalloendoproteases NEP and meprin B, which are known to cleave PYY1-36, are important in the breakdown of PYY3-36. Meprin-β potently degrades PYY3-36, acting at a number of acidic sites in the N terminus. Cleavage of the N terminus of PYY3-36 may be important in allowing further sequential proteolysis by other enzymes, or may enhance kidney filtration. Inhibition of meprin β in vivo allows PYY3-36 levels to remain elevated for longer and prolongs the effect of PYY3-36 on food intake by 2 hours. The effect of actinonin on physiological levels of PYY3-36, as well as other anorectic gut hormones, including GLP-1, OXM and PP, needs to be further investigated to determine whether actinonin may be useful in manipulating circulating levels of PYY, PYY3-36 and other gut hormones. NEP did not degrade PYY3-36 as potently as it did positive controls, or compared to its previously reported potency in degrading PYY1-36 (Medeiros and Turner, 1994). As PYY1-36 and PYY3-36 exhibit markedly different 3D conformations, the tertiary structure of PYY1-36 may be more prone to NEP-induced catabolism, though this remains to be investigated. Inactivation of NEP in vivo slightly prolongs the anorectic effect of PYY3-36. A recent study showed that NEP will only cleave the natriuretic hormone,
B-type natriuretic peptide (BNP) after it has been metabolised by meprin A (Pankow et al., 2007). Further work is required to investigate whether NEP may be important in sequential cleavage of bioactive fragments of PYY3-36, for example those produced after meprin B-induced hydrolysis. Taken together, these findings support a potential role for meprin β in the degradation of PYY3-36, and provide impetus for further investigation into using meprin inhibitors and/or designing meprin-resistant PYY3-36 analogues to prolong the anorectic effects of PYY3-36.

5.1.3 Use of αLT substitution to modify the 3D structure of PYY3-36 and Zn-precipitation systems to allow a slow release pharmacokinetic profile

These studies investigated whether modifying the two and three dimensional structure of PYY3-36 by substituting part of its α-helix with an epitope conserved between Lactroductus venom, Exendin-4 and GLP-1 would enhance the potency and/or longevity of PYY-36 (Fuhlendorff et al., 1990; Holz and Habener, 1998; Runge et al., 2008). Although this substitution slightly reduces Y2R affinity, PYY3-36-αLT results in a more sustained anorectic effect than PYY3-36, lasting up to 24h longer than PYY3-36 at the same dose. The effects of PYY3-36-αLT could not be attributed to behavioural aversion. Investigating the affinity of PYY3-36-αLT at the Y1R and Y5R will identify whether PYY3-36-αLT is a more selective Y2R agonist, and cAMP assays using Y1R/Y5R overexpressing cells may clarify whether PYY3-36-αLT acts as an antagonist at either of these receptors, which might explain the increased longevity of this analogue in reducing food intake compared to PYY3-36 (Kanatani et al. 1996). Indeed, there has been interest from pharmaceutical companies in the use of Y1R and Y5R antagonists as anti-obesity therapies (Parker et al., 2002). The putatively increased ability of PYY3-36-αLT to diffuse across the blood-brain barrier compared to PYY3-36, might also explain the enhanced bioactivity of PYY3-36-αLT (Banks, 2009).

Pro was added to the N terminus of PYY3-36-αLT as this substitution prolonged the anorectic effect of PYY3-36 in the analogue PYY3-36-NPro. Pro2 is conserved within members of the PP-fold family and has been shown by X-ray crystallography to form hydrophobic bonds with the conserved tyrosine residue at position 27,
stabilising the molecule (Glover et al., 1984; Nygaard et al., 2006). The analogue PYY3-36-NPro-αLT exhibited a slightly prolonged anorectic effect compared to PYY3-36-αLT. Interestingly, the plasma levels of PYY3-36-αLT and PYY3-36-NPro-αLT remain elevated for longer than PYY3-36, which may explain the prolonged bioactivity of these analogues.

A common side effect of the exogenous administration of gut peptides, including PYY3-36 (Le Roux et al., 2008), CCK (Greenough et al., 1998), OXM (Wynne et al., 2005) and GLP-1 (Naslund et al., 2004), is the induction of nausea at supraphysiological plasma concentrations. Nausea has also been reported following the administration of the stable GLP-1 analogues liraglutide (Astrup et al., 2009) and exenatide (Kolterman et al., 2003). However, by dose titration of exenatide (Fineman et al., 2004) and continued use of liraglutide (Astrup et al., 2009) the incidence of nausea in human volunteers was almost completely abated. This may not be possible for PYY3-36, as it has an extremely narrow therapeutic window. In order to circumvent the side effects such as nausea and gastrointestinal discomfort associated with supraphysiological doses of PYY3-36; I investigated the use of Zn in allowing slow-release and increased duration of action of PYY3-36 and PYY3-36 analogues in vivo. Such an approach has been previously described for insulin/Zn formulations, which result in the formation of an insulin-Zn precipitate complex at physiological pH, allowing the slow sustained release of insulin (Brange and Langkjaer, 1997; Owens et al., 2001). A similar use of Zn has also been described for a GLP-1 analogue, which prolongs its half-life and bioactivity (Qian et al., 2009).

PYY3-36-αLT and to PYY3-36-NPro-αLT are less soluble than PYY3-36 when combined with Zn at physiological pH. This suggests these substitutions may promote formation of a Zn-peptide complex and may thus be more likely to form a precipitate upon subcutaneous injection. This is in line with the finding that plasma levels of PYY3-36-αLT and PYY3-36-NPro-αLT took longer to return to baseline than PYY3-36 following subcutaneous injection. The effect of the addition of His residues to PYY3-36-NPro-αLT was therefore, investigated, in the hope that the precipitation profile might be further improved by the ability of Zn to interact with His residues (Brange and Langkjaer, 1997; Owens et al., 2001). His substitutions in PYY3-36-NPro-αLT-4H worsened Y2R affinity and bioactivity in vivo, but enhanced the ability
of the peptide to precipitate at physiological pH when administered with Zn in vitro. When administered in vivo, the analogue had an improved slow release pharmacokinetic profile, similar to that observed for the release of gut hormones through chronic osmotic minipumps. When chronically administered with Zn in vivo, PYY3-36-\(\alpha\)LT and PYY3-36-NPro-\(\alpha\)LT were equally effective in reducing body weight by around 10% in a rodent model of diet-induced obesity compared to PYY3-36, which did not significantly reduce food intake or body weight. In contrast, PYY3-36-NPro-\(\alpha\)LT-4H did not inhibit body weight gain, which may be due to a high amount of precipitation in the presence of Zn, coupled with its reduced Y2R affinity. Altering the ratio of Zn: peptide or the position of His residues may therefore improve the chronic bioactivity of this analogue. The fact that mice were still acutely sensitive to the effects of PYY3-36-NPro-\(\alpha\)LT-4H after around 1 month of daily injections suggest that the receptor desensitization and/or tolerance previously hypothesized to be associated with continuous administration of PYY and other gut hormones in osmotic minipumps, may not occur when following this administration regimen (Pittner et al., 2004; Donahey et al., 1998; Lukaszewski and Praissman, 1988; Crawley and Beinfeld, 1983; Tschop et al., 2004). Further work is warranted in species in which side effects are more evident, such as the dog or the human, in order to compare the effects of slow-release administration regimes and the effects of long-acting analogues such as PYY3-36-\(\alpha\)LT and PYY3-36-NPro-\(\alpha\)LT in their utility as potential anti-obesity therapies.

The narrow therapeutic window for PYY3-36 and the related obstacles in developing efficacious, yet tolerable treatments makes its use impractical to tackle the current obesity crisis. However, due to the markedly increased duration of anorectic action and the enhanced plasma longevity of the PYY3-36-\(\alpha\)LT-based analogues, these peptides may provide a framework for the design of further PYY3-36 analogues for the development of anti-obesity therapeutics.
References


Banks, W.A. (2009). Characteristics of compounds that cross the blood-brain barrier. BMC. Neurol. 9 Suppl 1, S3.


Ref Type: Thesis/Dissertation


References:


pancreatobiliary secretion, and gastric emptying of a mixed meal. Gastroenterology 95, 1344-1350.


Ref Type: Electronic Citation


Ref Type: Electronic Citation


Ref Type: Generic


## Appendices

### 6.1 Appendix I: Amino Acids

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6.2 Appendix II: Solutions used in Thesis

2M calcium chloride:
Dissolve 5.88g of CaCl2.2H2O in 20ml GDW and sterilise by passing through a 0.22μm filter. Store in 1ml aliquots at -20°C.

0.5M ethylenediaminetetra-acetic acid (EDTA):
Dissolve 186.1g C10H14H2O8Na2.2H2O in 800ml GDW and adjust to pH 8.0 with 1M NaOH. Make up to 1L with GDW.

GTE
Mix 2.5 ml 1M Tris-HCl, pH9.0, 2ml 0.5M EDTA and 5ml 18% glucose in GDW to a final volume of 100ml. Sterilise by passing through a 0.2μm filter.

Gel loading buffer
Mix 25% (v/v) glycerol, 0.1% (v/v) orange G, 25mM EDTA.

Isopropanol, Caesium Chloride saturated
Vigorously mix 100g CsCl2, 100mlGDW and 1L Isopropanol and leave to settle.

LB
Mix 10g sodium chloride, 10g tryptone and 5g yeast extract in GDW. Adjust pH to 7.5 with NaOH. Sterilise using an autoclave.

LB agar
Add 7g agar to 500ml LB, and then autoclave.

MPI:
Mix 30g sucrose, 4ml 0.5M EDTA and 5ml 1M Tris-HCL. Make up to 200ml with GDW.
0.1M 25kD PEI pH7
Dissolve 450mg PEI in 80ml GDW. Adjust to pH 7 with HCl. Make up to 100ml with water and filter solution.

3M potassium acetate:
dissolve 294.4g CH3COOK in 500ml GDW, add 115ml glacial acetic acid and GDW up to 1L.

10mg/ml RNase A:
dissolve 100mg RNase A in 10ml 10mM Tris-HCl, pH 7.5/15mM NaCl and boil for 15 mins. Allow to cool and distribute in to 1ml aliquots.

2M sodium acetate, pH 5.2:
dissolve 164.1g CH3COONa in 800ml GDW, adjust to pH 5.2 with glacial acetic acid and make up to 1L with GDW.

3M sodium acetate pH 4.6:
Dissolve 246.1g CH3COONa in 800ml GDW, adjust to pH 4.6 with glacial acetic acid and make up to 1L with GDW.

5M sodium chloride:
Dissolve 292.2g NaCl in 1L GDW.

20% sodium dodecyl sulphate (SDS):
Add 200g SDS to 800ml GDW, heat to 60°C while stirring. Allow to cool and make up to 1L with GDW.

10M sodium hydroxide:
Dissolve 400g of NaOH in 500ml GDW. Once dissolved, top up to 1L with GDW.

15% sucrose solution
Dissolve 150g sucrose in 1L GDW
50x TAE:
Dissolve 242g Tris in 843ml GDW and mix in 57ml glacial acetic acid and 100ml 0.5M C10H14H2O8Na2.2H2O.

100x TE:
Dissolve 121.1g Tris and 3.7g C10H14H2O8Na2.2H2O in 800ml GDW and adjust pH 7.5 with hydrochloric acid. Make up to 1L.

1M Tris-HCl, pH 7.5:
Dissolve 121.1g Tris in 800ml GDW and adjust to pH 7.5 with HCl. Make up to 1L.

2M Tris-HCl, pH 8.0:
Dissolve 121.1g Tris in 450ml GDW and adjust to pH 8.0 with HCl. Make up to 500ml.

TES
Mix 25ml 1M Tris-HCL pH 8, 5ml 5M NaCl and 5ml C10H14H2O8NA2.2H2O. Make up to 500ml with GDW.

6.3 Appendix III: Vectors

Y2R: Human cDNA clone (origene cDNA sequence generated by RT-PCR)
ATGGGTCCAATAGGTGCAGAGGCTGATGAGAACCAGACAGTGGAAGAAA
TGAAAGGTGGAAACATACGGGCCACAAAACACCTCAGAGGTGAACCTGGATCTGACCCTCCTGAGCCAGAGCTTATAGATAGTAC
AACTTCTCATATTGGCCTACTGCTCCATCATCTTGCTTGGGGTAATTG
GCAACTCCTTTGGTGATCCATGATCTTGAATTCAAGAGCAGTGCAGCACA
GTAACCAAACCTTTTTCCATTTGCAATCTGGGCTTGGGAGATCTTTGGTGGAAC
ACTCTGTGTACCCGTTCACACTTTACCTTAATTTGAGGAGTGGAAA
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CAAGTATCCACAATCCCTTGACAGTAATTGCCCTTGAGCCACAGGTG
CATCGTCTACCACCTAGAGAAGAATCTCAGCTCGTGTLCCAGTGCTTTTG
TTATGCGCCCTGAGCCCTGCAGTTATGCTGGCAGTTCCTGTCCTGCAACTTCCGCTGAGTATGCTGGGAGATCTTTGGTGAGCATCCTGTCCTGCTCTCTTCCGGTGCAGG
CCTGTAATCCAAAGGTGCCCTGGCAGGAGAAGACATCTATGGCAGTATGCTTTTGCTTGGCAGTATAT
Structure of CMV6-XL5 vector (taken from Origene website: www.origene.com)

An additional neomycin resistance gene (providing geneticin resistance) not shown in this diagram

6.4 Appendix iv: Radioimmunoassay

General Principles:

RIA is a technique that allows the detection and measurement of biological substances at very low concentrations from tissue or plasma samples. This method relies on the competition between radioactively-labelled and unlabelled antigen for a specific number of antibody binding sites. A fixed amount of antibody and $^{125}$I labelled antigen are added to the unlabelled antigen (samples or standard) and the reaction is allowed to reach equilibrium by incubation for a least 72 hours at 4°C. Samples with higher concentrations of antigen will result in less binding between the antibody
and labelled antigen. The antibody-bound antigen is then separated from the free antigen either by addition of dextran-coated charcoal (which binds free antigen in the charcoal pellet) or by complexing the primary antibody with a secondary antibody (free antigen remains in the supernatant) and then centrifuged to form the pellets. The supernatant and pellet are separated and the radioactivity in the bound and free components is measured in a ã-counter and the data used to construct a standard curve. By reference to the standard curve, the concentration of unknown samples can be calculated.

**Preparation of $^{125}\text{I}$-PYY Tracer**

All tracers were made in house by Prof M. Ghatei (Dept. of Investigative Medicine, Imperial College London). Peptides were iodinated using the iodogen method (Owji et al., 1995) and purified by HPLC using a C$_{18}$ column (Waters, Milford, CT, USA). In brief, the iodogen method adds a $^{125}\text{I}$ molecule onto a tyrosine residue in an oxidative reaction. Iodogen reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; Pierce Chemical Co., Rockford, IL, USA) is reacted with Na$^{125}\text{I}$ to oxidise the $^{125}\text{I}$ to the reactive iodine state, which subsequently incorporates into available tyrosine residues. The iodogen method requires the reaction (peptide in 0.2 M phosphate buffer (see appendix II), 37 MBq Na$^{125}\text{I}$, 23 nmol iodogen reagent) to be incubated at 22°C before the reaction products are purified by reverse-phase HPLC using a NovaPak C$_{18}$ column (Millipore, Milford, MA, USA). The column was equilibrated for 10 minutes with 15% (v/v) acetonitrile (AcN) solution in water containing 0.05% (v/v) trifluoroacetic acid (TFA), at a flow rate of 1 ml/min. The concentration of AcN was then gradually increased to 20-45% over the following 80 minutes and maintained at the final concentration of 45% for the next 10 minutes. Fractions were collected every 1.5 minutes into tubes containing 1 ml 20 mM HEPES, pH 11.0 (to neutralise the acidity of the collected fractions) and 0.3% (v/v) bovine serum albumin (BSA) (ICN Biochemicals Inc, Costa Mesa, CA, USA). The fractions were tested in an RIA and used at 1500 cpm/tube.
PYY3-36 antibody and PYY3-36 analogue antibodies

Plasma PYY1-36 and PYY3-36 were measured using a specific and sensitive RIA. The assay was performed using 0.06 M phosphate buffer with 0.3% BSA at a total volume of 700µl per tube and a sample volume of 1–100µl. The antiserum (Y21) was raised in rabbits against synthetic porcine PYY1-36 (Bachem Ltd. U.K), coupled to BSA, and used at a final dilution of 1:50,000. The Y21 antibody is specific for the C-terminal of PYY and reacts fully with human PYY1-36 and PYY3-36 and does not cross-react with PP, NPY or other known gut hormones. The PYY3-36 analogues had the following cross-reactivity with the Y21 antibody: PYY3-36-áLT and PYY3-36-NPro-áLT: 20-25% and PYY3-36-NPro-áLT-4H: 11%. Standard concentrations for the analogue RIAs were increased in order to allow the detection the respective peptides. A Standard concentration of 1pmol/ml was used for PYY3-36, 5pmol/ml was used for PYY-áLT and PYY3-36-NPro-áLT and 10pmol/ml was used for PYY3-36-NPro-áLT-4H. The assays were incubated for 3 days at 4ºC before separation of free and antibody-bound label by secondary antibody (sheep anti-rabbit antibody). The intra- and inter-assay coefficients of variation were 6% and 10% respectively.

PYY3-36/ PYY3-36 analogue assays were performed using 0.06M phosphate buffer and a standard curve was made for each assay using a solution of unlabelled antigen at a known concentration and made up to varying concentrations in the assay by adding in duplicate at volumes of 1, 2, 3, 5, 10, 20, 30, 50 and 100 µl to the assay buffer. All samples were assayed in duplicate. To measure for assay drift in antibody-antigen equilibrium, tubes with no sample (‘zero’ tubes) were placed at regular intervals throughout the assay and standard curves were performed at the beginning and end of each assay. Similar binding should be observed in the zero tubes and in both standard curves. The general structure of the RIA is outlined in the table below.
<table>
<thead>
<tr>
<th>Tubes</th>
<th>Content</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>NSB</td>
<td>No antibody is added to these tubes, allows to test for non-specific binding (NSB)</td>
</tr>
<tr>
<td>3-4</td>
<td>1/2 x</td>
<td>Half the amount of labelled antigen is added, allows to assess the integrity of the $^{125}\text{I}$ labeled antigen</td>
</tr>
<tr>
<td>5-6</td>
<td>2 x</td>
<td>Double the amount of labeled antigen is added, allows to assess the integrity of the $^{125}\text{I}$ labeled antigen. A large change in binding between 1/2 x, 2 x tubes and zero tubes suggest dissociation of $^{125}\text{I}$ from labeled antigen</td>
</tr>
<tr>
<td>7-10</td>
<td>Zero</td>
<td>Allows assessment of assay drift</td>
</tr>
<tr>
<td>11-12</td>
<td>Standard 1</td>
<td></td>
</tr>
<tr>
<td>13-14</td>
<td>Standard 2</td>
<td></td>
</tr>
<tr>
<td>15-16</td>
<td>Standard 3</td>
<td></td>
</tr>
<tr>
<td>17-18</td>
<td>Standard 4</td>
<td></td>
</tr>
<tr>
<td>19-20</td>
<td>Standard 5</td>
<td></td>
</tr>
<tr>
<td>21-22</td>
<td>Standard 6</td>
<td></td>
</tr>
<tr>
<td>23-24</td>
<td>Standard 7</td>
<td></td>
</tr>
<tr>
<td>23-26</td>
<td>Standard 8</td>
<td></td>
</tr>
<tr>
<td>27-28</td>
<td>Standard 9</td>
<td></td>
</tr>
<tr>
<td>29-30</td>
<td>Standard 10</td>
<td></td>
</tr>
<tr>
<td>31-32</td>
<td>Zero</td>
<td>Duplicate zeros were placed approximately every 50 tubes</td>
</tr>
<tr>
<td>33-</td>
<td>Samples</td>
<td></td>
</tr>
<tr>
<td>Final 2</td>
<td>Excess</td>
<td>Excess antibody added to assess immunological integrity of antibody</td>
</tr>
</tbody>
</table>

Table outlining the general structure of an RIA and tubes important for the assessment of performance of assay.

After incubation of RIAs for 72 hours at 4°C, free and bound peptides were separated using either charcoal adsorption or secondary antibody separation. For charcoal adsorption, the free fraction was separated using 4 mg charcoal/tube suspended in 0.06 M phosphate buffer with gelatin (Sigma-Aldrich) which was added immediately before centrifuging at 1500 g, 4°C for 20 minutes. For secondary antibody separation, 100 µl sheep anti-rabbit solid phase secondary antibody (Pharmacia Diagnostics, Uppsala, Sweden) was added to the samples and allowed to incubate for at least one hour. Immediately prior to centrifugation, 500 µl 0.01% Triton-X-100 solution was added to each tube. The samples were then immediately centrifuged at 1500 g, 4°C for 20 minutes. Bound and free label were separated and both the pellet and the supernatant counted for 180 seconds in a γ counter (model NE1600, Thermo Electro Corporation, Reading, Berks, UK). Peptide concentrations in the samples were calculated using a non-linear plot (RIA Software, Thermo Electron Corporation) and results calculated in terms of the standard.
Manuscripts and publications

Addison ML, Minnion JS, Shillito JC, Tan T, Field BC, Murphy KG, Ghatei MG & Bloom SR. Targeting meprin β metalloendopeptidase in prolonging the anorexigenic effect of PYY3-36 in mice (In submission to *Endocrinology*).


Hankir MK, Parkinson JRC, Minnion JS, Addison ML, Bloom SR & Bell JD. PYY3-36 and Pancreatic Polypeptide differentially regulate hypothalamic neuronal activity in mice in vivo as measured by Manganese Enhanced MRI (Accepted: *Journal of Neuroendocrinology*).

Presentations

British Society for Neuroendocrinology Meeting (June 2009: poster presented)
British Society for Endocrinology Annual Meeting (March 2010: poster presented)
Endo 2010: The 92nd Annual Meeting and Expo (June 2010: poster presented)