University of Dundee

DOCTOR OF PHILOSOPHY

Investigation of KATP channel function in response to metabolic and pharmacological manipulation, in the hypothalamic GT1-7 cell line

Haythorne, Elizabeth

Award date:
2014

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 10. Jan. 2021
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declarations</td>
<td>IV-V</td>
</tr>
<tr>
<td>Summary</td>
<td>VI</td>
</tr>
<tr>
<td>List of figures</td>
<td>VII-IX</td>
</tr>
<tr>
<td>List of tables</td>
<td>X</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>XI</td>
</tr>
</tbody>
</table>

### Chapter 1 - Introduction

1.1 Diabetes 2  
1.2.1 Hypoglycaemia 2  
1.2.2 Recurrent hypoglycaemia and hypoglycaemia unawareness 3  
1.3.1 Brain glucose-sensing 4  
1.4 The role of glucose-sensing neurons in nutrient sensing 9  
1.5.1 Metabolic-dependent glucose-sensing 10  
1.5.2 The role of central K\textsubscript{ATP} channels in glucose-sensing 12  
1.5.3 The role of glucose transport in central glucose-sensing 17  
1.5.4 The role of Glucokinase in central glucose-sensing 18  
1.5.5 The role of AMPK in central glucose-sensing 20  
1.5.6 The role of mitochondrial respiration in central glucose-sensing 23  
1.6 Non-metabolic-dependent glucose-sensing neurons 25  
1.7 Non-neuronal central glucose-sensing 27  
1.8 Aim of investigation 29

### Chapter 2 – Materials and Methods

2.1 Cell culture 32  
2.2.1 Cell treatments 34  
2.2.2 Recurrent hypoglycaemia, \textit{in vitro} 34  
2.3.1 Electrophysiological recordings 36  
2.3.2 Whole-cell patch clamp configuration 37  
2.3.3 Perforated patch clamp configuration 39  
2.3.4 Inside-out patch clamp configuration 39  
2.4 The Nernst equation 42  
2.5 \textsuperscript{3}H-2-Deoxyglucose uptake assay 43  
2.6 \textsuperscript{14}C-Glucose oxidation assay 43  
2.7 AMPK activity assay 44  
2.8 Seahorse XF24 Extracellular Flux Analyser 48  
2.9.1 RNA extraction 50  
2.9.2 CDNA synthesis 50  
2.9.3 Real-time PCR 51  
2.10 NADP\textsuperscript{+}/NADPH assay 51  
2.11 ROS detection assay 52  
2.12 ATP assay 53  
2.13 ATP/ADP assay 54  
2.14 Pyruvate dehydrogenase activity assay 54  
2.15 Isocitrate dehydrogenase assay 55  
2.16 Bradford protein assay 55  
2.17 Data analysis 55
Chapter 3 – *In vitro* modeling of recurrent hypoglycaemia in GT1-7 cells

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>58</td>
</tr>
<tr>
<td>3.2</td>
<td>Results</td>
<td>61</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Antecedent low glucose exposure attenuates glucose sensing,</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.2</td>
<td>Recurrent low glucose exposure attenuates glucose-sensing,</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.3</td>
<td>Recurrent low glucose exposure does not alter the availability of</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>the K&lt;sub&gt;ATP&lt;/sub&gt; channel to conduct maximally</td>
<td></td>
</tr>
<tr>
<td>3.2.4</td>
<td>Glucose uptake is not altered by recurrent low glucose exposure,</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.5</td>
<td>Glucose oxidation and incorporation are unaltered following</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>RH, in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.6</td>
<td>Recurrent low glucose exposure alters glucose utilisation,</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.7</td>
<td>Recurrent low glucose exposure does not alter hexokinase activity,</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.8</td>
<td>Mitochondrial efficiency is altered following recurrent low glucose</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>exposure, in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.9</td>
<td>Pyruvate dehydrogenase activity is reduced following recurrent low</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>glucose exposure, in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.10</td>
<td>Recurrent low glucose exposure prevents the ATP/ADP ratio from</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>falling during acute low glucose exposure and reduces ATP,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.11</td>
<td>Recurrent low glucose exposure blunts the ability of AMPKα1,</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>but not AMPKα2, to activate in response to acute low glucose,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.12</td>
<td>Lentiviral shRNA knock-down of AMPKα1 prevents reduced OCR</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>associated with recurrent glucose exposure, in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.13</td>
<td>Lentiviral shRNA knock-down of AMPKα1 does not prevent</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>attenuated hyperpolarisation to low glucose, following recurrent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>glucose exposure, in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.14</td>
<td>Lentiviral shRNA knock-down of AMPKα1 does not prevent failure of</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>ATP/ADP ratio to fall in response to acute low glucose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>following recurrent glucose exposure, in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.15</td>
<td>Inhibition of the pentose phosphate pathway reduces OCR and ECAR</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>in GT1-7 cells following both control and recurrent low glucose</td>
<td></td>
</tr>
<tr>
<td>3.2.16</td>
<td>Recurrent low glucose exposure results in increased levels of</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>NADP&lt;sup&gt;+&lt;/sup&gt; and reduced NADPH, in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.17</td>
<td>Acute low glucose causes an increase in ROS but this is not</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>exacerbated following recurrent low glucose exposure, in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.18</td>
<td>Isocitrate dehydrogenase activity is unaltered by recurrent low</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>glucose exposure, in GT1-7 cells</td>
<td></td>
</tr>
<tr>
<td>3.2.19</td>
<td>Recurrent low glucose exposure reduces K&lt;sub&gt;ATP&lt;/sub&gt; channel</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>sensitivity to activation by MgADP, in the presence and absence of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MgATP</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>Discussion</td>
<td>120</td>
</tr>
</tbody>
</table>
Chapter 4 – Chronic exposure of hypothalamic GT1-7 cells to the SUR1-selective agonist, NN414, induces a stable inactive state for $\text{K}_{\text{ATP}}$ channels

4.1 Introduction 137
4.2 Results 140
4.2.1 Continuous NN414 blunts glucose counter regulation 140
4.2.2 Acute NN414 application hyperpolarises GT1-7 cells 141
4.2.3 Continuous NN414 attenuates $\text{K}_{\text{ATP}}$ channel conductance, in GT1-7 cells 142
4.2.4 Short-term NN414 exposure attenuates $\text{K}_{\text{ATP}}$ channel conductance, in GT1-7 cells 144
4.2.5 Continuous NN414 exposure attenuates hypoglycaemia sensing, in GT1-7 cells 144
4.2.6 Short-term NN414 exposure attenuates hypoglycaemia sensing, in GT1-7 cells 145
4.2.7 Antecedent NN414 exposure attenuates hypoglycaemia sensing in GT1-7 cells but does not suppress $\text{K}_{\text{ATP}}$ channel conductance 146
4.2.8 Continuous Diazoxide exposure reduces $\text{K}_{\text{ATP}}$ channel conductance, in GT1-7 cells 147
4.2.9 Chronic NN414-associated attenuated $\text{K}_{\text{ATP}}$ channel conductance is not due to membrane potential hyperpolarisation 148
4.2.10 Continuous NN414 exposure does not alter glucose metabolism, in GT1-7 cells 167
4.2.11 Continuous NN414 exposure does not alter the ATP/ADP ratio, in GT1-7 cells 171
4.2.12 Tolbutamide prevents chronic NN414-associated attenuated $\text{K}_{\text{ATP}}$ channel conductance 179
4.2.13 Continuous NN414 exposure reduces $\text{K}_{\text{ATP}}$ channel sensitivity to activation by MgADP and acute NN414 182
4.3 Discussion 188

Chapter 5 – Final conclusions 171

Chapter 6 – Appendices 203

Chapter 7 – References 206
Candidates Declaration

I hereby declare that all results described in this thesis, unless otherwise stated, are entirely my own work. I further state that the composition of this thesis was performed by myself and none of the material has been submitted for any other degree. Lastly, I verify that all sources have been appropriately cited. This work was carried out in the Medical Research Institute, University of Dundee under the supervision of Professor Rory McCrimmon.

Elizabeth Haythorne
Supervisor’s Declaration

I certify that Elizabeth Haythorne has completed 8 terms of experimental research and has fulfilled conditions of Ordinance 39, University of Dundee, such that she is eligible to submit the following thesis in application for the degree of Doctor of Philosophy.

Professor Rory McCrimmon
Summary

Animal and human studies have consistently demonstrated that recurrent hypoglycaemia (RH) blunts both hormonal and behavioral counter regulatory responses (CRR) to further episodes of hypoglycaemia. It is now well established that the brain is involved in regulating whole-body glucose homeostasis, including the CRR to hypoglycaemia. The aim of the current study was to investigate if adaptations occur, following RH, which are intrinsic to glucose-sensing neurons in the absence of synaptic/glial inputs or signals from the periphery. Utilising the GT1-7 hypothalamic mouse cell line as an in vitro model of homogenous glucose-excited neurons, the current study has demonstrated that recurrent low glucose exposure reprograms intracellular metabolism towards a “hypometabolic state”. This result occurs in conjunction with an attenuated ability of the cells to hyperpolarise in response to low glucose and a reduction in the sensitivity of the $K_{ATP}$ channel to activation by MgADP. In an attempt to reverse the changes observed in $K_{ATP}$ channel activity, the SUR1-selective $K_{ATP}$ channel opener, NN414, was applied chronically to GT1-7 cells. However, chronic $K_{ATP}$ channel activation severely reduced channel conductance and sensitivity to activation by MgADP and further NN414 application. These results suggest that chronic activation of the $K_{ATP}$ channel leads to the induction of a negative feedback mechanism to reduce channel activity. This may be in an attempt to maintain neuronal membrane potential within a physiological range. These results also suggest activation of central $K_{ATP}$ channels during RH may be driving the resulting defective CRR. However, adaptations in metabolism following RH may also be altering the function of central $K_{ATP}$ channels.
List of figures

Chapter 1 – Introduction

Figure 1. Schematic diagram of a glucose-excited neuron

Chapter 2 – Materials and Methods

Figure 2.1 Schematic diagram of the recurrent hypoglycaemia in vitro protocol
Figure 2.2 Patch-clamp configurations

Chapter 3 – In vitro modeling of recurrent hypoglycaemia in GT1-7 cells

Figure 3.1 Antecedent low glucose exposure results in attenuated glucose-sensing at 0.5 mM glucose
Figure 3.2 Recurrent low glucose exposure results in attenuated glucose-sensing at 0.5 mM glucose
Figure 3.2 Recurrent low glucose exposure results in attenuated glucose-sensing at 0.1 mM glucose
Figure 3.4 Recurrent low glucose exposure does not alter $K_{\text{ATP}}$ channel conductance in response to dialysis of cell with 0 ATP
Figure 3.5 Glucose uptake in GT1-7 cells is linear over 12-25 minutes
Figure 3.6 Glucose uptake is not altered by recurrent low glucose exposure, in GT1-7 cells
Figure 3.7 Glucose oxidation and incorporation is not altered by recurrent low glucose exposure, in GT1-7 cells
Figure 3.8 Glucose utilisation is reduced following recurrent low glucose exposure, in GT1-7 cells
Figure 3.9 Glycolysis in response to 2.5 mM glucose is attenuated following recurrent low glucose exposure, in GT1-7 cells
Figure 3.10 Hexokinase activity was unaltered following recurrent low glucose exposure, in GT1-7 cells
Figure 3.11 Efficiency of ATP synthesis was altered following recurrent low glucose exposure, in GT1-7 cells
Figure 3.12 Spare respiratory capacity was unaltered following recurrent low glucose exposure, in GT1-7 cells
Figure 3.13 Pyruvate dehydrogenase activity is unaltered following recurrent low glucose exposure, in GT1-7 cells
Figure 3.14 Acute low glucose exposure reduces ATP/ADP ratios in control GT1-7 cells but not following recurrent low glucose exposure
Figure 3.15 Acute low glucose exposure reduces intracellular ATP concentration in control GT1-7 cells but not following recurrent low glucose exposure
Figure 3.16 Acute low glucose exposure increases AMPKα2 activity in GT1-7 cells and is unaltered following recurrent low glucose exposure
Figure 3.17 Acute low glucose exposure increases AMPKα1 activity in GT1-7 cells and but is attenuated following recurrent low glucose exposure
Figure 3.18 Lentiviral shRNA knockdown of AMPKα1 specifically reduces expression of AMPK complexes containing the α1-subunit
Glucose utilisation is reduced following recurrent low glucose exposure, in shControl but not shAMPKα1 GT1-7 cells.

Recurrent low glucose exposure results in attenuated glucose-sensing at 0.5 mM glucose, in GT1-7 cells transfected with control lentiviral vector.

Recurrent low glucose exposure results in attenuated glucose-sensing at 0.5 mM glucose in, in GT1-7 cells when AMPKα1 is reduced.

Acute low glucose reduces the ATP/ADP ratios, in cells transfected with control lentiviral vector, but not following recurrent low glucose exposure.

Acute low glucose reduces the ATP/ADP ratios, in cells transfected with control shAMPKα1 lentivirus, but not following recurrent low glucose exposure.

6-AN reduces oxygen consumption and extracellular acidification rates in GT1-7 cells.

DHEA reduces oxygen consumption and extracellular acidification rates in GT1-7 cells.

Recurrent low glucose exposure leads to increased NADP⁺ and reduced NADPH levels in GT1-7 cells.

Acute low glucose increases levels of ROS in GT1-7 cells at 3 hours.

Recurrent low glucose exposure does not alter isocitrate dehydrogenase activity, in GT1-7 cells.

Recurrent low glucose exposure reduces K⁺ATP channel sensitivity to activation by MgADP.

Recurrent low glucose exposure reduces K⁺ATP channel sensitivity to activation by 200 μM MgADP in the presence of MgATP.

Recurrent low glucose exposure reduces K⁺ATP channel sensitivity to activation by 500 μM MgADP in the presence of MgATP.

Schematic diagram of the regulation of PDH activity.

Hypothetical diagram of the adaptations which occur in glucose-excited neurons following recurrent hypoglycaemia.

 Continuous NN414 leads to higher GIRs after RH mediated by further suppression of adrenaline secretion.

NN414 activates K⁺ATP channels and hyperpolarises the cell membrane in GT1-7 cells.

8 days of NN414 exposure reduces the availability of the K⁺ATP channel to activate maximally in response to dialysis of cell with 0 ATP.

3 hour NN414 exposure reduces the availability of the K⁺ATP channel to activate maximally in response to dialysis of cell with 0 ATP.

24 hour N414 exposure results in a trend for attenuated glucose-sensing at 0.7 mM glucose.

24 hour N414 exposure results in a trend for attenuated glucose-sensing at 0.5 mM glucose.

3 hour N414 exposure results in a trend for attenuated glucose-sensing at 0.7 mM glucose.
Figure 4.8 3 hour N414 exposure results in a trend for attenuated glucose-sensing at 0.5 mM glucose
Figure 4.9 Antecedent NN414 exposure results in a trend for attenuated glucose-sensing at 0.7 mM glucose
Figure 4.10 Antecedent NN414 exposure results in a trend for attenuated glucose-sensing at 0.5 mM glucose
Figure 4.11 Antecedent NN414 exposure does not alter the availability of the $K_{ATP}$ channel to activate maximally in response to dialysis of cell with 0 ATP
Figure 4.12 24 hour Diazoxide exposure reduces the availability of the $K_{ATP}$ channel to activate maximally in response to dialysis of cell with 0 ATP
Figure 4.13 3 hour Diazoxide exposure reduces the availability of the $K_{ATP}$ channel to activate maximally in response to dialysis of cell with 0 ATP
Figure 4.14 High external [K+] prevents the membrane potential reaching $E_K$ in response to acute NN414 activation
Figure 4.15 24 hour NN414-associated reduced $K_{ATP}$ channel conductance is not a result of membrane hyperpolarisation
Figure 4.16 3 hour NN414-associated reduced $K_{ATP}$ channel conductance is not a result of membrane hyperpolarisation
Figure 4.17 Glucose uptake is not altered by chronic NN414 exposure
Figure 4.18 Glucose oxidation and incorporation is not altered by chronic NN414 exposure
Figure 4.19 Hexokinase activity in GT1-7 cells is not altered by chronic NN414 exposure
Figure 4.20 Cellular oxygen consumption is not altered by chronic NN414 exposure
Figure 4.21 Efficiency of ATP synthesis is not altered by chronic NN414 exposure
Figure 4.22 Spare respiratory capacity is not altered by chronic NN414 exposure
Figure 4.23 ATP/ADP ratio is not altered by chronic NN414 exposure
Figure 4.24 Acute Tolbutamide application prevents membrane hyperpolarisation by NN414
Figure 4.25 Tolbutamide prevents chronic NN414-associated attenuation of $K_{ATP}$ channel conductance
Figure 4.26 24 hour NN414 exposure reduces $K_{ATP}$ channel sensitivity to activation by MgADP
Figure 4.27 24 hour NN414 exposure reduces $K_{ATP}$ channel sensitivity to activation by acute application of NN414
Figure 4.28 $K_{ATP}$ channels silenced by 24 hour NN414 exposure are still present at the plasma membrane

Chapter 6 – Appendices

Appendix Figure 1 Glycolysis is more stable in response to 5 or 25 mM glucose in GT1-7 cells
Appendix Figure 2 Results of glucose metabolism gene array performed on GT1-7 cells following recurrent low glucose exposure
List of tables

Chapter 2 – Materials and Methods

Table 2.1 Chemicals, antibodies and reagents
Table 2.2 GT1-7 media components
Table 2.3 Electrophysiological recording solutions
Table 2.4 AMPK assay buffers

Chapter 4 – Chronic exposure of hypothalamic GT1-7 cells to the SUR1-selective agonist, NN414, induces a stable inactive state for $K_{ATP}$ channels

Table 4.1. Maximum $K_{ATP}$ channel conductance densities following chronic NN414 or vehicle treatment
Acknowledgements

Firstly I would like to thank my supervisors Professor Mike Ashford and Professor Rory McCrimmon for the opportunity of undertaking this research and providing supervision over the past 4 years.

I would like to thank all the members of the Ashford and McCrimmon labs, past and present, for providing an excellent research environment in which to carry out this project. I am especially grateful to Dr Lee Hamilton for words of encouragement, advice and for passing on his infectious enthusiasm for science. Thanks to my fellow PhD students, Susan, Geoff and Fiona for always being available to vent away the stresses of the PhD with a gin and a Nandos club. I would also like to thank my family for their support and encouragement over the past 4 years. They will be relieved that my ‘eternal student’ status is now over.

Finally I would like to thank John for his relentless support and faith in me. I am especially grateful for his patience and understanding when I was “just patching one more cell”!