2011

Neuroscience Research: Related to the Neuropathology of Schizophrenia, Neuroendocrinology of Obesity, and Brain Map

Xu-Feng Huang

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Neuroscience Research: Related to the Neuropathology of Schizophrenia, Neuroendocrinology of Obesity, and Brain Map

A thesis submitted in fulfilment of the requirements for the award of the degree

DOCTOR OF SCIENCE

by Academic Publications

From

School of Health Sciences, University of Wollongong, Australia

By

Xu-Feng Huang, MBBS, PhD

2011
CERTIFICATION

I, Xu-Feng Huang, declare that this thesis, submitted in fulfilment of the requirements for the award of A Higher Doctoral Degree - Doctor of Science, at the University of Wollongong, is a collective body of my research publications over the last 15 years. These full research papers have standing as significant and sustained contributions to the knowledge of Neuroscience, related to the neuropathology of schizophrenia, neuroendocrinology of obesity and cyto-and chemo-architecture of human and monkey brains.

These publications are original and have not been submitted for any qualification of any tertiary institution for any other claim for degrees.

Xu-Feng Huang

March, 2011
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I. Executive Summary

My case for a Higher Doctoral Degree, DOCTOR OF SCIENCE, by academic publications in Neuroscience and Metabolism is based upon the originality, significant contribution to scientific knowledge, and international recognition of my research publications.

Goal: My research goal is to find the means to prevent and treat schizophrenia, obesity and their related metabolic disorders. My research has largely focused on the regulation of body metabolism in normal and diseased situations. I have employed modern molecular biology techniques to study the pathologies of the diseases using human tissue, animal models and cell culture. I have carried out some translational research aiming to translate new findings from basic research into novel pharmacological and clinical interventions for the benefit of human diseases.

Career: I have a Doctor degree in Medicine and PhD degree in Neuroscience. I have gained broad experience in medical research through working at the Medical College in China (1983-1988), the Schools of Medicine and Psychology of University of New South Wales (1989-1995), the School of Health Sciences at the University of Wollongong (1995-present), and Illawarra Health and Medical Research Institute (2010-present).

Publications: I have produced 273 publications including 3 books, 8 book chapters, 103 international peer-reviewed journal articles, 21 letters to the editor and 138 conference proceedings since 1993. My research articles have been cited 1,505 times and my books have been cited 857 times. My research ‘h-Index’ is 22.

Grants: I have been awarded numerous research grants for my research, totalling $3.8 million dollars including 9 NHMRC, 1 ARC Linkage, 1 AINSE and 1 NISDA fellowship since 2002, consisting of 35 research proposals.

Leadership: Since 1996, I have mentored 7 post-doctoral fellows and 6 research assistants. I have been the principal or co-supervisor of 14 successful PhD/Master degree research students. I have coordinated and taught a number of subjects for undergraduate students including anatomy, motor control and dysfunction, neuroscience and pathophysiology. I have been awarded Highly Commended for the Vice Chancellor’s Excellence in Research Supervision Award in 2009, and received the Vice Chancellor’s Excellence in Research Supervision Award in 2010 at the University of Wollongong.

**Contributions to discipline:** Scientific Director for the Illawarra Health and Medical Research Institute Metabolic Theme from 2009 to present; Director of the Centre for Translational Neuroscience at the University of Wollongong, 1999 to present; Council Member of the Schizophrenia Research Institute and a Panel Member of the Developmental Neurobiology of the Neuroscience Institute of Schizophrenia, 2000 to present; Director of the Schizophrenia Research Institute-Wollongong Centre for Collaborative Human Brain Research from 1999 to present. Member of the University Academic Senate from 1997 to 1999; Member of the Faculty Research Committee from 2009-present; and Member of the BioSafety Committee. I hold memberships with several national and international professional bodies including the American Association for the Advancement of Science; Australian Neuroscience Society; Australian Obesity Society; Society for Neuroscience (USA); North American Association for the study of Obesity and Australian Neuroscience Society.

**Invited talks:** I have been invited to give numerous talks including: Title: Understanding the progressive central dysregulation caused by the chronic consumption of a high-energy diet, AstraZeneca Company Advisory Board meeting, Nice, France, March 2001; Title: Target identification of chemical coded neural networks for treating chronic diet-induced obesity, Garvan Medical Research Institute, Sydney, Australia, September 2002; Title: Glutamate and serotonin receptor ligand-binding in the human cortex, AstraZeneca Company, Wilmington, USA, October 2002; Title: Integrative approaches to identify the key targets for treating the late stages of obesity, AstraZeneca Company, Sweden, November 2003; Title: Neurotransmitter bases of pathology and therapy of schizophrenia, POWMRI, October 2009; Title: Neuropathology of schizophrenia and obesity, Intelligent polymer research institute (IPRI), March 2010; Title: Nanomaterial in cancer therapy, Institute for superconducting and electronic materials (ISEM), August 2010; Title: Brain to the mind and shape: Schizophrenia and obesity, University Research Showcase Series, September 2010; Title: The role of Histamine in Obesity, Australian and New Zealand Obesity Society Annual scientific Meeting, Sydney, October 2010.

**II. Studies on Schizophrenia**

Schizophrenia is a major mental health disorder affecting 1% of the population worldwide, with more than 26,000 children born with schizophrenia in Australia in the last decade alone. Schizophrenia is a research priority both nationally and internationally. I have published more than 40 international peer-reviewed research papers and over 50 conference presentations in this difficult research area.

1. **Neuropathology of Schizophrenia:** Over the last 15 years, I have spent substantial amount of time investigating the brain pathology of schizophrenia. This type of work is exclusively based on examination of post mortem brain tissue of schizophrenia. I have studied numerous neurotransmitter systems covering both excitatory and inhibitory systems of the brain. I have also studied those brain areas most relevant to schizophrenia, including the prefrontal cortex, anterior and posterior cingulate cortices, superior temporal gyrus and hippocampal formation. My studies have formed a unique body of literature, supporting the evidence that the brain of schizophrenia patients has imbalanced neurotransmission between the excitatory and inhibitory systems. In particular, the neurotransmitter systems that I
have studied are the glutamatergic, cannabinoid, muscarinic, serotonergic and GABAergic systems.

a. Glutamate Receptors: The glutamatergic system is the most important excitatory neurotransmitter system in the brain. The literature in this area is controversial in terms of the role of this system in the pathology of schizophrenia. My group has made significant contributions to understanding the role of the glutamatergic system within specific brain areas involved in the pathology of schizophrenia. We reported that there are increased binding sites for glutamatergic NMDA receptors in the anterior and posterior cingulate cortices of schizophrenia (Neuropsychopharmacology, 2002, 826-33, cited 33 times; NeuroReport, 2005, 1363-67, cited 9 times). On the other hand, we found no changes in other glutamatergic metabotropic receptors such as the binding sites for AMPA and Kainate receptors in these brain regions. The cingulate cortex is an important area of the limbic system in the brain and is involved in information processing relevant to the mood, learning and memory, which are altered in schizophrenia. Therefore, correcting altered NMDAR expression could be an important strategy for treating schizophrenia.

b. Cannabinoid System: CB1 is the most important receptor of the cannabinoid system in the brain and has a number of important functions. Its relevance to schizophrenia largely lies in its modulation of dopaminergic neuronal activity, which is one of the main central components of the neuropathology of schizophrenia. My group’s studies have confirmed the importance of the CB1 receptor in schizophrenia. We found a significant 64% increase in CB1 receptor binding sites in the anterior cingulate cortex (Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2004, 355-60, cited 80 times) and a 25% increase in the posterior cingulate cortex (Experimental Research, 2006, 556-560, cited 34 times) of schizophrenia compared to controls. An altered expression of CB1 receptors in the cingulate cortex could be related to the neuropathology of schizophrenia, since CB1 receptors regulate motivation and attention.

c. Muscarinic Receptors: Muscarinic receptors play an important role in the regulation of cognitive function. It is thought that deficits in muscarinic receptor function contribute to an altered cognitive function in schizophrenia. We identified that muscarinic receptors are significantly decreased in the superior temporal gyrus of schizophrenia. We further reported that it is M1/4 receptors rather than M2 receptors that are altered in schizophrenia. These results provided strong evidence to suggest that M1 receptors may potentially be a good target for the development of drugs for treating schizophrenia (Journal of Neuroscience Research, 2005; 883-890, cited 29 times).

d. GABA(A) Receptors: GABA is a major inhibitory neurotransmitter in the brain. Our study showed increased GABA(A) receptors in the superior temporal cortex and posterior cingulate cortex of schizophrenia patients (Experimental Brain Research, 2006, 587-590, cited 19 times; Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2007, 225-233, cited 18 times). I believe there is a deficiency in GABA transmission in the superior temporal cortex and posterior cingulate cortex in schizophrenia.
2. **Antipsychotic drug induced obesity:** The second generation of antipsychotic drugs (also known as ‘atypical’ antipsychotics) are widely used in treating a variety of mental illnesses including schizophrenia. Atypical antipsychotics are more effective than the first generation drugs in terms of their efficacy in improving cognitive function for schizophrenia. However, these effective antipsychotics can lead to significant weight gain and an increased risk for obesity-related metabolic disorders including dyslipidaemia, diabetes and cardiovascular disease. These problems contribute to a serious lack of compliance in taking medication, which leads to symptom relapse plus a worsened long-term outcome, and results in a huge cost for society and the families of patients. My contribution to this area of research is aimed at: 1) establishing an animal model mimicking the metabolic disorder of patients following atypical antipsychotic drug treatment; 2) identifying the key brain areas and neurotransmitter/receptors causing the metabolic side effects; and 3) developing new compounds for a better treatment of schizophrenia.

a. **Development of an animal model for the study of antipsychotic-induced obesity and metabolic disorders:** Not all species of rodents will develop obesity following antipsychotic treatment. In order to establish a suitable model similar to the patient situations, I have carried out a number of studies using different species of animal models. I tested their responses to antipsychotic treatments and examined the side effects. After many trials, we have successfully established a rat model suitable for the study of antipsychotic-induced obesity. We have systematically investigated the response of central neurotransmitter systems after antipsychotic drug treatment, including the 5-HT system (Behavioural Brain Research, 2006, 355-62, cited 21 times), neuropeptide Y system (Neuropeptides, 2006, 213-219, cited 14 times) and histaminergic system (Psychoneuroendocrinology, 2008, 619-625, cited 12 times). The animal model is now well accepted in the literature and has made some significant advances to our understanding of the mechanism of the therapeutic effects as well as the side effects of antipsychotics.

b. **Identification of the key brain areas and neurotransmitter and receptors causing metabolic side effects following antipsychotic treatment:** Approximately 38% of patients on atypical antipsychotic treatment develop metabolic disorders. After carefully studying brain neurotransmitter systems, we have found that the histamine H1 receptor in the ventromedial hypothalamic nucleus plays a critical role in causing obesity. After extensive analyses, we found the histamine H1 receptor to be an excellent target to work on for the prevention and treatment of antipsychotic drug-induced obesity and metabolic disorders.

c. **Development of new compounds for treating schizophrenia:** In collaboration with experts from medicinal chemistry at the University of Wollongong, we have used computer modelling techniques to search for and design a better compound for treating schizophrenia based on the chemical structure of olanzapine. We have focused on the histamine H1 receptor and its function in the ventromedial hypothalamic nucleus. With a structure modification, we have now removed the H1 receptor antagonist site from the olanzapine molecule (Australian provisional patent, No AU2010901520). Hopefully, the new compound developed in our laboratory will be able to remove the obesity side effect while at the same time retain the therapeutic efficacy of...
olanzapine. This compound is in the development of validation and further in vivo phase studies.

3. **NMDA receptor and animal model of schizophrenia.** My study in this area is largely focused on the hypotheses of ‘developmental neurobiology’ and ‘phencyclidine (PCP) blockade of NMDA receptor’ of schizophrenia. Disruption of brain development is known to play a critical role in schizophrenia. This is because abnormal brain development can lead the brain becoming fragile to late aversive impact. PCP is the best known pharmacological agent that can induce schizophrenia-like symptoms in humans and animals. We have published four peer reviewed papers in this area. We showed that early interruption of the NMDA receptor can alter brain development in the dopaminergic, muscarinic, glutamatergic and GABAergic systems. More importantly, we have found that PCP administration can alter NMDAR-neuregulin 1 signalling (via NMDAR-PSD95-ErbB4-NRG1 pathway). This has advanced our understanding of the mechanisms of brain development and is relevant to schizophrenia as neuregulin 1 and its receptor erbB4 are candidate genes for schizophrenia risk.

**III. Studies on Obesity**

Obesity is a major health problem today. My research largely focuses on neuroendocrine regulation of body weight and metabolism. I have been studying the basic physiology and pathological condition of metabolic disorders of obesity. I am extremely interested in using functional food and herb molecules to prevent and treat obesity and its related metabolic disorders. Using modern molecular and biological techniques I have investigated and answered a number of key issues.

1. **Identifying the localisation of leptin receptor expression in the brain:** Leptin signalling plays a key role in body weight regulation for both energy intake and expenditure. I, together with my group members, have made a significant contribution to this area. We were the first to report the location of leptin receptor mRNA expression in the neurons of the hypothalamic arcuate nucleus in mice (NeuroReport, 1996, 15-17; cited 92 times). We showed that leptin receptor in the hypothalamic arcuate nucleus can be down regulated in the late stages of diet-induced obesity in mice, which is accompanied by glucose intolerance peripherally. We also showed that the downstream regulatory molecules of the leptin receptor are neuropeptide Y and proopiomelanocortin, which respond to high-fat diet and contribute to the development of diet-induced obesity (Brain Research, 2000, 89-95; Cited 86 times).

2. **Central leptin resistance theory in the late stage of diet-induced obesity:** Leptin, produced from adipocytes, can act on the neurons in the hypothalamic arcuate nucleus and promote negative energy balance against weight gain. Paradoxically, obesity has increased blood leptin concentration, indicating the existence of leptin resistance. Our group was the first to report central leptin resistance in the late stages of diet-induced obesity using a diet-induced obese mouse model (International Journal of Obesity, 2000, 639-46, cited 121 times). We showed, in a systematic fashion, how central leptin resistance progressively develops: being initiated peripherally and then becoming central with a loss of food intake inhibition and dramatic failure of energy balance regulation. My
group was the first to show how leptin signals central neurotransmitter systems regulating energy balance including leptin-leptin receptor-neuropeptide Y-pro-opiomelanocortin in diet-induced obesity (Brain Research, 2000, 89-95, cited 86 times). These findings distinguished the differences between physiological and pathophysiological conditions of obesity at the early and late stages. The central leptin resistance theory has contributed to obesity research significantly because it opened up a new avenue for considering and designing treatment regimes for human obesity.

3. **Individual susceptibility to obesity development.** It is known that some develop obesity but others do not while on a high-energy diet in both humans and rodents. My group was the first to establish mouse models of diet-induced obese and diet-resistant mice under an isocaloric diet (Brain Research Bulletin, 2000, 235-242; Brain Research, 2003, 9-19; cited 63 times). As mice are on the same diet, comparing the differences in the regulatory system for energy balance between diet-induced obese and diet-resistant mice has allowed us to identify a number of neuromodulators that play significant roles in the prevention of diet-induced obesity. We reported the existence of differential regulations of neuropeptide Y and its Y2 receptors, 5-HT2a, and its transporter enzyme in the diet-induced obese mice.

4. **Some, but not all, fats are obesogenic:** We know that saturated fats are obesogenic while n-3 polyunsaturated fats are not. My group tested gene responses in the central nervous system to different types of dietary fats. We reported that saturated fats differentially affect the mRNA expression of the hypothalamic leptin receptor, neuropeptide Y and agouti-related peptide and proopiomelanocortin compared to n-3 and n-6 polyunsaturated fatty acid diet in mice. A high saturated fat diet reduces neuropeptide Y and agouti-related peptide mRNA expression while n-3 and n-6 polyunsaturated fat diets do not. Down regulated neuropeptide Y and agouti-related peptide are unable to effectively counteract the obesogenic drive derived from a high saturated fat diet. On the other hand, high fat diets emphasizing polyunsaturated fatty acids can protect against obesity (American Journal of Physiology-Endocrinology and Metabolism, 2002, 1352-1359; cited 77 times).

5. **Dietary fiber and obesity:** I am very interested in dietary fiber studies as dietary fiber has been reported to have numerous beneficial effects to obesity and diabetes. I am particularly interested in the structure and function of beta glucans, which are a soluble dietary fiber. Our studies have shown that oat beta-glucans (soluble dietary fiber) increase postprandial cholecystokinin levels, decrease insulin response and extend subjective satiety in overweight subjects (Molecular Nutrition and Food Research, 2009, 1343-1351). Furthermore, we showed that a high beta-glucan diet increases PYY production, which can down-regulate positive energy balance in overweight and obese subjects (Nutrition Research, 2009, 705-9).

**IV. Mapping the Human and Monkey Brains**

The Institute of Medicine (US Academy of Science) reported that, ‘Mapping the Brain and its Functions’ urges the construction of ‘standard’ atlases of the human, monkey, and rodent brain. In 1905, Brodmann constructed a map of the monkey cortex. The
rhesus monkey has been the principal non-human primate used to test theories of human brain organisation and function. However, no comprehensive map of the cortex, let alone the entire brain, of this species has been attempted by anyone in the last century. In this project, I undertook a trip to UCLA where the brain of a rhesus monkey was imaged using MRI. The entire monkey brain was then sectioned at 800 μm intervals. Prof. Paxinos and I examined the sectioned monkey brain stained for 11 different neurochemicals. After 6 years of work, our book ‘The Rhesus Monkey Brain in Stereotaxic Coordinates’ was published. This book is the ‘most comprehensive, detailed atlas of the monkey brain ever constructed’ (comment by Academic Press). Prof. Paxinos and I also published in 1996 (Academic Press, San Diego), the ‘Atlas of the Human Brainstem’ the first book of its kind on the human brain, since the work of Olszewski and Baxter (1954). In this work, we identified for the first time 47 human homologues to nuclei identified in other animals and it is the most authoritative work in its field. The identification of regions and homologues permitted us to propose organisational schemes, which are in harmony with functional data. Some new concepts have now been adopted in research publications as well as in text books based on our studies. ‘The Atlas of the Human Brainstem’ is a major accomplishment and a unique resource’ (Prof. C. Saper, of Harvard Medical School and Beth Israel Hospital). These two books are recognised as standard reference books in Neuroscience.

V. Curriculum Vitae

1. Personal Details
   Last Name: Xu-Feng, First Name: Huang; Title: Doctor; Current Position: Professor; Academic Unit: School of Health Sciences; Faculty: Health and Behavioural Sciences

2. Academic Qualifications

<table>
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<th>Degree</th>
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<td>Post-doc</td>
<td>Pathology</td>
<td>1994–1995</td>
<td>UNSW*</td>
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<tr>
<td>Post-doc</td>
<td>Psychology</td>
<td>1993–1994</td>
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<td>PhD</td>
<td>Neuroanatomy</td>
<td>1989–1993</td>
<td>UNSW</td>
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<tr>
<td>MBBS</td>
<td>Medicine</td>
<td>1977–1982</td>
<td>XuZhou Medical College</td>
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*UNSW: University of New South Wales, Australia

3. Awards, Fellowships & Scholarships

<table>
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<th>Year</th>
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<td>1989–1993</td>
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<tr>
<td>2009</td>
<td>Vice chancellor</td>
<td>Excellence in Research Supervision</td>
</tr>
<tr>
<td></td>
<td>UoW*</td>
<td>(Highly commended)</td>
</tr>
<tr>
<td>2010</td>
<td></td>
<td>Excellence in Research Supervision</td>
</tr>
</tbody>
</table>

#NHMRC: National Health and Medical Research Council of Australia

*UoW: University of Wollongong, Australia
4. Panel and Board Member
- Member of NHMRC Grant Review Panel, GRP-5E, 2010, Psychology, Psychiatry and Cognitive Sciences, 2010
- Member of the Research Evaluation Committee for ERA Cluster 7 (BCH), Excellence in Research in Australia, Australia Research Council, 2010
- Member of External Advisory Board, Australia Nuclear Science and Technology Organisation (ANSTO), 2010
- Council Member of Schizophrenia Research Institute, Australia, 2007–present
- Member of Developmental Neurobiology Panel, SRI, Australia, 1999–present
- Scientific Director of IHMRI Metabolic Theme, 2009–present

5. Memberships of Professional Societies
- 1999–present North American Association for the Study of Obesity
- 1998–present Australian Society for the Study of Obesity
- 1996–present American Association for the Advancement of Science
- 1991–present Neuroscience, USA
- 1989–present Australian Neuroscience Society

6. Positions Previously Held
- 2006–present Professor, School of Health Sciences, UoW
- 2002–2005 Associate Professor, Department of Biomedical Science, UoW
- 1997–2001 Senior Lecturer, Department of Biomedical Science, UoW
- 1995–1997 Lecturer, Department of Biomedical Science, UoW
- 1994–1995 Research Officer (RO2 & 3), NHMRC, Sch of Pathology, UNSW
- 1993–1994 Research Officer (RO1), NHMRC, School of Psychology, UNSW
- 1989–1991 Tutor (Part time), School of Anatomy, UNSW
- 1989–1991 Research Assistant (Part time), School of Anatomy, UNSW
- 1983–1987 Associate/Lecturer, XuZhou Medical College, China

7. Industrial/Commercial/Professional Activities and Other Relevant Experience
- University, Faculty and Department Committees/Responsibilities
  - University Bio-safety committee, member (2005-2008)
  - University of Wollongong – Anatomy License (2005)
  - University Academic Senate, Faculty Representative (1997–1999)
  - Faculty Research Committee, member (1997–1999)
  - Department Acting Head, Department of Biomedical Science in July 1998, December 1998, and July 2001
  - Course Coordinator for Medical Science (2001–2004)
  - Academic Advisor for the first year undergraduate students in the Department of Biomedical Science/School of Health Sciences (1997–2000)
  - Radioactive Safety Officer of the Department of Biomedical Science/School of Health Sciences (2005–2008)
- External to UoW
  - Uncle Toby’s Company of Australia, R & D Research Collaborator for the study of whole grain in prevention of obesity and health benefit (2005–2008)
• Director of NISAD-Wollongong Centre for Collaborative Human Brain Research (1999–present)
• Invited Reviewer of NHMRC Principal Research Fellowship and Promotion (2005)
• Invited Reviewer of National Heart Foundation (2000, 2001), and The Welcome Trust, UK (2000)
• Chair and Convenor, NISAD Satellite Meeting held in the University of Wollongong, 2005

8. Journal Reviewer and Editorial Board Member
• Reviewer for journal articles: American Journal of Physiology; Brain Research; Molecular Brain Research; Neurochemistry; Neuropsychopharmacology; American Journal of Physiology; Journal of Neuroscience Research; Molecular Carcinogenesis; Cancer Research
• Editorial board member: Members of Translational Neuroscience Editorial Board, 2010; International Journal of Obesity

9. Visiting Scientist/Professor
• Sabbatical, 2000, Obesity and Metabolism, AstraZeneca R&D Sweden
• Established industry collaborative research project ‘Differential expressed genes in the late stage of obesity’; published two peer reviewed articles.
• Sabbatical, 2005, Obesity and Metabolism, AstraZeneca R&D, Sweden
• Established industry collaborative research project ‘Cause and consequence genes of obesity’
• Sabbatical, 2005, Medicine, Xuzhou Medical College, PR China.
• Sabbatical, 2007, Beijing University, PR China

VI. Publications

1. Contributions including individual and joint collaborative work: Standard practice with respect to authorship in the discipline of neuroscience research and in my laboratory is to list the research fellow and doctoral student as the first authors, while the senior author is normally listed last on the author list. The senior author develops the original ideas, assists in analysis and interpretation of the results of the study, and provides funding and infrastructure for the study. As a senior author, my percentage of intellectual input (PI) ranges from 60 to 80% and my percentage of labour work (PL, e.g.: tissue sectioning, histology, feeding mice, statistics, etc.) approximates 20%. I am either the 1st or senior author for 80% (20 papers as the 1st Author, 56 papers as the last Author) of total 103 journal papers that I, together with my colleagues, have published. Among all 273 publications (including conference presentations), the works are about 82% derived from my laboratory in the School of Health Science, at the University of Wollongong. Those publications from my PhD work are not included in this application.

2. My journal article citations: Based on the collection of the ‘ISI’ Web of Knowledge” in March 2011, my journals articles have been cited 1,505 times in
international research articles. The figure below shows a sustained contribution to the knowledge as evidenced by yearly increased citations.

3. **My book citations (857 citations):** I, together with my colleagues, have published three brain atlases with Academic Press, San Diego, USA. They are now used as standard reference books by neuroscientists worldwide. ‘The Rhesus Monkey Brain in Stereotaxic Coordinates’ has been cited 698 times (Google scholar, 12/12/2010). ‘The Atlas of the Human Brainstem’ has been cited 159 times (Google scholar, 12/12/2010). The second edition of ‘The Rhesus Monkey Brain in Stereotaxic Coordinates’ has recently been published.

![Journal Article Citation Report of Xu-Feng Huang, UoW](image)

Summary:
ISI Web of Knowledge  
Key words: Huang Xu-Feng  
Day: March, 10th, 2011

Results found: 121  
Sum of the Times Cited: 1505  
Average Citations per Item: 11  
h-index: 22

4. **List of Publications**

**Books**


**Book Chapters**


Articles in International Refereed Journals

12. Dawson AE, Newell KA and Huang XF (2011). Reciprocal signalling between NR2 subunits of the NMDA receptor and Neuregulin1 and their role in schizophrenia. Progress in Neuro-Psychopharmacology and Biological Psychiatry, accepted for publication (ERA Journal Ranking: B; FoR1=17)


14. Frank E, Newell K and Huang XF (2011). Density of metabotropic glutamate receptors 2 and 3 (mGluR2/3) in the dorsolateral prefrontal cortex does not differ with schizophrenia diagnosis but decreases with age. Schizophrenia Research, accepted for publication (ERA Journal Ranking: A; FoR1=1109; Impact Factor=4.945)


28. du Bois T, Deng C and Huang XF (2009). Excitatory and inhibitory neurotransmission is chronically altered following perinatal NMDA


32. Huang XF and Chen J (2009). Obesity, the PI3K/Akt signal pathway and colon cancer. Obesity Reviews 10:610–616. (ERA Journal Ranking: A*; FoR1=1111; Impact Factor=5.086)


38. Yu YH, South T and Huang XF (2009). Inter-meal interval is increased in mice fed a high whey, as opposed to soy and gluten, protein diets. Appetite 52:372–379. (ERA Journal Ranking: B; FoR1=1701; Impact Factor=2.966)


41. du Bois T, Hsu CW, Li YL, Han M, Tan YY, Deng C and Huang XF (2008). Altered dopamine receptor and dopamine transporter binding and tyrosine hydroxylase mRNA expression following perinatal NMDA receptor blockade. *Neurochemical Research* 33:1224–1231. (ERA Journal Ranking: C; FoR1=1109; Impact Factor=2.392)


56. Deng C and **Huang XF** (2007). No changes in densities of cannabinoid receptors in the superior temporal gyrus in schizophrenia. *Neuroscience Bulletin* 23:341–347. (ERA Journal Ranking: C; FoR1=1109)


63. Rahardjo GL, **Huang XF**, Tan YY and Deng C (2007). Decreased plasma PYY accompanied by an elevated PYY and Y2 receptor binding sites in the


76. Deng C and Huang XF (2005). Decreased density of muscarinic receptors in the superior temporal gyrus in schizophrenia. *Journal of Neuroscience Research* 81:883–890. (ERA Journal Ranking: B; FoR1=1109; Impact Factor=3.190)


85. Huang XF, Xin X, McLennan P and Storlien LH (2004). Role of fat amount and type in ameliorating diet-induced obesity: insights at the level of hypothalamic arcuate nucleus, leptin receptor, neuropeptide Y and pro-opiomelanocortin mRNA expression. *Diabetes, Obesity and Metabolism* 6:35–45. (ERA Journal Ranking: B; FoR1=1103)


89. Huang XF, Han M, South T and Storlien LH (2003). Altered levels of POMC, AgRP and MC4-R mRNA expression in the hypothalamus and other parts of the limbic system of mice prone or resistant to chronic high-energy diet-induced obesity. *Brain Research* 992:9–19. (ERA Journal Ranking: B; FoR1=1109; Impact Factor=2.551)


**Letters to Editor**


116. Chen J and **Huang XF** (2010). The PI3K/Akt pathway may play a key role in social isolation-caused schizophrenia, comment re: Increased dopamine D2(High) receptors in rats reared in social isolation. *Synapse* 64:486-487. (ERA journal Ranking: B; FoR1=1109; Impact Factor=2.584)


120. Chen J and **Huang XF** (2010). Dietary flavonoids suppress azoxymethane-
induced colonic preneoplastic lesions in male C57BL/Ksj-db/db mice. *Chemico-Biological Interactions* 183:276-283. (ERA Journal Ranking: C; FoR1=0601)


125. Western-Green K, Huang XF and Deng C (2010). Sensitivity of the Female Rat to Olanzapine-Induced Weight Gain – Far From the Clinic? *Schizophrenia Research* 116:299-300. (ERA journal Ranking: A; FoR1=1109; Impact Factor=4.945)


Gastroenterology 58:1169. (ERA Journal Ranking: A*; FoR1=1103; Impact Factor=9.663)

134. **Huang XF** and Chen JZ (2009). Neuregulin 1, brain region specificity and PI3K/Akt in schizophrenia. European Archives of Psychiatry and Clinical Neuroscience 259:307–308. (ERA Journal Ranking: C; FoR1= 103; Impact Factor=3.149)


**National and International Conference Papers:**


143. du Bois TM, Newell KA and **Huang XF** (2010). NMDA receptor and neuregulin1 signalling in the perinatal PCP animal model of schizophrenia. Federation of European Neuroscience Societies Forum, Amsterdam, July 2010.


145. Frank E, Snikeris P, Pathy R and **Huang XF** (2010). Environmental enrichment affects PCP-induced behavioural and neurochemical symptoms of


149. Huang XF and Chen J (2010). Diet-induced obese mice are significantly more susceptible to develop colon cancer than diet-resistant mice after chronic azaoxymethane treatment. Obesity and cancer conference, Spain, October 2010.


182. Huang, X.F., South, T. Zavitsanou, K and Deng, C. A negative association between the CB1 receptor mRNA expression and high fat intake during the course of diet-induced obesity in mice. Cannabinoid Signalling in the Nervous System: 17th Neuropharmacology Conference. USA, 2007.

183. South, T. & Huang, X. The CB1 Receptor as a Target for the Preferential Reduction of High Fat Diet Intake. In 7th IBRO World Congress of Neuroscience Conference Proceedings; Melbourne, Australia, 2007; pp 282.

184. Weston-Green, K. L., Deng, C., Han, M. & Huang, X. Effects on Antipsychotic Drugs on Weight Gain and CB1 Receptors in the Dorsal Vagal
Complex. In 7th IBRO World Congress of Neuroscience Conference Proceedings; Melbourne, Australia, 2007; pp 144.


191. Rahardjo, G., Huang, X., Tan, Y. Y. & Deng, C. The Role of Pyy and H1 Histamine Receptor Expression in Chronic Diet-Induced Obese and Obese Resistant Mice. In 7th IBRO World Congress of Neuroscience Conference Proceedings; Melbourne, Australia, 2007; pp 283.


193. Deng C, Han M, Huang X-F. Antipsychotic drugs increase food intake and cannabinoid CB1 receptor mRNA expression in the brain of rats 17th Neuropharmacology Conference: Cannabinoid Signaling in the Nervous System Submitted October 31-November 2, 2007 San Diego USA.


195. Huang XF, du Bois TM, Newell KA, Zavitsanou K, Deng C A rodent model of NMDA receptor hypofunction for the study of schizophrenia 7th Biennial meeting of Chinese Society for Neuroscience, October 24-28, Hangzhou, China

197. Chao Deng, Mei Han, Kelly A. Newell and Xu-Feng Huang (2007) No changes in cannabinoid CB1 receptor binding density in the superior temporal gyrus in schizophrenia, Colorado, 2007-03-26.


199. Mei Han, Chao Deng, Kelly Newell, Xu-Feng Huang (2007) Histamine H1 mRNA expression is decreased in the rat hypothalamus following olanzapine treatment Colorado, 2007-03-26.


228. Huang, X.F., M. Han, YH Yu, X. Huang and K. Zavitsanou (2005) Differential expression of dopamine D2 and D4 receptor mRNA expression in the brain of chronic high-fat diet-induced obese mice. Society for Neuroscience, USA. 75.18 USA.


cortex in schizophrenia: Clues for abnormal neural circuitry. Australasian Schizophrenia Conference. 7:57.
263. Huang, X.F (1996) Leptin mRNA expression in the central nervous system. Society for Obesity. 5:26
5. Some publications in high impact journals or having high citations:


- Huang XF and J Chen (2009) Obesity, the PI3K/Akt signal pathway and colon cancer. Obesity Review, 10: 610-616. IF= 7.821 (A* journal). This review summarized existing knowledge, made critical hypotheses and pointed out a key therapeutic target addressing the link between obesity and bowel cancer.


- Huang XF and Chen JZ (2009) Adiponectin and signal pathways in obesity-induced colon cancer, Gut, 2009; Vol. 58, 1169; IF=10.0 (A* journal). This paper draws together a concept of how adiponectin as a cytokine plays a key role in prevention of obesity-associated colon cancer.


- Huang XF, Xin X, McLennan P and Storlien LH (2004) Role of fat amount and type in ameliorating diet-induced obesity: insights at the level of hypothalamic arcuate nucleus, leptin receptor, neuropeptide Y and pro-opiomelanocortin mRNA expression. Diabetes, Obesity and Metabolism. 6(1) 35-45. IF= 3.44. This paper provides a better understanding of dietary fat type and amount affecting specific neurotransmitters regulating energy balance (cited 33 times, ISI).


- Lin, S, Storlien, L, and Huang, X.F. (2000) Leptin receptor, NPY, POMC mRNA expression in the diet-induced obese mouse brain, Brain Research, 875:89-95. This paper investigated the regulatory relationship between peripheral signal (leptin and central control molecules, leading to high citations (cited 86 times).

- Lin, S., TC Thomas, LH Storlien and X.F. Huang (2000) Development of obesity and central leptin resistance in high-fat diet induced obese mice. International Journal of Obesity and Related Metabolic Disorders. 24639-646, IF= 3.6 (A journal). This paper reported for the first time that central leptin resistant is a key...
feature of chronic high-energy diet-induced obesity, leading to high citations (cited 120 times, ISI).

- Huang XF, I. Koutcherov, S. Lin, H.Q. Wang and L. Storlien (1996) Localisation of leptin receptor mRNA expression in mouse brain. NeuroReport. 7:2635-2638. This paper identified for the first time the location of the leptin receptor in the hypothalamus receiving fat signals, leading to high citations (cited 92 times, ISI).

VII. Successful Grants in Supporting My Research

In total, I have been awarded $5,170,301 in research funding in the last 16 years at UoW. I was the 1st Chief Investigator for 82% of funded grants.

NHMRC Funding
2. Mei Han (Postdoc) and XF Huang (Supervisor), 2010 NHMRC ID:635245, for NHMRC training fellowship, Understanding the role of neuregulin-1 genetic polymorphisms in patients with schizophrenia, 2010–2013, $334,171
3. Huang, XF and K. Newell, 2009 NHMRC project, ID:573426: The effects of phencyclidine on the NMDA receptor/neuregulin1 signalling complex: implications for schizophrenia, 2009–2011, $417,000
4. Yinghua Yu (Postdoc) and XF Huang (Supervisor) 2009 for NHMRC training fellowship, Evaluation of functional protein molecules in treating obesity, ID:573441, 2009–2012, $334,171
5. Huang, et al., NHMRC Equipment grant: Molecular Pathology Laboratory, 2008, $10,000
9. Huang, Deng, Zavitsanou, NHMRC Equip. ProgRes C10 digital microscope camera system. 2004, $5,000
10. Huang, XF, NHMRC Project Obesity induced by high-energy diet: Central influences in prevention, 2002–2005, $220,000
11. Huang XF, NHMRC equipment grant: Dedicated radioactive laboratory equipment. 2008, $5,480
12. Huang, XF, et al., NHMRC Equip. Digital Photo Lab. 1999, $7,000
13. Huang, XF, NHMRC Project Gene expression of leptin receptor in lean, obese and diabetic mouse Brain. 1997, $143,596

ARC-Linkage Project Funding
15. Huang, XF,ARC-small Brain regulation of energy balance. 2000, $10,500
16. Huang, XF, ARC/Small Expression of NMDA, 1998, $10,000
17. Huang, XF, ARC-small Molecule, chemical and weight control. 1997, $5,500

Neuroscience Institute of Schizophrenia and Allied Disorder Funding:
18. Huang, XF, Neuropathology of Schizophrenia; Schizophrenia Research Institute Funding, 2008–2010 ($140,000 × 3yrs). $420,000
19. Huang, XF, NISAD Equip. Beta-imager. 2004, $200,000
20. Huang, Tan, NISAD Project Neuropathology of Schizophrenia. 2005, $330,000
21. Huang, Deng, Zavitsanou, NISAD/UoW Beta-imager analysis of drug binding efficacy for treating schizophrenia. 2005, $20,000
23. Huang, Morris NISAD Project Schizophrenia and Neuropathology. 1999, $110,000

**AstraZeneca pharmaceutical company of Sweden funding**
24. Huang, XF, AstraZeneca Project Differential expressed genes in the late stage of obesity. 2003, $157,000
25. Huang, XF, AstraZeneca Project Development of obese mouse model. 2003, $25,000
26. Huang, XF, AstraZeneca Project Cause or consequence genes of obesity. 2006, $100,000

**University Research Council and Research Partnership Scheme funding**
27. Huang, XF and P Craig, URC Research Partnership Scheme, Development of a functional food combination to achieve synergistic satiety effects for the prevention of obesity. 2010, $40,000
28. Huang XF and C Deng. The role of histamine, NYP and melanocortin systems in the development and prevention of olanzapine-induced obesity; URC near miss grant. 2009, $15,000
29. Huang URC. New initiative of research strength. 2007, $40,000
30. Huang, XF. RIBG, Molecular Pathology Infrastructure. 2009, $73,000
31. Huang, XF. iiMH/UOW Assist in grant preparation, $2,000
32. Astheimer, Huang et al. RIBG Beta and Gamma Radiation Counters. 2005, $45,000
33. Else, Huang, et al. RIBG GC for fatty acid analyses. 2005, $35,474
34. Huang, et al. RIBG Acquisition system for physiology, 1999, $36,375
35. Huang, XF. URC, Hybridisation oven, 1998, $4,700
36. Huang, Zhang. URC Neural networks in weight control. 1996, $6,850
37. Huang, XF. Metabolic Research Centre, Neural networks and Metabolism. 1996, $2,293

**Australia Nuclear Science and Technology Organisation funding**
38. Huang XF and A Katsifits, AINSE, Peripheral Benzodiazapine as an Early Marker for Obesity and Inflammation: Implications for Development of Type II Diabetes Mellitus and Cancer, 2009, $14,000
39. Deng, Huang et al. AINSE Project PCP induced apoptosis in a rat model. 2005, $21,000

**National Food – Smart Food funding:**
40. Huang, et al. NCCEF Dietary protein and weight control. 2003, $50,481

**VIII. PhD, Masters and Honours Students and Research Fellows**

Experience and Success in Supervising Research Fellows and Students (PhD, Master, Honours and Research topic students): Table 1 summarises the various levels of fellows and students who have worked with me at the University of Wollongong since 1996. I
have supervised eight Research Fellows since 1999, ten PhD completions since 1997, as well as four Masters completions and 16 Honours completions since 1996. In addition, seven Visiting Research Fellows or Professors have worked in my laboratory since 1998. Many honours students after training from my centre were awarded PhD scholarships to continue their neuroscience research, while other students were accepted in Medical Schools (Sydney, Tasmania, Wollongong Universities). Among my PhD graduates, three are currently NHMRC Post-doctoral Fellows. It is common for PhD students finishing their research projects in my laboratory to have published three to five peer reviewed articles as the 1st author. Much of this work has been well-cited (for example, Dr Lin has now been cited 121 times).

Table 1. Primary Supervision/Co-Supervision of PhD, Masters Honours and Research Fellow Research Projects

<table>
<thead>
<tr>
<th>Degree Name</th>
<th>Year</th>
<th>Completion</th>
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<td>PhD C Xin</td>
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<td>PhD J Wickham</td>
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<td>PhD K Newell</td>
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<td>PhD T South</td>
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<td>2008</td>
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<td>PhD M Han</td>
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<td>PhD T du Bois</td>
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<td>PhD YH Yu</td>
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<td>PhD E Beck</td>
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<td>PhD Christopher Magee</td>
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<tr>
<td>MSc Somayeh Jafarj</td>
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<td>2008</td>
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<td>MSc G Rahardjo</td>
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<td>MS G. Tuess</td>
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<td>Hon A Howki.</td>
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<td>Hon M Hassi.</td>
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<td>Hon W Bell</td>
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<td>Hon B Klose</td>
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<td>Hon CW Hsu</td>
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<td>Hon D Weir</td>
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<td>Hon N Piyaratna</td>
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<td>Hon D Manassa</td>
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<td>Hon Asya</td>
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Senior research/Research fellows (SRF/RF)

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<td>1999</td>
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<td>32</td>
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<td>2001</td>
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<td>34</td>
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<tr>
<td>35</td>
<td>SRF</td>
<td>C Deng</td>
<td>2003</td>
<td>2010</td>
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<tr>
<td>36</td>
<td>RF</td>
<td>L Frank</td>
<td>2009</td>
<td>2010</td>
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Visiting Research Fellows

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<th></th>
<th>Type</th>
<th>Name</th>
<th>Year Start</th>
<th>Year End</th>
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<tbody>
<tr>
<td>37</td>
<td>A/Pr</td>
<td>YL Ge</td>
<td>1998</td>
<td>1998</td>
</tr>
<tr>
<td>38</td>
<td>Dr</td>
<td>Y Xia</td>
<td>1999</td>
<td>1999</td>
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<tr>
<td>39</td>
<td>Dr</td>
<td>M Han</td>
<td>2002</td>
<td>2004</td>
</tr>
<tr>
<td>40</td>
<td>Dr</td>
<td>X Huang</td>
<td>2003</td>
<td>2004</td>
</tr>
<tr>
<td>41</td>
<td>Dr</td>
<td>YH Yu</td>
<td>2003</td>
<td>2004</td>
</tr>
<tr>
<td>42</td>
<td>Dr</td>
<td>YX Lin</td>
<td>2003</td>
<td>2005</td>
</tr>
<tr>
<td>43</td>
<td>Dr</td>
<td>YL Li</td>
<td>2006</td>
<td>2006</td>
</tr>
</tbody>
</table>

IX. PhD, Master and Honours Theses Examination

- Honours thesis, Title: Amelioration of hyperthermia-induced birth defects through folic acid administration, Michael Conor Bulman, Thesis for Bachelor of Science (Honours), 2002
- Honours thesis, Title: Leptin kinetics and skin blood flow during rewarming, Cassandra Dale Haley, Bachelor of Science (Honours), 2003
- Honours thesis, Title: Does the speed of muscle belly displacement correlate to muscle fibre type Ms Laurel Snelson, Honours Project, 2003
- PhD thesis, Title: The role of hypothalamic neuropeptides in feeding behavior and obesity, Michelle Joan Hansen, Thesis for the degree of Doctor of Philosophy, 2003
- PhD thesis, Title: The role of PYY in regulating energy balance and glucose homeostasis, Dana Boey, PhD thesis, Garvan Medical Research Institute, 2004
- Honours thesis, Title: The effects of dietary calorie restriction on biochemical and physiological parameters in mice, Sally Faulks, Thesis for Bachelor of Science (Honours), 2004
- Honours thesis, Title: Description of dietary habits of people with and without schizophrenia, Deepa Ghedia, Honours, 2004
- PhD Thesis, Title: Neuropeptide Y and obesity by Dana Boey Garvan Institute of Medical Research, 2004
- Honours thesis, Title: Molecular analysis of membrane phospholipids species using a body mass comparison in mammals, Jessica Nealon, Thesis for Bachelor of Science (Honours), 2006
- Honours thesis, Title: Investigating the consequences to the neuregulin1/ErbB4 signalling pathway following perinatal disruption to the NMDA receptor: implications for schizophrenia. S. Ruthirakumar, Thesis for Bachelor of Science (Honours), 2008.
X. Acknowledgements

I would like to thank many people for their inspiration, encouragement, guidance and assistance with my research and achievements in Neuroscience Research.

I would like to thank Professor Istvan Tork and Professor George Paxinos who accepted me as a PhD student at the School of Anatomy in the University of New South Wales from 1989 to 1992. During three years of study, I published four papers in ‘The Journal of Comparative Neurology’, which is the top journal in the field (A* Journals). I would like to thank Professors Stan Catts, Philip Ward, Dennis Wakefield and Andrew Lloyd for providing me a post doctoral training position in the School of Pathology, University of New South Wales from 1993 to 1995. I learned numerous molecular biological techniques in their laboratory, which have been extremely useful in my subsequent research. I thank Professor Len Storlien for his encouragement and consistent support of my research over many years. He is inspirational and full of ideas, leading to our many excellent collaborations and publications. I would like to thank the heads of the School of Health Sciences (Faculty of Health and Behavioural Sciences, University of Wollongong) for supporting my research over the last 16 years. From 1995 to the present, these include Professor Len Storlien, Dr Mark Brown, Professor Paul Else, and Professor Julie Steel.

I would also like to thank the research funding bodies that have supported and provided funding for my research, including the NHMRC over the last 15 years, the ARC (3 years), NISAD over the last 9 years, and AstraZeneca Company of Sweden (6 years) as well as our University for providing URC, FRC, and other funds.

Finally, I would like to thank my family, particularly my wife, for supporting me carrying out these studies and allowing me to stay over time during the day and numerous weekends in my laboratory and in front of my computer writing up these publications. Without this help, this body of work would not have been possible.
A growing number of studies have identified chronic sleep restriction as a potential risk factor for obesity. This could have important implications for how obesity is prevented and managed, but current understanding of the processes linking chronic sleep restriction to obesity is incomplete. In this paper, we examined some of the pathways that could underlie the relationship between chronic sleep restriction and obesity. This involved exploring some of the potential environmental, health, behavioral, and sociodemographic determinants of chronic sleep restriction, which require further investigation in this context. Three pathways that could potentially link chronic sleep restriction to obesity were then examined: (1) altered neuroendocrine and metabolic function, (2) impaired glucose regulation, and (3) waking behavior. The selected pathways linking chronic sleep restriction to obesity reviewed in this paper are presented in a schematic representation; this may be used to guide future research in this area. This area of research is important because it may lead to more effective interventions and strategies to combat the present obesity epidemic.

1. Introduction

Obesity has increased at an alarming rate in recent decades, and it is currently estimated that between 25% and 30% of adults in countries such as the US and Australia are obese [1–3]. Obesity is a major health issue as it contributes to conditions such as diabetes, cardiovascular disease, osteoarthritis, and some metabolic-related cancers [3, 4]. Obesity has a multifactorial etiology comprising a range of genetic, metabolic, environmental, behavioral, and social/cultural factors [5]. However, the rapid rise in obesity suggests that the present epidemic is the result of recent social and environmental changes, with high fat diets and increasingly sedentary lifestyles identified as two of the main causes [3, 6]. There are, however, other factors that may be contributing to the present obesity epidemic which need to be addressed. One factor that is receiving increased attention is chronic sleep restriction (<7 hours sleep a night). This is based on a number of recent epidemiological studies reporting that shorter sleep durations are associated with overweight and obesity in adults; see Cappuccio et al. [7] for a review. Some longitudinal data also indicate that short sleep predicts weight gain over a period of several years [8–12]. Furthermore, several laboratory-based studies have demonstrated that sleep restriction affects hormones involved in regulating energy balance, in a manner that is consistent with weight gain [13–18]. Interestingly, Chaput et al. [19] recently examined the relative contribution of nine risk factors for obesity (e.g., diet, physical activity, and sleep duration) over a six-year period. They found that sleep duration along with low calcium consumption and high disinhibition and restraint eating behavior significantly predicted weight gain; energy intake and physical activity were not significant predictors of weight gain.

These findings suggest that chronic sleep restriction could be a possible risk factor for obesity, and this may have implications for obesity management and prevention. However, our understanding of the relationship between...
chronic sleep restriction and obesity is incomplete as the processes through which chronic sleep restriction contributes to obesity, and the extent and magnitude of these effects, remain unclear. Furthermore, an important gap in the literature is that the causes, or determinants, of chronic sleep restriction have been largely overlooked. In this area of research, chronic sleep restriction has tended to be viewed as a result of behavioral sleep curtailment or an underlying sleep disorder [20]. This is a problem because the causes of chronic sleep restriction are likely to be complex and encompass many factors. These need to be identified and understood because they could influence the nature of the relationship between sleep and obesity and would likely have treatment implications.

Therefore, the purpose of the present paper is to integrate two important bodies of literature and examine (1) the determinants of chronic sleep restriction and (2) selected pathways linking chronic sleep restriction to obesity. This paper is not intended to provide a comprehensive review of these areas, as the relationship between chronic sleep restriction and obesity is likely to be complex and involve a multitude of processes. Instead, we focus on the factors and pathways that we feel are most important based on our assessment of the available literature. For example, we explore the following three potential pathways linking chronic sleep restriction to obesity: (1) neuroendocrine and metabolic pathways, (2) glucose regulation, and (3) waking behavior.

It is also acknowledged that the association between chronic sleep restriction and obesity is likely to be bidirectional since obesity could also contribute to chronic sleep restriction (e.g., sleep apnea). However, this paper focuses primarily on chronic sleep restriction as a possible cause of obesity. We conclude the paper by presenting a schematic representation that integrates these pathways and provides an important framework that is currently lacking in the literature; this can be used to guide future research in this area.

2. Determinants of Chronic Sleep Restriction

Chronic sleep restriction is generally defined as habitual sleep durations that are less than 7 hours, but more than 4 hours, a night [21]. It is distinct from acute total sleep deprivation which refers to an absence of sleep over a minimum of 24 hours; this is neither a common nor chronic condition in humans. Chronic sleep restriction has become more common in recent decades, having more than doubled in the US since the 1960s [22, 23]. Approximately one-third of adults in the US currently report chronic sleep restriction and similar figures have been observed in other countries [24–26]. Chronic sleep restriction is increasingly recognized as a health concern because it is associated with motor vehicle and industrial accidents, health conditions such as hypertension, diabetes, obesity, and depression, and increased mortality [21, 27, 28].

Previous research investigating the link between chronic sleep restriction and obesity has not adequately addressed the underlying causes of chronic sleep restriction. Many studies have either overlooked the causes of chronic sleep restriction or viewed chronic sleep restriction as the result of voluntary sleep curtailment [20]. However, the determinants are likely to be complex and vary considerably between individuals. These need to be examined because the nature of the relationship between chronic sleep restriction and obesity could depend on, or vary according to, the precise cause of chronic sleep restriction. The potential to modify sleep duration to aid obesity prevention and management may also depend on the precise factor(s) that contribute to chronic sleep restriction. Therefore, the remainder of this section identifies some key determinants of chronic sleep restriction that require investigation in this context.

Sleep duration is influenced by a combination of genetic, health, sociodemographic, environmental, and behavioral factors; so there are likely a multitude of factors that could potentially contribute to chronic sleep restriction. Data from large-scale population studies have recently identified factors associated with chronic sleep restriction in adults at a population level. Most of these studies have been cross-sectional, but they do provide an insight into the factors that could be important predictors of chronic sleep restriction. In particular, sociodemographic factors such as increased age, lower education level, lower income, ethnicity, and nonmarried status have been identified as strong predictors of chronic sleep restriction [24, 25, 29–31]. Health behaviors such as smoking, excessive alcohol consumption, lower levels of physical activity, increased television viewing, shift work, long working hours, and increased time commuting to and from work have also been associated with shorter sleep durations [24, 25, 30–34]. Several studies also demonstrate that physical and mental health status strongly predict sleep duration. For example, chronic diseases such as diabetes or cardiovascular disease [24, 25, 29–31] and mental health conditions such as stress, depression, and sleep disorders (e.g., insomnia) have been associated with reduced sleep [35, 36]. Dramatic changes in our physical environments in recent decades may also be contributing to chronic sleep restriction. For example, it is argued that we increasingly live in a 24-hour society that is characterized by more artificial light; this has the potential to disrupt natural circadian rhythms and adversely affect sleep [37].

There are, therefore, many sociodemographic, behavioral/lifestyle, health, and environmental factors that could contribute to chronic sleep restriction. Importantly, the precise causes of chronic sleep restriction are likely to vary considerably between individuals. Some individuals may voluntarily limit the amount they sleep in order to meet work, social, or family demands, whilst others engage in behaviors such as cigarette smoking and excessive alcohol consumption that adversely impact on sleep duration. In other individuals, an underlying health condition, medication use, or factors associated with a low socioeconomic status could be the primary cause(s) of chronic sleep restriction.

If chronic sleep restriction is to be targeted as a modifiable risk factor for obesity, there is a need to better understand the underlying factors contributing to chronic sleep restriction in different populations. This is important for two main reasons. First, the nature and magnitude of
the relationship between chronic sleep restriction and obesity may depend on the underlying causes of chronic sleep restriction; this could influence the mechanisms or pathways linking chronic sleep restriction to obesity and also how susceptible an individual is to the effects of sleep restriction. Second, the potential to modify sleep through interventions may differ based on the specific cause(s) of chronic sleep restriction. For example, factors such as mental health problems and sleep disorders may require interventions from specialist practitioners. In contrast, factors such as work hours could be targeted by behavioral interventions that do not necessarily aim to reduce work hours (as this could have adverse consequences, such as a loss of income), but rather attempt to minimize the impact of work hours on sleep patterns. This could perhaps be achieved by addressing factors such as television viewing and time spent commuting to and from work which may be limiting sleep duration in people who work long hours [33, 34]. Thus it is important that research investigating the relationship between chronic sleep restriction and obesity examines the determinants of chronic sleep restriction.

3. Hypothesised Pathways Linking Chronic Sleep Restriction to Obesity

There are many processes or pathways through which chronic sleep restriction could contribute to obesity and it is not feasible to address all of these in a single review. Instead, we focus on the following three hypothesized pathways linking chronic sleep restriction to obesity: (1) neuroendocrine and metabolic pathways, (2) glucose regulation, and (3) waking behaviour. There is potential overlap between these pathways, but for the purposes of clarity, we discuss each of them separately.

The concept of energy balance is important in this context. A constant body weight depends on a balance between energy intake (diet) and energy expenditure (basal metabolic rate, physical activity, and thermogenesis). Under normal conditions energy balance is maintained by a complex regulatory system that involves multiple physiological pathways in the body which act on neural circuits to maintain body weight within a narrow range [38–40]. For example, the adipose tissue hormones leptin and adiponectin, the pancreatic hormone insulin, and the gastrointestinal hormones ghrelin, peptide YY3–36 (PYY), and glucagon-like peptide-1 (GLP-1) all act on hypothalamic circuits to influence energy balance. A chronic positive energy balance occurs when energy intake exceeds expenditure over a prolonged period of time; this has the potential to affect the processes involved in regulating body weight and can lead to obesity over time [39, 40].

3.1. Hypothesis 1. Sleep Restriction Alters Neuroendocrine and Metabolic Functioning. In a landmark series of experimental studies, Spiegel and colleagues [16–18, 41] demonstrated that short-term sleep restriction alters some neuroendocrine and metabolic hormones that are involved in the regulation of energy balance. In particular, six consecutive nights of sleep restriction (four hours sleep per night) were associated with increases in sympathetic nervous system (SNS) activity, evening cortisol levels and growth hormone levels (GH), and reductions in thyroid stimulating hormone (TSH), and leptin [16, 17, 41]. A follow-up study found that sleep restriction (four hours sleep per night) over two nights led to an 18% reduction in leptin and a 28% increase in ghrelin [18]. The increase in the leptin-to-ghrelin ratio corresponded with a 24% increase in hunger and a 23% increase in appetite that was mainly for energy dense foods. Other research groups have obtained similar results. For example, Guilleminault et al. [13] found that seven nights of sleep restriction (five hours sleep per night) led to a reduction in leptin rhythm amplitude. Schmid et al. [15] also found that a single night of 4.5-hour sleep led to an increase in ghrelin levels. Finally, Magee et al. [14] observed that two consecutive nights of five hours sleep led to a significant reduction in PYY levels and a corresponding decrease in satiety levels.

The profile of these hormonal changes is suggestive of increased energy intake, reduced energy expenditure and weight gain. For example, leptin, which is released in proportion to adipose tissue amount, acts on hypothalamic circuits to reduce energy intake and increase energy expenditure [42]. Insulin has many roles but also acts on hypothalamic circuits to reduce energy intake and increase energy expenditure [42, 43]. The reductions in leptin and insulin observed with sleep restriction are therefore suggestive of increased food intake and reduced energy expenditure. Gherelin is released primarily from the stomach when nutrient levels are low and acts on hypothalamic pathways to stimulate food intake [44, 45]. The PYY3–36 molecule is released from the gastrointestinal tract in response to ingested nutrients and acts on the hypothalamus to reduce food intake [46, 47]. Therefore, the elevations in ghrelin and reductions in PYY observed with sleep restriction may be predictive of increased food intake.

Similarly, the increases in evening cortisol and GH levels may also be suggestive of weight gain. The elevation in GH levels observed by Spiegel et al. [41] was the result of an extended period of nocturnal GH secretion. This may have increased the amount of exposure of peripheral tissues to GH; if prolonged this could impact on glucose regulation in a way that leads to obesity (this is discussed in more detail below) [41]. However, GH has been shown to promote lean tissue and reduce the accumulation of adipose tissue [48]. The precise implications of the increases in GH observed with sleep restriction are therefore unclear and require further investigation.

The elevations in cortisol following sleep restriction suggest greater activity of the hypothalamic-pituitary-adrenal gland (HPA) axis. This could reflect increased stress levels as the HPA axis plays an important role in regulating the stress response [49]. Importantly, elevated cortisol levels have been shown to promote increased food intake and the accumulation of visceral fat in humans [50]. Similarly, since TSH normally functions to stimulate basal metabolic rate, the reductions in TSH with sleep restriction are suggestive of a reduction in energy expenditure.
The findings reviewed above indicate that short-term sleep restriction under controlled laboratory conditions alters neuroendocrine and metabolic hormones in a manner that is consistent with weight gain and obesity. However, we only have partial understanding of these mechanisms and there are a number of issues that remain to be addressed. First, it is not clear which brain mechanisms link sleep restriction with the observed alterations in metabolic and neuroendocrine functioning. The activation of the SNS with sleep restriction is one possibility since increased SNS activity inhibits the release of leptin from adipose tissue and may inhibit insulin release [51]. Increased SNS activity also inhibits vagal nerve activity, which could account for the rise in ghrelin level observed with sleep loss [52, 53]. However, increased SNS activity is typically associated with reductions in energy intake and increased energy expenditure over time [54]. Since this pattern is predictive of weight loss, increased activation of the SNS may not be the predominant mechanism through which sleep restriction alters energy balance and leads to weight gain. Instead, other mechanisms involving disruptions in the functioning of the suprachiasmatic nucleus (SCN) or activation of the HPA axis could be more important.

The SCN is located in the anterior hypothalamus and regulates the circadian rhythms of several physiological systems, including sleep, and the secretion of hormones involved in energy balance regulation [55]. Alterations in hormones such as leptin, cortisol, and TSH and GH observed with sleep restriction could therefore be the result of disrupted SCN output. As noted earlier, the increases in cortisol secretion observed with sleep restriction suggest increased activation of the HPA-axis, which is indicative of the stress response. The stress response serves an important adaptive purpose by supplying extra energy to body tissues in anticipation of a fight or flight response [49, 50, 56]. This leads to the release of cortisol which through a series of feedback loops signals the HPA axis to reduce cortisol secretion [57]. However, chronic or frequent stress can desensitise the HPA axis such that cortisol remains elevated; over time this can lead to an increase in visceral body fat since cortisol promotes fat accumulation and also inhibits the release of leptin [49, 50, 58]. Activation of the HPA axis may also explain the increased SNS activity observed with sleep restriction. Thus it is possible that the physiological changes observed with sleep restriction are the result of increased HPA axis activity and/or altered SCN output, which have a cascading effect on a number of physiological systems.

The second important consideration is that it is not clear whether the physiological effects of sleep restriction observed under laboratory conditions over a period of a few days are equivalent to prolonged (or chronic) sleep restriction as it occurs in free-living individuals. In particular, it is feasible to assume some degree of physiological adaptation to the effects of sleep restriction, but one can only speculate on the extent and nature of this adaptation. Moreover, the effects of sleep restriction as observed in laboratory-based settings may differ to the effects of chronic sleep restriction in free living adults. These are all important considerations because, as noted above, the causes of chronic sleep restriction could differ considerably between individuals and the impact of sleep restriction on energy balance may depend on the underlying cause of chronic sleep restriction. Finally, few studies have examined whether sleep restriction alters components of energy expenditure. Schmid et al. [59] demonstrated that sleep restriction led to a reduction in physical activity under free-living conditions, but there were no significant changes in food intake, hunger and appetite, and levels of leptin and ghrelin. This suggests that sleep restriction might alter energy expenditure but this requires further investigation in studies that also examine other components of energy expenditure such as basal metabolic rate or nonexercise activity thermogenesis. This is important because examining both sides of the energy equation (i.e., energy intake and expenditure) will be critical to understanding the processes through which sleep restriction promotes obesity.

3.2. Hypothesis 2. Sleep Restriction Alters Glucose Regulation. Another potential pathway linking chronic sleep restriction to obesity could involve disruptions in the regulation of glucose levels; this may also have implications for diabetes, which are discussed by Spiegel et al. [60]. Alterations in glucose regulation have been linked with weight gain and obesity. For example, Boulé et al. [61] found that lower blood glucose concentrations at the end of an oral glucose tolerance test (OGTT) predicted weight gain over a 6-year period. These results were explained according to the Glucostatic Theory of Appetite Control, which postulates that glucose plays an important role in the regulation of satiety and appetite [62]. In particular, reduced glucose utilization in important regions of the brain leads to perception of hunger and increased food intake, whereas higher glucose utilization in these same areas promotes a decrease in hunger and a cessation of eating [62].

Sleep restriction has been shown to affect glucose levels in humans. Spiegel et al. [17] found that six nights of sleep restriction led to a 30% reduction in glucose effectiveness (i.e., noninsulin dependent glucose utilization) and a 40% reduction in glucose utilization following intravenous glucose administration. These results have been supported by cross-sectional and prospective data. For example, Chaput et al. [63] found that habitually short sleepers had higher levels of fasting plasma glucose and insulin concentrations and lower blood glucose concentrations at the end of an OGTT. Chaput et al. [64] also found that individuals reporting short sleep had increased glucose area below fasting glucose concentrations; this is indicative of reactive hypoglycemia and predicted diabetes/impaired glucose tolerance at six-year followup.

Thus it is possible that chronic sleep restriction contributes to obesity by disrupting the regulation of glucose in a manner that promotes increased food intake. Chronic sleep restriction could potentially exert these effects via activation of the SNS or disruptions in hormones such as cortisol or GH [63]. As with the neuroendocrine pathways discussed above, there is a need for more longitudinal data examining whether prolonged sleep restriction does promote fat accumulation and lead to obesity by impairing glucose regulation.

The third hypothesis discussed in this paper is that chronic sleep restriction contributes to obesity by affecting waking behavior, and in particular promoting patterns of behavior that cause weight gain. It is well documented, for example, that consumption of food with a high-energy content and sedentary behavior (e.g., television viewing, physical inactivity) are strong risk factors for obesity [6]. Chronic sleep restriction could lead to obesity by promoting these behaviors and this has received some empirical support.

Nedeltcheva et al. [65] for example, recently examined the effects of 14 consecutive days of sleep restriction on food intake, energy expenditure, and neuroendocrine hormones. In contrast to the studies conducted by Spiegel and colleagues [16–18], which involved a mild form of calorie restriction, Nedeltcheva et al. [65] provided food to participants ad libitum. Their results indicated that sleep restriction led to an increase in calorie consumption that was attributed to snacking particularly during the night when the individual would normally have been sleeping (this is unlikely to reflect the night eating syndrome). Food intake during meal time remained unchanged with sleep restriction, as did energy expenditure. The findings suggest that short sleepers could be more susceptible to weight gain because they have more time to eat. The increase in consumption because of greater exposure to food (rather than increased hunger) suggests that in addition to the homeostatic factors reviewed above, nonhomeostatic factors may also be involved in the relationship between chronic sleep restriction and obesity [66]. Thus, future studies will need to further investigate both homeostatic and nonhomeostatic pathways linking chronic sleep restriction to obesity.

Another plausible behavioral pathway linking chronic sleep restriction to obesity involves fatigue, since individuals who get insufficient sleep are more likely to experience fatigue and daytime sleepiness [67]. It is possible that individuals engage in behaviors such as consumption of high-energy drinks or food to counter the effects of fatigue. Fatigue may also render individuals less likely to engage in physical activity [68, 69] and more likely to engage in sedentary behaviors such as television viewing. This pattern of behavior could also promote a positive energy balance and may partially account for the association between chronic sleep restriction and obesity.

4. Integrating the Pathways Linking Chronic Sleep Restriction to Obesity

Chronic sleep restriction may be an important risk factor for obesity and could have implications for obesity prevention and management. However, current understanding of the processes underlying the relationship between chronic sleep restriction and obesity is limited. The purpose of the present paper was not to provide a definitive review of the literature, but rather to integrate two important aspects of this relationship which are summarized in Figure 1.

First, we examined potential determinants of chronic sleep restriction, which have been largely overlooked in the literature. We hypothesize that chronic sleep restriction could be the result of a range of factors including mental health status, lifestyle/behavioral factors, chronic disease, and sociodemographic status. It is important that these are examined further because the precise causes of chronic sleep restriction, and the potential to modify these in therapeutic settings, are likely to differ considerably between individuals. Second we examined the following three potential pathways through which chronic sleep restriction could potentially promote weight gain and obesity: (1) metabolic and neuroendocrine functioning, (2) glucose regulation, and (3)
waking behaviour. These pathways have the potential to promote a positive energy balance and may explain the mechanisms linking chronic sleep restriction to obesity. The first challenge for researchers will be to demonstrate that chronic sleep restriction does impact on these and other pathways not examined in this paper. This is important because the effects of short-term sleep restriction may not correspond with the effects of chronic sleep restriction, as there may be some form of physiological adaptation over time. Thus, more long-term prospective studies examining the associations between changes in sleeping patterns, body composition, and the pathways identified in Figure 1 are needed. There is also a need to investigate whether the magnitude of the association between chronic sleep restriction and obesity, and the underlying pathways, varies according to the causes of chronic sleep restriction.

It should be noted that the association between chronic sleep restriction and obesity is likely to be bidirectional and circular, and this is depicted in Figure 1. Therefore, although the primary purpose of this paper was to review evidence indicating that chronic sleep restriction contributes to obesity, it is also possible that obesity contributes to chronic sleep restriction. For example, symptoms of obesity such as pain and discomfort and comorbid conditions such as obstructive sleep apnea have been shown to impair and disrupt sleep. As a result, there is a need for more experimental or prospective research to delineate the magnitude of the effect of chronic sleep restriction on obesity.

A final consideration is that it is not clear whether chronic sleep restriction can be modified through interventions, and whether these changes are effective in preventing and managing obesity. Research addressing these issues will be important in determining not only the pathways linking chronic sleep restriction to obesity but also whether chronic sleep restriction is a risk factor that can be modified to treat and prevent obesity. Currently there is a 12-month randomized controlled trial being conducted in the US (clinicaltrials.gov register number NCT00261898) that is examining whether increasing sleep duration in obese individuals who report short sleep affects body weight and other related variables (e.g., glucose regulation, neuroendocrine hormones). This study may provide clarification as to whether chronic sleep restriction can be targeted as a modifiable risk factor for obesity, but the results of this study have not yet been published. This area of research is significant given that the obesity epidemic continues to grow and poses a number of major health, social, and economic problems; targeting the amount we sleep could be an important step in combating this health problem.

Disclosure Statement

The authors report no financial conflicts of interest.

References


Oat β-glucan supplementation does not enhance the effectiveness of an energy-restricted diet in overweight women

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Epidemiological evidence shows an inverse relationship between dietary fibre intake and body weight gain. Oat β-glucan, a soluble fibre alters appetite hormones and subjective satiety in acute meal test studies, but its effects have not been demonstrated with chronic consumption. The present study aimed to test the effects in women of two different doses of oat β-glucan on weight loss and hormones associated with appetite regulation. In a 3-month parallel trial, sixty-six overweight females were randomised into one of three 2 MJ energy-deficit diets: a control and two interventions including 5–6 g or 8–9 g β-glucan. Anthropometric and metabolic variables (blood glucose level, insulin, total cholesterol (TC), LDL, HDL, TAG and leptin), together with markers of appetite regulation (cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), ghrelin, peptide YY (PYY) and PYY3–36) were measured at baseline and at 3 months. After 3 months, all groups lost weight (P<0·001) and showed a reduced waist circumference (P<0·001). The study sample also showed reductions in TC, LDL, HDL, leptin, PYY, GLP-1 values (all P<0·001) and an increase in CCK levels (P<0·001). No significant differences were noted between the groups for all outcome values except PYY levels (P=0·018). In broad terms, the addition of oat β-glucan did not enhance the effect of energy restriction on weight loss in mildly overweight women, although wide variations in observed results suggests that individual responsiveness may be an issue.

β-1,3-D-Glucan: Weight loss: Weight control: Appetite hormones

Large bodies of epidemiological data show an inverse relationship between dietary fibre intake and body weight1–5 so that the logic and simplicity of including fibre in an energy-controlled diet remains tantalising. As a component of food, fibres are found in a fermentable (soluble) or non-fermentable (insoluble) form, but early research has been unable to show benefits from including either form in short term (3–4 weeks) ad libitum diets69. This was also the case when the form of fibre was mixed linkage β-glucan, a soluble fibre delivered by oats7, well recognised for its cholesterol8 and glucose lowering9 actions. In order to better expose the advantages apparent from epidemiological studies, more work is required in understanding the physical features of fibre, the food delivery system, and how these may work together to affect mechanisms associated with weight management, such as satiety, particularly over longer periods of time.

It is accepted that soluble fibre, by its viscous nature will not only increase upper gastrointestinal transit time, but also stimulate cholecystokinin (CCK) that will increase peristalsis10. The effects of other hormones are less clear, but the ileal brake formed by undigested foods in the distal gut (occurring with high fibre foods), in addition to the fermentation of soluble fibres in the large bowel are all seen as positive benefits of fibre. Meal studies are able to expose these mechanisms of action. This is done via assessment of biochemical markers and subjective measures of satiety, in addition to monitoring subsequent food intake after consumption of the test food. Studies of β-glucan have identified doses as low as 2 g may elicit acute lowering of glycaemia11, while others suggest a minimum of 4 g10 may be required for other gastrointestinal effects, such as those causing the release of appetite hormones. However, meal test studies only define an acute situation in very controlled conditions.

In addition to the time factor, the food delivery system requires consideration. For example, the clinical effectiveness of fibres such as β-glucan may be reduced in certain foods. It has been shown that in bread making, endogenous enzymes in the bread reduce the viscosity of the β-glucan thereby decreasing its clinical effectiveness12,13. However, a recent satiety study14 looking at high-dose (10 g) β-glucan and appetite hormones has showed that a lower viscosity drink (with viscosity lowered using β-glucanases) increased the levels of certain appetite hormones such as CCK and glucagon-like peptide-1 (GLP-1) compared with the high viscosity version of the same drink. Nevertheless, such contrasts once again do not define what may happen when soluble fibres are consumed over time, as a change in hormones over a few hours

Abbreviations: CCK, cholecystokinin; GLP-1, glucagon-like peptide-1; HBG, high-dose β-glucan; MBG, mid-dose β-glucan; PYY, peptide YY; RMANOVA, repeated-measures ANOVA; RTE, ready-to-eat; VAS, visual analogue scales.

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Oat β-glucan in energy-restricted diets

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does not necessarily translate to appetite and weight changes over a longer period of time.

The present study describes a 3-month randomised controlled dietary intervention trial, designed to review the specific effects of β-glucan from oat bran incorporated in an energy-restricted meal plan. The primary outcome was a difference in weight reduction between the control group and intervention groups. Secondary outcomes included a variety of biochemical measures linked with satiety or change in body weight, subjective satiety measures and perceived satisfaction with the product. Biochemical measures included fasting glucose, insulin, cholesterol, LDL, HDL, TAG, leptin, CCK, GLP-1, ghrelin, peptide YY (PYY) and PYY3–36. We hypothesised that the subjects receiving β-glucan would lose more weight than the subjects on the control diet, and that changes in appetite hormones may be detected due to mechanisms linking the satiety with ingestion of β-glucan.

Experimental methods

Subjects and recruitment

This was a 3-month parallel randomised controlled trial with female subjects, based on evidence that they may exhibit greater acute hormone changes with fibre intake. There were three arms to the study with all the groups receiving advice on energy restriction and all the subjects receiving cereal products to include in their diets. The control group had relatively high fibre products with no oat β-glucan, while the intervention groups had similar products with added β-glucan at a moderate (MBG; 5–6 g/d) and at a high (HGB; 8–9 g/d) level. A sample size of twenty subjects per group was based on data from a previous study showing a 1.8 kg difference between a group supplemented with β-glucan and a control group, where both groups were consuming an energy-deficient diet. For a power of 80%, seventeen subjects would be required in each group for this change to be significantly different at an α level of 0.05 and recruiting at least twenty subjects would allow for dropouts. Inclusion criteria advertised in local media were 19–45 years of age (pre-menopausal), BMI range from 25–32 kg/m², non-smokers, no known food allergies and of general good health. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the University of Wollongong, Human Ethics Committee (HE06/311). Written informed consent was obtained from all the subjects. The trial was registered with the Australian Clinical Trials Registry (ACTRN12607000126415).

The first seventy-seven of 215 enquiries were screened resulting in randomisation of sixty-six subjects, using a computer-generated sequence using random permuted blocks. All the subjects, but not dietitians were blinded to their randomisation status within the trial. The subjects attended the study centre for a total of five times. Visits included collection of background dietary data using a validated diet history interview and instruction on completion of a 3-d weighed food record, collection of fasting blood samples at baseline together with dietary education, two follow-up dietary visits and collection of fasting blood samples at 3 months. All the subjects had their height, weight and waist circumference recorded at each visit to the centre. The subjects were also contacted by telephone on one occasion in between the monthly visits to provide dietary review and support. Visual analogue scales (VAS) related to appetite, food records and the Baecke Physical Activity Questionnaire were all completed at baseline, half-way through the study and within the final week of the study. For the VAS, the subjects recorded their feelings on individual forms at six different time points throughout the day: immediately before each of the three main meals and 2 h after each of these meals. Markings by participants were measured in millimetres. All nutritional analysis was performed using FoodWorks 2007, version 5 (Xyris Software, Brisbane, QLD, Australia) with nutrient contents of study foods added as required.

Dietary intervention

A basal metabolic requirement for energy was calculated for each subject using the Schofield equation using BMI equal to 25 kg/m². A low activity factor (1.3) was chosen to estimate energy requirements and then 2000 kJ was subtracted from this level to a calculated weight loss of 0.5 kg/week. Intervention diets were designed to control for all macronutrients with the only variation in total fibre, primarily β-glucan such that a typical diet, as described in Table 1 only varied in the trial products given. Product development for cold and hot cereals as well as additional snack items took place in a pilot processing plant by qualified cereal food technologists. The source of β-glucan was a commercially available oat bran with 22% β-glucan. The subjects were instructed to eat the cereal provided at breakfast (ready-to-eat (RTE) cereal or porridge) and two snack items at afternoon tea (choice of two muesli bars, two cereal snack packs or one muesli bar and one cereal snack pack). Product consumption was evaluated for overall satisfaction, and positive and negative symptoms were observed using questionnaires at the completion of the study.

Products were tested for molecular weight, solubility, viscosity and both soluble and total β-glucan using methods described by Tosh et al. The amount of β-glucan in the control products was negligible (<0.2 g/serving). β-Glucan contents of the products, including soluble β-glucan are included in Table 2. The β-glucan in the RTE cereal and snack products had a higher solubility than the other products, with solubility improved by extrusion (porridges not extruded). The molecular weight of β-glucan extracted from all of the products showed only limited decreases due to effects of processing; highest where there was least processing (muesli bars and porridge) and lower in the extruded products (RTE cereal and cereal snack). The cereal and porridge samples with lower β-glucan contents (MBG samples) produced lower viscosity extracts (Table 2). The viscosity profiles of the HBG muesli bar and the HBG porridge had extracts with very similar viscosity profiles despite their differences in total β-glucan content. This is due to the greater solubility of the β-glucan in the muesli bar. The HBG RTE cereal had the greatest viscosity due to its high β-glucan and high solubility. All of the intervention products showed physicochemical characteristics which would be expected to produce
bioactivity. These products should have increased viscosity in the upper digestive tract which should modify the digestion and absorption rates of the nutrients.

Clinical indices

Fasting blood samples at 0 and 3 months were collected using Sarstedt Monovette blood collection tubes. Glucose, insulin, total cholesterol, HDL, LDL and TAG analysis was performed at an accredited pathology laboratory (Southern IML Pathology, Wollongong, NSW, Australia). A further sample was collected into tubes containing potassium EDTA (to achieve a concentration of 0.5–2 mg EDTA/ml of blood after collection). Both the tubes contained dipeptidyl peptidase IV (Millipore, Billerica, MA, USA) to level of 10 μl/ml of blood. One tube also contained aprotinin equivalent to 0.6 trypsin inhibitor units per ml of blood (Aprotinin from bovine lung Sigma Aldrich A1153 dissolved in normal saline with 0.9 % benzyl alcohol) for analyses of CCK and PYY. Together with the dipeptidyl peptidase IV inhibitor, the second tube contained 240 μl of complete protease inhibitor cocktail (Roche, Castle Hill, NSW, Australia) made to 25 x concentration in distilled water. Final concentrations met the recommendations for the manufacturers’ protocols for assays described later. The blood samples were then centrifuged at 4°C for 15 min at 1500 g. The plasma was stored at −80°C until further analysis could be completed.

Ghrelin, leptin, GLP-1 and total PYY were analysed using a Lincoplex Human Gut Hormone Panel (catalogue no. HGT-68K) according to manufacturer’s instructions. CCK analysis used Phoenix Peptides RIA (RK-069-04) for CCK octapeptide (CCK 26-33). The standard RIA protocol was applied but without extraction of the peptides before the

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**Fig. 1. Flow diagram of participation in the study. MBG, mid-dose β-glucan; HBG, high-dose β-glucan.**
assay. PYY3-36 analysis utilised a human specific RIA sourced from Linco (PYY-67HK) using the standard protocol.

Statistical analysis

Data for all anthropometry, blood analysis, VAS measurements and dietary intake were entered into SPSS for windows, version 15.0 (SPSS 15.0, Chicago, IL, USA). Repeated measures ANOVA (RMANOVA) using the general linear model with group (control, MBG and HBG) as the between-subjects factor, was used to identify primary changes in each parameter over time and also differences between the groups. Post hoc analysis using Bonferroni adjustments was reviewed to detect specific differences between the control and intervention groups. Log (base 10) transformations were also used as appropriate. Regression analysis was used to identify correlations between group anthropometric and biochemical indices. One-way ANOVA was used to review VAS and Baecke questionnaire results at various time points identifying any differences between the groups.

Results

Baseline data

Final numbers in each group were sixteen controls, twenty-one MBG and nineteen HBG (Fig. 1). There were two withdrawals from each of the MBG and HBG groups, but six were from the control group. Fifty-six subjects were included in the final data analysis. Some subjects did not complete all forms or blood was unable to be drawn for certain tests, so numbers in each calculation varied.

At baseline, there were no significant differences between group anthropometric and metabolic measures (Table 3). The subjects were overweight but overall they were not hyperlipidaemic (mean cholesterol 5·03 (SD 1·07) mmol/l). The only baseline biochemistry measure which showed significant differences between the groups was the fasting blood glucose level ($P=0·046$), where the level for the HBG group was lower than that of the other two treatments (Table 3).

Review of food records and diet histories at baseline indicated relatively well-matched groups. No significant differences were noted between energy, fibre, protein, fat or carbohydrate overall (Table 4). The only significant difference at baseline was the amount of MUFA consumed ($P=0·025$), where post hoc analysis using Bonferroni adjustments showed significantly less MUFA in the MBG group (24·8 (SD 9·8) g) compared with the control (32·5 (SD 8·0) g). The MUFA intake of the HBG group (28·8 (SD 6·8) g) was between these two levels.

Dietary intervention

Evaluation of the food records at mid-way through the study and at the end indicated little variation between the groups for macronutrient intakes at each time point with no significant differences between the groups. Review of the overall dietary composition of baseline diets compared with the mid-point of the study and the 3-month end-point showed a decrease in percentage energy from fat and an increase in the percentage energy from protein and carbohydrate (Table 4). General compliance with a weight reduction regimen was identified by overall weight loss (described later) and energy restriction detailed in the food records. Total energy

Table 1. Food serves for typical dietary study participant

<table>
<thead>
<tr>
<th>Food group</th>
<th>Serve size</th>
<th>Number of serves/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breads/cereal/starchy vegetables</td>
<td>1 slice bread or 1/2 cup pasta or 1 medium potato</td>
<td>4 serves</td>
</tr>
<tr>
<td>Other vegetables</td>
<td>1/2 cup cooked = 1 cup raw</td>
<td>2-5 cups cooked or 5 cups raw</td>
</tr>
<tr>
<td>Fruit</td>
<td>1 piece fresh or 3/4 cup canned</td>
<td>2 pieces</td>
</tr>
<tr>
<td>Milk/alternatives</td>
<td>150 ml light/200 ml skim, 100 g low-fat yoghurt</td>
<td>2–3 serves</td>
</tr>
<tr>
<td>Meat/alternatives</td>
<td>30 g meat/45–60 g fish/20 g low-fat cheese</td>
<td>3–4 serves</td>
</tr>
<tr>
<td>Fats</td>
<td>1 tsp oil or margarine or 1 tbsp avocado</td>
<td>3–4</td>
</tr>
<tr>
<td>Control or intervention products</td>
<td>Portion controlled pre-packed</td>
<td>Cereal + 2 snacks (muesli bar or snack pack of cereal)</td>
</tr>
</tbody>
</table>

Table 2. β-glucan and total fibre content of study products (allowing for moisture and fruit content, calculated for serve size)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
<th>Soluble β-glucan (g per serve)</th>
<th>Total β-glucan (g per serve)</th>
<th>Viscosity of extract (mPa x s)</th>
<th>Total fibre content (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>RTE cereal</td>
<td>Negligible</td>
<td>Negligible</td>
<td>Not done</td>
<td>4·0</td>
</tr>
<tr>
<td>Control</td>
<td>Porridge</td>
<td>Negligible</td>
<td>Negligible</td>
<td>Not done</td>
<td>1·2</td>
</tr>
<tr>
<td>Control</td>
<td>Extruded snack</td>
<td>Negligible</td>
<td>Negligible</td>
<td>Not done</td>
<td>0·6</td>
</tr>
<tr>
<td>Control</td>
<td>Muesli bar</td>
<td>Negligible</td>
<td>Negligible</td>
<td>Not done</td>
<td>2·9</td>
</tr>
<tr>
<td>MBG</td>
<td>RTE cereal</td>
<td>2·3</td>
<td>3·9</td>
<td>14·6</td>
<td>7·4</td>
</tr>
<tr>
<td>MBG</td>
<td>Porridge</td>
<td>1·2</td>
<td>3·0</td>
<td>19·0</td>
<td>6·6</td>
</tr>
<tr>
<td>HBG</td>
<td>RTE cereal</td>
<td>3·5</td>
<td>4·8</td>
<td>83·9</td>
<td>10·1</td>
</tr>
<tr>
<td>HBG</td>
<td>Porridge</td>
<td>1·7</td>
<td>4·4</td>
<td>58·9</td>
<td>9·7</td>
</tr>
<tr>
<td>HBG</td>
<td>Extruded snack</td>
<td>1·4</td>
<td>2·1</td>
<td>54·5</td>
<td>4·4</td>
</tr>
<tr>
<td>HBG</td>
<td>Muesli bars</td>
<td>0·9</td>
<td>1·7</td>
<td>57·0</td>
<td>4·6</td>
</tr>
</tbody>
</table>

RTE, ready-to-eat; MBG, mid-dose β-glucan; HBG, high-dose β-glucan.
intake was significantly lower at the mid-point (mean 6308 (SD 1068) kJ) of the trial and the end-point (mean 6000 (SD 1163) kJ) (P<0.001) compared with the baseline (mean 8725 (SD 1703) kJ) using RMANOVA with fibre level as the between-group effect and time as the within-subject variant. There was no interaction effect over time (P=0.192) indicating all the groups followed the energy-restricted diet to the same extent.

Dietary compliance with the study products measured by review of 3-d food records indicated that the use of breakfast cereal was very high (90% consumed), while compliance with the snacks was reasonable (74% consumed). Differences in dietary fibre intakes between the groups could be accounted for by the difference in fibre between the groups’ product supply. This includes both soluble (β-glucan) and insoluble fibre from the products. The groups achieved a significant difference in the amounts of dietary fibre consumed at mid-point (P<0.001, RMANOVA) and the last week of the study (P<0.001, RMANOVA; Table 4). There was limited change in the control group, indicating that the products supplied to the subjects (which especially in the case of the cereal were relatively high fibre) replaced foods of similar fibre content.

The only dietary variation between the groups was the monounsaturated fats which varied at baseline also. The levels were not different at mid-point and at 3 months, but rather the change from baseline produced an interaction effect for time × group (Table 4). Therefore, all anthropometric and biochemical results were also checked for correlation with the monounsaturated fat levels.

There were no significant differences in activity scores between the groups at any time point, indicating that changes in physical activity were not a factor in influencing other results.

**Clinical indices**

Almost all anthropometric and biochemical indices changed over time, although there were no significant differences between the groups for any measured clinical parameter over time (Table 5). The average weight loss for the study sample of 4·1 kg was significant (P<0.001; ranging from a 0·2 kg gain to a 14·9 kg loss). However, this is less than the anticipated 6–6·5 kg designed for the energy-restricted diet. It is also less than the change in kilojoule intake demonstrated in the food records would predict. The weight loss was not significantly different between the groups (P=0·921). Waist measurements decreased significantly (P<0·001). Fasting blood glucose results did not decrease, however, fasting insulin decreased significantly compared with baseline (P<0·001). However, no differences between the groups were identified (P=0·184; Table 5). Log transformation of insulin data showed the difference from baseline to 3 months was still significant (P=0·001) but not related to the intervention (P=0·113).

Regression analysis indicated that weight loss significantly predicted waist change (P=0·001), leptin (P=0·007), blood glucose level (P=0·031) and insulin (P=0·052). However, the predictions, other than the expected waist change (R² 0·480) were relatively weak (R² 0·130, 0·084 and 0·069, respectively). Adjusting changes in these parameters for weight loss, failed to identify any significant differences between the groups.

Lipid results were examined overall, between the groups and also reviewing data for those subjects who had elevated lipids at baseline. The reduction in total cholesterol was not significantly different between the groups, with mean decreases of 0·19 (SD 0·79), 0·31 (SD 0·55) and 0·56 (SD 0·61) mmol/l for the control, MBG and HBG, respectively (P=0·239). The general trend was as expected with the greatest decrease in cholesterol with the highest dose of β-glucan. Overall, there was a significant decrease in cholesterol over 3 months (P<0·001).

For all the subjects, HDL levels significantly decreased over time (P<0·001), LDL significantly decreased (P=0·028) and TAG did not change (P=0·353). Although the LDL results for RMANOVA did not show an overall effect between the groups, post hoc Bonferroni adjustments indicated a trend to differ between the control and high dose of β-glucan (P=0·077) with the greatest decrease in LDL in the HBG group (Table 5). No significant (P<0·05) differences were detected between the groups for the other lipid parameters.

Large standard deviations existed within all the datasets limiting the significance of results, especially hormonal

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**Table 3. Baseline characteristics of study subjects**

(Mean values and standard deviations)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n 16)</th>
<th>MBG (n 21)</th>
<th>HBG (n 19)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>37·1 ± 5·6</td>
<td>37·7 ± 6·0</td>
<td>37·4 ± 5·5</td>
<td>0·96</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>77·0 ± 7·8</td>
<td>80·9 ± 8·2</td>
<td>77·6 ± 6·5</td>
<td>0·17</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>83·8 ± 5·7</td>
<td>85·1 ± 8·0</td>
<td>82·4 ± 5·6</td>
<td>0·29</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/l)</td>
<td>4·7 ± 0·6</td>
<td>4·9 ± 0·5</td>
<td>4·5 ± 0·4</td>
<td>0·05*</td>
</tr>
<tr>
<td>Fasting insulin (µU/l)</td>
<td>10·7 ± 4·3</td>
<td>11·1 ± 5·8</td>
<td>12·1 ± 4·8</td>
<td>0·57</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5·2 ± 0·7</td>
<td>5·0 ± 1·1</td>
<td>4·9 ± 1·3</td>
<td>0·73</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>3·2 ± 0·5</td>
<td>2·9 ± 1·0</td>
<td>2·7 ± 0·9</td>
<td>0·17</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1·6 ± 0·4</td>
<td>1·6 ± 0·3</td>
<td>1·7 ± 0·3</td>
<td>0·63</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>1·3 ± 0·4</td>
<td>1·1 ± 0·5</td>
<td>1·0 ± 0·5</td>
<td>0·55</td>
</tr>
</tbody>
</table>

MBG, mid-dose β-glucan; HBG, high-dose β-glucan.

* Significant value was measured using ANOVA.
Table 4. Reported energy and macronutrient intakes at baseline, mid-point and at 3 months, with $P$ values for repeated-measures ANOVA between groups (Mean values and standard deviations)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline Mean (SD)</th>
<th>Mid-point Mean (SD)</th>
<th>3 months Mean (SD)</th>
<th>$P$-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MBG</td>
<td>HBG</td>
<td>Control</td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>9229 (1419)</td>
<td>8394 (2122)</td>
<td>8213 (1284)</td>
<td>6060 (910)</td>
</tr>
<tr>
<td>Protein (%E)</td>
<td>18.6 (3.5)</td>
<td>18.5 (5.1)</td>
<td>17.6 (5.6)</td>
<td>20.6 (2.7)</td>
</tr>
<tr>
<td>CHO (%E)</td>
<td>42.9 (6.5)</td>
<td>42.0 (16.4)</td>
<td>42.1 (8.8)</td>
<td>50.3 (7.7)</td>
</tr>
<tr>
<td>Total fat (%E)</td>
<td>34.9 (19.0)</td>
<td>32.1 (10.1)</td>
<td>34.3 (9.1)</td>
<td>22.6 (8.8)</td>
</tr>
<tr>
<td>SFA (%E)</td>
<td>13.3 (3.8)</td>
<td>12.7 (5.0)</td>
<td>14.5 (3.8)</td>
<td>8.4 (3.2)</td>
</tr>
<tr>
<td>PUFA (%E)</td>
<td>4.6 (1.4)</td>
<td>4.4 (1.7)</td>
<td>5.2 (1.8)</td>
<td>3.7 (1.6)</td>
</tr>
<tr>
<td>MUFA (%E)</td>
<td>13.1 (3.2)</td>
<td>10.9 (4.1)</td>
<td>13.0 (3.1)</td>
<td>8.1 (4.4)</td>
</tr>
<tr>
<td>Total fibre (g)</td>
<td>21.9 (4.6)</td>
<td>21.2 (6.0)</td>
<td>20.5 (6.8)</td>
<td>24.2 (5.4)</td>
</tr>
</tbody>
</table>

E, energy; CHO, carbohydrate.

* Significant values were measured using repeated-measures ANOVA.
Table 5. Changes in clinical indices overtime and between control, mid-dose β-glucan (MBG) and high-dose β-glucan (HBG) groups (Mean values and standard deviations)

<table>
<thead>
<tr>
<th>Changes in clinical indices</th>
<th>Baseline data</th>
<th>Mean change</th>
<th>P†</th>
<th>Control</th>
<th>Mean change</th>
<th>MBG</th>
<th>Mean change</th>
<th>HBG</th>
<th>Mean change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>78·4</td>
<td>−4·1</td>
<td>3·1</td>
<td>&lt; 0·001</td>
<td>77·6</td>
<td>7·8</td>
<td>−4·0</td>
<td>2·4</td>
<td>80·9</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>83·8</td>
<td>−4·1</td>
<td>2·8</td>
<td>&lt; 0·001</td>
<td>83·8</td>
<td>5·7</td>
<td>−4·0</td>
<td>2·6</td>
<td>85·3</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4·72</td>
<td>0·2</td>
<td>0·47</td>
<td>0·586</td>
<td>4·75</td>
<td>0·57</td>
<td>−0·01</td>
<td>0·41</td>
<td>4·89</td>
</tr>
<tr>
<td>Insulin (mmol/l)</td>
<td>11·37</td>
<td>−2·68</td>
<td>3·75</td>
<td>&lt; 0·001</td>
<td>10·74</td>
<td>4·29</td>
<td>−3·16</td>
<td>4·59</td>
<td>10·92</td>
</tr>
<tr>
<td>Cho (mmol/l)</td>
<td>5·0-3</td>
<td>−0·37</td>
<td>0·65</td>
<td>&lt; 0·001</td>
<td>5·21</td>
<td>0·73</td>
<td>−0·19</td>
<td>0·79</td>
<td>5·02</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>2·91</td>
<td>−0·23</td>
<td>0·2</td>
<td>&lt; 0·001</td>
<td>1·59</td>
<td>0·38</td>
<td>0·24</td>
<td>0·10</td>
<td>1·64</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>1·65</td>
<td>−0·14</td>
<td>0·48</td>
<td>0·028</td>
<td>3·23</td>
<td>0·47</td>
<td>−0·13</td>
<td>0·54</td>
<td>2·89</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>1·11</td>
<td>−0·09</td>
<td>0·43</td>
<td>0·353</td>
<td>1·27</td>
<td>0·40</td>
<td>−0·05</td>
<td>0·29</td>
<td>1·06</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>53·4</td>
<td>−6·4</td>
<td>29·5</td>
<td>0·217</td>
<td>45·4</td>
<td>21·0</td>
<td>−6·9</td>
<td>18·7</td>
<td>65·0</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>20·620</td>
<td>−7130</td>
<td>5720</td>
<td>&lt; 0·001</td>
<td>18·690</td>
<td>9340</td>
<td>−7050</td>
<td>6470</td>
<td>20·300</td>
</tr>
<tr>
<td>GLP (pg/ml)</td>
<td>47·95</td>
<td>−6·9</td>
<td>9·3</td>
<td>&lt; 0·001</td>
<td>49·6</td>
<td>16·7</td>
<td>−7·7</td>
<td>9·5</td>
<td>50·3</td>
</tr>
<tr>
<td>PYE (pg/ml)</td>
<td>85·80</td>
<td>−12·0</td>
<td>26·2</td>
<td>0·015</td>
<td>92·4</td>
<td>32·1</td>
<td>−5·6</td>
<td>11·8</td>
<td>89·1</td>
</tr>
<tr>
<td>CCK (pg/ml)§</td>
<td>73.23</td>
<td>6·1</td>
<td>21·0</td>
<td>0·195</td>
<td>71·4</td>
<td>11·7</td>
<td>2·5</td>
<td>18·2</td>
<td>71·0</td>
</tr>
<tr>
<td>CCK (pg/ml)§</td>
<td>218</td>
<td>177</td>
<td>170</td>
<td>&lt; 0·001</td>
<td>186</td>
<td>167</td>
<td>219</td>
<td>147</td>
<td>245</td>
</tr>
</tbody>
</table>

Cho, cholesterol; GLP, glucagon-like peptide; PYE, peptide YY; CCK, cholecystokinin.

* P values for ANOVA for change from 0 to 3 months.
† P values for repeated-measures ANOVA for changes between groups.
‡ P < 0·05 when compared to control using Bonferroni adjustments. No significant difference existed between MBG and HBG.
§ Results for CCK were higher than usually measured. Peptides were not extracted before analysis and this may have affected absolute results, however, as this is a repeated-measures analysis, trends can still be detected if they exist.
changes. The decrease in leptin levels for the study sample was significant over time ($P<0.001$) and regression analysis indicated the association with decreasing body weight ($P=0.007$). Between the groups, the differences approached significance ($P=0.078$) with the greatest decrease in leptin identified in the HBG group, even with the same body weight change.

Total PYY levels decreased significantly over time (RMANOVA, $P=0.015$), and this was significantly different between the groups ($P=0.041$). Post hoc Bonferroni adjustments indicated that the control group produced significantly different effects compared with both the MBG ($P=0.021$) and HBG ($P=0.050$) groups, where the smallest decrease in PYY was seen in the HBG group. The greatest change was in the MBG group so this would not seem related to dose of β-glucan. Even though levels of total PYY decreased in all the groups those for PYY$_{3-36}$ increased, although the latter were not statistically significant ($P=0.195$). PYY$_{3-36}$ levels increased more with the inclusion of β-glucan in the diet (8–10 pg/ml increase compared with 2.5 pg/ml in the control), but this was not significant between the groups ($P=0.807$). There was a significant decrease in GLP-1 over time ($P<0.001$) but no differences between the groups ($P=0.567$). Ghrelin levels did not significantly alter over time ($P=0.217$) and there were no between-group effects ($P=0.632$). CCK results seemed elevated most likely due to a lack of extraction of peptides, however, as this was a repeated-measures analysis, results were included. CCK levels increased significantly over time ($P<0.001$) but there was no group effect ($P=0.969$) with all the groups increasing the CCK levels at equal rates.

All variables were reviewed adjusting for weight loss and monounsaturated fat, but other than the correlations described previously, no significant trends were noted. The large standard deviations indicated that individual fluctuations are more varied than any overall effect of increased β-glucan.

**Subjective satiety**

VAS results were reviewed for individual time points to compare any differences which may have existed between the control and test groups (ANOVA). No significant differences were identified between the groups at any time point tested – that is all individuals showed similar hunger/fullness at the same test points (data not shown).

**Product evaluation**

The majority of subjects in the intervention groups reported some negative physical symptoms from the products (52 % for MBG and 63 % for HBG), although this was less than the numbers experiencing positive symptoms (67 % for MBG and 85 % for HBG). Negative symptoms included abdominal pain and changes in bowel patterns (both increased and decreased stool frequency). Positive associations with the study products included ‘feeling more full, for longer’, ‘healthy snacking avoiding unhealthy snacking’, ‘less peaks and lows’ in intake, ‘more regular bowel movements’ and ‘increased energy’. The products were also evaluated from an organoleptic perspective using questionnaires with Likert scales. Overall, all the RTE cereal products received favourable evaluations. The porridges overall were disliked and were consumed at a minimal level.

**Discussion**

Within the context of the present study, regardless of the diet subjects were on, they lost weight, with the expected changes in waist circumference, fasting insulin$^{21}$, lipids$^{22}$ and leptin$^{23}$. Even though weight loss was modest, the change of 5 % if maintained even at 3–4 % would most likely decrease the incidence of development of type 2 diabetes in this moderately overweight group, but at risk group$^{24}$. The waist circumference of the group as a whole decreased significantly and as a measure of abdominal adiposity indicated decreased risk of insulin resistance, glucose intolerance and dyslipidaemias$^{25,26}$. The fact that the subjects were only moderately overweight with limited progression towards metabolic syndrome, most likely limited the ability of the study to differentiate subtle differences that may have existed between the groups. Nevertheless, energy restriction alone remained effective for weight loss.

Much of the research with β-glucan as a functional ingredient focuses on improvements in parameters, such as glycaemic control and hypercholesterolaemia. The research here used healthy subjects whose ‘healthy overweight’ status ensured some elevated lipids but minimal insulin resistance. Greater effects may have been seen in a population with diabetes with greater metabolic dysfunction. Effects of β-glucan in a more overweight or obese sample with or without energy restriction cannot be inferred from the present study. The change in cholesterol, greatest for the HBG group may have been more exposed with more subjects, but this was a secondary outcome measure. Some literature on non-hypercholesterolaemic or mildly hypercholesterolaemic individuals tends to show limited response to β-glucan, so that the borderline significance of results would be expected, particularly given the observed weight loss. Greater than 10 % decrease in the HBG group together with evidence from other studies$^{27}$ still provides support for oat β-glucan as a dietary intervention agent in the management of hypercholesterolaemia. The observed decrease in HDL levels was unexpected, but the reported consumption of saturated fats was notably higher than polyunsaturated fats (Table 4), so the background diet may not have been favourable.

With the modest weight loss demonstrated here, there were still significant changes in PYY and GLP-1. Additionally, there was an overall decrease in PYY while maintaining or perhaps increasing the fractions involved with satiety PYY$_{3-36}$. It has been postulated that PYY has a role in the aetiology of human obesity due to negative correlation with BMI and the fact that injection of the active fraction of PYY, PYY$_{3-36}$ decreases food intake in human subjects$^{28}$. PYY is secreted in the same gut endocrine cells as GLP-1; both inhibit gastric emptying and promote satiety and are released in response to food. GLP-1 having an additional or additive role in its regulation of blood glucose via increasing insulin sensitivity$^{29}$.

The observed relationship between changes in hormones and weight loss was perplexing. Nevertheless, recent research has shown that with a very large weight loss, as seen in
surgical interventions for obesity, fasting levels of PYY increase but GLP-1 decreases, suggesting a less easily defined relationship between the two hormones and that they are not co-dependent(30). Studies using hypoenergetic diets to induce weight loss have identified a decrease in GLP-1 response to dietary stimuli with weight loss(31).

In clinical trials, fasting PYY and often PYY3-36 have been shown to be decreased in obesity. Roth identified decreased weight maintenance(32). Pfluger identified soluble fibre (35) that also could not find an intervention single fibre remain a difficult task within a human intervention. The results from the present study broadly mimic these results with an overall decrease in PYY, but an increase or at least maintenance in PYY3-36. Animal studies in our laboratory (unpublished results) have indicated an increase in PYY3-36 with increasing doses of β-glucan, and the trend in the present study warrants further investigation.

The group effect noted in fasting PYY, where both MBG and HBG results are different from the control, is difficult to interpret, given the smallest decrease was in the HBG and the largest decrease in the MBG. Other results which show similar trends (MBG a greater difference in one direction compared with the control or HBG such as with leptin) do not show significant correlations with the PYY results so it is difficult to infer a mechanism based on a U-shaped curve, where a certain dose would produce negative results but a greater dose would achieve desired outcomes. For example, in the present study no correlations existed with monounsaturated fat intake that was highest in the MBG group, so that the differences may just be statistical aberrations, in part created by the large variations in responses. Similarly, the larger change in leptin in the HBG group with the same weight loss seems positive, but the smallest change was with the MBG group and no dietary correlations exist.

We found a high overall acceptance of the high doses of fibre and good maintenance of the solubility, molecular weight and viscosity of the β-glucan after processing. No differences were identified with the subjective measures of satiety. The use of VAS is most likely to be accurate in a controlled situation such as in acute meal test studies in a laboratory situation(18). So it is perhaps not surprising that small differences from month to month are unlikely to be quantified by the subjects using the scales.

Despite attention to product attributes that theoretically affect satiety and tight dietary controls, the present study failed to confirm direct effects of set levels of β-glucan on weight loss in an energy-restricted diet. Epidemiological evidence strongly suggests that high fibre diets have positive effects on weight control(34). Separating out the effects of a single fibre remain a difficult task within an human intervention trial, and it would seem the major outcomes in the present study are similar to another recent intervention trial with soluble fibre(35) that also could not find an intervention effect with respect to weight loss. The lack of positive results such as these could certainly be used to support the notion that ‘fibre’ is not just an indigestible ingredient in a food but is a part of healthy diet that includes wholegrains, fruits and vegetables perpetuating good health. However, there are a number of confounding variables in the present study, which warrant consideration.

First the current trial was only of 3 months duration. Studies identifying positive effects of fibre, such as decreased weight gain usually last for a number of years(45). A 3-month intervention may not be long enough to separate out the differences between the subjects’ desire to comply and the actual effects of the dietary intervention. This difference is more likely to be obvious if the subjects were to follow a particular eating pattern over a longer timeframe. In a recent study, researchers have found that regardless of the dietary intervention, subjects who reported greater compliance with a weight loss regimen have lost a greater amount of weight(36). The authors conclude that strategies to increase adherence may be more important than dietary composition.

Second, the present study included products for the control group, which were still relatively high in fibre. Only the amounts of β-glucan varied within the diet. It seems likely that the effect of any one ingredient, even if positive, will be relatively small and hence showing a statistical difference between the groups will be difficult, especially over only 3 months. The numbers of subjects identified as necessary to detect a difference in the present study was based on the work of Solum(16), who used an energy-restricted diet with overweight women to determine the possible benefits of fibre supplementation. Control subjects used placebo tablets, while the intervention group had approximately 6 g of fibre sourced from grains and citrus fruit fibres. Over 12 weeks, the intervention group lost 1·8 kg more. The way the subjects consumed the fibre in the Solum study (capsules with water just before each meal) may have affected the outcomes. The overall greater weight loss of 6·7 and 8·5 kg for the control and intervention groups, respectively in this supplement study shows that adding food items to a dietary regimen (as in the currently reported study) may decrease weight loss to some degree. However, insisting subjects ate particular breakfast and afternoon tea items may have increased energy consumption at times when the subjects may have chosen to eat less in a non-directed environment.

Finally, it is difficult to control the intake of human subjects. Although food records and subjective discussions with the subjects indicated high compliance with consumption of study products overall, the subjects were less compliant with the energy restriction as evidenced by the modest weight loss. The greatest number of subjects withdrew from the control group, which could infer the greatest difficulty with compliance and a positive benefit of β-glucan, but this is unable to be confirmed within the constraints of the ethical requirements of the intervention trial.

In summary, although the groups experienced general improvement in measures such as fasting insulin and cholesterol, associated overall with weight loss, no effects seem specifically related to β-glucan dose. Most likely, some differences would have been realised if the study continued in the longer term, as generally, the subjects with higher fibre intakes will maintain weight more easily. However, within the timeframes of an intervention trial, and realistically, the time frames individuals may pursue a ‘weight loss diet’, there were no discernable differences between a regular energy-restricted diet and that which contained significant β-glucan.
Acknowledgements

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References


Letter to Editors:

**Title:** What is the mechanism for aripiprazole’s effect on reducing olanzapine-induced obesity?

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**Running title:** Aripiprazole, dopamine D2 receptor; obesity
Editors,

We read with great interest Henderson and colleagues’ paper in your journal (2009; 29:165–169), which reported that aripiprazole reduced olanzapine-induced overweight/obesity and hyperlipidemia in a 10-week placebo-controlled double-blind crossover study. This and their previous studies 1,2 provide a new way for controlling olanzapine- and clozapine-induced weight gain/obesity using another atypical antipsychotic, even without reducing the original olanzapine and clozapine doses, which is important particularly for treatment of refractory schizophrenia patients. The key issue is what are the mechanisms that underlie aripiprazole’s effects on body weight? Henderson et al. proposed that aripiprazole’s low histaminergic antagonism and 5-HT2C agonist activity may contribute to its effect on reducing olanzapine-induced weight gain 1. Several meta-analytical studies have indicated an association between histamine H1 antagonism properties in antipsychotic drugs and obesity side-effects 3,4. Consistent with these findings, both olanzapine and clozapine are potent H1 antagonists 5. A recent study found that, correlated with body weight gain, olanzapine treatment significantly down-regulated H1 receptor binding and mRNA expression in the rat hypothalamus, however, aripiprazole did not affect H1 receptor expression 6. These results suggest that aripiprazole’s effects in reducing olanzapine- and clozapine-induced weight gain/obesity should not be via H1 receptors, although histaminergic antagonism is a main cause of olanzapine- and clozapine-induced weight gain/obesity. We agree that 5-HT2C receptors may play a role, however, it should also be noted that aripiprazole has only a moderate affinity to 5-HT2C receptors 7. Aripiprazole was developed as a potent dopamine D2 partial-agonist, 5-HT1A partial agonist, and also 5-HT2A antagonist 7. A recent study has reported that both aripiprazole and olanzapine affect 5-HT1A receptor expression, but these changes
are not correlated with body weight. On the other hand, like aripiprazole, olanzapine and clozapine are 5-HT2A antagonists. Recent studies have suggested that aripiprazole is not a simple partial agonist, but a functionally selective drug that can act as a D2 agonist or D2 antagonist in different brain regions. We suggest that aripiprazole’s D2 agonistic property may account partly for the effect of aripiprazole in reducing olanzapine-induced overweight/obesity. Atypical antipsychotics such as olanzapine may increase appetite through the dopamine-mediated reward pathway. Dopamine D2 agonists have been reported to reduce food intake by acting in hypothalamic areas. Another possible mechanism of aripiprazole may be via the activation of the PI3K/Akt pathway. The PI3K/Akt pathway plays an important role in cellular proliferation, growth and metabolism. Over-expression of the pathway causes cancer but defects in the pathway could induce metabolic disorders. The PI3K/Akt pathway plays a key role in the action of insulin via control of Glu4, which transports glucose into the cells. The activity of the PI3K/Akt pathway in insulin-mediated Glu4 activation is impaired in olanzapine-induced obesity. Aripiprazole may have an effect on the activation of the PI3K/Akt pathway via its agonistic effect on D2 receptors. In fact, D2 receptor agonist (bromocriptine) has been reported to increase the PI3K/Akt pathway activity. It is possible that aripiprazole can restore the impairment of the PI3K/Akt pathway in insulin-mediated Glu4 activation caused by olanzapine so that the side-effect of weight gain is reduced. Further studies on these mechanisms will improve our understanding and management of atypical antipsychotic-induced weight gain/obesity.
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Review

The signal pathways in azoxymethane-induced colon cancer and preventive implications

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Key words: azoxymethane, colon cancer, signal pathway, K-ras, TGFβ, β-catenin, MAPK, PI3K/Akt

Colon cancer is the third most common cancer and third most common cause of cancer-related death in the USA according to 2008 American Cancer Society statistics. The carcinogenesis of colon cancer has been associated with both genetic and environmental factors. It has been found that several signal pathways, including K-ras, Src/PI3K/Akt, β-catenin, TGFβ and p53 play critical roles in its pathogenesis. The 5 year survival rate of metastatic colon cancer is below 10%. Thus, it is necessary to further understand its biology and search for effective therapy.

Azoxymethane (AOM) is a common model for colon cancer. It can specifically induce colon cancer similar to the pathogenesis of human sporadic colon cancer. Thus, it has been extensively used in the study of the molecular biology, prevention and treatment of colon cancer. After administration, AOM is metabolised into methylazoxymethanol by CYP2E1, which causes DNA mutations. Mutation of K-ras activates this pathway and its downstream PI3K/Akt pathway and MAPK pathway. Mutation of β-catenin also prevents it from being degraded by GSK-3 and accumulation of β-catenin leads to cell proliferation. TGFβ, a pro-apoptotic protein, is inhibited. All of these changes form the basis of AOM carcinogenesis. This model has been used in the study of the genetic deficiencies of colon cancer and in the prevention and treatment of the disease. For example, TGF-β2 and adiponectin knockout mice are more susceptible to AOM, while high amylose cornstarch, green tea and artemisia have protective effects.

Introduction

Colon cancer is the third most common cause of cancer-related death in USA according to 2008 American Cancer Society statistics. The carcinogenesis of colon cancer has been associated with both genetic and environmental factors. Many genetic defects in colon cancer have been found and these play important roles in the carcinogenesis of colon cancer. These mutations include: Apc (adenomatous polyposis coli), K-ras, PIK3CA, PTEN, the TGFβ receptor and TP53 genes. The corresponding signal pathways involved in colon cancer have also been characterised. Environmental factors, including obesity, diabetes and diet also play important roles in colon cancer. It has been recognised that the development of colon cancer proceeds in sequential stages from polyps to adenocarcinoma. It may take a long time for colon cancer to develop, and is estimated to be between 10–17 years. This lag time provides an opportunity to prevent the disease. Indeed, it has been demonstrated that colon cancer is the only cancer that can be prevented by selecting the appropriate foods and lifestyle. The most effective treatment for colon cancer is still surgical removal. Only those patients whose tumour cannot be removed will be treated using chemotherapy and radiotherapy. Unfortunately, the 5-year survival rate for metastatic colon cancer is below 10%. Thus, it is necessary to further understand the cancer biology and develop effective treatment. Colon cancer has been studied in many colon cancer cell lines. Several animal models have also been used to study molecular pathogenesis as well as preventive and therapeutic approaches. While the Apc mutation has been used as a model for the familial adenomatous polyposis colon cancer model, azoxymethane (AOM) is commonly used to mimic sporadic colon cancer.

The AOM colon cancer model is extensively used in the study of the underlying mechanisms of human sporadic colon cancer. AOM is a potent carcinogen causing a high incidence of colon cancer in rodents. Development of this cancer closely mirrors the pattern seen in humans. Repetitive intra-peritoneal treatment of rodents with AOM causes tumours specifically in the distal colon. This can be easily and reliably achieved, particularly in susceptible rats and mice, making it a useful model for colon cancer. Following AOM treatment, the epithelial cells undergo pathogenesis from minor lesion aberrant crypt foci (ACF), to adenoma and malignant adenocarcinoma. The in vivo metabolite of AOM causes DNA mutations, changing the nucleotides from G:C to A:T.
The duration of AOM-induced colon cancer takes 14 weeks in mice or rats.
In this review, we summarise the signal pathways activated by AOM for the carcinogenesis, and the application of the AOM model in the study of colon cancer molecular pathogenesis and preventive intervention.

**Metabolism of AOM**

Acrylomethane does not interact with DNA directly. It has to be activated in vivo to develop carcinogenesis. Acrylomethane is metabolised by cytochrome P450, specifically isoform CYP2E1.15 The first step is the hydroxylation of the methyl group of AOM to form methyloxacyzethanol (MAM). Methyloxacyzethanol then breaks down into formaldehyde and a highly reactive alkylating species, probably the methylazoniunium. This chemical actually causes alkylation of DNA guanine to O6-MEG and to O4-methylthymine.12 These mutations can initiate tumorigenesis through several key genes in intracellular signal pathways. The inhibition of CYP2E1 (for example by disulfiram, an agent used for avoidance therapy in alcohol abuse) has been shown to prevent chemical carcinogenesis.14 In CYP2E1 knockout mice, O6-MEG formation and colon polyp numbers decrease in response to AOM treatment.15

**Mechanisms for AOM Causing Colon Cancer**

Several activation pathways have been revealed to explain the mechanism of AOM-induced colon cancer (Fig. 1). These include K-ras, β-catenin and TGFβ. However, as yet, there is no unified explanation for the mechanism of this model.

**K-ras pathway.** K-ras plays an important role in the carcinogenesis of colon cancer. Using single cell isolation, the mutation of K-ras gene in colorectal cancer has been found to cause G1 to A1 at codon 12 deriving from O6-methyl-deoxyguanine adducts.12 It changes glycine to aspartic acid. This mutation causes the activation of the K-ras protein. K-ras is a small G-protein that regulates both MAPK and PI3K/Akt intracellular signal pathways, which, in turn, regulate cell growth, proliferation and glucose metabolism. Both pathways play important roles in the carcinogenesis of many types of cancers including colon cancer. This is similar to human colorectal cancer. A study showed that pEGFR, pAkt and pMAPK are increased in colon tumours compared to normal colon tissue.15

The PI3K/Akt pathway is important in colon cancer and 20% of patients have PI3KCA mutations.2,16,17 The activation of PI3K/Akt can increase cell survival pathways via phosphorylation of downstream targets, including NFκB, and Bcl-xL.18 PI3K/Akt also blocks p53 and the forkhead/Fas-ligand to decrease apoptosis.19,20 It has been shown that Fas is increased in colon cancer.21 In the cell cycle pathway, PI3K/Akt deactivates glycogen synthase kinase 3 (GSK3) and promotes cyclin D1 and myc to increase cell proliferation.22 In the cell growth pathway, PI3K/Akt activates the mammalian target of rapamycin (mTOR), a conserved Ser/Thr kinase to increase cell size.23 Whether mTOR activity is increased in the AOM model has not yet been studied. Downstream of PI3K/Akt, COX2 has also been shown to be involved in the carcinogenesis of AOM.24,25

**β-catenin pathway.** β-catenin plays an important role in cell adhesion and also is an oncogenic protein. It associates with cadherin or alpha-catenin to link the actin cytoskeleton. It is also a co-transcriptional activator of genes in the Wnt signal pathway. In the free form, it associates with the scaffolding proteins, axin and Apc, and is phosphorylated by GSK-3β resulting in degradation by the proteasome.29 In most colon cancer patients, there is a mutation in the Apc gene. For example, in 80% of sporadic colon cancer patients, a mutated Apc cannot bind to β-catenin for normal function.31 The N-terminus of β-catenin is also mutated in some cases, so that β-catenin cannot form the complex and be degraded.32 Thus, free β-catenin is increased and binds with the T-cell factor/lymphoid enhancer factor TCF/LEF to form a complex, which activates gene transcription and cell proliferation.33,34 It targets c-myc and cyclinD1 genes, which are well known carcinogens.35-34

Acrylomethane causes β-catenin mutations at codons 33 and 41, which are the serine and threonine residues that are targets for GSK-3β phosphorylation.35,36 This leads to the accumulation of β-catenin for the carcinogenesis. It has been shown that AOM

**Figure 1.** AOM is catalysed into MAM which makes DNA mutations. The mutation in TGFβ causes its inactivation, while mutation of β-catenin prevents degradation by GSK-3β and Ras is activated by mutation. These changes eventually lead to decreased apoptosis and an increased cell cycle for the carcinogenesis of colon cancer,

Activated Ras stimulates the serine/threonine-selective protein kinase, Raf kinase, which is an oncogene. The protein encoded has regulatory and kinase domains. Ras binds to CR1 in the regulatory region and phosphorylates CR2, which is rich in serine/threonine. This leads to activation of GR3 in the kinase region. It then, in turn, activates MAPK and ERK kinase (MEK), which activates mitogen-activated protein kinase (MAPK) and ERK. MAPK and ERK promote carcinogenesis via target proteins like c-myc, CREB, RSK, Mcl1, p16, Rb and cyclins.30 Inhibition of these pathways has been demonstrated to cause cancer cell death.37 Overexpression of cell cycle promoters, cyclin D1, may contribute to the AOM model as well.38 Cdhl has been found in the early stages in the AOM cancer induced mouse colon.28 **β-catenin pathway.** β-catenin plays an important role in cell adhesion and also is an oncogenic protein. It associates with cadherin or alpha-catenin to link the actin cytoskeleton. It is also a co-transcriptional activator of genes in the Wnt signal pathway. In the free form, it associates with the scaffolding proteins, axin and Apc, and is phosphorylated by GSK-3β resulting in degradation by the proteasome.29 In most colon cancer patients, there is a mutation in the Apc gene. For example, in 80% of sporadic colon cancer patients, a mutated Apc cannot bind to β-catenin for normal function.30 The N-terminus of β-catenin is also mutated in some cases, so that β-catenin cannot form the complex and be degraded.32 Thus, free β-catenin is increased and binds with the T-cell factor/lymphoid enhancer factor TCF/LEF to form a complex, which activates gene transcription and cell proliferation.33,34 It targets c-myc and cyclinD1 genes, which are well known carcinogens.35-34

**AOM-induced colon cancer**

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treatment increases both β-catenin and cyclin D. There are no reports to indicate that AOM causes APC mutations.

**TGF-β pathway.** Transforming growth factor-β (TGF-β) includes isoforms 1, 2, and 3 can inhibit cell growth, proliferation, and the cellular cycle progression and thus has an anti-tumour effect. Defects in TGF-β signalling have been found in 20–30% of colon cancer patients. The activity of the TGF-β pathway is decreased after AOM treatment, which mediates AOM-induced colon cancer. It has been demonstrated that the active form of TGF-β is decreased in AOM treated mice.

TGF-β induces apoptosis through several signal pathways. First, TGF-β forms dimers and binds to its type II receptor. This complex then associates and phosphorylates its type I receptor. The type I receptor in turn phosphorylates receptor-regulated SMAD (R-SMAD) to cause apoptosis. Second, the activated type II receptor binds to death-associated protein 6 to induce apoptosis. Third, TGF-β has also been shown to inhibit the phosphorylation of the p53 subunit of PI3K/Akt induced by GM-CSF in several myeloid leukemia cell lines, including MV4-11, TF-1 and TF-1a.

**Dose-Dependent Responses of AOM Treatment**

As mentioned previously, azoxymethane treatment is a model for non-familial colon cancer and is pathologically similar to sporadic colon cancer in the descending human colon. In the susceptible strain A/J mice, the dose 10 mg/kg was determined to be the maximum tolerated dose for tumorigenesis, causing an 80% incidence of colon cancer. The dose of 5 mg/kg initiates only 8% incidence. All A/J mice injected with a 20 mg/kg dose died after 1 week due to the toxicity of AOM. It has been shown that high dose of AOM causes fulminant hepatic failure in C57BL/6J mice, which are relatively resistant to AOM-induced colon cancer. It was also shown that oral administration of AOM is not effective in inducing colon cancer.

**Application of the AOM Model in the Study of the Biology of Colon Cancer and Therapy**

The utility of the AOM model of colon cancer in the study of genetic deficiency and the signal pathway in the carcinogenesis of colon cancer. It has been shown that polyposis numbers increased in TGFβR knockout mice treated with azoxymethane. Twenty percent to thirty percent of all colon cancer patients exhibit mutational inactivation of TGFβR2. Knockout of TGFβR2 significantly increases the number of AOM-induced adenomas and adenocarcinomas.

In adipopecin knockout mice, it has been shown that a genetic deficiency in the gene accelerates colon cancer development. The AOM model was used in three types of gene-deficient mice: adiponectin-deficient, adiponectin receptor 1-deficient, and adiponectin receptor 2-deficient. In adiponectin-deficient mice, the number of AOM-induced colon polyps was markedly increased compared with wild-type mice fed a high-fat diet, but not in mice fed a lab chow diet. This correlated with a significant increase in cell proliferative activity as well as mTOR pathway activity in the colonic epithelium of the adiponectin-deficient mice when compared with wild-type mice fed a high-fat diet.

**AOM and dietary prevention of colon cancer.** Azoxymethane has also been used in the study of the preventive effects of many foods and beverages on colon cancer, such as undigestable (resistant) starch. After feeding with 20% high amylose cornstarch, there is 50% decrease in the incidence of neoplasia induced by AOM in rats. It was demonstrated that high amylose cornstarch increased colon epithelial cell apoptosis and decreased their proliferation. This may be caused by increased fermentation so that butyrate is increased, which is known to have an anti-cancer effect. Other mechanisms could be also involved, including increased volume of faeces and changes in pH values as well as the properties of high amylose cornstarch itself.

Green tea, a popular beverage, has been shown to have anti-cancer effects in many animal models. Azoxymethane treatment has also been used to assess the effect of green tea on colon cancer. Rats were given AOM at 15 mg/kg for two weeks, followed by a 0.02% w/v crude green tea extract, using tap water as the control. The number of neoplastic lesions were reduced by 60% after administration of crude green tea extract with decreased COX2 compared with controls. In another experiment, 0.24% Polyphenon E (PPE, a standardised green tea preparation consisting of 65% of (−)-epigallocatechin-3-gallate and 22% other catechins) was found to significantly decrease the total number of ACF and the percentage of ACF with high grade dysplasia in rats. It has been shown that β-catenin and cyclin D1 in high-grade dysplastic ACF from a 0.24% PPE-treated group were decreased.

An extract of Atumisia used in traditional herbal medicine was demonstrated to have protective effect in this model. It was shown to decrease multiplicity of colon cancer polyps in a model established with one AOM injection followed by Dextran sulfate sodium administration (using a single intraperitoneal injection of AOM at 10 mg/kg body weight followed by 2.5% DSS in drinking water for seven consecutive days). The signal pathways of NFκB and β-catenin also decreased after atumisia administration. The aldose reductase inhibitor, sorbinil, was also demonstrated to inhibit iNos, Cox2, cyclin D1, β-catenin and NFκB and thus has preventive effect in AOM-induced colon cancer.

In the AOM model, red meat has been demonstrated to promote the development of colon cancer in rats—but white meat does not have such an effect. Supplementing haemin to the diet substantially increased both the size and number of ACF induced by AOM. This can be prevented by the addition of calcium, olive oil and antioxidants. Thus, a reduction in red meat consumption is thought to help prevent colon cancer.

**Summary**

Azoxymethane is a good model for the study of colon cancer. It effectively represents some types of colon cancers. It has been conveniently and widely applied to the study of the prevention of colon cancer and the cancer cell biology signaling pathways. However, it does have its limitations. Some changes can not be mimicked, like mutation of p53, which is an important pathway in the carcinogenesis of human colon cancer. Other changes
in signaling pathways, like GSK and mTor, need to be further characterized in the model.

Because the development of colon cancer spans about 10–15 years, there is a great opportunity to prevent this fatal disease. Many chemopreventive agents have been shown to have a positive preventative effect in preliminary studies. However, the optimal preventative needs to be identified. The AOM model could be adaptably used to screen potentially useful compounds.

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Aripiprazole differentially affects mesolimbic and nigrostriatal dopaminergic transmission: implications for long-term drug efficacy and low extrapyramidal side-effects

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Abstract

Aripiprazole has been used effectively to treat schizophrenia in the clinic; however, its mechanisms of action are not clear. This study examined how short- and long-term aripiprazole treatment affects dopaminergic transmission in mesolimbic and nigrostriatal pathways. For comparison, the effects of haloperidol and olanzapine treatment were also examined. Aripiprazole significantly increased D2 receptor mRNA expression and decreased tyrosine hydroxylase (TH) mRNA expression in the ventral tegmental area (VTA) after 1- and 12-wk treatment, but had no effect in substantia nigra (SN) and nucleus accumbens (NAc). Aripiprazole also decreased dopamine transporter (DAT) binding density in NAc (for 1- and 12-wk treatment) and VTA (1-wk treatment). In contrast, haloperidol significantly increased D2 receptor binding density and decreased DAT binding density in NAc and caudate putamen (CPu) after 1- and 12-wk treatment, and it also decreases DAT binding in VTA after 12-wk treatment. Olanzapine had less widespread effects, namely an increase in D2 receptor mRNA in VTA after 12-wk treatment and decreased DAT binding in NAc after 1-wk treatment. These results suggest that aripiprazole has selective effects on the mesolimbic dopaminergic pathway. Selectively reducing dopamine synthesis in VTA is a possible therapeutic mechanism for the long-term efficacy of aripiprazole in controlling schizophrenia symptoms with reduced extrapyramidal side-effects.

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Key words: Antipsychotics, dopamine D2 receptor, dopamine transporter, tyrosine hydroxylase, ventral tegmental area.

Introduction

Aripiprazole is a newly introduced antipsychotic drug, with clinical efficacy to control both positive and negative symptoms, which has been recommended as a first-line therapy for the treatment of schizophrenia (Cassano et al. 2007; DeLeon et al. 2004). An advantage of aripiprazole over existing antipsychotics is a favourable safety and tolerability profile, including a low incidence of extrapyramidal symptoms (EPS) compared with typical antipsychotics (such as haloperidol), and a low incidence of weight gain and other metabolic syndromes compared with other atypical antipsychotics (such as olanzapine) (DeLeon et al. 2004). However, the mechanisms underlying its clinical efficacy and improved safety and tolerability are not well understood. A unique pharmacology of aripiprazole has been proposed to explain its favourable clinical profile: unlike other antipsychotics, aripiprazole has both partial agonist and antagonist activity at the dopamine D2 receptor (Hirose et al. 2004; Shapiro et al. 2003). It is also a partial agonist to 5-HT1A receptors and a partial antagonist to 5-HT2A receptors, a so-called dopamine and 5-HT stabilizer. However, there is evidence that at therapeutic doses, aripiprazole exhibits low levels of 5-HT1A and 5-HT2A receptor occupancy and activity (Wolff et al. 2003; Wood & Reavill, 2007), and acts predominantly on
Dopaminergic neurons have two major projections which are derived from the mesencephalon: the mesolimbic pathway, in which the ventral tegmental area (VTA) projects to nucleus accumbens (NAc), and the nigrostriatal pathway, in which the substantia nigra (SN) projects to caudate putamen (CPu). Blockade of dopamine D₂ receptor activity in the mesolimbic pathway is the main mechanism of antipsychotic drug action (Kapur & Mamo, 2003). On the other hand, the EPS side-effects of typical antipsychotics (such as haloperidol) relates to blockade of D₂ receptors in the SN–striatal pathway (Stephen & Stahl, 2003). Whether aripiprazole exerts its effects solely through partial agonism on D₂ receptors (Burris et al., 2002; Wood & Reavill, 2007) or due to functionally selective activity at D₂ receptors (Lawler et al., 1999; Mailman, 2007; Shapiro et al., 2003; Urban et al., 2007) is controversial. Aripiprazole is a partial agonist at human D₂L receptors coupled to the inhibition of forskolin-stimulated cAMP accumulation (Burris et al., 2002). It has been suggested that aripiprazole is not simply a partial agonist, but a drug with functional selectivity, exerting effects differentially depending on the cellular location of the targeted receptor (Shapiro et al., 2003). For example, it acts as an agonist on pre-synaptic D₂ autoreceptors, whereas it also acts as an antagonist at post-synaptic D₂ receptors (Kikuchi, 1995; Shapiro et al., 2003). Recently aripiprazole has been found to act as a potent partial agonist at D₂ receptor-mediated signalling responses, such as the potentiation of arachidonic acid release, and as a weak partial agonist using MAPK (mitogen-activated protein kinase) phosphorylation, but lacked agonist activity on receptor internalization (Urban et al., 2007). These results support aripiprazole as being a functionally selective D₂ receptor ligand rather than a simple partial agonist (Mailman, 2007).

As mentioned above, aripiprazole has less EPS side-effects, which could be attributed to its partial agonism at D₂ receptors, in which some signals to post-synapses can be persistently generated although the post-synaptic D₂ receptors are fully occupied (Hirose et al., 2004). However, it is possible that aripiprazole is not only limited to straightforward receptor–drug interaction but, may also have differential effects on the mesolimbic and nigrostriatal dopaminergic pathways.

In-vivo microdialysis studies have found that acute aripiprazole treatment increases dopamine release in the medial prefrontal cortex and hippocampus (Bortolozzi et al., 2003; Zocchi et al., 2005) and elevates 3,4-dihydroxyphenylacetic acid (the major metabolite of dopamine) in the medial prefrontal cortex and striatum (Jordan et al., 2004). The dopamine transporter (DAT) regulates the re-uptake of free dopamine from the intra-synaptic cleft. It is the primary mechanism for inactivation of dopamine following its release into the synapse (Iversen, 1971). DAT binding density may therefore reflect levels of free dopamine in the intra-synaptic cleft. However, it is unclear whether aripiprazole, particularly chronic treatment, affects DAT binding density. Furthermore, although aripiprazole has been reported to reduce dopamine synthesis and tyrosine hydroxylase activity (TH; a rate-limiting enzyme for the synthesis of dopamine) in reserpine-treated rodents through action at pre-synaptic dopaminergic autoreceptors (Kikuchi, 1995), it is unclear whether aripiprazole affects TH mRNA expression. Therefore, in the present study, we examined the selective effects of short-term (1-wk) and chronic (12-wk) aripiprazole treatment on D₂ receptor and TH mRNA expression, and binding densities of D₂ receptors and DAT in the mesolimbic and nigrostriatal dopaminergic pathways. For comparison, the effects of haloperidol and olanzapine treatment were also examined.

**Methods**

**Animals and experimental procedures**

Sprague–Dawley rats (220–250 g) were obtained from the Animal Resource Center (Perth, Australia). Upon arrival, rats were housed individually in environmentally controlled conditions (temperature 22 °C, 12-h light/dark cycle, lights on 07:00 hours), and had *ad-libitum* access to water and standard laboratory chow diet. After 1 wk adaptation to the new environment, they were treated with aripiprazole (2.25 mg/kg.d; Eli Lilly, USA), haloperidol (0.3 mg/kg.d, Sigma, Australia), or vehicle (control) (Han et al., 2008a). The daily dosage was divided into three equal amounts and all rats were treated three times a day (06:00, 14:00, 22:00 hours) orally by specially prepared drug pellets as described previously (Han et al., 2008b; Huang et al., 2006). Each drug group was randomly subdivided into short-term (1-wk, n = 5) and chronic (12-wk, n = 5) treatment groups. Rats were sacrificed 48 h after the last drug treatment. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, and complied with the Australian Code of Practice for the Care and Use of Animal for Scientific Purposes.
Histology

All rats were sacrificed by carbon dioxide asphyxiation between 07:00 and 09:00 hours, in order to minimize the variation of circadian mRNA expression. Brains were immediately removed and frozen in liquid nitrogen. Coronal brain sections (14 μm) were cut at −17°C in a cryostat and thaw-mounted onto poly-L-lysine-coated slides. For in-situ hybridization, sections were immediately fixed in ice-cold phosphate buffer containing 4% paraformaldehyde. Acetylation was carried out in 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) for 10 min. Sections were then dehydrated in ethanol and stored at −20°C until use. For autoradiography, the sections were stored at −20°C after cutting until use without fixation. Identification of neuroanatomical structures was according to a standard rat brain atlas (Paxinos & Watson, 1997).

In-situ hybridization

The protocol followed that previously described (Huang et al. 2006). Briefly, the specific antisense hybridization probes for the D2 receptor were:

5′-cat gat aac ggt gca gag ttt cat gtc ctc agg ggt gtt gcc-3′
(NM010077, encoding bases 853–900),
5′-gac cca ttc aag ggc cgg ctc cag ttc tgc ctc ccc aga tca tca tc-3′
(NM010077, encoding bases 157–203), and for TH:

5′-tgg gtc agg gtt tgc agc tca tcc tgg acc ccc ttc aag gag cgg-3′
(NM009377, encoding bases 1437–1480).

No sequences bearing significant homology to the designed probes were found in the Gene Bank (NCBA). All oligonucleotide probes were terminally labelled with 10-fold molar excess of [35S]dATP (specific activity 100 Ci/mmol, Amersham, UK) and terminal transferase (Promega, USA), and purified over a MicroSpin G-50 column (Amersham). The probe concentration was $10^7$ cpm of 35S-labelled probes in 750 μl hybridization solution. Hybridization was performed by incubating sections in hybridization buffer [50% deionized formamide, 4 × SSC, 10% dextran sulfate, 1× Denhardt’s solution, 0.2% sheared salmon sperm DNA, 0.1% long-chain polyadenylic acid, 0.012% heparin, 20 mM sodium phosphate (pH 7.0), 10/75 μl labelled probe and 5% DTT] at 37°C for 16 h. Non-specific hybridization was determined by including 100-fold molar excess of non-labelled probes in the respective hybridization solution. After hybridization, sections were washed in 1× SSC buffer at 55°C for 3 × 20 min followed by 1 h incubation in 1× SSC buffer at room temperature. Finally, sections were dipped sequentially in Milli-Q water, 70% ethanol and 95% ethanol before air-drying and exposure to Hyper-β-max film (Amersham). After exposure (4 wk for D2 receptor and 2 wk for TH) in-situ hybridization films were developed using standard procedures. The sections containing positive signals were dipped in the emulsion solution (Amersham) and exposed (6 wk for D2 receptor and 4 wk for TH). This allows further examination of positive signals at cellular level and confirmation of the results from the film. As in our previous work (Huang et al. 2006), all films were analysed using a computer-assisted image analysis system, Multi-Analyst, connected to a GS-690 Imaging Densitometer (Bio-Rad, USA). Quantification of mRNA expression levels in various brain regions were performed by measuring the average density of each region. Values were then compared against a 35S-labelled autoradiographic standard (Amersham).

D2 receptor binding

Sections were preincubated in 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 0.1% ascorbic acid buffer (pH 7.4) for 30 min at room temperature to remove endogenous ligands. Sections were then incubated at room temperature for 1 h in the same buffer containing 5 nM [3H]raclopride (specific activity 60.1 Ci/mmol; PerkinElmer, USA). Non-specific binding was determined in the presence of 10 μM butaclamol. The sections were then washed 2 × 5 min in ice-cold buffer and rinsed briefly in ice-cold distilled water. Sections were then dried under a stream of cold air.

DAT binding

Sections were preincubated in 50 mM Tris–HCl containing 120 mM NaCl and 0.1% BSA (pH 7.4) for 20 min at 4°C and then incubated for 2 h in the same buffer containing 15 nM [3H]WIN 35,428 (specific activity 85.6 Ci/mmol; PerkinElmer). Non-specific binding was determined in the presence of 50 μM benztropine. The sections were washed 2 × 1 min in ice-cold buffer. After a brief rinse in ice-cold distilled water, the slides were rapidly dried under a stream of cold air.

Autoradiographic images were produced using a Beta image camera (BioSpace, France) as previously described (Deng & Huang, 2005). The exposure time was 3.5 h at a high-resolution setting.
sections with a known amount of ligand was used as a standard in all scans. Quantitative analysis of these images was performed with β-Image Plus (version 4; BioSpace). The density of the binding signal was first expressed in counts per minute per square millimetre (cpm/mm²) of area selected and was then converted into femtomoles of radioligand bound per milligram tissue equivalent (fmol/mg TE) by comparing with the standard.

**Statistical analysis**

The data was analysed statistically using SPSS version 15.0 software (SPSS Inc., USA). Data for each brain area were analysed by two-way ANOVA (drug treatment × treatment duration), followed by Tukey-Kramer–HSD post-hoc analyses. Pearson’s correlations were used to assess the relationships between D₂ receptor mRNA expression, TH mRNA expression, D₂ receptor binding and DAT binding.

**Results**

**D₂ receptor mRNA expression**

Differential expression of D₂ receptor mRNA was observed in NAc core (NAcC), NAc shell (NAcS), CPu, SN and VTA. Examples of D₂ receptor mRNA expression are presented in Fig. 1.

**CPu.** There were significant effects of drug treatment \(F_{3,32} = 5.712, p = 0.003\) and treatment duration \(F_{1,39} = 7.066, p = 0.012\) on D₂ mRNA expression in CPu, but no significant interaction between the two factors \(F_{3,32} = 0.697, p = 0.561\). After 1-wk treatment, only the aripiprazole group (237.9 ± 2.5 nCi/g TE) had significantly higher D₂ receptor mRNA expression compared to the control group (206.7 ± 10.7, \(p = 0.031\); Fig. 2d). However, after 12-wk treatment, no significant difference in D₂ receptor mRNA expression was observed between the aripiprazole and control groups (\(p = 0.471\); Fig. 2d). Olanzapine and haloperidol groups also did not significantly differ from controls in D₂ receptor mRNA expression (all \(p > 0.300\); Fig. 2d).

**VTA.** There were significant effects of drug treatment \(F_{3,32} = 11.243, p = 0.000\) and treatment duration \(F_{1,39} = 4.770, p = 0.036\) on D₂ mRNA expression in VTA. However, there was no significant interaction between the two factors \(F_{3,32} = 2.350, p = 0.091\). Compared to the control group, aripiprazole treatment significantly increased the level of D₂ receptor mRNA expression in the 1-wk (aripiprazole 256.8 ± 6.3 vs. control 203.1 ± 10.6, \(p = 0.008\); Fig. 1b–d and Fig. 2b) and 12-wk (aripiprazole 254.2 ± 10.2 vs. control 205.1 ± 5.1, \(p = 0.002\); Fig. 2b) treatment groups. However, compared to controls, olanzapine and haloperidol had significantly increased D₂ receptor mRNA expression only in the 12-wk treatment groups (olanzapine 246.7 ± 5.7 vs. control 205.1 ± 5.1, \(p = 0.008\); haloperidol 244.4 ± 9.1 vs. control, \(p = 0.013\); Fig. 2b) but not in the 1-wk treatment groups (all \(p > 0.299\); Fig. 2b). There were no significant effects of drug treatment on D₂ receptor mRNA expression in NAcC (Fig. 2a), NAcS, and SN (Fig. 2c).

**TH mRNA expression in VTA and SN**

There was a significant effect of drug treatment on the level of TH mRNA expression in VTA (\(F_{3,32} = 14.5.\)
p = 0.000), but no significant effect of treatment period (F_{1,39} = 1.2, p = 0.287). There was also no interaction between the two factors (F_{3,32} = 0.7, p = 0.554). Compared to the control group, TH mRNA expression levels were significantly decreased in the aripiprazole group after 1-wk (aripiprazole 319.6 ± 8.4 vs. control 460.3 ± 22.0, p = 0.001; Figs 1c–g, 2c) and 12-wk (aripiprazole 312.1 ± 8.4 vs. control 422.2 ± 19.9, p = 0.024; Fig. 2c) treatment. However, olanzapine and haloperidol treatment did not differ from controls in TH mRNA expression in SN (Fig. 2f). There was also no significant effect of drug treatment on TH mRNA expression in CPu (Fig. 2e). It is interesting that in the VTA, D2 receptor mRNA expression was negatively correlated with TH mRNA expression (r = −0.449, p = 0.004; Fig. 3a).

**D2 receptor binding**

Examples of D2 receptor binding are presented in Fig. 4c.

**NAcC and NAcS.** There were significant effects of drug treatment on D2 receptor binding densities in NAcC (F_{3,32} = 16.827, p = 0.000) and NAcS (F_{3,32} = 12.572, p = 0.000); however, there were no effects of treatment duration in NAcC (F_{1,39} = 0.054, p = 0.817) and NAcS (F_{1,39} = 0.062, p = 0.805). There was also no significant interaction between the two factors in the NAcC (F_{3,32} = 1.199, p = 0.326) and NAcS (F_{3,32} = 2.226, p = 0.104). Compared to the control group, only haloperidol treatment significantly increased binding densities in these brain areas after 1- and 12-wk treatment in these brain regions (NAcC, 1 wk: haloperidol 89.4 ± 4.1 fmol/mg TE vs. control 69.4 ± 2.1, p = 0.003; 12 wk: haloperidol 85.5 ± 4.7 vs. control 68.2 ± 1.9, p = 0.005; Fig. 5a. NAcS, 1 wk: haloperidol 79.9 ± 2.5 vs. control 65.2 ± 3.7, p = 0.030; 12 wk: haloperidol 79.1 ± 1.8 vs. control 58.9 ± 2.1, p = 0.002). CPu. There was a significant effect of drug treatment on D2 receptor binding density in CPu (F_{3,30} = 8.318, p = 0.000), but no effect on treatment duration (F_{1,30} = 1.622, p = 0.212). There was also no significant interaction between the two factors in CPu (F_{3,30} = 0.195, p = 0.899). Compared to the control group, only haloperidol treatment significantly increased binding densities after 1- and 12-wk treatment (1 wk: haloperidol 146.9 ± 5.0 vs. control 120.4 ± 4.6, p = 0.008; 12 wk: haloperidol 143.9 ± 8.1 vs. control 109.2 ± 4.6, p = 0.020; Fig. 5b).

However, haloperidol treatment had no effect on D2 receptor binding density in SN or VTA (Fig. 5c). Furthermore, aripiprazole and olanzapine treatments did not affect D2 receptor binding in any of these brain regions examined (Fig. 5a–c).
Examples of DAT binding are presented in Fig. 4d, e. There was a significant effect of drug treatment (F_{3,32} = 12.057, p = 0.000), but no significant effect of treatment period (F_{1,32} = 1.578, p = 0.218) on DAT binding in NAcC. There was also no significant interaction between the two factors (F_{3,32} = 0.789, p = 0.509). Compared to the control group, DAT binding density was significantly decreased in the 1- and 12-wk aripiprazole and haloperidol groups (Fig. 4e). However, in the olanzapine group, a significant difference was observed only after 1-wk treatment (olanzapine 29.7 ± 3.7 vs. control 41.2 ± 7.1, p = 0.018), although there was a tendency for binding to be decreased after 12-wk olanzapine treatment (olanzapine 30.3 ± 2.7 vs. control 36.0 ± 3.8, p = 0.109; Fig. 5d).

CPu. There was a significant effect of drug treatment (F_{3,32} = 5.338, p = 0.004), but no effect of treatment duration (F_{1,32} = 0.258, p = 0.615) on DAT binding in CPu. There was also no significant interaction between the two factors (F_{3,32} = 1.335, p = 0.280). As shown in Fig. 5e, compared to the controls, haloperidol significantly decreased DAT binding density after 1- and 12-wk treatment (Fig. 5e). However, aripiprazole and olanzapine treatments did not affect DAT binding in CPu (Fig. 5e).

VTA. There were significant effects of drug treatment on DAT binding in VTA (F_{3,32} = 4.214, p = 0.013) and no effect of treatment period (F_{1,32} = 0.044, p = 0.836). However, a significant interaction between drug treatment and treatment period was observed (F_{3,32} = 5.731, p = 0.003). After 1-wk treatment, the aripiprazole group (10.1 ± 0.6 fmol/mg TE) had significantly lower DAT binding density compared to the control group (13.7 ± 0.7 fmol/mg TE, p = 0.03; Fig. 5f), but was not significantly different from the other groups. After 12-wk drug treatment only the haloperidol (9.7 ± 0.7 fmol/mg TE) group had significantly lower DAT binding density than controls (12.5 ± 0.5, p = 0.048) (Fig. 5f). However, there were no effects of drug treatment and treatment period on DAT binding in NAcS and SN.

Discussion

This study has showed that aripiprazole has selective effects on mesolimbic vs. nigrostriatal dopaminergic pathways. Aripiprazole significantly increased D_2 receptor mRNA expression in VTA and CPu compared to controls after 1- and 12-wk treatment, but not in SN and NAc. Aripiprazole also significantly downregulated TH mRNA expression in the VTA, but not in SN, compared to controls. It is interesting that D_2 receptor mRNA expression is negatively correlated with TH mRNA expression in the VTA. Aripiprazole also decreased DAT binding sites in NAc (both 1- and 12-wk treatment) and VTA (1-wk treatment). In contrast, haloperidol had a significant influence on mesolimbic and nigrostriatal dopaminergic pathways. Haloperidol treatment increased D_2 receptor binding in CPu and NAc after 1- and 12-wk treatment, but decreased DAT binding in CPu and NAc (1- and 12-wk treatment), as well as VTA (12-wk treatment). Olanzapine had less widespread effects compared to the other two...
treatments, namely an increase in DAT binding in NAcC (1-wk treatment only) and D₂ receptor mRNA expression in VTA (12-wk treatment only).

A series of short-term (4–6 wk) and longer term (26 or 52 wk) clinical trials have revealed that aripiprazole not only has significant short- but also long-term efficacy in the treatment of positive and negative symptoms of schizophrenia (Cassano et al. 2007; DeLeon et al. 2004; Kane et al. 2007; Kasper et al. 2003; Travis et al. 2005). Compared with placebo, aripiprazole improved symptoms of schizophrenia as early as 1 wk after treatment (Travis et al. 2005), and this efficacy lasted up to 52 wk (Kasper et al. 2003). It has been suggested that the partial agonism of dopamine D₂ receptors is one of the mechanisms underlying the therapeutic effects of aripiprazole (Wood & Reavill, 2001).
However, it is worth noting that another D₂ receptor partial agonist (preclamol) also decreases positive and negative symptoms of schizophrenia, but its effects only last for 1 wk (Lahti et al. 1998). Therefore, the partial agonism of dopamine D₂ receptors cannot completely explain the long-term efficacy of aripiprazole. TH mRNA expression in VTA was decreased in the aripiprazole group in the present study. Since TH is the rate-limiting enzyme for the synthesis of dopamine, this indicates a reduction of dopamine synthesis in this brain region. The selective effects of aripiprazole on reducing dopamine production found in the present study may provide a mechanism to explain its long-term efficacy.

A reduction in dopamine synthesis may be mediated by D₂ autoreceptors (Wolf & Roth, 1990). Previously, in-vivo studies have found that aripiprazole has potent agonist activities at dopamine autoreceptors (Kikuchi, 1995). Aripiprazole may act on these D₂ autoreceptors in the VTA to reduce chronic dopamine synthesis, and continual treatment of aripiprazole would reduce dopamine release. Although a single low-dose (0.3 mg/kg) administration of aripiprazole increases dopamine release (Zocchi et al. 2005), a higher dose (2–40 mg/kg) decreases or has no effect on extracellular levels of dopamine in the rat cortex and striatum (Jordan et al. 2004; Zocchi et al. 2005). It follows that in our experiment, aripiprazole treatment (2.5 mg/kg.d) for 1 or 12 wk should decrease dopamine release. As a compensatory mechanism, D₂ autoreceptor synthesis in the VTA may be increased in response to the decrease of dopamine synthesis and release caused by aripiprazole treatment. Consistent with this, an increase in D₂ receptor mRNA expression was observed in VTA of the aripiprazole group. In fact, a negative correlation between D₂ receptor mRNA and TH mRNA expressions was found in this brain region. This suggests that aripiprazole may achieve its pharmacological effects by reducing dopamine production in VTA. Moreover, since the effects of aripiprazole on D₂ receptor mRNA and TH mRNA expression have been observed after 1- and 12-wk treatment, our theory may well explain its fast effects (1 wk) on improving symptoms and its long-term efficacy maintenance. It is interesting that aripiprazole had no effect on D₂ receptor mRNA expression and D₂ receptor binding in NAc. It is possible that aripiprazole reaches its long-term efficacy by reducing dopamine levels in the VTA–NAc pathway, but not directly through blocking post-synaptic D₂ receptors in the NAc.

In this study, like haloperidol, olanzapine increased D₂ receptor mRNA expression in VTA after 12-wk treatment, but it did not change in TH mRNA expression. Since olanzapine has relatively low affinity for D₂ receptors but high affinity for 5-HT₂A, 5-HT₂C and M₁ receptors (DeLeon et al. 2004), it has been suggested to achieve its pharmacological effects through its actions on these neurotransmission systems (Tyson et al. 2004). In fact, we have previously found that chronic olanzapine treatment down-regulated 5-HT₂A receptor mRNA expression in NAc (Huang et al. 2006) and up-regulated muscarinic M₁ receptor mRNA expression in the hippocampus (Han et al. 2008b).

It is an exciting finding that aripiprazole has differential effects on the VTA–NAc pathway compared to the SN–CPu pathway. Aripiprazole did not affect the expression of D₂ receptor mRNA or TH mRNA in SN. This result suggests that there are no changes in dopamine synthesis in SN, which may be due to the fact that D₂ autoreceptors in VTA are about 10-fold more sensitive to dopamine and D₂ receptor agonists than those in SN (Roth, 1979). Although 1-wk aripiprazole treatment slightly increased D₂ receptor mRNA expression in CPu compared with controls, this difference disappeared after 12-wk treatment. It is also interesting that 1- and 12-wk aripiprazole treatment did not affect D₂ receptor binding in CPu in this study. Therefore, the absence of changes to dopaminergic transmission in the SN–CPu pathway following chronic treatment of aripiprazole may partially explain why aripiprazole does not cause serious EPS after long-term treatment in the clinic. However, a recent human PET study using [¹⁸F]Fallypride has shown that aripiprazole occupies a very high percentage of the D₂/D₃ receptor in all brain regions investigated (including the putamen, caudate nucleus, thalamus, amygdala, and interior temporal cortex) (Grunder et al. 2008). Therefore, the more robust actions in mesolimbic dopamine neurons may be possibly due to the fact that the same amount of receptor binding of aripiprazole (a partial agonist) may cause different functional consequences in the nigrostriatal system.

It is important to note that a series of studies have shown aripiprazole is a functionally selective D₂ receptor ligand (Lawler et al. 1999; Mailman, 2007; Shapiro et al. 2003; Urban et al. 2007). The selective effects of aripiprazole on the mesolimbic and nigrostriatal systems might be due to its properties of functional selectivity. For example, as discussed in the Introduction, aripiprazole can act as a potent partial agonist at D₂ receptor-mediated signalling responses, such as the potentiation of arachidonic acid release, and as a weak partial agonist at MAPK
phosphorylation, but lacks agonist activity on receptor internalization (Urban et al. 2007). It is possible that aripiprazole may act as a potent agonist at dopamine D₂ autoreceptors in VTA to reduce dopamine synthesis, but as a weak agonist (or even short of agonist activity) in SN, therefore it modulates dopaminergic transmission mainly in the VTA–NAc pathway but not the SN–CPu pathway.

In contrast, haloperidol affected the mesolimbic and nigrostriatal pathways by blocking D₂ receptors. Haloperidol significantly increased D₂ receptor binding density in NAc and CPu after 1- and 12-wk treatment, which is in agreement with previous reports (Gross et al. 1991; Prosser et al. 1988; Sawda & Snyder, 2002; Stephen & Stahl, 2003). Some studies have also found that haloperidol (at a dose of 1.5–4 mg/kg) increases D₂ receptor mRNA expression in the striatum (Damask et al. 1996; Rogue et al. 1991); however, the present study did not observe any changes in D₂ receptor mRNA expression in CPu. Although haloperidol increased D₂ receptor mRNA expression in VTA after 12-wk treatment, it did not cause any change in TH mRNA expression. These results confirm previous findings that haloperidol improves schizophrenia symptoms by blocking D₂ receptors in NAc, while causing EPS side-effects by blocking D₂ receptors in CPu after chronic treatment (Kapur et al. 2000; Vohora, 2007). In this study, olanzapine did not affect D₂ receptor binding or expression of D₂ mRNA and TH mRNA in the CPu, which is consistent with a PET study showing that olanzapine had lower D₂ receptor blockade compared to haloperidol in the striatum (Xiberas et al. 2001). One study observed that olanzapine increased D₂ receptor density in the striatum of rats; however, much higher doses of olanzapine (5.0 mg/kg.d compared to 1.5 mg/kg.d in this study) were used (Tarazi et al. 2001). These results supported the observation that olanzapine does not cause EPS at common clinical dosages (Vohora, 2007).

Consistent with the changes of D₂ receptor and TH mRNA expression, selective effects of aripiprazole treatment on DAT binding densities were also observed in mesolimbic and nigrostriatal pathways. Since DAT mediates the re-uptake of free dopamine from the synaptic cleft (Iversen, 1971), decreased DAT density in NAc could be explained by decreased D₂ synthesis in this nucleus as discussed above. In contrast, haloperidol treatment reduced DAT binding densities in NAc and CPu. Although aripiprazole and haloperidol reduced DAT binding density, they might act through different mechanisms. Haloperidol has previously been reported to reduce the reuptake transport of dopamine in the striatum (McElvan & Schenck, 1992; Meiergerd et al. 1993). In addition, a negative correlation between D₂ receptor binding density and DAT binding density was found in NAc in this study (Fig. 3b). These results further suggest that haloperidol may control schizophrenia symptoms by D₂ receptor blockade in NAc, and produce EPS by D₂ receptor blockade and prolonged free dopamine reuptake in CPu. Olanzapine decreased DAT density in NAc only, which suggests that olanzapine might prolong dopamine activity in NAc.

DAT binding densities in VTA were decreased only after 1-wk aripiprazole treatment, but returned to a normal level after 12-wk treatment. It is interesting why only short-term treatment of aripiprazole affected DAT binding density. In contrast haloperidol had long-term effects on DAT binding in VTA. One possible explanation is the specific profile of aripiprazole as a selective D₂ receptor partial agonist. It was reported that D₂ receptor agonists may increase the re-uptake of dopamine via stimulating D₂ autoreceptors, but this effect could be reversed by D₂ receptor antagonists (Meiergerd et al. 1993; Parsons et al. 1993). Since aripiprazole has dual D₂ partial agonist and antagonist properties, it is possible that its dual effects may reach a balance after long-term treatment. However, haloperidol only has D₂ antagonist properties and needs long-term action to reduce DAT binding density in VTA.

In the literature, the dosages of aripiprazole, haloperidol, and olanzapine vary significantly in the animal studies. Similar doses to that used in this study have been used previously in the literature and have been shown to be pharmacologically and behaviourally effective. For example, aripiprazole has been used at doses ranging from 2 to 3 mg/kg (Kalinichev et al. 2005; Li et al. 2005; Schwabe & Koch, 2007). Haloperidol has frequently been utilized at a dose of 0.3 mg/kg (Pouzet et al. 2003; Wiley, 2008), while olanzapine treatments ranging from 1.2 mg/kg (Arjona et al. 2004; Huang et al. 2006) to 2.0 mg/kg (Cooper et al. 2005) have been used in rats. These selected doses all share a D₂ occupancy of ~70–80% in rats (Kapur et al. 2003; Natesan et al. 2006). Furthermore, antipsychotics were administrated orally three times a day in this study. We have shown previously that the doses of the drugs and treatment (at three times a day) used in this study affected central receptor systems relative to their pharmacological profiles (Han et al. 2008a,b; Huang et al. 2006), indicating the effectiveness of these treatments. Since rats were sacrificed 48 h after the last drug treatment, we could not completely rule out a possible drug withdrawal response observed 2 d after the last aripiprazole
treatment; however, it is unlikely. It has been shown that aripiprazole has a long elimination half-life (60–70 h) and exerts its effects on D2/D3 receptors for almost 1 wk after the last dose in humans (Grunder et al. 2008). Unfortunately, there is no data available on its half-life in rats. It is understandable that aripiprazole may have a different half-life in rats from that in humans; however, even assuming a 4- to 6-fold faster half-life of aripiprazole in rats, it may still have effects 1.5 d after the last treatment in rats. Further studies are necessary to measure the half-life of aripiprazole in rats (particularly in the brain) and to investigate changes of TH and D2 receptor mRNA expression a short period (e.g. a few hours) after the last aripiprazole treatment.

In conclusion, the present results suggest that aripiprazole, unlike other antipsychotics, has selective effects on dopaminergic pathways, in which both short- and long-term treatment predominantly modulates the dopaminergic neurotransmission in the mesolimbic but not nigrostriatal pathways. Selectively reducing dopamine synthesis in VTA (but not SN) is the possible therapeutic mechanism for long-term effects on dopaminergic pathways, in which both short- and long-term treatment predominantly modulates the dopaminergic neurotransmission in the mesolimbic but not nigrostriatal pathways. Selectively reducing dopamine synthesis in VTA (but not SN) is the possible therapeutic mechanism for long-term efficacy of aripiprazole in controlling schizophrenia symptoms with less extrapyramidal side-effects.

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Statement of Interest

None.

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A behavioural comparison of acute and chronic Δ⁹-tetrahydrocannabinol and cannabidiol in C57BL/6JArc mice

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Abstract
Cannabis contains over 70 unique compounds and its abuse is linked to an increased risk of developing schizophrenia. The behavioural profiles of the psychotropic cannabis constituent Δ⁹-tetrahydrocannabinol (Δ⁹-THC) and the non-psychotomimetic constituent cannabidiol (CBD) were investigated with a battery of behavioural tests relevant to anxiety and positive, negative and cognitive symptoms of schizophrenia. Male adult C57BL/6JArc mice were given 21 daily intraperitoneal injections of vehicle, Δ⁹-THC (0.3, 1, 3 or 10 mg/kg) or CBD (1, 5, 10 or 50 mg/kg). Δ⁹-THC produced the classic cannabinoid CB₁ receptor-mediated tetrad of hypolocomotion, analgesia, catalepsy and hypothermia while CBD had modest hyperthermic effects. While sedative at this dose, Δ⁹-THC (10 mg/kg) produced locomotor-independent anxiogenic effects in the open-field and light–dark tests. Chronic CBD produced moderate anxiolytic-like effects in the open-field test at 50 mg/kg and in the light–dark test at a low dose (1 mg/kg). Acute and chronic Δ⁹-THC (10 mg/kg) decreased the startle response while CBD had no effect. Prepulse inhibition was increased by acute treatment with Δ⁹-THC (0.3, 3 and 10 mg/kg) or CBD (1, 5 and 50 mg/kg) and by chronic CBD (1 mg/kg). Chronic CBD (50 mg/kg) attenuated dexamphetamine (5 mg/kg)-induced hyperlocomotion, suggesting an antipsychotic-like action for this cannabinoid. Chronic Δ⁹-THC decreased locomotor activity before and after dexamphetamine administration suggesting functional antagonism of the locomotor stimulant effect. These data provide the first evidence of anxiolytic- and antipsychotic-like effects of chronic but not acute CBD in C57BL/6JArc mice, extending findings from acute studies in other inbred mouse strains and rats.

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Key words: Antipsychotic, anxiolytic, cannabidiol, mouse, Δ⁹-tetrahydrocannabinol.

Introduction
The population risk for schizophrenia is increased by cannabis use (Henquet et al. 2005; Moore et al. 2007). This increase is reported to be greater in people with a predisposition to psychosis (Henquet et al. 2005). Δ⁹-tetrahydrocannabinol (Δ⁹-THC), the most abundant of the >70 cannabis constituents, produces the euphoric effects sought by recreational users, and produces psychotomimetic symptoms such as altered perception and disrupted working memory. Schizophrenia patients show increased susceptibility to these effects (D’Souza et al. 2005). Δ⁹-THC has partial agonist efficacy at cannabinoid CB₁ and CB₂ receptors and exerts many of its central effects via inhibition of neurotransmitter release by presynaptic CB₁ receptors. Cannabidiol (CBD), another cannabis constituent, does not produce the psychotropic effects of Δ⁹-THC and antagonizes the effects of CB₁/CB₂ receptor agonists (Pertwee, 2008). Interestingly, consumption of...
cannabis with little or no CBD content is associated with increased incidence of psychotic symptoms (Morgan & Curran, 2008; Rottanburg et al. 1982), suggesting that CBD may partially attenuate the psychotomimetic effects of Δ⁹-THC or other cannabis constituents. The therapeutic potential for CBD is supported by observations of its antipsychotic-like (Leweke et al. 2000; Zuardi et al. 2006a) and anxiolytic-like effects (Crippa et al. 2004, 2009) after acute oral administration in healthy volunteers. While preliminary data in treatment-resistant schizophrenia patients suggest that CBD monotherapy is not effective (Zuardi et al. 2006b), regular oral CBD administration reduces psychotic symptoms in Parkinson’s disease sufferers (Zuardi et al. 2008).

Reports of CBD effects in rodents are currently restricted to acute studies: CBD exerts anxiolytic-like (Campos & Guimaraes, 2008; Guimaraes et al. 1994; Moreira et al. 2006; Onaivi et al. 1990; Resstel et al. 2006) and antipsychotic-like (Long et al. 2006; Moreira & Guimaraes, 2005; Zuardi et al. 1991) effects in mice (Swiss, ICR) and rats (Wistar). Interestingly, CBD induces c-fos expression in the rat nucleus accumbens but not the dorsal striatum, in a pattern similar to that of clozapine (Guimaraes et al. 2004). To accurately model the effects of regular cannabis use in humans and to determine the potential for lasting clinical efficacy of cannabinoids such as CBD, chronic rodent behavioural studies are necessary. Furthermore, since a majority of mutant mouse models are generated on a C57BL/6J or mixed C57BL/6J × 129SvJ genetic background, studies in C57BL/6J mice are crucial for further research.

This study therefore aimed to directly compare the acute and chronic effects of Δ⁹-THC and CBD in C57BL/6J mice using a multi-tiered battery of schizophrenia- and anxiety-relevant behavioural tests. CBD effects were compared to the well-characterized effects of Δ⁹-THC in the classic cannabinoid CB₁ receptor agonist ‘tetrad’. Following this, mice were assessed using a comprehensive multi-tiered battery of behavioural models relevant to positive (spontaneous hyperactivity), negative (social withdrawal, anxiety) and cognitive (disrupted learning and working memory, impaired sensorimotor gating) symptoms of schizophrenia during repeated treatment with Δ⁹-THC or CBD. The antipsychotic effect of chronic CBD on drug-induced psychotomimetic behaviour was assessed using acute challenges with the non-competitive NMDA antagonist MK-801 and the catecholaminergic stimulant dexamfetamine (Dex). To enable comparison of the effects of repeated treatment, a follow-up study on acute anxiolytic and potentially anti-psychotic-like effects of CBD was conducted, using doses that were behaviourally active in the chronic study.

Methods

Animals

Test animals were 119 male C57BL/6Arc mice (aged 12–14 wk). Standard social interaction opponents were 12 male A/JArc mice (aged 9–10 wk) (Animal Resources Centre, Australia). Mice were maintained under a 12-h light/red light cycle (lights on 07:00 hours) and pair-housed in Macron cages (1144B: Tecniplast, Australia) containing paper tissues (Kimwipes®, Kimberly-Clark, Australia) as nesting material with ad libitum access to water and standard irradiated mouse feed (Gordon’s Specialty Stockfeeds, Australia). Chronic experiments were performed in four sets of 22–24 mice over 8 wk, with treatment randomized and counterbalanced across sets. Follow-up acute experiments with CBD were performed in a single set of 29 mice. Research and animal care procedures were approved by the Garvan Institute/ St Vincent’s Hospital Animal Experimentation Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Drug treatment

Δ⁹-THC and CBD (THC Pharm GmbH, Germany) were suspended in a 1:1:18 mixture of ethanol: Tween-80:saline. Dex and MK-801 (Sigma, Australia) were dissolved in saline. Dose ranges of Δ⁹-THC and CBD were based on previous studies (Boucher et al. 2007; Long et al. 2006). Drugs were injected at a volume of 10 ml/kg.

For chronic treatment, mice received 21 consecutive daily intraperitoneal (i.p.) injections of vehicle (1:1:18 ethanol:Tween-80:saline), Δ⁹-THC (0.3, 1, 3 or 10 mg/kg) or CBD (1, 5, 10 or 50 mg/kg) (n = 10). On day 7 all mice were injected with MK-801 (0.5 mg/kg i.p.) 35 min after cannabinoid injection. On day 21 all mice were injected with Dex (5 mg/kg i.p.) 45 min after cannabinoid injection.

Follow-up experiment

After observing key effects of chronic CBD we investigated the acute behavioural effects of CBD at relevant doses. Separate groups of age-matched male C57BL/6JArc mice received an i.p. injection of vehicle or CBD (1 or 50 mg/kg) (n = 9–10). Mice were tested in the elevated plus maze (EPM) (5 min) followed by
open-field (OF) testing of Dex-induced locomotor activity (45 min), in which all mice received an injection of Dex (5 mg/kg i.p.) 45 min after vehicle or CBD injection (in a manner identical to the Dex-induced OF testing after chronic CBD).

Behavioural testing
Injections commenced at the start of the light cycle (07:00 hours). On behavioural testing days injections were staggered within the light cycle to standardize intervals between injection and testing (Table 1). Mice were returned to the home cage following injection and behavioural testing. Environmental odours were removed from test apparatus between trials with 70% ethanol.

In conducting behavioural testing for schizophrenia, experiments need to target the range of behavioural domains that are affected in the disorder (Powell & Miyakawa, 2006). Furthermore, behavioural testing batteries over one or more days have previously yielded informative data on anxiety-related behaviour (Karl et al. 2008), sensory and motor function (Metz & Schwab, 2004) and defensive behaviour (Griebel et al. 1999). Ethical considerations prompted us to investigate more than one behavioural domain in each group of mice, and during repeated treatment, the order of behavioural testing proceeded from least to most aversive, such that tests involving an aversive stimulus (i.e. electric footshock in passive avoidance) were performed after tests of anxiety-like behaviour such as the EPM to avoid confounds related to the stress response of the mice.

Cannabinoid tetrad A: body temperature, catalepsy and nociception

Mice were assessed for the classical cannabinoid behavioural tetrad of hypothermia, catalepsy, hypolocomotion and nociception (Compton et al. 1993). Body temperature was measured 5 min before and 30 min after injection on days 1, 3, 5, 7, 12 and 14 using a lubricated rectal thermometer (SDR Clinical Technology, Australia). Catalepsy was measured 20 min after injection on days 1, 3, 5, 7, 12 and 14. The hindpaws of the mouse were placed on the bench and the forepaws were placed on a bar (0.2 cm diameter) raised 8 cm off the bench surface. The latency for the mouse to place both forepaws on the bench was recorded. Nociception was measured with the hot-water tail-flick test 45 min after injection on days 1, 14 and 20. Mice were gently wrapped in a towel leaving the tail unrestrained. The tail tip (1.5 cm) was placed in a beaker of water at room temperature (25 °C) for 10 s then placed immediately in a water bath at 52 °C. The latency for the mouse to flick its tail was recorded. Data are reported as the mean of three consecutive trials performed for each mouse, with 2-min inter-trial intervals during which mice were returned to the home cage.

Cannabinoid tetrad B: spontaneous and Dex-induced locomotor activity

Locomotor activity was measured by placing mice into an OF activity chamber (Med Associates Inc., USA). Horizontal (distance travelled) and vertical activity

<table>
<thead>
<tr>
<th>Day</th>
<th>Test Time</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>–2</td>
<td>07:00–10:00</td>
<td>Prepulse inhibition acclimatization</td>
</tr>
<tr>
<td>–1</td>
<td>07:00–10:00</td>
<td>Prepulse inhibition acclimatization</td>
</tr>
<tr>
<td>0</td>
<td>07:00–10:00</td>
<td>Body temperature, catalepsy, open field, tail flick, prepulse inhibition</td>
</tr>
<tr>
<td>1</td>
<td>07:00–11:00</td>
<td>Body temperature, catalepsy</td>
</tr>
<tr>
<td>3</td>
<td>07:00–10:00</td>
<td>Body temperature, catalepsy</td>
</tr>
<tr>
<td>5</td>
<td>07:00–10:00</td>
<td>Body temperature, catalepsy</td>
</tr>
<tr>
<td>7</td>
<td>07:00–11:00</td>
<td>Body temperature, catalepsy, MK-801 and prepulse inhibition</td>
</tr>
<tr>
<td>12</td>
<td>07:00–10:00</td>
<td>Body temperature, catalepsy</td>
</tr>
<tr>
<td>14</td>
<td>07:00–10:00</td>
<td>Body temperature, catalepsy, tail flick</td>
</tr>
<tr>
<td>15</td>
<td>07:00–12:00</td>
<td>Y-maze</td>
</tr>
<tr>
<td>17</td>
<td>07:00–13:00</td>
<td>Social interaction, prepulse inhibition</td>
</tr>
<tr>
<td>18</td>
<td>07:00–11:00</td>
<td>Social interaction, prepulse inhibition</td>
</tr>
<tr>
<td>19</td>
<td>07:00–11:00</td>
<td>Passive avoidance training</td>
</tr>
<tr>
<td>20</td>
<td>07:00–11:00</td>
<td>Passive avoidance retention test, tail flick</td>
</tr>
<tr>
<td>21</td>
<td>07:00–15:00</td>
<td>Dex and open field</td>
</tr>
</tbody>
</table>

Table 1. Chronic test biography of C57BL/6JArc mice

At 2-THC, Δ⁹-tetrahydrocannabinol; CBD cannabidiol, Dex, dexamphetamine.

Ninety mice were tested over a period of 8 wk. Mice were injected with either vehicle or Δ⁹-THC (0.3, 1, 3 or 10 mg/kg body weight) or CBD (1, 5, 10 or 50 mg/kg) once daily for 21 d (n=10). On day 7, all mice were injected with MK-801 (0.5 mg/kg) and on day 21 all mice were injected with Dex (5 mg/kg). The test schedule shows the order in which tests were completed. Test order and age at commencement of testing was standardized to avoid differential impact of repeated testing on the behavioural parameters investigated. Tests involving an aversive stimulus (i.e. electric footshock in passive avoidance) were performed after tests of anxiety-like behaviour such as the elevated plus maze to avoid confounds related to the stress response of the mice.

...
(rearing) in central and peripheral zones were measured (described previously; Karl et al. 2007). The ratio of central to total distance travelled (distance ratio) and time spent in the central zone were taken as measures of anxiety (Denenberg, 1969). On days 1 and 15 mice were placed in the OF 35 min after injection and allowed to explore freely for 10 min. On day 21 of the chronic study, and in the follow-up study, mice were placed in the OF 30 min after injection and allowed to explore freely for 15 min before they were removed, injected with Dex and immediately returned to the chamber for a further 30 min.

**Light–dark (LD) test**

Mice were placed into the opening of the dark compartment of the LD test apparatus (described previously; Karl et al. 2007) and allowed to explore freely for 10 min. The ratio of distance travelled in the light compartment to total distance travelled (distance ratio) and time spent in the light compartment were taken as measures of anxiety. Mice were tested with the LD test 30 min after injection on day 17.

**EPM**

Mice were placed onto the central platform of the EPM (described previously; Karl et al. 2007) facing an enclosed arm and allowed to explore freely for 5 min. Arm entries, time spent in arms and in the central platform, rearing and head dipping were scored. Arm entries were recorded when mice entered the arm with all four paws. Anxiety-related behaviour was measured by entries into the open arm from the centre, percentage of time spent on open arms (divided into inner and outer halves) and the percentage of open-arm entries (open-arm entry ratio) (Hogg, 1996; Pellow et al. 1985). Mice were tested in the EPM 45 min after injection on day 17 and 25 min after injection in the acute study.

**Social interaction (SI)**

SI between pairs of rodents is used to measure anxiety-like behaviours (File & Seth, 2003; Kask et al. 2001). Furthermore, reduction in SI models aspects of social withdrawal observed in schizophrenia patients (Ellenbroek & Cools, 2000; Rung et al. 2005). Test mice and weight-matched A/JArc standard opponents, with no prior exposure to either the test arena or to each other, were placed in opposite corners of a grey perspex arena (35 x 35 x 30 cm; illumination 50 lx) and allowed to explore freely for 10 min. Frequency and total duration of the active socio-positive behaviours general sniffing, anogenital sniffing, allogrooming, following and climbing over/under in each test mouse were recorded manually. Mice were tested for SI 35 min after injection on day 18.

**Prepulse inhibition (PPI)**

PPI, an operational measure of sensorimotor gating, is impaired in schizophrenia patients (Braff et al. 2001; Ludewig et al. 2003). Startle reactivity was measured using SR-Lab startle chambers (San Diego Instruments, USA). Mice were habituated to this apparatus for 30 min on two consecutive days. The next day, mice were tested for baseline PPI, and on the following day chronic drug treatment began. PPI testing commenced 60 min after injection on days 1 and 18 and 55 min after injection on day 7 (20 min after injection of MK-801). PPI test sessions consisted of 5-min acclimatization to 70-dB background noise followed by 105 trials presented in a pseudo-random order: 5 x 70-dB trials (background); 5 x 80-dB trials; 5 x 100-dB trials; 15 x 120-dB trials (startle). Percentage PPI (% PPI) was calculated as

\[
\frac{\text{startle response (120 dB)} - \text{PPI response}}{\text{startle response (120 dB)}} \times 100.
\]

% PPI was averaged across inter-stimulus (prepulse-startling pulse) intervals to produce a mean % PPI for each prepulse intensity.

**Y-maze**

The natural tendency of mice to alternate successive entry choices of arms in a Y-maze (spontaneous alternation) is used as an assessment of memory retention (Hughes, 2004). Mice were placed into the centre of a grey perspex Y-shaped maze (30 cm x 10 cm x 17 cm, arms joined by a triangular centre section) and allowed to explore freely for 8 min. Different patterns on each arm wall provided intra-maze directionality cues. Objects (e.g. a camera tripod) at the end of each arm provided extra-maze directionality cues. Arm entries (defined when all four paws were inside the arm) were recorded manually. The spontaneous alternation score was calculated as the number of novel triplet entries (three consecutive entries into different arms) expressed as a percentage of the maximum possible triplet entries (Hodges, 1996). Mice were tested 30 min after injection on day 16.
Passive avoidance (PA)

In PA, memory retention is indicated by avoidance of a naturally less aversive dark compartment after it is paired with an electrical footshock (Bovet et al. 1969). This hippocampus-dependent learning test is influenced by fear of highly illuminated areas and aversive stimuli (e.g. electrical footshock), and by nociception. Training and retention trials were performed 30 min after injection on days 19 and 20, respectively, using a shuttle box system (TSE Systems, Bad Homburg, Germany). Latency to enter the dark chamber on each trial was measured (cut-off time: 300 s) (previously described; Karl et al. 2008). Increased latency in the retention trial indicated memory of the aversive stimulus (0.4 mA footshock; 2 s). Due to apparatus malfunction several mice did not receive a footshock and were excluded from further analysis.

Statistical analysis

In analyses for all experiments the vehicle group was compared separately to the Δ⁹-THC (THC 0.3, THC 1, THC 3 and THC 10) and CBD (CBD 1, CBD 5, CBD 10 and CBD 50) groups. Differences between treatment groups were analysed with one-way (factor: treatment) or repeated-measures analyses of variance (ANOVA) [factors: treatment, 5-min block (Dex-induced locomotor activity), prepulse intensity and trial (PA)]. Main and interaction effects were identified when p < 0.05. Planned contrasts were used to determine which treatment groups were significantly different to vehicle (simple contrasts), to locate differences between levels of within-subjects factors (polynomial contrasts) and to locate interactions between levels of treatment groups and within-subjects factors (special interaction contrasts). Huynh–Feldt corrections for non-sphericity were applied. Degrees of freedom, F values and p values from ANOVA are presented in the Results, and p values from planned contrasts are indicated in the Figures and Tables by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001). Data are presented as mean ± standard error of the mean (S.E.M.). Analysis was performed using SPSS 16.0 for Windows (SPSS Inc., USA).

Results

Cannabinoid tetrad A: body temperature, catalepsy and nociception

Acute Δ⁹-THC (10 mg/kg) produced significant hypothermia [one-way ANOVA: F(4,49) = 9.3, p < 0.001] and catalepsy [one-way ANOVA: F(4,43) = 6.0, p < 0.001] (Table 2). There was a trend towards an effect of acute Δ⁹-THC on nociception (one-way ANOVA: p = 0.08). Δ⁹-THC (10 mg/kg) had a significant antinociceptive effect on day 14 [one-way ANOVA: F(4,44) = 3.9, p = 0.01] (Table 2), but not on day 20.

CBD (1 and 10 mg/kg) induced a hyperthermic response on day 7 [one-way ANOVA: F(4,49) = 3.5, p < 0.05] but had no effect on catalepsy or nociception (Table 2).

Cannabinoid tetrad B: spontaneous locomotor activity

Δ⁹-THC decreased overall distance travelled on day 1 [one-way ANOVA: F(4,49) = 3.5, p < 0.05] and day 15 [F(4,46) = 3.6, p < 0.05] at 10 mg/kg and on day 21 [F(4,44) = 13.3, p < 0.001] at 1, 3 and 10 mg/kg (Fig. 1a). Δ⁹-THC also decreased peripheral distance travelled in the OF test on day 1 [one-way ANOVA: F(4,49) = 3.1, p < 0.05] at 10 mg/kg and on day 21 [F(4,44) = 12.8, p < 0.001] at 1, 3 and 10 mg/kg (Fig. 1b). Δ⁹-THC also decreased the total distance travelled in the LD test [one-way ANOVA: F(4,46) = 9.6, p < 0.001] at 10 mg/kg (Fig. 1c) and the total number of arm entries in the EPM [one-way ANOVA: F(4,42) = 4.0, p < 0.01] at 3 and 10 mg/kg (Fig. 1d), confirming its locomotor suppressant effects.

In contrast, CBD had no effect on any locomotor activity measure in the OF test (one-way ANOVA: p > 0.05; Fig. 1e, f). CBD (10 mg/kg) significantly decreased the total distance travelled in the LD test [one-way ANOVA: F(4,44) = 2.6, p < 0.05] (Fig. 1g). There was a trend towards an effect of CBD on the total number of EPM arm entries (one-way ANOVA: p = 0.06; Fig. 1h).

Dex-induced locomotor activity

Chronic

There were main effects of chronic Δ⁹-THC (repeated-measures ANOVA: F(4,38) = 4.6, p < 0.01) and time (5-min block) [F(2.6, 97.8) = 196.2, p < 0.001] on the distance travelled in the OF test on day 21, and a moderate trend towards an interaction of Δ⁹-THC with time (p = 0.09) (Fig. 2a). An interaction contrast between the 15-min baseline (BL) and 30-min post-Dex periods showed that Dex increased the distance travelled in all Δ⁹-THC-treated groups [BL × post-Dex: F(1,38) = 284.7, p < 0.001]. The magnitude of this increase was dependent on Δ⁹-THC dose [BL × post-Dex × Δ⁹-THC interaction: F(4,38) = 2.8, p < 0.05]. Doses and time-points at which Δ⁹-THC attenuated the Dex-induced increase in locomotor activity were determined by special interaction contrasts (BL × specific post-Dex time-point × Δ⁹-THC) (Fig. 2a).
Table 2. Body temperature, catalepsy and nociception

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day</th>
<th>Vehicle</th>
<th>Δ⁹-THC 0.3</th>
<th>Δ⁹-THC 1</th>
<th>Δ⁹-THC 3</th>
<th>Δ⁹-THC 10</th>
<th>CBD 1</th>
<th>CBD 5</th>
<th>CBD 10</th>
<th>CBD 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermic response: difference in body temperature measured (°C)</td>
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<td>1.4±0.3</td>
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<td>2.3±0.4</td>
<td>1.2±0.4</td>
<td>−1.8±1.0</td>
<td>1.5±0.7</td>
<td>1.4±0.3</td>
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<td></td>
<td>3</td>
<td>1.4±0.3</td>
<td>1.3±0.3</td>
<td>1.6±0.1</td>
<td>1.5±0.2</td>
<td>0.8±0.4</td>
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<td>1.4±0.3</td>
<td>1.5±0.2</td>
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<tr>
<td></td>
<td>7</td>
<td>0.9±0.4</td>
<td>1.6±0.3</td>
<td>1.0±0.2</td>
<td>1.7±0.2</td>
<td>1.6±0.3</td>
<td>2.0±0.2**</td>
<td>1.0±0.3</td>
<td>1.8±0.3*</td>
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<tr>
<td></td>
<td>12</td>
<td>1.6±0.2</td>
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<tr>
<td>Catalepsy: latency to remove both forepaws, 20 min after injection (ms)</td>
<td>1</td>
<td>58.7±5.2</td>
<td>71.4±6.8</td>
<td>68.9±6.8</td>
<td>93.9±11.4</td>
<td>390.5±137.9*</td>
<td>50.4±4.5</td>
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<td>38.9±4.7</td>
<td>49.3±6.7</td>
<td>38.8±2.8</td>
<td>49.5±7.1</td>
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<tr>
<td>Nociception: mean latency to flick tail immersed in 52°C water, 45 min after injection (s), measured over three consecutive trials</td>
<td>1</td>
<td>2.4±0.1</td>
<td>2.6±0.2</td>
<td>2.6±0.2</td>
<td>2.9±0.3</td>
<td>3.5±0.4</td>
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<td>2.3±0.3</td>
<td>1.8±0.1</td>
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</table>

Δ⁹-THC, Δ⁹-tetrahydrocannabinol; CBD, cannabidiol.

Body temperature, catalepsy and nociception after injection with Δ⁹-THC (0.3, 1, 3 or 10 mg/kg) or CBD (1, 5, 10 or 50 mg/kg) on various days of treatment.

Data represent mean (±S.E.M.), n=8-10.

* p<0.05, ** p<0.01, *** p<0.001 vs. vehicle group (one-way ANOVA and planned contrasts).
There was a main effect of time (5-min block), but not CBD, on distance travelled in the OF on day 21 [repeated-measures ANOVA: $F(2.2, 92.9) = 225.8, p < 0.001$] (Fig. 2b). There was no CBD x time interaction ($p > 0.05$). An interaction contrast showed that Dex increased the distance travelled in all CBD-treated groups [BL x post-Dex: $F(1, 42) = 330.1, p < 0.001$]. The magnitude of this increase was independent of CBD dose (BL x post-Dex x CBD interaction contrast: $p > 0.05$).

Special interaction contrasts (BL x specific post-Dex time-point x CBD) showed that CBD (50 mg/kg) attenuated the Dex-induced increase in locomotor activity at 35–40 min and 40–45 min (Fig. 2b).

**Acute CBD**

In the follow-up experiment investigating the effect of acute CBD on Dex-induced locomotor activity there...
interaction contrasts (BL vs. D vertical activity in the OF test was decreased by
Exploratory activity
Vertical activity in the OF test was decreased by Δ²-tetrahydrocannabinol (THC) (0.3, 1, 3 or 10 mg/kg) or (b) cannabidiol (CBD) (1, 5, 10 or 50 mg/kg) during baseline (BL) and after injection of 5 mg/kg Dex (post-Dex) on day 21 of treatment. Data represent mean ±SEM. (n = 7–10). † p < 0.05 (Δ²-THC 0.3 vs. vehicle); + p < 0.05 (Δ²-THC 1 vs. vehicle); * p < 0.05, ** p < 0.01 (Δ²-THC 3 vs. vehicle); †† p < 0.05, ††† p < 0.001 (Δ²-THC 10 or CBD 50 vs. vehicle) (repeated-measures ANOVA and planned contrasts).

Exploratory activity
Vertical activity in the OF test was decreased by Δ²-tetrahydrocannabinol (THC) (1, 3 and 10 mg/kg) on day 1 [one-way ANOVA: F(4, 49) = 8.2, p < 0.001] and day 15 [F(4, 46) = 7.9, p < 0.001] and by Δ²-THC (3 and 10 mg/kg) on day 21 [F(4, 44) = 8.4, p < 0.001] (Table 3). This was confirmed by significant inhibition by Δ²-THC (10 mg/kg) of rearing in the LD test [one-way ANOVA: F(4, 46) = 3.5, p < 0.05; data not shown] and rearing [one-way ANOVA: F(4, 42) = 4.7, p < 0.01] and head dipping [one-way ANOVA: F(4, 42) = 3.1, p < 0.05] in the EPM (Table 3).

CBD did not affect vertical activity/rearing in the OF, LD or EPM tests (one-way ANOVA: p > 0.05). There was a trend towards an effect of CBD on head dipping in the EPM (one-way ANOVA: p = 0.06) (Table 3).

Anxiety measures
Chronic
Δ²-THC (10 mg/kg) significantly decreased the time spent in the central zone of the OF test on day 15 [one-way ANOVA: F(4, 46) = 2.8, p < 0.05] and day 21 [F(4, 44) = 3.4, p < 0.05] (Fig. 4a) and decreased the distance ratio on day 1 [F(4, 49) = 3.9, p < 0.01], day 15 [F(4, 46) = 3.1, p < 0.05] and day 21 [F(4, 44) = 8.6, p < 0.001] (Fig. 4b). Similarly, Δ²-THC (10 mg/kg) decreased the time spent in the light compartment [one-way ANOVA: F(4, 46) = 3.5, p < 0.05] and the distance...
Table 3: Exploratory activity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Δ-THC 0.3</th>
<th>Δ-THC 1</th>
<th>Δ-THC 10</th>
<th>Δ-THC 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertical activity (OF) (n)</td>
<td>1</td>
<td>107.9±10.5</td>
<td>80.0±10.5</td>
<td>93.2±10.6</td>
<td>95.7±9.9</td>
</tr>
<tr>
<td>Rearing (EPM) (n)</td>
<td>15</td>
<td>111.9±11.5</td>
<td>111.9±11.5</td>
<td>107.9±10.5</td>
<td>111.9±11.5</td>
</tr>
<tr>
<td>Head dipping (EPM) (n)</td>
<td>17</td>
<td>88.0±7.6</td>
<td>88.0±7.6</td>
<td>88.0±7.6</td>
<td>88.0±7.6</td>
</tr>
</tbody>
</table>

Δ-THC, Δ-tetrahydrocannabinol; CBD, cannabidiol; OF, open-field; EPM, elevated plus maze.

Frequency of vertical activity (OF test, 10 min) and rearing and head dipping (EPM, 5 min) after injection of Δ-THC (0.3, 1, 3 or 10 mg/kg) or CBD (1, 5, 10 or 50 mg/kg) on various days of treatment.

Frequency of vertical activity (OF test, 10 min) and rearing and head dipping (EPM, 5 min) after injection of Δ-THC (0.3, 1, 3 or 10 mg/kg) or CBD (1, 5, 10 or 50 mg/kg) on various days of treatment.

Acute CBD

In the follow-up experiment investigating the effect of CBD in the EPM there was no effect of CBD on total number of EPM arm entries, percentage time in the open arms, ratio of open-arm entries to total arm entries, time in the centre zone, head dipping, or rearing (one-way ANOVA: p > 0.05; data not shown).

Startle response and PPI

All mice displayed identical startle response and PPI at baseline testing 1 d prior to the first injection (data not shown). There was a main effect of prepulse intensity on PPI (p < 0.001) and a significant linear contrast between levels of prepulse intensity (p < 0.001) on all test days, indicating that PPI increased with increasing prepulse intensity.

Δ-THC (10 mg/kg) decreased the startle response on day 1 [one-way ANOVA: F(4,48) = 3.1, p < 0.05] and day 18 [F(4,44) = 3.1, p < 0.05] (Table 4). There was no effect of Δ-THC on the startle response when mice were treated with MK-801 (data not shown).

There was no effect of CBD on startle response on any day (one-way ANOVA: p > 0.05). There was no main effect of Δ-THC on PPI on days 1, 7 or 18 (repeated-measures ANOVA: p > 0.05, data not shown for day 7) (Fig. 5a, b). There was a significant interaction between Δ-THC and prepulse intensity on day 1 [F(6.5,71.4) = 5.0, p < 0.001] and day 18 [F(7.0, 70.2) = 5.6, p < 0.001]. Planned contrasts showed that acute Δ-THC (0.3, 3 and 10 mg/kg) significantly increased PPI (Fig. 5a).

There was a main effect of CBD on PPI on day 1 [repeated-measures ANOVA: F(4,44) = 4.9, p < 0.01]
Fig. 4. Anxiety-related measures after injection of Δ⁹-tetrahydrocannabinol (THC) (0.3, 1, 3 or 10 mg/kg) or cannabidiol (CBD) (1, 5, 10 or 50 mg/kg). -(a, e) Time spent in the central area (open-field test, 10 min) on days 1, 5 and 21 of treatment; (b, f) Distance ratio (open-field test); (c, g) Time spent in the light compartment (light–dark test, 10 min) on day 17; (d, h) Distance ratio (light–dark test). Data represent mean ± S.E.M. (n = 7–10). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle group (one-way ANOVA and planned contrasts).

(Fig. 5c), but not on day 7 (data not shown) or day 18 (p > 0.05) (Fig. 5d). There was no CBD x prepulse intensity interaction (p > 0.05). Planned contrasts showed that acute (1, 5 and 50 mg/kg) (Fig. 5c) and chronic (1 mg/kg) (Fig. 5d) CBD increased PPI.

**Social interaction**

Δ⁹-THC did not alter total SI time (one-way ANOVA: p > 0.05), but decreased the combined frequency of the social behaviours general sniffing, anogenital sniffing, allogrooming, following and climbing over/under [one-way ANOVA: F(4, 45) = 13.2, p < 0.001] (Table 5). Δ⁹-THC reduced general sniffing frequency [one-way ANOVA: F(4, 45) = 18.6, p < 0.001] at 3 and 10 mg/kg and its duration [one-way ANOVA: F(4, 45) = 3.9, p < 0.01] at 10 mg/kg (Table 5). Δ⁹-THC (10 mg/kg) also decreased anogenital sniffing frequency [one-way ANOVA: F(4, 45) = 4.5, p < 0.01].
There was no effect of CBD on SI (one-way ANOVA: \( p > 0.05 \)) (Table 5).

**Y-maze**

There was no effect of treatment on the percentage of novel entry triplets, indicating equal spontaneous alternation. Neither \( \Delta^9 \)-THC nor CBD affected the number of total arm entries (data not shown).

**Passive avoidance**

A main effect of trial on the latency to enter the dark chamber in vehicle- and \( \Delta^9 \)-THC-treated mice [repeated-measures ANOVA: \( F(1, 26) = 31.0, p < 0.001 \)] indicated that all mice learned to avoid the dark chamber after training (Fig. 6a). There was no main effect of \( \Delta^9 \)-THC (\( p > 0.05 \)) but there was a significant \( \Delta^9 \)-THC treatment \( \times \) trial interaction [\( F(4, 26) = 3.4, \]

### Table 4. Startle response

<table>
<thead>
<tr>
<th>Day</th>
<th>Vehicle</th>
<th>( \Delta^9 )-THC 0.3</th>
<th>( \Delta^9 )-THC 1</th>
<th>( \Delta^9 )-THC 3</th>
<th>( \Delta^9 )-THC 10</th>
<th>CBD 1</th>
<th>CBD 5</th>
<th>CBD 10</th>
<th>CBD 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58.1 ( \pm ) 4.0</td>
<td>49.8 ( \pm ) 3.4</td>
<td>50.4 ( \pm ) 6.2</td>
<td>48.8 ( \pm ) 4.3</td>
<td>35.7 ( \pm ) 4.6**</td>
<td>44.3 ( \pm ) 6.6</td>
<td>57.9 ( \pm ) 4.7</td>
<td>52.9 ( \pm ) 3.5</td>
<td>52.5 ( \pm ) 5.8</td>
</tr>
<tr>
<td>18</td>
<td>66.8 ( \pm ) 4.9</td>
<td>56.0 ( \pm ) 9.0</td>
<td>65.3 ( \pm ) 7.9</td>
<td>50.1 ( \pm ) 6.0</td>
<td>39.0 ( \pm ) 5.2*</td>
<td>54.3 ( \pm ) 10.5</td>
<td>71.0 ( \pm ) 8.1</td>
<td>64.3 ( \pm ) 4.9</td>
<td>65.6 ( \pm ) 6.1</td>
</tr>
</tbody>
</table>

\( \Delta^9 \)-THC, \( \Delta^9 \)-tetrahydrocannabinol; CBD, cannabidiol.

Startle response (arbitrary units) following 120 dB acoustic stimuli [prepulse inhibition (PPI), 30 min] after injection with \( \Delta^9 \)-THC (0.3, 1, 3 or 10 mg/kg) or CBD (1, 5, 10 or 50 mg/kg) on various days of treatment.

Data represent mean (\( \pm \) S.E.M.), \( n = 8–10 \).

* \( p < 0.05 \), ** \( p < 0.01 \) vs. vehicle group (one-way ANOVA and planned contrasts).
Duration and frequency of general sniffing, anogenital sniffing and total social interaction (SI) (SI test, 10 min) with a standard opponent A/JArc mouse after injection with Δ⁸-THC (0.3, 1, 3 or 10 mg/kg) or CBD (1, 5, 10 or 50 mg/kg) on day 18 of treatment. Data represent mean (±S.E.M.), n = 8–10.

- THC, -tetrahydrocannabinol; CBD, cannabidiol.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Δ⁸-THC 0.3</th>
<th>Δ⁸-THC 1</th>
<th>Δ⁸-THC 3</th>
<th>Δ⁸-THC 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>General sniffing duration (s)</td>
<td>30.6 ± 2.9</td>
<td>23.5 ± 1.2</td>
<td>28.2 ± 2.3</td>
<td>24.7 ± 1.7</td>
<td>18.7 ± 2.7**</td>
</tr>
<tr>
<td>General sniffing (o)</td>
<td>55.8 ± 3.6</td>
<td>48.1 ± 2.0</td>
<td>49.6 ± 4.1</td>
<td>43.1 ± 2.5*</td>
<td>21.4 ± 2.2***</td>
</tr>
<tr>
<td>Anogenital sniffing (o)</td>
<td>16.1 ± 2.0</td>
<td>16.3 ± 2.1</td>
<td>16.6 ± 2.9</td>
<td>13.6 ± 2.1*</td>
<td>5.7 ± 1.2***</td>
</tr>
<tr>
<td>Total social interaction duration (s)</td>
<td>50.2 ± 3.9</td>
<td>48.3 ± 3.6</td>
<td>48.3 ± 3.6</td>
<td>42.4 ± 4.6</td>
<td>4.1 ± 2.5***</td>
</tr>
<tr>
<td>Total social interaction (o)</td>
<td>84.3 ± 5.9</td>
<td>83.4 ± 6.4</td>
<td>79.3 ± 5.4</td>
<td>71.5 ± 6.0</td>
<td>11.6 ± 1.6***</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle group (one-way ANOVA and planned contrasts).

CBD-treated mice also learned to avoid the dark chamber after training, indicated by a main effect of trial [repeated-measures ANOVA: F(1, 31) = 37.6, p < 0.001] (Fig. 6b). There was no main effect of CBD or a CBD x trial interaction.

Discussion

This study is the first to compare acute and chronic effects of two cannabinoids, the prototypical psychoactive constituent Δ⁸-THC and the non-psychoactive constituent CBD, in a battery of schizophrenia and anxiety-relevant behavioural tests in male adult C57BL/6J Arc mice. The major findings were: (i) acute Δ⁸-THC and CBD enhanced PPI; (ii) chronic CBD, but not Δ⁸-THC, enhanced PPI; (iii) chronic Δ⁸-THC and CBD, but not acute CBD, attenuated Dex-induced hyperlocomotion and (iv) chronic, but not acute, CBD produced moderate anxiolytic-like effects.

Δ⁸-THC produced the cannabinoid ‘tetrad’ of acute effects (Compton et al., 1993), consistent with its accepted profile as a partial CB₁ agonist (Pertwee, 2008). In contrast, CBD had no effect on tetrad measures of catalepsy, nociception or locomotion and produced only modest hyperthermia, suggesting that acute CBD does not produce the sedative or hypolocomotor effects of Δ⁸-THC in C57BL/6J Arc mice (Compton et al., 1992; Onaivi et al., 1990; Varvel et al., 2006).

Our novel finding that Δ⁸-THC (acute) enhanced PPI in C57BL/6J Arc mice adds to an inconclusive database on the effects of Δ⁸-THC on PPI and represents the first such report of CBD effects. The results may be species- and strain-dependent since cannabinoid agonists have previously disrupted PPI in ddY mice (Nagai et al., 2006) and rats (Schneider & Koch, 2002), whereas in other animal models, Δ⁸-THC may disrupt (e.g., in socially isolated rats; Malone & Taylor, 2006) or enhance PPI (e.g., in neuregulin 1 mutant mice; Boucher et al., 2007). Importantly, we observed PPI effects in the absence of changes to the startle response except with 10 mg/kg Δ⁸-THC, which concomitantly enhanced PPI and decreased the startle response.

In contrast, few studies exist on the effects of CBD on PPI, and our findings that acute (1, 5 and 50 mg/kg) and chronic (1 mg/kg) CBD enhanced PPI represent the first such report of CBD’s effects. While these findings do not in themselves represent antipsychotic-like effects, it is interesting to speculate on whether such improved baseline sensorimotor gating as that
observed with acute and chronic CBD here might interact with the effect of genetic or pharmacological challenges on PPI. Previously, CBD alone had no effect on PPI in Swiss mice but dose-dependently increased the startle response and reversed an MK-801-induced PPI deficit at 5 mg/kg (Long et al. 2006). We aimed to assess whether repeated treatment with CBD could reverse the effects of MK-801, which dose-dependently disrupts PPI in rodents (Mansbach & Geyer, 1989; Varty et al. 2001; Yee et al. 2004) and mimics the glutamatergic hypofunction thought to occur in schizophrenia. However, MK-801 did not disrupt PPI in our vehicle group. Since mice experienced two PPI sessions prior to testing under MK-801, PPI enhancement may have masked its disruptive effect (Plappert et al. 2006). Future studies will confirm whether repeated CBD treatment can reverse the psychotomimetic effect of MK-801 on PPI.

We also investigated the effect of chronic Δ⁹-THC or CBD on the locomotor response to the psychostimulant Dex. Dex produced a typical increase in spontaneous locomotor activity that was attenuated in mice pre-treated with Δ⁹-THC (3 or 10 mg/kg) or CBD (50 mg/kg). Δ⁹-THC-treated mice showed decreased baseline activity, suggesting a functional antagonism by Δ⁹-THC of Dex-induced hyperlocomotion. In contrast, CBD attenuated Dex-induced hyperlocomotion without altering baseline locomotor activity. Importantly, acute CBD at low (1 mg/kg) or high (50 mg/kg) doses did not alter Dex-induced hyperlocomotion. Although previous studies report that at similar doses acute CBD reduced Dex- and ketamine-induced hyperlocomotion (Moreira & Guimarães, 2005), our divergent results may be due to genetic differences between mouse strains used. Further studies may elucidate potential cumulative effects of CBD on brain regions associated with the response to the psychostimulant. Indeed, changes in hypothalamic c-fos protein expression and catecholamine release in the nucleus accumbens core following intracerebroventricular CBD administration in rats (Murillo-Rodríguez et al. 2006) raise intriguing possibilities for mechanisms that may underlie the effects we observed. Our study is the first to report the effects of chronic CBD on behaviours related to schizophrenia and suggests that it might modulate such measures as PPI and Dex-induced hyperlocomotion.

Another area of therapeutic potential for CBD is in treating anxiety. We observed that low- and high-dose chronic CBD produced anxiolytic-like effects, in contrast to the anxiogenic effects observed with acute and chronic Δ⁹-THC. The significant effects of Δ⁹-THC and CBD on distance ratios in the OF and LD tests provide measures of anxiety-related behaviour that are relatively independent of locomotor activity. Interestingly, acute doses of CBD (1 and 50 mg/kg) that produced anxiolytic-like effects in the OF and LD tests after chronic administration had no effect in the EPM. This contrasts with previous reports of anxiolytic-like effects of acute CBD (Campos & Guimarães, 2008; Guimarães et al. 1994; Moreira et al. 2006; Onaivi et al. 1990) and may again be due to species and strain effects (Belzung, 2001). In the SI test, which measures anxiety and social withdrawal, Δ⁹-THC (10 mg/kg) reduced general sniffing and anogenital sniffing frequency, consistent with reports after high dose (van Ree et al. 1984) or chronic Δ⁹-THC treatment (Quinn et al. 2008). However, sedation might have reduced the overall activity of our Δ⁹-THC (10 mg/kg)-treated mice. In contrast, CBD did not alter baseline SI, consistent with previous reports in rats (Malone et al. 2009; van Ree et al. 1984). Overall, we provide novel...
evidence that chronic treatment with CBD produces anxiolytic-like effects in the LD test in C57BL/6JArc mice.

Learning and working memory are behavioural domains of therapeutic interest for schizophrenia, which is marked by cognitive deficits (Elvevag & Goldberg, 2000). In the present study Δ9-THC had no effect on spontaneous alternation or total arm entries in the Y-maze. Interestingly, Δ9-THC did not significantly reduce the number of arm entries, perhaps due to the less aversive nature of the Y-maze compared with the anxiogenic components of the LD or OF apparatus. In the PA test, Δ9-THC dose-dependently increased the latency to enter the aversive stimulus-paired dark chamber, an unexpected effect since higher doses of Δ9-THC generally disrupt short-term memory (Fadda et al. 2004; Quinn et al. 2008; Silva de Melo et al. 2005). Nevertheless, Δ9-THC no longer induced analgesia at this stage of treatment, excluding nociceptive interference with the aversive nature of the electric footshock, and any locomotor effects were controlled for in the statistical analysis. CBD did not affect spontaneous alternation in the Y-maze nor latency to enter the dark chamber in the PA test. These results, combined with previous observations that acute CBD had no effect in the radial arm maze (Lichtman et al. 1995) and that CBD-rich cannabis extracts had no effect in the Morris water maze (Fadda et al. 2006), suggest that CBD does not disrupt memory.

In conclusion, using a comprehensive behavioural phenotyping strategy in order to capture a number of features associated with schizophrenia and anxiety we provide the first report that chronic CBD facilitates sensorimotor gating, exerts anxiolytic-like effects and attenuates Dex-induced hyperlocomotion in C57BL/6JArc mice. Meanwhile, acute, but not chronic, Δ9-THC facilitates PPI. The chronic onset of the CBD effects and their complex dose-dependency are novel findings that raise intriguing avenues for further studies of its therapeutic potential. In particular, investigations using specific behavioural models of schizophrenia, particularly in genetic mouse models, will be useful in the future.

Acknowledgements

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Statement of Interest

None.

References


Quinn HR, Matsumoto I, Callaghan PD, Long LE, et al. (2008). Adolescent rats find repeated Delta(9)-THC less aversive than adult rats but display greater residual cognitive deficits and changes in hippocampal protein expression following exposure. Neuropsychopharmacology 33, 1113–1126.


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The effects of antipsychotics on the density of cannabinoid receptors in the dorsal vagal complex of rats: implications for olanzapine-induced weight gain

Katrina Weston-Green, Xu-Feng Huang, Mei Han and Chao Deng

Abstract

Some atypical antipsychotics clinically used to treat schizophrenia induce weight gain by unknown mechanisms. The dorsal vagal complex (DVC) of the brainstem and the endogenous cannabinoid system are implicated in the regulation of appetite signalling and food intake. We investigated whether antipsychotic drugs alter cannabinoid receptor-binding density in the DVC. Female Sprague–Dawley rats were treated with olanzapine, haloperidol, aripiprazole or vehicle for 1 wk (short-term) or 12 wk (chronic). Quantitative autoradiographic methods were employed to investigate the binding density of cannabinoid receptors in the DVC using a highly sensitive Beta Imager. Short-term olanzapine induced a significant 39% decrease in cannabinoid receptor binding compared to controls, whilst short-term aripiprazole and haloperidol had no significant effect. Chronic olanzapine treatment induced a significant 46% decrease in cannabinoid receptor binding compared to controls, aripiprazole slightly decreased cannabinoid receptor binding (12%), whilst haloperidol had no effect. Consistent with binding changes, short-term and chronic olanzapine treatment induced significant weight gain, but not aripiprazole or haloperidol. Cannabinoid receptor binding was negatively correlated to weight gain following chronic olanzapine treatment only \((r = -0.83, p = 0.01)\). In addition, only chronic olanzapine treatment increased food intake. These results show that olanzapine, an antipsychotic with a high risk of weight gain as a side-effect, significantly decreased cannabinoid receptor binding in the DVC, whilst aripiprazole and haloperidol, antipsychotics with a lower risk of weight gain had little or no effect on binding. These results suggest that a mechanism for antipsychotic-induced weight gain may be through the modulation of cannabinoid receptors in the DVC.

Key words: Cannabinoid receptor, dorsal vagal complex, olanzapine, weight gain.

Introduction

Antipsychotic drugs such as olanzapine, aripiprazole and haloperidol play a key role in the therapeutic treatment of schizophrenia. The clinical efficacies of the atypical antipsychotics olanzapine and aripiprazole are considered to be superior to conventional antipsychotics such as haloperidol in treating schizophrenia patients who are unresponsive to conventional drug therapy, with reduced risk of extrapyramidal symptoms (Keefe et al., 2006; Naber and Lambert, 2004). However, a significant side-effect of olanzapine is weight gain, which is of social and clinical importance as it may lead to further complications such as cardiovascular disease, diabetes and non-compliance to medication (Chagnon et al., 2004). Although the weight gain side-effect of aripiprazole is controversial, studies have shown that aripiprazole has less risk than olanzapine, but poses a higher risk than haloperidol (Chrzanowski et al., 2006; Naber and Lambert, 2004). Many suggestions have been made to explain the possible mechanisms underlying weight gained during antipsychotic drug-use (Atmaca et al., 2007; Baptista, 1999; Chagnon et al., 2004), however, this question appears to remain largely unanswered.
The dorsal vagal complex (DVC) is involved in gastrointestinal function and responds to hunger and satiety signals influencing energy homeostasis and appetite signalling; imbalances in this system can result in metabolic disturbances such as obesity and metabolic disorders (Blessing, 1997; Orr and Davy, 2005). The DVC comprises the dorsal motor nucleus of the vagus (DMN), nucleus tractus solitarius (NTS) and the area postrema (AP). The NTS and the hypothalamic arcuate nucleus (Arc) receive information about energy status via afferent signals from peripheral gut peptides such as ghrelin and leptin (Orr and Davy, 2005). This signalling results in alterations of neuronal activity in the brainstem and hypothalamus, which stimulate or suppress appetite and feeding accordingly (Orr and Davy, 2005).

The appetite-increasing effect of Cannabis sativa (marijuana) has been observed since AD 300. Administration of the endogenous cannabinoid, anandamide induces overeating in rats (Williams and Kirkham, 1999) and delta(9)-tetrahydrocannabinol (Δ9-THC), the main psychoactive component of marijuana, increases food consumption in mice (Wiley et al., 2005). Endogenous cannabinoids exert their effects through G-protein-coupled receptors, termed cannabinoid receptors CB1 and CB2 receptors and mRNA are widely distributed in the brain, including the DVC of rats and humans (Derbenev et al., 2004; Glass et al., 1997). A study by Miller et al. (2004) found that injections of CP-55940, a cannabinoid receptor agonist, into the hindbrain influenced feeding behaviour by increasing the intake of palatable food in rats. In addition, clinical studies have shown that the CB1 receptor antagonist, SR141716A (rimonabant hydrochloride; Sanofi Aventis, Paris, France), decreases appetite and body weight (Cota et al., 2003) and CB1 receptor knockout mice consume less food than their wild-type littermates, even after fasting (Di Marzo et al., 2001).

Despite the awareness of the influence of cannabinoid neurotransmission on appetite signalling and food intake, and the localisation of these receptors in the region of the brainstem involved in appetite signalling, the physiological effects of antipsychotics on cannabinoid neurotransmission in the DVC have not previously been explored. In addition, rimonabant (Acomplia®) has been approved by the European Medicines Agency (EMEA) for the treatment of obesity in patients with risk for Type 2 diabetes or heart disease, and may be a candidate for the treatment of antipsychotic-induced weight gain. However, it is unclear whether an interaction exists between antipsychotics and cannabinoid receptors in the brain centres involved in the regulation of food intake and body weight. Therefore, in the present study, we examined the binding of [3H]CP-55940 in the DVC of rats treated with olanzapine, aripiprazole, haloperidol or vehicle. We hypothesized that the antipsychotic known to clinically induce the most weight gain would induce the most significant change in cannabinoid receptor-binding density in the DVC, whilst the antipsychotic known to induce the lowest weight gain would cause little or no effect on receptor binding density.

**Methods**

**Animals, diet and experimental procedures**

Female Sprague–Dawley rats (226–250 g) were obtained from the Animal Resources Centre (Perth, WA, Australia). They were housed at 22 °C, on a 12-h light–dark cycle (lights on: 06:00 hours), and allowed ad libitum access to water and standard laboratory chow diet (3.9 kcal/g; 10% fat, 74% carbohydrate, 16% protein) throughout the study. Rats were randomly assigned to one of the following treatments: 0.3 mg/kg.d haloperidol (Sigma Aldrich, St Louis, MO, USA; n = 26), 1.5 mg/kg.d olanzapine (Eli Lilly, Indianapolis, IN, USA; n = 25), 2.25 mg/kg.d aripiprazole (Bristol–Myers Squibb, New York, NY, USA; n = 25), or vehicle (control, n = 25). Clinical studies have shown that haloperidol reaches a therapeutic level in the treatment of schizophrenia when 65–80% of D2 dopamine receptors are occupied in the brain, a level achieved by a dosage 0.3 mg/kg.d in rats (Kapur et al., 2000). In this study, dosages of olanzapine and aripiprazole were determined by comparing their clinical dose to that of haloperidol. Antipsychotics were orally administered via premixed drug-chocolate pellets, three times daily, as previously described by Deng et al. (2007). Weight gain and food intake were measured once weekly. After 7 d treatment, 12 rats from each treatment were sacrificed using carbon dioxide asphyxiation, 48 h after the last drug treatment, to examine short-term treatment effects. Treatment of the remaining rats (n = 13–14 for each treatment) was continued for a total of 12 wk, after which time they were sacrificed to examine chronic treatment effects. Therefore, there were eight treatment groups (4 drug treatments × 2 time-points). Six to eight rats per treatment group were used to examine [3H]CP-55940 binding in the DVC. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.
Histological procedures

Brain tissue was immediately removed after death and frozen using liquid nitrogen, and then stored at \(-80^\circ C\). Coronal sections were cryostatically cut (14 mm) at \(-18^\circ C\) from the level of Bregma \(-11.00\) mm to \(-14.60\) mm (Paxinos and Watson, 1998), thaw-mounted onto Polysine\textsuperscript{TM} Microscope Slides (Menzel GmbH & Co. KG, Braunschweig, Germany) and stored at \(-20^\circ C\).

Cannabinoid receptor binding using \([\text{^3}H]\text{CP-55940}\)

Cannabinoid receptor binding was performed as previously described by our group (Newell et al., 2006). Briefly, slides were air-dried then pre-incubated for 30 min in 50 mM Tris–HCl buffer (pH 7.4) containing 5% bovine serum albumin (BSA) at room temperature. Sections were incubated for 120 min with 10 nM \([\text{^3}H]\text{CP-55940}\) (168 Ci/mmol, PerkinElmer, Boston, MA, USA) in 50 mM Tris-HCl buffer (pH 7.4) containing 5% BSA to determine total binding, and non-specific binding was determined by incubating subsequent sections in 10 nM \([\text{^3}H]\text{CP-55940}\) in the presence of 10 \(\mu\)M CP-55940. Sections were then washed in 50 mM Tris–HCl buffer for 5 min at 4 \(^\circ\)C, dipped in cold milliQ H\textsubscript{2}O to remove buffer salts and gently dried in a stream of cool air.

Autoradiography and quantification of cannabinoid receptor binding

Cannabinoid receptor autoradiographic images were captured using a beta image camera (BioSpace, Paris, France), as previously described by Newell et al. (2006). Briefly, the level of radioactivity bound to the brain sections was counted directly from the amount of \(\beta\)-particles emitted from the tissue. Exposure time was 3.5 h at a high-resolution setting. Sections containing known amounts of tritium \([\text{^3}H]\) ligand were used to construct a standard curve. Quantitative analysis of images was performed using \(\beta\)-Image Plus software (version 4, BioSpace). Cannabinoid receptor-binding densities in the DVC and cerebellum were analysed by averaging samples from four Bregma levels: \(-13.24\) mm, \(-13.30\) mm, \(-13.68\) mm, \(-14.60\) mm (Figure 1) (Paxinos and Watson, 1998). Measurements of radioligand binding were converted to fmol/mg tissue equivalent using the aforementioned standards. Specific binding was obtained by subtracting non-specific binding values from total binding values. Anatomical positioning of the DVC was obtained by comparison with a set of corresponding Cresyl Violet-stained slides, assisted by the use of a standard rat brain atlas (Paxinos and Watson, 1998).

Quantitative data were statistically analysed using SPSS (version 13, SPSS, Chicago, IL, USA). Two-way ANOVAs (drug \times\) treatment duration) were employed to examine cannabinoid receptor-binding density in the DVC and cerebellum. Multiple comparisons were performed using post-hoc Tukey tests. Pearson’s correlations were used to determine the relationship between cannabinoid receptor binding and food intake and weight gain in overall groups. Spearman’s correlations were employed to examine correlations between specific treatment groups and food intake and weight gain due to the smaller sample size.

Figure 1. \([\text{^3}H]\text{CP-55940}\) binding in the dorsal vagal complex (DVC) of rats treated with vehicle [control (a, b), or olanzapine (c, d)] for 1 wk. (a, c) At the level of Bregma \(-14.60\) mm, (b) at the level of Bregma \(-13.30\) mm, and (d) at the level of Bregma \(-13.68\) mm (adapted from Paxinos and Watson, 1998).
Results

Figure 1 illustrates examples of [3H]CP-55940 binding in the brainstem. There was a significant effect of treatment \( (F_{3,49} = 64.94, p = 0.00) \) but not duration \( (F_{1,49} = 2.48, p = 0.12) \) on [3H]CP-55940 binding density in the DVC. In addition, there was no significant interaction between the two factors \( (F_{3,49} = 2.48, p = 0.07) \). Compared to controls short-term olanzapine treatment induced a significant \(-39\%\) decrease \( (p = 0.00, \text{Figure 2a}) \) in [3H]CP-55940 binding density in the DVC, whilst no significant change in [3H]CP-55940 binding was observed following short-term aripiprazole \( (p = 0.27, \text{Figure 2a}) \), or haloperidol treatment.
(p = 0.31, Figure 2a). Chronic olanzapine treatment induced a significant −46% decrease (p = 0.00, Figure 2b) in [3H]CP-55940 binding density in the DVC compared to controls, chronic aripiprazole treatment significantly decreased binding but to a lesser extent than olanzapine (−12%, p = 0.01, Figure 2b), whilst chronic haloperidol treatment had no effect on [3H]CP-55940 binding density (p = 0.37, Figure 2b). However, there was no significant effect of antipsychotic treatment on [3H]CP-55940 binding in the cerebellum (F_{3,48} = 0.241, p = 0.867) after both short-term and chronic treatment (Figure 2c, d).

A significant effect of antipsychotic treatment on weight gain was observed after 1 wk (F_{3,44} = 4.07, p = 0.01). Consistent with changes in [3H]CP-55940 binding density, short-term olanzapine treatment induced significant weight gain (p = 0.01, Figure 2e), but not short-term aripiprazole (p = 0.73, Figure 2e) or haloperidol (p = 0.59, Figure 2e) treatment compared to controls. However, short-term antipsychotic treatment had no significant effect on food intake compared to controls (all p > 0.05, Figure 2g). There was a significant effect of chronic drug treatment on weight gain (F_{3,49} = 3.172, p = 0.03). Chronic olanzapine treatment induced significant weight gain (p = 0.03, Figure 2f), whereas rats treated with chronic aripiprazole or haloperidol showed no significant weight gain compared to controls (aripiprazole vs. control: p = 0.87; haloperidol vs. control: p = 0.90; Figure 2f). Accordingly, there was a significant negative correlation between weight gain and [3H]CP-55940 binding density in the DVC following chronic olanzapine treatment only (r = −0.83, p = 0.01) (Figure 3a). In addition, there was a significant effect of chronic drug treatment on food intake (F_{3,49} = 8.63, p = 0.00). Chronic olanzapine induced a significant increase in food intake in rats (p = 0.04, Figure 2h), however, rats chronically treated with aripiprazole or haloperidol did not exhibit increased food intake compared to controls (aripiprazole vs. control: p = 0.99; haloperidol vs. control: p = 0.09; Figure 2h). There was a significant negative correlation between accumulated food intake and [3H]CP-55940 binding density in the DVC (r = −0.41, p = 0.03) following chronic antipsychotic treatment (Figure 3b).

**Discussion**

We investigated the effects of olanzapine, aripiprazole and haloperidol on the binding of [3H]CP-55940 in the DVC of rats, food intake and weight gain. A significant decrease in [3H]CP-55940 binding was observed following short-term and chronic olanzapine treatment compared to controls, whilst only a slight decrease in binding was noted following chronic aripiprazole treatment, and no significant change was apparent following treatment with haloperidol. In addition, although there was a high binding density of [3H]CP-55940 in the cerebellum, binding in this region was not affected by antipsychotic treatment. This result suggests that antipsychotic-induced alterations of cannabinoid receptor binding in the DVC is region-specific. In a similar trend, short-term olanzapine treatment significantly increased weight gain, and chronic olanzapine treatment increased weight gain and food intake. Whilst acute and chronic aripiprazole or haloperidol treatment had no effect on weight gain and food intake compared to controls. These results coincide with the clinical setting, in which patients treated with olanzapine exhibit higher weight gain.
than aripiprazole (Naber and Lambert, 2004) and haloperidol (Zipursky et al., 2005). For example, over an 8-wk period patients treated with olanzapine had an increase in body weight of 20% compared to aripiprazole (Naber and Lambert, 2004), and a 2-yr study by Zipursky et al. (2005) reported that olanzapine treatment induced significantly higher weight gain than haloperidol. Zipursky et al. (2005) also identified that patients treated with olanzapine had a five times greater risk for developing clinically significant weight gain than those treated with haloperidol.

In contrast to the high expression of cannabinoid CB1 receptors in the DVC (Glass et al., 1997), the distribution of CB2 receptors in the brainstem is controversial. Derbenev et al. (2004) reported that CB2 receptor protein was not present in the rat DVC following examinations using both reverse transcription–polymerase chain reaction (RT–PCR) and immunohistochemical methods, however, Van Sickle et al. (2005) identified CB2 receptor mRNA expression and immunoreactivity in the DMN, although at a much lower level. Since [3H]CP-55940 has a similar affinity for both CB1 and CB2 receptors (Pertwee, 1999), CB2 receptors cannot be completely excluded in the present study. However, due to the low abundance of CB2 receptors in the DVC, it is probable that [3H]CP-55940 binding observed in this study was mostly to the CB1 receptor.

To our knowledge, the present study is the first to observe the effects of antipsychotic drugs on the binding density of cannabinoid receptors in the brainstem and its relationship with weight gain. Previous studies have examined the effects of olanzapine and haloperidol on cannabinoid receptor-binding density in other regions of the brain; the outcome dependent on the region studied. For example, olanzapine and haloperidol had no effect on [3H]CP-55940 binding density in the hippocampus, frontal cortex, striatum and nucleus accumbens (Sundram et al., 2005), whilst haloperidol increased cannabinoid receptor mRNA levels in the caudate putamen (Mailleux and Vanderhaeghen, 1993), and [3H]CP-55940 binding density in the substantia nigra and striatum, but not the globus pallidus (Andersson et al., 2005). However, the DVC differs from these regions as it forms a direct component of the brain–gut axis, receiving signals from important peripheral satiety hormones such as ghrelin, leptin, peptide YY (3–36) and cholecystokinin (Orr and Davy, 2005). The absence of a blood–brain barrier on the medial and lateral aspects of the commissural NTS and the AP of the DVC allows greater access to circulating peptides and expedites appetite-signalling effects (Gross et al., 1990). In addition, neurons of the NTS send projections that converge with neurons derived from the Arc, which is traditionally thought to be the primary site for the regulation of food intake (Sainsbury et al., 2002).

As discussed previously, the appetite-enhancing effects of cannabinoid receptor agonists such as anandamide and Δ9-THC are well-documented (Miller et al., 2004; Wiley et al., 2005; Williams and Kirkham, 1999). In addition, previous studies show that an increase in endogenous cannabinoids decreases CB1 receptor mRNA (Rubino et al., 1994) and G-protein expression (Rubino et al., 1997) in the rat brain. In the present study, olanzapine treatment significantly induced weight gain and decreased [3H]CP-55940 binding. It is possible that antipsychotic treatment induces an increase in endogenous cannabinoid release, which leads to a compensatory decrease in cannabinoid receptor-binding density; the latter of which may be a result of decreased receptor expression and a possible functional decrease in G-protein activation.

Although the antipsychotics used in this study may not possess a profile to act directly on the cannabinoid system (Naber and Lambert, 2004; Richelson and Souder, 2000), they act on multiple neurotransmitter systems and may influence other pathways that can indirectly affect CB1 receptors. For example cannabinoids can presynaptically inhibit glutamatergic and GABAergic neurotransmission to anorexigenic pro-opiomelanocortin (POMC) neurons of the Arc (Nguyen and Wagner, 2007), which may result in positive energy balance. In fact, Ho and colleagues (2007) found a strong correlation between behavioural feeding changes induced by CB1 agonists (such as increasing meal frequency, duration and amount of food consumed) and the inhibition of glutamatergic neurotransmission presynaptically on anorexigenic POMC neurons of the Arc. However, studies by Corchero and colleagues (Corchero et al., 1997, 1999) found that administration of Δ9-THC increased POMC mRNA expression in the Arc of the rat, and that gonadal steroids could alter the effects of Δ9-THC on POMC regulation (Corchero et al., 2001). These studies suggest that the cannabinoid system can regulate Arc POMC neuron activation, which influences food intake and weight gain, however, further studies are required to reveal the exact mechanisms.

It is interesting that the NTS is the only nucleus besides the Arc to express POMC (Schwartz et al., 2000), however, the role of POMC neurons in the DVC is unclear. A study by Derbenev and colleagues (2004) found that cannabinoids act on CB1 receptors located on presynaptic terminals to inhibit GABAergic and
glutamatergic synaptic input to DMN neurons. Therefore, it is reasonable to predict that cannabinoid-induced presynaptic inhibition of POMC neurons observed in the Arc may also occur in the DVC. Taken together, these ideas provide a possible pathway for atypical antipsychotic-induced weight gain whereby a drug-induced over-expression of endocannabinoids may lead to the inhibition of anorexigenic POMC neurons in the DVC causing weight gain. As a compensatory mechanism, cannabinoid receptors located on the POMC neurons may decrease in an attempt to restore metabolic homeostasis. In fact, Derbenev et al. (2004) proposed that the orexigenic effects of cannabinoids may be via the suppression of inhibitory synapses from the NTS on neurons of the DMN, induced by the release of endocannabinoids from the active DMN. Further studies are required to confirm this mechanism.

The regulation of appetite signalling and energy homeostasis is highly complex and involves interactions between a variety of neurotransmitter systems. We previously found that olanzapine treatment decreases the binding density of muscarinic M_2 receptors in the DVC (Deng et al., 2007). Muscarinic M_2 and cannabinoid CB_1 receptors are co-localized in the DVC and it is possible that an interaction may exist between the muscarinic and endocannabinoid system in the regulation of energy balance. In the present study, aripiprazole induced a slight decrease in [H]CP-55940 binding but had no significant effect on weight gain or food intake, despite results indicating a slight elevation compared to controls. Although there was a significant change in [H]CP-55940 binding in the DVC, it may not have been sufficient to induce a physiological alteration in body weight and food intake. Contrary to olanzapine, aripiprazole does not act on muscarinic receptors (Naber and Lambert, 2004) and it is possible that an interaction between cannabinoid and muscarinic M_2 receptors is necessary to facilitate weight gain. In addition, olanzapine treatment decreases serotonin receptor mRNA in the rat hypothalamus (Huang et al., 2006) and chronic administration of the CB_1 receptor antagonist SR141716A results in increased central serotonin release (Darmani et al., 2003). Moreover, serotonin receptors are localized in the DVC (Browning and Travagli, 2001). Thus, another possible mechanism by which olanzapine may induce weight gain is through the combined modulation of cannabinoid and serotonergic neurotransmission systems, whereby these antipsychotics may decrease cannabinoid receptor-binding density in the DVC, resulting in a decrease in serotonin and an increase in food intake.

In conclusion, these results indicate that olanzapine, an antipsychotic with a high risk of weight gain as a side-effect decreased cannabinoid receptors in the DVC, whilst aripiprazole and haloperidol, i.e. drugs with less risk of weight gain had little or no effect on cannabinoid receptors in the DVC. This study supports a role for brainstem cannabinoid receptors in the mechanisms of antipsychotic-induced weight gain.

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Statement of Interest
None.

References


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Decreased Plasma Peptide YY Accompanied by Elevated Peptide YY and Y2 Receptor Binding Densities in the Medulla Oblongata of Diet-Induced Obese Mice

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It is well known that the peripheral peptide YY (PYY)-central neuropeptide Y (NPY) Y2 receptor axis plays an important role in promoting negative energy balance regulation. Both the hypothalamus and medulla oblongata express a high level of Y2 receptors; however, the functional role of this receptor in chronic high-fat diet-induced obesity has not been fully examined. Using quantitative autoradiography, this study measured binding densities of total [125I]PYY and Y2 receptors in the hypothalamus and medulla of chronic high-fat diet-induced obese (DIO), obese-resistant, and low-fat-fed mice. Plasma PYY was also measured using RIA after 22 wk of dietary intervention. The results revealed that body weight gain in chronic high-fat diet-induced obesity has not been fully examined. Using quantitative autoradiography, this study measured binding densities of total [125I]PYY and Y2 receptors in the hypothalamus and medulla of chronic high-fat diet-induced obese (DIO), obese-resistant, and low-fat-fed mice. Plasma PYY was also measured using RIA after 22 wk of dietary intervention. The results revealed that body weight gain was significantly higher in the obese mice, compared with the lean mice. Furthermore, PYY and NPY Y2 receptor binding densities in the medulla of the obese mice were significantly higher, compared with the lean mice, whereas the level of plasma PYY was significantly lower in the DIO mice than the low-fat-fed mice. In conclusion, this study showed that the DIO mice had low plasma PYY, which may have caused a compensatory up-regulation of PYY and Y2 receptor densities in the medulla. A low-level response of PYY-medullary regulation to positive energy balance may have contributed to the development of high-fat diet-induced obesity in DIO mice; conversely, a normal response of this regulatory axis in the obese-resistant mice may have contributed to the maintenance of body weight while on a high-fat diet. (Endocrinology 148: 4704–4710, 2007)

PEPTIDE YY (PYY) is a member of the pancreatic polypeptide family, which is structurally and functionally related to the neuropeptide Y (NPY) family (1, 2). PYY is mainly secreted from the endocrine L cells of the small and large intestine and released into the circulation in response to ingestion of food, especially in the presence of a fatty meal (3, 4). At present, it is known that the peripheral administration of PYY acutely inhibits food intake (5, 6). PYY has a high affinity to NPY Y2 receptors that is subsequently followed by NPY Y1 and Y5 receptors (7). Furthermore, it has been suggested that PYY works via the NPY Y2 receptor to suppress the amount of food intake (8).

In the hypothalamus, many studies have found that PYY acts on NPY Y2 receptors in the arcuate nucleus to decrease food intake (9–12). Additionally, it is known that the medulla has a high level of binding to PYY (13, 14). However, currently no information is available in regard to hypothalamic and medullary PYY and Y2 receptor regulation in diet-induced obesity. Using a chronic high-energy diet-induced obese (DIO) and diet-resistant (DR) mouse model, this study aimed to examine the levels of plasma PYY together with PYY and Y2 receptor binding density in the hypothalamus and medulla oblongata. It is hypothesized that differential regulation exists in the peripheral PYY and its hypothalamic and medullar binding densities between the mice prone or resistant to diet-induced obesity.

Materials and Methods

Animal model and diets

Forty-eight C57BL/6 mice aged 9 wk were obtained from the Animal Resource Centre (Perth, Western Australia) and kept in a temperature-controlled room at 22 C with a 12-h light, 12-h dark cycle. For the first week, all mice were given lab chow ad libitum to acclimatize them to their new surroundings. The mice were then placed in separate cages. Thirty-six mice were randomly chosen and fed a high-fat diet (Table 1) ad libitum and retrospectively assigned into one of three groups: DIO (n = 12), DR (n = 12), and intermediate, based on their body weight after 22 wk on this diet (15, 16). The other 12 mice were used as the control group (LF) and were given ad libitum low-fat diet (Table 1) for 22 wk. During the experiment, 24-h food intake and body weight were measured weekly. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Australia, and complied with the Australian Code of Practice for the Care and Use of Animal for Scientific Purposes.

Tissue preparation and body composition analysis

After 22 wk of feeding on the high- or low-fat diets, mice were given an overdose injection of sodium pentobarbitone (120 mg/kg, ip). All mice were killed between 0700 and 0900 h to minimize circadian variation. Blood samples were collected from the right ventricle of the heart. Brains were immediately removed after death and frozen in liquid nitrogen. Coronal brain sections (14 μm) were cut at −17 C using a cryostat and thaw-mounted onto polylysine microscope slides (Menzel GmbH & Co. KG, Braunschweig, Germany). Total body fat mass was measured via lipid extraction technique (17). Briefly, the mouse carcass was dried using a freeze drier (FD3 Freeze...
TABLE 1. The food composition of the diet used in the experiment

<table>
<thead>
<tr>
<th></th>
<th>LF diet</th>
<th>HF diet</th>
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<tbody>
<tr>
<td>Cornstarch, kcal, %</td>
<td>67.73</td>
<td>38.83</td>
</tr>
<tr>
<td>Sucrose, kcal, %</td>
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<td>5.39</td>
</tr>
<tr>
<td>Copha, kcal, %</td>
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<td>15.75</td>
</tr>
<tr>
<td>Beef tallow, kcal, %</td>
<td>9.75</td>
<td>8.07</td>
</tr>
<tr>
<td>Sunflower oil, kcal, %</td>
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<tr>
<td>Gelatine, kcal, %</td>
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</tr>
<tr>
<td>Casein, kcal, %</td>
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<td>51</td>
</tr>
<tr>
<td>Fiber, g/kg</td>
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<td>67</td>
</tr>
<tr>
<td>Minerals, g/kg</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

HF, High fat; LF, low fat.

drier; Dynavac Engineering, Sydney, Australia) and weighed before and after the drying process. After that, the body was cut into smaller portions and placed into cellulose thimbles. They were placed in a Soxhlet apparatus containing petroleum ether, which resulted in complete extraction of all neutral lipids. The total body fat mass was calculated by measuring the difference in body weight before and after the extraction.

$[^{125}]$I PYY binding autoradiography

PYY binding densities were visualized using $[^{125}]$I PYY as previously described (18). Sections were preincubated for 30 min in Krebs Henseleit Tris (KHT) buffer [118 mM NaCl, 4.8 mM KCl, 1.3 mM MgSO$_4$, 1.2 mM CaCl$_2$, 50 mM glucose, 15 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, 10 mM Tris (pH 7.3)]. Slides were then incubated for 120 min in KHT buffer containing 0.1% BSA, 0.05% bacitracin, and 25 pm $[^{125}]$I PYY (Sigma Aldrich, St. Louis, MO). Nonspecific binding was determined by incubating in the same incubation buffer plus 1 μM porcine NPY (Sigma Aldrich). Slides were then washed (3 × 5 min) in ice-cold buffer, dipped in ice-cold distilled water and dried under a gentle stream of cool air. Slides were stored overnight in desiccators and then apposed to X-OMAT AR film (Kodak, Rochester, NY) in the presence of standard microscales for 12 d. Autoradiographs were developed using Kodak D-19 developer and fixed with Ilford Hypham Rapid Fixer (Ilford Imaging, Clayton, Victoria, Australia).

Y2 receptor binding autoradiography

To measure the Y2 receptor binding density, [Leu$^{31}$, Pro$^{34}$]NPY (porcine; Sigma Aldrich) was included in the incubating solution to mask the NPY Y1 and Y5 receptors (19). Briefly, sections were preincubated for 30 min in KHT buffer. Slides were then incubated for 120 min in KHT buffer containing 0.1% BSA, 0.05% bacitracin, 100 nM [Leu$^{31}$, Pro$^{34}$]NPY, and 25 pm $[^{125}]$I PYY (Sigma Aldrich). Porcine NPY (1 μM) was used to determine nonspecific binding as mentioned above. The remaining methods were the same as for $[^{125}]$I PYY binding autoradiography.

Quantification

Autoradiography images were captured and analyzed using a computer-assisted image analysis system, Multi-Analysis, connected to a GS-690 imaging densitometer (Bio-Rad Laboratories, Hercules, CA), as the detailed description was given previously (20). The density of binding was calculated with the aid of the standard curve generated from the microscales, which then converted to nanocuries per milligram of tissue equivalent. Individual medullary nuclei were identified with reference to a standard mouse brain atlas (21).

Plasma PYY

A commercially available PYY (rat, mouse, porcine) RIA kit (Phoenix Pharmaceuticals, Belmont, CA) was used to measure the plasma level of PYY. The kit had 100% cross-reactivity with both circulating forms of PYY, PYY$_{1-36}$ and PYY$_{3-36}$ (22, 23).

Statistical analysis

Data were presented as means ± SD. We used the SPSS statistical package 13.0 (SPSS Inc., Chicago, IL) for our statistical analyses. A two-way repeated ANOVA (treatment × weeks as repeated measures) was used to analyze data of the weekly body weight and energy intake. Data of PYY binding density, Y2 receptor binding density, and plasma PYY measurements were assessed by one-way ANOVA, followed by a post hoc Tukey-Kramer honestly significant difference test for multiple comparisons among the groups. To analyze correlations between variables measured, a Pearson test was performed.

Results

Body weight, fat mass, and energy intake

A two-way repeated ANOVA revealed significant main effects of both treatment ($F_{2,33} = 70.19, P < 0.001$) and the repeated measurement weeks ($F_{11,363} = 387.82, P < 0.001$) on weight gains. There was also a significant interaction between the two factors ($F_{22,363} = 47.13, P < 0.001$). Although

![Fig. 1. Body weight (A), weekly energy intake (B), and average energy intake in 22 weeks (C) of chronic DIO, DR, and the control (LF) mice throughout 22 wk of feeding on the high- or low-fat diet. Data are represented as mean ± SD. Error bars are omitted when smaller than the symbol. *P < 0.05 DIO vs. LF; #, P < 0.05 DIO vs. DR.](image-url)
there was a consistent increase in the body weight in all groups, the DIO mice had significantly higher body weight gain than the DR (\(P = 0.007\)) and LF (\(P = 0.001\)) mice throughout the treatment period (Fig. 1A). The final body weight gain of the DIO group was 123 and 190% higher (42.7 ± 0.8 g) than the DR (31.6 ± 0.5 g) and LF mice, respectively (30.0 ± 0.8 g; F\(_{2,33} = 86.54, P < 0.001\); DIO vs. DR, \(P < 0.001\); DIO vs. LF, \(P < 0.001\)). Furthermore, the total fat mass of the DIO group was 129 and 192% heavier (13.3 ± 0.6 g) than the DR (6.5 ± 0.3 g) and LF mice, respectively (5.4 ± 0.4 g; F\(_{2,33} = 94.4, P < 0.001\); DIO vs. DR, \(P < 0.001\); DIO vs. LF, \(P < 0.001\)).

A two-way repeated ANOVA also revealed significant effects of treatment (F\(_{2,33} = 19.95, P < 0.001\)) and the repeated measurement weeks (F\(_{10,330} = 15.49, P < 0.001\)) on food intake along with a significant interaction between the two factors (F\(_{20,30} = 7.94, P < 0.001\)). Throughout the treatment period, the DIO mice had a significantly higher energy intake, compared with DR mice (\(P < 0.001\); Fig. 1B). Although there was a fluctuation in the energy intake of the LF mice on the various measurement days, overall, the DIO mice had a higher energy intake than the LF mice (\(P < 0.001\); Fig. 1B). No significant differences were found between the DR and LF mice. Furthermore, the average energy intake throughout the 22 wk of feeding was significantly higher in the DIO group (60.49 ± 0.87 kJ per 24 h), compared with the DR (53.03 ± 0.91 kJ per 24 h) and LF groups (55.13 ± 0.71 kJ per 24 h; F\(_{2,33} = 21.31, P < 0.001\); DIO vs. DR, \(P < 0.001\); DIO vs. LF, \(P < 0.001\); Fig. 1C).

**Plasma PYY**

The level of plasma PYY was significantly lower in the DIO group (32%), compared with the LF group (F\(_{2,21} = 6.26, P < 0.01\); DIO vs. LF, \(P = 0.007\); Fig. 2). No significant difference was found in the levels of plasma PYY between the DR and LF groups.

**PYY binding density and Y2 receptor binding density in the hypothalamus of DIO, DR, and LF mice**

Although there was a trend that the obese mice had a higher PYY binding density, compared with the lean mice, in the dorsomedial and ventromedial hypothalamus, these differences were not significant (Table 2). There was not any significant difference in PYY binding density in the arcuate nucleus and lateral hypothalamus. Furthermore, there were no significant differences of Y2 receptor binding density in any hypothalamic nuclei (Table 2) between the groups.

**PYY binding density in the medulla of DIO, DR, and LF mice**

A one-way ANOVA revealed that there were significant differences between the groups for PYY binding density in the dorsal vagal complex (DVC) containing the nucleus of solitary tract and dorsal motor nucleus of vagus nerve (F\(_{2,10} = 6.76, P = 0.019\)), intermediate reticular zone (IRt; F\(_{2,10} = 17.34, P = 0.001\)), and ventrolateral medulla (VLM; F\(_{2,10} = 7.60, P = 0.014\)) area (Figs. 3 and 4). In the DVC, the DIO mice had higher PYY binding density than that of the DR (68%, \(P = 0.015\)) and LF mice (37%, \(P = 0.079\)). Similar differences were also observed in the intermediate reticular zone, in which the DIO had a significantly higher binding density, compared with the DR (171%, \(P = 0.001\)) and the LF group (96%; \(P = 0.004\)). In the VLM, the DIO mice also had a significantly higher binding density than the DR (122%, \(P = 0.022\)) and the LF group (134%, \(P = 0.015\)).

**Y2 receptor density in the medulla oblongata of DIO, DR, and LF mice**

The DIO mice had significantly higher Y2 receptor binding density (Figs. 3 and 4), in the DVC, compared with the LF group (63%; F\(_{2,10} = 4.75, P = 0.044\); DIO vs. LF, \(P = 0.041\)). In the IRt, the DIO group had 47% higher binding density, compared with the DR (47%, \(P = 0.041\)) and LF (47%, \(P = 0.004\)) mice. In the VLM, there were no differences in the binding density among the DIO, DR, and LF groups.

### Table 2. The [\(^{125}\)I]PYY binding and Y2 receptor binding densities in various areas of the hypothalamus of chronic DIO, DR, and the control (LF) mice

<table>
<thead>
<tr>
<th>Brain area</th>
<th>DIO(^a)</th>
<th>DR(^a)</th>
<th>LF(^a)</th>
<th>F (2, 10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total binding with [(^{125})I]PYY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arc</td>
<td>284.8 ± 0.7</td>
<td>198.6 ± 66.5</td>
<td>195.7 ± 38.5</td>
<td>3.00</td>
<td>0.160</td>
</tr>
<tr>
<td>DMH</td>
<td>213.1 ± 27.7</td>
<td>123.7 ± 44.7</td>
<td>172.0 ± 20.9</td>
<td>4.39</td>
<td>0.079</td>
</tr>
<tr>
<td>LH</td>
<td>224.6 ± 30.1</td>
<td>131.2 ± 46.1</td>
<td>177.9 ± 42.7</td>
<td>3.02</td>
<td>0.138</td>
</tr>
<tr>
<td>VMH</td>
<td>248.60 ± 49.3</td>
<td>138.6 ± 41.4</td>
<td>168.3 ± 27.9</td>
<td>5.02</td>
<td>0.064</td>
</tr>
<tr>
<td><strong>Y2 receptor binding density</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arc</td>
<td>228.5 ± 17.2</td>
<td>154.3 ± 70.2</td>
<td>170.9 ± 35.5</td>
<td>1.36</td>
<td>0.337</td>
</tr>
<tr>
<td>DMH</td>
<td>206.2 ± 16.2</td>
<td>125.1 ± 58.9</td>
<td>154.3 ± 37.4</td>
<td>1.98</td>
<td>0.232</td>
</tr>
<tr>
<td>LH</td>
<td>222.1 ± 5.3</td>
<td>131.2 ± 51.0</td>
<td>162.6 ± 42.4</td>
<td>2.83</td>
<td>0.151</td>
</tr>
<tr>
<td>VMH</td>
<td>189.5 ± 30.9</td>
<td>139.9 ± 38.2</td>
<td>143.4 ± 23.7</td>
<td>1.72</td>
<td>0.269</td>
</tr>
</tbody>
</table>

Binding densities were quantified at the level of bregma −1.22, −1.70, and −2.18 mm. Arc, Arcuate nucleus; DMH, dorsomedial hypothalamus; LH, lateral hypothalamus.

\(^a\) Mean ± SD.
Correlation

A correlation analysis was carried out among the final body weight, body fat mass, energy intake of the last week of the dietary intervention, and the plasma PYY as well as the medullary PYY binding density and Y2 receptor binding density of the DIO, DR, and the control (LF) mice (Table 3). Final body weight was highly correlated to the plasma PYY and the PYY binding densities in all measured areas as well as the Y2 receptor binding densities in the IRt and VLM. Total body fat was also highly correlated to the plasma PYY and the PYY binding densities in all measured areas as well as the Y2 receptor binding densities in the IRt and VLM. The energy intake of the last week was significantly correlated to plasma PYY concentrations and the PYY binding densities and Y2 receptor binding densities in the IRt and the VLM.

Discussion

The results of this study revealed a significant increase of medullary PYY and Y2 receptor binding densities in the DIO mice, compared with the DR and LF mice. This increased binding density was accompanied by a decrease in plasma PYY level.

It is known that PYY acts on NPY Y2 receptors in the hypothalamic arcuate nucleus to decrease food intake (9–12). Furthermore, Y2 receptor conditional knockout mice have been shown to have a significant increase of food intake (24). Although there is an abundant amount of NPY receptor in the medulla (25, 26), to our present knowledge, no information is available in respect to whether the medullary NPY receptors are involved in the regulation of body weight in high-fat diet-induced obesity.

This study found that the PYY and Y2 receptor binding densities were significantly higher in the DIO mice compared to the DR and LF mice, indicating a potential role of these receptors in mediating the obesity phenotype.
densities in the obese mice were significantly higher than the lean DR and LF mice in most areas of the medulla regulating autonomic function (DVC, IRt, and VLM). This study also found that there was a positive correlation between PYY binding density in these areas and final body weight, energy intake, and body fat mass. Furthermore, although a similar trend was found in some of the hypothalamic nuclei (the dorsomedial and ventromedial hypothalamus), the differences between the obese and lean mice were not as significant as the findings in the medullary areas. These findings have demonstrated that PYY possibly acts to regulate energy balance via the medulla to control food intake rather than working exclusively in the arcuate hypothalamic nucleus.

The differences in the binding density in the VLM impl


dicating where the section was taken.

![Diagram](image)

FIG. 4. Photographs depicting the [125I]PYY bindings (B–D) and Y2 receptor bindings (B’ to D’) in the medulla of chronic DIO (B and B’), DR (C and C’), and the LF (control) group (D and D’). The line box (A) indicates where the section was taken.

TABLE 3. The correlation among the body weight, food intake, and plasma level of PYY and binding densities of [125I]PYY and Y2 receptor in the medulla nuclei

<table>
<thead>
<tr>
<th></th>
<th>Final body weight</th>
<th>Total body fat</th>
<th>Energy intake in last week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R value</td>
<td>P value</td>
<td>R value</td>
</tr>
<tr>
<td>Plasma PYY</td>
<td>−0.468</td>
<td>0.021</td>
<td>−0.475</td>
</tr>
<tr>
<td>Total binding with [125I]PYY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVC</td>
<td>0.738</td>
<td>0.010</td>
<td>0.738</td>
</tr>
<tr>
<td>IRt</td>
<td>0.842</td>
<td>0.001</td>
<td>0.838</td>
</tr>
<tr>
<td>VLM</td>
<td>0.803</td>
<td>0.003</td>
<td>0.817</td>
</tr>
<tr>
<td>Y2 receptor binding density</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DVC</td>
<td>0.495</td>
<td>0.122</td>
<td>0.468</td>
</tr>
<tr>
<td>IRt</td>
<td>0.727</td>
<td>0.011</td>
<td>0.747</td>
</tr>
<tr>
<td>VLM</td>
<td>0.618</td>
<td>0.045</td>
<td>0.634</td>
</tr>
</tbody>
</table>
a local effect of PYY neurons on PYY binding and Y2 receptor binding cannot be excluded. Further studies are needed to confirm this issue by measuring the levels of PYY mRNA and protein expression in the medulla of DIO mice.

Furthermore, the results of this study suggested that the elevation of the level of plasma PYY might be effective at decreasing food intake. However, previous studies have shown that there were different effects of the peripheral and central administration of PYY on food intake (40, 41). Peripheral injections of PYY caused significantly lower food intake in humans and rodents (including in a DIO mouse model) (2, 6, 11). Nevertheless, an intracerebroventricular injection of PYY induced higher food intake (42, 43). On the contrary, when PYY was injected directly into the hypothalamic arcuate nucleus, food intake was significantly decreased (6), an effect that was similar to that observed after a peripheral injection of PYY.

As for the difference in the effects of peripheral vs. central injections of PYY, one possible explanation could be that when PYY was injected peripherally, it was transported in the blood directly into areas in the brain with a high binding affinity to PYY, such as the hypothalamic arcuate nucleus (and possibly also the DVC), via the highly permeable blood-brain barrier. In these areas, PYY may have bound to the anorexigenic NPY Y2 receptor causing a decrease in food intake, which has been evidenced in Y2 knockout mice in which the anorectic effect of peripheral PYY injection was diminished (6). However, when injected centrally into the ventricle, it was likely that PYY bound to the more orexigenic NPY receptors, such as Y1 and Y5, caused an increase in food intake. This was also supported by the finding that the orexigenic effect of intracerebroventricular PYY was reduced in Y1 and Y5 receptor knockout mice (43).

In conclusion, it is clear that in diet-induced obese mice, there is a dysfunctional PYY regulation. Although there was an up-regulation in PYY and Y2 receptor binding in the medulla, the reduced amount of plasma PYY in the obese mice may have contributed to their high energy intake and subsequent weight gain.

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Critical insights into the etiology of insulin resistance have been gained by the use of animal models where insulin action has been modulated by strictly controlled dietary interventions not possible in human studies. Overall, the literature has moved from a focus on macronutrient proportions to understanding the unique effects of individual subtypes of fats, carbohydrates and proteins. Substantial evidence has now accumulated for a major role of dietary fat subtypes in insulin action. Intake of saturated fats is strongly linked to development of obesity and insulin resistance, while that of polyunsaturated fats (PUFAs) is not. This is consistent with observations that saturated fats are poorly oxidized for energy and thus readily stored, are poorly mobilized by lipolytic stimuli, impair membrane function, and increase the expression of genes associated with adipocyte proliferation (making their own home). PUFAs have contrasting effects in each instance. It is therefore not surprising that increased PUF intake in animal models is associated with improved insulin action and reduced adiposity. Less information is available for carbohydrate subtypes. Early work clearly demonstrated that diets high in simple sugars (in particular fructose) led to insulin resistance. However, again attention has rightly shifted to the very interesting issue of subtypes of complex carbohydrates. While no differences in insulin action have yet been shown, differences in substrate flux suggest there could be long-term beneficial effects on the fat balance of diets enhanced in slowly digested/resistant starches. A new area of major interest is in protein subtypes. Recent results have shown that rats fed high-fat diets where the protein component was from casein or soy were insulin-resistant, but when the protein source was from cod they were not. These are exciting times in our growing understanding of dietary factors and insulin action. While it has been clear for some time that ‘oils ain’t oils’, the same is now proving true for carbohydrates and proteins.

Dietary macronutrients: Energy balance: Insulin resistance

Background
Insulin resistance is a major player in the etiology of the metabolic syndrome cluster of diseases. Dietary factors undoubtedly influence insulin action, but it often appears that agreement stops with these two statements. Ambrose Bierce, in his wonderful tome The Devil’s Dictionary, defined a Trinitarian as ‘one who denies the divinity of a Unitarian’. Of course, a Unitarian was ‘one who denies the divinity of a Trinitarian’. In many ways the argument between the high carbohydrate versus fat-modified schools of dietary advice have fallen into Bierce’s dichotomous trap. Non-insulin-dependent or type 2 diabetes mellitus is the most linear disease descendent of insulin resistance. The prominent symptom, which provides the definition for the disease, is the inability to handle carbohydrate. The logical question is, why advocate a high-carbohydrate diet to individuals whose core problem is that macronutrient?

The answer, at least partially, concerns the macronutrients available to substitute for carbohydrate. There is limited ability to realistically manipulate the levels of protein, and therefore reducing carbohydrate comes at the cost of increasing fat. Since obesity is also part of the metabolic syndrome disease cluster, and insulin resistance is closely linked with adiposity, then the argument that ‘eating fat makes you fat’ comes into play. Fortunately, there is a way forward. Experimental animal investigations of insulin action, following manipulation not only of macronutrient proportions, but more particularly of macronutrient subtypes, is writing an exciting new story on diet and insulin action.

‘Oils ain’t oils’
The experimental animal literature on dietary fat subtypes is now quite large and coherent. There is evidence for both
direct effects on insulin action and indirect effects via induction of obesity. The literature on both has recently been reviewed, and only a summary is needed here with the focus on mechanistic insights.

‘Eating fat makes you fat’ is catchy but not well supported as a global concept. Overall there is now an enticing literature which links intake of saturated fats with development of obesity, but contrasts a neutral, or even positive, effect of polyunsaturated fatty acids (PUFAs) (Pan et al. 1994; Storlien et al. 1998). Compared to saturated fats, PUFAs are more readily used for energy when initially ingested (Leyton et al. 1987). This was demonstrated by observing labelled CO₂ production after oral administration of identical amounts of differently labelled fatty acids. Dramatic differences were observed over the subsequent 24 h, with only a small proportion of the saturated fats being fully used for energy compared to unsaturated fats (Leyton et al. 1987). The implication is that the saturated fats are being stored in adipose tissue depots. Once stored there, saturated fats are also less readily mobilized by lipolytic stimuli (Mougios et al. 1995; Halliwell et al. 1996; Raclot et al. 1997). Increasing the degree of unsaturation at a given carbon chain length increases the relative mobilizability of stored fats. This has an interesting implication for physically active individuals, where selective mobilization of unsaturated fats (Mougios et al. 1995), combined with the greater propensity of these fats for utilization as energy, means that such individuals are in danger of ‘saturating down’ their body lipid stores. As we shall see, this has potentially negative consequences for a number of aspects of metabolism.

Lipids are not just an efficiently stored energy reserve, they form the membranes of all cells and organelles. Here again, when saturated fats are incorporated into membranes they have the capacity to reduce metabolic rate and decrease receptor (e.g. beta adrenoceptor) binding (Matsuo & Suzuki, 1997) with the obvious implications for energy balance. Conversely, addition of n-6 PUFAs to the diet can increase beta adrenoceptor affinity (Nicolas et al. 1991).

In addition to their roles as energy and cell-structural elements, it is now clear that fatty acids act as potent gene regulators, notably in the current context on enzymes of endogenous lipid synthesis and adipocyte proliferation (Clarke et al. 1997). The beautiful pattern persists, with saturated fats up-regulating, and PUFAs down-regulating, these enzymes. Saturated fats are then not only adept at finding their way into the body’s stores and resistant to being winkled out, but proficient at making their own home – good friends in times of want but pernicious in the face of plenty. Finally, a more recent angle on fats and energy balance has been exposed (see Table 1). In mice fed diets high in saturated fats, neuronal activation was seen in the dorsal-lateral hypothalamus, the classic ‘feeding centre’, while activity in the ventromedial hypothalamic satiety centre was suppressed (Wang et al. 1999). This is in line with development of excess adiposity via a tilting of the autonomic nervous system balance to favour parasympathetic over sympathetic. This pattern was in contrast to PUFAs feeding where only an increase in ventromedial hypothalamic neuronal activity was observed, and even less fat accumulated than in low fat-fed control mice (Wang et al. 1999). It is important to link this new information with previous work which has demonstrated an increase in stress responsivity (indexed by blood glucose and corticosterone response, and hypothalamic noradrenaline turnover) with high-fat feeding (Pascoe et al. 1991).

The pattern of low sympathetic nervous system activity/basal metabolic rate and increased stress responsivity is a feature of many animal models of the metabolic syndrome. It is perhaps not surprising that the antiglucocorticoid RU486 (Kusunoki et al. 1995), and the alpha-2 adrenoceptor agonist clonidine (Rocchini et al. 1999), have both been shown to ameliorate the insulin resistance of high-fat feeding.

To complete this compelling pattern of dietary fat subtype effects, there is now evidence that ‘dietary fatty acid composition, independent of adipose tissue mass, is an important determinant of circulating leptin level’, with PUFAs-enriched diets leading to much higher leptin levels than those enriched in saturated fats (Cha & Jones, 1998). This suggests the possibility that there may even be an effect on the intake side of the energy-balance equation to help explain the anti-obesogenic effects of PUFAs.

As well as the indirect route via obesity, there is strong evidence that dietary fat subtypes are also able to modulate insulin action more directly (Storlien et al. 1996). Early in vitro studies provided evidence that changes in the composition of fatty acids within membrane phospholipids influenced insulin action, altering both insulin binding and

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Table 1. Effects of 1 week of feeding diets high in carbohydrate, saturated fat or polyunsaturated fats on neuronal (c-fos) activation (high-carbohydrate diet set at 100%)

<table>
<thead>
<tr>
<th>Energy balance</th>
<th>Lateral hypothalamus*</th>
<th>Ventromedial hypothalamus†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuronal activity (%)</td>
<td>Feeding</td>
<td>Parasympathetic</td>
</tr>
<tr>
<td>high carbohydrate diet</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>high saturated fat diet</td>
<td>367</td>
<td>33</td>
</tr>
<tr>
<td>High PUFAs</td>
<td>108</td>
<td>167</td>
</tr>
</tbody>
</table>

*The lateral hypothalamus has traditionally been viewed as a feeding centre and aligned with the parasympathetic arm of the autonomic nervous system (ANS). Activation of the lateral hypothalamus is thus associated with positive energy balance.

†The ventromedial hypothalamus is considered a sympathetic nervous system-linked satiety centre, activation of which will induce negative energy balance.

Adapted from Wang et al. 1999.
action. In general, the more saturated the fatty acids in membrane phospholipid, the more deleterious the effect (Grunfeld et al. 1981; Field et al. 1988). Further, there was some evidence that the highly unsaturated n-3 fatty acids were perhaps particularly beneficial (Sohal et al. 1992; Clandinin et al. 1993). These studies were followed by in vivo work combining the hyperinsulinaemic, euglycaemic clamp technique with bolus injection of labelled glucose and deoxyglucose to index insulin action in individual tissues after various feeding regimens (Storlien et al. 1991). Skeletal muscle was targeted as the single most important tissue of insulin-stimulated glucose uptake. These studies showed that feeding rats for short (3–4-week) periods on isocaloric diets, differing only in their fatty acid profile, was sufficient to provoke major insulin resistance in some, but not all, high fat-fed groups. Rats fed diets high in n-3 PUFAAs, and with a low n-6:n-3 PUFA ratio, maintained normal insulin action. Diets high in saturated and monounsaturated fats led to profound insulin resistance in numerous tissues, as did diets with a higher level of PUFAAs where those PUFAAs were of the n-6 class (Storlien et al. 1991).

A variable linked early with whether or not a diet group developed skeletal muscle insulin resistance was the fatty acid composition of membrane structural lipids in skeletal muscle (Storlien et al. 1991). This work has been replicated a number of times in rodent models and in a number of human populations (Storlien et al. 1996). In addition, pharmaceutical insulin sensitizers such as bezafibrate (Matsui et al. 1997) and benfluorex (L. H. Storlien, unpublished results) also unsaturate lipids, which may be an important part of their mode of action. However, the mechanistic basis of altered membrane lipid composition directly influencing insulin action is not entirely established. There are a number of possibilities, including effects on translocation and intrinsic activity of the insulin-regulatable glucose transporter GLUT4 (Zierath et al. 1997; Hansen et al. 1998). These workers have shown substantial impairment of insulin-stimulated glucose transport, along with reduced GLUT4 translocation to the membrane, following high-fat feeding in rats. However, no information is yet available regarding these metabolic elements and dietary fatty acid profiles which do not lead to insulin resistance. Specific diacylglycerol–protein kinase C interactions may be important. Activation of protein kinase C epsilon is associated with skeletal muscle insulin resistance in high-fat-fed rats (Schmitz-Peiffer et al. 1997), and it is possible that interaction with specific fatty acid subtypes in diacylglycerols modulates this activation. Also, there are possible mechanisms via influences on nitric oxide production, eicosanoid balance and ion flux. However, no specific work has yet borne fruit in these directions.

One final aspect of fatty acid profile which must be considered is the potential effects at the level of the pancreas. It is now clear that insulin secretion is powerfully, and differentially, influenced by individual fatty acids (Stein et al. 1997), with saturated fats in particular leading to hypersecretion of insulin. Hyperinsulinaemia per se may lead to insulin resistance.

A second variable identified in early studies as important for insulin action was muscle storage triglyceride (TG) levels, impaired insulin action being closely associated with high muscle TG levels (Storlien et al. 1991). This observation has now been replicated in human studies (Phillips et al. 1996; Pan et al. 1997). Some concern had been expressed about the location of the TG, but in vivo NMR studies have been used to show that the relevant TG was intracellular (Krssak et al. 1999). Using a more direct approach, we have recently measured insulin-stimulated glucose uptake in incubated soleus muscle using the 2-deoxyglucose method, and then measured TG in muscle fibres alone after stripping away all interstitial fat and connective tissue. Again, a highly significant inverse relationship was found between intramyocyte TG and insulin-stimulated glucose metabolism (T. C. Thomas & L. H. Storlien, unpublished results).

The possibility has been suggested that the concentration of long-chain fatty acyl-CoA is a marker for skeletal muscle TG hydrolysis, and accounts for the inhibitory effect of muscle TG levels on insulin action (Oakes et al. 1997). The observation that addition of cholate to a high-fat diet improves insulin is interesting (Ikemoto et al. 1997), in particular given the data showing that, at least in the liver, changes in TG accumulation paralleled the cholate-induced decline in acyl-CoA synthetase mRNA.

This brings into focus our lack of knowledge about what actually controls the accumulation and distribution of TG in skeletal muscle. It is clear that it is not just the total intramyocyte TG that modulates insulin action, but its distribution is also critical. Muscle TG is substantially elevated in trained, compared to sedentary, individuals, but insulin action is improved. Female rats, given access to running wheels, will build up to an average of over 10 km/d. We have exploited this chronic exercise model to investigate, using electron microscopy, the effect of ‘training’ on both amount and distribution of intramyocyte TG. While the work is in its early stages, we can confirm that intramyocyte TG is greatly elevated, with lipid droplets very tightly coupled to mitochondria (P.L. Else et al., unpublished results). What controls this increase in TG and its affinity for mitochondria and, in turn, why it is not deleterious to insulin action are questions of importance in our understanding of skeletal muscle insulin resistance.

Carbohydrate subtypes

Just as there are simple and complex carbohydrates, there are simple and complex arguments about the level and form of carbohydrate intake to optimize insulin action. From experimental animal work simple sugars, compared to complex carbohydrates, were found to have a negative influence on insulin action. These studies were conducted in rats and, as with the dietary fat subtype work described above, consisted of a series of pleasingly simple interventions of 3–4 weeks with caloric intake matched and only the carbohydrate type varied in the diet. Hyperinsulinaemic, euglycaemic clamps were performed with tracer administration to assess insulin action at the level of the liver and in individual skeletal muscles, and effects were found in both. Diets where starch was largely substituted by either sucrose or fructose were deleterious for insulin...
action (Storlien et al. 1988; Thorburn et al. 1989). The hypertriacylglycerolaemic repercussion of elevated fructose intake was seen as a likely intervening variable. Certainly, changing the fat source to emphasize n-3 fatty acids ameliorates both the insulin resistance and hypertriacylglycerolaemia of high sucrose feeding (Lombardo et al. 1996). Interestingly, glucose itself had no deleterious effect on insulin action compared to starch. This suggests that the rate of carbohydrate absorption does not, in the short term, alter insulin action.

What is even less explored, and potentially of much greater importance to the broader picture, are the effects of various forms of complex carbohydrates. However, there is considerable controversy even in relation to an acceptable classification framework (Cummings et al. 1997) when we start impinging on the grey area of resistant starches, soluble fibre and large-bowel digestion (fatty acids again, of the short-chain type).

The glycaemic index is a concept which has been around for many years but still is shrouded in confusion and capable of inciting some acrimony (Coulston & Reaven 1997; Wolever 1997). There is still no evidence for direct beneficial effects of low glycaemic-index diets on insulin action either in animal or human studies. However, there are a number of very logical bases for suggesting that there may well be a beneficial effect of slowly digested carbohydrates in the long run.

Long-term feeding of diets high in carbohydrate, differing only in type of starch, has shown a differential influence on the insulin response necessary to maintain essentially normal glucose tolerance. Rats fed on a diet with amylopectin as the starch are markedly hyperinsulinaemic during an intravenous glucose tolerance test compared to rats fed a high-starch diet emphasizing amylose (Byrnes et al. 1995; Higgins et al. 1996). This relative hyperinsulinaemic response becomes more pronounced the longer the feeding period. Amylose is a straight-chain polysaccharide which, by virtue of that configuration, is slowly digested. The branched-chain configuration of amylopectin allows multiple access points for alpha-glucosidase attack and rapid absorption.

At the molecular level, high-amylose starches have been shown to increase GLUT4, and decrease fatty acid synthase gene expression in rat epididymal tissue (Kabir et al. 1998b). In addition, maximal insulin-stimulated $^{14}$C-glucose oxidation was increased, whereas $^{14}$C-glucose incorporation into lipids was decreased (Kabir et al. 1998a), and glycogen synthesis in muscle was increased (Denyer et al. 1998), in amylose-fed compared to amylopectin-fed rats. Taken together, these results and those noted above are suggestive of potential effects of amylose-enriched starches on insulin secretion and/or on substrate repartitioning.

Finally, there are major developments in our understanding of the physiochemistry of starch and the increasingly blurred boundaries between resistant starches and fibre. There are few real data here in relation to insulin resistance, an important emerging field of research.

### Protein subtypes

Compared to fat and carbohydrate subtypes, proteins have been under-studied. Some years ago we noted that rats fed a synthetic diet were much more insulin-sensitive than those fed on laboratory chow (Storlien & Jenkins, 1996). This was puzzling as the synthetic diet had been prepared so as to closely match the chow macronutrient distribution and fatty acid profile. Since then, exciting new work has demonstrated that changing the protein source in synthetic diets can markedly dictate development, or not, of insulin resistance in medium- to high-fat-fed rats (Iritani et al. 1997). Follow-up work has shown that high-fat diets prepared with cod protein, as compared to soy or casein protein, do not lead to insulin resistance (Lavigne et al. 1999). Further mechanistic investigations showed that the cod protein improves GLUT4 translocation to skeletal muscle T-tubules, but not to the plasma membrane (Tremblay et al. 1999). The T-tubule GLUT4 protein correlates with insulin-stimulated glucose transport, and is most interesting in terms of the possibility that a specific protein might be critical in skeletal muscle insulin-stimulated glucose transport. The effect might be due to a specific protein which has a gene-specific effect at the intestinal level, a protein which escapes full digestion, a molecule which is co-extracted with protein, or indeed a particular amino acid pattern unique to cod. In this regard the observation that $L$-glutamine supplementation of a high-fat diet has beneficial effects on glycaemia and insulinemia in mice may be relevant (Opara et al. 1996). The exploration of these possibilities offers interesting new lines of research.

### Summary

Animal models have proven useful in studies of the influence of dietary variables and insulin action. This manuscript has focused on the macronutrients, and the overriding message is that we must look beyond the broad categories of fats, carbohydrates and proteins. A great deal of work has been done on fatty acid subtypes, and a harmonious pattern linking saturated fat intake, both indirectly and directly, to insulin resistance is evident. In contrast, PUFAs are, if anything, protective. Less is known about carbohydrate subtypes. While clear data have been obtained regarding the insulin resistance-inducing effects of sucrose, and in particular its fructose moiety, little other direct evidence is available on carbohydrate subtype–insulin action interactions. Finally, new work on protein sources and the profound differences in insulin action they induce is providing interesting new avenues to explore. What is now clear, and points in a particularly exciting direction, is that the macronutrients are all potent gene regulators – and subtype mix will undoubtedly be found important in the precise patterning of that gene regulation.

Research on experimental animals is important, for the most part, only in its capacity to inform directions for human work. This has happened in the field of diet and insulin action, and there is potential for new and exciting contributions. Our work has focused on macronutrient subtypes, inter alia from the recognition that persuading ‘free range’ individuals to introduce and sustain changes in the macronutrient proportions of their diet has proved remarkably difficult, at least in our hands. However, changing individual macronutrient subtype profiles is substantially
easier with the resultant increased capacity for a significant impact on the prevention and therapy of insulin resistance.

References


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