Roles of transient receptor potential canonical 4/5 channels in body weight and obesity

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Ion channels are proteins that stretch across the cell membrane allowing the passage of ions from one side of the membrane to the other. Calcium channels form calcium-permeable pores on plasma and intracellular membranes that can be opened by changes in membrane potential and/or chemical ligands. Playing crucial roles in many human diseases, particularly of the cardiac and nervous systems, including pain, seizure, hypertension and migraine.

Mammals store excess fat to keep themselves warm and insure against times of insufficient nutrition (Wang, 2006). The molecular mechanisms which enable this process is investigated in its relation to human obesity and metabolic syndrome.

The research in this project was to look at the effects of TRPC4 and TRPC5 in the setting of obesity. This will be observed in both TRPC KO mice and also with a small molecule inhibitor. Understanding the pathophysiological significance of TRPC4/5 in adipocytes may help in developing them as drug targets to improve adipose tissue phenotype in obesity.

In this series of studies, an in vivo approach was used to explore the effect of TRPC 4 and TRPC 5 knockout on mice fed standard chow or a 60% high fat diet (HFD). Metabolic profiling of genetically modified mice was performed which included glucose and insulin tolerance tests. The results of the this study found that in the HFD setting, TRPC knockout didn't have any significant effect on weight gain and adipose tissue.

Finally, a group of wild type C57BL/6 were injested with LDC204136 (C31) which is a TRPC1 TRPC4 and TRPC5 antagonist and compared to a vehicle only group. Promisingly, the mice injested with C31 displayed a reduction in weight gain and adipose tissue showing that TRPC channels may serve as a target for managing obesity.

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List of Abbreviations

BIN	Bis in die' (twice a day)
Ca	Calcium
Cl	Chlorine
eWAT	Epididymal white adipose tissue
GI	Gastrointestinal
GLM	Generalized linear model
GPCRs	G-protein-coupled receptors
GTT	Glucose Tolerance Test
HFD	High Fat Diet
iBAT	Intrascapular brown adipose tissue
ITT	Insulin Tolerance Test
I-V curve	Current-voltage characteristic curve
K	Potassium
Na	Sodium
NMDA	N-methyl-D-aspartate no mechanoreceptor potential C)
PVAT	Perivascular adipose tissue
SOCE	Store-operated calcium entry
TM	Transmembrane
TRP	Transient receptor potential
TRPA	Transient receptor potential (ankyrin)
TRPC	Transient receptor potential (canonical),
TRPM	Transient receptor potential (melastatin),
TRPML	Transient receptor potential (mucolipin)
TRPN	Transient receptor potential (NOMPC –
TRPP	Transient receptor potential (polycystin).

TRPV	Transient receptor potential (vanilloid),
VGCC	Voltage-gated Ca^{2+} channels
VSMC	Vascular smooth muscle cells
WT	Wild Type

Chapter 1

1.0 Introduction

1.1 Transient receptor potential (TRP) channels

Ion channels are proteins that are embedded in the cellular membranes allowing the passage of charged particles (ions) from one side of the membrane to the other. Calcium channels form calcium-permeable pores on plasma and intracellular membranes that can be opened by changes in mechanical stretch, membrane potential and/or chemical ligands (White 2000). Ion channels and transporters are critical regulators of cellular homeostasis and mediate important cell and organ functions such as inducing depolarization via calcium entry in muscle, neurons and other excitable cells which leads to contraction, signal transduction etc (Bean 2007). Calcium channels are usually multi-subunit proteins that may be encoded by a single or multiple genes. The resulting proteins often govern distinct functional roles within a given cell type and play crucial roles in many human diseases, particularly in the cardiac and nervous systems, including in pain, seizure, hypertension and migraine (Bean 2007).

The transient receptor potential (TRP) proteins' initial discovery was made in1969 by Cosens and Manning when they discovered a mutant strain of Drosophila melanogaster. A spontaneous mutation in the TRP gene led to a common fruit fly that did not respond to prolonged steady light, it only has a 'transient' response in contrast to the sustained response in the wild type flies with intact photoreceptors (Minke, Wu and Park 1975). Twenty years later, the mutant gene was cloned and the TRP non-selective cation channel was discovered and this was characterised as the Drosophila TRP locus (Montell and Rubin, 1989). Shortly after it was verified that the TRP gene product was an ion channel (Hardie and Minke, 1992). It is now known that there are at least 28 genes encoding TRP channels in mammals (Moran et al., 2004) (Fig.1) (Fleig and Penner,2004). These proteins have been divided into subfamilies based on their amino acid

sequence homology: TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPML (mucolipin) and TRPP (polycystin). A seventh subfamily, TRPN (NOMPC - no mechanoreceptor potential C), is absent from mammals (Clapham, 2003). Unlike most other ion channel families, the classification of TRP channels is based on sequence similarity as opposed to common functional features (Zheng, 2013). Due to this, members of the same subfamily may be functionally distinct or members from different subfamilies may share common features (Zheng, 2013).

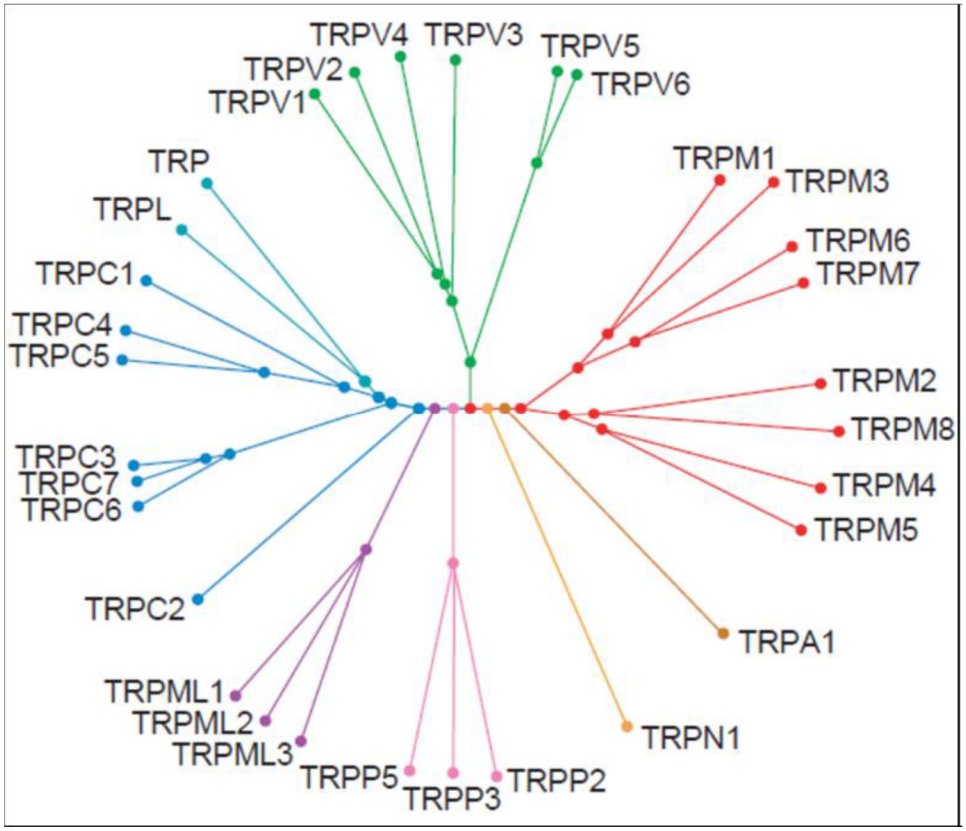


Figure 1 Phylogenetic family tree of mammalian TRP channels**.**

The TRP superfamily of ion channels is classified into three main homologous subfamilies (TRPC, TRPV and TRPM) and more-distantly related groups (TRPML, TRPP, TRPN and TRPA).

Adapted from Fleig and Penner (2004).

TRP proteins are comprised of six putative transmembranes (TM) domains (S1-S6) and have a pore loop region between segments S5 and S6 (Minke, 2010). The transmembrane regions are flanked by an intracellular amino-terminal and an intracellular carboxyl-terminal domain (Fig.2). They form a tetrameric quaternary structure, homo and hetero are possible (Fig.3) and each subunit contributes to a shared selectivity filter and ion-conducting pore, similar to that seen in potassium channels (Ramsey et al., 2006).

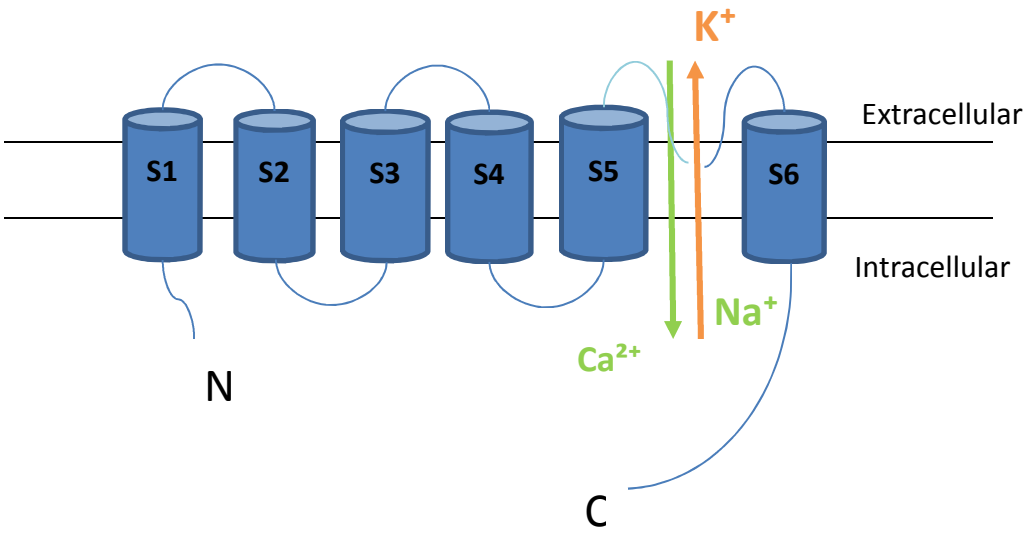


Figure 2 TRP monomer structure. Schematic of a TRP channel monomer, which is made up of 6 membrane-spanning domains, S1-S6. They have a pore region loop between transmembrane segments S5 and S6, Ca^{2+} and Na^{+}, can flow through. The transmembrane regions are flanked by an intracellular amino-terminal domain and an intracellular carboxyl-terminal domain.

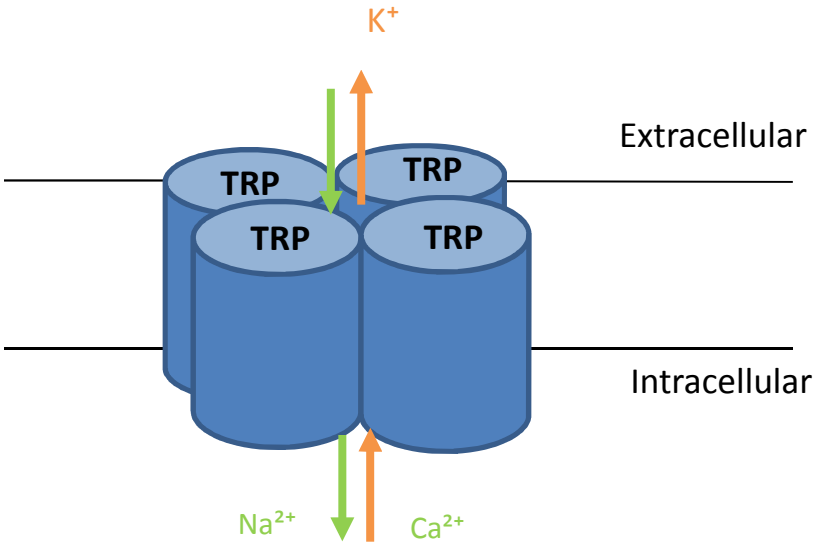


Figure 3 TRP channel structure. The four cylinders represent four TRP monomers, which form a tetrameric quaternary structure, with each subunit contributing to an ion-conducting pore enabling the influx of Ca^{2+} and Na^{+} ions, along with K^{+} efflux.

1.2 Function of TRP channels

The TRP superfamily consists of a diverse group of non-selective cation channels. The superfamily has a wide tissue distribution and is involved in many physiological processes including secretion of hormones, cell cycle modulation, and sensory perception. TRP channels are present in many cells and tissues including endothelial cells and vascular smooth muscle cells (VSMC). These channels have been implicated in the regulation of vascular wall permeability and angiogenesis (Moraes 2021). Furthermore, TRP channels are associated with cardiometabolic diseases, such as obesity and diabetes (Moraes 2021).

Great diversity in activation and inhibition is demonstrated in families of TRP ion channels. TRP ion channels function as signal integrators through their ion conductance properties, and in some cases kinase activity (Song, 2010). TRP channels have regulatory roles in all five senses - vision, taste, smell, hearing, and touch (Venkatachalam and Montell, 2007). The influx of cations into the cytoplasm is necessary for action potentials in excitable cells such as neurons. The existence of disease-causing mutations is evidence that TRP channels are important for human health (Winn et al, 2005). Genetic defects in TRP channels have been acknowledged as the direct underlying cause of hereditary diseases: TRPC6 - focal segmental glomerulosclerosis (Winn et al., 2005), TRPM6-hypomagnesemia with secondary hypocalcaemia (Walder et al., 2002). Although TRPC6 has been a major focus for drug discovery, more recent studies suggest that other TRPC family members play a role in the pathogenesis of glomerular disease processes and chronic kidney disease, and TRPML1 in mucolipidosis type IV (Nilius et al., 2007). Moreover, genetic variants of TRPM7 channel function and expression lead to various neuronal diseases such as Alzheimer's disease and Parkinson's disease (Sun, 2015). It is probable that, in the future, more diseases will be shown to be due to mutations or alterations in the activities of TRP channels. TRP channels are primarily expressed on the plasma membrane and hence are accessible to ion channel targeting drugs making them attractive candidate drug targets. (Stokłosa, et, al., 2020)

1.3 The canonical subfamily of TRP channels (TRPC)

The canonical subfamily of TRP channels (TRPC) form homo- or hetero-tetrameric, nonselective cation/calcium-permeable channels on the plasma and subcellular membranes of mammalian cells (Ramsey et al., 2006). There are 7 members in the TRPC family (TRPC1-7). The TRPC subfamily is divided into three groups based on sequence alignments and functional comparisons: TRPC1/4/5, TRPC3/6/7, and TRPC2 (Ramsey et al., 2006). Humans express only six of these; TRPC2 is a pseudogene in humans but is functional in other mammals (Zheng, 2013). One subgroup consists of TRPC3, TRPC6 and TRPC7 and the other of TRPC1, TRPC4 and TRPC5 and they contribute to a broad spectrum of cellular functions and physiological roles (Wang, et al. 2020). TRPC channels are expressed in most endothelial cells and VSMC, which is indicative of their role in vascular physiology and pathophysiology. Since TRPC channels can be stimulated by lipid and redox factors, which are elevated in obesity, it is reasonable to assume that TRPC channels are relevant to cardiovascular disease states aggravated by obesity (Beech, 2013). Studies indicate that there is augmented TRPC function in response to hypertension, hyperglycaemia and type-2 diabetes, which are all causative factors of coronary artery disease (Beech, 2013). TRPCs are activated by chemical and physical stimuli through the phospholipase C (PLC) signalling pathway. The subgroup TRPC1/4/5 can be found in either homotetramers or heterotetramers channels. These are described to be activated by Gq protein-coupled PLC and phosphatidylinositol 4, 5-bisphosphate (PIP^2) hydrolysis (Gao et al. 2021). This process generates inositol triphosphate (IP^3) that binds to the endoplasmic reticulum IP^3 receptor increasing extracellular Ca^{2+} entry called store-operated calcium entry (SOCE). However, the subgroup TRPC3/6/7 channels are unique in being directly activated by diacylglycerol (DAG), a degradation product of PIP^2, and the resultant calcium entry is known as receptor-operated calcium entry (ROCE) (Minke and Cook 2002; Jeon et al. 2012; Zheng 2013).

1.4 TRPC 1/4/5 channels

A few studies have suggested that the gating of TRPC channels containing TRPC1/4/5 is “polymodal” as they can be activated by many factors such as the stimulation of G-protein coupled receptors (GPCRs), as well as a change in intracellular Ca^{2+} concentration (Zheng and Phelan, 2014). Earlier studies of TRPC channels have gone through some struggles due to a lack of channel-specific agonists or antagonists (Kim et al. 2020). Developments of channel-specific pharmacological tools such as ML-204 (M.Miller et al. 2011), (-)-Englerin-A (or simply, Englerin-A) (Akbulut et al.2015) and Pico-145 (Rubaiy et al. 2017), has brought greater understanding of these TRPC channels. Advances in research have contributed greatly to the pharmacological knowledge of the TRPC channels and the mixture of channels in the plasma membrane is becoming clearer than ever.

The first member of the TRPC family to be identified and characterised was TRPC1 (Wes et al., 1995). TRPC1 lacks charged residues in the S4 transmembrane region that are required for voltage sensing in many voltage-gated ion channels (Wes et al., 1995). Thus TRPC1 is unlikely to be a voltage-sensitive channel (Pakhomov et al., 2009). TRPC1 is expressed in the adult heart, brain, testes and ovaries (Wes et al., 1995). In general, it is agreed that when TRPC1 is expressed in heteromeric complexes with TRPC3/4 or 5, it functions as part of a Gq/11 receptor-operated cation channel (Ramsey et al., 2006).

TRPC4 is widely expressed in the endothelium, smooth muscle, and kidneys and is particularly abundant in the brain (Pedersen et al., 2005; Mori et al., 1998). Freichel et al. (2001) showed that endothelial cells of mice deficient in TRPC4 have decreased agonist-induced Ca^{2+} entry and vasorelaxation. This indicates that TRPC4 channels directly provide a Ca^{2+} entry pathway that contributes to the regulation of blood vessel tone. As previous work showed that TRPC1/5 channels are constitutively active in adipocytes (Sukumar et al 2012), the use of small-

molecule inhibitors of TRPC4 and TRPC5 channels may improve adipocyte health in obesity.

TRPC5 is predominantly found in the central nervous system and has a high expression density in the limbic system (Strübing et al., 2001). It has also been reported that TRPC5 channels present in adipocytes can sense fatty acids (Sukumar et al., 2012). The group found that TRPC1 and TRPC5, together in a heteromultimeric channel in adipocytes, negatively regulate adiponectin (Sukumar et al., 2012).

Loss of TRPC1 may alter the regulation of cellular energy metabolism resulting in insulin resistance thereby leading to diabetes (Krout et al., 2017). Particularly native functions of TRPC4 include its role in the endothelium-dependent vasorelaxation and control of endothelial permeability (Pedersen et al., 2005; Mori et al., 1998).

TRPC4 and TRPC5, which contribute to the subgroup containing TRPC1, are highly homologous, TRPC4 and TRPC5 function as non-selective cation channels that are activated by the Gq/11 family GPCRs and receptor tyrosine kinases, independent of store depletion (Schaefer et al., 2000). Activation of these channels appears to require PLC activity, but neither Inositol-1, 4, 5-trisphosphate (IP^3) nor diacylglycerol (DAG) alone can stimulate TRPC4 or TRPC5. Suggesting that an unidentified PLC-dependent mechanism or a combination of messengers link membrane receptors to TRPC4 and TRPC5 activation (Clapham et al., 2001).

1.5 TRPC 3/6/7 channels

TRPC3, TRPC6 and TRPC7 constitute the second major subgroup of the TRPC subfamily. The three members of this subgroup can form either homotetrameric channels or heterotetrameric channels by complexing among themselves in both native and heterologous expression systems (Trebak et al, 2003). TRPC3 and TRPC7 are closely related and to a lesser degree to TRPC6. However, there is also evidence that TRPC3 forms a complex with TRPC1 (Liu et al 2005) or TRPC4 (Poteser et al, 2006). Therefore, the ability of TRPC3 to heteromerise with other TRPC members may be highly variable in different systems and under different conditions. TRPC3 is expressed in many tissues, but it is in the brain where the levels are high (Zhu et al 1996). In the brain, TRPC3 is most prominently expressed in the pituitary gland (Hartmann et al, 2008).

TRPC6 exhibits the highest expression in the lungs and brain (Montell, 2005) there are also significant levels of TRPC6 in the placenta, heart, pancreas and kidneys (Riccio et al, 2002). Furthermore, TRPC6 is expressed widley in the nervous system, TRPC6 exhibits expression in extrinsic fibres innervating intrinsic cardiac ganglia (Calupca et al, 2002), epithelium neurons (Elsaesser et al, 2005), retinal ganglion cells (Warren et al, 2006).

The greatest levels of TRPC7 are found in the heart, lung and eye and it is also detectable at lower levels in the brain, spleen, and testis (Okada et al., 1999). In 2001 Clapham et al, found receptor-operated non-selective cation channels that show rectification in both the inward and outward directions and can be functionally distinguished from the TRPC1/4/5 subgroup by the polarity of the effect of the secondary messenger DAG (Clapham et al.,2001). TRPC3 heterologously-expressed channels produce cationic currents with little selectivity for Ca^{2+} over Na^{+} (Zitt et al., 1997). These currents are constitutively active and are not enhanced by depletion of Ca^{2+} stores with IP3 (Rowell et al., 2010), TRPC7 which, like TRPC3, shows a level of constitutive activity (Trebak et al., 2003), can be activated by PLC-stimulating agonists (Lievremont et al., 2004).

1.6 Heteromerisation of TRPC channels

TRPC proteins can form heterotetramers, increasing the opportunity for more ion channels with differing properties and appearing to prefer particular associations between subunits (Beech, 2013). TRPC can form units with proteins from other subfamilies. TRPC1 is peculiar in that it forms channels poorly or not at all when expressed alone in vitro in heterologous systems (Xu et al., 2008). Some studies have shown signals when this subunit is expressed alone but these have been relatively small and may be explained by the protein forming heteromers with endogenous TRPs (Bon and Beech, 2013). Widespread expression of TRPC1 is shown and can form units with other TRPC subunits, including TRPC3/4/5/6 and 7, in vitro and/or in vivo to form functional heteromeric channels (Clapham et al., 2001; Strübing et al., 2001; Storch et al., 2012). Exchanging select amino acids in the putative pore-forming region of TRPC1 reduces Ca^{2+} permeability within these complexes, suggesting that TRPC1 does contribute to the channel pore (Storch et al., 2012). Subunits differ in the composition of heteromeric TRPC1, TRPC4, and TRPC5 channels resulting in channels with unique biophysical properties (Strübing et al., 2001). In comparison with homomeric TRPC4 or TRPC5 channels, heteromeric TRPC1/4 and TRPC1/5 channels display a simpler I-V curve, with a gently negative slope at negative potentials and smooth outward rectification (Ramsey et al., 2006). The single-channel conductance of TRPC1/5 channels was found to be significantly different than that of TRPC5 homomers (Ramsey et al., 2006). Strübing et al. (2001) revealed that TRPC1/TRPC5 heteromers displayed an 8-fold smaller conductance than TRPC5 homomers, implying that the heteromeric channel has altered permeation properties.

1.7 TRPC channels in disease

1.8 TRPC Channels in the Central nervous system

Studies have shown the complex roles of TRPC channels in neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, stroke, epilepsy, depression, and bipolar disorder thus impacting both medical and social problems due to the surge in their prevalence (Jeon et al, 2021). TRPC channels were originally identified as store-operated Ca^{2+} channels, it is now known that they can be receptor-operated (Zheng and Phelan, 2014).

Members of the transient receptor potential canonical (TRPC) channel family have been identified as a new class of Ca^{2+} channels, it could be anticipated that these channels could play important roles in neurodegenerative diseases, especially in Parkinson's disease (Sukumaran et al, 2017).

Riccio et al. reported that mice lacking TRPC4 displays decreased anxiety-like behavior and Gq/11-dependent responses (Riccio et al,2014). Also TRPC1/4/5 blocker, HC-070, alleviates anxiety and depression in mice (Just et al, 2018).

1.9 TRPC channels in cancer

In several forms of human cancers, high expression of TRPC proteins has been correlated with poor disease prognosis or poor therapeutic outcome (Santoni, et al.,2011). Ca^{2+} is a pivotal intracellular second messenger and plays a crucial role in living cells by regulating several processes from cell division to death. The Ca^{2+} homeostasis is related to many human diseases and pathological conditions.TRPC channels have been implicated in numerous cancers, mainly due to their roles in migration and proliferation (Shapovalov, et al., 2016). In 2009 Aydar et al targeted TRP channels in breast cancer, reportedly a specific activator of TRPC6 significantly reduced the growth and viability of the breast cancer cell lines but did not affect the non-cancerous breast cell line. TRPC6, along with TRPC3, was found to be significantly upregulated in various metastatic breast cancers, compared with normal breast tissue (Aydar et al., 2009). The TRPC1 ion channel is a key component of responses to hypoxia in breast cancer cells. TRPC protein expression correlated with a higher grade of the tumour (Zeng, Bo et al, 2013). P-glycoprotein has been identified as a transporter responsible for the efflux of cytotoxic drugs (Juliano and Ling, 1976). Therefore, a strategy to overcome multidrug resistance in cancer chemotherapy is to suppress P-glycoprotein. A study by Ma et al in 2012 shows that inhibition of TRPC5 reduced P-glycoprotein expression in an adriamycin-resistant breast cancer cell line, and as a result, reversed the adriamycin resistance. A subsequent paper published by the same group showed that TRPC5, is highly expressed in breast cancer after long-term chemotherapy and activates the transcription of vascular endothelial growth factor (VEGF) (Zhu et al., 2015). VEGF promote tumour angiogenesis and led to a poor chemotherapeutic outcome (Zhu et al., 2015). However, in 2020 Dong et al identified potential exosome-associated biomarkers in patients undergoing chemotherapy, concluding that increased circulating exosomes carrying TRPC5 after chemotherapy preceded cancer progression and predicted acquired chemotherapy resistance. Therefore, the detection of TRPC5-positive exosomes can be used to monitor chemotherapy resistance in real-time (Dong et al, 2020).

In colon cancer patients, increased expression of TPPC5 is correlated with tumour metastasis (Chen et al., 2017). In vitro overexpression of TRPC5, in patient-derived cancer cells, prompts an increase in intracellular Ca^{2+} and promoted a more metastatic phenotype (Chen et al., 2017). An increased expression of TRPC channels has been detected in other tumours and the case of non-small cell lung cancers, the expression of TRPC1, TRPC3, TRPC4 and TRPC6 correlate with the grade of the tumour (Jiang et al., 2013). The expression and function of plasma membrane Ca^{2+} permeable channels and sodium/calcium exchangers are frequently described in tumorigenesis and tumour development of the upper GI tract, including voltage-gated Ca^{2+} channels (VGCC), transient receptor potential (TRP) channels, store-operated channels (SOC) and Na^{+}/Ca^{2+} exchanger (NCX) (Rodrigues et al., 2019).

1.10 TRPC channels in cardiovascular disease

Ca^{2+} plays a crucial role in maintaining physiological functions in the cardiovascular system (Wu et al 2019). TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 are expressed in VSMC (Wang et al., 2004; Evans et al., 2009; Inoue et al., 2009; Mita et al., 2010) and endothelial cells (Gao et al.,2012; Sundivakkam et al., 2012) and in cardiac myocytes, (Flockerzi, V and B Nilius, 2014), indicating the importance of TRPC channels in vascular physiology and pathophysiology. Signalling pathways involving TRPC channels regulate vascular tone, for example, activation of TRPC1 and TRPC3 channels in the VSMC can cause depolarization and vasoconstriction (Reading et al., 2005; Wölfle et al., 2010). Furthermore, TRPC1, TRPC3, and TRPC4 stimulation in endothelial cells can induce vasodilation through increased Ca^{2+} (Freichel et al., 2001; Huang et al., 2011; Qu et al., 2017) and/or TRPC3 activation can induce endothelium-dependent hyperpolarization factor (EDHF)-mediated vasodilation (Kochukov et al., 2014). TRPC channels are known to be stimulated by particular lipid and redox factors, which are elevated in obesity (Beech, 2013).

1.11 My proposal

Mammals store excess fat to keep warm and insure themselves against times of dietary insufficiency (Wang et al, 2006). The molecular mechanisms which enable this process continue to be intensely investigated because of their relevance to human obesity and metabolic syndrome. Pilot data shows that genetically engineered TRPC4 and TRPC5 mice lack these ion channel-forming proteins, appear healthy and had normal body weights and adipose tissue masses when fed standard chow diet.

This project will look at the effects of high fat diet feeding on TRPC transgenic mice and also the effects of a small molecule inhibitor TRPC blocker. My hypothesis is that the TRPC KO animals will have a reduced body and adipose weight post fat feeding.

Chapter 2

2.0 Materials and Methods

2.1 Animals Husbandry

All animal use is authorized by the University of Leeds Animal Ethics Committee and The Home Office, UK, according to Home Office Project License to David Beech (P606320FB, issued 16 September 2016, expired 16 September 2021, “Calcium-permeable channels and their associated mechanisms and therapeutic potential”). Personal License Melanie Reay I6F5AF7AB and Simon Futers 154F67616 to carry out procedures.

2.2 Mice

TRPC 4 gene-disrupted (knockout) mice on the C57BL/6J background (B6.129P2-Trpc4tm1Dgen/H) were generated by Deltagen Inc. and supplied by the Medical Research Council Harwell, UK. The sequence spanning base 1272 to base 1330 of the *Trpc4* gene was deleted and inserted with a Lac-Z neo-cassette to create a detectable mutation in the mice.

TRPC 5 gene disrupted (knockout) C57BL/6 mice were generated as part of the International Mouse Phenotyping Consortium (IMPC) based on *Trpc5* gene-targeted ES cells originally created by the Knockout Mouse Project (KOMP) (Trpc5tm1b(KOMP)Wtsi) and provided by Riken BRC, Japan.

TRPC 4/5 (double knockout) mice were created by inter crossing mice from TRPC4 and TRPC5 knockout lines.

TRPC Wild type (WT) mice are C57BL/6J background mice taken from littermates of TRPC5/ TRPC4 crosses.

C57BL/6J 8 week old mice were purchased from Charles River. They arrived in 2 travel boxes, 5 mice in each compartment (n=20) these mice will be randomized according to their body weight on arrival into 2 homogenous groups (n=10/group).

2.3 Acclimatisation

Mice delivered from Charles River had at least 5 days of acclimatisation on arrival.

2.4 Genotyping

All mice used have been validated by genotyping.

Ear samples were taken and inserted into a 96 well plate. Genotyping was performed by Transnetyx, Inc.8110 Cordova Rd. Suite 119 Cordova, TN 38016 using real-time PCR.

2.5 Environment

Mice were weaned at 3 weeks of age onto *ad libitum* chow diet and water. Between 4 and 5 same-sex littermate mice were housed in the same cage. Animals were maintained in Tecniplast Green line GM500 individually ventilated cages with Aspen bedding (chip size (mm) 2x2x1) and environmental enrichment of domes and chew blocks provided by Datesand Ltd, Manchester, UK, cages were cleaned every other week. Mice were housed in normal 12 hours light cycle (at 06:30 pm lights off), temperature 21 ± 2 °C and 55 ± 10 % relative humidity. Only male mice were used in this study.

2.6 Food and water

During the acclimation phase standard chow diet and water was provided ad libitum. Chow diet was purchased from SDS product code 801722

Commencing the study the mice were then fed a 60% high fat diet with water ad libitum until the end of the experiment. 60% high fat diet was provided by Datesand product code F3282.

2.7 Treatment

Mice were treated by oral gavage BID (twice daily) (at ~08:30 morning and ~4:30pm afternoon) with vehicle or test item 30 $mg.kg^{-1}$ for 6 weeks. Dosing regime was calculated by the self life of the C31 drug and rate metabolised. Oral gavarge was demed the best route due to the suspention of the drug and digested via the gut.

2.8 Glucose tolerance test (GTT)

Mice were fasted overnight for 16 hours, weighed and injected intraperitoneal with glucose solution from Sigma- Aldrich (1g glucose/kg body weight dissolved in PBS). Blood glucose measurements were taken from the tail vein using a hand held SD Codefree Glucometer and disposable strips at, 0, 30, 60, 90 and 120 minutes after glucose injection.

Or

Mice were fasted overnight for 16 hour following with an intraperitoneal glucose injection (1g glucose/kg body weight dissolved in PBS). Blood glucose measured from the tail vein at, 0, 15, 30, 60, 90 and 120 minutes after glucose injection. A blood sample (15µL/EDTA) was also collected at time 15 minutes after glucose injection to measure plasma insulin.

2.9 Insulin Tolerance Test (ITT)

Mice were fasted for 2 hr prior to administration of insulin intraperitoneal injection from the NHS hospital pharmacy (concentrate of insulin in PBS 0.1 IU/ml 0.75 unit/kg of insulin solution). Blood glucose measured from the tail vein at, 0, 30, 60, 90 and 120 minutes after insulin injection.

Or

Mice were fasted for 2 hours followed by an intraperitoneal injection of insulin (1U/kg). Blood glucose was measured from the tail vein at 0, 15, 30, 60, 90, and 120 min after insulin injection.

2.10 Blood sampling

Blood collection (100µL/EDTA) for measurement of fasting blood glucose and plasma insulin.

2.12 Comprehensive Lab Animal Monitoring System (CLAMS)

Mice were given 48 hour acclimatisation before a 24 hour window was measured. Oxygen consumption (VO2), carbon dioxide production (VCO2) which can be used to calculate the respiratory exchange ratio - an indicator of substrate utilisation. Total energy expenditure, body temperature locomotor activity exercise tolerance and caloric intake can also be measured and used to provide extensive metabolic phenotyping of the knock out mice.

2.13 Plasma lipid level testing

Total cholesterol and triglycerides (TGs) and HDL cholesterol concentrations were measured with commercial kits (CHOD-PAP for cholesterol #87656, GPO-PAP for TGs #87319 and Cholesterol HDL-PTA for HDL cholesterol #86516; BIOLABO SA, Maizy, France). Non-esterified fatty acid (NEFA) concentrations were measured using a kit from WAKO chemicals.

Samples were measured in a Biotek plate reader at wavelength of 485nm for cholesterol and triglycerides and wavelength of 540nm for non-esterified fatty acid.

2.14 Statistical analysis

Origin 2015 software (OriginLab Corporation, Northampton, MA, USA) was used for statistical analysis and graphical presentation. All data were tested for normal distribution prior to appropriate use of parametric statistical tests. Data are shown in bar charts expressed as means ± standard error of the mean (SEM). To test for the effect of genotype on bodyweight, a one-way ANOVA was performed, followed by a post-hoc Tukey test. P values less than 0.05 were considered statistically significant. All significant differences are compared to wild type.

CLAMs data were analysed using CalR online software from Harvard.The analysis of covariance (ANCOVA) where appropriate, and the generalized linear model (GLM) where ANCOVA is not appropriate.

2.15 Treatment protocol

Mice were treated by oral gavage BID (at ~08:30 morning and ~4:30pm afternoon) with vehicle or test item 30 $mg.kg^{-1}$ for 6 weeks.

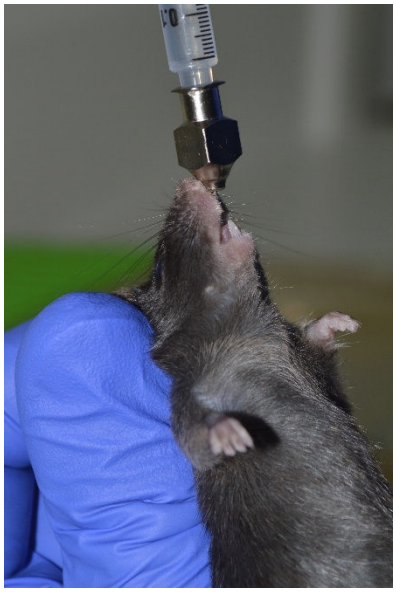


Figure 4 Oral gavage

Group N°	Nbr/group	Treatment	Route	Dose	Dose volume	Treatment duration
1	10	Vehicle BID	oral	/	10mL/kg	6 weeks
2	10	Test item BID	oral	30 $mg.kg^{-1}$ BID	10 $mL.kg^{-1}$	6 weeks

Table 1: Two treatment groups each holding 10 mice. One group to be given treatment drug and the other group to be given vehicle.

2.16 Test treatment

Name and dose administered (e.g. mg/kg)	LDC204136 at 30 $mg.kg^{-1}$ BID
Concentration in the formulation (e.g. mg/mL)	3 $mg.mL^{-1}$; 10 $mL.kg^{-1}$
Vehicle	0.5% Methylcellulose (w/v)
Required quantity	1200mg sent by Lead Discovery Center GmbH Germany
Physical aspect (powder, liquid, color etc.)	White to off-white powder
Conditions of storage	Powder can be stored at room temperature
Protocol of preparation	•addition of the required volume of 0.5% methyl cellulose (MC) solution to the pre-weighed amount of powder to get a final concentration of 3 $mg.mL^{-1}$ (dose 30 $mg.kg^{-1}$, dose volume 10 $ml.kg^{-1}$) •Stirring of the mixture in a water bath at 37°C and vortex mixing followed by 10-20 minutes of sonication at 37°C This should result in a milky suspension. In case the suspension still contains visible particles, the following manipulations will be performed to obtain a homogeneous suspension: •repeatedly push and pull the suspension through the gavage tube to minimize particle size •final sonication of the suspension for another 10 minutes at 37°C The formulation should be stirred until administration to avoid precipitation. Make sure that the compound precipitated during storage is thoroughly re-suspended so that a homogenous formulations is applied to the mice.
Frequency of preparation	Weekly
Conditions of formulation storage	4°C
Formulation shelf life	7 days

Table 2: Summary and preparation protocol of treatment drug.

2.17 Vehicle

Name	0.5% methyl cellulose
Code	M7140
Supplier	Sigma Aldrich,
Concentration	0.5% (w/v)
Conditions of storage	Room temperature
Protocol of preparation	Dissolve methyl cellulose at 0.5% in distilled water
Conditions of formulation storage	4°C
Frequency of preparation	Weekly
Formulation shelf life	7 days

Table 3: Summary and preparation protocol of vehicle

Chapter 3

Aims and Objectives

The overall aim of this project is to determine the metabolic roles of TRPC4 and TRPC5 channels *in vivo* at 8 weeks of age.

Objectives:

Establish baseline conditions in 8 week old transgenic mice of TRPC4 and TRPC5 genotype.

Investigate the metabolic characteristics in knockout animals before the studies commence.

3.1 Introduction

The primary investigation was to establish the metabolic characteristics of the global knockout mice of either TRPC4, TRPC5 or both ion channels up to the age 8 weeks, this is the age considered as an adult and the point the study commences. Knockout, double knockout and wild type mice appeared to have no observable differences either in reproduction and behaviour. Mice were fed a standard chow diet from weaning till 8 weeks of age. This would show an implication of TRPC channels for the control of body weight and establish baseline conditions for all TRPC transgenic mice. Also at the age of 8 weeks mice underwent basic metabolic phenotyping, this included both insulin and glucose tolerance testing to determine if TRPC4 or TRPC5 has a critical role in glucose and insulin sensitivity and metabolism.

3.2 Results

3.3 Baseline weights at 8 weeks of age

3 of the TRPC transgenic mice were harvested at 8 weeks of age to understand the physiological differences between all four genotypes. The body weights (Fig.5a) show that TRPC4 KO and TRPC4/5 double KO mice have reduced body weight and are significant in comparison to TRPC WT. The liver weight (Fig.5b) of TRPC mice follow the same trend as body weight with TRPC4 KO and TRPC4/5 double KO showing significant reduction. Furthermore, the weight of the epididymal white adipose tissue (eWAT) (Fig.5c) are similar in weight, as are the weight of the interscapular brown adipose tissue (iBAT) (Fig.5d) in all TRPC mice.

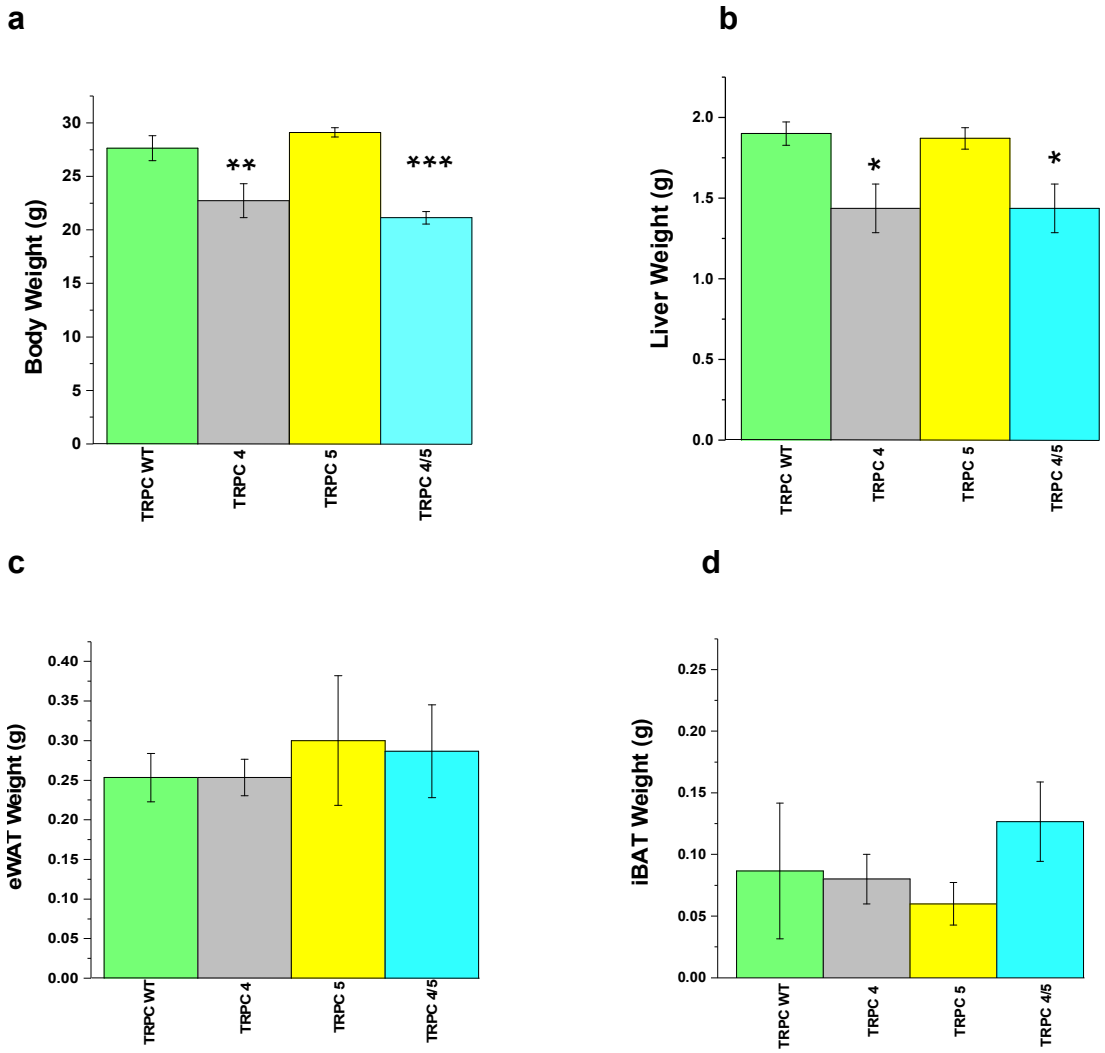


Figure 5 Body and organ weights of TRPC transgenic mice at 8 weeks of age (a) Body weight (b) Liver weight (c) eWAT weight (d) iBAT weight (n=3 for all mice). Statistical significances is indicated by * (P<0.05) ** (<P0.01) *** (<P0.001). Data shown as means ± standard error of the mean (SEM).

3.4 Glucose tolerance at 8 week of age

Fasting blood glucose level (Fig. 6a) shows no significant difference in the glucose level between genotypes after a 16 hour fast. After receiving a single intraperitoneal glucose injection, blood glucose was measured every 30 minutes. As expected, blood glucose rose after the first 30 minutes in all mice. TRPC4 KO and TRPC4/5 double KO transgenic lines glucose level was significantly lower over the 60, 90 and 120 time points compared to the TRPC WT animals (Fig.6b).

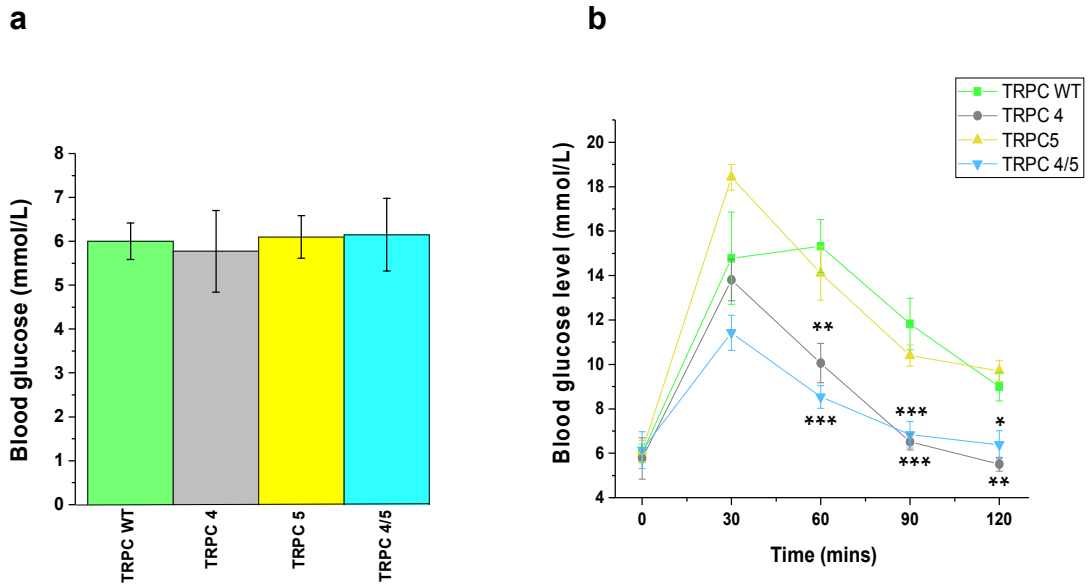


Figure 6 Glucose Tolerance Test. (a) Fasting blood glucose level in chow fed mice at 8 weeks of age. (b) GTT - blood glucose level at set time points after the administration of glucose injection (n =TRPC WT =10, TRPC4 KO =8, TRPC5 KO =7, TRPC 4/5 double KO =9). Statistical significances is indicated by * (P<0.05), ** (<P0.01), *** (<P0.001). Data shown as means ± standard error of the mean (SEM).

3.5 Insulin tolerance test at 8 weeks of age

Glucose levels after a 2 hour fast (Fig.7a) show that there are no significant difference between the groups. Strikingly after an intraperitoneal injection of insulin the blood glucose level of the TRPC5 KO mice showed significantly high sensitivity to insulin (Fig.7b) with a number of mice having to be removed from the study as the blood glucose level fell to dangerously low levels, less than 3mmol/L. TRPC5 KO mice withdrawn from the experiments were given a glucose injection to rise blood glucose levels and kept in a warming cabinet to recover.

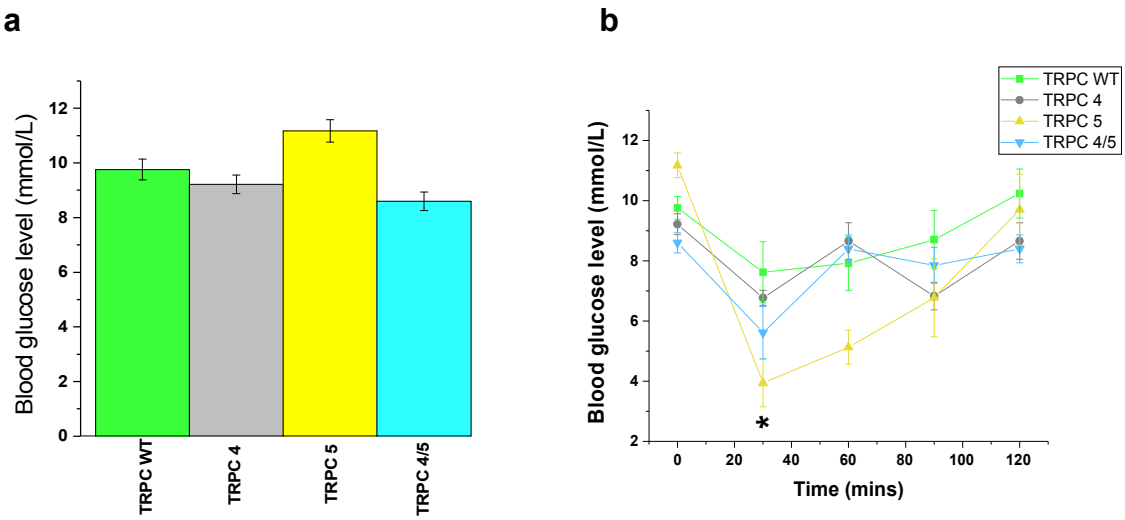


Figure 7 Insulin tolerance test (a) Fasting blood glucose level at 8 weeks of age. All mice were fasted for 2 hours. Blood glucose level was taken before a single insulin injection was administered. (b) Blood glucose level at set time points after the administration of insulin (n =TRPC WT =10, TRPC4 KO =8, TRPC5 KO =7, TRPC 4/5 double KO =9). Statistical significances is indicated by * ($P<0.05$). Data shown as means ± standard error of the mean (SEM).

3.4 Discussion

To understand the baseline metabolic conditions I firstly need to know the physiological difference between the TRPCs genotypes *in vivo*. In this chapter I show the difference between to genotypes at 8 weeks of age.

The study shows body weight (Fig.5a) of both the TRPC4 KO and the TRPC4/5 double KO mice were significantly lower than that of TRPC WT as were the weight of the liver (Fig.5b). However there was no difference for the eWAT (Fig.5c) or iBAT weights (Fig.5d).

After fasting for both ITT and GTT to establish a baseline, mice displayed no significant difference in fasted basal plasma glucose levels at 8 weeks of age. However TRPC4 KO and TRPC4/5 double KO show a significant reduction in blood sugar when compared to the TRPC WT control animals (Fig.6b), and appeared to have better glucose sensitivity and thus induction of insulin secretion. Furthermore TRPC5 KO mice showed to be significantly higher sensitivity to insulin (Fig.7b) after a single insulin injection.

Chapter 4

Aims and Objectives

The overall aim of this project is to determine the metabolic roles of TRPC4 and TRPC5 channels *in vivo.*

Objectives:

Investigate the metabolic characteristics of TRPC4 and TRPC5 knockout animals .

Investigate the physiological functions of TRPC4 and TRPC5 channels in adipose tissue and their pathophysiological importance.

4.1 Introduction

In this chapter I will look at the TRPC animals fed chow diet. Mice were fed a standard chow diet from weaning to the end of the study at 16 weeks of age. Weekly weighing commenced at 8 weeks and continued until the end of study to assess the weight gain. This would show an implication of TRPC channels for the control of body weight. In the last week of study the TRPC transgenic mice also underwent extensive metabolic profiling in the Comprehensive Lab Animal Monitoring System (CLAMs). Single housed mice were placed in the CLAMs caging and after a period of acclimatisation, a 24 hour reading was taken to establish the metabolic differences between genotypes. Energy expenditure and food consumption was also recorded. To conclude the study, body and organ weights were collated, weighed and plasma lipid measurement were done.

4.2 Metabolic phenotyping at 16 weeks of age.

4.3 Marked reduction in weight gain

Mice were weighed at the same time point every week and the data shows (Fig.8a) that there is a significant reduction of body weight of the TRPC4/5 double KO compared to the TRPC WT throughout the 8 weeks. The endpoint weight at 16 weeks (Fig.8b) shows a slight increase in weight of the TRPC5 KO however this was not significant.

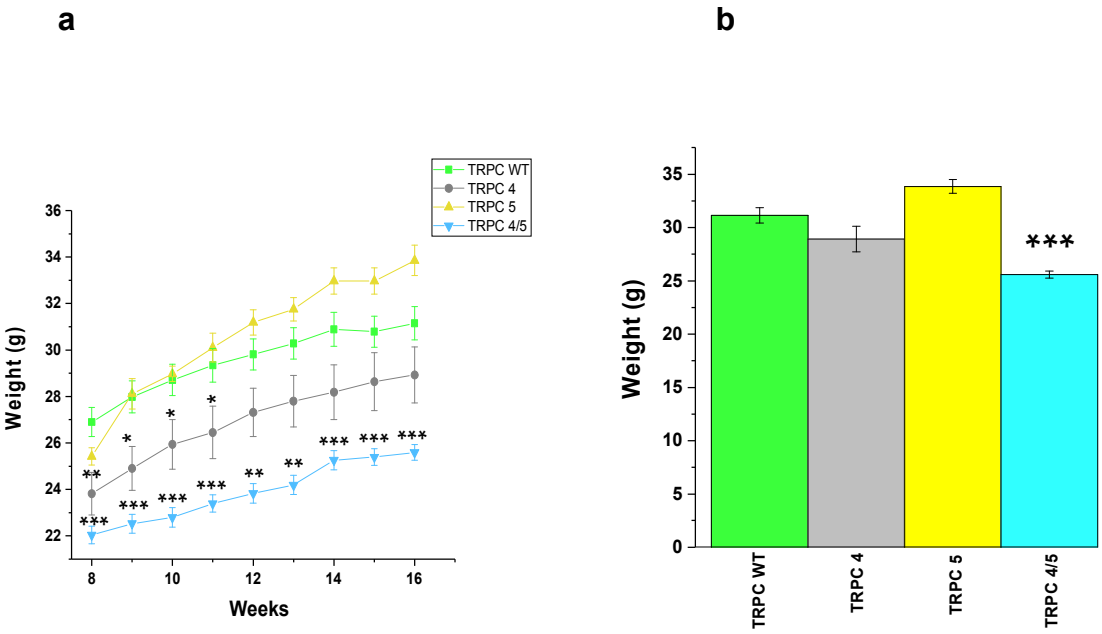
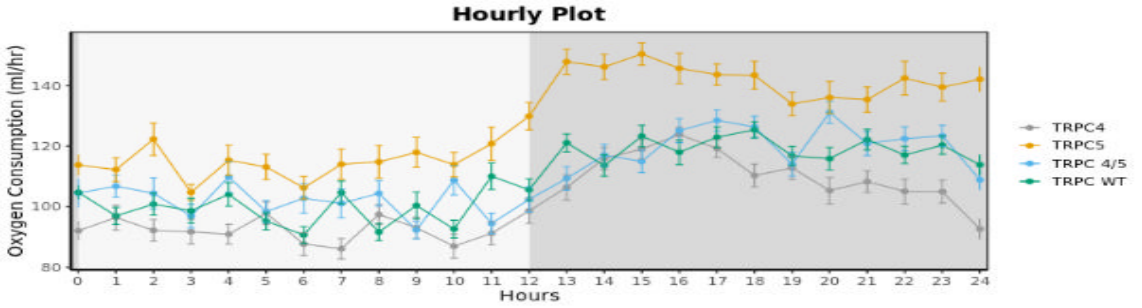


Figure 8 Weekly body weights and average end weights of TRPC genotypes at 16 weeks of age after been fed on chow diet (a) Weekly body weight taken at the same point every week (b) Body weights of TRPC genotypes at 16 weeks of age after been fed on chow diet (n =TRPC WT =10, TRPC4 KO =8, TRPC5 KO =7, TRPC 4/5 double KO =9). Statistical significance is indicated by * (P<0.05) ** (<P0.01) *** (<P0.001). Data shown as means ± standard error of the mean (SEM).

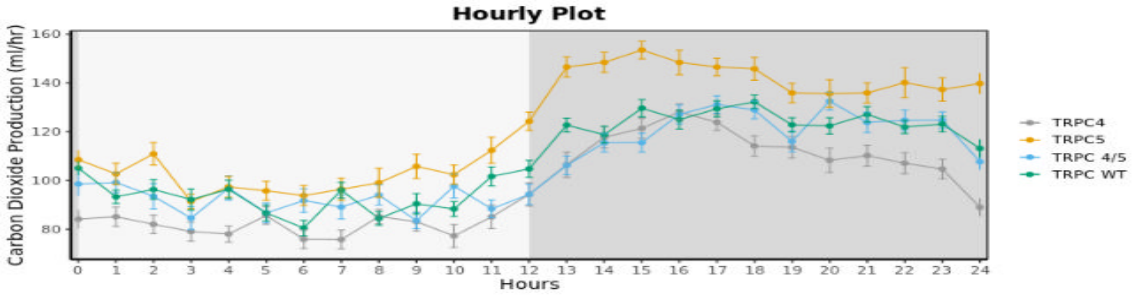
4.4 Comprehensive Lab Animal Monitoring System (CLAMs)

After 48 hours of acclimation, various parameters were recorded in CLAMs cages. Overall data was plotted in a 24-hour cycle 0- 12 hours in the light and 12 – 24 in the dark. The TRPC5 KO mice consume more oxygen (Fig.9a) during the dark cycle compared to the WT and showed an increase at P<0.001; However across the light and dark cycles it averaged to <P0.01 (Fig.9). TRPC5 KO mice also showed a significant increase in the production of carbon dioxide and energy expenditure (Fig.9b and 9c) in the dark cycle

a



b



c

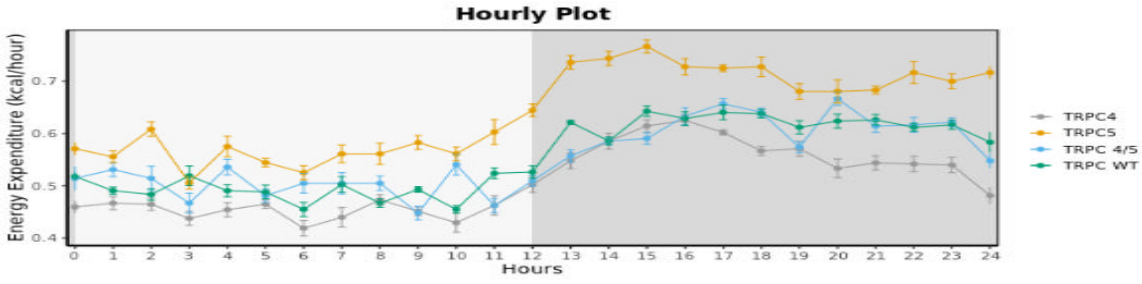
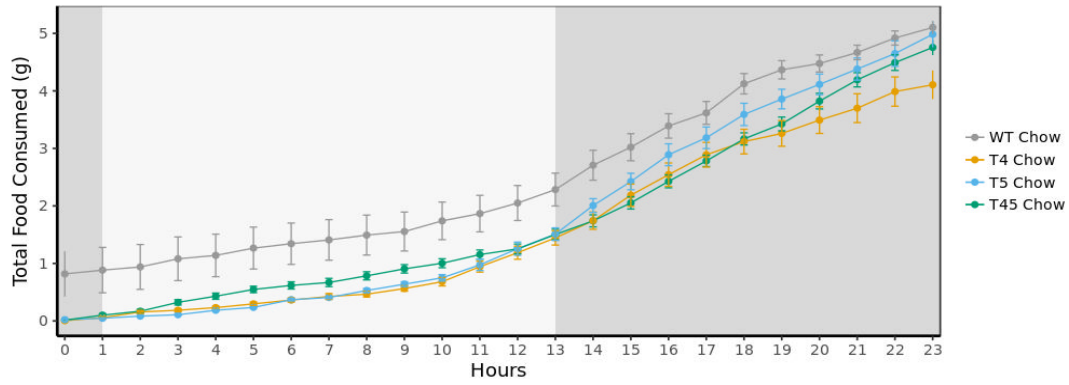


Figure 9 Comprehensive Lab Animal Monitoring System (CLAMs). All TRPC genotypes over a period of 24 hours during the light and dark cycle (n=7-8) (a) Oxygen consumption (b) The Carbon dioxide production (c) The Energy expenditure.

4 .5 Food consumption measured in CLAMs

Data shows the consumption of food per hour (Fig.10a and 10b) during the light and dark cycles. TRPC WT continuously consume more diet thought out the whole 24 hour period, with the TRPC 4 KO eating the least.

a



b

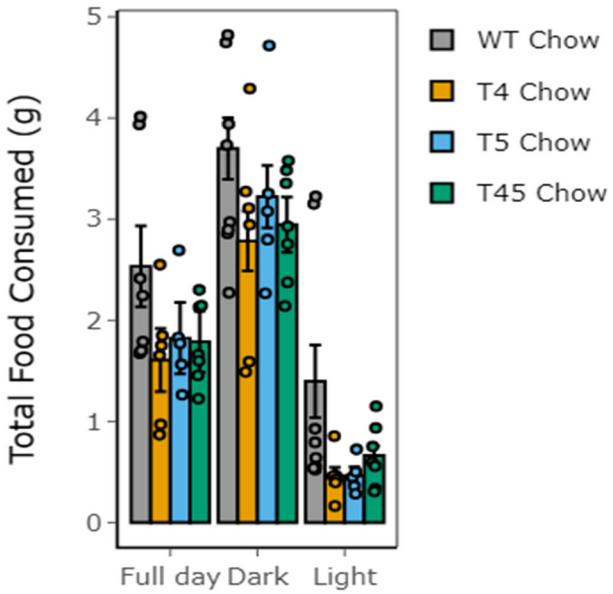


Figure 10 Comprehensive Lab Animal Monitoring System (CLAMs).

All TRPC genotypes over a period of 24 hours during the light and dark cycle (n =TRPC WT =10, TRPC4 KO =8, TRPC5 KO =7, TRPC 4/5 double KO =9). T4 representing TRPC4, T5 = TRPC5, T45 = TRPC4/5 double KO. (a) Total food consumption in hours over a 24 hour period. (b) Total food consumption full day (dark and light cycle).

4.6 CLAMS Exercise activity

Data shows the locomotor activity (Fig.11a) and the ambulatory activity (Fig.11b) in hour during the light and dark cycles. TRPC 4/5 double Ko showing the least activity thought out the whole 24 hour period.

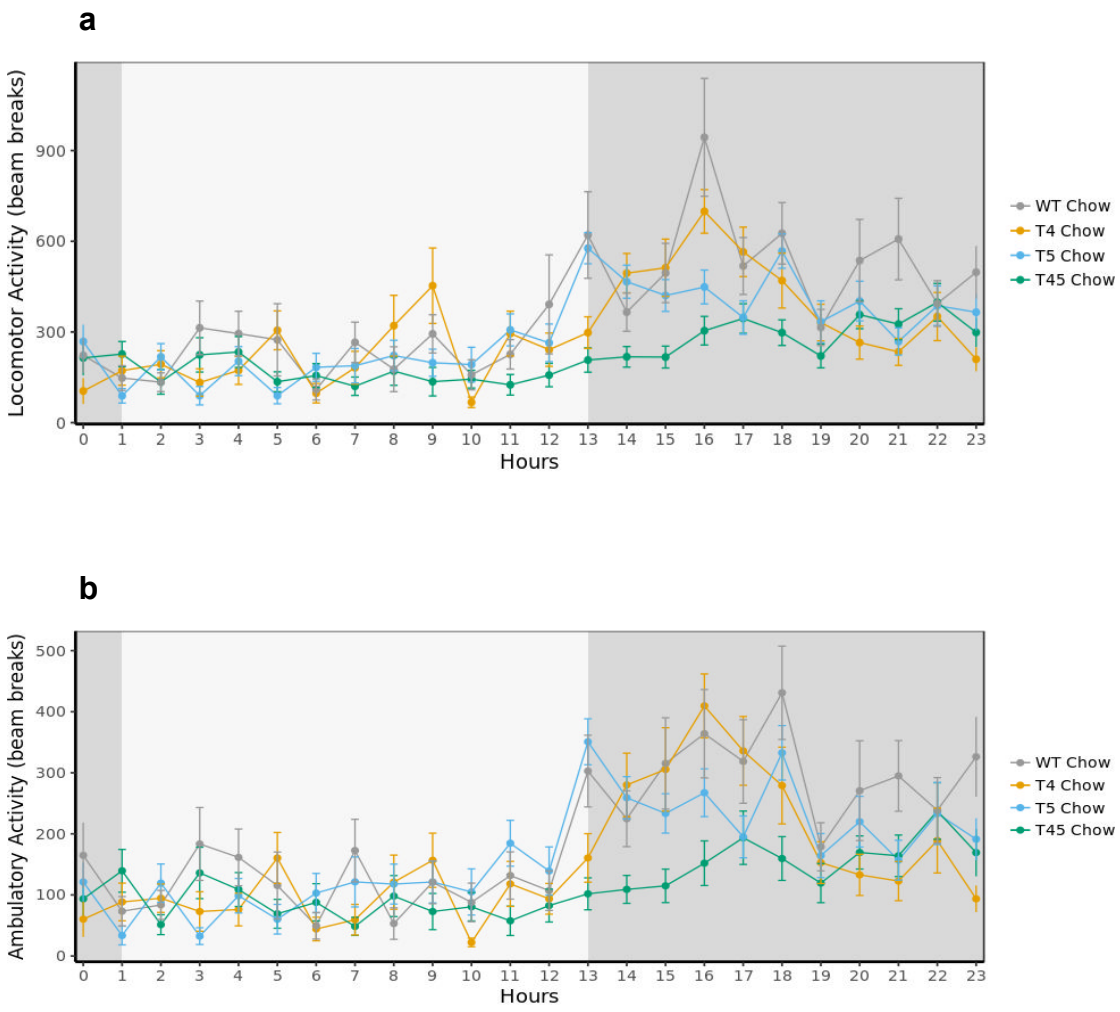


Figure 11. Exersise data from the CLAMs.

All TRPC genotypes over a period of 24 hours during the light and dark cycle (n =TRPC WT =10, TRPC4 KO =8, TRPC5 KO =7, TRPC 4/5 double KO =9). T4 representing TRPC4, T5 = TRPC5, T45 = TRPC4/5 double KO. (a) The total locomotor activity (beam break) in hours over a 24 hour period. (b) Total ambulatory activity (beam breaks) in a full day (dark and light cycle).

4.7 CLAMs Statistics

Data was divided into light /dark and full day cycles. Each TRPC group's data was compared to TRPC WT (Fig.12) and CLAMs data were analysed using CalR online software from Harvard.

GLM (T4 Chow vs WT Chow)

	Full Day		Light		Dark	
Effect	Mass	Group	Mass	Group	Mass	Group
Oxygen Consumption (ml/hr)	0.3861	0.3960	0.6310	0.8049	0.2598	0.1996
Carbon Dioxide Production (ml/hr)	0.5668	0.3001	0.8584	0.4441	0.3689	0.3025
Energy Expenditure (kcal/hour)	0.4545	0.3537	0.4868	0.1843	0.3027	0.2215
Hourly Food Consumed (g)	0.8097	0.2379	0.5025	0.9893	0.3401	0.1314
Total Food Consumed (g)	0.6372	0.1056	0.4490	0.0708	0.8139	0.2694

GLM (T5 Chow vs WT Chow)

	Full Day		Light		Dark	
Effect	Mass	Group	Mass	Group	Mass	Group
Oxygen Consumption (ml/hr)	0.3861	0.0030 **	0.6310	0.0227 *	0.2598	<0.001 ***
Carbon Dioxide Production (ml/hr)	0.5668	0.0774	0.8584	0.1530	0.3689	0.0335 *
Energy Expenditure (kcal/hour)	0.4545	0.0075 **	0.4868	0.1274	0.3027	0.0024 **
Hourly Food Consumed (g)	0.8097	0.9929	0.5025	0.4863	0.3401	0.7980
Total Food Consumed (g)	0.6372	0.5945	0.4490	0.4064	0.8139	0.9306

GLM (T45 Chow vs WT Chow)

	Full Day		Light		Dark	
Effect	Mass	Group	Mass	Group	Mass	Group
Oxygen Consumption (ml/hr)	0.3861	0.5069	0.6310	0.3435	0.2598	0.7410
Carbon Dioxide Production (ml/hr)	0.5668	0.9758	0.8584	0.7999	0.3689	0.8883
Energy Expenditure (kcal/hour)	0.4545	0.6117	0.4868	0.0496 *	0.3027	0.8294
Hourly Food Consumed (g)	0.8097	0.8406	0.5025	0.7449	0.3401	0.8215
Total Food Consumed (g)	0.6372	0.1896	0.4490	0.1722	0.8139	0.3610

Figure 12 Data from the CLAMs analysed by CalR online software from Harvard. T4 representing TRPC4, T5 = TRPC5, T45 = TRPC4/5 double KO. Mass = Probability and Group = Mean. GLM Statistical significances is indicated by * (P<0.05) ** (<P0.01) *** (<P0.001).

4.8 Glucose tolerance test

After feeding standard chow for a total of 16 week glucose tolerance test (GTT) were performed again. It (Fig.13a) shows that the TRPC4/5 double KO had a reduced level of fasting blood glucose compared to the TRPC WT mice after a 16 hour fast. However after a single glucose injection all TRPC genotypes show no significant difference over the course of the test (Fig.13b).

a **b**

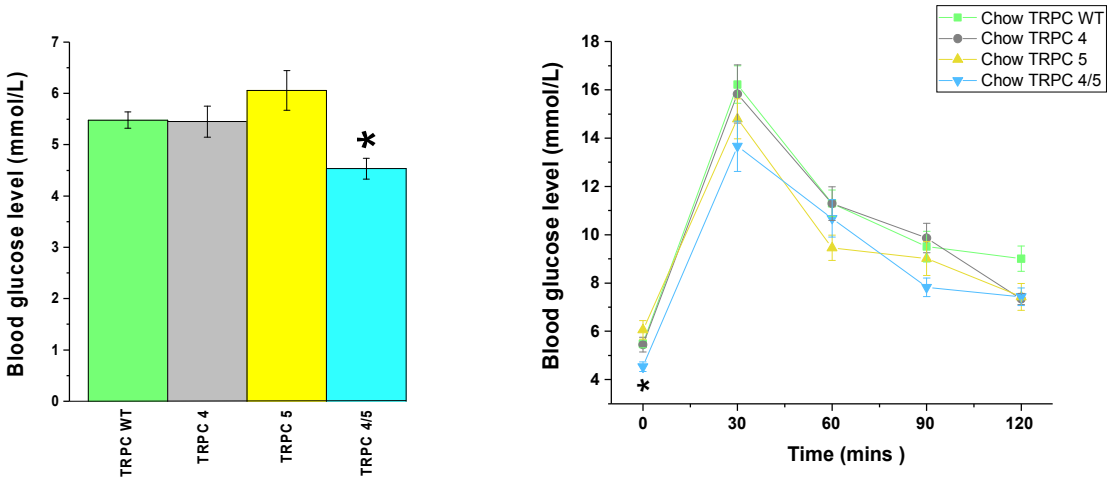


Figure 13. Glucose Tolerance Test (a) Fasting blood glucose levels in chow fed mice at 16 weeks of age (b) Blood glucose levels at 16 weeks of age fed on a chow diet set time points after the administration of glucose injection (n =TRPC WT =10, TRPC4 KO =8, TRPC5 KO =7, TRPC 4/5 double KO =9).Statistical significances is indicated by * ($P<0.05$). Data shown as means ± standard error of the mean (SEM).

4.9 Insulin tolerance test

Insulin tolerance tests were carried out after a 2 hour fast. Blood glucose levels (Fig.14a) show there are no significant difference between groups. After an insulin injection (Fig.14b) TRPC5 KO and TRPC4/5 double KO show greater sensitivity with a rapid drop in blood glucose after insulin injection in the first 30 minutes.

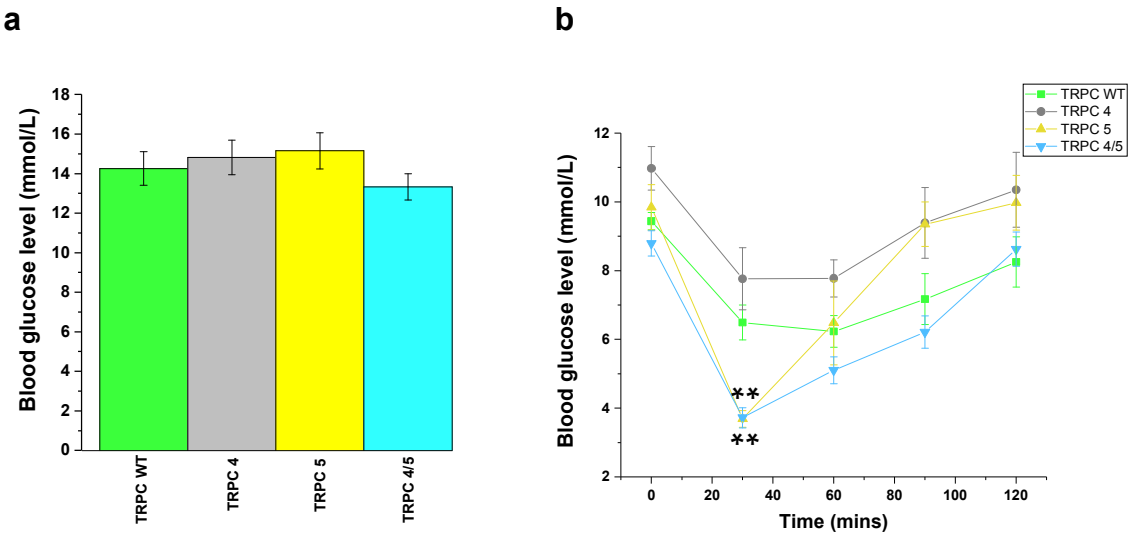


Figure 14. Insulin tolerance test. (a) Fasting blood glucose level at 16 weeks of age. (b) Blood glucose level at set time points after the administration of insulin injection (n =TRPC WT =10, TRPC4 KO =8, TRPC5 KO =7, TRPC 4/5 double KO =9). Statistical significances is indicated by ** (<P0.01). Data shown as means ± standard error of the mean (SEM).

4.10 Organ weight at 16 weeks

After 16 weeks of chow feeding, mice were harvested and organs weighed. TRPC5 KO show increased liver weight (Fig.15a) in relation to all other genotypes; however no significant difference in eWAT (Fig.15b) or iBAT was observed (Fig.15c).

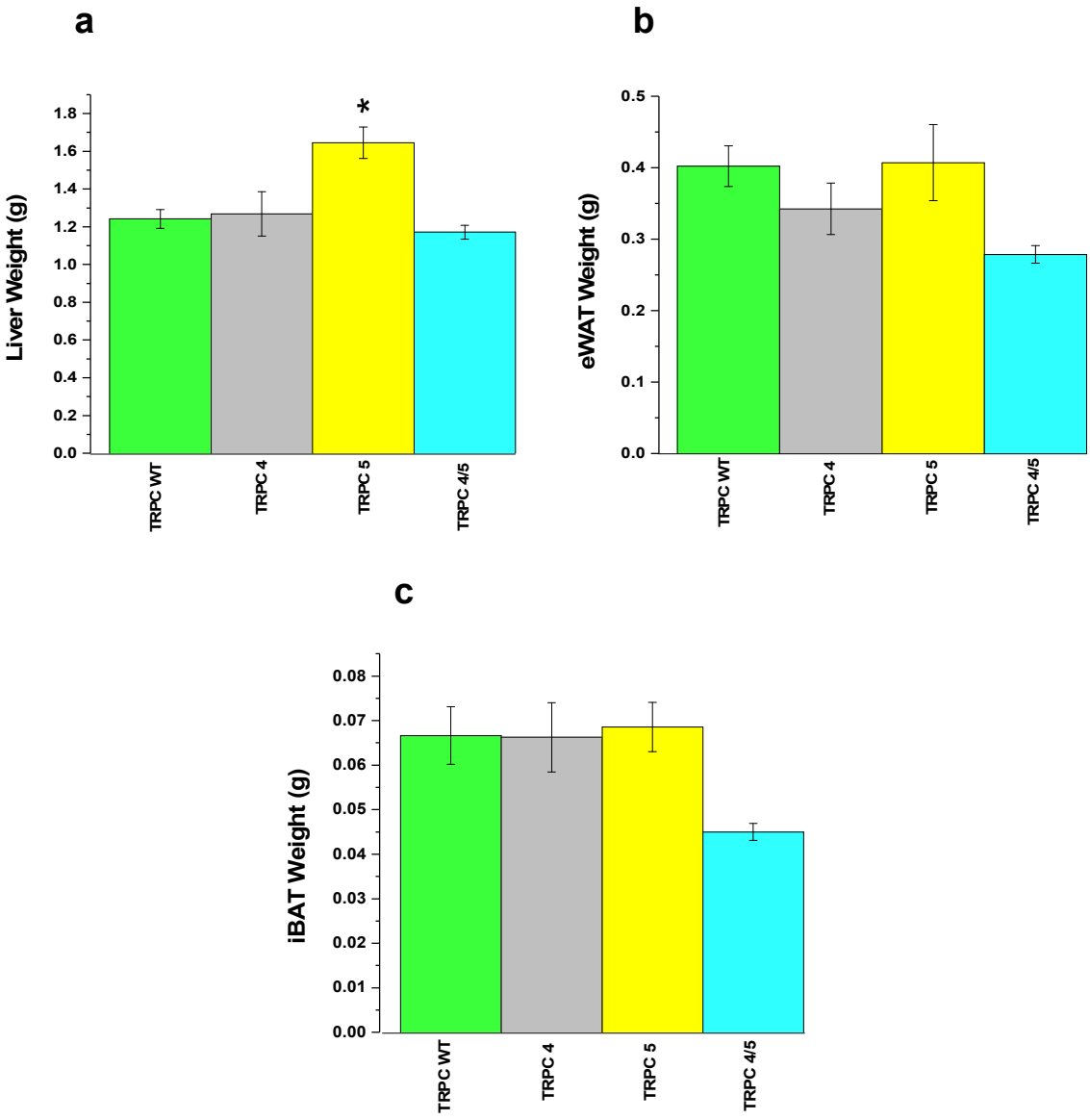


Figure 15. Harvest weights of TRPC genotypes at 16 weeks of age after been fed on chow diet (a) liver weight (b) eWAT weight (c) iBAT (n =TRPC WT =10, TRPC4 KO =8, TRPC5 KO =7, TRPC 4/5 double KO =9). Statistical significance is indicated by * ($P<0.05$). Data shown as means ± standard error of the mean (SEM).

4.11 Plasma lipid levels at 16 weeks

Cholesterol (Fig.16a), high-density lipoprotein cholesterol (HDL) (Fig. 16b) and the non-esterified fatty acid (Fig.16c) measurements showed no significant difference between the TRPC genotypes. However, triglyceride level (Fig.16d) in TRPC4 KO and TRPC4/5 double KO were significantly lower compared to TRPC WT.

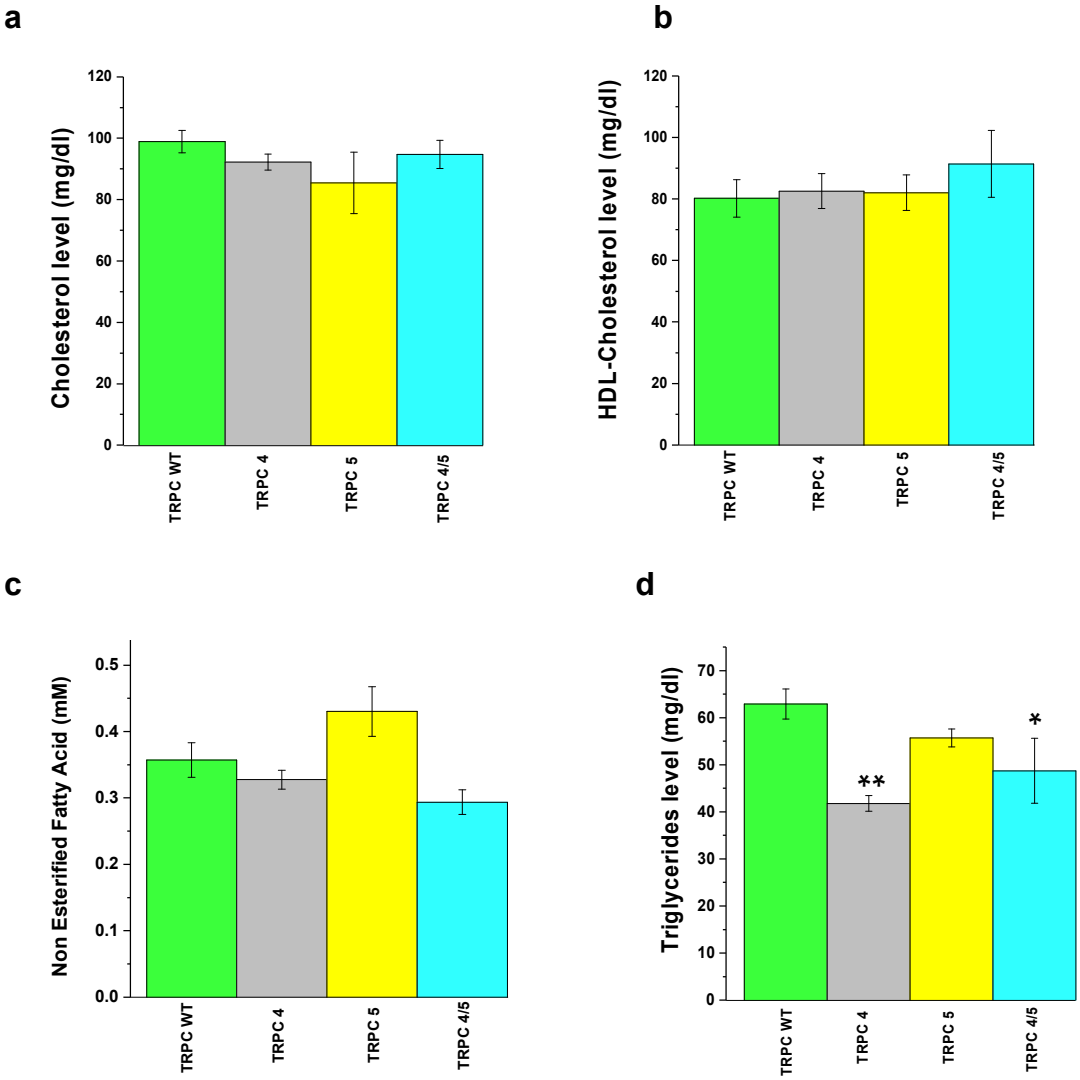


Figure 16. Plasma lipid levels of TRPC genotypes at 16 weeks of age after been fed on chow diet (a) cholesterol levels (b) high-density lipoprotein (HDL) cholesterol levels (c) non-esterified fatty acid levels (d) triglyceride levels. Statistical significances are indicated by * (P<0.05) ** (<P0.01). Data shown as means ± standard error of the mean (SEM).

4.12 Discussion

In vivo data presented in this chapter shows in weeks 8-12 TRPC4 KO mice display a significant reduction in body weight when compared to TRPC WT (Fig.8a) however as the study progressed the difference became not significant. Whereas the TRPC4/5 double KO body weights are significantly reduced throughout.

The data from the CLAMs system has shown that the TRPC4 KO and TRPC4/5 double KO consumed less food over 24 hour period. However the TRPC 4 KO mice were the most active. Studies have shown TRPC4 and TRPC5 have been implicated in central nervous system response to the hormone leptin, which regulates the sensation of hunger and has also been associated with anti-diabetic effects (Qiu et al,.2010).

GTT (Fig.13) show that the fasting blood glucose levels of TRPC4/5 double KO are significantly lower than TRPC WT after a 16 hour fast. Furthermore the sensitivity after the administration of glucose that we saw at 8 weeks was lost at 16 weeks, indicating resistance to insulin. However this is not the case in ITT (Fig.14) the TRPC5 KO mice continue to be sensitive to insulin. Furthermore TRPC4/5 double KO has also developed a response to insulin sensitivity.

At the end of the study, TRPC5 KO mice have an increased weight in the liver (Fig.15a). Plasma lipid levels (Fig.16) showed no significance apart from the triglyceride levels (Fig. 16d) of the TRPC4 KO and TRPC4/5 double KO mice this indicates that the TRPC4 KO and the TRPC4/5 double KO mice were better at burning fat and reducing plasma lipids.

Chapter 5

Aims and objectives

Investigate the metabolic characteristics of TRPC4 and TRPC5 knockout animals in the setting of obesity.

Investigate the physiological functions of TRPC4 and TRPC5 channels in adipose tissue and their pathophysiological importance in obese mice.

5.1 Introduction

Having established the baseline conditions in TRPC transgenic mice fed on chow diet, I next considered the situation of high calorie availability, with those on 60% high-fat diet (HFD). This was to investigate the affects in an obese setting. At 8 weeks of age, 60% HFD feeding commenced this continued for 8 weeks until the mice reached 16 weeks of age. Record of body weight was documented at the same time point weekly. At 16 weeks the TRPC transgenic mice underwent glucose and insulin tolerance testing to observe if the levels of sensitivity had changed in obese mice. After recovering from the metabolic profiling mice were then place in the CLAMs cages, as before the TRPC transgenic mice had an acclimatisation faze before a 24 hour time period was taken to analyse. TRPC transgenic mice were then harvested, organ weights recorded and blood take for lipid analysis.

5.2 Results

5.3 Metabolic phenotyping at 16 weeks of age.

HFD-fed mice were weighed at the same time point every week (Fig.17a) and the data shows (Fig.17b) that there is no significant difference in weights across 4 genotypes over 8 weeks of feeding 60% HFD.

a **b**

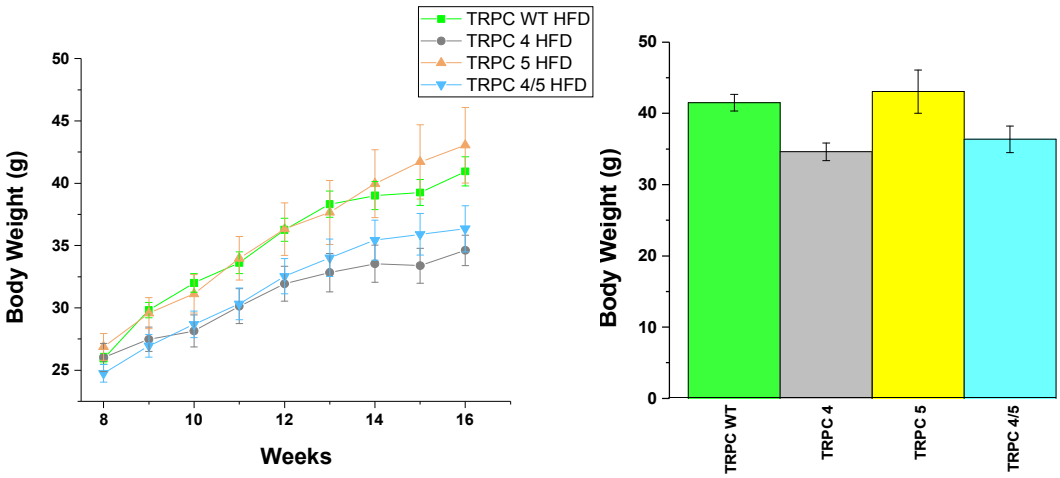


Figure 17. Weekly body weights and overall body weights of TRPC genotypes at 16 weeks of age after been fed on HFD diet (a) Weekly body weight taken at the same point every week (n =TRPC WT =10, TRPC4 KO =7, TRPC5 KO =8, TRPC 4/5 double KO =8). (b) Body weights of TRPC genotypes at 16 weeks of age after been fed on HFD diet. Data shown as means ± standard error of the mean (SEM).

5.4 Glucose tolerance test

After feeding HFD for a total of 8 weeks, glucose tolerance test (GTT) were performed. Fasting blood glucose (Fig.18a) showed that the TRPC5 KO had a higher level of glucose in relation to the all other TRPC transgenic mice in this study. GTT (Fig.18b) results showed a similar increase in blood glucose levels in all TRPC transgenic mice KO regardless of genotype at 30 and 60 minutes after glucose was administered. However TRPC4 KO and TRPC5 KO show a significantly increase in blood glucose at 90 and 120 minutes respectably.

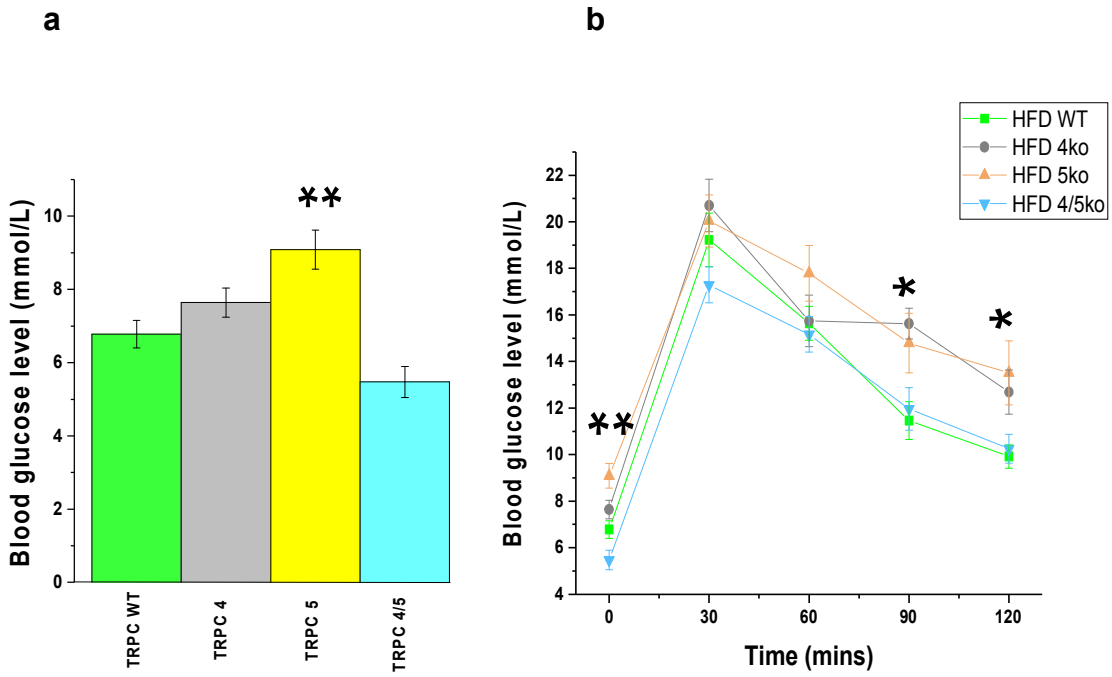


Figure 18. Glucose tolerance test (a) Fasting blood glucose level in HFD fed mice at 16 weeks of age (b) Blood glucose level at 16 weeks of age fed on a 60%HFD at set time points after the administration of glucose injection (n =TRPC WT =10, TRPC4 KO =7, TRPC5 KO =8, TRPC 4/5 double KO =8). Statistical significances is indicated by * (P<0.05) ** (P<0.01) *** (P<0.001). Data shown as means ± standard error of the mean (SEM).

5.5 Insulin tolerance test

Blood glucose levels (Fig.19a) show that all TRPC transgenic mice show a similar level of blood glucose after fasting for 2 hours and the similar insulin sensitivity was shown throughout the 120 minutes during ITT (Fig.19b).

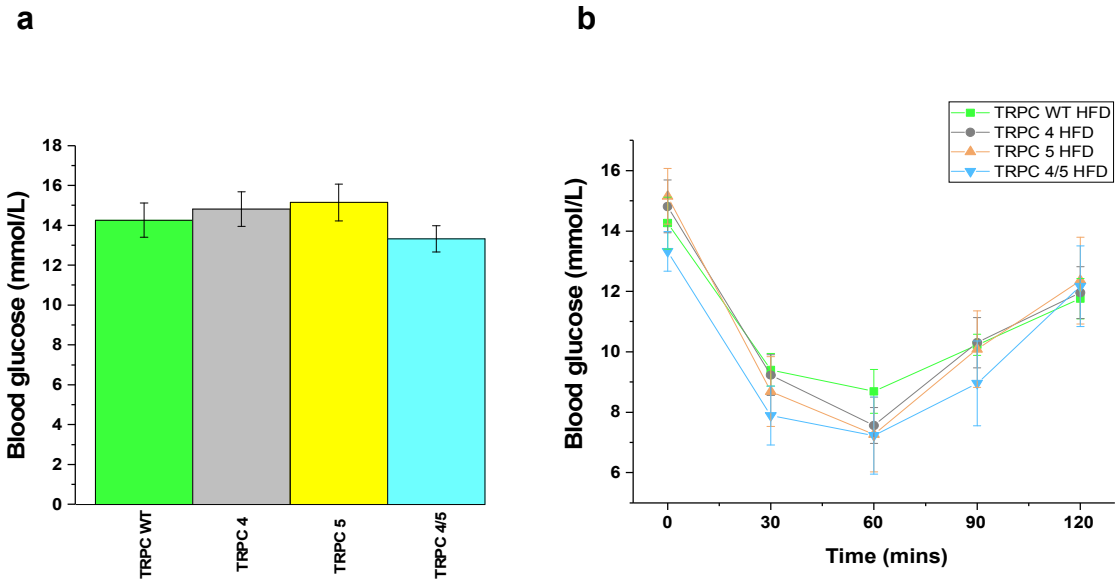
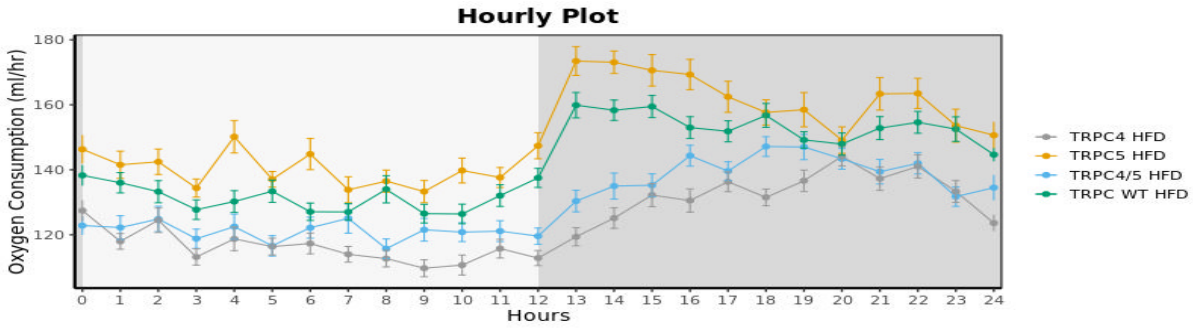


Figure 19. Insulin tolerance test (a) fasting blood glucose level at 16 weeks of age on 60% HFD fed mice. (b) Blood glucose level at set time points after the administration of insulin injection to check of sensitivity (n =TRPC WT =10, TRPC4 KO =7, TRPC5 KO =8, TRPC 4/5 double KO =8). Data shown as means ± standard error of the mean (SEM).

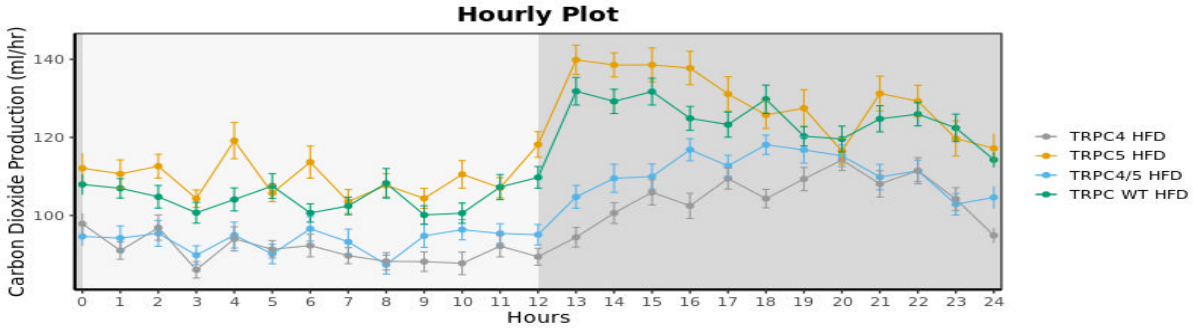
5.6 Comprehensive Lab Animal Monitoring System (CLAMs)

At 16 weeks of age mice were put into the CLAMs and allowed to acclimatise for 48 hour before a 24-hour cycle 0- 12 hours in the light and 12 – 1 in the dark period was plotted. The TRPC4 KO mice consumed significantly less oxygen in the dark and displayed less carbon dioxide production and this was also the case in the energy expenditure, (Fig.20a, 20b and 20c)

a



b



c

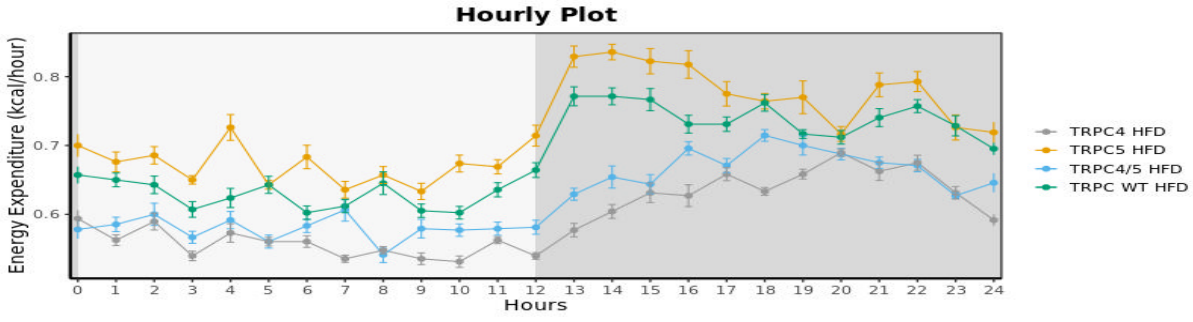
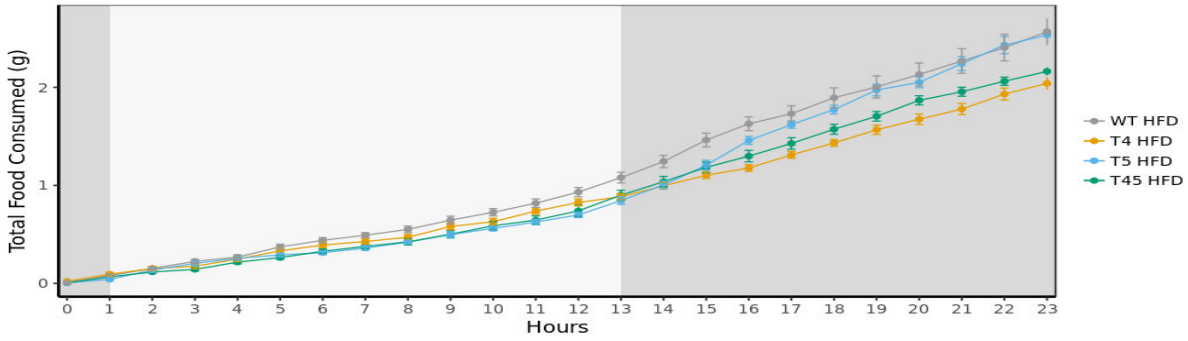


Figure 20. Comprehensive Lab Animal Monitoring System (CLAMs). TRPC genotypes over a period of 24 hours during the light and dark cycle (n=7-8) (a) Oxygen consumption (b) Carbon dioxide production (c) The energy expenditure

5.7 Food consumption

The consumption of food per hour (Fig.21a and 21b) during the light and dark cycles showed that TRPC WT continuously consume more food thought out the whole 24 hour period, with the TRPC4 KO eating significantly less food over a 24hour period.

a



b

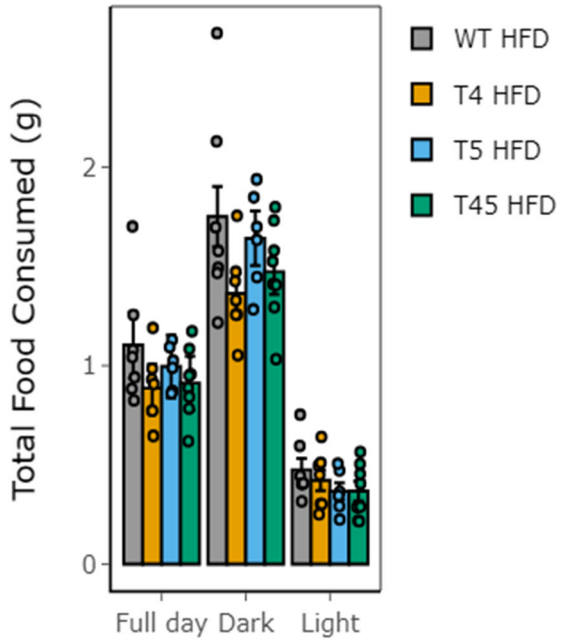


Figure 21. Comprehensive Lab Animal Monitoring System (CLAMS). TRPC genotypes over a period of 24 hours during the light and dark cycle (n =TRPC WT =10, TRPC4 KO =7, TRPC5 KO =8, TRPC 4/5 double KO =8). T4 representing TRPC4, T5 = TRPC5, T45 = TRPC4/5 double KO. (a) Total food consumption in hours over a 24 hour period. (b) Total food consumption full day, dark and light cycle.

5.8 CLAMS Exercise activity

Data shows the locomotor activity (Fig.22a) and the ambulatory activity (Fig.22b) in hour during the light and dark cycles. TRPC WT and TRPC5 KO become more active once the light cycle changes from light to dark.

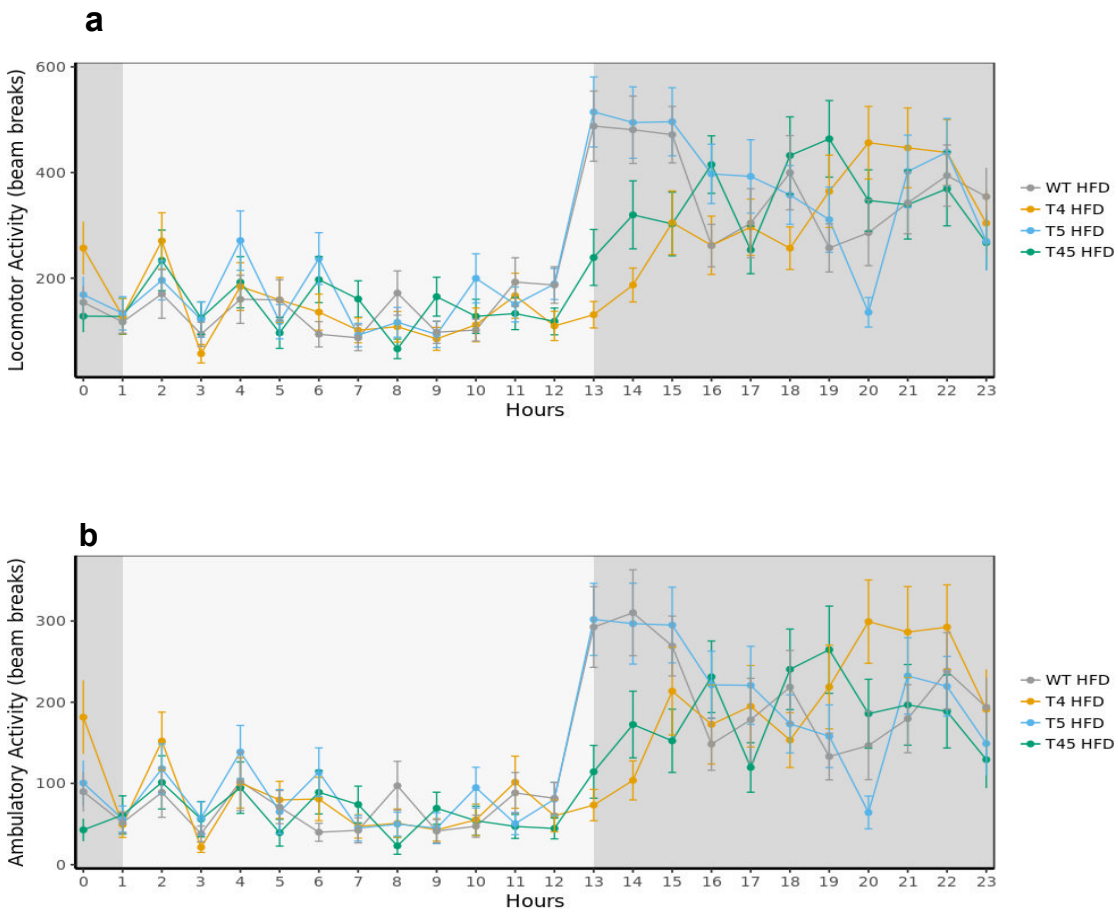


Figure 22. Exersise data from the CLAMs.

All TRPC genotypes over a period of 24 hours during the light and dark cycle (n =TRPC WT =10, TRPC4 KO =8, TRPC5 KO =7, TRPC 4/5 double KO =9). T4 representing TRPC4, T5 = TRPC5, T45 = TRPC4/5 double KO. (a) The total locomotor activity (beam break) in hours over a 24 hour period. (b) Total ambulatory activity (beam breaks) in a full day (dark and light cycle).

5.9 CLAMs Statistics

Data was divided into light /dark and full day cycles. Each TRPC group's data was compared to TRPC WT (Fig.23) CLAMs data were analysed using CalR online software from Harvard.

GLM (T4 HFD vs WT HFD)

	Full Day		Light		Dark	
Effect	Mass	Group	Mass	Group	Mass	Group
Oxygen Consumption (ml/hr)	0.0012 **	0.0249 *	<0.001 ***	0.2304	0.0163 *	0.0057 **
Carbon Dioxide Production (ml/hr)	0.0026 **	0.0027 **	<0.001 ***	0.0194 *	0.0241 *	0.0030 **
Energy Expenditure (kcal/hour)	0.0012 **	0.0154 *	<0.001 ***	0.1601	0.0168 *	0.0042 **
Hourly Food Consumed (g)	0.3073	0.1250	0.4034	0.2832	0.1323	0.2772
Total Food Consumed (g)	0.8578	0.0934	0.7557	0.4367	0.7153	0.0680

GLM (T5 HFD vs WT HFD)

	Full Day		Light		Dark	
Effect	Mass	Group	Mass	Group	Mass	Group
Oxygen Consumption (ml/hr)	0.0012 **	0.1691	<0.001 ***	0.1898	0.0163 *	0.2333
Carbon Dioxide Production (ml/hr)	0.0026 **	0.4556	<0.001 ***	0.4861	0.0241 *	0.5230
Energy Expenditure (kcal/hour)	0.0012 **	0.2062	<0.001 ***	0.1964	0.0168 *	0.3015
Hourly Food Consumed (g)	0.3073	0.9342	0.4034	0.1570	0.1323	0.5006
Total Food Consumed (g)	0.8578	0.3495	0.7557	0.1822	0.7153	0.5294

GLM (T45 HFD vs WT HFD)

	Full Day		Light		Dark	
Effect	Mass	Group	Mass	Group	Mass	Group
Oxygen Consumption (ml/hr)	0.0012 **	0.8850	<0.001 ***	0.3669	0.0163 *	0.2752
Carbon Dioxide Production (ml/hr)	0.0026 **	0.2435	<0.001 ***	0.5246	0.0241 *	0.1836
Energy Expenditure (kcal/hour)	0.0012 **	0.7386	<0.001 ***	0.4540	0.0168 *	0.2342
Hourly Food Consumed (g)	0.3073	0.3478	0.4034	0.1356	0.1323	0.8414
Total Food Consumed (g)	0.8578	0.1863	0.7557	0.1674	0.7153	0.2652

Figure 23. Data from the CLAMs analysed by using CalR online software from Harvard. P values of mice for a 24 hour period. T4 representing TRPC4 KO, T5 = TRPC5 KO, T45 = TRPC4/5 double KO. Mass = Probability and Group = Mean. GLM statistical significances is indicated by * (P<0.05) ** (<P0.01) *** (<P0.001).

5.10 Organ weight at 16 weeks

After harvesting the TRPC transgenic mice at 16 weeks old, the liver weight (Fig. 24a) show an increase weight however has not reached statistical significance. Furthermore the TRPC4/5 double KO has a reduction in liver weight but also hasn't reached a statistical significance. The eWAT weight (Fig.24b) show the TRPC4/5 double KO had significant reduction in weight. The iBAT weight (Fig. 24c) shows that TRPC4/5 double KO has a tendency towards reduced weight, but it has not reached statistical significance.

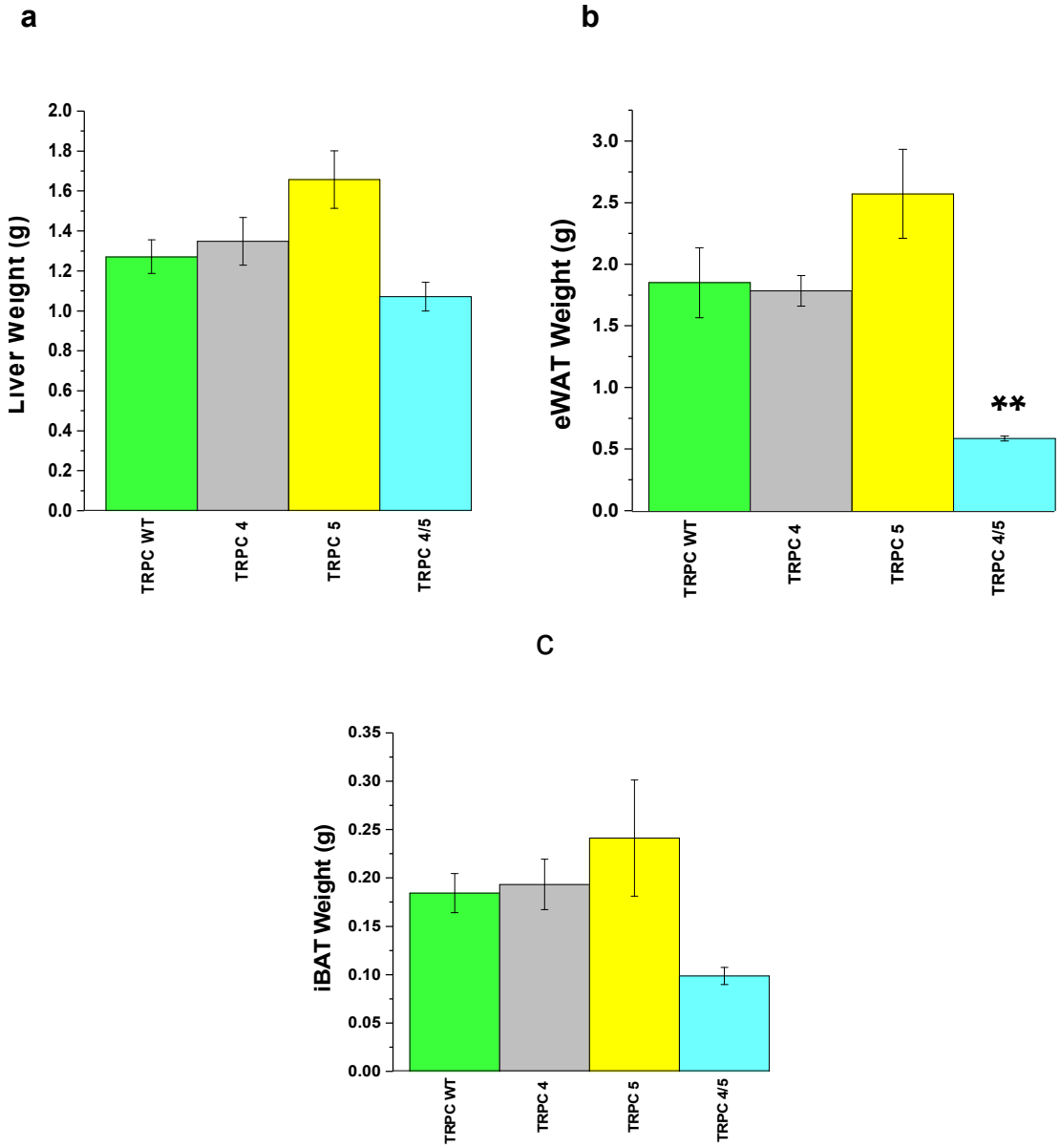


Figure 24. Harvest weights of TRPC genotypes at 16 weeks of age after been fed on 60% HFD for 8 weeks (a) Liver weight (b) eWAT weight (c) iBAT (n =TRPC WT =10, TRPC4 KO =7, TRPC5 KO =8, TRPC 4/5 double KO =8). Statistical significances are indicated by * (P<0.05) ** (<P0.01). Data shown as means ± standard error of the mean (SEM).

5.11 Plasma lipid levels

All TRPC transgenic mice have an increased level of cholesterol when compared to TRPC WT (Fig.25a) however this did not reach statistical significant. The high-density lipoprotein cholesterol levels (HDL) (Fig.25b) and the non-esterified fatty acid (Fig.25c) levels are also not significantly different. However (Fig.25d) the TRPC4/5 double KO have a significant decreased level of triglyceride.

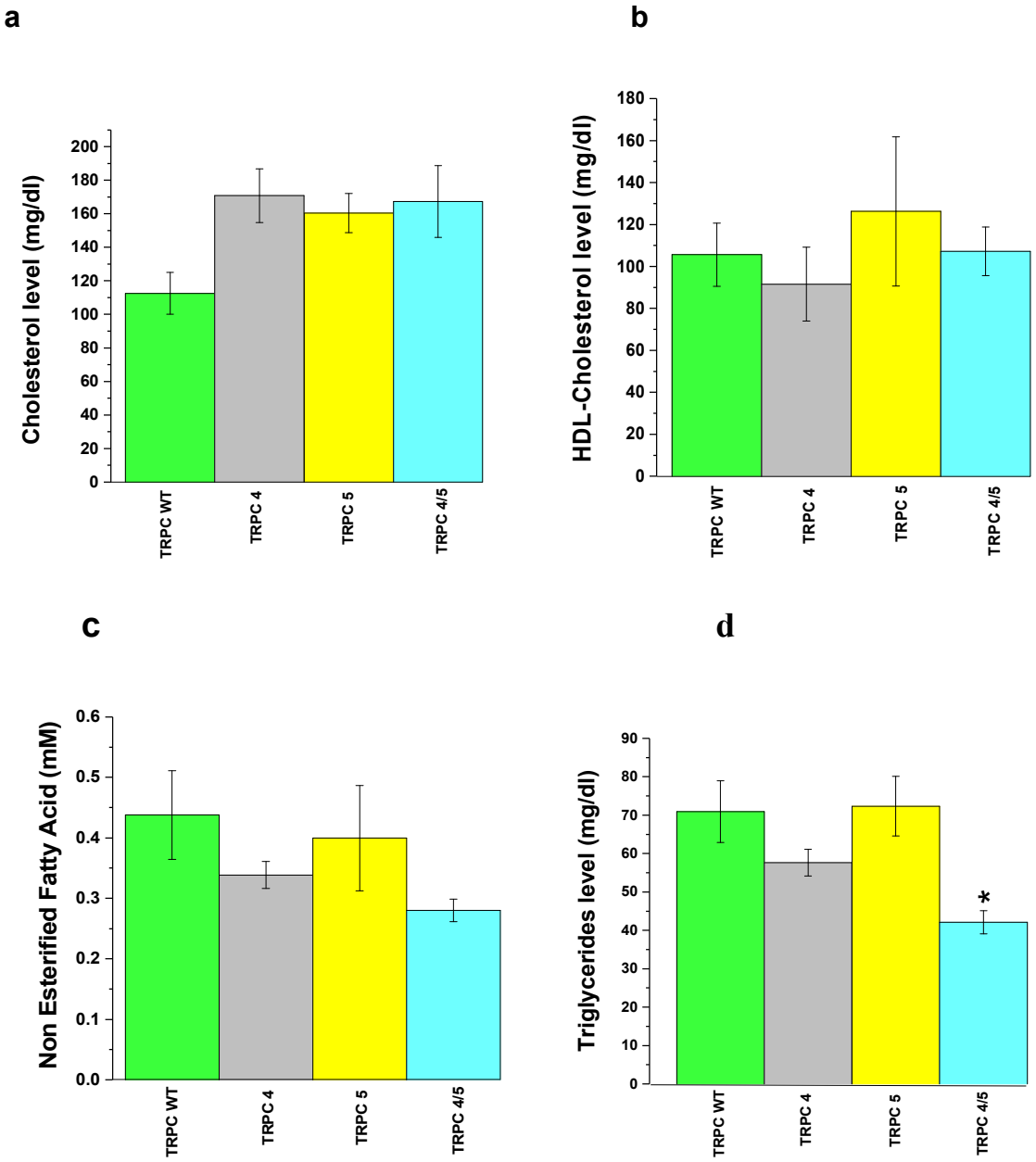


Figure 25. Plasma lipid levels of TRPC genotypes at 16 weeks of age after been fed on 60% High Fat diet (a) Cholesterol levels (b) High-density lipoprotein (HDL) cholesterol levels (c) Non-esterified fatty acid (d) Triglyceride level. Data shown as means ± standard error of the mean (SEM).

5.12 Discussion

Mice were fed a 60% high fat diet for 8 weeks and male litter-mates were compared to TRPC WT mice. No obvious adverse effects on health were evident.

All TRPC transgenic mice were shown to display no significant difference in body weight (Fig.17) compared to TRPC WT controls. However, the data shows TRPC4 KO and TRPC4/5 double KO were slower to gain weight and the difference between the groups was increasing as the study progressed. Furthermore both the TRPC4 KO and the TRPC4/5 double KO consumed less HFD than the WT. The study also found that the TRPC5 KO mice weighed more than the TRPC WT at the end of experimental period. This data is in conflict with other studies on TRPC5 KO (Rode, B. et al. 2019), and surprisingly consumed less diet in the 24 hour period in the CLAMs. Therefore it would be useful to increase the length of the study and waited for the mouse weights to reach a plateau to conclude. However on HFD the The TRPC5 KO mice were more active.

After fasting mice for 16 hours for GTT (Fig.18a) the data shows the fasting blood glucose levels were higher in the TRPC5 KO mice. ITT results show there was a greater sensitivity to insulin by the TRPC5 KO and TRPC4/5 double KO (Fig.19b).

There was no significant difference in the organ weights (Fig.24) among the 4 TRPC genotypes with the exception of the eWAT weights (Fig.24b) of the TRPC4/5 KO, which was significantly lower. Furthermore, TRPC 4/5 double KO also showed significantly decreased level of triglyceride (Fig.25d) which suggests those are less likely to be associated with cardiovascular disease and type 2 diabetes. However analysis of the other plasma lipid tests (Fig.25) showed no significant differences among the TRPC transgenic mice.

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Chapter 6

Aims and objectives

To evaluate the effects of the small molecule TRPC1/4/5 inhibitor LDC204136 (C31/ Pico145) on high fat fed C57BL/6J mice.

6.1 Introduction

6.2 Introduction to small molecule inhibiter

LDC204136 (C31) is a Transient Receptor Potential Canonical 1, 4 and 5 (TRPC1 TRPC4 and TRPC5) antagonist. It has no effect on other TRP channels tested, including TRPC3, TRPC6, TRPV1, TRPV4, and TRPA1, TRPM2 and TRPM8 or store-operated Ca^{2+} entry mediated by Orai1. This suggests C31 is most likely a specific molecule for TRPC1/4/5 inhibition (Rubaiy et *a*l., 2017). C31 is also known as Pico 145 (Rubaiy, Ludlow, Bon, & Beech, 2017).

In this chapter I will look at the effects of C31 in the setting of obesity.

Mice were fed a high fat diet containing 60% fat and treated orally, BID, with test compound or vehicle for 6 weeks. Body weights were measured twice weekly until the end of the experiment. At 3 weeks of treatment mice were weighed and fasted for 2 hours before 100µL of blood was taken to measure glucose and plasma insulin levels. At 5 weeks of treatment mice were fasted for 16 hours, blood collected and GTT commenced. After 72 hours mice underwent fasting for 2 hours followed by ITT. At 6 weeks of treatment mice were sacrificed and organs harvested.

6.3 Results

6.4 Bodyweight

Body weights were recorded twice a week from the start of experimentation (Fig.26a). All mice on both the vehicle and C31 groups lost weight during the initial few weeks of the study due to the oral gavage however the vehicle group recovered quickly. At week 3 the C31 group showed a significant lower weight when compared to vehicle however this was lost in time point 3.5 and 4. By week 4.5 the significant difference was again evident between the 2 groups. In weeks 5, 6 and 6.5 there was a significance reduction of weight in the C31 group when compared to the vehicle group ($P<0.01$). Furthermore we can see a reduction in weight in both groups at 5.5 as a result of fasting for both groups ITT and GTT. Overall body weights at the endpoint of experiment (Fig.26b) show that vehicle mice weigh more than the C31 mice.

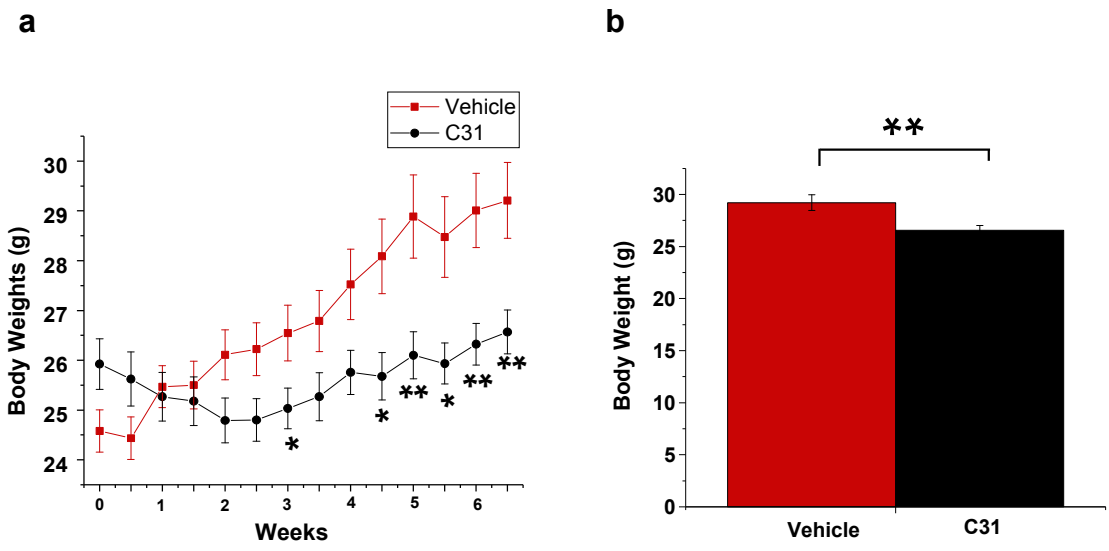

Figure 26. Weekly body weights and overall end weights of Vehicle and C31 groups. (a) Twice weekly body weight taken at the same point every week. (b) Weights of mice at 14 weeks old (n =10). Statistical significance is indicated by * ($P<0.05$) ** ($P<0.01$). Data shown as means ± standard error of the mean (SEM).

6.5 Metabolic Profiling

At 2, 3 and 4 weeks of diet and treatment, mice were weighed and fasted at 08:00am for 2 hours prior to the collection blood (100µL/EDTA) at 10.00am. Fasting blood glucose measurement taken and recorded. At 4 weeks on 60% HFD and treatment the group receiving C31 show a significant lower level of blood glucose (Fig.27).

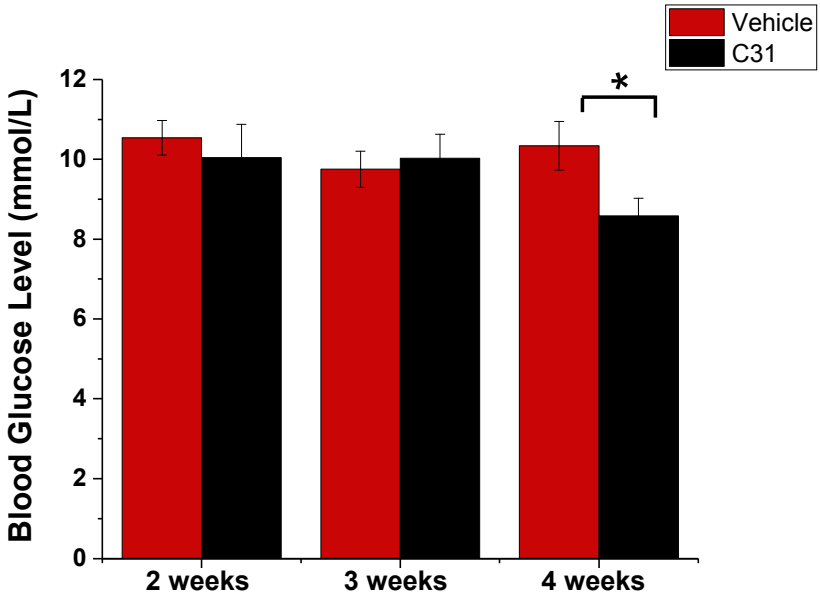

Figure 27. Blood glucose levels at 2,3,and 4 weeks of vehicle and C31 groups (n = 10) Statistical significance are indicated by * (P<0.05). Data shown as means ± standard error of the mean (SEM).

6.6 Glucose tolerance test

At 5 weeks of 60% HFD diet and oral treatment, mice were weighed and fasted overnight for a glucose tolerance test (GTT). Fasting blood glucose levels (Fig.28a) were significantly lower in C31 group after a 16 hours fasting. After an intraperitoneal glucose injection, blood glucose was measured from the tail tip bleed at 15, 30, 60, 90 and 120 minutes (Fig.28b) which revealed a significantly lower glucose levels (P<0.05) at 30 and 60 minutes in C31 group.

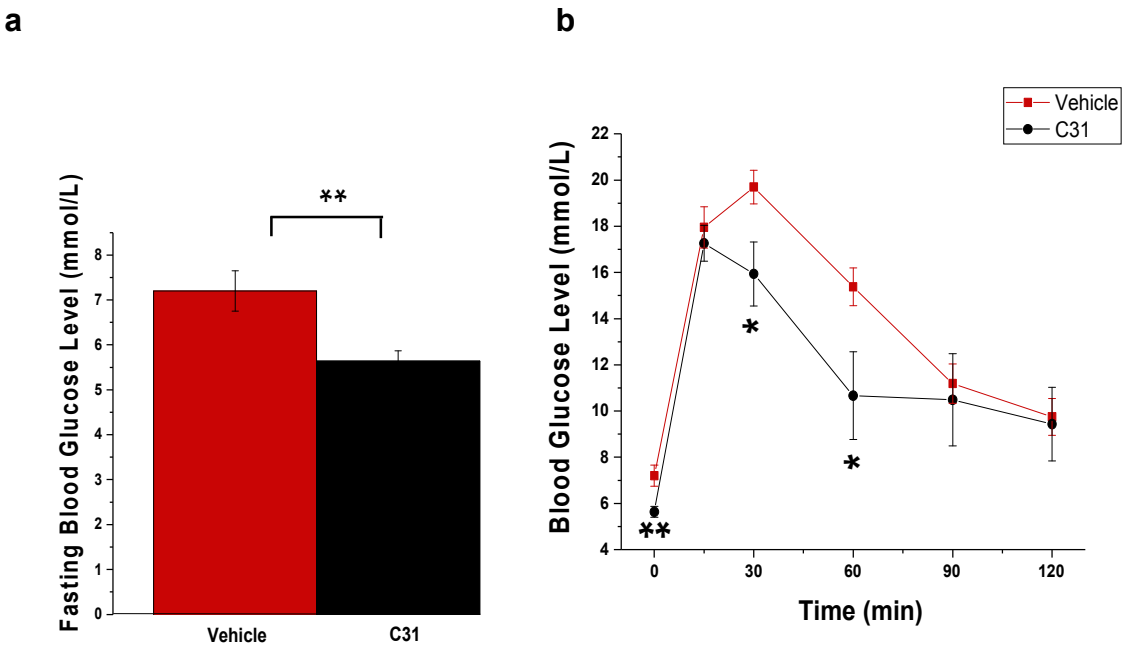

Figure 28. Glucose tollerance test of vehicle and C31 groups. (a) Fasting blood glucose level. (b) Blood glucose level at set time points after a single glucose injection (n = 10). Statistical significances are indicated by * (P<0.05) ** (P<0.01). Data shown as means ± standard error of the mean (SEM).

6.7 Insulin tolerance test

Also at 5 weeks of 60% HFD diet and oral treatment, mice were fasted for 2 hours prior to an insulin tolerance test (ITT). Fasting blood glucose levels (Fig.29a) show the group receiving C31 treatment were significantly higher ($P<0.001$) after fast. A single intraperitoneal insulin injection was administered and blood glucose was measured from the tail tip bleed at 15, 30, 60, 90 and 120 minutes (Fig.29b). Blood glucose level show that after 30 minutes the C31 group had a significant lower reading ($P<0.05$).

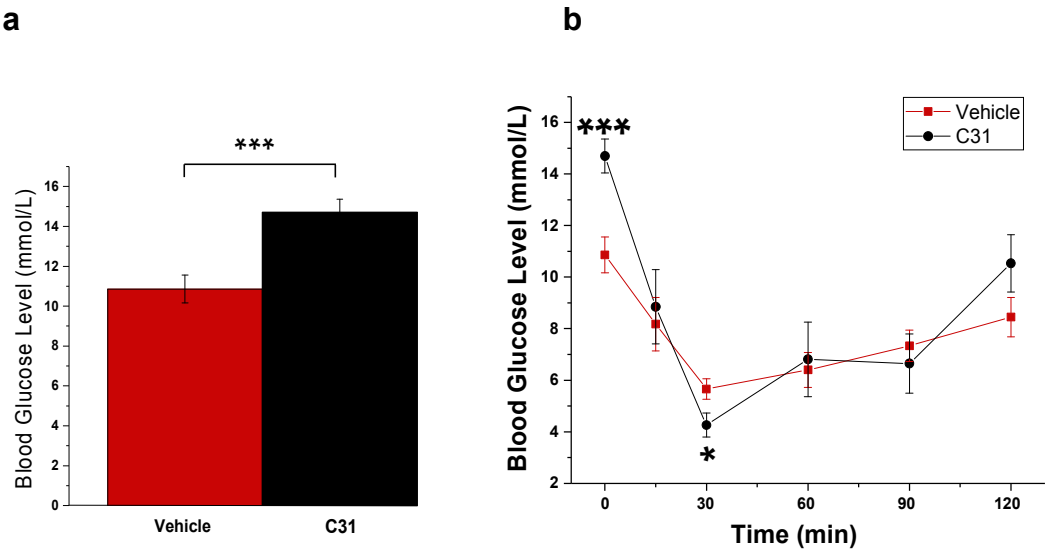

Figure 29. Insulin tolerance test of vehicle and C31 groups. (a) Fasting blood glucose level. (b) Blood glucose level at set time points after a single insulin injection (n = 10). Statistical significances is indicated by * ($P<0.05$) *** ($P<0.001$). Data shown as means ± standard error of the mean (SEM).

6.8 Organ weights

After harvesting the vehicle and C31 mice the inguinal fat (Fig.30a) and epididymal fat (Fig.30b) were weighed. Both results show that C31 group were significantly lower in weight (P<0.01) lower in weight when compared with the vehicle group. However the liver (Fig.30c) of the C31 mice show a significant increase in weight (P<0.01) more than the vehicle group.

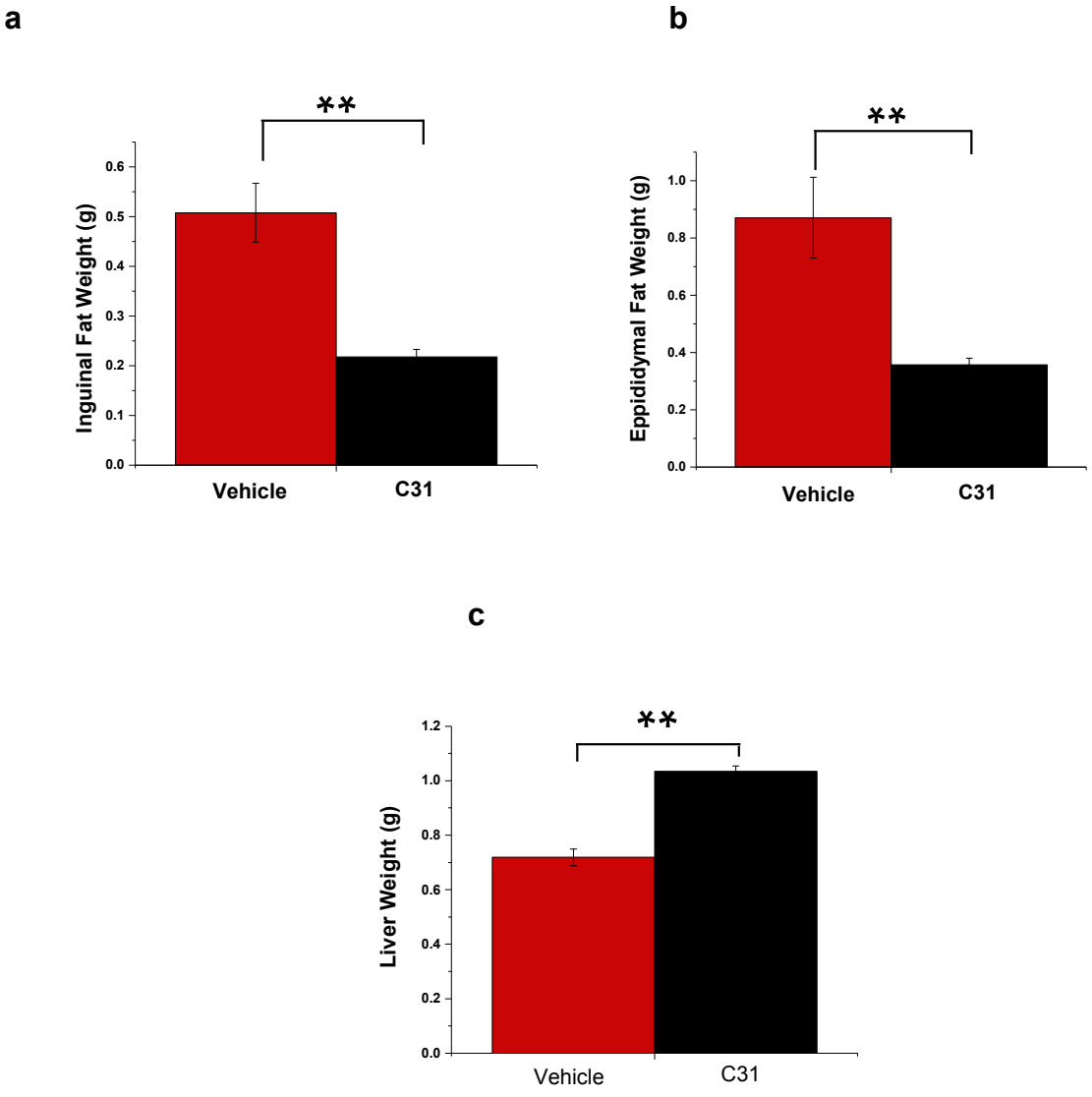

Figure 30. Harvest weight of adipose tissue and liver of the vehicle and C31 groups (n = 10). (a) Inguinal fat. (b) Epididymal fat. (c) Liver. Statistical significances is indicated by ** (P<0.01). Data shown as means ± standard error of the mean (SEM).

6.9 Discussion

The biweekly weighing data show that at the end of the study the C31-treated mice were significantly lighter ($P<0.01$) when compared to the vehicle group (Fig.26).

GTT was conducted at 5 weeks from the start of study and shows (Fig.28) the C31 group to have a lower fasting blood glucose level and tolerated glucose injection better. ITT (Fig.29) was also completed at 5 weeks and shows the blood fasting level of the C31 group was significantly higher after the 2 hour fasting however after a single dose of Insulin the blood glucose level dropped showing high sensitivity to insulin. Furthermore the adipose tissue weights were significantly lighter in the C31 group but the liver weights were significantly more ($P<0.01$) at the end of the study (Fig.30).

Chapter 7

7.1 Conclusion

Members of the TRPC channel subfamily 1, 4, 5 have been associated with various pathologies, ranging from cancer to cardiovascular disease. Therefore, it is unsurprising that TRPC channels have been viewed as potential molecular targets of interest. The data from the study shows an exciting possibility that the TRPC 1, 4, 5 channels could be pursued as molecular targets for managing metabolic disorders.

For the study containing 60% high fat diet (HFD), it was found that when HFD was introduced and fed for an 8 week period, the effects on weight gain and adipose tissue was not significant between the TRPC genotypes. Although as expected all HFD groups did experience weight and adipose tissue gain when compared to the same genotypes fed on chow diet. However TRPC4 KO and the TRPC4/5 double KO showed a trend towards reduction in weight gain over the 8 weeks of high fat feeding it was not consistent as previous groups have found. TRPC5 was heavier in body weight and adipose weight than the TRPC wt but was not significant. The data shown in this chapter contradicts what other groups have reported (Ma et al.2022, Sukumar et al 2012). Analysing some of the data as ratios (rates of change over time) rather than endpoint may give other interpretations. If the data regarding weight gained had been normalised to 0 and the rates were measured, it maybe that the TRPC5 KO mice significantly gained weight.

Under normal chow fed conditions the TRPC5 KO mice were more sensitive to insulin and heavier than the TRPC WT mice. However it also appeared to be more active when observing the data collected for the CLAMs, the TRPC5 KO did trigger more beam breaks. This could be further

validated by using the free running wheel attachment in the CLAMs system where animals are free to exercise if chose.

There are broadly-speaking two types of adipose tissue, white and brown which have distinct developmental origins and functions. White adipose tissue plays a pivotal role in maintaining whole-body energy homeostasis by storing triglycerides when energy is in surplus, releasing free fatty acids as a fuel during energy shortage. (Feng et al., 2013). Obesity is characterised by the expansion of white adipose tissue (Feng et al., 2013) and results from imbalance in energy accrued through eating and drinking relative to energy burned through physical activity (Millward, 2013). This report shows that TRPC 4/5 double KO causes lower levels of triglycerides and weight of white adipose tissue.

The results of the small molecule inhibiter C31 show a significant reduction in body weight of wild type mice over the course of the study (6 weeks treatment). There was also a reduction in the weight of inguinal and epididymal fat. The data from this chapter is consistent with finding from other group (Ma 2022), as C31 is most likely a specific molecule for TRPC1/4/5 inhibition (Rubaiy et *a*l., 2017).

The data observed from my studies suggest that inhibition of TRPC4 and TRPC5 channels didn't protect against excess weight gain in HFD conditions associated with sustained excess calorie intake as predicted, contradicting the C31 study result where the mice did show a reduction in body and adipose tissue weight.

Furthermore the C31 mice it was observed that as well as an increase in liver weight the liver appeared to have a marbling effect. This could be a redistribution of adipose in these mice, however this was not robustly inverstigated in this study but in the future histologial analyses of the liver should be performed.

The results from the C31 study suggests ion pore blockers of TRPC channels could protect against obesity, however the results of the HFD study contradicted this. The potential explanations could be the combination of TRPC4, TRPC5 and TRPC1 is what is needed to protect against excess body fat storage.

7.2 Limitations

In the transgenic TRPC mice I did not see the effects of the reduction in weight gain in animals fed on 60% HFD. The limiting factor was that the mice were still putting on weight with a similar curve to the chow diet however it was slower. If we had have waited until the mice had plateaued in body weight there could have been a difference in genotypes.

Having had more time available in the laboratory, it would have been favourable to have some histology and staining on the different TRPC genotypes adipose tissue. The size and number of adipocytes should be measured to give a greater understanding to what the baseline condition are and changes in the setting of obesity.

There are also limitations with using global knockouts when looking at obestity. The gene is is not specifically investigating a specific role in adipose tissue therefore could have affect other tissues for example skeletal, muscular and brain and this could contribute to affecting metabolism and therefore possibly confound some of the results observed.

7.3 Further work

In future studies, there is the potential to further investigate the link between TRPC 1, 4 and 5 and obesity/adipocyte metabolism *in vivo*. This would consist of sourcing a genetically modified TRPC1 KO mouse and transgenic breeding.

Firstly, to generate mouse models for TRPC1/4 double KO, TRPC1/5 double KO and a TRPC 1, 4, 5 triple KO. The mice should then be placed on a protocol where two studies would take place. One on transgenic mice TRPC1/4 double KO, TRPC1/5 double KO and a TRPC 1, 4, 5 triple KO to be observed on standard chow diet starting at 8 weeks of age. Baseline conditions should be documented for both glucose tolerance and insulin tolerance. Weekly weights should be recorded and metabolic profiling must be done and analysed in the CLAMs. Organs would then be weighed and analysed further.

The studies would then be repeated in the setting of obesity. This would include the transgenic mice TRPC1/4 double KO, TRPC1/5 double KO and the TRPC1, 4, 5 triple KO. 60% HFD feeding would commence at 8 weeks of age however as stated in the limitations, it would be advantageous to wait for the mouse weights to plateaued before concluding.

The conclusion of these experiments would give us a greater understanding in the role TRPC 1 plays alongside TRPC 4 and TRPC 5 in vivo as a subunit. Studies using these mice would then eliminate the effects of other factors and show a more accurate representation of the effects HFD on TRPC 1, 4, 5 in *vivo.*

References

Akbulut, Y., Gaunt, H.J., Muraki, K., Ludlow, M.J., Amer, M.S., Bruns, A., Vasudev, N.S., Radtke, L., Willot, M., Hahn, S., Seitz, T., Ziegler, S., Christmann, M., Beech, D.J. and Waldmann, H. 2015. (-)-Englerin A is a potent and selective activator of TRPC4 and TRPC5 calcium channels. *Angew Chem Int Ed Engl.* **54** (12), pp.3787-91.

Aydar, E., Yeo, S., Djamgoz, M. and Palmer, C. 2009. Abnormal expression, localization and interaction of canonical transient receptor potential ion channels in human breast cancer cell lines and tissues: a potential target for breast cancer diagnosis and therapy. *Cancer Cell Int.* **9**, p23.

Bean, B.P. 2007. The action potential in mammalian central neurons. *Nature Reviews Neuroscience*, **8** (6), pp.451–465.

Beech, D.J. 2013. Characteristics of transient receptor potential canonical calcium-permeable channels and their relevance to vascular physiology and disease. *Circ J.* **77**(3), pp.570-9.

Bon, R.S. and Beech, D.J. 2013. In pursuit of small molecule chemistry for calcium-permeable non-selective TRPC channels – mirage or pot of gold? *British Journal of Pharmacology.* **170**(3), pp.459-474.

Calupca, M.A., Locknar, S.A. and Parsons, R.L. 2002, TRPC6 immunoreactivity is colocalized with neuronal nitric oxide synthase in extrinsic fibers innervating guinea pig intrinsic cardiac ganglia. J. Comp. Neurol., **450**: 283-291.

Chung, Hye Soo. Soon Young Hwang, Ju Hee Choi, Hyun Jung Lee, Nam Hoon Kim, Hye Jin Yoo, Ji-A Seo, Sin Gon Kim, Nan Hee Kim, Sei Hyun Baik, Kyung Mook Choi, 2018. Implications of circulating Meteorin-like (Metrnl) level in human subjects with type 2 diabetes, Diabetes Research and Clinical Practice,**136**,pp 100-107.

Clapham, D.E. 2003. TRP channels as cellular sensors. *Nature.* **426** (6966), pp.517-24.

Clapham, D.E., Runnels L.W. and Strübing C. 2001. The trp ion channel family. *Nat Rev Neurosci.* **2**(6), pp.387-396.

Cosens, D.J. and Manning, A. 1969. Abnormal electroretinogram from a Drosophila mutant. *Nature.* **224**(5216), pp.285-7.

Ding Jian Hong, Zhe Jin, Xiaoxu Yang, Jun Lou, Weixi Shan, Yanxia Hu, Qian Du, Qiushi Liao, Jingyu Xu, Rui Xie, 2020, Plasma membrane Ca2+-permeable channels and sodium/calcium exchangers in tumorigenesis and tumor development of the upper gastrointestinal tract, 2020, Cancer Letters. 475, pp 14-21.

Dong, X., Bai, X., Ni, J. *et al.* 2020. Exosomes and breast cancer drug resistance. *Cell Death Dis* **11,** 987.

Elsaesser R, Montani G, Tirindelli R, Paysan J 2005 Phosphatidyl-inositide signalling proteins in a novel class of sensory cells in the mammalian olfactory epithelium. Eur J Neurosci **21**(10): pp 2692–2700.

Evans, J. F., Lee, J. H., and Ragolia, L. 2009. Ang-II-induced Ca^{2+} influx is mediated by the 1/4/5 subgroup of the transient receptor potential proteins in cultured aortic smooth muscle cells from diabetic Goto-Kakizaki rats. *Mol. Cell. Endocrinol.* **302**, pp 49–57.

Feng, B., Zhang, T., & Xu, H. 2013. Human adipose dynamics and metabolic health. Annals of the New York Academy of Sciences, **1281**: 160–177.

Fleig, A. and Penner, R. 2004. The TRPM ion channel subfamily: molecular, biophysical and functional features. *Trends Pharmacol Sci.* **25**(12), pp.633-9.

Flockerzi, V.; Nilius, B. 2014 TRPs: Truly remarkable proteins. Handb. Exp. Pharmacol. **222**, 1–12. pp 55.

Freichel, M., Suh, S.H., Pfeifer, A., Schweig, U., Trost, C., Weissgerber, P., Biel, M., Philipp, S., Freise, D., Droogmans, G., Hofmann, F., Flockerzi, V. and Nilius, B. 2001. Lack of an endothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in TRP4-/- mice. *Nat Cell Biol.* **3**(2), pp.121-7.

Gao, YY., Tian, W., Zhang, HN. *et al.* 2012 Canonical transient receptor potential channels and their modulators: biology, pharmacology and therapeutic potentials. *Arch. Pharm. Res.* **44,** 354–377 (2021).

Hardie, R.C. and Minke, B. 1992. The trp gene is essential for a light-activated Ca^{2+} channel in Drosophila photoreceptors. *Neuron.* **8**(4), pp.643-51.

Hartmann, J., Dragicevic, E., Adelsberger, H., Henning, H. A., Sumser, M., Abramowitz, J., et al. 2008. TRPC3 channels are required for synaptic transmission and motor coordination. *Neuron* **59**, 392–398.

Huang, J.-H., He, G.-W., Xue, H.-M., Yao, X.-Q., Liu, X.-C., Underwood, M. J., et al. 2011. TRPC3 channel contributes to nitric oxide release: significance during normoxia and hypoxia–reoxygenation. *Cardiovasc. Res.* **91**, pp 472–482.

Inoue, R., Jensen, L. J., Jian, Z., Shi, J., Hai, L., Lurie, A. I., et al. 2009. Synergistic activation of vascular TRPC6 channel by receptor and mechanical stimulation via phospholipase C/diacylglycerol and phospholipase A2/ω-Hydroxylase/20-HETE Pathways. *Circ. Res.* **104**, 1399–1409.

Istock (2020). *Ligand-gated ion channel. channel proteins which open to ions Na, K, Ca, or Cl*. *Istockphoto.com*.

Jeon JP, Hong C, Park EJ, Jeon JH, Cho NH, Kim IG, Choe H, Muallem S, Kim HJ, So I . 2012 Selective Galphai subunits as novel direct activators of transient receptor potential canonical (TRPC)4 and TRPC5 channels. J Biol Chem **287**: pp 17029–17039.

Jeon,Jaepyo, Bu,Fan, Sun,Guanghua, Tian,Jin-Bin, Ting,Shun-Ming, Li,Jun, Aronowski,Jaroslaw, Birnbaumer,Lutz, Freichel,Marc, Zhu,Michael X. 2021. Contribution of TRPC Channels in Neuronal Excitotoxicity Associated With Neurodegenerative Disease and Ischemic Stroke. **8**. 1755.

Jiang, H.-N., Zeng, B., Zhang, Y., Daskoulidou, N., Fan, H., Qu, J. and Xu, S. 2013. Involvement of TRPC Channels in Lung Cancer Cell Differentiation and the Correlation Analysis in Human Non-Small Cell Lung Cancer. *PLoS ONE.* **8**(6), pe67637.

Juliano, R.L. and Ling, V. 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* **455**(1), pp.152-162.

Just S, Chenard BL, Ceci A, Strassmaier T, Chong JA, Blair NT, Gallaschun RJ, Del Camino D, Cantin S, D'Amours M, Eickmeier C, Fanger CM, Hecker C, Hessler DP, Hengerer B, Kroker KS, Malekiani S, Mihalek R, McLaughlin J, Rast G, Witek J, Sauer A, Pryce CR, Moran MM (2018) Treatment with HC-070, a potent inhibitor of TRPC4 and TRPC5, leads to anxiolytic and antidepressant effects in mice. PLoS ONE 13:e0191225.

Kim, J.; Ko, J.; Hong, C.; So, I. 2020 Structure–Function Relationship and Physiological Roles of Transient Receptor Potential Canonical (TRPC) 4 and 5 Channels. *Cells*, ***9***, 73.

Kochukov, M. Y., Balasubramanian, A., Abramowitz, J., Birnbaumer, L., and Marrelli, S. P. 2014. Activation of endothelial transient receptor potential C3 channel is required for small conductance calcium-activated potassium channel activation and sustained endothelial hyperpolarization and vasodilation of cerebral artery. *J. Am. Heart Assoc.* **3**:e000913.

Krout, D., Schaar, A., Sun, Y., Sukumaran, P., Roemmich, J.N., Singh, B.B. and Claycombe-Larson, K.J. 2017. The TRPC1 Ca2+-permeable channel inhibits exercise-induced protection against high-fat diet-induced obesity and type II diabetes. *Journal of Biological Chemistry*, [online] **292**(50), pp.20799–20807.

Lievremont, JP., Bird, G.S. and Putney, J.W. 2004. Canonical transient receptor potential TRPC7 can function as both a receptor- and store-operated channel in HEK-293 cells. *Am J Physiol Cell Physiol.* **287** (6), pp.C1709-16.

Liu, X., Wang, W., Singh, B.B., Lockwich, T., Jadlowiec, J., O'Connell, B., Wellner, R., Zhu, M.X. and Ambudkar, I.S. 2000. Trp1, a candidate protein for the store-operated Ca(2+) influx mechanism in salivary gland cells. *J Biol Chem.* **275** (5), pp.3403-11.

Ma, H., He, C., Li, L. *et al.* TRPC5 deletion in the central amygdala antagonizes high-fat diet-induced obesity by increasing sympathetic innervation. *Int J Obes* (2022)

Ma, X., Cai, Y., He, D., Zou, C., Zhang, P., Lo, C.Y., Xu, Z., Chan, F. L., Yu, S., Chen, Y., Zhu, R., Lei, J., Jin, J. and Yao, X. 2012. Transient receptor potential channel TRPC5 is essential for P-glycoprotein induction in drug-resistant cancer cells. *Proc Natl Acad Sci U S A.* **109** (40), pp.16282-7.

Miller, M., Shi, J., Zhu, Y., Kustov, M., Tian, J. Stevens, A., Wu, M., Xu, J., Long, S., Yang, P., Zholos, A.V., Salovich, J.M., Weaver, D.C., Hopkins, C.R., Lindsley, C.W., McManus, O., Li, M. and Zhu, M.X. 2011. Identification of ML204, a Novel Potent Antagonist That Selectively Modulates Native TRPC4/C5 Ion Channels. *The Journal of Biological Chemistry.* **286**(38), pp.33436-33446.

Millward, D. J. 2013. Energy balance and obesity: a UK perspective on the gluttony v. sloth debate. Nutrition Research Reviews, **26**(2): 89–109.

Minke, B. 2010. The history of the Drosophila TRP channel: The birth of a new channel superfamily. *Journal of neurogenetics.* **24**(4), pp.216-233.

Minke, B. and Cook, B. 2002. TRP Channel Proteins and Signal Transduction. *Physiological Reviews.* **82**(2), pp.429-472.

Minke, B., Wu, C.-F., and Pak, W. L. 1975. Induction of photoreceptor voltage noise in the dark in *Drosophila* mutant. *Nature* **258**, pp 84–87.

Mita, M., Ito, K., Taira, K., Nakagawa, J. I., Walsh, M. P., and Shoji, M. 2010. Attenuation of store-operated Ca^{2+} entry and enhanced expression of TRPC channels in caudal artery smooth muscle from Type 2 diabetic Goto-Kakizaki rats. *Clin. Exp. Pharmacol. Physiol.* **37**, pp 670–678.

Montell, C. 2005. The TRP Superfamily of Cation Channels. *Science Signaling.* **2005** (272), pp.re3-re3.

Montell, C. and Rubin, G.M. 1989. Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. *Neuron.* **2**(4), pp.1313-23.

Moraes Raiana dos Anjos, Webb R. Clinton, Silva Darízy Flávia Vascular Dysfunction in Diabetes and Obesity: Focus on TRP Channels Frontiers in Physiology 12, 2021.

Moran, M.M., Xu, H. and Clapham, D.E. 2004. TRP ion channels in the nervous system. *Curr Opin Neurobiol.* **14**(3), pp.362-9.

Mori, Y., Takada, N., Okada, T., Wakamori, M., Imoto, K., Wanifuchi, H., Oka, H., Oba, A., Ikenaka, K. and Kurosaki, T. 1998. Differential distribution of TRP Ca^{2+} channel isoforms in mouse brain. *Neuroreport.* **9** (3), pp.507-15.

Nilius, B., Owsianik, G., Voets, T. and Peters, J.A. 2007. Transient Receptor Potential Cation Channels in Disease. *Physiological Reviews.* **87** (1), pp.165-217.

Okada, T., Inoue, R., Yamazaki, K., Maeda, A., Kurosaki, T., Yamakuni, T., Tanaka, I., Shimizu, S., Ikenaka, K., Imoto, K. and Mori, Y. 1999. Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. Ca(2+)-permeable cation channel that is constitutively activated and enhanced by stimulation of G protein-coupled receptor. *J Biol Chem.* **274** (39), pp.27359-70.

Pakhomov. Andrei G, Angela M. Bowman, Bennett L. Ibey, Franck M. Andre, Olga N. Pakhomova, Karl H. Schoenbach, Lipid nanopores can form a stable, ion

channel-like conduction pathway in cell membrane, Biochemical and Biophysical Research Communications, Volume 385, Issue 2, 2009,Pages 181-186.

Pedersen, S.F., Owsianik, G. and Nilius, B. 2005. TRP channels: An overview. *Cell Calcium.* **38**(3–4), pp.233-252.

Premanand C. Sundivakkam, Marc Freichel, Vandana Singh, Joseph P. Yuan, Stephen M. Vogel, Veit Flockerzi, Asrar B. Malik and Chinnaswamy Tiruppathi Molecular Pharmacology April 2012, **81** (4) pp.510-526.

Qiu j, Yuan Fang, Oline K. Rønnekleiv and Martin J. Kelly **L**eptin Excites Proopiomelanocortin Neurons via Activation of TRPC Channels Journal of Neuroscience 27 January 2010, 30 (4) 1560-1565.

Qu, Y.-Y., Wang, L.-M., Zhong, H., Liu, Y.-M., Tang, N., Zhu, L.-P., et al. 2017. TRPC1 stimulates calcium-sensing receptor-induced store-operated Ca^{2+} entry and nitric oxide production in endothelial cells. *Mol. Med. Rep.* **16**, pp 4613–4619.

Ramsey, I.S., Delling, M. and Clapham, D.E. 2006. An introduction to TRP channels. *Annu Rev Physiol.* **68**, pp.619-47.

Reading, S. A., Earley, S., Waldron, B. J., Welsh, D. G., and Brayden, J. E. 2005. TRPC3 mediates pyrimidine receptor-induced depolarization of cerebral arteries. *Am. J. Physiol. Circ. Physiol.* **288**, pp 2055–2061.

Riccio A, Li Y, Tsvetkov E, Gapon S, Yao GL, Smith KS, Engin E, Rudolph U, Bolshakov VY, Clapham DE (2014) Decreased anxiety-like behavior and Gαq/11-dependent responses in the amygdala of mice lacking TRPC4 channels. J Neurosci 34:3653–3667.

Riccio A, Medhurst AD, Mattei C, Kelsell RE, Calver AR, Randall AD, Benham CD, Pangalos MN (2002) mRNA distribution analysis of human TRPC family in CNS and peripheral tissues. Brain Res Mol Brain Res **109**(1–2): pp 95–10.

Rode, B., Yuldasheva, N.Y., Baxter, P.D. Sedo, A., Ainscough, J.F., Shires, M., Kearney, M.T., Bailey, M.A., WheatcroftS.B., Beech D.J., TRPC5 ion channel permeation promotes weight gain in hypercholesterolaemic mice. *Sci Rep* **9,** 773 (2019).

Rodrigues.T, Gabriela Nohemi Nunez Estevez, Ivarne Luis dos Santos Tersariol, $Na+/Ca^{2+}$ exchangers: Unexploited opportunities for cancer therapy?,Biochemical Pharmacology, Volume 163, 2019,Pages 357-361.

Rowell, J., Koitabashi, N. and Kass, D.A. 2010. TRP-ing up Heart and Vessels: Canonical Transient Receptor Potential Channels and Cardiovascular Disease. *Journal of cardiovascular translational research.* **3**(5), pp.10.

Rubaiy, H.N.; Ludlow, M.J.; Bon, R.S.; Beech, D.J. Pico145—Powerful new tool for TRPC1/4/5 channels. *Channels (Austin)* **2017**, *11*, 362–364.

Santoni, Giorgio; Farfariello, Valerio Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets -Immune, Endocrine & Metabolic Disorders), Volume **11**, Number 1, 2011, pp. 54-67(14).

Schaefer, M., Plant, T.D., Obukhov, A.G., Hofmann, T., Gudermann, T. and Schultz, G. 2000. Receptor-mediated regulation of the nonselective cation channels TRPC4 and TRPC5. *J Biol Chem.* **275**(23), pp.17517-26.

Shapovalov, G., Ritaine, A., Skryma, R. *et al.* Role of TRP ion channels in cancer and tumorigenesis. *Semin Immunopathol* **38,** 357–369 (2016).

Song, Michael & Yuan, Jason. 2010. Introduction to TRP Channels: Structure, Function, and Regulation. Advances in experimental medicine and biology. **661**. pp.99-108.

Stokłosa, Paulina, Borgström, Anna, Kappel, Sven, Peinelt, Christine. 2020. TRP Channels in Digestive Tract Cancers. International Journal of Molecular Sciences **21** (5) .pp 1877.

Storch, U., Forst, A.L., Philipp, M., Gudermann, T. and Mederos y Schnitzler, M. 2012. Transient receptor potential channel 1 (TRPC1) reduces calcium permeability in heteromeric channel complexes. *J Biol Chem.* **287**(5), pp.3530-40.

Strübing, C., Krapivinsky, G., Krapivinsky, L. and Clapham, D.E. 2001. TRPC1 and TRPC5 Form a Novel Cation Channel in Mammalian Brain. *Neuron.* **29**(3), pp.645-655.

Sukumar, P., Sedo, A., Li, J., Wilson, L.A., O'Regan, D., Lippiat, J.D., Porter, K.E., Kearney, M.T. Ainscough, J.F. and Beech, D.J. 2012. Constitutively active TRPC channels of adipocytes confer a mechanism for sensing dietary fatty acids and regulating adiponectin. *Circ Res.* **111**(2), pp.191-200.

Sukumaran, P., Sun, Y., Schaar, A., Selvaraj, S., Singh, B.B. (2017). TRPC Channels and Parkinson's Disease. In: Wang, Y. (eds) Transient Receptor Potential Canonical Channels and Brain Diseases. Advances in Experimental Medicine and Biology, vol 976. Springer, Dordrecht.

Sun Y, Sukumaran P, Schaar A, Singh BB. TRPM7 and its role in neurodegenerative diseases. Channels (Austin). 2015. **9** (5), pp 253-61.

Trebak, M., Vazquez, G., Bird, G.S. and Putney Jr, J.W. 2003. The TRPC3/6/7 subfamily of cation channels. *Cell Calcium.* **33**(5-6), pp.451-61.

Trebak, M., Vazquez, G., Bird, G.S. and Putney Jr, J.W. 2003. The TRPC3/6/7 subfamily of cation channels. *Cell Calcium.* **33**(5-6), pp.451-61.

Venkatachalam, Kartik, and Craig Montell. "TRP channels." *Annual review of biochemistry* vol. 76 (2007): 387-417.

Walder, R.Y., Zheng, F. and Gill, D.L. 2002. Mutation of TRPM6 causes familial hypomagnesemia with secondary hypocalcemia. *Nat Genet.* **31**(2), pp.171-4.

Wang, J., Shimoda, L. A., and Sylvester, J. T. 2004. Capacitative calcium entry and TRPC channel proteins are expressed in rat distal pulmonary arterial smooth muscle. *Am. J. Physiol. Cell. Mol. Physiol.* **286**, pp L848–L858.

Wang, T. et al. 2017 Increasing circulating exosomes-carrying TRPC5 predicts chemoresistance in metastatic breast cancer patients. *Cancer Sci.* **108**, 448–454.

Wang, T., Hung, C.C. & Randall, D.J. The comparative physiology of food deprivation: from feast to famine. *Annu Rev Physiol* **68**, 223-251 (2006).

Wang, Z., Yang, J., Qi, J., Jin, Y., and Tong, L. 2020. Activation of NADPH/ROS pathway contributes to angiogenesis through JNK signaling in brain endothelial cells. *Microvasc. Res.* **131**, 104012.

Warren, E. J., C. N. Allen, L. Brown and D. W. Robinson 2006 The light-activated signaling pathway in SCN-projecting rat retinal ganglion cells. *Eur. J. Neurosci.* **23**(9), 2477– 2487.

Wes, P.D., Chevesich, J., Jeromin, A., Rosenberg, C., Stetten, G. and Montell, C. 1995. TRPC1, a human homolog of a Drosophila store-operated channel. *Proc Natl Acad Sci U S A.* **92**(21), pp.9652-6.

White P J, Calcium channels in higher plants, Biochimica et Biophysica Acta (BBA) - Biomembranes, Volume 1465, Issues 1–2, 2000,Pages 171-189.

Winn, M.P., Conlon, P.J., Lynn, K.L., Farrington, M.K., Creazzo, T., Hawkins, A.F., Daskalakis, N., Kwan, S.Y., Ebersviller, S., Burchette, J.L., Pericak-Vance, M.A., Howell, D.N., Vance, J.M. and Rosenberg, P.B. 2005. A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science.* **308**(5729), pp.1801-4.

Wölfle, S. E., Navarro-Gonzalez, M. F., Grayson, T. H., Stricker, C., and Hill, C. E. 2010. Involvement of nonselective cation channels in the depolarisation initiating vasomotion. *Clin. Exp. Pharmacol. Physiol.* 37, 536–543.

Wong, C.O., Sukumar, P., Beech, D.J. and Yao, X. 2010. Nitric oxide lacks direct effect on TRPC5 channels but suppresses endogenous TRPC5-containing channels in endothelial cells. *Pflugers Arch.* **460** (1), pp.121-30.

Wu, X., Ren, G., Zhou, R. *et al.* 2019. The role of Ca^{2+} in acid-sensing ion channel 1a-mediated chondrocyte pyroptosis in rat adjuvant arthritis. *Lab Invest* **99,** 499–513.

Xu, S.Z., Sukumar, P., Zeng, F., Li, J., Jairaman, A., English, A., Naylor, J., Ciurtin, C., Majeed, Y., Milligan, C.J., Bahnasi, Y.M., Al-Shawaf, E., Porter, K.E., Jiang, L.H., Emery, P., Sivaprasadarao, A. and Beech, D. J. 2008. TRPC channel activation by extracellular thioredoxin. *Nature.* **451** (7174), pp.69-72.

Zeng, Bo; Yuan, Cunzhong; Yang, Xingsheng; L. Atkin, Stephen; Xu, Shang-Zhong Current Cancer Drug Targets, Volume **13**, Number 1, 2013, pp. 103-116(14).

Zheng, F. and Phelan, K.D. 2014. The Role of Canonical Transient Receptor Potential Channels in Seizure and Excitotoxicity. *Cells.* **3** (2), pp.288-303.
Zheng, J. 2013. Molecular Mechanism of TRP Channels. *Comprehensive Physiology.* **3** (1), pp.221-242.

Zhu, Y., Pan, Q., Meng, H., Jiang, Y., Mao, A., Wang, T., Hua, D., Yao, X., Jin, J. and Ma, X. 2015. Enhancement of vascular endothelial growth factor release in long-term drug-treated breast cancer via transient receptor potential channel 5-Ca2+-hypoxia-inducible factor 1α pathway. *Pharmacological Research.* **93**, pp.36-42.

Zitt, C., Obukhov, A.G., Strübing, C., Zobel, A., Kalkbrenner, F., Lückhoff, A. and Schultz, G. 1997. Expression of TRPC3 in Chinese Hamster Ovary Cells Results in Calcium-activated Cation Currents Not Related to Store Depletion. *The Journal of Cell Biology.* **138**(6), pp.1333-1341.