UNIVERSITÄT ZU LÜBECK

Institute for Neurobiology
of the University of Lübeck
Director: Prof. Dr. rer. nat. Henrik Oster

"Circadian regulation of murine hypothalamic insulin actions"

Dissertation
for Fulfilment of Requirements
for the Doctoral Degree
of the University of Lübeck

From the Department of Natural Sciences

Submitted by

Ankita Galinde
from Pune, India

Lübeck, Germany 2025

First referee: Prof. Dr. rer. nat. Henrik Oster

Second referee: Prof. Dr. rer. nat. Henriette Kirchner

Date of oral examination: October 21, 2025

Approved for printing: Lübeck, October 22, 2025

Declaration

Herewith, I confirm that I have written the present PhD thesis independently and with no other sources and aids than quoted.

Lübeck, June 2025

Ankita Galinde

“What makes you, you?

I am the relationship between my neurons.

…

We’re not fixed.

From cradle to grave, we’re works in progress.”

- Dr. David Eagleman

Table of Contents

Abstract

Insulin, a key pleiotropic hormone plays a vital role in both peripheral metabolism and central nervous system functions, including appetite regulation and cognition. Emerging evidence suggests that insulin signalling and circadian timing mechanisms interact closely in a bidirectional way. However, it remains unclear whether actions of insulin in brain are modulated by the circadian clock. This PhD thesis investigates whether regulatory functions of insulin in murine hypothalamus are temporally gated, focusing on its role in maintaining metabolic homeostasis and supporting neuronal health.

Using a combination of immortalised hypothalamic neuronal cultures and tissue preparations from arcuate nucleus-median eminence (Arc-ME) region, this study first validated the presence of robust, self-sustained circadian oscillations in both models. Insulin receptor expression was confirmed across systems. Comprehensive sets of dose- and time-response experiments were conducted to assess insulin-induced transcriptional changes in metabolic neuropeptides. However, these targeted approaches revealed minimal effects, suggesting that insulin's central metabolic actions may be either subtle or dependent on a more complex physiological context.

To overcome the limitations of targeted approach, transcriptomic profiling (3' BRB-seq) was performed on the Arc-ME tissue preparations treated with insulin at peak and trough circadian phases. The data revealed that time-of-day exerted a stronger influence on gene expression than insulin treatment. No interaction between circadian phase and insulin response was detected. With treatment-effect alone, fourteen insulin-responsive genes were identified, three of which, neurofilament medium polypeptide (*Nefm*), transient receptor potential cation channel, subfamily C, member 3 (*Trpc3)* and oligodendrocytic myelin paranodal and inner loop protein (*Opalin)*, were predominantly expressed in the brain and associated with neuronal development, structural maintenance and myelination. These genes showed modest but consistent upregulation in response to insulin, independent of circadian phase, suggesting a circadian-independent, tonic regulation.

Taken together, the findings of this thesis support a model in which insulin may exert its central effects via a dual regulatory mode; a circadian gated mechanism potentially governing metabolic functions, and a time-independent, tonic mechanism supporting neuronal health and maintenance. The study marks the complexity in investigating insulin's role in central metabolic homeostasis, while the identification of neuroprotective, insulin-responsive genes offers a valuable foundation to study insulin's multifaceted actions in the brain.

Zusammenfassung

Insulin, ein zentrales pleiotropes Hormon, spielt eine entscheidende Rolle sowohl im peripheren Stoffwechsel als auch in den Funktionen des zentralen Nervensystems, einschließlich der Appetitregulation und der Kognition. Neue Erkenntnisse deuten darauf hin, dass Insulinwirkung und zirkadiane Zeitmechanismen in einer bidirektionalen Weise eng miteinander interagieren. Dennoch ist bislang unklar, ob die Wirkung von Insulin im Gehirn durch die zirkadiane Uhr moduliert wird. Diese Dissertation untersucht, ob die regulatorische Funktion von Insulin im murinen Hypothalamus zeitlich gesteuert ist, mit besonderem Fokus auf seine Rolle bei der Aufrechterhaltung der metabolischen Homöostase und der Unterstützung der neuronalen Gesundheit.

Unter Verwendung von immortalisierten hypothalamischen Zelllinien und Gewebepräparationen des Nucleus Arcuatus–Eminentia mediana (Arc-ME) wurde zunächst das Vorhandensein robuster, selbsttragender zirkadianer Oszillationen in beiden Modellen bestätigt. Die Expression des Insulinrezeptors konnte in beiden Systemen nachgewiesen werden, und es wurden umfassende Dosis- und Zeitverlaufsexperimente durchgeführt, um insulininduzierte transkriptionelle Veränderungen klassischer metabolischer Neuropeptide zu analysieren. Diese gezielten Ansätze zeigten jedoch nur minimale Effekte, was darauf hinweist, dass die zentralen metabolischen Wirkungen von Insulin entweder subtil sind oder von einem komplexeren physiologischen Kontext abhängen könnten.

Um die Grenzen des gezielten Ansatzes zu überwinden, wurde ein transkriptomisches Profiling (3'-BRB-seq) an den mit Insulin behandelten Arc-ME-Gewebepräparaten zu den zirkadianen Hoch- und Tiefphasen durchgeführt. Die Daten zeigten, dass die Tageszeit einen stärkeren Einfluss auf das Genexpressionsmuster hatte als die Insulinbehandlung, wobei keine Interaktion zwischen zirkadianer Phase und Insulinantwort festgestellt wurde. Unter Berücksichtigung des alleinigen Behandlungseffekts wurden vierzehn insulinresponsive Gene identifiziert, von denen drei, *Nefm*, *Trpc3* und *Opalin,* vorwiegend im Gehirn exprimiert wurden und mit neuronaler Entwicklung, struktureller Erhaltung und Myelinisierung assoziiert sind. Diese Gene zeigten eine moderate, aber konsistente Hochregulation durch Insulin, unabhängig von der zirkadianen Phase, was auf eine zirkadian-unabhängige, tonische Regulation hindeutet.

Zusammenfassend stützen die Ergebnisse dieser Arbeit ein Modell, in dem Insulin seine zentralen Wirkungen über zwei verschiedene Regulationsmodi ausübt: einen zirkadian-gesteuerten Mechanismus, der möglicherweise metabolische Funktionen steuert, und einen zirkadian-unabhängigen, tonischen Mechanismus, der die neuronale Gesundheit und Stabilität unterstützt. Die Studie zeigt die Komplexität der Untersuchung von Insulins Rolle in zentralen Stoffwechselfunktionen auf, während die Identifikation neuroprotektiver, insulinresponsiver Gene eine wertvolle Grundlage für die Erforschung der vielseitigen Wirkungen von Insulin im Gehirn bietet.

Chapter 1: Introduction

Maintaining energy balance and optimal brain function relies on a complex integration of hormonal signals and intrinsic circadian rhythms. Among these, insulin stands out as a crucial pleiotropic hormone, fundamental to both peripheral metabolic control and central nervous system processes, including appetite regulation and cognition. Growing evidence points to significant bidirectional interactions between insulin signalling and circadian timing, with broader implications for metabolic and neuronal health. However, it remains unclear whether insulin's actions in the brain are modulated by the circadian clock. This PhD thesis aims to address this by investigating the circadian gating of insulin's regulatory roles in the murine hypothalamus.

1.1 Insulin

1.1.1 The discovery

Around a century ago, chronic high sugar levels in the blood was a global rising burden. In 1889, two German scientists, Joseph von Mering and Oskar Minkowski discovered that excision of pancreas in animals caused symptoms of diabetes and related deaths. This observation highlighted the organ's critical role in carbohydrate metabolism and started a search for an important 'pancreatic substance' responsible for this regulation (Mering & Minkowski, 1890). Subsequent experiments narrowed this search to Ilets of Langerhans, when in 1915, Sir Edward Albert Sharpey-Schafer proposed to name this important substance as 'insulin' (Latin *insula*, meaning island). Building on these findings, in 1921, Frederick Banting and Charles Best successfully isolated pancreatic tissue extracts that included insulin and administered it in a diabetic dog, who soon showed improved health conditions and reduced diabetic symptoms (Banting & Best, 1992). However, the challenge of purifying insulin remained a concern. In later years Canadian biochemist James Collip contributed significantly to refine the extraction process of insulin from cattle pancreases, enabling its clinical use (Banting et al., 1991). A 14-year boy dying from severe diabetes became the first person in 1921 to receive pharmacological insulin, which drastically improved the young boy's health within 24 hours (King, 2003). The news of this successful intervention spread globally, and in 1923, Banting and Macleod received the Nobel Prize in Physiology or Medicine, which they shared with Best and Collip. This groundbreaking work, achieved through collaborative efforts, marked the beginning of insulin's role in diabetes treatment and revolutionized medical care for diabetic patients (King, 2003).

1.1.2 The structure

Insulin is a peptide hormone consisting of two polypeptide chains: A and B- chains containing 21 and 30 amino acids, respectively, and linked by two cysteine bridges. Within the A-chain lies an additional intrachain disulphide bond (Abel, 1926). It is initially synthesized in the cytosol as a single-chain precursor called preproinsulin, which

upon further cleavage in the endoplasmic reticulum, forms proinsulin (Chan & Steiner, 1977; Steiner et al., 1967). This undergoes additional processing of C-peptide removal in the Golgi apparatus, that yields the mature active insulin molecule (Steiner & Oyer, 1967). The mature insulin compound has strong structure-function characteristics, thus accurate formation and folding of disulphide bonds are critical for its structural stability and functionality (Blundell et al., 1972). With increasing concentrations of synthesized insulin and the optimal surrounding pH levels (~6.0), monomers of active insulin molecule aggregate to form dimers and further to form hexamers . The hexameric form of insulin is stabilised with zinc ions for efficient storage in secretory granules (Blundell et al., 1972; Smith et al., 2003). While the monomeric form is the active form of insulin, the hexamers form the storage compound (Fu et al., 2013). Additionally, the hexameric structure facilitates its controlled release in response to glucose fluctuations in the blood (Blundell et al., 1972).

1.1.3 Insulin secretion

The secretion of insulin by pancreatic β cells, is regulated primarily by blood glucose levels and modulated by an array of other metabolic, hormonal and neural signals (Henquin et al., 2003). After consuming a meal, carbohydrates are broken down increasing blood glucose levels. The glucose is then transported into the pancreatic β cells through the glucose transporter GLUT2 (Unger, 1991). Inside the cell, glucose is rapidly phosphorylated by glucokinase, and the resulting glucose-6-phosphate undergoes glycolysis and the tricarboxylic acid (TCA) cycle, producing ATP. Increasing ATP levels cause ATP to bind to ATP-sensitive potassium channels (K_{ATP}), leading to their closure (Davis & Lazarus, 1976). As a result, intracellular potassium accumulates causing membrane depolarisation. The change in membrane potential triggers opening of the voltage-gated calcium channels, allowing calcium ions (Ca^{2+}) to flow inside the cell. The influx of Ca^{2+} facilitates the insulin-containing vesicles to move towards the cell membrane causing insulin's release into the bloodstream (Davis & Lazarus, 1976). Conversely, when blood glucose levels decrease, ATP production is lowered, leaving open K_{ATP} channels. This facilitates efflux of potassium outside the cell which maintains a negative membrane potential. The voltage-gated calcium channels remain closed preventing Ca^{2+} influx, thereby restricting insulin secretion (MacDonald & Wheeler, 2003). Notably, pancreatic insulin secretion follows a rhythmic pattern that is primarily synchronised with feeding cycles (Dibner & Schibler, 2015; Perelis et al., 2015).

1.1.4 Insulin signalling pathways

Insulin, by binding to its receptor, regulates multiple cellular processes and signalling events essential for glucose homeostasis, metabolism and gene expression (Figure 1). The insulin receptor (IR) is a heterodimeric protein composed of two extracellular α-subunits and two intracellular β-subunits (Jacobs & Cuatrecasas, 1981; Yip, 1993). When the insulin molecule binds to the α-subunit, it triggers autophosphorylation of the β-

subunit, which further recruits and phosphorylates insulin receptor substrate (IRS) proteins (White, 1997). This phosphorylation event activates two major downstream signalling pathways. First, the phosphatidylinositol-3 kinase/ protein kinase B (PI3K/Akt) pathway which regulates glucose metabolism and food intake, and the second, mitogen-activated protein kinase (MAPK) pathway, which is involved in cell proliferation and differentiation (Figure 1).

The PI3K pathway is activated when the tyrosine phosphorylation of IRS proteins results in the activation of PI 3-kinase that catalyses the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Shepherd et al., 1998; Stokoe et al., 1997). Elevated PIP3 induces activation of phosphoinositide-dependent kinase (PDK) and protein kinase B (Akt) (Shepherd et al., 1998; Stokoe et al., 1997). Once activated, Akt phosphorylates key metabolic regulators, including glycogen synthase kinase 3 (GSK3) and forkhead box protein O1 (FOXO1) (Hers et al., 2011). Phosphorylation of GSK3 inhibits its activity, promoting glycogen synthesis (Sutherland et al., 1993). Meanwhile, FOXO1 phosphorylated by Akt, is inactivated by nuclear exclusion upon insulin stimulation (Van Der Heide et al., 2004). Inactivated FOXO1 leads to suppression of orexigenic peptides– neuropeptide Y (*Npy)* and agouti- related protein (*Agrp*), with an intermediate component G-protein coupled receptor 17 (*Gpr17)* (Ren et al., 2012; Schwartz et al., 1992). Simultaneously, it relieves the inhibition of anorexigenic peptides, proopiomelanocortin (*Pomc*) and cocaine-and-amphetamine-regulated transcript (*Cart)* via carboxypeptidase E (*CpE*), thereby exerting an overall anorexic effect (Hill, 2010; Plum et al., 2009). The Akt–FOXO1 signalling pathway thus plays a crucial role in regulating appetite and feeding behaviour (Kim, 2012) (Figure 1).

Along with IRS, the IR also phosphorylates Src homology and collagen protein (Shc), activating the MAPK signalling pathway (Giorgetti et al., 1994; Sasaoka & Kobayashi, 2000). This event makes Shc recruit growth factor receptor binding protein 2 (Grb-2), which in turn activates the GTP-exchange factor Son of sevenless (SOS) and small GTP protein Ras. Activated Ras triggers a cascade of kinase activations, ultimately leading to the full activation of MAPKs. This pathway is responsible in regulating insulin-induced gene expression, cell growth and mitogenesis (Morrison, 2012) (Figure 1).

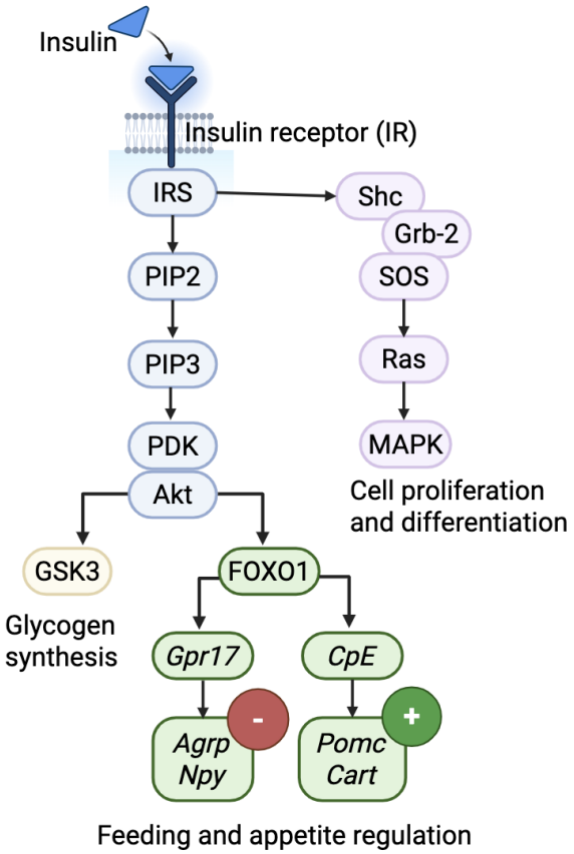


Figure 1. Insulin signalling pathways.
Upon insulin stimulation, the IR undergoes autophosphorylation and recruits IRS proteins, initiating the PI3K/Akt pathway. This cascade leads to phosphorylation of GSK3 to promote glycogen synthesis and FOXO1, to regulate appetite and feeding behaviour. Inactivated FOXO1 suppresses expression of orexigenic neuropeptides, *Agrp (*via Gpr17) and Npy. Simultaneously, it promotes the expression of anorexigenic neuropeptides, *Pomc* (via CpE) and Cart. These actions together contribute to appetite suppression. In parallel, IR also activates the MAPK pathway through phosphorylation of Shc, which recruits Grb-2, SOS, and Ras, culminating in MAPK activation that governs cell proliferation and differentiation. Created with Biorender. (IR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphatidylinositol-3 kinase; Akt, protein kinase B; GSK3, glycogen synthase kinase 3; FOXO1, forkhead box protein O1; Agrp, Agouti- related protein; Gpr17, G-protein coupled receptor 17; Npy, Neuropeptide Y; Pomc, Proopiomelanocortin; Cpe, Carboxypeptidase E; Cart, cocaine-and-amphetamine-regulated transcript; MAPK, mitogen-activated protein kinase ; Shc, Src homology and collagen protein ; Grb-2, growth factor receptor binding protein 2; SOS, Son of sevenless).

1.1.5 Physiological functions of insulin

Insulin's primary physiological function is to regulate blood glucose levels and facilitate glucose uptake into cells for energy use. Through the above explained pathways, insulin exerts its diverse physiological effects on multiple peripheral tissues, playing a crucial role in regulating multiple metabolic processes.

After ingesting a meal, rising level of insulin in blood stream promotes glucose storage as glycogen via glycogenesis in the liver (Miller & Larner, 1973). It also stimulates hepatic glucose utilisation through glycolysis (Weber et al., 1966). Simultaneously, it suppresses glucose production by inhibiting gluconeogenesis and glycogenolysis (Bach & Holmes, 1937; Sutherland & Cori, 1948) (Figure 2). These coordinated actions together help restore baseline blood glucose levels. There are both direct and indirect mechanisms that

mediate insulin's effects on liver function (Girard, 2006). Directly, insulin binds to the hepatic IRs and activates the PI3K/Akt signalling pathway to regulate glucose metabolism. This leads to the suppression of hepatic gluconeogenesis and enhancement of glycogen synthesis (Claus & Pilkis, 1976; Marks & Botelho, 1986; Sindelar et al., 1996). Indirectly, insulin influences liver functions by decreasing pancreatic glucagon secretion (Ito et al., 1995) and by inhibiting fat lipolysis (Sindelar et al., 1997) that in turn impacts hepatic metabolism. Both direct and indirect mechanisms are essential for insulin's regulation over liver function, with their relative contributions varying depending on physiological conditions.

Skeletal muscles play a critical role in glucose homeostasis, that accounts for a substantial portion of post prandial glucose disposal. Insulin acts as one of the key regulator of glucose uptake in skeletal muscles (Zierath et al., 1996) (Figure 2). Increased insulin levels after a meal promote translocation of glucose transporter GLUT4 to the myocyte membrane, thereby stimulating glucose uptake. GLUT4 translocation facilitates glucose entry into muscle cells causing a significant lowering of blood glucose levels (Zierath et al., 1996). In addition to this primary role, insulin also influences branched-chain amino acid, non-esterified fatty acid metabolism and muscle mitochondrial ATP production (Boden & Shulman, 2002; Harper et al., 1970; Kelley et al., 2002).

While skeletal muscle is the dominant site of insulin-stimulated glucose uptake, adipose tissue also contributes significantly. It accounts for approximately one-tenth of the total whole-body glucose uptake, although this proportion likely varies with individual adiposity (Smith, 2002). Insulin in adipose tissue primarily regulates lipid metabolism (Dimitriadis et al., 2011) (Figure 2). It also regulates adipocyte development and differentiation (Cignarelli et al., 2019). Although adipocytes themselves are relatively independent of glucose uptake, they are dependent on insulin for the uptake of free fatty acids (FFAs), which insulin liberates into the bloodstream. These FFAs are then utilized by other organs, such as the heart (Smith, 2002). Beyond the classical metabolic tissues, insulin also extends its direct or indirect influence on renal function (Hale & Coward, 2013), reproductive organ competence (Sliwowska et al., 2014), endothelial health (Muniyappa & Sowers, 2013) and central nervous system.

For insulin to exert its effects in the brain, it must first navigate the highly selective blood brain barrier (BBB). This transport process of endogenous peripheral insulin into the brain is tightly regulated, involving both saturable and non-saturable mechanisms (Gray & Barrett, 2018). The primary route of entrance is through a receptor-mediated transcytosis across the BBB, facilitated by IRs expressed on brain endothelial cells. Insulin binds to these receptors on the blood-facing, luminal side of the endothelial cells triggering endocytosis. This subsequently facilitates the transport of the hormone to the brain-facing, abluminal side, where it is released into the brain interstitial fluid (Baura et al., 1993; Duffy & Pardridge, 1987; Schwartz et al., 1991). In addition to the receptor-mediated transcytosis, insulin may also traverse the BBB through non-saturable mechanisms. This can be as a passive diffusion across regions of BBB with compromised integrity or through circumventricular organs (CVOs) that lack a fully developed BBB

(Duvernoy & Risold, 2007; Gray & Barrett, 2018). Among these, the receptor-mediated transport is highly regulated and responsive to peripheral insulin fluctuations. Once inside the brain, insulin interacts with IRs widely spread across different brain tissues, where it regulates diverse functions including appetite control, glucose metabolism, synaptic plasticity etc., as explored in the next section.

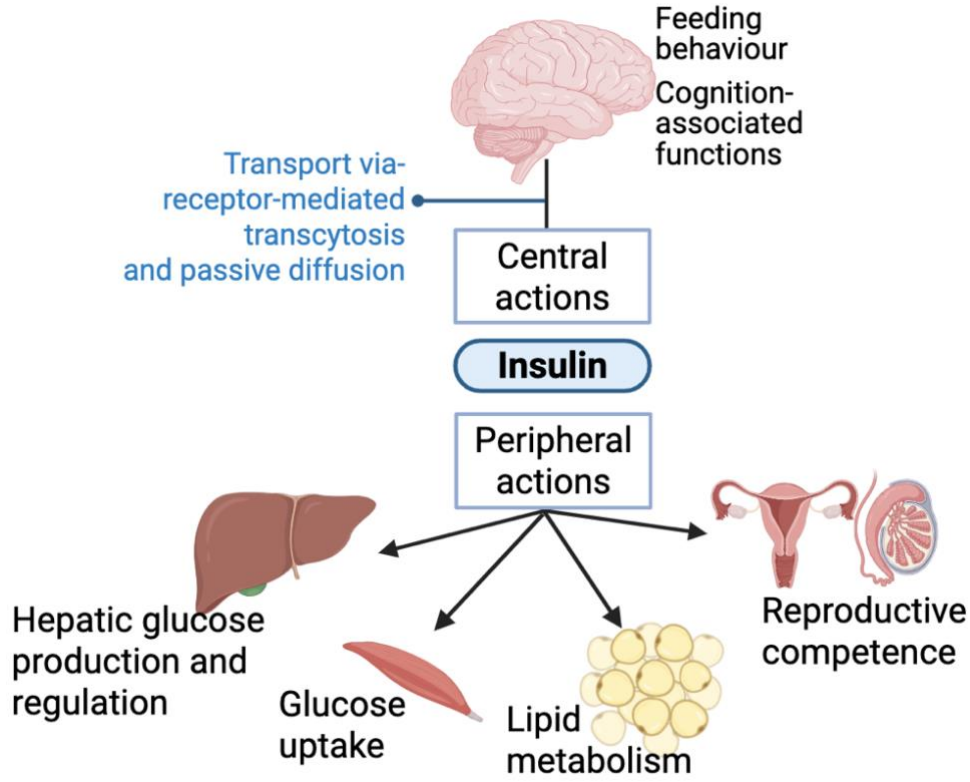


Figure 2. Central and peripheral actions of insulin:
Insulin exerts multiple functions in the body. Peripherally, it regulates key metabolic processes, including regulation of hepatic glucose production, glucose uptake in skeletal muscle, lipid metabolism in adipose tissue, and reproductive competence. It can cross the blood–brain barrier via receptor-mediated transcytosis and passive diffusion to enter the brain. In the brain, insulin has two major roles, modulating feeding behaviour and supporting cognition-associated functions. Created with Biorender.

1.1.6 Functions of insulin in the brain

For a long time following the discovery of insulin's critical role in peripheral physiology, its presence and functional significance in the CNS remained debated. Early evidence of the brain's insulin sensitivity came from studies that showed widespread expression of IRs throughout the brain, notably, in hypothalamus, cortex and cerebellum (Hopkins & Williams, 1997). Since then, insulin's actions in the brain are largely associated to feeding behaviour and cognition-associated functions (Hallschmid & Schultes, 2009) (Figure 2). The primary difference between peripheral and brain insulin action is the regulation of glucose transporters. In peripheral tissues like muscle and adipose, insulin promotes glucose uptake through activation of the Akt pathway which triggers the translocation of GLUT4 to the plasma membrane (Leto & Saltiel, 2012). In contrast, in the brain GLUT4

shows limited expression confined to hippocampus and hypothalamus, resulting in a relatively small impact on glucose uptake (Leloup et al., 1996; Reno et al., 2017).

The role of insulin in regulating feeding behaviour was first demonstrated in 1979, when intracerebroventricular (ICV) infusion of insulin in baboons was shown to significantly reduce food intake and thus decrease body weight (Woods et al., 1979). Subsequent studies in rodents revealed that insulin administration into the third ventricle suppresses food intake by decreasing the expression of orexigenic and increasing the expression anorexigenic neuropeptides in the arcuate nucleus (Arc) of the hypothalamus (Schwartz et al., 2000). This insulin-mediated modulation of hunger-regulating neuropeptides further leads to enhanced activity of α-melanocyte stimulating hormone (α-MSH) in the paraventricular nucleus (PVN), increasing appetite suppression (Schwartz et al., 2000). Conversely, insulin deficient streptozotocin (STZ)-treated mice show elevated orexigenic neuropeptide levels and reduced anorexigenic activity, developing a state of hyperphagia. In line with this, brain-specific deletion of the IR in mice led to the increased food intake and mild obesity (Brüning et al., 2000). The anorexigenic effects of insulin are mediated, in-part, by PI3K-dependent activation of ATP-sensitive potassium channels in the hypothalamus which hyperpolarizes orexigenic neurons (Könner et al., 2007).

Insulin resistance in the hypothalamus has been shown to disrupt both energy and glucose metabolism. Rodents injected in hypothalamus with IR-specific antisense oligodeoxynucleotides to induce hypothalamic insulin resistance exhibit impaired suppression of hepatic glucose production and food intake (Obici, Feng, et al., 2002; Obici, Zhang, et al., 2002). Consistently, selective knockdown of the IR in rat hypothalamus increases body weight and adiposity (Grillo et al., 2007). In addition, chronic reduction of IR expression in the ventromedial hypothalamus (VMH) of rats leads to pancreatic islet dysfunction, causing glucose intolerance without affecting body adiposity (Paranjape et al., 2011). Insulin also modulates feeding behaviour through its actions in the ventral tegmented area (VTA). Direct infusion of insulin into the VTA reduces food intake (Liu et al., 2016), while loss of VTA-specific IR results in hyperphagia and obesity (Könner et al., 2011).

Roles of insulin in cognitive and neuronal health functions have recently been documented. In the CNS, insulin functions as a growth factor, promoting synaptogenesis and neuronal growth (Nelson et al., 2008). Consequently, insulin resistance or impaired insulin signalling can cause cognitive decline (Hoyer, 1998, 2004). Peripheral insulin resistance and metabolic syndrome are recognised risk factors for Alzheimer's disease (AD) (Luchsinger et al., 2004). Furthermore, emerging evidence suggests that AD is heavily associated with deficiency in brain insulin signalling. AD is also evidently accompanied by reduced levels of insulin, IRs and C-peptide in the brain (Frölich et al., 1998). Intranasal insulin administration has been shown to enhance verbal memory and modulate plasma amyloid-beta (Aβ) levels in AD patients (Craft et al., 1999). Diet-induced insulin resistance exacerbates amyloidosis in a transgenic mouse model of AD suggesting that impaired insulin signalling may worsen Aβ metabolism (Ho et al., 2004).

Additionally, both insulin and Aβ are substrates for insulin degrading enzyme (IDE) and Aβ has shown to compete with insulin to bind to IR (Qiu et al., 1998; Xie et al., 2002). Similarly to insulin mediated hepatic clearance of plasma Aβ, low-density lipoprotein receptor-related protein 1 (LRP1) also mediates the brain-to-blood clearance of Aβ raising the possibility that CNS insulin may influence Aβ efflux (Deane et al., 2004, 2008).

Importantly, beyond its role in Aβ metabolism and AD, insulin also heavily influences other aspects of brain functions. It is known to play a critical role in promoting neuronal survival and protection under stress conditions. In primary cell cultures of rat hippocampus, IR signalling has been shown to protect neurons from oxygen-glucose deprived (OGD) cell death (Mielke et al., 2006). This neuroprotective effect is speculated to be achieved in part by enhancing γ-aminobutyric acid (GABA) signalling. Insulin also protects embryonic retinal cells during development by reducing the levels of pro-apoptotic proteins (Valenciano et al., 2006). Mediators of insulin signalling pathways are implicated in dendritic arbor formation. IRSp53, a brain-specific IRS protein, localises to the post-synaptic density and is speculated to stabilize cytoskeletal scaffolding proteins (Abbott et al., 1999). Overexpression of IRSp53 promotes dendritic arbor development in neuronal cultures, while RNAi against it inhibits this process (Choi et al., 2005; Govind et al., 2001). Insulin also regulates synaptic plasticity by modulating the trafficking of post-synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors. In cultured rat hippocampal neurons, insulin promotes the endocytosis of GluR2-containing AMPA receptors, a process implicated in long-term depression (LTD) (Beattie et al., 2000; Man et al., 2000). Additionally, IR signalling enhances trafficking of GABA receptors to the post-synaptic membrane, highlighting insulin's role in balancing excitatory and inhibitory neurotransmission (Wan et al., 1997).

Lastly, insulin action is also closely linked to the learning and memory processes. Behavioural studies in rodents, like water maze training, have shown that IR levels in the hippocampus are dynamically regulated during memory consolidation (Dou et al., 2005; W. Zhao et al., 1999). To note, the changes in the IR levels occur independently of alterations in NMDA or AMPA receptor expression (Dou et al., 2005). Intranasal insulin administration in mice has been shown to improve cognition as evident by enhanced short- and long-term object recognition (Benedict, 2004; Reger et al., 2008).

Taken together, functions of insulin in the brain extend far beyond feeding behaviour and energy homeostasis, encompassing critical effects on neuronal survival, synaptic plasticity, dendritic maturation and cognitive processes. While these roles have been mapped across various brain regions, it is equally important to consider how insulin exerts its effects at the cellular level, as discussed in the next section.

Insulin functions in different brain-cell types

IR is expressed across various cell types in the brain, hinting that insulin might exert functions that are cell-type specific. Neuronal populations in the hypothalamus, hippocampus and cortex exhibit the highest levels of IR expression. Among these, the actions of insulin are particularly well characterised in the orexigenic and anorexigenic neurons in terms of food intake and energy expenditure (Morton et al., 2006). Insulin promotes satiety by acting on these neuronal populations with opposing mechanisms. In orexigenic-AgRP/NPY neurons, insulin acts to inhibit their activity and reducing the hunger signals while it de-inhibits and subsequently activates the activity of POMC neurons to increase the anorexigenic signals (Morton et al., 2006). Several transgenic approaches have provided insights into insulin's effect in these populations. For instance, mice lacking IRS2 in POMC neurons exhibit impaired glucose tolerance, signs of obesity and altered melanocortin action (Choudhury et al., 2005). Insulin depolarises POMC neurons by activating TRPC5 channels and inhibits their activity via PI3K activation (Qiu et al., 2014, 2018). A study in mice revealed that insulin signalling in AgRP neurons regulates hepatic insulin action, with minimal impact on adipose tissue, while deletion of IRs in POMC neurons selectively impairs insulin's effects in adipose tissue (Shin et al., 2017). Conversely, overexpression of IR in POMC neurons enhances energy expenditure and locomotor activity, while also exacerbating insulin resistance and HGP (Lin et al., 2010). In addition to feeding regulation, insulin action in AgRP neurons is critical in glucose metabolism. Studies involving AgRP knockout mice demonstrate that insulin actions leads to increased appetite. Additionally, in the AgRP knock in mice, insulin signalling restrains hepatic glucose production, emphasizing its role in metabolic control (Lin et al., 2010; Shin et al., 2017). Multiple studies have further shown that insulin actions in these neuronal populations regulate neuronal plasticity (Dodd et al., 2018), adipose lipolysis (Shin et al., 2017), and fertility (Hill et al., 2010).

In addition to studies using transgenic animal models, effects of insulin action on neuronal functions have also been extensively investigated using *in vitro* systems. For instance, insulin application on the mouse hypothalamic embryonic cell line 46 (mHypoE46) shows dose dependent suppression of *Npy* and *Agrp* mRNA expression, indicating its regulatory role in hypothalamic neuropeptide signalling (Mayer et al., 2009). While *in vivo* studies have provided valuable insights, they are often limited by the cellular heterogeneity and the extensive arborised neuronal networks in the hypothalamus, making it challenging to isolate direct effects of insulin on specific neuronal populations (Belsham et al., 2004; Mayer et al., 2009). In the early 2000's, Denise Belsham's laboratory established a comprehensive panel of immortalised hypothalamic neuronal cell lines through mass immortalisation of primary hypothalamic cells derived from mice and cultured them in monolayers (Belsham et al., 2004). These clonal neuronal populations were further analysed for neuroendocrine markers, providing robust *in vitro* models to investigate the molecular mechanisms underlying hypothalamic functions, including energy homeostasis, circadian biology, neuronal plasticity and glucose sensing (Belsham et al., 2004; Dalvi et al., 2011a). Building on this

foundation, the present study leverages these immortalised hypothalamic cell lines to further elucidate the role of insulin action on hypothalamic functions.

Insulin signalling also plays critical role in other neuronal populations. For instance, the whole-body knockout of melanin-concentrating hormone (MCH) in mice results in hypophagia and reduced body weight (Shimada et al., 1998). But, deletion of IR specifically in MCH expressing neurons, although it does not alter metabolic phenotype in the lean mice, improves insulin sensitivity and decreases locomotor activity in obese mice (Hausen et al., 2016). Orexin is another key peptide involved in glucose and energy homeostasis that interacts with insulin signalling. Modulation of orexin-producing neurons or orexin-dependent neuronal activity induces hepatic insulin resistance and alters feeding behaviour (Inutsuka et al., 2014; Tsuneki et al., 2013).

Astrocytes are critical in maintaining synaptic plasticity and neurotransmission (Khakh & Deneen, 2019). They express IR and exhibit functional insulin signalling which plays an important part in hypothalamic glucose sensing. Studies using glial fibrillary acidic protein (GFAP)-driven Cre/LoxP systems to create astrocyte-specific IR knockout mice (GIRKO) demonstrated that insulin regulates SNARE complex formation, a process that facilitates ATP exocytosis modulating synaptic plasticity at dopaminergic axonal terminals (Cai et al., 2018). In addition, loss of astrocytic insulin signalling reduces evoked dopamine release in the dorsal striatum and nucleus accumbens (NAc), leading to anxiety- and depression-like behaviours in GIRKO mice (Cai et al., 2018). GIRKO mice also show reduced glucose-induced activation of POMC neurons and impaired physiological responses to glucose availability (García-Cáceres et al., 2016). A recent study demonstrated that astrocytic insulin signalling regulates the circadian free-running period of locomotor activity in female and circadian food entrainment in male mice (González-Vila et al., 2023). In addition, disruption of the IR in hypothalamic astrocytes affects energy homeostasis and alters dopamine metabolism (González-Vila et al., 2023). Role of insulin signalling have also been studied in tanycytes- a group of non-neuronal cells that share some attributes with astrocytes. In contrast to its role in endothelial cells, IR in tanycytes may be of paramount importance in regulating insulin transport into the Arc (Porniece Kumar et al., 2021).

A role for insulin action in microglia, the resident immune cells of the brain, is still emerging. Intranasal insulin treatment reduces Aβ levels and microglial activation. This has been shown to restore impaired insulin signalling in an AD mouse model (Chen et al., 2014). Aβ clearance induction by isoproterenol is inhibited by insulin in microglial cell lines (Kong et al., 2010). Microglial overactivation is observed in insulin-resistant rats. This effect was further shown to decrease with antidiabetic drugs associated with enhancing cognition (Gad et al., 2016). Endothelial cells in the brain express IR and exhibit functional insulin signalling. A study has shown that conditional knockout of IR in the neuro-vascular endothelium can delay insulin signalling onset in multiple brain regions including hippocampus, hypothalamus and prefrontal cortex (Konishi et al., 2017). Loss of endothelial IR also impairs BBB function and can lead to systemic insulin resistance and mild obesity (Konishi et al., 2017). Lastly, the information on insulin

actions in oligodendrocytes, compared to other glial cells, is scarce; however, some clinical evidence hints at its potential involvement. In patients with multiple system atrophy (MSA), increased phosphorylation of IRS-1 at serine 312 (a clinical marker of insulin resistance) has been observed (Bassil et al., 2017). Another study shows that both, neurons and oligodendrocytes but not astrocytes and microglia, exhibit insulin resistance in MSA (O'Grady et al., 2019), hinting at a cell-specific regulation of insulin action.

Having explored the diverse, region-specific roles of insulin in the brain, as well as its actions across distinct brain cell types, restrengthens the argument that insulin is integral not only to metabolic regulation but also to neuronal health, synaptic plasticity, and behavioural control. However, when insulin signalling in the brain is disrupted, whether due to systemic metabolic imbalance or local resistance, these finely tuned processes can become dysregulated, as is explored briefly in the next section.

1.1.7 Insulin in pathology

Impairments in insulin action contribute to various physiological and neurological ailments, manifesting into multiple metabolic and neurodegenerative diseases.

Insulin deficiency, as seen in type1 diabetes, leads to impaired glucose and lipid metabolism. In individuals receiving insulin treatment, excessive fatty food consumption can induce mitochondrial dysfunction and oxidative stress, thereby, exacerbating insulin resistance (Ruegsegger et al., 2018). Pathologically, both insulin resistance and deficiency alter plasma glucose levels (Ferrannini, 1998). Obese and insulin resistant patients are often encountered with overproduction of glucose and heavy buildup of lipids in the liver (Bazotte et al., 2014). Increased insulin resistance is also an important factor in diabetic microvascular issues (Ramzy et al., 2018). Hyperinsulinemia, a condition of having higher insulin levels in the blood than usual, is also often caused by prolonged insulin resistance and is linked with obesity, type 2 diabetes mellitus (T2DM) and cardiovascular complications (Kuzuya & Matsuda, 1997; Thomas et al., 2019). When undiagnosed and untreated, this condition can lead to myocardial insulin signalling damage, mitochondrial dysfunction and oxidative stress further leading to cardiac dysfunction (Jia et al., 2018). Chronic hyperinsulinemia is associated with polycystic ovarian syndrome (PCOS) (Otto-Buczkowska et al., 2018). Hyperglycemia, defined by elevated blood glucose levels, arises from islet dysfunction and reduced insulin secretion (Giugliano et al., 2008; Simon & Wittmann, 2019; Yari et al., 2020). It is a key feature of T2DM and is also associated with cardiovascular disease risk (Coutinho et al., 1999). Prolonged hyperglycemia can lead to life-threatening complications like ketoacidosis and hyperosmolar syndrome (Gosmanov et al., 2000).

Pathologies related to insulin signalling frequently manifest in the CNS as brain insulin resistance (BIR). It is often referred to as type 3 diabetes and is implicated in Alzheimer's (AD) and Parkinson's disease (PD). In AD, BIR disrupts glucose metabolism, increase oxidative stress and promotes neuroinflammation leading to Aβ accumulation and cognitive decline (Kandimalla et al., 2017; Salkovic-Petrisic & Hoyer, 2007). As also

mentioned in previous sections, microglial activation and astrocyte dysfunction further exacerbate neuroinflammation and Aβ pathology (Kaur et al., 2019; Liddelow et al., 2017). In PD, BIR is associated with mitochondrial dysfunction, Alpha-synuclein accumulation and dopaminergic neuron loss (Kakoty et al., 2023; Schapira et al., 2014). In addition, insulin signalling impairment is also linked to mood disorders and depression (Kleinridders et al., 2015; Nguyen et al., 2018).

Taken together, understanding insulin's role in both peripheral and central metabolic regulation lays the foundation for recognizing how disruptions in its signalling contribute to the pathology of various metabolic and neurological disorders. However, insulin does not operate in isolation. Its secretion, sensitivity, and downstream actions are coordinated with the body's internal timing system which orchestrates daily rhythms in physiology and behaviour. To fully grasp the complexity of insulin regulation in both health and disease, we further explore the circadian system that governs its temporal dynamic.

1.2 The biological clock

1.2.1 Biological rhythms and the circadian clock

Internal timekeeping is essential for living organisms. To have a working timekeeping mechanism, organisms leverage certain biological rhythms to adapt to predictable changes in environmental demands. These include, but are not limited to, the daily light-dark cycle, seasonal changes, changes in the ambient temperature, food availability or to protect themselves from predators at specific times. So, internal timekeeping is essential for living organisms, primarily to increase their chances of survival.

There are different types of biological rhythms. These include circannual rhythms, that regulate events occurring on an annual basis, circalunar rhythms, which span approximately one-month, circadian rhythms, which align with a 24-hour period, and lastly, rhythms shorter than 24 hours grouped under ultradian rhythms. Among these different categories, circadian rhythms are most prominently studied (Copinschi et al., 1999). The term, derived from the Latin *circa diem* ("about a day"), reflects their alignment with Earth's day-night cycle (Partch et al., 2014). These rhythms regulate key processes such as the sleep-wake and the feeding-fasting behaviour. Organisms rely on circadian clocks to maintain these internal cycles.

The concept of circadian rhythms has interesting historical roots. It was first described by a French interdisciplinary researcher and philosopher, Jean Jacques d'Ortous de Mairan in 1729. He observed that the *Mimosa pudica* plant showed a rhythmic opening and closing of leaves even when placed in a dark cupboard, suggesting the presence of an internal clock independent of external stimuli (de Mairan, 1729). Over evolutionary time, circadian clocks have developed across virtually all species and are highly conserved, indicating their fundamental importance (Dunlap, 1999; Stanton et al., 2022). For instance, clock mechanisms have been identified in prokaryotes, like cyanobacteria,

which were among the earliest organisms to evolve circadian rhythms (Dvornyk et al., 2003).

Jürgen Aschoff, a German physician and biologist, pioneered the study of circadian rhythms in humans by conducting experiments in isolation chambers (or "bunkers"). His research showed self-sustained periodicity in body temperature and sleep-wake cycles in the tested subjects. These cycles were sustained in absence of environmental signals but interestingly, he also found that these rhythms could desynchronize from the 24-hour day, with a free-running period slightly longer than 24 hours (Aschoff, 1965; Aschoff, 1986). Of note, mice, in contrast, have a slightly shorter free-running period of approx. 23.6 hours (Bunger et al., 2000).

As of now, modern circadian biology research defines three core properties of circadian clocks: entrainment, self-sustainment, and temperature compensation (Buhr et al., 2010). These core properties are studied not only in mammals but also in simpler organisms like cyanobacteria (Nakajima et al., 2005). Entrainment refers to the synchronization of the internal circadian clock to external environmental cues such as light or temperature, i.e., *zeitgebers* (German, time givers). Self-sustainment ensures that circadian rhythms persist even in the absence of such external signals, leading to endogenous free-running periods. Finally, temperature compensation distinguishes circadian rhythms from other physiological processes, because their kinetics are stable across a wide range of temperatures (Bruce, 1972; Nakajima et al., 2005; Pittendrigh, 1954).

These fundamental properties of circadian clocks establish a robust and evolutionarily conserved timekeeping system. Interestingly, beyond simply keeping time, circadian clocks actively shape physiology through 'circadian gating' and 'tuning', mechanisms by which sensitivity to signals (such as hormones and nutrients) is modulated depending on the time of day (De Assis et al., 2024; Oster et al., 2006). These mechanisms are often considered as functional consequences of clock regulation which ensure that biological responses are not only rhythmic but also optimally timed. Subsequent chapters will explore these concepts in more detail.

1.2.2 The mammalian molecular circadian clock

In mammals, circadian rhythms are regulated by cellular circadian clocks composed of autoregulatory transcription-translation feedback loops (TTFLs). They result in the rhythmic production and degradation of proteins, forming the foundation of the circadian clock mechanism (Ko & Takahashi, 2006).

The primary feedback loop is composed of two interdependent components. The positive arm involves brain and muscle ARNT-like 1 protein (BMAL1, also known as ARNTL) and circadian locomotor output cycles kaput (CLOCK) or its paralog neuronal PAS domain protein 2 (NPAS2) (Figure 3). These proteins form heterodimers that bind to E-box (enhancer box) sequences within the promoters of *Period* (*Per1–3*) and *Cryptochrome* (*Cry1/2*) genes, initiating their transcription (Buhr & Takahashi, 2013; Gekakis et al.,

1998; Hogenesch et al., 1998). CLOCK and BMAL1 belong to the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) family of transcription factors, with their interaction mediated by PAS domains (Huang et al., 2012; King et al., 1997).

The negative arm of the clock comprises PER1–3 and CRY1/2 proteins (Buhr & Takahashi, 2013; Kume et al., 1999) (Figure 3). Upon being synthesized, these proteins accumulate in the cytoplasm and form complexes with casein kinase 1 delta or epsilon (CK1δ/ε). These complexes shuttle into the nucleus where they suppress the transcriptional activity of CLOCK:BMAL1, which in-turn inhibits their own expression (Aryal et al., 2017; Finger & Kramer, 2021). In addition to forming complexes, CK1δ/ε also regulates the stability and activity of PER proteins (Lee et al., 2009). In the cytoplasm, CK1δ/ε phosphorylates PER 1 and PER2 , which recruits the adapter protein beta transducing repeat-containing E3 ubiquitin protein ligase (ßTrCP), targeting PERs for proteasomal degradation (Camacho et al., 2001; Eide et al., 2005; Keesler et al., 2000; Shirogane et al., 2005). CK1ε phosphorylation of PER1 also masks its nuclear localization signal, preventing it to translocate into the nucleus (Vielhaber et al., 2000). Like PER proteins, stability of CRY proteins is also tightly regulated. CRY1 and CRY2 are phosphorylated by AMPK and other kinases, which can either stabilize or target them for proteasomal degradation via f-box and WD repeat domain-containing 7 (FBXL3), thereby contributing to precise timing of the negative feedback loop (Hirano et al., 2013). Notably, CK1ε-mediated phosphorylation of PER proteins is important for determining the circadian period, as it directly influences generation of the circadian rhythm (Lowrey et al., 2000; Ralph et al., 1990; Ralph & Menaker, 1988).

Beyond the core loop, there are additional interlocked 'auxiliary feedback loops' that act as support players to refine and stabilize the circadian system. One of such loops involves reverse erythroblastosis virus α and β (REV-ERBα/β) and retinoic acid receptor-related orphan receptors α/β/γ (RORα/β/γ) (Figure 3). The promoter regions of both, *Reverb* and *Ror* contain E-box sequences that are leveraged by CLOCK:BMAL1 for binding and activating gene transcription (Sato et al., 2004; Triqueneaux et al., 2004). REV-ERB and ROR subsequently compete for binding to ROR response elements (RREs) in the promoter region of *Bmal1,* with RORs activating and REV-ERBs repressing *Bmal1* transcription, creating another regulatory layer (Guillaumond et al., 2005; Ko & Takahashi, 2006; Preitner et al., 2002). *Reverbα/β* influence circadian period and can cause phase shifts in the rhythms while RORs can modulate amplitude (Liu et al., 2008; Preitner et al., 2002).

Additionally, CLOCK:BMAL1 regulates other downstream gene products including D-site albumin promoter-binding protein (DBP) and nuclear factor interleukin 3-regulated protein (NFIL3, also called E4BP4). These modulate the expression of *Rors*, *Rev-erbs*, and *Pers* via D-box elements, further shaping clock dynamics (Finger & Kramer, 2021; Mitsui et al., 2001).

In mammals, these molecular clocks are ubiquitously present, functioning in nearly all nucleated cells (O'Neill & Reddy, 2011). This intricate molecular clock mechanism also

regulates a vast array of clock-controlled genes (CCGs) in multiple brain regions and peripheral tissues (Figure 3). Leveraging E-boxes, D-boxes or ROREs in their promotor regions, circadian clocks regulate the expression of these genes in a tissue-specific manner to drive circadian variations in vital physiological processes (Dibner et al., 2010; Korenčič et al., 2014; Lamia et al., 2008).

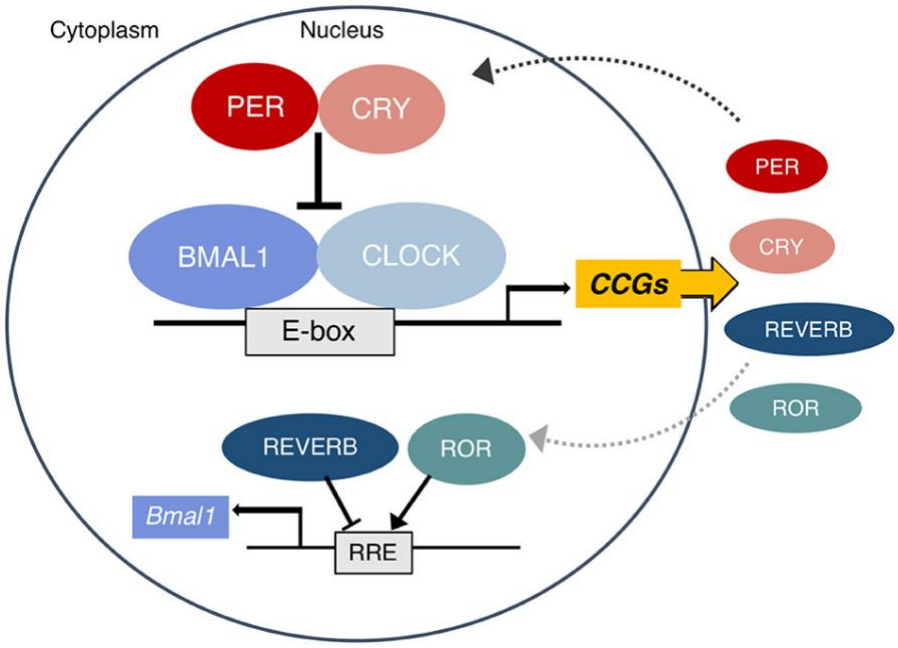


Figure 3. The molecular circadian clock.
The core circadian clock operates through a transcriptional–translational feedback loop, where the CLOCK:BMAL1 heterodimer rhythmically binds to E-box elements to drive expression of CCGs, including *Per1–3* and *Cry1, 2*. The resulting PER and CRY proteins translocate to the nucleus and inhibit CLOCK:BMAL1 activity, closing the negative feedback loop. These repressors are eventually degraded via the proteasome, allowing the cycle to restart roughly every 24 hours. A secondary feedback loop involves *Rev-erbα/β* and *RORα/β/γ*, which repress and activate *Bmal1* transcription, respectively. In addition to regulating rhythmic gene expression, certain CCGs participate in metabolic pathways and feedback to modulate the clock itself. Figure referred from Pickel & Sung, 2020. (CLOCK, Circadian locomotor output cycles kaput ; BMAL1, Brain and muscle ARNT-like 1 protein; E-box, Enhancer box; CCGs, Clock-controlled genes; Per, Period; Cry, Cryptochrome; Rev-erbα/β, Reverse erythroblastosis virus α and β; RORα/β/γ, Retinoic acid receptor-related orphan receptors α/β/γ).

1.2.3 The clock network

To ensure that the physiological processes of our body are synchronised, tissue clocks within an organism must be coordinated with one another and adjusted to environmental cues throughout the day (Finger & Kramer, 2021). Early research demonstrated the critical role of the suprachiasmatic nucleus (SCN) in generating and maintaining circadian rhythms across the body. The SCN lesions in rats led to the loss of circadian locomotor activity, drinking behaviour, and corticosterone oscillations (Moore & Eichler, 1972; Stephan & Zucker, 1972). Located in the hypothalamus, just above the optic chiasm, the SCN receives light input from a specific subset of retinal ganglion cells via the retinohypothalamic tract (Moore et al., 1995; Moore & Lenn, 1972). In later experiments, transplanting a foetal SCN into SCN-lesioned hamsters restored free-running locomotor activity rhythms in dark conditions (Lehman et al., 1987). A pivotal experiment by Ralph

et al. showed the SCN's role as the master circadian pacemaker. They conducted reciprocal SCN transplants between wildtype and heterozygous *tau* mutant hamsters and observed that the recipient adopted the circadian period of the transplanted SCN rather than sustaining a period of its original genotype (Ralph et al., 1990). In another set of experiments, foetal SCN transplants into *Clock* mutant or *Cry1/2* double knockout mice successfully restored rhythmic locomotor activity in constant darkness (Sujino et al., 2003). Based on these findings, the circadian system has traditionally been described as a hierarchical model in which the SCN functions as the central pacemaker, aligning peripheral and extra-SCN clocks with environmental light cues (Dibner et al., 2010).

However, an alternative, more decentralized (or federal) model has been proposed using mice that carry a genetically dysfunctional SCN clock (Husse et al., 2015). Studies on SCN-specific *Bmal1* knockout mice revealed that while these animals maintained behavioral and tissue-molecular rhythmicity under a standard 12/12-hour light-dark cycle, they became arrhythmic in dark conditions. This suggests that the SCN is only essential to synchronise the peripheral clocks in the absence of external light cues (Husse et al., 2011, 2014, 2015). These mice also showed body weight gain and impaired glucose metabolism, but only in constant darkness (Kolbe et al., 2019). Similar findings were reported in forebrain-specific *Bmal1* knockout mice, which also lack a functional SCN clock. Although peripheral tissues maintained circadian rhythms, they became desynchronized in constant darkness (Izumo et al., 2014).

The SCN exerts its influence on various brain regions through direct and indirect projections. It communicates with hypothalamic areas such as the Arc and dorsomedial hypothalamus (DMH), as well as other regions including the paraventricular nucleus (PVN), the supraoptic nucleus, the preoptic area, and the medial amygdala (Aston-Jones et al., 2001; Deurveilher et al., 2002; Guzmán-Ruiz et al., 2014; Hattar et al., 2006; Kalsbeek et al., 1993; Novak et al., 2000; Watson et al., 1995). Notably, rhythmic neuronal communication from SCN to Arc persists in fasted rats, suggesting that these projections also contribute to physiological outputs, such as the sleep-wake cycle (Guzmán-Ruiz et al., 2014). The Arc in turn, provides feedback to the SCN, integrating signals from peripheral metabolic hormones (Yi et al., 2006a). Structurally, the SCN is divided into core and shell regions, where the core processes incoming information and coordinates outputs, while the shell synchronizes peripheral tissue clocks and extra-SCN oscillators (Evans et al., 2015).

In addition to neuronal projections, the SCN relies on hormonal signals to synchronize peripheral clocks. For instance, a key hormone under SCN control is melatonin, which is secreted rhythmically during the dark phase and influences circadian behaviours (Takahashi et al., 1980; Turek et al., 1976). Similarly, rhythmic glucocorticoid secretion plays a role in aligning peripheral tissue clocks (Balsalobre et al., 2000; Cuesta et al., 2015).

Overall, the SCN serves as the principal circadian pacemaker, orchestrating both central and peripheral clocks through neuronal and humoral signals. At the same time, a

decentralized network of clocks can interact and function independently under certain conditions.

1.2.4 Extra-SCN clocks in the brain

In addition to the SCN, circadian clocks are distributed across various brain regions, with most of them relying on the master clock to sustain rhythmicity. The retina was the first neuronal tissue, apart from the SCN, shown to have endogenous circadian rhythms, specifically in melatonin synthesis (Tosini & Menaker, 1996). Subsequent studies using clock gene reporters revealed that many other brain regions of mice maintain rhythmicity, although they dampen quickly without a consistent SCN input, and thus were classified to be as secondary or slave oscillators (Guilding & Piggins, 2007). One exception was found for the olfactory bulb, that functions as an autonomous, entrainable and temperature-compensated pacemaker (Granados-Fuentes et al., 2004). It responds to light and odour cues with rhythms persisting even in SCN-lesioned mice (Granados-Fuentes et al., 2006; Hamada et al., 2011). Regions in the cortex exhibit SCN-dependent clock gene expression rhythms, although few studies showed that time-restricted feeding can induce rhythms in the SCN-ablated mice (Rath et al., 2013, 2014; Wakamatsu et al., 2001). The hippocampus with critical memory functions rhythmically expresses core clock components (Jilg et al., 2010; Otalora et al., 2013; Wang et al., 2009). These rhythms persists in constant darkness (Wang et al., 2009). Also, while melatonin has been shown to reset hippocampal clocks, pinealectomy has minimal effects (Amir et al., 2006; Jilg et al., 2019). Amygdala also shows robust clock protein rhythms dependent on the SCN and that are entrainable by GCs (Segall et al., 2006). The nucleus accumbens (NAc) and caudate putamen exhibit rhythmicity influenced by mood and diet, though exact entrainment mechanisms remain unclear (Landgraf et al., 2016). Lateral habenula (LHb) appears semi-autonomous, with *ex vivo* oscillations persisting without SCN input, although SCN input is required for the synchronisation between distinct LHb clocks (Baño-Otálora & Piggins, 2017). Midbrain and hindbrain clock oscillations are less characterised, with some evidence of rhythmicity in the substantia nigra and thalamus (Abe et al., 2002a; Natsubori et al., 2014). And lastly, many hypothalamic regions apart from SCN, such as Arc, DMH, LH and VMH, show weaker but persisting rhythms mainly entrained by feeding and metabolic cues (Buijs et al., 2017; Guilding et al., 2009; Mieda et al., 2006; Wang et al., 2017) (Figure 4).

1.2.5 Convergence of circadian system and metabolism

The hypothalamus serves as a key hub, a critical regulatory region where two physiological systems of circadian rhythms and metabolic homoeostasis converge (Figure 4). While the SCN functions as the master clock, orchestrating circadian rhythms throughout the body, extra-SCN hypothalamic regions play essential roles in metabolic regulation and are largely entrained by feeding and nutrient-derived cues. The close

anatomical and functional interplay makes it plausible that metabolic feedback and circadian regulation are integral and interdependent processes.

Lesion studies from as early as the 1950s demonstrated that different hypothalamic regions play distinct roles in feeding behaviour and energy balance. Disruption of the mediobasal hypothalamus (MBH) results in hyperphagia and obesity, whereas lesions in the LH lead to cessation of overall food consumption (Anand & Brobeck, 1951; Dietrich & Horvath, 2009). Within the Arc, orexigenic (NPY and AgRP) and anorexigenic (POMC and CART) neuronal populations play a critical role in energy homeostasis. These neurons project to the PVN, where AgRP acts as an antagonist and α-MSH as an agonist of melanocortin receptors, thereby modulating feeding behaviour (Fan et al., 1997; Lu et al., 1994). Notably, the activity of these hypothalamic neuropeptides is under circadian control. NPY oscillates rhythmically, with its release influenced by serotonin and GABA in the SCN (Glass et al., 2010; Xu et al., 1999). Also, AgRP neuronal activity follows a daily rhythm *in vivo* and is synchronised to the light phase during *ad libitum* food access (Sayar-Atasoy et al., 2024). Serotonin enhances NPY secretion, while GABA inhibits it, and both display circadian fluctuations in the brain (Aguilar-Roblero et al., 1993; Ciarleglio et al., 2011), The melanocortin system is likewise tightly linked to metabolic and circadian systems. Knockout models of melanocortin receptors, MCR-3 and MCR-4, promote obesity (reviewed in Yanik & Durhan, 2025). Interestingly, melanocortin neurons in the brain regulate rhythmic anticipatory activity in response to food availability (Begriche et al., 2009).

Metabolic hormones like ghrelin, leptin, adiponectin and insulin function as key messengers between the metabolic and circadian systems. Ghrelin exerts its hunger effects through the growth hormone secretagogue receptor (GHSR), which is highly expressed in hypothalamus. Activation of this receptor leads to AMPK-mediated mitochondrial fatty acid oxidation in NPY neurons and GABAergic inhibition of POMC neurons, ultimately stimulating feeding behaviour (Kojima et al., 1999; Kola et al., 2005). Ghrelin secretion follows a circadian rhythm, rising before meal times and decreasing postprandially (LeSauter et al., 2009). In addition, fatty acid oxidation associated with ghrelin signalling relies on the cofactor NAD+, which has also been shown to oscillate in other metabolic tissues (Ramsey et al., 2009). Leptin, an anorexigenic hormone also exhibits diurnal fluctuations, regulated by the master clock (Kalsbeek et al., 2001). The SCN modulates the sensitivity of the Arc neurons to circulating leptin, ensuring that energy intake and expenditure are appropriately regulated across the day-night cycle (Kalsbeek et al., 2001; Kettner et al., 2015). Adiponectin, too, follows a circadian rhythm (Barnea et al., 2015). Unlike leptin, which suppresses AMPK activity in the hypothalamus, adiponectin activates it, promoting food intake and reducing energy expenditure (Kubota et al., 2007). The kinase activity of AMPK is itself rhythmic and regulates circadian rhythms of energy metabolism in a tissue-specific manner (Um et al., 2011). It is speculated that a leptin-adiponectin-AMPK axis relays metabolic cues to the master clock via the Arc (Greco & Sassone–Corsi, 2019). Furthermore, AMPK regulates the targets of the mTOR pathway, a nutrient-sensing system that also oscillates in a circadian manner

in several tissues, including the SCN (Cao et al., 2013). Other studies have also shown that modulation of mTOR signalling directly impacts circadian timing, reinforcing the link between nutrient availability and the body's internal clock (Ramanathan et al., 2018; Zheng & Sehgal, 2010).

Lastly, insulin, a key satiety hormone, is increasingly emerging as a crucial player in circadian-metabolic interaction. To reiterate from previous sections, pancreatic insulin secretion follows a circadian pattern aligning with the feeding rhythm (Dibner & Schibler, 2015). Recent evidence suggests that insulin can influence central circadian processes by acting directly on hypothalamic neurons (Crosby et al., 2019; Mayer et al., 2009). Given the role of the hypothalamus as a hub for both, metabolic and circadian, regulators, and insulin being one of its key modulators, its ability to influence hypothalamic functions places it at the heart of this intricate crosstalk. The following chapter will delve into the bidirectional relationship between insulin signalling and the circadian system, examining how insulin not only responds to but also actively influences circadian rhythms.

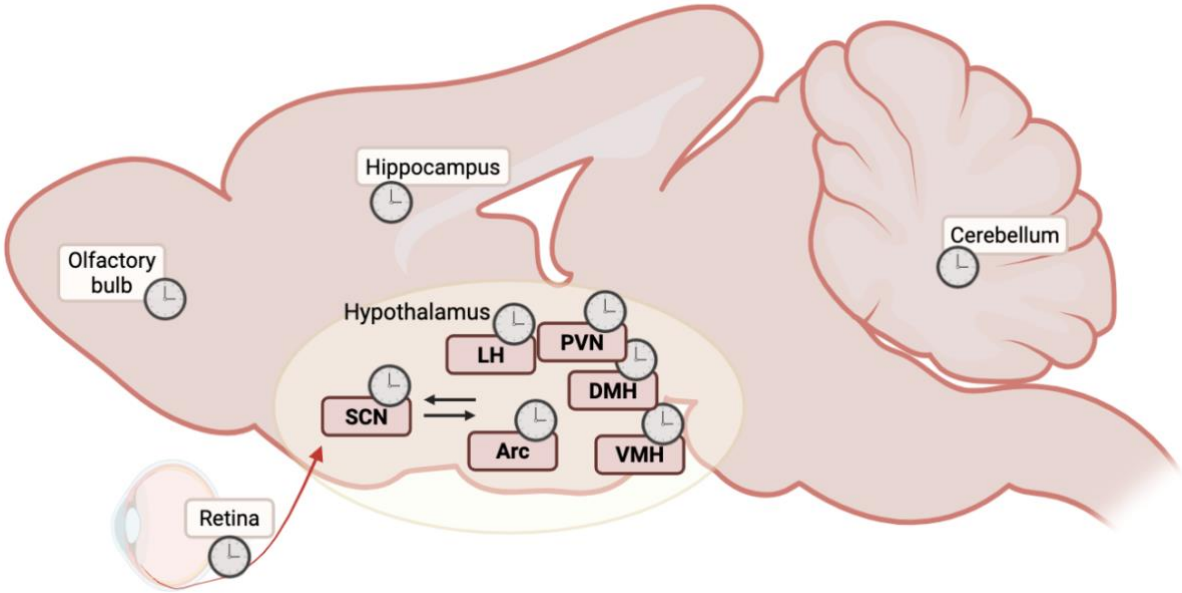


Figure 4. Interaction of circadian system and metabolism.
Brain consists of multiple extra-SCN clocks distributed in the regions like cerebellum, hippocampus, olfactory bulb and hypothalamus. In hypothalamus, the master clock, SCN is synchronised by light input from retina. It communicates with other hypothalamic nuclei including Arc, DMH, VMH, LH and PVN, which contain local clocks that regulate feeding and energy homeostasis. The bidirectional interaction of these nuclei converges two vital bodily processes of metabolic regulation and circadian system. Created with Biorender. (SCN, Suprachiasmatic nucleus; Arc, Arcuate nucleus; DMH, Dorsomedial hypothalamus; VMH, Ventromedial hypothalamus; LH, Lateral hypothalamus; PVN, Paraventricular nucleus).

1.3 Crosstalk between insulin signalling and circadian system

1.3.1 Insulin influences the circadian system

The circadian system orchestrating various physiological processes over the 24-hour cycle is intertwined with metabolic regulation. Insulin has been increasingly recognised

as a modulator of circadian rhythms which possibly influences both, molecular clock components and behavioural rhythmicity. It's role in circadian regulation is potentially mediated through its ability to interact with clock genes, synchronise peripheral oscillators and regulate feeding-driven entrainment of circadian rhythms.

Insulin plays a significant role in directly modulating the master clock. Insulin administration into the SCN disrupts circadian rhythms of food intake, decreasing consumption during the subjective active period while increasing during the inactive period (Mori et al., 1985; Nagai et al., 1982). At the cellular level, insulin primarily inhibits neuronal activity (Shibata et al., 1986). SCN insulin injection impairs rhythms in plasma glucose levels (Mori et al., 1985). In neuronal cells, insulin has been shown to induce phase shifts in PER2 oscillations (Crosby et al., 2019). Deletion of IR in hypothalamic astrocytes leads to alterations in the free-running period of locomotor activity in female and affects food-entrained behaviours in male mice. This indicates a sexual-dimorphic influence of insulin on the circadian system (González-Vila et al., 2023). In a study, insulin was shown to modulate both the firing patterns of SCN neurons and the functional input these neurons receive from other metabolic regions of the hypothalamus, suggesting a role for insulin in influencing the circadian regulation of metabolism and feeding behaviour (Inyushkin et al., 2023). In addition, insulin can act as a *zeitgeber*, particularly when delivered between midday and the early night phase, indicating that endogenous insulin may contribute to circadian entrainment, especially in the absence of light input (Inyushkin et al., 2013).

Insulin plays a significant role in synchronising peripheral oscillators, especially in the liver, adipose tissue and pancreas that are highly sensitive to insulin. Its secretion is inherently rhythmic, following feeding-induced fluctuations and serving as a metabolic *zeitgeber* that entrains peripheral clocks to nutrient availability (Boden et al., 1996; Sato et al., 2014). Studies have shown that insulin directly regulates the phase entrainment of hepatocyte oscillators (Yamajuku et al., 2012). It increases the expression of clock genes *Per1* and *Per2* in goldfish liver explants, likely via the PI3K/Akt pathway (Saiz et al., 2023). Insulin post-transcriptionally modulates *Bmal1*, also through Akt-mediated phosphorylation, leading to its nuclear exclusion and suppression of transcriptional activity (Dang et al., 2016). Another study has shown that insulin regulates the liver clock by inducing acute changes in the expression of *Per2* and *Rev-erbα* (Tahara et al., 2011). Furthermore, hepatocytic IR is essential for programming the liver clock and rhythmic gene expression in response to time-restricted feeding schedules (Fougeray et al., 2022). Further, insulin directly influences the circadian clock in adipose tissue by modulating the expression of *PER2* and *PER3* (Tuvia et al., 2021).

Moving forward, just as insulin is shown to influence circadian system in both, central and peripheral tissues, the biological clock, in turn modulates insulin signalling and action, as will be discussed in the next section.

1.3.2 Circadian gating and regulation of insulin sensitivity and action

The circadian system, playing a critical role in regulating metabolic homeostasis, modulates insulin sensitivity and action across central and peripheral tissues. There is growing evidence that insulin secretion, glucose uptake and tissue responsiveness follow a circadian rhythm for metabolic efficiency. Rhythmic control of insulin sensitivity and action is orchestrated by the intrinsic biological cycles and the molecular components of the circadian clock. First, a functional circadian clock is a pre-requisite for optimal insulin secretion by pancreatic beta cells (Saini et al., 2016). On the molecular level, whole-body deletion of CLOCK leads to metabolic dysregulation, including hyperinsulinemia and hyperglycemia with impaired glucose-stimulated insulin secretion (Marcheva et al., 2010). Mice lacking BMAL1 show glucose intolerance and hypoinsulinemia (Marcheva et al., 2010). Deletion of CRY in rodents increases gluconeogenic gene expression in the liver and fasting blood glucose levels (Zhang et al., 2010). CRY deficiency has also been shown to cause higher potency for high-fat diet induced obesity and impaired insulin sensitivity in adipose tissues (Barclay et al., 2012). In addition, REV-ERB α and β double knockout mice exhibit hyperglycemia (Cho et al., 2012). Organ specific clock disruptions further confirm the circadian clock's role in regulating insulin-related metabolic processes . For instance, deletion of BMAL1 in pancreas leads to hyperglycemia, impaired glucose tolerance and reduced insulin secretion, highlighting clock's clear role in insulin production (Lee et al., 2013). REV-ERBα deficiency in skeletal muscles impair insulin-stimulated glucose uptake by reducing Akt phosphorylation and disrupting mitochondrial function, thereby decreasing insulin sensitivity (Woldt et al., 2013).

In addition to the direct core clock components, clock-regulated hormone secretion can modulate insulin sensitivity and actions. For instance, altered circadian secretory profiles in cortisol impair systemic insulin action and phase-shift insulin responsiveness (Van Cauter et al., 1997). Ghrelin, that oscillates diurnally, enhances hepatic glucose production via a gut-brain-liver neurocircuitry and suppresses insulin secretion in human islet cells *ex vivo* (Lindqvist et al., 2020). It also inhibits IR expression and Akt phosphorylation (Lin et al., 2019). Additionally, experiments in mice lacking ghrelin signalling reveal disruptions in glucose rhythms, suggesting its influence on insulin signalling cascades (Cummings & Overduin, 2007). Lastly, melatonin, promotes energy consumption and limits WAT accumulation by modulation of insulin signalling (Cipolla-Neto et al., 2014). When melatonin is administered at non-physiological times, on the other hand, it impairs insulin secretion (Garaulet et al., 2020). In addition, variants of the melatonin receptor gene (*MtnR1b)* are associated with reduced early-phase insulin release and an increased risk of T2DM (Garaulet et al., 2020).

Together with the clock-regulated modulation, the process of 'circadian gating' adds another temporal layer of control over insulin sensitivity and action. Circadian gating refers to the time-dependent modulation of physiological responsiveness of hormone target tissues, thereby creating periods of heightened or dampened sensitivity. An array of experiments provides insights into the circadian gating of insulin action. In an SCN-lesion study, for example, it was proven that outputs from the master clock control the

diurnal rhythm in whole-body insulin sensitivity (La Fleur et al., 2001). In humans, glucose tolerance and insulin-stimulated glucose uptake are higher in the morning compared to the evening following the normal meal rhythm, however this pattern is also shown to be independent of food intake and physical activity (Poggiogalle et al., 2018; van Cauter et al., 1991). Other studies show that feeding during the inactive/rest phase leads to altered rhythmic expression of insulin-responsive genes, despite equal calorie intake (Hatori et al., 2012). Conversely, time-restricted eating confined to the active phase in men, restores insulin sensitivity, demonstrating that meal timing aligned with circadian rhythms leverages optimal windows of insulin action (Sutton et al., 2018a). In addition to whole-body insulin responsiveness, individual peripheral metabolic tissue clocks affect circadian gating of insulin sensitivity. For instance, skeletal muscle exhibits diurnal variations in the expression of insulin-responsive components such as GLUT4, tightly regulated by the muscle clock (Dyar et al., 2014). Adipose tissue also follows an intrinsic circadian rhythm in insulin sensitivity and signalling (Carrasco-Benso et al., 2016), and in some cases, this is influenced by the clock-controlled secretion of adipokines such as adiponectin (Gómez-Abellán et al., 2010).

Although peripheral insulin sensitivity exhibits temporal gating, evidence for similar rhythms in the brain remains limited. Insulin is transported into the hypothalamus via IRs in tanycytes (Porniece Kumar et al., 2021), and there is experimental evidence that tanycytes respond to timing cues from the SCN to mediate circadian variations in brain glucose uptake (Imbernon et al., 2022; Rodríguez-Cortés et al., 2022). However, whether insulin transport into the brain via tanycytes is circadian-gated, remains to be shown. In addition, neuronal populations in the Arc integrating hormonal and nutrient cues to regulate feeding behaviour exhibit circadian rhythmicity in neuronal activity (Jamali & Tramu, 1997; Sayar-Atasoy et al., 2024; Van Drunen & Eckel-Mahan, 2021a). Although, insulin signalling plays a paramount role in Arc-mediated feeding control, it is plausible that hypothalamic insulin sensitivity is under circadian regulation, though this has not yet been demonstrated directly.

Interestingly, neuronal functions like synaptic plasticity, learning and memory consolidation are known to follow circadian rhythms, with distinct time-of-day variations in performance and underlying molecular processes (Eckel-Mahan & Storm, 2009; Frank, 2016; Van Drunen & Eckel-Mahan, 2023). Insulin's role in these functions have been well established (De Felice & Benedict, 2015; Huang et al., 2010; Yaribeygi et al., 2023), however, whether insulin actively contributes to the temporal patterns in these functions remains largely unclear. It has been therefore suggested that insulin's neuromodulatory actions in the brain may operate independently of circadian timing, highlighting a critical gap in our understanding that warrants further investigation.

Taken together, intrinsic circadian clocks are crucial for modulating insulin sensitivity and action. In addition, as a functional consequence of clock regulation, circadian gating adds an extra layer of temporal control over tissue responsiveness to insulin. While metabolic actions of insulin appear to be circadian gated, for insulin-related brain functions such as neuronal health and cognition there is limited evidence so far

supporting temporal control. Emerging evidence highlights that insulin-clock crosstalk is not only evident under normal physiological conditions but also becomes increasingly apparent in states of chronodisruption, such as shift work or jet lag, as well as in metabolic disturbances.

1.3.3 Insulin resistance and circadian desynchrony

Metabolic health is optimal when different daily rhythms, including the sleep-wake cycle, feeding-fasting cycles, hormonal rhythms, autonomic nervous system outputs and central-peripheral clock rhythms, oscillate in synchrony with each other. Modern lifestyle factors such as artificial light exposure at night, erratic eating patterns, irregular working schedules like shift work, and jet lag are increasingly leading to a state of chronodisruption. This term refers to a situation where daily bodily processes are not well synchronised to the environmental cycles. Such misalignment between internal and environmental timing can further manifest itself into deeper levels of disrupted states such as inter-tissue or intra-tissue circadian desynchrony (Galinde et al., 2023). Desynchronised states reduce resilience and may therefore result in adverse health outcomes. They are increasingly recognised as a significant contributor to metabolic diseases, including insulin resistance, thus linking the circadian system and insulin in pathological conditions.

Considering the strong convergence of circadian system and metabolism, one could point out primarily to erratic eating patterns and mistimed food intake as causes circadian dysregulation, further leading to issues in insulin sensitivity and glucose homeostasis. For instance, in both humans and rodents, glucose tolerance is highest at the start of the active phase and worsens when food is primarily consumed during the resting phase (Bolli et al., 1984; Moran-Ramos et al., 2016). Feeding in inactive phases also severely impairs insulin responses in rodents (De Goede et al., 2019; Moran-Ramos et al., 2016). Mistimed feeding disrupts metabolism by desynchronising peripheral-tissue clocks. In rodents, daytime feeding shifts the liver clock by 12 hours, dampens clock gene expression in the LH and brown adipose tissues and completely diminishes muscle clock rhythmicity (de Goede et al., 2018; Opperhuizen et al., 2016). This misalignment is speculated to cause insulin resistance by disturbing the coordination between glucose production in liver and uptake in the muscle. Within-tissue misalignment has also been observed where lipid metabolism in skeletal muscle remains aligned with the light-dark cycle, while carbohydrate metabolism follows feeding time (Goede et al., 2022). In a study where mice were fed a snack during the early rest phase (i.e., morning), increased serum glucose levels were observed indicating hyperglycemia (Begemann et al., 2023).

In humans, too, mealtime can impact insulin sensitivity. Earlier meal timing has been corelated with lower fasting glucose and HOMA-IR (Homeostatic model assessment of insulin resistance) (Ali et al., 2023), while a later first meal bout (in this study, after 9:00 am) is associated with a higher risk of T2DM (Palomar-Cros et al., 2023). In a randomised controlled trial, an early eating window (8.00 to 19:00) improved fasting glucose, insulin

and HOMA-IR, while a delayed one (12:00 to 23:00) drastically worsened these markers (Allison et al., 2021). Shift workers, who are often prone to eat at night, exhibit reduced glucose tolerance and increased insulin resistance, both risk factors of the metabolic syndrome (Bahinipati et al., 2022; Centofanti et al., 2025).

Another major contributor to circadian misalignment is artificial light exposure at night. It is proven that constant light exposure in mice increases fasting glucose levels and induces insulin resistance (Borck et al., 2022; Russart et al., 2019; Yamamuro et al., 2020). Even brief light pulses at night impair glucose tolerance in rodents and alter liver gene expression (Masís-Vargas et al., 2020; Opperhuizen et al., 2019). During gestation, if rats are exposed to light at night, it disrupts corticosterone rhythms and impairs glucose tolerance and insulin sensitivity in both mothers and offspring (Dzirbíková et al., 2022; Mendez et al., 2016). This holds true for pregnant women, too. Exposure of pregnant women to strong light during three hours before sleep increases the risk of gestational diabetes (Kim et al., 2023).

Chronic exposure to light at night eventually causes sleep disturbances which can further impinge on metabolic functions. In addition, sleep disturbances caused due to (travel) jet lag, social jet lag, shift work or insomnia have been shown to affect glucose metabolism and insulin sensitivity. Individuals with later chronotypes show lower peripheral insulin sensitivity (Remchak et al., 2022; Zhang et al., 2022). Both, very short and long sleep durations are associated with an increased prevalence of T2DM (Mokhlesi et al., 2019; Shan et al., 2015). In fact, social jetlag and sleep variability appear to be strong predictors of increased HbA1c levels in diabetic individuals (Brouwer et al., 2020; Kelly et al., 2020). Experiments in animal models of shift work and jet lag support these findings (Wang et al., 2025). Shift work generally combines multiple circadian disturbances including mistimed light exposure, food intake, sleep and physical activity. In humans, circadian disruption due to shift work is strongly associated with metabolic syndrome, a condition that encompasses T2DM and peripheral insulin resistance (Lowden et al., 2010; Sooriyaarachchi et al., 2022; Yang et al., 2021). Evidence suggests that metabolic syndrome further increases the risk of brain insulin resistance, also referred to as type 3 diabetes (Kakoty et al., 2023). Impaired insulin sensitivity in such condition is shown to contribute to the development of neurodegenerative disorders like AD and PD (Hölscher, 2019; Kakoty et al., 2023; Vinuesa et al., 2021).

While circadian disruption is a known contributor to insulin resistance, emerging evidence suggests that the reverse is also true, insulin resistance can impair the functioning of the circadian clock. For instance, high-fat diet induced insulin resistance in mice disrupts behavioural rhythm of locomotor activity and impairs clock gene expression in the brain as well as in peripheral tissues (Kohsaka et al., 2007). Insulin resistance is associated with increased oxidative stress and inflammation, which further influence core clock functioning (Evans et al., 2005; Lananna & Musiek, 2020). In human patients, a study has reported that T2DM is associated with impaired peripheral clock gene expression (Ando et al., 2009), however, another study have found no significant

alterations (Otway et al., 2011), suggesting that these effects may be context-dependent or vary with the tissue-type or disease state.

Taken together, disrupted clocks may contribute to the pathological condition of insulin resistance. Vice versa, the insulin resistant physiological state may worsen the symptoms of a disrupted circadian system. Understanding this bi-directional relationship in healthy and pathological states is therefore valuable to develop therapeutic strategies for maintaining metabolic health.

1.3.4 Involvement of insulin in circadian medicine

Circadian medicine is an approach that aims to align medical treatments or mitigating strategies with the body's natural biological rhythms and internal circadian clock. This includes lifestyle interventions that promote circadian synchrony. In humans, these measures typically involve time-restricted eating, prioritizing larger meals during the day and fasting during the evening and night, exposure to bright light in the early morning, and regulating sleep–wake behaviour (reviewed in detail in Speksnijder et al., 2024; Stenvers et al., 2019). For instance, early time-restricted eating have been shown to improve insulin sensitivity and fasting glucose levels, even in the absence of weight loss (Sutton et al., 2018b). In addition, morning light exposure is proven to improve insulin action by reinforcing circadian alignment (Aras et al., 2019).

A complementary and increasingly explored approach involves administering medications in synchrony with the body's circadian rhythms to enhance therapeutic efficacy and reduce side effects. In the context of insulin-related metabolic and neurological diseases, circadian-based therapy seeks to optimize the timing of insulin (or its analogues) administration to correspond more closely with the body's natural insulin secretion and sensitivity patterns. For example, administering basal insulin formulations such as neutral protamine Hagedorn (NPH) insulin at bedtime led to improved glycaemic control in patients with T2DM. However, this regimen posed a notable risk of nocturnal hypoglycaemia due to the peak activity of NPH insulin during sleep (Seigler et al., 1992). The circadian dynamics of insulin medication involves a complex interplay between insulin secretion, sensitivity and tissue-specific responses. Serum insulin levels typically follow a diurnal rhythm, peaking in alignment with feeding times, so often between noon and early evening (Boden et al., 1996). At the same time, insulin sensitivity, particularly in peripheral tissues like skeletal muscle, is generally highest in the morning and declines across the day (van cauter, 1991, Poggiogalle et al., 2018). This apparent mismatch highlights the need to consider both the timing of insulin secretion and time-of-day responsiveness of target tissues. Efforts to optimize insulin therapy have explored the timing of basal administration. For instance, Standl et al. found that morning administration of insulin glargine yielded better glycaemic control than bedtime dosing (Standl et al., 2005). However, early studies argued that increased insulin requirements in the morning may result not from decreased sensitivity, but rather from elevated hepatic glucose output due to the dawn phenomenon (Blackard et al., 1989; Wolfsheimer,

1990). Time-of-day-aware insulin dosing shows a higher potential for improving health conditions of patients, although currently not many studies have validated the outcome.

The principles of chronotherapy could extend beyond insulin itself to other antidiabetic medications. Metformin, a well-established first-line treatment for T2DM, has been studied for its time-of-day- and chronotype-dependent effects (Türk et al., 2023). In a model of circadian disruption and obesity, it has been shown that co-administration of melatonin and metformin prior to the inactive phase restores glucose tolerance and insulin sensitivity (Thomas et al., 2016). Peroxisome proliferator-activated receptors (PPARs), key regulators of circadian rhythms and metabolism, also play a critical role in this context (Chen et al., 2014). The PPARα ligand bezafibrate, used to reduce insulin resistance, was significantly more effective when administered during the night compared to daytime injections (Duez & Staels, 2010; Oishi et al., 2008). Fenofibrate, the human analogue of bezafibrate, has likewise shown enhanced efficacy when administered during the sleep phase (Chew et al., 2008; Duez & Staels, 2010).

Glyburide, another antidiabetic agent, was found to be more effective when taken before bedtime compared to morning administration, resulting in improved fasting blood glucose levels (Hennessey et al., 1994). While most circadian-aligned medications focus on peripheral insulin resistance, recent research highlights centrally acting agents. Bromocriptine, a dopamine agonist in a quick-release formulation, has been shown to exert significant time-dependent effects on glycaemic control. Morning administration, timed to coincide with the body's natural dopaminergic peak, yields more pronounced metabolic improvements (Ezrokhi et al., 2021). Additionally, bromocriptine is effective in suppressing key hypothalamic neuropeptides such as NPY and AgRP, and influences the expression of genes associated with neuronal plasticity (Ezrokhi et al., 2021).

Taken together, circadian-based pharmacotherapy represents a promising strategy for optimizing the treatment of insulin-related metabolic disorders by aligning medication administration with the body's biological rhythms. This approach has the potential to enhance therapeutic efficacy and minimize side effects, ultimately contributing to improved health outcomes.

1.4 Rationale and hypothesis

Insulin plays a pivotal role in the central nervous system influencing appetite regulation and cognition-associated functions (Hallschmid & Schultes, 2009; Woods et al., 1979; Zhao et al., 1999). The hypothalamus acts as a central regulator of essential bodily processes including metabolic homeostasis and the body's internal timing. This makes it a crucial site for the convergence of metabolic cues (like insulin) and circadian signals. Emerging evidence highlights a bidirectional interplay between the circadian clock and insulin signalling. Circadian clocks modulate insulin sensitivity, while insulin itself can impact the rhythmicity of molecular clocks (Marcheva et al., 2010; Mori et al., 1985; Nagai et al., 1982; Zhang et al., 2010). These interactions are relevant not only under physiological conditions but also in states of circadian disruption or metabolic stress,

where dysregulated insulin action may contribute to pathological outcomes (Speksnijder et al., 2024; Stenvers et al., 2019).

Understanding the circadian modulation or gating of insulin action in the brain represents an important step toward deciphering the integration of metabolism and the circadian system. In the long term and with further research, such knowledge could help refining chrono-therapeutic interventions aimed at restoring metabolic and neurological balance. Given the increasing prevalence of insulin resistance and circadian disruption in modern lifestyles, elucidating how insulin's effects are temporally regulated in the hypothalamus was the fundamental rationale for this research project.

Accordingly, I hypothesized that insulin elicits differential effects in the hypothalamus depending on the circadian phase of administration. With respect to insulin's role in regulating feeding behaviour, I hypothesized that insulin selectively alters gene expression of hypothalamic neuropeptides involved in metabolic homeostasis, with greater sensitivity during the activity/feeding phase. In contrast, for its cognition-associated functions, I hypothesized that insulin influences the expression of genes associated with neuronal health and cognitive processes, independent of circadian time, suggesting a tonic mode of regulation (Figure 5).

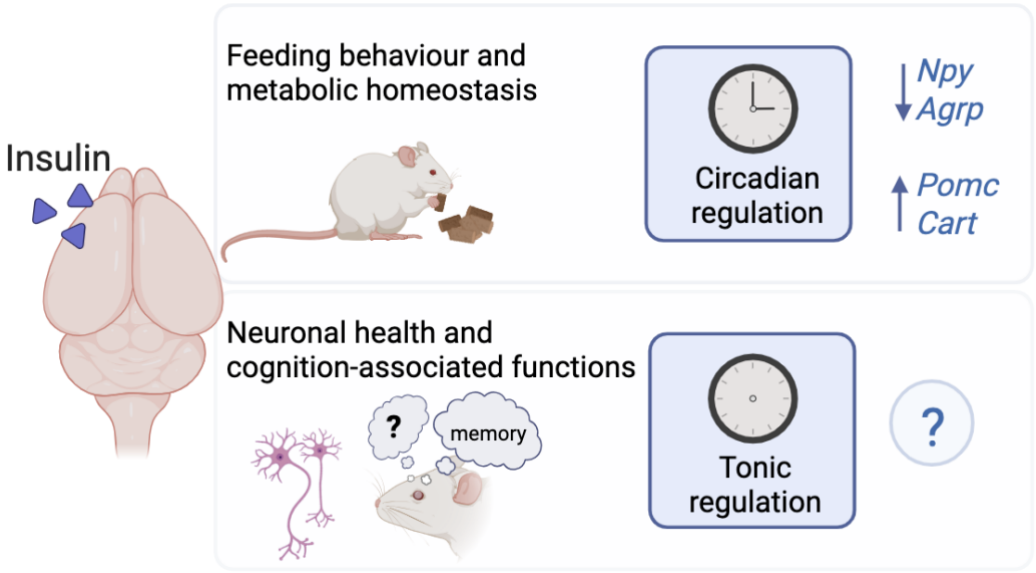


Figure 5. Schematic representation of the proposed hypothesis.
Insulin plays a vital role in the brain influencing feeding behaviour and cognition-associated functions. The hypotheses of the project were that insulin exerts effects on the metabolic genes in a circadian-regulated manner while it influences the genes involved in neuronal health and cognitive processes in a time-independent, i.e., tonic fashion. Created with Biorender. (Npy, Neuropeptide Y; Agrp, Agouti- related protein; Pomc, Proopiomelanocortin; Cart, cocaine-and-amphetamine-regulated transcript).

Chapter 2. Materials and Methods

2.1 Mice sacrifice and hypothalamic tissue culture

All experiments involving mice were conducted in accordance with German Animal Welfare Law, under institutional approval for animal sacrifice (License Ids: 4_2020-09-23_Oster and 4_2021-12-13_Oster). The experimental animals were housed and maintained in the animal facility at University of Lübeck. The study used adult male C57BL/6J mice (7–12 weeks old) obtained from Janvier and heterozygous PER2::LUC (*Period2::Luciferase*) mice, which were bred in-house on a C57BL/6J background. These PER2::LUC mice express a PER2-LUCIFERASE fusion protein under the control of the native *Per2* promoter, allowing real-time bioluminescence-based tracking of PER2 expression (Yoo et al., 2004).

All animals were housed in groups of 5–6 per cage in individually ventilated cages (IVCs) under controlled conditions (21–23°C, 55–65 % humidity) with a 12/ 12-hour light/dark cycle. The time of "lights on" was designated as ZT0, and "lights off" as ZT12. Animals were given ad libitum access to standard chow (Altromin) and water.

Mice were sacrificed by cervical dislocation, followed by brain extraction in ice-cold Hank's Balanced Salt Solution (1X HBSS). Four coronal brain slices of the hypothalamus (-1.80 mm to -2.10 mm relative to bregma) were prepared at 300 µm thickness using a vibratome (Thermo Scientific, Microm HM 650V). Precise dissection of the Arc-ME complex was then conducted under a dissection microscope (Leica) based on coordinates and visual markers from the Paxinos Mouse Brain Atlas, third edition.

Each dissected section was placed on a culture insert (Merck, Millipore) in 35-mm Petri dishes (Merck, Sigma Aldrich) containing 2 mL of culture media (DMEM with 4.5 g/L glucose and 2 mM GlutaMax, supplemented with 10 % FBS and 1 % penicillin-streptomycin). Cultures were incubated at 37 °C with 5 % CO_2 until further experimental use.

2.2 Cell culture and cell line maintenance

Hypothalamic neuronal cell lines – mHypo E44, E41, E25, and A2/23 (Cedarlane Labs, USA) – were cultured and maintained in standard growth media for the course of the experiments. The base medium used was Dulbecco's Modified Eagle Medium (DMEM) with phenol red, supplemented with 4.5 g/L glucose and 2 mM GlutaMAX (Gibco). Additionally, 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin cocktail were added to the medium to support cell growth. For circadian bioluminescence experiments, these cell lines were stably transduced using a lentiviral vector to express luciferase under control of a clock gene promoter, *Bmal1.* Following transduction, the cells were polyclonally selected using puromycin to establish stable *Bmal1-luc* reporter cell lines. This procedure was previously performed and established for circadian bioluminescence studies in our lab by Anthony Tsang (Tsang et al., 2020). All experiments

were conducted with cell lines maintained between passage numbers 20 and 40. The cell lines were incubated at 37 °C in a humidified atmosphere with 95 % humidity and 5 % CO_2.

2.3 Bioluminescence recordings

For circadian bioluminescence experiments, the protocol was adapted from established methods by (Tsang et al., 2020 and Srimani et al., 2022). Briefly, *Bmal1-luc* neuronal cell lines were seeded at a density of 2 × 10^5 cells per well in 35-mm culture dishes with 2 mL of standard DMEM culture media. Cells were incubated for 24–48 h at 37 °C and 5 % CO_2 until they reached approximately 70-80 % confluency. Once confluency was reached, cells were synchronized by treatment with 100 nM dexamethasone for 2 h. Following synchronization, the DMEM culture media was replaced with 2 mL of recording medium (Pilorz et al., 2020, composition detailed below), specifically prepared to be phenol-red-free to avoid luminescence artifacts.

For bioluminescence measurements of the Arc-ME complex, tissue slices were obtained from heterozygous PER2::LUC mice, as established (Yoo et al., 2004; Tsang et al., 2020). Arc-ME slices were dissected from coronal slices of the hypothalamic region and cultured on filter inserts in 35-mm Petri dishes (as described in Section 2.1) containing 2 mL of recording medium.

Once recording media were added, light exposure in the culture hood was reduced, and all further procedures were performed under dim light. Culture dishes containing cells or tissue slices were covered with glass coverslips and sealed with silicone grease. Bioluminescence recordings were conducted using the Lumicycle system (Actimetrics, 32 Luminometer) over a period of 5 days, maintained at 37 °C in constant darkness.

Composition of recording medium:

DMEM low-glucose (Sigma Aldrich),

10 mM D-glucose (Sigma Aldrich),

3 mM 7.5 % sodium bicarbonate (Roth),

10 mM Hepes (pH 7.2) (Thermo Fisher Scientific),

1% penicillin/streptomycin (Thermo Fisher Scientific),

2% B-27 (Thermo Fisher Scientific),

0.1 mM luciferin (Promega)

2.4 Insulin dose response curve (DRC) and response-time curve (RTC) experiments

2.4.1 Dose response curve

An insulin dose-response curve was determined to identify an optimal pharmacological insulin concentration that elicits measurable effects on metabolic gene expression in

hypothalamic neuronal cell lines (E44 and A2/23) and Arc-ME tissue slices. Pharmacological insulin (4 mg/mL, human recombinant, zinc solution; Gibco) was dissolved in PBS and stored at -20 °C until experimental use, following the manufacturer's instructions. For each experiment, insulin was diluted in PBS to prepare final concentrations ranging from 1 to 500 nM.

Neuronal cell lines were seeded in 12-well culture plates at a density of 2 × 10^5 cells per well and cultured in standard growth media for 24–48 hours at 37 °C with 5 % CO_2 until they reached 90–95 % confluency (as described in Section 2.2). Once confluent, cells were washed with PBS, and the culture medium was replaced with medium containing insulin (or PBS) according to the designated treatment groups. Fresh insulin solutions were prepared by diluting the stock solution and adding it to the standard growth medium to achieve final concentrations of 2, 10, 50, or 100 nM in a total volume of 2 mL. Control samples were treated with the corresponding volumes of 1X PBS in standard growth medium. A total of four wells (n=4) were maintained per concentration group. Cells were treated for 1 hour and then processed for downstream applications. For RNA isolation and gene expression analysis, cells were harvested in 500 µL of TRIzol reagent (ThermoFisher). For immunocytochemistry, cells were washed and fixed with 4 % paraformaldehyde (PFA, Electron Microscopy Sciences).

For Arc-ME slices, a similar setup was implemented. Immediately after the sacrifice of mice, brains were harvested in ice-cold 1X HBSS, and the Arc-ME complex was micro-dissected from the coronal slices of the hypothalamic region. These slices were cultured on tissue-culture inserts in Petri dishes containing 2 mL of standard growth medium as described in section 2.1. They were acclimatized to *ex-vivo* conditions at 37 °C with 5 % CO_2 for 1 hour. Following acclimatization, the medium was replaced with 2 mL of medium containing insulin at final concentrations of 1, 10, 100, or 500 nM. Control slices were treated with 1X PBS. Five to six slices were included per condition. Due to the intact tissue interactions in Arc-ME slices compared to cell cultures, the insulin exposure time was reduced to 30 minutes. After insulin treatment, slices were harvested in 500 µL of TRIzol (ThermoFisher) reagent for further analysis.

Notably, the insulin dosages used in this study were chosen based on previous reports investigating insulin action in similar experimental models (Mayer & Belsham, 2009; Sato et al., 2005; Choi et al., 2010; Dhillon & Belsham, 2011). The selected dose range included both physiologically relevant and pharmacologically effective concentrations, ensuring an effective scan to identify the optimal dose capable of inducing quantifiable molecular effects. Similarly, the choice of treatment duration was guided by findings from prior studies, which demonstrated that the selected timeframes are sufficient for pharmacological hormone doses to elicit measurable transcriptional changes in gene expression (Mayer & Belsham, 2009; Sato et al., 2005; Choi et al., 2010; Dhillon & Belsham, 2011).

2.4.2 Response-time curve

To investigate temporal kinetics of insulin action and to find an optimal treatment time for insulin to produce a measurable response in metabolic gene expression, a response-time curve was determined on both model systems used in this study, hypothalamic neurons and Arc-ME slices of the mouse brain.

Neuronal cells lines (E44 and A2/23) and Arc-ME tissue preparations were cultured and processed similarly as mentioned for the DRC experimental setup above. Initial findings from the DRC in neuronal cells (see Section 3.3 A-D) indicated that a 1-hour insulin treatment was insufficient to produce clear changes in the expression of metabolic genes. Thus, an expanded time range was introduced to capture both rapid and sustained insulin effects in the neurons. For E44 cells, the strongest tested dosage of insulin, 100 nM, was implemented for 30 minutes, 1, 2, 4, 6, 12, and 24 hours. A2/23 cells, after insulin treatment, were additionally sampled at intervals of 5 and 10 minutes to capture transient, immediate insulin action. Each response time included a PBS treated control sample for comparison. Additionally, the setup used a 0-hour (baseline) time point control for normalization across different response times for insulin and PBS treated samples. After insulin treatment for the mentioned time intervals, cells were harvested in 500 µL TRIzol for following analyses. Of note, gene expression data from the untreated control group was analysed for rhythmicity using the CircWave V1.4 computational tool. This software fits a standard 24-hour cosine function to averaged, de-trended time-series data. Rhythmicity was defined based on a significance threshold of $p < 0.05$.

Findings from the RTC in neuronal cells (refer to Section 3.3 E and F) revealed comparable gene expression patterns at both, the early (5, 10, 15 minutes) and late time points (12 and 24 h). Therefore, for Arc-ME slices, only moderate treatment times (1, 3, and 6 hours), were selected to align with insulin's potential molecular response timeline in the tissue. Arc-ME slices were treated with the highest effective insulin dose (500 nM) based on the DRC results. Following each treatment duration, slices were harvested in TRIzol and stored at -80 °C until further analysis.

2.5 Immunocytochemistry

The experimental protocol for insulin DRC and RTC to observe insulin-mediated pFOXO1 nuclear exclusion was carried out on an 8-well chambered-glass slide (Millipore, Merck). After the incubation times of insulin in individual experimental setups, the culture medium was aspirated, and the cells were fixed in 4 % paraformaldehyde (Electron Microscopy Sciences) in 1X PBS at 4 °C for 30 mins. Post-fixed cells were blocked for non-specific binding sites with 5 % goat serum (Gibco) and 0.4 % Triton X-100 (Sigma Aldrich) at 4 °C for 2 h. After a quick wash with PBS, the cells were incubated overnight with pFOXO1 primary rabbit antibody (Invitrogen, ThermoFisher Scientific) at 1:250 dilution at 4 °C. The following day, a secondary antibody (goat anti-rabbit IgG, 1:200 dilution) labelled with Alexa fluor 488 (Abcam) was applied for 2 h at room temperature. Finally, the individual chambers were taken off and the slide was mounted with DAPI

Vectashield anti-fade mounting medium (Biozol) and covered with a glass coverslip. Images were obtained via an inverted fluorescence microscope (Zeiss) with DAPI (excitation 358 and emission 463 nm) and FITC (excitation 495 and emission 519 nm) filters. Images were exported and signals were quantified by the FIJI (ImageJ2 enhanced version) software, using a built-in 'Measure ROI intensity' feature.

2.6 RNA extraction, reverse transcription and quantitative real-time (q)PCR

Total RNA was extracted from cultured cells or from dissected Arc-ME slices (described in section 2.4). The cells were scraped from the culture dishes/ multi-well plates after addition of 500 µL TRIzol (ThermoFisher). The mixture was then collected in a 1.5-mL nuclease-free reaction tube and homogenised by vigorous pipetting. For the Arc-ME slices, samples were directly transferred to screw-cap tubes containing 500 µL TRIzol solution (ThermoFisher) and 5-6 ceramic beads (1.4-mm ceramic beads, Omni International). The slices were homogenized in a bench homogenizer (Omni Bead Ruptor, Omni International) for 5 cycles at a speed of 2.9 m/s. Each cycle lasted for 20 s with a break of 10 s between consecutive cycles.

For both sample types, homogenates were incubated at room temperature for 5 mins to facilitate continuation of cell membrane disintegration. 100 µL of chloroform (Honeywell, ThermoFisher) was added to the homogenate, and samples were incubated for 10 mins on ice, followed by centrifugation at 14,000 rpm for 15 mins at 4 °C. The upper aqueous phase containing RNA was carefully collected in a new tube and mixed with 250 µL isopropanol (Roth). This mixture was incubated at -20 °C for 2 hrs and followed by centrifugation for 45 mins with the same settings as for the previous step. The supernatant was discarded, and the RNA pellet was washed twice with 500 µL of 70 % EtOH with 10-minute centrifugation cycles for each wash (at the same temperature and speed settings as before). Pellets were briefly air-dried for 5-10 min and dissolved in 15 µL nuclease-free water. RNA concentrations and purities were determined by absorption at 260 and 280 nm on a spectrophotometer (Epoch Microplate Spectrophotometer, BioTek Instruments) and analysed using Gen5 software (version 2.0, BioTek Instruments). Samples were either used directly for reverse transcription or stored at -80 °C until further use.

To obtain cDNA, extracted mRNA was reverse-transcribed using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems). Total RNA yields of 1.5 to 2 µg from the neuronal cell samples were obtained in the process while the RNA yield from Arc-ME slices was very low, hence only 1 µg per sample was processed further. The respective volumes of mRNA were adjusted to a total of 10 µL with nuclease-free water. Next, a 10 µL of RT master mix (Table 1) was added to this mixture (per reaction tube) to initiate the process of reverse transcription for the mentioned incubation periods (Table 2). Obtained cDNA was diluted 1:5 for neuronal cells and 1:10 for tissue preparations and stored at -20 °C for further use.

Reaction component	Volume (µL)
RT Buffer 10X	2.0
dNTP 25X	0.8
Random Primer	2.0
Multiscribe reverse transcriptase	1.0
Water	4.2

Table 1. Reaction components for reverse transcription reaction

Temperature (°C)	Time (mins)
25	10
37	120
85	5

Table 2. Program for thermocycler

Real-time PCR from cDNA preparations was conducted using 2.5 µL of diluted cDNA with 2.5 µL of primer mix (1.4 µM, Table 3) and 5 µL Go-Taq qPCR Master Mix (Promega) on a 96-well PCR plate (Bio-Rad). Plates were sealed with adhesive tape (Biozym Biotech Trading) and measured in a real-time PCR detection system from BioRad (CFX96 thermocycler) with the following temperature cycles (Table 3).

Temperature (°C)	Time	No. of cycles
94	5 mins	
94	15 sec	40 X
60	15 sec	40 X
72	20 sec	40 X
72	5 mins	

Table 3. Program for qPCR thermocycler

Genes	Forward primer sequence	Reverse primer sequence
Eef1A	TGCCCCAGGACACAGAGACTTCA	AATTCACCAACACCAGCAGCAA
Npy	CTCCGCTCTGCGACACTA C	GGAAGGGTCTTCAAGCCTTGT
Agrp	ATGCTGACTGCAATGTTGCTG	CAGACTTAGACCTGGGAACTC
Pomc	CTTTCCGCGACAGAGACTAGG	GCACCAGCTCCACACATCTAT
Cart	GCGCTATGTTGCAGATCGAA	TCTCTGAGGGGAACGCAAAC
Gpr17	AGTCAAGCCTTCCTCCTACAG	CCTCCAGACCGTTCATCTTGT
Cpe	CGGCATCTCCTTCGAGTACC	CACCTCGGTGTATCTGCTCC
Nefm	AGCTGCAGTCCAAGAGCATC	AACTGCTGGATGGTGTCCTG
Trpc3	AGGCGCAGCAGTATGTGGA	GCCCAAAGCTCTCGTTTGC
Opalin	CTGCCTCTCACTCAACATCA	GCTGGATCAAAGTAAACAGC

Table 4. qPCR primer sequences

Ct values were exported from the BioRad software and, to normalize targeted gene expression, *Eukaryotic elongation factor 1 α (Eef1α)* expression was used as reference. Relative gene expression levels were then calculated using the Pfaffl ΔΔCT method (Pfaffl, 2001). Of note, technical outliers, as determined by Grubb's tests with significance level of < 0.05, were excluded from the analyses.

2.7 Insulin resetting of PER2::LUC rhythms in hypothalamic tissue preparations and validating insulin-stripped culture conditions

Heterozygous PER2::LUC mice maintained on an *ad-libitum* chow diet in 12/12-hour light-dark conditions were sacrificed for brain tissue collection. The hypothalamic region was sectioned using a vibratome, and the Arc-ME complex was microdissected as described in the section 2.1. Tissue preparations were cultured on a membrane insert in a 35-mm Petri dish containing 2 mL of standard DMEM growth medium supplemented with 4.5 g/L glucose and 2 mM GlutaMax. Cultures were acclimatized at 37 °C with 5 % humidity for 1 hour.

After acclimatization, the slices were segregated into three experimental groups (n= 4 per group). In the first group, the medium was replaced with recording medium (composition as detailed below) containing 500 nM insulin. In the second group, recording medium was supplemented with 2 % insulin-stripped B27 supplement (Gibco, B27 supplement, minus insulin). The third group served as the control and was maintained in standard recording medium. To minimize light exposure, dishes were handled under dim conditions, and 200 µM luciferin (Promega) was added to each culture

dish prior to recording. Bioluminescence was recorded using the Lumicycle system (Actimetrics, 32 Luminometer) for 5 days at 37 °C in constant darkness. A schematic of protocol is outlined in (Figure 6).

Composition of recording medium:

DMEM low-glucose (Sigma Aldrich),

10 mM D-glucose (Sigma Aldrich),

3 mM 7.5% sodium bicarbonate (Roth),

10 mM Hepes (pH 7.2) (Thermo Fisher Scientific),

1% penicillin/streptomycin (Thermo Fisher Scientific),

2% B-27 (Thermo Fisher Scientific, for group 1 and 3).

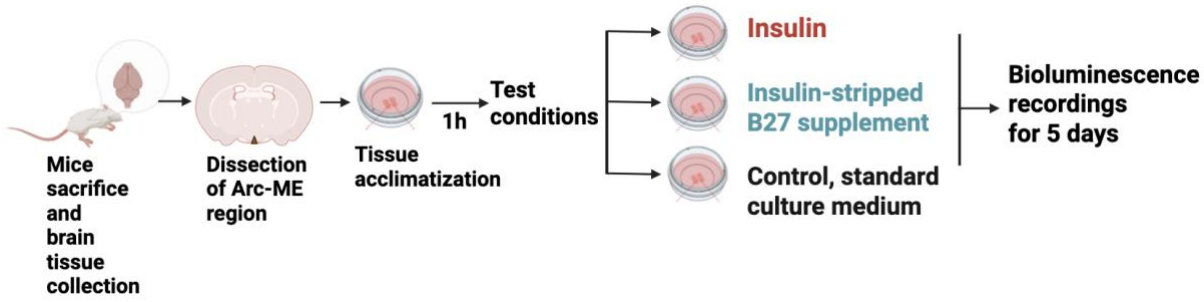


Figure 6. Protocol for the insulin resetting of PER2::LUC rhythms in hypothalamic tissue preparations and validating insulin-stripped culture conditions.
PER2::LUC mice were sacrificed, and the Arc-ME region was microdissected following vibratome sectioning. Tissues slices were cultured and divided into 3 groups: 1. Insulin treated 2. Insulin deprived and 3. Control, standard culture conditions. After 1 h acclimation period, luciferin was added, and samples were subjected to bioluminescence recordings at 37 °C in constant dark conditions for 5 days. Created with Biorender. (Arc-ME, Arcuate nucleus- Median eminence).

2.8 Phase-dependent insulin treatment of Arc-ME slices

The objective of this experiment was to investigate the circadian regulation of the insulin response in the hypothalamic Arc-ME slices of male mice at the transcriptomic level. Male heterozygous PER2::LUC mice were housed under a 12/12-hour light-dark and provided with *ad-libitum* access to a standard chow diet. On the day of the experiment, mice were sacrificed by cervical dislocation to collect brain tissue, and the Arc-ME complex was micro-dissected from the hypothalamic region as described in detail in Section 2.1. Tissue slices were cultured at 37 °C with 5 % humidity in standard DMEM supplemented with 4.5 g/L glucose and 2 mM GlutaMax. To eliminate any external insulin influence, the culture medium was supplemented with 2 % synthetic, insulin-free B27, replacing foetal bovine serum. The presence of circadian rhythms in the Arc-ME slices in this media formulation was validated in section 3.7.

Cultured slices were divided into two experimental groups based on the phase-specific timing of substance treatment. The rhythmic expression of the PER2::LUC fusion protein

was used as a temporal reference, derived from prior bioluminescence recordings. In this context, the time '0 h' marked the onset of bioluminescence monitoring, corresponding to when tissue preparations were initially cultured under standard conditions. The treatment time points were then determined relative to the peak (maximum) and trough (minimum) expression phases of PER2. To prepare the tissues for phase-dependent insulin response assessment, all slices underwent a 6-hour glucose starvation period prior to substance treatment. This was achieved by replacing the standard culture medium with DMEM containing 1 g/L glucose while keeping all other media components constant.

In the experimental timeline for Group 1, tissue slices were cultured for 3 hours, followed by 6 hours of glucose starvation. Substance treatment was administered at the peak phase of PER2 expression, which occurred at ca. 9 hours into the cultivation. In contrast, Group 2 slices were maintained in standard medium for 14 hours, followed by the 6-hour glucose starvation period. These slices received substance treatment at the trough phase of PER2 expression, ca. 21 hours after the start of cultivation (which was 12 hours apart from the peak-phase treatment). As for substance treatment, both groups received an insulin dosage of 100 nM; corresponding control samples were treated with 1X PBS. Tissue preparations were treated with the respective substance for a treatment period of 1 hour. After treatment duration, slices were harvested directly in the TRIzol reagent and were processed for RNA extraction. The protocol is outlined in Figure 7.

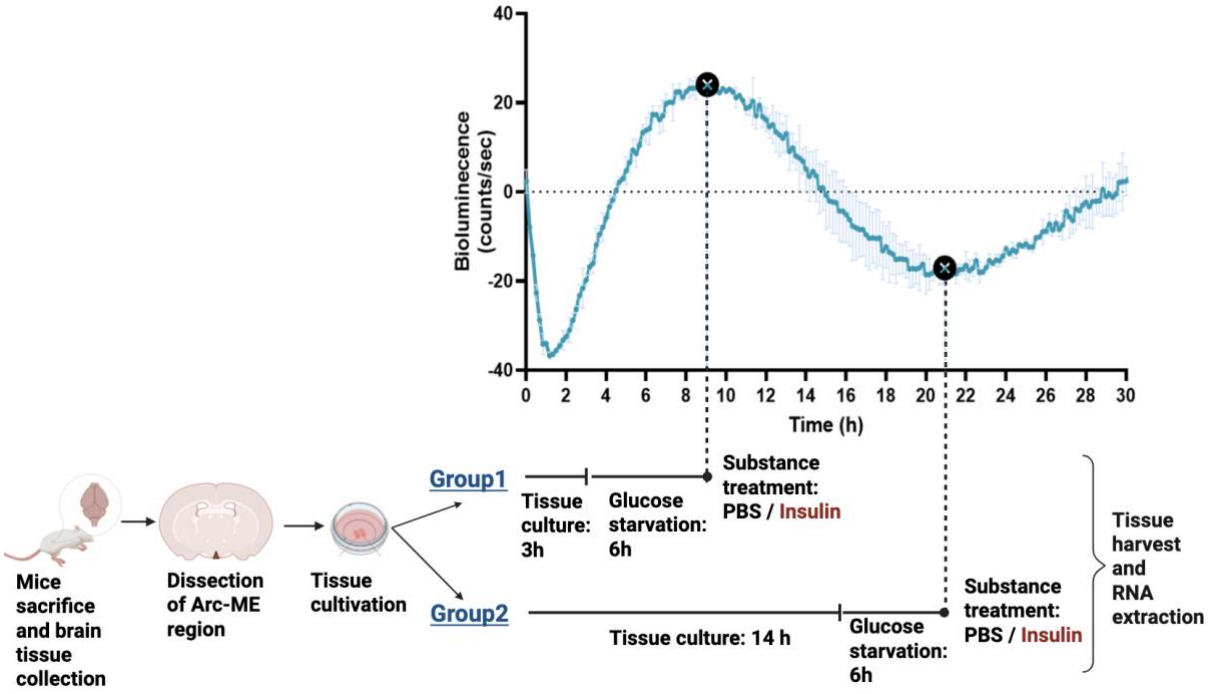


Figure 7. Protocol to investigate circadian regulation of insulin responses in Arc-ME slices of male mice. Heterozygous PER2::LUC male mice were sacrificed by cervical dislocation for brain tissue collection. Micro-dissected Arc-ME slices were cultured in DMEM with 4.5 g/L glucose, 2 mM GlutaMax, and 2 % insulin-free B27 supplement. Samples were divided into two groups for phase-specific treatment based on PER2::LUC expression peaks and troughs, determined from prior bioluminescence data. All slices underwent a 6-hour glucose starvation period (DMEM with 1 g/L glucose) prior to treatment. Group 1 was treated with 100 nM insulin (or PBS) at the PER2 peak (9 h), while Group 2 received treatment 12 hours apart, at the trough phase (21 h). Post 1-h substance treatment, slices were harvested

in TRIzol for RNA extraction and further transcriptomic analysis. Created with Biorender. (Arc-ME, Arcuate nucleus-Median eminence).

2.9 3' Bulk RNA barcoding and sequencing (BRB-seq)

A total of 20 samples were harvested in TRIzol reagent for RNA extraction (procedure as detailed in Section 2.6). The quality and concentration of extracted RNA were determined by measuring absorbance on a spectrophotometer (Epoch Microplate Spectrophotometer, BioTek Instruments) and analysed using Gen5 software (version 2.0, BioTek Instruments). Samples with absorbance 260/280 ratios higher than 1.8 were selected for downstream processing. In addition, RNA functionality was verified by qPCR amplification of known genes relevant to the experimental premises (*Eef1α, Agrp, Per1* and *18S rRNA*). Total RNA of ~100 ng obtained from each sample was sent to Alithea Genomics for library preparations and transcriptome analysis.

The cDNA libraries were prepared using the Mercurius Transcriptomics tools and sequenced on the Illumina NovaSeq 6000 platform. Transcriptomics profiling was done using bulk RNA barcoding and sequencing (BRB-seq) with high-quality sequenced reads aligned to the *Mus musculus* genome (mm10, Ensembl release 102) using the STAR alignment algorithm (RRID:SCR_004463, Dobin et al., 2013). Count matrices were subsequently generated using the HTseq tool (RRID:SCR_005514, Anders et al., 2015). The technical details of library sequencing and data analysis, as provided by the company, are summarized in the Table 5.

Technical information	**Measurements**
Average RNA concentration per sample	86.99 ng
Average demultiplexed reads per sample	12 million reads
Percentage demultiplexed reads	98 %
Percentage mapped to genome	76.4 %
Percentage mapped to exons	61.27 %
Percentage gene biotypes across samples	91.75 % protein coding genes

Table 5. Summary of technical details concerning library sequencing and data analysis

2.10 DESeq2 and downstream bioinformatic analysis

DESeq2 analysis: To determine differentially expressed genes (DEGs), analysis was performed in RStudio (version 2023.06.2, Build 561) using the DESeq2 work package (RRID:SCR_000154, Love et al., 2014). Raw counts from the count matrices and the sample set information were incorporated in the script. In place of the standard approach of global analysis (Treatment, Timepoint, Interaction), a 2x2 matrix of *design factors* was

implemented to tailor the analysis as per the experimental groups. All the *design factors* leveraged Wald's test for statistical evaluation. Expressed genes with a sum count higher than 10 were selected for downstream processing, while those with counts lesser than 10 were discarded. A significance level of adjusted *p*-value (false discovery rate, FDR) of 0.05 was applied to the data set in all the design factors. A null log fold-change (Lfc) threshold was applied for filtering DEGs. The matrix strategy employed here generated distinct DEG output for each design scheme.

Table ***6*** summarises the design schemes and the number of genes obtained as an output.

DESeq2 design factors	**Sample group**	**Output of DEGs**
Treatment	1. peak	19,917
	2. trough	21,433
Timepoint	3. PBS	20,306
	4. insulin	21,203

Table 6. Summary of DESeq2 designs and output number of DEGs

Each output file obtained was further filtered for the up- and down-regulated genes according to the positive or negative Lfc, respectively. These filtered DEGs were used as input files to generate a Venn diagram in RStudio for visualizing the unique and overlapping gene sets among the experimental sample groups (RRID:SCR_002414, Hanbo Chen, 2011). All subsequent analyses were conducted using log2-transformed, normalized expression values obtained through the variance stabilizing transformation (vst) function.

Enrichment analysis: Enrichment analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) (RRID:SCR_001881, Huang et al., 2009) and Enrichr-KG (Enrichment-Knowledge Graph) (RRID:SCR_001575, Chen et al., 2013) tools to provide a comprehensive coverage for gene set analysis. For treatment effect genes (section 3.9), a list of 14 DEGs was annotated using the 2021 KEGG pathway database in the DAVID tool. An annotated pathway was considered significant if it contained a gene count greater than 2 and adjusted *p*-value (*q* value) of ≤ 0.1. To determine statistically significant biological annotations, the DEGs were compared against a custom background list of all genes identified in this experimental data set. For graphical visualisation, *q* values were converted to a -Log10 scale.

For temporal effect DEGs (section 3.13), gene set enrichment was carried out using the Enrichr-KG tool with the 2021 GO Biological Process database. A standard significance threshold of an adjusted *p* value (*q* value) of ≤ 0.1 was applied. DEGs were compared

against a custom background list containing all identified DEGs of this experimental dataset. Using a criterion of at least one link per gene and per term, a knowledge graph was generated for the top-25 terms. The graph and the corresponding enrichment table were exported for further downstream processing, including clustering and overarching pathway analysis.

Two enrichment tools were utilised because downstream analysis of temporal effect DEGs required visualisation of links between the enriched biological pathways and the DEGs involved in these processes. Thus, in addition to the standard and widely used DAVID tool, the knowledge graphs generated by the Enrichr-KG tool were employed.

Clustering and overarching pathway analysis: For the clustering and overarching pathway analysis, enrichment results were first obtained using DAVID and Enrichr-KG tools. The knowledge graph generated by Enrichr-KG was used to visualize connections, where nodes represented DEGs and/or enriched biological processes, and edges indicated relationships or shared involvement between them. To reduce redundancy in the enriched GO terms, the REVIGO tool (Reduce + Visualize Gene Ontology) (RRID:SCR_005825, Supek et al., 2011) was employed. The enriched GO terms along with their adj. p values were incorporated in the REVIGO system, using which the system foremost removed redundant GO terms. The output list was then aligned to the experimental species, *Mus musculus* and with the redundancy score of 0.5, the system summarised and organised GO terms based on semantic similarity. Clustering of biological processes was performed manually by grouping processes that shared functional similarities. A literature-informed approach was adopted to categorize similar processes and group them into meaningful biological themes.

Tissue-specific gene expression analysis: Tissue-specific gene expression data for treatment-effect DEGs (Section 3.10) was obtained from the NCBI Gene database, that utilizes information from the Mouse ENCODE transcriptome project as the primary source. This dataset is derived from RNA-seq alignments across a broad range of mouse tissues and provides normalized expression values reported as RPKM (reads per kilobase of transcript per million mapped reads) for quantified gene expression. To facilitate comparisons across multiple genes and tissues in this dataset, the raw RPKM values for each gene were normalized relative to their highest expression values provided in the database. The final values were expressed as percentages. For clarity and ease of interpretation in downstream analyses, specific tissues with overlapping or similar biological functions were grouped into broader categories. For instance, the large intestine, small intestine, and duodenum were combined into a single category and the tissue with the highest expression in this group was represented in the data figure. The normalized data were subsequently used to create a heatmap using GraphPad Prism, to illustrate the relative tissue-specific expression of the genes of interest.

Tissue- localisation and brain cell-type specific gene expression analysis: To investigate the tissue localization and brain cell-type specific expression of DEGs from the temporal-effect dataset, an automated pipeline was created and employed. The DEG list

comprised 721 genes, for which would be extensive to apply queries manually for tissue-specific expression in the NCBI Gene database. To surpass this, publicly available datasets were utilized for the automated analysis.

A published study (Cai et al., 2021) leveraged single-cell profiled data of 20,921 individual cells in the Arc-ME (and its surrounding) region of adult mice fed under ad-libitum standard chow diet (Campbell et al., 2017). With this data, the authors identified gene sets enriched for specific brain cell-types for their further analysis. The output file from this study, which is available publicly, includes expression levels of DEGs enriched for 5 different brain cell types including neurons, oligodendrocytes, astrocytes, microglia and endothelial cells. The lists of DEGs from this output file was intersected with the DEGs identified in our RNA-seq analysis using a base 'intersect' function in an R-based script. The intersected output categorized DEGs from our study into five distinct brain cell-types.

DEGs from our RNA-seq dataset that did not overlap with the cell-type-specific gene sets were either considered to lack detectable expression in the Arc-ME region or to represent genes not yet annotated in the single-cell database. To narrow down and streamline this analysis, DEGs belonging to either of these conditions were neglected whereas DEGs from the intersected output, identified for a particular brain cell type, were prioritised.

2.11 Overall pipeline for differential gene expression analysis

Differential expression analysis was conducted to assess treatment effects (insulin vs. PBS) and temporal variation (peak vs. trough). DESeq2 in R Studio was used to identify DEGs for both comparisons. For the treatment group, DEGs were annotated using DAVID to identify enriched KEGG pathways (Figure 8.1B). Genes without pathway enrichment were further investigated via literature and GeneCards. To ensure hypothalamic relevance, DEGs were filtered using tissue expression data from the NCBI Mouse ENCODE Gene database, and brain-enriched genes were validated by qPCR (Figure 8.1 C-D).

For temporal group, DEGs were first filtered by tissue expression (Figure 8.2 B), then classified by cell type using publicly available Arc-ME single-cell RNA-seq datasets (Fig. 3.2 C). Cell-type specific DEG groups (neurons, glial and endothelium) were analysed for functional enrichment using DAVID and Enrichr KG (Figure 8.2 D). Redundant GO terms were summarized via REVIGO and manually curated into broader pathways based on literature (Figure 8.2 E), enabling interpretation of cell-type specific, time-dependent gene expression dynamics.

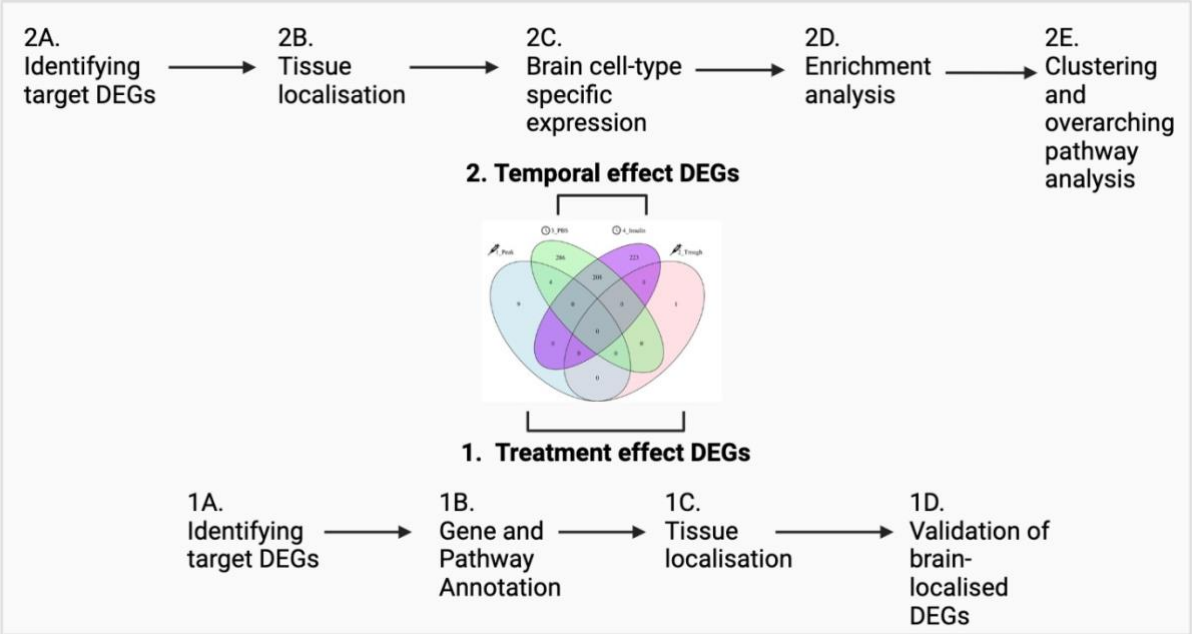


Figure 8. Pipeline for Differential gene expression analysis. For details see text.

2.12 Data analysis

Bioluminescence recordings- Bioluminescence recordings were analysed using the Lumicycle Actimetrics software, which provides a detailed workflow for adjusting and refining raw data. First, a 'best-fit' curve was applied to the raw bioluminescence data using a damped sine fit. Only fits with a 'goodness of fit' score above 75% were processed further. To remove background noise, a 'running-average' function was applied, which subtracted a baseline curve from the raw values. This baseline adjustment was calculated by subtracting from each value a running-average value spanning both the preceding and following 12 hours, for a total coverage of 24 hours. Both the raw and baseline-subtracted data were exported to Excel, and the respective rhythm graphs were visualised using GraphPad Prism. The Lumicycle Actimetrics software also calculated key circadian parameters, including period, amplitude, and damping rate, based on sine fitting of baseline-subtracted data. The calculated values were exported to Excel for additional processing and comparative analysis using the feature of 'fit parameters (and export)' in the software.

Immunocytochemistry- Images captured from the microscope were processed using the FIJI software (ImageJ2 enhanced version, 1.53t). Each image was first converted to a 16-bit grayscale format for uniform analysis. A neuronal cell in the image was traced using the polygonal selection tool to measure whole-cell pixel intensity. Similarly, regions surrounding the neuron were traced to obtain background intensity values. Nuclear intensity was quantified by selecting the nuclear region within each neuron, guided by the corresponding DAPI-stained image for accurate nuclear localization. For each experimental condition, 20-30 neurons (from at least three distinct regions of staining)

were measured, and raw intensity values (in arbitrary units) were automatically exported to an Excel sheet, which was used for further calculations.

Cytoplasmic intensity for each neuron was derived by subtracting nuclear intensity from the whole-cell intensity. Both nuclear and cytoplasmic intensity values were corrected for background intensity by subtracting corresponding background pixel values. Finally, a nucleus-to-cytoplasm intensity ratio was calculated for each neuron. A ratio greater than 1 indicated a stronger nuclear signal. Conversely, a ratio less than 1 indicated active nucleus-to-cytoplasmic shuttling of pFOXO1. For comparative analysis, all intensity ratios from control and treatment groups were plotted in GraphPad Prism, allowing treatment or time duration-based analysis of pFOXO1 translocation activity.

2.13 Statistical analysis

All data are presented as means ± standard errors of the mean (SEMs). Statistical analysis was performed using GraphPad prism software (version 10.2.0). Student's t-test was used for comparison of a single variable between two groups. One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests was applied for comparison of one variable among the control and two or more test conditions. A standard significance threshold of 0.05 was applied to each test. To identify and confirm significant outliers, Grubb's test with a significance threshold of 0.05 was applied, and positive data were excluded from further analyses.

Chapter 3. Results

This PhD project investigated the circadian gating of insulin action in the hypothalamus region of the male mouse brain. To address this research question, experiments were conducted on embryonic- and adult-mouse hypothalamic neuronal cell lines and adult-mouse hypothalamic tissue preparations. The experimental plan involved: 1. Establishing circadian rhythmicity of a clock gene reporter and identifying the time durations that correspond to its maximum (peak) and minimum (trough) expression; 2. Determining the optimum dosage and response time of insulin to investigate its effects on the molecular targets; and 3. Treating the cells/tissues with insulin at the identified peak and trough time points to quantify the phase dependency of insulin action.

3.1 Characterisation of murine hypothalamic neuronal cell lines

To establish a suitable working model for addressing the gating of molecular insulin action, four immortalised neuronal cell lines from the hypothalamic region of embryonic mice, E25, E41, E44 and adult mice, A2/23 (Loganathan & Belsham, 2016; Mayer et al., 2009) were analysed for the expression of insulin receptor (IR) and metabolic neuropeptide transcripts. Of the two insulin receptor isoforms (IR-A and-B), both are distributed in the brain, but at the cellular level, neurons predominantly express IR-A (Moruzzi et al., 2021; Pomytkin et al., 2018). In this investigation, all the cell lines tested expressed transcripts of *IR-A* (normalised to the housekeeping gene *Eef1α*, Figure 9 A). Embryonic cell lines- E25, E41 and E44 showed comparable expression levels while the adult cell line- A2/23 showed significantly higher *IR-A* expression.

The hypothalamus is a brain region involved in regulation of energy homeostasis, housing a heterogeneous population of neurons. Among these neuronal populations, orexigenic neurons, which include those expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY), are key drivers of hunger and food-seeking behaviour. In contrast, anorexigenic neurons, which express pro-opiomelanocortin (POMC) *and* cocaine- and amphetamine-regulated transcript (CART) promote satiety and suppress food intake (Graunke & Argyropoulos, 2003; Valassi et al., 2008). Normalised gene expression analysis showed that *Agrp* levels were significantly higher in the A2/23 cell line compared to E25, E41 and E44. E25 and E41 showed comparable expression while E44 had markedly lower Agrp expression (Figure 9 B). A similar pattern was observed for Npy, with the highest levels in A2/23 and lower expression levels in E44 cells (Figure 9 C).

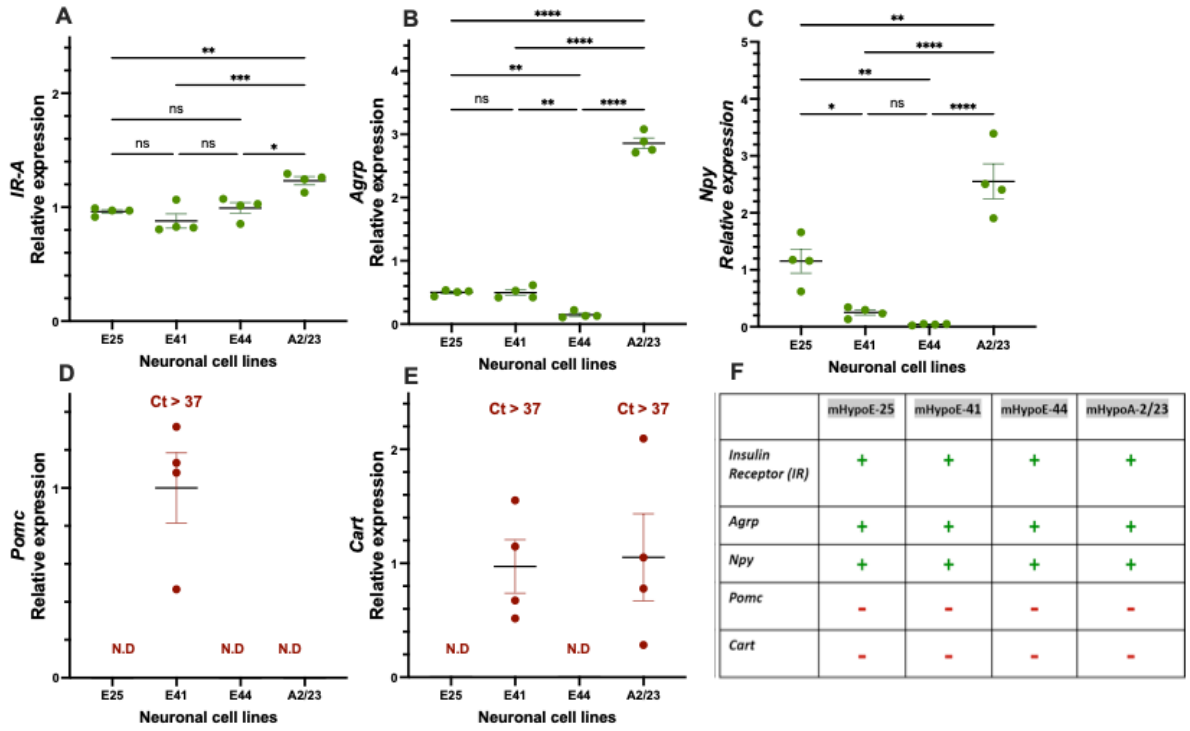


Figure 9. Tested cell lines show characteristics of orexigenic neurons.
Gene expression analysis across four neuronal cell lines from embryonic/adult mice hypothalamic region of (A) IR (B and C) orexigenic neuropeptides- *Npy, Agrp* and (D and E) anorexigenic neuropeptides- *Pomc, Cart*. (F) Summary of metabolic characterisation across cell lines. n = 3-4. One way ANOVA with Tukey's multiple comparison tests, * $p < 0.05$, **** $p < 0.0001$. The blue dotted line represents the Ct value limit of detection. N. D. - not detectable. The data are presented as mean ± SEM. (IR, Insulin receptor; Npy, Neuropeptide Y; Agrp, Agouti-related peptide; Pomc, Pro-opiomelanocortin; Cart, Cocaine- and amphetamine-regulated transcript).

With a limit of detection set at Ct > 37, the Pomc mRNA transcript, detected only in the E41 cell line with a Ct value of 38.86 ± 0.22, was considered as not expressed (Figure 9 D). Similarly, the Cart transcript, identified in the E41 (38.22 ± 0.21) and A2/23 (37.72 ± 0.23) cell lines, was also deemed not expressed (Figure 9 E). Thus, Pomc and Cart transcripts were not reliably detected in any of the cell lines. The conclusive results of neuropeptide expression profiles across four tested cell lines are summarized in the Figure 9 F.

Taken together, all working cell models expressed transcripts for insulin receptor and orexigenic neuropeptides and, thus, they were characterized as resembling orexigenic neurons, making them suitable models for studying the metabolic action of insulin in the hypothalamus.

3.2 Characterisation of circadian parameters of Bmal1-luc expression in murine hypothalamic neuronal cell lines

Most neuronal clonal cell lines express components of the circadian machinery. However, not all non-SCN neuronal cell models have been studied to exhibit rhythmic behaviour (Dalvi et al., 2011b; Mayer et al., 2009). To monitor circadian oscillations in real-time, the cell lines used in this study were formerly modified to stably express a bioluminescence

reporter for circadian rhythms, i.e. they were expressing luciferase under control of the human *Bmal1* promoter (Bmal1-luc) (Tsang et al., 2020; Brown et al., 2005). After synchronization with 100 nM dexamethasone for 2 hours, cells were monitored for Bmal1-luc activity for 5 days. Raw bioluminescence files were further processed in Lumicycle Actimetrics software for downstream analysis.

Of the four cell lines, E44 and A2/23 showed periodic oscillations with 2-3 cycles applicable for quantitative analysis (Figure 10 A). They also displayed a physiologically relevant period length of 22.03 ± 0.19 h and 23.83 ± 1.10 h, respectively. E25 had a period length of 27.60 ± 3.8 h, while E41 displayed a period of 52.00 ± 1.4 h, both of which were considered as being outside the circadian range (Figure 10 B). Amplitude reflects the strength or robustness of the rhythm (Bano-Otalora et al., 2021). The cell lines E44 and A2/23 showed significantly higher amplitude compared to the other two, E41 and E25 (Figure 10 C). To assess the stability of detected rhythms, the 50-% damping rates of the peak expression magnitudes of damped sine wave regressions were calculated (Srimani et al., 2022). Among the tested cell lines, E44 had the slowest damping rate of 2.43 ± 0.8 days, whereas E25 dampened fastest with a damping rate of around one day (Figure 10 D).

To confirm the rhythmic strength, R^2 fitting of the damped sine wave regressions was used to quantify how well the data fit a robust oscillatory pattern. E44 showed the highest R^2 value (0.61), followed by A2/23 (0.37), indicating reasonably stable rhythmicity. E25, however with a comparable R^2 value of 0.25, displayed weaker amplitude and unstable rhythms, while E41 had the lowest R^2 (0.08), classifying it as arrhythmic.

Taken together, E44 emerged as the most robust circadian model, followed by A2/23. E25 was excluded due to its variable period length, modest amplitude, and rapid damping. E41, which showed non-circadian behaviour, was also excluded. Thus, E44 and A2/23 were chosen as cell models to study the circadian gating of insulin action.

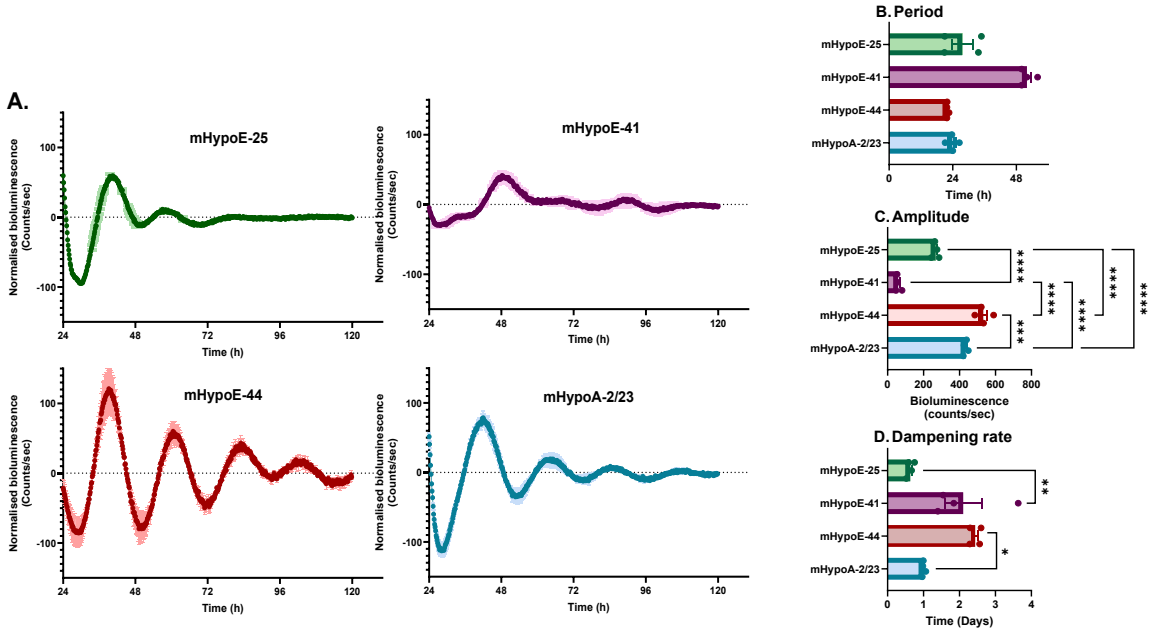


Figure 10. Out of the four tested cell lines, mHypoE44 and A2/23 displayed robust circadian properties.
(A) Normalised bioluminescence recordings of the tested neuronal cell lines, mHypoE25, E41, E44 and A2/23 with Bmal1-luc circadian reporter, for 5 days after synchronisation with dexamethasone for 2 h. The dotted line represents the running-average of the bioluminescence counts. (B) Avg. period length (C) Avg. amplitude and (D) Avg. damping rate over 5 days for each cell line. n = 4 samples per cell line. The data are presented as mean ± SEM values. *p < 0.05,** p < 0.01, *** p < 0.001, **** p < 0.0001.

3.3 Insulin shows no significant effects on gene expression levels of metabolic neuropeptides in E44 and A2/23 cells

Insulin exerts satiety effects on the hypothalamus and suppresses food intake (Porte & Woods, 1981). It has a direct action on orexigenic neurons where it down-regulates *Agrp* and *Npy* expression (Mayer & Belsham, 2009). Since the hypothalamus contains a heterogeneous population of cell types, the use of clonal cell models facilitates the observation of the direct effects of insulin on individual neuronal cells. To determine the optimal insulin concentration for experimental treatments, a DRC was performed using pharmacological doses of insulin (2, 10, 50, and 100 nM) on the characterized orexigenic cell lines, E44 and A2/23. The cells were incubated with insulin for 1 h and processed for gene expression analysis (for details, refer to Section 2.4.1)

Gene expression levels of Npy in insulin treated cells (E44 and A2/23) were comparable to those in untreated control preparations and also among all the doses tested (Figure 11 A and B). A similar absence of effects was observed for Agrp expression (Figure 11 C and D). Of note, a higher variance was detected in Npy expression among the biological replicates.

Immortalised cell lines are a simulation of an *in vivo* system that can lack complexities of cellular networks (Mayer et al., 2009). Thus, caution is considered when extrapolating the kinetics of physiological insulin action to cell models. To investigate insulin's response time in an *in-vitro* system, the two selected cell lines (E44 and A2/23) were treated with 100 nM insulin for a period of 24 hours. The cells were harvested for a response-time curve (RTC) after 30 min and 1, 2, 4, 6, 12, and 24 h. Due to a high variance in *Npy* gene expression in the DRC, this experiment only investigated the expression levels of *Agrp*. The RTC revealed no significant effect of insulin on *Agrp* expression at any of the tested time points, neither in the E44 nor A2/23 cell line. It cannot be excluded that the kinetics of insulin action at the molecular level are more rapid than previously reported in the literature, as cell models allow for a simulated environment facilitating direct insulin action at a cellular level. In the adult cell line (A2/23), effects of insulin were therefore further investigated at 5 and 10 min of incubation. However, no significant difference was observed between untreated and insulin-treated groups (Figure 11 F). Gene expression data for the untreated control group were passed through rhythmicity analysis using the CircWave V1.4 computational tool. With a borderline *p*-value of 0.076, the *Agrp* gene expression pattern was not validated to be rhythmic in this experimental cell model.

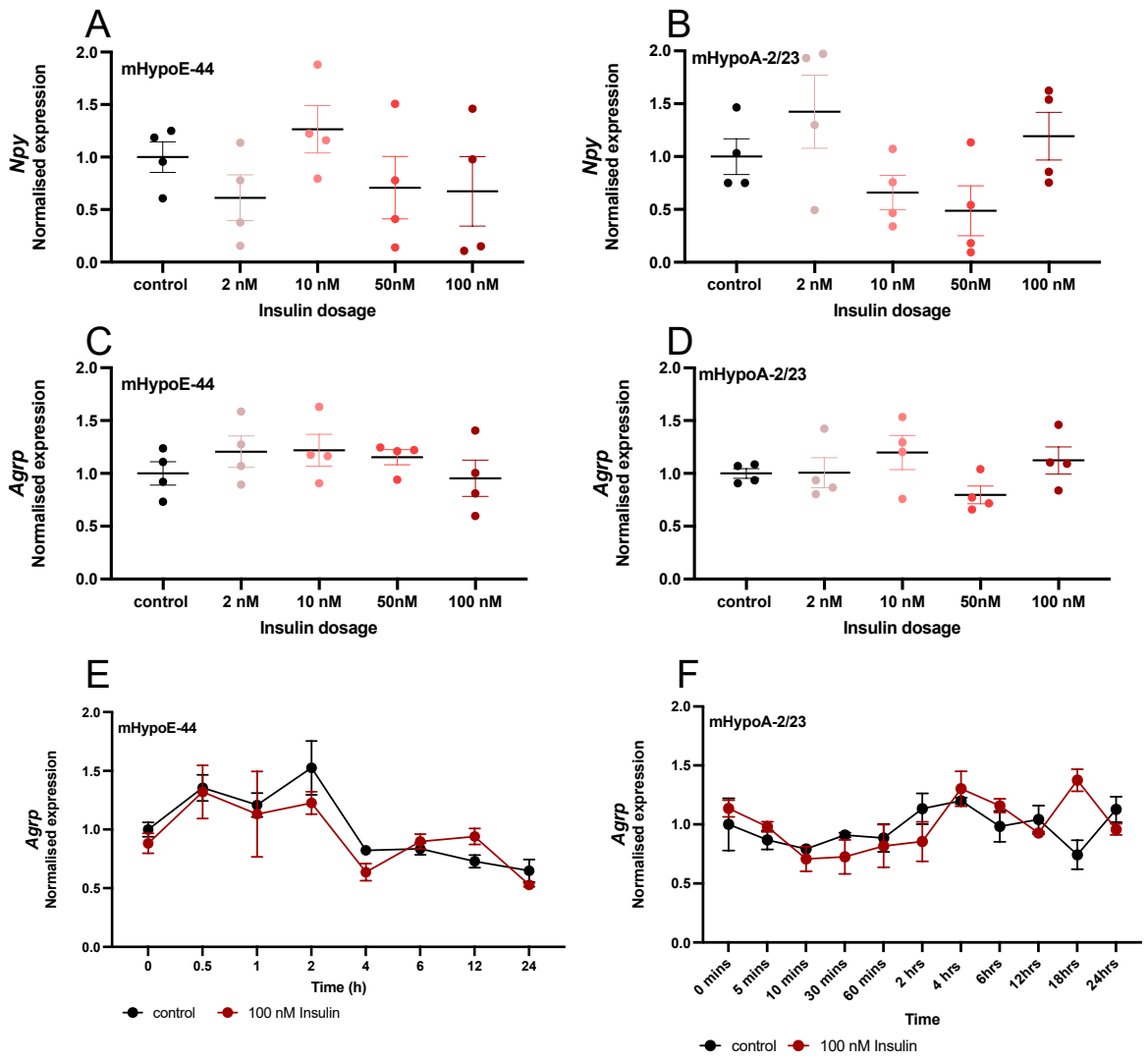


Figure 11. No effects are observed on the gene expression levels of orexigenic neuropeptides in the two selected cell lines.
(A-D) Dose response analysis of insulin on the gene expression levels of *Npy* and *Agrp* in E44 and A2/23 neurons. Data are normalised to the control condition of the individual subset. (E and F) Response-time analysis of insulin action on the Agrp gene expression level in the two tested cell lines. Data are normalised to the 0 h of control condition. n = 4 per condition. The data are presented as mean ± SEM values. (Npy, Neuropeptide Y; Agrp, Agouti-related peptide).

3.4 Insulin-mediated pFOXO1 nuclear exclusion was not observed in insulin-treated A2/23 neurons

Since the effects of insulin on gene expression levels were not successfully observed, we aimed to identify protein-level targets in the central insulin signalling pathway. Downstream of the PI3K-AKT phosphorylation cascade, FOXO1, a key regulatory transcription factor, undergoes nucleocytoplasmic shuttling upon phosphorylation by activated AKT (Fukuda et al., 2008). The nuclear exclusion property of phosphorylated FOXO1 was exploited to investigate the action of insulin. A2/23 cells were treated with varying insulin doses (DRC- 20, 50 and 100 nM) and harvested at different treatment times (RTC- 5, 15, 30 and 60 mins). Subsequently, immunofluorescence staining was performed using a pFOXO1 antibody. Fluorescence intensity was quantified in both the

nucleus and cytoplasm of individual neurons. The shuttling activity of pFOXO1 was confirmed by calculating the ratio of nuclear to cytoplasmic intensity (Fukuda et al., 2008).

Contrary to the anticipated outcome, pFOXO1 translocation into the cytoplasm was not reflected in insulin-treated cells. Comparable ratios between the insulin-treated and untreated groups confirmed that insulin-induced nuclear exclusion of pFOXO1 was not observed in the A2/23 cell model. This observation was consistent for both the DRC (Figure 12 A) and the RTC (Figure 12 B) experiments.

In addition to insulin, other proteins including epidermal growth factor (EGF) and nerve growth factor have been reported to phosphorylate FOXO1 via AKT-dependent signalling (Jackson et al., 2000; Morris et al., 2005; Wen et al., 2011). To confirm the functionality of the experimental technique, a test experiment was performed by treating A2/23 cells with 100 ng/mL EGF for 15 mins, followed by immunofluorescence using the same pFOXO1 antibody. Previous studies have shown that EGF treatment induces AKT-dependent FOXO1 phosphorylation in different cell types. In human breast cancer cells, an EGF treatment of 50 ng/mL yields a significantly higher phosphorylation of FOXO1 at 10 mins (Jackson et al., 2000) while, in neonatal rat cardiomyocytes, around 60 ng/mL EGF induces maximal FOXO1 phosphorylation at 30 mins (Morris et al., 2005). Lacking information in neuronal models and based on the above findings, a concentration of 100 ng/mL EGF for a treatment duration of 15 mins was selected in this study, as these parameters fall within the range of previously reported effective conditions.

In the performed experiment, the ratio of nucleus:cytoplasmic fluorescence intensity was comparable between the EGF-treated cells and the untreated control cells (Figure 12 C). Since EGF-induced nucleocytoplasmic shuttling via AKT phosphorylation was not observed, the functionality of the experimental technique was not positively validated.

Its proximity to the median eminence, which contains fenestrated capillaries and lacks a complete blood–brain barrier, allows Arc neurons to sense circulating hormones, nutrients, and metabolic molecules.

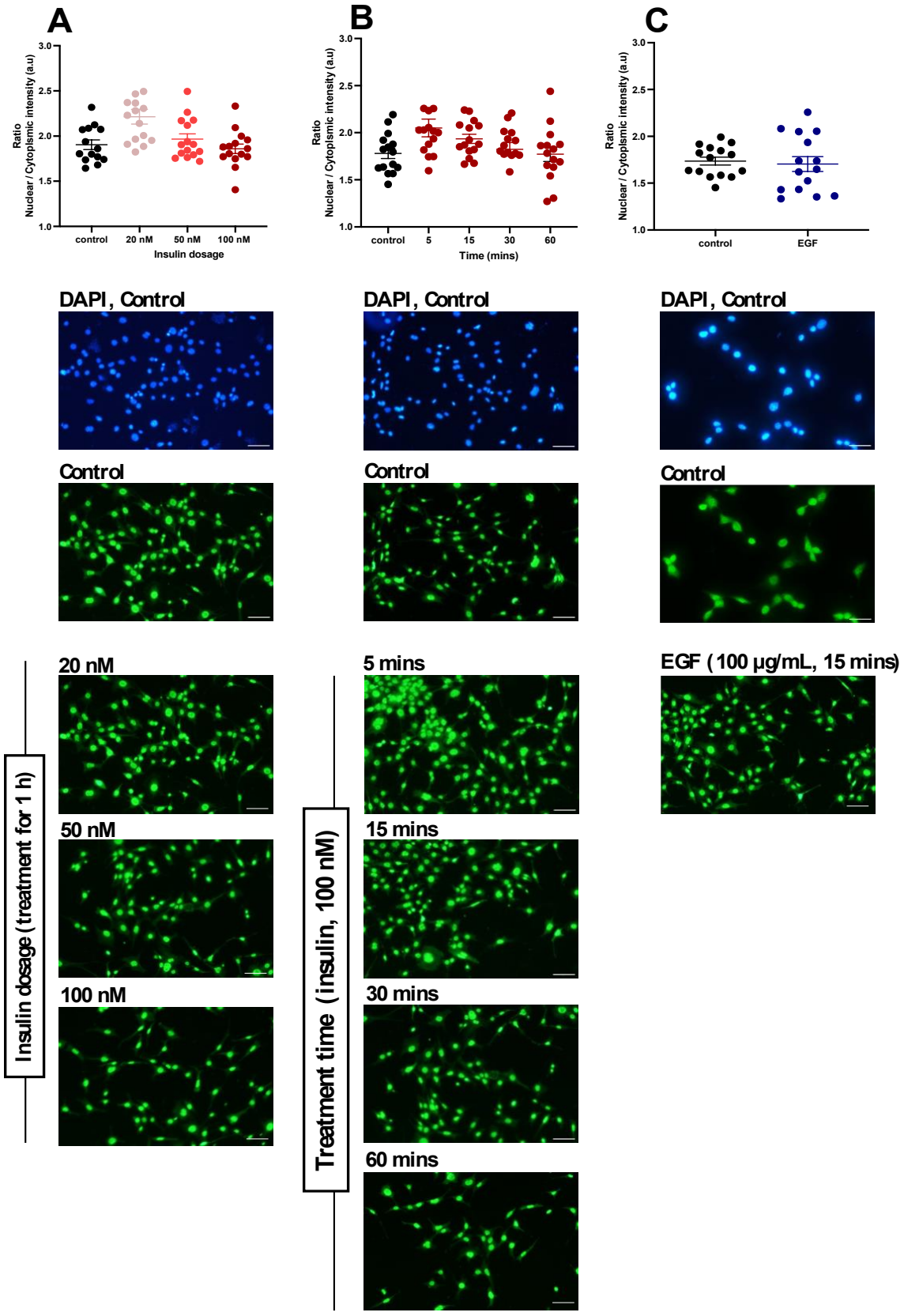


Figure 12. Insulin-induced nuclear shuttling of pFoxo1 in A2/23 cells.
(A) Dose-response analysis: cells treated with 20, 50, or 100 nM insulin for 1 h. (B) response-time analysis: cells treated with 100 nM insulin for 5–60 min. (C) Positive control: 100 µg/mL EGF for 15 min. Cells were stained with DAPI (nuclei, blue) and pFOXO1 antibody (green). Quantified nuclear-to-cytoplasmic intensity ratios are shown in left panel. Imaging: 20×; scale bars: 10 µm. Data are presented as mean ± SEM.

3.5 Robust circadian parameters are established in the Arc-ME region of the mouse brain

Insulin action was not detected in the immortalized hypothalamic cell models. Thus, further experimental setups were focused on establishing an *ex vivo* system of hypothalamic tissue to study the predominant action of insulin in feeding control. The Arc of the hypothalamus houses receptors for various endocrine hormones, including insulin, leptin, and ghrelin (Yi et al., 2006b). Its proximity to the ME, which contains fenestrated capillaries and lacks a complete BBB, allows Arc neurons to sense circulating hormones, nutrients, and metabolic molecules (Sainsbury et al., 2002). In addition, there lies a reciprocal connection between the SCN and Arc, a critical framework for integrating metabolic information into the circadian system, thereby allowing the adaption of circadian rhythms in hypothalamic functions of energy homeostasis (Méndez-Hernández et al., 2020; Yi et al., 2006b).

Dissected Arc-ME slices were isolated from the hypothalamus of PER2::LUC mice. They were cultured in a bioluminescence-optimized growth medium and monitored for PER2 expression patterns over five days. The observed activity patterns were compared to the circadian parameters obtained from SCN tissue slices. Arc-ME (Bregma, -1.80 mm to -2.10 mm) and SCN (Bregma, -0.22 to -0.82 mm) slices were dissected from the mice of the same experimental batch to avoid batch effects in the comparisons. However, due to functional loss or a decline in tissue viability in two SCN samples during the preparation or culturing process, the final sample size for Arc-ME slices was four, while the SCN preparations were limited to two.

Arc-ME tissue preparations exhibited rhythmic PER2::LUC expression with notable similarity to SCN oscillations in certain circadian parameters. The period of oscillation in Arc-ME slices was closely matching the SCN showing no statistically significant difference (Figure 13 B). The amplitude of PER2::LUC oscillations in Arc-ME was slightly lower than the SCN amplitude; however, this difference was not substantial, indicating that both regions maintain comparable oscillatory robustness (Figure 13 C).

A key difference was observed in the damping rates of PER2::LUC oscillations between the two regions. SCN slices displayed robust and sustained rhythmicity, with an average damping rate of 4.40 ± 0.15 days, in contrast to Arc-ME slices that exhibited a significantly faster damping rate of 3.19 ± 0.19 days ($p = 0.015$) (Figure 13 D). This suggests that, under the applied experimental conditions, the Arc-ME tissues lose rhythmicity more quickly than the SCN slices.

Despite the faster damping rate, the Arc-ME slices were retained as a viable model for subsequent studies due to their comparable period length and sufficient amplitude during the initial cycles of rhythmicity. Importantly, the research focus of this thesis, lies within the early stages of circadian oscillations (within the first 48 h post-dissection), during which Arc-ME slices display stable and physiologically relevant rhythmic parameters. This makes them suitable for investigating temporal gating of molecular insulin action in future experiments. The rapid damping in Arc-ME slices, while a

limitation for long-term studies, does not impede the objectives of the current experimental framework.

Of note, although a t-test was used to compare circadian parameters between the Arc-ME and SCN slices, it is important to acknowledge that the SCN sample size (n = 2) limits the robustness of the statistical conclusions. While this approach provides a preliminary comparison, caution is taken when interpreting the results.

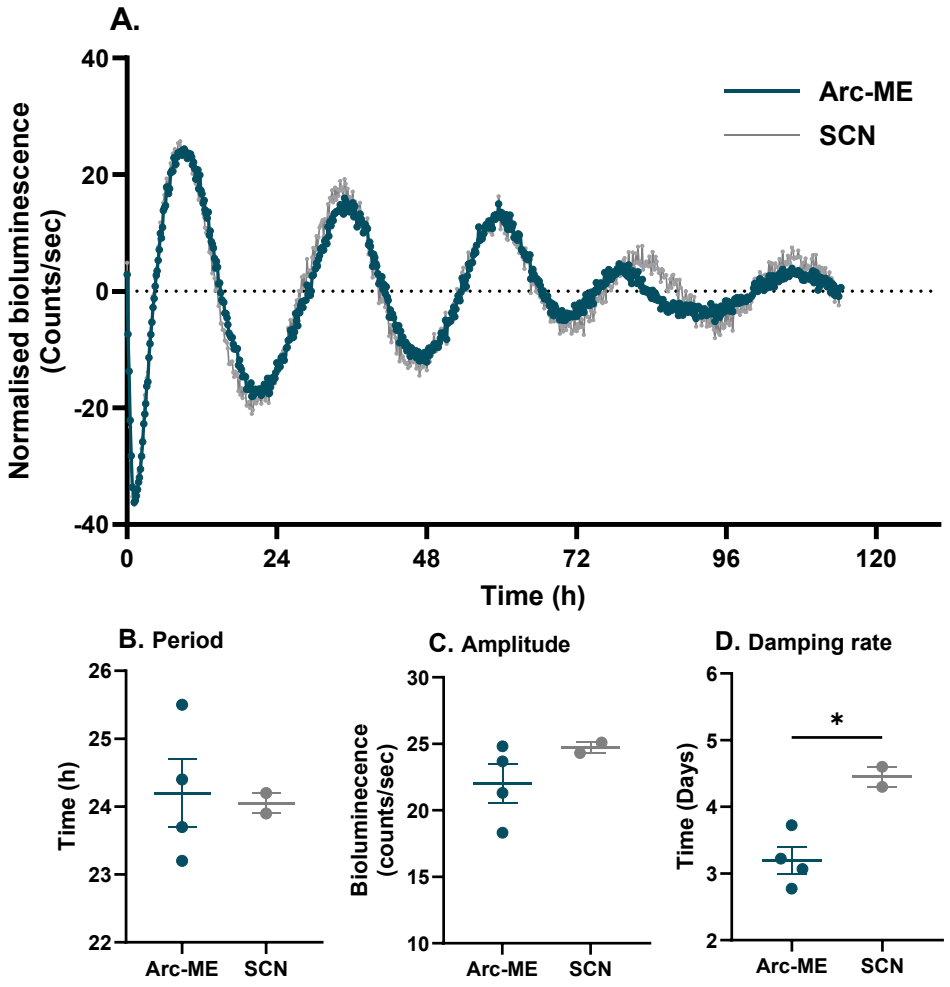


Figure 13. Adult mice Arc-ME tissue slices show comparable circadian properties to the SCN slices.
(A) Normalised bioluminescence recordings of the Arc-ME region of adult mouse harbouring a PER2::LUC circadian reporter. SCN, the master clock slices are used as a comparative circadian reference. The dotted line represents running average of the bioluminescence counts. (B) The avg. period length (C) Amplitude and (D) Damping rate of Arc-ME and SCN slices monitored for 5 days. Students t-test, *p < 0.05. n =4 slices of Arc-ME region and 2 for SCN region. The data are presented as mean ± SEM values. (Arc-ME, Arcuate nucleus-Median eminence; SCN, Suprachiasmatic nucleus).

3.6 Insulin shows no significant effects on gene expression levels of metabolic neuropeptides in cultured Arc-ME slices

3.6.1 Dose response analysis

The effects of insulin were explored on the gene expression level of metabolic neuropeptides in cultured Arc-ME slices. The tested metabolic neuropeptides included

orexigenic (*Npy, Agrp* and *Gpr17)* and anorexigenic (*Pomc, Cart* and *Cpe)* genes. A DRC was determined using increasing concentrations of insulin (1, 10, 100 and 500 nM) for 0.5 h. A RTC analysis was performed to access the temporal dynamics of insulin responses over 1, 3, and 6 h of insulin (500 nM) treatment. Additionally, *cFos* expression was monitored for both analyses as positive control.

The DRC analysis did not reveal any significant effects of insulin treatment on the expression levels of neither of the six genes investigated (Figure 14 A-F).

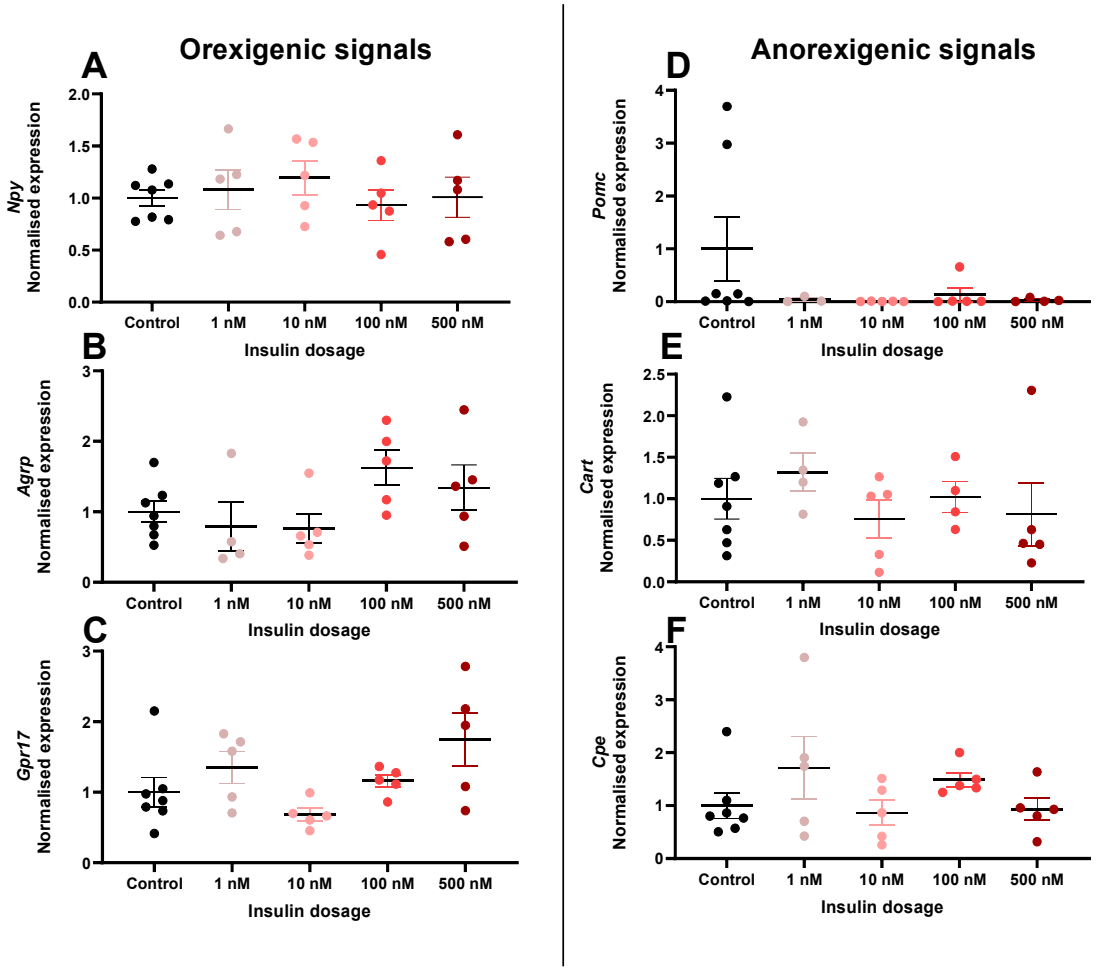


Figure 14. No significant effects on gene expression levels of metabolic neuropeptides were observed in the Arc-ME tissue preparations at any of the insulin doses tested.
Tissue preparations were treated with varying concentrations of insulin (1, 10, 100 and 500 nM) for 0.5 h. Effects were investigated on the mRNA levels of (A-C) orexigenic (*Npy, Agrp* and *Gpr17)* and (D-F) anorexigenic (*Pomc, Cart* and *Cpe)* neuropeptides. Values for insulin dosages were normalised to the PBS-control condition. n = 5. The data are presented as mean ± SEM values. (Arc-ME, Arcuate nucleus-Median eminence; Agrp, Agouti- related protein; Gpr17, G-protein coupled receptor 17; Npy, Neuropeptide Y; Pomc, Proopiomelanocortin; Cpe, Carboxypeptidase E; Cart, cocaine-and-amphetamine-regulated transcript).

3.6.2. Response-time analysis

In contrast to the DRC results, the RTC analysis did yield a single instance where insulin treatment elicited a significant response. One of the hunger-promoting genes (Gpr17) (Figure 15 C) exhibited a decrease in the expression following 1 h, 3 h and 6 h of insulin

treatment as compared to the 0 h control. However, this observation for Gpr 17 gene was limited to a single trial (Trial 1) (Figure 15 C) and could not be replicated in the following trial (Trial 2) (Figure 16 C) of the same experiment set up. Different from Gpr17, other genes displayed comparable and indifferent expression levels among the tested treatment times in the RTC analysis of insulin treatment.

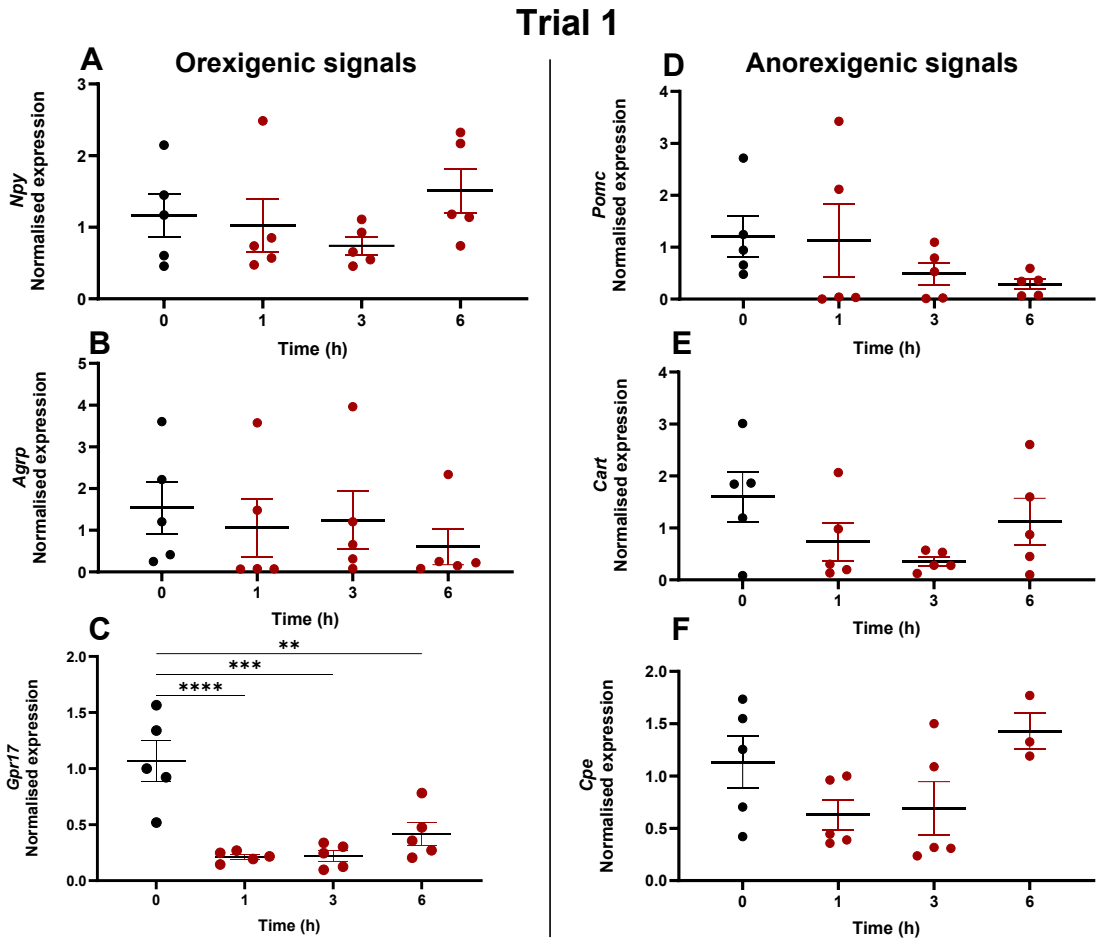


Figure 15. Insulin elicits a response in Gpr17 gene expression level at 1, 3 and 6 h after treatment.
Arc-ME tissue slices were treated with 500nM of insulin for varying time durations (1, 3 and 6 h). Effects of insulin on its molecular targets was not observed in mRNA levels of any of the neuropeptides, *Agrp, Npy, Pomc, Cart* and *Cpe* (A, B, D, E and F) except for *Gpr17* (C). The down regulatory effect on the orexigenic *Gpr17* was consistent at all the time durations tested. One-way ANOVA with Dunnett's multiple comparison tests, ** p =0.001, *** p = 0.0001, **** p < 0.0001. n = 5. The data are presented as mean ± SEM values. (Arc-ME, Arcuate nucleus-Median eminence; Agrp, Agouti-related protein; Gpr17, G-protein coupled receptor 17; Npy, Neuropeptide Y; Pomc, Proopiomelanocortin; Cpe, Carboxypeptidase E; Cart, cocaine-and-amphetamine-regulated transcript).

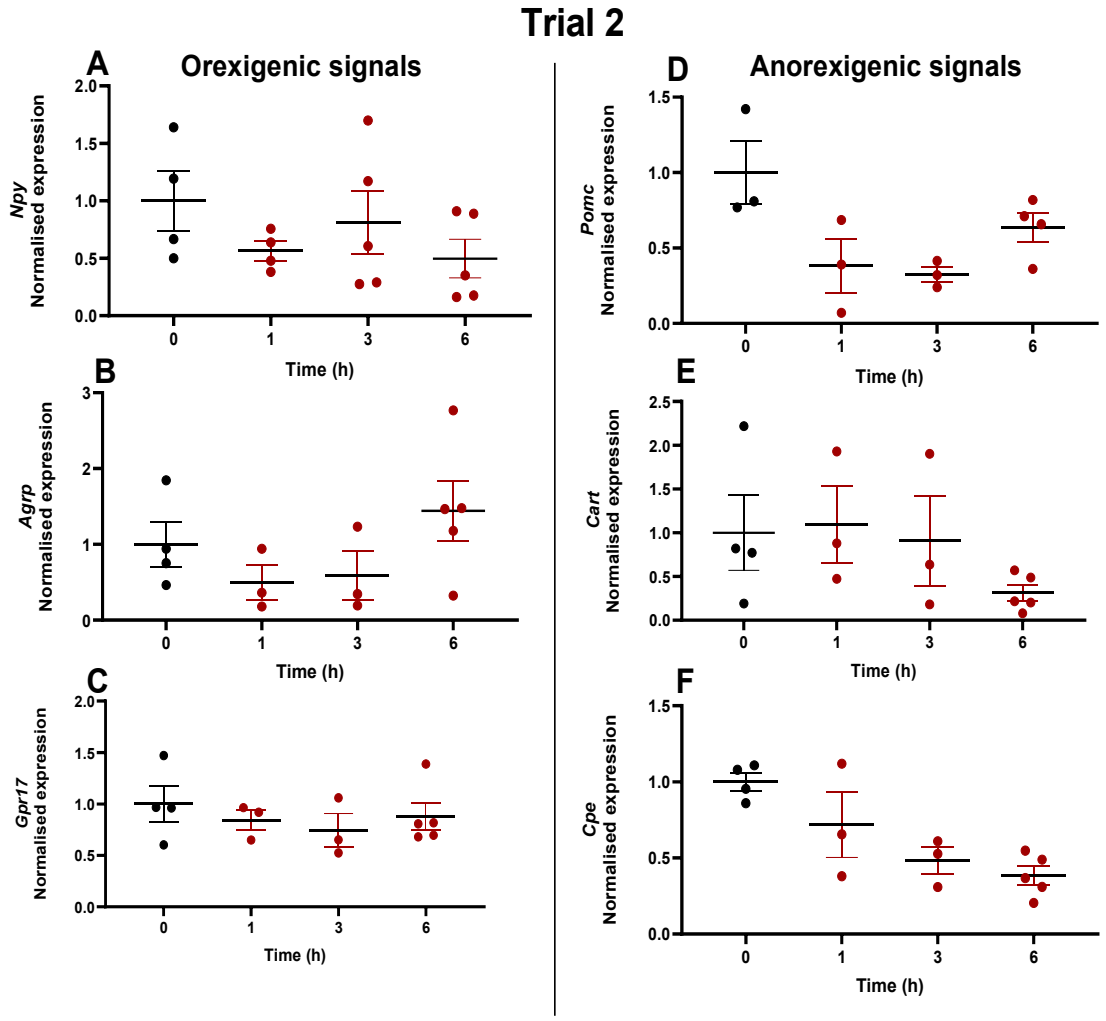


Figure 16. The down regulatory effect of insulin on the expression level of *Gpr17* gene was not reproduced in the trial 2 of the same experimental set up.
Arc-ME preparations were treated with insulin as in (Fig. 7). Expected effect of insulin was not observed on tested metabolic neuropeptides, *Agrp, Npy, Pomc, Cart* and *Cpe* (A, B, D, E, F). Also, the insulin-influenced down regulation of *Gpr17* gene expression as observed in trial 1, was not reproduced in this trial (C). n = 5. The data are presented as mean ± SEM values. (Arc-ME, Arcuate nucleus-Median eminence; Agrp, Agouti- related protein; Gpr17, G-protein coupled receptor 17; Npy, Neuropeptide Y; Pomc, Proopiomelanocortin; Cpe, Carboxypeptidase E; Cart, cocaine-and-amphetamine-regulated transcript).

3.6.3. *cFos* expression as a positive control to assess the functionality of the experimental set up

cFos was investigated as a standard positive control for the neuronal activity between the untreated control and the treated groups. Since hunger induces *cFos* expression, insulin being the satiety hormone, we expected it to cause a down-regulation of *cFos* gene. In the DRC analysis for a time duration of 0.5 h, no effect of insulin treatment was observed on the cFos expression. (Figure 17 A). The gene expression levels did not vary among the different doses of insulin with respect to the normalised untreated control samples. This observation concluded that insulin treatment of varying concentrations over a period of 0.5 h was not enough to elicit a response on the gene expression levels and thus prompted to employ a RTC analysis of insulin treatment for increasing treatment durations. In

contrast to the DRC, *cFos* expression in the RTC analysis (Figure 17 B and C) with 500 nM of insulin treated for 1, 3 and 6 h- Trial 1, displayed a downregulation in response to insulin-treated for 1 h, 3 h and 6 h as normalised to the untreated control group (Figure 17 B). The downregulatory effect of *cFos* after insulin treatment remained consistent in Trial 2 (Figure 17 C), despite of the fact that the expression differences in the orexigenic and anorexigenic signals were lost in the same set up. This observation indeed confirmed the functionality of the experimental setup; however, it also raised potential shortcomings of detecting insulin-mediated changes in gene expression levels of metabolic neuropeptides using this approach.

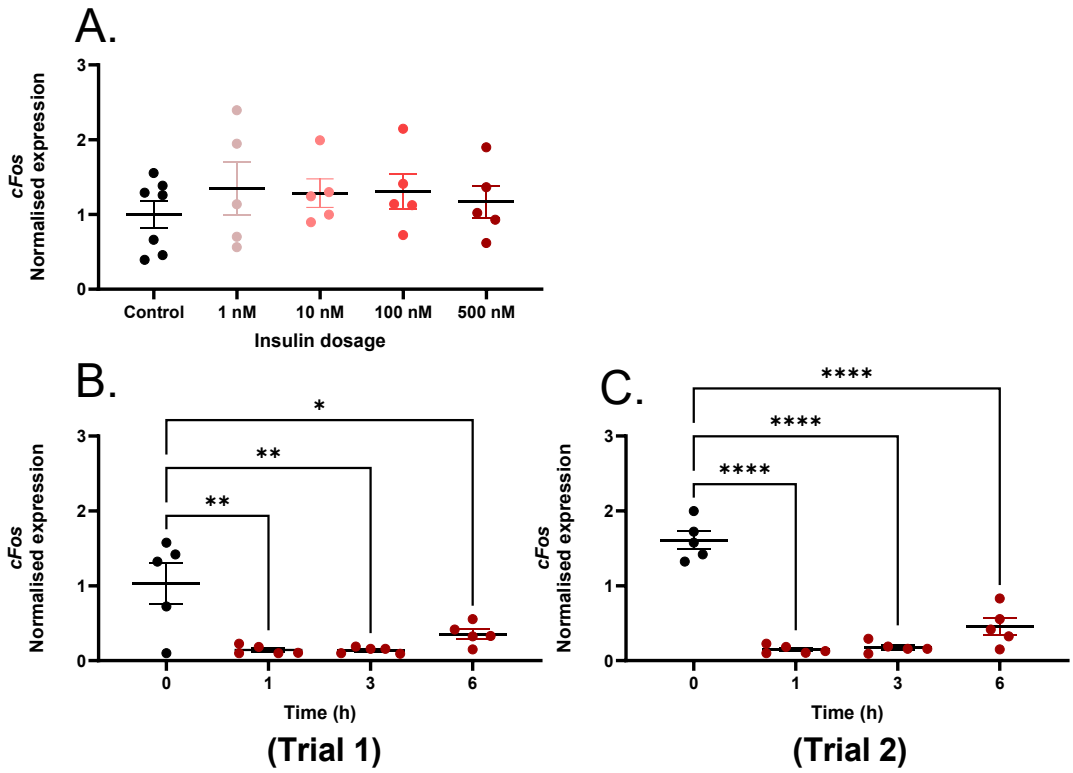


Figure 17. *cFos* expression levels were investigated as a positive control to validate effects of insulin on its molecular targets.
(A) Insulin dose response analysis for *cFos* expression levels showed no observable alterations at none of the insulin concentration (1, 10, 100, 500 nM) tested. (B and C) Response-time analysis of insulin action tested with 500 nM for 1, 3 and 6 h showed significant down regulation of *cFos* expression at all the tested time durations. n = 5. One-way ANOVA with Dunnett's multiple comparison tests, * p< 0.05, ** p < 0.01, **** p < 0.0001. The data are presented as mean ± SEM values.

3.7 Insulin resetting of PER2::LUC rhythms in hypothalamic tissue preparations and validating insulin-stripped culture conditions.

A targeted approach of testing insulin effects on specific metabolic gene expression levels encountered few limitations. This raised an initial concern regarding the functional integrity of the exogenous insulin compound used for substance treatment. To address this, the biological activity of the insulin compound was verified by evaluating its ability to induce phase-shifts in the PER2::LUC rhythm of hypothalamic tissue preparations, a

response well characterised in the literature (Crosby et al., 2019). This validation step was paired with assessing the suitability of insulin-stripped B27 supplement, which is used as a serum-alternative to supply essential nutrients and vitamins in the culture medium. Importantly, this supplement was integral to this experimental design as it minimizes the influence of endogenous insulin in the medium and prevents a potential ceiling effect when exogenous insulin is applied as a substance treatment. Thus, the overall aim of this experiment was twofold: first, to confirm the efficacy of the insulin compound used in this study. This thereby, rules out the possibility that a lack of effect on the gene expression could be the reason of non-functional insulin treatment. And second, to verify that the use of insulin-stripped B27 supplement as an alternative for serum in the culture medium, is appropriate to cultivate tissue preparations. The outcome of this experiment was valuable to validate the technical set up for subsequent transcriptomic-based investigations used to systematically explore insulin's molecular effects.

Arc-ME tissue slices from PER2::LUC mouse model were cultured in standard DMEM medium for 24 hours, followed by one of three treatments: (1) transfer to insulin-free medium- to ensure that the medium condition is suitable for cultivation and maintenance of tissue preparations. Because, in addition to the essential growth factors in the culture medium, insulin of adequate concentrations is crucial for cell survival and cell growth (Rhee et al., 2013) (2) treatment with external insulin- to validate the functionality of the insulin compound, and (3) a control group, maintained in the standard DMEM medium. After treatment, bioluminescence recordings of PER2 expression in the Arc-ME were obtained using a Lumicycle system.

The bioluminescence recordings revealed that all three groups exhibited rhythmic expression of PER2 over 96 h in culture (Figure 18 A). They displayed comparable circadian periods of approximately 24 hours (Figure 18 B). Tissue slices maintained in standard regular medium and those treated with external insulin showed robust amplitudes, indicating optimal tissue health and rhythmic stability. In contrast, slices cultured in insulin-free B27 medium displayed significantly reduced amplitudes, suggesting a potential reduction in circadian rhythmic strength or viability (Figure 18 C).

To assess phase modulation, phase values for each test group were calculated relative to the control group, which was used as the baseline reference (normalized to 0 h). Accordingly, tissue slices in insulin-free medium showed no phase modulation as compared to the control group. Importantly, insulin-treated tissue slices exhibited a phase-delay of 6.7 ± 0.29 h, an observation which is consistent with the previous findings demonstrating insulin's ability to modulate and delay circadian phase (Crosby et al., 2019) (Figure 18 D).

These observations confirm that the external insulin compound is functional and effective for experimental treatments. While the insulin-free B27 medium led to a reduction in amplitude and potentially increased the damping rate, it was selected for subsequent experiments to prevent potential ceiling effects from endogenous insulin

present in the culture medium. The reduced amplitude in the absence of insulin might also facilitate the detection of differential gene expression responses to exogenous insulin treatment by minimizing baseline insulin signalling, thereby enhancing the sensitivity of the experimental model. Using the insulin-free B27 medium creates a baseline condition where insulin's effects can be more precisely measured and provides a clearer understanding of insulin-mediated changes in circadian regulation within the Arc-ME preparations.

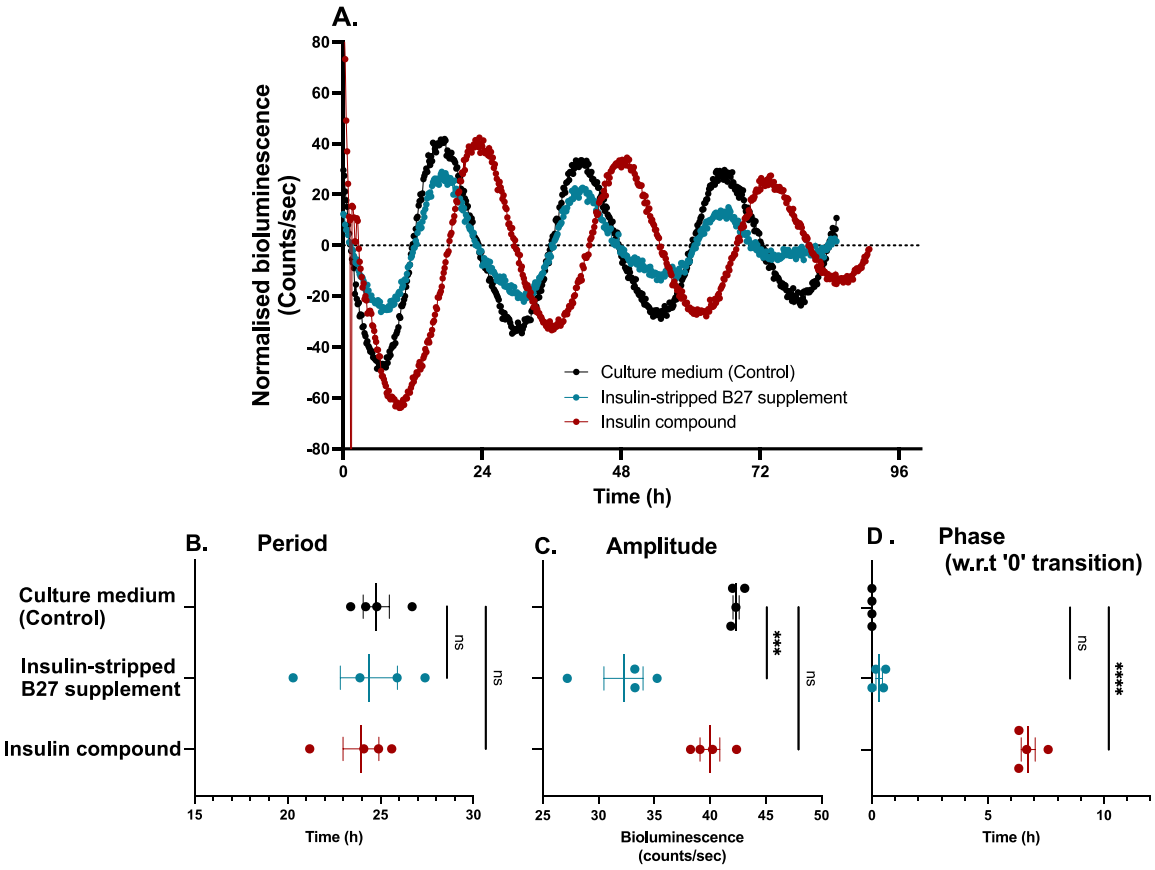
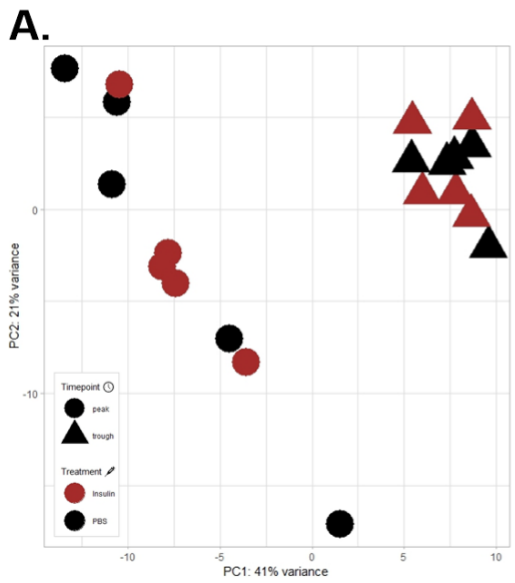


Figure 18. Functional validation of insulin treatment and insulin-stripped medium conditions in Arc-ME tissue preparations.
(A) Normalised bioluminescence recordings of Arc-ME tissue slices (harbouring PER2::LUC reporter) recorded for a period of 4 days in three conditions- insulin-treated medium , insulin-stripped b27 supplemented medium and standard culture medium (control). The dotted line represents the running average of the bioluminescence counts. (B) the avg. period length- (C) Amplitude- and (D) Phase shifts- derived from the same bioluminescence recordings. Phase values were calculated relative to the control group (set as the 0 h baseline). One way ANOVA with Dunnett's multiple comparison tests, *** p< 0.001, ****p < 0.0001, ns= non-significant. n= 4 slices per condition. The data are presented as mean ± SEM values. (Arc-ME, Arcuate nucleus-Median eminence).

3.8 Exploratory transcriptomic analysis reveals a higher time than insulin effect on gene expression in Arc-ME slices.

To investigate the time-dependent effects of insulin in the hypothalamus, we performed experiments on the Arc-ME tissue slices treated with insulin at peak or trough timepoints of PER2 expression. Samples were collected one hour post-treatment, and the extracted RNA was subjected to RNA sequencing.

Initial exploratory principal component analysis (PCA) of the RNAseq data revealed distinct clustering of samples. The first two principal components (PC1 and PC2) explained 41% and 21% of the total variance, respectively. PC1 primarily separated samples based on sampling time, while no clear separation by insulin treatment was observed along PC2. One outlier in the analysis was visually identified in the 'peak' sample group and was excluded to ensure robust downstream analyses (Figure 19 A). These observations suggest that time had a much stronger influence on gene expression patterns than insulin treatment in this dataset.



B.

	Groups (DESeq2 designs)	UP regulated genes	DOWN regulated genes	Total
Treatment effect	1. Peak	4	9	13
	2. Trough	1	0	1
Temporal effect	3. PBS	173	325	498
	4. Insulin	120	311	431

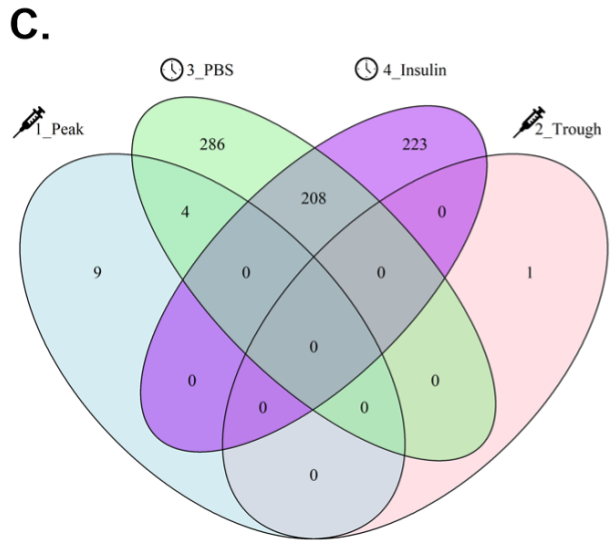


Figure 19. Exploratory transcriptomic analysis reveals stronger temporal effect and subtle treatment effect in the experimental groups.
(A) Principal component analysis (PCA) plot depicts variance in gene expression patterns across different experimental conditions. The samples are color-coded by treatment and shaped according to treatment timepoints. PCA reveals a clear clustering of samples based on temporal effect and a subtle effect of treatment. (B) Differential gene expression analysis conducted by DESeq2 statistical model in RStudio summarises differentially expressed genes (DEGs) w.r.t. treatment and temporal design strategies. (C) A Venn diagram illustrating number of DEGs identified separately in each design group as well as the overlap of DEGs between them.

Differential gene expression analysis was conducted using the DESeq2 package in R-studio, leveraging the Wald test for statistical evaluation. DEGs were selected based on an adjusted p-value of 0.05 and a null log fold change (Lfc) threshold. Since the samples were grouped by the experimental conditions, *designs* of DESeq2 were tailored to allow subsequent cross-comparisons. Consistent with PCA findings, temporal comparisons (peak vs. trough) yielded the largest sets of DEGs, identifying 498 and 431 genes in the PBS- and insulin-treated samples, respectively. Conversely, treatment effects (PBS vs. insulin) resulted in a limited number of 14 DEGs, with 13 identified in the peak group and only 1 DEG identified in the trough group (Figure 19 B).

Finally, a Venn diagram visualizing the overlap and uniqueness of gene sets between the groups revealed that no genes were shared across all four conditions globally, meaning no single gene exhibited both temporal and treatment effects simultaneously across the experimental groups. However, 208 DEGs were common to the temporal comparisons (peak vs. trough) in both PBS and insulin-treated samples, while 286 and 223 DEGs were unique to the temporal comparisons in PBS and insulin groups, respectively. Regarding the treatment effect, 9 DEGs were specific to the peak group, 1 DEG was unique to the trough group, and 4 DEGs exhibited an effect only within the PBS condition. These results highlight the distinct gene expression patterns associated with temporal and treatment factors, with no consistent overlap across all experimental groups (Figure 19 C).

In summary, analysis of this dataset identified DEGs based on two key experimental variables- insulin treatment and temporal effects. With no gene identified to show concurrent effects of both factors, gene expression patterns were predominantly driven by intrinsic temporal variations, with minimal influence from external insulin treatment.

3.9 Insulin influenced DEGs are involved in peripheral and central functions

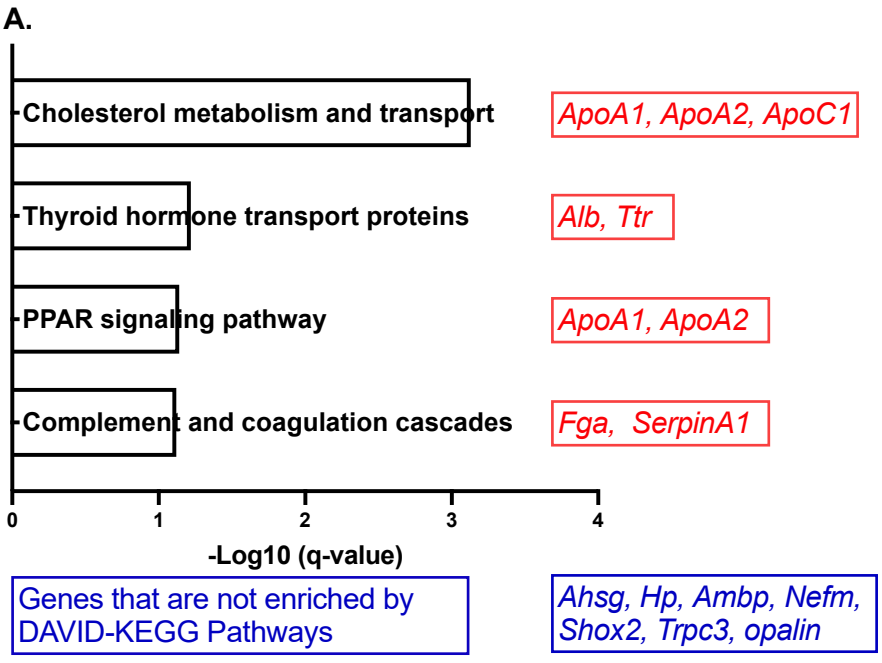
The fourteen DEGs influenced by insulin treatment were analysed for pathway enrichment using the 2021 KEGG pathway database in the DAVID tool. Significant pathways (adj. p-value < 0.1, counts ≥ 2) included cholesterol metabolism and transport (apolipoproteins A1, A2 and C1(*ApoA1, ApoA2, ApoC1)*), thyroid hormone transport proteins (albumin (*Alb)* and transthyretin*, (Ttr*)), PPAR signalling (apolipoproteins A1, A2 (*ApoA1, ApoA2*)), and complement and coagulation cascade (fibrinogen alpha chain (*Fga),* and Serpin family A1 *(Serpina1b*)) (Figure 20 A). However, seven DEGs were not enriched in KEGG pathways. To gain insights into their functions, literature searches and GeneCards database queries were conducted.

Neurofilament medium chain (*Nefm)* encodes a key subunit of the neurofilament polymer, an integral component of the neuronal cytoskeleton. Neurofilament proteins are widely used as biomarkers for axonal integrity, and their elevated levels in blood and cerebrospinal fluid (CSF) serve as indicators of axonal damage in conditions such as traumatic brain injuries (Martínez-Morillo et al., 2015; Yuan & Nixon, 2021). Short stature homeobox 2 (*Shox2)* encodes a transcription factor involved in bone and pattern formation. It has also been implicated in brain development, with mutations in the gene

linked to cerebral impairment and motor coordination deficits (Rosin et al., 2015). Transient Receptor Potential Cation Channel Subfamily C Member 3 (*Trpc3)* is a non-selective cation channel highly expressed in the brain, particularly in oligodendrocytes. It is involved in calcium signalling and plays diverse roles in physiological processes such as glucose detection and energy homeostasis in the hypothalamus, and the regulation of hippocampal excitability and contextual fear memory (Chrétien et al., 2017; Cole & Becker, 2023; Neuner et al., 2015). Oligodendrocytic Myelin Paranodal and Inner Loop Protein (*Opalin)*, also known as *Tmem10*, is a transmembrane protein that plays a critical role in oligodendrocyte differentiation and the regulation of myelin gene expression (Golan et al., 2008). Alpha-2-HS-Glycoprotein (*Ahsg)*, alpha-1-Microglobulin/Bikunin Precursor (*Ambp)*, and haptoglobin (*Hp)* are glycoproteins involved in the acute phase response, where their serum levels fluctuate in response to inflammation or infection. Beyond their acute-phase properties, these proteins exhibit high affinities for specific ligands, *Hp* binds haemoglobin, while *Ahsg* and *Ambp* have affinities for steroids and growth factors. Functionally, these proteins are involved in modulating inflammatory responses and immune activity (Tyagi et al., 2002; Wöltje et al., 2006; Yerbury et al., 2005).

Based on the functional information gathered, *Nefm*, *Shox2*, *Trpc3*, and *Opalin* were categorized under the biological function of Neuronal development and protection, while *Ahsg*, *Ambp*, and *Hp* were classified under Inflammatory activity (Figure 20 E and F).

Interestingly, with respect to gene regulation following insulin treatment, a distinct pattern was observed. While genes involved in other biological processes were downregulated (Figure 20 B, C, D and F), genes associated with neuronal development and protection, *Nefm, Shox2, Trpc3,* and *Opalin*, exhibited subtle but significant upregulation (Figure 20 E).



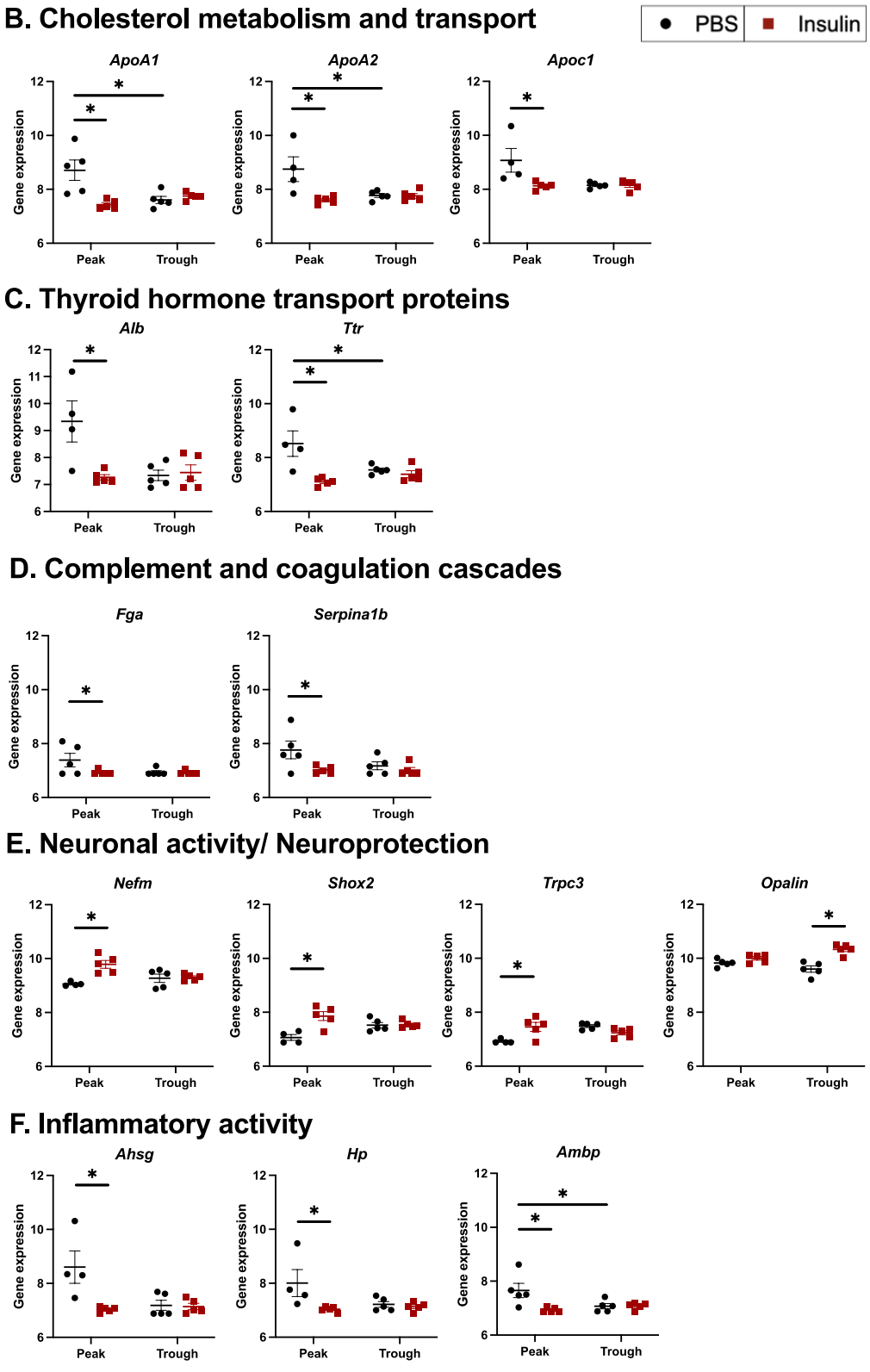


Figure 20. Differential expressed genes (DEGs) with prominent insulin effect were enriched for peripheral and central biological pathways.
(A) Out of fourteen DEGs, half the number were enriched for peripheral functions while the other seven DEGS were not assessed through the database and were studied via literature queries. Enrichment analysis was performed using DAVID's KEGG pathway database with adj. $p < 0.1$. The adj. p values (q values) from the output of DAVID are converted to a scale of -Log 10 values to enhance scale visualisation. (B-F) Gene expression plots of the individual DEGs grouped under the enriched biological pathways. n= 5 per condition. DESeq2, Wald test, adj. $p< 0.05$. The data are presented as mean ± SEM values.

3.10 Three insulin influenced DEGs are predominantly expressed in the brain

Given that RNA sequencing was conducted on hypothalamic preparations, the DEGs were filtered based on their predominant localization and expression in the brain to maintain relevance with the experimental tissue. Tissue-specific expression data was sourced from the NCBI Gene database, utilizing information from the Mouse ENCODE transcriptome project. This dataset, computed from RNA-seq alignments of various mouse tissues, is normalized and presented as RPKM values (Reads Per Kilobase of transcript per Million mapped reads) (Yue et al., 2014). To facilitate comparison across multiple genes and tissues, expression levels for each gene were further normalized to their highest RPKM value and expressed as a percentage. For clarity in data visualization, certain tissues, such as the large and small intestines, duodenum, and adrenals, were grouped to generate a more concise list of tissues for analysis.

A majority of DEGs (*ApoA1, ApoA2, ApoC1, Ahsg, Hp, Ambp, Alb, Ttr, Fga, and Serpina1b*) were predominantly expressed in the liver. Few DEGs also exhibited tissue-specific expression patterns, such as *ApoA1* in the intestine, *Hp* in the lung and subcutaneous fat, *ApoA1* and *Ttr* in the placenta and *Hp, Shox2* and *Trpc3* in endocrine glands. Importantly, *Nefm*, *Trpc3*, and *Opalin* were primarily expressed in the brain, aligning with their postulated roles in neuronal development and protection (Figure 21). These brain-localized DEGs were subsequently prioritized for further analysis.

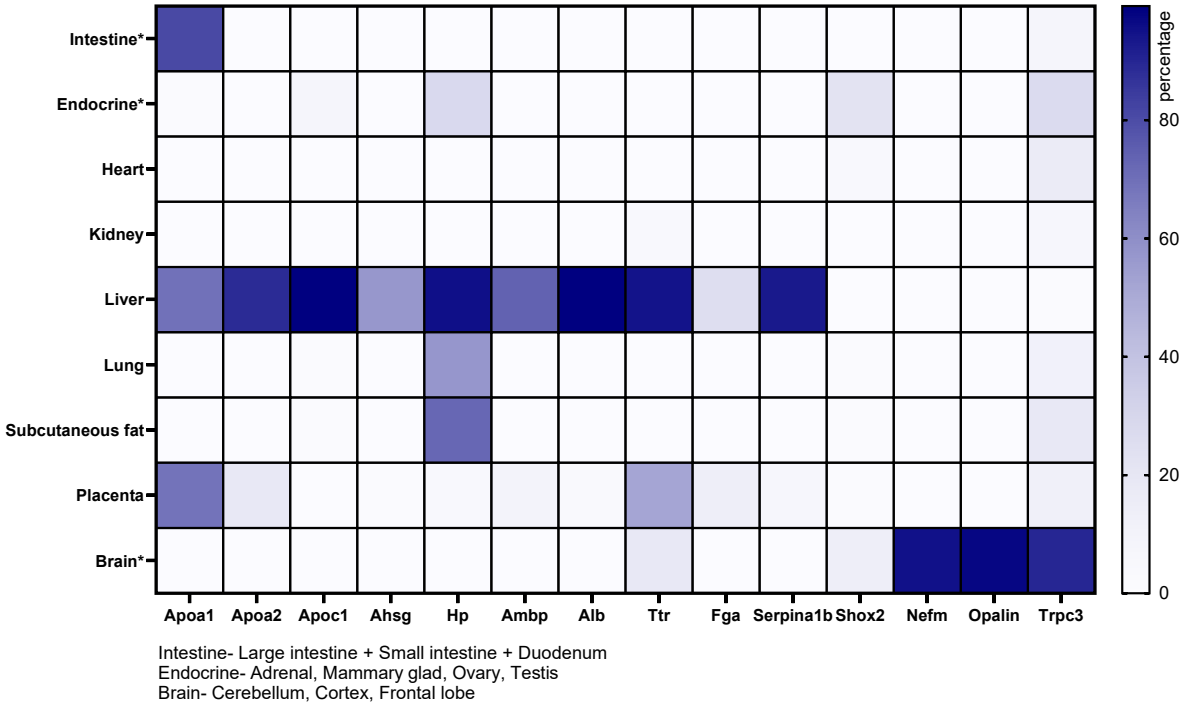


Figure 21. Tissue-specific expression of insulin-influenced DEGs.
Although, maximum DEGs had liver (and other peripheral tissue)-specific localisation, three genes were predominantly localised and expressed in the brain as relevant to the experimental hypothalamic tissue. Expression data was sourced from Mouse ENCODE transcriptome project via NCBI Gene Database.

3.11 Molecular validation of brain-expressed DEGs via qPCR

To validate the RNA-seq results, qPCR was performed on the prioritised brain-localised DEGs- *Nefm, Trpc3* and *Opalin*. Of note, the cDNA material processed for qPCR analysis was transcribed from the RNA used for sequencing to avoid batch effects. *Nefm* and *Trpc3* gene expression showed an increasing trend in the insulin-treated samples at the peak time point, with a near-significant statistical difference of p= 0.07 and 0.06, respectively (Figure 22 A and B). However, no difference was observed in the insulin-treated samples at trough timepoint for the expression of *Opalin* gene as compared with the RNAseq data (presented as an in-box panel for individual genes) (Figure 22 C).

Overall, while no statistical significance was reached, these qPCR results correspond to the trends observed in the RNAseq data, particularly for *Nefm* and *Trpc3*, suggesting a potential influence by insulin treatment.

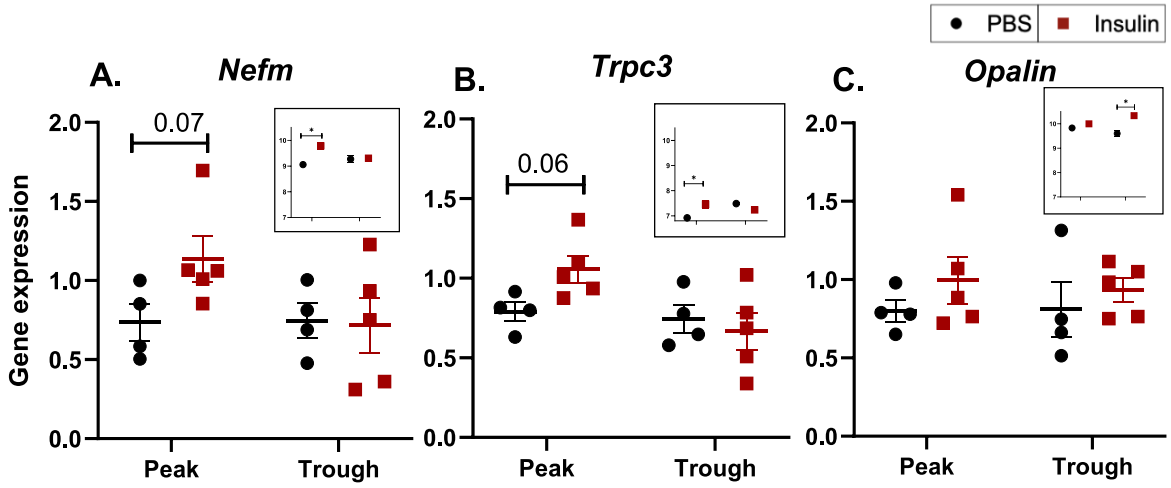


Figure 22. Insulin influenced brain-localised DEGs were validated by qPCR gene expression analysis.
(A) *Nefm* and (B) *Trpc3* mRNA levels showed an upregulation trend (with near-significant statistical threshold) in the insulin treated samples, as consistent with the RNAseq data. (C) Effect on *Opalin* expression was not validated. n = 5 per condition, Two-way ANOVA with Tukey multiple comparison test. The in-box in individual panel represents the mean data of respective DEG expression from RNAseq dataset. The data are presented as mean ± SEM values.

In summary, treatment effect analysis identified fourteen DEGs influenced by insulin, with pathway enrichment revealing five major functional categories: cholesterol metabolism, thyroid hormone synthesis, neuronal development/ protection and inflammatory activity. Notably, while most of the genes were downregulated, genes associated with neuronal development/ protection (*Nefm*, *Trpc3*, and *Opalin*) showed subtle but significant upregulation following insulin treatment, suggesting a potential role for insulin in promoting neuronal resilience under the tested conditions. Tissue-localisation query confirmed that these genes are predominantly expressed in the brain.

3.12 Temporal effect DEGS are segregated based on tissue-localisation and brain-cell type specific expression

Although the differential expression analysis using DESeq2 was performed with four distinct design models to assess both, treatment and temporal effects, the analysis of DEGs associated with the temporal effect (peak vs. trough) was conducted without segregating samples into PBS or insulin-treated groups. This approach was based on the premise that insulin treatment was administered for only one hour at specific circadian timepoints (peak or trough), making it unlikely for insulin to influence intrinsic temporal gene expression patterns significantly. Also, as described in previous sections, overall insulin had a very minor effect in this dataset. Thus, the observed differences in gene expression are primarily attributed to the inherent time-related changes. To clarify further, the downstream enrichment analysis is conducted without segregating the insulin/ PBS groups while the gene expression patterns presented in the graphs still includes four subgroups to align and remain consistent with the DESeq2 output.

DESeq2 identified a total of 721 DEGs with a significant temporal effect (peak vs. trough) across the combined PBS and insulin-treated samples. Similar to the treatment effect DEG analysis, to ensure consistency with the experimental tissue, these DEGs were further filtered to prioritize those predominantly expressed in the brain. The tissue-specific expression data were curated using a single-cell profiling dataset of the Arc-ME region (Campbell et al., 2017). This filtering process identified 117 DEGs as 'brain-localized' (Figure 23 A), while the remaining 604 DEGs could not be definitively categorized based on the current dataset (Figure 23 B). These uncategorized DEGs may include genes with ubiquitous expression across multiple tissues, low-abundance brain expression not captured in the dataset, or peripheral expression.

In addition, acknowledging the brain's complex cellular composition, the brain-localised set of 117 DEGs were identified and segregated for cell type-specific expression. Of these, 24 DEGs showed predominant neuronal expression, while the remaining 93 DEGs were associated with glial cells. A more detailed breakdown revealed 45 DEGs were expressed in microglia, 27 DEGs were astrocytic, oligodendrocyte-expressed were 17 DEGs and only 4 DEGs were expressed in the endothelial cells (Figure 23 A).

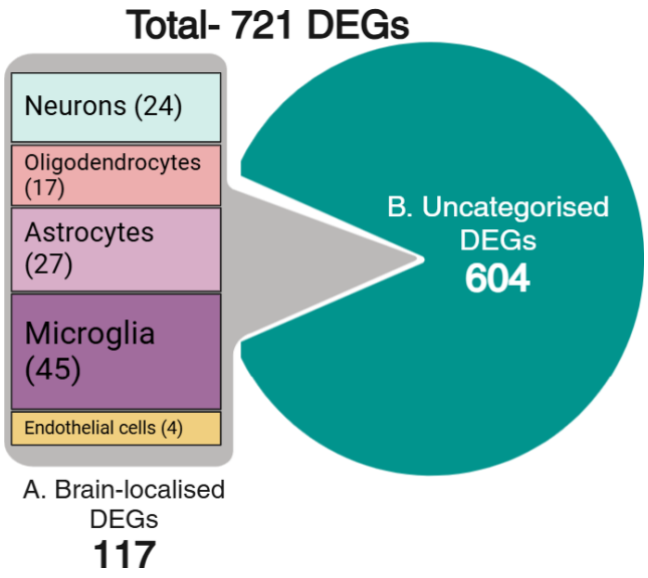


Figure 23. Temporal effect DEGs were filtered for tissue specific localisation to maintain relevance with the experimental tissue.
Temporally influenced 117 DEGs were predominantly localised and expressed in the brain, while 604 DEGs were uncategorised in the current dataset. The brain-localised DEGs were further segregated based on the brain-cell type (neurons, oligodendrocytes, astrocytes, microglia and endothelial cells) expression. The filtering was conducted using a publicly available single-cell dataset profiled for mice Arc-ME region (Campbell et al., 2017).

3.13 Enrichment analysis for DEGs in different brain cell types

The brain, being a complex tissue is rich in diverse cell types, each contributing to its unique functionality. It mainly includes neuronal and glial cells. Neurons are the primary signalling units that form complex circuits responsible for transmitting information through electrical and chemical signals. These circuits exhibit plasticity, allowing for learning and memory formation (Berridge, 2014; Powers et al., 2023). Glial cells that include oligodendrocytes (OLs), astrocytes and microglia support and modulate neuronal activity. OLs are primarily responsible for myelin production and strengthening, but also provide metabolic support to neurons and may have immunomodulatory functions (Han et al., 2022; Kuhn et al., 2019). Astrocytes, once considered mere support cells, are now recognised as crucial players in maintaining the neural microenvironment, to guide neuronal migration, and support synaptic activity (Montgomery, 1994; Sidoryk-Wegrzynowicz et al., 2011). Microglia are the primary immune cells of the CNS performing housekeeping and defence functions but also playing role in brain development, homeostasis (Colonna & Butovsky, 2017; Hickman et al., 2018). And lastly, endothelial cells are vital for vascular homeostasis and angiogenesis within the brain (Michiels, 2003).

To further explore the brain-localized temporal effect DEGs, enrichment analysis was conducted separately for each brain-cell type group of DEGs. The enrichment analysis utilized the GO Biological Pathway 2021 database in the Enrichr KG tool, applying its standard statistical framework for ranking pathways. Results were filtered based on a standard significance threshold of adj. $p < 0.1$, reported in the Enrichr KG output.

For neuronal DEGs, the top enriched pathways were- Positive regulation of intracellular protein transport, Regulation of neuron projection development, Regulation of ion transmembrane transport activity, Protein methylation and Negative regulation of cell projection organization (Figure 24 A). These pathways reflect potential functions of neuronal DEGs in transport activities, protein modifications and neuron projection development. OL-specific DEGs were enriched for Cell-cell junction assembly, Tight junction organization, Positive regulation of toll-like receptor 9 signalling pathway, Regulation of morphogenesis of a branching structure and Pentose-phosphate shunt (Figure 24 B). These pathways were linked to cell-cell communication, immune signalling and some metabolic processes. Similarly, astrocytic DEGs were enriched for Positive regulation of cholesterol esterification, Cellular response to cholesterol, Neurotransmitter uptake, Regulation of cholesterol esterification and Negative regulation of amyloid fibril formation (Figure 24 C). These pathways for astrocytes summarize roles in cellular responses and regulation of lipids, neurotransmitter uptake and protein aggregation. Lastly, enrichment analysis for microglial DEGs resulted in Neutrophil degranulation, Neutrophil activation involved in immune response, Neutrophil mediated immunity and Synapse pruning (Figure 24 E), strongly suggesting role in immune functions and synaptic maintenance. Of note, because fewer DEGs were identified to be localised in endothelial cells (Figure 24 D), they were not subjected to enrichment analysis but will be studied individually in the subsequent sections.

In conclusion, this analysis revealed distinct, cell-type-specific functional roles for temporal effect DEGs. Neurons show involvement in intracellular transport and projection development, OLs in cell communication and metabolism, astrocytes in lipid regulation and neurotransmitter processing, and microglia mainly in immune functions.

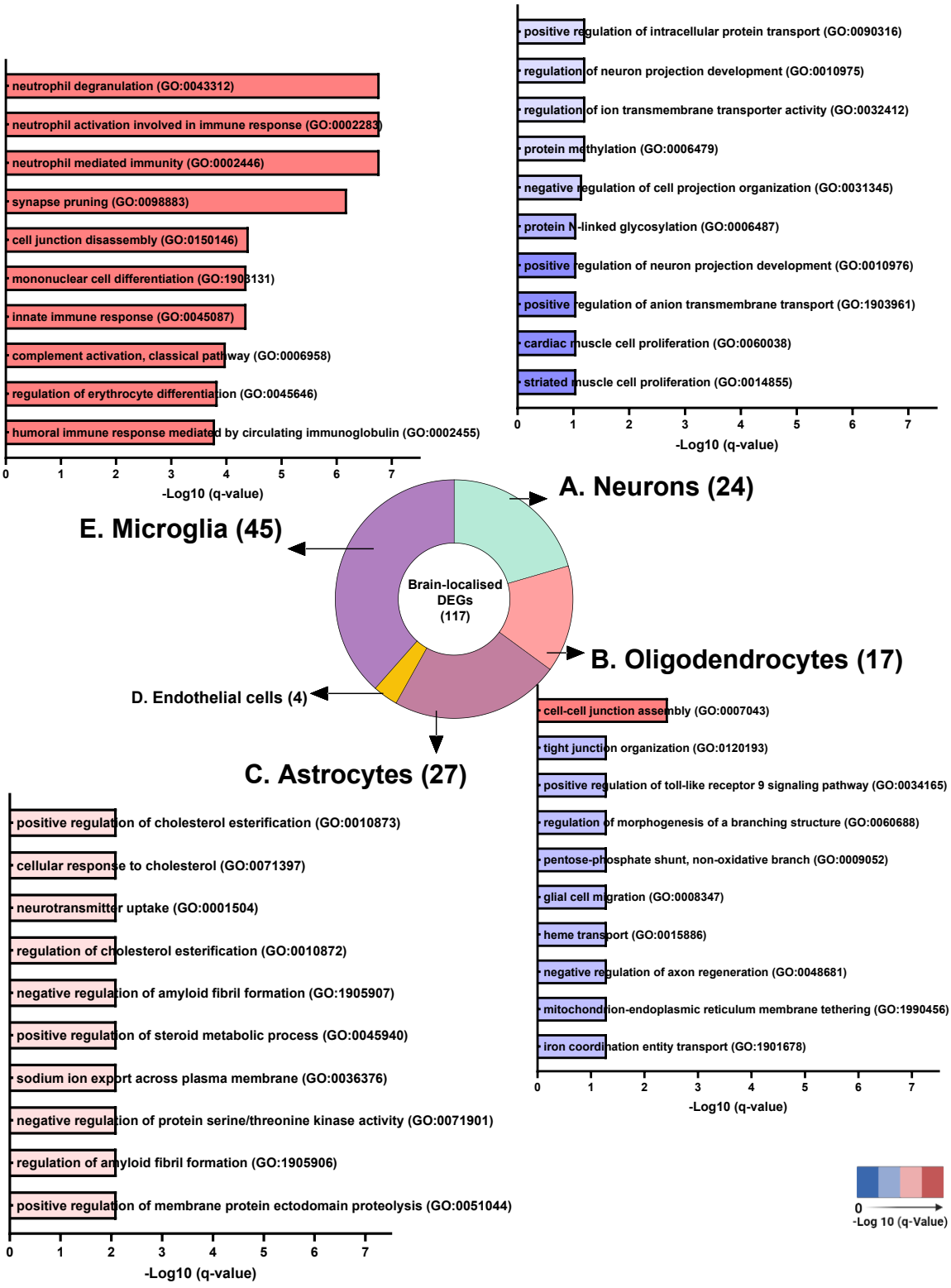


Figure 24. Enrichment analysis for the brain-localised DEGs.
Cell-type specific DEGs were enriched using the 2021 GO Biological Pathway database of Enrichr-KG. (A-E) Bar plots representing the top enriched pathways for each brain cell type- (A) Neurons, (B) Oligodendrocytes, (C) Astrocytes, (E) Microglia and (D) number of endothelial cells. The adj. p values (q values) from the output of Enrichr-KG are converted to a scale of -Log 10 values to enhance scale visualisation. The central pie chart represents segregation of brain-localised DEGs based on the cell-type specific expression as in Fig. 23.

3.14 Clustering and overarching pathway analysis for top-enriched pathways in different brain cell types

After performing brain cell type-specific enrichment analysis, Enrichr KG generates a knowledge graph that visualizes the top enriched biological processes and the key DEGs contributing to them. In this graph, enriched pathways are represented as 'nodes,' while the associations between them are depicted as 'edges'. Using the standard settings of at least one connection per gene and per pathway, knowledge graphs were constructed for each set of enriched biological pathways corresponding to each brain cell type. Utilising term reduction feature in the REVIGO tool, related biological pathways were further grouped into clusters based on literature-based associations. These clusters were then organized into overarching pathways that encompass broader biological themes. This approach helps connect functionally related genes and enriched pathways, thereby facilitating the identification of key expression patterns within complex datasets and simplifying their biological interpretation.

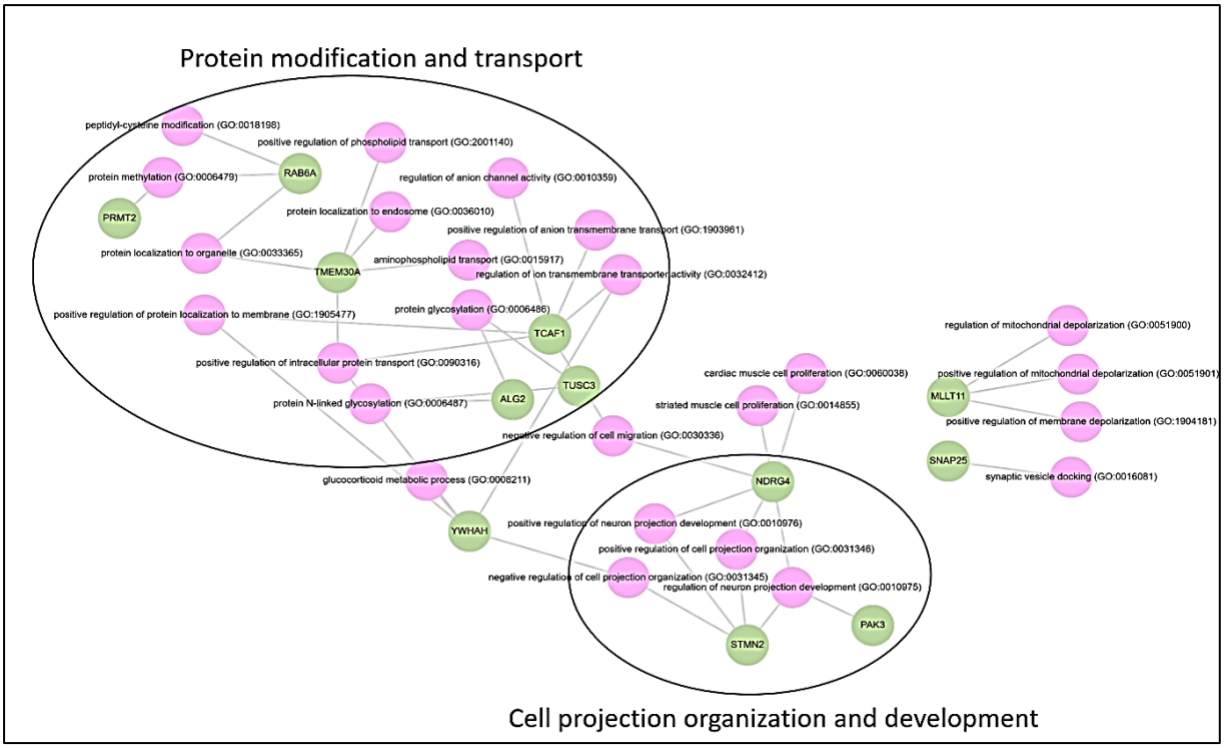
3.14.1 Neurons

For the top enriched biological pathways of the neuronal DEGs, two distinct clusters were identified. The first, larger cluster encompassed pathways such as Protein methylation**,** Protein localization to organelle, Protein glycosylation, and Intracellular protein transport. These pathways were unified under the broader theme of "Protein Modification and Transport" (Figure 25 A). The primary DEGs contributing to this cluster include *Prmt2* (Protein arginine methyltransferase 2), *Rab6a* (Ras-related protein Rab-6A), *Tmem30a* (Transmembrane protein 30A), *Alg2* (Alpha-1,3/1,6-mannosyltransferase), *Tcaf1* (TRPM8 Channel Associated Factor 1), and *Tusc3* (Tumor suppressor candidate 3).

The second, smaller cluster consisted of pathways such as Positive regulation of neuron projection development, Positive regulation of cell projection organization, and Regulation of neuron projection development. This cluster was categorized under the overarching theme of "Cell Projection Organization and Development", involving DEGs such as *Ndrg4* (N-myc downstream-regulated gene 4), *Stmn2* (Stathmin 2), and *Pak3* (p21-activated kinase 3) (Figure 25 A).

Interestingly, temporal regulation patterns for the key DEGs involved in these pathways showed upregulation during the PER2::LUC trough phase in only PBS-treated samples, except *Mlt11*, which showed upregulation in both PBS and insulin-treated samples (Figure 25 B,C and D).

A.



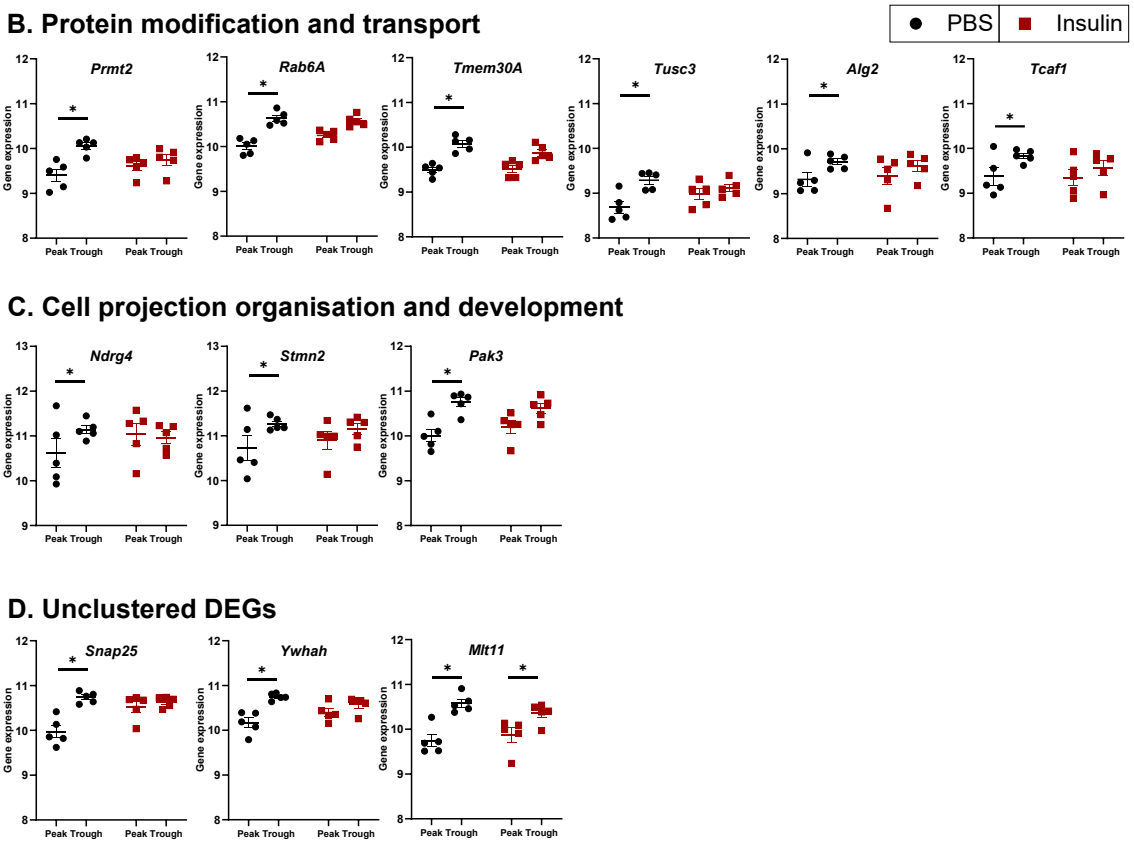
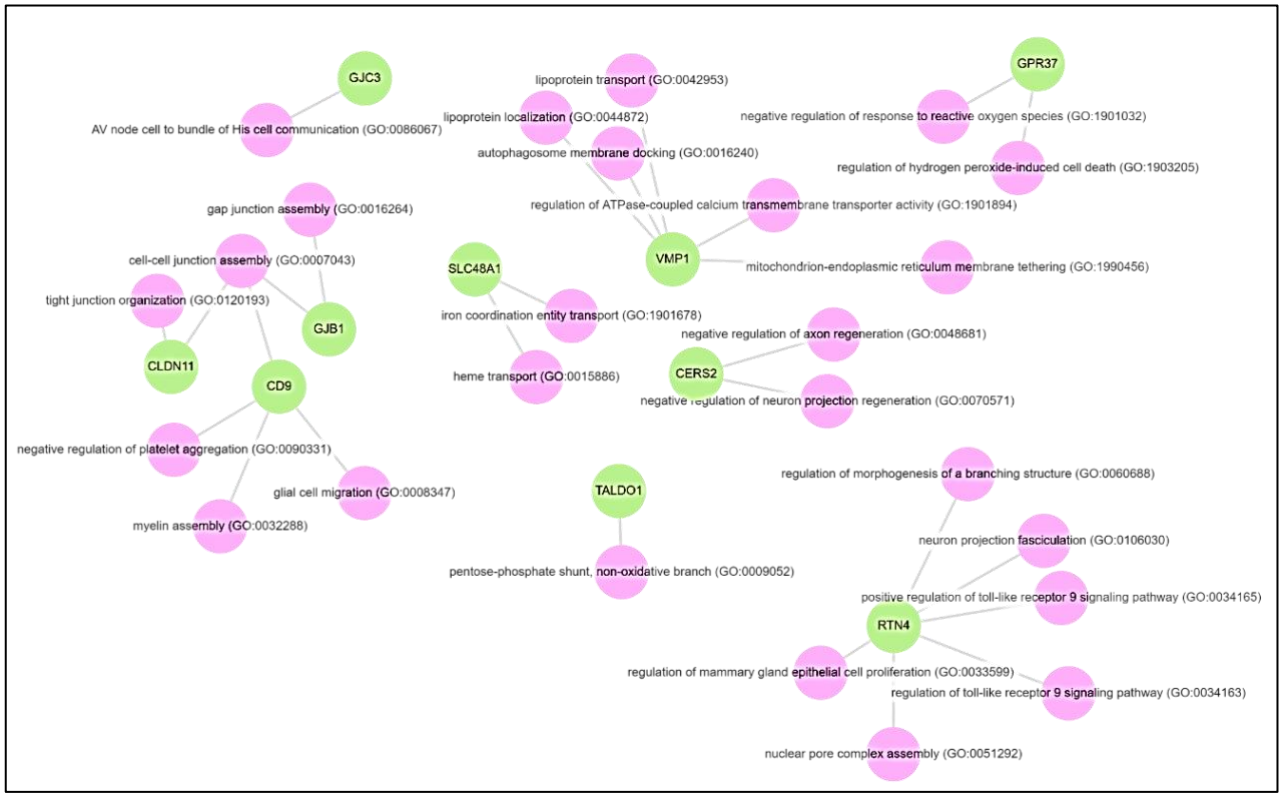


Figure 25. Knowledge graph-based pathway clustering and temporal expression analysis of neuronal DEGs. (A) Knowledge graph constructed from Enrichr KG for neuronal DEGs shows enriched biological pathways (nodes) and their contributing genes (edges). (B and C) Gene expression plots of the key DEGS belonging under 2 identified clusters. n=5 per condition. DESeq2, Wald test, p adj.< 0.05. The data are represented as mean ± SEM values.

3.14.2 Oligodendrocytes

The knowledge graph analysis for oligodendrocyte DEGs interestingly revealed no significant connectivity among the enriched pathways, as no edges were observed between any of the nodes (Figure 26 A). The key DEGs also displayed diverse temporal expression patterns. *Cd9* (Cluster of Differentiation 9) was downregulated during the trough phase in the PBS-treated group, while *Gjlc3* (Gap Junction Lambda 3), *Vmp1* (Vesicle-associated Membrane Protein 1), *Cers2* (Ceramide Synthase 2), and *Gpr37* (G-protein Coupled Receptor 37) were upregulated at the trough phase in the insulin-treated group. Other DEGs, such as *Cldn11* (Claudin 11), *Slc48a1* (Solute Carrier Family 48 Member 1), and *Taldo1* (Transaldolase 1), were consistently upregulated at the trough phase across both PBS and insulin groups, whereas *Gjb1* (Gap Junction Beta 1) and *Rtn4* (Reticulon 4) were downregulated under the same conditions (Figure 26 B).

A.



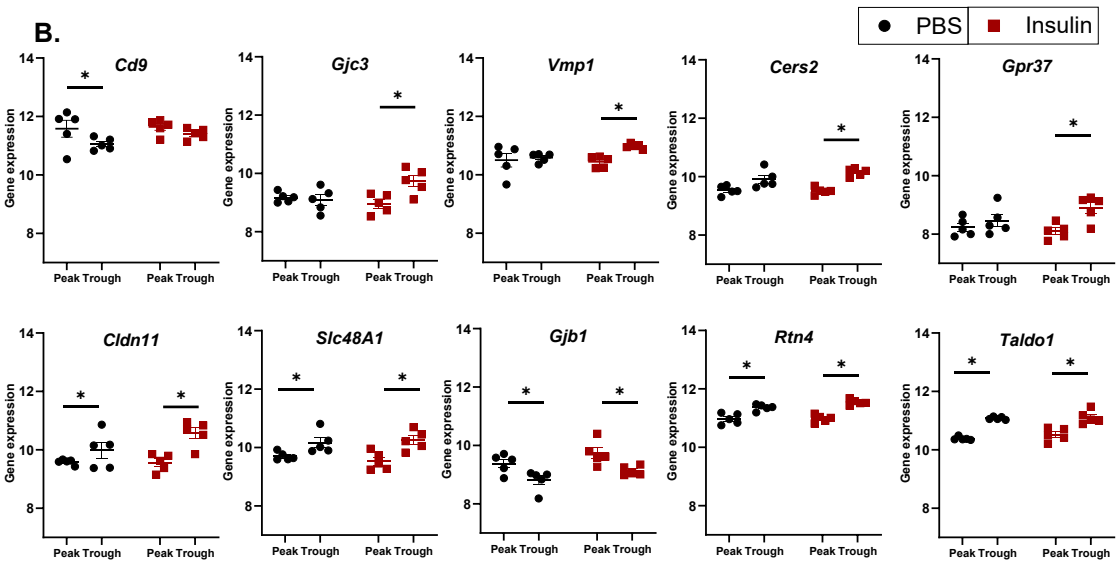


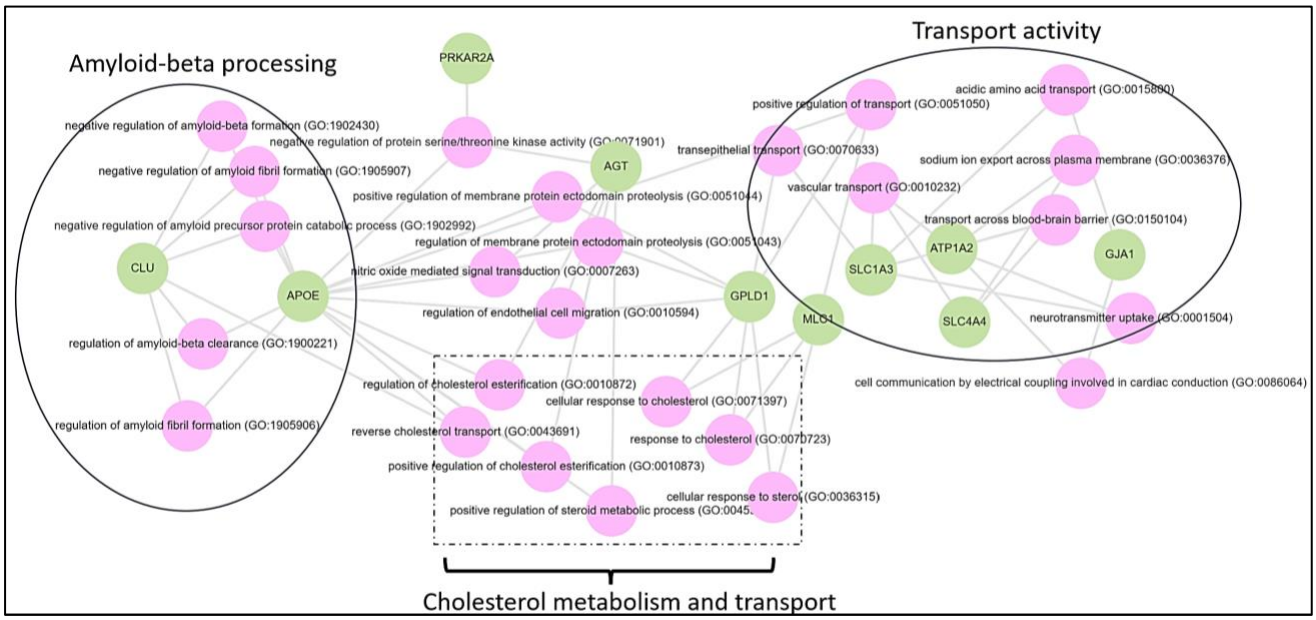
Figure 26. Knowledge graph-based pathway clustering and temporal expression analysis of oligodendrocytic DEGs.
(A) The top-enriched pathways for oligodendrocyte-specific DEGs showed no significant connection among each other- the key DEGs represented as nodes showed no edges between them, thus, no cluster was identified. (B and C) Gene plots of the key DEGs belonging to the top enriched pathways. n=5 per condition. DESeq2, Wald test, p adj. < 0.05. The data are represented as mean ± SEM values.

3.14.3 Astrocytes

For the top enriched biological pathways identified in astrocytic DEGs, two distinct clusters emerged. The first cluster, involving two key genes, *Clu* (Clusterin) and *Apoe* (Apolipoprotein E), was associated with pathways such as Negative regulation of amyloid-beta formation, Regulation of amyloid-beta clearance, and Negative regulation of amyloid fibril formation. These pathways were collectively linked to the broader theme of "Amyloid-beta processing". The second cluster included enriched terms like Positive regulation of transport, Vascular transport and Transport across the blood-brain barrier. These were grouped under the overarching category of "Transport activity" (Figure 27 A) and involved five genes (Figure 27 C): *Mlc1* (Myosin Light Chain 1), *SlcA3* (Solute Carrier Family A3 Member 1), *SlcA4* (Solute Carrier Family A4 Member 1), *Gja1* (Gap Junction Alpha-1), and *Atp1a2* (ATPase Na+/K+ Transporting Alpha 2 Subunit).

Most of the overrepresented genes under these pathways were downregulated during the trough phase in both PBS and insulin-treated groups, except for *Mlc1* and *Gja1*, which showed downregulation at the trough phase only in the insulin-treated samples (Figure 27 B, C, D).

A.



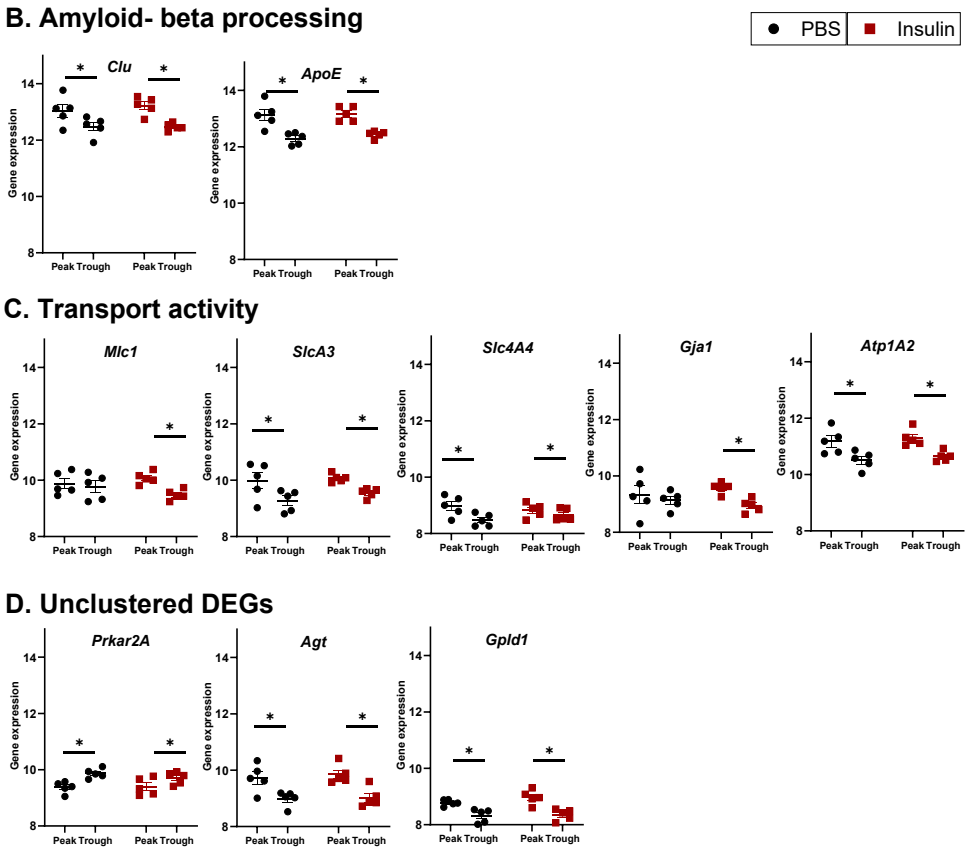
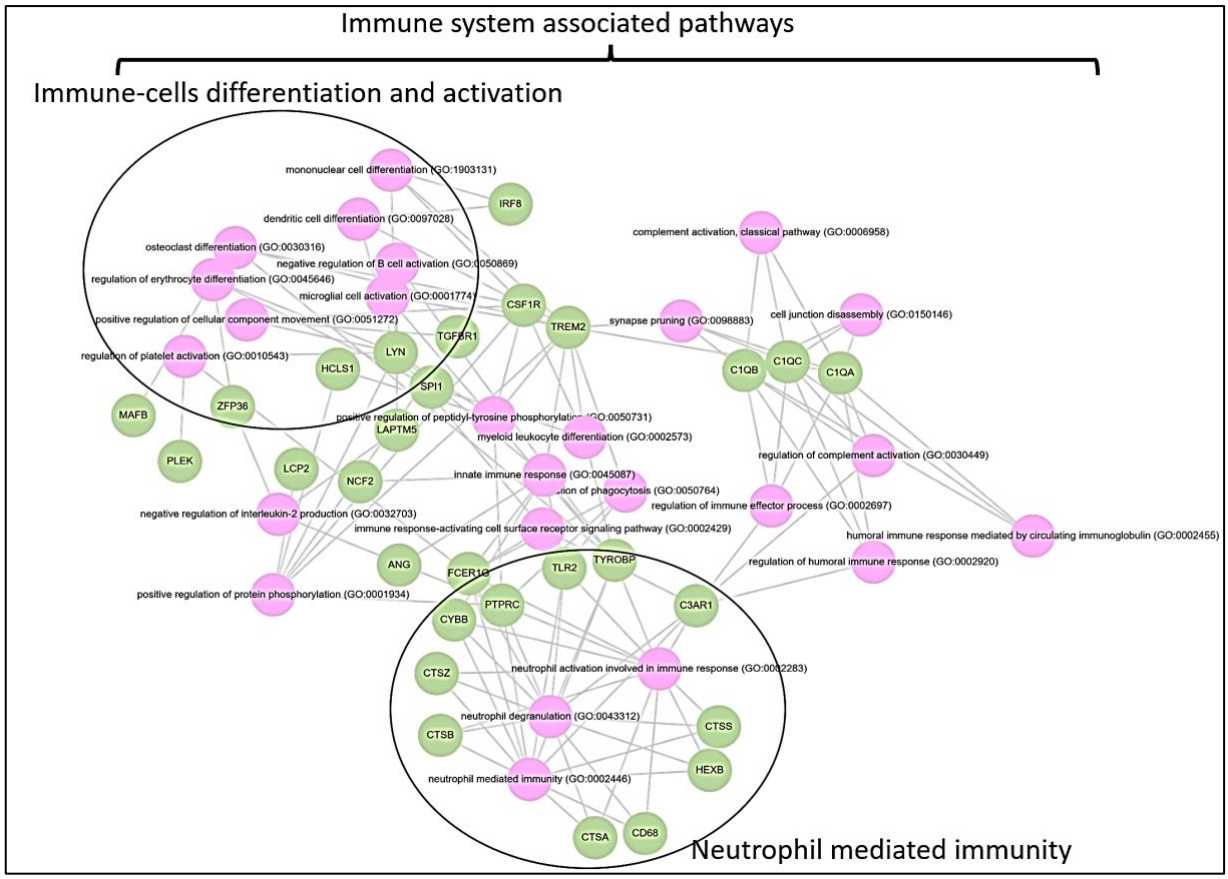


Figure 27. Knowledge graph-based pathway clustering and temporal expression analysis of astrocytic DEGs. (A) Cluster and overarching pathway analysis for the astrocytic- specific DEGs identified two major clusters with nodes connected by edges among the functionally similar pathways (represented as solid-line circles). In addition, a group of functionally similar pathways was identified without any connecting nodes and was assigned to a boarder theme of biological function (represented as a dotted square). (B, C and D) Gene expression plots of the key DEGs involved under the identified clusters. n=5 per condition. DESeq2, Wald test, p adj. < 0.05. The data are represented as mean ± SEM values.

3.14.4 Microglia

Similarly, microglial-specific DEGs were enriched for pathways linked to immune responses, such as Regulation of erythrocyte differentiation, Innate immune response, Neutrophil degranulation, and Regulation of complement activation. Although two subclusters were identified, namely, Immune-cell differentiation and activation and Neutrophil-mediated immunity, all enriched pathways were collectively categorized under the broader theme of "Immune-system associated pathways" (Figure 28 A). Multiple genes were overrepresented, including key immune-related genes such as *Lyn* (Tyrosine-protein kinase-Lyn), *Irf8* (Interferon regulatory factor 8), *Tlr2* (Toll-like receptor 2), *Cd68* (Cluster of differentiation 68), and *C1qa* (Complement component 1, q subcomponent alpha chain). Notably, all genes within this overarching pathway exhibited the same pattern: downregulation at the trough phase in both PBS and insulin-treated groups. Representative gene expression plots for this regulatory pattern are shown in Figure 28 B.

A.



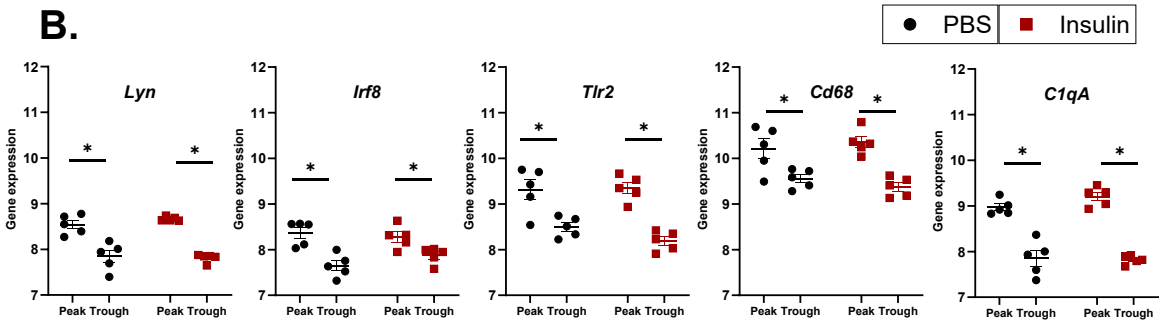


Figure 28. Knowledge graph-based pathway clustering and temporal expression analysis of microglial DEGs. (A) Microglial- specific DEGs enriched for significant biological pathways, resulted in the annotations belonging under an overarched biological function of immune system associated pathways. These pathways were further sub-clustered in two other pathways based on functionally connected nodes and edges. (B) Gene expression plots of the representative key genes identified in the overarched biological function. n=5 per condition. DESeq2, Wald test, p adj. < 0.05. The data are represented as mean ± SEM values.

3.14.5 Endothelial cells

Lastly, DEGs expressed in the endothelial cells displayed either down regulation (*Pltp* (Phosphatidylcholine Transfer Protein) *and Grap* (Grb2-associated binder 1) or upregulation (*Cldn5* (Claudin 5) *and Tspan13* (Tetraspanin 13) in the gene expression at the trough phase in both the PBS and insulin groups (Figure 29).

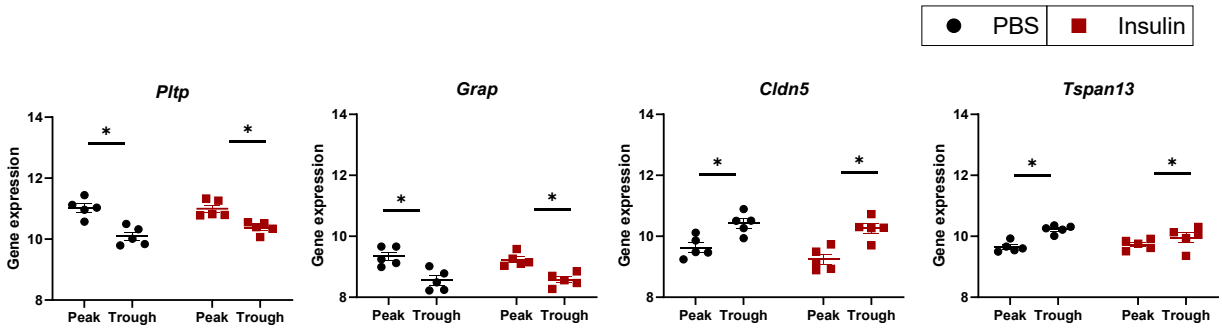


Figure 29. Gene expression plots of the endothelial cell-specific DEGs.
n=5 per condition. DESeq2, Wald test, p adj.< 0.05. The data are represented as mean ± SEM values.

3.15 Enrichment analysis for uncategorised DEGs

The 604 DEGs that were not identified under any of the brain-cell types were marked as 'uncategorised'. These uncategorised DEGs potentially include genes with ubiquitous expression, meaning that they are expressed across multiple tissues rather than being restricted to specific cell types. Such genes often play critical roles in fundamental cellular processes, such as metabolism, transcription, or protein synthesis etc, which are essential across various tissues. Or they include DEGs with low-abundance brain expression that fall below the detection threshold or genes with peripheral expression that could have relevance to systemic biological processes or disease states.

Enrichment analysis for these group of genes using Enrichr KG tool with standardised significance threshold of p adj. < 0.1, resulted in the top enriched pathways associated with Immune responses. The enriched pathways included, Cytokine-mediated signalling pathway, Cellular response to lipopolysaccharide, Cellular response to cytokine stimulus, etc (Figure 30). These findings show the possibility that the uncategorised DEGs may act as mediators of immune-related processes, either within the brain's neuro-immune axis or through systemic immune pathways.

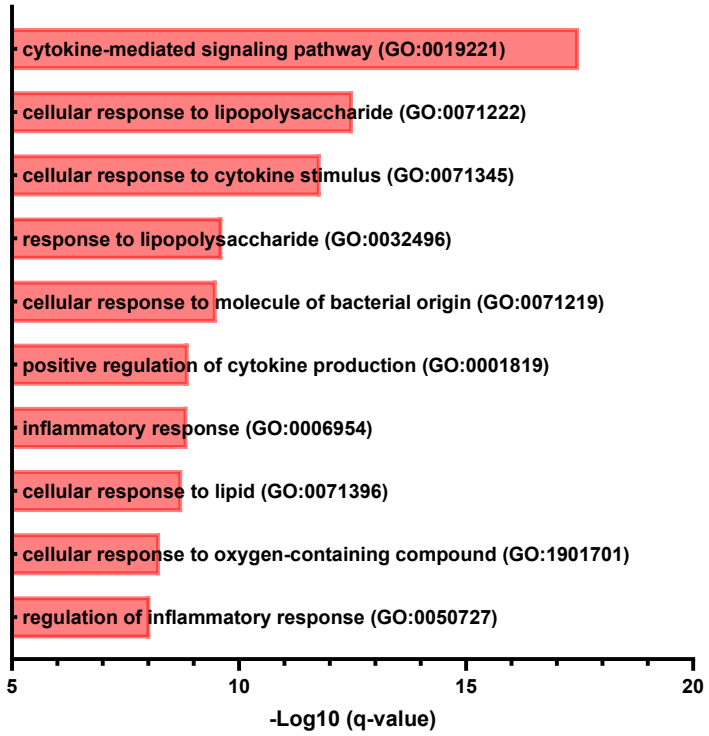


Figure 30. Enrichment analysis of the uncategorised, temporal-effect DEGs.
Conducted using 2021 GO Biological Pathway database of Enrichr-KG tool with a significance threshold of p adj. < 0.1. Bar plots represent the top enriched pathways where the q values for the enriched pathways are converted to a scale of -Log 10 values to simplify scale visualisation.

In summary, DESeq2 identified 721 DEGs associated with temporal variation. To align with the experimental tissue context, these DEGs were filtered for tissue localisation using reference datasets, yielding 117 brain-localised and 604 uncategorised DEGs. The uncategorised set potentially included DEGs with ubiquitous or peripheral expression. The brain-localised DEGs were further segregated by cell-type (neuronal, glial and endothelium) specific expression. Each cell-type group was further subjected to pathway enrichment analysis that hinted a presence of time-of-day dependent gene expression in the Arc-ME region.

Chapter 4: Discussion

This thesis investigated whether insulin's actions in the murine hypothalamus are under circadian regulation. By examining two fundamental roles of insulin in the brain, metabolic homeostasis and cognition-associated functions, the project examined how insulin's effects may vary depending on the circadian phase of exposure. Using immortalized hypothalamic neuronal cultures and *ex vivo* tissue preparations, the study first confirmed that robust circadian rhythmicity was preserved across both model systems, validating their use for temporal studies. While targeted gene expression analysis revealed limited transcriptional responses to insulin, transcriptomic profiling uncovered a set of insulin-responsive genes linked to neuronal development and protection, unaffected by circadian phase. These findings suggest a possible dual mode of insulin action in the brain: circadian-gated effects on metabolism and tonic regulation of neuronal health and maintenance.

4.1. Clocks are retained in neuronal cultures and Arc-ME slice preparations, but limited metabolic effects are observed

4.1.1 Circadian integrity is maintained in both model systems

Clear evidence of circadian rhythmicity was observed in both model systems used in this study. Immortalised hypothalamic neurons exhibited robust *Bmal1*-Luc oscillations, showing high-amplitude and sustained rhythms with constant period length (Section 3.2). In parallel, Arc-ME preparations from PER2::Luc reporter mice maintained self-sustained rhythms over multiple days. The rhythms were observed under constant conditions and without external entrainment, confirming the autonomous nature of the circadian machinery (Section 3.5). These observations provide strong support for the preservation of intrinsic circadian timing mechanisms outside the whole-organism context, consistent with prior work showing that extra-SCN brain regions retain clock gene rhythms *in vitro* (Abe et al., 2002b; Chun et al., 2015).

The retention of clock functionality is a critical feature that validates relevance of using these models for studying circadian regulation. Circadian rhythms are essential for coordination of hypothalamic functions like feeding and metabolic homeostasis (Eckel-Mahan & Sassone-Corsi, 2013; Van Drunen & Eckel-Mahan, 2021b). For the current study, which focuses on whether insulin action is modulated by circadian gating, it was particularly critical to demonstrate that the endogenous oscillator remained intact. The research surrounding circadian gating presumes that cells and tissue respond differently to a stimulus depending on internal circadian phase. Therefore, without demonstration of functional rhythms, the concept could not be tested. Thus, the confirmation of rhythmicity in isolated cells and tissue, without systemic signals, serves not only as a positive control, but as a necessary foundation for interpreting the observations of this study, particularly in understanding why insulin responses may (or may not) exhibit time-of-day variation.

Furthermore, these model systems allow for tightly controlled determination of circadian phase for stimulus exposure, enabling a level of experimental precision that is often difficult to achieve *in vivo* (Pang & Chiba, 1994). Such isolated systems avoid the complexity of systemic signals, feedback loops, behavioural masking and metabolic cues that often confound interpretation in whole-animal models (Pang & Chiba, 1994). Prior studies have similarly used *ex vivo* tissue preparations and circadian-synchronised cell cultures to study phase-dependent gene expression and hormonal sensitivity (Crosby et al., 2019; Lincoln et al., 2024), reinforcing the relevance of this experimental approach. In addition, despite known challenges such as rhythm damping in long term cultures (Mihelakis et al., 2022), detection of rhythmic oscillations in both models sustained for multiple days validates the technical robustness of the experimental systems used in this work.

In summary, the observed circadian rhythmicity in both immortalized hypothalamic neurons and Arc-ME tissue slices confirms that these models are well-suited for investigating phase-dependent insulin responses**.** Importantly, by establishing that the clock is functional**,** this section surpasses the later observation that any absence of insulin effects on metabolic gene expression levels is not due to clock failure but likely reflects low-amplitude or context-dependent features of insulin action in the brain itself.

4.1.2 Challenges in detecting the metabolic effects of central insulin action

In this study, insulin's transcriptional effects on hypothalamic metabolic neuropeptides were minimal across both *in vitro* and *ex vivo* models. Importantly, IR transcript was detected in both models confirming that the primary molecular machinery for eliciting insulin signalling and its actions is present. Also, as described in the previous section, both systems demonstrated robust circadian rhythmicity indicating that the internal timing machinery was retained and was suitable for exploring circadian gating. Despite these validations, insulin treatment did not produce significant transcriptional alterations in classical hypothalamic neuropeptides (*Npy*, *Agrp*, *Pomc*, and *Cart*) associated with metabolic regulation (Section 3.3 and 3.6).

From a technical standpoint, several factors could have limited our ability to detect such effects. To comprehensively test insulin's potential effects, DRC experiments were employed. The chosen doses were supraphysiological to overcome sensitivity thresholds in isolated model systems. In parallel, RTC experiments covered up to 24 hrs of insulin exposure, with multiple time-points ensuring that both acute and delayed transcriptional responses could be detected. Despite the primary meticulous experimental set up, transcriptional changes in classical metabolic neuropeptides remained largely unresponsive. This suggests that technical insufficiency alone could not explain the muted metabolic effects of insulin.

Moving on to the biological constraints, the gene targets analysed in this study were primarily limited to a panel of hypothalamic neuropeptides (*Npy*, *Agrp*, *Pomc*, and *Cart*), which are the terminal transcriptional outputs of the insulin signalling cascade involved

in central metabolic regulation. To cover the transcriptional scope, this study attempted to examine terminal transcriptional outputs as well as intermediate ones such as *Gpr17* and *Cpe*. However, these additional targets also did not reveal any substantial improvement in the detection of insulin-induced transcriptional changes. Insulin's central metabolic effects have also been studied through post-transcriptional mechanisms, including phosphorylation of intracellular signalling intermediates such as AKT and FOXO1 (Carvalheira et al., 2003; Fukuda et al., 2008; Niswender et al., 2003; Saneyasu et al., 2018) and modulation of neuronal excitability (Chen et al., 2019; Rafiei et al., 2024). In line with this, the present study attempted to assess insulin-induced phosphorylation of FOXO1, as a proxy for pathway activation, by monitoring its nuclear exclusion (Section 3.4). However, due to methodological constraints, likely stemming from limitations in detection sensitivity, the expected translocation of phosphorylated FOXO1 could not be reliably confirmed. Addressing these limitations, further attempts to gain insights of metabolic regulation in the brain, studies could employ techniques such as patch-clamp electrophysiology or calcium imaging to measure activity of specific neuronal subtypes, like AgRP and POMC neurons (Chen et al., 2015; De Solis et al., 2024). These methods provide real-time, cell-type-resolved insights into the hormone's influence on neuronal excitability, circumventing the need for large transcriptional changes.

There's also a possibility that the model systems employed in this study, may have lacked the cellular diversity and integrative network context required for insulin's functional effect. Cell cultures, though useful for controlled mechanistic assays, suffer from certain inherent limitations. The hypothalamic neuronal cell lines used in this study (mHypoE27, E41, E44 and A2/23) were immortalised using retroviral transfer of SV40 T-antigen (Belsham et al., 2004). These immortalised cell lines despite expressing IR may lack mature downstream signalling dynamics or hormone-sensitive gene regulatory environments. Furthermore, these cultures were composed predominantly of orexigenic neurons, expressing *Agrp* and *Npy*, and lack the representation of anorexigenic components, *Pomc* or *Cart*. Literature suggests that insulin's anorectic effect in the hypothalamus results from dual and opposing modulation, inhibition of orexigenic neurons and activation of anorexigenic ones (De Solis et al., 2024; Morton et al., 2006). Thus, one-sided or half representation of only orexigenic signals could have shunted insulin's anorectic effect. In addition, the cell lines used were purely composed of neurons and did not include glial cells. Recent studies have shed light upon presence of IR in astrocytes and their involvement in energy homeostasis (Chen et al., 2016; Yang et al., 2015). Their absence may have blunted cellular cross-talk and signal integration.

The lack of integrative components discussed above, prompted the usage of tissue slices to study insulin's metabolic effects in hypothalamic region. While the use of Arc-ME tissue preparations attempted to circumvent few limitations by preserving regional brain architecture, it represented new challenges. Importantly, hypothalamic regulation of feeding and energy homeostasis relies on interconnected neural networks, modulated by a multi-hormonal milieu (Morton et al., 2006; Timper & Brüning, 2017). Insulin in the

brain does not operate in isolation and is only one of many players, acting in concert with leptin, ghrelin, adiponectin and glucose-sensing pathways to influence hypothalamic function (Morton et al., 2006; Timper & Brüning, 2017; Xu et al., 2005). In simplified *in vitro* or *ex vivo* systems, many of these interacting inputs are absent. Isolated insulin application may therefore have failed to elicit full physiological responses, not because the hormone lacked potency, but because key contextual signals were missing.

This goes in line with a broader body of literature that has reported challenges in studying effects of these individual hormones in the brain (Jessen et al., 2010; Woods & Begg, 2015). Woods and colleagues were among the first to demonstrate insulin's anorectic action via central administration (Woods et al., 1979), yet in subsequent decades, their own laboratory, as well as others, faced repeated difficulties in reliably reproducing this effect under standardized conditions (Woods & Begg, 2015; Woods & Langhans, 2012). For example, Jessen et al. reported that third ventricle insulin injections failed to consistently suppress food intake, even when dose, route, animal strain, and housing conditions were tightly controlled (Jessen et al., 2010). Woods, Begg and Langhans described such observations as 'fickle' and 'iffy' reporting how insulin's efficacy in the brain appears fragile and highly sensitive to experimental nuance, highlighting their persistence even when conditions were tightly regulated (Woods & Begg, 2015; Woods & Langhans, 2012). In the context of this project, while transcriptional responses in classical neuropeptides remained negligible across all tested conditions, one instance of hunger-promoting gene (*Gpr17)* exhibited a transient reduction in expression following insulin treatment in Arc-ME slices; however, this effect was not reproducible in a subsequent experimental trial. A few other inconsistent results were also observed during the RTC and DRC analyses but were excluded from interpretation due to their lack of reproducibility. These fluctuations align with the reports of ambiguous or inconsistent assessment of food intake. Reports have often highlighted factors such as hormones and prior feeding state of the organism, stress levels, and circadian phase to play a major role in modulating such inconsistent effects of hormones on central metabolic regulation (Woods & Langhans, 2012).

Notably, while one might argue that whole-animal models offer a more "complete" environment to study central metabolic regulation, it is important to recognize that many of the inconsistent or fickle insulin effects described in the literature have emerged precisely from *in vivo* experiments (Jessen et al., 2010; Woods & Langhans, 2012). Even when systemic integration is intact, the brain's response to hormones such as insulin can remain elusive. Thus, while isolated models like neuronal cultures and tissue slices are limited in terms of complexity, whole-animal studies are not exempted from these challenges. The intact organism introduces additional variables, including behaviour, endocrine feedback loops, and environmental stressors, all of which can confound the detection of discrete hormone effects (Woods & Langhans, 2012).

Taken together, the lack of robust insulin-induced transcriptional responses in this study reflects the combined limitations of model system simplification, biological incompleteness, and methodological sensitivity. While our experimental design was

reasonably comprehensive in its treatment parameters, and the models themselves retained functional circadian clocks and insulin signalling machinery, the central metabolic effects of insulin remained below detection threshold. This observation is not unique but rather is observed as a broader challenge in the field (Woods & Langhans, 2012). Detecting its effects requires not only technically sensitive assays but also biologically integrative systems with neuronal subtypes, glial populations, and the full spectrum of metabolic and behavioural signals. Thus, future studies could prioritize post-transcriptional signalling assays over transcript-level measurements, particularly in simplified models where transcriptional responses are low-amplitude and transient. Techniques such as Western blotting or phospho-specific ELISAs targeting activated intermediates (e.g. pAkt, pFOXO1 or pERK) could offer more robust and reproducible readouts of insulin signalling dynamics (Kumar et al., 2007; Matsuzaki et al., 2003; Ramalingam et al., 2016) . Notably, beyond the classical PI3K-Akt-FOXO1 axis, studies could also explore other established insulin-induced pathway such as the MAPK/ERK cascade (Mayer & Belsham, 2009b). Furthermore, to address the limitation of lacking glial representation and anorexigenic signals, studies would benefit from co-culture model systems that incorporate neuronal subtypes and glial cells, enabling a more physiologically relevant context that includes cellular crosstalk required for metabolic homeostasis (Ioannou et al., 2019; Park et al., 2001).

4.2. Moving beyond targeted approaches: Why systematic global profiling was necessary

4.2.1 Narrow insights through targeted classical gene markers prompted a need for an unbiased, transcriptomic profiling approach

The initial approach in this project to study circadian gating of insulin in central metabolic regulation focused on the transcriptional modulation of classical hypothalamic neuropeptides that are widely accepted as successful readouts in the literature. These genes (*Npy*, *Agrp*, *Gpr17, Cpe, Pomc*, and *Cart)* are established downstream targets of multiple hormonal and nutrient-related signalling pathways and are often considered reliable indicators of hypothalamic activity in response to metabolic stimuli (Morton et al., 2006; Ono, 2019). However, despite their relevance, none of these genes showed consistent or reproducible changes in response to insulin treatment, across both model systems, for all tested timepoints and insulin dosages. The lack of regulation in these well-characterized metabolic markers suggests a couple of possibilities: either insulin could not elicit any effect in these models, or its effects lie outside the scope of this narrow gene panel. Given that IR expression was confirmed and presence of respective neuropeptide gene expression was validated, the subsequent molecular machinery of insulin-associated metabolic pathway in the brain was confirmed to be intact. Thus, the more plausible interpretation was that the targeted approach, while mechanistically logical, was limited in scope to capture the full range of insulin's transcriptional influence**.** The tendency to rely on a small set of well-known molecular targets, which may lead to under-detection of non-canonical or low-amplitude responses, was challenged. It prompted a

shift toward an unbiased, global profiling approach, with the goal of capturing a more comprehensive and an umbrella view of insulin's action in hypothalamic tissue.

4.2.2 Transcriptomic profiling validated subtle and low-amplitude effects of central insulin action

The transcriptomics method used in this study employed a bulk RNA-seq approach applied to intact hypothalamic tissue preparations after acute insulin exposure at two circadian phases. This method offered an unbiased, umbrella view of gene expression changes across the experimental tissue, capturing the integrated effects of heterogeneous cell populations. Compared to targeted qPCR, which is limited to predefined genes and requires a priori assumptions, bulk RNA-seq allowed for a presumption-free discovery of both expected and novel gene targets. This shift from targeted to global analysis provided a chance to determine whether the lack of effects observed in earlier assays reflected biological reality or limitations in gene selection panel.

The transcriptomic analysis revealed several key insights. First, when testing for interaction between insulin treatment and time-of-day effect, no genes were differentially expressed. This suggests that insulin's effects, in this experimental set-up, at least at the transcriptional level, did not vary significantly across the two circadian phases. In contrast, when analysed independently, time-of-day had a stronger influence on gene expression than insulin treatment (Section 3.8).

On one hand, while the analysis was limited to two time points, the differential expression results pointed toward subtle but consistent temporal variation in gene regulation. To explore this further, enrichment analysis of temporally regulated DEGs was carried out in a brain cell-type specific approach. The findings indicated time-dependent modulation of gene sets associated with various neural and glial cell populations. While intriguing, these results are interpreted with caution, as the temporal resolution of the data was very limited. With only two circadian phases sampled, the study makes modest observations about rhythmicity or temporal gating of cell-type functions in the brain. However, these initial observations suggest that future studies involving multiple circadian sampling could provide valuable insights into time-of-day-dependent regulation of hypothalamic cell populations.

On the other hand, analysis of insulin treatment alone yielded 14 DEGs, confirming the low-amplitude nature of insulin's transcriptional impact in this model. Importantly, none of the DEGs belonged to classical metabolic pathways tested in earlier targeted gene assays. The absence of known metabolic regulators among the insulin-responsive DEGs reinforces the conclusion that insulin's transcriptional influence on hypothalamic metabolism is either weak with low-amplitude, indirect, or could be detected via non-transcriptional routes in these in isolated and reduced model systems. Interestingly, the insulin-responsive genes showed no overlap with the temporal DEGs, indicating that their regulation was strictly associated with insulin treatment and not influenced by time-of-day. Enrichment analysis of these genes revealed functional associations with

both central and peripheral biological processes, hinting at a broader role of insulin beyond classical metabolic regulation. These findings prompted the study to explore non-metabolic roles of insulin in the hypothalamus, including potential effects on neuronal health and cognition-associated functions.

4.3 Involvement of insulin in maintaining neuronal health and protection, with a time-independent, tonic regulation

4.3.1 Brain localised insulin-responsive DEGs suggest a role in neuronal health and maintenance

The transcriptomic analysis identified 14 genes that were differentially expressed in response to insulin treatment in hypothalamic slice preparations. These DEGs were enriched for both peripheral and central functions. Enrichment of peripheral function genes, despite the hypothalamic origin of the experimental tissue, may be attributed to the interconnected nature of biological processes across different tissues. Or it may result from the systemic signals (in this case, insulin) influencing gene expression of metabolic processes in distant organs.

Among these, 4 DEGs, *Nefm*, *Trpc3*, *Opalin*, and *Shox2*, were associated with neuronal health and development. Notably, *Nefm*, *Trpc3*, and *Opalin* were confirmed to have predominant localization and expression in the brain, as evidenced by tissue-specific expression data from the Mouse ENCODE transcriptome project (Section 3.10). A particularly compelling observation was that while most insulin-influenced genes were downregulated, those associated with neuronal development and protection exhibited a subtle but significant upregulation. This observation of RNAseq data was further validated by checking their expression levels by qPCR (Section 3.11). The expression trend (specifically for *Nefm* and *Trpc3*) was confirmed with near-significant *p* values, hinting at a potential neurotrophic or neuroprotective role of insulin under the opted experimental conditions. Furthermore, these insulin-influenced expression changes showed no interaction effect with time of day, suggesting a more consistent or baseline regulation.

Insulin's role in neuronal health and cognition-associated functions is well established (De Felice & Benedict, 2015; Huang et al., 2010; Yaribeygi et al., 2023). Insulin signalling modulates the expression of multiple genes and proteins involved in neuronal protection and survival (Apostolatos et al., 2012; Duarte et al., 2008; Lee et al., 2005; Van Der Heide et al., 2006), learning and memory (De Felice & Benedict, 2015; W.-Q. Zhao et al., 2004), synaptic plasticity (Izumi et al., 2003; Van Der Heide et al., 2005) and myelination (Rachana et al., 2016). In line with insulin's influence on the transcriptomic level, three prioritized DEGs (*Nefm*, *Trpc3*, and *Opalin)* in this study can be potentially viewed as downstream effectors or indirect targets of insulin's neurotrophic or neuroprotective action. *Nefm*, encoding a critical subunit of the neuronal cytoskeleton, is essential for maintaining axonal calibre and structural integrity. Neurofilaments are generally the most abundant components of myelinated axons (Garcia et al., 2003). These proteins are

often used as biomarkers for monitoring neurological diseases (Martínez-Morillo et al., 2015; Yuan & Nixon, 2021). Although, to the best of our knowledge, strong evidence of insulin's direct influence on *Nefm* gene expression is missing, a study has pointed out the insulin-deficient conditions result in abnormal neurofilament phosphorylation and aggregation via dysregulated stress kinases, ultimately compromising neuronal structure and cognitive function (Schechter et al., 2005). *Trpc3*, encoding cation channels have prominent roles in cerebellum and hippocampal neurons regulating neuronal excitability and synaptic transmission (Hartmann et al., 2008; Neuner et al., 2015). Although again, direct transcriptional regulation by insulin remains unclear, however, insulin is known to modulate TRPC channels (particularly TRPC5) in hypothalamic POMC neurons to facilitate their anorectic activity (Qiu et al., 2018b). Lastly, *Opalin*, encoding specific myelin proteins, is critical for myelin sheath formation and maintenance (Teng et al., 2024). Insulin and insulin-like growth factor I (IGF-I) are known to promote oligodendrocyte maturation and myelin protein expression (Rachana et al., 2016; Saneto et al., 1988), suggesting that *Opalin* may be indirectly involved in this promyelinating cascade .

Taken together, these findings align with established roles of insulin in supporting cognition-associated functions, with potentially adding the identified DEGs to the list of insulin's downstream targets involved in neuronal maintenance and protection.

4.3.2 Circadian gated and tonic regulation of insulin action

The observed time-independent upregulation of genes associated with neuronal health and maintenance prompted in a direction that insulin may possibly act through both circadian-dependent and -independent mechanisms, suggesting a potential dual mode of regulation.

Insulin's role in maintaining metabolic homeostasis is closely aligned to external behavioural rhythms of feeding and physical activity which follow a predictable circadian pattern. As such, it is reasonable to presume that insulin's metabolic function in the brain are temporally gated by the circadian clock. This circadian dependency would ensure that insulin's regulatory actions governing metabolic neuropeptide expression are synchronised with organism's activity phase, when nutrient intake and energy demands are highest. In contrast, insulin's role in maintaining neuronal health, promoting synaptic plasticity and supporting cognitive functions, appears to be more constant and less directly influenced by external timing cues. These functions are fundamental to persisting brain health and must be sustained across the 24-hour cycle to preserve neuronal stability and cognitive performance. This then suggests that insulin's influence on cognition-associated functions may be driven in a circadian-independent, or tonic mode of regulation. Tonic action may ensure a steady-state background level of activity and sustain neuroprotective mechanisms regardless of circadian phase.

Such a dual mode of regulation is not unique to insulin. The most studied hormone, in this context is melatonin. For years it has been widely studied that melatonin regulates sleep

cycles through circadian mechanisms (Dijk & Cajochen, 1997; Lavie, 1997). Circulating melatonin levels exhibit a pronounced daily rhythm, aligning peripheral clocks to the day–night schedule, thereby, maintaining the body's internal clock (Pevet & Challet, 2011). Along with its circadian role, it also exerts tonic antioxidant and immune effects. It scavenges free radicals and reduces oxidative stress in neurons, contributing to cellular maintenance and longevity (Malhotra et al., 2004; Reiter, 1995; Reiter et al., 2000). Another example is cortisol, which displays expression surge before onset of the active period, preparing the organism for wakefulness and activity (Contreras & Gutierrez-Garcia, 2018; Spencer et al., 2018). In addition, also maintaining a basal level that regulate metabolism and anti-inflammatory activity (reviewed in detail in Uchoa et al., 2014)).

In this framework, to reiterate, while insulin's metabolic actions may be acutely aligned with circadian cycles, its cognitive-related functions appear to operate in a continuum and consistent manner. These observations, thus, suggest the possibility of insulin exerting a dual mode of regulation, though this study requires additional, in-depth research for making stronger claims.

4.4 Temporal complexities of brain insulin action warrants further research

4.4.1 Reflection on Hypotheses

This study first aimed to test the hypothesis that insulin's metabolic effects in the hypothalamus are modulated by circadian phase, that is, whether central insulin sensitivity exhibits circadian gating. Both neuronal cultures and Arc-ME tissue slices demonstrated robust circadian rhythmicity, validated through clock gene reporter oscillations. These findings confirmed that the experimental systems retained endogenous timekeeping and were therefore suitable for assessing phase-dependent hormonal responses. However, the critical limitation emerged from the lack of detectable metabolic gene regulation in response to insulin, across both models and all tested conditions. Without a measurable insulin effect, there was no biological signal upon which assessment of circadian variation could be made. The outcome of this investigation thus represents a non-conclusive result with respect to Hypothesis 1. It is important to emphasize that these findings do not refute the hypothesis, rather, they put forward the difficulty of testing it under simplified experimental conditions (Figure 31).

In the second part, this study hypothesizes that insulin influences cognition-associated functions in the brain independent of circadian timing, consistent with a sustained, homeostatic role in maintaining neuronal health. The findings of this study support this hypothesis with modest observations. It demonstrated that insulin could modulate the expression of genes involved in neuronal health and maintenance (*Nefm*, *Trpc3*, and *Opalin),* with no time-of-day effect, suggesting a circadian-independent regulation. While the effects observed were sparse and satisfactory, they provide a foundation for further exploration into insulin's neuronal health and cognitive-related functions (Figure 31).

Taken together, although the circadian-gated regulation of insulin mediated metabolic homeostasis is not validated in this study, the hypothesis itself is not refuted and remains

scientifically applicable. In addition, modest modulation of genes associated with neuronal maintenance under apparent tonic conditions, suggests the possibility that insulin action in the brain may be governed by a dual mode of temporal regulation.

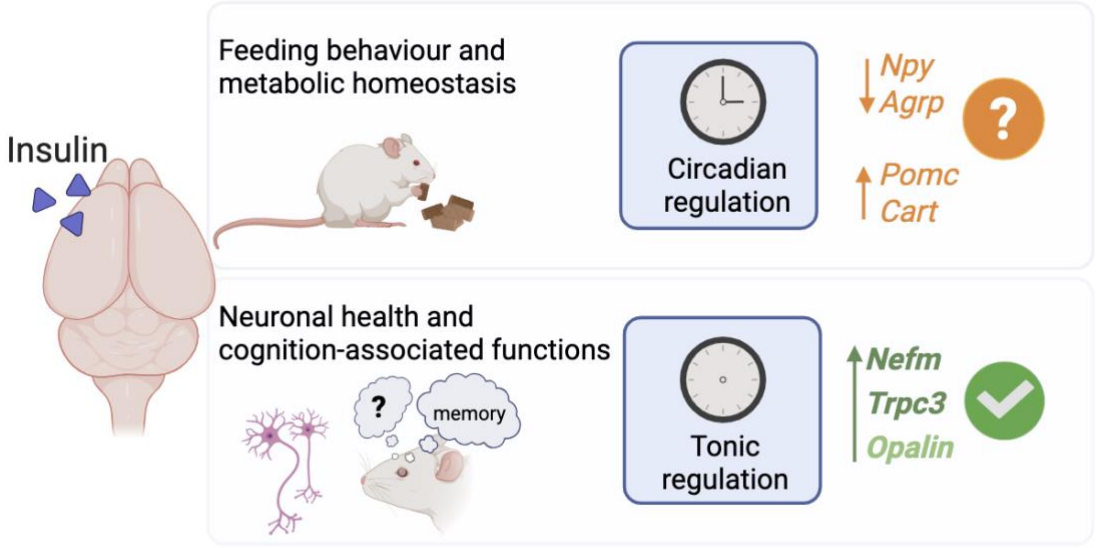


Figure 31. Summary of experimental outcomes in relation to the proposed hypothesis.
Insulin plays a vital role in the brain influencing feeding behaviour and cognition-associated functions. The hypothesis of the project is that insulin exerts effects on the metabolic genes in a circadian-regulated manner while it influences the genes involved in neuronal health and cognitive processes in a circadian-independent, tonic regulation. Created with Biorender.

4.4.2 Acknowledging limitations of the study

While the identification of insulin-responsive DEGs associated with neuronal health, *Nefm*, *Trpc3*, and *Opalin*, offers intriguing insight into potential tonic functions of insulin in the brain, several limitations must be acknowledged to contextualize these finding.

A relatively small sample size opted for each experimental set up definitely affects the significance of the findings. This study relies on reduced and isolated *in vitro* cell cultures and *ex vivo* hypothalamic tissue preparations. These simplified models, while useful for mechanistic exploration, do not fully capture the complexity of the *in vivo* brain environment.

Importantly, the observed gene expression changes in the transcriptomic analysis were modest in number and magnitude. While statistically significant, and additionally validated with qPCR analysis, the subtle upregulation of these neuronal genes warrants cautious interpretation, as small fold changes may or may not translate into meaningful functional outcomes. Moreover, this study is inherently exploratory (for transcriptomic part) and correlative in nature. Although associations between insulin treatment and gene expression were observed, it does not confirm the causative relationship without further functional validation.

Looking ahead, future research should aim to address these limitations to better characterize the physiological relevance of these transcriptional changes. In-depth

follow-up studies would strengthen the conclusions drawn here and would be fundamental to clarify insulin's role in the central regulation of metabolic homeostasis and neuronal health.

4.5 Conclusion and outlook

In this PhD project, I aimed to investigate the potential modulation of hypothalamic insulin action by circadian gating, a concept suggesting that the brain's responsiveness to physiological cues varies depending on the time of day. Focusing on insulin's well studied central roles in metabolic regulation and cognition-associated functions, this project sought to determine if these actions exhibit circadian regulation.

Using immortalised hypothalamic neuronal cultures and tissue preparations, the study first successfully confirmed the preservation of robust circadian rhythmicity in both models. This was crucial to validate their suitability for studying time-dependent modulation of insulin action. While targeted gene expression analyses revealed minimal transcriptional response to insulin in classical metabolic neuropeptides, the observation was consistent across the tested circadian phases and experimental conditions. The preserved IR expression and circadian clock function in both systems suggested that the lack of metabolic response was more likely due to biological complexity than technical limitations, which further prompted for an unbiased, transcriptome-wide profiling.

Transcriptomic analyses did offer valuable insights. Insulin-time interaction showed no effect and interestingly, time-of-day alone had a stronger influence on gene expression than insulin treatment. Analysis of insulin treatment alone yielded a modest set of fourteen DEGs. Notably, three genes, *Nefm, Trpc3* and *Opalin* exhibited brain-predominant expression and were associated with neuronal development, structural maintenance and myelination. These genes were subtly but consistently upregulated upon insulin exposure and were further validated by qPCR. Moreover, their differential expression appeared independent of timing, suggesting that insulin may act through circadian-independent mechanisms.

Insulin's central role in metabolic homeostasis is closely aligned with circadian rhythms of activity and feeding behaviour, suggesting a plausible circadian-gating of its metabolic functions. In contrast, insulin's influence in maintaining neuronal health and cognition-associated functions demands to be operated in a stable and consistent manner, hinting at a circadian-independent, tonic regulation. Although not conclusively demonstrated, the former remains scientifically applicable, and the latter aligns with the findings of this study offering promising future direction. Taken together this study raises the possibility of a dual mode of temporal regulation for insulin action in the brain.

The study's exploratory and corelative nature necessitates cautious interpretations. However, the identification of brain-expressed, insulin-responsive genes related to neuronal health and maintenance adds a meaningful layer to our understanding of central insulin actions. From a future research perspective, the subtle yet consistent

upregulation of *Nefm, Trpc3* and *Opalin,* invites targeted *in vivo* validation. Further studies could employ insulin administration via ICV infusion to ensure direct central delivery, or intranasal routes to explore translational feasibility. Using mouse models with gene knockdowns or reporter constructs for these targets, researchers could investigate whether insulin-induced transcriptional changes translate into measurable functional outcomes, such as alterations in neurofilament density, myelin protein expression, or susceptibility to axonal damage. Alternatively, future work could assess whether insulin's effect on these genes are mediated through canonical signalling pathways such as PI3K/Akt or MAPK/ERK, using pathway inhibitors or phosphoproteomic profiling. *Nefm, Trpc3* and *Opalin* have been previously implicated in neurodegenerative conditions and myelin-related disorders (Kittur et al., 1994; Selvaraj et al., 2010; Tang et al., 2022; Yuan & Nixon, 2021). In parallel, prior studies support insulin's broader role in neuroprotection, synaptic plasticity and myelination (Apostolatos et al., 2012; Rachana et al., 2016; Van Der Heide et al., 2005). These studies, in conjunction with observations of this thesis, offer a rational foundation for further mechanistic exploration. Elucidating whether insulin's transcriptional effects lead to functional neurobiological outcomes could provide valuable understanding into pathophysiology of insulin-associated neurodegenerative conditions or metabolic-cognitive comorbidities.

References

Abbott, M.-A., Wells, D. G., & Fallon, J. R. (1999). The Insulin Receptor Tyrosine Kinase Substrate p58/53 and the Insulin Receptor Are Components of CNS Synapses. *The Journal of Neuroscience*, *19*(17), 7300–7308. https://doi.org/10.1523/JNEUROSCI.19-17-07300.1999

Abe, M., Herzog, E. D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., & Block, G. D. (2002a). Circadian Rhythms in Isolated Brain Regions. *The Journal of Neuroscience*, *22*(1), 350–356. https://doi.org/10.1523/JNEUROSCI.22-01-00350.2002

Abe, M., Herzog, E. D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., & Block, G. D. (2002b). Circadian Rhythms in Isolated Brain Regions. *The Journal of Neuroscience*, *22*(1), 350–356. https://doi.org/10.1523/JNEUROSCI.22-01-00350.2002

Abel, J. J. (1926). Crystalline Insulin. *Proceedings of the National Academy of Sciences of the United States of America*, *12*(2), 132–136. https://doi.org/10.1073/pnas.12.2.132

Aguilar-Roblero, R., Verduzco-Carbajal, L., Rodríguez, C., Mendez-Franco, J., Morán, J., & De La Mora, M. P. (1993). Circadian rhythmicity in the GABAergic system in the suprachiasmatic nuclei of the rat. *Neuroscience Letters*, *157*(2), 199–202. https://doi.org/10.1016/0304-3940(93)90736-5

Ali, M., Reutrakul, S., Petersen, G., & Knutson, K. L. (2023). Associations between Timing and Duration of Eating and Glucose Metabolism: A Nationally Representative Study in the U.S. *Nutrients*, *15*(3), 729. https://doi.org/10.3390/nu15030729

Allison, K. C., Hopkins, C. M., Ruggieri, M., Spaeth, A. M., Ahima, R. S., Zhang, Z., Taylor, D. M., & Goel, N. (2021). Prolonged, Controlled Daytime versus Delayed Eating Impacts Weight and Metabolism. *Current Biology*, *31*(3), 650-657.e3. https://doi.org/10.1016/j.cub.2020.10.092

Amir, S., Harbour, V. L., & Robinson, B. (2006). Pinealectomy does not affect diurnal PER2 expression in the rat limbic forebrain. *Neuroscience Letters*, *399*(1–2), 147–150. https://doi.org/10.1016/j.neulet.2006.01.041

Anand, B. K., & Brobeck, J. R. (1951). Hypothalamic control of food intake in rats and cats. *The Yale Journal of Biology and Medicine*, *24*(2), 123–140.

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics*, *31*(2), 166–169. https://doi.org/10.1093/bioinformatics/btu638

Ando, H., Takamura, T., Matsuzawa-Nagata, N., Shima, K. R., Eto, T., Misu, H., Shiramoto, M., Tsuru, T., Irie, S., Fujimura, A., & Kaneko, S. (2009). Clock gene expression in peripheral leucocytes of patients with type 2 diabetes. *Diabetologia*, *52*(2), 329–335. https://doi.org/10.1007/s00125-008-1194-6

Apostolatos, A., Song, S., Acosta, S., Peart, M., Watson, J. E., Bickford, P., Cooper, D. R., & Patel, N. A. (2012). Insulin Promotes Neuronal Survival via the Alternatively Spliced Protein Kinase CδII Isoform. *Journal of Biological Chemistry*, *287*(12), 9299–9310. https://doi.org/10.1074/jbc.m111.313080

Aras, E., Ramadori, G., Kinouchi, K., Liu, Y., Ioris, R. M., Brenachot, X., Ljubicic, S., Veyrat-Durebex, C., Mannucci, S., Galié, M., Baldi, P., Sassone-Corsi, P., & Coppari, R. (2019). Light Entrains Diurnal Changes in Insulin Sensitivity of Skeletal Muscle via Ventromedial Hypothalamic Neurons. *Cell Reports*, *27*(8), 2385-2398.e3. https://doi.org/10.1016/j.celrep.2019.04.093

Aryal, R. P., Kwak, P. B., Tamayo, A. G., Gebert, M., Chiu, P.-L., Walz, T., & Weitz, C. J. (2017). Macromolecular Assemblies of the Mammalian Circadian Clock. *Molecular Cell*, *67*(5), 770-782.e6. https://doi.org/10.1016/j.molcel.2017.07.017

Aschoff, J. (1965). Circadian Rhythms in Man: A self-sustained oscillator with an inherent frequency underlies human 24-hour periodicity. *Science*, *148*(3676), 1427–1432. https://doi.org/10.1126/science.148.3676.1427

Aston-Jones, G., Chen, S., Zhu, Y., & Oshinsky, M. L. (2001). A neural circuit for circadian regulation of arousal. *Nature Neuroscience*, *4*(7), 732–738. https://doi.org/10.1038/89522

Bach, S. J., & Holmes, E. G. (1937). The effect of insulin on carbohydrate formation in the liver. *Biochemical Journal*, *31*(1), 89–100. https://doi.org/10.1042/bj0310089

Bahinipati, J., Sarangi, R., Pathak, M., & Mohapatra, S. (2022). Effect of night shift on development of metabolic syndrome among health care workers. *Journal of Family Medicine and Primary Care*, *11*(5), 1710–1715. https://doi.org/10.4103/jfmpc.jfmpc_375_21

Balsalobre, A., Brown, S. A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H. M., Schütz, G., & Schibler, U. (2000). Resetting of Circadian Time in Peripheral Tissues by Glucocorticoid Signaling. *Science*, *289*(5488), 2344–2347. https://doi.org/10.1126/science.289.5488.2344

Bano-Otalora, B., Martial, F., Harding, C., Bechtold, D. A., Allen, A. E., Brown, T. M., Belle, M. D. C., & Lucas, R. J. (2021). Bright daytime light enhances circadian amplitude in a diurnal mammal. *Proceedings of the National Academy of Sciences*, *118*(22), e2100094118. https://doi.org/10.1073/pnas.2100094118

Baño-Otálora, B., & Piggins, H. D. (2017). Contributions of the lateral habenula to circadian timekeeping. *Pharmacology Biochemistry and Behavior*, *162*, 46–54. https://doi.org/10.1016/j.pbb.2017.06.007

Banting, F. G., & Best, C. H. (2007). The internal secretion of the pancreas. 1922. *The Indian Journal of Medical Research*, *125*(3), 251–266.

Banting, F. G., Best, C. H., Collip, J. B., Campbell, W. R., & Fletcher, A. A. (1991). Pancreatic extracts in the treatment of diabetes mellitus: Preliminary report. 1922. *CMAJ: Canadian Medical Association Journal = Journal de l'Association Medicale Canadienne*, *145*(10), 1281–1286.

Barclay, J. L., Husse, J., Bode, B., Naujokat, N., Meyer-Kovac, J., Schmid, S. M., Lehnert, H., & Oster, H. (2012). Circadian Desynchrony Promotes Metabolic Disruption in a Mouse Model of Shiftwork. *PLoS ONE*, *7*(5), e37150. https://doi.org/10.1371/journal.pone.0037150

Barnea, M., Chapnik, N., Genzer, Y., & Froy, O. (2015). The circadian clock machinery controls adiponectin expression. *Molecular and Cellular Endocrinology*, *399*, 284–287. https://doi.org/10.1016/j.mce.2014.10.018

Bassil, F., Canron, M.-H., Vital, A., Bezard, E., Li, Y., Greig, N. H., Gulyani, S., Kapogiannis, D., Fernagut, P.-O., & Meissner, W. G. (2017). Insulin resistance and exendin-4 treatment for multiple system atrophy. *Brain*, *140*(5), 1420–1436. https://doi.org/10.1093/brain/awx044

Baura, G. D., Foster, D. M., Porte, D., Kahn, S. E., Bergman, R. N., Cobelli, C., & Schwartz, M. W. (1993). Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. *Journal of Clinical Investigation*, *92*(4), 1824–1830. https://doi.org/10.1172/JCI116773

Bazotte, R. B., Silva, L. G., & Schiavon, F. P. (2014). Insulin resistance in the liver: Deficiency or excess of insulin? *Cell Cycle*, *13*(16), 2494–2500. https://doi.org/10.4161/15384101.2014.947750

Beattie, E. C., Carroll, R. C., Yu, X., Morishita, W., Yasuda, H., Von Zastrow, M., & Malenka, R. C. (2000). Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. *Nature Neuroscience*, *3*(12), 1291–1300. https://doi.org/10.1038/81823

Begemann, K., Heyde, I., Witt, P., Inderhees, J., Leinweber, B., Koch, C. E., Jöhren, O., Oelkrug, R., Liskiewicz, A., Müller, T. D., & Oster, H. (2023). Rest phase snacking increases energy resorption

and weight gain in male mice. *Molecular Metabolism*, *69*, 101691. https://doi.org/10.1016/j.molmet.2023.101691

Begriche, K., Sutton, G. M., Fang, J., & Butler, A. A. (2009). The role of melanocortin neuronal pathways in circadian biology: A new homeostatic output involving melanocortin-3 receptors? *Obesity Reviews*, *10*(s2), 14–24. https://doi.org/10.1111/j.1467-789X.2009.00662.x

Belsham, D. D., Cai, F., Cui, H., Smukler, S. R., Salapatek, A. M. F., & Shkreta, L. (2004). Generation of a Phenotypic Array of Hypothalamic Neuronal Cell Models to Study Complex Neuroendocrine Disorders. *Endocrinology*, *145*(1), 393–400. https://doi.org/10.1210/en.2003-0946

Benedict, C. (2004). Intranasal insulin improves memory in humans. *Psychoneuroendocrinology*, *29*(10), 1326–1334. https://doi.org/10.1016/j.psyneuen.2004.04.003

Berridge, M. J. (2014). Module 10: Neuronal Signalling. *Cell Signalling Biology*, *6*, csb0001010. https://doi.org/10.1042/csb0001010

Blackard, W. G., Barlascini, C. O., Clore, J. N., & Nestler, J. E. (1989). Morning Insulin Requirements: Critique of Dawn and Meal Phenomena. *Diabetes*, *38*(3), 273–277. https://doi.org/10.2337/diab.38.3.273

Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., & Mercola, D. A. (1972). Three-Dimensional Atomic Structure of Insulin and Its Relationship to Activity. *Diabetes*, *21*(Supplement_2), 492–505. https://doi.org/10.2337/diab.21.2.S492

Boden, G., Ruiz, J., Urbain, J. L., & Chen, X. (1996). Evidence for a circadian rhythm of insulin secretion. *American Journal of Physiology-Endocrinology and Metabolism*, *271*(2), E246–E252. https://doi.org/10.1152/ajpendo.1996.271.2.E246

Boden, G., & Shulman, G. I. (2002). Free fatty acids in obesity and type 2 diabetes: Defining their role in the development of insulin resistance and β-cell dysfunction. *European Journal of Clinical Investigation*, *32*(s3), 14–23. https://doi.org/10.1046/j.1365-2362.32.s3.3.x

Bolli, G. B., Feo, P. D., Cosmo, S. D., Perriello, G., Ventura, M. M., Calcinaro, F., Lolli, C., Campbell, P., Brunetti, P., & Gerich, J. E. (1984). Demonstration of a Dawn Phenomenon in Normal Human Volunteers. *Diabetes*, *33*(12), 1150–1153. https://doi.org/10.2337/diab.33.12.1150

Borck, P. C., Rickli, S., Vettorazzi, J. F., Batista, T. M., Boschero, A. C., Vieira, E., & Carneiro, E. M. (2022). Effect of nighttime light exposure on glucose metabolism in protein-restricted mice. *Journal of Endocrinology*, *252*(2), 143–154. https://doi.org/10.1530/JOE-21-0133

Brouwer, A., Van Raalte, D. H., Rutters, F., Elders, P. J. M., Snoek, F. J., Beekman, A. T. F., & Bremmer, M. A. (2020). Sleep and HbA1c in Patients With Type 2 Diabetes: Which Sleep Characteristics Matter Most? *Diabetes Care*, *43*(1), 235–243. https://doi.org/10.2337/dc19-0550

Brown, S. A., Fleury-Olela, F., Nagoshi, E., Hauser, C., Juge, C., Meier, C. A., Chicheportiche, R., Dayer, J.-M., Albrecht, U., & Schibler, U. (2005). The Period Length of Fibroblast Circadian Gene Expression Varies Widely among Human Individuals. *PLoS Biology*, *3*(10), e338. https://doi.org/10.1371/journal.pbio.0030338

Bruce, V. G. (1972). MUTANTS OF THE BIOLOGICAL CLOCK IN *CHLAMYDOMONAS REINHARDI*. *Genetics*, *70*(4), 537–548. https://doi.org/10.1093/genetics/70.4.537

Brüning, J. C., Gautam, D., Burks, D. J., Gillette, J., Schubert, M., Orban, P. C., Klein, R., Krone, W., Müller-Wieland, D., & Kahn, C. R. (2000). Role of Brain Insulin Receptor in Control of Body Weight and Reproduction. *Science*, *289*(5487), 2122–2125. https://doi.org/10.1126/science.289.5487.2122

Buhr, E. D., & Takahashi, J. S. (2013). Molecular Components of the Mammalian Circadian Clock. In A. Kramer & M. Merrow (Eds.), *Circadian Clocks* (Vol. 217, pp. 3–27). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-25950-0_1

Buhr, E. D., Yoo, S.-H., & Takahashi, J. S. (2010). Temperature as a Universal Resetting Cue for Mammalian Circadian Oscillators. *Science*, *330*(6002), 379–385. https://doi.org/10.1126/science.1195262

Buijs, F. N., Guzmán-Ruiz, M., León-Mercado, L., Basualdo, M. C., Escobar, C., Kalsbeek, A., & Buijs, R. M. (2017). Suprachiasmatic Nucleus Interaction with the Arcuate Nucleus; Essential for Organizing Physiological Rhythms. *Eneuro*, *4*(2), ENEURO.0028-17.2017. https://doi.org/10.1523/ENEURO.0028-17.2017

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., & Bradfield, C. A. (2000). Mop3 Is an Essential Component of the Master Circadian Pacemaker in Mammals. *Cell*, *103*(7), 1009–1017. https://doi.org/10.1016/S0092-8674(00)00205-1

Cai, W., Xue, C., Sakaguchi, M., Konishi, M., Shirazian, A., Ferris, H. A., Li, M. E., Yu, R., Kleinridders, A., Pothos, E. N., & Kahn, C. R. (2018). Insulin regulates astrocyte gliotransmission and modulates behavior. *Journal of Clinical Investigation*, *128*(7), 2914–2926. https://doi.org/10.1172/JCI99366

Cai, W., Zhang, X., Batista, T. M., García-Martín, R., Softic, S., Wang, G., Ramirez, A. K., Konishi, M., O'Neill, B. T., Kim, J. H., Kim, J. K., & Kahn, C. R. (2021). Peripheral Insulin Regulates a Broad Network of Gene Expression in Hypothalamus, Hippocampus, and Nucleus Accumbens. *Diabetes*, *70*(8), 1857–1873. https://doi.org/10.2337/db20-1119

Camacho, F., Cilio, M., Guo, Y., Virshup, D. M., Patel, K., Khorkova, O., Styren, S., Morse, B., Yao, Z., & Keesler, G. A. (2001). Human casein kinase Iδ phosphorylation of human circadian clock proteins period 1 and 2. *FEBS Letters*, *489*(2–3), 159–165. https://doi.org/10.1016/S0014-5793(00)02434-0

Campbell, J. N., Macosko, E. Z., Fenselau, H., Pers, T. H., Lyubetskaya, A., Tenen, D., Goldman, M., Verstegen, A. M. J., Resch, J. M., McCarroll, S. A., Rosen, E. D., Lowell, B. B., & Tsai, L. T. (2017). A molecular census of arcuate hypothalamus and median eminence cell types. *Nature Neuroscience*, *20*(3), 484–496. https://doi.org/10.1038/nn.4495

Cao, R., Robinson, B., Xu, H., Gkogkas, C., Khoutorsky, A., Alain, T., Yanagiya, A., Nevarko, T., Liu, A. C., Amir, S., & Sonenberg, N. (2013). Translational Control of Entrainment and Synchrony of the Suprachiasmatic Circadian Clock by mTOR/4E-BP1 Signaling. *Neuron*, *79*(4), 712–724. https://doi.org/10.1016/j.neuron.2013.06.026

Carrasco-Benso, M. P., Rivero-Gutierrez, B., Lopez-Minguez, J., Anzola, A., Diez-Noguera, A., Madrid, J. A., Lujan, J. A., Martínez-Augustin, O., J. L. Scheer, F. A., & Garaulet, M. (2016). Human adipose tissue expresses intrinsic circadian rhythm in insulin sensitivity. *The FASEB Journal*, *30*(9), 3117–3123. https://doi.org/10.1096/fj.201600269RR

Carvalheira, J. B. C., Ribeiro, E. B., Ara□jo, E. P., Guimar□es, R. B., Telles, M. M., Torsoni, M., Gontijo, J. A. R., Velloso, L. A., & Saad, M. J. A. (2003). Selective impairment of insulin signalling in the hypothalamus of obese Zucker rats. *Diabetologia*, *46*(12), 1629–1640. https://doi.org/10.1007/s00125-003-1246-x

Centofanti, S., Heilbronn, L. K., Wittert, G., Dorrian, J., Coates, A. M., Kennaway, D., Gupta, C., Stepien, J. M., Catcheside, P., Yates, C., Grosser, L., Matthews, R. W., & Banks, S. (2025). Fasting as an intervention to alter the impact of simulated night-shift work on glucose metabolism in healthy adults: A cluster randomised controlled trial. *Diabetologia*, *68*(1), 203–216. https://doi.org/10.1007/s00125-024-06279-1

Chan, S. J., & Steiner, D. F. (1977). Preproinsulin, a new precursor in insulin biosynthesis. *Trends in Biochemical Sciences*, *2*(11), 254–256. https://doi.org/10.1016/0968-0004(77)90217-1

Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G. V., Clark, N. R., & Ma'ayan, A. (2013). Enrichr: Interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics*, *14*, 128. https://doi.org/10.1186/1471-2105-14-128

Chen, N., Sugihara, H., Kim, J., Fu, Z., Barak, B., Sur, M., Feng, G., & Han, W. (2016). Direct modulation of GFAP-expressing glia in the arcuate nucleus bi-directionally regulates feeding. *eLife*, *5*, e18716. https://doi.org/10.7554/eLife.18716

Chen, Y., Essner, R. A., Kosar, S., Miller, O. H., Lin, Y.-C., Mesgarzadeh, S., & Knight, Z. A. (2019). Sustained NPY signaling enables AgRP neurons to drive feeding. *eLife*, *8*, e46348. https://doi.org/10.7554/eLife.46348

Chen, Y., Lin, Y.-C., Kuo, T.-W., & Knight, Z. A. (2015). Sensory Detection of Food Rapidly Modulates Arcuate Feeding Circuits. *Cell*, *160*(5), 829–841. https://doi.org/10.1016/j.cell.2015.01.033

Chen, Y., Zhao, Y., Dai, C., Liang, Z., Run, X., Iqbal, K., Liu, F., & Gong, C.-X. (2014). Intranasal insulin restores insulin signaling, increases synaptic proteins, and reduces Aβ level and microglia activation in the brains of 3xTg-AD mice. *Experimental Neurology*, *261*, 610–619. https://doi.org/10.1016/j.expneurol.2014.06.004

Chew, G. T., Watts, G. F., Davis, T. M. E., Stuckey, B. G. A., Beilin, L. J., Thompson, P. L., Burke, V., & Currie, P. J. (2008). Hemodynamic Effects of Fenofibrate and Coenzyme Q10 in Type 2 Diabetic Subjects With Left Ventricular Diastolic Dysfunction. *Diabetes Care*, *31*(8), 1502–1509. https://doi.org/10.2337/dc08-0118

Cho, H., Zhao, X., Hatori, M., Yu, R. T., Barish, G. D., Lam, M. T., Chong, L.-W., DiTacchio, L., Atkins, A. R., Glass, C. K., Liddle, C., Auwerx, J., Downes, M., Panda, S., & Evans, R. M. (2012). Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β. *Nature*, *485*(7396), 123–127. https://doi.org/10.1038/nature11048

Choi, J., Ko, J., Racz, B., Burette, A., Lee, J.-R., Kim, S., Na, M., Lee, H. W., Kim, K., Weinberg, R. J., & Kim, E. (2005). Regulation of Dendritic Spine Morphogenesis by Insulin Receptor Substrate 53, a Downstream Effector of Rac1 and Cdc42 Small GTPases. *The Journal of Neuroscience*, *25*(4), 869–879. https://doi.org/10.1523/JNEUROSCI.3212-04.2005

Choi, S. J., Kim, F., Schwartz, M. W., & Wisse, B. E. (2010). Cultured hypothalamic neurons are resistant to inflammation and insulin resistance induced by saturated fatty acids. *American Journal of Physiology. Endocrinology and Metabolism*, *298*(6), E1122-1130. https://doi.org/10.1152/ajpendo.00006.2010

Choudhury, A. I., Heffron, H., Smith, M. A., Al-Qassab, H., Xu, A. W., Selman, C., Simmgen, M., Clements, M., Claret, M., MacColl, G., Bedford, D. C., Hisadome, K., Diakonov, I., Moosajee, V., Bell, J. D., Speakman, J. R., Batterham, R. L., Barsh, G. S., Ashford, M. L. J., & Withers, D. J. (2005). The role of insulin receptor substrate 2 in hypothalamic and β cell function. *Journal of Clinical Investigation*, *115*(4), 940–950. https://doi.org/10.1172/JCI24445

Chrétien, C., Fenech, C., Liénard, F., Grall, S., Chevalier, C., Chaudy, S., Brenachot, X., Berges, R., Louche, K., Stark, R., Nédélec, E., Laderrière, A., Andrews, Z. B., Benani, A., Flockerzi, V., Gascuel, J., Hartmann, J., Moro, C., Birnbaumer, L., … Fioramonti, X. (2017). Transient Receptor Potential Canonical 3 (TRPC3) Channels Are Required for Hypothalamic Glucose Detection and Energy Homeostasis. *Diabetes*, *66*(2), 314–324. https://doi.org/10.2337/db16-1114

Chun, L. E., Woodruff, E. R., Morton, S., Hinds, L. R., & Spencer, R. L. (2015). Variations in Phase and Amplitude of Rhythmic Clock Gene Expression across Prefrontal Cortex, Hippocampus, Amygdala, and Hypothalamic Paraventricular and Suprachiasmatic Nuclei of Male and Female Rats. *Journal of Biological Rhythms*, *30*(5), 417–436. https://doi.org/10.1177/0748730415598608

Ciarleglio, C. M., Resuehr, H. E. S., & McMahon, D. G. (2011). Interactions of the serotonin and circadian systems: Nature and nurture in rhythms and blues. *Neuroscience*, *197*, 8–16. https://doi.org/10.1016/j.neuroscience.2011.09.036

Cignarelli, A., Genchi, V. A., Perrini, S., Natalicchio, A., Laviola, L., & Giorgino, F. (2019). Insulin and Insulin Receptors in Adipose Tissue Development. *International Journal of Molecular Sciences*, *20*(3), 759. https://doi.org/10.3390/ijms20030759

Cipolla-Neto, J., Amaral, F. G., Afeche, S. C., Tan, D. X., & Reiter, R. J. (2014). Melatonin, energy metabolism, and obesity: A review. *Journal of Pineal Research*, *56*(4), 371–381. https://doi.org/10.1111/jpi.12137

Claus, T., & Pilkis, S. (1976). Regulation by insulin of gluconeogenesis in isolated rat hepatocytes. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *421*(2), 246–262. https://doi.org/10.1016/0304-4165(76)90291-9

Cole, B. A., & Becker, E. B. E. (2023). Modulation and Regulation of Canonical Transient Receptor Potential 3 (TRPC3) Channels. *Cells*, *12*(18), 2215. https://doi.org/10.3390/cells12182215

Colonna, M., & Butovsky, O. (2017). Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annual Review of Immunology*, *35*(1), 441–468. https://doi.org/10.1146/annurev-immunol-051116-052358

Contreras, C. M., & Gutierrez-Garcia, A. G. (2018). Cortisol Awakening Response: An Ancient Adaptive Feature. *Journal of Psychiatry and Psychiatric Disorders*, *02*(01), 29–40. https://doi.org/10.26502/jppd.2572-519x0038

Copinschi, G., Van Reeth, O., & Van Cauter, E. (1999). [Biologic rhythms. Circadian, ultradian and seasonal rhythms]. *Presse Medicale (Paris, France: 1983)*, *28*(17), 933–935.

Coutinho, M., Gerstein, H. C., Wang, Y., & Yusuf, S. (1999). The relationship between glucose and incident cardiovascular events. A metaregression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years. *Diabetes Care*, *22*(2), 233–240. https://doi.org/10.2337/diacare.22.2.233

Craft, S., Asthana, S., Newcomer, J. W., Wilkinson, C. W., Matos, I. T., Baker, L. D., Cherrier, M., Lofgreen, C., Latendresse, S., Petrova, A., Plymate, S., Raskind, M., Grimwood, K., & Veith, R. C. (1999). Enhancement of Memory in Alzheimer Disease With Insulin and Somatostatin, but Not Glucose. *Archives of General Psychiatry*, *56*(12), 1135. https://doi.org/10.1001/archpsyc.56.12.1135

Crosby, P., Hamnett, R., Putker, M., Hoyle, N. P., Reed, M., Karam, C. J., Maywood, E. S., Stangherlin, A., Chesham, J. E., Hayter, E. A., Rosenbrier-Ribeiro, L., Newham, P., Clevers, H., Bechtold, D. A., & O'Neill, J. S. (2019). Insulin/IGF-1 Drives PERIOD Synthesis to Entrain Circadian Rhythms with Feeding Time. *Cell*, *177*(4), 896-909.e20. https://doi.org/10.1016/j.cell.2019.02.017

Cuesta, M., Cermakian, N., & Boivin, D. B. (2015). Glucocorticoids entrain molecular clock components in human peripheral cells. *The FASEB Journal*, *29*(4), 1360–1370. https://doi.org/10.1096/fj.14-265686

Cummings, D. E., & Overduin, J. (2007). Gastrointestinal regulation of food intake. *Journal of Clinical Investigation*, *117*(1), 13–23. https://doi.org/10.1172/JCI30227

Dalvi, P. S., Nazarians-Armavil, A., Tung, S., & Belsham, D. D. (2011a). Immortalized neurons for the study of hypothalamic function. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *300*(5), R1030–R1052. https://doi.org/10.1152/ajpregu.00649.2010

Dalvi, P. S., Nazarians-Armavil, A., Tung, S., & Belsham, D. D. (2011b). Immortalized neurons for the study of hypothalamic function. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *300*(5), R1030–R1052. https://doi.org/10.1152/ajpregu.00649.2010

Dang, F., Sun, X., Ma, X., Wu, R., Zhang, D., Chen, Y., Xu, Q., Wu, Y., & Liu, Y. (2016). Insulin post-transcriptionally modulates Bmal1 protein to affect the hepatic circadian clock. *Nature Communications*, *7*(1), 12696. https://doi.org/10.1038/ncomms12696

Davis, B., & Lazarus, N. R. (1976). An in Vitro system for studying insulin release caused by secretory granules-plasma membrane interaction: Definition of the system. *The Journal of Physiology*, *256*(3), 709–729. https://doi.org/10.1113/jphysiol.1976.sp011347

De Assis, L. V. M., Harder, L., Lacerda, J. T., Parsons, R., Kaehler, M., Cascorbi, I., Nagel, I., Rawashdeh, O., Mittag, J., & Oster, H. (2024). Tuning of liver circadian transcriptome rhythms by thyroid hormone state in male mice. *Scientific Reports*, *14*(1), 640. https://doi.org/10.1038/s41598-023-50374-z

De Felice, F. G., & Benedict, C. (2015). A Key Role of Insulin Receptors in Memory. *Diabetes*, *64*(11), 3653–3655. https://doi.org/10.2337/dbi15-0011

De Goede, P., Foppen, E., Ritsema, W. I. G. R., Korpel, N. L., Yi, C.-X., & Kalsbeek, A. (2019). Time-Restricted Feeding Improves Glucose Tolerance in Rats, but Only When in Line With the Circadian Timing System. *Frontiers in Endocrinology*, *10*, 554. https://doi.org/10.3389/fendo.2019.00554

de Goede, P., Sen, S., Oosterman, J. E., Foppen, E., Jansen, R., la Fleur, S. E., Challet, E., & Kalsbeek, A. (2018). Differential effects of diet composition and timing of feeding behavior on rat brown adipose tissue and skeletal muscle peripheral clocks. *Neurobiology of Sleep and Circadian Rhythms*, *4*, 24–33. https://doi.org/10.1016/j.nbscr.2017.09.002

De Solis, A. J., Del Río-Martín, A., Radermacher, J., Chen, W., Steuernagel, L., Bauder, C. A., Eggersmann, F. R., Morgan, D. A., Cremer, A.-L., Sué, M., Germer, M., Kukat, C., Vollmar, S., Backes, H., Rahmouni, K., Kloppenburg, P., & Brüning, J. C. (2024). Reciprocal activity of AgRP and POMC neurons governs coordinated control of feeding and metabolism. *Nature Metabolism*, *6*(3), 473–493. https://doi.org/10.1038/s42255-024-00987-z

Deane, R., Sagare, A., & Zlokovic, B. (2008). The Role of the Cell Surface LRP and Soluble LRP in Blood-Brain Barrier Aβ Clearance in Alzheimers Disease. *Current Pharmaceutical Design*, *14*(16), 1601–1605. https://doi.org/10.2174/138161208784705487

Deane, R., Wu, Z., Sagare, A., Davis, J., Du Yan, S., Hamm, K., Xu, F., Parisi, M., LaRue, B., Hu, H. W., Spijkers, P., Guo, H., Song, X., Lenting, P. J., Van Nostrand, W. E., & Zlokovic, B. V. (2004). LRP/Amyloid β-Peptide Interaction Mediates Differential Brain Efflux of Aβ Isoforms. *Neuron*, *43*(3), 333–344. https://doi.org/10.1016/j.neuron.2004.07.017

Deurveilher, S., Burns, J., & Semba, K. (2002). Indirect projections from the suprachiasmatic nucleus to the ventrolateral preoptic nucleus: A dual tract-tracing study in rat. *European Journal of Neuroscience*, *16*(7), 1195–1213. https://doi.org/10.1046/j.1460-9568.2002.02196.x

Dhillon, S. S., & Belsham, D. D. (2011). Leptin differentially regulates NPY secretion in hypothalamic cell lines through distinct intracellular signal transduction pathways. *Regulatory Peptides*, *167*(2–3), 192–200. https://doi.org/10.1016/j.regpep.2011.01.005

Dibner, C., & Schibler, U. (2015). A pancreatic clock times insulin release. *Science*, *350*(6261), 628–629. https://doi.org/10.1126/science.aad5412

Dibner, C., Schibler, U., & Albrecht, U. (2010). The Mammalian Circadian Timing System: Organization and Coordination of Central and Peripheral Clocks. *Annual Review of Physiology*, *72*(1), 517–549. https://doi.org/10.1146/annurev-physiol-021909-135821

Dietrich, M. O., & Horvath, T. L. (2009). Feeding signals and brain circuitry. *European Journal of Neuroscience*, *30*(9), 1688–1696. https://doi.org/10.1111/j.1460-9568.2009.06963.x

Dijk, D.-J., & Cajochen, C. (1997). Melatonin and the Circadian Regulation of Sleep Initiation, Consolidation, Structure, and the Sleep EEG. *Journal of Biological Rhythms*, *12*(6), 627–635. https://doi.org/10.1177/074873049701200618

Dimitriadis, G., Mitrou, P., Lambadiari, V., Raptis, S. A., & Maratou, E. (2011). Insulin effects in muscle and adipose tissue. *Diabetes Research and Clinical Practice*, *93*, S52–S59. https://doi.org/10.1016/S0168-8227(11)70014-6

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

Dodd, G. T., Michael, N. J., Lee-Young, R. S., Mangiafico, S. P., Pryor, J. T., Munder, A. C., Simonds, S. E., Brüning, J. C., Zhang, Z.-Y., Cowley, M. A., Andrikopoulos, S., Horvath, T. L., Spanswick, D., & Tiganis, T. (2018). Insulin regulates POMC neuronal plasticity to control glucose metabolism. *eLife*, *7*, e38704. https://doi.org/10.7554/eLife.38704

Dou, J.-T., Chen, M., Dufour, F., Alkon, D. L., & Zhao, W.-Q. (2005). Insulin receptor signaling in long-term memory consolidation following spatial learning. *Learning & Memory*, *12*(6), 646–655. https://doi.org/10.1101/lm.88005

Duarte, A. I., Santos, P., Oliveira, C. R., Santos, M. S., & Rego, A. C. (2008). Insulin neuroprotection against oxidative stress is mediated by Akt and GSK-3β signaling pathways and changes in protein expression. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1783*(6), 994–1002. https://doi.org/10.1016/j.bbamcr.2008.02.016

Duez, H., & Staels, B. (2010). Nuclear Receptors Linking Circadian Rhythms and Cardiometabolic Control. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *30*(8), 1529–1534. https://doi.org/10.1161/ATVBAHA.110.209098

Duffy, K. R., & Pardridge, W. M. (1987). Blood-brain barrier transcytosis of insulin in developing rabbits. *Brain Research*, *420*(1), 32–38. https://doi.org/10.1016/0006-8993(87)90236-8

Dunlap, J. C. (1999). Molecular Bases for Circadian Clocks. *Cell*, *96*(2), 271–290. https://doi.org/10.1016/S0092-8674(00)80566-8

Duvernoy, H. M., & Risold, P.-Y. (2007). The circumventricular organs: An atlas of comparative anatomy and vascularization. *Brain Research Reviews*, *56*(1), 119–147. https://doi.org/10.1016/j.brainresrev.2007.06.002

Dvornyk, V., Vinogradova, O., & Nevo, E. (2003). Origin and evolution of circadian clock genes in prokaryotes. *Proceedings of the National Academy of Sciences*, *100*(5), 2495–2500. https://doi.org/10.1073/pnas.0130099100

Dyar, K. A., Ciciliot, S., Wright, L. E., Biensø, R. S., Tagliazucchi, G. M., Patel, V. R., Forcato, M., Paz, M. I. P., Gudiksen, A., Solagna, F., Albiero, M., Moretti, I., Eckel-Mahan, K. L., Baldi, P., Sassone-Corsi, P., Rizzuto, R., Bicciato, S., Pilegaard, H., Blaauw, B., & Schiaffino, S. (2014). Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock. *Molecular Metabolism*, *3*(1), 29–41. https://doi.org/10.1016/j.molmet.2013.10.005

Dzirbíková, Z., Stebelová, K., Kováčová, K., Okuliarová, M., Olexová, L., & Zeman, M. (2022). Artificial Dim Light at Night during Pregnancy Can Affect Hormonal and Metabolic Rhythms in Rat Offspring. *International Journal of Molecular Sciences*, *23*(23), 14544. https://doi.org/10.3390/ijms232314544

Eckel-Mahan, K. L., & Storm, D. R. (2009). Circadian rhythms and memory: Not so simple as cogs and gears. *EMBO Reports*, *10*(6), 584–591. https://doi.org/10.1038/embor.2009.123

Eckel-Mahan, K., & Sassone-Corsi, P. (2013). Metabolism and the Circadian Clock Converge. *Physiological Reviews*, *93*(1), 107–135. https://doi.org/10.1152/physrev.00016.2012

Eide, E. J., Woolf, M. F., Kang, H., Woolf, P., Hurst, W., Camacho, F., Vielhaber, E. L., Giovanni, A., & Virshup, D. M. (2005). Control of Mammalian Circadian Rhythm by CKIε-Regulated Proteasome-Mediated PER2 Degradation. *Molecular and Cellular Biology*, *25*(7), 2795–2807. https://doi.org/10.1128/MCB.25.7.2795-2807.2005

Evans, J. A., Suen, T.-C., Callif, B. L., Mitchell, A. S., Castanon-Cervantes, O., Baker, K. M., Kloehn, I., Baba, K., Teubner, B. J. W., Ehlen, J. C., Paul, K. N., Bartness, T. J., Tosini, G., Leise, T., & Davidson, A. J. (2015). Shell neurons of the master circadian clock coordinate the phase of tissue clocks throughout the brain and body. *BMC Biology*, *13*(1), 43. https://doi.org/10.1186/s12915-015-0157-x

Evans, J. L., Maddux, B. A., & Goldfine, I. D. (2005). The Molecular Basis for Oxidative Stress-Induced Insulin Resistance. *Antioxidants & Redox Signaling*, *7*(7–8), 1040–1052. https://doi.org/10.1089/ars.2005.7.1040

Ezrokhi, M., Zhang, Y., Luo, S., & Cincotta, A. H. (2021). Time-of-Day-Dependent Effects of Bromocriptine to Ameliorate Vascular Pathology and Metabolic Syndrome in SHR Rats Held on High Fat Diet. *International Journal of Molecular Sciences*, *22*(11), 6142. https://doi.org/10.3390/ijms22116142

Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., & Cone, R. D. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature*, *385*(6612), 165–168. https://doi.org/10.1038/385165a0

Ferrannini, E. (1998). Insulin Resistance versus Insulin Deficiency in Non-Insulin-Dependent Diabetes Mellitus: Problems and Prospects. *Endocrine Reviews*, *19*(4), 477–490. https://doi.org/10.1210/edrv.19.4.0336

Finger, A., & Kramer, A. (2021). Mammalian circadian systems: Organization and modern life challenges. *Acta Physiologica*, *231*(3), e13548. https://doi.org/10.1111/apha.13548

Fougeray, T., Polizzi, A., Régnier, M., Fougerat, A., Ellero-Simatos, S., Lippi, Y., Smati, S., Lasserre, F., Tramunt, B., Huillet, M., Dopavogui, L., Salvi, J., Nédélec, E., Gigot, V., Smith, L., Naylies, C., Sommer, C., Haas, J. T., Wahli, W., … Guillou, H. (2022). The hepatocyte insulin receptor is required to program the liver clock and rhythmic gene expression. *Cell Reports*, *39*(2), 110674. https://doi.org/10.1016/j.celrep.2022.110674

Frank, M. (2016). Circadian Regulation of Synaptic Plasticity. *Biology*, *5*(3), 31. https://doi.org/10.3390/biology5030031

Frölich, L., Blum-Degen, D., Bernstein, H.-G., Engelsberger, S., Humrich, J., Laufer, S., Muschner, D., Thalheimer, A., Türk, A., Hoyer, S., Zöchling, R., Boissl, K. W., Jellinger, K., & Riederer, P. (1998). Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. *Journal of Neural Transmission*, *105*(4), 423. https://doi.org/10.1007/s007020050068

Fu, Z., R. Gilbert, E., & Liu, D. (2013). Regulation of Insulin Synthesis and Secretion and Pancreatic Beta-Cell Dysfunction in Diabetes. *Current Diabetes Reviews*, *9*(1), 25–53. https://doi.org/10.2174/157339913804143225

Fukuda, M., Jones, J. E., Olson, D., Hill, J., Lee, C. E., Gautron, L., Choi, M., Zigman, J. M., Lowell, B. B., & Elmquist, J. K. (2008). Monitoring FoxO1 Localization in Chemically Identified Neurons. *The Journal of Neuroscience*, *28*(50), 13640–13648. https://doi.org/10.1523/JNEUROSCI.4023-08.2008

Gad, E. S., Zaitone, S. A., & Moustafa, Y. M. (2016). Pioglitazone and exenatide enhance cognition and downregulate hippocampal beta amyloid oligomer and microglia expression in insulin-resistant rats. *Canadian Journal of Physiology and Pharmacology*, *94*(8), 819–828. https://doi.org/10.1139/cjpp-2015-0242

Galinde, A. As., Al-Mughales, F., Oster, H., & Heyde, I. (2023). Different levels of circadian (de)synchrony -– where does it hurt? *F1000Research*, *11*, 1323. https://doi.org/10.12688/f1000research.127234.2

Garaulet, M., Qian, J., Florez, J. C., Arendt, J., Saxena, R., & Scheer, F. A. J. L. (2020). Melatonin Effects on Glucose Metabolism: Time To Unlock the Controversy. *Trends in Endocrinology & Metabolism*, *31*(3), 192–204. https://doi.org/10.1016/j.tem.2019.11.011

Garcia, M. L., Lobsiger, C. S., Shah, S. B., Deerinck, T. J., Crum, J., Young, D., Ward, C. M., Crawford, T. O., Gotow, T., Uchiyama, Y., Ellisman, M. H., Calcutt, N. A., & Cleveland, D. W. (2003). NF-M is an essential target for the myelin-directed 'outside-in' signaling cascade that mediates radial axonal growth. *The Journal of Cell Biology*, *163*(5), 1011–1020. https://doi.org/10.1083/jcb.200308159

García-Cáceres, C., Quarta, C., Varela, L., Gao, Y., Gruber, T., Legutko, B., Jastroch, M., Johansson, P., Ninkovic, J., Yi, C.-X., Le Thuc, O., Szigeti-Buck, K., Cai, W., Meyer, C. W., Pfluger, P. T., Fernandez, A. M., Luquet, S., Woods, S. C., Torres-Alemán, I., … Tschöp, M. H. (2016). Astrocytic Insulin Signaling Couples Brain Glucose Uptake with Nutrient Availability. *Cell*, *166*(4), 867–880. https://doi.org/10.1016/j.cell.2016.07.028

Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., & Weitz, C. J. (1998). Role of the CLOCK Protein in the Mammalian Circadian Mechanism. *Science*, *280*(5369), 1564–1569. https://doi.org/10.1126/science.280.5369.1564

Giorgetti, S., Pelicci, P. G., Pelicci, G., & Van Obberghen, E. (1994). Involvement of Src-homology/collagen (SHC) proteins in signaling through the insulin receptor and the insulin-like-growth-factor-I-receptor. *European Journal of Biochemistry*, *223*(1), 195–202. https://doi.org/10.1111/j.1432-1033.1994.tb18983.x

Girard, J. (2006). Insulin's effect on the liver: 'Direct or indirect?' continues to be the question. *Journal of Clinical Investigation*, *116*(2), 302–304. https://doi.org/10.1172/JCI27743

Giugliano, D., Ceriello, A., & Esposito, K. (2008). Glucose metabolism and hyperglycemia. *The American Journal of Clinical Nutrition*, *87*(1), 217S-222S. https://doi.org/10.1093/ajcn/87.1.217S

Glass, J. D., Guinn, J., Kaur, G., & Francl, J. M. (2010). On the intrinsic regulation of neuropeptide Y release in the mammalian suprachiasmatic nucleus circadian clock. *European Journal of Neuroscience*, *31*(6), 1117–1126. https://doi.org/10.1111/j.1460-9568.2010.07139.x

Goede, P., Wüst, R. C. I., Schomakers, B. V., Denis, S., Vaz, F. M., Pras-Raves, M. L., Weeghel, M., Yi, C., Kalsbeek, A., & Houtkooper, R. H. (2022). Time-restricted feeding during the inactive phase abolishes the daily rhythm in mitochondrial respiration in rat skeletal muscle. *The FASEB Journal*, *36*(2). https://doi.org/10.1096/fj.202100707R

Golan, N., Adamsky, K., Kartvelishvily, E., Brockschnieder, D., Möbius, W., Spiegel, I., Roth, A. D., Thomson, C. E., Rechavi, G., & Peles, E. (2008). Identification of *Tmem10/Opalin* as an oligodendrocyte enriched gene using expression profiling combined with genetic cell ablation. *Glia*, *56*(11), 1176–1186. https://doi.org/10.1002/glia.20688

Gómez-Abellán, P., Gómez-Santos, C., Madrid, J. A., Milagro, F. I., Campion, J., Martínez, J. A., Ordovás, J. M., & Garaulet, M. (2010). Circadian Expression of Adiponectin and Its Receptors in Human Adipose Tissue. *Endocrinology*, *151*(1), 115–122. https://doi.org/10.1210/en.2009-0647

González-Vila, A., Luengo-Mateos, M., Silveira-Loureiro, M., Garrido-Gil, P., Ohinska, N., González-Domínguez, M., Labandeira-García, J. L., García-Cáceres, C., López, M., & Barca-Mayo, O. (2023). Astrocytic insulin receptor controls circadian behavior via dopamine signaling in a sexually dimorphic manner. *Nature Communications*, *14*(1), 8175. https://doi.org/10.1038/s41467-023-44039-8

Gosmanov, A. R., Gosmanova, E. O., & Kitabchi, A. E. (2000). Hyperglycemic Crises: Diabetic Ketoacidosis and Hyperglycemic Hyperosmolar State. In K. R. Feingold, S. F. Ahmed, B. Anawalt, M. R. Blackman, A. Boyce, G. Chrousos, E. Corpas, W. W. de Herder, K. Dhatariya, K. Dungan, J. Hofland, S. Kalra, G. Kaltsas, N. Kapoor, C. Koch, P. Kopp, M. Korbonits, C. S. Kovacs, W. Kuohung, … D. P. Wilson (Eds.), *Endotext*. MDText.com, Inc. http://www.ncbi.nlm.nih.gov/books/NBK279052/

Govind, S., Kozma, R., Monfries, C., Lim, L., & Ahmed, S. (2001). Cdc42hs Facilitates Cytoskeletal Reorganization and Neurite Outgrowth by Localizing the 58-Kd Insulin Receptor Substrate to Filamentous Actin. *The Journal of Cell Biology*, *152*(3), 579–594. https://doi.org/10.1083/jcb.152.3.579

Granados-Fuentes, D., Saxena, M. T., Prolo, L. M., Aton, S. J., & Herzog, E. D. (2004). Olfactory bulb neurons express functional, entrainable circadian rhythms. *European Journal of Neuroscience*, *19*(4), 898–906. https://doi.org/10.1111/j.0953-816X.2004.03117.x

Granados-Fuentes, D., Tseng, A., & Herzog, E. D. (2006). A Circadian Clock in the Olfactory Bulb Controls Olfactory Responsivity. *The Journal of Neuroscience*, *26*(47), 12219–12225. https://doi.org/10.1523/JNEUROSCI.3445-06.2006

Graunke, D., & Argyropoulos, G. (2003). Deciphering Transcriptional Regulation Relevant to Eating Behavior. *Current Genomics*, *4*(7), 623–633. https://doi.org/10.2174/1389202033490150

Gray, S. M., & Barrett, E. J. (2018). Insulin transport into the brain. *American Journal of Physiology-Cell Physiology*, *315*(2), C125–C136. https://doi.org/10.1152/ajpcell.00240.2017

Greco, C. M., & Sassone–Corsi, P. (2019). Circadian blueprint of metabolic pathways in the brain. *Nature Reviews Neuroscience*, *20*(2), 71–82. https://doi.org/10.1038/s41583-018-0096-y

Grillo, C. A., Tamashiro, K. L., Piroli, G. G., Melhorn, S., Gass, J. T., Newsom, R. J., Reznikov, L. R., Smith, A., Wilson, S. P., Sakai, R. R., & Reagan, L. P. (2007). Lentivirus-mediated downregulation of hypothalamic insulin receptor expression. *Physiology & Behavior*, *92*(4), 691–701. https://doi.org/10.1016/j.physbeh.2007.05.043

Guilding, C., Hughes, A. T., Brown, T. M., Namvar, S., & Piggins, H. D. (2009). A riot of rhythms: Neuronal and glial circadian oscillators in the mediobasal hypothalamus. *Molecular Brain*, *2*(1), 28. https://doi.org/10.1186/1756-6606-2-28

Guilding, C., & Piggins, H. D. (2007). Challenging the omnipotence of the suprachiasmatic timekeeper: Are circadian oscillators present throughout the mammalian brain? *European Journal of Neuroscience*, *25*(11), 3195–3216. https://doi.org/10.1111/j.1460-9568.2007.05581.x

Guillaumond, F., Dardente, H., Giguère, V., & Cermakian, N. (2005). Differential Control of Bmal1 Circadian Transcription by REV-ERB and ROR Nuclear Receptors. *Journal of Biological Rhythms*, *20*(5), 391–403. https://doi.org/10.1177/0748730405277232

Guzmán-Ruiz, M., Saderi, N., Cazarez-Márquez, F., Guerrero-Vargas, N. N., Basualdo, M. C., Acosta-Galván, G., & Buijs, R. M. (2014). The Suprachiasmatic Nucleus Changes the Daily Activity of the Arcuate Nucleus α-MSH Neurons in Male Rats. *Endocrinology*, *155*(2), 525–535. https://doi.org/10.1210/en.2013-1604

Hale, L. J., & Coward, R. J. M. (2013). Insulin signalling to the kidney in health and disease. *Clinical Science*, *124*(6), 351–370. https://doi.org/10.1042/CS20120378

Hallschmid, M., & Schultes, B. (2009). Central nervous insulin resistance: A promising target in the treatment of metabolic and cognitive disorders? *Diabetologia*, *52*(11), 2264–2269. https://doi.org/10.1007/s00125-009-1501-x

Hamada, T., Honma, S., & Honma, K. (2011). Light responsiveness of clock genes, Per1 and Per2, in the olfactory bulb of mice. *Biochemical and Biophysical Research Communications*, *409*(4), 727–731. https://doi.org/10.1016/j.bbrc.2011.05.076

Han, S., Gim, Y., Jang, E.-H., & Hur, E.-M. (2022). Functions and dysfunctions of oligodendrocytes in neurodegenerative diseases. *Frontiers in Cellular Neuroscience*, *16*, 1083159. https://doi.org/10.3389/fncel.2022.1083159

Hanbo Chen. (2011). *VennDiagram: Generate High-Resolution Venn and Euler Plots* (p. 1.7.3) [Dataset]. https://doi.org/10.32614/CRAN.package.VennDiagram

Harper, A. E., Benevenga, N. J., & Wohlhueter, R. M. (1970). Effects of ingestion of disproportionate amounts of amino acids. *Physiological Reviews*, *50*(3), 428–558. https://doi.org/10.1152/physrev.1970.50.3.428

Hartmann, J., Dragicevic, E., Adelsberger, H., Henning, H. A., Sumser, M., Abramowitz, J., Blum, R., Dietrich, A., Freichel, M., Flockerzi, V., Birnbaumer, L., & Konnerth, A. (2008). TRPC3 channels are required for synaptic transmission and motor coordination. *Neuron*, *59*(3), 392–398. https://doi.org/10.1016/j.neuron.2008.06.009

Hatori, M., Vollmers, C., Zarrinpar, A., DiTacchio, L., Bushong, E. A., Gill, S., Leblanc, M., Chaix, A., Joens, M., Fitzpatrick, J. A. J., Ellisman, M. H., & Panda, S. (2012). Time-Restricted Feeding without Reducing Caloric Intake Prevents Metabolic Diseases in Mice Fed a High-Fat Diet. *Cell Metabolism*, *15*(6), 848–860. https://doi.org/10.1016/j.cmet.2012.04.019

Hattar, S., Kumar, M., Park, A., Tong, P., Tung, J., Yau, K., & Berson, D. M. (2006). Central projections of melanopsin-expressing retinal ganglion cells in the mouse. *Journal of Comparative Neurology*, *497*(3), 326–349. https://doi.org/10.1002/cne.20970

Hausen, A. C., Ruud, J., Jiang, H., Hess, S., Varbanov, H., Kloppenburg, P., & Brüning, J. C. (2016). Insulin-Dependent Activation of MCH Neurons Impairs Locomotor Activity and Insulin Sensitivity in Obesity. *Cell Reports*, *17*(10), 2512–2521. https://doi.org/10.1016/j.celrep.2016.11.030

Hennessey, J. V., Bustamante, M. A., Teter, M. L., Markert, R. J., & McDonald, S. D. (1994). Bedtime Dosing of Glyburide and the Treatment of Type II Diabetes Mellitus. *The American Journal of the Medical Sciences*, *308*(4), 234–238. https://doi.org/10.1097/00000441-199430840-00004

Henquin, J. C., Ravier, M. A., Nenquin, M., Jonas, J. C., & Gilon, P. (2003). Hierarchy of the β-cell signals controlling insulin secretion. *European Journal of Clinical Investigation*, *33*(9), 742–750. https://doi.org/10.1046/j.1365-2362.2003.01207.x

Hers, I., Vincent, E. E., & Tavaré, J. M. (2011). Akt signalling in health and disease. *Cellular Signalling*, *23*(10), 1515–1527. https://doi.org/10.1016/j.cellsig.2011.05.004

Hickman, S., Izzy, S., Sen, P., Morsett, L., & El Khoury, J. (2018). Microglia in neurodegeneration. *Nature Neuroscience*, *21*(10), 1359–1369. https://doi.org/10.1038/s41593-018-0242-x

Hill, J. W. (2010). Gene Expression and the Control of Food Intake by Hypothalamic POMC/CART Neurons. *Open Neuroendocrinology Journal (Online)*, *3*, 21–27.

Hill, J. W., Elias, C. F., Fukuda, M., Williams, K. W., Berglund, E. D., Holland, W. L., Cho, Y.-R., Chuang, J.-C., Xu, Y., Choi, M., Lauzon, D., Lee, C. E., Coppari, R., Richardson, J. A., Zigman, J. M., Chua, S., Scherer, P. E., Lowell, B. B., Brüning, J. C., & Elmquist, J. K. (2010). Direct Insulin and Leptin Action on Pro-opiomelanocortin Neurons Is Required for Normal Glucose Homeostasis and Fertility. *Cell Metabolism*, *11*(4), 286–297. https://doi.org/10.1016/j.cmet.2010.03.002

Hirano, A., Yumimoto, K., Tsunematsu, R., Matsumoto, M., Oyama, M., Kozuka-Hata, H., Nakagawa, T., Lanjakornsiripan, D., Nakayama, K. I., & Fukada, Y. (2013). FBXL21 Regulates Oscillation of the Circadian Clock through Ubiquitination and Stabilization of Cryptochromes. *Cell*, *152*(5), 1106–1118. https://doi.org/10.1016/j.cell.2013.01.054

Ho, L., Qin, W., Pompl, P. N., Xiang, Z., Wang, J., Zhao, Z., Peng, Y., Cambareri, G., Rocher, A., Mobbs, C. V., Hof, P. R., & Pasinetti, G. M. (2004). Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. *The FASEB Journal*, *18*(7), 902–904. https://doi.org/10.1096/fj.03-0978fje

Hogenesch, J. B., Gu, Y.-Z., Jain, S., & Bradfield, C. A. (1998). The basic-helix–loop–helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proceedings of the National Academy of Sciences*, *95*(10), 5474–5479. https://doi.org/10.1073/pnas.95.10.5474

Hölscher, C. (2019). Insulin Signaling Impairment in the Brain as a Risk Factor in Alzheimer's Disease. *Frontiers in Aging Neuroscience*, *11*, 88. https://doi.org/10.3389/fnagi.2019.00088

Hopkins, D. F. C., & Williams, G. (1997). Insulin receptors are widely distributed in human brain and bind human and porcine insulin with equal affinity. *Diabetic Medicine*, *14*(12), 1044–1050. https://doi.org/10.1002/(SICI)1096-9136(199712)14:12<1044::AID-DIA508>3.0.CO;2-F

Hoyer, S. (1998). Is sporadic Alzheimer disease the brain type of non-insulin dependent diabetes mellitus? A challenging hypothesis. *Journal of Neural Transmission*, *105*(4), 415. https://doi.org/10.1007/s007020050067

Hoyer, S. (2004). Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. *European Journal of Pharmacology*, *490*(1–3), 115–125. https://doi.org/10.1016/j.ejphar.2004.02.049

Huang, C.-C., Lee, C.-C., & Hsu, K.-S. (2010). The role of insulin receptor signaling in synaptic plasticity and cognitive function. *Chang Gung Medical Journal*, *33*(2), 115–125.

Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, *4*(1), 44–57. https://doi.org/10.1038/nprot.2008.211

Husse, J., Eichele, G., & Oster, H. (2015). Synchronization of the mammalian circadian timing system: Light can control peripheral clocks independently of the SCN clock: Alternate routes of entrainment optimize the alignment of the body's circadian clock network with external time. *BioEssays*, *37*(10), 1119–1128. https://doi.org/10.1002/bies.201500026

Husse, J., Leliavski, A., Tsang, A. H., Oster, H., & Eichele, G. (2014). The light-dark cycle controls peripheral rhythmicity in mice with a genetically ablated suprachiasmatic nucleus clock. *The FASEB Journal*, *28*(11), 4950–4960. https://doi.org/10.1096/fj.14-256594

Husse, J., Zhou, X., Shostak, A., Oster, H., & Eichele, G. (2011). Synaptotagmin10-Cre, a Driver to Disrupt Clock Genes in the SCN. *Journal of Biological Rhythms*, *26*(5), 379–389. https://doi.org/10.1177/0748730411415363

Imbernon, M., Dehouck, B., & Prevot, V. (2022). Glycemic control: Tanycytes march to the beat of the suprachiasmatic drummer. *Current Biology*, *32*(4), R173–R176. https://doi.org/10.1016/j.cub.2022.01.038

Inutsuka, A., Inui, A., Tabuchi, S., Tsunematsu, T., Lazarus, M., & Yamanaka, A. (2014). Concurrent and robust regulation of feeding behaviors and metabolism by orexin neurons. *Neuropharmacology*, *85*, 451–460. https://doi.org/10.1016/j.neuropharm.2014.06.015

Inyushkin, A. N., Mistryugov, K. A., Gromova, D. S., Pugachev, E. I., Belyakov, V. I., & Inyushkina, E. M. (2013). Effects of Insulin on the Circadian Rhythm of Voluntary Locomotor Activity in Rats. *Neuroscience and Behavioral Physiology*, *43*(3), 401–408. https://doi.org/10.1007/s11055-013-9746-8

Inyushkin, A. N., Mistryugov, K. A., Ledyaeva, O. V., Romanova, I. D., Isakova, T. S., & Inyushkin, A. A. (2023). The Effects of Insulin on Spike Activity of the Suprachiasmatic Nucleus Neurones and Functional State of Afferent Inputs from the Arcuate Nucleus in Rats. *Российский*

Физиологический Журнал Им И М Сеченова, *109*(2), 192–213. https://doi.org/10.31857/S086981392302005X

Ioannou, M. S., Liu, Z., & Lippincott-Schwartz, J. (2019). A Neuron-Glia Co-culture System for Studying Intercellular Lipid Transport. *Current Protocols in Cell Biology*, *84*(1). https://doi.org/10.1002/cpcb.95

Ito, K., Maruyama, H., Hirose, H., Kido, K., Koyama, K., Kataoka, K., & Saruta, T. (1995). Exogenous insulin dose-dependently suppresses glucopenia-induced glucagon secretion from perfused rat pancreas. *Metabolism*, *44*(3), 358–362. https://doi.org/10.1016/0026-0495(95)90166-3

Izumi, Y., Yamada, K. A., Matsukawa, M., & Zorumski, C. F. (2003). Effects of insulin on long-term potentiation in hippocampal slices from diabetic rats. *Diabetologia*, *46*(7), 1007–1012. https://doi.org/10.1007/s00125-003-1144-2

Izumo, M., Pejchal, M., Schook, A. C., Lange, R. P., Walisser, J. A., Sato, T. R., Wang, X., Bradfield, C. A., & Takahashi, J. S. (2014). Differential effects of light and feeding on circadian organization of peripheral clocks in a forebrain Bmal1 mutant. *eLife*, *3*, e04617. https://doi.org/10.7554/eLife.04617

Jackson, J. G., Kreisberg, J. I., Koterba, A. P., Yee, D., & Brattain, M. G. (2000). Phosphorylation and nuclear exclusion of the forkhead transcription factor FKHR after epidermal growth factor treatment in human breast cancer cells. *Oncogene*, *19*(40), 4574–4581. https://doi.org/10.1038/sj.onc.1203825

Jacobs, S., & Cuatrecasas, P. (1981). Insulin Receptor: Structure and Function. *Endocrine Reviews*, *2*(3), 251–263. https://doi.org/10.1210/edrv-2-3-251

Jamali, A. K., & Tramu, G. (1997). Daily cycle of fos expression within hypothalamic POMC neurons of the male rat. *Brain Research*, *771*(1), 45–54. https://doi.org/10.1016/S0006-8993(97)00767-1

Jessen, L., Clegg, D. J., & Bouman, S. D. (2010). Evaluation of the lack of anorectic effect of intracerebroventricular insulin in rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *298*(1), R43–R50. https://doi.org/10.1152/ajpregu.90736.2008

Jia, G., Whaley-Connell, A., & Sowers, J. R. (2018). Diabetic cardiomyopathy: A hyperglycaemia- and insulin-resistance-induced heart disease. *Diabetologia*, *61*(1), 21–28. https://doi.org/10.1007/s00125-017-4390-4

Jilg, A., Bechstein, P., Saade, A., Dick, M., Li, T. X., Tosini, G., Rami, A., Zemmar, A., & Stehle, J. H. (2019). Melatonin modulates daytime-dependent synaptic plasticity and learning efficiency. *Journal of Pineal Research*, *66*(3), e12553. https://doi.org/10.1111/jpi.12553

Jilg, A., Lesny, S., Peruzki, N., Schwegler, H., Selbach, O., Dehghani, F., & Stehle, J. H. (2010). Temporal dynamics of mouse hippocampal clock gene expression support memory processing. *Hippocampus*, *20*(3), 377–388. https://doi.org/10.1002/hipo.20637

Kakoty, V., Kc, S., Kumari, S., Yang, C.-H., Dubey, S. K., Sahebkar, A., Kesharwani, P., & Taliyan, R. (2023). Brain insulin resistance linked Alzheimer's and Parkinson's disease pathology: An undying implication of epigenetic and autophagy modulation. *Inflammopharmacology*, *31*(2), 699–716. https://doi.org/10.1007/s10787-023-01187-z

Kalsbeek, A., Fliers, E., Romijn, J. A., La Fleur, S. E., Wortel, J., Bakker, O., Endert, E., & Buijs, R. M. (2001). The Suprachiasmatic Nucleus Generates the Diurnal Changes in Plasma Leptin Levels. *Endocrinology*, *142*(6), 2677–2685. https://doi.org/10.1210/endo.142.6.8197

Kalsbeek, A., Teclemariam-Mesbah, R., & Péavet, P. (1993). Efferent projections of the suprachiasmatic nucleus in the golden hamster (*Mesocricetus auratus*). *Journal of Comparative Neurology*, *332*(3), 293–314. https://doi.org/10.1002/cne.903320304

Kandimalla, R., Thirumala, V., & Reddy, P. H. (2017). Is Alzheimer's disease a Type 3 Diabetes? A critical appraisal. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1863*(5), 1078–1089. https://doi.org/10.1016/j.bbadis.2016.08.018

Kaur, D., Sharma, V., & Deshmukh, R. (2019). Activation of microglia and astrocytes: A roadway to neuroinflammation and Alzheimer's disease. *Inflammopharmacology*, *27*(4), 663–677. https://doi.org/10.1007/s10787-019-00580-x

Keesler, G. A., Camacho, F., Guo, Y., Virshup, D., Mondadori, C., & Yao, Z. (2000). Phosphorylation and destabilization of human period I clock protein by human casein kinase Iε: *NeuroReport*, *11*(5), 951–955. https://doi.org/10.1097/00001756-200004070-00011

Kelley, D. E., He, J., Menshikova, E. V., & Ritov, V. B. (2002). Dysfunction of Mitochondria in Human Skeletal Muscle in Type 2 Diabetes. *Diabetes*, *51*(10), 2944–2950. https://doi.org/10.2337/diabetes.51.10.2944

Kelly, R. M., Finn, J., Healy, U., Gallen, D., Sreenan, S., McDermott, J. H., & Coogan, A. N. (2020). Greater social jetlag associates with higher HbA1c in adults with type 2 diabetes: A cross sectional study. *Sleep Medicine*, *66*, 1–9. https://doi.org/10.1016/j.sleep.2019.07.023

Kettner, N. M., Mayo, S. A., Hua, J., Lee, C., Moore, D. D., & Fu, L. (2015). Circadian Dysfunction Induces Leptin Resistance in Mice. *Cell Metabolism*, *22*(3), 448–459. https://doi.org/10.1016/j.cmet.2015.06.005

Khakh, B. S., & Deneen, B. (2019). The Emerging Nature of Astrocyte Diversity. *Annual Review of Neuroscience*, *42*(1), 187–207. https://doi.org/10.1146/annurev-neuro-070918-050443

Kim, D.-H. (2012). Hypothalamic Akt PKB signaling in regulation of food intake. *Frontiers in Bioscience*, *S4*(3), 953–966. https://doi.org/10.2741/s311

Kim, M., Facco, F. L., Braun, R. I., Wolf, M. S., Garcia-Canga, B., Grobman, W. A., Zee, P. C., & Reid, K. J. (2023). The association between light exposure before bedtime in pregnancy and the risk of developing gestational diabetes mellitus. *American Journal of Obstetrics & Gynecology MFM*, *5*(8), 100922. https://doi.org/10.1016/j.ajogmf.2023.100922

Kittur, S., Hoh, J., Endo, H., Tourtellotte, W., Weeks, B. S., Markesbery, W., & Adler, W. (1994). Cytoskeletal Neurofilament Gene Expression in Brain Tissue from Alzheimer's Disease Patients. I. Decrease in NF-L and NF-M Message. *Journal of Geriatric Psychiatry and Neurology*, *7*(3), 153–158. https://doi.org/10.1177/089198879400700305

Kleinridders, A., Cai, W., Cappellucci, L., Ghazarian, A., Collins, W. R., Vienberg, S. G., Pothos, E. N., & Kahn, C. R. (2015). Insulin resistance in brain alters dopamine turnover and causes behavioral disorders. *Proceedings of the National Academy of Sciences*, *112*(11), 3463–3468. https://doi.org/10.1073/pnas.1500877112

Ko, C. H., & Takahashi, J. S. (2006). Molecular components of the mammalian circadian clock. *Human Molecular Genetics*, *15*(suppl_2), R271–R277. https://doi.org/10.1093/hmg/ddl207

Kohsaka, A., Laposky, A. D., Ramsey, K. M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F. W., & Bass, J. (2007). High-Fat Diet Disrupts Behavioral and Molecular Circadian Rhythms in Mice. *Cell Metabolism*, *6*(5), 414–421. https://doi.org/10.1016/j.cmet.2007.09.006

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., & Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, *402*(6762), 656–660. https://doi.org/10.1038/45230

Kola, B., Hubina, E., Tucci, S. A., Kirkham, T. C., Garcia, E. A., Mitchell, S. E., Williams, L. M., Hawley, S. A., Hardie, D. G., Grossman, A. B., & Korbonits, M. (2005). Cannabinoids and Ghrelin Have Both Central and Peripheral Metabolic and Cardiac Effects via AMP-activated Protein Kinase. *Journal of Biological Chemistry*, *280*(26), 25196–25201. https://doi.org/10.1074/jbc.C500175200

Kolbe, I., Leinweber, B., Brandenburger, M., & Oster, H. (2019). Circadian clock network desynchrony promotes weight gain and alters glucose homeostasis in mice. *Molecular Metabolism*, *30*, 140–151. https://doi.org/10.1016/j.molmet.2019.09.012

Kong, Y., Ruan, L., Qian, L., Liu, X., & Le, Y. (2010). Norepinephrine Promotes Microglia to Uptake and Degrade Amyloid β Peptide through Upregulation of Mouse Formyl Peptide Receptor 2 and Induction of Insulin-Degrading Enzyme. *The Journal of Neuroscience*, *30*(35), 11848–11857. https://doi.org/10.1523/JNEUROSCI.2985-10.2010

Konishi, M., Sakaguchi, M., Lockhart, S. M., Cai, W., Li, M. E., Homan, E. P., Rask-Madsen, C., & Kahn, C. R. (2017). Endothelial insulin receptors differentially control insulin signaling kinetics in peripheral tissues and brain of mice. *Proceedings of the National Academy of Sciences*, *114*(40). https://doi.org/10.1073/pnas.1710625114

Könner, A. C., Hess, S., Tovar, S., Mesaros, A., Sánchez-Lasheras, C., Evers, N., Verhagen, L. A. W., Brönneke, H. S., Kleinridders, A., Hampel, B., Kloppenburg, P., & Brüning, J. C. (2011). Role for Insulin Signaling in Catecholaminergic Neurons in Control of Energy Homeostasis. *Cell Metabolism*, *13*(6), 720–728. https://doi.org/10.1016/j.cmet.2011.03.021

Könner, A. C., Janoschek, R., Plum, L., Jordan, S. D., Rother, E., Ma, X., Xu, C., Enriori, P., Hampel, B., Barsh, G. S., Kahn, C. R., Cowley, M. A., Ashcroft, F. M., & Brüning, J. C. (2007). Insulin Action in AgRP-Expressing Neurons Is Required for Suppression of Hepatic Glucose Production. *Cell Metabolism*, *5*(6), 438–449. https://doi.org/10.1016/j.cmet.2007.05.004

Korenčič, A., Košir, R., Bordyugov, G., Lehmann, R., Rozman, D., & Herzel, H. (2014). Timing of circadian genes in mammalian tissues. *Scientific Reports*, *4*(1), 5782. https://doi.org/10.1038/srep05782

Kubota, N., Yano, W., Kubota, T., Yamauchi, T., Itoh, S., Kumagai, H., Kozono, H., Takamoto, I., Okamoto, S., Shiuchi, T., Suzuki, R., Satoh, H., Tsuchida, A., Moroi, M., Sugi, K., Noda, T., Ebinuma, H., Ueta, Y., Kondo, T., … Kadowaki, T. (2007). Adiponectin Stimulates AMP-Activated Protein Kinase in the Hypothalamus and Increases Food Intake. *Cell Metabolism*, *6*(1), 55–68. https://doi.org/10.1016/j.cmet.2007.06.003

Kuhn, S., Gritti, L., Crooks, D., & Dombrowski, Y. (2019). Oligodendrocytes in Development, Myelin Generation and Beyond. *Cells*, *8*(11), 1424. https://doi.org/10.3390/cells8111424

Kumar, N., Afeyan, R., Sheppard, S., Harms, B., & Lauffenburger, D. A. (2007). Quantitative analysis of Akt phosphorylation and activity in response to EGF and insulin treatment. *Biochemical and Biophysical Research Communications*, *354*(1), 14–20. https://doi.org/10.1016/j.bbrc.2006.12.188

Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., Maywood, E. S., Hastings, M. H., & Reppert, S. M. (1999). mCRY1 and mCRY2 Are Essential Components of the Negative Limb of the Circadian Clock Feedback Loop. *Cell*, *98*(2), 193–205. https://doi.org/10.1016/S0092-8674(00)81014-4

Kuzuya, T., & Matsuda, A. (1997). Classification of Diabetes on the Basis of Etiologies Versus Degree of Insulin Deficiency. *Diabetes Care*, *20*(2), 219–220. https://doi.org/10.2337/diacare.20.2.219

La Fleur, S. E., Kalsbeek, A., Wortel, J., Fekkes, M. L., & Buijs, R. M. (2001). A Daily Rhythm in Glucose Tolerance. *Diabetes*, *50*(6), 1237–1243. https://doi.org/10.2337/diabetes.50.6.1237

Lamia, K. A., Storch, K.-F., & Weitz, C. J. (2008). Physiological significance of a peripheral tissue circadian clock. *Proceedings of the National Academy of Sciences*, *105*(39), 15172–15177. https://doi.org/10.1073/pnas.0806717105

Lananna, B. V., & Musiek, E. S. (2020). The wrinkling of time: Aging, inflammation, oxidative stress, and the circadian clock in neurodegeneration. *Neurobiology of Disease*, *139*, 104832. https://doi.org/10.1016/j.nbd.2020.104832

Landgraf, D., Long, J. E., & Welsh, D. K. (2016). Depression-like behaviour in mice is associated with disrupted circadian rhythms in nucleus accumbens and periaqueductal grey. *European Journal of Neuroscience*, *43*(10), 1309–1320. https://doi.org/10.1111/ejn.13085

Lavie, P. (1997). Melatonin: Role in Gating Nocturnal Rise in Sleep Propensity. *Journal of Biological Rhythms*, *12*(6), 657–665. https://doi.org/10.1177/074873049701200622

Lee, C.-C., Huang, C.-C., Wu, M.-Y., & Hsu, K.-S. (2005). Insulin Stimulates Postsynaptic Density-95 Protein Translation via the Phosphoinositide 3-Kinase-Akt-Mammalian Target of Rapamycin Signaling Pathway. *Journal of Biological Chemistry*, *280*(18), 18543–18550. https://doi.org/10.1074/jbc.m414112200

Lee, H., Chen, R., Lee, Y., Yoo, S., & Lee, C. (2009). Essential roles of CKIδ and CKIε in the mammalian circadian clock. *Proceedings of the National Academy of Sciences*, *106*(50), 21359–21364. https://doi.org/10.1073/pnas.0906651106

Lee, J., Moulik, M., Fang, Z., Saha, P., Zou, F., Xu, Y., Nelson, D. L., Ma, K., Moore, D. D., & Yechoor, V. K. (2013). Bmal1 and β-Cell Clock Are Required for Adaptation to Circadian Disruption, and Their Loss of Function Leads to Oxidative Stress-Induced β-Cell Failure in Mice. *Molecular and Cellular Biology*, *33*(11), 2327–2338. https://doi.org/10.1128/MCB.01421-12

Lehman, M., Silver, R., Gladstone, W., Kahn, R., Gibson, M., & Bittman, E. (1987). Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. *The Journal of Neuroscience*, *7*(6), 1626–1638. https://doi.org/10.1523/JNEUROSCI.07-06-01626.1987

Leloup, C., Arluison, M., Kassis, N., Lepetit, N., Cartier, N., Ferré, P., & Pénicaud, L. (1996). Discrete brain areas express the insulin-responsive glucose transporter GLUT4. *Molecular Brain Research*, *38*(1), 45–53. https://doi.org/10.1016/0169-328X(95)00306-D

LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D. W., & Silver, R. (2009). Stomach ghrelin-secreting cells as food-entrainable circadian clocks. *Proceedings of the National Academy of Sciences*, *106*(32), 13582–13587. https://doi.org/10.1073/pnas.0906426106

Leto, D., & Saltiel, A. R. (2012). Regulation of glucose transport by insulin: Traffic control of GLUT4. *Nature Reviews Molecular Cell Biology*, *13*(6), 383–396. https://doi.org/10.1038/nrm3351

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., Bennett, M. L., Münch, A. E., Chung, W.-S., Peterson, T. C., Wilton, D. K., Frouin, A., Napier, B. A., Panicker, N., Kumar, M., Buckwalter, M. S., Rowitch, D. H., Dawson, V. L., Dawson, T. M., … Barres, B. A. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature*, *541*(7638), 481–487. https://doi.org/10.1038/nature21029

Lin, H. V., Plum, L., Ono, H., Gutiérrez-Juárez, R., Shanabrough, M., Borok, E., Horvath, T. L., Rossetti, L., & Accili, D. (2010). Divergent Regulation of Energy Expenditure and Hepatic Glucose Production by Insulin Receptor in Agouti-Related Protein and POMC Neurons. *Diabetes*, *59*(2), 337–346. https://doi.org/10.2337/db09-1303

Lin, J. W., Ju, W., Foster, K., Lee, S. H., Ahmadian, G., Wyszynski, M., Wang, Y. T., & Sheng, M. (2000). Distinct molecular mechanisms and divergent endocytotic pathways of AMPA receptor internalization. *Nature Neuroscience*, *3*(12), 1282–1290. https://doi.org/10.1038/81814

Lin, Y., Liang, Z., He, L., Yang, M., Liu, D., Gu, H. F., Liu, H., Zhu, Z., Zheng, H., Li, L., & Yang, G. (2019). Gut ghrelin regulates hepatic glucose production and insulin signaling via a gut-brain-liver pathway. *Cell Communication and Signaling*, *17*(1), 8. https://doi.org/10.1186/s12964-019-0321-y

Lincoln, K., Zhou, J., Oster, H., & De Assis, L. V. M. (2024). Circadian Gating of Thyroid Hormone Action in Hepatocytes. *Cells*, *13*(12), 1038. https://doi.org/10.3390/cells13121038

Lindqvist, A., Shcherbina, L., Prasad, R. B., Miskelly, M. G., Abels, M., Martínez-Lopéz, J. A., Fred, R. G., Nergård, B. J., Hedenbro, J., Groop, L., Hjerling-Leffler, J., & Wierup, N. (2020). Ghrelin suppresses insulin secretion in human islets and type 2 diabetes patients have diminished islet ghrelin cell number and lower plasma ghrelin levels. *Molecular and Cellular Endocrinology*, *511*, 110835. https://doi.org/10.1016/j.mce.2020.110835

Liu, A. C., Tran, H. G., Zhang, E. E., Priest, A. A., Welsh, D. K., & Kay, S. A. (2008). Redundant Function of REV-ERBα and β and Non-Essential Role for Bmal1 Cycling in Transcriptional Regulation of Intracellular Circadian Rhythms. *PLoS Genetics*, *4*(2), e1000023. https://doi.org/10.1371/journal.pgen.1000023

Liu, S., Globa, A. K., Mills, F., Naef, L., Qiao, M., Bamji, S. X., & Borgland, S. L. (2016). Consumption of palatable food primes food approach behavior by rapidly increasing synaptic density in the VTA. *Proceedings of the National Academy of Sciences*, *113*(9), 2520–2525. https://doi.org/10.1073/pnas.1515724113

Loganathan, N., & Belsham, D. D. (2016). Nutrient-sensing mechanisms in hypothalamic cell models: Neuropeptide regulation and neuroinflammation in male- and female-derived cell lines. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *311*(2), R217–R221. https://doi.org/10.1152/ajpregu.00168.2016

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Lowden, A., Moreno, C., Holmbäck, U., Lennernäs, M., & Tucker, P. (2010). Eating and shift work – effects on habits, metabolism and performance. *Scandinavian Journal of Work, Environment & Health*, *36*(2), 150–162. https://doi.org/10.5271/sjweh.2898

Lowrey, P. L., Shimomura, K., Antoch, M. P., Yamazaki, S., Zemenides, P. D., Ralph, M. R., Menaker, M., & Takahashi, J. S. (2000). Positional Syntenic Cloning and Functional Characterization of the Mammalian Circadian Mutation *tau*. *Science*, *288*(5465), 483–491. https://doi.org/10.1126/science.288.5465.483

Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R. P., Wilkison, W. O., & Cone, R. D. (1994). Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature*, *371*(6500), 799–802. https://doi.org/10.1038/371799a0

Luchsinger, J. A., Tang, M.-X., Shea, S., & Mayeux, R. (2004). Hyperinsulinemia and risk of Alzheimer disease. *Neurology*, *63*(7), 1187–1192. https://doi.org/10.1212/01.WNL.0000140292.04932.87

M King, K. (2003). A history of insulin: From discovery to modern alternatives. *British Journal of Nursing*, *12*(19), 1137–1141. https://doi.org/10.12968/bjon.2003.12.19.11801

MacDonald, P. E., & Wheeler, M. B. (2003). Voltage-dependent K + channels in pancreatic beta cells: Role, regulation and potential as therapeutic targets. *Diabetologia*, *46*(8), 1046–1062. https://doi.org/10.1007/s00125-003-1159-8

Malhotra, S., Sawhney, G., & Pandhi, P. (2004). The therapeutic potential of melatonin: A review of the science. *MedGenMed: Medscape General Medicine*, *6*(2), 46.

Man, H.-Y., Lin, J. W., Ju, W. H., Ahmadian, G., Liu, L., Becker, L. E., Sheng, M., & Wang, Y. T. (2000). Regulation of AMPA Receptor–Mediated Synaptic Transmission by Clathrin-Dependent Receptor Internalization. *Neuron*, *25*(3), 649–662. https://doi.org/10.1016/S0896-6273(00)81067-3

Marcheva, B., Ramsey, K. M., Buhr, E. D., Kobayashi, Y., Su, H., Ko, C. H., Ivanova, G., Omura, C., Mo, S., Vitaterna, M. H., Lopez, J. P., Philipson, L. H., Bradfield, C. A., Crosby, S. D., JeBailey, L., Wang, X., Takahashi, J. S., & Bass, J. (2010). Disruption of the clock components CLOCK and BMAL1 leads

to hypoinsulinaemia and diabetes. *Nature*, *466*(7306), 627–631. https://doi.org/10.1038/nature09253

Marks, J. S., & Botelho, L. H. (1986). Synergistic inhibition of glucagon-induced effects on hepatic glucose metabolism in the presence of insulin and a cAMP antagonist. *The Journal of Biological Chemistry*, *261*(34), 15895–15899.

Martínez-Morillo, E., Childs, C., García, B. P., Álvarez Menéndez, F. V., Romaschin, A. D., Cervellin, G., Lippi, G., & Diamandis, E. P. (2015). Neurofilament medium polypeptide (NFM) protein concentration is increased in CSF and serum samples from patients with brain injury. *Clinical Chemistry and Laboratory Medicine (CCLM)*, *53*(10). https://doi.org/10.1515/cclm-2014-0908

Masís-Vargas, A., Ritsema, W. I. G. R., Mendoza, J., & Kalsbeek, A. (2020). Metabolic Effects of Light at Night are Time- and Wavelength-Dependent in Rats. *Obesity*, *28*(S1). https://doi.org/10.1002/oby.22874

Matsuzaki, H., Daitoku, H., Hatta, M., Tanaka, K., & Fukamizu, A. (2003). Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(20), 11285–11290. https://doi.org/10.1073/pnas.1934283100

Mayer, C. M., & Belsham, D. D. (2009a). Insulin directly regulates NPY and AgRP gene expression via the MAPK MEK/ERK signal transduction pathway in mHypoE-46 hypothalamic neurons. *Molecular and Cellular Endocrinology*, *307*(1–2), 99–108. https://doi.org/10.1016/j.mce.2009.02.031

Mayer, C. M., & Belsham, D. D. (2009b). Insulin directly regulates NPY and AgRP gene expression via the MAPK MEK/ERK signal transduction pathway in mHypoE-46 hypothalamic neurons. *Molecular and Cellular Endocrinology*, *307*(1–2), 99–108. https://doi.org/10.1016/j.mce.2009.02.031

Mayer, C. M., Fick, L. J., Gingerich, S., & Belsham, D. D. (2009). Hypothalamic cell lines to investigate neuroendocrine control mechanisms. *Frontiers in Neuroendocrinology*, *30*(3), 405–423. https://doi.org/10.1016/j.yfrne.2009.03.005

Mendez, N., Halabi, D., Spichiger, C., Salazar, E. R., Vergara, K., Alonso-Vasquez, P., Carmona, P., Sarmiento, J. M., Richter, H. G., Seron-Ferre, M., & Torres-Farfan, C. (2016). Gestational Chronodisruption Impairs Circadian Physiology in Rat Male Offspring, Increasing the Risk of Chronic Disease. *Endocrinology*, *157*(12), 4654–4668. https://doi.org/10.1210/en.2016-1282

Méndez-Hernández, R., Escobar, C., & Buijs, R. M. (2020). Suprachiasmatic Nucleus–Arcuate Nucleus Axis: Interaction Between Time and Metabolism Essential for Health. *Obesity*, *28*(S1). https://doi.org/10.1002/oby.22774

Mering, J., & Minkowski, O. (1890). Diabetes mellitus nach Pankreasexstirpation. *Archiv für Experimentelle Pathologie und Pharmakologie*, *26*(5–6), 371–387. https://doi.org/10.1007/BF01831214

Michiels, C. (2003). Endothelial cell functions. *Journal of Cellular Physiology*, *196*(3), 430–443. https://doi.org/10.1002/jcp.10333

Mieda, M., Williams, S. C., Richardson, J. A., Tanaka, K., & Yanagisawa, M. (2006). The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker. *Proceedings of the National Academy of Sciences*, *103*(32), 12150–12155. https://doi.org/10.1073/pnas.0604189103

Mielke, J. G., Taghibiglou, C., & Wang, Y. T. (2006). Endogenous insulin signaling protects cultured neurons from oxygen–glucose deprivation-induced cell death. *Neuroscience*, *143*(1), 165–173. https://doi.org/10.1016/j.neuroscience.2006.07.055

Mihelakis, M., Ndikung, J., Oelgeschläger, M., & Ertych, N. (2022). The 4th dimension of in vitro systems – Time to level up. *Environment International*, *164*, 107256. https://doi.org/10.1016/j.envint.2022.107256

Miller, T. B., & Larner, J. (1973). Mechanism of control of hepatic glycogenesis by insulin. *The Journal of Biological Chemistry*, *248*(10), 3483–3488.

Mitsui, S., Yamaguchi, S., Matsuo, T., Ishida, Y., & Okamura, H. (2001). Antagonistic role of E4BP4 and PAR proteins in the circadian oscillatory mechanism. *Genes & Development*, *15*(8), 995–1006. https://doi.org/10.1101/gad.873501

Mokhlesi, B., Temple, K. A., Tjaden, A. H., Edelstein, S. L., Utzschneider, K. M., Nadeau, K. J., Hannon, T. S., Sam, S., Barengolts, E., Manchanda, S., Ehrmann, D. A., Van Cauter, E., The RISE Consortium, Ehrmann, D. A., Temple, K. A., Rue, A., Barengolts, E., Mokhlesi, B., Van Cauter, E., … Leschek, E. W. (2019). Association of Self-Reported Sleep and Circadian Measures With Glycemia in Adults With Prediabetes or Recently Diagnosed Untreated Type 2 Diabetes. *Diabetes Care*, *42*(7), 1326–1332. https://doi.org/10.2337/dc19-0298

Montgomery, D. L. (1994). Astrocytes: Form, Functions, and Roles in Disease. *Veterinary Pathology*, *31*(2), 145–167. https://doi.org/10.1177/030098589403100201

Moore, R. Y., & Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Research*, *42*(1), 201–206. https://doi.org/10.1016/0006-8993(72)90054-6

Moore, R. Y., & Lenn, N. J. (1972). A retinohypothalamic projection in the rat. *Journal of Comparative Neurology*, *146*(1), 1–14. https://doi.org/10.1002/cne.901460102

Moore, R. Y., Speh, J. C., & Patrick Card, J. (1995). The retinohypothalamic tract originates from a distinct subset of retinal ganglion cells. *Journal of Comparative Neurology*, *352*(3), 351–366. https://doi.org/10.1002/cne.903520304

Moran-Ramos, S., Baez-Ruiz, A., Buijs, R. M., & Escobar, C. (2016). When to eat? The influence of circadian rhythms on metabolic health: are animal studies providing the evidence? *Nutrition Research Reviews*, *29*(2), 180–193. https://doi.org/10.1017/S095442241600010X

Mori, T., Nagai, K., Hara, M., & Nakagawa, H. (1985). Time-dependent effect of insulin in suprachiasmatic nucleus on blood glucose. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *249*(1), R23–R30. https://doi.org/10.1152/ajpregu.1985.249.1.R23

Morris, J. B., Kenney, B., Huynh, H., & Woodcock, E. A. (2005). Regulation of the Proapoptotic Factor FOXO1 (FKHR) in Cardiomyocytes by Growth Factors and α1-Adrenergic Agonists. *Endocrinology*, *146*(10), 4370–4376. https://doi.org/10.1210/en.2005-0162

Morrison, D. K. (2012). MAP kinase pathways. *Cold Spring Harbor Perspectives in Biology*, *4*(11), a011254. https://doi.org/10.1101/cshperspect.a011254

Morton, G. J., Cummings, D. E., Baskin, D. G., Barsh, G. S., & Schwartz, M. W. (2006). Central nervous system control of food intake and body weight. *Nature*, *443*(7109), 289–295. https://doi.org/10.1038/nature05026

Moruzzi, N., Lazzeri-Barcelo, F., Valladolid-Acebes, I., Moede, T., Paschen, M., Leibiger, B., Berggren, P., & Leibiger, I. B. (2021). Tissue-specific expression of insulin receptor isoforms in obesity/type 2 diabetes mouse models. *Journal of Cellular and Molecular Medicine*, *25*(10), 4800–4813. https://doi.org/10.1111/jcmm.16452

Muniyappa, R., & Sowers, J. R. (2013). Role of insulin resistance in endothelial dysfunction. *Reviews in Endocrine and Metabolic Disorders*, *14*(1), 5–12. https://doi.org/10.1007/s11154-012-9229-1

Nagai, K., Mori, T., Nishio, T., & Nakagawa, H. (1982). EFFECT OF INTRACRANIAL INSULIN INFUSION ON THE CIRCADIAN FEEDING RHYTHM OF RATS[1]. *Biomedical Research*, *3*(2), 175–180. https://doi.org/10.2220/biomedres.3.175

Nakajima, M., Imai, K., Ito, H., Nishiwaki, T., Murayama, Y., Iwasaki, H., Oyama, T., & Kondo, T. (2005). Reconstitution of Circadian Oscillation of Cyanobacterial KaiC Phosphorylation in Vitro. *Science*, *308*(5720), 414–415. https://doi.org/10.1126/science.1108451

Natsubori, A., Honma, K., & Honma, S. (2014). Dual regulation of clock gene *Per2* expression in discrete brain areas by the circadian pacemaker and methamphetamine-induced oscillator in rats. *European Journal of Neuroscience*, *39*(2), 229–240. https://doi.org/10.1111/ejn.12400

Nelson, Thomas. J., Sun, M.-K., Hongpaisan, J., & Alkon, D. L. (2008). Insulin, PKC signaling pathways and synaptic remodeling during memory storage and neuronal repair. *European Journal of Pharmacology*, *585*(1), 76–87. https://doi.org/10.1016/j.ejphar.2008.01.051

Neuner, S. M., Wilmott, L. A., Hope, K. A., Hoffmann, B., Chong, J. A., Abramowitz, J., Birnbaumer, L., O'Connell, K. M., Tryba, A. K., Greene, A. S., Savio Chan, C., & Kaczorowski, C. C. (2015). TRPC3 channels critically regulate hippocampal excitability and contextual fear memory. *Behavioural Brain Research*, *281*, 69–77. https://doi.org/10.1016/j.bbr.2014.12.018

Nguyen, T. T. L., Chan, L. C., Borreginne, K., Kale, R. P., Hu, C., & Tye, S. J. (2018). A review of brain insulin signaling in mood disorders: From biomarker to clinical target. *Neuroscience & Biobehavioral Reviews*, *92*, 7–15. https://doi.org/10.1016/j.neubiorev.2018.05.014

Niswender, K. D., Morrison, C. D., Clegg, D. J., Olson, R., Baskin, D. G., Myers, M. G., Seeley, R. J., & Schwartz, M. W. (2003). Insulin Activation of Phosphatidylinositol 3-Kinase in the Hypothalamic Arcuate Nucleus. *Diabetes*, *52*(2), 227–231. https://doi.org/10.2337/diabetes.52.2.227

Novak, C. M., Harris, J. A., Smale, L., & Nunez, A. A. (2000). Suprachiasmatic nucleus projections to the paraventricular thalamic nucleus in nocturnal rats (Rattus norvegicus) and diurnal nile grass rats (Arviacanthis niloticus). *Brain Research*, *874*(2), 147–157. https://doi.org/10.1016/S0006-8993(00)02572-5

Obici, S., Feng, Z., Karkanias, G., Baskin, D. G., & Rossetti, L. (2002). Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. *Nature Neuroscience*, *5*(6), 566–572. https://doi.org/10.1038/nn0602-861

Obici, S., Zhang, B. B., Karkanias, G., & Rossetti, L. (2002). Hypothalamic insulin signaling is required for inhibition of glucose production. *Nature Medicine*, *8*(12), 1376–1382. https://doi.org/10.1038/nm1202-798

O'Grady, J. P., Dean, D. C., Yang, K. L., Canda, C., Hoscheidt, S. M., Starks, E. J., Merluzzi, A., Hurley, S., Davenport, N. J., Okonkwo, O. C., Anderson, R. M., Asthana, S., Johnson, S. C., Alexander, A. L., & Bendlin, B. B. (2019). Elevated Insulin and Insulin Resistance are Associated with Altered Myelin in Cognitively Unimpaired Middle-Aged Adults. *Obesity*, *27*(9), 1464–1471. https://doi.org/10.1002/oby.22558

Oishi, K., Uchida, D., & Ishida, N. (2008). Circadian expression of *FGF21* is induced by PPARα activation in the mouse liver. *FEBS Letters*, *582*(25–26), 3639–3642. https://doi.org/10.1016/j.febslet.2008.09.046

O'Neill, J. S., & Reddy, A. B. (2011). Circadian clocks in human red blood cells. *Nature*, *469*(7331), 498–503. https://doi.org/10.1038/nature09702

Ono, H. (2019). Molecular Mechanisms of Hypothalamic Insulin Resistance. *International Journal of Molecular Sciences*, *20*(6), 1317. https://doi.org/10.3390/ijms20061317

Opperhuizen, A., Wang, D., Foppen, E., Jansen, R., Boudzovitch-Surovtseva, O., De Vries, J., Fliers, E., & Kalsbeek, A. (2016). Feeding during the resting phase causes profound changes in

physiology and desynchronization between liver and muscle rhythms of rats. *European Journal of Neuroscience*, *44*(10), 2795–2806. https://doi.org/10.1111/ejn.13377

Opperhuizen, A.-L., Foppen, E., Jonker, M., Wackers, P., Van Faassen, M., Van Weeghel, M., Van Kerkhof, L., Fliers, E., & Kalsbeek, A. (2019). Effects of Light-at-Night on the Rat Liver – A Role for the Autonomic Nervous System. *Frontiers in Neuroscience*, *13*, 647. https://doi.org/10.3389/fnins.2019.00647

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffmann, M. W., & Eichele, G. (2006). The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metabolism*, *4*(2), 163–173. https://doi.org/10.1016/j.cmet.2006.07.002

Otalora, B. B., Hagenauer, M. H., Rol, M. A., Madrid, J. A., & Lee, T. M. (2013). *Period* Gene Expression in the Brain of a Dual-Phasing Rodent, the *Octodon degus*. *Journal of Biological Rhythms*, *28*(4), 249–261. https://doi.org/10.1177/0748730413495521

Otto-Buczkowska, E., Medical Specialist Centre in Gliwice, Grzyb, K., Medical University of Silesia in Katowice, Poland, Jainta, N., & Medical University of Silesia in Katowice, Poland. (2018). Polycystic ovary syndrome (PCOS) and the accompanying disorders of glucose homeostasis among girls at the time of puberty. *Pediatric Endocrinology Diabetes and Metabolism*, *24*(1), 40–44. https://doi.org/10.18544/PEDM-24.01.0101

Otway, D. T., Mäntele, S., Bretschneider, S., Wright, J., Trayhurn, P., Skene, D. J., Robertson, M. D., & Johnston, J. D. (2011). Rhythmic Diurnal Gene Expression in Human Adipose Tissue From Individuals Who Are Lean, Overweight, and Type 2 Diabetic. *Diabetes*, *60*(5), 1577–1581. https://doi.org/10.2337/db10-1098

Palomar-Cros, A., Srour, B., Andreeva, V. A., Fezeu, L. K., Bellicha, A., Kesse-Guyot, E., Hercberg, S., Romaguera, D., Kogevinas, M., & Touvier, M. (2023). Associations of meal timing, number of eating occasions and night-time fasting duration with incidence of type 2 diabetes in the NutriNet-Santé cohort. *International Journal of Epidemiology*, *52*(5), 1486–1497. https://doi.org/10.1093/ije/dyad081

Pang, K. S., & Chiba, M. (1994). Metabolism: Scaling-up from In Vitro to Organ and Whole Body. In P. G. Welling & L. P. Balant (Eds.), *Pharmacokinetics of Drugs* (Vol. 110, pp. 101–187). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-78680-8_5

Paranjape, S. A., Chan, O., Zhu, W., Horblitt, A. M., Grillo, C. A., Wilson, S., Reagan, L., & Sherwin, R. S. (2011). Chronic reduction of insulin receptors in the ventromedial hypothalamus produces glucose intolerance and islet dysfunction in the absence of weight gain. *American Journal of Physiology-Endocrinology and Metabolism*, *301*(5), E978–E983. https://doi.org/10.1152/ajpendo.00304.2011

Park, L. C. H., Zhang, H., & Gibson, G. E. (2001). Co-culture with astrocytes or microglia protects metabolically impaired neurons. *Mechanisms of Ageing and Development*, *123*(1), 21–27. https://doi.org/10.1016/s0047-6374(01)00336-0

Partch, C. L., Green, C. B., & Takahashi, J. S. (2014). Molecular architecture of the mammalian circadian clock. *Trends in Cell Biology*, *24*(2), 90–99. https://doi.org/10.1016/j.tcb.2013.07.002

Perelis, M., Marcheva, B., Moynihan Ramsey, K., Schipma, M. J., Hutchison, A. L., Taguchi, A., Peek, C. B., Hong, H., Huang, W., Omura, C., Allred, A. L., Bradfield, C. A., Dinner, A. R., Barish, G. D., & Bass, J. (2015). Pancreatic β cell enhancers regulate rhythmic transcription of genes controlling insulin secretion. *Science*, *350*(6261), aac4250. https://doi.org/10.1126/science.aac4250

Pevet, P., & Challet, E. (2011). Melatonin: Both master clock output and internal time-giver in the circadian clocks network. *Journal of Physiology-Paris*, *105*(4–6), 170–182. https://doi.org/10.1016/j.jphysparis.2011.07.001

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), 45e–445. https://doi.org/10.1093/nar/29.9.e45

Pickel, L., & Sung, H.-K. (2020). Feeding Rhythms and the Circadian Regulation of Metabolism. *Frontiers in Nutrition*, *7*, 39. https://doi.org/10.3389/fnut.2020.00039

Pilorz, V., Kolms, B., & Oster, H. (2020). Rapid Jetlag Resetting of Behavioral, Physiological, and Molecular Rhythms in Proestrous Female Mice. *Journal of Biological Rhythms*, *35*(6), 612–627. https://doi.org/10.1177/0748730420965291

Pittendrigh, C. S. (1954). ON TEMPERATURE INDEPENDENCE IN THE CLOCK SYSTEM CONTROLLING EMERGENCE TIME IN DROSOPHILA. *Proceedings of the National Academy of Sciences*, *40*(10), 1018–1029. https://doi.org/10.1073/pnas.40.10.1018

Plum, L., Lin, H. V., Dutia, R., Tanaka, J., Aizawa, K. S., Matsumoto, M., Kim, A. J., Cawley, N. X., Paik, J.-H., Loh, Y. P., DePinho, R. A., Wardlaw, S. L., & Accili, D. (2009). The obesity susceptibility gene Cpe links FoxO1 signaling in hypothalamic pro-opiomelanocortin neurons with regulation of food intake. *Nature Medicine*, *15*(10), 1195–1201. https://doi.org/10.1038/nm.2026

Poggiogalle, E., Jamshed, H., & Peterson, C. M. (2018). Circadian regulation of glucose, lipid, and energy metabolism in humans. *Metabolism*, *84*, 11–27. https://doi.org/10.1016/j.metabol.2017.11.017

Pomytkin, I., Costa-Nunes, J. P., Kasatkin, V., Veniaminova, E., Demchenko, A., Lyundup, A., Lesch, K., Ponomarev, E. D., & Strekalova, T. (2018). Insulin receptor in the brain: Mechanisms of activation and the role in the CNS pathology and treatment. *CNS Neuroscience & Therapeutics*, *24*(9), 763–774. https://doi.org/10.1111/cns.12866

Porniece Kumar, M., Cremer, A. L., Klemm, P., Steuernagel, L., Sundaram, S., Jais, A., Hausen, A. C., Tao, J., Secher, A., Pedersen, T. Å., Schwaninger, M., Wunderlich, F. T., Lowell, B. B., Backes, H., & Brüning, J. C. (2021a). Insulin signalling in tanycytes gates hypothalamic insulin uptake and regulation of AgRP neuron activity. *Nature Metabolism*, *3*(12), 1662–1679. https://doi.org/10.1038/s42255-021-00499-0

Porniece Kumar, M., Cremer, A. L., Klemm, P., Steuernagel, L., Sundaram, S., Jais, A., Hausen, A. C., Tao, J., Secher, A., Pedersen, T. Å., Schwaninger, M., Wunderlich, F. T., Lowell, B. B., Backes, H., & Brüning, J. C. (2021b). Insulin signalling in tanycytes gates hypothalamic insulin uptake and regulation of AgRP neuron activity. *Nature Metabolism*, *3*(12), 1662–1679. https://doi.org/10.1038/s42255-021-00499-0

Porte, D., & Woods, S. C. (1981). Regulation of food intake and body weight by insulin. *Diabetologia*, *20*(S1), 274–280. https://doi.org/10.1007/BF00254493

Powers, R. M., Hevner, R. F., & Halpain, S. (2023). The Neuron Navigators: Structure, function, and evolutionary history. *Frontiers in Molecular Neuroscience*, *15*, 1099554. https://doi.org/10.3389/fnmol.2022.1099554

Preitner, N., Damiola, F., Luis-Lopez-Molina, Zakany, J., Duboule, D., Albrecht, U., & Schibler, U. (2002). The Orphan Nuclear Receptor REV-ERBα Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator. *Cell*, *110*(2), 251–260. https://doi.org/10.1016/S0092-8674(02)00825-5

Qiu, J., Wagner, E. J., Rønnekleiv, O. K., & Kelly, M. J. (2018a). Insulin and leptin excite anorexigenic pro-opiomelanocortin neurones via activation of TRPC 5 channels. *Journal of Neuroendocrinology*, *30*(2), e12501. https://doi.org/10.1111/jne.12501

Qiu, J., Wagner, E. J., Rønnekleiv, O. K., & Kelly, M. J. (2018b). Insulin and leptin excite anorexigenic pro-opiomelanocortin neurones via activation of TRPC5 channels. *Journal of Neuroendocrinology*, *30*(2). https://doi.org/10.1111/jne.12501

Qiu, J., Zhang, C., Borgquist, A., Nestor, C. C., Smith, A. W., Bosch, M. A., Ku, S., Wagner, E. J., Rønnekleiv, O. K., & Kelly, M. J. (2014). Insulin Excites Anorexigenic Proopiomelanocortin Neurons via Activation of Canonical Transient Receptor Potential Channels. *Cell Metabolism*, *19*(4), 682–693. https://doi.org/10.1016/j.cmet.2014.03.004

Qiu, W. Q., Walsh, D. M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M. B., Rosner, M. R., Safavi, A., Hersh, L. B., & Selkoe, D. J. (1998). Insulin-degrading Enzyme Regulates Extracellular Levels of Amyloid β-Protein by Degradation. *Journal of Biological Chemistry*, *273*(49), 32730–32738. https://doi.org/10.1074/jbc.273.49.32730

Rachana, K. S., Manu, M. S., & Advirao, G. M. (2016). Insulin influenced expression of myelin proteins in diabetic peripheral neuropathy. *Neuroscience Letters*, *629*, 110–115. https://doi.org/10.1016/j.neulet.2016.06.067

Rafiei, N., Mitchell, C. S., Tedesco, C. R., Chen, J., Choi, E. A., Roughley, S., Jean-Richard-dit-Bressel, P., Kumar, N. N., McNally, G. P., Herzog, H., & Begg, D. P. (2024). Chemogenetic activation of arcuate nucleus NPY and NPY/AgRP neurons increases feeding behaviour in mice. *Neuropeptides*, *107*, 102454. https://doi.org/10.1016/j.npep.2024.102454

Ralph, M. R., Foster, R. G., Davis, F. C., & Menaker, M. (1990). Transplanted Suprachiasmatic Nucleus Determines Circadian Period. *Science*, *247*(4945), 975–978. https://doi.org/10.1126/science.2305266

Ralph, M. R., & Menaker, M. (1988). A Mutation of the Circadian System in Golden Hamsters. *Science*, *241*(4870), 1225–1227. https://doi.org/10.1126/science.3413487

Ramalingam, M., Kwon, Y.-D., & Kim, S.-J. (2016). Insulin as a Potent Stimulator of Akt, ERK and Inhibin-βE Signaling in Osteoblast-Like UMR-106 Cells. *Biomolecules & Therapeutics*, *24*(6), 589–594. https://doi.org/10.4062/biomolther.2016.030

Ramanathan, C., Kathale, N. D., Liu, D., Lee, C., Freeman, D. A., Hogenesch, J. B., Cao, R., & Liu, A. C. (2018). mTOR signaling regulates central and peripheral circadian clock function. *PLOS Genetics*, *14*(5), e1007369. https://doi.org/10.1371/journal.pgen.1007369

Ramsey, K. M., Yoshino, J., Brace, C. S., Abrassart, D., Kobayashi, Y., Marcheva, B., Hong, H.-K., Chong, J. L., Buhr, E. D., Lee, C., Takahashi, J. S., Imai, S., & Bass, J. (2009). Circadian Clock Feedback Cycle Through NAMPT-Mediated NAD^+ Biosynthesis. *Science*, *324*(5927), 651–654. https://doi.org/10.1126/science.1171641

Ramzy, A., Mojibian, M., & Kieffer, T. J. (2018). Insulin-Deficient Mouse β-Cells Do Not Fully Mature but Can Be Remedied Through Insulin Replacement by Islet Transplantation. *Endocrinology*, *159*(1), 83–102. https://doi.org/10.1210/en.2017-00263

Rath, M. F., Rohde, K., Fahrenkrug, J., & Møller, M. (2013). Circadian clock components in the rat neocortex: Daily dynamics, localization and regulation. *Brain Structure and Function*, *218*(2), 551–562. https://doi.org/10.1007/s00429-012-0415-4

Rath, M. F., Rovsing, L., & Møller, M. (2014). Circadian oscillators in the mouse brain: Molecular clock components in the neocortex and cerebellar cortex. *Cell and Tissue Research*, *357*(3), 743–755. https://doi.org/10.1007/s00441-014-1878-9

Reger, M. A., Watson, G. S., Green, P. S., Baker, L. D., Cholerton, B., Fishel, M. A., Plymate, S. R., Cherrier, M. M., Schellenberg, G. D., Frey Ii, W. H., & Craft, S. (2008). Intranasal Insulin Administration Dose-Dependently Modulates Verbal Memory and Plasma Amyloid-β in Memory-Impaired Older Adults. *Journal of Alzheimer's Disease*, *13*(3), 323–331. https://doi.org/10.3233/JAD-2008-13309

Reiter, R. J. (1995). The role of the neurohormone melatonin as a buffer against macromolecular oxidative damage. *Neurochemistry International*, *27*(6), 453–460. https://doi.org/10.1016/0197-0186(95)80002-3

Reiter, R. J., Tan, D., Osuna, C., & Gitto, E. (2000). Actions of Melatonin in the Reduction of Oxidative Stress. *Journal of Biomedical Science*, *7*(6), 444–458. https://doi.org/10.1159/000025480

Remchak, M.-M. E., Heiston, E. M., Ballantyne, A., Dotson, B. L., Stewart, N. R., Spaeth, A. M., & Malin, S. K. (2022). Insulin Sensitivity and Metabolic Flexibility Parallel Plasma TCA Levels in Early Chronotype With Metabolic Syndrome. *The Journal of Clinical Endocrinology & Metabolism*, *107*(8), e3487–e3496. https://doi.org/10.1210/clinem/dgac233

Ren, H., Orozco, I. J., Su, Y., Suyama, S., Gutiérrez-Juárez, R., Horvath, T. L., Wardlaw, S. L., Plum, L., Arancio, O., & Accili, D. (2012). FoxO1 Target Gpr17 Activates AgRP Neurons to Regulate Food Intake. *Cell*, *149*(6), 1314–1326. https://doi.org/10.1016/j.cell.2012.04.032

Reno, C. M., Puente, E. C., Sheng, Z., Daphna-Iken, D., Bree, A. J., Routh, V. H., Kahn, B. B., & Fisher, S. J. (2017). Brain GLUT4 Knockout Mice Have Impaired Glucose Tolerance, Decreased Insulin Sensitivity, and Impaired Hypoglycemic Counterregulation. *Diabetes*, *66*(3), 587–597. https://doi.org/10.2337/db16-0917

Rhee, Y.-H., Choi, M., Lee, H.-S., Park, C.-H., Kim, S.-M., Yi, S.-H., Oh, S.-M., Cha, H.-J., Chang, M.-Y., & Lee, S.-H. (2013). Insulin concentration is critical in culturing human neural stem cells and neurons. *Cell Death & Disease*, *4*(8), e766–e766. https://doi.org/10.1038/cddis.2013.295

Rodríguez-Cortés, B., Hurtado-Alvarado, G., Martínez-Gómez, R., León-Mercado, L. A., Prager-Khoutorsky, M., & Buijs, R. M. (2022). Suprachiasmatic nucleus-mediated glucose entry into the arcuate nucleus determines the daily rhythm in blood glycemia. *Current Biology*, *32*(4), 796-805.e4. https://doi.org/10.1016/j.cub.2021.12.039

Rosin, J. M., McAllister, B. B., Dyck, R. H., Percival, C. J., Kurrasch, D. M., & Cobb, J. (2015). Mice lacking the transcription factor SHOX2 display impaired cerebellar development and deficits in motor coordination. *Developmental Biology*, *399*(1), 54–67. https://doi.org/10.1016/j.ydbio.2014.12.013

Ruegsegger, G. N., Creo, A. L., Cortes, T. M., Dasari, S., & Nair, K. S. (2018). Altered mitochondrial function in insulin-deficient and insulin-resistant states. *Journal of Clinical Investigation*, *128*(9), 3671–3681. https://doi.org/10.1172/JCI120843

Russart, K. L. G., Chbeir, S. A., Nelson, R. J., & Magalang, U. J. (2019). Light at night exacerbates metabolic dysfunction in a polygenic mouse model of type 2 diabetes mellitus. *Life Sciences*, *231*, 116574. https://doi.org/10.1016/j.lfs.2019.116574

Saini, C., Petrenko, V., Pulimeno, P., Giovannoni, L., Berney, T., Hebrok, M., Howald, C., Dermitzakis, E. T., & Dibner, C. (2016). A functional circadian clock is required for proper insulin secretion by human pancreatic islet cells. *Diabetes, Obesity and Metabolism*, *18*(4), 355–365. https://doi.org/10.1111/dom.12616

Sainsbury, A., Cooney, G. J., & Herzog, H. (2002). Hypothalamic regulation of energy homeostasis. *Best Practice & Research Clinical Endocrinology & Metabolism*, *16*(4), 623–637. https://doi.org/10.1053/beem.2002.0230

Saiz, N., Velasco, C., De Pedro, N., Soengas, J. L., & Isorna, E. (2023). Insulin Controls Clock Gene Expression in the Liver of Goldfish Probably via Pi3k/Akt Pathway. *International Journal of Molecular Sciences*, *24*(15), 11897. https://doi.org/10.3390/ijms241511897

Salkovic-Petrisic, M., & Hoyer, S. (2007). Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: An experimental approach. In M. Gerlach, J. Deckert, K. Double, & E. Koutsilieri (Eds.), *Neuropsychiatric Disorders An Integrative Approach* (pp. 217–233). Springer Vienna. https://doi.org/10.1007/978-3-211-73574-9_28

Saneto, R. P., Low, K. G., Melner, M. H., & De Vellis, J. (1988). Insulin/insulin-like growth factor I and other epigenetic modulators of myelin basic protein expression in isolated oligodendrocyte

progenitor cells. *Journal of Neuroscience Research*, *21*(2–4), 210–219. https://doi.org/10.1002/jnr.490210213

Saneyasu, T., Fujita, S., Kitashiro, A., Fukuzo, S., Honda, K., & Kamisoyama, H. (2018). Hypothalamic Akt-mediated signaling regulates food intake in chicks. *Neuroscience Letters*, *670*, 48–52. https://doi.org/10.1016/j.neulet.2018.01.032

Sasaoka, T., & Kobayashi, M. (2000). The Functional Significance of Shc in Insulin Signaling as a Substrate of the Insulin Receptor. *Endocrine Journal*, *47*(4), 373–381. https://doi.org/10.1507/endocrj.47.373

Sato, I., Arima, H., Ozaki, N., Watanabe, M., Goto, M., Hayashi, M., Banno, R., Nagasaki, H., & Oiso, Y. (2005). Insulin Inhibits Neuropeptide Y Gene Expression in the Arcuate Nucleus through GABAergic Systems. *The Journal of Neuroscience*, *25*(38), 8657–8664. https://doi.org/10.1523/JNEUROSCI.2739-05.2005

Sato, M., Murakami, M., Node, K., Matsumura, R., & Akashi, M. (2014). The Role of the Endocrine System in Feeding-Induced Tissue-Specific Circadian Entrainment. *Cell Reports*, *8*(2), 393–401. https://doi.org/10.1016/j.celrep.2014.06.015

Sato, T. K., Panda, S., Miraglia, L. J., Reyes, T. M., Rudic, R. D., McNamara, P., Naik, K. A., FitzGerald, G. A., Kay, S. A., & Hogenesch, J. B. (2004). A Functional Genomics Strategy Reveals Rora as a Component of the Mammalian Circadian Clock. *Neuron*, *43*(4), 527–537. https://doi.org/10.1016/j.neuron.2004.07.018

Sayar-Atasoy, N., Aklan, I., Yavuz, Y., Laule, C., Kim, H., Rysted, J., Alp, M. I., Davis, D., Yilmaz, B., & Atasoy, D. (2024). AgRP neurons encode circadian feeding time. *Nature Neuroscience*, *27*(1), 102–115. https://doi.org/10.1038/s41593-023-01482-6

Schapira, A. H. V., Olanow, C. W., Greenamyre, J. T., & Bezard, E. (2014). Slowing of neurodegeneration in Parkinson's disease and Huntington's disease: Future therapeutic perspectives. *The Lancet*, *384*(9942), 545–555. https://doi.org/10.1016/S0140-6736(14)61010-2

Schechter, R., Beju, D., & Miller, K. E. (2005). The effect of insulin deficiency on tau and neurofilament in the insulin knockout mouse. *Biochemical and Biophysical Research Communications*, *334*(4), 979–986. https://doi.org/10.1016/j.bbrc.2005.07.001

Schwartz, M. W., Bergman, R. N., Kahn, S. E., Taborsky, G. J., Fisher, L. D., Sipols, A. J., Woods, S. C., Steil, G. M., & Porte, D. (1991). Evidence for entry of plasma insulin into cerebrospinal fluid through an intermediate compartment in dogs. Quantitative aspects and implications for transport. *Journal of Clinical Investigation*, *88*(4), 1272–1281. https://doi.org/10.1172/JCI115431

Schwartz, M. W., Sipols, A. J., Marks, J. L., Sanacora, G., White, J. D., Scheurink, A., Kahn, S. E., Baskin, D. G., Woods, S. C., & Figlewicz, D. P. (1992). Inhibition of hypothalamic neuropeptide Y gene expression by insulin. *Endocrinology*, *130*(6), 3608–3616. https://doi.org/10.1210/endo.130.6.1597158

Schwartz, M. W., Woods, S. C., Porte, D., Seeley, R. J., & Baskin, D. G. (2000). Central nervous system control of food intake. *Nature*, *404*(6778), 661–671. https://doi.org/10.1038/35007534

Segall, L. A., Perrin, J. S., Walker, C.-D., Stewart, J., & Amir, S. (2006). Glucocorticoid rhythms control the rhythm of expression of the clock protein, Period2, in oval nucleus of the bed nucleus of the stria terminalis and central nucleus of the amygdala in rats. *Neuroscience*, *140*(3), 753–757. https://doi.org/10.1016/j.neuroscience.2006.03.037

Seigler, D. E., Olsson, G. M., & Skyler, J. S. (1992). Morning versus Bedtime Isophane Insulin in Type 2 (Non-insulin dependent) Diabetes Mellitus. *Diabetic Medicine*, *9*(9), 826–833. https://doi.org/10.1111/j.1464-5491.1992.tb01901.x

Selvaraj, S., Sun, Y., & Singh, B. (2010). TRPC Channels and their Implications for Neurological Diseases. *CNS & Neurological Disorders - Drug Targets*, *9*(1), 94–104. https://doi.org/10.2174/187152710790966650

Shan, Z., Ma, H., Xie, M., Yan, P., Guo, Y., Bao, W., Rong, Y., Jackson, C. L., Hu, F. B., & Liu, L. (2015). Sleep Duration and Risk of Type 2 Diabetes: A Meta-analysis of Prospective Studies. *Diabetes Care*, *38*(3), 529–537. https://doi.org/10.2337/dc14-2073

Shepherd, P. R., Withers, D. J., & Siddle, K. (1998). Phosphoinositide 3-kinase: The key switch mechanism in insulin signalling. *Biochemical Journal*, *333*(3), 471–490. https://doi.org/10.1042/bj3330471

Shibata, S., Liou, S. Y., Ueki, S., & Oomura, Y. (1986). Inhibitory action of insulin on suprachiasmatic nucleus neurons in rat hypothalamic slice preparations. *Physiology & Behavior*, *36*(1), 79–81. https://doi.org/10.1016/0031-9384(86)90077-6

Shimada, M., Tritos, N. A., Lowell, B. B., Flier, J. S., & Maratos-Flier, E. (1998). Mice lacking melanin-concentrating hormone are hypophagic and lean. *Nature*, *396*(6712), 670–674. https://doi.org/10.1038/25341

Shin, A. C., Filatova, N., Lindtner, C., Chi, T., Degann, S., Oberlin, D., & Buettner, C. (2017). Insulin Receptor Signaling in POMC, but Not AgRP, Neurons Controls Adipose Tissue Insulin Action. *Diabetes*, *66*(6), 1560–1571. https://doi.org/10.2337/db16-1238

Shirogane, T., Jin, J., Ang, X. L., & Harper, J. W. (2005). SCFβ-TRCP Controls Clock-dependent Transcription via Casein Kinase 1-dependent Degradation of the Mammalian Period-1 (Per1) Proteinm. *Journal of Biological Chemistry*, *280*(29), 26863–26872. https://doi.org/10.1074/jbc.M502862200

Sidoryk-Wegrzynowicz, M., Wegrzynowicz, M., Lee, E., Bowman, A. B., & Aschner, M. (2011). Role of Astrocytes in Brain Function and Disease. *Toxicologic Pathology*, *39*(1), 115–123. https://doi.org/10.1177/0192623310385254

Simon, K., & Wittmann, I. (2019). Can blood glucose value really be referred to as a metabolic parameter? *Reviews in Endocrine and Metabolic Disorders*, *20*(2), 151–160. https://doi.org/10.1007/s11154-019-09504-0

Sindelar, D. K., Balcom, J. H., Chu, C. A., Neal, D. W., & Cherrington, A. D. (1996). A Comparison of the Effects of Selective Increases in Peripheral or Portal insulin on Hepatic Glucose Production in the Conscious Dog. *Diabetes*, *45*(11), 1594–1604. https://doi.org/10.2337/diab.45.11.1594

Sindelar, D. K., Chu, C. A., Rohlie, M., Neal, D. W., Swift, L. L., & Cherrington, A. D. (1997). The Role of Fatty Acids in Mediating the Effects of Peripheral Insulin on Hepatic Glucose Production in the Conscious Dog. *Diabetes*, *46*(2), 187–196. https://doi.org/10.2337/diab.46.2.187

Sliwowska, J. H., Fergani, C., Gawałek, M., Skowronska, B., Fichna, P., & Lehman, M. N. (2014). Insulin: Its role in the central control of reproduction. *Physiology & Behavior*, *133*, 197–206. https://doi.org/10.1016/j.physbeh.2014.05.021

Smith, G. D., Pangborn, W. A., & Blessing, R. H. (2003). The structure of T_6 human insulin at 1.0 Å resolution. *Acta Crystallographica Section D Biological Crystallography*, *59*(3), 474–482. https://doi.org/10.1107/S0907444902023685

Smith, U. (2002). Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance—Is insulin resistance initiated in the adipose tissue? *International Journal of Obesity*, *26*(7), 897–904. https://doi.org/10.1038/sj.ijo.0802028

Sooriyaarachchi, P., Jayawardena, R., Pavey, T., & King, N. A. (2022). Shift work and the risk for metabolic syndrome among healthcare workers: A systematic review and meta-analysis. *Obesity Reviews*, *23*(10), e13489. https://doi.org/10.1111/obr.13489

Speksnijder, E. M., Bisschop, P. H., Siegelaar, S. E., Stenvers, D. J., & Kalsbeek, A. (2024). Circadian desynchrony and glucose metabolism. *Journal of Pineal Research*, *76*(4), e12956. https://doi.org/10.1111/jpi.12956

Spencer, R. L., Chun, L. E., Hartsock, M. J., & Woodruff, E. R. (2018). Glucocorticoid hormones are both a major circadian signal and major stress signal: How this shared signal contributes to a dynamic relationship between the circadian and stress systems. *Frontiers in Neuroendocrinology*, *49*, 52–71. https://doi.org/10.1016/j.yfrne.2017.12.005

Srimani, S., Schmidt, C. X., Gómez-Serranillos, M. P., Oster, H., & Divakar, P. K. (2022). Modulation of Cellular Circadian Rhythms by Secondary Metabolites of Lichens. *Frontiers in Cellular Neuroscience*, *16*, 907308. https://doi.org/10.3389/fncel.2022.907308

Standl, E., Maxeiner, S., Raptis, S., Karimi-Anderesi, Z., Schweitzer, M. A., & the HOE901/4009 Study Group. (2005). Good Glycemic Control With Flexibility in Timing of Basal Insulin Supply. *Diabetes Care*, *28*(2), 419–420. https://doi.org/10.2337/diacare.28.2.419

Stanton, D., Justin, H. S., & Reitzel, A. M. (2022). Step in Time: Conservation of Circadian Clock Genes in Animal Evolution. *Integrative and Comparative Biology*, *62*(6), 1503–1518. https://doi.org/10.1093/icb/icac140

Steiner, D. F., Cunningham, D., Spigelman, L., & Aten, B. (1967). Insulin Biosynthesis: Evidence for a Precursor. *Science*, *157*(3789), 697–700. https://doi.org/10.1126/science.157.3789.697

Steiner, D. F., & Oyer, P. E. (1967). THE BIOSYNTHESIS OF INSULIN AND A PROBABLE PRECURSOR OF INSULIN BY A HUMAN ISLET CELL ADENOMA. *Proceedings of the National Academy of Sciences*, *57*(2), 473–480. https://doi.org/10.1073/pnas.57.2.473

Stenvers, D. J., Scheer, F. A. J. L., Schrauwen, P., La Fleur, S. E., & Kalsbeek, A. (2019). Circadian clocks and insulin resistance. *Nature Reviews Endocrinology*, *15*(2), 75–89. https://doi.org/10.1038/s41574-018-0122-1

Stephan, F. K., & Zucker, I. (1972). Circadian Rhythms in Drinking Behavior and Locomotor Activity of Rats Are Eliminated by Hypothalamic Lesions. *Proceedings of the National Academy of Sciences*, *69*(6), 1583–1586. https://doi.org/10.1073/pnas.69.6.1583

Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R. J., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., & Hawkins, P. T. (1997). Dual Role of Phosphatidylinositol-3,4,5-trisphosphate in the Activation of Protein Kinase B. *Science*, *277*(5325), 567–570. https://doi.org/10.1126/science.277.5325.567

Sujino, M., Masumoto, K., Yamaguchi, S., Van Der Horst, G. T. J., Okamura, H., & Inouye, S.-I. T. (2003). Suprachiasmatic Nucleus Grafts Restore Circadian Behavioral Rhythms of Genetically Arrhythmic Mice. *Current Biology*, *13*(8), 664–668. https://doi.org/10.1016/S0960-9822(03)00222-7

Supek, F., Bošnjak, M., Škunca, N., & Šmuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. *PloS One*, *6*(7), e21800. https://doi.org/10.1371/journal.pone.0021800

Sutherland, C., Leighton, I. A., & Cohen, P. (1993). Inactivation of glycogen synthase kinase-3 β by phosphorylation: New kinase connections in insulin and growth-factor signalling. *Biochemical Journal*, *296*(1), 15–19. https://doi.org/10.1042/bj2960015

Sutherland, E. W., & Cori, C. F. (1948). Influence of insulin preparations on glycogenolysis in liver slices. *The Journal of Biological Chemistry*, *172*(2), 737–750.

Sutton, E. F., Beyl, R., Early, K. S., Cefalu, W. T., Ravussin, E., & Peterson, C. M. (2018a). Early Time-Restricted Feeding Improves Insulin Sensitivity, Blood Pressure, and Oxidative Stress Even without Weight Loss in Men with Prediabetes. *Cell Metabolism*, *27*(6), 1212-1221.e3. https://doi.org/10.1016/j.cmet.2018.04.010

Sutton, E. F., Beyl, R., Early, K. S., Cefalu, W. T., Ravussin, E., & Peterson, C. M. (2018b). Early Time-Restricted Feeding Improves Insulin Sensitivity, Blood Pressure, and Oxidative Stress Even without Weight Loss in Men with Prediabetes. *Cell Metabolism*, *27*(6), 1212-1221.e3. https://doi.org/10.1016/j.cmet.2018.04.010

Tahara, Y., Otsuka, M., Fuse, Y., Hirao, A., & Shibata, S. (2011). Refeeding after Fasting Elicits Insulin-Dependent Regulation of *Per2* and *Rev-erbα* with Shifts in the Liver Clock. *Journal of Biological Rhythms*, *26*(3), 230–240. https://doi.org/10.1177/0748730411405958

Takahashi, J. S., Hamm, H., & Menaker, M. (1980). Circadian rhythms of melatonin release from individual superfused chicken pineal glands in vitro. *Proceedings of the National Academy of Sciences*, *77*(4), 2319–2322. https://doi.org/10.1073/pnas.77.4.2319

Tang, R., Vargas-Medrano, J., Ramos, E., Thompson, P., & Gadad, B. (2022). Gene Expression Analysis of CCL2, MOBP and OPALIN in Major Depressive Disorder and Suicidality (P5-6.005). *Neurology*, *98*(18_supplement). https://doi.org/10.1212/wnl.98.18_supplement.3266

Teng, X.-Y., Hu, P., Zhang, C.-M., Zhang, Q.-X., Yang, G., Zang, Y.-Y., Liu, Z.-X., Chen, G., & Shi, Y. S. (2024). OPALIN is an LGI1 receptor promoting oligodendrocyte differentiation. *Proceedings of the National Academy of Sciences*, *121*(32). https://doi.org/10.1073/pnas.2403652121

Thomas, A. P., Hoang, J., Vongbunyong, K., Nguyen, A., Rakshit, K., & Matveyenko, A. V. (2016). Administration of Melatonin and Metformin Prevents Deleterious Effects of Circadian Disruption and Obesity in Male Rats. *Endocrinology*, *157*(12), 4720–4731. https://doi.org/10.1210/en.2016-1309

Thomas, D. D., Corkey, B. E., Istfan, N. W., & Apovian, C. M. (2019). Hyperinsulinemia: An Early Indicator of Metabolic Dysfunction. *Journal of the Endocrine Society*, *3*(9), 1727–1747. https://doi.org/10.1210/js.2019-00065

Timper, K., & Brüning, J. C. (2017). Hypothalamic circuits regulating appetite and energy homeostasis: Pathways to obesity. *Disease Models & Mechanisms*, *10*(6), 679–689. https://doi.org/10.1242/dmm.026609

Tosini, G., & Menaker, M. (1996). Circadian Rhythms in Cultured Mammalian Retina. *Science*, *272*(5260), 419–421. https://doi.org/10.1126/science.272.5260.419

Triqueneaux, G., Thenot, S., Kakizawa, T., Antoch, M. P., Safi, R., Takahashi, J. S., Delaunay, F., & Laudet, V. (2004). The orphan receptor Rev-erbα gene is a target of the circadian clock pacemaker. *Journal of Molecular Endocrinology*, *33*(3), 585–608. https://doi.org/10.1677/jme.1.01554

Tsang, A. H., Koch, C. E., Kiehn, J.-T., Schmidt, C. X., & Oster, H. (2020a). An adipokine feedback regulating diurnal food intake rhythms in mice. *eLife*, *9*, e55388. https://doi.org/10.7554/eLife.55388

Tsang, A. H., Koch, C. E., Kiehn, J.-T., Schmidt, C. X., & Oster, H. (2020b). An adipokine feedback regulating diurnal food intake rhythms in mice. *eLife*, *9*, e55388. https://doi.org/10.7554/eLife.55388

Tsuneki, H., Tokai, E., Sugawara, C., Wada, T., Sakurai, T., & Sasaoka, T. (2013). Hypothalamic orexin prevents hepatic insulin resistance induced by social defeat stress in mice. *Neuropeptides*, *47*(3), 213–219. https://doi.org/10.1016/j.npep.2013.02.002

Turek, F. W., McMillan, J. P., & Menaker, M. (1976). Melatonin: Effects on the Circadian Locomotor Rhythm of Sparrows. *Science*, *194*(4272), 1441–1443. https://doi.org/10.1126/science.1006311

Türk, D., Scherer, N., Selzer, D., Dings, C., Hanke, N., Dallmann, R., Schwab, M., Timmins, P., Nock, V., & Lehr, T. (2023). Significant impact of time-of-day variation on metformin

pharmacokinetics. *Diabetologia*, *66*(6), 1024–1034. https://doi.org/10.1007/s00125-023-05898-4

Tuvia, N., Pivovarova-Ramich, O., Murahovschi, V., Lück, S., Grudziecki, A., Ost, A.-C., Kruse, M., Nikiforova, V. J., Osterhoff, M., Gottmann, P., Gögebakan, Ö., Sticht, C., Gretz, N., Schupp, M., Schürmann, A., Rudovich, N., Pfeiffer, A. F. H., & Kramer, A. (2021). Insulin Directly Regulates the Circadian Clock in Adipose Tissue. *Diabetes*, *70*(9), 1985–1999. https://doi.org/10.2337/db20-0910

Tyagi, S., Salier, J.-P., & Lal, S. K. (2002). The Liver-Specific Human α1-Microglobulin/Bikunin Precursor (AMBP) Is Capable of Self-Association. *Archives of Biochemistry and Biophysics*, *399*(1), 66–72. https://doi.org/10.1006/abbi.2001.2745

Uchoa, E. T., Aguilera, G., Herman, J. P., Fiedler, J. L., Deak, T., & De Sousa, M. B. C. (2014). Novel Aspects of Glucocorticoid Actions. *Journal of Neuroendocrinology*, *26*(9), 557–572. https://doi.org/10.1111/jne.12157

Um, J.-H., Pendergast, J. S., Springer, D. A., Foretz, M., Viollet, B., Brown, A., Kim, M. K., Yamazaki, S., & Chung, J. H. (2011). AMPK Regulates Circadian Rhythms in a Tissue- and Isoform-Specific Manner. *PLoS ONE*, *6*(3), e18450. https://doi.org/10.1371/journal.pone.0018450

Unger, R. H. (1991). Diabetic Hyperglycemia: Link to Impaired Glucose Transport in Pancreatic β Cells. *Science*, *251*(4998), 1200–1205. https://doi.org/10.1126/science.2006409

Valassi, E., Scacchi, M., & Cavagnini, F. (2008). Neuroendocrine control of food intake. *Nutrition, Metabolism and Cardiovascular Diseases*, *18*(2), 158–168. https://doi.org/10.1016/j.numecd.2007.06.004

Valenciano, A. I., Corrochano, S., De Pablo, F., De La Villa, P., & De La Rosa, E. J. (2006). Proinsulin/insulin is synthesized locally and prevents caspase- and cathepsin-mediated cell death in the embryonic mouse retina. *Journal of Neurochemistry*, *99*(2), 524–536. https://doi.org/10.1111/j.1471-4159.2006.04043.x

Van Cauter, E., Blackman, J. D., Roland, D., Spire, J. P., Refetoff, S., & Polonsky, K. S. (1991). Modulation of glucose regulation and insulin secretion by circadian rhythmicity and sleep. *Journal of Clinical Investigation*, *88*(3), 934–942. https://doi.org/10.1172/JCI115396

Van Cauter, E., Polonsky, K. S., & Scheen, A. J. (1997). Roles of Circadian Rhythmicity and Sleep in Human Glucose Regulation*. *Endocrine Reviews*, *18*(5), 716–738. https://doi.org/10.1210/edrv.18.5.0317

Van Der Heide, L. P., Hoekman, M. F. M., & Smidt, M. P. (2004). The ins and outs of FoxO shuttling: Mechanisms of FoxO translocation and transcriptional regulation. *Biochemical Journal*, *380*(2), 297–309. https://doi.org/10.1042/bj20040167

Van Der Heide, L. P., Kamal, A., Artola, A., Gispen, W. H., & Ramakers, G. M. J. (2005). Insulin modulates hippocampal activity-dependent synaptic plasticity in a *N*-methyl-D-aspartate receptor and phosphatidyl-inositol-3-kinase-dependent manner. *Journal of Neurochemistry*, *94*(4), 1158–1166. https://doi.org/10.1111/j.1471-4159.2005.03269.x

Van Der Heide, L. P., Ramakers, G. M. J., & Smidt, M. P. (2006). Insulin signaling in the central nervous system: Learning to survive. *Progress in Neurobiology*, *79*(4), 205–221. https://doi.org/10.1016/j.pneurobio.2006.06.003

Van Drunen, R., & Eckel-Mahan, K. (2021a). Circadian Rhythms of the Hypothalamus: From Function to Physiology. *Clocks & Sleep*, *3*(1), 189–226. https://doi.org/10.3390/clockssleep3010012

Van Drunen, R., & Eckel-Mahan, K. (2021b). Circadian Rhythms of the Hypothalamus: From Function to Physiology. *Clocks & Sleep*, *3*(1), 189–226. https://doi.org/10.3390/clockssleep3010012

Van Drunen, R., & Eckel-Mahan, K. (2023). Circadian rhythms as modulators of brain health during development and throughout aging. *Frontiers in Neural Circuits*, *16*, 1059229. https://doi.org/10.3389/fncir.2022.1059229

Vielhaber, E., Eide, E., Rivers, A., Gao, Z.-H., & Virshup, D. M. (2000). Nuclear Entry of the Circadian Regulator mPER1 Is Controlled by Mammalian Casein Kinase I ε. *Molecular and Cellular Biology*, *20*(13), 4888–4899. https://doi.org/10.1128/MCB.20.13.4888-4899.2000

Vinuesa, A., Pomilio, C., Gregosa, A., Bentivegna, M., Presa, J., Bellotto, M., Saravia, F., & Beauquis, J. (2021). Inflammation and Insulin Resistance as Risk Factors and Potential Therapeutic Targets for Alzheimer's Disease. *Frontiers in Neuroscience*, *15*, 653651. https://doi.org/10.3389/fnins.2021.653651

Wakamatsu, H., Yoshinobu, Y., Aida, R., Moriya, T., Akiyama, M., & Shibata, S. (2001). Restricted-feeding-induced anticipatory activity rhythm is associated with a phase-shift of the expression of *mPer1* and *mPer2* mRNA in the cerebral cortex and hippocampus but not in the suprachiasmatic nucleus of mice. *European Journal of Neuroscience*, *13*(6), 1190–1196. https://doi.org/10.1046/j.0953-816x.2001.01483.x

Wan, Q., Xiong, Z. G., Man, H. Y., Ackerley, C. A., Braunton, J., Lu, W. Y., Becker, L. E., MacDonald, J. F., & Wang, Y. T. (1997). Recruitment of functional GABAA receptors to postsynaptic domains by insulin. *Nature*, *388*(6643), 686–690. https://doi.org/10.1038/41792

Wang, A., Foppen, E., Rumanova, V. S., Kool, T., Kalsbeek, A., & Stenvers, D. J. (2025). Circadian phase inversion causes insulin resistance in a rat model of night work and jet lag. *Scientific Reports*, *15*(1), 9687. https://doi.org/10.1038/s41598-025-91485-z

Wang, D., Opperhuizen, A.-L., Reznick, J., Turner, N., Su, Y., Cooney, G. J., & Kalsbeek, A. (2017). Effects of feeding time on daily rhythms of neuropeptide and clock gene expression in the rat hypothalamus. *Brain Research*, *1671*, 93–101. https://doi.org/10.1016/j.brainres.2017.07.006

Wang, L. M. C., Dragich, J. M., Kudo, T., Odom, I. H., Welsh, D. K., O'Dell, T. J., & Colwell, C. S. (2009). Expression of the Circadian Clock Gene *Period2* in the Hippocampus: Possible Implications for Synaptic Plasticity and Learned Behaviour. *ASN Neuro*, *1*(3), AN20090020. https://doi.org/10.1042/AN20090020

Watson, R. E., Langub, M. C., Engle, M. G., & Maley, B. E. (1995). Estrogen-receptive neurons in the anteroventral periventricular nucleus are synaptic targets of the suprachiasmatic nucleus and peri-suprachiasmatic region. *Brain Research*, *689*(2), 254–264. https://doi.org/10.1016/0006-8993(95)00548-5

Weber, G., Lea, M. A., Fisher, E. A., & Stamm, N. B. (1966). Regulatory Pattern of Liver Carbohydrate Metabolizing Enzymes: Insulin as Inducer of Key Glycolytic Enzymes. *Enzymologia Biologica et Clinica*, *7*(1–2), 11–24. https://doi.org/10.1159/000457201

Wen, Q., Duan, X., Liao, R., Little, P., Gao, G., Jiang, H., Lalit, S., Quirion, R., & Zheng, W. (2011). Characterization of intracellular translocation of Forkhead transcription factor O (FoxO) members induced by NGF in PC12 cells. *Neuroscience Letters*, *498*(1), 31–36. https://doi.org/10.1016/j.neulet.2011.04.055

White, M. F. (1997). The insulin signalling system and the IRS proteins. *Diabetologia*, *40*(0), S2–S17. https://doi.org/10.1007/s001250051387

Woldt, E., Sebti, Y., Solt, L. A., Duhem, C., Lancel, S., Eeckhoute, J., Hesselink, M. K. C., Paquet, C., Delhaye, S., Shin, Y., Kamenecka, T. M., Schaart, G., Lefebvre, P., Nevière, R., Burris, T. P., Schrauwen, P., Staels, B., & Duez, H. (2013). Rev-erb-α modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy. *Nature Medicine*, *19*(8), 1039–1046. https://doi.org/10.1038/nm.3213

Wolfsheimer, K. J. (1990). Problems in diabetes mellitus management. Insulin resistance. *Problems in Veterinary Medicine*, *2*(4), 591–601.

Wöltje, M., Tschöke, B., von Bülow, V., Westenfeld, R., Denecke, B., Gräber, S., & Jahnen-Dechent, W. (2006). CCAAT enhancer binding protein β and hepatocyte nuclear factor 3β are necessary and sufficient to mediate dexamethasone-induced up-regulation of alpha2HS-glycoprotein/fetuin-A gene expression. *Journal of Molecular Endocrinology*, *36*(2), 261–277. https://doi.org/10.1677/jme.1.02001

Woods, S. C., & Begg, D. P. (2015). Food for Thought: Revisiting the Complexity of Food Intake. *Cell Metabolism*, *22*(3), 348–351. https://doi.org/10.1016/j.cmet.2015.08.017

Woods, S. C., & Langhans, W. (2012). Inconsistencies in the assessment of food intake. *American Journal of Physiology-Endocrinology and Metabolism*, *303*(12), E1408–E1418. https://doi.org/10.1152/ajpendo.00415.2012

Woods, S. C., Lotter, E. C., McKay, L. D., & Porte, D. (1979). Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature*, *282*(5738), 503–505. https://doi.org/10.1038/282503a0

Xie, L., Helmerhorst, E., Taddei, K., Plewright, B., Van Bronswijk, W., & Martins, R. (2002). Alzheimer's β-Amyloid Peptides Compete for Insulin Binding to the Insulin Receptor. *The Journal of Neuroscience*, *22*(10), RC221–RC221. https://doi.org/10.1523/JNEUROSCI.22-10-j0001.2002

Xu, A. W., Kaelin, C. B., Takeda, K., Akira, S., Schwartz, M. W., & Barsh, G. S. (2005). PI3K integrates the action of insulin and leptin on hypothalamic neurons. *Journal of Clinical Investigation*, *115*(4), 951–958. https://doi.org/10.1172/JCI24301

Xu, B., Kalra, P. S., Farmerie, W. G., & Kalra, S. P. (1999). Daily Changes in Hypothalamic Gene Expression of Neuropeptide Y, Galanin, Proopiomelanocortin, and Adipocyte Leptin Gene Expression and Secretion: Effects of Food Restriction[1]. *Endocrinology*, *140*(6), 2868–2875. https://doi.org/10.1210/endo.140.6.6789

Yamajuku, D., Inagaki, T., Haruma, T., Okubo, S., Kataoka, Y., Kobayashi, S., Ikegami, K., Laurent, T., Kojima, T., Noutomi, K., Hashimoto, S., & Oda, H. (2012). Real-time monitoring in three-dimensional hepatocytes reveals that insulin acts as a synchronizer for liver clock. *Scientific Reports*, *2*(1), 439. https://doi.org/10.1038/srep00439

Yamamuro, D., Takahashi, M., Nagashima, S., Wakabayashi, T., Yamazaki, H., Takei, A., Takei, S., Sakai, K., Ebihara, K., Iwasaki, Y., Yada, T., & Ishibashi, S. (2020). Peripheral circadian rhythms in the liver and white adipose tissue of mice are attenuated by constant light and restored by time-restricted feeding. *PLOS ONE*, *15*(6), e0234439. https://doi.org/10.1371/journal.pone.0234439

Yang, L., Qi, Y., & Yang, Y. (2015). Astrocytes Control Food Intake by Inhibiting AGRP Neuron Activity via Adenosine A1 Receptors. *Cell Reports*, *11*(5), 798–807. https://doi.org/10.1016/j.celrep.2015.04.002

Yang, X., Di, W., Zeng, Y., Liu, D., Han, M., Qie, R., Huang, S., Zhao, Y., Feng, Y., Hu, D., & Sun, L. (2021). Association between shift work and risk of metabolic syndrome: A systematic review and meta-analysis. *Nutrition, Metabolism and Cardiovascular Diseases*, *31*(10), 2792–2799. https://doi.org/10.1016/j.numecd.2021.06.007

Yanik, T., & Durhan, S. T. (2025). Pro-Opiomelanocortin and Melanocortin Receptor 3 and 4 Mutations in Genetic Obesity. *Biomolecules*, *15*(2), 209. https://doi.org/10.3390/biom15020209

Yari, Z., Behrouz, V., Zand, H., & Pourvali, K. (2020). New Insight into Diabetes Management: From Glycemic Index to Dietary Insulin Index. *Current Diabetes Reviews*, *16*(4), 293–300. https://doi.org/10.2174/1573399815666190614122626

Yaribeygi, H., Maleki, M., Butler, A. E., Jamialahmadi, T., & Sahebkar, A. (2023). Brain insulin signaling and cognition: Possible links. *EXCLI Journal; 22:Doc237; ISSN 1611-2156*. https://doi.org/10.17179/EXCLI2023-5841

Yerbury, J. J., Rybchyn, M. S., Easterbrook-Smith, S. B., Henriques, C., & Wilson, M. R. (2005). The Acute Phase Protein Haptoglobin Is a Mammalian Extracellular Chaperone with an Action Similar to Clusterin. *Biochemistry*, *44*(32), 10914–10925. https://doi.org/10.1021/bi050764x

Yi, C.-X., Van Der Vliet, J., Dai, J., Yin, G., Ru, L., & Buijs, R. M. (2006a). Ventromedial Arcuate Nucleus Communicates Peripheral Metabolic Information to the Suprachiasmatic Nucleus. *Endocrinology*, *147*(1), 283–294. https://doi.org/10.1210/en.2005-1051

Yi, C.-X., Van Der Vliet, J., Dai, J., Yin, G., Ru, L., & Buijs, R. M. (2006b). Ventromedial Arcuate Nucleus Communicates Peripheral Metabolic Information to the Suprachiasmatic Nucleus. *Endocrinology*, *147*(1), 283–294. https://doi.org/10.1210/en.2005-1051

Yip, C. C. (1993). Insulin Receptor: Aspects of its Structure and Function. In C. G. Östenson, S. Efendić, & M. Vranic (Eds.), *New Concepts in the Pathogenesis of NIDDM* (Vol. 334, pp. 79–88). Springer US. https://doi.org/10.1007/978-1-4615-2910-1_6

Yoo, S.-H., Yamazaki, S., Lowrey, P. L., Shimomura, K., Ko, C. H., Buhr, E. D., Siepka, S. M., Hong, H.-K., Oh, W. J., Yoo, O. J., Menaker, M., & Takahashi, J. S. (2004). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proceedings of the National Academy of Sciences*, *101*(15), 5339–5346. https://doi.org/10.1073/pnas.0308709101

Yuan, A., & Nixon, R. A. (2021). Neurofilament Proteins as Biomarkers to Monitor Neurological Diseases and the Efficacy of Therapies. *Frontiers in Neuroscience*, *15*, 689938. https://doi.org/10.3389/fnins.2021.689938

Yue, F., Cheng, Y., Breschi, A., Vierstra, J., Wu, W., Ryba, T., Sandstrom, R., Ma, Z., Davis, C., Pope, B. D., Shen, Y., Pervouchine, D. D., Djebali, S., Thurman, R. E., Kaul, R., Rynes, E., Kirilusha, A., Marinov, G. K., Williams, B. A., … The Mouse ENCODE Consortium. (2014). A comparative encyclopedia of DNA elements in the mouse genome. *Nature*, *515*(7527), 355–364. https://doi.org/10.1038/nature13992

Zhang, E. E., Liu, Y., Dentin, R., Pongsawakul, P. Y., Liu, A. C., Hirota, T., Nusinow, D. A., Sun, X., Landais, S., Kodama, Y., Brenner, D. A., Montminy, M., & Kay, S. A. (2010). Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. *Nature Medicine*, *16*(10), 1152–1156. https://doi.org/10.1038/nm.2214

Zhang, R., Cai, X., Lin, C., Yang, W., Lv, F., Wu, J., & Ji, L. (2022). The association between metabolic parameters and evening chronotype and social jetlag in non-shift workers: A meta-analysis. *Frontiers in Endocrinology*, *13*, 1008820. https://doi.org/10.3389/fendo.2022.1008820

Zhao, W., Chen, H., Xu, H., Moore, E., Meiri, N., Quon, M. J., & Alkon, D. L. (1999). Brain Insulin Receptors and Spatial Memory. *Journal of Biological Chemistry*, *274*(49), 34893–34902. https://doi.org/10.1074/jbc.274.49.34893

Zhao, W.-Q., Chen, H., Quon, M. J., & Alkon, D. L. (2004). Insulin and the insulin receptor in experimental models of learning and memory. *European Journal of Pharmacology*, *490*(1–3), 71–81. https://doi.org/10.1016/j.ejphar.2004.02.045

Zheng, X., & Sehgal, A. (2010). AKT and TOR Signaling Set the Pace of the Circadian Pacemaker. *Current Biology*, *20*(13), 1203–1208. https://doi.org/10.1016/j.cub.2010.05.027

Zierath, J. R., He, L., Gumà, A., Wahlström, E. O., Klip, A., & Wallberg-Henriksson, H. (1996). Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia*, *39*(10), 1180. https://doi.org/10.1007/BF02658504

Abbreviations

AD	Alzheimer's disease
adj. p-value / q values	Adjusted p value
AgRP	Agouti- related protein
Agt	Angiotensinogen
Ahsg	Alpha-2-HS-Glycoprotein
Akt	Protein kinase B
Alb	Albumin
Alg2	Alpha-1,3/1,6-mannosyltransferase
Ambp	Alpha-1-Microglobulin/Bikunin Precursor
AMPA	Alpha -amino-3-hydroxy-5-methyl-4-isoxazolepropionic
ANOVA	Analysis of variance
ApoA1, A2, C1	Apolipoproteins A1, A2, C1
Apoe	Apolipoprotein E
Arc	Arcuate nucleus
ATP	Adeniosine tri phosphate
Atp1a2	ATPase Na+/K+ Transporting Alpha 2 Subunit
Aβ	Amyloid-beta
BBB	Blood brain barrier
bHLH	Basic helix-loop-helix
BIR	Brain insulin resistance
BMAL1	Brain and muscle ARNT-like 1 protein
C1qa	Complement component 1, q subcomponent alpha chain
CART	Cocaine-and-amphetamine-regulated transcript
CCGs	Clock-controlled genes
Cd68	Cluster of differentiation 68
Cd9	Cluster of Differentiation 9
Cers2	Ceramide Synthase 2
CK1δ/ε	Casein kinase 1 delta or epsilon
Cldn11	Claudin 11
Cldn5	Claudin 5
CLOCK	Circadian locomotor output cycles kaput
Clu	Clusterin
CNS	Central nervous system
CpE	Carboxypeptidase E
Cry	Cryptochrome
CSF	Cerebrospinal fluid
CVO	Circumventricular organs
DAVID	Database for Annotation, Visualization and Integrated Discovery
DBP	D-site albumin promoter-binding protein
DEGs	Differentially expressed genes
DESeq2	Differentially expression from sequencing
DMEM	Dulbecco's Modified Eagle Medium
DMH	Dorsomedial hypothalamus
DRC	Dose response curve
Eef1α	Eukaryotic elongation factor 1 α
EGF	Epidermal growth factor
Enrichr-KG	Enrichment-Knowledge Graph
FBS	Fetal bovine serum

FBXL3	f-box and WD repeat domain-containing 7
FDR	False discovery rate
FFA	Free fatty acids
Fga	Fibrinogen alpha chain
FOXO1	Forkhead box protein O1
GABA	Gamma aminobutyric acid
GCs	Glucocorticoids
GFAP	Glial fibrillary acidic protein
GHSR	Growth hormone secretagogue receptor
GIRKO	Glial-insulin receptor knockout
Gja1	Gap Junction Alpha-1
Gjb1	Gap Junction Beta 1
Gjlc3	Gap Junction Lambda 3
GO	Gene Ontology
Gpld1	Glycosylphosphatidylinositol Anchored Lipid-Dependent Protein 1
GPR17	G-protein coupled receptor 17
Gpr37	G-protein Coupled Receptor 37
Grap	Grb2-associated binder 1
Grb-2	Growth factor receptor binding protein
GSK 3	Glycogen synthase kinase 3
HBSS	Hank's Balanced Salt Solution
HOMA-IR	Homeostatic model assessment of insulin resistance
Hp	Haptoglobin
ICV	Intracerebroventricle
IDE	Insulin degrading enzyme
IR	Insulin receptor
Irf8	Interferon regulatory factor 8
IRS	Insulin receptor substrate
IVC	Individually ventilated cages
KEGG	Kyoto Encyclopedia of Genes and Genomes
Lfc	Log fold-change
LHb	Lateral habenula
LRP1	Lipoprotein receptor-related protein 1
LTD	Long-term depression
Lyn	Tyrosine-protein kinase-Lyn
MAPK	Mitogen-activated protein kinase
MBH	Mediobasal hypothalamus
MCH	Melanin-concentrating hormone
MCR	Melanocortin receptors
mHypoE/A	Mouse hypothalamic embryonic/ adult
Mlc1	Myosin Light Chain 1
Mlt11	MLLT11 transcription factor 7 cofactor
MSA	Multiple system atrophy
MtnR1b	Melatonin receptor gene
mTOR	Mammalian target of rapamycin
Nac	Nucleus accumbens
NAc	Nucleus accumbens
NCBI	National Center for Biotechnology Information
Ndrg4	N-myc downstream-regulated gene 4

Nefm	Neurofilament medium chain
NFIL3, E4BP4	Nuclear factor interleukin 3-regulated protein
NMDA	N-methyl-D-aspartate
NPAS2	Neuronal PAS domain protein 2
NPH	Neutral protamine Hagedorn
NPY	Neuropeptide Y
OGD	Oxygen-glucose deprived
Ols	Oligodendrocytes
Opalin	Oligodendrocytic Myelin Paranodal and Inner Loop Protein
Pak3	p21-activated kinase 3
PAS	PER-ARNT-SIM
PCA	Principal component analysis
PCOS	Polycystic ovarian syndrome
PD	Parkinson's disease
PDK	Phosphoinositide-dependent kinase
Per	Period
PFA	Paraformaldehyde
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PIP3K	Phosphatidylinositol-3 kinase
Pltp	Phosphatidylcholine Transfer Protein
POMC	Proopiomelanocortin
Prkar2a	Protein Kinase AMP-Activated Catalytic Subunit Alpha 2
Prmt2	Protein arginine methyltransferase 2
PVN	Paraventricular nucleus
PVN	Paraventricular nucleus
qPCR	Quantitative polmerase chain reaction
Rab6a	Ras-related protein Rab-6A
REV-ERBα/β	Reverse erythroblastosis virus α and β
REVIGO	Reduce + Visualize Gene Ontology
ROR	Retinoic acid receptor-related orphan receptors
RPKM	Reads per kilobase of transcript per million mapped reads
RTC	Response-time curve
Rtn4	Reticulon 4
SCN	Suprachiasmatic nucleus
Serpina1b	Alpha-1-antitrypsin, Serpin family A member 1
Shc	Src homology and collagen protein
Shox2	Short stature homeobox 2
Slc48a1	Solute Carrier Family 48 Member 1
SlcA3 / A4	Solute Carrier Family A3 Member 1 / A4 Member 1
SOS	Son of sevenless
ßTrCP	Beta transducing repeat-containing E3 ubiquitin protein
Stmn2	Stathmin 2
STZ	Streptozotocin
T2DM	Type2 diabetes mellitus
Taldo1	Transaldolase 1
Tcaf1	TRPM8 Channel Associated Factor 1
Tlr2	Toll-like receptor 2
Tmem30a	TRPM8 Channel Associated Factor 1

Tmem30a	Transmembrane protein 30A
Trpc3	Transient Receptor Potential Cation Channel Subfamily C Member 3
Tspan13	Tetraspanin 13
TTFL	Transcription-translation feedback loops
Ttr	Transthyretin
Tusc3	Tumor suppressor candidate 3
VMH	Ventromedial hypothalamus
Vmp1	Vesicle-associated Membrane Protein 1
vst	Variance stabilizing transformation
VTA	Ventral tegmented area
α-MSH	Alpha-melanocyte stimulating hormone

List of Figures

List of tables

Acknowledgements

"I cannot pretend I am without fear. But my predominant feeling is one of gratitude.
I have loved and been loved."

-Dr. Oliver Sacks

My sincere gratitude to all who have provided me with invaluable support throughout my doctoral studies and made it a memorable phase of my life. Many thanks, firstly to my supervisor, Prof. Henrik Oster. I am grateful that you gave me an opportunity to work in the institute. I highly appreciate your continouus support and respect your work-ethics. Your productive feedback and discussion meetings were very valuable to shape this project. Also beyond work, I have enjoyed our conversations. Your words, "*keep an eye on opportunities, they are always along the way*", would stay with me for a long time to come.

Thank you to my co-supervisor, Dr. Carla Schulz. You provided some early insights in the project that were helpful to me. I admire your joyful nature. Huge thanks to Prof. Henriette Kirchner and other members of the examination committee for your time and willingness to examine my work. Thanks to the graduate program GRK1957 and team for the interesting events, workshops, summer schools and coaching. Thanks to Chaoqun and Katja for coordinating this.

A very heartfelt thanks to all the former and present members of the Institute of Neurobiology. It was your unwavering support and belief in me that gave me the strength to show up each day and keep going. Thanks Violetta, for always seeing my potential and highlighting my qualities. You are one of the finest lecturer and story-teller! Dear Leo, how do I thank you enough? For your supervision, encouragement, care and friendship. Much love to you and sweet Mari. For almost adopting me, *may the force be with you! Obrigada*. Heyya Iwona, *Dzięki* for being the biggest support during the moments of my self-doubt. Your feedback during the writing process was very valuable. I fancy your wittiness;) Dear Isa, I admire your work-discipline. *Danke* for valuable inputs during the experiments. With board-game evenings, dinners and office talks, I value our friendship! *Danke* Kimberly, for checking on me in the office, regularly. I cherish our time during the EBRS Zurich trip and summer schools. Heyyo Xenia (xe xe), my team-partner, *Danke*, for all your positive encourgement and imparting a spirit of not giving up! Dear Carina, *fellow-INFJ*, it was lovely to discuss *'life'* with you, best wishes! Dear Lucie, Misa, Jazmin, Marianne, Brinja, Faheem, Federic, Sophia, Amrei, Marie, Tjale and Emelie, thank you for supporting me! I miss our lunch table coversations! Dear Dorothea, your kindness and care is very comforting, *Danke! Danke* Nadine, for helping me with mice-colony organisation. *Danke* Ludmila, for helping me with Lab and exprimental works.

I am grateful to Prof. Aarti Jagannath and lab colleagues for hosting me at University of Oxford. You all made my research-stay experience very enriching and enjoyable!

A heartfelt thanks to my lovely friends, Marie and Léo. Your understanding and company over last years is most cherished. Much love! Dear Mojgan, how do I thank you for walking side-by-side with me every single day! For taking care of my spiralling mind. The last steps of this journey would have been extremely long without you. Thanks, for your unconditional support, my *Ehsani-fabulous friend:)* Thank you to my childhood friends overseas. We look out for eachother from a far. May distance never diminish the bond we have. Cheers to other lovely friends I met in Germany.

And lastly, deepest gratitude to my family. Dear Mumma, your unwavering belief in me has been my strength at every step. Thank you for instilling in me the value of patience and persistence. Dear Pappa, you have taught me the dignity of self-sufficiency and the value of hard work. And to my dear brother, Ashutosh, you are the most compassionate person in my life. *Everything I have, and everything I am, carries a part of you all.*

-Namaste (for something you have given me, I bow to the provider in you)

Ankita