Neuro Modulated Diabetes Control
(NeuMeDiC)
Decoding the neural pathways to achieve a better control of diabetes.

By
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Supervised by Dr Pantelis Georgiou

DEPARTMENT OF ELECTRICAL AND ELECTRONIC ENGINEERING
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ABSTRACT

Diabetes is a disease caused by a breakdown in the glucose metabolism resulting in abnormal blood glucose fluctuations. Traditional therapies involve external insulin injection in response to elevated blood glucose, which faces limitations such as delays in insulin action and the non-physiological absorption of insulin into the systemic circulation. Recently, bioelectronic medicine has emerged as a powerful strategy for treating chronic diseases, such as diabetes, by electrically interfacing with peripheral nerves and organs.

The research conducted in this thesis combines different strategies to investigate the development of novel neuromodulation-pharmaceutical therapeutic technology for people with diabetes. Following a scientific-based approach, the thesis presents studies that allowed increasing our understanding of the physiological mechanisms underlying the neural glycemic regulation. In vivo experiments in rodents provided new insights into the impact of vagus nerve stimulation (VNS) frequency on blood glucose, insulin and glucagon concentrations. To further characterize this neuro-metabolic interaction, the first mathematical model describing the physiological metabolic events after cervical VNS was developed and validated.

Using an application-based strategy, the thesis investigates the opportunities for incorporating bioelectronic medicine to traditional diabetes technology in clinical scenarios. In particular, one study presents the proof of concept of a novel closed-loop glucose controller that regulates the insulin and glucagon dose delivery, and drive the insulin sensitivity (SI) of virtual patients using neuromodulation. In silico experiments demonstrated improved safety and efficacy compared to traditional controllers. An additional study introduces an original data-driven approach to predict the quality of overnight glycaemia based on the application of binary classifiers on metabolic data.

To fully exploit bioelectronic medicine as an alternative or complementary therapy for diabetes, advances in minimally invasive technology and promotion of our knowledge of the field are needed. The work presented in this thesis opens the door to further research that promises to transform the current healthcare scenario in diabetes.
To my family.

Thank you for always being by my side.
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<td>AUC</td>
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<td>BAT</td>
<td>brown adipose tissue</td>
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<td>BiAP</td>
<td>bio-inspired artificial pancreas</td>
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<td>CAP</td>
<td>compound action potentials</td>
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<td>CCK</td>
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<td>DMNX</td>
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<td>ETC</td>
<td>extended tree classifier</td>
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<td>FDA</td>
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FN  false negatives
FP  false positives
GE  glucose-excited
GI  glucose-inhibited
GMEAN  geometric mean
GRP  gastrin-releasing polypeptide
HEX  hexamethonium
HBGI  high blood glucose risk index
HOS  hold-out set
HGP  hepatic glucose production
HPT  hypothalamic-pituitary-thyroid
InSiG  insulin sensitivity glucose controller
LBGI  low blood glucose risk index
LHN  lateral hypothalamic nucleus
LLR  linear logistic regression
LSTM  long short-term memory networks
MAPE  mean absolute percentage error
MC2R  melanocortin 2 receptor
MC4R  melanocortin 4 receptor
MdAPE  median absolute percentage error
MPC  model predictive control
MRA  multiresolution analysis
NA  noradrenaline
NPs  neuropeptides
NPY  neuropeptide Y
NPY/AgRP  neuropeptide Y/agouti-related peptide
NT  neurotransmitter
NTS  nucleus of the tractus solitarius
PACAP  pituitary adenylate cyclase activating polypeptide
PID  proportional-integrative-derivative
POMC  pro-opiomelanocortin
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<th>Description</th>
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<td>pro-opiomelanocortin/cocaine-amphetamine related transcript</td>
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<td>PVN</td>
<td>paraventricular nucleus</td>
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<tr>
<td>RFC</td>
<td>random forest classifier</td>
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<td>RMSPE</td>
<td>root mean square percentage error</td>
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<tr>
<td>RMSE</td>
<td>root mean square error</td>
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<td>subcutaneous</td>
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<td>SCN</td>
<td>suprachiasmatic nucleus</td>
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<td>SENS</td>
<td>sensitivity</td>
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<tr>
<td>SI</td>
<td>insulin sensitivity</td>
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<tr>
<td>SMOTE</td>
<td>synthetic minority over-sampling</td>
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<tr>
<td>SPEC</td>
<td>specificity</td>
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<tr>
<td>sPD</td>
<td>standard proportional-derivative</td>
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<tr>
<td>SVM</td>
<td>support vector machine</td>
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<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
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<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
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<tr>
<td>TH</td>
<td>tirosine hydroxylase</td>
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<tr>
<td>TN</td>
<td>true negatives</td>
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<tr>
<td>TP</td>
<td>true positives</td>
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<tr>
<td>VIP</td>
<td>vasoactive intestinal polypeptide</td>
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<tr>
<td>VMH</td>
<td>ventromedial nucleus of the hypothalamus</td>
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<tr>
<td>VNS</td>
<td>vagus nerve stimulation</td>
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<tr>
<td>WAT</td>
<td>white adipose tissue</td>
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INTRODUCTION

1.1 Research in context

A key biological process of every living system is the maintenance of homeostasis to ensure stability via continuous and rapid self-adjustments of the physiological state. The nervous system has a major role in preserving homeostasis using closed-loop mechanisms called neural reflexes. These include sensing, integration and effector circuitries that modulate the organ function. Interfacing with the neural reflexes may allow for direct and efficient access to the rapidly changing internal conditions of the organism.

In recent decades, modulation of the nervous system through the delivery of electrical or pharmaceutical agents has allowed the development of effective treatments to several severe clinical conditions [1]. Furthermore, we have seen the introduction of bioelectronic medicine, an evolution of neuromodulation, which aims to provide real-time and patient-specific diagnosis and therapies by recording and modulating the activity of specific peripheral nerves to improve or restore impaired biological function of specific organs [2, 3].

Initial research on bioelectronic medicine focused on the use of neuromodulation for treating inflammatory diseases by interfering with the neuro-immune reflex [4, 5, 6]. Previous research confirmed that electrical stimulation of the vagus nerve modulates the production of inflammatory cytokines, and ultimately reduces the uncontrolled inflammatory activity associated with several disorders. This strategy has successfully improved the treatment of rheumatoid arthritis and colitis in clinical trials [7], encouraging the application of bioelectronic medicine for the management of other chronic and severe diseases such as diabetes.

Diabetes is a chronic metabolic disease that results in elevated blood glucose. Its prevalence has alarmingly increased worldwide in the last decades. It currently affects over 460 million people (20-79 years old) worldwide, which implies a four-fold rise of its prevalence since 1980, and is forecast to affect 700 million people in 2045 [8]. Persistently elevated glucose levels (hyperglycaemia) due to insufficient treatment is of increasing concern given its association with many secondary complications such as ketoacidosis, blindness, nephropathy and heart disease [9, 10]. Furthermore, deficient treatments may also increase the frequency of low blood glucose (hypoglycaemic) events [11]. In 2013, the risk of mortality due to insulin-induced hypoglycaemia was reported to be between 6 and 10% of patients [12]. Moreover, in 2019 there
CHAPTER 1. INTRODUCTION

were 4.2 million deaths worldwide due to diabetes [8], which is expected to be the 7th leading cause of death in 2030 [13]. These figures illustrate the impact of diabetes and justify the worldwide societal need to develop technology to acutely and/or chronically regulate blood glucose concentration in people with this disease.

Diabetes is generally classed as type 1 or type 2 depending on the cause of the disease. Type 1 diabetes mellitus (T1DM) is an autoimmune disease that causes destruction of the pancreatic \( \beta \)-cells. This results in the absence of insulin secretion, the hormone responsible for glucose absorption, and therefore poor regulation of plasma glucose levels. Over the years, diverse technologies have been developed to improve the control of glucose for T1DM using an external device. These systems will be reviewed in more extent in the next chapter.

The second class, known as Type 2 diabetes mellitus (T2DM), is characterised by chronic hyperglycaemia resulting from defects in insulin action, which leads to a reduction of SI and insulin resistance. Consequently, several methods have been formulated to modulate SI in these patients to reverse the disease. Among them, changes in nutrition and exercise [14] and drug therapies oriented to take control of the inflammatory processes that underlie the insulin resistance [15] are the most widely studied treatments. However, as reviewed by Gao and Ye (2012) the efficacy of anti-inflammatory treatments has not been proven yet [16].

Therapies involving insulin administration are indispensable for people with T1DM. For T2DM, such therapies are sometimes used in early stages, and are critical at later stages of the disease. Despite being essential, the costs associated with the insulin market are untenable, growing from $232 billion spent worldwide in 2007, to $727 billion spent in 2017 for adults aged 20–79 years, based on the IDF Diabetes Atlas 2019 [8]. The American Diabetes Association also reported in March 2018 that the direct medical costs of treating people diagnosed with diabetes have risen to $237 billion in 2017 from $175 billion in 2012 [17, 10]. Besides the economic impact, diabetes management poses a great scientific and clinical challenge due to the great complexity of the biological mechanisms for glucose control, where many biological
substances, including hormones and neurotransmitters, interact among each other to guarantee a robust and adequate regulation of blood glucose fluctuations [18]. Among them, neural reflexes have a major role in preserving homeostasis [19, 20, 21]. Hence, significant efforts have been focused on improving diabetes management through the introduction of new technology and drugs to improve glucose control by reducing hyper and hypoglycaemic events.

The application of bioelectronic medicine to treat diabetes was previously criticized by some members of the scientific community, who claimed that the complexity of the underlying biological processes was unapproachable and questioned the legitimacy of preliminary simulations [22]. Recent work, however, supports the opportunities of modulating the peripheral nerve signals to improve the metabolic dysregulation [23, 24]. These studies also demonstrate the potential benefits of VNS to improve diabetes co-morbidities (e.g. cardiovascular diseases), and have initiated new research exploring the opportunities of bioelectronic medicine for improving glucose control in people with diabetes, including regulation of gastric emptying, insulin sensitivity, and secretion of pancreatic hormones.

![Figure 1.2: A depiction of future closed-loop neuromodulation systems for diabetes management.](image)

Metabolic biomarkers and neurophysiological recordings from a variety of peripheral nerves can be used to automatically control the stimulation dosage to be delivered back to the peripheral nervous system or directly to the organs to modulate their function.

The therapeutic impact of bioelectronic medicine can be boosted by replicating the body’s closed-loop mechanisms: metabolic and neurophysiological biomarkers can be recorded and analysed in real-time to accordingly adjust the characteristics of the electrical stimulation delivered to the peripheral nerves or directly to the organs to modulate their function, as illustrated in Figure 1.2 [25, 26]. Advances in bioelectronic medicine towards these closed-loop systems are supported by the development of new algorithms and implantable technology, which enable a safe, effective and minimally invasive interface with the nervous system [27, 28]. This strategy promises to provide real-time, autonomous and patient-specific therapies, which are crucial in chronic diseases such as diabetes.
1.2 RESEARCH HYPOTHESIS AND QUESTIONS

Furthering this emerging field, this Ph.D. thesis builds upon the hypothesis that:

*The incorporation of bioelectronic technology into current pharmaceutical-based diabetes closed-loop systems can outperform conventional approaches and enhance the safety and efficiency of glucose control.*

This thesis, combines work from a scientific, i.e. based on providing basic research and understanding, and an industrial, i.e. based on applications, approaches to bring some light to this hypothesis by answering the following questions:

- What is the impact of neuromodulation of the peripheral pathways on glucose fluctuations?
- Which organs and biological mechanisms are implicated in these neuro-stimulated metabolic responses?
- Is it possible to replicate *in silico* the neural mechanisms involved in the control of glucose homeostasis?
- What are the open-loop and closed-loop opportunities for introducing bioelectronics to current pharmaceutical-based diabetes technology?
- What would be the technological challenges for implementing these new hybrid bioelectronic-pharmaceutical systems?

The first three questions have been mainly addressed from a scientific approach, because it is important to lay a strong ground-floor of knowledge and understanding of the underlying physiology upon which new technology can be developed. The last two questions have been tackled from an industrial approach, by proposing novel technology whose performance has been discussed.

1.3 DEFINITION OF OBJECTIVES

Objective 1: To study the impact of VNS parameters on the hormonal secretion from the endocrine pancreas and the glucose levels in the healthy state.

*Rationale:* The parasympathetic control of the liver and pancreas and its impact glucose metabolism have been extensively studied throughout the years. Yet, there are few comprehensive quantitative studies describing the relationship, in time and magnitude, between the VNS parameters and the kinetics of change of pancreatic hormones and glucose levels in blood.
1.3. DEFINITION OF OBJECTIVES

Approach: *In vivo* experiments were carried out in rodents to evaluate the impact of electrical stimulation of the vagus nerve using different configurations of parameters on several metabolic markers, including insulin, glucagon and blood glucose levels. The stimulation was performed at the cervical vagus nerve, although the feasibility of stimulating closer to the pancreas (i.e. pancreatic branch) to improved specificity was also assessed.

Objective 2: To develop mathematical models of the neural control of glucose homeostasis.

Rationale: The neural mechanisms through which the brain controls glucose homeostasis are still unclear and disregarded in existing simulators. Hence, to the best of my knowledge, the important contribution of the neural signalling on metabolic processes has never been described mathematically.

Approach: Outcomes from previous literature and the results from the *in vivo* experiments were used to develop a mathematical model of the neural-metabolic interaction. The ultimate goal was to create a unified bio-inspired neural metabolic model that reproduces the physiology of glucose regulation to a greater extend.

Objective 3: To study the opportunities of neuromodulation for diabetes management through the development of novel closed-loop technology.

Rationale: Incorporation of bioelectronic medicine technology in diabetes will require to extend current closed-loop systems (artificial pancreas) to take into consideration the neural control and state signals (i.e. descending signals from the central nervous system (CNS) and ascending signals from digestive organs, respectively) in addition to blood biomarkers (i.e. glucose levels), to advance towards an effective control of insulin delivery [29]. These neural signals may also allow control systems to predict and pre-empt potential high glucose excursions, thereby preparing the system to future glucose challenges. Despite extensive research is available in the context of neuromodulation of diabetes-related metabolic disorders, especially on the control of appetite and food intake in obesity, there are few studies focused on the introduction of neuromodulation for T1DM management.

Approach: To fill this gap, it would be ideal to carry out *in vivo* animal experimental studies to target a T1DM-induced cohort, whose CNS is functional but whose pancreas is not able to produce enough insulin to regulate glucose. A highly selective stimulation system could then be used to study the impact of VNS on glucose homeostasis and the opportunities to control glucose levels through stimulation. The complexity of this experimental design required a team of experts and sufficient time to obtain successful results. As a consequence, *in silico*
experiments were performed instead to bring some light into the opportunities of modulating the neural signals for T1DM control by incorporating novel neuromodulation-pharmaceutical closed-loop technology.

1.4 Contributions to each objective

Contribution 1: experimental study of the impact of VNS parameters on glucose metabolism.

Three experimental studies using two experimental setups were carried out in healthy rodent models to investigate the differential impact of VNS frequency on the metabolic and hemodynamic responses: stimulation in the intact cervical vagus nerve for afferent and efferent activation, and in the distal end of the ligated nerve for efferent activation. The influence of afferent and efferent fibres was also assessed and discussed based on the obtained outcomes. All the experiments were performed in accordance with the guidelines of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), and approved by the Johns Hopkins Animal Care and Use Committee.

Contribution 2: development of a neural-metabolic mathematical model of the impact of VNS on glucose metabolism.

In this thesis, I present a novel mathematical model that describes the physiological events that occur after VNS at the cervical level, by employing the quantitative knowledge that is available in the literature and the findings obtained from in vivo experiments. The model structure comprises different subsystems, including models of the nerve activation as a function of different stimulation parameters, neurotransmitters secretion from nerve terminals, endogenous glucose production, glucose utilization, and insulin and glucagon secretion and kinetics. The resulting unified neural-metabolic model is the major contribution of this thesis. Its high scientific value arises from its ability to highlight the current knowledge about this specific neuro-metabolic system and determine where we should direct experimental research to acquire new knowledge to fill in the missing gaps [30]. When fully developed, it will provide a virtual platform for studying and testing new diabetes technology that incorporates neuromodulation as an additional or alternative functional component.

Contribution 3: proof-of-concept of a new neuromodulation-pharmaceutical closed-loop system for diabetes management.

The opportunities of incorporating bioelectronic medicine through neuromodulation in a person with T1DM were analysed in an in silico study. In particular, this thesis presents the design and initial evaluation of a proof of concept for a new hybrid closed-loop glucose controller
that regulates (i) the insulin and glucagon doses delivered using a pump and (ii) the value of insulin sensitivity of the patient (i.e. the sensitivity of the liver and peripheral tissues to the action of insulin), which would be modulated through electrical stimulation of the nervous system. The proposed controller achieved improved glucose control with an increased percentage of time of glucose levels within target and decreased hormonal delivery when compared with conventional glucose controllers (standard proportional/derivative (sPD) controller [31] and coordinated bi-hormonal bio-inspired artificial pancreas (BiAP) [32]).

Additional contributions

This thesis presents an additional contribution to current diabetes technology, in particular to decision support systems (DSS). These systems support self-management control in diabetes, for example, by suggesting the administration of meal insulin boluses or corrective insulin boluses to mitigate hyperglycaemia [33, 34]. Despite the large amount of ongoing research in this field, few studies focus on their use for improving overnight glycaemic control. To fill this gap, a novel data-driven approach was conceptualised to predict the quality of overnight glycaemic control in people with T1DM based on the application of binary classifiers on commonly gathered data during the day-time period (continuous glucose monitoring data, meal intake and insulin boluses). Further work in this field would take into consideration features extracted from recorded neural signals to/from the brain through the use of bioelectronic medicine.

1.5 Thesis organization

Chapter 2 describes the past and present evolution of technology in diabetes and motivates the introduction of bioelectronic medicine to overcome the challenges that arise from purely metabolic and pharmaceutical approaches. Then, an introduction to the most relevant bioelectronic technology, from computational modelling to neurostimulation systems, is presented. Finally, the chapter provides an overview of current applications of invasive and non-invasive stimulation of the vagus nerve and other neuromodulation strategies for treating chronic diseases, with special interest on diabetes.

Chapter 3 provides an overview of neuromodulation of the peripheral nervous system. It first presents the peripheral nervous pathways innervating the pancreas, with special interest in the gross and microscopical anatomy of the vagus nerve. This is followed by a review of the strategies used to study the impact of the neural innervation on the secretion of pancre-
atic hormones and on the hemodynamic and respiratory responses. These include electrical stimulation of the vagus nerve and the use of neural blockers.

Chapter 4 presents the rationale and analysis of the in vivo experiments carried out at Johns Hopkins University in rodent models. To begin with, the chapter justifies the experimental design implemented based on a review of experimental protocols reported in previous literature. Then, three studies are presented with the ultimate objective of examining the differential impact of different VNS parameters on the metabolic and hemodynamic responses. In particular, studies one and two provide insight into the impact of stimulation frequency on the metabolic and hemodynamic responses; study three analyses the differential impact of stimulation on afferent and efferent fibres, and only on efferent fibres; and study four provides a thorough analysis on the hemodynamic responses due to VNS.

Chapter 5 details the mathematical model representing the kinetics of the neural control of glucose, insulin and glucagon blood levels. The model represents each biological process using a compartmental approach. Results obtained during the experimental phase (reported in Chapter 4) and quantitative data found in previous literature have been used to identify and validate the proposed model.

Chapter 6 moves forward from the fundamental research presented in the previous chapters and motivates a potential future application of a system that incorporates neuromodulation to conventional pharmaceutical-based technology as a therapeutic strategy for T1DM. In particular, a new generation of hybrid hormonal-insulin sensitivity glucose controllers is presented, which include additional control over the insulin sensitivity using neuromodulation to achieve improved glucose management.

Chapter 7 contributes to current diabetes technology by introducing a novel decision support system for predicting overnight glycaemia in people with T1DM based on data from the preceding day. Different binary classifiers are trained and compared using features extracted from day-time windows of different lengths. Despite the proposed system currently lacks the neural input, it lays the ground for incorporating bioelectronic information and technology in the future to achieve better performance.

To conclude, Chapter 8 summarizes the presented work, provides an overview of future directions of research, and discusses potential challenges to be addressed to see a successful evolution of bioelectronic medicine within diabetes technology.
This chapter presents an overview of the evolution of diabetes technology over time (see Figure 2.1), where traditional care for patients with diabetes requiring insulin therapy has become safer, more efficient, and cost-effective. Despite the adoption of new pharmaceutical-based technology is associated with improved metabolic control, more time in range, less time in hypoglycaemia, reduced anxiety, and improved quality of life, glucose control is still far from the optimal glucoregulation observed in health. The introduction of bioelectronic medicine has the potential to make a step forward towards improved glycaemic control in patients with diabetes.

![Timeline of Diabetes Technology](image)

**Figure 2.1: Timeline of Diabetes Technology**

### 2.1 Introduction

Glucose is the body’s source of energy and is obtained from the carbohydrates contained in the food. It is stored in the form of glycogen and is used through aerobic respiration. It is also the primary source of energy for the brain. Therefore, it is crucial to ensure that blood glucose levels are kept within a controlled narrow range that guarantees its availability for vital processes.

Healthy people maintain glucose levels between 70 and 180 mg/dl through several mechanisms that prevent undesirable blood glucose fluctuations that may result in dangerous hyperglycaemic or hypoglycaemic situations. These robust mechanisms comprise both chemical and neural signalling involving many biological substances including hormones, neurotransmitters
and signalling particles that interact with each other to ensure tight control of blood glucose. This regulation presents a characteristic attribute that makes it extremely effective, which is redundancy by having multiple control layers on the glucoregulatory response. As a result, if any of the control layers fails in the response, other regulatory mechanisms are in place to prevent a deep fall in blood glucose. There are certain situations, however, where our bodies are not able to restore homeostasis resulting in a breakdown in the glucose metabolic process that characterizes diabetes mellitus. Without treatment, diabetes can lead to fatal consequences and eventually death.

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that causes destruction of the \( \beta \)-cells in the islets of Langerhans in the pancreas, being unable to provide the necessary level of insulin to control plasma glucose levels. Insulin is an anabolic peptide hormone that is secreted in response to increased blood levels of glucose and amino acids following the ingestion of a meal. Insulin regulates the metabolism of carbohydrates and fats by promoting the absorption of glucose from the blood to skeletal muscle and fat tissue, and fats storage. It also inhibits glucagon secretion from pancreatic \( \alpha \)-cells, and hepatic glycogenolysis and gluconeogenesis [35]. The discovery of insulin in 1921 was therefore a breakthrough for diabetes control, as insulin therapy became the standard treatment for T1DM, and for many people with Type 2 diabetes mellitus (T2DM). This event can be defined as the first contribution to diabetes technology, but was definitely not the last one in the run to develop an external device that replaces the lost endocrine functionality of the pancreas of people with T1DM and improves metabolic control (see Figure 2.1).

2.2 Short overview of diabetes technology timeline

The American Diabetes Association defines Diabetes Technology as ‘the hardware, devices, and software that people with diabetes use to help manage their condition, from lifestyle to blood glucose levels’ [36]. In the 1960s, the first dual-hormone pump for insulin and glucagon delivery was developed [37], and towards the end of the decade the first portable glucose meter was available, the Ames Reflectance meter [38]. In the 1970s, the first subcutaneous insulin pump, the AutoSyringe, was introduced in the market and successfully tried in the first trials of continuous subcutaneous insulin infusion (CSII) [39, 40]. Since then, the insulin pump market has achieved the USD 4.15 billion in 2019 and is projected to grow at an average of 9.4% until 2027 [41]. Late in the same decade, diabetes technology experienced two major advances. To begin with, the first closed-loop system, known as the artificial pancreas, was developed in 1979 by using a mathematical algorithm that responds to changes in blood glucose concentrations by auto-
matically modulating insulin delivery \cite{32, 42, 43, 44, 45}. It was called the Biostator Glucose Controller and involved intravenous routes for both glucose sensing and insulin delivery \cite{46}. While the Biostator had the ideal intravascular route for closing the loop, the lack of portability of the entire system prevented widespread use in patients with T1DM in a free-living situation \cite{47}. For this reason, later generations of the artificial pancreas include the subcutaneous (SC) route for both glucose sensing and insulin delivery. The SC, contrary to the intravenous route, is easy-to-access and safe, making it a more suitable path for hormonal delivery. However, this compartment is not physiological and suboptimal, presenting several limitations including time delays on insulin actions during meals (15-20 min) \cite{48}, and on glucose sensing (up to 15 min), which result in reduced accuracy of glucose measurements \cite{49}, and insulin precipitation as a result of changes in local pH and insulin complex formation \cite{50}.

In the same year, 1979, Bergman and Cobelli presented the first mathematical Minimal Model of Glucose Kinetics, which laid foundations for the development of other algorithms that replicate the endocrine function of a healthy pancreas and calculate the optimal doses to be delivered based on glucose readings from a sensor \cite{51}. It was not until 2009 when the first implementation of the pancreatic $\beta$-cell electrophysiology in silicon electronics was proposed, enabling the development of low power, miniaturized, and low-cost closed-loop systems \cite{52}.

The extensive research in designing new algorithms for insulin delivery and glucose control made having an \textit{in silico} testing platform a major requirement. In 2008, the Food and Drug Administration (FDA) approved the first computer simulator of T1DM as a substitute from animal trials for the preclinical testing of control strategies in artificial pancreas studies \cite{53}. This simulator has been used in Chapter 6 to validate a novel controller for insulin sensitivity in people with T1DM.

In addition to a subcutaneous insulin pump and a control algorithm, an artificial pancreas requires a last component to be minimally invasive and autonomous: a sensor for continuous glucose measurements (CGM) (Figure 2.3). We had to wait until the end of the previous century, in 1999, when Medtronic produced the first subcutaneous CGM system called Minimed CGMS \cite{54}. Since then, the CGM device market size was valued at USD 437.0 million in 2018 and is estimated to reach USD 1.25 billion by 2026, growing at an average rate of 13.9\% (see Figure 2.2) \cite{55}. Yet, self-monitoring of blood glucose from capillary blood using glucometers is still considered a fundamental part towards an effective and individualized management of T1DM, that gives the users the ability to follow their metabolic status and act accordingly \cite{56, 57}.  
Despite the early availability of an artificial pancreas, its transition to everyday clinical use is happening recently. This delay for clinical use arises from the complexity of closed-loop systems, relying on the correct functioning of hardware and software to control the challenging glucoregulatory situations present in daily tasks. Therefore, still today, the most widely adopted approach relies on the external subcutaneous injection of insulin multiple times a day with the use of an insulin pen (multiple daily injections), or continuously through an infusion pump (continuous subcutaneous insulin infusion) [42, 45]. Despite these approaches are effective in lowering glycated haemoglobin (HbA1C) levels, which is a marker of blood glucose, they are still open-loop (i.e. it is not an automated system) requiring a lot of manual effort from the user, and finger picking is reported to be very unpleasant for the patients [43]. As a result, most patients still have suboptimal glycaemic control.

### 2.3 Current new features in diabetes technology

Refinements in mathematical algorithms to control continuous subcutaneous insulin infusion, CGMs, and insulin pumps, along with furthering miniaturization and portability of these de-
2.3. CURRENT NEW FEATURES IN DIABETES TECHNOLOGY

Vices, have enhanced the practicality and usability of contemporary close-loop devices, making them fully wearable and minimally invasive [58]. In some cases, other physiological information such as heart rate, accelerometry, or skin temperature collected from additional sensors are also included in the algorithms to improve dose calculations [59, 60, 61]. Current artificial pancreas also include filters and fault detection algorithms to guarantee correct functioning of the controllers [62, 63]. As a result, the state-of-the-art artificial pancreas are now providing real-time, long-term optimal control in patients’ everyday life with reduced risk of nocturnal hypoglycaemia in comparison with previous techniques [42, 43].

Furthermore, recent systems incorporating both insulin and glucagon infusions have extended the concept from an artificial $\beta$-cell to the actual artificial endocrine pancreas. Nowadays there are insulin pumps that, with relatively minor modifications, are already capable of accurately pumping either glucagon or insulin [64]. This dual-hormone artificial pancreas system has higher complexity than the single-hormone system, resulting in improved glycaemic control, especially for reducing the risk and time spent in hypoglycaemia [42, 65, 66], and for recovering from this state [67]. However, there is no consensus on whether dual-hormone is more effective and practical to use than single-hormone artificial pancreas. Single-hormone systems are easier to use and also achieve the desired control of hypoglycaemia during the night [42]. Moreover, excessive glucagon administration can lead to side effects, including hyperglycaemia and nausea, [67], and glucagon storage is limited by its inability to remain stable in solution. In this sense, there is ongoing research into creating improved glucagon formulations [64].

Improvements in CGM technology have enabled daily and continuous evaluation of the glycaemic state of the patients, making other metrics related with the average glycaemia and blood glucose fluctuations the standard markers in clinical control [56]. In more detail, metrics such as time-in-target, risk values and glucose variability can be computed with a temporal resolution of minutes/hours, compared to the time scale of days/weeks obtained with self-monitoring of blood glucose, and months considering the glycated haemoglobin [45].

Finally, the extensive amount of data generated by diabetes technology and the growth of machine learning and big data algorithms [68] have enabled the development and incorporation of decision support systems (DSS). DSS are used to help the users to make decisions on specific situations by, for example, suggesting the administration of meal insulin boluses or corrective insulin boluses to mitigate hyperglycaemia [34, 69, 70], recommending the intake of carbohydrates (CHO) to tackle hypoglycaemia (rescue CHO) [71, 72, 73], or providing suggestions to prevent exercise-induced hypoglycaemia [74]. The use of DSS systems for diabetes
management and a novel DSS for improving control of overnight hypoglycaemia are further presented in Chapter 7.

Currently, one of the most widely employed artificial pancreas systems is the Medtronic 670G (Medtronic, Northridge, CA, USA), which is the first commercial hybrid closed-loop, with an automated basal rate of insulin infusion, and manually administered boluses. It is approved by the US Food and Drug for use in people with T1DM over 14 years of age [75], and is also CE Mark approved for use in people over 7 years of age within Europe. Despite the improved glycaemic control recently reported in clinical trials [76, 77], glucose profiles are still far from acquiring the optimal regulation observed in healthy individuals (see Figure 2.4). In addition, the system is still not fully autonomous and requires user input, especially during meal intake [48, 78].

![Figure 2.4: Comparative of glucoregulation in healthy individuals and T1DM patients with an artificial pancreas.](image)

(a) Mean continuous interstitial glucose profile from a healthy volunteer under everyday life conditions. Figure adapted with permission from Figure 2 in [79]. (b) CGM profile from a person with T1DM using the Medtronic 670G. Figure adapted with permission from Figure 2 in [80]

## 2.4 Incorporating bioelectronic medicine in diabetes technology

Current diabetes therapies are, as described before, mainly based on the administration of drugs, which is the most common form of treatment for most diseases. However, this form of therapy involves important drawbacks that limit its use, such as the development of pharmaceutical drug resistance, its low specificity and existence of systemic effects that may affect wide body regions indiscriminately with the associated side effects, and the problems arising from forgetting to take the medication, among others. Despite the pharmaceutical industry has improved drugs to selectively interact with the target of interest, a common drawback of current...
insulin therapies remains being that subcutaneous insulin is non-physiologically absorbed into the systemic circulation, and due to its chemical nature, it can be metabolized or decomposed into other compounds that may alter unrelated processes [65]. This is associated with risks such as peripheral hyper-insulinemia, weight gain and hypoglycemia. Another major hurdle to the treatment of diabetes is its heterogeneous nature. In particular, intra- and inter-patient variability can affect the efficacy of insulin because of patient changes in insulin sensitivity due to illness, stress, circadian rhythm, dawn phenomenon, among other factors. For these reasons, the development of more targeted therapies that act specifically in the desired places is needed [81].

As a result, other non-pharmaceutical strategies have been investigated in recent years for treating diabetes, such as gene therapies. Suppression of β-cell apoptosis, stimulation of β-cell growth, and induction of β-cell differentiation and regeneration are common approaches for both types of diabetes that have the potential to replace insulin [82]. The diabetes gene therapy has already been tested in mice showing restoration of normal blood glucose levels for an extended period of time, typically around four months [83]. At present, however, these strategies are technically difficult to implement.

This thesis motivates and justifies the creation of other technological solutions involving bioelectronic medicine that move away from a purely pharmaceutical approach to using a combination of technological tools like mathematical modelling and bioelectronics.

2.4.1 Computational modelling

Modelling of biological systems is a highly valuable scientific tool that is widely used in the fields of diabetes and bioelectronic medicine. Computational models provide a mean to realize what information is missing while trying to reproduce the behaviour of a specific system, allowing to direct experimental research towards new directions that are necessary to complete this understanding. This directionality of research lays the foundation for the interplay between deductive and inductive scientific reasoning that is critical to advance in human knowledge, as conceptualised in Figure 2.5. To serve this purpose, developed models should be continuously updated to incorporate new knowledge as it becomes available.

In diabetes, a plethora of model architectures have been proposed to represent and predict the complex processes involved in glucose metabolism. These include from black-box models that use insulin and meal as inputs to predict future glucose levels, such as linear [84] or fuzzy logic models [85], to explainable physiological models that characterize, with greater or lesser detail, the dynamics of the biological mechanisms of interest [86, 87, 88, 89, 90, 91, 49]. Each type of model presents its advantages and limitations, and their use depends on the specific
Figure 2.5: Interplay between modelling and experiments. Modelling provides a strategy to generate a hypothesis of the underlying physiology, which can then be tested using an experimental approach. The acquired new knowledge should then be incorporated to obtain a continuously updated model.

Application [92]. For example, black-box models typically outperform physiological models as they can capture high non-linearities and interactions between features. For this reason, this type of data-driven models is generally used in control systems. On the other hand, black-box models lack the ability to explain the underlying processes, which are clearly defined when implementing explainable models [93]. As a result, explainable models are mainly used to provide simulation environments that give insight to the physiological processes, while providing a platform to monitor interventions.

In bioelectronic medicine, the physiological response to a given stimulation configuration can suffer from significant inter and intra-patient variability. The development of computational models allows to study the complex activation mechanisms of the peripheral nerves and facilitate real-time and personalised predictions of the physiological responses to neuromodulation. Models of peripheral nerves that integrate physiological insights (i.e. properties of axons, spontaneous activity patterns, organ responses, among others) enable faster and more detailed analysis of peripheral nerve stimulation and recording, and accelerate the design of interfaces while reducing the experimentation load [94]. Higher-level computational models have also been developed for both implantable VNS [95, 96, 97] and nVNS [98, 99, 100], with the aim of finding an optimal stimulation configuration to maximize the therapeutic effect while minimizing unwanted side effects. Despite the advances in modelling in the field of neuromodulation, these systems have yet to be put into the context of glucose control and diabetes. Chapter 5 provides the starting point towards this type of hybrid models.
2.4. INCORPORATING BIOELECTRONIC MEDICINE IN DIABETES TECHNOLOGY

2.4.2 Background on bioelectronics and neural stimulation

During peripheral nerve stimulation, at least two electrodes are needed to deliver the electrical pulses to the neural tissue, one acting as reference and the other as stimulation electrode. In the electrode-tissue interface, the injected electrical charge (electrons) is translated into chemical processes that modify the balance of ions inside and outside the nerve, therefore changing the membrane potential. There are two primary mechanisms of charge transfer at the electrode/electrolyte interface that can be represented using an equivalent circuit model as illustrated in 2.6. The first mechanism comprises non-faradaic or capacitive reactions involving a reversible redistribution of charge where no electrons are transferred between the electrode and electrolyte. The second mechanism comprises faradaic reactions that involve electrons transfer between the electrode and electrolyte, resulting in the reduction or oxidation of chemical species [101]. More detail on electrode designs and configurations is given in Section 4.1.2 in Chapter 4.

![Figure 2.6: The electrode/electrolyte interface showing faradaic charge transfer (top) and capacitive redistribution of charge (bottom) as the electrode is driven negative: (a) physical representation, (b) equivalent electrical circuit representation.](image)

When the injected charge is enough to depolarize the membrane above a certain threshold, it leads to the generation and propagation of action potentials. As a result, activation of neural tissue (i.e. fibre recruitment) is primarily affected by the perceived electric field, which is intimately related to the charge density:

\[
\text{Charge density} = \frac{Q}{A} = \frac{I \cdot PW}{A},
\]  

(2.1)
where $Q$ is the injected charge per phase ($\mu$C), $A$ is the stimulation surface ($cm^2$), $I$ is the pulse amplitude ($mA$) and $PW$ is the pulse width ($ms$).

Neuromodulation can be performed by activating the neural tissue, which is known as excitatory stimulation, or by blocking the neural activity, which is known as inhibitory stimulation. For example, high-frequency electrical stimulation is used to provide a safe and rapidly reversible block of nerve activity in peripheral nerves that are pathologically over-activated. Specific examples of both types of neuromodulation are reviewed in the following sections.

There are different methods for controlling charge injection in neurostimulation, with constant current and constant voltage being the most common ones. Constant current control is usually the preferred method because a constant current source directly controls the amount of charge density delivered into the tissue. The injected current is not affected by the tissue impedance, making it safer to use [102, 103]. However, this mode will always be limited by the system maximum voltage. In constant voltage control, a constant voltage is applied between the cathode and anode. In this case, the current flowing between the electrodes is influenced by the tissue impedance, challenging the exact determination of the injected charge.

Irrespective of the control mode, different waveforms can be applied. Monophasic pulses are generally very efficient for creating action potentials. However, the lack of a recovery phase increases the risk of irreversible reactions taking place in the electrode-tissue interface, leading to tissue and electrode damage. As a result, charge-balanced biphasic pulses are generally preferred for their efficiency and reduced tissue damage (allow full reversion). Moreover, inclusion of an interphase delay between pulses allows the tissue to restore its resting potential, making the stimulation more efficient and safer. Among all the parameters that can be controlled for determining pulse waveform, pulse amplitude and pulse width/duration are the most remarkable due to their direct influence on the injected charge. Pulse amplitude controls the volume of the region that is being activated, whereas pulse duration determines the number of fibres recruited within that region. The relationship between the two parameters is usually represented by the Lapicque formula as follows:

\[ I_{th} = \frac{I_{rh}}{1 - e^{\frac{PW}{\tau_m}}}, \]  

\[(2.2)\]

where $I_{th}$ (mA) is the threshold current at each pulse width, $I_{rh}$ (mA) is the rheobase current (intensity when using an infinitely long pulse width), $PW$ (ms) is the pulse width and $\tau_m$ (ms) is the membrane time constant. The graphical representation of this relationship is called strength-duration curves, and determine the pulse amplitude required for a specific pulse duration to activate a nerve.
2.4.3 Vagus nerve stimulation as a therapeutic approach for chronic diseases

In 1997, the US Food and Drug Administration (FDA) approved an implanted cervical VNS device (NeuroCybernetic Prosthesis System, Cyberonics, Inc, Houston, TX, USA - now Livanova) for use in conjunction with drugs or surgery as an adjunctive treatment for adults and adolescents over 12 years of age with medically refractory partial onset seizures [104]. In 2005, the FDA approved the same VNS device for the adjunctive long-term treatment of chronic or recurrent depression for patients over 18 years with a major depressive episode without adequate response to antidepressant treatments [105]. More recent studies have focused on the use of neuromodulation for treating inflammatory diseases by interfering with the neuro-immune reflex [4, 5, 6]. Although the signalling pathway and mechanism by which VNS affects inflammation remain unknown, preclinical VNS has shown promising results in treating chronic inflammatory disorders such as sepsis, lung injury, pain management, cardiovascular disease, stroke or traumatic brain injury, obesity, fibromyalgia, migraines, rheumatoid arthritis (RA), and diabetes [106]. Moreover, clinical implantable VNS devices have improved outcomes in several inflammatory diseases mainly, RA [7], Crohn’s disease [107], and fibromyalgia [108], with some patients achieving disease remission. However, implantation of a VNS device is associated with complications such as bradycardia, asystole, delayed arrhythmias, syncope, cough, paraesthesia, pain, sleep apnoea, surgical or vagus nerve trauma with unilateral vocal cord dysfunction and dyspnoea, and thermal injury to the vagus nerve and adjacent structures due to radio-frequency exposure [109, 110].

Non-invasive VNS. To avoid surgical implant-related complications, researchers have developed non-invasive VNS (nVNS) devices that stimulate the vagus nerve through either transcutaneous auricular stimulation (TA-VNS) or transcutaneous cervical stimulation (TC-VNS). Application of nVNS in hemicrania continua [111], asthma [112], chronic pelvic pain [113], pain perception [114], migraines [115], and RA [116] have all shown positive results in small sample sizes with no significant serious device-related adverse events reported.

2.4.4 Neuromodulation as a therapeutic approach for controlling glucose metabolism in diabetes

The majority of research related to glucose control and neuromodulation has occurred in the context of metabolic disorders, specifically in the control of appetite and food intake in managing obesity [106, 117, 118, 119]. Direct stimulation of the gastric myoelectrical activity with gastric pacemakers, known as gastric pacing, is one of the most exploited techniques [120, 121, 122]. For example, delivery of high-frequency and short-pulse width vagal stimulation in human clinical studies with the Transcend gastric pacemaker (Transneuronix, Mt. Ar-
NEMOS is a transauricular VNS device that stimulates the cymba chonica. GammaCore is a transcervical, self-administered VNS device stimulating through the neck to the cervical vagus nerve. 

In Livingston, NJ, inhibited the efferent vagal activity to the stomach [120, 121, 123], which caused increased gastric distension and slowed gastric emptying. These changes led to earlier satiety, and eventually a reduction of food intake. These studies reported a 20% to 40% of weight loss, depending on the patient. These findings on gastric pacing support further research in direct modulation of vagal activity for obesity management, which is known as vagal pacing. In this regard, a human study in patients already using VNS to treat epilepsy reported a variety of responses, with slightly more than 50% of the patients losing between 5% and 10% of body weight [124]. More advanced strategies are being developed to automatically modulate vagal activity in response to stomach distension and/or presence of satiating hormones, among other markers [125, 126, 127, 128, 129, 130]. As an example, a recent study in rats successfully reported up to a 38% of body weight reduction by delivering biphasic electric pulses based on the peristalsis measured on the stomach [129]. Bilateral VNS applied abdominally during 12 weeks recovered the insulin sensitivity and hepatic glucose uptake rate of obese mini-pigs to the values obtained from lean animals [131].

Conversely, research on bioelectronic medicine for direct control of diabetes has emerged recently, with two main areas of interest: i) recording from the peripheral nerves to extract metabolic information and ii) modulating their electrical activity to improve glycaemic fluctuations. Regarding the former, recent work aims to use the vagus nerve as a glucometer to identify hypoglycaemic events from neural readings [132, 133]. In more detail, the neural activity presents a negatively correlated response to glycaemia [132]. The complexity of the recorded data requires advanced strategies to identify and decode neural signals related to glycaemic levels. In particular, Masi et al. (2019) have developed a decoding algorithm that recreates blood glucose levels based on the identified neural events with high accuracy using regression models with regularization to avoid over-fitting [132]. The preliminary results are promising
and encourage the application of their method for glucose monitoring and control in the near future, especially for T1DM.

Neuromodulation by selective electrical blockade or activation of specific vagal fibres to specific organs is a promising way to impact different metabolic processes in diabetes resulting in different glycaemic outcomes. One strategy that is traditionally used in animal experiments to achieve vagal blocking of afferent/efferent fibres consists in direct transection of the vagus nerve [134], however, this methodology cannot be translated into clinical research in humans. As a result, ongoing research is focused on finding the combination of parameters that can selectively block specific fibre types, or the afferent/efferent traffic [106]. One such technique uses high-frequency stimulation targeting a variety of nerves and locations. For example, this strategy has shown to successfully block nerves conveying pain signals, making it an alternative efficient method to conventional spinal cord stimulation in the treatment of chronic pain [135]. In the field of diabetes treatment, Shikora et al. (2013) used intermittent vagal blocking at the level of the abdomen in people with T2DM, and reported a reduction in body weight and improved glycaemic control based on enhanced levels of HbA1c, which is a marker for diabetes development [136]. Conde et al. (2018) and Sacramento et al. (2016, 2019) also successfully restored insulin sensitivity and glucose tolerance in people with T2DM by blocking the activity of the carotid sinus nerve using kilohertz frequency alternating current (KHFAC) modulation [137, 138, 139].

A recent study has also explored the impact of VNS on glucose levels in type 2 diabetic rats [140]. In more detail, the dorsal subdiaphragmatic vagus nerve was stimulated with different combinations of stimulation parameters (amplitude: 2mA and 3mA; pulse width: 0.3 ms and 3 ms; frequency: 5Hz, 14Hz, 40Hz, and 5KHz). They found a variety of interesting results, such as that shorter pulse widths of 0.3ms and lower stimulation frequencies of 5Hz are more effective in reducing blood glucose levels during an oral glucose tolerance test (OGTT). Interestingly, their results also suggest that intermittent stimulation results in greater responses than continuous stimulation. They did not report any difference between unilateral and bilateral VNS.

The impact of neurostimulation on T1DM has been less explored, but recent research from Guyot et al. (2019) suggests that stimulation of the pancreatic sympathetic nerves projecting to the pancreatic lymph nodes inhibits the progression of the disease, at least in mice [141]. Activation of these nerves with a stimulation frequency of 10 Hz and 450 µA of amplitude resulted in a reduction of pro-inflammatory cytokines and a proliferation of autoreactive T cells, therefore delaying the onset of the disease and limiting its progression. By carrying out a
preliminary optimization of the stimulation parameters, they eliminated undesired effects such as blood flow alteration and axonal excitability exhaustion, making it suitable for therapeutic use. These results demonstrate the important, and yet generally disregarded, contribution of the nervous system in the pathology of T1DM. Together with previous findings of electrical stimulation of peripheral nerves for neural regeneration, these motivate the use of peripheral neuromodulation as a strategy to restore the neural signalling dysregulation in T1DM.

It is also possible to directly stimulate the organs of interest to regulate their function. For example, acute electrical stimulation of the parenchyma tissue of the liver for 90 min resulted in a reduction of both fasting and postprandial glucose levels in the healthy state and type 1 and type 2 diabetic rats [142]. Moreover, continuous stimulation for 8 hours during 4 consecutive days not only resulted in decreased glucose levels in the three groups, but also delayed gastric empty and increased plasma glucagon-like peptide-1 (GLP-1) level. Adverse effects were not reported in either case, making it a feasible and safe approach for glycaemic control.

Finally, inflammation has been found to be related to obesity-induced insulin resistance in people with T2DM [143], as inflammatory cytokines such as TNF can directly induce insulin resistance [144, 145]. In addition, reduced vagal activity has been reported in these patients [144]. As a result, modulation of the immune reflex arc using vagal activation might provide a pathway to alleviate both the inflammatory and the metabolic dysregulation of these patients [143, 144].

2.5 Conclusion

In conclusion, diabetes technology has evolved extensively over the last century and it is currently entering into the stage of fully automated portable systems: in silico models provide detailed descriptions of the human metabolic system; physiological signals are available continuously and in real-time, including continuous glucose monitoring; insulin delivery is automated; and advanced control algorithms are capable of controlling blood glucose fluctuation even in challenging everyday life scenarios. However, limitations such as delays in external insulin action [48] and the risk of hypoglycaemia [146], still have to be resolved in order to achieve the best glycaemic control. Incorporating the opportunities that bioelectronic medicine offers for diabetes will enable improved pre-emptive computation of the insulin doses, increased autonomy, and personalized glucose control. The following chapters enlarge current understanding on the impact of electrical modulation of the vagus nerve on glucose metabolism, and present some of the clinical fields where diabetes therapy can benefit from incorporating bioelectronic technology.
The previous chapter has shown that there is potential for improved glucose control using bio-electronic medicine by interfacing at various levels in the central and peripheral nervous system to impact glucose control. Neuromodulating the central nervous system to control glucose homeostasis, although being a promising field, is out of the scope of this thesis. The reader can find a short review of opportunities and ongoing research in Appendix B. This chapter provides a review of the metabolic control acquired through interfacing with the peripheral nervous system. To begin with, Section 3.1 provides a general overview of the neural peripheral pathways that are involved in the hormonal secretion by the endocrine pancreas. This review is extended in Section 3.2 with an overview of the techniques that are commonly used to study the vagal control of the pancreatic endogenous secretion and hemodynamic processes, including vagus nerve stimulation and the use of neural blockers.

3.1 Neural innervation of the pancreas

The peripheral nervous system ultimately controls the activity of the endocrine pancreas, including glucagon and insulin secretion, and α and β-cell number and proliferation [147, 148]. There are two major pathways identified in regulating the endocrine pancreatic function, the parasympathetic and sympathetic pathways (see Figure 3.1). There are other branches formed by sensory nerves, nitric oxide and cholecystokinin (CCK) nerves that are also suggested to take part in regulating the pancreatic function.

3.1.1 Parasympathetic innervation

Many studies have demonstrated an extensive parasympathetic innervation of the pancreatic cells, which mainly involve the vagus nerve. This is the tenth cranial and is also known as the pneumogastric nerve because it targets the heart, lungs, and digestive tract. It increases the islets secretion through two main mechanisms: cholinergic regulation via the release of acetylcholine (ACh) and non-cholinergic mechanisms mediated by neuropeptides such as the vasoactive intestinal polypeptide (VIP), the pituitary adenylate cyclase activating polypeptide (PACAP), or the gastrin-releasing polypeptide (GRP). These neurotransmitters convey the neural signals by binding to specific receptors that are present on the β-cells and α-cells.
CHAPTER 3. REVIEW OF PERIPHERAL NEUROMODULATION OF THE PANCREAS

Figure 3.1: Schematic of the most significant neural connections between the brain and the pancreas. The neural pathways to the α and β cells include postganglionic parasympathetic (green colour range) and sympathetic (red colour range) nerves. Afferent connections from the pancreas to the brain are also depicted (grey).

Cholinergic Mechanisms

The preganglionic cholinergic efferent fibres of the bilateral vagus nerve that innervate the intramural ganglia associated with thoracic and abdominal viscera (heart, the lungs, and the gastrointestinal tract) are originated in the dorsal motor nucleus of the vagus nerve (DMNX) [149, 150, 106]. This area shows a viscerotropic organization, meaning that neurons projecting to different parts of the gastrointestinal tract are located in anatomically different mediolateral columns. In more detail, neurons in the lateral columns of the DMNX columns predominately form the right bundle of the cervical vagus nerve. In contrast, the preganglionic fibres that project to the head and body of pancreas occupy the two medial columns of the DMNX and form part of the left cervical vagus nerve. From its origin in the DMNX the vagus nerve runs caudal to the glossopharyngeal nerve and superficial to the internal jugular vein [151] towards the jugular foramen. At the cervical level, the vagus nerve continues descending between the internal jugular vein and the internal and external carotid arteries, and bifurcates into different branches innervating the larynx, bronchi, lungs, heart, and esophagus [106, 125]. In the lower esophagus, the left vagus runs ventrally whereas the right bundle runs dorsally. After cross-
3.1. NEURAL INNERVATION OF THE PANCREAS

ing the diaphragm, the dorsal (or posterior) subdiaphragmatic vagus nerve ramifies into the dorsal gastric and the dorsal celiac branches. On the other hand, the ventral subdiaphragmatic vagus divides into the ventral (or anterior) gastric and celiac branch, and the common hepatic branch [152]. Both the ventral and dorsal gastric branches innervate the stomach and proximal duodenum, whereas the celiac branches innervate the distal duodenum and colon [125]. The hepatic branch further bifurcates into the main hepatic branch that innervates the liver, and the gastroduodenal branch, that innervates the duodenum and proximal pancreas [106] (see Figure 3.2. Once in the pancreas, the preganglionic fibres make presynaptic connections with the cholinergic postganglionic fibres in the intrapancreatic ganglia. These postsynaptic nerves then penetrate within the islets close to the pancreatic cells [149, 150]. Interestingly, the vagal afferent circuitry that conveys information about the physiological state of the target organs presents remarkable plasticity, showing a great degree of adaptability in response to the large number of local changes that arise in the short and long terms, such as synaptic structural remodelling and changes in membrane excitability [153]. At the level of the brain, the vagal brainstem nuclei establish multiple central connections in regions involved in interoceptive reflexive functions that are ultimately related to glucose metabolism as described in Appendix B.

Figure 3.2: Schematic of the vagus nerve gross anatomy. The red line shows the neural pathway from the DMNX to the pancreas. Adapted from [154].
In summary, when the vagus nerve is activated, the terminals of the preganglionic nerves in the intrapancreatic ganglia release ACh that binds to nicotinic receptors in the postganglionic nerves. In turn, this binding causes the release of ACh from the postganglionic terminals into the islets [155, 156], where it binds to muscarinic receptors in the pancreatic β-cells eliciting insulin secretion [157, 158]. The vagus nerve then conveys information from the pancreas via afferent sensory fibres projecting directly from this organ to the solitary and spinal trigeminal nuclei of the brainstem, whose cell bodies are located in the inferior and superior cervical ganglia [153]. Interneurons within the brain finally close the loop by integrating the sensory information from afferent fibres and conveying control actions through efferent fibres to maintain body homeostasis.

Structural anatomy of the vagus nerve

The gross anatomy of the vagus nerve, as described in Section 3.1.1, is well studied, yet its fascicular functional anatomy remains almost completely unknown. The vagus nerve is a heterogeneous nerve that comprises efferent fibres (∼20%) and a great proportion of afferent sensory fibres (∼80%), with the specific morphology depending on the location of the nerve [159, 160, 161]. It therefore enables bi-directional communication between the brain and the different parts of the body, transmitting both sensory and motor information (see Figure 3.1). Vagal fibres can be further described according to the classification by Erlanger and Gasser (1937) [162] based on their nerve conduction properties in A, B and C fibres (see Table 3.1) [150, 160, 134, 163]. Each type of fibre carries different physiological information: large myelinated A-fibres mostly convey somatic afferent and efferent signals, small myelinated A-fibres visceral transmit afferent signals, B-fibres provide efferent sympathetic and parasympathetic preganglionic innervation, and small unmyelinated C-fibres comprise most of the afferent visceral innervation [150]. Their size, and therefore conduction properties, affect the excitation thresholds at which they respond to electrical stimulation. The stimulation threshold is determined by the total charge delivered to the nerve fibre, which depends on the stimulation current, the pulse duration, the stimulation frequency (i.e. the rate at which these pulses are applied) and the waveform, among others [164, 165]. In general, higher stimulation currents, longer pulse durations, and/or higher stimulation frequencies are needed to activate smaller nerve fibres. As a result, A-fibres are recruited first with the lowest stimulation current, followed by B-fibres and C-fibres with increasing stimulation intensities [150, 161, 134, 164]. In conclusion, the physiologic response to vagal nerve stimulation greatly depends on the recruitment of fibres based on the different stimulation parameters.
3.1. NEURAL INNERVATION OF THE PANCREAS

Table 3.1: Characterization of vagus nerve fibres.

<table>
<thead>
<tr>
<th>Type of fibre</th>
<th>Size (diameter)</th>
<th>Myelinated</th>
<th>Velocity range (m/s)</th>
<th>Transmitted information</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-fibre</td>
<td>Large (5–20um)</td>
<td>Yes</td>
<td>&gt;4.5</td>
<td>Afferent visceral information and efferent motor signals</td>
</tr>
<tr>
<td>B-fibre</td>
<td>Mid-size (1–3um)</td>
<td>Yes</td>
<td>2-4.5</td>
<td>Most of the efferent parasympathetic signals</td>
</tr>
<tr>
<td>C-fibre</td>
<td>Small (0.4–2um)</td>
<td>Yes</td>
<td>&lt;2</td>
<td>Mainly afferent visceral information</td>
</tr>
</tbody>
</table>

Noteworthy, the electrical properties of the vagus nerve fibres vary depending on the nerve projection of interest [166, 102]. For example, the pancreatic branch differentiates from gastric-projecting neurons in that they have i) longer duration of action potentials, ii) longer after-hyperpolarization decay time, iii) smaller soma area and iv) larger diameter. Moreover, when compared to the intestinal branch, pancreatic neurons have i) higher input resistance, ii) smaller after-hyperpolarization amplitude and iii) a higher firing rate in response to current injections [166].

Non-cholinergic pathways

Previous studies in dogs and pigs have reported that insulin secretion mediated by vagal signalling is sometimes resistant to the muscarinic inhibition with atropine, which suggests that there are other non-cholinergic mechanisms involved through the action of peptides [167]. As a result, activation of the vagus nerve enhances islets hormone secretions through both cholinergic and non-cholinergic mechanisms [168]. Up to date, three neuropeptides, VIP, GRP and PACAP, have been found in the terminals of pancreatic ganglia nerves and are thought to be released after vagal activation using VNS in rats and pigs [18, 167, 168]. These neuropeptides increase both insulin and glucagon secretion in vivo and in vitro in several species including humans [168]. Interestingly, VIP and PACAP have been revealed to stimulate insulin secretion in a glucose-dependent manner [168].

3.1.2 Sympathetic innervation

The preganglionic sympathetic nerves originate in the hypothalamus and leave the spinal cord at the level of C8 to L3. The fibres then pass through the lesser and greater splanchnic nerves to reach the paravertebral ganglia chain or the celiac and mesenteric ganglia where they synapse with postganglionic fibres [166]. These can be either adrenergic containing noradrenaline (NA) or non-adrenergic involving other neurotransmitters, and eventually innervate the intrapancreatic ganglia and the islets. Preganglionic fibres can also directly enter the pancreas without an intermediate synapse [168, 169].
Adrenergic Mechanisms

Electrical stimulation of adrenergic nerves elicits the release of NA into the pancreatic veins. By binding to \( \alpha \)-adrenoceptors, NA inhibits both basal and glucose-stimulated insulin secretion \([147, 170]\). This response has been demonstrated \textit{in vivo} in dogs and calves, and in the isolated pancreas of rats, pigs and humans \([168, 169]\). Interestingly, NA can also stimulate insulin secretion by two different actions: through the activation of the islet \( \beta_2 \)-adrenoceptors, and through an indirect action on the \( \alpha \)-cells, probably mediated by both \( \alpha_2 \)-adrenoceptors and \( \beta_2 \)-adrenoceptors that stimulate glucagon secretion, which in turn stimulate insulin secretion. These mechanisms have primarily been observed in rats, although an increase in insulin basal levels was also reported during sympathetic nerve stimulation in pigs \([171]\). In summary, the net effect of noradrenaline on insulin secretion might depend on the relative abundance or activity of \( \alpha \)-adrenoceptors compared with \( \beta \)-adrenoceptors on the \( \beta \)-cells \([172]\), but the overall effect is an inhibition of insulin release.

Regarding glucagon secretion, sympathetic innervation mainly stimulates it through activation of the \( \alpha \)-cell \( \beta_2 \)-adrenergic receptors \([147, 170]\). Both central and peripheral glucose sensors that detect hypoglycaemia are greatly involved in the neural reflex that controls this process \([147]\). For example, the hepato-portal vein region contains glucose sensitive nerve terminals that ultimately control glucagon secretion \([173]\).

Non-adrenergic Mechanisms

The inhibitory action of sympathetic nerves on insulin secretion is also mediated by non-adrenergic neuropeptides, such as galanin and neuropeptide Y (NPY), which are localised in the sympathetic nerve terminals in the islet and in the nerve cell bodies in the celiac ganglion. However, as reported in Table 3.2, the effect of these neuropeptides on insulin release substantially varies among animal species \([168]\). Further studies have to be carried out to elucidate the contribution of these peptides to the pancreatic secretion.

**Table 3.2:** Summary of the effects of non-adrenergic neuropeptides on different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Effect on insulin secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogs</td>
<td>Galanin greatly inhibits insulin secretion. No sign of NPY mediated inhibition.</td>
</tr>
<tr>
<td>Rats</td>
<td>Great NPY mediated inhibition.</td>
</tr>
<tr>
<td>Humans</td>
<td>No sign of galanin inhibition.</td>
</tr>
<tr>
<td>Pigs</td>
<td>Both galanin and NPY has demonstrated to stimulate insulin secretion.</td>
</tr>
</tbody>
</table>

A summary of the impact of each peripheral neural pathway on glucose metabolism and pancreatic endogenous secretion can be found in Table 3.3.
3.2 Techniques to study the neural control of the endocrine pancreas

Many techniques have been implemented to study the neural mechanisms that mediate the pancreatic secretion. These normally consist either of an assessment of the responses elicited by VNS or on a direct interruption of the neural innervation involved, therefore expecting an abolishment of the responses. Studies focused on the general understanding of the neural control of the endocrine pancreas most often use the former technique to extract conclusions. On the other hand, studies that aim to identify the specific neural circuitry involved in the secretion of insulin and glucagon most commonly apply interruption of neural signalling.

3.2.1 Vagus nerve stimulation

Impact on insulin and glucagon secretion

Back in 1967, Kaneto et al. already suggested that the neural pathways could be used to regulate insulin secretion [174]. In one study, they showed that stimulation of the right and left cervical vagus and the dorsal vagal trunk induced an immediate significant elevation in in-
sulin secretion, with no decrease in blood glucose, contrary to stimulation of the ventral vagal trunk. Further experiments in the field have reinforced the idea that artificial stimulation of the vagus nerve overall increases insulin secretion from the $\beta$-cells in many species, including rats [149, 134], pigs [175, 176, 177] and dogs [178]. In fact, vagotomy results in an inhibition of insulin secretion despite VNS [178]. The results of these studies using stimulation are complemented with recent observations from experiments that record from the vagus nerve before and after glucose and insulin dosage. Interestingly, these studies found an increase in vagal activity within 10 minutes after the administration of insulin, and a decrease in vagal signalling after glucose disposal [179, 132]. It is also hypothesised that basal afferent signalling in the vagus nerve conveys information related to the hypoglycaemic state [132]. Overall, these changes in the vagal firing rate may mediate the secretion of insulin from the $\beta$-cells and the counteregulatory response to hypoglycaemia. It is noteworthy that the impact of VNS in some animal models sometimes differ from the responses observed in humans. For example, a recent study in human patients suffering rheumatoid arthritis reported that VNS applied before meal intake (30 minutes) resulted in reduced levels of the early postprandial insulin secretion (first 60 min after meal intake), but not in the total postprandial plasma insulin levels [180]. Therefore, more research in animal models that resemble the human neurophysiology and neuroanatomy is needed to fully understand the neural control of metabolic processes and to eventually translate the stimulation paradigms to humans, as explained in Section 4.1.2 in the next chapter.

There are other factors that influence the results of the experiments, including 1) the direction of propagation of the action potentials induced by the stimulation (i.e. afferent or efferent), 2) the basal glycaemic state, 3) the site of stimulation, and 4) the use of health or disease models. These are described in more detail in the following lines.

To begin with, vagal stimulation of afferent and efferent branches results in different outcomes. Selective efferent parasympathetic activation, achieved by stimulating the distal end of the sectioned cervical vagus nerve in fed rats, resulted in a small temporary increase in blood glucose levels followed by a reduction of its concentration, associated with an increase in serum insulin levels [134, 164] and a decrease in hepatic glucose release [134]. These results are in agreement with the increase in glucose and insulin levels after stimulation of the distal stump of the left vagus nerve in fed rats previously reported by Peitl et al. (2005) [181]. On the other hand, afferent parasympathetic activation, either selective or combined with efferent stimulation, caused a sustained and strong increase of blood glucose levels as a result of an increase of hepatic glucose production and inhibition of insulin secretion [134].
Secondly, both *in vitro* and *in vivo* studies have reported dependence of insulin secretion after vagal stimulation on blood glucose levels [182, 183, 184]. In fact, researchers have observed a higher efficacy of ACh on promoting insulin release with higher plasma glucose concentrations. This enhanced response with glucose concentration has also been reported for GIP and neurotensin secretion, whereas the release of glucagon and PP has unexpectedly been found to be reduced under these conditions [184].

Thirdly, the site of stimulation and its accessibility greatly conditions the experimental design and the observed responses. Stimulation at the cervical level is systemic, virtually impacting all visceral organs, yet it is more accessible from the surgical perspective, and also the nerve is bigger facilitating the electrode implantation. As we descend the nerve closer to the organs of interest, the stimulation is more targeted, but the access is more challenging and the nerve diameter is greatly reduced. One study by Rozman et al. (2002) in the intact pancreas found that stimulation of the vagus nerve at the cervical level resulted in a significant increase in insulin secretion and a decrease in the secretion of C-peptide, whereas no significant changes were observed in glucagon levels [185]. On the contrary, stimulation of the pancreatic nerve did not cause any change in the levels of any of the hormones. They also reported an increase in glucagon release, with no changes in insulin or C-peptide levels after splanchnic nerve stimulation. These discrepancies may arise from the non-specificity that characterizes cervical VNS, which results in many simultaneous confounding systemic effects, such as hemodynamic changes as explained in the following chapter and efferent signalling to the liver and stomach. These off-target effects may affect glucagon and ghrelin secretion, thereby indirectly impacting the outcomes of pancreatic neuromodulation [50].

Finally, it is worth highlighting the impact of using healthy vs disease models. Healthy organisms have counterregulatory mechanisms that are fully functional for maintaining homeostasis that may therefore affect the expected outcomes. For example, the experimental procedure presented in the previous paragraph was repeated under T1DM conditions resulting in very different outcomes [185]. Firstly, vagal nerve stimulation caused a marked increase in both insulin and glucagon secretion, with a minor rise in C-peptide. Secondly, the stimulation of the pancreatic branch was found to result in a small increase in glucagon and C-peptide. Finally, activation of the splanchnic nerve resulted in a small decrease in insulin secretion, a small increase in C-peptide and a marked increase in glucagon levels.

Regarding the neural contribution to glucagon secretion, there are studies that reveal an increase in the secretion of glucagon after vagal stimulation in several species [186, 170] including humans [147, 187], although the specific neural mechanisms that are involved vary
among species. In dogs, this response is suggested to be mediated by non-muscarinic mechanisms (probably peptidergic and nicotinic) [188]. In rodents, however, glucagon secretion is thought to be mediated by M3 muscarinic receptors [186, 170]. Finally, a recent interesting study in humans suggests that, in contrast to rodents’, human α-cells do not express any type of muscarinic receptors that lead to increases glucagon secretion [189].

**Impact on the hemodynamic and respiratory responses**

In addition to the metabolic responses, it is worth studying the impact of VNS on vital processes such as heart rate, blood pressure and breathing. The heart rate response to vagal nerve stimulation is primarily mediated by B-fibres that carry most of the efferent parasympathetic signals. As previously mentioned, the recruitment of these fibres depends on whether the stimulation parameters surpass their threshold of activation. For example, it has been reported that for a voltage of 3V and a stimulation frequency of 5Hz, a pulse duration of 0.5msec is required to recruit B-fibres [164]. However, shorter pulse durations can also surpass the threshold for B-fibres by increasing the stimulation frequency or amplitude. In the same study, the respiratory response was found to have a biphasic response with increasing pulse durations. Up to a pulse duration of 0.5msec, respiration rate increased with increasing pulse widths, decreasing afterwards with longer pulse durations.

Recent studies have also demonstrated a differential response in blood pressure, heart rate and respiratory rate depending on the side of stimulation [164]. Left cervical vagal nerve stimulation in rats resulted in a greater bradycardic (decreased heart rate), hypotensive (decreased blood pressure), and tachypneic (increased breathing) effects than right nerve stimulation.

**3.2.2 Techniques based on interruption of the neural pathways**

The techniques based on interruption of the neural pathways can be classified based on their invasiveness. Among the minimally-invasive techniques, the most exploited methodology is the use of neural blockers that allow to selectively inhibit the neural branches involved in the mechanisms of interest. These are explained in more detail in the next section. Another less invasive technique is called vagal cooling and allows to carry out reversible cervical vagotomy [175]. Invasive techniques include sub-diaphragmatic vagotomy for complete surgical denervation of the pancreas [7, 190, 158], or selective denervation of the β-cells[158]. The latter seems to be most precise as it allows a selective denervation of the β-cells while leaving intact the innervation to other islet cells and the gastrointestinal tract [158]. However, it is associated with many surgical complications that challenge its use.
3.2. TECHNIQUES TO STUDY THE NEURAL CONTROL OF THE ENDOCRINE PANCREAS

Neural blockers

The use of neural blockers is one of the most popular methods for understanding and identifying the neural mechanisms that mediate the hormonal secretion from the pancreas. Pre-ganglionic sites in both the sympathetic and parasympathetic nervous systems are regulated by nicotinic acetylcholine receptors. Postganglionic sympathetic nerves are usually regulated by norepinephrine acting on adrenergic receptors, whereas postganglionic parasympathetic nerves are controlled by acetylcholine acting on muscarinic receptors. It is worth noting that some postganglionic sympathetic neurons also release acetylcholine. Given these mechanisms of actions, different neural blockers can be used to inhibit the pathways of interest (see Figure 3.3). A summary of the most used neural blockers is presented in Table 3.4. Other neural blockers that have been previously reported are phenoxybenzamine chloride [176], α-bungarotoxin (ATX) or curarine chloride, which works as a nicotinic receptor blocker [191], and the Na+ channel blocker TTX [155].

Table 3.4: Review of the most remarkable neural blockers and their action.

<table>
<thead>
<tr>
<th>Neural blocker</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimethaphan</td>
<td>Ganglionic nicotinic blocker that prevents stimulation of postsynaptic receptors by competing with acetylcholine (i.e. cholinergic transmission), therefore blocking both the sympathetic and the parasympathetic signalling mechanisms. It is widely used to fully remove the parasympathetic post ganglionic signalling during the cephalic and absorptive phases.</td>
<td>[192, 193]</td>
</tr>
<tr>
<td>Atropine</td>
<td>Competitive reversible antagonist of muscarinic acetylcholine receptors. As a result, it only blocks the contribution of the muscarinic cholinergic signalling.</td>
<td>[156, 157, 167, 191, 192, 176, 177, 178, 181, 184, 193, 194]</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>Non-selective α-adrenergic blocker. It has been found to inhibit epinephrine-induced hyperglycaemia and increase portal venous insulin concentration [178]. It has also been used to counteract the inhibitory effect of circulating catecholamines on the β-cells [183].</td>
<td>[156, 167, 191, 177, 178, 183]</td>
</tr>
<tr>
<td>Hexamethonium (HEX)</td>
<td>Ganglionic nicotinic receptor blocker, therefore acting on receptors at preganglionic sites in both the sympathetic and parasympathetic nervous systems, and blocking its contribution.</td>
<td>[155, 167, 191, 176, 177, 188]</td>
</tr>
<tr>
<td>Propranolol</td>
<td>Non-selective β-adrenergic receptor blocker, with negligible effects on α-adrenergic and muscarinic receptors.</td>
<td>[149, 156, 191, 176, 177]</td>
</tr>
<tr>
<td>Methylatropine</td>
<td>Muscarinic blocker.</td>
<td></td>
</tr>
<tr>
<td>Timolol</td>
<td>Non-selective β-adrenoceptor blocker that prevents action of epinephrine (i.e. sympathetic signalling).</td>
<td></td>
</tr>
<tr>
<td>6-hydroxydopamine</td>
<td>Selectively destroys dopaminergic and noradrenergic neurons, resulting in sympathectomies.</td>
<td>[195]</td>
</tr>
</tbody>
</table>
Interestingly, some studies have reported only a partial inhibition by atropine of the vagal-induced hormonal secretion [168]. Moreover, the secretion of insulin and glucagon induced via vagal mechanisms in pigs is completely resistant to atropine [167]. In these cases, nicotinic ACh antagonists, like hexamethonium (HEX), are used to guarantee the inhibition of the nerves of interest [168]. Nicotinic neural blockers are indeed more effective because they act directly in the ganglia blocking all postganglionic neurotransmission, which is fully mediated by acetylcholine [167].

Finally, muscarinic blockers, such as atropine, have to be used with care as they can indirectly impact the results in studies of the metabolic impact of meal intake or the cephalic phase by (i) inducing an immediate increase in heart rate [157], (ii) causing a marked reduction in salivation, which can interfere with food appreciation [157], and (iii) significantly delaying gastric emptying, which can result in a reduction of insulin release [194].

### 3.3 Executive summary

#### Neural innervation of the pancreas
3.4 Conclusion

- There are two major pathways identified in regulating islet secretion and hepatic function, the parasympathetic primarily through the vagus nerve, and the sympathetic.

- The vagus nerve increases the islets secretion of insulin and glucagon through two main mechanisms: cholinergic regulation via the release of ACh and non-cholinergic mechanisms mediated by neuropeptides (vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase activating pituitary adenylate cyclase activating polypeptide peptide (PACAP), or gastrin-releasing peptide (GRP)). Vagal signalling is sometimes resistant to muscarinic inhibition with atropine, which suggests that other non-cholinergic mechanisms also play a major role in enhancing hormone secretion from the pancreas.

- Sympathetic innervation is divided into two types of signalling mechanisms: adrenergic mechanisms that elicit the release of noradrenaline, and non-adrenergic mechanisms mediated by galanin and/or NPY.

Vagal control of metabolic and hemodynamic processes

- Nerve stimulation threshold is determined by the total charge delivered to the nerve fibre, which mainly depends on the stimulation current, the pulse duration, the stimulation frequency, and the pulse waveform. Large A-fibres have the lowest stimulation threshold and therefore are recruited first, followed by medium size B-fibres, and small C-fibres.

- When studying the impact of electrical stimulation on the vagus nerve on glucose control it is important to consider 1) the animal model, 2) the direction of propagation of the action potentials induced by the stimulation (i.e. afferent or efferent), 3) the basal glycaemic state, 4) the site of stimulation, and 5) the use of health or disease models.

- Cervical vagus nerve stimulation has an impact on heart rate, blood pressure and breathing. Therefore, special care has to be taken on monitoring these processes during the experiments to guarantee the safety of the animals and its transferability to the clinic.

3.4 Conclusion

This chapter has provided a detailed review of the gross and microscopic anatomy of the peripheral nerves that innervate the pancreas and how they are involved in the control of the secretion of insulin and glucagon and, therefore, in glucose homeostasis.
IN VIVO EXPERIMENTS: IMPACT OF VNS ON GLUCOSE HOMEOSTASIS

With the aim to increase the current knowledge on neuromodulation of glucose metabolism presented in the previous chapter, this chapter presents an overview of the experiments carried out during two research internships at Johns Hopkins University under the supervision of a cross-divisional team of experts, composed by Prof. Etienne-Cummings’ time, Dr. Sheng Bi, and Prof. Nitish Thakor. The chapter first describes the most relevant considerations that were taken into account for designing a rigorous and successful experimental protocol (Section 4.1.2). Then, Sections 4.2 to 4.7 present and discuss three studies aiming at studying the effects of changing the stimulation waveform parameters on the regulation of glucose fluctuations during electrical stimulation of the vagus nerve. These experimental results were used to validate the neural-metabolic mathematical model presented in the following chapter. Part of the results have been previously published in the Journal Diabetes Technology & Therapeutics (Güemes et al, 2020 [196]) and presented in the Society of Medical Research meeting Bioelectronic Medicines: Past, Present and Future (London, 2019).

4.1 Introduction

4.1.1 Rationale for the experiments

Previous studies have investigated the impact of various stimulation parameters on the hemodynamic response (heart rate and blood pressure) [164, 197, 198]. Others have tried to find the combination of parameters that can selectively activate or block specific fibre types connecting to specific organs as a way to impact different metabolic processes [106]. One such technique uses high-frequency stimulation targeting a variety of nerves and locations. For example, Shikora et al. (2013) used intermittent vagal blocking at the level of the abdomen in people with type 2 diabetes, and reported a reduction in body weight and improvement of glycaemic control based on enhanced levels of HbA1c, which is a marker for diabetes development [136]. Further research has targeted the activity of the carotid sinus nerve [137, 138, 139]. In this domain, Conde et al. (2018) successfully restored insulin sensitivity and glucose tolerance in people with type 2 diabetes by blocking the activity of the carotid sinus nerve using kilohertz frequency alternating current (KHFAC) modulation [138, 139]. Meyers et al. (2016) investigated the differential impact of selective afferent or efferent, or combined cervical vagus nerve stimulation (VNS) on the metabolic (blood levels of glucose, insulin and glucagon) and
the hemodynamic responses [134]. Finally, Jiman et al. (2018) targeted the activity of renal 
nerves using electrical stimulation to assess its impact on blood glucose levels in healthy [199] 
and diabetes-induced rats [200].

Despite this research, nobody has ever done a comprehensive study on the impact of stimu-
lation parameters, such as stimulation frequency, amplitude and pulse width, on glucose, 
insulin and glucagon blood concentrations. Realising this could be breakthrough, because re-
searchers currently use unjustified stimulation configurations seeking a response in glucose 
levels. Stimulation patterns are generally selected by blindly experimenting with different pa-
rameters without monitoring the evoked signalling in the nerve [179]. The work presented 
in this chapter aims to bring some light to this research question, by studying the impact of 
stimulation frequency on the metabolic response.

4.1.2 Towards a rigorous experimental design: a practical review

Using information extracted from a total of more than 90 manuscripts, this section includes 
important good practice considerations for designing a successful and rigorous experiment on 
the metabolic and neurostimulation scenarios.

Animal model

Research on the neural control of the pancreas and glucose homeostasis has been investi-
gated in different animal models, from rodents to big animals, such as pigs and dogs. When 
selecting the animal model, it is important to understand the physiological characteristics of 
each model in the context of the experimental objectives and the feasibility for clinical trans-
lation to humans. The ultimate goal of the experiments presented in the following sections 
is to gain some insight into the physiological mechanisms for the neural control of glucose 
homeostasis, and to reproduce them using mathematical models that can be extrapolated to 
humans. In this respect, the animal model should provide a good representation of the human 
mechanisms both in glucose metabolism and neuromodulation.

To begin with, recent studies have determined that the islet architecture in the rodents pan-
creas is different from the one in humans [189, 201]. In particular, two main differences are 
highlighted. First, insulin secretion by ACh is mainly stimulated by activation of the M3 recep-
tor subtype, which is presented in large concentrations [170, 202, 203], whereas in humans it is 
activated through M3 and M5 receptors [189]. Secondly, the different types of endocrine cells 
in the human islet are highly combined, which allows blood vessels to irrigate all the different 
types of cells, whereas their distribution in rodents follows aggregates. Hence, not the struc-
tural nor functional information can be theoretically extrapolated to humans models. On the
4.1. INTRODUCTION

Table 4.1: Comparative anatomy of the cervical vagus nerve between mouse, rat, canine, pig (12 kg) and human.

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Nerve diameter</th>
<th>Number of fascicles</th>
<th>Epineurium thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>~200um</td>
<td>1-2</td>
<td>No epineurium</td>
</tr>
<tr>
<td>Rat</td>
<td>~300-500um</td>
<td></td>
<td>No epineurium</td>
</tr>
<tr>
<td>Dog</td>
<td>~3mm</td>
<td>1</td>
<td>Large</td>
</tr>
<tr>
<td>Pig (12kg)</td>
<td>~2-3mm</td>
<td>&gt;10</td>
<td>~0.1mm</td>
</tr>
<tr>
<td>Human (adult)</td>
<td>~3-5mm</td>
<td>~10</td>
<td>~0.2mm</td>
</tr>
</tbody>
</table>

...other hand, pigs have been found to have a similar pancreatic architecture to humans [204], a similar response to feeding [205] showing also a cephalic phase [204].

Regarding the neuroanatomy of the vagus nerve, previous literature has reported different thoracic and abdominal branches arising from the left and right vagal trunks in different species, with afferent and/or efferent axons from one side crossing over to the other side. In the rat, however, there is little evidence for the crossing of efferent axons, and only around 20% of afferents cross from one side to another at the thoracic level [206]. In this context, early research on the field suggested that the right vagus nerve was not a major contributor to the vagal stimulation of insulin secretion, at least in rats [149]. However, there is evidence that the dorsal diaphragmatic vagus nerve sends fibres to the splenic end of the pancreas [166], which presents a larger number of Langerhans islets based on a histological study of the rat pancreas [207]. In contrast, the ventral subdiaphragmatic vagus mainly innervates the head and body of the pancreas. The composition and size of the vagus nerve also differ between species, as gathered in Table 4.1. The pig presents the most similar neuroanatomy of the vagus nerve to that of the human [208, 209], although the vagus nerve composition of the rat at the cervical and abdominal levels with respect to myelinated and unmyelinated fibres is also similar to humans [209].

All these features make big animals, such as pigs, an appropriate animal model for studying VNS [209]. Nonetheless, rats are the preferred animal model for basic research, pilot and safety testing experiments following the general guiding rules for Replacement, Refinement and Reduction of Animals in animal research to reduce the number of big animals involved (e.g. pigs or piglets) until experimental protocols are optimized. Large or relevant animals are then used for testing the efficacy of new therapies, before being eventually implemented in clinical studies. Therefore, the use of rats as animal model to provide a first characterization of the metabolic mechanisms involved after vagus nerve stimulation is justified for the pilot experiments presented hereby.
Fasting period

Unexpectedly, it has been found that the fasting conditions of the cohorts before the experiments onset greatly impact the integrity of the observed results, especially in studies of the cephalic phase [210]. After an overnight of fasting, plasma glucose levels become a function of the rate of hepatic glucose production (HGP), which primarily reflects the gluconeogenic activity (although renal gluconeogenesis may also contribute to overnight hepatic glucose production (HGP)) [211]. As a result, many experiments start in the pre-absorptive state after an overnight of fasting (8h-18h), when there is no absorption of nutrients from the gastrointestinal tract [157, 192, 212, 193, 213, 214, 215]. It is worth highlighting the results from Bellisle et al. (1983) [216], where they consciously set a normal deprivation time of 4 hours between breakfast and lunch to reproduce the normal daily metabolic profile. Despite the importance of providing fasting information for experimental reproducibility, many papers do not describe it [217, 218, 219, 220]. For those that start in the morning, one could hypothesize an overnight of fasting, but this cannot be confirmed. Following this consideration, the experiments presented in this thesis clearly specify the fasting period and the time of the surgery onset in the respective sections.

Anaesthesia

In metabolic studies, the type and level of anaesthesia used can differentially affect the concentrations of many blood metabolites [134, 184, 221]. The most widely used anaesthetics in rats models is isoflurane given the simplicity of its utilization and control during surgery [222]. Isoflurane at a concentration of 2% has been found to inhibit insulin secretion from the rat pancreatic islets of Langerhans [223]. However, at lower concentrations (1.5%) the secretion of insulin was not found to be blocked [134]. Despite these considerations, isoflurane is the preferred option over other anaesthetics such as ether, pentobarbitone sodium and fentanyl plus droperidol, as these have been observed to increase blood glucose concentrations [221]. This response was similarly observed after the use of ketamine/xylazine in fed, but not fasting, rats, which resulted in acute hyperglycaemia associated with decreased insulin, adrenocorticotropic hormone and corticosterone concentrations [221].

For neural recording experiments, the level of isoflurane anaesthesia was also found to impact the recording of spontaneous basal neural activity [222]. High levels of 2% almost completely blocked the neural activity, whereas using low levels of 1.5% the neurograms were masked with a large amount of background noise due to the reduced effect of anaesthesia in the body. A level of 1.75% was selected as the optimal to acquire the anaesthetic effect while recording baseline activity. Given these challenges, intraperitoneally injected urethane (20 mg/kg) is
the preferable choice of anaesthetic in studies involving neural stimulation and recording because it does not affect peripheral nerve activity [163]. The main limitation is the complications in dose adjustments, which are associated with a greater risk of overdose.

Blood collection

Biomarkers

The changes in concentration of a substance in blood reflect the temporary imbalance between the rates at which that substance enters and leaves the vascular space. Any rise in concentration of a particular biomarker could therefore be due to either an increased release or a decreased utilization/degradation. As a result, the interpretation of the data obtained in vivo must be restricted by the characteristics of the experimental design. This is of special importance for the quantitative interpretation of observed increases in blood concentration of insulin. This increase can only be directly associated with an increase in insulin secretion when the rate of flow of blood through the pancreas is known. However, when blood is only sampled from arteries and veins the origin of such increase cannot be warranted [224]. Tables 4.2 and 4.3 provide a summary of the most relevant biomarkers and related information that have been reported in the reviewed papers in addition to the plasma concentrations of glucose and insulin. Given the high costs and technical difficulties related to measuring each hormone, and the scope of the experiments presented in this chapter, which is elucidating the effects of VNS frequency on blood glucose, insulin and glucagon levels, only the plasma levels of these metabolites were measured and analysed as presented in Section 4.2.3.
Table 4.2: Overview of frequently measured biomarkers.

<table>
<thead>
<tr>
<th>Location</th>
<th>Biomarker</th>
<th>Description</th>
<th>Remarkable studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>Saliva</td>
<td>It is the first response during the cephalic phase. Atropine causes a marked reduction of its secretion</td>
<td>[157]</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Glucagon</td>
<td>Released during glycopenic stress (e.g. hypoglycaemia or neuroglycopenia) [168]. It acts in the liver enhancing HGP and peripherally inhibiting glucose uptake.</td>
<td>[134, 184, 138, 219, 220]</td>
</tr>
<tr>
<td></td>
<td>Pancreatic polypeptide (PP)</td>
<td>Released mainly after food ingestion, but also in prolonged fasting, diabetes and exercise [225]. It acts increasing gastric emptying and gut motility. Levels of PP are a sensitive indicator of the vagal activation to food stimuli and cholinergic input to the pancreas because its release is controlled exclusively by vagal mechanisms (atropine inhibits its secretion totally in dogs [167] and largely but not fully in pigs and calves [167, 226, 176, 213].</td>
<td>[192, 177, 193, 194, 219, 214]</td>
</tr>
<tr>
<td></td>
<td>C-peptide</td>
<td>Used as a measure of the release of endogenous insulin from the pancreas because it is secreted in equimolar amounts to insulin and, contrary to insulin, it is not rapidly metabolized by the pancreas [227, 228].</td>
<td>[157, 210, 217, 185, 138, 194, 220]</td>
</tr>
<tr>
<td></td>
<td>Somatostatine</td>
<td>Secreted by the delta-cells (located in the pancreas and the gastrointestinal tract among other locations). It inhibits insulin and glucagon secretion. Vagal nerve stimulation and cholinergic agonists in pigs inhibit its secretion, and this response is blocked by atropine [167].</td>
<td>[220]</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>VIP</td>
<td>Released after vagal stimulation in anaesthetized pigs, cats and calves [184]. A less significant rise has been observed in conscious animals, probably due to the effect of anaesthesia on the clearance of VIP from the gastrointestinal tract. VIP release is also resistant to atropine [184].</td>
<td>[184]</td>
</tr>
<tr>
<td>(stomach and gut)</td>
<td>Ghrelin</td>
<td>Also known as the &quot;hunger hormone&quot;, it is produced in the stomach and acts as a neuropeptide regulating appetite. It is secreted when the stomach is empty to prepare the body for food intake and its release stops when the stomach is stretched.</td>
<td>[157]</td>
</tr>
<tr>
<td></td>
<td>GIP</td>
<td>Secreted in the gastrointestinal tract, it promotes the secretion of insulin from the pancreas.</td>
<td>[157, 192, 193, 220]</td>
</tr>
<tr>
<td></td>
<td>GLP-1</td>
<td>Secreted in the gut, it induces insulin secretion and inhibits glucagon release. Together with GIP belong to the group of incretins, which result in a reduction of glucose levels. It has been reported to have a cephalic phase [229].</td>
<td>[157, 192, 193]</td>
</tr>
</tbody>
</table>

Pancreatic polypeptide (PP), Vasoactive intestinal peptide (VIP), Gastric inhibitory polypeptide (GIP), Glucagon-like polypeptide 1 (GLP-1), hepatic glucose production (HGP).
### Table 4.3: Overview of frequently measured biomarkers (continuation).

<table>
<thead>
<tr>
<th>Location</th>
<th>Biomarker</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other organs</td>
<td>FFA and NEFA</td>
<td>Secreted by adipose tissue, they are normally used as a marker of insulin resistance [230].</td>
<td>[210, 231, 219, 214, 215]</td>
</tr>
<tr>
<td></td>
<td>Catecholamines (epinephrine and norepinephrine)</td>
<td>Secreted by the adrenal glands in stress conditions. They overall inhibit insulin secretion [227, 232]. However, epinephrine has also been found to increase insulin release by acting on β2-adrenoreceptors [213], being possibly involved in meal-related stimulation of insulin secretion. In addition, epinephrine can promote HGP through the neuropeptide galanin [219]. All of these effects might result in an indirect rise of glucose levels.</td>
<td>[192, 210, 213]</td>
</tr>
<tr>
<td></td>
<td>Corticosteroids</td>
<td>Steroid hormones produced in the adrenal cortex. Among them, glucocorticoids such as cortisol, are released in conditions of stress (including glycopenic stress [168]) and are involved in metabolism of carbohydrate, fat and protein.</td>
<td>[138, 204, 214, 215]</td>
</tr>
</tbody>
</table>

Free fatty acids (FFA), non-esterified free fatty acids (NEFA), pancreatic norepinephrine spillover (PNESO), hepatic glucose production (HGP).
Blood collection method and location

The selection of the blood collection method depends on different factors, including the instrumental available to the investigator, the type of experiment (i.e. acute or chronic), and the blood volume requirements. A summary of the characteristics of the most widely used blood sampling methods in the field of diabetes is shown in Table 4.4. Sampling from the tail tip is the preferred choice in short metabolic studies, such as the ones presented in this thesis, given its simplicity and low risks, and therefore was selected as the blood collection method.

<table>
<thead>
<tr>
<th>Sampling method</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail tip</td>
<td>-Simple procedure, especially for rodents</td>
<td>-The sample can contain both arterial and venous blood, along with tissue product contamination [233].</td>
</tr>
<tr>
<td></td>
<td>-Can be carried out in any laboratory</td>
<td>-Large samples require prolonged massaging (up to 2 min) causing more stress (increased catecholamine levels, HGP and glucose disappearance)</td>
</tr>
<tr>
<td></td>
<td>-Does not require catheterization</td>
<td>-Delays of interstitial readings compared to plasma changes, especially with rapid changes.</td>
</tr>
<tr>
<td></td>
<td>Convenient for glucose measurements using a glucometer (only a drop is required)</td>
<td>-Variation in the readings due to local fluctuations in blood flow.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inflammation induced by sensor resulting in encapsulation by fibrous tissue. This may limit the time-frame in which the sensor can be used.</td>
</tr>
<tr>
<td>Implantable telemetry systems</td>
<td>-Continuous monitoring</td>
<td>-Invasive method</td>
</tr>
<tr>
<td></td>
<td>-High accuracy</td>
<td>-Can result in the loss of animals prior to the study</td>
</tr>
<tr>
<td></td>
<td>-Low stress induced</td>
<td>-Risk of blood clots formation that may eventually result in stroke [234]</td>
</tr>
<tr>
<td>Arterial or venous catheter</td>
<td>-Provides vascular access during an experiment without introducing additional stress</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-Allows taking large volumes of sample</td>
<td>-</td>
</tr>
</tbody>
</table>

In experiments involving repeated sample collection, it is important to carefully design the blood sampling procedure so that it does not exceed the volume limits recommended for each specie. For example, rats have around 64 ml of blood per kg of body weight [233, 235]. Following the guidelines of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) [235] and the Animal Care and Use Committee at Johns Hopkins University [236], up to 10% of the circulating blood volume can be taken on a single occasion from a normal healthy animal, and up to 15% in 28 days. For repeated sampling at shorter intervals, a maximum of 10% of an animal’s circulating blood volume can be removed every 24 hours [237]. If the limits were exceeded it would be necessary to substitute the blood and fluid losses (e.g. infusion of dextran in pigs [176]).
Regarding the blood withdrawal location, it is worth highlighting that the concentration of insulin and glucagon measured in peripheral plasma is much smaller than when measured directly from the portal vein. The reason is that up to 50% of the insulin is removed by the liver and the remaining concentration is further diluted by the extra-hepatic venous blood [158, 194, 221, 224]. Finally, it is important to determine the type of sample that is required, such as whole blood, plasma or serum. For whole blood collection, an anticoagulant must be added to the sample. Plasma contains fibrinogen and other clotting factors obtained from their separation from red blood cells after centrifugation of anti-coagulated samples. Serum, on the other hand, is obtained from blood collected without an anticoagulant and centrifuged afterwards [233].

Selecting the appropriate electrodes and stimulation design

A critical aspect of neuromodulation is choosing the most appropriate electrode design and characteristics and the stimulation parameters, as these can greatly influence the results and contribute to failures in the experiments. To begin with, the electrode design greatly contributes to defining the characteristics of the applied stimulation. Different peripheral nerve electrode architectures have been proposed including surface electrodes (cuff and hook electrodes), penetrating electrodes (LIFE, TIME and USEA) and regenerative electrodes, as reviewed in [238] (see Figure 4.1).

![Figure 4.1: A schematic drawing of (A) the peripheral nerve; (i) epineurium, (ii) fascicle containing axons and (iii) blood vessels; (B) regenerative, (C) intra-fascicular, (D) inter-fascicular and (E) surface electrodes for electrical interfacing with peripheral nerves. Reused with permissions from Russell et al. (2019) [238].](image-url)
This section provides a comparison only on surface electrodes, which is summarized in Table 4.5. Despite cuff electrodes provide, in overall, a better performance, the lack of in-house fabrication and the challenges that arose from its implantation made it necessary to use hook electrodes for the experiments presented in this thesis.

Table 4.5: Comparative surface electrode architectures.

<table>
<thead>
<tr>
<th>Electrode type</th>
<th>Advantages</th>
<th>Limitations</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sling cuff</td>
<td>Allow uniform delivery of current to the nerve</td>
<td>Electrode implantation</td>
<td>KHFAC stimulation [138]</td>
</tr>
<tr>
<td></td>
<td>-More control and improved reliability</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Inherently isolator from the surrounding tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Better confinement of the current into the nerve, making them appropriate for chronic implantation [239].</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Prevent current leakage</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Significantly reduce background noise and increase the signal to noise ratio in recordings.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Design customization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cuff</td>
<td>Easy to implant</td>
<td>Electrode implantation</td>
<td>Acute &amp; chronic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hook</td>
<td>Easy to implant</td>
<td>Need to be immersed in oil pools (e.g. paraffin oil) or isolating substances (e.g. vaseline) to isolate the electrode site from the surrounding tissue and to avoid extracellular fluid to accumulate in the contact site, which may lead to failed stimulation and/or recording.</td>
<td>Only acute</td>
</tr>
</tbody>
</table>

Different design specifications can be customized in cuff electrodes to best fit the target nerve, including the electrode material and flexibility, number of contacts, length, inner and outer diameters, weight and type of surgical sutures, among other parameters. As an example, it is desired for acute stimulation that the inner diameter of the cuff electrode matches the nerve the best possible. If the electrode is larger than the nerve, stronger stimulations would be required to compensate for the lack of contact between the nerve bundle and the electrode. For chronic implants, it is recommended to use thinner electrodes to keep the leads as light and flexible as possible. In these cases, the electrode’s stiffness and weight are critical because implanting too heavy or stiff cuffs can pull or twist the vagus nerve too much resulting in premature animal death, as this nerve is necessary for respiration and heart rate control.
4.1. INTRODUCTION

The electrode impedance is another feature of interest that can reveal malfunctions of the system and tissue damage (e.g. scar formation). Therefore, to ensure the integrity of the results, authors recommend tracking the electrode impedance several times along the experiment course, from immediately after the surgery to before the termination of the animals [142]. In addition, to easily check the correct location of the electrode at the cervical level, it is a common practice to monitor respiratory and/or heart rates for short periods of time (e.g. 2 seconds) [134, 138]. Other authors have confirmed the correct implantation of electrodes for stimulating the pancreas by measuring the exocrine secretion of pancreatic juice [177].

The selection of stimulation parameters (i.e. waveform, pulse amplitude, pulse width, frequency, among others), as previously explained, determine the fibres that will respond to stimulation (see Section 3.1.1), and therefore will be recruited. Table 4.6 provides an overview of some of the stimulation protocols previously used. The large variability found in each parameter challenges a fair comparison of the results of the studies. It is therefore required to comprehensively study the effects of changing the stimulation parameters. One final consideration to bear in mind when exposing and isolating the nerve is that the surgery may increase the excitability of the tissue, which might in turn result in undesirable spurious stimulations [178]. Therefore, it is recommended to allow from thirty to sixty minutes of stabilization before the onset of the experiment.
Table 4.6: Comparison of VNS designs and parameters.

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Site</th>
<th>Waveform</th>
<th>Amplitude</th>
<th>Pulse duration</th>
<th>Frequency</th>
<th>Duration</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Right cervical VN</td>
<td>Proximal &amp; distal</td>
<td>3V</td>
<td>1msec</td>
<td>5Hz</td>
<td>2h</td>
<td>[134]</td>
</tr>
<tr>
<td>Rat</td>
<td>Bilateral cervical VN</td>
<td>Intact</td>
<td>3V</td>
<td>1msec</td>
<td>5Hz</td>
<td>2h</td>
<td>[240]</td>
</tr>
<tr>
<td>Rat</td>
<td>Left cervical VN</td>
<td>Distal</td>
<td>25V</td>
<td>0.5msec</td>
<td>5Hz</td>
<td>15min</td>
<td>[181]</td>
</tr>
<tr>
<td>Rat</td>
<td>Left cervical VN</td>
<td>Intact</td>
<td>250uA</td>
<td>50usec</td>
<td>5Hz</td>
<td>10sec</td>
<td>[241]</td>
</tr>
<tr>
<td>Rat</td>
<td>Bilateral cervical VN</td>
<td>Distal</td>
<td>0.5-0.7mA</td>
<td>1msec</td>
<td>4Hz</td>
<td>10min</td>
<td>[149]</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td>1-2 mA</td>
<td></td>
<td>20-50kHz</td>
<td>10min</td>
<td>[138]</td>
</tr>
<tr>
<td>Dog</td>
<td>Ventral &amp; dorsal branches of thoracic VN</td>
<td>Intact</td>
<td>13.5mA</td>
<td>5msec</td>
<td>10Hz</td>
<td>10min</td>
<td>[188]</td>
</tr>
<tr>
<td>Dog</td>
<td>Left and right cervical VN</td>
<td>Distal</td>
<td>3mA (4-6V)</td>
<td>8msec</td>
<td>60Hz</td>
<td>15-30-60min</td>
<td>[178]</td>
</tr>
<tr>
<td>Dog</td>
<td>Supradiaphragmatic VN</td>
<td>Distal</td>
<td>14V</td>
<td>5msec</td>
<td>100 Hz</td>
<td>3 (10-sec bursts alternating with 5-sec intervals)</td>
<td>[174]</td>
</tr>
<tr>
<td>Dog</td>
<td>Left cervical, splanchnic &amp; pancreatic VN</td>
<td>Intact</td>
<td>1mA</td>
<td>200usec</td>
<td>20Hz</td>
<td>-</td>
<td>[185]</td>
</tr>
<tr>
<td>Cat</td>
<td>Hepatic nerves</td>
<td>Proximal</td>
<td>15V</td>
<td>1msec</td>
<td>8Hz</td>
<td>15min</td>
<td>[242]</td>
</tr>
<tr>
<td>Pigs</td>
<td>VN above diaphragm and below heart.</td>
<td>Proximal</td>
<td>8mA</td>
<td>5msec</td>
<td>0.25-20Hz</td>
<td>5min</td>
<td>[176]</td>
</tr>
<tr>
<td>Calf</td>
<td>Bilateral VN below heart</td>
<td>Proximal</td>
<td>10-15V</td>
<td>0.5msec</td>
<td>10Hz</td>
<td>10min</td>
<td>[184]</td>
</tr>
</tbody>
</table>
4.2 Experimental methods

The previous sections have provided an overview of the most relevant methodologies and experimental considerations, and explained the choices made for the experiments presented hereby (see 4.2), including the selection of the animal model, the type and concentration of anaesthesia, the measured metabolites and sampling methodology, and the electrode type. The preliminary experiments and the experiments corresponding to the first study were carried out during a first 5-month internship, and the experiments corresponding to the second study were performed during a second 3-month internship. This second research stay was planned to last 6 months, but the Covid-19 pandemic hindered its completion. The challenges that intrinsically arise from setting a laboratory for a new set of experiments, combined with the time it took me to learn the surgeries and prepare and optimize the experimental setup, caused a great delay for the beginning of the experiments. This is reflected by the reduced sample size that is presented for each cohort, despite the initial experimental plan accounted for a minimum of 8 rats based on previous similar experiments [240, 134]. More experimental...
research has been already planned to fulfil the original proposal and provide a more robust analysis of the results.

4.2.1 General surgical procedure

All surgical procedures were performed in accordance with the guidelines of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), and approved by the Johns Hopkins Animal Care and Use Committee. Wistar rats (male, 300g-350g) were housed under 12-h light/12-h dark conditions, temperature of 22-24°C, and with access to food and water ad libitum. The animals were allowed to acclimatize for 2 weeks prior to experiment. Food, but not water, was withheld 4h, 8h, or 12h (overnight) before the surgery onset. All experimental cohorts followed the same surgical procedures.

To induce unconsciousness, the animal was held in a transparent chamber containing a mixture of 4.5% isoflurane, 45.5% oxygen and 50% nitrogen gas at 4L/min. Following observed unconsciousness, the animal was removed from the transparent chamber and maintained at 2.0% isoflurane during surgery through a nosecone. After vagus nerve exposure, anesthesia was maintained for the length of the experiments at 1.25% to guarantee it had no influence on the pancreatic secretion (see Section 4.1.2), and adjusted if necessary. Heart rate was constantly monitored throughout the experiments via a RX5 TDT device (Tucker Davis Technologies, Alachua, FL). Signals were amplified and digitized with an RA16PA preamplifier (Tucker-Davis Technologies, Alachua, FL).

Exposure of the left vagus nerve was done following the surgical procedure described elsewhere [243]. The surgical site in the neck was shaved with a border of at least 1 cm. The site was then scrubbed with a germicidal scrub (povidone-iodine, chlorhexidine) from the centre of the site towards the periphery. The site was then rinsed with 70% alcohol, sterile water, or sterile saline. Three alternating preparations of germicidal scrub and rinse were considered adequate. A 15 mm longitudinal cervical midline incision through subcutaneous tissue and platysma was made. The thyroid glands were separated apart to expose the underlying muscles. A self-retaining retractor was positioned between the left digastric, sternomastoid and sternohyoid muscles. The omohyoid muscle was retracted downward to expose the left carotid artery and left vagus nerve. Precise dissection of the perivascular structures (fascia, adventitia and sympathetic plexus) around the carotid artery using sharp curved forceps was performed. Great care was taken in this step to avoid excessive manipulation or lesion of the surrounding neurra before the vagus nerve was exposed free (see panel (a) in Figure 4.3). Hooked bipolar electrodes (PBAD08100, Frederick Haer & Co.) were then placed around the left cervical vagus nerve for recording and/or stimulation in all the experimental cohorts (including control).
4.2. EXPERIMENTAL METHODS

the first experimental setup the nerve was left intact for combined efferent and afferent stimulation (see left panel in Figure 4.2). In the second experimental setup the nerve was mechanically ligated by placing a suture to hold it and cutting the nerve distally from the stimulation electrode, for selective efferent stimulation (see right panel in Figure 4.2). In either case the cervical cavity containing the vagus nerve was filled with paraffin mineral oil to keep the nerve moist and to create an insulation medium.

(a) Left cervical vagus nerve

Figure 4.3: Surgical exposure of (a) the cervical left vagus nerve, (b) the supradiaphragmatic vagus nerve, and (c) the subdiaphragmatic vagus nerve.

(b) Supradiaphragmatic vagus nerve

(c) Subdiaphragmatic vagus nerve

In a subset of the animals, a heparinized catheter (Intramedic Non-Radiopaque Polyethylene Tubing PE-50 catheters, Becton Dickinson) was inserted in the femoral vein for blood pressure monitoring and, in study 1, for intermittent blood sampling of insulin and glucagon concentrations (two samples). For implanting the catheter, the skin above the left thigh was shaved, cleaned with povidone-iodine solution and a 2 cm ventral skin incision was made. Af-
ter visualization and exposure of the left femoral vein, clamps were used to occlude the ends of the vessel and a small incision was made for inserting the cannula tube. In these animals, arterial blood pressure was also constantly monitored throughout the experiments via the RX5 TDT device (Tucker Davis Technologies, Alachua, FL).

Stimulation was performed to the left vagus nerve because this branch mostly innervates the ventral trunk, which eventually gives off the hepatic and anterior gastric branches that innervate proximal and central part the pancreas. However, it is worth noting that the splenic end of the pancreas is also innervated by neurons from the celiac branch, which arises from the right vagus nerve [166]. In addition, stimulation of the left cervical branch has been found to overall result in a stronger bradycardic and hypotensive response in rodents [164], which was used as visual feedback for a correct stimulation. It is worth noting that in humans, on the contrary, the right vagus branch has more projections to the heart [161], so clinical VNS is generally applied to the left branch to limit undesired cardiovascular responses. During the training period, the vagus nerve was also exposed above and below the diaphragm to assess the feasibility of stimulating closer to the pancreas (see panels (b) and (c) in Figure 4.3). However, given the absence of visual feedback for a successful stimulation and the high risks associated with this surgery, these approaches were discarded and the stimulation was performed cervical.

After the surgery, the animals were left undisturbed for a period of 30-60 minutes before the onset of the experiments to minimize the indirect effects of the surgery in glucose and hormonal levels. All experiments were acute, and animals were euthanised with an overdose of isoflurane, death being confirmed in accordance with approved guidelines.

4.2.2 General stimulation procedures

The Intan 128ch Stimulation/Recording Controller (Intan Technologies Los Angeles, Ca) was connected to a bipolar hooked electrode through a RHS2000 16-channel stim/record headstage with 18-pin electrode connector (Intan Technologies Los Angeles, Ca) (see Figure 4.4)). Electrode impedance was measured with the INTAN processor at 1 kHz in the nerve immediately after implantation, and during and at the end of the experiments. The controller was digitally triggered using an arbitrary waveform generator set to the desired stimulation frequency (model 33120A, 15MHz, Hewlett Packard, San Jose, Ca). Bipolar, biphasic (cathodic-first), balanced-charged, current driven stimulation pulses with different parameters were applied to the vagus nerve in experimental cohorts, whereas control animals were sham-stimulated. The specific stimulation protocols used in each study are covered in the corresponding sections.
4.2. EXPERIMENTAL METHODS

4.2.3 Blood sampling and radioimmunoassay studies

Blood samples for glucose, insulin, and glucagon measurements were collected from the tail vessels. The specific time points for blood withdrawal can be found in the corresponding section for each study. To reduce the variability in determining glucose concentrations, the mean value obtained from applying a blood drop from the cut tip of the tail to two handheld glucometers was calculated (FreeStyle Lite, Abbott Laboratories Co.). For repeated glucose samples the tail tip was wiped to reinitiate blood flow. For plasma insulin and glucagon concentrations, the tail was gently massaged from the body to the tip until obtaining the necessary volume of blood for each sample (200 ul). To facilitate the blood flow, a heating pad was placed beneath the tail and lower body resulting in vasodilation of tail vessels. The blood samples were collected using 250 uL micro blood heparinized collecting tubes (Fisher Scientific, Pittsburgh, PA). They were centrifuged for 10 min at 5,000 rpm (4° air temperature) to separate the plasma from the hematocrit. The decanted plasma was stored in ice until analysed with the Ultra-Sensitive Rat Insulin ELISA, Ultra-Sensitive Rat Glucagon ELISA, for insulin and glucagon assays respectively (Crystal Chem; Downers Grover, IL). The total amount of withdrew blood complies with the limits of animal welfare for blood sampling for rats of 300-350g.

The analysis of the results was performed using the change in magnitude between baseline, stimulation and post-stimulation periods. ANCOVA is generally the method of choice for analysing the results of these types of experiments given its high statistical power, yet it could not be used in this case due to the small dataset. The percentage of change from baseline, despite being the most popular metric in clinical environments, is statistically less efficient and may not correct for imbalance between groups at baseline [244], so its used was limited.

4.2.4 Software

Python and Matlab (2018b, Matworks, Natick, MA) were employed for analysing the results. A number of libraries in Python were used, including Pandas for data handling [245],
Matplotlib and Seaborn for data visualization [246] and Statsmodels for the statistical analysis [247].

4.3 Preliminary study

Preliminary stimulation experiments were initially carried out to determine the optimal stimulation setup for the consecutive studies. Rats were fasted overnight for 14h. The system was initially connected to an oscilloscope to verify that the desired waveforms were being delivered. When a stimulation of 1.5mA was applied, the voltage pulse depicted in the screen of the oscilloscope was around 3.5V, which is in accordance with an overall impedance (tissue and electrode impedances) of 2-4KOhms. Then, an initial stimulation protocol (ISP) of increasing pulse durations (0.05, 0.1, 0.25, 0.5, 1 ms) and increasing stimulation amplitudes (0.25, 0.5, 0.75, 1, 1.5, 1.75 mA) at 5Hz frequency was used to investigate the impact of VNS on the bradycardic response. The configurations that resulted in a consistent bradycardic response were then selected to evaluate their impact on glucose levels in a subsequent set of preliminary experiments (PE). The minimum stimulation time needed to obtain a glucose response was also studied by increasing the stimulation duration in each preliminary experiment (1, 5, 15 and 30 min in PE 1 to 4, respectively). A summary of the stimulation configurations used in each preliminary experiment is presented in Table 4.7. Detailed results and discussion of the preliminary study can be found in Appendix C.

Table 4.7: Preliminary experiments: stimulation protocols.

<table>
<thead>
<tr>
<th>Preliminary experiment (PE)</th>
<th>Rat ID</th>
<th>Stimulation protocols</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE 1</td>
<td>'002'</td>
<td>Protocol 1: 5Hz, 0.05ms, 0.75mA, 1min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocol 2: 5Hz, 0.05ms, 1.5mA, 2min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocol 3: 5Hz, 0.5ms, 0.75mA, 1min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocol 4: 5Hz, 0.5ms, 1.75mA, 1min</td>
</tr>
<tr>
<td>PE 2</td>
<td>'003'</td>
<td>Protocol 1: 5Hz, 0.5ms, 1.5mA, 5min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocol 2: 5Hz, 1ms, 1.75mA, 5min †</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocol 1: 5Hz, 1ms, 1.75mA, 15min †</td>
</tr>
<tr>
<td>PE 3</td>
<td>'004'</td>
<td>Protocol 2: 10Hz, 1ms, 1.75mA, 15min ‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocol 3: 5Hz, 0.5ms, 12.5mA, 15min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocol 4: 5Hz, 0.5ms, 12.5mA, 15min ‡</td>
</tr>
<tr>
<td>PE 4</td>
<td>'005'</td>
<td>Protocol 1: 5Hz, 1ms, 1.75mA, 30min ‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protocol 2: 10Hz, 1ms, 1.75mA, 30min ‡</td>
</tr>
</tbody>
</table>

† Similar stimulation configuration as in Meyers et al. (2016) [134].
‡ Similar stimulation configuration as in Meyers et al. but using 10Hz instead [134].
⋆ Similar stimulation configuration as in Peitl et al. (2005) [181].
4.4 STUDY 1: IMPACT OF STIMULATION FREQUENCY ON THE INTACT NERVE

4.4.1 Stimulation protocol

The two configurations that had the greatest impact on glucose levels in the preliminary experiments were selected (see Table 4.8). In particular, two stimulation frequencies of 5Hz and 10Hz, with otherwise same configuration parameters (biphasic, symmetric, charge-balanced square pulses of 1.75mA pulse amplitude and 0.5ms pulse width - i.e. 1ms pulse duration) were applied for 20 minutes to the intact left cervical vagus nerve in fasted anaesthetized rats for combined afferent and efferent VNS. Three experimental cohorts were designed, as shown in Table 4.8. To reduce the variability that arises from the 24-hour variations in glucose and metabolic hormones [234], all experiments were scheduled to start at the same time of the day (surgery onset at 12:30). Food was withdraw 4 hours before the onset of the surgery. Glucose, insulin and glucagon plasma concentrations were measured at the following time points: 0 (baseline), 15, 20, 25, 30, 35 (stimulation), 75 and 100 (post-stimulation).

Table 4.8: Study 1: Description of experimental cohorts

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat ID</th>
<th>Stimulation protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>'08' and '09'</td>
<td>Sham-stimulation</td>
</tr>
<tr>
<td>Group 1</td>
<td>'11' and '13'</td>
<td>5Hz, 1ms, 1.75mA, 20min</td>
</tr>
<tr>
<td>Group 2</td>
<td>'12', '15' and '16'</td>
<td>10Hz, 1ms, 1.75mA, 20min</td>
</tr>
</tbody>
</table>

4.4.2 Results

Metabolic results

As illustrated in Figure 4.5 (b) and (c), both frequencies resulted in an immediate and sustained increase in glucose concentration, with the highest frequency having the greatest change over baseline (10Hz-stimulation: 53.69 ± 10.86 mg/dL; 5Hz-stimulation: 38.05 ± 13.73 mg/dL). The peak was obtained between 3 and 10 minutes after stimulation cessation and glucose levels returned to baseline 35 minutes after. It is worth noting that the glucose levels show big changes between consecutive readings. This fluctuation arises from the initial use of a single glucometer. To overcome this limitation, the mean value obtained from two glucometers was subsequently used to reduce the instrumental error as previously introduced.

In neither case this increase was accompanied by a meaningful change in insulin before and during stimulation (10Hz-stimulation: 0.34 ± 0.41 ng/mL increment over baseline; 5Hz-stimulation: 0.98±1.13 ng/mL increase over baseline). However, at the individual results, some animals presented a peak during stimulation period, including in the control group, which becomes indiscernible on the mean population response. These peaks can be observed at the
beginning (‘ID 08’ - control, ’ID 11’ - group 1, ’ID 15’ - group 2), or at the end of stimulation (‘ID 12’ - group 2) (see Appendix C). The rest of the animals exhibited a sustained decay of insulin concentration during the whole stimulation period. Interestingly, in both stimulation cohorts there was a peak in insulin around 5-10 minutes after stimulation cessation, which most probably was a result of a glucose-dependent increase in insulin secretion due to the increase in plasma glucose. Regarding glucagon, the analysis showed a decrease in levels during the 10Hz-stimulation period (\(-0.58 \pm 0.09\) pg/mL decrease related to baseline). This decrease, although less marked, was also observed during the 5Hz-stimulation (\(-0.19 \pm 0.12\) pg/mL related to baseline). The results from the control group are summarized in Table 4.9, and visualized Figure 4.5 (a). In Figure 4.5, it is interesting to note a sudden drop in insulin and, less marked, in glucagon concentrations observed in the green line (corresponding to ’Rat ID 09’) at around 45min. The origin of this pronounced decrease remains unclear and research in a larger sample size should be carried out to elucidate whether it arises from a physiological or experimental source.

Table 4.9: Study 1: Results from control group.

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mg/dL)</th>
<th>Insulin</th>
<th>Glucagon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>132.98 ± 5.57</td>
<td>3.11 ± 0.35</td>
<td>1.50 ± 0.13</td>
</tr>
<tr>
<td>Sham-stimulation</td>
<td>117.83 ± 4.83</td>
<td>3.06 ± 0.08</td>
<td>1.28 ± 0.11</td>
</tr>
<tr>
<td>Post sham-stimulation</td>
<td>118.77 ± 7.54</td>
<td>2.15 ± 0.32</td>
<td>1.31 ± 0.13</td>
</tr>
</tbody>
</table>

Values refer to mean value ± std.

Hemodynamic results: impact of VNS on heart rate and blood pressure

Stimulation with 10Hz frequency consistently resulted, as expected [164, 241], in a decrease in the heart rate (percentage of change with respect to basal of \(-20.28 \pm 10.42\%\)) that increased over basal levels after stimulation ended (25.54 \pm 6.64%), as depicted in Figure 4.6. Similarly, blood pressure was reduced with stimulation and greatly increased afterwards (\(-7.81 \pm 10.84\%\) stimulation vs 13.61 \pm 5.33% post-stimulation). The hemodynamic results from the 5Hz-stimulation group are not reliable because in one of the animals (‘ID 13’) recordings were extremely noisy and no useful information could be extracted and analysed. In the control group (Figure 4.6 (a)), blood pressure fluctuated around baseline during the whole experimental period, whereas heart rate increased with time (percentage of change with respect to basal of 7.69 \pm 4.52% stimulation, 12.04 \pm 6.1% post-stimulation).
4.4. STUDY 1: IMPACT OF STIMULATION FREQUENCY ON THE INTACT NERVE

Figure 4.5: Study 1: Metabolic population results. (a) Control group (sham-stimulation), (b) 5Hz-stimulation group, (c) 10Hz-stimulation group. From top to bottom: glucose concentration (mg/dL), change in glucose concentration with respect to baseline (mg/dL), plasma insulin concentration (ng/mL), change in plasma insulin concentration with respect to baseline (ng/mL), plasma glucagon concentration (pg/mL), change in plasma glucagon concentration with respect to baseline (pg/mL).
Figure 4.6: Study 1: Hemodynamic population results. (a) Control group (sham-stimulation), (b) 5Hz-stimulation group, (c) 10Hz-stimulation group. Top: percentage of change in heart rate (HR) with respect to baseline (%); Bottom: percentage of change in blood pressure (BP) with respect to baseline (%).
4.5 STUDY 2: IMPACT OF STIMULATION FREQUENCY ON THE LIGATED NERVE

4.5.1 Stimulation protocol

In this study, three stimulation frequencies of 8Hz, 10Hz, and 15Hz, with otherwise same configuration parameters (biphasic, symmetric, charge-balanced square pulses of 1.75mA pulse amplitude and 0.5ms pulse width - i.e. 1ms pulse duration) were applied for 15 minutes to the distal end of the left cervical vagus nerve after ligation in fasted anaesthetized rats for efferent VNS. Note that stimulation waveform, amplitude and pulse width remained the same as in the previous study. Four experimental cohorts were designed in this case, as shown in Table 4.10. It is worth remarking that in this study the nerve was mechanically ligated and stimulated distally for selective efferent stimulation.

The experiments were scheduled to happen two times per day (surgery onsets at 7:30 and 16:00). Food was withdraw 8-12 hours before the onset of the surgery. Glucose, insulin and glucagon serum concentrations were measured at the following time points: 2, 14 (baseline), 20, 25, 29 (stimulation), 35, 45 and 60 (post-stimulation).

Table 4.10: Study 2: Description of experimental cohorts

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat ID</th>
<th>Stimulation protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>‘D6A’, ‘D6M’, ‘D7M’</td>
<td>Sham-stimulation</td>
</tr>
<tr>
<td>Group 1</td>
<td>‘D3A’, ‘D4A’, ‘D4M’</td>
<td>8Hz, 1ms, 1.75mA, 15min</td>
</tr>
<tr>
<td>Group 2</td>
<td>‘D1A’, ‘D1M’, ‘D5M’</td>
<td>10Hz, 1ms, 1.75mA, 15min</td>
</tr>
<tr>
<td>Group 3</td>
<td>‘D2A’, ‘D2M’, ‘D5A’</td>
<td>15Hz, 1ms, 1.75mA, 15min</td>
</tr>
</tbody>
</table>

4.5.2 Results

Stimulation on the ligated nerve consistently resulted in an immediate and sustained increase in all the measured metabolites (see Figure 4.7), with the highest frequency (15Hz) having the greatest and most consistent change over baseline. Glucose concentration increased during the whole stimulation period for all frequencies (see Table 4.11). As a result, the mean glucose derivative obtained during the stimulation period also increased with increasing frequencies. Interestingly, levels never returned back to baseline during post-stimulation period in either experimental protocol. Responses in insulin and glucagon were robust with stimulation, with a clear and immediate increase in both hormones that vanished after stimulation ceased. Insulin and glucagon levels achieved the maximum value after 10-13 minutes of stimulation, and started to decrease afterwards. In the control group (see Figure 4.7 (a)), there was again a sustained increase throughout the whole experiment of glucose concentration, characterized by a positive rate of change of glucose and glucagon concentrations. Insulin levels, on
the contrary, slightly decreased during sham-stimulation period and towards the end of the experiment.

Table 4.11: Study 2: Analysis of glucose levels. In the 'Maximum change related to baseline during stimulation period', values refer to mean value±std.

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Control</th>
<th>8Hz</th>
<th>10Hz</th>
<th>15Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum change related to baseline during stimulation period (mg/dL)</td>
<td>−0.3 ± 1.75</td>
<td>20.05 ± 3.73</td>
<td>46.45 ± 31.95</td>
<td>55.84 ± 17.33</td>
</tr>
<tr>
<td>Mean glucose derivative during the stimulation period (mg/dL/s)</td>
<td>0.0033</td>
<td>0.0025</td>
<td>0.0380</td>
<td>0.0539</td>
</tr>
<tr>
<td>Mean glucose derivative during the post-stimulation period (mg/dL/s)</td>
<td>0.0035</td>
<td>0.0027</td>
<td>−0.0004</td>
<td>−0.0094</td>
</tr>
</tbody>
</table>

Table 4.12: Study 2: Analysis of insulin and glucagon levels. Values refer to mean value ± std.

<table>
<thead>
<tr>
<th>Maximum change related to baseline during stimulation period</th>
<th>Insulin (ng/mL)</th>
<th>Glucagon (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−0.4 ± 0.13</td>
<td>15.29 ± 6.70</td>
</tr>
<tr>
<td>8Hz</td>
<td>1.25 ± 1.12</td>
<td>57.15 ± 23.21</td>
</tr>
<tr>
<td>10Hz</td>
<td>2.28 ± 1.92</td>
<td>88.88 ± 83.47</td>
</tr>
<tr>
<td>15Hz</td>
<td>4.95 ± 2.12</td>
<td>234.27 ± 77.93</td>
</tr>
</tbody>
</table>

Table 4.13: Study 2: Analysis of rate of change of measured metabolites in the control group.

<table>
<thead>
<tr>
<th>Rate of change</th>
<th>Glucose (md/dL/s)</th>
<th>Insulin (ng/mL/s)</th>
<th>Glucagon (pg/mL/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.0069</td>
<td>−0.0003</td>
<td>0.0075</td>
</tr>
<tr>
<td>Sham-stimulation</td>
<td>0.0033</td>
<td>−0.0002</td>
<td>0.0084</td>
</tr>
<tr>
<td>Post sham-stimulation</td>
<td>0.0035</td>
<td>−0.0001</td>
<td>0.0003</td>
</tr>
</tbody>
</table>
Figure 4.7: Study 2: Metabolic population results. (a) Control group (sham-stimulation), (b) 8Hz-stimulation group, (c) 10Hz-stimulation group, (d) 15Hz-stimulation group. From top to bottom: glucose concentration (mg/dL), change in glucose concentration with respect to baseline (mg/dL), plasma insulin concentration (ng/mL), change in plasma insulin concentration with respect to baseline (ng/mL), plasma glucagon concentration (pg/mL), change in plasma glucagon concentration with respect to baseline (pg/mL).
CHAPTER 4. IN VIVO EXPERIMENTS: IMPACT OF VNS ON GLUCOSE HOMEOSTASIS

4.6 Study 3: Comparative intact versus ligated nerve

4.6.1 Analysis

The small number of samples in each group (up to n=3) makes it difficult to perform a meaningful statistical study to compare the obtained results from stimulating with 10Hz in the intact (study 1) and ligated (study 2) nerves, with otherwise same stimulation parameters and experimental conditions. As a result, statistical tests could not be applied and only a visual and quantitative comparison could be done at this stage based on the mean and standard deviation of each group. Nonetheless, I designed and implemented the pipeline that will be required for running a thorough statistical analysis when enough samples are acquired. The required sample size to obtain a statistical power of 80% will be determined, following the guidelines of the NC3Rs, based on a power analysis setting a significance level of 5%, and using an estimate of the standard deviation from these pilot experiments. To begin with, the Shapiro-Wilk test will be applied to verify that the data comes from a normal distribution. Then, the Levene test will be used to check for homogeneity of variance (homoscedasticity). This is a requirement for running the t-test to determine if there is a significant difference between the means of two groups, which will be then applied if appropriate. If normality or homoscedasticity cannot be assumed, then the non-parametric Wilcoxon test will be performed. Significance level will be set at p<0.05. For this study, the following metrics were compared between scenarios: mean baseline concentrations, maximum change with respect to basal, and mean trend values (derivative) obtained during stimulation and post-stimulation periods, for the glucose, insulin and glucagon profiles.

4.6.2 Results

Table 4.14: Study 3: Comparative intact versus ligated nerve

<table>
<thead>
<tr>
<th>Metric</th>
<th>Intact nerve</th>
<th>Ligated nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline concentration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>107.69 ± 6.70</td>
<td>105.79 ± 2.39</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>2.45 ± 0.22</td>
<td>1.88 ± 0.07</td>
</tr>
<tr>
<td>Glucagon (pg/mL)</td>
<td>1.52 ± 0.05</td>
<td>19.01 ± 4.23</td>
</tr>
<tr>
<td><strong>Maximum change (peak) during stimulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>53.69 ± 14.11</td>
<td>46.45 ± 40.04</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.34 ± 0.41</td>
<td>2.28 ± 1.92</td>
</tr>
<tr>
<td>Glucagon (pg/mL)</td>
<td>0.03 ± 0.16</td>
<td>88.88 ± 83.47</td>
</tr>
<tr>
<td><strong>Mean rate of change during stimulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL/s)</td>
<td>0.0383</td>
<td>0.0380</td>
</tr>
<tr>
<td>Insulin (ng/mL/s)</td>
<td>0.0005</td>
<td>0.0012</td>
</tr>
<tr>
<td>Glucagon (pg/mL/s)</td>
<td>-0.0003</td>
<td>0.0647</td>
</tr>
<tr>
<td><strong>Mean rate of change post-stimulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL/min)</td>
<td>-0.0103</td>
<td>-0.0004</td>
</tr>
<tr>
<td>Insulin (ng/mL/s)</td>
<td>-0.0003</td>
<td>-0.0005</td>
</tr>
<tr>
<td>Glucagon (pg/mL/s)</td>
<td>0.0001</td>
<td>-0.0129</td>
</tr>
</tbody>
</table>
4.7 DISCUSSION

Glucose concentration baseline, maximum peak and rate of increase during stimulation period, were similar in both experimental conditions. The rate of recovery to basal concentrations during post-stimulation time, however, was higher in the intact nerve (-0.0103 mg/dL/s) than in the ligated nerve, where it was close to 0 (-0.0004 mg/dL/s). Despite the resemblance in glucose profiles, insulin and glucagon concentrations and rates of change were consistently lower in the intact conditions (excluding baseline concentrations of insulin).

4.7 Discussion

Table 4.15: Summary of experimental design, objective and key outcomes of the three studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Study 1</th>
<th>Study 2</th>
<th>Study 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats per group</td>
<td>2-3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Groups</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Stimulation directionality</td>
<td>Afferent and efferent</td>
<td>Efferent</td>
<td>Comparative</td>
</tr>
<tr>
<td>Stimulation frequencies</td>
<td>0Hz, 5Hz, 10Hz</td>
<td>0Hz, 8Hz, 10Hz, 15Hz</td>
<td>10Hz</td>
</tr>
<tr>
<td>Duration of stimulation</td>
<td>15min</td>
<td>20min</td>
<td></td>
</tr>
<tr>
<td>Objective</td>
<td>To study the metabolic and hemodynamic impact of VNS frequency applied to the intact nerve</td>
<td>To study the metabolic and hemodynamic impact of VNS frequency applied to the ligated nerve (efferent stimulation)</td>
<td>To compare the differential responses of afferent and efferent stimulation and purely efferent stimulation</td>
</tr>
<tr>
<td>Key outcomes</td>
<td>- Immediate and sustained increase in glucose concentration</td>
<td>- Immediate and sustained increase in glucose concentration</td>
<td>- Similar metrics of glucose concentration</td>
</tr>
<tr>
<td></td>
<td>- No meaningful change in insulin concentration</td>
<td>- Glucose levels did not return back to baseline during post-stimulation</td>
<td>- Recovery of glucose to baseline during post-stimulation was higher stimulating in the intact nerve</td>
</tr>
<tr>
<td></td>
<td>- A decreasing trend in glucagon concentration</td>
<td>- Increase in insulin and glucagon concentrations with stimulation</td>
<td>- The values of the metrics of insulin and glucagon were lower stimulating in the intact nerve</td>
</tr>
</tbody>
</table>

The parasympathetic control of the liver and pancreas and its impact on glucose metabolism have been extensively studied throughout the years, as introduced in Chapter 3. There are, however, few comprehensive quantitative studies describing the relationship, in time and magnitude, between VNS parameters and the kinetics of change of pancreatic hormones and glucose levels in blood. The previous sections have reported the impact of VNS frequency on blood concentrations of glucose, insulin and glucagon. The influence of afferent and efferent
fibres using two experimental setups - stimulation in the intact cervical vagus nerve for afferent and efferent activation, and in the distal end of the ligated nerve for efferent stimulation - have also been presented. A summary of some characteristics of the experimental design, and the objective and key outcomes of the three studies can be found in Table 4.15.

Both experimental scenarios resulted in an immediate and sustained hyperglycaemia with VNS. The changes observed in the pancreatic hormones in both conditions, on the other hand, were very different for insulin, and even opposite for glucagon concentrations. In a previous study on the metabolic impact of cervical VNS, Meyers et al. (2016) reported an increase in glucose levels after stimulation on the intact cervical vagus nerve (using a similar experimental setup), whereas this increase was almost absent during stimulation of the ligated nerve [134]. They suggested that the observed hyperglycaemia was mainly a result of the inhibition of insulin secretion, and the increase in glucagon secretion after stimulating afferent fibres. I found, however, that glucose increase was comparable during VNS in the intact (afferent and efferent) and ligated (efferent only) nerves. This suggests that other mechanisms are taking place. Given the prompt rise in glucose, I hypothesise that the liver is being activated by electrical stimulation of efferent fibres of the cervical vagus nerve, which promotes endogenous glucose production and the subsequent rise in blood glucose. One of the most surprising results of this study is that hyperglycaemia was maintained only after efferent stimulation, while levels return to baseline quicker after afferent and efferent stimulation. This indicates the presence of a negative feedback control involving afferent fibres from the liver that inhibits glucose production during post-stimulation. The absence of this neural feedback in the second study might explain the observed outcomes.

The hyperglycaemia observed during combined afferent and efferent VNS in the first study was not found to be accompanied by a meaningful increase in insulin concentration. On the other hand, insulin and glucagon levels consistently and markedly increased with stimulation of efferent fibres (ligated nerve), with values returning to baseline after stimulation. This suggests that this increase in pancreatic hormones is completely caused by stimulation of efferent fibres, and not affected by afferent stimulation or glucose levels. This is in agreement with previous studies demonstrating that efferent fibres stimulate insulin secretion, whereas afferent fibres inhibit it [167, 171, 134]. Meyers et al. (2016) suggested that this negative feedback mechanism involves vagal afferent fibres originating in the pancreas that project to the nucleus of the tractus solitarius (NTS), which then connect with hypothalamic regions that are involved in regulation of food intake, pancreatic secretion and insulin sensitivity (see Appendix B for more detail). The sympathetic splanchnic nerve fibres may eventually close the neural reflex.
4.7. DISCUSSION

loop by inhibiting insulin secretion (see Chapter 3) [171, 134]. I additionally suggest that other neural reflexes originated by indirect stimulation of the liver and other organs in the gastrointestinal tract may also be involved in preventing insulin levels to increase. It is worth noting that insulin concentration remained approximately constant during post-stimulation at a value that was greater than baseline. This indicates that glucose secreted during stimulation which did not return to baseline was, in turn, stimulating insulin secretion after VNS.

Interestingly, insulin and glucagon concentrations reached the maximum after 10-13 minutes of a 15 minute stimulation, and started to decrease before stimulation ceased. A similar response has been previously reported in studies in vitro on the isolated pancreas [183], and in vivo in dogs [248], and also in vivo in pigs [171]. In all the cases the insulin peak was observed within 3 to 5 minutes of a 10 minute stimulation in the former experiments and 5 minute stimulation in the latter. One hypothesis is that vagal stimulation cause the release of insulin from a “labile” compartment which can be related with the first phase (or cephalic phase) of insulin release [183]. This hypothesis, however, does not hold when more than one stimulation is used. In fact, higher frequencies have been found to result in greater amounts of insulin secreted due to stimulation both in the experiments reported in this thesis and in previous literature [171]. Another explanation may be the activation of counter-balance mechanisms that allow to restore homeostasis in healthy animals. Interestingly, this ‘early-peak’ response was not observed in experiments in rats using longer continuous stimulations (120 minutes), where peaks were obtained toward the end of the stimulation period [134]. This divergence between results may arise due to the different animal model used, as rodents have a different pancreatic and neural architecture compared to other animals such as pigs and dogs [189, 204, 208, 209]. It is worth reminding that we are assuming that the increase in hormones in all these studies is a result of an increased release, but, being rigorous, this cannot be confirmed without knowing the pancreatic blood flow [224]. Glucagon levels were found to be greatly reduced during stimulation of the intact nerve, while its concentration markedly increased when stimulating only the efferent fibres. These outcomes could be a result of inhibition of glucagon secretion mechanisms during stimulation of afferent fibres, although systematic errors should not be discarded.

The hemodynamic responses recorded during the first study provide some insight into the state of the animals throughout the experiments. It is interesting to highlight the increase in heart rate observed after the stimulation period in the three cohorts (sham, 5Hz and 10Hz stimulation). This is probably a result of the intrinsic stress associated to the experiments. I also hypothesize that sympathetic counterregulatory mechanisms counter-balancing the vagal stimulation could be involved. There was also a small increase in heart rate observed in the
beginning, which was probably a result of the surgical stress, but could also be due to the reduction of anaesthesia from 2% after surgery to 1.25% during the trials.

Despite all precautions were considered to ensure the quality of the experiments, some sources of variability have been identified and explained throughout the previous sections. Among them, the reduced sample size, the use of hook electrodes, and the presence of instrumental and systematic errors (e.g. use of one vs two glucometers), are foregrounded. In addition, the sustained and slow increase in glucose and glucagon levels in the control group throughout the length of the second study suggests that the stress of the animals during the procedures might also have impacted the results. This could be a result of an over-activation of sympathetic pathways, which serve as counter-balance mechanisms. More research on a larger sample size and the use of other electrode architecture, such as cuff electrodes, will allow quantifying the contribution of each source of variability on the results.

4.8 Executive summary and future work

The experimental studies presented in this chapter bring some light into the differential metabolic and hemodynamic responses obtained after cervical VNS. Some interesting outcomes have been found:

- Higher stimulation frequencies result in more consistent and greater responses (with otherwise same stimulation parameters). Variability in the results is larger for smaller frequencies.

- Responses in glucose concentration during stimulation on the intact (afferent and efferent stimulation) and ligated (only efferent stimulation) nerves with the same stimulation parameters are very similar. The post-stimulation period, however, differs among groups, with glucose levels not returning immediately to baseline after efferent stimulation. This suggests the presence of a negative-feedback carried by afferent fibres to restore homeostasis after stimulation.

- The immediate increase in glucose concentration with stimulation seems to be a result of direct activation of hepatic glucose production, rather than an indirect (and slower) action of glucagon levels.

- The maximum increase in insulin and glucagon is reached before the end of the stimulation period. Two hypotheses are posed to explain this outcome: 1) the completion of the ‘labile’ pool of hormones that is ready for release from the pancreatic cells before stimulation ends, and 2) the activation of counter-balance mechanisms to restore homeostasis.
4.9 Conclusion

The study on the hemodynamic responses suggests that stress and sympathetic activation may be present during the experiments.

These outcomes and hypotheses require further analysis and study in larger experimental sample sizes. Other considerations for future work are:

- To investigate methodologies to acquire more spatial specificity and selectivity in stimulation and to reduce the number of off-target effects (e.g. stimulating closer to the pancreas or the use of neural blockers). Towards this objective, bigger animals such as piglets or pigs would be ideal to facilitate the access to branches at deeper levels of the vagus nerve.

- Use diabetic animal models (either type 1 or type 2), which won’t present the counter-regulatory mechanisms that are fully functioning for maintaining homeostasis in healthy individuals. Novel strategies to improve glycaemic control on these disease models could be investigated.

4.9 Conclusion

The effects of VNS on the metabolic response have been previously studied in vivo in different animal models [167, 171, 134], yet a comprehensive analysis on the impact, in time and magnitude, of different stimulation parameters was lacking. This chapter has provided insight into the impact of stimulation frequency on glucose, insulin and glucagon blood concentrations, by presenting the results obtained from in vivo experiments in healthy rats. These results have also been used to validate the proof of concept of a novel unified neural-metabolic mathematical model, which is presented in the next chapter.

More research is needed to further confirm the results, investigate the presented hypothesis, and analyse the impact of changing other waveform parameters. This investigation, however, should not focus on finding the optimal set of parameters, but on expanding our understanding of the range of outcomes. The reason arises from the fact that optimal stimulation parameters will greatly vary depending on the animal model and the stimulation site, and it remains to be elucidated whether these parameters could be directly translated into clinically relevant parameters for human neuromodulation [50]. Therefore, pre-clinical animal data should be used for gaining insight into the stimulation parameters, but further experiments in humans are required for identifying the ideal clinical parameters [30, 50].
A UNIFIED NEURAL-METABOLIC MATHEMATICAL MODEL

This chapter presents a mathematical model describing the physiological process underlying the impact of cervical vagus nerve stimulation on glucose, insulin, and glucagon blood levels. In this model, the pancreas secretion is no longer controlled solely by hormones and metabolites, as current models do, but also includes the essential impact of the neural mechanisms on glucose control, as illustrated in Figure 5.1.

Figure 5.1: Unified compartmental neural-metabolic model during vagus nerve stimulation. (a) Physiological scenario. Diagram of the biological interaction among the most important organs involved in glucose control during meal intake. (b) Scheme of the unified neural-metabolic model. Each of the boxes represents one subsystem of the model. The pink shaded boxes depict some of the novel subsystems that have been introduced to the current metabolic models (grey shaded boxes). Insulin-related action and mass fluxes (solid black lines) and glucose-related action and mass flux (dotted black lines) are depicted $G(t), I(t), N(t)$ relate to plasma glucose, insulin, and glucagon, respectively.
The use of explainable computational models, such as the one presented in this chapter, allows normalizing the research approach to neuroscience and diabetes, stepping away from purely experimental protocols towards a hypothesis-based verification process. It is not a matter of transferring mathematical tools to a biological context, but of creating them ad hoc, derived from the very nature of the biological process to be studied. As a result, this discipline is essential to understand and navigate the complex landscape that characterizes the biological processes of interest over time, and to identify those processes that are still unknown to direct new experimental research to elucidate them. It also enables the development of physiologically detailed simulator environments to evaluate neuromodulation protocols and medical devices, such as the one used in the following chapter.

Figure 5.2 depicts the work-flow followed for developing the unified neural-metabolic mathematical model, which is explained in detail along the chapter. To begin with, Section 5.1 introduces a new methodology to identify and describe the vagus nerve activation (i.e. fibre recruitment) as a function of the stimulation parameters (pulse amplitude and width). Section 5.2 incorporates the obtained model of nerve activation into a model of neurotransmitter secretion from the nerve terminals as a function of the stimulation frequency. Section 5.3 presents the unified neural-metabolic model by incorporating this neural model into existing metabolic models. Different datasets have been used in each step, as shown in the diagram. Finally, Section 5.5 provides a thorough discussion of the overall performance of the unified neural-metabolic model and its current limitations.

Figure 5.2: Diagram of the workflow followed for developing the unified neural-metabolic mathematical model. Boxes in grey depict the datasets and the models (the purely metabolic model) taken from previous literature. Boxes in white represent the method and models implemented in this work.
5.1 Strategies for identification of neural events

5.1.1 Background

The future of bioelectronic medicine rests on the development of closed-loop systems that take advantage of the latest technology on neural interfaces to record and decode useful information extracted from neural signals, and to use it to modulate the peripheral nerves activity through stimulation. Recently, research on peripheral stimulation is gaining ground with a plethora of studies reporting successful neuromodulation of the organs of interest. Recording, on the other hand, faces challenges that hinder its rapid evolution, such as the sub-millimetre nature of autonomic nerves, the low-amplitude waveforms generated from them, and the low signal-to-noise ratio characterizing neurograms. Therefore, the acquisition system of a closed-loop neuromodulation device should include analogue components, such as amplifiers and circuits for signal pre-processing including filtering to remove non-neural signals (e.g. electrical interferences) or physiological signals from cardiac and muscle activity, to ensure signals are within the requirements for digitization. Then, an analogue-to-digital converter (ADC) will digitize the signals at an appropriate sampling rate. These digital signals will be eventually analysed using a processor unit that will extract, in real time, the relevant features from the neural activity of interest, combine them to provide an estimate of the physiological state of the system, and generate a certain output that is eventually delivered through a programmable neurostimulator or a drug delivery system, such as an artificial pancreas.

There has been remarkable progress in offline and online processing and classification of evoked responses [249] that has supported the optimization of stimulation paradigms, yet there is no consensus on the best strategy to extract information from peripheral nerves. In addition to the small-voltage and noisy nature of the recorded peripheral neural signals, neurograms are characterized for being non-stationary, and therefore the frequency content varies with time. In these cases, traditional signal analysis using Fourier Transform to decompose the signal into a sum of sinusoids to detect events, such as naturally-occurred action potentials or evoked compound action potentials, cannot be used because the temporal information about the changes in frequency is lost. Four methodologies are generally exploited instead to detect and classify the meaningful events from the background noise of peripheral neurograms: 1) statistical tests applied to sliding windows [250, 251], 2) the short-term Fourier transform [252], 3) spike-sorting methods based on spatio-temporal patterns [249, 253, 254], and 4) wavelet analysis [255].

Statistical tests applied to sliding windows are based on detecting the structures in the signal whose duration is known but whose shape is unknown. They have been successfully
used for automatically extracting and classifying events from time series without any predefined characteristics of events in terms of their magnitude or geometry, but with the nature of the background noise known in advance, such as from real-world atmospheric datasets [251]. This method therefore greatly relies only on the temporal information, which challenges the identification of complex-shape signals such as neurograms. The short-term Fourier transform applies the Fourier transform to shorter segments of equal length separately, resulting in a different Fourier spectrum for each segment. These two methods require previous knowledge of the signal of interest to select the appropriate window length for analysis, hindering its application for processing neurograms. Despite the limitations, short-term Fourier transform has been previously applied for spike detection in EEGs from the brain and peripheral neurograms, showing improved detection of true spikes when compared to simple-thresholding methods [252].

The use of spike-sorting methods using machine learning algorithms such as clustering is becoming a standard approach for identifying evoked compound action potentials (CAP)s from peripheral nerves [249, 253, 254]. In short, this methodology is based on classifying the activity elicited from fibres with different conduction velocities and locations based on its spatio-temporal profile. Recorded waveforms with similar amplitude and shape are therefore assigned to the same group, which would correspond to activation of a specific group of fibres. This algorithm is very popular for decoding motor signals from peripheral nerves in the development of closed-loop neuroprostheses [256, 257].

Finally, wavelet analysis allows detecting self-similar oscillatory events that are localized in time and resemble wavelets. Different wavelet-based strategies can be implemented to identify action potentials. A direct approach is to carry out a multiresolution analysis of the signal by filtering and reconstructing the bandwidths of interest for the neural signals. This has the advantage of reducing the pre-processing steps by directly de-noising the signal while isolating the structures of interest. Other techniques are based on wavelet optimization or Wavelet Best Basis, which consists of finding the optimal representation of the signal using fixed or adaptive wavelets by minimizing a cost function [258, 259]. This strategy is of special interest for the detection of action potentials, as it assumes that signals are comprised of independent events of a known shape. Furthering this idea, a recent paper has proposed a new method for analysing time series that consist of rescaled, phase-shifted, isolated replicates of a previously specified time-localized function using the generalized Morse wavelet family [255].

The following lines present the study carried out to provide a comparison of three offline strategies to denoise and detect neural events, in this case evoked CAPs, from neurograms.
5.1. STRATEGIES FOR IDENTIFICATION OF NEURAL EVENTS

These strategies could be adapted in the future to decode neural activity in real time, although for the scope of this study implementing an offline methodology was adequate. The first strategy is based on a purely temporal analysis by setting a simple threshold, whereas the other two exploit the opportunities and benefits of wavelet analysis both in its continuous and discrete forms. The final goal of this study is not to perform fibre classification, but to provide a metric to estimate the vagus nerve activation (i.e. fibre recruitment) as a function of the range of stimulation parameters considered (pulse amplitude and width).

5.1.2 Methods

Data

Neurograms containing the CAP recordings were obtained from in vivo experiments carried out by Dr. Mirza and colleagues (2018) [163]. CAP waveforms represent the sum of action potentials originated from individual fibres. A short description of their experimental protocol in Wistar rats is provided in this paragraph to understand the origin of these recordings. A set of recording and stimulating electrodes were implanted separated by a distance of $8 \pm 0.5$ cm. A pseudo-tripolar cuff electrode was placed at the left cervical vagus nerve for stimulating, and a pair of bipolar platinum were used for recording: the active one was placed around the subdiaphragmatic gastric branch, and the reference was placed in the subdiaphragmatic cavity to capture in-band interferences. Five pulse widths ($PW$) were used for the stimulation protocols ($PW = 0.1, 0.2, 0.5, 1$ and $5$ ms). For each pulse width, stimulation current ($I$) was varied through 16 different values: for pulse widths $0.1$ ms and $0.2$ ms, the stimulation current ranged from $0.2$ mA up to $3$ mA ($I = 0.2, 0.5, 0.8, 1$-$1.8$ mA (every $0.1$ mA), $1.8$-$3$ mA (every $0.2$ mA)). For the greatest pulse widths ($0.5$ ms, $1$ ms, $5$ ms) the stimulation current ranged from $0.1$ mA up to $2$ mA ($I = 0.1$-$1.2$ mA (every $0.1$)). Each stimulation protocol was repeated for $10$ times. Neurograms were then averaged and pre-processed to remove artefacts.

Proposed strategies for identification of CAPs

As reviewed in Chapter 3, the vagus nerve is composed of different types of fibres, mainly A, B, and C fibres, which conduct action potentials at different velocities. Consequently, if stimulation and recording sites are sufficiently separated, then the CAPs originated from different fibres can be resolved temporally. In other words, peaks in the CAP recordings represent the activation of fibres with a specific conduction velocity. Therefore, activation of different fibre types can be obtained by dividing the neurograms into different velocity regions and identifying the CAP peaks in each of them. The larger the stimulation current, the larger the number of fibres that are recruited resulting in more peaks of larger amplitude on the neurograms.
In the present study, the activation of each fibre type after stimulation was analysed by dividing the recorded neurograms into three main regions, corresponding to A, B, and C fibres. The temporal limits for each region were easily calculated from the velocity conductions range (see Table 3.1), and the separation length between the stimulation and recording electrodes \((8 \pm 0.5 \text{ cm})\).

Three strategies were implemented and compared to identify CAPs from the recordings background noise and intrinsic neural activity: 1) simple-thresholding by setting a fixed voltage threshold, 2) performing a time-frequency analysis using continuous wavelet transformation (CWT), and 3) performing a multiresolution analysis (MRA) using discrete wavelet transformation (DWT). All the analysis was carried out in Matlab (2018b, Matworks, Natick, MA).

For the first strategy, a simple thresholding method using a fixed threshold of \(V_{\text{th}} = 5\mu V\) was applied to the entire signal. This value was selected from previous literature [163]. Figure 5.3 shows a neurogram where negative peaks below the voltage threshold are detected as CAP. This method, despite its simplicity, greatly relies on the value of the selected threshold making it prone to missing events or identifying false CAPs.

To overcome this limitation, two methods based on wavelet denoising were implemented to filter background noise and increase the identifiability of the signal of interest. Wavelet analysis
is very useful for processing non-stationary signals, such as neural recordings, as it provides information about events in time and frequency, therefore allowing for a multiresolution analysis. In both methods, each neurogram was divided into a reference signal comprising the region preceding the stimulation artifact, and an evoked signal comprising the regions corresponding to each fibre type observed after the stimulation artefact. The reference signal provides a good representation of the noise and basal neural activity of each neurogram. Both wavelet-based strategies also relied on a two-step approach for noise removal: 1) signal reconstruction based on the frequency characteristics of the signal of interest, and 2) setting a threshold corresponding to the maximum amplitude obtained from the reference part of the signal. Each strategy, however, implemented a specific methodology for each step.

On the first wavelet-based strategy, CWT was used to perform the time-frequency analysis of the evoked signal, which was then compared with that obtained from the reference signal. Daubechies orthogonal wavelets of order 3 (db3) were selected given the similarity of their shape with that of the CAPs. The CWT calculates the amount of overlap of the wavelet as it is shifted and dilated/compressed over the signal. Where the signal matches the wavelet, the resulting coefficients have a higher magnitude. Using wavelets that are differently dilated allows defining the evolution of the scale (frequency) content of the signal over time.

In these neurograms, the reference signal centred most of its energy in high-frequency components (i.e. low scales, shrank wavelets), whereas, overall, the evoked signal focused its energy in a band of 250-550Hz, which unsurprisingly corresponds to the length of the CAPs (1.5 to 3.2ms). As a result, filtering the frequencies that were not in that bandwidth eliminated most of the noise from the signals of interest. This filtering was done by obtaining the coefficients of the CWT of both signals at the range scales corresponding to the bandwidth of frequencies containing the maximum energy, scales 30 to 64, which was calculated using the following relationship:

\[ F_{eq} = \frac{C_f \times f_s}{s \times dt}, \]  

(5.1)

where \( F_{eq} \) is the equivalent frequency, \( C_f \) is the central frequency of the wavelet (in this case db3), \( f_s \) is the sampling frequency and \( s \) is the scale. The greater the coefficients, the larger the overlap (i.e. similarity) between the signal and the wavelet at each scale. Therefore, an optimal reconstruction of the reference and the evoked signal was carried out by selecting, in each time step, the wavelet scale that yielded the maximum overlap with the signal.

For the second step towards noise removal, it is common practice in the wavelet domain to apply a universal threshold \( th_{uni} \) to all of the wavelet detail constituents, which removes additive Gaussian noise with high probability [260] (see Equation 5.3). This threshold tends
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to result in overly smooth results when its value becomes big given its dependency on the number of samples.

\[ th_{uni} = \sigma \sqrt{2 \ln(n)}, \quad (5.2) \]

where \( n \) is the sample size and \( \sigma \) is the robust median estimator. To overcome this limitation, the minimax threshold method can be used to perform a less aggressive denoising leading to smaller thresholds \( (\theta) \) as compared to the universal threshold [261]:

\[ \theta = \sigma (0.3936 + 0.1829 \log_2(N)), \quad (5.3) \]

where \( n \) is again the sample size and \( \sigma \) is the standard deviation of the noise.

In this study, however, a novel threshold approach was followed to ensure that baseline neural activity was also discarded. The proposed strategy consists of selecting the value of the maximum coefficient obtained from the reference period for each neurogram as the threshold for identifying the evoked CAPs during the subsequent signal. The activity over this noise threshold in the CWT coefficients signals was identified as CAPs (see Figure 5.4), therefore eliminating the intrinsic neural activity.

![Figure 5.4: CAPs identification performing a time-frequency analysis using CWT. Left panel: CWT transformation of the reference part of the neurogram. The maximum coefficient obtained in this region was set as the threshold for noise reduction. Right panel: CWT transformation of the signal of interest. CAPs were detected as events over the threshold previously identified.](image)

The second wavelet-based method was based on a multiresolution analysis of the reference and evoked signals using the DWT. The DWT decomposes a signal using orthogonal wavelet functions applied in the form of a filter bank. This allows analysing single events on the signal at different scales (frequencies). In short, at each level \( n \) of the DWT, the signal is both high-pass filtered to obtain the detail coefficients \( (D_n) \) at the scale \( 2^n \) and low-pass filtered to obtain the approximation coefficients \( (A_n) \). The approximation signal is further decomposed following the same methodology until detail coefficients are obtained at the desired level. In this
work, reference and evoke signals were decomposed using $db3$ wavelets up to level 7 (based on preliminary analysis). The first three levels (scales $2^1$ to $2^3$) contained the highest-frequency content of the signals, mostly related to noise. The detail coefficients of these levels obtained from the reference signal captured most of its energy (around 80% in all reference signals). On the other hand, the contribution of these levels to the total energy of evoked signals was almost negligible (less than 5%). As a result, the reference and evoked signals were reconstructed using the detail coefficients of the four largest levels, therefore filtering out the noise from the signal of interest. A similar approach to that of the previous wavelet-based method was then taken for the second step for noise removal, where the maximum amplitude of the reference signal was selected as a threshold to identify the evoked activity. In this case, however, it is the amplitude of the reference signal itself, and not the amplitude of the coefficients, which was used to compute the threshold (see Figure 5.5.)

**Figure 5.5: CAPs identification performing a multivariate analysis using DWT.** Left panel: multivariate reconstruction of the reference part of the neurogram after removing the high-frequency components. The maximum amplitude obtained in this region was set as the threshold for noise reduction. Right panel: multivariate reconstruction of the signal of interest after removing the high-frequency components. CAPs were detected as events over the threshold previously identified (marked with a red triangle).

**Developing a metric for nerve activation**

Once the CAPs were identified from the surrounded noise and intrinsic neural activity, a detailed analysis of the nerve activation was carried out. Different metrics were implemented and compared to assess the nerve activation elicited by each stimulation protocol. The lack of knowledge about the absolute maximum activation of the nerve made it necessary to pro-
vide metrics of activation that are relative to the maximum activation obtained from the stimulation protocols that were used (percentage metrics). The min-max normalization strategy was implemented for this purpose. Each percentage metric is based on a different feature of the identified CAPs: 1) number of detected peaks, 2) amplitude, 3) width, 4) area under the curve (AUC). The geometric mean (GMEAN) of these individual metrics was also computed to get a more robust assessment of the nerve activation. The geometric mean was selected as a measure of the central tendency as it is less affected by extreme values in skewed data and it is an appropriate measure for analysis that involves ratios. These five metrics were applied individually to each conduction velocity region, to analyse the percentage of activation of each fibre type, and overall to the complete neurograms to study the percentage of activation of the whole nerve bundle.

5.1.3 Results

Wavelet filtering and reconstruction

Filtered and reconstructed signals obtained with each method are shown in Figure 5.6. Reconstruction of evoked signals using the MRA analysis by applying the DWT perfectly reproduced the activity in the region of interest and the background noise was clearly reduced. Reconstruction from the CWT decomposition resulted in CAP peaks whose amplitude was greater than the amplitude of the original evoked signal, making them easily identifiable. Similarly, high-frequency noise was successfully filtered as shown in the reference part of the signal.

Figure 5.6: Wavelet signal reconstruction. Signal reconstructed after CWT and DWT (red and blue lines, respectively) on the reference part of the neurogram (left panel) and the region with the evoked response (right panel). The green line plots the original signal before denoise.
Comparison of proposed metrics for nerve activation

Figure 5.7: Comparison of the proposed metrics for nerve activation for each CAP identification method. This figure depicts the distribution of values obtained by computing the proposed metrics to the CAPs identified with each method for each stimulation protocol (pulse width - amplitude): fixed temporal threshold (top panel), CWT (left bottom panel), and MRA with DWT (right bottom panel). The variability between the metrics using the fixed thresholding method is less noteworthy than using the wavelet-based methodologies, especially for short pulse widths. Denoising based on the wavelet analysis allowed to detect smaller neural events that increased the variability of the activation metrics computed on them.

The percentage of activation of the whole nerve bundle based on 1) the number of detected peaks, 2) amplitude, 3) width, 4) AUC, and 5) GMEAN of the metrics, for each stimulation protocol and method is presented in Figure 5.7. The variability between the metrics when using the fixed thresholding method is less noteworthy than using the wavelet-based methodologies, especially for short pulse widths. Setting a fixed threshold to completely remove the background noise for all the neurograms resulted in the identification of only the most salient CAPs, particularly on the noisier recordings obtained with shorter pulse widths. Hence, calculating the
percentage activation metrics on these salient events led to similar results. On the other hand, computing an adaptable threshold to each specific neurogram based on the wavelet analysis allowed the identification of events that were otherwise masked in background noise. In these events, the features used for computing the activation metrics were less consistent leading to a greater variability. Similar graphs obtained for each fibre type can be found in Figures D.1, D.2, and D.3 in Appendix D.

The robustness of each metric was studied by analysing the variation along the three CAP identification strategies previously presented for each stimulation protocol. As depicted in Figure 5.8, the metric that provided the most consistent results, i.e. the least variability along strategies, was the AUC. For example, focusing at a pulse width of 0.1ms and amplitude of 3mA (first row, last value on each column, i.e. metric), the number of peaks presents a large variation of its value between strategies of $\pm 21.3\%$, whereas when computing the AUC it is reduced to $\pm 2.1\%$, and this is consistently shown for all the parameter combinations. As a result, this metric is proposed as an appropriate indicator of nerve activation.
5.1. STRATEGIES FOR IDENTIFICATION OF NEURAL EVENTS

Figure 5.8: Variation of metrics across CAP identification methods. Each column shows the variation of the value of each metric obtained after on the three CAP identification methods (fixed threshold, CWT, DWT) on each stimulation protocol (pulse width varies from 0.1ms to 5ms from top to bottom rows). The metric that provided the least variability along strategies was the AUC, which motivates computing it as a robust indicator of nerve activation.
Analysis of proposed methods for nerve activation

Once the most robust metric was identified, the AUC, the performance of the three identification strategies was studied regarding 1) the percentage of nerve activation for each stimulation protocol (combination of amplitude and pulse width stimulation parameters) (see Figure 5.9), and 2) the strength-duration curves (see Figure 5.10). Strength-durations curves were modelled using the Lapicque formula 5.4, where $I_{th}$ (mA) is the threshold current at each pulse width ($PW$) (ms). The rheobase current ($I_{rh}$) (mA) for each fibre type was defined as the minimum stimulation to elicit a CAP with an amplitude over the threshold in the corresponding region.

$$I_{th} = \frac{I_{rh}}{1 - e^{\frac{PW}{\tau_m}}},$$ (5.4)

In general, the larger the injected charge density (larger pulse width and amplitude), the higher the percentage of recruited fibres (see Figure 5.9). This behaviour, however, reversed for the stimulation protocol using the pulse width of 0.5ms, where an increased number of fibres were recruited. This suggests that pulse widths of 0.5ms are optimal for fibre recruitment. For a detailed overview of the performance of each metric for each fibre type using the three identification methods see Figures D.4, D.5 and D.6 in Appendix D.

![Figure 5.9: 3D surface plots of the percentage of nerve activation and recruitment during each stimulation protocol.](image)
5.1. STRATEGIES FOR IDENTIFICATION OF NEURAL EVENTS

![Strength-duration curves](image)

**Figure 5.10: Strength-duration curves.** Plotted by fitting the Laplacian formula to the obtained minimum pulse amplitudes required for each pulse duration to elicit nerve activation. In each panel, CAPs were identified by applying a different method to the recorded neurograms: fixed temporal thresholding (top panel), decomposition and filtering using CWT (left bottom panel), and decomposition, filtering, and reconstruction using DWT (right bottom panel). The smallest rheobase currents ($I_{rh}$) were obtained using the wavelet-based methods, probably because of using a two-step noise removal strategy that allowed to reduce only the background noise and not the smallest CAPs, which were otherwise masked and removed when setting the fixed threshold. This is of special importance for the C fibres.

From strength-duration curves in Figure 5.10, it can be observed that rheobase currents ($I_{rh}$) for A, B and C fibres slightly varied depending on the employed method (see red line in Figure 5.10). The smallest values were obtained with the MRA method using DWT, followed by the CWT and the fixed temporal threshold, especially for the C fibres. The reason may be that most of the noise and background activity were removed in the first two methods by using...
the two-step noise removal strategy. On the other hand, by setting a fixed temporal threshold, in this case 5\(\mu V\), small evoked CAPs below the threshold were masked in the noise and were possibly removed as well. This is mainly the case for small C-fibres activation, making it look like higher currents were needed for activation of these fibres. In any case, the behaviour was as expected, A-fibres had the lowest activation threshold, therefore being recruited first, followed by B-fibres, and C-fibres (see Chapter 3).

5.1.4 Discussion and conclusion of the strategies for identification of neural events and nerve activation

Closed-loop bioelectronic medicine is becoming a promising strategy for diagnosis and treatment of several chronic diseases, such as diabetes. Research in neural decoding algorithms, coupled with advances in data analysis techniques, are allowing us to extract information about the state of nerves and related organs [2, 254]. This work advances in this field by comparing three offline strategies to identify evoked compound action potentials on the sub-diaphragmatic gastric branch of the vagus nerve, with the ultimate goal of defining a metric of its activation as a function of the stimulation parameters (pulse amplitude and width).

The results indicate that using wavelet analysis, both in its continuous and discrete approaches, is a reliable strategy to identify CAP in recorded neurograms. In this case, MRA reconstruction using DWT seems to provide better filtering of undesired signals, which allows detecting smaller CAPs that are otherwise screened by external noise and intrinsic nerve activity. The strategies suggested in this paper overcome the limitations of windows-based methodologies, such as short-term Fourier transform, by analysing the whole length of the signals. Furthermore, they lack the computational complexity and cost that characterizes wavelet optimization algorithms. Finally, the two wavelet-based strategies that are presented implement a two-step denoising approach that allows to completely filter out the noise and background neural activity of the neurograms, therefore improving the identification of the signals of interest, while making them more robust to noisy signals. These are crucial characteristics in the design of real-time closed-loop devices. A quantitative comparison of the methods proposed in this study to other previously published methods applied to the same database of neurograms [163] is planned to further validate the analysis and consolidate the results.

This work also presents an alternative methodology for computing the quantitative estimation of the fibre recruitment and nerve activation that has the potential to replace the computational complex spike-sorting methods for cases where sorting the detected CAPs is not a requirement. Among the five metrics that have been proposed based on different features of the identified CAPs, the AUC provided the most robust results, showing the least variability
among identification methodologies. Having a robust metric for nerve activation is important until a scientific consensus is acquired on the best strategy to decode neural signals, as it would be less affected by the used methodology and will facilitate the comparison of different results.

Contrary to other studies that employ artificially synthesized neurograms [252, 255], this work utilized experimentally recorded data using a stimulation/recording paradigm that allowed to successfully resolve different fibre types [163]. Despite the limitations that this dataset may encounter, in terms of signal-to-noise ratio and the presence of artefacts, it provides a true scenario for developing and comparing strategies for CAP identification. Further analysis on the sensitivity and specificity of each of the proposed methods will be carried out by calculating the true and false detection rates in different experimental datasets to confirm our results. This analysis should also be extended to compare their performance to other previously reported methods, such as wavelet optimization.

The analytics flow presented in this study is limited to experimental protocols that involve stimulation and recording of evoked action potentials separated by a certain distance that allows to recognize groups of fibres based on their conduction velocities. The analysis of neurogram acquired using other experimental setups may require an additional processing step to identify the group of fibres that originate the detected CAP waveforms. The denoising and event detection methodology explained in the previous lines can be extended with classification algorithms that allow associating CAP waveforms with similar shape characteristics to distinct individual or groups of fibres. This will be implemented in future work.

For the scope of the thesis and the computational model, this study provides a valid model for determining an estimate of the nerve activation based on the stimulation amplitude and pulse width, which will be combined with the models developed in the following sections.

5.2 Mathematical Model of Neurotransmitter Secretion from Nerve Terminals

Once the percentage of fibres within the nerve that is activated for a certain stimulation pulse width and amplitude was obtained, the next step was to determine the amount of neurotransmitters that are released based on the stimulation frequency. I recently developed a model of ACh secretion from the vagus nerve terminals in the endocrine pancreas and its impact on insulin secretion (see Güemes et al. (2019) [262]). This model was limited by the scarcity of in vivo experiments measuring the continuous secretion of ACh in the pancreas elicited by vagal stimulation, which made it necessary to assume similar kinetics of ACh secretion from nerve terminals in different organs. In that case, the model proposed by Dexter and colleagues [263]
was selected to reproduce the secretion of ACh from vagus nerve terminals on the heart. To the best of my knowledge, no further research has been carried out in this regard, so a similar model is presented in this work with slight variations from my previous work. The most relevant difference is that the ultimate mathematical model that is developed in this thesis takes into account the whole parasympathetic innervation, the cholinergic mediated by ACh, and the non-cholinergic mediated by neuropeptides (NPs). For the latter, there is also a lack of studies describing the kinetics of release of non-cholinergic neurotransmitters that impact the pancreatic secretion of hormones. It is therefore inevitable to assume that all neurotransmitter (NT) follow a similar model of secretion from the nerve terminals.

In this model, the activation of the vagus nerve depletes a pool of NT vesicles from the terminals, where \( 0 \leq V(t) \leq 1 \) (dimensionless) is the normalized quantity of NT vesicles available for release. The change in NT concentration in the interstitial space is given by:

\[
\dot{V}(t) = -\rho V(t)s(t) + K_R(1 - V(t)) \text{ with } V(0) = 1, \\
R(t) = m\rho V(t)s(t), \\
\dot{N}T(t) = -K_D N_T(t) + R(t) \text{ with } N_T(0) = 0,
\]

where \( N_T(t) \) (nM) represents the interstitial concentration of NT; \( K_D \) (min\(^{-1}\)) is the rate of NT enzymatic degradation; \( s(t) \) is the vagal firing pattern of action potentials (APs); \( K_R \) (min\(^{-1}\)) represents the rate of renewal of vesicles; \( \rho \) (unitless) is the fraction of the total vesicles \( V \) released by each vagal stimulation; and \( m \) (nM) is the maximal concentration of NT that can be released per stimulus.

Vagal firing patterns consist of a burst of action potentials (APs) or spikes (0 or 1 events) with a certain firing frequency. It is well studied that for low stimulation frequencies (approximately up to 50 Hz), neurons in the CNS fire APs at the frequency of the stimulation [264]. Higher frequencies can weaken the phase-locked relationship between unit spikes and stimulation pulses increasing randomness in neuronal firing [264]. In this work, only frequencies lower than 10Hz are considered, therefore it can be assumed for modelling purposes that the firing frequency is the same as the stimulation frequency. In summary, the vagal firing pattern was modelled as a train of squared pulses \( s(t) = 1 \) during the pulse and \( s(t) = 0 \) otherwise), each of them with a duration \( \delta \) (ms) that resembles that of the real action potential (see panel (a) in Figure 5.12).

It is worth noting that the NT concentration (nM) in the interstitial space follows a first-order Boltzmann dynamics, eventually reaching a mean plateau value with time (with some superimposed ripple) that increases with greater stimulation frequencies. Only the value at the steady-state reflects the neural response at the temporal scale of the metabolic responses.
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(minutes, hours). It is therefore reasonable to simplify the model by defining the mean concentration of NT in the steady-state:

\[ V = \frac{KR}{\rho \text{mean}(s(t)) + KR}, \]  
\[ NT = \frac{m \rho V \text{mean}(s(t))}{KD}, \]  
(5.8)  
(5.9)

This simplified neural model solely depends on the firing frequency of the stimulus. Under this scenario, the higher the stimulation frequency, the higher the mean of the stimulus along time, resulting in a higher steady-state value for each neurotransmitter (ACh and NPs) concentration. Pulse amplitude and duration determine, as detailed in the previous section, the number of fibres that are being recruited, and therefore the percentage of the nerve that is being activated. Assuming that the amount of released NTs obtained with the model for a specific frequency refers to the maximum secretion, the percentage of activated nerve directly determines the amount of NTs that are eventually released to the pancreatic interstitial space.

5.2.1 Experimental data

The experimental data reported by Dexter et al. (1989) [263] in a study of the changes in heart rate elicited by vagal stimulation were used to identify the model (see Figure 5.11). Data were extracted from the graphs published in the literature using the software ScanIt [265].

![Figure 5.11: Mean interstitial concentration of acetylcholine (nM) over time. Data extracted from [263].](image)

5.2.2 Results

The values of the parameters of the neural model of NT secretion are reported in Table D.2 in Appendix D. Figure 5.12 illustrates the profiles of vesicles secretion and ACh concentration in the interstitial space, together with the correspondent stimulus, using an exemplary train of pulses (stimulus) with a frequency of 10 Hz, pulse duration of 4.2ms and total train duration of 15s (as previously published in 2019 [262]). It can be observed how the concentration of neu-
neurotransmitters, in this case ACh, in the interstitial space increases until reaching a plateau level with some ripple as long as the stimulus is present, and decreases exponentially afterwards.

![Neural model of neurotransmitter release.](image)

Figure 5.12: Neural model of neurotransmitter release. (a) Neural stimulus of 10Hz and 15s of overall duration and zoom to visualize the train of pulses and its discretization. (b) Profile of general neurotransmitter concentration in the interstitial space. (c) Profile of vesicle secretion (V(t)) from the nerve terminals. (Material from: Güemes et al. [262].)

5.3 Unified Neural-Metabolic Model

5.3.1 Introduction

Existing metabolic mathematical models are implemented using differential equations that describe the cellular dynamics for insulin release from the β-cells and glucagon release from α-cells using exclusively glucose levels and other metabolites as inputs [49, 266, 88]. On the other hand, there are mathematical models of the neuroregulation of other physiological processes, including modelling of the respiratory system [267], blood pressure [268], or heart rate amongst others [263]. To the best of my knowledge, models describing the effects of the neural regulation on the pancreatic function have not been reported. Hence, the important neural-
pancreatic interaction is currently disregarded, although it is crucial to achieving optimal glucose control.

To bridge this gap, I have combined for the first time metabolic and neural mathematical models in a unified physiological model to reproduce to a great extent the ideal glucoregulation seen in healthy subjects. This unified model represents the components and interactions of the complex dynamical behaviour of glucose homeostasis over time using a compartmental approach. The analysis and model developed in the previous sections allow to determine the amount of NTs that are eventually released to the pancreatic interstitial space, and act on specific organs neuromodulating their function. The following sections first provide an overview of the purely metabolic subsystem models found in the literature, which are then extended with new equations characterizing the interaction and impact of the NTs on the pancreas and liver.

5.3.2 Methods

Process-based modelling

The model design and selection were carried out following the process-based modelling paradigm. In short, process-based modelling employs formalized knowledge about the dynamical systems in the domain of interest and experimental measurements of the observed system [269]. The model structure that best reconstructs the observed system, i.e. fits the experimental data, is chosen as the most likely and physiologically accurate.

There are two types of uncertainties that challenge the modelling process that depend on the amount of previous available knowledge and measurements [269]. The first type of uncertainty is related to the model structure: the less available knowledge about the system, the larger the number of different model structures to consider, and the larger the uncertainty. Resolving the structural uncertainty means selecting the optimal model structure, and therefore the most appropriate set of model components and modelling assumptions on the kinetics of the interactions. In this study, the scarce knowledge of the dynamical behaviour of the neuregulation of glucose fluctuations and pancreatic secretion greatly increased the structural uncertainty. Therefore, a variety of model designs (i.e. sets of components and interactions between them) were taken into account.

The second type of uncertainty is called parametric uncertainty and is related to the values of the parameters in the models, including the kinetic rates and initial values. To resolve the parametric uncertainty, the values of the parameters are estimated using optimization algorithms that minimize the differences between the measured data and the results obtained with the model simulations. This is explained in more detail in Subsection Parameter identification.
CHAPTER 5. A UNIFIED NEURAL-METABOLIC MATHEMATICAL MODEL

Experimental data

The model design detailed in the following lines builds on recent experimental and modelling work to determine structure and parameter values of glucose, insulin, and glucagon kinetics during vagus nerve stimulation (VNS). Two experimental datasets were used, one for building the most appropriate model structure, and the second to further evaluate the performance of the chosen model structure.

The first dataset consists of the plasma glucose, insulin, and glucagon concentrations obtained during bilateral thoracic, subcardiac, vagal nerve stimulation protocol in dogs [248]. In more detail, the dataset includes experimental data from 23 dogs obtained in the presence and absence of the ganglionic blockers atropine, which is a muscarinic antagonist, and hexamethonium, a nicotinic antagonist. Atropine allows the inhibition of the direct action of ACh in the pancreatic cells, whereas hexamethonium results in blockage of the whole postsynaptic signalling (both cholinergic and non-cholinergic) providing results that approach a purely metabolic scenario. A summary of the experimental conditions and stimulation protocol can be found in Table 5.1. This experimental data were primarily used to develop a variety of model structures based on different hypothesis on the secretion and action of hormones and neurotransmitters, and to identify the most physiologically suitable. Further information on the methodology followed for the model selection and parameter identification can be found in the following sections.

Table 5.1: Details of the experimental protocol in the dog model used for the development and identification of the mathematical model.

<table>
<thead>
<tr>
<th>Mean Body Weight (Kg)</th>
<th>Type of stimulation</th>
<th>Place of stimulation</th>
<th>VNS characteristics</th>
<th>Total stimulation duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>Squared-wave</td>
<td>Subcardiac, thoracic</td>
<td>Amplitude (mA) 13.5</td>
<td>10</td>
</tr>
</tbody>
</table>

The second dataset comprises the plasma glucose, insulin and, glucagon concentrations obtained after electrical stimulation of the distal terminal of the ligated cervical vagus nerve of Wistar rats (see Section 4.5 in Chapter 4). By using this dataset, I could assess the ability of the selected model structure to resemble experimental data from other animal species. It is worth noting that the proposed model aims to replicate the impact of stimulation in the intact vagus nerve. The results from the first experimental setup, which reflect the metabolic impact of VNS on the intact nerve, would be desirable in this case, yet the limitations and inconsistencies of that dataset hindered its use for model evaluation purposes. To overcome this challenge and to analyse the model’s ability to replicate the impact of VNS at different frequencies, the results obtained using 10Hz and 15Hz stimulation on the distal terminal of the ligated cervical nerve...
were eventually used. A description of the characteristics of this experiment can be found in Table 5.2.

Table 5.2: Details of the experimental protocol in the rat model used for evaluation of the mathematical model.

<table>
<thead>
<tr>
<th>Mean Body Weight (Kg)</th>
<th>Type of stimulation</th>
<th>Place of stimulation</th>
<th>VNS characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.308</td>
<td>Biphasic, cathodic first cervical</td>
<td>1.75</td>
<td>1</td>
</tr>
<tr>
<td>0.32</td>
<td>Biphasic, cathodic first cervical</td>
<td>1.75</td>
<td>1</td>
</tr>
</tbody>
</table>

Metabolic system

Figure 5.13 depicts the diagrams of the glucose, insulin and glucagon subsystem comprising the metabolic model, which are described in more detail in the following lines. The fluxes of mass (continuous lines) and control signals (dashed lines) are visualised to provide a comprehensive overview of the relation between the different compartments.

a) Glucose subsystem

The model originally developed by Bergman et al. (1979) [51] was used as the core system for describing the interaction between insulin and glucose. This model was extended to account for the contribution of plasma glucagon concentration as it is depicted in the top panel in Figure 5.13.

The model describes the whole body as a single compartment with basal concentrations of glucose, insulin, and glucagon. Glucose production and disappearance are influenced by two remote insulin and glucagon compartments that depend on insulin and glucagon, respectively (see the interaction among blocks \( G_p \), \( X_N \), and \( X_I \) in Figure 5.13 upper panel). The equation characterizing plasma glucose concentration is derived from the balance between glucose utilization and glucose production as reported by Dalla Man et al. (2014) [270]:

\[
\dot{G}_p(t) = HGP(t) + Ra(t) - U_id - k_1 G_p(t), \quad (5.10)
\]

\[
G(t) = G_p(t)/V_G, \quad (5.11)
\]

where \( G_p(t) \) (mg/kg) and \( G(t) \) (mg/dl) are the plasma glucose mass and concentration respectively, \( HGP(t) \) is the endogenous hepatic glucose production (mg/kg/min), \( Ra(t) \) (mg/kg/min) is the rate of appearance of carbohydrates after meal intake, \( U_id \) (mg/kg/min) is the insulin-dependent glucose utilization in the tissues, \( k_1 \) (min\(^{-1}\)) defines the rate of glucose degradation, and \( V_G \) (dL/Kg) is the glucose distribution volume.

Despite previously published models are mainly focused on the impact of meal intake [51, 266, 88], this is not the scope of this work. Therefore, for the sake of simplicity, the equa-
CHAPTER 5. A UNIFIED NEURAL-METABOLIC MATHEMATICAL MODEL

Figure 5.13: Diagram of the metabolic compartmental model. Diagram of the glucose subsystem (top panel), insulin secretion subsystem (left middle panel), insulin kinetics subsystem (right middle panel), and glucagon subsystem (bottom panel) comprising the metabolic part of the model. Fluxes of mass are depicted with continuous lines and the control signals with dashed lines.
5.3. UNIFIED NEURAL-METABOLIC MODEL

tions related to the gastrointestinal subsystem (carbohydrate absorption and rate of appearance $Ra(t)$) have not been included in the following lines (for a detailed description of these equations see [262]).

Furthering Eq. 5.10, the endogenous hepatic glucose production and insulin-dependent glucose utilization are described by the following equations:

$$HGP(t) = B_0 - k_5 Gp(t) - k_6 X_I(t) Gp(t) + k_9 X_N(t) Gp(t), \text{ with } HGP(0) = B_0$$  \hspace{1cm} (5.12)

$$\dot{X}_I(t) = -k_3 X_I(t) + k_2 (I(t) - I_b),$$  \hspace{1cm} (5.13)

$$\dot{X}_N(t) = -k_8 X_N(t) + k_7 (N(t) - N_b),$$  \hspace{1cm} (5.14)

$$U_{id} = -k_4 X_I(t) Gp(t),$$  \hspace{1cm} (5.15)

where $B_0$ (mg/kg/min) is the basal glucose production, $k_5$ (min$^{-1}$) control the action of glucose per se to enhance its own hepatic production, $X_I(t)$ (pmol/L) is the remote compartment describing the interstitial concentration of insulin, characterized by the rate constants $k_2$ and $k_3$ (min$^{-1}$), and $X_N(t)$ (pg/mL) is the remote compartment describing the interstitial concentration of glucagon, characterized by the rate constants $k_7$ and $k_8$ (min$^{-1}$); $k_6$ (min$^{-1}$ per pmol/L) and $k_9$ (min$^{-1}$ per pg/mL) describe the effect of the remote insulin and glucagon, respectively, on glucose uptake by the tissue, and $k_4$ (min$^{-1}$ per pmol/L) controls the action of the remote insulin on inhibition of glucose production from the liver.

Constants $k_1$-$k_6$ are generally simplified into two main parameters: the glucose effectiveness $S_G$ (min$^{-1}$), which is defined as the glucose ability per se to promote its own disposal and inhibit its production [49], i.e., independently of insulin, and the insulin sensitivity $S_I$ (min$^{-1}$ per pmol/L), defined as the ability of insulin to increase glucose effectiveness [49] and characterizes the effect of insulin in the balance of glucose production and uptake. The equations that describe this relationship are the following:

$$S_G = k_1 + k_5,$$  \hspace{1cm} (5.16)

$$S_I = \frac{k_2 (k_4 + k_6)}{k_3}.$$

b) Insulin Secretion

The model describing the glucose-dependent insulin secretion is founded on the one proposed by Toffolo et al. (2001) [91] and Breda et al. (2001) [271], and lately reported by Dalla Man et al. (2007) [88] as follows:
\[ S^I(t) = \gamma I_{po}(t), \quad (5.18) \]
\[ \dot{I}_{po}(t) = -\gamma I_{po}(t) + I_{p1}(t) + I_{p2}(t) + S^I_b \text{ with } I_{po}(0) = I_{po b}, \quad (5.19) \]
\[ I_{p1}(t) = \begin{cases} 
    k_{D1}\dot{G}(t) & \text{if } \dot{G} > 0, \\
    0 & \text{otherwise},
\end{cases} \quad (5.20) \]
\[ \dot{I}_{p2}(t) = \begin{cases} 
    -\alpha [I_{p2}(t) - \beta (G(t) - h)] & \text{if } \beta (G(t) - h) \geq -S^I_b \quad I_{p2}(0) = 0, \\
    -\alpha [I_{p2}(t) + S^I_b] & \text{otherwise}
\end{cases} \quad (5.21) \]

where \( S^I(t) \) (pmol/Kg/min) is the glucose-dependent secreted insulin, subindex \( b \) refers to the basal state; \( \gamma \) (min\(^{-1}\)) is the transfer rate constant between portal vein and liver; \( I_{po}(t) \) (pmol/Kg) represents the amount of insulin in the portal vein; \( I_{p1}(t) \) (pmol/Kg) is responsible for the first phase of secretion; and \( I_{p2}(t) \) (pmol/Kg) characterises the slower second phase of secretion; \( k_{D1} \) (pmol/Kg per mg/dL) is the pancreatic responsivity to the glucose rate of change; \( \alpha \) is the delay between glucose signal and insulin secretion; \( \beta \) (pmol/Kg/min per mg/dL) is the pancreatic responsivity to glucose and \( h \) (mg/dL) is the threshold level of glucose above which the \( \beta \)-cells initiate to produce new insulin. The glucose threshold for triggering insulin secretion, \( h \), was set to the basal glucose concentration \( (G_b) \) as stated in [88] to guarantee the steady-state in basal conditions.

c) Insulin kinetics

The model of insulin kinetics is based on the two-compartment model developed by Dalla Man et al. (2007) [88]. This model takes the total insulin secretion from the \( \beta \)-cell (both glucose- and neural-dependent insulin) as the input and determines the plasma insulin as the output. The two compartments represent the insulin degradation that occurs in both the liver and the periphery.

The set of differential equations that describes this subsystem corresponds to the right middle panel in Figure 5.13 as follows:

\[
\begin{align*}
I_I(t) &= -(m_1 + m_3(t))I_I(t) + m_2 I_p(t) + S^I(t) \quad I_I(0) = I_{I b}, \\
I_p(t) &= -(m_2 + m_4)I_p(t) + m_1 I_I(t) \quad I_p(0) = I_{p b}, \\
I'(t) &= \frac{I_p}{V_I} \quad I(0) = I_b,
\end{align*}
\]

where \( I_p \) and \( I_I \) (pmol/Kg) are insulin masses in plasma and liver respectively; \( I \) (pmol/L) is the plasma insulin concentration; \( I_b \) represents the basal state; \( S^I(t) \) (pmol/Kg/min) is the total insulin secretion from \( \beta \)-cell; \( V_I \) (L/Kg) is the insulin distribution volume; and \( m_1, m_2, \) and \( m_4 \) (min\(^{-1}\)) are rate parameters.
The hepatic extraction of insulin ($HE$), which is defined as the insulin flux that leaves the liver irreversibly divided by the total insulin flux leaving the liver has been previously described as \[88\]:

\[
HE(t) = -m_5 S_T(t) + m_6 \quad \text{with } HE(0) = 0,
\]

\[5.23\]

\[
m_3(t) = \frac{HE(t)m_1}{1 - HE(t)}.
\]

\[5.24\]

For the sake of simplicity, in this model $HE$ was considered to be time-invariant and set to the basal value, and therefore:

\[
HE(t) = HE_b,
\]

\[5.25\]

\[
m_3 = HE_b m_1 \frac{1}{1 - HE}.
\]

\[5.26\]

d) Glucagon secretion and kinetics

The model of glucagon secretion from the $\alpha$-cell is derived from the model developed by Dalla Man et al. (2014) \[270\] and incorporated in the Type 1 UVA-Padova simulator. The release of glucagon has two components, a slower response ($N_s$) that depends directly on the blood glucose levels and a faster one ($N_f$) that is proportional to the rate of glucose change. The equations describing this subsystem are:

\[
S^N(t) = N_s(t) + N_f(t),
\]

\[5.27\]

\[
N_f(t) = \delta \max(-\dot{G}(t), 0),
\]

\[5.28\]

\[
\dot{N}_s(t) = \begin{cases} 
-N_s - \max(\sigma_2(h - G(t)) + S_b^N, 0) & \text{if } G(t) \geq h, \\
-N_s - \max\left(\frac{2(h - G(t))}{1 + \frac{G(t)}{G_b}} + S_b^N, 0\right) & \text{otherwise},
\end{cases}
\]

\[5.29\]

where $S^N(t)$ (pg/Kg/min) is the total glucose-dependent glucagon secretion, subindex $b$ refers to the basal state; $\delta$ (pg/Kg per mg/dL) is the $\alpha$-cell responsivity to the glucose rate of change; $\rho$ (min$^{-1}$) accounts for the delay between glucose signal and glucagon secretion; $\sigma$ and $\sigma_2$ (pg/Kg/min per mg/dL) is the $\alpha$-cell responsivity to glucose and $h$ (mg/dL) is the threshold level of glucose below which the $\beta$-cells initiate to release glucagon, which has been set to $(G_b)$ to guarantee the steady-state in basal conditions.

Glucagon kinetics are very rapid and can be described with a one-compartment model that follows the equation:

\[
\begin{cases} 
\dot{N}_p(t) = -n N_p(t) + S^N(t) & N_p(0) = N_{pb}, \\
N(t) = \frac{N_p}{V_N} & N(0) = N_b,
\end{cases}
\]

\[5.30\]
where $N_p(t) \text{ (pg/Kg)}$ is the glucagon mass in plasma; $N(t) \text{ (pg/mL)}$ is the plasma glucagon concentration; subindex $b$ represents the basal state; $n \text{ (min}^{-1}\text{)}$ is the clearance rate; and $V_N \text{ (mL/Kg)}$ is the glucagon distribution volume.

Unified neural-metabolic model

Given the large structural uncertainty, several model structures for the neurostimulation-induced insulin and glucagon secretions were built and evaluated, including direct action models, and uni-compartmental and bi-compartmental models to add delays to the control. Three representative models that were initially designed but eventually discarded are summarized in Figure 5.15.

Figure 5.14: Summary of some of the tested models of neurostimulation-induced hormonal secretion. Note that the secretion of hormones is now also under neural control (pink and grey boxes).

<table>
<thead>
<tr>
<th>Insulin Secretion</th>
<th>Glucagon Secretion</th>
<th>Model description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model I</strong></td>
<td></td>
<td>ACh and non-cholinergic neurotransmitters elicit the release of a specific pool of vesicles ($V_{\text{ins}}$ and $V_{\text{gl}}$) which increases the interstitial concentrations of insulin and glucagon, respectively. These contribute to the glucose-dependent secretion of hormones ($S^I$ and $S^N$).</td>
</tr>
<tr>
<td><strong>Model II</strong></td>
<td></td>
<td>ACh and non-cholinergic neurotransmitters, and their rate of change, directly elicit the release of insulin and glucagon.</td>
</tr>
<tr>
<td><strong>Model III</strong></td>
<td></td>
<td>ACh and non-cholinergic neurotransmitters act directly on the pancreatic cells stimulating the release of insulin and glucagon. The rate of change of the neurotransmitters act with a delay introduced using and additional compartment.</td>
</tr>
</tbody>
</table>
e) Neurostimulation-induced insulin secretion

![Diagram of Insulin and Glucagon Secretion](image)

Figure 5.15: Scheme of the unified neural-metabolic insulin secretion (left panel) and glucagon secretion (right panel) subsystems. Note that the secretion of hormones is now also under control of ACh (pink boxes) and neuropeptide signals (grey boxes).

Previous literature has reported a significant dependence on plasma glucose levels in the impact of ACh, and therefore vagal stimulation, on insulin secretion [171, 182, 183]. Both in vitro and in vivo studies have shown that the efficacy of vagal stimulation and ACh concentration on inducing insulin secretion increases over a certain level of blood glucose, generally on the lower limits of normoglycaemia. Despite no literature was found describing experiments analysing the same insulin response for other non-cholinergic neurotransmitters, I hypothesize this phenomenon is not exclusive for ACh. On the contrary, this response may arise from the intrinsic responsiveness of $\beta$-cells to glucose levels, which generally allows the release of insulin.

The structure that was eventually selected for representing the experimental data to the greater extend describes a scenario where insulin secretion is induced by the concentration of the neurotransmitters and their rate of change, using a single compartment to characterize its kinetics (see the left panel in Figure 5.15). The glucose-dependency mechanism that triggers the secretion of insulin after vagal stimulation, as described in the previous paragraph, was also considered in the model. The resulting equations defining the action of the neurotransmitters on the neurostimulation-induced insulin release are:
where $S^I(t)$ (pmol/Kg/min) is the total amount of insulin secreted by the $\beta$-cells; $\gamma$ (min$^{-1}$) is again the transfer rate constant between portal vein and liver; $I_{po}$ (pmol/Kg) represents the amount of insulin in the portal vein due to vagal stimulation; $I_{p1}(t)$ and $I_{p2}(t)$ (pmol/Kg) characterise the first and second phase of glucose-dependent secretion; and $I_n1(t)$ and $I_n2(t)$ (pmol/Kg) account for the kinetics of insulin release elicited by the interstitial concentration of $ACh$, and its rate of change $\dot{ACh}$; $k_{d1}^I$ (pmol/min per nM) and $k_{d2}^I$ (pmol/min per nM/s) are the delay between the $ACh$ and $ACh$ signals and insulin secretion, respectively; $k_{d2}^I$ (pmol/Kg/min per nM), $k_{d2}^I$ (pmol/Kg per nM/s), and $K_{NP}$ (pmol/Kg/min per nM), are the $\beta$-cell responsiveness to the concentration of $ACh$, $ACh$ rate of change, and NPs concentration, respectively, modulated by $K_{ACH}^I$ and $k_{d3}^I$ (unitless); $th$ is the glucose threshold for the stimulation to induce insulin secretion.

**f) Neurostimulation-induced glucagon secretion**

VNS induces secretion of glucagon by both cholinergic and non-cholinergic mechanisms, through the action of $ACh$ and neuropeptides (NPs), respectively [167, 168] (see Chapter 3). Some studies, however, indicate that the neurostimulation-induced secretion of glucagon is mainly mediated by NPs, such as VIP, rather than $ACh$ [248, 184, 188]. Following this observation and comparing the model responses implementing different structures, a biphasic secretion of glucagon release mainly controlled by NPs concentration was considered (see the right panel in Figure 5.15). Equation 5.27 was updated to account for the neurostimulation-induced secretion of glucagon as follows:

$$S^N(t) = S^N + N^N_n(t),$$

$$\dot{N}^N_{n1}(t) = -k_{n1}^N N^N_{n1}(t) + k_{n2}^N N^N(t),$$

$$\dot{N}^N_{n2}(t) = -k_{d1}^N N^N_{n2}(t) + k_{d2}^N N^N(t),$$

$$N^N_n(t) = k_{ACH}^N ACh(t) + k_{NP}^N N^N_{n1}(t) + k_{d3}^N N^N_{n2}(t),$$

where $S^N(t)$ (pg/Kg) is the total amount of glucagon secreted by the $\alpha$-cells; and $N_{n1}(t)$ and $N_{n2}(t)$ (pg/Kg) comprise the kinetics of glucagon release elicited by the interstitial concen-
5.3. **UNIFIED NEURAL-METABOLIC MODEL**

...tion of \( NPs \), and its rate of change \( \dot{NP} \); \( k_{n1}^N \) (pg/min per nM) and \( k_{d1}^N \) (pg/min per nM/s) describe the delay between the \( NP \) and \( \dot{NP} \) signals and glucagon secretion, respectively; \( k_{n2}^N \) (pg/Kg per nM), \( k_{d2}^N \) (pg/Kg per nM/s), \( K_{NP}^N \) (pg/Kg/min per nM), are the \( \alpha \)-cell responsivity to the concentration of \( NPs \), \( NPs \) rate of change, and ACh concentration, respectively, modulated by and \( K_{ACh}^I \) (pg/Kg/min per nM) and \( k_{d3}^N \) (unitless).

g) Neurostimulation-induced hepatic glucose production

![Figure 5.16: Scheme of the unified neural-metabolic glucose subsystem.](image)

Figure 5.16: Scheme of the unified neural-metabolic glucose subsystem. Note that the hepatic production of glucose is now also under the control of ACh (pink boxes) and neuropeptide signals (grey boxes).

Results from previous literature report an increase in glucose levels that may be independent of glucagon secretion [167]. This observation was confirmed in the experimental study carried out at Johns Hopkins University in rats, where the increase in glucose concentration began with the stimulation onset and increased consistently until stimulation was over (see previous Chapter 4). These results suggest that electrical stimulation of the vagus nerve at the cervical and abdominal levels activates both cholinergic and non-cholinergic mechanisms that enhance endogenous glucose production from the liver. This hypothesis was included in the model by implementing the action of the concentration of neurotransmitters in the liver using a first-order Boltzmann equation (see Figure 5.16). As a result, Equation 5.12 of the endogenous glucose production was updated to:


\[ \text{HGP}(t) = B_0 + (-k_5 - k_6 X_I(t) + k_9 X_N(t) + k_{n3} X_{ACh}^G(t) + k_{n6} X_{NP}^G(t)) G_p(t), \] (5.39)

\[ \text{HGP}(0) = HGP_b, \] (5.40)

\[ \dot{X}_{ACh}^G(t) = -k_{n2} X_{ACh}^G + k_{n1} ACh(t), \] (5.41)

\[ \dot{X}_{NP}^G(t) = -k_{n5} X_{NP}^G + k_{n4} NP(t), \] (5.42)

where \( k_{n3} \) and \( k_{n6} \) (\( \text{min}^{-1} \) per \( nM \)) are the parameters governing the amplitude of the delayed \( \text{ACH} \) and \( \text{NPs} \) action on the liver, \( X_{ACh} \) and \( X_{NP} \) (nM), respectively, whose kinetics are determined by parameters \( k_{n1}, k_{n2}, k_{n4} \) and \( k_{n5} \) (\( \text{min}^{-1} \)).

### Parameter identification

Mathematical models using ordinary differential equations (ODEs) are widely used to describe complex dynamic systems following scientific principles. These models, however, are very sensitive to the value of the ODEs parameters, which are typically unknown and have to be estimated from noisy measurements of the dynamic systems.

In this study, as previously described, the results published on the study of the impact on VNS of the hormonal secretion in dogs were used for selecting the most appropriate model structure and identifying its parameters [248]. The data were extracted from the graphs reported in the literature using the open software PlotDigitizer [272]. The parameters of all the model structures that were initially proposed were at first manually identified to obtain an approximation of their overall performance. The model that resembled the most the kinetics of the physiological data based on the value of two accuracy metrics was then selected, as described in the previous lines, and its parameters \( \theta \) were automatically identified.

The two accuracy metrics were calculated to analyse the quality of the data fit for each curve. To begin with, the mean absolute percentage error (MAPE) is one of the most common metrics used in model evaluation for its simple interpretation as it measures the percentage of deviation from the actual data (see equation 5.43). However, one of the major drawbacks of this metric is that it is sensitive to extreme values or outliers. For this reason, the median absolute percentage error (MdAPE), which is more robust to outliers, was preferentially chosen (see equation 5.44). In any case, both MAPE and MdAPE values have to be carefully analysed given its asymmetry with respect to over and under-forecasting (values are higher on negative errors) [273]. The second metric is the root mean square percentage error (RMSPE), which takes the square root of the errors and computes the average fitting error in percentage (see Equation 5.45) [274]. With either metric, reporting the percentage error by normalizing by the values of the actual data makes them independent from the magnitude of the variables of interest, allowing for a better comparison among models. Squaring the errors using RMSPE
also allow for more accurate results, as the negative and positive errors do not cancel out each other, thus adding more accuracy to the result. On the other hand, it makes the metric more sensitive to outliers. The use of metrics that normalise by the actual values is limited to the cases where actual values are not zero. When this requirement was not be met (when considering increments with respect to baseline) other metrics were used, such as the root mean square error (RMSE) that provides a measure of the average magnitude of the residuals (see Equation 5.46). The goodness of the fit of the model was evaluated by the closeness of the MdAPE and the RMSPE to 0%, or, alternatively, the RMSE to 0 (concentration units).

\[
\begin{align*}
MAPE &= \text{mean} \left( \frac{|\hat{y} - y|}{|y|} \right) \times 100, \\
MdAPE &= \text{median} \left( \frac{|\hat{y} - y|}{|y|} \right) \times 100, \\
RMSPE &= \sqrt{\text{mean} \left( \frac{|\hat{y} - y|^2}{|y|^2} \right)} \times 100, \\
RMSE &= \sqrt{\text{mean}(|\hat{y} - y|^2)}.
\end{align*}
\]

where \(\hat{y}\) refers to each of the predicted concentrations, and \(y\) relates to the experimental concentrations, respectively.

The data fitting was done by numerical approximation of an initial value problem using a procedure known as the non-linear least squares method [275]. In short, a numerical method algorithm is used to approximate the solution given a trial set of parameter values and initial conditions, a.k.a. simulation. The fit value is inserted into an optimization algorithm that updates parameter estimates by minimizing a cost function, defined as the “distance” between the model estimates (\(\hat{y}\)) and the actual observations (\(y\)). There were three curves to be simultaneously identified (glucose, insulin and glucagon concentrations), therefore the cost function was defined as the sum of the least squares error functions of each curve \(c\):

\[
\hat{\theta} = \arg\min_{\theta} \sum_{c=1}^{3} \sum_{i=1}^{N} (\hat{y}_c^i(\theta, t) - y_c^i(t))^2.
\]

In this study, an Evolution Strategy with Covariance Matrix Adaptation (CMA-ES) algorithm for non-linear global minimization was employed for the identification process [276, 277]. The models were numerically integrated using a fourth-order Runge-Kutta method with a fixed integration step size of 0.1 min.

Given the large number of parameters to be simultaneously identified, the fitting was progressively done in three steps. Firstly, the data obtained in the presence of the neural blocker hexamethonium were used to identify the parameters that provided stability to the purely metabolic model (equations 5.10 to 5.30). Then, the data in presence of atropine were employed to fit the kinetics of the response of plasma insulin, glucagon and glucose (equations
5.31 to 5.42) to the non-cholinergic (peptidergic) neural signalling (muscarinic contribution equals 0). Finally, the control data were used to identify the parameters explaining the contribution of the muscarinic ACh influence on the pancreatic islets. The parameters were also constrained to lie within a range of values with physiological significance and, when possible, within the range of values reported in the literature, as shown in Table D.1 in Appendix D.

5.3.3 Results

The final estimation values of the parameters can be found in Table D.3 in Appendix D. The left panel in Figure 5.17 shows graphically the fitting to the corresponding experimental data in the group that was pre-treated with atropine. Hence, the concentration of ACh in the interstitial space is absent, and the increase in glucose, insulin and glucagon comes from the action of non-cholinergic mechanisms (neuropeptides, NPs). The right panel in Figure 5.17 depicts the comparison of the results from the proposed model and the experimental data in the control group. In this case, all the neural signalling mechanisms are present (both cholinergic and non-cholinergic). A summary of the metric errors obtained in the estimation of glucose, insulin and glucagon, for each group, can be found in Table 5.3.

Table 5.3: Accuracy of the model fitting to the experimental data in dogs.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Atropine group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose profile</td>
<td>Insulin profile</td>
</tr>
<tr>
<td>MdAPE (%)</td>
<td>32.08%</td>
<td>4.51%</td>
</tr>
<tr>
<td>RMSPE (%)</td>
<td>3.57%</td>
<td>0.58%</td>
</tr>
<tr>
<td>RMSE</td>
<td>1.19mg/dL</td>
<td>7.49 ng/mL</td>
</tr>
</tbody>
</table>

*In this dataset, glucose profiles are increments with respect to baseline, therefore the MdAPE and RMSPE could not be computed. RMSE are shown instead to provide a reference of the accuracy of the model. The model parameters that lead to these results can be found in Table D.3 in Appendix D.
5.4 MODEL STRUCTURE EVALUATION

The ability of the selected model structure presented in the previous sections to generalize and resemble the experimental data from other species was evaluated by using the stimulation protocol and results obtained in Wistar rats during Study 2 (see Chapter 4).

Due to the existing anatomical and physiological differences between species in terms of nerve conduction properties and glucose metabolism, some parameters were re-identified to address the challenge of model transferability between species (see Table D.4 in Appendix D). Nonetheless, parameters were constrained to lie within the same range of values as previously detailed (see Table D.1 in Appendix D). A comparison between the model’s performance and the experimental results using the two stimulation frequencies in the rat model can be found in Figure 5.18 and Table 5.4, and are further discussed in the following Section 5.5.

Table 5.4: Accuracy of the model fitting to the experimental data in rats.

<table>
<thead>
<tr>
<th>Metric</th>
<th>10Hz stimulation</th>
<th>15Hz stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose profile</td>
<td>Insulin profile</td>
</tr>
<tr>
<td>MdAPE (%)</td>
<td>2.27%</td>
<td>13.14%</td>
</tr>
<tr>
<td>RMSPE (%)</td>
<td>0.36%</td>
<td>2.93%</td>
</tr>
</tbody>
</table>

The model parameters that lead to these results can be found in Table D.4 in Appendix D.
**5.5 Discussion**

The proposed model succeeds to replicate the kinetics of the neural control of glucose homeostasis, as reported in experimental results found in the literature [167, 171]. Access to data describing the response of glucose, insulin, and glucagon under the presence of neural blockers, such as atropine, allows to clearly identify the contribution of the muscarinic cholinergic innervation and the non-muscarinic, mainly peptidergic, transmission. This enables the development and identification of a more comprehensive model of the physiological mechanisms that induce hormonal secretion from the pancreas and control glucose levels.

The most noteworthy novelty of the model is its ability to describe the physiological responses to VNS at all levels, from the activation of peripheral nerves to the replication of the metabolic changes. In more detail, the model comprises: 1) characterization of vagus nerve activation, 2) secretion of cholinergic and non-cholinergic neurotransmitters (ACh and NPs, respectively), 3) glucose-dependent hormonal secretion from the pancreas, 4) hormonal-dependent glucose control, 5) hormonal secretion elicited by the interstitial concentration of ACh and NPs, and 6) neurally-induced hepatic glucose production. Considering distinct cholinergic and non-cholinergic pathways is another original contribution to the model, since previous experimental literature has reported that insulin secretion is not completely abolished by...
5.5. DISCUSSION

Atropine, whereas glucagon secretion induced by vagal stimulation is mainly mediated by neuropeptides, such as VIP, rather than by ACh [168, 248].

Overall, the accuracy of the proposed model in resembling the kinetics of glucose and hormonal profiles in dogs is very acceptable, with the largest error obtained in the insulin curve in presence of atropine (RMSPE of 3.57%, see Table 5.3). Glucose profiles are represented as increments over baseline, resulting in presence of zero values at basal state (0% of change). The metrics based on percentage errors (MdAPE and RMSPE), despite having the advantage of normalizing the scale of the values, always induce some errors in this situation. As a consequence, the RMSE was computed in this case giving minor deviations of around 1mg/dL from actual observations. Overall, the kinetics of the responses after VNS are well characterized, with the peaks amplitude, and rising and decreasing constants, resembling the experimental results.

The evaluation of the model structure in results from rats produced a fair reproduction of the shape of the peaks in the glucose, insulin and glucagon concentration curves for the two stimulation protocols (i.e. 10Hz and 15Hz). In particular, the kinetics of glucose plasma concentration obtained when stimulating with both 10Hz and 15Hz frequencies are very tightly reproduced (10Hz stimulation: RMSPE=0.36%, 15Hz stimulation: RMSPE=0.21%). Insulin and glucagon concentration profiles under 10Hz stimulation are also closely matched and stay within the shaded region of variability of individual results. Greater differences are observed in the hormonal concentration profiles under 15Hz stimulation, where the model resembles the shape of the hormonal curves, but fails to provide enough magnitude of change. Given the good performance of the model for matching the temporal kinetics (i.e. shape) of experimental results in dogs, I suggest this deficiency may arise from the large variability obtained in the in vivo experiments in rats (notice the broad orange shaded region in Figure 5.18). One interesting aspect to highlight is that the proposed model has been designed to replicate the physiological processes activated after stimulation of the intact cervical vagus nerve for efferent and afferent activation. The experimental results from rats used as reference, however, were obtained after distal stimulation on the ligated nerve for efferent activation. As discussed in the previous chapter, solely efferent stimulation results in sustained hyperglycaemia that is not associated with an increase in insulin concentration post-stimulation. Since this purely efferent stimulation mechanism is not considered in the proposed model, some differences arise between the model and experimental results during the post-stimulation period.

As with all models, there are some limitations. The most important in this case is the lack of knowledge about the specific metabolic processes triggered after electrical stimulation of the
vagus nerve, which hinders the development of more comprehensive mathematical models of the underlying physiology and challenges the model transferability between species, and between nerves and tissues within the same species. As a consequence, it was necessary to consider some assumptions and simplifications to build the model.

To begin with, differences in the anatomical and conduction properties among peripheral nerve fibres affect their sensitivity and response to electrical stimulation. For example, the pancreatic branch differs from the gastric-projecting neurons in that they have i) longer duration of action potentials, ii) longer after-hyperpolarization decay time, iii) smaller soma area and iv) larger diameter [102, 278]. Differences in these properties become more significant between species. These limit the development of generic models for nerve activation, yet there is a lack of studies providing a comprehensive view of the similarities/divergences of nerve parameters between nerve fibres and species. Therefore, it is only possible to hypothetically extrapolate the model structure between animal models until more research is carried out in this context. As a consequence, I assumed equal activation mechanisms of the vagus nerve at the cervical level for the rat and dog models for this work.

A second assumption of the neural part of the model specifies that the vagus nerve terminals in different organs have similar kinetics of ACh secretion, which are also the same among different neurotransmitters. This assumption seems to be valid since the secretion and re-absorption of vesicles filled with neurotransmitters in the interstitial space are generally modelled using the same mathematical equations. Further experimental research at the molecular scale is needed to identify the characteristics of neurotransmitters release to the interstitial space and their action to the pancreas and liver.

Even more significant is the challenge of transferability of the metabolic model, since there is enormous variability in the metabolism of glucose between species, within the same species, and even throughout the day within the same subject. Consequently, it is essential to use a large experimental dataset to identify and validate the model that reflects this inter- and intra-subject variability. In this regard, the available experimental dataset used in this work was insufficient becoming one of the most critical limitations of the study.

Finally, the neural-metabolic system is, in fact, very complex, so describing it using various sub-models or compartments is one of its more remarkable characteristics since it allows us to better understand the underlying physiological processes. Yet, the sole availability of plasma glucose, insulin and glucagon concentrations does not allow to build a reliable simulation model, since one can obtain a good description of plasma glucose, insulin and glucagon concentrations with many different descriptions of the underlying fluxes in the system, where
5.6 OVERALL MODEL DISCUSSION AND CONCLUSION

structural errors in some unit process models compensate those in others [279]. Thus, plasma metabolic concentrations are insufficient to reliably identify the model by directly using traditional identification techniques, such as non-linear least squares [280], maximum likelihood, or collocation methods like the generalized profiling method, which uses a non-parametric function to approximate the solutions of the dynamic process [275]. Moreover, advanced techniques in Bayesian statistics, such as the Markov chain Monte Carlo method [281] or the Maximum a Posteriori Bayesian approach [279] could not be applied due to the high dimensionality of the parameters to sample, the absence of a large and complete experimental dataset, and the limited prior knowledge about the processes to be modelled [281]. Given the compartmental structure of the model, a good identification strategy requires virtually model-independent measurements of the various glucose, insulin and glucagon fluxes occurring during VNS, such as the profiles of glucose production and hormonal secretions [88]. This extensive metabolic analysis can be experimentally achieved by using a triple-tracer protocol during meal intake, as previously reported by Dalla Man et al. (2007) [88]. The absence of such measurements of internal fluxes and the reduced available dataset, made it necessary to implement a three-stepwise strategy to reduce the number of simultaneously estimated parameters, and therefore the degrees of freedom of the model, and consecutively apply a global non-linear least squares method as explained in Subsection 5.3.2.

5.6 Overall model discussion and conclusion

Existing models of glucose homeostasis currently neglect the neural contribution, therefore disregarding the physiological basis of the interaction between the brain and the pancreas and liver, and its importance in the control of glucose homeostasis. To the best of my knowledge, the unified neural-metabolic model outlined in this chapter is the first attempt at defining a physiological model of the impact of vagus nerve stimulation in glucose homeostasis. The model describes a comprehensive and complete compartmental scenario that includes from nerve activation based on the stimulation parameters to the ultimate impact of this neural stimulation on blood glucose, insulin and glucagon concentrations.

Notwithstanding the limitations of the proposed model, the results closely represent the physiological effect of VNS on glucose metabolism observed in previous experiments in animal models. This is important as computational models are a good platform to optimise systems and provide insight into the underlying mechanisms of the nervous control of glucose metabolism without the need for real evaluation on animals [282, 283]. It also allows identifying missing knowledge in the field and guide experimental research towards new directions.
that complete this understanding (see Figure 2.5) [30]. This is even of greater importance in the
field of bioelectronic medicine, where a purely experimental evidence-based approach is not
efficient given the reduced knowledge about the biological targets, and it is very costly [30].
Computational models therefore become an essential tool to provide preliminary validation
of the systems and reduce the number of experimental trials. To serve this purpose, computa-
tional models should never be static, but be continuously updated as new knowledge and more
specific/selective stimulation technology to interface with peripheral nerves become available.

Detailed physiological models, such as the one presented in this chapter, also lay the foun-
dation for the development of pathophysiological models that reproduce the complex regu-
lation of glucose homeostasis in animal models with diabetes, becoming a powerful tool for
evaluating medical devices. Towards this objective, it is necessary to update and adapt the
model to characterize the human behaviour, making the availability of clinical data to test and
validate the models a requirement. In the future, this will allow existing simulators of diabetes
mellitus to provide improved environments for testing treatments and monitoring interven-
tions, opening the door to more personalized and predictive medicine [270, 284] as discussed
in the next chapter.

In addition to explainable models, data-driven models, are becoming increasingly used
within software platforms as more efficient machine learning algorithms are developed, serv-
ing as clinical decision support tools and being embedded in medical devices. This type of
models, however, work as black-boxes and lack the ability to explain the underlying physio-
logical processes. This intrinsic feature of data-driven models challenges their application for
guiding research and enabling simulation platforms, and therefore justifies the need and de-
development of detailed physiological models of the systems of interest.

In conclusion, the proposed work gives insight into the physiological basis of the nervous
control of glucose metabolism with the design of an extensive compartmental computational
model, and confirms the benefit of considering the neural contribution in this field. Stepping
away from purely experimental protocols towards a hypothesis-based verification process, the
development and continuous update of computational models enable an optimal interplay be-
tween deductive and inductive scientific reasoning, which I believe is the way science should
be performed.
IN SILICO APPLICATION OF BIOELECTRONIC MEDICINE FOR IMPROVED TYPE 1 DIABETES MANAGEMENT

The previous chapters present studies of the neural-metabolic interaction on glucose control using an experimental (Chapter 4) and a computational (Chapter 5) approach. This fundamental research lays the required ground for advancing in the field of bioelectronic medicine for diabetes. This chapter moves forward from this initial research to present an idea of how bioelectronic medicine can be applied to a closed-loop system enabling novel neuromodulation-pharmaceutical therapeutic technology for people with diabetes. To completely test the feasibility of this hypothesis in humans, it would be necessary to study, in vivo, the impact of highly selective vagus nerve stimulation (VNS) on glucose homeostasis in people with diabetes whose nervous systems is functional. However, the research on this field is too preliminary to be assessed in clinical studies. Computational models of the human physiology and pathophysiology, such as the one presented in the previous chapter, are essential to bridge the gap between basic and clinical research, and enable to test the feasibility of new devices which would be otherwise impracticable.

In this context, this chapter presents the proof of concept of the first closed-loop system that incorporates electrical modulation of the nervous system using an external device to target the organs and tissues that impact insulin sensitivity (SI) and provide further control of glucose fluctuations after meal intake. This work makes up the foundations for developing a new generation of hybrid hormonal-neural controllers.

Further details on the work presented in this chapter can be found in the conference paper published in the 2019 IEEE International Symposium on Circuits and Systems (Güemes et al., 2019 [285]), and in Diabetes Technology & Therapeutics (Güemes et al., 2019 [286]).


6.1 Background

Insulin sensitivity (SI) quantifies how sensitive the tissue is to the action of insulin. Recent research on the field has demonstrated that central and peripheral nervous mechanisms are
involved in modifying the SI of a person, by affecting the glucose production in the liver and its uptake in other peripheral tissues [19, 287, 166, 190, 288, 289]. As a result, nowadays there is a growing interest in interfacing with the nervous system for neuromodulation of SI for T2DM treatment [29, 138].

The opportunities offered by applying bioelectronic medicine to increase SI should not be restricted solely to T2DM. Modulating the action of the exogenous insulin that is injected on tissue could significantly improve glucose control in people with T1DM. As technology advances to realize bioelectronic medicine, we will one day be able to directly stimulate the central and peripheral nervous pathways and modulate the neural signals to change and control SI for T1DM and T2DM treatment using an external non-invasive device.

The therapeutic impact of bioelectronic medicine would benefit of implementing closed-loop devices that incorporate real-time monitoring and analysis of metabolic and neurophysiological biomarkers to automatically adjust the electrical stimulation delivered to the peripheral nerves or directly to the organs to modulate their function [25, 26]. This strategy promises to provide real-time, autonomous, and patient-specific therapies, which are crucial in chronic diseases such as diabetes.

The following sections present an overview of a new generation of hybrid hormonal-insulin sensitivity glucose controller (InSiG) that extend the existing bi-hormonal bio-inspired artificial pancreas (BiAP) controller developed by Herrero et al. (2017) [32], by including additional control over the action of insulin in the liver and peripheral tissues that impact the SI of people with T1DM to achieve improved glucose management (see Figure 6.1. These findings should
serve as motivation to pursue the field of bioelectronic medicine to develop non-invasive devices to directly modulate SI through electrical stimulation of the nervous system for type 1 and type 2 diabetes mellitus treatment.

6.2 Methodology

For T1DM, existing closed-loop controllers vary on the complexity of the control algorithm used to calculate the doses of the drugs (e.g. insulin) to be injected. Examples of closed-loop controllers include proportional-integrative-derivative (PID) algorithms [31, 290, 291, 292, 293], model predictive control (MPC) [294], fuzzy logic [295], and more complex systems like the BiAP controller, which try to mimic the physiology of the pancreatic secretion [296]. All of them can be either uni-hormonal or bi-hormonal systems [297, 298]. In the latter, the glucagon dose is specified in addition to insulin.

Two strategies were implemented to show the potential benefits of modulating insulin delivery for glucose homeostasis. The first approach involved the design of a uni-hormonal hybrid controller that comprises a standard proportional-derivative (sPD) controller as the core module for insulin delivery and an additional sPD for tuning the SI (uni-hormonal sPD-InSiG). The promising preliminary results suggested that using more complex core algorithms would allow obtaining tighter control of glucose fluctuations after meal intake. Hence, the coordinated bi-hormonal BiAP controller was then selected as the core control algorithm and combined with the sPD for adjusting the SI (bi-hormonal BiAP-InSiG). All controllers used values from continuous glucose monitoring (CGM) every 5 minutes as inputs.

6.2.1 Subjects

In both strategies, the UVa-Padova T1DM simulator (version 3.2) [270] was employed to implement, identify and test the performance of all the controllers. In particular, a virtual population of 10 adults and 10 adolescent subjects with T1DM was used. These cohorts are a sample of a population with 100 adults and 100 adolescents that have been accepted by the Food and Drug Administration (FDA) as a truthful representation of the range of physiological parameters shown in people with T1DM. Among these, two parameters of the model of the virtual subjects in the simulator define the insulin sensitivity: \( k_{p3} \) and \( V_{mx} \). The first one modulates the inhibitory action of insulin on hepatic glucose production, whereas the second one specifies the stimulatory action on glucose uptake by the peripheral tissues.
6.2.2 Controllers design

Core module for hormonal delivery

*Uni-hormonal sPD-InSiG* The sPD controller has the following form [31]:

\[
  u(t) = K_P(G(t) - G_{ref}) + K_D(d(G(t) - G_{ref}))/dt, \tag{6.1}
\]

where \( u(t) \) represents the deviation of the control variable from its basal value (original value of the parameter in each virtual subject), \( G(t) \) is the blood glucose measurement (one every 5 minutes) and \( G_{ref} \) is the target glucose concentration (set-point), which was set to 120 mg/dl. Parameters \( K_P \) and \( K_D \) are the proportional and derivative weights respectively. The initial glucose concentration of the virtual subjects was set to 100mg/dl. In this case, \( u(t) \) represents the insulin dose to be delivered.

*Bi-hormonal BiAP-InSiG* The core of the coordinated bi-hormonal BiAP for insulin and glucagon delivery is based upon the biologically-inspired mathematical models of hormonal secretion, including a positive feedback loop to account for the potentiation effect that glucagon has on insulin secretion [32] (see the left panel in Figure 6.2).

**InSiG module**

The sPD controllers for determining the desired SI were designed following Equation 6.1, where \( u(t) \) represents in this case the control variables \( kp3 \) and \( Vmx \). In this context, three versions of the InSiG controllers were designed to account for the differences in glucose control achieved through the independent closed-loop modulation of each control variable \( kp3 \) and \( Vmx \) (InSiG-kp3 and InSiG-Vmx controllers, respectively) and the combined modulation of them (InSiG-Full controller). A summary of the design of each controller concerning each control variable is shown in Table 6.1.

**Table 6.1:** Comparison of the three design versions of the InSiG controller.

<table>
<thead>
<tr>
<th></th>
<th>Hormonal delivery</th>
<th>kp3</th>
<th>Vmx</th>
</tr>
</thead>
<tbody>
<tr>
<td>InSiG - kp3</td>
<td>Closed-loop</td>
<td>Closed-loop</td>
<td>Open-loop</td>
</tr>
<tr>
<td>InSiG - Vmx</td>
<td>Closed-loop</td>
<td>Open-loop</td>
<td>Closed-loop</td>
</tr>
<tr>
<td>InSiG - Full</td>
<td>Closed-loop</td>
<td>Closed-loop</td>
<td>Closed-loop</td>
</tr>
</tbody>
</table>

Existing controllers for insulin and glucagon delivery can be tested *in silico* because the models in the simulator replicate the physiological delays of the exogenous insulin and glucagon. However, the dynamics of the effect of acute neural stimulation on modifying the sensitivity of the liver and peripheral tissues to the action of insulin (i.e. the SI) of a subject have not been defined in the literature and are not included in computational models yet. Based on the rapid changes observed after stimulation of the vagus nerve on *in vivo* experiments [299, 171] and
the conduction velocities of sympathetic and parasympathetic nerves in healthy and diabetic rats [300], we have assumed that the dynamics of change of SI occur in the order of milliseconds to seconds, and therefore were considered instantaneous in the time scale of the models (minutes-hours). As a result, the value of the SI-related parameters \((kp3\) and \(Vmx\)) specified by the controllers was instantaneously changed in the model and was assumed to return to the basal value as soon as the stimulus disappeared.
Figure 6.2: Graphical representation of the InSiG controllers and the simulator environment.
6.2. Methodology

6.2.3 Simulation scenario

As previously introduced, the uni-hormonal sPD-InSiG controller was initially implemented to demonstrate whether there was a potential benefit for automatically adjusting the SI while injecting the insulin dose. For this controller, the tuning of the parameters was done with a standard 24-hour three meal-scenario of 40g, 80g, and 60g of CHO given at 8am, 1pm, and 9pm (breakfast, lunch and dinner time, respectively). Given the promising outcomes that were obtained, we created a more challenging scenario of 80g, 100g, and 90g of CHO given at 8am, 2pm, and 9pm for tuning the bi-hormonal BiAP-InSiG controller. In both cases, the scenario used for testing the controllers performance comprised similar CHO content, but different conditions of noise and intra-person variability.

6.2.4 Control tuning

The set of parameters $K_P$ and $K_D$ for each of the control variables $kp3$ and $V_{mx}$, and the percentage of the basal insulin that 1) maximized the percentage of time in range while 2) minimized the total insulin delivered for each cohort was identified using an adaptive grid search algorithm. The identified controllers were then tested in a different scenario (as previously described), and the resulting metrics of glucose variability amplitude and timing were analysed as a measure of the safeness and efficacy of the controllers. In particular, the following metrics were considered: mean blood glucose (BG) (mg/dl); percentage of time below range in hypoglycaemia ($BG < 70$ mg/dl); percentage of time within the 70–180 mg/dl of target range; percentage of time above range in hyperglycaemia ($BG > 180$ mg/dl); low blood glucose risk index (LBGI); high blood glucose risk index (HBGI); blood glucose risk index (BGRI); insulin delivered per day (ins/day) (U); glucagon delivered per day (mg); mean $kp3$ (mg/kg/min per pmol/L); mean $V_{mx}$ (mg/kg/min per pmol/L). Finally, control variability grid analysis (CVGA) plots were used to show the glucose variability (i.e. the effectiveness of the closed-loop controllers) at a population level [291, 296].

6.2.5 Statistical analysis

A comparison in the performance of the uni-hormonal sPD-InSiG and bi-hormonal BiAP-InSiG controllers with the traditional core controllers for hormonal delivery alone (sPD and bi-hormonal BiAP, respectively) was carried out for giving insight into the glucose control achieved with the proposed hybrid controllers. The statistical analysis of the differences between them was evaluated using a paired t-test implemented with Matlab (2017b, Matworks, Natick, MA). Differences were considered significant at a p<0.01.
6.3 Results

Uni-hormonal sPD-InSiG

The proposed controller significantly increased ($p < 0.01$) the percentage of time with glucose levels within target (70-180 mg/dL) to $98.5 \pm 2.5\%$ in adults (sPD: $92.9 \pm 7.1\%$) and to $91.7 \pm 5.8\%$ in adolescents (sPD: $83.7 \pm 11.2\%$). In addition, the total risk index significantly decreased ($p < 0.01$) to $1.24 \pm 0.35$ in adults (sPD: $2.25 \pm 1.66$) and to $2.13 \pm 1.05$ in adolescents (sPD: $4.1 \pm 3.2$). Figure 6.3 illustrates the smoother glucose control over time obtained on the average adult subject with the hybrid controller in comparison with the sPD one.

![Uni-hormonal sPD-InSiG vs sPD: comparison of glucose profiles. Average glucose (mean adult cohort) for sPD (upper panel) vs sPD-InSiG (lower panel). Meal is given at minute 5. Red line: plasma glucose (mg/dl), blue line: continuous glucose monitoring (mg/dl).](image)

Bi-hormonal BiAP-InSiG

Table 6.2 shows the set of parameters identified for each controller that resulted in the best glycaemic control regarding the two metrics previously mentioned (i.e. maximum percentage of time in range and minimum insulin delivered). A representative comparison between the bi-hormonal BiAP and the InSiG-kp3 controllers regarding the CGM profiles (mg/dl) and delivered insulin and glucagon is depicted in Figure 6.4. A detailed overview of the metrics related to the amplitude and timing of the glucose variability obtained with each controller for the adult and adolescent populations can be found in Tables 6.3 and 6.4.

Using a more challenging scenario did not hinder the glucose control obtained with the bi-hormonal BiAP-InSiG controller. In fact, the percentage of time with glucose levels within target (70-180 mg/dL) was found to be significantly increased up to $99.4 \pm 1.0\%$ in adults (using the bi-hormonal InSiG-Full controller (bi-hormonal BiAP: $90.38 \pm 5.33\%$) and, particularly remarkable, up to $96.4 \pm 4.6\%$ in adolescents using the bi-hormonal InSiG-kp3 controller.
Table 6.2: Identified parameters leading to the best glycaemic control with each controller.

<table>
<thead>
<tr>
<th></th>
<th>InSiG-kp3 Adults</th>
<th>InSiG-kp3 Adolescents</th>
<th>InSiG-Vmx Adults</th>
<th>InSiG-Vmx Adolescents</th>
<th>InSiG-Full Adults</th>
<th>InSiG-Full Adolescents</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{P_{kp3}}$</td>
<td>200</td>
<td>200</td>
<td>-</td>
<td>-</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>$k_{D_{kp3}}$</td>
<td>100</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>150</td>
<td>125</td>
</tr>
<tr>
<td>$k_{P_{Vmx}}$</td>
<td>-</td>
<td>-</td>
<td>200</td>
<td>300</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>$k_{D_{Vmx}}$</td>
<td>-</td>
<td>-</td>
<td>150</td>
<td>300</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Percentage of basal insulin delivery (%)</td>
<td>95</td>
<td>95</td>
<td>80</td>
<td>85</td>
<td>85</td>
<td>90</td>
</tr>
</tbody>
</table>

Figure 6.4: Bi-hormonal BiAP-InSiG vs bihormonal BiAP:. Comparison between bi-hormonal BiAP (left panel) and InSiG-kp3 (right panel) for the mean adult cohort. (a) CGM (mg/dl); (b) Insulin delivery from the pump (U/min); (c) Glucagon delivery from the pump boluses (mg/Kg/min); (d) value of $kp3$ (mg/min/kg per pmol/l).

(bi-hormonal BiAP: $77.22 \pm 12.14\%$) ($p < 0.01$), while not increasing hypoglycaemia nor hyperglycaemia.

In addition, the percentage of basal insulin that was needed to achieve good glycaemic control with the bi-hormonal BiAP-InSiG controller was found to be reduced up to 80% of the basal insulin amount that is normally required. This reduction resulted in a significant decrease in the total insulin delivered by the BiAP-InSiG in comparison with the bi-hormonal BiAP in both populations ($p < 0.01$). Interestingly, the glucagon dose per day was also significantly reduced ($p < 0.01$).

Glucose risk indexes were also found to be significantly lower in all the comparison pairs for the two populations ($p < 0.01$), except for the HBGI obtained with InSiG-Vmx in the adult cohort (differences were significant at $p < 0.05$). The total risk index was similarly found to be significantly reduced ($p < 0.01$).
The controllers performance at the population level is depicted in the CVGA plots (see Figure 6.5) showing 100% of the time in zones A and B for the controllers InSiG-Vmx and InSiG-Full for both cohorts. Controller InSiG-kp3 presented a 10% of zone Lower D in the adult population arisen from the short time spent in hypoglycaemia. In all the cases, the glucose variability achieved with the bi-hormonal BiAP-InSiG controllers is improved in comparison with the bi-hormonal BiAP.

<table>
<thead>
<tr>
<th>Table 6.3: Bi-hormonal BiAP-InSiG vs bihormonal BiAP: amplitude and timing metrics of the glucose variability for the adult population.</th>
</tr>
</thead>
<tbody>
<tr>
<td>InSiG-kp3</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>BG</td>
</tr>
<tr>
<td>% Time &lt;70</td>
</tr>
<tr>
<td>% Time in range</td>
</tr>
<tr>
<td>% Time &gt;180</td>
</tr>
<tr>
<td>LBGI</td>
</tr>
<tr>
<td>HBGI</td>
</tr>
<tr>
<td>RI</td>
</tr>
<tr>
<td>Insulin/day (U)</td>
</tr>
<tr>
<td>Glucagon/day (mg)</td>
</tr>
<tr>
<td>kp3 (*10^-2)</td>
</tr>
<tr>
<td>Vmx (*10^-2)</td>
</tr>
</tbody>
</table>

* statistical difference between each InSiG controller and the bi-hormonal BiAP (p<0.01). † statistical difference between each InSiG controller and the bi-hormonal BiAP (p<0.05).

<table>
<thead>
<tr>
<th>Table 6.4: Bi-hormonal BiAP-InSiG vs bihormonal BiAP: amplitude and timing metrics of the glucose variability for the adolescent population using all controllers.</th>
</tr>
</thead>
<tbody>
<tr>
<td>InSiG-kp3</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>BG</td>
</tr>
<tr>
<td>% Time &lt;70</td>
</tr>
<tr>
<td>% Time in range</td>
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<tr>
<td>LBGI</td>
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<tr>
<td>HBGI</td>
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<tr>
<td>RI</td>
</tr>
<tr>
<td>Insulin/day (U)</td>
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<td>Glucagon/day (mg)</td>
</tr>
<tr>
<td>kp3 (*10^-2)</td>
</tr>
<tr>
<td>Vmx (*10^-2)</td>
</tr>
</tbody>
</table>

* statistical difference between each InSiG controller and the bi-hormonal BiAP (p<0.01). † statistical difference between each InSiG controller and the bi-hormonal BiAP (p<0.05).
6.4 Discussion

The work presented in this chapter serves as the starting point for designing a system that automatically stimulates the neural pathways based on the values of SI-related parameters ($k_{p3}$ and $V_{mx}$) specified by the proposed InSiG controllers with the objective of keeping the SI of the people with T1DM within a controlled target range. This preliminary work strengthens future opportunities for applying bioelectronic medicine for diabetes management. The first proposed version of these hybrid controllers, the uni-hormonal InSiG-sPD, successfully evidenced an improved control of glycaemic fluctuations compared to the core module of insulin delivery (sPD) alone. Furthermore, the second family of InSiG controllers, the bi-hormonal BiAP-InSiG, showed an improved metabolic control in terms of efficacy and safety metrics during a meal in
comparison with the bi-hormonal BiAP, whose performance was considered as reference. As it is depicted in Figure 6.4, an increased SI during meals (represented by a rise in the value of $kp3$ and $Vmx$) resulted in a boosted effect of the meal boluses on keeping glucose levels within the target range. This finding, while preliminary, supports the idea that enhancing the action of the exogenous insulin may greatly improve control of glucose fluctuations in T1DM subjects.

In both populations, exclusive control over the insulin action on hepatic glucose production (i.e. parameter $kp3$) using the InSiG-kp3 controller was overall enough to achieve a safe and efficient glucose control, leading to similar results to those obtained with the InSiG-Full controller. This outcome indicates that modifying the insulin action on the liver, for example with a prompt inhibition of the hepatic glucose production, might have a more powerful effect in glucose homeostasis than modulating the glucose uptake by the peripheral tissues.

Increasing the sensitivity of tissues to insulin action also allowed a reduction in the basal insulin, which resulted in a significant decrease in the total insulin delivered. In addition, tighter control of glucose levels allowed also a reduction of the total glucagon delivery. Less insulin and glucagon doses will significantly reduce the costs associated with diabetes. For reference, in the United Kingdom the cost of insulin for each T1DM patient rises to £532 per year [301] and accounts for around 10% of the NHS expenditures associated with this disease [302]. Therefore, reducing the use of drugs in therapies, insulin in particular, is of significant priority especially in those countries where people with T1DM who are uninsured or underinsured are changing from insulin pumps (closed-loop systems) back to insulin pens (open-loop systems) to reduce their insulin-related bills [303].

It is interesting to note that adolescents generally have a decreased SI, which is correlated with an increase in the growth hormone secretion during puberty. The growth hormone acts antagonizing the insulin action on the peripheral tissues, therefore increasing the hepatic glucose production and decreasing the glucose uptake [304]. This puberty-induced defects in SI contribute, among other causes, to hinder diabetes management in adolescents [305]. Our results show that this challenge can be overcome by taking direct control over the SI of adolescents with T1DM. In fact, the glucose control achieved in the adolescent cohort using the InSiG controllers was found to be similar to the control achieved in the adult population. This finding gives support to the benefits of modifying SI as a complementary therapy to drug delivery in T1DM.

The generalizability of these results, although promising, is subject to certain limitations. The main restriction of this study is the lack of knowledge about the relationship between the magnitude of stimulation of the nervous system and the dynamics of change of insulin sensi-
6.5. CONCLUSION

As a result, to be able to translate this proof of concept into a real device, more experimental studies are needed to determine the delays between the stimulation and its effect on the insulin action on the tissues, and the duration and magnitude of these changes. It is also required to include the model of the dynamics of these changes into existing simulators of T1DM. Once again, the development of physiological models that more accurately reproduce the complex regulation of glucose homeostasis, like the one presented in the previous chapter, will allow existing simulators to provide improved environments for designing and testing controllers.

Finally, despite the employed simulator has been approved by the FDA, a larger subject data set and further \textit{in silico} validations for different scenarios are needed for a more accurate identification of the controller’s parameters. Notwithstanding these limitations, this study suggests a new approach for managing T1DM by controlling not only the insulin to be delivered but also its effect on the organs it targets and reveals that an enhanced glycaemic control is possible.

6.5 Conclusion

This chapter presents the design and \textit{in silico} validation of a new generation of controllers that specify insulin and glucagon doses and the optimal value of the sensitivity of tissues to the action of insulin, i.e. SI, of T1DM patients based on continuous glucose measurements. The simulations have shown that keeping the SI within the desired range using neuromodulation results in a reduction of the insulin and glucagon delivered per day and an improvement of the safety and efficacy of glucose control.

These results provide the first evidence for supporting the thesis hypothesis that bioelectronic medicine can be used for improving diabetes management. Other ideas of closed-loop neuromodulation-pharmaceutical systems that improve current therapeutic technology for people with diabetes are presented in Chapter 8. In this context, the development of new technology for non-invasive interface with the nervous system becomes one of the key catalysts to allow the progression of bioelectronic medicine for optimal glucose control. The next chapter presents another strategy to manage glycaemic control, which is based on decision support systems, that can also benefit from incorporating bioelectronic medicine technology and knowledge.
This chapter presents an additional contribution of the thesis which motivates the adoption of decision support systems (DSS) to acquire greater control of glucose fluctuations, with special focus on reducing undesired nocturnal hypoglycaemic events in patients using continuous glucose monitoring (CGM) systems. The development of semi-automatic systems to modulate insulin pump delivery are becoming a reality, but their elevated cost and performance below user expectations are hindering their adoption. Decision support systems, are therefore becoming an attractive alternative for people with T1DM on multiple daily injections or insulin pump therapy to avoid undesirable overnight blood glucose fluctuations (hyper- or hypoglycaemic).

The novel data-driven approach presented in this chapter incorporates easy-to-access metabolic data for computing the predictions, including CGM, meal intake and insulin boluses. Looking beyond employing purely metabolic information, these systems may benefit from incorporating new data sources, such as information exacted from neural recordings through the use of bioelectronic medicine. As a result, this work opens the doors to developing platforms where bioelectronic medicine can be incorporated into current pharmaceutical technology. The work described in this chapter has been previously published in IEEE Journal of Biomedical and Health Informatics (Güemes et al. 2019 [306]).


7.1 Decision Support Systems for glycaemic control in T1DM

Decision support systems (DSS) are software tools designed to help people make decisions on situations that may be unknown. Their use has notably increased over the last decades as it is being adopted in every field. In particular, DSS allow people with diabetes to improve blood glucose (BG) control in their daily routine. For instance, a typical DSS consists of alerts notifying the user of potential future adverse events, such as hypoglycaemia and hyperglycaemia [33]. These systems might also suggest the administration of meal or corrective insulin
boluses to mitigate hyperglycaemia [34, 69, 70], recommend the intake of CHO to tackle hypoglycaemia (rescue CHO) [71, 72, 73], or provide suggestions to prevent exercise-induced hypoglycaemia [74]. Another less studied application where T1DM management can benefit from DSS is for improving overnight glycaemic control [307]. There is a significant clinical evidence that overnight glycaemic control is affected by the behaviour of the person with diabetes during the day [308]. Therefore, a DSS that recommends actions to the patients, such as adjusting overnight basal insulin dosing, or whether rescue CHO should be taken before going to bed, could be a very useful tool.

A significant amount of research has been done for forecasting blood glucose levels within a short to mid-term horizon (15min-2hours) [309]. Some of this work has been translated to commercial products, such as the predictive low-glucose insulin suspension systems (Medtronic 640G and Tandem Basal-IQ). However, such a short prediction horizon might not always be sufficient to prevent overnight hypoglycaemia and do not prevent the user from waking up during the night in the case of a prediction alert being triggered.

Machine learning techniques are becoming increasingly popular to solve many T1DM management problems, such as glucose forecasting [310], optimal insulin dosing [34, 69], patient risk stratification [311], and CGM fault detection [63], as a result of their ability to represent complex non-linear input-output relationships, such as the glucose-insulin dynamics. They can also be used to classify the quality of glycaemic control. Preliminary work on this subject was proposed by Bertachi et al. (2018) looking at predicting nocturnal hypoglycaemia using artificial neural networks [312]. However, this work just focuses on hypoglycaemia prediction and evaluates only one classification algorithm.

The hypothesis presented in this work is that it is possible to predict the quality of overnight glycaemic control of a person with T1DM by analysing the features extracted from CGM and commonly user-reported data (CHO and insulin) from a predefined temporal window within the preceding day-time period (see Figure 7.1). In particular, three independent binary classification problems were defined to determine, at bedtime, the probabilities of three metrics associated with the quality of overnight glycaemic control: 1) the percentage of time spent in target range, 2) the presence of nocturnal hypoglycaemia, and 3) the presence of nocturnal hyperglycaemia. To do so, different commonly used machine learning models (binary classifiers) were evaluated on a clinical dataset and their performance was assessed in terms of their classification accuracy. Finally, a discussion of the best strategies and their possible integration in a DSS is presented at the end of the chapter.
Figure 7.1: Predicting the quality of overnight glycaemic control in people with type 1 diabetes by using binary classifiers on common metabolic data gathered during the day-time period (continuous glucose monitoring data, meal intake and insulin boluses). Methods: raw data was pre-processed to eliminate corrupted data, features in the temporal and frequency domains were extracted, and a number of popular established machine learning algorithms for binary classification were evaluated and compared on a publicly available clinical dataset (i.e. OhioT1DM).

7.2 Materials and methods

7.2.1 Data

The publicly-available OhioT1DM dataset was used in this study to evaluate the proposed approach [313]. This dataset contains 8 weeks of data corresponding to six participants with T1DM between 40 and 60 years old, all of them on insulin pump therapy and CGM. During the 8 weeks of study, participants used the Medtronic 530G insulin pump and the Medtronic Enlite CGM sensor (Medtronic Diabetes, Northridge, CA, US). Participants also reported life-event data, such as meal times and CHO intake, using a smartphone app. Finally, physiological data were collected from a Basis Peak fitness band (Intel, Santa Clara, CA, US). Among all the data available in this dataset, the following physiological data were used in this study: CGM blood glucose level sampled every five minutes; insulin doses, both bolus and basal; user self-reported meal times with carbohydrate estimates; time and amount of rescue CHO, and time of self-reported hypoglycaemic events.
7.2.2 Pre-processing of raw data

Despite the robust data collection methodology, the OhioT1DM dataset still contains some data corruption, mainly due to erroneous manual inputs and missing data. The following steps were followed to clean the raw data:

- Missing data from the CGM, which might happen due to sensor failure or data transmission problems, were addressed by discarding days with two consecutive hours of missing data. For shorter periods of time, missing data was interpolated using the spline method. Moreover, the first and last day for each patient were also discarded as they were generally incomplete.

- Outliers in the user self-reported CHO content estimation were identified and corrected (e.g. three times beyond the standard deviation). These outliers were replaced by the mean of the CHO intake over the 8 weeks of data collection.

To reduce the errors arising from using the discrete time series of CHO intake and insulin boluses, some data transformations based on physiological models were performed to convert them into continuous time series. CHO intake was converted to the glucose rate of appearance into the systemic circulation using the gastrointestinal absorption model developed by Hovorka et al. (2004) [266]. Moreover, plasma insulin concentration due to subcutaneous insulin infusion was estimated using a validated model of subcutaneous insulin absorption [266].

Finally, data was segmented as follows. The 8 weeks of continuous profiles were divided into individual days, each of them starting at 5am. This time was selected after visual inspection of the daily profiles, concluding that the majority of participants woke up between 5am to 6am. Then, these daily-profiles were further divided into day-time and night-time. Night-time was defined from 1am to 5am in order to exclude the impact of the postprandial excursion after dinner and breakfast times. The period defined as day-time was chosen by using windows of different lengths (1h, 3h, 5h, 8h, 12h and 18h) before 11pm (see Figure 7.2). The period from 11pm to 1am was not considered for feature extraction as it coincides with the postprandial excursion after dinner.

7.2.3 Feature extraction and labelling

A set of 19 features was extracted from each day-time and night-time periods (see Table 7.1). This set of input features includes: i) 8 indices to describe glucose variability in the time domain; ii) 8 indices to describe glucose variability in the frequency domain (Fast Fourier Transform, FFT), which comprise the 4 highest amplitudes and their corresponding frequencies (discarding the DC component); and iii) features related to meal intake, insulin dosage
7.2. MATERIALS AND METHODS

Figure 7.2: Segmentation of daily profiles into day and night periods. The blue boxes represent the temporal windows used to extract the day-time features (e.g., 1h, 8h or 18h windows). The green boxes represent the temporal windows used to extract the night-time features. Note that night-time features are used to compute the category label for three independent binary classification problems: night-time within glucose range, with hypoglycaemia and with hyperglycaemia.

and presence of self-reported hypoglycaemic events. Note that features extracted from the frequency domain of the CGM signal contain valuable information that contributes to acquiring a better characterization of the signal [314]. A sensitivity analysis was carried out in a preliminary study to assess the impact of the features extracted from the FFT on the overnight glycaemic control quality, and the indices that demonstrated the greatest impact were incorporated into the features set. As a result, the complete set of input features was deemed to provide enough information to evaluate the risk of suffering severe glucose fluctuations, i.e. hypoglycaemia or hyperglycaemia, during day and night times. Note that other features such as exercise, stress, and illness might also significantly affect glycaemic control. In this work, however, it is hypothesised that it is possible to predict the quality of overnight glycaemic control by only using CGM, meal intake, and insulin data.

The complete set of 19 features extracted from the day-time period was used as input to the classification problem. Then, some of the features extracted from the night-time period were used to define the binary labels for each problem. In the first classification problem (Night_in), nights with more than 80% time in the glucose target range [70 – 180] mg/dl (given by time_in) with no nocturnal hypoglycaemic events detected or reported (given by hypo_correct), were labelled as On-target. Otherwise, nights were labelled as Off-target. In the second classification problem (Hypo_night), nights were labelled as Hypo when a hypoglycaemic event was detected or reported by the user. Hypoglycaemic events were considered when there was a self-reported hypoglycaemic event or when CGM measurements were below 70 mg/dL for at least 10 min, or below 55 mg/dL for at least 30 min, as defined by Maahs et al. (2016) [315]. Otherwise, nights were labelled as Non-hypo. In the third classification problem (Hyper_night), nights were labelled as Hyper when the percentage of time above 180 mg/dL (given by time_above) during the night-time period was more than 30%. Otherwise, nights were labelled as Non-hyper. A description of the features and thresholds used for each type of label is reported in Table 7.2.
Table 7.1: Calculated features from the CGM, meal intake and insulin dosage data

<table>
<thead>
<tr>
<th>Category</th>
<th>Feature</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temporal domain</td>
<td>Mean CGM</td>
<td>mean_cgm</td>
</tr>
<tr>
<td></td>
<td>Standard deviation CGM</td>
<td>std_cgm</td>
</tr>
<tr>
<td></td>
<td>Risk index</td>
<td>ri</td>
</tr>
<tr>
<td></td>
<td>Low blood glucose index</td>
<td>lbgi</td>
</tr>
<tr>
<td></td>
<td>High blood glucose index</td>
<td>hbgi</td>
</tr>
<tr>
<td></td>
<td>Percentage of time in target (70-180mg/dL)</td>
<td>time_in</td>
</tr>
<tr>
<td></td>
<td>Percentage of time below target</td>
<td>time_under</td>
</tr>
<tr>
<td></td>
<td>Percentage of time above target</td>
<td>time_above</td>
</tr>
<tr>
<td>Frequency domain</td>
<td>Four largest Fourier coefficients</td>
<td>$P_1, P_2, P_3, P_4$</td>
</tr>
<tr>
<td></td>
<td>Frequencies corresponding to largest coefficients</td>
<td>$f_1, f_2, f_3, f_4$</td>
</tr>
<tr>
<td>Insulin dosage</td>
<td>Mean plasma insulin concentration</td>
<td>ins</td>
</tr>
<tr>
<td>Meal intake</td>
<td>Mean CHO rate of rate of glucose appearance (Ra)</td>
<td></td>
</tr>
<tr>
<td>Hypoglycaemic events</td>
<td>Total amount of rescue CHO</td>
<td>hypo_correc</td>
</tr>
</tbody>
</table>

Table 7.2: Definition of the three classification problems for predicting overnight glycaemic control quality

<table>
<thead>
<tr>
<th>Classification problem</th>
<th>Abbreviation</th>
<th>Binary labels and definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Night in target</td>
<td>$Night_{in}$</td>
<td>On-target: $time_{in} &gt; 80% &amp; hypo_{correc} = 0 &amp; no reported hypo event</td>
</tr>
<tr>
<td>Nocturnal hypoglycaemia</td>
<td>$Hypo_{night}$</td>
<td>Hypo: hypo_{correc} = 0 &amp; no reported hypo event</td>
</tr>
<tr>
<td>Nocturnal hyperglycaemia</td>
<td>$Hyper_{night}$</td>
<td>Hyper: $time_{above} &lt; 30%$</td>
</tr>
</tbody>
</table>
7.2. MATERIALS AND METHODS

In summary, the feature extraction and labelling steps produced, for each classification problem, a set of observations consisting of 19 features extracted from the day-time period and three binary labels determined from the following night-time period. Figure 7.4 depicts the three previously described binary classification problems.

Figure 7.3: Description of the features vector and labels: metadata (green), feature vector (orange) and labels (red).

7.2.4 Selected binary classification methods

A binary classifier is a type of supervised learning algorithm that is used to classify the elements of a given set into two groups on the basis of a classification rule [316]. In this study, three binary classifiers were used to classify the quality of overnight glycaemic control into: On-target/Off-target nights, Hypo/Non-hypo nights, and Hyper/Non-hyper nights. In particular, the following commonly used binary classifiers were selected for comparison purposes: random forest classifier (RFC) [317], artificial neural networks (ANN) [318], support vector machine (SVM) [319], linear logistic regression (LLR) [320], and extended tree classifier (ETC) [317]. Unlike other machine learning techniques (e.g. deep learning), the selected techniques are well-suited to deal with relatively small datasets, such as the OhioT1DM dataset.

7.2.5 Pre-processing of the feature vectors

Some pre-processing techniques were applied to the feature vectors before building the models to address some issues that might arise from working with clinical datasets:

- Class imbalance: to handle class imbalance the synthetic minority over-sampling (SMOTE) technique was used, which performs over-sampling of the minority class to equalize the number of samples in all the classes and acquire better performance [321].

- Data scaling: it is a common pre-processing step for many classifiers that can affect the classifiers performance. Data standardization was performed on the input feature vectors. The advantage of using standard scaling is to centralize the data distribution so that it becomes of zero mean and unit variance.
### 7.2.6 Evaluating performance for model selection

The work-flow proposed by Hernandez et al. (2017) was used to build and evaluate the models (see Figure 7.4) [322]. In this process, the data was initially divided into cross-validation set (CVS) and hold-out set (HOS), with the latter dataset comprising the 25% of the observations. It is worth noting that data-sampling was performed exclusively within the CVS training dataset. Otherwise, if applied before it would negatively affect the results by leading to overfitting and/or generation of artificial observations that would be used for testing. In this study, 10-fold stratified cross-validation was used to assess the model’s performance on an independent dataset. Models were tested on both balanced and imbalanced versions of the testing fold to reduce the risk of over-fitting. Finally, to assess the potential of translation of the results into a clinical decision support system, the models were validated on the HOS, which contains observations that were completely unseen during the model training. The overall performance score for each model was obtained by averaging the results obtained on the HOS across the 10 folds.

**Figure 7.4: Diagram of the work-flow followed in the study.** First, raw-data pre-processing was performed, including removal of outliers, division in daily profiles and feature extraction. The observations are then split into 10-fold CVS and HOS. Sampling and scaling were carried out after this division. The performance of the models was evaluated using completely unseen observations from HOS. T: equation to scale new observations; M: built models.

### 7.2.7 Evaluation metrics

A set of widely-accepted scores to evaluate the performance of binary classifiers was used. In particular, to describe and compare the model’s performance, the following metrics were employed: the area under the receiver operating characteristic curve \( (AUC_{ROC}) \), that quantifies
class separability (unitless); sensitivity (SENS), that measures the proportion of true positives that are correctly classified as such (unitless); specificity (SPEC), that measures the proportion of true negatives that are correctly classified as such (unitless); and their GMEAN (unitless). It is worth highlighting that these metrics are not affected by class imbalance. In particular, the $AUC_{ROC}$ enables evaluation of the classifier’s performance along its whole operating range, therefore providing greater insight into the classifier’s general performance. For each window, the model with the highest combined performance score (CS) (unitless), as defined in Equation 7.1, was selected as the best model.

$$CS = \sqrt{AUC_{ROC}^2 + GMEAN^2}$$ (7.1)

Finally, the number of true positives (TP), false positives (FP), true negatives (TN) and false negatives (FN), were also considered for the selection of the best models.

7.2.8 Statistical Analysis

The statistical significance of the differences between the classifiers scores was determined using the non-parametric test Kruskal-Wallis one-way ANOVA on ranks to account for the heterogeneity of variance. Post-hoc analysis using Tukey’s test was performed to determine pairwise differences. Significance level was set at $p<0.05$.

7.2.9 Software

Python was used in this study. The implementation of the models and their evaluation was done using the models and performance scores from the library Scikit-learn [323]. Moreover, the sampling techniques were used from the library Imbalanced-learn [324], data handling was done with Pandas [245] and data visualization using Matplotlib and Seaborn [246]. Finally, the statistical analysis was performed using Statsmodels [247].

7.3 Results

7.3.1 Data insight

The number of observations (i.e. daily profiles) before and after the raw-data pre-processing (i.e., after profiles with too many missing data were discarded) for each of the subjects in the dataset (#559, #563, #570, #575 #588, #591) and for the different time windows lengths defining the day-time (1h, 3h, 5h, 8h, 12h, 18h) is shown in Table 7.3. The distribution of binary labels for each day-time window and classification problem is depicted in Figure 7.5.
CHAPTER 7. PREDICTING QUALITY OF OVERNIGHT GLYCAEMIC CONTROL IN TYPE 1 DIABETES USING BINARY CLASSIFIERS

Table 7.3: Number of observations (daily profiles) for each subject and window length defining the day-time period

<table>
<thead>
<tr>
<th>Subject</th>
<th>#559</th>
<th>#563</th>
<th>#570</th>
<th>#575</th>
<th>#588</th>
<th>#591</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw-data</td>
<td>52</td>
<td>56</td>
<td>51</td>
<td>56</td>
<td>56</td>
<td>55</td>
<td>326</td>
</tr>
<tr>
<td>1h</td>
<td>45</td>
<td>49</td>
<td>44</td>
<td>46</td>
<td>52</td>
<td>45</td>
<td>281</td>
</tr>
<tr>
<td>3h</td>
<td>43</td>
<td>48</td>
<td>42</td>
<td>46</td>
<td>52</td>
<td>44</td>
<td>275</td>
</tr>
<tr>
<td>5h</td>
<td>42</td>
<td>46</td>
<td>41</td>
<td>45</td>
<td>52</td>
<td>44</td>
<td>270</td>
</tr>
<tr>
<td>8h</td>
<td>41</td>
<td>45</td>
<td>39</td>
<td>45</td>
<td>51</td>
<td>43</td>
<td>264</td>
</tr>
<tr>
<td>12h</td>
<td>39</td>
<td>43</td>
<td>39</td>
<td>42</td>
<td>49</td>
<td>39</td>
<td>251</td>
</tr>
<tr>
<td>18h</td>
<td>32</td>
<td>39</td>
<td>33</td>
<td>36</td>
<td>43</td>
<td>37</td>
<td>220</td>
</tr>
</tbody>
</table>

Figure 7.5: Overview of the class label distribution within each temporal window for each classification problem.

7.3.2 Prediction of quality of overnight glycaemic control

Table 7.4 presents the model with the best performance based on the CS of $AUC_{ROC}$ and GMEAN (Equation 7.1) for each classification problem ($Night_{in}$, $Hypo_{night}$, $Hyper_{night}$) and temporal window. In addition, Figure 7.6 depicts the scores ($AUC_{ROC}$, GMEAN, SPEC and SENS associated with these classifiers for the different windows lengths and problem. As shown in Figure 7.6, the best $AUC_{ROC}$ score for the first two classification problems (glucose in target – $Night_{in}$, and presence of nocturnal hypoglycaemia – $Hypo_{night}$) was achieved with a window of 18h using the ETC and SVM classifiers respectively. These classifiers also presented the highest GMEAN values (around 0.65 in both cases), but the results were not significantly different from the performance in other windows. Regarding the classification of night in target ($Night_{in}$), the specificity and sensitivity were moderate (between 0.5 and 0.65). On the other hand, for the classification of nocturnal hypoglycaemia ($Hypo_{night}$), the specificity is greater than the sensitivity for every window length. Focusing on the results of the third classification problem for predicting nocturnal hyperglycaemia ($Hyper_{night}$), the best model was found to be the RFC classifier in the window of 8h. While the sensitivity was lower for all the window lengths (0.5 to 0.6), the $AUC_{ROC}$ (0.73) and the specificity (0.8) obtained with this classifier were significantly higher than those obtained by the other classifiers.
Table 7.4: Overview of the best classifiers for each temporal window and classification problem.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Window</th>
<th>Best model</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Night _ in</td>
<td>1h</td>
<td>RFC</td>
<td>Minimum number of samples per leaf: 1; Number of trees: 50</td>
</tr>
<tr>
<td></td>
<td>3h</td>
<td>RFC</td>
<td>Minimum number of samples per leaf: 10; Number of trees: 20</td>
</tr>
<tr>
<td></td>
<td>5h</td>
<td>ANN</td>
<td>Activation: logistic sigmoid function; $\alpha=0.0001$; Learning rate: 0.001; Solver: stochastic gradient-based optimizer</td>
</tr>
<tr>
<td></td>
<td>8h</td>
<td>ETC</td>
<td>Minimum number of samples per leaf: 50; Number of trees: 50</td>
</tr>
<tr>
<td></td>
<td>12h</td>
<td>ETC</td>
<td>Minimum number of samples per leaf: 5; Number of trees: 50</td>
</tr>
<tr>
<td></td>
<td>18h</td>
<td>ETC</td>
<td>Minimum number of samples per leaf: 50; Number of trees: 50</td>
</tr>
<tr>
<td></td>
<td>1h</td>
<td>ANN</td>
<td>Activation: logistic sigmoid function; $\alpha=0.1$; Learning rate: 0.001; Hidden layer sizes: (10, 10); Solver: stochastic gradient-based optimizer</td>
</tr>
<tr>
<td>Hypo _ night</td>
<td>3h</td>
<td>ANN</td>
<td>Activation: logistic sigmoid function $\alpha=0.1$; Learning rate: 0.001; Hidden layer sizes: (5,0); Solver: stochastic gradient-based optimizer</td>
</tr>
<tr>
<td></td>
<td>5h</td>
<td>SVM</td>
<td>Decision function shape: one vs rest; Kernel coefficient: 0.01; Kernel: Radial-basis function</td>
</tr>
<tr>
<td></td>
<td>8h</td>
<td>RFC</td>
<td>Minimum number of samples per leaf: 5; Number of trees: 10</td>
</tr>
<tr>
<td></td>
<td>12h</td>
<td>LLR</td>
<td>Optimization algorithm: Newton-CG</td>
</tr>
<tr>
<td></td>
<td>18h</td>
<td>SVM</td>
<td>Decision function shape: one vs rest; Kernel coefficient: 0.01; Kernel: Radial-basis function</td>
</tr>
<tr>
<td>Hyper _ night</td>
<td>1h</td>
<td>ETC</td>
<td>Minimum number of samples per leaf: 5; Number of trees: 10</td>
</tr>
<tr>
<td></td>
<td>3h</td>
<td>ETC</td>
<td>Minimum number of samples per leaf: 50; Number of trees: 10</td>
</tr>
<tr>
<td></td>
<td>5h</td>
<td>ETC</td>
<td>Minimum number of samples per leaf: 5; Number of trees: 50</td>
</tr>
<tr>
<td></td>
<td>8h</td>
<td>RFC</td>
<td>Minimum number of samples per leaf: 10; Number of trees: 50</td>
</tr>
<tr>
<td></td>
<td>12h</td>
<td>ETC</td>
<td>Minimum number of samples per leaf: 50; Number of trees: 100</td>
</tr>
<tr>
<td></td>
<td>18h</td>
<td>ETC</td>
<td>Minimum number of samples per leaf: 50; Number of trees: 100</td>
</tr>
</tbody>
</table>
7.4 Discussion

The pre-processing of the data used for training and evaluating the binary classifiers, resulted in a considerable reduction of the number of observations when compared to the original dataset. This is an indicator of the significant number of missing CGM data points. There was also a decrease in the number of observations with the increase in the day-time window length. This outcome was expected, because the longer the considered time interval, the higher the probability of having two-hour CGM gaps. Moreover, using splines to interpolate missing CGM data could have introduced variability affecting the classification performance. Future work could take advantage of more advanced interpolation techniques, such as the use of T1DM physiological models to account for the glucose dynamics. It is interesting to note that in the first classification problem (Night_in), there was a balanced distribution of the labels (i.e. normoglycaemia vs. abnormal glycaemia) along all the windows (see Figure 7.5). However, there was some imbalance in the distributions in the second classification problem based on presence of a nocturnal hyperglycaemic event (around 40% of nights were hyperglycaemic).
This imbalance was more remarkable when classifying based on the presence of hypoglycaemic nights (only around 10% of nights had hypoglycaemic events).

The impact of the window length in each classification problem was extensively studied. As depicted in Figure 7.6, better results were obtained with larger windows, which might be explained by the fact that they have more information from the events that occurred throughout the day. However, note that the larger the window, the smaller the dataset used for training and evaluating the models. Hence, the impact of the size of the dataset on the results is something that should be further evaluated in future studies.

In terms of model selection, as reported in Table 7.4, the best model for each window length greatly varied for each classification problem ($Night_{in}$, $Hypo_{night}$, $Hyper_{night}$). As an example, for window lengths of 1h and 3h the best models were RFC, ANN and ETC for each problem respectively. Moreover, within each classification problem there was also a great variation on the best model across window lengths, except when classifying based on nocturnal hyperglycaemia. In this case, the best model for almost all windows, excluding the one of 8h, was found to be an ETC with different parameters’ configuration. For the 8h window the best model was an RFC classifier, presenting significantly higher $AUC_{ROC}$ and specificity in comparison with the best models on other windows.

Interestingly, for the classification problems based on the percentage of time in target and the presence of nocturnal hypoglycaemia, the highest $AUC_{ROC}$ and GMEAN scores were achieved in the window of 18h using an ETC and SVM classifiers, respectively. In these classification problems, the sensitivity achieved with the best model for each window ranged from 0.3 up to almost 0.7, suggesting a great dependence in the dataset used. Finally, the obtained specificity was overall higher, especially for classification based on nocturnal hypo and hyperglycaemia, where values up to 0.8 were obtained.

Despite the promising results suggesting that different aspects of the quality of overnight glycaemia can be predicted, there are some limitations that need to be addressed before translating this methodology into real practice. The main drawback is associated with the scarce amount of available data, which greatly restricts the training and validation of the classifiers. As an example, for the first classification problem ($Night_{in}$), in the testing set for the window of 18 hours there were only 3 observations with the hypoglycaemia label from a total of 58 observations. It is very difficult for the algorithms to infer patterns for further classification from such a small dataset. Having a larger dataset will also enable the evaluation of more advanced classification algorithms suited for processing sequences of data, such as long short-term mem-
ory networks (LSTM). Other methods that could be investigated are the development of multi-
class and assembled classifiers, which could potentially improve the results.

Another way to improve the results is the inclusion of additional features. For example,
incorporating information about changes in the basal insulin profile could help improving
the classification of overnight glycaemic control. Moreover, non-stationary frequency analy-
sis methods, such as wavelets transform, which provide temporal-specific glycaemic features,
could also potentially improve the classification results. Such potential improvement will be
investigated in future work. The OhioT1DM dataset includes other physiological data, such as
exercise and stress, which could also be included to improve the prediction of overnight gly-
caemic control. However, initial studies [325], indicate that the inclusion of these parameters
do not yield better results. Finally, if reliably measured, the quality of sleep over the preceding
ights could also be accounted as a predictor for the glucose control of the current night.

In terms of practical implementation of the proposed technique in a real-life setting, this
could be embedded within a mobile app, or within a sensor-augmented insulin pump, that
interfaces to a CGM device. Then, by indicating the bedtime to the app, the user would get
back information about the probability of having a night within target, having hypoglycaemia,
or having hyperglycaemia. In addition, based on these probabilities, a simple heuristic rule
could be implemented to recommend the required actions to the user in order to have a smooth
night (e.g., reducing the basal insulin by 30%).

7.5 Conclusion

This chapter has introduced a novel data-driven approach to predict the quality of overnight
glycaemic control in people with T1DM by analysing common metabolic data gathered dur-
ing the day-time period (CGM, meal intake and insulin boluses). The proposed approach is
able to predict whether overnight glucose concentrations are going to remain within or out-
side the target range, and therefore allows the user to take appropriate preventive actions (e.g.
snack or change in basal insulin). These results encourage advanced research in algorithms
and incorporation of new data sources for predicting overnight glycaemia to avoid risky situa-
tions and improve T1DM management. Examples of additional data are heart rate and sweat,
which can be obtained from external wearable sensors [59, 61, 326, 327]. Moreover, given the
amount of relevant metabolic information contained in neural signals, bioelectronic medicine
offers a plethora of opportunities to record, decode and use them to upgrade the glycaemic con-
trol. This enables the development of new diabetes technology that incorporates bioelectronic
medicine to overcome the challenges presented by current pharmaceutical technology.
CONCLUSIONS AND FUTURE PERSPECTIVES

Modulation of the nervous system by delivering electrical or pharmaceutical agents has contributed to the development of novel treatments to serious health chronic disorders. Recent advances in multidisciplinary research have enabled the emergence of a new powerful therapeutic approach called bioelectronic medicine. As previously introduced, bioelectronic medicine exploits the fact that every organ in our bodies is neurally innervated and thus electrical interfacing with peripheral nerves can be a potential pathway for diagnosing or treating diseases such as diabetes. In this context, a plethora of studies have confirmed the important role of the nervous system in maintaining a tight regulation of glucose homeostasis. These have motivated new research exploring the opportunities of bioelectronic medicine for improving glucose control in people with diabetes, including regulation of gastric emptying, insulin sensitivity (SI), and secretion of pancreatic hormones. The work presented in this thesis supports the hypothesis that "the incorporation of bioelectronic technology into current pharmaceutical-based diabetes closed-loop systems can outperform conventional approaches and enhance the safety and efficiency of glucose control".

This work comprises both scientific-based and application-based studies that are closely related to each other as conceptualised in Figure 8.1. To begin with, scientific-based studies were implemented in vivo and in silico to obtain new knowledge of the mechanisms of the neuro-metabolic interaction in the context of glucose control, and identify missing understanding in the field. Then, an application-based strategy was followed to assess the benefits of incor-
porating bioelectronic medicine to traditional diabetes technology in clinical scenarios. This chapter summarizes the novel contributions of this thesis, outlines future lines for research, and exposes the most important challenges to be addressed to translate these strategies into real closed-loop therapies. Some of the ideas presented in this chapter have been published in the Journal of Bioelectronic Medicine (Güemes et al., 2020) [26].

8.1 Contributions

- Chapter 2 provided a review of the past and present evolution of technology in diabetes from a metabolic and pharmaceutical approach. The limitations of this therapeutic approach were then foregrounded to motivate the introduction of bioelectronic medicine through the combination of neuromodulation and pharmacological therapeutic strategies to overcome these challenges and further improve glycaemic control. The most relevant bioelectronic technology, from computational modelling to neurostimulation systems, was then presented. Finally, the chapter provided an overview of current applications of invasive and non-invasive stimulation of the vagus nerve and other neuromodulation strategies for treating chronic diseases, with the special case of diabetes.

- Chapter 3 introduced an overview of the peripheral nervous pathways innervating the pancreas, with especial interest in the gross and microscopical anatomy of the vagus nerve. In this context, the chapter continued with a review of the strategies used to study the impact of the neural innervation on the secretion of pancreatic hormones and the hemodynamic and respiratory responses, such as electrical stimulation of the vagus nerve or the use of neural blockers.

- Chapter 4 contributes to current experimental research on the impact of vagus nerve stimulation (VNS) parameters on glucose metabolism and pancreatic endogenous secretion. To begin with, the chapter presented a review of good practice considerations for designing a reliable and successful experimental protocol reported in previous studies. Motivated and justified by this experimental review, the chapter presented two studies arising from implementing two experimental setups using healthy rat models aiming to investigate the differential impact of VNS frequency on the metabolic and hemodynamic responses: stimulation in the intact cervical vagus nerve for afferent and efferent activation, and in the distal end of the ligated nerve for efferent stimulation. By comparing these two scenarios, it was also possible to analyse and discuss the influence of afferent and efferent fibres.
8.2. FUTURE OPPORTUNITIES

- Chapter 5 introduced the major contribution of this thesis, a novel unified neural-metabolic model that describes the physiological metabolic events following VNS at the cervical level. The system incorporates models of the nerve activation as a function of different stimulation parameters, ACh secretion from nerve terminals, endogenous glucose production, glucose utilization, and insulin and glucagon secretion and kinetics. The value of this model arises from being the first attempt to mathematically characterize this specific neuro-metabolic system, showing the current understanding and identifying the missing knowledge that requires further research. When fully developed, it will provide a virtual platform for studying and testing new diabetes technology that incorporates neuromodulation as an additional or alternative functional component.

- Chapter 6 advanced upon the previous chapters focused on fundamental research towards clinical implementation of a neuromodulation-pharmaceutical closed-loop system. In particular, it introduced the proof of concept for a new hybrid closed-loop glucose controller that regulate not only the insulin and glucagon doses delivered through a pump but also the SI of the virtual patient through electrical stimulation of the nervous system. This study assessed for the first time, in silico, the clinical opportunities of applying bioelectronic medicine in a person with T1DM.

- Finally, Chapter 7 presented a novel data-driven approach to predict the quality of overnight glycaemic control in people with T1DM based on the application of binary classifiers on commonly gathered data during the day-time period, including continuous glucose monitoring data, meal intake and insulin boluses. Incorporating this methodology into current decision support systems would boost self-management control in diabetes. Despite not directly related to the other chapters in this thesis, Chapter 7 motivated another example where diabetes technology, in this case decision support systems, could be improved by taking into consideration features extracted from recorded neural signals to/from the brain.

8.2 Future opportunities

This section presents an overview of the most remarkable opportunities for applying closed-loop control by incorporating bioelectronic medicine as an alternative or an upgrade to traditional therapeutic approaches for people with diabetes.

From a purely bioelectronic control, neuromodulation can be used to target key metabolic organs, such as the liver or the pancreas, to regulate their function as desired. For example, modulating the neural signals to the liver would allow reducing the risk of hypoglycaemic
events by enhancing endogenous glucose production, which would rapidly increase glucose levels. This would be of special importance for people with T1DM, whose continuous risk of hypoglycaemia is recognized as one of the principal impediments to optimal glycaemic control [59]. Moreover, modulating the neural pathways to the pancreas can impact the secretion of insulin and glucagon in patients with T2DM, early stages of T1DM, where there is still a considerable number of \( \beta \)-cells intact, and even in progressed stages of the disease, as healthy \( \beta \)-cells have been observed in the islets of the patients decades after disease onset [328]. Therefore, it would be possible to neuromodulate the remaining healthy \( \beta \)-cells through electrical stimulation to promote or inhibit insulin release on demand [50]. This can be particularly desirable in scenarios where glucose control is challenging, such as during excessive meals - which may result in hyperglycaemia - and during exercise or excessive stress - leading to hypoglycaemia. Following this approach, hypoglycaemia could be restricted through a prompt inhibition of insulin secretion and promotion of counterregulatory hormones secretion, such as glucagon, before starting exercise.

Neuromodulation of the pancreatic secretion could be further applied in patients with stem cell-derived \( \beta \)-cell transplants. This strategy has improved the glycaemic control in pre-clinical trials, but many challenges are yet to be resolved including the restoration of vascularization and neural innervation. Successful advances are being made in the former, but full restoration of the functionality of the transplanted islets is still far from optimal. To address this issue, Seicol et al. (2019) propose the development of biocircuit-augmented islet transplants containing mature, vascularized and neural innervated \( \beta \)-cells, where neurons will grow inside hydrogel-based micro tissue engineered neural network (micro-TENN) scaffolds [329]. I propose bringing this idea one step further and using closed-loop neuromodulation of neural bio-circuits to restore the physiologic response to neural signalling that is present in healthy individuals [29].

The above-mentioned strategies present bright alternative opportunities for improving glycemic control. I believe, however, that their combination with conventional pharmaceutical technologies promises to transform the current therapeutical scenario in diabetes, as conceptualised in Figure 8.2. One technology that is regarded as cutting-edge in glucose management for diabetes is the artificial pancreas [58, 78, 330, 331, 332]. As a reminder, an artificial pancreas comprises a sensor for continuous glucose monitoring, an insulin pump and an algorithm that replicates the endocrine function of a healthy pancreas and calculates the optimal dose to be delivered based on the real-time glucose readings from the sensor [49, 333]. This pharmaceutical approach faces many limitations such as delays in external insulin action [48] after
meal intake and the non-physiological insulin absorption into the systemic circulation, which are associated with risks of hyper and hypoglycemia. Incorporating the bioelectronic control will enable improved pre-emptive computation of the insulin doses, increased autonomy, and personalized glucose control.

Figure 8.2: Integration of closed-loop neuromodulation systems in artificial pancreas. Metabolic biomarkers and neurophysiological recordings from a variety of peripheral nerves will be recorded and analysed in real-time to accordingly adjust the insulin delivery through an insulin pump and the characteristics of the electrical stimulation delivered to the peripheral nerves to modulate their function.

One such novel idea of a hybrid neuromodulation-pharmaceutical closed-loop system has been motivated and presented in Chapter 6, where the control of SI through neuromodulation was incorporated into an artificial pancreas to improve glycaemic outcomes. This type of hybrid controllers could become a relevant strategy for improved glycaemic control for both types of diabetes. T2DM is characterized by a reduction of SI, therefore neuromodulation of SI emerges as a logical approach for reversing the disease. Patients with T1DM, that require the external injection of the insulin to lower glucose levels can also benefit from neuromodulation of the SI to increase the effect of this exogenous insulin in lowering blood glucose [286].

Moreover, the detection of the neural signals that trigger the cephalic phase of hormonal secretion (CPHS) from decoding neurograms would allow a fast identification of the meal onset, which could then be used to automatically activate insulin controllers. In short, the CPHS refers to the pre-absorptive secretion of insulin and glucagon triggered by neural signals rather than to changes in plasma glucose concentrations. This strategy expects to increase the user autonomy and reduce the delays for responding to meal intake. Extending this idea, it would be interesting to assess whether the cephalic phase is dependent on the type/amount of meal, and which are the most appropriate biomarkers to characterize this relation. If dependent, this information could also be extracted from neural recordings and be used to optimize injection dosing.
Furthering this area, metabolic information from neural recordings and other monitored biomarkers can be integrated into different patient-specific control strategies. From an open-loop perspective, relevant features extracted from neural signals can be included in decision support systems (see Chapter 7), to enhance the accuracy of the predictions. This would allow users to make better decisions to respond and even avoid their exposure to risky glycaemic events. On the other hand, integration of nerve recordings into closed-loop systems can be used to compute the optimal output of the controller, which can be the insulin dose, as previously presented, the characteristics of the neural stimulation parameters to selectively drive the organs functionality to restore metabolic homeostasis, ideally a combination of both.

A different approach for a hybrid electrical-pharmaceutical system has been recently proposed, which involves direct control of gene expression using electrical signals. This bioelectronic device, designed by Krawczyk et al. (2020), contains a microchip that activates a capsule with engineered human $\beta$-cells that respond to membrane depolarization by releasing insulin from intracellular storage vesicles [334]. In more detail, an external signal temporarily depolarizes the cell membrane causing calcium ions to flow into the cell, and potassium ions flow out. The temporary depolarization activates the insulin-producing gene and the release of insulin. The ultimate goal would be to implant this electro-triggered vesicular release system subcutaneously and control its activation using a phone app. I believe this innovative system could become completely autonomous by using meal information extracted from neural recordings to automatically trigger the electro-genetic cellular insulin release system.

Finally, the development of detailed mathematical models of the complex regulation of the healthy endocrine pancreas that incorporate the neural-metabolic interaction, like the one presented in Chapter 5, help to bridge the gap between biology and electronics. Moreover, development of simulators of diabetes mellitus incorporating the neural contribution will enable improved environments to optimise systems, monitor interventions, and provide safety and efficiency testing without the need for initial validation on animals [282, 283].

8.3 Technological challenges

The previous section has presented ideas for incorporating bioelectronic technology to pharmaceutical technology, such as the artificial pancreas. This roadmap, although innovative and promising, faces some important technological challenges that must be addressed to translate these strategies into real closed-loop therapeutic approaches.

Selective activation of the specific neural fibres that target the biological processes of interest is a major requirement to minimize the risk of undesired activation of other tissues, organs,
and processes. This relies on having a clear picture of the anatomy of the peripheral nervous system and the role of the individual fibres in healthy and disease conditions. Investigating the functional anatomy of the vagus nerve entails the development of novel stimulation strategies and devices to access and modulate the activity of individual fibres \[159, 208, 209\]. The fibre-to-organ mapping for studying sensorimotor signals is generally done by asking patients to describe sensations or to move specific parts of the body or prosthetic devices. This strategy, however, is not possible for mapping the neural innervation to visceral organs. In this context, some techniques are currently foregrounded due to their success in localizing individual fibres, such as anterograde or retrograde tracing \[335\], electrophysiology with multielectrode arrays, computerised tracing with microCT, and fast and non-invasive neural electrical impedance tomography (EIT) \[159\]. In the clinical scenario, successful stimulation of the processes of interest will additionally require the real-time and continuous monitoring of biological markers, such as the hormonal secretion.

Having such a map of the functional anatomical organisation of the vagus nerve at different locations will scale down the challenge of transferability, not only between species but also between nerves and tissues in the same species \[209\]. This challenge arises from the anatomical and conduction differences of nerve fibres that can affect their sensitivity and response to electrical stimulation \[102\]. As a result, pre-clinical experiments in healthy and disease animal models that resemble the most the human neurophysiology are of great value as proof of concept studies, yet more extensive clinical research is required to translate the stimulation paradigms to humans.

It is also necessary to acquire a better understanding of the electrode-nerve interface and the design of new electrode materials and fabrication strategies that ensure a safe stimulation, reliability during chronic recording, full biostability and biocompatibility with the surrounding tissue and long term preservation of their functionality \[102, 254, 336, 337\]. Minimizing the size of the electrodes while increasing the channels density will open the doors to a direct and specific organ control preventing off-target stimulation. The success of bioelectronic medicine as a therapy ultimately relies on the development of non-invasive technology with high temporal and spatial resolution that avoids the risks of surgical procedures. Non-invasive VNS has been presented as a promising therapeutic strategy to treat chronic disorders, such as rheumatoid arthritis, and its use for T1DM will be studied in clinical trials in the upcoming months. In addition to the therapeutic outcomes, special attention will be taken to the presence of side effects that may arise from a non-targetted, systemic stimulation of the vagus nerve. To overcome this limitation, peripheral focused ultrasound stimulation (pFUS) is being introduced as
a promising tool for non-invasive selective modulation of neural circuitries involved in glucose homeostasis by targeting specific end-organ locations [338]. In one study, Cotero et al. (2019) explored sites contain peripheral nerve soma and synapses at the liver, the pancreas and the intestine, and observed a differential metabolic response depending on the targeted location [339]. One last important requirement to guarantee the clinical translation of neurostimulation deals with deepening our knowledge about how our bodies adapt and tolerate peripheral neural stimulation, as its effects may decrease over time or even change due to neural plasticity [50].

**Recording** still faces challenges that hinder its rapid evolution, such as the sub-millimetre nature of peripheral autonomic nerves, the low-amplitude waveforms and the low signal-to-noise ratio, and the non-stationary nature of neural signals. Improved neural decoding algorithms, coupled with advances in data analysis techniques, are allowing us to extract real-time information about the state of the nerves and the related organs [2, 254]. Combining the information extracted from these neural recordings with data acquired during continuous monitoring of other biomarkers, and capitalizing on the use of machine learning algorithms, present a promising strategy to characterize the state of the biological processes of interest in great detail.

**Current artificial pancreas systems** also have several challenges that need to be addressed if they are to be used in conjunction with bioelectronic medicine. Among them, the delays on interstitial glucose sensing and hormonal subcutaneous absorption [48, 50, 78, 330, 340], failures in the insulin pump [63], and glycaemic control during meals [44] and exercise [341] are the most critical. Despite these challenges, the use of the artificial pancreas is associated with high levels of user satisfaction and quality of life, and reduced fear of hypoglycaemic events [78]. These positive outcomes have also been facilitated by the ongoing strategies for educating the users to understand and operate these closed-loop systems [342, 343, 344]. Overall, this scenario suggests that novel technology integrating closed-loop neuromodulation and metabolic systems can be efficiently implemented and successfully adopted by the diabetes community.

A final challenge involves integrating all the hardware and software into patient-specific closed-loop systems, where innovative solutions for **data transmission and storage, and power supply** are also needed.

8.4 Conclusion

The work presented in this thesis motivates the incorporation of bioelectronics to traditional pharmaceutical-based technology as a promising new pathway for diabetes treatment. In summary, bioelectronic medicine promises the following improvements in diabetes[3]:

[168]
8.4. CONCLUSION

- Patient-tailored treatments that can be continuously adjusted in real-time by using automatic closed-loop systems.
- Targeted therapies that avoid or greatly reduce the use of drugs with off-target adverse effects.
- Access to a large amount of personalized data that will present an unprecedented opportunity for analytics to improve healthcare.

To fully exploit the potential benefits of bioelectronic medicine as an alternative or adjunctive therapy for diabetes, medicine and technology should work in synchrony. Advances in minimally invasive technology for organ-specific neuromodulation are needed to take us one step closer to using bioelectronic therapies for optimal metabolic control in daily life. Accordingly, it is crucial to fully understand the neurophysiology and neuroanatomy that underlie the glucose-response reflex to enable the creation of more efficient and safe systems. As conceptualized in Figure 8.2, incorporating the benefits of neuromodulation to current closed-loop systems for glucose control in diabetes, like the artificial pancreas, promises to artificially reproduce the efficient control of glucose homeostasis present in healthy individuals. The work presented in this thesis lays the ground for the development of novel diabetes technology for improved glucose control, increased autonomy and a reduction in diabetes-associated complications that promise to transform the current challenging healthcare scenario in diabetes.
BIBLIOGRAPHY


[41] Fortune Business Insights, “Insulin Pump Market Size, Share & Industry Analysis, By Product Type (Pumps (Tethered Pumps, Patch Pumps, and Others), and Consumables), By Disease Indication (Type 1 Diabetes and Type 2 Diabetes), By Distribution Channel (Hospital Pharmacy Retail Pharmac,” tech. rep., Fortune2020, 2020.


[133] A. Bedows, “When a nerve can be your glucometer,” 2018.


[154] “Vagus Nerve - Location, Stimulation, Disorders and Test.”


[192] B. Ahrén and J. J. Holst, “The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia,” *Diabetes*, vol. 50, pp. 1030–1038, may 2001.


189


[265] AmsterCHEM, “ScanIt.”


193


[347] Patrick Dougherty, “Hypothalamus: Structural Organization (Section 4, Chapter 1) Neuroscience Online: An Electronic Textbook for the Neurosciences | Department of Neurobiology and Anatomy - The University of Texas Medical School at Houston.”


LIST OF PUBLICATIONS ARISING FROM THIS WORK

The publications are sorted according to each chapter of this thesis, although some publications may have been presented in more than one chapter.

Chapter 3 and Appendix B: Literature review of the neural control of glucose metabolism.


Chapter 4: *In vivo* experiments - impact of vagus nerve stimulation on glucose homeostasis.


Chapter 5: Unified neural-metabolic mathematical model.


Chapter 6: *In silico* application of bioelectronic medicine for improved type 1 diabetes management.


Chapter 7: Predicting quality of overnight glycaemic control in type 1 diabetes using binary classifiers.


Chapter 8: Review of opportunities for closed-loop neuromodulation in diabetes.

REVIEW OF THE CNS IN THE CONTROL OF GLUCOSE METABOLISM

Figure B.1: Overview of key hypothalamic nuclei and other areas involved in glucose homeostasis. Representation of a sagittal section of a human brain, where the most relevant nuclei of the hypothalamus and the brainstem involved in the control of glucose production and uptake are depicted.

This Appendix provides a summary of the most important regions in the central nervous system (CNS) and neural signalling pathways that are involved in glucose homeostasis. Particular attention is given to their impact on insulin sensitivity (i.e. the effect of insulin on glucose production and uptake in the tissues) and on the hormonal secretion by the endocrine pancreas. Some of the sections included in this chapter have been previously published in the Journal of Bioelectronic Medicine (Güemes et al., 2018 [29]).

B.1 Principal CNS regions and neuronal populations

One of the brain areas that contributes the most in the regulation of glucose homeostasis is the hypothalamus, located in the diencephalon [24]. There are eleven major nuclei in the hypothalamus (see Figure B.1), out of which four have been found to have key roles in the neuroendocrine regulation: i) the arcuate nucleus of the hypothalamus (ARC), located next to the third ventricle in the mediobasal hypothalamus, ii) the paraventricular nucleus (PVN), located in the periventricular zone, iii) the ventromedial nucleus of the hypothalamus (VMH), and iv) the lateral hypothalamic nucleus (LHN) [345, 346]. The suprachiasmatic nucleus (SCN), involved in circadian timing, together with the anterior hypothalamic nucleus (AHN) and the
lateral and medial preoptic nuclei (LPN and MPN, respectively), which are involved in control of the autonomic nervous system, also play a role in the control of energy homeostasis [347]. As presented in Table B.1, the different nuclei, and within them the different population of neurons, contribute to different aspects of glucose regulation. The most interesting areas for this project are those implicated in hepatic glucose metabolism, insulin sensitivity and pancreatic secretion, which are reviewed in more detail throughout the chapter. In addition to the hypothalamus, there are other brain regions, such as the brainstem, that contain nuclei that are also implicated in the regulation of the body’s energy homeostasis [346].

Table B.1: Role of neural populations on glucose metabolism: differential involvement of neural populations from the same nuclei on glucose metabolic processes.

<table>
<thead>
<tr>
<th>Metabolic process</th>
<th>ARC</th>
<th>VMH</th>
<th>LHN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake and energy expenditure</td>
<td>AgRP &amp; NPY</td>
<td>SF1 &amp; Glut</td>
<td>LHN</td>
</tr>
<tr>
<td>Gluconeogenesis and HGP</td>
<td>AgRP</td>
<td>VMH</td>
<td>LHN</td>
</tr>
<tr>
<td>Insulin release</td>
<td>AgRP</td>
<td>Glut</td>
<td></td>
</tr>
<tr>
<td>Glucagon release</td>
<td>POMC</td>
<td>VMH</td>
<td></td>
</tr>
<tr>
<td>Insulin action (insulin sensitivity)</td>
<td>AgRP &amp; POMC</td>
<td></td>
<td>LHN (in BAT)</td>
</tr>
<tr>
<td>Glucose utilization</td>
<td>POMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipolysis</td>
<td>POMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermogenesis</td>
<td>NPY</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.1.1 Arcuate nucleus of the hypothalamus

The ARC has important implications in the control of glucose homeostasis. This region has discrete neuronal populations that express neurotransmitters that mediate effects on food intake and energy expenditure, which are regulated by specific signals of the nutritional state [348, 349, 350]. Among them, two populations of neurons stand out: the orexigenic (i.e. appetite stimulating) neuropeptide Y/agouti-related peptide (NPY/AgRP) neurons and the anorexigenic pro-opiomelanocortin/cocaine-amphetamine related transcript (POMC/CART) neurons. Recently, a new population of neurons in the ARC have been discovered, the tirosine hydroxylase (TH) neurons, which have orexigenic effects [24]. Finally, the ARC also possesses gluco sensing neurons [348, 351] that take part in the neural circuitry of energy control.

NPY/AgRP neurons. The contribution of NPY/AgRP neurons to metabolic homeostasis has been widely studied and is well characterized. This population synthesises and secretes two neuropeptides, agouti-related peptide (AgRP) and NPY, that have potent orexigenic effects [352, 353]. Recent studies have described that acute activation of AgRP neurons can cause insulin resistance through an impairment of the insulin-stimulated glucose uptake into brown
adipose tissue (BAT). Furthermore, a recent relevant study has demonstrated that hypothalamic injection of fibroblast growth factor 1 (FGF-1) contributes to T2DM remission. Intact AgRP neurons were found to respond the most after FGF-1 injection, and since these neurons function as endogenous inhibitors of melanocortin signalling, the researchers concluded that an intact melanocortin network is required for the antidiabetic action of FGF1 [354]. Additionally, NPY derived from the ARC is critical in the control of sympathetic outflow and BAT function [353]. In fact, a recent study has reported that an increase in NPY from the ARC decreases the sympathetic outflow that controls the BAT thermogenesis via TH neurons [350]. NPY neurons have also been shown to promote HGP, probably by reducing hepatic insulin sensitivity through an increase in the sympathetic tone to the liver [350, 355].

**POMC neurons.** The α-melanocyte-stimulating hormone (α-MSH) is the most important pro-opiomelanocortin (POMC)-derived peptide involved in feeding and metabolism. It is a non-selective full agonist of all the melanocortin receptors except from the melanocortin 2 receptor (MC2R), which is exclusive for the adrenocorticotropic hormone (ACTH). In one study, the intracerebral infusion of α-MSH resulted in increased gluconeogenesis and hepatic glucose production [350, 356]. It is remarkable that the two major populations of neurons in the ARC, AgRP/NPY and POMC, have antagonistic effects with regards to glucose homeostasis. This antagonizing function is of great importance to adapt the hypothalamic-pituitary-thyroid (HPT) axis to the predominant energy status. For example, in fasting conditions it is required a reduction in α-MSH and an increase in AgRP [357].

**TH neurons.** Arcuate TH neurons are involved in many neuronal circuits related to energy homeostasis. For example, studies of optogenetic stimulation of TH neurons of the ARC in mice showed an increase in food intake [24]. In more detail, arcuate TH cells have projections to the PVN and when the former were stimulated, there was a co-release of the neurotransmitters dopamine and GABA to the neurons in the PVN. In the PVM, dopamine excited orexigenic neurons that synthesize AgRP and NPY, but inhibited anorexigenic neurons that synthesize POMC (i.e. powerfully stimulating feeding) [24].

### B.1.2 Ventromedial nucleus of the hypothalamus

The VMH is a key brain region involved in glucose regulation and energy homeostasis in mammals [358, 359, 360, 361]. In particular, the VMH has a crucial role in detecting hypoglycaemic events and initiating the physiological counterregulatory responses to return low blood glucose levels into the normal range by causing the release of glucagon and epinephrine [362, 299, 363, 364, 365, 366]. Studies of electrical stimulation of the VMH further support this evidence reporting a marked increase in the circulating level of glucagon, together with a rapid
rise in glucose levels [367, 368]. In another study, electrical stimulation of the VMH resulted in an increase in glucose utilization in the interscapular BAT, heart, and skeletal muscle, but did not significantly affect the white adipose tissue (WAT), the brain or the diaphragm [359]. The increased rate of glucose uptake in the BAT after VMH stimulation was suppressed almost completely by local sympathetic denervation, indicating that the effect of VMH stimulation is mediated by sympathetic nerves to the tissue [363]. The fact that VMH stimulation does not increase insulin secretion from the pancreas [367] suggests that its effect on tissue glucose uptake is not mediated by insulin. On the contrary, it is probably mediated by sympathetic nerve action, and therefore an insulin-independent mechanism of glucose transport.

The VMH contains a heterogeneous population of neurons. A subset of VMH neurons expresses the leptin receptor [369], and most of these neurons also express the steroidogenic factor-1 (SF1) receptor [370, 371]. Leptin is a hormone predominantly made in adipose cells that helps to regulate energy balance by i) inhibiting hunger, ii) stimulating glucose uptake in some peripheral tissues, including red-type skeletal muscle, and iii) enhancing endogenous glucose production by inducing glycogen phosphorylase activity in the liver. Thereby, it affects insulin sensitivity and helps to maintain blood glucose levels in the appropriate range [372, 373].

Furthermore, SF1 neurons in the VMH are required for maintenance of normal glucose and energy metabolism [359] and also mediate the anorexic and metabolic effects of leptin [359, 370, 371]. Moreover, neurons expressing the SF1 receptor are key components of the neural circuit that activates during insulin-induced hypoglycaemia and extends from the lateral parabrachial nucleus (LPBN) to the anterior bed nucleus of the stria terminalis (aBNST) [366].

### B.1.3 Lateral hypothalamic nucleus

The LHN contains the primary orexinergic nucleus within the hypothalamus [374]. Many studies of stimulation of the LHN have shown an activation of this area over hepatic glucose metabolism [350, 363]. In fact, stimulation of the LHN, contrary to VMH activation, resulted in increased hepatic glycogenesis by activation of the key enzyme glycogen synthetase [350, 363]. Moreover, evidence of the interaction between the LHN region and the pancreas via the pancreatic parasympathetic branch are broadly found in experiments in the literature [375, 186]. Stimulation of different areas within the LHN resulted in a differential pattern of activation of the vagus nerve [375]. As an example, stimulation of the ventral LHN enhanced pancreatic vagus activity [375, 186]. Finally, up to date, electrical stimulation of the LHN has not exhibited any significant direct effect on glucose uptake from either adipose or skeletal tissue [363, 367]. However, activation of the melanocortin 4 receptor (MC4R) expressed in the LHN has been
found to cause an increase in glucose uptake specifically into BAT, suggesting that $MC4R^{LHN}$ signalling enhances BAT glucose utilization via sympathetic pathways [376].

**B.1.4 Suprachiasmatic nucleus and paraventricular nucleus**

The SCN is a small region of the hypothalamus situated directly above the optic chiasm. It is well known for regulating many different body functions in a 24-hour cycle (circadian rhythms), including the generation of the 24-h rhythm of plasma glucose [377]. Studies of electrical stimulation of the SCN have found to induce hyperglycaemia, which could be prevented by the use of sympathetic blockers or denervation [350, 378, 379]. This suggests a sympathetic mediation of the effects of SCN activation. Similarly, hyperglycaemic events were also reported in experiments where the inhibitory input from the SCN to the PVN was removed, revealing an implication of the latter in generating the 24-h oscillations of plasma glucose [380].

Overall, this hyperglycaemia is a result of i) direct and indirect stimulatory effects on glycogenolysis by increasing hepatic glucose production [377, 378] and promoting pancreatic secretion [381], respectively, ii) an increase in the activity of the hepatic enzyme glycogen phosphorylase alpha, which is involved in the glycogenolysis, and iii) a significant decrease in the liver glycogen content [377, 381].

**B.1.5 Extrahypothalamic regions: nucleus of the tractus solitarius**

The integration of information related to the body’s energy homeostasis does not occur solely in the hypothalamus. For example, meal-related satiety information is conveyed to the nucleus of the NTS in the medulla, where vagally transmitted signals from the gastrointestinal tract converge [346]. Some NTS neurons are glucose sensitive, while others express POMC, leptin receptors or the MC4R, giving evidence to its important function as an integration centre of metabolic signals. The NTS also sends a dense projection to the LHN, reinforcing its contribution in the control of body energy [346].

**B.2 METABOLIC AND HORMONAL SIGNALLING IN THE BRAIN**

**B.2.1 Glucose signalling**

The ability of sensing glucose is of great importance for maintaining optimal glucose homeostasis, being in fact a major signal for integrating and regulating the whole body metabolism [382, 147]. Glucosensing cells can be found in the mouth, the hepatoporal vein area, the brainstem and the hypothalamus and are linked to each other by nervous connections. These neurons can be either glucose-excited (GE) or glucose-inhibited (GI) neurons that increase
and decrease, respectively, their action potential frequency as extracellular glucose levels increase throughout the physiological range [348]. They are thought to control the activity of the parasympathetic and sympathetic innervation of the pancreatic $\alpha$ and $\beta$-cells, regulating their hormonal secretion and cell number [147]. Depending on their location, glucosensing neurons have different functions on the glucoregulatory process:

**Hypothalamic ARC nuclei.** GI neurons are found predominantly in the medial ARC, whereas GE neurons are mainly located in the lateral ARC and are found to be intermixed with POMC neurons [348]. Some studies have demonstrated that GE neurons change their firing rate in a dose-dependent fashion to physiological changes in extracellular glucose [348, 383]. Under physiological conditions, brain glucose levels are generally approximately 20-30% of the plasma levels [383, 384]. Therefore, except in extreme hyperglycaemic diabetic conditions, it is unlikely that extracellular brain glucose levels ever exceed 5 mmol/l. However, the blood-brain barrier has a higher permeability in the ARC [348, 385, 386], so GE neurons in this area can be exposed to higher glucose levels than the rest of the brain. In fact, some studies have shown that glucosensing neurons in this region respond to glucose levels over 5 mmol/l [348].

It is noteworthy the modulatory effect of other hormones and neurotransmitters over the activity of ARC GE neurons. To begin with, insulin has been found to increase their firing pattern in a glucose-dependent fashion [348, 387]. NPY and $\alpha$-MSH also regulate their activity in a way that is consistent to the roles of these peptides in the regulation of food intake, and energy balance [24, 348, 352, 388, 389]: NPY inhibits and $\alpha$-MSH stimulates ARC GE neurons [348, 390]. On the other hand, leptin has no effect on ARC GE neurons [348]. In conclusion, ARC GE neurons have a pivotal position as integrators of central and peripheral information of energy homoeostasis.

**Hypothalamic VMH nuclei.** Glucosensing neurons in the VMH are key elements in the counterregulatory response. Apart from the inherently glucose sensing neurons (both GE and GI neurons) [348, 361, 391], other neurons in the VMH were found to be able to alter their firing rate in response to a variety of extracellular glucose concentrations [348]. However, the changes in their activity were caused by presynaptic inputs from other glucosensing neurons whose cell bodies may reside outside the VMH, as opposed to directly sensing glucose levels. One location of these presynaptic glucosensing inputs to the VMH has been reported to be the ARC [348].

**Brainstem nuclei.** The brainstem might also be a physiologically relevant site for hypoglycaemia detection and counterregulation [147, 391].
To conclude, some studies suggest that impairment of these glucose-sensing mechanisms might be the origin of the metabolic deregulations that lead to obesity and T2DM, such as overeating, reduced energy expenditure, impaired suppression of glucagon secretion and reduced first phase of insulin secretion [147].

### B.2.2 Central insulin signalling

Both insulin and leptin have an important signalling role in central nervous mechanisms of glucose homeostasis and energy balance, illustrated by the large number of receptors that are found in specific areas of the brain [369, 392, 393, 394], including the ARC [29, 345, 350, 352, 395]. Insulin is able to cross from the periphery to the brain via an insulin receptor-mediated transport process involving brain capillary endothelial cells in the choroid plexus [287]. Within the brain, insulin action results in a great range of effects depending on the targeted nuclei, including control of food intake, hepatic processes [345, 356, 396, 397, 398], hepatic insulin sensitivity [350] and glucose uptake [23]. Moreover, some studies suggest that brain insulin might also be involved on the neural reflex that occurs during the cephalic phase [345, 350, 396, 399], by affecting parasympathetic and sympathetic activity, and therefore influencing pancreatic secretion [29, 345, 350, 287, 396, 399]. It is surprising that the action of insulin in the brain does not alter peripheral insulin signalling, but influences insulin action in peripheral tissues [396, 399, 400]. Thus, hypothalamic insulin uses signalling pathways other than peripheral insulin in target organs such as WAT and liver to regulate insulin action [401]. As an example, insulin suppresses hepatic glucose production and lipolysis in adipose tissue through signalling in the mediobasal hypothalamus that alters parasympathetic and sympathetic outflow to these tissues, respectively [345, 396, 402].

### B.2.3 Central leptin signalling

Leptin action in the brain is involved in hepatic glucose control by redistributing the intrahepatic glucose fluxes, increasing gluconeogenesis and suppressing glycogenolysis. However, it does not change total glucose production by the liver [403, 404] in contrast to central insulin that acts by inhibiting glucose production [345, 396]. The effects of hypothalamic leptin signalling on hepatic insulin sensitivity could be blocked by selective hepatic vagotomy [350] and the expression of leptin receptors in the ARC improved peripheral insulin sensitivity by promoting the suppression of glucose production [405]. Finally, it was shown that leptin deficiency induced severe insulin resistance that could be corrected with external infusion of this hormone [349].
B.2.4 FAs signalling

In addition to leptin and insulin, there are other metabolic substances such as fatty acids that are also directly sensed by the hypothalamus and carry out a central role via hypothalamic mechanisms on insulin action and on stimulating hepatic glucose production \[350, 397, 406\]. However, it remains to be clarified where and the extent to which level fatty acids elicit this effect \[350\].

B.3 Therapeutic opportunities for affecting insulin sensitivity through stimulation of the central nervous system

Table B.2 summarizes the many opportunities for glucose control and diabetes management arising from modulating the activity of the central nervous system, which could be used in the future as therapies through bioelectronic medicine.

For example, through modulation of the neural signals we could control the course of insulin-independent and insulin-dependent mechanisms (i.e. influencing the effectiveness of insulin, $S_I$) of hepatic glucose production and glucose uptake to reach optimal glucose control. This is very important for T1DM, where there is not an endogenous production of insulin from the pancreas. In these subjects, the insulin that is needed to lower glucose levels after a meal intake is externally delivered by either an insulin pen or a pump. In this case, modulation of the neural circuits could increase the effect of exogenous insulin in lowering blood glucose and we would be able to reduce post-prandial glucose excursions, therefore reducing hyperglycaemia. In addition, external stimulation of hypothalamic regions such as the VMH could help to reduce hypoglycaemic events in people with T1DM, as its activation promotes activation of hepatic gluconeogenesis and glucagon release from the pancreas, which in turn elevate blood glucose levels.

Despite the potential benefits of using bioelectronic medicine in this sense, there are still many limitations that need to be overcome before becoming a reality for diabetes treatment. Among them, acquiring a full understanding of the central neural circuitries and their impact on glucose levels stands out.

B.4 Executive summary

- The hypothalamus is one of the regions of the central nervous system presenting a key role in neuroendocrine regulation. In particular, the arcuate (ARC), the ventromedial (VMH), and the lateral hypothalamic nuclei (LHN) are foregrounded.
### B.4. EXECUTIVE SUMMARY

Table B.2: Central mechanisms involved in glucose homeostasis and benefit of modulating them for diabetes management.

<table>
<thead>
<tr>
<th>Region</th>
<th>Population of neurons</th>
<th>Peripheral mechanisms</th>
<th>Impact on glucose homeostasis</th>
<th>Ref.</th>
<th>Opportunities for diabetes management</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC</td>
<td>AgRP</td>
<td>Food entrained oscillator</td>
<td>Regulate feeding behaviour</td>
<td>[407]</td>
<td>Predicting cephalic phase</td>
</tr>
<tr>
<td></td>
<td>AgRP (\rightarrow) aBNST(^{vl})</td>
<td>(↑) BAT myostatin expression</td>
<td>Insulin resistance</td>
<td>[23]</td>
<td>Control of SI</td>
</tr>
<tr>
<td>ARC</td>
<td>AgRP (\rightarrow) LHN</td>
<td>(↑) Appetite</td>
<td>(↑) HGP</td>
<td>[23]</td>
<td>Control of food intake</td>
</tr>
<tr>
<td></td>
<td>AgRP(^{IR})</td>
<td>(↓) Hepatic vagal tone</td>
<td>(↔) Peripheral glucose uptake</td>
<td>hepatic SI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NPY</td>
<td>(↑) Appetite</td>
<td>(↑) HGP</td>
<td>[345, 350, 396]</td>
<td>Control of hepatic SI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(↑) Hepatic sympathetic tone</td>
<td>(↑) HGP</td>
<td>[350]</td>
<td>Control of hepatic SI</td>
</tr>
<tr>
<td>VMH</td>
<td>VMH(^{FF1})</td>
<td>↑ Glucose uptake</td>
<td>↑ HGP</td>
<td>[359]</td>
<td>Control of hepatic SI</td>
</tr>
<tr>
<td></td>
<td>VMH(^{Glut})</td>
<td>↑ Glucose uptake</td>
<td>↑ HGP</td>
<td>[367, 368]</td>
<td>Control of hypoglycaemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Glucose uptake</td>
<td>↑ Insulin</td>
<td>[367]</td>
<td>Control of hypoglycaemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Glucose uptake</td>
<td>↑ Tissue uptake</td>
<td>[359]</td>
<td>Control of blood glucose levels</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Glucose uptake</td>
<td>↑ Response to hypoglycaemia</td>
<td>[386]</td>
<td>Control of hypoglycaemia</td>
</tr>
<tr>
<td>LHN</td>
<td>LHN(^{MC4R})</td>
<td>↑ Parasympathetic tone</td>
<td>↓ HGP</td>
<td>[363]</td>
<td>Control of blood glucose levels</td>
</tr>
<tr>
<td></td>
<td>SCN (\rightarrow) PVN</td>
<td>↑ Sympathetic tone</td>
<td>↑ HGP</td>
<td>[376]</td>
<td>Control of SI</td>
</tr>
</tbody>
</table>

This table summarizes the impact of activating the most relevant brain regions involved in glucose homeostasis and the potential benefit of changing their activity for modulating insulin sensitivity (SI) and food intake in the context of type 1 and type 2 diabetes mellitus management. In particular, their effect on plasma glucose levels \((G(t))\), hepatic glucose production (HGP) and glucose uptake are shown.

- The different nuclei, and within them the different population of neurons, are involved in different aspects of glucose regulation, including the counterregulatory response to hypoglycaemia, hepatic glucose metabolism, insulin sensitivity and pancreatic secretion.
- The brain exhibits a great ability to sense glucose and hormones, including insulin and leptin. This is crucial for integrating and processing the metabolic changes experienced in the rest of the body, and triggering the necessary mechanisms to respond to them.
- Neuromodulation of these brain regions is arising as a promising therapeutic opportunity to acquire better control of glucose levels in both type 1 and type 2 diabetes mellitus.
B.5 Conclusion

This appendix has presented an overview of the most relevant central signalling mechanisms that are involved in control of glucose homeostasis. It also provides a discussion on the therapeutic opportunities of using bioelectronic medicine through electrical modulation of the neural activity of these brain regions to modify and restore lost biological processes that are essential for keeping a good balance of glucose levels.
C.1 Preliminary experiments

The first preliminary experiments using short stimulation durations (1 and 5 min-stimulation) lack of a consistent bradycardic response (Figures C.1 and C.2), which may suggest that stimulation was not being properly delivered. This would explain the lack of a meaningful change in glucose concentration during or after these stimulation protocols. The fluctuations that are observed in the glucose profiles are most likely a result from the intrinsic variability in basal glucose levels and measurement errors.

On the other hand, using longer stimulation periods of 15 and 30 minutes resulted in a small increase in glucose levels with respect of basal at the end of stimulation and right after its cessation (Figures C.3 and C.4). The increase in glucose concentration was greater using the higher frequency of 10Hz.

Figure C.1: Glucose-response protocol - rat 002. Impact of stimulation on glucose. In each sub-figure, from top to bottom: pulse amplitude (mA), pulse duration (ms), stimulation frequency (Hz), heart rate (bpm), glucose concentration (mg/mL).
APPENDIX C. EXTENDED RESULTS FROM EXPERIMENTS

Figure C.2: Glucose-response protocol - rat 003. Impact of stimulation on glucose. Left: protocol 1; Right: protocol 2. From top to bottom: pulse amplitude (mA), pulse duration (ms), stimulation frequency (Hz), heart rate (bpm), glucose concentration (mg/mL).

Figure C.3: Glucose-response protocol - rat 004. Impact of stimulation on glucose. Left to right are consecutively depicted protocols 1 to 4 respectively. From top to bottom: pulse amplitude (mA), pulse duration (ms), stimulation frequency (Hz), heart rate (bpm), glucose concentration (mg/mL).
C.1. PRELIMINARY EXPERIMENTS

Figure C.4: Glucose-response protocol - rat 005. Impact of stimulation on glucose. Left: protocol 1 (5Hz-stimulation); Right: protocol 2 (10Hz-stimulation). From top to bottom: pulse amplitude (mA), pulse duration (ms), stimulation frequency (Hz), heart rate (bpm), glucose concentration (mg/mL).

Discussion

The presence of a peak after the termination of the 15-minutes and 30 minutes stimulation is an interesting result that, to the best of our knowledge, has not been previously reported. Several hypothesis of the origin of the outcome has been discussed. To begin with, the stimulation of the parasympathetic pathways could result in an over-stimulation of the sympathetic pathways to activate peripheral counteracting mechanisms, such as a promotion of hepatic glucose production. This bounce could also be due to the long overnight fast (14h), which might have caused the animals to be very unstable and reactive to compensate to changes. Finally, the peak could be a result of the delayed effect of the glucagon secreted during stimulation. This last hypothesis could be assessed in the succeeding experiments by measuring the glucagon concentration in blood samples (see results in Chapter 4).
D.1 Comparison of metrics for specific fibre type recruitment

Figures D.1, D.2 and D.3 present the distribution of the proposed metrics for computing the activation of each specific fibre type obtained by applying the three proposed methods for compound action potential (CAP) identification on the recordings using each stimulation protocol: fixed temporal thresholding, decomposition and filtering applying the continuous wavelet transform (CWT), filtering and reconstruction using multiresolution analysis (MRA) by applying the discrete wavelet transform (DWT), respectively.

Figure D.1: Comparison of proposed metrics for activation of each fibre type applying temporal thresholding as the CAP identification method. The figure shows the distribution of values obtained by computing each metric of fibre recruitment on the region corresponding to A-fibres (left panel), B-fibres (middle panel), C-fibres (right panel) for each stimulation protocol (pulse width - amplitude).
Figure D.2: Comparison of proposed metrics for activation of each fibre group using decomposition and filtering applying CWT as the CAP identification method. The figure shows the distribution of values obtained by computing each metric of fibre recruitment on the region corresponding to A-fibres (left panel), B-fibres (middle panel), C-fibres (right panel) for each stimulation protocol (pulse width - amplitude).

Figure D.3: Comparison of proposed metrics for activation of each fibre group using multiresolution analysis applying DWT as the CAP identification method. The figure shows the distribution of values obtained by computing each metric of fibre recruitment on the region corresponding to A-fibres (left panel), B-fibres (middle panel), C-fibres (right panel) for each stimulation protocol (pulse width - amplitude).
D.2 NERVE ACTIVATION OF SPECIFIC FIBRE TYPES

Figures D.4, D.5 and D.6 show the 3D plots of the activation of each specific fibre type for each stimulation protocol obtained using the three proposed methods for CAP identification: fixed temporal thresholding, decomposition and filtering using CWT, filtering and reconstruction using multiresolution analysis applying DWT.

As expected, the greater the injected charge density (bigger stimulation parameters pulse width and amplitude), the higher the percentage of fibres recruited. Surprisingly, this behaviour reversed for the stimulation protocol using the pulse width of 0.5ms, where there was an increased of the recruited fibres compared to using the pulse width of 1ms. This situation was particularly remarkable on the activation of B fibres, which suggests that a pulse width of 0.5ms is optimal for recruiting of these fibres.

![3D surface plots of the percentage of each fibre type activation and recruitment during each stimulation protocol](image)

Figure D.4: 3D surface plots of the percentage of each fibre type activation and recruitment during each stimulation protocol. The fibre recruitment was calculated by computing the AUC of the CAP identified by applying fixed temporal thresholding.
Figure D.5: 3D surface plots of the percentage of each fibre type activation and recruitment during each stimulation protocol. The fibre recruitment was calculated by computing the AUC of the CAP identified by applying decomposition and filtering using CWT.

Figure D.6: 3D surface plots of the percentage of each fibre type activation and recruitment during each stimulation protocol. The fibre recruitment was calculated by computing the AUC of the CAP identified by applying filtering and reconstruction using DWT to the recorded neurograms.
## D.3 Neural-metabolic model identification: overview of parameters

Table D.1: Constraints used in the parameter identification process and their explanation.

<table>
<thead>
<tr>
<th>Process</th>
<th>Parameter</th>
<th>Constraints</th>
<th>Units</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neural Model of NT release</td>
<td>$\delta$</td>
<td>4.2</td>
<td>ms</td>
<td>Absolute refractory period of APs is 1ms and the maximum duration 5ms [411]</td>
</tr>
<tr>
<td>Glucose subsystem</td>
<td>$V_G$</td>
<td>1.88</td>
<td>dL/Kg</td>
<td>Reported by Dalla Man et al. [88]</td>
</tr>
<tr>
<td>Insulin Secretion</td>
<td>$k_{Di}$</td>
<td>1-3</td>
<td>pmol/Kg per mg/dL</td>
<td>Covers the values reported by Dalla Man et al. [88]</td>
</tr>
<tr>
<td></td>
<td>$\alpha$</td>
<td>0.01-0.5</td>
<td>min$^{-1}$</td>
<td>Covers the values reported by Dalla Man et al. [88]</td>
</tr>
<tr>
<td></td>
<td>$\beta$</td>
<td>0.1-0.2</td>
<td>pmol/Kg per mg/dL</td>
<td>Covers the values reported by Dalla Man et al. [88]</td>
</tr>
<tr>
<td>Insulin Kinetics</td>
<td>$V_I$</td>
<td>0.02-0.07</td>
<td>L/Kg</td>
<td>Covers the values reported by Dalla Man et al. [88]</td>
</tr>
<tr>
<td></td>
<td>$m_1$</td>
<td>0.19 ± 0.01</td>
<td>min$^{-1}$</td>
<td>5% variation range from value reported by Dalla Man et al. [88]</td>
</tr>
<tr>
<td></td>
<td>$m_2$</td>
<td>0.48 ± 0.025</td>
<td>min$^{-1}$</td>
<td>5% variation range from value reported by Dalla Man et al. [88]</td>
</tr>
<tr>
<td></td>
<td>$m_4$</td>
<td>0.194 ± 0.01</td>
<td>min$^{-1}$</td>
<td>5% variation range from value reported by Dalla Man et al. [88]</td>
</tr>
<tr>
<td></td>
<td>$m_5$</td>
<td>0.015-0.025</td>
<td>min$^{-1}$·Kg/pmol</td>
<td>Covers the values reported by Dalla Man et al. [88] &amp; to ensure stability in the steady state</td>
</tr>
<tr>
<td></td>
<td>$m_6$</td>
<td>0.6-0.7</td>
<td>min$^{-1}$·Kg/pmol</td>
<td>Covers the values reported by Dalla Man et al. [88] &amp; to ensure stability in the steady state</td>
</tr>
</tbody>
</table>
Table D.2: Overview of the parameters of the neural model.

<table>
<thead>
<tr>
<th>Process</th>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neural Model of NT release</td>
<td>$K_D$</td>
<td>0.00139†</td>
<td>$min^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$K_R$</td>
<td>0.0035†</td>
<td>$min^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$\rho$</td>
<td>0.6†</td>
<td>Dimensionless</td>
</tr>
<tr>
<td></td>
<td>$m$</td>
<td>55†</td>
<td>$nM$</td>
</tr>
<tr>
<td></td>
<td>$\delta$</td>
<td>4.2</td>
<td>$ms$</td>
</tr>
</tbody>
</table>

The identification methodology is described by Güemes et al. [262].
### Table D.3: Parameters of the unified neural-metabolic model identified using the experimental data from dogs.

<table>
<thead>
<tr>
<th>Process</th>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete glucose subsystem</td>
<td>$V_g$</td>
<td>1.88</td>
<td>$dL/Kg$</td>
</tr>
<tr>
<td></td>
<td>$k_1$</td>
<td>$10^{-3}$</td>
<td>$\text{min}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_2$</td>
<td>0.2</td>
<td>$\text{min}^{-1}$ per $\text{pmol/mL}$</td>
</tr>
<tr>
<td></td>
<td>$k_3$</td>
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<td>$\text{min}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_4$</td>
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<td>$\text{min}^{-1}$ per $\text{pmol/L}$</td>
</tr>
<tr>
<td></td>
<td>$k_5$</td>
<td>$10^{-3}$</td>
<td>$\text{min}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_6$</td>
<td>$10^{-4}$</td>
<td>$\text{min}^{-1}$ per $\text{pmol/L}$</td>
</tr>
<tr>
<td></td>
<td>$k_7$</td>
<td>0.2</td>
<td>$\text{min}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_8$</td>
<td>0.28</td>
<td>$\text{min}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$k_9$</td>
<td>$10^{-3}$</td>
<td>$\text{min}^{-1}$ per $\text{pg/mL}$</td>
</tr>
<tr>
<td></td>
<td>$k_{n1}$</td>
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<td>$\text{min}^{-1}$</td>
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<td>$k_{n2}$</td>
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<td>$\text{min}^{-1}$</td>
</tr>
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<td>$\text{min}^{-1}$ per $\text{nM}$</td>
</tr>
<tr>
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<td>$k_{n4}$</td>
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<td>$\text{min}^{-1}$</td>
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<td>$k_{n5}$</td>
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<td>$\text{min}^{-1}$</td>
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<td></td>
<td>$k_{n6}$</td>
<td>$4 \times 10^{-4}$</td>
<td>$\text{min}^{-1}$ per $\text{nM}$</td>
</tr>
<tr>
<td>Complete insulin secretion subsystem</td>
<td>$\rho$</td>
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<td>$\text{min}^{-1}$</td>
</tr>
<tr>
<td></td>
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<td>$\text{pmol/Kg}$ per $\text{mg/dL}$</td>
</tr>
<tr>
<td></td>
<td>$\alpha$</td>
<td>0.015</td>
<td>$\text{min}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$\beta$</td>
<td>0.15</td>
<td>$\text{pmol/Kg}$ per $\text{mg/dL}$</td>
</tr>
<tr>
<td></td>
<td>$k_{I ACh}$</td>
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</tr>
<tr>
<td></td>
<td>$k_{I NP}$</td>
<td>0.22</td>
<td>$\text{pmol/Kg}$ per $\text{nM}$</td>
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<td>$k_{I 1}$</td>
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</tr>
<tr>
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<td>$k_{I 2}$</td>
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<tr>
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<td>$k_{I 5}$</td>
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<td>$\text{pmol/Kg/min}$ per $\text{nM}$</td>
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<tr>
<td>Insulin kinetics subsystem</td>
<td>$V_I$</td>
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<td>$L/Kg$</td>
</tr>
<tr>
<td></td>
<td>$m_1$</td>
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<td>$\text{min}^{-1}$</td>
</tr>
<tr>
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<td>$\text{min}^{-1}$</td>
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<tr>
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<td>$m_4$</td>
<td>1.5</td>
<td>$\text{min}^{-1}$</td>
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<tr>
<td></td>
<td>$HE_b$</td>
<td>0.08</td>
<td>Dimensionless</td>
</tr>
<tr>
<td>Complete glucagon secretion subsystem</td>
<td>$\rho$</td>
<td>0.05</td>
<td>$\text{min}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$\sigma$</td>
<td>0.02</td>
<td>$\text{pg/Kg/min}$ per $\text{mg/dL}$</td>
</tr>
<tr>
<td></td>
<td>$\sigma_2$</td>
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<td>$\text{pg/Kg/min}$ per $\text{mg/dL}$</td>
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<tr>
<td></td>
<td>$\delta$</td>
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<tr>
<td>Glucagon kinetics subsystem</td>
<td>$V_N$</td>
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<tr>
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<td>$n$</td>
<td>0.311</td>
<td>$\text{min}^{-1}$</td>
</tr>
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</table>

Note that the parameters of the model in presence of atropine are the same, except for $k_{I ACh}$ and $k_N^{ACh}$ which are 0.
Table D.4: Parameters of the unified neural-metabolic model validated using the experimental data from rats.

<table>
<thead>
<tr>
<th>Process</th>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete glucose subsystem</td>
<td>( V_g )</td>
<td>1.88</td>
<td>dL/Kg</td>
</tr>
<tr>
<td></td>
<td>( k_1 )</td>
<td>0.02</td>
<td>( \text{min}^{-1} )</td>
</tr>
<tr>
<td></td>
<td>( k_2 )</td>
<td>0.01</td>
<td>( \text{min}^{-1} ) per pmol/mL</td>
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<tr>
<td></td>
<td>( k_3 )</td>
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</tr>
<tr>
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<td>( k_4 )</td>
<td>( 9.6 \times 10^{-4} )</td>
<td>( \text{min}^{-1} ) per pmol/L</td>
</tr>
<tr>
<td></td>
<td>( k_5 )</td>
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<td>( \text{min}^{-1} )</td>
</tr>
<tr>
<td></td>
<td>( k_6 )</td>
<td>( 9.9 \times 10^{-4} )</td>
<td>( \text{min}^{-1} ) per pmol/L</td>
</tr>
<tr>
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<td>( k_7 )</td>
<td>0.0056</td>
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<td>( k_8 )</td>
<td>( 10^{-4} )</td>
<td>( \text{min}^{-1} )</td>
</tr>
<tr>
<td></td>
<td>( k_9 )</td>
<td>( 10^{-3} )</td>
<td>( \text{min}^{-1} ) per pg/mL</td>
</tr>
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<td>( k_{n1} )</td>
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<tr>
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<tr>
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<td>( k_{n6} )</td>
<td>( 10^{-4} )</td>
<td>( \text{min}^{-1} ) per nM</td>
</tr>
<tr>
<td>Complete insulin secretion subsystem</td>
<td>( \gamma )</td>
<td>0.99</td>
<td>( \text{min}^{-1} )</td>
</tr>
<tr>
<td></td>
<td>( k_{Di} )</td>
<td>1.15</td>
<td>pmol/Kg per mg/dL</td>
</tr>
<tr>
<td></td>
<td>( \alpha )</td>
<td>0.015</td>
<td>( \text{min}^{-1} )</td>
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<tr>
<td></td>
<td>( \beta )</td>
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<td>( k_{NP}^I )</td>
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<td>( k_{I2}^I )</td>
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<td>pmol/Kg/min per nM</td>
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<tr>
<td>Insulin kinetics subsystem</td>
<td>( V_I )</td>
<td>0.04</td>
<td>L/Kg</td>
</tr>
<tr>
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<td>( m_4 )</td>
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<tr>
<td></td>
<td>( HE_b )</td>
<td>0.1</td>
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</tr>
<tr>
<td>Complete glucagon secretion subsystem</td>
<td>( \rho )</td>
<td>0.05</td>
<td>( \text{min}^{-1} )</td>
</tr>
<tr>
<td></td>
<td>( \sigma )</td>
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<tr>
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<td>( \sigma_2 )</td>
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<tr>
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<td>( \delta )</td>
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<td>( k_{ACh}^N )</td>
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<td>pg/min per nM</td>
</tr>
<tr>
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<td>( k_{NP}^N )</td>
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<td>( k_{NP}^N )</td>
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<td>( n )</td>
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</table>

Note that only some parameters differ from the values reported in Table D.3 using a different animal model.