Peripheral Hypoglycaemic Neuropathy in Type 1 Diabetic Rats
Morphologic and Metabolic Studies

Reza Jamali
Cover picture is a digitally designed electromicrograph of a neuropathological characteristic called *collagen pocket*.

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To My Wonderful Wife

Elham

On life

What seems to grow fairer to me as life goes by is the love and the grace and tenderness of it; not its wit and cleverness and grandeurs of knowledge -grand as knowledge is- but just the laughter of children and the friendship of friends, and the cozy talk by the fire, and the sight of flowers, and the sound of music.

Eleanor Leah Woods
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


IV. Jamali R. and Mohseni S. Simultaneous glucose measurement in blood, peripheral nerve, muscle and skin in control and diabetic rats. Manuscript
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Abstract

Hyperglycaemia caused by insulin deficiency is believed to play a major role in the development of neuropathy in diabetic patients. The clinical and pathological features of diabetic neuropathy vary considerably, although sensory and autonomic dysfunctions are the most common characteristics. Normalisation of the blood glucose level by effective insulin treatment decreases the incidence of diabetic neuropathy in patients. However, intensive insulin therapy may result in more frequent hypoglycaemic episodes than are provoked by less ambitious diabetes control. Neuropathy might also be induced by severe hypoglycaemia in diabetes or insulinoma. Accordingly, it seems that the diversity in clinical symptoms of diabetic neuropathy may be due to the combined effects of hyperglycaemia and hypoglycaemia. Based on that assumption, the general aim of this project was to study the relationship between severe hypoglycaemia and peripheral neuropathy in diabetic rats. To understand how the development of neuropathy is related to glycaemic control, we needed to be aware of the glucose dynamics in the animal model that we used. The aim was to ascertain whether the diabetic rats were similar to type 1 diabetic patients with regard to such dynamics. To achieve that goal, we used a MiniMed continuous glucose monitoring system (CGMS®) to measure subcutaneous glucose in freely moving rats over a period of 72 hours. The glucose monitor worked well, and it showed that the insulin-treated diabetic BB/Wor rats with a hyperglycaemic insulin regimen have a glycaemic status similar to that of type 1 diabetic patients with poor glycaemic control. The diabetic rats with a hypoglycaemic regimen generally had low blood glucose levels.

Prolonged hypoglycaemia led to axonal de- and regeneration of large myelinated fibres in vagus nerve destined to the laryngeal muscle. Axonal de- and regeneration was also observed in the gastrocnemius and sural nerves, although the frequency of degeneration was much lower in the sural nerve. Small myelinated and unmyelinated nerve fibres were normal in these nerves. These results suggest that hypoglycaemia preferentially damages muscle-related nerve fibres. In contrast, in the diabetic rats exposed to prolonged hyperglycaemia, only the sural nerve exhibited decreased myelinated fibre diameter in the absence of obvious axonal degeneration.

In situ glucose measurements by microdialysis showed that the glucose concentrations in blood and subcutaneous tissue were similar in healthy, diabetic hyperglycaemic, and diabetic hypoglycaemic rats. In the healthy and hyperglycaemic animals, the lowest glucose level was found in the peripheral nerve. Moreover, in controls, the glucose level was lower in muscle than in blood. In hypoglycaemic rats, there were no significant differences in glucose concentrations between different tissues.
### Abbreviations

<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BB/Wor</td>
<td>BioBreeding/Worcester</td>
</tr>
<tr>
<td>CGMS®</td>
<td>Medtronic MiniMed Continuous Glucose Monitoring System</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>cpm</td>
<td>Count per minute</td>
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<tr>
<td>D</td>
<td>Fibre diameter (including myelin)</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>GN</td>
<td>Gastrocnemius nerve</td>
</tr>
<tr>
<td>g -ratio</td>
<td>Axon/fibre diameter ratio</td>
</tr>
<tr>
<td>$^3$H</td>
<td>Tritium</td>
</tr>
<tr>
<td>HbA$_{1c}$</td>
<td>Glycosylated haemoglobin</td>
</tr>
<tr>
<td>L</td>
<td>Internodal length</td>
</tr>
<tr>
<td>LGN</td>
<td>Lateral gastrocnemius nerve</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>MDP</td>
<td>Myelin degradation product</td>
</tr>
<tr>
<td>MGN</td>
<td>Medial gastrocnemius nerve</td>
</tr>
<tr>
<td>mOsm</td>
<td>Milliosmolar</td>
</tr>
<tr>
<td>OsO$_4$</td>
<td>Osmium tetroxide</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PG</td>
<td>Paraganglionic tissue</td>
</tr>
<tr>
<td>RR</td>
<td>Relative recovery</td>
</tr>
<tr>
<td>RL</td>
<td>Relative loss</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SN</td>
<td>Sural nerve</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
</tbody>
</table>
Background

The human nervous system is a complex and well-coordinated network that controls perceptive and responsive activities in the body. Not only does it monitor and respond to the internal and external environment, but it is also responsible for higher cognitive functions such as learning, remembering, pleasure and joy, decision making, and many other characteristic that are unique to each human beings. In other words, the nervous system is what makes us who we are.

The peripheral nervous system (PNS) is composed of all parts of the nervous system other than the brain and spinal cord (CNS), that is, the spinal nerves, most of the cranial nerves, and peripheral components of the autonomic nervous system. The PNS can be divided into a sensory and a motor division (Figure 1).

![Figure 1. The diagram illustrates the major components of the peripheral nervous system.](image)

Peripheral Nerves

The peripheral nerves composed of bundles and fascicles of nerve fibres that are collected together with a specified collagenous connective tissue (see below). The nerve fibres here denote axon with its associated Schwann cells. These cells enwrap the axon segment with which they are associated with an electrically insulating multilayered cell membrane specialisation, the myelin sheath. The sensory (afferent) axons can be either visceral or somatic. The somatic sensory nerve fibres convey information from the skin, muscle, and joints; the visceral sensory fibres send the
monitoring information from internal organs, such as the cardiovascular and digestive systems to the CNS. The motor (efferent) axons carry signals from the CNS toward the voluntary muscles (somatic) and the involuntary muscles or glands (visceral or autonomic; Figure 1).

Peripheral nerve tissue is relatively rich in blood and consists of axons, Schwann cells, blood vessels, lymphatic vessels, connective tissue, macrophages, and mast cells (Figure 2). Each single nerve fibre is surrounded by a loose connective tissue called the endoneurium. A group of fibres can be enwrapped by perineurium to form a fascicle, and an example of this is the sural nerve in the human at the level of ankle, which may comprise 6 to 14 fascicles. The perineurium is in turn covered with a tough fibrous sheath called the epineurium. In the peripheral nerve, axons constitute only a small fraction of the total volume of a nerve trunk (Figure 2).

![Diagram of a peripheral nerve](image)

**Figure 2.** The picture shows the structural details of a typical peripheral nerve.

An axon consists of neuronal cytoplasm (axoplasm) that is delimited by the neuronal cell membrane which is called the axolemma. The axoplasm consists primarily of cytoskeleton made up of microtubules, neurofilaments, and microtubular matrix, and it also contains organelles such as mitochondria, axoplasmic...
BACKGROUND

reticulum, dense lamellar bodies, multivesicular bodies, vesiculotubular profiles, membranous cisterns, and granular materials.

The axons in the PNS are accompanied by Schwann cells, and are classified either as unmyelinated or myelinated. Unmyelinated axons are distributed alone or in groups in longitudinal troughs formed by the Schwann cells. In a myelinated fibre, a single axon is associated with several longitudinally arranged Schwann cells. The meeting points between two such cells appears as a 1-µm-long myelin-free segment called the node of Ranvier. Such junctions contain a large number of voltage-sensitive sodium channels and can therefore support the fast depolarisation/repolarisation process that is necessary for saltatory conduction of action potential. The fibre segment between two nodes (internode) corresponds to the extension of one Schwann cells, and it can range in length for approximately 200 to 2000 µm. The length of the internodes is positively correlated with the size of the axon. In the PNS, myelinated axons can be more than 1–2 µm thick, whereas unmyelinated axons have a diameter of less than 2 µm. During development, a Schwann cells proliferates enormously and creates a one-to-one relationship with prospective myelinated fibres. However, once myelin production is initiated, the Schwann cells do not proliferate unless it is further induced by a pathologic process.

Peripheral Neuropathy

Peripheral neuropathy is a collective term for a spectrum of morbid conditions in the PNS, and it represents one of the most common diseases of the nervous system. The prevalence is between 0.8% and 8%, depending on the age group in question. In the elderly population with glucose intolerance, the prevalence increases from 8% to about 11%, and a rise from 32% to 50% is seen in diabetic patients. The peripheral neuropathies have a wide range of aetiologies and diverse clinical presentations. Because every peripheral nerve has a specialized function in a specific part of the body, the clinical manifestations of peripheral neuropathy vary widely. Mononeuropathy refers to damage of only one nerve, which causes sensory loss and/or muscle weakness in the territory of that nerve. However, peripheral neuropathy usually involves multiple nerves, and such cases are called polyneuropathies, many of which lead to sensory, motor, and autonomic dysfunction. These disorders are often symmetric and primarily affect extremities. This wide variation makes it difficult to obtain a universally accepted nomenclature. The classifications can be based on the type of nerve that is damaged, for example, they can consider sensory, motor, or autonomic neuropathy. Some conditions influence predominantly one type of nerve fibres, as in vitamin B12 deficiency, which injures sensory nerves. However, the most common picture is a mixed pattern of sensorimotor neuropathy, with or without autonomic components. The classification can also be based on the aetiology of the condition of interest (e.g., diabetic neuropathy
or nutritional neuropathy) or, as mentioned above, it can be categorised according to the pattern of nerves involved (i.e., mononeuropathy or polyneuropathy). Table 1 illustrates one approach to classification of peripheral neuropathies and accompanying aetiologies in humans.

Table 1. Classification of human peripheral neuropathy along with many of the causes

<table>
<thead>
<tr>
<th>Type of Neuropathy</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mononeuropathies</strong></td>
<td></td>
</tr>
<tr>
<td>Compression</td>
<td>Median carpal tunnel syndrome</td>
</tr>
<tr>
<td>Hereditary</td>
<td>Familial liability to pressure palsies</td>
</tr>
<tr>
<td>Inflammation; infection</td>
<td>Facial: Bell's palsy; herpes simplex</td>
</tr>
<tr>
<td>Multiple mononeuropathies (mononeuritis multiplex)</td>
<td>Vasculitis</td>
</tr>
<tr>
<td><strong>Polyneuropathy</strong></td>
<td></td>
</tr>
<tr>
<td>Hereditary</td>
<td>Charcot-Marie-Tooth disease(s)</td>
</tr>
<tr>
<td>Metabolic</td>
<td>Diabetes, uraemia, porphyria</td>
</tr>
<tr>
<td>Infections</td>
<td>Leprosy; diphtheria</td>
</tr>
<tr>
<td>Postinfectious (autoimmune)</td>
<td>Guillain-Barré syndrome</td>
</tr>
<tr>
<td>Toxic</td>
<td>Lead toxicity</td>
</tr>
<tr>
<td>Drug</td>
<td>Amiodarone, pyridoxine, toluene toxicity</td>
</tr>
</tbody>
</table>

Peripheral neuropathy can involve myelin and/or axons, (myelinopathy and axonopathy). In most cases that are due to systemic conditions (e.g. renal insufficiency), or are related to drugs and toxins, it is primarily the axons that are damaged \(^4, 5\). One of the underlying mechanisms in axonopathy entails impaired axonal transport, an effect that results in altered synthesis and delivery of neurofilaments, which leads to reduced axon calibre \(^6, 7\) and thereby causes axonal atrophy \(^8, 9\). Changes in axonal diameter and viability may in turn, give rise to secondary demyelinations and remyelinations \(^10, 11\). Neuropathy can also be induced by primary demyelination, which is believed to be due to autoimmunity \(^12, 13\). Of all the diseases that result in demyelination, chronic inflammatory demyelinating polyneuropathy and multiple sclerosis have been studied most extensively. Tsunoda and Fujinami \(^14\) focused on multiple sclerosis and considered the possibility of primary axonal pathogenesis, and indeed, it is known that primary demyelination gradually leads to secondary axonal loss \(^15\). Thus, the final picture of most neuropathic conditions will not be pure axonal or demyelinating neuropathy.

Electrophysiological testing is an integral part of the initial evaluation of peripheral neuropathy. Applying electrophysiological methods, after nerve biopsy examina-
tion, is the most accurate way of distinguishing between axonal neuropathy and demyelinating neuropathy (Fig 3)\(^4\), \(^5\). Axonal degeneration reduces both the compound muscle action potential and the sensory action potential, but the conduction velocity can be normal or only slightly reduced\(^16\). Demyelinating neuropathies, on the other hand, lead to reduced conduction velocities, protracted distal motor latencies, prolonged F-wave latencies, conduction block, and abnormal temporal dispersion\(^17\). Besides electrophysiological testing, there are many other methodologies, such as laboratory and imaging studies (not discussed here), that can be used for analysis of patients with peripheral neuropathy. Notwithstanding, 20–25% of the cases remain undiagnosed after all investigations\(^16\), \(^18\).

**Figure 3.** Typical pattern of muscle action potential after distal and proximal stimulation of a nerve. The upper trace of each pair is the record after distal stimulation. In demyelinating diseases the distal motor latency is prolonged and nerve conduction velocity slowed to less than 80% of normal. In axonal neuropathy, the muscle action potential is reduced, but the distal motor latency and nerve conduction velocity are essentially unaffected.

**Diabetic Neuropathy**

Neurological damage in patients with diabetes mellitus was first reported by Marchal de Calvi in 1864, and a hundred years later, diabetic neuropathy was still considered to be a single “homogeneous” condition\(^19\). It is now generally accepted that diabetic neuropathies are a group of “heterogeneous” neurological syndromes that can occur in both type 1 and type 2 diabetes. This is one of the most common complications of long standing diabetes and it is the main cause of hospitalisation of these patients. The frequency of this condition varies between 5% and 100%, depending on the definition and diagnostic methods used\(^20\), \(^21\). Diabetic neuropathy is
also the most common form of peripheral neuropathy in developed countries\(^{(21, 22)}\). Once symptomatic, the patients may complain of abnormal sensations mostly in the extremities (more common), as well as chronic pain, motor weakness and muscle atrophy (less common), and various symptoms of autonomic dysfunction. According to the San Antonio Conference\(^{(23)}\), the main clinical presentations of neurological disturbances in diabetes mellitus are as follows:

- Subclinical neuropathy, determined by abnormalities in electrodiagnostic and quantitative sensory testing.
- Diffuse clinical neuropathy with distal symmetric sensorimotor and autonomic syndromes.
- Focal syndromes.

Neurological complications in diabetes can also be classified on the basis of the different patterns of the PNS involvement (Table 2)\(^{(24, 25)}\).

**Table 2.** Major diabetic neuropathies

<table>
<thead>
<tr>
<th>Symmetric polyneuropathies</th>
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<tbody>
<tr>
<td>Sensory and sensorimotor polyneuropathy</td>
</tr>
<tr>
<td>Autonomic polyneuropathy</td>
</tr>
<tr>
<td>Acute painful neuropathy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Focal and multifocal neuropathies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial neuropathy</td>
</tr>
<tr>
<td>Thoraco-abdominal neuropathy</td>
</tr>
<tr>
<td>Focal limb neuropathy (including entrapment neuropathy)</td>
</tr>
<tr>
<td>Proximal diabetic neuropathy (diabetic amyotrophy)</td>
</tr>
</tbody>
</table>

| Mixed forms                                       |

Electrophysiological assessment of peripheral nerves can show the occurrence of neuropathy even before the onset of symptoms, but such evaluations cannot effectively reveal abnormalities in unmyelinated nerve fibres\(^{(26, 27)}\). These fibres are involved in aspects such as transmission of pain and thermal sensations. Generally, the pattern of nerve conduction irregularities in diabetic neuropathy is the result of the involvement or preservation of various peripheral nerves\(^{(21, 27)}\). Decreased sensory and motor response amplitudes, reduced conduction velocity, and conduction blocks are common electrophysiological abnormalities in diabetic neuropathy\(^{(26)}\).

Early diabetes-related changes in the morphology of peripheral nerves include Schwann cell abnormalities, degeneration/regeneration of unmyelinated fibres, and
microangiopathy\(^ {(28)} \). Later in the disease process, the dominant picture includes degeneration/regeneration, and demyelination/remyelination of myelinated axon\(^ {(29-31)} \). Although, these pathological changes have been studied extensively in both humans and animals, there is no general agreement regarding their aetiology, timing, and pattern of occurrence\(^ {(28, 32, 33)} \). Involvement of the PNS in diabetes is well recognised, whereas involvement of the CNS is a rather recent discovery\(^ {(34-37)} \).

It is assumed that the group of neurological syndromes that constitute diabetic neuropathies are induced by several pathogenic mechanisms, and the most important hypotheses in this context include the following:

- Metabolic alterations, such as increased polyol pathway flux\(^ {(38)} \), oxidative stress\(^ {(39, 40)} \) and non-enzymatic protein glycation\(^ {(41, 42)} \).
- Vascular dysfunction, leading to decreased blood flow and subsequent hypoxia in nerves\(^ {(43, 44)} \).
- Alterations in neurotrophic support\(^ {(45)} \).
- Defective axonal transport\(^ {(46)} \).
- Apoptosis associated with mitochondrial dysfunction\(^ {(47)} \).
- \( \text{Ca}^{2+} \) dysregulation as a common final pathway of neuronal damage\(^ {(48)} \).

Hyperglycaemia is believed to be the causative factor behind all of these mechanisms. Large prospective multi-centre studies in the United States and Europe have revealed that the onset and progression of neuropathic changes in both type 1 and type 2 diabetes can be delayed by improving the glycaemic control to attain glucose and HbA\(_{1c} \) levels that are close to normal\(^ {(49, 50)} \).

**Hypoglycaemic Neuropathy**

The term “hypoglycaemic neuropathy” denotes a situation in which very low blood glucose level triggers functional and structural abnormalities in the CNS and/or PNS, regardless of the underlying diseases. Hypoglycaemic neuropathy occurs predominantly in patients with insulin-producing adenomas (insulinoma)\(^ {(51-53)} \) or less commonly, in patients with diabetes who have experienced periods of low blood glucose due to accidental over dosage of insulin or insulin secretagogues agents\(^ {51, 54, 55} \). In latter condition, an imbalance between the antidiabetic medication used, food intake, and physical activity is a precondition for development of hypoglycaemic episodes.

Autonomic or neurogenic symptoms (e.g., sweating, tremor, and palpitation) and neuroglycopenic symptoms (e.g., confusion, mood changes, and diplopia) first appear when the blood glucose level falls below 3 mmol/l in healthy individuals,
whereas these alarming reactions occur at a much lower blood glucose concentrations in some of diabetic and insulinoma patients\(^{(52, 56, 57)}\). In a study by McAuley and colleagues\(^{(56)}\), almost 90% of insulin-treated diabetic patients experienced hypoglycaemic episodes. Furthermore, an increase in the occurrence of severe hypoglycaemia has been observed in diabetic patients receiving intensive insulin treatment compared to those given conventional treatment\(^{(58, 59)}\). According to Cryer and co-workers\(^{(60)}\), 2–4% of deaths in diabetic patients are associated with hypoglycaemia. The fear of hypoglycaemia has a negative impact on the everyday life of the patients, and it may also causes both patients and physicians to deliberately aim at less ambitious glycaemic control\(^{(61)}\).

According to most studies\(^{(62-66)}\) but not all studies\(^{(67)}\), a severe hypoglycaemia initially results in neuropsychological impairments such as memory and cognitive derangements. The symptomatic neuropathies in the PNS caused by severe hypoglycaemia is most common in insulinoma patients, but even in that group not more than 50 cases have been reported in the literature\(^{(52, 53, 68)}\). The symptoms are typically symmetrical, predominantly distal, and usually involve mainly the upper limbs. The patients initially complain of burning and tingling paraesthesias in hands and feet, although objective sensory loss is rare. Proximal muscle weakness is also one of the frequent presentations. Interestingly, after correction of the condition by removal of the insulin-producing adenoma, the sensory problems are completely resolved, but not the motor problems\(^{(53)}\).

Available knowledge about the development of hypoglycaemic neuropathy is very limited. It is recognised that many metabolic alterations can occur in hypoglycaemia, but their importance in the causation of peripheral nerve damage are not known. It has been established that increases in the activity of coagulation factor VIII\(^{(69)}\), rises in the concentration of fibrinogen, enhancement of adenosine diphosphate (ADP) induced platelet aggregation\(^{(70)}\), and changes in plasma volume\(^{(71)}\), are among the abnormalities that ensue in the course of hypoglycaemia. The low glucose level leads to diminished ATP synthesis\(^{(72)}\), which in turn decreases the ability of neurons to maintain the intracellular/extracellular electrolyte balance\(^{(73, 74)}\). This imbalance has various grave consequences, such as loss of membrane potential, which leads to an isoelectric EEG\(^{(75, 76)}\), and also the abundance of intracellular Ca\(^{+2}\), which is a common dead-end in various cytotoxic processes\(^{(40, 77)}\). However, the CNS and PNS differ both quantitatively and qualitatively with respect to the energy metabolism. The peripheral nerves are less dependent on glucose because they can utilize amino acids and fatty acids\(^{(78, 79)}\). Disturbed axonal transport in response to hypoglycaemia has also been observed\(^{(80-83)}\). In addition, microvascular insufficiency has been detected before the occurrence of structural changes in nerve fibres\(^{(84-86)}\).
The threshold and the minimum duration of a low blood glucose level that are necessary to initiate nerve damage are fairly known in laboratory animals but have not yet been identified in humans. Ohshima and Nukada (86) detected microvascular changes in the sciatic nerve of Spargue Dawley rats after 1–3 hours of hypoglycaemia (< 3 mmol/l). In another study (85), persistence of a low blood glucose concentration (mean 1.4 mmol/l) for more than 12 hours, but not ≤ 11 hours, resulted in fibre degeneration. In our experiments (87, 88), neuropathological changes were obvious after prolonged (3–4 months) hypoglycaemia (mean blood glucose 3–4 mmol/l) in diabetic rats.

Severe hypoglycaemia may lead to irreparable brain damage, mainly in the neocortex, hippocampus, thalamus, and hypothalamus, and the picture includes apoptosis and necrotic neuronal loss (89-91). In some investigations, neurons have been found to display chromatolysis and degenerative changes in the form of swelling, fragmentation, and atrophy of dendrites, and there are also reports of glial alterations such as proliferation and degeneration of astrocytes and swelling of oligodendrocytes (92). Agardh et al (93) observed minimal pathological changes in cerebral cortex in rats after 60 min of severe hypoglycaemia, whereas Auer and co-workers (76) were able to detect neuronal loss in the cortex of hypoglycaemic rats only after the onset of EEG isoelectricity, regardless of a state of severe hypoglycaemia.

Pathological sequelae in the PNS comprise de- and regenerations (52, 53, 94), and light and electron microscopic examinations have revealed axonal de- and remyelination in the sciatic, tibial, and sural nerves in hypoglycaemia (95, 96). The pattern and distribution of these pathological findings are not fully clear. The occurrence of regenerative sprouts has been observed in affected nerves, which indicates that at each given level, the proximal part is preserved (87, 88, 97). In addition, Mohseni found nerve fibre degeneration in diabetic eu-/ hypoglycaemic rats at distal level, but only some of those animals showed degeneration in ventral root axons (98). These evidences suggest that there is distal-to-proximal spreading of hypoglycaemic neuropathy.
Objectives

The goal of this study was to find new information about peripheral hypoglycaemic neuropathy in diabetes. In order to do this, we have tried to answer the following questions:

- How is the glycaemic state in our diabetic animals during insulin treatment (I)?
- How similar or different is the glycaemic state in our diabetic hyperglycaemic and hypoglycaemic rats compared to diabetic patients with poor glycaemic control (I)?
- Which types of peripheral nerve fibres are predominantly affected by severe hypoglycaemia (II, III)?
- Are there any differences between the impact of hyperglycaemia and hypoglycaemia on peripheral nerves (II, III)?
- Is the blood glucose level similar to that in other peripheral tissues such as nerve, muscle and skin (IV)?
- Is there any direct relationship between low tissue glucose level and the occurrence of peripheral hypoglycaemic neuropathy (IV)?
Methodological Considerations

This section describes the animal model and the techniques used in the present study, rather than give detailed information about the animals, materials and methods. Therefore, the reader may refer to separate papers (I–IV) for full, systematic descriptions.

Animal Model (I–IV)

We used a genetically defined type 1 diabetic rat that was originally called the BioBreeding or BB rat\(^{99, 100}\). This animal model was first described 1974 after occurrence of spontaneous diabetes in a group of offspring of Wistar rats at the BioBreeding Laboratories in Ottawa, Canada. Approximately 70–80% of these rats develop type 1 diabetes after autoimmune inflammation of the pancreas (insulitis), which results in complete destruction of pancreatic β cells, and thus the animals are dependent on exogenous insulin for survival. Glycosuria and hyperglycaemia as the first clinical manifestations of diabetes occur between the age of 40 and 120 days, with a mean of 65 to 90 days, according to different studies\(^{88, 97, 99, 101, 102}\). The onset of insulitis is seen 2–3 weeks before the clinical manifestation of diabetes. The pathogenesis of the disease in BB/Wor rats closely resembles human type 1 diabetes, and hence, these animals constitute a good model for studying spontaneous autoimmune diabetes in humans\(^{101}\).

We obtained the BB/Wor rats when they were about one month old, after they had been genetically labelled as either controls (heterozygous) or pre-diabetics (homozygous)\(^{100}\), which made it possible to reduce the number of animals that had to be purchased. Heterozygous siblings do not develop diabetes, and were used as healthy controls. The pre-diabetic rats in our studies showed the first signs of diabetes when they were about 65 days old, indicated by cessation of weight gain or weight loss and mild hyperglycaemia (7–10 mmol/l). According to available literature, this animal model has severe lymphopenia, which means the rats are more susceptible to infections\(^{103}\). However, we did not observe any clinical manifestation of infections in the rats we used. In order to maintain a stable glycaemic status, administration of insulin should be adjusted on a day-to-day basis. However, we used a subcutaneous insulin implant, which released a constant daily amount of insulin (see Diabetes Management). Thus, in order to obtain a more stable blood glucose level during treatment periods, we used female rats when it was possible, because they show slower weight gain and more stable insulin needs than the males.
The NOD (Non-Obese Diabetic) mouse is another valuable model for studying human type 1 diabetes\(^{104, 105}\). In the female animals, insulin-dependent diabetes appears by the age of three months and around 80–90% of each litter develops diabetes by the age of 30 weeks. In male NOD mice diabetes is delayed, and only 40–50% have the disease by the age of 30 weeks. We decided to use BB/Wor rats because they are larger than NOD mice. It was important for practical reasons, in two of the studies: the application of glucose sensors (I) and microdialysis probes (IV) would have been much more difficult in mice due to the smaller body size.

There are also other rat models available for studying diabetes such as streptozotocin (STZ) and alloxan-intoxicated rats, which have been used extensively in diabetes research\(^{106, 107}\). Both those compounds exhibit potent diabetogenicity, and pancreatic β cell death is induced by fragmentation of nuclear DNA in the case of STZ\(^{106}\). Nevertheless, these toxically induced animal models of diabetes are not ideal for investigating human type 1 diabetes.

**Diabetes Management (I–IV)**

There are several methods for administering insulin to diabetic animals, for example insulin injection, osmotic minipump or implant. Daily insulin injection has been used in many studies. However, that technique is time consuming, and it can result in more extensive fluctuation of the blood glucose level compared to the results of continuous delivery of insulin. We treated our rats for almost three months in the neuropathological studies (II, III). To achieve a relatively stable hyperglycaemic or hypoglycaemic state over that long period, we used a method involving continuous insulin delivery rather than insulin injection. We choose the subcutaneous insulin implant (Linplant\(^{®}\)), because it functions for four to six weeks, has small size (7 x 2 mm), and it is easy to insert. The implant is made of micro-recrystallised palmitic acid, and it releases about 2 U bovine insulin per day\(^{108}\).

In our studies, the goal of insulin treatment was to maintain constant high and low glucose levels in hyperglycaemic and hypoglycaemic rats, respectively. Therefore, the animals received insulin implants of different lengths depending on the current blood glucose level, the desired glycaemic state, and the body weight. Hyperglycaemic rats usually received a small piece of implant (3–4 mm) and hypoglycaemic animals often received a whole implant (7 mm). For rats in the hyperglycaemic group, we delayed the implant insertion a few days after the onset of clinical diabetes, to avoid unwanted hypoglycaemia, although on some occasions that happened anyway. Both hyperglycaemic and hypoglycaemic animals showed marked fluctuations in glucose levels during the first few days after implant insertion. In addition, the pattern of diurnal fluctuation we observed in the hyperglycaemic rats was very
similar to that seen in young type 1 diabetic patients with poor glycaemic control (I). Such diurnal fluctuation was less prominent in hypoglycaemic rats.

In hypoglycaemic rats, there were a few high blood glucose values during the insulin treatment period, which resulted in an unrepresentatively high mean blood glucose value. This phenomenon was more obvious in the second study (II), in which the mean and the median blood glucose level for the hypoglycaemic rats were 6.8 and 3.8 mmol/l, respectively. Therefore, to obtain a more representative estimation of the rats’ glycaemic status, we reported both the mean and the median glucose level (II, III). It should be mentioned that administration of a similar amount of insulin to different rats resulted in different blood glucose levels. Those disparities were probably the result of the current status of the animals with regard to glucose level, weight, amount of food intake and individual variation in metabolism. This was more pronounced in hypoglycaemic rats, in which the blood glucose should have been reduced from about 30 mmol/l to less than 3 mmol/l (Table 3).

**Table 3.** Biological information on control and diabetic female BB/Wor rats used in third study (III)

<table>
<thead>
<tr>
<th>Animals</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (day)</td>
<td>157</td>
<td>155</td>
<td>155</td>
<td>149</td>
<td>148</td>
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<tr>
<td>Weight (g)</td>
<td>244</td>
<td>222</td>
<td>239</td>
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<td>263</td>
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<td><strong>Hyperglycaemic group</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age (day)</td>
<td>157</td>
<td>168</td>
<td>158</td>
<td>160</td>
<td>167</td>
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<tr>
<td>Weight (g)</td>
<td>218</td>
<td>189</td>
<td>240</td>
<td>220</td>
<td>228</td>
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<tr>
<td>Days with BG ≥ 8 mmol/l</td>
<td>74</td>
<td>90</td>
<td>96</td>
<td>103</td>
<td>65</td>
</tr>
<tr>
<td>Days with BG ≥ 15 mmol/l</td>
<td>51</td>
<td>62</td>
<td>75</td>
<td>81</td>
<td>57</td>
</tr>
<tr>
<td><strong>Hypoglycaemic group</strong></td>
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<tr>
<td>Age (day)</td>
<td>149</td>
<td>172</td>
<td>179</td>
<td>141</td>
<td>148</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>225</td>
<td>253</td>
<td>275</td>
<td>244</td>
<td>225</td>
</tr>
<tr>
<td>Days with BG ≤ 3.5 mmol/l</td>
<td>49</td>
<td>47</td>
<td>46</td>
<td>31</td>
<td>63</td>
</tr>
<tr>
<td>Days with BG ≤ 2.5 mmol/l</td>
<td>27</td>
<td>22</td>
<td>22</td>
<td>10</td>
<td>38</td>
</tr>
</tbody>
</table>

Weight was measured at the end of the experiment. Diabetic animals were treated with insulin for 3–4 months. BG, blood glucose.

We also measured HbA$_1c$ at the end of the experiments in two studies (II, III). The results confirmed that the poor glycaemic control in our hyperglycaemic rats was associated with significantly higher HbA$_1c$ levels. However, there was no significant difference in HbA$_1c$ values between the control and the hypoglycaemic rats, which agrees with other studies showing that the HbA$_1c$ value is not a proper indicator of past hypoglycaemic episodes$^{(61, 94, 109)}$. 


Taken together, our findings show that it was appropriate to use insulin implants to manage diabetes in this animal model. The glycaemic control observed in the hyperglycaemic animals was very similar to that seen in young diabetic patients with poor glycaemic control (I). However, the severity and duration of hypoglycaemic episodes in our hypoglycaemic rats were not comparable to levels of those aspects in the majority of diabetic patients\(^{61, 102}\). Interestingly, almost all rats survived long-term severe hypoglycaemic periods. The only observed abnormality in the surviving animals was a characteristic grunting sound related to partial denervation of laryngeal muscles in those animals with lowest mean blood glucose levels (II, III).

**Anaesthesia (I–IV)**

In our studies animals were anaesthetised at following stages:

- At the time of insertion of insulin implants (< 1 min; I–IV).
- During application of the glucose sensor (< 30 min; I).
- Before perfusion (II, III).
- During the microdialysis procedure (> 3 hrs; IV).

Use of proper anaesthetics is an important ethical and practical issue in experimental research. There are various types of anaesthetic compounds available on the market, including barbiturates (e.g., pentobarbital and Inactin\(®\)), ketamine (Ketalar\(®\)), xylazine (Rumpon\(®\)), and volatile anaesthetics such as methoxyflourane (Metofane\(®\)) and isoflurane (Forene\(®\)). Unfortunately, all of these increase blood and tissue glucose levels to varying extents\(^{110-112}\). In addition, pentobarbital has a narrow therapeutic range\(^{113, 114}\), and there is always a risk that intraperitoneal injection of this drug will either not provide adequate anaesthesia or kill the animal. This risk was much higher in our metabolically unstable diabetic rats, and hence we used pentobarbital only as a pre-perfusion anaesthesia in two studies (II, III). When the rats were anaesthetised for longer periods, we used inhalation anaesthetics such as metofane (I, II) or isoflurane (III, IV). Isoflurane is a halogenated volatile anaesthetic that induces rapid loss of consciousness and allows rapid recovery due to its low solubility in blood and body tissues\(^{115}\). Isoflurane is always administered mixed with air and/or oxygen. In the healthy rats, but not the diabetic animals, the glucose level increased after the initiation of isoflurane anaesthesia. By meticulous adjustment of inhalatory isoflurane, we were able to reduce the effects of this compound on the blood glucose level and at the same time, achieve adequate analgesia. Briefly, by using the above mentioned anaesthetics in different studies, we got the desired results without loss of many animals.
**Continuous Glucose Monitoring System (CGMS®) (I)**

We investigated the effect of long-term hyperglycaemia and hypoglycaemia on the peripheral nerves (II–III) and also on the metabolic status in different tissues (IV). Therefore, it was important to check the glycaemic state of the animals, which we did by measuring the blood glucose level several times a week. We used a Glucometer Elite® to measure glucose in capillary blood obtained from the tail of healthy control and diabetic rats. This portable device offers reliable accuracy, and it is convenient to use in the setting of an animal laboratory (115-117). However, we measured blood glucose only during daytime, which is the resting time for the rats, and thus we could not gain a representative picture of the animals’ glycaemic state over a period of 24 h. To determine the glucose dynamics in our rats, we needed to achieve more precise and continuous long-term monitoring of blood glucose levels, but as far as we knew at that time no system existed for such analysis in small laboratory rodents. However, Medtronic Minimed did market a small device for diabetic patients, called the Continuous Glucose Monitoring System (CGMS®) (118), and it occurred to us that it might be possible to use that system for monitoring our rats. The CGMS is a glucose peroxide sensor that measures the subcutaneous (s.c.) glucose level every 10 s and records a mean value every 5 min over a period of three days (118, 119). The sensor is composed of a thin probe and a 1 x 2 cm plastic plate. This sensor system has been constructed on the premise that the blood and s.c. tissue have the same concentration of glucose (IV) (120-122). According to manufacturers, the monitor should be calibrated by registration of a capillary blood glucose value at least three times a day. The system has a sensitivity range of 2 to 22 mmol/l, and the glucose values outside that range cannot be used for calibration.

The glucose sensor was sutured to the back (interscapular area) of an animal while it was under light methoxyflurane anaesthesia, and the monitor was suspended above the cage (Figure 4). The attachment of the sensor was done in such a way that the animals could move and feed freely without confinement and stress during the three-day experiment. To find out the similarities and differences between glycaemic state of our rats and type 1 diabetic patients, we also analysed the CGMS data on twelve young type 1 diabetic patients with poor glycaemic control, which were randomly selected from records kept at the paediatric clinic of Linköpings Universitets Hospital (see Major Findings and Discussion).

The rats accepted the presence of the sensor after about half an hour and thereafter behaved normally during the rest of the monitoring time. On average the control and hyperglycaemic rats lost only 7 g of their body weight during the experiment, but no weight loss was detected in hypoglycaemic animals. There was no sign of skin inflammation or infection due to sensor attachment. The blood glucose level and the corresponding s.c. glucose concentration were statistically the same.
Figure 4. This photograph shows a rat with a MiniMed continuous glucose monitoring sensor at work. The lid of the cage has a longitudinal slot (arrowhead) that allows free movements of the cable. The monitor (arrow) is suspended in such a way that it can rotate freely.

Several studies have shown that there is a time difference between changes in blood and s.c. glucose\textsuperscript{(123, 124)}, and the CGMS software compensate for that difference by automatically delaying calibration of the monitor by 10 min\textsuperscript{(118, 119)}. We conclude that it was appropriate to use CGMS monitoring to investigate the glycaemic conditions in our control and diabetic rats. Two recent studies conducted by Wiedmeyer and colleagues have shown that the use of CGMS is also valid in healthy and diabetic dogs, cats, and horses\textsuperscript{(125, 126)}.

Light and Electron Microscopy (II, III)

We studied pathological changes in peripheral nerves by light microscopic (LM) examination of teased preparations (III) and electron microscopic (EM) evaluation of nerve cross-sections (II, III). The nerve samples were taken after perfusion of pentobarbital anaesthetised rats. Fixation of the tissues by perfusion is the best method, because it ensures penetration of the fixative (5% glutaraldehyde in phosphate buffer) to the deepest parts of a sample\textsuperscript{(127)}. After perfusion, nerve samples were carefully taken under a surgical microscope. In the study presented in paper II, the left vagus nerve was exposed at the level of the aortic arch, and samples (3 mm
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long) for EM assessment were taken (1) immediately rostral to the emergence of the recurrent laryngeal nerve and (2) just caudal to that level, as well as (3) from the left recurrent laryngeal nerve itself, just distal to its branching off from the vagus nerve (Figure 5). In the study reported in paper III, samples were collected from the lateral gastrocnemius nerve (LGN) for LM investigation of teased preparations and from the medial gastrocnemius nerve (MGN) for EM examination. Two samples (3 mm each) of the sural nerve (SN) were taken at the same level as in the gastrocnemius nerves (GNs) for LM and EM analyses. All samples were then post-fixed in a 2% osmium tetroxide (OsO4) solution. Fixation in glutaraldehyde in phosphate buffer followed by OsO4 is the best combination for preservation of structural details.

Figure 5. This schematic picture illustrates the sites of specimen collection along the left vagus nerve.

To process the teasing preparations (III), the nerve samples were treated with glycerine after post-fixation in OsO4. The nerve fibres separated from each other under a dissecting microscope, using two small sewing needles. The internodal length and diameter of 100 internodes were measured in a LM, because that approach is believed to give a representative picture of neuropathological alterations. Such LM examination of teased nerve fibres is probably the best way to studying the morphology of individual nerve fibres. It allows qualitative and quantitative
evaluation of consecutive internodes of the same myelinated fibres. These measurements of internodal lengths and fibre diameter can be done to detect axonal degeneration and regeneration, as well as segmental demyelination and remyelination. Irregularities in the myelin sheath and sprouting can also be visualised. However, the teasing procedure can produce artefacts, because the needles can cause excessive stretching and mechanical damage of the fibres. Therefore, it is important to recognise the artefacts and ignore the mechanically damaged fibres. Pretreatment of nerve samples with collagenase before osmication makes it easier to separate fibres from each other without causing mechanical damage\textsuperscript{(130)}.

For EM examination (II, III), nerve samples were dehydrated gradually in ethanol and embedded in Epoxy resin. Semi-thin cross sections (1 µm) were stained with toluidine blue for control of the entirety and the overall quality of the samples. Large myelin degradation products caused by degeneration of myelinated nerve fibres were visible at this stage. Ultra-thin (60–80 nm) sections of the samples were then collected on Formvar-coated copper grids\textsuperscript{(131)}. In an electron microscope biological material shows very weak contrast due to its limited ability to scatter electrons. Thus, the aim of “staining” of samples for electron microscopy is to improve the contrast by increasing its capacity to deflect electrons. Such staining can be achieved by increasing the electron density of the material by adding heavy metals, such as lead and uranyl. We used uranyl acetate and lead citrate to increase the contrast of our tissue sections. These two compounds are fairly unspecific with regard to how they react with different tissue components, although uranyl acetate does show greater affinity for structures that contain nucleic acids, like chromatin, ribosomes, or the mitochondrial matrix, and lead citrate is more specific for membranes and glycogen granules\textsuperscript{(132)}.

In addition to qualitative assessment of nerve cross sections, we used morphometric methods to evaluate nerve damage. Quantitative analysis of peripheral nerves is an important tool for investigation and classification of neuropathy. Morphometric assessments can be performed to determine aspects such as the diameter distribution of myelinated fibres, axonal area and diameter, the number of myelin sheath lamellae, the density of myelinated fibres, and the relationship between axonal and myelin thickness in health and disease. In morbid conditions, the number and diameter distribution of myelinated fibres can be altered due to axonal atrophy, degeneration and regeneration, as well as demyelination and remyelination. Counting the total number of myelinated and unmyelinated fibres can be done to evaluate nerve fibre loss caused by axonal degeneration. In the studies described in papers II and III, the total number of unmyelinated fibres was counted directly in the electron microscope in recurrent laryngeal nerve and the MGN. In the vagus and sural nerves, however, the number was calculated by sample count due to the presence of numerous unmyelinated axons. Measuring the myelinated fibre diameter (D, includ-
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Estimation of the ratio between axon diameter (d) and fibre diameter (D), called the $g$-ratio, is an indicator of the presence or absence of axonal atrophy. Myelinated fibre occupancy is the percentage of the cross sectional area of a nerve that consists of myelinated fibres and a decreased value may indicate the presence of oedema or replacement of the fibres by collagen. Considering myelinated fibre density, that is, the number of fibres per unit area, a decreased value can also indicate fibre loss, whereas a high density suggests the presence of many small myelinated fibres.

**Microdialysis (IV)**

*In vivo* microdialysis is performed to measure the chemical composition of the interstitial fluid. The method is based on extraction of soluble molecules from the intercellular space by using a semi-permeable membrane\(^{(133-135)}\). It was initially developed to measure concentration of different neurotransmitters in the CNS *in vivo*\(^{(136)}\). However, this technique has now found its place in various research fields, including the study of carbohydrate metabolism and diabetes\(^{(137, 138)}\). Theoretically, presence of a microdialysis tube (made of a semi-permeable membrane) in a water-based environment leads to exchange (diffusion) of molecules between the inside and outside of the tube\(^{(139)}\). The different molecules move independently of each other, and the direction of this traffic is determined by concentration gradient of each type of compound. After a while, a state of equilibrium will be reached, which means that the concentration of molecules is equal on both sides of the membrane. The pore size of the membrane determines what molecules can pass through, and this is expressed as the “cut-off size” given in Daltons (Da). For example, only particles that are 5000 Da or smaller, can pass through a membrane that has a cut-off size of 5000 Da. The electrical charge of the molecules and their stickiness in relation to the membrane are other factors that are involved in the permeability of a membrane for a specific compound\(^{(135)}\).

There are different types of microdialysis probes (Figure 6)\(^{(140)}\). The most common types are made up of a simple tube in which the two ends are glued to an inert nylon hose, or they consist of a concentric double-layered probe in which the semi-permeable membrane forms the outer covering that is in contact with tissue. We used the latter type, specifically, a CMA/20 probe, which has a 4 or 10 mm long membrane and a cut-off size of 20,000 Da. While functioning, the inlet of the probe is connected to a micro-syringe in a precision pump, and there is a persistent flow of a physiologic solution (perfusate) inside the probe. The perfusate enters the exchange area, where the semi-permeable membrane is in contact with a water-based environment (*in vitro or in vivo*), and it flows back through the outlet of the probe to

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arrive at collecting vials. This continuous flow prevents molecules on the inside and outside of the probe from reaching complete equilibrium. Thus, depending on the flow rate, the perfusate can “recover” a fraction of metabolites from the outside environment.

**Figure 6.** This schematic picture shows structural basis of two types of microdialysis probes. **A**, perfusate; **B**, dialysate.

The proportion of a certain substance that a probe can recover from an environment is called the relative recovery (RR). The RR of a specific compound, for example glucose, can be calculated as following:

\[ RR = \frac{\text{glucose}_{\text{dialysate}}}{\text{glucose}_{\text{medium}}} \times 100 \]

in which glucose_{dialysate} is the concentration of glucose in the sample obtained from the microdialysis probe, and glucose_{medium} is the glucose concentration in the environment outside the probe (in vitro or in vivo). In addition to the factors mentioned above, there are many other aspects that impinge on the RR for a specific probe and a specific molecule, and some of the most important ones are the chemophysical properties of the membrane, the composition and flow rate of the perfusate, the water content of the tissue, the temperature, and the structural complexity and solid elements of the external environment\(^{140, 141}\). The question is how can we define the RR of a desired substance in a specific setting, because that value is needed to find the “true” tissue concentration of the target substance. In other words, we have to “calibrate” the raw results obtained from collected samples (i.e. the dialysate). However, there is a certain setting that does not need to be calibrated, namely when using a probe with a long membrane (> 20 mm) and a very low perfusion rate (< 0.5 µl/min) to study small molecules (e.g. glucose, urea or some neurotransmitters). In this setting, it is assumed that the concentration of a compound is the same in the
tissue and the dialysate, and hence the RR approaches 100% (i.e. > 95%)\(^{(142-144)}\). Although, this is the preferred method in some studies, it has two disadvantages. First, due to the very low flow rate, the collecting periods should be long in order to obtain adequate amounts of sample for required analyses. However, as a result of the long sampling period, it is not possible to detect the dynamics of changes in concentration of the target substance\(^{(145, 146)}\). The second disadvantage is that there is not always enough space for a 20–30-mm long probe in some tissues, particularly in small laboratory animals.

For studies \textit{in vivo}, several approaches for calibration of a microdialysis probes have been described in the literature\(^{(134)}\). In the study reported in paper IV, we employed the internal reference method\(^{(147-149)}\), which is based on the assumption that the RR of each substance under certain conditions is similar to the proportion of the molecules of the same compound that move from the perfusate towards the tissue. This value is called relative loss (RL), and to measure the RL of a compound, the radiolabeled form of the same molecule is added in the perfusate. The following formula can be used to calculate the RL:

\[
\text{RL} = \frac{\text{cpm}_{\text{perfusate}} - \text{cpm}_{\text{dialysate}}}{\text{cpm}_{\text{perfusate}}} \times 100
\]

in which cpm\(_{\text{perfusate}}\) and cpm\(_{\text{dialysate}}\) (cpm = counts per minute) represent the radioactivity of the perfusate and the dialysate fluids, respectively. The complexity of the microdialysis procedure and the difficulties involved in calculating the RR \textit{in vivo} make it necessary to use an individual functional setting (e.g. concerning the type of probe and the calibration method) for each experiment. Thus, we performed trials both \textit{in vitro} and \textit{in vivo}, and the former experiments were used to ascertain whether the RR and RL of glucose were the same in our experiments. Briefly, the probes were placed in glass beakers containing a glucose solution and were perfused with a physiologic fluid supplemented with radiolabeled glucose. Thereafter, the glucose level and the amount of radioactivity were measured in dialysate. The perfusate were analysed for its radioactivity. We even measured the glucose level in the glucose solution in the glass beakers. By calculating the RL of radioactivity and measuring the RR of glucose, we were able to verify the reliability of our experimental setting. The results showed that the RR and the RL \textit{in vitro} were the same. The mean RR and RL were 32.3 ± 2.0% vs. 33.4 ± 2.0% for the 4-mm probe (1 \(\mu\)l/min) and 46.4 ± 2.2% vs. 47.9 ± 2.5% for the 10-mm probe (1.5 \(\mu\)l/min). In addition, the mean glucose concentration in the solution in the beakers (measured directly in those containers) was similar to the mean concentration calculated from microdialysis analysis (5.1 ± 0.03 vs. 5.3 ± 0.08 mmol/l). These results showed that the probes obtained a reliable RR \textit{in vitro}; they also clearly demonstrated the effect of membrane length and flow rate on RR. For detection of tissue glucose concentration, we used \textit{in vivo} calibration (see below).
In the study described in paper IV, we performed microdialysis in healthy rats, diabetic hyperglycaemic rats, and diabetic hypoglycaemic rats. After initiation of anaesthesia, a probe (CMA/20, 4-mm-long membrane) was inserted in the right jugular vein. Other probes (CMA/20, 10-mm-long membrane) were inserted in the sciatic nerve, gastrocnemius muscle, and skin of the hind paw. Heart rate and blood oxygen saturation were monitored during the operation. After 30 min of stabilisation, perfusion of all probes was started at a rate of 1 µl/min using a perfusate supplemented with radiolabeled glucose (\(^3\)H-glucose; 1600 cpm/µl). Four samples (4 x 10 min) were collected for each tissue probe, and one blood glucose measurement was done in the middle of each sampling period using a drop of blood collected from the tail and the Glucometer Elite device. The radioactivity in the perfusate and dialysate samples was analysed in a liquid scintillation counter. The rest of the samples were stored at \(-20^\circ\)C pending measurement of glucose on a CMA600® system.

The RR of the probes in vivo proved to be stable over time (IV). In addition, the mean RR of jugular vein probes was similar to the corresponding RR in vitro. However, for the probes placed in other tissues (nerve, muscle, and skin), the RR in vivo was significantly lower than that in vitro. This difference was expected, and it reflected the effect of interstitial factors (e.g., water content and structural properties of the tissue) on the RR of the probe. The results of our experiments in vivo clearly showed that the method overestimated the blood glucose concentration when calibrated data were compared with the results obtained using the Glucometer Elite. This problem has also been observed by other investigators\(^\text{149}\), and suggests that the RR and RL in vivo are not necessarily the same, and the internal reference calibration does not give a correct interstitial glucose concentration in certain experimental settings. The reason is that the internal reference method results in an underestimation of RR in vivo.

In conclusion, we believe that the microdialysis method, with its advantages and disadvantages, is a valuable technique for monitoring of the chemistry of the extracellular space in different tissues in vivo. It is less invasive than taking tissue biopsies, and it does not disturb tissue homeostasis. Nevertheless, the task of calibrating and converting of the data obtained into real tissue values do not seem to be completely resolved, and thus the results should be interpreted cautiously.
Major Findings and Discussion

Continuous Glucose Monitoring in Control and Diabetic Rats, and in Type 1 Diabetic Children (I)

The blood glucose in nondiabetic rats fluctuated around a median of 6 mmol/l, within a relatively narrow range (4 and 9 mmol/l), and they created the normal pattern in our BB/Wor rats. The blood glucose data for the hyperglycaemic rats varied considerably more, with levels over 9 mmol/l in more than 50% of the measurements, and below 4 mmol/l in about 10%. In contrast, there was less variation in the hypoglycaemic animals with blood glucose levels below 4 mmol/l on more than 50% of occasions, and rarely over 9 mmol/l. (Table 4; Figure 7).

Table 4. Capillary blood glucose and subcutaneous glucose levels in control and diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Blood glucose (mmol/l)</th>
<th>Subcutaneous glucose (mmol/l)</th>
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<tr>
<td></td>
<td>Range</td>
<td>Median</td>
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<tr>
<td>Control rats (n=6)</td>
<td>4.9-10.4</td>
<td>6.0</td>
</tr>
<tr>
<td>Hyperglycaemic Rats (n=6)</td>
<td>3.2-28.9</td>
<td>11.0</td>
</tr>
<tr>
<td>Hypoglycaemic Rats (n=6)</td>
<td>1.1-11.5</td>
<td>3.2</td>
</tr>
<tr>
<td>Type 1 diabetic Patients (n=12)</td>
<td>2.3-31.0</td>
<td>10.5</td>
</tr>
</tbody>
</table>

* Blood glucose concentrations measured with a Glucometer Elite.  
  b Since the Medtronic MiniMed glucose monitor has a limited sensitivity range (2.2-22.0 mmol/l), median values are more accurate than mean±SD.

Each sensor on average recorded subcutaneous (s.c.) glucose values during 65 h out of a total of 72 h of continuous monitoring. In control rats, the values fluctuated in a range of 4.0 to 9.0 mmol/l for about 90% of the time. The fluctuations had a short-wave pattern with mean amplitude of less than 1 mmol/l. The s.c. glucose values correlated with the corresponding blood glucose values (r = 0.7). The s.c. glucose in hyperglycaemic rats was more than 9.0 mmol/l for about 70% of the time. However, it oscillated significantly and during a typical day was below 4.0 mmol/l for about half an hour. The s.c. glucose values agreed with the corresponding blood glucose values (r = 0.9). In the hypoglycaemic rats, the s.c. glucose concentration fluctuated less, and values above 9.0 mmol/l were rarely found. The s.c. glucose level was
below 4.0 mmol/l for about 70% of the time. The blood glucose levels were consistent with the corresponding s.c. glucose values ($r = 0.9$).

**Figure 7.** Representative examples of subcutaneous glucose recordings in (A) a normal control rat, (B) an insulin-treated diabetic rat with a hyperglycaemic regime, (C) an insulin-treated diabetic rat with a hypoglycaemic regime and (D) a young Type I diabetic patient. [•] indicates time points when the monitor was calibrated with blood glucose values. In Figure D [♦] indicates meals and [◆] indicates insulin injections.

In type 1 diabetic patients the blood glucose concentration was at a hyperglycaemic level (>$8.0$ mmol/l) on 60% of the occasions, and about 5% of the measurements showed a hypoglycaemic level ($<3.0$ mmol/l). In the average patient, the sensor recorded s.c. glucose values over a period of approximately 65 h. The s.c. glucose
values fluctuated markedly, and the levels were above 8.0 mmol/l for about 65% of
the time, and they were occasionally below 3 mmol/l. The corresponding s.c. glu-
cose and blood glucose levels were in accordance ($r = 0.8$).

In this study, we analysed the glycaemic state over a period of three days in con-
trol and insulin-treated diabetic rats and in type 1 diabetic patients with poor
glycaemic control. We did not find any similar investigation in the literature, which
is surprising, considering the extensive use of rats and other rodents in diabetes re-
search.

In hyperglycaemic diabetic rats, the s.c. glucose concentration exhibited obvious
short-wave fluctuations during the monitoring time. This was not unexpected, since
the release of insulin from the implant is constant, but the rats’ physical activity and
eating behaviour vary over a period of 24 h. The long-wave fluctuations of the s.c.
glucose level (Figure 7 B) might be due to diurnal variations or some other physio-
logical parameters. All of the diabetic patients we studied were hyperglycaemic
most of the time. Interestingly, the hyperglycaemic rats and the type 1 diabetic pa-
tients had very similar pattern of glycaemia (Figure 17 B, D) indicating that diabetic
BB/Wor rats treated according to our protocol represent a valuable animal model
for investigating the disease. In our hypoglycaemic rats, the blood and s.c. glucose
levels were very low almost all the time. It is known that intensive insulin treatment
is associated with significant increase in the incidence of severe hypoglycaemia in
type 1 diabetic patients when it compared to conventional insulin therapy, but the
severity and duration of hypoglycaemia in our rats may not be representative for
diabetic patients in general$^{53, 150, 151}$. Nevertheless, diabetic patients with hypogly-
caemia unawareness or insulinoma patients may have glycaemic states similar to
those seen in our hypoglycaemic rats$^{53, 68}$.

**Hyperglycaemic Neuropathy**

**The Vagus Nerve (II)**

Hyperglycaemic rats (mean glucose level = 18.8 mmol/l) were similar to controls
with regards to the qualitative EM picture of the proximal part of the nerve (Figure
8). The presence of a few myelin-like bodies in some animals was judged to be
normal. The cross sections of the proximal part of the vagus nerve could be divided
into three different parts, which we designated A, B, and C based on nerve fibre
composition (Figure 8). Part A was composed mainly of large myelinated axons,
along with some medium-sized and a few small myelinated axons. There were also
a few groups of unmyelinated axons at the outer margins, close to the perineurium.
MAJOR FINDINGS AND DISCUSSION

Figure 8. Survey electron micrographs showing complete cross-section from the proximal level of the left vagus nerve in a control rat. The interrupted lines indicate the approximate course of the borders between areas A, B and C. Note the distinct presence of thick myelinated axons in area A. Some large myelinated axons are also present in areas B and C.

Figure 9. Survey electron micrograph illustrating the anatomy of the left vagus nerve, distal to the level of the recurrent branch in a normal control rat. In terms of fibre composition the picture resembles that seen in part C at the proximal level of the nerve.

Part B contained mainly small myelinated axons, and it formed a zone adjacent to part A that separated it from the rest of the nerve. In addition, part B included a few scattered medium-sized and large myelinated axons and groups of unmyelinated axons. Part C was composed primarily of unmyelinated axons, and there were also many small and medium-sized myelinated axons and few large myelinated fibres.
MAJOR FINDINGS AND DISCUSSION

Sections from the distal level of the vagus nerve displayed a microscopic anatomy similar to that seen in part C of the proximal level (Figure 9). In addition, the recurrent laryngeal nerve was composed of fibres from part A and B at the proximal level of the vagus nerve (Figure 8, 10). The mean total number of axons and the mean diameter of myelinated axons in hyperglycaemic rats in the vagus and recurrent laryngeal nerves were normal (Table 5). Paraganglionic tissues were found at all levels examined.

Figure 10. Survey electron micrographs showing complete cross section from the recurrent laryngeal branch of the left vagus nerve in a normal control rat. The interrupted lines indicate the approximate course of the borders between area A and B (PG = paraganglionic tissue). Note that areas A and B in this section resemble areas A and B at the proximal level of the vagus nerve trunk in terms of fibre composition.

The vagus nerve is the tenth cranial nerve, and as such it is the major parasympathetic autonomic branch innervating the thoracic and abdominal organs. Proximal to the aortic arch, the vagus nerve gives off the recurrent laryngeal nerve, which is a mixed nerve containing motor fibres that innervate all laryngeal muscles, except the cricothyroid, via motoneurons in the nucleus ambiguous. In addition, the recurrent nerve mediates afferent axons from the infraglottic area of the larynx to the nucleus tractus solitarii. The vagus nerve continues its way to thoracic and abdominal organs at its distal part of the nerve. Thus, the nerve has an interesting microscopic anatomy at the level of the aortic arch. The accessibility of the vagus nerve at distal (abdominal) and proximal (cervical) levels makes it suitable for studies of the autonomic nervous system.

The analysis of vagus nerve specimens from our hyperglycaemic rats showed no pathological changes. In the thoracic part of the vagus nerve in hyperglycaemic BB/Wor rats, Yagihashi and Sima and Zhang et al. found increased numbers of axonal glycogenosomes and axonal sequestration, as well as decreased mean fibre size in both myelinated and unmyelinated axons. On the other hand, Sharma and
MAJOR FINDINGS AND DISCUSSION

Thomas\textsuperscript{(159)} did not observe any pathological features in the vagus nerve of STZ-induced hyperglycaemic diabetic rats. It is conceivable that the varying presence of signs of neuropathy in different studies can be explained in terms of the severity and/or the temporal extension of hyperglycaemia, and inherent differences in various animal models used. We conclude that the hyperglycaemic regime used in our study does not elicit pathological alterations at the examined level of the vagus nerve.

Table 5. Morphological data from the left vagus and recurrent laryngeal nerves

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hyperglycaemic</th>
<th>Hypoglycaemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
</tr>
<tr>
<td></td>
<td>(mean±SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
</tr>
<tr>
<td>Proximal vagal level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of myelinated axons</td>
<td>1 578 ± 173</td>
<td>1 584 ± 122</td>
<td>1 535 ± 196</td>
</tr>
<tr>
<td>Diameter of myelinated axons (µm)</td>
<td>part A 5.7 ± 2.1</td>
<td>5.9 ± 2.2</td>
<td>3.9 ± 1.7 (p&lt;0.001)\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal vagal level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of myelinated axons</td>
<td>993 ± 146</td>
<td>1 064 ± 194</td>
<td>1 022 ± 159</td>
</tr>
<tr>
<td>Number of unmyelinated axons</td>
<td>15 790 ± 1 940</td>
<td>15 853 ± 2 575</td>
<td>17 042 ± 2 267</td>
</tr>
<tr>
<td>Diameter of myelinated axons (µm)</td>
<td>part A 2.9 ± 1.1</td>
<td>3.0 ± 1.1</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>Recurrent laryngeal nerve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of myelinated axons</td>
<td>458 ± 73</td>
<td>389 ± 110</td>
<td>468 ± 103</td>
</tr>
<tr>
<td>Number of unmyelinated axons</td>
<td>454 ± 81</td>
<td>355 ± 127</td>
<td>418 ± 109</td>
</tr>
<tr>
<td>Diameter of myelinated axons (µm)</td>
<td>part A 5.7 ± 2.0</td>
<td>5.3 ± 2.0</td>
<td>3.7 ± 1.5 (p&lt;0.001)\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Significantly different from control and hyperglycaemic values. \textsuperscript{b} Significantly different from hyperglycaemic value. \textsuperscript{c} (n = 5)

The Lateral and Medial Gastrocnemius and Sural Nerves (III)

The average hyperglycaemic rat (mean glucose level = 22 mmol/l), weighed significantly less than the controls. The qualitative evaluations of the LM and EM preparations of MGN (Figure 11 A, B) and SN (Figure 11 D, E) showed a similar picture as in the controls. There were no signs of degeneration and regeneration, demyelination, or nodal pathology in myelinated fibres. The EM analyses indicated that unmyelinated nerve fibres, and other elements, such as perineurium, endoneurial connective tissue, and blood vessels, were similar to the controls. In addition, quantitative analyses of the MGN resulted in normal values (Table 6).
### Table 6. Morphometric data on lateral and medial gastrocnemius (LGN and MGN) and sural nerves (SN) of control and diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>LGN (LM)</th>
<th>MGN (EM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L (µm)</td>
<td>D (µm)</td>
</tr>
<tr>
<td><strong>Control</strong> (n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>708.7±45.6</td>
<td>8.3±0.6</td>
</tr>
<tr>
<td><strong>Diabetic hyperglycaemic</strong> (n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>702.6±61.7</td>
<td>8.4±0.6</td>
</tr>
<tr>
<td><strong>Diabetic hypoglycaemic</strong> (n=5)</td>
<td>496.5±136.6 (p=0.01)</td>
<td>6.5±1.1 (p=0.01)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>SN (LM)</th>
<th>SN (EM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L (µm)</td>
<td>D (µm)</td>
</tr>
<tr>
<td><strong>Control</strong> (n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>602.8±48.8</td>
<td>6.3±0.6</td>
</tr>
<tr>
<td><strong>Diabetic hyperglycaemic</strong> (n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>521.6±76.0</td>
<td>5.2±0.4 (p=0.008)</td>
</tr>
<tr>
<td><strong>Diabetic hypoglycaemic</strong> (n=5)</td>
<td>495.8±41.2 (p=0.006)</td>
<td>5.4±0.3 (p=0.01)</td>
</tr>
</tbody>
</table>

The diabetic animals were treated with insulin implants during three months. LM, light microscopy; EM, electron microscopy; L, internodal length; D, fibre diameter including myelin sheath; MDP, mean number of myelin degradation products seen in nerve cross-sections; MF, no. of myelinated fibres; UMF, no. of unmyelinated fibres; g-ratio, axon/fibre diameter ratio; %, percentage of total no. of fibres; p, significant difference compared to control *, and hyperglycaemic rats b. Data are presented as mean ± SD.
In the SN, internodes were significantly thinner \((p = 0.008)\) compared to controls (Figure 12 D, E). The fibre diameter distribution histogram obtained from EM measurements was shifted to the left compared to controls (III). Moreover, the decreased mean fibre diameter in individual animals was correlated with reduced body weight \((r = 0.8)\). The number of myelinated and unmyelinated fibres and the level of myelinated fibre occupancy and the \(g\)-ratio were normal in both the MGN and SN. Density of myelinated fibres was normal in the MGN but increased in the SN (Table 6).

In our hyperglycaemic animals, the mean diameter of myelinated fibres in the SN, but not in the GN, was significantly smaller compared to controls. In contrast to what was anticipated, we did not observe any signs of axonal degeneration and regeneration or demyelination and remyelination, which are typical findings in diabetic polyneuropathy\((28-30)\). Thus, the smaller diameter must have been caused by some other factors. Decreased nerve fibre diameter in the absence of axonal degeneration and regeneration in diabetic animals has also been reported by other researchers\((159-162)\), who have suggested that the underlying factors could be hypero-
smolarity, deranged axonal metabolism, loss of neurofilaments, or retarded growth\textsuperscript{(160, 163, 164)}. We recorded normal $g$-ratios in both the SN and MGN in different groups of animals, indicating that there were no axonal atrophy. In addition, the occupancy of myelinated fibres in both the MGN and SN of the hyperglycaemic rats was normal, whereas the density of myelinated fibres in the SN was significantly higher than normal. Taken together, these observations seem to imply that the smaller fibre diameter and higher myelinated fibre density in the SN of these rats might have been due to retarded growth or abnormal metabolism. Whatever the mechanisms were, the results do suggest that hyperglycaemia affects somatic sensory nerves but not somatic motor nerves in diabetic BB/Wor rats\textsuperscript{(165)}.

**Hypoglycaemic Neuropathy**

**The Vagus Nerve (II)**

The mean and median blood glucose levels in diabetic hypoglycaemic rats during the entire treatment period were 6.4 and 3.8 mmol/l, respectively. In part A of the
proximal level of the left vagus nerve, the occurrence of large myelinated axons was reduced to varying degrees compared with control and diabetic hyperglycaemic rats (Figure 13). Many Schwann cell-like or macrophage-like cells were associated with MDP (Figure 14 A) or with lipid droplets (Figure 14 B). In addition, unmyelinated and/or small myelinated axons formed structures similar to regeneration unit (Figure 14 B, C). We occasionally found Schwann cells containing large myelin aggregates, some of which were devoid of axons and/or included collagen pockets (Figure 14 D). The number of myelinated fibres in these samples was similar to what was seen in control and hyperglycaemic rats. In hypoglycaemic rats, the mean myelinated fibre diameters in part A was significantly subnormal (see Table 5). The size distribution was unimodal and skewed to the left (II).

Figure 13. The picture illustrates complete cross-section from the proximal level of the left vagus nerve in a diabetic hypoglycaemic rat. The interrupted lines indicate the approximate course of the borders between areas A, B and C. This has been one of the most severely affected hypoglycaemic rats. Areas A, B and C contain very few large-diameter myelinated axons.

The samples from the distal part of the vagus nerve showed no evident pathology, except for the presence of a few myelin-like profiles. There was no sign of axonal loss or statistically significant decreased mean fibre diameter (Table 5). Myelinated fibres in the recurrent nerve in hypoglycaemic rats often exhibited de- and regenerative features, and these were much more frequent in part A than in part B (Figure 15). The occurrence of such features varied between individual rats. The morphol-
ogy of unmyelinated axons in these nerves was normal. In part A, but not part B, the mean diameter of myelinated fibres was subnormal. The size distribution histogram of myelinated axons in part A shifted to the left, indicating the disappearance of larger fibres.

**Figure 14.** Electron micrographs illustrating some representative pathological features observed in transverse sections from part A of the proximal vagus nerve segment or part A in the recurrent laryngeal branch in hypoglycaemic rats. **A.** Proximal vagus nerve segment. This electron micrograph shows a large myelin degradation product (MDP) enclosed within a basal-lamina covered Schwann cell. Note the presence of a myelinated axon in direct association with this cell (asterisk). Two unmyelinated axons are related to a separate Schwann cell profile in the vicinity (arrows). Altogether, this seems to represent a small regenerating unit. **B.** Proximal vagus nerve. The picture shows a basal lamina-covered putative Schwann cell loaded with lipid droplets (L). A myelinated axon (asterisk) and an unmyelinated axon (arrow) are associated with separated Schwann cell profile in the vicinity. **C.** Recurrent laryngeal branch. The picture presents an example of the frequently encountered regenerating units (SC = Schwann cell). This unit includes five myelinated axons (asterisks) and five unmyelinated axons (arrows). **D.** Proximal vagus nerve. The picture shows an orphan basal lamina-covered putative Schwann cell (SC) that is devoid of axons and exhibits so called collagen pockets (arrowhead).

Examination of the pathological changes in the vagus and recurrent nerves in the hypoglycaemic rats revealed extensive degeneration of large myelinated fibres. These neuropathologies were positively related to degree and duration of hypogly-
caemia, which has also been reported by other investigators (85). As discussed earlier, most of the large myelinated fibres in the rat vagus nerve are motor axons en route to laryngeal muscles (152, 166). Hence, the de- and regenerative features observed in part A at the proximal level and in part A of the recurrent nerve must have affects the laryngeal muscles. At the same time, the presence of many regenerating compounds suggests that an active regeneration process was in progress in these nerves, which is confirmed by the fact that the total number of myelinated fibres was not reduced. The regenerating fibres found in proximity to remnants of large myelinated fibres indicated that the degeneration spared the proximal part of the affected axons, which was then able to emit regenerating sprouts. This picture resembles the pattern that has previously been observed in hind limb nerves and lumbar ventral roots in hypoglycaemic rats (99). The absence of obvious signs of neuropathological changes in small myelinated and unmyelinated fibres may indicate that sensory and autonomic components of the vagus nerve were not affected. It is not known whether the presence or absence of pathology depends on fibre type, fibre calibre, or the nature of the different target tissues.

Figure 15. This electron micrograph showing complete cross section from the recurrent laryngeal branch of the left vagus nerve in a severely affected hypoglycaemic diabetic rat. The interrupted lines indicate the approximate course of the borders between area A and B (PG = paraganglionic tissue). Note that areas A and B in this section resemble areas A and B at the proximal level of the vagus nerve trunk in terms of fibre composition. It illustrates many degenerative features and that area A is devoid of large-size myelinated axons. These seem to have been replaced by myelinated axons with small/medium-sized diameters. Area B has also lost all large-diameter myelinated axons.
The Lateral and Medial Gastrocnemius and Sural Nerves (III)

Qualitative assessment of teased LGN and SN from hypoglycaemic rats, with mean and median blood glucose levels of 4.1 and 3.1 mmol/l, respectively, revealed numerous signs of past and ongoing degeneration of large myelinated nerve fibres. Many larger myelin sheaths were wrinkled or had disintegrated into large ovoids or rows of smaller ovoids and clusters of myelin debris. However, the SN had markedly fewer pathological features than the LGN. Paranodal and segmental demyelination or intercalated nodes were not observed in the nerves. Internodes in both the LGN and SN were shorter and thinner compared to controls (Figure 12 C, F; Table 6). The graphic visualisation of the relationship between fibre diameter and internodal length resulted in two seemingly separate populations (Figure 12 C, F). One of these was a population composed of fibres with small diameter and short internodes, and the number of such fibres was obviously increased compared to control and diabetic hyperglycaemic rats. The other population of fibres has large diameters and internodal lengths, and they were remarkably less frequent in hypoglycaemic animals than in control and diabetic hyperglycaemic rats (Figure 12 C, F).

Ultrastructural examination of the MGN in hypoglycaemic rats showed a marked reduction in the number of large and medium-sized myelinated axons (Figure 11 C), as well as variety of pathological features similar to those seen in the vagus nerve (Figure 14 A–D). All hypoglycaemic animals exhibited these characteristics, although the frequency varied between animals and was positively related to the duration of severe hypoglycaemia. The picture of unmyelinated nerve fibres was normal in all samples. Furthermore, the total number of myelinated fibres was normal, but the mean fibre diameter was significantly reduced compared to the controls (Table 6). The size distribution histograms showed a marked increase in the number of small fibres and a decrease in large myelinated ones. In addition, there was a strong correlation ($r = -0.9$) between duration of severe hypoglycaemia ($\leq 2.5$ mmol/L) and the mean fibre diameter. The mean g'-ratio was normal.

Electron micrographs of the SN in the hypoglycaemic animals exhibited a picture essentially comparable to that seen in control and hyperglycaemic rats, although they did exhibit a few isolated neuropathological characteristics. There were few MDPs, and there were few Schwann cells that lacked associated axons and possessed collagen pockets (Figure 11 F). No other abnormalities were observed in endoneurial or perineurial nerve elements. Neither did we find any obvious alterations in the morphology of unmyelinated fibres. As in the MGN, the fibre count was unremarkable, but the mean diameter in these rats was significantly less than in controls and displayed a unimodal distribution pattern with distinct reduction in the number of large myelinated fibres. Occupancy, but not density of myelinated fibres

43
was reduced in both MGN and SN compared to controls which implies that small regenerated fibres had compensated for the loss of large myelinated fibres (Table 6).

The neuropathological alterations were more evident in muscle-related nerves (LGN and MGN) than in skin-related nerves (SN). Absence of axonal loss despite obvious signs of axonal degeneration suggests the presence of an active regeneration process. The occurrence of Wallerian-like axonal degeneration and the lack of demyelination imply that hypoglycaemia primarily damages the axons, and destruction of myelin should be considered as secondary demyelination. Degeneration of myelinated axons seems to be a consequence of hypoglycaemia, whereas the increased densities of small myelinated axons might be induced by hyperinsulinemia\textsuperscript{(167)}. However, the endoneurial microvascular abnormalities that other investigators have observed in hypoglycaemic rats did not occur in our animal model\textsuperscript{(86)}. The functional aspect of these pathological changes is not known. Inasmuch as hypoglycaemia has been found to cause obvious changes in nerve conduction characteristics of the peripheral nerves\textsuperscript{(94, 168)}, undertaking some sort of electrophysiological testing might add valuable information to strengthen our results.

The rat MGN contains intrafusal and extrafusal somatic motor fibres, as well as sensory and sympathetic fibres\textsuperscript{(169)}. The alpha motor nerve fibres to the extrafusal muscle and the Ia afferent axons are the largest myelinated fibres. In our experiments, hypoglycaemia had the greatest impact on the large myelinated fibres in the MGN, thus we conclude that hypoglycaemic neuropathy is preferentially a somatic motor neuropathy. The rat SN supplies sensory and sympathetic fibres to the skin, and it also innervates foot muscles via the lateral plantar nerve\textsuperscript{(169, 170)}. Thus, it is likely that those few degenerated large fibres in the SN were related to muscle. It is plausible that hypoglycaemia damages large-diameter fibres, but it is also possible that it preferentially damages fibres that innervate the muscles. The consequence in both cases is that hypoglycaemia would be more likely to affect skeletal muscle than skin. The severity and duration of hypoglycaemia in our rats may be more similar to the corresponding aspects in diabetic patients with “hypoglycaemia unawareness”\textsuperscript{(150)} or insulinoma patients\textsuperscript{(52, 53)} than in representative type 1 diabetic patients. Nevertheless, it is possible that the cumulative effect of occasional severe hypoglycaemia on structure of peripheral nerves seen in patients with long-term type 1 diabetes is the same as we observed in our rats.

Many experimental and clinical studies have indicated that the glucose level is lower in muscle than in skin\textsuperscript{(171, 172)}. If that is the case, the glucose concentration in hypoglycaemic rats may drop to an even lower level in muscle tissue than in skin and thereby cause more extensive axonal damage at target organ. The aim of our
next study was to investigate the glucose concentration in various peripheral tissues (IV).

**Tissue Glucose Levels in Control and Diabetic Rats (IV)**

In this investigation, the microdialysis method was used to compare glucose concentration *in vivo* in blood, sciatic nerve, gastrocnemius muscle, and s.c. tissue in control, diabetic hyperglycaemic, and diabetic hypoglycaemic rats. According to measurements made on tail blood using the Glucometer Elite device, the mean glucose concentration in control rats, but not diabetic rats, increased markedly after initiation of anaesthesia. Notably, in all groups of animals, the blood (in the superior vena cava) glucose levels measured by microdialysis method were significantly higher than the values provided by the Glucometer Elite (Table 7). The mean glucose concentration resulted from microdialysis, were similar in blood and s.c. tissue in all groups. In control rats, the glucose values in blood were significantly higher than that of the nerve and muscle (Table 8). Considering the hyperglycaemic animals, blood glucose level was significantly higher than nerve, and muscle glucose level was lower than s.c. tissue. In hypoglycaemic animals, the average RR of glucose was lowest in muscle (Table 8). There was no difference between mean glucose level of blood compared to nerve and muscular tissues. These relationships between glucose levels of different tissues have temporal variations over the sampling periods in all groups, as shown in figures 16 A–C. For example, in the hypoglycaemic group (Figure 16 C), the nerve glucose level was lower than that in blood in the second and third collected samples.

**Table 7.** Blood glucose concentrations (mmol/l) measured by the Glucometer Elite device and by microdialysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-operation <em>a</em> (Glucometer Elite)</th>
<th>During operation <em>a</em> (Glucometer Elite)</th>
<th>During operation Microdialysis <em>b</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.6±0.1</td>
<td>7.1±0.3 <em>(p &lt;0.001)</em></td>
<td>12.5±0.5 <em>(p &lt;0.001)</em></td>
</tr>
<tr>
<td>Hyperglycaemic</td>
<td>18.5±1.1</td>
<td>18.2±2.5</td>
<td>28.4±4 <em>(p &lt;0.001)</em></td>
</tr>
<tr>
<td>Hypoglycaemic</td>
<td>2.4±0.3</td>
<td>2.4±0.5</td>
<td>4.2±0.7 <em>(p=0.004)</em></td>
</tr>
</tbody>
</table>

*a* Capillary blood from tail. *b* Probes were placed in the superior vena cava. Significantly different from *c* pre-operation value, and *d* during operation value. The values are means ± SEM.
Table 8. Relative recovery (%) of microdialysis probes and mean glucose levels (mmol/l)

<table>
<thead>
<tr>
<th></th>
<th>Blood $^a$</th>
<th>Sciatic nerve</th>
<th>Gastrocnemius muscle</th>
<th>Subcutaneous tissue $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Relative recovery</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32.7±2</td>
<td>23.6±1.4</td>
<td>16.1±0.7</td>
<td>26.9±0.9</td>
</tr>
<tr>
<td>Hyperglycaemic</td>
<td>27.8±1.9</td>
<td>24.8±0.8</td>
<td>10.3±0.5</td>
<td>21.7±1</td>
</tr>
<tr>
<td>Hypoglycaemic</td>
<td>34.7±1</td>
<td>35.2±0.9</td>
<td>13.4±0.9</td>
<td>26.0±1</td>
</tr>
<tr>
<td><strong>Mean glucose value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.5±0.5</td>
<td>9.1±1.2 ($p=0.03$) $^c$</td>
<td>10.7±0.6 ($p=0.04$) $^c$</td>
<td>11.6±0.6</td>
</tr>
<tr>
<td>Hyperglycaemic</td>
<td>28.4±4</td>
<td>20.4±3.1 ($p=0.02$) $^c$</td>
<td>24.3±2.2 ($p&lt;0.05$) $^d$</td>
<td>27.1±3</td>
</tr>
<tr>
<td>Hypoglycaemic</td>
<td>4.2±0.7</td>
<td>3.7±0.5</td>
<td>4.4±0.7</td>
<td>3.4±0.4</td>
</tr>
</tbody>
</table>

A CMA/20 probe with a 4-mm membrane (blood) and 10-mm membrane (other tissues) was used. Microdialysis probes were placed $^a$in the superior vena cava and $^b$under the skin of the left hind paw. $^c$Significantly different from blood value. $^d$Significantly different from subcutaneous glucose level. The values given are means ± SEM.

Hypoglycaemia has different effects on motor and sensory peripheral nerves, according to the results of our studies (II, III) and investigations conducted by other groups$^{165}$. This raised the question of whether the target organ plays a role in the development of peripheral neuropathy. Our hypothesis was that the abnormal glucose level in the target tissue (muscle and skin) may have detrimental effects on nerve fibre endings, and initiate “dying back” neuropathy$^{173}$.

As previously mentioned, microdialysis method overestimated the blood glucose concentration. Therefore, if we had been interested in the absolute glucose level in various tissues, using the results of this technique might have led to erroneous conclusions. However, our objective was to determine the relationship between different tissues in terms of glucose concentration. According to both experimental$^{174}$ and clinical reports$^{175, 176}$, blood and s.c. glucose concentrations are statistically the same. Since we did not detect any differences between interstitial glucose concentrations in these tissues, we assumed that the rate of overestimation caused by microdialysis is equal in different tissues. Hence, the relationship between glucose levels in different tissues indicated by microdialysis in this study may reflect the situation in vivo.

In control animals, the mean glucose level in blood was similar to that in s.c. tissue and significantly higher than that in muscle (Table 8), and those findings agree with the results of other studies$^{172, 174-176}$. However, in hypoglycaemic animals, we did not observed any significant differences in glucose levels between different tissues. One possibility is that there are very small differences between various tissues,
Figure 16. Blood glucose level obtained from Glucometer Elite and glucose level in different tissues obtained by microdialysis in controls (A, n = 7) and in diabetic hyperglycaemic (B, n = 7) and hypoglycaemic (C, n = 5) rats. □, Blood from Glucometer Elite®; O, blood (superior vena cava) from microdialysis; Δ, the sciatic nerve; ◊, muscle; ●, subcutaneous tissue. * Significant difference between blood and nerve, and † between blood and muscle. All values are means ± SEM.
hence we could not detect it. The other possibility is that there is no difference at all. Anyhow, the results do not support our idea that a lower glucose level in muscle than in skin may damage nerve fibre endings in the former tissue more severely under hypoglycaemic conditions. In fact, the glucose in muscle was equal to that in blood and s.c. tissue. Moberg and colleagues (171) reported lower glucose levels in adipose tissue than in plasma in healthy insulin-induced hypoglycaemic patients between one and two hours after provoking the condition (171). Our animals were hypoglycaemic for almost four weeks before the experiments and their glucose levels in s.c. tissue were 19% (0.8 mmol/l) lower than in blood and 23% (1 mmol/l) lower than in muscle. If the local glucose concentration at the target level had in any way caused damage to nerve endings, then the lower glucose level in s.c. tissue compared to muscle should have a greater negative impact on nerve endings in the former tissue. That was not, however, the case in our investigation of hypoglycaemic neuropathy (II, III).

Taken together, our results show that:

- Blood and s.c. glucose concentrations are equal in healthy and diabetic animals in hyper- or hypoglycaemic conditions.
- The glucose concentration is lower in muscle than in blood in healthy rats.
- The peripheral nerve has the lowest glucose concentration of all the tissue types analysed in present study.
- The glucose concentration varies slightly between different tissues during hypoglycaemia.
Conclusions

- Hypoglycaemia in diabetic BB/Wor rats causes degenerative changes in large myelinated axons in somatic sensory and motor nerves as well as in somatic parts of the vagus nerve. Such degeneration has a more severe impact on somatic motor nerve fibres than on somatic sensory fibres.

- The absence of signs of neuropathy in small myelinated and unmyelinated axons suggests that the sensory and autonomic components of those nerves that we examined were less affected by hypoglycaemia.

- Hyperglycaemia in diabetic BB/Wor rats reduces the diameter of myelinated axons in somatic sensory nerves but not in somatic motor nerves.

- Blood and subcutaneous glucose levels are similar, regardless of glycaemic state.

- The concentration of glucose at the target organs does not seem to affect the development of distal axonal damage in hypoglycaemic rats.

Neurological complications in patients with diabetes mellitus are diverse, and their pattern and mechanisms of development are not fully understood. Abnormalities in glucose metabolism, and its pathophysiological consequences, have been recognised as important causative factors for development of neuropathy. In this context, much attention has been paid to hyperglycaemia, despite the fact that hypoglycaemia also has detrimental effects on both the CNS and PNS in humans. Hypoglycaemic neuropathy seems to be primarily a somatic motor neuropathy. Hyllienmark and his colleagues found mainly motor dysfunction in type 1 diabetic patients being on intensive insulin therapy. In fact, hypoglycaemia in type 1 diabetic patients is a condition that occurs more often when strict glycaemic control is applied. Our conclusion is that the diverse picture of diabetic neuropathy may be the result of both hyperglycaemia and hypoglycaemia, and hence, both conditions should be avoided in order to prevent development of neuropathy.
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References


