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THE ROLE OF GLUTAMATE SIGNALLING IN DIABETIC NEUROPATHY

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Under the Supervision of Professor Thomas E. Adrian

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Declaration of Original Work

I, Nadia Hussain, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled *"The role of glutamate signalling in diabetic neuropathy,"* hereby, solemnly declare that this dissertation is an original research work that has been done and prepared by me under the supervision of Professor Thomas E. Adrian in the College of Medicine and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or a similar title at this or any other university. The materials borrowed from other sources and included in my dissertation have been properly cited and acknowledged.

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Abstract

The majority of diabetics develop neuropathy, which can be debilitating, but the underlying pathophysiological mechanisms are poorly understood. Diabetic neuropathy progresses in a distal to proximal manner. Previous studies have shown that glutamate, the most common excitatory neurotransmitter, plays a role in the pathogenesis of neuropathy. The reason why the role of glutamate in nociception becomes a problem in diabetes and the mechanisms that are involved are unknown. Based on the preliminary data, the hypothesis was that glutamate pathways are likely to be involved in diabetic neuropathy particularly neuropathic pain. Pathways were investigated to look for changes that might reflect neuropathic pain and fit with previously established pharmacological evidence. The aim of this project was to identify changes in expression of genes and their protein products that are involved in glutamate signalling in diabetes. This will help to further the understanding of the mechanisms of diabetic neuropathy. In diabetic rats, there were consistent changes in expression, particularly in the lumbar and sacral dorsal root ganglia of the spinal cord and in the sympathetic ganglia. The changes were consistent between the different groups of animals as well as between adjacent groups of ganglia. The most prominent changes in both the GK groups included marked upregulation of Gria4 (ionotropic AMPA receptor), downregulation of Grik3 and Grik4 (both ionotropic, kainite receptors) and Grin1 and Grin2A (both ionotropic, NMDA receptors), activation of all of which has been shown to induce hyperalgesia; downregulation of Slc1a6 (excitatory amino acid transporter 4) and upregulation of Slc1a1 (excitatory amino acid transporter 3), both of which mediate neural reuptake of glutamate from the synaptic cleft; and upregulation of Gclc (glutathione synthase), which reflects a response to protect against oxidative damage.

Despite many theories existing about the pathogenesis of diabetic neuropathy, there is no unifying hypothesis. It is possible that changes in glutamate signalling can contribute to these other mechanisms and possibly unify these different theories. A better understanding of the role that glutamate plays in development of diabetic neuropathy may pave the way for future therapeutic intervention.

Keywords: Diabetes, Diabetic Neuropathy, Glutamate Signalling, Dorsal Root Ganglia, Sympathetic Ganglia.

Title and Abstract (in Arabic)

دور إشارات الجلوتاميت في الاعتلال العصبي لمرض السكري

الملخص

أغلبية مرضى السكري يعانون من الإعتلالات العصبية اللتي تسبب الوهن . ولكن بالرغم من ذلك , فإن اليات الفسيولوجية المرضية لهذه الإعتلالات غير مفهومة . الإعتلال العصبي لمرض السكري يتطور في المرضى من لاأطراف باتجاه الجذع . الدراسات السابقة أظهرت أن الجلوتاميت وهو الناقل العصبي المحفز الأكثر شيوعا , يلعب دورا بنشوء هذا الإعتلال . السبب وراء دور الجلوتاميت بالاحساس بالألم عند مرضى السكري واليات ارتباطه غير معروفة . الهدف من هذه الدراسة هو تحديد التغيرات بالتعابير الجينية والمنتوج البروتيني وارتباطه بالإشارات العصبية للجلوتاميت بمرض السكري . هذه الدراسة ستساهم في تأييد فهمنا لاليات الإعتلال العصبي لمرض السكري . لقد وجدنا في الجرذان المصابة بالسكري تغييرات ثابتة بالتعابير الجينية بالعقد الجذرية العصبية القطنية والعجزية للحبل الشوكي بالإضافة إلى العقد الجذرية العصبية للجهاز الودي السمبثاوي . هذه التغيرات كانت ثابتة بين مجموعات الحيوانات بالإضافة إلى المجموعات العقدية المتجاورة . أبرز هذه التغيرات في مجموعات حيوانات "جي كيه" (GK) تضمنت ارتفاع ملحوظ في مستقبلات "Gria4" من نوع مستقبلات "ionotropic AMPA" , وانخفاض في مستقبلات "Grik3" و "Grik4" كليهما من نوع مستقبلات "ionotropic kainite" , و انخفاض في مستقبلات "Grin1" و "Grin2A" كليهما من نوع مستقبلات "ionotropic NMDA" . تفعيل جميع هذه المستقبلات أظهرت حثها لفرط التألم . بالإضافة لذلك أظهرت الدرساة انخفاض التعبير الجيني ل "Slca6" وهو ناقل الأحماض الأمينية من نوع 4 , وارتفاع التعبير الجيني ل "Slc1a1" وهو ناقل الأحماض الأمينية من نوع 3 , وكليهما تتواسط لإعاده امتصاص الجلوتاميت في المشابك العصبية (synaptic cleft) . وأظهرت الدراسة أيضا ارتفاع التعبير الجيني ل "Gclc" وهو انزيم سنثاز جلوتاثايون (glutathione synthase) والذي يعكس الاستجابة للحمايه من التلف التأكسدي .

بالرغم من النظريات الحالية عن التطور المرضي للاعتلال العصي لمرض السكري , فإنه لا يوجد إلى الان أي نظرية مجمع عليها . من المحتمل أن التغيرات للإشارات العصبية للجلوتاميت تسهم في هذه الاليات ومن المحتمل أن توحد هذه النظريات المختلفة . الفهم الأفضل للدور الذي تلعبه الغلوتاميت في تطور اعتلال الأعصاب السكري قد يمهد الطريق لتدخل علاجي في المستقبل.

الكلمات المفتاحية : مرض السكري , الاعتلال العصبي لمرض السكري , الإشارات العصبية للجلوتاميت , العقد الجذرية العصبية , العقد الجذرية للجهاز الودي السيمبثاوي .

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Dedication

To my loving parents and sisters, my wonderful husband and daughter Fatima.

To my two little birds in heaven

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List of Abbreviations

AGE	Advanced Glycation End Products
DAN	Diabetic Autonomic Neuropathy
DN	Diabetic neuropathy
DNA	Deoxyribonucleic acid
EKG	Electrocardiogram
FFA	Free Fatty Acids
GLUT	Glucose transporter
HbA1C	Hemoglobin
HDL	High density Lipoprotein
HLA	Human Leukocyte Antigen
IDDM	Insulin Dependent Diabetes Mellitus
IRS	Insulin receptor substrate
LDL	Low density lipoprotein
NDDG	National Diabetes Data Group
NIDDM	Non-Insulin Dependent Diabetes Mellitus
OGTT	Oral Glucose Tolerance Test
PKC	Protein Kinase C
PP	Pancreatic Polypeptide
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphisms
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

Chapter 1: Introduction

1.1 Diabetes Mellitus

The term Diabetes mellitus comprises a group of metabolic disorders characterized by the presence of excess blood glucose; hyperglycaemia (A. D. Association, 2014). The defect in diabetes is characterized by defects in insulin secretion or action, or a combination of both. This disturbs the carbohydrate, fat and protein metabolism. The resulting hyperglycaemia leads to long-term damage, dysfunction and consequent failure of several organs. These complications are debilitating enough that affect the quality of life and mortality (Alberti, Zimmet, & Consultation, 1998). Complications and their consequences occur such as retinopathy lead to blindness, nephropathy to renal failure, neuropathy to foot ulcers or amputation, autonomic dysfunction and its variety of manifestations. Diabetics are prone to cardiovascular, peripheral vascular and cerebrovascular disease.

Patients with diabetes often present with characteristic symptoms such as excessive thirst, the passing of excessive urine (polyuria), blurred vision and loss of weight. Some patients present in the ketoacidosis or non-ketotic hyperosmolar state that can lead to stupor, coma and in extreme cases death. Unless dramatic, the absence of symptoms or unalarming symptoms can go unchecked and lead to the development of complications before diabetes is diagnosed. These symptoms are usually because of the presence of hyperglycemia (Alberti, 1998).

1.2 Statistics and Diabetes

Diabetes and other forms of glucose intolerance; impaired glucose tolerance (IGT) and impaired fasting glucose (IFG), are now found in every population and there is epidemiological evidence suggesting the burden of diabetes is increasing globally and continuing to do so. Diabetes places a heavy toll on the socioeconomic

fabric of society due to the large number of people affected (Wild, Roglic, Green, Sicree, & King, 2004). From 175 million diabetics in 2000 (Yach, Stuckler, & Brownell, 2006) to 371 million currently affected (IDF 2014), diabetes is a pandemic of epic proportions.

Worldwide rank wise, Kuwait 3rd, Qatar 6th, Saudi Arabia 7th, Bahrain 8th and United Arab Emirates 10th all have places in the top 10 countries with prevalence of diabetes at 20-79 years of age in 2011. These positions are expected to ascend by 2030 considering the current prevalence rate (D. Chen & Wang).

In the United Arab Emirates the ENDCAD study in 2000, 19.6% of the total population was found to be diabetic; 24% locals and 17.4% expatriates (ENCAD UAE, 2009). Diabetes is one of the major causes of mortality in UAE, causing 2 – 3% of deaths (*Ministry of Health strategic plan till 2010,* 2000).

1.3 Classification of Diabetes Mellitus:

This often depends on how the patient presents at the time of diagnosis and although some do not easily fit into a single category, the classification is important from a therapeutic point of view. This allows better understanding of the pathogenesis of the hyperglycemia and leads towards better treatment options.

Although the majority of the diabetic individuals fall into two broad etiopathogenetic categories, as discussed below, assigning a type of diabetes to an individual depends on the circumstances present at the time of diagnosis. Many diabetic individuals do not easily fit into any single class. For example, a female patient who has gestational diabetes mellitus may continue to be hyperglycaemic after delivery and in fact have type 2 diabetes. An individual who acquires diabetes because of large doses of steroids may become normoglycaemic when the steroids are discontinued but later on develop diabetes after instances of pancreatitis.

Another example is a person treated with thiazides who develops diabetes years later and it could be because the person had type 2 diabetes exacerbated by the drug. For the clinician and the patient, having the correct label for the type of diabetes leads to better understanding of the pathogenesis of the hyperglycaemia and effective treatment strategies.

One of the earliest formal attempts to classify diabetes uniformly was by the 1964 World Health Organization Expert Committee. It was recognized that clear classification would aid epidemiological approaches and advance clinical research in developing of evidence based therapies. A symptomatic classification was adopted and the age of onset was an additional criteria. The age characterization was as follows (Mellitus & Organization, 1965):

1. Infantile or childhood diabetes (0-14 years of age)
2. Young diabetes (15-24 years of age)
3. Adult diabetes (25-64 years of age)
4. Elderly diabetes (65 and above years of age)

However, the terms type 1 diabetes and type 2 diabetes emerged following the National Diabetes Data Group (NDDG) in the USA and the second report of the WHO Expert Committee on Diabetes Mellitus.

Two main classes of diabetes were suggested: insulin-dependent diabetes mellitus (IDDM/type 1) and non-insulin dependent diabetes mellitus (NIDDM/type 2). ‘Other types’ and gestational diabetes mellitus were also added to the list. The condition of impaired glucose tolerance (IGT) also appeared, replacing ‘borderline’ diabetes ("WHO Expert Committee on Diabetes Mellitus: second report," 1980).

The 1999 classification retained the main groups of type 1, type 2, other specific types, and gestational diabetes, but the terms ‘insulin dependent’ and ‘non-insulin dependent’ were presumed to be confusing and so dropped (Alberti, 1998)

Currently ("WHO, Diabetes report" 2013) diabetes is generally classified into Type 1, Type 2 and other types of diabetes. Type 1 results from the destruction of beta cells of pancreas leading to total insulin deficiency. Type 2 is a combination of progressive secretory defect combined with insulin resistance. Other types include gestational diabetes and diabetes occurring due to genetic defects of beta cell function or insulin action or diseases of the pancreas itself or due to chemicals or drugs (Gabir, 2000).

1.4 Physiology of Insulin and Glucose control

Insulin's name is derived from the Latin *insula* for "island". Insulin is produced by the beta cells that lie in the middle of the pancreatic islets. These islets, which range from one million to three million, form the 2% endocrine part of the pancreas – a predominantly exocrine gland. Within the islets, the beta cells constitute 65–80% of all the cells. The beta cells also secrete amylin (Hall, 2011).

The pancreatic islets are also where the alpha, delta and PP cells reside. The alpha cells secrete glucagon, the delta cells secrete somatostatin and the PP cells secrete pancreatic polypeptide. These cells are closely related and the proximity allows direct control of hormone secretion by the other hormones. Such as insulin inhibiting the secretion of glucagon, amylin inhibiting insulin and somatostatin inhibiting insulin and glucagon (Hall, 2011).

Insulin is the main hormone for food derived energy storage and is coded for on chromosome 11. Insulin is a protein and in humans has a molecular weight of 5808. It consists of two amino acid chains bound by disulfide bonds. Insulin is first made by the endoplasmic reticulum as preproinsulin which is cleaved to form proinsulin and cleaved into the A and B chain that are bound together and the C chain that is separated in the Golgi apparatus. The insulin and C peptide are packed into secretory granules. When insulin is released into the blood it is an unbound

form and has a plasma half-life of six minutes. Within fifteen minutes it is cleared from the circulation. The portion of insulin that is not bound to receptors is degraded by insulinase. In the liver, 50% of it is degraded, the rest is cleared by the kidneys (Hall, 2011).

The liver achieves a constant level of glucose despite variations of food intake and energy usage by processes such as glucose storage and gluconeogenesis. Tissue specific glucose utilization requires glucose transporters which ordinarily depend on insulin. The brain however does not need insulin although it is the main consumer of the body's glucose. Muscle and fat tissues are facultative glucose consumers (Barrett E K, 2012; Cahill, Rossini, & Aoki, 1974; Rodney A. Rhoades, 2014; Rossini, Like, Chick, Appel, & Cahill, 1977).

The insulin receptor is a combination of four subunits that are linked with each other by disulfide bonds. The two alpha subunits are extracellular and the two beta subunits are transmembrane. Insulin binds to the alpha subunits and consequently the beta subunits are activated and become autophosphorylated. This activates a local tyrosine kinase which causes phosphorylates other enzymes including a group called insulin-receptor substrates. Different types of these substrates are expressed in different tissue. Activating these, in turn, activates some enzymes while inactivating others. Insulin directs the metabolic machinery in cells to bring about the desired effects on carbohydrates, fat and proteins.

After insulin binds, the cells increase their uptake of glucose. This is of particular importance for muscle cells and adipose cells. The increase of glucose levels in the cells leads to phosphorylation and providing a substrate for the carbohydrate metabolic functions. The glucose is taken up by the increased availability of intracellular vesicles to the cell surface. Insulin causes cells to become permeable to amino acids, potassium and phosphate ions. Insulin also affects

translation of messenger RNAs at the ribosomes for new protein production and also alters rate of DNA transcription. Insulin thus remolds much of the cellular enzymes to achieve its metabolic goals (Hall, 2011).

The resting muscle membrane is only slightly glucose permeable but on insulin stimulation glucose is allowed to enter the muscle. During exercise and when large amounts glucose is available after a meal, the extra insulin secreted by the pancreas stimulates the muscle to take up glucose. During much of the day, muscle depends on fatty acids rather than glucose for its energy requirements.

In muscle, especially during periods of inactivity, the glucose that is taken up is stored as glycogen or broken into lactate which fuels hepatic gluconeogenesis. In fat, glucose is a source of energy and a substrate for triglyceride synthesis (Gale EAM, 2002).

One of the most important effects of insulin is to cause the liver to store glucose that is available after a meal. Insulin brings about these actions by enhancing the activity of enzymes such as glucokinase and glycogen synthase. This helps form glycogen. Insulin suppresses activity of enzymes such as phosphorylase and glucose phosphatase. The net effect of these actions increases the glycogen content in the liver. Insulin also prevents gluconeogenesis by suppressing enzymes and amino acid release from extra hepatic tissues such as the liver. However, hours after a meal the levels of insulin and glucose fall in the blood and liver glycogen breaks down to provide the required glucose. If the liver cells cannot store more glycogen, insulin promotes converting the excess glucose to fatty acids. After the liver glycogen content reaches approximately five to six percent, further glycogen synthesis is inhibited and all additional glucose forms fatty acids and triglycerides to store. Insulin also encourages fat storage into adipose cells by enhancing glucose transport into cells and inhibiting lipase. The lack of insulin causes lipolysis of stored

fats and release of fatty acids, increases plasma cholesterol levels and in extreme cases can lead to ketosis and acidosis. The effect of protein metabolism by insulin includes stimulating amino acid transport into cells, increasing messenger RNA translation, increasing the rate of transcription of selected DNA genetic sequences to allow more RNA and thus protein to form and inhibiting protein catabolism (Hall, 2011).

Glucose transporters are classed as the GLUT family and have distinct substrate specificities, coding genes kinetic properties, and tissue distributions determining roles and expression. GLUT-4 is the main insulin responsive glucose transporter and is mainly located in muscle cells and adipocytes. Insulin binds to the receptor, activating tyrosine kinase phosphorylation and a series of cellular events ensue allowing glucose inside the cell (Shepherd & Kahn, 1999). A member of the glucose transporter (GLUT 2) is found in beta cells and allow a rate of glucose to enter these cells that is proportional to the blood concentration in the physiological range. Once inside the beta cells, glucose is converted to glucose-6-phosphate by glucokinase. This is the rate limiting step for glucose metabolism in the beta cell and is thought to be the main mechanism for glucose sensing and adjustment of the amount of secreted insulin to glucose levels (Hall, 2011).

1.5 Type 1 Diabetes

Type 1 Diabetes affects 5-10% of diabetics and arises from the immune mediated destruction of the beta cells and is commonly associated with other autoimmune disorders (Amer Diabet, 2011). In some cases there may be an inherited tendency for beta cell degeneration without the involvement of viral infections or autoimmune disorders (Hall, 2011).

The commonest form of diabetes is Type 2 with up to 90% of diabetics having this type, although Type 1 is also on the increase (Patterson, 2002).

Type 1 diabetes stems from the immune mediated destruction of the beta cells. Markers of this destruction include autoantibodies such as; islet cell autoantibodies, insulin autoantibodies, and autoantibodies to GAD and tyrosine phosphatases IA-2 and IA-2b. Most diabetics have one or more of these autoantibodies upon diagnosis. These patients are also more prone to other autoimmune disorder such as Graves' disease, vitiligo, myasthenia gravis and so forth.

Type 1 diabetes has strong HLA associations, with linkage to DQA and DQB genes and is also under the influence of DRB genes. These HLA-DR/DQ alleles can be either predisposing or protective of diabetes (A. D. Association, 2014).

The term Type 1 diabetes however does not encompass the forms of beta cell destruction or failure to which specific causes can be attributed such as cystic fibrosis (Alberti, 1998). There are other forms of Type 1 diabetes that have unknown etiologies. Some of these diabetics have permanent lack of insulin and are at increased risk of developing ketoacidosis but have on indication of autoimmunity. These individuals are rare and more often of African or Asian descent. They experience ketoacidosis at intervals and have varying degrees of insulin deficiency. The requirement for insulin to be replaced in their bodies may come and go (A. D. Association, 2014). This form is often classed as Type 1B – a rarer form of Type 1 diabetes. Type 1A is the commoner form, resulting from the autoimmune destruction of beta cells (Daneman 2004).

The autoimmunity of type 1 diabetes is a concept that has been backed by several important research studies and one of the most persuasive evidence was provided by case reports of diabetes developing in people who received bone marrow from type 1 diabetics. The recipients first underwent bone marrow ablation as treatment for conditions such as haematological cancers to remove all immune

cells. To bring back their immune system they received bone marrow donations from siblings with type 1 diabetes. However, they themselves developed type 1 diabetes sever years later. This provides evidence for the theory that immune cells were transferred in the bone marrow destroyed the beta cells (Wass A.H J, 2011).

Autoreactive T cells against the pancreatic cells are produced and expand. Due to the deficient regulation of T cell response, clonal expansion of these particular T cells continues unchecked and triggers a response of beta antigen specific (T cell) immune and other inflammatory responses that destroy the beta cells. Ordinarily, T cells the react against self-antigens are checked and halted during development and eventually eliminated by clonal deletion. However in type 1 diabetes, as in other autoimmune conditions, this censoring process is not effective.

One of the methods to interrupt and reverse the autoimmunity of type 1 diabetes before end organ damage occurs has been the advent of bone marrow transplant as a treatment option. However, this has its own complications and difficulties (Domenick & Ildstad, 2001).

Autoimmunity does not exist in all forms of type 1 diabetes. In some patients this is lacking as is the WHO diabetes classification of the type 1B category. In Japan, this form of diabetes has been described as affecting 15-20% of type 1 diabetes and marked in the absence of insulitis and diabetes-related antibodies, an abrupt onset with preceding of common cold like and gastrointestinal symptoms, a surprisingly near normal level of HbA1c despite the high levels of glucose, and high levels of serum pancreatic enzyme concentrations (Imagawa, Hanafusa, Miyagawa, & Matsuzawa, 2000).

The mechanism of beta cell death appears to differ in this particular type of diabetes compared to the autoimmune category. While the beta cells are destroyed in autoimmune diabetes over a span of time, often several years – this destruction is

extremely rapid in the fulminant form of type 1 diabetes. This process is postulated to be triggered by viral infection (Willcox, 2008).

In the 1980s, Eisenbarth proposed the current model to explain the development of Type 1A diabetes and today the basic idea of this model persists. The model hypothesizes that every person has a susceptibility to develop this type of diabetes but some more so than others. Susceptibility is inherited, residing in the HLA genotypes DR and DQ and to a lesser extent in other genetic loci termed IDDM susceptibility genes. 50% of the genetic susceptibility stems from the HLA focus and 15% from two genes; IDDM2 and IDDm12 with minor participation from the other IDDM genes. The susceptibility genes are thought to be involved in the regulation of immune response.

Exposure to an environmental trigger that alters immune function leads to beta cell destruction. Triggers such as viruses; enteroviruses, coxsackie, congenital rubella), environmental toxins such as nitrosamines or food such as early exposure to cow milk proteins and gluten have been linked to varying degrees. The over activation of the T cell mediated immune system in those individuals with increased susceptibility experience insulitis and a B cell response the produces antibodies to beta cell antigens. As the beta cells are destroyed, insulin levels drop till below a critical level a state of absolute insulin deficiency ensues. The so called ‘honeymoon’ period occurs after initiation of insulin therapy begins when some insulin is secreted by few of the last remaining beta cells but these eventually fail as well.

The connection to autoimmunity also arises from evidence that these patients are also more prone to developing other autoimmune diseases such as Graves’ disease, Addison’s disease, myasthenia gravis and so on. There is one central theory of cause of type 1 diabetes and occasionally interesting hypotheses

have cropped up such as the hygiene hypothesis that postulates early exposure to infections in children builds immunity and prevents type 1 diabetes. Another interesting hypothesis is the accelerator hypothesis where the two types of diabetes are only distinguished by the rate of beta cell loss. Three accelerators are postulated in the accelerator hypothesis; the high rate of beta cell apoptosis, insulin resistance that stresses the collapsing beta cells, immunologically vulnerable beta cells in genetically susceptible individuals. However these hypotheses need more investigation (Daneman 2004).

Understanding the allelic architecture i.e. identifying disease associated gene variants, how frequently these occur and the risk attached to each variant, of type 1 diabetes will help identify new targets for treatment. Individuals are able to manage with 20% of the beta cell mass and this offers a window of opportunity for interventions before the diabetes sets in (Wass A.H J, 2011).

Type 1 diabetes is increasing, a recent study by the EURODIAB Study group that assessed 20 population based registries in 17 European countries, showed that there has been a 3.9% increase of incidence between 1989 and 2003 in children aged 5 - 15 and a 5.4% rise per year in the under 5 age group.

Type 1 diabetes results from a complex interaction of several genes and environmental factors, which can promote the disease and occasionally protect from it. At least four chromosomal regions are implicated to contribute to the risk of developing type 1 diabetes, the HLA region on chromosome 6p21 IDDM1, the insulin gene region at 11p15 IDDM2, the CTLA4 locus at 2q33 IDDM12 and the PTPN22 locus at 1p13. There is however no standard for defining what is or is not a risk locus for developing type 1 diabetes but there is consistent evidence that supports a role for the genes in these regions to implicate them as conferring an

increased risk of developing type 1 diabetes in individuals (Concannon, Rich, & Nepom, 2009).

The pancreatic pathological lesion that is characteristic of type 1 diabetes is insulitis. This term describes the immune cell infiltration surrounding the islets with additional immune cells scattered around them. This appears to increase as the beta cells die off. In later stages, this infiltration subsides. The beta cell proliferative capacity appeared to be unchanged despite the ongoing destructive processes. Insulitis is commonly observed in new-onset patients with diabetes of new onset but it does not affect all insulin containing islets equally. This is perhaps due to the differences in islet function; factors including sensitivity to glucose, insulin release and so on could play a role. This also suggest that during periods of physiologic demand for increased insulin such as during puberty or under the influence of high sugar intake, there might be a population of islets more vulnerable to dysfunction and cell death that attract APCs and promote insulitis in certain genetically susceptible patients. These functional differences between the islets could explain why autoreactive lymphocytes target some islets more than others (Rowe A. Patrick, 2011).

Analysis of the immune cell infiltrate in T1D islets show that during the initial period of beta cell death, the most abundant immune cells are the $CD8^+$ cytotoxic T cells.

Once the decline in beta cells is established, the larger share of population consists of the $CD20^+$ cells. However, once the beta cells are completely destroyed, immune cells decrease dramatically in the islets and this suggests that their role is only while viable beta cells exist. The stimulus for insulitis declines after the death of beta cells. Glucagon staining is normal in such islets and this shows that alpha cells are unaffected (Willcox, 2008).

Type 1 diabetes mellitus develops in stages (Wass A.H J, 2011):

Stage 1 – in this stage, a trigger allows the T lymphocytes to develop the ability to recognize β cell autoantigens that emerge from the thymus. This lets the peripheral immune system from birth contain adaptive immune cells with the ability to attack the beta cells. Immunogenetic studies on type 1 diabetes have backed this information. HLA genes that increase risk of developing type 1 diabetes could possibly involve the ineffective functions of the thymus such as reducing the ability of it to delete beta cell specific T lymphocytes.

Stage 2 – inflammation damages the beta cells. Autoantigens to beta cells are released and absorbed by dendritic cells which migrate to the pancreatic lymph nodes and, in combination with defective thymic function, the islet proteins are presented to naïve beta cell specific T lymphocytes with the relevant T cell receptors. Viruses have often been thought to be culprits in the initiation.

Stage 3 – the imbalance in immune regulation allows the beta cell specific T lymphocytes to activate and mature to form the pro-inflammatory phenotype. This results in a greater propensity to activate the harmful T lymphocytes.

Stage 4 – autoreactive T lymphocytes migrate to the inflamed islets, further adding to the inflammation and beta cell damage. Autoreactive B lymphocyte recruitment is also a feature and autoantibodies may help the inflammation by forming immune complexes. The beta cells are systematically destroyed from islet to islet.

Stage 5 – chronic inflammation settles and in this stage the presence of autoantibodies are established. Relapses and remissions also occurs in this stage and these could be influenced by events irrelevant to the initial trigger. The beta cell destruction spreads till the disease is fully established.

1.6 Type 2 diabetes

Type 2 diabetes is the result of the complicated interactions of genetic and environmental factors. Although changes such as an increasingly sedentary lifestyle and excessive consumption of calories seem to be triggering factors, genetic elements are involved (Stumvoll, Goldstein, & van Haeften, 2005). One of the hallmarks of type 2 diabetes is insulin resistance; which is the inability of insulin to be effective at its normal circulating concentrations (Wass A.H. J, 2011). The presence of hyperglycaemia, characteristic of diabetes, adds to insulin resistance in skeletal muscle by glucose toxicity (Rossetti, Giaccari, & DeFronzo, 1990) and impairs insulin-stimulated glucose utilization and glycogen synthesis in skeletal muscle (Tomas, 2002).

Insulin and glucagon act for opposing goals. Glucagon increases the glucose output from the liver to increase circulating glucose by increasing gluconeogenesis and glycogenolysis. An excessive or imbalanced amount of glucagon contributes to the post meal hyperglycaemia present in type 2 diabetes since glucagon is not suppressed normally (Dunning & Gerich, 2007). The incretin effect, observation that gut derived factors enhance glucose stimulated insulin secretion from the pancreatic beta cells more than parenteral glucose infusions, is also significantly blunted in type 2 diabetics which could be due to the decrease in sensitivity of islets to the incretin hormones. Glycaemic control partially compensates for this defect (Drucker & Nauck).

Type 2 diabetes is the quintessential multifactorial trait, where individual risk is determined by complicated interactions of genetic and environmental factors. Although changes such as an increasingly sedentary lifestyle and excessive consumption of calories seem to be triggering factors, genetic elements are also involved in the pathogenesis of this disorder. A positive family history increases the

risk to develop type 2 diabetes by 2.4 fold. 15-25% of first degree relatives of patients with type 2 diabetes develop diabetes or the state of impaired glucose tolerance. If one parent has type 2 diabetes, the lifetime risk for the offspring is calculated to be 38% and this increases to 60% if both parents are afflicted.

Twin studies have also added to this knowledge. Dizygotic twins share 50% of their genes, unlike monozygotic twins. Concordance rates in the latter twins have been used to differentiate between genetic and non-genetic contributions. In people older than 60 years of age, concordance rates for diabetes were 35–58% in monozygotic twins and 17–20% in dizygotic twins (Stumvoll, 2005).

This is based on the 'equal environment assumption' since twins share similar environments, the increased concordance rates for disease in monozygotic twins compared with dizygotic twins indicate the importance of genetic factors that contribute to type 2 diabetes. The understanding of the molecular basis of this complex disease is hindered at several levels. Several features of this complicated disease is at root such as the variable age of onset, the varying degrees of severity that makes phenotyping difficult, locus and allelic heterogeneity which implicates several genes across multiple biological pathways interact with the environment to confer risk or offer protection against the disease. Each susceptibility gene and environmental factor contributes to the disease with varying results in different populations so replicating these results in other populations is difficult. Also several minor contributing genes make traditional linkage based approaches difficult and large studies are needed to find these genes.

Linkage studies in type 2 diabetes have encountered lack of replication of peaks of lineage and difficulties in identifying the underlying genes. In fact, the only type 2 diabetes gene identified using linkage and position cloning is calpain 10 and this too has not been widely accepted. The doubt arises because linkage to

CAPN10 on chromosome 2q was only detected in Mexican Americans, SNPs associated with type 2 diabetes were a set of functionally unclear variants, the original findings with CAPN10 were not replicated in other populations and its actual function and mechanism in relation to diabetes was unclear. Some loci have been identified and found to be in different populations as well; however none have strong evidence in their favour. Study of the intermediate phenotypes of type 2 diabetes, rather than when the disease is fully established, has uncovered other loci.

Association studies have also been employed, especially of the candidate gene type, in studying the genetics of type 2 diabetes. These studies have often focused on genes encoding pathways of glucose induced insulin secretion from beta cells, peripheral insulin led uptake of glucose by fat and muscle and also the influence of insulin on the liver's gluconeogenic pathways. These studies have revealed certain genes and polymorphisms such as the common Pro12 allele which has a frequency range 80–90% in different populations of the Pro12Ala polymorphism in PPARG, the E23K variant of KCNJ11 and lately the involvement of the HNF4A gene (Barroso, 2005).

Type 2 diabetes has proven to be very heterogeneous and genetic studies have shown very diverse results. Two of the most popular methods used to study genetic factors involved in disease include the candidate gene approach and the genome wide scan approach. The candidate gene approach examines specific genes with a plausible role in the disease process. This is done by testing the statistical association of a given allele and a phenotype in unrelated people. The genome wide scan or linkage basis approach locates genes through their genomic position and rationales that related individuals sharing a specific phenotype will also share chromosomal regions surrounding the gene involved (Stumvoll, 2005).

However, the candidate gene approach has not had very much success. There are several reasons for this which includes limitations inherent to the method but also to the era in which this type of research was being widely used. A fundamental requirement is to have a detailed knowledge of the disease when candidate genes are being selected and since type 2 diabetes is an incredibly complex disease, any single candidate gene will have a low chance in affecting susceptibility to the disease. Also study designs using this technique depend on the sample size and phenotypic characterization of the studied sample. Thousands of samples are needed and statistics are often applied to compensate for the lack of sample sizes. Meta-analysis is often used but this is subject to publication bias where negative reports often go unpublished and so skew meta-analysis and other biases and heterogeneity can influence the outcome. Variables such as ethnicity, age, gender also contribute to heterogeneity (Wass & Stewart, 2011).

One major goal for elucidating the genetic backdrop of type 2 diabetes is to improve treatment by identifying patients more likely than others to benefit from specific therapeutic agents. Neonatal diabetic patients with the KCJN11 mutation, when switched from insulin to sulfonylurea showed improved symptoms especially their neurological manifestations that are often severe (Ridderstråle & Groop, 2009).

Type 2 diabetes is generally characterized by four major metabolic abnormalities. These are obesity, the impaired action of insulin, dysfunction of insulin secretion and the increased output of glucose. The first three are present in most patients before onset of the disease, the sequence of development and the contributions to the progress from normal to impaired glucose tolerance to full blown diabetes has been difficult to ascertain. People who develop diabetes may manifest primary defects in both insulin action and secretion which predispose them to diabetes. These defects occur early during development towards established

diabetes and worsen as glucose tolerance deteriorates. The inability to compensate for either defective insulin action or secretion distinguishes individuals who develop diabetes than those who maintain normal glucose tolerance (Weyer, Bogardus, Mott, & Pratley, 1999).

Insulin resistance is a strong indicator of type 2 diabetes but eventual development of the disease and hyperglycemia associated with diabetes is associated with deficient insulin secretion. This defect appears to be determined by genetics. Insulin resistance is the inability of insulin to be effective at its normal circulating concentrations. In relation to glucose, it leads to impaired suppression of endogenous glucose production, in the basal state and after food consumption, when insulin rises according the glucose absorption from gut and stop further glucose production by liver.

In muscle and adipocytes, insulin resistance reduces the uptake of glucose and localized storage of glycogen and triglycerides. For the liver cells, insulin resistance results in a decrease in the synthesis of glycogen and glycogen storage. Also glucose production is no longer repressed by the ineffective insulin and more glucose is present in the blood. The term Insulin resistance usually refers to the reduced glucose-lowering effects of insulin.

Insulin resistance has tissue specific consequences. Reduced ability of insulin to suppress VLDL (very low density lipoprotein) production from the liver increases serum triglycerides, which leads to a decrease in HDL cholesterol and helps form LDL (low density lipoprotein) that contributes to atherosclerosis. In adipose tissue, insulin resistance increases the flux of non-esterified fatty acids (Ishiyama, Taguchi, Yamamoto, & Murakami) to the liver and skeletal muscle and impairs the action of insulin on the glucose metabolism within these tissues (Wass A.H. J, 2011).

The most important site of insulin resistance appears to be the peripheral tissues (DeFronzo, Ferrannini, Hendler, Felig, & Wahren, 1983). When insulin is applied as therapy, hepatic insulin sensitivity, as measured directly by insulin's ability to suppress production of glucose by the liver, significantly increases. FFA concentrations and rates of lipid oxidation decrease. Based on the assumption that insulin inhibits lipolysis, observations that liver fat reduces and hepatic insulin sensitivity increases could be due to the reduced FFA movement through the liver (Juurinen, Tiikkainen, Häkkinen, Hakkarainen, & Yki-Järvinen, 2007).

Insulin resistance affects the liver differently during the overnight fast periods and in the postprandial state. After an overnight fast, normally, insulin inhibits endogenous glucose production. In type 2 diabetics, the insulin resistance adds to the increase in basal endogenous production. Excess blood glucose and increased levels of insulin normally inhibit endogenous glucose production so insulin resistance is implicated in increasing production of basal endogenous glucose (Wass A.H. J, 2011).

Once the liver is insulin resistant, the action of insulin is impaired and leads to hyperglycaemia accompanied by stimulation of insulin secretion. Additionally, the more the level of resistance at the liver, the higher is the need for endogenous and exogenous insulin.

It is postulated that one of the reason for individual variation in insulin requirements in type 2 diabetes is the variation in insulin action. The variation in hepatic fat content may influence the need for insulin by influencing the sensitivity of endogenous glucose production to insulin (Ryysy, 2000).

In the postprandial state, under normal conditions, the endogenous glucose production is suppressed due to the increase in insulin and decrease in glucagon. The major effect of insulin in liver is the suppression of glucose output. In type 2

diabetics, the glucose production is not effectively suppressed due to hepatic resistance, deficiency in insulin and the presence of excess glucose. Continual production of glucose by the liver adds to the post meal hyperglycaemia. The rate of glucose usage in type 2 diabetics is normal because the presence of hyperglyacemia compensates for the impaired action of insulin to increase glucose uptake into tissues (Wass A.H. J, 2011).

Insulin is an important regulator of several aspects of adipocyte biology since adipocytes are one of the most insulin responsive cells. Insulin promotes triglyceride storage in adipocytes by assisting the differentiation of preadipocytes to adipocytes and, in mature adipocytes, by stimulating the transport of glucose, lipogenesis and inhibiting lipolysis. Functional defects in type 2 diabetes affects adipocyte regulation and these defects, in adipocytes, can occur from downregulation of the major insulin responsive glucose transporter; GLUT4. Also insulin binding to its receptor, phosphorylation of the receptor and consequent tyrosine kinase activity and IRS phosphorylation are all reduced. This occurs in muscle and adipose tissue. Particularly in adipose tissue, type 2 diabetics have adipocytes that have reduced IRS-1 expression that leads to decreased P13K activity and PI3 turns to depend on IRS-2 as the main docking protein. In muscle cells, although the levels of IRS 1 and 2 are normal, PI3K activity is impaired (Kahn & Flier, 2000).

Insulin resistance in adipose tissue is important because lipolysis is sensitive to insulin levels. Adipose tissue is also affected by insulin resistance as is evidenced by observations that there is increased triglyceride breakdown and higher fatty acid concentrations in type 2 diabetics compared to normal subjects at similar insulin levels. The increased rate of post meal free fatty oxidation could also lead to impaired glucose output by the liver and defective stimulation of glucose oxidation.

Excessive lipolysis leading to ketoacidosis does not occur in type 2 diabetes because the insulin deficiency is not profound enough to do so (Groop C L, 1989).

1.7 Diagnosis of Diabetes

Correct diagnosis and confirmation of the diagnosis is required especially considering the impact on the individual is considerable and lifelong. The requirements for diagnostic confirmation differ from individual to individual and are often based on the presenting symptoms such as severe symptoms and gross hyperglycemia to patients presenting with blood glucose values just above the cut off value. Severe glycaemia detected under conditions such as acute traumatic or infective stress could be transitory and cannot be considered as diagnostic of diabetes. A single abnormal blood glucose value in patient experiencing no symptoms is also not enough for a concrete diagnosis of diabetes. In such cases, one additional blood glucose test with a value in the diabetic range [fasting/random] or from the oral glucose tolerance test (OGTT) is required for diagnostic confirmation. In the face of unclear diagnosis, periodic re testing is advisable till the situation is clarified (Alberti, 1998; Consultation, 1999)

1.7.1 Diagnostic Criteria Values:

A1C ≥ 6.5% OR FPG 126 mg/dl [7.0 mmol/l]. Fasting is defined as no caloric intake for at least 8 h but plain water is allowed OR 2-h plasma glucose ≥ 200mg/dl (11.1mmol/l) during an OGTT OR In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥ 200 mg/dl (11.1 mmol/l) (A. D. Association, 2014)

The OGTT test, for all intents and purposes, should be resorted to only if the blood glucose values are between levels establishing or excluding the diagnosis of diabetes and fasting are below diabetic diagnostic values. If this test is performed it

is enough to measure the blood glucose values while fasting and thereafter 2 hours after a 75 gm oral glucose load by dissolving anhydrous glucose in water. For children this is calculated according to body weight: 1.75 gm/kg although the diagnostic criteria remain the same as for adults. It should be given in the morning after 3 days of unrestricted eating. The overnight fast is usually for 8 – 14 hours with water permitted (Alberti, 1998)

Although previously thought to be an unreliable diagnostic tool for diabetes due to lack of standardization and availability (Alberti, 1998; Consultation, 1999) glycated haemoglobin (HbA1C) is now considered to be a diagnostic tool that closely resembles other diagnostic tools currently used for the correct diagnosis of diabetes. Current recommendations and data show that the A1C cutoff of 6.5% is highly specific and reasonably sensitive in diagnosing diabetes. Repeating fasting glucose tests can minimize incorrect diagnosis of diabetes, the high reliability of A1C suggests a single measurement is enough for diagnosis (Selvin, Steffes, Gregg, Brancati, & Coresh, 2011).

An International Expert Committee, comprising members from the European Association for the Study of Diabetes, the International Diabetes Federation and the American Diabetes Association reported values of HbA1c ≥ 6.5% (48 mmol/mol) as the cut-off point for diagnosing diabetes. The United Kingdom Department of Health recommends using algorithms for diabetes screening in high-risk individuals that include traditional glucose diagnostic criteria or, alternatively, HbA1c measurements combined or not with glucose measurements. In non-symptomatic patients, a confirmed HbA1c ± 6.5% (48 mmol/mol) is enough to diagnose Type 2 diabetes. But patients with HbA1c levels ≥ 6.0% (42 mmol/mol) and < 6.5% (48 mmol/mol) should undergo the OGTT to determine the diagnosis of diabetes. However, no further testing is required for patients with HbA1c < 6.0% (42 mmol/mol). Until recently, the

lack of HbA1c standardization prevented the use of HbA1c as part of a potential screening and diagnostic program. The introduction of a new reference method improved HbA1c assay standardization worldwide and its role in the screening and diagnosis of Type 2 diabetes (Hu, 2010; WHO, 2011).

1.8 Diabetic Complications

Several serious complications are associated with long term diabetes of either type which increases morbidity and mortality in diabetics, whether acute or chronic. In terms of the tissues affected, complications can be divided into microvascular and macrovascular, based on arterial size affected (Hermans, 2007).

The level of hyperglycaemia increases the risk of microvascular damage such as retinopathy, nephropathy and neuropathy. Incidences of macrovascular complications such as ischaemic heart disease, stroke and peripheral vascular disease are also increased.

Although some complications are more specific to the different types of diabetes, most chronic complications affect both types of diabetes (Gale EAM, 2002).

There are several well studied mechanisms by which hyperglycaemia inflicts damage such as the polyol pathway, Advanced glycation end product formation, Protein kinase C activation (PKC), overactive hexosamine pathway and oxidative stress.

In the polyol pathway, the enzyme aldose reductase shunts the extra glucose into forming sorbitol that is later oxidized to fructose. This consumes NADPH which is critical for regenerating intracellular oxidants such as glutathione which defends against oxidative stress, another critical contributor to cell damage.

The precursors of AGE products damage cells by modifying intracellular proteins that regulate gene transcription, modifying nearby extracellular matrix molecules and proteins leading to cellular dysfunction and activation of inflammatory pathways. In the protein kinase C pathway, the cellular hyperglycaemia increases production of diacylglycerol that activates PKC and has varying effects on gene expression.

Hyperglycaemia also leads to increased flux through the hexosamine pathway which also has damaging consequences (Brownlee, 2005). The hexosamine pathway is implicated in glucose toxicity that contributes to insulin resistance in the muscle, an important pathological mechanism in Type 2 diabetes (Shepherd & Kahn, 1999).

1.9 Diabetic Neuropathy

Diabetic neuropathy (DN) is one of the most common complications of diabetes, with up to 50% of patients developing it and altering patients' quality of life, mentally and physically (M. J. Young, Boulton, Macleod, Williams, & Sonksen, 1993).

An internationally accepted definition of diabetic polyneuropathy for clinical practice is the "presence of and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes".

Factors such as poor control of blood glucose levels, aberrant lipid levels, hypertension and obesity can be linked to the development of DN (Solomon Tesfaye, 2010; Thomas, 1997).

Patients experience numbness, tingling, pain, or weakness that often begin in the feet and spread proximally in a length-dependent manner leading to chronic pain, ulcer formations, amputations, balance issues and falls. Although DN can

manifest at any stage of the diabetes, the prevalence increases with age and duration of diabetes with the highest rates appearing amongst diabetes of twenty five years (Boulton, 2005b).

Diabetic neuropathy is characterized by diffuse or localized damage to autonomic or peripheral nerve fibres. Broadly, diabetic neuropathy is classed into two major groups as shown in Table 1 based on Boulton and Thomas (Boulton, 2005b; Solomon Tesfaye ., 2010).

Generalized symmetric polyneuropathies	**Focal and multifocal neuropathies**
Acute sensory	Cranial
Chronic sensorimotor	Truncal
Autonomic	Focal limb
	Proximal motor amyotrophy
	Coexisting Chronic Inflammatory Demyelinating Polyneuropathy (CIDP)

Table 1: Classification of diabetic neuropathy

The typical pattern, and the most common, is of a symmetrical and length-dependent sensorimotor polyneuropathy; distal sensory polyneuropathy (DSPN) (Thomas, 1997). Focal neuropathies are less common. As the duration and severity of diabetes progresses, in peripheral neuropathy, there is a 'stocking-glove' distribution of sensory deficits ahead of any motor dysfunction observed. The signs and symptoms depend on the type of fibers involved and their functions. Large fiber

involvement affects light touch sensation and proprioception (Kleggetveit & Jørum, 2010) while small fiber involvement affects pain and temperature leading to paresthesias, dysesthesias and neuropathic pain (Aminoff, 2006). With more advanced neuropathy, complications such as ulceration and diabetic arthropathy emerge. Diabetic arthropathy, also known as Charcot neuroarthropathy, affects the bones, joints and soft tissues of the ankle and foot. Charcot first described the relationship between loss of sensation and arthropathy in 1868 (Kumar, Aslinia, Yale, & Mazza, 2011). Although it can occur because of various peripheral neuropathies, diabetes appears to be the commonest cause. The interaction of diabetes, sensory-motor neuropathy, autonomic and sensory-motor neuropathy, metabolic derangements of the bones and trauma result in the acute localized condition that leads to bone destruction, subluxation, dislocation, and deformity (Rogers, 2011).

Many diabetics develop foot ulcers that are neuropathic in origin, often accompanied by cellulitis or osteomyelitis. Poor wound healing often provides the background for severe infections to set in which could complicate to gangrene and often surgical amputation is required to conserve viable tissue (Ramsey, 1999). Neuropathy also a contributor to diabetic mortality (Coppini, Bowtell, Weng, Young, & Sonksen, 2000).

Diabetic autonomic neuropathy (DAN) is a form of diffuse diabetic neuropathy and can target sympathetic and parasympathetic functions that encompass genitourinary, cardiovascular and gastrointestinal dysfunctions. DAN highly prevalent yet poorly understood and has a negative impact on quality of life and mortality (Coppini, 2000; Freeman, 2005).

In the more focal forms of diabetic neuropathy, the damage can be to single or multiple peripheral nerves, cranial nerves or regions of plexuses or nerve roots.

The most common are the single nerve damage, or peripheral nerve mononeuropathies, affecting the medial and ulnar nerves. Carpal tunnel syndrome is more common in diabetics (Chammas, 1995) with approximately 30% of diabetics demonstrating some evidence of this on electrophysiological testing (Dyck, 1993). Focal diabetic neuropathies often have an acute onset, are self-limiting and relatively uncommon. The occulomotor nerve is most commonly affected amongst the cranial neuropathies and manifests as unilateral headache, diplopia, and ptosis without pupillary involvement.

This is termed as diabetic ophthalmoplegia and often coexistences with other diabetic complications and cardiovascular risk factors (Greco, Gambina, & Maggio, 2009).

Nerve plexus and nerve root involvement in diabetics often manifests lumbosacral plexopathy and polyradiculopathy and is termed diabetic amyotrophy. It is more prevalent in elderly patients with type 2 diabetics and is associated with unilateral thigh pain innervated by L2 to L4 (L. Smith, Burnet, & McNeil, 2003). Thoracic radiculopathy often manifests as sharp pains experienced in the thoracic or abdominal area (Chokroverty, Reyes, Rubino, & Tonaki, 1977).

Overall, diabetic polyneuropathy and diabetic autonomic neuropathy are common, most often diffuse and often progressive. The focal neuropathies are quite often rare, appear suddenly and have a self-limiting course (Thomas, 1997; Vinik, Mehrabyan, Colen, & Boulton, 2004).

The criteria required to diagnose diabetic peripheral neuropathy is based on the presence of neuropathic symptoms, signs and is reinforced by the presence of abnormal electrophysiological results – although the latter is more important for disease progression rather than diagnosis (England, 2005).

It is apparent that peripheral neuropathy is highly prevalent in diabetics and appears to increase with the duration of the disease and glycaemic control. Tight glycaemic control reduces the incidence and progression of neuropathy (Dyck, 1993; S. Tesfaye, 1996; M. J. Young, 1993). While diabetic peripheral neuropathy affects at least 50% of older type 2 diabetic patients, its pathogenesis is not clearly understood.

Diabetics can experience a range of symptoms. For some there are extremely painful symptoms but others, usually those with a more marked deficit, there may be no symptoms at all. Some of the typical neuropathic symptoms include; painful symptoms including burning sensations, knife-like pain, electric sensations of shock, throbbing pain and allodynia. The nonpainful symptoms including feeling the part of the body is asleep or ‘dead’, numbness, tingling and prickling sensations (Boulton, 2005).

The biochemical imbalances that underlie diabetic neuropathy are similar to those of other complications. A few have been mentioned previously and other mechanisms include activation of mitogen-activated protein kinases and inducible nitric oxide synthase, elevated cytokines such as tumour necrosis factors, interleukin 16, 18, hypoxia, ischemia and deficiencies of growth factors such as nerve growth factor (Chan, Terashima, Urabe, Lin, & Kojima, 2011).

The progression of diabetic neuropathy occurs in a ‘stocking glove’ pattern and has various stages (Table 2) (Thomas, 1997)

There are also various clinical types of peripheral diabetic neuropathy (Table 3) (Boulton, 2005a)

Stage of neuropathy	**Symptoms**	**Signs**
No neuropathy	None	None
Chronic painful	Shooting, stabbing and burning sensations Pin and needle sensations	Absent sensation to several modalities Reduced/absent reflexes
Acute painful	Above symptoms including hyperesthesia	Minor or absent signs
Painless with complete/partial sensory loss	Numbness/deadness of feet; painless injury,	Reduced/absent sensation Reduced sensitivity to heat/cold Absent reflexes
Late complications	Foot lesions; neuropathic deformity; amputation	Various

Table 2: Stages of diabetic peripheral neuropathy

Large fiber neuropathy	**Small fiber neuropathy**	**Proximal motor neuropathy**	**Acute mono neuropathies**	**Pressure palsies**
Sensory loss 0 → +++ (Touch, vibration) Pain + → +++ Tendon reflex N → ↓↓↓	Sensory loss 0 → + (Thermal, allodynia) Pain + → +++ Tendon reflex N → ↓	Sensory loss 0 → + Pain + → +++ Tendon reflex N → ↓↓↓	Sensory loss 0 → +++ Pain + → +++ Tendon reflex N	Sensory loss in nerve distribution + → +++ Pain + → ++ Tendon reflex N

Table 3: Clinical types of peripheral diabetic neuropathy

1.9.1 Impact of Diabetic Neuropathy

The criteria required to diagnose diabetic peripheral neuropathy is based on the presence of neuropathic symptoms, signs and is reinforced by the presence of abnormal electrophysiological results – although the latter is more important for disease progression rather than diagnosis (England, 2005). It is apparent that peripheral neuropathy is highly prevalent in diabetics and appears to increase with the duration of the disease and glycaemic control. Tight glycaemic control reduces the incidence and progression of neuropathy (M. J. Young, 1993), (Dyck, 1993), (S. Tesfaye, 1996).

Diabetic autonomic neuropathy is poorly understood. All organs receive innervation from the autonomic nervous system so every organ system can potentially be affected. Most organs have dual innervation with parasympathetic and sympathetic input. Most of the parasympathetic activity is mediated by the vagus

nerve which is the longest autonomic nerve as well (Berthoud & Neuhuber, 2000). Since the longest nerve fibers are first affected in diabetic neuropathy, early symptoms usually involve the parasympathetic system and are widespread (Ewing & Clarke, 1982). Subclinical neuropathy is usually present within a short time of diagnosing diabetes. The diagnosis of diabetic autonomic neuropathy depends on clinical and physiological assessments. Although many systems are affected, the cardiovascular system is the most commonly tested for variations in heart rate in relation to deep breathing, position changing and breathing out against pressure are measured by EKG (Pfeifer, 1984).

The risk of autonomic neuropathy increases with poor glycaemic control and duration of diabetes. The presence of other factors that have been linked to the development of DN include hypertension, an altered lipid profile and complications such as retinopathy (Cohen, Jeffers, Faldut, Marcoux, & Schrier, 1998; Gerritsen, 2001; Ziegler, 1993).

The autonomic nervous system practically regulates every bodily function and when it becomes derailed, the consequences are wide and varied. From irritating to deadly, diabetic autonomic neuropathy has a significant impact on patients' lives. Cardiac manifestations are related to increased mortality. In the early stages there is resting tachycardia and loss of heart rate variation and as the sympathetic function is affected later cardiac adrenergic sensitivity increases that can lead to death. Other complications include exercise intolerance, orthostatic hypotension, increased intraoperative death and silent myocardial ischaemia (Freeman, 2005). Gastrointestinal complications of diabetic autonomic neuropathy can affect any part of the gut. From heartburn and dysphagia in esophageal involvement (Freeman, 2005) to gastroparesis (Kong, Horowitz, Jones, Wishart, & Harding, 1999) and diarrhea (P. A. Low, 2004) – the symptoms are myriad.

The genitourinary involvement of diabetic autonomic neuropathy manifests as altered urinary frequency (Clarke, Ewing, & Campbell, 1979), bladder disorders such as urine retention or overflow incontinence (Buck, Reed, Siddiq, Chisholm, & Russell Fraser, 1976), erectile dysfunction (Kolodny, Kahn, Goldstein, & Barnett, 1974), vaginal dryness and decreased libido (Freeman, 2005).

Sudomotor dysfunction of diabetes often begins as a loss of thermoregulatory sweating in a glove and stocking distribution and progresses in a length-dependant manner typical of diabetic neuropathy. It can predispose to heat stroke and hyperthermia and lead to a compensatory central hyperhidrosis (Fealey, Low, & Thomas, 1989; Tentolouris, 2009)

1.9.2 Theories on the Pathogenesis of Diabetic Neuropathy

Several etiologies have been implicated in diabetic neuropathy. Hyperglycaemia is clearly an important factor for the development and progression of diabetic neuropathy. Several pathways have been identified and studied; most of these are glucose metabolic pathways linked to hyperglycaemia that are related to the cellular metabolic and/or redox status (Norman E Cameron & Cotter, 1997). These metabolic pathways include glucose flux through the polyol pathway, the hexosamine pathway, production of the protein kinase C isoforms and accumulation of advanced glycation endproducts (R. S. Clements, 1979; Toth ., 2008). The additive imbalance of these pathways leads to an altered state of mitochondrial redox state of the cell and forms high levels of reactive oxygen species (Phillip A Low, Nickander, & Tritschler, 1997; Andrea M. Vincent, Brownlee, & Russell, 2002), (Norman E Cameron & Cotter, 1997). The increased level of reactive oxygen species within the cells also activates the Poly-ADP ribose polymerase (PARP) pathway (Irina G Obrosova, 2005; Irina G. Obrosova, 2004). This is involved in expression of the genes that determine inflammatory reactions that are also involved

in neuronal dysfunction (Ilnytska, 2006). Other etiologies involved include altered metabolism of lipids, amino acids, vascular insufficiency, abnormal axon transport and reduced neurotrophism (Feldman, Stevens, & Greene, 1997). Hyperglycaemia has been implicated in damaging nerve cells directly and via ischaemia.

1.9.2.1 Polyol Pathway

The polyol pathway was first identified in the seminal vesicles (Hers, 1956) and demonstrated to convert blood glucose into fructose, an important source of energy for sperm cells. Sorbitol was later identified to be present in diabetic rat lens (Hohman, Nishimura, & Robison Jr, 1989) and the link between aldose reductase, the polyol pathway and the development of diabetic complications was postulated (Ramana & Srivastava, 2010; Van Heyningen, 1959). Aldose reductase in rat is highly expressed in the lens, the retina, and sciatic nerve (Hohman, 1989). These are all major targets for diabetic complications.

Aldose reductase is an enzyme that reduces glucose to sorbitol, which is converted to fructose by the enzyme sorbitol dehydrogenase (SDH) (Blakley, 1951). These enzymes are abundant in tissues more sensitive to hyperglycaemic damage. Aldose reductase is present in most cells (Yabe-Nishimura, 1998) and in the peripheral nerves it is localized in Schwann cells. This pathway is activated by mass action of excess glucose and high levels of sorbitol are produced (D. A. Greene, Lattimer, & Sima, 1988). This resulting cellular hypertonicity causes osmolytes such as *myo*-inositol (Finegold, Lattimer, Nolle, Bernstein, & Greene, 1983) and taurine (Nakamura, 1999) to leave the cell in an effort to compensate the osmotic imbalance. NADPH, which is essential for regenerating reduced glutathione, is consumed by the over activity of aldose reductase and the formation of excess fructose. This contributes to oxidative stress in the cell. Activation of aldose reductase increases the formation of diacylglycerol which, in turn, activates the

protein kinase C pathway. The rise in NADH/NAD^+ ratio could also facilitate the formation of diacylglycerol by increasing the availability of dihydroxyacetone phosphate and favouring its reduction to glycerol 3-phosphate, an intermediate of diacylglycerol synthesis (Pugliese, Tilton, & Williamson, 1991).

In normoglycemia, most cellular glucose is phosphorylated into glucose 6-phosphate by hexokinase as it shunts glucose into the glycolytic pathways (Yabe-Nishimura, 1998). Overactivity of the polyol pathways is associated with a decrease in energy flux in the diabetic nerve.

1.9.2.2 Hexosamine Pathway

The hexosamine pathway deals with excess fructose-5 phosphate, a metabolic intermediate from the glycolytic pathway, and converts it to glucosamine-6 phosphate by glutamine fructose-6 phosphate amidotransferase. The result is the formation of uridine diphosphate-N-acetyl glucosamine - a molecule that attaches to the serine and threonine residues of transcription factors (Kornfeld, 1967).

Hyperglycaemia allows excess amounts of uridine diphosphate-N-acetyl glucosamine to be produced and in turn this alters gene expression (Chou, 2004). Transcription factors such as Sp1 assist the process. Sp1 alters expression of genes involved in glucose control such as transforming growth factor β1 (TGF – β1) (Sysa, Potter, Liu, & Mezey, 2009) and plasminogen activator inhibitor 1 (PAI – 1) (X.-L. Du, 2000). The overexpression of TGF – β1 leads to an increased production of collagen matrix that culminates in endothelial fibrosis and decreased mesangial cell proliferation. PAI – 1 overexpression is involved in atherosclerosis because it encourages vascular smooth muscle cell proliferation (Y.-Q. Chen, 1998). It is also overexpressed by the protein kinase C (PKC) pathway and hexosamine pathway.

The presence of hyperglycaemia produces excess NADP and overloads the mitochondrial electron transport chain leading to oxidative stress, mitochondrial

damage and activation of PARP. PARP acts with the hexosamine and PKC pathway to induce inflammation and aberrant neuronal function.

GlcNAc also impairs beta cell function by adding to the oxidative stress of the cell (K. Liu, Paterson, Chin, & Kudlow, 2000). Increased glutamine fructose-6 phosphate amidotransferase or glucosamine levels has been linked to insulin resistance (Hebert Jr, 1996), increased hydrogen peroxide levels, reduced expression of insulin, glucose transporter protein 2 and glucokinase genes (Kaneto, 2001).

1.9.2.3 Protein Kinase C Pathway

Hyperglycaemia is intrinsically involved in damaging tissues prone to complications. The protein kinase C pathway is no exception. Increased levels of glucose stimulate diacylglycerol which activates PKC. Excess levels of PKC β isoform has been linked to increased levels of the angiogenic protein vascular endothelial growth factor (VEGF), PAI-1, nuclear factor κB (NF-κB), TGFβ and other diabetic complications such as retinopathy, nephropathy and cardiovascular complications (Arikawa, 2007; Das Evcimen & King, 2007; Geraldes & King, 2010; Veves & King, 2001).

The activation of the PKC pathway also affects capillary permeability and vasoconstriction. This can lead to hypoxia, angiogenesis, endothelial proliferation and thickening of the basement membrane (Williams, Gallacher, Patel, & Orme, 1997). These changes in the neurovascular blood flow are most likely how PKC is involved in neuropathy (Way, Katai, & King, 2001). The sodium potassium ATPase pump and other enzymes that are critical for nerve function are also targeted. PKC isoforms negatively affect sodium potassium ATPase pumps (D. A. Greene, Lattimer, & Sima, 1987; Ishii, Koya, & King, 1998; Koya & King, 1998). Inhibiting PKC inhibition improves both nerve blood flow and nerve conduction velocity (Ishii,

1996), (Das Evcimen & King, 2007), (Way, 2001). PKC isoforms also influence insulin resistance (Griffin, 1999; J. K. Kim ., 2004).

1.9.2.3 Advance Glycation End Product Formation

These result from non-enzymatic reactions between reducing sugars and proteins or lipids (Goh & Cooper, 2008). The AGE precursors are also known as reactive dicarbonyls and are particularly harmful. Most commonly, the by-product of these irreversible reactions form Schiff bases that degrade into additionally harmful Amadori products or fructosamine. Reactive dicarbonyls such as glyoxal and methyloxal can also modify proteins, lipids, and nucleic acids and are more damaging. Thus reactive dicarbonyls are one of the main mechanisms to produce AGEs, add to carbonyl stress and lead to complications (Jack & Wright, 2012),(Huebschmann, Regensteiner, Vlassara, & Reusch, 2006). Methylglyoxal, has been linked to increasing the susceptibility to vascular damage in endothelial cells (Yao, 2007).

AGEs go on to modify components of the cell and act via the receptor; RAGE (Schmidt , 1996) which is expressed in a multitude of tissues (Brett, 1993). The interaction of AGE with its receptors has consequences such as the activation of the transcription factor nuclear factor κB (NF-κB). Upregulation of this factor can increase expression of proinflammatory genes and alter neuronal function (Sugimoto, Yasujima, & Yagihashi, 2008). Increased levels of AGE and RAGE are found in diabetic tissue (Tanji, 2000). Diabetic mice with RAGE knockout showed an improvement in the neuropathic state and had decreased expression of NF-κB and PKC (Toth, 2008).

AGEs also result in decreased blood flow of nerves by altering vessel wall structure and patency. AGEs are also implicated in affecting neurons by reducing neurotrophic support (Wada & Yagihashi, 2005).

1.9.2.5 PolyADP-ribose Polymerase Pathway

The enzyme poly ADP-ribose polymerase (PARP) is a nuclear membrane enzyme that synthesizes poly ADP-ribose. This represents a cellular response mechanism to DNA damage (S. Smith, 2001). PARP is activated by oxidative stress and also appears to contribute to oxidative stress (Irina G Obrosova, 2005). The action of PARP involves transfer of ADPribose from nicotinamide adenine dinucleotide to nuclear proteins. This results in depleting NAD^+, changes gene transcription and expression, increases oxidative stress and shunts the intermediates of the glycolytic pathways to pathological pathways PKC and advanced glycation end product formation (Erdelyi, Bakondi, Gergely, Szabo, & Virag, 2005; Soriano ., 2001)

The functional domains of PARP include a DNA binding domain at the amino terminus, a catalytic domain at the carboxyl terminus and a central automodification domain which accepts polyADP-ribose. The DNA binding domain recognizes and binds to DNA strand breaks and has two zinc—finger motifs which are similar to the motifs in DNA ligase III and DNA polymerase. When PARP binds to breaks in DNA strands it triggers the polyADP ribosylation reaction (Jeggo, 1998; Süsse, Scholz, Bürkle, & Wiesmüller, 2004)

Abnormalities that have been linked to PARP manifest clinically as decrease in nerve conduction velocity, neurovascular abnormalities, small fiber neuropathy, thermal and mechanical hyperalgesia, and tactile allodynia (F. Li, Drel, Szabó, Stevens, & Obrosova, 2005; Irina G Obrosova, 2007; Pacher, 2002).

1.9.2.6 Oxidative Stress and Apoptosis

Most of the pathological mechanisms discussed above contribute to oxidative stress in the cell. AGE and the polyol pathways reduce the redox capacity of the cell by forming ROS or reducing availability of glutathione. The hexosamine, PKC and PARP pathways cause damage via increased expression of inflammatory

proteins. Based on the observation that diabetic neuropathy progresses in an axon length-dependent manner (Said, 2007), the damage could origin in the axon. These sites are more susceptible to damage by hyperglycaemia because of the large number of mitochondria that they possess. Against the backdrop of ischaemia, hyperglycaemia overloads the capacity of the mitochondria and sets the stage for oxidative stress. This further enhances mitochondrial damage which in turn leads to axon degeneration and death (Leinninger, Edwards, Lipshaw, & Feldman, 2006).

Mitochondria are vulnerable to damage by reactive oxygen and nitrogen species. Reactive oxygen species are produced by the mitochondria and usually removed by cellular agents such as glutathione, catalase and superoxide dismutase. Hyperglycaemia accentuates ROS production in the cell via mitochondrial activity (Friederich, Hansell, & Palm, 2009; Leinninger ., 2006; Andrea M Vincent, Russell, Low, & Feldman, 2004).

The main reactive nitrogen species, peroxynitrite is formed from the reaction of nitric oxide and superoxide ion. Peroxynitrate is inherently unstable and results in the formation of nitrate through isomerisation. Reactive nitrogen species have several cell damaging effects; including PARP activation and protein nitrosylation (Patel, 1999).

The presence of excess superoxide ion inhibits GAPDH and upstream glycolytic intermediates accumulate in the cell. These in turn enhance the Aldose reductase, hexosamine, PKC pathway and AGE product formation increase cell injury (Nishikawa, 2000),(X.-L. Du, 2000).

The vicious cycle of injury in cells prone to diabetic complication continues. Hyperglycaemia leads to over activation of metabolic pathways and produce cellular oxidative stress. Against the backdrop of decreased nerve blood flow and ischaemia, the tissue suffers more injury. The production of reactive oxygen species

produces oxidative stress which leads to further injury and also impairs the protective antioxidative mechanisms of the cell.

Mitochondria are also important in determining cell viability (Friederich 2001). The presence of oxidative stress initiates signalling pathways that destroy local mitochondria. One of these utilizes the dynamin-related protein 1 (Drp 1). The usual process of mitochondrial fission and fusion, a delicately balanced process essential for mitochondrial viability (Knott & Bossy-Wetzel, 2008) is often disrupted by Drp1 which translocates to the mitochondrial surface and tips the balance towards fission. Irregular fission is associated with mitochondrial death and apoptosis. Diabetic neuropathy is associated with increased levels of Drp1 (Figueroa-Romero, Sadidi, & Feldman, 2008; Frank, 2001; Knott & Bossy-Wetzel, 2008). There is evidence of apoptosis in the cell body of neurons and the presence of neuroaxonal dystrophy (Russell, Sullivan, Windebank, Herrmann, & Feldman, 1999). Recurrent injury in the face of hyperglycaemia activates cell death pathways which are beyond the scope of glial support and repair. This initiates mitochondrial damage and redistribution till the axons die back towards the cell body (Srinivasan, Stevens, & Wiley, 2000), (Leinninger, 2006).

1.9.2.7 Inflammation

Inflammation is linked to diabetic neuropathy, substances such as C-reactive protein and plasma levels of TNF-α are in higher concentrations in diabetics and appear to correlate with the incidence of diabetic neuropathy, amongst other neuropathies, and are also linked to other diabetic complications. HSP27, an intermediate in the TNF-α induction of the inflammatory mediators; cyclooxygenase-2 (COX-2), Interleukin 6 (IL-6), and Interleukin 8 (IL-8), is also increased in diabetic neuropathy (Empl ., 2001; González-Clemente, 2005; Gruden, 2008; Jager, 1999; Makino, 2005; Schalkwijk, 1999).

Excess glucose is shunted to metabolic pathways that increase expression of NF-κB and TGF-β (Esposito, 2002; G. L. King & Loeken, 2004). AGE modifications bring about their own contributions, one of which includes the formation of methylglyoxal which decreases the binding of transcription factors such as Sp3 which is an angiotensin II repressor (Yao, 2007). Hyperglycaemia increases tissue angiotensin II and activates vascular endothelial cells, which leads to inflammatory cell recruitment, cytokine formation, ischaemia, increased ROS formation etc (Lawrence J Coppey, 2006). Other AGE products utilize RAGE to increase intracellular inflammatory signalling to upregulate NF-κB (Toth, 2008).

NF-kB is a very important transcription factor and regulates the expression of many genes that are involved in inflammation such as COX-2 (L. A. J. O'Neill & Kaltschmidt, 1997). COX-2 is upregulated in diabetic neuropathy and is a potential target for treatment. COX-2, in turn, generates prostaglandin E2 and reactive oxygen species that further activate NF-κB. Inhibition of COX-2 appears to prevent harmful changes to peripheral nerves such as nerve conduction deficits, reduction of blood flow, GSH depletion and increases in TNF-α production (Pop-Busui, Kellogg, & Cheng, 2008).

NF-κB also regulates another enzyme involved in inflammation; inducible nitric oxide synthase (eNOS) which in turn induces NF-κB further and similar to COX-2 sets off a vicious cycle of inflammation (Y. Kim ., 2008). The nitric oxide (NO) produced by iNOS increases nerve blood supply, modulates post injury changes in microvasculature and is linked to neuropathic pain. High levels of NO in inflammation could damage growth cones and axons (N. E. Cameron, Eaton, Cotter, & Tesfaye, 2001; McDonald, Cheng, Martinez, & Zochodne, 2007; Whittle, 1995; Zochodne & Levy, 2005)

NF-κB is important in the inflammatory pathways of diabetic neuropathy. With chronic activation of NF-κB, blood vessels and nerve cells are more prone to

injury in ischaemia reperfusion. This is followed by infiltration of monocytes, macrophages, and granulocytes into the peripheral nerves (Yanping Wang, Schmeichel, Iida, Schmelzer, & Low, 2006). This macrophage recruitment is trigged by the cytokine production induced by NF- κB in endothelial cells, Schwann cells and neurons. Macrophages in turn contribute to diabetic neuropathy by producing ROS, cytokines and proteases that degrade myelin and increase oxidative stress. This also contributes to the impaired nerve regeneration in diabetic neuropathy (Conti, 2002; Kennedy & Zochodne, 2005; Tesch, 2007; Yamagishi, 2008).

1.9.2.8 Growth Factors

Growth factors promote the growth and survival of neurons and determine neurite outgrowth (Chiarelli, Santilli, & Mohn, 2000; Leinninger, Vincent, & Feldman, 2004; Tomlinson, Fernyhough, & Diemel, 1997). Since diabetic neuropathy exhibits evidence of neuronal degeneration and Schwann cell damage (Russell, 1999), alterations in growth factors such as nerve growth factor, insulin-like grown factor 1 (IGF-1) and neurotrophin 2 could be involved in the pathogenesis. These factors all bind to tyrosine kinase receptors. The nerve growth factor (NGF) receptor is made up of p75NTR and a specific trk tyrosine kinase, which confers ligand specificity.

Many growth factor levels are altered in diabetic neuropathy. Nerve growth factor, the most widely studied factor, is produced by muscle cells and keratinocytes. The nerve growth factor receptor; trkA receptor is expressed on sensory and sympathetic neurons (Anand, 1996). Animal models of diabetic neuropathy have shown altered expression of several growth factors and both NGF levels and retrograde transport of the growth factor are reduced (R Hellweg & Hartung, 1990).

The transport of NGF to the stoma is required for its neurotrophic effects to occur (Rainer Hellweg, Raivich, Hartung, Hock, & Kreutzberg, 1994). However, when glucose levels were normalized, NGF levels also return to normal suggesting

a link between hyperglycaemia to growth factor levels (Rainer Hellweg, 1991). Administering insulin appears to bring growth factor levels of several such as IGFI and II back towards normal levels (Migdalis., 1995).

1.9.2.9 Lipid Abnormalities

The mechanisms by which plasma lipids influence neuronal injury have not been fully elucidated but certain factors have been implicated. Correlations between an altered lipid profile and the increased incidence of neuropathy have been identified in both type1 and type 2 diabetics.

In animal models, a high fat diet fed to mice results in overexpression of 12/15 lipoxygenase in peripheral nerves even though these animals are glucose tolerant but not overly diabetic (Irina G. Obrosova, 2007). Along with hyperglycaemia dyslipidaemia initiates neuronal injury (Rockenfeller, 2010). These molecular altercations can also activate the endoplasmic reticulum unfolded protein response in many types of cells, which can also lead to cell death(McAlpine, Bowes, & Werstuck, 2010). It is unclear if elevated lipid levels have direct effects on the peripheral neurons. Factors such as permeabilization of the lysosomal membrane by cathepsin L leading to damage of the mitochondria and oxidative stress could be important (Rockenfeller, 2010).

In diabetes, plasma lipoproteins are exposed to an oxidizing environment. Peripheral neurons express scavenger receptors for oxidized LDLs including oxidized LDL receptor 1 (LOX1) (Ishiyama, 2010) and Toll-like receptor 4 (Geng, 2010; Nowicki ., 2010; A. M. Vincent, 2009). These neurons also express RAGE, which binds glycated LDL and internalizes oxidized LDL and glycated LDL, releasing potentially injurious triglycerides and fatty acids within the cell that increase damage (Honjo, 2008; Stielow, 2006; Andrea M. Vincent, Callaghan, Smith, & Feldman, 2011; A. M. Vincent, 2007). NADPH oxidase is also activated and adds to the oxidative stress. Besides the other damaging effects, oxidative stress in diabetes

enhances expression of oxLDL and RAGE via p38 mitogen-activated protein kinase MAPK signalling and adds to the damage (Toth, 2008), (Ishiyama, 2010).

Although treatments have been developed that target these pathogenetic mechanisms (Figure 1) there is no clear evidence to suggest remarkable benefits and thus early institution of optimal glycemic control remains the only available measure with proven efficacy in preventing or slowing progression of diabetic neuropathy (Boucek, 2006).

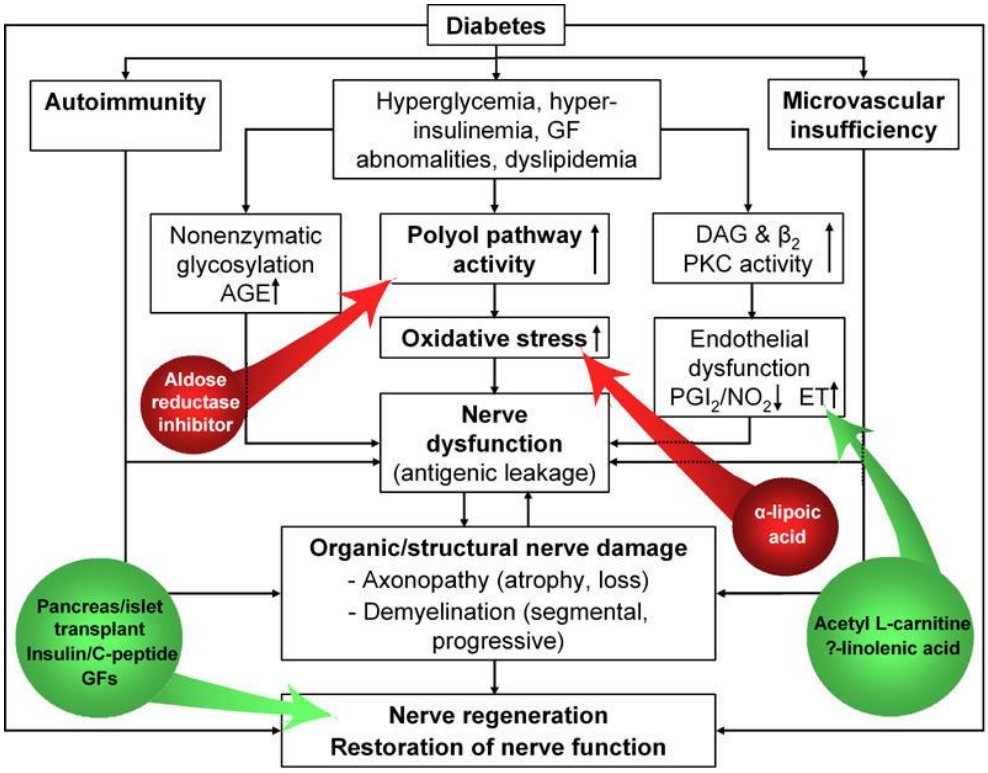

Figure 1: Therapeutic interventions based on hypothetic pathogenetic mechanisms of diabetic neuropathy

Interventions with inhibitory effects are marked in red, with mainly supportive effects in green. AGE: advanced glycation end products. GF: growth factor. DAG: diacylglycerol. PKC: protein kinase C. PG: prostaglandin. NO: nitric oxide. ET: endothelin (Boucek, 2006)

1.10 Anatomy of the Autonomic Nervous System

The autonomic nervous system is a conduit between the central nervous system and the viscera. It is organized in groups of ganglia, which can be grouped schematically into paravertebral, prevertebral, paravisceral, and intramural (Paxinos, 2004). Ganglia are networks of bundled or connected somata and dendritic structures which serve as relay points for the nervous system (Per, 2010).

Dorsal root ganglion (DRG), also called spinal ganglion, is the ganglion of the posterior root of each spinal segmental nerve, containing the cell bodies of the unipolar primary sensory neurons. These also lie on the side of the vertebral column in close association with the spinal cord (Paxinos, 2004). These ganglia reside at each segmental level of the spinal column within, the intervertebral foramen. The DRG is interposed between the dorsal root that resides within the subarachnoid space and the spinal nerve that proceeds to peripheral receptive fields. The sensory neuron somata for all sensory modalities and types of fibers reside in the DRG (Koopmeiners, Mueller, Kramer, & Hogan, 2013). The cell body of each sensory neuron in the dorsal root ganglion has a long axon which extends from the dendrites such as the skin, to the cell body in the dorsal root ganglion and then continues to the spinal cord. The section of the axon that runs from the ganglion to the spinal cord is bundled with similar axons. These bundles are called dorsal roots (Hart, 2010).

The axons of posterior root ganglion neurons are known as afferents because they relay sensory information into the central nervous system (i.e. the brain and the spinal cord). These neurons are of the pseudo-unipolar type; the axon has two branches that act as a single axon and are termed as a distal process and a proximal process. Unlike the majority of neurons found in the central nervous system, an action potential in posterior root ganglion neuron may initiate in the distal

process in the periphery, bypass the cell body, and continue to propagate along the proximal process until reaching the synaptic terminal in the posterior horn of spinal cord(R, 2010).

The distal section of the axon may either be a bare nerve ending or encapsulated by a structure such as a Meissner's corpuscle or Pacinian corpuscle to relay specific information to nerve. For example, corpuscle may encapsulate the nerve ending, rendering the distal process sensitive to mechanical stimulation. The dorsal root ganglia develops in the embryo from neural crest cells instead of the neural tube. This gives rise to theories that regard the dorsal root ganglia as gray matter of the spinal cord that became translocated to the periphery(R, 2010).

The beginning of somatosensory information transmission occurs when peripheral receptors of the primary afferent neurons are activated. In cutaneous and visceral nerves of the rat, the Aβ class of nerves is the fastest-conducting large myelinated sensory fibers; the Aδ group is the slower-conducting, thinly myelinated fibers and the slowest-conducting unmyelinated small fibers are C-fibers. C fibers respond to stronger and possible painful stimuli. Several neurotransmitters are postulated to be released from the afferent neurons after a painful stimulus. Evidence points towards the excitatory amino acids (EAAs), aspartate and glutamate and substance P amongst others (Budai, 2000). The transmission of impulses from nociceptive afferents to the central nervous system, essential to perceive pain depends on the release of certain neurotransmitters. These include glutamate, substance P and calcitonin gene-related peptide (Duggan, 2002).

The autonomic ganglia house the cell bodies of sympathetic or parasympathetic motor neurons. The paravertebral ganglia are organized as the sympathetic chains that lie on other side of the vertebral column. These neurons in these ganglia receive synaptic input from preganglionic autonomic neurons whose

cell bodies are located in the CNS. The autonomic motor neurons in the ganglia send efferent fibers (postganglionic autonomic nerve fibers) to innervate cardiac muscle fibers of the heart and smooth muscle fibers of body organs and glands.

Compared to the dorsal root ganglia, the neuron cell bodies in autonomic ganglia are more widely dispersed, with a meshwork of nerve fibers lying between them, and the nerve fibers generally are not as well organized. Unlike the dorsal root ganglia, which have no synapses and therefore no neuropil, in sympathetic ganglia many preganglionic sympathetic fibers from the spinal cord synapse on the sympathetic neurons, and others travel through the ganglia without synapsing. The cell bodies of sympathetic neurons are smaller than those of sensory neurons in the dorsal root ganglion. The cells are multipolar with eccentrically placed nuclei. The satellite cells (glial cells) are sparse and less apparent.

The pelvic ganglia, in the male rat, are usually located on the side of the prostate, closely apposed to its fascia. The rat pelvic ganglia contain neurons projecting to several urogenitary organs such as the bladder (Gu, 1984), penis (Y.-Q. Ding, Takada, Kaneko, & Mizuno, 1995),(Y.-q. Ding, Wang, Qin, & Li, 1993) and prostate (Kepper & Keast). The ganglion contains some cells bodies with the characteristics of both adrenergic and non-adrenergic neurons. The latter contain Vasoactive Intestinal Polypeptide and Galanin. Another group of neurons are the small intensely fluorescent cells also termed as SIF cells (Paxinos, 2004) that contain transmitters such as Substance P and Galanin (Dail, Galindo, Leyba, & Barba, 1997; Keast, 1991). Substance P along with CCK are present in presynaptic terminals at different sites in the ganglion. Two types of nerve endings, containing SOM and/or CCK are preferentially associated with the non-noradrenergic Neuropeptide Y neurons (Keast, 1991), and Substance P is present in axon terminals on other non-noradrenergic neurons as well (Keast & Chiam, 1994).

1.10.1 The Pain Pathway

The endogenous somatosensory system transduces sensory information from the periphery to the central nervous system using a three neuron relay system. The first order neurons are located in the peripheral nervous system with their cell bodies in the dorsal root ganglia. Peripheral sensory neuron cell bodies give rise to a pseudounipolar axon which is a single axon that terminates in sites within the central and peripheral nervous systems. Pseudounipolar cells have two axons rather than an axon and dendrite. One axon extends centrally toward the spinal cord; the other axon extends toward the skin or muscle. Peripheral terminals of primary afferent neurons innervate cutaneous and muscle tissues and transduce both innocuous and noxious sensory information. Noxious (physical/mechanical), thermal, or chemical stimuli preferentially activate specialized types of primary afferent neurons which are termed nociceptors. Nociceptive primary afferent neurons signal to the spinal cord, terminating on second order neurons in the gray matter of the dorsal horn. The somatosensory system is a contralateral system, and nociceptive signals cross the midline in the anterior white commissure at the level of the spinal cord. The information that travels via axons in the lateral funiculus of the ventral horn and one of its major termination sites is the thalamic nuclei. From the thalamus, neurons project to multiple sites within the brain including the somatosensory cortex, prefrontal cortex and anterior cingulate cortex which are regions important for integrating, interpreting, and coordinating a relevant response to the nociceptive information. In parallel to the ascending nociceptive system there is a descending system that provides inhibitory and excitatory pain control. The descending modulation of pain is when the integrated efferent message from the brain is modulated by brainstem areas before reaching reaches the spinal cord. This descending system provides communication, either directly or indirectly, from the brain to the spinal cord to modulate pain and can promote both endogenous

analgesia as well as facilitation of chronic pain states. The ascending and descending pain modulatory systems code for both sensory/discriminative and affective/emotional aspects of pain (Edelmayer, Brederson, Jarvis, & Bitner, 2014). The figure below delineates the pain pathway (Edelmayer, Brederson, Jarvis, & Bitner, 2014)

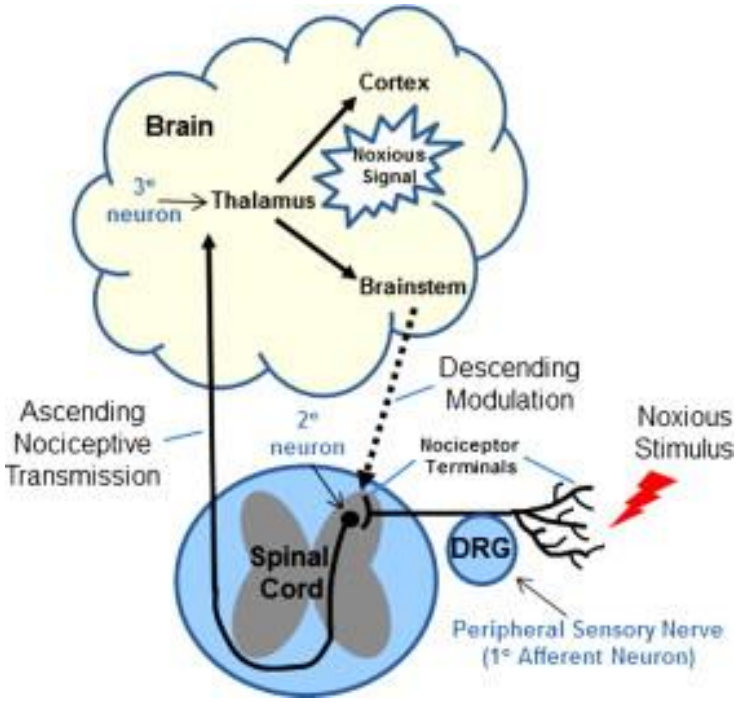

Figure 2: The pain pathway

In the most basic sense, the ascending somatosensory pathway consists of a three neuron relay system. The first order neuron resides in the DRG. DRG cell bodies give rise to a single pseudounipolar axon, which extends to peripheral and central termination sites. In the periphery, nociceptors are a specialized type of primary afferent neuron that is preferentially activated by noxious stimulation and transmits nociceptive signals to the central nervous system. The afferent fibers synapse on second order neurons in the gray matter in the dorsal horn region of the spinal cord. Second order neurons cross the mid line and nociceptive signals continue to be relayed to thalamic neurons. Third order neurons in the thalamus

have multiple terminal projection sites. The descending system consists of brainstem nuclei that project to the spinal cord, either directly or indirectly, to influence endogenous analgesia.

Several sensory abnormalities occur in conditions of chronic pain, hyperalgesia and allodynia. These have been linked to the possible plasticity of dorsal horn circuits. Sensitization of primary afferent fibers can be linked to primary hyperalgesia while allodynia could be due to sensitization of neurons in the dorsal horn. Hyperalgesia, allodynia and chronic pain sensation after peripheral tissue injury depends on the increase in sensitivity of primary afferent nociceptors at the site of the injury; peripheral sensitization, and on an increase in the efficiency of the synapses between primary afferent fibers and the dorsal horn neurons; central sensitization (Yaksh, Hua, Kalcheva, Nozaki-Taguchi, & Marsala, 1999)

1.11 The Glutamate Signalling Pathway

The main excitatory neurotransmitter of the nervous system is glutamate that acts via its receptors. The released glutamate from the presynaptic terminal is transduced to biochemical and electrical events in the postsynaptic neuron for functions such as neuronal excitability modulation and synaptic transmission.

The excitatory action of glutamate in the mammalian brain and spinal cord has been known since the 1950s (Curtis & Watkins, 1960). Since it is present in high concentration and is relatively evenly distributed throughout the different areas of the brain, it was only recognized as a neurotransmitter by the 1970s. It is one of the main excitatory neurotransmitters in the central nervous system of mammals (Meldrum, 2000). By the mid-1980s, glutamate was shown to have largely filled the four main criteria to be classified as a neurotransmitter; presynaptic localization, release by physiologic stimuli, identical in action to a naturally occurring transmitter and mechanism for quick termination of its action. Further studies strengthened the

concept that glutamate is a neurotransmitter. These included the demonstration of ATP-dependent selective transport of glutamate into synaptic vesicles (Shigetaka Naito & Tetsufumi Ueda, 1985) , the presence of high concentrations of glutamate in synaptic vesicles isolated from the brain (Orrego & Villanueva, 1993) and a calcium dependent exocytotic release of glutamate from isolated nerve terminals(Nicholls, 1995).

1.11.1 The Biochemistry of Neurotransmitter Glutamate

The metabolism of glutamate is complicated as it involves neurons and glial cells. The neurotransmitter glutamate can be synthesized through different metabolic pathways, and different populations of glutamatergic neurons may differ in different aspects of glutamate metabolism.

The formation and degradation of glutamate is part of the general energy metabolism of the brain because glucose is the main substrate for the brain. Glucose is one of the most important precursors for transmitter glutamate via the tricarboxylic (TCA) cycle.

The cerebral TCA cycle activity is 15-20nmol min^{-1} mg^{-1} protein (Mason, Rothman, Behar, & Shulman, 1992) and this activity corresponds to the whole brain activity of alpha ketoglutarate dehydrogenase, and it is lower than all other enzyme activities of the TCA cycle as measured *in vitro*. Alpha dehydrogenase which converts alpha ketoglutarate into succinyl-CoA, is a rate limiting step of the TCA cycle (Lai, Walsh, Dennis, & Clark, 1977). This bottleneck allows alpha ketoglutarate to accumulate. Alpha ketoglutarate is transaminated to glutamate by the highly active transaminases such as aspartate aminotransferase and alanine aminotransferase (Mason, 1992). These enzymes use aspartate or alanine as the amino group donor (Mason, 1992). Alanine is exported from astrocytes, taken up by neurons and then metabolized to glutamate (Westergaard ., 1993). Other amino

group donors such as leucine from the circulation are also used (Yudkoff, 1997). The large pool of glutamate present in the glutamatergic neurons is maintained by the bottleneck function of alpha ketoglutarate dehydrogenase in the TCA cycle, the very high activities of transaminases compared to alpha ketoglutarate dehydrogenase and the abundant supply of amino group donors in transamination actions.

Glutamate is the precursor of GABA. However, glutamate is present in low levels in the GABAergic neurons and astrocytes and this could be because the alpha ketoglutarate dehydrogenase bottleneck is bypassed in these cells. In GABAergic neurons glutamate enters the GABA shunt and is converted successively into GABA, succinic semialdehyde and succinyl-coA. In astrocytes, glutamate is diverted from the bottleneck of alpha ketoglutarate dehydrogenase by the formation of glutamate which then exits the cells. This leads to the levels of both glutamate and aspartate to be low in astrocytes (Ottersen & Storm-Mathisen, 1985).

Thus all brain cells contain glutamate as a byproduct of energy metabolism. A neuron can be identified as glutamatergic on an immunocytochemical basis only after detection of glutamate in synaptic vesicles.

Glutamate is pooled in various parts of the brain and these pools communicate with each other, as and when required. The transmitter pool is located in the vesicles of glutamatergic terminals, the GABA precursor pool that is present in GABAergic neurons, the glutamine precursor pool that is located in glia and the lastly the metabolic pool of glutamate which is present in all cells. These pools communicate in various instances; when glutamate is diverted form the metabolic pool to become transmitter or precursor of GABA and glutamine, or when the amino acid transmitters return to the metabolic pool and are processed to become carbon dioxide and water. There is also extensive transport of glutamate and its derivatives, GABA and glutamine, between cell types(Fonnum, 1993). Glutamate is

synthesized directly from glutamine, 1-pyrroline-5-carboxylate (P5C) or alpha ketoglutarate (2-oxoglutarate) in the CNS. The formation of glutamate from glutamine is an energy saving process that is catalyzed by phosphate-activated glutaminase (PAG), which could play a major role in the production of transmitter glutamate. P5C is derived from ornithine through glutamic semialdehyde by the catalysis of ornithine delta-aminotransferase (OAT) or from proline by proline oxidase (PO), then converted to glutamate by P5C dehydrogenase (P5CDH).

Glutamine is an important precursor for transmitter glutamate. The transmitter pool of glutamate is preferentially supplied from glutamine (Bradford, Ward, & Thomas, 1978). In the brain, glutamine synthetase is located in the astroyctes and oligodendrocytes (Martinez-Hernandez, Bell, & Norenberg, 1977; Tansey, Farooq, & Cammer, 1991). Approximately 60% of alpha ketoglutarate formed in astrocytes is converted to glutamate and then to glutamine (Akiyama, Kaneko, Mizuno, & McGeer, 1990). Glutamine is formed from glutamate by amidation.

The enzyme glutaminase is located on the external aspect of the inner mitochondrial membrane and is an amidohydrolase enzyme that generates glutamate from glutamine. Since glutamine is an important energy substrate for neurons, most of the glutamate that is formed from glutamine enters the mitochondria (Bradford, 1978). After glutamine is converted to glutamate, it is packed in the secretory vesicles and awaits release.

The astrocytic export of glutamine implies a continuous loss of alpha ketoglutarate from the astrocytic TCA cycle but this is not the case. The intermediates of the TCA cycle have restricted access across the blood-brain barrier and so the anaplerotic process of pyruvate carboxylation continually replenishes the loss. Anaplerosis is the process of converting pyruvate, which is derived from glucose via glycolysis, to oxaloacetate or malate. Astrocytes express the enzymes

pyruvate carboxylase and cytosolic and mitochondrial malic enzyme (A. C. Yu, Drejer, Hertz, & Schousboe, 1983). Observations that neurons and subpopulations of neurons have the ability to replenish their TCA cycle by carboxylating pyruvate can explain why some glutamatergic pathways have a low level of glutaminase, whereas others have high levels, and it explains how transmitter glutamate can be formed from neuronal precursors (Hassel & Brathe, 2000a). Neurons can thus carboxylate pyruvate and not depend on glutamine as a precursor for transmitter glutamate.

The glutamine cycle, the 1:1 exchange between astrocytes and neurons of glutamine for glutamate and GABA was conceptualized in the 1970s (van den Berg & Garfinkel, 1971). In this cycle, astrocytes take up glutamate released from neurons, convert it to glutamine in an energy dependent process and then supply glutamine to the extracellular fluid to maintain a high concentration of glutamine which is approximately 0.3mM. Neurons consume glutamine and convert it to glutamate by glutaminase.

1.11.2 Vesicular Uptake of Transmitter Glutamate

The transmitter pool of glutamate is estimated to be about 20-30% of the total brain glutamate content (Karlsen & Fonnum, 1978). Glutamate formed in the nerve terminals is transported into the vesicles via the vesicular transporter. This transporter has a low affinity for glutamate with a Km around 1mM (S. Naito & T. Ueda, 1985). This is approximately 1000 times higher than the Km of the plasma membrane transporters, which correlates with the concentration of glutamate being 1000-fold higher in the cytosol than extracellular fluid. Glutamate transport into vesicles is driven by an electrochemical gradient. This is generated by a proton pump that depends on ATP and magnesium and is stimulated by a chloride concentration of 4-10mM, similar to the cytosolic release (S. Naito & T. Ueda, 1985).

Glutamate in the vesicles has a concentration of approximately 100mM (Burger, 1989).

1.11.2 Mechanism of Glutamate Release

The depolarization of glutamatergic neurons allows calcium to enter the terminal and trigger glutamate exocytosis. The vesicular release of glutamate is a Ca^{2+} dependent mechanism that involves N- and P/Q-type voltage-dependent Ca^{2+} channels (Birnbaumer, 1994) and these channels appear to be closely linked to vesicle docking sites. The vesicular membrane fuses with the plasma membrane proteins, an ATP dependent process (Esser, 1998) that is regulated by protein phosphorylation (Hanson, Heuser, & Jahn, 1997). Rapid application of glutamate to neuronal membrane patches at a concentration that is estimated to be similar to a physiologic synapse, brings about the postsynaptic response when excitatory synapses are activated (Bergles, Diamond, & Jahr, 1999; J. D. Clements, Lester, Tong, Jahr, & Westbrook, 1992).

Releasing a single release vesicle produces an excitatory postsynaptic potential (EPSP) that is related primarily to glutamate receptor activation. The synaptic release of glutamate is controlled by a wide range of presynaptic receptors. After its release, glutamate must be cleared from the synaptic cleft. It is rapidly cleared from the cleft and internalized into astrocytes. It can now enter two major biochemical pathways. First, glutamate may become amidated to glutamine by glutamine synthetase in the cytosol. This pathway is important, which is indicated by the presence of glutamine synthetase in astrocytic processes that are near glutamatergic synapses (Derouiche & Frotscher, 1991). Second, glutamate may enter the mintochondria of astrocytes to become transaminated (by aminotransferases) or deaminated (by glutamate dehydrogenase) to alpha ketoglutarate and may be oxidized successively to succinate, fumarate and malate. Malate, after leaving the mitochondria, may then be decarboxylated to pyruvate by

cytosolic malic enzyme (Kurz, Wiesinger, & Hamprecht, 1993). The magnitude of the flux of the transmitter glutamate from neurons to astrocytes may be roughly estimated from the formation of glutamine from transmitter glutamate. GABA and alpha ketoglutarate derived from the astrocytic TCA cycle also contribute to glutamine formation.

Astrocytes are metabolically coupled with excitatory neurons and serve them by supplying precursor glutamine and by de novo synthesis of glutamate from alpha ketoglutarate and ammonia by using energy. Inhibitory neurons appear to use glutamate formed from alpha ketoglutarate as the immediate precursor of GABA. Glutaminase is mostly found in excitatory neurons, although it is also found in a limited number of GABAergic neurons in certain areas of the brain (Kaneko, Shigemoto, & Mizuno, 1988).

Forming and degrading transmitter glutamate is an ATP dependent process. The fusion of the vesicular membrane with the plasma membrane needs protein phosphorylation and needs ATP. The uptake of glutamate by astrocytes is coupled to the influx of three molecules of sodium which are cleared by the Na/K-ATPase, leading to the use of one molecular of ATP per molecule of internalized glutamate. Formation of glutamine from glutamate requires one ATP per molecule (Levy, Warr, & Attwell, 1998). The uptake of glutamine across the neuronal plasma membrane goes against a concentration gradient and is sodium dependent. One molecule of glutamine needs the entry of three sodium or hydrogen ions, which implies that one ATP is used up by the Na/K-ATPase. Therefore, one transmitter glutamate cycle of vesicular uptake and release, astrocytic uptake and amidation, and neuronal uptake of glutamine, could use at least four molecules of ATP per molecule of glutamate; two in neurons and two in astrocytes. This calculation does not include the ATP expenditure inherent in the depolarization of presynaptic membrane which triggers

the transmitter release and the depolarization of postsynaptic membranes caused by glutamate receptor activation.

Assuming a flux of transmitter to glutamate to astrocytes, which makes up approximately 30% of the cerebral TCA cycle rate, it means that less than 3% of the energy extracted form serum glucose is used in the handling of transmitter glutamate. The uptake of glutamate into cultured astrocytes increases Na/K-ATPase activity and also an increase in glucose uptake and lactate export (Magistretti, Pellerin, Rothman, & Shulman, 1999).

In summary, glutamate is synthesized from glutamine and also from neuronal precursors that are supplied by neuronal pyruvate carboxylation. Transmitter glutamate is mostly taken up into astrocytes for conversion to glutamine, but some part of the glutamate is metabolized by the astrocytic TCA cycle either fully to CO_2 and water or only partially to form malate that is converted to pyruvate and lactate. The uptake of glutamate into astrocytes stimulates the astrocytic uptake of serum glucose and exports lactate. Glutamine is shunted to neurons where it is mostly converted to CO_2 and water and some of it is converted to transmitter glutamate. The uptake processes that are related to the handling of transmitter glutamate and glutamine, in addition to the formation of glutamine uses up approximately 3% of the total energy of the serum glucose that is taken up by the brain.

The location of glutaminase, on the external side of the inner mitochondrial membrane, could play a role in events after cell damage occurs. When trauma or hypoxia damages the cell, the enzyme can leak out of neurons and into the extracellular space where it can covert extracellular glutamine to glutamate. This can contribute to a continuous and excitotoxic glutamatergic stimulation of neurons. This has been demonstrated in vitro and may occur in vivo as well (Driscoll, Deibler, Law, & Crane, 1993).

1.12 Glutamate Receptors

The glutamate receptors transduce the glutamate released from the presynaptic terminal into biochemical and electrical events in the postsynaptic neuron. Glutamate receptors are critical for transducing the vast amount of excitatory neurotransmission and regulating the strength of both excitatory and inhibitory transmission in the nervous system (Vandenberg, 1998).

The glutamate receptors are broadly classified into the ligand-gated ionotropic glutamate receptors (iGluRs) and G protein-coupled metabotropic (Meldrum, 2000) glutamate receptors (mGluRs).

The ionotropic glutamate receptors were the first to be discovered and mediate the fast excitatory transmission in the postsynaptic neuron. These receptors comprise of a diverse group of ion channels that are mainly gated by the binding of glutamate. The metabotropic glutamate receptors are composed of seven transmembrane-domain proteins that depend on heterotrimeric guanosine triphosphate (GTP)-binding proteins to activate intracellular signalling pathways (Greenamyre & Porter, 1994). The glutamatergic systems are dysfunctional in most neuropathologies and receptor dysfunction appears to play a role in many neurologic disorders. In depth information about these receptors in terms of structure and function could play an important role.

1.12.1 Ionotropic Glutamate Receptors

In the early 1990s, the cloning of cDNAs that encoded the glutamate receptor subunits helped the field immensely (Hollmann & Heinemann, 1994). Ionotropic glutamate receptors are classified as; NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid) and kainate receptor classes, named originally according to their preferred, synthetic, agonist.

Further studies showed that the receptors are encoded by at least six gene families based on sequence homology. Further details are in Table 4.

The similarity in sequence indicates a common evolutionary origin for these receptors. The gene families consist of a single family for AMPA receptors, two for kainate, and three for NMDA. The genes are located over several chromosomes but GRIA4 and GRIK4 genes are located on the long arm of chromosome 11 and GRIK5 and GRIN2D on chromosome 19. The table below is for the glutamate receptor subunits and corresponding genes (Dingledine, Borges, Bowie, & Traynelis, 1999)

Group	Receptor Family	Subunit	Gene	Chromosome human	GenEMBL Accession No.		
					Mouse	Rat	Human
1	AMPA	GluA1	*GRIA1*	5q33	X57497	X17184	I57354
1	AMPA	GluA2	*GRIA2*	4q32-33	X57498	M85035	A46056
1	AMPA	GluA3	*GRIA3*	Xq25-26		M85036	X82068
1	AMPA	GluA4	*GRIA4*	11q22-23		M36421	U16129
2	Kainate	GluK1	*GRIK1*	21q21.1-22.1	X66118	M83560	U16125
2	Kainate	GluK2	*GRIK2*	6q16.3-q21	D10054	Z11715	U16126
2	Kainate	GluK3	*GRIK3*	1p34-p33		M83552	U16127
3	Kainate	GluK4	*GRIK4*	11q22.3		X59996	S67803
3	Kainate	GluK5	*GRIK5*	19q13.2	D10011	Z11581	S40369
4	NMDA	GluN1	*GRIN1*	9q34.3	D10028	X63255	X58633
5	NMDA	GluN2A	*GRIN2A*	16p13.2	D10217	D13211	U09002
5	NMDA	GluN2B	*GRIN2B*	12p12	D10651	M91562	U28861
5	NMDA	GluN2C	*GRIN2C*	17q24-q25	D10694	D13212	
5	NMDA	GluN2D	*GRIN2D*	19q13.1qter	D12822	D13214	U77783
6	NMDA	GluN3A	*GRIN3A*	9q31.1		L34938	
7	NMDA	GluN3B	*GRIN3B*	19p13.3			
8	Delta	δ	*GRID1*		D10171	Z17238	
9	Delta	δ	*GRID2*	4q22	D13266	Z17239	

Table 4: Glutamate receptor subunits and their genes

Ionotropic glutamate receptors are integral membrane proteins that have four large subunits. These subunits that constitute the receptors have more than 900 residues and together form a central ion channel pore. Receptor heterogeneity within each class arises from the homo-oligomeric, or hetero-oligomeric, assembly of distinct subunits into cation-selective tetramers. The subunits contain four domains; the extracellular amino-terminal domain (ATD), the extracellular ligand-binding domain (LBD), the transmembrane domain (TMD), and an intracellular

carboxyl- terminal domain (CTD). The receptor has a 2-fold symmetry perpendicular to the plane of the membrane. The glutamate receptor TMD consists of three transmembrane helices which are M1, M3 and M4 and a cytoplasm-facing re-entrant membrane loop; M2. The M2 segment of the NMDA receptors is somewhat similar to potassium channels and contains residues that influence the permeation properties of the ion channel. M1, M2 and M3 form a structure that is similar to an inverted K^+ channel pore and M4 mainly makes contacts with the TMD of an adjacent subunit (Dingledine, 1999).

The C terminus faces intracellularly while the N terminus extracellularly. The mechanism by which a receptor protein is threaded through the membrane during synthesis determines which segments will face the extra or intracellular areas and this in turn specifies the protein domains that will be available for ligand recognition, cytoplasmic modification, phosphorylation and interactions between the receptor and cytoplasmic proteins (Dingledine, 1999).

Receptors, ion channels and several other types of proteins that are involved in transmembrane signalling are usually composed of different subunits. This varies based on the cell state, availability of subunit types which depends in turn on protein production and subcellular localization. Based on subunit availability and interaction affinity, complexes may form in one or more fixed stoichiometries or form with heterogeneous stoichiometries. Based on the subunit composition, it is clear that multiple subtypes exist within the ionotropic receptor families. Glutamate receptors are grouped into four distinct classes based on pharmacology and on structural homology. These include the AMPA receptors (GluA1-GluA4), the kainate receptors (GluK1-GluK5), the NMDA receptors (GluN1, GluN2A-GluN2D, GluN3A and GluN3B), and the δ receptors (GluD1 and GluD2). Please refer to Table 5 for previously utilized nomenclature.

NMDARs are composed of various combinations of GluN1 and GluN2 or GluN2A–GluN2D subunits and, in some cases, GluN3 or GluN3A and GluN3B subunits. These subunits differ in the length of their C-termini, with the longest ones found in GluN2A and GluN2B and the shortest in GluN1 and GluN3B. GluN2 subunits are the major determinants of the functional properties of NMDARs, including single-channel conductance, calcium permeability, magnesium sensitivity, agonist affinity and deactivation kinetics. The non-GluN1 subunits determine unique physiological properties that lead to functional diversity. GluN2 subunits bind glutamate and form GluN1/GluN2 receptors that require glycine and glutamate for activation. GluN3 subunits bind glycine and GluN1/GluN3 receptors are gated by glycine alone; however, excitatory glycine receptors have not yet been observed in neurons expressing the GluN3 subunit. The genes encoding GluN1, GluN2 and GluN2A–GluN2D are GRIN1, GRIN2A GRIN2B, GRIN2C, GRIN2D and GRIN3A (glutamate receptor, ionotropic, NMDA) (Stephen F Traynelis, 2010).

The AMPA receptor subunits GluA1 to GluA4 can form both homo- and heteromers. The subunits combine to form tetramers. Most AMPA receptors are heterotetrameric, consisting of symmetric 'dimer of dimers' of GluA2 and either GluA1, GluA3 or GluA4. AMPA receptor subunits differ most in their C-terminal sequence, which determines their interactions with scaffolding proteins. All AMPA receptors contain post synaptic density protein (PDZ)-binding domains, but which PDZ domain they bind to differs. The AMPA receptors permeability to calcium and other cations, such as sodium and potassium, is governed by the GluR2 subunit. If an AMPAR lacks a GluA2 subunit, then it will be permeable to sodium, potassium, and calcium. The presence of a GluA2 subunit will almost always render the channel impermeable to calcium. The receptor subunits of AMPARs are encoded for by separate but related genes that form a single gene family. The receptor subunits are encoded by similarly distinct families of genes. The genes encoding GluA1-4 are

GRIA1-4 (glutamate receptor, ionotropic, AMPA). The overall size of these genes is likely to be >200 kilobases and the translated protein subunits contain approximately 850-900 amino acids (Stephen F Traynelis, 2010).

Kainate (KA) receptors are tetramers made up of homo or heteromeric association of the subunits; GluK1-5. GluK1-3 homomers are functional kainate gated ion channels, GluK4 and GluK5 only form functional channel as heteromers. The Kainate recetors are formed from the products of encoding genes denoted GRIK1 – GRIK5 (gluatamate receptor, ionotropic, kainate) (Stephen F Traynelis, 2010).

The receptors classified as Delta (δ) ionotropic glutamate receptors form a tetrameric stoichiometry. GluD2 has been found to form heteromers with AMPA or kainate receptors and can modify the channel properties of these receptors. Genes encoding these receptors are GRID1 and GRID2 (Glutamate receptor, ionotropic, Delta). The δ1 and δ2 subunits of GRID1 and GRID2 have only 18–25% amino acid identity of the other glutamate receptor subunits. These subunits do not form functional channels themselves or modify the function of other subunits. The importance of the δ2 subunits was highlighted in knockout mice that showed loss of activity-related depression of the parallel fiber-Purkinje cell synapse (Kashiwabuchi, 1995). The genetic mouse model of excitotoxcity; Lurcher has been shown to be caused by a mutation of the δ2 which makes the receptor into a constitutively leaky cation channel (Vogel, Caston, Yuzaki, & Mariani, 2007). Heteromeric assembly of edited and unedited subunits is a mechanism of functional modulation.

There have been many different nomenclature schemes for the ionotropic glutamate receptors. The following table gives the new nomenclature, agreed in Paris in 2008 by the IUHPAR (International Union of basic and clinical pharmacology) (S. F. Traynelis, 2010).

NMDA	**AMPA**	**Kainate**	**Delta**
NR1 = **GluN1** NR2A = **GluN2A** NR2B = **GluN2B** NR2C = **GluN2C** NR2D = **GluN2D** NR3A = **GluN3A** NR3B = **GluN3B**	GluRA = GluR1 = **GluA1** GluRB = GluR2 = **GluA2** GluRC = GluR3 = **GluA3** GluRD = GluR4 = **GluA4**	GluR5 = GLUK5 = **GluK1** GluR6 = GLUK6 = **GluK2** GluR7 = GLUK7 = **GluK3** KA1 = GLUK1 = **GluK4** KA2 = GLUK2 = **GluK5**	δ1 **= GluD1** δ2 **= GluD2**

Table 5: Nomenclature for Ionotropic glutamate receptors

Studies have implicated the NMDA receptors in many neurologic disorders and neurodegenerative diseases including the seizure spread in epilepsy (Kullmann, Asztely, & Walker, 2000) ischemic brain damage, Huntington's chorea, and amyotrophic lateral sclerosis (Heath & Shaw, 2002; Kawahara & Kwak, 2005; Kwak & Kawahara, 2005), Parkinson's (M. J. O'Neill, 2005) and Alzheimer's diseases (Marenco & Weinberger, 2006). These receptors have also been linked to several neurodevelopmental disorders such as Fragile X mental retardation (Bear, Huber, & Warren, 2004) schizophrenia (Marenco & Weinberger, 2006), and the proliferation of glioblastoma tumours (Maas, Patt, Schrey, & Rich, 2001) depression, and Rasmussen's syndrome (Granata, 2003).

Glutamate at elevated concentrations transitions from being a neurotransmitter to a neurotoxin (Gill & Pulido, 2001). The neurotoxicity of glutamate depends on the cellular expression of the glutamate receptors and their excessive activation (Choi, 1992; Gasic & Hollmann, 1992; Peng, Taylor, Finch, Switzer, & Ramsdell, 1994). A neurotoxic phenomenon ensues, termed as excitotoxicity.

Diabetes is linked to altered activity and regulation of neurotransmitter receptors and cellular enzymes. Certain gene expression studies in diabetic rats have shown interesting outcomes such as the down-regulation of the enzyme glutamate decarboxylase (GAD) mRNA and glutamate aspartate transporter (GLAST). GAD is the rate limiting enzyme that catalyses the decarboxylation of glutamate. There are studies that report glutamate mediated excitoxicity linked to neuronal damage and to diabetic complications such as retinopathy (Delyfer, 2005).

GLAST contains cysteine residues that develop cysteine bridges in the presence of oxidative stress and leads to glutamate flux inhibition through the transporters (Trotti, Danbolt, & Volterra, 1998). The presence of hydrogen peroxide, superoxide anion and peroxynitrite anion can inhibit glutamate uptake through GLAST (Zeng, 2010).

Hyperglycaemia has also been linked to diminished blood flow to nerves and neural ischaemia is an important factor contributing to diabetic neuropathy (L. J. Coppey, Davidson, Dunlap, Lund, & Yorek, 2000). This is due to the deranged mitochondrial function, which is further damaged by the presence of excitotoxic glutamate (Srinivasan, 2000). Over activity of glutamate induced by ischaemia is linked to neuronal damage in neurological conditions such as stroke, amytorphic lateral sclerosis and neuropathic pain. Limitation of toxic glutamate receptor activation is expected to be neuroprotective (Zhang, 2002).

The glutamate receptors, via a common injury pathway, are involved in inflammation and cellular injury. In the CNS, receptor overstimulation allows excess calcium influx into the neuron and subsequent neuronal injury (Choi, 1992; Erdő, 1992; Lipton, 1993; Lipton & Rosenberg, 1994; Meldrum, 1994). The iGluRs permit entry of ions such as Na^+, K^+ and Ca^{2+} and sustained stimulation allows excessive ion and water entry leading to osmotic injury. The increased intracellular calcium activates several enzyme pathways and signalling cascades including

phospholipases, protein kinase C, proteases, protein phosphatases, nitric acid synthases and generates free radicals (Asztely & Gustafsson, 1996; Barb, Campbell, Armstrong, & Cox, 1996; G. Bertrand, Gross, Puech, Loubatieres-Mariani, & Bockaert, 1992; H.-O. Bertrand, Bessis, Pin, & Acher, 2002; Bruni, Bose, Pinsky, & Glavin, 1991; Burns, Stephens, & Benson, 1994; Butcher, Sandberg, Hagberg, & Hamberger, 1987; Carlton, Hargett, & Coggeshall, 1995; Castelli, Ingianni, Stefanini, & Gessa, 1999; Chaudhari, 1996; Dierkes, Hochstrate, & Schlue, 1996; Lipton, 1993; Lipton & Rosenberg, 1994; Meldrum, 1994). Protein kinase C translocates from the cytoplasm to the membrane and this leads to phosphorylation of membrane proteins via PKC. This further destabilizes the regulatory mechanisms for calcium homeostasis and toxicity (Choi, 1992). Arachidonic acid is generated when phospholipase A2 is activated. Platelet-activating factors increase the calcium levels in neurons by stimulating glutamate release. Arachidonic acid potentiates NMDA evoked currents and inhibits glutamate reabsorption into astrocytes and neurons. This feeds forward when free radicals are formed during arachidonic acid metabolism and further phospholipase A2 activation occurs. This increases the concentration of extracellular glutamate which activates the GluRs (Choi, 1992; Conn & Pin, 1997).

As a consequence, cysteine transport is then inhibited and this decreases intracellular reducing sulphydryls, more free radicals are produced and cell death ensues. Nuclear enzymes such as endonucleases are also activated and lead to nuclear chromatin condensation, DNA fragmentation and nuclear breakdown. This leads to apoptosis. The presence of free radicals adds to DNA fragmentation. The raised calcium concentration activates nitric oxide synthetases and produces more nitric oxide and free radicals. Nitric oxide has been observed in peripheral tissue, ganglion cells, nerve fibers and so on. These are also sites where glutamate receptors are present (Gill & Pulido, 2001).

Pain is one of the most common symptoms of diabetic neuropathy and is experienced in varying types and degrees of intensity. Glutamate has been implicated in the generation and maintenance of neuropathic pain. Certain drugs that modulate glutamate neurotransmission have been linked to neuropathic pain relief in animal models of peripheral neuropathy (Bordi & Quartaroli, 2000; Bruno ., 1995). In animal models, chronic diabetic pain is postulated by increased spontaneous firing of afferent neurons (Burchiel, Russell, Lee, & Sima, 1985). This is associated with the presence of increased voltage-dependent calcium currents in dorsal root ganglion cells and leads to increased calcium concentrations in the neurons. This is added to by the opening of glutamate-regulated calcium channels (Kawamata & Omote, 1996; Thayer & Wang, 1995; White, 2000).

However, depending on the type of mGluR subtype activated the consequence can be neuroprotective or neurotoxic (Glaum & Miller, 1994; Lin, Chong, & Maiese, 2002; Trombley & Westbrook, 1992). In animal models, activation of certain mGluR subtypes such as Group II and III, protects cells against neuronal insults such as NMDA excitotoxicity, brain injury due to trauma, stroke, glucose deprivation, oxygen deprivation and oxidative stress (Aarts & Tymianski, 2003; Baskys & Blaabjerg, 2005; Berent-Spillson, 2004; Blaabjerg, Fang, Zimmer, & Baskys, 2003; Cai, Xiao, Fratkin, & Rhodes, 1999; Chong, Li, & Maiese, 2005; Kalda, Kaasik, Vassiljev, Pokk, & Zharkovsky, 2000; Leker & Shohami, 2002; Lin, Vincent, Shaw, Maynard, & Maiese, 2000; Sagara & Schubert, 1998; A. M. Vincent, Mohammad, Ahmad, Greenberg, & Maiese, 1997; Zhu, DeCoster, & Bazan, 2004). A role of these receptors in preventing hyperglycaemia induced neuronal injury has been observed (Berent-Spillson, 2004; Berent-Spillson & Russell, 2007).

Group II and III inhibit voltage gated calcium entry and regulate glutamate release. These receptors act as neuroprotective presynaptic autoreceptors that control glutamate release from presynaptic terminals. Group II mGluRs regulate

glutathione (GSH), an important component of cellular antioxidant defence. The glutathione homeostasis is maintained by oxidation reduction pathways that recycle GSH from oxidized glutathione disulfide and synthesis by the gamma glutamyl cycle. Cysteine uptake and the formation of cysteine is important in the GSH cycle. This is linked to glutamate transporters (Baker, Xi, Shen, Swanson, & Kalivas, 2002; Bender, Reichelt, & Norenberg, 2000; Moran, Melendez, Baker, Kalivas, & Seamans, 2003; Shanker & Aschner, 2001; Tang & Kalivas, 2003). Glia synthesize GSH and support nearby neurons. The released GSH is degraded to precursors that are taken up by neurons and synthesized. Activating mGluR3 on Schwann cells and glia, where these receptors are prevalent, increases neuronal GSH. The GSH synthesis inhibitor; buthionine sulfoximine blocks the protective action of mGluR3 (Berent-Spillson & Russell, 2007).

The enzyme glutamate carboxypeptidase II, formerly NAALADase is a neuropeptidase primarily expressed in glial cells. The enzyme converts N-acetyl-aspartyl-glutamate (NAAG) to NAA. NAAG protects neurons by acting as an mGluR3 agonist. Direct and indirect activation of mGluR3 receptor by inhibiting glutamate carboxypeptidase II (GCPII) protects from glucose induced neuronal injury. The GCP II inhibitor; 2- phosphonomethyl pentanedioic acid (2-PMPA) protects the dorsal root ganglia neurons against glucose-induced cell injury and neurite degeneration (Berent-Spillson ., 2004), (Spillson & Russell, 2003). Another agonist APDC has activity at mGluR2 as well as mGluR3, suggesting mGluR2 could also be neuroprotective. In animal models of diabetic neuropathy, evidence of neuroprotection is observed. GCPII inhibitors have shown to be beneficial against the hyperalgesia and neurophysiological and structural degenerative diabetic neuropathy changes in inbred type 1 diabetic BB/Wor rats (Zhang, 2002).

1.13 Glutamate Transporter Proteins

Glutamate is continuously being released from cells and is continuously being removed from the extracellular fluid. It is of critical importance that the extracellular glutamate concentration is kept low. Its receptors are found on most of the cellular elements such as dendrites, nerve terminals, neuron cell bodies and glial cells. The importance of keeping glutamate at a low concentration is not just limited to the synaptic clefts.

Maintaining a low extracellular glutamate concentration is essential to preventing glutamate neurotoxicity that may occur under many pathological conditions such as diabetic neuropathy. A low extracellular glutamate concentration is required for a high signal-to-noise (background) ratio in synaptic as well as in extrasynaptic transmission. The clearance of extracellular glutamate via glutamate metabolism or diffusion is not significant (Danbolt, 2001), the regulation of extracellular glutamate depends on a highly efficient glutamate transporter system consisting of high-affinity membrane-bound glutamate transporters. The transporter proteins represent the only significant mechanism to remove glutamate from the extracellular fluid and their importance for the long-term maintenance of low and non-toxic concentrations of glutamate is now well documented.

1.14 Gene Expression

Gene expression is a process involving fundamental cell processes such as; transcription, mRNA degradation, translation and protein degradation. Each step is controlled by gene regulatory events (Schwanhausser, 2013). Gene expression profiling is the measurement of the activity or the expression of thousands of genes at once, to create a global picture. Changes in gene expression in diabetes presents a different pathway involved in diabetic pathogenesis. The results of alterations in gene expression lead to molecular and consequently functional changes that

malfunction as time goes on. Studying gene expression might indicate how therapies can be developed that can prevent or at the least slow down the complications of chronic diabetes (Klein & Waxman, 2003).

For experimental work, single stranded DNA; complementary DNA or cDNA is often used because it is more stable than RNA. It is generated by reverse transcription which uses single stranded RNA as a template and the ability of the enzyme reverse transcriptase to synthesis the DNA from the 3' end of the primer using the RNA sequence as template. Primers are integral to this process and there are several types available, the choice depends on the end result required. An oligo(dT) primer will allow the synthesis of cDNA from most mRNAs because it can bind to the poly(A) tail. Random primers can be used for non-polyadenylated RNA because they anneal throughout the target RNA. Gene specific primers are used to reverse transcribe specific mRNA (Life science technologies, 2013).

1.15 Hypothesis and Aim of the Study

Based on the preliminary data, the hypothesis was that glutamate pathways are likely to be involved in diabetic neuropathy particularly neuropathic pain. Pathways were investigated to look for changes that might reflect neuropathic pain and fit with previously established pharmacological evidence. The aim of this project was to identify changes in expression of genes and their protein products that are involved in glutamate signalling in diabetes. This will help to further the understanding of the mechanisms of diabetic neuropathy.

1.16 Animal Models

Animal models allow control in vivo the genetic and environmental factors that influence the development of diabetes and its complications. Rodents are preferable due to advantages such as short generation intervals, economic viability,

ease of care and housing compared to larger models. Animal models develop diabetes either spontaneously or by using chemical, surgical, genetic or other techniques (Chatzigeorgiou, Halapas, Kalafatakis, & Kamper, 2009). For type 1 diabetes, the animal models range from spontaneously developing autoimmune diabetes to chemical ablation of pancreatic beta cells. Type 2 diabetes is modeled in both obese and non-obese animal models with varying degrees of insulin resistance and beta cell failure (A. J. King, 2012). For the importance of relevance to the study, the characteristics of the animal model should reflect the pathophysiology and natural history of diabetes or the model should develop diabetic complications with an etiology similar to humans (Cefalu, 2006). However, no one animal model fits all these criteria. Using several different models to check for changes that affect a broad range of diabetic models can be considered a suitable approach.

In type 2 diabetes, a popular model is the Goto-Katazaki (GK) rat which is a nonobese and has a decrease beta cell mass. It is characterized, similar to humans, by the presence of liver and skeletal muscle insulin resistance. The fasting blood glucose shows only a moderate increase (Cefalu, 2006). The streptozotocin (STZ) model is created by injecting the rats with drugs such as streptozotocin to destroy the Langerhans islets beta cells. It represents a model, with limitations, for type 1 diabetes and its subsequent complications (Akbarzadeh, 2007) (Rossini, 1977).

Although diabetic complications such as neuropathy are typically associated with a chronic duration of the diabetes itself, studies have also indicated the early presence of abnormalities which could represent a premature development of these complications in autonomic and peripheral nerves (Pfeifer, 1984), (R. J. Young, Ewing, & Clarke, 1983), (Schaan, 2004).

In the STZ model, early neuropathic changes are detected as early as 2 weeks of developing diabetes, as can be linked to mechanical hyperalgesia and

thermal hypoalgesia, tested by observing as paw withdrawal thresholds to mechanical stimuli or tail flick latencies to thermal stimuli (Sugimoto, Rashid, Shoji, Suda, & Yasujima, 2008).

The GK model showcases a more chronic scenario of neuropathy. This is similar to humans as there is a strong correlation between duration of the diabetes and development of neuropathy (Dyck, 1999), (Dyck, 2011). Early stages of this model show little change in nerve conduction velocity and few morphometric abnormalities such as atrophy or axonal loss. However, at later stages abnormalities in these areas begin to manifest and are indicators of neuropathic changes. Decreased expression of nerve growth factor, its receptor, susbstance P and calcitonin gene-related protein can be observed in the dorsal root ganglia (Murakawa, 2002).

Chapter 2: Material and Methods

2.1 Ethical Approval

The protocol of the experiment was approved by the Faculty of Medicine and Health Sciences Animal Ethics Committee, which uses protocols based on those of the National Institutes of Health in the USA.

2.2 Groups of Animals and Conditions of Animal Care

Proper housing and management of the animals are essential to the well-being of the animals, the quality of research data and testing. Good management of the animals also helps to minimize the variations that affect research results (Research, 1996).

Blood glucose levels were measured in all groups of rats by Accu-check performa blood glucose Monitoring System (Roche Diagnostics) using ~2 µl of whole blood collected from the tail tip after a 25-G needle puncture of unrestrained rat.

All animals were maintained under specific pathogen free conditions at 22–25°C ambient temperature and relative humidity at 50% with a 12 h light-dark cycle (1800-0600 h). The cages were polycarbonate cages with wood chip bedding. There was free access to water and Harlan-Teklad rat chow was provided.

The rats belonged to different rat models; streptozotocin treated and Goto-Kakizaki.

2.2.1 Streptozotocin Treated Rats

Experiments were performed on tissues of adult male Wistar rats (200–250 g; bred inhouse) which had been given intraperitoneal injection of STZ (Sigma, St Louis, MO, USA) at the dose 60 mg/kg at 8 weeks of age. The STZ was dissolved in a citrate buffer solution (0.1 mol/l citric acid, 0.1mol/l sodium citrate; pH 4.5). Age-matched controls received an equivalent volume of the citrate buffer solution alone. Both groups of animals were maintained on the same diet and water ad libitum until they were used.

Blood glucose (mg/dl) for the control rats were (78.3±2.5, n=9) and STZ rats (366.7±23.8, n=9). The body weights of the diabetics were significantly less than the controls, by approximately 35% at 12 weeks and 24% at 20 weeks of age.

2.2.2 Goto-Kakizaki Rats

Young adult male (5 weeks) GK rats and age-matched Wistar controls with initial body weights of 150–180 g were obtained from a commercial colony (Taconic City, Germantown, NY, USA).

Blood glucose concentrations 120 min following a glucose challenge in GK rats (236.27 ± 15.12, n = 15, mg/dl) was significantly ($p<0.01$) higher than blood glucose in sedentary controls (93.93 ± 2.32, n = 15, mg/dl). Body weight and non-fasting blood glucose, measured immediately before experiments, were significantly ($p<0.01$) increased in GK compared to control rats

The control blood glucose concentrations were in the range 53–70 mg/dL (mean 62 mg/dL) and the diabetic levels were 125–375 mg/dL (mean 225 mg/dL).

The body weights of the diabetics were more than the controls, by approximately 12% at 10months. This was also the point when the animals were sacrificed.

2.2.3 Goto-Kakizaki Rats – Sucrose Fed

Five week GK rats were obtained (Taconic City, Germantown, NY, USA). The seven-week-old GK rats were fed with 100 mM for 4 weeks, 200 and 300 mM sucrose for 4 weeks each and 400 mM sucrose solutions thereafter till termination. The objective was to induce more severe hyperglycemia. Their condition was compared with that of age-matched GK rats that were not given sucrose were used for comparison. All rats received normal rat chow and drinking water *ad libitum*. Body weight, non-fasting blood glucose, food and water consumption were measured periodically.

At the beginning of the study, the GK Sucrose rats had a non-fasting blood glucose value of 142 mg dl-1 compared with 115 mg dl−1 in GK Control rats. By the end of the study, the blood glucose had risen to 254 mg dl−1 in GK Sucrose rats, compared with 143 mg dl−1 in GK control rats.

The non-fasting blood glucose in GK Sucrose rats (254.25 ± 20.51 mg dl−1, n = 5) was significantly ($P < 0.05$) higher than that in GK (143.06 ± 6.67 mg dl−1, n = 5). The animals were sacrificed at 10months of age. Gene expression experiments began after 8 months of dietary intervention.

The body weights were; GK Sucrose (445.08 ± 8.98 g, n = 5) and GK rats (442.38 ± 6.08 g, n = 5). Liquid consumption in GK Sucrose rats was (164.65 ± 5.76 ml per rat day−1, n = 5) compared to GK (50.06 ± 0.97 ml per rat day−1, n = 5).

A glucose tolerance test (GTT) was performed at the end of the experiment (after 12 weeks of treatment) for each rat. All the rats were made to fast overnight

(at least 18hr) prior to the test. Glucose 10ml/kg/wt, was given intraperitoneally to the animals on the day of the GTT. Blood samples were collected from the tail veins of the rat at time 0 (prior to the glucose load), 30, 60, 120 and 180min after glucose load. All animals are weighed before termination. Tissues were removed with the utmost rapidity possible after dissection to ensure RNA quality and samples placed in RNALater RNAlater (AM7021; Applied Biosystems, Carlsbad, CA, USA) and kept overnight at 4ºC to allow thorough penetration of the tissue. The samples were then stored in -20°C till further processing.

Neural tissue
Spinal cord; Cervical, Upper thoracic, Lower thoracic, Lumbar, Sacral
Thoracic dorsal root ganglia (TDRG); 1-3, 4-6, 9-12
Thoracic sympathetic ganglia (TSG); 1-6, 7-12
Pelvic ganglia
Lumbar dorsal root ganglia (LDRG)
Sacral dorsal root ganglia (SDRG)
Non neural tissue
Retina and Lens
Penis
Bladder
Kidney
Tail skin and foot skin

Table 6: The list of tissue samples harvested from animals

2.3 Dissection Technique

Animals are sacrificed using the guillotine method (Suckow A M). Decapitation yields tissues uncontaminated by chemical agents. A specialized rodent guillotine was used after ensuring it is in good, clean condition with sharp

blades. If correctly handled, rats do not show evidence of hypothalamic-pituitary-adrenal axis activation from this method or from being present when other rats are similarly terminated (A. V. M. Association).

Many autonomic ganglia can be recognized without a microscope as swelling or protrusions along nerve trunks or as knots within a mesh of nerves. Certain ganglia are connected in a sequence or ganglionated chain such as the paravertebral sympathetic chain, while other ganglia are connected by a mesh of nerve trunks with which they form a plexus. Many microscopic ganglia are buried within a nerve or embedded in the wall of the viscera. In contrast the sensory component consists mainly of neurons located in dorsal root ganglia (and in the nodose ganglion of the vagus nerve); in addition, afferent neurons are present within enteric ganglia.

2.3.1 Thoracic Sympathetic Ganglia

In the thorax, the sympathetic chains lie ventral to the head of the ribs and dorsal to the parietal pleura. Each chain is made up of some 10-12 ganglia. The lowermost ganglion lies opposite the 10th intercostal space. In the abdomen the sympathetic chain is retroperitoneal and is embedded in the psoas muscle. Once identified under the dissecting scope, the sympathetic chain was isolated from its adherent tissue. The use of micro dissecting forceps was of the utmost importance. The chain was divided based by counting the ribs.

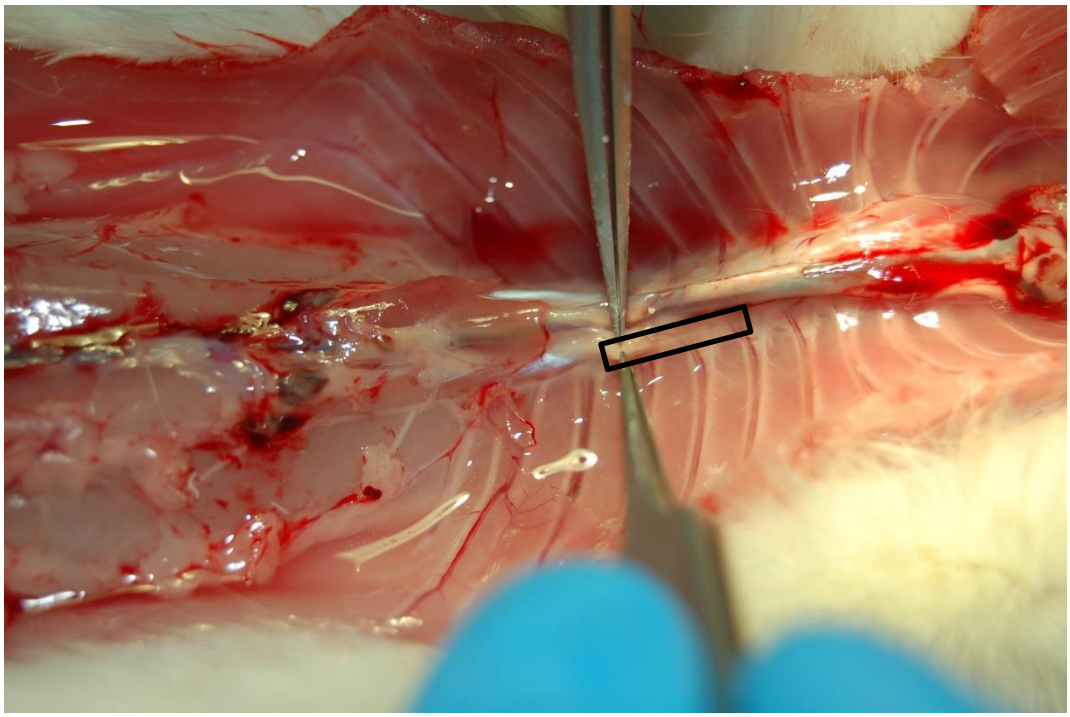

Figure 3: Removal of thoracic sympathetic ganglia.

This is beginning from the start of the rib cage. The black rectangle represents the location of the sympathetic ganglia before removal.

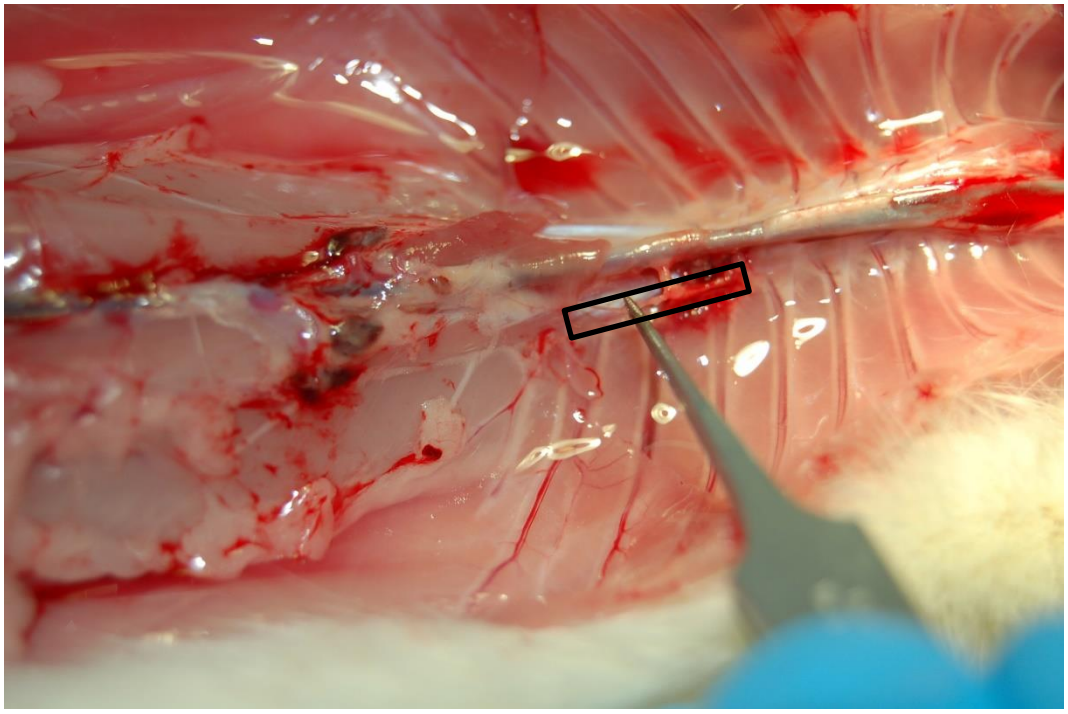

Figure 4: Removal of thoracic sympathetic ganglia from its adherent tissue

2.3.2 Pelvic Ganglia

In the male rat, the main component of the pelvic plexus is a single, large, bilaterally symmetric ganglion, the right and left pelvic ganglia. The ganglion is diamond-shaped, measuring about 2×4 mm, and lies on the side of the prostate, closely apposed to its fascia, ventral to the rectum, and caudal to the ureter and vas deferens. It shows lobulations that protrude in the direction of the surrounding organs. The ganglion is accompanied by a few, small, accessory ganglia, mainly related to the seminal vesicles and the vas deferens. Once identified, the ganglia was separated as much as possible from its adherent tissue and isolated. The encircled area represents the location in the picture below.

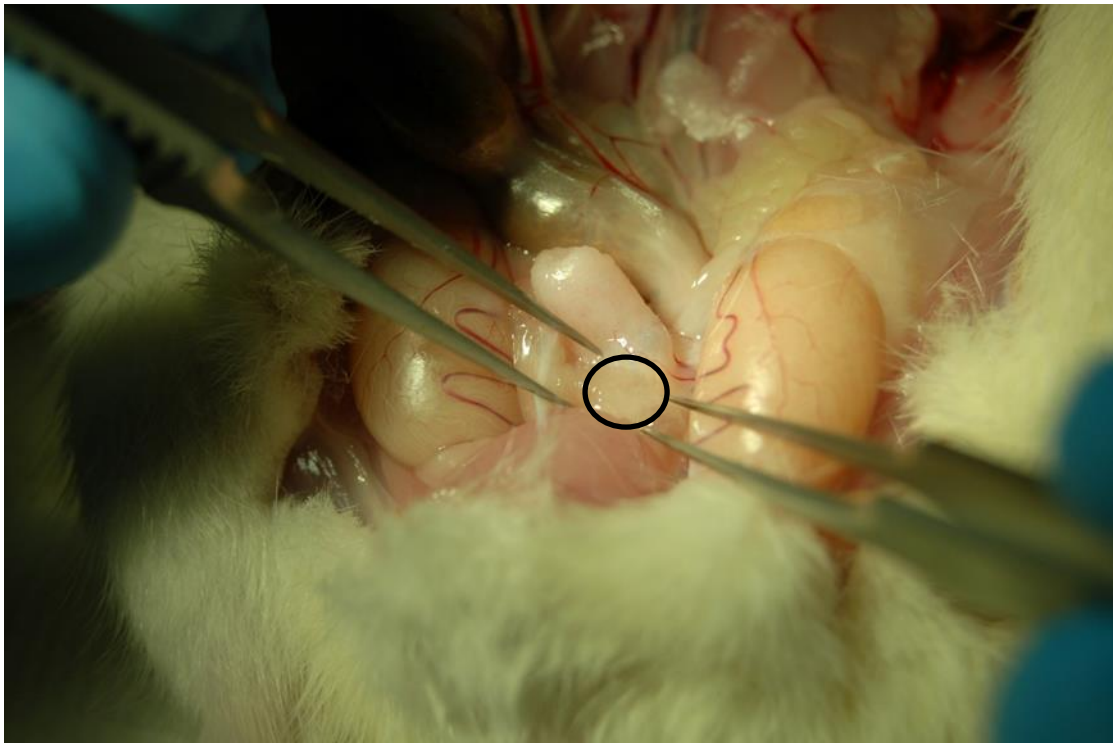

Figure 5: Location of pelvic ganglia.

2.3.3 Dorsal Root Ganglia

Parallel cuts using micro-dissecting scissors were made through the verterbrae adjacent to the spinal cord and any overlying muscle and bone were removed. The entry point for the scissors was between the vertebral column and spinal canal at rostral end and this allowed easier access to cut the vertebral column. The right and left halves of the vertebral column were separated to expose the spinal cord and DRGs. The spinal cord was lifted from the dorsal structures by grasping the spinal cord at the rostral end while carefully cutting behind and around the dorsal root ganglia to sever the adherent tissues. The dorsal root ganglia were present along either side of the spinal column, in small “pockets” in the bone. Each dorsal root ganglion was identified under the dissecting scope as being a round structure with small pieces of nerve root on either side. The dorsal roots are very short at cervical and upper thoracic levels, and the ganglia lie closely opposed to the spinal cord. The last thoracic ganglion (T13) and the L4 ganglion which is the largest ganglion were identified for orientation with respect to the whole rat. The thoracolumbar transition is easily identified because T13 is just caudal to the last rib. L4 is generally the largest lumbar ganglion. The dorsal root ganglia were grasped with micro forceps, then cut root on either side with micro scissors. All visible nerve roots were snipped away before removing dorsal root ganglia from cords. Using established anatomical landmarks and visual identification, the dorsal root ganglia were divided into thoracic, lumbar and sacral ganglia.

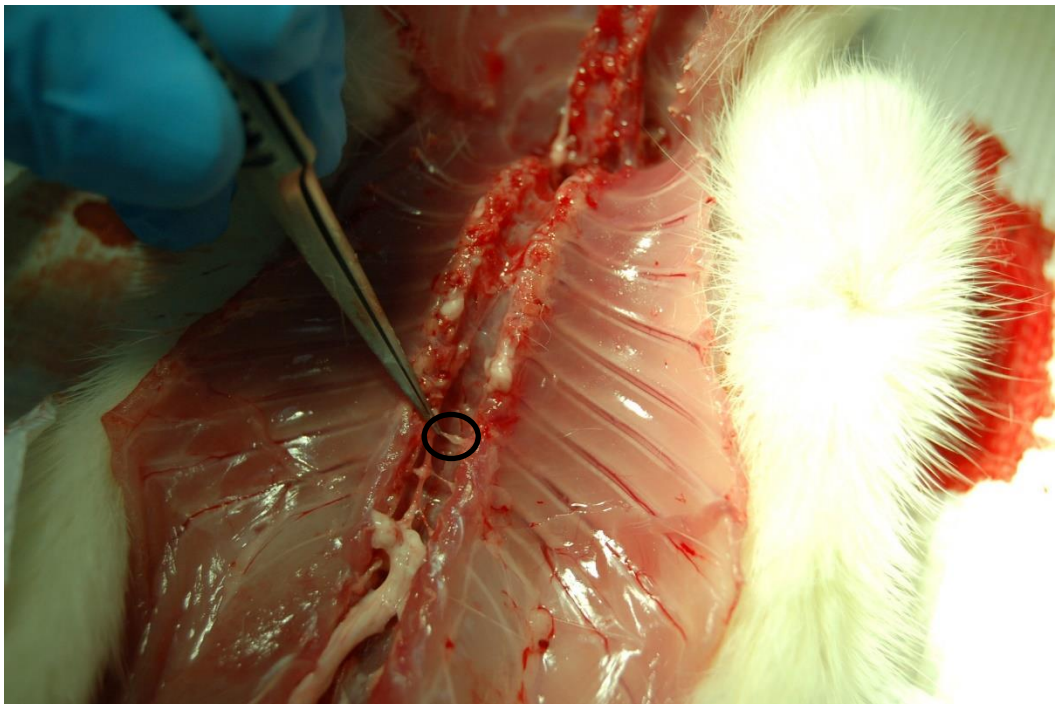

Figure 6: Removal of dorsal root ganglia from its location.

2.4 RNA Extraction

Extracting RNA allows the study of genes of interest amongst the myriad of coding and non-coding sequences is by following the mRNAs that correspond to the genes of interest. At the moment of extraction, being able to identify a particular mRNA means its corresponding gene is being expressed (Peirson & Butler, 2007).

Tissue homogenization was using the Polytron mechanical homogenizer (POLYTRON® PT 2100 Homogenizers, Kinematica, Switzerland). Total RNA was extracted by using the Trizol method (15596-026, TRIzol® Reagent, Life Technologies, Thermo Fisher Scientific, USA) which allows the simultaneous isolation of RNA, DNA, and protein from the same sample. This is an important consideration because most of the samples of ganglia weigh 1mg and less.

Sample homogenizations were done at intermittent intervals with samples kept in ice in between to avoid RNA and protein degradation. Intermittent homogenization is one of the best methods available to assist complete retrieval of RNA and protein from such small sample (Haimov-Kochman, Fisher, & Winn, 2006). Homogenization was done until till no clear visible homogenate particles were observed. The generator was cleaned in between samples in the following sequence; DNase/RNase free distilled water, 70% ethanol in DNase/RNase free distilled water, followed by DNase/RNase free distilled water. This was to prevent cross-contamination.

The total RNA was then precipitated using ethanol and processing of RNA isolation was completed using the SV Total RNA Isolation System (Promega, Madison, USA) according to manufacturer instructions.

2.4.1 RNA Purity

The concentration and purity of RNA samples was measured by checking absorbance at 260 nm A260 and the ratio of the absorbance at 260 and 280 nm using ND-1000; NanoDrop (ND-1000 NanoDrop, Thermo Scientific, Wilmington, DE, USA). Only ratios ≥ 1.8 were accepted. RNA samples were kept on ice during processing and quality check and then stored at -80º C pending further processing.

Purity of the extracted RNA was also checked by running RNA samples on a 1% agarose gel and observing the 18S and 28S bands. These ribosomal RNA bands are 5.0 kb and 1.9 kb in size respectively. The RNA was also treated with the DNase protocol by Invitrogen which should improvement in the purity of the RNA. Post DNase treated RNA was also run on a gel for quality check (Wieczorek D, 2012).

2.5 Reverse Transcription

Using a High Capactiy cDNA Reverse Transcription Kit by Applied Biosystems (High Capacity cDNA Reverse Transcription Kit; 4374966; Applied Biosystems, Carlsbad, CA, USA), using the manufacturer's instructions. The total RNA was converted into cDNA. Samples were diluted to make a final concentration of 4ng in a 30 µl PCR reaction.

Reverse Transcription Master Mix Components	**Volume** **1 x**
10 x RT Buffer	3.0 µl
25 x dNTP Mix	1.2 µl
10 x RT Random Primers	3.0 µl
Multiscribe™ Reverse Transcriptase	1.0 µl
RNASE Inhibitor	1.0 µl
RNase Free H_2O	= 30 - 9.2 - vol of RNA template used µl
Total Volume Reaction	30.0 µl

Table 7: Reverse Transcription Master Mix Components

The above reaction gives a final cDNA concentration of = 1ug/30µl = 16ng/µl. Each cDNA sample were then diluted to 4ng/µl and stored in -20º C.

Reverse transcription reaction was carried out in a Veriti thermal cycler (Life Technologies, Applied Biosystems, USA) using the following parameter values:

	Step 1	Step 2	Step 3	Step 4
Temp ºC	25 ºC	37 ºC	85 ºC	4 ºC
Time	10 minutes	120 minutes	5 minutes	∞

Table 8: Reverse Transcription Reaction paramater values

2.6 Protein Extraction

The organic phase that was obtained during trizol extraction of RNA was processed using the manufacturer's recommended method. Samples were then quantified using BCA method (Pierce) and stored in -80 till further processing.

2.7 Low Density Arrays

These allowed screening to identify genes that showed altered expression. Gene expression arrays were performed by first loading 100ng of cDNA (RNA equivalent) with an equal amount of TaqMan© Gene Expression Master Mix for a total of 100 µl/port. The cDNA and master mix were mixed by inversion and spun briefly in an Eppendorf microcentrifuge. After the cards reached room temperature, 100 µL of the pooled control and pooled diabetic samples was loaded into each of the 8 ports on the TaqMan Low-Density Array. The cards were placed in Sorvall® Custom Buckets and centrifuged for 1 min at 1580g. This was repeated after checking the fluid level in the cards. Immediately after centrifugation, the cards were sealed using an array sealer to prevent cross-contamination and run on the ABI 7900HT Fast Real-Time PCR system with thermal cycling conditions:

Purpose of step	**Activate uracil-DNA glycosylase**	**Activation**	**Denaturation**	**Annealing and Extension**
Temp ºC	50 ºC	94.5 ºC	59.7 ºC	59.7 ºC
Time	02 minutes	10 minutes	30 sec cycles	1 minute

Table 9: Fast Real-Time PCR system thermal cycling conditions

Analyses were restricted to genes that were expressed at detectable levels.

2.8 Real Time Polymerase Chain Reaction (RT–PCR) Gene Expression Assay

Gene expression is a process involving fundamental cell processes such as; transcription, mRNA degradation, translation and protein degradation. Each step is controlled by gene regulatory events (Schwanhausser ., 2013). Gene expression profiling is the measurement of the activity or the expression of thousands of genes at once, to create a global picture. Changes in gene expression in diabetes presents a different pathway involved in diabetic pathogenesis. The results of alterations in gene expression lead to molecular and consequently functional changes that malfunction as time goes on. Studying gene expression might indicate how therapies can be developed that can prevent or at the least slow down the complications of chronic diabetes (Klein & Waxman, 2003).

For experimental work, single stranded DNA; complementary DNA or cDNA is often used because it is more stable than RNA. It is generated by reverse transcription which uses single stranded RNA as a template and the ability of the enzyme reverse transcriptase to synthesis the DNA from the 3' end of the primer using the RNA sequence as template. Primers are integral to this process and there are several types available, the choice depends on the end result required. An oligo(dT) primer will allow the synthesis of cDNA from most mRNAs because it can bind to the poly(A) tail. Random primers can be used for non-polyadenylated RNA

because they anneal throughout the target RNA. Gene specific primers are used to reverse transcribe specific mRNA (technologies, 2013).

Low density arrays are effective approaches to gene expression profiling. These are more sensitive and focused than gene chips and offer higher throughput than more established approaches (Goulter, Harmer, & Clark, 2006).

The TaqMan© Low Density Array is an easy-to-use micro fluidic card for real-time PCR. The micro fluidic technology utilizes eight sample-loading ports, each connected to 48 reaction chambers. These function as an array of reaction vessels for the PCR step. The wells contain custom TaqMan© Gene Expression Assays and are used to detect the real-time amplification of user-specified targets. Relative levels of gene expression will be determined from the fluorescence data generated during PCR (Biosystems, 2010).

The expression levels of mRNA were analyzed using target specific TaqMan® gene expression assays and performed in a 7900HT Fast ABI Prism 7900HT Sequence Detection System.

On a 96 well optical reaction plate (4306737, Applied Biosystems, USA), 2ng of cDNA is used in a 10ul reaction and the following components were added; 20x TaqMan© Gene Expression Assay, 2x TaqMan© Gene Expression Master Mix, and the endogenous control or gene of interest. This was then run in the 7900HT Fast Real-Time PCR system (Applied Biosystems, USA).

Analyzing the data from TaqMan© Gene Expression Assays required viewing the amplification plots for the entire plate, setting the baseline and threshold values and then using the relative standard curve or the comparative CT method to analyze the data.

The TaqMan® primers and probes kits were from Applied Biosystems:

	Assay ID	Gene symbol	Gene name
1	Rn00689046_m1	Gclc, rCG26075	glutamate-cysteine ligase, catalytic subunit
2	Rn00583547_m1	Gria3, rCG53210	glutamate receptor, ionotropic, AMPA 3
3	Rn00709588_m1	Gria1, rCG34336	glutamate receptor, ionotropic, AMPA 1
4	Rn00561341_m1	Grin2a, rCG49797	glutamate receptor, ionotropic, N-methyl D-aspartate 2A
5	Rn00583322_m1	Grip1, rCG49164	glutamate receptor interacting protein 1
7	Rn00570130_m1	Slc1a3, rCG50959	solute carrier family 1 (glial high affinity glutamate transporter), member 3
8	Rn00583283_m1	Slc1a6, rCG29419	solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6
9	Rn00564705_m1	Slc1a1, rCG48035	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1
10	Rn00561285_m1	Gls, rCG22604	Glutaminase
11	Rn00566625_m1	Grm1, rCG57330	glutamate receptor, metabotropic 1
12	Rn01436038_m1	Grin1, rCG45539	glutamate receptor, ionotropic, N-methyl D-aspartate 1
13	Rn00710532_m1	Grik3, rCG30794	glutamate receptor, ionotropic, kainate 3
14	Rn00561331_m1	Grik4, rCG57905	glutamate receptor, ionotropic, kainate 4
15	Rn00568514_m1	Gria2, rCG62437	glutamate receptor, ionotropic, AMPA 2
16	Rn00568544_m1	Gria4, rCG24522	glutamate receptor, ionotrophic, AMPA 4
17	Rn00515053_m1	Grid2, rCG55329	glutamate receptor, ionotropic, delta 2
18	Rn00691548_m1	Slc1a2, rCG26600	solute carrier family 1 (glial high affinity glutamate transporter), member 2
19	Rn00561244_m1	Gad2, rCG40828	glutamate decarboxylase 2

Table 10: TaqMan® primers and probes kits

The reaction mix of 10µl containing a total cDNA of 4ng/reaction was prepared using TaqMan® H Fast Universal PCR Master Mix, No AmpErase H UNG (Life Technologies #4352042, Applied Biosystems, USA) as follows:

Real Time PCR Master Mix Reaction	**Volume** **1 x**
DNase/RNase free H_2O	3.5 µl
cDNA (stock concentration 2ng/µl)	1 µl
2 X TaqMan H Fast Universal PCR Master Mix	5 µl
20 X Gene of Interest (GOI)	0.5 µl

Table 11: Real Time PCR Master Mix Reaction Components

Quantitative real-time PCR assay for the gene of interest was performed in duplicates and in a singleplex PCR reaction.

The PCR thermal cycling parameters were run in fast mode as follow:

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temp ºC	50º C	95 º C	40 cycles of 95 º C	60 º C
Time	2 minutes	20 seconds	1 second	20 seconds

Table 12: Real Time PCR Master Mix Reaction Components

Results were initially analyzed with the ABI Prism 7900HT SDS program v2.4, all remaining calculations and statistical analysis were performed by the SDS RQ Manager 1.1,4 software using the $2^{-\Delta\Delta Ct}$ method with a relative quantification RQmin/RQmax confidence set at 95%.

The endogenous control used was rat β-actin. Other expression related guidelines were followed from the MIQE guidelines (Bustin ., 2009).

2.9 Statistics

Results are expressed as the means ± S.E.M. of ‘n' observations. Statistical comparisons will use either Student's unpaired t test or ANOVA followed by Bonferroni corrected t tests for multiple comparisons, as appropriate. A value of $P < 0.05$ will be considered to indicate a significant difference. Statistical analyses were done using the GraphPad Prism Software and SPSS (v21).

Chapter 3: Results

3.1. General Characteristics of Animal Groups Studied

3.1.1 General Characteristics of Control and Goto-Kakizaki Rats; GK Group

The general characteristics of the animals are shown in Table 13.

The bodyweights of GK (443.64 ± 7.94 g, n=18) were significantly ($p<0.05$) higher than Control (371.73 ± 11.80 g, n=18) rats.

The non-fasting blood glucose in GK rats (161.29 ± 12.77, n=18) was significantly ($p<0.05$) higher than Control (89.67 ± 1.58 mg/dl, n=18).

	Control	**GK**
Bodyweight (g)	371.73 ± 11.80	443.64 ± 7.94
Non-fasting blood glucose (mg/dl)	89.67 ± 1.58	161.29 ± 12.77

Table 13: Body weight measurements and fasting blood glucose values of GK group

Data are mean±SEM. Number of animals in each group = 18. Significance values refer to numbers in parenthesis.

3.1.2 General Characteristics of Control and Goto-Kakizaki Rats; GK Sucrose Group

The general characteristics of the animals are shown in Table 14.

	Control Sucrose	GK Sucrose
Bodyweight (g)	362.33±17.91	444.33 ± 18.37
Non-fasting blood glucose (mg/dl)	106.67±15.81	223.00 ± 30.04

Table 14: Body weight measurements and fasting blood glucose values of GK Sucrose group

Data are mean±SEM. Number of animals in each group = 5. Significance values refer to numbers in parenthesis.

The bodyweights of GK Sucrose (445.08±8.98 g, n=5) were significantly ($p<0.05$) higher than Control Sucrose (421.28±13.36 g, n=5) rats.

The non-fasting blood glucose in GK Sucrose (254.25±20.51, n=5) was significantly ($p<0.05$) higher than Control Sucrose (110.93±1.76 mg/dl, n=5).

3.1.3 General Characteristics of Control and Streptozotocin Rats; Streptozotocin 20wk Group

The general characteristics of the animals are shown in Table 15.

Body weights were significantly ($P<0.05$) lower in STZ 20wks rats (259.05±8.67 g, n=9 rats) compared to Controls (351.05±8.47 g, n=10 rats).

Non-fasting blood glucose was significantly ($P< 0.05$) elevated in STZ rats (440.38±24.56 mg/dl, n =9 rats) compared to Controls (98.00±2.47 mg/dl, n =10 rats).

	Control	**STZ 20wk**
Bodyweight (g)	351.05±8.47 g	259.05±8.67 g
Non-fasting blood glucose (mg/dl)	98.00±2.47 mg/dl	440.38±24.56 mg/dl

Table 15: Body weight measurements and fasting blood glucose values of STZ20wks

Data are mean±SEM. Number of animals in each group = 9. Significance values refer to numbers in parenthesis.

3.1.4 General Characteristics of Control and Streptozotocin Rats; Streptozotocin 30wk Group

The general characteristics of the animals are shown in Table 16.

Body weight was significantly ($P<0.001$) lower in STZ 30wk rats (266±10 g, n=5) as compared to control (426±13 g, n=5) rats.

	Control	**STZ 30wk**
Bodyweight (g)	426±13 g	266±10 g
Non-fasting blood glucose (mg/dl)	110±4 mg/dl	554±25 mg/dl

Table 16: Body weight measurements and fasting blood glucose values of STZ30wks

Data are mean±SEM. Number of animals in each group = 5. Significance values refer to numbers in parenthesis.

3.2 Low Density Array Results

GENE NAME	GENE	TDRG 9-12	LDRG	SDRG
Neuropeptides and enzymes regulating neurotransmitters production, metabolism or transport				
Calcb	calcitonin-related polypeptide, beta	**0.62**	**3.73**	**2.76**
CCK	Cholecystokinin	**0.61**	**3.73**	**2.79**
Vip	vasoactive intestinal polypeptide	**0.22**	0.24	**6.39**
Tac2	tachykinin 2	0.57	**4.27**	1.93
Npy	neuropeptide Y	0.79	1.56	**3.05**
Nos1	nitric oxide synthase 1, neuronal	0.92	1.69	**3.08**
Nos3	nitric oxide synthase 3, endothelial cell	**0.41**	0.93	1.48
Maob	monoamine oxidase B	**0.49**	0.97	1.64
Slc6a2	solute carrier family 6 (neurotransmitter transporter), member 2	**0.31**	1.37	**3.90**
Slc6a4	solute carrier family 6 (neurotransmitter transporter), member 4	NA	0.81	**4.03**
Slc18a3	solute carrier family 18 (vesicular acetylcholine transporter), member 3	**0.40**	1.82	**8.54**
Receptors for neuropeptides and neurotransmitters				
Chrna3	cholinergic receptor, nicotinic, alpha 3 (neuronal)	**0.45**	0.72	0.86
CCKar	cholecystokinin A receptor	**0.59**	1.31	1.58
Npyr1	neuropeptide Y receptor Y1	**0.45**	0.70	**2.74**
Sstr2	somatostatin receptor 2	**0.18**	1.66	**3.99**
Tacr2	tachykinin receptor 2	**0.25**	0.92	**5.31**
Vipr2	vasoactive intestinal peptide receptor 2	**6.42**	1.32	2.53
P2rx4	purinergic receptor P2X, ligand-gated ion channel 4	**0.45**	1.31	1.78
P2rx7	purinergic receptor P2X, ligand-gated ion channel, 7	**0.44**	0.93	1.57
Ion channels				
Accn2	acid-sensing (proton-gated) ion channel 1	**0.42**	1.73	**2.26**
Scn3a	sodium channel, voltage-gated, type III, alpha subunit	**0.39**	0.79	**2.33**

GENE NAME	GENE	TDRG 9-12	LDRG	SDRG
Ion channels				
Trpv1	transient receptor potential cation channel, subfamily V, member 1	**0.45**	**2.16**	1.65
Slc12a5	solute carrier family 12 (potassium-chloride transporter), member 5	NA	**4.74**	**4.48**
Growth factors and their receptors				
Ntrk1	neurotrophic tyrosine kinase, receptor, type 1	NA	1.78	**2.06**
Ntrk2	neurotrophic tyrosine kinase, receptor, type 2	**0.37**	1.34	**2.40**
Ntrk3	neurotrophic tyrosine kinase, receptor, type 3	**0.41**	1.47	**2.34**
Microtubule associated proteins				
Map1a	microtubule-associated protein 1A	**0.43**	1.65	**2.02**
Map2	microtubule-associated protein 2	**0.32**	1.24	1.67
Mapt	microtubule-associated protein tau	**0.32**	0.94	**2.35**
Nrtn	Neurturin	NA	**6.39**	**3.04**
Pdyn	Prodynorphin	NA	**2.95**	1.09

Table 17: STZ 20wks low density array results

GENE	TDRG 9 - 12	LDRG	SDRG	Pelv G
Gad2 Glutamate decarboxylase	**2.17**	**6.28**	**8.95**	NE
Grip2 glutamate receptor interacting protein 1	1.04	1.26	1.14	**0.45**
Grip1 glutamate receptor interacting protein 2	1.24	1.45	**2.65**	**3.93**
Grm5 Glutamate receptor 5	1.48	**3.88**	1.95	NE
Grin1 glutamate receptor, ionotropic	1.12	1.77	1.22	**0.36**
Grin2a glutamate receptor, ionotropic	1.62	0.61	1.58	NE

GENE	TDRG 9 - 12	LDRG	SDRG	Pelv G
Grin2b glutamate receptor, ionotropic	1.03	1.36	1.22	NE
Gria1 glutamate receptor, ionotropic, AMPA1	0.89	1.23	1.41	NE
Gria2 glutamate receptor, ionotropic, AMPA2	0.98	0.59	0.77	0.67
Grik3 glutamate receptor, ionotropic, kainate 3	0.99	1.43	1.09	0.47

Table 18: STZ 20wks results of genes involved in glutamate signalling pathways.

Thoracic dorsal root ganglia 9 – 12 TDRG 9 – 12, Lumbar dorsal root ganglia LDRG, Sacral dorsal root ganglia SDRG, Pelvic ganglia PelvG with fold changes

3.3 Individual Gene Expression Results

CLASSIFICATION	**GENE**
GLUTAMATE RECEPTORS	
Ionotropic glutamate receptors AMPA	Gria1 Gria2 Gria3 Gria4
Ionotropic glutamate receptors Kainate	Grik3 Grik4
Iontropic glutamate receptors NMDA	Grin1 Grin2A
Ionotropic glutamate receptors Delta 2	Grid 2
GLUTAMATE TRANSPORTERS	
Solute carrier family	Slc1a1 Slc1a2 Slc1a3 Slc1a6
ENZYMES INVOLVED IN THE GLUTAMATE CYCLE & GLUTAMATE RECEPTOR INTERACTING PROTEIN	
Glutamate decarboxylase	Gad 2
Glutamate-cysteine ligase	Gclc
Glutaminase	Gls
Glutamate receptor interacting protein	Grip1

Table 19: Classification of genes that were studied for levels of specific mRNA

3.3.1 Genes expressing Glutamate Receptors – Ionotropic AMPA

Genes encoding for the ionotropic glutamate receptors AMPA are Gria1, Gria2, Gria3 and Gria4. These are shown in Figures 7 – 12. Figure 7 and 8 shows gene expression of individual genes; Gria1 and Gria3 in GK rats (diabetic vs control). Figures 9 - 12 show gene expression of individual genes; Gria2 and Gria4 in GK rats (diabetic vs control) in comparison to GK Sucrose rats (diabetic vs control).

Gria1 showed a significant decrease in expression ($p<0.05$) in TDRG1-4 and TDRG5-8. There was a significant increase in expression ($p<0.05$) in the LDRG of GK 10months compared to control rats. Gria1 showed no significant change in expression in GK Sucrose rats compared to control rats.

Gria2 showed a significant decrease in expression ($p<0.05$) in TSG1-6 of GK compared to control rats.

Gria3 showed a significant increase in expression ($p<0.05$) in LDRG of GK compared to control rats.

Gria4 showed a significant increase in expression ($p<0.05$) in both TDRG5-8 of GK compared to control rats and in TDRG9-12 in GK Sucrose compared to control rats.

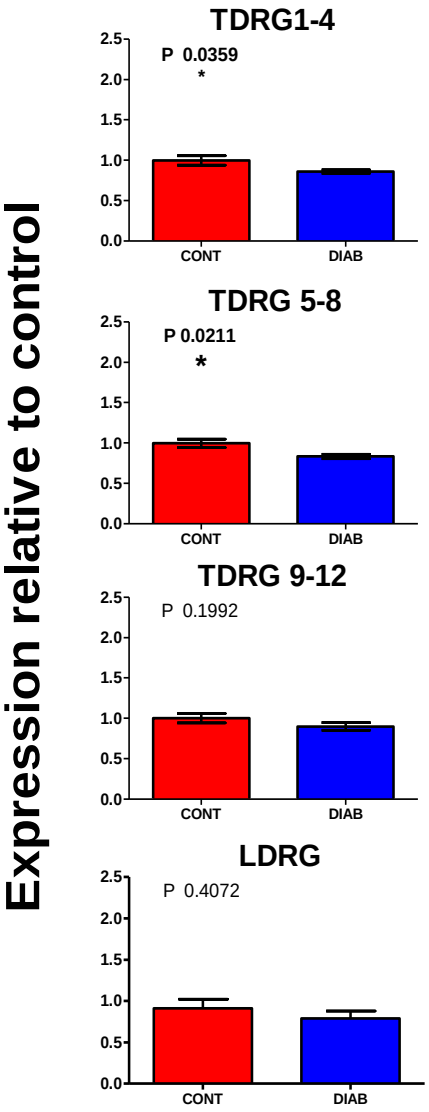

Figure 8: Expression of Gria1 in dorsal root ganglia in GK rats and controls

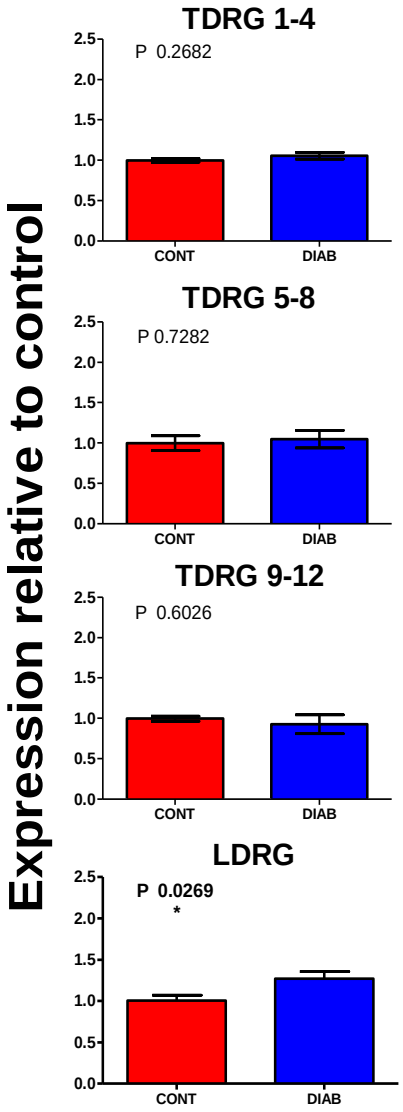

Figure 7: Expression of Gria3 in dorsal root ganglia in GK rats and controls

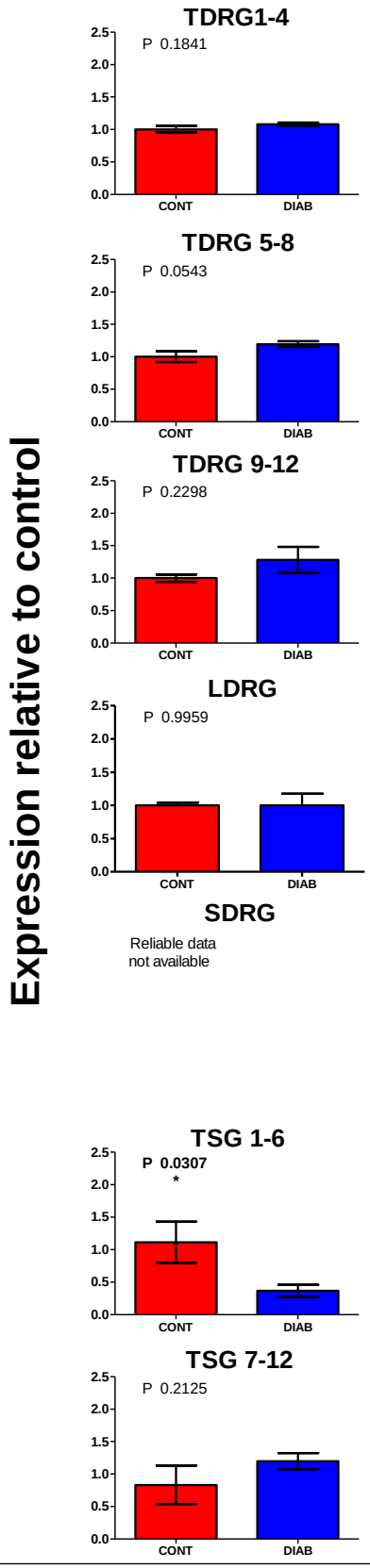

Figure 9: Expression of Gria2 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

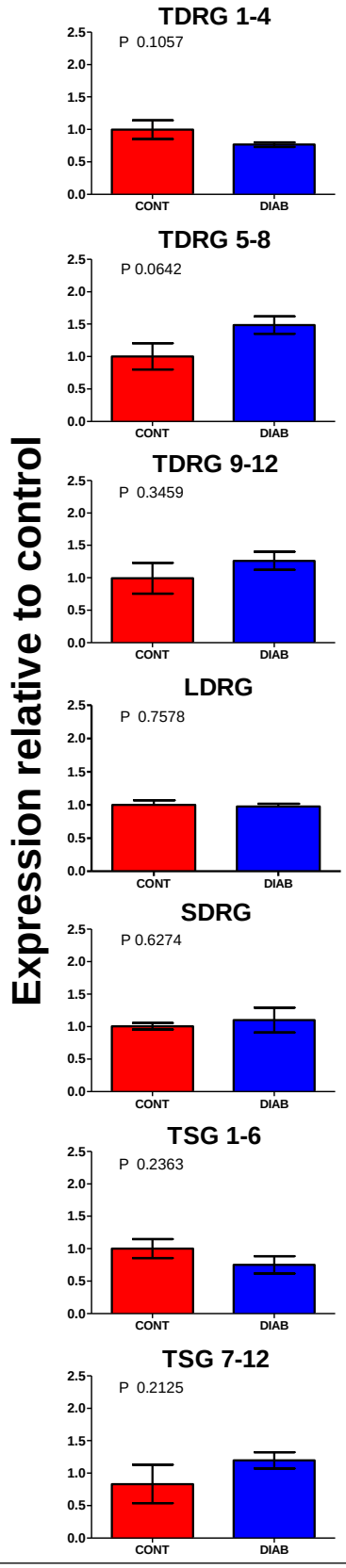

Figure 10: Expression of Gria2 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

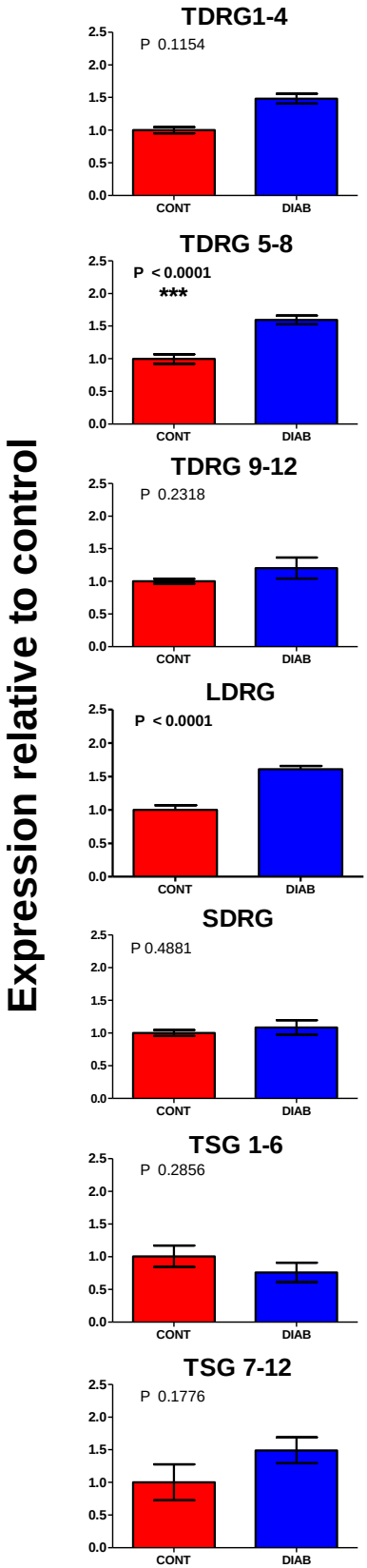

Figure 12: Expression of Gria4 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

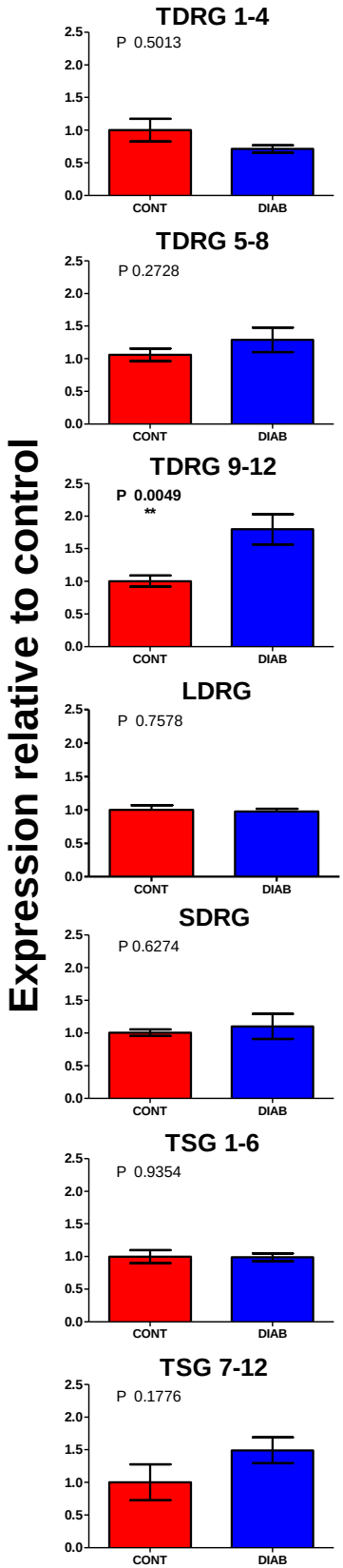

Figure 11: Expression of Gria4 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

3.3.2 Gene Expressing Glutamate Receptors – Ionotropic KAINATE

Genes encoding for the ionotropic glutamate receptors kainate are Grik3 and Grik4. These are shown in Figures 13 – 16. The figures show gene expression of individual genes; Grik3 and Grik4 in GK rats (diabetic vs control) in comparison to GK Sucrose rats (diabetic vs control).

Grik3 showed a significant decrease in expression ($p<0.05$) in TDRG1-4, TDRG5-8, TDRG9-12, LDRG, SDRG, TSG1-6 and TSG7-12 of both GK 10months rats and GK Sucrose rats in comparison to control rats.

Grik4 showed a significant decrease in expression ($p<0.05$) in TDRG1-4, TDRG5-8, TDRG9-12, LDRG and SDRG of both GK rats and GK Sucrose rats in comparison to control rats. Grik4 did not show a significant change in expression in TSG1-6 and TSG7-12 of both GK and GK Sucrose rats compared to control rats.

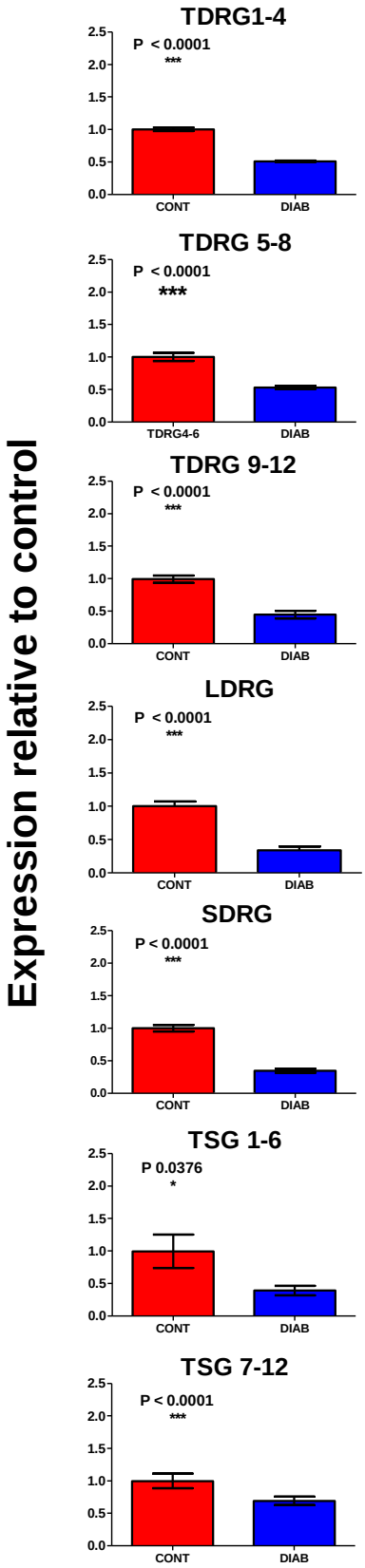

Figure 14: Expression of Grik3 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

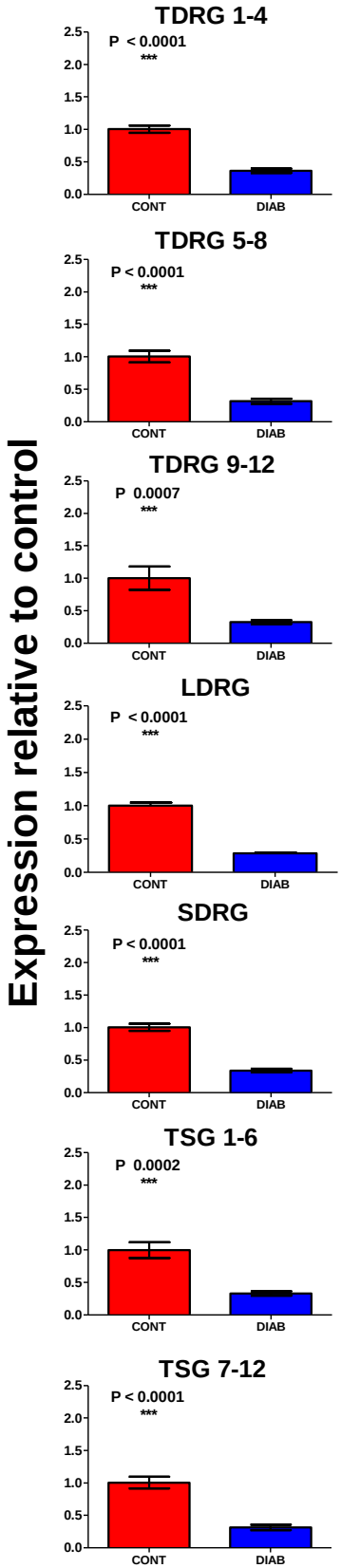

Figure 13: Expression of Grik3 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

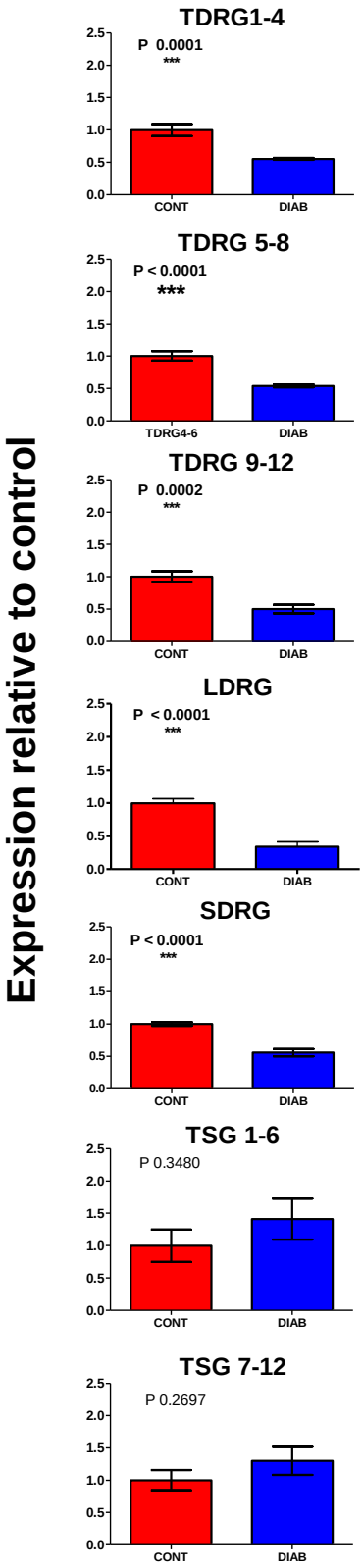

Figure 15: Expression of Grik4 in dorsal root ganglia and thoracic sympathetic ganglia in GK fed rats and controls

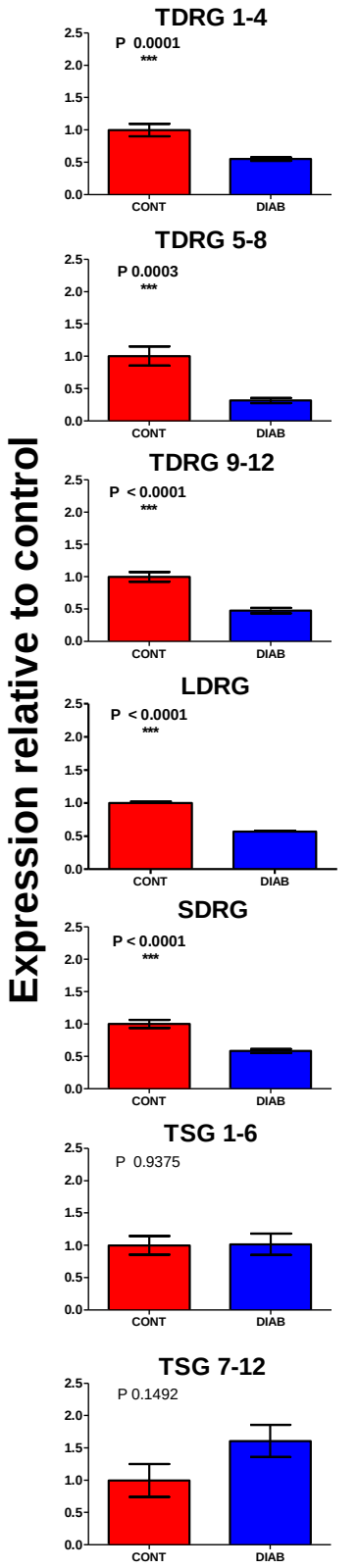

Figure 16: Expression of Grik4 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

3.3.3 Gene Expressing Glutamate Receptors – Ionotropic NMDA and DELTA 2

Genes encoding for the ionotropic glutamate receptors NMDA are Grin1 and Grin2A. The gene encoding for the ionotropic glutamate receptor Delta 2 is Grid2. These are shown in Figures 17 – 20. Figure 17 shows gene expression of individual genes; Grin1 in GK (diabetic vs control) in comparison to control rats. Figure 18 shows Grid2 in GK (diabetic vs control) in comparison to control rats. Figure 19 shows Grin2A in GK rats (diabetic vs control) in comparison to Figure 20 which shows Grin2A in GK Sucrose rats (diabetic vs control).

Grin1 showed a significant decrease in expression ($p<0.05$) in TDRG1-4, LDRG and SDRG of GK rats in comparison to control rats.

Grid2 showed a significant increase in expression ($p<0.05$) in TDRG5-8 in GK rats in comparison to control rats.

Grin2A showed a significant decrease in expression ($p<0.05$) in TDRG1-4, TDRG5-8, TDRG9-12, LDRG and SDRG of both GK rats and GK Sucrose rats in comparison to control rats.

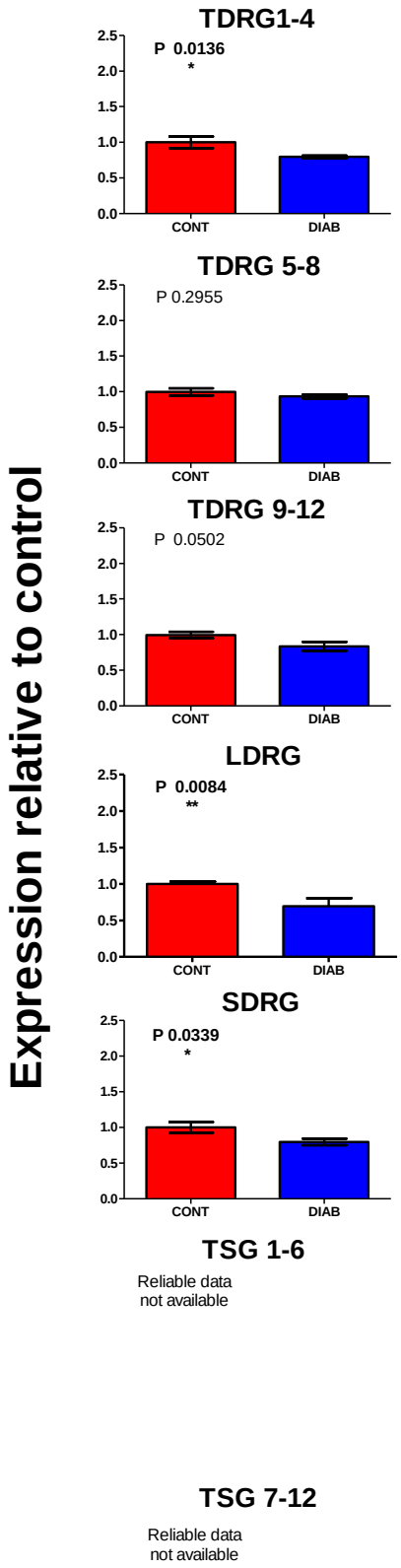

Figure 17: Expression of Grin1 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

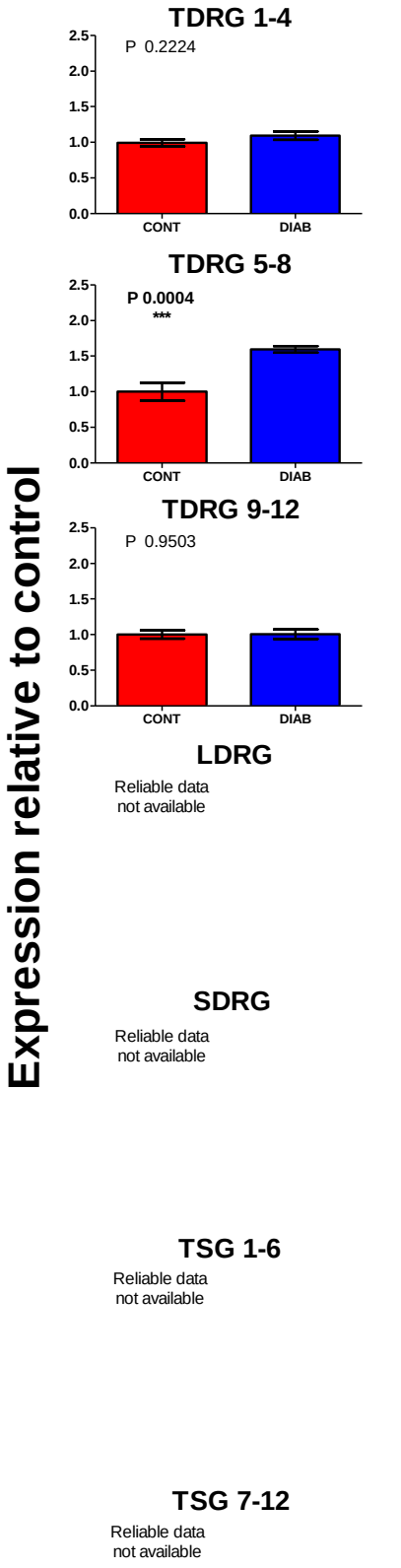

Figure 18: Expression of Grid2 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

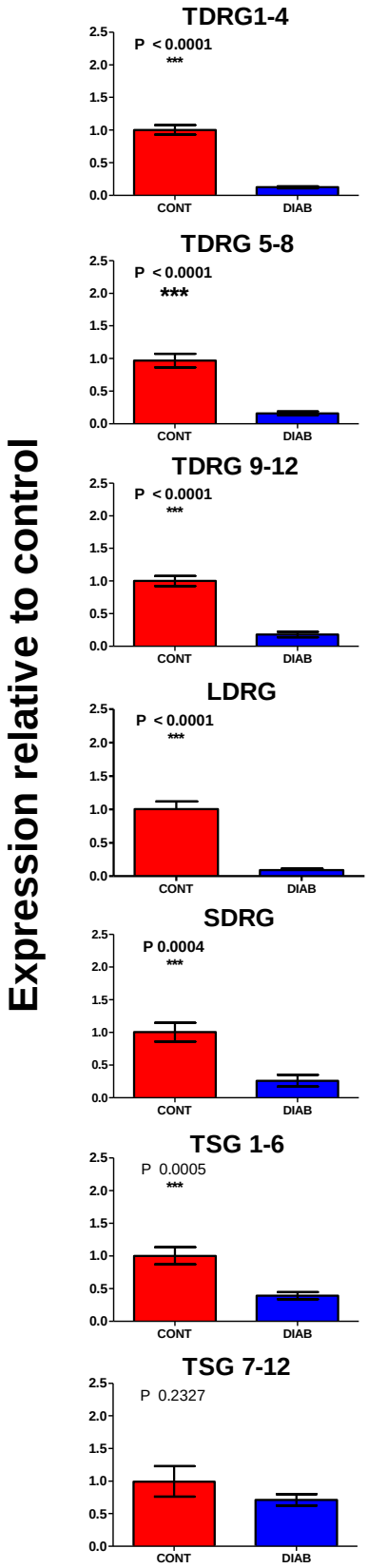

Figure 19: Expression of Grin2A in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

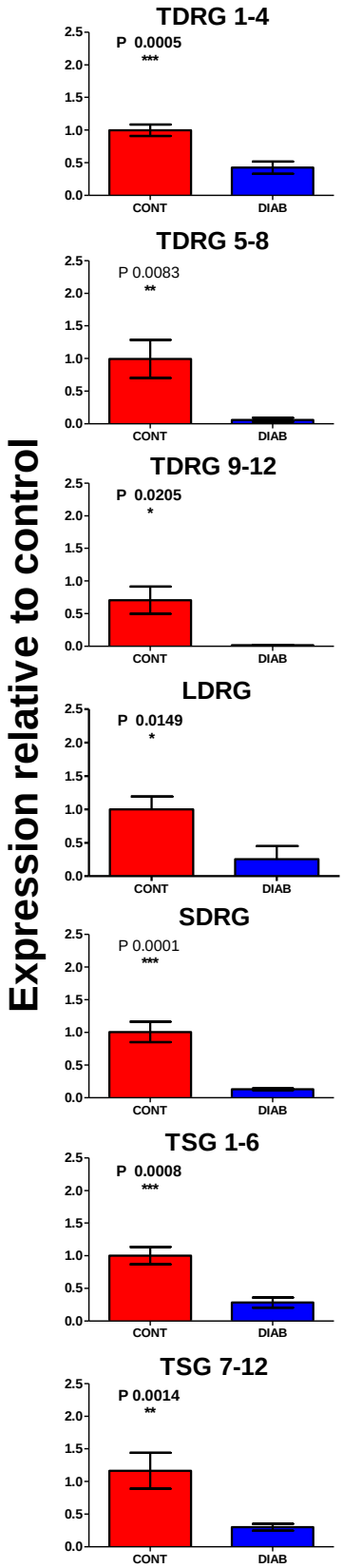

Figure 20: Expression of Grin2A in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

3.3.4 Genes Expressing Glutamate Transporters

Genes encoding for glutamate transporters are Slc1a1, Slc1a2, Slc1a3 and Slc1a6. These are shown in Figures 21 – 27. Figure 21 shows gene expression of individual genes; Slc1a1 in GK (diabetic vs control) and Figure 22 shows GK Sucrose in comparison to control rats. Figure 23 shows the expression of Slc1a2 in GK (diabetic vs control) in comparison to control rats. Figure 24 and 25 show the expression of Slc1a3 in both GK rats (diabetic vs control) and GK Sucrose rats (diabetic vs control). Figure 26 and 27 show the expression of Slc1a6 in both GK rats (diabetic vs control) and GK Sucrose rats (diabetic vs control).

Slc1a1 showed a significant increase in expression ($p<0.05$) in TDRG1-4 of GK rats in comparison to control rats. Slc1a1 showed a significant increase in expression ($p<0.05$) in TDRG5-8 and TDRG9-12 in both GK and GK Sucrose rats in comparison to control rats. Slc1a1 showed a significant increase in expression ($p<0.05$) in LDRG, SDRG and TSG1-6 in GK Sucrose rats compared to control.

Slc1a2 showed no significant change in expression in GK rats compared to control rats.

Slc1a3 showed a significant increase in expression ($p<0.05$) in TDRG1-4 and TSG1-6 in GK rats compared to control. Slc1a3 showed a significant increase ($p<0.05$) in expression in TDRG5-8 in both GK and GK Sucrose rats compared to control rats. Slc1a3 showed a significant increase in expression ($p<0.05$) in TDRG9-12 and SDRG in GK Sucrose rats compared to control rats.

Slc1a6 showed a significant decrease in expression ($p<0.05$) in TDRG1-4 and LDRG in GK and GK Sucrose rats compared to control rats. Slc1a6 showed a significant decrease in expression ($p<0.05$) in TDRG5-8 and TDRG9-12 in GK rats

compared to control rats. Slc1a6 showed a significant decrease in expression ($p<0.05$) in TSG1-6 in the GK Sucrose rats compared to control.

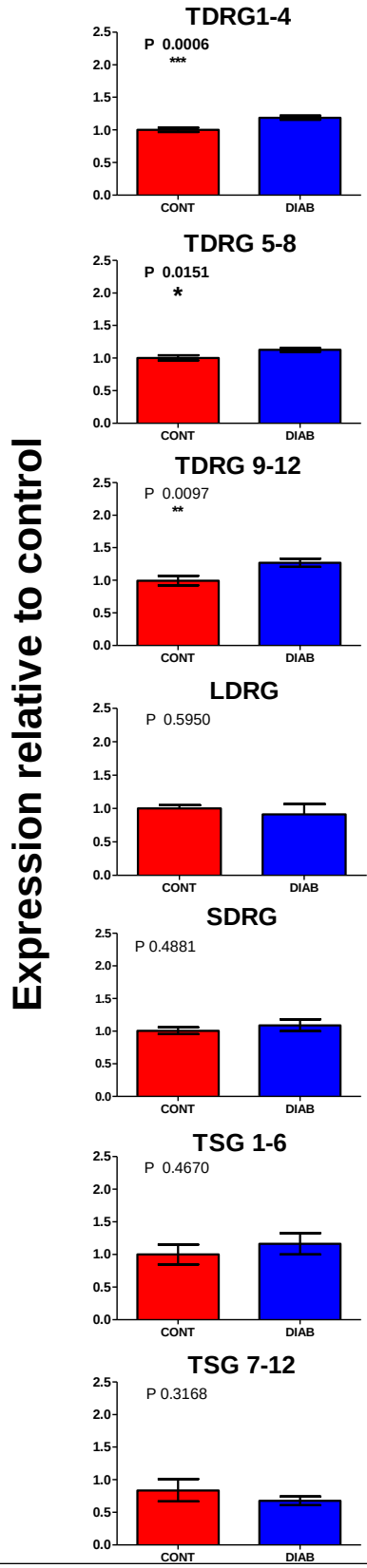

Figure 21: Expression of Slc1a1 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

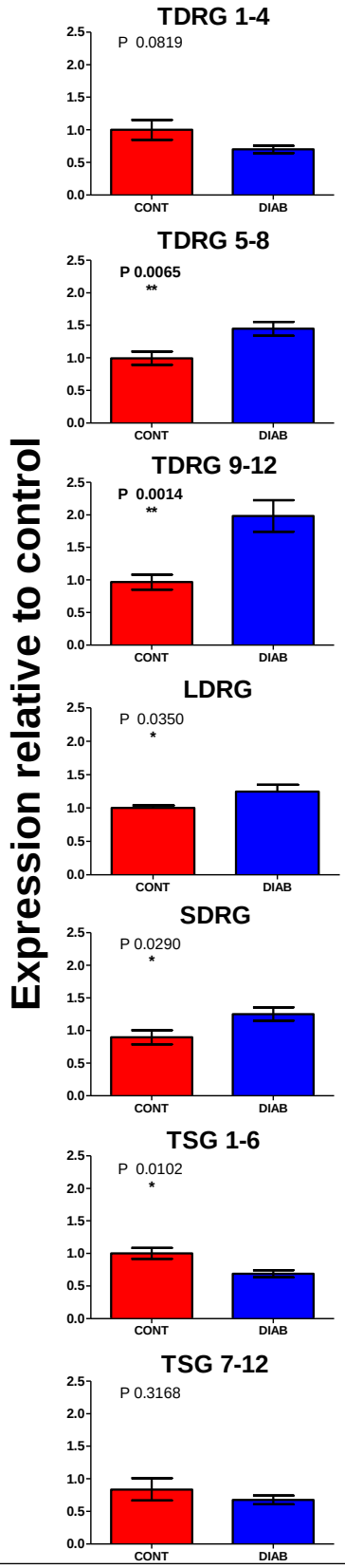

Figure 22: Expression of Slc1a1 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

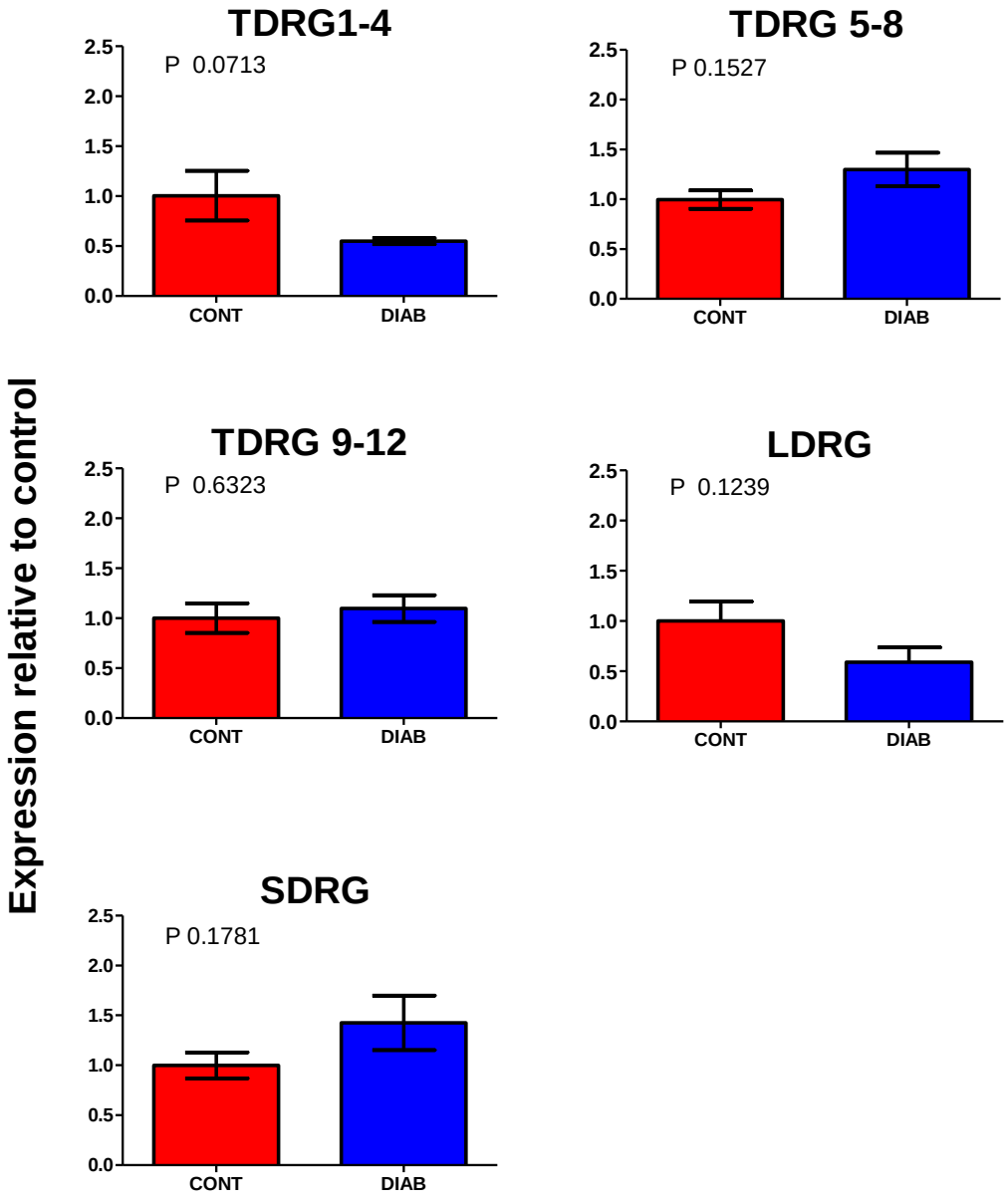

Figure 23: Expression of Slc1a2 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

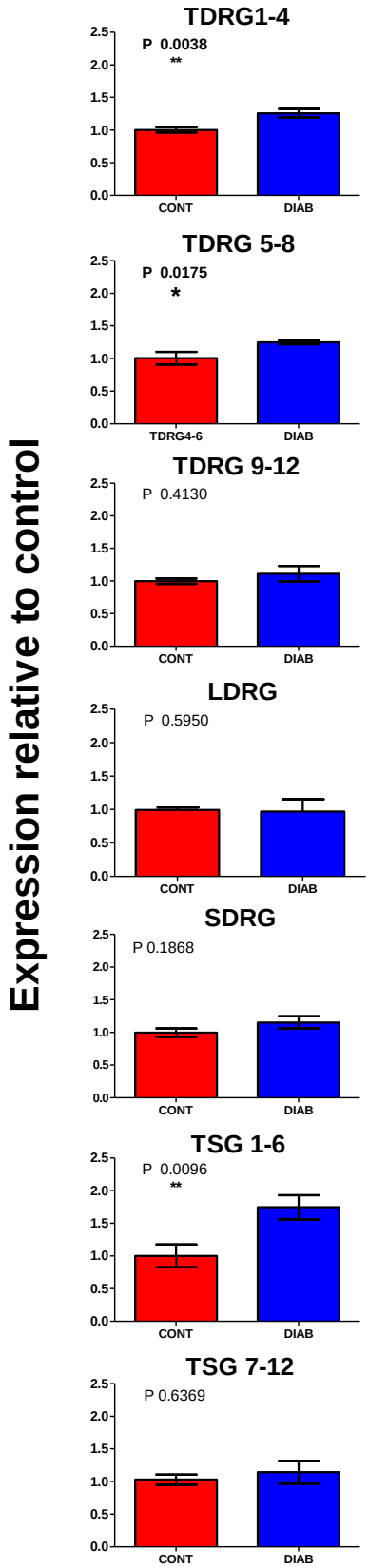

Figure 24: Expression of Slc1a3 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

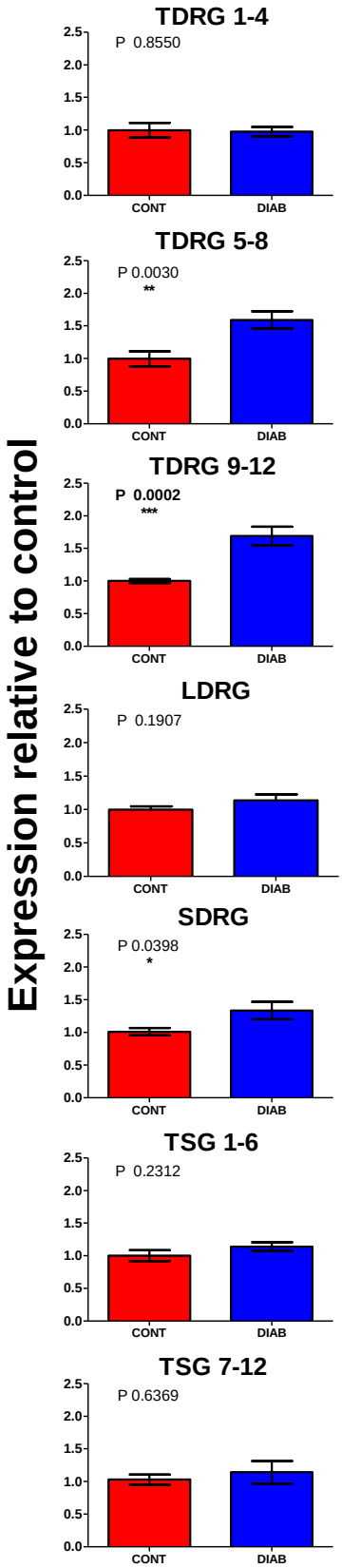

Figure 25: Expression of Slc1a3 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

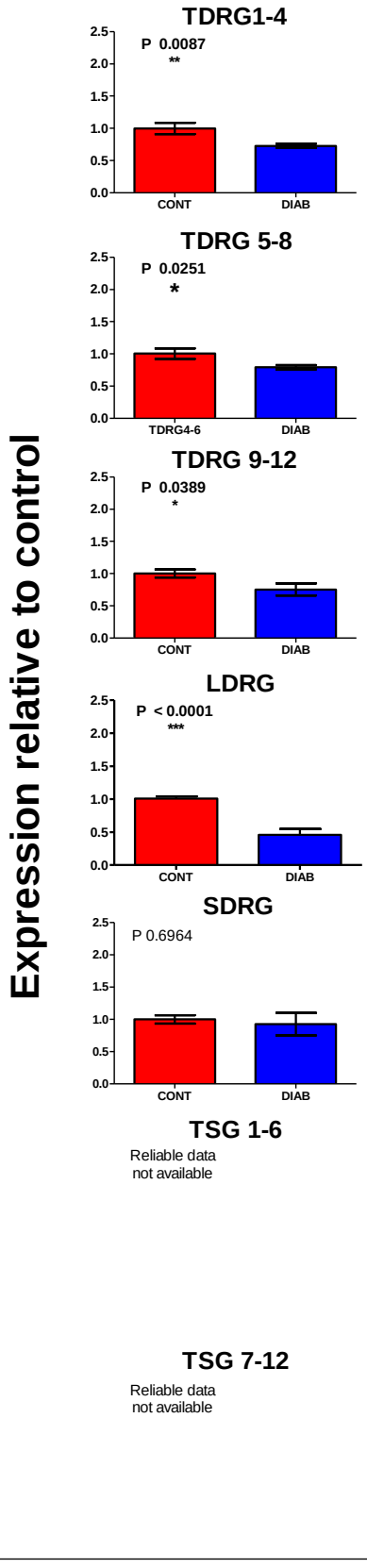

Figure 26: Expression of Slc1a3 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

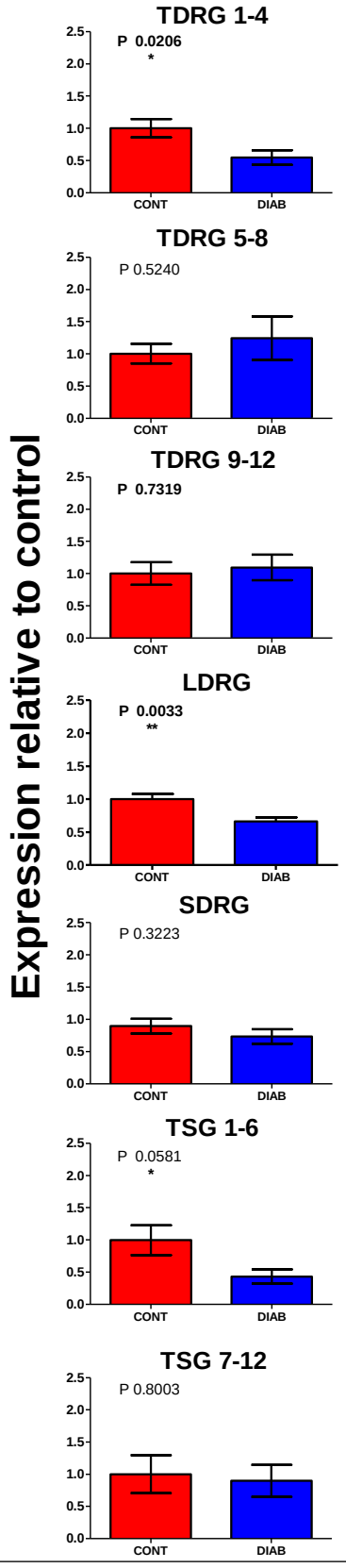

Figure 27: Expression of Slc1a3 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

3.3.5 Gene Expressing Enzymes involved in the Glutamate Cycle and Glutamate Receptor Interacting Proteins

Genes encoding for enzymes that are involved in the synthesis glutamate and metabolism of glutamate include; Gad2, Gclc and Gls. These are shown in Figures 26 – 33. Figure 26 and 27 shows gene expression of individual genes; Gad2 in GK (diabetic vs control) and GK Sucrose in comparison to control rats. Figure 28 and 29 shows the expression of Gclc in GK (diabetic vs control) and GK Sucrose in comparison to control rats. Figure 30 shows the expression of Gls in GK rats (diabetic vs control) in comparison to figure 31 that shows GK Sucrose rats (diabetic vs control). Figure 32 shows the expression of Grip1 in GK rats (diabetic vs control) in comparison figure 33 that shows GK Sucrose rats (diabetic vs control).

Gad2 showed a significant decrease in expression ($p<0.05$) in TDRG1-4 and TDRG9-12 of GK rats compared to control rats. Gad2 showed a significant decrease in expression ($p<0.05$) in TDRG5-8 and a significant increase in expression ($p<0.05$) in TSG7-12 in GK Sucrose rats compared to control rats.

Gclc showed a significant increase in expression ($p<0.05$) in TDRG1-4, TDRG5-8, LDRG and SDRG in GK rats and GK Sucrose rats compared to control rats.

Gls showed a significant decrease in expression ($p<0.05$) in TDRG9-12 in GK rats compared to control rats. Gls showed a significant decrease in expression ($p<0.05$) in LDRG and TSG1-6 in GK Sucrose rats compared to control rats. Gls showed a significant increase in expression ($p<0.05$) in TSG7-12 in GK rats compared to control rats.

Grip1 showed a significant decrease in expression ($p<0.05$) in TDRG1-4 of GK rats compared to control rats. Grip1 also showed a significant decrease in expression ($p<0.05$) in LDRG and TSG1-6 in both GK and GK Sucrose rats compared to control rats.

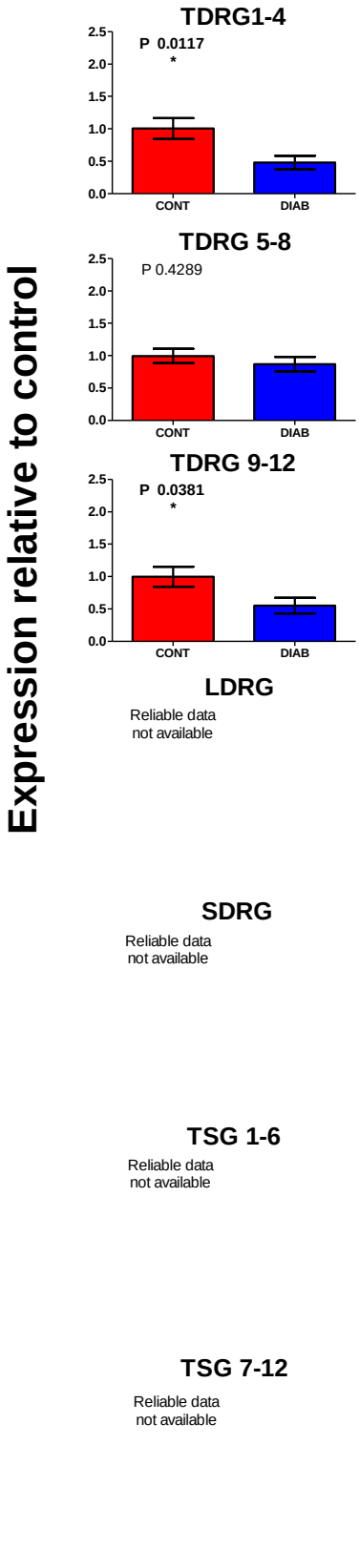

Figure 29: Expression of Gad2 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

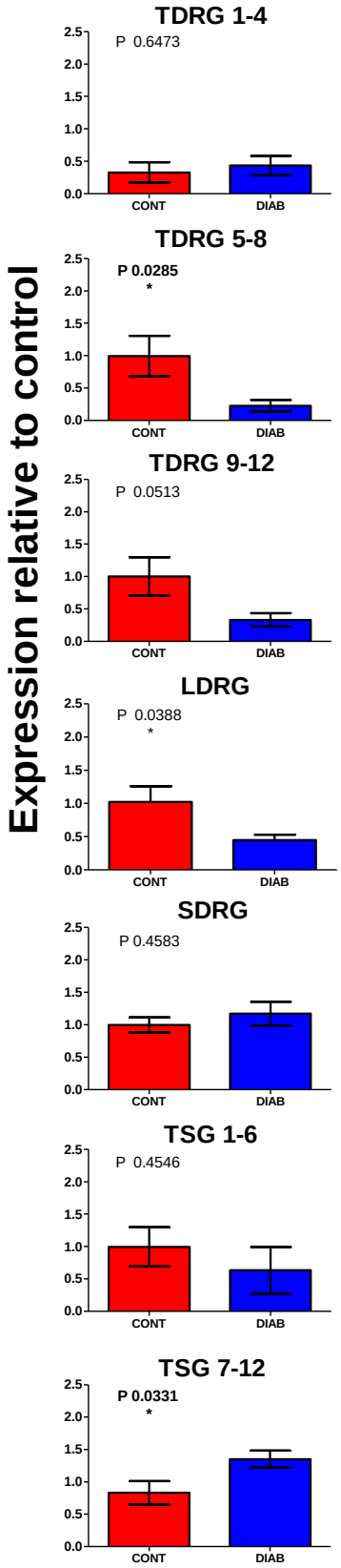

Figure 28: Expression of Gad2 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

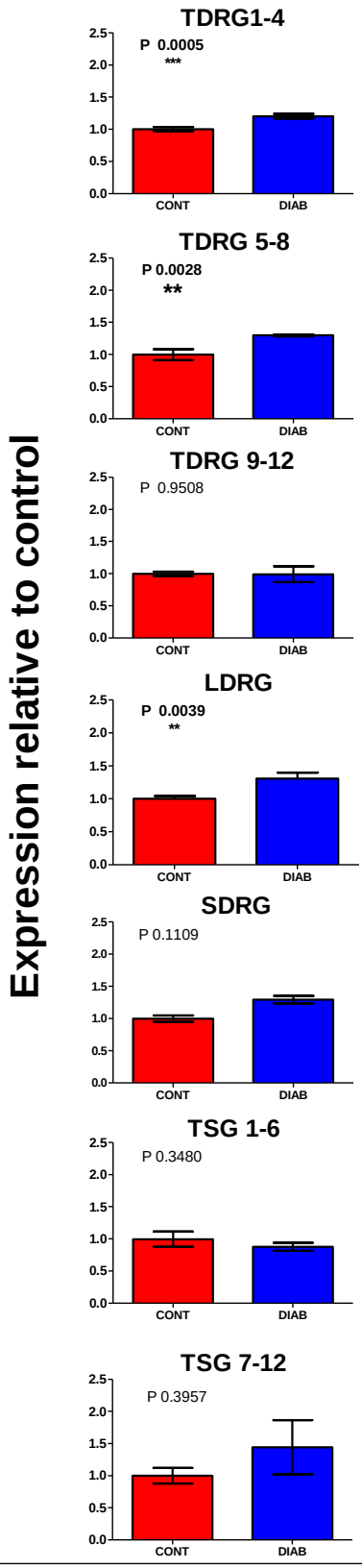

Figure 30: Expression of Gclc in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

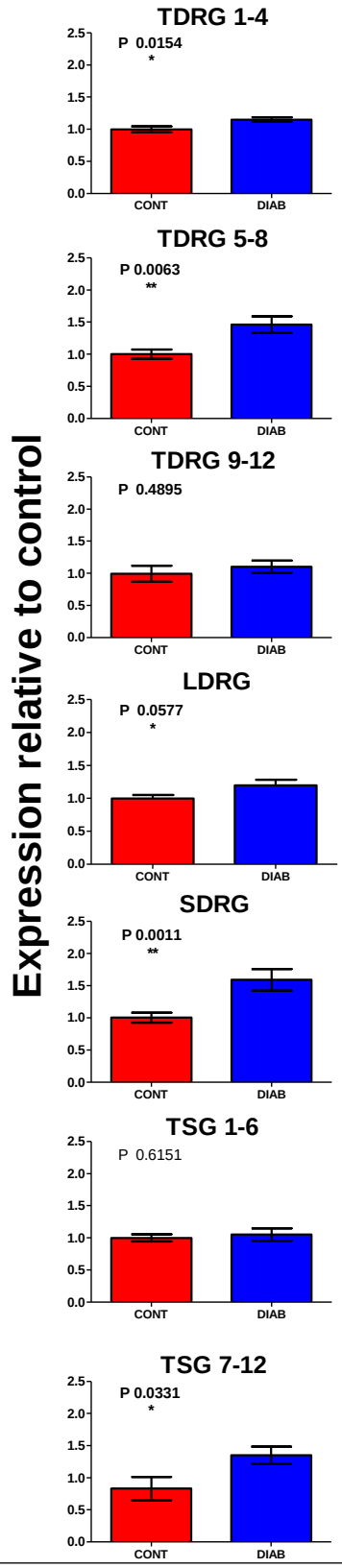

Figure 31: Expression of Gclc in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

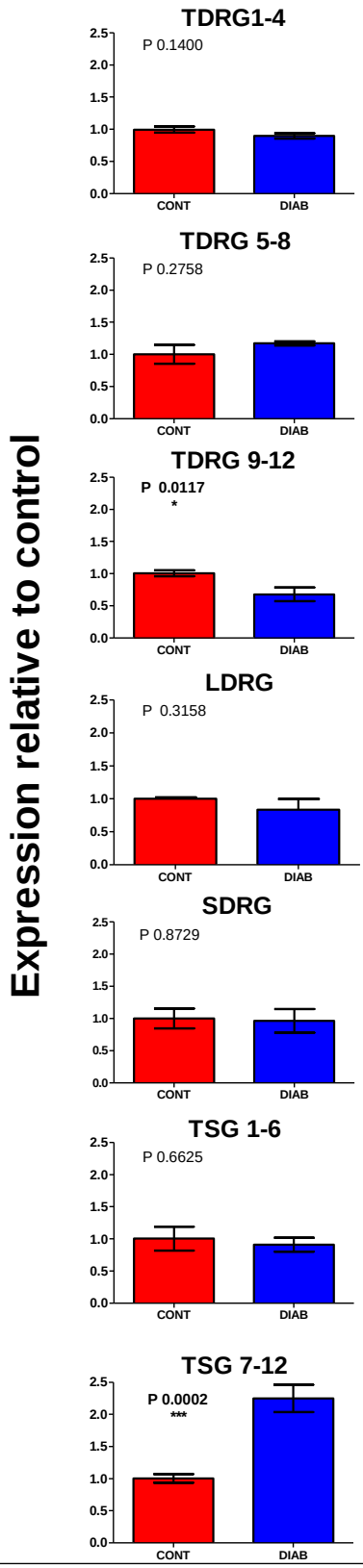

Figure 33: Expression of Gls in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

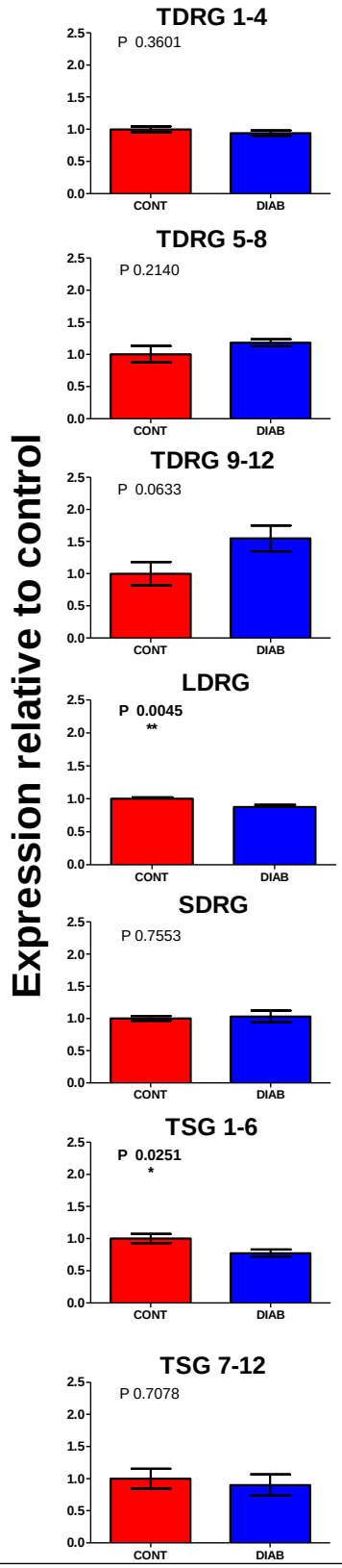

Figure 32: Expression of Gls in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

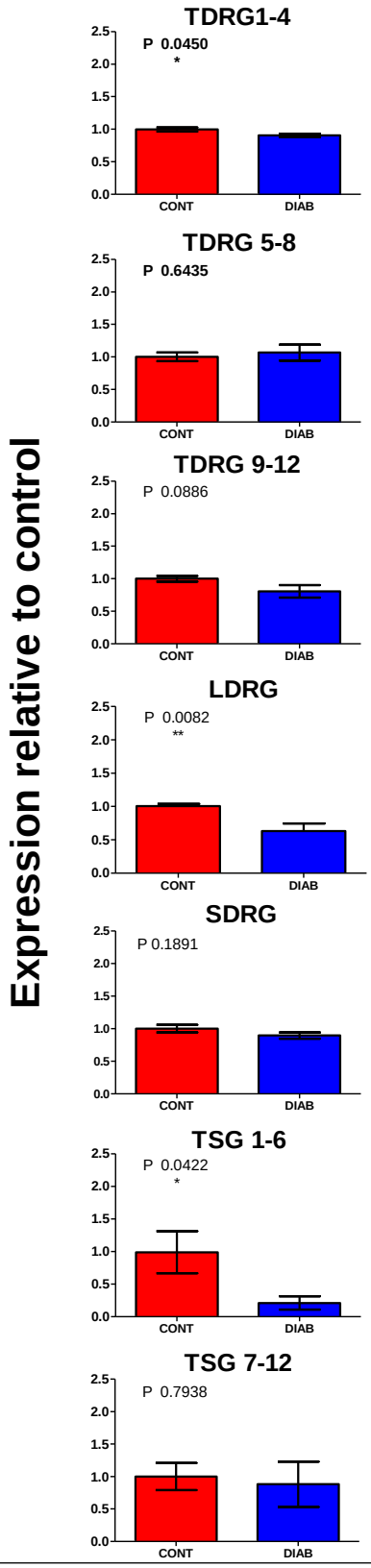

Figure 34: Expression of Grip1 in dorsal root ganglia and thoracic sympathetic ganglia in GK rats and controls

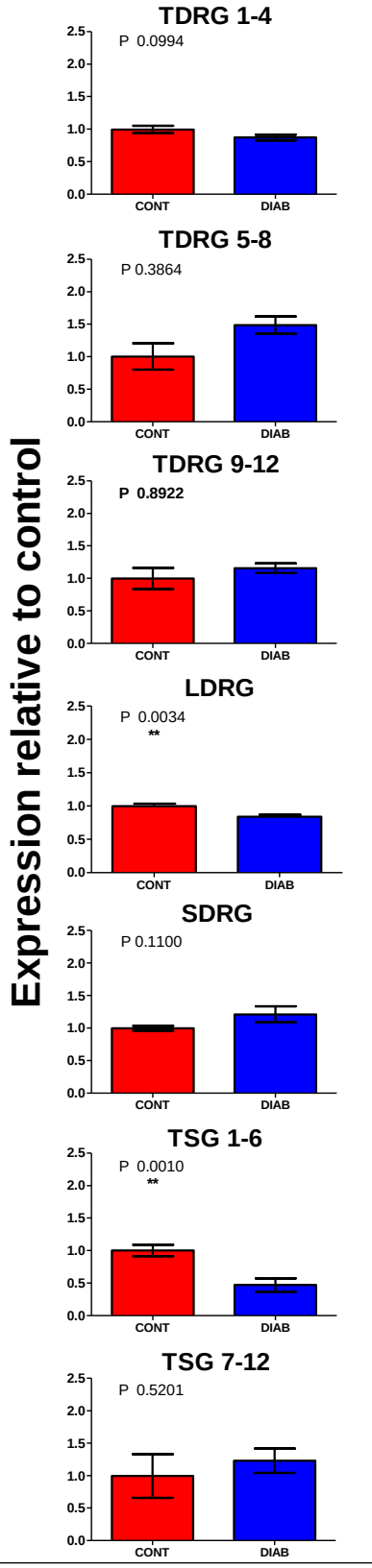

Figure 35: Expression of Grip1 in dorsal root ganglia and thoracic sympathetic ganglia in GK sucrose fed rats and controls

3.4 Analysis of Variance

Using ANOVA followed by Bonferroni post hoc test for multiple comparisons, the following results were obtained. P <0.05 was considered to indicate a significant difference (IBM SPSS statistics, v21).

Gene	**F value**	**P value**
Gria2	107.159 (4, 160)	0.000
Gria4	44.603 (4, 200)	0.001
Slc1a1	24.684 (4, 200)	0.000
Slc1a3	5.445 (4,200)	0.831
Slc1a6	5.350 (4,200)	0.000
Grik3	2805.8 (4,200)	0.000
Grik4	50.002 (4,200)	0.000
Grin2A	2.675 (4, 200)	0.115
Gclc	64.227 (4, 200)	0.000
Gls	43.895 (4, 200)	0.000
Grip1	12.779 (4, 200)	0.000
Gad2	3.303 (4, 120)	0.888

Table 20: Analysis of variance for the effect of dorsal root ganglia on gene expression

In the case of Gria2, concentration in SDRG were different from all levels of the dorsal root ganglia.

For Gria3 and Gria4, concentration in LDRG were different from all levels of the dorsal root ganglia.

For Slc1a1, concentration in LDRG and SDRG were different from all levels of the dorsal root ganglia.

For Slc1a3 and Slc1a6, Grik3, Grip1 and Grin2A there was no significant difference in the any of the dorsal root ganglia across the levels.

For Grik4 and Gclc and Gls, concentration in TDRG 5-8 were different from all levels of the dorsal root ganglia.

Chapter 4: Discussion

While diabetic peripheral neuropathy affects at least 50% of older type 2 diabetic patients, its pathogenesis is not clearly understood. Considering the large number of individuals afflicted with diabetes, this is an issue of epic proportions. Diabetic patients suffering from neuropathic complications can experience a range of symptoms. For some there are extremely painful symptoms but others, usually those with a more marked deficit, there may be no symptoms at all. Typical neuropathic symptoms can include burning sensations, knife-like pain, electric sensations of shock, throbbing pain and allodynia. On the other end of the spectrum, patients can experience sensations such as feeling the part of the body is asleep or 'dead', numbness, tingling and prickling sensations.

The diabetic patients that experience pain frequently exhibit one or more kinds of stimulus-evoked pain, including increased responsiveness to noxious stimuli (hyperalgesia) as well as a hyper-responsiveness to normally innocuous stimuli (allodynia) that are often concurrent with a paradoxical loss of stimulus-evoked sensation.

Clinical examination of patients with chronic diabetic peripheral neuropathy usually reveals a symmetrical sensory loss to all modalities in a stocking distribution. This may well extend into the mid-calf level and may also affect the upper limbs in more severe cases.

Changes in the expression of mRNA, caused by diabetes and/or changes in diet, if translated into changes in proteins may cause alterations to the architecture of glutamate receptors which may in turn have implications for diabetic neuropathy.

Studies involving gene expression suggest that chronic neuropathic pain is associated with strong activation of certain neuronal genes and defining these gene

expression profile can further the understanding of neuropathic pain etiology(Rodriguez Parkitna, 2006).

The pathogenesis of diabetic neuropathy has several theories that endeavor to explain it. When several theories orbit a condition, it suggests that there is no unifying hypothesis. The pathological signalling pathways associated with diabetic neuropathy are complex and this influences the development of targeted therapies. Despite extensive investigations and development of multiple theories the mechanism involved in the development of diabetic neuropathy are still not completely understood. In order to shed light on this process we decided to investigate changes in gene expression in the ganglia that is crucial for the neuropathic process. Preliminary investigations using low density arrays comprised of genes involved in neurotransmitter production and degradation, neuropeptides, receptors, post receptor signalling and transport showed several promising results. The most interesting results involved the genes that are involved in the glutamate pathway. Genes for glutamate receptor expression, glutamate processing enzymes and glutamate transporter proteins showed significant changes in expression.

Furthermore, existing studies have focused predominantly on the spinal cord and peripheral tissue, the dorsal root ganglia have not received such in-depth attention and scrutiny. This could be due to the inherent difficulty in harvesting the tissue intact from animal models because of the extremely small size of the ganglia. The results in this study are unique in addressing the role of genes involved in the glutamate signalling pathway in diabetic neuropathy. The changes observed will be discussed in relation to symptoms of diabetic neuropathy, particularly in light of the substantial evidence that glutamate is involved in neuropathic pain. The cellular and molecular signalling pathways revealed in this study could provide strategies for the development of analgesics and management of diabetic neuropathy. This study

identifies that the genes involved in the expression of glutamate receptors, genes that are involved in the expression of enzymes regulating glutamate concentrations and transporter of the neurotransmitter glutamate could play a role in the development of diabetic neuropathy and its physical manifestations.

4.1 Animal Model

This study investigated the expression of genes involved in glutamate signalling and their possible role in the development of diabetic neuropathy. The experiments utilized an animal model that represents type 2 diabetes; the most common type of diabetes in humans.

Two of the animal groups studied were Goto-Kakizaki diabetic animals who exhibit modestly elevated glucose levels and their litter mate controls. In order to investigate the effect of increased hyperglycaemia, we also studied the same two groups of animals following a sucrose enriched diet. Thus there are four study groups overall: Control animals (Cont), GK diabetic animals (GK), Control Sucrose fed (Cont-Suc), GK Sucrose fed (GK-Suc). The longer the duration of exposure to risk factors, the worse are the effects of T2DM on the nervous system as well as the whole body. The combination of subtle and pronounced changes in expression of genes encoding the receptors, enzymes and transport proteins involved in the glutamate signalling pathway suggests an important role that these play in the development of diabetic neuropathy.

4.2 Glutamate Receptors and Neuropathic Pain

The metabotropic glutamate receptors (mGluRs) have received more attention that the fast acting ionotropic glutamate receptors (iGluRs) in terms of their role in neuropathic pain. The fast acting iGluRs have not been as explored in their potential roles in causing the symptoms of diabetic neuropathy. Furthermore the

iGluRs are also involved in glutamate toxicity while certain subtypes of mGluRs are actually neuroprotective. Since most fast excitatory synaptic neurotransmission in the mammalian nervous system is mediated by glutamate, it is plausible that many neurological disorders may be associated with abnormalities in the glutamatergic signalling system, and that therapeutic benefits might be attainable through the manipulation of this system. We therefore conducted a study to characterize the expression of messenger RNAs (mRNAs) coding for subunits of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, N-methyl-d-aspartate (NMDA) receptors, Kainate receptors, glutamate transporters and enzymes involved in glutamate regulation such as glutaminase and glutamate cysteine ligase in the dorsal root ganglia in two rat models of diabetic neuropathy.

It is generally assumed that neuropathic pain is caused by changes in expression and function of receptors, enzymes, and voltage-dependent ion channels in peripheral nerves and DRG neurons. The changes at synapses in the nociceptive pathway in the central nervous system also contribute(W. Wang, Gu, Li, & Tao, 2011)

There are possibly a variety of modified alterations in pain-related gene expression in DRG neurons that underlie the neural plasticity in neuropathic pain. What role does the glutamatergic system play in diabetic peripheral neuropathy? This is akin to asking how large a hole can be made in a ship without sinking it. What the ship tolerates depends on the capacity of its pumps to remove water, the energy for the pumps, the weather, the mass and properties of the cargo, the skills of the crew as well as a number of other factors. As long as one variable is not extreme, it will be the combination of several factors that will determine whether the ship will sink.

Collectively, the different responses of glutamate and its receptors in DRG neurons to neuropathic pain could be promoting or conquering the overexcited neuronal transmission that may be substantially associated with corresponding gene expression modifications. Alterations in gene expression may function as pathological contributor to the development and maintenance of neuropathic symptoms in diabetic neuropathy.

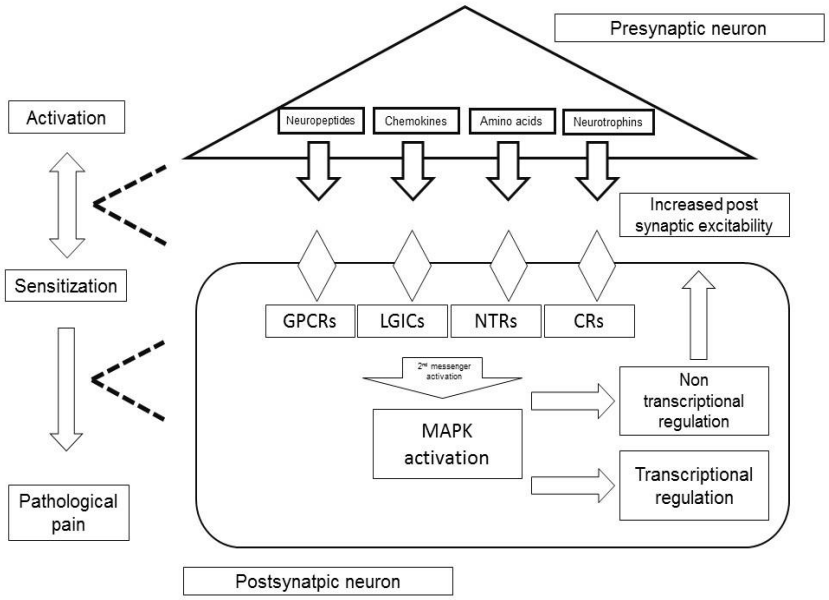

Figure 36: MAP-kinase signalling along pain pathways in experimental rodent models.

Pathological pain signalling can be a dual process of activation and sensitization which has the potential to become self-perpetuating and ultimately bidirectional. Activation begins with an increase in the presynaptic release of nociceptive transmitter-mediators from presynaptic neuron terminals or glia. These include neuropeptides (substance P or calcitonin gene related product) chemokines (fractalkine), amino acids (glutamate), neurotrophins (nerve growth factor or brain-derived neurotrophic factor). Further activation occurs due to the postsynaptic stimulation of nociceptors located on neurons or glia that include: GPCRs (G-protein

coupled receptors) that bind neuropeptides and chemokines, LGICs (ligand-gated ion channels) that bind amino acids (such as glutamate; NTR (neurotrophin receptor) tyrosine kinases that bind the neurotrophins (e.g. NGF and BDNF) and cytokine receptors. Nociceptor stimulation activates of various second-messenger signal transduction cascades that converge to ‘‘turn-on’’ MAPKs via phosphorylation, regarded as a key component in nociceptive sensitization. Activation (phosphorylation) of MAPKs leads to transcriptional regulation on DNA producing gene products, as well as non-transcriptional regulation that enhances the excitatory properties of nociceptive activation and sensitization. The combination of these factors underlie the generation and maintenance of chronic pain(Edelmayer, 2014).

4.3 Mechanisms of Neuropathic Pain

Neuropathic pain can be caused by changes in the expression and function of receptors, enzymes, and ion channels in peripheral nerves and DRG neurons, as well as at the synapses in the nociceptive pathway of the central nervous system. There dynamic interactions between peripheral and central mechanisms are implicated in neuropathic pain(Gracely, Lynch, & Bennett, 1992)

The dorsal root ganglia express many different kinds of ion channels and receptors. Broadly their functions are; transduction (sodium channels and ATP-sensitive receptors expressed in the peripheral terminals of DRG neurons that transduce noxious stimuli into electric impulses), conduction (e.g., sodium and potassium channels are involved action potential propagation) and synaptic transmission modulation (e.g. glutamate receptors expressed on presynaptic terminals of the primary afferents in dorsal horn to regulate neurotransmitter release). The abnormal activity of neurons in conditions of neuropathic pain could be

related to changes in the density, distribution, and functional activities of glutamate receptors in the dorsal root ganglia neurons.

The most important changes responsible for the induction of neuropathic pain are found in the altered gene/protein expression in primary sensory neurons. After damage to peripheral sensory fibers, upregulated expression of certain receptors can contribute to hyperalgesia; while the downregulation of other receptors in unmyelinated neurons is responsible for the phenotypic switch in pain transmission (Sapunar, Kostic, Banozic, & Puljak, 2012).

The major cellular mechanisms that have been studied in diabetic neuropathy include ectopic or spontaneous nerve activity, peripheral and central hyperexcitability, phenotypic changes in the pathways that conduct pain and morphological reorganization. Understanding the relationship between symptoms and key cellular and molecular mechanisms is vital to develop strategies to direct rational drug therapy rather than following clinical serendipity. Antinociceptive gene therapies that involve the glutamate signalling pathway to treat diabetic peripheral neuropathies have been met with some success in reducing neuropathic pain in animal models of diabetes after gene transfer to the dorsal root ganglion(Y. Wang, 2013)

Mechanistic and molecular-based understanding of chronic neuropathic pain has relied heavily on animal studies using a disease or mechanism-related models that emphasize key symptomatic characteristics such hyperalgesia and mechanical allodynia. Most of these models have involved peripheral nerve lesions such as sciatic nerve ligation or L4 and L5 spinal nerve ligation. However, there are relatively fewer studies from other disease-related models such as diabetic neuropathy to provide a balanced view about the ubiquity of key mechanisms.

The pathogenesis of peripheral and central neurological complications of diabetes is likely to share many features. The pathogenesis of peripheral neuropathy is multifactorial and involving metabolic alterations, such as increased polyol pathway flux, oxidative stress and non-enzymatic protein glycation. There is also evidence for vascular dysfunction, leading to decreases in nerve blood flow and hypoxia and alterations in neuronal functions. Increases in polyol pathway flux, leading to accumulation of sorbitol and fructose, accumulation of advanced glycation end products and increased concentrations of the by-products of lipid peroxidation have all been demonstrated in the neural tissue of diabetic rats.

Neuropathic pain most often originates from a lesion in the nervous system and diabetic neuropathy is one type. A characteristic of neuropathic pain is that the aberrance leading to pain must directly involve the nociceptive pathways. Painful diabetic neuropathy is length dependent which means the longest axons in the peripheral nerve are most vulnerable. Patients also complain of bilateral burning pain in the toes and feet. Quantitative sensory testing reveals decreased pain sensibility (with or without decreased touch sensibility).

Although physiological pain has a protective effect, pathological pain is pain that has lost its original biological signalling function and does not serve the survival. Pathological pain is characterized by exaggerated responses to all suprathreshold and eventually subthreshold stimuli. The pain is elicited by stimuli which is otherwise innocuous and the pain spreads from the site of injury to the surrounding unaffected area. This type of pain also persists even in the absence of any apparent stimuli.

4.4 Hyperalgesia and Allodynia

In physiological terms hyperalgesia and allodynia refer to a dramatic decrease in the threshold of pain elicited by unmyelinated C-fibre afferents

(hyperalgesia) and that of Aβ myelinated fibres that are activated normally only by mechanical stimuli and carry non-nociceptive information.

However, hyperalgesia and allodynia are two major forms of sensitized behavioural responses assessed in animal models of neuropathic pain. Diabetic patients often experience hyperalgesia which is abnormally heightened sensitivity to pain and can be classified into primary and secondary. Primary hyperalgesia occurs at tissue injury site and is mediated by primary afferent nociceptor sensitization which shows as increased response to stimuli such as heat. Secondary hyperalgesia occurs in uninjured tissue surrounding site of injury and is linked to central sensitization and shows an increased response to mechanical stimuli. Mechanical hyperalgesia can manifest as pain to light-stroking stimuli (i.e. allodynia) and enhanced pain to punctate stimuli. In summary hyperalgesia is when nociceptive responses to noxious stimuli are significantly enhanced while in allodynia, previously non-noxious stimuli such as cold stimuli or mechanical touch leads to nociceptive responses. Hyperalgesia and allodynia are often concurrent with a paradoxical loss of stimulus-evoked sensation.

The pain in diabetic neuropathy is complex and involves numerous cellular receptors, channels and chemicals. Hyperexcitability of primary afferent neurons after injury could be due to a change in the expression of ion channels(Passmore, 2005). This makes ion channel subunits, particularly those predominantly expressed in nociceptive neurons, novel targets to better understand the mechanisms behind diabetic neuropathy and for future therapeutic targeting. Most studies of ion channels have been carried out in dissociated DRG neurons in culture because of the ease with which neurons in culture can be experimentally manipulated. Studies done on DRG harvested from various diabetic models such as the Goto-Kakizaki and Streptozotocin rat model are relatively uncommon.

4.5 The Role of Dorsal Root Ganglia in the Pain Pathway

The cell body of sensory neurons is an interesting model to study the properties of afferent terminals. Experimentally, the soma of neurons is more accessible that the terminals and may serve as a model of mechanisms that are at play in the terminals. Studying the dorsal root ganglia cells are unique because the responses of the somas of these cells to neurotransmitters may reflect those of the synaptic terminals of the primary afferent neurons. The dorsal root ganglia have also been subject to being used as targets for cell therapy treating neuropathic pain(H. Yu, 2015). Many mediators like neurotransmitters, ionic channels and cytokines have been identified being involved in the regulation of pain through functioning on the DRG neurons. As the entrance site of pain transmitted into the CNS, DRG possesses a pivotal role in the functional regulation of context of chronic pain.

Alterations in the expression of neurotransmitters and corresponding receptors play a significant role in sensitizing or desensitizing DRG neurons. The imbalanced levels of excitatory and inhibitory transmitters including their receptors make the whole DRG be easier to sensitize to stimulation due to a reduced threshold or spontaneous activity.

In a regional approach to understanding the role of pain signalling though the glutamate pathways, the dorsal root ganglia present unique targets. The DRG is located between the dorsal root and the spinal nerve and contains pseudounipolar neurons that convey sensory information from the periphery to the CNS. Following nerve injury or inflammation, these neurons may become an important source of increased nociceptive signalling through increased neuronal excitability and the production of ectopic discharges. The DRG also lacks a protective surrounding capsular membrane and has a permeable connective tissue capsule which can

make it a good choice for drug application. Its permeability is due to a very high density of blood capillaries in the ganglion tissue(Sapunar, 2012).

When nociceptive signals are produced at distal nerve fibers, the signals travel through DRG neurons to the CNS. When the nerves are injured, the DRG neurons become hyperexcitable, generating spontaneous action potentials or abnormal high-frequency activity that contributes significantly to neuropathic pain. The development of chronic pain was considered as a mixed result of multiple factors that involve the neurons and neurons and glia. Supporting glial cells of the peripheral nervous system include satellite cells of dorsal root ganglia and Schwann cells of peripheral nerves. Pathological alterations in the plasticity and homeostasis of the neural micro-environment give way to hypersensitivity.

Theories such as the phenotypic switch predict neurons repeatedly stimulated by noxious stimuli activate genes resulting in transcription factors that enhance the expression of various neurotransmitters and receptors which perpetuate the dysfunction(Neumann, Doubell, Leslie, & Woolf, 1996).

The neurons in DRG are typically divided according to the size of their cell soma; small, medium and large. The neurons with a cell body diameter less than 30 μm are putative nociceptors and large diameter neurones (>30 μm) are regarded as non-nociceptive. Non-myelinated C-fibres, that transmit noxious information, arise from neurons with small cell bodies, while myelinated Aα- and Aβ-fibres, which convey low-threshold non-noxious information, are from large cell body neurons. Aδ-fibres, which can also be nociceptive, are from neurons with cell bodies that are medium in size(Passmore, 2005).

Glutamate has been shown to play a role also in the transduction of sensory input at the periphery and its involvement in peripheral neuropathies has been suggested on the basis of experimental studies in animals.

At spinal level the most intense glutamate immunoreactivities can be found in the superficial laminae (I, II) of the dorsal horn, in the dorsal root ganglion cells and their efferent axons(Szekely, Torok, & Mate, 2002)

In neuropathic pain, neurons are important but so are the glia cell responses that alter neuronal function in the peripheral and central nervous system. Neurons in the DRG are surrounded by satellite glial cells (SGCs), which carry receptors for numerous neuroactive agents and can therefore receive signals from other cells and respond to changes in their environment. Activating these cells can influence nearby neurons, and thus SGCs may participate in signal processing and transmission in sensory ganglia. Damage to the axons of sensory ganglia contributes to neuropathic pain. Such damage also affects SGCs so these cells have a role in the pathological changes in the ganglia(Hanani, 2005)

4.6 The Role of Ionotropic Glutamate Receptors in Neuropathic Pain

Glutamate receptors (GluRs) are widely expressed in the membrane of spinal neurons postsynaptic to nociceptive afferents. AMPA, KA and NMDA receptors are expressed in the rat DRG neurons(Sato, Kiyama, Park, & Tohyama, 1993). In addition to this, subunits of both ionotropic and metabotropic glutamate receptors that are translated and synthesized in dorsal root ganglion (DRG) neurons are transported to peripheral and central locations(Carlton, 1995; Coggeshall & Carlton, 1998; Lu, Hwang, Phend, Rustioni, & Valtschanoff, 2003). The peripheral transport of GluRs is suggested by immunoreactivity for NMDA, kainate, or AMPA receptors detected on nerve fibers and endings in the rodent and human skin(Carlton ., 1995; Coggeshall & Carlton, 1998; Kinkelin, Brocker, Koltzenburg, & Carlton, 2000). Peripheral GluRs can be activated by using agonists and these receptors function as sensors of glutamate released by peripheral terminals, contributing to sensitization after injury. The central terminals of DRG neurons

release glutamate, so presynaptic iGluRs expressed by these terminals may act as autoreceptors. Activation of presynaptic autoreceptors in central endings of DRG neurons decreases the release of glutamate from these terminals (Bardoni, Torsney, Tong, Prandini, & MacDermott, 2004; Kerchner, Wilding, Huettner, & Zhuo, 2002; C. J. Lee ., 2002).

AMPA-receptor antagonists, NMDA-receptor antagonists, and mGluR antagonists have all been shown to ameliorate correlates of hyperalgesia and allodynia in other animal models of diabetes such as the streptozotocin model (Calcutt & Chaplan, 1997; Gupta, Singh, Sood, & Arora, 2003; Malcangio & Tomlinson, 1998).

Ionotropic glutamate receptors are essential to the appropriate function of the mammalian CNS. They mediate chemical synaptic transmission at the vast majority of excitatory synapses, underlie cellular models of learning and memory, modulate neuronal network excitability, and are required for maturation of synaptic connections during early development.

4.6.1 AMPA Ionotropic Glutamate Receptors and Neuropathic Pain

In the dorsal root ganglia there were no significant changes in expression of Gria1, Gria2 and Gria3. With regard to Gria4, statistically significant but modest increase in expression was seen in TDRG 5-8 and LDRG of the GK group and in TDRG 9-12 of the GK Sucrose group. The other levels of DRG showed a trend for increased expression but did not reach statistical significance. Therefore, the biological importance of the significant changes might be minimal.

The modest increase in mRNA expression of Gria4 in the lumbar dorsal root ganglia, considering its role of presynaptic inhibition, could be an adaptive response. This suggests the possibility of a compensatory mechanism to reduce the increased

activity of these receptors. This would serve the purpose of reducing the synaptic sensitivity to glutamate and tempering the information flow from the peripheral nervous system to the central nervous system. Increasing Gria1 containing AMPA receptors at progressively more distal synapses can help to normalize synaptic strength by compensating for increased dendritic filtering of synapses more distal to the cell body. This down-regulation might be related to epigenetic gene silencing. This information suggests that the development of sensitization could require glutamate transmission and it also suggests mechanistic similarities to other forms of neuronal plasticity.

Antagonists that target AMPA receptors are more effective in abolition of hyperalgesia seen during experimental inflammation although NMDA antagonists are more potent in experimental models of neuropathic pain (Székely, Torok, & Mate, 2002).

In low doses glutamate receptor antagonists have been observed to elevate the threshold of the physiological pain sensation. This leads to suppression of pathological sensitisation i.e. lowering of the pain threshold seen upon excessive or lasting stimulation of C-fiber afferents. Both NMDA and AMPA/kainate receptors inhibit wind up i.e. lasting activation of the polymodal, second-order sensory neurones in the deeper layers of the dorsal horn. During sensitisation the resting Mg^{++} blockade of transmembrane Ca^{2+} channels is abolished, certain second messenger pathways are activated, the transcription of many genes is enhanced leading to overproduction of glutamate and other excitatory neurotransmitters and expression of Na^{+} channels in the primary sensory neurons activated at lower level of depolarisation. This cascade of events can lead to increased excitability of the pain pathways (Szekely, 2002).

AMPA receptors are transported to the central terminals of DRGs and are present in different DH laminae. The AMPA receptors are most prominently located near central terminals of primary afferent fibers. This is unusual for ionotropic receptors on DRG neurons since most other families of ionotropic receptor are found both at the terminals and on the cell bodies (C. J. Lee ., 2002). The AMPA receptors are expressed by small diameter dorsal root ganglion neurons (C. J. Lee ., 2002) and these neurons are involved in nociception. These receptors are detected at the level of the soma and are strongly expressed near the primary afferent central terminals. Glutamate is released from the terminals of these central primary afferents, upon nociceptor stimulation, onto the dorsal horn neurons. Prolonged stimulation of nociceptors, such as after nerve damage, can lead to the continuous release of glutamate.

Activation of presynaptic AMPA receptors inhibits the release of glutamate from the terminals which suggests that primary afferent depolarization is also mediated by glutamate acting on presynaptically localized AMPA receptors (C. J. Lee, 2002). Thus AMPA receptors on primary afferent central terminals mediate primary afferent depolarization and modulate glutamate release.

Glutamate released from central terminals of primary afferents into the spinal cord in response to nociceptor stimulation primarily activates the AMPA receptors on dorsal horn neurons. Prolonged stimulation of nociceptors that often occurs after nerve damage can lead to a continuous release of glutamate that can cause long-lasting depolarization of the nerve membrane and consequently activation of NMDA receptors by glutamate (Chizh, 2002).

The AMPA receptors play a very important role in presynaptic inhibition. The mechanism that drives AMPA receptor-mediated presynaptic inhibition at the primary afferent to dorsal horn neuron synapse is probably primary afferent

depolarization (PAD) (Rudomin & Schmidt, 1999) and is accompanied by an enhanced excitability of afferent fiber terminals. This is a slow negative potential and when it reaches firing threshold it produces dorsal root reflexes (DRRs) and is also implicated in generating neurogenic inflammation (C. J. Lee ., 2002; Lobanov & Peng, 2011; Willis, 1999). It tempers the flow of information from the peripheral nervous system to the central nervous system.

Besides, $GABA_A$ receptors, evidence now suggests that ionotropic glutamate receptors at the presynaptic terminals of afferent fibers also contribute to PAD (Rudomin & Schmidt, 1999). The expression of AMPA and kainate receptors by nociceptors makes it plausible to predict that these receptors participate in activity-dependent generation of PAD in Aδ and C fibers. The mechanism can be blocked by $GABA_A$ and non-NMDA glutamate receptor antagonists (Evans & Long, 1989; Willis, 1999). Directly activating these presynaptic AMPA receptors results in a strong inhibition action on the amount of glutamate released from some primary afferent terminals in the superficial dorsal horn of the spinal cord.

The pain system in the spinal cord is phylogenetically primitive and exhibits behavioral changes such as hyperalgesia in response to inflammation. The use of AMPA receptor antagonists has been linked to suppressing inflammation-induced structural changes in the dendrites in the spinal dorsal horn(Matsumura, Taniguchi, Nishida, Nakatsuka, & Ito, 2015).

Glutamate signalling through the AMPA receptors plays important roles in central pain circuits. The subunits of AMPA receptors are dynamic at synaptic sites and trafficking to the plasma membrane has been implicated in pathological conditions. The protein product of Gria1 and its accumulation has been hinted at playing a role in sensory modulation at the level of the spinal cord. Pharmacologically induced down regulation of the Gria1 receptor in the spinal dorsal

horn can help ameliorate hyperaglesia (Y. Z. Li ., 2014). Pharmacological compounds such as AMPAkines have been linked to help alleviate pain hypersensitivity in rat models of persistent neuropathic pain and inflammatory associated pain (Le, Lee, Su, Zou, & Wang, 2014).

Several studies suggest that receptor encoded for by Gria1 may play distinct roles in the regulation of AMPA receptor trafficking and synaptic plasticity (Toyoda, 2009). The Gria1 receptor is required for NMDA receptor-dependent synaptic delivery of AMPA receptors in LTP (Yang, Wang, Frerking, & Zhou, 2008). Long-term potentiation (LTP) in nociceptive spinal pathways also shares several features with hyperalgesia and has been proposed to be one of the cellular mechanisms of pain amplification in chronic pain states.

Previous studies have suggested that peripheral Gria1 receptors located at sensory nerve endings in the skin are involved in nociceptor activation and sensitization. Using an antibody that recognizes the three low-affinity kainite receptor subunits Gria1/2/3 that one or more of these receptor subunits is likely expressed on 28% of unmyelinated fibers in the digital nerve from normal rats, and that this increases to roughly 40% acutely following Freund's adjuvant-induced inflammation. In addition to this, injection of kainate in the hindpaw induced acute mechanical hypersensitivity, and that an AMPA/Kainate receptor antagonist (CNQX) injected in the paw could reduce inflammation-induced mechanical hypersensitivity (J. Du, Zhou, & Carlton, 2006).

Gria1 containing AMPA receptors are thought to be involved in the regulation of synaptic strength. The Gria1 containing receptors are also the predominant type of ionotropic glutamate receptor expressed by the small-diameter sensory neurons in dorsal root ganglia (Qiu ., 2011).

There is a pool of AMPA receptors that recycle rapidly between the plasma membrane and intracellular vesicles, the processes of exocytosis and endocytosis have important roles in transporting these receptors to and from synapses. Studies on subunit specific AMPA receptor transportation routes suggest Gria2/3 subunit containing receptors are delivered by direct insertion and are continuously recycled within the synapse. This is important to determine basal synaptic strength. Gria1/2 subunit containing receptors, on the other hand, are thought to initially insert into the extrasynaptic membrane and then move laterally into the postsynaptic density depending on the activity pattern of the synapse (Andrásfalvy, Smith, Borchardt, Sprengel, & Magee, 2003). AMPA receptor trafficking in complex and involves vesicular trafficking, lateral diffusion and protein-protein interactions to determine the distribution of AMPA receptors on the neuronal surface.

The pain pathway also involves the higher centers in the brain and includes one of the most significant parts; the acute cingulate cortex. Certain pathological conditions such as peripheral chronic inflammation or nerve injury is linked to a long term potentiation of excitatory synaptic transmission in the acute cingulate cortex. AMPA subunits are dynamic at synaptic sites and their trafficking onto the plasma membrane has shown to be important in synaptic potentiation such as long term potentiation (T. Chen, 2014). Long term potentiation (LTP) is a major form of synaptic plasticity and is linked to chronic pain (Sandkuhler, 2007). It results in a long term increase in synaptic strength and has two stages that depend on the duration and involvement of signal transduction; early and late. While the early phase lasts only for a few hours, the late phase involves protein synthesis and involves structural changes at synapses that can last the life span of the animal. LTP can be expressed pre- and/or postsynaptically. This means the synaptic strength can increase if the neurotransmitter release is enhanced or if the postsynaptic effects of the neurotransmitter are enhanced. Recent studies have

shown that LTP can also be induced in pain pathways and may contribute to hyperalgesia caused by inflammation, trauma or neuropathy. Induction of LTP at C fiber synapses also requires the activation of ionotropic glutamate receptors and the influx of calcium via the AMPA receptors (Hartmann ., 2004). When glutamate binds to the AMPA receptors, it allows sodium to flow into the postsynaptic cell and bring about depolarization. NMDA receptors which also play a role in LTP cannot open directly because at resting membrane potential these receptors are blocked by Mg^{2+} ions and only open when a depolarization from the AMPA receptor activation leads to repelling of the Mg^{2+} cation out into the extracellular space and the pore can then pass current. The NMDA receptors are permeable to both Na^{+} and Ca^{2+} and when Ca^{2+} that enters the cell it triggers the upregulation of AMPA receptors to the membrane, which results in a long-lasting increase in excitatory postsynaptic potential size underlying LTP. The calcium entry also phosphorylates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which phosphorylates AMPA receptors and increases their single-channel conductance. AMPA receptors increase in ratio to NMDA receptors after high-frequency stimulation. A nonsynaptic pool of AMPA receptors is present adjacent to the postsynaptic membrane and without the need for protein synthesis in early LTP, these receptors are rapidly trafficked into the postsynaptic membrane when the appropriate LTP-inducing stimulus arrives (Malinow, 2003). Gria1 has been implicated in playing a role in cortical plasticity and persist pain using the ERK-dependent signalling pathway.

During long term LTP, transcribed mRNA of AMPA receptors must reach the intended dendritic spine and can be transported even to synapses that did not receive the LTP-inducing stimuli. The neuronal soma can synthesize receptor mRNA in the nucleus, package it within a transport ribonucleoprotein complex (a subtype of an RNA complex), initiate transport, prevent translation during transport, and deliver the complex to the appropriate dendritic spine (Bramham & Wells,

2007). The synaptic tagging hypothesis partly explains how mRNA is trafficked to the appropriate dendritic spine and signalling the mRNA-RNP complex to dissociate and enter the dendritic spine (Martin & Kosik, 2002).

At the synaptic level, hyperalgesia and allodynia may be mimicked by changes in synaptic transmission in spinal cord dorsal horn or cortical synapses. For hyperalgesia, enhanced synaptic responses, mainly by postsynaptic modification of AMPA receptors, may directly contribute to enhanced sensory responses to stimuli. A synaptic amplifier of nociception has been identified at the synapses between primary afferent C-fibres, many of which are nociceptive, and neurons in the superficial dorsal horn of the spinal cord in rodents(R. Ruscheweyh, Wilder-Smith, Drdla, Liu, & Sandkuhler, 2011). In the case of allodynia, the recruitment of 'silent' synapses, or increases in synaptic AMPA receptors may explain why subthreshold sensory stimulation can activate nociceptive neurons.

Central sensitization is an enhanced responsiveness of nociceptive neurons in the CNS to their normal afferent input. Some forms of central sensitization could contribute to hyperalgesia and/or allodynia (Sandkuhler, 2007). Initiation and maintenance of central sensitization is a consequence of evoked or spontaneous and this is suggested by the ability of transient local anesthesia to end hyperalgesia in animal models of central sensitization (Reichling & Levine, 2009) Sensitization and its role in neuropathic pain can be understood at some fundamental mechanism such as increased release of excitatory neurotransmitter (e.g., glutamate) and/or enhanced synaptic efficacy. These mechanisms may relate to several cellular events that are divided into changes that are at the presynaptic, postsynaptic changes and interneuron level. There is also evidence of sensitization being subject to changes in descending modulation of pain (Campbell & Meyer, 2006)

Summation determines whether or not an action potential will be triggered by the combined effects of postsynaptic potentials. Spatial summation requires the input from multiple presynaptic cells while temporal summation is when a high frequency of action potentials in the presynaptic neuron elicits postsynaptic potentials that overlap and summate with each other. In terms of nociceptive stimulation, spatial summation integrates painful input from large areas while temporal summation integrates repetitive nociceptive stimuli. Widespread and long lasting pain are characteristics of many chronic pain syndromes such as diabetic neuropathy and this suggests that both types of summation could play a role. Through pressure stimulation experiments, it has been shown that spatial summation facilitates temporal summation of nociceptive inputs, specifically pressure pain. The AMPA receptor-mediated excitatory postsynaptic potentials (EPSPs) have a half-time of less than 10 ms (Swanson & Sakai, 2009). This allows the receptors to be available for re-activation during periods of relatively high frequency input. The ionotropic glutamate receptors respond to both spatial and temporal summation.

Along with the presynaptic kainate receptors, AMPA receptors should prove to be an important regulator of synaptic transmission in the pain pathway as well as an important modulator of non-noxious sensory transmission.

Activation of presynaptic ionotropic glutamate receptors may reduce neurotransmitter release. As autoreceptors in terminals of Aδ and C afferent fibers in the spinal cord laminae I–III, presynaptic ionotropic glutamate receptors may play a role in inhibiting nociception. As heteroreceptors in GABAergic terminals in the same laminae, on the other hand, presynaptic ionotropic glutamate receptors may have an opposite role and contribute to central sensitization and hyperalgesia (Lu ., 2005).

4.6.2 NMDA Ionotropic Glutamate Receptors and Neuropathic Pain

In the present study, a marked and highly significant reduction in Grin2A expression was seen at all levels of the DRG. In addition there was a small reduction in expression of Grin1 throughout the DRG which reached statistical significance in TDRG 1-4, LDRG and SDRG. These reductions in expression may reflect adaptive changes that protect the neurons from excitotoxicity and overstimulation.

In neurons, alterations in the level of intracellular calcium regulates electrical activity, neuropeptide secretion, synaptic transmission, and gene expression. The sensory neurons located in the dorsal root ganglia express numerous voltage-gated and ligand-gated calcium channels important in transducing nociceptive signals from the periphery to the spinal cord. Studies point towards a prolonged, small increase in basal $[Ca^{2+}]_i$ that exists in the DRG neurons of diabetic animal and a reduction in the Ca^{2+} dynamics of diabetic animals (Biessels, ter Laak, Hamers, & Gispen, 2002).

Glutamate is released simultaneously with substance P (SP) and calcitonin gene related peptide (CGRP) since they are stored in the same terminals. The wind up in sensitization could be because of the concerted actions of glutamate with neuropeptides such as substance P and CGRP. The short-lasting excitation induced by glutamate could be extended and amplified by the co-released peptides. The EPSPs induced by them lead to enough depolarisation to abolish the voltage dependent Mg^{++} blockade of NMDA receptor controlled channels. These channels upon opening allow Ca^{2+} influx into the cell, activating intracellular biochemical cascades that lead to long lasting depolarisation of the postsynaptic cell membrane i.e. hyperexcitability (Szekely, 2002).

The NMDA receptors deserve particular attention in the study of neuropathic pain due to their crucial roles in excitatory synaptic transmission, plasticity, and

neurodegeneration in the nervous system. NMDA receptors have certain unique properties that distinguish them from other ligand-gated ion channels. These receptors controls a cation channel that is highly permeable to monovalent ions and calcium. Second, simultaneous binding of glutamate and glycine, the coagonist, is required for efficient activation of NMDA receptors. Third, at resting membrane potential the NMDA receptor channels are blocked by extracellular magnesium and open only on simultaneous depolarization and agonist binding.

The NMDA receptors are regulated by protein kinases as well and protein kinase C mediated channel phosphorylation can either enhance or suppress the activity of NMDA receptors depending on the Grin2 subunits and the Grin1 splice variants involved. Phosphorylation can alter the ion channel kinetics or alter the number of functional receptors on the cell surface by trafficking of channels in and out of the plasma membrane. The NMDA receptors are also able to couple to other signal transduction pathways such as PKC, calmodulin kinase II, nitric oxide synthase, and mitogen-activated protein kinases (MAP kinases) (Chaban, 2004). Experimental pain models have shown sustained MAPK activation in nociceptive primary sensory neurons located in dorsal root ganglia (DRG) (Reichling & Levine, 2009) and initial MAPK activation has been postulated to be involved in the initiation and maintenance of pathological pain. As the diabetic neuropathy progresses the decrease in MAPK phosphorylation observed in relevant tissues such as DRG from experimental neuropathic models can be viewed as a biochemical marker/phenotype of adaptive nociceptive signalling.

In the peripheral nervous system receptors NMDA receptors are expressed on the central and peripheral terminals of primary afferent neurons which are involved in nociception. Nerve injury can result in altered peptide expression in the dorsal root ganglia and in the dorsal horn of the spinal cord. The mRNA of different

ion channels can be downregulated at the nerve membrane and the dorsal root ganglion.

The situation that arises in pain experienced by diabetics is even more complex by the phenomenon of central sensitization if it is induced. Excitation of unmyelinated C fibers produces slow excitatory postsynaptic potentials that release glutamate which then acts on its receptors such as NMDA receptors. The NMDA receptor is an important receptor in the mechanisms of chronic neuropathic pain. The receptor consists of a Grin1/Grin2 heterodimer and physiologically the receptor and its associated ion channel is blocked my magnesium. When the membrane is depolarized and sensitized, magnesium is removed and different ions pass through that induce an intracellular second messenger and result in pain. The variability in the modulation of these neural processes has made it difficult for therapeutic interventions to be effective (Devulder & De Laat, 2000).

Studies show that nociceptor sensitization depends on glutamate release at the DRG and subsequent NMDA receptor activation in satellite glial cells (Ferrari, 2014) which supports the idea that peripheral hyperalgesia is an event that is modulated by the DRG glutamatergic system.

NMDA receptors containing Grin2A or Grin2B subunits contribute to most of the NMDA receptor currents (Zhuo, 2014). Physiologically, the expression of Grin2A expression increases with age (Shipton & Paulsen, 2014).

Evidence suggests that Grin1-containing NMDARs are localized synaptically while Grin2A-containing receptors are usually localized perisynaptically or extrasynaptically (Townsend, Yoshii, Mishina, & Constantine-Paton, 2003). Studies have shown that during over activity, calpain cleaves the Grin2 subunits of NMDA receptors that are on the cell surface. This serves to down regulate the function of

NMDA receptors which provides a neuroprotective mechanism against NMDA receptor overstimulation (Andrásfalvy, 2003).

Wind-up is a frequency-dependent increase in the excitability of spinal cord neurons that occurs due to electrical stimulation of C-fiber primary afferent nerves. The low-frequency stimulation of C fiber afferents leads to an increased response in the dorsal horn cell. This increase is excitability is due to temporal summation of slow postsynaptic potentials and spatial summation because of the convergence of the afferent fibers of different modalities on the same population of postsynaptic neurons. Windup lasts tens of seconds and represents a short-term form of sensitization. It amplifies the nociceptive message that arrives from peripheral nociceptors. The receiving neurons become consequently responsive to otherwise subthreshold stimulation induced by afferents that carry even non-nociceptive information. The fact that these second order multireceptive cells demonstrate wind up shows that the basis of this phenomenon is simultaneous activation of afferents of different modalities. However, not all the multireceptive neurons are sensitised, some actually become desensitised upon repeated stimulation and this has been shown by directly applying glutamate or kainate to the primary sensory neurons of the dorsal root ganglia (Szekely ., 2002).

Blocking NMDA receptors with ketamine or (2R)-amino-5-phosphonopentanoate can inhibit the wind-up phenomenon of spinal dorsal horn neurons and reduce the hypersensitivity of spinal dorsal horn neurons in neuropathic pain models (Dickenson & Sullivan, 1987).

In neuropathic pain, nociceptors with unmyelinated axons (C-nociceptors) are sensitized such that they discharge spontaneously, respond to normally innocuous stimuli, and have a supernormal response to noxious stimuli. Nociceptor sensitization underlies the spontaneous pain and hypersensitivity of the injured site.

The area surrounding the injured site also becomes a source of pain and hypersensitivity, and this is now known to be due, at least in part, to an NMDA receptor-mediated hyperexcitability in spinal neurons evoked by C-nociceptor input from the injured site. The chronic pain syndromes due to peripheral neuropathies are now believed to be related, in part, to a similar NMDA receptor-mediated hyperexcitability in spinal neurons. Axotomized C-nociceptors begin to discharge ectopically, and this has the same effect as the discharge of sensitized C-nociceptors it evokes NMDA receptor-mediated central hyperexcitability. NMDA receptor antagonists block neuropathic pain in animals, and early clinical trials suggest that they are effective drugs for human painful peripheral neuropathies. High levels of activity at NMDA receptors in the nervous system are known to lead to neuronal death or dysfunction. There is evidence that pain-evoked glutamate release is excitotoxic to small, presumed inhibitory interneurons, in the spinal dorsal horn.

Mitochondrial dysfunction, which is implicated in diabetic neuropathy, can lead to secondary excitotoxicity. Metabolic impairment depletes ATP, depresses Na^{+}/K^{+}-ATPase activity and can lead to graded neuronal depolarization. This relieves the voltage-dependent Mg^{2+} block of the NMDA receptor and allows apparently innocuous levels of glutamate to become toxic. Mitochondrial impairment also disrupts cellular calcium homeostasis. Increased activation of the NMDA receptor, in turn, leads to further mitochondrial impairment and damage. The calcium that enters a neuron through NMDA receptors now has can access to mitochondria and lead to free radical production and mitochondrial depolarization. This suggests the presence of a feed-forward cycle where the mitochondrial dysfunction activates NMDA receptor and to further mitochondrial impairment. In this scenario, NMDA receptor antagonists could possibly be neuroprotective (Ankarcrona, 1995)

4.6.3 Kainate Ionotropic Glutamate Receptors and Neuropathic Pain

In the present study, there was a highly significant and marked downregluation in expression of both Grik3 and Grik4 in all the dorsal root ganglia in both groups of animals versus their control counterparts. Furthermore, these reductions were seen at all levels of the dorsal root ganglia. Marked Grik3 downregulation was also seen in the thoracic sympathetic ganglia (TSG 1-6, TSG 7-12). However, no significant change was seen of Grik4 in these sympathetic ganglia. This consistent finding is in line with the possibility that downregulation of the genes expressing these receptors is an adaptive response. Adaptive responses are appropriate reactions to a cellular event and at the gene level, this is very important for the neuronal cell bodies to cope with sustained stress. Kainate receptor activation is linked to neuronal excitability and subsequent reduction in firing threshold and KA antagonists reduce mechanical allodynia and thermal hyperalgesia. The location of KARs in DRG neurons, specifically in neurons that give rise to thinly myelinated or unmyelinated Aδ and C fibers is highly suggestive of the role that these receptors play in pain. The presynaptic location of KARs also suggests a role in inhibition of neurotransmission. Since pre-synaptic KA receptors is also expressed on spinal cord inhibitory interneurons and activate inhibitory transmission, a downregulation in the production of KARs could further propagate the phenomenon of sensitization along the pain pathway and increase the perception of pain to the lightest stimuli. This is manifested as hyperalgesia and allodynia.

Kainate receptors are specifically localized on small to intermediate diameter DRG neurons that give rise to thinly myelinated or unmyelinated Aδ and C fibers including nociceptive fibers. This information paved the way to study the role of KA receptors in the transmission of nociception and the perception of pain. Behavioural

studies also show antinociceptive effects of specific KA receptor antagonists in models of persistent pain. SYM2081, a KA antagonist, reduces mechanical allodynia and thermal hyperalgesia in the chronic constriction injury model and cold injury model of neuropathic pain. LY293558 has been shown in human studies to reduce spontaneous pain and mechanical allodynia after capsaicin injection into the skin. The permeability to calcium of some KA receptors increases the intracellular concentration of calcium. This brings about reactions such as excitoxicity and synaptic plasticity.

The Kainate receptors show functional diversity from the subunit composition and also their association with auxiliary subunits such as the neuropilin and tolloid-like proteins.

Besides depolarizing neuronal membranes when glutamate binds, there is a second signalling mode unique to KARs and this is mediated by G protein-coupled activation of protein kinases. Although the metabolic pathways are not clearly understood it is known that this dual mode of signalling, ionotropic and metabotropic, plays a role in modulating neural circuits. Some of the mechanisms of this modulation arise from postsynaptic depolarizing of a subset of synapses, presynaptic modulation of glutamate and GABA releases as well as direct alteration of voltage gated ion channels that regulate action potential firing.

Kainate receptors located on the central endings of dorsal root ganglia neurons regulate glutamatergic transmission. Kainate receptors are located both postsynaptically (mainly mediating excitatory neurotransmission) and presynaptically (modulating excitatory and inhibitory transmissions). Pre-synaptic KA receptors expressed on spinal cord inhibitory interneurons activate inhibitory transmission (F. Wang, Stefano, & Kream, 2014).

Pain begins from a series of signalling events that occurs at multiple levels of the nervous system. Acute pain signals begin when the peripheral stimulation of A delta and C-fiber nociceptors are activated. These action potentials are propagated through the cell soma of the DRG neurons to the dorsal horn of the spinal cord, thalamus and higher order processing centers. This pathway also plays a role in analgesia through the stimulation of endogenous opiod receptors and modulating the inhibitory GABA tone on nociceptive dorsal horn neurons. This sensory transduction pathways allows nociceptors to respond to noxious stimuli and assists us in protecting ourselves. The perception of pain is altered by modulating the signal transduction in peripheral and nociceptive pathways. Persistent pain arises from pathologic increases in excitability, or sensitization, of one or more of the peripheral or central parts of pain pathways. Peripheral sensitization is due to a reduction in firing threshold and an increased responsiveness of peripheral nociceptors. This is linked to neurogenic factors such as inflammatory factors; calcitonin gene related peptide, substance P, ATP and serotonin. Central sensitization can be when the dorsal horn neurons in the spinal cord become hyperexcitable and this can be because of pre and postsynaptic changes and changes in the postsynaptic membrane excitability. Long-term alteration in neuronal excitability is linked to persistent pain and can be from adaptive changes in structure and function of several brain regions that are implicated in the higher processing of pain related input (Bhangoo & Swanson, 2013).

Activation of ionotropic glutamate receptors increases energy consumption because the resultant influx of Na^+ and Ca^{2+} requires energy to be pumped out of the cell again. Neurons become more vulnerable to glutamate after energy deprivation or after inhibition of the Na^+K^+-ATPase. Early mitochondrial damage plays a key role in induction of glutamate neurotoxicity and glucose level fluctuations that result in hypoglycaemia and hyperglycaemia are also harmful (Novelli, Reilly,

Lysko, & Henneberry, 1988). Too high glucose levels also increase the neuronal vulnerability to ischemia (Kelleher, Chan, Chan, & Gregory, 1993). Glutamate receptor activation also enhances the rates of generation of reactive oxygen species and potentiates the toxic effects of H_2O_2 and of mutant superoxide dismutase.

Metabolic inhibition predisposes neurons to glutamate mediated damage and pathological conditions such as diabetic neuropathy can be associated with a hypersensitivity to glutamate due to for example impaired cellular energy metabolism or aberrant expression of glutamate receptors (J. G. Greene & Greenamyre, 1996). Several different primary events or changes may share a final common pathway.

Vicious cycles can ensue if the glutamatergic system malfunctions. High extracellular glutamate can activate glutamate receptors causing further glutamate release. Glutamate receptor activation increases energy consumption and free radical production which may impair energy production and glutamate uptake and lead to even reversing the transporters and causing even more glutamate to be released. The ion influx can lead to cell edema, ischemia and a compromised energy supply and so on.

4.6.4 Ionotropic Glutamate Receptor – Delta 2

Expression of Grid2 was significantly in TDRG 5-8 of the GK rats. The receptor that this gene codes for forms heteromeric assemblies with other types of iGluRs including AMPA and Kainate receptors to form functional channels. Since it requires the involvement of the types of iGluRs that are implicated in neuropathic pain, it is tempting to speculate the increased expression of this gene might also contribute to neuropathic pain.

4.7 The Role of Glutamate Transporters in Glutamate Toxicity

Some changes were seen in expression of the genes coding for glutamate transporter proteins. Slc1a1 expression was significantly increased in TDRG 1-4, 5-8, 9-12, LDRG and SDRG. We found the mRNA levels of Slc1a3 to be significantly upregulated in TDRG1-4, TDRG 5-8 and TSG1-6 of GK animals and TDRG 5-8, TDRG9-12 and SDRG of GK Sucrose animals. It was also significantly upregulated in TDRG5-8 of both GK and GK Sucrose animals. Expression of Slc1a2 was not altered. Slc1a6 was downregulated in TDRG 1-4, 5-8, 9-12 in GK animals and in TDRG 1-4 and LDRG of GK Sucrose.

Besides the essential glutamate removal role, the glutamate transporters also play a role in neurotransmission modulation. These transporters modify the time course of synaptic events, the extent and pattern of activation and desensitization of receptors outside the synaptic cleft and at neighboring synapses (intersynaptic cross-talk). The glutamate transporters provide glutamate for synthesis of important molecules such as GABA and glutathione. Glutamate uptake can be modulated on all possible levels i.e. DNA transcription, mRNA splicing and degradation, protein synthesis and targeting, and actual amino acid transport activity and associated ion channel activities. A variety of molecules besides glutamate (such as cytokines and growth factors) also influence glutamate transporter expression and activities.

A decrease in expression of glutamate transporters would lead to an extracellular build-up of glutamate which would, via glutamate receptors, increase Na^+ and Ca^{2+} influx, energy consumption and free radical production.

However, extracellular glutamate overload occurs after CNS traumatic injury and causes excessive Ca^{2+} influx into neurons through overactivation of neuronal ionotropic glutamate receptors, leading to neuronal excitotoxicity. Additionally,

numerous glutamate-induced CNS neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), epilepsy, ischemia and traumatic brain injury, have been reported to associate with dysfunction of astrocytic glutamate transporters. Therefore, the function of glutamate transporters, especially astrocytic glutamate transporters especially the astrocytic glutamate transporters (glutamate aspartate transporter; GLAST/EAAT-1) and glutamate transporter-1 (GLT-1/EATT-2) is of great importance for protecting neurons from glutamate-induced excitotoxicity.

Studies demonstrate that rat peripheral sensory terminals and dorsal root ganglia (DRG) express molecules which are involved in glutamate signalling, including high-affinity membrane-bound glutamate transporters Slc1a1 (EAAC1, EAAT3), Slc1a2 (GLT-1, EAAT2), Slc1a3 (EAAT1, GLAST) and Slc1a6 (EAAT4)(Berger & Hediger, 2000). Evidence suggests in DRG, satellite cells express Slc1a2 whereas sensory neurons were positive for Slc1a1. Slc1a3 is localized in both neurons and satellite cells (V. A. Carozzi, 2008). EAAC1, GLAST, and GLT-1 are particularly relevant for regulating glutamate uptake in many of the central nervous system regions including the spinal cord (Danbolt, 2001).

Transporters located inside the synaptic cleft directly affect the glutamate concentration there and transporters located outside the cleft, such as on glia or non-synaptic parts of neurons indirectly affect glutamate concentrations by reducing or increasing the amount of extrasynaptic glutamate that can diffuse back into the synaptic clefts.

Defects in glutamate uptake due to alterations in the function and/or in the expression of these transporters have been implicated in several neurologic and neurodegenerative disorders in the central nervous system (V. Carozzi, 2011) but there are not extensive studies about the uptake activity of the glutamate receptors in the peripheral nervous system in diabetic neuropathy.

Depending on whether transporters are located in relation to the synapse, these transporters either take up glutamate to concentrate into presynaptic vesicles or recycle glutamate through intracellular metabolic reactions to return back to the neurons (nerve endings) in the form of a precursor such as glutamine. The transport of glutamate and precursors of glutamate across plasma membranes is a strategically positioned mechanism to terminate glutamate and also be part of its recycling. Several protein molecules act as glutamate transporters and regulate the function of the glutamatergic synapses. Glutamatergic neurotransmission can be weakened if glutamate is removed/inactivated before it can adequately interact with its postsynaptic receptors. Thus, hyperactive glutamate transport can contribute to temper excessive glutamate neurotransmission. The expression of glutamate transporters is influenced by a number of neuropeptides and growth factors (Figiel, Maucher, Rozyczka, Bayatti, & Engele, 2003).

Alterations in the expression and/or functionality of glutamate transporters have been implicated in several models of peripheral neuropathy, neuropathic pain and hyperalgesia. Thus the effects of altered expression and glutamate uptake activity of glutamate transporters in dorsal root ganglia is likely to be mediated via changes in regional glutamate homeostasis. Evidence demonstrates that functionally active glutamate transporters can be studied in dorsal root ganglia and help to further understand the role of glutamatergic transport in the peripheral nervous system (V. Carozzi ., 2011).

Under physiological conditions, the extracellular concentration of glutamate strongly depends on its re-uptake into cells through specific transporters located in neurons such as Slc1a1 and on the membrane of glial cells Slc1a2 and Slc1a3 (V. A. Carozzi, 2008).

Slc1a1 is considered to be the predominant transporter in neural tissues outside the blood-brain barrier and in the peripheral nervous system. Slc1a1 expression has been observed in the satellite cells surrounding the ganglion neurons and these cells are the corollary to astrocytes in the peripheral nervous system. Expression of Slc1a1 in DRG has been localized in the nuclei and cytoplasm of sensory neurons (V. A. Carozzi, 2008). The function of Slc1a1 expression by the dorsal root ganglia neurons is likely to take up the glutamate that is released from glutamatergic nerve terminals innervating the ganglion neurons (Berger & Hediger, 2000)

Previous studies have demonstrated Slc1a3 mRNA was expressed by satellite cells in DRG (Berger & Hediger, 2000).

The overactivity of glutamate transporters has been implicated in the pathophysiology of schizophrenia and other mental illnesses, whilst underactivity is seen in ischemia and traumatic brain injury. Several mechanisms could contribute to increased expression of the glutamate transporters. The pathological release of glutamate from primary afferents may serve as a positive feedback on glutamate transporter expression. Expression of neurotrophic factors in dorsal root ganglia and spinal cord is also upregulated dramatically under pathological conditions. Neurotrophic factors such as Brain derived neurotrophic factor which modulate N-methyl-D-aspartate (NMDA)-evoked responses and nociceptive sensory inputs in the spinal cord (Ha, Kim, Hong, Kim, & Cho, 2001).

Glial cells that express these transporters are found between neighboring synapses and may prevent the released glutamate from activating receptors at neighboring synapses (cross-talk). However a pathological increase in the density of glutamate transporters can give rise to paradoxical effects. A high density of transporters on glial cells can actually trap the glutamate and prevent it from leaving

the cleft and allow glutamate the opportunity to re-enter the cleft upon unbinding from these transporters. The transporters can also mediate release of glutamate via reversed uptake (Danbolt, 2001). Reversed uptake is when dissipation of the ion gradients that are driving force for the uptake causes the high intracellular concentration of glutamate to drive the transporters backwards releasing glutamate. This has been observed in ischemia (Danbolt, 2001). So though glutamate binding to the transporter reduces the extracellular concentration of free glutamate, the binding also slows down the diffusion of glutamate away from the release site.

Glutamate escaping from the synaptic cleft may then reach and activate presynaptic receptors thus modulating its own release, this suggests an indirect involvement of glutamate transporters because of they control the amount of glutamate that can access the presynaptic receptors. Glutamate bound to GLAST has a higher probability of unbinding than of being transported but it is the opposite case for glutamate bound to GLT and EAAT4. Thus, the paradoxical effects may be more important in those locations where GLAST rather than GLT-1 is the predominant transporter (Danbolt, 2001).

Before being released into the synaptic cleft, cytosolic glutamate first need cross the vesicular membrane via the activity of vesicular glutamate transporters (VGLUTs). These transporters also belong to the family of glutamate transporters and their expression is up-regulated in the DRG neurons after peripheral nerve injury and evidence suggests that there is an increase in glutamate release by VGLUT up-regulation that contributes to the elevated glutamatergic transmission (Moechars, 2006).

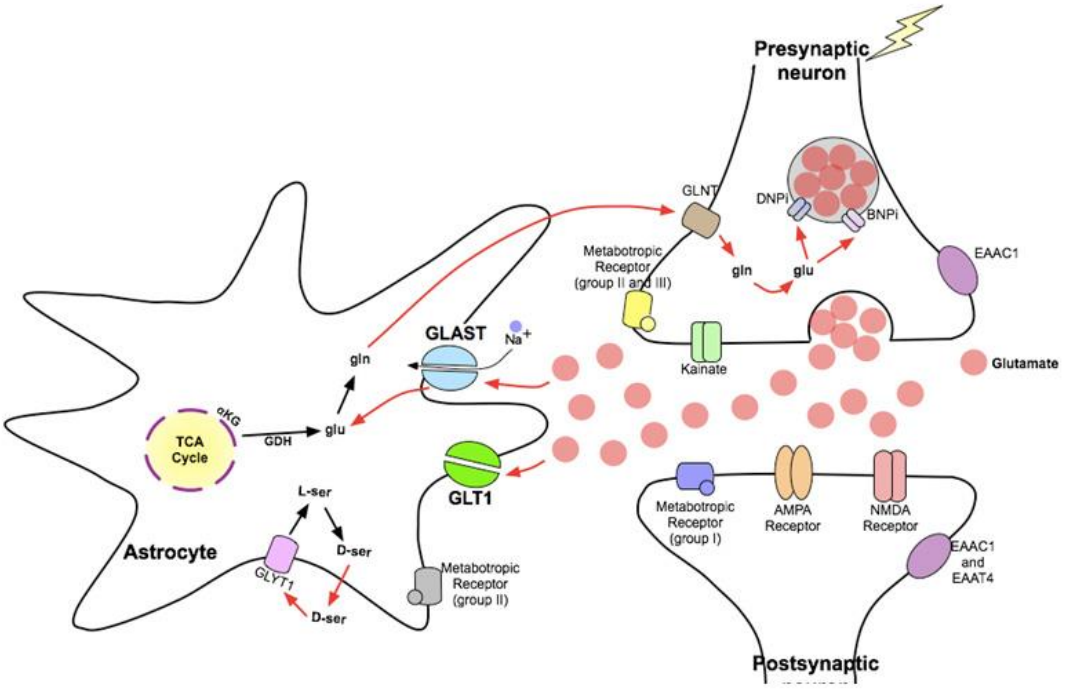

Figure 37: Glutamate transporters in astrocytes are responsible for synaptic glutamate homeostasis.

Modified from (Y. P. Liu, Yang, & Tzeng, 2008)

4.8 The Role of Enzymes Involved in the Glutamate Cycle and Glutamate Receptor Interacting Proteins in Diabetic Neuropathy

We found the mRNA expression of Grip1 to be significantly decreased in TDRG1-4 of GK and also in the LDRG and TSG1-6 of both GK and GK Sucrose animals in comparison to control.

Since a decrease in interactions of Grip1 with its respective AMPA receptors could reduce sensitization, these findings suggest a compensatory mechanism is in place to help reduce the increase in neuronal sensitivity and hyperexcitability that manifests as some of the symptoms of diabetic neuropathy.

Glutamate is toxic in increased concentrations because of how important it is. Since it is the main mediator of excitatory signals and also involved in nervous

system plasticity, including cell elimination, it follows that glutamate should be present at the correct concentrations in the right places and at the right time. Cells should have the correct sensitivity to glutamate and be able to withstand normal stimulation, and that glutamate should be removed with the right speed from the right locations.

Glutamatergic neurotransmission is not an independent process. It regulates other processes and is regulated by other systems including itself. Many, if not all, structures that are important for glutamatergic neurotransmission such as cells, synapses and dendritic spines are dynamic and subject to regulation. In pathological situations, a change in one area of the nervous system often leads to changes at other sites. In consequence of this, the primary cause of a disease may induce several secondary changes. These changes can be part of adaptive responses that affect the progression of the disease and the compensatory alterations try to counteract the primary defect or changes. The secondary changes themselves can aggravate the condition and thereby become problems in themselves.

Glutamine expression showed some minor changes which were statistically significant. However, since these are not reproduced between the GK group and the GK-Sucrose group and because these changes are generally very minor these are probably of little biological importance. Glutamine is recycled form the glial cell back to the presynaptic nerve terminal where the enzyme glutaminase converts it to glutamate for packaging into secretory granules ready to be released at the synapse (Bradford, 1978). The astrocytic export of glutamine implies a continuous loss of alpha ketoglutarate from the astrocytic TCA cycle but this is not the case. The intermediates of the TCA cycle have restricted access across the blood-brain barrier and so the anaplerotic process of pyruvate carboxylation continually replenishes the loss. Anaplerosis is the process of converting pyruvate, which is derived from

glucose via glycolysis, to oxaloacetate or malate. Astrocytes express the enzymes pyruvate carboxylase and cytosolic and mitochondrial malic enzyme (Kurz, 1993; Salganicoff & Koeppe, 1968; A. C. Yu, 1983). Observations that neurons and subpopulations of neurons have the ability to replenish their TCA cycle by carboxylating pyruvate can explain why some glutamatergic pathways have a low level of glutaminase, whereas others have high levels, and it explains how transmitter glutamate can be formed from neuronal precursors (Hassel & Brathe, 2000a, 2000b). Neurons can thus carboxylate pyruvate and not completely depend on glutamine as a precursor for transmitter glutamate.

The glutamine cycle, the 1:1 exchange between astrocytes and neurons of glutamine for glutamate and GABA was conceptualized in the 1970s (van den Berg & Garfinkel, 1971). In this cycle, astrocytes take up glutamate released from neurons, convert it to glutamine in an energy dependent process and then supply glutamine to the extracellular fluid to maintain a high concentration of glutamine which is approximately 0.3mM. Neurons consume glutamine and convert it to glutamate by glutaminase.

In dorsal root ganglia glutamine and glutamine related enzymes are enriched in the satellite cells that surround the neurons. In the peripheral nervous system, the glial tricarboxylic and glutamine cycles produces glutamine for neuronal cell uptake and conversion to glutamate for synaptic transmission. These cycles may also function in peripheral glia similar to central nervous system astrocytes in terms of providing support for the energy demands of the neurons (Miller, Richards, & Kriebel, 2002).

The glutamine cycle is often described occurring at the synaptic terminal but evidence suggests that glutamine is also taken up and converted to glutamate for synaptic use in the cell bodies and axons of DRG neurons. Glutamine and related

enzymes in the satellite cells of the DRG may facilitate glutamate production in DRG neurons for synaptic transmission in the spinal dorsal horn (Johnson, 1974).

There is a trend for reduction in expression of GAD2 in the DRG which reaches statistical significant in TDRG 1-4, 9-12 of GK group and in TDRG 5-9 and LDRG in the GK-Sucrose group. The decrease in mRNA expression may be due to a reduced transcription/translation of GAD65. The consequence of this would be a decrease in GABA concentrations and could contribute to neuropathic pain.

Glutamic acid decarboxylase (GAD) is an enzyme that catalyzes the decarboxylation of glutamate to GABA and CO_2. GAD exists in two isoforms encoded by two different genes - GAD1 and GAD2. These isoforms are GAD67 and GAD65 and have molecular weights of 67 and 65 kDa, respectively (Erlander, Tillakaratne, Feldblum, Patel, & Tobin, 1991). GAD65 and GAD67 synthesize GABA at different locations in the cell, at different times during development and for functionally different reasons. This explains why GAD67 is spread evenly throughout the cell while GAD65 is localized to nerve terminals. GAD67 synthesizes cytosolic GABA for neuron activity that is not related to neurotransmission such as synaptogenesis and protection from neural injury. GAD65, however, synthesizes a synaptic GABA for vesicular release and neurotransmission and would be required at nerve terminals and synapses. Post translation regulation of Gad65 involves activation by phosphorylation and thus regulated by protein kinase C synapses(Pinal & Tobin, 1998).

Gamma-aminobutyric acid (GABA), the product by glutamic acid decarboxylase (GAD), is a principal inhibitory neurotransmitter and plays a crucial role in modulating synaptic circuits. The loss of GAD65-producing GABAergic neurons in the dorsal horn of the spinal cord that results in GABA hypo-function has been well documented in various pain models. The exogenous introduction of the

GAD gene to the dorsal root ganglia (DRG) in genetic therapy has been shown to increase GABA concentrations in the spinal dorsal horn and helped to reduce symptoms of pain (J. Kim, Kim, Lee, & Chang, 2009; B. Lee, Kim, Kim, Lee, & Chang, 2007).

Transfecting DRG neurons with vectors that express GAD65 and GAD67 have been found to reduce diabetes-induced mechanical allodynia more effectively than gabapentin. Studies suggest that either GAD65 or GAD67 vectors are the most effective in the treatment of diabetic pain (Y. Wang, 2013).

We observed an increase in the mRNA expression of Gclc in the TDRG1-4 and 5-8, LDRG and SDRG in both GK and GK Sucrose rats compared to controls. This would be expected if there was an increase in production of this enzyme to counteract the oxidative stress that is implicated in diabetic neuropathy.

Clinical and experimental studies have documented that chronic complications, including diabetic neuropathy, persist and progress even with good glycaemic control. Hyperglycaemia induced oxidative stress has been implicated in diabetic neuropathy (Andrea M Vincent ., 2004). Glutamate cysteine ligase catalyzes the first and rate-limiting step in de novo glutathione synthesis and the regulation of expression and activity of Gclc is critical for glutathione homeostasis(Meister, 1983). This enzyme is responsible for maintaining homeostasis of the intracellular antioxidant status. Mitochondria are vulnerable to damage by reactive oxygen and nitrogen species. Reactive oxygen species are produced by the mitochondria and usually removed by cellular agents such as glutathione, catalase and superoxide dismutase. Hyperglycaemia accentuates ROS production in the cell via mitochondrial activity (Friederich, 2009; Leinninger, 2006; Andrea M Vincent, 2004).

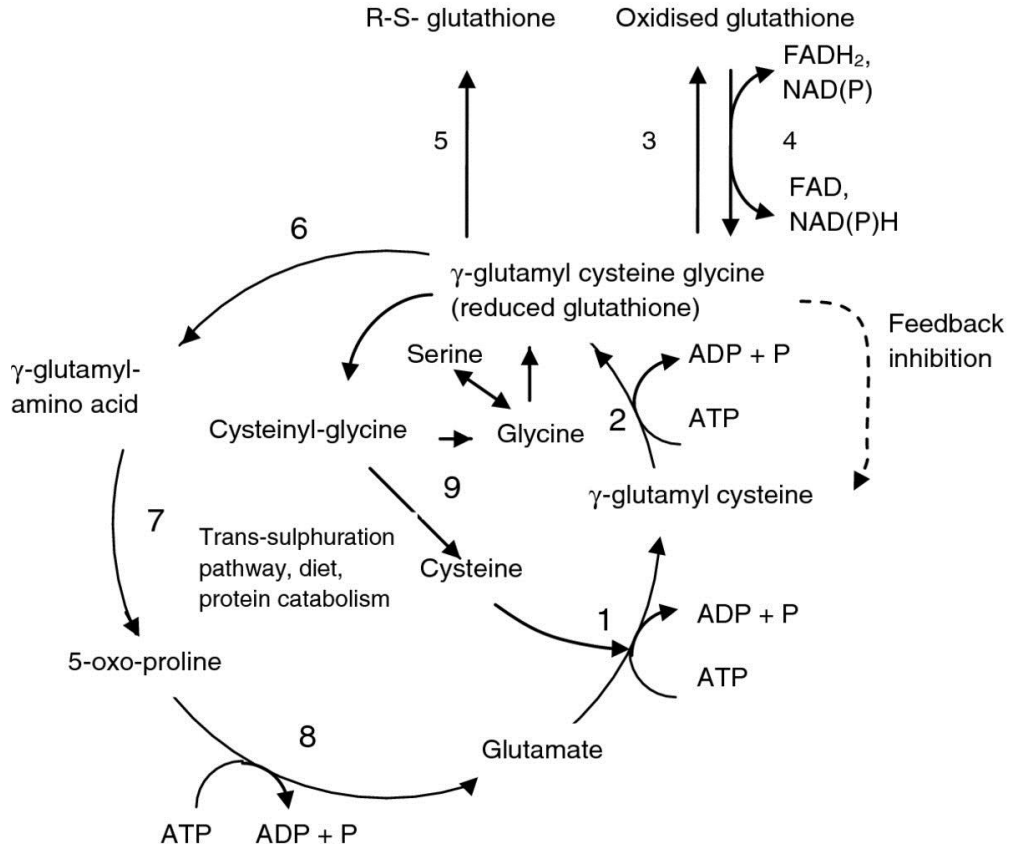

Figure 38: The gamma glutamyl cycle.

(γ-Glutamyl cycle, 1 Glutamate cysteine ligase (GCL), 2 Glutathione synthetase (GS), 3 Glutathione peroxidase, 4 Glutathione reductase, 5 Glutathione-S-transferases (detoxification reactions), 6 γ-glutamyl-transferase (GGT), 7 γ-glutamyl cyclotransferase (GCT), 8 5-Oxoprolinase, 9 Dipeptidase, R Protein, FAD, FADH2 Flavin-adenine dinucleotide, NAD, NAD+ Nicotinamide-adenoside dinucleotide, NADP, NADPH+ Nicotinamide-adenoside dinucleotide phosphate, ADP, ATP Adenosine diphosphate, Adenosine triphosphate(Main, Angley, O'Doherty, Thomas, & Fenech, 2012).

Glutaminase (Gclc or GLS) is an enzyme that generates glutamate from glutamine. The enzyme plays an important role in glial cells. The glutamate released into the synapse for neurotransmission is rapidly taken up by nearby glial cells and converted to glutamine. This glutamine is supplied to the presynaptic terminals of

the neurons, where glutaminase converts it back to glutamate for loading into synaptic vesicles. GLS is also expressed in the brain, spinal cord, and dorsal root ganglia neurons.

We observed a decrease in mRNA levels of Gls in TDRG9-12 in GK and in LDRG and TSG1-6 in the GK Sucrose animals compared to control rats. Decrease levels of mRNA of Gls from the cell body can translate into decreased amounts of Gls produced from the DRG cell bodies to axons. This could be a compensatory mechanism to counteract the pathological levels of glutamate implicated in neuronal hyperexcitability and central sensitization. We postulate that a decrease in the amount of GLS during chronic diabetic neuropathy leads to decreased production and release of glutamate. Decreased production and release of these substances could adaptive mechanisms to ameliorate neuronal hypersensitivity that contributes towards many of the symptoms of diabetic neuropathy. A decrease in GLS production could affect the peripheral terminals as well because glutamate that is released from the peripheral afferents that also have glutamate receptors. A decrease in glutamate production and release could reduce the activation of these terminals to compensate for the sensitization of primary afferents. A cycle of decrease glutamate production and release, decrease in numbers of axons with glutamate receptor expression on axons could lead to a decrease in sensitization of peripheral nerve terminals and help to ameliorate the process of chronic pain and hyperaglesia that is prevalent in diabetic neuropathy. Glutaminase inhibition has been postulated to represent an ‘upstream’ mechanism of glutamate regulation that could reduce transmission at all glutamatergic receptors and antagonists for this enzyme have been been shown to provide analgesia which further supports the theory that a reduction in glutamate production by inhibiting glutaminase enzyme activity might provide pain relief in neuropathic pain (Hoffman & Miller, 2010).

Glutamate carboxypeptidse II (N-acetylasparatylglutamate peptidase) is a membrane bound metalloenyzme that cleaves the abundant neuropeptide NAAG to N-acetylaspartate (NAA) and glutamate. GCP II is abundant in the spinal cord and DRG. NAAG is one of the most common peptide transmitters in the brain and is also a type 3 metabotropic glutamate receptor (mGluR3) agonist. GCP II inhibitors increase extracellular NAAG, decrease glutamate and have been implicated in reducing excess glutamate transmission in pathological conditions such as ischemia/stroke, motoneuron disease, brain and spinal cord injury and peripheral neuropathy.

It showed that daily oral intake of the drug protected against the sensory and motor loss and neurodegeneration that plauges neuropathy. Administering GCP II inhibitor improved the locomotor, sensory and thermal sensitivity deficits. GCP II inhibition also protects against neurodegeneration. The mechanism could be by reducing excitoxicity that is induced by excess glutamate concentrations. GCP II inhibition also increases NAAG levels. NAAG partially antagonizes NMDA receptors and could also provide neuroprotection. GCP II inhibition has also been protective in diabetic and chemotherapy induced neuropathy. GCP II inhibitors have been well tolerated in humans and appears to affect increased glutamate concentration levels rather than baseline levels(Potter, Wozniak, Callizot, & Slusher, 2014).

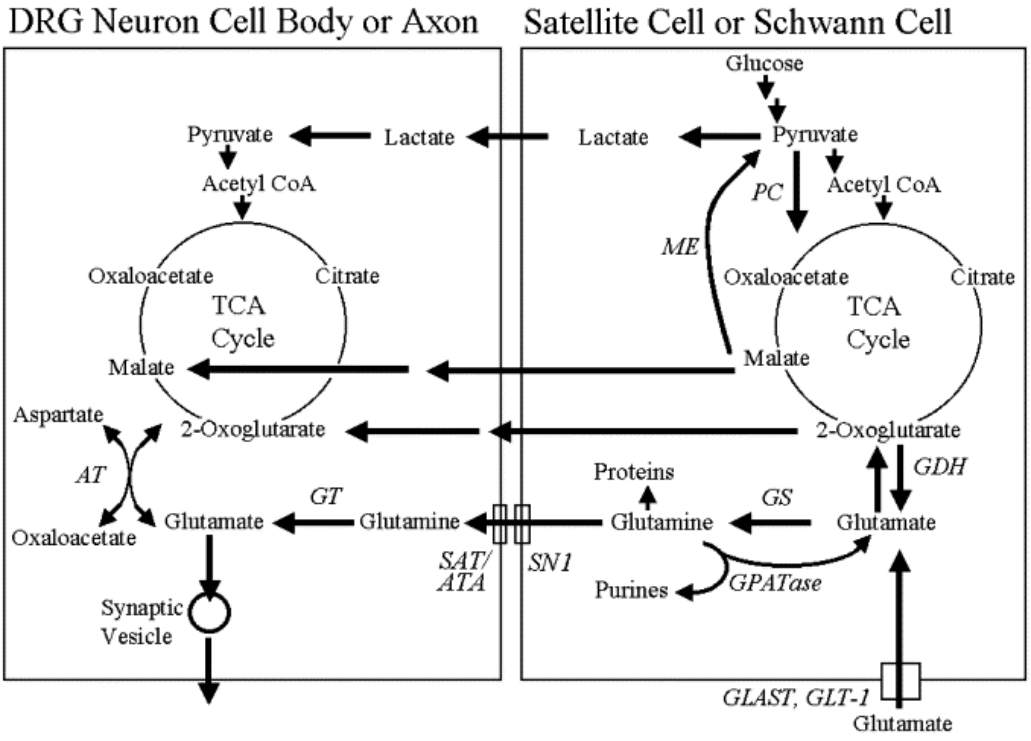

Figure 39: Glial cell metabolism is intricately related to neuronal metabolism.

This diagram illustrates that glutamine, glutamine synthetase, glutamate dehydrogenase, and pyruvate carboxylase are located in the peripheral nervous system in satellite cells of the DRG and Schwann cells of the peripheral nerve. These enzymes have major roles in supporting peripheral neuronal metabolism and neurotransmission. Glial cells take up glutamate from the extracellular milieu via transporters (GLAST, GLT-1) and glutamate synthetase converts it to glutamine. Glutamine can be shuttled out of glial cells by the SN1 glutamine transporter and taken up by neurons via the SAT/ATA glutamine transporters for use by glutaminase (GT) in the glutamine cycle. In addition, glutamine is an important branch point substrate for purine synthesis via GPATase. Glutamate dehydrogenase is a bidirectional enzyme that can either add glutamate for GS in the glutamine cycle or convert glutamate to 2-oxoglutarate for the TCA cycle. 2-Oxoglutarate and other TCA intermediates such as malate can be shuttled from glia for use in neurons. Malate also can be converted to pyruvate via malic enzyme (ME). Pyruvate can be

converted to lactate and used in neuronal metabolism. Pyruvate carboxylase is an anaplerotic enzyme that refills the glial TCA cycle with carbon as TCA intermediates are used for other purposes (Miller, 2002).

The Grip1 gene encodes for a member of the glutamate receptor interacting protein family. This encoded scaffold protein binds to and mediates the trafficking and membrane organization of several transmembrane proteins. Prototypical scaffolding proteins such as the glutamate receptor interacting protein 1 (GRIP1) contain several PSD-95/Discs-large/ZO-1 homology (PDZ) domains operate as molecular adapters in neuronal synapses. The PDZ-containing scaffolds assemble specific proteins into large molecular complexes at defined locations in the cell and in the postsynaptic density of neuronal excitatory synapses organize glutamate receptors and their associated signalling proteins. This helps to determine the size and strength of synapses. The PDZ scaffolds also play a role in the dynamic trafficking of synaptic proteins by assembling cargo complexes that are then transported by molecular motors (E. Kim & Sheng, 2004).

GRIP1 has been implicated in playing a role in the synaptic clustering of AMPA receptors and unmasking silent glutamatergic synapses in somatosensory inputs (P. Li, 1999). Its role in neuropathic pain has been suggested by studies that showed reducing interactions of the Grip1 protein with the product of Gria2 (GluR2 AMPA receptor) has inhibited the neuropathic reflex sensitization. This suggests an involvement of Grip1, if expressed, in neuropathic pain (Garry, 2003).

Chapter 5: Conclusion

Diabetic neuropathy is the most common and debilitating complication of diabetes. It manifests itself as pain, decreased motility, and often results in amputation. Diabetic neuropathy encompasses a variety of forms whose impact ranges from discomfort to death.

Amongst the myriad of therapeutic options available, glucose control remains the single most important factor that can modify the disease (Leinninger, 2006). Despite the wide reaching implication of diabetic neuropathy when considering the number of people afflicted, the pathogenesis of diabetic neuropathy has many theories that surround it. Despite extensive investigations and development of multiple theories the mechanism involved in the development of diabetic neuropathy are still not completely understood. When several theories orbit a condition, it suggests that there is no unifying hypothesis.

The expression and regulation of genes gives the cell control over cellular structure and function. It is the basis for cellular differentiation, morphogenesis and is also crucial for the versatility and adaptability of any organism. The timing, location and amount of gene expression can have a profound effect on the functions of the gene. Analysing genomic and proteomic data from suitable animal models and diabetic neuropathy patients can provide insight into disease pathogenesis and possibly identify therapeutic targets for disease modifying treatments. Developing symptomatic and disease-modifying therapeutic interventions depends on a comprehensive and pertinent understanding of the pathogenesis and pathophysiology of diabetic neuropathy.

The genes that are involved in human pathology require study to improve the outcome of disease processes and reveal therapeutic targets. Since genes and

gene functions have been so highly conserved throughout evolution, the study of less complex model organisms reveals critical information about similar genes and processes in humans. The selection of appropriate animal models for gene expression studies is of the utmost importance. Animal models allow control in vivo the genetic and environmental factors that influence the development of diabetes and its complications. Rodents are preferable due to advantages such as short generation intervals, economic viability, ease of care and housing compared to larger models. In our study we included the Goto-Kakizaki (GK) and Goto-Kakizaki Sucrose (GK Sucrose) animal groups. The Goto-Kakizaki (GK) rat is a non-obese Wistar substrain which develops Type 2 diabetes mellitus early in life. The GK rat is one of the best characterized animal models of spontaneous T2DM and has proved to be a valuable tool offering sufficient commonalities to study this aspect.

Comprehensive studies of gene expression provide a layer of information that is useful for predicting gene function. Information about a gene's function can be deduced by identifying genes that share its expression pattern. Gene expression studies that focus on sensory and sympathetic neurons from different animal models of diabetic peripheral neuropathy and autonomic neuropathy will show differentially regulated transcripts. These expression profiles will allow data to define categories of genes that are functionally regulated in diabetic neuropathy such as genes involved in metabolism or nerve regeneration.

On a broad level it can help to gain greater knowledge about how individual mechanisms of the disease fit together as a whole and generate novel hypotheses concerning the pathogenesis, diagnosis and progression of diabetic neuropathy. This can also help pinpoint targets for disease regulation and therapeutic intervention. Relevant pathways related to functional gene categories can be pinpointed for new information of disease mechanisms and targets. Genes that are

differentially expressed between healthy and diseased tissue can be put into the context of cellular pathways and used to predict regulatory elements that control the changes observed. Those regulatory elements that are identified can be utilized later as drug targets and also used to predict the downstream effects of gene expression. These can also pave the way for developing biomarkers.

For expression profiling, or high-throughput analysis of many genes within a sample, quantitative PCR is a popular and reliable method to study hundreds of genes simultaneously. In preliminary studies, low density arrays revealed many interesting findings but it was not possible to follow all of these changes. Once a set of genes involved in a particular biological process has been identified, the next logical step is to further study the genes individually. Determining when a gene acts can facilitate the reconstruction of entire genetic or biochemical pathways, and such studies have been central to our understanding of pathological processes. The focus was honed to the genes involved in the glutamate signalling pathway because of there were several changes in the pathways of these genes. Genes for glutamate receptor expression, glutamate processing enzymes and glutamate transporter proteins showed significant changes in expression. This was also in light of the literature that indicated the glutamate pathway was involved in neuropathic pain including diabetic neuropathy.

The presence of excess glucose activates multiple biochemical pathways that lead to damage. Amongst the myriad of therapeutic options available, glucose control remains the single most important factor that can modify the disease (Leinninger, 2006). However, no therapeutic agent aids in eliminating the root cause of diabetic neuropathy. Understanding the root cause of neuropathy will illuminate the path towards therapeutic intervention at a stage when complications have not been fully established.

Observing patterns of changes in gene expression using different animal models of diabetes will help pave the way for further studies in individual gene analysis, protein characterization and functional studies.

Using informatics to analyze genomic and proteomic data from suitable animal models and DN patients can provide insight into disease pathogenesis and possibly identify therapeutic targets for disease modifying treatments. Developing symptomatic and disease-modifying therapeutic interventions depends on a comprehensive and pertinent understanding of the pathogenesis and pathophysiology of diabetic neuropathy.

The low density arrays showed several interesting results of which the most prominent changes were the genes that are involved in the glutamate pathway. Changes in gene expression were confirmed by fast real-time RT-PCR. In GK rats, these studies showed consistent changes in expression, particularly in the lumbar and sacral dorsal root ganglia of the spinal cord and in the sympathetic ganglia. The changes were consistent between the different groups of animals as well as between adjacent groups of ganglia. The most prominent changes in both the GK groups included marked upregulation of Gria4 (ionotropic AMPA receptor), downregulation of Grik3 and Grik4 (both ionotropic, kainite receptors) and Grin1 and Grin2A (both ionotropic, NMDA receptors), activation of all of which has been shown to induce hyperalgesia; downregulation of Slc1a6 (excitatory amino acid transporter 4) and upregulation of Slc1a1 (excitatory amino acid transporter 3), both of which mediate neural reuptake of glutamate from the synaptic cleft; and upregulation of Gclc (glutathione synthase), which reflects a response to protect against oxidative damage.

The immense physical, psychological, and economic cost of diabetic neuropathy underscore the need for better understanding of the pathogenesis at an early interventional level.

The pathological signalling pathways associated with diabetic neuropathy are complex and this influences the development of targeted therapies.

Chapter 6: Future directions

The most prominent and interesting changes in these animal groups were those involving; Gria4 (ionotropic AMPA receptor), Grik3 and Grik4 (both ionotropic, kainite receptors) and Grin2A (both ionotropic, NMDA receptors). Future studies could be focused in many directions such as incorporating more animal models, further gene analysis and involving human studies. Obvious directions include confirmation in other diabetic animal models for example Zucker fatty rats. Zucker animals have been obtained and these studies are now underway. Earlier time points of the GK model can also be investigated. Characterization of protein expression needs to be investigated by western blot and/or immunohistochemistry. In addition, it would be worthwhile to broaden the investigation of gene expression to include genes that are colocalized with glutamate in the secretory granules. These include those that code for genes such as CGRP, tachykinins (substance P, neurokinin A etc) which are known to be involved in the pain pathways.

Analyzing gene expression patterns can provide clues about gene function but further understanding require mutants that lack a particular gene may quickly reveal the function of the protein that it encodes. Other directions include manipulation of gene expression using knockout mice and also overexpression of the respective genes and investigating the effects of that on diabetes in these animal groups.

It may also be possible to undertake limited investigation using autopsy tissues from patients with and without diabetic neuropathy.

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