Diabetes-induced depression of synaptic transmission in autonomic ganglia and in adrenal medulla

Alona Rudchenko

Department of Physiology

McGill University, Montréal

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to Lionel
to my parents and to Dima

ABSTRACT

Synapses in the autonomic ganglia form a vital link between the activity of the central nervous system and the peripheral tissues. The fast neurotransmission in these autonomic synapses is mediated by the postsynaptic nicotinic acetylcholine receptors (nAChRs) and is remarkably robust, producing suprathreshold excitatory postsynaptic potentials. However, in diabetes, the sympathetic synaptic transmission becomes depressed when hyperglycemia elevates reactive oxygen species (ROS) and ROS inactivate the postsynaptic nAChRs containing the α3 subunit. Because dysautonomias result from such synaptic depression, the important questions are whether the synaptic depression happens in all 3 branches of the autonomic nervous system – the sympathetic, the parasympathetic, and the adrenal medulla, and whether it is possible to reverse this autonomic synaptic depression, or prevent it in diabetes.

To address these questions, I studied sympathetic and parasympathetic ganglia and the adrenal medulla using intracellular recordings of synaptic transmission and whole-cell patch clamp combined with imaging of ROS in primary neuronal cultures and in the intact ganglia.

I hypothesized the following. 1) The autonomic ganglia will develop depressed synaptic transmission in diabetes provided that the postsynaptic nAChRs are α3 subunit-containing and intracellular ROS become elevated. 2) If ROS are lowered in diabetes *in vivo* by reversing hyperglycemia with insulin, the nAChR function would recover in the neurons and synaptic transmission would recover *in vivo*. 3) If ROS in autonomic neurons are decreased in a mutant mouse strain which shows improved

ROS detoxification, synaptic depression will not develop in autonomic ganglia in diabetes.

To find out whether α3 subunit-containing nAChRs mediate synaptic transmission in different autonomic ganglia and the adrenal medulla, I used mice with a deletion in the α3 subunit gene. To study the effects of diabetes, I induced hyperglycemia by destroying the pancreatic β cells in mice with streptozotocin injections, as well as a genetic mouse model of diabetes, Akita mouse, which has a mutation in the insulin 2 gene. To reverse hyperglycemia, I implanted insulin pellets in diabetic mice. And to test the effects of lowered ROS levels, I used the mutant mouse strain WLD^s, a mouse strain which previously showed improved detoxification of intracellular ROS in peripheral neurons.

My results demonstrated that in all autonomic ganglia studied and in the adrenal medulla, EPSPs were mediated almost entirely by α3-containing nAChRs. Only in the parasympathetic ganglion, about 10% of the postsynaptic response on average was mediated by α7-containing nAChRs. During hyperglycemia, the ROS became elevated and the postsynaptic nAChRs were inactivated in the sympathetic neurons and the adrenal gland, depressing the synaptic transmission. However, parasympathetic synaptic transmission remained normal in diabetes and the neurons were resistant to elevation in ROS. The studies on the nAChR recovery after inactivation in cultured neurons showed that the function of the receptor returns within 24-48 hours, and may not require new protein synthesis. *In vivo*, reversing hyperglycemia with insulin restored the synaptic transmission fully after short diabetes duration (2 weeks), but only partially after longer diabetes durations. When the effects of lowered ROS in diabetes were

explored in WLD^s mouse strain, remarkably, normal synaptic transmission was maintained despite 2 months of hyperglycemia in these mice.

Together, these results reveal that 1) although α3-containing nAChRs underlie most of the sympathetic and parasympathetic synaptic transmission, the 2 autonomic branches differ in vulnerability to diabetes; 2) reversing the synaptic depression is possible by correcting hyperglycemia with insulin, although a critical period exists for the full recovery to happen, and 3) if ROS detoxification can be improved, as may happen in the WLD^s mouse strain, the synaptic depression can be prevented in diabetes.

RÉSUMÉ

Les synapses dans les ganglions autonomes constituent un lien vital entre l'activité du système nerveux central et les tissus périphériques. La neurotransmission rapide dans ces synapses autonomes est effectuée par les récepteurs nicotiniques de l'acétylcholine post-synaptiques (nAChRs). Cette neurotransmission est remarquablement robuste, produisant des potentiels post-synaptiques excitateurs supraliminaires. Toutefois, en diabète, la transmission synaptique sympathique devient réduite à cause de l'augmentation des espèces réactives de l'oxygène (ROS) causée par l'hyperglycémie quand les ROS désactivent les nAChRs post-synaptiques contenant la sous-unité α3. Comme des dysautonomies se produisent à cause d'une telle dépression synaptique, les questions importantes sont : est-ce que la dépression synaptique se produit dans les trois branches du système nerveux autonome - le sympathique, le parasympathique, et la médullosurrénale; et est-il possible de renverser ou prévenir cette dépression synaptique autonome en diabète.

Pour répondre à ces questions, j'ai étudié les ganglions sympathiques et parasympathiques et la médullosurrénale utilisant des enregistrements intracellulaires de la transmission synaptique et la cellule entière patch clamp combinée avec l'imagerie de ROS dans des cultures primaires de neurones et dans les ganglions entiers.

J'ai émis les hypothèses suivantes. 1) Les ganglions autonomes développeront une transmission synaptique réduite en diabète à condition que les nAChRs post-synaptiques contiennent la sous-unité α3 et que les ROS deviennent élevés dans les cellules. 2) Si les ROS sont abaissés en diabète *in vivo* en traitant l'hyperglycémie avec de l'insuline, la fonction des nAChRs pourra récupérer dans les neurones, et la

transmission synaptique va récupérer *in vivo*. 3) Si les ROS dans les neurones autonomes sont diminués dans une souche de souris mutante avec la détoxification des ROS améliorés, il n'y aura pas de réduction de la transmission synaptique dans les ganglions autonomes en diabète.

Pour déterminer si les nAChRs α3 effectuent la transmission synaptique dans différents ganglions autonomes et la médullosurrénale, j'ai utilisé des souris qui possèdent une perturbation du gène de la sous-unité α3. Pour étudier les effets du diabète, l'hyperglycémie était produite par la destruction des cellules β du pancréas chez les souris avec des injections de streptozotocine. J'ai aussi utilisé un modèle de souris génétique du diabète, Akita, qui possède une mutation dans le gène de l'insuline 2. Pour inverser l'hyperglycémie, j'ai implanté des granules d'insuline chez des souris diabétiques. Pour tester les effets des ROS diminués, j'ai utilisé des souris mutantes WLD^s, une souche qui démontre la désintoxication des ROS améliorée dans les neurones périphériques.

Mes résultats démontrent que dans tous les ganglions autonomes que j'ai étudié et dans la médullosurrénale, des potentiels post-synaptiques excitateurs sont médiatisés presque entièrement par les nAChRs contenant α3. Seulement dans le ganglion parasympathique, en moyenne environ 10 % des potentiels post-synaptiques étaient effectués par les nAChRs contenant α7. Au cours de l'hyperglycémie, les ROS sont devenus élevés et les nAChR post-synaptiques ont été inactivés dans les neurones sympathiques et la glande surrénale, réduisant la transmission synaptique. Cependant, la transmission synaptique parasympathique n'était pas réduite en diabète, et les neurones étaient résistants à l'élévation de ROS. Les études sur la récupération

des nAChR après qu'ils sont inactivés dans des neurones en culture ont montré que la fonction des récepteurs revient dans 24 à 48 heures, et nécessite pas de production de nouvelles protéines. *In vivo*, l'inversion de l'hyperglycémie avec l'insuline a complètement rétabli la transmission synaptique après une courte durée du diabète (deux semaines), mais uniquement partiellement après des durées de diabète plus longues. Dans la souche de souris WLD^s, c'était remarquable que la transmission synaptique normale a été maintenu en dépit de deux mois de l'hyperglycémie.

Ensemble, ces résultats indiquent que 1) même si les nAChR contenant α3 effectuent la plupart des transmissions synaptiques sympathiques et parasympathiques, les deux branches autonomes diffèrent dans la vulnérabilité au diabète; 2) il est possible d'inverser la réduction de la transmission synaptique autonome en corrigeant l'hyperglycémie avec de l'insuline, mais il existe une période critique pour une récupération complète ; et 3) si la désintoxication des ROS peut être améliorée, comme illustré pour la souche de souris WLD^s, il est possible de prévenir la réduction de la transmission synaptique en diabète.

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CONTRIBUTIONS TO SCIENCE

My experiments follow up on previous findings that ROS elevations in sympathetic neurons inactivate postsynaptic nAChRs and depress synaptic transmission in sympathetic superior cervical ganglion in diabetes.

In Chapter 3, I show that α3 subunit-containing nAChRs mediate synaptic transmission in 2 sympathetic ganglia, in the adrenal medulla and a parasympathetic ganglion. In addition, I discovered that α7 subunit-containing nAChRs have a small contribution to synaptic transmission in the parasympathetic ganglion. I also demonstrate that in diabetes, hyperglycemia inactivates the postsynaptic nAChRs only in sympathetic ganglia and the adrenal medulla, but not in the parasympathetic ganglia. Together with Dr Eli Akude, we show that the neurons in these ganglia differ in their vulnerability to elevations in ROS. All the electrophysiology and cell culture experiments in this chapter were performed by me, including synaptic transmission and patch clamp recordings. The experiments that combine the imaging of ROS and electrophysiology were performed together with Dr Eli Akude. All the experiments were performed with the supervision and guidance of my PhD supervisor, Dr Ellis Cooper. The manuscript based on this chapter has been accepted for publication at the Journal of Neuroscience with revisions.

In Chapter 4, I find that the depression of synaptic transmission induced by diabetes in the sympathetic ganglion, SCG, is reversible with insulin treatment after a short duration of diabetes. The findings reveal that a critical period appears to exist for the recovery of synaptic transmission, and its full recovery does not happen in mice diabetic for more than 2 weeks prior to insulin treatment. In cultured neurons, I

demonstrate that after the inactivation of the nAChRs by ROS, the receptor function recovers in the cells within 24-48 hrs. I also show that after irreversible inactivation of nAChRs through alkylation, the receptor function recovers by 24-48hrs and requires new protein translation, consistent with previous findings in cultured bovine chromaffin cells. All the experiments in this chapter were performed by me, including electrophysiological recordings of synaptic transmission and whole-cell patch clamp, cell culture, and mouse surgery. Inducing diabetes in mice, breeding and genotyping of diabetic Akita mice were performed together with Dr Eli Akude. All the experiments were performed with the supervision and guidance of my PhD supervisor, Dr Ellis Cooper.

In Chapter 5, I show that diabetes-induced depression of sympathetic synaptic transmission is prevented in the mouse strain WLD^s. I also find that homozygous but not heterozygous WLD^s mice show protection from the synaptic depression in diabetes. All the electrophysiology experiments in this chapter were performed by me. Breeding and genotyping of the WLD^s mice were performed together with Dr Eli Akude. An additional experiment measuring ROS elevations in cultured neurons of WLD^s mice is described in the discussion of the chapter and was performed by Dr Eli Akude. All the experiments were performed with the supervision and guidance of my PhD supervisor, Dr Ellis Cooper.

LIST OF ABBREVIATIONS

ACh	acetylcholine
Anti-A	antimycin A
Bromo-ACh	bromo-acetylcholine
CNS	central nervous system
CNTF	ciliary-derived neurotrophic factor
Cys	cysteine
DCCT	Diabetes Control and Complications Trial
DMXB	3-[2,4-Dimethoxybenzylidene]anabaseine
DTNB	5,5'-Dithiobis-(2-Nitrobenzoic Acid)
DTT	dithiothreitol
EPSP	excitatory postsynaptic potential
ETC	electron transport chain
GDNF	glia-derived neurotrophic factor
GLUT	glucose transporter
HbA1c	hemoglobin A1c
HBSS	Hanks Balanced Salt Solution
Hex	hexamethonium
HPLC	high performance liquid chromatography
IRS	insulin receptor substrate
mAChR	muscarinic acetylcholine receptor
MLA	methyllycaconitine

MODY	maturity onset diabetes of the young
nAChR	nicotinic acetylcholine receptor
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NGF	nerve growth factor
NMNAT	nicotinamide mononucleotide adenylyl transferase
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
ROS	reactive oxygen species
SCG	superior cervical ganglion
SMG	superior mesenteric ganglion
STZ	streptozotocin
Subm	submandibular ganglion
UKPD	UK Prospective Diabetes Study
WLDS	wallerian degeneration slow

Table of Contents

CHAPTER 1

INTRODUCTION

When our internal or external environments change, the activity of organs in our body is carefully adjusted by the autonomic nervous system. For instance, when we detect danger in our environment, our body is quickly mobilised for action. Working without our voluntary control, our autonomic neurons spare us from conscious decisions about tasks such as digestion, heart rate, sweating, or salivation. Although these tasks can be routine, how well the autonomic nervous system works to control them can be vital. For example, if one was suddenly attacked by a dangerous animal, getting increased blood supply to muscles, elevated blood glucose for energy and improved alertness can improve one's response and help survival.

Fortunately, the autonomic nervous system usually functions very well. The autonomic neurons transmit signals from the brain to the peripheral organs, carrying these signals very reliably across the synapses within the autonomic ganglia.

Despite this good function under normal conditions, the autonomic control of different organs becomes abnormal in diabetic people, producing dysautonomias. The dysautonomias are common in diabetes and can be debilitating, yet their causes are not well understood.

Recent work showed that when glucose levels become abnormally elevated, autonomic neurons of the superior cervical ganglion show increased intracellular reactive oxygen species (ROS). Elevated ROS inactivate the postsynaptic nicotinic receptors in this ganglion, depressing synaptic transmission and causing dysautonomias in diabetic mice (Campanucci et al., 2008, Campanucci et al., 2010). With the wish to learn more about this synaptic defect and hoping to better address dysautonomias in diabetes, I followed up on these findings with my studies. I asked

whether different autonomic ganglia have depressed synaptic transmission in diabetes. I then tested if it is possible to reverse or prevent the depression of autonomic synaptic transmission in diabetes.

As an introduction to these studies, in this chapter I will review how glucose levels are regulated and will describe the disease of diabetes mellitus. Then, I will describe diabetic neuropathies, focusing on the autonomic nervous system. Finally, I will outline the objectives and the hypotheses for my studies.

REGULATION OF GLUCOSE LEVELS

Blood glucose levels are highly regulated and normally remain between 5 and 6mM, but they can increase up to about 9mM after a meal (Rizza et al., 1980) or can decrease to about 3mM after prolonged exercise (Wahren et al., 1978). A drop in glucose of 1.1mM stimulates processes of glucose release into circulation, while an increase in glucose by 0.5mM stimulates processes of glucose removal.

To increase circulating glucose, glucagon is secreted from the α cells of pancreatic islets of Langerhans. To lower circulating glucose, insulin is secreted from the β cells of the pancreatic islets. (Gerich, 1988). While the cells in pancreatic islets of Langerhans directly sense the changing glucose levels, other factors such as hormones, amino acids, as well as innervation by the sympathetic and parasympathetic autonomic nerves influence the release of glucagon and insulin (Ahren, 2000, Thorens, 2011).

In addition to affecting insulin and glucagon release, the activity of sympathetic nerves and the adrenal gland can increase circulating glucose through effects on the

liver, the kidney, the skeletal muscle and fat tissue (Shimazu and Fukuda, 1965, Nonogaki, 2000). The activity of parasympathetic nerves that innervate the liver can have glucose-lowering effects (Shimazu, 1967, Lautt and Wong, 1978). Circulating glucose is also increased by the insulin-opposing actions of growth hormone, released from the pituitary gland. Incretins, secreted from the intestinal mucosa, have effects that lower circulating glucose. Glucose levels are also modulated by amylin, which is co-secreted with insulin from pancreatic β cells and acts to slow the appearance of glucose in the circulation after meals (Gerich, 1993).

Glucose Uptake

Glucose is removed from circulation through its uptake by cells. For this to happen, glucose can be transported across cellular membranes in insulin-dependent and insulin-independent ways.

Insulin-dependent glucose uptake

Fat and skeletal muscle cells take up glucose in an insulin-dependent way. The glucose uptake in these cells can be initiated by the binding of insulin to the insulin receptor, a member of the tyrosine kinase family, a dimer composed of 2 α and 2 β subunits. The 2 extracellular α subunits are disulfide-linked, while 2 transmembrane β subunits are disulfide-linked to α subunits and include cytoplasmic tyrosine kinase domains (Sparrow et al., 1997). On binding of insulin, the conformational change in the insulin receptor leads to transphosphorylation and activation of the tyrosine kinase

domains, initiating intracellular signalling cascades (Taha and Klip, 1999, Hubbard, 2013).

Among these initiated signalling cascades, the following are the main steps to increase glucose uptake. The insulin receptor's activated tyrosine kinase domains recruit and phosphorylate the insulin receptor substrate (IRS) proteins (Quon et al., 1994), which then bind and activate phosphatidylinositol 3-kinase (PI3K) (Okada et al., 1994, Tsakiridis et al., 1995). The activation of the PI3 kinase of class 1a leads to the phosphorylation of protein kinase B (PKB). PKB activation then leads to increased translocation of the facilitative glucose transporters of GLUT4 subtype from intracellular pools to the plasma membrane (Wang et al., 1998). In addition to PKB, atypical protein kinases C (PKC), PKCλ and PKCζ, are activated downstream of PI3K, and contribute to increasing the translocation of GLUT4 to the plasma membrane (Standaert et al., 1997, Kotani et al., 1998).

The GLUT4 transporters in the intracellular pools continuously move to and from the plasma membrane, but when not stimulated by insulin their endocyosis is higher than exocytosis and 90% of GLUT4 are intracellular (Satoh et al., 1993). When insulin signalling increases GLUT4 exocytosis and decreases the endocytosis, GLUT4 density in plasma membrane increases up to 10-20 fold, which increases glucose uptake (Watson and Pessin, 2001).

Insulin-independent glucose uptake

Glucose is also taken up by many cells in an insulin-independent way through GLUT1 and other glucose transporters constitutively present in the plasma membrane

(Pessin and Bell, 1992). Among the cells taking up glucose in an insulin-independent way are neurons. It was shown for example that forebrain neurons in primary culture take up radioactively-labelled glucose analog 2-deoxyglucose at the rate that is independent of whether insulin is present in culture medium (Heidenreich et al., 1989). Similarly, in a study with peripheral sensory neurons, Patel and colleagues found no influence of insulin on uptake of a glucose analog (Patel et al., 1994). Consistent with these findings, the main glucose transporters expressed by many neurons are of subtypes GLUT1, GLUT3 (Duelli and Kuschinsky, 2001), and GLUT8 (Reagan et al., 2002) which are not regulated by insulin. This suggests that during hyperglycemia of diabetes, despite decreased effects of insulin, high extracellular glucose likely leads to increased intracellular glucose in these cells.

Once glucose is intracellular, it is mainly used by cells for energy production. Glucose is also used for glycogen synthesis in liver and muscle cells, and for triglyceride synthesis in liver and fat cells (Silverthorn, 2001). When intracellular glucose becomes abnormally elevated, it can also be used in intracellular pathways that cause toxicity.

When glucose control by insulin is abnormal

When glucose levels are not regulated properly by insulin, abnormally high blood glucose or hyperglycemia develops. A decrease in the insulin's effect on the liver is one cause of hyperglycemia. When there is a lack of insulin-dependent inhibition of hepatic glucose production, and also a lack of insulin-dependent conversion of glucose into glycogen by the liver, circulating glucose increases (Burcelin et al., 1995, Michael et al.,

2000). In addition, circulating glucose becomes further elevated when it is not taken up in an insulin-dependent way by fat and muscle cells (Lauro et al., 1998). Hyperglycemia also develops because of high glucagon output by pancreatic α cells, which is normally suppressed by insulin (Unger and Orci, 1975, Kawamori et al., 2009).

DIABETES MELLITUS

The disease and its main types

Diabetes mellitus is a metabolic disease that results in abnormal glucose homeostasis. Although diabetes has affected people since ancient times (Gemmill, 1972), its prevalence is high today and is quickly increasing. In 2012 diabetes affected 371 million people worldwide, including 2.7 million Canadians. By 2030, the number of diabetics is predicted to reach half a billion worldwide (International Diabetes Federation).

The diagnosis of diabetes mellitus is done using 3 main measures: blood glucose levels during fast, blood glucose after consuming a standard amount of glucose (commonly at 2 hours after consuming 75g glucose), and glycation of haemoglobin HbA1c ("Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia. IDF/WHO", 2006). Although the specific cut-off values can vary, they are usually based on the association with diabetes complications.

High circulating glucose in diabetes develops for 2 main reasons: 1) either insulin is insufficient, or 2) insulin sensitivity is low. The disorder is traditionally divided into: type 1, type 2, gestational, and other diabetes types (1979).

Type 1 diabetes

Type 1 diabetes includes 5-10% of all cases and is caused by autoimmune destruction of insulin-producing pancreatic β cells by lymphocytes and macrophages (Foulis et al., 1991). Once about 75% of β cells are destroyed, not enough insulin is produced for glucose regulation (Fauci et al., 2008). Patients develop hyperglycemia, which can be severe and insulin treatment is needed. Type 1 diabetes often involves genetic susceptibility (Barrett et al., 2009, Zoka et al., 2013), and its onset can be facilitated by environmental triggers such as an infection. While the mechanism of the disease onset is unclear, it is thought that the cytotoxic T cells lose tolerance to some β cell antigens and the β cells are destroyed. The loss of autotolerance could be promoted by factors such as elevated inflammatory signals and tissue damage in pancreatic islets and increased expression by β cells of molecules such as major histocompatibility complex I (Coppieters et al., 2012, Roep and Peakman, 2012).

Type 1 diabetes usually starts in childhood, but can also happen in adults, known as Latent Autoimmune Diabetes in Adults. Autoantibodies to β cell antigens can usually be detected in type 1 diabetes and can be used for diagnosis (Batstra et al., 2001).

Type 2 diabetes

About 90% of diabetes cases are of type 2. This diabetes type involves insulin resistance and defective insulin secretion and is often associated with obesity. High levels of fatty acids in obesity can cause high adipokine (Fried et al., 2000, Steppan et al., 2001) and low adiponectin secretion by adipose cells (Hu et al., 1996) and abnormal lipid metabolites in muscle cells (Adams et al., 2004). The infiltration of adipose tissue

by macrophages causes a pro-inflammatory state (Galic et al., 2010). These events are linked to insulin resistance in muscle and adipose cells (Diez and Iglesias, 2003, Eckardt et al., 2011). In addition to lifestyle, genetics strongly contributes to developing type 2 diabetes: more than 40 genes are linked to abnormal β cell function and development, or predispose to obesity (Ashcroft and Rorsman, 2012). At the beginning of the disease, decreased insulin signalling is often compensated by its higher release (Chiles and Tzagourn.M, 1970, Warram et al., 1990) and only postprandial hyperglycemia develops. With progressive β cell dysfunction insulin release becomes insufficient and patients then show fasting hyperglycemia (Weyer et al., 1999).

Gestational diabetes

Gestational diabetes happens in 2 to 18% of pregnancies (Linnenkamp et al., 2013) usually during the second half. It involves decreased insulin sensitivity by up to 80% in response to elevation of placental lactogen and other hormonal changes (Boden, 1996). While possibly an adaptive mechanism to provide good supply of glucose to the fetus, it can cause complications for the mother and the developing child (Reece and Eriksson, 1996, Jovanovic, 1998).

Other diabetes mellitus types

Many other types of diabetes mellitus exist and are caused by: 1) genetic defects that affect β cell function, such as maturity onset diabetes of the young (MODY), 2) genetic defects affecting insulin action, such as with insulin receptor mutations, 3)

diseases of pancreas, such as pancreatitis, 4) endocrine disorders, such as Cushing's syndrome, 5) drugs, 6) infections, 7) immune reactions, 8) and other genetic causes.

Although there are many causes of diabetes mellitus, all types of the disease are characterized by defective regulation of blood glucose levels.

COMPLICATIONS OF DIABETES MELLITUS

Short-term effects of hyperglycemia

Hyperglycemia can cause acute problems as well as long-term complications. Acutely, in cases of extremely high blood glucose (above 33mM), a hyperosmolar hyperglycemic state can develop. In this state, high blood glucose causes blood hyperosmolarity, and an osmotic gradient pulls water from tissues into the blood, which dehydrates many tissues. High glucose is also not properly reabsorbed by kidney, causing glucose to appear in the urine. With glucose present in the urine, the kidney is less able to preserve water by concentrating urine, and high amounts of water are excreted, causing severe dehydration (Nugent, 2005).

Long-term effects of hyperglycemia

Hyperglycemia can also cause damage to many cells and tissues over long term, producing lasting complications. The complications of diabetes can affect the retina, kidney, heart and vasculature, gastrointestinal system, liver, skin, and nervous system. These complications have serious consequences for diabetic people. For example, retinopathy that commonly develops in diabetes can lead to blindness (Roy et al., 2004); the skin and sensory nerve problems can produce severe damage to the feet

and lead to amputation (Jeffcoate and Harding, 2003); autonomic neuropathy can deregulate many organs increasing the risk of mortality (Gerritsen et al., 2001). The development and progression of complications is strongly associated with hyperglycemia.

Glucose level control helps prevent diabetic complications

It is well established today that to avoid the complications of diabetes, blood glucose needs to remain as close as possible to normal levels. This fact first became known in the late 1980s as a result of large-scale clinical studies with diabetic people. One major study the Diabetes Control and Complications Trial (DCCT) was done starting in 1983 with 1,441 type 1 diabetic people in the United States and Canada. In this study, one group of diabetic people was treated intensively with insulin to strictly maintain blood glucose levels below 10mM (intensive treatment group). Another group of diabetics underwent a milder insulin treatment and had varying blood glucose levels that averaged at 13mM (conventional group). By 6.5 years after the beginning of the DCCT study it was clear that diabetes complications were much less likely to start or to progress in diabetic people with intensive compared to conventional treatment. The intensively-treated group had a lower risk of developing and progression of retinopathy by 76% and 50% respectively, a 60% lower risk of developing neuropathy, and a 54% lower risk of nephropathy.

Similarly, reduced risk for developing complications of type 2 diabetes was observed in a large-scale study the UK Prospective Diabetes Study (UKPD). This study was conducted with 5,102 people who had type 2 diabetes in the UK between the years

1977 and 1997. During the study, diabetic patients were either in an intensive or in a conventional group. Intensively-treated people had their blood glucose maintained close to normal levels by using insulin or by stimulating insulin release from pancreatic β cells with sulfonylureas. For people in the conventional group, blood glucose was lowered less intensively with diet only. The study's results showed that, compared to diabetic people who were treated with diet alone, those treated intensively had lower risks of retinopathy, nephropathy, neuropathy, and myocardial infarction, stroke and amputations. In addition to the DCCT and the UKPD, many other studies also showed the association of high glucose levels with the development of complications (Mattila and de Boer, 2010). Importantly, it is still unclear today, despite these results, whether established complications can be reversed by normalizing glucose.

Microvascular and macrovascular complications of diabetes

Blood vessels can show many pathological changes in diabetes. They can accumulate fatty and calcium deposits, can show inflammation (Kanter and Bornfeldt, 2013) and high oxidative stress (Schaffer et al., 2012). The vascular smooth muscle of the blood vessels becomes thicker and atherosclerotic plaques develop in diabetes (Furchgott and Zawadzki, 1980).

One common way to classify various diabetic complications is to view them as either microvascular or macrovascular. The complications of diabetes which involve pathological changes in large blood vessels, such as arteries and veins, are classified as macrovascular. These complications include coronary artery disease and stroke (Ettinger and Regan, 1989), which are 2-4 times higher in diabetic than in nondiabetic

people (Bornfeldt and Tabas, 2011), as well as peripheral vascular disease or circulation problems.

The complications of diabetes which show pathological changes in small blood vessels, such as capillaries, are classified as microvascular, and include diabetic neuropathies, nephropathy, and retinopathy. For example, arterioles in the retinas of diabetic people can become leaky, causing small haemorrhages. Such haemorrhages are linked to the degeneration of the cells that make up the blood-retinal barrier (Joussen et al., 2007). Some blood vessels also become occluded causing retinal hypoxia. As new blood vessels form in compensation, retinal traction can happen which can lead to its detachment (Stitt et al., 2013). When blood vessels become leaky in the macula, the region of the highest visual acuity in the retina, this can cause very poor vision and even blindness (Joussen et al., 2007).

Researchers who have studied biopsies from diabetic people with neuropathy observe that the capillaries supplying peripheral nerves have thickened walls (Malik et al., 1989). Studies also showed a correlation between abnormalities in blood vessels supplying sensory sural nerve and neuropathy symptoms in diabetics (Yasuda and Dyck, 1987, Britland et al., 1990).

Peripheral nerves in diabetes were shown to have decreased blood flow. Tesfaye and colleagues measured blood flow in the sensory sural nerve of diabetics using in-vivo quantification of fluorescein injected intravenously. They found that blood flow was lower in diabetics with sensory or motor neuropathy compared to people without diabetes or diabetics without neuropathy (Tesfaye et al., 1993). Similarly to observations with the sural nerve, reduced blood flow was also seen in autonomic

superior cervical ganglia as well as sensory dorsal root ganglia in diabetic rats. Using autoradiography to quantify the blood in sections of the ganglia, Sasaki and colleagues observed a 40% reduction in diabetic compared to healthy animals (Sasaki et al., 1997). Despite these findings, there is evidence that the decrease in blood flow is not the only cause of diabetic neuropathies.

Nonvascular problems contribute to diabetic neuropathies

A number of abnormalities in peripheral neurons in diabetes are intrinsic to neurons themselves and are not directly caused by decreased blood flow. For example, it is known that sensory neurons of dorsal root ganglia develop high oxidative stress and show cell death when cultured in hyperglycemic conditions (Russell et al., 2002, Vincent et al., 2005). Since no vasculature is present in these neuronal cultures, these problems must originate from neurons. In addition, the pattern of sensory nerve degeneration seen in diabetes *in vivo* does not resemble degeneration caused by ischemia. Sensory nerve degeneration in diabetes happens in the distal parts of the sensory nerves, while ischemia instead would affect the large nerve trunks (Zochodne et al., 2008).

It is also possible that the problems in blood vessels supplying peripheral nerves are themselves produced by a dysfunction of autonomic neurons that innervate these blood vessels. Beggs and colleagues examined biopsies of peripheral nerves of diabetic people using ultrastructural analysis and immunocytochemistry. They found that arterioles supplying these nerves were often denervated in diabetes (Beggs et al., 1992).

DIABETIC PERIPHERAL NEUROPATHIES

The complications of diabetes that affect sensory, motor, and autonomic neurons can cause pain, movement problems, can lead to foot ulcers and even amputations. Some peripheral neuropathies can deregulate the function of many organs and can be life-threatening. More than 50% of diabetic people have neuropathies after 5 years of diabetes (Adler et al., 1997), while 10 to 20% of diabetics have neuropathies already at the time of diabetes diagnosis (Boulton et al., 2005, Ziegler, 2008).

In the following sections I will briefly describe the effects of diabetes on sensory and motor neurons. Then I will review the autonomic neurons in more detail, because the autonomic neurons in diabetes have been studied very little and these neurons are the focus of experiments presented in my thesis.

Diabetic sensory neuropathies

Diabetic sensory neuropathies result from damage to neurons of the dorsal root ganglia, particularly the terminals. Among the first sensory neurons to be affected by diabetes are the ones with the longest and smallest axons, including unmyelinated C fibers and thinly myelinated Að fibers. The conduction velocity of sensory nerves' axons gets slower and myelinated axons can lose myelin (Behse et al., 1977). Commonly, these axons also develop dystrophy or swellings (Lauria et al., 2003, Zherebitskaya et al., 2009). These pathological changes likely cause defective temperature and pressure sensation in many diabetic patients (Tesfaye, 2011).

In addition to losing myelin, the axons of sensory neurons degenerate, which sometimes progresses to cell body degeneration (Kamiya et al., 2006). This nerve

degeneration decreases the innervation of the skin which is clearly seen in skin biopsies of diabetics (Herrmann et al., 1999a, b). Some regeneration of axons happens, although it happens at a slower than normal rate (Dyck et al., 1988, Bradley et al., 1995, Polydefkis et al., 2004, Said et al., 2008).

While the degeneration of the sensory nerves produces numbness, the defective regeneration is likely causing burning, pain and hypersensitivity symptoms in diabetics (Brown et al., 1976, Sinnreich et al., 2005, Ziegler, 2008).

The degeneration progresses in a “stocking and glove pattern” moving from feet to knees, then trunk, eventually the head. When diabetic people lose sensation they can easily get injured, especially at their feet. When injury sites become infected, ulcers develop and in some cases even lead to amputations (Perez and Kohn, 1994, Gordois et al., 2003, Margolis et al., 2011).

Diabetic motor neuropathies

Motor neurons, with their cell bodies in the spinal cord and axons innervating skeletal muscle, are usually affected less and later than sensory neurons in diabetes (Boulton and Ward, 1986, Ramji et al., 2007).

The conduction velocity of motor neurons’ axons becomes slower in diabetics (Zochodne and Ho, 1992). However, unlike with sensory neurons, the slowing of conduction velocity in these neurons is not accompanied by loss of myelin (Ramji et al., 2007, Zochodne et al., 2008). The axonal degeneration in motor neurons is also less common than what is seen for sensory neurons. In addition, the cell bodies of motor neurons usually remain unaffected (Ramji et al., 2007).

When the degeneration of axons of the motor neurons does happen, some muscles become denervated. This is observed as a decrease in estimated numbers of motor units (motor neurons together with muscle they innervate) in studies of leg and foot muscles of diabetics. For example, in their studies using electromyography, Allen and colleagues observed a 30-60% decrease in estimated number of motor units in diabetic compared to healthy humans (Allen et al., 2013a, Allen et al., 2013b). Evidence shows that motor neurons probably reinnervate denervated muscles through collateral sprouting in diabetes. Andersen and colleagues estimated the density of muscles innervated by the same motor neuron using single fiber electromyography in diabetic people. They observed an increase in this density of muscles innervated by the same nerve fiber in diabetic patients who had neuropathy, and an even further increase in those with muscle weakness (Andersen et al., 1998). Despite muscle reinnervation, the muscles show decreased contractile properties and sometimes atrophy in diabetes (Sander and Chokroverty, 1996, Fahim et al., 1998).

Diabetic autonomic neuropathies

Autonomic neuropathies affect about 60% of diabetic people and predispose them to mortality, often from a sudden cardiac problem or renal failure (Ewing et al., 1980, O'Brien et al., 1991, Rathmann et al., 1993, Gerritsen et al., 2001, Maser et al., 2003).

The autonomic nervous system, which includes sympathetic and parasympathetic neurons as well as the adrenal medulla, regulates every internal organ

of the body and the skin. This regulation becomes disrupted in diabetes and many organs can function abnormally.

Before describing some autonomic problems in diabetes I will review how the autonomic nervous system functions.

The autonomic circuit

The autonomic nervous system regulates the activity of various organs in the body to adjust this activity to changes in the internal and external environment. It consists of a circuit where signals from the brain stem, the hypothalamus, and the forebrain are transmitted through sympathetic and parasympathetic neurons to the target tissues. The sensory signals from different tissues are in turn detected by sensory neurons and are then carried by them to the nucleus of the solitary tract in the medulla. This provides feedback to the central nervous system and completes the circuit (Kandel, 2000).

Both sympathetic and parasympathetic branches of the autonomic nervous system transmit signals to target organs by 2 orders of autonomic cells – pre-ganglionic, which originate in the spinal cord, and postganglionic, which are found in ganglia in the periphery (Fig 1.1).

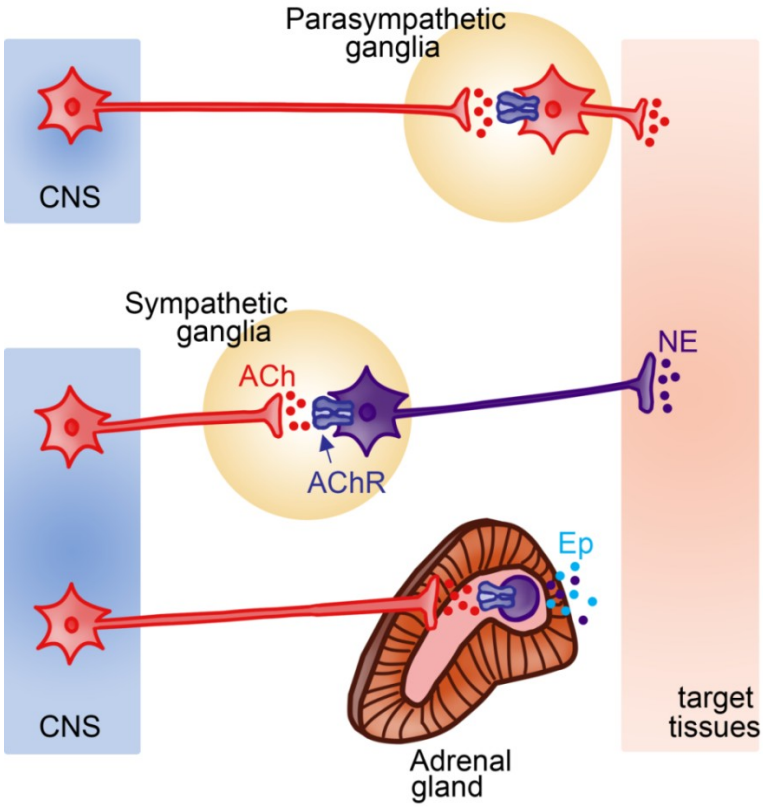


Figure 1.1 Synapses in autonomic ganglia link the CNS activity to regulation of target tissues.

Autonomic preganglionic neurons synapse on the postganglionic neurons in autonomic ganglia which innervate target tissues. Some sympathetic preganglionic neurons innervate adrenal chromaffin cells

ACh: acetylcholine; NE: norepinephrine; Ep: epinephrine;
AChR: acetylcholine receptor

The sympathetic division

The sympathetic preganglionic neurons are found in the thoracic and lumbar sections of the spinal cord forming a column that is positioned intermediolaterally. These neurons send their axons through the spinal cord's ventral roots and form synapses on:

1) paravertebral ganglia which are arranged in the sympathetic chain running along the vertebrae;

2) prevertebral ganglia which are located in the abdominal region away from the vertebrae;

3) chromaffin cells in the medulla of the adrenal gland.

The neurons in the paravertebral and prevertebral ganglia send axons to synapse on target organs. The adrenal chromaffin cells, on the other hand, release transmitter molecules directly into bloodstream.

The parasympathetic division

The parasympathetic preganglionic neurons are found in several brain stem nuclei associated with cranial nerves III, VII, IX, and X, and in the sacral region of the spinal cord. The axons of the preganglionic neurons synapse on:

1) ganglia located in the head

2) ganglia located within or near the target organ

In both sympathetic and parasympathetic autonomic branches, the autonomic ganglia contain the synapse between the pre- and postganglionic neurons which is the crucial link between the central nervous system and the peripheral targets.

Synaptic transmission in autonomic ganglia

The synaptic transmission between the pre- and post-ganglionic autonomic neurons happens when an action potential triggered in preganglionic neurons depolarizes their axon terminals, leading to Ca^{2+} entry and exocytosis of acetylcholine (ACh) from synaptic vesicles into the synaptic cleft. The ACh then binds to its receptors on the postsynaptic cell membrane to cause postsynaptic potentials. The ACh receptors that produce fast excitatory postsynaptic potentials (EPSPs) on autonomic neurons are the nicotinic receptors (nAChRs).

Postsynaptic receptors

Nicotinic acetylcholine receptors

The nAChRs are ion channels that open in response to the binding of ACh and conduct positively-charged ions. There are 16 different structurally homologous subunits of nAChR known (Albuquerque et al., 2009). Each individual nAChR receptor is made up of 5 subunits, which, in the case of the neuronal nAChR, include 2 subunits of α subtype and 3 subunits of the β subtype (Fig 1.2) (Cooper et al., 1991, Sargent, 1993). The muscle nAChRs consist of 2 α subunits, β, δ, and γ or ε subunits (Karlin, 2002).

Each subunit of the receptor contains a large conserved extracellular N-terminal domain, 4 transmembrane domains, a variable cytoplasmic loop, and short variable C-terminus. The receptor's extracellular domain is made up of β sheets that fold in an antiparallel way into a β barrel structure. The transmembrane domains are α-helical,

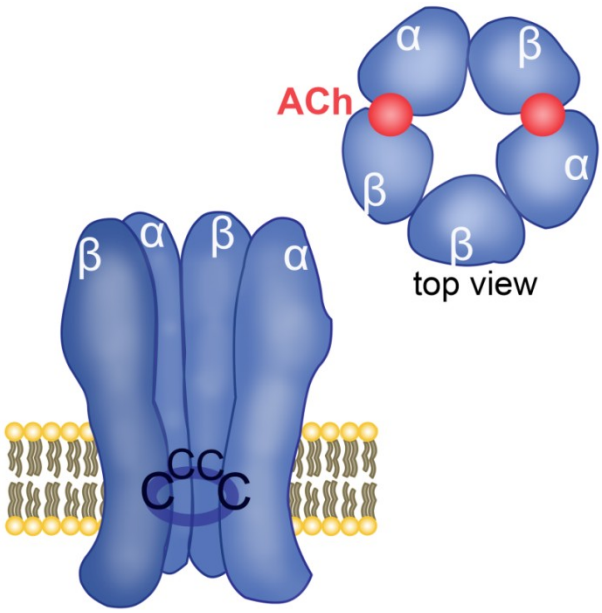


Figure 1.2 Neuronal nicotinic receptor, viewed from the side and from the top.

Acetylcholine bound to the receptor is shown in the top view. Cysteine residues at position 239 found in the intermediate region of the receptor's pore are shown (C) in the side view. These Cysteine resides make the receptor vulnerable to oxidation by intracellular reactive oxygen species.

arranged around a hydrophobic pore that is lined by transmembrane region 2 (Brejc et al., 2001).

The α subunit of nAChRs contains the ligand-binding site in its N-termunus (Kao et al., 1984). The ligand binds in a pocket between an α subunit and its adjacent subunit, leading to a conformational change of the receptor, with a clockwise rotation in the extracellular domain (Sine, 2002). This moves the receptor's pore region replacing hydrophobic residues by hydrophilic ones and allowing ions to pass through (Unwin, 2005).

Disruptions of nicotinic receptor function

Among the important residues within the ligand-binding site of the nAChR is a pair of Cysteine (C) residues at positions 192 and 193 (C^{192}, C^{193}) which normally remain crosslinked through a disulfide bridge in the oxidising extracellular environment. If this pair of C residues is reduced by dithiothreitol, this prevents ligand binding (Pike and Loring, 1992) and abolishes ACh-evoked currents through the receptor, making nAChR non-functional (Derkach et al., 1991).

Previous work at the Cooper lab showed that the nAChR pore can become blocked by intracellular polyamines, such as spermine, in a voltage-dependent way (Haghighi and Cooper, 1998). This work showed that at positive membrane potentials, intracellular polyamines interact with negatively-charged amino acids at the intermediate location in the receptor's pore, blocking it. This produces inward rectification, allowing the channel to conduct ions inwardly at negative membrane potentials, but preventing the outward movement of the ions when the membrane potential is positive.

An interesting finding was also that removing negative charge from the intermediate region of the receptor pore by mutating glutamic acid residue at position 240 (E^{240}) prevented inward rectification, but also reduced the channel's permeability to Ca^{2+} and its conductance dramatically (Haghighi and Cooper, 2000). This suggests that changes to the residues in this region of the nAChR pore could disrupt the receptor's function.

nAChR subunit composition in autonomic ganglia

The exact subunit composition of nAChRs in autonomic ganglia is not known. A number of subunits are expressed in the superior cervical ganglion, including α3, α5, α7, β2, β4 (Mandelzys et al., 1994, De Koninck and Cooper, 1995). However, these subunits may not all contribute to functional receptors that mediate synaptic transmission in different ganglia. The mRNA for the α3 and β4 subunits was found to be the highest (De Koninck and Cooper, 1995). It is thought that these subunits combine together, as well as possibly with β2 and α5, to form nAChRs in the autonomic ganglia (Covernton et al., 1994, Mandelzys et al., 1994).

Importantly, when the α3 subunit was genetically deleted in mice, this caused severe problems with autonomic regulation (Xu et al., 1999). The mice also had no synaptic transmission in the superior cervical ganglion (Rassadi et al., 2005). Whether α3-containing nAChRs are required for synaptic transmission in other autonomic ganglia is not known.

Some studies suggested that nAChRs containing α subunits other than α3 may be present in various autonomic ganglia. For example, it was reported that

parasympathetic ciliary ganglion expresses α7 nAChR subunit at the embryonic stage (Corriveau and Berg, 1993, Zhang et al., 1996, Chang and Berg, 1999, Pardi and Margiotta, 1999). Adrenal chromaffin cells were reported to express α7 and α9 subunits (Lopez et al., 1998, Colomer et al., 2010). So it remains unclear if α3-containing nAChRs mediate synaptic transmission throughout the autonomic nervous system.

Muscarinic acetylcholine receptors

In addition to activating postsynaptic nAChRs on autonomic neurons, ACh can activate muscarinic receptors (mAChRs). The mAChRs are 7-transmembrane domain proteins coupled to G-proteins, which activate intracellular signalling cascades. Five subtypes of mAChRs are known, M1-M5 (Caulfield and Birdsall, 1998), belonging to 2 classes. The M1, M3, and M5 couple to G_q family of G proteins, while M2 and M4 couple to G proteins of $G_{i/o}$ family.

In autonomic neurons, muscarinic activation is known to produce slow EPSPs. These slow EPSPs happen when muscarinic signalling leads to closing of the M-type K^+ channels, which is also known as the "M current" (Brown and Adams, 1980). The mAChR subtype that produces this response in autonomic neurons is not known. It is thought that large amounts of ACh need to be released to activate mAChRs on autonomic neurons, as these receptors are found extrasynaptically (Brown and Selyanko, 1985).

While the activation of postsynaptic nAChRs by ACh can cause large postsynaptic depolarizations of >20mV, firing action potentials and causing exocytosis of norepinephrine in the postsynaptic cells (Greene and Rein, 1978, Dolezal et al.,

1995), mAChR activation has a much smaller effect. The muscarinic responses have been recorded in sympathetic neurons in response to 10-20Hz preganglionic nerve stimulation (Morita and Suzuki, 2001, Rassadi et al., 2005). The EPSPs that were produced in response were only up to 5mV in amplitude and lasted 20-30s, and these EPSPs did not trigger action potentials. So the most likely role of mAChRs in autonomic ganglia is to modulate the effects of the fast strong EPSPs produced by nAChRs, without mediating synaptic transmission on their own.

Postsynaptic neurotransmitter synthesis and vulnerability to oxidative stress

The postganglionic sympathetic neurons and adrenal chromaffin cells release noradrenaline and epinephrine, while postganglionic parasympathetic neurons release acetylcholine. The synthesis of these neurotransmitters in the 2 autonomic divisions is quite different.

In parasympathetic postganglionic neurons, acetylcholine is synthesized in axons and their terminals by enzyme choline acetyltransferase from substrates acetyl-CoA and choline. In sympathetic postganglionic neurons and the adrenal chromaffin cells, the synthesis of noradrenaline and epinephrine involves conversion of L-tyrosine to DOPA by tyrosine hydroxylase. Tyrosine hydroxylase is an iron-containing monooxygenase enzyme which shows uncoupling of hydroxylation of L-tyrosine, producing H_2O_2. It was suggested that the iron that is contained in this enzyme can catalyze a Fenton-type reaction producing hydroxyl radicals (OH•) – highly reactive and oxidising species (Haavik et al., 1997). In addition, catecholamines norepinephrine and epinephrine can be degraded in the sympathetic postganglionic axon terminals by oxidative deamination

catalyzed by the intracellular monoamine oxidase. One end-product of this reaction is H_2O_2, which can contribute to oxidative stress (Houslay and Tipton, 1973). Therefore, the synthesis and degradation of epinephrine and norepinephrine might predispose sympathetic cells to increased oxidative stress, making them vulnerable in diseases with elevated ROS.

Inactivation of α3 subunit-containing nAChRs by ROS

Previous work showed that the α3 subunit-containing nAChRs expressed in the sympathetic neurons of SCG can be oxidised by ROS (Campanucci et al., 2008, Campanucci et al., 2010). This work showed that Cysteine residues at position 239 (C^{239}) at the pore of the α3 subunit-containing nAChR are targets of ROS. When the nAChRs are oxidised, they are inactivated in a use-dependent way.

The C^{239} residues are present on each of the 5 nAChR subunits – these residues are located in the pore region of the receptor, forming a ring (Fig 1.2). Possibly, oxidation causes cross-linking of 2 or more subunits, changing the receptor's conformation (Campanucci et al., 2010). This work also showed that the oxidation of the C^{239} on the α3 subunits is sufficient to inactivate the nAChR. When the C^{239} were mutated to Alanine (A^{239}) only on α3 subunits, the inactivation of the nAChR by ROS was prevented. In contrast, muscle-type nAChRs that do not contain α3 subunits and do not have a ring of Cysteine residues in the receptor's pore, did not become inactivated by ROS (Campanucci et al., 2008).

Once the nAChR is oxidised by ROS, one way for the receptor to recover might be by remaining in the plasma membrane and being reduced. Another possibility is that

the nAChR is removed from the plasma membrane, is degraded, and then is replaced by functional receptors. It is also possible that the nAChR becomes reduced in the process of recycling, regains its function and is reinserted into the plasma membrane.

Relevant to how the nAChR may recover after inactivation by ROS, several groups of researchers inactivated the nAChRs by alkylation or with an antibody, and then estimated the changes in functional nAChRs at the plasma membrane over time (Higgins and Berg, 1988a, b, Free et al., 2005).

Free and colleagues indirectly estimated the functional nAChRs in bovine adrenal chromaffin cell plasma membrane by measuring the secretion of [^{3}H]norepinephrine in response to incubation of cells with nicotine and by measuring receptor binding using epibatidine (Free et al., 2005). They showed that after inactivating the nAChRs at the plasma membrane irreversibly using cell-impermeable alkylation, the nicotine-stimulated secretion of [^{3}H]norepinephrine by the cells and the binding of epibatidine recovered to close to pre-alkylation levels within 24 to 48 hours. In addition, the authors found that this recovery could be prevented by inhibiting translation in the cells with puromycin. This indicated that new protein synthesis - presumably of the nAChRs, was needed for the recovery to happen. Moreover, protein synthesis was needed in the cells continuously to maintain the estimated nAChRs in the memebrane. When the chromaffin cells were treated with puromycin without nAChR alkylation, there was a gradual decrease in [^{3}H]norepinephrine secretion and epibatidine binding over time (Free et al., 2005).

Useful findings about the turnover of nAChRs also come from studies in bovine chromaffin cells by Higgins and Berg (Higgins and Berg, 1988a, b). The researchers

inactivated the nAChRs by crosslinking them with an antibody mAb35. Using nicotine-induced release of [^{3}H]norepinephrine to estimate the functional nAChRs, they then estimated that the receptor is removed from the membrane at a rate of ~3% per hour, and the receptor half-life is ~24 hours. The authors estimated that the rate of the nAChR insertion in cell membrane is about the same as its removal, ~3% per hour (Higgins and Berg, 1988b, a).

It is possible that the way nAChRs turn over in chromaffin cells described by these studies applies to nAChR turnover in other autonomic cells, however this is presently unknown.

Autonomic problems in diabetes

Many organs show abnormal regulation by the autonomic nervous system in diabetes. The following are examples of autonomic problems in the gastrointestinal system (receiving innervation from the superior mesenteric ganglion), problems with hyperglycemia awareness (which is mediated by the adrenal medulla), problems with the function of the pupil (innervated by the superior cervical ganglion), and the saliva production (stimulated by input from the submandibular ganglion) (Fig 1.3).

Gastrointestinal tract

Gastrointestinal (GI) tract, which receives sympathetic innervation from superior mesenteric ganglion, shows very disordered function in diabetes. Sympathetic dysfunction in diabetes contributes to gastroparesis, or decreased motility of the GI tract and slower digestion (Kong et al., 1999, Jones et al., 2001, Faraj et al., 2007).

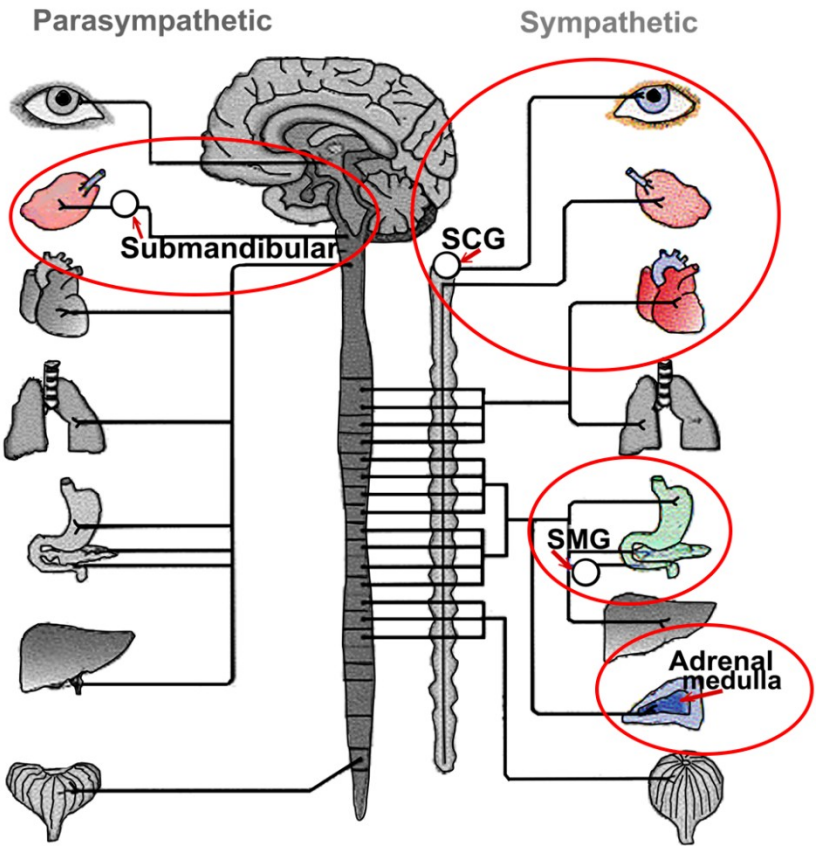


Figure 1.3 Sympathetic and parasympathetic branches of the autonomic nervous system.

The organs innervated by the ganglia investigated in this thesis and the adrenal medulla are circled.

With decreased GI tract activity, there can be residual food that favours bacterial growth and causes nausea, as well as postprandial hypoglycaemia (Said, 2007). Sympathetic dysfunction is also known to cause diabetic diarrhea (Saslow and Camilleri, 1995). This results from decreased sympathetic control that normally stimulates absorption of solutes and water (Chang et al., 1985, Chang et al., 1986, Valdovinos et al., 1993, Zietz et al., 2000). Sympathetic dysfunction in diabetes can also cause incontinence when sympathetic control of resting anal sphincter pressure is defective (Wald and Tunuguntla, 1984).

Hypoglycemia awareness

A very serious complication of diabetes is difficulty detecting hypogycemia, which happens as a result of sympathetic dysfunction. Normally, hypoglycemia activates the sympathetic nervous system to release epinephrine from the adrenal medulla, increasing sweating and increasing heart rate, creating awareness of hypoglycaemia. In diabetes, such sympathetic response to hypoglycaemia is decreased, and this makes it difficult for diabetic people to detect hypoglycaemia (Amiel et al., 1988, Dagogo-Jack et al., 1993). In addition, glucagon is normally released from pancreatic α cells in response to hypoglycemia, but in diabetic people the glucagon release in hypoglycemia is lost. This prevents compensatory glucose increase in hypoglycemia (Taborsky et al., 1998, Cryer, 2001).

Problems with detecting and correcting hypoglycemia in diabetes are very dangerous, especially in patients using insulin to lower blood glucose. The brain, which

normally relies exclusively on glucose for energy can show abnormal function if blood glucose levels become too low (less than ~2.8 mM). If the low blood glucose remains undetected and hypoglycemia is prolonged, neuronal necrosis can happen, which can lead to coma and death (Mitrakou et al., 1991).

Pupillary problems

Normally, the pupil dilates in dark conditions so that more light can enter the eye. This effect is mediated by sympathetic stimulation from neurons of the superior cervical ganglion which causes contraction of the pupil's radial muscle. In diabetic people pupil dilation is decreased, which often makes it difficult for them to see at night (Pittasch et al., 2002). When tested clinically, pupil dilation in the dark is decreased by up to 37% in diabetic people compared to control (Ferrari et al., 2010).

Saliva production

The parasympathetic submandibular ganglion innervates the submandibular gland which controls the production of saliva. Some studies showed that saliva production is decreased in diabetic people compared to healthy controls (Sreebny et al., 1992, Moore et al., 2001, Lopez et al., 2003, Mata et al., 2004). However, in other studies, it was found that saliva production remains normal in diabetes (Dodds et al., 2000, Chavez et al., 2001).

Both parasympathetic and sympathetic activity can increase saliva production. To examine the contribution of these inputs separately, Anderson and colleagues directly stimulated these separate nerve inputs to the submandibular gland in

anesthesized diabetic and healthy control rats and examined the production of saliva. The saliva produced in response to parasympathetic stimulation did not differ in its total amount or protein output between the diabetic and control rats (Anderson et al., 1993). This finding argues against parasympathetic dysfunction in diabetes.

As with the saliva production, it is also not clear if parasympathetic control of other organs is affected by diabetes. For example, bladder dysfunction such as its incomplete emptying and overdistension are thought to happen in diabetes because of decreased parasympathetic control (Norden et al., 1988, Kaplan et al., 1995). To test this idea, Tompkins and colleagues studied the function of the parasympathetic pelvic ganglion which innervates the bladder. The researchers recorded synaptic transmission in the pelvic ganglion of diabetic and control mice, but found that the synaptic transmission in this ganglion remained normal in diabetes (Tompkins et al., 2013). It remains unclear if parasympathetic neurons are affected by diabetes.

Neuronal populations differ in their susceptibility to diabetes

It appears that some neurons are less vulnerable than others to the complications of diabetes. The central neurons of the brain and the spinal cord are relatively unaffected by the hyperglycemia of diabetes. Although some researchers found associations of diabetes with cognitive dysfunction (Reaven et al., 1990, Cukierman-Yaffe et al., 2009), it is not common to find serious abnormalities such as degeneration affecting central neurons in diabetes. The likely reason for this is that central neurons are protected from hyperglycemia by the blood-brain barrier. The blood-brain barrier is made up of closely-packed endothelial cells and pericytes with tight

junctions and has to be crossed, which has an effect of lowering the glucose levels (Brightman and Reese, 1969). For example, the glucose levels in the extracellular fluid of the brain in normoglycemic rats were measured at 1-2mM (McNay and Gold, 1999) or even less (Fellows et al., 1992), much lower than the normal blood glucose levels of 5-6mM. Brain glucose levels during hyperglycemia were measured by Silver and Ereciska, who used substrate-specific microelectrodes in anesthesized rats. They found that when blood glucose levels were at 15mM, the glucose levels in the extracellular fluid of the brain were only at 4.5mM (Silver and Erecinska, 1994).

On the other hand, peripheral neurons do not have an effective blood-nerve barrier. It was shown that the endothelial cells of the blood vessels supplying peripheral ganglia have fenestrations of up to 70 nm (Jacobs, 1977), through which glucose, being about 10Å in diameter, should easily pass. In fact experiments with horseradish peroxidase, which is larger in size than glucose, showed that the molecule penetrates well into the interneuronal spaces of the dorsal root ganglia (Jacobs et al., 1976, Arvidson, 1979), the autonomic ganglia as well as the adrenal medulla (Jacobs, 1977) within minutes of its intravenous injection in rats, mice and guinea pigs. The blood vessels supplying the ventral roots of the spinal cord which contain motor neuron axons also allow penetration of albumin (1.4nm in diameter) injected intravenously in rats, showing that no effective blood brain barrier is present (Olsson, 1968).

Most peripheral neurons are affected by diabetes to some degree, as I have described, but they show differences in vulnerability. The cell bodies of motor neurons are affected less severely than those of sensory, likely because they are contained within the spinal cord. Similarly to the brain, hyperglycemia for the motor neurons' cell

bodies is prevented by the blood-nerve barrier. However, neither the axons of sensory neurons nor those of motor neurons have an effective blood-nerve barrier, and therefore other factors must contribute to their different vulnerabilities (Ramji et al., 2007). High metabolic demand of the sensory neurons' axons is one explanation offered to account for these axons' vulnerability (Kadekaro et al., 1985, Zochodne et al., 2008), although the exact reason has not been established.

Comparing sympathetic and parasympathetic function in diabetes, it is not clear whether both autonomic branches are affected similarly. Almost all organs receive dual innervation (with the exception of the adrenal medulla, the sweat glands and the skin which receive sympathetic input only) and therefore both sympathetic and parasympathetic dysfunction could disrupt the regulation of various organs. There are no studies exploring possible differences in vulnerability of sympathetic and parasympathetic neurons in diabetes.

Oxidative Stress in Diabetes

Many cells and tissues of diabetic people show evidence of oxidative stress. This is usually detected as increased amounts of end products of oxidation in their tissue, plasma, or urine. For example, the urine of diabetics shows a 2-fold elevation of lipid peroxidation product molondialdehyde (Hoeldtke et al., 2011). Interestingly, the authors report that high molondialdehyde amounts were correlated with sympathetic nerve dysfunction in patients, measured by a decrease in sweating. The urine and leukocytes of diabetics also show increased amounts of oxidative DNA damage product, 8-

hydroxydeoxyguanosine, which correlates with developing nephropathy and retinopathy (Hinokio et al., 2002).

Importantly, evidence of increased oxidative stress can be seen in peripheral neurons in diabetes. Researchers report elevated hydroperoxides and lower levels of reduced glutathione in sympathetic and sensory nerves of diabetic animals (Nickander et al., 1994, Nagamatsu et al., 1995, Low et al., 1997). Zherebitskaya and colleagues report oxidative damage and mitochondrial dysfunction in sensory neurons of diabetic rats (Zherebitskaya et al., 2009).

Hyperglycemia elevates neuronal ROS in diabetes

It is known that hyperglycemia causes an elevation in ROS in diabetes. Mice that are fed with high glucose diet develop hyperglycemia and show oxidative stress (Folmer et al., 2002). Hyperglycemic cultures of vascular endothelial cells and renal mesangial cells also show oxidative stress (Giardino et al., 1996, Catherwood et al., 2002). There could be multiple mechanisms by which hyperglycemia elevates ROS. The most studied mechanisms for diabetes-induced ROS elevation in neurons are the following.

Brownlee proposed that hyperglycemia increases the traffick of electrons through the mitochondrial electron transport chain, causing a high mitochondrial potential. As a result, the electron transfer at the complex III slows down, causing electrons to leak to molecular oxygen, producing superoxide, $O^{\bullet -}$(Brownlee, 2001, 2005). Superoxide can be then converted by manganese superoxide dismutase to hydrogen peroxide, H_2O_2. If H_2O_2 is not detoxified by enzymes such as catalase, in the presence of iron, highly reactive hydroxyl radical can be produced.

Polyol pathway becomes overactivated in hyperglycemia. When glucose levels become elevated, glucose is used by aldose reductase as a substrate. NADPH is a co-factor of aldose reductase but also a co-factor of many anti-oxidant enzymes, including glutathione reductase. With high aldose reductase activity, decreasing levels of NADPH cause oxidative stress. Several studies with diabetic rats showed that inhibitors of aldose reductase led to improvements in neuropathy, such as improved nerve conduction velocity and lower oxidative stress (Stevens et al., 1994, Obrosova et al., 2002). However, clinical trials of several aldose reductase inhibitors in diabetic humans with neuropathy showed little effect: some studies showed small improvements in nerve conduction velocity while others showed no improvements (Schemmel et al., 2010).

Advanced glycation products also contribute to oxidative damage of hyperglycemia. They form when glucose slowly reacts with amines to form a Schiff base, which can rearrange and produce glycated proteins. When these glycated proteins become oxidized, cross-linked advanced glycation end-products form and can change the structure and function of proteins (Brownlee, 2001, Neves, 2013). Hemoglobin glycation is an example of this process, and it is commonly used as a measure of hyperglycemia in diabetic patients (Lyons and Basu, 2012).

Although it is not known whether any of these mechanisms cause oxidative stress in autonomic neurons, there is evidence that hyperglycemia elevates ROS at least in some autonomic neurons. Using cultured neurons from SCG, and elevating glucose from 5mM to 25mM, Campanucci, Krishnaswamy and Cooper observed an increase in ROS, as measured by an oxidation-sensitive dye H2DCFDA (Campanucci

et al., 2010). Whether parasympathetic neurons similarly show elevated ROS in hyperglycemic conditions is not known.

What is known about the causes of autonomic neuropathies

Recent research identified some structural and functional defects in some autonomic ganglia in diabetes.

Structural changes in autonomic ganglia in diabetes

Schmidt and colleagues studied sympathetic ganglia of diabetic rodents and humans. They found that many morphological abnormalities happen in prevertebral sympathetic ganglia including superior mesenteric ganglion. For example, abnormalities were seen in mitochondria, autophagosomes, the nuclei of neurons; there were accumulations of multivesicular bodies, and enlarged axon terminals. However, the researchers did not see any such abnormalities in paravertebral SCG (Schmidt et al., 1993, Schmidt et al., 2003, Schmidt et al., 2008, Schmidt et al., 2009). This finding is surprising, because organs innervated by paravertebral ganglia, such as the pupil of the eye do show sympathetic dysfunction in diabetes (Smith and Smith, 1983). It is then possible that functional problems rather than structural ones in these ganglia are responsible for such dysautonomias in diabetes.

Functional defects in autonomic ganglia in diabetes

There is evidence that the function of some autonomic ganglia is abnormal in diabetes. For example, Gallego and colleagues measured norepinephrine and epinephrine in different tissues of diabetic rats using HPLC. The authors saw that

norepinephrine was decreased in stellate ganglia and in the serum, however was increased in cardiac ventricles. Epinephrine was reduced in the serum but increased in the adrenal gland (Gallego et al., 2003). One explanation of these results could be a reduced activity of sympathetic ganglia and adrenal medulla in diabetes, causing less norepinephrine and epinephrine to be released. However, this causes relatively higher amounts of these molecules to be detected in the sympathetic axon terminals at target organs and in the adrenal gland.

Recent findings from the Cooper lab showed that in diabetes, hyperglycemia causes an elevation in intracellular ROS in the SCG, and these ROS oxidise and inactivate the postsynaptic nAChR in a use-dependent way (Fig 1.4). As a result, synaptic transmission in the SCG becomes depressed in diabetic mice, and the mice develop dysautonomias (Campanucci et al., 2010).

Whether parasympathetic and other autonomic ganglia have a disruption of synaptic transmission in diabetes is not clear. In a recent study, Tompkins and colleagues studied synaptic transmission in the parasympathetic pelvic ganglion in diabetic mice, but found no depression of synaptic transmission in this ganglion (Tompkins et al., 2013).

Learning whether different autonomic ganglia show synaptic dysfunction in diabetes could help in understanding and treating neuropathies. In addition, to treat autonomic neuropathies, it is important to know if the abnormalities can be reversed once they have appeared. Interest in these issues motivated the studies that are part of this thesis.

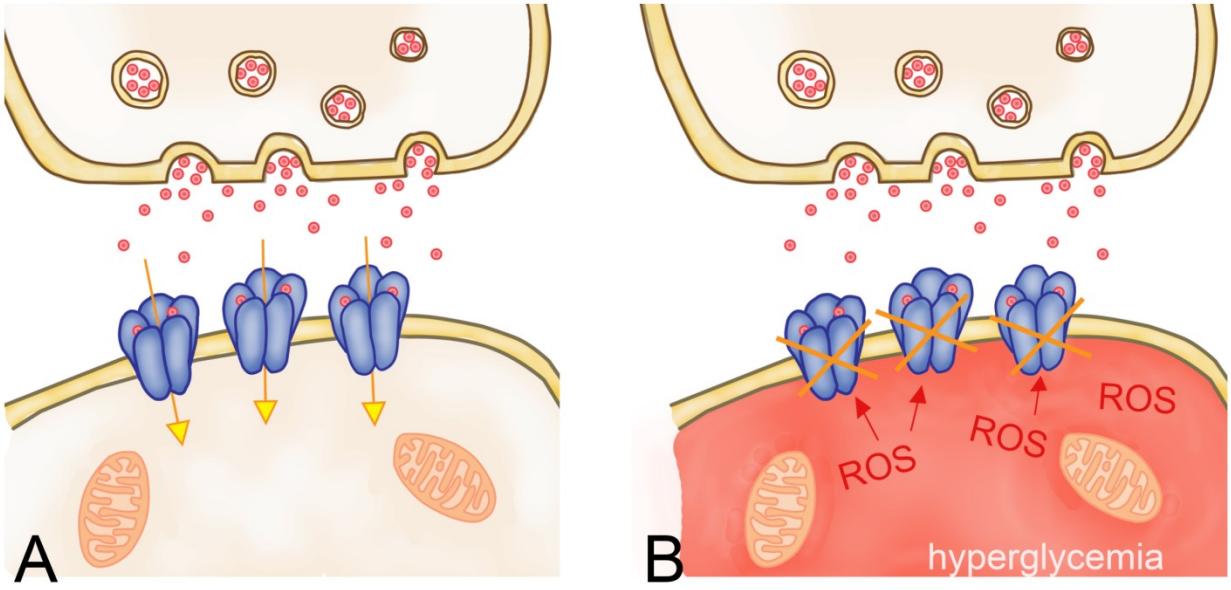


Figure 1.4 Synapse in a sympathetic superior cervical ganglion is shown.

A: In healthy conditions, acetylcholine released from the presynaptic terminal binds to the postsynaptic nicotinic acetylcholine receptors. The activated receptors conduct ions, producing excitatory postsynaptic potentials.

B: In hyperglycemia, ROS become elevated in postganglionic neurons. These elevated intracellular ROS inactivate the nicotinic acetylcholine receptors and depress the excitatory postsynaptic potentials in the postganglionic neuron.

OBJECTIVES

Although autonomic neuropathies are very serious and common complications of diabetes, much about autonomic neuropathies is not understood. It is not clear why the parasympathetic dysfunction is less pronounced than sympathetic in diabetes. It is not known whether autonomic dysfunction can be reversed or prevented.

While many factors may contribute to how neuropathies develop and progress, in my experiments I focused on studying synaptic transmission in autonomic ganglia.

I have the following aims:

- To find out if inactivation of synaptic transmission happens in different sympathetic and parasympathetic ganglia of diabetic mice
- To test if this inactivation of synaptic transmission is reversible
- To test if it can be prevented by lowered oxidative stress in a mutant mouse strain WLD^s.

HYPOTHESES

I hypothesize the following:

- All autonomic ganglia will have depressed synaptic transmission in diabetes. This will happen provided that 2 conditions are met: 1) α3-containing nAChRs mediate synaptic transmission, and 2) intracellular ROS become elevated.
- If ROS levels return to normal levels after a period of hyperglycemia, the function of the nAChR should recover at the plasma memebrane and synaptic transmission should recover *in vivo*.

- If ROS levels are lowered in the SCG, there will be no depression of synaptic transmission with diabetes.

Specific Questions

1) Do the prevertebral sympathetic superior mesenteric ganglion, the adrenal medulla, and the parasympathetic submandibular ganglion show a depression of synaptic transmission in diabetes?
2) Once nAChRs on cultured sympathetic neurons become inactivated by ROS, how soon does their function recover at the plasma membrane? When hyperglycemia is reversed *in vivo* in diabetic mice treated with insulin, does synaptic transmission recover?
3) When WLD^s mouse strain, known to have lower ROS levels, is made diabetic, does synaptic transmission become depressed in the SCG?

CHAPTER 2

MATERIALS AND METHODS

Animals

We maintained the mice for the experiments at the McGill Animal Resource Centre. The mice had access to standard mouse chow and water and were on a 12h dark-12h light cycle. When breeding mice, we weaned them at 3 weeks of age. For experiments with wild type mice, mice of the C57BL/6J strain and mice on the mixed C57BL/6J x CD1 background were used. All procedures for animal handling were performed according to the guidelines of the Canadian Council on Animal Care.

Diabetes Mouse Models

To study the effects of diabetes, 2 mouse models were used: Akita mutant mice and streptozotocin (STZ) - injected diabetic mice.

Akita mice

Akita mice were on the C57BL/6J background and had a missense mutation in the insulin 2 gene (*Ins2)*, inherited in an autosomal dominant way. These mice were purchased from the Jackson Laboratory. Previous studies showed that among homozygous Akita mice, both male and female develop severe diabetes, however do not survive well. Among heterozygous Akita mice, males show more severe diabetes than females, and develop high blood glucose by 3-4 weeks of age (Yoshioka et al., 1997). Since homozygous Akita mice have low survival, we used the heterozygous male Akita mice in our studies. We bred mice for experiments by crossing heterozygous female Akita mice with wild-type C57BL/6/J male mice. The offspring of these mice were genotyped using PCR to detect the *Ins2* mutation. We measured blood glucose by obtaining a small drop (<5 µl) of blood from mouse tail and testing it with Bayer Contour

glucose test strips. We used mice that developed blood glucose >20mM by 4 weeks of age.

Streptozotocin (STZ) injections

We made wild type mice diabetic with STZ injections. We injected mice at 3-4 weeks of age intraperitoneally with STZ (40-60mg/kg) dissolved in Na^+ citrate buffer. Prior to these injections, the mice were deprived of food for 6 hrs. The food was returned immediately after the injection. Blood glucose levels were monitored at regular intervals with Bayer Contour glucose test strips. Within 48 hrs of STZ injection, blood glucose levels were >20mM in approximately 80% of mice and remained elevated.

Genotyping

DNA extraction

We obtained DNA samples from mouse tail tissue (~2-3mm). The tail tissue was digested with Proteinase K (10mg/ml) in Lysis Buffer at 55 °C overnight, after which the samples were boiled to inactivate Proteinase K. DNA from the sample was then amplified with PCR and products run on an agarose gel.

PCR - Akita mice

We used the forward primer: 5'-TGC TGA TGC CCT GGC CTG CT-3' and the reverse primer 5'-TGG TCC CAC ATA TGC ACA TG-3' to amplify a 280bp PCR product of the *Ins2* gene. We ran the PCR using a thermal controller using the following

program: 94° C, 3min; 12 cycles of: 94° C, 20 s; 64° (with each cycle, lower this by 0.5° C), 30 s; 72° C, 35 s; then repeat for 25 more cycles using the annealing t° of 58°C.

Enzymatic digestion

Following PCR, we digested the amplified 280bp product overnight (>6hrs) at 37 °C with the restriction enzyme, Fnu4HI. The wild type allele product is cut by Fnu4HI producing 140bp product. In the mutant *Ins2* gene, the restriction site is absent and the product remains at 280bp. We ran the products on a 2.5% agarose gel at 90V for 50min.

Primary Cell Culture

The ganglia or the adrenal medulla were removed from postnatal day 5 (P5) to P20 mice under sterile conditions and were cleaned them from non-neuronal tissues. We incubated them in Hanks Balanced Salt Solution (HBSS) containing trypsin (1mg/ml, Worthingtin, Freehold, NJ), and buffered with HEPES (adjusted to pH 7.4 with NaOH) at 37°C for up to 45 min. For adrenal medullae, we used Ca^{2+} and Mg^{2+} -free HBSS. We then dissociated the tissue by passing it through a fire-polished glass pipette. The resulting cell suspension was washed twice in serum-containing medium and plated on laminin-coated coverslips attached to modified 35mm culture dishes (Campanucci et al., 2008). Sympathetic neurons were grown in media consisting of L-15 supplemented with 5 mM glucose, vitamins, cofactors, penicillin-streptomycin, 5% rat serum and 25ng/ml Nerve Growth Factor (NGF). Adrenal chromaffin cells were grown in the same media, but without NGF. For parasympathetic neurons, we supplemented the

growth media with ciliary-derived neurotrophic factor (CNTF), glia-derived neurotrophic factor (GDNF), and neurturin, all at 10 ng/ml.

To reduce the growth of non-neuronal cells, cultures were treated with cytosine arabinoside (10 μM; Sigma, St. Louis, MO) for the first 48-72 hrs after plating. Cultures were maintained at 37 °C in an incubator with humidified atmosphere of 95% air and 5% CO_2, and fresh growth media was added every 3-4 days. To investigate the effects of hyperglycemia, we elevated glucose in the media from 5 mM to 25 mM.

Electrophysiology

Whole cell patch clamp

Equipment setup

The cell culture dishes were mounted on an inverted microscope (Axiovert 200M, Zeiss, Oberkochen, Germany) and viewed cells through a 40x objective. The dishes were perfused continuously at 1ml/min with extracellular recording solution containing (in mM): 140 NaCl, 5.4 KCl, 0.33 NaH2PO4, 0.44 KH2PO4, 2.8, CaCl2, 0.18 MgCl2, 10 HEPES, 5.6 glucose, 2 glutamine, with pH adjusted to 7.4 with NaOH. We recorded membrane currents with a VE-2 amplifier (Alembic Instruments, Montreal, Quebec, Canada) at room temperature (23-25 °C). The patch electrodes had resistances of 2-5 MΩ and the series resistance was reduced by 85-100%. The electrodes were filled with a solution containing (in mM) 65 KF, 55 KAc, 5 NaCl, 0.2 CaCl2, 1 MgCl2, 10 EGTA, and 10 HEPES, and pH was adjusted to 7.2 with KOH.

Recording

To record ACh-evoked currents, ACh (100 μM) was dissolved in the perfusion recording solution and applied to neurons by pressure ejection from pipettes with tip diameters of 2-5 μm positioned 20-30 μm from the cell body (Fig 2.1) (Campanucci et al., 2008). To increase cytosolic ROS in individual neurons, we dissolved antimycin-A in the recording solution at 1 μM, 2 μM, and 10 μM (a 1:10000, 2:10000, and 1:1000 dilutions, respectively, from a 10 mM stock dissolved in EtOH). As control, we used an identical solution (0.01, 0.02, and 0.1% EtOH) without antimycin-A. As a measure of rundown of the ACh-evoked currents we plotted the ratio of the peak current (I) in response to the 30th or 60th application (I_{30} or I_{60}) to the first application (I_1) in the series. The data were acquired using Igor Pro software (WaveMetrics, Lake Oswego, OR), and were filtered and analyzed with Igor Pro offline.

Synaptic transmission recordings

Dissections

Each ganglion or adrenal medulla was dissected while being perfused continuously with the oxygenated modified Tyrode's (2.5 mM Ca^{2+}) solution. For each ganglion and the adrenal medulla, the preganglionic nerve was carefully freed from surrounding tissue. For the adrenal gland, the medullary chromaffin cells were exposed by cutting off a portion of the adrenal cortex tissue on adrenal gland's ventral side. Some tissue surrounding each ganglion was kept for pinning during the recording.

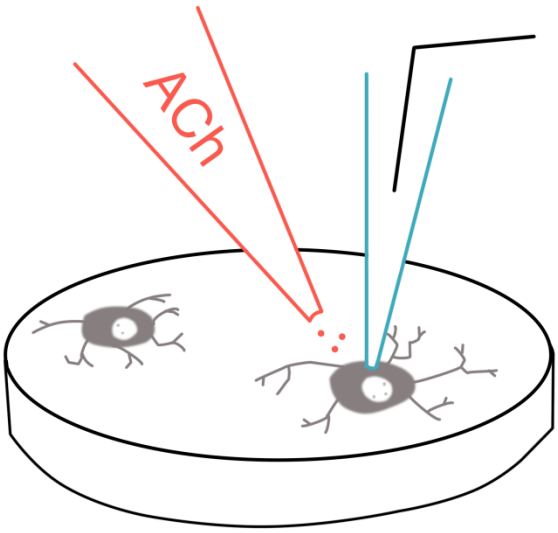


Fig 2.1

Figure 2.1 Schematic of the whole cell patch clamp recording of acetylcholine-evoked currents.

Equipment setup

The ganglia and the adrenal medullae were pinned down to the Sylgard floor (Dow Corning, Midland, MI) of a recording chamber (1.5 ml volume) mounted on a fixed stage of a dissecting microscope (SMZ-10; Nikon, Tokyo, Japan). The chamber was perfused continuously at 3-4 ml/min with a modified oxygenated Tyrode's (2.5 mM Ca^{2+}) solution at 33-36 °C. We used 60-100 MΩ glass microelectrodes (G150F-4; Warner Instruments) made with a DMZ universal puller (Zeitz Instruments, Munich, Germany) as described previously (Rassadi et al., 2005). To get stable recordings, a high inertial precision microdrive (Inchworm 8200; EXFO, Vanier, Quebec, Canada) was used, attached to a micromanipulator (SM11; Narshige, Tokyo, Japan) that drove the electrode through the ganglia or the medulla. The recording electrode was filled with 1M KAc and connected by a thin silver chloride wire to the head stage of an Axoclamp 2A amplifier (Molecular Devices, Sunnyvale, CA) used in current-clamp mode. The preganglionic nerve was stimulated using a stimulator (410 ORTEC dual channel; EG&G, Gaithersburg, MD).

The data were aquired using Igor Pro software (WaveMetrics, Lake Oswego, OR), and analyzed offline using Igor Pro.

Recording

Synaptic transmission in autonomic ganglia and the adrenal medullae was measured by recording nerve-evoked EPSPs with intracellular electrodes while stimulating the preganglionic nerve. For each ganglion and the adrenal medulla, the preganglionic nerve (sympathetic trunk for the SCG; left splanchnic nerve for the SMG;

the splanchnic nerve for the adrenal medulla; the lingual nerve for the submandibular ganglion) was connected to a stimulator with a suction electrode and stimulated with brief (0.1-0.3 ms) voltage pulses. To quantify synaptic transmission, the preganglionic nerve was stimulated at 0.5-1Hz and individual EPSPs were integrated. For suprathreshold EPSPs, the area bound by the action potential (AP) spike was integrated and then subtracted from the total area; the area under the AP was usually <2% of the total area. Recordings were made only from neurons with membrane potentials greater than -45 mV, and neurons were held at -80 to -85 mV to minimize any afterhyperpolarizations. To elevate ROS in some intracellular recordings, we added antimycin-A (100 μM, Sigma) to the recording electrode solution (a 1:100 dilution from a 10 mM stock dissolved in EtOH); as control, we recorded from neurons in identical solution (1% EtOH) without antimycin-A.

Statistics

To test for statistical significance we used ANOVA or the Student's t test; for comparisons of the I_{30}/I_1 or I_{60}/I_1 ratios or EPSP areas, we used the nonparametric Mann-Whitney test.

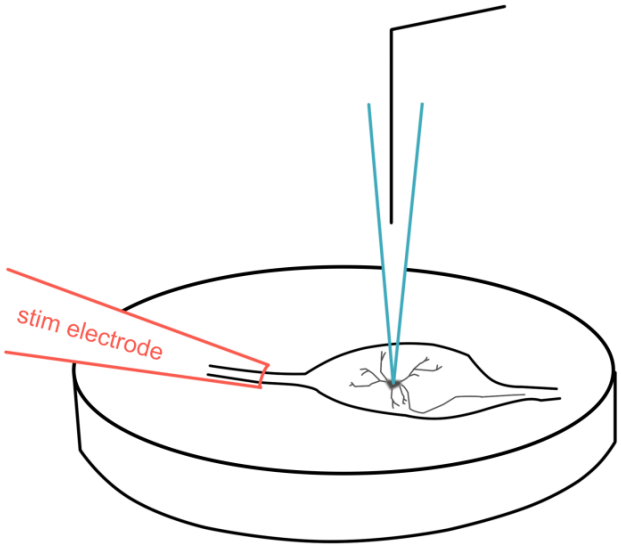


Fig 2.2

Figure 2.2 Schematic of the intracellular recording of nerve-evoked EPSPs in autonomic ganglia.

CHAPTER 3

Synapses on sympathetic neurons and parasympathetic neurons differ in their vulnerability to diabetes

ABSTRACT

Synapses in autonomic ganglia represent the final output of various structures in the central nervous system that regulate the function of the periphery. Normally, these excitatory cholinergic-nicotinic synapses produce large suprathreshold EPSPs on sympathetic and parasympathetic neurons to convey signals from the central nervous system. In certain disease states, however, synaptic transmission in autonomic ganglia is depressed and the periphery becomes deregulated. For example, previous work demonstrated that hyperglycemia depresses EPSPs on sympathetic neurons, and disrupts sympathetic reflexes, by causing a ROS-dependent inactivation of the postsynaptic nAChRs. What is not clear, however, is whether some autonomic neurons are more vulnerable to hyperglycemia than others. One possibility is that sympathetic neurons may be more prone than cholinergic parasympathetic neurons to hyperglycemia-induced elevations in cytosolic ROS because sympathetic neurons contain several pro-oxidant molecules involved in noradrenaline metabolism. To test this hypothesis, we recorded synaptic transmission from different mouse sympathetic and parasympathetic ganglia, as well as from the adrenal medulla. In addition, we used cellular imaging to measure hyperglycemia-induced changes in cytosolic ROS and whole-cell recordings to measure the use-dependent rundown of ACh-evoked currents. Our results demonstrate that hyperglycemia depresses synaptic transmission on sympathetic neurons and adrenal chromaffin cells, and elevates cytosolic ROS. On the other hand, hyperglycemia has little effect on synaptic transmission at synapses on parasympathetic neurons. We conclude that sympathetic neurons and adrenal

chromaffin cells are more vulnerable to diabetes than parasympathetic neurons, a finding that may have implications for both long-term diabetic autonomic neuropathies, as well as for insulin-induced hypoglycemia, a serious complication of diabetes.

INTRODUCTION

Most people with diabetes develop problems with their autonomic nervous system that severely affect their quality of life and shorten their life expectancy. Symptoms range in severity and include cardiac arrhythmias, orthostatic hypotension, gastrointestinal abnormalities, and poor control of the circulation (Vinik et al., 2003, Freeman, 2005, Vinik and Ziegler, 2007, Kuehl and Stevens, 2012). There is much about this complication that is poorly understood.

One puzzling aspect is that diabetes does not seem to affect all autonomic nerves in the same way. For example, research on patients with diabetes shows that many sympathetic nerve endings in the prevertebral superior mesenteric and celiac ganglia are significantly enlarged and appear dystrophic (Schmidt, 1996); however, there are no structural abnormalities in the paravertebral sympathetic ganglia, ganglia involved in regulating the cardiovascular system (Schmidt et al., 2008, Schmidt et al., 2009). These findings suggest that some autonomic neurons are more sensitive than others to the detrimental effects of hyperglycemia. Since there are no morphological changes in paravertebral ganglia, these findings also suggest that diabetic cardiovascular dysautonomia results from functional rather than structural defects. Consistent with this idea, we discovered recently that diabetic mice have functional defects in the paravertebral superior cervical ganglia (SCG). Briefly, we showed that hyperglycemia elevates reactive oxygen species (ROS) in SCG neurons; ROS then act on highly conserved cysteine residues located in α3, α2 and α4 nicotinic acetylcholine receptor (nAChR) subunits near the intracellular mouth of the receptor pore to depress

ganglionic synaptic transmission and reduce the function of sympathetic reflexes (Campanucci et al., 2010, Krishnaswamy and Cooper, 2012).

The question is: how widespread is this mechanism in the autonomic nervous system? Answers to this question may provide clues to the underlying causes of the dysautonomia. Specifically, does hyperglycemia inactivate postsynaptic nAChRs and depress synaptic transmission in other autonomic ganglia, or the adrenal medulla, a gland that is directly involved in the defense against insulin-induced hypoglycemia. For this to be the case, two criteria must be satisfied: one, the postsynaptic nAChRs must contain the conserved cysteine residues; and two, hyperglycemia should lead to an elevation in cytosolic ROS.

Concerning postsynaptic nAChRs, for neurons in many autonomic ganglia, the nAChR subtypes have not been fully resolved. While there is good evidence that α3-containing nAChRs are present on many autonomic neurons, there is evidence that synaptic transmission in parasympathetic neurons in chick is mediated by both α3-containing nAChRs and α7-containing nAChRs (Zhang et al., 1996, Ullian et al., 1997), a subtype that lacks the conserved cysteine (Krishnaswamy and Cooper, 2012). As for the adrenal medulla, there is evidence that these cells express α7 or α9 nAChR subunits as well as α3 (Criado et al., 1997, Colomer et al., 2010), however, the alpha subunit composition of the postsynaptic receptors has not been resolved.

Nor is there much information about hyperglycemia-induced elevations in cytosolic ROS in autonomic neurons. Do all autonomic neurons respond similarly? Or, for example, are noradrenergic sympathetic neurons and adrenal chromaffin cells rendered more sensitive to hyperglycemia than cholinergic parasympathetic neurons, in

part, because they contain highly reactive products related to catecholamine synthesis and metabolism that compromise their defenses against oxidative stress (Coyle and Puttfarcken, 1993)?

To address these issues, we have investigated synaptic transmission in three branches of the autonomic nervous system: a prevertebral sympathetic ganglion, the superior mesenteric; a parasympathetic ganglion, the submandibular; and the adrenal medulla. In all three, we find that the nerve-evoked EPSPs are mediated by α3-containing nAChRs. Synaptic transmission is markedly depressed in the superior mesenteric ganglia and in the adrenal medulla within 1 week of diabetes. Unexpectedly, however, synaptic transmission in the parasympathetic submandibular ganglion is only marginally affected by diabetes, even after 4 months. Using combined ROS imaging and electrophysiology, we show that this differential effect on synaptic transmission occurs because sympathetic neurons are more vulnerable to hyperglycemia-induced oxidative stress than parasympathetic neurons.

METHODS

Animals

In addition to using diabetic mice, which are described in Chapter 2, we used mice with a deletion in the α3 nAChR subunit gene.

α3 nAChR subunit gene-null mice

(gift from Dr.A. Beadeut, Baylor College of Medicine).

We used 1-5 month old wild-type and homozygote α3 nAChR subunit gene-null mice (α3 KO) on out-crossed C57BL/6/J x CD1 background. To obtain these animals, we mated male and female heterozygous (α3 +/-) mice and genotyped the offspring.

Genotyping α3 KO mice

PCR was done with:

a wild-type forward primer, 5'-GTGGATCCCTCCGGCCATCTTTAAGAG;

wild-type reverse primer, 5'-GACTGTG-ATGACAATGGACAAGGTGAC;

mutant reverse primer, 5'-TGGCGCGAAGGGACCACCAAAGAACGG.

Intracellular ROS measurements

To monitor ROS levels, we loaded cultures with the redox-sensitive dye CM-H2DCFDA, an acetoxymethyl ester (Molecular probes, Burlington, Ontario), which was dissolved in perfusion solution at 10 μM. The cultures were incubated for 30 min at 37 °C, then washed three times with recording perfusion solution. The cultures were then placed on the stage of an inverted microscope (Axiovert 200M; Zeiss, Oberkochen,

Germany) and viewed though a 40X (1.3 numerical aperture) Plan Neofluor oil-immersion objective (Zeiss). The cells were perfused continuously and whole-cell recordings were done as described earlier. For simultaneous ROS measurements, we acquired DIC and fluorescent images (Campanucci et al., 2008). We excited the dye at wavelengths of 450-480 nm for 200 ms using a 150W xenon arc lamp (LAMBDA DG-4; Sutter Instruments, Novato,CA), and the signal was detected at an emission wavelength of 510-550 nm (filter ser 31001;Chroma Technology, Brattleboro, VT) with a cooled CCD camera (CoolSnap HQ; PhotoMetrics, Tucson, AZ) controlled by Metafluor software (Universal Imaging, West Chester, PA). On the DIC images, we defined regions of interest (neuronal cell body, excluding the nucleus) and transferred these to the fluorescent images of the same field. We quantified changes in mean CM-H2DCFDA fluorescence intensity over time by acquiring images every 25 s. The background fluorescence (F) was determined from neighbouring area. For each neuron, we subtracted its initial fluorescent intensity from final fluorescent intensity to obtain the change in fluorescent intensity (ΔF) and expressed it as ΔF/F.

For other details on methodology, see Chapter 2.

RESULTS

Superior mesenteric ganglion

To investigate the effects of diabetes on synaptic transmission in the superior mesenteric ganglion (SMG), we recorded with intracellular electrodes from SMG neurons of 1-2 month old mice while stimulating the left splanchnic nerve. Some neurons in the anterior part of the ganglion send axons out through the splanchnic nerve and become excited antidromically (Miller et al., 1996). To avoid this, we recorded mainly from neurons located in the posterior part of the ganglion (Fig 3.1A). Stimulating the splanchnic nerve evoked large, suprathreshold fast EPSPs on SMG neurons; these EPSPs were reversibly blocked by hexamethonium (100 μM), indicating that these synapses were cholinergic-nicotinic (Fig 3.1B). To determine whether these EPSPs were mediated by α3-containing nAChRs, we recorded from superior mesenteric ganglia of α3 nAChR subunit null (α3 KO) mice (Xu et al., 1999, Rassadi et al., 2005). Splanchnic nerve stimulation failed to evoke EPSPs in over 90% of the neurons (Fig 3.1C), indicating that the EPSPs are mediated by α3-containing nAChRs.

Next, we investigated synaptic transmission in SMG one week and one month after the onset of diabetes. We found that EPSPs were markedly reduced on SMG neurons from diabetic mice compared to those in age-matched control mice (Fig 3.1D). On average the EPSPs were depressed by ~65% 1 week after the onset of diabetes, and this depression persisted for at least 1 month (Fig 3.1D).

The results in Fig 3.1D show that diabetes depresses synaptic transmission at synapses on prevertebral SMG neurons. Next, we asked whether hyperglycemia leads to an inactivation of the postsynaptic nAChRs on these neurons, as is the case for

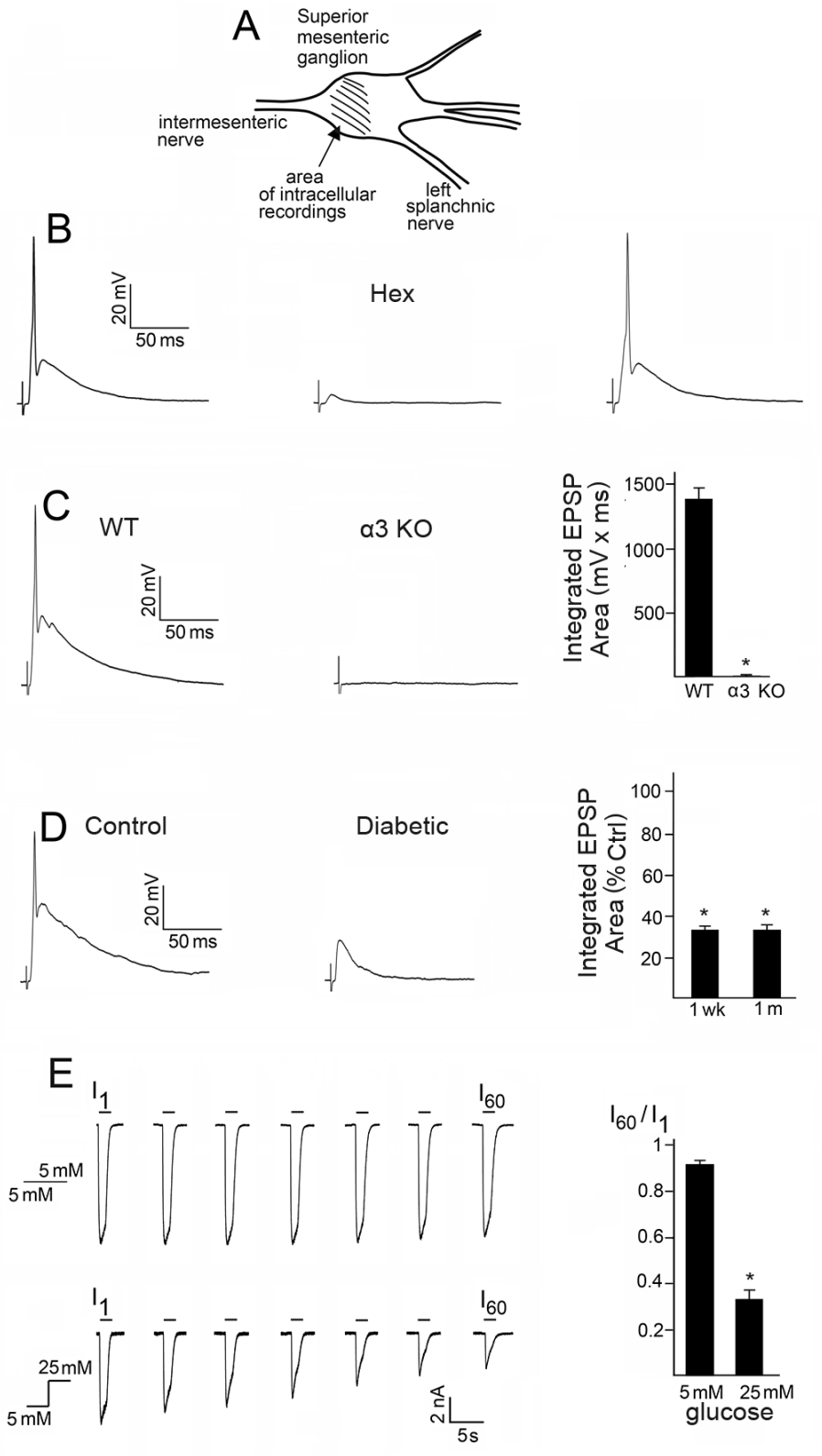

A
Superior mesenteric ganglion
intermesenteric nerve
area of intracellular recordings
left splanchnic nerve
B
20 mV
50 ms
Hex
C
WT
α3 KO
20 mV
50 ms
Integrated EPSP Area (mV x ms)
1500
1000
500
*
WT α3 KO
D
Control
Diabetic
20 mV
50 ms
Integrated EPSP Area (% Ctrl)
100
80
60
40
20
*
*
1 wk
1 m
E
I1
I60
5 mM
5 mM
25 mM
5 mM
2 nA
5s
I60 / I1
1
0.8
0.6
0.4
0.2
*
5 mM
25 mM
glucose

Fig 3.1

Fig 3.1 Synaptic transmission in sympathetic superior mesenteric ganglion (SMG) is mediated by α3-containing nAChRs and becomes depressed in diabetes.

(A) A schematic of the SMG showing the region of the ganglion used for recording.
(B) Left: nerve-evoked EPSPs in SMG in response to splanchnic nerve stimulation; Middle: response in hexamethonium (Hex, 100 μM); Right: response after washing out Hex.
(C) Left: nerve-evoked EPSPs in SMGs from 1 month-old WT and α3 KO age-matched mice. Right: average integrated EPSP size in SMGs of WT (n=60) and α3 KO mice (n=31). The values are means ±SEM. * represents $p<0.0001$.
(D) Left: nerve-evoked EPSPs in SMGs of 2 month-old WT control and 2 month-old Akita mice (5 wk after the onset of diabetes). Right: average integrated EPSPs in SMGs from mice 1 week (n=94) and 1 month (n=70) after the onset of diabetes; the data are % of EPSPs in SMG of age-matched WT controls (n=60 for 1 week; n=16 for 1 month). The data from diabetic mice include both Akita mice and WT mice made diabetic with STZ injections. n=60 for age-matched control SMG neurons to compare with those in mice that were diabetic for 1 week, and n=16 to compare with those in mice diabetic for 1 month. The values are the mean ±SEM and are significantly different from age-matched controls ($p<0.0001$).
(E) Elevated (25 mM) extracellular glucose causes rundown of ACh-evoked inward currents on cultured SMG neurons. Left: ACh-evoked currents on cultured SMG neurons in response to 1s application of ACh (100 μM) delivered at 15s intervals. (Top) cultured in 5 mM glucose for 2 weeks. (Bottom) cultured in 5mM glucose for the first week and then in 25mM glucose for the second week. Every fifth trace is shown for clarity. Right: ratio of the 60th ACh-evoked current to the first in the series (I_{60}/I_1) for neurons cultured in 5 mM glucose for 2 weeks (n=21) and for neurons cultured in 5mM glucose for the first week, then in 25mM glucose for the second week (n=15). The values are the mean ±SEM; * represents $p<0.0001$.

sympathetic neurons in the paravertebral superior cervical ganglion (SCG) (Campanucci et al., 2010). To address this, we recorded ACh-evoked inward currents on SMG neurons that developed in culture under hyperglycemic conditions. Neurons from 2-3 week-old mice were cultured for 7 d in 5 mM glucose and then glucose was elevated to 25 mM for 7 d; the controls were maintained in 5 mM glucose throughout the 14 day period. A single application of ACh (100 μM applied for 1s) evoked large (~5-7 nA) inward currents when measured at a holding potential of -60 mV (Fig 3.1E). On control neurons (neurons cultured in 5 mM glucose for 14 days), these ACh-evoked currents were remarkably stable in response to a series of repetitive ACh applications at 15 s intervals (Fig 3.1E). In contrast, we observed an irreversible rundown of the ACh-evoked currents on neurons cultured in 25 mM glucose; after 30 applications the peak I_{ACh} was ~50% of the first application, and reached a plateau of ~35% after 50-60 applications (Fig 3.1E).

These results indicate that hyperglycemia causes a use-dependent rundown of the ACh-evoked currents on prevertebral SMG neurons, similar to what it does on paravertebral sympathetic neurons in the SCG (Campanucci et al., 2010).

Adrenal medulla

Next, we investigated the effects of diabetes on synaptic transmission at synapses on chromaffin cells in the intact adrenal medulla. Stimulating the greater splanchnic nerve evoked large, cholinergic-nicotinic EPSPs on chromaffin cells, recorded intracellularly, that were reversibly blocked by hexamethonium (100 μM) (Fig 3.2A). These EPSPs were large enough to evoke action potentials on approximately

50% of the chromaffin cells. If these EPSPs are mediated by α3-containing nAChRs then they could be targets of elevated ROS; however, the subtype of nAChRs present postsynaptically at synapses between preganglionic terminals and chromaffin cells had not been fully established. Therefore, to determine whether the nerve-evoked EPSPs are mediated by α3-containing nAChRs, we recorded nerve-evoked EPSPs on chromaffin cells in intact adrenal medulla from α3 KO mice. In contrast to WT adrenal medullae, stimulating the greater splanchnic nerve innervating adrenal medullae from 1 month old α3 KO mice failed to evoke EPSPs on chromaffin cells, although all chromaffin cells evoked action potentials when stimulated directly. These results indicate that α3-containing nAChRs are the major postsynaptic receptors at preganglionic - chromaffin synapses in mice.

To determine whether diabetes depresses synaptic transmission in the adrenal medulla, we recorded nerve-evoked EPSPs on chromaffin cells 1 week and 1 month after the onset of diabetes. Within 1 week after the onset of diabetes, the nerve-evoked EPSPs were depressed by approximately 50% compared to those in age-matched control mice (Fig 3.2C), and this depression persisted for at least 1 month (Fig 3.2C).

The results in Fig 3.2C show that diabetes induces a depression in synaptic transmission at synapses on adrenal chromaffin cells. To determine whether hyperglycemia inactivates the postsynaptic nAChRs on chromaffin cells, we recorded ACh-evoked inward currents on chromaffin cells that developed in culture initially in 5 mM glucose for 7 days and then were switched to 25 mM glucose for 7 days; as control, we recorded from chromaffin cells that were cultured in 5 mM glucose throughout the 14 day period.

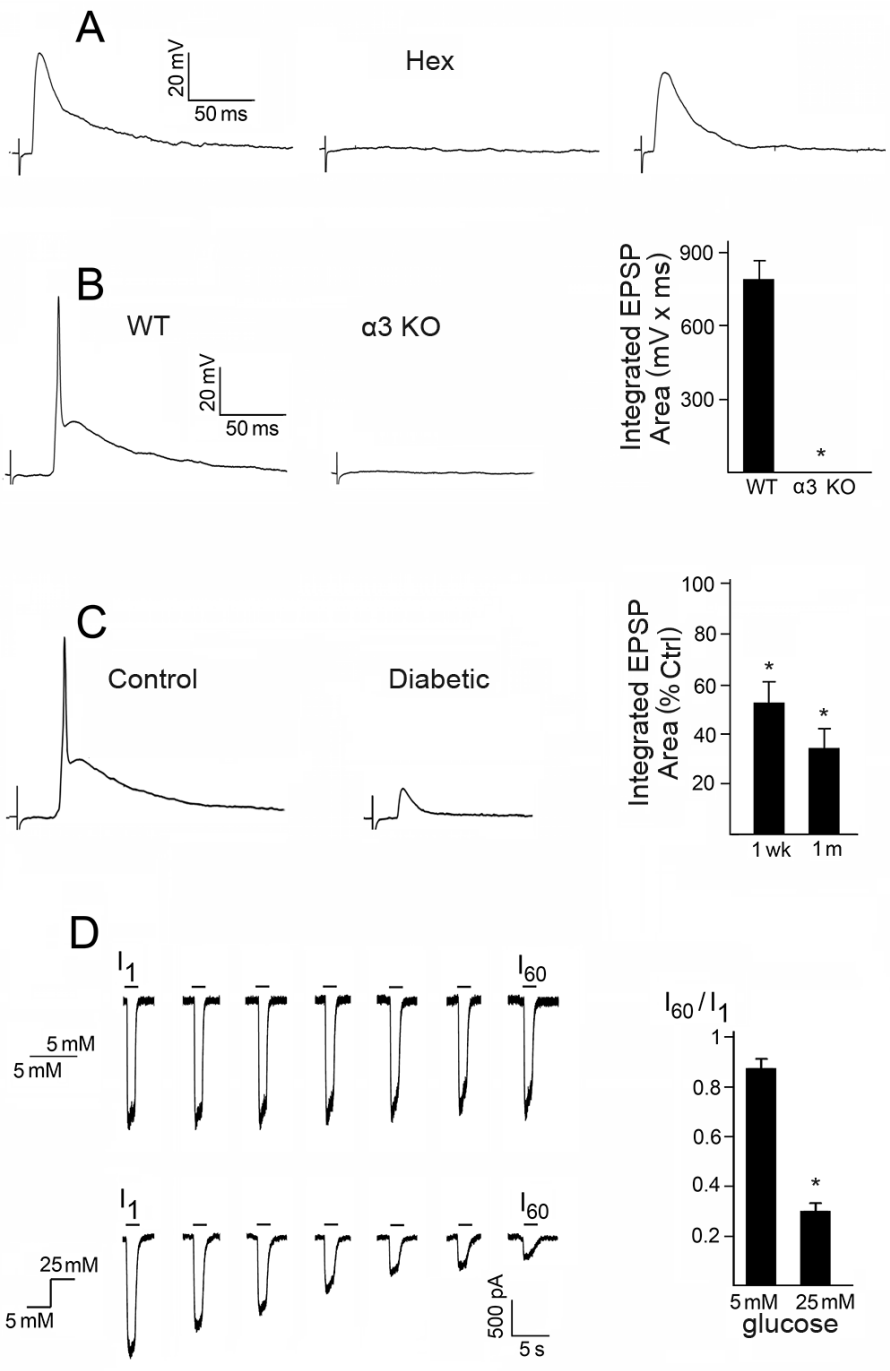

A
20 mV
50 ms
Hex
B
WT
α3 KO
20 mV
50 ms
Integrated EPSP
Area (mV x ms)
900
600
300
*
WT α3 KO
C
Control
Diabetic
Integrated EPSP
Area (% Ctrl)
100
80
60
40
20
*
*
1 wk 1 m
D
I1
I60
5 mM
5 mM
I1
I60
25 mM
5 mM
500 pA
5 s
I60 / I1
1
0.8
0.6
0.4
0.2
*
5 mM 25 mM
glucose

Fig 3.2

Fig 3.2 Synaptic transmission in adrenal medulla is mediated by α3-containing nAChRs and becomes depressed in diabetes.

(A) Left: nerve-evoked EPSPs recorded intracellularly in adrenal medulla in response to splanchnic nerve stimulation; Middle: response in Hex (100 μM); Right: response after washing out Hex.

(B) Left: nerve-evoked EPSPs at synapses on WT and α3 KO adrenal chromaffin cells. Right: average integrated EPSPs on chromaffin cells in adrenals from WT (n= 26) and α3 KO mice (n=10). The values are means ±SEM. * represents $p<0.0001$.

(C) Left: nerve-evoked EPSPs at synapses on a chromaffin cell from a 1 month-old WT and from age-matched diabetic (Akita) mice. Right: average integrated EPSPs at synapses on chromaffin cells from mice 1 week (n=50) and 1 month (n=21) after the onset of diabetes; the data are expressed as a % of EPSPs at synapses on chromaffin cells from age-matched WT mice. The data from diabetic mice include both Akita mice and WT mice made diabetic with STZ injections. n=45 for age-matched control chromaffin cells to compare with those in mice that were diabetic for 1 week, and n=27 to compare with those in mice diabetic for 1 month. The values are the mean ±SEM; * represents $p<0.0001$.

(D) Elevated (25 mM) extracellular glucose causes rundown of ACh-evoked inward currents on cultured chromaffin cells. Left: ACh-evoked currents on cultured adrenal chromaffin cells in response to 1s application of ACh (100 μM) delivered at 15s intervals. (Top) cultured in 5 mM glucose for 2 weeks; (Bottom) cultured in 5 mM glucose for the first week and then in 25 mM glucose for the second week. Every fifth trace is shown for clarity. Right: ratio of the 60th ACh-evoked current to the first in the series (I_{60}/I_1) for neurons cultured in 5 mM glucose for 2 weeks (n=10) and for neurons cultured in 5 mM glucose for the first week, then in 25 mM glucose for the second week (n=11). The values are the mean ±SEM; * represents $p<0.0001$.

ACh applications (100 μM for 1s) evoked large (~1-3 nA) inward currents when measured at a holding potential of -60 mV (Fig 3.2D). On chromaffin cells in control media (5 mM glucose for 14 days), these ACh-evoked currents were remarkably stable in response to a series of repetitive ACh (100 μM) applications at 15 s intervals (Fig 3.2D). In contrast, we observed an irreversible rundown of the ACh-evoked currents on chromaffin cells cultured in 25 mM glucose for 7 d (Fig 3.2D); after the 30th application the peak I_{ACh} was ~50% of the first application, and reached a plateau of ~25% after 50-60 applications. These results indicate that hyperglycemia causes a use-dependent rundown of ACh-evoked currents on adrenal chromaffin cells, similar to its effects on the ACh currents on sympathetic neurons in both paravertebral SCG and prevertebral SMG.

Parasympathetic neurons

Synaptic transmission in ganglia throughout the autonomic nervous system is mediated by cholinergic-nicotinic synapses. Therefore, we had expected that diabetes would similarly depress synaptic transmission in parasympathetic ganglia, as it does in sympathetic ganglia and the adrenal medulla. To test this idea, we recorded intracellularly from parasympathetic neurons in intact submandibular ganglion of 1-2 month old mice while stimulating the preganglionic lingual nerve. As expected, stimulating the lingual nerve evoked large, suprathreshold fast EPSPs on submandibular neurons; these EPSPs were reversibly blocked by hexamethonium (100 μM) indicating that these synapses were cholinergic-nicotinic.

To determine whether these EPSPs are mediated by α3-containing nAChRs, we recorded nerve-evoked responses on submandibular ganglion neurons from α3 KO mice (Fig 3.3A). In many neurons, we observed a small EPSP of several mV in size. In a small number of α3 KO neurons, the nerve-evoked EPSPs were >10 mV (Fig 3.3A). We found that these EPSPs were mediated by α7 nAChRs, because we could not detect any EPSPs in α3 α7 double KO mice (Suppl Fig 1). In addition, we could block these EPSPs in α3 KO mice by methyllycaconitine (MLA) (Suppl Fig 1).

To establish whether diabetes depresses synaptic transmission in parasympathetic ganglia, we recorded nerve-evoked EPSPs on neurons in submandibular ganglia, 5 weeks after the onset of diabetes. In contrast to what we observed in sympathetic ganglia and the adrenal medulla, we found no statistical difference between nerve-evoked EPSPs on parasympathetic neurons from diabetic animals compared to those on age-matched controls (Fig 3.3B). To determine whether parasympathetic neurons require longer exposure to hyperglycemia before synaptic transmission becomes depressed, we recorded synaptic transmission in submandibular ganglia from mice up to 4.5 months after the onset of diabetes. Yet, even after 4.5 months of diabetes, the nerve-evoked EPSPs at synapses on parasympathetic neurons were no different statistically from those on age-matched controls (Fig 3.3B). These unexpected results indicate that synaptic transmission on parasympathetic neurons is less sensitive to diabetes compared to that at synapses on sympathetic neurons or adrenal chromaffin cells.

Either hyperglycemia does not elevate ROS in parasympathetic neurons as it does in sympathetic neurons, and consequently, the postsynaptic, α3-containing

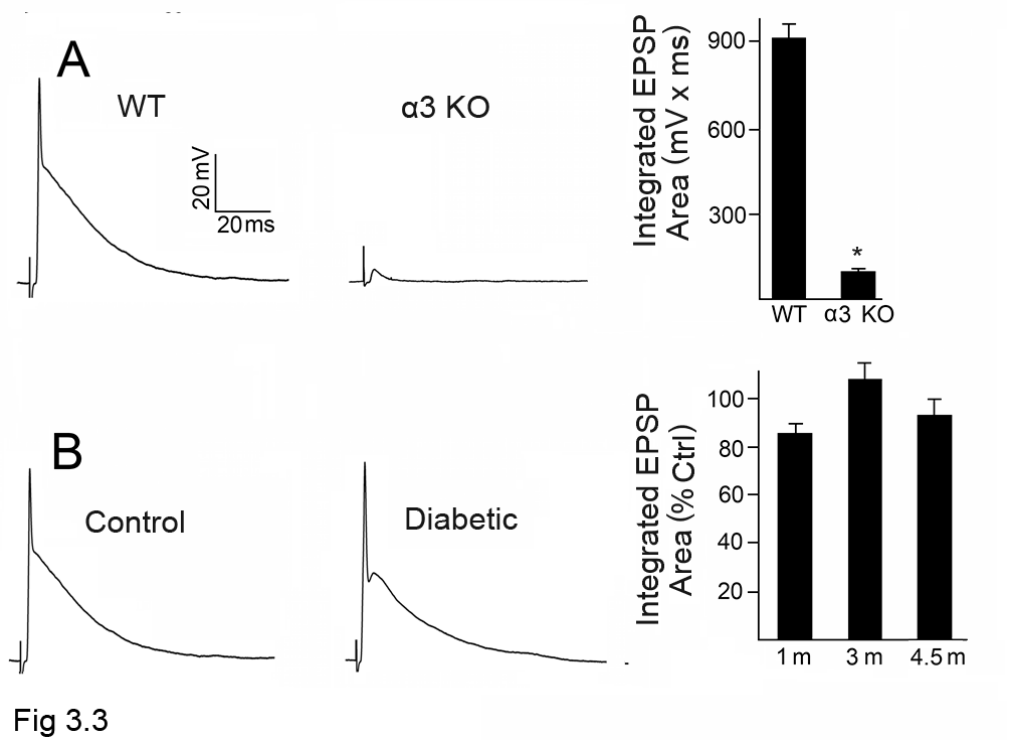

A
WT
α3 KO
20 mV
20 ms
Integrated EPSP Area (mV x ms)
900
600
300
*
WT
α3 KO
B
Control
Diabetic
Integrated EPSP Area (% Ctrl)
100
80
60
40
20
1 m
3 m
4.5 m

Fig 3.3

Fig 3.3 Synaptic transmission in parasympathetic submandibular (Subm) ganglion is mediated mainly by α3-containing nAChRs and is not affected by diabetes.

(A) Left: nerve-evoked EPSPs at synapses on WT and α3 KO Subm ganglion neurons in response to the preganglionic nerve stimulation. Right: average integrated EPSPs on Subm ganglion neurons in intact ganglia from WT (n= 40) and α3 KO mice (n=55). All values are means ±SEM. * represents $p<0.0001$.

(B) Left: nerve-evoked EPSPs in Subm ganglia of 2 month-old WT control and 2 month-old Akita mice (5 wk after the onset of diabetes). Right: average integrated EPSPs in Subm ganglia from mice with 1 month (n=52), 3 months (n=57), and 4.5 months (n=32) of diabetes; the data are % of EPSPs in Subm ganglia of age-matched WT control (n=40 for 1 month, n=9 for 3 months, and n=26 for 4.5 month). The data from the diabetic mice include both Akita mice and WT mice made diabetic with STZ injection. The values are the mean ±SEM. $p=0.02$ at 1month; $p>0.05$ at 3 and 4.5 months.

nAChRs on parasympathetic neurons are not inactivated by hyperglycemia. Or, α3-containing nAChRs on parasympathetic neurons are somehow different from those on sympathetic neurons and do not become inactivated by elevations in cytosolic ROS.

To examine the first possibility, we injected parasympathetic neurons through the recording electrode with antimycin-A (Anti-A), a drug known to increase ROS production by blocking complex III of the mitochondrial electron transport chain (ETC). We chose this approach because previous work showed that similar treatment when recording from SCG ganglia depresses the nerve-evoked EPSPs by ~ 50% (Campanucci et al., 2008). Unlike synapses on sympathetic neurons, however, the nerve-evoked EPSPs were depressed by less than 10% when recording from parasympathetic neurons with anti-A-containing electrodes (Fig 3.4 A,B). This lack of effect with anti-A suggests that parasympathetic neurons have decreased elevations in ROS than sympathetic neurons. Next, we asked if pairing hyperglycemia with Anti-A might elevate ROS sufficiently to depress synaptic transmission. Interestingly, we found that the nerve-evoked EPSPs were depressed by > 30% when we recorded from parasympathetic neurons from diabetic mice with Anti-A in the electrode (Fig 3.4 A,B), a greater depression than either treatment alone. This result suggests that a partial block of complex III in parasympathetic neurons, when coupled with hyperglycemia, will produce a sufficient elevation in cytosolic ROS to inactivate the postsynaptic nAChRs.

Elevating ROS in parasympathetic neurons inactivates α3-containing nAChRs

To test directly whether elevating cytosolic ROS in parasympathetic neurons causes a use-dependent inactivation of nAChRs, we planned to examine isolated

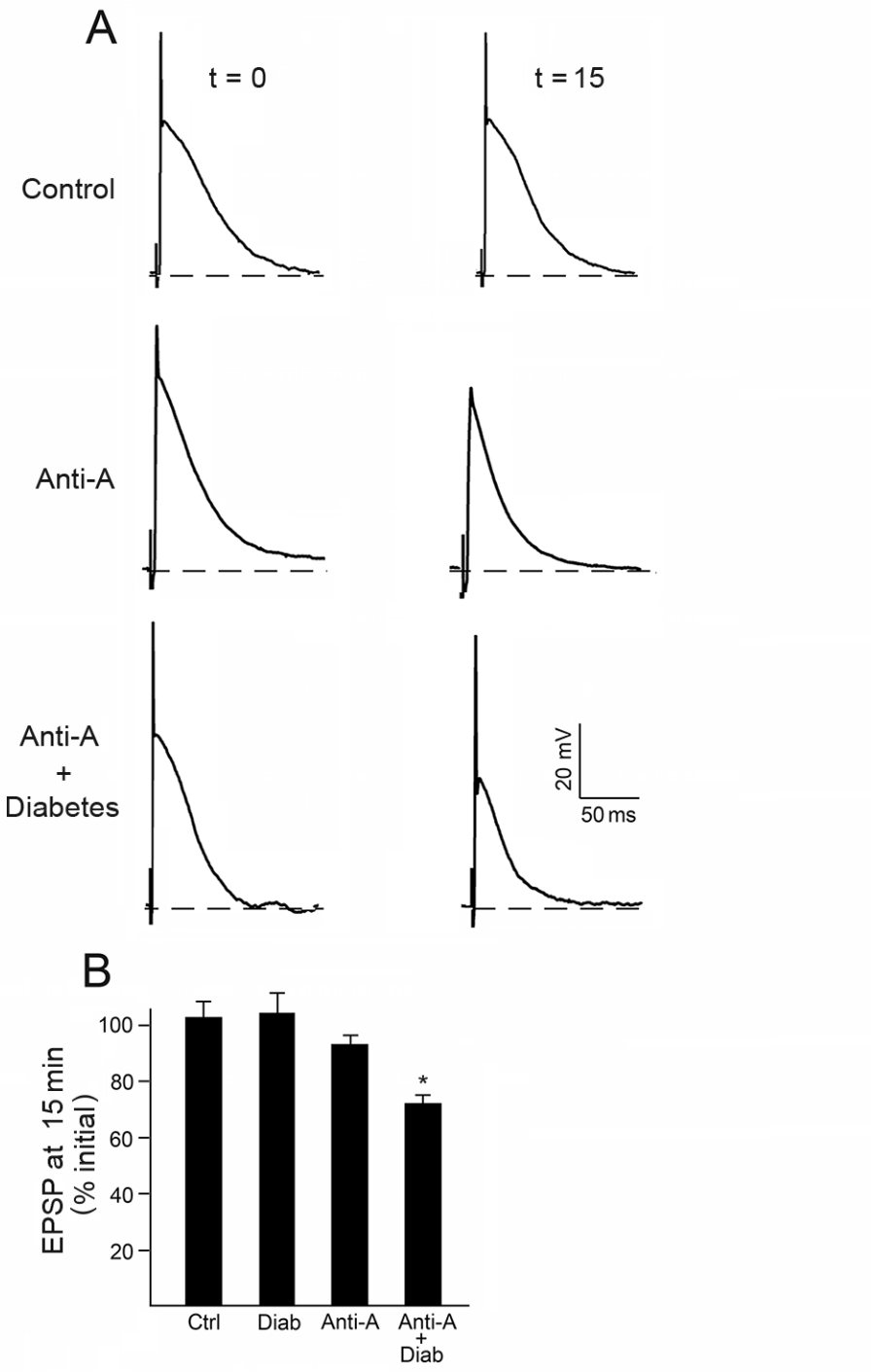

A
t = 0
t = 15
Control
Anti-A
Anti-A
+
Diabetes
20 mV
50 ms
B
EPSP at 15 min
(% initial)
100
80
60
40
20
*
Ctrl
Diab
Anti-A
Anti-A
+
Diab

Fig 3.4

Fig 3.4 Hyperglycemia combined with blockade of mitochondrial ETC complex III depresses synaptic transmission in parasympathetic submandibular ganglion.

(A) Nerve-evoked EPSPs at synapses on parasympathetic Subm ganglion neurons in response to the preganglionic nerve stimulation at 1Hz for 15 min. Top pair: recordings from a ganglion in a 2 month-old WT control mouse with control solution in the recording electrode at the beginning (t=0) and the end (t=15) of the 1 Hz train. Middle: recordings from a ganglion in a 2 month-old WT control mouse with anti-A (100 μM) in the recording electrode. Bottom: recording from a ganglion in an Akita mouse 1 month after onset of diabetes with anti-A in the recording electrode.

(B) Average integrated EPSPs measured at 15 min and expressed as a % of the initial EPSPs in Subm ganglia of WT control mice and recorded with control solution in the electrode (Ctrl; n=8), in Subm ganglia of Akita diabetic mice (~ 5 weeks after the onset of diabetes) and recorded with control solution in the electrode (Diab; n=11), in Subm ganglia of WT control mice recorded with anti-A in the electrode (anti-A; n=10), and in Subm ganglia of Akita diabetic mice with anti-A containing electrodes (n=11). The values are the mean ±SEM. p=0.06 for anti-A vs Ctrl, * represents $p<0.001$ for (anti-A + Diab) vs Ctrl.

neonatal mouse parasympathetic neurons in culture. We observed that dissociated neurons from young (P5-P20) mouse submandibular ganglia grew well in culture for at least 2-3 weeks (Fig 3.5 A). These cultured parasympathetic neurons had large, stable ACh-evoked inward currents (Fig 3.5 B), and when ACh (80-100 μM) was applied repetitively at 15 s intervals, we saw no significant decline in the ACh-evoked currents (Fig 3.5 C). Moreover, we could not detect any response when applying ACh (100 μM) on to submandibular ganglion neurons from α3 nAChR KO mice (Fig 3.5 B), indicating that the inward currents on these cultured parasympathetic neurons were mediated by α3-containing nAChRs.

To determine whether elevating cytosolic ROS inactivates nAChRs on parasympathetic neurons, first we loaded neurons with the ROS indicator, DCFDA, and elevated ROS by including Anti-A in the recording electrode during whole-cell recording. Then we simultaneously imaged neurons to monitor changes in ROS-induced fluorescence, and recorded whole-cell ACh-evoked currents in voltage-clamp. As control, we recorded from neurons in the same microscopic field but without Anti-A in the pipette solution.

Including 1 μM Anti-A in the recording electrode to inhibit partially the ETC complex III failed to produce a significant change in DCFDA fluorescence (Fig 3.6 A,B). Also, the ACh-evoked inward currents on these neurons were stable in response to repetitive ACh application (Fig 3.6 C, D); the peak inward current to the 30th application (I_{30}) was not significantly different from the peak current to the first ACh application (I_1) (Fig 3.6 C, D). These results are in contrast to those from sympathetic neurons: we observed ~5-10-fold increase in ROS-induced DCFDA fluorescence (Fig 3.6

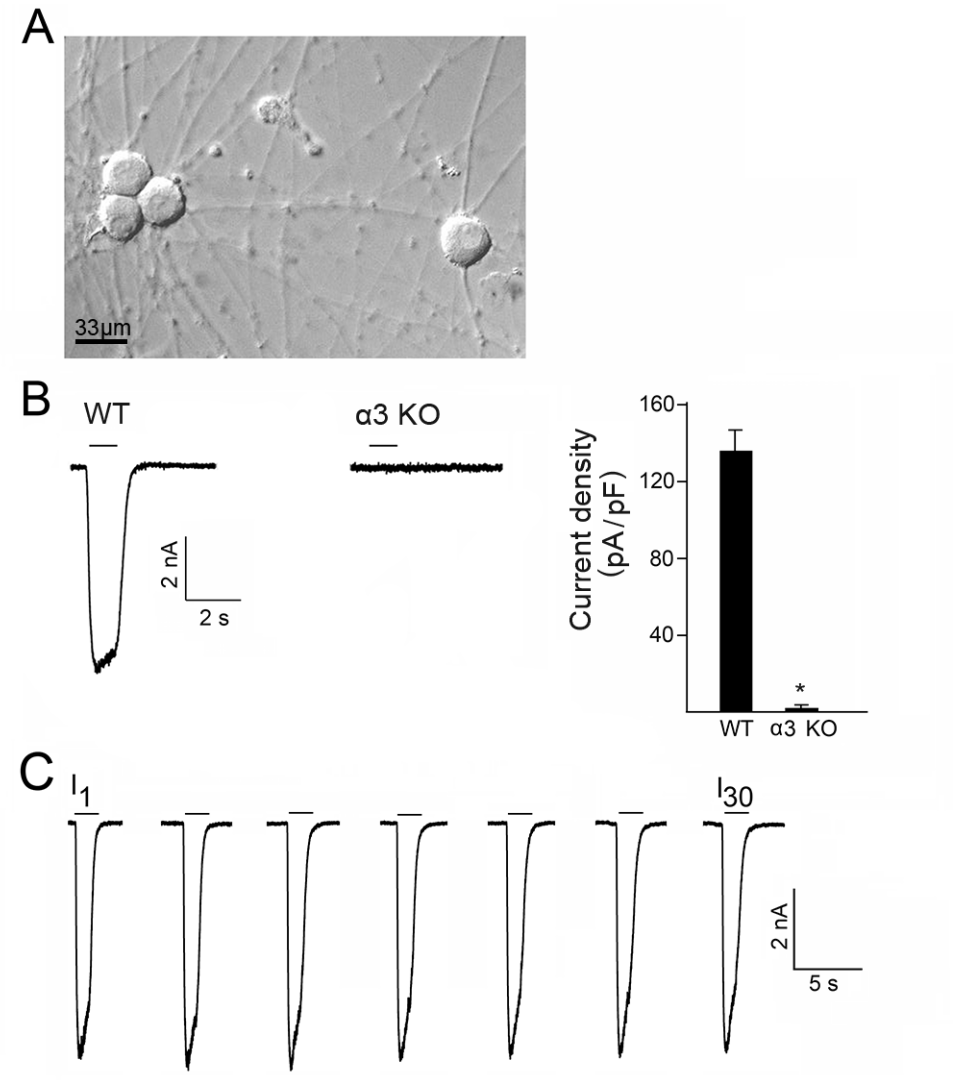

A
33µm
B
WT
α3 KO
2 nA
2 s
Current density (pA/pF)
160
120
80
40
*
WT
α3 KO
C
I1
I30
2 nA
5 s

Fig 3.5

Fig 3.5 Cultured submandibular ganglion neurons have stable ACh-evoked inward currents mediated by α3-containing nAChRs.

(A) Photomicrograph of dissociated mouse parasympathetic Subm ganglion neurons in culture for 2 weeks.

(B) Left: ACh-evoked inward currents on cultured Subm ganglion neurons from WT mice in response to a 1 s application of ACh (100 μM). Middle: No ACh-evoked currents on cultured Subm ganglion neurons from α3 KO mice. Right: Average ACh-evoked currents on cultured parasympathetic neurons from WT (n=28) and α3 KO (n=12) mice. The values are the mean ±SEM; * represents $p<0.0001$.

(C) ACh-evoked inward currents on cultured Subm ganglion neurons from WT mice in response to a 1 s applications of ACh (80 μM) at 15 s intervals. These data show that the ACh-evoked currents on cultured parasympathetic neurons are stable.

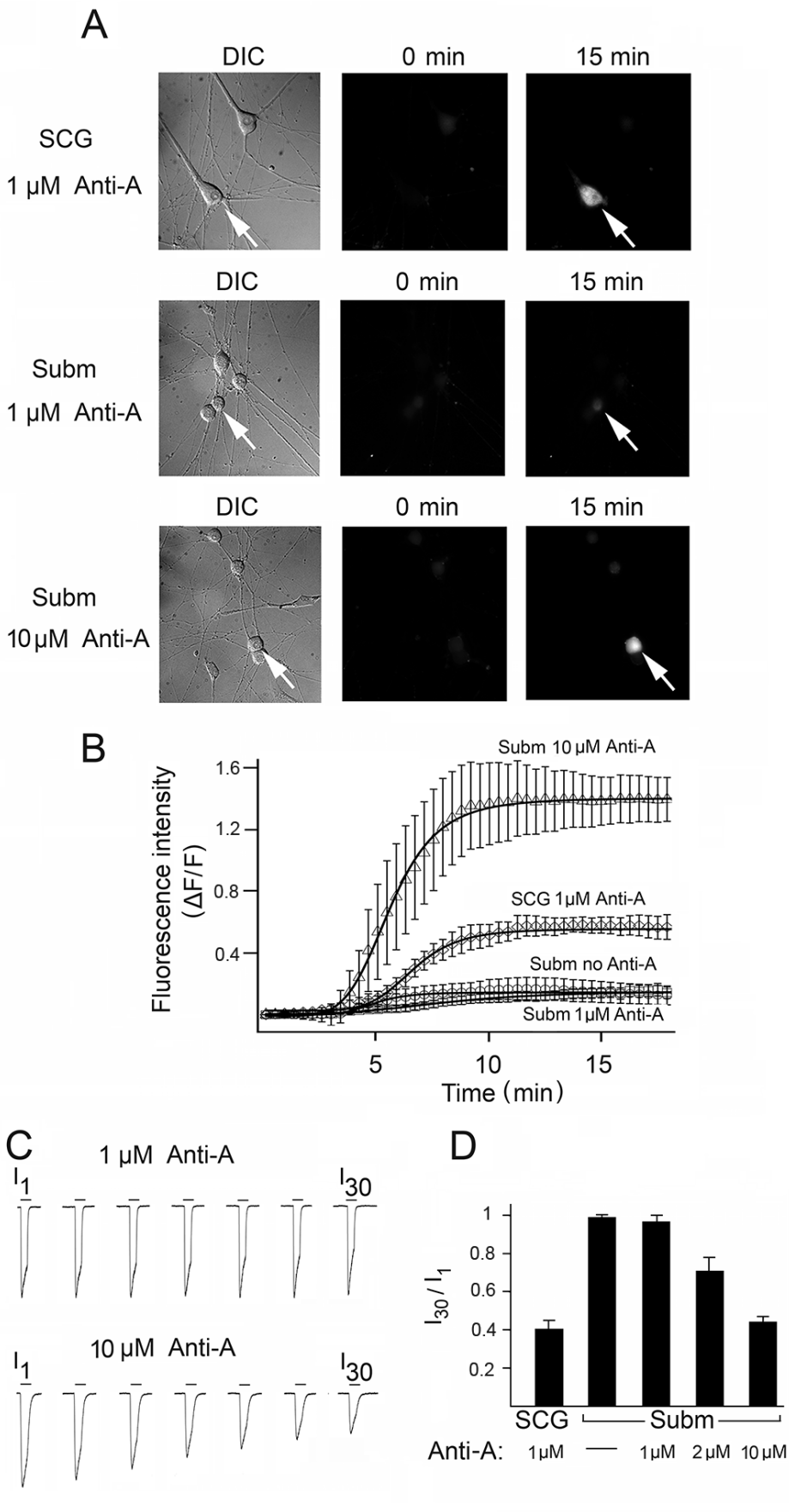

A
DIC
0 min
15 min
SCG
1 μM Anti-A
DIC
0 min
15 min
Subm
1 μM Anti-A
DIC
0 min
15 min
Subm
10 μM Anti-A
B
Subm 10 μM Anti-A
SCG 1μM Anti-A
Subm no Anti-A
Subm 1μM Anti-A
Fluorescence intensity
(ΔF/F)
1.6
1.2
0.8
0.4
5
10
15
Time (min)
C
1 μM Anti-A
I1
I30
10 μM Anti-A
I1
I30
D
I30 / I1
1
0.8
0.6
0.4
0.2
SCG
Subm
Anti-A:
1μM
1μM
2μM
10μM

Fig 3.6

Fig 3.6 Blocking mitochondrial ETC complex III elevates cytosolic ROS on parasympathetic neurons and inactivates nAChRs.

(A) DIC (left) and DCFDA-induced fluorescent images (right) of cultured sympathetic (top) and parasympathetic neurons (middle and bottom) immediately or 15 min after perfusing the neurons through the recording electrode with either 1 μM Anti-A (top and middle) or 10 μM Anti-A (bottom). Fluorescence intensities were measured at 25 s intervals for a period of 15 minutes. In all experiments, neurons were pre-loaded with CM-H2DCFDA for 30 min and treated acutely with Anti-A through the recording electrode.

(B) Average increases in ROS-induced DCFDA fluorescence for sympathetic SCG neurons (n=6) perfused intracellularly through the recording electrode with 1 μM Anti-A, and parasympathetic neurons perfused without Anti-A (n=6), 1 μM Anti-A (n=16), or 10 μM Anti-A (n=21). The data represent the mean ΔF/F ±SEM and fit with a Hill function (solid lines). For Subm ganglion neurons (1 μM Anti-A) vs. SCG (1 μM anti-A), and for Subm ganglion neurons (1 μM Anti-A) vs Subm ganglion neurons (10 μM Anti-A), $p < 0.001$; for Subm ganglion neurons (no Anti-A) vs Subm ganglion neurons (1 μM Anti-A), $p > 0.2$.

(C) ACh-evoked inward currents on cultured parasympathetic neurons from WT mice in response to a 1 s application of ACh (80 μM) at 15 s intervals ~15 min after perfusing neurons intracellularly with either 1 μM Anti-A (top) or 10 μM Anti-A (bottom). Every fifth trace is shown for clarity.

(D) Ratio of the 30th ACh-evoked current (I_{30}) to the first (I_1) 15-20 min after perfusing neurons through the recording electrode with Anti-A: Sympathetic SCG neurons (1 μM Anti-A) (n=6); parasympathetic Subm ganglion neurons perfused intracellularly with control solution (Ctrl) (n=6); 1 μM Anti-A (n=8); 2 μM Anti-A (n=6), 10 μM Anti-A (n=6). The values are the mean ±SEM. For Subm ganglion neurons (10 μM and 1 μM) vs. SCG (1 μM), $p < 0.05$; for Subm ganglion neurons (10 μM Anti-A) vs. SCG (1 μM Anti-A), $p > 0.1$.

A,B), and an irreversible rundown of the ACh-evoked inward currents when recording from sympathetic neurons with 1 μM Anti-A in the recording electrode (Fig 3.6 C,D), similar to what was reported previously (Campanucci et al., 2008).

To determine whether a stronger block of the ETC complex III would elevate cytosolic ROS in parasympathetic neurons, we recorded from submandibular ganglion neurons with 2 μM and with 10 μM Anti-A in the recording electrode; 2 μM Anti-A produced a significant increase in ROS-induced DCFDA fluorescence, which increased even further with 10 μM Anti-A (Fig 3.6 A,B). Moreover, when cytosolic ROS were elevated with 10 μM Anti-A, repeated application of ACh (80 μM) caused the ACh-evoked inward currents to run down; the peak ACh-evoked current to the 30th application (I_{30}) was ~ 40% of the first (I_1) (Fig 3.6 C,D). These results indicate that an increase in cytosolic ROS in parasympathetic neurons leads to a use-dependent inactivation of nAChRs, as it does on sympathetic neurons.

Sympathetic neurons are more vulnerable to hyperglycemia than parasympathetic neurons

Our results with partial block of the ETC complex III suggest that parasympathetic neurons differ from sympathetic neurons in their ability to buffer ROS from mitochondria. Moreover, our results from recording nerve-evoked EPSPs in intact ganglia suggest that postsynaptic nAChRs on sympathetic neurons exposed to hyperglycemia are inactivated whereas those on parasympathetic neurons are stable. To test directly whether hyperglycemia causes a use-dependent rundown of ACh-evoked currents on parasympathetic neurons, we grew parasympathetic neurons from

2-3 week-old mice in culture for 7 d in 5 mM glucose and then glucose was elevated to 25 mM for 7 d while keeping the controls in 5 mM glucose throughout the 14 day period. For comparison, we grew sympathetic neurons from the SCG under identical conditions. For sympathetic neuron cultures, the steady-state ROS-induced DCFDA fluorescence in neurons in 25 mM glucose was ~ 2-fold greater than those in control 5 mM (1.87 ± 0. 2 (mean ± s.e.m), n=18), whereas, for parasympathetic cultures, the steady-state DCFDA levels in neurons in 25 mM glucose were not significantly different from those in control 5 mM (1.18 ± 0.2 (mean ± s.e.m), n=15).

Consistent with these ROS-induced DCFDA measurements, the ACh-evoked inward currents on sympathetic neurons maintained in 25 mM glucose ran down, whereas those on parasympathetic neurons were stable (data not shown). These results on ROS levels and ACh-evoked currents indicate that sympathetic neurons are more vulnerable to hyperglycemia than parasympathetic neurons.

On the other hand, in diabetic mice, we found that including Anti-A in the recording electrode depressed synaptic transmission at synapses on parasympathetic neurons in diabetic mice (see Fig 3.4). Therefore, we asked whether Anti-A would elevate ROS in parasympathetic neurons when cultured in hyperglycemic conditions. To test this, we included 1 μM Anti-A while recording from cultured parasympathetic submandibular ganglion neurons maintained in 25 mM glucose for 5-7 days. We found a significant 12-20 fold increase in ROS-induced DCFDA fluorescence (Fig 3.7 A). Equally relevant, the ACh-evoked inward currents on these neurons ran down, whereas, those on neighbouring neurons recorded without Anti-A were stable (Fig 3.7 B).

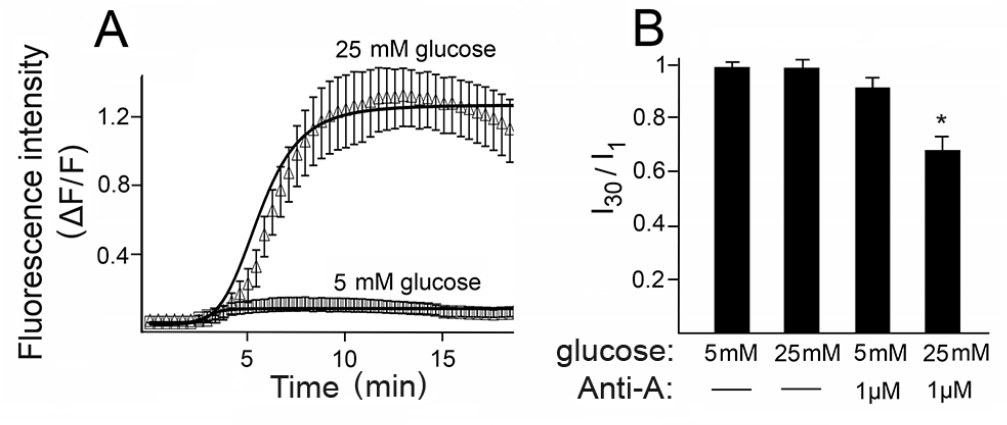


Fig 3.7

Fig 3.7 Hyperglycemia together with partial blockade of mitochondrial ETC complex III elevates cytosolic ROS on parasympathetic neurons and inactivates nAChRs.

(A) Average increase in ROS-induced DCFDA fluorescence in parasympathetic Subm ganglion neurons cultured in 5 mM glucose for 14 d, or in 5 mM glucose for 7 d and 25 mM glucose for 7 d (n=6) and then perfused through the recording electrode with 1 μM Anti-A. The data represent the mean ΔF/F ±SEM and fit with a Hill function (solid lines), $p < 0.001$.

(B) Ratio of the 30th ACh-evoked current (I_{30}) to the first (I_1) after perfusing neurons through the recording electrode with 1 μM anti-A for 15-20 min. (n=12 for 5 mM glucose; n=14 for 25 mM glucose; n= 9 for 5 mM glucose and 1 μM Anti-A; n=18 for 25 mM glucose and 1 μM anti-A). I_{30}/I_1 was significantly less for neurons grown with 25 mM glucose and perfused intracellularly with 1 μM anti-A compared to those in 5 mM glucose with or without Anti-A, or those grown in 25 mM glucose without Anti-A ($p < 0.05$).

DISCUSSION

To learn more about diabetes-induced dysautonomia, we focused on synaptic transmission in autonomic ganglia. The activity of cholinergic-nicotinic synapses in autonomic ganglia represents the final output of various CNS structures that regulate autonomic function; when synaptic transmission in autonomic ganglia is depressed, dysautonomia ensue. Previous work demonstrated that hyperglycemia depresses synaptic transmission in the SCG by causing a ROS-dependent inactivation of the postsynaptic nAChRs (Campanucci et al., 2010). This inactivation involves the highly conserved cysteine residues on the α3 nAChR subunit located near the intracellular mouth of receptors (Campanucci et al., 2010, Krishnaswamy and Cooper, 2012).

In this study, we demonstrate that sympathetic and parasympathetic ganglia differ in their vulnerability to diabetes. Hyperglycemia depresses synaptic transmission in prevertebral and paravertebral sympathetic ganglia and in the adrenal medulla, but surprisingly hyperglycemia has relatively little effect on synaptic transmission in parasympathetic ganglia, even though synaptic transmission in both types of ganglia and in the adrenal medulla is mediated by α3-containing nAChRs. In line with our findings, it was reported recently that hyperglycemia does not depress synaptic transmission on parasympathetic neurons in the pelvic ganglia (Tompkins et al., 2013).

To investigate this differential effect of hyperglycemia on autonomic ganglia, we combined ROS imaging with electrophysiology. Focusing on postganglionic neurons, our results show that hyperglycemia produces considerably smaller elevations in cytosolic ROS in parasympathetic neurons compared to sympathetic neurons, a result

suggesting that sympathetic neurons have lower ROS buffering capacity than parasympathetic neurons. We observed similar results when blocking complex III of the mitochondrial electron transport chain with antimycin-A. At 1 μM, Anti-A produced greater elevations in cytosolic ROS in sympathetic neurons than in parasympathetic neurons. A likely explanation for this differential response to mild oxidative stress is that these neurons use different neurotransmitters. Specifically, sympathetic neurons synthesize catecholamines and are at high risk for oxidative stress because molecules involved in the synthesis of the catecholamines, such as tyrosine hydroxylase and monoamine oxidase, produce H_2O_2 as normal by-products of their activities (Coyle and Puttfarcken, 1993). In addition, catecholamines undergo auto-oxidation and produce H_2O_2. Accumulated H_2O_2 slowly decomposes to the highly reactive hydroxyl radical, a process that is accelerated markedly in the presence of Fe^{2+} by the Fenton reaction. When subjected to additional oxidative stress, possibly from enhanced glucose metabolism in people with diabetes, sympathetic neurons have difficulty keeping ROS in balance. On the other hand, the neurotransmitter used by parasympathetic neurons is acetylcholine whose by-products are not pro-oxidant. As such, it is likely that parasympathetic neurons are better able to buffer ROS and require a greater block of complex III and higher concentration of Anti-A to elevate cytosolic ROS.

With parasympathetic neurons, neither hyperglycemia nor 1 μM Anti-A alone produced a significant elevation in cytosolic ROS, and the ACh-evoked currents on these neurons are stable; however, when 1 μM Anti-A is applied to parasympathetic neurons grown in hyperglycemic conditions, there is a strong synergistic elevation in ROS, and these elevated ROS cause an irreversible, use-dependent rundown of the

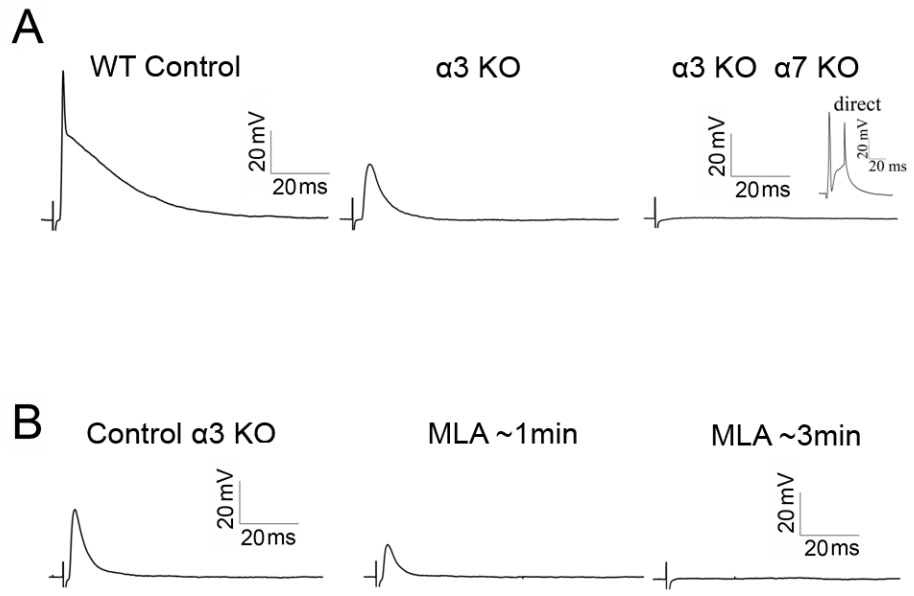
ACh-evoked currents. Similarly, synaptic transmission in parasympathetic ganglia in diabetic animals is not depressed, nor is it significantly depressed when recording with Anti-A in the electrode. On the other hand, recording synaptic transmission from parasympathetic ganglia of diabetic mice with Anti-A in the recording electrode significantly depressed nerve-evoked EPSPs. These results imply that while diabetes alone does not affect synaptic transmission in parasympathetic ganglia, it will depress synaptic transmission if a subject succumbs to a disease that either elevates ROS or compromises the neuron's anti-oxidant pathways.

While our experiments demonstrate that diabetes does not disrupt synaptic transmission in parasympathetic ganglia, they do not rule out the possibility that parasympathetic function is perturbed. For example, diabetes may alter transmitter release from parasympathetic nerve terminals, as it does for motor nerve terminals at the neuromuscular junction (Kimura et al., 1993, Ramji et al., 2007, Souayah et al., 2009). And/or, diabetes may alter synaptic drive on parasympathetic preganglionic neurons in the dorsal motor nucleus of the vagus (Sohn et al., 2013). Although we have not investigated the parasympathetic nervous system's overall performance in these diabetic mice, we saw little evidence of abnormal parasympathetic function, as is seen in α3 KO mice that have no synaptic transmission in any of the autonomic ganglia or the adrenal medullae.

In addition to sympathetic and parasympathetic ganglia, we investigated synaptic transmission between the preganglionic nerve and adrenal chromaffin cells, a topic that has received little attention. Adrenal chromaffin cells have been shown to express a number of different nAChR subtypes (Lopez et al., 1998, Di Angelantonio et al., 2003,

Sala et al., 2008), and there has been some uncertainty about which subtypes are present at synapses with preganglionic nerve terminals. Our experiments with α3 KO mice resolve this issue by establishing that the nerve-evoked EPSPs on adrenal chromaffin cells are mediated by α3-containing nAChRs. Relevantly, we show that these synapses are depressed significantly in diabetic animals.

The depression of synaptic transmission in the adrenal medulla and in the prevertebral sympathetic ganglia may have implications for insulin-induced hypoglycemia, a serious, acute complication of diabetes (Cryer et al., 1986, Havel and Ahren, 1997, Taborsky et al., 1998, Cryer, 2013). Briefly, to protect against a fall in blood glucose, pancreatic α-cells are stimulated to release glucagon. The major stimulants to α-cells are norepinephrine released from sympathetic nerves originating from neurons in prevertebral ganglia, and circulating epinephrine released from chromaffin cells in the adrenal medullae. In diabetics, it is likely that the functional sympathetic innervation to α-cells is reduced because synaptic transmission in prevertebral ganglia is depressed. In addition, a defect in nerve-evoked synaptic transmission at synapses on chromaffin cells results in a decrease in circulating epinephrine. Reduced activity in the sympathoadrenal system in diabetics would have severe consequences for glucagon release by pancreatic α-cells, and by implication, impair the major counter-regulatory mechanisms that defend against insulin-induced hypoglycemia (Havel and Ahren, 1997, Taborsky et al., 1998, Cryer, 2013).



Suppl Fig 1

Suppl Figure 1 nAChRs containing α7 subunit contribute to some EPSPs in parasympathetic neurons of the submandibular ganglion.

(A) Nerve-evoked EPSPs at synapses on WT, α3 KO, and α3 α7 KO Subm ganglion neurons in response to the preganglionic nerve stimulation.

(B) Nerve-evoked EPSP in α3 KO Subm ganglion with control extracellular recording solution (left), then 1 min after MLA was added to extracellular perfusion solution (middle), and 3 min after MLA was added to extracellular perfusion solution (right) while recording in the same cell.

CHAPTER 4

Critical period exists for cholinergic synaptic transmission to recover in sympathetic ganglia in diabetes, although postsynaptic nicotinic receptors readily recover in cultured neurons.

ABSTRACT

Recent work showed that one cause of autonomic neuropathy in diabetes is the depression of synaptic transmission in the superior cervical ganglion (SCG). This synaptic depression in the SCG develops when hyperglycemia elevates reactive oxygen species (ROS) which oxidise and inactivate the postsynaptic nicotinic receptors (nAChRs). In this study, I investigate the recovery of the nAChRs following their oxidation by ROS. I test the hypothesis that the nAChR function will recover if the reduced nAChRs are restored at the membrane of the sympathetic neurons and ROS return to normal levels. If this is true, restoring glucose to normal level in diabetic mice with insulin implants should correct the defect in synaptic transmission.

To study the recovery of the nAChR function after inactivation, I inactivated nAChRs in cultured SCG neurons in a ROS-dependent way (using mitochondrial electron transport blocker antimycin A) as well as a ROS-independent way (receptor alkylation with bromoacetylcholine). I then measured the timecourse of recovery by recording nAChR current densities. To test whether new gene transcription or new protein synthesis were required for recovery, I blocked transcription with actinomycin D or blocked protein translation with puromycin in the cultured neurons during recovery from inactivation. To study the recovery of synaptic transcription *in vivo*, I lowered glucose in diabetic mice with insulin implants.

The full recovery of nAChR currents from both ROS-dependent and ROS-independent inactivation happened by 24-48 hours. Blocking mRNA translation during the first 3 hours of recovery, prevented the nAChR current recovery from alkylation, but did not affect the recovery from ROS-induced inactivation. Therefore, although new

protein synthesis is necessary for nAChR to recover from alkylation, the nAChR are able to recover from oxidation by ROS without new protein synthesis at the 3 hour time point. In diabetic mice lowering glucose with insulin led to recovery of synaptic transmission only after short (2 weeks) but not longer (1, 2, 3.5 months) duration of diabetes. These results suggest that the recovery of the nAChR function after inactivation by ROS is possible in cultured cells, but there is a critical period for the recovery of synaptic transmission *in vivo*.

INTRODUCTION

Autonomic neuropathy is among the most dangerous complications of diabetes. It can deregulate many organs in the body, and increases mortality for diabetic people (Sampson et al., 1990, O'Brien et al., 1991, Rathmann et al., 1993). Normalising glucose levels with insulin can prevent or slow down this complication (1993). However, once autonomic neuropathy develops, reversing it may be difficult (Navarro et al., 1997, Pop-Busui et al., 2010). For example, in a study by Navarro and colleagues, diabetic patients who received insulin or pancreatic transplant showed very little or no improvement on autonomic tests during the 10 years of treatment (1997). It is not clear why despite normalising glucose, autonomic neuropathy does not get reversed.

One problem may be that treatment with insulin is not started early enough. Studies with diabetic animals show that there is often a critical period for starting insulin treatment to reverse complications such as retinopathy (Engerman and Kern, 1987, Hammes et al., 1993), motor neuron (Spadella et al., 2012) and vascular dysfunction (Roy et al., 1990). It is not known if a critical period exists for treating autonomic neuropathies.

In addition, not very much is known on how autonomic neurons can restore their function after they are affected by diabetes. Schmidt and colleagues observed that diabetic rats and humans have structural changes in neurons of the sympathetic ganglia that innervate digestive organs (superior mesenteric and celiac ganglia) (Schmidt et al., 1993, Schmidt et al., 2003, Schmidt et al., 2008, Schmidt et al., 2009). They also found that such structural changes can be partially reversed in mice treated with insulin for 1 month after 2 months of diabetes (Schmidt et al., 2011). However, it is not clear if

reversing structural changes in the autonomic ganglia allows the function of the ganglia to recover.

Recent work has shown that the sympathetic superior cervical ganglion (SCG) shows depressed synaptic transmission in diabetes. This work showed that hyperglycemia elevates reactive oxygen species (ROS) in the SCG which oxidise the postsynaptic nicotinic receptors (nAChRs) at the conserved cysteine residues at position 239 in the pore of the receptor (Campanucci et al., 2008). The oxidation of the postsynaptic nAChRs inactivates them in a use-dependent way. As a result, synaptic transmission becomes depressed in the SCG and mice develop dysautonomias (Campanucci et al., 2010). Therefore to reverse diabetic autonomic neuropathies one would need to restore the synaptic transmission in sympathetic ganglia.

I hypothesize that sympathetic synaptic transmission should recover when the nAChRs are restored in their reduced state and when ROS return to their normal levels. For the receptors to return to their reduced state, they either: 1) become reduced while remaining in the membrane, 2) are degraded and replaced by newly-synthesized receptors, and/or 3) are reduced during recycling. In addition, if a critical period exists for the recovery of sympathetic synaptic transmission, as was found with several complications of diabetes (Engerman and Kern, 1987, Roy et al., 1990, Hammes et al., 1993, Spadella et al., 2012), then synaptic transmission will recover when insulin treatment starts before this period but not after.

To test these predictions, I measured the time course of nAChR current recovery after inactivating them in cultured SCG neurons. I found that nAChRs could recover from inactivation by ROS within several days, and the recovery did not require new

protein synthesis. I then used mice which were diabetic for 2 weeks, 1, 2, or 3.5 months. I normalised their blood glucose with insulin for 1 week – time that is longer than what was needed for nAChRs to recover in cultured neurons. The sympathetic synaptic transmission recovered in mice that had been diabetic for 2 weeks, but not in mice diabetic for longer times, suggesting that a brief critical period exists for the synaptic transmission to recover in diabetes.

METHODS

nAChR inactivating treatments

Inactivation of nAChRs by Bromo-Acetylcholine

To study the turnover of functional nAChRs in SCG neurons, nAChRs were inactivated using Bromoacetylcholine (Bromo-ACh), similar to the method described by Mandelzys (1992). The following solutions were used.

DTT Solution was prepared by dissolving 0.2 mg/ml of Dithiothreitol (DTT) in extracellular recording solution. The pH was adjusted to 8 using NaOH.

Bromo-ACh Solution was prepared from a 100mM stock of Bromoacetylcholine bromide (Sigma Aldrich) dissolved in water, which was diluted as 1:1000 in extracellular patch solution for a final concentration of 100μM. The pH was adjusted to 7.2 with HCl.

DTNB Solution was made by dissolving 0.2 mg/ml of 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) in extracellular recording solution. The pH was 7.2.

Neurons were treated in the biosafety cabinet at room temperature. Growth medium was removed and the DTT solution was added to each dish (~2ml/dish), for a total of 3 DTT rinses to remove growth medium. Cells were left in the DTT solution for 25 min. After removing the DTT solution, Bromo-ACh solution (~2ml/dish) was added, and was used to rinse cells 3 times, then left for 15 min. After removing the BAC solution, DTNB solution (~2ml/dish) was added very carefully for no longer than 3 min. Care and short treatment time had to be used when applying DTNB to prevent cell detachment from the dish. After rinsing off the DTNB solution with growth medium (2ml/dish) 3 times, the culture dishes were returned to the incubator. They remained in

the incubator at 37 °C and in humidified atmosphere of 95% air and 5% CO_2 for the durations of recovery, after which whole-cell currents were recorded.

Use-dependent inactivation by ROS

To inactivate the nAChRs in cultured SCG neurons by oxidation with ROS, intracellular ROS were elevated by treating the neurons with Antimycin A (10 μM, Anti-A), which was included in the growth medium for 1 hour. Anti-A crosses cell membranes and blocks the mitochondrial electron transport chain at complex III. This elevates ROS by causing electrons to leak to molecular oxygen and producing superoxide. Since the nAChR oxidation by ROS is use-dependent, nAChRs were stimulated with ACh. To measure the inactivation of nAChRs, ACh-evoked currents were recorded and were expressed as current densities by dividing each cell's current amplitude by its capacitance. I applied concentrated ACh (2mM) to cultured dishes of SCG neurons with plastic pipettes which had tip diameters of 2-3mm every 30 sec for a total of 60 applications. Simultaneously, the dishes were perfused with extracellular solution at ~5ml/min. Following the treatment, each dish was washed and refilled with growth medium and returned to the incubator for recovery. Control dishes were used immediately after the treatment to verify nAChR inactivation.

Puromycin and Actinomycin D treatments

Translation or transcription was blocked for 4 hours before inactivation and during 3 hours of recovery. Cells were treated with puromycin (50 μM) or with Actinomycin D (4 μM) dissolved in culture medium during these time periods.

Surgery to implant insulin

To study the effects of reversing hyperglycemia on autonomic synaptic transmission, diabetic Akita and STZ-injected mice were maintained for 2 weeks, 1, 2, or 3.5 months after the onset of hyperglycemia (blood glucose >20mM). Then, mice were implanted with insulin pellets subcutaneously (Linbit implant, LinShin Canada) at the recommended dose. During surgery to implant insulin, mice were anesthetized with isofluorane gas delivered through a nose cone. They were injected subcutaneously with local analgesic Carprofen (1μL/g, 1mg/ml) in the implant area and were injected with saline at 0.2 to 0.5mL/10g body weight to replentish body fluids. Mice were placed on a heated pad during the procedure and the back area was shaved and disinfected with chlorhexidine. A ~4-5mm incision was made in the skin and, after inserting the insulin pellets subcutaneously, the incision was sutured using Vicryl 3.0 thread. Local anesthetic Lidocaine/Bupivacaine (2-3 drops) was applied in the incision area. After the procedure, blood glucose of the mice was monitored 3 times daily. Once blood glucose fell below 7mM (usually 2-3 days after insulin implant) mice were maintained for the treatment periods of 1, 2, or 4 weeks. The control animals were left untreated for the same duration as the insulin treatment. A common problem was hypoglycemia, with glucose sometimes dropping below 1-2mM, unfortunately causing some mice to die. For mice which became hypoglycemic, glucose was added to the drinking water (up to 2M) to normalize blood glucose.

After insulin treatment, the mice were euthanized with CO_2 gas and decapitated. The SCGs of the insulin-treated and control mice were dissected and synaptic transmission recordings were made.

For other details on methodology, see Chapter 2.

Statistics

For comparisons of current densities or EPSP areas, we used the Student's t test. All procedures for animal handling were performed according to the guidelines of the Canadian Council on Animal Care.

RESULTS

Oxidised nAChRs do not recover while remaining in the membrane

I hypothesized that the recovery of sympathetic synaptic transmission from a diabetes-induced depression *in vivo* will require postsynaptic nAChRs to return to a reduced state. To study the recovery of nAChR function after oxidation by ROS, I fist asked if the nAChRs can recover by being reduced in the membrane. To elevate intracellular ROS, I treated cultured SCG neurons with Antimycin A (10μM, Anti-A) by dissolving it in the growth medium for 1 hour. I recorded whole cell currents evoked by 30 puffs of ACh (100μM) applied repeatedly every 15s. When pretreated with Anti-A, the ACh-evoked currents ran down (Fig 4.1 A). The ratio of the 30^{th} current amplitude to the 1^{st} (I_{30}/ I_1) was ~0.2 in neurons treated with Anti-A. In contrast, the I_{30}/ I_1 was ~1 for untreated control neurons (Fig 4.1 B). To ensure that the electrode solution was reducing, I used a strong reducing agent dithiothreitrol (DTT) dissolved in the solution of the patch electrode. Including DTT in the electrode did not prevent the current rundown (Fig 4.1 B). In addition, in all cases the ACh-evoked currents did not recover when tested after waiting for 10 min (Fig 4.1 A). This suggests that it is difficult to reduce the nAChRs while the receptors remain in the membrane.

As control, I verified that DTT can reduce cysteine residues on the nAChR. The nAChR has a pair of extracellular cysteine residues (Cys^{192}, Cys^{193}) within its ligand-binding site, which normally remain oxidised (Kao et al., 1984, Kao and Karlin, 1986).

It was shown previously that reducing these cysteine residues by applying DTT in the extracellular solution prevents ACh from binding and prevents currents through the nAChR (Derkach et al., 1991). Indeed, applying DTT extracellularly caused ACh-evoked

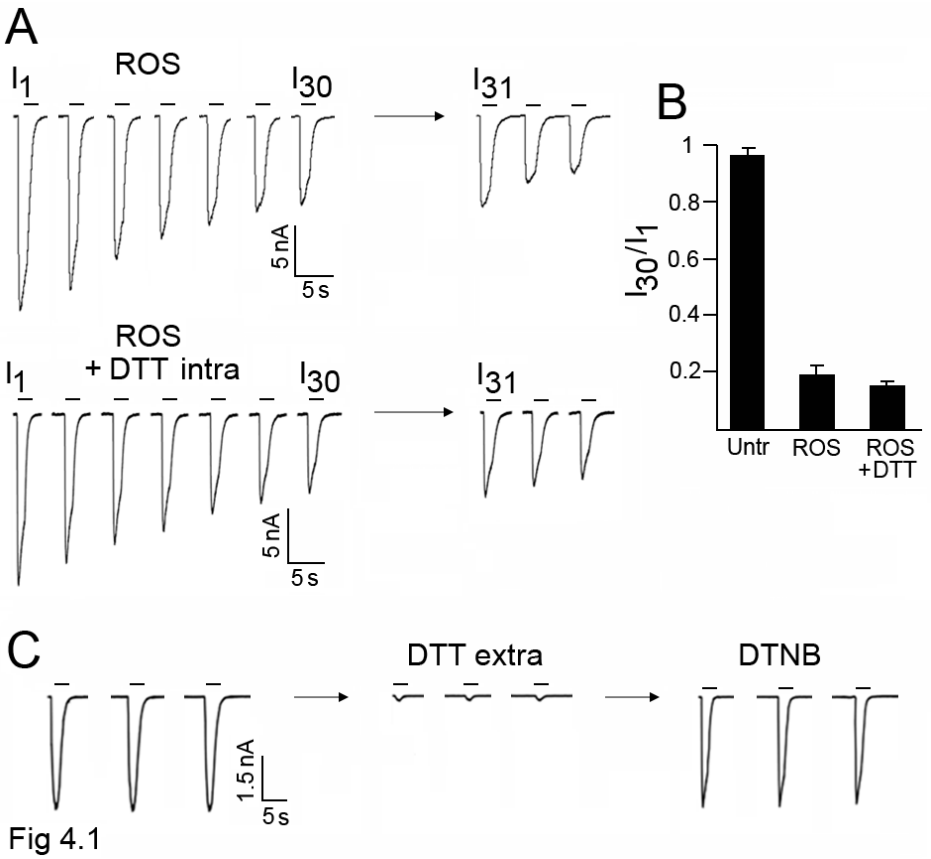

A
ROS
I1
I30
I31
5 nA
5 s
ROS
+ DTT intra
I1
I30
I31
5 nA
5 s
B
I30/I1
1
0.8
0.6
0.4
0.2
Untr
ROS
ROS
+DTT
C
DTT extra
DTNB
1.5 nA
5 s

Fig 4.1

Figure 4.1 nAChRs do not recover from inactivation by ROS by being reduced in the membrane.

A) Inward currents on cultured SCG neurons in response to 1 s applications of ACh (100μM) at 15 s intervals. Top panel: The currents in neurons with elevated ROS are running down over time. Bottom panel: The currents in neurons with elevated intracellular ROS while dithiothreitol (DTT, 2mM) is included in the patch electrode.
B) The ratio of the 30^{th} ACh-evoked current to the 1^{st} in control neurons (n=10), in neurons with elevated ROS (n=10), and in neurons with elevated ROS while recording with DTT in the patch electrode (n=14).
C) Inward currents on cultured SCG neurons in response to 1 s applications of ACh (100μM) given at 15 s intervals. Left: Currents recorded with regular extracellular solution. Middle: Currents recorded in the same cell after perfusing with extracellular solution containing DTT (2mM). Right: Currents recorded in the same cell after perfusing with extracellular solution containing (5 5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 2mM) (n=3).

currents to disappear almost completely. This was then reversible in the same cell by applying an oxidising agent DTNB (Fig 4.1 C). This control experiment confirmed that DTT could reduce cysteine residues of the nAChR.

nAChRs inactivated by Bromo-ACh recover within 48 hours in SCG neurons

I next examined the time course of nAChR recovery after inactivation. Before inactivating the nAChRs with ROS, I tested how long the turnover of the nAChRs takes after irreversible inactivation by Bromo-acetylcholine (Bromo-ACh). To do this, DTT was applied extracellularly to reduce the pair of cysteines in the ligand-binding site of the nAChR, after which Bromo-ACh was applied. The Bromo-ACh covalently binds the receptor's ligang-binding site and disrupts it permanently. To verify the effect, I measured current density after the Bromo-ACh treatment, which became ~8% of the current densities in the untreated neurons (Fig 4.2 A). Control treatments excluding DTT did not reduce ACh-evoked currents. Also, excluding Bromo-ACh but treating neurons with DTT and DTNB to reduce and then reoxidise the pair of cysteines in the nAChR ligand-binding site did not reduce ACh-evoked currents (Fig 4.2 A).

To find out how long it takes for nAChRs to recover from the irreversible inactivation by Bromo-ACh, I inactivated nAChRs, returned the cultured neurons to the incubator, and recorded ACh-evoked current densities at different times. The current densities recovered to 36% of control values within 3 hours after inactivation. The current densities reached 74% of control by 12 hours and recovered completely by 24 hours (Fig 4.2 B). Interestingly, current densities of 130-140% of control size were

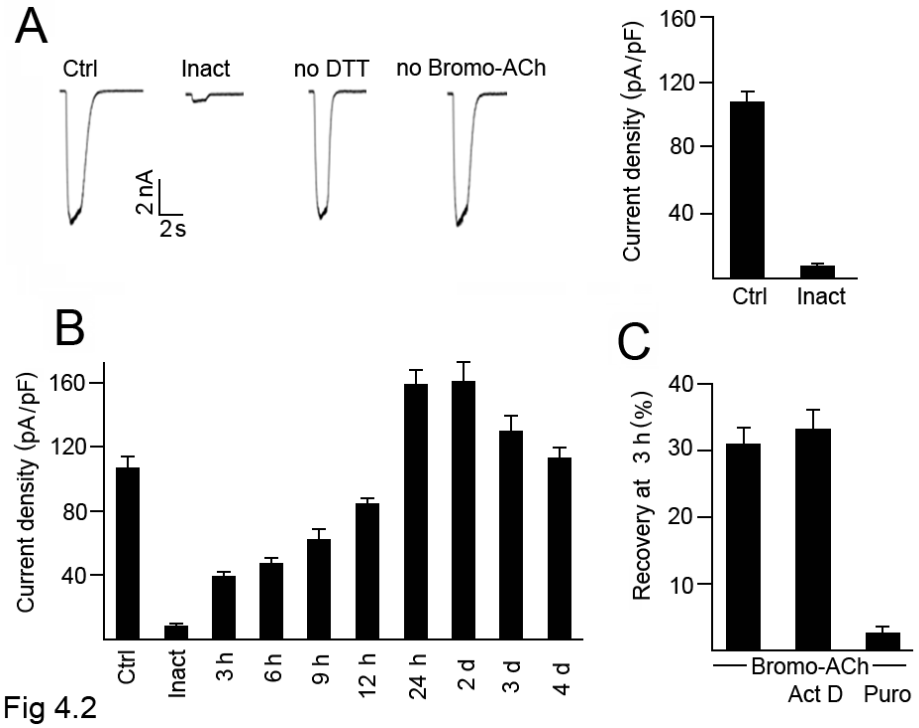


Fig 4.2

Figure 4.2 The recovery of nAChRs from inactivation by Bromo-ACh happens within 24 hrs and depends on protein translation but not transcription.

A) Left panel: Representative inward currents on cultured SCG neurons without inactivation, after inactivation with Bromo-acetylcholine (Bromo-ACh), after inactivation during which DTT was omitted (n=10), and after inactivation during which Bromo-ACh was omitted (n=18). Right panel: Averaged current densities (current amplitude divided by cell capacitance) recorded in neurons without inactivation (n=21) and after inactivation with Bromo-ACh (n=30).

B) Current densities recorded in cultured neurons in control conditions (n=21), after inactivation with Bromo-ACh (n=30), and at 3h (n=17), 6h (n=18), 9h (n=12), 12h (n=23), 24h (n=24), 2d (n=14), 3d (n=10), and 4d (n=12) after inactivation.

C) Current densities recorded 3 h after inactivation with Bromo-ACh with no treatment during recovery (n=12), with Actinomycin D (Act D) treatment during recovery (n=17), or with Puromycin (Puro) treatment during recovery (n=16). Current densities are expressed as % of total recovery.

observed at 1 and 2 days after inactivation, and these current densities returned to the same size as control by 4 days (Fig 4.2 B). These results suggest that approximately 1 day is sufficient for the function of nAChR to recover from irreversible inactivation by Bromo-ACh in cultured SCG neurons.

Recovery of nAChR function after Bromo-ACh inactivation requires protein translation but not new gene transcription

To learn more about the turnover of the nAChRs in the SCG neurons, I determined whether new gene transcription or new protein synthesis were necessary for nAChR recovery after Bromo-ACh inactivation. SCG neurons were treated with Actinomycin D (4 μM) to block transcription. Actinomycin D was applied with growth medium 4 hours before Bromo-ACh treatment. Previous work showed that in cultured adrenal chromaffin cells, it took about 3 hours for newly-synthesized nAChRs to appear at the membrane (Higgins and Berg, 1988b). Therefore a 4 hour pretreatment with Actinomycin D was used to avoid currents from nAChRs synthesized before the Actinomycin D treatment during recovery. The nAChRs were then inactivated by Bromo-ACh, and Actinomycin D was included in the growth medium during 3 hours of recovery. The results showed that ACh-evoked current densities were not different in these neurons from those recovering without the Actinomycin D treatment (Fig 4.2 C). This suggests that gene transcription is not necessary for nAChR recovery at 3 hours after Bromo-ACh treatment.

To test whether protein translation was necessary for nAChR recovery from Bromo-ACh inactivation, I blocked translation with puromycin (50 μM). Puromycin was

added to the culture medium for 4 hours before the Bromo-ACh treatment and then during the 3 hours after inactivation. The recorded current densities were not significantly different from current densities recorded right after Bromo-ACh inactivation (Fig 4.2 C). Therefore, blocking translation prevented the recovery of nAChRs at 3 hours after Bromo-ACh inactivation.

nAChRs oxidised by ROS recover within 48 hours

Next, I examined the recovery of nAChRs from inactivation by ROS. To elevate ROS, cells were treated with Anti-A (10 μM) in the growth medium for 1 hour. Since the nAChRs are inactivated by ROS in a use-dependent way (Campanucci et al., 2008), ACh was applied from a large pipette over culture dishes while perfusing them at fast speed (~5ml/min). After applying ACh every 30 sec for a total of 60 times, ACh-evoked current densities decreased to ~38% of normal size (Fig 4.3 A). In control dishes treated with only ACh puffs but no Anti-A pretreatment there was no significant decrease in current densities (Fig 4.3 A). Anti-A treated dishes that did not have ACh applications were not significantly different from control (Fig 4.3 A).

The recovery of current densities was then examined at different times after this inactivation. At 3 hours after inactivation, current densities reached 57% of normal size, which was 19% of total recovery (Fig 4.3 B). The current density reached 82% of control, or 45% of total recovery by 12 hours. The recovery was about complete by 24 hours, reaching 95% of normal current density (Fig 4.3 B).

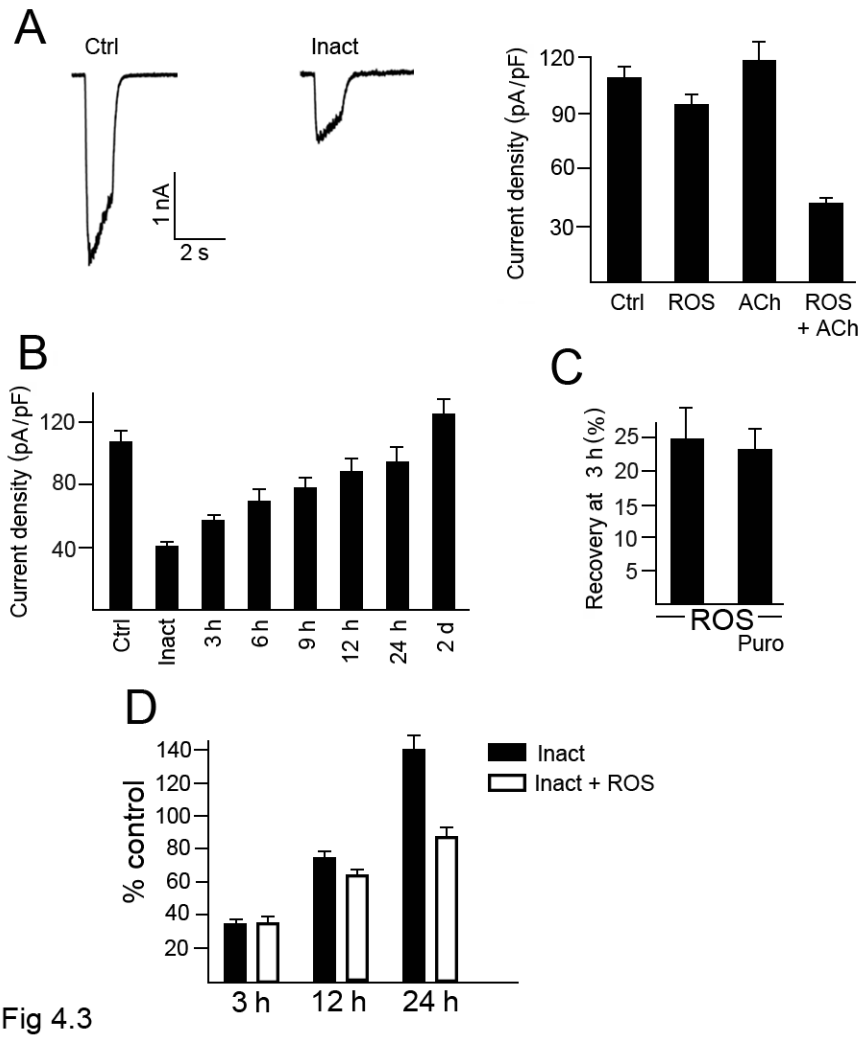

A
Ctrl
Inact
1 nA
2 s
Current density (pA/pF)
120
90
60
30
Ctrl
ROS
ACh
ROS
+ ACh
B
Current density (pA/pF)
120
80
40
Ctrl
Inact
3 h
6 h
9 h
12 h
24 h
2 d
C
Recovery at 3 h (%)
25
20
15
10
5
ROS
Puro
D
% control
140
120
100
80
60
40
20
Inact
Inact + ROS
3 h
12 h
24 h

Fig 4.3

Figure 4.3 The recovery of nAChRs from inactivation by ROS happens by 48 hrs and does not depend on new protein synthesis.

A) Inward currents on cultured SCG neurons after inactivation of nAChRs with ROS, done by a 1 h pretreatment of neurons with Antimycin-A (Anti-A) followed by 60 puffs of ACh. Left panel: Representative currents on cultured neurons without inactivation and after inactivation with ROS. Right panel: Averaged current densities recorded in neurons without inactivation (n=21), in neurons treated only with Anti-A (n=12), in neurons treated only with ACh puffs (n=13), or in neurons where Anti-A pretreatment was combined with ACh puffs (n=16).
B) Current densities recorded on cultured neurons in control conditions (n=21), after inactivation with ROS (n=16), and 3h (n=16), 6h (n=16), 9h (n=17), 12h (n=12), 24h (n=13), and 2d (n=13) after inactivation.
C) Current densities recorded 3h after inactivation with ROS with no treatment during recovery (n=16), or with puromycin treatment during recovery (n=24). Current densities are expressed as % of total recovery.
D) Current densities recorded 3h, 12h, or 24h after inactivation with Bromo-ACh either without Anti-A pretreatment (black bars, n=16, n=12, n=13 respectively) or with Anti-A pre-treatment to elevate ROS (white bars, n=12, n=26, n=16 respectively). Current densities are expressed as % of total recovery.

These results suggest that, similarly to what is seen after nAChR inactivation with Bromo-ACh, approximately 1-2 days is sufficient for the receptor function to recover fully after oxidation by ROS. Unlike the recovery from Bromo-ACh, the current densities were not above normal at 24 hours, however they did become larger than normal values (116% of control size) by 2 days after inactivation.

nAChRs can recover from inactivation by ROS without new protein synthesis

Does the recovery of nAChRs from ROS-induced inactivation require protein synthesis? To test this I treated SCG neurons with puromycin for 4 hours before the inactivating treatment, and then during the 3 hours of recovery after inactivation.

Unlike inactivation with Bromo-ACh, the current densities recovered to normal levels despite the puromycin treatment, and were not significantly different from those that recovered without the puromycin treatment (Fig 4.3 C).

ROS can slow down the recovery of nAChRs from inactivation

The current densities after recovery from inactivation by ROS appeared smaller compared to those inactivated by Bromo-ACh, particularly at later time points. I asked whether ROS may have effects that decrease the recovery of nAChRs. To explore this, I pre-treated the cultured SCG neurons with Antimycin-A (10 μM) for 1 hour prior to inactivation with Bromo-ACh and recorded ACh-evoked current densities at different times after recovery. I expressed the current densities as % of control values. At 3 hours

after inactivation, the Anti-A-pre-treated neurons showed a similar recovery to neurons not pre-treated with Anti-A and instead pre-treated with growth medium. However, the current densities in Anti-A pre-treated neurons were smaller than in medium-treated neurons at later time points (Fig 4.3 D). At 12 hours of recovery, the Anti-A-pre-treated neurons had current densities that were 64% of control, unlike 75% seen in growth medium-pre-treated neurons. By 24 hours, the Anti-A-pre-treated neurons recovered to 87% of control, while those pre-treated with growth medium had current densities that were 140% of control (Fig 4.3 D).

nAChR currents stop running down 48 hrs after glucose of hyperglycemic SCG cultures is normalised

Being interested in the recovery of nAChRs *in vivo* in the context of diabetes after blood glucose has been lowered, I estimated the time that it takes for ROS levels to return to normal once glucose levels are normalized. To do this, I used hyperglycemic cultures of SCG neurons. When glucose is elevated from 5mM to 25mM in cultures of SCG neurons, there is an intracellular elevation of ROS and ACh-evoked currents run down (Campanucci et al., 2010). Since ACh-evoked currents run down reliably when ROS are elevated (Campanucci et al., 2008), I used current rundown as an indication of elevated ROS. Using hyperglycemic SCG cultures with 25mM glucose in culture medium, I lowered glucose back to 5mM and recorded ACh-evoked currents at different times. I reasoned that when ACh-evoked currents become stable, ROS will have returned to lower levels. At 24 hours after normalizing glucose, the ACh-evoked

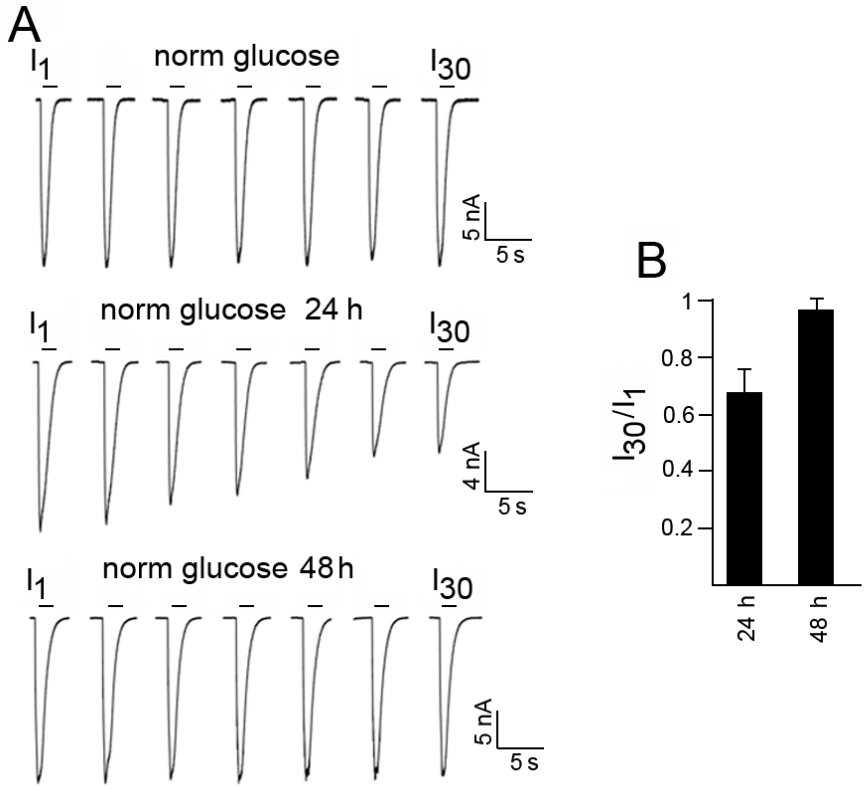


Fig 4.4

Figure 4.4 When the SCG cultures hyperglycaemic for 1 week are returned to normal glucose, the rundown of nAChRs currents stops within 48 hrs.

A) Inward currents on cultured neurons in response to 1 s applications of ACh (100μM) at 15 s intervals. Top panel: ACh-evoked currents in neurons cultured in growth medium containing 5mM glucose for 2 weeks and 2 days (n=8). Middle panel: ACh-evoked currents in neurons cultured with 5mM glucose for the 1 week, then treated with growth medium containing 25mM glucose for the 2nd week, and returned to growth medium with 5mM glucose for 24hrs (n=11). Bottom panel: ACh-evoked currents in neurons cultured with 5mM glucose for the 1st week, then treated with growth medium containing 25mM glucose for the 2nd week, and returned to growth medium with 5mM glucose for 48hrs (n=12).

B) The ratio of the 30th ACh-evoked current to the 1st in neurons cultured in 5mM glucose during the 1st week, then treated with growth medium containing 25mM glucose during the 2nd week, then returned to growth medium with 5mM glucose for 24 (n=11) or 48 hours (n=12).

currents showed a run-down (Fig 4.4 A). The I_{30}/ I_1 at 24 hours of normal glucose was ~0.7, which indicates a milder rundown than usually seen in hyperglycemic cultures that show an I_{30}/ I_1 of ~0.5. When tested 48 hours after glucose was normalised, the ACh-evoked currents were stable (Fig 4.4 A, B), suggesting that ROS levels have decreased.

Synaptic transmission recovers in SCG of diabetic mice when treatment with insulin begins no later than 2 weeks after diabetes onset

If ROS levels in SCG neurons of mice change with a similar time-course to cultured SCG neurons, then, in diabetic mice in which glucose is normalised with insulin, the ROS levels should return to normal after ~48 hours (according to results in Fig 4.4). In addition, knowing that 24-48 hours is sufficient for nAChR function to recover from inactivation by ROS (according to results in Fig 4.3 B), a total of ~3-4 days should be enough for nACh to recover when hyperglycemia is reversed in SCG *in vivo*. This suggests that in diabetic mice, nicotinic synaptic transmission should recover after glucose is normalized by insulin well by 1 week. However, if there is a critical period, recovery could depend on the duration of diabetes prior to insulin treatment.

I recorded EPSPs in mice which were diabetic for 2 weeks and which were treated with insulin for 1 week. Unlike diabetic mice which on average have EPSPs depressed to ~50% of control, these insulin-treated mice had EPSPs of the same size as in healthy mice, showing full recovery (Fig 4.5 A, B). However, interestingly, with longer diabetes durations of 1, 2, or 3.5 months, treatments with insulin did not produce

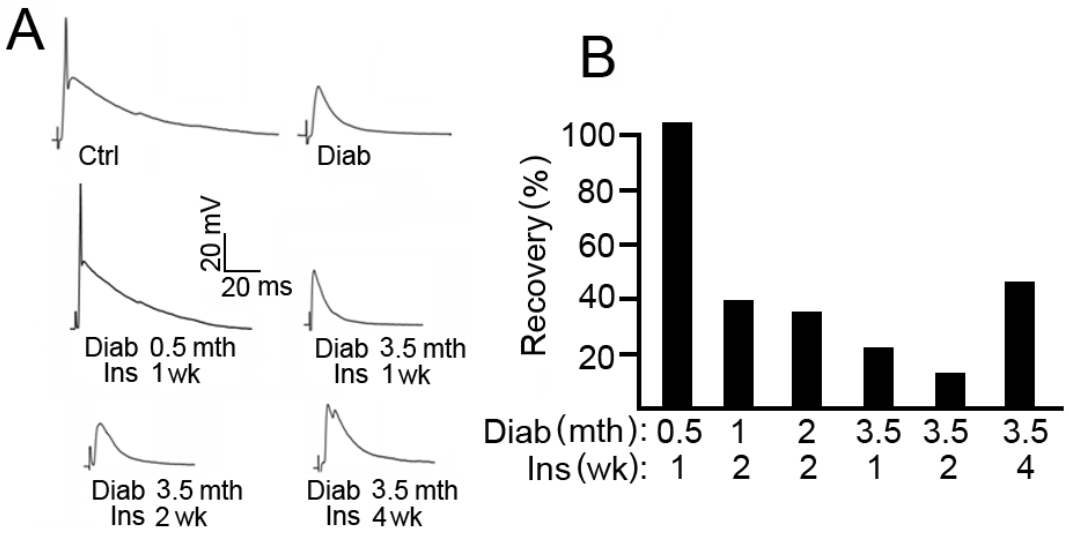


Fig 4.5

Figure 4.5 Insulin treatment to lower glucose restores normal synaptic transmission in mice treated after 2 weeks of diabetes, but the recovery is partial in mice treated with insulin after 1, 2, or 3.5 months of diabetes.

A) EPSPs recorded in response to stimulation of preganglionic nerve in SCGs of healthy control mice, in mice diabetic for 2 wk, 1, 2, 3.5mth and implanted with insulin for 1, 2 or 4 wks.
B) Recovery of synaptic transmission (measured as integrated EPSP areas and expressed as % of full recovery needed to reach the EPSP size of control mice) in mice diabetic for 2 wk treated with insulin for 1 week (n=3 mice, n=46 neurons), diabetic for 1mth treated with insulin for 2 weeks (n=2 mice, n=35 neurons), diabetic for 2 mths treated with insulin for 2 weeks (n=1 mouse, n=20 neurons), or diabetic for 3.5 mths and treated with insulin for 1 wk (n=3 mice, n=61 neurons), diabetic for 3.5 mths and treated with insulin for 2 wks (n=2 mice, n=45 neurons), or diabetic for 3.5 mths and treated with insulin for 4 wks (n=1 mouse, n=39 neurons).

a complete recovery of synaptic transmission. With 2 weeks of insulin treatment, mice previously diabetic for 1 month showed only 39% of full recovery, and mice previously diabetic for 2 months showed 35% of full recovery. After 3.5 months of diabetes, insulin treatments for 1, 2, and 4 weeks produced 22%, 13%, and 46% of full recovery respectively (Fig 4.5 A, B).

DISCUSSION

Autonomic neuropathies can disrupt the function of many organs and, in some cases, become life-threatening. From previous work (Campanucci et al., 2010), we know that an important cause of autonomic neuropathies in diabetes is the depression of synaptic transmission in the sympathetic ganglia. The synaptic depression in sympathetic ganglia happens when ROS inactivate the postsynaptic nAChRs. We wanted to find out whether this defect in nAChR function and synaptic transmission is reversible.

I first addressed this question in cultured SCG neurons, in which I examined the recovery of nAChRs after inactivation by ROS. I saw no evidence that nAChR can recover in the membrane in the short term (~10min), even when strong reducing agent was used in the recording electrode. It is possible that once oxidised, the nAChR folds into a conformation in which the Cys^{239} in its pore is not accessible to reducing. In addition, it is possible that despite getting reduced, the nAChR is unable to fold back into a functional conformation while remaining in the membrane.

I then examined the recovery of nAChRs after oxidation by ROS over the longer term of several days in cultured SCG neurons. As a reference, I also examined the turnover of nAChRs after alkylating them irreversibly with Bromo-ACh. I found that the nAChRs recovered from Bromo-ACh inactivation by 24 hours, similarly to what was previously seen in chromaffin cells (Free et al., 2005), and this recovery required new protein synthesis. Interestingly, new gene transcription was not needed for nAChRs to recover at 3 hours after inactivation, which suggests that the mRNAs of the nAChRs are

quite stable. Therefore, protein translation is needed but new gene transcription is not necessary for the recovery of nAChRs 3 hours after Bromo-ACh inactivation.

When the recovery of the nAChRs from oxidation by ROS was examined, this recovery did not require new protein synthesis, unlike what was seen with inactivation by Bromo-ACh. The nAChRs were able to recover normally at 3 hours after inactivation despite the block of translation with puromycin. One possible explanation of this finding may be that the nAChRs can recover through recycling. Another possibility is that premade nAChRs, such as those found in intracellular pools, may be inserted to allow nAChR function to recover.

Importantly, the results showed that after oxidation by ROS, the function of the nAChRs in cultured SCG neurons can recover within 24-48 hours. I then studied the recovery of nAChRs after inactivation by ROS *in vivo*. I estimated that the time needed for ROS to become normalized once glucose returns to normal levels after hyperglycemia in cultured SCG neurons is ~48 hours. In total then, 3-4 days could be enough for nAChR to recover in the sympathetic ganglia *in vivo* if hyperglycemia is reversed with insulin treatment.

In contrast to this estimate is evidence from diabetic humans whose autonomic neuropathy is difficult to reverse even with many years of insulin treatment (Navarro et al., 1997, Pop-Busui et al., 2010). Indeed, when diabetic mice were implanted with insulin, the depression of synaptic transmission did not fully recover, unless diabetes duration was very short (2 weeks). This result is consistent with the phenomenon of metabolic memory, previously seen in studies of diabetic complications (Pirola et al., 2010, Ranjit Unnikrishnan et al., 2011). In such studies, unless hyperglycemia is

reversed early, the complications of diabetes usually persist despite normalizing glucose. For example, in diabetic dogs, retinopathy could be reversed by insulin treatment only in dogs that were diabetic for 2 months but not in those diabetic for 2.5 years (Engerman and Kern, 1987). In diabetic rats, retinopathy could be reversed by pancreatic islet transplant after 6 weeks but not after 12 weeks of diabetes (Hammes et al., 1993). Diabetic rats also showed improved conduction velocity of the sciatic nerve if they received a pancreas transplant 1 or 3 months but not 6 months after diabetes onset (Spadella et al., 2012).

According to studies of molecular mechanisms of metabolic memory, oxidative stress is the important mediator (Ceriello et al., 2005, Ihnat et al., 2007b). ROS produced in hyperglycemia are linked to epigenetic changes such as post-translational modifications of histones (Cooper and El-Osta, 2010). It is not known whether and which epigenetic changes happen in autonomic neurons in diabetes, and exploring this in future studies would be interesting. Possibly, metabolic memory effects mediated by ROS could interfere with nAChR recovery. Consistent with this, we observed that elevating ROS in cultured SCG neurons slows down the recovery of nAChRs from inactivation by Bromo-ACh (Fig 4.3 D). If ROS are elevated during long periods, as in long durations of diabetes, metabolic memory effects could prevent the recovery of synaptic transmission.

It is also possible that ROS simply do not decrease when glucose is normalized after long durations of diabetes as has been observed previously (Kowluru, 2003, Kowluru et al., 2004, Ihnat et al., 2007b). For example, damage to mitochondria after long diabetes durations could lead to persistent elevation of ROS (Pieczenik and

Neustadt, 2007). In such case, high ROS would continue to inactivate the nAChRs in synapses of sympathetic ganglia. The results in cultured SCG neurons showed that ROS decreased by 48 hours after lowering glucose after hyperglycaemia that lasted for 1 week. However, ROS might not decrease in neurons that remained hyperglycemic for longer durations. For example, a study by Kowluru and colleagues showed that markers of oxidative stress in urine and kidneys of diabetic rats remained elevated even after 7 months of insulin treatment if this treatment began following 6 months of diabetes. However, when insulin treatment started 3-4 days after diabetes onset, the oxidative stress markers became normalized (Kowluru et al., 2004).

Taken together, these results suggest that for synaptic transmission to recover in sympathetic ganglia in diabetes, it is important to start insulin treatment as early as possible. Therefore, the most likely implication for diabetic people is that an early and quick diagnosis of diabetes is needed, and glucose-lowering treatments should not be delayed.

It would be interesting to see if combining glucose-lowering with antioxidant treatments could reverse the depression of synaptic transmission even after long durations of diabetes. Promising results were seen with vascular endothelial cells and retinal cells that remained hyperglycaemic for 2 weeks. After lowering glucose for 1 week, oxidative stress markers remained elevated in these cells. However, when 1 week of lowered glucose was combined with the antioxidant α-lipoic acid, the markers of oxidative stress decreased (Ihnat et al., 2007a). Similarly in their study, the researchers observed beneficial effects of the antioxidant for whole retina. The retina of rats diabetic for 2 weeks showed increased oxidative stress even after glucose was lowered with

insulin implants for 1 week. Only when insulin was combined with injections of α-lipoic acid during the 1 week of treatment, oxidative stress markers normalised in the retina of rats (Ihnat et al., 2007b).

Unfortunately, antioxidant treatments in diabetic humans are usually ineffective (Cocheme and Murphy, 2010, Halliwell, 2012). Early glucose control in diabetes is therefore very important to reverse the depression of synaptic transmission in sympathetic ganglia. Possibly, new treatments could be developed when more is known about the mechanisms of metabolic memory in sympathetic neurons.

CHAPTER 5

Diabetes-induced depression of synaptic transmission in sympathetic ganglia is prevented in WLD^s mice

ABSTRACT

Among the important causes of peripheral neuropathies of diabetes is an elevation of reactive oxygen species (ROS) induced by hyperglycemia in this disease. In the sympathetic superior cervical ganglion (SCG), elevated ROS cause a depression of synaptic transmission, which develops as a result of the inactivation of postsynaptic nicotinic receptors by ROS.

Whether it is possible to prevent diabetic neuropathies by lowering ROS has been difficult to determine, in part because antioxidants *in vivo* can be ineffective. It has been reported that in the mutant mouse strain Wallerian degeneration slow (WLD^s), oxidising treatments or axonal damage to peripheral nerves result in ROS elevations that are lower than normally observed. Using the WLD^s mouse strain, I examine possible effects of decreased ROS for the synaptic depression that happens in the SCG in diabetes. I test the hypothesis that decreased ROS levels in the SCG of WLD^s mice can prevent the depression of synaptic transmission in this sympathetic ganglion in diabetes.

To examine this hypothesis, homozygous and heterozygous WLD^s mice and wild type controls were made diabetic, and following 2 months of diabetes, nerve-evoked EPSPs were recorded in the SCG with intracellular electrodes. It was found that, while the diabetic wild-type mice had EPSPs that were depressed by >60% compared to age-matched control mice, the EPSPs remained normal in diabetic homozygous WLD^s mice. However, the EPSPs in diabetic heterozygous WLD^s mice became depressed and did not differ from those in diabetic wild types.

These results suggest that the synaptic depression in the sympathetic ganglia in diabetes is preventable. Both copies of the WLD^s mutation are needed to protect the sympathetic synapses in diabetes, suggesting that the amount of the WLD^s protein is important for protection.

INTRODUCTION

Damaging effects of reactive oxygen species (ROS) are involved in many diseases, especially those affecting the nervous system - Parkinson's disease (Gandhi and Abramov, 2012), Alzheimer's disease (Smith et al., 2000), Amyotropic Lateral Sclerosis (Barber and Shaw, 2010). In diabetes, oxidative stress is linked to peripheral neuropathies (Pop-Busui et al., 2006, Hosseini and Abdollahi, 2013), among the most serious of which are diabetic autonomic neuropathies. In many diabetic patients, autonomic neuropathies cause abnormal function of major organs, including the heart, the digestive organs, and the bladder, increasing the risk of mortality (Gerritsen et al., 2001, Vinik and Erbas, 2013).

Recent work at the Cooper lab showed that hyperglycemia elevates ROS in sympathetic superior cervical ganglion (SCG) of diabetic mice, and elevated ROS inactivate the postsynaptic nicotinic acetylcholine receptors (nAChRs) in this ganglion. As a result of the nAChR inactivation, synaptic transmission in the SCG becomes depressed and causes dysautonomias (Campanucci et al., 2010). These findings bring a question of whether one could prevent diabetic dysautonomias by lowering ROS levels. One way to address this question is to lower ROS with antioxidants, but *in vivo* attempts to lower ROS with antioxidants are often ineffective (Cocheme and Murphy, 2010, Halliwell, 2012). Another way to test if lowering ROS can prevent diabetic dysautonomias is by using animal models which have improved ROS detoxification *in vivo*.

The mouse strain Wallerian degeneration slow (WLD^s) has a mutation that slows the degeneration of their peripheral nerves, which was suggested to result from

decreased ROS levels in these nerves (Press and Milbrandt, 2008). When the axons of peripheral nerves are severed from the cell body in WLD^s mice, the axons can survive for over a week, instead of degenerating within 12 to 24 hours as in wild type mice (Perry et al., 1990).

The WLD^s mutation causing this phenotype is a spontaneous tandem triplication of an 85kb region that spans several genes on distal chromosome 4 (Conforti et al., 2000, Mack et al., 2001). As a result, a unique WLD^s protein is produced which at its C-terminal includes all 285 amino acids of an enzymatically acitve nicotinamide mononucleotide adenylyl transferase1 (NMNAT1). The N-terminus of the protein contains a portion (70 of a total of 1,173 amino acids) of an E4-type ubiquitin ligase Ube4b, which lacks its ligase activity. The WLD^s protein also includes a unique sequence of 18 amino acids produced by a read-through of the 5' UTR of NMNAT1 (Fig 5.1 A).

The WLD^s mutation shows a gene dosage effect: in heterozygous mutants or transgenic animals with fewer WLD^s mutation copies, there is decreased preservation of axons from degeneration (Mack et al., 2001).

Though it is not known how the mutation in WLD^s mice causes slower axonal degeneration, there is evidence that WLD^s protein reduces elevations of ROS that normally happen in injured axons. For example, O'Donnell and colleagues examined ROS elevations in sensory neurons of transgenic zebrafish that overexpress the WLD^s protein. These zebrafish also expressed a GFP molecule that was sensitive to reduction-oxidation and was targeted to mitochondria (mito-roGFP). When axons of wild-type zebrafish neurons were severed, there was a 3-fold elevation in mitochondrial

ROS by 2 hours after axonal damage. In contrast, in neurons of zebrafish overexpressing the WLD^s protein, ROS levels remained normal throughout the 2 hour period (O'Donnell et al., 2013).

Press and Milbrandt also observed decreased elevations of ROS in cultured mouse dorsal root ganglia neurons transfected with NMNAT3, the mitochondria-localized isoform of the NMNAT enzyme which gets overexpressed as part of WLD^s fusion protein. The researchers used oxidising treatments – rotenone, which increases mitochondrial ROS by blocking complex I of the mitochondrial electron transport chain, and H_2O_2, and used a ROS-sensitive dye CM-H2DCFDA to measure ROS. They saw smaller and delayed ROS elevation in NMNAT3-transfected neurons in response to rotenone. Both rotenone and H_2O_2 treatments also caused less axonal damage in the NMNAT3-transfected neurons than they did in control neurons (Press and Milbrandt, 2008).

The WLD^s mutation has been shown to have beneficial effects for peripheral nerves in the context of diabetes (Zhu et al., 2011). In the study by Zhu and colleagues, 7 weeks of diabetes caused conduction velocity to slow down in sensory and motor nerves of wild type mice by ~40-55%, but in WLD^s mice the conduction velocity slowed down by only ~12-20%.

It is not known if the WLD^s mutation has benefits for the autonomic synaptic transmission, which becomes depressed in diabetes and is associated with serious autonomic neuropathies (Campanucci et al., 2010). To investigate this, we studied autonomic synaptic transmission in the superior cervical ganglia (SCG) of diabetic WLD^s mice. We hypothesized that decreased ROS levels in SCG of WLD^s mice will

prevent the inactivation of postsynaptic nAChRs and prevent the depression of synaptic transmission in diabetes.

If WLD^s does protect the SCG synapses in diabetes, we also asked whether gene dosage effect of WLD^s will be seen, as previously observed for axonal degeneration. We predicted that if lower amount of WLD^s protein in heterozygous (+/-) WLD^s mice is not enough to lower ROS levels, there will be lower or no protection of synaptic transmission in their SCG in diabetes than in homozygous (+/+) WLD^s mice.

METHODS

Animals

We used 3 month old WLD^s mice on C57BL background and wild type C57BL and C57BL/6/J x CD1 mice. Both male and female mice were used.

Streptozotocin injections

Some animals were made diabetic at 3-4 weeks of age by an injection of streptozotocin (STZ) dissolved in Na^+ citrate buffer at 40-60mg/kg. Mice were deprived of food for 6 hours before the STZ injection, and food was returned immediately after. Zhu and colleagues observed that fewer WLD^s than wild type mice became diabetic when injected with multiple low doze STZ and more pancreatic β cells in these mice survived the destruction by STZ (Zhu et al., 2011). While we did not observe a difficulty inducing diabetes in WLD^s mice, we only used mice which developed glucose levels >20mM within 48-72 hours after the STZ injection for experiments.

Akita x WLD^s mice

Diabetic heterozygous (+/-) WLD^s mice were obtained by crossing a ♂ Akita mouse which is +/- for the ins2 mutation and -/- for the WLD^s mutation with a ♀ mouse which is +/+ for the WLD^s mutation and -/- for the ins2 mutation. The litters of this cross gave mice which were all WLDs +/- and half of which on average were ins2 +/-. We genotyped the mice to detect those that were ins2 +/-. We also genotyped the mice to confirm that the WLD^s mutation was present. The ♂ Akita mice develop more severe

hyperglycemia than ♀ Akita mice (Toyoshima et al., 2007), and therefore we used ♂ mice which were ins2 +/- and WLDs +/-. These mice developed glucose levels >20mM by 4 weeks of age.

PCR

The WLD^s mice were identified by amplifying a region in the WLD^s mutation formed by the adjacent triplicated units (Fig 5.1 A, B) (Mi et al., 2002). PCR with the following conditions was used: 94° C, 2min; 94° C, 45 s; 58.5° C, 45 s; 72° C, 45 s; 35 cycles; 72° C, 10min. The following primers were used:

Forward: 5' CGTTGGCTCTAAGGACAGCAC3'

Reverse: 5'CGTTGGCTCTAAGGACAGCTGCAGCCCCCACCCCTT 3'.

The PCR amplified a 182bp band in WLD^s mice and no band in wild type mice (Fig 5.1 C).

For other details on methodology, see Chapter 2.

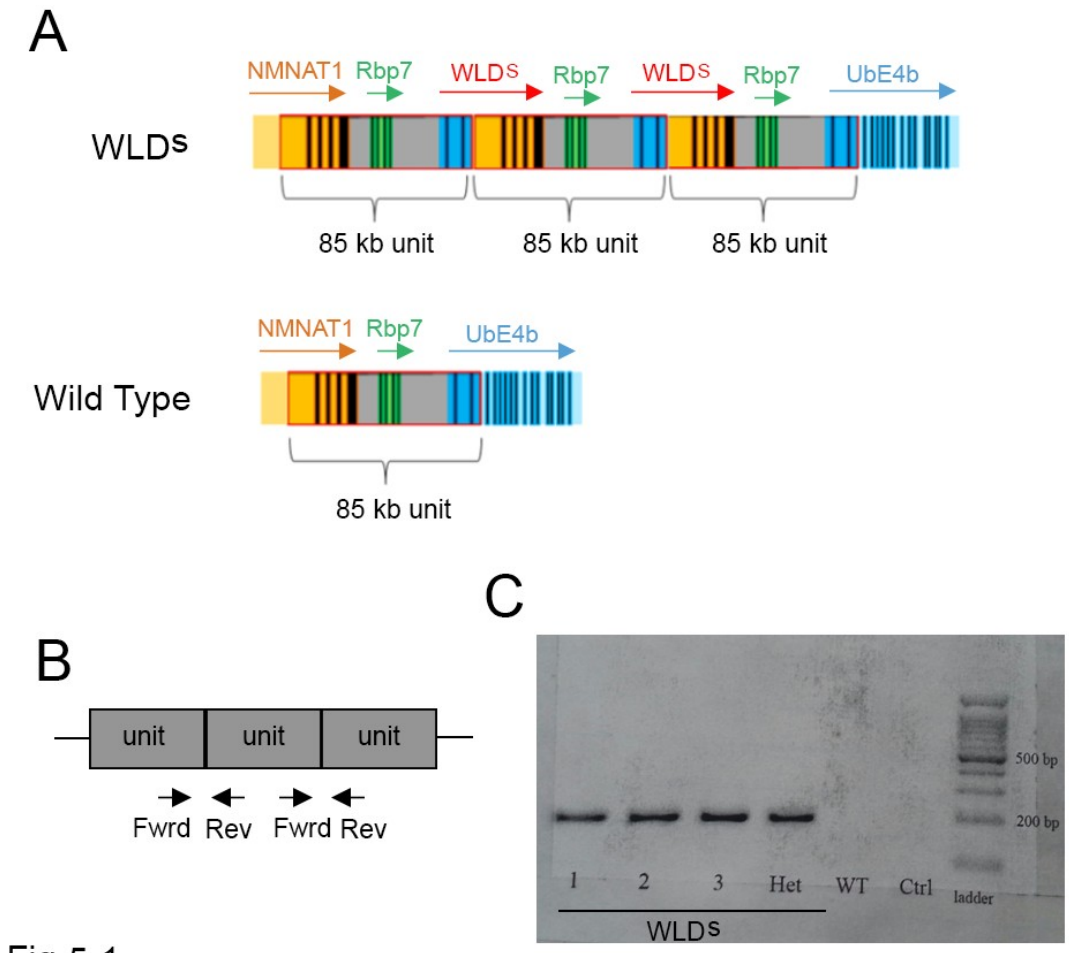


Fig 5.1

Figure 5.1 WLD^s mutation and its detection by PCR.

A) A schematic of the tandem triplication of an 85kb region in WLD^s mice (Top) and the same region in wild type mice (Bottom). Genes of NMNAT1, Rbp7, and Ube4b are indicated in color. Exons are shown as black bands. WLD^s gene is indicated, formed from NMNAT1 and Ube4b gene regions brought together at adjacent units.

B) A schematic showing the position of the primers for PCR amplifying the unique region formed by the adjacent triplicated units in WLD^s mice.

C) An example of a WLD^s PCR gel showing an 182bp band amplified in homozygous WLD^s mice (columns 1-3) as well as in the heterozygous WLD^s (Het).

RESULTS

EPSPs in SCG of WLD^s mice do not differ from EPSPs in wild type mice

Our interest was to find out if the depression of synaptic transmission that happens in SCG in diabetes is prevented in WLD^s mice. First, we asked whether synaptic transmission in SCG of nondiabetic WLD^s mice is the same as in SCG of nondiabetic wild type mice. Wishart and colleagues evaluated the development and histology of internal organs of the WLD^s mice and found no abnormalities. They also did a histological assessment of the sympathetic ganglia of WLD^s mice and found no differences from wild type mice (Wishart et al., 2009). Knowing that structurally the sympathetic ganglia were normal, we verified if their function in the WLD^s mice was also similar to wild type mice.

To test this, I recorded synaptic transmission in the SCGs of WLD^s mice and of age-matched control wild type mice. In both WLD^s and wild-type mice, the nerve-evoked EPSPs were over 20mV in size and usually suprathreshold (Fig 5.2 A). The size of the EPSPs, measured by integrating the area bound by the EPSP curve, did not differ between WLD^s and wild type mice (Fig 5.2 A, B).

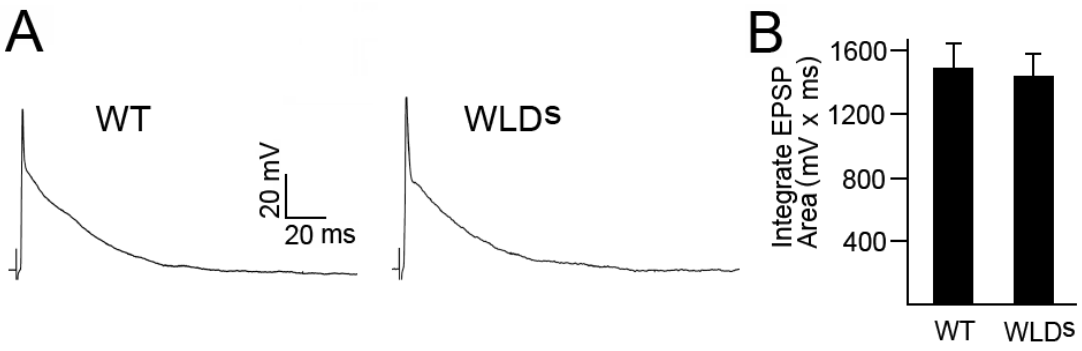


Fig 5.2

Figure 5.2 The EPSPs in superior cervical ganglion (SCG) of WLD^s mice do not differ from EPSPs in wild type mice.

A) Nerve-evoked EPSPs at SCG synapses of 3 month old wild type and WLD^s mice.
B) Averaged integrated EPSP areas of 3 month old wild type (n=3 mice, n=36 neurons) and WLD^s mice (n=3 mice, n=22 neurons). The values are means ±SEM.

Synaptic transmission in SCG of WLD^s mice does not become depressed in diabetes

Previous work from the Cooper lab showed that hyperglycemia leads to an elevation of intracellular ROS in SCG neurons, which oxidise and inactivate the postsynaptic nAChRs in SCG. As a result of hyperglycemia in diabetic mice, the EPSPs in SCG are significantly depressed (Campanucci et al., 2010). We wanted to find out if lower ROS in SCG neurons of WLD^s mice would prevent the depression of synaptic transmission in SCG of these mice in diabetes.

To test this, we made WLD^s and wild type mice diabetic with an injection of STZ, and the mice developed blood glucose levels >20mM within 48-72hrs. After 2 months of diabetes, we recorded nerve-evoked EPSPs in SCG of these mice. We observed that the EPSPs were significantly depressed in the SCGs of wild type diabetic mice compared to age-matched control mice. The integrated EPSP areas in SCG of these diabetic mice were 38% of those in control mice (Fig. 5.3 A, B).

Along with inducing diabetes in wild-type mice, age-matched homozygous WLD^s mice were also made diabetic with STZ. I recorded EPSPs in SCG of the WLD^s mice which were either diabetic or healthy control, and found that diabetes did not decrease the EPSP size in the WLD^s mice (Fig 5.3 C, D). The averaged integrated EPSP areas in diabetic WLDs mice were not significantly different from EPSP areas in healthy control mice. This suggests that synaptic transmission remained normal in homozygous WLD^s mice despite 2 months of diabetes.

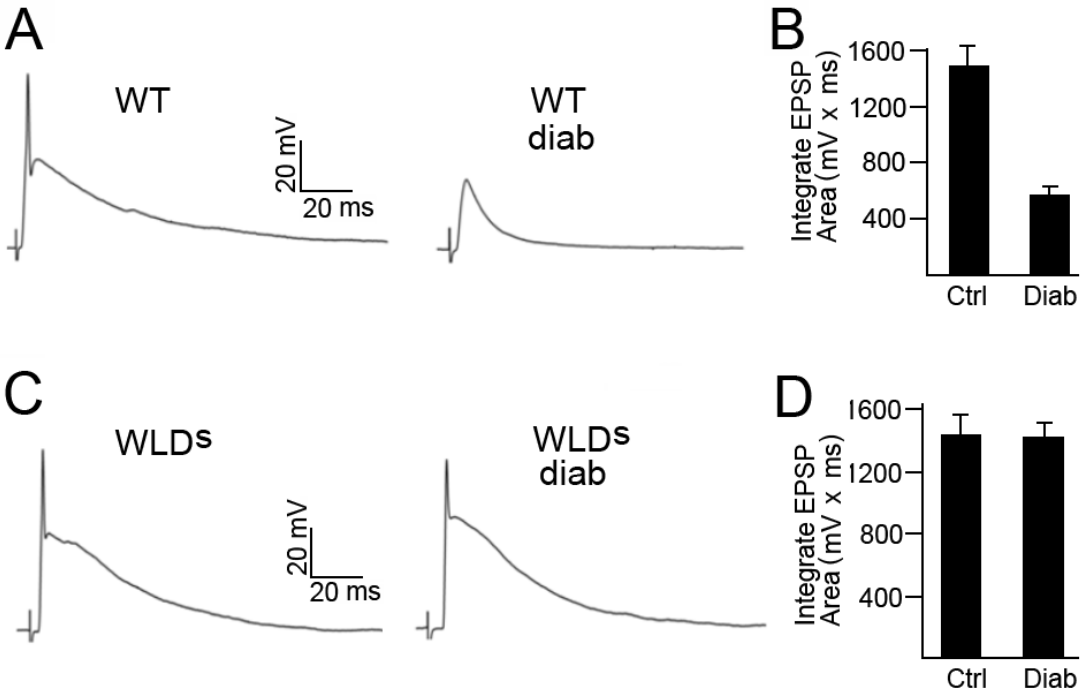


Fig 5.3

Figure 5.3. Synaptic transmission in SCG of WLD^s mice does not become depressed in diabetes.

A) Nerve-evoked EPSPs at SCG synapses of wild type mice diabetic for 2 months and age-matched control.
B) Averaged integrated EPSP areas of wild type mice diabetic for 2 months (n=4 mice, n=45 neurons) and age-matched control mice (n=3 mice, n=36 neurons). The values are means ±SEM.
C) Nerve-evoked EPSPs at SCG synapses of WLD^s mice diabetic for 2 months or control nondiabetic WLD^s mice.
D) Averaged integrated EPSP areas of diabetic (n=3 mice, n=40 neurons) and control (n=3 mice, n=22 neurons) WLD^s mice. The values are means ±SEM.

In heterozygous WLDs mice, the diabetes-induced depression of synaptic transmission in SCG is not prevented.

Previous studies showed that the WLD^s mutation had a gene dosage effect: fewer copies of WLD^s gene provided less protection from axonal degeneration (Perry et al., 1990). To determine whether one copy of WLD^s is sufficient to prevent a depression of synaptic transmission in SCG in diabetes, we compared synaptic transmission in SCG of diabetic homozygous and heterozygous WLD^s mice.

We obtained diabetic heterozygous WLD^s mice through breeding. We used offspring of one parent which was WLD^s +/+ and ins2 -/- crossed with another parent which was WLD^s -/- and ins2 +/-. We confirmed the presence of WLD^s (Fig. 5.1 C) and Akita mutations by PCR. All mice were WLD^s +/- and we selected those mice which were ins2 +/- and were male, because male mice with ins2 mutation develop more severe hyperglycemia than female (Toyoshima et al., 2007). Blood glucose of these mice became elevated >20mM by 4 weeks of age, and I recorded nerve-evoked EPSPs in the SCGs at 2 months after the onset of diabetes.

I found that unlike in SCGs of homozygous WLD^s mice, the EPSPs in SCGs of heterozygous WLD^s mice were depressed to 45% of normal size after 2 months of diabetes (Fig 5.4 A), and did not differ significantly from EPSPs in diabetic wild type mice ($p>0.05$). This suggests that one copy of the WLD^s mutation in heterozygous mice is not sufficient to protect diabetic mice from a depression of synaptic transmission.

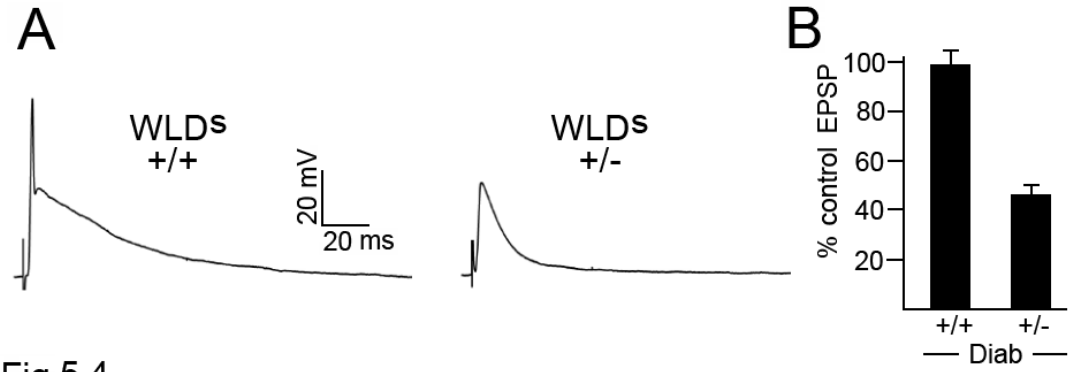


Fig 5.4

Figure 5.4 Synaptic transmission in SCG of +/- WLD^s mice becomes depressed in diabetes.

A) Nerve-evoked EPSPs at SCG synapses of mice that are WLD^s +/+ or WLD^s +/- and diabetic for 2 months.
B) Averaged integrated EPSP areas of WLD^s +/+ (n=3 mice, n=40 neurons) or WLD^s +/- (n=2 mice, n=24 neurons) that were diabetic for 2 months. The values are means ±SEM.

DISCUSSION

The results in this study showed that it is possible to prevent the diabetes-induced depression of synaptic transmission in the SCG. While SCG of wild-type mice had EPSPs that were reduced by >60% in diabetes, the EPSPs remained normal in diabetic mice that had a WLD^s mutation.

The WLD^s mutation was previously shown to decrease ROS in zebrafish sensory neurons (O'Donnell et al., 2013) and we think that mice with the WLD^s mutation have reduced elevations of ROS in SCG neurons in response to hyperglycemia. We expect that there is reduced or no oxidative stress in synapses of SCG of these mice in diabetes, which is preventing the inactivation of the postsynaptic nAChRs and preventing the depression of synaptic transmission.

Consistent with this idea, recent evidence from the Cooper lab showed that ROS elevations are reduced in SCG neurons of WLD^s mice when ROS production is induced from mitochondria by blocking complex III of the electron transport chain with antimycin-A (anti-A) (Akude and Cooper, unpublished). In this work, the neurons were loaded with a ROS-sensitive dye CM-H2DCFDA, and after being treated with anti-A, were imaged for 20min. In response to 1 μM Anti-A, the neurons of wild-type mice had a ~5-fold elevation in CM-H2DCFDA fluorescence, but the neurons of WLD^s mice had no significant ROS elevation (Fig 5.5). When the concentration of Anti-A applied to neurons of WLD^s mice was increased to 10 μM, there was an elevation in ROS but it remained significantly smaller than in wild-type mice (Fig 5.5).

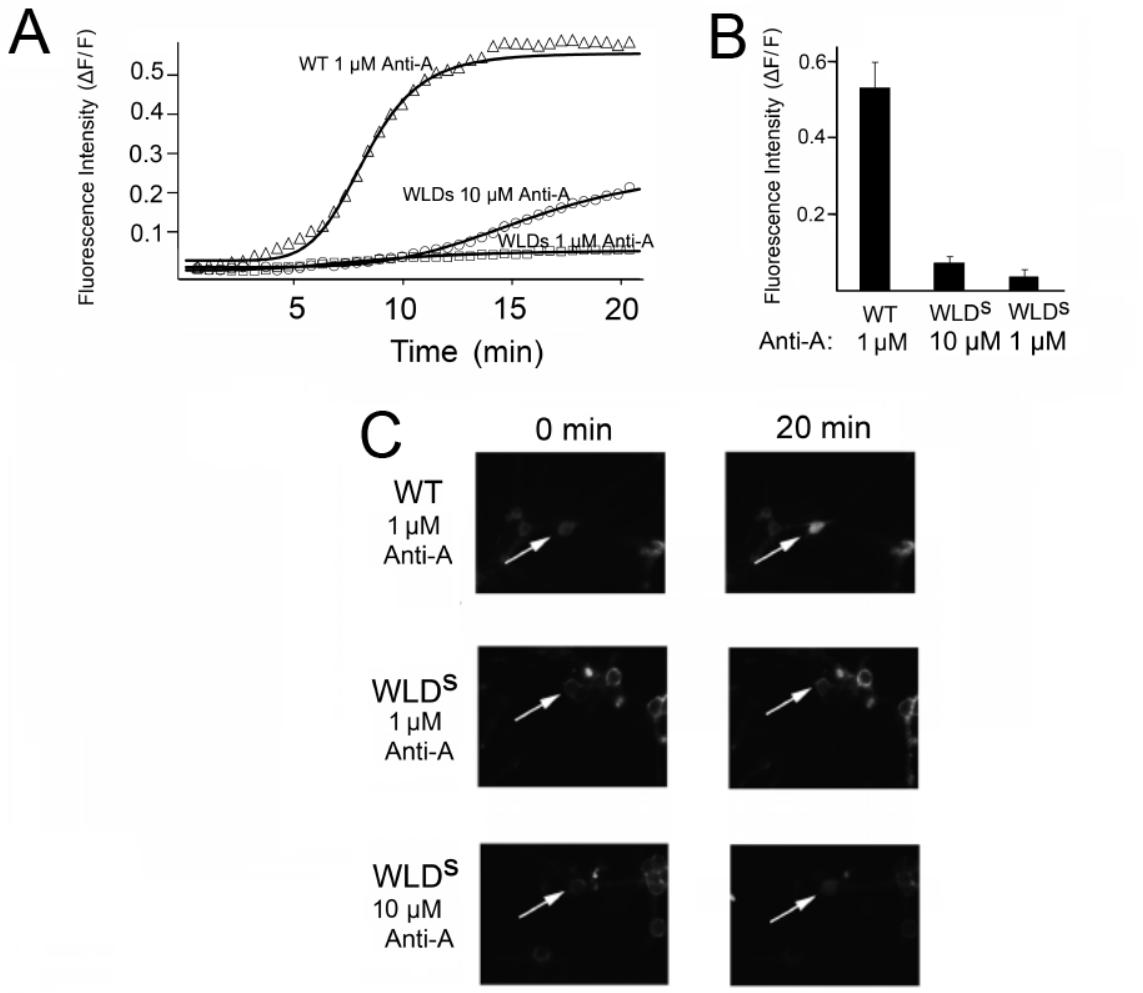

A
Fluorescence Intensity (ΔF/F)
0.5
0.4
0.3
0.2
0.1
WT 1 μM Anti-A
WLDs 10 μM Anti-A
WLDs 1 μM Anti-A
5
10
15
20
Time (min)
B
Fluorescence Intensity (ΔF/F)
0.6
0.4
0.2
WT
WLDS
WLDS
Anti-A:
1 μM
10 μM
1 μM
C
0 min
20 min
WT
1 μM
Anti-A
WLDS
1 μM
Anti-A
WLDS
10 μM
Anti-A

Fig 5.5

Figure 5.5 Blocking the mitochondrial ETC complex III with antimycin-A causes lower intracellular ROS in cultured SCG neurons of WLD^s mice than wild type mice.

A) The CM-H2DCFDA fluorescence imaged over the course of 20 min after perfusing cultured SCG neurons of wild type mice (top trace) or neurons of WLD^s mice with 1 μM anti-A (bottom trace), or perfusing neurons of WLD^s mice with 10 μM anti-A (middle trace). Fluorescence intensities were measured at 25 s intervals for a period of 20 minutes. In all experiments, neurons were pre-loaded with CM-H2DCFDA for 30 min prior to stimulation.
B) Quantification of fluorescence intensity in A at 15 min.
C) Representative images of CM-H2DCFDA fluorescence in cultured SCG neurons immediately or 20 min after perfusing cultured SCG neurons with anti-A. Neurons of wild type mice perfused with 1 μM anti-A are shown in top panels. Neurons of WLD^s mice perfused with 1 μM anti-A are shown in the middle panels. Neurons of WLD^s mice perfused with 10 μM anti-A are shown in the bottom panels.

This work showed that ROS elevations are indeed reduced in WLD^s neurons. More specifically it suggested that ROS produced by mitochondria were lower in these neurons, because stimulating mitochondrial ROS production with anti-A caused much lower ROS elevation in neurons of WLD^s compared to wild type mice. This suggests that the WLD^s protein has effects in the mitochondria. The NMNAT1, which becomes overexpressed as a part of the WLD^s protein, contains a nuclear localization signal (NLS), localizes to the nucleus, and the WLD^s protein is abundant in the nucleus (Fang et al., 2005). However, the WLD^s protein is found outside of the nucleus as well. Yahata and colleagues used subcellular fractionation analysis of brain tissue and showed that WLD^s protein that was outside the nucleus was localised to the mitochondrial matrix and mitochondrial inner membrane (Yahata et al., 2009). Avery and colleagues also used subcellular fractionation to examine WLD^s protein in synapses of striatal neurons. They found that the WLD^s protein was present in synaptic fractions containing mitochondria, but not in synaptic fractions without mitochondria (Avery et al., 2012). This evidence suggests that within synapses, the WLD^s protein may work specifically in mitochondria. When NMNAT3, the isoform of the NMNAT enzyme that localizes to the mitochondria, was overexpressed in transgenic mice, there was a strong protection from axonal degeneration in the mice (Yahata et al., 2009). Therefore, it is possible that WLD^s protein works at the mitochondria, and has protective effects by reducing mitochondrial ROS levels.

Exactly how the WLD^s protein protects the axons, or how it reduces ROS is not known. Wishart and colleagues used electron microscopy to examine the mitochondria of WLD^s and wild type mice. They saw no difference in the morphology, location or

number of the mitochondria of the WLD^s mice (Wishart et al., 2007). Yahata and colleagues also found no difference in the levels of the mitochondrial electron transport proteins between the WLD^s and wild type mice (Yahata et al., 2009). In the context of axonal protection from degeneration, there is evidence that enzymatic activity of NMNAT is required. NMNAT is the enzyme that catalyzes a key step for synthesizing NAD. WLD^s mice were shown to have a 4-fold increase in the enzymatic activity of NMNAT (Mack et al., 2001). When a mutation (W258A) which disrupts the NAD-synthesizing activity of NMNAT was introduced into the WLD^s protein and expressed in transgenic mice, the axonal degeneration in the mice did not differ from wild type (Yahata et al., 2009).

One possibility is that elevated NMNAT enzymatic activity in cells of WLD^s mice elevates NAD, which is a precursor to NADPH. In turn, NADPH is a co-factor for many antioxidant enzymes, including glutathione reductase and thioredoxin reductase. Possibly, as a result of high NMNAT enzymatic activity, the WLD^s cells have increased levels of NADPH which increases antioxidant capacity in these cells (see Fig 5.6 A). Such increased antioxidant capacity at the mitochondria could be especially effective, because mitochondria are a major source of cellular ROS. In diabetes, increased NADPH levels may be very important, because the overactivation of the polyol pathway in hyperglycemia is known to deplete NADPH (see Fig 5.6 B) (Brownlee, 2001). It would be interesting to measure the levels of NADPH in neurons of WLD^s mice, particularly in diabetes. While there are no reports of measures of NADPH levels in WLD^s mice, NAD levels in WLD^s mice did not differ when compared to wild type NAD levels (Mack et al., 2001). However, treating cultured neurons with 1mM NAD slowed down axonal

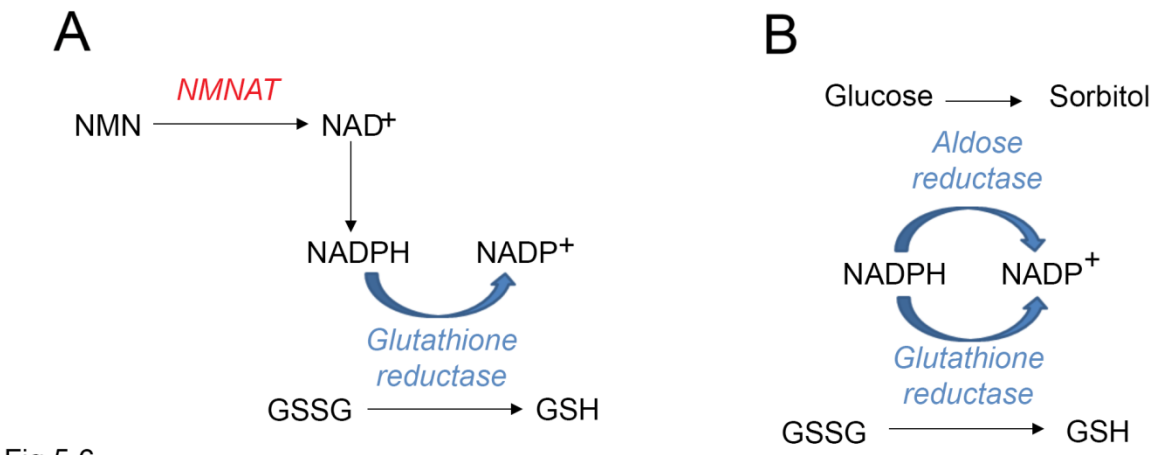


Fig 5.6

Figure 5.6 Possible mechanism of reduced oxidative stress in WLD^s mice.

A) As a result of increased NMNAT activity in WLD^s mice, more NAD may be produced, which is converted in NADPH. As NADPH serves as a co-factor for many antioxidant enzymes, such as glutathione reductase, oxidative stress is reduced.
B) Polyol pathway overactivation in diabetes is an important source of oxidative stress induced by hyperglycemia. Hyperglycemia in diabetes causes glucose to be used as substrate of aldose reductase. The reaction consumes NADPH, which reduces the amount of NADPH available as co-factor for antioxidant enzymes.

degeneration, similarly to the effect of the WLD^s mutation (Araki et al., 2004, Wang et al., 2005). It is possible that increased NAD that results from high NMNAT activity of WLD^s is converted to NADPH, which leads to antioxidant effects. This possibility has not been tested and could be addressed in future experiments.

My results in this study showed that only homozygous but not heterozygous WLD^s mice were protected against the synaptic depression in the SCG in diabetes. Similarly, already at the time of the discovery of the WLD^s mouse strain, Perry and colleagues (1989) observed that axonal degeneration in homozygous WLD^s mice was slower than in heterozygous mice (Perry et al., 1990). Mack and colleagues produced transgenic mice with different levels of WLD^s protein. They saw that the amount of WLD^s protein correlated with the amount of protection for motor neurons and neuromuscular junctions from degeneration (Mack et al., 2001). Therefore the number of WLD^s gene copies is important for both the axonal protection and the prevention of synaptic depression in diabetes in WLD^s mice.

Taken together, the findings in this study show that the defect in synaptic transmission in sympathetic ganglia in diabetes can be prevented and this may prevent diabetic autonomic neuropathies from developing. Preventing the depression of synaptic transmission in diabetes could be done by lowering ROS elevations in sympathetic neurons. If we can learn more on how WLD^s lowers mitochondrial ROS, this may help develop treatments for diabetic neuropathies and other diseases with elevated ROS.

CHAPTER 6

General discussion and conclusions

In experiments presented in this thesis, I investigated a complication of diabetes affecting the autonomic nervous system. This complication develops when hyperglycemia-induced ROS inactivate postsynaptic nAChRs in sympathetic ganglia, disrupting synaptic transmission and producing dysautonomias (Campanucci et al., 2010).

In my studies, I had the following aims: 1) To find out if the diabetes-induced depression of synaptic transmission affects all 3 divisions of the autonomic nervous system: sympathetic and parasympathetic ganglia and the adrenal medulla; 2) To test if the depression of sympathetic synaptic transmission in diabetes is reversible; and 3) To test if the depression of sympathetic synaptic transmission in diabetes can be prevented by lowered oxidative stress in the mutant mouse strain WLD^s.

The following are the main conclusions from these studies.

Findings on whether synaptic depression happens throughout the autonomic nervous system in diabetes

I hypothesized that synaptic transmission would become depressed in autonomic ganglia or in adrenal medulla when two conditions are met: 1) the postsynaptic nAChRs are α3-containing, and 2) ROS become elevated in diabetes. The results showed that α3-containing nAChRs indeed mediated synaptic transmission almost entirely in these ganglia. In the parasympathetic submandibular ganglion, however, on average about 10% of EPSP size was mediated by α7-containing nAChRs. Nevertheless, we expected that this synaptic transmission mediated by α3-containing nAChRs would become depressed in diabetes, provided that ROS became elevated.

The results showed that diabetes depressed synaptic transmission in sympathetic ganglia and in the adrenal medulla. Consistent with this, ROS became elevated when these sympathetic neurons were treated with the mitochondrial ETC blocker Anti-A. In contrast, in parasympathetic submandibular ganglion, Anti-A at the same concentration did not significantly elevate ROS. And, as we predicted, synaptic transmission did not become depressed in this ganglion in diabetes. These findings bring a question of whether synapses in all parasympathetic ganglia or only in the submandibular ganglion are protected from the effects of ROS in diabetes.

Do all parasympathetic ganglia have reduced ROS in diabetes?

In a recent study, Tompkins and colleagues (2013) recorded synaptic transmission in the parasympathetic pelvic ganglion of diabetic mice. Similar to our findings with the submandibular ganglion, the researchers found no depression of synaptic transmission in the pelvic ganglion in diabetes (Tompkins et al., 2013). Possibly then, synapses in other parasympathetic ganglia in addition to the submandibular show decreased vulnerability in diabetes.

How are ROS elevations prevented in the parasympathetic ganglion?

One difference between the postganglionic sympathetic and parasympathetic neurons is that they synthesize different neurotransmitters. There is evidence that the synthesis of norepinephrine that happens in postganglionic sympathetic neurons promotes formation of ROS, because of a Fenton-like reaction associated with the enzyme tyrosine hydroxylase (Haavik et al., 1997). On the other hand, the synthesis of

acetylcholine in postganglionic parasympathetic neurons is not expected to be prooxidant. There could be other reasons for different susceptibility of sympathetic and parasympathetic neurons to elevations of ROS. For example, an interesting observation by my colleague Yumaine Chong is that the submandibular ganglion has higher autofluorescence in the blue-green spectrum than sympathetic SCG when the ganglia are imaged with confocal microscopy. It is known that the intracellular reduced pyridine nucleotides NADH and NADPH account for the majority of cellular autofluorescence (Dellinger et al., 1998, Chance, 2004), and their excitation (at 340-360nm) and emission (at 430-450nm) spectra (Chance et al., 1962, Aubin, 1979) generally correspond to the signal observed. Possibly, high autofluorescence in the submandibular ganglion results from elevated levels of NADPH. Since NADPH is a cofactor for many antioxidant enzymes, its high level could allow parasympathetic neurons to detoxify ROS efficiently. Elevated NADPH levels could be particularly beneficial in the context of diabetes, because depletion of NADPH happens in hyperglycemia as a result of aldose reductase overactivation (Fig 5.6 B) (Brownlee, 2001).

We verified the observation of high autofluorescence in the submandibular ganglion together with Yumaine Chong by imaging cultured neurons of sympathetic SCG and parasympathetic submandibular neurons using excitation at 405 nm and emission at 430-470 nm. The results showed that autofluorescence was significantly higher in parasympathetic than in sympathetic neurons (Fig 6.1). Future studies could test whether NADPH levels are in fact elevated in the parasympathetic submandibular ganglion neurons.

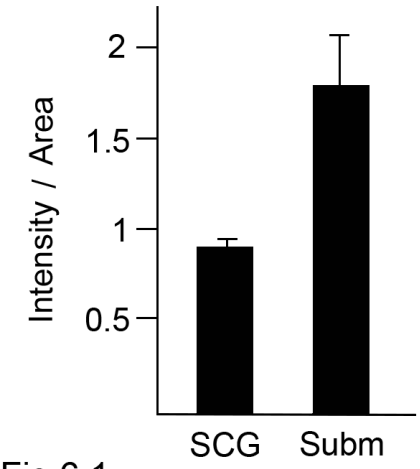


Fig 6.1

Figure 6.1 Autofluorescence corresponding to NAD(P)H is higher in Subm than in the SCG.

Quantification of autofluorescence imaged using the excitation of 405nm and emission of 430-470nm in cultured neurons of submandibular ganglion (Subm) and superior cervical ganglion (SCG). Fluorescence intensity was measured in cells excluding the nucleus area and was divided by the measured area.

Is the presence of α7-containing nAChRs in parasympathetic ganglia involved in the protection from synaptic depression in diabetes?

One could ask if the contribution of the α7-containing nAChR to the synaptic transmission in the submandibular ganglion is involved in preventing the synaptic depression in this ganglion in diabetes. We know that α7-containing nAChRs do not become inactivated by ROS, since these receptors do not contain the cysteine residues targeted by ROS (Campanucci et al., 2010). However, the α7-containing nAChRs are responsible for only 10% of the average EPSP size in submandibular ganglion neurons. It is unlikely that this contribution would mask the synaptic depression of >50% that happens in sympathetic ganglia in diabetes. Nevertheless, one could imagine that the α7 nAChR contribution could become larger through some compensatory process in response to inactivation of α3-containing nAChRs in diabetes. To verify this, while recording EPSPs from submandibular ganglia of diabetic mice, I applied methyllyconitine (MLA) to block the α7-containing nAChRs. I compared the EPSPs size before and after applying MLA. If α7-containing nAChRs had a large contribution to EPSPs as a result of compensation in diabetic animals, I expected that blocking these receptors with MLA would decrease the EPSP size. However, I did not observe any obvious change in EPSP size after applying MLA (data not shown).

Finally, one can ask if the α7 nAChRs could have an antioxidant role. Several studies showed that agonists of α7-containing nAChRs can improve neuronal survival and decrease ROS. For example, Pugh and Margiotta observed that activation of nAChRs sensitive to α-Bungarotoxin improved the survival of cultured parasympathetic neurons from the chick ciliary ganglion (Pugh and Margiotta, 2000). In a study by Li and

colleagues, an α7 nAChRs agonist DMXB decreased ROS-induced DCFDA fluorescence in adrenal PC12 cells in response to ethanol, and the effect could be blocked by an α7 nAChR antagonist MLA (Li et al., 2000). An α7 nAChRs agonist PNU282987 also reduced DCFDA fluoresnce in hippocampal microglia, and the effect was reversed by blocking α7 nAChRs with bungarotoxin (Parada et al., 2013). However, the antioxidant effect of α7 nAChRs is unlikely, because in the experiments with cultured submandibular neurons, no agonist was present to activate the α7 nAChRs. In spite of this, hyperglycaemia did not cause ROS-mediated rundown of ACh-evoked currents in parasympathetic neurons in culture (Fig 3.7 B) although it did so in the sympathetic neurons (Fig 3.1 E, Fig 3.2 D). Similarly, parasympathetic neurons maintained in culture without ACh had lower ROS and had stable currents (Fig 3.6) in response to an Anti-A treatment, in contrast with the sympathetic neurons.

Findings on reversing the diabetes-induced depression of synaptic transmission with insulin

I predicted that after synaptic transmission in sympathetic ganglia becomes depressed in diabetes, it should recover when the nAChRs are back in their reduced state and when ROS return to their normal levels. The results showed that when studied in cultured sympathetic neurons, nAChRs readily recover from inactivation by ROS within 48 hours. Interestingly, this recovery did not require new protein synthesis, at least when measured at 3 hours after inactivation. Possibly, the oxidised nAChRs can be reduced and can recover during the process of recycling, possibly in an endosomal compartment.

Since the nAChR recovery was possible in cultured cells, this suggested that nAChRs should also recover *in vivo* in diabetic mice once glucose is lowered with insulin and ROS become normalized. I observed that insulin treatment that began 2 weeks after diabetes onset but not after longer diabetes durations could reverse the depression of synaptic transmission in SCG. This suggests that a critical period as short as 2 weeks exists for insulin treatment in mice. It is possible that after longer diabetes durations, ROS do not become normalized despite insulin treatment. For example, damage to mitochondria after a certain period of hyperglycemia could cause continuously elevated ROS production. In addition, epigenetic changes can be induced by prolonged hyperglycemia, and many such changes cause elevated ROS. Therefore, in future work, it will be useful to find out if ROS remain elevated or become lowered in sympathetic ganglia when insulin treatment begins after different periods of hyperglycemia. For instance, do ROS become normalized when insulin treatment starts after 3.5 months as well as 2 weeks of diabetes?

The finding that there is a critical period for starting insulin treatment has important implications for human diabetic patients. It suggests that studies could be done to determine how long such critical period may be in humans, but such studies may be difficult for ethical reasons.

Findings on prevention of the diabetes-induced synaptic depression in the mutant mouse strain WLD^s

I tested the possibility that the mutant mouse strain WLD^s would not develop the depression of synaptic transmission in SCG despite diabetes due to improved

detoxification of ROS in neurons of these mice. In fact, the SCG of WLD^s mice maintained normal synaptic transmission in diabetes, but only in the homozygous and not heterozygous WLD^s mice. This finding is generally in agreement with previous findings that transgenic mice with more copies of the WLD^s mutation had increased protection from the axonal degeneration (Mack et al., 2001). However, in these studies, heterozygous WLD^s mice did show some, although decreased, protection when compared to homozygous WLD^s mice. What could be the reason that heterozygous WLD^s mice in our experiments had no protection of sympathetic synaptic transmission in diabetes? Possibly, a decrease in ROS levels that results from the WLD^s mutation in heterozygous mice is not sufficient to prevent inactivation of postsynaptic nAChRs. Indeed, previous work showed that even mild elevations of ROS can inactivate the nAChRs (Campanucci et al., 2008). To better examine this issue in future studies, it will be useful to measure ROS in neurons of heterozygous and homozygous WLD^s mice during diabetes.

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