UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Neurexins in Insulin Exocytosis from Pancreatic Beta Cells

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Merrie Mosedale

Committee in charge:

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Professor Fred Levine
Professor Palmer Taylor
Professor Nicholas J. Webster

2012

The Dissertation of Merrie Mosedale is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2012

DEDICATION

In loving memory of Ward Mosedale.
You would have been very proud!

EPIGRAPH

When you are passionate, you always have your destination in sight, and you are not distracted by obstacles. Because you love what you are pursuing, things like rejection and setbacks will not hinder you in your pursuit. You believe that nothing can stop you!

Coach K

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LIST OF ABBREVIATIONS

AUC	area under the curve.
BR-MyoVa	brain myosin Va
CaV1.2	L-type voltage-gated calcium channel
Cbln	cerebellin precursor protein
CycD1	cyclin D1
GABA	γ-aminobutyric acid
GluD2	glutamate receptor δ2
GLUT2	glucose transporter 2
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IP	immunoprecipitation
ISR	insulin secretion rate
K_{ATP} channel	ATP-sensitive K^+ channel
LNS domain	laminin A, neurexin, sex hormone-binding domain
LRRTM	leucine-rich repeat transmembrane proteins
NL-1	neuroligin-1
NL-2	neuroligin-2
NRXN	neurexin
qPCR	real-time quantitative PCR
SEAP	secreted alkaline phosphatase
SLMV	synaptic-like microvesicle
VAMP2	vesicle associated membrane protein 2
VDCC	voltage dependent Ca^{2+} channel

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PRESENTATIONS

Mosedale, M., Egodage, S., Calma, R.C., Chessler, S.D. A possible role for neurexin in the docking of insulin granules at the β-cell membrane. American Diabetes Association Scientific Sessions, San Diego, CA. June 26, 2011.

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ABSTRACT OF THE DISSERTATION

The Role of Neurexins in Insulin Exocytosis from Pancreatic Beta Cells

by

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Doctor of Philosophy in Biomedical Sciences

Professor Steven D. Chessler, Chair

Neurexins are a family of synaptic adhesion molecules that in neurons bind to constituents of the secretory machinery, play a key role in the organization and stabilization of the presynaptic active zone, and help mediate docking of synaptic vesicles. Neurexins, like many protein constituents of the neurotransmitter exocytotic machinery, are also expressed in pancreatic β cells. I hypothesized that neurexins in β cells help to mediate insulin granule docking and thereby inhibit secretion.

The results from this project demonstrate that human, rat, and mouse islets express neurexin protein, but a more restricted pattern of neurexin transcripts than neurons. Neurexin-1α in INS-1E β cells is expressed on the β-cell membrane and

interacts with several components of the secretory granule docking machinery, including the secretory granule-associated protein granuphilin. EM and secretion studies showed that siRNA knockdown of neurexin-1α reduces granule docking at the β-cell membrane and improves insulin secretion. These results were confirmed in primary islets isolated from neurexin-1α KO mice, and perifusion of the isolated islets demonstrated that loss of neurexin-1α resulted in a significant increase in second-phase insulin secretion with a trend towards an increase in first-phase secretion. Upon glucose stimulation, neurexin-1α protein levels decrease. This glucose-induced neurexin down-regulation may enhance stimulated insulin secretion. My hypothesis that changes in neurexin expression contribute to β-cell dysfunction in type 2 diabetes has thus far tested negative.

I demonstrated that neurexin-1α is a component of the β-cell secretory machinery that contributes to secretory granule docking, most likely through interactions with granuphilin. My work supports the notion that docking is inhibitory to insulin secretion and is the first to identify a transmembrane component of the insulin granule docking machinery. These findings provide new insights into the mechanisms of insulin granule docking and exocytosis. Building on these findings, additional work may enable the development of drugs that improve insulin secretion (type 2 diabetes) or the development and function of islets for transplantation (type 1 diabetes). Because neurexins are β-cell specific and cell-surface proteins, they are also promising targets for noninvasive β-cell *in vivo* imaging. This technology could be used to monitor β-cell mass in both disease and therapy conditions and therefore enhance the development of new therapies to prevent β-cell loss or recover β-cell mass.

CHAPTER 1:

INTRODUCTION

Many factors contribute to the pathogenesis of type 2 diabetes mellitus, but β-cell failure is now recognized as the determining factor in the transition from prediabetes to clinically overt disease (1-3). As a result, preservation of β-cell function is a critical target in the treatment of diabetes (4-6). The development of new therapies to prevent β-cell failure and restore secretory capacity will require a greater understanding of both the mechanisms involved in insulin exocytosis and the pathways that become affected in the pathogenesis of diabetes. A family of synaptic adhesion molecules called neurexins (NRXNs) may a be a critical component of the insulin exocytotic machinery, but their presence in the β cells has only recently been discovered (7-9), and their role in the β cell has yet to be defined. Therefore, the objective of this thesis was to identify the role of NRXNs in exocytosis of insulin from the pancreatic β cell and to investigate how the function of these proteins may become impaired in the development of type 2 diabetes. This introduction serves to familiarize the reader with the background, significance, objective, and hypotheses of this thesis project.

A. β cells are Similar to Neurons

β cells share many similarities with neurons, including the expression of many of the same scaffolding and exocytotic proteins (10,11). These proteins primarily mediate the exocytosis of neurotransmitters from neurons and insulin in β cells (12). In neurons, an action potential induces depolarization of the axon and opening of voltage dependent Ca^{2+} channels (VDCCs). Entry of Ca^{2+} into the cell results in neurotransmitter-containing synaptic vesicle fusion with the presynaptic membrane and release of the neurotransmitter contents into the synaptic cleft. A high temporal and spatial fidelity is needed in order for the neurotransmitter to reach its target receptor sites on the postsynaptic density (13,14). In β cells, glucose stimulation leads to sugar metabolism, a change in the ATP/ADP ratio, closure of the ATP-sensitive K^+ (K_{ATP}) channels, membrane depolarization and opening of VDCCs. The subsequent increase in cytosolic free Ca^{2+} activates the exocytotic machinery, promotes membrane fusion of insulin-containing secretory granules and release of insulin into the extracellular space (11).

Microdomains of secretion

In addition to the expression and use of overlapping secretory proteins, the exocytosis of secretory granules from β cells also exhibits the neurosecretory phenotype of synaptic-like microdomains, areas of Ca^{2+} entry and secretion which occur around VDCCs (15-17). As in neurons, exocytotic release does not occur randomly over the cell surface, but rather colocalizes with sites of Ca^{2+} entry (18,19). VDCCs are also unevenly distributed over the plasma membrane (20) and tend to associate with the exocytotic machinery (21,22). Although the rationale for this coupling seems obvious, as it would

ensure that the Ca^{2+}-sensitive exocytotic machinery is exposed to very high concentrations of Ca^{2+} (23) and that secretory granules are able to undergo a rapid release (24), the complete complex of proteins involved in assembling the exocytotic microdomain have yet to be identified.

Synaptic-like microvesicles in β cells

In β cells, exocytosis primarily involves secretion from insulin-containing secretory granules or large dense core vesicles. However, distinct synaptic-like microvesicles (SLMVs) have also been identified in the pancreatic islets (25,26). These vesicles contain membrane proteins involved in neurotransmitter exocytosis and are different from insulin-containing secretory granules in appearance and size (27,28), as well as pharmacologic properties such as release rate and threshold for Ca^{2+} stimulation (29). SLMVs are similar to the small synaptic vesicles in neurons (25) which mainly secret classical neurotransmitters. It is thought that these vesicles are responsible for the transport and release of neurotransmitters, such as γ-aminobutyric acid (GABA) from β cells (30).

GABAergic phenotype of β cells

GABA is the key inhibitory neurotransmitter in brain (31,32). It is expressed not only in the central nervous system, but also in peripheral tissues, however the concentration is typically about 1% of that in brain. An exception to this is the pancreatic islets where considerably greater amounts of GABA have been found to be stored in microvesicles (33). Two isoforms of glutamic acid decarboxylase, GAD65 and GAD67,

which catalyze the formation of GABA from glutamate are also present in β cells (25,27) as is the vesicular inhibitory amino acid transporter of GABA, VIAAT (34). More recently it was discovered that GABA receptors, which are also present in β cells and other islet cell types, play a role in regulating pancreatic islet hormone secretion, although it is still unclear how GABA secretion is regulated and how GABA regulates autocrine and paracrine signaling (35). Work from our lab has demonstrated that the synaptic adhesion molecule neuroligin-2 (NL-2), which is specific to GABAergic synapses (36) is expressed by pancreatic β cells and plays a role in insulin secretion (9). Because of its role in GABAergic differentiation, it is possible that the presence of GABA and related proteins in β cells is due to an influence of NL-2 on β-cell maturation.

Because of the many similarities shared by β cells and neurons, advances in the neurobiology field regarding synaptic maturation and function frequently offer important clues regarding overall β-cell function and the mechanisms underlying insulin secretion. Special attention is paid to understanding exocytosis in inhibitory neurons because β cells expresses all of the machinery essential for GABA synthesis and secretion and are thus frequently described as GABAergic.

B. Neurexin Structure and Function at the Synapse

NRXNs were originally discovered as the functional receptors for α-latrotoxin, a component of venom from black widow spiders (37-39), but further investigation into these proteins allowed for the identification of their primary function as synaptic adhesion molecules, neuronal cell-surface receptors and signaling proteins (36,40-42). There are three NRXN genes (NRXN1-3), and each gene has an upstream promoter which produces a longer α-isoform (α-NRXN) and a downstream promoter which generates a shorter β-isoform (β-NRXN) (43).

Structure of NRXN isoforms

All of the NRXNs are highly spliced and glycosylated (44-48), adding to their diversity. However, the general structure of the respective isoforms is consistent among the splice variants. Both isoforms are single transmembrane proteins with conserved transmembrane and intracellular domains (40). The extracellular N-terminal sequences of NRXNs, however, are distinct. The extracellular portion of the β-NRXNs have a glycosylation site and just a single laminin A, neurexin, sex hormone-binding protein (LNS; also known as a laminin G) domain before a short, β-NRXN-specific domain (49). The extracellular portion of α-NRXNs are much longer and consist of a glycosylation site and then three pairs of LNS domains, each sandwiching an epidermal growth factor (EGF)-like sequence. Both isoforms localize to the presynaptic membrane (50,51) and bind to a diverse array of secreted molecules, postsynaptic adhesion molecules, receptors, and exogenous factors.

NRXN binding partners

Both NRXN isoforms, via their LNS domains, bind dystroglycan in a Ca^{2+}-dependent manner (52). This interaction links the intracellular actin cytoskeleton to the extracellular matrix, connecting cells mechanically in the context of an overall tissue.

α-NRXNs bind to the secreted ligand neurexophilins at the second LNS domain in also in a Ca^{2+}-dependent manner (53,54). There are at least four genes encoding neurexophilins (53,54) and they generate a product with a neuropeptide-like structure (55). There is no direct evidence for a signaling function of the NRXN-neurexophilin interaction, but it has been suggested that neurexophilins serve as neuropeptides while NRXNs act as their receptors (56).

The neuroligins are cell adhesion molecules and bind the neurexins in a Ca^{2+}-dependent manner (57,58). Five neuroligin genes are expressed in humans, and each gene can be alternatively spliced at multiple positions (59). In culture studies, NRXNs on the presynaptic density interact with neuroligins on the postsynaptic density to induce pre and postsynaptic assembly, formation, and specializations (60-66). However, phenotypic analyses of neuroligin and NRXN KO mice is at odds with the culture studies and suggest that these molecules are dispensable for synapse formation *in vivo* (67,68). Nevertheless, it is possible that KO mice maintain expression of other synaptogenic proteins with overlapping functions that compensate for the loss of NRXNs and neuroligins in these studies.

For instance, another family of synaptogenic proteins, leucine-rich repeat transmembrane neuronal proteins (LRRTMs), were recently found to regulate synaptic differentiation (69). LRRTM1 and 2 bind NRXNs with a similar affinity but distinct code

from the code for binding neuroligin-1 (NL-1) (70). Whereas NL-1 binds to NRXNs 1, 2, and 3β but not α-variants, regardless of splicing, LRRTMs binds to all NRXNs specifically lacking an insert at splice site 4. In neuron cultures, LRRTMs are more potent than neuroligins in promoting synaptic differentiation (70). However, these two families of NRXN-binding partners cooperate in an additive or synergistic manner. It is possible that NRXNs are master regulators of the cooperative activities of LRRTMs and neuroligins (71). While there is functional redundancy between neuroligins and LRRTMs during early synapse development, functional divergence occurs at later stages of synapse maturation, and therefore NRXN ligands in vivo form a dynamic synaptic cell adhesion network (72).

NRXN has also been recognized to have synaptogenic activity through its indirect interaction with the glutamate receptor δ2 (GluD2). The N-terminal domain of this receptor interacts with presynaptic NRXNs through cerebellin precursor protein (Cbln) to promote cerebellar synapse formation. (73). Cblns are secreted from cerebella granule cells and highly accumulated in the synaptic cleft of parallel fiber-Purkinje cell synapses. Cbln1 and Cbln2, but not Cbln4 bind both NRXN isoforms, serve as bidirectional synaptic organizers, directly induce presynaptic differentiation and indirectly serve as a postsynaptic organizer by binding GluD2 (74). NRXN, GluD2 and Cbln1 will form a Ca^{2+}-independent tripartite complex that helps to cluster NRXN and GluD2 and organize presynaptic and postsynaptic densities

Not all NRXN transcellular interactions are synaptogenic. In fact, both NRXN isoforms bind the G-protein coupled receptor CIRL1/latrophilin-1 in trans, but this interaction does not contribute to synapse formation (75). However it may provide

additional synaptic cell adhesion complexes and promote synapse stabilization. Or, as latrophilin-1 is also a receptor for α-latrotoxin, it may contribute to α-latrotoxin toxicity.

Extracellular domains of NRXN also bind exogenous ligands in addition to α-latrotoxin. Recently, it was discovered that a peptide sequence on the specific N-terminal region of β-NRXNs is the high affinity binding partner for a cell-wall anchored protein, SdrC, of pathogenic gram-positive bacteria (76).

The intracellular, C-terminal region of NRXNs contain a PDZ-binding domain (40) and interact with a number of exocytotic proteins, including the scaffolding proteins Mint1 and Velis (77,78), the Sec1/Munc18-like protein Munc18 (78), the Ca^{2+}/calmodulin-dependent-kinase containing membrane-associated guanylate kinase CASK (77,79-81), the Ca^{2+} sensor synaptotagmin 1 (82-84), and t-SNARE syntaxin 1 (83). It is likely that NRXNs also interact indirectly with, the t-SNARE SNAP-25 (85) and the v-SNARE synaptobrevin/vesicle associated membrane protein 2 (VAMP2) (86).

Role of NRXNs in neurons

In vitro, the transsynaptic interactions of NRXNs and neuroligins are important for synaptic differentiation. NRXNs expressed in COS cells or on beads cocultured with neurons induce the formation of postsynaptic densities at points of contact (64,87,88) and vice versa, neuroligins expressed in cocultures with neurons induce NRXN clustering and the formation of presynaptic densities (65,89). However, this phenomenon seems to be unique to *in vitro* models. *In vivo*, the development of double and triple α-NRXN KO mice (α-NRXN DKO and TKO) mice has uncovered a unique role for the α-NRXNs in the differentiation of inhibitory as opposed to excitatory synapses, instead of synapse

formation itself (67). These studies are supported by *in vitro* work indicating that α-NRXNs and NRXNs specifically containing splice site 4 (+SS4) contribute to the formation of GABAergic synapses (90,91). The ability to drive inhibitory or excitatory differentiation is likely dependent on structural changes in different isoforms and splice variants of NRXN that regulate NRXNs ability to partner with specific extracellular proteins such as neuroligins, LTTRMS, etc. For instance, α-NRXNs and NRXNs +SS4 bind Clbns, NL-2 and NL-1 lacking a splice insert at splice site B (47,74,91-95). Whereas NRXNs -SS4 preferentially bind to latrophilin-1, excitatory-synapse inducing neuroligins-1,-3,-4, neuroligins carrying splice insert B, and LRRTMs (70,75,96,97). Although it is not well understood what drives variations in gene and isoform expression in different areas of the brain, recent work has indicated that alternative splicing of SS4 can be regulated by depolarization (90).

α-NRXN KO mice also demonstrate the importance of NRXN for maintenance of synapse function and neurotransmission (98,99). More specifically, α-NRXNs are responsible for linking Ca^{2+} channels to the secretory machinery and to release-ready synaptic vesicles allowing for rapid stimulus-secretion coupling (67,100). The extracellular domain of α-NRXNs is essential for the regulation of VDCCs (83,101), whereas the intracellular domain regulates exocytotic function.

NRXN mutations in neurodevelopmental disorders

Mutations in all 3 NRXN proteins have been linked to neurodevelopmental disorders such as autism (102), schizophrenia (103,104), mental retardation (105), early onset epilepsy (106), Parkinson's disease (107), addiction (108-111), congenital dental

anomalies (112), and language and communication difficulties (113). Disease-inducing mutations can result from protein misfolding or structural variations (103,114), complete loss of NRXN protein (104,115), and protein translocation (116). While most of these aberrations appear to be genetic, some are induced by environmental impacts such as exposure to nicotine *in utero* (117), ischemic injury (118), and radiation exposure (119). This neurodevelopmental association with synaptogenic protein mutations is also true for several NRXN binding partners. For example, mutations in LRRTM1 were recently linked to both schizophrenia and handedness (120,121). And a similar range of deletions, variations, and mutations in neuroligins are associated with autism (122), fragile X syndrome (123), Asperger syndrome, mental retardation (124,125), Tourette syndrome (126), and behavior abnormalities (113,127-129).

C. Neurexins in the β cell

We previously found that β cells express NRXNs (9). Immunofluorescent staining of rat pancreas indicated that NRXN was specific to the β-cell membrane and not expressed by other islet cell types. In keeping with the GABAergic phenotype of the β cell, we found that β cells only express the inhibitory-synapse-associated NRXNs +SS4. We also found that β cells express the NRXN postsynaptic binding partners, neuroligins, and that these proteins have a role in insulin exocytosis. The functional presence of neuroligins led us to question the possibility that NRXNs also contribute to β-cell function and formulate a hypothesis as to how that might occur.

As previously discussed, studies with α-NRXN KO mice indicated that α-NRXNs are essential for the organization and stabilization of the presynaptic machinery (67,98-100). *In vitro* work has demonstrated that NRXNs in neurons interact with rabphilin-3A via CASK (130) and contribute to synaptic vesicle docking (82,84). β cells also express CASK (9) and a rabphilin-3-like protein called granuphilin which associates with secretory granules (131) and promotes secretory granule docking (132). Granuphilin-mediated docking is inhibitory to insulin secretion (132-134). Although this finding is at odds with studies suggesting that docking precedes or facilitates insulin exocytosis (22,135,136), it is in line with other studies showing attenuation of insulin secretion by proteins that promote granule docking but inhibit SNARE-mediated fusion of secretory granules with the plasma membrane. For instance α-synuclein promotes secretory granule association with K_{ATP} channels and similarly inhibits insulin secretion (137). To some extent this is also true for exophilin8 which restricts the motion of insulin granules at a

region deeper than granuphilin (138). However exophilin8-induced immobility of insulin granules is only transient and is eliminated upon secretagogue stimulation.

Granuphilin in the β cells

Granuphilin expression is specific to the pancreatic β cells and the pituitary gland (131) where, at least in β cells, rabphillin-3 is not expressed (139,140). Granuphilin protein contains an N-terminal zinc-finger motif and C-terminal C_2-domains, similar to that of rabphilin-3 (131). There are two granuphilin isoforms: the larger isoform, granuphilin-a, which contains two C_2-domains and the smaller isoform, granuphilin-b, which has only the first C_2-domain. The first C_2-domain binds phospholipids in a Ca^{2+}-dependent manner, but the second C_2-domain does not. Both granuphilin isoforms are expressed in INS-1 cells at similar levels and are reported to function in the same manner (141). In β cells, granuphilin associates with the surface of insulin granules, but not with SLMVs, and therefore differs from its rabphillin-3 counterpart, which associates with SLMVs in neurons (131,141).

In β cells, granuphilins bind the monomeric small GTPases Rab3 (139,141) and Rab27a (133). Granuphilins also bind the brain-spliced isoform of Myosin Va (BR-MyoVa), which plays a role in the transport of secretory granules to the plasma membrane via a complex with Rab27a (142), and Rim2α, which determines priming states in insulin granule exocytosis via Rab3a (143). Both Rab3a and Rab27a specifically localize on secretory granules (144,145). Rab27a KO mice have impaired insulin secretion from isolated islets but do not exhibit the docking defect (144,146). GTPase-deficient mutants of the Rab3 isoforms decrease insulin release (139,145). This is

possibly explained by interaction of granuphilin with Rab3 occurring only with the protein is in its GTP-bound form (141). As a result of the GTPase deficiency, Rab3 and granuphilin interactions would not be reversible. Complete loss of Rab3 binding enhances exocytosis, however, the granuphilin/Rab3 complex alone is not sufficient to mediate the decrease of exocytosis, suggesting the existence of additional binding partners (141). Disruption of granuphilin-BR-MyoVa complex results in a perinuclear accumulation of granules, which increases insulin secretion from β cells (142).

Granuphilin and secretory granule docking

Numerous studies have more closely investigated the role of granuphilin in insulin secretion and secretory granule docking in β cells. Overexpression of granuphilin in insulin-secreting cell lines inhibits stimulated insulin exocytosis (141), whereas knockdown- or KO-mediated ablation of granuphilin improved insulin secretion in β cells (132,147). Furthermore, transcription factors that promote granuphilin expression, such as PGC-1β, SREBP-1c, and FOXA2, regulate insulin secretion inversely to granuphilin expression. (147,148). These alterations in insulin secretion are ascribed to granuphilin's role in secretory granule docking. Granuphilin is essential for docking granules at the β-cell membrane, but as previously mentioned, this docking is inhibitory toward insulin secretion (132,149).

Granuphilin also interacts with the exocytotic proteins Munc-18 and syntaxin 1a (133,134,141,150) possibly to mediate granule docking by linking Rabs on the granule membrane (151) with the syntaxin la/Munc18-1 complex on the plasma membrane (132). This is further evidenced by the fact that Munc18-1-null embryonic chromaffin cells

show a defect in granule docking (152) and Munc18-1-depleted β cells exhibit defective docking of insulin granules at the plasma membrane (134). Docking has not been investigated in Munc18-1-deficient β cells because Munc-18 KO mice are not viable at birth. Munc18 is also required for the specific interaction of insulin granules with syntaxin 1 (134). A decrease in the formation of the fusion-incompetent syntaxin-1a–Munc18-1 complex, with which granuphilin normally interacts, enhances insulin secretion (132,133). On the other hand, loss of syntaxin 1a does not cause a significant granule-docking defect (153). It is possible that this is due the remaining presence of other syntaxin isoforms or other proteins involved in this process in the β cell. NRXN has not been well studied in the β cell, and therefore the contribution of NRXN to granuphilin-mediated, secretory granule docking is not known. However, based on NRXNs role in docking in neurons, it would seem like a plausible candidate to serve as granuphilin's membrane-associated binding partner and therefore another factor that contributes to secretory granule docking

D. Significance

β cell function becomes impaired in type 2 diabetes

A key contributor to the pathogenesis of type 2 diabetes is β cell failure – including dedifferentiation, impaired secretory capacity, and eventually loss of β-cell mass (154-158). To develop new therapies to treat type 2 diabetes, it is critical to gain a better understanding of the β-cell exocytotic pathway in normal, healthy cells as well as uncover the mechanism driving the β-cell failure that occurs in type 2 diabetes. The mechanisms responsible for loss of β-cell function are likely to be multifactorial, but models of diabetes, including hyperglycemia, have helped demonstrate that the glucotoxicity, lipotoxicity and inflammation that occur in diabetes lead to decreased expression and function of exocytotic proteins that, in neurons, interact directly or indirectly with NRXNs (159-162). Therefore, it is possible that loss of exocytotic function in type 2 diabetes may be secondary to defects NRXNs themselves.

Neurexins and diabetes

Some evidence of NRXN dysfunction in diabetes already exists as it has been reported that the NRXN DKO mouse exhibits hyperglycemia and has hypomorph β cells with impaired secretory capacity (unpublished observations in (67)). Furthermore, as was previously discussed, NRXN mutations are linked to neurodevelopmental diseases such as autism and schizophrenia. It has been shown that independent of pharmacological intervention, the prevalence of diabetes in people with cognitive disease is higher than the prevalence of diabetes in the general population (163-169). Also, variants of NRXN3 have been correlated with increased obesity, waste circumference, and BMI (170,171),

and are therefore precursors to insulin resistance, metabolic syndrome and type 2 diabetes. Further studies are needed to confirm the putative role of NRXN3 in the pathophysiology of obesity.

As a result of the clear links between NRXNs and exocytosis of insulin as well as NRXN dysfunction in impaired β-cell secretory capacity, developing a better understanding of NRXN function in the β cell is a significant and innovative project that may help provide a novel candidate therapeutic target for the treatment of diabetes.

E. Objective of the Dissertation

In conclusion, NRXNs are a family of synaptic adhesion molecules that in neurons bind to constituents of the secretory machinery, play a key role in the organization and stabilization of the presynaptic active zone, and help mediate docking of synaptic vesicles. We have previously shown that NRXNs, like many protein constituents of the neurotransmitter exocytotic machinery, are also expressed in pancreatic β cells. A role for NRXNs in β cells has yet to be defined.

The **objective** of this thesis project was to: (1) Characterize the expression, localization, and binding partners of NRXNs in pancreatic β cells; (2) Identify the contribution of NRXNs to insulin secretion; and (3) Understand the role of NRXN dysfunction in type 2 diabetes.

I **hypothesize** that NRXNs in β cells play a comparable role to that in neurons, participating in the granuphilin-mediated docking of insulin granules at the β-cell membrane and thereby inhibiting insulin secretion. In *in vivo* and *in vitro* models of type 2 diabetes β cells lose expression and function of exocytotic proteins that, in neurons, interact directly or indirectly with NRXNs. Therefore, I also **hypothesize** that NRXN expression is altered in β cells when exposed to chronically elevated levels of glucose. This NRXN dysfunction contributes to the impairment of the late steps of insulin exocytosis characteristic of type 2 diabetes.

The following chapters of this dissertation describe the expression, localization, and binding partners of NRXNs in β cells (Chapter 2), the contribution of NRXN to secretory granule docking and exocytosis in INS-1E β cells (Chapter 3) and mouse islets (Chapter 4), and the regulation of NRXN mRNA expression in diabetic models (Chapter

5). Chapter 6 will present the conclusions, remaining questions, and proposed future work from this project.

CHAPTER 2:

NEUREXINS ARE PRESENT IN β CELLS AND INTERACT WITH DOCKING AND SUBMEMBRANE-RESIDENT EXOCYTOTIC PROTEINS

A. Summary

Neurexins are a family of transmembrane, synaptic adhesion molecules. In neurons, neurexins bind to both sub-plasma-membrane and synaptic-vesicle-associated constituents of the secretory machinery, play a key role in the organization and stabilization of the presynaptic active zone, and help mediate docking of synaptic vesicles. We hypothesized that neurexins, like many protein constituents of the neurotransmitter exocytotic machinery, are also expressed in pancreatic β cells and interact with docking and exocytotic proteins. In the current study, we demonstrate that neurexin protein is present in human, rat, and mouse β cells. β cells express a more restricted pattern of neurexin transcripts than neurons, with a clear predominance of neurexin-1α expressed in isolated islets. Using a custom pan-neurexin antibody, we found that neurexin protein is expressed on the surface of INS-1E β cells and neurexin-1α interacts with membrane-bound components of the secretory granule-docking and -exocytotic machinery. We conclude that neurexin-1α is a component of the β-cell secretory machinery that possibly contributes to secretory granule docking through interactions with granuphilin. Neurexin-1α is the only transmembrane component of the docking machinery identified thus far. Our findings provide new insights into the expression of neurexins outside of the central nervous system.

B. Introduction

β cells share many similarities with neurons, including the expression of many of the same scaffolding and exocytotic proteins (10,11). These proteins mediate the exocytosis of synaptic vesicles in neurons and insulin granules in β cells (12). Advances in the neurobiology field regarding synaptic maturation and function thus frequently offer important clues regarding overall β-cell function and the mechanisms underlying insulin secretion.

Neurexins (NRXNs) are a family of synaptic adhesion molecules that function as neuronal cell-surface receptors and signaling proteins (36,40,41). They are encoded by three genes (NRXN1-3), each using an upstream promoter to produce the longer α-isoform (α-NRXNs) and a downstream promoter to generate a shorter β-isoform (β-NRXNs) (43). The α- and β-isoforms of each NRXN are single-pass transmembrane proteins maintaining identical transmembrane and intracellular domains but distinct N-termini (40). NRXNs in neurons localize to the presynaptic membrane and bind transsynaptically to postsynaptic adhesion molecules (50,70,172,173) and receptors (73). The intracellular, C-terminal regions of NRXNs contain PDZ-binding domains (40). Studies in neurons have demonstrated that the intracellular domains of NRXNs interact with a number of exocytotic proteins, including the scaffolding proteins Mint1 and Velis (77,78), the Sec1/Munc18-like protein Munc18-1 (78), the t-SNARE syntaxin 1 (83), the calcium sensor synaptotagmin 1 (82,83), and the calcium/calmodulin-dependent-kinase containing membrane-associated guanylate kinase CASK (79,81).

We previously found that β cells express NRXNs and one of their major postsynaptic binding partners, neuroligins (9). NRXNs and neuroligins were also

identified as some of the most highly-expressed transcripts of membrane-associated, islet-specific proteins in humans (8). Studies with double and triple α-NRXN knockout mice have indicated that α-NRXNs in neurons are essential for the organization and stabilization of the presynaptic machinery (67,98,100). *In vitro* work suggests that NRXNs interact with the synaptic vesicle-associated protein rabphilin-3A via CASK (130) and contribute to synaptic vesicle docking (82,84). β cells also express CASK (9) and, in addition, granuphilin, a rabphilin-3 homologue implicated in insulin granule docking (131,132). Based on the similarities between neurons and β cells, we hypothesized that NRXNs in the β cell are constituents of the insulin secretory and docking machinery perhaps via interactions with granuphilin.

The present study examined the expression, localization and binding partners of NRXNs in β cells. Our data show that NRXN protein is present in human, rat, and mouse β cells. The expression profile of NRXN transcripts in β cells is different than that of brain, with NRXN1α transcripts predominating in human, rat and mouse islets and NRXN1α and 2β predominating in INS-1E β cells. The development of a custom, pan-NRXN antibody allowed us to demonstrate that NRXN protein is also present on the cell membrane of INS-1E β cells, and that NRXN1α is an integral component of the secretory granule docking machinery. Our findings provide new insights into the expression of NRXNs outside of the central nervous system.

C. Experimental Procedures

Antibodies

The following antibodies were obtained commercially: goat anti-NRXN1 (P-15), goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology); rabbit anti-granuphilin (Atlas Antibodies); mouse anti-syntaxin 1 and -GAPDH (Sigma-Aldrich); mouse anti-synaptophysin, -CASK and -Munc18 (BD Biosciences); rabbit anti-GFP/CFP (Molecular Probes); guinea pig anti-NRXN1α (Millipore); guinea pig anti-insulin (Dako); biotinylated goat anti-guinea pig IgG (Vector Laboratories); and IRDye 680-conjugated goat anti-mouse IgG and IRDye 800CW-conjugated goat anti-rabbit IgG (LI-COR).

In order to detect NRXN expression by immunoblotting, we raised a polyclonal, pan-NRXN antibody against a previously described NRXN peptide (83) and a custom designed NRXN peptide. Rabbits were injected with keyhole limpet hemocyanin-conjugated peptides 1 (CAKSANKNKKNKDKEYYV) and 2 (CVKEKQPSSAKSANKNKK), and serum was affinity purified (Open Biosystems). The antibody was validated for peptide binding by ELISA. The antibody was also validated by Western blot and detects all six CFP-tagged NRXN isoforms (91). The identity of the bands was confirmed with an anti-GFP/CFP antibody. Competition assay was used to confirm the peptide antigen. The antibody was incubated with an excess of control FLAG peptide (Sigma-Aldrich), peptide 1, or peptide 2 for one hour before use in Western blotting.

Histology

Pancreas tissue was obtained from adult male Sprague-Dawley rats, C57BL/6 mice and NRXN1α KO mice, fixed for 24 h in Pen-Fix (Thermo Fisher Scientific) and embedded in paraffin. All procedures were approved by the University of California, San Diego Institutional Animal Care and Use Committee. Human pancreases from healthy donors were obtained from the National Disease Research Interchange, fixed in 10% buffered formalin, and embedded in paraffin. Sections (6 μm) of human, rat, and mouse pancreases were prepared by the University of California, San Diego histology core. Deparaffinized sections were subjected to antigen retrieval by treating with a citrate buffer. Immunostaining with the Vectastain Elite ABC kit and NovaRED substrate (Vector Laboratories) was performed as previously described (174). Controls for immunostaining included tissue stained exclusively with secondary antibody. Images were captured using a Nikon Eclipse E800 microscope and SPOT RT SE camera at 20x.

Cell culture and transfection

INS-1E cells (175) and 832/13 cells (176) were a gift from Dr. Pierre Maechler (Geneva University) and Dr. Christopher B. Newgard (Duke University) respectively. Both lines were cultured in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and 0.25 μg/ml amphoterecin B. Islets were cultured in the same medium except without 2-mercaptoethanol. COS-7 cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml

streptomycin (177). All cells were maintained in a humidified 37^{0}C incubator with 5% CO_2.

Absolute real-time quantitative PCR (qPCR)

Brain tissue was obtained from adult male Sprague-Dawley rats. Freshly isolated islets from adult male Sprague-Dawley rats and Swiss Webster mice were provided by the University of Washington Diabetes and Endocrinology Research Center Islet Cell and Functional Analysis Core. Human islets isolated as previously described (178) were obtained from the Southern California Islet Cell Consortium and the University of Alabama at Birmingham through the National Institutes of Health-sponsored Islet Cell Resources Basic Science Islet Distribution Program and flash frozen after 1 to 3 days in culture. Pancreases were procured from heart-beating, cadaveric, female donors ages 48 and 49 with body mass indices of 34 and 23 respectively. Islet preparations were deemed ≥ 80% purity and ≥ 90% viability. Total RNA was isolated and reverse transcribed as previously described (9). Gene- and isoform-specific primers (see Figure 2.1 for approximate locations) were used to perform absolute qPCR with Power SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Each sample was analyzed in triplicate along with no-RT and no-template controls. Standard curves of human, rat, and mouse NRXN gene- and isoform-specific amplicons were generated by PCR and gel purified with a QIAquick gel extraction kit (Quiagen). Normalization was carried out using total RNA values and confirmed with qPCR of 18S RNA. NRXN primers were designed to avoid major splice

variant regions. All primers were designed using Primer 3 (179) or PerlPrimer (180) software and are described in Table 2.1.

Plasmid constructs

The cDNA construct encoding CFP was previously described (181). The cDNA constructs encoding each of the six isoforms of CFP-tagged NRXN (91) were a gift from Dr. Ann Marie Craig (University of British Columbia).

Western blotting

Total cell extract was made by lysing cells in RIPA buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM PMSF, and 1% Protease Inhibitor Cocktail (Sigma-Aldrich). Plasma membrane was separated from cytosolic fractions as previously described by differential centrifugation and detergent/aqueous partitioning using the Plasma Membrane Protein Extraction Kit (BioVision) per the manufacturer's instructions (182). Proteins were quantified using the D_C Protein Assay (Bio-Rad Laboratories). NuPAGE LDS Sample Buffer and NuPAGE Reducing Agent (Invitrogen) were added to protein at a 1x final concentration. Protein was then incubated at 70^0C for 10 min and electrophoresed on either 4-12% Bis-Tris or 3-8% Tris-Acetate NuPAGE Gels (Invitrogen). Protein was transferred to PVDF membrane, blocked with 5% milk in PBS and probed with antibodies in 5% milk in PBS containing 0.1% Tween 20. Membranes probed with HRP-labeled secondary antibodies were incubated with PS-3 chemiluminescent detection reagent (Lumigen) and the chemiluminescent signal captured by HyBlot CL

autoradiography film (Denville Scientific). Membranes probed with IRDye-conjugated secondary antibodies were directly imaged with the Odyssey Infrared Imaging System and Software (LI-COR).

Immunoprecipitation (IP)

Cells were lysed in a buffer containing 150 mM NaCl, 1% Nonidet P-40, 50 mM Tris (pH 8), 1 mM PMSF, and 1% Protease Inhibitor Cocktail. Lysate was precleared with rec-Protein G-Sepharose (Invitrogen) and then incubated overnight at 4^0C with 5 μg of goat anti-NRXN1 P-15 antibody or purified goat IgG (Millipore). The reactions were incubated with rec-Protein G-Sepharose at 4^0C for 2 h and beads were thoroughly washed with lysis buffer. Samples were combined with LDS Sample Buffer and Reducing Agent for Western blotting as described above.

D. Results

NRXN protein is present in β cells

We previously demonstrated the presence of NRXN in the rat pancreatic β cell by immunofluorescence (9). β-cell expression of NRXN protein was confirmed by immunostaining of human, rat, and mouse pancreas sections (Figure 2.2).

NRXN transcript levels in β cells

We next sought to identify the NRXN isoforms present in β cells. Because existing antibodies cannot distinguish between the three NRXN genes, we used absolute RT-qPCR to quantify transcripts of each isoform in rat islets, rat brain, as well as the rat β-cell lines INS-1E and 832/13 (Figure 2.3A). Brain expresses similar levels of all NRXN isoforms except for a slight enrichment of NRXN1α. Islets and β cells demonstrated a distinctly different pattern, showing a clear predominance of one or two isoforms. In INS-1E and 832/13 β cells, NRXN 1α and 2β predominate. In rat islets, NRXN1α is the predominant transcript (Figure 2.3A, inset). Human (Figure 2.3B) and mouse (Figure 2.3C) islets displayed a similar expression pattern, with NRXN1α being most abundant, followed by lower expression of NRXN 2β. Because NRXN1α is the most abundant isoform in primary islets, it was chosen as the focus for further investigations.

Presence of NRXN protein on the INS-1E β-cell plasma membrane is confirmed by Western blotting with custom, polyclonal pan-NRXN antibody

In order to detect NRXN expression by immunoblotting, we raised a polyclonal, pan-NRXN antibody against a previously described NRXN peptide (1, CAKSANKNKKNKDKEYYV) (83) and a custom designed NRXN peptide (2, CVKEKQPSSAKSANKNKK). The antibody was validated by Western blot (Figure 2.4A). NRXN1α protein expression in INS-1E β cells was confirmed by immunoprecipitation of NRXN1α from cell lysate with a commercial antibody followed by immunoblotting using our custom, pan-NRXN antibody (Figure 2.4A, lane 5). Immunoblot analysis of INS-1E cells demonstrated the presence of α-NRXN in the plasma membrane fraction, consistent with the plasma membrane localization of NRXN observed in our previous immunofluorescent staining (9). NRXN was not detected in the cytosolic fraction (Figure 2.4A, lanes 1-2).

As part of validation of the pan-NRXN antibody, it was also used to immunoblot CFP-tagged NRXN expressed in transfected COS cells (Figure 2.4A, lanes 8-9). The expected increase in NRXN molecular weight due to the CFP tag is not obvious due to the high molecular weight of α-NRXNs (~180 kDa) relative to CFP (~27 kDa). The identity of the CFP-tagged NRXN bands was confirmed with an anti-GFP/CFP antibody. CFP-tagged NRXN bands as well as a CFP overexpression construct were detected at the appropriate molecular weights with the anti-GFP/CFP antibody (Figure 2.4B, lanes 3-9). No bands were detected with the anti-GFP/CFP antibody in the total lysate from INS-1E or COS cells (Figure 2.4B, lanes 1-2). Competition assay was used to identify the peptide antigen. NRXN was detected on immunoblots using pan-NRXN antibody incubated with control FLAG peptide or peptide 2, but not peptide 1 (Figure 2.4C), indicating the peptide 1 is the primary antigen for the custom NRXN antibody.

Coimmunoprecipitation of NRXN1α with docking and submembrane-resident exocytotic proteins

To identify NRXN-interacting proteins in INS-1E cells, we immunoprecipitated NRXN1α and analyzed coprecipitating proteins by Western blot. In neurons, NRXN seeds the assembly of the exocytotic machinery through interactions with Mint 1 (77), Velis (77,78), CASK (79,81,183), syntaxin 1 (83), and Munc18 (78). Consistent with NRXN being a constituent of the insulin granule submembrane secretory apparatus in β cells, we found coprecipitation of NRXN1α with the submembrane exocytotic proteins syntaxin 1, CASK, and Munc18 (Figure 2.5). In contrast, no co-precipitation with synaptophysin was detected even in overexposed immunoblots, suggesting that NRXN does not bind directly or indirectly to this vesicular protein. These results suggest NRXN is a component of the multiprotein complex that regulates the plasma-membrane docking and exocytosis of insulin granules.

In neurons, NRXNs likely contribute to synaptic vesicle docking via interactions with rabphilin-3A (130). β cells express the rabphilin-3-like protein granuphilin, which associates with secretory granules (131). Granuphilin on the surface of insulin granules directly interacts with syntaxin-1 and participates in secretory granule docking and inhibition of SNARE-mediated insulin release (132,133). Both isoforms of granuphilin, the full-length granuphilin-a and the shorter splice variant granuphilin-b, are expressed in INS-1 cells at similar levels and are reported to function in the same manner (141). Immunoprecipitation of NRXN1α revealed its interaction with both isoforms of granuphilin in β cells (Fig 2.5, bottom panel).

E. Discussion

The present study describes the presence of NRXN in the β cell and specifically as a binding partner of the insulin granule docking machinery at the β-cell membrane. Consistent with our prior immunohistology study of rat pancreas (9), we found that NRXN protein is present in human, rat, and mouse islets. NRXN transcripts in β cells have a very different, more restricted expression profile than in the brain. In rat, human, and mouse islets, NRXN1α transcript expression predominates. NRXN1α is also highly expressed in INS-1E cells. There are also substantial levels of NRXN2β transcripts in INS-1E cells and, to a lesser degree, in mouse and human islets.

The development of a custom, pan-NRXN antibody allowed us to confirm that NRXN is also present on the membrane of the INS-1E β-cell, consistent with the exclusive β-cell membrane staining of NRXN demonstrated in our prior immunohistology study of rat pancreas (9). As we hypothesized, the proteins that interact with NRXN1α in β cells seem to mirror those in the neuronal presynaptic active zone. This is further evidence that β cells and neurons employ highly conserved machineries to mediate regulated secretion. As in neurons, we found that NRXN1α in β cells binds to components of the submembrane exocytotic machinery. NRXN1α also associates with granuphilin, a granule-associated protein that plays a key role in secretory granule docking (132). This suggests that NRXN is a component of the plasma membrane-associated protein complex that mediates insulin granule docking.

Granuphilin is associated with the insulin granule surface and is essential for mediating docking to the submembrane, syntaxin 1A/Munc18-containing docking site (132,146). Granuphilin-mediated docking is inhibitory to insulin secretion (132-134).

Although this finding is at odds with studies suggesting that docking precedes or facilitates insulin exocytosis (22,135,136), it is in line with a recent study showing attenuation of insulin secretion by α-synuclein, another β-cell protein that promotes secretory granule association with the membrane (137). Because of NRXNs presence in the β-cell and interactions with constituents of the docking apparatus, it is possible that NRXN also constrains insulin exocytosis, halting secretory granules en route to SNARE complex formation and subsequent insulin release.

It is thought that insulin granule docking occurs via interactions of granuphilin with syntaxin 1 beneath the β-cell plasma membrane (133). However, loss of syntaxin 1 had no effect on secretory granule docking (153), suggesting the presence of an alternative submembrane receptor for granuphilin. The findings presented here suggest that NRXN1α may be the membrane-associated protein that anchors the docking complex at a subplasmalemmal site. NRXN1α associates, either directly or indirectly, with granuphilin (Figure 2.5). And given the extensive resemblance between regulated secretion in neurons and in the β cell, the known importance of interactions involving NRXN in synaptic vesicle docking at the presynaptic membrane suggests a similar role for NRXN in β cells (82,84,130). In neurons, the interaction between NRXN and the granuphilin-related protein rabphilin-3 is mediated by the intermediary protein CASK (130), a known NRXN binding partner (79,81,183). We have found that NRXN in β-cells also interacts with CASK. Overall, as would be extrapolated from findings in the synapse, our findings suggest that secretory granule docking in β cells is mediated by granuphilin interacting with NRXN1, either directly or indirectly (perhaps via CASK).

NRXNs in the β cell are also interesting in the context of identifying cell surface, β-cell specific markers for imaging healthy islet cell masses in humans. In type 1 diabetes, β-cells are selectively destroyed by an autoimmune process. There is growing evidence that β-cell loss contributes to the onset of type 2 diabetes as well (1,2). As a result, preservation and expansion of β-cell mass is a critical target in the treatment of diabetes. The development of new therapies to prevent β-cell loss and recover β-cell mass will require a greater understanding of β-cell fate in disease and therapy conditions (184). One possible way to build this knowledge would be through monitoring β-cell mass with noninvasive *in vivo* imaging (184,185). Identification of β-cell-specific membrane targets will be critical to the success of this technology (186,187). Our lab and others (8) have identified NRXNs as possible imaging candidates. Although some literature suggests that NRXNs can be found in multiple tissues outside of the central nervous system (119,188-191), our work indicates that NRXNs outside of the central nervous system are β-cell specific (9). Development of labeled ligands to target these molecules for imaging could pave the way for new islet replacement and regeneration therapies.

In summary, our data demonstrate that NRXN1 is a component of the β-cell machinery that regulates insulin exocytosis and insulin granule docking. NRXN1α interacts with constituents of the submembrane exocytotic and docking protein machinery and with the secretory granule-associated protein granuphilin. Chapters 3 and 4 will address the functional role of NRXN1α in insulin secretion and insulin granule docking.

F. Acknowledgements

Chapter 2, in part, is a reprint of the material as it appears in Mosedale, M., Egodage, S., Calma, R.C., Chi, N.W., Chessler, S.D. (2012) Neurexin-1α contributes to insulin-containing secretory granule docking. *J Biol. Chem*. 287(9): 6350-61. The dissertation author was the primary investigator in the development and execution of the study, and the principal author of this paper. I thank Challise Sullivan for help with validation of the custom, pan-NRXN antibody.

G. Tables

Table 2.1. Sequences of primers used for PCR and qPCR.

Transcript		Forward Primer	Reverse Primer
Primers used to generate NRXN PCR products Copies were quant-ified and used to make standard curves for absolute qPCR	Rat NRXN1α	CGTGAAACTCACGGTCAATC	GGGATGTTGTCTTGAATTGGA
	Rat NRXN1β	ACCACATCCACCATTTCCAT	TCAATGGCGATGTCATCTGT
	Rat NRXN2α	CCAGAGCAGCACTGATGAGA	GTCCCCTTCCTTAGCCAGTC
	Rat NRXN2β	GTCTCGTCCAGCCTCAGC	AAAAATCACCCCAACAGTGC
	Rat NRXN3α	CTGCTTCCTCTTGGATGGTC	CATTCACTGGCTCCACAATG
	Rat NRXN3β	CTGGACACTTGGGATCTGGT	TCGTTGACAGGGGTTCTCTC
	Mouse NRXN1α	CGGTGTGGTGGCTTTTAAGT	CAAGCCACCCAGGTACAACT
	Mouse NRXN1β	CTCTGGATAGTCCCGCTCAC	AGACTCTTCGATGGCGATGT
	Mouse NRXN2α	ACGAGATCACACTGGCCTTC	GTCCCCTTCCTTAGCCAGTC
	Mouse NRXN2β	GTCTCGTCCAGCCTCAGC	GCCACCACTTCGAGTAAAGC
	Mouse NRXN3α	GCGTGTCAAGCTCATGGTTA	GGTCAGGTAGCTGCTCTTGG
	Mouse NRXN3β	AGCAGGAACACCATTTCCAC	TTTACCATTTGGAGGCGTTC
	Human NRXN1α	AAGGACTGCAGCCAAGAAGA	AAGTCATCAGACCCCAGCAT
	Human NRXN1β	CTTTGGATAGTCCCGCTCAC	GGATTCTTCAATGGCGATGT
	Human NRXN2α	GTCTGGCTGGTCATCAACCT	TCGGGACTCTCAAAGGTCAC
	Human NRXN2β	CTCCTCCAGCCTCAGCAC	CAGGCGCTCGTTATCAAAGT
	Human NRXN3α	GCGGACACCAAGATGAAAAT	ATGTCTCCTTCCAGGTCCAG
	Human NRXN3β	GTTGACCATGCACCTGAGAA	TGGAGGAAGTCACCAAGTCC
Primers for absolute qPCR	Rat NRXN1α	AACTGTAATTCCAGCAAAGGTC	GGCAACATAGCTTGATTTGG
	Rat NRXN1β	GCCTATTGCAATCTACAGGTC	TTTCCTTGGTGTATGTGCAG
	Rat NRXN2α	CTAACTACGTCAACCTGTCC	TTGTAGACCACGTCCTTGAG
	Rat NRXN2β	CACTTCCACAGCAAGCAC	CTGGTCAATGTGCAGCTG
	Rat NRXN3α	CTCAGAAGATGTCAGTCAAGG	AGGTCTTAAAGGAAAGGGTG
	Rat NRXN3β	GTGTGGAGTTCTTCTAATGTAGC	TTTCCCTTGTTCTATGTGAAGC
	Mouse NRXN1α	ATTTGATTTCCGTACAACGGAG	ACAGAAATGGTACCTGACCG
	Mouse NRXN1β	GCCTATTGCAATCTACAGGTC	GCCACTTATATGTAATCTGTCCAC
	Mouse NRXN2α	GTCAATGGCAAGTTCAACGA	TTGTAGACCACGTCCTTGAG
	Mouse NRXN2β	ATCGCCATCAACCGCA	GTAATGTCGTCCGTGCC
	Mouse NRXN3α	CTGTAACTCCAGCAAAGGAC	GTACAGTAAGCCATTGAACATGAG
	Mouse NRXN3β	CTCTGTGCCTATTTCTATCTATCG	TTTCCCTTGTTCTATGTGAAGC
	Human NRXN1α	CAACAATGTGGAAGGTCTGG	ATATTGTCACCATAGCGTGTC
	Human NRXN1β	CACATCCACCATTTCCATGG	TTTCCCTGGTGTATATGCAG
	Human NRXN2α	GTCAATGGCAAGTTCAACGA	CTTATAGACCACGTCCTTGAG
	Human NRXN2β	CCACCACTTCCACAGCAAG	GCTGCAGGTAGTCTCCAAGG
	Human NRXN3α	GCGAAGTTGTGTTTAAGTGTG	CTGATATAGTACCTGATCTGCCA
	Human NRXN3β	TGTAGCTTCCTCCTCCTCCA	ACCAAGATGCCATCCTTCAC
	18S	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC

H. Figures

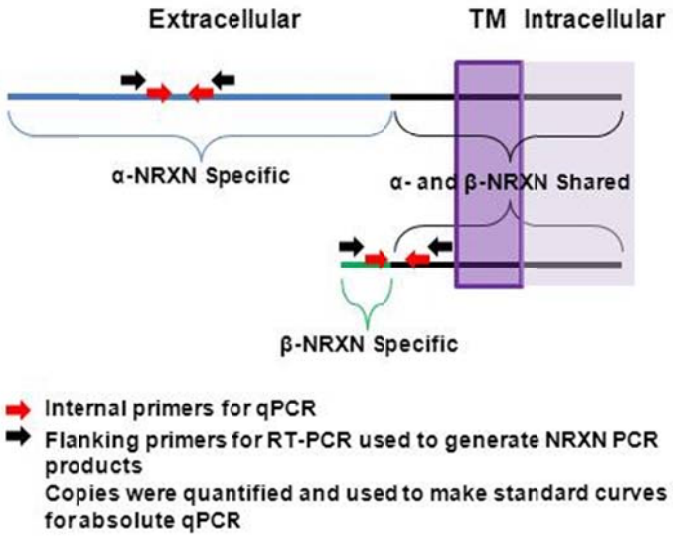

Figure 2.1. Schematic of approximate primer locations for RT-PCR and qPCR. Approximate locations of PCR primers used to amplify specific α- and β-NRXN transcripts. Internal primers were used for absolute and relative qPCR. Flanking primers were used for RT-PCR and to generate standard curves for absolute qPCR.

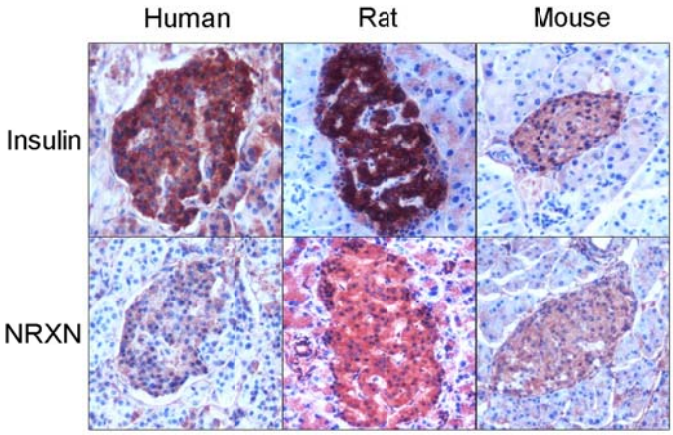

Figure 2.2. NRXN protein is present in β-cells. Serial sections of human, rat and mouse pancreas were stained for insulin (top) and NRXN (bottom). Omission of primary antibody in parallel control experiments abolished the observed islet staining (data not shown).

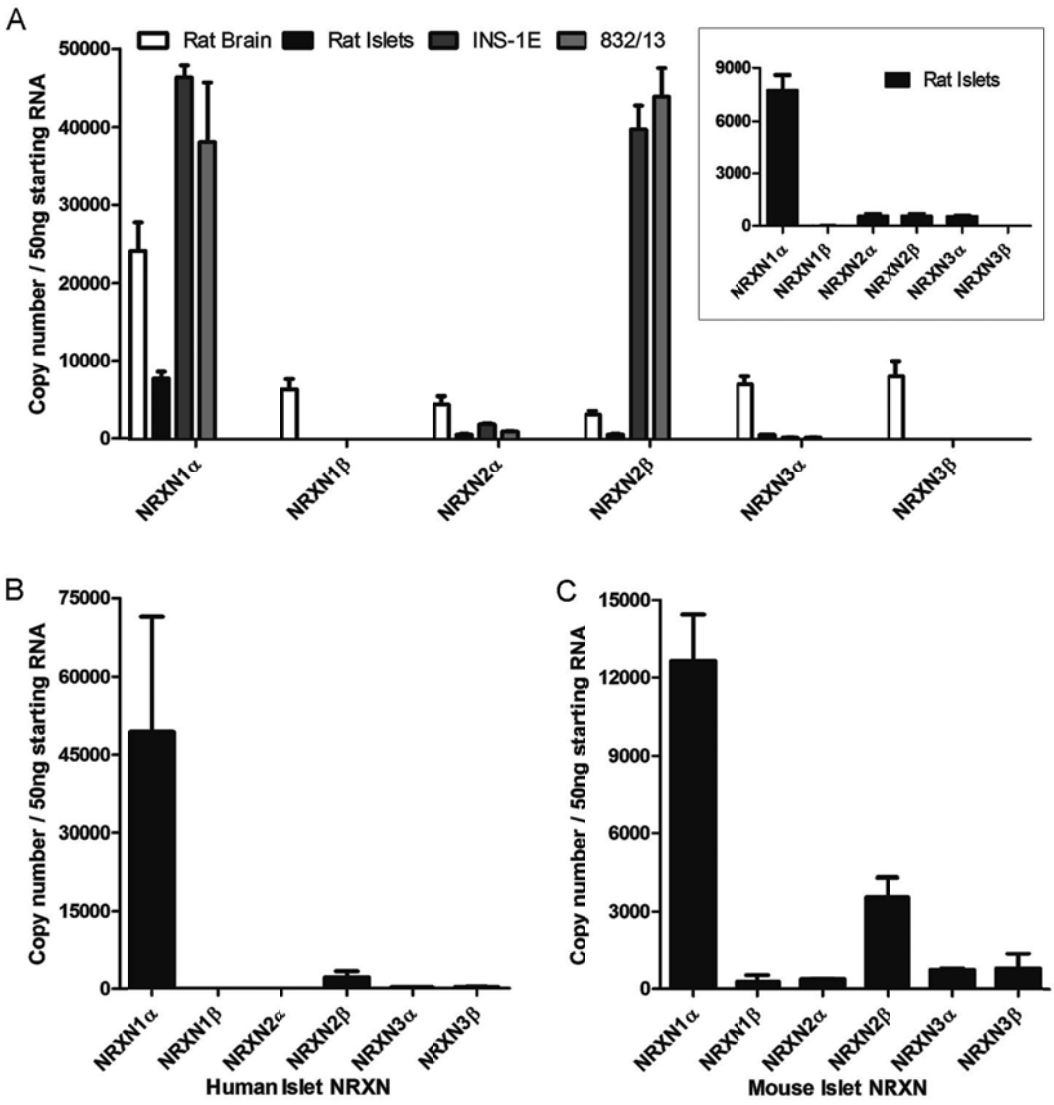

Figure 2.3. Quantification of NRXN transcripts in β cells by absolute RT-qPCR. NRXN mRNA transcript numbers in 50 ng of starting RNA from A, rat islets, the rat β-cell lines INS-1E and INS-1 832/13, and rat brain as well as B, human islets and C, mouse islets were quantified by absolute qPCR using a set of standard curves for each reaction with known NRXN copy numbers. Rat islet data is also represented as an inset in panel A. Data are represented as mean ± SEM from three individual tissue, islet or culture preparations assayed in triplicate.

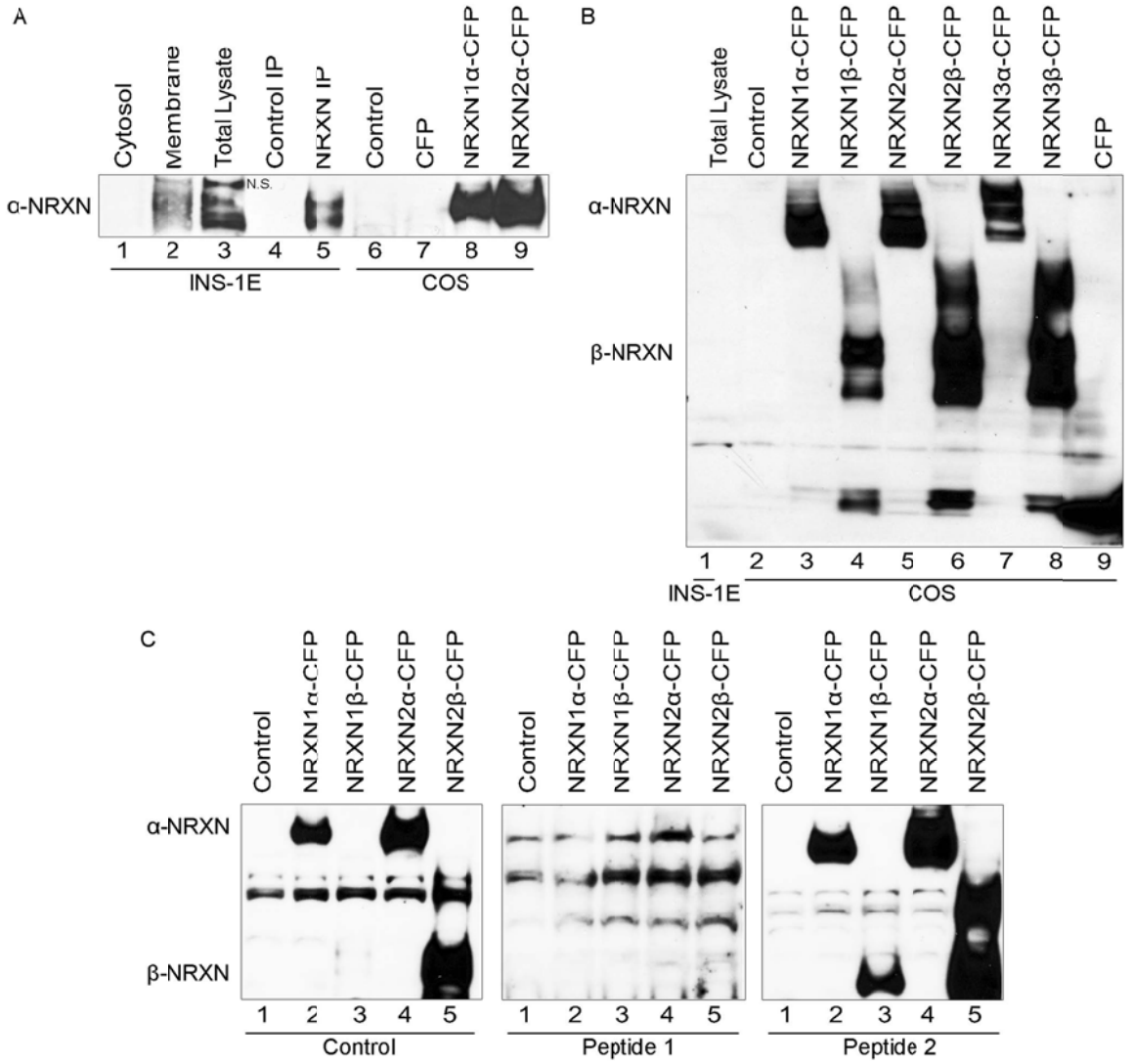

Figure 2.4. α- and β-NRXN protein from INS-1E β cells is detected on Western blots with custom NRXN antibody. A, Cytosolic and plasma membrane fractions (10 µg each, lanes 1-2) of INS-1E cells as well as total lysate (30 µg, lane 3) were immunoblotted with a custom pan-NRXN antibody. Protein immunoprecipitated from INS-1E cell extracts with a NRXN1 antibody (NRXN IP, lane 5) or an isotype control antibody (Control IP, lane 4) was analyzed on the same blot. Lysates of control COS-7 cells (Control) and COS-7 cells overexpressing CFP or CFP-tagged NRXN1α and NRXN2α (lanes 6-9) were immunoblotted in parallel to further validate the pan-NRXN antibody. N.S. indicates a non-specific band detected by the pan-NRXN antibody in the INS-1E lysate (lane 3) but not in anti-NRXN1 immunoprecipitates (lane 5). B, The identity of the bands was confirmed with an anti-GFP/CFP antibody. C, Lysates of control COS-7 cells (Control) and COS-7 cells overexpressing CFP-tagged NRXN1α, 1β, 2α, and 2β (lanes 2-5) were immunoblotted with the pan-NRXN antibody after incubation of the antibody with control peptide (Control), peptide 1 or peptide 2.

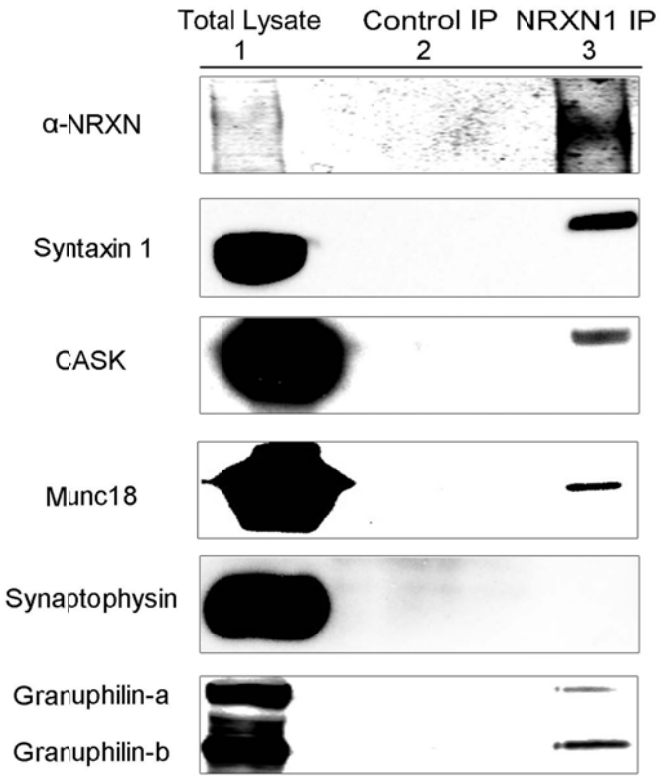

Figure 2.5. NRXN interacts with exocytotic and docking proteins in the INS-1E β-cell line. Anti-NRXN1 immunoprecipitates from INS-1E cells were immunoblotted for the indicated proteins (NRXN1 IP, lane 3) using isotype control immunoprecipitates as controls (Control IP, lane 2). Total lysate is represented in lane 1. The experiments were repeated with similar results.

CHAPTER 3:

NEUREXIN-1α CONTRIBUTES TO INSULIN-CONTAINING SECRETORY GRANULE DOCKING IN INS-1E β CELLS

A. Summary

Neurexins are a family of transmembrane, synaptic adhesion molecules. In neurons, neurexins bind to both sub-plasma-membrane and synaptic-vesicle-associated constituents of the secretory machinery, play a key role in the organization and stabilization of the presynaptic active zone, and help mediate docking of synaptic vesicles. We previously demonstrated that neurexins are expressed in the pancreatic β cells, where they interact with the insulin granule secretory machinery and the secretory granule docking protein granuphilin. We hypothesized that neurexins in β cells, like many other protein constituents of the neurotransmitter exocytotic machinery**,** play an analogous role in β cells, helping to mediate insulin granule docking and secretion. Using INS-1E β cells, we found that neurexin-1α is necessary for normal secretory granule docking. Decreased expression of neurexin-1α, like decreased expression of granuphilin, reduces granule docking at the β-cell membrane and improves insulin secretion. The constitutive secretory pathway, in contrast, is not affected. Upon glucose stimulation, neurexin-1α protein levels are decreased. Since insulin granule docking is inhibitory to insulin secretion, this glucose-induced neurexin down-regulation may enhance glucose-stimulated insulin secretion. We conclude that neurexin-1α is an integral component of the β-cell secretory machinery and contributes to secretory granule docking, most likely through interactions with granuphilin. Our findings provide new insights into the mechanisms of insulin granule docking and exocytosis.

B. Introduction

We previously found that β cells express neurexins (NRXNs), a family of synaptic adhesion molecules that in neurons function as neuronal cell-surface receptors and signaling proteins (36,40,41). Three genes encode NRXNs, each with an upstream promoter to produce the longer α-isoform (α-NRXNs) and a downstream promoter to generate a shorter β-isoform (β-NRXNs) (43). The α- and β-isoforms of each neurexin are single-pass transmembrane proteins maintaining identical transmembrane and intracellular domains but having distinct (i.e., long and short) extracellular domains (40). Unlike neurons which express all of the NRXN isoforms at similar levels, we demonstrated that β cells express a more restricted pattern of NRXN transcripts with NRXN1α predominating in rat, human, and mouse islets (see Figure 2.3). NRXN1α mRNA is also highly expressed in INS-1E cells.

The development of mice lacking α-NRXN genes (α-NRXN KO) has uncovered a unique role for the α-NRXNs in the differentiation of inhibitory as opposed to excitatory synapses (67). These studies are supported by *in vitro* work indicating that α-NRXNs and NRXNs specifically containing splice site 4 (SS4) contribute to the formation of GABAergic synapses (91). Interestingly, β cells have been described as GABAergic, since they expresses all of the machinery essential for GABA synthesis and secretion (33-35,174,192,193). β cells also only express NRXNs containing SS4 (9).

α-NRXNs in neurons are essential for the organization and stabilization of the presynaptic machinery (67,98,100). In vitro work suggests that NRXNs interact with the synaptic vesicle-associated protein rabphilin-3A via CASK (130) and contribute to synaptic vesicle docking (82,84). We also found that in β cells, NRXN1α binds a similar

array of neuronal binding partners including syntaxin 1, CASK, and Munc18 (see Figure 2.5) (78,79,81,83). These proteins mediate the exocytosis of synaptic vesicles in neurons and insulin granules in β cells (12). NRXNs in β cells also interact with the β-cell specific protein granuphilin (see Figure 2.5, bottom panel), a rabphilin-3 homologue implicated in insulin granule docking (131,132). Granuphilin-mediated secretory granule docking has been shown to be inhibitory toward insulin secretion (132,141,146,153). Based on the similarities between neurotransmitter exocytosis and insulin secretion, we hypothesized that as constituents of the insulin secretory machinery, NRXNs may help to mediate secretory granule docking via interactions with granuphilin.

In the present study we examined the role of NRXN1α in INS-1E β-cell function. Our data show that NRXN1α is an integral component of the secretory granule docking machinery. Like other proteins that contribute to the granuphilin-mediated docking of secretory granules at the β-cell membrane, NRXN1α inhibits insulin secretion (139,145). Our findings provide new insights into the mechanisms of insulin granule exocytosis.

C. Experimental Procedures

Antibodies

In order to detect NRXN expression by immunoblotting, we raised a polyclonal, pan-NRXN antibody against a previously described NRXN peptide (83). Rabbits were injected with the keyhole limpet hemocyanin-conjugated peptide CAKSANKNKKNKDKEYYV, and serum was affinity purified (Open Biosystems). The antibody was validated for peptide binding by ELISA and competition assay. The antibody was also validated by Western blot and detects all six CFP-tagged NRXN isoforms (91). The identity of the bands was confirmed with an anti-GFP/CFP antibody (see Figure 2.4).

The following antibodies were obtained commercially: rabbit anti-CaV1.2 (L-type voltage-gated calcium channel) (Alomone Labs); rabbit anti-granuphilin (Atlas Antibodies); mouse anti-Munc18 (BD Biosciences); mouse anti-syntaxin 1 and rabbit anti-VAMP2 (synaptobrevin) (Abcam); mouse anti-syntaxin 1 and -GAPDH (Sigma-Aldrich); goat anti-granuphilin (G-17), goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology); rabbit anti-GFP/CFP (Molecular Probes); and IRDye 680-conjugated goat anti-mouse IgG and IRDye 800CW-conjugated goat anti-rabbit IgG (LI-COR).

Cell culture and transfection

INS-1E cells (175) were a gift from Dr. Pierre Maechler (Geneva University) and were cultured in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 0.05 mM

2-mercaptoethanol and 0.25 μg/ml amphoterecin B. Cells were maintained in a humidified 37^0C incubator with 5% CO_2. For transfections, INS-1E cells were plated onto 12- or 24-well plates in antibiotic-free media and transfected with DNA constructs or siRNA duplexes using Lipofectamine 2000 (Invitrogen) or Pepmute (SignaGen Laboratories) according to manufacturers' protocols. Cells were stimulated or harvested 48 h or 72 h after transfection.

RNAi

For NRXN1 RNAi experiments, two pools of siRNAs targeting NRXN1 (NRXN1 pools 1 and 2) and two non-targeting control pools of siRNA (non-targeting pools 1 and 2) comprised of completely different siRNAs were purchased from Dharmacon Research (Thermo Fisher Scientific). For NRXN2 RNAi experiments, a pool of siRNAs targeting NRXN2 (NRXN2 pool A) was purchased from Sigma-Aldrich and a pool of non-targeting control siRNAs (non-targeting pool A) was purchased from Dharmacon Research (Thermo Fisher Scientific). All sequences are listed in supplemental Table 3.1.

Real-time quantitative PCR (qPCR)

Total RNA was isolated and reverse transcribed as previously described (9). Gene-specific primers were used to perform qPCR with Power SYBR Green PCR Master Mix (Applied Biosystems), PerfeCTa SYBR Green FastMix (Quanta BioSciences) or 2x qPCR Master Mix (BioPioneer) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Each sample was analyzed in triplicate along with no-RT and no-template controls. Values were normalized to 18S RNA. NRXN primers were designed to be gene

specific and avoid major splice variant regions (see Figure 2.1). All primers were designed using Primer 3 (179) or PerlPrimer (180) software and are described in Tables 2.1 and 3.2.

Western blotting

Total cell extract was made by lysing cells in RIPA buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM PMSF, and 1% Protease Inhibitor Cocktail (Sigma-Aldrich). Proteins were quantified using the D_C Protein Assay (Bio-Rad Laboratories). NuPAGE LDS Sample Buffer and NuPAGE Reducing Agent (Invitrogen) were added to protein at a 1x final concentration. Protein was then incubated at 70^0C for 10 min and electrophoresed on either 4-12% Bis-Tris or 3-8% Tris-Acetate NuPAGE Gels (Invitrogen). Protein was transferred to PVDF membrane, blocked with 5% milk in PBS and probed with antibodies in 5% milk in PBS containing 0.1% Tween 20. Membranes probed with HRP-labeled secondary antibodies were incubated with PS-3 chemiluminescent detection reagent (Lumigen) and the chemiluminescent signal captured by HyBlot CL autoradiography film (Denville Scientific). Membranes probed with IRDye-conjugated secondary antibodies were directly imaged and bands quantified with the Odyssey Infrared Imaging System and Software (LI-COR).

Plasmid constructs

The cDNA vector pSEAP2 encoding secreted alkaline phosphatase (SEAP) was from Clontech. The cDNA construct encoding eGFP-tagged NRXN1α (51) was a gift

from Dr. Markus Missler (University of Munster). The NRXN1α expression vector, the GFP expression vector and an empty vector control were derived from the pCMV-eGFP-NRXN1α vector by removing regions encoding eGFP, NRXN1α and eGFP-NRXN1α respectively. The cDNA vector pXGH5 encoding human growth hormone (HGH) was from Nichols Institute Diagnostics.

Glucose stimulation

INS-1E cells were switched to antibiotic-free media containing 5 mM glucose for approximately 18 h (unless otherwise noted) and then to 2.75 mM glucose Krebs-Ringer bicarbonate buffer for 1 h. Fresh Krebs-Ringer buffer containing 2.75 mM, 15 mM, or 16.7 mM glucose was then applied for 1 h as previously described (9). Insulin in cell lysate and in conditioned media was measured using a rat insulin RIA (Millipore). For SEAP co-transfection studies, cell lysate and conditioned media were also assayed for SEAP as described (194). For HGH co-transfection studies, cell lysate and conditioned media were also assayed for HGH using the Growth Hormone Ultrasensitive EIA (ALPCO Diagnostics). Insulin, SEAP, and HGH secretion are represented as media content normalized to intracellular content. Total cellular insulin content in lysate was normalized to total cellular protein.

Immunoprecipitation (IP)

Cells were cross-linked with 5 mM DTBP crosslinker for 30 min on ice and then incubated with 100 mM Tris (pH 8) for 10 min to stop crosslinking. Cells were lysed in a buffer containing 150 mM NaCl, 1% Nonidet P-40, 50 mM Tris (pH 8), 1 mM PMSF, and

1% Protease Inhibitor Cocktail. Lysate was precleared with rec-Protein G-Sepharose (Invitrogen) and then incubated overnight at 4^0C with 5 μg of goat anti-granuphilin G-17 antibody or purified goat IgG (Millipore). The reactions were incubated with rec-Protein G-Sepharose at 4^0C for 2 h and beads were thoroughly washed with lysis buffer. Cross-linked product was treated with 150 mM DTT at 37^0C for 30 min to reverse crosslinking. Samples were combined with LDS Sample Buffer and Reducing Agent for Western blotting as described above.

EM

INS-1E cells in normal culture media were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for at least 4 h, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h and stained *en bloc* in 1% uranyl acetate for 1 h. Samples were dehydrated in ethanol, embedded in epoxy resin, sectioned at 60 to 70 nm and stained with uranyl acetate and lead nitrate. Grids were viewed using a transmission electron microscope (1200EX II, JEOL) and photographed using a digital camera (Gatan). Cells were chosen at random. Distances were determined by drawing a straight line from the granule center to the nearest plasma membrane. All measurements were performed in ImageJ (195).

Lysosomal inhibition

INS-1E cells were switched to antibiotic-free media containing 5 mM glucose for approximately 18 h and then to 2.75 mM glucose Krebs-Ringer bicarbonate buffer with or without a pool of lysosomal inhibitors for 1 h. The lysosomal inhibitor pool contained

200 μM choloroquin (Sigma-Aldrich), 20 mM ammonium chloride (Mallinckrodt), and 100 μM leupeptin (Sigma-Aldrich). Controls included equal volumes of inhibitor diluents. Fresh Krebs-Ringer buffer containing 2.75 mM or 15 mM glucose with inhibitors or controls was then applied for 1 h. Cells were lysed in RIPA buffer and lysates used for Western blotting as described above. Membranes probed with IRDye-conjugated secondary antibodies were directly imaged and bands quantified with the Odyssey Infrared Imaging System and Software (LI-COR). α-NRXN bands were normalized to GAPDH loading control bands and expressed relative to 2.75 mM glucose- and control-treated cells.

D. Results

Knockdown of NRXN1α in INS-1E cells increases insulin secretion

We previously demonstrated that NRXN interacts with the insulin granule docking protein, granuphilin (see Figure 2.5). Granuphilin-mediated secretory granule docking is thought to act as a "temporal constraint" on insulin secretion, inhibiting SNARE-mediated fusion of insulin granules with the plasma membrane (132,146). Because of this, experimental knockdown and knockout of granuphilin increased insulin secretion (134,146). If NRXN is also necessary for docking, its depletion should similarly increase insulin secretion. We thus used siRNA to knockdown NRXN1 in INS-1E cells and determined the effect on glucose-stimulated insulin secretion. siRNA treatment of INS-1E cells resulted in a 78% decrease in NRXN1α transcript levels (Figure 3.1A) without increasing the levels of other α-NRXN transcripts (data not shown). A similar depletion of NRXN1α protein was also observed (Figure 3.1B; the top band identified by "N.S." reacted nonspecifically with the pan-NRXN antibody). NRXN1 silencing resulted in a 50% increase in insulin secretion at high glucose (Figure 3.1C) and a 62% increase in the stimulation index (the ratio of insulin secretion at high vs. low glucose) (Figure 3.1D). Basal insulin secretion was not significantly changed. Insulin secretion experiments were repeated with similar results using a completely different pool of both control and NRXN1 siRNAs.

To confirm that NRXN1 knockdown selectively impacts the regulated secretory pathway, we also measured its effect on the secreted alkaline phosphatase (SEAP), a recombinant cargo commonly used to track the efficiency of the constitutive secretory pathway (194,196). We found that NRXN1 knockdown did not affect SEAP secretion at

high glucose (Figure 3.1E), suggesting that insulin secretion was selectively modulated by NRXN1.

Knockdown of NRXN1α in INS-1E cells increases insulin content and insulin 2 gene expression

Interestingly, the increased insulin secretion (measured as % of total cellular content) after NRXN1 knockdown was accompanied by up-regulation of cellular insulin content and insulin 2 mRNA by 31% and 37% over control, respectively (Figure 3.2 A-B). Insulin content and mRNA were measured in INS-1E cells after an overnight incubation in 5 mM glucose. Under prolonged stimulatory conditions (> 12 h), both insulin content and insulin mRNA levels are increased (197,198). Since 5 mM glucose would still promote insulin secretion (175), we hypothesized that the increased secretion observed at high glucose in NRXN siRNA-treated cells (Figure 3.1C) was causing a feedback loop and driving the observed increases in insulin content and mRNA.

To determine the effect of glucose, and thereby increased stimulation, on the observed increases in cellular insulin content, NRXN1 knockdown cells were incubated for 18 h in different glucose concentrations prior to insulin assay. 2.8 mM glucose was used to replicate basal secretion where no difference in insulin secretion was observed in siRNA-treated cells (Figure 3.1C, black bars). 11.2 mM glucose was used to simulate a stimulatory condition, where NRXN1 siRNA-treated cells have increased insulin secretion compared to control siRNA-treated cells (Figure 3.1C, white bars). We found that 2.8 mM glucose, but not 11.2 mM glucose recapitulated the increase in insulin content (Figure 3.2C).

Knockdown of NRXN1α alters the expression and interaction of exocytotic and docking proteins INS-1E cells

To get a better understanding of the NRXN1 siRNA-induced changes that could contribute to the observed increases in insulin secretion and content, we first looked for changes in protein and mRNA expression of several components of the secretory granule docking and exocytotic complex. CaV1.2 is essential for glucose-stimulated insulin secretion, and its expression and function is regulated by several components of the exocytotic machinery (199). The scaffolding proteins CASK and Mint1 have also been shown to interact with voltage dependent Ca^{2+} channels in neurons (200). Ca^{2+} channel activity is reduced in NRXN KO mice, and it has been proposed that this is due to the importance of neuronal α-NRXNs for coupling presynaptic calcium channels to other parts of the secretory machinery (67). We previously showed that granuphilin, Munc18, and syntaxin 1 interact with NRXN1α (see Figure 2.5). These three proteins are all involved in secretory granule docking where they initially function as inhibitors to insulin secretion (133,134,141,201,202). But Munc18 is also a positive regulator of secretion through its involvement in priming (134,203), as is syntaxin 1 and its t-SNARE binding partner SNAP25 by promoting SNARE complex assembly and fusion (204,205). VAMP2 is a v-SNARE expressed on the vesicle membrane that interacts with SNAP25 and syntaxin 1 to promote insulin granule exocytosis (206-208).

Western blots of various exocytotic and docking proteins confirmed that protein levels of granuphilin-a, Munc18, and VAMP2 were all increased after NRXN1 knockdown (Figure 3.3A-B). Levels of CaV1.2, granuphilin-b, and syntaxin 1 were not statistically different in NRXN1 siRNA-treated cells. We then used qPCR to examine

changes in the exocytotic machinery at the mRNA level and found that granuphilin-a and Munc18-1 transcripts were also increased, whereas CASK and Mint1 mRNA levels were decreased (Figure 3.3C).

Alterations in granuphilin and Munc18 levels after NRXN1 knockdown prompted us to investigate these proteins in more depth. Granuphilin is expressed on the secretory granule membrane whereas Munc18 is associated with the β-cell membrane (134,141,205). Because of the coprecipitation NRXN with both granuphilin and Munc18 (see Figure 2.5), we hypothesized that NRXN1 could promote the interaction of these two proteins. A reduction in the critical interactions of granuphilin and Munc18 after NRXN1 siRNA treatment, and therefore a reduction in granule docking, might be contributing to the increase in insulin secretion. Therefore, in spite of the increased levels of both Munc18 and granuphilin-a, we predicted that their binding would be reduced after NRXN1 knockdown. We immunoprecipitated granuphilin from INS-1E cells after control or NRXN1 siRNA treatment and immunoblotted for coprecipitating Munc18 (Figure 3.3D). Contrary to our expectations, Munc18 and granuphilin interactions were actually increased in cells with reduced levels of NRXN1 (Figure 3.3E).

Knockdown of NRXN1 impairs secretory granule docking

Munc18 is not an integral membrane protein, and therefore, its interactions with granuphilin might not be indicative of secretory granule docking. Therefore, it is possible that NRXN siRNA-induced increases in insulin secretion could still be related to reduced β-cell-membrane, secretory granule docking.

We next asked whether NRXN1, like granuphilin, contributes to secretory granule docking at the β-cell membrane. Using EM, we examined the distribution of secretory granules in INS-1E cells with and without NRXN1 siRNA treatment (Figure 3.4A). Given their average diameter of ~350 nm, secretory granules whose centers reside within 100-200 nm of the β-cell membrane are considered docked (22,132,144). We categorized granules into four groups based on their distance from the plasma membrane. Compared to control siRNA-treated cells, NRXN1 siRNA-treated cells had 32% fewer granules in the docked (100-200 nm) category but significantly more in the >400 nm category (Figure 3.4B). Consistent with the finding of greater insulin content after NRXN1 siRNA treatment (Figure 3.2A), knockdown also increased insulin granule number per cell cross section by 57% (Figure 3.4C). To confirm that the increased granule number was not related to alterations in β-cell size, we also measured the area and perimeter of cell cross sections. There was no difference in area or perimeter of β cells after NRXN1 siRNA treatment relative to control siRNA-treated cells (Figure 3.4D).

Knockdown of NRXN2β in INS-1E cells also increases insulin secretion and insulin content

To determine if NRXN isoforms other than NRXN1α also regulate insulin secretion and content, we conducted similar knockdown experiments using siRNAs targeting NRXN2β, the other abundant isoform in INS-1E cells (see Figure 2.3). In INS-1E cells where NRXN2β transcript was depleted by 72% (Figure 3.5A), we found a 67% increase in insulin secretion at high glucose (Figure 3.5B), a 91% increase in the

stimulation index (Figure 3.5C), and 26% increase in cellular insulin content (Figure 3.5D). Basal insulin secretion was not significantly changed.

NRXN protein levels decrease at high glucose

In order to understand how β cells would compensate for NRXN as a negative regulator of insulin secretion, we next asked whether glucose affects NRXN1α protein levels. We found that after a 1 h treatment with high glucose, endogenous NRXN1α protein levels were decreased by 33% compared to low-glucose-treated controls (Figure 3.6A-B left panels). High glucose also decreased the expression of transfected NRXN1α protein by a similar 40% (Figures 3.6A-B, right panels). Though this decrease in NRXN1α was unexpected, it is noteworthy that glucose transporter 2 (GLUT2), another β-cell membrane protein, has been reported to undergo internalization and degradation upon glucose stimulation (209).

GLUT2 turnover is thought to occur via lysosomal degradation (209). To see if this was also the case for NRXNs, we treated INS-1E cells with a pool of lysosomal inhibitors prior to and during glucose stimulation and then immunoblotted for α-NRXN (Figure 3.6C). Compared to low glucose-treated, control samples, high glucose-treated control samples had a 28% (and statistically significant) reduction in α-NRXN protein which was ameliorated by treatment with the pool of lysosomal inhibitors (Figure 3.6D).

Overexpression of NRXN1α has no effect on glucose-stimulated insulin secretion

To complement the knockdown study, we also overexpressed NRXN1α to investigate its effect on insulin secretion. Transient transfection of INS-1E cells with a

NRXN1α overexpression vector resulted in an increase in NRXN1α protein (Figure 3.7A) but had no effect on basal or high glucose-stimulated insulin secretion (Figure 3.7B) and therefore, no effect on the stimulation index (Figure 3.7C). We also investigated the effect of NRXN1α overexpression on insulin content, but found that elevated levels of NRXN1α had no effect (Figure 3.7D).

Preliminary experiments examining the transfection efficiency of our NRXN1α overexpression construct revealed that as few as 30% of cells were expressing the recombinant protein (data not shown). To evaluate insulin secretion in only NRXN1α-overexpressing cells we cotransfected our overexpression construct with human growth hormone (HGH), a recombinant protein that gets targeted to secretory granules and cosecreted with insulin (141,210,211) and measured the effect of NRXN1α overexpression on HGH secretion. During these experiments we determined that transfection and expression of the cotransfected protein impacted total HGH content (data not shown). As a result, we used an equal molar amount of a GFP overexpression construct supplemented with an equal μg amount of empty vector carrier DNA as a control. Transient transfection of the GFP control and the NRXN1α overexpression constructs resulted in an increase in the respective proteins (Figure 3.7E). However, compared to the GFP overexpressing control, NRXN1α overexpression had no effect on basal or high glucose-stimulated HGH secretion (Figure 3.7F) and therefore no effect on the stimulation index (Figure 3.7G).

E. Discussion

The present study establishes a role for NRXN1 in insulin secretion and specifically in insulin granule docking at the β-cell membrane. As we hypothesized, NRXN1α, like granuphilin (132,141,146,153) is inhibitory to insulin secretion and impairs secretory granule docking. The insulin secretion and EM results presented herein, indicate that NRXN is a component of the plasma membrane-associated protein complex that mediates insulin granule docking.

Granuphilin is associated with the insulin granule surface and is essential for mediating docking to the submembrane, syntaxin 1A/Munc18-containing docking site (132,146). Granuphilin-mediated docking is inhibitory to insulin secretion (132-134). Although this finding is at odds with studies suggesting that docking precedes or facilitates insulin exocytosis (22,135,136), it is in line with a recent study showing attenuation of insulin secretion by α-synuclein, another β-cell protein that promotes secretory granule association with the membrane (137). By providing another example of a constituent of the docking apparatus that inhibits insulin secretion, our results support the idea that docking serves to constrain insulin exocytosis, halting secretory granules en route to SNARE complex formation and subsequent insulin release.

To clarify the role of NRXN1 in exocytosis, we used siRNA to deplete NRXN1 in INS-1E cells. Loss of NRXN1—as was previously observed with loss of granuphilin (134,146)—increased glucose-stimulated insulin secretion (Figure 3.1A). Also, as in the case with granuphilin (141), NRXN1 knockdown did not impact basal insulin secretion, nor did it affect constitutive secretion, indicating that the knockdown did not cause a general impairment of β-cell secretory function.

The parallel between the effects of NRXN1α and granuphilin levels on insulin secretion is consistent with NRXN, like granuphilin, being essential to the docking mechanism. Interestingly, NRXN1 knockdown caused an increase in insulin content and transcript levels. Electron micrographs also show increased numbers of secretory granules in NRXN1 siRNA-treated cells. The increase in insulin levels was most likely not due to increased insulin secretion caused by NRXN knockdown. Insulin levels were significantly increased in INS-1E cells treated with NRXN1 siRNA and incubated overnight in non-stimulatory glucose levels compared to NRXN1-siRNA treated cells incubated at high glucose and non-targeting-siRNA treated cells incubated at either glucose level. However, we cannot rule out that the increase in glucose levels suppresses protein knockdown in the NRXN1 siRNA-treated cells or destabilizes NRXN1 protein in control cells (see Figure 3.6), thus masking the effect of NRXN1 knockdown on insulin content. As a result, the mechanism whereby NRXNs influence β-cell insulin content remains to be uncovered. It is noted, however, that in NRXN1 siRNA-treated INS-1E cells, the increases in insulin secretion cannot be explained by increased insulin content since insulin secretion in our static culture studies was normalized to total cellular insulin content and also the magnitudes of the increases in secretion resulting from loss of NRXN1α exceeded those of the increases in content.

In addition to increasing insulin secretion and content, knockdown of NRXN1 altered protein and mRNA expression of several components of the secretory granule docking and exocytotic complex. For example levels of docking proteins granuphilin and Munc18, which we previously showed interact with NRXN1α in INS-1E β cells (see Figure 2.5), were increased. This finding was surprising because docking is known to be

inhibitory to insulin secretion (146) and in granuphilin KO mice, the reduction of granuphilin is followed by reduced levels of Munc18 that in combination increase glucose-stimulated insulin secretion.

It is possible, however, that NRXN1α serves to mediate the interactions between NRXN on the β-cell membrane and granuphilin on the secretory granule. When NRXN levels are reduced, levels of these proteins are increased in compensation. We found that NRXN1 knockdown did not affect granuphilin-Munc18 binding, however, it is possible that these interactions are no longer taking place at the β-cell membrane. Munc18 is soluble protein with multiple cytosolic binding partners (203). If this is the case, loss of NRXN1 would still impact docking without decreasing levels or binding of other docking proteins. Furthermore, levels of CASK and Mint1 mRNA were decreased in NRXN1 siRNA treated cells, indicating that NRXN may be an important nucleating factor for these scaffolding proteins.

It is thought that insulin granule docking occurs via interactions of granuphilin with syntaxin 1 beneath the β-cell plasma membrane (133). However, loss of syntaxin 1 had no effect on secretory granule docking (153), suggesting the presence of an alternative submembrane receptor for granuphilin. The findings presented here suggest that NRXN1α may be the membrane-associated protein that anchors the docking complex at a subplasmalemmal site. First, we demonstrated previously that NRXN1α associates, either directly or indirectly, with granuphilin (see Figure 2.5). Second, NRXN1 knockdown in INS-E cells impairs secretory granule docking. Third, given the extensive resemblance between regulated secretion in neurons and in the β cell, the known importance of interactions involving NRXN in synaptic vesicle docking at the

presynaptic membrane suggests a similar role for NRXN in β cells (82,84,130). In neurons, the interaction between NRXN and the granuphilin-related protein rabphilin-3 is mediated by the intermediary protein CASK (130), a known NRXN binding partner (79,81,183). We have found that NRXN in β-cells also interacts with CASK. Overall, as would be predicted by extrapolation of findings in the synapse, our results suggest that secretory granule docking in β cells is mediated by the interaction of granuphilin with NRXN1, either directly or indirectly (perhaps via CASK).

While it seems likely that NRXNs are essential for secretory granule docking, granule docking was not completely abolished in INS-1E cells treated with NRXN1 siRNA. Possible explanations for the persistence of docked granules in these studies include incomplete NRXN1 gene silencing in the siRNA-treated cells and functional redundancy with NRXN2β or other NRXN isoforms (Figure 3.5). Alternatively, the remaining granules that appear to be docked by morphological criteria may be stochastically located close to the plasma membrane without being molecularly docked, as has been proposed to account for the approximately 30% of granules that remain in the 100-200 nm distance category in granuphilin null mice (132).

We also found that glucose stimulation causes a reduction in NRXN protein, possibly via lysosmal degradation. This is similar to other membrane proteins involved in glucose-stimulated insulin secretion such as GLUT2 (209). Since NRXN1 down-regulation increases glucose-stimulated insulin secretion, NRXN degradation may enhance the insulin secretory response to elevated glucose levels.

Overexpression of NRXN1α had no effect on insulin secretion, suggesting that the maximal effect of NRXN1α is already achieved at endogenous levels, perhaps, for

example, due to limited availability of key binding partners at supraphysiological expression levels. In fact, it has been shown via EM and quantitative stereological techniques that docking sites are limited (212). In any case, this lack of significant effect on insulin secretion would be expected after increasing expression of a protein—such as we now believe NRXN1α to be—normally expressed at levels not rate-limiting for insulin exocytosis. An example of this is GLUT2, knockdown of which impairs glucose-stimulated insulin secretion (213) but overexpression of which has no effect (214).

In summary, our data demonstrate that NRXN1 has a functional role in β cells as a component of the machinery for regulated exocytosis and, more specifically, is essential for insulin granule docking. NRXN1α interacts with constituents of the submembrane exocytotic and docking protein machinery and with the secretory granule-associated protein granuphilin. Decreased expression of NRXN1 in β cells increases glucose-stimulated insulin secretion, most likely by decreasing secretory-granule docking. This increase in insulin secretion after NRXN knockdown provides further evidence that docking acts, as has been proposed by others, as a "temporal constraint" on insulin secretion (146). Under stimulatory conditions, NRXN1α protein levels are decreased, and this glucose-dependent regulation of NRXN could allow the β cell an additional level of control over insulin secretion. Chapter 4 will address the functional role of NRXN1α in primary mammalian β cells.

F. Acknowledgements

Chapter 3, in part, is a reprint of the material as it appears in Mosedale, M., Egodage, S., Calma, R.C., Chi, N.W., Chessler, S.D. (2012) Neurexin-1α contributes to insulin-containing secretory granule docking. *J Biol. Chem*. 287(9): 6350-61. The dissertation author was the primary investigator in the development and execution of the study, and the principal author of this paper. I thank Leyla Kaplan for assistance evaluating siRNA transfection reagents.

G. Tables

Table 3.1. Sequences of NRXN siRNAs.

Pool	NRXN1 siRNA	Sequence
1	1	GCUCAACUAUGGCUACGUA
	2	GGGACGACGUAUAUCUUUA
	3	CAUAAUAAUUGGCGGGAAA
	4	GGACUAAUUCUGUAUAACA
2	1	UCUCAAAGAGGUCGUAUAU
	2	AGACAUGGGAUCAGGUACU
	3	CAUACAAAUCGUUGCCGAA
Pool	**Non-targeting siRNA**	**Sequence**
1	1	AUGAACGUGAAUUGCUCAA
	2	UAAGGCUAUGAAGAGAUAC
	3	AUGUAUUGGCCUGUAUUAG
	4	UAGCGACUAAACACAUCAA
2	1	UGGUUUACAUGUCGACUAA
	2	UGGUUUACAUGUUGUGUG
	3	UGGUUUACAUGUUUUCUGA
	4	UGGUUUACAUGUUUUCCUA
Pool	**NRXN2 siRNA**	**Sequence**
1	1	CACGGAUGACAUUACAAUU
2	2	CAACCUGCCCGCGGGCAAA

Table 3.2. Sequences of rat primers used for qPCR.

Transcript	Forward Primer	Reverse Primer
Insulin 2	AGGCTTTTGTCAAACAGCACCT	AAGAATCCACGCTCCCCAC
Granuphilin-a	GTGATGAAGAAGACCTTGAATCC	GTCCCATACAGTCAGTTCCA
Granuphilin	GATGTCTGCTCCCAAATCTC	GTCAATGTCCTCTTCCTCCT
Munc18-1	TCCTGAAGAACGGTATCACTG	GAGCGGGTAGAGATGTATGG
CASK	ACACTGCTGGATCTGTATGAC	GCACTTTCTGGAGAATCACC
Mint1	GAATGGAATTATCTGTAGCCTC	ATTGGAGAGTATGTGGACGA
Syntaxin 1	CTAAAGAGCATCGAGCAGAG	GAGTCCATGATGATCCCAGAG

H. Figures

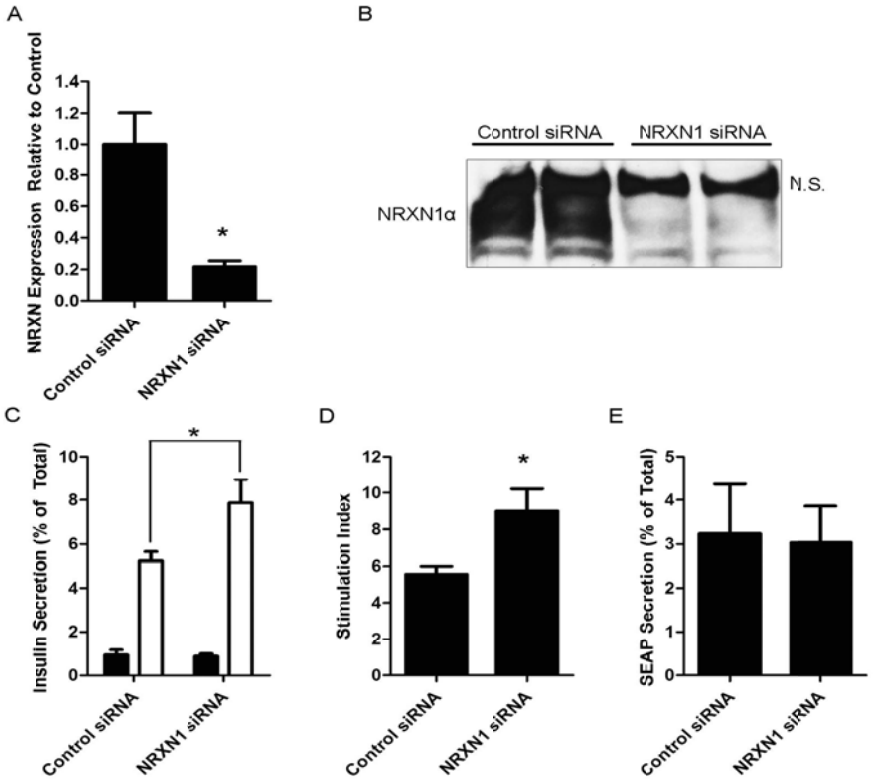

Figure 3.1. siRNA-mediated knockdown of NRXN1 in INS-1E cells increases glucose-stimulated insulin secretion. INS-1E cells were transfected for 72 h with a pool of either non-targeting control or NRXN1 siRNAs. A, mRNA was isolated and NRXN1α gene expression was determined using RT-qPCR, normalized to 18S RNA. B, Equal protein amounts of total cell lysates were immunoblotted for NRXN to confirm knockdown at the protein level. Lysates from two independent culture wells are shown for both control and NRXN1 siRNA. N.S. indicates a non-specific band detected by the pan-NRXN antibody. C, Cells treated with control or NRXN1 siRNA were incubated for 1 h in Krebs-Ringer solution containing either 2.75 mM (black bars) or 16.7 mM glucose (white bars). Insulin in the conditioned media was determined by RIA. D, The glucose stimulation index as defined by the ratio of insulin secretion (% of total) at 16.7 mM to secretion at 2.75 mM glucose is represented. E, INS-1E cells were transfected with a secreted alkaline phosphatase (SEAP) construct in addition to control or NRXN1 siRNA and treated with 15 mM glucose as in C. Conditioned media and cell lysates were assayed for SEAP content. The percent of total SEAP secreted is depicted. All data are represented as mean ± SEM from six samples, and each experiment was repeated three times with similar results. Insulin secretion experiments were repeated an additional three times with similar results using a completely different pool of control and NRXN1 siRNAs. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to non-targeting siRNA transfected controls.

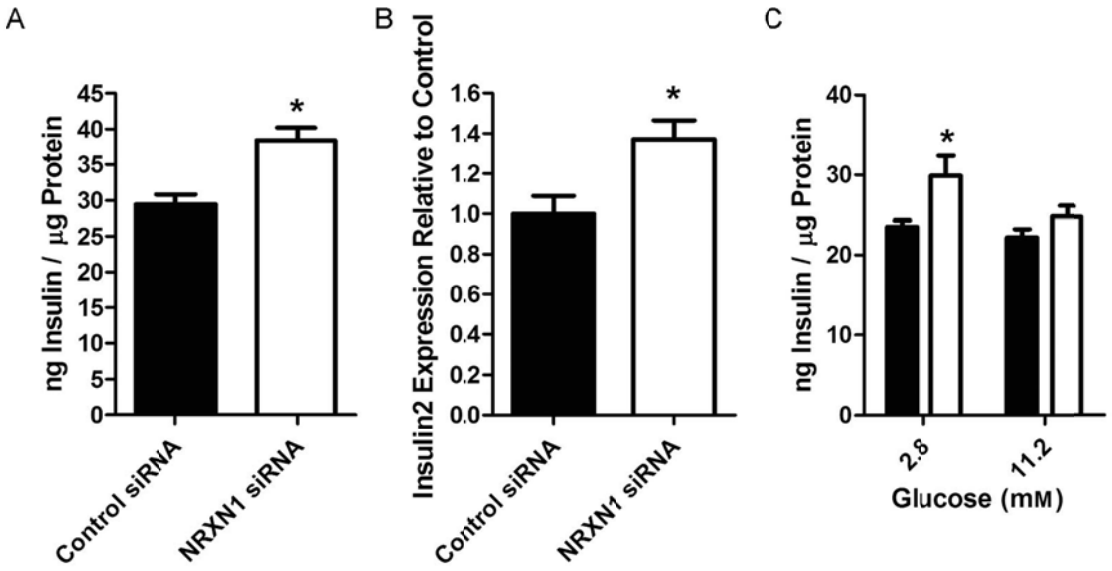

Figure 3.2. siRNA-mediated knockdown of NRXN1 in INS-1E cells increases insulin content. INS-1E cells were transfected for 72 h with a pool of either non-targeting control or NRXN1 siRNAs. A, Total lysate from siRNA-treated cells incubated for 24 h at 5 mM glucose was assayed for insulin by RIA. Insulin content normalized to total protein is depicted. B, Insulin 2 gene expression in siRNA-treated cells incubated at 5 mM was determined by qPCR and normalized to 18S RNA. C, Insulin content normalized to total protein was determined for control (black bars) or NRXN (white bars) siRNA-treated cells incubated for 24 h at 2.8 mM or 11.2 mM glucose. All data are represented as mean ± SEM from six samples, and experiments at 5 mM glucose were repeated three times with similar results. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to non-targeting siRNA transfected controls.

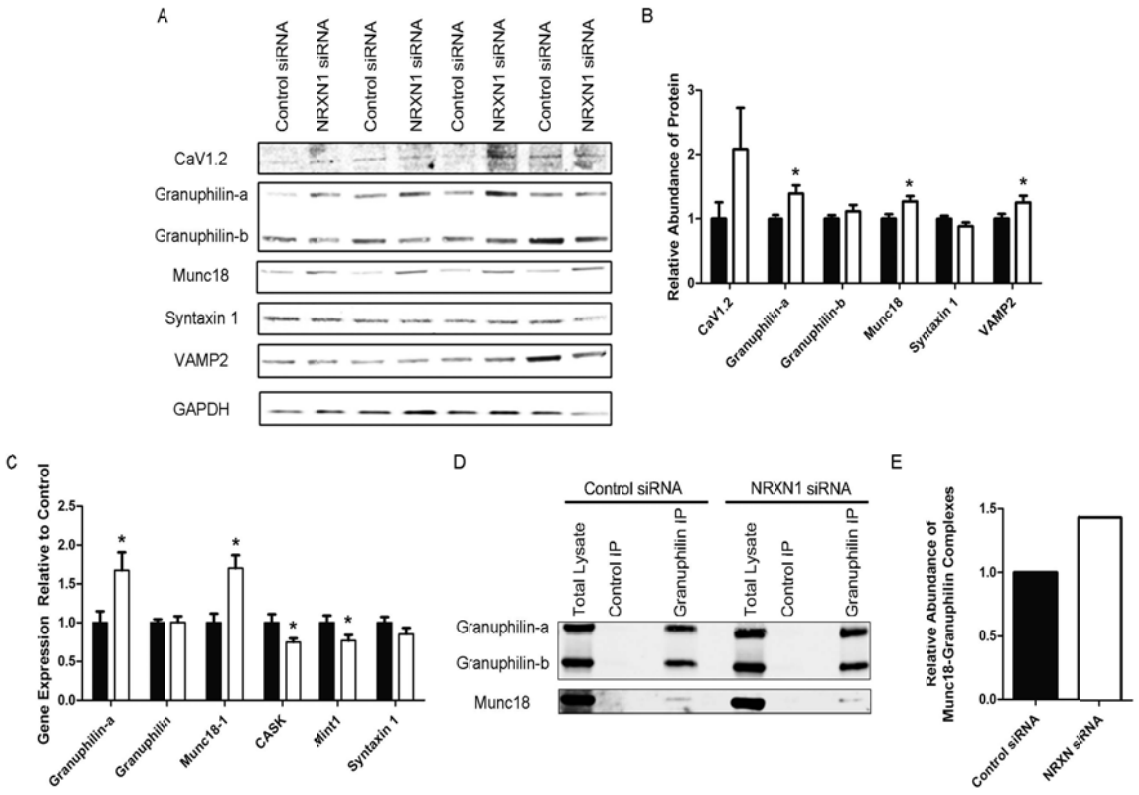

Figure 3.3. Expression profile and protein-protein interactions of insulin granule exocytotic machinery are altered by siRNA-mediated knockdown of NRXN1 in INS-1E cells. INS-1E cells were transfected for 72 h with a pool of either non-targeting control or NRXN1 siRNAs. A, Equal protein amounts of total lysate from siRNA-treated cells were immunoblotted for the indicated proteins. Lysates from four independent culture wells are shown for both control and NRXN siRNA. B, Intensity of bands for control (black bars) and NRXN1(white bars) siRNA-treated cells was normalized to the GAPDH loading-control band and quantified for three independent experiments. C, Gene expression in the siRNA-treated cells was determined by qPCR and normalized to 18S RNA. D, Anti-granuphilin immunoprecipitates from control or NRXN1 siRNA-treated INS-1E cells were immunoblotted for the indicated proteins (Granuphilin IP) using isotype control immunoprecipitates as controls (Control IP). Total lysate is also represented. E, Intensity of bands for Munc18 from granuphilin IP lanes for control (black bars) and NRXN1 (white bars) siRNA-treated cells was normalized to the granuphilin IP band and quantified for the single experiment. Western blots were repeated with similar results. Protein and gene expression data is represented as mean ± SEM from 12 samples. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to non-targeting siRNA transfected controls.

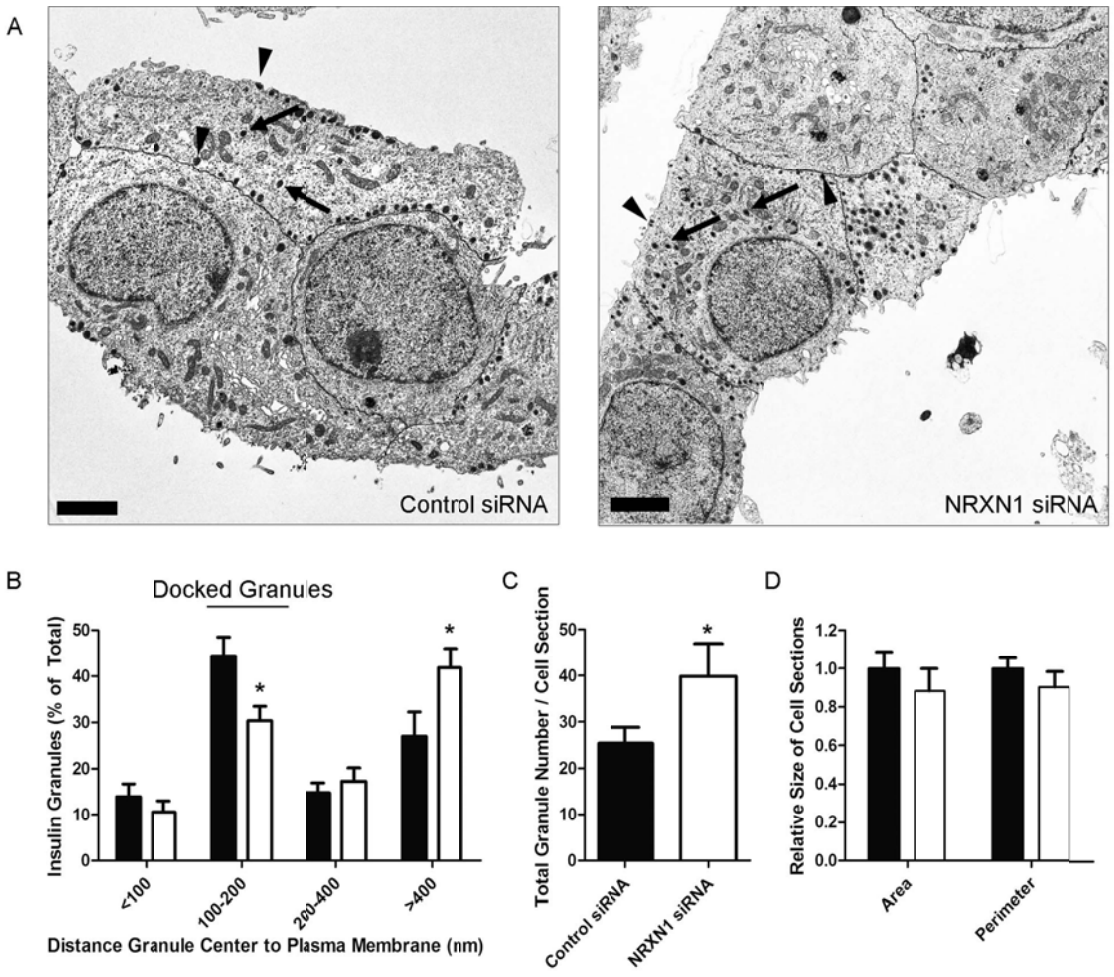

Figure 3.4. NRXN1 knockdown in INS-1E cells reduces the percentage of insulin granules docked at the plasma membrane. INS-1E cells were treated with NRXN1 or control siRNAs. Cells were fixed, pelleted, and sectioned for EM. A, Representative electron micrographs of control siRNA- and NRXN1 siRNA-treated cells are shown. Cell borders are traced in black. Scale bars indicate 2 μm. Arrows point to examples of undocked granules. Arrowheads point to examples of docked granules. B, In cells treated with control siRNA (black bars) and NRXN1 siRNA (white bars), granules were classified into four groups based on the distance from the center of each granule to the plasma membrane. Data is represented as the distribution of granules among the four groups. C, The average total granule number per cell cross section after control or NRXN1 siRNA treatment is depicted. D, The average area and perimeter of cell cross sections relative to control siRNA-treated cells after control (black bars) or NRXN1 (white bars) siRNA treatment is depicted. All data are represented as mean ± SEM from 20 cells. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the comparison of control siRNA and NRXN1 siRNA-transfected cells.

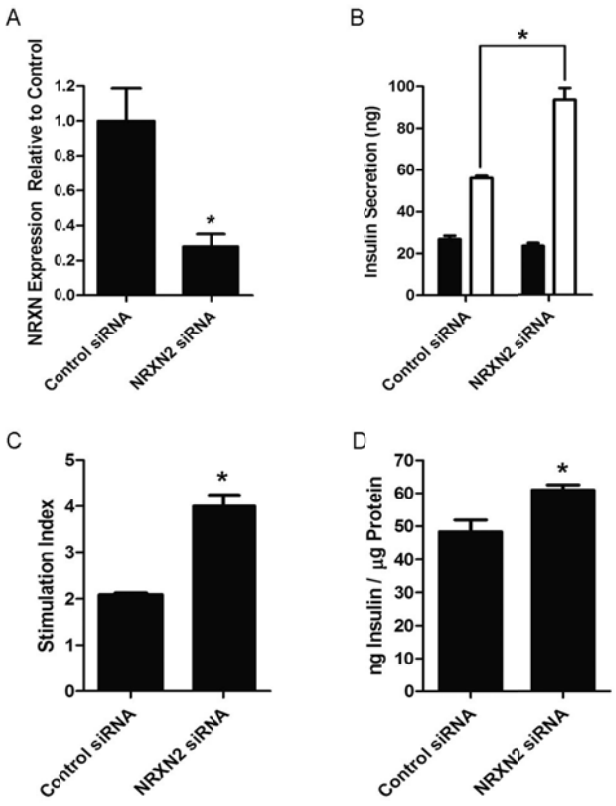

Figure 3.5. siRNA-mediated knockdown of NRXN2 in INS-1E cells increases glucose-stimulated insulin secretion. INS-1E cells were transfected for 48 h with a pool of either non-targeting control or NRXN2 siRNAs. A, mRNA was isolated and NRXN2β gene expression was determined using RT-qPCR, normalized to 18S RNA. B, Cells treated with control or NRXN2 siRNA were incubated for 1 h in Krebs-Ringer solution containing either 2.75 mM (black bars) or 16.7 mM glucose (white bars). C, The glucose stimulation index as defined by the ratio of insulin secretion (% of total) at 16.7 mM to secretion at 2.75 mM glucose is represented. D, Total lysate from siRNA-treated cells was assayed for insulin content. All data are represented as mean ± SEM from three samples, and each experiment was repeated three times with similar results. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to non-targeting siRNA-transfected controls.

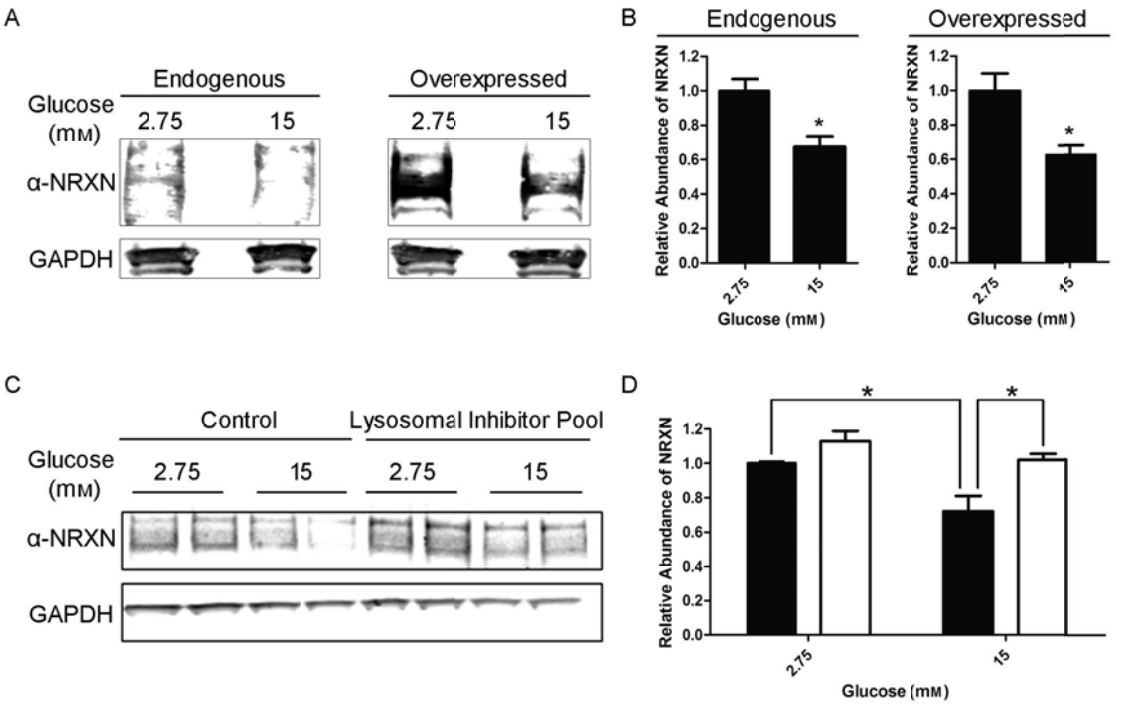

Figure 3.6. Glucose stimulation causes a decrease in NRXN protein content. A, INS-1E cells were either untransfected (Endogenous, left panel) or transfected for 48 h with a NRXN1α overexpression construct (Overexpressed, right panel). Cells were treated for 1 h with a Krebs-Ringer solution containing 2.75 mM (low) or 15 mM (high) glucose. Total cell lysates were immunoblotted for NRXN and the loading control GAPDH. Representative Western blot images show lysate pooled from six different untransfected (Endogenous) and NRXN1α-transfected (Overexpressed) samples treated with low or high glucose concentration. B, Intensity of bands was normalized to the GAPDH loading-control band and expressed relative to low glucose samples. C, INS-1E cells were treated for 1 h with a pool of lysosomal inhibitors (Lysosomal Inhibitor Pool) or vehicle controls (Control). Cells were then treated for 1 h with inhibitors or controls and a Krebs-Ringer solution containing 2.75 mM (low) or 15 mM (high) glucose as in A. Total cell lysates were immunoblotted for NRXN and the loading control GAPDH. Representative Western blot images show lysates from two different wells of each condition. D, Intensity of bands for NRXN from control (black bars) and lysosomal inhibitor (white bars) treated cells was normalized to the GAPDH and quantified relative to control- and low glucose-treated samples. Western blots were repeated with similar results. Protein data in B is represented as mean ± SEM from seven experiments. Protein data in D is represented as mean ± SEM from four samples. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to controls.

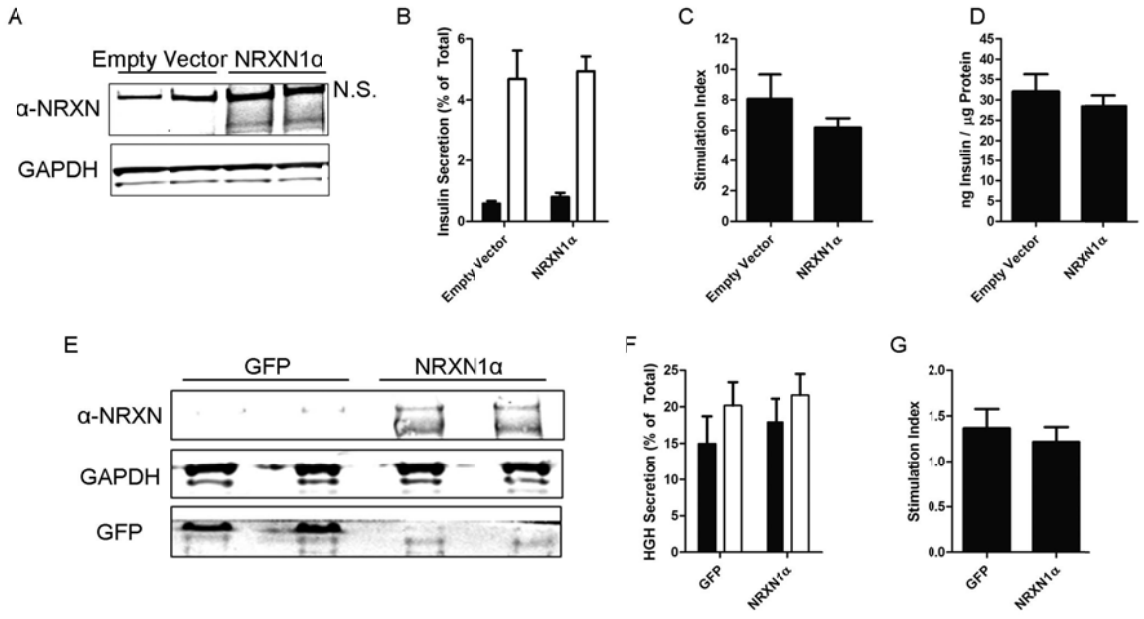

Figure 3.7. Overexpression of NRXN1α has no effect on insulin secretion. A, INS-1E cells were transfected for 48 h with an empty vector (Empty Vector, left panel) or a NRXN1α overexpression construct (NRXN1α, right panel). Total cell lysates were immunoblotted for NRXN and the loading control GAPDH. Representative Western blot images show lysates from two individual wells for each condition. N.S. indicates a non-specific band detected by the pan-NRXN antibody. B, Transfected cells were incubated for 1 h in Krebs-Ringer solution containing either 2.75 mM (black bars) or 16.7 mM glucose (white bars). Insulin in the conditioned media was determined by RIA. C, The glucose stimulation index as defined by the ratio of insulin secretion (% of total) at 16.7 mM to secretion at 2.75 mM glucose is represented. D, Total lysate from transfected cells incubated for 24h at 5 mM glucose was assayed for insulin by RIA. Insulin content normalized to total protein is depicted. E, INS-1E cells were transfected with a human growth hormone (HGH) construct in addition to GFP or NRXN1α overexpression constructs. Total cell lysates were immunoblotted for NRXN, GAPDH, and GFP. Representative Western blot images show lysates from two individual wells for each condition. F, Transfected cells were incubated for 1 h in Krebs-Ringer solution containing either 2.75 mM (black bars) or 15 mM glucose (white bars) as in B. HGH in the conditioned media was determined by ELISA. G, The glucose stimulation index as defined by the ratio of HGH secretion (% of total) at 15 mM to secretion at 2.75 mM glucose is represented. All data are represented as mean ± SEM from six samples, and each experiment was repeated two times with similar results. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to non-NRXN overexpressing controls.

CHAPTER 4:

NEUREXIN-1α CONTRIBUTES TO INSULIN-CONTAINING SECRETORY GRANULE DOCKING IN MOUSE β CELLS

A. Summary

Neurexins are a family of transmembrane, synaptic adhesion molecules that play a role in synaptic vesicle docking. We have previously shown that neurexins, like many other protein constituents of the neurotransmitter exocytotic machinery, are expressed in pancreatic β cells. We also demonstrated that neurexin-1α is high expressed in INS-1E β cells where it helps to mediate insulin granule docking and impairs insulin secretion. Here we demonstrate that loss of neurexin-1α expression in islets of neurexin-1α knockout mice reduces granule docking at the β-cell membrane and improves insulin secretion. The same phenomenon is observed in islets from granuphilin knockout mice. Perifusion of neurexin-1α knockout mouse islets revealed a significant increase in second-phase insulin secretion with a trend towards increased first-phase secretion. We conclude that neurexin-1α is a component of the β-cell secretory machinery and contributes to secretory granule docking, most likely through interactions with granuphilin. Neurexin-1α is the only transmembrane component of the docking machinery identified thus far. Our findings provide new insights into the mechanisms of insulin granule docking and exocytosis.

B. Introduction

We previously demonstrated that neurexins (NRXNs) in INS-1E β cells interact with both membrane-bound components of the secretory granule-docking machinery and with the granule-associated protein granuphilin. We also showed that NRXN1α is abundantly expressed in INS-1E β cells, inhibits insulin secretion, and contributes to the docking of secretory granules at the β-cell membrane.

Islets from granuphilin KO mice have impaired secretory granule docking and improved glucose-stimulated insulin secretion (146,153). It has been proposed that this is due to the "temporal constraint" of docking on secretory granule exocytosis (146). It thought that docking occurs via interactions of granuphilin on the granule with syntaxin 1 at the β-cell membrane (133). However, a recent report demonstrated that loss of syntaxin 1 in islets of syntaxin 1 KO mice had no effect on secretory granule docking (153). We previously demonstrated that NRXN1α interacts with granuphilin and syntaxin 1 in the β cell, so we hypothesized that NRXN1α may be the membrane-bound binding partner of granuphilin and therefore contribute to secretory granule docking in primary mammalian β cells. It is possible that NRXN on the β-cell membrane interacts with granuphilin on the granule to allow for secretory granule docking at the β-cell membrane.

Studies with double and triple α-NRXN knockout mice have focused on the role of NRXNs in neural development and have indicated that α-NRXNs in neurons are essential for the organization and stabilization of the presynaptic machinery (67,98,100). *In vitro* work suggests that NRXNs interact with the synaptic vesicle-associated protein rabphilin-3A via CASK (130) and contribute to synaptic vesicle docking (82,84). Granuphilin, which is expressed by β cells is a rabphilin-3 homologue implicated in

insulin granule docking (131,132). β cells also express CASK (9). Based on the similarities between neurotransmitter exocytosis and insulin secretion, and our previous observations about the role of NRXNs in INS-1E β cells, we hypothesized that islets from α-NRXN knockout mice would also have impaired secretory granule docking and improved glucose-stimulated insulin secretion.

In the present study we examined the role of NRXN1α in isolated mouse islets. Our data from NRXN1α KO mice confirms that NRXNs have a role in secretory granule docking in the β-cell. Like other proteins that contribute to the granuphilin-mediated docking of secretory granules at the β-cell membrane, NRXN1α inhibits insulin secretion (139,145) and loss of NRXN1α impairs secretory granule docking. Our findings provide new insights into the mechanisms of insulin granule exocytosis.

C. Experimental Procedures

Animals

NRXN1α KO mice were bred from previously described NRXN1α and 2α heterozygous knockout mice (67) purchased from Jackson Laboratory. Genotyping was performed by PCR using primer pairs described on Jackson's website and was confirmed by RT-PCR using RNA isolated from brain and islets as previously described (9). All primers were designed using Primer 3 (179) or PerlPrimer (180) software and have been described in Table 2.1. A different primer for NRXN1α with the following sequences was also used: Forward: ATTTCTCTGTGAAGGTGTCCA; Reverse: CAGAAGATGGAGAAGCTGAG.

Islets were isolated from age-matched KO and WT mice at ages 10-14 weeks as previously described (215). Islets were handpicked and cultured overnight in RPMI 1640 medium containing 8 mM glucose, 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 0.25 μg/ml amphoterecin B in a humidified 37^0C incubator with 5% CO_2 before use in experiments. All procedures were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Antibodies

The following antibodies were obtained commercially: Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes); donkey anti-guinea pig-Cy3 (Millipore); guinea pig anti-insulin (Dako); mouse anti-glucagon (Sigma Aldrich); and biotinylated goat anti-guinea pig IgG (Vector Laboratories).

Histology

Pancreas tissue was obtained from adult WT or NRXN1α KO mice, fixed for 24 h in Pen-Fix (Thermo Fisher Scientific) and embedded in paraffin. Pancreas tissue fixed in 4% paraformaldehyde in 0.1 M PBS from WT mice and mice lacking both NRXN1α and 2α (α-NRXN DKO) was a gift from Dr. Markus Missler (University of Munster). DKO and corresponding WT pancreases were embedded in OCT compound (Tissue-Tek) and frozen. Sections (6 μm) of mouse pancreases were prepared by the University of California, San Diego histology core. Deparaffinized sections were subjected to antigen retrieval by treating with a citrate buffer. H&E staining was performed using standard methods. Immunostaining with the Vectastain Elite ABC kit and NovaRED substrate (Vector Laboratories) was performed as previously described (174). Immunofluorescence was performed using 1-5% serum in PBS or PBS-T for blocking and antibody dilutions. Primary antibodies (4^{0}C overnight), secondary antibodies (room temperature for 1 h), and DAPI (room temperature for 5 min) were applied in a humidified chamber. Controls for immunostaining and immunofluorescence included tissue stained in parallel with secondary antibody alone. Images were captured using a Nikon Eclipse E800 microscope and SPOT RT SE camera at 20x. Islets were chosen at random, and morphometry was analyzed using Image-Pro (Media Cybernetics).

Insulin secretion and glucose stimulation

For static studies, islets were transferred into 2.75 mM glucose Krebs-Ringer bicarbonate buffer for 1 h and then 10 similar-sized islets (per well in 96-well plates) were transferred again into fresh Krebs-Ringer buffer containing 2.75 mM or 16.7 mM

glucose. Insulin in the cell lysate and media was measured using a rat insulin RIA (Millipore) or ultrasensitive rat insulin ELISA kit (Crystal Chem). Insulin secretion is represented as media content normalized to total intracellular content. Perifusion studies were performed by the University of Washington Diabetes and Endocrinology Research Center Islet Cell and Functional Analysis Core as previously described (216).

EM

Isolated islets in normal culture media were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for at least 4 h, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h and stained *en bloc* in 1% uranyl acetate for 1 h. Samples were dehydrated in ethanol, embedded in epoxy resin, sectioned at 60 to 70 nm and stained with uranyl acetate and lead nitrate. Grids were viewed using a transmission electron microscope (1200EX II, JEOL) and photographed using a digital camera (Gatan). Cells were chosen at random and morphometry was analyzed after deidentification of experimental groups. Distances were determined by drawing a straight line from the granule center to the nearest plasma membrane. All measurements were performed in ImageJ (195).

D. Results

Gene expression and body weight in NRXN1α KO mice

We previously demonstrated that knockdown of NRXN1α in INS-1E β cells improves glucose-stimulated insulin secretion. We hypothesized that NRXNs, in concert with granuphilin, serve to mediate secretory granule docking and constrain insulin secretion, inhibiting SNARE-mediated fusion of insulin granules with the plasma membrane (132,146). To further examine the role of NRXNs in insulin secretion we sought to evaluate insulin secretion from islets of NRXN KO mice. We chose to conduct *in vitro* experiments with islets isolated from NRXN1α KO mice and age-matched WT controls.

The NRXN2α KO allele was bred out of previously described NRXN1α and 2α heterozygous knockout mice (67). All mice were homozygous for the WT NRXN2 allele. Islets were isolated from NRXN1α KO and WT controls. RT-PCR was performed using RNA from the isolated islets to confirm the absence of NRXN1α transcript and the presence of both NRXN2α and 2β transcripts (Figure 4.1A). Bodyweight measurements were taken from the mice prior to islet isolation and revealed a slight but significant decrease in body weight of the NRXN1α KO male mice compared to WT controls, but no difference in body weight of female mice (Figure 4.1B).

Phenotypic differences in islets of α-NRXN KO mice

Immunohistochemistry of pancreas sections from NRXN1α mice revealed normal islet architecture despite loss of NRXN1α (Figure 4.2A). A slight increase in islet size in the NRXN1α KO mice was noted observationally, but a quantitative comparison was not

performed. We hypothesized that phenotypic differences in these mice might be moderated by the persistence of other α-NRXN transcripts (see Figure 4.1A). Therefore, we performed a more detailed analysis of β-cell size and area on pancreas sections mice lacking both NRXN1α and 2α (α-NRXN DKO). α-NRXN DKO mice also appeared to have normal islet architecture (Figure 4.2B), but quantitative measurements revealed that islet size as measured by insulin staining was larger in the DKO mice compared to controls. β-cell size as measured by nuclei per insulin area was not different between the groups.

Loss of NRXN1α increases insulin secretion in isolated mouse islets

We then measured insulin secretion from NRXN1α KO islets in static culture. Islets from KO mice had a 184% increase in insulin secretion at high glucose (Figure 4.3A) and a 143% increase in the stimulation index (Figure 4.3B). Basal secretion was not significantly changed. Because insulin content was increased NRXN1 siRNA-treated cells (see Figure 3.2A), we also measured total insulin in the islets from NRXN1α KO mice, but found insulin content was not different from WT controls.

Both first- and second-phase insulin secretion is increased in islets isolated from NRXN1α KO mice

Isolated islets were perifused to compare insulin secretion rates over time after high-glucose stimulation. Overall, the insulin secretion rate (ISR) of NRXN1α KO islets was higher than that of WT controls after glucose stimulation (Figure 4.4A). Although the increase in the area under the curve (AUC) of first-phase (0-5 min) secretion by KO

islets was not statistically significant, the AUC of second-phase secretion (10-40 min) by the KO islets was significantly (79%) higher than with WT islets (Figrure 4.4B). A separate, post-hoc analysis of insulin secretion during the peak of first phase secretion revealed that the ISR of KO islets was significantly greater than the ISR of WT islets (mean 40% increase, p=0.02) during the interval consisting of the 3 to 5 min time points.

Islets from NRXN1α KO mice have reduced secretory granule docking

We next sought to determine if, as was the case in siRNA-treated INS-1E cells, docking is reduced in NRXN1α KO mouse islets. Using EM, we examined the distribution of secretory granules in islet cells from WT and NRXN1α KO mice (Figure 4.5A). Given their average diameter of ~350 nm, secretory granules whose centers reside within 100-200 nm of the β-cell membrane are considered docked (22,132,144). We categorized granules into four groups based on their distance from the plasma membrane. Analysis of granule distribution by EM revealed that, compared to WT islets, KO islets had 39% fewer granules in the 100-200 nm distance category (Figure 4.5B). Consistent with our previous findings of greater insulin content (see Figure 3.2A) and granule number after NRXN1 knockdown in INS-1E cells (see Figure 3.4C), NRXN1α KO islets also had a 46% increase in insulin granule number per cell cross section compared to WT controls (Figure 4.5C). To confirm that the increased granule number was not related to alterations in β-cell size, we also measured the area and perimeter of cell cross sections. Unlike NRXN1 siRNA-treated cells where β-cell size was not different from control (see Figure 3.4D), analysis of β-cell area and perimeter revealed that both were increased in islets from NRXN1α KO mice compared to WT controls (Figure 4.5D). As a result, we

reevaluated insulin granule number normalized to β-cell area, but found that insulin granule number was still 23% greater in the islets from NRXN1α KO mice compared to WT controls (Figure 4.5E).

E. Discussion

The present study supports a role for NRXN1 in insulin secretion and specifically in insulin granule docking at the β-cell membrane. Consistent with our prior study of NRXN1α in INS-1E β-cells, we found that loss of NRXN1α increases glucose-stimulated insulin secretion and impairs secretory granule docking in isolated mouse islets.

We chose the NRXN1α KO mouse model to study the role of NRXNs in primary mammalian β cells for several reasons. First, we previously demonstrated that NRXN1α was the most abundant NRXN transcript in mouse islets (see Figure 2.3C). Second, siRNA experiments in INS-1E β cells in our prior study indicated a role for NRXN1α in insulin secretion (see Figure 3.1) and docking (see Figure 3.4) despite the presence of other NRXN transcripts with overlapping function (see Figure 3.5). Finally, of the readily available mouse models, a single α-NRXN KO was the only breeding strain that consistently reached maturity without strong developmental impairment (67). Triple α-NRXN KO mice die within 24 h of birth and mice lacking both α- and β-NRXNs exhibit major neurological impairment, reduced bodyweight, and a high degree of perinatal lethality (Jackson Laboratory website).

Ideally we would have performed *in vivo* insulin secretion analysis, on the NRXN1α KO mice. However, even though these mice reach maturity, they present an altered neural phenotype (217) and males have reduced bodyweight (Figure 4.1B), which could impair our ability to differentiate secretory and whole-body metabolic effects. At the time of this study's initiation no β-cell specific, cre-expressing mice without leaky neuronal cre expression and activity were readily available to generate a β-cell specific,

NRXN KO mouse (218). Therefore, we chose to conduct *in vitro* experiments with islets isolated from NRXN1α KO mice and age-matched WT controls.

Islets isolated from NRXN1α KO maintained expression of other NRXN isoforms despite the loss of full length NRXN1α. We demonstrated that at least NRXN2α and 2β are also present (Figure 4.1A). It should be noted that RT-PCR was conducted strictly to determine the presence or absence of NRXN transcripts and was not quantitative. Primer efficiency and cycle number were different for the different isoform reactions. Therefore, the band intensity is not reflective of the absolute or relative transcript quantities. However the presence of NRXN2 is likely attenuating the effects of NRXN1α KO as we previously demonstrated that NRXN2 has an overlapping function (see Figure 3.5). This would explain why the neural phenotype of multiple α-NRXN KO mice is more severe than single α-NRXN KOs (67).

Even though NRXN1α KO mice have a milder phenotype than their α-NRXN DKO counterparts (67,217), we still found that at 10-weeks of age when we isolated islets and conducted static secretion experiments, male mice had a slightly reduced bodyweight. It is well known that alterations in body mass and glycaemia can induced changes in the secretory capacity of islets even when controlled for size and stimulated *ex vivo* (219). However, bodyweight changes alone are not sufficient to alter insulin secretion (220) and ß cells that have been exposed to high glucose and are allowed to recover under normal glucose conditions have insulin content partially restored (221). We do not believe that bodyweight differences are the sole explanation for insulin secretory differences from islets of NRXN1α KO mice. First, weight loss was only observed in NRXN1α KO males. We evaluated insulin secretion from islets of both male and female

mice, and insulin secretion was increased in both sexes. Second, we picked similar size islets for our static incubations and allowed them to recover overnight in culture. Third, we did not observe any obvious differences in glycaemia in the NRXN1α KO mice (data not shown), nor has this been reported in other studies of NRXN1α KO (217). And finally, we previously demonstrated that the same increase in insulin secretion is observed after transient knockdown of NRXN1α *in vitro* in INS-1E cells (see Figure 3.1).

Another phenotypic difference shared by NRXN1α siRNA-treated INS-1E cells and islets from NRXN1α KO mice was an increase in insulin content. Although differences in content were not observed in islets from the static cultures – likely because only 10 similar size islets were selected per well, and small differences may have been difficult to observe under those conditions—EM of KO islets indicated an increase in insulin granule number. Electron micrographs also show increased numbers of secretory granules in NRXN1 siRNA-treated cells. The increase in insulin levels was most likely not due to increased insulin secretion because we previously showed that insulin content increased in INS-1E cells treated with NRXN1 siRNA even at non-stimulatory glucose levels at which NRXN1 silencing did not increase insulin secretion (see Figure 3.2). The mechanism, then, whereby NRXNs influence β-cell insulin content remains to be uncovered. In NRXN1 siRNA-treated INS-1E cells and islets from NRXN1α KO mice, the increases in insulin secretion cannot be explained by increased insulin content since insulin secretion in our static culture studies was normalized to total cellular insulin content and also the magnitudes of the increases in secretion resulting from loss of NRXN1α exceeded those of the increases in content.

Instead, we believe that the increases in insulin secretion are due to the loss of secretory granule docking. It is thought that insulin granule docking occurs via interactions of granuphilin with syntaxin 1 beneath the β-cell plasma membrane (133). However, loss of syntaxin 1 had no effect on secretory granule docking (153), suggesting the presence of an alternative submembrane receptor for granuphilin. The findings presented here suggest that NRXN1α may be the protein that anchors the docking complex. First, NRXN1α associates, either directly or indirectly, with granuphilin (see Figure 2.5). Second, our EM results demonstrate that NRXN1 knockdown in INS-E cells and KO in mouse islets impairs secretory granule docking. Third, given the similarities between regulated secretion in neurons and β cells, the previously demonstrated role for NRXN in synaptic vesicle docking in the synapse suggests a similar role for NRXN in β cells (82,84,130). In neurons, the interaction between NRXN and the granuphilin-related protein rabphilin-3 is mediated by CASK (130), a known NRXN binding partner (79,81,183). We also previously demonstrated that NRXN in β-cells interacts with CASK. As a result, our findings suggest that secretory granule docking in β cells may be mediated by the interaction of granuphilin with NRXN1, either directly or indirectly (perhaps via CASK).

However, it is of note that secretory granule docking was not completely abolished in INS-1E cells treated with NRXN1 siRNA or in islets from NRXN1α KO mice. Possible explanations for the persistence of docked granules in these studies include incomplete NRXN1 gene silencing in the siRNA-treated cells and functional redundancy with NRXN2β or other NRXN isoforms (see Figures 3.5 and 4.1). Alternatively, the remaining granules that appear to be docked by morphological criteria

may be stochastically located close to the plasma membrane without being molecularly docked, as has been proposed to account for the approximately 30% of granules that remain in the 100-200 nm distance category in granuphilin null mice (132).

As in the case with granuphilin (141), islets from NRXN1α KO mice had increased glucose stimulated insulin secretion but no differences in basal insulin secretion. Our previous findings also demonstrated that knockdown of NRXN1 did not affect constitutive secretion (see Figure 3.1E), indicating that the knockdown did not cause a general impairment of β-cell secretory function. Results from perifusion of KO and control mouse islets revealed a significant effect on second-phase secretion as well as evidence of increased first-phase secretion. These results resemble those observed with granuphilin KO mice, in which both first and second phase secretion were increased (153). It is possible that the continued expression of other NRXN isoforms (see Figures 3.5 and 4.1) moderated the effect observed here of NRXN1α KO on first (and second) phase secretion.

The persistence of the secretory and content phenotypic difference demonstrate that these are likely related to functional contributions of NRXN1α to secretion and docking. Another phenotypic difference that was observed in islets of the NRXN KO mice but not in NRXN1-siRNA treated INS-1E cells was a difference in insulin area (islet size) and β-cell size. Larger islets contain more β-cells and therefore secrete more insulin than smaller islets (220), and similarly larger β cells generally contain more insulin and also have a greater secretory capacity (222).

Increases in β cell size and area were not observed under all conditions, for instance immunofluorescent analysis of α-NRXN DKO mice indicated an increase in β-

cell mass but not size (Figure 4.2) whereas EM analysis of NRXN1α KO islets indicated an increase in β-cell size. Furthermore, unpublished observations reported in a study of α-NRXN DKO (67) indicated that β cells of α-NRXN DKO mice were actually smaller than their WT counterparts. Quantitative immunofluorescent analysis of β-cell mass and size was not performed in NRXN1α KO islets or NRXN1-siRNA treated INS-1E cells. The differences in these findings could be explained by the ability of each respective method to accurately detect small changes in β-cell size and mass as well as divergent functions of different α-NRXN genes.

A possible explanation for the increase in β-cell mass and size in NRXN KO mice is feedback of the increased secretory capacity observed in the NRXN1α KO islets on cell and islet size. In fact the unpublished observations about the α-NRXN DKO (67) that suggested β cells in these mice were smaller indicated that these mice had impaired secretory capacity. The opposite would be true in NRXN1α KO mice. Dynamic adaptation of β-cell size has been observed in many cases such as an increase in β-cell size during pregnancy (223) and in persistent hyperglycemia (224). It has been proposed that the modulation of cell size in these cases is related to activity of a serine/threonine kinase, liver kinase B1 (222). However, since we did not notice a change in β-cell size after NRXN1 knockdown in siRNA-treated INS-1E cells, and it seems more likely that alterations in β-cell mass and size are due to developmental changes in the islets of NRXN1α KO mice. NRXNs help initiate and guide synaptic differentiation (42), so it is also possible that NRXN1, in addition to its role in exocytosis, contributes to the development and functional maturation of the β cell.

NRXN1 is different from most established mediators of docking and exocytosis in that it is a transmembrane protein with a large extracellular domain that binds to multiple partners. Some of these binding partners, such as neuroligins and α-dystroglycan, are expressed on the β-cell surface and perhaps also by the islet vasculature (9,189,225,226). NRXN1 thus provides a direct link between the exocytotic machinery and the β-cell surface and could influence the localization of the exocytotic microdomains beneath the plasma membrane (18). It is also possible that trans interactions of NRXN with extracellular binding partners contribute to the functional architecture of the islet by juxtaposing beta cells with endothelial cells to promote cell β-cell development, secretory function (227) and polarized orientation (228) as well as maintaining cell-cell contacts important for signaling and regulated secretion (229). Further work is needed to determine whether NRXN1α has a role in β-cell maturation and whether its extracellular interactions determine the docking sites of insulin granules or contribute to the functional architecture of the β-cell.

In summary, our data demonstrate that NRXN1 has a functional role in primary mammalian β cells as a component of the machinery for regulated exocytosis and, more specifically, is essential for insulin granule docking. NRXN1α interacts with constituents of the submembrane exocytotic and docking protein machinery and with the secretory granule-associated protein granuphilin. KO of NRXN1 in mouse islets increases glucose-stimulated insulin secretion, most likely by decreasing secretory-granule docking. This increase in insulin secretion after NRXN knockdown provides further evidence that docking acts, as has been proposed by others, as a “temporal constraint” on insulin secretion (146).

F. Acknowledgements

Chapter 4, in part, is a reprint of the material as it appears in Mosedale, M., Egodage, S., Calma, R.C., Chi, N.W., Chessler, S.D. (2012) Neurexin-1α contributes to insulin-containing secretory granule docking. *J Biol. Chem*. 287(9): 6350-61. The dissertation author was the primary investigator in the development and execution of the study, and the principal author of this paper. I thank Charlie Zhang and Shokufeh Nourollahi for help with the immunofluorescence of the DKO mice and Drew Couron and Ian Sweet at University of Washington for their help with the perifusion experiments.

G. Figures

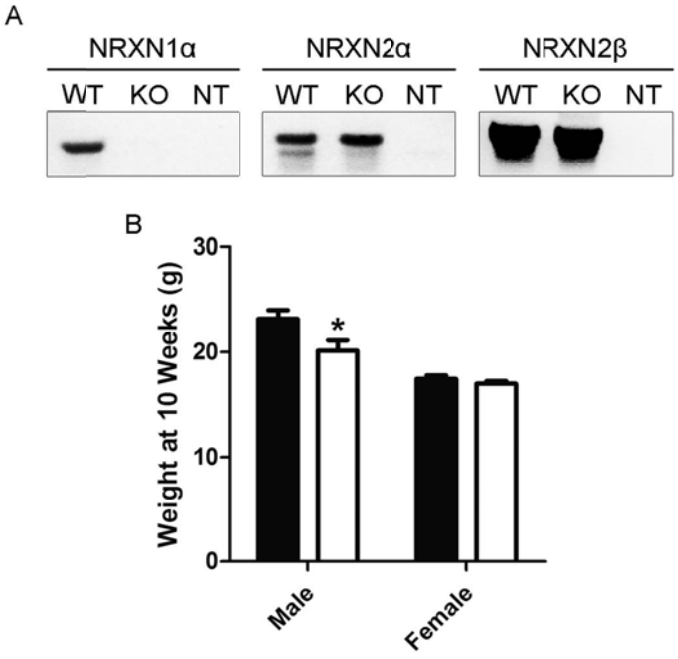

Figure 4.1. NRXN1α KO mice express NRXN2α and 2β, but have reduced body weight. A, RNA was isolated from WT and NRXN1α KO mouse islets. NRXN gene expression was determined using RT-PCR. No-template (NT) controls were assayed in parallel. B, Weights of WT (black bars) and NRXN1α KO (white bars) mice were recorded at 10 weeks of age. Data is represented as mean ± SEM from 6-12 mice per group. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to WT controls.

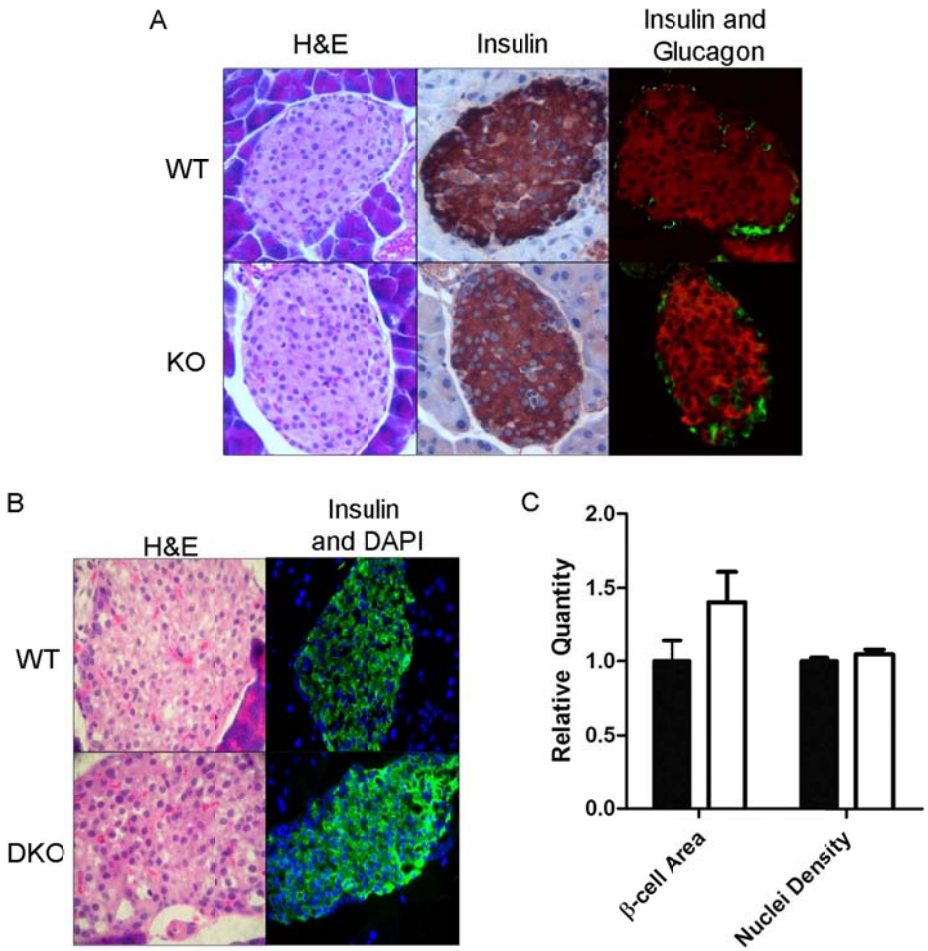

Figure 4.2. Islets from NRXN1α KO and NRXN1α/2α DKO mice have normal morphology. A, H&E (left panels), immunostaining for insulin (center panels), and immunofluorescence for insulin (red) and glucagon (green) (right panels) were performed on pancreas sections from WT (top panels) and NRXN1α KO (bottom panels) mice. B, H&E (left panels) and immunofluorescence for insulin (green) and DAPI (green) (right panels) were performed on pancreas sections from WT (top panels) and NRXN1α/2α DKO (bottom panels) mice. C, Insulin and DAPI staining of WT (white bars) DKO (black bars) mice was used to quantify β-cell area and nuclei density (number of nuclei per insulin area) relative to WT controls. Data is represented as mean ± SEM from 40-42 islets total from four mice per group. Statistical significance was determined using a Student's t-test.

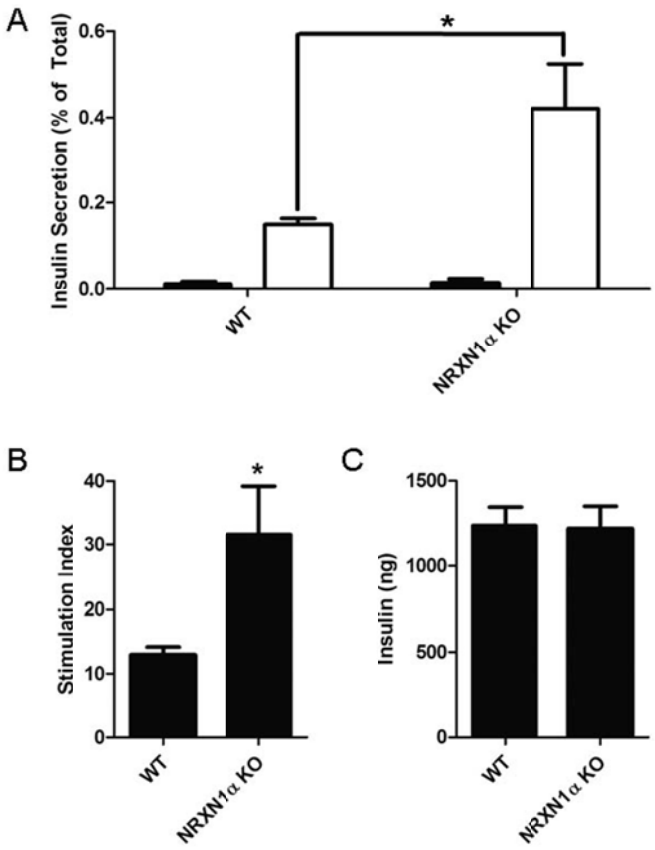

Figure 4.3. Islets from NRXN1α KO mice have increased glucose-stimulated insulin secretion. Islets were isolated from WT or NRXN1α KO mice and allowed to recover overnight in culture. A, Static cultures of islets were incubated for 1 h in Krebs-Ringer solution containing either 2.75 mM (black bars) or 16.7 mM glucose (white bars). Insulin in the conditioned media was determined by ELISA. B, The glucose stimulation index as defined by the ratio of insulin secretion (% of total) at 16.7 mM to secretion at 2.75 mM glucose is represented. C, Total islet lysate was assayed for insulin content. Data are represented as mean ± SEM from three samples, and each experiment was repeated three times with similar results. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the comparison of secretion from WT and NRXN1α KO islets.

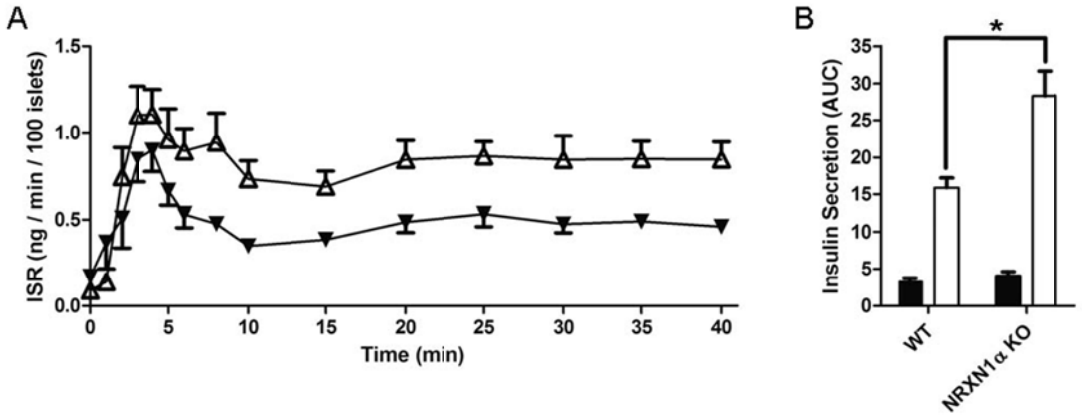

Figure 4.4. Second phase insulin secretion is increased in islets from NRXN1α KO mice. Islets were isolated from WT or NRXN1α KO mice and allowed to recover overnight in culture. A, Islets were perifused in Krebs-Ringer solution containing 3 mM glucose and then switched at time 0 to 20 mM glucose in Krebs-Ringer solution for 40 min. Insulin secretion rate (ISR) from WT (black triangles) and NRXN1α KO islets (white triangles) was measured every minute for the first 6 min, every 2 min until minute 10 and then every 5 min until minute 40. B, Area under the curve (AUC) was calculated for the first 5 min to determine first-phase insulin secretion (black bars) and then from minute 10 to 40 to determine second-phase insulin secretion (white bars). Data is represented as mean ± SEM from six islet preparations of each type. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the comparison of secretion from WT and NRXN1α KO islets.

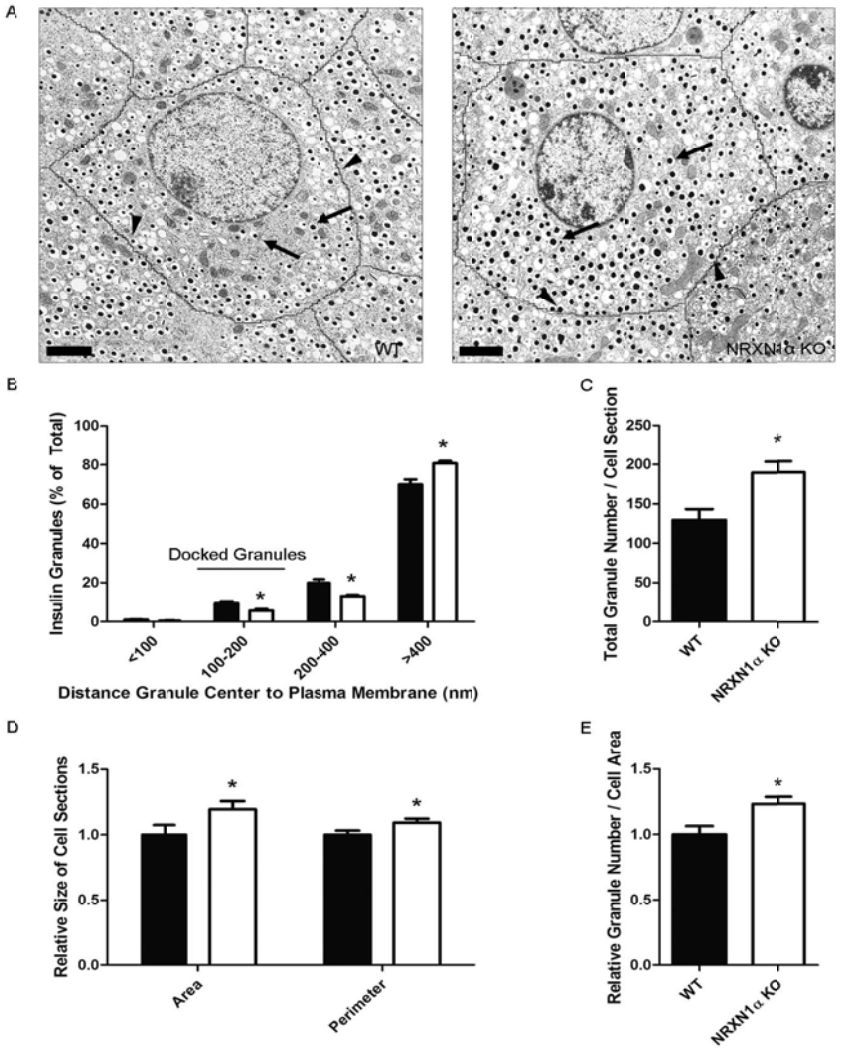

Figure 4.5. Islets from NRXN1α KO mice have a reduced percentage of insulin granules docked at the plasma membrane. Islets isolated from WT or NRXN1α KO mice were allowed to recover overnight in culture. The next day, islets were fixed, pelleted, and sectioned for EM. A, Representative electron micrographs of WT and NRXN1α KO islets are shown. Cell borders are traced in black. Scale bars indicate 2 μm. Arrows point to examples of undocked granules. Arrowheads point to examples of docked granules. B, The distance from the center of each granule to the plasma membrane was measured for WT (black bars) and KO (white bars) cells and used to assign granules to one of four distance groups. Data is represented as the distribution of granules among the four groups. C, The average total granule number per cell cross section is depicted. D, The average area and perimeter of cell cross sections relative to control siRNA-treated cells after control (black bars) or NRXN1(white bars) siRNA treatment is depicted. E, The average total granule number per cell area relative to WT controls is depicted. All data are represented as mean ± SEM from 20 cells. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the comparison of cells from WT and NRXN1α KO islets.

CHAPTER 5:

THE POSSIBLE CONTRIBUTION OF NEUREXIN DYSFUNTION TO THE DEVELOPMENT OF DIABETES MELLITUS

A. Summary

Neurexin is a neuronal presynaptic protein also found within pancreatic islet β cells. We previously demonstrated that neurexin is a critical component of the insulin secretory granule docking and exocytotic machinery β cells. A key contributor to the pathogenesis of type 2 diabetes is β cell failure – including dedifferentiation, impaired secretory capacity, and eventually loss of β-cell mass. Models of diabetes have helped demonstrate that the glucotoxicity, lipotoxicity and inflammation that occur in diabetes leads to decreased expression and function of exocytotic proteins that, in neurons and β cells, interact directly or indirectly with neurexins. Therefore, it is possible that loss of exocytotic function in type 2 diabetes may be secondary to defects neurexins themselves. The objective of this study was to understand how neurexin expression is altered in models of type 2 diabetes. In this study we demonstrate that NRXN1α mRNA expression in INS-1E β cells and isolated mouse islets is not altered by short term glucolipotoxicity. Furthermore, islets from ob/ob obese mice have normal levels of NRXN1α mRNA. However, we cannot rule out the possibility that NRXN dysfunction plays a role in the development of diabetes. Further work is needed to understand how NRXN protein levels and function are altered in disease states and whether or not NRXN could be a therapeutic target for the treatment of diabetes.

B. Introduction

The prevalence of type 2 diabetes is increasing at an alarming rate. A key contributor to the pathogenesis of this disease is β cell failure – including dedifferentiation, impaired secretory capacity, and eventually loss of β-cell mass (154-158). As a result, preservation of β-cell function is now a critical target in the treatment of type 2 diabetes (4-6). The development of new therapies to prevent β-cell failure and restore secretory capacity will require a greater understanding of both the mechanisms involved in the insulin exocytosis and the pathways that become affected in the pathogenesis of this disease.

We have previously shown that a family of synaptic adhesion molecules called neurexins (NRXNs) are a critical component of the insulin exocytotic and docking machinery. The mechanisms responsible for loss of β-cell function are likely to be multifactorial, but models of diabetes, including hyperglycemia, have helped demonstrate that the glucotoxicity, lipotoxicity and inflammation that occur in diabetes lead to decreased expression and function of exocytotic proteins that, in neurons, interact directly or indirectly with NRXNs (159-162). Therefore, it is possible that loss of exocytotic function in type 2 diabetes may be secondary to defects NRXNs themselves.

Some evidence of NRXN dysfunction in diabetes already exists as the α-NRXN double KO mouse has been reported to exhibit hyperglycemia and hypomorph β cells with impaired secretory capacity (unpublished observations in (67)). Furthermore, NRXN mutations are linked to cognitive diseases such as autism (61,114,230) and schizophrenia (103,104,231), and it has been shown that independent of pharmacological intervention,

the prevalence of diabetes in people with cognitive disease is higher than the prevalence of diabetes in the general population (164-169).

As a result of the role of NRXNs in insulin secretion and the clear links between exocytotic dysfunction and diabetes, developing a better understanding of NRXN dysfunction in the diseased β cell may help provide a novel candidate therapeutic target for the treatment of type 2 diabetes. The objective of this study was to determine the role of NRXN dysfunction (focusing on aberrantly expressed NRXN transcript levels) in the development of diabetes. Because we previously demonstrated that NRXNs are a component of the insulin granule docking machinery that impair insulin secretion, we hypothesized that NRXN expression would be increased in β cells when exposed to chronically elevated levels of glucose. The increased presence of NRXNs might contribute to the impairment of the late steps of insulin exocytosis characteristic of type 2 diabetes. In this study we determined that NRXN1α mRNA expression in INS-1E β cells or isolated mouse islets is not altered by short term glucolipotoxicity nor is it altered in islets from ob/ob obese mice. However, we cannot rule out the possibility that NRXN dysfunction still plays a role in the development of diabetes.

C. Experimental Procedures

Animals

10-week old C57BL/6 mice were purchased from Jackson Laboratory. Islets were isolated as previously described (215), hand-picked and cultured overnight in 8 mM glucose before use in experiments. All procedures were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Cell culture

INS-1E cells (175) were a gift from Dr. Pierre Maechler (Geneva University). They were cultured in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and 0.25 μg/ml amphoterecin B. Islets were cultured in the same medium except without 2-mercaptoethanol. All cells were maintained in a humidified 37^0C incubator with 5% CO_2.

Glucolipotoxicity

For glucolipotoxicity experiments, INS-1E cells were plated onto 24-well plates in media with 11.2 mM glucose and cultured to 50% confluency. Fresh media with 5 mM glucose was then applied for 24 h. Similar size islets were plated 10 per well in 96-well plates. At time 0, islets and INS-1E cells were cultured in either 5 mM glucose media with 0 mM palmitate and vehicle control or 25 mM glucose media with 0.5 mM palmitate for 72 h. Fresh media was applied every 24 h.

Real-time quantitative PCR (qPCR)

Total RNA was isolated and reverse transcribed as previously described (9). Islet RNA from adult male lean or ob/ob obese mice on a C57BL/6 background was provided by Dr. Mark Huising (Salk Institute). Gene-specific primers were used to perform qPCR with Power SYBR Green PCR Master Mix (Applied Biosystems), PerfeCTa SYBR Green FastMix (Quanta BioSciences) or 2x qPCR Master Mix (BioPioneer) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Each sample was analyzed in triplicate along with no-RT and no-template controls. Values were normalized to 18S RNA. NRXN primers were designed to avoid major splice variant regions (see Figure 2.1). All primers were designed using Primer 3 (179) or PerlPrimer (180) software and are described in supplemental Tables 2.1, 3.2, and 5.1.

D. Results

Glucolipotoxicity has no effect on NRXN gene expression in INS-1E β cells

β cells are exposed to chronically elevated levels of both glucose and free fatty acids in type 2 diabetes mellitus (232,233). As a result, glucolipotoxicity is commonly used as an *in vitro* model for this disease. β-cell lines and islets cultured in glucolipotoxic conditions present many of the same phenotypic changes as β cells from diabetic humans and rodents such as altered expression of exocytotic proteins (161,234). In *in vivo* and *in vitro* models of type 2 diabetes, β cells lose expression and function of exocytotic proteins that in neurons and β cells interact directly or indirectly with NRXNs (159-162,235). It is possible that loss of exocytotic function in type 2 diabetes may be secondary to defects NRXNs themselves.

To determine if NRXN1α expression is altered in type 2 diabetes, we cultured INS-1E cells for 72 h in media with high fat and glucose, and then evaluated changes in gene expression of several components of the exocytotic machinery. After 72 h, INS-1E cells cultured in high fat and glucose had a 79% decrease in insulin 2 mRNA compared to control, confirming the glucolipotoxic effect of the media (162,235-237). The expression of NRXN1α, granuphilin-a, SNAP25, and CaV1.2 (L-type voltage dependent Ca^{2+} channel) were not significantly changed.

Glucolipotoxicity has no effect on NRXN gene expression in isolated mouse islets

To confirm that this effect (or lack thereof) was not specific to INS-1E cells, we also evaluated NRXN1α and granuphilin-a expression in isolated mouse islets cultured under glucolipotoxic conditions. After 72 h, islets cultured in high fat and glucose had a

58% decrease beta-actin and an 18.3-fold increase in insulin 2 mRNA compared to control. No changes were observed in either NRXN1α or granuphilin-a expression.

NRXN gene expression in not altered in diabetic mouse islets

We also evaluated NRXN1α gene expression in islets isolated from the ob/ob obese mouse model (238,239). Islets were isolated between 12-14 weeks of age when ob/ob mice on the C57BL/6 background are typically hyperglycemic (Jackson Laboratory website). The ob/ob mice used for these experiments were reported to be hyperglycemic with frank diabetes (communication from Dr. Mark Huising).

Islets from ob/ob mice had no significant change beta-actin gene expression, but a 61% increase in the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) compared to control. Cyclin D1 (CycD1), which has been reported to increase in islets of ob/ob mice (240) was also elevated by 124%. Granuphilin-a mRNA was decreased in ob/ob islets by 70%, and no change was observed in NRXN1α. Insulin 2 mRNA displayed a trend toward increasing in islets from ob/ob mice, but was not significantly different from control.

E. Discussion

The present study examined the potential role of NRXN1α dysfunction in the development of diabetes. In *in vivo* and *in vitro* models of type 2 diabetes, β cells lose expression and function of exocytotic proteins that in neurons and β cells interact directly or indirectly with NRXNs (159-162,235). We have shown previously that loss of NRXN1α increases levels of granuphilin-a (see Figure 3.3), a protein that is known to be elevated in diabetic models (147). Therefore, we hypothesized that loss of exocytotic function in type 2 diabetes may be secondary to alterations in the expression level of NRXNs themselves.

We evaluated INS-1E cells and islets cultured under glucolipotoxic conditions and islets from the ob/ob obese mouse, but no changes in NRXN1α gene expression were observed. This suggests three possibilities: first, NRXN1α dysfunction does not occur or contribute to diabetes; second, NRXN1α dysfunction may occur, but is not indicated by alterations in gene expression, third, NRXN1α levels are affected in type 2 diabetes but our diabetic model systems were not appropriate to detect these alterations.

It is possible that NRXN1α dysfunction does not occur or contribute to diabetes. There are several lines of evidence to indicate that NRXN dysfunction is correlated with diabetes: the relationship between mutations in NRXNs, neurodevelopmental disorders, and the development of diabetes (61,103,104,114,164-169,230,231); the known alterations of exocytotic protein levels under glucolipotoxic conditions (161,234,241); and the previously reported hyperglycemia of the α-NRXN DKO mice (observation in (67)). However, levels of other exocytotic proteins like Munc18 that interact with NRXN1α (see Figure 2.5) and contribute to maintaining normal secretory capacity

(134,203) have not been reported to contribute to alterations in secretory capacity in type 2 diabetes (161).

Lack of NRXN mRNA alterations in our models of type 2 diabetes doesn't rule out the possibility that NRXN dysfunction still contributes to the development of this disease. It is possible that NRXN mRNA levels remain normal, but NRXN splicing, protein levels, and/or protein function are compromised. Alternative splicing of NRXN in neurons is known to induce functional differences in the protein (43,46). For instance, α-NRXNs and NRXNs specifically containing splice site 4 (+SS4) contribute to the formation of GABAergic synapses (91), and preferentially interact with neuroligin 2 (93). Interestingly, β cells have been described as GABAergic, since they expresses all of the machinery essential for GABA synthesis and secretion (33-35,174,192,193). β cells also only express NRXNs containing SS4 (9).

Depolarization of neurons has been reported to shift the splicing of NRXNs toward splice site 4 negative transcripts (-SS4) (90). Under glucolipotoxic conditions, the β cell is in a constant state of depolarization, and therefore the balance of NRXN1 +SS4 to NRXN1 -SS4 could be changing. Since our primers were specifically designed to avoid SS4, we would not have picked up on this shift. Such a shift would also alter NRXN protein function. For instance addition or subtraction of SS4 induces structural changes in NRXN1 that makes biding with certain neuroligins more or less favorable (93). The SS4- splice variant would be more likely to bind neuroligin 1 and induce the formation of a glutamatergic phenotype (57,64,94), which is not normally represented in β cells.

We may also have missed changes in NRXN1α protein levels, even if NRXN mRNA levels were unchanged. For many glucose-sensitive proteins, translation is the first process to become affected (197,198). Therefore, 72 h of elevated glucose may not have been a long enough glucose exposure to observe changes at the mRNA level of NRXN.

Another possibility is that NRXN1α levels are affected in type 2 diabetes but our diabetic model systems were not appropriate to detect these alterations. We chose a 72 h incubation in high glucose and fat because this method is commonly used and has been shown to induce changes in exocytotic protein levels similar to those observed in human diabetic pancreas (161,234). However, under glucolipotoxic conditions, levels of SNAP25 are reported to increase (161,162,234) and CaV1.2 are reported to decrease (161). We observed small increases and decreases in levels of SNAP25 and CaV1.2 mRNA respectively, but they were not significant. This indicates that 72 h may not be long enough to see changes in the level of mRNA in these molecules and therefore the same may be true for NRXN1α. A similar phenomenon occurred in islets from ob/ob mice, which are reported to have significantly more insulin (240) than WT controls. In our study, there was an increase in insulin mRNA, but again it was not statistically significant. This indicates that changes may have occurred in NRXN1α mRNA or protein that were not detectable in this particular model system at the point of study. Furthermore, it has been reported that islets from C57BL/6 are resistant to glucolipotoxicity *in vitro*. Even after one week of culture in 28 mM glucose, insulin content, mRNA, and secretion at high glucose were actually increased in these islets when compared to control (242).

Regardless of whether or not NRXN dysfunction occurs in diabetes and or whether that dysfunction promotes a loss of β-cell secretory capacity, NRXN still remains a promising therapeutic target for the treatment of this disease. We have previously demonstrated that NRXN1α is a component of the β-cell secretory machinery that contributes to secretory granule docking, most likely through interactions with granuphilin. Our work supports the notion that docking is inhibitory to insulin secretion. Additional work may enable the development of drugs that improve insulin secretion by inhibiting the docking function of NRXN. We also believe that outside of the central nervous system, NRXN is only expressed in pancreatic β cells. Furthermore, NRXN is a transmembrane protein with a large extracellular domain (40). As a result, it remains a promising drug target for enhancing insulin secretory capacity in type 2 diabetes.

NRXNs have been implicated in neuronal development where they are thought to help initiate and guide synaptic differentiation (42). In fact, culture of neurons with extracellular NRXN binding partners has been shown to induce the formation of the presynaptic density and arrangement of the exocytotic machinery (65,89). Therefore, it is also possible that NRXN1, in addition to its role in exocytosis, contributes to the development and functional maturation of the β cell. NRXNs are different from most established mediators of docking and exocytosis in that they are transmembrane proteins with extracellular domains that bind to multiple partners. Some of these binding partners, such as neuroligins and α-dystroglycan, are expressed on the β-cell surface and perhaps also by the islet vasculature (9,189,225,226). NRXN1 thus provides a direct link between the exocytotic machinery and the β-cell surface and could influence the localization of the exocytotic proteins beneath the plasma membrane (18). Trans interactions of NRXN

with extracellular binding partners could also contribute to the functional architecture of the islet by juxtaposing beta cells with endothelial cells to promote cell β-cell development, secretory function (227) and polarized orientation (228) as well as maintaining cell-cell contacts important for signaling and regulated secretion (229). Because of its potential role in β-cell and islet development, it is possible that NRXN itself or molecules that induce NRXN expression could be used to enhance the development and function of islets for transplantation and therefore be used to treat type 1 diabetes. Further work is needed to determine whether NRXN1α has a role in β-cell maturation and whether its extracellular interactions determine the docking sites of insulin granules or contribute to the functional architecture of the β-cell.

Finally, the development of new therapies to prevent β-cell loss and recover β-cell cell mass could be enhanced by the monitoring β-cell mass in both disease and therapy conditions. Because neurexins are β-cell specific and cell-surface proteins, they are promising targets for noninvasive β-cell *in vivo* imaging.

In summary, our data demonstrate that NRXN1α mRNA expression in INS-1E β cells or isolated mouse islets is not altered by short term glucolipotoxicity. Furthermore, islets from ob/ob obese mice have normal levels of NRXN1α mRNA. However, we cannot rule out the possibility that NRXN dysfunction plays a role in the development of diabetes. Further work is needed to understand how NRXN protein levels and function are altered in disease states and whether or not NRXN could be a therapeutic target for the treatment of diabetes.

F. Acknowledgements

I thank Christian Calma and Shokufeh Nourollahi for help with the glucolipotoxicity studies and Mark Huising at the Salk Institute for help with ob/ob mouse islet experiments.

G. Tables

Table 5.1. Sequences of rat and mouse primers used for qPCR.

Transcript	Forward Primer	Reverse Primer
Beta Actin	AGCCATGTACGTAGCCATCCA	TCTCCGGAGTCCATCACAATG
Rat CaV1.2	ACCCCCGCTCAAAGTCTGTAG	GTGGTTTGTTCTTGCTTTCGAA
Rat SNAP25	CTGGCTCTTGTTGATCACCATCT	TCTCAGCAATTTGGTTGTGCAT
Mouse Insulin 2	AGGTTATTGTTTCAACATGGCC	GGTCTAGTTGCAGTAGTTCTC
Mouse Granuphilin-a	GGATCAAAGAAGCCAAGAACC	CATTACTGATCCCAGTGCCA
Mouse HPRT	GTGATAGATCCATTCCTATGACTG	ACAAACGTGATTCAAATCCCTG
Mouse CycD1	CCTCTAAGATGAAGGAGACCA	AAATGAACTTCACATCTGTGGC

H. Figures

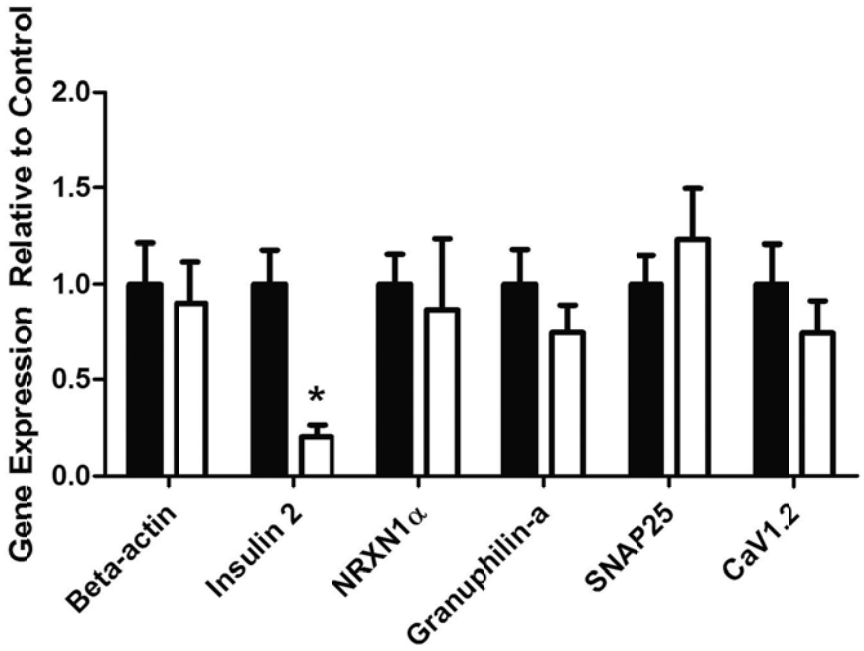

Figure 5.1. NRXN1α transcript expression in INS-1E cells is not affected by acute glucolipotoxicitiy. INS-1E cells were cultured for 72 h in media containing either 5 mM glucose alone (black bars) or 25 mM glucose and 0.5 mM palmitate (white bars). mRNA was isolated and gene expression in the treated cells was determined using RT-qPCR, normalized to 18S RNA. Data is represented as mean ± SEM from six samples, and experiments were repeated three times with similar results. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to 5 mM glucose controls.

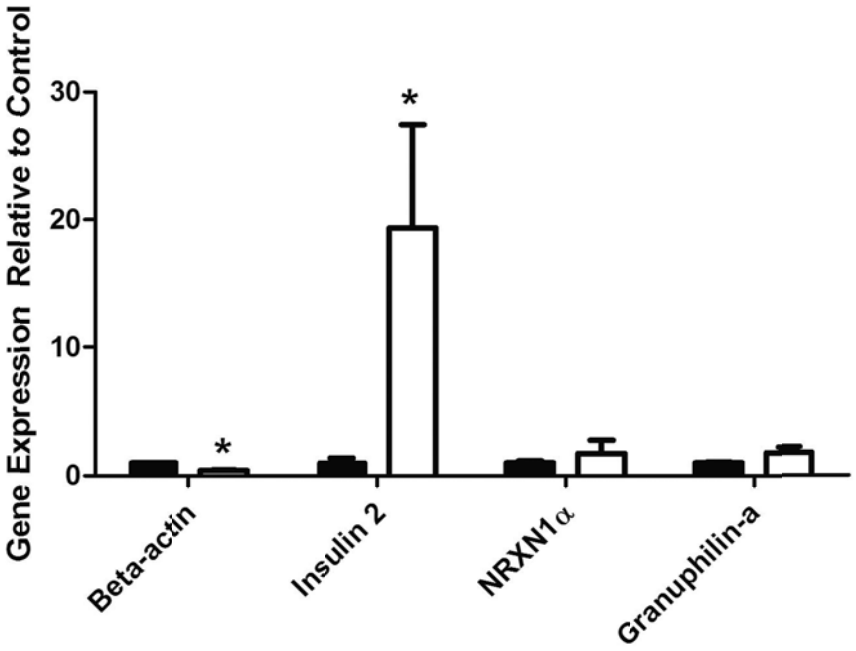

Figure 5.2. NRXN1α transcript expression in mouse islets is not affected by acute glucolipotoxicitiy. Islets were isolated from WT mice and allowed to recover overnight in culture. Islets were then cultured for 72 h in media containing either 5 mM glucose alone (black bars) or 25 mM glucose and 0.5 mM palmitate (white bars). mRNA was isolated and gene expression in the treated islets was determined using RT-qPCR, normalized to 18S RNA. Data is represented as mean ± SEM from three samples, and experiments were repeated three times with similar results. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to 5 mM glucose controls.

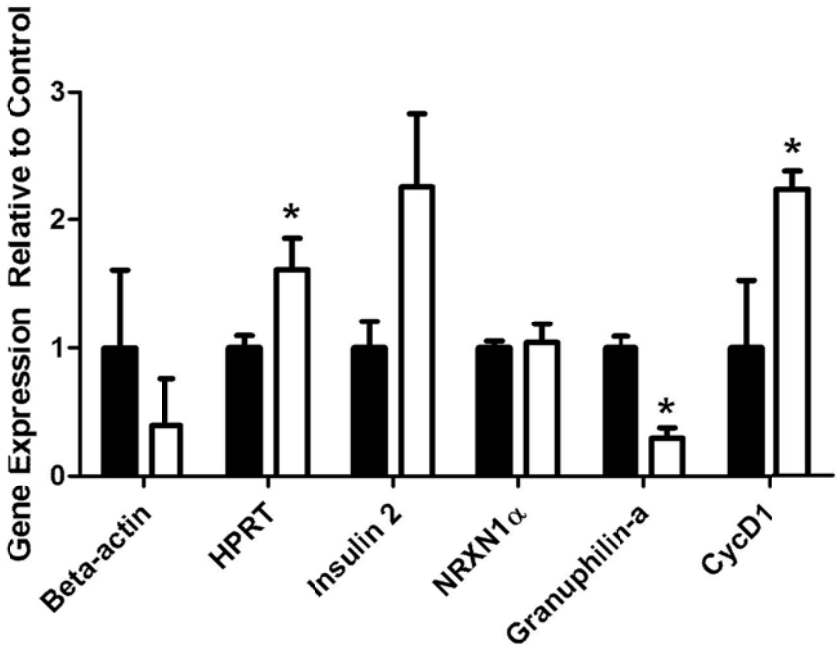

Figure 5.3. NRXN1α transcript expression is not altered in ob/ob diabetic mouse islets. Islets were isolated from WT (black bars) or ob/ob diabetic (white bars) mice and allowed to recover overnight in culture. mRNA was isolated and gene expression in the islets was determined using RT-qPCR, normalized to 18S RNA. Data is represented as mean ± SEM from three mice. Statistical significance was determined using a Student's t-test. *$p<0.05$ indicates the difference compared to WT glucose controls.

CHAPTER 6:

CONCLUSIONS

The results from this thesis project demonstrate that human, rat, and mouse islets express NRXN protein, but a more restricted pattern of NRXN transcripts than neurons. We developed a custom NRXN antibody that allowed us to demonstrate that NRXN1α in INS-1E β cells is expressed on the β-cell membrane and interacts with several components of the secretory granule docking machinery, including the secretory granule-associated protein granuphilin. Through EM and secretion studies we showed that siRNA knockdown of NRXN1α, like decreased expression of granuphilin, reduces granule docking at the β-cell membrane and improves glucose-stimulated (but not basal) insulin secretion. We confirmed these results in primary mammalian islets isolated from NRXN1α KO mice. We used perifusion of the isolated islets to demonstrate that loss of NRXN1α resulted in a significant increase in second-phase insulin secretion with a trend towards an increase in first-phase secretion. To better understand how β-cells compensate for the negative regulation of insulin secretion by neurexins, we demonstrated that upon glucose stimulation, NRXN1α protein levels decrease. This glucose-induced NRXN down regulation may enhance stimulated insulin secretion.

A. Implications

NRXNs impact on β cell biology

Our findings provide new insights into the mechanisms of insulin granule docking and exocytosis. We demonstrated that NRXN1α is a component of the β-cell secretory machinery. NRXN1 is a transmembrane protein with a large extracellular domain that binds to multiple partners (40). Some of these binding partners, such as neuroligins and α-dystroglycan, are expressed on the β-cell surface and perhaps also by the islet vasculature (9,189,225,226). NRXN thus provides a direct link between the exocytotic machinery and the β-cell surface and could influence the localization of the exocytotic microdomains beneath the plasma membrane (18) as well as the regulation of intracellular docking and exocytosis by extracellular signals.

Much like neurons maintain synaptic contact between axons and dendrites and alignment of presynaptic exocytotic machinery and postsynaptic receptors help to enhance rapid synaptic transmission (13,14), cell-to-cell contact (243,244) and specific localization of the exocytotic microdomains (18,19) are essential for normal glucose-stimulated insulin secretion from pancreatic β cells. A polarized orientation in β cells has also been shown to be important for signaling and regulated secretion (229). It is possible that NRXN via interactions with neuroligins, dystrogylcan, or yet uncovered extracellular binding partners on islet and vascular cells could help to organize the exocytotic microdomains, regulate β-cell orientation, and establish cell-cell connections that allow for normal β-cell function. The identification of NRXNs as components of the exocytotic machinery provides an additional area of investigation for uncovering both proteins essential for cellular organization and signaling.

We also demonstrated that NRXN1α contributes to secretory granule docking, most likely through interactions with granuphilin. Our work supports the notion that docking is inhibitory to insulin secretion. It is thought that insulin granule docking occurs via interactions of granuphilin with syntaxin 1 (133). However, loss of syntaxin 1 had no effect on secretory granule docking (153), suggesting the presence of an alternative submembrane receptor for granuphilin. Additional syntaxins were suggested to have overlapping function, and thus fill this role. However our work suggests that NRXNs may be the membrane-associated protein that anchors the docking complex at a subplasmalemmal site. Additional work will identify the direct and indirect components of the granuphilin-NRXN docking complex.

Therapeutic benefit of targeting β-cell NRXNs

With our findings, additional work may enable the development of drugs that improve insulin secretion (type 2 diabetes) or the development and function of islets for transplantation (type 1 diabetes). Furthermore, the development of new therapies to prevent β-cell loss and recover β-cell mass could be enhanced by the monitoring β-cell mass in both disease and therapy conditions. Because neurexins are β-cell specific and cell-surface proteins, they are promising targets for noninvasive β-cell *in vivo* imaging.

First, our work supports the notion that docking is inhibitory to insulin secretion. Additional work may enable the development of drugs that improve insulin secretion by inhibiting the docking function of NRXN. We also believe that outside of the central nervous system, NRXN is only expressed in pancreatic β cells. Furthermore, NRXN is a transmembrane protein with a large extracellular domain (40). As a result, it remains a

promising drug target for enhancing insulin secretory capacity in type 2 diabetes.

Next, NRXNs have been implicated in neuronal development and synaptogenesis where they are thought to help initiate and guide synaptic differentiation (42). *In vitro* culture of NRXNs extracellular binding partners with neurons has been shown to induce the formation of the presynaptic density and arrangement of the exocytotic machinery (65,89). Therefore, it is also possible that NRXN1, in addition to its role in exocytosis, contributes to the development and functional maturation of the β cell. As previously mentioned NRXNs are transmembrane proteins and some of their extracellular binding partners are expressed on the β-cell surface and perhaps also by the islet vasculature (9,189,225,226). Trans interactions of NRXN with extracellular binding partners could also contribute to the functional architecture of the islet by juxtaposing beta cells with endothelial cells to promote cell β-cell development, secretory function (227) and polarized orientation (228) as well as maintaining cell-cell contacts important for signaling and regulated secretion (229). Because of its potential role in β-cell and islet development, it is possible that NRXN itself or molecules that induce NRXN expression could be used to enhance the development and function of islets for transplantation and therefore be used to treat type 1 diabetes. Further work is needed to determine whether NRXN1α has a role in β-cell maturation and whether its extracellular interactions determine the docking sites of insulin granules or contribute to the functional architecture of the β-cell.

Finally, NRXNs in the β cell are also interesting in the context of identifying cell surface, β-cell specific markers for imaging healthy islet cell masses in humans. Type 1 and type 2 diabetes result in β-cell loss (1,2). As a result, preservation and expansion of

β-cell mass is a critical target in the treatment of diabetes. Monitoring of β-cell mass with noninvasive *in vivo* imaging could be used to build a greater understanding of β-cell fate in disease and therapy conditions and develop new therapies to prevent β-cell loss and recover β-cell mass (184,185). NRXNs are possible candidates for β-cell-specific membrane targets, which are critically needed in order for this technology to be successful (186,187). Our work indicates that NRXNs outside of the central nervous system are β-cell specific (9). Development of labeled ligands to target these molecules for imaging could pave the way for new islet replacement and regeneration therapies.

B. Remaining Questions

While the findings of this thesis project have enhanced our understanding of the mechanisms of insulin granule docking and exocytosis, and contributed to the efforts to understand β-cell development and treat β-cell dysfunction, many questions remain. Some of these questions are addressed in the following paragraphs.

Why does NRXN knockdown and KO increase insulin content?

NRXN1 knockdown caused an increase in insulin content and transcript levels. Electron micrographs also show increased numbers of secretory granules in NRXN1 siRNA-treated cells and islets from NRXN1 α KO mice. We mostly ruled out the possibility that the increase in insulin levels were due to a feedback loop of reduced NRXN1 levels increasing insulin secretion. Insulin levels were significantly increased in INS-1E cells treated with NRXN1 siRNA and incubated overnight in non-stimulatory glucose levels compared to NRXN1-siRNA treated cells incubated at high glucose and non-targeting-siRNA treated cells incubated at either glucose level. However, we cannot eliminate the possibility that the increase in glucose levels suppresses protein knockdown in the NRXN1 siRNA-treated cells or destabilizes NRXN1 protein in control cells, thus masking the effect of NRXN1 knockdown on insulin content. Either way, the mechanism whereby NRXNs influence β-cell insulin content remains to be uncovered.

Since we only observed NRXN staining on the β-cell, it is unlikely that NRXN itself is serving as a transcriptional regulator. It is possible however, that NRXN interacts with other proteins that regulate insulin expression and content. One such factor is neuroligin. Unpublished observations from our lab have demonstrated that neuroligin

expressing cells in culture with β cells can promote expression of insulin transcription factors and insulin itself through a NRXN-independent mechanism. Since NRXN is known to interact with neuroligin, perhaps NRXN competes with neuroligin's non-NRXN binding partner and when NRXN levels are reduced, neuroligin, now independent of NRXN, enhances insulin expression and therefore insulin content.

How does NRXN turnover enhance insulin secretion?

We also found that glucose stimulation causes a reduction in NRXN protein, possibly via lysosmal degradation. This is similar to other membrane proteins involved in glucose-stimulated insulin secretion such as the glucose transporter 2 (209). However, we did not investigate this regulation further to determine if NRXN degradation may enhance the insulin secretory response to elevated glucose levels. Since NRXN down-regulation enhances insulin secretion, this may be a mechanism whereby the β cell has enhanced control over glucose-stimulated insulin exocytosis.

Do NRXNs contribute to the exocytosis of SLMV?

NRXNs in neurons interact with rabphilin-3A via CASK (130) and contribute to synaptic vesicle docking (82,84). We demonstrated that in β cells, NRXN interacts with the rabphilin-3-like protein granuphilin, likely via CASK. Granuphilin associates with the surface of insulin granules, but not with SLMVs, and therefore differs from its rabphilin-3 counterpart, which associates with SLMVs in neurons (131,141). Because of NRXNs role in synaptic vesicle docking in neurons, the question still remains whether or not NRXNs in β cells also contributes to SLMV exocytosis. Since rabphilin-3 is not

expressed in β cells (139,140) and granuphilin does not interact with SLMVs, another question as to what SLMV-associated binding partner would function as a NRXN interacting protein would also have to be answered.

Do NRXNs perform completely overlapping functions in the β cell?

Secretory granule docking was not completely abolished in islets from NRXN1α KO mice. Possible explanations for the persistence of docked granules in these studies include functional redundancy with NRXN2β or other NRXN isoforms. In fact, we demonstrated that knockdown of NRXN2β has a similar effect on insulin secretion as knockdown of NRXN1α. This begs the question as to whether or not all β-cell NRXNs have overlapping functions. It seems unlikely since NRXNs in neurons are differentially expressed and their functional binding partners are dependent on both isoform and splice variation(90) (47,74,91-95). However, the complete array of NRXN β-cell isoforms, binding partners, and function has yet to be uncovered.

Do NRXNs play a role in islet and β-cell development?

Finally, the known contribution of NRXNs to neuronal development, synaptogenesis, and synaptic differentiation (64,67,87,88) hints at the possibility that NRXN is involved in islet and β-cell development. We observed that islets of the NRXN KO mice had an increase in insulin area (islet size) and β-cell size. A possible explanation for the increase in β-cell mass and size in NRXN KO mice is feedback of the increased secretory capacity observed in the NRXN1α KO islets on cell and islet size. Dynamic adaptation of β-cell size has been observed in many cases such as an increase in

β-cell size during pregnancy (223) and in persistent hyperglycemia (224). However, since we did not notice a change in β-cell size after NRXN1 knockdown in siRNA-treated INS-1E cells, it seems more likely that alterations in β-cell mass and size are due to developmental changes in the islets of NRXN1α KO mice.

As thoroughly discussed, it is possible that NRXN, via interactions with binding partners on the β-cell surface and the islet vasculature provides a direct link between the exocytotic machinery and the β-cell surface and could influence the localization of the exocytotic microdomains beneath the plasma membrane. NRXN may also contribute to the functional architecture of the islet by juxtaposing beta cells with endothelial cells to promote cell β-cell development, secretory function (227) and polarized orientation (228) as well as maintaining cell-cell contacts important for signaling and regulated secretion (229).

C. Future Studies

Future studies are proposed to address some of these remaining questions about the role of NRXNs in insulin exocytosis and β-cell development. Future experiments will use various neurexin KO mouse models to determine whether NRXN has a role in β-cell maturation, whether its extracellular interactions determine the docking sites of insulin granules, and whether it is a potential therapeutic target for modulating β-cell function in diabetes.

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