Transcriptional Control of *Stim1* gene by transcription factor NEUROD2

by

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ABSTRACT

Neurogenic differentiation factor 2 (NEUROD2) transcription factor, a member of basic helix-loop-helix transcription factor family, have essential roles in brain development. Studies performed in our laboratory have revealed *Stim1* gene among the top targets of NEUROD2. STIM1 functions as a sensor of endoplasmic reticulum (ER) calcium store levels. Upon store depletion, STIM1 activates store-operated calcium entry (SOCE). NEUROD2 binds to an intronic element within *Stim1* gene and regulates its expression.

My thesis is composed of three major parts. In the first part, I investigated the functions of NEUROD2 by binding to *Stim1* intronic element. I found that NEUROD2 does not function as a repressor by binding to *Stim1* intronic element. Indeed, my results suggest that NEUROD2 might function as part of an insulator complex.

In the second part, I investigated the effect of NEUROD2 on SOCE in cortical neurons by calcium imaging. My results indicated that the overexpression of *Neurod2* does not cause a change in neuronal SOCE response at 5 day *in vitro* (5DIV). I also tested the effect of *Neurod2* knock-down on neuronal SOCE response at 5DIV but my results were inconclusive.

In the last part, I aimed to delete *Stim1* intronic region comprising clustered E-boxes where NEUROD2 binds to by using CRISPR-Cas9 genome editing system. A better understanding of functions of NEUROD2 regulation of *Stim1* expression can be developed by examining the effects of this deletion in the context of cortical development.

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NOMENCLATURE

APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
BME	Basal Medium Eagle
bp	Basepair
cDNA	Complementary Deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiothreitol
ER	Endoplasmic reticulum
EDTA	Ethylenediaminetetraacetic acid

FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-Hydroxyethly)piperazine-1-ethanesulfonic acid
HRP	Horseradish peroxidase
kDa	Kilodalton
LB	Luria Broth
MEM	Minimum Essential Media
mRNA	Messenger Ribonucleic acid
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNK	Polynucleotide kinase
P/S	Penicillin/Streptomycin
Q-PCR	Quantitative Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SERCA	Sarco/endoplasmic Ca2+ ATPase
shRNA	Small hairpin ribonucleic acid
sgRNA	Single guide RNA
SOCE	Store-operated calcium entry
TBS-T	Tris Buffered Saline-Tween
TEMED	Tetramethylethyleneamine
TG	Thapsigargin
TTX	Tetradotoxin

Chapter 1

INTRODUCTION

The brain performs several tasks that include sensing the environment, handling physical movement, and creating new ideas. Coordination and regulation of these tasks is possible because of the large networks of connecting neurons. Therefore, correct wiring of the brain is crucial for its proper functioning. The basic map of brain wiring is completed during embryonic and postnatal development. However, the exact mechanism of how neurons find their final position in the brain and make the proper connections remains unknown to a great extent.

Each cell type of the brain originates from neuroepithelial (NE) cells that are found in the neural tube [1]. NE cells behave as neural stem cells while not exhibiting neuronal identity. In early development, NE cells divide symmetrically to increase their number. With the onset of neurogenesis, they divide asymmetrically to transform into radial glial cells (RGCs) and neural progenitor cells (NPCs) and neurons are generated [2]. Neuronal differentiation is a highly regulated process to give rise to a correct brain architecture needed for proper brain function.

Transcription factors have an essential contribution in the regulation of neurogenesis [3]. Basic helix-loop-helix (bHLH) transcription factors are key determinants of many steps in neurogenesis. Correct spatiotemporal expression of each bHLH protein that plays a role in the regulation of neurogenesis is crucial for neuronal differentiation [4] [5]. NEUROD2 (Neurogenic differentiation factor 2) is an example of neuronal bHLH protein that regulates

neurogenesis [6]. Previous chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq) experiments by using embryonic day 14.5 (E14.5) and postnatal day 0 (P0) mouse cortices conducted in our laboratory have revealed genome-wide *in vivo* developmental targets of NEUROD2 [7] [[8]. A binding site mapping to the second intron of the *Stim1* (Stromal interaction molecule 1) gene has been ranked among the highest scoring NEUROD2 binding sites in both E14.5 and P0 ChIP-Seq data. This binding site comprises 4 consensus E –box motifs that bHLH proteins including NEUROD2 can recognize and bind. STIM1 protein is an essential component of store-operated calcium entry (SOCE) which allows replenishing of ER calcium stores upon depletion [9].

In this study, I aimed to elucidate the functions of NEUROD2 binding to *Stim1* intron. My thesis is composed of three major parts. In the first part, I investigated whether NEUROD2 functions as a repressor by binding to *Stim1* intronic element. Previous studies conducted in our laboratory showed that knocking-down *Neurod2* expression increases *Stim1* expression in cultured cortical neurons. One explanation for this phenotype might be that NEUROD2 functions as a repressor by binding to *Stim1* intronic element inhibiting *Stim1* gene expression. To investigate this, I used a luciferase reporter system where I examined the influence of NEUROD2 on reporter gene expression upon binding to *Stim1* intronic element. However, I found that NEUROD2 significantly activates gene expression when it is bound to *Stim1* intronic element. I also investigated the effect of *Neurod2* overexpression on *Stim1* gene expression in different cell lines. However, I didn't observe a decrease in *Stim1* expression levels upon *Neurod2* overexpression. These results together suggest that NEUROD2 does not function as a repressor by binding to *Stim1* intronic element.

In the second part, I investigated the effect of NEUROD2 on SOCE in cortical neurons by calcium imaging. My results indicated that the overexpression of *Neurod2* does

not cause a change in neuronal SOCE response at 5 day *in vitro* (5DIV) different than its previously reported suppressive effect at 7-9DIV [8].

I further aimed to delete *Stim1* intronic region comprising clustered E-boxes where NEUROD2 binds to by using CRISPR-Cas9 genome editing system to show that NEUROD2 regulates *Stim1* levels by binding to its intronic region. A better understanding of functions of NEUROD2 regulation of *Stim1* expression can be developed by examining the effects of this deletion in the context of cortical development.

In chapter 2, literature review on NEUROD2 and its roles in neurons, STIM1 protein and SOCE mechanism, and their roles in neurons can be found. Chapter 3 contains the materials and methods used in the experiments performed. Chapter 4 contains the results of the experiments and Chapter 5 contains their critique and implications.

Chapter 2

LITERATURE REVIEW

2.1 Overview

The brain performs various functions that include sensing the environment, controlling motor movement, and processing cognitive tasks. Coordination and regulation of these tasks is only possible with the large networks of properly connected neurons. Therefore, proper brain development is essential to form the correct brain architecture that allows proper brain functioning.

During early stages of animal embryonic development, three different germ layers named as endoderm, mesoderm, and ectoderm form. Each germ layer will give rise to different body organs. For example, the ectoderm gives rise to skin and the nervous system. Following the formation of germ layers, neurulation occurs to form the neural tube: by folding of a part of the ectoderm inward that creates a groove. This folding causes formation of an isolated tube down the dorsal midsection of the embryo. Central nervous system including brain and spinal cord is derived from the neural tube [1].

Neural tube contains neuroepithelial (NE) cells that function as neural stem cells which can give rise to all cell types in the brain. At the beginning of neurogenesis, NE cells give rise to radial glial cells (RGC) which are non-stem cell progenitors having more restricted cell fates [10]. RGCs are polarized cells with their cell bodies residing in the ventricular zone (VZ). RGCs can divide both symmetrically and asymmetrically giving rise

to different daughter cells according to division type. RGCs increase their cell number by symmetric division. On the other hand, asymmetric division results into one daughter cell which is either an immature neuron or a basal progenitor (BP) while the other daughter stays as RGC. BPs are the major producers of cortical neurons [2]. RGCs serve as scaffold for the migration of newborn neurons to their final position in the brain since newborn neurons migrate along the radial glial fibers. Subventricular zone (SVZ) located right above VZ is the place where BP cells migrate to and further differentiate into neurons [11].After neurogenesis is completed, remaining RGC population either differentiate into glial cells or is preserved through adulthood as neuronal progenitor cells [10].

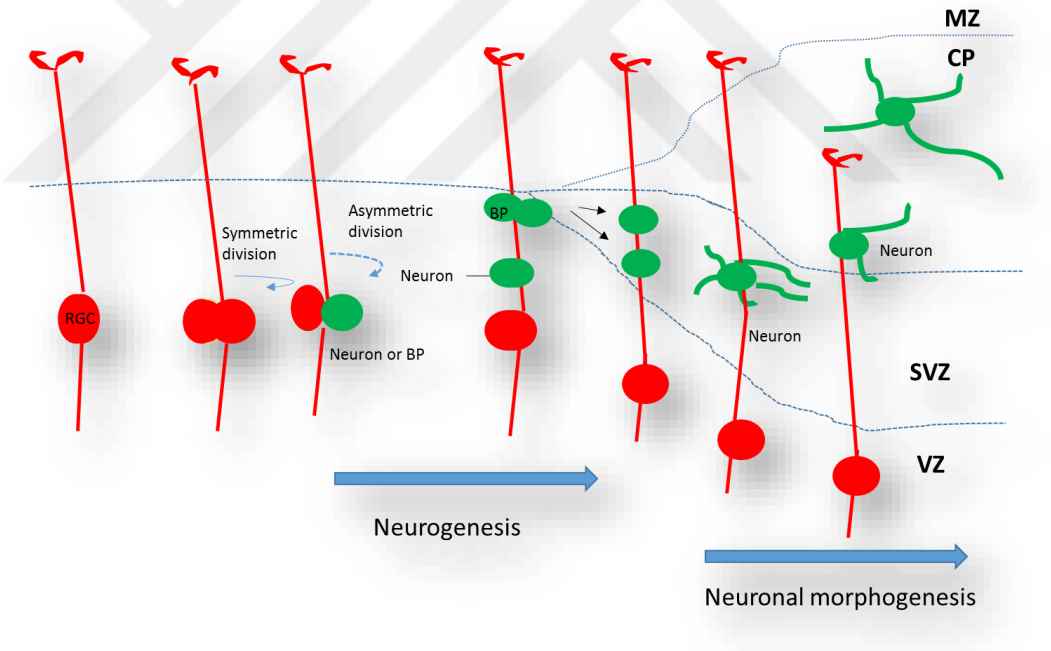

Figure 2.1: Schematic representation of neural development of the mammalian neocortex [12]. During neurogenesis, neural progenitors proliferate and differentiate to generate neurons in a highly regulated manner. Differentiated neurons migrate towards

their final position as neuronal morphogenesis continues. RGCs are progenitor cells which generate the majority of the neurons in the developing cortex. RGCs divide symmetrically in VZ to increase their numbers in early development. Later on, they begin to divide asymmetrically to generate one self-renewing progenitor and one post-mitotic neuron or one BP. BPs migrate to SVZ and divide symmetrically to form neurons. CP, cortical plate; MZ, marginal zone.

During neurogenesis, various types of neurons are produced from NE cells. The brain is able to perform complex tasks due to the different neural networks formed by the correct interactions between these different cell types in their proper positions. Among various functions of the brain, higher order functions such as cognition, motor movement generation and sensory perception are controlled by the neocortex. The neocortex is organized into six layers labelled I to VI in which layer I is the outermost and layer VI is the innermost. Neurons in each layer have distinct morphological and physiological properties from each other [13]. The layers of neocortex have a heterogeneous cell population consisting of both excitatory projection neurons and inhibitory interneurons. Inhibitory interneurons originate from ganglionic eminence located at the ventral ventricular zone of telencephalon and tangentially migrate towards neocortex. On the other hand, excitatory projection neurons originate from the dorsal telencephalon and radially migrate to their final position at neocortex. Six-layered structure of the neocortex is achieved by the radial migration of these neurons in an inside-out fashion meaning that the late-born neurons migrate on top of the early-born neurons. Therefore, the later-born neurons are found in the superficial layers whereas the earlier-born neurons are found in the deep layers [13].

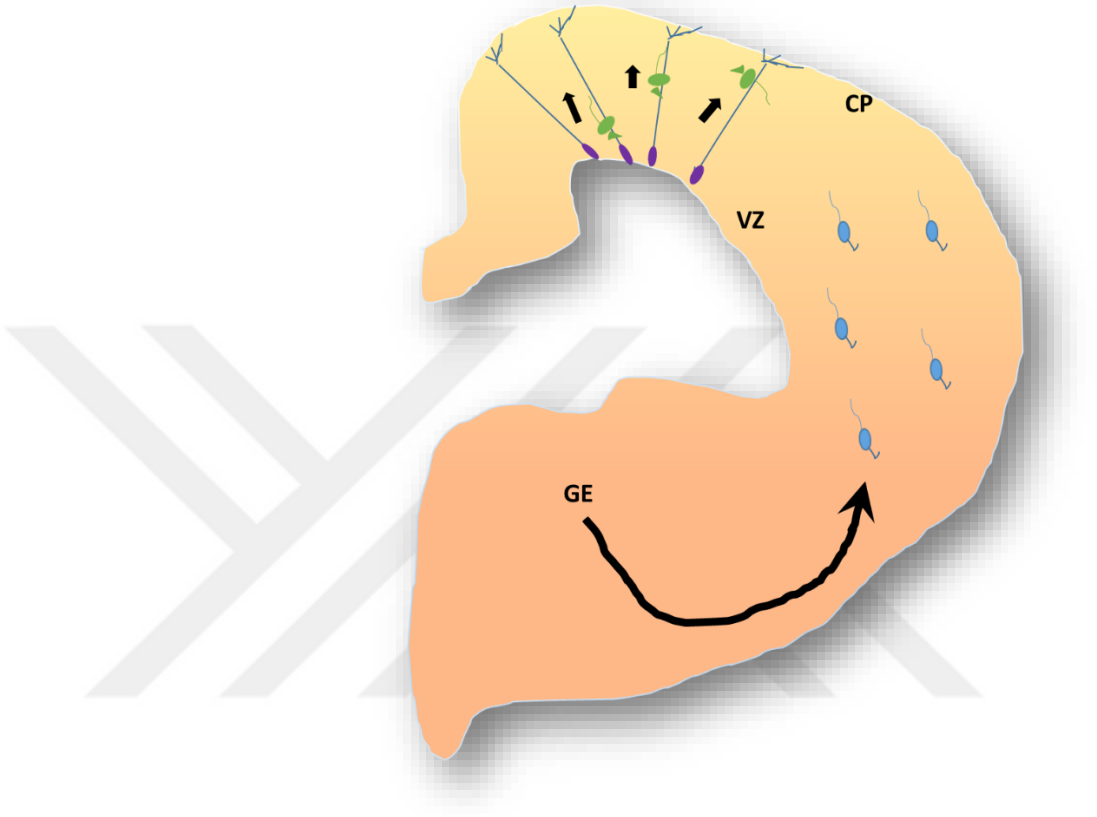

Figure 2.2: Migration of projection neurons and interneurons. Projection neurons born at ventricular zone (VZ) and migrate radially to cortical plate (CP), whereas interneurons born at ganglionic eminence (GE) and migrate tangentially.

Every stage of neurogenesis from the neural stem cell proliferation to neuronal migration is highly regulated by various developmental cues. Transcription factors have an essential contribution in the regulation of neurogenesis [3]. Basic helix-loop-helix (bHLH) transcription factors are key determinants of many steps in neurogenesis and their expression at the correct time and place is crucial for neuronal differentiation. In the early stages of neurogenesis, *Hes*, *Hey* and *Id* families of bHLH proteins promote progenitor cell

self-renewal and proliferation while repressing differentiation. At later stages, neurogenins (*Neurog1-2*) and *Asc11* promote differentiation of excitatory projection neurons and inhibitory interneurons respectively by triggering a cascade of downstream bHLH protein expression [4], [5].

Cellular Ca^{2+} concentration is another key regulator of neural development. Intracellular Ca^{2+} levels is an important determinant of progenitor cell proliferation in early neurogenesis. Defective store-operated calcium entry (SOCE) which is one of the main calcium influx mechanisms in neurons results in a decrease in the progenitor cell population by inhibiting proliferation [14]. SOCE is an important component of cellular calcium signaling and cellular calcium homeostasis that functions in refilling of ER calcium store levels upon depletion. STIM1 and ORA1-2 are the key players of SOCE. STIM1 protein is located in ER membrane and senses the ER Ca^{2+} levels and ORAI1-2 channels are Ca^{2+} channels found in cell membrane. If any depletion occurs in the stored Ca^{2+} levels it physically interacts with ORAI1-2 to activate them. Refilling of Ca^{2+} stores is achieved by Ca^{2+} influx via active ORAI1-2 channels. Suppression or deletion of *Stim1* and *Orai1-2* expression results in a significant reduction in progenitor cell proliferation both *in vitro* and *in vivo* [14].

2.2 Calcium regulation of neuronal gene expression

Activity-dependent Ca^{2+} entry and spontaneous Ca^{2+} oscillations are essential to control neuronal differentiation. Activity-dependent Ca^{2+} entry via voltage-gated calcium channels activates various transcription factors that contribute to cortical development. For example, CREST (Calcium-responsive transactivator) protein which is an important regulator of dendritic growth is activated by activity-dependent Ca^{2+} entry into maturing neurons [15] .

The common mechanisms for activity-dependent signaling pathway regulation of gene transcription include modification of the function, localization, or expression of transcriptional regulators in the nucleus [16]. Calmodulin or other calcium sensor bound calcium activates several calcium-regulated intracellular signaling cascades including the members of calcium-calmodulin kinase (CaMK) and Ras/mitogen-activated protein kinase (MAPK) families [17]. Changes in the activity of these signaling intermediates can alter posttranslational modifications on transcription factors [18]. For example, *CaMKIV* (Ca^{2+}/ Calmodulin dependent protein kinase IV) and *CREB* (cyclic adenosine monophosphate (cAMP) responsive element binding) regulated transcription is important for dendrite growth in mouse cortical neurons [19]. Both CaMKIV inhibition and dominant-negative form of CREB cause dendrite growth inhibiton [19]. NEUROD2 (Neurogenic differentiation factor 2) is another example of calcium-activated neuronal transcription factors that regulates neurogenesis [20].

2.3 NEUROD2 Transcription Factor

NEUROD2 is a member of the NeuroD family of neuronal bHLH proteins. The other members of the family are NEUROD1, NEUROD4 and NEUROD6. Neur*od2* gene was identified based on its sequence similarity to previously identified *Neurod1* [21]. NEUROD2 is able to induce neurogenic differentiation in non-neuronal cells since ectopic induction of neurogenesis is observed in *Xenopus laevis* embryos when *Neurod2* is overexpressed [21]. NEUROD2 heterodimerizes with E-proteins and binds to E-box motifs on the genome which defined as CANNTG where N can be any nucleotide [21].

2.4 Expression pattern of *Neurod2* in the mouse brain

In mouse brain, *Neurod2* expression starts at embryonic day 11 (E11) when progenitors exit the cell cycle and sustained through the adulthood [6], [21]. Its expression is regulated by Neurogenins which are the upstream transcription factors as *Neurog1/2*

double knock-out mice shows decreased *Neurod2* expression [22]. At E12, *Neurod2* expression is observed in the neocortex and in the spinal cord. As development continues, its expression is expanded so that *Neurod2* is present in various regions consisting of the neocortex, cerebellum, spinal cord, amygdala, brain stem and optic tectum at E18. In adulthood, its expression is restricted to hippocampus, cerebellum and cerebral cortex at low abundance [6].

2.5 Roles of NEUROD2 in neurons

2.5.1 Neuronal Induction

NEUROD2 is sufficient for the induction of neuronal differentiation in non-neuronal cell lines. When expressed in P19 embryonal carcinoma cell line, NEUROD2 is able to induce expression of neuronal markers [23]. If E12 protein which is the dimerization partner of NEUROD2 is co-expressed with NEUROD2, neurite extensions and electrical excitability can be observed in P19 cells. Induction of neuronal cell fate by NEUROD2 expression is also observed in mouse embryonic stem cells and mouse neuroblastoma cell lines N18-RE-105 and N1E-115 in different studies [24]–[26].

2.5.2 Brain development

NEUROD2 regulates various fundamental features of brain development. *Neurod2* knockout mice exhibit shrinkage in brain size compared to wild type mouse brain especially at the regions where *Neurod2* is abundantly expressed such as the cerebellum [6]. *Neurod2* knockout mice also show increased neuronal death[6]. Thalamocortical axons terminals were shown to be unable to form properly in *Neurod2* knock-out mice together with the defective thalamocortical synapses[20].

NEUROD2 functions in the regulation of amygdala development and emotional learning in mice [27]. The lateral and basolateral amygdala was not properly developed in *Neurod2* knockout mice and NEUROD2 heterozygous mice exhibit fewer neurons in this region. Moreover, NEUROD2 heterozygous mice showed serious deficiency in emotional learning when compared to wild-type controls in terms of decreased anxiety behaviors in unconditioned fear tests[27].

NEUROD2 functions in the maintenance of excitatory/inhibitory balance in developing neurons [28]. NEUROD2 regulates the postmitotic expression of genes such as Gastrin releasing peptide and SK2 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2) to promote inhibitory synapse development and restrain intrinsically generated neuronal excitability[28]. Indeed, *Neurod2* knockout mice exhibit low seizure threshold and they are prone to spontaneous epilepsy [6].

Neurod2 overexpression in cortical neural progenitors causes early differentiation by initiating premature cell cycle exit. [3]. During embryonic development, the regulation of various components of neuritogenesis and radial migration by NEUROD2 is also revealed by target gene analyses and gene expression studies [3], [7].

2.6 NEUROD2 binding sites in genome

In vivo targets of NEUROD2 have been identified by chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq) in our laboratory by using dissected mouse cortex [7], [8]. The experiment was conducted at two different developmental time points selected as embryonic day 14.5 (E14.5) and postnatal day 0 (P0) to gain a better insight about the functions of NEUROD2 during cortical development. The gene ontology analyses of E14.5 ChIP-Seq experiment has revealed that various genes that play roles in cortical radial migration and axon guidance are NEUROD2 targets. The most enriched

functional categories found in P0 ChIP-Seq data are dendrite development and synapse organization [7], [8]. Interestingly, a binding site mapping to the second intron of the *Stim1* gene has been ranked among the highest scoring NEUROD2 binding sites in both E14.5 and P0 ChIP-Seq data [7], [8].

Table 2.1: Top 10 highest scoring NEUROD2 binding sites revealed by P0 ChIP-Seq [8]

Gene symbol	Gene name	Genomic region	Score
Krtap16-1	Keratin-associated protein 16-1	Promoter	8439.73 0
n/a	n/a	Intergenic	6366.16 0
Prss36	Polyserase-2 precursor	Promoter	6362.44 0
Man1c1	Mannosidase alpha class 1C member 1	Exon	5669.73 0
n/a	n/a	Intergenic	5018.18 0
Tecr	Synaptic glycoprotein SC2	Intron	5011.04 0
Stim1	Stromal Interaction molecule 1	Intron	4889.58 0
Hlcs	Holocarboxylase synthetase	Promoter	4595.15
Btbd17	BTB/POZ domain-containing protein 17	Promoter	4541.48 0
Erg	Erythroblast transformation specific transcription factor	Intron	4390.44 0

Score = -10log10(p value)
n/a: not applicable

2.7 Stromal Interaction Molecule 1 (STIM1)

STIM1 and its homologue STIM2 are Ca^{2+} binding single pass transmembrane proteins that are located on ER membrane with their N –termini extended into the ER lumen and their C-termini extended into cytosol [29]. STIM proteins are ubiquitously expressed in various cell types. In most of the tissue types STIM1 expression level is higher while STIM1 and STIM2 are found in similar levels in the brain with the exception of hippocampus where STIM2 is the predominantly expressed form [30]–[32]. STIM1 senses the ER calcium store levels and mediates store-operated calcium entry (SOCE) by gating of store-operated Ca^{2+} influx channels to refill the stores after depletion [33], [34].

While STIM1 protein is ubiquitously expressed in cells, its alternatively spliced long variant STIM1L shows a restricted expression pattern including human skeletal muscle mouse skeletal muscle, heart, and brain and neonatal rat cardiomyocytes [35], [36]. STIM1L results from an alternative splicing of exon 11 which contains an extra 106 residues (aa 515-620). This extra domain functioning as an actin binding domain is inserted in the cytosolic domain of the protein. STIM1L is able to form permanent clusters by interacting with ORAI1 channels [36].

2.8 Structure of STIM1 and store-operated calcium entry mechanism

The two major domains of ER luminal portion of STIM1 are the EF-hand domain and a sterile alpha motif (SAM) domain. EF-hand domain is bound to a single Ca^{2+} ion when STIM1 is in its inactive state. As Ca^{2+} ions are released from the ER lumen, STIM1 changes its state to the active form which is no longer bound to Ca^{2+} ions. The recent model for STIM1 activation postulates that hydrophobic regions in the EF-SAM domains dimerize with a neighboring STIM1 N-terminus upon store depletion [37].

There are three coiled coil domains (CC1, CC2, and CC3) in the C-terminus of STIM1 corresponding to the cytosolic portion of the protein. After store depletion, CC2 and CC3 but not CC1 are required for STIM1 oligomerization needed for STIM1-ORAI interactions and calcium release-activated channel (CRAC) activation. The region that comprises CC2 and CC3 is alternatively called as the CRAC activation domain (CAD) (aa 342-448), STIM1−ORAI activating region (SOAR) (aa 344-442), or coiled-coil domain b9 (CCb9) (aa 339-444). This domain is sufficient for CRAC channel activation if expressed as soluble protein. The lysine-rich residue at the end of SOAR domain is able to interact with ORAI channels to initiate Ca^{2+} influx upon store depletion [37].

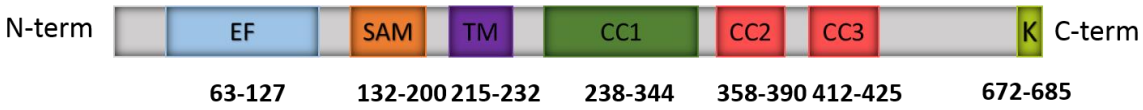

Figure 2.3: Representation of STIM1 domains. EF-hand domain (EF), sterile alpha motif domain (SAM), transmembrane region (TM), coiled-coil domains 1−3 (CC1−3), and lysine-rich region (K) are labeled.

At resting conditions, Ca^{2+} ion concentration is kept less than 100 nM in cytosol while resting levels of luminal calcium is 250-600 μM [29]. When Ca^{2+} levels are high in ER, Ca^{2+}-bound EF- hand motif is in a close association with SAM domain. This close confirmation of EF-SAM domain prefers STIM1 to be in monomeric state and STIM1 is uniformly distributed throughout ER membrane [37]. When an extracellular stimulation triggers phospholipase C-β (PLC-β) activation by binding to its g-protein coupled receptor, inositol triphosphate ($InsP_3$) is produced. Production of $InsP_3$ results in the depletion of $InsP_3$-sensitive Ca^{2+} stores via activated $InsP_3R$ channels found in ER membrane. Consequently, Ca^{2+} dissociates from EF domain causing STIM1 oligomerization and translocates into punctate clusters in regions close to the plasma membrane. Oligomerized STIM1 binds to and gates ORAI1 so that SOCE is activated.

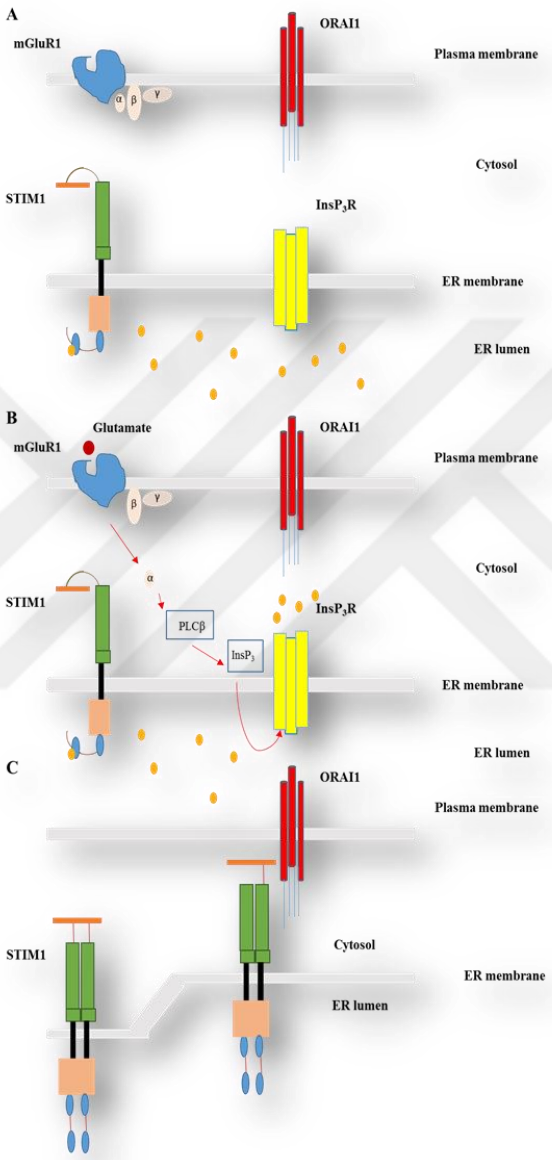

Figure 2.4: Mechanism of SOCE activation. (A) In steady-state condition, Ca^{2+} ions are present in ER in high concentration (orange spheres). STIM1 is in Ca^{2+}-bound state and is inactive. ORAI1 channels are not open. (B) When a signal that leads to ER Ca^{2+} store usage

is received, such as glutamate which activates its GPCR receptor (mGluR1), activated receptor causes dissociation of G protein, and G_{α} gets released. G_{α} activates phospholipase C- β (PLC-β), and leads to inositol triphosphate ($InsP_3$) production. $InsP_3$ binds to $InsP_3R$ found on ER membrane. This binding causes the opening of $InsP_3R$ channel and Ca^{2+} ions release to cytosol from ER via this channel. (C) Decrease in ER Ca^{2+} levels causes Ca^{2+} to dissociate from STIM1. STIM1 oligomerizes. STIM1 oligomers interact with ORAI1 on the plasma membrane. Orai1 channels open and extracellular Ca^{2+} enters the cytosol via ORAI1 channels**.**

2.9 STIM1 and SOCE in neural development

SOCE is the main calcium entry mechanism in non-excitable cells. However, excitable cells such as neurons have other calcium uptake pathways including voltage-gated calcium channels and ligand-gated calcium channels used as major routes for calcium entry. Therefore, the role of SOCE in excitable cells are understudied compared to its role in non-excitable cells.

The role of STIM1 in growth cone navigation is shown as inhibition of STIM1 causes perturbation in growth cone turning response to guidance cues [38]. SOCE also plays an essential role in neural progenitor cell proliferation. If STIM1 or ORAI1 are not present the proliferation rate of neuronal progenitor cells decreases as shown both *in vitro* in cultured neural progenitor cells and *in vivo* in the subventricular zone [14]. STIM1 also inhibits voltage-gated calcium channel $Ca_v1.2$. STIM1 causes the internalization of those channels by repressing their calcium intake[39]. Consistent with these findings, in the course of cortex development voltage-gated calcium influx is upregulated while SOCE is downregulated as the progenitors differentiate into mature neurons [14], [40], [41].

2.10 NEUROD2 regulation of *Stim1*

NEUROD2 binds to the second intron of *Stim1* gene [8]. This binding site exhibits a higher level of sequence conservation compared to random intronic sequences indicating a potential function [8]. However, it is not enriched in histone modifications associated with either transcriptionally active (H3K4me3, H3K4me1, and H3K27ac) or repressive marks (H3K36me3, H3K9me3, and H3K27me3) [8].

NEUROD2 controls *Stim1* expression levels. Knockdown of *Neurod2* expression increases *Stim1* expression levels in cultured cortical neurons as shown in both mRNA and protein levels. NEUROD2 also regulates SOCE since knockdown of *Neurod2* expression causes an upregulation whereas overexpression of *Neurod2* causes a downregulation in SOCE response in cultured cortical neurons [8]. However, the exact molecular mechanism of how NEUROD2 regulates STIM1 and SOCE remains to be elucidated.

Chapter 3

MATERIAL AND METHODS

3.1 Luciferase Assay

HEK293T cells were transfected in 24-well plates using Lipofectamine 2000 (Invitrogen). Each well was cotransfected with 200 ng of pXPG luciferase reporter construct (Bert et al., 2000), 200 ng of empty or NEUROD2-expressing pcDNA4 plasmid and 100 ng of Renilla pRL-null plasmid (Promega). Twenty-four hours after transfection, the cells were taken from the incubator and washed with 350 µl cold PBS. After washing, they were lysed in 1X PLB buffer (Promega) for 15 minutes at room temperature. 20 µl of the lysate was used to measure the activity of Firefly and Renilla luciferases by Dual-Luciferase Reporter Assay System (Promega).

3.2 Maintenance and Transfection of HEK293T cells

Cells were maintained in high glucose DMEM containing 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate and 0.5% L-glutamate and passaged when they reach confluency. Transfection was performed when cells reach 70-80% confluency. For one well in a 24 well plate, two tubes with 50 µl OptiMEM ® were prepared. To one tube, 500 ng plasmid DNA was added, while to the other tube 1 µl Lipofectamine 2000 was added. After 5 minutes incubation at room temperature, the two solutions were mixed. The mixed solution was incubated at room temperature for 20 minutes. After that, it was added

on the corresponding well dropwise. The transfected cells were used for luciferase assay twenty-four hours after transfection.

3.3 WT-253 and WT-570 construct preparation

To prepare WT-570 and WT-253 constructs in pGL5 backbone vector, a 570 bp fragment encompassing the NEUROD2 binding site in *Stim1* intron 2 and a shorter fragment within *Stim1* intronic element that was 253 bp in length were amplified by PCR using mouse BALB/C genomic DNA as template. 150 µg purified PCR product and 750 µg pGL5 backbone vector were digested with 10 units of KpnI (New England Biolabs, Cat. #R0142S) and 10 units of XbaI (New England Biolabs, Cat. # R0145S) enzymes in 1X NEBuffer2.1 (New England Biolabs, Cat. #B7202S) separately. Digestion was performed 2 hours at 37 °C. Digested samples were purified by GENEJET PCR Purification kit (Thermo Scientific, Cat. #K0701) and used for ligation. 3 µl of digested fragment and 5 µl of digested vector were mixed. 5 unit T4 DNA ligase (Thermo Scientific, Cat. # EL0014) and 1x T4 DNA ligase buffer (Thermo Scientific, Cat. #B69) were added to the reaction. The ligation reaction was incubated in room temperature for 2 hours.

To prepare WT-570 constructs in pXPG backbone vector, a 570 bp fragment encompassing the NEUROD2 binding site in *Stim1* intron 2 was amplified by PCR using mouse BALB/C genomic DNA as template.150 µg purified PCR product and 750 µg pXPG backbone vector were digested with 10 units of KpnI (New England Biolabs, Cat. #R0142S) and 10 units of HindIII (New England Biolabs, Cat. #R0104S) enzymes in 1X NEBuffer2.1 (New England Biolabs, Cat. #B7202S) separately. Digestion was performed 2 hours at 37 °C. Digested samples were purified by GENEJET PCR Purification kit (Thermo Scientific, Cat. #K0701) and used for ligation. 3 µl of digested fragment and 5 µl of digested vector were mixed. 5 unit T4 DNA ligase (Thermo Scientific, Cat. # EL0014)

and 1x T4 DNA ligase buffer (Thermo Scientific, Cat. #B69) were added to the reaction. The ligation reaction was incubated in room temperature for 2 hours.

Ligation product was transformed to chemically competent DH5α *E.coli* cells as described in the section 3.4. Plasmid DNA was extracted from the bacterial culture started from a single colony selected from the transformation plate. The extraction was performed using GeneJET Plasmid Miniprep Kit (Thermo Scientific, Cat. #K0503). Positive samples were detected by diagnostic digestion using 10 units of KpnI (New England Biolabs, Cat. #R0142S) and 10 units of HindIII (New England Biolabs, Cat. #R0104S) enzymes in 1X NEBuffer2.1 (New England Biolabs, Cat. #B7202S) for 5ul isolated plasmid DNA. The reaction was incubated 2 hours at 37^{o}C. 6X gel loading dye (New England Biolabs, Cat. # B7021S) was added into the digested samples to obtain 1X final dilution. After that, the samples were loaded on 1 % agarose gel stained with ethidium bromide. The gel was run at 100 V for 30 minutes and observed under UV light. Positive samples having DNA bands corresponding to the insert size of either 570 or 253 bp were observed and were confirmed by Sanger sequencing. Then, maxiprep culture was started in 200 ml LB containing 200 µl ampicillin by using 1.5 ml sample culture. After overnight incubation of the culture, the plasmid DNA was extracted by PureLink Maxiprep kit (Invitrogen, Cat. # K210006). The isolated high concentration plasmid DNA was stored in – 20 ^{o}C.

3.4 Plasmid DNA Transformation into chemically competent bacteria

Chemically competent DH5α *E.coli* cells were taken from -80^{o}C store and directly put onto ice. The cells were allowed to thaw on ice. 1 µl ligation product was added onto the bacteria. The tube was incubated for 10 minutes on ice. Then, the cells were heat shocked for 1 minutes at 42^{o}C. 500 µl LB was added onto the tube and the cells were incubated at 37^{o}C for 1 hour. After incubation, the cells were spread on LB plates containing the appropriate antibiotics for plasmid selection. The plates were incubated at

37^{o}C overnight. Next day, single colonies were selected to grow in LB media with the appropriate antibiotics. Plasmid DNA can be isolated from the cultures for further use.

3.5 MUT-570 construct preparation by site-directed mutagenesis

All four E-boxes in WT-570 construct were destroyed one by one to create MUT-570 construct by PCR-based site-directed mutagenesis. PCR reaction mixture contained 1X Phusion HF reaction buffer (New England Biolabs, Cat. #B0518S), 200 µM dNTPs, 0.5 µM forward primer and 0.5 µM reverse primer 3% DMSO, 10 ng template DNA and 1 Unit Phusion Hot Start Flex DNA Polymerase (New England Biolabs, Cat. #M0535L). During PCR reaction, samples were subjected to 98^{o}C for 45 seconds, 60^{o}C for 2 minutes and 72 ^{o}C for 7 minutes in sequence for 30 cycles. PCR products were purified by GENEJET PCR Purification kit (Thermo Scientific, Cat. #K0701). After DNA concentration was measured by Nanodrop2000c (Thermo Scientific), phosphorylation reaction was set up for the products as follows: A final volume of 50 µl mixture was prepared to have 1 mM ATP (New England Biolabs, Cat. #P0756S), 1X Polynucleotide Kinase Buffer A (New England Biolabs Cat. # B0201S), 10 Unit Polynucleotide Kinase (New England Biolabs Cat. # M0201S) and 50 ng purified PCR product. The mixture was incubated at 37 ^{o}C for 60 minutes for phosphorylation reaction. Right after phosphorylation, the mixture was incubated at 65 ^{o}C for 25 minutes for enzyme deactivation. Circularization of the phosphorylated products was achieved by ligation reaction carried out in 10 µl mixture containing 5 unit T4 DNA ligase (Thermo Scientific, Cat. # EL0014), 1x T4 DNA ligase buffer (Thermo Scientific, Cat. #B69) and 5 µl of phosphorylated product at room temperature for 2 hours. Transformation of the circularized product was performed as described in the section 3.4.

Plasmid DNA was extracted from the bacterial culture started from a single colony selected from the transformation plate. The extraction was performed using GeneJET

Plasmid Miniprep Kit (Thermo Scientific, Cat. #K0503). Positive samples were detected by diagnostic digestion using 10 units of KpnI (New England Biolabs, Cat. #R0142S) and 10 units of HindIII (New England Biolabs, Cat. #R0104S) enzymes in 1X NEBuffer2.1 (New England Biolabs, Cat. #B7202S) for 5ul isolated plasmid DNA. The reaction was incubated 2 hours at 37^{o}C. 6X gel loading dye (New England Biolabs, Cat. # B7021S) was added into the digested samples to obtain 1X final dilution. After that, the samples were loaded on 1 % agarose gel stained with ethidium bromide. The gel was run at 100 V for 30 minutes and observed under UV light. A positive sample, where a 570 bp band corresponding to the insert size was observed, was confirmed by Sanger sequencing. When a mutation confirmed by sequencing, the miniprep DNA was used as template for another round of PCR reaction. When, all 4 E-boxes were mutated maxiprep culture was started in 200 ml LB containing 200 µl ampicillin by using 1.5 ml sample culture. After overnight incubation of the culture, the plasmid DNA was extracted by PureLink Maxiprep kit (Invitrogen, Cat. # K210006). The isolated high concentration plasmid DNA was stored in – 20 ^{o}C.

3.6 SDS-PAGE and Western Blotting

The composition of SDS-PAGE gel used for Western blotting experiments as follows: 12% separating gel (12% Acrylamide-Bisacrylamide (29:1), 375 mM Tris-HCl, pH 8.8, 0.2% APS, 0.2% SDS, 0.1% TEMED) and 5% stacking gel (5% Acrylamide-Biasacrylamide (29:1), 126mM Tris-HCl, pH 6.8, 0.1% APS, 0.1% SDS, 0.1% TEMED). Gel chamber was sealed with agarose to prevent any leakage. Water saturated butanol was applied after casting the separating gel. After separating gel polymerized, buthanol was removed and stacking gel was applied. A comb was placed in stacking gel to create the wells needed to load samples. After stacking gel polymerized, the gel was placed in SDS running buffer (25 mM Tris Base, 250 mM glycine, 0.1% SDS, pH 8.3). Samples were

mixed in equal volume with 2x SDS-Loading Buffer (100mM Tris-HCl pH 6.8, 2% β-mercaptoethanol, 4% SDS and 0.02% bromophenol blue, 20% glycerol), and incubated at 95 ^{o}C for 5 minutes. Samples were spun down and loaded onto SDS-PAGE gel. GangNam-STAIN (iNtRON Biotech, Cat. # 24052) loaded as protein size reference. The gel was run at 80V for 30 minutes and at 110V for 90 minutes.

After running, the gel was blotted on membrane by wet-transfer method. Polyvinyl difluoride membrane (PVDF, Thermo Scientific, Cat. #88518) was used for the transfer. PVDF membrane was cut according to required size and briefly kept in methanol. The membrane was covered on SDS-PAGE gel in transfer buffer (100 mM glycine, 12 mM Tris base, 15% methanol, at 4^{o}C.). Transfer performed at 110 mA current for 2 hours at 4 ^{o}C.

After transfer, the membrane was incubated in blocking solution containing TBS-T (10mM Tris-HCl pH7.5, 150mM NaCl, 0.05% Tween) with 5% non-fat dried milk powder (AppliChem, Cat. #A0830) for 1 hour at room temperature. Primary antibody was prepared by adding desired amount of antibody into TBS-T containing 5% non-fat dried milk powder. The membrane was incubated with primary antibody solution either for 2 hours at room temperature or overnight at 4 ^{o}C. The membrane was washed three times with TBS-T for 20 minutes. HRP-coupled secondary antibody was added to TBS-T containing 5% non-fat dried milk powder. Secondary antibody treatment was performed for 1 hour at room temperature. The membrane was washed three times with TBS-T for 20 minutes. HRP reagent SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific, Cat. # 34096) was diluted with water (1:2 reagent:water ratio) and applied onto membrane. The blot was imaged using Gel Logic 2200 Imaging System (Molecular Imaging System CareStream Health Inc.). The images were quantified using ImageJ software.

Table 3.1: Primary antibodies used in western blotting

Antibody	Host	Company	Catalogue Number	Dilution
myc	Mouse	Santa Cruz	sc-40	1:1000
STIM1	Rabbit	Cell Siganlling	9715	1:1000
BETA-ACTIN	Rabbit	Thermo Scientific	PA5-16914	1:2000

Table 3.2: Secondary antibodies used in western blotting

Antibody	Host	Company	Catalogue Number	Dilution
Anti-Mouse IgG HRP-linked	Horse	Cell Signalling	7076S	1:2500
Anti-Rabbit IgG HRP-linked	Goat	Abcam	ab6721	1:2500

3.7 Protein lysate collection from cells

The medium of the cells was aspirated. Cells were washed with 1x ice-cold PBS solution. 1X RIPA lysis buffer containing protease inhibitor was applied onto cells. Cells were incubated on shaker for 10 minutes at 4^{o}C. The lysate was collected from the plate and transferred into an Eppendorf tube. Lysates were centrifuged for 20 min at 14,000 rpm, in 4°C. The supernatant was transferred into a clean Eppendorf tube. Lysates were stored at -20 ^{o}C.

3.8 Calcium Imaging

Primary neuronal cultures from mouse embryonic day 14 (E14) embryos were prepared as explained in chapter 3.10 and used in calcium imaging. 300,000 cells were seeded per each coated well in 24-well plates. At 2 DIV, 1 μg shRNA-mCherry plasmid (expressing either *Neurod2* targeting or non-silencing shRNA) was transfected into the cells for knock-down experiments. For rescue experiments, co-transfection was performed at same time point (2 DIV), with 1 μg shRNA-mCherry plasmid and 2 μg of either shRNA-resistant *Neurod2* or empty plasmid. After transfection and at every following three days after transfection, media of cultured neurons were changed with 50% BNBF + 50% conditioned media until the day of calcium imaging.

At the day of imaging, calcium-sensitive dye was loaded. 1 mM stock solution of calcium-sensitive dye was prepared by adding pure DMSO to one vial of 50 μg Fluo-3 AM (Life Technologies, Cat: # F1242). The vial was covered with aluminum foil to block light exposure to the fluorescent dye. A non-ionic detergent Triton X-100 added on the stock solution (0.5% by volume) to enhance dye entry into the cells. The solution was briefly vortexed after detergent addition. The stock solution of calcium-sensitive dye diluted in Ringer solution (155 mM NaCl, 10 mM d-Glucose, 5 mM HEPES (pH 7.4), 4.5 mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, pH 7.4) to 4 μM final concentration. Cells were washed two times with Ringer solution before dye loading. An equal amount of dye was loaded onto washed wells and the plate was covered with aluminum foil to prevent light exposure. The plate was incubated at 37ºC for 30 minutes. After incubation, cells were washed with Ringer solution two times to remove the dye, and incubated for an additional 30 minutes at 37ºC. After the second incubation, cells were again washed two times with Ringer solution. After the last wash, tetrodotoxin (TTX) was added to the Ringer solution on the cells to reach 1 μM final concentration.

Calcium dye loaded plate was placed on the stage of live imaging microscope (Xcellence Pro®, Olympus). The microscope stage was enclosed and set to 37ºC, 5% CO_2. Red channel (595 nm) image was taken to detect shRNA-mCherry transfected cells and green channel (495 nm) was used to image calcium dye. Images were taken every 6 seconds until imaging finished in 10 minutes. First, baseline calcium levels were measured in first two minutes of the experiments to start examining store-operated calcium entry response of the cells. After two minutes, the medium of the cells was changed with Ca^{2+} -free Ringer solution (155 mM NaCl, 10 mM d-Glucose, 5 mM HEPES (pH 7.4), 4.5 mM KCl, 1mM EGTA , 1mM $MgCl_2$, pH 7.4), containing 5 μM thapsigargin (for depleting ER calcium stores) and 1 μM TTX to deplete ER Ca^{2+} stores. Ca^{2+}-free Ringer solution was applied for 5 minutes for complete store depletion. Then, Ca^{2+}-free media was removed and Ringer solution containing 5 μM thapsigargin and 1 μM TTX was added so that cells were allowed to uptake calcium from the newly added medium. Imaging was completed after capturing images for 3 minutes following the second media change. All images were taken under 40x magnification. The images were quantified with ImageJ software based on mean signal intensity measured on soma of the neurons. The quantified images were plotted as $\Delta F / F_o$ after background subtraction. F_o is the minimum signal obtained in the images after background signal is subtracted. The minimum signal is taken as 1 and other measurements (ΔF) plotted in ratio to this minimum signal.

3.9 Plate coating for primary neuronal culture

As coating solution, 0,6 μM Poly-L-lysine Hydrobromide (Sigma Life Sciences, Cat. #P9155) and 15 nM Mouse Laminin (Millipore, Cat. #CC095) was added in 1x cold PBS. For 24-well plates, 500 μl of the coating solution was added into a single well. Coating solution containing plates were incubated for 16 hours at 37°C, 5% CO_2 in the tissue culture incubator. After 16 hours, coated plates were washed three times with ddH_2O

and let for air dry in the tissue culture hood. Plates were used immediately or kept at 4^{o}C for later use.

3.10 Preparation of primary neuronal culture

Before the operation, all the equipment were sterilized with ethanol. Cervical dislocation was performed to euthanized pregnant mice to obtain embryos at embryonic day 14 (E14). Heads of the embryos were removed immediately and put in ice-cold 1x HBSS (Gibco, Cat. #14185-045) with 10 mM HEPES (pH 7.4). Brains of the embryos were taken out of the skull, and telencephalic hemispheres were separated. Meninges was separated from the brain. Hippocampus and olfactory bulbs were taken out, and cortex was separated. The separated cortices were taken and put in enzyme solution containing cysteine (3.2 mg) and 200 unit papain (Worthington, Cat. # LS003126) in dissociation media (82 mM Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 0.252 mM $CaCl_2$, 1mM HEPES (pH 7.4), 20mM glucose, 0.001% phenol red, 0.2 mN NaOH in ddH_2O). The cortices were incubated in enzyme solution for 15 minutes at 37°C. The cortices were gently mixed in the middle of incubation duration. The cortices were transferred to light inhibitor solution (10 mg BSA and 10 mg trypsin inhibitor in 10 ml dissociation media, pH adjusted and filtered) and incubated for 1 minute. Then, cortices were put into heavy inhibitor solution (50 mg BSA and 50 mg trypsin inhibitor in 5 ml dissociation media, pH adjusted and filtered) and were incubated for 1 minute again. The cortices were washed in BF solution composed of 5%FBS added to BME (Lonza, Cat. #BE12-105F) containing 1% P/S and 1% Ultra-glut (Lonzo, Cat. #BE17-605E/U1). Washed cortices were taken into another tube containing BF, and triturated by serological pipette several times for mechanical dissociation of the cells from the cortex tissue. After trituration, solution was let to settle down, allowing large tissue fragments to settle. The upper part of the sample was taken and passed through a cell strainer. Cells were placed on a hemocytometer and counted. 300.000 cells were seeded in

0.5 ml BF for one well of a coated 24-well plate. Cells were put into the incubator for 2 hours. After 2 hours, BF media was changed to 500 µl BNBF (5% FBS, 2% B-27 supplement, 1% N-2 supplement in BME). For every 2-3 days in vitro (DIV), medium was changed with 50% conditioned-50% fresh BNBF medium.

3.11 Transfection of primary neuronal cultures

Transfection of the primary neuronal cultures was performed at 2DIV. The media of the neuronal culture was collected into a tube and 500 µl OptiMEM® media was added per well for 24-well plates. Collected media was filtered and kept in 37oC water bath as conditioned media. In one Eppendorf tube, 2 µl Lipofectamine 2000 solution was added into 250 µl OptiMEM® per well. In another eppendorf, 1-3 µg plasmid DNA (varies with the conditions of the experiment) was mixed with 250 µl OptiMEM®. After 10 minutes incubation at room temperature, two mixtures were gently mixed. The mixture was incubated for 20 minutes in room temperature. After incubation, the mixture was added onto the cells dropwise. After 2 hours incubation in 37^{o}C incubator, media of the cells was changed with 50% conditioned media + 50% fresh BNBF (BF media containing 1x N2 (Gibco Life Technologies, Cat. #17502-048) and 1x B-27 (Gibco Life Technologies, Cat. #12587-010)).

3.12 Total RNA isolation

Total RNA isolation from nucleofected primary neuronal cultures was performed using Absolutely RNA Microprep Kit (Agilent Technologies, Cat. #400805). To lyse the cells, 0,7 µl β-mercaptoethanol was added to 100 µl of lysis solution supplied with the kit. To prevent contamination, the addition was performed under the hood and solutions were only opened under the hood. Lysis solution containing β-mercaptoethanol was added onto the cells per well in 12-well plate. The cells were triturated and the lysate was collected into a clean tube. The lysate can be stored for -80^{o}C for further use or can be directly used. 70%

ethanol was added into lysate in equal volume. After vortexing for 5 seconds, ethanol-lysate mixture was transferred into an RNA-binding spin column. The column was centrifuged for 1 minute at maximum speed. The filtrate was discarded and 600 µl of 1x Low Salt Wash Buffer was added into column. The column was centrifuged for another 1 minute at maximum speed and the filtrate was discarded. The column was centrifuged for 2 minutes at maximum speed to remove residual solution. DNase solution (5 µl DNase I enzyme with 25 µl DNase Digestion Buffer) was added onto the filter inside the column. The filter was incubated in DNase solution at 37ºC for 15 minutes. DNase solution was washed with 500 µl 1X High Salt Wash Buffer and centrifuged for 1 minute at maximum speed. The filtrate was discarded and 600 µl 1X Low-Salt Wash Buffer was added. After centrifuging 1 minute at maximum speed, 300 µl 1X Low-Salt Wash Buffer was added. The column was centrifuged for 2 minutes at maximum speed. After completing the washing steps, the filter was put into a clean eppendorf tube. 30 µl Elution Buffer was added onto the filter and incubated for 2 minutes at room temperature. After incubation, tube was centrifuged for 1 minute at maximum speed. The purified RNA was either directly used in cDNA synthesis or stored for later use. The purified RNA can be stored at -20°C up to one month or at -80°C for longer periods.

To extract total RNA from N2A cells, 1 ml of Qiazol (Qiagen, Cat. #79306) was added onto cells. After Qiazol addition, the plate was shaken to dissociate cells and incubated for 5 minutes at room temperature. Dissociated cells were taken into a clean tube and 200 µl of chloroform was added. Then, the tube was shaken vigorously for 15 seconds and incubated for 3 minutes at room temperature to settle-down. The sample was centrifuged at 14000 rpm for 15 minutes at 4°C. After centrifugation, the upper phase was transferred into a clean tube. 500 µl of isoproponal was added into the mixture, vortexed, and incubated for 10 minutes at room temperature. Another centrifugation was carried out at 14000 rpm for 10 minutes at 4°C. The supernatant was discarded and RNA pellet was

washed with 1 ml of 75% ethanol without pipetting. The mixture was centrifuged at 10000g for 5 minutes at 4°C. The supernatant was discarded and pellet was air-dried for 20 minutes. After air dry, the pellet was dissolved with 50 µl of nuclease free water. The concentration was measured by Nanodrop2000c (Thermo Scientific). Extracted RNAs were stored at -80°C.

3.13 cDNA synthesis from total RNA samples

To synthesize cDNA from total RNA samples isolated from nucleofected primary neuronal cultures, Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Cat. #05081955001) was used. All steps of the protocol was performed on ice to avoid RNA degradation. In a PCR tube, 10 ng RNA and 2 µl random hexamer primers (600 µM) were mixed with water. Water was added to reach a final volume of 11.4 µl. The sample was denatured by incubation at 65ºC for 10 minutes. After incubation, the sample was taken on ice and following components of cDNA synthesis reaction were added as follows; 4 µl of 5x Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 0,5 µl Protector RNase inhibitor, 2 µl dNTP mix, 1 µl DTT (dithiothreitol) and 1,1 µl Transcriptor High Fidelity Reverse Transcriptase. Then, the reaction mixture was placed into block cycler with heated lid and treated at 29°C for 10 minutes, 48°C for 1 hour and 85°C for 5 minutes. The cDNA samples can be directly use or can be stored at -20ºC for later use.

To synthesize cDNA from total RNA samples isolated from N2A cells, Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Cat. #05081955001) was used. All steps of the protocol was performed on ice to avoid RNA degradation. In a PCR tube, 100 ng RNA and 2 µl random hexamer primers (600 µM) were mixed with water. Water was added to reach a final volume of 11.4 µl. The sample was denatured by incubation at 65ºC for 10 minutes. After incubation, the sample was taken on ice and following components of cDNA synthesis reaction were added as follows; 4 µl of 5x Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 0,5 µl Protector RNase inhibitor, 2 µl dNTP mix, 1 µl DTT

(dithiothreitol) and 1,1 µl Transcriptor High Fidelity Reverse Transcriptase. Then, the reaction mixture was placed into block cycler with heated lid and treated at 50°C for 30 minutes and 85°C for 5 minutes. The cDNA samples can be directly use or can be stored at -20ºC for later use.

3.14 Quantitative PCR (qPCR) with cDNA template

Luminaris HiGreen qPCR Master Mix (Thermo scientific, Cat. #K0991) was used for quantitative real time PCR (qRT-PCR). All the protocol was performed on ice. Master mix was prepared by mixing 7,8 µl PCR-grade water with 0,6 µl of respective forward and reverse primers for each reaction. 2x Luminaris Color q-PCR Master Mix was added into the master mix as 10 µl for each reaction. Master mix was covered from the light since Luminaris Color q-PCR Master Mix is light sensitive. 19 µl of the prepared mix was aliquoted to 8-strip PCR tubes with flat caps. 1 µl cDNA was added into the PCR tubes and mixed gently. Samples were briefly centrifuged and placed into qPCR block cycler. Applied cycle program was as follows: denaturation at 95oC for 15 seconds, annealing at 59-64oC for 30 seconds (depending on the primer pair used) and extension 72˚C for 30 seconds. The annealing temperature was changed according to primers used in the experiment. The cycle was repeated for 45 times. GAPDH primers were used in all experiments as reference. The cycle threshold (CT value) was recorded by the software. Three replicates for each reaction was prepared. The average of three technical replicates were calculated and normalized to GAPDH average read values. The normalized values were calculated by the formula $2^{(\text{GAPDH-Sample})}$.

3.15 Maintenance and Transfection of N2A cells

Cells were maintained in MEM containing 10% FBS, 1% penicillin/streptomycin, and 0.5% L-glutamate and passaged when they reach 80% confluency. Transfection was performed when cells reach 50-60% confluency. For one well in a 6-well plate, 2 tubes with 125 µl OptiMEM® were prepared. On one tube, 2500 ng plasmid DNA (500 ng of

Cas9nickase bearing vector and 500 ng of each sgRNA bearing vectors) and 5 µl P3000 reagent were added, while on the other tube 5 µl Lipofectamine 3000 was added. After 5 minutes incubation at room temperature, the two solution were mixed. The mixed solution was incubated at room temperature for 20 minutes. After the incubation, prepared solution was added onto the corresponding well dropwise.

3.16 Cloning of sgRNAs into b2-HU6A vector

Separate phosphorylation reactions were set-up for each forward and reverse oligos by incubating 10 µl oligo stock solution and 10 Unit Polynucleotide Kinase (New England Biolabs Cat. # M0201S) in 1X T4 DNA Ligase Buffer (containing ATP) (Thermo Scientific, Cat. #B69) for 1 hour at 37 ^{o}C and 20 minutes at 65 ^{o}C. 300 ng of backbone b2HU6-A vector was digested using 10 units of NotI-HF (New England Biolabs, Cat. # R3189S) in 1X CutSmart (New England Biolabs, Cat. #B7204S) for 2 hours at 37 oC. An additional 1 hour incubation was performed after 1 unit of FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific, Cat. # EF0654) was added. Annealing of the phosphorylated oligos to each other was performed by putting 5 µl of forward and reverse oligo into 40 µl oligo annealing buffer composing of 100 mM NaCl and 50 mM HEPES (pH 7.4) and incubating the reaction at 94 oC for 4 minutes and gradually decreasing the temperature to 40 ^{o}C. The incubation time was kept as 4 minutes for each step. Then the reaction was incubated at 37 ^{o}C for 20 minutes. Ligation reaction was set-up by mixing 2 µl of annealing product, 2 µl of digested vector, 5 unit T4 DNA ligase (Thermo Scientific, Cat. # EL0014) and 1x T4 DNA ligase buffer (Thermo Scientific, Cat. #B69). Transformation was performed as explained in the section 3.4 by using STBL3 bacteria. Plasmid DNA was extracted from the bacterial culture started from a single colony selected from the transformation plate. The extraction was performed using GeneJET Plasmid Miniprep Kit (Thermo Scientific, Cat. #K0503). Miniprep DNAs were directly sent to Sanger sequencing. After the sequences were confirmed, maxiprep culture was started by

adding 1.5 ml sample culture in 200 ml LB containing 200 µl ampicillin. After overnight incubation of the bacterial culture, the plasmid DNA was extracted by PureLink Maxiprep kit (Invitrogen, Cat. # K210006). The isolated high concentration plasmid DNA was stored in – 20ºC.

3.17 Generation of knock-out cells

After cells were transfected as described in section 3.12, puromycin selection was performed as follows until all untransfected cells died: One day after transfection, puromycin solution was added onto cells to reach 3 µg/ml final concentration. The media of the cells were changed with fresh puromycin containing media in every two days until selection was terminated. After puromycin selection, cells were transferred to 96-well plate for single cell isolation by serial dilution. First, cells were washed 3 times with 1X PBS. Then they detached by trypsin incubation for 5 minutes at 37 oC. Serially diluted cells were seeded into 96-well plate to reach 1 cell per well. However, the calculations were done as to have 2 cells per well to increase the possibility. 4 days after seeding the cells 100 µl MEM medium was added onto cells and. After the addition of 100 µl, medium was changed with fresh medium in every 4 days without washing the cells to avoid any harm. 2 weeks after seeding the cells into 96 well plate, cells were detached again by trypsin and split into 2 and seeded again in 96-well plates. One plate was used for PCR and the other was kept for propagation. Cells were lysed for PCR reaction after reaching 50% confluency by the addition of 50 µl Lysis Buffer (50 Mm KCl, 10 Mm Trris-HCl pH 8.8, 0.1 % Triton-X 100, freshly added 0,4 mg/ml Proteinase K) and incubated at 55 ºC for 1 hour followed by 10 minutes incubation at 95 ºC. 2 ul of the lysate was directly used as template for PCR reaction and the rest of the lysate can be kept in -20 ºC . PCR reaction mixture contained 1X Taq reaction buffer with KCl (Thermo Fisher, Cat. #B38), 200 µM dNTPs, 2 mM MgCl2, 0.1 µM forward primer and 0.1 µM reverse primer, 2 µl template DNA and 1 Unit Taq DNA Polymerase (Thermo Fisher , Cat. #18038042) as total reaction volume

arranged to 25 µl.). PCR conditions for denaturing, annealing and extension were 95oC for 10 seconds, 55ºC for 30 seconds and 72ºC for 1 minutes, respectively for 40 cycles. PCR products were analyzed by agarose gel electrophoresis. The expected sizes for products were 2400 bp for wild-type product and 2200 bp for the deletion product.

3.18 *In vitro* transcription of sgRNAs

DNA template for in vitro transcription was amplified from sgRNA expressing plasmids using T7_sgRNA forward and reverse primers. RNA was transcribed as the manufacturer's protocol by HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Cat. #E2040S). 1 ul of transcribed RNA was analyzed by agarose gel electrophoresis while the rest of it was used for RNA purification. RNA was purified by phenol/chloroform isoamyl extraction as following: The volume of RNA product was completed to 200 µl by adding necessary amount of nuclease-free water. Then, 20 ul of 3 M sodium acetate solution pH 5.2 was added to the mixture. 200 ul phenol/chloroform/isoamyl alcohol solution 25:24:1 (AppliChem, Cat. #3F002476) was added to the tube. The mixture was slowly vortexed for 5 minutes. Then, it was centrifuged for 5 minutes at 4 ºC at 14000 rpm. After centrifugation, the upper phase was collected into a clean tube and the extraction process was repeated. Then, 2 volume of 100% ethanol was added to the solution. The tube was incubated at -20 ºC for 2 hours. Then the pellet was collected by centrifugation at 14000 rpm for 5 minutes at 4ºC. The supernatant was removed and the pellet was rinsed with 500 µl of cold 70% ethanol without disturbing. The solution was centrifuged at 10000 rpm for 5 minutes at 4 ºC. The supernatant was discarded and the tube was air-dried for 30 minutes. After that, RNA pellet was resuspended in 50 µl of 0.1 mM EDTA solution pH 8.0. The concentration was measured and sgRNAs were diluted to 500 ng/µl in nuclease free injection buffer (5 mM Tris-HCl pH 7.4, 0.1 mM EDTA pH 8.0). Aliquoted solutions were stored at -80 ºC to be used for microinjections.

Chapter 4

RESULTS

4.1 NEUROD2 recruitment to *Stim1* intronic element significantly increases reporter gene expression in HEK293T cells

I cloned *Stim1* intronic element into pGL5_luc backbone vector in front of E4T minimal promoter. I co-transfected HEK293T cells with this vector and NEUROD2 expressing vector to observe the changes in luciferase reporter gene expression levels. I cloned two fragments of *Stim1* intronic element in different lengths into backbone vector separately to observe the effect of fragment length on reporter gene expression levels. Both fragments encompass four E-box elements found in *Stim1* intronic element. The longer fragment is 570 bp long which corresponds to full sequence of strong NEUROD2 peak located on intron 2 obtained from ChIP-Seq whereas the other fragment is 253 bp long (Figure 4.1).

I conducted the experiment in two different time points as 24 hours after transfection and 48 hours after transfection to test if the change in expression levels varies with time. NEUROD2 causes a significant upregulation in luciferase activity when recruited to a site upstream of a minimal promoter directly followed by reporter gene (Figure 4.2 and 4.3).

This result was not unexpected since NEUROD2 is known to act as transcriptional activator when bound to either endogenous promoter sequences or promoters cloned immediately upstream of reporter genes [7], [20], [21], [42] . Although 253 bp fragment leads to a higher firefly luciferase activity compared to Renilla luciferase, fold changes of luciferase activity are similar in both fragments when NEUROD2 is recruited. This result

again is not unexpected since both fragments encompass four E-box elements. Although the changes are more robust when the luciferase activity was measured 48 hours after transfection, 24 hours is sufficient to observe the changes in luciferase activity. Therefore, following experiments were performed at 24 hours after transfection.

Neurod2 knock-down have been shown to cause an increase in *Stim1* mRNA levels and STIM1 protein levels [8]. It might be argued that NEUROD2 might act as repressor so that its knock-down causes an increase in the expression levels which otherwise is repressed by NEUROD2 binding to *Stim1* intronic element. However, my luciferase assay results indicate that NEUROD2 binding to *Stim1* intronic element does not cause a general repression in gene expression levels suggesting NEUROD2 does not function as repressor binding to *Stim1* intronic element.

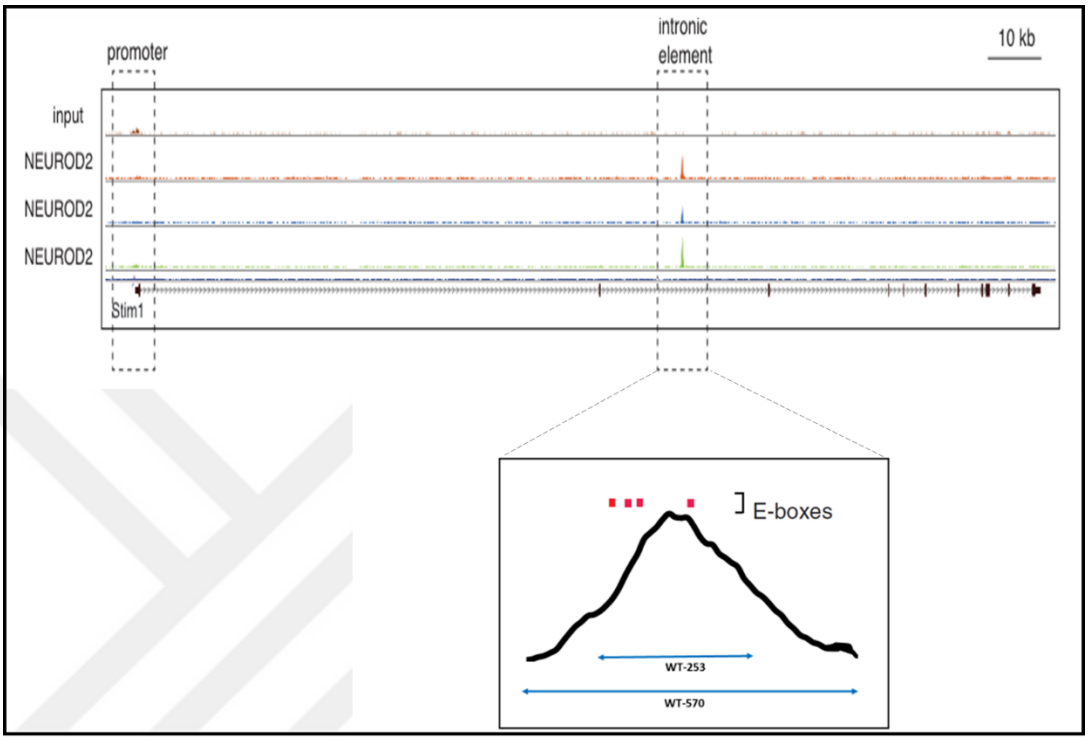

Figure 4.1: Representation of genomic locations of the fragments of *Stim1* intronic element cloned into luciferase reporter vector. The upper part of the figure shows NEUROD2 binding peak obtained from P0 ChIP-Seq experiment can be seen on the second intron of *Stim1* gene obtained from 3 different NEUROD2 antibodies shown in red, blue, and green separately. The lower part of the figure represents the genomic location of cloned fragments into WT-570 and WT-253 constructs. WT-570 encompasses the full binding site for NEUROD2 on *Stim1* intron 2. WT-253 have a shorter fragment of the intronic element while still encompassing all 4 E-box elements. Black curve represents the binding peak of NEUROD2 on *Stim1* intronic element. Red boxes represent the locations of E-box elements within *Stim1* intronic element.

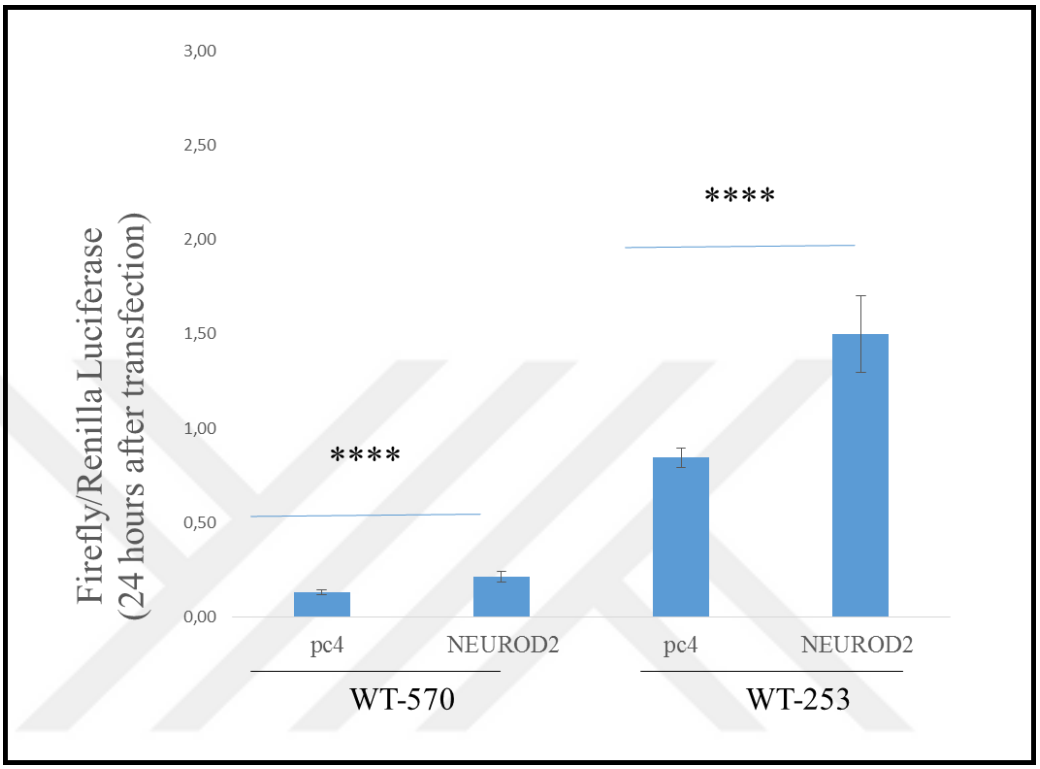

Figure 4.2: Quantification of luciferase activity measured from HEK293T cell lysates 24 hours after transfection. Cells were transfected either with a luciferase reporter downstream of a full fragment of *Stim1* intronic element (WT-570) or a shorter fragment (WT-253). Also, cells were co-transfected with either an empty (pc4) or a NEUROD2 expressing (NEUROD2) pcDNA4 vector. Firefly luciferase signal was normalized to Renilla luciferase signal. Data represent three independent experiments with each sample measured in quadruplets (n=12). Two-tailed Student's test was applied for statistical analysis. (**** $p < 0{,}0005$)

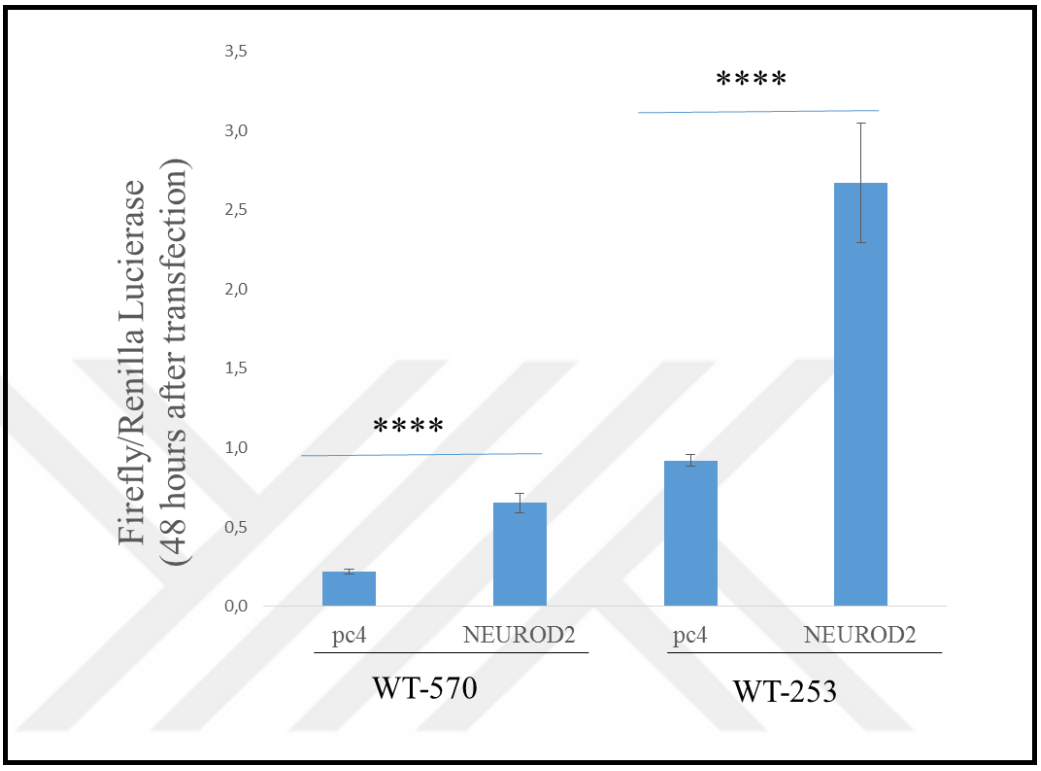

Figure 4.3: Quantification of luciferase activity measured from HEK293T cell lysates 48 hours after transfection. Cells were transfected either with a luciferase reporter downstream of a full fragment of *Stim1* intronic element (570) or a shorter fragment (253) 48 hours after transfection. Also, cells were co-transfected with either an empty (pc4) or a NEUROD2 expressing (NEUROD2) pcDNA4 vector. Firefly luciferase signal was normalized to Renilla luciferase signal. Data represent three independent experiments with each sample measured in quadruplets (n=12). Two- tailed Student's test was applied for statistical analysis. (**** $p < 0{,}0005$)

4.2 Creating MUT-570 construct by site directed mutagenesis

After showing that NEUROD2 recruitment to Stim1 intronic element increases reporter gene expression, I created a mutant construct where I mutated all E-box elements by site directed mutagenesis to show that NEUROD2's effect on luciferase activity does not result from an unspecific binding instead result from binding on Stim1 intronic element. For this purpose, I cloned 570 bp fragment of *Stim1* intronic element upstream of the transcription start site of the luciferase gene in pXPG backbone vector. pXPG vector does not contain any promoter sequence upstream of luciferase gene. I used pXPG vector to prevent unwanted effects of NEUROD2 on luciferase activity levels by binding to its promoter region. Then, I destroyed E-box elements one by one by changing the nucleotide sequences by site-directed mutagenesis and the mutations were confirmed by Sanger sequencing (Figure 4.4).

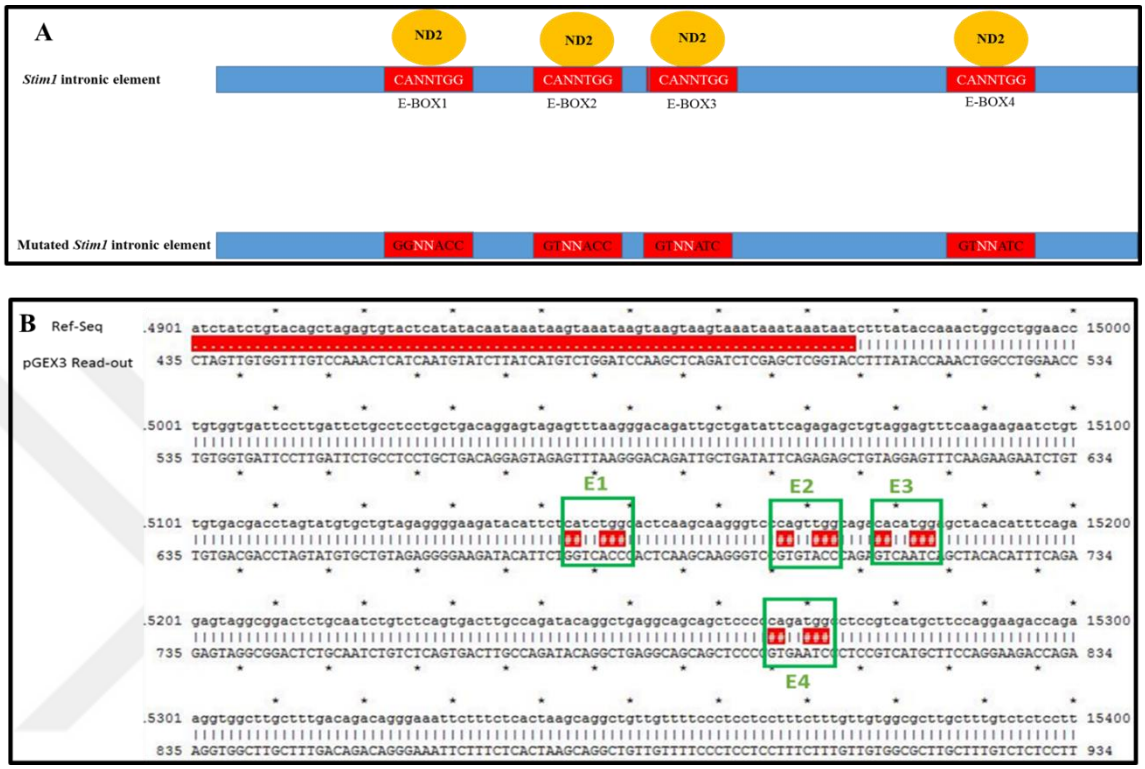

Figure 4.4: Preparation of MUT-570 construct by site-directed mutagenesis **A.** Schematic representation of mutations created by site-directed mutagenesis on E-box elements within *Stim1* intronic element. NEUROD2 is no longer able to bind *Stim1* intronic element after mutations. ND2: NEUROD2 **B.** Alignment of *Stim1* intronic element within created mutant construct to reference sequence of mouse *Stim1* intronic element taken from UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9) Assembly. pGEX3 primer was used for Sanger sequencing. E1: E-box1, E2: E-box2, E3: E-box3, E4: E-box4

4.3 E-boxes are required for NEUROD2 recruitment to *Stim1* intronic element

I cloned 570 bp long *Stim1* intronic element into pXPG backbone vector upstream of transcription start site of the luciferase gene (WT-570). To test if E-box elements were required for NEUROD2 recruitment to this site, I created a mutant construct by site-directed mutagenesis as described in section 4.2 (MUT-570). I co-transfected HEK293T cells with the reporter construct along with either an empty or a NEUROD2 expressing construct and I measured luciferase activity 24 hours after transfection. NEUROD2 is not capable to influence reporter gene expression when MUT-570 construct was used indicating that E-box element are required for NEUROD2 recruitment to this site (Figure 4.5).

NEUROD2 recruitment to wild type *Stim1* intronic element located upstream of luciferase reporter gene increases reporter activity when different constructs that have either pGL5 or pXPG backbone were used. However, since NEUROD2 binding site on *Stim1* is located in an intronic region, far from transcription start site, this reporter construct most likely was not able to recapitulate the effect of NEUROD2 on endogenous *Stim1* expression.

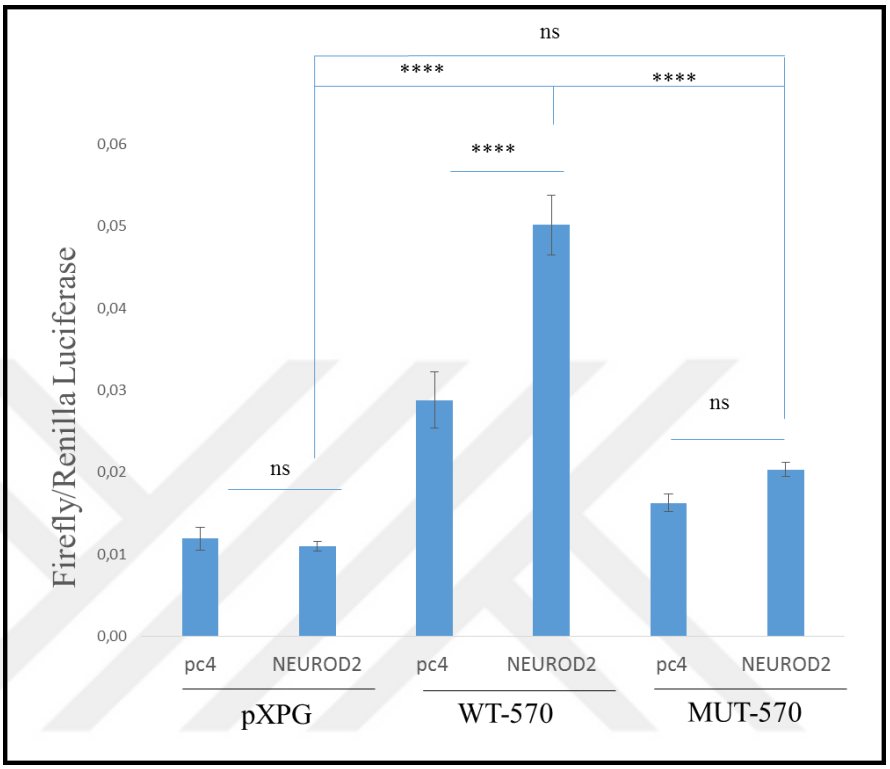

Figure 4.5: Quantification of luciferase activity measured from HEK293T cell lysates transfected with mutant construct. Cells were transfected either with an empty luciferase reporter plasmid (pXPG) or with a luciferase reporter downstream of a wild-type (WT-570) or mutated 570 bp fragment (MUT-570) *Stim1* intronic element. Also, cells were co-transfected with either an empty (pc4) or a NEUROD2 expressing (NEUROD2) pcDNA4 vector. Firefly luciferase signal was normalized to Renilla luciferase signal. Data represent seven independent experiments with each sample measured in triplicates (n=21). Two-tailed Student's test was applied for statistical analysis. (**** $p < 0{,}0005$) ns: nonsignificant)

4.4 Effect of *Neurod2* overexpression on *Stim1* expression levels

After showing that NEUROD2 increases reporter gene expression by luciferase assay, I tested the effect of *Neurod2* overexpression in endogenous *Stim1* expression levels in both cortical neurons and N2A cells by RT-qPCR.

For that purpose, first, I nucleofected mouse primary cortical neurons with NEUROD2-myc expressing plasmid and measured *Stim1* mRNA levels in two different time points as 2 days *in vitro* (2DIV) and 4 days *in vitro* (4DIV) . I observed a significant increase in *Stim1* mRNA levels at both 2DIV and 4DIV samples upon *Neurod2* overexpression. Although knock-down of *Neurod2* was reported to cause an increase in *Stim1* expression in mouse primary cortical neurons [8], an overexpression might cause an imbalance for the cells that might explain why Stim1 mRNA levels were not downregulated in mouse primary cortical neurons nucleofected with NEUROD2-myc expressing construct.

I also tested the effect of *Neurod2* overexpression on *Stim1* mRNA levels in N2A cells by transfecting them with NEUROD2-myc expressing plasmid and measured *Stim1* mRNA levels. I did not observe a significant change in *Stim1* mRNA levels upon *Neurod2* overexpression in N2A cells. These results together suggest that again NEUROD2 do not function as repressor when binds to *Stim1* intronic element.

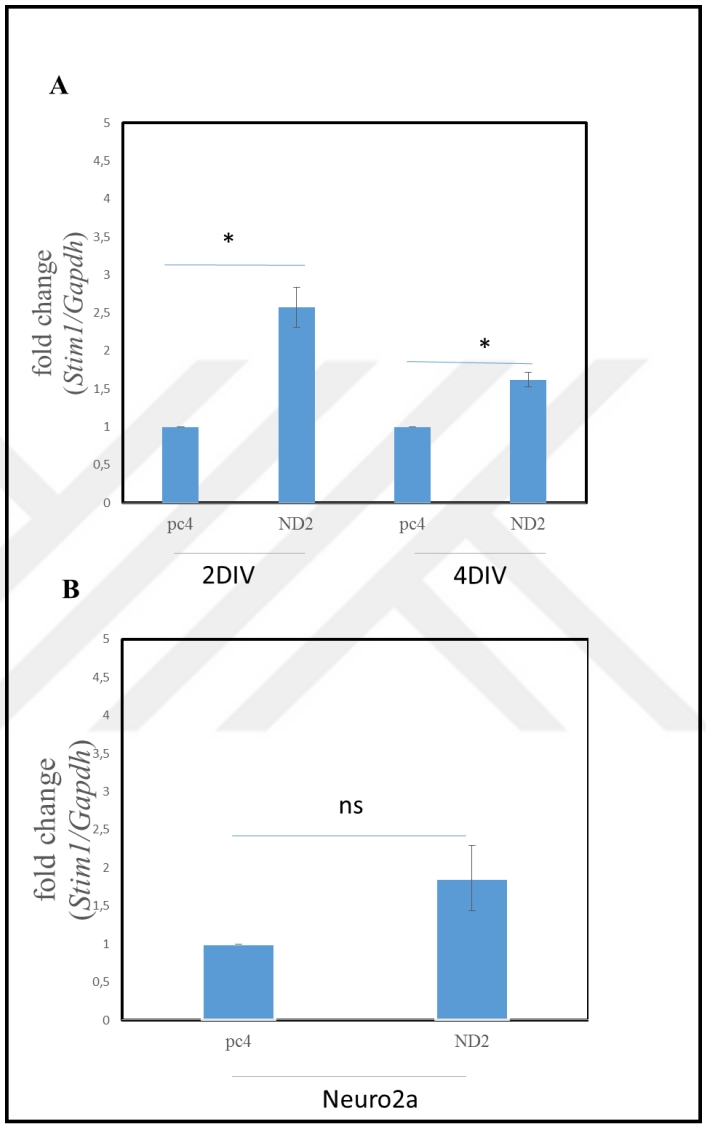

Figure 4.6: Effect of *Neurod2* overexpression in *Stim1* mRNA levels **A.** *Neurod2* overexpression causes an increase in *Stim1* mRNA levels in cortical neurons. RT-qPCR results of *Stim1* mRNA levels normalized to *Gapdh* mRNA in mouse primary cortical neurons transfected with either NEUROD2-myc expressing construct (ND2) or empty backbone (pc4). Total mRNAs were isolated in either 2 days *in vitro* (2DIV) or 4 days *in*

vitro (4DIV). Data represent three technical replicates. Two-tailed Student's test was applied for statistical analysis. **B.** *Stim1* mRNA levels do not change upon *Neurod2* overexpression in N2A cells. RT-qPCR results of *Stim1* mRNA levels normalized to *Gapdh* mRNA in N2A cells transfected with either NEUROD2-myc expressing construct (ND2) or empty backbone (pc4). Total mRNAs were isolated 48 hours after transfection. Data represent three biological replicates each composed of three technical replicates. Two-tailed Student's test was applied for statistical analysis.

4.5 STIM1 levels do not change upon *Neurod2* overexpression in either N2A or HEK293T cells

After testing the effect of *Neurod2* overexpression on *Stim1* mRNA levels, I also tested its effect on STIM1 protein levels. For this purpose, I transfected both N2A cells and HEK293T cells with either a NEUROD2-myc expressing plasmid or an empty plasmid and measured STIM1 levels by immunoblotting. I did not observe any significant change in STIM1 levels upon *Neurod2* overexpression in either N2A cells or HEK293T cells (Figure 4.7) There might be cell type specific binding partners of NEUROD2 that are either not present or not functioning in either N2A cells or HEK293T cells. This might explain why *Stim1* expression levels change upon changes in *Neurod2* levels in primary cortical neurons as previously shown in section 4.4 but not in either N2A or HEK293T cells.

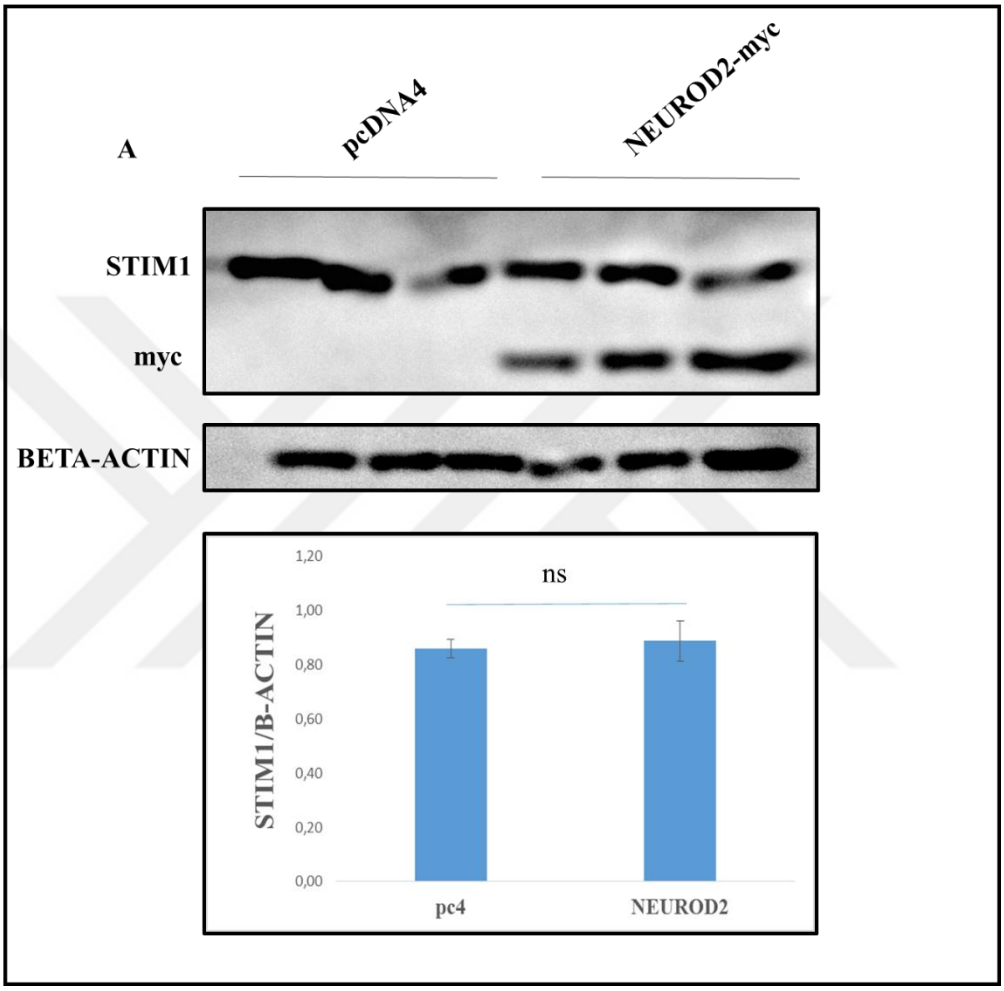

Figure 4.7: Effect of *Neurod2* overexpression on STIM1 levels in N2A cells **A.** Western blot result of STIM1 levels upon *Neurod2* overexpression in N2A cells. Lysates were collected from transfected N2A cells with Neurod2-myc expressing construct and blotted. The backbone vector pcDNA4 was transfected as control. **B.** Quantification of STIM1 protein levels. Western blot results were quantified by Image J software based on mean signal intensity of bands. STIM1 protein levels were normalized to BETA-ACTIN loading control. Two-tailed Student's test was applied for statistical analysis (p=0.32)

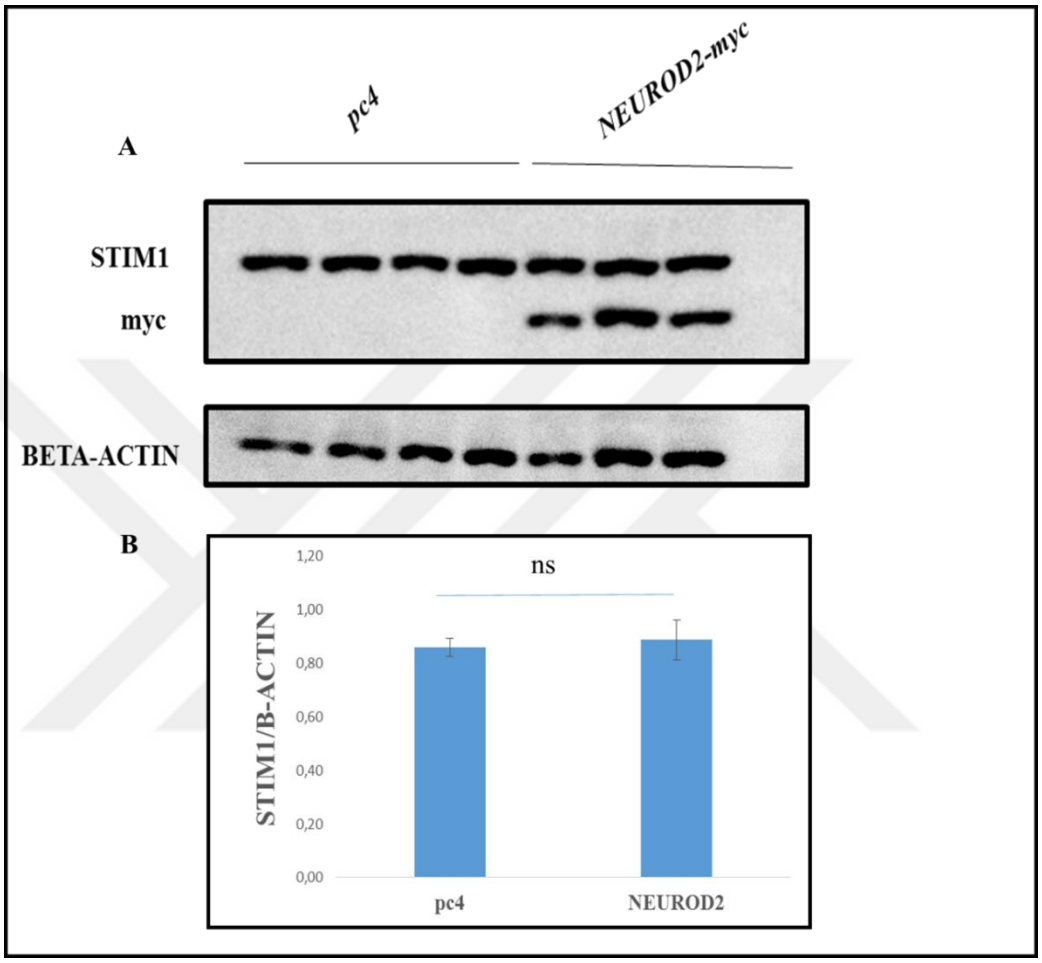

Figure 4.8: Effect of *Neurod2* overexpression on STIM1 levels in HEK293T cells **A.** Western blot result of STIM1 levels upon *Neurod2* overexpression in HEK293T cells. Lysates were collected from transfected HEK293 cells with Neurod2-myc expressing construct and blotted. The backbone vector pcDNA4 was transfected as control. **B.** Quantification of STIM1 protein levels. Western blot results were quantified by Image J software based on mean signal intensity of bands. STIM1 protein levels were normalized to BETA-ACTIN loading control. Two-tailed Student's test was applied for statistical analysis (p=0.97)

4.6 Effect of NEUROD2 on SOCE in mouse primary cortical neuorons

Since previous data from our lab showed that knocking down *Neurod2* resulted in an upregulation in STIM1 levels[8], the effect of NEUROD2 on neuronal SOCE was also previously tested by calcium imaging experiments conducted at 7-9 days *in vitro* and an upregulation of SOCE was observed upon *Neurod2* depletion. Additionally, the effect of *Neurod2* overexpression on SOCE was also tested again at 7-9 days *in vitro* and a reduction of SOCE was observed upon *Neurod2* overexpression [8]. To test if NEUROD2 regulation of SOCE would still be observed at an earlier time point as similar to 7-9DIV, I performed calcium imaging experiments at 5 days *in vitro* (5DIV). Towards this aim, I transfected mouse primary cortical neurons prepared from E14.5 embryos with either *Neurod2* shRNA or non- silencing shRNA expressing pSuperneo-mCherry plasmid at 2DIV. For rescue experiments, I co-transfected resND2 construct in pcDNA4 together with shRNAs.

Calcium imaging protocol that I used for the experiments can be divided into three phases. In the first phase, basal calcium signal was recorded from the cell as reference. For the second phase, cells were treated with thapsigargin and the medium was changed with a calcium-free medium. Thapsigargin blocks SERCA channels sitting on ER membrane and therefore blocks the ability of the cell to pump calcium inside ER. Thapsigargin application causes a small peak in recorded signals because of the calcium release from ER. Due to the lack of calcium in the medium, cells cannot replenish ER calcium stores during the second phase. Calcium was added in the third phase so that the extracellular calcium can be taken inside the cell via SOCE. Calcium entry causes a rapid increase in signals recorded from the cell in this last phase.

I did not observe any significant change in SOCE response between *Neurod2* silencing shRNA transfected neurons (ND2 shRNA) and the neurons transfected with rescueNeurod2 expressing construct along with *Neurod2* silencing shRNA (rND2) as can

be seen on the second peak of the graph (Figure 4.9.A). The initial peak height of SOCE response and the area under the peak region was further quantified .The similarity between ND2 shRNA SOCE and rND2 SOCE was also shown by plotting normalized peak height and peak area (Figure4.9.B, C). This similarity might be explained by potentially not sufficient duration between the time of transfection (2DIV) and time of imaging (5DIV) to observe the effect of *Neurod2* overexpression on SOCE response. This argument by itself may not be sufficient to explain the higher SOCE response observed in non-silencing shRNA transfected neurons (NS) compared to either ND2 shRNA or rND2. However, when I analyzed the first peak indicating the initial ER calcium release upon thapsigargin treatment, I observed that both ND2 shRNA and rND2 have lower initial ER calcium release (Figure 4.9.D). This suggests that the health of the recorded cells was not good causing to record a lower SOCE response during the experiments.

After testing the effect of *Neurod2* silencing on SOCE response of neurons in 5DIV time point, I also tested the effect of *Neurod2* overexpression on SOCE response of neurons. Again, I did not observe any difference in SOCE response between *Neurod2* overexpressing cells transfected with rNeurod2 construct along with non-silencing shRNA (NS +ND2) and cells transfected with empty vector along with non-silencing shRNA (NS+pc4) (Figure 4.10 A, B, C). The initial calcium release of NS +ND2 and NS+pc4 was in similar levels (Figure 4.10 D, E). This result suggest that the effect of *Neurod2* overexpression on SOCE response shown before at 7-9DIV [8] starts at a later point than 5DIV.

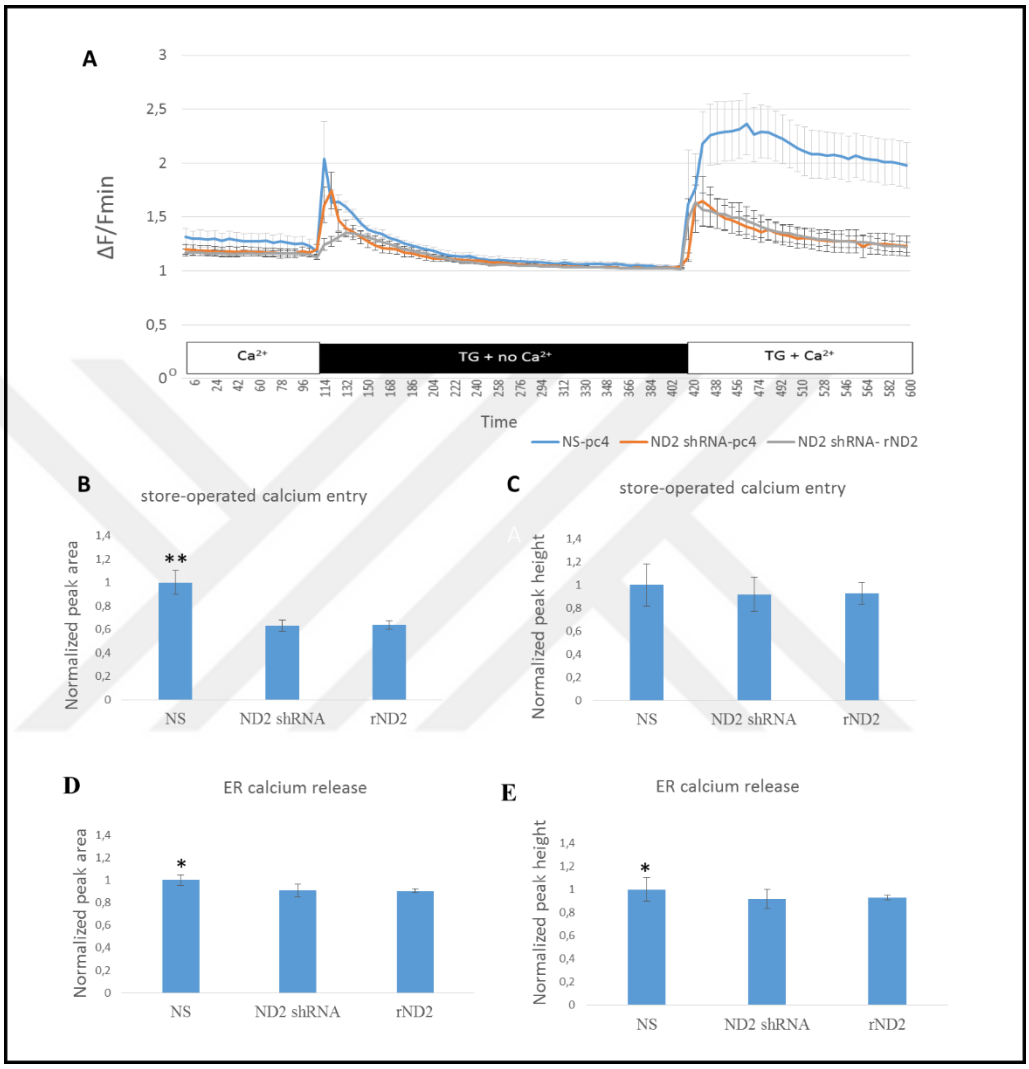

Figure 4.9: Effect of *Neurod2* knock-down on SOCE response at 5DIV mouse cortical neurons **A.**SOCE response in *Neurod2* shRNA treated neurons. Mouse primary cortical neurons were prepared by dissecting E14.5 mice cortices. 300.000 cells/well were seeded into 24-well plate. ImageJ software was used to measure signal. Background corrected signal normalized to minimum signal (Fmin) obtained form that sample, and the normalized values (ΔF/Fmin) are plotted. Ca^{2+} indicates Ca^{2+} containing medium, TG + no

Ca^{2+} indicates thapsigargin treatment in calcium lacking medium, TG + Ca^{2+} indicates Ca^{2+} containing medium with thapsigargin. Ca^{2+} concentration is 2 mM in the medium. Average of three biological replicates represented in the plot. n= 8 for non-silencing shRNA, n=10 for *Neurod2* silencing shRNA, n= 15 for *Neurod2* shRNA + rNeurod2. X axis represents time in seconds. Bars in the plot indicate standard error of mean. **B.** Quantification of the total area under the peaks corresponding store-operated calcium response of cells. Normalized values were plotted. Two-tailed Student's test was applied for statistical analysis by comparing each condition with each other. ($p<0.01$) **C.** Quantification of initial peak heights of store-operated calcium entry. Normalized values were plotted. Two-tailed Student's test was applied for statistical analysis by comparing each condition with each other. **D.** Quantification of the total area under the peaks corresponding initial ER calcium release of cells. Normalized values were plotted. Two-tailed Student's test was applied for statistical analysis by comparing each condition with each other. ($p<0.05$) **E.** Quantification of initial peak heights of ER calcium release. Normalized values were plotted. Two-tailed Student's test was applied for statistical analysis by comparing each condition with each other. ($p<0.05$)

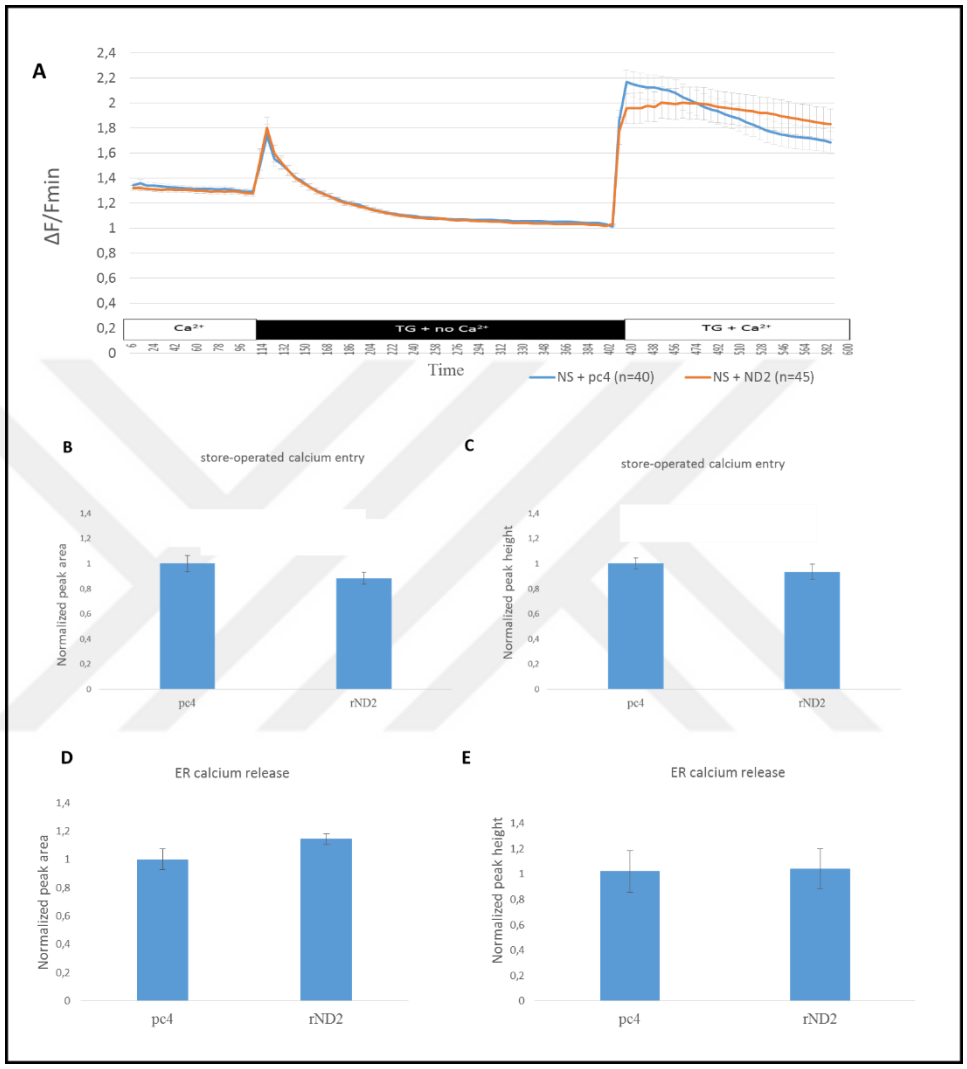

Figure 4.10: Effect of *Neurod2* overexpression on SOCE response at 5DIV mouse cortical neurons **A.** SOCE response in shRNA resistant *Neurod2* overexpressed neurons. Mouse primary cortical neurons were prepared by dissecting E14.5 mice cortices. 300.000 cells/well were seeded into 24-well plate. ImageJ software was used to measure signal. Background corrected signal normalized to minimum signal (Fmin) obtained form that sample, and the normalized values (ΔF/Fmin) are plotted. Ca^{2+} indicates Ca^{2+} containing

medium, TG + no Ca^{2+} indicates thapsigargin treatment in calcium lacking medium, TG + Ca^{2+} indicates Ca^{2+} containing medium with thapsigargin. Ca^{2+} concentration is 2 mM in the medium. Average of three biological replicates represented in the plot. n=40 for non-silencing shRNA + pc4, n=45 for non-silencing shRNA + rNeurod2. X axis represents time in seconds. Bars in the plot indicate standard error of mean. **B.** Quantification of the total area under the peaks corresponding store- operated calcium response of cells. Normalized values were plotted. Two-tailed Student's test was applied for statistical analysis. (p=0.20) **C.** Quantification of initial peak heights of store-operated calcium entry. Normalized values were plotted. Two-tailed Student's test was applied for statistical analysis. (p=0.40) **D.** Quantification of the total area under the peaks corresponding initial ER calcium release of cells. Normalized values were plotted. Two-tailed Student's test was applied for statistical analysis. (p=0.18) **E.** Quantification of initial peak heights of ER calcium release. Normalized values were plotted Two-tailed Student's test was applied for statistical analysis. (p=0.74)

4.7 Cloning of sgRNAs into b2-HU6A vector

To show that NEUROD2 regulates *Stim1* expression levels by directly binding to its intronic region, I aimed to mutate E-box elements located on *Stim1* intronic region in their genomic loci by CRISPR/Cas9 genome editing system. For that aim, I transfected N2A cells with a Cas9 protein expressing vector along with the vectors expressing single guideRNAs (sgRNA) that direct Cas9 protein to DNA sequences of interest. To perform transfection, I cloned sgRNAs targeting *Stim1* intronic element separately into b2-HU6A vector.

I designed two sgRNA pairs (named as sgRNA1, sgRNA2, sgRNA3, and sgRNA4) to target both the start and the end of *Stim1* intronic element. Therefore, the entire region encompassing all 4 E-box elements can be deleted at once. I used b2HU6A vector to

express sgRNAs under human U6 promoter. I digested b2HU6A vector with NotI restriction enzyme to complement the ends of the sgRNA oligonucleotides. Then, I inserted annealed oligonucleotides into digested b2HU6A vector. To confirm the insertions, I directly sent the plasmid DNA extracted from miniprep cultures to Sanger sequencing without any diagnostic digestion step since NotI site was destroyed after ligation.

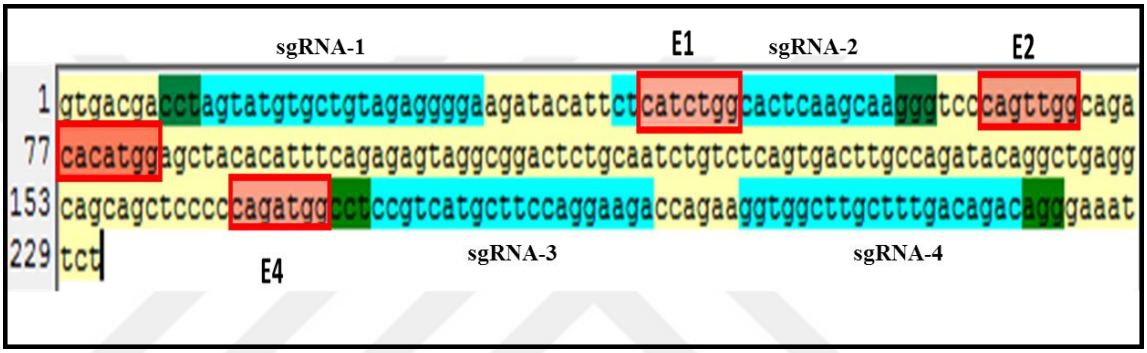

Figure 4.11: Representation of targeted DNA sequences by corresponding sgRNAs on *Stim1* intronic element. Blue boxes indicate target sequences of sgRNAs. Green boxes indicate protospacer adjacent motifs (PAM sequences) necessary for Cas9 protein binding on DNA. Red boxes indicate E-box elements (E1-E2-E3-E4).

4.8 Deletion of *Stim1* intronic element in N2A cells by CRISPR/Cas9 genome editing

After I created sgRNA expressing plasmid, I transfected N2A cells with a Cas9 protein expressing vector along with the vectors expressing sgRNAs to delete the region of Stim1 intronic element encompassing E-box elements in their genomic loci. For transfection, I seeded 300.000 N2A cells/well in 6-well plates. 24 hours after seeding, I transfected cells with either a Cas9 protein expressing vector along with vectors expressing each sgRNA cloned or with a vector expressing a modified version of Cas9 protein called Cas9nickase along with vectors expressing each sgRNA. While wild-type Cas9 protein creates double-stranded breaks, Cas9nickase creates a nick in one strand. To delete a large

region of DNA (such as in our case 200 bp), a pair of sgRNAs are required when a wild-type Cas9 protein is used. However, two pairs of sgRNAs are required if Cas9nickase protein is used instead of wild-type Cas9 protein. Since Cas9nickase requires additional sgRNAs, the specificity of the deletions created increases while the efficiency decreases.

24 hours after transfection, I added puromycin into the medium as a selection antibiotics for cells. Both wild-type Cas9 and Cas9nickase expressing vectors express puromycin resistance gene as selection marker. Therefore, I continued puromycin selection until I observed no live cell in untransfected control cells. For selection, I optimized puromycin concentration to 3 μg/ μl after I tested different concentrations to determine a certain concentration which cause the death of almost all untransfected cells within 5 days.

Wild-type Cas9 expressing vector (wt-Cas9) that I used in the experiments has a lentiviral backbone. Transient transfection of wt-Cas9 yielded a lower transfection efficiency compared to transfection of Cas9nickase expressing vector as I compared the number of cells remained after puromycin selection. Following the termination of puromycin selection, I detached the cells and transferred them into 96-well plates for single cell isolation. To achieve one cell seeded per well, I counted the cells and I performed the required dilutions accordingly. Cells were able to reach 30-40% confluency at the end of 2 weeks while I changed the medium to a fresh medium in every 4 days. Then, I detached the cells again to create two replicates for each single cell colony. After they reached 50% confluency, I extracted DNA from the cells. I set-up a PCR reaction using that template DNA to detect if the region of interest was successfully deleted. 31 single cell colonies in total from three different biological replicates were analyzed to detect deletion. However, neither a homozygous nor a heterozygous deletion was observed since a 2200 bp fragment was not encountered during PCR screen.

Table 4.1: Number of single cell colonies tested/will be tested for deletion

	wt-Cas9	Cas9-nickase
Number of negative colonies	2	29
Number of colonies that will be tested	12	40
Total number of colonies	14	69

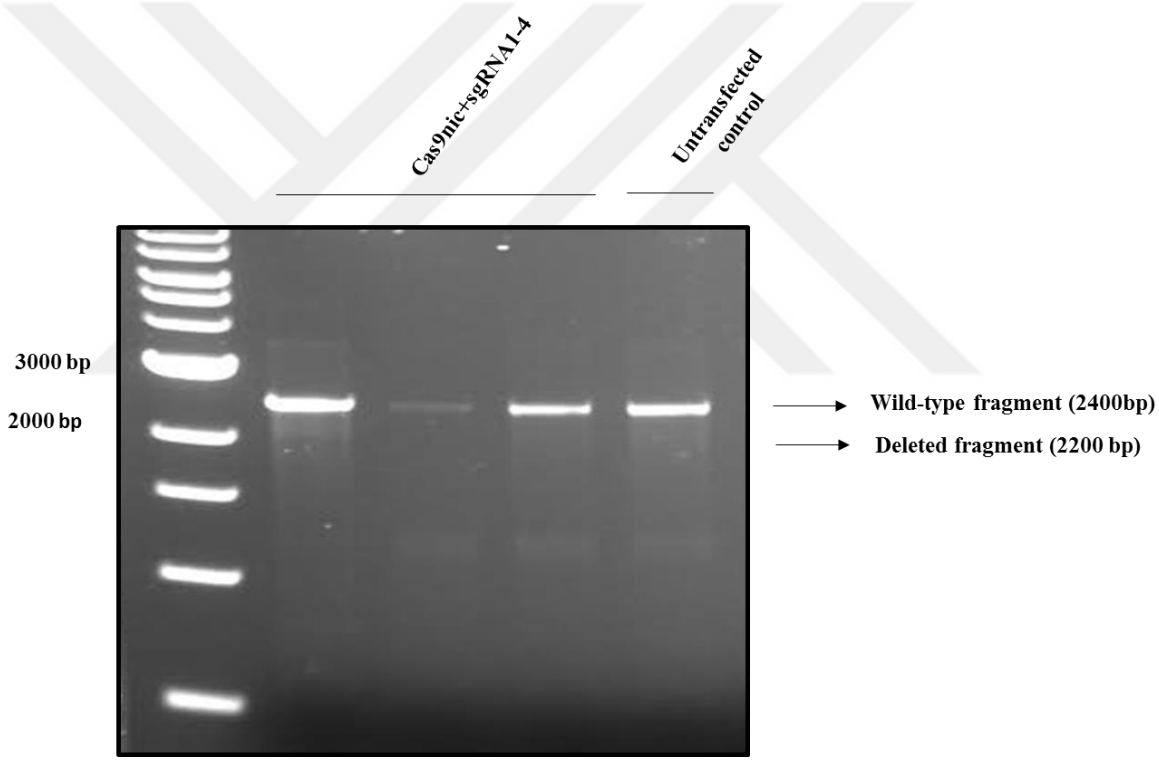

Figure 4.12: Gel electrophoresis image of DNA extracted from single cell colonies. Colonies were isolated from N2A cells transfected with guideRNA expressing vectors (sgRNA1, sgRNA2, sgRNA3, sgRNA4) along with Cas9nickase expressing vector. 2400 bp fragments represents non-deleted wild type fragment as present in untransfected control

line. The size of expected fragment upon deletion was 2200 bp. However, a deleted fragment was not observed among the examined single cell colonies.

4.9 *In vitro* transcription of sgRNAs

In addition to aiming to delete *Stim1* intronic element in cell line, we also aimed to delete it *in vivo* in mouse embryos by CRISPR/Cas9 genome editing system to generate genetically modified mice carrying the deletion. For this purpose, we planned to microinject sgRNAs and commercially available purified Cas9 protein into fertilized oocytes rather than microinjecting the vectors expressing Cas9 protein and sgRNAs into the nucleus to increase the efficiency. Therefore, I performed *in vitro* transcription to produce sgRNAs using T7 RNA Polymerase after I prepared DNA template for *in vitro* transcription. To prepare DNA template, I used oligonucleotides carrying minimal T7 promoter sequence in the correct orientation to amplify sgRNA sequences from sgRNA expressing vector. After transcription, I analyzed the transcribed RNAs by agarose gel electrophoresis (Figure 4.13). Gel electrophoresis revealed that some unspecific RNAs longer than the expected size of sgRNAs were also produced by *in vitro* transcription. However, I continued with the purification of sgRNAs. After purification, I diluted sgRNAs to a final concentration of 500 ng/ µl and stored at -80 ^{o}C to be used for microinjections into mouse embryos.

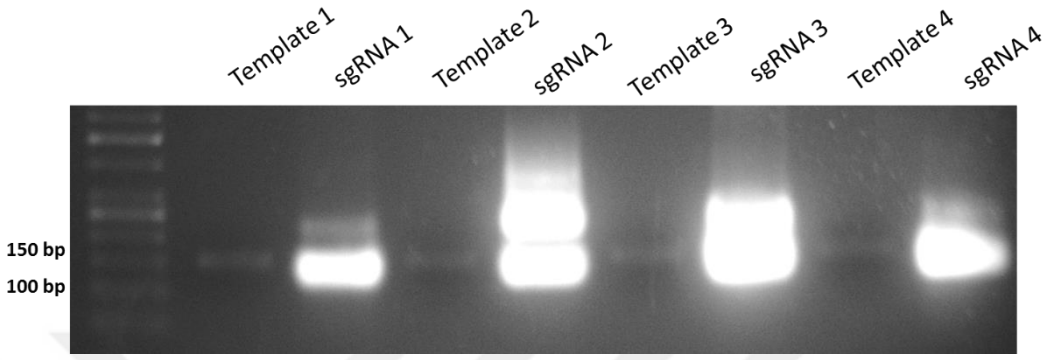

Figure 4. 13: Gel electrophoresis image of *in vitro* transcribed sgRNA1-4. The expected size for transcribed sgRNAs was 100 bp whereas the expected size for template DNAs was 128 bp due to the additional T7 promoter sequence. Lane 2, 4, 6, and 8 represent template DNA for sgRNA1-4 respectively. Template DNAs were loaded proportional to amount of sgRNAs to be able to compare the transcribed amount of RNA.

Chapter 5

DISCUSSION

In this study, the function of NEUROD2 transcription factor binding on an intronic region of *Stim1* gene have been analyzed. *Stim1* gene encodes for STIM1 protein which senses the ER calcium stores level and activates store-operated calcium entry. It has been showed that *Neurod2* knock down results an increased neuronal SOCE response whereas *Neurod2* overexpression leads to a decreased neuronal SOCE [8]. Analyzing the function of this intronic binding of NEUROD2 on *Stim1* gene is important to elucidate the mechanism of NEUROD2 regulation on store-operated calcium entry.

Although the increase in STIM1 protein levels as well as in *Stim1* mRNA levels was previously shown upon *Neurod2* knock-down, the effect of *Neurod2* overexpression on STIM1 levels have not been reported before. My results suggest that *Neurod2* overexpression does not cause a significant repression in STIM1 levels. Therefore, the regulation of Stim1 expression by NEUROD2 may require additional factors leading to a complex regulation mechanism. Interestingly, a binding site of insulator protein CTCF (CCCTC-binding factor) that overlapped with NEUROD2 binding site within *Stim1* intronic element have been revealed by ChIP-Seq analysis conducted in P0 mouse forebrain tissue [43]. CTCF is a transcriptional factor that act as transcriptional repressor by binding to chromatin insulators and preventing interaction between promoter and nearby enhancers [44]. Therefore, it is possible that *Stim1* intronic element may function as an insulator. Hence, the influence of inappropriate enhancer activity on *Stim1* promoter may be blocked upon NEUROD2 binding on *Stim1* intronic element. Possible protein-protein

interactions between NEUROD2 and CTCF can be examined in future experiments to test if NEUROD2 functions as a part of an insulator complex.

By using a reporter system, I have shown that clustered E-box elements within *Stim1* intronic element is required for NEUROD2 recruitment to this site since the effect of NEUROD2 on reporter gene expression significantly diminishes when E-box elements are mutated. Additionally, deletion of *Stim1* intronic element in its genomic locus would reveal if this sequence is required for regulation of *Stim1* gene expression by NEUROD2. If this sequence is required for NEUROD2 regulation, silencing *Neurod2* would not change *Stim1* gene expression levels in neurons in which *Stim1* intronic element is deleted.

Stim1 intronic element must be deleted in neurons to test the effect of NEUROD2 on neuronal SOCE. Since cultured neurons cannot be passaged due to the inability of neurons to divide, generating a mouse model lacking *Stim1* intronic element and using it for primary neuronal culture preparation is an effective way to study the effects of NEUROD2 on neuronal SOCE upon *Stim1* intronic element deletion. Additionally, this mouse model can also be used to test if the deletion of *Stim1* intronic element has any effect on cortical development. SOCE is known to play a significant role in neural progenitor cell proliferation in early phases of cortical development [14]. If NEUROD2 limitation on SOCE is prevented by the deletion of *Stim1* intronic element, neural progenitor cell proliferation may last longer than needed. This may cause anomalies in neuronal differentiation; hence, it may cause abnormalities in cortical development. Therefore, it would be interesting to investigate the effects of *Stim1* intronic element deletion on cortical development by using a mouse model. CRISPR/Cas9 genome editing system significantly reduces the required time and the cost of genetically modified mouse generation compared to the traditional methods based on homologous recombination. I have aimed to delete *Stim1* intronic region in N2A cell line in addition to *in vivo* experiments that we conducted in collaboration to confirm the efficiency of generated sgRNAs.

If *Neurod2* overexpression does not cause a repression in *Stim1* expression levels as my results suggest, it may be argued that the inhibitory effect of *Neurod2* overexpression on neuronal SOCE as previously reported [8] may not be directly related to the changes in STIM1 protein levels. As SOCE dependent calcium signaling is important in the regulation of cell function, the components of SOCE including STIM1 are expected to be under tight regulation by various mechanisms. In fact, additional mechanisms for SOCE regulation can rely on regulation of STIM1 localization since translocation of STIM1to ER-plasma membrane junctions is crucial to start SOCE. Tian et al. described a cAMP and protein kinase A dependent mechanism that regulates STIM1 puncta formation [45]. Moreover, cytoplasmic adaptor Homer proteins have been reported to enhance STIM1 and ORAI1 interaction [46]. STIM1 tyrosine phosphorylation is also required for the association between STIM1 and ORAI1 [47]. Another mechanism of STIM1-mediated SOCE regulation revealed depends on cholesterol-enriched lipid rafts [48]. Additionally, STIM1-mediated SOCE is further regulated by mitochondria buffering ER calcium release; hence, enhancing store depletion [49]. Therefore, it may be possible that *Neurod2* overexpression causes a change in SOCE response via another mechanism rather than controlling STIM1 protein levels but it remains to be elucidated.

Ca^{2+} ions play central role in many different cellular processes. Elucidating the mechanism of NEUROD2 regulation of SOCE would enhance our understanding of these processes including learning and memory. As being an ER Ca^{2+} sensor, STIM1 can potentially operate synaptic strength. Indeed, Garcia-Alvarez et al. have demonstrated that *Stim1/Stim2* double knockout mouse shows an increased long-term potentiation of excitatory synaptic transmission (LTP) [50]. An increase in LTP have been also reported upon the suppression of constraints in cyclic adenosine monophosphate (cAMP) signaling [51], [52] while STIM1 was shown to affect cytosolic cAMP levels [53]. Interestingly, this

process occurred independently of changes in cytosolic Ca2+, which is characteristic of SOCE.

To date, the role of SOCE in cortical development have not extensively studied. Recently, Rash and his colleagues have showed that Ca^{2+} fluxes propagate bidirectionally within the elongated fibers of radial glial cells (RGCs) using an optogenic sensor in the mouse embryonic forebrain [54]. Their findings provide a novel communication mechanism that links the proliferative and postmitotic zones before the onset of synaptogenesis. Ca^{2+} activity along RGC fibers is crucial for this communication mechanism providing feedback information along the radial migratory pathway. These results revealed a novel role for Ca^{2+} activity during early cortical development that influence neurogenesis and migration. Burst of Ca^{2+} activity in migrating neurons including RGC fiber Ca^{2+} activity causes induction of saltatory migratory movements that gives a motivational force to migratory neurons to exit the proliferative zones and to reach their destined cortical layers [54]. The specific role of SOCE in this Ca^{2+} regulated mechanism remains to be elucidated.

Dysregulation of ER Ca^{2+} homeostasis have been also associated with elimination of dendritic spines during neurodegenerative diseases, such as Alzheimer's disease (AD). Elimination of dendritic spines results in synaptic failure and memory loss [55]. An increase in ER Ca^{2+} concentration causes a compensatory increase in inositol phospho three receptor (IP_3R) and ryanodine receptor (RyR) expression and a down-regulation in SOCE in familial AD cases with mutations in presenilin genes PSEN1 and PSEN2 which are the sub-component of gamma secretase that is responsible for the cutting of amyloid precursor protein APP [55], [56]. In another study that associates SOCE to FAD pathogenesis, Stim2- SOCE-CaMKII pathway has been shown to be impaired in hippocampal neurons isolated from the PS-1 M146V knock-in (KI) mouse model of FAD [57]. Therefore,

understanding SOCE regulation in neurons potentially lead novel therapeutic strategies for the treatment of neurodegenerative diseases.

Here, this work has contributed to efforts put in to understand the function of NEUROD2 binding on *Stim1* intronic element which eventually is required to uncover the mechanism of NEUROD2 regulation of neuronal SOCE. Deletion of *Stim1* intronic element may reveal its possible insulator function. To date NEUROD2 have been reported as transcriptional activator with the exception of Guner et al. suggesting a possible function of NEUROD2 in an insulator complex [8]. Experimental evidence for this hypothesis can be obtained by examining the effects of *Stim1* intronic element deletion *in vivo*.

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VITA

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Appendix: Primer List

Primer Name	Primer Sequence
stim long kpn1 forward	5’ AAAGGTACCTTTATACCAAACTGGCCTGG3’
stim long xba1 reverse	5’AAATCTAGATTAACTCAGAATTTGTTCTTCTTTTTGTTAC3’
stim short kpn1 forward	5’ AAAGGTACCACATTCTCATCTGGCACTC 3’
stim short xba1 reverse	5’AAATCTAGAAGCAAGCGCCACAACAAAG3’
stim long HindIII reverse	5’AAAAAGCTTTTAACTCAGAATTTGTTCTTCTTTTTGTTAC 3’
stim short HindIII reverse	5’AAAAAGCTTAGCAAGCGCCACAAC 3’
stim1_e1 mut forward on e2	5’ CACTCAAGCAAGGGTCCGT 3’
stim1_E1 mut reverse	5’GGTGACCAGAATGTATCTTCCC 3’
stim1_E2 mut forward	5’ CAGACACATGGAGCTACAC3’
stim1_E2 mut reverse	5’ GGTACACGGACCCTTGCTTG3’
stim1_E3 mut forward	5’AGCTACACATTTCAGAGAGTAGGC 3’
stim1_e3 mut reverse on e2	5’GATTGACTCTGGGTACACGGACC 3’
stim1_E4 mut forward	5’ CCTCCGTCATGCTTCCAGGA3’
stim1_E4 mut reverse	5’ GATTCACGGGGAGCTGCTGCCTC3’
T7 sgRNA 1 F	5’GAAATTAATACGACTCACTATAGGGAGAAGTATGTGCTGT AGAGGGGACGGCCGCAGAAGACCTGTTTTAGA 3’
T7 sgRNA 2 F	5’GAAATTAATACGACTCACTATAGGGAGATTGCTTGAGTGC CAGATGAGCGGCCGCAGAAGACCTGTTTTAGA 3’

T7 sgRNA 3 F	5’GAAATTAATACGACTCACTATAGGGAGACCGTCATGCTTC CAGGAAGACGGCCGCAGAAGACCTGTTTTAGA 3’
T7 sgRNA 4 F	5GAAATTAATACGACTCACTATAGGGAGAGTCTGTCAAAGC AAGCCACCGGCCGCAGAAGACCTGTTTTAGA 3’
sgRNA R	5’AAAAAAGCACCGACTCGGTG3’
forward nested primer	5’CCCACTGATAGACTGATGAGAAACTAT 3’
reverse nested primer	5’ GCTCCTAACATACCTCAGTCAAAGTAG 3’