

Neurogenic transcription factor control of store-operated calcium entry in cortical neurons

by

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This is to certify that I have examined this copy of a master's thesis by

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To My Dearest Family...

ABSTRACT

Neurogenic differentiation factor 2 (NEUROD2) transcription factor, a member of basic helix-loop-helix transcription factors, is a factor with essential roles in brain development. Upon calcium binding, NEUROD2 mediates its transcriptional role and promotes neurogenesis. Studies performed in our laboratory have identified genes that are targeted by NEUROD2, and verified that *Stim1* gene is among the top targets of NEUROD2.

My thesis is composed of three major parts. In the first part, I investigated the effect of NEUROD2 on its targets with known functions in calcium signaling. Specifically, I found NEUROD2 targets several genes present in Ca^{2+} / Calmodulin-dependent protein kinase (CaMK) pathway. Also, I investigated whether a suppression of *Neurod2* expression causes defects in the transcription of its targets.

In the second part, I focused on one specific target of NEUROD2 called *Stim1*. We confirmed NEUROD2 binding to a conserved sequence within the second intron of *Stim1*. Further, a suppression of *Neurod2* expression by shRNA transfection was associated with upregulation of STIM1 protein and mRNA levels. Since STIM1 is a sensor of ER Ca^{2+} levels, I investigated the role of NEUROD2 in store operated Ca^{2+} entry (SOCE). I demonstrated that knockdown of *Neurod2* caused an upregulation of neuronal SOCE, which was rescued by expression of shRNA-resistant form of *Neurod2*.

In the last part of my experiments, I studied a potential regulatory mechanism of NEUROD2 activity. I demonstrated that NEUROD2 is composed of cytosolic and nuclear fractions. Further, the cytoplasmic fraction was associated with a higher molecular weight NEUROD2, most likely representing a post-translational modification (PTM). I tested whether this PTM was a SUMO moiety, however my results were inconclusive.

Taken together, my results suggest that NEUROD2 acts as a general regulator of neuronal calcium signaling. Future experiments will focus on identifying specific mechanisms of how NEUROD2 influences SOCE.



ÖZET

Bazik heliks-lup-heliks yazılım faktörü familyasından olan Neurogenic differentiation factor 2 (NEUROD2) yazılım faktörü, beyin gelişiminde önemli bir role sahiptir. NEUROD2, kalsiyum bağlanmasıyla, yazılım faktörü rolünü gerçekleştirir ve nöron oluşumuna öncülük eder. Laboratuvarımızdaki çalışmalar NEUROD2'nin genomda hangi genleri hedef aldığını saptamıştır. Bu hedef genler arasında yer alan *Stim1* en yüksek skorlardan birine sahiptir.

Tezim üç ana kısımdan oluşmaktadır. İlk kısımda, NEUROD2'nin kalsiyum sinyalinde rol aldığı bilinen hedef genleri üzerindeki etkisini inceledim. NEUROD2'nin Ca^{2+} / Calmodulin dependent protein kinase (CaMK) metabolic yolunda bulunan birkaç geni hedef aldığını buldum. Ayrıca *Neurod2* seviyesini baskılayanın bu genlerin anlatımı üzerinde bir etkisi olup olmadığını inceledim.

İkinci kısımda, özellikle NEUROD2'nin *Stim1*adlı hedef genine odaklandım. NEUROD2'nin *Stim1* geninin intronunda bulunan korunmuş bir sekansa bağlandığını bulduk. Ayrıca NEUROD2 seviyesini baskılayanın STIM1'de protein ve mRNA seviyesinde artışa yol açtığını gözlemledik. STIM1 ER'daki kalsiyum iyonu seviyesini algılayan bir protein olduğundan, ben NEUROD2'nin store-operated calcium entry (SOCE) üzerindeki görevini inceledim. *Neurod2* seviyesini baskılayanın nöronlarda SOCE'yi arttırdığını ve bu artışın shRNA dirençli bir *Neurod2* ile kurtarıldığını gözlemledim.

Son kısımda, NEUROD2 üzerinde olası bir control mekanizmasını çalıştım. NEUROD2'nin sitoplazma ve çekirdekte bulunduğunu gözlemledim. Ayrıca, sitoplazmadaki NEUROD2'nin moleküler ağırlığı normalden daha fazla olan bir formu bulunduğunu gözlemledim. Bu ağır form büyük bir olasılıkla bir çevirim sonrası değişim (ÇSD)

olayını göstermektedir. Bu ÇSD'nin SUMO olup olmadığını test ettim, fakat sonuçlarım bir kanıya varmak için yetersizdi.

Özet olarak, sonuçlarım NEUROD2'nin genel bir nöronal kalsiyum sinyali regülatörü olduğunu göstermektedir. Gelecek deneyler NEUROD2'nin SOCE'yi nasıl etkilediğini bulmaya yoğunlaşacaktır.



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NOMENCLATURE

AM	Acetoxymethyl
APS	Ammonium persulfate
BME	Basal medium eagle
Bp	Base pair
BSA	Bovine serum slbumin
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DIV	Days in vitro
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiothreitol
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
HBSS	Hank's balanced salt solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IF	Immunofluorescence
IP	Immunoprecipitation
Kb	Kilobase
kDa	Kilodalton
LB	Lysogeny broth

MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
Na-DOC	Sodium deoxycholate
NEM	N-ethylmaleimide
NLS	Nuclear localization signal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNK	Polynucleotide kinase
PVDF	Polyvinyl difluoride
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Real time quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	Sarco/endoplasmic Ca^{2+} ATPase
shRNA	Small hairpin ribonucleic acid
TBS-T	Tris buffered saline-tween
TEMED	Tetramethylethylenediamine
TG	Thapsigargin
TTX	Tetradotoxin

Chapter 1

INTRODUCTION

The brain is able to execute various different functions, from controlling motor movement to forming abstract thoughts. This functional diversity of the brain is achieved by complex connections between billions of neurons. Precise localization and connections of these neurons are essential for a properly functioning brain. It is largely unknown how the neurons can find their proper location and connection targets among this vastly complex structure.

All the cells found in the brain arise from neuroepithelial (NE) cells found in the neural tube [1]. These cells are in pluripotent state and do not show neuronal identity. During development, the pluripotent cell population gradually decrease as more cells attain neuronal properties. They lose their ability to divide, start to extend neurites and form neuronal connections called synapses. All these steps in neurogenesis are tightly regulated to achieve the complex and well defined layout of the brain.

Transcription factors are among the important elements in regulation of neurogenesis. Sequential expression of transcription factor cascades allow regulation of neuronal development [2]. Basic helix loop helix (bHLH) transcription factors are good examples for sequential transcriptional regulation. bHLH transcription factors maintain progenitor cell

population in early development by promoting self-renewal. Later in development, glial or neuronal cell fate determination is also regulated by bHLH transcription factors [3].

Besides transcription factors, secondary messenger molecules are also important elements of neurogenesis. Among these secondary messengers, the Ca^{2+} ion is especially important for neurons, regulating many downstream target pathways. Due to its importance, intracellular Ca^{2+} concentration is tightly regulated, both spatially and temporally, by a variety of Ca^{2+} buffer proteins and Ca^{2+} channels [55]. In neurogenesis, Ca^{2+} is involved at different stages, from cell fate determination to synapse formation and neurite extension [4][5].

Neurod2, a member of the NeuroD family bHLH transcription factors, is involved in neuronal differentiation. *Neurod2* can induce neurogenesis when ectopically expressed in non-neuronal cells [6]. NEUROD2 mediated transcription is activated upon Ca^{2+} entry, allowing activity dependent regulation of transcription in neurons [7]. The role of NEUROD2 is not limited to the neurogenesis period, as its expression pattern is developmentally regulated and sustained through adulthood [6][8].

In order to elucidate gene targets of NEUROD2 in a developmental context, chromatin immunoprecipitation (ChIP) experiments were performed on embryonic day 14 (E14) and postnatal day 0 (P0) mouse cortices [9][10]. The intronic region of *Stim1* gene was identified as one of the most prominent NEUROD2 target in the whole genome. *Stim1* is involved in the store-operated calcium entry (SOCE) mechanism, allowing refilling of ER Ca^{2+} stores when they are depleted. Additionally, Ca^{2+} / calmodulin kinase activation pathway appear as a NEUROD2 targeted pathway in P0 ChIP analysis [10].

In this study, I aimed to identify the functional role of NEUROD2 binding to *Stim1* intron. Since STIM1 regulates SOCE, I examined if there is any change in SOCE response

in the absence of NEUROD2. Briefly, I knocked down *Neurod2* in primary cultured neurons by transfecting neurons with *Neurod2*-targeting shRNA expressing plasmids. I examined the SOCE with live calcium imaging by loading calcium sensitive dyes on the shRNA-treated primary cultured neurons. The neurons treated with *Neurod2* shRNA showed higher SOCE response. This indicates absence of NEUROD2 may increase the expression level of STIM1. To ensure the observed SOCE phenotype was specifically due to knockdown of *Neurod2*, but no other off target effect of *Neurod2* shRNA, I expressed shRNA-resistant form of *Neurod2* in shRNA treated neurons. Expression of shRNA-resistant *Neurod2* rescued the knockdown phenotype. High SOCE response observed in *Neurod2* shRNA treatment is reduced upon shRNA-resistant *Neurod2* expression. In parallel, *Neurod2* overexpression have resulted in lowered SOCE response in the neurons.

Additionally, by quantitative polymerase chain reaction (qPCR) using genomic DNA fragments obtained by NEUROD2 ChIP on P0 cortices, I confirmed that NEUROD2 binds to genes involved in Ca^{2+} / calmodulin kinase activation pathway. However, my reverse transcription quantitative polymerase chain reaction (qRT-PCR) analysis done on total RNAs isolated from *Neurod2* knockdown primary culture neurons did not show a significant change in expression levels of the genes involved in this pathway.

Furthermore, upon obtaining preliminary results showing that NEUROD2 might be also present in the cytoplasm of the neurons, I performed subcellular fractionation on mice cortices collected at different developmental stages. Western blot results of the cytosolic and nuclear fraction showed that NEUROD2 is indeed present in the cytoplasm of the neurons. Cytosolic presence of NEUROD2 does not appear to be dependent of developmental stage. The fractionation experiments also suggested a possible SUMOylation on NEUROD2. I performed immunoprecipitation (IP) experiment by immunoprecipitating NEUROD2 and checking SUMO protein by western blotting. NEUROD2 does not appear to be

SUMOylated, as no NEUROD2 specific SUMO signal obtained from western blotting of IP samples.

In the chapter 2 of the thesis, 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 and 5 contains the results of the experiments and their critique and implications.



Chapter 2

LITERATURE REVIEW

2.1 Overview

The brain is responsible for performing many complex tasks, such as detecting sensory information, regulating motor movements and executing cognitive functions. Proper architecture and connections of an enormous number of neurons in the brain are essential in controlling these complicated functions. Thus, development of the brain to accurately form these structures is crucial for its function.

The brain and other parts of the central nervous system originated from the neural plate which is derived from ectoderm, the outermost germ layer. After forming from the ectoderm, the neural plate lengthens in the rostroradial direction while it mediolaterally narrows down. The elongated neural plate starts folding inside, and this bent neural plate creates a structure named the neural tube. These events leading to the formation of the neural tube are called neurulation [11].

The neural plate and the neural tube are composed of neuroepithelial cells (NE) [1]. These cells are the progenitors of all cell types found in the brain. NE cells divide and cover the surface of the ventricle. At the beginning of neurogenesis, NE cells start to turn into radial glial cells (RGC). RGCs have a polarized structure. Their cell body is found in the ventricular zone (VZ) while the cells extend to the pial surface. Importantly, these cells have the ability to divide both symmetrically and asymmetrically. Symmetric divisions allow the number of

RGCs to increase. In asymmetric division, one daughter develops to a more differentiated state, into immature neurons or basal progenitors (BP), while the other daughter cell remains as RGC [12]. The newly formed immature neurons start to migrate toward the pial surface via protrusions of RGCs, while the BP cells migrate to the subventricular zone (SVZ), which is located immediately above VZ. The BP cells in the SVZ further divide and differentiate into neurons [12]. The neuron population in the brain is supplied by these events. After neurogenesis terminates, the remaining population of RGCs at VZ mostly become glial cells, while some fraction of RGCs remain through adulthood and preserve their neurogenic ability.

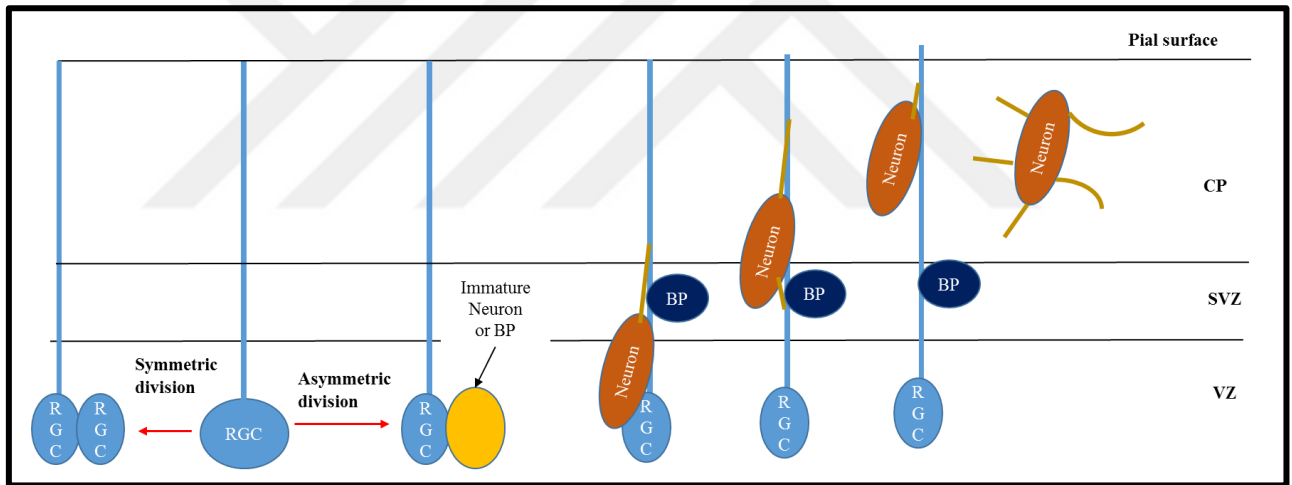


Figure 2.1: Representation of neuronal migration and differentiation. Symmetric divisions increase RGC population while asymmetric divisions create differentiated cells. Abbreviations: RGC, radial glial cells; BP, basal progenitor; VZ, ventricular zone; SVZ, subventricular zone; CP, cortical plate; MZ.

Although originated from single type of cell – from NE cells – the brain is composed of diverse types of neurons and glial cells. Accurate localization and interactions of these diverse types of cells can form networks that perform complex functions. For example, the neocortex found in the mammalian cerebral cortex is the site responsible for higher functions, such as voluntary movement, sensory perception, and complex cognitive abilities like reasoning, speaking and mathematical skills.

The neocortex is organized as six distinct layers, and each layer is morphologically different from the others [13]. Within these layers, there is a fine tuned balance of excitatory neurons (projection neurons) and inhibitory neurons (interneurons). Projection neurons usually have a pyramidal shape while interneurons have more circular cell body. The projection neurons use glutamate as neurotransmitter unlike interneurons which use γ -Aminobutyric acid (GABA) as neurotransmitter [14]. These two neuron types also originate from different sites within the brain. Projection neurons are born at the dorsal telencephalon, and migrate radially. Interneurons, on the other hand, form at the ventral telencephalon, at a structure named ganglionic eminence (GE). The GE is located far from the interneurons' destination; thus, these neurons migrate a long tangential route to the ventricle [14][15]. Radial migration of the neurons are important in the formation of the six-layered organization. The newly born cells migrate on top of the existing neurons and locate there, forming a new layer. Thus, the newborn neurons are found in the outer layers (layer II) while the first born neurons create layer VI. Layers are functionally distinct from each other; for example, layer IV contains excitatory neurons that projects to thalamocortical neurons, while layer II/III neurons make intercortical projections [16].

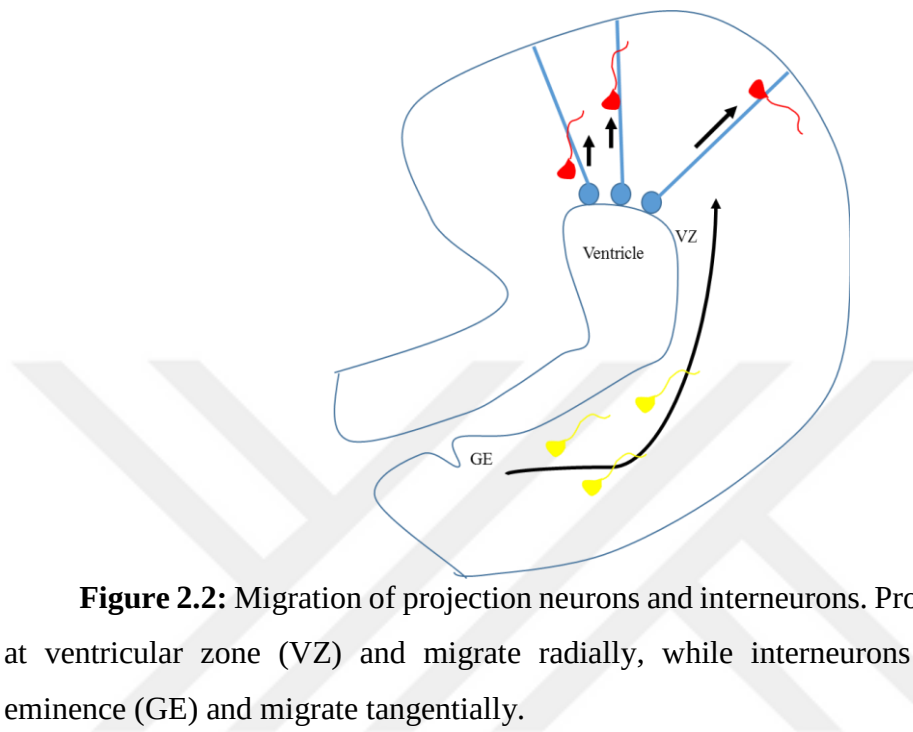


Figure 2.2: Migration of projection neurons and interneurons. Projection neurons born at ventricular zone (VZ) and migrate radially, while interneurons born at ganglionic eminence (GE) and migrate tangentially.

All these steps of neurogenesis occur under tight control via developmental cues. Transcription factors are crucial intracellular factors regulating neurogenesis. The basic helix-loop-helix (bHLH) transcription factors are known to be important in the process. Early in neurogenesis, progenitor cells should be kept in a non-differentiated, stem cell like state in order to increase their numbers to meet the necessary cell number. bHLH member Hes, Hey and Id family genes suppress the differentiation and promote proliferative state of the progenitor cells. Expression of neurogenins (*Neurog1* and *Neurog2*) and *Ascl1*, on the contrary, promote the neuronal fate. *Ascl1* is expressed in ventral telencephalon, where the inhibitory interneuron is born, and takes a role in inhibitory neuron differentiation. Similarly, *Neurog1/2* expressed in the dorsal telencephalon, promote excitatory projection neuron differentiation. Another bHLH factor *Olig 1/2* promotes oligodendrocyte formation, a type

of glia cells [3]. During development, bHLH proteins act in a cascade during their regulation over neurogenesis, one causing activation of the other bHLH proteins. These transcriptional waves control the differentiation of newborn neuron in neocortex. For example, expression of *Neurog2* is followed by *Tbr1* and *Neurod2* expression, which are neuronal bHLH transcription factors [2][17].

Along with the transcriptional machinery driving neurogenesis, signaling molecules and secondary messengers also play important roles for neurogenesis. For example, the inhibitory effect of Notch over RGC differentiation prevents early differentiation of these cells [17], maintaining the balance of proliferation and differentiation during neurogenesis. Cellular Ca^{2+} concentration has been shown to be effective in various steps during neural development. In early neurogenesis, intracellular Ca^{2+} levels are important in neuronal fate determination. Neuronatin, a protein found in the ER membrane, induces neuronal cell fate via regulating the intracellular Ca^{2+} levels by inhibiting ER- Ca^{2+} ATPase protein. The knockdown of neuronatin in embryonic stem cells decreases the proportion of cells that undergo neuronal differentiation. This knockdown phenotype is alleviated by presenting thapsigargin, which is known to block ER- Ca^{2+} ATPase, acts in parallel with neuronatin [4]. Proliferation of the neuronal stem cells are also under the influence of intracellular Ca^{2+} levels. Dysfunctions in store operated calcium entry (SOCE) of these cells leads to their impaired proliferation [18]. SOCE is known to be a component Ca^{2+} homeostasis within the cells, by facilitating ER refilling when Ca^{2+} levels in ER goes down. ORAI and STIM proteins are mediators of SOCE. When a nonfunctional *Orai1* mutant is presented or *Orai1* is knocked down, the proliferation rate of neural stem cells decreases [18].

Both spontaneous Ca^{2+} oscillations and activity dependent Ca^{2+} entry are necessary for neuronal differentiation. Spontaneous Ca^{2+} waves in the retina allows proper connections within neural circuits, before sensory input induces activation in these neurons [19]. Activity

dependent Ca^{2+} entry via voltage gated channels can facilitate transcription factors that regulate gene transcription important for neuronal differentiation. *CaMKIV* (Ca^{2+} /Calmodulin dependent protein kinase IV) and *CREB* (cyclic adenosine monophosphate (cAMP) responsive element binding) regulated transcription is shown to be effective in dendrite growth in mouse cortical neurons [5]. *CaMKIV* inhibition and dominant-negative form of *CREB* both can decrease dendrite growth [5]. *Neurod2* is another example for neuronal transcription factors that utilize activity dependent Ca^{2+} entry for facilitating its transcriptional role [7].

2.2 *Neurod2* Transcription Factor

Neurod2 is a member of the NeuroD family transcription factors, along with *Neurod1*, *Neurod4* and *Neurod6*. *Neurod2* was discovered by its sequence similarity to *Neurod1*, the first identified NeuroD family member. *NEUROD2*, like *NEUROD1*, can ectopically induce neuron formation, as overexpression of either *Neurod2* or *Neurod1* can induce neurogenesis in *Xenopus laevis* ectoderm. These two transcription factors bind to E-box motifs on genome, by forming heterodimers with E-proteins [6].

2.3 *Neurod2* expression pattern in mouse brain

Neurod2 expression starts at embryonic day 11 (E11), and its expression continues through adulthood in mice [6]. Early in development, at E12, *Neurod2* is present in spinal cord and the neocortex. At E18, *Neurod2* is found in many regions: neocortex, amygdala, cerebellum, spinal cord, optic tectum and brainstem. However, in adults, the expression of *Neurod2* is limited to the hippocampus, Purkinje cells in the cerebellum and in the cerebral cortex with low abundance. *Neurod2* expression was not observed in peripheral neurons [8] (Figure 2.1).

Expression of *Neurod2* is regulated by upstream transcription factors *Neurog1/2*. Microarray analysis has shown that the level of *Neurod2* expression decreases in *Neurog2* knockouts, and this expression decrease shown to be more severe when a double knockout of *Neurog1/2* is examined [20]. *Neurog1* regulation over *Neurod2* is also shown by a luciferase reporter assay, where *Neurod2* gene promoter followed by luciferase gene is presented together with different bHLH transcription factors. *Neurog1* was the only transcription factor that can express luciferase, indicating NEUROG1 can bind to *Neurod2* gene and induce its expression [21].

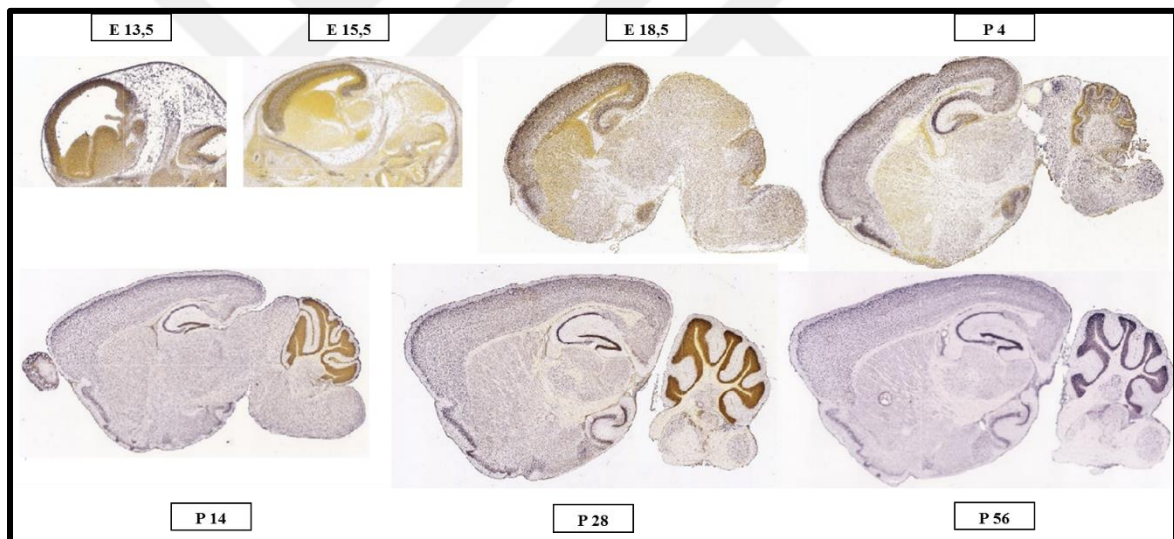


Figure 2.3: Expression pattern of *Neurod2*. Displayed by in situ hybridization in mouse brain at different ages, from embryonic day 13,5 to adulthood (Allen Brain Atlas).

2.4 Transcriptional activation of NEUROD2

Interactions and the activity of neurons are crucial for development of the brain, as was postulated in 1949, by Donald Hebb. Activity patterns in a neuron network refines the neuronal connections, enabling development of a properly functioning circuit. Ca^{2+} is an important secondary messenger for relaying this neuronal activity. With Ca^{2+} - regulated transcription factors, long term effects, such as neuronal development, can be linked to short action potential events. NEUROD2 is a Ca^{2+} - regulated transcription factors, activating its transcriptional activity in the presence of Ca^{2+} influx [7][25].

2.5 Roles of NEUROD2 in neurons

2.5.1 Neuronal Induction

NEUROD2 is able to induce neuronal differentiation in nonneuronal cell lines. Farah et al. assayed different neuronal bHLH proteins and observed their neurogenic ability. NEUROD2 was revealed as a prominent bHLH transcription factor for neuronal induction. NEUROD2 can effectively induce expression of neuron specific markers in the P19 embryonal carcinoma cell line. Neuronal induction efficiency of *Neurod2* is enhanced when it is co-expressed with E12 protein, dimerization partner of NEUROD2. *Neurod2-E12* co-expressed P19 cells also showed neurite extensions and gained electrical excitability properties, which are indications of neuronal phenotype [22]. Ability of NEUROD2 to induce neuronal differentiation is not only shown in P19 cell line, mouse neuroblastoma cell line N1E-115 also shown to undergo neuronal differentiation upon *Neurod2* expression [23].

2.5.2 Brain development

Observations showed that *Neurod2* knockout mice have smaller brains in size, compared to wild-type mice. The smaller size is especially observed in the regions where *Neurod2* expression is high, such as the cerebellum [8]. *Neurod2* knockout mice also displayed an increase in neuronal cell death. Higher rate of seizures, ataxia and motor deficits are seen in these *Neurod2* knockout mice, indicating a defective brain. *Neurod2* knockout mice have decreased levels of brain-derived neurotrophic factor (BDNF). Absence of BDNF is shown to cause ataxia and cerebellar cell death, similar to *Neurod2* knockouts [8]. Thus the cerebellar phenotype observed in *Neurod2* knockout mice might be partially related with the low expression of BDNF [8].

2.5.3 Thalamocortical connections

Thalamocortical connections function in transmitting sensory information gathered via sensory systems to the sensory cortex of the brain. The sensory information passes through the brainstem and thalamus before reaching the cortex. These connections are well-defined and their location on the cortex is precise. For example, each whisker of rodents correspond to a unique and discrete location within the somatosensory cortex at layer IV, called a barrel. Each whisker correspond to single barrel, and the distribution of the barrel cortex is highly similar with the positions of the whisker follicles [24].

In *Neurod2* knockout mice, these barrel formations are shown to be disturbed. The thalamocortical axons terminals were unable to form properly, and the thalamocortical synapses were shown to be defective. These deficiencies in thalamocortical connection on *Neurod2* knockout mice shows importance of NEUROD2 for proper thalamocortical development [25].

2.5.4 Synaptogenesis

Excitatory and inhibitory neurons can be distinguished by the neurotransmitter they have utilized in the synaptic transmission. Inhibitory neurons utilize GABA, while excitatory neurons use glutamate as neurotransmitter. These neurotransmitters are recognized by the receptors at the post-synaptic terminals. For GABA, GABAA and GABAB receptors are found, the former is an ionotropic channel and the latter is a metabotropic channel. For glutamate, three ionotropic receptors are found, NMDA, AMPA and kainate. NMDA receptors are shown to be important for early in synapse formation for proper connections, while AMPA receptors are important in mature synapses [26]. In *Neurod2* knockout mice, AMPA:NMDA ratio is lower compared to a wild type thalamocortical synapse, suggesting NEUROD2 functions in synaptic maturation [25].

Synapses of mossy fibers, which allows transmission from dentate gyrus to CA3 area of hippocampus, are also affected in *Neurod2* knockout mice. The number of thorny excrescences, dendritic sites of mossy fibers, are lower in the *Neurod2* knockout mice. Also, similar with observations made in thalamocortical synapses, AMPA:NMDA ratio at mossy fibers synapses of *Neurod2* knockout mice were lower than wild-type mice. *Neurod2* knockout mice have reduced expression of post-synaptic protein PSD-95, which may be the cause of synapse loss observed in absence of NEUROD2 [27].

Recently, it is shown that NEUROD2 also take role in inhibitory synapse formation. Chen et al. used *Neurod2* overexpression and knockdown primary cortical cultures at 18-20 DIV (days in vitro) for examining the role of NEUROD2 on synaptic excitation/inhibition balance. They found that *Neurod2* overexpression leads increased miniature inhibitory post-synaptic current (mIPSP) frequency, and increased number of inhibitory synapses. To identify potential NEUROD2 regulated genes that have role on synaptogenesis, Chen et al. performed microarray screen and compared the transcripts of *Neurod2*knockout and wild-

type neuronal culture. Gastrin-releasing peptide (GRP), a peptide known to involve in inhibitory neurotransmission with its receptor GRP receptor (GRPR), found to be significantly reduced in *Neurod2* knockout neurons. Applying GRPR inhibitor drug to *Neurod2* overexpressed cells have partially rescued the increased mIPSP frequency phenotype, suggesting the effect of NEUROD2 on inhibitory synapse formation might be partially due to GRP levels [28].

2.5.5 Commissural fiber tract formation

Two hemispheres of the brain connects via commissural fiber tracts. These fibers are composed of axon bundles. There are three commissural fiber tracts, corpus callosum, hippocampal commissure and anterior commissure. These commissural fibers originate from pyramidal projecting neurons in the cortex, and extend to other hemisphere.

Analysis done in *Neurod2* / *Neurod6* double mutant mice revealed that corpus callosum and anterior commissure was defective in absence of NEUROD2 and NEUROD6. These findings showed that NEUROD2 and NEUROD6 together are required for formation of commissural fiber tracts [29].

2.5.6 Emotional learning and memory

Emotional learning and memory is shown to require proper neuronal activity in amygdala. Amygdala is essential for formation of fear conditioning, which is a paradigm used for studying emotional learning and memory [30]. Observations made in *Neurod2* +/- mice revealed that the number of neurons on amygdala is found lower compared to wild-type mice. When there is no NEUROD2 is present, as in *Neurod2* -/- mice, lateral and basolateral nuclei of the amygdala was absent [31]. Fear conditioning experiments done on *Neurod2* heterozygous mice revealed the defects in emotional memory formation of these mice.

AMPA receptors of lateral amygdala take part in fear conditioning, as they possess important function in long-term potentiation event. During fear conditioning, the AMPA receptors (AMPA) localize to synapses in lateral amygdala. Reduced levels of AMPARs in amygdala is observed in *Neurod2* heterozygous mice [31]. These findings showed haploinsufficiency of *Neurod2* causes amygdala deficiencies in mice, leading to defects in emotional memory formation.

2.6 NEUROD2 binding sites in genome

Studies done on *Neurod2* knockout mice helped to reveal genes that are targeted by NEUROD2. For example, GAP-43 protein, a protein expressed in presynaptic axon sites within neurons, was found to be decreased in *Neurod2* knockout mice [6][25]. Similarly, PSD-95, a synaptic protein acting as a scaffold at postsynaptic sites, is also shown to be upregulated by NEUROD2. *Neurod2* knockout mice have reduced expression of PSD-95 [27]. J.Olson et al. performed a comprehensive investigation on *Neurod2* knockout mice, by examining expression levels of 6000 different mRNA molecules in P21 cerebellum with microarray. They found 27 among 6000 mRNAs have significantly decreased in *Neurod2* knockout mice cerebellum at P21. Among these mRNAs, brain-derived neurotrophic factor (*BDNF*) *tyro3/sky*, *ras-p21*, *MAPK*, *c-fos* and *c-jun* are found, which have roles on neuron survival in cerebellum [8].

In a genome-wide screen to identify the targets of *Neurod2*, Fong et al. performed *Neurod2* transduction by lentivirus on P19 pluripotent mouse cell line that have been converted to neurons. Findings showed that 532 genes were upregulated and 278 genes were downregulated upon *Neurod2* expression in the cell line. Gene ontology analysis of the upregulated genes showed neuronal development and differentiation related genes are increased upon presence of NEUROD2 [32]. However, use of neurons derived from pluripotent cell line is not the perfect representation of *in vivo* NEUROD2 targets. Previous

work done in our laboratory have addressed that problem, by performing chromatin immunoprecipitation followed by high-throughput sequencing (CHIP-Seq) on dissected mouse cortex [9][10]. To have a better understanding for roles of NEUROD2 in cortex development, CHIP-Seq was performed for two developmental time points, on embryonic day 14 (E14) and postnatal day 0 (P0).

In the study done with E14 mice cortex, 94,621 peaks obtained by CHIP-Seq were mapped on the mouse genome. Each peak corresponds to NEUROD2 binding event to that site of the genome. These peaks converted to gene scores by ClosestGene method, which quantifies the peaks based on their closeness to the transcriptional start site. Calculated ClosestGene score normalized by subtracting a random sample's score from NEUROD2 score. Gene ontology analysis of the high-scoring genes revealed that cortical radial migration and axon guidance is under influence of NEUROD2. Reelin signaling pathway, which is known to have role in neuronal migration and actin cytoskeleton rearrangements, were also found to be influenced by NEUROD2 [9]. We performed another CHIP-Seq experiment with using P0 mice cortex [10]. This experiment suggested Ca^{2+} / Calmodulin dependent protein kinase (CaMK) activation pathway is targeted by NEUROD2. Additionally, the most prominent NEUROD2 binding on genome is found on second intron of stromal interaction molecule 1 (*Stim1*) [10].

2.7 Ca^{2+} / Calmodulin dependent protein kinases

Ca^{2+} is an important secondary messenger molecule that is involved in various biological processes. Calcium binding proteins are key elements that relay this secondary messenger molecule into a physiological response. Calmodulins (CaMs) are well-known calcium binding proteins that regulate many different biological events [56][57]. CaM family is composed of three isoforms CaM1, CaM2, and CaM3. CaMs are a well-conserved protein family, they have been found in all vertebrates with very high sequence similarity. CaMs

have the 5th highest conversion score among species in all proteins, after histone H3 and H4, ubiquitin and β -actin [58]. Four EF-hand motifs present in the CaMs enables them to bind Ca^{2+} with high affinity. When Ca^{2+} binds to CaM, conformational changes in the CaM allow its ability to bind its target proteins.

Ca^{2+} / Calmodulin dependent protein kinases (CaMKs) are serine/threonine kinases that are activated by CaMs. CaMKs are found in all tissues but they are highly expressed in the brain. In parallel to their high expression in brain, CaMKs have important roles in neurons. CaMKII is a well-characterized protein for its key role in learning and memory [34][35]. Also CaMKII has been shown to be involved in many other neuronal processes, such as neurotransmitter release and synthesis, ion channel regulation, neurite growth control, and gene expression [35].

CaMKII is a multifunctional Ca^{2+} / Calmodulin dependent protein kinase, able to phosphorylate not a single protein but it has a range of protein targets. Other multifunctional CaMKs are involved in CaMK cascade. In the CaMK cascade, three CaMKs are found, CaMKI, CaMKIV and CaMKK (CaMK kinase). CaMKK phosphorylates and activates CaMKI and CaMKIV. Active CaMKI and CaMKIV phosphorylate their target proteins. Best characterized target of CaMK cascade is the transcription factor cyclic adenosine monophosphate (cAMP) responsive element binding (CREB) (Figure 2.2). CREB is involved in many neuronal functions. It is a key protein in transcription- dependent phase of long term potentiation, which is an essential process for learning and memory. CREB also known to regulate expression of neuronal immediate early genes, genes that are expressed immediately after neuronal stimulation, such as c-Fos and BDNF [36]. CaMKI can also affect the Ras/Erk pathway, and with this crosstalk it can influence calcium dependent transcription in neurons [37].

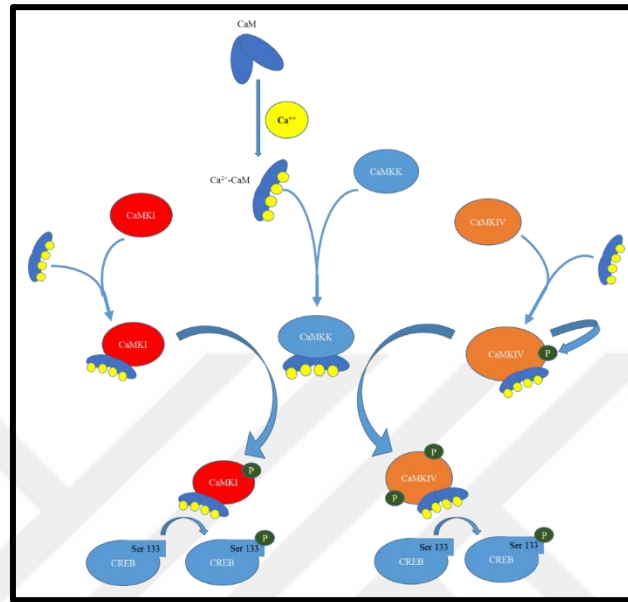


Figure 2.4: CaMK cascade scheme. Calmodulin (CaM) found in inactive conformation in Ca^{2+} unbound form. When bound to Ca^{2+} , it interacts with CaMKK, CaMKI and CaMKIV in CaMK cascade, and activates the proteins. CaMKIV-CaM binding cause autophosphorylation of CaMKIV. CaM-CaMKK is able to phosphorylate its target genes, including CaMKI and CaMKIV. CaMKK phosphorylation of these proteins induce their kinase activity, and they phosphorylate Ser133 residue on CREB.

2.8 STIM1 and store-operated calcium entry

STIM proteins are composed of two members, STIM1 and STIM2. STIMs are single-pass transmembrane proteins located on the ER membrane. They are type I transmembrane proteins with N-terminus found in ER lumen while C-terminus is extended into cytosol. STIMs are widely expressed proteins that are found in various tissues [38]. In brain, STIM1 and STIM2 is widely expressed. In the cerebellum thalamus, cortex and amygdala, STIM1 and STIM2 proteins found in similar levels. Hippocampus is the exception, where STIM2 expression is higher compared to STIM1 [39].

RNA interference screen studies have identified the role of STIM proteins in store-operated calcium entry (SOCE). SOCE is responsible for calcium uptake when the ER calcium stores are depleted. The molecular components of SOCE were unknown, until two concurrent study investigated calcium refilling ability of cells, of which calcium stores were depleted by drugs. Study performed by Liou et al. assayed over 2,000 siRNAs and screened the calcium refilling ability of HeLa cells. To study SOCE event, they depleted ER calcium stores by thapsigargin (TG), which blocks sarco/endoplasmic Ca^{2+} -ATPase (SERCA) pumps found on ER membrane. From the screen of TG treated HeLa cells, siRNAs targeting STIM1 and STIM2 appeared to cause defects in calcium refilling ability of the cells, showing the role of STIM1 and STIM2 in SOCE [40]. Roos et al. applied a similar approach in *Drosophila* S2 cells, by using siRNAs targeting 170 different genes. Their study identified STIM1 as an essential element in SOCE [41]. STIM1 interacts with ORAI1, a Ca^{2+} selective channel protein on plasma membrane, and this interaction activates SOCE [59].

2.9 Structure of STIM1

STIMs are targeted to ER by signaling peptide sequence found at the N-terminus of the protein. N-terminus of STIMs also bear a EF-SAM domain. The EF-SAM domain is composed of three motifs; canonical EF-hand motif (cEF), noncanonical EF-hand motif (ncEF) and a sterile alpha motif (SAM). EF-hand motif, composed of 12 aminoacids, allows Ca^{2+} binding via its acidic residues. This motif is present in many other Ca-binding proteins such as calmodulins, which contain 4 EF-hand motifs [42]. The noncanonical EF-hand motif contains few amino acid changes from the canonical EF-hand motif, which may alter its Ca^{2+} binding affinity. SAM is a motif known to be part of many protein-protein interactions. These three motifs found adjacent to each other creates EF-SAM domain, which mediates Ca^{2+} binding function of STIMs in ER lumen.

Following the transmembrane domain located within the ER membrane, five motifs are found in the cytosolic part of STIMs. Three coiled-coil regions (CC1-CC2-CC3), followed by a proline/serine rich (P/S) region and a lysine rich region at the C-terminus. Binding site of STIM1 to ORAI1 is found on the CC2-CC3 region. Ca^{2+} release activated Ca^{2+} (CRAC) activation domain (CAD), which is shown to be sufficient for activating CRAC via binding to Orai1 found in CC2-CC3 region of STIM1 [43][44]. The lysine rich residue found at C-terminus creates a basic region which interacts with the phosphate groups of phospholipids found on the plasma membrane (PM), allowing STIMs to localize to PM-ER junctions [43] (Figure 2.3). Additionally, it has been shown that the region between residues 470-491 of STIM1 is required for fast Ca^{2+} dependent inactivation (CDI) of CRAC channels [44].

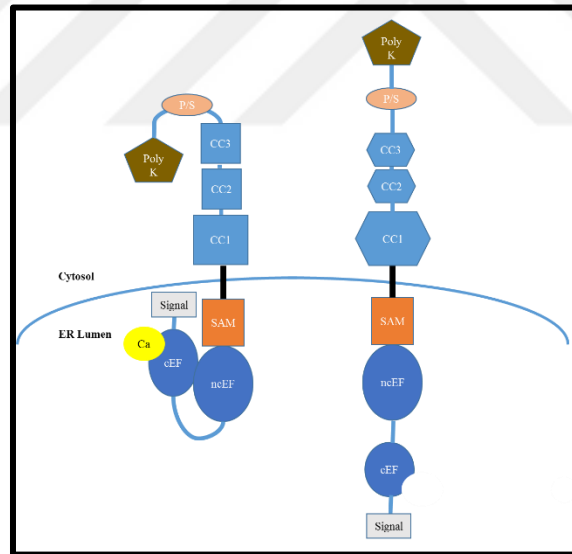


Figure 2.5: Ca^{2+} bound (left) and unbound (right) structures of STIM1. Signal peptide sequence targets STIM1 to ER membrane. In Ca -bound state, EF-SAM domain found in close association and poly-K site located close to CC 1-2-3. When Ca^{2+} released, SAM domain is exposed and can induce oligomerization. Conformational changes at CC 1-2-3 cause poly-K to extend, enabling its interaction with plasma membrane.

2.10 Molecular mechanisms of SOCE

In normal conditions, when there is no ER calcium channels activation, Ca^{2+} ion concentration in ER is higher compared to cytoplasm. High Ca^{2+} ion concentration in ER allow Ca^{2+} ion binding to EF-hand motif of the STIM1 in ER lumen. Ca^{2+} -bound EF-hand motif is found in close association with SAM domain. This close confirmation of EF-SAM domain keep STIM1 protein in a monomeric state. Thus presence of Ca^{2+} in ER lumen favors monomeric state of STIM1 [40].

Upon Ca^{2+} depletion from ER, Ca^{2+} concentration in ER lumen decrease and this causes Ca^{2+} ions to dissociate from EF-hand motif of STIM1. Ca^{2+} dissociation from EF-hand motif destabilizes EF-SAM domain, allowing oligomerization of STIM1 proteins via SAM motif. Ca^{2+} dissociation from EF-SAM domain also causes conformational changes in cytosolic part of the protein. Lysine-rich domain is exposed upon conformational changes in three coiled coil domains (CC1-CC2-CC3). Exposed lysine-rich domain binds to negatively charged phosphoinositides found on the plasma membrane, facilitating STIM1 to localize proximal to plasma membrane. Ca^{2+} release activated Ca^{2+} (CRAC) activation domain (CAD), found in CC2-CC3 domain of STIM1, and mediates interaction with Orai1 on plasma membrane. STIM1 binding to ORAI1 through CAD activates ORAI1 channels, and activated Orai1 channels allow calcium ion inflow to the cytoplasm, allowing ER stores to refill their calcium stores from cytosol [39][42] (Figure 2.4).

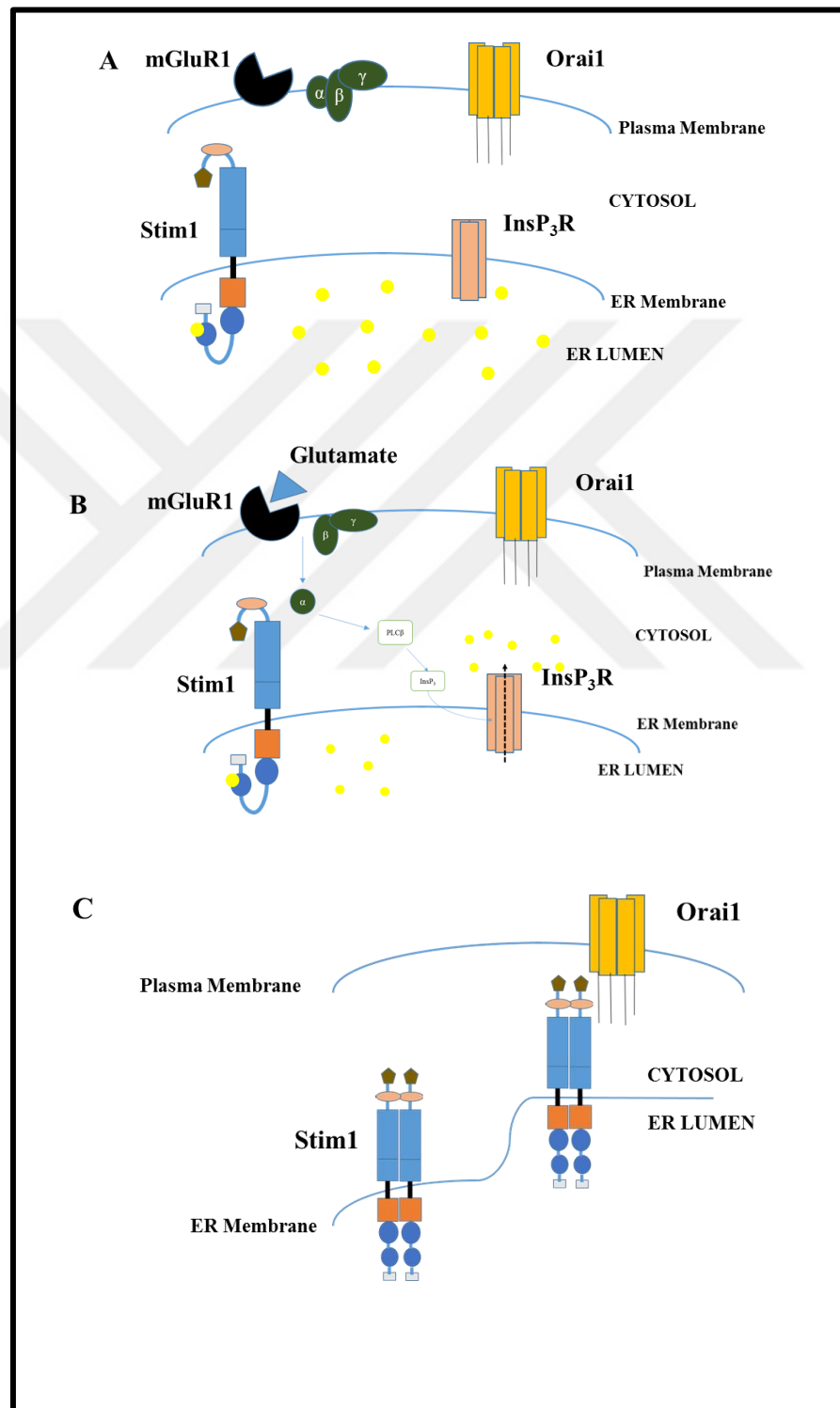


Figure 2.6: Mechanism of SOCE activation. (A) In steady-state condition, Ca^{2+} ions present in ER in high concentration (yellow spheres). STIM1 is in Ca-bound state and is inactive. Orai1 channels does not intake Ca^{2+} . (B) Upon a signal that lead to ER Ca^{2+} store usage, in our example, GPCR activation by glutamate binding to its receptor. Activated receptor cause G protein to dissociate, and release G_α . G_α activates phospholipase C (PLC), Leading to inositol triphosphate (InsP_3) production. InsP_3 activates InsP_3R found on ER membrane, and Ca^{2+} in ER flow to cytoplasm via this channel. (C) Decrease in ER Ca^{2+} levels cause Ca^{2+} to dissociate from STIM1. Ca-unbound STIM1 oligomerize via SAM domain. CAD domain in STIM1 oligomers interact with Orai1 on the plasma membrane. Orai1 channel opens and calcium is taken up by the cell.

2.11 SOCE and STIMs in neurons

STIM, and its partner in SOCE, Orai are ubiquitously expressed and found in many cell types. Especially STIM-Orai proteins are extensively studied in immune cells, which are non-excitable cells that utilize calcium for their activity and require SOCE as major pathway to uptake calcium. Role of SOCE in excitable cells, such as neurons, are overlooked until recently, as neurons have voltage-gated calcium channels and ligand-gated calcium channels (e.g. n-methyl d-aspartate (NMDA) receptors) as major routes of calcium entry into the cell.

However, role of SOCE and STIM1 within neurons have been elucidated. Spontaneous neurotransmitter release in hippocampal synaptic boutons are found to be related to SOCE [45]. Another study at hippocampal neurons have showed that NMDA-receptor mediated synaptic plasticity is decreased when SOCE is inhibited [46]. Absence of STIM1 in mouse cerebellar Purkinje neurons leads to impairments in glutamatergic synaptic potentials and in cerebellar motor behavior [47]. SOCE is important for proliferation of neuronal progenitor cells. STIM1 or ORAI1 absence attenuated the proliferative ability neuronal progenitor cells, both in vitro at NPC cultures, and in vivo at subventricular zone

[18]. SOCE also shown to alter gene transcription in mouse cerebellar granule cells, by causing degradation of Sp4 transcription factor [48]. STIM1 was found to inhibit voltage-gated calcium channel $Ca_v1.2$. Inhibition acutely represses calcium uptake from the channels, and in long term, causes internalization of the channel [49]. This study shows importance of STIM1 on calcium dynamics of neurons, not only by its role in SOCE but also regulating other routes of calcium uptake.

SOCE impairments have been reported in neurological diseases. Familial Alzheimer's disease (FAD) phenotype in hippocampal neurons have been associated with SOCE. *Presenilin-1* gene, which functions in ER calcium store refilling, is commonly found mutated in FAD [50]. SOCE is also proposed as a potential drug target for epilepsy, as SOCE inhibitor drugs can reduce epileptic activity in chronic epileptic hippocampal slices [51]. In a screen performed with Huntington's disease model *Drosophila* flies, chemicals improving the condition of the flies were shown to inhibit SOCE in neurons [52].

Chapter 3

MATERIALS AND METHODS

3.1. Calcium Imaging

Primary neuronal cultures prepared from mouse E14 embryos were used in calcium imaging (see chapter 3.3). 300,000 cells were seeded for each coated well in 24-well plates. At 2 DIV, 1 μ g shRNA-mCherry plasmid (*Neurod2* targeting or non-silencing shRNA) was transfected into the cultured cells for knockdown experiments. For rescue experiments, co-transfection was performed at same timepoint (2 DIV), with 1 μ g shRNA-mCherry plasmid and 2 μ g of shRNA-resistant *Neurod2* (or vehicle) plasmid. After transfection and at every following three days after transfection, media of cultured neurons were changed with 50% BDNF + 50% conditioned media until the day of calcium imaging. Around 7 DIV (between 5-9 DIV), calcium-sensitive dye was loaded. Initially, 1 mM stock solution of calcium-sensitive dye was prepared by adding pure DMSO to one vial of 50 μ g Fluo-3 AM (Life Technologies, Cat: # F1242). The vial was covered to block light exposure to the fluorescent dye. A non-ionic detergent Triton X-100 added on the stock solution (0.5% by volume) for enhancing dye entry into the cells. Solution vortexed briefly 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 μ M

final concentration. Cultures were washed three times with Ringer solution prior to dye loading. An equal amount of dye loaded on washed wells and cells covered for protection from light and incubated at 37°C for 30 minutes. Cells were washed with Ringer solution two times to remove the dye, and incubated for an additional 30 minutes at 37°C. Two times wash with Ringer solution was repeated after the second incubation period was over. After the last wash, tetrodotoxin (TTX) was added to the Ringer solution on the cells (final concentration: 1 μ M). Calcium dye loaded plate placed on the stage of live imaging microscope (Xcellence Pro®, Olympus). The microscope stage was enclosed and set to 37°C, 5% CO₂. Initially, the red channel (595 nm) was image taken to detect shRNA-mCherry transfected cells. Green channel (495 nm) was used for imaging the calcium dye, and images were taken every 6 seconds until imaging finished in 10 minutes. For examining store-operated calcium entry in the cells, following imaging protocol was used. The baseline calcium levels measured in first two minutes of the experiments. After two minutes, media of the cells were removed and Ca²⁺-free Ringer solution (155 mM NaCl, 10 mM d-Glucose, 5 mM HEPES (pH 7.4), 4.5 mM KCl, 1mM EGTA, 1mM MgCl₂, pH 7.4), containing 5 μ M thapsigargin (for depleting ER calcium stores) and 1 μ M TTX was added to deplete ER Ca²⁺ stores. Ca²⁺-free Ringer solution was applied for 5 minutes, to completely deplete Ca²⁺ ions in ER. Ca²⁺-free media was removed and Ringer solution (containing 5 μ M thapsigargin and 1 μ M TTX) added, allowing cells to uptake calcium which is present in this newly added medium. Imaging was terminated after capturing images for 3 minutes after second media change. All images were captured with 40x magnification. The images were quantified with ImageJ software, based on mean signal intensity measured on soma of the neurons. The quantified images plotted as $\Delta F / F_o$ after background subtraction. F_o is the minimum signal obtained in the images after background subtraction is performed. This minimum signal is taken as 1, and other measurements (ΔF) plotted in ratio to this minimum signal.

3.2. Plate coating for primary neuronal cultures

As coating solution, 0,6 μ M Poly-L-lysine Hydrobromide (Sigma Life Sciences, Cat. #P9155) and 15 nM Mouse Laminin (Millipore, Cat. #CC095) added in 1x PBS. For immunofluorescence experiments, autoclaved and ethanol washed cover glasses (Heinz Herenz Medizinalbedarf GmbH, Cat. #1051204) were put into wells before coating solution were applied. For other experiments, coating solution was directly added to the well. For 24-well plates, 0.5ml of the coating solution was added to single well. Coating solution containing plates were incubated for 16 hours at 37°C, 5% CO₂ in the tissue culture incubator. After 16 hours, coated plates were washed three times with ddH₂O, and let for air dry in the tissue culture hood. Plates were used immediately or kept at 4°C for later use.

3.3. Preparation of primary neuronal culture

All the equipment used were ethanol sterilized before the operation. Cervical dislocation performed to euthanized pregnant mice to obtain embryos between embryonic day 14 to 18 (E14-18). 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 (3.2mg) and 200 unit papain (Worthington, Cat. # LS003126) in dissociation media (82mM Na₂SO₄, 30mM K₂SO₄, 5.8mM MgCl₂, 0.252mM CaCl₂, 1mM HEPES (pH 7.4), 20mM glucose, 0.001% phenol red, 0.2mN NaOH in ddH₂O). 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 (10mg BSA and 10mg trypsin inhibitor in 10 ml dissociation media, pH adjusted and filtered) and incubated for 1 minute. Then, cortices were put into heavy inhibitor solution (50mg BSA and 50mg

trypsin inhibitor in 5 ml dissociation media, pH adjusted and filtered) and were incubated for 1 minute again. The cortices 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 falcon containing BF, and triturated by serological pipette several times for mechanical dissociation of the cells from the cortex tissue. After trituration, sample was let for settle down, allowing large tissue fragments to settle. The upper part of the sample was taken and passed through cell strainer. Cells were placed on 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 incubator for 2 hours. After 2 hours, BF media was removed and 0.5 ml BNBF, BF media containing 1x N2 (Gibco Life Technologies, Cat. #17502-048) and 1x B-27 (Gibco Life Technologies, Cat. #12587-010), was added to each well. For every 2-3 days in vitro (DIV), media change was made by removing half of the media from the wells and replacing it with freshly prepared BNBF.

3.4. Transfection of primary neuronal cultures

Transfection of the primary neuronal cultures was performed at 2-3 DIV. The media of the neuronal culture was collected and replaced with OptiMEM® media, 500 µl per well for 24-well plates. The collected media was filtrated and kept in 37°C water bath as conditioned media. Two separate eppendorfs were prepared for transfection of neurons. In one Eppendorf, 2 µl Lipofectamine 2000 solution was added into 250 µl OptiMEM® per well. And in other eppendorf, 1-3 µg plasmid DNA (varies with the conditions of the experiment) was mixed with 250 µl OptiMEM®. Both mixtures were incubated separately for 10 minutes at room temperature. Incubated solutions were gently mixed with each other. The mixture was incubated for 20 minutes in room temperature. After incubation period was over, the mixture was added to the cells found in OptiMEM® media dropwise. The cells were incubated for 2 hours in 37°C incubator. Media of the cell was changed with 50%

conditioned media + 50% fresh BNBf (5% FBS, 2% B-27 supplement, 1% N-2 supplement in BME) after 2 hour incubation period is over.

3.5. Nucleofection of primary neuronal cultures

Suspended neuronal cells dissected from the cortex of E14 mice were used in nucleofection. For this reason, a similar protocol to primary neuronal culture was applied (see section 3.3). Only differences were, in nucleofection, 500 μ l BF (5% FBS in BME) was added on the coated wells before dissection starts, and cells were suspended in BME media, instead of BF media.

From the cell suspension, volume corresponding to 1,5 million cells was taken and put in 15 ml falcon tube. The tube was centrifuged for 3 minutes at 1500rpm, in 4°C. The supernatant was removed and cells were suspended in 100 μ l P3 nucleofection solution (Lonza, Cat. #V4XP-3024) gently. The suspended cells were transferred to nucleofection cuvette. CU133 program of the nucleofection machine was used on the cuvettes. Cells were incubated for 10 minutes on room temperature after nucleofection. Nucleofected cells were suspended in 1 ml BF solution, and 500 μ l of the cell suspension was added on the wells of the BF-containing plate, corresponding to 750,000 cells per well. Two hours after seeding the cells, the media was changed to BNBf (2% B-27 and 1% N-2 in BF media) media. Media of the cells were refreshed with 50% conditioned media collected from the well and 50% fresh BNBf at 3DIV. Cells were used for total RNA isolation, or for western blotting at 5 DIV.

3.6. Protein lysate collection from cultured cells

Cells were taken from incubator put on ice. The media of the cells was removed, and cells were washed with 1x cold PBS solution once. 1X RIPA lysis buffer containing protease inhibitor was applied to the cells and let for 10 minutes shaking on 4°C. The lysate was collected from the plate and transferred into an Eppendorf tube. Lysates were centrifuged for 20 min at 14,000 g, in 4°C. The supernatant was taken and put into a new Eppendorf tube. Lysates kept at -20°C for storage.

3.7. Maintenance and transfection of cell lines

For Neuro2A cells, MEM (Gibco Life Technologies, Cat. #31095029) containing 10% FBS, 1% penicillin/streptomycin, 0,5% L-glutamate was used. For HEK293 cells, high-glucose DMEM containing 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate and 0,5% L-glutamate was used. Cell lines were maintained in their corresponding media and passaged when they reach confluency. Transfection was performed when cells reach 70-80% confluency.

Cell lines were transfected with Lipofectamine 2000 as follows. For single well in a 24-well plate, two tubes with 50 µl OptiMEM® media were prepared. On one tube, 1 µg plasmid DNA added, while on the other tube, 2 µl Lipofectamine 2000 was added. For 60 mm plates, all the amounts given above were used by multiplying with six. The two tubes were incubated in room temperature for 5 minutes. After incubation was finished, two tubes were mixed, and the mixture was incubated in room temperature for 20 minutes. The incubated mixture was added over the cells dropwise. The transfected cells were used in experiments 48 hours after transfection.

3.8. Immunofluorescence (IF) of cell lines and primary neuronal cultures

Cells that were seeded for imaging plated on 24-well plates, with coverslips found at the bottom of the wells. Cell fixation was done by equal volume of 4% paraformaldehyde addition on the media of the cells. Cells were incubated in paraformaldehyde for 20 minutes, at room temperature. Paraformaldehyde use must be done under hood for safety. Paraformaldehyde was removed and cells were washed with 1x PBS for three times. At this stage, cells can be either kept in 4°C with adding 2 % sodium-azide onto PBS solution or directly used for immunofluorescence (IF) staining. For IF staining, the coverslips were taken in a covered chamber that blocks the light exposure. Primary antibodies diluted in IF blocking solution (3% BSA, 0.5 % Triton-X-100 in 1x PBS) was added over coverslips, to cover all surface area of the coverslip. Coverslips were incubated with primary antibodies for 2 hours, in room temperature. After incubation period is over, cells were washed three times with 1x PBS for 5 minutes. Fluorophore conjugated secondary antibody was selected according to used primary antibody and diluted in IF blocking solution. Secondary antibody was added over the cells, and incubated for 1 hour in room temperature. To avoid bleaching of the fluorophore, samples were kept in dark. After incubation, 1:1000 diluted DAPI (in 1x PBS) was applied on the cells, and kept for 10 minutes. The cells were washed two times with 1x PBS for 5 minutes. One drop of mounting media (Thermo Scientific, Cat. #TA-060-UG) was placed on a glass slide. The coverslips were carefully placed on mounting media by avoiding any bubble under the coverslip. Mounted slides were stored at 4°C at dark. For imaging the slides, Nikon Eclipse 90i confocal microscope was used. The captured images were analyzed and quantified by ImageJ software.

Antibody	Host	Company	Catalogue Number	Dilution
GMP-1 (SUMO)	Mouse	Invitrogen	33-2400	1:1000
Myc	Mouse	Santa Cruz	sc-40	1:1000
Neurod2	Rabbit	Abcam	ab104430	1:1000
Neurod2	Rabbit	Abcam	ab109406	1:1000
Neurod2	Mouse	Abcam	ab168932	1:2500
Histone H3	Rabbit	Cell Signaling	9715	1:1000
GFP	Rabbit	Santa Cruz	H0612	1:1000
Beta-Actin	Rabbit	Thermo Scientific	PA5-16914	1:1000
Myc	Mouse	Santa Cruz	Sc-40	1:1000
Turbo-GFP	Rabbit	Evrogen	AB513	1:1000
STIM1	Rabbit	Cell Signaling	5668	1:1000

Table 3.1: List of primary antibodies used for IF and western blotting

Antibody	Host	Company	Catalogue Number	Dilution
Anti-Mouse IgG (H+L) Alexa Fluor®488	Donkey	Invitrogen	A21202	1:1000
Anti-Rabbit IgG (H+L) Alexa Fluor®488	Donkey	Invitrogen	A21206	1:1000
Anti-Rabbit IgG (H+L) Alexa Fluor®594	Donkey	Invitrogen	A21207	1:1000
Anti-Mouse IgG (H+L) Alexa Fluor®594	Donkey	Invitrogen	A21203	1:1000
Anti-Mouse IgG HRP-linked	Horse	Cell Signalling	7076S	1:2500
Anti-Rabbit IgG HRP-linked	Goat	Abcam	ab6721	1:2500

Table 3.2: List of secondary antibodies used in IF and western blotting

3.9. Total RNA isolation

Total RNA isolation from nucleofected primary neuronal cultures was performed by Absolutely RNA Microprep Kit (Agilent Technologies, Cat. #400805). For lysis of the cells, 0,7 μ l β -mercaptoethanol was added to 100 μ l lysis solution of the kit. To avoid contamination, this addition step was performed under hood, and solutions were only opened under the hood. Lysis solution containing β -mercaptoethanol was added on the cells found in one well of 12-well plate. The cells were homogenized by trituration, and lysate was collected. The lysate can be stored for -80°C for storage, or can be directly used. 70% ethanol was added into lysate in equal volume. Ethanol-lysate mixture was vortexed for 5 seconds, and transferred to RNA-binding spin column. The column was centrifuged for 1 minute, at maximum speed. The filtrate was discarded and 600 μ l of 1x Low Salt Wash Buffer was added in column. Again, the column was centrifuged for 1 minute, at maximum speed and the filtrate was discarded. The column was centrifuged 2 minutes at maximum speed to remove residual solution left on the filter. DNase solution (5 μ l DNase I enzyme with 25 μ l DNase Digestion Buffer) was added on the filter inside the column. The filter was incubated in DNase solution at 37°C for 15 minutes. DNase solution was washed by adding 500 μ l 1X High Salt Wash Buffer and centrifuged for 1 minute at maximum speed. The filtrate was discarded and 600 μ l 1X Low-Salt Wash Buffer was added. After 1 minute centrifuge at maximum speed, 300 μ l 1X Low-Salt Wash Buffer was added. The column was centrifuged for 2 minutes at maximum speed. After the wash, the filter was taken into a clean eppendorf tube. 30 μ l 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.

3.10. cDNA synthesis from total RNA samples

For synthesizing cDNA from isolated total RNA samples, Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Cat. #05081955001) was used. All steps of the protocol was done on ice to avoid RNA degradation. In a PCR tube, 10 ng RNA and 2 μ l random hexamer primers (600 μ M) were mixed with water. Water was added to final volume of 11.4 μ l. The sample was denatured with incubating at 65°C for 10 minutes. Sample was taken on ice after incubation and following components of the cDNA synthesis reaction were added as follows; 4 μ l of 5x Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 0,5 μ l Protector RNase inhibitor, 2 μ l dNTP mix, 1 μ l DTT (dithiothreitol) and 1,1 μ l Transcriptor High Fidelity Reverse Transcriptase. Then, the reaction mixture was put 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 -20°C for later use.

3.11. 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 prepared by mixing 7,8 μ l PCR-grade water with 0,6 μ l of respective forward and reverse primers for each reaction. 2x Luminaris Color q-PCR Master Mix was added into the master mix, 10 μ l for each reaction. Master mix was covered from the light, because Luminaris Color q-PCR Master Mix is light sensitive. 19 μ l of the prepared mixture was aliquoted to 8-strip PCR tubes with flat caps. 1 μ l cDNA was added into the PCR tubes and mixed gently. Samples were centrifuged briefly and placed into qPCR block cycler. Following cycle plan was applied, denaturation at 95°C for 15 seconds, annealing at 59-64°C 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 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 of each reaction is made. These three technical replicates were averaged. These average CT values from the samples were normalized to GAPDH average read values. The normalized values were calculated by $2^{(GAPDH-Sample)}$.

3.12. Quantitative PCR (qPCR) with ChIP DNA template

Equal volume of ChIP and input DNA were used in ChIP-qPCR experiments. Input DNA was prepared by using standard phenol-chloroform extraction method. 10% of the cross-linked lysate was used for preparation of the input DNA. 1 μ l of DNA sample was used for quantitative PCR experiments. Primers designed to amplify ~200 base pair region of the targeted part of the genome were used in final concentration of 3 μ M. Luminaris HiGreen qPCR Master Mix (Thermo scientific, Cat. #K0991) was added to reaction solution, and dH₂O was added to reach final concentration of reactants. Reaction mixtures were prepared on ice and kept away from the light. Log₂10 (~3.32) was subtracted from the Ct value read from input sample to get adjusted input Ct value, as correction for 1:10 dilution. Normalization of ChIP DNA data was performed by following equation: $100 \times 2^{(Adjusted\ input\ Ct - ChIP\ Ct)}$

3.13. Subcellular fractionation of mouse brain cortex

Mice from desired time point were dissected and brain of the mice were removed. The cortex was removed from the dissected brain and placed into an eppendorf tube. Dissected cortex can be directly used or can be stored in -80°C for later use. Tissue was dissociated in 750 µl 1x PBS with dounce homogenizer. Suspended cells were pelleted by 10 second spindown at 4°C. The cell pellet was resuspended by NP-40/PBS solution (0,1% NP-40, 20 mM n-ethylmaleimide (NEM), 1x protease inhibitor in PBS). Volume of the NP-40/PBS solution determined according to weight of the tissue. For example, 0.05g brain tissue was dissolved in 100 µl NP-40/PBS solution. To keep the nucleus intact, resuspension in this step was done gently, with several trituration performed with a cut pipette tip. Half of the resuspension was separated as whole cell lysate sample. The remaining half was centrifuged for 10 second at 4°C. The pellet in the tube is nuclear fraction and supernatant is cytosolic fraction. The supernatant was carefully taken, without disturbing the pellet. To avoid nuclear contamination, supernatant was taken into another eppendorf tube and centrifuged again, for 10 second at 4°C, and supernatant was taken as cytosolic fraction. The pellet, which was the nuclear fraction, was washed two times with 1x PBS to avoid cytosolic contamination. After washes, half volume of initially added NP-40/PBS solution was added over nuclear pellet. Pellet was suspended in the solution, this time, faster trituration performed to disrupt the nuclear membrane. Nuclear fraction and whole cell lysate were sonicated two times, in each time the samples were sonicated for 5 second with 50% output. Sonicated samples were centrifuged for 10 second at 4°C. Supernatant was transferred to another Eppendorf and labeled as nuclear fraction or whole cell lysate. On all three fractions, one volume of 2x RIPA-SDS buffer (37,5 mM Tris-HCl pH 8.0, 75 mM Tris-HCl pH 6.8, 75mM NaCl, 0,75% Na-DOC, 0,75% NP-40, 2,5% SDS, 50% glycerol in ddH₂O) containing 20 mM n-ethylmaleimide (NEM) + 1x protease inhibitor was added. The fraction lysates were stored in -80 °C.

3.14. SDS-PAGE and Western blotting

SDS-PAGE gel used for Western blotting experiment was composed of 12% separating gel (12% Acrylamide-Bisacrylamide (29:1), 375mM Tris-HCl, pH 8.8, 0.2% APS, 0.2% SDS, 0.1% TEMED) and 5% stacking gel (5% Acrylamide-Bisacrylamide (29:1), 126mM Tris-HCl, pH 6.8, 0.1% APS, 0.1% SDS, 0.1% TEMED). Gel chamber was sealed with agarose to avoid leakage of separating gel before polymerization. Water saturated butanol was applied after separating gel have cast. After polymerization of separating gel finished, butanol was removed and stacking gel was applied. A comb was placed in stacking gel to create the wells for sample loading. After stacking gel was 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 kept at 95°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 90V for 1 hour and 110V for 2 hours. After running was finished, wet-transfer method was used for blotting the gel on membrane. Polyvinyl difluoride membrane (PVDF, Thermo Scientific, Cat. #88518) was used for the transfer. PVDF membrane was cut according to required size and kept in methanol briefly. PVDF membrane was covered on SDS-PAGE gel in transfer buffer (100 mM glycine, 12 mM Tris base, 15% methanol, at 4°C). Transfer performed at 100 mA current for 2 hours, at 4°C. After transfer was finished, the membrane was blocked with TBS-T (10mM Tris-HCl pH7.5, 150mM NaCl, 0.05% Tween) containing 5% non-fat dried milk powder (AppliChem, Cat. #A0830) for 1 hour. Primary antibody prepared in desired amount of antibody was added into TBS-T containing 5% non-fat dried milk powder, and applied on the membrane after blocking. Primary antibody was treated for either 2 hours at room temperature or overnight at 4°C. Membrane was washed with TBS-T for 15 minutes for three times. HRP coupled secondary antibody selected against the primary antibody used

was added in TBS-T containing 5% non-fat dried milk powder. Secondary antibody treatment was performed for 1 hour at room temperature. Three time TBS-T wash were repeated, for 15 minutes for each wash. HRP reagent SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific, Cat. # 34096) was diluted with water (1:3 reagent:water ratio) and applied onto membrane. The blot was imaged under Gel Logic 2200 Imaging System (Molecular Imaging System CareStream Health Inc.). The images were quantified using ImageJ software.

3.15. Immunoprecipitation

P7 mice cortex was taken and fractionation applied as described in section 3.12. Only one hemisphere was used for the experiment. The cytosolic fraction was either lysed in 0,1% NP-40 – PBS buffer or in RIPA-SDS buffer. 50 μ l of Protein A/G magnetic beads (Pierce, Cat. # 88803) were washed with these two buffers separately for 3 times. Samples and beads using same buffer were mixed together, and incubated for 1 hour in cold room on roller. Samples were put on magnetic strand and lysates removed, while the beads were discarded. Samples were separated into two, one for Neurod2 antibody (ab104430) incubation and other for control incubation with turboGFP antibody. Antibodies were added in 1:200 dilution. Antibody-lysate mixture was incubated overnight at cold room on roller. Again, 50 μ l of Protein A/G magnetic beads were washed with these two buffers separately for 3 times. The antibody-lysate mixture was added onto its respective beads. Beads and lysate were incubated for 1 hour at cold room on roller. The samples were placed on magnetic stand and supernatant collected as post-IP sample. The beads were washed with their respective buffer for three times, each was for 15 minutes on roller at cold room. The beads were eluted by 10 μ l SDS-PAGE loading buffer and boiled for 5 minutes at 95°C. Resulting sample was used in western blotting.

3.16. Transformation of plasmid DNA to chemically competent bacteria

The ligation products were transformed into chemically competent DH5a *E.coli* cells. Competent bacteria were taken from -80°C store and let on ice until the frozen cells thaw. 30 µl of bacteria were taken in another pre-chilled tube and 1 µl ligation product added onto cells. Cells were incubated for 10 minutes on ice after ligation product was added. Heat shock applied to cells for 1 minute by 42°C heat block. After heat shock, 500 µl LB added over the cells and incubated at 37°C for 1 hour. Cells were plated on LB plate containing the antibiotic that the plasmid have resistant gene for. The plates were incubated at 37°C overnight. From the colonies formed on the plate, several of them was selected and grown in LB media containing their respective antibiotics. These single colony cultures can be used for isolating plasmid DNA, starting maxiprep cultures or preparing glycerol stocks.

3.17. *Neurod2* targeting shRNA cloning

For preparation of the shRNA expressing plasmids, pSUPER vector system (OligoEngine, Cat. # VEC-PBS-0006) was used. Oligonucleotides were designed as 60mers, each oligonucleotide contains a 19 base-pair mRNA target sequence and its perfect complement sequence to form a hairpin RNA. The loop region, allowing RNA to form a hairpin, was found in the center of the oligonucleotides, in between complementing target sequences. Oligonucleotides contain sequences complementary to restriction enzyme cut site sequences to clone into the pSUPER-neo-GFP vector. Oligonucleotides were used as pairs, each oligonucleotide was complementing the other perfectly except the flanking sequences

that are left as sticky ends for ligating into the digested vector. A schematic representation of pSUPER-neo-GFP vector is given in Figure 3.1.

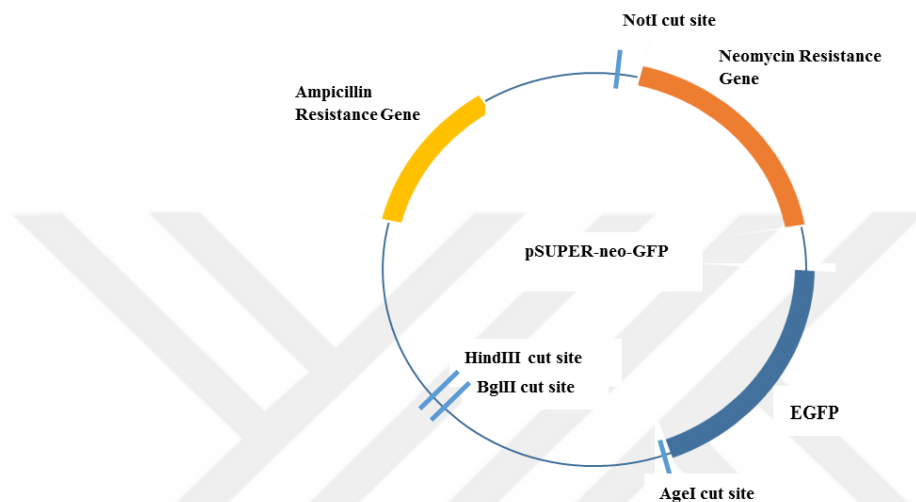


Figure 3.1: Schematic representation of pSUPER-neo-GFP vector. Vector contains EGFP reporter gene, and ampicillin and neomycin resistance genes for selection. HindIII-BglII site was used for inserting shRNA oligonucleotides. AgeI and NotI was used in Section 3.18 for creating pSUPER-mCherry vector.

300 μ g pSUPER-neo-GFP vector was digested with 10 units of BglII (New England Biolabs, Cat. #R0144S) and 10 units of HindIII (New England Biolabs, Cat. #R0104S) enzymes, in 1x NEBuffer 3 (New England Biolabs, Cat. #B7003S). Digestion performed for 3 hours at 37°C. After digestion, the reaction was incubated at 80°C for 20 minutes to inactivate restriction enzymes. Meanwhile, oligonucleotides were annealed by mixing 1,6 μ l of oligonucleotide pairs (100 μ M) in 46,8 μ l oligonucleotide annealing buffer (100 mM NaCl and 50 mM HEPES pH 7.4 in water). The oligonucleotide mixture was heated to 94°C and incubated for 4 minutes, and gradually cooled to 90°C and incubated 4 minutes again. The

cooling and incubation period were repeated, with decreasing the temperature 5°C in each step and incubating for 4 minutes. After incubation at 40°C was finished, samples were incubated at 37°C for 20 minutes. Both digested vector and annealed oligonucleotides were immediately used for ligation. 1 µl of digested pSUPER-neo-GFP vector and 2 µl of annealed oligonucleotides were mixed in 5 µl distilled water, and 5 unit T4 DNA ligase (Thermo Scientific, Cat. #EL0014) added together with 1x T4 DNA ligase buffer (Thermo Scientific, Cat. #B69). No annealed oligonucleotide was added for negative control ligation. The ligation reactions were incubated either in room temperature for 2 hours, or at 16°C for overnight incubation.

Ligation product was transformed to chemically competent bacteria as mentioned in this section (section 3.15). The plasmid DNA of the bacteria found in the culture was isolated by GeneJET Plasmid Miniprep Kit (Thermo Scientific, Cat. #K0503). Diagnostic digestion to detect positive samples was made by 6 units of EcoRI-HF (New England Biolabs, Cat. #R3101S) and 6 units of HindIII-HF (New England Biolabs, Cat. #R3104S) enzyme with 1x CutSmart (New England Biolabs, Cat. #B7204S) and 5 µl isolated plasmid DNA digested. Reactions were incubated 1 hour at 37°C. After incubation, 6x gel loading dye (New England Biolabs, Cat. #B7021S) was added to reach 1x final dilution. Samples were loaded on 1,5% agarose gel stained with ethidium bromide. 120 V was applied to gel for 40 minutes, and gel was imaged under UV. Two possible outcome was expected from the digestion. If the oligonucleotide pair ligated into the vector, a band with 281 bp in size was expected. Otherwise, if the result was negative, a band with 240 bp in size was observed. One of the LB culture samples that have correct size of insert selected. Upon confirmation by sequencing, to obtain high concentration and transfection quality plasmid DNA, maxiprep culture was started in 150 ml LB containing 150 µl ampicillin by adding 1,5 ml sample culture. For maxiprep, PureLink Maxiprep kit (Invitrogen, Cat. # K210006) was used and the kit protocol followed. The isolated plasmid DNA was stored in 20°C.

3.18. pSUPER-mCherry construct preparation

In order to use Fluo-3 dye together with our shRNA expressing pSUPER-neo-GFP vector, EGFP encoding gene was replaced with mCherry gene in pSUPER-neo-GFP backbone. Both pSUPER-neo-GFP and mCherry vector were digested with 3 unit AgeI (Thermo Scientific, Cat. #ER1461) and 3 unit NotI (Thermo Scientific, Cat. #ER0591) restriction enzymes in 1x Buffer O (Thermo Scientific, Cat. #BO5) containing distilled water. The digested vectors were run on 1% agarose gel. Short fragment of mCherry digestion sample, corresponding to mCherry gene, and long fragment of pSUPER-neo-GFP digestion sample, corresponding to pSUPER-neo-GFP backbone without EGFP gene, were extracted from agarose gel. Gel extraction was performed by EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Cat. #BS353-50). Extracted DNA fragments were ligated with 5 unit T4 DNA ligase (Thermo Scientific, Cat. #EL0014) added together with 1x T4 DNA ligase buffer (Thermo Scientific, Cat. #B69). Ligation product was transformed to chemically competent bacteria, and competent cells were plated on LB agar plate with ampicillin. Transformation to competent bacteria was done according to protocol given in section 3.16. Plasmid DNA obtained by GeneJET Plasmid Miniprep Kit (Thermo Scientific, Cat. #K0503). Control digestion was done by the same restriction digestion setup made for digesting the pSUPER-neo-GFP and mCherry. One of the positive colonies was selected and shipped for sequencing. Upon confirmation by sequencing, 150 ml LB culture of confirmed sample was grown, in presence of ampicillin. The culture was grown overnight and plasmid DNA isolated by PureLink Maxiprep kit (Invitrogen, Cat. # K210006). The isolated plasmid DNA stored in -20°C.

3.19. myc- *Neurod2* - Δ NLS cloning

A putative nuclear localization signal (NLS) was predicted by NLS Mapper online software [53]. A primer pair was designed such that, the pair will recognize the sites flanking the predicted NLS sequence as their 3' end extend away from the NLS sequence. With this method, whole plasmid will be cloned by skipping the NLS sequence. Due to high GC content of the NLS flanking sites, additional three base pairs from both ends were also not covered by the primers. PCR reagents were added as follows; 10 μ l 5x GC buffer, 0,4 μ l dNTP (25mM), 2,5 μ l forward and reverse primer (from 10 μ M stock), 2 μ l template DNA, 0,5 μ l Phusion Hot Start Flex DNA Polymerase (New England BioLabs, Cat. #M0535), 1,5 μ l DMSO, and PCR-grade water up to 50 μ l. PCR cycle was set up as, 98°C for 45 seconds, 60°C for 2 minutes and 72°C for 7 minutes. This cycle was repeated 25 times. Before cycle begins, 98 °C treatment was applied for 2 minutes to the sample. After all cycles were finished, PCR mix was treated at 72 °C for 7 minutes. PCR product was purified by EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc. Cat. #BS363). Purified product was phosphorylated by PNK treatment. PNK treatment was performed by 5 μ l PNK Buffer A, 5 μ l ATP (10nM), 1 μ l PNK enzyme (Thermo Scientific, Cat # EK0031) applied to 50 ng purified PCR product, in distilled water up to 50 μ l. PNK treatment was incubated at 37 °C for 1 hour, and at 65°C for 25 minutes. Reaction kept on ice after incubation period was over. 5 μ l of PNK reaction mixture was directly taken and put into ligation reaction, containing 5 unit T4 DNA ligase (Thermo Scientific, Cat. #EL0014) added together with 1x T4 DNA ligase buffer (Thermo Scientific, Cat. #B69). Reaction was incubated for two hours in room temperature and transformed into competent bacteria as described (Section 3.16). Miniprep culture started and the plasmid DNA was isolated from colonies. The DNA was digested with 5 unit Pst1 and 5 unit NotI enzyme. Colonies with correct band size was sent for sequencing. After the sequences were confirmed, maxiprep was performed and high concentration plasmid DNA isolated and kept at -80 °C.

Chapter 4

RESULTS

4.1. **CHIP-Seq reveals CaMK pathway genes and *Stim1* intronic region targeted by NEUROD2**

CHIP-Seq performed on P0 mice cortices revealed NEUROD2 binding sites on the genome. Three different antibodies were used against NEUROD2 for CHIP, and the overlapping peaks were selected. Using this method, 2071 peaks were revealed, corresponding to 1111 genes. Gene ontology analysis of the identified genes showed that NEUROD2 has a role in development of the synapse and dendrites in P0 mouse brain cortex (Table 4.1) [10]. Bioinformatics analysis of the NEUROD2 target genes revealed different results, based on the database used. The BIOCARTA database identified the Ca^{2+} / Calmodulin dependent protein kinase (CaMK) activation pathway as the most significant NEUROD2 target (Table 4.2), while the KEGG database shows axon guidance and long-term potentiation pathways as NEUROD2 targets (Table 4.3). The difference may be caused by the type of the data these two databases contain. BIOCARTA tends to have pathways that are smaller in gene number compared to KEGG. Also, the two databases do not complement each other. For example, CaMK activation pathway is not found in the KEGG database.

Among the NEUROD2 targeted genes, one of the most prominent NEUROD2 binding in the genome was found on the second intron of the stromal interaction molecule 1 (*Stim1*) gene (Table 4.4). Further analysis was performed on this intronic NEUROD2 binding site by looking at the histone marks and conservation of the targeted sequence. The binding was observed with in all three NEUROD2 antibodies (Figure 4.1). However, the binding site does not correlate with any available activator or repressor histone marks. Interestingly, although found in a non-coding region, the binding site appears to be highly conserved among placental mammals, suggesting a biological importance of the sequence [10].

Gene ontology	Fold Enrichment	P-value
Dendrite morphogenesis (GO:0048813)	6,39	$2,23 \times 10^{-4}$
Dendrite development (GO:0016358)	4,13	$5,92 \times 10^{-3}$
Regulation of synapse organization (GO:0050807)	4,07	$9,15 \times 10^{-4}$
Regulation of synapse structure or activity (GO:0050803)	3,9	$1,08 \times 10^{-9}$
Synapse organization (GO:0050808)	3,86	$1,14 \times 10^{-3}$

Table 4.1: Gene ontology analysis of the NEUROD2 target genes at P0 mice cortex. Gene ontology ID numbers are given in parenthesis. Terms with $p < 0,01$ selected.

Pathway name (BIOCARTA)	P-value
Ca ²⁺ / Calmodulin-dependent Protein Kinase Activation	$3,7 \times 10^{-3}$
Nitric Oxide Signaling Pathway	$6,3 \times 10^{-3}$
Map Kinase Inactivation of SMRT Corepressor	$1,4 \times 10^{-2}$

Table 4.2: The pathways found to be targeted by NEUROD2 in BIOCARTA database. NEUROD2 most significantly targets Ca²⁺/ Calmodulin dependent protein kinase activation pathway.

Pathway name (KEGG)	P-value
Axon guidance	$1,7 \times 10^{-5}$
Long-term potentiation	$2,3 \times 10^{-4}$
Cell adhesion molecules (CAMs)	$3,4 \times 10^{-3}$

Table 4.3: The pathways found to be targeted by NEUROD2 in KEGG database. The pathways discovered from P0 NEUROD2 data. NEUROD2 most significantly targets Ca^{2+} /Calmodulin dependent protein kinase activation pathway.

Gene ID	Total score -10 * log10 (p value)	Location
<i>Krtap16-1</i>	8439,73	promoter
intergenic site	6366,16	intergenic
<i>Prss36</i>	6362,44	promoter
<i>Man1c1</i>	5669,73	exon
Intergenic site	5018,18	intergenic
<i>Tecr</i>	5011,04	intron
<i>Stim1</i>	4889,58	intron
<i>Hlcs</i>	4595,15	promoter
<i>Btbd17</i>	4541,48	promoter
<i>Erg</i>	4390,44	intron

Table 4.4: The top ten highest scoring genes found in P0 NEUROD2 CHIP-Seq. The score calculated as $-10 * \log_{10}(\text{p value})$ [10]. Location of the binding sites on the genes are shown in the table.

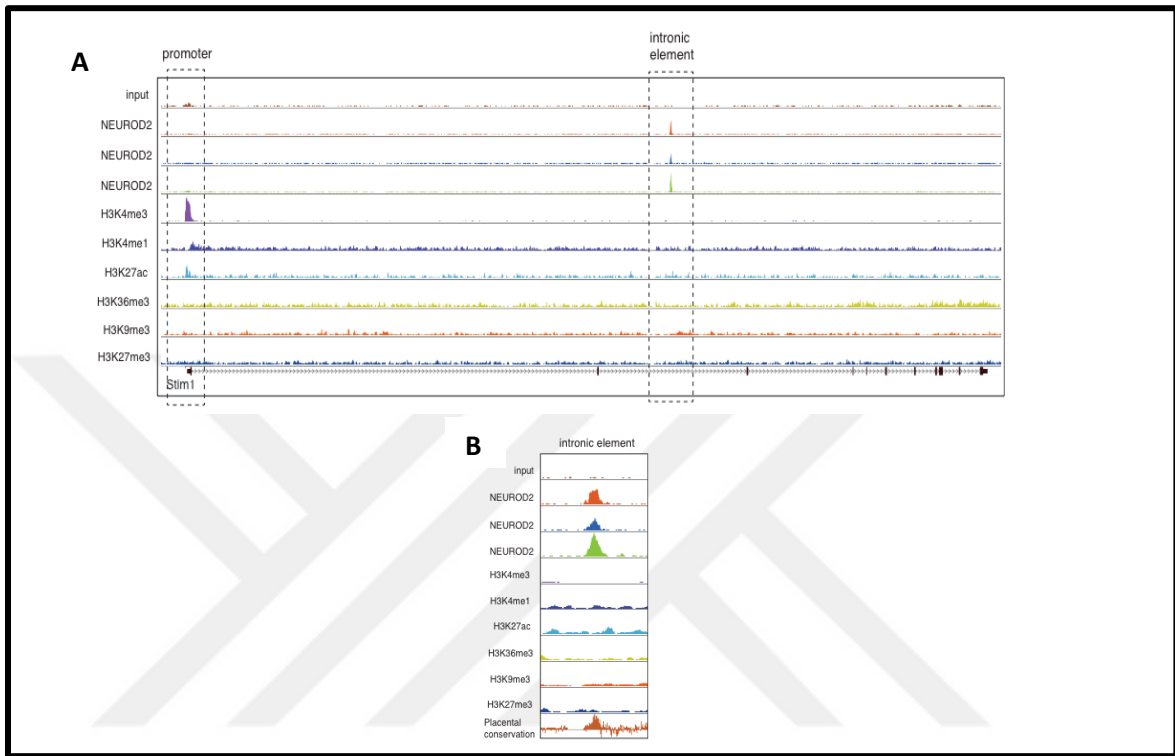


Figure 4.1: NEUROD2 binding sites mapped on *Stim1* gene. Input, ChIP result of three NEUROD2 antibody and histone marks are displayed. (A) *Stim1* gene represented at the bottom of the figure. All three NEUROD2 antibodies shows the binding of NEUROD2 to second intron of *Stim1* gene. Binding site does not correlate with any activator or repressor histone mark given in the figure. Promoter of *Stim1* gene enriched in transcriptional start site marker H3K4me3. (B) Intronic element of (A). No histone marks colocalize with NEUROD2 peaks. Placental conservation at the bottom indicates that the NEUROD2 bound site of the genome is conserved among placental mammals. Histone mark data for P0 mouse cortex were acquired from the ENCODE project (www.encodeproject.org). H3K4me3 is a promoter region marker, H3K4me1 is an enhancer region marker, H3K27ac is an active element marker, H3K36me3 is an active gene body marker, H3K9me3 and H3K27me3 are repression markers.

4.2. NEUROD2 target genes in Ca^{2+} / Calmodulin dependent protein kinase activation pathway

As discussed earlier, NEUROD2 ChIP-seq results indicated NEUROD2 targets the Ca^{2+} / Calmodulin dependent protein kinase activation pathway. To confirm the binding of NEUROD2 to genes involved in this pathway, I performed qPCR using P0 NEUROD2 ChIP DNA. Four genes involved in the pathway were selected, including all three calmodulin genes present in the mouse (*Calm1*, *Calm2*, *Calm3*) and *CamK1d* (Figure 4.2).

GFP ChIP DNA was used as a control, to confirm that the NEUROD2 antibody did not pull the DNA fragment due to unspecific interactions coming from antibody chain. The reads of the qPCR were plotted in ratio to input DNA, and given as percent input. The results in Figure 4.2 shows that all four genes assayed are indeed targeted by NEUROD2.

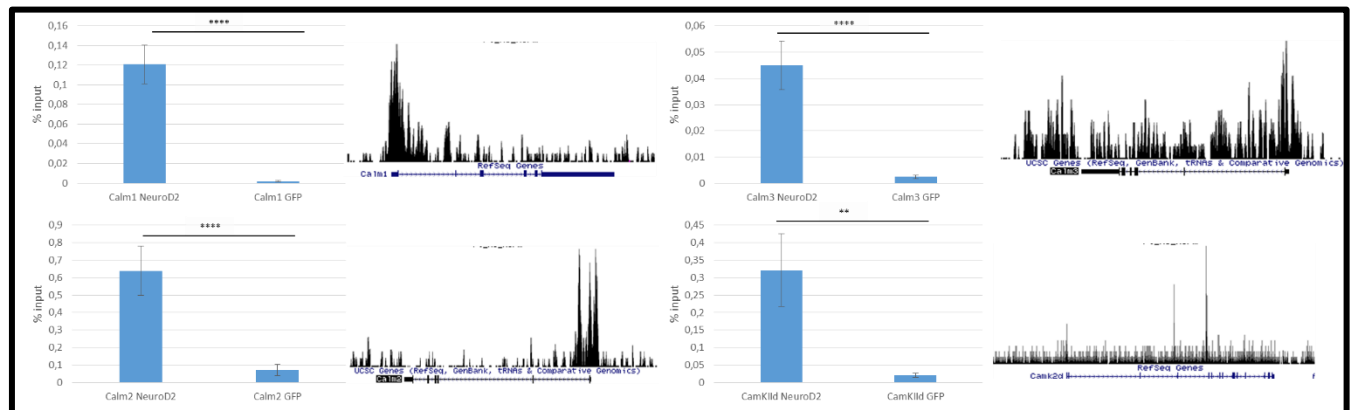


Figure 4.2: Four members of Ca^{2+} / Calmodulin dependent protein kinase activation pathway were confirmed to be bound by NEUROD2 on P0 cortex genome. (** p < 0,05 , **** p < 0,0005). The ChIP-qPCR reads were normalized to the total input reads and plotted as ‘% input’. The CHIP NEUROD2 binding profile is given on the right side of the plots.

4.3. NEUROD2 binding on gene expression of CaMK activation pathway genes

After confirming the NEUROD2 binding to genes of the CaMK activation pathway, I performed qPCR analysis to reveal if this binding has any transcriptional role on these genes. Along with 4 genes that were shown to be targeted by NEUROD2 (*Calm1*, *Calm2*, *Calm3* and *CamKIId*), *CaMKIV*, another gene found in the CaMK activation pathway, was also subjected to qPCR for detecting mRNA levels. To understand the effect of NEUROD2 on the expression of these five genes, primary neuronal cultures were nucleofected by *Neurod2* shRNA, or NS shRNA as control. Nucleofection is an efficient electroporation method that increases the transfection yield. High yield is required, since for qPCR analysis, total RNA from the whole plate is isolated, introducing RNA from many non-transfected neurons into the experiment. RNA was isolated from nucleofected samples at 5 DIV and used in cDNA synthesis. This cDNA was used as template DNA in qPCR. Three technical replicates were performed for each reaction. GAPDH was used as reference. Three different biological replicates were used, except for *Calm1* and *Calm3*, which were repeated with 7 biological replicates.

Results shown in Figure 4.3 revealed that the variation between experiments are very high, and thus no significant change was observed in mRNA levels of the selected five genes.

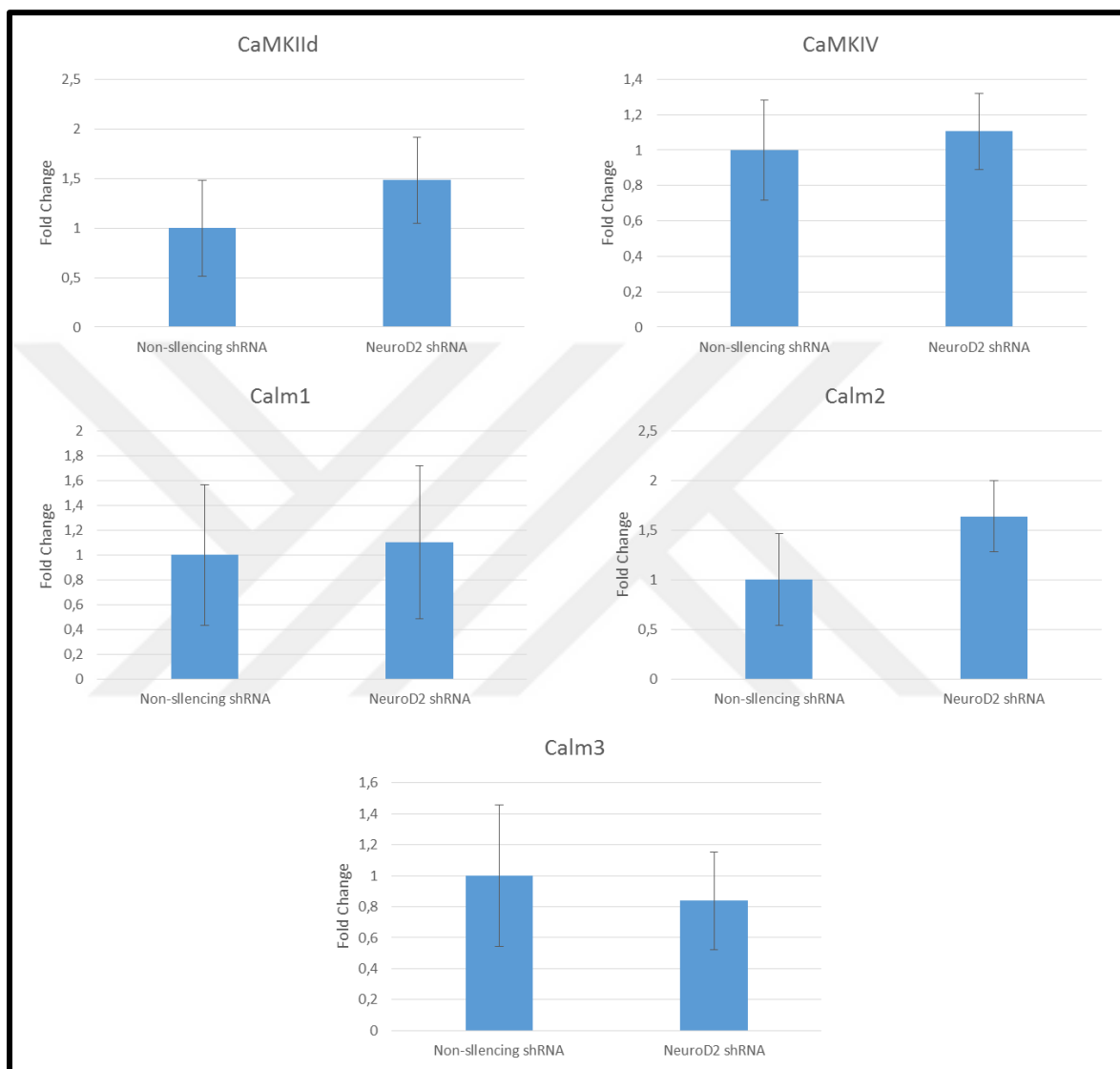


Figure 4.3: qPCR results of CaMK activation pathway genes. The reads were normalized to GAPDH signal. *Neurod2* shRNA fold change levels were calculated by normalizing to non-silencing shRNA measurements. No significant difference between NS shRNA and *Neurod2* shRNA was observed in all five samples.

4.4. Cloning of *Neurod2* shRNA into pSUPER-neo-GFP vector

In order to elucidate the role of intronic binding of NEUROD2 on the *Stim1* gene, I planned to perform knockdown of *Neurod2* and compare the *Neurod2* knockdown cells with mock-treated cells. To perform the knockdown, I cloned *Neurod2* targeting shRNA into pSUPER-neo-GFP vector.

pSUPER-neo-GFP vector is used to express shRNAs by a H1 promoter. The pSUPER-neo-GFP vector is digested with HindIII and BglII restriction enzymes to complement the ends of the shRNA oligonucleotides. For single shRNA, two complementing oligonucleotides are annealed and inserted into HindIII and BglII digested pSUPER-neo-GFP vector. Control of the successful ligation is performed by restriction digestion. During ligation, the BglII site is lost, thus EcoRI is used for control. EcoRI – HindIII digestion creates a 281 bp long DNA fragment if the oligonucleotide is inserted to the vector. If the oligonucleotide insertion failed, EcoRI – HindIII digestion results in 227 bp long DNA fragment.

I used shRNAs designed against *Neurod2* and cloned them into the pSUPER-neo-GFP vector. Oligonucleotides designed for *Neurod2* were annealed and ligated into the digested vector. The ligation product was transformed to bacteria and plated. Colonies formed on the ampicillin resistant plate were selected and their plasmid DNA was extracted. I treated the extracted plasmid with EcoRI – HindIII. Results shown in the Figure 4.4 show that I successfully created a *Neurod2*-shRNA pSUPER vector. Following the same protocol, a non-silencing shRNA that does not have any target in the mammalian genome cloned into pSUPER-neo-GFP vector to use as a control (unpublished data, I. Kotan)

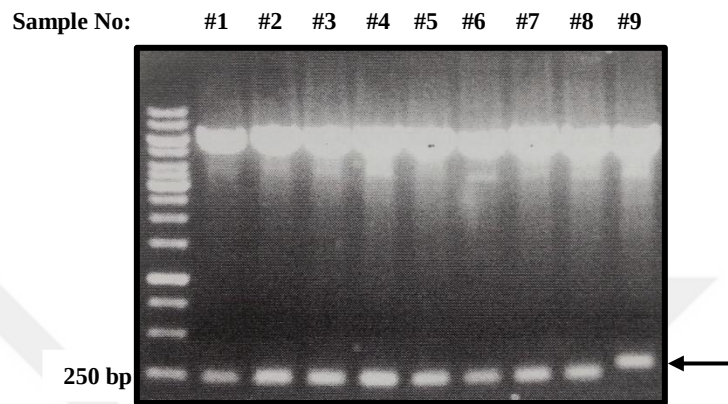


Figure 4.4: EcoRI – HindIII double digestion of the *Neurod2* shRNA-pSUPER ligation. Run performed on 0.8% agarose gel. The bottom band of the ladder represents 250 bp. Arrow indicates the expected size from a positive colony. Only colony #9 (last lane) have band larger than 250 bp, showing the oligonucleotide is present in that sample.

4.5. Knockdown of *Neurod2* by *Neurod2*-shRNA

To validate the designed shRNA can knockdown *Neurod2*, I tested the shRNA on HEK293 cells. HEK293 cells co-transfected with a myc- *Neurod2* vector and the *Neurod2* shRNA (or empty shRNA vector). 3 μ g of each of the two vectors were transfected into HEK293 cells on 60mm plates and the cell lysate was collected two days after the transfection. The lysates were run on SDS-PAGE, and the blot was treated with anti-myc antibody. The same blot was stripped and treated with anti- β -actin antibody. The stripping protocol was repeated and anti-GFP treatment was performed on the same blot. The western blot results of these experiments are shown in Figure 4.5.

The western blot results show that *Neurod2* shRNA was able to knock down *Neurod2*. The effect is not due to pSUPER-neo-GFP vector since only expressing myc- *Neurod2* alone and expressing myc- *Neurod2* together with empty pSUPER-neo-GFP vector show no differences. A slight intensity decrease in myc- *Neurod2* + pSUPER-neo-GFP compared to myc-Neurod2 is likely due to loading amount of the sample, since β -actin levels show a similar reduction. The reduction upon *Neurod2* shRNA is not related with the loading amount, since β -actin levels are not different enough to justify the observed reduction. Thus, the *Neurod2* shRNA is the reason for the decreased amount of myc- *Neurod2* in the lysate.

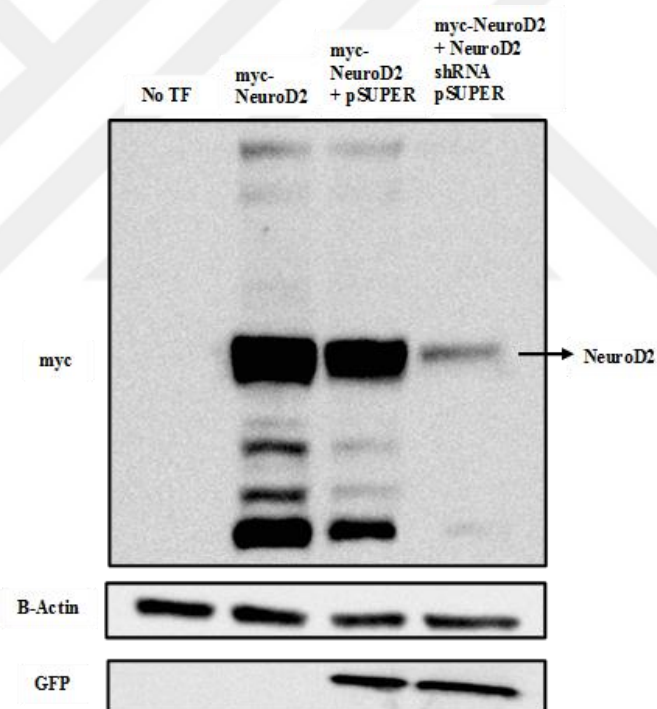


Figure 4.5: Western blot results of *Neurod2* shRNA knockdown of *Neurod2*. Lysate collected from transfected HEK293 cells are blotted. No TF sample is the lysate collected from non-transfected cells. pSUPER-neo-GFP is the vector that shRNAs are cloned into. The arrow on the right side indicates the expected size of NEUROD2 protein.

4.6. Creating pSUPER-mCherry vector

The pSUPER-neo-GFP shRNA constructs are not suitable for live imaging experiments, because the GFP present in the pSUPER-neo-GFP vector interferes with the signal recorded from Fluo-3, both of which are captured in the green channel. To overcome this problem, I removed the GFP gene from the pSUPER-neo-GFP vector and inserted a mCherry cassette. In this way, I would still be able to select transfected cells in red channel, without interfering with the Fluo-3 signal.

Two suitable restriction digestion sites were present in mCherry and pSUPER-neo-GFP vector. Both genes were flanked by an AgeI restriction enzyme site in the 5' end and a NotI site at the 3' end. Since the same restriction sites were located at same 5' and 3' of the genes, the inserted mCherry will not be reverted. Double digestion was performed with AgeI and NotI to both pSUPER-neo-GFP and mCherry vectors. Digestion products were run on an agarose gel. The results are displayed in Figure 4.6. The large fragment in the pSUPER-neo-GFP lane corresponding to the vector backbone, and the small fragment in mCherry lane corresponds to the mCherry gene. In pSUPER-neo-GFP vector, the neo resistance gene was also removed because it was located in the removed GFP fragment after digestion. These fragments were purified from the agarose gel. Fragments were ligated and transformed into competent DH5 α E.coli bacteria. Bacteria were plated and colonies were selected from the plate. Control digestion was again performed by AgeI and NotI enzymes. Colonies having mCherry band were selected and a maxiprep was performed to obtain plasmid DNA.

I transfected the pSUPER-mCherry construct to N2A cell line seeded on coverslips to ensure the plasmid is able to express mCherry by immunofluorescence. N2A cells were fixed two days after transfection. Figure 4.7 shows that mCherry attached pSUPER vector can express mCherry, thus can be used in imaging experiments.

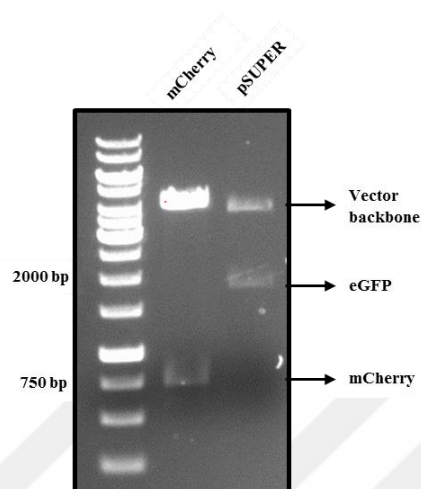


Figure 4.6: Gel electrophoresis result of AgeI-NotI digestion of mCherry and pSUPER vector. 750 bp fragment represents the mCherry cassette. Approximately 2 kb fragment shows EGFP-neo is removed from the pSUPER-neo-GFP vector. The small band in mCherry and large band in pSUPER were extracted from the gel and ligated.

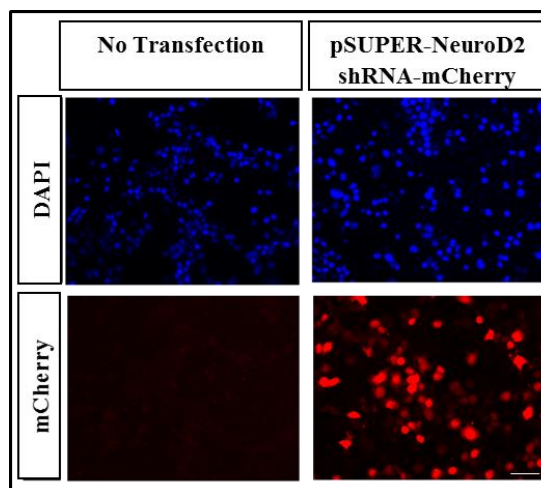


Figure 4.7: pSUPER-mCherry construct can express mCherry. N2A cells were either transfected with pSUPER- *Neurod2* shRNA-mCherry vector or not transfected. DAPI (blue), mCherry (red). Scale bar, 10 μ m.

4.7. STIM1 levels increase upon *Neurod2* knockdown

With the ChIP-Seq results showing NEUROD2 binding to *Stim1* intron (Figure 4.1) (Table 4.4), we wanted to examine how this binding affects STIM1 levels. The intronic binding is confirmed by ChIP-qPCR using DNA collected from P0 and E14,5 ChIP (Figure A.1) The effect of the binding on STIM1 levels was tested by using *Neurod2* targeting shRNAs. *Neurod2* shRNAs caused an increase in expression of STIM1. Increased STIM1 expression was shown in mRNA level (Figure A.2) and in protein level, with western blotting and IF (Figure A.2 and A.4) at 5 days after transfection (Gizem Güzelsoy, unpublished data).

A rescue experiment was conducted to ensure the phenotype is due to a decrease in NEUROD2 levels. *rNeurod2* (shRNA resistant *Neurod2*) was able to express NEUROD2 even in the presence of *Neurod2* shRNA (Figure A.3). Expression of *rNeurod2* in neurons have reduced the STIM1 signal from IF to control levels, showing that the effect is NEUROD2 specific (Figure A.4) [10]. .

4.8. Optimization of live calcium imaging

After cloning all required constructs for shRNA transfection, I aimed to optimize the imaging protocol before using primary neuronal cultures for the experiments. As the first step of the optimization for establishing a standard calcium imaging protocol, I tested if I can obtain a fluorescence signal from Fluo-3 dye within live cells. To get a calcium-induced signal from the cells, Fluo-3 dye has to enter into the cells and should respond to intracellular Ca^{2+} level changes. I used N2A cells to test these parameters. Ionomycin was used for altering the Ca^{2+} levels in these cells. Ionomycin opens pores in the plasma membrane that allows Ca^{2+} passage. Due to high extracellular Ca^{2+} concentration, Ca^{2+} ions diffuse into the cells via these pores. Therefore, if our calcium sensitive dye loading is successful, after applying ionomycin, there should be an increase in fluorescence levels due to intake of Ca^{2+} .

I treated N2A cells with four different dye concentrations (2 μ M, 3 μ M, 4 μ M, 5 μ M) and three different drug concentrations (5 μ M, 10 μ M, 20 μ M) to find in which concentration range the dye is responsive to intracellular calcium changes. The quantified cells were plotted in Figure 4.8. Not all the conditions were able to be quantified due to problems that are not related with dye or drug treatment, such as focus shift during imaging.

The results in Figure 4.8 showed that Fluo-3 dye successfully entered the cell and responded to calcium changes induced by ionomycin. In all tested dye concentrations, an intracellular Ca^{2+} level increase induced by ionomycin could be detected. In all tested Fluo-3 concentrations, threefold increase in the fluorescence signal was observed, indicating that Fluo-3 dye is responsive in all tested conditions. Only condition where such intensity increase is not observed is the condition with the lowest concentration of ionomycin (2 μ M Fluo-3 / 5 μ M ionomycin). This phenotype is likely due to low ionomycin concentration, as 2 μ M Fluo-3 / 10 μ M ionomycin sample has similar fluorescent signal intensity with all other conditions.

Ionomycin was shown to induce a rapid calcium uptake inside the cell just after it is applied. Following this rapid increase, intracellular calcium levels quickly go down. This decrease ceases at a level above the initial calcium levels. After this point, the cell starts to take up calcium gradually [54]. The decrease in calcium levels mentioned above is not observed in the 5 μ M Fluo-3 condition. In 2 μ M Fluo-3 and 3 μ M Fluo-3, the gradual calcium uptake is not seen as clearly as in 4 μ M Fluo-3 condition (Figure 4.8). Therefore in following experiments, Fluo-3 dye concentration was used as 4 μ M.

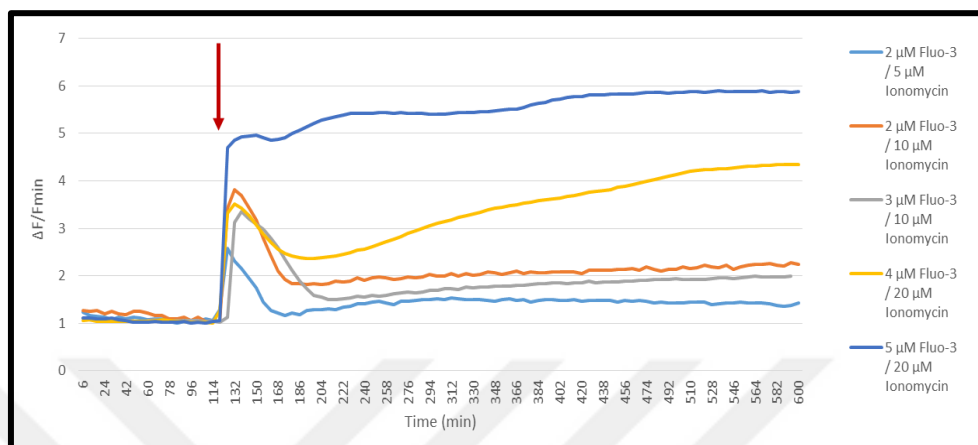


Figure 4.8: Fluorescent signal obtained from N2A cells loaded with different concentrations of Fluo-3 dye, upon different concentrations of ionomycin treatment. The background signal level was subtracted from the fluorescent signal measured from the cell. Background corrected signal was normalized to minimum signal (F_{min}) obtained from that sample, and the normalized values ($\Delta F/F_{min}$) are plotted. The time point where ionomycin added is indicated by the arrow. Sample size for each condition are; 2 μ M Fluo-3 / 5 μ M Ionomycin $n=2$, 2 μ M Fluo-3 / 10 μ M Ionomycin $n=2$, 3 μ M Fluo-3 / 10 μ M Ionomycin $n=2$, 4 μ M Fluo-3 / 20 μ M Ionomycin $n=8$ and 5 μ M Fluo-3 / 20 μ M Ionomycin $n=1$.

4.9. SOCE on *Neurod2* knockdown primary culture neurons

To examine the role of NEUROD2 binding on *Stim1* intron, I observed the SOCE response of the primary neuronal cultures prepared from E14 mice cortex. As discussed earlier, STIM1 protein is a key player in the SOCE mechanism. Together with ORAI channels, they allow calcium entry upon store depletion. Thus, I hypothesized that this NEUROD2 binding on the STIM1 intron may alter expression of the *Stim1* mRNA, leading to a change in STIM1 protein levels. The change in STIM1 protein levels can cause a phenotype in SOCE of the neurons.

There are many previous studies that have examined SOCE in different cell types. With these studies, a well-established SOCE experimentation protocol has been created for calcium imaging [60]. This protocol can be separated into three phases. In the first phase, basal calcium signal from the cell is recorded as a reference. Then in the second phase, thapsigargin (TG) is applied with calcium-free media. TG blocks the SERCA channels on the ER and diminishes the calcium stored in the ER. A small peak is observed due to calcium released from the ER. Since there is no calcium in the media, calcium deprived cell cannot refill its ER stores in this phase. In the third phase, calcium is supplied to the media. The extracellular calcium is taken up in the cell via the SOCE mechanism, causing a rapid increase in calcium concentration in the cell.

Optimizations done in N2A cell line determined the proper experimental conditions for live imaging in primary neuronal cultures. To elucidate the role of NEUROD2 on SOCE, I performed *Neurod2* knockdown by shRNA targeting *Neurod2* mRNA, on primary neuronal cultures prepared from E14 mice cortices. A non-silencing shRNA that does not complement any known mRNA in mammals was used as a control. Both shRNA constructs contain a mCherry cassette, allowing selection of transfected cells by red fluorescence. 1 μ g shRNA was transfected into cultured neurons at 2-3 DIV. At ~7DIV, imaging was initiated by loading Fluo-3 dye into neurons. 4 μ M Fluo-3 was prepared in Ringer solution and loaded into the neurons. Calcium-free solution was prepared for second phase of the experiment. 5 μ M TG was added to both calcium-free solution and solution used in the third phase. Quantification of the results are shown in Figure 4.9.

Results showed that knockdown of *Neurod2* significantly elevated the calcium uptake in ER calcium store-depleted neurons. This high calcium uptake response may suggest that the level of STIM1 protein is increased. And the increase in STIM1, in turn, leads to this amplified response. Since this phenotype is observed upon decreased levels of NEUROD2, this finding may indicate that NEUROD2 binding to intronic region of STIM1 decreases the expression rate of STIM1 mRNA, leading to decreased protein levels for STIM1. Relieving this inhibition by knocking down *Neurod2* leads to higher STIM1 levels and SOCE within neurons.

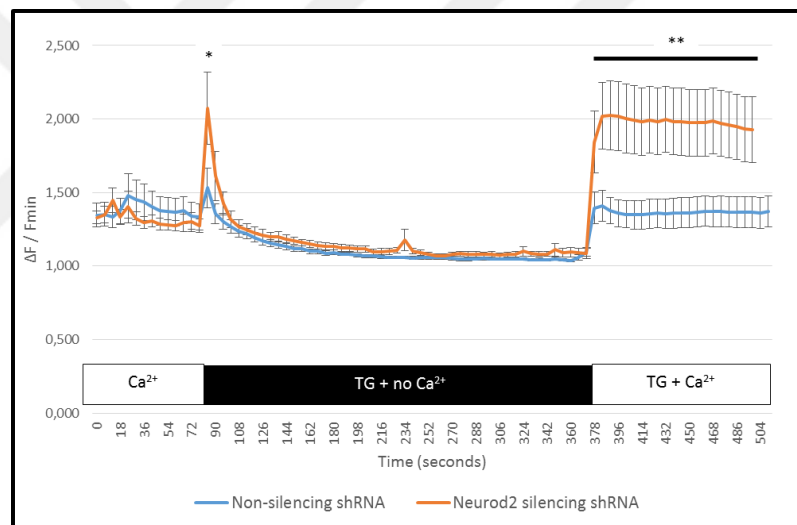


Figure 4.9: Increased SOCE response in *Neurod2* shRNA treated neurons. E14 mice cortices dissected and 300,000 cell/well seeded in 24-well plate. Background corrected signal normalized to minimum signal (F_{min}) obtained from that sample, and the normalized values ($\Delta F/F_{min}$) are plotted. Timing of the media changes and drug addition indicated in the black and white bar above x axis. Added Ca^{2+} concentration is 2 mM. Average of three biological replicates represented in the plot. $n=18$ for non-silencing shRNA, $n=21$ for *Neurod2* silencing shRNA. Bars in the plot indicate standard error of mean. Two tailed Student's test applied for statistical analysis. (** $p < 0,05$, * $p < 0,1$)

4.10. Immunofluorescence analysis for determination of rescue conditions

Knockdown of *Neurod2* was shown to increase calcium uptake upon ER calcium store depletion in neurons. However, it is possible that this observed phenotype may not be linked with NEUROD2. The shRNA used for knocking down *Neurod2* may have off-target effects. These off-target genes, if present, might be the underlying reason of the observed phenotype. To test this possibility, I used shRNA-resistant *Neurod2* (*rNeurod2*) on shRNA treated neurons. The shRNA-resistant *Neurod2* was formed by three point mutations targeted to *Neurod2* shRNA binding sequence. These three point mutations were designed such that they do not cause any change in the amino acid composition of NEUROD2 [10] (Figure A.3).

To find the optimal plasmid composition for successful *Neurod2* knockdown and rescue, I performed immunofluorescence analysis on cultured primary neurons, transfected with different amounts of *Neurod2* shRNA and *rNeurod2* plasmids.

E14 mice cortices are used for the primary neuronal cultures. Cells are plated on coverslips containing coated wells. At 2 DIV transfection is performed. Transfection performed with a combination of three different *rNeurod2* (0 – 0,5 – 1 µg) and three different shRNA (0,5 – 1 – 1,5 µg) conditions. Both non-silencing and *Neurod2* shRNA are used in these conditions. The transfected cells were fixed at 7 DIV. The cells treated with anti-*Neurod2* antibody, and green-fluorophore linked anti-mouse secondary antibody were used for detecting anti-*Neurod2* antibody. Red signal shows mCherry, which indicates that the cell was able to take up the pSUPER vector and expressed the plasmid.

Captured images are displayed in Figure 4.10, Figure 4.11 and Figure 4.12. The quantification of all measured images is found in Figure 4.13.

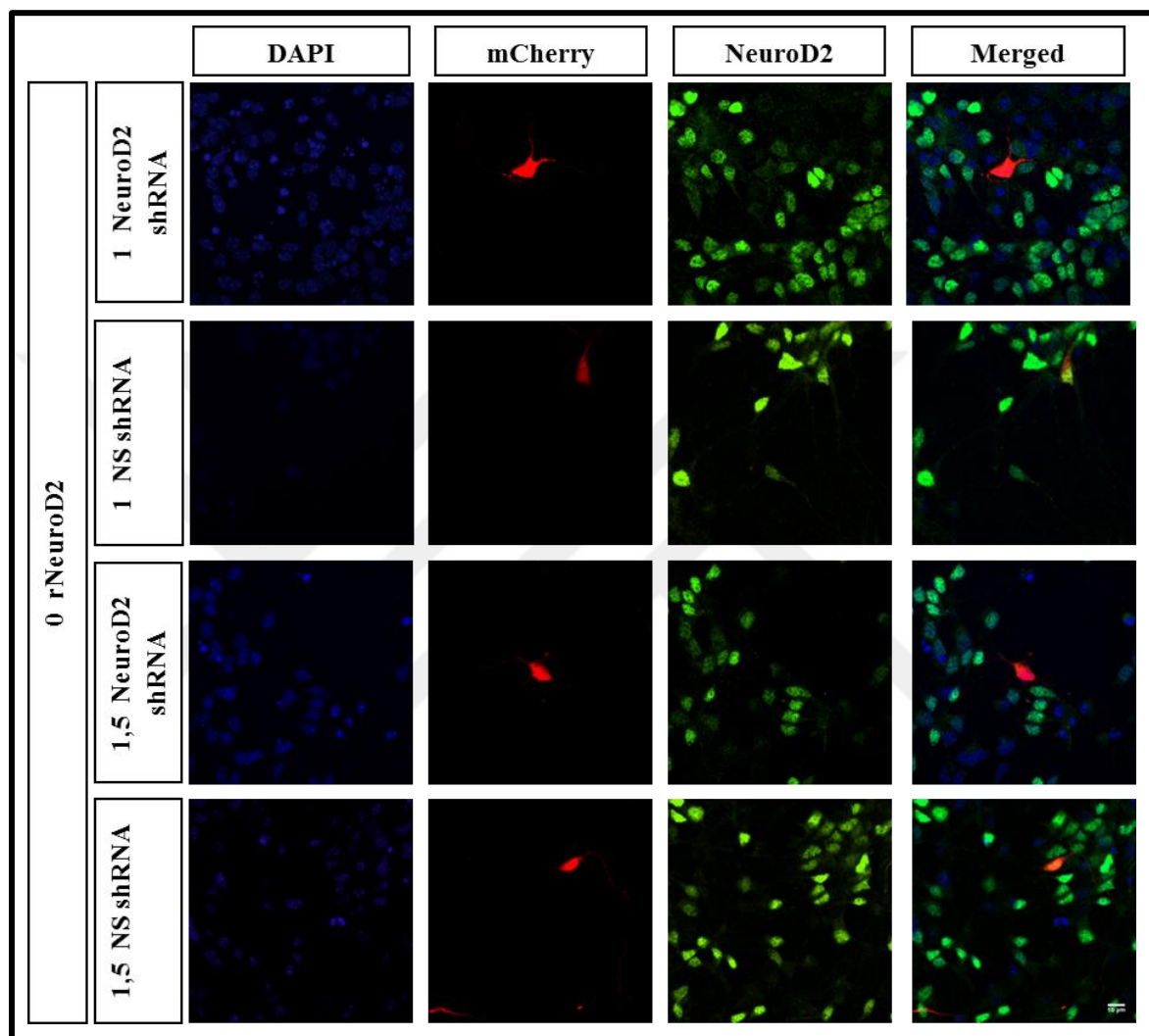


Figure 4.10: Immunofluorescence results of *Neurod2* shRNA-mCherry and non-silencing (NS) shRNA- mCherry transfected neurons. No shRNA-resistant *Neurod2* transfection was performed in this conditions, indicated as 0 rNeurod2. The number on the labels found at left side indicates how much DNA is transfected, in μg . anti-Neurod2 used in immunostaining to determine the effect of *Neurod2* knockdown on NEUROD2 signal. DAPI (blue), mCherry (red), NEUROD2 (green) Scale bar, 10 μm . Quantification done by ImageJ software.

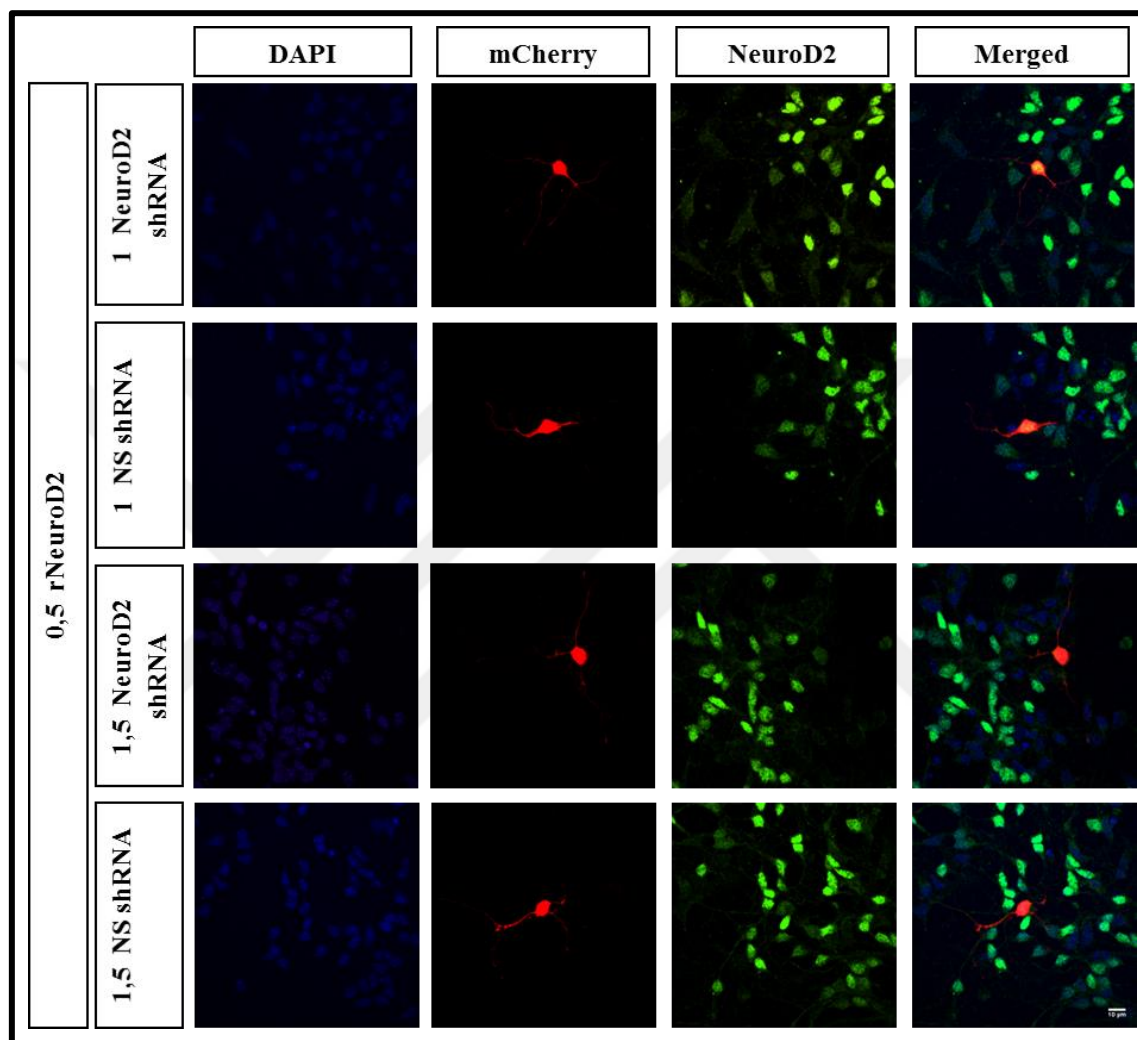


Figure 4.11: Immunofluorescence result of *Neurod2* shRNA-mCherry and non-silencing (NS) shRNA- mCherry transfected neurons, in presence of 0,5 μ g rNeurod2 . The number on the labels found at left side indicates how much DNA is transfected, in μ g. anti-Neurod2 used in immunostaining to determine the effect of *Neurod2* knockdown on NEUROD2 signal. DAPI (blue), mCherry (red), NEUROD2 (green) Scale bar, 10 μ m. Quantification done by ImageJ software.

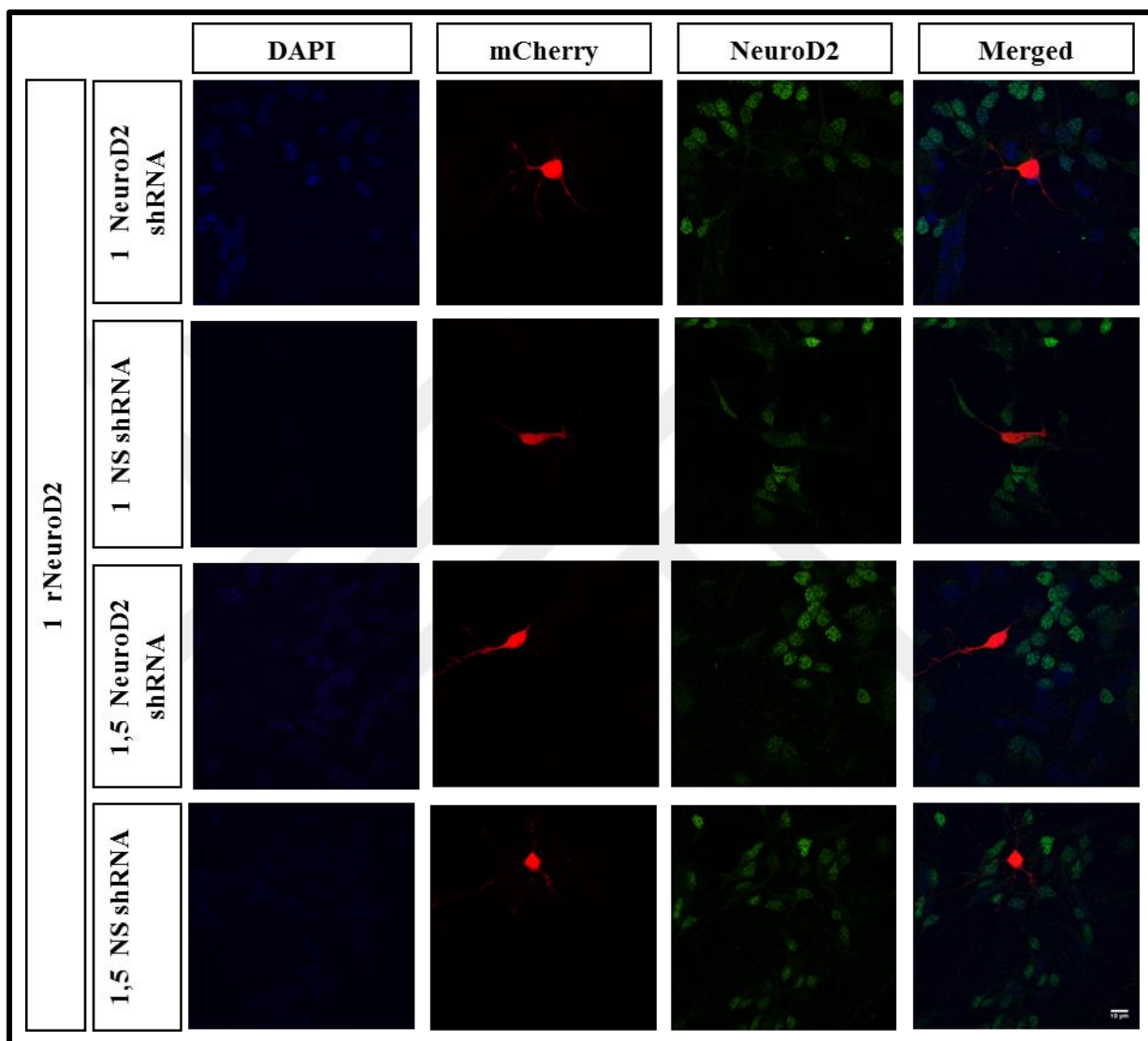


Figure 4.12: Immunofluorescence result of *Neurod2* shRNA-mCherry and non-silencing (NS) shRNA- mCherry transfected neurons, in presence of 1 μ g rNeurod2. The number on the labels found at left side indicates how much DNA is transfected, in μ g. anti-Neurod2 used in immunostaining to determine the effect of *Neurod2* knockdown on NEUROD2 signal. DAPI (blue), mCherry (red), NEUROD2 (green) Scale bar, 10 μ m. Quantification done by ImageJ software.

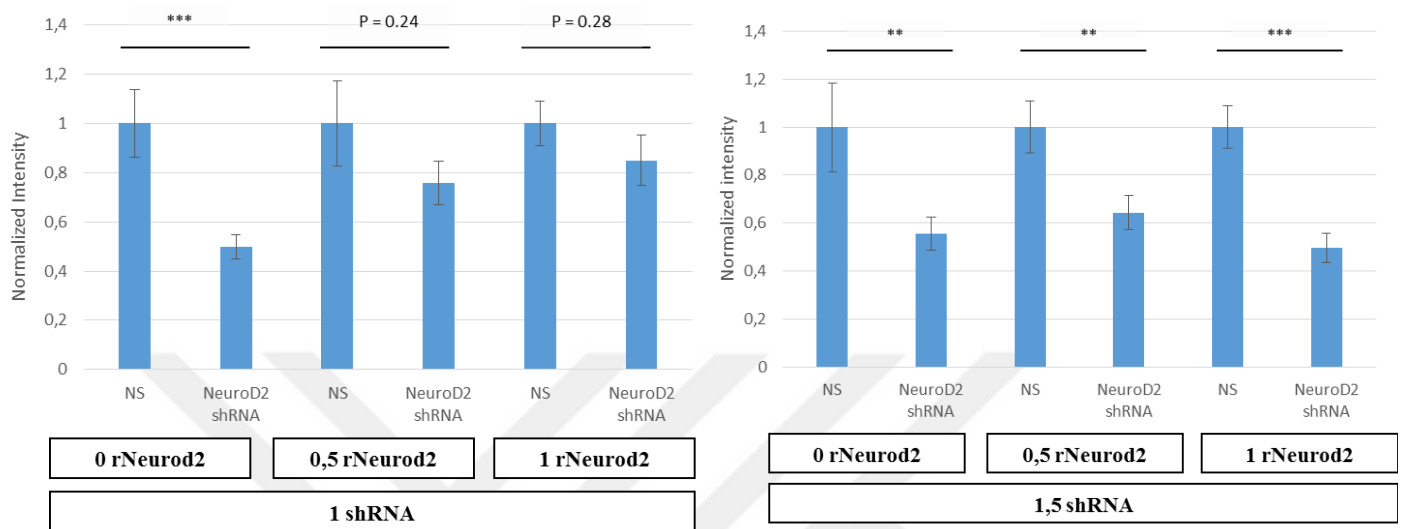


Figure 4.13: Quantification of IF signal intensity of the samples of the experiment in Figure 4.10, Figure 4.11 and Figure 4.12. Amount of transfected plasmid given in the boxes found below o of the plot, in µg. On left graph, 1 µg shRNA condition is found. And on the right part, 1,5 µg shRNA condition is found. All Neurod2 shRNA values are normalized to their NS, which is subjected to same transfection conditions. (** $p < 0,05$, *** $p < 0,005$). Error bars on the graph indicates standard error of mean.

Using 1 µg shRNA appeared to be more suitable for rescue experiments. In 1 µg shRNA condition, NEUROD2 intensity responds to increases in rNeurod2 plasmid amounts. However, in 1,5 µg shRNA condition, rNeurod2 increase does not improve the expressed NEUROD2 amounts. Thus, 1 µg shRNA is used in imaging experiments. The amount of rNeurod2 transfection in 1 µg shRNA conditions, however, does not appear to rescue the phenotype completely. Even in the best rescue condition, 18% reduction from NS control is observed. To overcome this, I repeated the experiment with 2 µg rNeurod2 + 1 µg shRNA conditions.

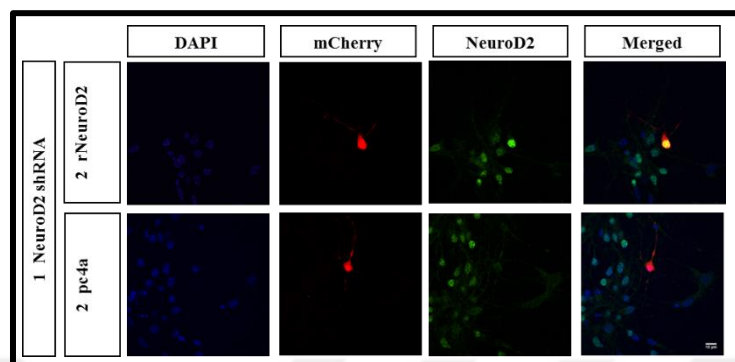


Figure 4.14: Immunofluorescence result of *Neurod2* shRNA-mCherry and empty rNeurod2 vector (pc4a) transfected neurons. The number on the labels found at left side indicates how much DNA is transfected, in μg . anti-Neurod2 used in immunostaining to determine the effect of *Neurod2* knockdown on NEUROD2 signal. DAPI (blue), mCherry (red), NEUROD2 (green) Scale bar, 10 μm . Quantification done by ImageJ software.

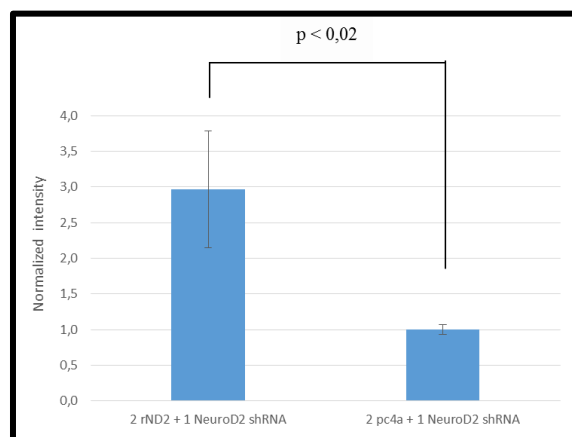


Figure 4.15: Quantification of IF signal intensity of the samples of the experiment on Figure 4.14. Sample numbers from left to right are; n= 16, 17. The signal intensity normalized to 2 pc4a + 1 *Neurod2* shRNA sample. Two tailed Student's test applied for statistical analysis.

As seen in Figure 4.14 and 4.15, increasing the used rNeurod2 amount has increased NEUROD2 levels threefold. The *Neurod2* shRNA was shown to decrease NEUROD2 levels by 50%, so a threefold increase is able to overcome this reduction in Neurod2 expression. Thus, in rescue experiments during calcium imaging, 2 μ g rNeurod2 + 1 μ g *Neurod2* shRNA transfection condition is used.

4.11. Recovery of SOCE increase by shRNA-resistant *Neurod2* expression

The shRNA-resistant *Neurod2* (rNeurod2) construct was co-transfected with *Neurod2* targeting (or non-silencing) shRNA into neurons. If the phenotype observed with *Neurod2* targeting shRNA is due to off-target effects of the shRNA, expression of shRNA-resistant *Neurod2* should not show an effect in calcium uptake phenotype. In the other case, if the phenotype is linked with the reduced levels of NEUROD2, expression of shRNA-resistant *Neurod2* should rescue the phenotype and reduce the calcium uptake.

Neurons were co-transfected with 1 μ g shRNA (*Neurod2* targeting or non-silencing) and 2 μ g rNeurod2 vector (or empty vector) at 2-3 DIV, as determined in section 4.10. Three different co-transfection conditions were used (non-silencing shRNA + vector, *Neurod2* silencing shRNA + vector, and *Neurod2* silencing shRNA + rNeurod2). For studying SOCE of transfected neurons, I repeated the same experimental procedure used in observing the effect of *Neurod2* shRNA. Different from the previous experiment, 1 μ M tetratodoxin (TTX) was present in all the media used in live imaging. TTX binds to voltage gated sodium channels and blocks spontaneous action potential firing of the neurons, hence it will eliminate the possibility of action potential involvement in the phenotype I have observed. Live imaging was performed at 6-8DIV. Results of the experiments are given in Figure 4.17.

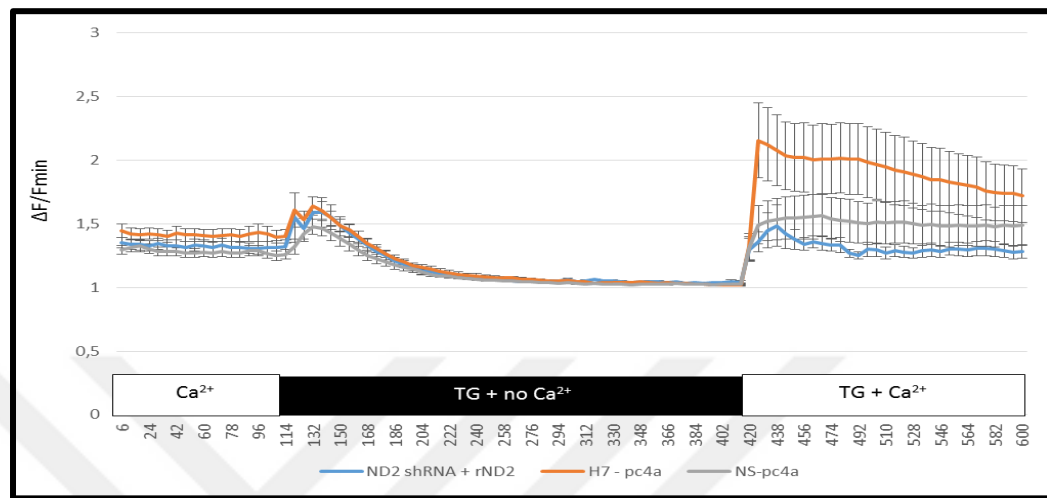


Figure 4.16: shRNA resistant *Neurod2* able to rescue increased calcium uptake phenotype upon store depletion. E14 mice cortices dissected and 300,000 cell/well seeded in 24-well plate. Background corrected signal normalized to minimum signal (Fmin) obtained from that sample, and the normalized values ($\Delta F/F_{min}$) are plotted. Timing of the media changes and drug addition indicated in the black and white bar above x axis. Average of three biological replicates represented in the plot. NS stands for son-silencing shRNA and pc4a is the vehicle vector. The number of quantified cells are; n= 35 for *Neurod2* shRNA + rNeurod2, n=51 for *Neurod2* shRNA + pc4a and n=21 for NS shRNA + pc4a. Bars in the plot indicate standard error of mean.

Figure 4.16 shows that the rapid calcium uptake seen in *Neurod2* knockdown cells can be reverted by expressing rNeurod2. This result rules out the possibility of shRNA off-target effect, showing that the decrease in NEUROD2 levels is indeed responsible for the observed phenotype.

To better understand the effect of Neurod2 on SOCE, I performed more detailed analysis of the plot. The areas under the first and second peak are calculated and the highest $\Delta F/F_{min}$ values of these peaks are examined in Figure 4.17. I examined the second peak caused by addition of calcium containing media as early and late response. Early response represents the initial calcium inflow into the cell while late response shows the equilibration of the calcium ions within the cell. Results of this analysis are given in Figure 4.17.

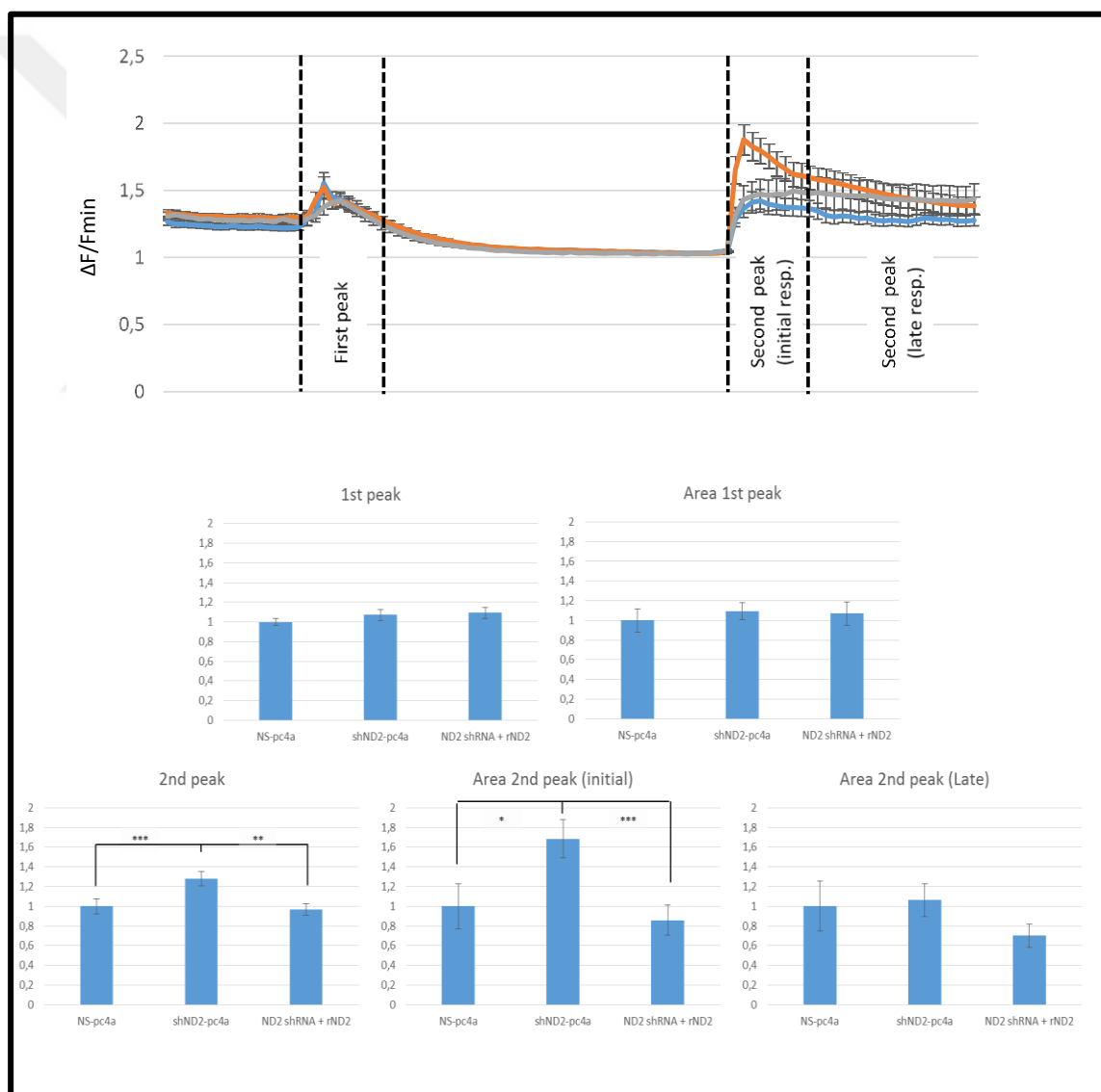


Figure 4.17: Quantification of peak signals and peak areas of the plot in figure 4.16. The peak names are labeled on the plot. The peak observed in first media change named as 1st peak and 1st rise peak and 2nd rise peak stands for the maximum $\Delta F/F_{min}$ value obtained with the corresponding peak. All the plots are normalized NS-pc4a sample (non-silencing shRNA + pc4a). The number of quantified cells are; n= 35 for *Neurod2* shRNA + r*Neurod2*, n=51 for *Neurod2* shRNA + pc4a and n=21 for NS shRNA + pc4a. Bars in the plot indicate standard error of mean. Two tailed Student's test applied for statistical analysis (** p < 0,05, *** p < 0,005, **** p < 0,0005, the differences are not significant if not indicated).

The quantification of the plot at Figure 4.17 indicated that the peak response of the second peak is significantly higher in *Neurod2* shRNA neurons than the control neurons. This effect is diminished upon r*Neurod2* expression. There is no significant change observed in the first peak and the late phase of the second peak. The signal observed at the first peak should be arising from the internal calcium stores, since there is no outside calcium source present. There is no significant change observed in the first peak, suggesting that the steady state ER Ca^{2+} levels are not regulated by *Neurod2*. Similarly, the difference in calcium signal levels for the late phase response were not significantly different between each condition, suggesting that *Neurod2* has no effect on the adjustment of intracellular calcium levels after high calcium uptake. A high initial phase area of the second peak and high maximum second peak signal of *Neurod2* knockdown sample can be proposed to be linked with increased STIM1 levels. Increased STIM1 presence on the ER can allow activation of more ORAI channels on the plasma membrane, allowing more calcium to be taken up by the neuron.

4.12. Overexpression of rNeurod2 further suppresses SOCE

After observing that absence of NEUROD2 protein can enhance SOCE, I examined how SOCE response of the neurons are affected when Neurod2 is overexpressed. I expressed 1 μ g rNeurod2 plasmid with 1 μ g NS shRNA. shRNA transfection allowed selection of the successfully transfected neuron in live imaging. As control, Neurod2 vehicle vector is co-transfected with 1 μ g NS shRNA. The calcium imaging protocol performed on previous sections are repeated. In concordance with knockdown experiments, overexpression of NEUROD2 displayed opposite effect of what have seen in shRNA treatment (Figure 4.18).

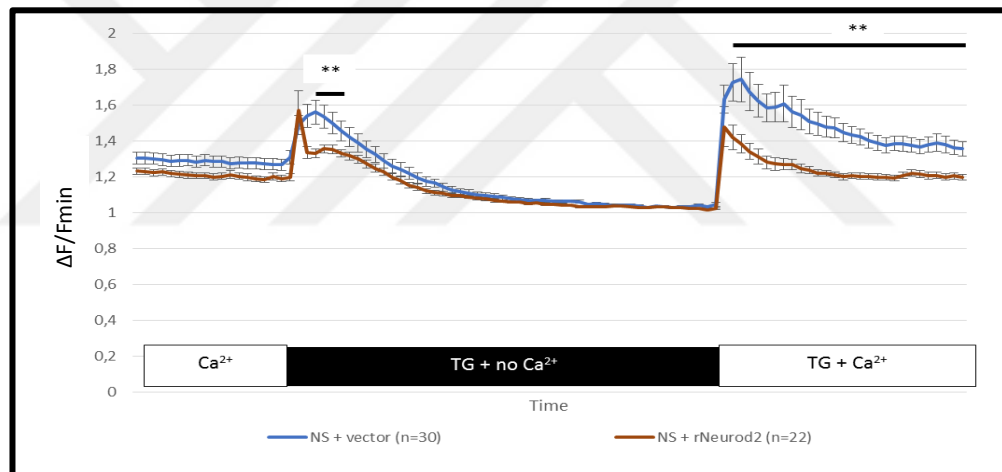


Figure 4.18: shRNA resistant *Neurod2* overexpression further blocks calcium uptake phenotype upon store depletion. E14 mice cortices dissected and 300,000 cell/well seeded in 24-well plate. Background corrected signal normalized to minimum signal (F_{min}) obtained from that sample, and the normalized values ($\Delta F/F_{min}$) are plotted. Timing of the media changes and drug addition indicated in the black and white bar above x axis. Average of three biological replicates represented in the plot. NS stands for non-silencing shRNA. The number of quantified cells are; $n = 22$ for rNeurod2 + NS shRNA, $n = 39$ for NS shRNA + pc4a. Bars in the plot indicate standard error of mean. (** $p < 0,05$)

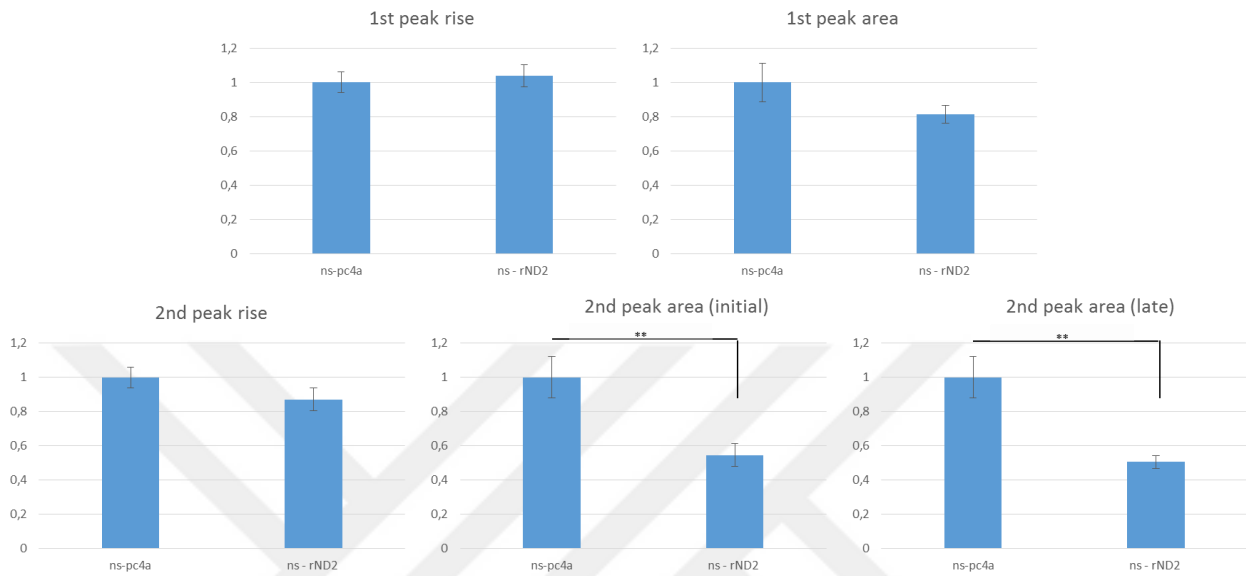


Figure 4.19: Quantification of peak signals and peak areas of the plot in figure 4.18. The peak names are labeled on the plot. The peak observed in first media change named as 1st peak and 1st rise peak and 2nd rise peak stands for the maximum $\Delta F/F_{min}$ value obtained with the corresponding peak. All the plots are normalized NS-pc4a sample (non-silencing shRNA + pc4a) (** p < 0,05).

No difference is observed in the first peak and area of the first peak, similar to what we have seen in Figure 4.17. This shows that the steady state ER Ca^{2+} levels are not regulated by *Neurod2*. Both knockdown and overexpression of *Neurod2* was not affecting the steady state ER Ca^{2+} levels. Second peak, however, diminished with *Neurod2* overexpression. Unlike the knockdown experiment, peak value is not significantly affected and late response is significantly reduced. Decrease in initial response shows SOCE inhibition. The decrease in late response might be linked with the low amount of Ca^{2+} entry, as it will be easier to buffer low amount of ions flowing in.

4.13. Developmental expression pattern of NEUROD2

NEUROD2 expression starts at E11 and continues through adulthood [6]. And the expression pattern of Neurod2 in the brain changes as mice continues to develop.

I conducted a western blot experiment with using mice cortices collected at different time periods during development, to see how the cortical NEUROD2 levels change in course of development. I tested seven different time points, from E14 to P28.

Expression of NEUROD2 in cortex is high at embryo stage and until P0. Levels of NEUROD2 at cortex decreases after P0 stage. (Figure 4.20)

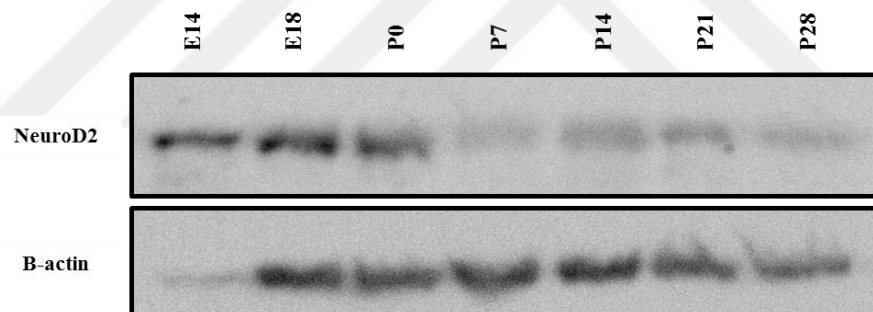


Figure 4.20: Developmental expression pattern of Neurod2. β -actin used as loading control. 1:1000 mouse anti-Neurod2 and 1:2500 rabbit anti-actin antibody used for blotting.

4.14. Developmental subcellular localization pattern of NEUROD2

Preliminary observations made in our lab by immunofluorescence suggested that Neurod2 might be present in cytoplasm. To investigate this possibility, I performed fractionation to separate cytosolic and nuclear components of the cortex of the mice. I collected cortices from mice at different developmental time points, to assess if Neurod2 localization is developmentally regulated. I grouped the western blot results coming from different timepoints according to their developmental stage. The data collected under three groups, embryonic, early postnatal and late postnatal.

Fractionation achieved by using low detergent containing buffer (0.1% NP-40). Low detergent concentration allows nuclear membrane to keep its integrity while plasma membrane is disturbed, allowing separation of cytoplasm from the nucleus of the cells. As a control for fractionation, Histone H3 antibody used as nuclear marker and HSP-90 antibody used as cytosolic marker.

The western blot shown in figure 4.21 is one of the three western blots performed. Quantification of all fractionation western blots displayed in figure 4.22. Quantification revealed that Neurod2 is indeed found in the cytoplasm, as 20% of Neurod2 protein located. There is no significant developmental regulation on Neurod2 localization seen in the results, although presence of Neurod2 in cytoplasm appear to be increasing.

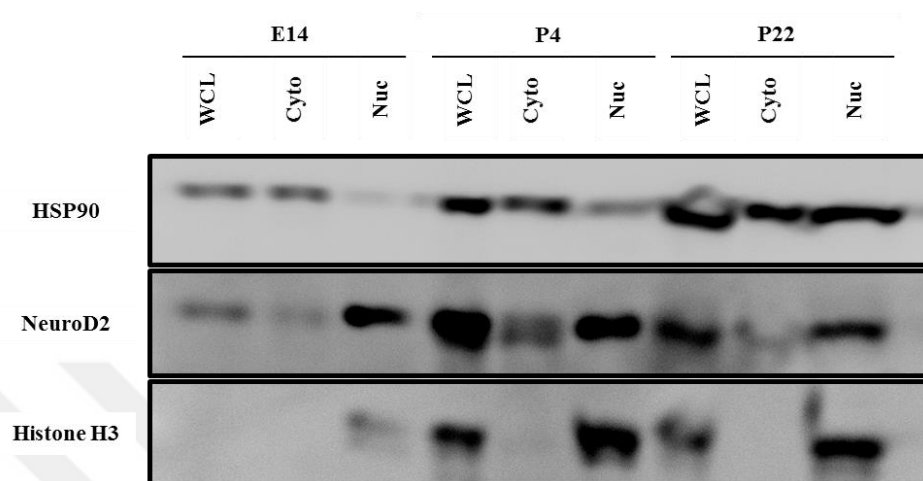


Figure 4.21: Developmental NeuroD2 expression pattern in nuclear and cytosolic fractions. Subcellular fractionation performed on samples from different developmental timepoints. HSP-90 antibody used as cytosolic control and Histone H3 antibody used as nuclear control. NeuroD2 antibody (ab168932) used. WCL: whole cell lysates taken from the samples before fractionation performed.

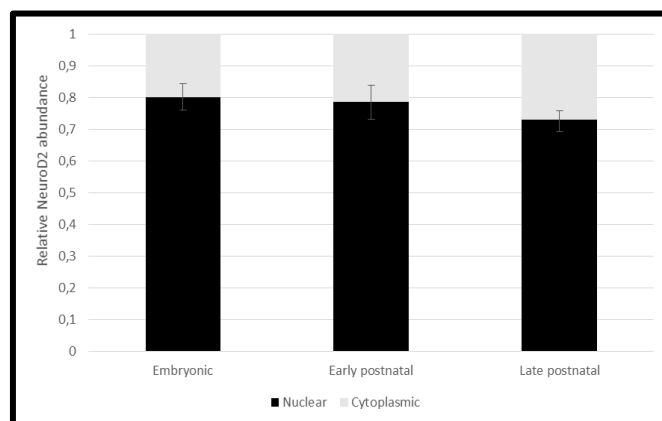


Figure 4.22: Neuronal NeuroD2 composition in embryonic (E14), early postnatal (P0-P7) and late postnatal (P7-adult) stages. Sample number n=2 for embryonic and late postnatal sample, and n=3 for early postnatal sample.

4.15. Possible SUMOylation of Neurod2

Western blotting experiments done on nuclear-cytosolic fractions of the cortex at different timepoints revealed an unexpected ~70kDa band at Neurod2 antibody treated membranes. The ~70kDa band predominantly found in cytosolic fraction of the samples, at P7 and P14 samples (Figure 4.23). The condition in sample preparation suggested a covalently linked modification, because noncovalent bindings should dissociate when boiled in SDS-Loading buffer. Also, the modification should be approximately 20 kDa in mass, since the shift in expected Neurod2 band size is ~20 kDa. Upon these observations, SUMOylation of Neurod2 became a plausible option. Bioinformatics analysis supported the view and showed putative SUMOylation sites on Neurod2 (Figure 4.24).

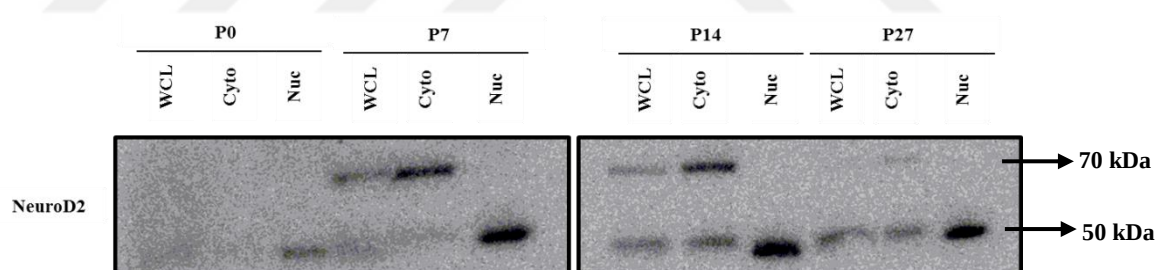


Figure 4.23: Western blot performed with fractionated samples from mice cortex. Lower bands correspond to expected size of Neurod2, while upper bands present in P7 and P14 cytosolic fraction.

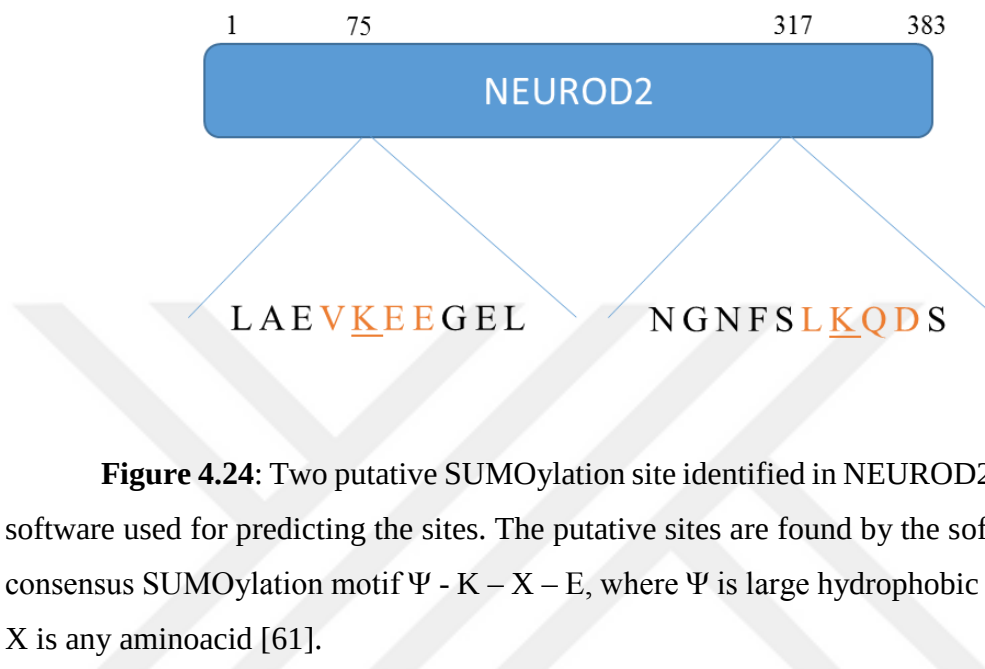


Figure 4.24: Two putative SUMOylation site identified in NEUROD2. SUMOplot™ software used for predicting the sites. The putative sites are found by the software based on consensus SUMOylation motif Ψ - K - X - E, where Ψ is large hydrophobic amino acid and X is any aminoacid [61].

To test whether Neurod2 is SUMOylated, I conducted immunoprecipitation experiments. Briefly, I immunoprecipitated NEUROD2 and followed the IP by anti-SUMO western blotting. Cytosolic fraction of the P7 mice brain is used since the ~70kDa band was present in that sample. The samples split into two and incubated with Neurod2 antibody or turboGFP antibody as negative control. Antibodies incubated in lysate collected with protein A/G magnetic beads, Antibody bound proteins eluted and run on western blot. The membrane treated with mouse α -GMP-1 to detect SUMO proteins. There was no observable difference between tGFP and Neurod2 antibody immunoprecipitated samples, thus I was not able to detect SUMO on Neurod2 (Figure 4.25).

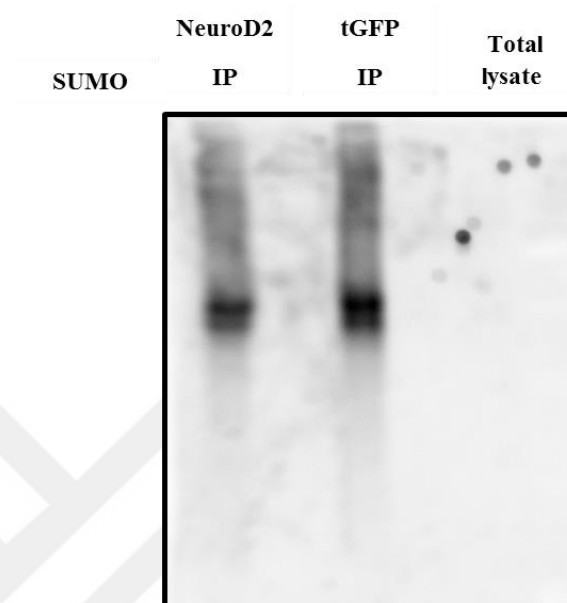


Figure 4.25: Neurod2 and tGFP immunoprecipitated samples blotted with α -GMP-1 antibody targeting SUMO. No signal could be obtained from total lysate. The pattern in Neurod2 and tGFP samples appear similar.

4.16. Δ NLS-Neurod2 cloning

After observing that Neurod2 is present in the cytoplasm, I created a Neurod2 construct that can mimic cytosolic Neurod2 and examine the phenotype associated with cytosolic Neurod2. To do that, I found a putative nuclear localization signal and designed two primers that will clone the rest of the vector by skipping NLS sequence. The resulting construct named Δ NLS-Neurod2 (Figure 4.26).

Δ NLS-Neurod2 expressed in primary cultured neurons to see if the construct really can express cytosolic Neurod2. However, green signal is also observed from the nucleus, indicating Δ NLS-Neurod2 was not only present in the cytoplasm (Figure 4.27)

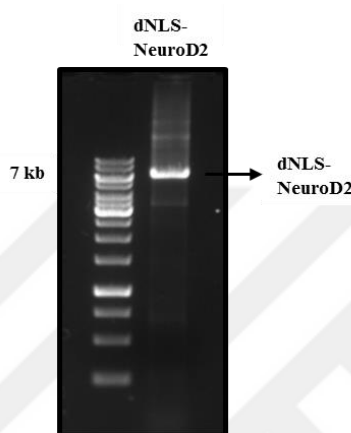


Figure 4.26: Δ NLS PCR result. PCR band observed at ~ 7 kb, which is size of backbone + Neurod2 coding sequence.

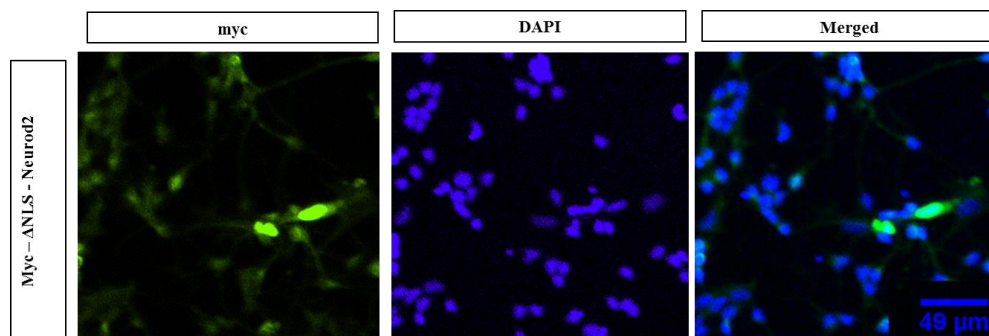


Figure 4.27: Δ NLS-Neurod2 is present in nucleus. Primary cultured neurons transfected with 2,5 μ g Δ NLS-Neurod2 plasmid. Myc tag linked Neurod2 stained with green.

Chapter 5

DISCUSSION

In this study, a previously unknown link between store operated calcium entry and NEUROD2 transcription factor have been elucidated. Reduction in NEUROD2 levels by knockdown have shown to significantly enhance SOCE. Similarly, overexpression of NEUROD2 further decreases the existing SOCE in neurons. These two experiments showed that levels of NEUROD2 directly regulate calcium store refilling ability of neurons. Moreover, the phenotype observed upon *Neurod2* shRNA treatment is rescued by expression of rNeurod2, indicating that Neurod2 shRNA phenotype is mediated via *Neurod2* and no other nonspecific events. As NEUROD2 is known to be activated by calcium influx [7][25], NEUROD2's regulatory role over SOCE might indicate a feedback control mechanism over calcium dynamics within the neurons. Different feedback mechanisms likely to be present in calcium homeostasis of neurons. Calmodulins, together with STIM1 was shown to inhibit ORAI1 channels, acting as a negative feedback mechanism upon calcium entry by SOCE [62]. STIM1 can also inhibit calcium entry via voltage gated-calcium channels, regulating other calcium entry routes apart from SOCE [63]. These findings show that there is also a temporal regulation on SOCE. Calmodulin and STIM1 creates a quick response upon SOCE initiation, while NEUROD2 mediates a long term transcriptional response.

Korkorin et al. showed that use of thapsigargin reduces the spine number in cultured hippocampal neurons [64]. Decrease in spine numbers upon thapsigargin treatment indicates the role of SOCE in the process. Similarly, reduced spine phenotype was observed in hippocampal neurons of *Neurod2* knockout mice [27]. However, findings in this study suggest an increase in SOCE in the absence of NEUROD2. This suggests a complex regulation mechanism on spine formation, as the phenotype cannot be simplified only to SOCE. Our gene ontology analysis have revealed processes closely related with spine density, such as regulation of synapse organization, dendrite morphogenesis and development (Table 4.1). Thus, it is likely that NEUROD2 regulates the spine density on many levels, not merely by its regulation over SOCE. In such case, manipulations on SOCE should affect the spine density in *Neurod2* knockout neurons to a lesser extent. The hypothesis can be tested by manipulating SOCE in *Neurod2* knockout neurons, either by using thapsigargin to inhibit SOCE or using the newly identified SOCE potentiation agent methoxy diethylborinate (MDEB) [65].

Although my results suggest that NEUROD2 inhibits SOCE, it is still unknown that how this inhibition is achieved. NEUROD2 regulation on SOCE mediated via STIM1 is a plausible option. Both western blot experiments and immunofluorescence data show that STIM1 levels increase upon *Neurod2* knockdown (Figure A.2) (Figure A.4). NEUROD2 binding on second intron of *Stim1* may physically block transcription, or NEUROD2 may recruit other proteins to repress STIM1 expression. It is reported that binding of transcription factors onto intronic sites of the genes may suppress the expression of the bound gene [66]. It is not very likely that NEUROD2 have a transcriptional role inside the STIM1 intron since there are no activity associated histone marks are found. These facts pointing out the STIM1 intronic region as a site where STIM1 expression is inhibited. However, further validation is required. With newly developing elegant gene editing tools, such as CRISPR/Cas9, the *Neurod2* binding site in STIM1 intron can be deleted. In such case, if NEUROD2 inhibits

the STIM1 levels by intronic binding, we expect to see an increase in STIM1 levels. If NEUROD2 regulation of SOCE solely dependent on this intronic site, no phenotype change should occur in SOCE upon *Neurod2* knockdown or overexpression. Additionally, if *Neurod2* knockdown phenotype mediated via increased levels of STIM1 protein, STIM1 targeting shRNA should act as a rescue for the increased SOCE.

NEUROD2 targeting to CaMK genes have been validated by ChIP-qPCR. However, qPCR analysis to detect expression levels failed to find significant change in gene expression due to high variation. It is likely that NEUROD2 is not the only determining transcription factor in the expression of CaMK pathway genes and that redundancy exists. Calmodulins, for example, known to play role in diverse biological processes and interact with many different proteins [56][57]. Expression of such multifunctional gene is not likely to be influenced by knockdown of a single transcription factor. NEUROD2 and CaMK pathway are known to share some target genes. BDNF and c-fos expression is increased by both NEUROD2 [8] and CaMK target CREB [36]. Additionally, CaMKII and CREB are well characterized proteins with their role in long term potentiation, molecular mechanism of learning [35][36]. Our results showed that long term potentiation pathway is targeted by NEUROD2 (Table 4.3). Given the role of NEUROD2 in learning and memory [31], these information may suggest NEUROD2 and CaMK are in same or at least in parallel pathways. Decreasing the variation in qPCR analysis by controlling the knockdown efficiency, with measuring GFP or *Neurod2* mRNA levels, might be helpful to assess whether NEUROD2 regulates CaMK pathway.

The fractionation experiment that I have performed revealed a cytosolic presence of NEUROD2. And its cytosolic presence seems to be sustained throughout development. However, the function of cytosolic NEUROD2 remains to be elucidated. There are different examples on transcription factors functioning in cytoplasm. Serum response factor (SRF)

transcription factor shown to be involved in axon regeneration is also localized to the cytoplasm [66]. Local synaptic inputs can be relayed to nucleus directly by transcriptional regulation [67]. TFII-I shown to reduce calcium entry by directly interfering with TRPC channels [68]. The transcription factors can have variety of roles in cytoplasm as these examples suggest. Use of Δ NLS-Neurod2 could be helpful to resolve the function of cytosolic NEUROD2, however my Δ NLS-Neurod2 construct was present in nucleus. NEUROD2 localization might be not dependent on NLS sequence. There may be NLS independent nuclear import mechanisms, as there are examples of proteins that does not depend on NLS while going into the nucleus [69].

Here, this work revealed a previously unknown function of NEUROD2, on regulation of SOCE response. As a calcium activated transcription factor, the role of Neurod2 on SOCE elaborates the calcium regulatory mechanism of the neurons. The role of Neurod2 on calcium dynamics is further expanded by demonstrating its ability to bind CaMK pathway genes. Additionally, cytoplasmic presence of NEUROD2 may uncover undiscovered roles for NEUROD2.

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VITA

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APPENDIX A: List of primers used

Primer Name	5' - Primer Sequence -3'
CaMKIId_CHIP_F	5'-GTGGCATCATTTAAAGGACCTCATG-3'
CaMKIId_CHIP_R	5'-ATCTAGTGGCTACAGCAGAAGTTTG-3'
CaMKIId_QPCR_F	5'-CATCTTGACAACTATGCTGGCTACG-3'
CaMKIId_QPCR_R	5'-CAATGCTTCAGGTTCAAAGGCAGTG-3'
CaMKIV_QPCR_F	5'-CGAAGATGCTCAAAGTCACGGTG-3'
CaMKIV_QPCR-R	5'-CTGACTCCACCTCGAAGAAATCG -3'
CALM1-F	5'- CCGTGGTGCCGTTACTCG -3'
CALM1-R	5'- GAACGGAGCAGGCGAGG -3'
CALM2-QPCR-F	5'- GGCGGAGGGAGGTGTTTATG -3'
CALM2-QPCR-R	5'- GCTGCAAGGGCTACGGG -3'
CALM3-F	5'- CTGCGGGCAAAGAGTGTG -3'
CALM3-R	5'- GAGGCACCGGTATCCGGAG -3'
NEUROD2_SHRNA-F	5'GATCCCCAAGACAAGAGATTCTCGGATTCAAGAGATCC GAGAATCTCTTGTCTTTTTTA -3'
NEUROD2_SHRNA-R	5'AGCTTAAAAAAGACAAGAGATTCTCGGATCTCTTGAA TCCGAGAATCTCTTGTCTTGGG -3'
CALM2_CHIP_F	5'-CTAGGTTCCGTTATCTGGATGAG-3'
CALM2_CHIP_R	5'-CAATCCGCTTCAGCATCTTTC-3'
Δ NLS_ND2_F	5'- CGTCTGGAGCGCTCCAAG -3'
Δ NLS_ND2_R	5'- CCCGCGCTTCTTGGGC -3'
Gapdh-QPCR-F	5'-CGACTTCAACAGCAACTCCCACTCTTCC-3'
Gapdh-QPCR-R	5'-TGGGTGGTCCAGGGTTTCTTACTCCTT-3'

Table A.1: List of primers used

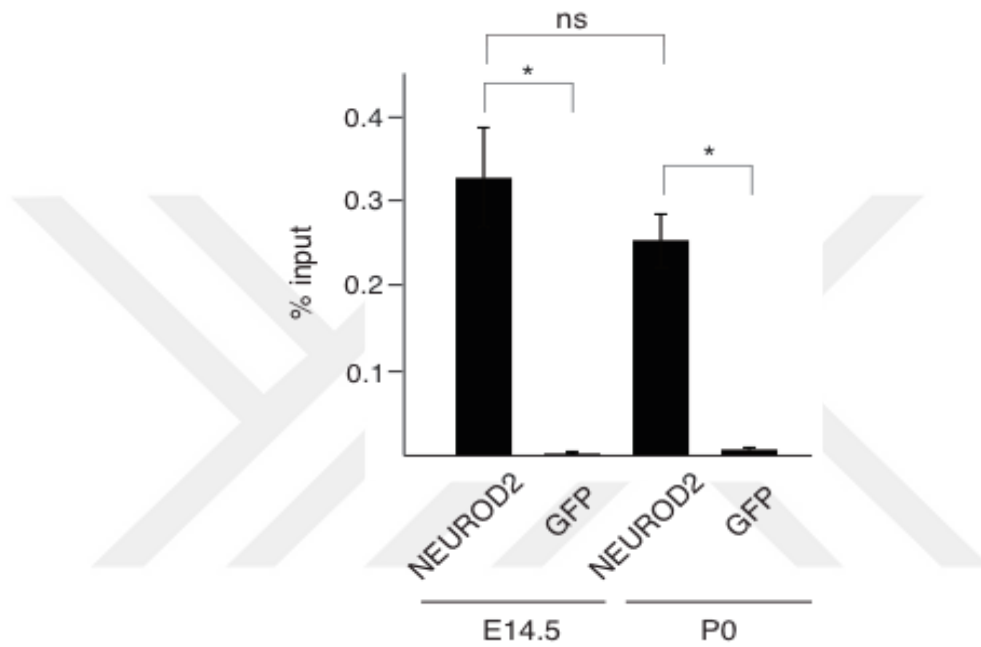
APPENDIX B: NEUROD2 binding to *Stim1* intron

Figure A.1: NEUROD2 binding to intronic region of STIM1 in E14.5 and P0 cortex. The ChIP-qPCR reads normalized the reads from total input and plotted as % input (unpublished data, Gizem Güzelsoy).

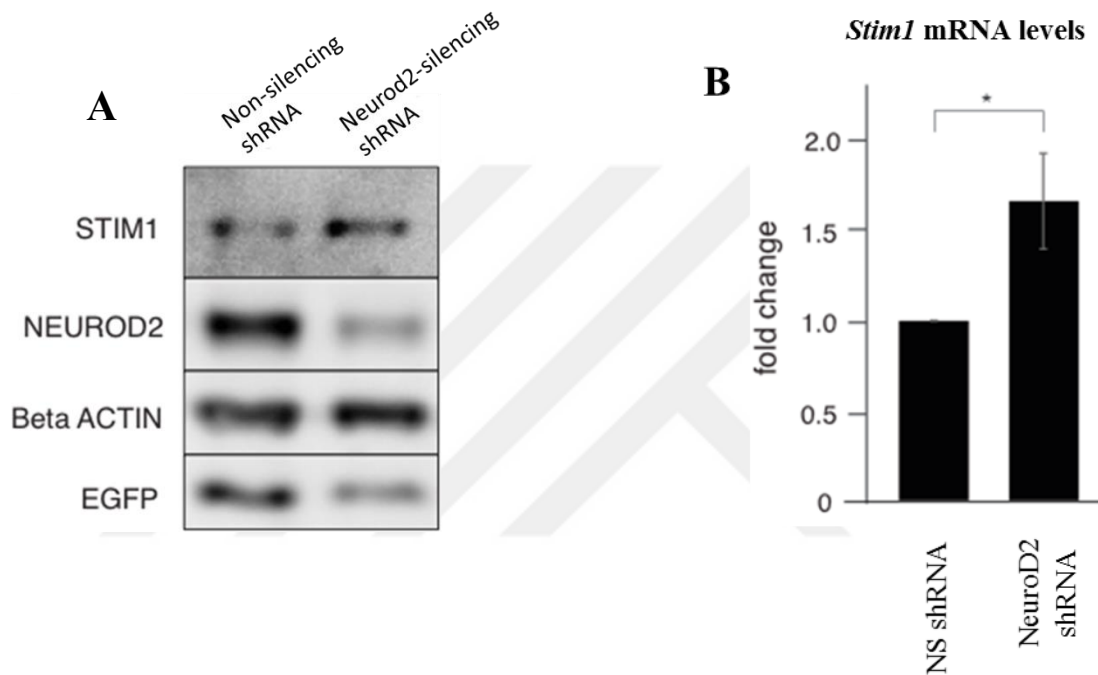
APPENDIX C: *Neurod2* knockdown cause STIM1 increase

Figure A.2: *Neurod2* knockdown cause increase in STIM1 protein and mRNA levels. (A) Western blot result of NS and *Neurod2* shRNA nucleofected samples. (B) Increase in mRNA levels upon *Neurod2* knockdown, NS shRNA sample set to 1 and data normalized accordingly (unpublished data, Gizem Güzelsoy).

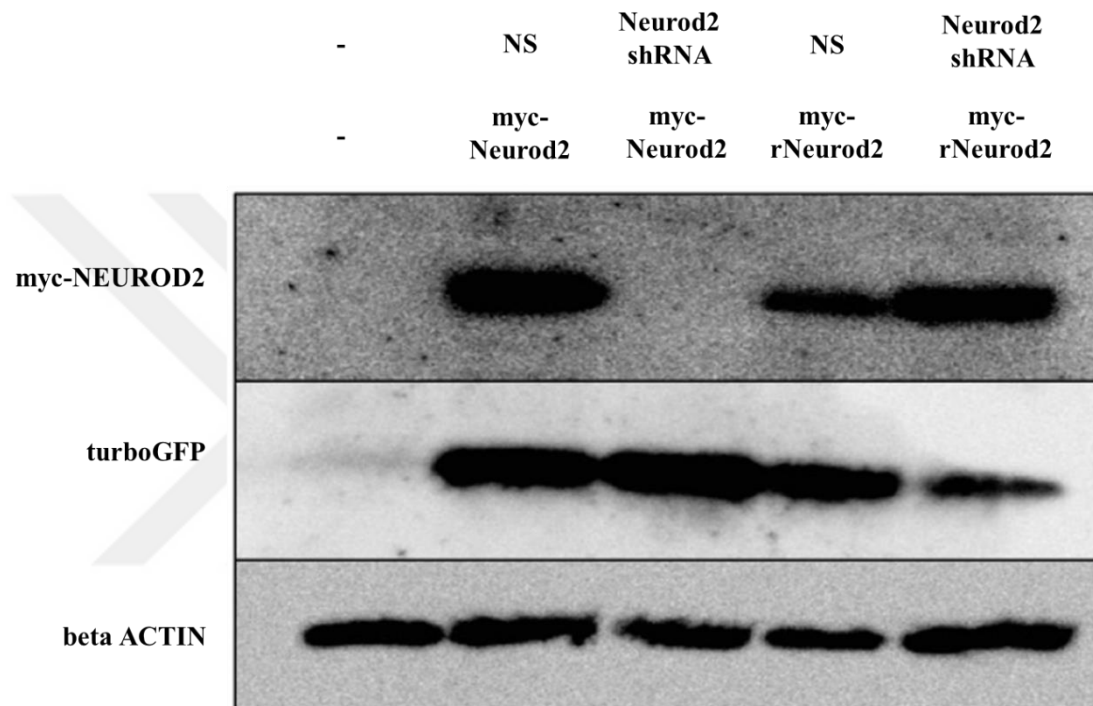
APPENDIX D: rNeurod2 can resist *Neurod2* shRNA

Figure A.3: shRNA-resistant *Neurod2* (rNeurod2) did not showed decrease upon shRNA treatment [10].

APPENDIX E: rNeurod2 can decrease the STIM1 levels increased by *Neurod2* shRNA

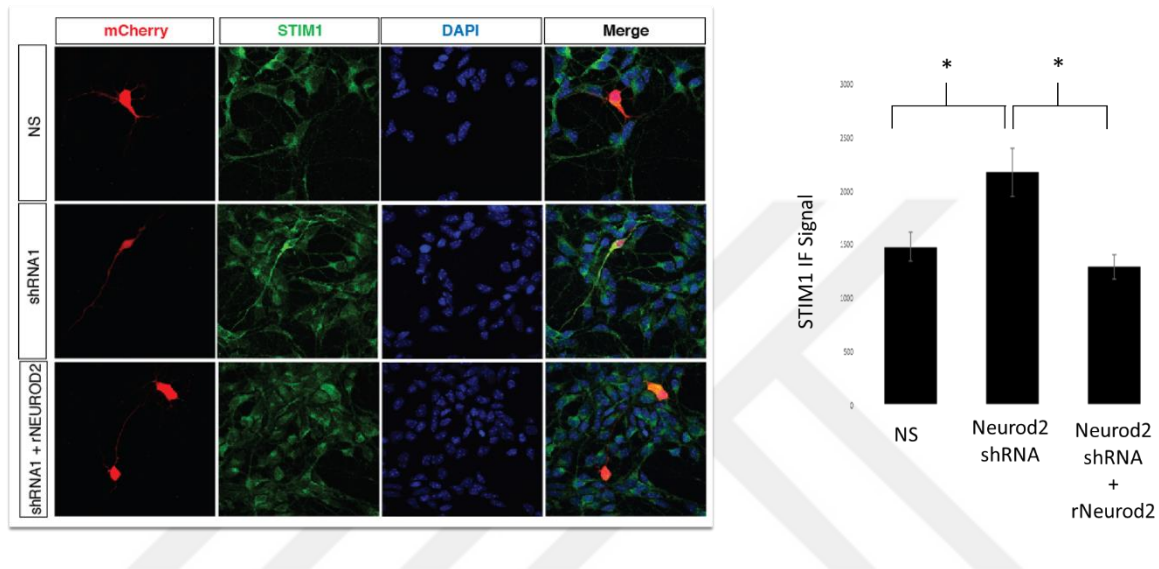


Figure A.4: STIM1 IF signal increased by *Neurod2* shRNA expression reduced to control levels upon rNeurod2 expression (unpublished data, Gizem Güzelsoy).