

Roles of Calmodulin and a N-methyl-D-aspartate Receptor - Pannexin-1 signaling complex in Connexin-36 Plasticity

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ABSTRACT

The synergic actions of calcium, calmodulin, and calmodulin kinase II are the central mechanism for synaptic plasticity. Until the last two decades, chemical synapses through N-methyl D-aspartate receptors were thought to be the only synapse that demonstrates plasticity. Our research has shown previously that electrical synapses use CaM/CaMKII interaction in a similar Ca^{2+} -dependent process for plasticity. New evidence suggests that other channels and kinases have modulatory roles in the whole mechanism. This thesis explores the contributions and roles that different kinases, receptors, and channels have on electrical synaptic plasticity. **The over-arching hypothesis is that the activation of N-methyl-D-aspartate receptors and pannexin1 channels drives Ca^{2+} change for physiological signaling of Cx36 plasticity.** Here, I introduce a novel modality for CaM electrical synapse regulation and unify the electro-chemical transmission mechanism. I demonstrate that both N-methyl-D-aspartate receptors and pannexin1 channels influence the plasticity at the gap junction nexus. These findings opened up new avenues to expand on mechanisms for synaptic plasticity.

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PREFACE

As one searches their memories a few decades ago, many neurons in the brain communicate with one another to recall that memory. It is fascinating how the brain organizes and orchestrates these neuronal networks in such a confined space. In general, neuronal communication is accomplished through chemical (release of neurotransmitters) and electrical (direct contact) synapses. Although the mechanism of brain plasticity has long been attributed to chemical synapses, the impacts of nearby electrical synapses, channels, and receptors are important elements to the molecular pathways.

Many neurological diseases have been linked to overactive receptor activity (i.e., N-methyl-D-aspartate receptors leading to excitotoxicity) or overstimulated channels (i.e., pannexin1 and connexin36 channels in Alzheimer's disease). The interconnectedness of different molecular pathways presents a complicated obstacle in drug discovery for specific diseases. Hence, this thesis examines a previously unexplored path in neuronal communication by shedding some light on the intertwined molecular pathways linking both chemical and electrical synapses. It is important to emphasize that, despite my confidence in the conclusions of this thesis, I have only touched the surface of the intermingled pathways that may contribute to the whole story of mixed synaptic plasticity.

TABLE OF CONTENTS

ABSTRACT	II
ACKNOWLEDGEMENTS	III
PREFACE	V
TABLE OF CONTENTS	VI
LIST OF TABLES	X
LIST OF FIGURES AND ILLUSTRATIONS	X
LIST OF SUPPLEMENTARY FIGURES AND ILLUSTRATIONS	XII
LIST OF APPENDICES FIGURES AND ILLUSTRATIONS	XIII
LIST OF SYMBOLS, ABBREVIATIONS	XIV
 CHAPTER ONE: INTRODUCTION	 1
1.1) Brain Plasticity	1
1.1.1) Activity-Dependent Plasticity leading to Synaptic Plasticity	1
1.2) Types of Synapses	3
1.3) Chemical Synapses	5
1.4) Electrical Synapses (Gap Junctions)	7
1.5) Family of Connexins	8
1.6) Connexins	8
1.7) Pannexins	10
1.8) Ca²⁺/Calmodulin & Calmodulin Kinase II	12
1.9) Studying subcellular dynamics and interactions	14
1.10) Hypothesis and Research Aims	19
Aim 1: Determining the mechanism of CaM and Cx36 interaction	19
Aim 2: Determining how NMDAR-Src-Panx1 signaling modulates the interactions at electrical synapses.....	20
 CHAPTER TWO: Experimental Procedures	 21
2.1) Plasmid constructs and site-directed mutagenesis	21
2.2) Cell culture, transient transfection and western blot	22
2.3) Immunohistochemistry (IHC)	23
2.4) TALEN Panx1 KD Cells	24
2.5) Confocal Microscopy	24
2.6) FRET Analysis	25

2.7) Ethidium Bromide Uptake and Recovery after Photobleaching Assay.....	26
2.8) Dynamic Ca ²⁺ Imaging.....	27
2.9) Arclight Fluorescent Voltage Probe Imaging	27
2.10) Pharmacology.....	28
2.11) Protein Expression and Purification.....	29
2.12) NMR spectroscopy.....	30
2.13) Structure Determination.....	30
2.14) Databases.....	30
2.15) Isothermal Titration Calorimetry (ITC)	31
2.16) Statistics.....	31
 CHAPTER THREE: Experimental Materials.....	 32
3.1) Biosafety	32
3.2) Organisms.....	32
3.2.1) Bacterial strains	32
3.2.2) Eukaryotic cells.....	32
3.3) Kits for recombinant DNA applications.....	33
3.4) Plasmids and Oligonucleotides	33
3.4.1) Plasmids.....	33
3.4.2) Oligonucleotides.....	34
3.5) Solutions	34
3.5.1) Solutions and media for cultivating bacteria	34
3.5.2) Solutions for Molecular Biology.....	34
3.5.3) Solutions for Protein Biochemistry	35
3.5.4) Solutions for Cell Culture	35
3.5.5) Solutions for Stable/Transient Transfection	36
3.6) Consumables	36
3.6.1) General Consumables.....	36
3.6.2) Cell Culture Consumables	36
3.7) Equipment	37
3.7.1) Molecular Biology Equipment.....	37
3.7.2) Protein Biochemistry Equipment.....	37
3.7.3) Cell Culture Equipment.....	38
3.8) Software.....	38

CHAPTER FOUR: Structural and Functional Consequences of Connexin 36 (Cx36) Interaction with Calmodulin.....	39
4.1) Abstract	40
4.2) Introduction	40
4.3) Results.....	42
4.3.1) Cx36-CaM at gap junctions in Neuro2a cells.....	42
4.3.2) Mutation W277A alters subcellular distribution and exchange between cell pairs	45
4.3.3) Cx36-CaM binding is strengthened by elevated intracellular calcium at intracellular vesicles.....	47
4.3.4) W277 is critical for calcium dependent Cx36-CaM binding	50
4.3.5) Reduced dye-redistribution in Neuro2a cells expressing W277A	51
4.3.6) A high-resolution solution structure of Cx36 and CaM.....	53
4.4) Discussion	57
4.4.1) The Cx36-CaM binding motif is distinct from other connexins.....	58
4.4.2) Calmodulin confers calcium signaling to Cx36 outside the GJP	59
4.4.3) Ménage a trios: competition or cooperation?	59
4.5) Supplementary Figures	63
 CHAPTER FIVE: An NMDA receptor-pannexin1 signaling complex transforms the interaction of CaMKII with Connexin36	 69
5.1) Graphical Abstract.....	70
5.2) Abstract	70
5.3) Introduction	71
5.4) Results.....	73
5.4.1) Calcium modulates proximity among Cx36 channels in GJPs	73
5.4.2) Calcium signals converge onto Cx36 through interaction with Ca²⁺-CaM/CaMKII.....	75
5.4.3) Calcium ions orchestrate the interaction of Cx36 with CaMKIIα at the GJP ...	78
5.4.4) NMDA receptor activation modulates Cx36 interaction with CaMKII.....	80
5.4.5) Pannexin 1 modulates the interaction between Cx36 and CaMKII	84
5.4.5) Overexpression of Panx1 rescued the Cx36-CaMKII interaction in Panx1 knock-down cells.....	87
5.4.7) The compactness of the Cx36 -CaMKII interaction is modulated by an NMDA receptor-Src family kinase – Panx1 signaling complex	90
5.5) Discussion	93

5.6) Supplementary Figures	98
5.7) Figure Z-statistics	103
CHAPTER SIX: General discussion	109
6.1) Summary of findings	109
6.2) Multifaceted role of Calmodulin	111
6.3) Panx1 and its modulatory role on electrical synapses.....	113
6.4) Ionotropic vs. metabotropic NMDA receptor signaling complex	115
6.5) Role of magnesium in plasticity.....	117
6.6) Proposing a unified signaling pathway	119
6.7) Significance.....	122
BIBLIOGRAPHY	124
APPENDICES	145
A.1) Supplemental figures relating to <i>Chapter 4</i> (NMDAR)	145
A.1.1) AMPA/Kainite receptor inhibition modulates Cx36-CaMKII α	145
A.1.2) Membrane potentials are reduced through NMDAR suppression	148
A.1.3) NMDAR-Src kinase-pannexin 1 signaling affects connexin 36 potentiation ...	149
A.1.4) Knocking down Panx1 reduces NMDAR-Src kinase signaling	151
A.2: Supplemental figures relating to <i>Chapter 4</i> (Panx1).....	154
A.2.2) Cx36 and Panx1 protein co-localization in interneurons	155
A.2.3) Neuro2a cells express functional pannexin 1 channels.....	159
A.3: Supplemental DNA Construct Maps	161
PUBLICATIONS	167
CONFERENCES & POSTERS	168

LIST OF TABLES

Table 1.1: Oligonucleotides for mutagenesis of CaMKII, Cx36 and Panx1.....	34
Table 4.3.1: Statistics for the ensemble of 20 structures of the CaM-Cx36 hybrid protein.	56

LIST OF FIGURES AND ILLUSTRATIONS

Figure 1.1: Synaptic mechanism of long-term depression (LTD) and long-term potentiation (LTP).....	2
Figure 1.2: Two modes of synaptic transmission (chemical and electrical synapses).	4
Figure 1.3: Simplified representation of FRET fluorescence microscopy (acceptor bleach).	16
Figure 1.4: Theoretical FRAP recovery curve.	17
Figure 1.5: Schematic of gapFRAP showing regions of analysis and sample trace for each region of a cell pair.	18
Figure 4.3.1 Expression, localization and gap junction plaque formation of Cx36 in Neuro2a cells.....	44
Figure 4.3.2 Colocalization of Cx36 with organelle markers indicating interaction occurring as early as at the endoplasmic reticulum.	46
Figure 4.3.3 FRET analysis of Cx36 and CaM interaction.....	49
Figure 4.3.4 Alanine scanning mutagenesis reveals W277 as the critical site for Cx36-CaM binding.	51
Figure 4.3.5 Ethidium bromide dye uptake and redistribution after photobleaching.	53
Figure 4.3.6 Structural features of the Cx36-CaM complex.....	54
Figure 4.3.7 Isothermal titration calorimetry (ITC) of Cx36 peptides and CaM.....	57
Figure 4.4.1: Model of calmodulin (CaM), Ca ²⁺ /calmodulin dependent kinase 2 α (CaMKII α) and connexin 36 (Cx36) in plasticity of the electrical synapse.....	61
Figure 5.1: Schematic summary of NMDAR-Src-Panx1 signaling pathways leading to connexin 36 plasticity.....	70

Figure 5.3.1: Calcium modulates proximity between Cx36 channels when CaM/CaMKII binding is intact.	75
Figure 5.3.2: Calcium-induced compaction of Cx36 located in GJPs requires CaM/CaMKII binding.	77
Figure 5.4.3: Calcium manipulation influences Cx36-CaMKIIα interaction.	79
Figure 5.4.4: Impact of NMDA receptor activation on the modulation of the compactness of the Cx36-CaMKIIα interaction complex.	84
Figure 5.4.5: The activation of endogenous Panx1 modulates the interaction between Cx36 and CaMKII at the GJP.	87
Figure 5.4.6: Knocking down Panx1 channel expression in Neuro2a cells or blocking its function reduced the compaction of Cx36-CaMKII in response to K_{Glu} and low ATP concentration.	90
Figure 5.4.7: A Src kinase is involved in modulating the Cx36-CaMKII interaction.	92
Figure 6.1: Balance of Mg²⁺, Ca²⁺ and ATP dependent on cellular state.	118
Figure 6.2: Proposed molecular pathway for asymmetric functionality of mixed synapses (AMPA_Rs, NMDA_Rs, Panx1 and Cx36).	120

LIST OF SUPPLEMENTARY FIGURES AND ILLUSTRATIONS

Supplementary Figure 4.5.1: Representative 3D images of Neuro2a cells expressing wild-type Cx36, tagged with EGFP, or DsRed monomer.	63
Supplementary Figure 4.5.2: Representative 3D images of Neuro2a cells expressing the Cx36 W277A mutant.	64
Supplementary Figure 4.5.3: Western blot detection of ER and Golgi tagged EGFP after transient expression in Neuro2a cells (in red, position indicated by lines).	64
Supplementary Figure 4.5.4: Representative 2D co-localization images of Neuro2a cells ..	65
Supplementary Figure 4.5.5: Representative 3D images of Neuro2a cells expressing wild-type Cx36-ECFP (in red) and DsRed monomer tagged mitochondria (in light turquoise). 66	
Supplementary Figure 4.5.6: A ¹⁵N-edited HSQC spectrum of ¹³C,¹⁵N-labeled CaM-Cx36 hybrid protein acquired at 700 MHz.	67
Supplementary Figure 4.5.7: Superposition of the ensemble of twenty CaM-Cx36 NMR solution structures deposited in the Protein Data Bank (2N6A).	68
Supplementary Figure 5.6.1: CaMKII and Cx36 mutants showed normal expression in Neuro2a cells.	98
Supplementary Figure 5.6.2: Chelation of calcium reduced gap junction plaque (GJP) frequency but not size of Neuro2a cells expressing ECFP-CaMKIIα-ECFP and Cx36-DsRed.	99
Supplementary Figure 5.6.3: Subunits of NMDA receptors are expressed in Neuro2a cells and respond to stimulants and pharmacological blockers.	100
Supplementary Figure 5.6.4: TALEN Panx1 knockdown (KD) strategy for Neuro2a cells.	101
Supplementary Figure 5.6.5: Triply transfected WT and TALEN knockdown Panx1 Neuro2a cells show normal protein expressions.	102

LIST OF APPENDICES FIGURES AND ILLUSTRATIONS

Figure A.1.1: Non-competitive inhibitors of AMPAR showed reduced Ca²⁺ signals and interaction of Cx36-CaMKIIα.	147
Figure A.1.2: Detecting voltage change with Arclight S249 under different conditions over five minutes.	148
Figure A.1.3: Inhibition of Src kinase or Panx1 channels impedes the Cx36-CaMKIIα interaction	150
Figure A.1.4: Knocking down Panx1 channel expression with TALEN reduces effective binding of CaMKIIα and Cx36.	153
Figure A.2.1: Localization of Panx1 and Cx36 in Neuro2a cells.	154
Figure A.2.2: Localization of Cx36 and Panx1 in C57BL/6 hippocampal coronal slices...	156
Figure A.2.2.1: Localization of Cx36, CaMKII and Panx1 in C57BL/6 hippocampal coronal slices.	157
Figure A.2.2.2: Localization of Panx1 and Cx36 in C57BL/6 mouse retinal slices.	158
Figure A.3.1: Gene map of pDsRed2-Calmodulin (CaM) indicating insert and various regions of expressions.	161
Figure A.3.2: Gene map of pEGFP-Connexin36 (Cx36) indicating insert and various regions of expressions.	162
Figure A.3.3: Gene map of pECFP-mCaMKII (WT) indicating insert and various regions of expressions.	163
Figure A.3.4: Gene map of pEGFP-mPanx1 indicating insert and various regions of expressions.	164
Figure A.3.5: Gene map of E3-Crimson-mPanx1 indicating insert and various regions of expressions.	165
Figure A.3.6: Gene map of Arclight S249 indicating insert and various regions of expressions.	166

LIST OF SYMBOLS, ABBREVIATIONS

Symbol	Definition
[X] _E	Extracellular concentration
[X] _I	Intracellular concentration
¹⁰ Panx	Mimetic peptide inhibitor of Panx1 (WRQAAFVDSY)
AC	Adenyl cyclase
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATP	adenosine-5'-triphosphate
BAPTA-AM	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)
Ca ²⁺	Calcium ion
CaM	Calmodulin
CaMKII	Calmodulin-dependent kinase II
CaMKII α	Calmodulin-dependent kinase II alpha subunit
cAMP	3'-5'-cyclic adenosine monophosphate
Cl ⁻	Chloride ion
CTRL	Control
Cx	Connexin
Cx36	Connexin 36
D-APV/AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
DIC	Differential interference contrast microscopy
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DsRed	Discosoma Red
ECFP	Enhanced cyan fluorescent protein
EDTA	Ethylenediaminetetraacetate
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
FRET _{dis}	FRET distance
FRET _{eff}	FRET efficiency
gapFRAP	Gap junction fluorescence recovery after photobleaching
GJC	Gap junction channel
GJP	Gap junction plaque
Iono	Ionomycin
IP ₃	Inositol trisphosphate
K ⁺	Potassium ion
kDa	Kilodaltons
KN93	N-[2-[[[3-(4-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]- N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide
LTD	Long term depression
LTP	Long term potentiation

Mg ²⁺	Magnesium ion
MgSO ₄	Magnesium sulphate
min	Minute
Mito	Mitochondria
MK-801	Dizocilpine; (5S,10R)-(+)-5-Methyl-10,11-dihydro-5Hdibenzo[a,d]cyclohepten-5,10-imine maleate
mL	Millilitre
mM	Millimolar
n	Sample size
Na ⁺	Sodium ion
Neuro2a	Neuroblastoma 2a
nm	Nanometer
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
OGB	Oregon green BAPTA
P2X7R	Purinergic Receptor
Panx1	Pannexin-1
PBS	Phosphate buffered saline
PKA	Protein kinase A or cAMP-dependent protein kinase
PKC	Protein kinase C
PP2	3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin- 4-amine
PP3	1-Phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine
ROI	Region of interest
s	Second
^{SC} Panx	Scrambled inactive version of ¹⁰ Panx (FSVYWAQADR)
SEM	Standard error of the mean
TALEN	Transcription activator-like effector technology
TIRF	Total internal reflection fluorescence
WT	Wildtype
μL	Microlitre
μM	Micromolar
μm	Micrometer

CHAPTER ONE: INTRODUCTION

1.1) Brain Plasticity

The nervous system can reorganize itself throughout life by forming new and removing unused connections. This ability to remold and shape is known as plasticity. In this thesis, I will focus on brain plasticity. There are multiple types of plasticity, but here I will focus on cellular synaptic plasticity.

1.1.1) Activity-Dependent Plasticity leading to Synaptic Plasticity

Throughout one's life, circuits in the brain are sensitive to different experiences, which influence the function and structure of connections. Activity or experience-dependent plasticity occurs when neuronal activation leads to intracellular signaling pathways that modulate gene expression (Carulli, Foscari, and Rossi 2011). The result stemming from such gene modification by selectively reinforcing and weakening connections leads to changes for information processing. These synaptic changes play a vital role in learning and memory. Short-term memory relies on the strengthening and weakening of pre-existing connections, while long-term memory generates and reorganizes the neural network (Bailey and Kandel 1993). The ability to fortify and modify synaptic strengths is known as synaptic plasticity. The mechanism of synaptic plasticity involves many key ions, receptors, kinases, and gene expression (**Figure 1.1**).

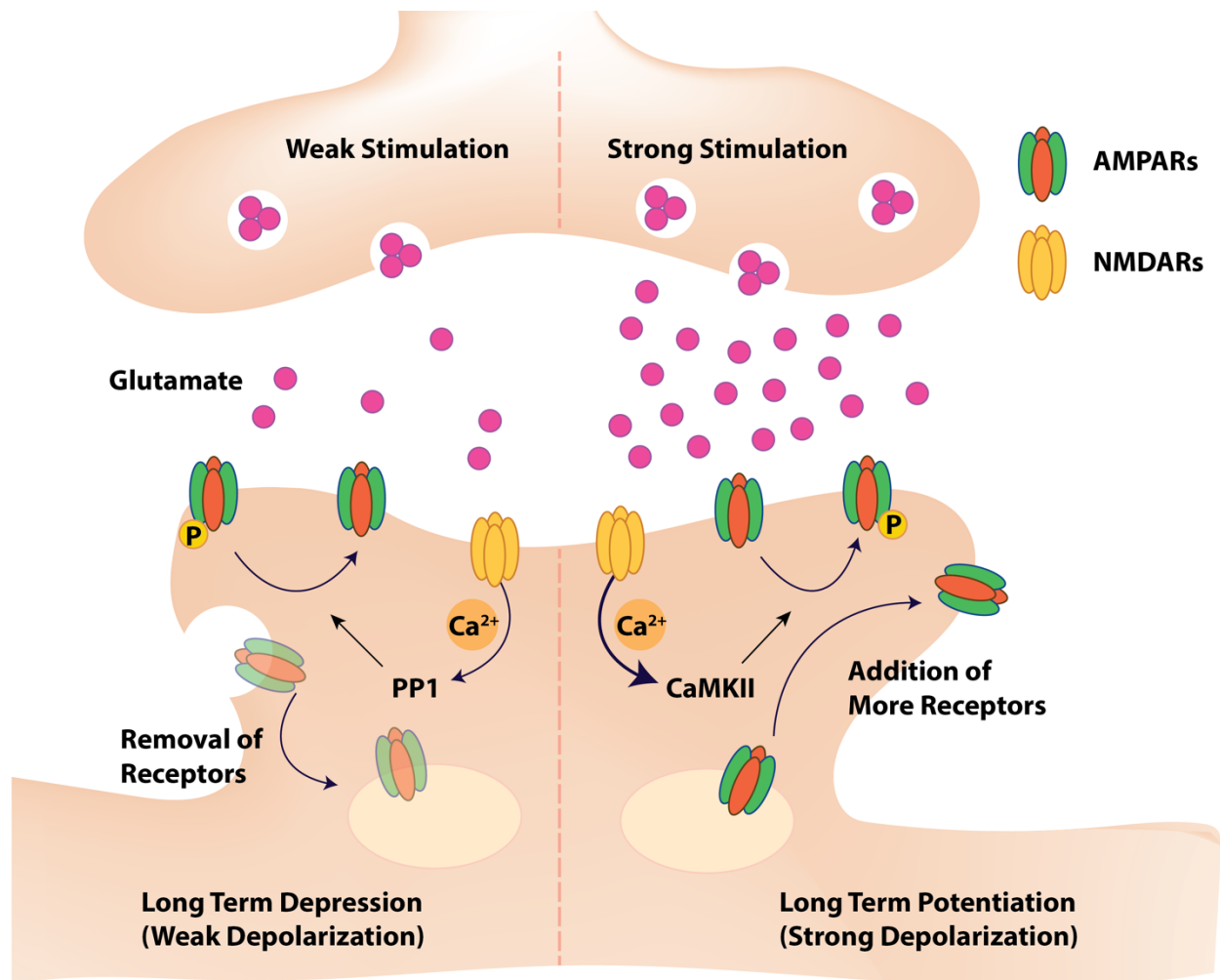


Figure 1.1: Synaptic mechanism of long-term depression (LTD) and long-term potentiation (LTP). Weak stimulation from presynaptic neuron releases less glutamate, which produces weak depolarization at the postsynaptic terminal and low calcium influx from N-Methyl-D-aspartate (NMDA) receptors. This leads to phosphatases (i.e., PP1) to dephosphorylate α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and thus promote endocytosis (removal of AMPA receptors), which triggers LTD. Strong activation, on the contrary, yields strong depolarization and increased influx of calcium ions. This leads to addition of AMPA receptors to the post-synaptic membrane and thus promote LTP. (Note: concept of the figure is from (Lüscher and Malenka 2012), which has been redrawn and modified)

The molecular process of synaptic plasticity through experience-dependent changes can be described through long-term potentiation (LTP) or long-term depression (LTD) depending on the strength of stimulation. Both LTP and LTD are known to be the underlying processes of learning and memory (Lüscher and Malenka 2012). Both LTP and LTD are induced based on activity patterns (Malenka 1994). For LTP, both presynaptic and postsynaptic neurons must be strongly

depolarized. The surge of glutamate binding to inactivated receptors leads to increased synaptic efficacy. Conversely, LTD is induced through repeated low frequencies, which leads to lower levels of glutamate release. Receptors are activated, but less receptors are engaged leading to reduced synaptic efficacy and weakening of the synapse.

1.2) Types of Synapses

There are billions of neurons in the brain that are vital for everyday brain functions. To maintain order amongst the billions of neurons, efficient neuronal communication is mandatory. Neuronal communication is an electrochemical process that allows the transfer of both electrical and chemical signals from one cell to another at identifiable regions known as synapses. There are primarily two modes of synaptic communication: chemical and electrical. Chemical synapses allow the delivery of neuronal signals (i.e., neurotransmitters; NTs) to adjacent neurons in the vicinity, while electrical synapses rely on the direct connections (known as gap junctions) between cells to transfer both electrical and molecular signals. Historically, chemical synapses were extensively studied over the past decades to establish a better understanding of synaptic plasticity. More recent research has shown that the formation of both electrical and chemical synapses through development has common regulatory mechanisms, which involve interacting with scaffolding proteins (Miller et al. 2015).

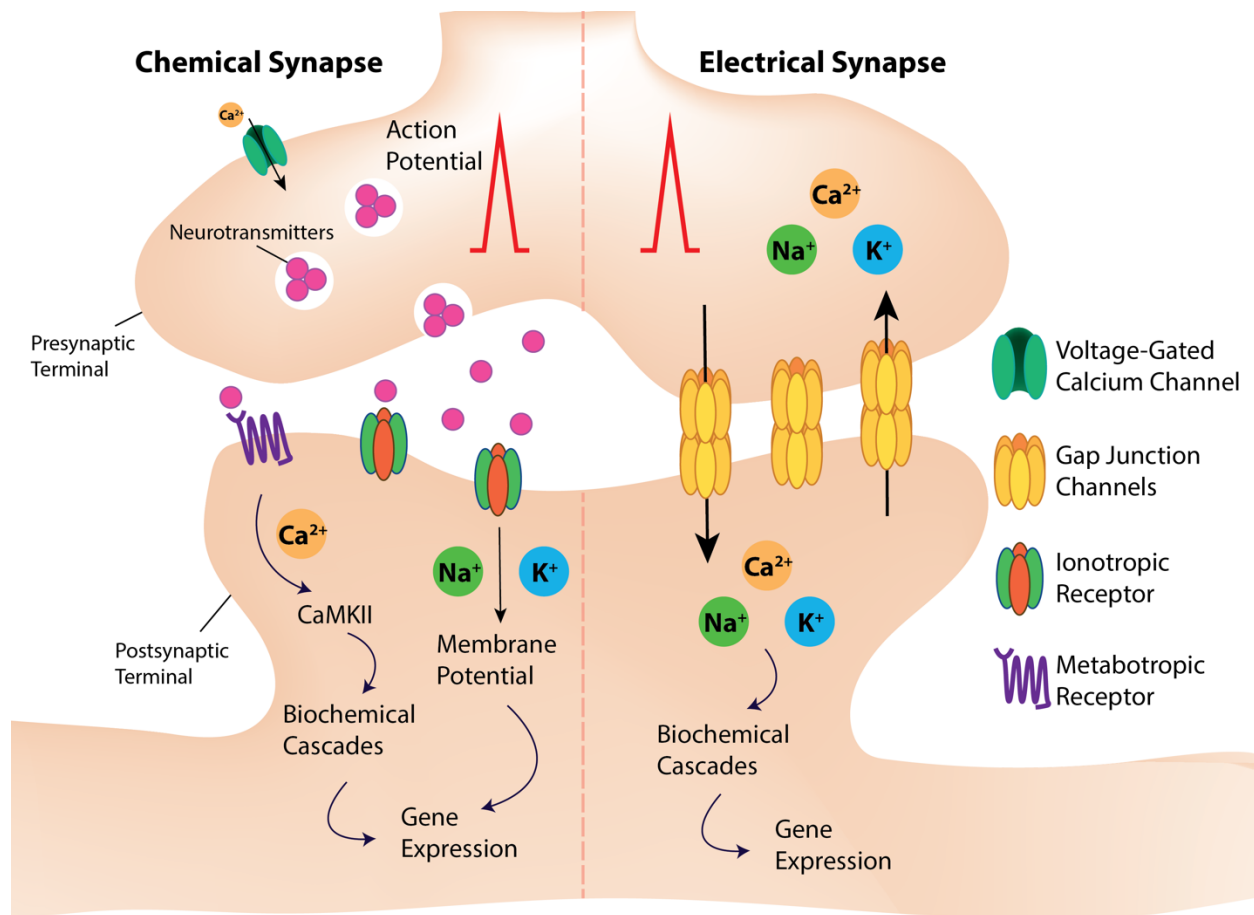


Figure 1.2: Two modes of synaptic transmission (chemical and electrical synapses). Chemical synaptic communication (left) requires an electrical signal (action potential) to depolarize the presynaptic terminal leading to the activation of voltage-gated calcium channels (VGCCs) and influx of calcium ions. Vesicular release of neurotransmitters (i.e., glutamate) promote the activation of ionotropic and metabotropic receptors to translate messages from the presynaptic terminal to the postsynaptic terminal. This leads to different postsynaptic events such as change in membrane potential to gene expression. Electrical synaptic communication (right) is mediated by intercellular channels known as gap junctions that link adjacent cells to enable bidirectional passage of electrical and ionic signals, cellular messengers and other small metabolites. This leads to similar biochemical events and gene expression as chemical synapses. (Note: concept of the figure is from (Alberto E Pereda 2014), which has been redrawn and significantly modified.)

1.3) Chemical Synapses

Chemical synapses allow signal transmission from neuron to neuron. The sequential events of different biochemical reactions lead to synaptic potentiation (**Figure 1.2; left**). When an impulse of electrical signal travels to the presynaptic terminal, presynaptic voltage gated Ca^{2+} channels (VGCCs) allow the influx of Ca^{2+} . Elevated levels of Ca^{2+} regulates NT-filled vesicles travel to the terminal end and release its contents to the synaptic cleft through exocytosis. NTs diffuse across the synaptic cleft and bind to the receptors on the post-synaptic membrane. Ligand-gated ion channels change confirmation to permit ions (i.e., Ca^{2+} , K^{+} , or Na^{+}) into the post-synaptic terminal (Unwin 1993). The surge of ion influx generates membrane potential change, which leads to the strengthening of the synapse (Murthy 1998). Amongst different types of ionotropic receptors expressed in neurons, there are three main types of ionotropic glutamate receptors: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), kainite receptor (KARs), and *N*-methyl-D-aspartate receptor (NMDARs).

AMPA receptors are membrane-bound ionotropic receptors, which mediate signal transmission upon glutamate activation. There are four subunits: GluA1, GluA2, GluA3, and GluA4, which combine to form a heteromeric tetrameric unit (I. Song and Huganir 2002). Each of the four subunits is accessible to glutamate, which allows the opening of the pore. Depending on the composition of the heteromeric tetrameric unit, the permeability of cations (i.e., Ca^{2+} , K^{+} , Na^{+}) will differ (Hollmann 1994). For example, AMPARs with GluA2 subunits are mostly impermeable to Ca^{2+} , while the absence of GluA2 makes the channel permeable to other cations, including Ca^{2+} . Phosphorylation regulates the function and potentiation of the receptors leading to signal transmission. Several different kinases directly phosphorylate serine, threonine, and tyrosine

residues, for example CaMKII, PKA, and PKC (Carvalho, Duarte, and Carvalho 2000). The flow of Na^+ into the cell causes depolarization, which leads to NMDARs activation.

Kainate or kainic acid receptors (KARs) are similar to AMPARs which are ionotropic receptors that are sensitive to glutamate activation. There are five subunits: GluK1, GluK2, GluK3, GluK4 and GluK5 (Collingridge et al. 2009). KARs are tetrameric ionotropic receptors that are permeable to Na^+ , K^+ , and Ca^{2+} . Its role in plasticity is not as well documented compared to both AMPAR and NMDARs, but antagonistic treatments on GluK1 have shown to block LTP in CA3 hippocampal neurons (at the mossy fibres) (Bortolotto et al. 1999; More et al. 2004). The expression of KARs at presynaptic terminals suggest its role in modifying the probability of glutamate release to the synaptic bouton. These receptors have notable relevance in different disorders, including epilepsy. By knocking out GluK2 subunit or blockage of KARs, seizure susceptibility has significantly reduced, which suggested KARs role in regulating excitatory transmissions (Grosenbaugh et al. 2018).

NMDARs are another type of ionotropic glutamate receptor active in the presence of glutamate and glycine or other glutamate agonists. There are three different subunits: NR1 (NR1a, NR1b), NR2 (NR2A, NR2B, NR2C, NR2D) and NR3 (NR3A, NR3B) (Paoletti, Bellone, and Zhou 2013) forming a heteromeric tetrameric unit similar to AMPARs. NR1 and NR3 subunits are involved in glycine reception, while the NR2 subunits are known for glutamate. The receptor channel is voltage-gated with an Mg^{2+} blockade and is regulated by levels of Zn^{2+} (Paoletti, Ascher, and Neyton 1997). Upon cellular depolarization generated by Na^+ influx through AMPARs, Mg^{2+} is expelled and influxes a surge of cations (i.e., Ca^{2+} , Na^+ , K^+). The flow of Ca^{2+} triggers downstream signaling pathways involving different kinases (CaMKII, PKA, and Src family kinases (SFK))

(Woolfrey and Dell'Acqua 2015; Mao et al. 2014; Weiler, Tang, and Thompson 2012). NMDARs activation can lead to excitotoxicity or promote plasticity depending on the duration of exposure. As mentioned previously, the activation of NMDARs are the source of Ca^{2+} . Repeated activation of these receptors is necessary for long-term potentiation (LTP). Dysregulation of such receptors leads to various disease phenotypes (i.e., Alzheimer's disease, developmental delays, Huntington's, and schizophrenia), which have been reviewed in 2013 by Zhou & Sheng (Q. Zhou and Sheng 2013).

1.4) Electrical Synapses (Gap Junctions)

Unlike chemical synapses, electrical synapses are direct interactions between adjacent neurons. The membranes of both neurons come into proximity allowing a transfer of small molecules (up to 1kDa) and signals through a conduit known as a gap junction (reviewed in 2014 by Pereda 2014), which includes Ca^{2+} , IP_3 or ATP (**Figure 1.2; right**). Unlike chemical synapses, electrical synapses allow synchrony amongst cells. Typical gap junction channels consist of two hemichannels known as connexons to form a complete channel. Tens of thousands of connexons aggregate in defined membrane domains to form what is known as the gap junction plaque (GJP). The interaction of connexins with other cellular complexes (i.e., occluding junctions and anchoring junctions) form the gap junction nexus. These complexes allow the anchoring of connexins at the postsynaptic density zone (PDZ). Connexin aggregates with associated complexes are stable but retain fluidity for rearrangement (reviewed in: Duffy, Delmar, and Spray 2002). The nexus is where the direct exchange of metabolites and ions takes place between adjacent cells. Every connexon is composed of six homomeric or heteromeric subunits of connexin (Cx) proteins forming a ring-like structure (Sohl and Willecke 2004). The heteromeric nature of some gap junction channels gives rise to the more complex function and specificity. In general, each

connexin protein has a similar fold of peptide chains following four transmembrane domains (M1-4) with two extracellular loops (E1-2). The C-termini is located on the cytoplasmic side, where phosphorylation and regulation of the protein channel occur (Söhl and Willecke 2004). Many connexins are relevant in diseases including but not limited to Cx43 in ODDD (Kelly et al. 2016), Cx26 in deafness (Murgia et al. 1999), and Cx32 in Charcot-Marie-Tooth disease (Seeman et al. 2001).

1.5) Family of Connexins

Intercellular communication amongst cells is vital for their function and survival. As mentioned previously, electrical synapses allow a direct exchange of molecules. Vertebrates express connexins and pannexins (functional hemichannels only), while invertebrates express innexins. Connexins share structural and membrane topology with innexins, while both pannexins and innexins share evolutionary homology (Baranova et al. 2004).

1.6) Connexins

To date, there are twenty mouse and twenty-one human connexin genes with unique specifications and functional properties. The nomenclature of these proteins is preceded with “Cx” notation, followed by their molecular mass of the polypeptide in kilodaltons. Expression of connexins ranges between the liver (Cx26, Cx32), skin (Cx31, Cx30, Cx26), brain (Cx43, Cx36), eye/ lens (Cx36, Cx46, Cx50), immune cells (Cx43, Cx37, Cx40), and heart (Cx45, Cx43, Cx40) (Urban, Rozental, and Spray 1999; Dobrowolski and Willecke 2009). Although each isoform of connexin is important, the focus will be placed on connexins expressed in the brain, namely in glia (Cx43 mentioned) and neurons (Cx36 being the focus).

Both Cx30 and Cx43 are expressed in astrocytes, with Cx43 being the most abundant (Schulz et al. 2015). Cx43 can function both as gap junctions and as uncoupled hemichannels. Coupled as gap junctions, these junctions facilitate homeostasis of positive ions (i.e., K^+), glycine, and glutamate (Lapato and Tiwari-Woodruff 2018). While uncoupled, these hemichannels regulate the release of ATP and glutamate into the extracellular space and thus influencing neighboring neurons (Sáez et al. 2003). Astrocytic Cx43 allows the dispersion of oxidative stress and thus making neurons more resilient to insults. Through gliotransmitters like ATP and glutamate, Cx43 plays a role in Ca^{2+} waves, synaptic plasticity, and memory consolidation (Stehberg et al. 2012; Meunier et al. 2017). Since Cx43 can be activated in both physiological and pathological states, pathologic activation of these hemichannels can lead to neuronal damage or neuronal death. These pathological stimulants include but are not limited to, oxidative stress, acidosis, and inflammasomes. Cx43 has been linked to neurodegenerative diseases (i.e., Alzheimer's disease (AD)), brain ischemia, and chronic pain (J I Nagy et al. 1996; Morioka et al. 2015; Choi et al. 2017). Due to its widespread health implications, Cx43 is among the most studied gap junction isoforms.

On the other hand, Cx45 and mainly Cx36 are primarily expressed in neurons (Belousov and Fontes 2013). Similar to other connexins, Cx36 allows the transfer of small molecules and metabolites (around or under 1 kDa) from one cell to another. Cx36 has been found extensively in the retina, olfactory bulb, inferior olive, and hippocampus (Blankenship et al. 2011; Teubner et al. 2000). Functionally coupled Cx36 proteins establish neuronal connections, which serve importance in memory consolidation, visual processing, and learning (Allen et al. 2011; Güldenagel et al. 2001). To date, there have not been many reports noting human mutations of Cx36. Knockout of Cx36 in mice has shown near-complete ablation of neuronal gap junctions in

the CNS (Long et al. 2002; Hormuzdi et al. 2001). Phenotypes of these mice were mild, which included visual defects (Güldenagel et al. 2001). Cx36 has been associated with different diseases, including refractive errors and degenerative myopia, seizures and epilepsy, and AD. A genome-wide study has noted Cx36 as a candidate gene responsible for refractive error and myopia due to its important role in eye growth and lens maturation in mice (Solouki et al. 2010; Rong et al. 2002). Reports have shown that Cx36-deficient mice hippocampal slices were less susceptible to 4-aminopyridine-induced seizure activity (Maier et al. 2002). The discoveries opened up opportunities for treatments targeting Cx36. Of the diseases, Alzheimer's disease has been associated with Cx36 and pannexin1 channels. The accumulation of amyloid plaques over-activates neurons expressing Cx36 and pannexin1 and induces uncontrolled depolarization (Orellana et al. 2011). On the contrary, Cx36 has been mainly reported to be functional as coupled entities, but some suggest its protective role as hemichannels. Reports have suggested that neuronal tolerance to insults is due to ATP's release through Cx36 hemichannels (Schock et al. 2008).

1.7) Pannexins

Pannexins consist of three family members (Pannx1, Pannx2, and Pannx3), which are glycoproteins that form single integral membrane channels (Panchina et al. 2000). Although pannexins have no sequence homology to connexins, they have similar topology with four helical transmembrane domains, two extracellular loops, one intracellular loop (new structure suggest two intracellular loops in frogs) and both N and C-terminal in the cytoplasm (Baranova et al. 2004) (Michalski et al. 2020). Recently, the cryo-EM structure of Pannx1 has been published, demonstrating a heptameric arrangement rather than a hexameric channel (Michalski et al. 2020). The large pore of the channels allows signaling molecules (i.e., ATP, ions, and amino acids) to exchange with extracellular space.

Pannexins play important roles in cellular communication through the response of different stimuli. These responses include but not limited to pH (Whyte-Fagundes and Zoidl 2018), mechanical stimulation (Taylor, Wright, and Mahaut-Smith 2015), voltage, calcium levels, and many others. The function of pannexin channels was previously reviewed in 2013 (review: (Penuela, Gehi, and Laird 2013)).

Distribution of the pannexin-1 is ubiquitously expressed in tissues, including the brain, heart, liver, skin, lungs, and many more to name (Vanden Abeele et al. 2006). Pannexin-1 is also extensively expressed in the central nervous system, including the cerebellum, cortex, hippocampus, retina, neurons, and glial cells (Zoidl et al. 2007; Baranova et al. 2004). On the other hand, pannexin-2 is expressed mainly in the central nervous system, including medulla, cerebral cortex, and putamen (Ray et al. 2006). Pannexin-3 is expressed in bone-related tissues, including osteoblasts, fibroblasts, and cartilage (Ishikawa et al. 2011; Bond et al. 2011).

Pannexins have diverse functional applications from immune response, ischemic apoptosis to development. In terms of immune responses, Panx1 has been involved in responses through interaction with P2X₇ receptors and ATP-activated release of cytokine interleukin 1 β , which further activates caspase-1 (Pelegrin and Surprenant 2006). These active cytokines lead to inflammatory responses. Both Panx1 and Panx2 have been shown to play a role in tumorigenesis suppression. Overexpression of Panx1 in mice has reduced tumor growth in glioma compared to controls (Charles P.K. Lai et al. 2007; C P K Lai, Bechberger, and Naus 2009). Ischemic conditions have been reported to open Panx1 channels, which leads to ionic imbalance. The dysregulated opening of Panx1 channels through SFK phosphorylation and uncontrolled activation of NMDA

receptors lead to epileptic seizures and ischemic cell death (Thompson, Zhou, and MacVicar 2006; Santiago et al. 2011). The role of Panx3 remains elusive, but the expression of Panx3 in chondrocytes has shown supportive roles in altering the state from proliferation to differentiation (Iwamoto et al. 2010).

1.8) Ca²⁺/Calmodulin & Calmodulin Kinase II

Calcium (Ca²⁺) ions are secondary messengers that are involved in most eukaryotic activities. Ca²⁺ ions are involved in transmitting depolarizing signals, activate different biochemical pathways, and contribute to synaptic communication. In the case of neurons, Ca²⁺ is crucial for neurogenesis, synaptic plasticity, and downstream gene transcription (Brini et al. 2014). In resting cells, intracellular levels are kept relatively low (around 100nM), which is approximately four times lower than extracellular Ca²⁺. Intracellular Ca²⁺ storage is mainly in the endoplasmic reticulum (ER) and can be over 100μM. Upon cellular stimulation, Ca²⁺ stores are released through inositol 1,4,5 triphosphate (IP₃) receptors and ryanodine receptors (RyR) (Takeshima, Venturi, and Sitsapesan 2015). Calcium is used through the mitochondrial matrix to generate necessary ATP through the citric acid cycle (Wan et al. 1989). Ca²⁺ influx from extracellular space is mediated by voltage-gated Ca²⁺ channels, ionotropic NMDAs, AMPARs and among others (Brini et al. 2014). The increase of NMDA receptor activation leads to a further influx of Ca²⁺. During synaptic strengthening (i.e., long term potentiation (LTP) in learning and memory), AMPA receptors provide the surge of positive ions (i.e., potassium and sodium ions) leading to membrane depolarization and initiating potentiation. Depolarization and the binding of neurotransmitters (both glycine and glutamine) activates NMDARs. With the help of AMPA receptors, the inhibiting magnesium ion is removed from NMDA receptors and allow an influx of calcium. Intracellular

calcium levels are critical for initiating reaction cascades for gene transcription, protein modification, and channel modulations (Dingledine et al. 1999) (**Figure 1.2; left**).

The essential mediator to initiate calcium response is calmodulin (CaM), a 148 amino acid protein with a molecular weight of 16.7kDa (Cheung 1980). CaM has four EF-hands with two forming the N-terminal and the other pair forming the C-terminal. Both domains exhibit different conformations depending on the presence or absence of Ca^{2+} (Chin and Means 2000). It is a calcium-modulated protein, which is sensitive to calcium level changes. CaM is involved in various pathways, including contraction and motility of the cytoskeleton system, cell proliferation, cellular metabolism, proteolysis, and both protein phosphorylation and dephosphorylation (Sharma and Parameswaran 2018). The intracellular Ca^{2+} sensor determines the transmission to other calcium-sensitive enzymes (i.e., calmodulin kinases), ion channels (i.e., ligand gated channels), or other proteins (Orellana et al. 2012; Evans and Martin 2002). Among CaM targets, Ca^{2+} /CaM-dependent kinase II (CaMKII) is the most prominent, which is pivotal in synaptic changes and normal LTP (M. K. Bennett, Erondur, and Kennedy 1983). On the other hand, a constant small increase in postsynaptic Ca^{2+} promotes CaM to activate calcineurin (an abundant protein phosphatase) and promote LTD (Mulkey et al. 1994). Since potential CaM-binding proteins are much more abundant than CaM itself, CaM is a limiting factor for which signaling pathway is to be activated (Xia and Storm 2005).

CaMKII is one of the most important kinases for synaptic plasticity. The kinase forms a complex dodecameric holoenzyme assembly (over 600kDa) that phosphorylates a range of proteins in the nervous system (Kanaseki et al. 1991). There are four subtypes of CaMKII (α , β , γ , and δ), with α

and β being found exclusively in the nervous system (Yamauchi 2005). CaMKII α is known to transmit the Ca^{2+} concentration signal downstream. CaMKII β , on the other hand, is known for F-actin transport and guiding CaMKII α to postsynaptic terminals (Sanabria et al. 2009; Shen et al. 1998). Structurally, CaMKII proteins are composed of three main domains: N-terminal catalytic domain, regulatory domain, and C-terminal domains (Fink and Meyer 2002). The catalytic domain contains the sites for target protein interaction, which catalyzes phosphotransferase reactions. The autoinhibitory nature defines how CaMKII can operate like a switch (from an inhibitory state to an active state). Initially, the enzyme is inhibited through the autoinhibitory domain in its regulatory or autoinhibitory region (Alev et al. 2008). A region in this domain resembles protein substrates to maintain the inactivated state (like a hairpin). With activated Ca^{2+} /CaM binding to CaMKII at the C-terminus, the interaction activates kinase activity. Upon initial phosphorylation, activated CaMKII (exposure of Thr286) can maintain phosphorylation through its autophosphorylation nature by increasing the binding affinity for CaM hundreds of folds (E. Yang and Schulman 1999). The change in binding affinity, in turn, decreases the CaM dissociation rate from activated CaMKII (Shifman et al. 2006). Its role includes but is not limited to the regulation of ion channel activity, phosphorylation of various proteins, and neurite extension (Fink et al. 2003). The calcium dependent CaMKII is part of the molecular basis of synaptic plasticity and LTP.

1.9) Studying subcellular dynamics and interactions

The development and combination of molecular and cellular tools with advanced imaging techniques provide means to study processes in living cells. Many of these methods stemmed from fluorescence microscopy techniques, but the speed, resolution, and analysis methodology have greatly advanced in the last decades. Amongst the many methods, Förster/ Fluorescence resonance

energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) are sophisticated techniques to capture complex cellular events and structures for qualitative and quantitative analyses.

FRET allows the analysis of molecular interactions in many systems based on the distance between proteins (Gautier et al. 2001). FRET can provide information about protein conformation and determine their binding affinity. The method is diverse and can be used both *in vitro* and in living cells. The mechanism behind FRET is the transfer of energy from one structure to another through dipole-dipole coupling (Sekar and Periasamy 2003). By utilizing overlapping excitation wavelength of fluorescently tagged proteins, the protein of interest will fluoresce if their proximity is less than 10nm. On the contrary, FRET will not occur in tagged structures with a distance greater than 10nm (refer to **Figure 1.3**). Since FRET requires fluorescently labelled proteins to interact in close proximity, one must ensure the increased protein size do not affect protein interaction, localization or expression.

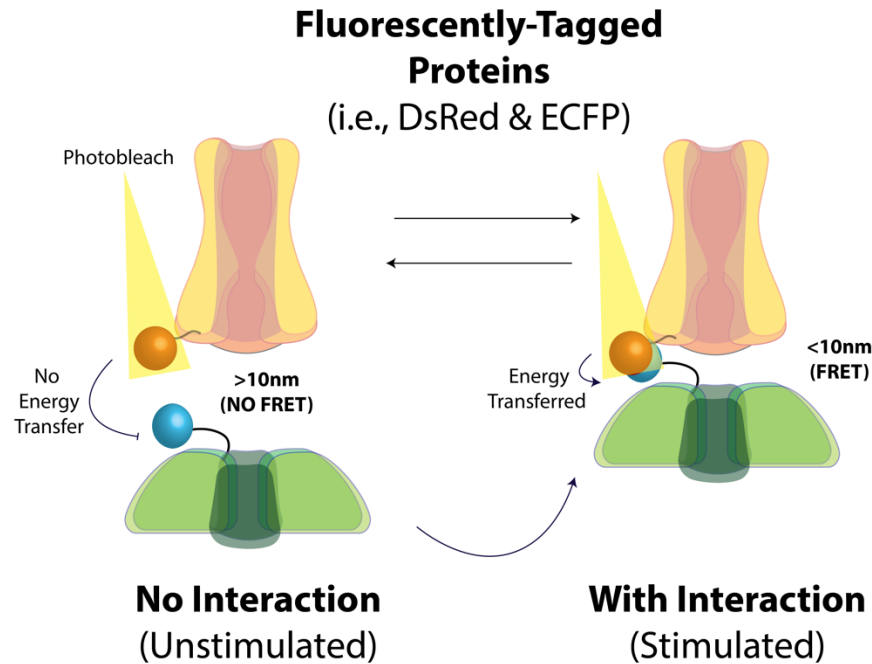


Figure 1.3: Simplified representation of FRET fluorescence microscopy (acceptor bleach). Fluorescently labeled proteins (example FRET pair; DsRed and ECFP) show no energy transfer if fluorescent tags are over 10nm apart (i.e., under unstimulated state) after photobleaching. Fluorescent tags with less than 10nm apart will enable effective energy transfer upon strong laser bleaching (i.e., in a stimulated state).

In recent years, FRAP has been used to study the mobility of protein and protein binding that may influence movement (Day et al. 2012; Sprague and McNally 2005). This technique is used in tandem with other fluorescently labeled proteins. When excited by a specific wavelength of light (depending on the fluorophore), one can track the protein's fluorescence. In typical experimental designs, FRAP initiates by capturing a series of images to establish a baseline before an intense laser is shortly applied to the region of interest (ROI). The intensity of the laser will bleach the fluorescent proteins in the area of the application, causing the loss of fluorescence. The speed in which the fluorescently labeled proteins replace the bleached molecules will be recorded over time within the ROI (Sprague and McNally 2005; Carisey et al. 2011). The change in intensity over time can be documented as a recovery curve, as shown in **Figure 1.4**.

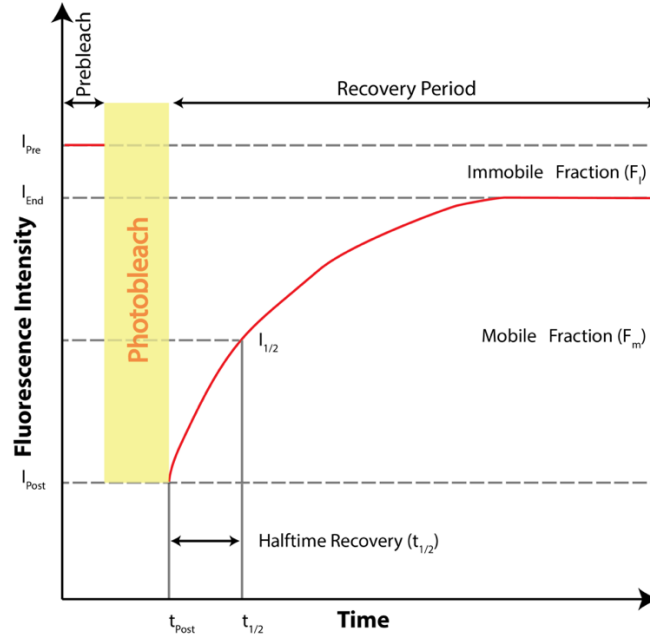


Figure 1.4: Theoretical FRAP recovery curve. Curve of a theoretical FRAP experiment for fluorescent tags or dyes. (I_{pre} : prebleach intensity, I_{end} : intensity after recovery, I_{post} : postbleach intensity, $I_{1/2}$: intensity after half recovery, t_{post} : time after photobleach, $t_{1/2}$: time after half recovery, F_i : immobile fraction, F_m : immobile fraction)

The immobile fraction determines the portion of proteins in the ROI bound to an immobile structure (i.e., cellular receptor or channel) that cannot be easily displaced by diffusion. The rate of recovery will be slowed by a factor correlated to the association and disassociation coefficients of binding (Day et al. 2012; Sprague et al. 2004) and can be exploited for protein binding investigations.

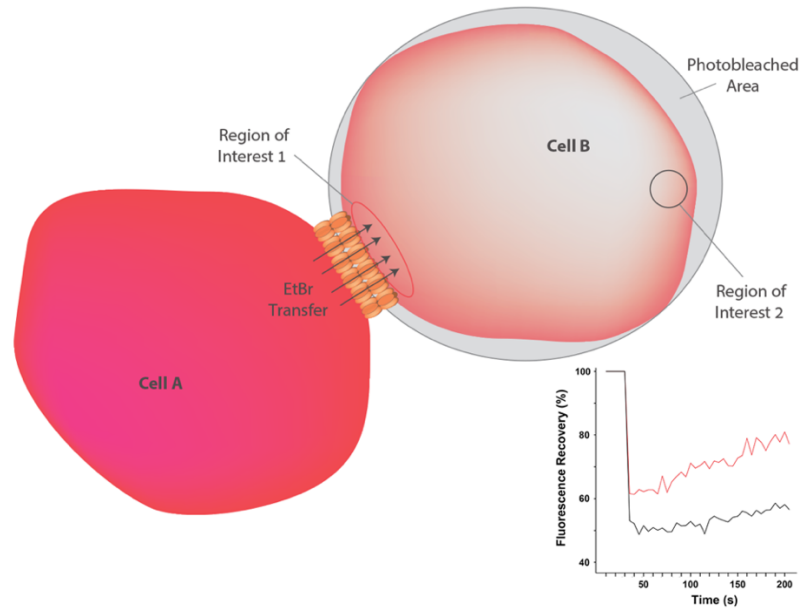


Figure 1.5: Schematic of gapFRAP showing regions of analysis and sample trace for each region of a cell pair. Cell B is photobleached with a powerful laser (100%) to eradicate fluorescence. Region of interest 1 (ROI 1) is located under the gap junction plaque of the bleached cell, while the ROI 2 is near the edge of the cell (by the membrane). Fluorescent dye from Cell A allow flow through the gap junction and replenish Cell B overtime. The sample trace captures the events from prebleach of 100%, post bleach (around 50%) and recovery over time (in seconds).

Recently, a modified version of the dye uptake assay previously demonstrated in (Prochnow et al. 2009; Kurtenbach et al. 2013) was coined as gapFRAP (Siu et al. 2016) in order to determine the functionality of gap junction channels using dyes (i.e., ethidium bromide). The same FRAP concept is utilized, but the ROI is confined to the area immediately under the gap junction plaque (please refer to **Figure 1.5**). As one of the cells in the pair is photobleached, dye from the unbleached cell will travel through the gap junction plaque and displace the bleached dye. The fluorescence recovery % over a certain time period (i.e., five minutes) determines the gap junction's functionality. High recovery rates suggest increased activity/ active state of the gap junctions, and low recovery rates suggest an inactive or inhibited state.

1.10) Hypothesis and Research Aims

Aim 1: Determining the mechanism of CaM and Cx36 interaction

Electrical synapses synchronize neural activity in cooperative interaction with chemical synapses and contribute to the mechanism for synaptic plasticity. The system utilizes key regulators to activate receptors (i.e., NMDA receptors) and channels (i.e., Cx36 channels), leading to calcium-dependent plasticity. The interaction of calmodulin and CaMKII is vital in both chemical and electrical synapses. At chemical synapses, the synergistic interaction of both calmodulin and NMDA receptors enable CaMKII to remain in an active state upon initial Ca^{2+} influx. In electrical synapses, the interaction of Cx36 and CaMKII defines functional plasticity, but competing calmodulin overlaps the CaMKII binding motif. In this section, I proposed a model predicting sequential interaction between calmodulin, CaMKII, and Cx36. I hypothesized that CaM-Cx36 interaction is favoured at the initial rise of calcium preceding the arrival of Cx36 protein at the GJP. In summary, this section demonstrates the critical priming of Cx36 by calmodulin in plasticity at electrical synapses.

Data documenting this aim was published in *Frontiers in Molecular Neuroscience* (Siu et al. 2016) titled as Structural and Functional Consequences of Connexin 36 (Cx36) Interaction with Calmodulin, which is Chapter 4 of this thesis. Several sub-aims in this chapter include:

- I. Validate CaM-Cx36 interaction dependence on Cx36 localization
- II. Determine the residue(s) of Cx36 critical for CaM-Cx36 interaction
- III. Resolve the structural features of CaM and Cx36 in relation to other ligands

Aim 2: Determining how NMDAR-Src-Panx1 signaling modulates the interactions at electrical synapses

The synergistic actions of calcium, calmodulin (CaM), and calmodulin kinase II (CaMKII) constitute a central mechanism for synaptic plasticity of chemical synapses. Research has shown previously that connexin-36 (Cx36) uses CaM/CaMKII interaction in a similar Ca^{2+} -dependent process for plasticity of electrical synapses. In this chapter, I hypothesized that beyond utilizing a similar molecular machinery, both types of synapses communicate efficiently. I explored the possibility of NMDA receptors' influence on the interaction between CaMKII and Cx36. Here, I report the discovery of an ionotropic N-methyl-D-aspartate receptor - Src family kinase – Pannexin1 signaling complex, which facilitates CaMKII-Cx36 interaction.

Data documenting this aim is part of a manuscript ready for submission titled as An NMDA receptor – Pannexin1 signaling complex adjusts the interaction of CaMKII with Connexin36, which is Chapter 5 of this thesis. Several sub-aims in this chapter include:

- I. Understand how Ca^{2+} impacts the proximal distance of Cx36 proteins in different physiological conditions
- II. Determine residues crucial for the Cx36-CaMKII interaction
- III. Explore how NMDA receptors, Src kinases, and Panx1 channels contribute to the Cx36-CaMKII interaction

CHAPTER TWO: Experimental Procedures

To provide ease of reading and conciseness, all experimental procedures and materials have been consolidated from *Chapter Four*, *Chapter Five* and *appendices* into this section. It should be noted that some experiments have been performed either solely or in collaboration with contributors. Specific author contributions are outlined either within this chapter or in figure legends.

2.1) Plasmid constructs and site-directed mutagenesis

Expression vectors contained full-length rat Cx36 (NM_019281, amino acids (aa) 1-321), CaM (NM_031969, aa 1-149), and CaMKII α (NM_012920.1, aa 1-478), mouse Panx1 (NM_019482.2, aa 1-426), Cx43 (NM_012567, aa 1-382) in variants of pEGFP-N1 expression vectors (Clontech Laboratories Inc., Mountain View, CA, USA). Full-length in-frame cloning of CaM, the calcium insensitive mutant CaM_{E1234Q} (Maunes, Kleeq, and Beckinghams 1992; VanScyoc et al. 2002), CaMKII α , the calcium insensitive mutants: CaMK_{T286A}, CaMK_{T286D}, CaMK_{F293A}, CaMK_{R296A} (Bayer et al. 2001; Singla et al. 2001), and Cx36, the CaMKII α binding mutant: Cx36_{R278A}, Cx36_{K279A}, Cx36_{K281A} (Alev et al. 2008), Panx1, the Src kinase insensitive mutants: Panx1_{Y308F}, Panx1_{Y308A} (Weilinger et al. 2016), Panx1_{Y198F}, Panx1_{Y198A} (DeLalio et al. 2019) as well as genetically encoded organelle markers (pER-DsRed2, pGolgi/DsRed2, pMito-DsRed2,) (Fliegel et al. 1989; Watzele and Berger 1990; Rizzuto et al. 1989) were completed in two steps. The open reading frames were synthesized as gBlocks (Integrated DNA Technologies Inc. (IDT), Coralville, IA, USA) and cloned into the TA cloning vector pJet1.2 (Thermo Fisher Inc., Mississauga, ON, Canada). Then the coding regions were isolated by restriction digest and cloned in-frame into pEGFP-N1, pEYFP-N1, pECFP-N1 or the pDsRed2-monomer expression vector. Mutant Cx36 plasmids (G276A, W277A, R278A, K279A, K281A), CaMKII (T286A,

T286D, F293A, R296A) were generated using the Q5TM Site-Directed Mutagenesis Kit according to the manufacturer's protocol (New England Biolabs Inc. (NEB), Boston, MA, USA). Oligonucleotides were designed using the NEBaseChanger tool (NEB) and synthesized by IDT. Table I summarizes the oligonucleotides utilized in this study (mutations depicted in bold and underlined). All plasmid constructs used on this study were sequence verified (Eurofins MWG Operon LLC, Huntsville, AL, USA).

2.2) Cell culture, transient transfection and western blot

Neuroblastoma 2a (Neuro2a) cells (Olmsted et al. 1970) were cultivated in DMEM with 2mM glutamine, 1% non-essential amino acids (NEAA), 1% penicillin and streptomycin (PS) and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO₂. Cells were seeded in 24-well plates or glass-bottom dishes (MatTek Corporation, Ashland, MA, USA) and transfected with 200 ng (single transfection) or 400ng (double transfection) endotoxin-free plasmid DNA, using the Effectene transfection protocol (Qiagen Inc., Valencia, CA, USA). For western blot, whole cell protein lysates were prepared 48 hrs after transfection. 20 µg protein was separated by 10% SDS-PAGE, transferred to 0.2 µm Midi format nitrocellulose membrane and processed using the iBindTM Western System (Bio-Rad Inc., Mississauga, ON, Canada). Primary antibodies were diluted 1:1,000 (mouse anti-GFP, Roche; rabbit anti-GFP (FL), Santa Cruz Biotechnologies, TX, USA; rabbit anti-Panx1, Invitrogen, 488100) and 1:15,000 (mouse anti-β-actin; Sigma-Aldrich Chemie GmbH, Munich, Germany). The secondary antibodies (LI-COR Biosciences, St. Lincoln, NE, USA) were diluted 1:20,000 (donkey anti-rabbit IRDye680LT) or 1:20,000 (goat anti-mouse IRDye800CW). Signals were detected using the Odyssey[®] CLx Infrared Imaging System (LI-COR Biosciences).

2.3) Immunohistochemistry (IHC)

Four-weeks-old C57BL/6 mice were humanely euthanized and quickly followed by full body perfusion prior to head separation. The whole brain was carefully isolated from the skull and placed in physiological Ringers solution (138 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 10 mM Glucose, pH 7.4). The brain was fixed in 4% PFA overnight and then cryopreserved with 30% sucrose at 4°C ON before sectioning. Ten micrometers thick cryosections were prepared using a Leica cryostat under -18°C. Antigen retrieval was completed with 1% SDS for 5 min, followed by three washes each for 5 min with PBS. The IHC methods documented previously by our lab (Whyte-Fagundes et al. 2018) was followed. Primary antibodies (polyclonal anti-Panx1, ACC-234, Alomone Labs, Jerusalem, Israel, dilution 1:300 (Beckel et al. 2015); monoclonal anti-parvalbumin, P 3088, Sigma-Aldrich, MO, USA, dilution: 1:500 (Vatsa et al. 2019; Miri et al. 2018); monoclonal CaMKII alpha (6G9), MA1-048, Invitrogen, dilution: 1:500 (Biro et al. 2017); monoclonal anti-connexin36, 1E5H5, Thermo Scientific, dilution: 1:300 (Ciolofoan et al. 2007); polyclonal anti-VGlu1, ABN1647, Millipore, dilution: 1:1000 (Hammoum et al. 2018) were applied in buffer with 1% BSA in PBS containing 0.1% Triton X-100, and incubated at 4°C ON in a humidified environment. After washing in PBS for 30 min, secondary goat anti-rabbit Alexa Fluor 488 and 568 (Invitrogen, Canada) diluted in 1xPBS (1:5000) and applied for 40 min at RT in the dark. After three 10 min washes with PBS-Tween 20 (PBS-T), sections were mounted, stained with Fluoroshield™ with DAPI (Sigma Aldrich, Canada), sealed, and kept at 4°C ON in the dark. Confocal microscopy was performed using ZEISS LSM 700 microscope. All imaging parameters were controlled by the ZEISS ZEN 2010 software. Imaging was performed with 5x (NA 0.16; M27 DIC), 40x or 63x (NA 1.4; M27 DIC). Images were exported and processed using ImageJ and Photoshop CS6.

2.4) TALEN Panx1 KD Cells

Transcription Activator-Like Effector Technology (TALEN) cloning protocols were carried out described by Reyon and colleagues (Reyon et al. 2012). The ZiFit Targeter software (<http://zifit.partner.org/ZiFiT/>) was used to design constructs targeting a single NcoI restriction site located at close to the start of the coding region in exon 1 of the mouse Panx1 gene (Ensembl:ENSMUSG00000031934). Constructs were assembled in the expression vectors JDS74 and JDS78 using the protocol explained by Reyon and colleagues (Reyon et al. 2012). Neuro2a cells were transiently transfected as described previously with both TALEN constructs, while selecting for transfected cells with nucleoside antibiotics blasticidin (10µg/mL). Upon reaching 80-100% confluency, genomic DNA was extracted using the GeneJET Genomic DNA purification kit (Thermo Fisher Inc., Mississauga, ON, Canada). PCR amplification of the genomic DNA confirmed the loss of the NcoI site at the start of the Panx1. PCR products were cloned into pJET1.2 plasmid (Thermo Fisher Inc., Mississauga, ON, Canada) and sequenced for confirmation of the mutation.

2.5) Confocal Microscopy

Transfected cells were fixed with 4% paraformaldehyde for 20 min at RT, washed with PBS, and mounted with ProLong[®] Antifade Mountant (Thermo Fisher Inc., Mississauga, ON, Canada) for imaging. Samples were visualized using a Zeiss LSM 700 confocal microscope with a Plan-Apochromat 63x/1.4 Oil DIC M27 objective and the ZEN 2010 program to control all hardware parameters. Images were collected by line averaging (4x) at high resolution (2048x2048pixel) using single planes or z-stacks. The GJ frequency was quantified as described previously (Qu, Gardner, and Schrijver 2009), by counting the number of EGFP-expressing cell pairs against the number of cell pairs having GJPs in images from randomly selected, non-

overlapping visual fields collected from experimental replicates ($n \geq 3$). The GJ area was quantified using ImageJ. Here, the ImageJ free hand tool was used to draw the contours of GJs between cell pairs, followed by quantification of the GJ area using the measure tool. Mander's overlap coefficients were calculated using the co-localization analysis of the ZEN 2010 program (Manders, Verbeek, and Aten 1993). Images were exported and further processed using ImageJ or the Imaris Scientific 3D/4D Image Processing & Analysis Software (Bitplane USA, Concord, MA, USA). Images were combined using Adobe Photoshop CS6 for presentation.

2.6) FRET Analysis

The acceptor bleach protocol was reported previously (Siu et al. 2016; Timonina, Kotova, and Zoidl 2020a). Neuro2a cells were transfected with DsRed-monomer and CFP-tagged expression vectors. Baseline readings were established before initiating the acceptor bleach protocol (five initial readings). In this protocol, DsRed-tagged proteins were photobleached using the 555 nm laser line at 100% intensity, while the intensity change of CFP or GFP tagged proteins were recorded using the 405 or 488 nm laser line respectively. The experimental protocol was terminated upon reaching 10% of the acceptor channel baseline intensity. FRET efficiency was then calculated by using the FRET efficiency formula:

$$FRET\ Efficiency\ (E; \%) = \frac{(D_{Post} - D_{Pre})}{D_{Post}}$$

where D_{post} is the average intensity after bleaching, and D_{pre} is the average intensity before the bleach (Okamoto et al. 2004) after subtracting the values of the background (noise). The FRET distance ($FRET_d$) between the interacting proteins was calculated using the formula:

$$FRET\ Distance\ (R; nm) = R_0 \left(\frac{1}{E} - 1 \right)^{\frac{1}{6}}$$

where R_0 is the previously referenced distance between the two fluorescent tags (DsRed and ECFP: 5.09; DsRed and EGFP: 5.76)(Erickson, Moon, and Yue 2003), and E is the FRET efficiency. The average values of the fluorescent intensity change, and distance were calculated and represented in graphical formats. Error bars denote the standard error of the mean (SEM) of each protein-protein interaction.

2.7) Ethidium Bromide Uptake and Recovery after Photobleaching Assay (gapFRAP)

The assay has been introduced (Siu et al. 2016; Whyte-Fagundes et al. 2018) as a variation to the ethidium bromide uptake assay previously reported (Prochnow et al. 2009; Kurtenbach et al. 2013). Transfected Neuro2a cells cultured for 48 h in 3.5 cm MatTek cell culture dishes were incubated with 10 μ M of ethidium bromide in supplemented growth medium for 10 min at 37°C and 5% CO₂. MatTek dishes were placed in a live-cell imaging chamber and imaged at 37°C using a Zeiss 700 confocal microscope. Cx36–EGFP expressing cell pairs were selected, and a time-lapse baseline image was recorded (defined as 100%). One of the cell pairs was bleached using the 555 nm laser line with 100% laser power intensity. The number of iterations (~40 iterations at 100% intensity) in the bleaching protocol was terminated upon achieving 40–50% reduction in fluorescence. A voxel of 36.2 μ m $\times$ 36.2 μ m $\times$ 1.6 μ m was bleached using a non-confocal imaging mode. The recovery of ethidium bromide fluorescence in the center plane of this voxel after bleaching was measured over time (~3 minutes) in confocal imaging mode. In all experiments, three regions of interest (ROI: R0, R1, R2) were selected for analysis. R0 was placed outside the cell pair to determine background the fluorescence. ROIs were placed inside the bleached cell close (R1) and distant (R2) to the GJP. The fluorescence recovery in percent was calculated using the formula:

$$\text{Fluorescence Recovery (\%)} = \frac{(F_{\text{Average(3 min Post Bleach)}} - F_{\text{Bleach}})}{(F_{\text{Average(Pre Bleach)}} - F_{\text{Bleach}})}$$

2.8) Dynamic Ca²⁺ Imaging

Transfected Neuro2a cells were incubated in DMEM⁽⁻⁾phenol red with Ca²⁺ indicators Oregon Green 488 BAPTA-AM Cell Permeant (OGB) (Thermo Fisher Inc., Mississauga, ON, Canada) for 30 minutes on MekTek plates. Cells were washed with PBS^{+/+} before incubating for another 30 minutes in DMEM⁽⁻⁾phenol red. Cell pairs were imaged using a Zeiss Observer Z1 Spinning Disk Confocal Microscope equipped with live cell imaging chamber set to 37°C and 5% CO₂. Evolve TIRF camera with a Plan-Apochromat 63x/1.4 Oil DIC M27 objective was used to measure the Ca²⁺ signal changes over 300 cycles at 1 sec intervals. The 488nm laser line at 20% laser intensity were used to measure the cells. Drug treatments were delivered after the first 10 cycles with tubing and syringe. Images were processed and analyzed in ImageJ with the Heatmap Histogram and Time Series Analyzer V3 plugin after background subtraction.

2.9) ArcLight Fluorescent Voltage Probe Imaging

Dually transfected Neuro2a cells with DsRed-Cx36 and ArcLight S249 (Jin et al. 2012) were imaged 48h post-transfection. Cells were imaged using a Zeiss Observer Z1 Spinning Disk Confocal Microscope equipped with live cell imaging chamber set to 37°C and 5% CO₂. Evolve TIRF camera with a Plan-Apochromat 63x/1.4 Oil DIC M27 objective was used to measure the fluorescence change of ArcLight S249 over 300 cycles at 2 sec intervals. The 488nm and 561nm laser lines were at 15% and 10% laser intensity respectively during the experiments. The first 30 cycles were used to establish the baseline for each experimental run, while drug treatments were delivered through tubing with a syringe during the 31st - 40th cycle. The definite focus strategy

was used to maintain the plane of view over the course of the experiment. Images were processed and analyzed in ImageJ with the Time Series Analyzer V3 plugin after background subtraction.

The fluorescence scaled to baseline was calculated using the formula,

$$Fluorescence\ (Scaled) = \frac{F_{Time}}{F_{Average(Baseline)}}$$

and the fluorescence change ($\Delta F/F$) was calculated using the formula,

$$Fluorescence\ Change\ (\Delta F/F) = \frac{(F_{Average(300)} - F_{Average(Baseline)})}{(F_{Average(Baseline)})}$$

where F_{Time} is the fluorescence at the given time, $F_{Average(Baseline)}$ is the average of fluorescence in the first 30 cycles and $F_{Average(300)}$ is the average of fluorescence in the last 30 cycles to 5 minutes.

2.10) Pharmacology

Prior to imaging transfected cells were treated with simulants or blockers in a final concentration supplemented with complete growth medium: BAPTA-AM (24 μ M; 10 mins; Thermo Fisher; (Ueda and Shah 1992)), ionomycin (2 μ M; 5 mins; (Liu and Hermann 1978)), carbenoxolone (CBX; 50 μ M; 10 mins; (J. Li et al. 2001)), CaM antagonist W-7 (10 μ M; 10 mins) (Weiss, Prozialeck, and Wallace 1982), NMDA (50 μ M; 5 mins; (Nong et al. 2003)), caffeine (10mM; 1 mins; (Carrasco, Jaimovich, and Kemmerling 2004)), potassium gluconate (40mM; 5 mins; (Lan et al. 2014)), glutamate and glycine (10mM & 2mM; 1 mins; (Cummings and Popescu 2015)), EDTA (10 μ M; 5 mins; (Glasgow and Johnson 2007)), MgSO₄ (5mM; 10 mins; (Clerc et al. 2013)), carvedilol (1 μ M; 30 mins; (Q. Zhou et al. 2011)), thapsigargin (1 μ M; 10 mins; (Lytton, Westlin, and Hanley 1991)), DNQX (10 μ M; 30 mins; (Weilinger et al. 2016)), kynurenic acid

(KYNA; 2mM, 30 mins; (Cruikshank et al. 2004), MK801 (20 μ M; 30 mins; (Mantuano et al. 2015; Weilinger et al. 2016), AP5 (100 μ M; 10 mins; (Alpert and Alford 2013)), PP2 (10 μ M; 30 mins; (Weilinger et al. 2016)), PP3 (10 μ M; 30 mins; (Weilinger et al. 2016)), ¹⁰Panx (100 μ M; 10 mins), ^{SC}Panx (100 μ M; 10 mins; (Thompson et al. 2008; Weilinger et al. 2016)), BB-FCF (10 μ M; 10 mins), Bt₂-cAMP (100 μ M; 15 mins; (Dugan et al. 1999)), H89 (10 μ M; 10 mins; (Allagnat et al. 2008; Herbst, Allen, and Zhang 2009)) at 37°C and 5% CO₂. The final solvent concentration of DMSO in treated cells was 0.002% - 0.02%. All reagents were supplied by Sigma-Aldrich unless other specified.

2.11) Protein Expression and Purification

A pET15 based (EMD Millipore, Etobicoke, ON, Canada) plasmid for expression of hexahistidine-tagged human calmodulin (His₆-CaM) was a gift from Prof. Mitsu Ikura (Univ. of Toronto). From a 1 L fermentation in Luria broth, milligram quantities of >95% pure His₆-CaM were obtained by a two-step Ni-NTA affinity chromatography (Qiagen Inc., Toronto, ON, Canada) and gel filtration chromatography (Sephadex-75 HiLoad 16/60 column, GE Life Sciences) procedure. Final buffer conditions were 5 mM Tris-HCl, 100 mM NaCl, 0.05% NaN₃, pH 7.5. The CaM-Cx36 complex pursued for structural determination was expressed as a 169 aa fusion protein consisting of His₆-tagged human CaM (5-150), a short linker (GASTAAGS), and residues 276-292 of rat Cx36. A gene fragment encoding this fusion protein was synthesized by DNA2.0 (Menlo Park, CA, USA) and supplied in a T5-based expression vector (pJ441-kan). ¹⁵N, ¹³C labeled His₆-CaM-Cx36 was obtained from a 1 L fermentation in M9 media containing 1 g (99%, ¹⁵N) ammonium chloride, 3 g (99%, ¹³C-glucose) and 0.5 g (98%, ¹³C-¹⁵N) algal extract (Cambridge Isotopes, Tewksbury, MA, USA). Final buffer conditions for NMR spectroscopy were 1.5 mM protein in 5 mM Tris-HCl, 100 mM NaCl, 5 mM CaCl₂, 0.05% NaN₃, pH 7.5 with 10% (v/v) ²H₂O.

2.12) NMR spectroscopy

Spectra were acquired at 298 K with a 700 MHz Bruker Avance spectrometer equipped with a TXI cold probe. Backbone and side chain chemical shift assignments were obtained using a suite of standard heteronuclear, triple resonance experiments (HNCACB, CBCACONH, HNCO, HNCACO, HNCA, CCH-TOCSY, HCCH-TOCSY) with non-uniform Poisson gap sparse sampling at 10-15%. Distance restraint data were obtained from ^{15}N -edited and ^{13}C -edited 3D-NOESY spectra (120 ms mixing time). All datasets were processed with NMRPipe (Delaglio et al. 1995) and analyzed with CcpNmr Analysis (Skinner et al. 2015). Dr. L. Donaldson performed and analyzed the data for all NMR spectroscopy and protein production.

2.13) Structure Determination

CcpNmr Analysis was used to integrate NOEs and calculate distance restraints based on an r^{-6} function spanning 1.8 – 5.5 Å with a reference distance set at 3.2 Å. These restraints were used as input to CYANA (Buchner and Guntert 2015) along with Ca^{2+} coordination distance restraints, hydrogen bonding distance restraints and torsion angle restraints predicted by the DANGLE method embedded in CCPN Analysis. Allowed backbone torsion angles and side chain rotamers were enforced by the ramaaco and rotameraco methods embedded in the CYANA suite. The final representative ensemble of 20 structures based on lowest CYANA target function was selected from an initial calculation of 400 structures. As this ensemble demonstrated good geometry and bonding, no additional refinement was pursued.

2.14) Databases

The structural ensemble was deposited in the Protein Data Bank under the accession number 2N6A.

2.15) Isothermal Titration Calorimetry (ITC)

ITC measurements were performed on MicroCal VP-ITC calorimeter (MicroCal Inc., Northampton, MA). The recombinant H₆-CaM (affinity purified as described above) was titrated with Cx36 derived peptides reflecting the wild type (GSGWRKIKLAVRGAQAKRKSVYEIR; CanPeptide Inc., Montréal, Quebec, Canada) or the W227A mutant ((GSGARKIKLAVRGAQAKRKSVY; BioBasic, Markham, ON, Canada). An optimal titration was achieved with 25 μ M CaM in the reaction cell and 250 μ M Cx36 peptide in the syringe, each in a buffer containing 50 mM NaCl, 5 mM BisTris pH 7.0, 5 mM CaCl₂. After an initial injection of 2 μ L, the bulk of the titration consisted of 34 successive 8 μ L injections with an equilibration delay of 300 s. Heats of dilution were determined by titrating the same peptide solutions into buffer alone. Titration profiles corrected for the heat of dilution were fitted into one-binding-site model using MicroCal Origin vv5.0. All values reported were calculated based on three individual experiments. Dr. E. Smirnova performed and analyzed the data for the ITC experiments.

2.16) Statistics

Statistical analysis and data presentation were performed using the IBM-SPSS and R-software packages. Results shown derive from at least three independent experimental replicates. All data were analyzed with the Shapiro-Wilk normality test and Levene's homogeneity of variance test. Data (mean $\pm$ SEM) with normal distribution and equal variance were subjected to paired or independent T-tests (2-tailed), and non-normal distributed data were subjected to Mann-Whitney *U* (2-tailed) tests with statistical significance threshold set to 5% ($p < 0.05$). Outliers were determined and shown on figures but have been excluded from statistical analyses.

CHAPTER THREE: Experimental Materials

3.1) Biosafety

The research project in this thesis was performed in accordance with federal, provincial and institutional regulations for the containment Level 2 laboratories located at the Life Science Building (LSB), Department of Biology.

3.2) Organisms

3.2.1) Bacterial strains

Escherichia coli (*E. coli*) DH5 α (Invitrogen, Burlington, Canada)

Genotype: F $^{-}$ Φ 80*lacZ* Δ M15 Δ (*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rK $^{-}$, mK $^{+}$)
phoA supE44 λ^{-} thi-1 gyrA96 relA1

E. coli NEB 5-alpha competent (New England Biolabs, Whitby, Canada)

Genotype: *fhuA2 Δ (argF-lacZ) U169 phoA glnV44 Φ 80 Δ (lacZ)M15 gyrA96 recA1
relA1 endA1 thi-1 hsdR17*

3.2.2) Eukaryotic cells

Neuroblastoma 2a cells (Neuro2a cells) were derived from *Mus musculus*, which were developed by Klebe and Ruddle in 1967 from a strain A albino mouse spontaneous tumour. Neuro2a cells used in this thesis was generously provided by Dr. David C. Spray (Albert Einstein College, NY, USA).

3.3) Kits for recombinant DNA applications

Polymerase Chain Reaction	Q5 [®] High Fidelity DNA Polymerase PCR Kit (New England BioLabs)
QIAquick Gel Extraction Kit	Gel Elution of DNA fragments (QiaGen)
Ligation of DNA fragments	CloneJET [™] PCR Cloning Kit (Thermo Scientific)
Plasmid DNA Purification	QIAprep Spin Miniprep Kit (QiaGen)
Re-ligation of vector DNA	Rapid DNA Ligation Kit (Thermo Scientific)
Restriction of DNA	Fast digestion Top Fermentas Kit (Thermo Scientific)
Site-directed mutagenesis	Q5 [®] Site-directed Mutagenesis Kit (New England Biolabs)
iBind Western Blot	iBind Solution Kit (Life Technologies)

3.4) Plasmids and Oligonucleotides

3.4.1) Plasmids

pJET1.2	Thermo-Fisher
E2-crimson	Addgene
pECFP-N1	Clontech
pEGFP-N1	Clontech
pEYFP-N1	Clontech
pDsRed-monomer	Clontech
Arclight S249	Addgene

(See appendix for plasmid DNA maps.)

3.4.2) Oligonucleotides

Table 1.1: Oligonucleotides for mutagenesis of CaMKII, Cx36 and Panx1.

Gene	Mutation	Forward Primers	Reverse Primers
CaMKII α	T286A	CAGACAGGAG G CCGTGGACTGCC	TGCATGCAGGAGGCCACAG
	T286D	CAGACAGGAG GAC GTGGACTGCCTG	TGCATGCAGGAGGCCACA
	F293A	CCTGAAGAAG G CCAATGCCAGGAGG	CAGTCCACGGTCTCCTGT
	R296A	GTTCAATGCC GCG AGGAACTGAAGGG	TTCTTCAGGCAGTCCACG
Cx36	G276A	TAACCATCTG GCA TGGCGGAAGA	AGTTCAGCCAGATTGAGC
	W277A	CCATCTGGGAG GCG CGGAAGATCAAAC	TTAAGTTCAGCCAGATTGAG
	R278A	TCTGGGATGG GCG AAGATCAAACCTGGC	TGGTTAAGTTCAGCCAGATTG
	K279A	GGGATGGCGG GCG ATCAAACCTGG	AGATGGTTAAGTTCAGCC
	K281A	GCGGAAGATC GCA CTGGCTGTCC	CATCCAGATGGTTAAGTTC
Panx1	Y308F	GTGTATGAAAT TTT TGCCACCTTC	TTTGAGAATGTCCGTTTTTC
	Y308A	GTGTATGAAAG GCG TGCCACCTTC	TTTGAGAATGTCCGTTTTTC
	Y198F	CGTGGAGCAG TTCT TGAAGACAA	ATTGGGTACTTGAAGTGG
	Y198A	CGTGAAGCAG GCC TTGAAGACAAAAA	ATTGGGTACTTGAAGTGG

3.5) Solutions

3.5.1) Solutions and media for cultivating bacteria

LB Media	1% bacto trytone, 0.5% yeast extract, 0.5% NaCl, 50 μ L/mL of kanamycin or 100 μ L/mL of ampicillin
LB agar plates	LB medium (1% agar), 50 μ L/mL of kanamycin or 100 μ L/mL of ampicillin
SOC medium	2% bacto tryptone, 0.5% bactoyeast extract, 10mM NaCl, 2.5 mM KCl, 10mM MgCl ₂ , 10mM MgSO ₄ , 20 mM glucose

3.5.2) Solutions for Molecular Biology

DNA Loading Buffer	10x FastDigest Green buffer (Fermentas)
1x TAE Gel Loading Buffer	40mM Tris, 20mM acetic acid, 1mM EDTA

3.5.3) Solutions for Protein Biochemistry

Laemmli Sample Buffer	2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.1% Orange G, 50mM Tris-HCl, pH 6.8
Laemmli Running Buffer	192mM glycine, 0.1% SDS, 25mM Tris-HCl pH 8.3
Staining Solution	Coomassie PAGE BLUE (BioRad)
Blocking Solution	Odyssey Blocking Buffer (Li-Cor Bioscience)
Phosphate Buffered Saline (PBS)	130mM NaCl, 28mM KCl, 10mM Na_2HPO_4 , 1.8mM KH_2PO_4 , pH 7.4

3.5.4) Solutions for Cell Culture

10% Formalin	Sigma-Aldrich
Trypsin	Sigma-Aldrich
PBS with/without calcium/magnesium	Sigma-Aldrich
Penicillin and Streptomycin	BioShop
FBS (Fetal Bovine Serum)	Gibco
NEA (Non-essential Amino Acids)	Sigma-Aldrich
DMEM	Sigma-Aldrich
Mounting Solution: Fluoroshield with DAPI	Sigma-Aldrich
ProLong TM Gold antifade reagent (no DAPI	Invitrogen

3.5.5) Solutions for Stable/Transient Transfection

Effectene®	QiaGen
EC Buffer	QiaGen
Enhancer	QiaGen
DMEM	Sigma-Aldrich
PBS with/without calcium/magnesium	Sigma-Aldrich
Blasticidin	Sigma-Aldrich

3.6) Consumables

3.6.1) General Consumables

Bent Glass Rods	Sigma-Aldrich
Glass Pipettes	Eppendorf
Plastic Agar Plates	Fisher Scientific
Plastic Consumables	Sarstedt and Fisher Scientific
Plastic Cuvettes	VWR

3.6.2) Cell Culture Consumables

Petri Dish (Coated) for Cell Culture	Falcon®
24 Well TC-Treated Cell Polystyrene Permeable Support Companion Plate with Lid (Sterile)	Falcon®
MatTek Dish (Coated) for Cell Culture	MatTek Corp.
Glass Pipettes (10mL and 5mL)	Fisher Scientific
Cover Slips	Fisher Scientific
Glass Slides	Fisher Scientific

3.7) Equipment

3.7.1) Molecular Biology Equipment

Bacterial Shaker	Innova40 (New Brunswick)
Incubator	HERA THERM (Thermo Scientific)
Incubator	Single cell electroporator (Eppendorf)
Lab Centrifuge	Sorvall Legend Micro 21R (Thermo Scientific)
PCR Machine	Mastercycler Nexus X1 (Eppendorf)
UV Transilluminator	UV Transilluminator TFM-20V (UVP)
UV Gel Documentation System	Alpha Imager HP System (Fisher Scientific)
Water Bath	Isotemp 210 Fisher Scientific
Weighing scale	Navigator OHAUS
Magnetic Stirrer and Hot plate	Isotemp Fisher Scientific
Electrophoresis Tank and Current Supply	BIO RAD
Nanodrop 2000 Photospectrometer	Thermo Scientific

3.7.2) Protein Biochemistry Equipment

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	Mini-PROTEAN Tetra system (BioRad)
Western Device	iBind Western Device (Thermo Scientific)
Blotting Chamber	Trans-Blot Turbo Transfer System (BioRad)
Infrared Western Blot Scanning System	Infrared: Odyssey Infrared Imaging System (LI-COR Biosciences)
Blotting Membrane	Nitrocellulose Starter Kit (BioRad)
Protein Purification System	QIAcube System (Qiagen)

3.7.3) Cell Culture Equipment

Water Bath	VWR
Fume Hood	1300 Series A2 (Thermo Scientific)
Light Microscope	CKX41 (Olympus)
Confocal Microscope	LSM 700 (Zeiss)
Spinning Disk Confocal	Observer Z1 Spinning Disk Confocal Microscope (Zeiss)
Incubator	Formula Steri-Cycle CO ₂ Incubator (Thermo Scientific)
Hemocytometer	La fontaine

3.8) Software

Image Processing	ImageJ, Adobe Photoshop and Illustrator 2020,
Primer Design and PCR Analysis	NCBI/Primer-BLAST
Analyses of DNA Sequences and Sequence Data	SnapGene Viewer
Restriction Analyses	EnzymeX
Microscope Image Software	ZEN 2010, ZEN Blue, (Carl Zeiss Microscopy)
Statistical Analysis	IBM SPSS Statistics 24, Prism 8 (GraphPad), R-statistics packages

CHAPTER FOUR: Structural and Functional Consequences of Connexin 36 (Cx36) Interaction with Calmodulin

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Author Contributions

Ryan C.F. Siu have performed and analyzed all experiments pertaining to the quantification of GJPs (**Figure 4.3.1**), colocalization studies (**Figure 4.3.2**), FRET analyses (**Figure 4.3.3 & Figure 4.3.4**), gapFRAP (**Figure 4.3.5**), 3D representative images (**Supplementary Figure 4.5.1, Supplementary Figure 4.5.2 & Supplementary Figure 4.5.5**), western blot analysis (**Supplementary Figure 4.5.3**), and 2D colocalization (**Supplementary Figure 4.5.4**). Ryan was involved in preparing and finalizing the manuscript.

4.1) Abstract

Functional plasticity of neuronal gap junctions involves the interaction of the neuronal connexin36 with calcium/calmodulin-dependent kinase II. The important relationship between Cx36 and CaMKII must also be considered in the context of another protein partner, Ca²⁺ loaded calmodulin, binding an overlapping site in the carboxy-terminus of Cx36. We demonstrate that CaM and CaMKII binding to Cx36 is calcium-dependent, with Cx36 able to engage with CaM outside of the gap junction plaque. Furthermore, Ca²⁺ loaded calmodulin activates Cx36 channels, which is different to other connexins. The NMR solution structure demonstrates that CaM binds Cx36 in its characteristic compact state with major hydrophobic contributions arising from W277 at anchor position 1 and V284 at position 8 of Cx36. Our results establish Cx36 as a hub binding Ca²⁺ loaded CaM and they identify this interaction as a critical step with implications for functions preceding the initiation of CaMKII mediated plasticity at electrical synapses.

4.2) Introduction

Electrical and chemical synapses can form mixed synapses conferring synchronous activity among coupled neurons in the vertebrate nervous system (Hamzei-Sichani et al. 2012; J. I. Nagy et al. 2013; Rubio and Nagy 2015). This enables synchronous oscillations that temporally correlate firing patterns in otherwise heterogeneous networks (Galarreta and Hestrin 1999; Deans et al. 2001; M. V. L. Bennett and Zukin 2004; Vervaeke et al. 2010; Haas, Zavala, and Landisman 2011; Postma et al. 2011). In many regions of the nervous system, synchronous rhythmic oscillations serve as a mechanism to entrain the activity of distributed neuronal networks, and promote processes related to memory formation and retrieval, sensory perception, motor control, attention and plasticity (Deans et al. 2001; Hormuzdi et al. 2004; Van Der Giessen et al. 2008; Alberto E Pereda 2014).

Neurons express connexin36 (Cx36) and form gap junctions, which are the molecular substrate of electrical synapses. Functional plasticity of neuronal gap junctions in goldfish Mauthner cells (A E Pereda et al. 1998; A. Pereda, O'Brien, Nagy, Smith, et al. 2003; Alberto E. Pereda et al. 2004; Flores et al. 2010), and in both teleost and rodent retina (H. Li, Chuang, and O'Brien 2009; Cachope and Pereda 2012; Kothmann et al. 2012) involves interaction of Cx36 with the calcium/calmodulin-dependent kinase (CaMKII). In this process, CaMKII binds Cx36 in a manner analogous to binding of this kinase to the NR2B subunit of the NMDA receptor and phosphorylates the connexin protein both *in situ* and *in vitro* (Alev et al. 2008). Blocking of Cx36-CaMKII binding and phosphorylation by decoy inhibition, and deletion of CaMKII binding sites, as well as inhibition of phosphorylation sites through site-directed mutagenesis and peptide inhibition, leads to a significant loss of the “run up” activity, the term coined by us to describe the plasticity of the process (Zoidl et al. 2002; Del Corssio et al. 2012).

Interaction of Cx36 with CaMKII endows electrical synapses with functional plasticity but is in competition with binding of calmodulin (CaM), another multifunctional Ca^{2+} signaling protein (Burgoyne 2007), to a region overlapping with the CaMKII binding motif (Burr et al. 2005). At chemical synapses both CaM and the NMDA receptor subunit NR2B synergize to lock CaMKII in an active conformation conferring calcium-dependent plasticity (Bayer et al. 2001; Lisman, Schulman, and Cline 2002; Bhattacharyya et al. 2016). A similar mechanism involving Cx36, CaM and CaMKII appears unlikely due to the overlapping binding site. Addressing this conflict, we have proposed a model predicting sequential, competitive processing of interaction between CaM, CaMKII and Cx36 (Alev et al. 2008). To resolve this conflict, the neuroblastoma cell line Neuro2a

was used to interrogate how calcium signaling converges on Cx36 through CaM binding. Using Förster resonance energy transfer analysis (FRET), we demonstrate that formation of the Cx36-CaM binding complex is dependent on the capacity of CaM to bind calcium and strengthened when intracellular calcium concentrations rise. The formation of the Cx36-CaM complex precedes arrival of the Cx36 protein at the gap junction plaque (GJP) or is established after removal of Cx36 from the GJP. Functional dye transfer/dye recovery assays in cell pairs under physiological conditions showed that Cx36-CaM binding endows GJPs with the capacity to exchange a fluorescent dye between adjacent cells. Pharmacological blocking of CaM or mutation of the critical amino acid W277 within the core CaM binding motif of Cx36 significantly diminished this property. This observation led us to propose a revised model of calcium signaling where the localization of Cx36 in the cell affects the participants (CaM and/or CaMKII) and consequently, the outcomes arising from these interactions. This model is additionally supported by detailed structural and kinetic view of the Cx36-CaM interaction using a combination of the NMR and calorimetric approaches. In summary, this study demonstrates that priming of Cx36 by CaM represents a critical stage in the creation of plasticity at electrical synapses.

4.3) Results

4.3.1) Cx36-CaM at gap junctions in Neuro2a cells

Previous work (Burr et al. 2005) and the sequence alignment of Cx36 orthologs representing major vertebrate phyla ranging from primitive fish to humans suggested the existence of a highly conserved potential CaM binding motif in the carboxy-terminus of Cx36 resembling a variation of the 1-8-14 subclass. This motif overlapped with a binding site for the calcium-dependent calmodulin kinase II (**Figure 4.3.1A**) (Alev et al. 2008).

The role of W277 in position 1 of the putative CaM-binding site was investigated after sequential alanine-scanning mutagenesis of positions 276 to 278 of the rat Cx36 protein. Western blot analysis confirmed exogenous expression of Cx36 wild type and Cx36 mutants (G276A, W277A, and R278A) in single transfected Neuro2a cells 48 hrs post transfection (**Figure 4.3.1B**). A similar result was obtained with CaM and the Ca^{2+} insensitive mutant of CaM (CaM_{E1234Q} (Maunes, Kleeq, and Beckinghams 1992)) generated for this study (**Figure 4.3.1B**). The localization of Cx36, Cx36 mutants, and CaM was investigated in double-transfected cells 48 hrs post transfection. The formation of GJPs was observed in cells co-expressing CaM with wild-type Cx36 and mutant proteins (**Figure 4.3.1C**). A quantification of the GJP size revealed no significant differences in the GJP sizes by the mutations introduced (**Figure 4.3.1D**) (*values in μm^2* ; WT: 1.72 ± 0.20 , G276A: 1.22 ± 0.09 , $p=0.05$, W277A: 1.37 ± 0.11 , $p=0.19$, R278A: 1.13 ± 0.11 , $p=0.01$). The number of cells with GJP formation in transfected cells was significantly reduced for mutant W277A, but not when WT was compared to G276A and R278A (**Figure 4.3.1E**) (*values in %*; WT: 13.68 ± 2.52 , G276A: 11.24 ± 2.03 , $p=0.5$, W277A: 3.63 ± 0.72 , $p=1.9 \times 10^{-5}$, R278A: 8.69 ± 1.55 , $p=0.12$).

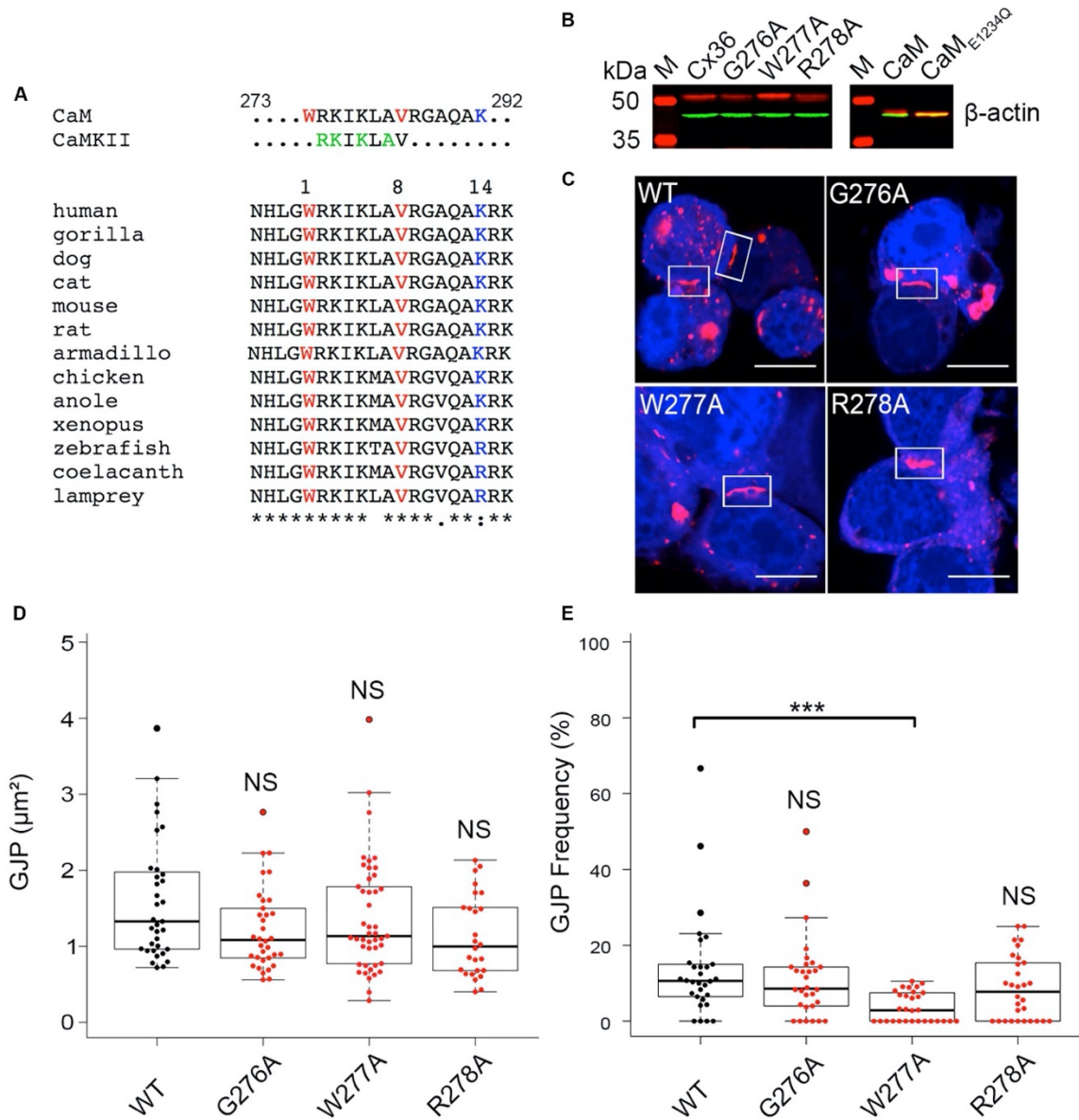


Figure 4.3.1 Expression, localization and gap junction plaque formation of Cx36 in Neuro2a cells. **A)** Sequence alignment of Cx36 orthologs from major vertebrate phyla showing a conserved CaM-binding domain overlapping with the CaMKII pseudosubstrate binding site. The 1-8-14 positions of the CaM-binding motif are highlighted. **B)** Western blot analysis of transiently transfected Neuro2a cells expressing Cx36 wild type, Cx36 mutants, CaM, and CaME_{1234Q}. Expressed proteins were detected using an anti-EGFP antibody (in red), and an anti-beta-actin antibody (in green) as the control. M indicated the protein standard. **C)** Rectangles indicate representative gap junction plaques in Neuro2a cells double transfected with CaM (blue), Cx36 (red) wild type, and mutant Cx36 proteins. Scale bar: 10μm. **D-E)** Quantification of gap junction size and frequency demonstrating that gap junction size is not affected by mutations of the core CaM binding region, but mutation W277A affects GJP frequency. Error bars box plot: maximum and minimum range with median; Mann-Whitney U test, *** p<0.001

4.3.2) Mutation W277A alters subcellular distribution and exchange between cell pairs

Two types of experiments were performed to clarify the distribution of Cx36 and W277A in Neuro2a cells. When single transfected Neuro2a cells expressing Cx36-EGFP or Cx36-DsRed monomer were paired and analyzed 48 hrs after transfection, 3D imaging of cell pairs showed mixed gap junction plaques (GJPs, see inset in Figure 2A and Supplementary View Figure 1), and limited presentation of Cx36 in mixed vesicles. Cell pairs expressing the W277A mutation showed mixed GJPs (see Supplementary Figure 2), and an extensive display of vesicular structures exchanged between cell pairs, suggesting that the mutation altered GJP stability (**Figure 4.3.2A**).

Next, the subcellular localization of Cx36 was visualized using Neuro2a cells double transfected with EGFP tagged Cx36 and genetically encoded cell organelle markers for endoplasmic reticulum (ER), Golgi, and mitochondria (**Figure 4.3.2B, Supplementary Figure 4.5.3**). Wild-type Cx36 was predominantly localized in vesicles and at GJPs. Outside of GJPs, both 2D images (data not shown) and 3D reconstructions of images from double transfected cells showed Cx36 in vesicles of variable size embedded in or attached to ER and Golgi (Supplementary Figure 4). Co-localization analysis revealed a significant overlap of Cx36 with ER (0.695 ± 0.0413 , $n=30$, $p=9.837 \times 10^{-6}$) and Golgi organelles (0.651 ± 0.0355 , $n=30$, $p=4.209 \times 10^{-5}$). The Mander's overlap coefficient for Cx36 and mitochondria was significantly lower (0.356 ± 0.0413 , $n=30$) demonstrating that exogenously expressed, tagged wild-type Cx36 is not redistributed into this organelle type (**Figure 4.3.2C, Supplementary Figure 4.5.5**). The distribution of Cx36 in cell pairs, together with the localization of tagged Cx36 in ER, Golgi and GJPs is consistent with the subcellular distribution of other connexin proteins. When Cx36-W277A was expressed, the Mander's overlap coefficient was significantly higher than wild-type Cx36 in all organelles (ER:

0.814±0.012, p=0.009; Golgi: 0.811±0.017, p=1.6 x10⁻⁵; mitochondria: 0.812±0.010, p= 2.22 x10⁻⁹). The distinct subcellular distribution of the W277A mutant suggests a mechanism that interferes with the transport, formation and/or stabilization of GJPs in transfected Neuro2a cells.

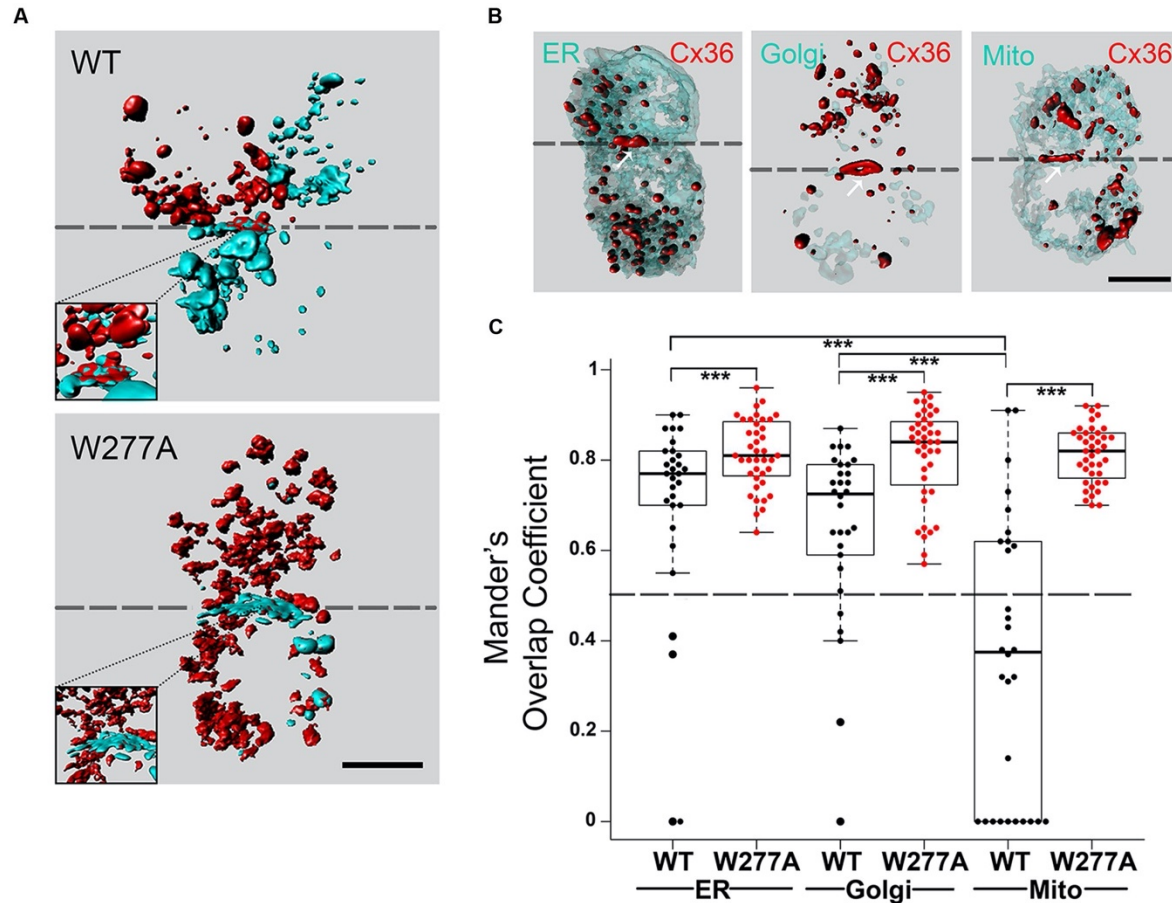


Figure 4.3.2 Colocalization of Cx36 with organelle markers indicating interaction occurring as early as at the endoplasmic reticulum. **A)** Representative 3D images of Neuro2a cells expressing wild-type Cx36 or the W277A mutant. Note that cell pairs derived from single independent transfections tagging the proteins of interest with EGFP or DsRed monomer. The increased presentation of mixed red and turquoise vesicles in W277A transfected cell pairs suggests reduced GJP stability and increased protein turnover. Insets represent magnifications of the GJP region. **B)** Neuro2a cells expressing wild-type Cx36-ECFP (in red) and DsRed monomer tagged organelle markers (endoplasmic reticulum, Golgi apparatus, and mitochondria, in light turquoise). The localization of Cx36 plaques is indicated with white arrows. **C)** Mander's overlap coefficients calculated with the ZEN 2010 program for Cx36 WT (n=30) and W277A (n=40) with the different organelle markers are shown with error bars indicating the maximum and minimum range. 3D images in A and B were created with Bitplane Imaris 7.6.4. Scale bars in A and B: 10 μ m. Error bars box plot: maximum and minimum range with median; Mann–Whitney U test, *** p<0.01.

4.3.3) Cx36-CaM binding is strengthened by elevated intracellular calcium at intracellular vesicles

The interaction of Cx36-DsRed with CaM-ECFP in living cells was investigated using Förster resonance Energy Transfer (FRET) (**Figure 4.3.3**). The Cx36-ECFP and Cx36-DsRed assembly into mixed GJPs (see **Figure 4.3.2A**) served as a positive FRET control. The FRET efficiency value (FRET_{eff} expressed in %) was 11.50 ± 0.93 . The interaction of Cx36 with the Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) has been shown previously (Alev et al. 2008; Del Corosso et al. 2012). Here, Cx36-ECFP and CaMKII-EYFP served as the second positive control demonstrating that intracellular calcium elevation by treatment with 2mM extracellular calcium $[\text{Ca}^{2+}]_{\text{E}}$ and 2 μM ionomycin increased the FRET_{eff} significantly at GJPs (values in %: non-stimulated: 5.1 ± 0.4 ; stimulated: 8.1 ± 0.5 , $p=0.0002$), but not at vesicular structures (non-stimulated: 6.7 ± 0.9 ; stimulated: 8.1 ± 0.9 , $p=0.33$) (**Figure 4.3.3A**).

The FRET_{eff} for Cx36-DsRed and CaM-ECFP was comparable at GJPs and in cytoplasmic vesicles under physiological conditions without calcium stimulation (GJP: 3.23 ± 0.26 ; vesicles: 3.65 ± 0.54 , $p=0.54$) (**Figure 4.3.3B**). When the fluorescent protein tags (FRET pair I: Cx36-DsRed, CaM-ECFP. FRET pair II: Cx36-ECFP, CaM-DsRed) were switched (GJP: 3.35 ± 0.49 , vesicles: 3.09 ± 0.40 , $p=0.82$) FRET_{eff} was not significantly different, suggesting that the protein tags had equal impact.

No significant changes to FRET_{eff} were observed at GJPs when intracellular calcium levels were raised (non-stimulated: 3.3 ± 0.5 , stimulated: 2mM $[\text{Ca}^{2+}]_{\text{E}}$ with 2 μM ionomycin; 2.8 ± 0.6 , $p=0.33$). Intracellular calcium reduction (24 μM BAPTA-AM; 2.8 ± 0.9 , $p=0.42$), or blocking of CaM with

10 μ M (W-7; 2.7 ± 0.6 , $p=0.44$) had no impact on FRET_{eff}. Co-transfection with the calcium insensitive CaM_{E1234Q} mutant (non-stimulated: 1.1 ± 0.3 ; stimulated: 1.3 ± 0.4 , $p=0.85$) reduced FRET_{eff} (**Figure 4.3.3C**). At vesicles, raising intracellular calcium ($[Ca^{2+}]_i$) levels significantly increased FRET_{eff} (non-stimulated: 3.10 ± 0.4 , stimulated: 6.38 ± 0.71 , $p=0.002$), and led to a significant reduction after BAPTA-AM (0.30 ± 0.30 , $p=0.9 \times 10^{-4}$), or W-7 application (0.88 ± 0.56 , $p=3.1 \times 10^{-5}$) (**Figure 4.3.3D**). Similar to our measurements at the GJP, CaM_{E1234Q} at the vesicles showed a significantly reduced response upon $[Ca^{2+}]_i$ increase (non-stimulated: 2.18 ± 0.54 , stimulated: 0.45 ± 0.27 , $n=21$, $p=0.005$) (**Figure 4.3.3D**).

Next, FRET-Distance (FRET_{dis}) for Cx36-CFP and CaM-DsRed was compared at GJPs and the vesicles. Typically, if two interacting proteins are within 10nm distance, FRET can be achieved (threshold indicated by the dashed line). No significant difference at the GJPs (non-stimulated: 9.11 ± 0.24 , stimulated: 9.61 ± 0.33 , $p=0.33$), but a significant reduction in FRET_{dis} was observed at cytoplasmic vesicles when $[Ca^{2+}]_i$ was increased, (non-stimulated: 9.27 ± 0.22 , stimulated: 8.09 ± 0.19 , $p=0.002$). When CaM_{E1234Q} was used to test the interaction with Cx36 WT-CFP, neither at the GJP (non-stimulated: 10.93 ± 0.63 , stimulated: 10.13 ± 0.53 , $p=0.21$) nor the vesicles (non-stimulated: 9.96 ± 0.58 , stimulated: 10.81 ± 0.46 , $p=0.23$) was significantly different (**Figure 4.3.3E**). All values were below 10nm except for CaM_{E1234Q}, which indicated FRET was not achieved between wild-type Cx36 and CaM_{E1234A}. We concluded that changes in $[Ca^{2+}]_i$ are relayed to Cx36 before reaching GJPs, and that the interaction between Cx36 and CaM is critically dependent on the capacity of CaM to bind calcium.

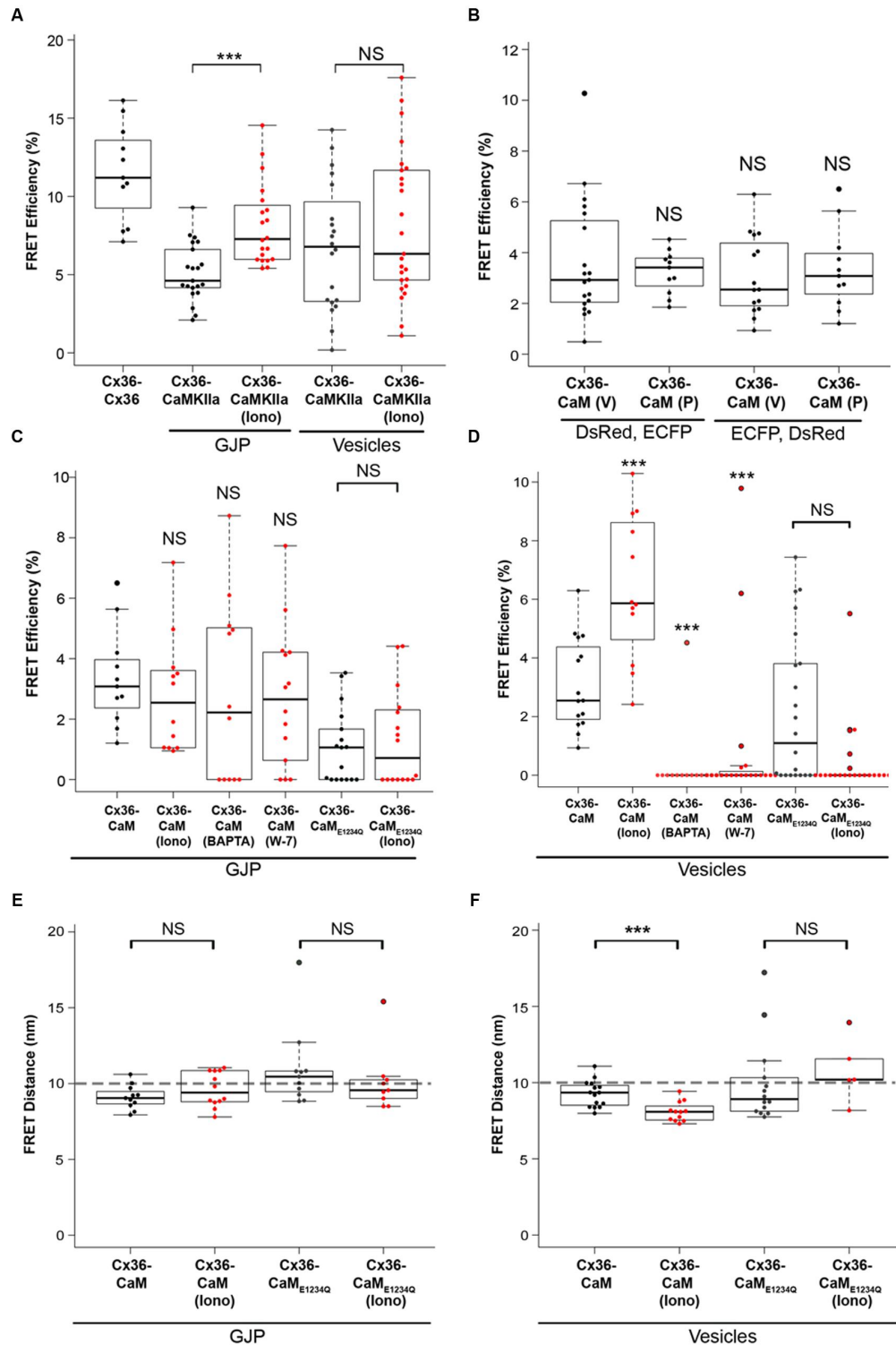


Figure 4.3.3 FRET analysis of Cx36 and CaM interaction. Neuro2a cells were transiently co-transfected, and FRET efficiencies (FRET_{eff}) determined 48hrs post transfection. **A)** FRET_{eff} of Neuro2a cells expressing active FRET control pairs. **B)** DsRed and ECFP protein tags are interchangeable and with minimal impact on FRET_{eff} . **C)** and **D)** FRET_{eff} at GJPs and vesicles

without and with 2mM extracellular calcium $[Ca^{2+}]_E$ and 2 μ M ionomycin in the presence of the pharmacological calmodulin blocker W-7 (10 μ M), the calcium chelator BAPTA-AM (24 μ M), or the Ca^{2+} insensitive CaM_{E1234Q} mutant. **E)** and **F)** FRET distance (FRET_{dis}) highlighting that calcium-dependent binding of Cx36 and CaM is significantly shortened at vesicles. Abbreviations: Iono, treatment with 2mM $[Ca^{2+}]_E$ and 2 μ M ionomycin. V = vesicles. GJP = Gap junction plaque. Error bars box plot: maximum and minimum range with median; *Mann–Whitney U test* significance, *** $p < 0.01$, NS = not significant.

4.3.4) W277 is critical for calcium dependent Cx36-CaM binding

The role of the amino acid tryptophan in position 277 (W277) of Cx36 was investigated in calcium-dependent CaM binding together with the flanking amino acids 276 and 278. FRET_{eff} was determined for tagged Cx36 wild-type and Cx36 mutants in vesicles at physiological conditions, and after increasing $[Ca^{2+}]_I$ by stimulation with 2mM $[Ca^{2+}]_E$ and 2 μ M ionomycin. The FRET pair Cx36-ECFP and Cx36-DsRed served as the positive control (**Figure 4.3.4**). FRET_{eff} significantly increased in Neuro2a cells co-expressing CaM-DsRed and wild-type Cx36-ECFP (all values in %; non-stimulated: 3.65 ± 0.54 , stimulated: 8.82 ± 1.19 , $p = 0.0003$), the Cx36 mutants G276A (non-stimulated: 6.61 ± 0.63 , stimulated: 9.74 ± 1.02 , $p = 0.02$), or R278A (non-stimulated: 2.84 ± 0.29 , $n = 15$; stimulated: 6.54 ± 1.02 , $n = 17$, $p = 0.003$) (**Figure 4.3.4A**). In contrast, FRET_{eff} for W277A and CaM-DsRed were unaffected by the intracellular Ca^{2+} change (non-stimulated: 3.18 ± 0.39 , $n = 18$; stimulated: 2.90 ± 0.69 , $p = 0.45$).

This result was consistent with changes to the FRET distances (FRET_{dis}) between tagged Cx36 and CaM after stimulation with 2mM $[Ca^{2+}]_E$ and 2 μ M ionomycin. Wild-type Cx36 (all values in nm; non-stimulated: 9.15 ± 0.26 , stimulated: 7.69 ± 0.22 , $p = 0.0003$), mutant G276A (non-stimulated: 8.16 ± 0.23 , stimulated: 7.48 ± 0.15 , $p = 0.02$), or mutant R278A (non-stimulated: 9.33 ± 0.19 , stimulated: 8.26 ± 0.24 , $p = 0.003$) showed a significant decrease in FRET_{dis}, indicating that binding

between both protein pairs increased (**Figure 4.3.4B**). In contrast, the FRET_{dis} for the Cx36 mutant W277A and CaM increased when intracellular Ca²⁺ was raised (non-stimulated: 9.22±0.20, stimulated: 10.61nm±0.91, p=0.45). In summary, the FRET analysis suggested that tryptophan 277 is critical for CaM binding and that binding is sensitive to intracellular Ca²⁺ levels.

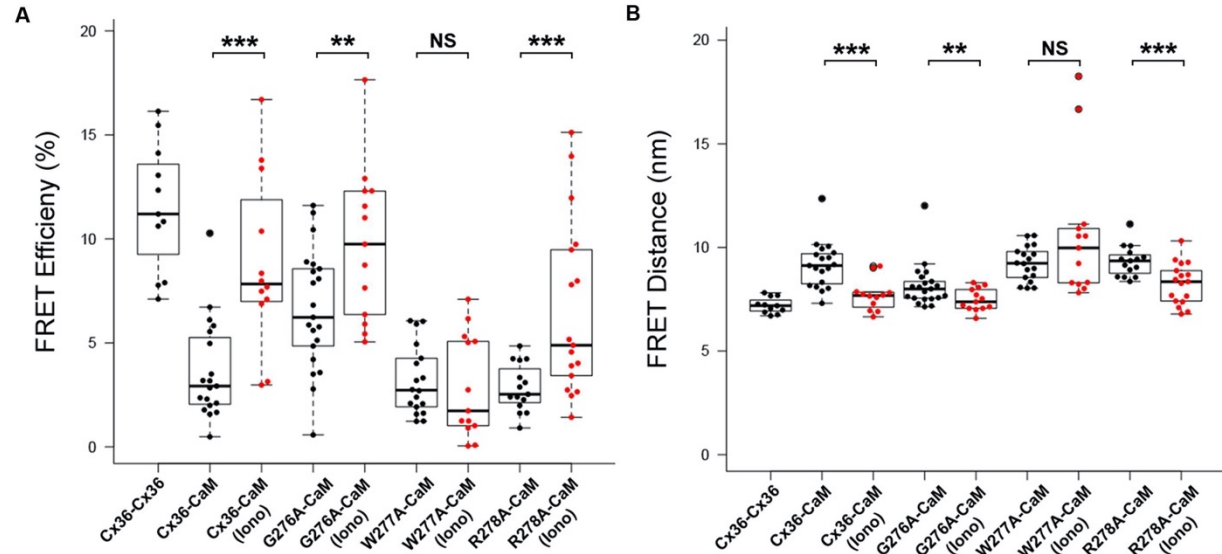


Figure 4.3.4 Alanine scanning mutagenesis reveals W277 as the critical site for Cx36-CaM binding. **A)** FRET_{eff} at vesicles in Neuro2a cells. Cells expressed fluorescently tagged Cx36-ECFP and Cx36-DsRed monomer as the control. Wild-type Cx36-DsRed monomer and Cx36 mutants (G276A, W277A, R278A) in the presence of CaM were stimulated with 2μM of ionomycin for 10 minutes. **B)** FRET distance with the same parameters as FRET efficiency. The dashed line indicates the FRET distance threshold at 10nm. Error bars box plot: maximum and minimum range with median; *Mann–Whitney U test*, **p<0.05, ***p<0.01)

4.3.5) Reduced dye-redistribution in Neuro2a cells expressing W277A

The W277A mutation decreased the GJP frequency, but not the GJP size (see **Figure 4.3.1D,E**), and caused a reduced response to [Ca²⁺]_i elevation (**Figure 4.3.4A,B**). To investigate impaired gap junction communication prompted by the W277A mutation, the non-invasive uptake and redistribution of ethidium bromide across GJPs under normal cell growth conditions was quantified using a method based on gap-FRAP (Abbaci et al. 2008). Pairs of Neuro2a cells expressing Cx36-ECFP or W277A-ECFP and forming GJPs were selected for imaging (**Figure**

4.3.5A-D, left column). Sample traces for each experimental condition show the fluorescence in regions of interest (R1, red trace; R2, black trace) over the entire time course (**Figure 4.3.5A-D, right column**). Regions of interest were placed close to the GJP (R1) and at a more distant location (R2) in the same cell to capture recovery of ethidium bromide after photobleaching. Quantifications shown below (**Figure 4.3.5E**) demonstrated that treatment with 2mM $[Ca^{2+}]_E$ and 2 μ M ionomycin caused a significant ethidium bromide fluorescence recovery in Neuro2a cells expressing the Cx36-ECFP protein (values in %: non-stimulated: 5.82 ± 4.22 , stimulated: 47.54 ± 13.56 , $p=0.0045$). Blocking of CaM with W-7 led to a significant reduction of fluorescence recovery when intracellular calcium levels were increased suggesting that CaM binding is critical for this response (non-stimulated: 16.60 ± 8.01 , stimulated: 3.77 ± 3.41 , $p=0.1427$). Treatment with the gap junction blocker carbenoxolone (CBX) modulated fluorescence recovery (non-stimulated: -8.66 ± 4.65 , stimulated: 8.91 ± 1.89 , $p=0.0032$). When Neuro2a cells expressed Cx36-W277A, no significant recovery was seen after the same treatment (non-stimulated: 8.12 ± 4.21 , stimulated: -0.55 ± 8.35 , $p=0.425$). It was concluded that W277A expression impairs dye redistribution between cell pairs in a way similar to a gap junction blocker. Control experiments using non-transfected Neuro2a cells with and without ionomycin treatment showed limited recovery (Neuro2a without ionomycin treatment: -15.72 ± 17.52 , Neuro2a with ionomycin treatment: 3.40 ± 1.23 , $p=0.001$). The recovery observed after CBX or ionomycin treatment likely reflects alterations to the biophysical properties of the cell membranes caused by the compounds applied.

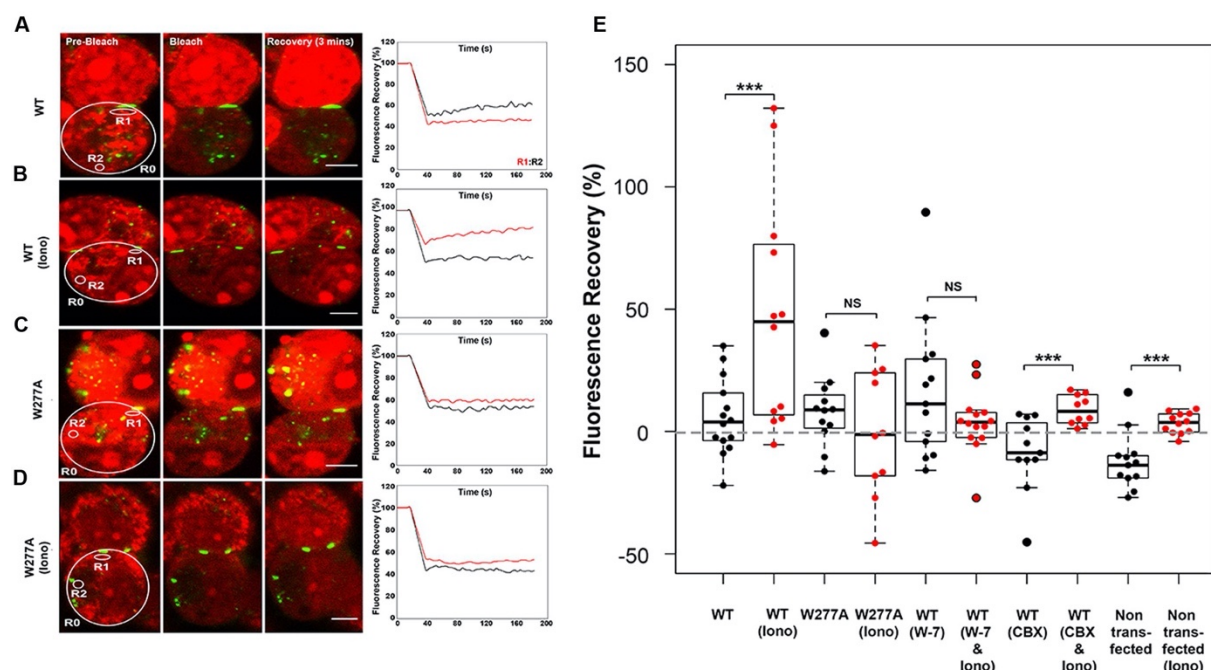


Figure 4.3.5 Ethidium bromide dye uptake and redistribution after photobleaching. A-D) show cell pairs expressing Cx36 wild-type proteins or the W277A mutant. The left column highlights examples for each treatment condition, with the cells selected for photobleaching encircled (large circles) and cell positions of regions of interest indicated (small circles; R1, R2, and R0 = background). The right column shows traces representing fluorescence over time for regions of interests. Scale bars: 10μm **E)** Quantification of fluorescence recovery in regions of interest. Treatments: Iono (2mM $[Ca^{2+}]_E$ and 2μM ionomycin), W-7 (10μM W-7, calmodulin inhibitor), CBX (50μM carbenoxolone, gap junction blocker). Error bars display the maximum and minimum range. NS, not significant. *Paired T-test*, p-values ** < 0.01, *** < 0.005.

4.3.6) A high-resolution solution structure of Cx36 and CaM

Following a strategy used successfully in earlier structure determination (Ogura et al. 2012), a single-chain protein hybrid consisting of human CaM, a short glycine-rich linker, and the CaM binding region of rat Cx36 (**Figure 4.3.6A**) was expressed and uniformly ^{13}C , ^{15}N labeled for NMR spectroscopy. Amide 1H , ^{15}N HSQC spectra of this CaM-Cx36 hybrid protein (Supplementary figure 6) and a sample of ^{15}N -labeled CaM in a 1:1 complex with a wild type 25 amino acid long Cx36 carboxy-terminal tail peptide were similar suggesting that the structure of the CaM-Cx36 hybrid was an accurate representation of a binary Cx36-CaM interaction.

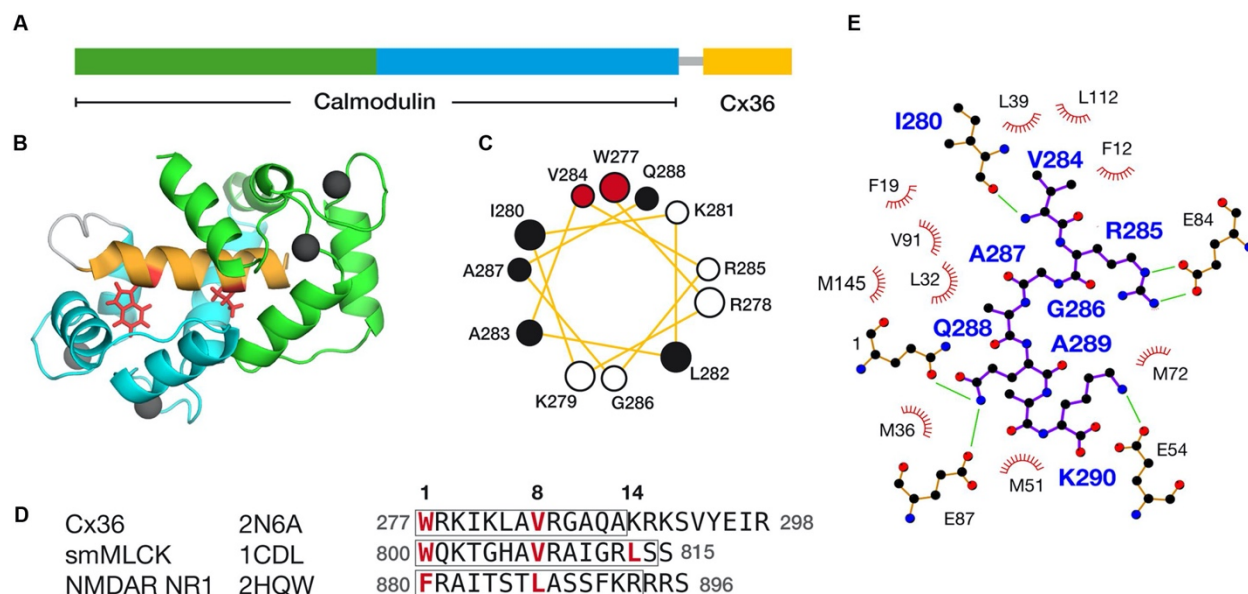


Figure 4.3.6 Structural features of the Cx36-CaM complex. **A)** A schematic representation of the hybrid Cx36-CaM protein used for the NMR structural determination. The N- and C-terminal lobes are colored for reference, followed by a short linker leading into a region in the carboxy-terminal cytoplasmic tail of Cx36. **B)** Cartoon representation of the lowest energy model in the ensemble of twenty solutions. Four calcium ions are depicted as balls and other features colored consistently with previous panels. **C)** Helical wheel representation of Cx36 bound to CaM as determined by experimental observations. W277 and V284 at anchor positions 1 and 8 are colored red and other hydrophobic amino acids colored black. **D)** Sequence alignment of Cx36, smooth muscle myosin light chain kinase (smMLCK), and NMDA receptor NR1 (NMDAR NR1). Key contact sites between CaM and these ligands are made at positions 1, 8 and 14. Boxes indicate the helical amino acids in each ligand. The ligand may extend amino-terminally before anchor position 1 but for clarity, only amino acids from position 1 are shown. **E)** Ligand (Wallace, Laskowski, and Thornton 1995) of key interactions observed in the Cx36-CaM complex. Green lines denote hydrogen bonds and ionic interactions.

The Cx36-CaM hybrid protein structure was determined using 1100 distance restraints from ^{15}N - and ^{13}C -edited 3D-NOESY spectra, supplemented with a ^{12}C -filtered, ^{13}C -edited 2D-NOESY of a ^{13}C -CaM / ^{12}C -Cx36 peptide complex (**Table 4.3.1**). The ensemble of 20 lowest energies structures is shown in supplementary figure 7. From the representative structure of the Cx36-CaM complex, CaM binds Cx36 in a well-known compact form (**Figure 4.3.6B**) with both Ca^{2+} saturated lobes enveloping a helical ligand with amphipathic features (**Figure 4.3.6C**). Based on the spacing of hydrophobic amino acids with W277 at anchor position 1, Cx36 resembles a 1-8-

14 class ligand except that position 14 is occupied by K280 where a hydrophobic acid would typically be found. Accordingly, the Cx36-CaM protein structure compares favorably to other crystal structures of compact CaM complexes bearing 1-8-(14) ligands from smooth muscle myosin light chain kinase (Meador, Means, and Quiococho 1992) (smMLCK; PDB:1CDL, 1.63 Å) and NMDA receptor NR1 C1 region (Ataman et al. 2007) (NMDAR NR1, PDB:2HQW, 1.81 Å) (**Figure 4.3.6D**). A contact map of Cx36-CaM from position 8 onwards is shown in **Figure 4.3.6E**. The guanidino group of arginine at position 9 (R285 in Cx36) is balanced by ionic contacts from E84 in CaM. Likewise, the hydrophobic amino acid position 11 (A287 in Cx36) is supported by F19, L32 and V91 in CaM. At the penultimate contact position 14, the helix of Cx36 terminates earlier than smMLCK and NMDAR NR1 ligands. Despite this difference, three methionines in CaM (M36, M51, and M72) still contribute important hydrophobic contacts to Cx36. A lysine at position 14 in Cx36 is protruding from the CaM binding cleft toward a series of acidic amino acids in CaM (E47, D50 and E54). Taken together, a continuous network of contacts is observed between CaM and Cx36 thereby establishing Cx36 as bona fide ligand of CaM.

Table 4.3.1: Statistics for the ensemble of 20 structures of the CaM-Cx36 hybrid protein

NOE restraints		
Total		1275
Intraresidue		432
Sequential		303
Medium range		269
Long range		271
Additional Restraints		
H-bond (H–O/N–O pairs)		58
Dihedral angle (ϕ/ψ pairs)		154
Ca ²⁺ upper/lower restraint pairs		48
RMS violations		
NOE restraints (Å)		0.0223 ± 0.020
Dihedral angles (°)		0.77 ± 0.20
van der Waals clashes		5.9 ± 0.2
Ramachandran plot (%)		
Most favored regions		83.1
Additional regions		16.7
Generous regions		0.2
Disallowed regions		0.0
Structural precision RMSD		
Backbone atoms in ordered residues (Å)		0.63 ± 0.18
Heavy atoms in ordered residues (Å)		1.17 ± 0.14
<i>RMSDs for residues 11–18, 29–38, 46–55, 67–75, 82–92, 102–112, 118–126, 138–146, 156–168.</i>		

Finally, isothermal titration calorimetry (ITC) was performed to determine the affinity of CaM for a 25 amino acid long peptide derived from the Cx36 cytoplasmic tail (**Figure 4.3.7**). As predicted from the NMR structure, the Cx36 peptide bound Ca²⁺-CaM stoichiometrically with a K_d of 0.53 ± 0.05 μ M (n = 3). Highlighting the role of the amino acid W277 at the position 1 of the 1-8-(14) motif, no binding was observed for the peptide bearing a W277A substitution (n = 3). Consistent with the structure and biological assays described throughout this study, peptide binding was only observed with Ca²⁺ saturated CaM.

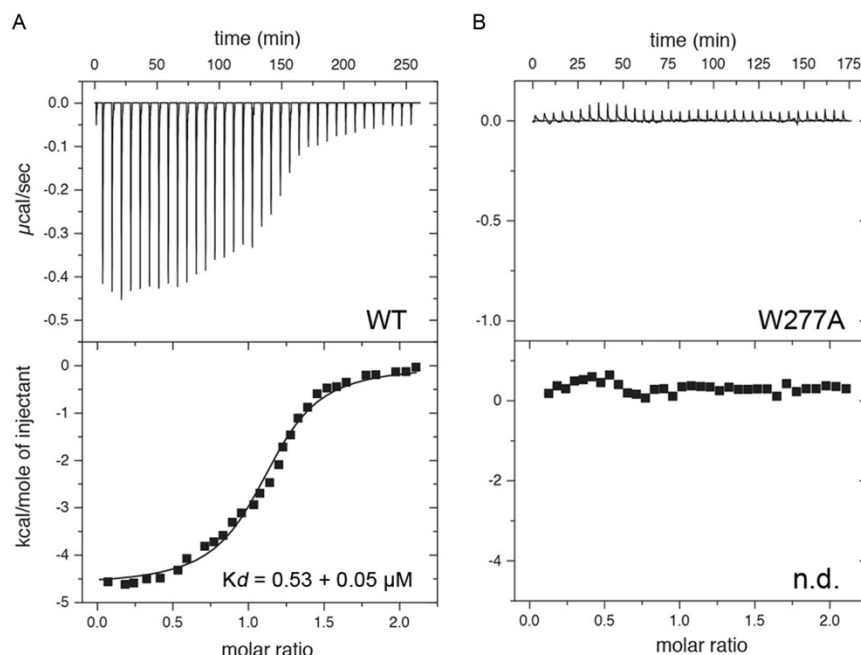


Figure 4.3.7 Isothermal titration calorimetry (ITC) of Cx36 peptides and CaM. Calorimetric traces and integrated isotherms acquired at 30°C for A) Calcium saturated CaM titrated with a Cx36 derived peptide, and B) Calcium saturated CaM titrated with the same peptide bearing a W277A substitution. No binding was detected between CaM and W277A mutant peptide. Abbreviations: K_d , dissociation constant; n.d., not detected.

4.4) Discussion

The interaction of the neuronal gap junction protein Cx36 with CaM was investigated in Neuro2a cells and the amino acid W277 identified as a critical residue in the CaM binding motif. The binding of Cx36 to CaM was demonstrated in intracellular vesicles and at GJPs, but only the interaction at vesicles showed a calcium-dependent increase, revealed by pharmacological blocking of CaM, manipulation of intracellular calcium or overexpression of a calcium insensitive CaM protein. Mutation W277A, as well as pharmacological blocking of CaM caused a significant reduction in dye transfer between cell pairs, implicating CaM binding to Cx36 in gap junction communication. *In vitro*, CaM binds Cx36 with a comparable affinity to other connexins with contacts that follow a 1-8-14 motif found in other CaM-binding proteins such as smMLCK, NMDAR-NR1 and MARCKS.

4.4.1) The Cx36-CaM binding motif is distinct from other connexins

The novel details of CaM binding to rat Cx36 build on the original findings using mouse Cx36 and two perch Cx35 variants (Burr et al. 2005). The Cx36 binding motif is highly conserved across species from humans to lower vertebrates, like lamprey and zebrafish, and overlapping with a CaMKII binding site (Alev et al. 2008). In contrast, the CaM binding sites of Cx35/Cx36 are distinct from other connexins including Cx32, Cx43, Cx44, Cx46, and Cx50 in terms of ligand class (1-8 versus 1-5-10), location of the ligand binding sites, and resemble to date the only connexins activated by CaM (Zhou et al. 2007; Dodd et al. 2008; Myllykoski, Kuczera, and Kursula 2009; Zhou et al. 2009; Y. Chen et al. 2011; Xu et al. 2012; Mruk et al. 2014).

The similarity of the Cx36 binding motifs across different species is reflected in comparable dissociation kinetics and thermodynamic characteristics found in mouse, the two perch Cx35 variants (Burr et al. 2005) and the rat Cx36 protein sequence investigated in this study. In more general terms, connexins bind CaM with dissociation kinetics and thermodynamic characteristics covering more than a 100-fold range (Dodd et al. 2008; Myllykoski, Kuczera, and Kursula 2009; Zhou et al. 2009; Y. Chen et al. 2011). Together, the specific localization of binding motifs, distinct subclasses and different dissociation kinetics suggest functional adaptations specific for each connexins protein.

The low affinity of Cx36 to CaM, at ~500nM, may have arisen due to the substitution of K290 at position 14 of typical 1-8-14 ligands like smMLCK, NMDAR-NR1, or MARCKS, which CaM encounters in the compact state. Furthermore, the less extensive array of hydrophobic contacts arising from the shorter side chains throughout Cx36, namely A283 and A287, cannot be

compensated by larger hydrophobic amino at position 14 (K290 in Cx36). Regardless of the precise structural basis, a lower affinity may help Cx36 quickly sample CaM, CaMKII and possibly other proteins, depending upon the numerous signaling contexts it participates in throughout the life cycle of this protein.

4.4.2) Calmodulin confers calcium signaling to Cx36 outside the GJP

Generally, CaM binding to connexins has been investigated in the context of gap junction communication, mainly focusing on implications for channel functions in the cell membrane. On the basis of the subcellular distribution of Cx36 and mutants thereof, we have expanded this focus, showing that binding already occurs before Cx36 reaches the cell membrane forming gap junctions, or after removal from the GJP. Interestingly, the W277A mutation caused retention in vesicles and showed a reduced gap junction coupling, reflecting in part similar observations made for Cx43 (Zhou et al. 2007). Further, gap junctions made by W277A are less frequent and more prone to removal as annular plaques found distributed in cell pairs, suggesting that decorating Cx36 with CaM is critical and most likely serving multiple purposes during the life cycle of this connexin.

4.4.3) Ménage a trios: competition or cooperation?

We have previously proposed a model predicting the sequential, cooperative processing of interaction between CaM, CaMKII and Cx36 at the GJP (Alev et al. 2008). The main results of this study are more consistent with a sequential and competitive process, sequestering calcium-dependent actions between vesicles and GJPs. To emphasize the new findings, we propose a revised model for plasticity at electrical synapses. As highlighted in **Figure 3.4.1**, both CaM and CaMKII have the capacity to bind Cx36 at vesicles, with CaMKII binding at physiological calcium levels, and CaM binding only when intracellular calcium is elevated. At GJPs, CaMKII is calcium-

responsive, with CaM showing no further strengthening of binding in response to calcium increase. This sequence of actions differs significantly from the activation of NMDA receptors (Bayer et al., 2001), and lead us to conclude that electrical synapses share a portfolio of interacting proteins, but mechanisms of achieving plasticity do not appear to fully recapitulate those found at chemical synapses. Instead, CaMKII binding at Cx36 at GJP seems to be the critical interaction conferring calcium-dependent plasticity at electrical synapses, while calcium-dependent sensitization of CaM appears restricted to spatially separate compartments. This could endow electrical synapses with a tunable molecular machinery using calcium as the main regulator for activation through CaMKII, while interaction of Cx36 with CaM open new avenues for linking this channel to intracellular calcium signaling pathways.

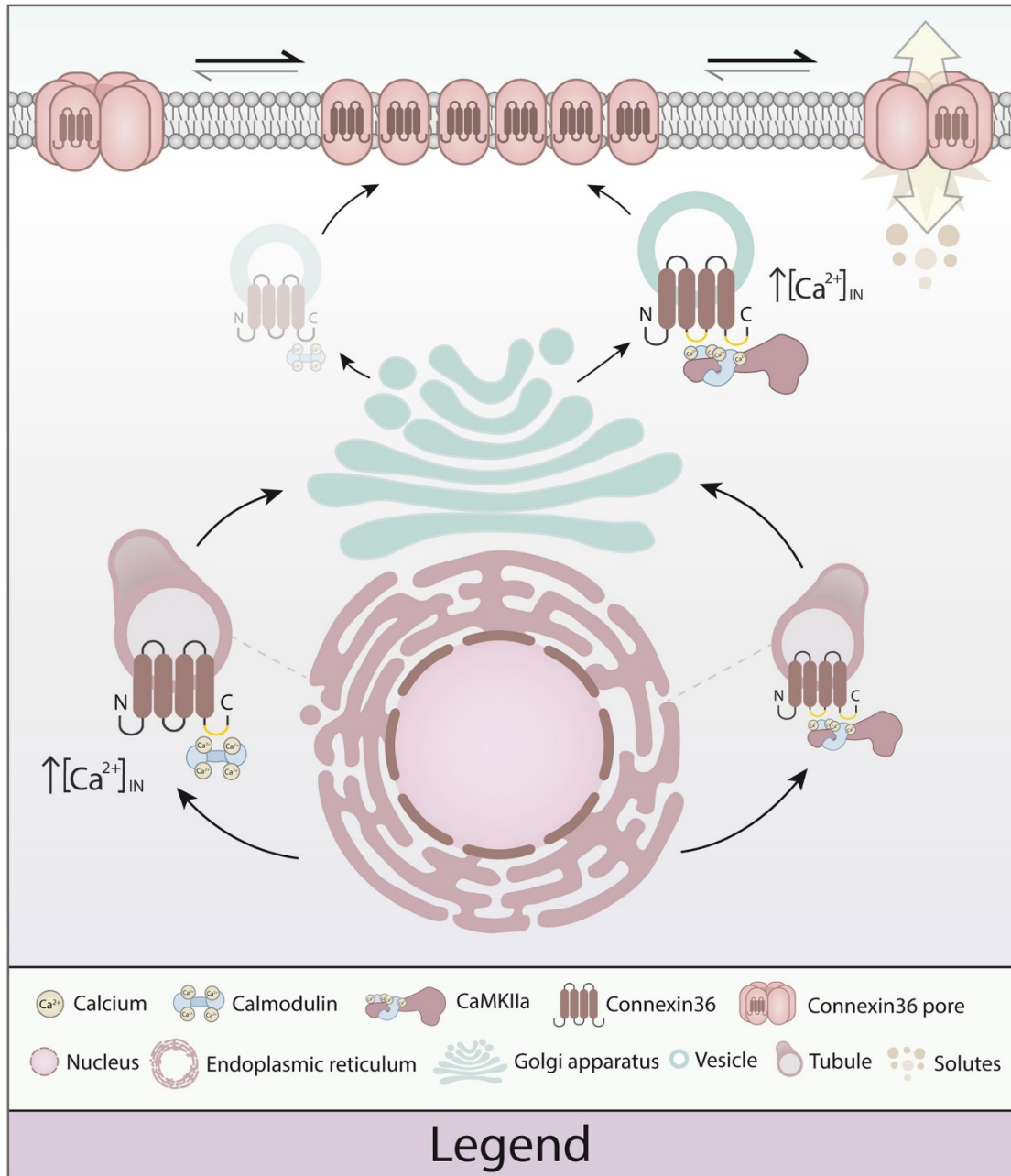
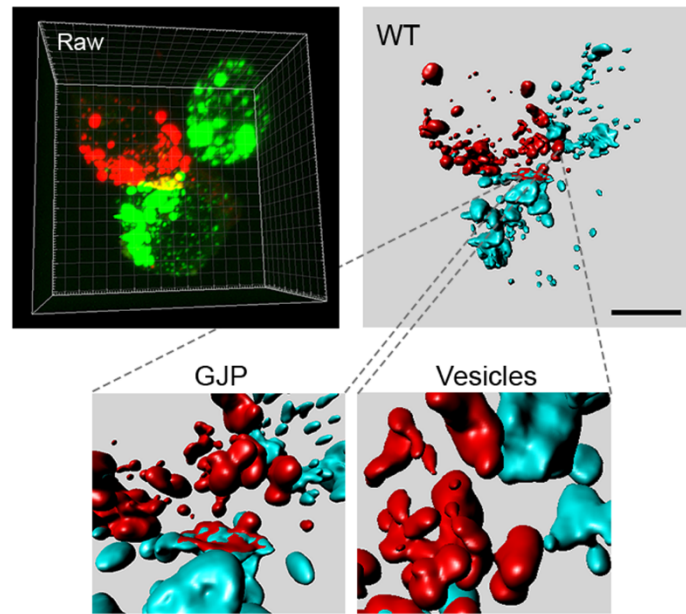


Figure 4.4.1: Model of calmodulin (CaM), Ca^{2+} /calmodulin dependent kinase 2a (CaMKII α) and connexin 36 (Cx36) in plasticity of the electrical synapse. Different to our original model, Cx36 can either bind calcium-activated CaM (right) or interact with CaMKII α (left) at the vesicular level. The initial binding of CaM and Cx36 is critically dependent on elevated calcium concentrations. As the Cx36-CaM loaded vesicles traffic to the cellular membrane, the response to intracellular calcium activation shifts towards CaMKII α interaction by calcium elevation. At the nexus, CaM serves as a kinase activator bound directly to CaMKII α , and CaMKII α plays an active part in modulating Cx36 nexus conductance (Illustration by Lesia Szyca, 2016).

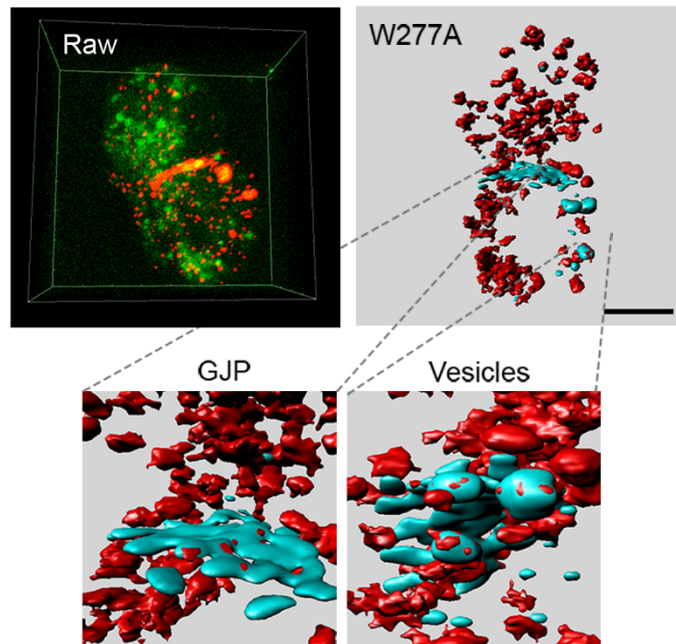
What could be the function(s) of CaM binding to Cx36? The work by Burr et al. (2005) and our data suggest that CaM binding to Cx36 will occur when $[Ca^{2+}]_{in}$ levels are elevated. Such conditions typically are found during synaptic activity in neurons (Gamble and Koch 1987). Since elevation of $[Ca^{2+}]_{in}$ increases Cx36-CaMKII, but not Cx36-CaM interaction at GJP, we conclude that calcium signals converge through CaM onto Cx36 before the protein reaches the GJP, ruling out a major role in plasticity.

It is reasonable to speculate that Cx36-CaM binding at vesicles may be sensitive to competition with other proteins with higher CaM affinity, suggesting that CaM binding could be orchestrated and/or sequestered by calcium affinities of CaM relative to CaMKII, or other, yet unidentified calcium binding proteins, or those activated or sensitized by calcium. Prior to its arrival at GJPs, Cx36-CaM could promote vesicular transport to the cell membrane and/or post-translational modifications affecting the oligomerization of connexins in the Golgi complex (Musil and Goodenough 1993), as well as the assembly or disassembly of Cx36 into GJPs, potentially in a way similar to glutamate receptor trafficking to dendritic spines (Korkotian and Segal 2007; Loo et al. 2014). Alternatively, calcium-dependent binding of CaM to Cx36 could link functional Cx36 channels in the ER directly to intracellular calcium signaling as suggested for Panx1, Panx3 or the transient receptor potential vanilloid 1 channel (TRPV1) (Vanden Abeele et al. 2006; Gallego-Sandin et al. 2009; Ishikawa et al. 2011) or by limiting calcium leakage from the ER as exemplified by CaM binding to Sec61 alpha (Erdmann et al. 2011). Addressing these consequences in future experiments will close this important knowledge gap.

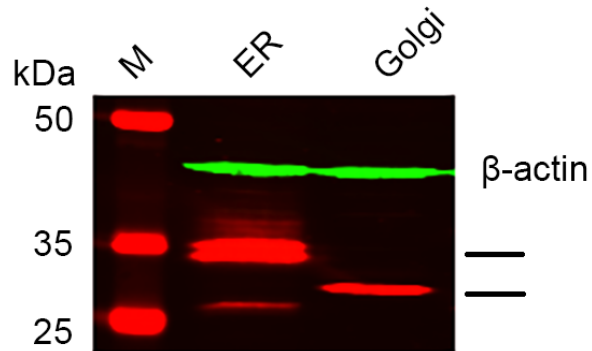
4.5) Supplementary Figures



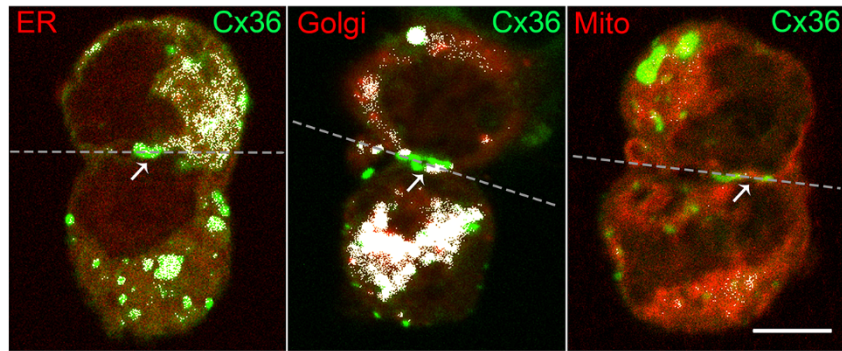
Supplementary Figure 4.5.1: Representative 3D images of Neuro2a cells expressing wild-type Cx36, tagged with EGFP, or DsRed monomer. The original projection based on the raw Z-stack before processing is shown top left. By rotating the 3D image and zooming into structural details of the Cx36 distribution it is possible to highlight the exchange of Cx36 proteins between adjacent cells connected by a gap junction plaque. Note that GJPs share a mixture of EGFP (in turquoise) and DsRed monomer (in red) tagged Cx36 proteins, whereas redistribution between cells as indicated by red and turquoise vesicles is low (Scale bar: 10 μ m).



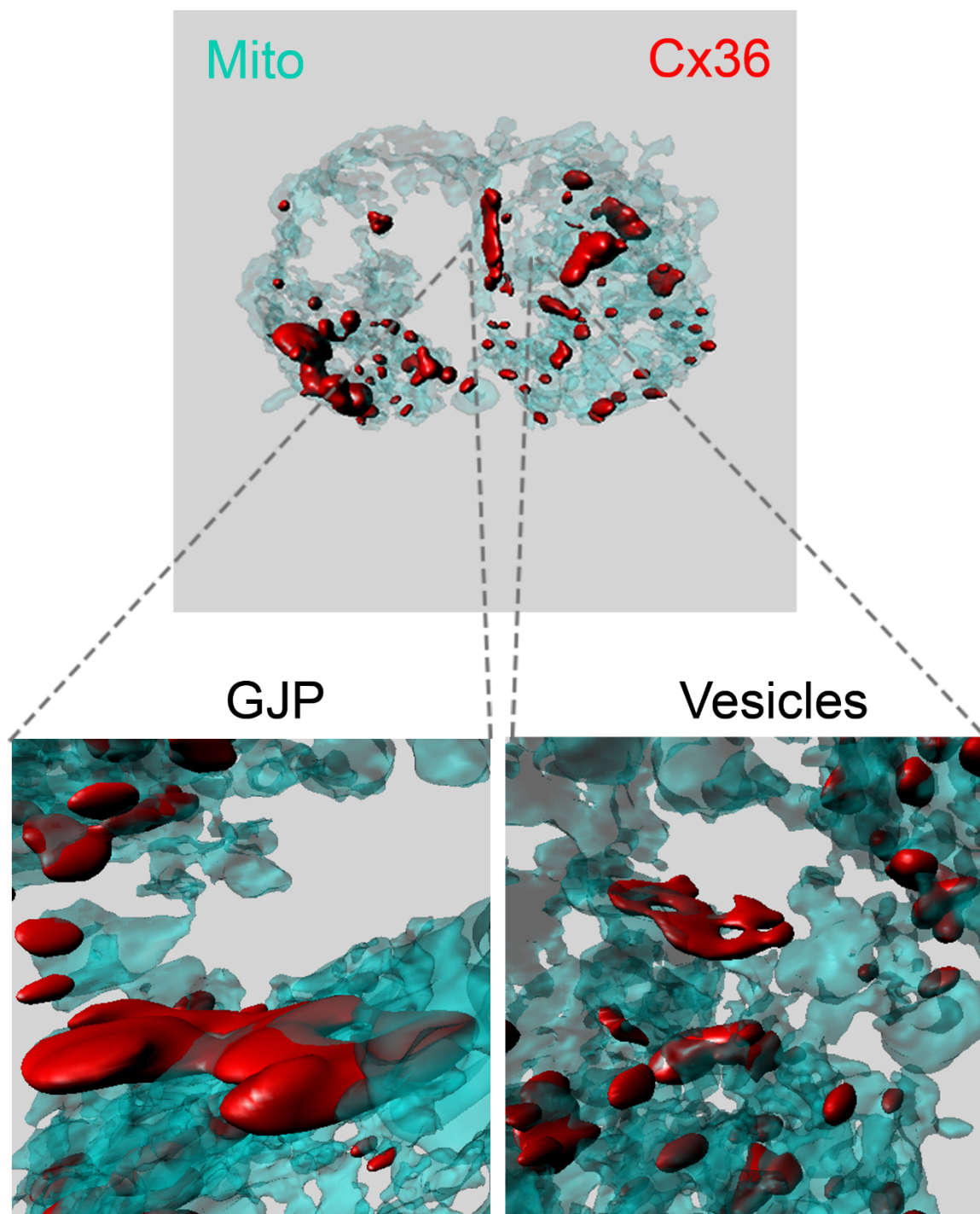
Supplementary Figure 4.5.2: Representative 3D images of Neuro2a cells expressing the Cx36 W277A mutant. The original projection based on the raw Z-stack before processing is shown top left. Note the mixed red and turquoise GJP and the increased redistribution of red and turquoise vesicles between adjacent cells indicating the reduced stability of the GJP and increase in protein turnover (Scale bar: 10 μ m).



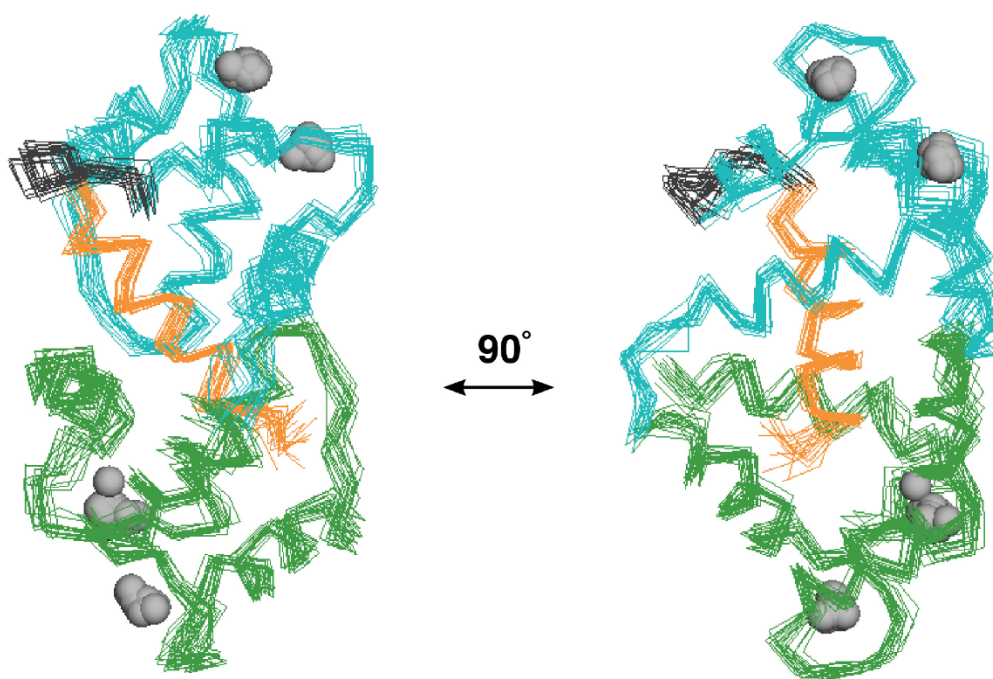
Supplementary Figure 4.5.3: Western blot detection of ER and Golgi tagged EGFP after transient expression in Neuro2a cells (in red, position indicated by lines). β -actin expression was used as internal protein standard.



Supplementary Figure 4.5.4: Representative 2D co-localization images of Neuro2a cells expressing wild-type EGFP tagged Cx36 and genetically encoded cell organelle markers for endoplasmic reticulum (ER), Golgi, and mitochondria Cx36. White areas indicate co-localization between Cx36 and organelle markers. (Scale bar: 10 μ m).



Supplementary Figure 4.5.5: Representative 3D images of Neuro2a cells expressing wild-type Cx36-ECFP (in red) and DsRed monomer tagged mitochondria (in light turquoise). This image is a typical example showing the lack of co-localization between Cx36 and mitochondria (Scale bar: 10 μ m).



Supplementary Figure 4.5.7: Superposition of the ensemble of twenty CaM-Cx36 NMR solution structures deposited in the Protein Data Bank (2N6A). The protein is presented as a $\text{C}\alpha$ ribbon (CaM lobe 1, green; CaM lobe 2, blue; linker, grey; Cx36, orange) and the calcium ions are presented as spheres.

CHAPTER FIVE: An NMDA receptor-pannexin1 signaling complex transforms the interaction of CaMKII with Connexin36

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Author Contributions

Ryan C.F. Siu have performed and analyzed all experiments pertaining to microscopy analysis (FRET, gapFRAP, protein localization, Arclight, Ca²⁺ imaging), cell culturing, site-directed mutagenesis, plasmid construction, TALEN KD of Panx1, and western blot analysis. These contributions attributed to all figures (**Figure 5.4.1 – Figure 5.4.7**) and supplementary figures (**Supplementary Figure 5.6.1 – Supplementary Figure 5.6.5**). Ryan was involved in preparing and finalizing the manuscript.

5.1) Graphical Abstract

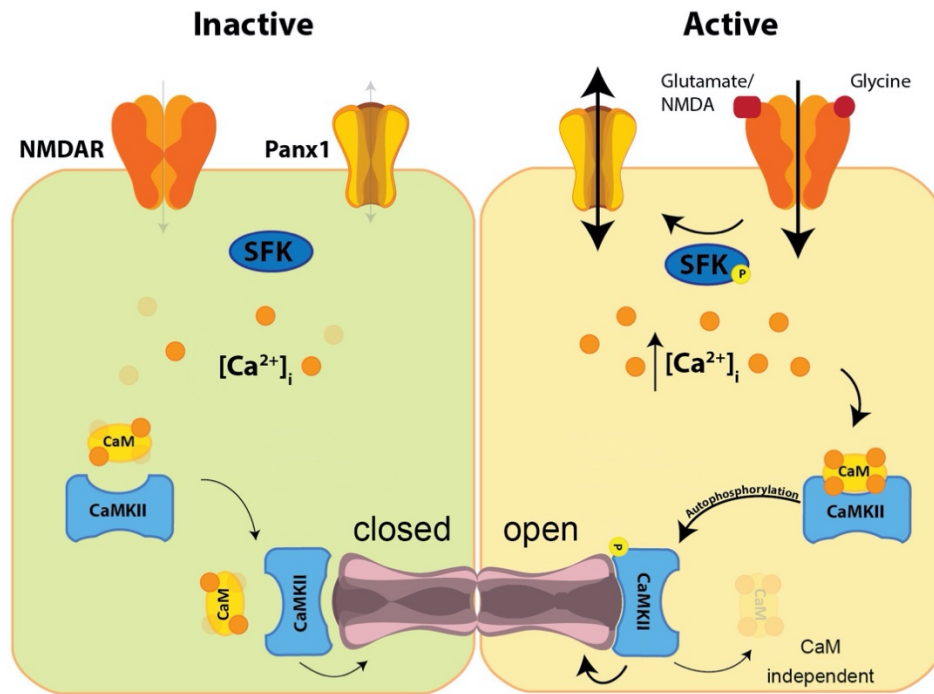


Figure 5.1: Schematic summary of NMDAR-Src-Panx1 signaling pathways leading to connexin 36 plasticity. (Left) Inactive synapse maintain a low intracellular Ca^{2+} level, which favours the closed state of connexin 36. (Right) Active synapses have NMDARs active and increases intracellular Ca^{2+} levels. Through increase SFK activities, Panx1 gets phosphorylated and further drives Ca^{2+} into the cell. The increased intracellular Ca^{2+} promotes CaM activities, which allows CaMKII to be CaM independent through autophosphorylation. The compact interaction of CaMKII with connexin 36 allows the channel to open.

5.2) Abstract

In the mammalian brain, electrical and chemical synapses exist in mixed synapses. How both types of synapses interact and share activity-dependent functional plasticity through molecular mechanisms involving calcium triggered interactions between N-methyl-D-aspartate receptors and Ca^{2+} /calmodulin-dependent protein kinase II is incompletely understood. Here, we

report the discovery of an ionotropic N-methyl-D-aspartate receptor - Src family kinase – pannexin1 signaling complex, which facilitates this interaction. The signaling complex was revealed by genetic and pharmacological interference with proteins' functions at multiple steps using fluorescent-tagged proteins expressed in a neuroblastoma cell line. Manipulations influencing the compactness of the connexin 36 - Ca^{2+} /calmodulin-dependent protein kinase II interaction complex were detected at nm-resolution using Förster resonance energy transfer. Functional dye coupling assays confirmed changes in the communication efficacy of connexin 36 channels. The data support a framework for asymmetric functionality of electrical synapses and molecular signaling between electrical and chemical synaptic plasticity.

5.3) Introduction

Electrical synapses in neurons coexist alongside chemical synapses in mixed synapses throughout the vertebrate nervous system (James I. Nagy, Pereda, and Rash 2018; James I Nagy, Pereda, and Rash 2019). In the mammalian brain, most electrical synapses are composed of connexin 36 (Cx36) gap junction channels (GJC) (Rash et al. 2013). Cx36 GJCs show functional plasticity similar to chemical synapses (Alcamí and Pereda 2019; Alberto E Pereda 2016), in which the interaction of Cx36 with Ca^{2+} -activated calmodulin (CaM) and calmodulin protein kinase II (CaMKII) is considered analogous to the interaction of ionotropic N-methyl-D-aspartate (NMDA) receptors with CaM/CaMKII (Alev et al. 2008).

We here describe the identification of a signaling complex in which activation of ionotropic N-methyl-D-aspartate (NMDA) receptors modulates the fundamental interaction between Cx36 and CaMKII. Individual steps in the signaling process include activation of the ATP-release channel

pannexin1 (Panx1) (Dahl 2015; Whyte-Fagundes and Zoidl 2018; Dahl 2018), Src-family kinases (SFKs) (Salter and Kalia 2004), and elevated intracellular calcium ($[Ca^{2+}]_i$). Förster resonance energy transfer (FRET) was used to identify steps at which signaling converges onto the Cx36-CaMKII interaction complex. This technology allowed the determination of the compactness of the three-dimensional gap junction plaque (GJP) superstructure at nm-resolution when the interaction between the dodecameric Cx36 GJC and the dodecameric CaMKII holoenzyme was tuned to activation or deactivation. Functional dye transfer assays and gap junction protein fluorescence recovery after photobleaching (gapFRAP) (Siu et al. 2016) were used to correlate functional changes with structural rearrangements in the protein complexes.

Results emphasize that the ionotropic NMDA receptor-mediated rise in intracellular calcium was an essential signal requirement to change the compactness of the Cx36-CaMKII complex and open Cx36 channels. Mutations in protein domains targeting the interaction between Cx36 and either CaM or CaMKII, and pharmacological interventions disrupting the NMDA receptor or CaMKII activation abolished intracellular calcium signaling opening of Cx36 channels located in gap junction plaques (GJPs). Inhibition of Src-family kinases (SFK), main actors in pathways leading to the generation of Ca^{2+} signals, and modulators of NMDA receptor (Salter and Kalia 2004), the CaMKII holoenzyme (Soderling and Derkach 2000), and Panx1 (Lohman et al. 2019), blocked Cx36 channel opening in this study. The genetic ablation of Panx1 was equally effective in closing Cx36 as was the blockade of NMDA receptors, SFK or CaMKII, an effect that was rescued by overexpression of Panx1. In primary neurons, a signaling complex involving ionotropic NMDA receptors and Panx1 is expected to be restricted to the postsynaptic cell membrane based on known protein localization data. We conclude that the asymmetric distribution of Panx1 and ionotropic

NMDA receptors provides flexibility for mixed chemical and electrical synapses to control synaptic outputs.

5.4) Results

5.4.1) Calcium modulates proximity among Cx36 channels in GJPs

Opening and closing Cx36 channels was expected to affect the space occupied in the three-dimensional crystal-like structure of GJPs (**Figure 5.4.1a**). Fluorescence resonance energy transfer (FRET) was used to compare the short-range structural changes between wildtype Cx36 with that of the CaM/CaMKII binding deficient mutant Cx36 R278A (Siu et al. 2016), in response to increased $[Ca^{2+}]_i$. At normal cell growth conditions, the FRET efficiency ($FRET_{eff}$) of EGFP-Cx36 and DsRed-Cx36 pairs were significant, suggesting proximity of the fluorescent protein tags when Cx36 channels were packed in GJPs. Elevation of intracellular Ca^{2+} by treatment with $2\mu M$ ionomycin in the presence of $2mM$ extracellular calcium $[Ca^{2+}]_E$ decreased $FRET_{eff}$, consistent with an increase in distance between the fluorescent protein tags corresponding to the expected opening of Cx36 GJ channels (values in %, non-stimulated: 10.3 ± 0.9 , $n=21$; ionomycin: 6.8 ± 0.6 , $n=20$, $p=7.0 \times 10^{-3}$). Co-expressed CaM/CaMKII binding deficient mutants EGFP-Cx36 R278A and DsRed-Cx36 R278A had low $FRET_{eff}$ regardless of Ca^{2+} stimulation (non-stimulated: 3.2 ± 0.5 , $n=20$; ionomycin: 1.6 ± 0.6 , $n=20$, $p=3.0 \times 10^{-3}$) (**Figure 5.4.1b,c**).

Next, we tested the functional coupling of wildtype Cx36 and the R278A mutant using gapFRAP (Siu et al. 2016; Timonina, Kotova, and Zoidl 2020a). Neuro2a cell pairs expressing Cx36-EGFP or Cx36-R278A-EGFP GJPs were selected to quantify the recovery of ethidium bromide fluorescence in the cytoplasm adjacent to GJPs after photobleaching one side of the channels

(**Figure 5.4.1c**). Neuro2a cells treated with ionomycin showed significant ethidium bromide fluorescence recovery in this region of interest only when expressing Cx36-EGFP (**Figure 5.4.1d**) (Fluorescence Recovery in %: non-stimulated Cx36-EGFP: 5.8 ± 3.0 , $n=20$; ionomycin: 29.6 ± 8.6 , $n=22$, $p=0.04$; non-stimulated Cx36 R278A: 11.5 ± 1.2 , $n=22$; ionomycin: 10.3 ± 2.4 , $n=24$, $p=0.08$).

Potential confounding factors, such as changes to GJP size and frequency caused by modulation of $[Ca^{2+}]_i$ were excluded. Both GJP size (**Figure 5.4.1e, f**) (all values in μm^2 ; wild-type non-stimulated: 3.6 ± 0.5 , $n=21$; ionomycin: 3.3 ± 0.3 , $n=19$, $p=0.90$) and GJP frequency (all values in %; WT: wild-type non-stimulated: 15.6 ± 2.5 , $n=20$; ionomycin: 11.8 ± 1.3 , $n=20$, $p=0.74$) showed no significant difference with elevated $[Ca]_i$. EGFP-Cx36 R278A and DsRed-Cx36 R278A mutant channels also formed GJPs and the frequency was unaffected by $[Ca^{2+}]_i$ stimulation (GJP size: all values in μm^2 ; R278A: non-stimulated: 1.6 ± 0.2 , $n=20$; ionomycin: 1.8 ± 0.2 , $n=20$, $p=0.75$; GJP frequency: R278A: non-stimulated: 16.3 ± 2.9 , $n=20$; ionomycin: 17.2 ± 3.5 , $n=20$, $p=0.75$). The GJP size of mutants was significantly smaller than wild-type Cx36, suggesting that loss of CaM/CaMKII binding affected Cx36 recruitment to GJPs. The results demonstrated proximity changes between Cx36 channels caused by increased $[Ca^{2+}]_i$, which require the CaM/CaMKII binding motif to converge calcium signals onto Cx36 GJCs.

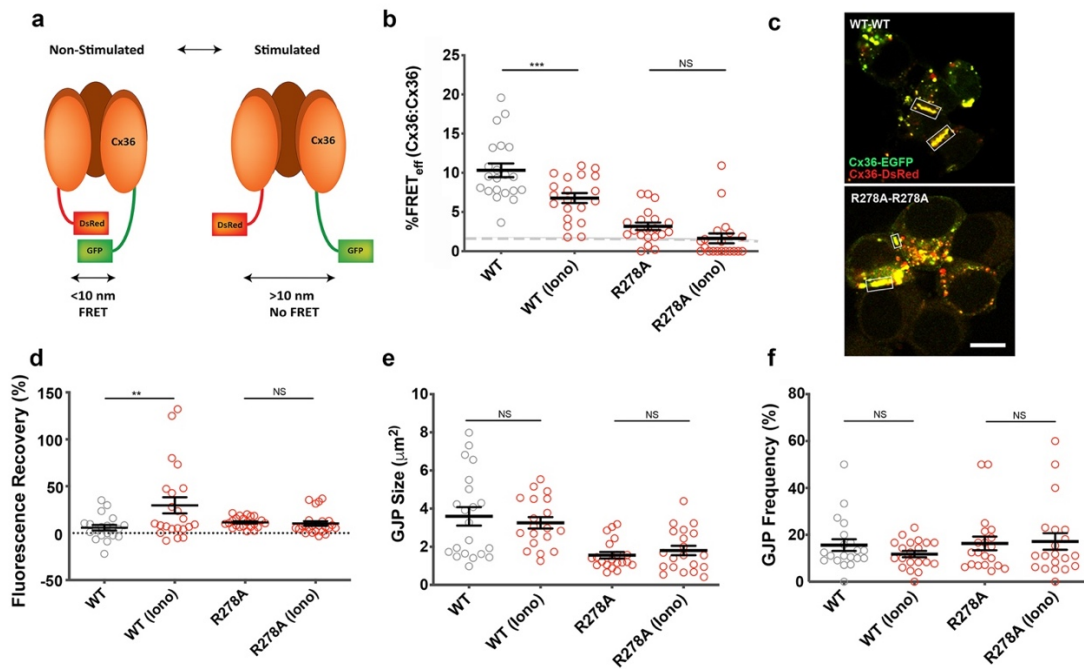


Figure 5.4.1: Calcium modulates proximity between Cx36 channels when CaM/CaMKII binding is intact. **a)** Anticipated changes to the compactness of Cx36 channels located in GJPs when the distance between FRET pairs increases after channels open. **b)** FRET_{eff} of wildtype (WT) or the mutant R278A with and without treatment with 2μM ionomycin and 2mM extracellular calcium. The dashed line indicates the 1.7% or 10 nm limit of FRET. **c)** Expression and localization of Cx36-DsRed/Cx36-EGFP and Cx36-DsRed/Cx36 R278A in double transfected Neuro2a cells. GJPs are highlighted with rectangles. Scale bar: 10μm. **d)** Functional assessment of Cx36 channel opening by photobleach recovery of cytoplasmic ethidium bromide (gapFRAP). **e,f)** Quantification of gap junction plaque size and frequency. Beeswarm graphs show mean ± SEM; Mann-Whitney U (2-tailed) significance test, ****p* < 0.01; abbreviations: Iono, ionomycin; NS, not significant.

5.4.2) Calcium signals converge onto Cx36 through interaction with Ca²⁺-CaM/CaMKII

Short-range structural changes altering the compactness of the Cx36-CaMKIIα interaction complex response to experimentally manipulated [Ca²⁺]_i were investigated next. Cx36 shares with CaMKIIα a conserved CaM binding region found across CaMKII isoforms (**Supplementary Figure 5.6.1a**). Ca²⁺ effects on proximity of Cx36 to wildtype and mutant CaMKII were used to investigate the step at which Ca²⁺ activated CaM binds CaMKIIα (**Figure 5.4.2a**). Elevation of [Ca²⁺]_i with 2μM ionomycin caused significant FRET_{eff} responses (FRET_{eff} in %: CTRL: 5.1±0.4,

n=21; ionomycin: $8.1\% \pm 0.5$, n=20, $p=2.13 \times 10^{-4}$) for Cx36 binding to CaMKII α , but not with the CaMKII α F293A mutant (non-stimulated: 2.9 ± 0.6 , n=20; stimulated: 2.0 ± 0.3 , n=20, $p=0.52$) or CaMKII α R296A (non-stimulated: 3.1 ± 0.5 , n=20; stimulated: 3.0 ± 0.6 , n=20, $p=0.51$) (**Figure 5.4.2b**). The increase in compactness when CaM binds to CaMKII to form the calcium-activated CaM/CaMKII holoenzyme complex suggested a tighter binding to Cx36.

Cx36, similar to NMDA receptors, can bind the calcium-activated CaM/CaMKII complex, and autophosphorylation at amino acid T286 is required for orchestrating the binding of CaMKII α to Cx36. Pharmacological intervention with the CaMKII α inhibitor KN93 decreased FRET_{eff} to nearly the detection (non-stimulated: 5.1 ± 0.4 , n=21; inhibited: 2.8 ± 0.5 , n=20, $p=1.0 \times 10^{-3}$) (**Figure 5.4.2b**), likely through changes in the CaMKII α structure relaxing the binding complex. The autophosphorylation negative CaMKII α mutant T286A lacked response to the $[Ca^{2+}]_i$ increase (non-stimulated: 8.9 ± 1.0 , n=20; stimulated: 5.5 ± 0.7 , n=22, $p=0.01$). Both the negative control CaMKII α T286D and the wild-type protein showed a similar response to $[Ca^{2+}]_i$ increase (non-stimulated: 4.1 ± 0.5 , n=22; stimulated: 6.8 ± 0.7 , n=22, $p=4.0 \times 10^{-3}$) (**Figure 5.4.2b**). We conclude that Ca^{2+} -induced structural changes caused by the binding of Cx36 to CaMKII require CaMKII α autophosphorylation at T286.

Cx36 point mutations at R278 and K281 disrupt binding to CaMKII α . We found no change in FRET intensity of either mutant with CaMKII when $[Ca^{2+}]_i$ was increased, consistent with a lack of compaction of the Cx36-CaMKII complex (R278A, non-stimulated: $6.1\% \pm 0.8$, n=20; stimulated: $6.3\% \pm 0.6$, n=21, $p=0.95$; K281A, non-stimulated: $5.7\% \pm 0.6$, n=20; stimulated:

6.6%±0.5, n=20, p= 0.09). Gap junction plaque frequency and size showed no significant differences when mutants Cx36-R278A and K281A were co-expressed with CaMKII. The mutant Cx36-K279A showed the reduced GJP frequency reported previously (Brown et al. 2019) (Supplementary Figure 5.6.1e,f).

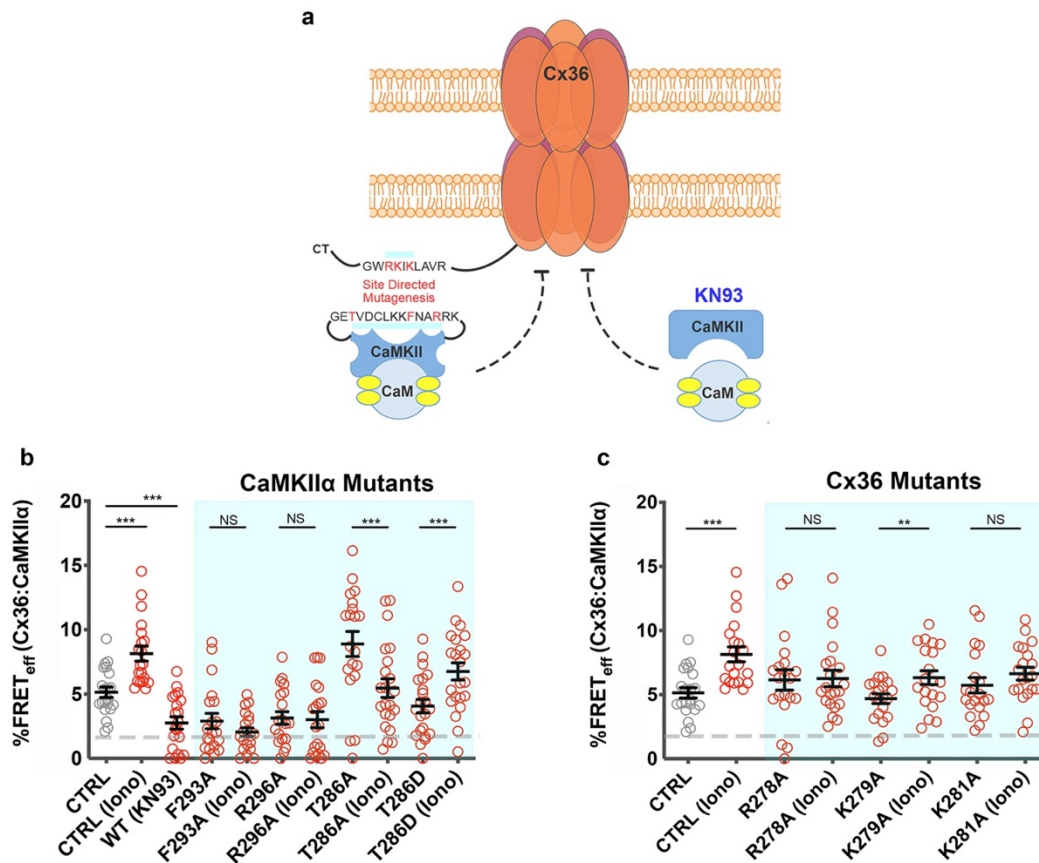


Figure 5.4.2: Calcium-induced compaction of Cx36 located in GJPs requires CaM/CaMKII binding. **a**) Visual summary of the interactions targeted pharmacologically (KN93) or genetically (site-directed mutagenesis) (blunt arrow = blockage/ inhibition). **b**) FRET_{eff} of Cx36-DsRed and WT or mutant CaMKIIα-ECFP in response to Ca²⁺ and pharmacological blocking of CaMKII with KN-93. **c**) FRET_{eff} of WT and mutant Cx36-DsRed and WT and CaMKIIα-ECFP in response to Ca²⁺. Treatments in b,c: Iono (2 mM [Ca²⁺]_E and 2 μM ionomycin), KN93 (50 μM). Beeswarm graphs with mean±SEM; Mann-Whitney U (2-tailed) significance, ****p* < 0.01, NS, not significant.

5.4.3) Calcium ions orchestrate the interaction of Cx36 with CaMKII α at the GJP

The fluorescent calcium reporter Oregon Green BAPTA-AM (OGB) was used next to visualize changes to intracellular Ca²⁺ levels after treatment. Sample traces of treated Neuro2a cells, reporting fluorescence changes over 120s, confirmed ionomycin induced Ca²⁺ influx (boxed to show the most substantial changes). DMSO or BAPTA treatments caused negligible differences compared to the control (**Figure 5.4.3a**). Together, the results demonstrated a causal relationship between [Ca²⁺]_i and the strength of the Cx36-CaMKII α interaction at GJPs.

[Ca²⁺]_i elevation is considered the critical step to initiate the binding of calmodulin to its CaMKII α binding site, followed by binding the complex to Cx36 (**Figure 5.4.3b**). We used FRET to understand better how Ca²⁺ orchestrates short-range molecular changes of the Cx36-CaMKII α complex at the GJP. Under normal cell growth conditions, Cx36-DsRed and CaMKII α -ECFP co-expressed in Neuro2a cells showed significant FRET_{eff}, which increased after treatment with Ca²⁺ ionophore (FRET_{eff} in %: non-stimulated: 5.6 $\pm$ 0.5, n=20; ionomycin: 9.9 $\pm$ 1.1, n=19, p=5.9 x 10⁻³) (**Figure 5.4.3c**). A low [Ca²⁺]_i condition was established with 24 μ M BAPTA-AM, which abolished the interaction between Cx36 and CaMKII α indicated by a FRET_{eff} of less than 1.7% (1.6 $\pm$ 0.3, n=20, p= 6.0 x 10⁻⁶). Treatment with the solvent DMSO alone did not significantly alter Cx36-CaMKII FRET (DMSO: 6.6 $\pm$ 0.8, n=23, p=0.66) (**Figure 5.4.3c**). None of the above treatments caused significant differences in gap junction plaque size or frequency in double transfected cells expressing both tagged Cx36 and CaMKII α (**Supplementary Figure 5.6.2a-c**).

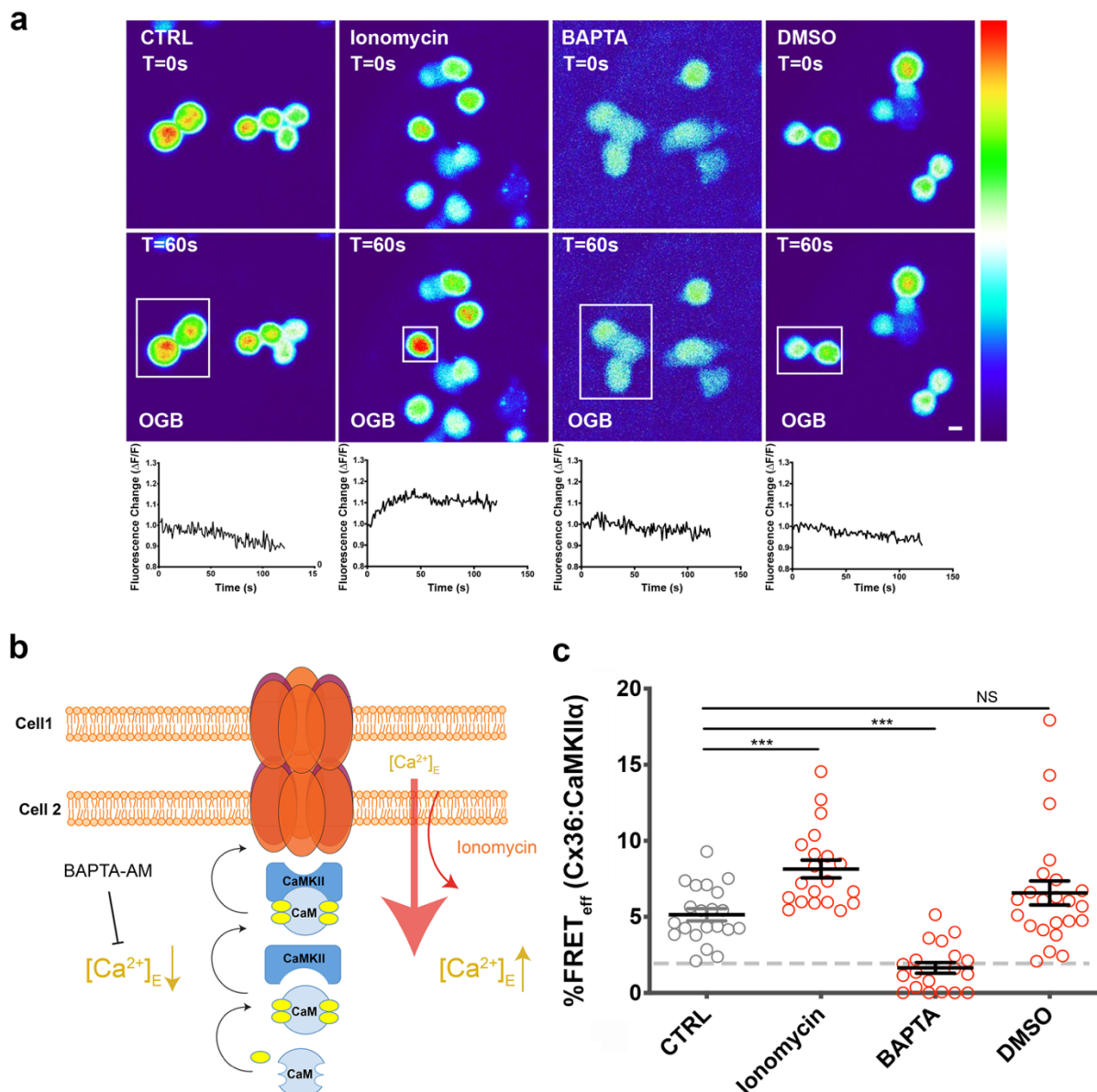


Figure 5.4.3: Calcium manipulation influences Cx36-CaMKII α interaction. **a)** Neuro2a cells labeled with Oregon green BAPTA (OGB) showing calcium influx in response to pharmacological stimulants used in **b**. The bottom row shows sample traces of relative fluorescence ($\Delta F/F$) for ~ 120 s of selected cells (indicated by boxes) showing responses to treatments. Scale bar = $10\mu\text{m}$. **b)** Neuro2a cells were co-transfected with Cx36-DsRed and CaMKII α -ECFP and FRET efficiency (FRET_{eff}) was determined 48h post-transfection. The interaction between Cx36 and CaMKII α was impacted by manipulating intracellular calcium levels (sharp arrows = direction of processes; blunt arrow = blockage/ inhibition). **c)** Transfected Neuro2a cells with drugs added for 5 mins (ionomycin) or 10 minutes (BAPTA) or 10 minutes DMSO as a negative control. Beeswarm graphs with mean $\pm$ SEM; dashed line indicates the 1.7% limit of FRET; Mann-Whitney U (2-tailed) significance, *** $p < 0.01$, ** $p < 0.05$, NS, not significant.

5.4.4) NMDA receptor activation modulates Cx36 interaction with CaMKII

Neuro2a cells expressed low but detectable mRNA levels of multiple ionotropic NMDA receptor subunits and responded to NMDA stimulation (50 μ M) with a rapid increase in $[Ca^{2+}]_i$ (**Supplementary Figure 5.6.3a,b**). The response to NMDA was used to correlate receptor activation with structural changes of the Cx36-CaMKII interaction complex (**Figure 5.4.4**). Stimulation of the glycine recognition site of the NR1 subunit (1 mM glycine) and the glutamate recognition site of NR2 subunit (10 mM glutamate) (**Figure 5.4.4a**) caused a significant increase in FRET_{eff} of Cx36-DsRed and CaMKII α -ECFP implying that CaMKII bound more tightly to Cx36, and in a dose-dependent manner when compared to ionomycin treatment (**Supplementary Figure 5.6.3c**) (FRET_{eff} in %: non-stimulated: 1.7 ± 0.5 , n=20; glycine: 4.5 ± 0.8 , n=21, p= 6.3×10^{-4} ; glutamate: 5.3 ± 0.6 , n=20, p= 1.50×10^{-4} ; glycine & glutamate: 6.2 ± 0.5 , n=19, p= 6.79×10^{-6} ; NMDA: 7.0 ± 0.7 , n=23, p= 3.25×10^{-6}). The agonist NMDA showed effects similar to the co-stimulation with glutamate and glycine. Treatment with NMDA did not affect Cx36 protein expression (**Supplementary Figure 5.6.3d**).

Experimentally reduced extracellular Mg^{2+} levels activate NMDARs allowing a surge of Ca^{2+} influx into the cell, whereas high Mg^{2+} levels limit the probability of NMDAR opening. Treatment with 10 μ M EDTA to reduce Mg levels led to an increase in FRET_{eff} that was similar to that following activation by NMDA (non-stimulated: 3.6 ± 0.3 , n=20; NMDA: 4.9 ± 0.4 , n=22, p=0.02; EDTA: 5.3 ± 0.5 , n=19, p= 1.3×10^{-2}). Simultaneous addition of both EDTA and NMDA achieved the most substantial change in Cx36-CaMKII α interaction (EDTA & NMDA: 6.8 ± 0.2 , n=19, (to CTRL) p= 2.0×10^{-7}). Conversely, 5mM $MgSO_4$ treatment reduced FRET_{eff} when compared to

control (MgSO₄: 2.6 ± 0.4 , n=19, p=0.02) and blocked potentiation upon NMDA stimulation (MgSO₄ & NMDA: 3.7 ± 0.3 , n=20, p=0.82) (**Figure 5.4.4b**). The results indicate that extracellular Mg²⁺ and NMDA levels alter the compaction of Cx36-CaMKII, consistent with their activation of the NMDAR.

Membrane potential changes caused by activation of NMDA receptors were detected using the green fluorescent protein sensor ArcLight S249 as a surrogate for electrophysiology. After chelating [Mg²⁺] with EDTA alone, or in tandem with NMDA stimulation membrane depolarization was detected by an increase in ArcLight S249 fluorescence (**Figure 5.4.4c**). The quantification of fluorescence changes is summarized in **Figure 5.4.4d** (values in % fluorescence change; baseline (CTRL): -1.6 ± 0.9 , n=27; EDTA: 20.6 ± 5.9 , n=20; p= 2.15×10^{-4} ; EDTA & NMDA: 49.9 ± 6.0 , n=25, p= 2.0×10^{-9}). The non-competitive NMDAR antagonist MK801 (dizocilpine) reduced the effects of both EDTA and NMDA significantly (EDTA & NMDA & MK801: 23.4 ± 5.1 , n=20, p= 2.0×10^{-6}). The DMSO solvent control caused no fluorescence change (DMSO: 3.1 ± 2.9 , n=21; p= 0.1).

FRET measurements revealed the relationship between NMDAR activation and the binding of CaMKII α to Cx36 after treatment with the NMDAR antagonists MK801 or AP5/APV (**Figure 5.4.4e, Supplementary Figure 5.6.3d**). MK801 targets the NMDA receptor pore formed by NR1 and NR2 subunits, and we found that MK801 treatment altered the proximity of the Cx36-DsRed and CaMKII α -ECFP complex, but it blocked the increased FRET due to NMDA stimulation (MK801: 3.3 ± 0.5 , n=24; p= 0.64; MK801 & NMDA: 4.5 ± 0.5 , n=32, p=0.16). Targeting the NR2B binding site with the competitive antagonist D-AP5 (APV) had a similar effect, augmenting

the basal FRET between Cx36-DsRed and CaMKII α -ECFP but abolishing its response to NMDA activation (D-AP5: 5.8 ± 0.5 , n=20, p= 2.0×10^{-3} ; D-AP5 & NMDA: 4.7 ± 0.5 , n=20, p=0.06).

Next, gapFRAP evaluated the impact on the gap junction function of activation and inhibition of NMDA receptors. Neuro2a cells expressing Cx36-EGFP and DsRed-CaMKII showed significant ethidium bromide redistribution after stimulation of NMDA receptors (values in %: non-stimulated: 10.3 ± 3.6 , n=21; NMDA: 37.8 ± 7.0 , n=22, p= 3.0×10^{-3}). The pharmacological blockade of NMDARs with MK801 suppressed the ethidium bromide (EtBr) transfer through GJPs and inhibited the enhanced EtBr transfer caused by NMDA (MK801: 13.1 ± 4.8 , n=20, p= 0.94; MK801 & NMDA: 17.9 ± 4.9 , n=20, p=0.36 (**Figure 5.4.4f**). The quantification of the gap junction plaque frequency showed a significant reduction when NMDARs were blocked for 30 minutes with MK-801, or 10 minutes with AP5, suggesting that $[Ca^{2+}]_i$ changes can modulate GJ communication rapidly (**Supplementary Figure 5.6.3e**). The data establish NMDA receptors as a gateway for calcium entry that enhances the binding of Cx36 to the CaM/CaMKII holoenzyme.

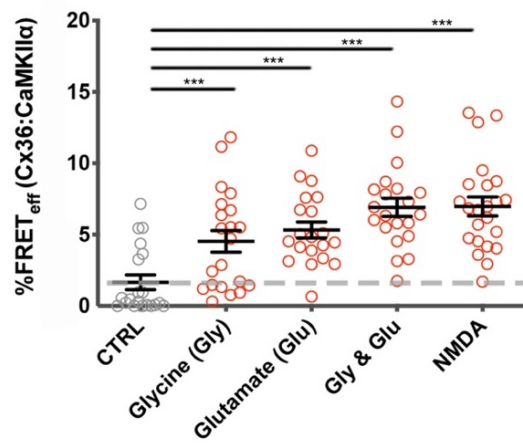
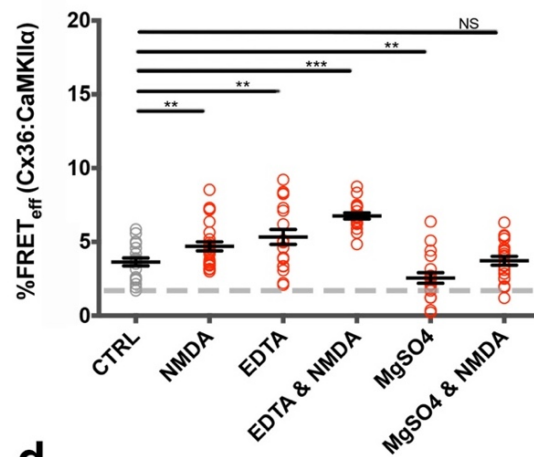
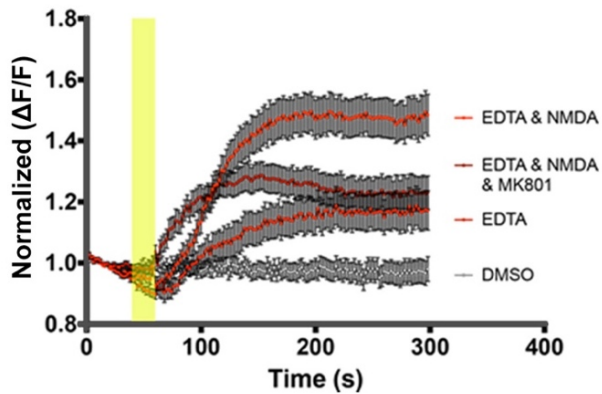
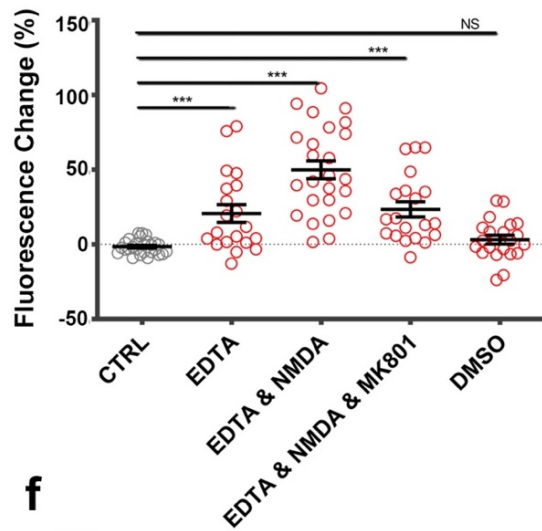
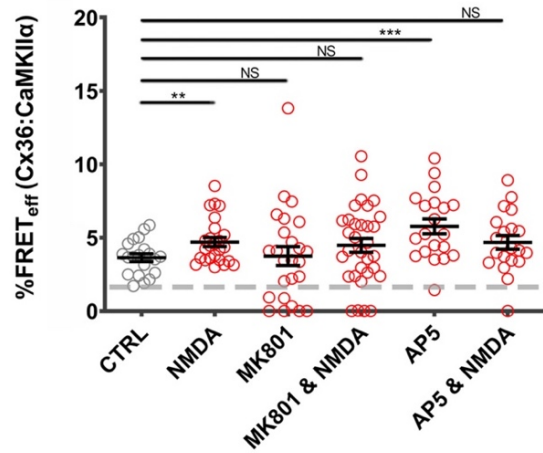
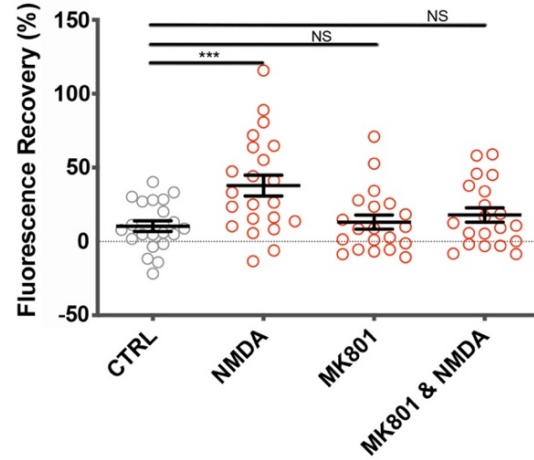
a**b****c****d****e****f**

Figure 5.4.4: Impact of NMDA receptor activation on the modulation of the compactness of the Cx36-CaMKII α interaction complex. In **a,b,e**) Neuro2a cells co-transfected with Cx36-DsRed and CaMKII α -ECFP. The FRET efficiency (FRET_{eff}) was determined at 48hrs post-transfection. **a**) FRET_{eff} after treatment with glycine (1mM), glutamic acid (10mM), and NMDA (50 μ M). **b**) FRET_{eff} after treatment with EDTA (3mM), NMDA (50 μ M), and MgSO₄ (5mM). **c-d**) Neuro2a cells expressing Arclight S249 and Cx36-DsRed. **c**) Cell images contrasting Arclight S249 fluorescence changes after treatment with EDTA, EDTA & NMDA, EDTA & NMDA & MK801, and control for the solvent DMSO. Cells were imaged at one minute (baseline fluorescence) and after five minutes of treatment (post-treatment). Scale bar = 10 μ m. **c**) Arclight fluorescence change over five minutes with a yellow panel indicating the time window when compounds were added (n >25, mean $\pm$ SEM). **d**) Quantitative analysis of Arclight fluorescence changes in response to drug treatments (Beeswarm graphs with mean $\pm$ SEM for n>25 cells; Mann-Whitney U significance, ***p < 0.01, **p < 0.05, NS, not significant). **e**) FRET_{eff} following treatment with NMDA receptor antagonists MK801 and AP5. **f**) GapFRAP analysis of Cx36-EGFP expressing Neuro2a cells treated with NMDAR antagonists. Beeswarm graphs with mean $\pm$ SEM; dashed line indicates the 1.7% limit of FRET; Mann-Whitney U (2-tailed) significance, ***p < 0.01, **p < 0.05, NS, not significant.

5.4.5) Pannexin 1 modulates the interaction between Cx36 and CaMKII

Neuro2a cells express low levels of Panx1 and respond to potassium gluconate stimulation (40 mM KGlu) (Whyte-Fagundes et al. 2018), as shown here using the genetically encoded membrane potential probe Arclight S249 (**Figure 5.4.5a,b**; all values in % fluorescence change; baseline (CTRL): -1.5 $\pm$ 1.5, n=22; KGlu: 47.4 $\pm$ 5.4, n=22; p= 3.0 x 10⁻⁸). The fluorescence of Arclight S249 in the presence of KGlu was reduced when Panx1 channels were blocked with the blue food dye BB-FCF (J. Wang, Jackson, and Dahl 2013), or the ¹⁰Panx peptide (Pelegrin and Surprenant 2006) (compared to CTRL; BB-FCF: 21.1 $\pm$ 5.7, n=20; p= 3.8 x 10⁻³; ¹⁰Panx: 3.4 $\pm$ 3.9, n=26; p= 0.66). The scrambled control peptide ^{SC}Panx had no effect (^{SC}Panx: 53.7 $\pm$ 4.2, n=25; p= 1.13 x 10⁻⁸).

Treatment of Neuro2a cells cotransfected with ECFP-Cx36 and DsRed-Cx36 with 2mM [Ca²⁺]_E and 40mM KGlu decreased FRET_{eff} at GJPs, indicating Cx36 channel opening (all values in %: non-stimulated: 8.5 $\pm$ 0.7, n=28; KGlu: 4.2 $\pm$ 0.6, n=23, p=4.9 x 10⁻⁵) (**Figure 5.4.5c,d**). The Panx1

channel blockers $^{10}\text{Panx}$ peptide or BB-FCF significantly reduced the effects of KGlu stimulation, while the scrambled version of $^{10}\text{Panx}$ ($^{\text{SC}}\text{Panx}$) had no noticeable effect ($^{10}\text{Panx}$ & KGlu: 7.0 ± 0.6 , $n=20$, $p=0.14$; $^{\text{SC}}\text{Panx}$ & KGlu: 5.1 ± 0.5 , $n=22$, $p=9.3 \times 10^{-4}$; BB-FCF & KGlu: 9.6 ± 0.8 , $n=24$, $p=0.16$).

KGlu stimulation increased FRET_{eff} at GJPs significantly when Neuro2a cells co-expressed $\text{CaMKII}\alpha$ -ECFP and Cx36-DsRed (non-stimulated: 5.1 ± 0.4 , $n=21$; K-Glu: 9.3 ± 1.0 , $n=23$, $p=1.4 \times 10^{-2}$) (**Figure 5.4.5e,f**). The effect of KGlu was suppressed with $^{10}\text{Panx}$ and BB-FCF ($^{10}\text{Panx}$ & K-Glu: 2.5 ± 0.4 , $n=20$, $p=1.0 \times 10^{-3}$; BB FCF & KGlu: 6.4 ± 0.8 , $n=21$, $p=0.38$), but was unchanged by $^{\text{SC}}\text{Panx}$ treatment ($^{\text{SC}}\text{Panx}$ & K-Glu: 8.2 ± 0.8 , $n=20$, $p=0.02$). This result suggested that Panx1 channels modulate the binding of Cx36 to CaMKII.

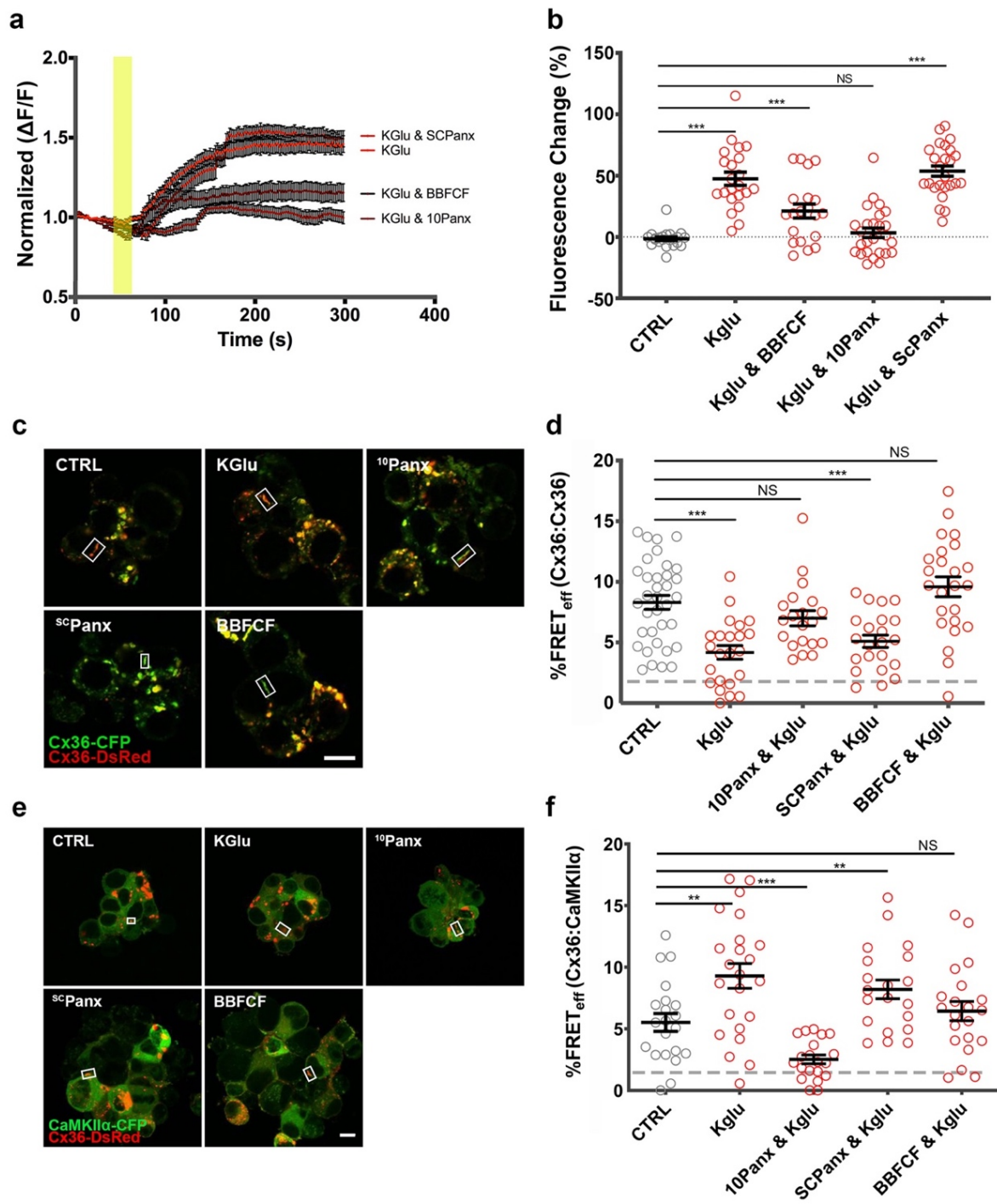


Figure 5.4.5: The activation of endogenous Panx1 modulates the interaction between Cx36 and CaMKII at the GJP. **a)** Arclight fluorescence change over five minutes after treatment with KGlu, KGlu & 10 Panx, and KGlu & SC Panx. The yellow panel indicates the time at which treatments were added (values: mean $\pm$ SEM). **b)** Arclight fluorescence change quantification by comparing baseline (before one minute) to the final five minutes with different drug treatments. **c)** Neuro2a cells double transfected with DsRed-Cx36 and ECFP-Cx36. GJPs are highlighted with rectangles. **d)** FRET_{eff} in response to treatments at GJPs. **e)** Neuro2a cells double transfected with DsRed-Cx36 and ECFP-CaMKII α . GJPs are highlighted with rectangles. **f)** Treatments altered FRET_{eff} of Cx36-DsRed and Cx36-ECFP WT and various blockers with and without 40 μ M potassium gluconate (KGlu). Beeswarm graphs in **d,f** with mean $\pm$ SEM; dashed line indicates the 1.7% or 10 nm limit of FRET; Mann-Whitney U (2-tailed) significance, ***p < 0.01, NS, not significant; scale bars in **c,e** = 10 μ m).

5.4.5) Overexpression of Panx1 rescued the Cx36-CaMKII interaction in Panx1 knock-down cells

Neuro2a cells with targeted knock-down of Panx1 (KD-Panx1) were generated using TALEN technology (**Supplementary Figure 5.6.4a-c**). Neuro2a cells expressing Cx36 and endogenous Panx1 showed enhanced gapFRAP after KGlu stimulation that was blocked by ATP (% recovery in non-stimulated WT CTRL: 9.2 $\pm$ 3.2, n=21; KGlu: 32.1 $\pm$ 8.4, n=19, p= 0.04; KGlu & 3mM ATP: 5.8 $\pm$ 3.1, n=19, p= 0.44), but neither the increased coupling or the block by ATP were observed in TALEN KD-Panx1 cells when compared to WT CTRL (TALEN KD-Panx1: 14.2 $\pm$ 5.5, n=21, p= 0.95; KGlu: 2.63 $\pm$ 4.6, n=19, p= 0.09; KGlu & 3mM ATP: 10.1 $\pm$ 6.3, n=22, p= 0.92) (**Figure 5.4.6b**).

The high/low ATP paradigm for closing/opening Panx1 channels (Bao, Locovei, and Dahl 2004; Qiu and Dahl 2009) was used to demonstrate the impact of Panx1 on the CaMKII α -Cx36 interaction. In Neuro2a cells FRET_{eff} of Cx36-CaMKII α increased with KGlu treatment (all values in %: WT CTRL: 4.0 $\pm$ 0.6, n=19; KGlu: 6.6 $\pm$ 0.4, n=21, p= 2.8 x 10⁻³), 0.1mM ATP (6.8 $\pm$ 0.5, n=21,

$p=3.3 \times 10^{-3}$) or both (0.1mM ATP & KGlu: 6.5 ± 0.6 , $n=22$, $p=2.1 \times 10^{-3}$), while 3mM ATP showed a reduction in FRET_{eff} , both without (3.5 ± 0.3 , $n=21$, $p=0.80$) and with KGlu (4.7 ± 0.5 , $n=20$, $p=0.26$) (**Figure 5.4.6c**). KD-Panx1 cells showed no response of the Cx36-CaMKII α complex to any of these treatments (non-stimulated: 4.9 ± 0.8 , $n=19$; KGlu: 4.7 ± 0.2 , $n=18$, $p=0.54$; 0.1mM ATP (4.9 ± 0.3 , $n=21$, $p=0.43$); 3mM ATP (4.9 ± 0.3 , $n=25$, $p=0.44$); 0.1mM ATP & KGlu (5.2 ± 0.6 , $n=20$, $p=0.40$), or 3mM & KGlu (4.7 ± 0.5 , $n=19$, $p=0.56$) (**Figure 5.4.6d**). The responsiveness to KGlu was at least in part rescued by transfecting Panx1-Crimson in both WT (CTRL with Panx1-Crimson non-stimulated: 2.1 ± 0.3 , $n=20$; CTRL with KGlu: 5.3 ± 0.5 , $n=21$, $p=4.0 \times 10^{-5}$) and KD-Panx1 cells when compared to WT CTRL (TALEN with Panx1-Crimson non-stimulated: 1.72 ± 0.4 , $n=21$; TALEN with KGlu: 3.2 ± 0.4 , $n=22$, $p=0.02$) (**Figure 5.4.6e**).

We speculated that overexpression of Panx1 channels provided a condition of Ca^{2+} entry and that blocking Panx1 with $^{10}\text{Panx}$ or chelating Ca^{2+} with BAPTA-AM should attenuate the effects. Indeed, at baseline treatment with $^{10}\text{Panx}$ or BAPTA-AM, abolished any significant FRET effect indicating interaction between CaMKII α and Cx36, whereas the scrambled control Panx1 peptide had no effect (CTRL: 2.1 ± 0.3 , $n=20$; $^{10}\text{Panx}$: 2.0 ± 0.3 , $n=21$, $p=0.9$; $^{\text{SC}}\text{Panx}$: 2.7 ± 0.4 , $n=21$, $p=0.14$; BAPTA-AM: 1.9 ± 0.3 , $n=21$, $p=0.94$). The scrambled control Panx1 peptide did not block KGlu induced interaction between CaMKII α and Cx36 ($^{\text{SC}}\text{Panx}$ & KGlu: 4.7 ± 0.5 , $n=21$, $p=1.8 \times 10^{-4}$), and FRET_{eff} was not changed when KGlu was added in the presence of $^{10}\text{Panx}$ or BAPTA-AM ($^{10}\text{Panx}$ & KGlu: 3.1 ± 0.5 , $n=20$, $p=0.12$; BAPTA-AM & KGlu: 1.8 ± 0.4 , $n=22$, $p=0.25$) (**Figure 5.4.6f**).

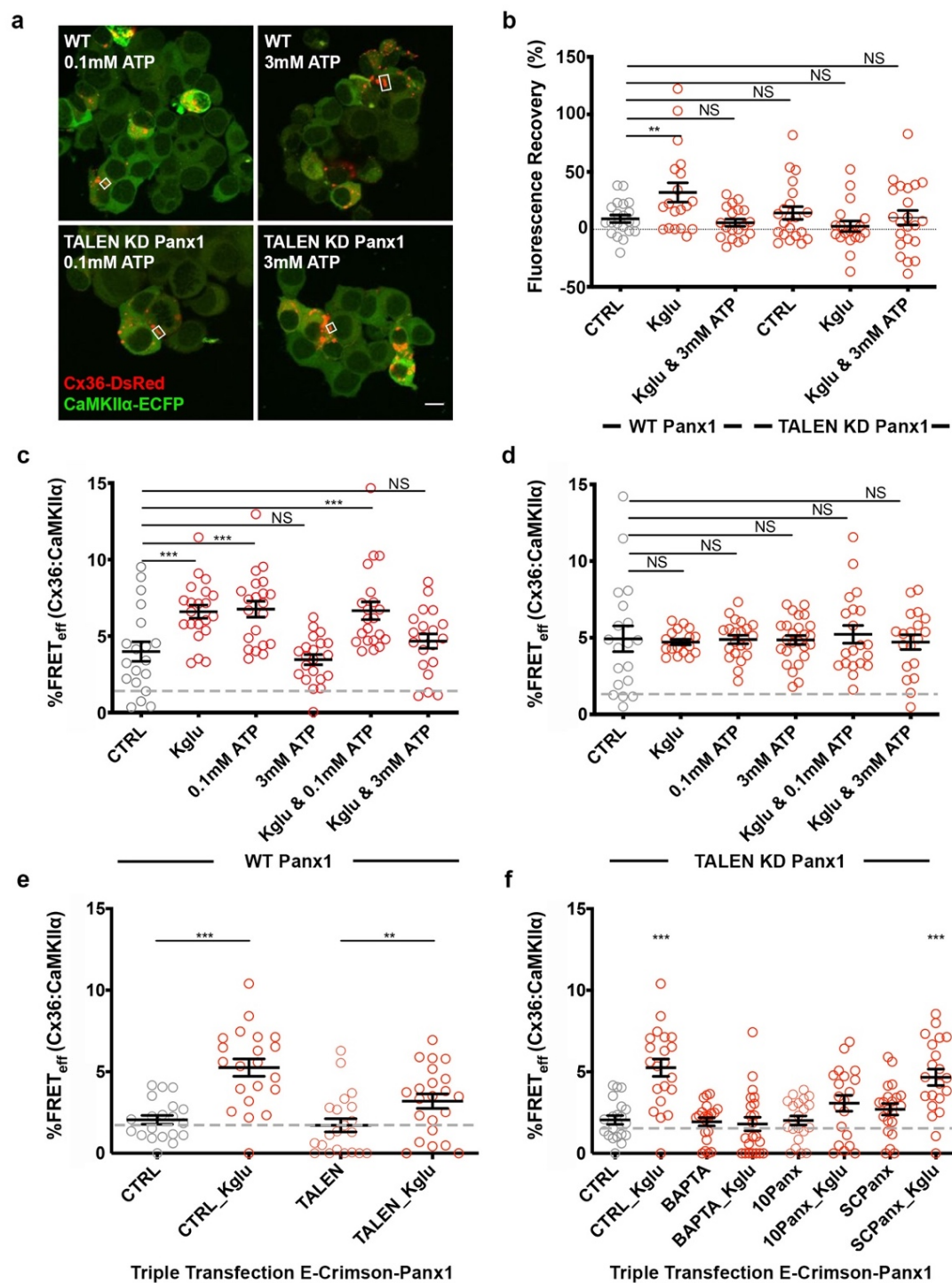


Figure 5.4.6: Knocking down *Panx1* channel expression in Neuro2a cells or blocking its function reduced the compaction of Cx36-CaMKII in response to KGlu and low ATP concentration. Neuro2a cells (WT and TALEN KD *Panx1*) were either transfected doubly with Cx36-DsRed and CaMKII α -ECFP or singly transfected with Cx36-EGFP for gapFRAP. **a)** Normal GJP formation in both WT and TALEN *Panx1* KD cells (GJPs indicated by white boxes; scale bar = 10 μ m). **b)** gapFRAP of WT Neuro2a cells or TALEN KD *Panx1* cells treated with potassium gluconate (KGlu) or 3mM ATP. **c,d)** FRET analysis of doubly transfected WT and TALEN cells treated with KGlu, or 0.1mM or 3mM ATP. **e)** Triply transfected CTRL or TALEN *Panx1* KD cells with E-Crimson-*Panx1* were analyzed with and without KGlu. **f)** Triply transfected cells were analyzed after being treated with BAPTA, 10 Panx, or 8C Panx with or without KGlu. (Beeswarm graphs with mean $\pm$ SEM; dashed line indicates the 1.7% limit of FRET; Mann-Whitney U (2-tailed) significance, **p < 0.05, ***p < 0.01, NS, not significant).

5.4.7) The compactness of the Cx36 -CaMKII interaction is modulated by an NMDA receptor-Src family kinase – *Panx1* signaling complex

Initial experiments were directed towards calcium entry through ionotropic NMDA receptors and how this affects CaMKII-Cx36 interactions. In a separate investigation line, we turned to whether posttranslational modification of *Panx1* by Src family kinases (SFKs) affects CaMKII-Cx36 interactions and increases coupling after stimulation of ionotropic NMDA receptors. This change in focus was motivated by research demonstrating a signaling pathway between metabotropic NMDA receptors and *Panx1*, and the regulation of *Panx1* channel function by Src family kinase-mediated phosphorylation at the two tyrosine residues Y308 and Y198 (Weilinger et al. 2016). In gapFRAP experiments, activation of Cx36 GJC by stimulation of the NMDA receptor increased the redistribution of ethidium bromide between Neuro2a cell pairs after photobleaching (values in %: non-stimulated: 5.8 $\pm$ 3.0, n=20; NMDA: 32.7 $\pm$ 7.4, n=23, p= 1.4 x 10 $^{-3}$) (**Figure 5.4.7a**). The increase in dye in the photobleached regions of interest below GJPs was suppressed by co-application of the Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) (PP2: 11.8 $\pm$ 4.5, n=22, p= 0.15; PP2 & NMDA: 9.7 $\pm$ 5.7, n=20, p= 0.89). The negative control 4-Amino-1-phenyl-1H-pyrazolo[3,4-d] pyrimidine (PP3) had

no effect on ethidium bromide redistribution (PP3: 9.0 ± 4.7 , $n=23$, $p=0.76$; PP3 & NMDA: 35.1 ± 6.2 , $n=22$, $p=2.6 \times 10^{-4}$). Gap junction frequency (all values in %: CTRL: 12.8 ± 2.0 , $n=22$; PP2: 6.6 ± 1.3 , $n=20$, $p=6.0 \times 10^{-3}$; PP3: 11.0 ± 2.0 , $n=20$, $p=0.34$), but not the size of gap junction plaques was affected by PP2 treatment (all values in nm: CTRL: 3.1 ± 0.4 , $n=22$; PP2: 3.5 ± 0.6 , $n=21$, $p=0.66$; PP3: 3.8 ± 0.3 , $n=24$, $p=0.02$) (**Figure 5.4.7b,c**). Double-transfected Neuro2a cells treated with PP2 and PP3 showed regular cell morphology (**Figure 5.4.7d**).

SFKs also modulated the compactness of the Cx36-DsRed and CaMKII α -ECFP complex. Treatment with PP2, a Src kinase inhibitor, reduced FRET_{eff} to control levels, while PP3, a negative control for PP2 was not effective (CTRL: 5.9 ± 0.9 , $n=19$; PP2: 3.1 ± 0.8 , $n=21$; $p=1.3 \times 10^{-2}$; PP3: 6.9 ± 1.1 , $n=20$, $p=0.55$), (**Figure 5.4.7e**).

FRET_{eff} of the Cx36-CaMKII interaction complex in KD-Panx1 cells was significantly increased after KGlu stimulation only when Panx1-Crimson was overexpressed (**Supplementary Figure 5.6.5**) (non-stimulated: 2.0 ± 0.3 , $n=25$; KGlu: 5.3 ± 0.7 , $n=24$, $p=7.5 \times 10^{-5}$). Expression of mutants Panx1-Y308F and Panx1-Y198A showed no rescue of the phenotype (Panx1-Y308F: 1.8 ± 0.4 , $n=21$, $p=0.65$; Panx1-Y308F & KGlu: 1.6 ± 0.3 , $n=22$, $p=0.46$; Panx1-Y308A: 2.7 ± 0.6 , $n=21$, $p=0.53$; Panx1-Y308A & KGlu: 2.8 ± 0.5 , $n=22$, $p=0.46$). The mutants Panx1-Y198F and -Y198A reduced Cx36-CaMKII interaction (Panx1-Y198F: 1.0 ± 0.3 , $n=23$, $p=0.11$; Panx1-Y198F & KGlu: 1.9 ± 0.4 , $n=22$, $p=0.08$; Panx1-Y198A: 1.3 ± 0.3 , $n=21$, $p=0.01$; Panx1-Y198A & KGlu: 1.22 ± 0.26 , $n=21$, $p=0.78$) (**Figure 5.4.7f,g**). The results demonstrated a role of SFK phosphorylation of Panx1 in modulating the interacting complex of Cx36 and CaMKII.

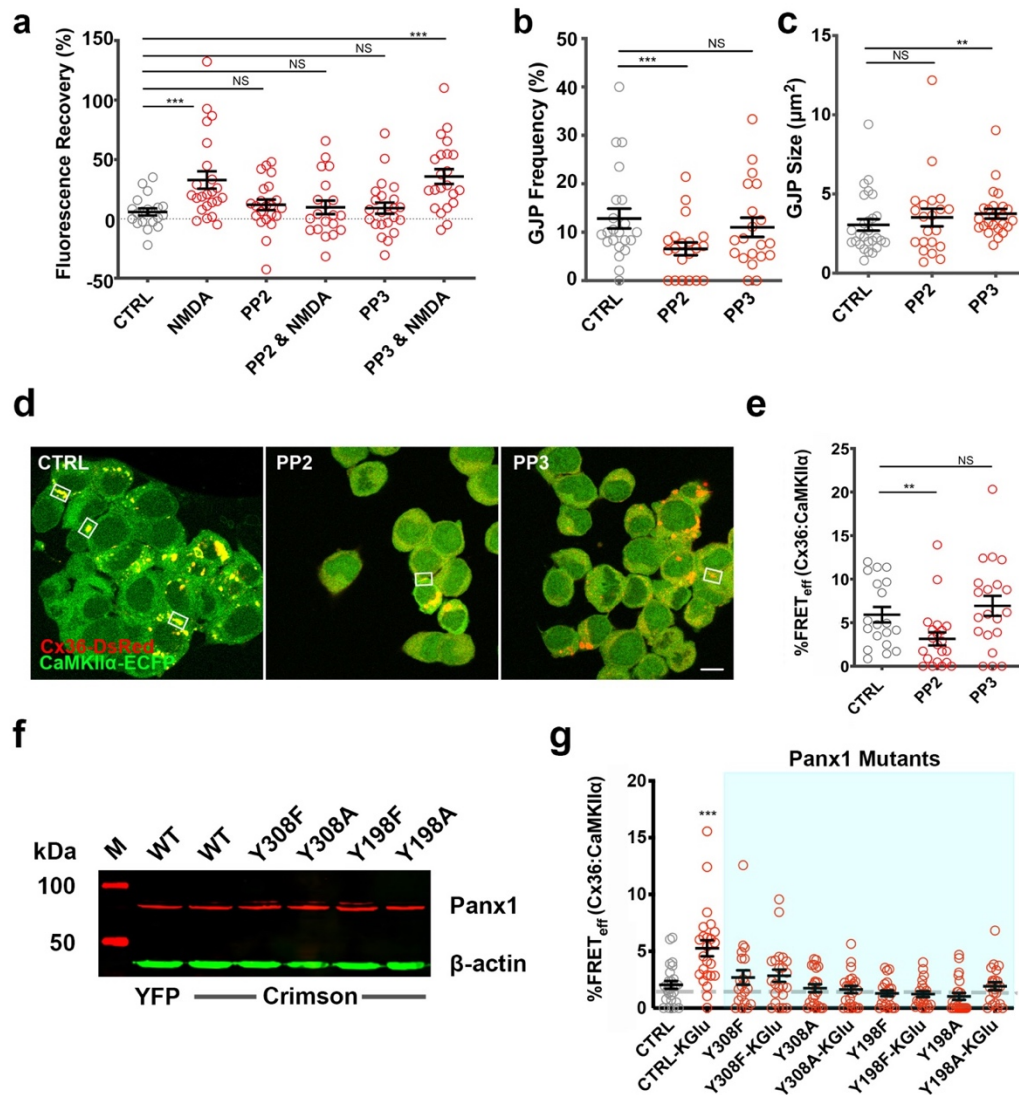


Figure 5.4.7: A Src kinase is involved in modulating the Cx36-CaMKII interaction. **a)** gapFRAP fluorescence recovery of ethidium bromide through Cx36-EGFP gap junctions under the effects of PP2 or PP3 (negative control for PP2) with or without NMDA stimulation. **b & c)** Quantification of gap junction frequency and size with the addition of PP2 or PP3. **d)** Neuro2a cells expressing Cx36-DsRed and CaMKII α -ECFP showing normal gap junction expression with PP2 or PP3. **e)** FRET efficiency of Cx36-DsRed and CaMKII α -ECFP under the effects of PP2 or PP3. **f)** western blot confirming expression of Panx1 WT and mutant (Y308F, Y308A, Y198F, and Y198A) tagged with YFP or Crimson in 48h post-transfected Neuro2a cells (Panx1= red; β -actin= green). **g)** FRET efficiency of Cx36 with CaMKII α interaction in triply transfected (Cx36-DsRed, CFP-CaMKII α , and Panx1-E2-Crimson (WT, Y308F, Y308A, Y198F, and Y198A) Neuro2a cells upon potassium gluconate (KGlu) stimulation. (Beeswarm graphs with mean $\pm$ SEM; dashed line indicates the 1.7% limit of FRET; Mann-Whitney U (2-tailed) significance, ***p < 0.01, NS, not significant).

5.5) Discussion

The activity-dependent strengthening or weakening of chemical synaptic transmission is regarded as a fundamental mechanism by which neural circuits are formed or broken. A principal molecular mechanism in which excitatory synapses are strengthened is through interaction of CaMKII and NMDA receptor subunits. Evidence for similar use-dependent facilitation of electrical synapses strength was first revealed in the goldfish Mauthner cell - giant motor fiber synapse (Korn and Faber 1976; X. D. Yang, Korn, and Faber 1990). Like chemical synaptic potentiation, this facilitation was shown to depend on CaMKII binding to the channel protein, in this case, one or more teleost homologs of Cx36 (A. Pereda, O'Brien, Nagy, Bukauskas, et al. 2003). We previously demonstrated that the mammalian neuronal gap junction protein Cx36 also exhibits use-dependent facilitation (so-called "run-up") (Zoidl et al. 2002; Del Corso et al. 2012). The protein interacts with and is phosphorylated by CaMKII *in vitro*, similar to the CaMKII interaction with the NR2B subunit of ionotropic NMDA receptors (Alev et al. 2008). We further showed that phosphorylation by CaMKII strengthens junctional currents of Cx36 channels, thereby conferring functional plasticity on electrical synapses formed of this protein.

Here, using neuroblastoma cells as an efficiently manipulatable neural cell line, we identify components of a signaling complex that modifies the CaMKII interaction with Cx36 at the gap junction plaque. The novel signaling complex consists of ionotropic NMDA receptors, SFK, and Panx1 and is distinct from protein interactions that we and others have previously reported involving Cx36, such as the binding to Ca²⁺ loaded CaM (Burr et al. 2008), which mainly occurs at the ER/Golgi complex (Siu et al. 2016), the interaction with tubulin facilitating microtubule-

dependent transport to the GJP (Brown et al. 2019), or the removal of Cx36 from the GJP by binding to caveolin-1 (Kotova, Timonina, and Zoidl 2020). Our present interpretation is that these interactions primarily contribute to on-demand transport to the GJP and endocytosis of Cx36.

The findings reported here were accomplished with FRET measurements of fluorescent protein-tagged construct pairs as a sensitive method to detect pathway proteins' interactions at the cell membrane. This method revealed increased compactness of protein-protein complexes, interpreted as tighter interactions between the pairs, when intracellular Ca^{2+} was elevated, either with Ca^{2+} ionophore or by activation of ionotropic NMDA receptors. The result made in our minimalistic neuroblastoma cell model provides mechanistic insight into how a critical function of electrical synapses is modulated by crosstalk with molecular components that are generally hallmarks of chemical synapses. Neuronal gap junctions are often found in close proximity to glutamatergic synapses, where they collectively are termed mixed synapses (James I Nagy, Pereda, and Rash 2019). Using established methods of manipulating NR1/NR2B subunits' activation (by relieving the magnesium block (Burnashev et al. 1992; Mori et al. 1992), inhibition or activation of extracellular receptor sites, or pharmacological modulation (X. Song et al. 2018; A. Yu and Lau 2018)), we found that ionotropic NMDA receptors constitute an important entry point for extracellular calcium. The increase in intracellular calcium was identified as essential driver of the transition of the Cx36-CaMKII complex to a compact state.

Since the '90s, electrophysiological studies in lower vertebrates demonstrated NMDA receptor-mediated strengthening of electrical coupling between neurons in mixed synapses (A E Pereda et al. 1998; X. D. Yang, Korn, and Faber 1990) and implicated CaMKII (A E Pereda et al. 1998).

Later, regulation and plasticity of electrical transmission by NMDA receptor-initiated sequences of events were found in the mammalian brain in synaptic loci and at extra-synaptic loci proximal to gap junctions (Turecek et al. 2014). Our previous reports of “run-up plasticity” in which Cx36 binds Ca^{2+} -activated CaMKII *in vitro*, using conserved binding domains, similarly to the NR2B subunit of the NMDA receptor were evidence for a shared molecular basis of use-dependent plasticity (Alev et al. 2008; Del Corso et al. 2012). The new results identify short-range structural rearrangements which increase the compactness of the Cx36-holoenzyme complex as a fundamental requirement to open Cx36 channels. We predict that recent advances in cryoEM technologies provide an opportunity to solve the differences of compact and open states of the Cx36-holoenzyme complex at atomic resolution.

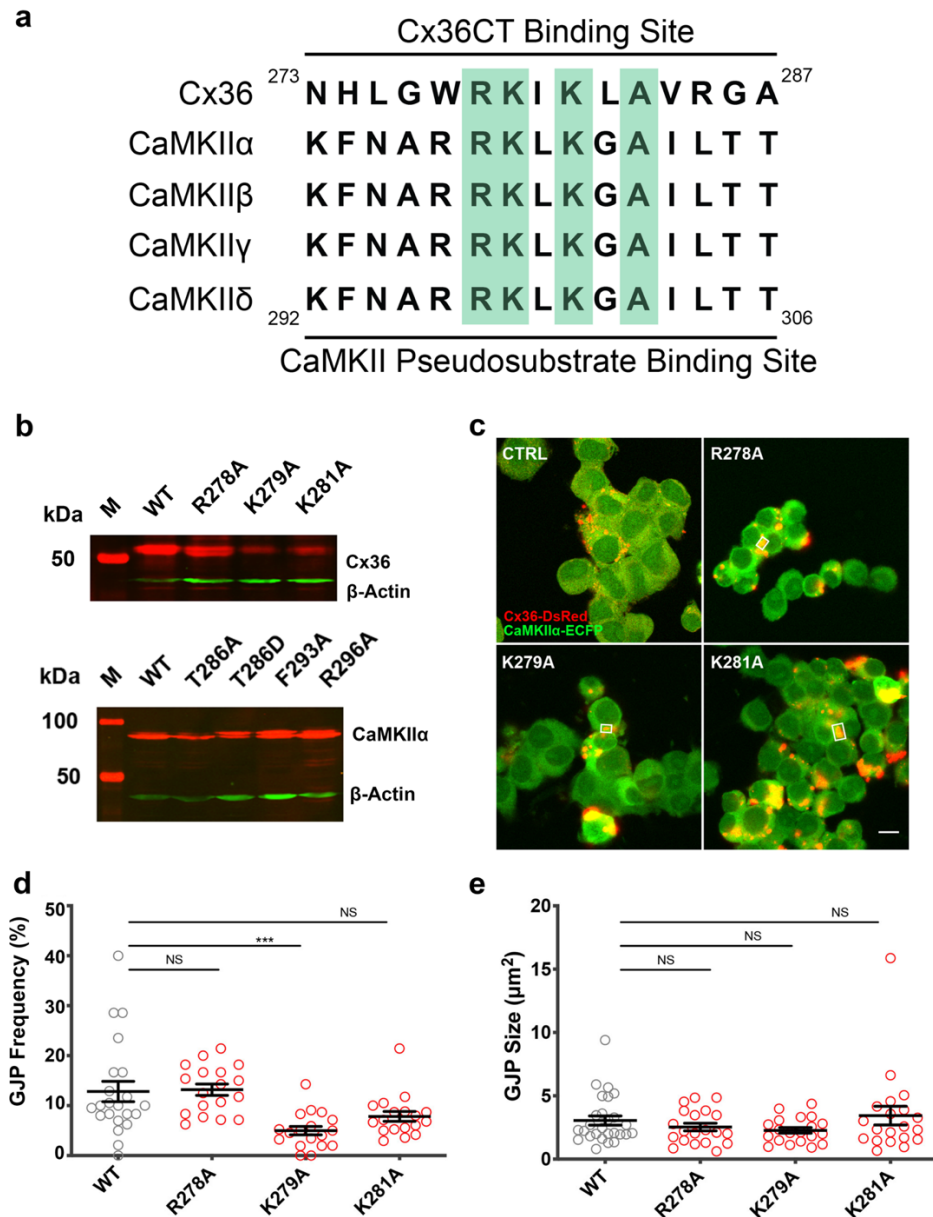
An additional way in which activation of the signaling complex may be modulated is through intracellular Mg^{2+} , which was shown by Palacios-Prado et al. to inhibit Cx36-dependent gap junctional intercellular communication (GJIC) in cell lines exogenously expressing Cx36, and in brain slice recordings from neurons in the mesencephalic nucleus known to express endogenous Cx36 (Palacios-Prado et al. 2014). The authors argued that Mg^{2+} concentration changes sufficient to alter coupling might occur physiologically by ATP fluctuations, which reduces Mg^{2+} and thus increases coupling. It was suggested that the effect of Mg^{2+} on coupling strength was due to direct interaction with the channel. However, Mg^{2+} also competes with Ca^{2+} for binding to calmodulin (Malmendal et al. 1999), and the effects reported are consistent with an interference with the step-wise assembly of the Cx36-CaMKII signaling complex.

The involvement of Panx1 in regulating Cx36 was unexpected. It is to our knowledge the first observation of functional interaction between members of the connexin and pannexin gene families. Previously, several stimuli have been reported to activate Panx1 channels (Chekeni et al. 2010; Chiu et al. 2017; Vanden Abeele et al. 2006), including ionotropic NMDARs activated during anoxia or following energy deprivation (Isakson and Thompson 2014; Weilingner et al. 2013). The Thompson group also reported that Panx1 activation required Src family kinase (SFK), which corroborated the link between NMDARs and Panx1 were SFKs (Weilingner, Tang, and Thompson 2012). The discovery that activation of ionotropic NMDA receptors, Panx1 and SFKs increase intracellular calcium levels which drive Cx36 bound to CaMKII to an open state demonstrates molecular and perhaps mechanistic interaction between chemical and electrical communication in nerve cells. They also suggest a possible involvement of Cx36 in the postsynaptic metabotropic NMDARs, Panx1, and SFKs signaling complex reported by the Thompson group.

Ionotropic NMDA receptors are located close to Cx36 at mixed synapses in excitatory neurons (James I. Nagy, Pereda, and Rash 2018; James I Nagy, Pereda, and Rash 2019). Panx1 proteins were found in rodent hippocampal and cortical principal neurons accumulating in proximity to postsynaptic densities (Zoidl et al. 2007). In contrast, CaMKII and SFKs can be found in both pre- and postsynaptic compartments. The restricted localization of both NMDA receptors and Panx1 in postsynaptic compartments suggests that the signaling complex in CNS neurons is not a simple mirror image across the gap junction plaque. This finding adds to growing evidence for functional asymmetry caused by differences in posttranslational modifications of individual Cx36 proteins (Bazzigaluppi et al. 2017b), or in the complement of Cx36-associated proteins found at the

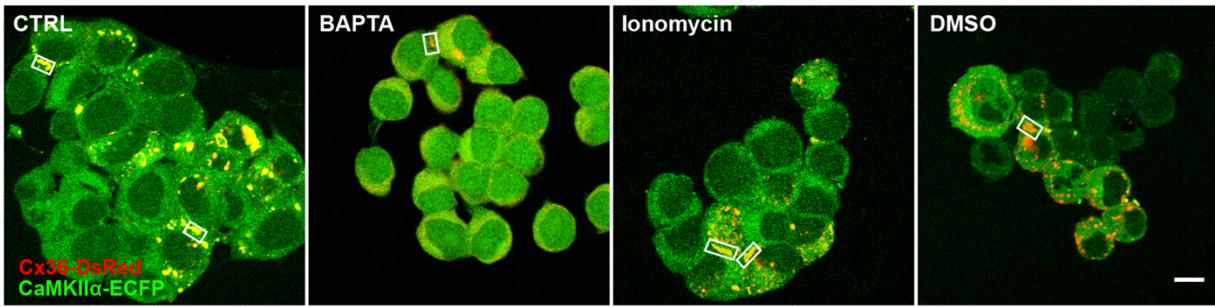
electrical synapse density (ESD) (J I Nagy and Lynn 2018; X Li, Lynn, and Nagy 2012; Xinbo Li et al. 2004). Taken together, outcomes of this study have the potential to transform the traditional view of electrical and chemical communication as separate and functionally independent. Instead, we propose that in mixed synapses regulation of coupling strength and synaptic output involves cooperation between electrical and chemical synapses.

5.6) Supplementary Figures

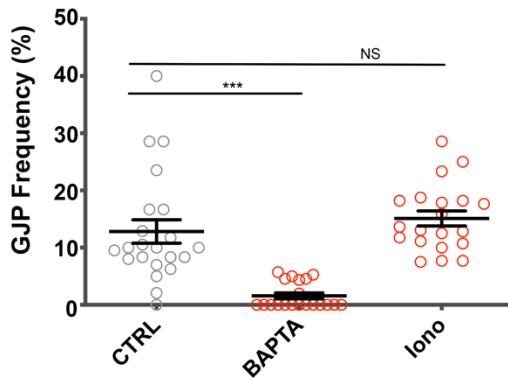


Supplementary Figure 5.6.1: CaMKII and Cx36 mutants showed normal expression in Neuro2a cells. **a)** Pseudosubstrate binding region sequence alignment of all CaMKII isoforms (291 to 311) to Cx36 CT (272 to 292). Reserved region across all isoforms are indicated in green. **b)** Western blot analysis of double transfected Neuro2a cells with wild type CaMKII α (WT), CaMKII mutants (T286A, T286D, F293A and R296A), Cx36 (WT) and Cx36 mutants (R278A, K279A and K281A). (Red channel: CaMKII α -EGFP or Cx36-EGFP; green channel = β -actin control) **c)** Images of Neuro2a cells doubly transfected as outlined in b. Scale bar = 10 μm . **(d,e)** GJP frequency and size for wild type Cx36 (WT) and the Cx36-mutants. Beeswarm graphs with mean $\pm$ SEM; Mann-Whitney U (2-tailed) significance, *** $p < 0.01$, ** $p < 0.05$, NS, not significant.

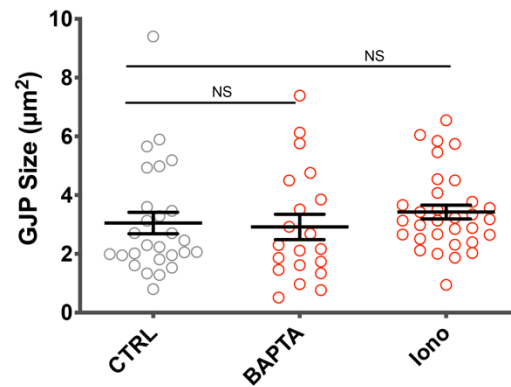
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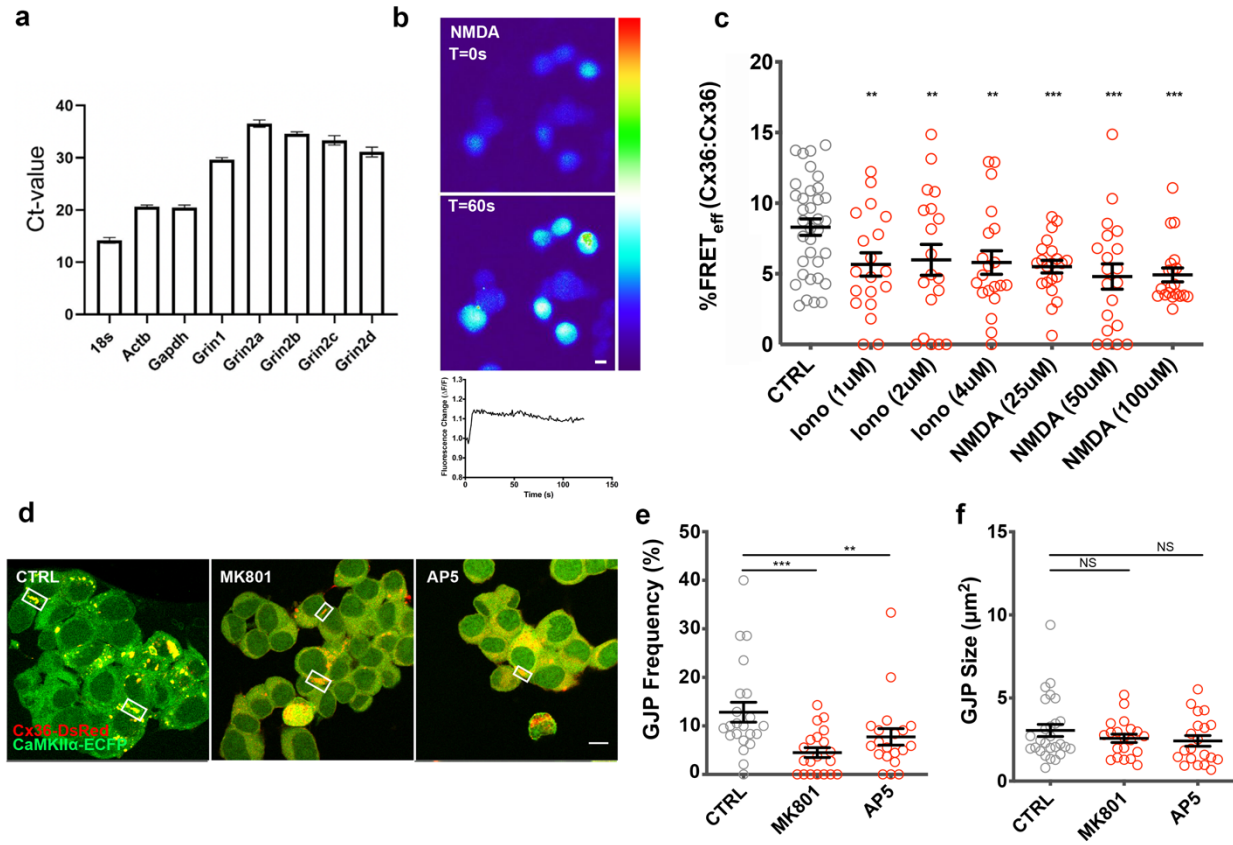
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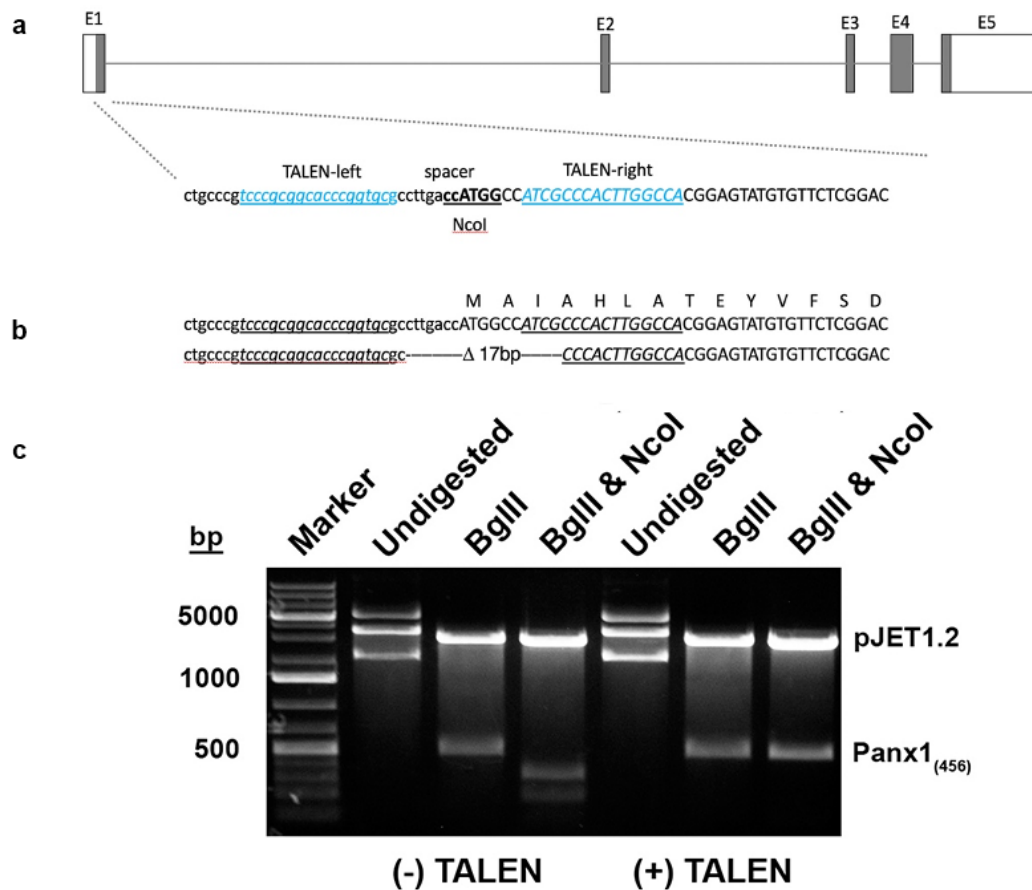
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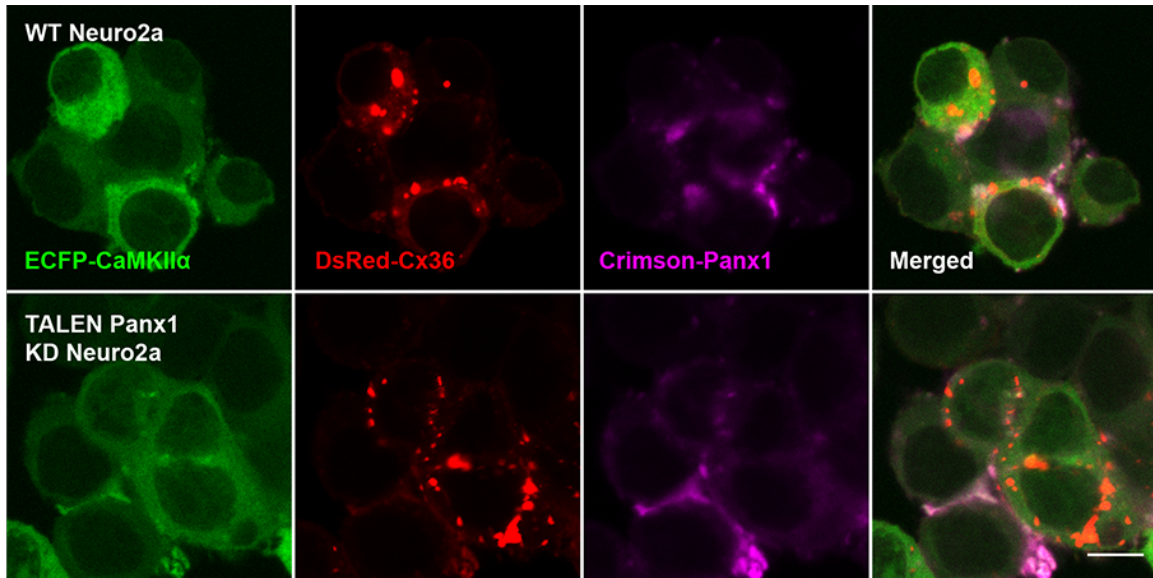
Supplementary Figure 5.6.2: Chelation of calcium reduced gap junction plaque (GJP) frequency but not size of Neuro2a cells expressing CaMKIIα-ECFP and Cx36-DsRed. a) Doubly transfected Neuro2a cells after treatment with BAPTA, ionomycin, and DMSO. Gap junction plaques indicated with a white box. Scale bar = 10μm. **b & c)** GJP frequency and size for treatments outlined above. Beeswarm graphs with mean±SEM; Mann-Whitney U (2-tailed) significance, ***p < 0.01, **p < 0.05, NS, not significant.



Supplementary Figure 5.6.3: Subunits of NMDA receptors are expressed in Neuro2a cells and respond to stimulants and pharmacological blockers. **a)** mRNA levels of different ionotropic NMDA receptor subunits in Neuro2a cells. **b)** Rapid influx of calcium (OGB) following NMDA stimulation of NMDA receptors. Sample trace represent relative fluorescence ($\Delta F/F$) of $[Ca^{2+}]_i$ in ROI. Scale bar = $10\mu m$ **c)** Dose-dependent decrease in FRET_{eff} contrasting non-selective (ionomycin) and selective (NMDA) increase of $[Ca^{2+}]_i$. **d)** Images of double-transfected Neuro2a cells without and with NMDA receptor antagonist treatments. GJPs were highlighted in rectangles. Scale bar = $10\mu m$. Quantification of gap junction plaque (GJP) frequency **e)** and size **f)** of double-transfected Neuro2a cells expressing CaMKII α -ECFP and Cx36-DsRed post-treatment with NMDA receptor blockers. Beeswarm graphs in **b,e,f)**: mean $\pm$ SEM; Mann-Whitney U (2-tailed) significance, *** $p < 0.01$, ** $p < 0.05$, NS, not significant.



Supplementary Figure 5.6.4: TALEN Panx1 knockdown (KD) strategy for Neuro2a cells. a,b) TALEN approach and results in generating Panx1 knockdown. Sequence alignment shows a deletion of 17bp for the TALEN Panx1 (KD) cells. **c)** Restriction digest test for WT and TALEN Panx1 (KD) Neuro2a cells. WT cells show two bands with BglII and NcoI digest, while TALEN Panx1 KD cells show abolishment of NcoI site.



Supplementary Figure 5.6.5: Triply transfected WT and TALEN knockdown Panx1 Neuro2a cells show normal protein expressions. Neuro2a cells (WT and TALEN) expressing ECFP-CaMKII α , Cx36-DsRed, and Crimson-Panx1.

5.7) Figure Z-statistics

Figure 1		Average	SEM	Number	U	W	Z	p
b	WT	10.3	0.9	21				
	WT (Iono)	6.8	0.6	20	106	316	-2.713	7.0×10^{-3}
	R278A	3.2	0.5	20				
	R278A (Iono)	1.6	0.6	20	90	300	-3.007	3.0×10^{-3}
d	WT	5.8	3.0	20				
	WT (Iono)	29.6	8.6	22	140	350	-2.015	0.04
	R278A	11.5	1.1	22				
	R278A (Iono)	10.3	2.4	24	184	484	-1.759	0.08
e	WT	3.6	0.5	21				
	WT (Iono)	3.6	0.4	20	195	426	-0.122	0.90
	R278A	1.6	0.2	20				
	R278A (Iono)	1.8	0.2	20	188	398	-0.325	0.75
f	WT	15.6	2.5	20				
	WT (Iono)	11.8	1.3	20	168.5	378.5	-0.337	0.74
	R278A	16.3	2.9	20				
	R278A (Iono)	17.2	3.5	20	152	323	-0.317	0.75

Figure 2		Average	SEM	Number	U	W	Z	p
b	CTRL	5.1	0.4	21				
	CTRL (Iono)	8.1	0.5	20	68	299	-3.704	2.1×10^{-4}
	CTRL (KN93)	2.8	0.5	20	88	298	-3.183	1.0×10^{-3}
	F293A	2.9	0.6	20				
	F293A (Iono)	2.0	0.3	20	176	386	-0.649	0.52
	R296A	3.1	0.5	20				
	R296A (Iono)	3.0	0.6	20	175	385	-0.663	0.51
	T286A	8.9	1.0	20				
	T286A (Iono)	5.5	0.7	22	116	369	-2.169	0.01
	T286D	4.1	0.5	22				
c	T286D (Iono)	6.8	0.7	22	119	372	-2.887	4.0×10^{-3}
	CTRL	5.1	0.4	21				
	CTRL (Iono)	8.1	0.5	20	68	299	-3.704	2.1×10^{-4}
	R278A	6.1	0.8	20				

	R278A (Iono)	6.3	0.6	21	168	378	-0.061	0.95
	K279A	4.7	0.4	20				
	K279A (Iono)	6.3	0.5	20	126	336	-2.002	0.05
	K281A	5.7	0.6	20				
	K281 (Iono)	6.6	0.5	20	136	346	-1.731	0.09

Figure 3		Average	SEM	Number	U	W	Z	p
c	CTRL	5.6	0.5	20				
	CTRL (Iono)	9.9	1.1	19	92	302	-2.754	5.9×10^{-3}
	BAPTA	1.6	0.3	20	29	219	-4.526	6.0×10^{-6}
	DMSO	6.6	0.8	23	212	422	-0.438	0.66

Figure 4		Average	SEM	Number	U	W	Z	p
a	CTRL	1.7	0.5	20				
	Gly	4.5	0.8	21	79	289	-3.420	6.27×10^{-4}
	Glu	5.3	0.6	20	60	270	-3.791	1.50×10^{-4}
	Gly & Glu	6.9	0.5	19	30	240	-0.493	6.79×10^{-6}
	NMDA	7.0	0.7	23	39	249	-4.654	3.25×10^{-6}
b	CTRL	3.6	0.3	20				
	NMDA	4.9	0.4	22	124.5	334.5	-2.405	0.02
	EDTA	5.3	0.5	19	102	312	-2.473	1.3×10^{-2}
	EDTA & NMDA	6.8	0.2	19	5	215	-5.198	2.0×10^{-7}
	MgSO4	2.6	0.4	19	105	295	-2.389	0.02
	MgSO4 & NMDA	3.7	0.3	20	191.5	401.5	-0.230	0.82
d	CTRL	-1.6	0.9	27				
	EDTA	20.6	5.9	20	98	476	-3.701	2.15×10^{-4}
	EDTA & NMDA	49.9	6.0	25	10	388	-5.998	2.0×10^{-9}
	EDTA & NMDA & MK801	23.4	5.1	20	49	427	-4.755	1.98×10^{-6}
	DMSO	3.1	2.9	21	204	582	-1.652	0.1
e	CTRL	3.6	0.3	20				
	NMDA	4.9	0.4	22	124.5	334.5	-2.405	0.02
	MK801	3.3	0.5	24	220	520	-0.472	0.64
	MK801 & NMDA	4.5	0.5	32	245.5	455.5	-1.402	0.16
	AP5	5.8	0.5	20	86	296	-3.084	2.0×10^{-3}

	AP5 & NMDA	4.7	0.5	20	131	341	-1.867	0.06
f	CTRL	10.3	3.6	21				
	NMDA	37.8	7.0	22	109	340	-2.964	3.0×10^{-3}
	MK801	13.1	4.8	20	207	417	-0.078	0.94
	MK801 & NMDA	17.9	4.9	20	175	406	-0.913	0.36

Figure 5		Average	SEM	Number	U	W	Z	p
b	CTRL	-1.5	1.5	22				
	Kglu	47.4	5.4	22	0	210	-5.541	3.0×10^{-8}
	Kglu & BBFCF	21.1	5.7	20	93	303	-2.894	3.8×10^{-3}
	Kglu & 10Panx	3.4	3.9	26	240	450	-0.443	0.66
	Kglu & ScPanx	53.7	4.2	25	0	210	-5.710	1.1×10^{-8}
d	CTRL	8.5	0.7	28				
	Kglu	4.2	0.6	23	147	423	-4.061	4.9×10^{-5}
	Kglu & 10Panx	7.0	0.6	20	265	475	-1.487	0.14
	Kglu & ScPanx	5.1	0.5	22	183	436	-3.311	9.3×10^{-4}
	Kglu & BBFCF	9.6	0.8	24	330	960	-1.389	0.16
f	CTRL	5.1	0.4	21				
	Kglu	9.3	1.0	23	129	339	-2.459	0.01
	Kglu & 10Panx	2.5	0.4	20	78	288	-3.301	1.0×10^{-3}
	Kglu & ScPanx	8.2	0.8	20	112	322	-2.380	0.02
	Kglu & BBFCF	6.4	0.8	21	176	386	-0.887	0.38

Figure 6		Average	SEM	Number	U	W	Z	p
b	CTRL	9.2	3.2	21				
	Kglu	32.1	8.4	19	123	354	-2.072	0.04
	Kglu & 3mM ATP	5.8	3.1	19	171	361	-0.772	0.44
	CTRL	14.2	5.5	21	218	449	-0.063	0.95
	Kglu	2.6	4.6	19	136	326	-1.720	0.09
	Kglu & 3mM ATP	10.1	6.3	22	227	480	-0.097	0.92
c	CTRL	4.0	0.6	19				
	Kglu	6.6	0.4	21	89	279	-2.993	2.8×10^{-3}
	0.1mM ATP	6.8	0.5	21	91	281	-2.939	3.3×10^{-3}
	3mM ATP	3.5	0.3	21	190	421	-0.257	0.80

	Kglu & 0.1mM ATP	6.5	0.6	22	86	276	-3.074	2.1×10^{-3}
	Kglu & 3mM ATP	4.7	0.5	20	150	340	-1.124	0.26
d	CTRL	4.9	0.8	19				
	Kglu	4.7	0.2	18	159	369	-0.614	0.54
	0.1mM ATP	4.9	0.3	21	180	390	-0.782	0.43
	3mM ATP	4.9	0.3	25	216	426	-0.777	0.44
	Kglu & 0.1mM ATP	5.2	0.6	20	169	379	-0.839	0.40
	Kglu & 3mM ATP	4.7	0.5	19	169	379	-0.590	0.56
e	CTRL	2.1	0.3	20				
	CTRL & Kglu	5.3	0.5	21	52.5	262.5	-4.108	4.0×10^{-5}
	TALEN	1.7	0.4	21				
	TALEN & Kglu	3.2	0.4	22	132	363	-2.410	0.02
f	CTRL	2.1	0.3	20				
	CTRL & Kglu	5.3	0.5	21	52.5	262.5	-4.108	4.0×10^{-5}
	BAPTA	1.9	0.3	21	207	417	-0.078	0.94
	BAPTA & Kglu	1.8	0.4	22	174.5	427.5	-1.150	0.25
	10Panx	2.0	0.3	21	205	415	-0.130	0.90
	10Panx & Kglu	3.1	0.5	20	143	353	-1.542	0.12
	ScPanx	2.7	0.4	21	153	363	-1.487	0.14
	ScPanx & Kglu	4.7	0.5	21	66.5	276.5	-3.743	1.8×10^{-4}

Figure 7		Average	SEM	Number	U	W	Z	p
a	CTRL	5.8	3.0	20				
	NMDA	32.7	7.4	23	99	309	-3.190	1.4×10^{-3}
	PP2	11.8	4.5	22	163.5	373.5	-1.423	0.15
	PP2 & NMDA	9.7	5.7	20	195	405	-0.135	0.89
	PP3	9.0	4.7	23	217.5	427.5	-0.304	0.76
	PP3 & NMDA	35.1	6.2	22	75	285	-3.652	2.6×10^{-4}
b	CTRL	12.8	2.0	22				
	PP2	6.6	1.3	20	111.5	321.5	-2.741	6.0×10^{-3}
	PP3	11.0	2.0	20	182	392	-0.968	0.34
c	CTRL	3.1	0.4	22				

	PP2	3.5	0.6	21	262	640	-0.447	0.66
	PP3	3.8	0.3	24	199	577	-2.359	0.02
e	CTRL	5.9	0.9	19				
	PP2	3.1	0.8	21	108	339	-2.479	0.01
	PP3	6.9	1.1	20	168.5	358.5	-0.604	0.55
g	CTRL	2.0	0.3	25				
	CTRL & Kglu	5.3	0.7	24	102	427	4.000	7.5×10^{-5}
	Y308F	1.8	0.4	21	242	567	-0.452	0.65
	Y308F & Kglu	1.6	0.3	22	240	565	-0.747	0.46
	Y308A	2.7	0.6	21	234	465	-0.630	0.53
	Y308A & Kglu	2.8	0.5	22	240	493	-0.747	0.46
	Y198F	1.0	0.3	23	190	421	-1.601	0.11
	Y198F & Kglu	1.9	0.4	22	182	413	-1.776	0.08
	Y198A	1.3	0.3	21	161	437	-2.631	0.01
	Y198A & Kglu	1.2	0.3	21	262	515	-0.277	0.78

Figure S1		Average	SEM	Number	U	W	Z	p
a	WT	0.1	0.0	22				
	R278A	0.1	0.0	18	161	414	-1.008	0.31
	K279A	0.1	0.0	18	69.5	240.5	-3.497	4.7×10^{-4}
	K281A	0.1	0.0	18	120.5	291.5	-2.109	0.03
b	WT	3.1	0.4	27				
	R278A	2.5	0.3	20	231	441	-0.839	0.40
	K279A	2.3	0.3	20	214	424	-1.205	0.23
	K281A	3.4	0.7	20	270	480	0.000	1.00

Figure S2		Average	SEM	Number	U	W	Z	p
a	CTRL	0.1	0.0	22				
	BAPTA	0.0	0.0	20	22	232	-5.084	3.7×10^{-7}
	Iono	0.2	0.0	20	146	399	-1.865	0.06
b	CTRL	3.1	0.4	27				
	BAPTA	2.9	0.4	30	253	463	-0.366	0.71
	Iono	3.4	0.2	32	306	684	-1.917	0.06

Figure S3		Average	SEM	Number	U	W	Z	p
a	CTRL	0.1	0.0	35				
	Iono (1uM)	0.1	0.0	19	200	390	-2.400	0.02
	Iono (2uM)	0.1	0.0	20	236.5	446.5	-1.987	0.05
	Iono (4uM)	0.1	0.0	20	210	420	-2.450	0.01
	NMDA (25uM)	0.1	0.0	21	197	428	-2.886	3.9×10^{-3}
	NMDA (50uM)	0.0	0.0	20	175.5	385.5	-3.053	2.3×10^{-3}
	NMDA (100uM)	0.0	0.0	20	153.5	363.5	-3.438	5.9×10^{-4}
b	CTRL	0.1	0.0	22				
	MK801	0.0	0.0	20	84	294	-3.438	5.9×10^{-4}
	AP5	0.1	0.0	20	126.5	336.5	-2.359	0.02
c	CTRL	3.0	0.4	27				
	MK801	2.6	0.3	20	248	458	-0.473	0.64
	AP5	2.4	0.3	20	206	416	-1.377	0.17

CHAPTER SIX: General discussion

6.1) Summary of findings

The encompassing discovery of this thesis is the interconnected signaling pathway between NMDA receptors, Src kinase, Panx1 channels, connexin 36 channels, and Ca^{2+} /CaM/CaMKII. Analysis of these signaling pathways was achieved through live and fixed cell imaging (including FRET, gapFRAP, Ca^{2+} imaging, and more to name) as well as molecular biology and biochemical analyses. The novel findings outlined in this thesis are as follows: data in *Chapter 4* revealed the importance of CaM directly interacting and regulating Cx36 outside of the gap junction plaque (namely in Golgi apparatus and endoplasmic reticulum). CaM was originally thought to be the secondary messenger of Ca^{2+} signaling for various protein kinases, including CaMKII. However, the competitive binding region of CaM and CaMKII allow Cx36 to have dual-modality (outside of GJP favors CaM, while at the GJP favors CaMKII). In addition, the NMR solution structure revealed the importance of Cx36-W277 anchor and CaM-Cx36 structure supports a 1-8-14 binding motif similar to other proteins like NMDARs and smMLCK. These findings show the importance of this CaM-Cx36 interaction as a critical step preceding CaMKII-mediated plasticity at the electrical synapses.

Chapter 5 revealed the discovery of a signaling pathway consisting of ionotropic NMDA receptors, SFK, and Panx1 that impacts the Cx36-CaMKII interaction at the GJP. By utilizing cellular imaging (as previously mentioned), the critical residues for CaM (F293 and R296 of CaMKII) were important to preserve the interaction with Cx36. This emphasized the importance of CaM and activated CaMKII for Cx36 interaction. In addition to the importance of CaM, *Chapter 5* is also an exploration of the mechanistic interactions and influence of NMDA receptors within the

Ca²⁺/CaM/CaMKII-Cx36 signaling cascade. Through pharmacological blocking of NMDA receptors, the interaction of CaMKII and Cx36 is weakened. NMDA receptors provide a portal for Ca²⁺ entry upon stimulation, which increases protein interactions at the GJP. Also, Panx1 channels are known for their role in regulating cellular responses based on external stimuli. Previous reports have documented that the signaling complex between NMDA receptors and the Panx1 channel is achieved through phosphorylation by Src kinases (Weilinger et al. 2016). Stimulation of NMDA receptors induces phosphorylation of Src at Y416, which leads to the phosphorylation of Y308 and Y198 of Panx1. Activated Panx1 channels allow different ionic and metabolite exchanges, including Ca²⁺, which ramps up at the GJP through CaM and CaMKII. Pharmacological blocking of Panx1 using ¹⁰Panx peptide or blue food dye cuts off a source of Ca²⁺ needed for strengthening the Cx36-CaMKII interaction. The NMDA receptors, SFK, and Panx1 channel may not be directly influencing plasticity at electrical synapses but play a role in modulating the activities leading to plasticity interweaving chemical and electrical synapses.

The notion that both NMDA receptors and Panx1 channels can modulate Cx36 is relevant in physiology and pathology. As pannexins were originally thought to be the same family as connexins (Panchina et al. 2000), the distinction of pannexins from connexins have since been studied in isolation. Results from this thesis bridge the gap between pannexins and connexins in the events leading to synaptic plasticity. The results obtained from *Chapter 4* and *5* led us to formulate molecular pathway weaving events occurring at the vesicles (i.e., modification of Cx36 at the Golgi) and the membrane (i.e., NMDAR-Src-Panx1-Cx36 signaling pathway). The proposed pathway provided us with the avenue to visit outstanding questions in these studies. In the following discussion, I will identify potential questions for future studies and attempt to propose

experiments to address the questions. Furthermore, I will discuss the implications of this thesis and its relevance in current synaptic communication research.

6.2) Multifaceted role of Calmodulin

Calmodulin is well-known as a secondary messenger that regulates different kinase activities upon Ca^{2+} binding. In the last decades, CaM have been shown to interact with other channels and receptors. The data in *Chapter 4* described the novel role of calmodulin in regulating Cx36 outside of the GJP. A recent paper by John O'Brien's group pointed out a conflict with CaM affecting coupling of connexin 35/36 (Aseervatham et al. 2020). In the paper, the group concluded that the binding of CaM to Cx35 uncouples the gap junction. Although our results did not emphasize on the conduction of gap junctions, our results support the conclusion from O'Brien's paper. In *Chapter 4*, we concluded that CaM binding to Cx36 is favoured at the vesicular level, while CaMKII binding is favoured at the gap junction. The paper also pointed out the importance of timely analysis. Increase of intracellular Ca^{2+} through ionomycin can vastly differ depending on the timepoint of analysis. For example, the initial stimulation with ionomycin increase of Ca^{2+} and increases coupling within the first 1-2 min (Moore et al. 2020). By analyzing this timepoint, the increase in coupling and Ca^{2+} -driven activities can be analyzed. On the other hand, the analysis of a later timepoint with prolonged stimulation of ionomycin may cause stress to the cells. Although cell death is not often found in cells treated for 10 minutes, the accumulation of ROS and starts inducing necrosis after 1 hour at $1\mu\text{M}$ of ionomycin (Nakamura et al. 2016). The higher dosage of ionomycin used in Aseervatham et al. may expedite the accumulation of ROS due to increased intracellular Ca^{2+} through mitochondria and ER. This may result in uncoupling of connexins at the gap junction.

The direct binding of CaM on premature proteins (i.e., Cx36) in the ER-Golgi network suggested a regulatory role limiting further protein interactions before transport. This hypothesis is further supported by other studies documenting CaM's inhibitory influence on NMDA receptors (Leonard et al. 1999; C. Wang et al. 2008) and Sec61 complex in the ER (Erdmann et al. 2011). NMDA receptors are known for their role in LTP and Ca^{2+} -permeability. Interestingly, increasing Ca^{2+} levels promotes noncanonical CaM to inactivate the ionotropic NMDA receptor channels. This paradoxical occurrence may be a regulatory mechanism preventing excessive Ca^{2+} influx, which may cause excitotoxicity (Ataman et al. 2007). The direct binding of CaM allows fine control of Ca^{2+} levels at the synaptic membrane and at the ER. On a similar note, ER is one of the main reservoirs for Ca^{2+} in the cell, which must be regulated. CaM regulates protein activity of ion channels, especially ones involved in calcium balance (i.e., IP_3 receptors (Yamada et al. 1995), voltage-gated calcium channels (Dick et al. 2008), ryanodine receptors (Yamaguchi, Xin, and Meissner 2001), and Sec61 (Erdmann et al. 2011)). For example, the aqueous Sec61 complex pore has been associated with Ca^{2+} leakage, and uncontrolled leakage of Ca^{2+} will interfere with the capacity of ER to maintain Ca^{2+} concentration (four to five folds difference) to the cytosol (R. Yu and Hinkle 2000). The interaction of CaM with Sec61 triggers closure of the channel and prevents leakage of Ca^{2+} in the ER (Erdmann et al. 2011).

Panx1 channels are expressed both in the cell membrane and ER as well. Their physiological function allows the exchange of Ca^{2+} between extracellular space, cytosol, and ER with the cytosol. These channels have been associated with the Ca^{2+} leakage and intracellular Ca^{2+} homeostasis (Vanden Abeele et al. 2006). As mentioned previously, CaM is involved in regulating Ca^{2+} levels by directly binding to the receptors or channels. It is unclear whether Panx1 undergoes a similar

regulation by CaM but having the inhibition at the ER before transport would serve physiological advantage similar to that of both NMDA receptors and Cx36. To determine the effects of CaM on Panx1 at the membrane and ER, one approach similar to FRET but having higher signal/background noise is BRET. This approach can be done through the typical BRET pairs Renilla luciferase (Rluc) as the donor and YFP or GFP as the acceptor. When Panx1-Rluc is treated with coelenterazine, Rluc will emit light at around 400nm wavelength. If CaM-YFP or CaM-GFP is interacting (less than 10nm), energy will be transferred, and CaM will fluoresce in respective wavelength. These experiments can be done in Neuro2a cells with stimulatory (i.e., ionomycin) and inhibitory (i.e., W-7) protocols. The increase in BRET signals could then be recorded to determine the interaction between CaM-Panx1 under physiological, stimulated, or suppressed conditions. In order to detect the Ca^{2+} signals at the ER level, one can use Ca^{2+} indicators, for instance, X-Rhod-1 for more vesicular Ca^{2+} signaling, to observe the vesicular Ca^{2+} levels while Panx1 is blocked (i.e., $^{10}\text{Panx}$ or BB-FCF) or stimulated (i.e., potassium gluconate or ATP). These data will provide a better understanding of Panx1 signaling complex and its mechanism leading to plasticity.

6.3) Panx1 and its modulatory role on electrical synapses

How Panx1 modulates the activities at the GJP remains an area of research worth investigating. Most literature emphasizes the interaction of Panx1 with P2X7 receptors and its relationship with both AMPA and NMDA receptors. Little to no reports have shown a relationship with connexins. In *Chapter 5*, we have demonstrated that the blockage of Panx1 channels with mimetic peptide $^{10}\text{Panx}$ or blue food dye (BB-FCF) affect the potentiation at the GJP. How complexes with Panx1 influence the activation of the GJP remains unresolved.

Panx1 is known to form clusters with P2X7 receptors and is influenced by NMDA receptors through Src phosphorylation. P2X7 receptors are ligand-gated cation channels that are sensitive to ATP. Activation of the P2X7 receptors leads to the recruitment of Panx1, which allows ATP and other ions (i.e., Ca^{2+}) to move based on concentration (Iglesias et al. 2008). Activated P2X7 receptors and Panx1 also allows the spread of depolarization (S.-P. Chen et al. 2017). The release of ATP generates a feedback loop to sustain the activation of purinergic receptors (Scemes et al. 2007). Aside from the Ca^{2+} entry through purinergic P2XR, the transient receptor potential vanilloid receptors (i.e., TRPV4) activated by mechanical stress can also introduce Ca^{2+} into the cells (Ryskamp et al. 2011). The interaction between TRPV4 and Panx1 have been reported. TRPV4 can transduce stretch signals to Panx1 channels, which releases ATP (Shahidullah, Mandal, and Delamere 2012). In addition, Panx1 has also been reported to interact with voltage dependent potassium channel ($\text{Kv}\beta 3$) physically (Bunse et al. 2005). This subtype of potassium channels has shown to regulate Panx1 and attenuates different blockers (i.e., carbenoxolone and Probenecid) (Bunse et al. 2009). The results suggest that $\text{Kv}\beta 3$ serves as an essential regulator for Panx1 activation. The interaction with these channels and receptors further emphasizes the importance of regulating Panx1.

In *Chapter 5*, I have demonstrated a possible indirect relationship between Panx1 and Cx36. Although there have not been any reports showing connections between both channels, PKA and PKC (Penuela et al. 2007) are candidates for regulating the interaction between Cx36 and Panx1. PKA phosphorylation regulates the potentiation properties of Cx36 (Kothmann, Massey, and O'Brien 2009) and also the release of ATP by Panx1 (da Silva-Souza et al. 2014). The conversion of intracellular ATP to cAMP relays the signals from outside the cell to trigger downstream

signaling cascades, which include regulating the activation of both channels. Interestingly, some reports have shown that activation of Cx36, which leads to an increase in Ca^{2+} , activates PKC (Protzek et al. 2014). This suggests the possibility of mutual regulation between Cx36 and Panx1 through activated PKC.

To study the phosphorylation events that regulate Cx36 or Panx1, one could utilize the GCaMP3 construct designed and described in Moore and coauthors in 2020 to report Ca^{2+} changes in the microenvironment under different physiological conditions. By point mutating conserved sites for PKA or PKC on Cx36 or Panx1, one can determine the importance of particular phosphorylation events controlling the states of the channels. For example, Neuro2a cells can be transfected with Cx36-GCaMP3 and have mutated Panx1 tagged with mPlum (far-red construct). Different stimulatory protocols can determine the importance of these phosphorylation sites and their impacts on either Cx36 or Panx1 regulation.

6.4) Ionotropic vs. metabotropic NMDA receptor signaling complex

NMDA receptors are ionotropic glutamate receptors known for their ligand-gated properties to allow cations (i.e., K^+ , Na^+ , and Ca^{2+}) to the postsynaptic neuron. The flow of cations into the postsynaptic neuron increases the EPSP, causing an increase in intracellular calcium. A substantial increase in Ca^{2+} elicits LTP, while a small increase promotes LTD. The calcium concentration change acts as second messengers to promote intracellular cascades. Of the different processes, Ca^{2+} increase activates calmodulin, which has many binding partners, including c-Src and CaMKII. Reports have shown that the direct interaction of Ca^{2+} activated CaM with c-Src at the unique domain causes Src activity to increase. On the contrary, apo-CaM showed no interaction

with the c-Src unique domain, which suggests intracellular Ca^{2+} levels regulate these processes (Pérez et al. 2013). Src kinases drive Panx1 activation through phosphorylation and thus modulating the levels of Ca^{2+} in a feedback loop (Weilinger et al. 2016).

Recently, reports from the last decade have shown that NMDA receptors can induce LTD without the ion-flow through the channel (i.e., pharmacologically block the ion channel; MK-801 or APV) (Ghasemi and Schachter 2011). By blocking the ionic flow, binding of glutamate to NMDAR causes conformational change resulting in LTD (reviewed by: (Dore, Aow, and Malinow 2016)). The signaling complex involving NMDAR, Panx1, and Src kinase is linked with cellular dysfunction and neuronal damaging independent of ion flux (Weilinger et al. 2016). Peptides that suppressed Src kinase or interfered with Panx1 phosphorylation were effective in reducing neuronal damage in stroke models.

In *Chapters 5*, we documented the ionotropic NMDA receptor-Src kinase- Panx1 facilitating electrical synaptic plasticity through intracellular Ca^{2+} level. Blocking NMDA receptors with MK-801 or APV provided evidence for ionotropic signaling complex rather than metabotropic. MK-801 is a non-competitive inhibitor that blocks the ion channel but retains metabotropic properties. As MK-801 impaired the interaction at the GJP, data suggests the ionotropic properties of NMDA receptors to be essential for driving processes at the GJP. AP5, a competitive inhibitor of NMDA receptors, blocks both ionotropic and metabotropic properties of NMDA receptors. AP5 showed similar effects on Cx36-CaMKII interaction, which further supported a Ca^{2+} driven interaction. It is worth investigating the relevance of metabotropic NMDA receptors and LTD on electrical synaptic plasticity. Metabotropic NMDA receptor activation leads to the loss of AMPA and

NMDA receptors through endocytosis. It will be interesting to observe the changes to Panx1 and Cx36 through metabotropic NMDA receptor-triggered LTD.

6.5) Role of magnesium in plasticity

Mg²⁺ regulates NMDA receptors activation by blocking the channel and preventing Ca²⁺ to enter the cell. This control over NMDA receptor activation is important physiologically due to its involvement with synaptic plasticity and memory formation. Recently, reports have shown a Mg²⁺-dependent electrical synaptic plasticity in cells expressing Cx36 (Palacios-Prado et al. 2013, 2014). Prado et al. in 2013 and 2014 have demonstrated that synaptic transmission of GJPs expressing Cx36 was reduced with high intracellular Mg²⁺ and while a reduction in Mg²⁺ increased conduction. Since most intracellular Mg²⁺ are bound to ATP (Pecoraro, Hermes, and Cleland 1984), both free Mg²⁺ and ATP are dependent on the cellular metabolism state (i.e., increase metabolism demands results in more ATP release from mitochondria). Their findings can be incorporated into the current model with NMDA receptors, Panx1 and Cx36, which we have described in this thesis.

During physiological state, metabolic demands are low and ATP levels remain stable. As metabolic demands increase, more ATP is generated to meet cellular demands. When NMDA receptors are activated through depolarization, Ca²⁺ rushes into the cell and increases Cx36 conductance through Ca²⁺/CaM/CaMKII signaling. The increase of intracellular ATP resulted from increased metabolic demands reduces free Mg²⁺ and thus promotes increase in Cx36 conduction with neighboring cells. Panx1 channels open and release ATP into the extracellular space to regulate intracellular Ca²⁺ levels (Weilinger, Tang, and Thompson 2012). As ATP hydrolyzes, intracellular Mg²⁺ levels increase and tunes down Cx36 conduction. During

repolarization, NMDA receptors return to an inactive state, Panx1 channels close and intracellular ATP binds with Mg^{2+} to return to physiological state (**Figure 6.1**).

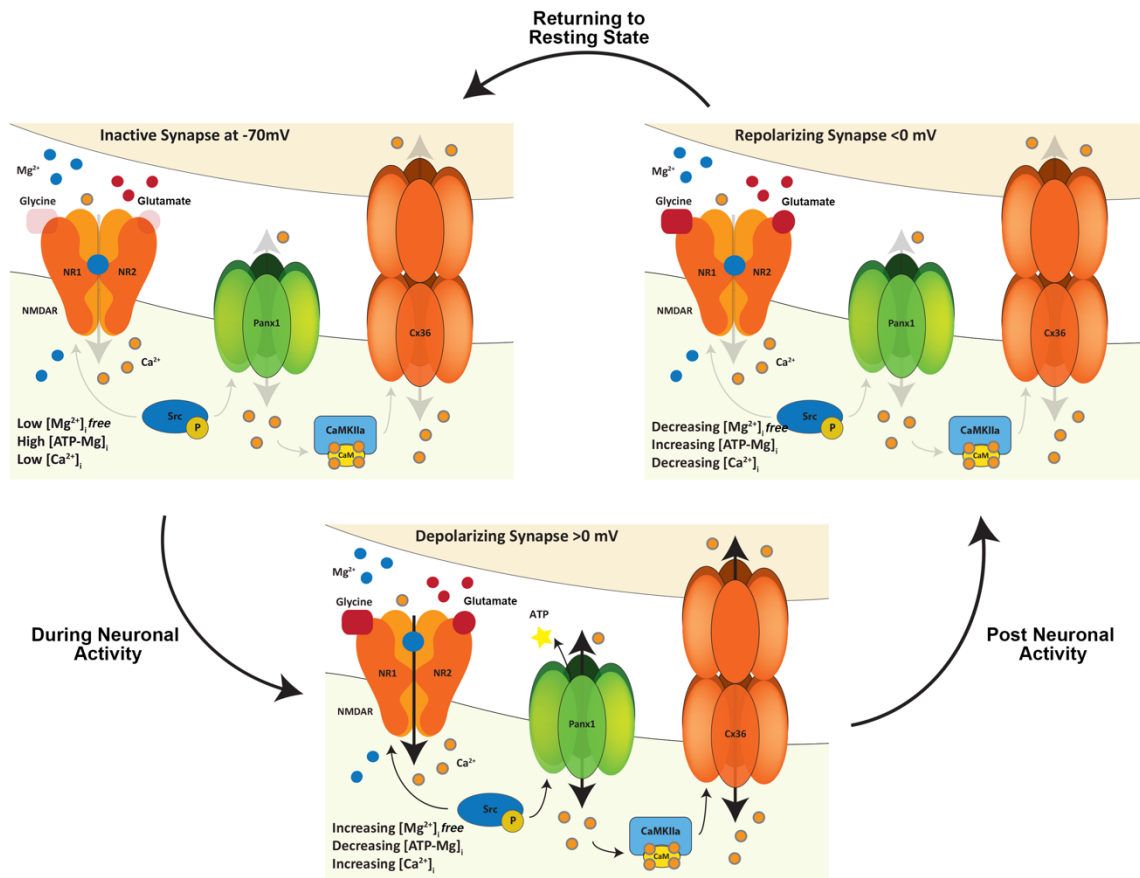


Figure 6.1: Balance of Mg^{2+} , Ca^{2+} and ATP dependent on cellular state. Inactive synapses under physiological state have NMDARs blocked with Mg^{2+} and both Panx1 and Cx36 closed. During demands of neuronal activity, synapses depolarize and thus activate NMDARs and opening of Panx1 and Cx36 through increased kinase activities (i.e., Src kinase and CaMKII). During repolarization, NMDARs inactivate and both Panx1 and Cx36 close. The closure and inactivation return the synapses to physiological state preparing for the next cycle.

6.6) Proposing a unified signaling pathway

Both chemical and electrical synaptic communication is often studied as separate means of neuronal communication, until the discovery of mixed synapses in Mauthner cells (Alberto E. Pereda et al. 2004). Previously, the run-up phenomenon has been described by our lab as the physiological signature of plasticity at the electrical synapses (Del Corso et al. 2012) analogous to the chemical synapses. The run-up phenomenon's focus was based on CaMKII driving the whole process of plasticity at the Cx36 nexus (Alev et al. 2008). In *Chapters 4 and 5* of this thesis, we have expanded the pathway to incorporate the roles of CaM, NMDA receptors, and Panx1 channels preceding the plasticity at electrical synapses. Here, we propose a mechanistic representation collating the results from the thesis to unify both chemical synaptic proteins (AMPA receptors and NMDARs) with electrical synaptic proteins (Cx36), along with Panx1 channels through kinase-mediated pathways (**Figure 6.2**).

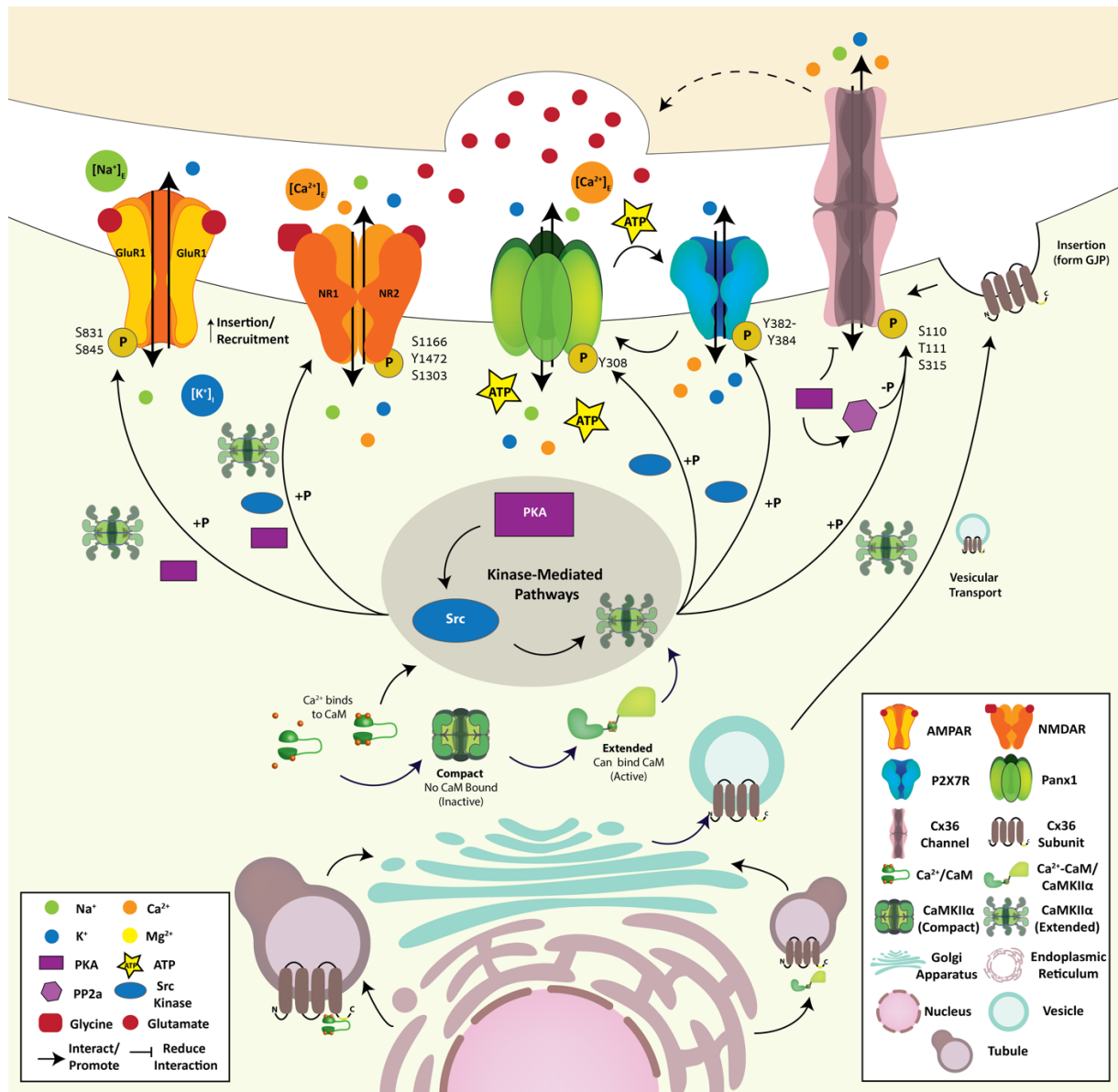


Figure 6.2: Proposed molecular pathway for asymmetric functionality of mixed synapses (AMPA, NMDAR, Panx1 and Cx36).

Action potential from the presynaptic terminal (calcium influx regulates the transport of vesicular transport) signals the release of vesicular neurotransmitters (i.e., glutamate) into the synaptic cleft. Receptors in the post-synaptic membrane are activated and allow ionic exchange (influx of positive ions: Na^+ and Ca^{2+}). The surge of Ca^{2+} activates CaM, which activates various kinase-mediated pathways (through PKA, Src and CaMKII). The increased activity of CaM promotes the modification of connexin 36 (Cx36) in the endoplasmic reticulum and Golgi apparatus. These proteins are transported to the membrane and inserted to form gap junctions. As CaM activates CaMKII, the holoenzyme complex moves from a compact state to an extended state upon CaM binding. The extended active form of CaMKII promotes further activation of other channels and receptors (including AMPA and NMDA receptors and Cx36). Activated Src kinase responds similar to CaMKII by phosphorylation NMDA receptors, Panx1, and P2X7 receptors, which promotes entry of Ca^{2+} . The influx of Ca^{2+} through P2X7Rs positively feedback to Panx1, while

the release of ATP through Panx1 feedback to activate more P2X7 receptors. PKA regulates AMPA and NMDA receptor through phosphorylation and modulate activities of Cx36 (through phosphorylation or dephosphorylation of PP2a)) (pointed arrow = promotes/ activates; blunt arrow = blocks/ inhibits).

Electrical signals travel to the presynaptic terminal and activate voltage gated Ca^{2+} channels (VGCCs) allowing the influx of Ca^{2+} (not shown in figure). The influx of Ca^{2+} regulates the vesicular release of glutamate (or NMDA agonist) into the synaptic cleft, which allows the activation of AMPA receptors at the postsynaptic terminal. Upon the ligand-binding activation of receptors, Na^+ rushes into the post-synaptic terminal, while extracellular K^+ increases (**Figure 6.2; left**). The shift in ionic equilibrium allows the removal of NMDA receptors internal Mg^{2+} blockade, and thus allow Ca^{2+} to influx into the cells. The increased Ca^{2+} levels allow calmodulin to deliver the signal to different proteins in different areas of the cell. Of the many locations, CaM can interact with immature NMDA receptors and Cx36 in the endoplasmic reticulum and the Golgi apparatus. This direct binding of CaM allows the immature proteins to remain inactivated before arriving at the membrane. In terms of Cx36, CaM binding is highly favored, while CaMKII interactions are limited at the ER-Golgi complex (Siu et al. 2016). Receptors and channels are trafficked through vesicular transport to their designated membrane locations. In addition, elevated intracellular Ca^{2+} levels allow calmodulin (CaM) to bind with high affinity and thus promote the production of cAMP from ATP at the adenylyl cyclase (AC) (MacNeil, Lakey, and Tomlinson 1985) and interact with CaMKII and Src kinases (Stateva et al. 2015). The CaMKII, Src kinases, and PKA feedback loop phosphorylate (+P) different channels and receptors at different serine or tyrosine residues. Phosphorylation of AMPARs by CaMKII or PKA (S831 (Kristensen et al. 2011) or S845 (Roche et al. 1996), respectively) further increases the recruitment of AMPARs (Makino and Malinow 2009). Similarly, activated CaMKII, PKA and Src kinase (B. S. Chen and Roche 2007; Y. Zhou et al. 2007; Murphy et al. 2014; Salter and Pitcher 2012) phosphorylate the NR2B

subunit of NMDARs at Y1303, S1166, and Y1472 respectively to further drive the influx of Ca^{2+} into the cell. As demonstrated previously by our lab, activated holoenzyme CaMKII (with CaM bound) phosphorylates Cx36 at S110, T111, and S315 to promote conduction (Alev et al. 2008). Recent research has shown PKA having modulatory effects on the opening probability through direct phosphorylation (Bazzigaluppi et al. 2017a) or dephosphorylation by PP2a (Kothmann, Massey, and O'Brien 2009), which suggests various enzymes regulate the process. As Panx1 allows various ions to be exchanged between cells and the extracellular space, increased activity of Src kinases encourages an increase in ionic exchange after phosphorylation at Y308. The release of ATP via Panx1 activates P2X7 receptors, while the influx of Ca^{2+} feedback to the activation of Panx1 (Shestopalov and Slepak 2014; Locovei et al. 2007). While ATP activates P2X7 receptors extracellularly, the increased activities of Src kinases also phosphorylate P2X7 receptors at Y382-Y384 to further ramp up the activation process (Kopp et al. 2019). Whether Panx1 plays a direct or indirect role in regulating the run-up and influencing the activation of NMDARs remains unresolved. Also, how the feedback (positive or negative) through Cx36 can influence presynaptic signaling pathways remain elusive.

6.7) Significance

Despite extensive research in understanding synaptic plasticity mechanisms, we have yet to fully understand the molecular pathways leading to strengthening or weakening of synapses. Ever since S. Ramon y Cajal and C. Sherrington solidified the stage for modern neuroscience in the early 1900s, the field debated the nature of synaptic transmission on whether neuronal signals were mediated by chemical or electrical synapses (Haider 2007). With more recent research emerging in the last few decades, it is an indisputable fact that both chemical and electrical

synapses coexist and contribute to transmissions leading to synaptic change. A knowledge base of different proteins and channels involved in plasticity has been established but combining all of the pathways involved will indeed be a challenge. In this thesis, we have attempted to combine the mechanism for electro-chemical transmission and demonstrated molecular asymmetry at electrical synapses. Data uncovers a mechanistic link between NMDARs, Src kinase, Panx1, and Cx36. This non-canonical signaling complex of NMDAR, Panx1, and Cx36 is not only novel for physiology, it may also further our understanding of their role(s) in other diseases. For instance, NMDA receptors, Panx1, and Cx36 activities have been implicated in diseases like Huntington's, Alzheimer's diseases, and ischemia (Lakhan, Caro, and Hadzimichalis 2013; Penuela et al. 2014; Yamasaki 2018). Drugs that target specific receptors, for example memantine blocking NMDARs in Alzheimer's disease, may utilize the pathway to better understand the true impacts on the system and to avoid side effects. In conclusion, this thesis unraveled mysteries in mixed synaptic transmission and laid a new layer of foundation for synaptic communication that spans across neuroscience.

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APPENDICES

A.1) Supplemental figures relating to *Chapter 4* (NMDAR)

In this section, I will present unpublished results that complement findings in *Chapter 4* catering to the importance of NMDA receptors. These findings further provide the interconnectedness of NMDARs, Panx1, and Cx36. Here, supplementary experiments were performed to highlight the impact on GJP through pharmacological blocking of AMPA receptors, Src kinase or the Panx1 channel with NMDA as the activator. The suppression of AMPA receptors, Panx1 (by TALEN knockdown or mimetic peptide) or Src kinase negatively impacts potentiation at the GJP. Data suggests a disruption of the signaling cascade from NMDARs-Src-Panx1 converging to Cx36.

A.1.1) AMPA/Kainite receptor inhibition modulates Cx36-CaMKII α

Indirect activation of NMDA receptors and initiation of calcium signaling by was tested using the competitive AMPA/Kainite receptor antagonists 6,7-dinitroquinoxaline-2,3-dione (DNQX) and kynurenic acid (KYNA). Blocking with both DNQX and KYNA reduced $[Ca^{2+}]_i$ (CTRL: -0.01 ± 0.006 , $n=16$; BAPTA: -0.10 ± 0.02 , $n=16$, $U=10$, $W=146$, $Z=-4.447$, $p=9.0 \times 10^{-6}$; DNQX: -0.09 ± 0.01 , $n=22$, $U=11$, $W=264$, $Z=-4.878$, $p=1.0 \times 10^{-6}$; KYNA: -0.12 ± 0.01 , $n=18$, $U=16$, $W=187$, $Z=-4.416$, $p=1.0 \times 10^{-5}$) similar to treatment of cells with BAPTA in our cell culture model (**Figure A.1.1a,b**). Cx36 GJP frequency (all values in %: CTRL: 12.82 ± 2.04 , $n=22$; DNQX: 5.85 ± 1.02 , $n=20$, $U=103.5$, $W=313.5$, $Z=-2.941$, $p=3.0 \times 10^{-3}$; KYNA: 6.07 ± 2.04 , $n=20$, $U=101.5$, $W=311.5$, $Z=-3.001$, $p=3.0 \times 10^{-3}$), but not size of GJPs was reduced in double-transfected Neuro2a cells (all values in nm: CTRL: 3.05 ± 0.36 , $n=22$; DNQX: 3.256 ± 0.43 , $n=20$,

U= 248, W= 626, Z= -0.473, p= 0.64; KYNA: 2.41 ± 0.32 , n=20, U= 223, W= 601, Z= -1.011, p= 0.31) (**Figure A.1.1c,d,e**).

The interaction between Cx36 and CaMKII α strengthened upon NMDA receptor agonist stimulation (all values in %: non-stimulated: 1.66 ± 0.5 , n=20; NMDA: 7.0 ± 0.7 , n=23, U= 39, W= 249, Z= -4.654, p= 0.29) and was suppressed when AMPARs were blocked with DNQX (DNQX: 2.33 ± 0.9 , n=20, U= 190.5, W= 400.5, Z= -0.260, p= 0.79; DNQX & NMDA: 2.80 ± 0.7 , n=20, U= 152, W= 362, Z= -1.306, p= 0.19) (**Figure A.1.1e**). The functional gap-FRAP assay showed that dye transfer was stimulated by NMDA (all values in %: non-stimulated: 5.78 ± 3.0 , n=20; NMDA: 44.84 ± 14.1 , n=24, U= 99, W= 309, Z= -3.190, p= 1.42×10^{-3}) and that this increase was reduced in the presence of DNQX (DNQX: 9.56 ± 5.2 , n=25, U= 207, W= 417, Z= -0.982, p= 0.33; DNQX & NMDA: 32.96 ± 7.67 , n=20, U= 93, W= 303, Z= -2.894, p= 3.80×10^{-3}) (**Figure A.1.1f**), suggesting that AMPA/Kainite-receptors contributed to signaling events that modulate the compactness of the Cx36-CaMKII α complex.

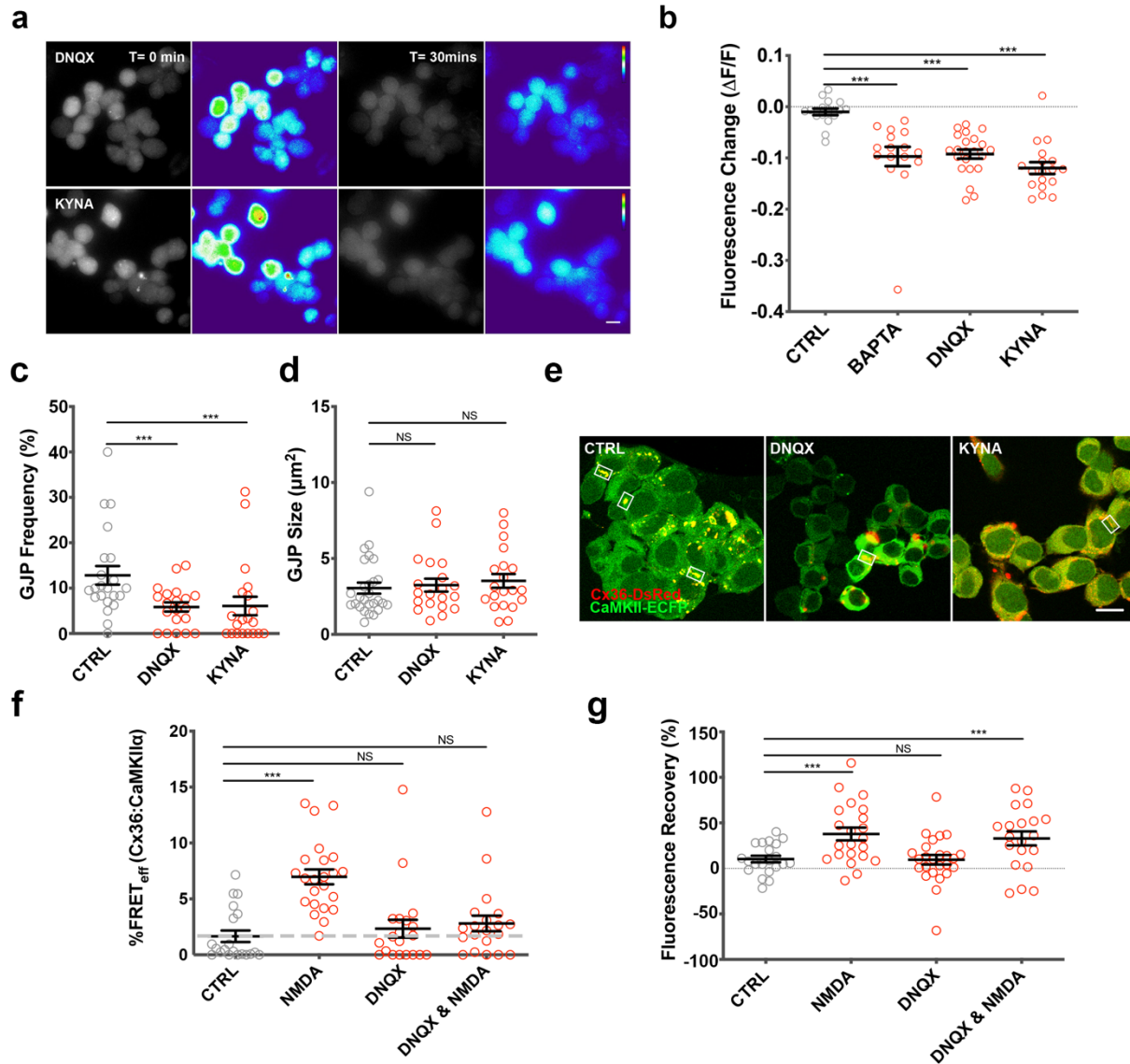


Figure A.1.1: Non-competitive inhibitors of AMPAR showed reduced Ca^{2+} signals and interaction of Cx36-CaMKII α . **a)** Neuro2a cells were treated with Ca^{2+} indicator OGB for 30 minutes before imaging with DMSO (CTRL), BAPTA (Ca^{2+} chelator) or inhibitors (DNQX, kynurenic acid (KYNA)). Images show Ca^{2+} intensities at T= 0 mins and T= 30 mins with thermopseudocolouring to indicate Ca^{2+} intensities; red= high Ca^{2+} , blue=low Ca^{2+} . **b)** Quantification of $[Ca^{2+}]$ with OGB comparing before and after intensities ($\Delta F/F$) for cells treated with DMSO, BAPTA, DNQX or KYNA. **c & d & e)** Quantification and expression of gap junction plaque (GJP) frequency and size of Neuro2a cells expressing CaMKII α -ECFP (green) and Cx36-DsRed (red) with inhibitors (DNQX or KYNA). **f)** FRET_{eff} of Cx36-DsRed and CaMKII α -ECFP with AMPARs antagonist DNQX (10 μ M) with and without 50 μ M NMDA. **g)** gapFRAP Fluorescence recovery of ethidium bromide with Cx36-EGFP expressing cells with DNQX AMPAR blocker with/without NMDA stimulation. Beeswarm graphs with mean $\pm$ SEM; dashed line indicates the 1.7% limit of FRET; Mann-Whitney U significance, ***p < 0.01, NS, not significant.

A.1.2) Membrane potentials are reduced through NMDAR suppression

Activation of neurons causes membrane potential changes as positive ions (i.e. K^+ and Ca^{2+}) travel across various membrane channels. Since NMDARs provide a surge of positive ions exchange through the cell (K^+ and Ca^{2+} respectively), the change in membrane potentials can signify activation of the cell. By utilizing the green fluorescent protein sensor Arclight S249, we were able to detect the changes in membrane potential through fluorescent changes over time with various pharmacological treatments (EDTA, EDTA & NMDA, EDTA & NMDA & MK801, and DMSO) (**Figure A.1.2**). By chelating $[Mg^{2+}]_E$ with EDTA alone or in tandem with NMDA stimulation, NMDARs allow the surge of Ca^{2+} into the cell and thus increased membrane potential. With the suppressive drug MK801, the effects of EDTA and NMDA has been significantly reduced. DMSO was used as a negative control to show the fluorescent change was caused by the drugs alone and not the solvent.

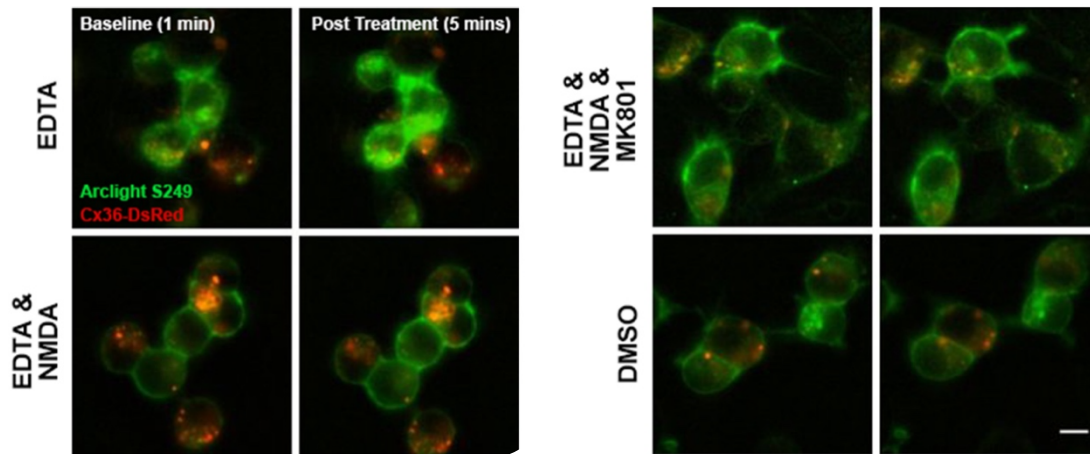


Figure A.1.2: Detecting voltage change with Arclight S249 under different conditions over five minutes. Neuro2a cells expressing Arclight S249 and Cx36-DsRed with various drug treatments (EDTA, EDTA & NMDA, EDTA & NMDA & MK801, and DMSO control) at one minute and five minutes (scale bar = 10 μ m).

A.1.3) NMDAR-Src kinase-pannexin 1 signaling affects connexin 36 potentiation

N-methyl-D-aspartate (NMDA) receptors in the central nervous system undergo complex regulation by phosphorylation. Src family kinases (SFKs), such as Src, closely associate with their substrate, the NR2 subunits of NMDA receptors, and regulate channel opening and ion flux (**Figure A.1.3A**). Previous research has shown the communication between NMDARs and Panx1 channels through Src kinases. As we have mentioned Panx1 channel being a contributor to membrane potential changes, we also tested the possibility of Panx1 channels providing a source of Ca^{2+} for driving the $\text{CaMKII}\alpha$ -Cx36 interaction. To test this hypothesis, the Panx1 inhibitory peptide $^{10}\text{Panx}$ and its control $^{\text{SC}}\text{Panx}$ were used in presence of the NMDA agonist (**Figure A.1.3B**). The control presented significant increase of FRET_{Eff} after NMDA stimulation (non-stimulated: 5.1 ± 0.4 , $n=21$; NMDA: $7.0\% \pm 0.7$, $n=23$, $U=39$, $W=249$, $Z=-4.654$, $p=3.25 \times 10^{-6}$). The effects of NMDA agonist were suppressed with $^{10}\text{Panx}$ ($^{10}\text{Panx}$ & NMDA: 3.09 ± 0.4 , $n=18$, $U=77$, $W=248$, $Z=-3.011$, $p=3.0 \times 10^{-3}$), but maintained with $^{\text{SC}}\text{Panx}$ ($^{\text{SC}}\text{Panx}$ & NMDA: 5.84 ± 0.2 , $n=19$, $U=119$, $W=329$, $Z=-1.995$, $p=0.05$) (**Figure A.1.3B**).

FRET data showed the negative impact on Cx36 and $\text{CaMKII}\alpha$ interaction when Panx1 was blocked. Data from gapFRAP also supported the hypothesis of Panx1 contributing to the dynamics of GJCs. Cells inhibited with $^{10}\text{Panx}$ had no significant recovery upon NMDA activation (with NMDA: 15.33 ± 6.96 , $n=21$, $U=186$, $W=396$, $Z=-0.626$, $p=0.53$), while the scrambled version of $^{10}\text{Panx}$, $^{\text{SC}}\text{Panx}$, had no impairment in fluorescence recovery (with NMDA: 22.26 ± 3.87 , $n=19$, $U=78$, $W=288$, $Z=-3.147$, $p=2.0 \times 10^{-3}$). Since NMDA was prepared using DMSO, the solvent made no significant impact on the ethidium bromide transfer (DMSO: 14.40 ± 3.80 , $n=26$, $U=192$, $W=402$, $Z=-1.507$, $p=0.132$) (**Figure A.1.3C**).

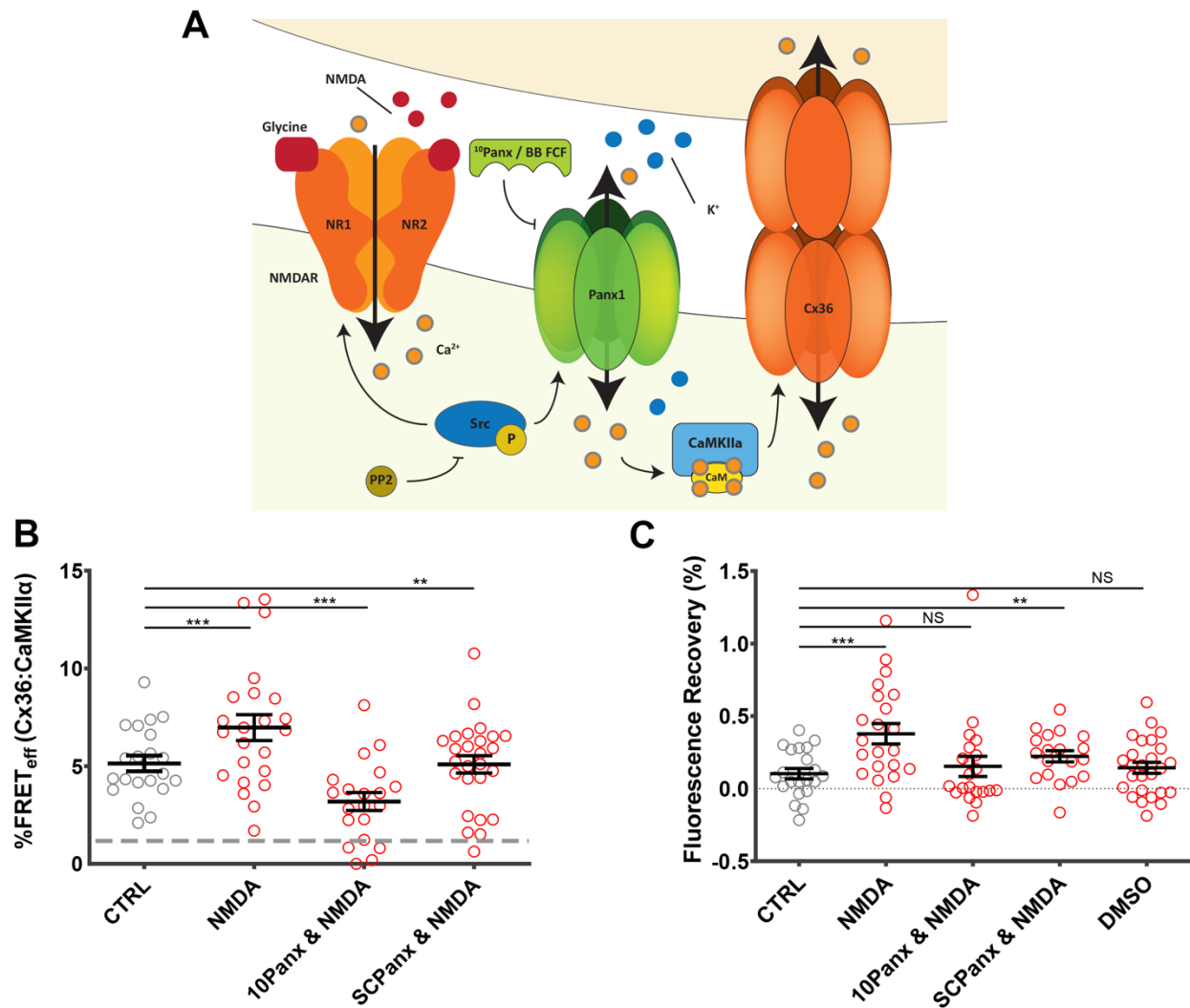


Figure A.1.3: Inhibition of Panx1 channels impedes the Cx36-CaMKII α interaction Neuro2a cells were either transfected doubly with Cx36-DsRed and CaMKII α -ECFP for FRET analysis or singly with Cx36-EGFP for gapFRAP analysis. **(A)** Illustration of mixed synaptic communication with Panx1 channel as an intermediate channel for Ca^{2+} influx upon kinase activation (pointed arrow= activation; blunt arrow= inhibition). **(B)** FRET_{eff} of Cx36-DsRed and Cx36-EGFP with various selective blockers of Panx1 ($^{10}\text{Panx}$ or $^{\text{SC}}\text{Panx}$) with 50mM NMDA. **(C)** gapFRAP redistribution of ethidium bromide with panx1 channel blockers $^{10}\text{Panx}$, scrambled blocker $^{\text{SC}}\text{Panx}$ or negative control of DMSO. Beeswarm graphs with mean $\pm$ SEM; dashed line indicates the 1.7% limit of FRET; Mann-Whitney U significance, **p < 0.05, ***p < 0.01, NS, not significant.

A.1.4) Knocking down Panx1 reduces NMDAR-Src kinase signaling

By abolishing Panx1 expression in our Neuro2a cells using TALEN, the interaction with CaMKII α and Cx36 had no significant effect after NMDA stimulation and was comparative to the increase in FRET_{eff} as seen in WT cells (WT non-stimulated: 1.66 ± 0.5 , n=20; NMDA: 6.97 ± 0.7 , n=23, U= 89, W= 279, Z= -2.671, p= 7.6×10^{-3} ; TALEN non-stimulated: 2.08 ± 0.4 , n=20; NMDA: 4.88 ± 0.7 , n=20, U= 89, W= 279, Z= -2.671, p= 7.6×10^{-3}). Although knocking down Panx1 expression in cells did not suppress the activation with NMDA, TALEN Panx1 KD cells responded significantly less after NMDA stimulation (U= 134, W=344, Z= -2.338, p= 0.02) (**Figure A.1.4A**).

The importance of Panx1 channels were further supported through ethidium bromide dye redistribution in TALEN KD Panx1 cells. Wildtype cells had significant fluorescence recovery after photobleaching post NMDA stimulation (non-stimulated: 10.31 ± 3.61 , n=21; NMDA: 37.80 ± 7.03 , n=22, U= 39, W= 249, Z= -4.654, p= 3.3×10^{-6}). While TALEN Panx1 KD cells showed no significant differences after NMDA stimulation (TALEN non-stimulated: 15.60 ± 6.61 , n=21; NMDA: 6.58 ± 5.13 , n=20, U= 79, W= 289, Z= -3.273, p= 1.1×10^{-3}) (**Figure A.1.4B**).

Since knocking down Panx1 expression affected the overall effectiveness of NMDA stimulation, overexpression of Panx1 should potentially reverse effects of the knockdown. When Neuro2a cells were transfected to overexpress Panx1-Crimson, both WT and TALEN Panx1 KD cells has increased FRET_{eff} prior to any stimulation (WT with Panx1-Crimson non-stimulated: 6.93 ± 0.3 , n=20; U= 12, W= 222, Z= -5.006, p= 5.5×10^{-7} ; TALEN non-stimulated: 4.44 ± 0.3 , n=20; U= 73, W= 283, Z= -3.128, p= 1.8×10^{-3}). Stimulation with NMDA showed marginal differences for WT cells (WT NMDA: 5.61 ± 1.0 , n=20, U= 83, W= 273, Z= -2.846, p= 4.0×10^{-3}), but significant increase for TALEN KD Panx1 cells (TALEN NMDA: 7.58 ± 0.5 , n=20, U= 25, W= 235, Z= -4.531, p= 5.9×10^{-6}) (**Figure A.1.4C**).

As results suggest, overexpression of Panx1 channels provide a source of Ca^{2+} entry to the cell similar to stimulation, blocking Panx1 with $^{10}\text{Panx}$ or chelate Ca^{2+} with BAPTA should attenuate the effects. When cells were treated with $^{10}\text{Panx}$ or BAPTA, FRET_{eff} was lower when compared to triply transfected WT cells ($^{10}\text{Panx}$: 6.1 ± 0.7 , $n=21$, $U=126$, $W=336$, $Z=-1.798$, $p=0.07$; BAPTA: 4.9 ± 0.7 , $n=20$, $U=32$, $W=203$, $Z=-4.224$, $p=2.4 \times 10^{-5}$). When cells were stimulated with NMDA post-treatment, significant increase of interacting $\text{CaMKII}\alpha$ and Cx36 was seen with $^{10}\text{Panx}$ ($^{10}\text{Panx}$ & NMDA: 8.4 ± 0.7 , $n=20$, $U=102$, $W=312$, $Z=-2.473$, $p=0.01$), but remained unchanged with BAPTA (BAPTA & NMDA: 4.3 ± 0.5 , $n=20$, $U=165$, $W=336$, $Z=-0.439$, $p=0.66$) (**Figure A.1.4D**). This suggests that overexpression of Panx1 may favour activities at chemical synapses rather than electrical synapses unless blocked by peptides or other compounds. Collectively, results indicated communication between chemical and electrical synapses involve a multitude of different receptors and channels, including Panx1 channel.

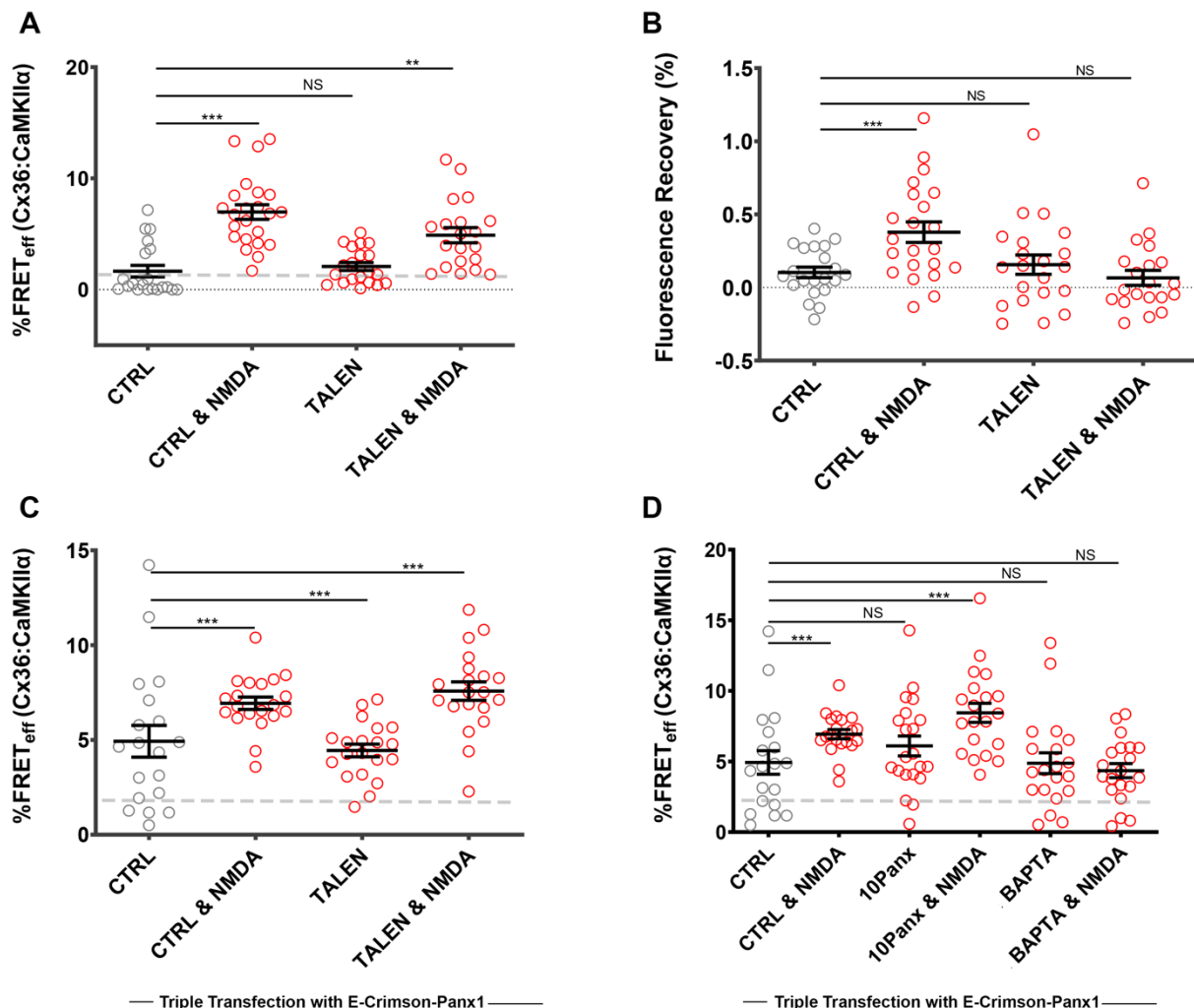


Figure A.1.4: Knocking down Panx1 channel expression with TALEN reduces effective binding of CaMKIIα and Cx36. Neuro2a cells (WT and TALEN KD Panx1) were either transfected doubly with Cx36-DsRed and CaMKIIα-ECFP or triply with the addition of Panx1-Crimson for FRET analysis or singly transfected with Cx36-EGFP for gapFRAP. **(A)** FRET analysis of Doubly transfected WT and TALEN cells with and without NMDA stimulation. **(B)** gapFRAP of WT Neuro2a cells or TALEN KD Panx1 cells stimulated NMDA. **(C)** Triply transfected WT or TALEN cells with and without NMDA stimulation. **(D)** Triply transfected WT cells in the presences of 10Panx or BAPTA with or without NMDA stimulation. Beeswarm graphs with mean±SEM; dashed line indicates the 1.7% limit of FRET; Mann-Whitney U significance, **p < 0.05, ***p < 0.01, NS, not significant.

A.2: Supplemental figures relating to *Chapter 4* (Panz1)

In this section, both cellular (transfected; **Figure A.2.1**) and tissue (IHC slices; **Figure A.2.2**, **Figure A.2.2.1**, **Figure A.2.2.2**) to support the findings in the chapter. Neuro2a cells were doubly transfected with EYFP-Panz1 and DsRed-Cx36. As immunohistochemical slices of the mouse brain were prepared and analyzed, we have also performed similar experiments on mouse retina to confirm the colocalization of Cx36 and Panx1. Dr. Liang Zhang generously donated wildtype retinal tissues and Panx1 KO tissues from UHN. Prominent colocalization found amongst Cx36 and Panx1 is the photoreceptor layer and also the ganglion cell layer.

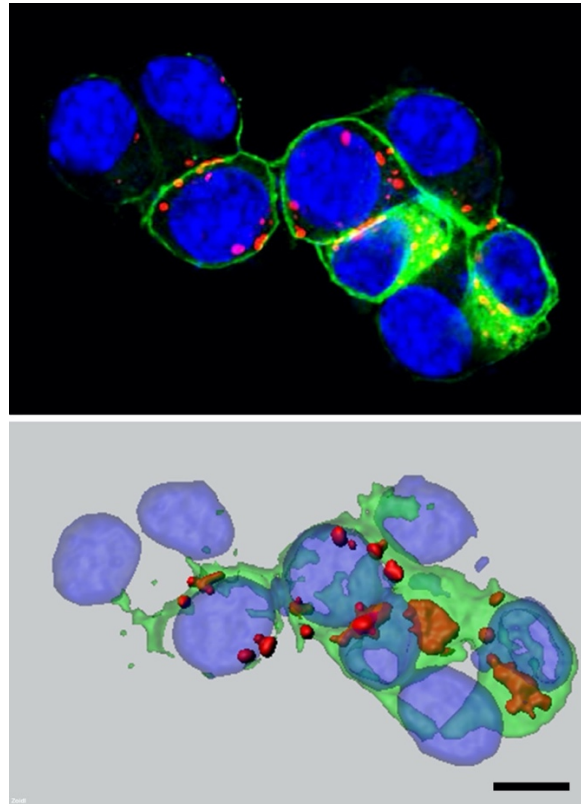


Figure A.2.1: Localization of Panx1 and Cx36 in Neuro2a cells. Cells were transfected with EYFP-Panz1 (green) and DsRed-Cx36 (red) and nuclei were denoted with DAPI (blue) (Top) (Scalebar= 10 μ m). (Bottom) 3D-reconstructed image of transfected Neuro2a cells expressing Panx1 and Cx36.

A.2.2) Cx36 and Panx1 protein co-localization in interneurons

Our previous work has shown prominent colocalization of Cx36 and Panx1 mRNA in the CA1 and CA3 regions of the hippocampus (Ray et al. 2005). Here, Cx36 and Panx1 proteins were identified in the hippocampus, thalamus, and the cortex (**Figure A.2.2A**). Colocalization of Cx36 and Panx1 was primarily restricted to interneurons identified by shape, localization (**Figure A.2.2B**), and immunoreactivity with parvalbumin (**Figure A.2.2.1**). 3-D reconstruction of images in CA1, CA3, cortex (CTX), and lateral dorsal thalamus (TH_{LD}) showed proximal expression of Cx36 and Panx1 within the same cell. Using a pan-CaMKII antibody revealed colocalization of Cx36 and CaMKII in interneurons, suggesting that Panx1 proteins are expressed alongside Cx36 and CaMKII proteins (**Figure A.2.2.1**). All CaMKII isoforms share the CaMKII binding motif CTB2 (Alev et al. 2008; Del Corso et al. 2012) required for the formation of the Cx36-CaMKII binding complex.

Similarly, colocalization of Cx36 and Panx1 can be found in the pigment layer with the photoreceptors and the ganglion cell layer. Panx1 knockout mice slices were used as negative control, which showed significant reduction of Panx1 in both the pigment and ganglion cell layers. Slices with no primary antibodies were used to test the specificity of the antibodies (**Figure A.2.2.2**).

Previous reports have shown the expression of Panx1 in interneurons and pyramidal cells in the cortex and hippocampus (Ray et al. 2005). Although Cx36 has been mainly documented in neuronal cells, some reports have shown Cx36 expressed in parvalbumin-positive interneurons into CA3 of the hippocampus (Kinjo et al. 2014). Research has shown the importance of Cx36

coupling in interneurons for short-term spatial memory. Cx36-coupling of interneurons are involved in spatial selectivity and cognition of hippocampal place cells (pyramidal cells). Inhibitory interneurons regulate the firing of these place cells (Allen et al. 2011). Others have shown the importance of parvalbumin-positive interneurons in CA1 social memory (Deng et al. 2019), preventing memory loss and hyperexcitability networks in mice (Hijazi et al. 2019). Since Panx1 and Cx36 have been documented expression in interneuron (notably in CA3), the possibility of Panx1 having a regulatory role on Cx36 at the system level is valid.

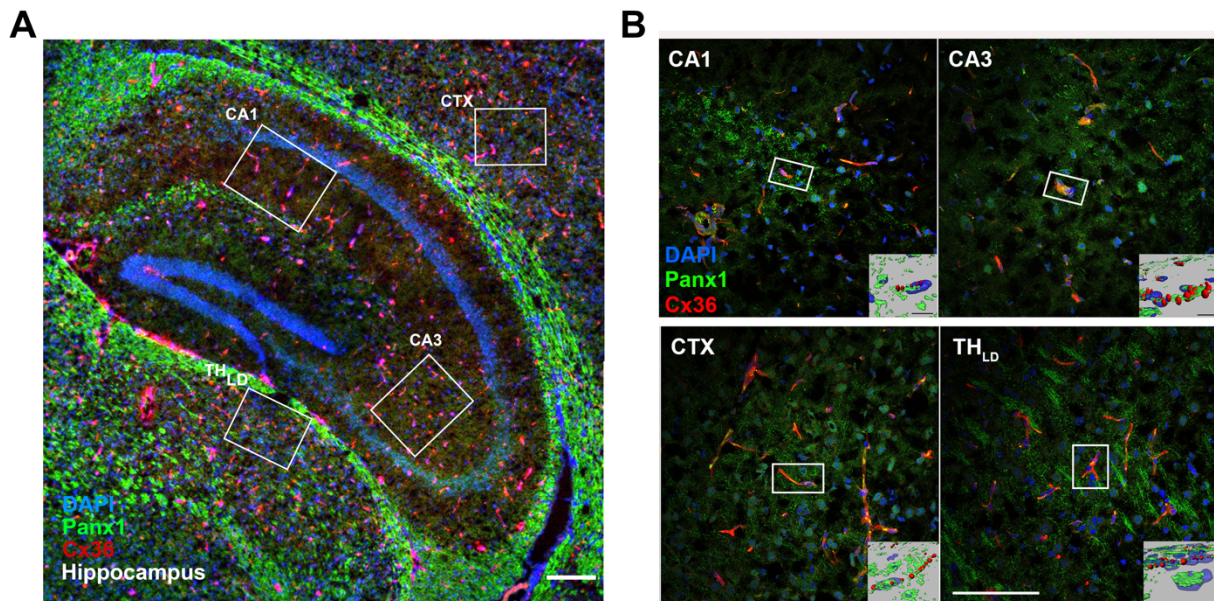


Figure A.2.2: Localization of Cx36 and Panx1 in C57BL/6 hippocampal coronal slices. Four weeks old mice whole brain 12 μ m cryosections were used for immunohistochemistry. **(A)** An overview of the hippocampus with nuclei labelled in blue (DAPI), Panx1 is labelled in green (Alexa488), and Cx36 is in red (Alexa568). **(B)** Different regions of interests (ROIs) are indicated with white boxes on the overview (CA1, CA3, cortex (CTX) layer 6/5 and Lateral dorsal nucleus of thalamus (TH_{LD})) with corresponding images. A 3D-reconstructed image of an area within the ROI showed the proximity of Panx1 and Cx36 (overview was imaged under 10x magnification stitched with four separate images; scale bar = 200 μ m; specific ROI images were imaged under 63x magnification; scale bar = 100 μ m; 3-D reconstruction scale bar = 30 μ m).

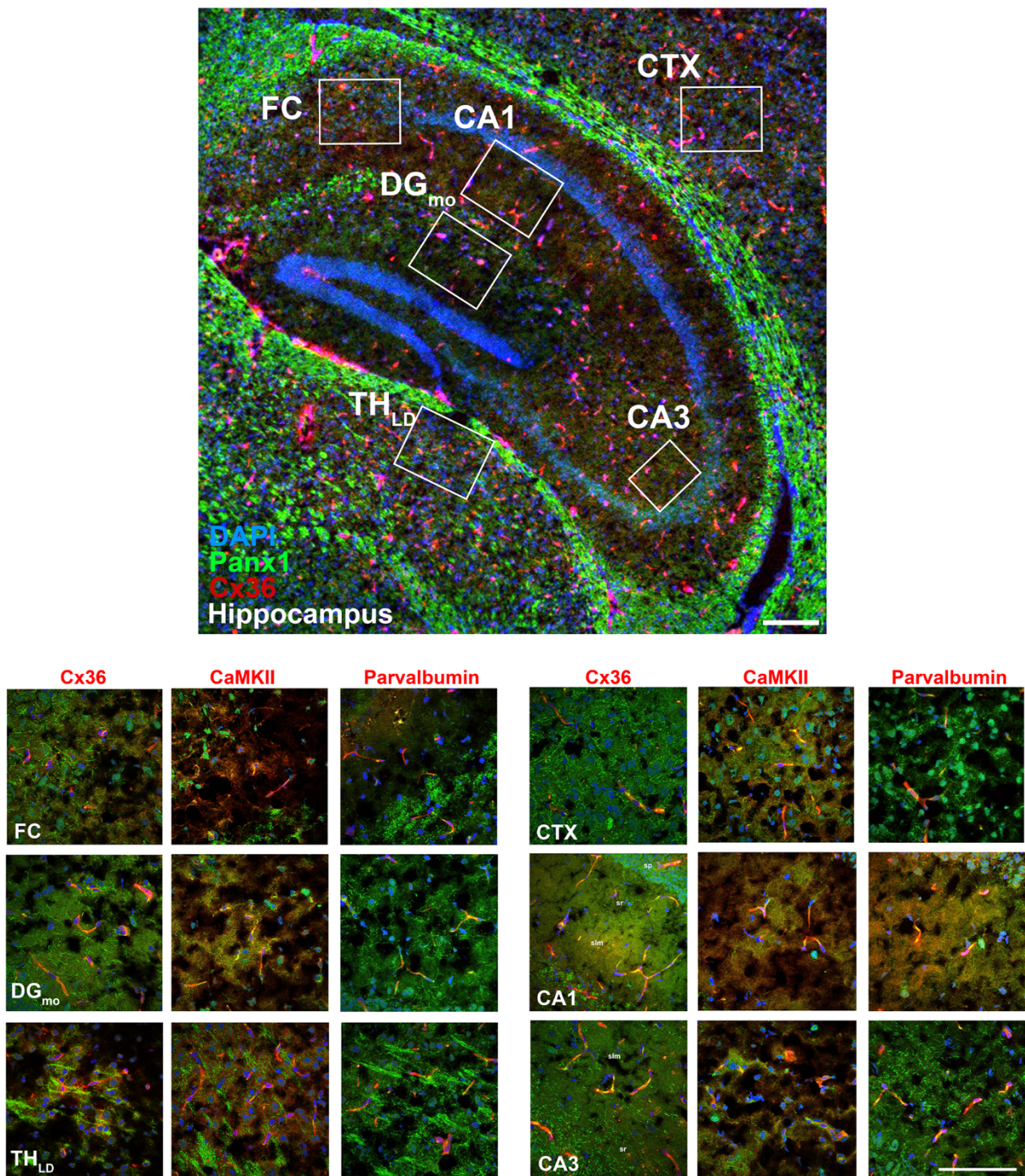


Figure A.2.3.1: Localization of Cx36, CaMKII and Panx1 in C57BL/6 hippocampal coronal slices. Four weeks old mice whole brain 12 μ m cryosections were used for immunohistochemistry. An overview of the hippocampus with nuclei labelled in blue (DAPI), Panx1 is labelled in green (Alexa488), and Cx36, pan-CaMKII or parvalbumin are in red (Alexa568). Different regions of interests (ROIs) are indicated with white boxes (i.e. Fasciola cinerea (FC), CA1, CA3, DG

molecular layer (DG_{mo}), cortex (CTX) layer 6/5 and Lateral dorsal nucleus of thalamus (TH_{LD})) with corresponding images. (Overview was imaged under 10x magnification stitched with four separate images; scale bar = 200 μ m; specific ROI images were imaged under 63x magnification; scale bar = 100 μ m).

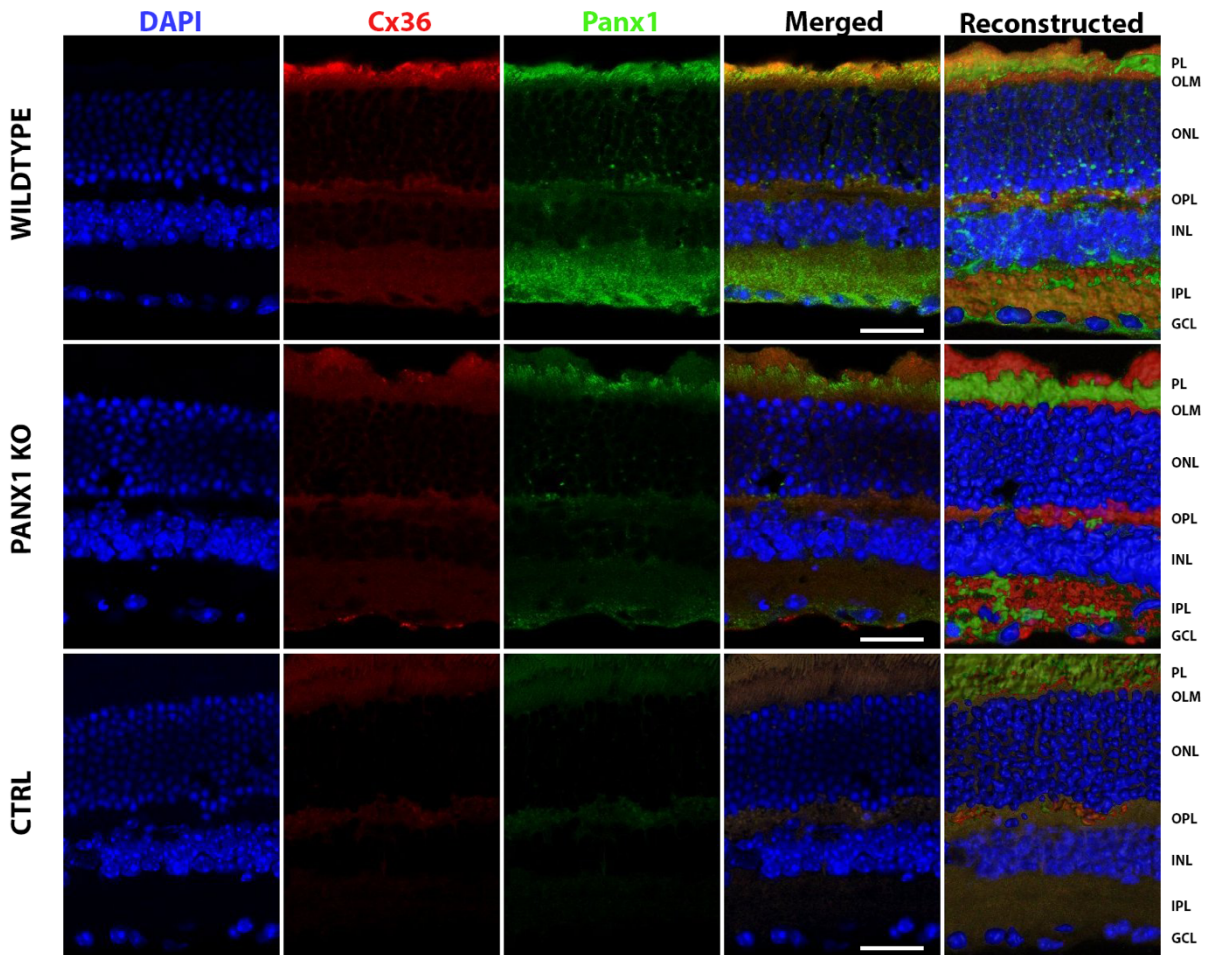


Figure A.2.4.2: Localization of Panx1 and Cx36 in C57BL/6 mouse retinal slices. Mouse retinal immunohistochemical slices showing Panx1 (green) and Cx36 (red) expressions and nuclei are highlighted with DAPI (blue). Retinal slices from wildtype and Panx1 KO mice were used to demonstrate the location of each protein. CTRL with no primary antibody was used as a negative CTRL. (Scalebar= 10 μ m, PL: photoreceptor layer, OLM: outer layer matrix, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer and GCL: ganglion cell layer).

A.2.3) Neuro2a cells express functional pannexin 1 channels

Neuro2a cells express low levels of Panx1 and respond to potassium gluconate stimulation (40 mM KGlu) (Timonina, Kotova, and Zoidl 2020b), as shown here using the genetically encoded membrane potential probe Arclight S249 (**Figure A.2.3**). Stimulation treatment significantly increased the fluorescence of the genetically encoded membrane potential probe Arclight S249. Functional Panx1 channels can be controlled through pharmacological stimulants and blockers.

Cellular membrane potential regulates the gating properties of Panx1, which is dependent on the direction and amplitude of ion fluxes through the channel (Nomura et al. 2017). Since ions can travel in both directions, the direction and magnitude of the ion flow will determine whether the channel is driven towards the open or closed state. In addition, potassium channels like Kv β 3 have been shown to interact with the Panx1 channel in neurons of rat brains (Bunse et al. 2009). When positive ions (i.e., K⁺ with the addition of KGlu) are introduced to cells expressing Panx1 channels, an increase of K⁺ elevates the membrane potential and activates Panx1 channels. Blocking of Panx1 channels with ¹⁰Panx or BB-FCF reduces a means of elevating membrane potentials and limiting the effects of K⁺.

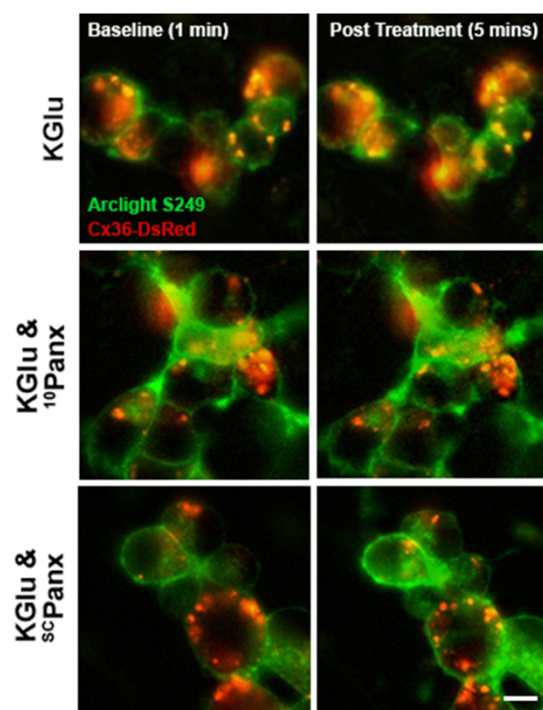


Figure A.2.3: Detecting membrane potential change with Arclight S249 under different conditions over five minutes. (A) Neuro2a cells expressing Arclight S249 and Cx36-DsRed with various drug treatments (Kglu, Kglu & ¹⁰Panx and Kglu & ^{sc}Panx) at one minute and five minutes (scale bar = 10μm)

A.3: Supplemental DNA Construct Maps

The following are DNA constructs generated for the purpose of the studies. Variations of pEGFP-N1 (pECFP-N1 and pEYFP-N1) have been used, but only one variation of the constructs are shown to avoid repetitiveness. These construct maps have been generated from SnapGene Viewer.

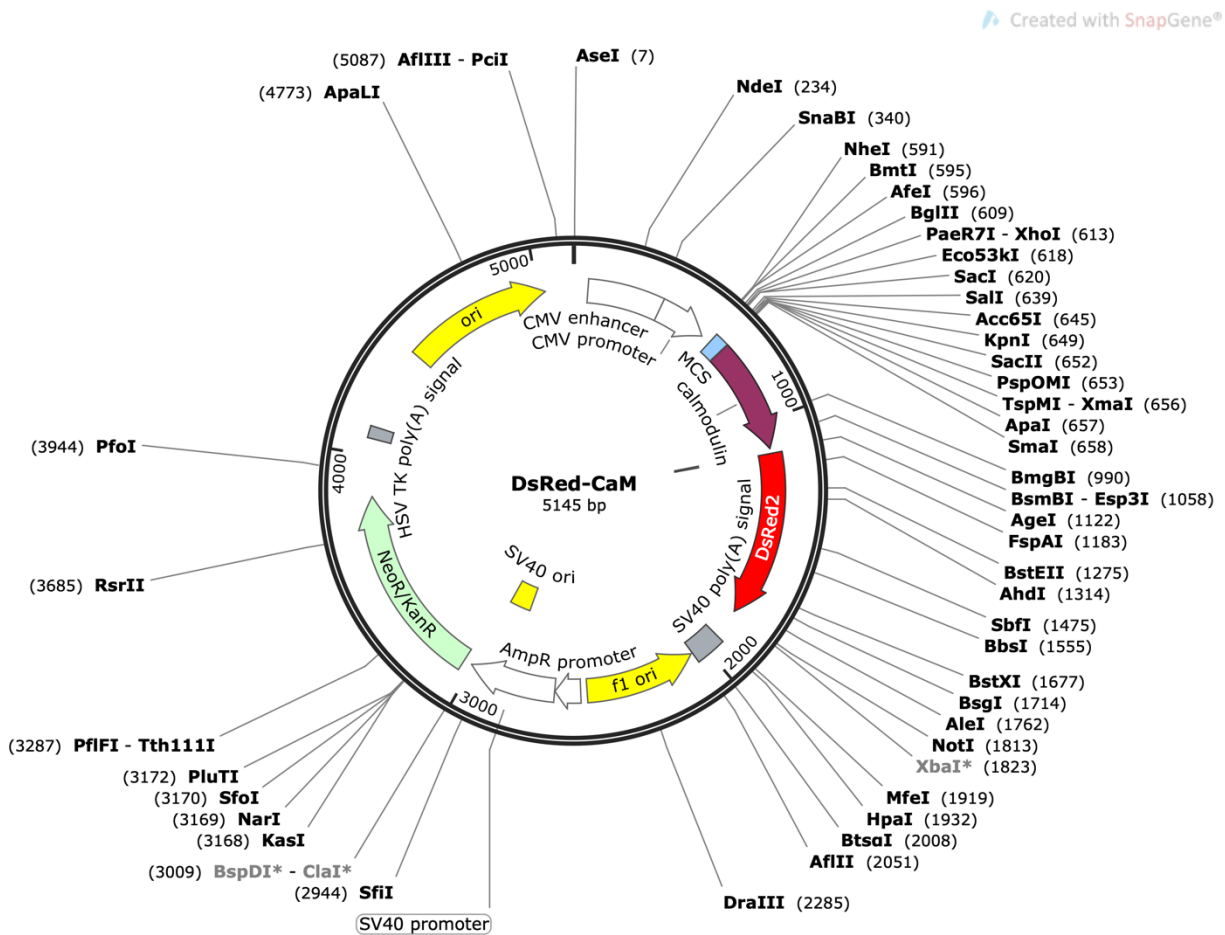


Figure A.3.1: Gene map of pDsRed2-Calmodulin (CaM) indicating insert and various regions of expressions.

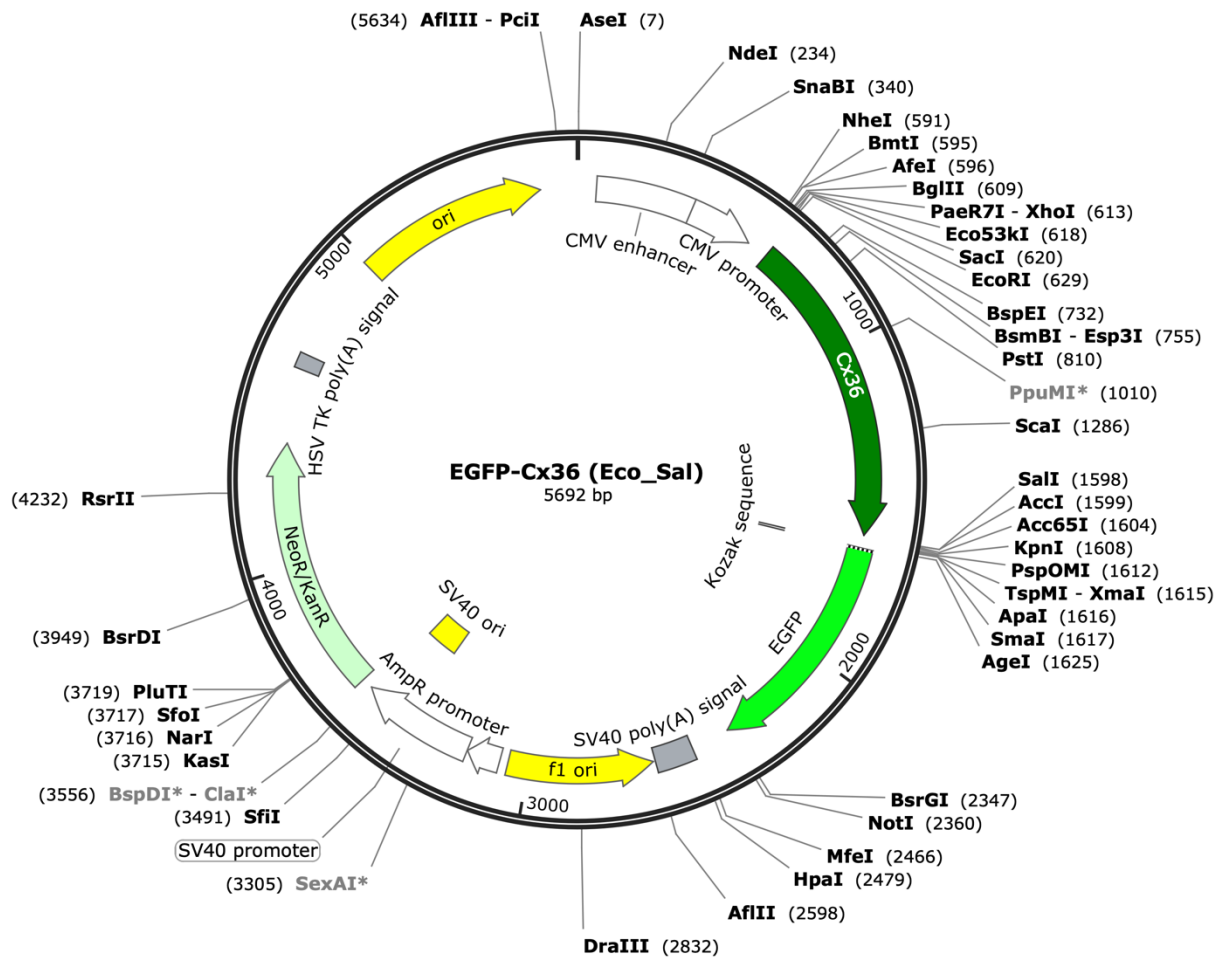


Figure A.3.2: Gene map of pEGFP-Connexin36 (Cx36) indicating insert and various regions of expressions.

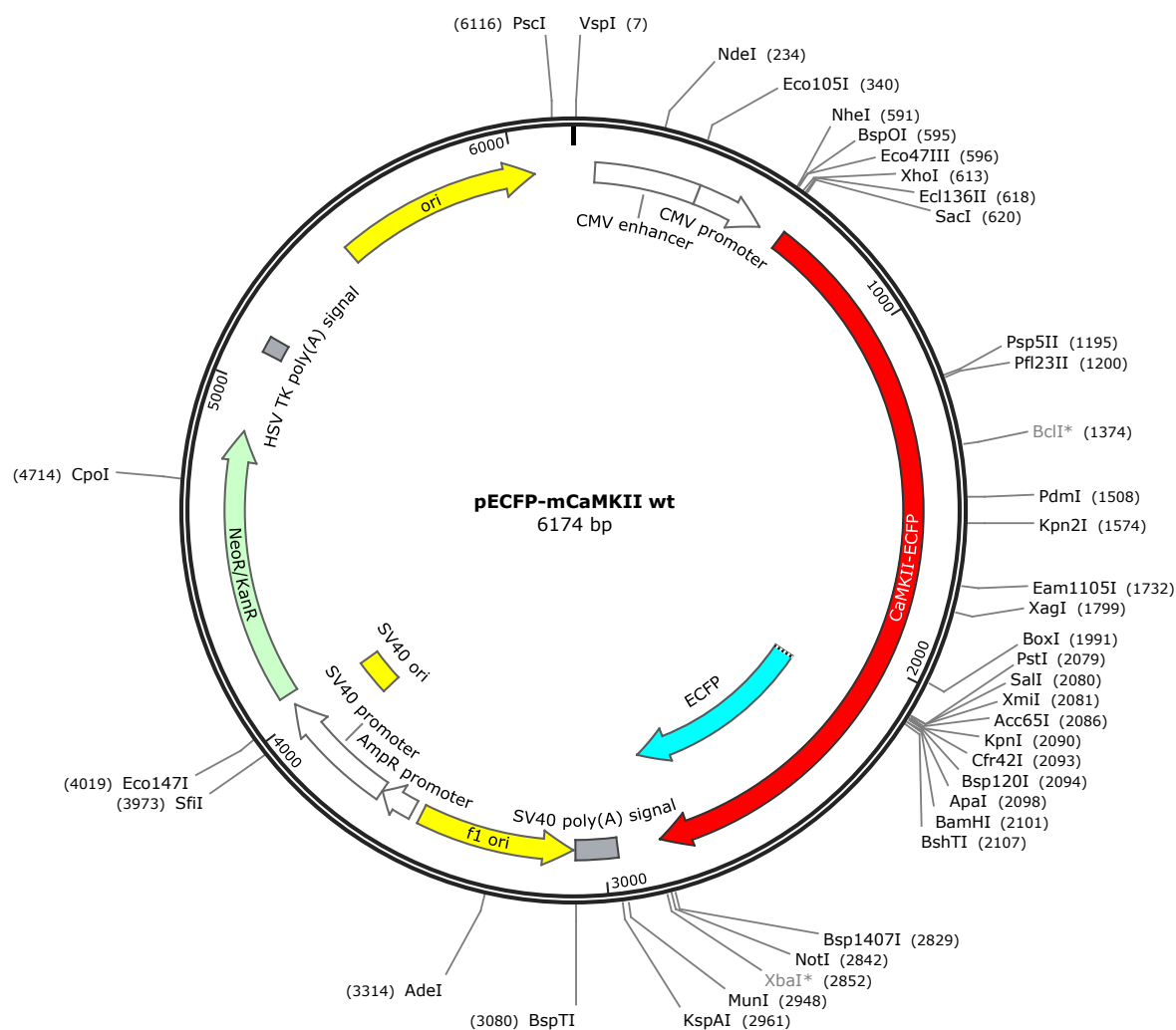


Figure A.3.3: Gene map of pECFP-mCaMKII (WT) indicating insert and various regions of expressions.

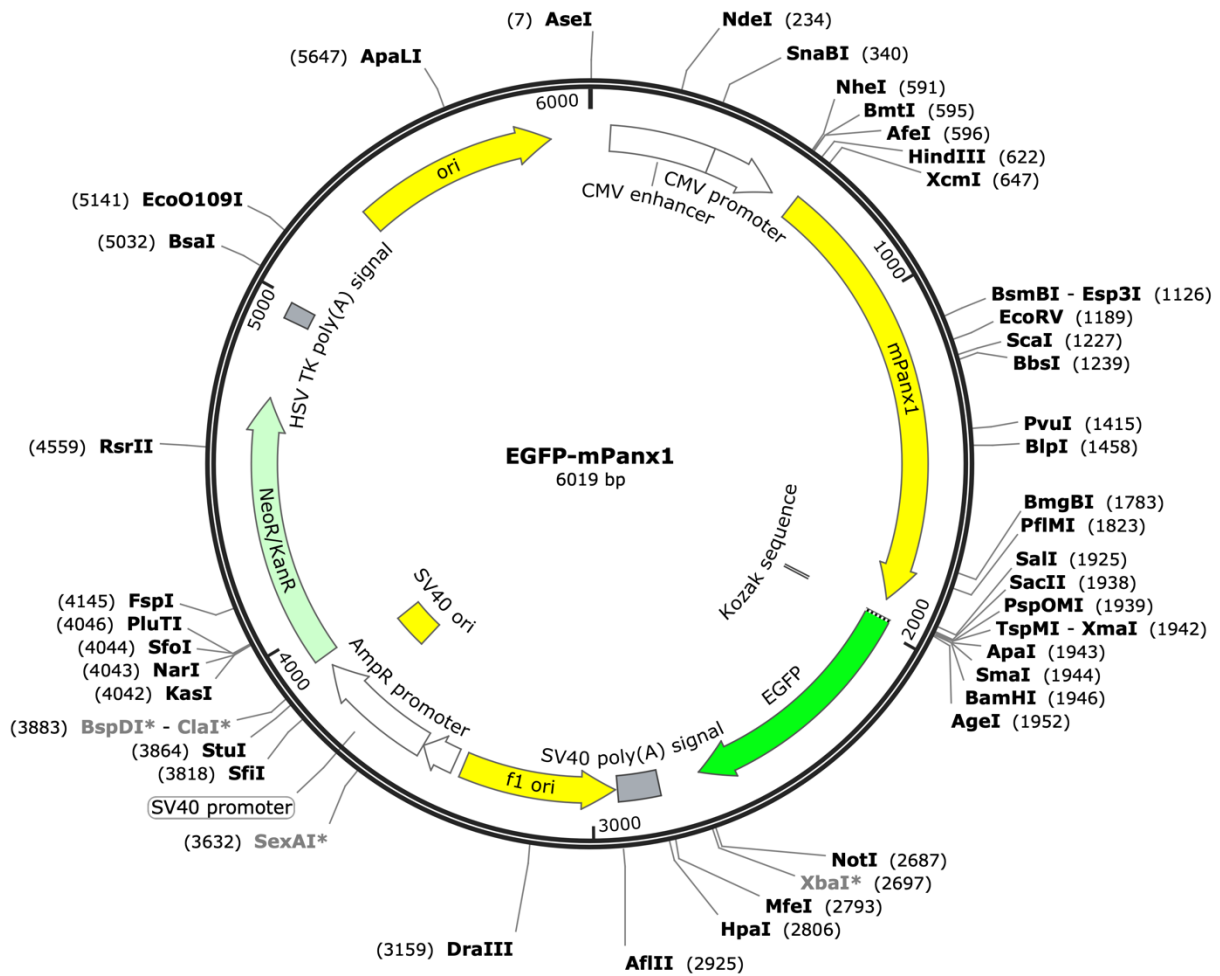


Figure A.3.4: Gene map of pEGFP-mPanx1 indicating insert and various regions of expressions.

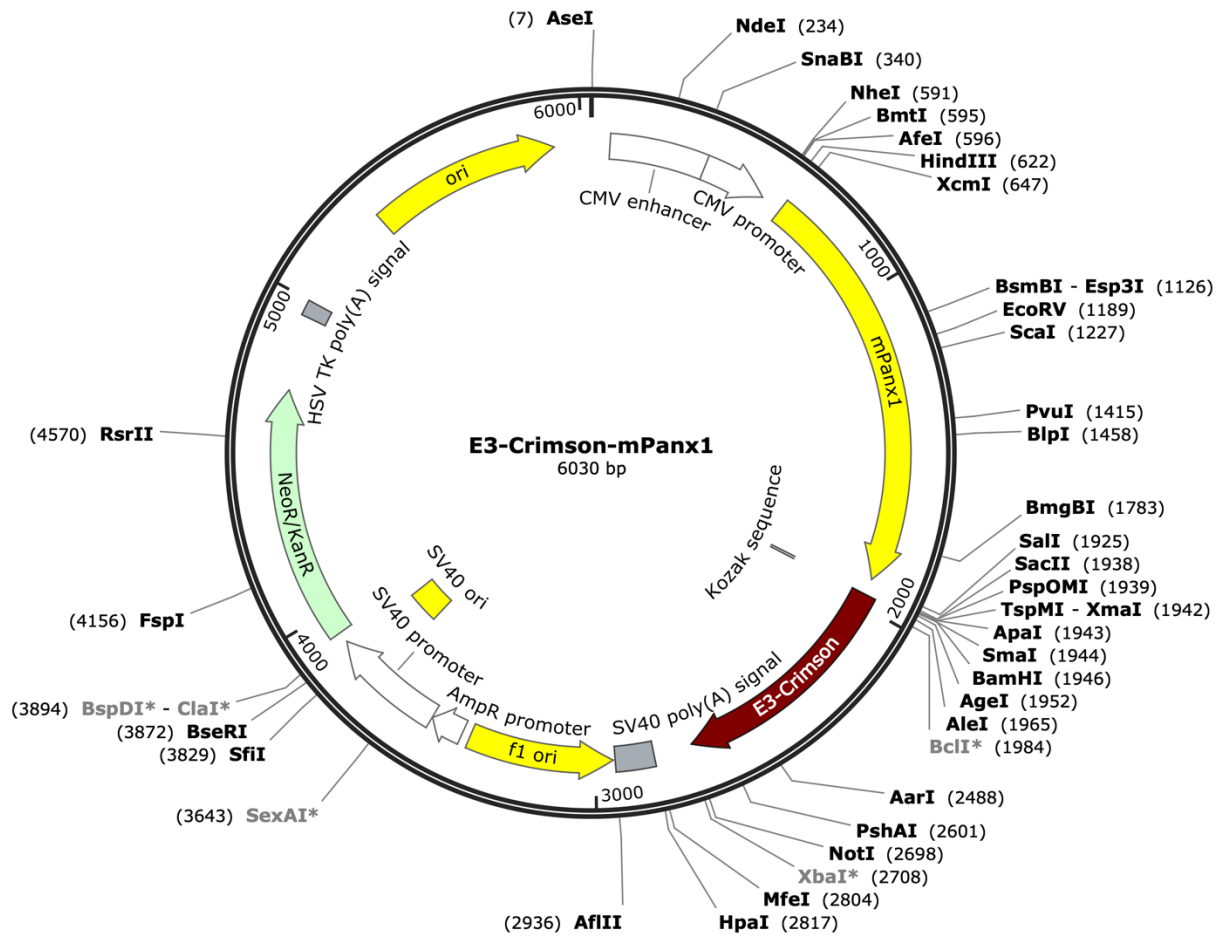


Figure A.3.5: Gene map of E3-Crimson-mPanx1 indicating insert and various regions of expressions.

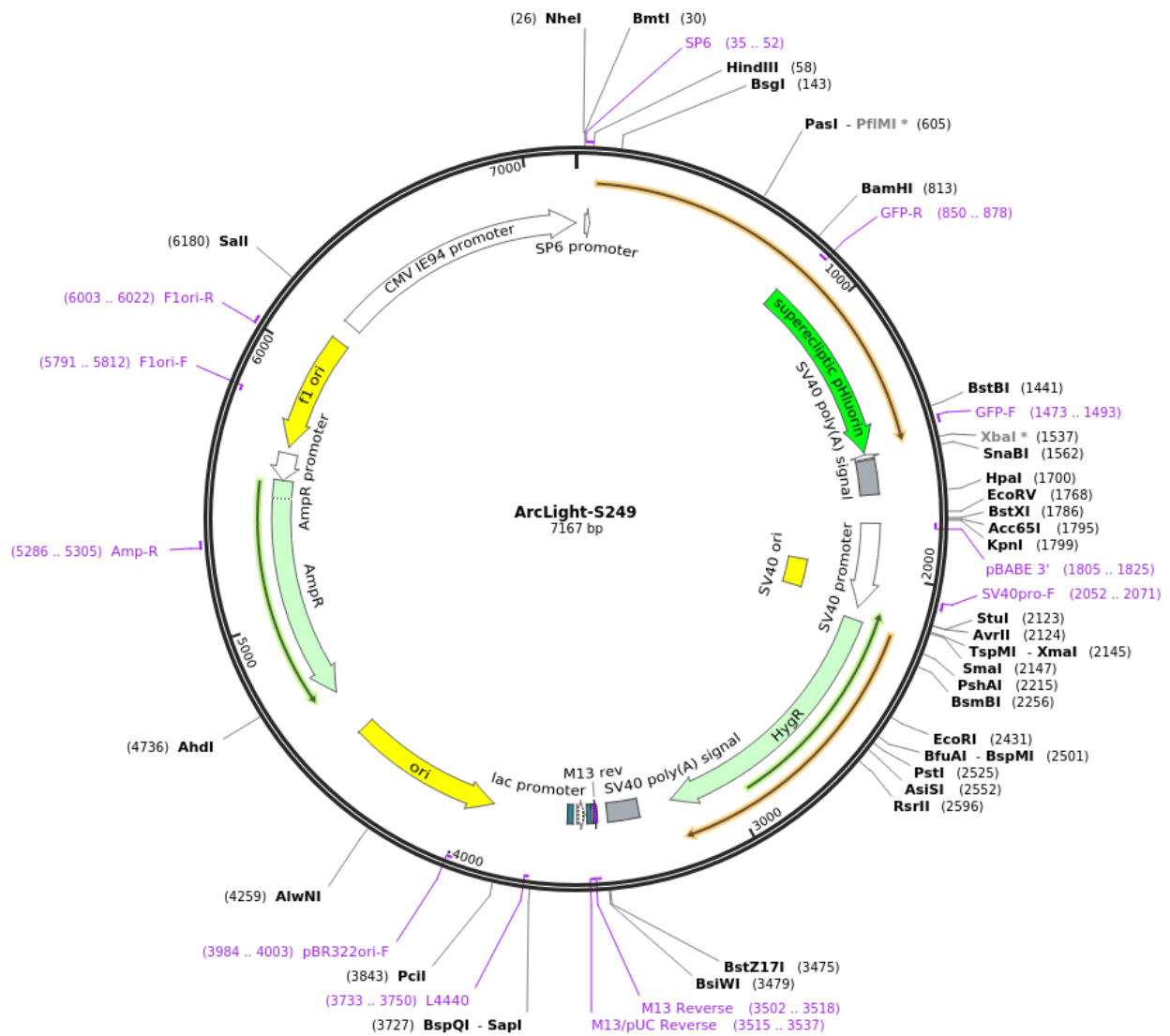


Figure A.3.6: Gene map of ArcLight S249 indicating insert and various regions of expressions.

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[3] **Ryan CF. Siu***, Cherie A Brown, Christiane Zoidl, David C Spray, Logan W Donaldson and Georg Zoidl. (2017). CaM, CaMKII and Cx36 at Electrical Synapses. International Gap Junction Conference (IGJC 2017). (International; Oral Presentation; PhD Work).

[4] **Ryan CF. Siu***, Ekaterina Smirnova, Cherie A. Brown, Logan W. Donaldson and Georg Zoidl. (2016). Dissecting the Role of Connexin 36 and Calmodulin in the Plasticity of Electrical Synapses. 10th Annual Canadian Association for Neuroscience Conference. (National; Poster presentation; PhD Work).

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[6] **Ryan CF. Siu***, Cherie A. Brown, Georg Zoidl. (2015). Dissecting the Role of Connexin 36 and Calmodulin in Learning and Memory. 50th Biology Day (Institutional; Poster presentation; Masters Work).