IN-VITRO MODELS FOR STUDYING NEURODEGENERATIVE DISEASES BASED ON SH-SY5Y CELL LINE

by

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ABSTRACT

IN-VITRO MODELS FOR STUDYING NEURODEGENERATIVE DISEASES BASED ON SH-SY5Y CELL LINE

The main objective of this study was to use a variety of biophysical assays such as electrophysiological recordings and flourescent microscopy to evaluate an in-vitro model based on differentiated SH-SY5Y neuroblastoma cell lines. To validate the model a demonstration study was conducted to investigate effects of Brain Derived Neurotropic Factor (BDNF) on state of transmembrane currents during neurodegeneration caused by Amyloid beta peptides. Two protocols were developed to use BDNF as an adjacent drug to a control group differentiated by Retinoic Acid (RA). Morphological and fluorescent assays, including F-actin staining, were used to verify and characterize cell differentiation and uptake of amyloid beta peptides by the cells. Upon uptake of amyloid beta, whole cell patch clamp technique was used to observe changes in transmembrane current in control and BDNF groups. Furthermore a Hodgkin Huxley based stochastic search engine was employed to extract ion channel composition data from whole cell recording experiments. The results demonstrated that however BDNF significantly increase cell survivability against amyloid beta, the transmembrane currents would be highly altered, indicating possible functional alterations of survived cells in Alzheimers disease. Lipid coated recording which has been presented in this thesis uses a bi layer of synthesized lipids such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) to obtain gigaseals before touching the cells with glass micro pipettes. results presented in this thesis represent an initial optimization phase and proof of concept for further development of the technology and increase in its applications.

Keywords: Neurodegeneration, Electrophysiology, Neuroblastoma, Brain Derived Neurotrophic Factor.

ÖZET

TEZİN TÜRKÇE BAŞLIĞI

Sunulan çalışmanın temel amacı, farklılaşmış SHSY5Y nöroblastoma hücrelerinde elektrofizyolojik kayıtlar, floresan mikroskobu ve in-siliko araçlar gibi çeşitli biyofiziksel analizler yaparak in-vitro bir model tasarlamaktir. Modeli doğrulamak için, Beyinden Türetilmiş Nörotropik Faktörün (BDNF) amiloid beta peptitlerden kaynaklanan nörodejenerasyon sırasında transmembran akımlarının üzerindeki etkilerini araştırmak için bir demonstrasyon çalışması yapılmıştır. Retinoik Asit (RA) ile farklılaşan kontrol grubuna BDNF'in de farklılaşma ajanı olarak eklendiği iki protokol geliştirilmiştir. F-aktin ve DAPI çekirdek boyaması dahil olmak üzere morfolojik ve floresan analizleri, hücre farklılaşmasını ve hücreler tarafından amiloid beta peptitlerinin alınmasını doğrulamak ve karakterize etmek için kullanılmıştır.Sonuçlar, BDNF'in amiloid beta'ya karşı hücre canlılığını önemli ölçüde arttırdığını, ancak transmembran akımlarının sağ kalan hücrelerde büyük ölçüde değiştiğini göstermekte ve Alzheimer hastalığında hayatta kalan hücrelerde fonksiyonel değişiklikler olduğuna işaret etmektedir. Ayrıca tasarlanan model söz konusu hücrelerden yeni bir elektrofizyolojik kayıt tekniğinin geliştirilmesi için kullanılmıştır. Bu tezde sunulan lipit kaplı kayıt, cam mikro pipetler ile hücrelere dokunmadan önce gigaseal elde etmek için 1-palmitoil-2-oleoil-sn-glisero-3-fosfokolin (POPC) gibi bir sentetik çift katmanlı lipit tabakası kullanılmıştır. Bu çalışmadaki sunulan sonuçlar, teknolojinin daha fazla geliştirilmesi ve uygulamalarında artış için bir ilk optimizasyon aşamasını ve konsept kanıtını temsil etmektedir.

Nörodejenerasyon, Elektrofizyoloji, Nöroblastoma, Beyin Türevli Nörotropik Faktör.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

R	Resistance
C	Capacitance
I_t	Total TransmembraneCurrent
I_{Na}	Transmembrane Sodium Current
I_k	Transmembrane Potassium Current
G	Gating function
τ	Channel Deactivattion time
E	Channel Activation Threshold
U	Holding Potential
m,n,h	Defined intermediate variables
t	Time

LIST OF ABBREVIATIONS

AD	Alzheimers Disease
ND	NeuroDegeneration
RA	Retinoic Acid
BDNF	Brain Derived Neurotropic Factor
Abeta	Amyloid Beta
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DPHPC	1,2-diphytanoyl-sn-glycero-3-phosphocholine
DOPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine

1. INTRODUCTION

Neurodegeneration (ND) is defined as the loss of structure or function of a neuron. Too often it is suggested that the loss of function of a neuron comes before observable loss of structure [1]. Consequently existing perplexities surrounding specific functions of individual neurons has cast a long shadow of uncertainty over neurodegeneration studies. Under such ambiguities, one instrumental approach to perform objective studies is to isolate individual neurons in a standard predefined environment to have uncluttered and enhanced view on detailed structure and functions of the neuron by applying multiple assessment tools.

Such models, called in-vitro neurodegeneration modes, have a series of advantages and disadvantages which should be considered before delving into specifics of design. In-vitro models provide an indispensable tool to investigate molecular mechanisms however they do not incorporate effects of extra-cellular environment on triggering and progression of the disease. In particular regarding to neurodegeneration, intercellular signaling, be it in the form of synaptic transmission or mechanotransduction, is mostly omitted in in-vitro models.

This particular limitation is mostly due to inadequate technologies at present time for orientation and identification of cell to cell connections. Such shortcomings may be addressed through emerging organotypic culture methods which not only considers cell to cell connections but presence of multiple cell types, extracellular matrix and functional phenotypes of the tissue [2]. Organotypic culture requires precise control over differentiation of the stem cells which is also a present challenge in the field of development of in-vitro disease models.

Verification is particularly important when the model is obtained by differentiation of neural stem cells rather than isolation of a particular type from a pathological sample. One recent review discovered that only 54 precent of studies on in-vitro parkin-

sondisease models have verified the type of the cell in the study. Due to high costs of genetic verification of the models, the verification procedure is usually done by observation of cell morphology and fluorescent tagging of different neuro transmitters [3].

Many physiological properties of the cells of the in-vitro models can not be verified by chemical assays. Particularly membrane properties such as the exact types of ion channels, reversibility of ion flows, sensitivity of receptors and oscillatory properties are prone to evade chemical assays [4]. To address these elusive properties controlled sources of the cell types and rigorous verification procedures are needed. We aim to produce such protocols using powerful electrophysiological measures during this project. However Once an invitro model established, it could be used to assess effects of a wide variety of the ND agents, given the context and the objective of the study, In this effort we focused on a model for Alzheimers disease to explore full potential of the model to derive and verify insights to the molecular pathways of the disease.

1.1 Alzheimer's Disease

Alzheimer's Disease (AD) is the most prevalent sub type of neurodegenerative diseases. In 2015, 80 percent of all reported cases of dementia worldwide were suspected of Alzheimerâs disease [5]. At the same time perio, 200,000 cases of early onset Alzheimer's, showing pathological signs before the age of 65 had been identified while the number of patients older than 65 years surpasses 5 million. By 2050, the number of Alzheimer's disease patients is projected to be more than 15 million worldwide.

The most well known symptom of the Alzheimers disease is memory loss. Although a vast range of memory types are vulnerable to in the disease, inability to form and consolidate and lose existing episodic memory is the most prominent sign of the disease [1]. Alzheimer's disease is also associated with a vast range of behavioral and psychological disorders, states of which are correlated to progression and sub-types of the disease.

Paranoia, delusions, anxiety, aggression, seizures and movement impairments are told to peak at the medial stages of the disease. Other subtle cognitive impairment such as spatial cognition impairment and even olfactory impairments have also been reported to appear before clinical diagnosis of Alzheimerâs disease [6].

Anatomically, AD is characterized by frontotemporal atrophy of the brain. Areas such as entorhinal cortex in hippocampus, neocortex and nucleus basalis show more volume loss in Alzheimerâs disease [7]. However morphological changes associated with Alzheimerâs disease are explicit, network impairments are more complicated and less intuitional.

A current body of research efforts focuses on combining cognitive tasks involving memory and problems solving with functional imaging to pinpoint network dynamic alterations associated with AD onset. One interesting finding in this field is the higher prefrontal activity in onset of the disease during memory and repetition tasks which may underline role of memory in processing and problem solving [8].

The accepted hypothesis for underlying causes of AD is accumulation of Amyloid-beta plaques in the brain region associated with the disease. The production cycle of amyloid plaques starts with production of Amyloid Precursor Protein (APP) [9]. APP is a transmembrane protein produced all across the body and all through the life. It has between 695 to 750 protein an is encoded by a gene located on the 21st chromosome with 290 Kbps. The most common type of APP in the brain is APP695 which is produced chiefly by the neurons.

After production APP will be cleaved by three types of enzymes which are named after the transmembrane domain they operate on. The alpha-secretase enzyme, cleaves the Alpha-domain of the protein and produces alpha-APP. It has been indicated that the Alpha-APP production correlates with neural activity. Beta and Gamma-secretase enzymes can both act on the transmembrane protein or the internalized alpha-APP oligomers . While Beta-Secretase enzyme action may result in production of less toxic Abeta-30/32 and maybe other forms, action of Gamma-secretase enzyme, whether

on transmembrane protein or products of Alpha/Beta-secretase, produces the toxic Abeta-1-40/42 isoforms.

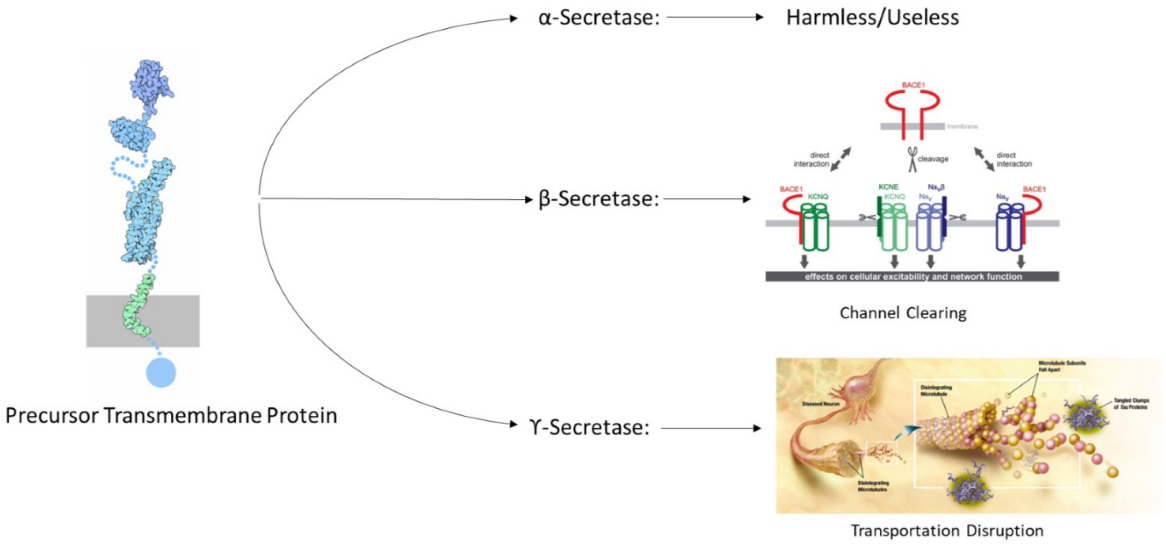

Figure 1.1 Different Secretase Enzymes Acting on APP and Implications of Their Products [5-7].

Recently the profound effects of secretase enzyme family on electrophysiological activity of neurons have come to attention. Beta-secretase enzyme in particular can break the beta-subunit of transmembrane proteins and in particular voltage activated sodium channels [10]. bta-secretase enzyme can also replace the Beta-subunits and, in case of voltage activated sodium channels, interact with the pore-forming alpha-subunit directly.

Exact implication of the interactions between secretase enzymes and ion channels remain poorly understood. It has shown that upregulation of beta-secretase enzyme would increase firing rate and reduce adoptability of Purkinje cells but given the abundance of voltage activated sodium channels and the vast variety of the roles they undertake, more precise understanding of the exact effects of such dynamic would require intensive research on the subject.

However different forms of Amyloid beta peptides constantly is extracted and cleared from neurons by insulin-degrading enzyme, Neprilysin and such, the imbalance

between clearance and production would result in accumulation and further destructive effects of Amyloid beta. Toxic forms of Amyloid beta trigger multiple cascade inside the neurons. Along with activating receptors regulating apoptotic gene expression and else, Abeta-1-40/42 reacts with tau proteins present in cytoskeletal structure of the cells and form neurofibrillary tangles in axons and dendrites. These plaques are found extensively in the areas of the brain which show severe atrophy in AD.

1.2 SH-SY5Y Cell Line

In-vitro models may be acquired by harvesting cells from human or animal samples or differentiating stem cells. Harvesting cells from tissue samples, however under optimum conditions provides a more exact and relevant model, is more labor intensive and costly. Furthermore, due to diversity of the cells acquired in the procedure, the end results of such perpetrates may not entirely represent the intended disease [11]. Therefore, to construct disease models, it is customary to use standard stem cell lines provided by verified reservoir centers.

In this study we used SH-SY5Y neuroblastoma cell line provided by ATCC. SH-SY5Y is an established line of human neural stem cells which was driven from metastatic neuroblastoma of bone marrow biopsy of a 4-year old female in mid-1970s. This cell line demonstrates a series of genetic anomalies, due to its cancerous origins, but has been verified to keep most of dysfunctional genes/pathways in neurodegenerative conditions, such as in Parkinson's disease, intact [12]. SH-SY5Y, in culture medium, exhibits two sub types; the S-subtype which are suspended and more granular in shape and proliferate to the point of adhesion and the N-subtype which are more pyramidal in shape, quasi-differentiated, hence expressing more neuron-like factors.

General culture protocol for neuroblastoma is fairly routine with a growth medium composed of mostly DMEM/F12 with 15 percent FBS supplement and other optional additives. Cells can attach to the plate in 24 hours and reach confluence in 3-4 days. Cultures on both glass and untreated polymer substrates in forms of coverslips

have been reported [12, 13, 14, 15]. This cell line could be used in both multi-potent and terminally differentiated states to model different types of neurons. While undifferentiated cells are considered relevant in a range neurological studies, the major advantage of using SH-SY5Y cell line is the broad spectrum of cell linage obtainable from the cell line depending on the differentiation agents used. Two particular factors stand out among utilized differentiation factors in the literature:

1. Retinoic Acid (RA):

   Retinoic Acid, a Vitamin A derivative, is the most common factor for differentiation of SH-SY5Y cells. Use of RA could be compared to effects of Vitamin A, the lack of which results in squamous metaplasia in endothelial tissues. RA not only promotes adhesion and differentiation of the undifferentiated cells, but also improves cells resilience to presence of some of the toxic factors [14, 15]. Treatment of cells with 10uM/L of RA for 3 to 5 days results in cholinergic neurons. Further more mature dopaminergic neurons could be acquired by further treatment of the cells with phorbol esters [16]. One of the most important roles of RA is promotion of Kinase protein receptor TKrB which binds to Brain Derived Neurotrophic Factor and promotes cell survival and enhanced differentiation.

2. Brain Derived Neurotrophic Factor (BDNF):

   BDNF is the best known and most characterized member of neurotrophic factors. BDNF has several roles in neurogenesis and cell preservation including stem cell survival, axonal guidance and branching, guidance and maintenance of dendritic trees and spines . BDNF is also implicated in facilitating synaptic plasticity by increasing voltage activated sodium channels and facilitating memory formation. BDNF has also long been implicated in protection against AD [17]. However exact mechanism of BDNFs protective effects against Amyloid beta toxicity is unknown one working hypothesis is selective phosphorylation of tau proteins which is a critical step in formation of neurofibrillary tangles. It has been shown that combined treatment of SHSY5Y cell line with RA and BDNF increase expression of various kinase proteins which may be involved in tau protein phosphorylation [12].

The differentiation factors used on SH-SY5Y cell line are not restricted to the two aforementioned agents. TPA for example, a phorbol ester, promotes differentiation of SH-SY5Y stem cells and induces appearance of long, straight and uneven neurite process along with cytoplasmic neurosecretory granola. Treatment of cells with 0.16 uM/L TPA for 4 days has shown to induce such effects [18]. The major difference between outcomes of RA and TPA treatment is 200-fold increase in cellular noradrenaline content. Therefore neurons differentiated by use of TPA should be considered as adrenergic.

Identification of numerous factors which control differentiation of SH-SY5Y cell line, has led to significant use of these cells in modeling of different kinds of diseases. Due to prominent expression of dopaminergic factors most of the usage has been reported in Parkinson's disease related studies [3]. This not to say that SH-SY5Y hasn't been a significant factor in study of other diseases. For example a land mark study used this cell line to verify the theory that Amiloyd Beta type1 would trigger mitosis cycle and halt at C phase resulting in loss of neurons, providing a great establishing ground for the main cause of Alzheimers disease [19].

Electrophysiological characteristics of SH-SY5Y cell line has been extensively studied [20]. Evidence exists of changes of trans membrane current dynamic after differentiation. Increased inward sodium current and reduced deactivation of potassium channels has been reported to contribute to rise of action potential-like spikes in RA differentiated cells [21]. No observation of voltage activated potassium channels have been reported in the aforementioned studies. There has been also a stark notion of extreme heterogeneity in the electrophysiological characteristics of the individual cells in SH-SY5Y line [22].

Challenges still remain in utilization of SH-SY5Y cell line in in-vitro disease models. As mentioned before cells' may keep some of the cancerous properties after differentiation. Furthermore electrophysiological properties of the cells have been reported to vary even in individual culture samples.

Electrophysiological properties of neuroblastoma would be even more critical considering the fact that majority of current neurodegenerative diseases are associated with membrane factors, regulating electrophysiological responses of the neuron. Furthermore membrane factors control cell-cell communications which affect in-network aspect of cell performance creating a rippling effect in case of degeneration.

1.3 Study Design

The complex nature of neuroscience requires deployment of complex methodology and precise planning to be able to acquire unambiguous insights from the raw data. Such complex paradigm required rigorous verification of methods and protocol. To have a measure of our success and establish a reliable framework, we define three clear questions to answer before tackling any specific question regarding;

1. Is the SH-SY5Y capable of differentiating to the type of neuron required for the disease?
2. Are the differentiated cells affected by the ND stimulants the same way tissue samples do?
3. Does electrophysiological behavior of the cell changes with the induction of the ND state?

We suggested study Alzheimer' s disease through the protocol described in [3] as the starting point of the study with the possibility of expansion to other known states, pending on the results of the first induced ND state. To answer the questions above our initial suggestion is to take 6 steps:

1. Successful Differentiation of SH-SY5Y neuroblastoma stem cells
2. Induction of neurodegeneration state

3. Recording of action potentials (AP) and determination of oscillatory properties of the cells

4. Recording of inward and outward flows in presence of appropriate blockers

5. Interpretative modeling of the recorded currents for further processing

We propose following time table for completion of mentioned steps:

Table 1.1
Time Table of the Project.

Step	1st Month	2nd Month	3rd Month	4th Month	5th Month	6th Month	7th Month	8th Month
Differentiation	■							
ND Induction	■	■						
NT Characterization		■	■					
AP Recording		■	■	■	■	■		
Patch-Clamp Records					■	■	■	
Modeling							■	■

Electrophysiological recording was used to assess main characteristics of the culture cells and the main target of the study, namely alterations in composition of ion channels during different neurodegenerative diseases. The established set-up, although optimized for electrophysiological recording, can be used for micro injection of different viral, peptide, nucleotide and visual agents, thus bringing a vast range of possibilities for future studies.

Morphological and fluorescent assays were employed to screen for effects of differentiation and validate molecular events which would lead to unambiguous morphological hallmarks. The study also was planned to make extensive use of numerical methods to acquire sub-level events of experimental recordings.

2. MATERIALS AND METHODS

The entire range of methods employed in this study can be described in two categories, model development and model evaluation. Model development was based on previously existing literature to maximize use of open-source data, therefore the model development methods would be discussed and referenced in the result section. As mentioned before three main methods were used in this study to evaluate the results of the model.

2.1 Electrophysiology

Electrophysiology is a broad term used to describe any method of direct measurement of electrical activity in biological samples. Electrophysiology means vary from large head-sized arrays of electrodes to small proteins transcribed inside selective population of neurons which induce or indicate electrophysiological activity.

Single cell electrophysiology is based on the promise of spatial segregation of recording field to acquire detailed insight about micro-dynamics of different constituents of neural systems. This is usually achieved by use of a micro-fabricated glass tube. The micro tube is usually filled with an electrolyte solution compatible with the test condition. An electrode is situated inside this special purpose built tube. The electrode is in turn connected to an amplifier which would induce the input signal to the cell and amplify the cell's response so it could be translated by the A/D card and transmitted to the operating software.

Micro-manufacturing of the glass micro tube better known as micropipette is done by heating a glass tube and then stretching the tube. Precise arrangement of heat and pulling speed of the glass would determine the tip diameter of the micropipette and the profile of glass contraction toward the tip. Different choices of glass tubes are available including silicon glass and Pyrex glass. While silicon glass is well suited

for production of precise micropipettes with uniform and specific tip openings, pyrex glass which would be well suited for long and strong pipettes designed to penetrate deep inside the target tissue and perform recordings. Additional features such as supporting threads and tip coating could be also employed to improve recording quality such as capacitance reduction which would be discussed later in this text. The small electrode inside the micropipette is usually formed of silver. Silver electrodes are usually chlorinated to eliminate of the chance of electrode polarization and alteration of the natural respond acquired from the cell. Another electrode is usually situated inside the container of the biological sample. These two electrodes complete the circuit which include the cells, experiment medium better known as the bath.

The patch pipette and the electrode can be used in different arrangement in the bath to acquire electrical characterization of a specimen. Common arrangements are cell attached, whole cell and cell free excited patch recordings. In cell attached configuration the pipette is moved adjacent to the cell and a small patch of the cell is pulled inside the pipette by application of small suction pressure. This arrangement is suitable for recording excitable properties of a small population of Ion channels on the pulled patch. In the cell free excited patch recording method the small patch of membrane would be torn apart from the cell after aspiration inside the glass micropipette. This isolated patch then would form a cover on top of the tip opening making it possible to record activity of the accumulated population of the ion channels on the patch or otherwise incorporate desired elements inside the patch.

The most commonly used configuration of patch clamp experiment is whole cell recording. In this configuration first the micro pipette would be lowered to gently touch the target cell. As the small tip opening of the micropipette would limit the movement of ions between the electrode inside the micro pipette and inside the bath, there would be an electrical resistance caused by this constraint usually known as tip opening resistant otherwise known as tip resistance or pipette resistance.

As the pipette would touch the cell, a small suction pressure would be applied to pull the cell membrane inside the micropipette and the tip resistance would increase as

the cell would cover the tip opening. The final resistance of the cell-covered tip would amount to several giga-ohms. As depicted by figure 2.1. This configuration is best known as giga-seal formation. Obtaining a giga seal is essential in ensuring elimination of external noise inside the bath and precise injection of stimulation pulse to the cell.

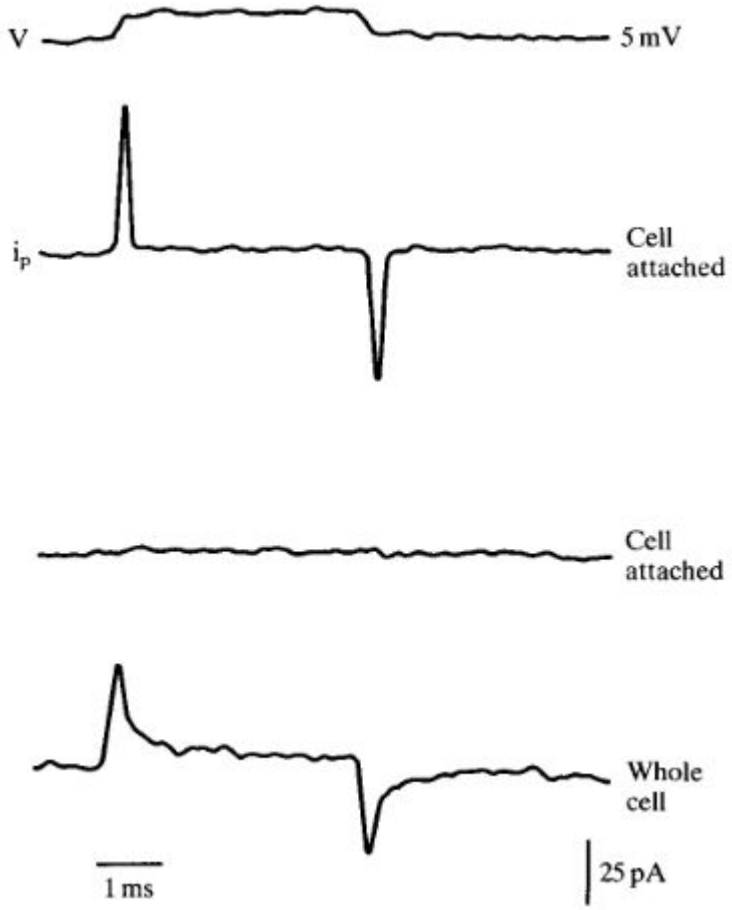

Figure 2.1 Signal Variation in Whole Cell and Cell-Attached configurations [23] .

After formation of the giga seal application of additional pressure would rupture the cell membrane, granting direct access to the cell cytoplasm. This procedure is controlled by manitoring behavior of a square input signal. A square voltage pule (usually 10mV) would elicit a corresponding square pulse current corresponding to the tip resistance. The current square pulse would decrease and eventually diminish while obtaining giga seal. As the membrane ruptures the current pulse would appear again, but in the shape of two curves leading and exiting to and from the pulse square (figure.2).

The curves at the beginning and the end of the pulse are due to a phenomenon known as cell capacitance. The cell membrane which integrates to the micropipette after membrane rupture acts as an insulator and holds positive and negative charges on the different sides of the membrane. This physical phenomenon is modeled as an electrical capacitance, producing an electrical circuit given by figure.2.2

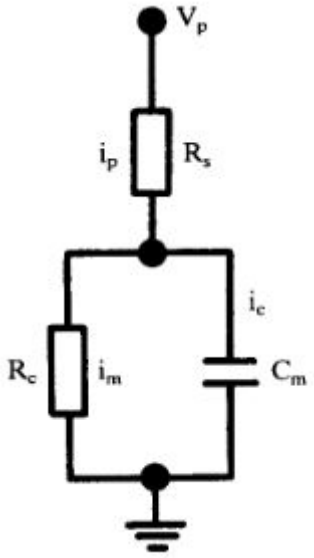

Figure 2.2 Equivalent Electrical Circuit to Whole Cell Configurations [23].

Solving the mentioned circuit results in:

$$E(t) = E(1 - e^{-t/\tau}) \tag{2.1}$$

Where E is electrical potential, and τ is the time constant of the curve given by:

$$\tau = RC \tag{2.2}$$

However cell capacitance is an important indicator for size and static properties of cell membrane, it can obscure crucial trans-membrane current dynamics during the experiments. Multiple hardware and software approaches are developed to eliminate cell capacitance.

2.2 Numerical Modeling

During the firing of an action potential, changes in membrane potential cause various types of ion channels to open up in a stochastic manner. In this stochastic process each channel opens at an unspecific time point after the stimulation and then closes after a slightly less variable period of time. Assume one of the study samples points to higher inward current which in turns could be translated to increased number of sodium channels open at the onset of the action potential, the fact that the outward current remains the same between the two groups implies number of open potassium channels have remained the same.

To reach the final level of outward current, the same number of potassium channels should pass a higher number of ions at the same time period, which could be done only with opening faster and for a longer period of time. This could be further investigated by a closer look to time derivative of the current profiles, from the profiles it could be observed that the rate of increase in outward current is higher in the onset of outward climb and it drops rapidly pointing to maximal steady opening of the channels.

A variety of numerical models have been developed to expand the results of electrophysiological recordings. Such models first provide a hypothesis on arrangement of function dependency of transmembrane Ion channels and then proceed to formulate the hypothesis. Eventually the formula presented by the hypothesis is fitted to capture the experimentally recorded functions. Resulting constants would be presented as the physiological characteristics of the ion channels.

The Hodgkin-Huxley model is the first model presented for representation of dynamics of ion channels . This model presumes linear voltage current relations in ion channels and no cooperative effects between Ion channels. Collective sodium and potassium channels could be seen as figure 2.3.

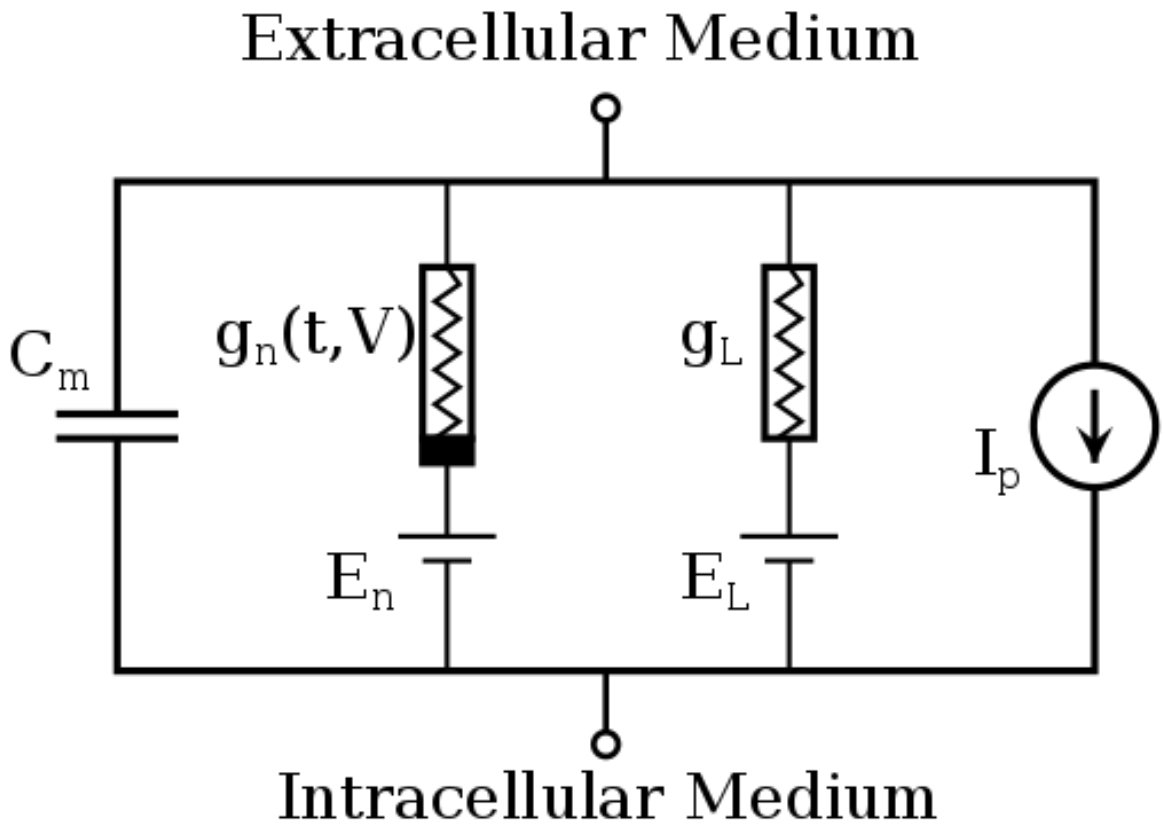

Figure 2.3 Schematics of Hodgkin Huxeley Model.

$$C\frac{du}{dt} = I_c(t) + \sum_k I_k(t) \tag{2.3}$$

According to Hodgkin and Huxley, performance of each channel type is controlled by a simple equation:

$$I_x = g_x(u - E_x) \tag{2.4}$$

In which G roughly represents gating characteristic of Ion channels and E is the activation potential of each channel type. Hodgkin Huxley recommended following gating characteristics for sodium, potassium and leak currents:

$$\sum_K I_k = g_{Na}m^3h(u - E_{Na}) + g_K n^4(u - E + K) \tag{2.5}$$

In which m,h, and n if considered as x is governed by:

$$\frac{\partial x}{\partial t} = -\frac{1}{\tau_x(u)} \tag{2.6}$$

Solving for $I(t)$then gives:

$$m(t) = m_0(u_1) + [m_0(u_0) - m_0(u_1)]exp[\frac{-(t-t_0)}{\tau_m(u_1)}] \tag{2.7}$$

$$n(t) = n_0(u_1) + [n_0(u_0) - n_0(u_1)]exp[\frac{-(t-t_0)}{\tau_n(u_1)}] \tag{2.8}$$

$$h(t) = h_0(u_1) + [h_0(u_0) - h_0(u_1)]exp[\frac{-(t-t_0)}{\tau_h(u_1)}] \tag{2.9}$$

Simple action potential current could be described by this equation. Common algorithms use different optimization methods such as Levenberg-Magrath, Genetic or other stochastic algorithms. After introduction HH model countless similar models, deterministic or probabilistic, have been introduced. There is an extensive debate on biological relevance of data acquired by these methods [24], although verification of either side of that debate is out of scope of this work, we took one lesson away which was to verify the results of modeling before relying on those results to provide insight on underlying biological procedures.

2.3 Fluorescent and Morphological Assay:

Previous studies have provided extensive data on morphological effects of differentiation of SH-SY5Y cell line under various differentiation agents. Neurite elongation, various states of polarization as well as synaptic contact probability has been described as variables uniquely tied to cell differentiation . To verify these phenotypes and consequently verify differentiation we used light microscopy for evaluation of cell morphology and florescent morphology to investigate further connection of morphological signs to molecular events.

In this study use of anti-body conjugated fluorescent agents was avoided. F-actin staining was done with Phalloidin. This agent provides easy and reliable staining of F-actin with high illumination power and low background noise. Such characteristics are essential for capturing F-actin uptake which is one of the known results of BDNF uptake.

2.4 Neurotechnology Proof of Concept: Lipid Coating

Patch clamp experiments start with obtaining giga-seals which are prone to cause damage to cells, are unstable amid mechanical interaction with the cell, render micropipettes unusable after a single recording and require a precise approach to a suitable harbor on cell. Although extremely limiting, giga-seals are essential for isolating the electrode inside the pipette from the ambient noise and provide precise current injection. The need for having giga-seals during whole cell recording may be remedied by a similar phenomenon, sealing of glass micropipette tips by wrapping them in a lipid bilayer coating.

Laffafian and Hallett reported this technic in 1996 [25] and used it to inject neutrophil's. This effort followed a remarkably simple protocol: first a stock solution of POPC, a lipid found in neutrophilâs bilayer membrane, was formed by dissolving POPC in chloroform then micropipettes were dipped into diluted solutions of the stock POPC while the vials were kept on ice. Upon evaporation of chloroform a lipid coating was formed on the micropipettes. This coat was inflamed upon entering the bath containing salt-water solution and formed a seal on the tip of pipettes. When slammed against neutrophils, lipid fusion formed an opening between the cell and the pipette causing substance transfer between to the cell (Figure 3.8).

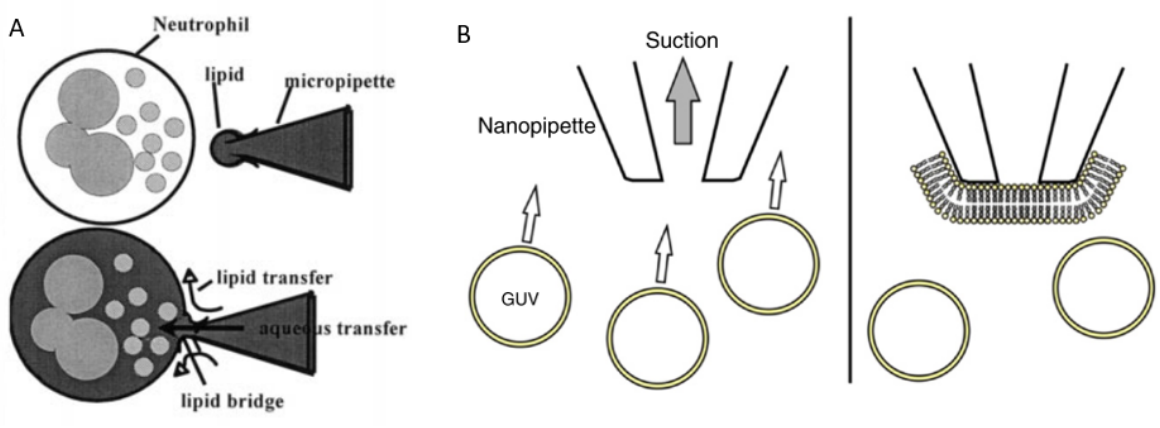

Figure 2.4 formation of an opening between the coated microneedle/micropipette and neutrophils as described by Laffafian and Hallett [25]. B: Lipid coating formation by slamming GUVs to micropipette tip as described by Gutsmann et al [26].

A later review on lipid coating of small cavities, including micropipette tips, to record from single Ion channels provides more elaborate protocols [26]. Gutsmann et al. suggest a solution combined of DPhPC, cholesterol, Sorbitol and bio-beads to be first dissolved in hexadecane, then dried and rehydrated in phosphate buffer solution and electroswelled to form Giant Unilamellar Vesicles (GUV), ultimately the UGVs would be slammed to the opening of the micropipette by aspiration to form a bilayer coating. Gutsmann et al actually observe formation of giga-seal as a measure of formation of the lipid coating.

The protocol described by Laffafian and Hallett [25] was recreated in Cellular Imaging and Electrophysiology (CIE) laboratory of Bogazici university in a single cell patch clamp set up. Upon coating of the micropipettes with POPC formation of giga-seals was observed and by approaching the micropipettes toward SH-SY5Y neuroblastoma cells (differentiated with Retinoic acid according to established protocols) a small opening in the tip was observed and attempts were made to record electrophysiological properties of the cells.

To achieve this, a vial of 100mg POPC (>0.99) was purchased from Sigma Aldrich. Due to the various risks in solution perpetration's, instead of preparing a full

stock solution, 1mM concentration of POPC in chloroform (according to Laffafian and Hallett) was formed from the beginning and kept in -20C. Glass micropipettes were dipped inside the solution for less than 5 minutes and dried for less than a minute before being immersed inside the bath.

3. RESULTS

3.1 Alzheimers Disease

SH-SY5Y cells were obtained from ATCC and were thawed by 37 degree bath and mildly heated growth medium. Undifferentiated cells were seeded on 12 well microplates on a polylysine substrate to enhance cell adhesion. Incubation for 48 hours in 37degrees using Dulbecco's Modified Eagle's Medium (DMEM/F-12) with 12% Fetal Bovine Serum (FBS) caused confluence of cells. Differentiation was initiated by withdrawing FBS from the growth medium and adding either 15mM of RA or combined 15mM of Ra and 5uM of BDNF. All protocols were extracted from previously reported works to allow for most broad use of open source data.

The first step after differentiation was documentation of morphological changes after differentiation. Undifferentiated SH-SY5Y cells appear granular with no linear structure which may resemble neurites. For 20 samples (10 undifferentiated and 10 differentiated) Onset of differentiation causes and immediate drop in cell count (normalized mean=0.241, $t<0.01$, $P<0.001$), as Figure.3.1 demonstrates this drop does not continue in following days of differentiation. Neurite length in RA differentiated cells reach the steady state after day 5 (mean: 261um) and does not significantly change afterward (Paired-$t=0.15$, $P<0.35$).

BDNF mediated differentiation didnt cause any decrease in cell count drop immediately after differentiation ($t=0.08$, $P<0.12$, $n=10$). A significant increase on neurite length (mean=447um $t=0.014$, $P<0.008$) however could be identified in BDNF treated cells. Cell polarization (mean=3.1 $t=0.04$, $P<0.03$) also increased in the BDNF treated set. Furthermore f-actin staining demonstrated higher actin uptake in BDNF sets ($t=0.003$, $P<0.001$) which is consistent with expected effect of BDNF [27].

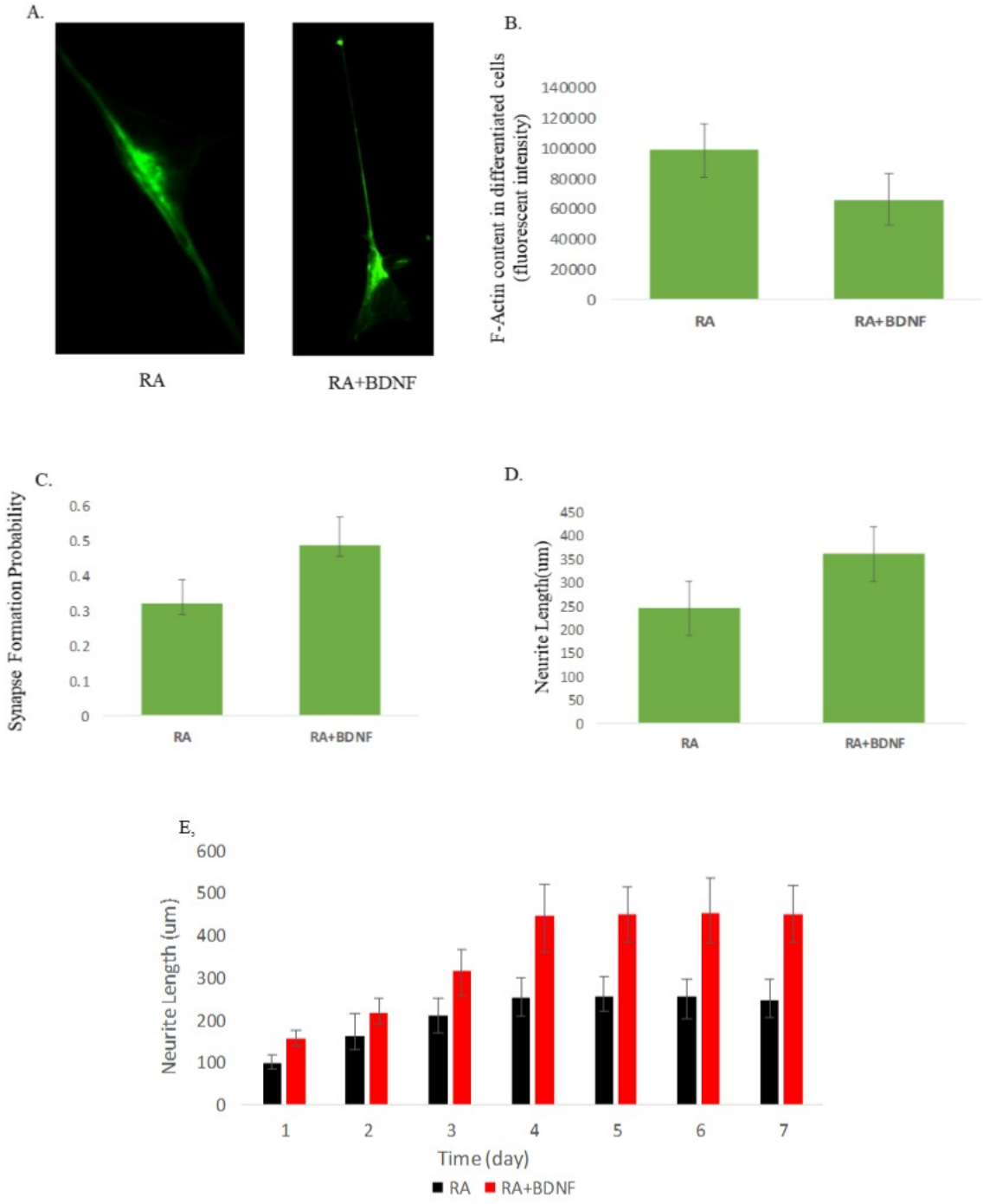

Figure 3.1 Morphological Changes during SH-SY5Y cell differentiation demonstrates a significant increase in neurite length (C,D), F-actin uptake also increases significantly in BDNF treated group compared to the control RA group (A,B). Morphological changes plateau for both samples at day 4 (E).

As one of the main objectives of this study was to establish an in-vitro model

for further research use, first a general screening of the electrophysiological properties of the cells was conducted. Undifferentiated cells were recorded at day 3 of cell culture and all differentiated cells were recorded at day 15 of differentiation. As figure 3.2 and 3.3 demonstrate, results indicated 5 electrophysiological subtypes prior differentiation (n=32) with some exhibiting inward outward action potential-like currents (n=5). Electrophysiological diversity of the cells decreased significantly after differentiation and only two electrophysiological sub-types were identifiable after differentiation (n=25).

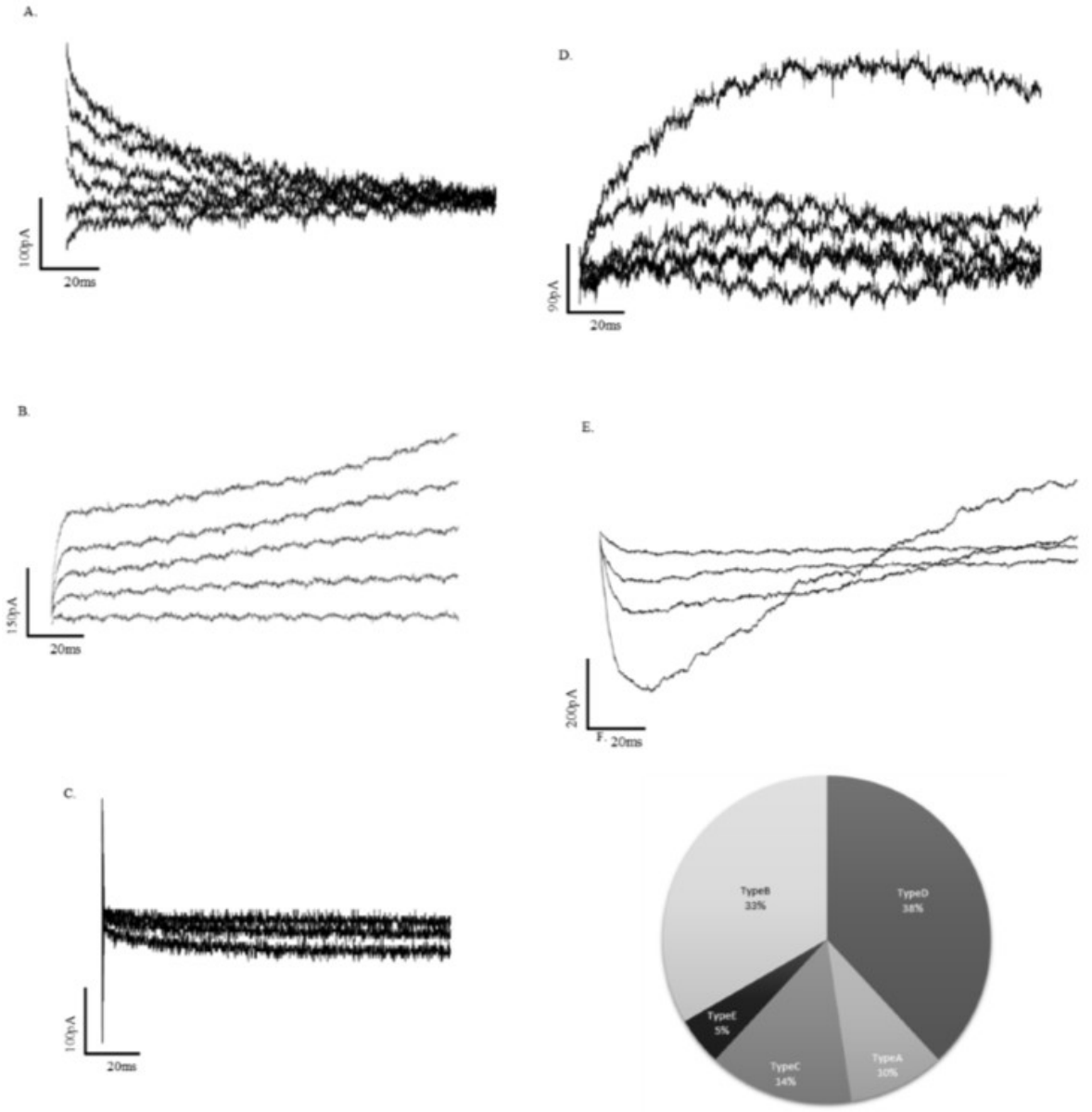

Figure 3.2 Five electrophysiological subtypes identified for the undifferentiated celd (A-E) and the significant heterogeneity of undifferentiated SH-SY5Y cells shown as the fractional distribution of each electrophysiological subtype (F).

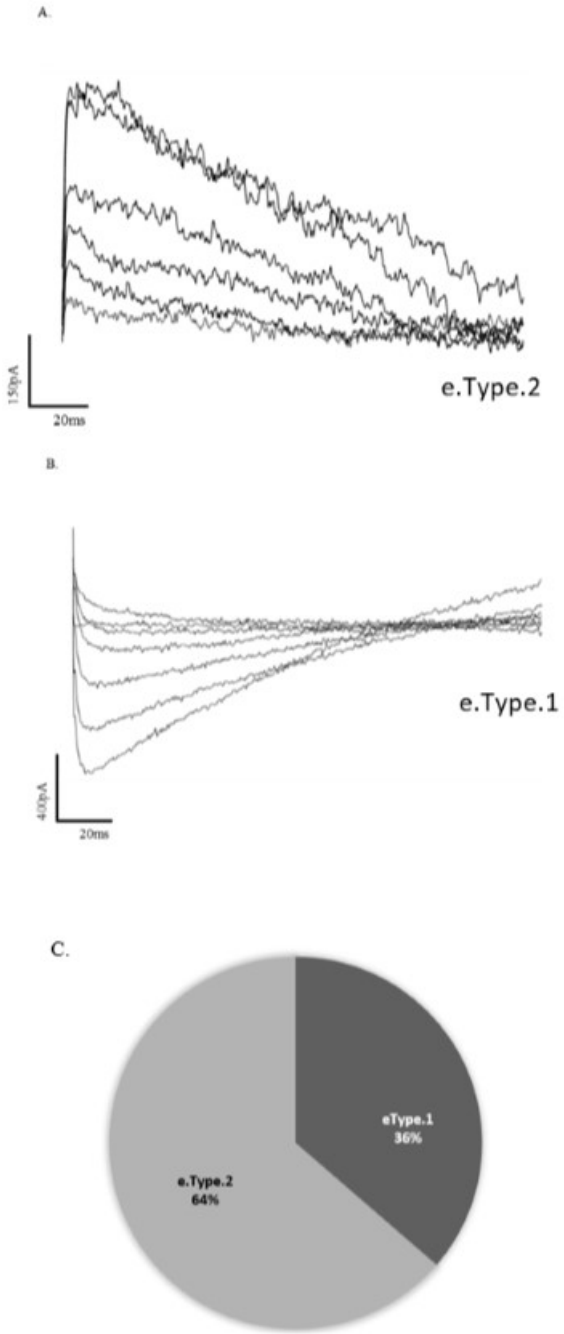

Figure 3.3 Upon differentiation heterogeneity of electrophysiological properties of the cells decrease significantly. In the differentiated sets two types of electrophysiological activity is observable, the most prevelant type consists of a prominent outward current (A,C), a minority of cells show a classic inward-outward current (B, C) reminescence of action potentials.

We observed that, after treatment with BDNF, the fraction of cells over-expressing potassium channels was increased from 18.1 percent at RA control group compared with 10 in RA+BDNF group ($p<0.043$, two-sample t-test). In total 22 recording attempts were made from which 5 recordings from RA group and 8 recordings from the BDNF

group showed full inward outward current.

A brief comparison between I-V diagrams depicted in figure 3.4, of the two groups shows that the mean activation threshold of the BDNF group is slightly less than the RA group but the results do not imply a significant difference ($P<0.1$ two-sample t-test). But there is a significant difference between the observed maximum inward currents ($P<8e\text{-}16$ for Klomogrov-Smirnov test, $p<0.0063$ for two-sample t-test with a power of 1) which increases from mean -302 Pico Amperes (pA) in RA group to a mean of 1406pA in BDNF group (effect size of 18SD). However the observed increase of mean maximum outward current of the BDNF group was assessed to be insignificant ($p<0.8$, two-sample t-test), the outward current's slope was significantly increased from a statistical perspective ($p<0.015$) from 30 to 450, the same significant difference also was observed for the inward currents. Derivatives of current profiles with regards to time (dI/dt) also showed a significant difference between two groups ($P<0.01$, two-sample t-test) implying an increase among the BDNF group.

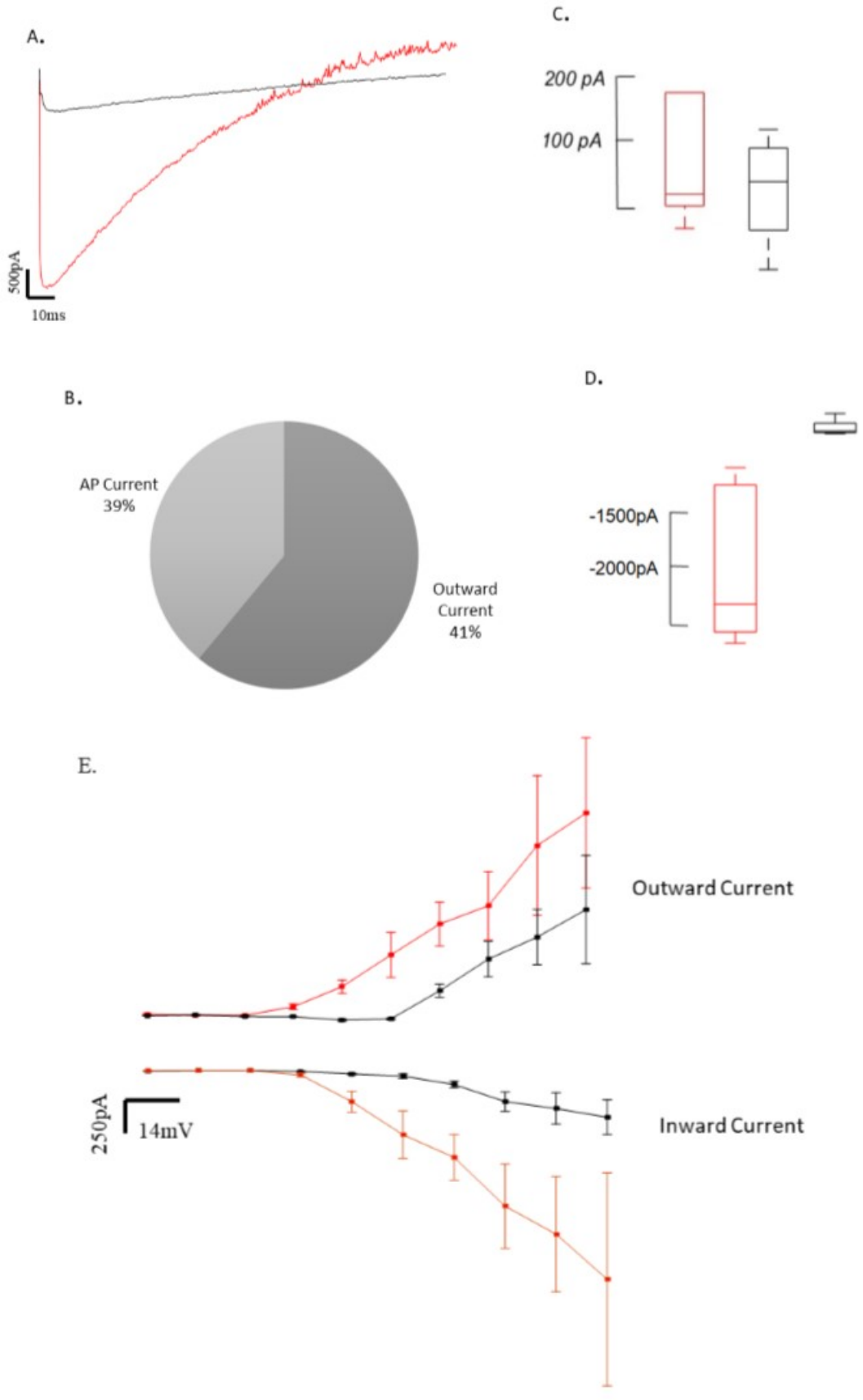

Figure 3.4 Whole Cell Recording of Transmembrane Currents Demonstrated a significant increase in inward current of BDNF treated group(A, D) while outward current didnt show any significant changes(D). Fraction of firing cells also didnt change in the BDNF group (B) chnges in the activation dynamics of Ion channels was evident in the voltage current profiles (E).

To assert the previous observation and obtain detail kinetics of channel types, a Hodgkin Huxley model with one type of sodium and one type of potassium channel was fitted to the highest voltage step (142mV). It is established that Hodgkin Huxley model maps a series of possible populations of ion channels corresponding to the observed current profiles, rather than a precise population composition. To acquire the mean of possible populations the fitting procedure was repeated five times and then population of each constant was examined for a significant change. The fitting algorithm was based on using a genetic algorithm to satisfy a least square criterion. All fitted models had a goodness of fit value of less than 0.2 (quality of which is demonstrated in figure.3.5).

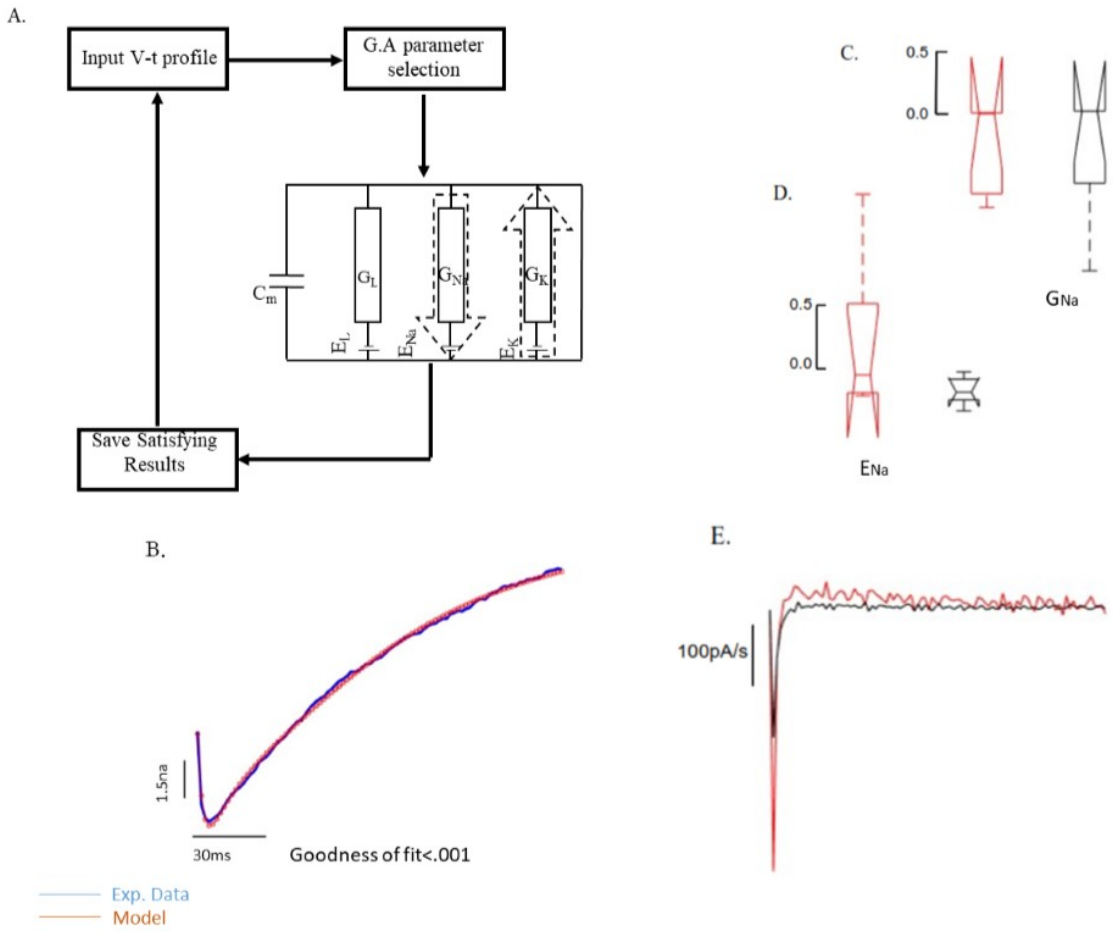

Figure 3.5 A genetic algorithm coupled with Hodgkin Huxley model (A) was used to model the dynamics of ion channels. comparison of the possible populations generated for gating characteristics (C) and deactivation times(D) contradicted with experimental data desipte achievement of desirabole fit quality (B), and observation of dynamic changes in time derivatives of transmembrane currents(E).

Despite the raw observations of increased inward currents and faster outward currents but at the same magnitude, Hodgkin Huxley model failed to affirm this observation. Comparison of sodium conductance values did not even exhibit a significant difference ($P<0.4$, two-sample t-test), the same indifference was also observed for activation times of potassium channels ($P<0.8$, two-sample t-test). The raw observations are also in line with previous molecular studies which have concluded that [2,3] channels were expressed more due to presence of BDNF asserting incompetence of the model in illustration of the observed results.

With disqualification of numerical approach to understand the underlying causes of changes in ion channels, the work proceeded to examine effects of selected neurodegenerative agent. However several agents were employed including rotenone, ceramide and amyloid beta. 50mM of Amyloid beta 1-42 purchased from sigma was added to the growth medium at day 15 of differentiation.

Morphological characterization was employed to assess cell survival initially. In the RA group, number of cells dropped dramatically to less than 20 percent ($n=10$, $t<0.001$, $p<0.001$), in the first day and almost all the cells died after the third day ($n=10$, $t<0.001$, $p<0.001$). In the BDNF group however a significant increase in cell survivability, with cell count dropping to roughly 80 percent ($n=7$, $t=0.015$, $p<0.007$) and remained at the same level for the next days until one week after Amyloid beta treatment ($n=7$, $t=0.08$, $p<0.09$).

As depicted by figure 3.6, new morphological signatures were identified in the BDNF cells which corresponds to known effects of amyloid beta on culture cells. Observation of microbeads on neurites which indicated neurofibrillary tangles significantly increased after amyloid beta treatment ($n=7$, $t=0.008$, $p<0.001$) and synaptic distortion, characterized by convergence angle at the end of neurite, was also significantly increased after amyloid bet treatment ($n=7$, $t=0.012$, $p<0.018$). Further f-actin staining also confirmed increased presence of actin in microbeads observed on the neurites.

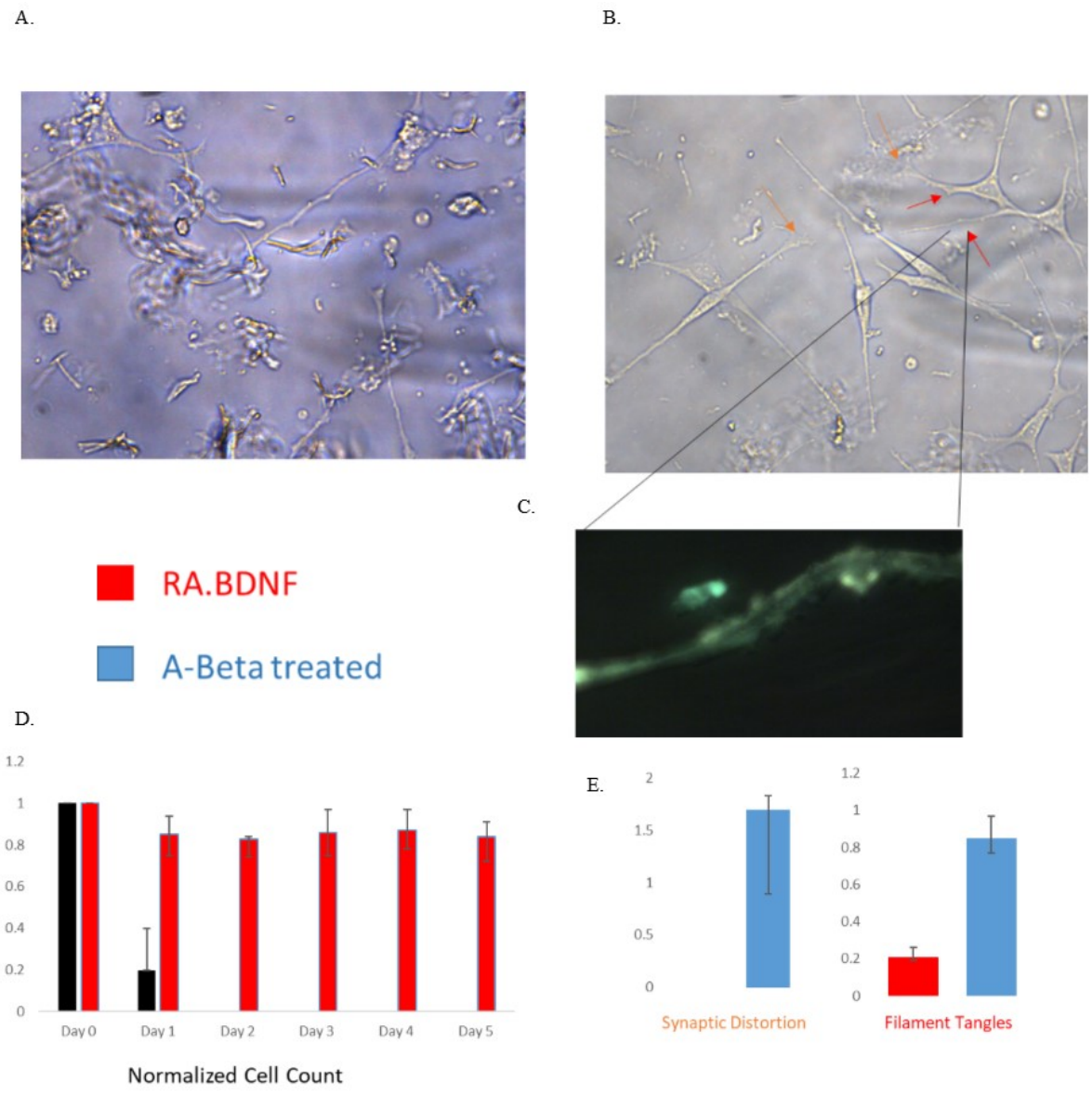

Figure 3.6 Morphological evaluation of cell response to Amyloid Beta toxication showed that RA differentiated cells demonstrate little resistance to Amyloid Beta (A) while BDNF treated group show significant resistance to amyloid beta efffects (B,D), accumulation of micro beads and synaptic distortion (E) was used as hall marks of Amyloid beta uptake by BDNF cells, furthermoe relation of microbeads to neurifibrillary tangles was demonstrated by F-actin staining of the cells.

Whole cell recording of the BDNF samples treated by amyloid beta was performed at the first day of treatment of the cells with amyloid beta. Recordings (n=11) uncovered a new electrophysiological subtype among the treated cells (n=2) with a prominent depolarizing current after the initial inward-outward currents which is depicted in figure 3.7. Furthermore a significant reduction in maximum inward current

in the sets was observed compared with nor amyloid beta treated BDNF cells (n=11, t<0.001, p<0.001). the same increase was observed in outward potassium current of the first electrical subtype of Amilooyd-beta treated BDNF cells.

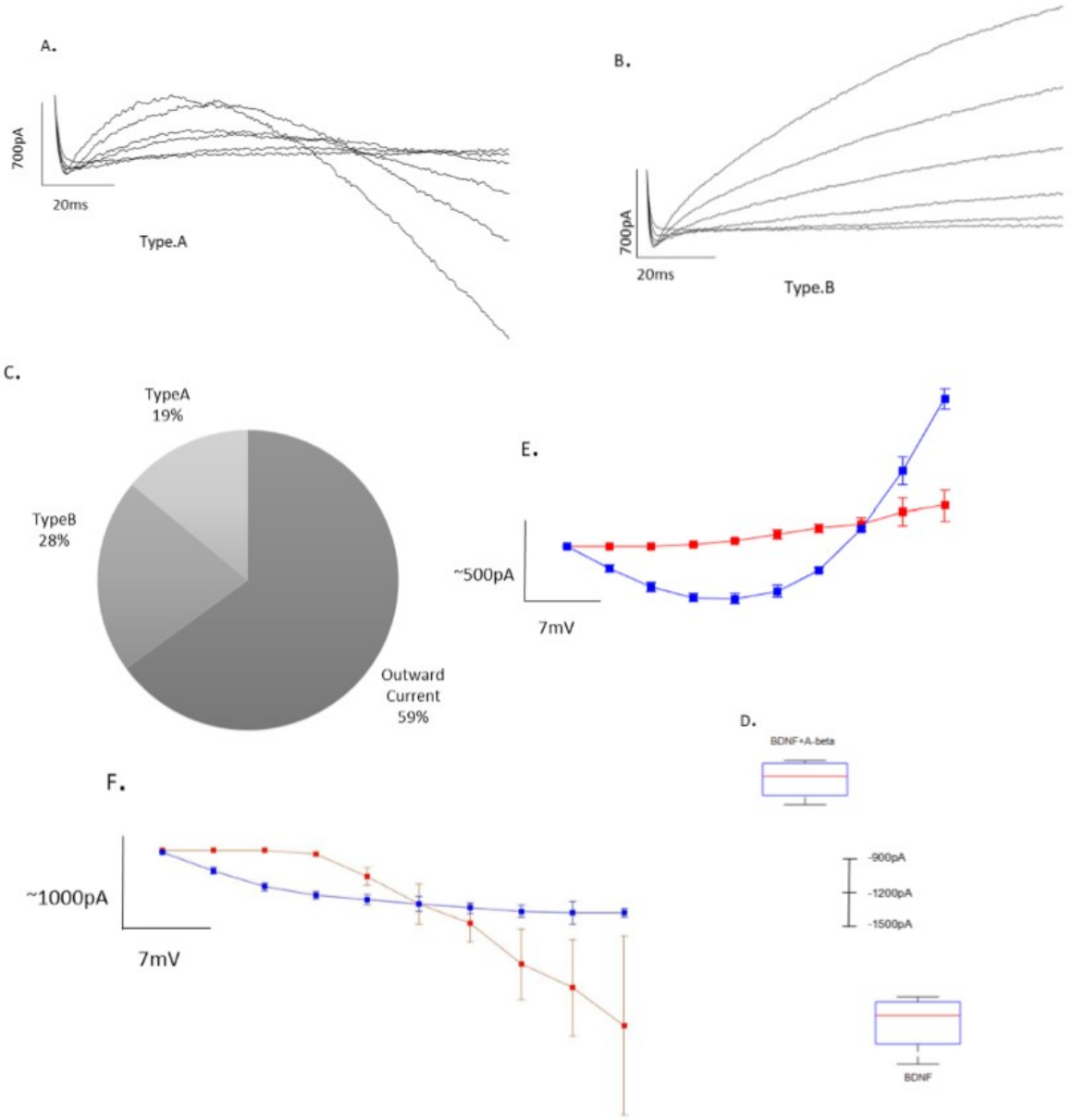

Figure 3.7 Transmembrane currents of BDNF differentiated cells treated by Amyloid beta demonstrated two distinct action potential firing current (A,B), while the total fraction of firing cells to non firing cells remained the same(C), Current-voltage diagrams demonstrated a significant alteration in activation profiles of inward (F) and outward (E) currents, a significant difference between maximum inward current of BDNF cells prior and after Differentaiation by Amyloid-betaeta was aslo demonstarted (D).

3.2 Lipid Coating

A total number of +70 coating attempts were made, 30 of which was from the first batch which contained 3Ml of 1mM POPC in chloroform, from the 30 initial coating, 24 resulted in formation of a giga seal seconds (10-60s) after being immersed in the bath containing Artificial Cerebrospinal Fluid (150Mm NaCl, 4 mM KCl, 2mM CaCl, 1.2 mM MgSO4, 10mM HEPES at PH=7.4). The ability to form giga-seals in bath degraded significantly after the first batch (10 observations in next 40 coatings). However, lipid coating of the micropipettes were confirmed by observation of a thick lipid layer on the bath solution (similar to situations where a giga-seal was observed, a DIL(X) dye was not available to further confirm lipid transfer) in +65 cases of coating attempts.

A total of 10 recording attempts was made from SH-SY5Y neuroblastoma cell line differentiated by 10mM Retinoic Acid (RA) at day +15. Average access resistance was recorded to be 350 Mohm. In two cases, two consequent recording from one cell was attempted, in both cases the cell did not detach from the petri dish (extremely rare in neuroblastoma cells).

Stability of lipid coating was determined by applying voltage steps and recording membrane capacitance until the point of poration of the membrane. Both membrane capacitance and poration threshold showed significant variation pointing to significant variation between quality of the coatings. Laffafian and Hallett, in their 1996 paper, described this variation as varying number of cells which could be injected with a single coated needle).

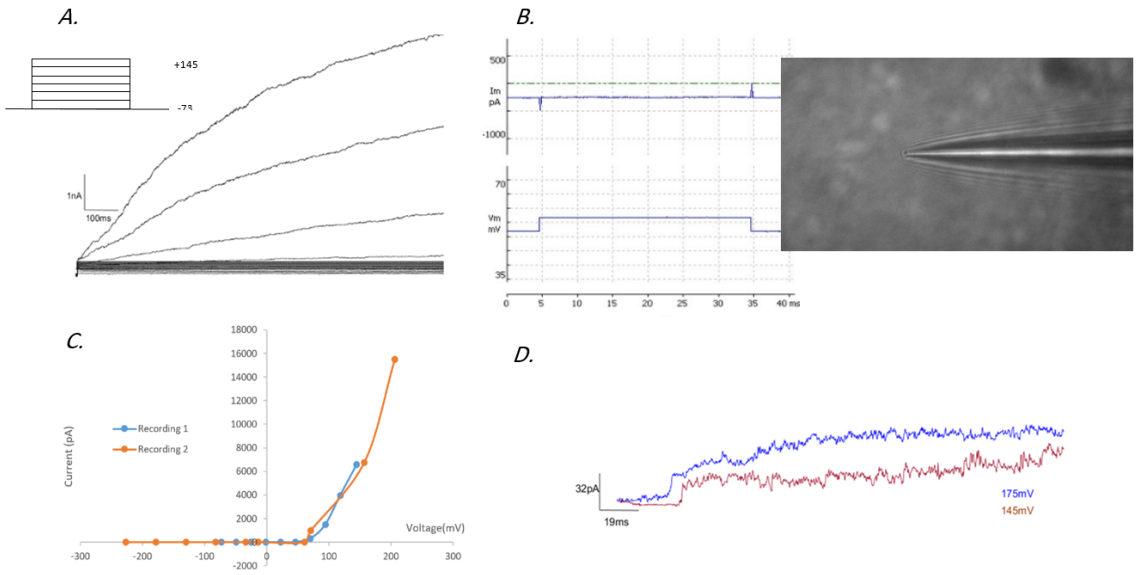

Figure 3.8 Voltage clamp recording of a POPC bilayer showing incoming current after electroporation. B: lipid coating and formation of giga-seal in the tip of the micropipette. C: disparity between two voltage clamp recordings, D: observation of Opening in Electroporation of a POPC membrane due to two different voltage steps.

One other significant observation of this study was the delayed opening of the lipid coating in cases which exhibited strong coating. A total 0f 10 recording from 3 coatings exhibited such characteristics. In all cases the delay required for poration of coating decreased nonlinearly with increasing the voltage step. Further post processing and data analysis of the results was deemed not useful as so many parameters were not under control during the recordings, the least of which was temperature of the bath.

This technique was further improved by the generous support provided by Laboratory of Neural Micro Circuitry in EPFL under Prof. Henry Markram and Dr. Rodrigo Perin. During this optimization attempt, 4 different types of lipids (POPC (sigma), DOPC(sigma), DOPS(sigma) and DPHPC(Avanti)) in addition to cholestrol were used to optimize lipid formation, stability and othe relevant parameters.

Results pointed that spread coating as indicated by [28], using DPHPC lipid type would result in more than 85 percent coating probability with feasible stability of

the lipid coating, the preliminary results are presented in Figure 3.9.

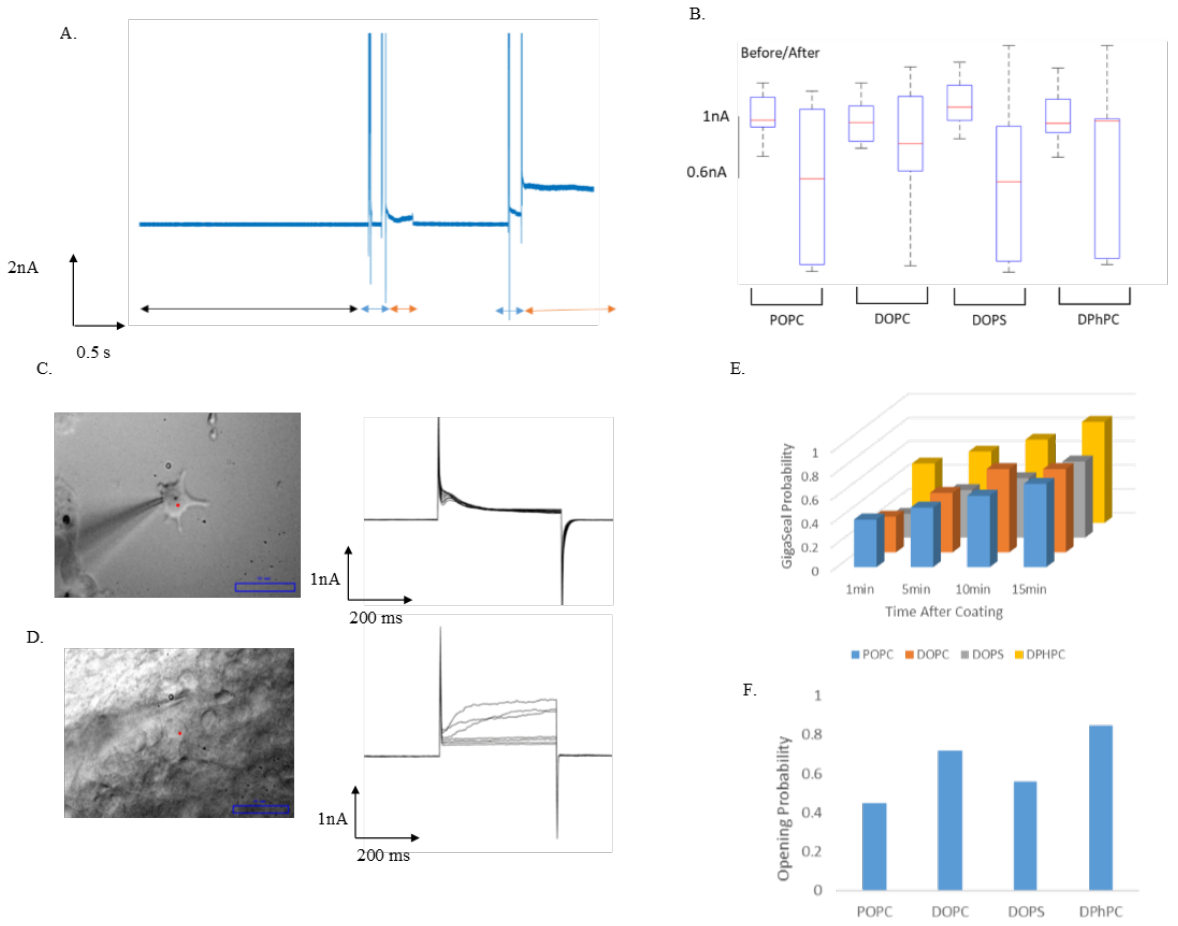

Figure 3.9 Optimization of coating protocols by different lipid types and variation of different parameters demonstrates the immense potential of the technique to be enhanced. A: general trace of a lipid coated pipette subjected to zap pulses (blue arrows) which causes opening in the coating (red arrows), B: avrage pipette current passing through the pipette before and after coating, C: Sample recording from culture cells, D: sample voltage clamp recording trace from a random cortical cell, E: optimization effects on each of the lipid times through time after coating initiation, F. probability of each coating being opened after a zap pulse.

To verify the recording ability, two separate sets of recordings was conducted; one in cell culture slices and the other in acute tissues. The achieved sample size was deemed not big enough (5 successful recording from culture cells and 2 successful recording from the slices. however, the optimized protocol and successful recordings can act as a proof of concept to develop the technique in collaborative efforts between the labs in future.

4. DISCUSSIONS AND CONCLUSIONS

4.1 Implications and Discussions on Results

The general results of this project could be described in two categories. First the procedure and verification of establishment of an in-vitro model resulting on a viable model to investigate neurodegenerative agents. And secondly a demonstration of application of the established model to investigate a biologically viable question.

For the first part, it was demonstrated that the existing cell line in Bogazici university institute of biomedical engineering is capable of demonstrating neural like behavior upon application of certain protocols. These protocols were as such that they enabled maximum use of open source data and no case specific modification was required to make them compatible with the existing instrumentations in the lab. All protocols then was shown to be repeatable and reliable in their outcome as it was evident by the considerable sample sizes in each group of study.

Furthermore it was shown that the existing cell line is responsive to neuro degenerative agents and it could be characterized by using the electrophysiological and microscopic instrumentation available in the lab. Minimal requirements for execution of the principles of the project means that the presented model could be modified extensively for future projects.

A vast body of research utilizes undifferentiated SH-SY5Y cell line in order to investigate different constituents of neuro-degenerative disease [29, 30, 31]. Numerous problems have been associated with this mass assay approach including unreliability of electrophysiological data [3]. By presenting a categorical classification of undifferentiated and differentiated neurons, we demonstrated that consistent data sets with reasonable variety could be generated for each subject sample.

However the in-silico means utilized in this study proved to be inadequate in providing more insight to molcular underpinnings of the observed changes, this failure could be seen from the prospect of using the in-vitro model to develop other in-silico models to enhance understanding from electrophysiological dynamics in different Neuro-degenerative diseases.

There are extensive debates on viability of different models to capture transmembrane current dynamics [25, 32, 33], which mostly discuss the possible populations of ion channels produced by models from the experimental results. Yet the in-vitro model, due to its accommodation of several differentiation protocols and the detailed previous characterization proved non trivial and challenging to the currently known numerical models.

Verification of the presented model is the result of successful execution of the demonstration study on counter effects of amyloid beta and brain derived neurotrophic factor. However a demonstration study, the acquire result on amyloid beta deserve a discussion in their own right.

Current understanding of the effects of amyloid beta on transmembrane current of neurons is largely based on the secretase hypothesis presented in the introduction section of this text. This hypothesis states that the reduction in original transmembrane currents is largely due to interference of APP cleavage procedure with the beta subunits of transmembrane channels. We bypassed this model by directly using amyloid beta peptides rather than amyloid precursor proteins.

We also challenged a new line of studies which focus on protective effects of BDNF against effects of amyloid beta intoxication. Indeed our results demonstrated that BDNF significantly boosts cell survival in confrontation with amyloid beta toxicities [34, 35, 36]. But at the same time changes in transmembrane currents of the survived cells demonstrate the disparity between morphological and functional survival of the neural cells.

Introduction of the new functional-morphological dynamics in cell survival against amyloid beta effects opens the path for future work which can benefit from a vast range of single cell and network level techniques. For example single cell transcriptomic techniques can shed light on the molecular events leading to decline in transmembrane current demise and multipatch techniques could be employed to investigate network level effects of alteration in ion channel composition.

The model presented in this study has the potential of being applied to various degenerative states,

Beside pharmacological studies on neurodegenerative diseases, the established model also proved to be capable of evaluating novel neurotechnologies. development of lipid coating approach for electrophysiological recording of the cells exemplifies this fact.

The challenging process of optimizing lipid coating, proved additional standard parameters are required in presentation of lipid coating studies. This study presented probability of coating which was not present in any of the previous work [37, 28, 26]. Results obtained in this project with collaboration of Laboratory of neural microcircuitry in Lausanne, Switzerland, demonstrated that the concept of achieving lipid coatings to form gigaseals inside the bath and then perform whole cell electrophysiological recordings is attainable but needs further modification and optimization to reach operational status.

4.2 Future Work

The three main achievements of this study could be further expanded to, establish more robust models, illuminate unknown aspects of neurodegenerative diseases and development of new neurotechnologies.

Addition of other cell line such as induced pluripotent stem cells, HEK cells

and other available cell lines to provide redundantly verifying results to any present question, Finding survival boost agents for rotenone and ceramide would also add two active models to investigate in the present SH-SY5Y substrate.

Further work is also required to establish lipid coated patching as a novel form of electrophysiological recording. After stabilization of the coating the major remaining challenge is to facilitate smooth opening between the cell and the pipette. This can be achieved by incorporating large transmembrane molecules in the coating or use of cholesterol as a destabilizing factor .

4.3 List of publications produced from the thesis

1. M. Abbaszadeh, A. Ozgun, B, Garipcan, H. Saybasili "BDNF enhances the inward current in SH-SY5Y neuroblastoma cell Line" poster presentation, FENS2018, Berlin, 2018.

2. M. Abbaszadeh, A. Ozgun, B, Garipcan, H. Saybasili "Braind Dericved Neurotropic Factor Enhances Morphological Survivability but not Functional characteristics of SH-SY5Y neuroblastoma Cells" (Under Preperation).

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