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DIPLOMARBEIT

GENES RELATED TO CALCIUM SIGNALING AND THEIR ASSOCIATED DISEASES

A BIOINFORMATICAL AND EXPERIMENTAL STUDY

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ABSTRACT

The calcium ion (Ca^{2+}) is one of the key components of inter- and intracellular signaling. From the first cell division, to the contraction of muscle fibres, to the formation of memories, to cell death, all these processes are governed by changing amounts of intracellular Ca^{2+}. Despite the central function of this ion, little is known about the genetic background of the Ca^{2+} signaling toolkit, especially its link to human diseases.

This thesis, separated into two parts, approaches genes connected to Ca^{2+} signaling from two different stand points.

In the first part, a bioinformatic approach was chosen, to determine all genes with a possible connection to Ca^{2+} signaling, as well as diseases associated with these genes. Here we present the Calcium Gene Database (CaGeDB), the first attempt to establish a comprehensive database linking genes to Ca^{2+} signaling and their associated diseases. Initially, possible gene database annotation terms were selected. These were then used to query two genome databases. After merging the resulting genes, a database mapping genes to diseases was queried. To improve the handling of the data, another database which provides a hierarchical structure for diseases was used. All data is acquired automatically by scripts written for this thesis.

CaGeDB contains 1597 genes which are connected to 1409 different diseases and disease categories and is available at uhlenlab.org/cagedb.

For the second part, an experimental approach using cell lines to examine the relation between the gene SNCA, which is involved in Parkinson's Disease and Ca^{2+} signaling was chosen. Here we show that cells with a suppressed SNCA expression had a significantly higher rate of Ca^{2+} induced cell death compared to normal cells. In order to show this, HeLa cells were cultured and then transfected with DNA to suppress the expression of SNCA. Afterwards the effects of this suppression with respect to Ca^{2+} signals and especially Ca^{2+} induced cell death were tested using wide field fluorescence microscopy.

Because the concentration of Ca^{2+} in the mitochondria is a key component in the initiation of cell death, the results strengthen the hypothesis that α-synuclein is involved in the mitochondrial Ca^{2+} homeostasis.

Both parts, the first one as a general discovery tool and the second one providing further knowledge about a specific gene and its role in Ca^{2+} signaling shed new light on the role genes play with respect to Ca^{2+} signals.

Kalzium, genauer gesagt das Kalzium Ion Ca^{2+}, stellt eines der grundlegendsten Signale innerhalb einer einzelnen Zelle aber auch zwischen mehreren benachbarten Zellen dar, dabei fungiert der Kalziumspiegel als Informationsträger. Die erste Zellteilung, die Kontraktion von Muskelfasern, die Speicherung von Erinnerung und der programmierte Zelltod, all das sind nur einige Beispiele der unzähligen Vorgänge in einem Organismus, die durch den Kalziumgehalt in Zellen gesteuert werden. Trotz dieser zentralen Rolle, ist wenig über den genetische Hintergrund des Kalziumsignalfunktionen bekannt, insbesondere in Hinsicht auf
Krankheiten.
Diese Arbeit besteht aus zwei Teilen, die die Verbindung zwischen Genen und Kalziumsignalen aus zwei verschiedenen Richtungen betrachtet.
Im ersten Teil wurden ein bioinformatischer Ansatz gewählt um alle Gene, die mit Kalziumsignalen verknüpft sein könnten, als auch die mit ihnen verbundenen Krankheiten zu bestimmen. Die daraus entstandene Datenbank CaGeDB stellt dabei den ersten Versuch dar, eine Datenbank zu erstellen, die Gene, die mit Kalziumsignalen in Verbindung gebracht werden, sowie den mit diesen Genen in Verbindung gebrachten Krankheiten, zu sammeln. Dazu wurden zuerst für Kalziumsignale relevante Begriffe, die in Gendatenbanken verwendet werden um die Genfunktion zu beschreiben, ausgewählt. Anhand dieser wurden dann zwei Gendatenbanken abgefragt und die daraus resultierenden Ergebnisse miteinander abgeglichen. Anschließend wurde eine weitere Datenbank verwendet um Krankheiten, die mit diesen Genen in Verbindung gebracht werden, zu finden. Um diese Krankheiten besser handhaben zu können, wurde eine zusätzliche Datenbank benutzt, mittels derer die Krankheitsbegriffe gruppiert werden konnten. Insgesamt umfasst die resultierende Datenbank, CaGeDB, 1597 Gene und 1409 verschiedene Krankheiten und ist unter uhlenlab.org/cagedb erreichbar.
Im zweiten Teil der Arbeit wurden experimentelle Mittel gewählt um eine mögliche Verbindung zwischen dem Gen SNCA und Kalziumsignalen genauer zu untersuchen. Das Gen SNCA stellt das Rezept für das Protein α-Synuclein dar, welches eine Rolle bei der Parkinson-Krankheit spielt. Die genaue Funktion dieses Proteins ist noch unbekannt, es gibt jedoch Indizien die darauf Hinweisen, dass es eine Rolle in der Regulierung das Kalziumspiegels innerhalb der Mitochondrien spielt. Dieser Aspekt wurde in dieser Arbeit genauer untersucht, dabei wurde ausgenutzt, dass die Mitochondrien nicht nur Energie für die Zellen produzieren, sondern auch den programmierten Zell-

tod einleiten. Dazu wurden zuerst sogenannte HeLa Zellen, eine sehr robuste Zellkultur menschlichen Ursprungs, gezüchtet und anschließend Genmaterial eingeschleust, welches die korrekte Auslese des Gens SNCA unterdrückt. Danach wurde per Fluoreszenzmikroskop untersucht, wie sich diese Veränderung auf den durch Kalziumüberschuss eingeleiteten Zelltods auswirkt. . Hierbei zeigte sich, dass jene Zellen bei denen SNCA unterdrückt wurde, signifikant häufiger den programmierten Zelltod einleiten. Nachdem dies auf eine gestörte Regulation des Kalziumspiegels zurückgeführt werden kann, stellt dieses Resultat ein weiteres Indiz dafür da, dass α-Synuclein an der Kontrolle des Kalziumhaushalts beteiligt ist.

ACKNOWLEDGMENTS

The thesis in front of you is not the result of my work alone, but was only possible with the support by numerous people during my six years of studies.

First of all, I am grateful to my parents whose support I could always relay on.

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Thanks to all other members of the Per Uhlén group who were always open and helpful to me. Special thanks to Songbai Zhang for showing me everything I needed to know in the cell culture lab.

This thesis would also have not been possible without my university colleagues who led me to and through the masters programme of biomedical engineering and without whom I would have not been able to bear the content and stress of my studies.
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CONTENTS

1

INTRODUCTION TO CALCIUM SIGNALING

The basic component behind Ca^{2+} signaling is the changing amount of intracellular Ca^{2+}. Under physiological conditions the intracellular Ca^{2+} concentration is approximately 100nM, which is raised into the micromolar range during a signal peak [1]. Since the prolonged exposure to such high Ca^{2+} levels is toxic for the cell and because of similarly high extracellular Ca^{2+} concentrations, intricate cellular mechanism have evolved to control these fluctuations [2].
Ca^{2+} signals are used in every cell type in numerous ways, for example during fertilization, where the fusion of the sperm cell with the ovum rapidly elevates the egg cells Ca^{2+} concentration starting from the point of the fusion and travelling as a wave through the whole with multiple effects, from the elevation of the fertilization envelope to the start of the cell cycle [3]. But as mentioned above, it is also used during cell death, controlling both the apoptotic pathway, i.e., programmed cell death, as well as necrosis [4]. It governs the axis and pattern formation in embryos as the asymmetrical spreading and locations of Ca^{2+} signals lead to asymmetrical gene expression [3]. Ca^{2+} signals are also involved in the formation of memories, by facilitating the release of neurotransmitters as well as through regulation of genes and thereby strengthening and weakening synapses [5].
These are just a few examples of the numerous processes utilizing Ca^{2+} in an organism.
To illustrate what Ca^{2+} signals look like, an example of a Ca^{2+} signal controlling the contraction of skeletal muscle cells is given below. It was chosen, because it is well established that Ca^{2+} is multiple times involved in this process with different effects. Although the pathway is not exactly the same for all muscle types, Ca^{2+} is involved in all of them. For an illustration of the description below, see Figure 1 and Figure 2. Note that the figure only shows the mechanisms increasing the intracellular Ca^{2+} concentration and its effects.

Ca^{2+} signals are involved in multiple ways in this process. First, when an action potential, i.e., an electrical signal, which has originated somewhere in the central nervous system, reaches the connection between neurones and muscle fibres. The nerve ending, i.e., the synapse, contains voltage gated Ca^{2+} channels, which are opened by the action potential causing extracellular Ca^{2+} to enter the target cell. There, Ca^{2+} is bound to synaptotagmin, which is located between the plasma membrane and a vesicle containing acetylcholine. Upon binding of Ca^{2+} to synaptotagmin undergoes a conformational change which allows a partial insertion into the plasma membrane. This insertion of synaptotagmin increases the local curvature of the plasma membrane and

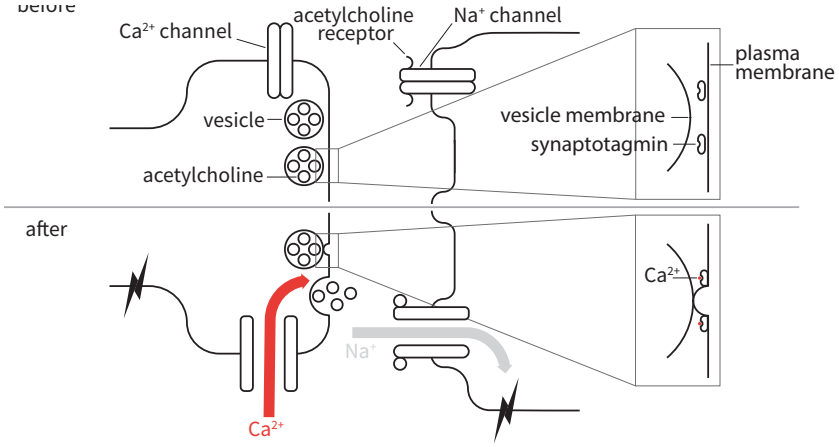

Figure 1: Before (top) and after (bottom) the start of the Ca^{2+} signal required for the contraction of skeletal muscle fibres at the synaptic junction. An action potential activates voltage gated Ca^{2+} channels at the synaptic head. A zoom-in of the reaction involving Ca^{2+} is shown on the right side. The imported Ca^{2+} binds to synaptotagmin, which leads to a partial insertion into the plasma membrane, increasing the local curvature and initiating membrane fusion between the plasma membrane and the membrane of the vesicle containing acetylcholine. At the postsynaptic membrane acetylcholine the released acetylcholine binds to receptors which activate Na+ channels, initiating an action potential.

reduces the distance between the two bilipid layers, both effects increase the probability of membrane fusion [6]. Shortly after the Ca^{2+} channels open, plasma membrane Ca^{2+} ATPase is activated and decreases the Ca^{2+} concentration by pumping Ca^{2+} out of the synapse, thereby stopping the release of further vesicles.

After the fusion of the membranes, acetylcholine is released, diffuses across the synaptic cleft and binds onto acetylcholine receptors on the postsynaptic membrane. These receptors open sodium channels, resulting in an influx of sodium. This leads to a less negativ membrane potential, which triggers an action potential.

The action potential spreads across the muscle fibre network via T-tubules and cause the activation of dihydropyridine receptor L-type Ca2+ channel (DHPR), a voltage gated Ca^{2+} channels, in the membrane of the T-tubules. But the Ca^{2+} gating mechanism of these channels seems to be not their main mode of operation in this case, since the signal transduction works even in the absence of external Ca^{2+} [7]. The signal seems rather to be dependent on a physically linkage between DHPR on the T-tubules and Ca^{2+} release channels on the membrane of the sarcoplasmic reticulum [8]. These release channels are called ryanodine receptor 1 (RyR1) and release Ca^{2+} from the sarcoplasmic reticulum to the cytoplasm.

There, Ca^{2+} binds to troponin, which is connected to tropomyosin. Tropomyosin is wound around the actin filaments and held by troponin above the binding sites for the myosin heads. Upon binding of

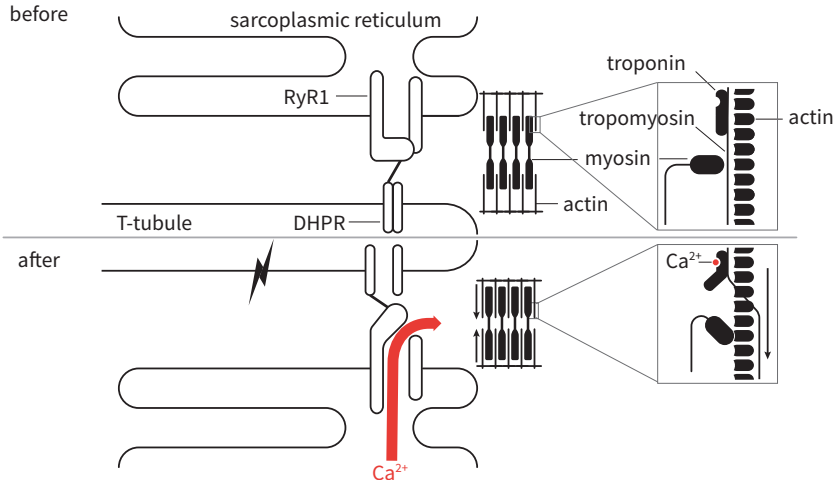

Figure 2: Before (top) and after (bottom) the start of the Ca^{2+} signal required for the contraction of skeletal muscle fibres inside the muscle fibre. A zoom-in of the reaction involving Ca^{2+} is shown on the right side. The action potential originating in the postsynaptic membrane travels through the T-tubule and activates the voltage gated channel DHPR. This activation opens the is physically coupled Ca^{2+} channel RyR1, which is located on the sarcoplasmic reticulum. The released Ca^{2+} binds to troponin, inducing a conformational change, which allows tropomyosin to reveal the binding sites on the actin filament. The myosin head binds to actin filament and changes is conformation, which leads to the contraction of the muscle fibre.

Ca^{2+}, which takes 1 ms [9], troponin changes its conformation, allowing tropomyosin to move. This allows the myosin heads to bind on the actin filament and change their confirmation, which moves the actin filament relative to the myosin, leading to the contraction of the fibre. As soon as Ca^{2+} is released from the sarcoplasmic reticulum Ca^{2+}-ATPase starts to pump it back into the sarcoplasmic reticulum, thereby limiting the repetitions of this cycle. Of course Ca^{2+} is not only bound by synaptotagmin and troponin in this process, but interacts with numerous other proteins, which can indirectly influence muscle plasticity and activity [9].

In this example we see mechanisms (voltage gated Ca^{2+} channels) increasing the cytosolic Ca^{2+} concentration. Processes reducing the Ca^{2+} level in the cytosol (Ca^{2+} pumps) and the effect of the signal on cellular processes (binding of myosin to actin via binding of Ca^{2+} to troponin).
This view can be applied to all Ca^{2+} signaling pathways and can therefore be used to group different components involved in this system. Berridge introduced the terms ON-reactions, OFF-reactions and signaling functions for them [10].
In all three groups exist additional ways of these reactions:

- In the group of ON-reactions, i.e. reactions increasing the amount of Ca^{2+} in the cytosol, there exist also ligand operated Ca^{2+} channel, e.g., N-methyl-D-aspartate (NMDA). This Ca^{2+} channel is activated for example in the brain by the neurotransmitter Glutamate [2]. But Ca^{2+} itself can intensify the signal via Inositol trisphosphate receptors ($InsP_3Rs$), which release sequestered Ca^{2+} from the endoplasmic reticulum and played an important role in the history of Ca^{2+} signaling research [2].

- Mitochondrial uniporters are another possible component of OFF-reactions, i.e., reactions lowering the level of cytosolic Ca^{2+}. These channels enable the Ca^{2+} to be buffered in the mitochondria and later on transported to the sarco/endoplasmic reticulum. The electrochemical gradient created by the proton pump during the respiratory cycle is the driving force for the Ca^{2+} influx into the inner mitochondrial membrane [11]. But this mechanism is also dangerous, since a prolonged high Ca^{2+} level in the mitochondria blocks the production of ATP and induces cell death [4, 12].

- There exist numerous ways how the Ca^{2+} signal is translated, e.g., via other messenger proteins, calmodulin being the most prominent and abundant example. Binding of Ca^{2+} to this protein's four binding sites induces a conformational change, exposing hydrophobic groups, which facilitates interactions with other proteins. For example it can activate a Ca^{2+}-ATPase pump, controlling the amount of intracellular Ca^{2+} in this way [13].

Such Ca^{2+} increases can happen singularly or be rapidly repeated, as so called Ca^{2+} oscillations [2]. In this oscillations the information of the signal is not only encoded in the change of the Ca^{2+} concentration itself, but also in its frequency. These oscillations act as a compromise between a certain amount of Ca^{2+} needed for slower processes and the cytotoxicity of a prolonged and high Ca^{2+} concentration in the cytosol [14]. The target proteins, e.g. Ca^{2+}/calmodulin-dependent kinase II (CaM-kinase II), act in this case as integrators of the signals and show therefore activation in a magnitude a singular peak could not accomplish. This signal can also be transported between cells via gap junctions.
This thesis looks into the genetic background governing all these processes from two different angles. In the first part, a database collecting all genes connected to Ca^{2+} signaling and associated diseases is established. The second part takes a deeper look into one specific gene, SNCA, and examines its role in cellular Ca^{2+} homeostasis using HeLa cells.

2

BIOINFORMATIC STUDY: CaGeDB - A DATABASE FOR GENES RELATED TO CALCIUM SIGNALING AND THEIR ASSOCIATED DISEASES

2.1 INTRODUCTION

Since Ca^{2+} signaling is used in virtually every cell type with widely different applications [15], numerous reports of genes related to these signals exists, either involved in the increase of the intracellular Ca^{2+} concentration, i.e. ON-reactions, in its decrease, i.e., OFF-reactions or involved in its signaling function [15, 16].
There already exist general genome databases, e.g., ensembl [17] and NCBI-Gene [18], but because of their broad approach to collect every aspect of a gene, relations to Ca^{2+} signaling are often indistinct. More focused gene database for example on cancer related genes, e.g., Cancer Genetics Web [19]) exist as well, but no gene database focused on Ca^{2+} signaling has yet been established. Therefore the goal of this part of the thesis was the creation of the Calcium Gene Database (CaGeDB), a database containing all genes related to Ca^{2+} signaling as well as their associated diseases.
The straight forward approach would be to search for all scientific reports documenting genes connected to Ca^{2+} signaling. But in the last year alone over a thousand articles where published on this topic, therefore the collecting of these results has to be automated. But this presents another problem since there exists a large variety of terms used to describe Ca^{2+} signals.
Therefore a controlled gene annotation vocabulary had to be used. The choice fell on Gene Ontology (GO) terms, since these are widely used in genome databases and cover also Ca^{2+} signaling pathways. This also allowed to fully rely on and be comparable with already established genome databases, which are build on a well developed information extrusion infrastructure. Besides general genome databases, this also includes databases specialised on gene-disease associations.
Similar challenges arose for the names of diseases associated with the Ca^{2+} genes. In order to better interpret the results, terms from the Medical Subject Headings (MeSH) database, a controlled disease vocabulary were used. With this approach and the chosen databases, their provided APIs, i.e. functions to automatically access a database, could be used to create the database.
The complete database is updated automatically by scripts written in ruby and is available at www.uhlenlab.org/cagedb.

2.2 METHODS

Five databases were queried to create the database of genes related to Ca^{2+} signaling. A flow graph of the data acquisition is shown in figure 3. Each query is automatically executed by a ruby script and the results are saved in an SQLite database.

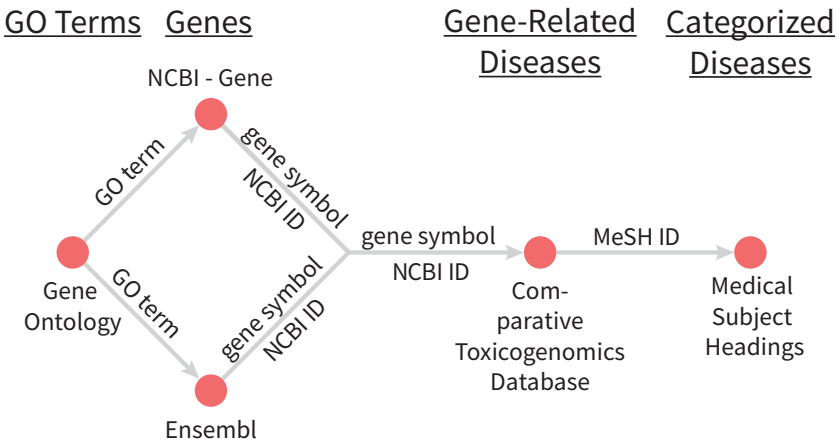

Figure 3: Overview of the data acquisition for the CaGeDB database. First, Gene Ontology (GO) terms describing the Ca^{2+} signaling pathway were chosen. These were used to query the databases NCBI-Gene and Ensembl. The results were merged based on their gene symbols and NCBI IDs. The resulting list of gene symbols together with NCBI IDs were used to query the Comparative Toxicogenomics Database to acquire diseases associated to the genes. In the last step these entries were connected with terms from the Medical Subject Headings (MeSH) database to categorize the diseases, based on the MeSH Tree hierarchy.

2.2.1 *GO Terms*

The Gene Ontology (GO, geneontology.org, [20]) is a database curated by the Gene Ontology consortium with the goal to establish a species-independent standardized description of biological processes and is the de-facto standard for protein function annotations [21].
The Gene Ontology consortium, which consists of several large databases, curates the ontology and its connected gene database. Each GO annotation contains beside its GO accession number, a GO term and an evidence code. This evidence code describes the source on which the annotation is based on, e.g., experimental data. These evidence codes are not directly connected to the annotation quality, but have some influence on it [21]. Because of this and the general approach of CaGeDB, each GO term gene connection was saved with the corresponding evidence code, but no filter based on them was applied. See section 2.3.2 for a more detailed description of the evidence codes which occurre in CaGeDB.

All database entries containing “Cal” were used as a starting point for CaGeDB. Afterwards these terms were manually filtered with respect to their relevance for Ca^{2+} signaling. The reason for this particular query term was that it enabled results not only containing the word “calcium” but also GO terms for Ca^{2+} regulated proteins like calmodulin. The full list of used GO terms is shown in Appendix A.
For a better overview of these terms, they were sorted using the categorization by Berridge of “signaling functions”, “ON-reactions”, “OFF-reactions”, and, if the term describes both of the two latter categories it was categorized as “ON-OFF-reactions” [1]. Except for the category signaling functions, the categories were further divided based on their localization into “intracellular”, “extracellular” and “at unspecified site”, if no specific location could be derived from the term or its description.

2.2.2 *Genes*

The following two gene databases were used to collect genes annotated with the selected GO terms.

- *Ensembl:* The Ensembl database (www.ensembl.org) is one of the largest platforms providing gene annotations for different species [17]. It is maintained by the Wellcome Trust Sanger Institute and the European Bioinformatics Institute.
  For this work Ensembl version 79 and the human genome assembly GRCh38.p2 were used.

- *NCBI - Gene:* This gene database is curated by the National Center for Biotechnological Information (NCBI,www.ncbi.nlm.nih.gov/gene) and updated weekly [18].
  The data used in this work was last updated on April 22, 2015.

These two databases were chosen, because they represent the most established genome databases containing annotations.
The results of the GO term based queries were merged based on their HGNC gene symbol, if available, otherwise based on their NCBI ID, using a mapping between the two database IDs provided by ensembl.

2.2.3 *Gene-related Diseases*

One of the largest databases collecting gene-disease-associations is the Online Mendelian Inheritance in Man (OMIM, omim.org, [22]) database, which collects scientific reports of linkages between diseases and genes. It is curated at the McKusick-Nathans Institute of Genetic Medicine, The Johns Hopkins University School of Medicine.
Since the API is not suited for queries based on gene symbols the Comparative Toxicogenomic Database (ctd, ctdbase.org, [23]) was used as a middle layer. This database is curated by the Department of Bioin-

formatics at the MDI Biological Laboratory and the Department of Biological Sciences at the North Carolina State University and collects besides other things also entries from the OMIM database. The queries were based on HGNC gene symbols and NCBI IDs.

2.2.4 *Categorized Diseases*

The Comparative Toxicogenomics Database also provides a mapping between the OMIM and the Medical Subject Headings (MeSH, www.nlm.nih.gov/mesh/) database. MeSH is an attempt by the National Library of Medicine to establish a streamlined and controlled medical vocabulary. Compared to OMIM terms, the MeSH terms consist of more generic names for the diseases [24]. Each term has an unique MeSH ID and a MeSH Tree number. This MeSh Tree organizes the terms in a tree structure and provides therefore a categorization for the disease terms. A mapping between the MeSH IDs and the MeSH Tree numbers is provided by the MeSH database site. This was used to categorize the disease terms obtained from the ctd.

2.3 RESULTS

Interactive and continuously updated versions of these figures are available at uhlenlab.org/cagedb.

Table 1 shows the results of each query based on the last database update on April 22nd, 2015.

Table 1: Number of results for each query.

GO-terms	243
NCBI Gene/Ensembl	1466 / 1556
Genes after merging	1597
gene-diseases associations	4676
diseases	1409

The following sections will look at the different aspects of these results.

2.3.1 *Comparison of NCBI Gene and Ensembl*

To examine the necessity of quering two different genome databases, a deeper look was taken into their individual contributions to CaGeDB, see Figure 4. The query with the 243 selected GO terms led to 1556

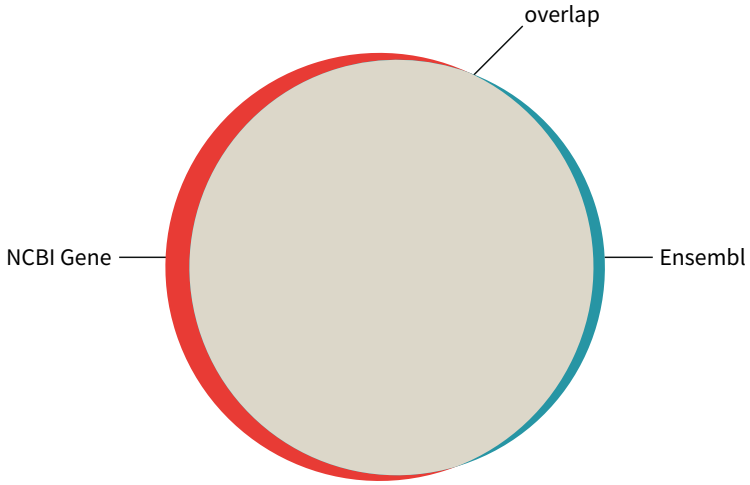

Figure 4: Venn diagram of the genes annotated with the selected GO terms in the Ensembl (red) and NCBI Gene (turquoise) databases.

genes in the Ensembl database. Out of these, 7 did not have a mapping to a NCBI Gene ID or a gene symbol and could therefore not be used for the query to the Comparative Toxicogenomics Database. In the case of the NCBI Gene database, 1466 genes were found based on their annotations. Both database shared 1425 genes, which led to a fi-

nal amount 1597 genes after merging both databases based on gene symbols and NCBI IDs.

2.3.2 *Distribution of GO Evidence Codes*

Since all GO term annotations are connected to an evidence code indicating the source on which the annotation is based on, their distribution in CaGeDB is examined in this section.
As shown in Figure 5, more than half of the annotations were auto-

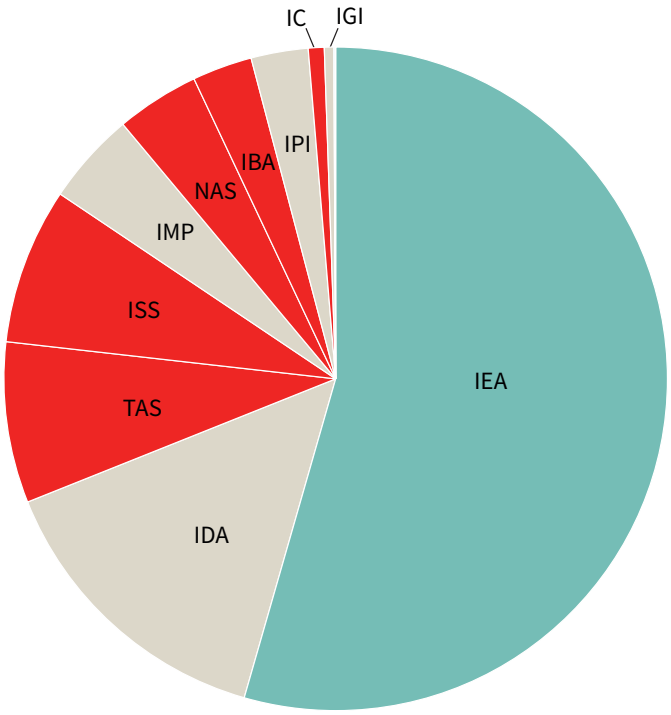

Figure 5: Pie graph of the GO term evidence codes supporting the gene-GO term associations. Evidence codes for fully automatic annotations are turquoise, codes directly based on experimental data are in gray and curated non-experimental evidence codes are in red.
The abbreviations stand for: IEA - Inferred from Electronic Annotation, IDA - Inferred from Direct Assay, TAS - Traceable Author Statement, ISS - Inferred from Sequence or structural Similarity, IMP - Inferred from Mutant Phenotype, NAS - Non-traceable Author Statement, IBA - Inferred from Biological aspect of Ancestor, IPI - Inferred from Physical Interaction, IC - Inferred by Curator, IGI - Inferred from Genetic Interaction;

matically assigned, i.e. they had the evidence code IEA. The rest is evenly shared between annotations from experimental data and curated non-experimental data.
A short explanation of each evidence code in order of their occurrence in CaGeDB: “Inferred from Electronic Annotation” (IEA) is for example applied, if annotations are automatically transferred from other databases or the originate from unreviewed sequence studies.

The second most common source for annotation are direct assays (IDA), e.g., binding assays. Followed by "Traceable Author Statements" (TAS), which typically originate from reviews, where original evidence is only referenced. The evidence code "Inferred from Sequence or structural Similarity" (ISS) indicates manually reviewed sequence studies. "Inferred from Mutant Phenotype" (IMP) is used for annotations derived from singular allele-based differences. The code "Non-traceable Author Statement" (NAS) denotes data from unreferenced sources, e.g., from statements in the abstract or the introduction of papers without any supporting references. If data is derived from a characterization in an ancestral gene it has the evidence code "Inferred from Physical Interaction" (IBA). The evidence code "Inferred from Physical Interaction" (IPI) is a special form of ISS, where the target of the interaction, e.g., the binding partner, is known. If the annotation is not based on direct data but can be reasonably inferred by a curator it is labeled with the evidence code "Inferred by Curator" (IC). This is used for example if the location of a specific protein is unknown, but it is associated with a specific nuclear RNA polymerase and it can therefore be assumed to be located in the nucleus. Is the annotation based on a comparison between two strands with multiple mutations, the evidence code "Inferred from Genetic Interaction" (IGI) is used.

2.3.3 *Ca^{2+} Signaling Categories and their Associated Genes and Diseases*

Because all GO terms were sorted according to Berridge's Ca^{2+} signaling categories, the same could be done for the resulting genes and their associated diseases. This could give a deeper insight into the importance of different aspects of Ca^{2+} signals.
While the number of genes and diseases associations related to the GO term category "signaling functions" was almost equal, the latter has a lower percentage in the overall number of diseases. As shown in Figure 6, this was not true for all the other categories, which had both in numbers and percentages more diseases than genes connected to them, except for the smallest two categories "extracellular ON-reactions" and "extracellular OFF-reactions". Note that not all genes in the database were connected to diseases and some had multiple associations.

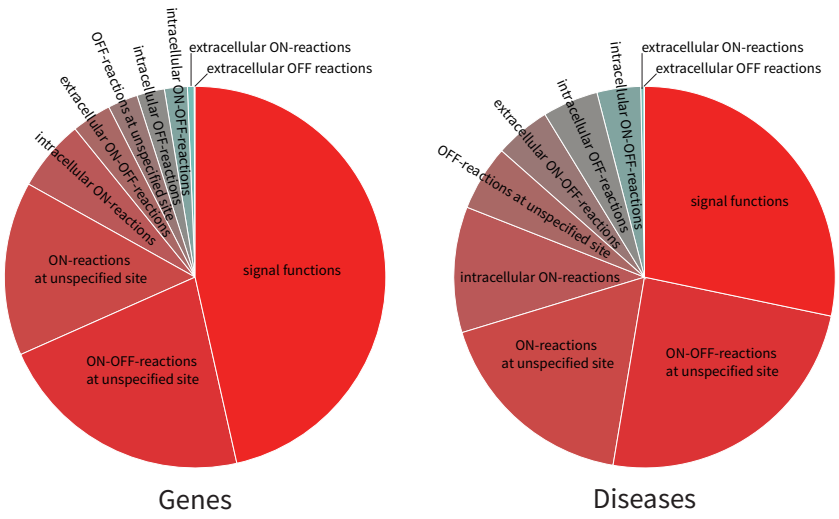

Figure 6: Pie graph of GO term categories with respect to genes annotated to them (left) and their associated diseases (right).

2.3.4 *Gene-Disease Associations*

Two interesting aspects of the diseases associated with genes related to Ca^{2+} signaling are how the diseases are distributed among the genes and if the associated diseases occur in specific disease categories or are more common in specific organs.
Of all the 1513 genes with gene symbols, 712 have no disease association. Of the remaining 801 genes, 27 account for 25% of all gene-disease associations. One quarter of all 4674 gene disease associations are accounted by 21 genes. See Figure 7 for the distribution of disease association with respect to the genes related to Ca^{2+} signaling.

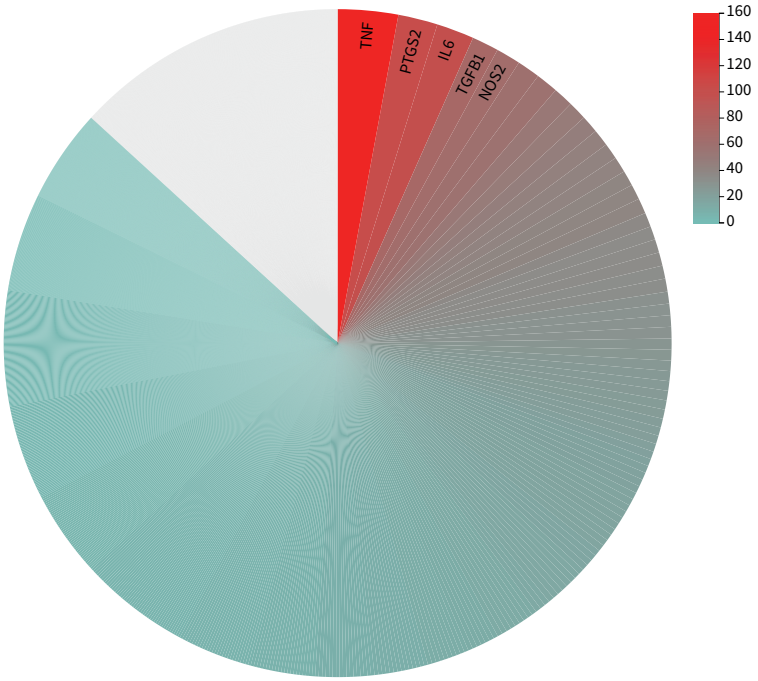

Figure 7: Pie chart of all genes based on the number of disease connections indicated by color and size. The five genes with the most disease associations are labeled.

The gene with the most reported disease relations is TNF with 157 associated MeSH terms. This gene is part of the database based on the GO term annotation “calcium mediated signaling”. The evidence code “Inferred from Electronic Annotation” and the supporting references suggest, that this annotation was automatically derived from a gene annotation for rats. This annotation is based on a report by Kim et al., where they describe TNF induced apoptosis in rat liver cancer cells by an increased Ca^{2+} release from the ER and a gene expression suppression of a gene connected to the reuptake of Ca^{2+} by the endoplasmic reticulum [25]. Since TNF’s product is a proinflammatory cytokine, it is involved in many diseases, e.g., Asthma [26].

The second most common gene is PTGS2 with 103 MeSH terms, followed by IL6 with 98, TGFB1 with 70 and NOS2 with 64 associated diseases terms.

To examine the distribution of gene associations in disease categories and localizations, the categorization provided by the MeSH Tree was applied on the disease terms.

Out of 11721 MeSH terms describing human diseases and mental disorders, 2159 (approximately 18%) have at least one reported association with a gene related to Ca^{2+} signaling, see Figure 8. The category of neoplasms, i.e., abnormal growth in a body, e.g., tumors, is the most common one with 871 gene connections to it and its children. Many disease terms occur multiple times in different categories, e.g., lung neoplasms are part of the categories for neoplasms as well as for respiratory tract diseases. Therefore term associations in different cate-

gories should only be compared with caution. The disease term with the most genes associated is “prostatic neoplasm”, with 67 gene connections.

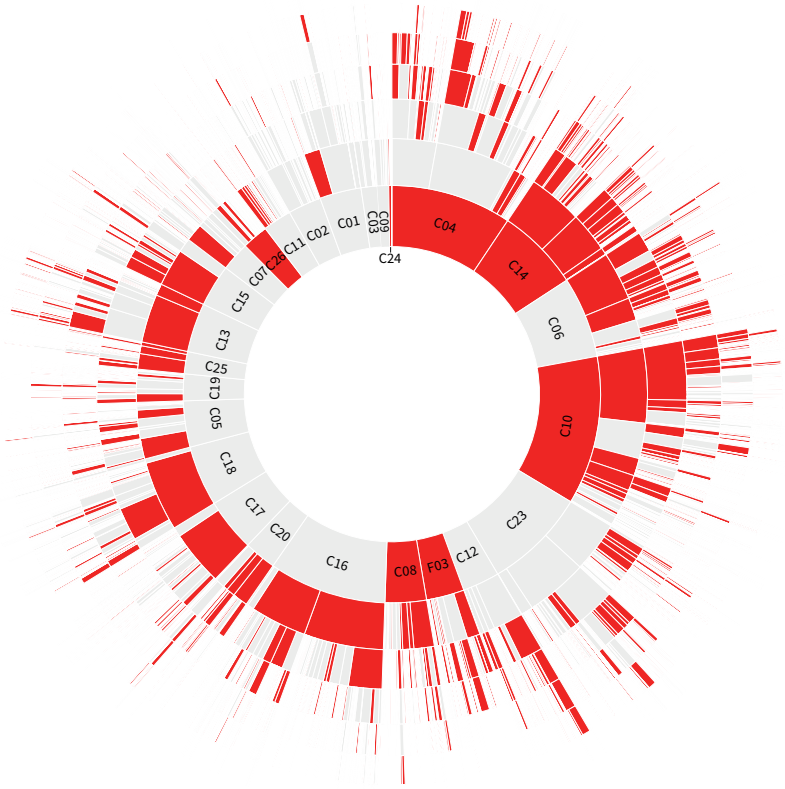

Figure 8: Sunburst diagram of the MeSH terms for diseases and mental disorders associated with at least one Ca^{2+} signaling gene (red) or without associations (grey). The angle each bar covers corresponds to the number of children the term includes. Subterms are further away from the center than there parent terms.
The parent categories with the number of genes associations to them and their subterms in a descending order are: C04 - neoplasms (871), C14 - cardiovascular diseases (856), C06 - digestive system diseases (757), C10 - nervous system diseases (746), C23 - pathological conditions, signs and symptoms (729), C12 - urologic and male genital diseases (437), F03 - mental disorders (393), C08 - respiratory tract diseases (355), C16 - congenital, hereditary, and neonatal diseases and abnormalities (295), C20 - immune system diseases (244), C17 - skin and connective tissue diseases (240), C18 - nutritional and metabolic diseases (235), C05 - musculoskeletal diseases (235), C19 - endocrine system diseases (228), C25 - Chemically-Induced Disorders (221), C13 - female genital diseases and pregnancy complications (209), C15 - hemic and lymphatic diseases (136), C07 - stomatognathic diseases (106), C26 - Wounds and Injuries (77), C11 - eye diseases (77), C02 - virus diseases (54), C01 - bacterial infections and mycoses (34), C03 - parasitic diseases (23), C09 - otorhinolaryngologic diseases (22), C24 - occupational diseases (13)

A more detailed look of the neurodegenerative disease category and terms therein linked with genes related to Ca^{2+} signaling is given in Figure 9. Alzheimer Disease is the term with the most gene connections, with 17 reported connections. This is followed with Parkinson's Disease (10 gene assocations) and Lewy Body Disease (7), which share four common genes, including SNCA, which is examined in the second part of this thesis (see Chapter 3).

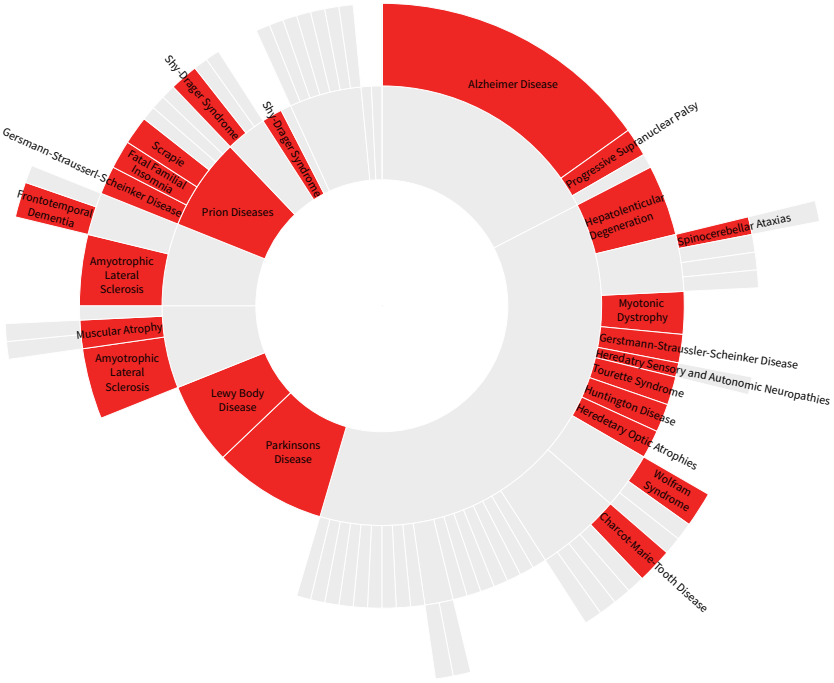

Figure 9: Sunburst diagram of the MeSH terms for neurodegenerative diseases associated with at least one Ca^{2+} signaling gene (red), including their title, or without associations (grey). The angle each bar covers corresponds to the number of children the term includes as well as to the number of associated genes. Subterms are further away from the center than there parent terms

2.4 DISCUSSION

The established database represents the first attempt to collect all genes related to Ca^{2+} signaling and their associated diseases. This database could help scientists focusing on one gene to expand their research with respect to related diseases, as well as provide a possible link to Ca^{2+} signaling for a disease researchers.
The following points will discuss the specific tentpoles of the database, i.e. GO-terms, genes, gene-disease associations and categorized disease terms.

2.4.1 *GO Terms*

The GO vocabulary is not complete, in the sense that a missing annotation does not mean that a gene's product does not have certain features, it simply can be the case that no connection between a scientific discovery and the gene has been made [27]. This could be mitigated by applying text-mining algorithms on scientific publications, as was done for example by Bayés et al for gene disease connections and diseases [28] . But this was beyond the scope of this work.
The relatively larger number of genes with the not as specific GO terms categories in Figure 6, which than also impacts the association between disease terms and GO-term categories, is probably due to automatic gene annotations, since they tend to apply less specific annotations [21].
Although more than half of the genes in the CaGeDB database are based on automatically assigned annotation, i.e. they have the evidence "IEA", as shown in Figure 5, this is not so unfavorable considering that 98% of all GO annotations were based on this evidence code in 2010 [29]. The fact that a quarter of all annotations in this database are based on experimental data, which was shown to have a higher reliability [21], support the validity of the connections between Ca^{2+} signaling and the collected genes.

2.4.2 *Genes*

The differences between the Ensembl and the NCBI Gene database could be due to different update cycles as well as different methods of automatically produced annotations. Although there exists a high consensus between the two of them, the fact there are differences supports the approach being based on two different databases.

2.4.3 *Gene-Disease Associations*

Interestingly none of the associations is between a disease and a gene without a gene symbol. This could be because OMIM, which is the ba-

sis for the ctd, maps diseases to genes based only on gene symbols and their synonyms. But since scientific reports, which are the base for the database, relay more frequently on the use of gene symbols or synonyms to reference a gene than on database IDs or other nomenclatures, the number of missed results is assumably low.
The quality of this part of the database is heavily impacted by the fact that although GO terms are the most common way to annotate protein function, researchers often do not annotate their findings. Most of the annotations for experimental data are later on added by a curator, which leads to less specific and outdated annotations [29]. An example for this problem is shown in Section 3.4.

2.4.4 *Categorized Disease Terms*

Genes related to Ca^{2+} signaling are reported to be connected to wide spread of diseases types and diseases (see Figure 8). This scatter could be because of weak connections between the diseases and the genes, which therefore appear with various diseases. But it could also be due to the abundance of Ca^{2+} signals for a multitude of cellular processes, where a faulty component could lead to vastly different effects. Indeed going through the list of neurodegenerative diseases associated with Ca^{2+} signaling, as shown in Figure 9, one can find reports of Ca^{2+} signaling defects related to the diseases in this category for example in the case of amyotrophic lateral sclerosis [30], Parkinson's disease [31], skeletal muscle atrophy [32], familial frontotemporal dementia [33], Alzheimer's disease [34], scrapie [35], progressive supranuclear palsy [36], Tourette syndrome [37], Huntington's disease [38] and Wolfram syndrome [39].
Like many vocabularies for biological systems, the MeSH database constricts the view on diseases, by merging several disease subtypes, yet this constrictions give in this case a better view of the diseases, compared to the less generic terms used in OMIM [24]. This is unfavorable for the database presented in this thesis, since it would hinder the discoverability of diseases connections.

2.4.5 *General Limitations*

It can not be assumed that every disease in this database is actually related to Ca^{2+} signalling, since their relation to a specific gene is often weak [40]. This association to the Ca^{2+} signaling pathways is further weakened by the fact that this is not even secured for the genes themselves. But the aim of this database is to be a discovery tool for possible relationships between genes and diseases and therefor it was decided to risk the inclusion of false positives, i.e. genes and diseases that are not actually related to Ca^{2+} signaling.
Another factor which could be seen as limiting the CaGeDB database is that not all available databases are used, since some do not provide

an API suited for the CaGeDB database, e.g. they have a query limit as is the case with KEGG or in the case of OMIM, they can't be directly queried, since they do not provide a satisfying API to get gene-disease relations based on gene symbols or NCBI IDs. Because of this, the size of the database as well as the wish to automatically update the database influenced the work flow and therefore the resulting database. By the usage of derived databases, e.g., the Comparative Toxicogenomics Database, which collects its data from OMIM, and due to the synchronization between KEGG and OMIM the quality of the CaGeDB database is not too strongly impaired by this.

3

GENETIC STUDY: α-SYNUCLEIN PLAYS A VITAL ROLE IN MITOCHONDRIAL CALCIUM HOMEOSTASIS

3.1 INTRODUCTION

The interest in the 140 amino acids long protein α-synuclein started, when two studies showed a strong connection between α-synuclein and Parkinson's disease (PD). First, Polymeropoulos et al. showed that a missense mutation in the gene SNCA, which codes for α-synuclein, causes a familial form of PD [41]. Two months later Spillantini et al. reported that α-synuclein was a main component of Lewy bodies, a defining pathological accumulation found in brains of PD patients [42]. Since then, additional reports support the key role of SNCA besides other genes as well environmental factors in the development of PD [31]. PD is the most common neurodegenerative disease in the western world, affecting 1-2% of the population over 50, and there is still no cure available, although a vaccine which reduces α-synuclein is currently in clinical trial [31, 43, 44].
The physiological function of α-synuclein is still largely unknown, but evidence suggests that it interacts with phospholipid layers, e.g. it was shown to be important for vesicle dynamics at synapses [45, 46]. Additionally it is also important in mitochondria dynamics, e.g., it was reported that α-synuclein effects the mitochondrial fragmentation [47]. Recent results suggest also an involvement of α-synuclein in the Ca^{2+} exchange between mitochondria and endoplasmic reticulum [48].
The goal of this part of the thesis was to further investigate this path. Therefore, the following hypothesis was tested. If α-synuclein supports the Ca^{2+} exchange between mitochondria and the endoplasmic reticulum, then cells, where the production of this protein is suppressed, should show a lower resistance to mitochondrial Ca^{2+} stress, i.e., they should be more susceptible to Ca^{2+} induced cell death.

3.1.1 *Methological Background*

The following subsection provides deeper information on some of the experimental procedures used for this study.

3.1.1.1 *HeLa Cells*

In 1951 George Gey established the first stable human cell line, which were extracted from the unusual aggressive cervical cancer of Henriette Lacks. They were proliferated without her consent and she died a

few months later of the cancer. In order to partially anonymise them, Gey called them HeLa [49].
This cell line marked a revolution in medicine and cell biology, since it was the first time human tissue could be repeatedly tested and is still one of the most popular cell lines in research [49].

3.1.1.2 *Transfection*

To insert DNA into the cells we used the method of transfection. To be more precise we used lipofection, i.e., we utilized positively charged liposomes for DNA insertion. Liposomes are spheres formed by a lipid bilayer [50]. In lipofection, their positively charged head groups interact with the DNA backbone and encapsulate the DNA. This complex then binds to the cell wall either via receptors or through electrostatic interactions between the positively charged complex and the negatively charged cell surface. Afterwards it enters the cell via endocytosis, i.e. the DNA-liposome complex is engulfed by the plasma membrane and stored inside an endosome.
Motor proteins transport this endosome to the nucleus where the complex escapes it [51]. Thereafter the DNA is released from the liposome and enters the nucleus using two different but not exclusive pathways. It can be incorporated during cell division or through the nuclear pores with the help of nuclear transport mediators. There it is translated, in the case of this study, producing RFP and shRNA silencing SNCA.

3.1.1.3 *Applied Fluorophores*

The following three fluorphores were used to identify the cells and cell states.

- Ca^{2+} marker: Ca^{2+} was observed with Fluo-4. This fluorophore is initially able to passively diffuse across the plasma membrane. Once inside the cell, its AM group is cleaved of by esterase, whereby it loses its ability to permeate the cell membrane, which leads to an accumulation inside the cell. Upon binding Ca^{2+} its fluorescence intensity is increased by the factor 100 [52], with its excitation/emission maxima at 494/516 nm.

- General cell marker: In order to measure the whole cell population Hoechst 33342 was used. This fluorophore binds to the minor groove of the DNA, thereby labelling the whole nucleus. It is excited using ultraviolett light (maximum at 361 nm) and emits bright blue light (maximum at 497 nm).

- Transfection marker: In front of the segment coding for SNCA-silencing shRNA, a segment coding for RFP was placed on the transfected plasmid. Therefore successfully transfected cells emitted orange-red light (maximum at 583 nm), if excited with green light (maximum at 553 nm).

- Apoptosis marker: To mark the onset of apoptosis, a DNA dye initially blocked by a peptide was applied. Caspase-3/7, a protein activated at the onset of programmed cell death, cleaves this peptide, releasing the DNA binding site of the fluorophore, which emits green light (maximum at 530 nm) if excited with blue light (maximum at 502 nm).

3.1.1.4 *shRNA Induced Gene Silencing*

Short hairpin RNAs (shRNA) are approximately 50 nucleotide long RNA strands, folded into a sharp hairpin due to complementary base pairs on their ends [53]. In order to silence a specific gene, a plasmid containing DNA coding for the shRNA is transfected. After the translation of this DNA, the shRNA forms it hair pin structure and leaves the nucleus via exportin 5. Once in the cytosole, the RNAse III enzyme Dicer docks to it and cleaves it into a 21-23 nucleotide long double stranded short interfering RNA (siRNA), where one strand, the so called guide, is homologues to the a part of the gene targeted for silencing and is therefore complementary to the gene's mRNA. The other strand is called passenger. The double stranded siRNA is then incorporated in the so called RNAi induced silencing complex (RISC). There the passenger strand is degraded, while the other strand is used by the RISC to guide it to the mRNA by binding to it. Once bound, the target mRNA is cleaved by the RISC or its transcription is blocked by it. In both cases the mRNA is eventually degraded, without producing the protein it codes for.

3.1.2 *ATP Induced Ca^{2+} release*

If extracellular ATP binds to P2Y receptors on the plasma membrane, it releases its coupled G-protein [54]. This activates an enzyme, which hydrolizes phosphatidylinositol bisphosphate (PIP_2) to form inositol trisphosphate (IP_3) [2]. IP_3 binds to receptors on the membrane of the ER activating thereby Ca^{2+} release channels, which creates the Ca^{2+} signal.

3.1.2.1 *Staurosporine Induced Apoptosis*

The exact mechanism how staurosporine, a broad spectrum protein kinase inhibitor, induces apoptosis is still unknown. But cells treated with staurosporine show a sustained increase in cytosolic Ca^{2+}, which is coupled to the release of cytochrome c, which starts the apoptotic pathway by activating caspase 9 [55–57]. Caspase-9 activates caspase-3 and and caspase-7, which are highly active proteases, able to cleave a multitude of substrates and thereby destroying the cell.

3.2 METHODS

The following steps were undertaken to examine the effects of a suppression of SNCA with respect to mitochondrial Ca^{2+} homeostasis.

3.2.1 *Cell cultures*

HeLa cells were grown in Basal Medium Eagle (Invitrogen) with 10 % fetal bovine serum and 2% penicilin and streptomycin. They were suspended in 35mm dishes 4 days prior to imaging and maintained at 37°C, 5% CO_2, and 95% humidity. The medium was changed daily including a washing with proton buffer solution (PBS). Approximately 10^5 cells were seeded to accomplish a dish coverage of approximately 80% at the day of imaging.

3.2.2 *Transfection*

The plasmid containing RFP and SNCA surpressing SHRNA was transfected 48 h prior to imaging, using Lipofectamin 2000 (Invitrogen) and Opti-MEM and a penicillin and streptomycin free medium. This time span was chosen, because α-synuclein shows a half life of 48 hours [58]. 6 hours after the transfection the medium was exchanged for a medium with penicillin and streptomycin.

3.2.3 *Control Experiment*

Since an elevation of intracellular Ca^{2+} was needed for our experiment, the influence of SNCA silencing on it was tested by the addition of ATP, a common Ca^{2+} signal inducing event. 6 Petri dishes with transfected and non-transfected HeLa cells were loaded with Fluo-4 (Invitrogen) 30 min prior to imaging. This allowed a measurement of the intracellular Ca^{2+} levels. A baseline of approximately three minutes was acquired before 200 ml of 10 μM ATP were added. The imaging was carried out at 37°C with a cooled back-illuminated EMCCD camera (Cascade II; Photometrics, Tucson, AZ) mounted on an inverted microscope (Zeiss Axiovert 100M; Carl Zeiss, Jena, Germany) equipped with a 25 × /0.8NA water objective (Carl Zeiss). Excitation was carried out using a Lambda LS xenon-arc lamp equipped with a SmartShutter (Sutter Instrument, Novato, CA). To capture transfection a wavelength 545 nm was used, for the measurement of Ca^{2+} signals the samples were excited at 480 nm. The computer software MetaFluor (Molecular Devices, Sunnyvale, CA) was used to control all devices.
Sections with a confluency of approximately 80% and a transfection rate of 50% were preferred.

3.2.4 *Treatment with Staurosporine*

The medium in all cell dishes was replaced with Krebs-Ringer's buffer containing 119.0 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 20.0 mM HEPES (pH 7.4), and 11.0 mM dextrose. For dishes in the STS group, the Krebs-Ringer's buffer contained additionally 600 nM staurosporine. This dosage as well as the treatment duration of 30 minutes, after it showed in several test series to be sublethal for non-transfected cells, while being lethal to transfected ones, similar to [59]. The apoptosis marker CellEvent™Caspase-3/7 Green ReadyProbes® Reagent (Invitrogen) was added 30 minutes before the imaging. After 20 minutes the nuclear label Hoechst 33342 was added. Before the first images were taken the cells were washed twice with Krebs-Ringer's buffer. After the washing, 2ml Krebs-Ringer's buffer containing STS if the dish was not part of the control group, were added into the dish.
The imaging was carried out at 37°C with a cooled back-illuminated EMCCD camera (Cascade II; Photometrics, Tucson, AZ) mounted on an inverted microscope (Zeiss Axiovert 100M; Carl Zeiss, Jena, Germany) equipped with a 25×/0.8NA water objective (Carl Zeiss). Excitation was carried out at three different wavelengths, one to identify the transfected cells (545 nm), one to detect the apoptotic cells (480 nm) and one to detect all cells in the field of view (380) using a Lambda LS xenon-arc lamp equipped with a SmartShutter (Sutter Instrument, Novato, CA). The computer software MetaFluor (Molecular Devices, Sunnyvale, CA) was used to control all devices.
The images were selected based on the Hoechst staining to reduce any bias. Sections with dens but not overcrowded cell population were preferred.

3.2.5 *Image and Data Analysis*

The images were processed in the image processing software ImageJ [60]. The three images of the different wave lengths were automatically merged and colored. Afterwards the cells were counted manually based on both the composite and the single color images.
The results were analysed using Microsoft Excel (Microsoft, Redmond, Washington, United States) and MATLAB 2014b (The MathWorks, Inc., Natick, Massachusetts, United States.). The data were statistically examined using a student t-test. Differences were declared significant if $p < 0.05$.

3.3 RESULTS

3.3.1 *Control Experiment*

The cells were loaded 30 min before the imaging with the calcium marker Fluo-4. Transfected cells were labeled with RFP. The first 3 minutes were used as a baseline, after which 200 ml 10 μM ATP were added. No differences in the ability to initiate ATP-induced calcium signals could be observed between transfected and non transfected cells. An example sequence is shown in Figure 10.

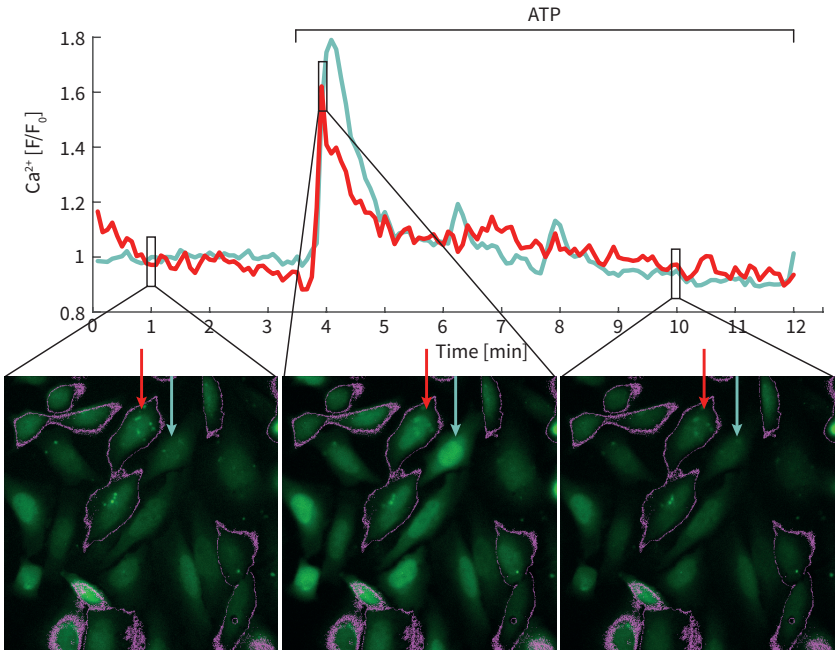

Figure 10: ATP-induced Ca^{2+} signal in HeLa cells. Top: The Ca^{2+} level of one transfected (red) and one non-transfected (turquoise) cell, ATP was applied at minute 3.5. Bottom: Fluorescent microscopy images at minute 1 (left), minute 4 (middle) and minute 10 (right). The edges of the transfected cells are marked magenta, calcium is labeled green.

3.3.2 *Treatment with Staurosporine*

Apoptosis was determined via a Caspase 3/7 marker. Transfection was marked with RFP and every cell nucleus was labeled with Hoechst staining. An example image is shown in Figure 11 with two transfected cells, colored in magenta and three apoptotic cells, coloured in green, every cell nucleus is visible in blue.
Some Petri dishes had to be excluded because of a too high signal-to-noise ratio with respect to the green marker. In the end, 15 dishes were examined, 7 of them with added STS and 8 were used as a control, i.e., they were incubated without staurosporine. The control showed an

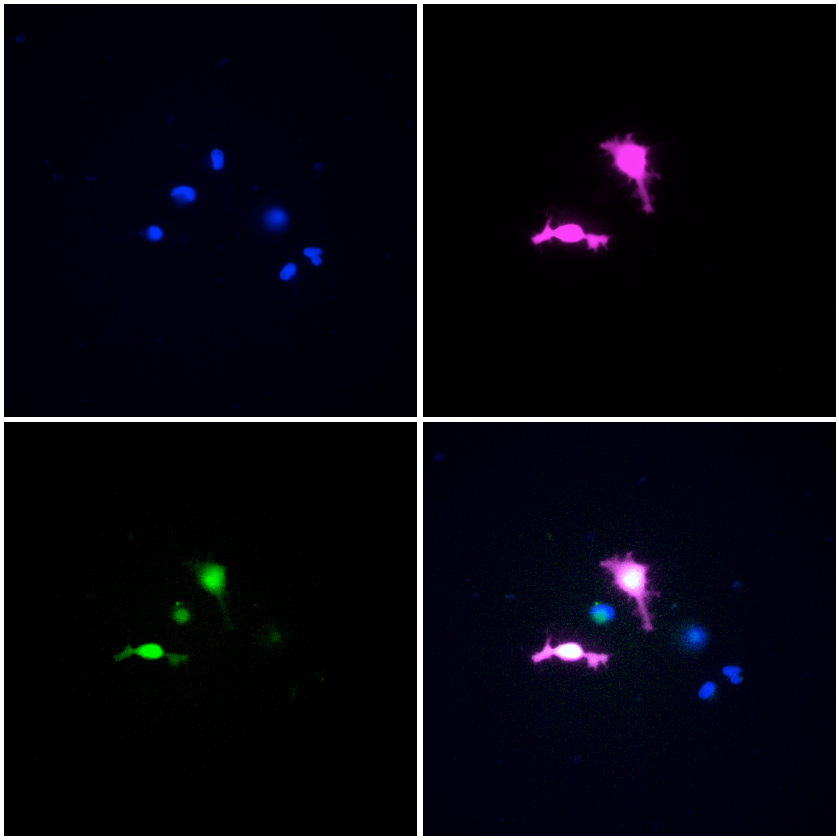

Figure 11: HeLa cells filtered for Hoechst staining (top left), for RFP (top right), for the Caspase 3/7 marker (bottom left) and all three images merged (bottom right).

average transfection rate of 20% (std = 4%). In average 24% (std = 2%) of the cells treated with STS were transfected. According to a two sample Student's t-test these differences are not significant ($p = 0.12$).

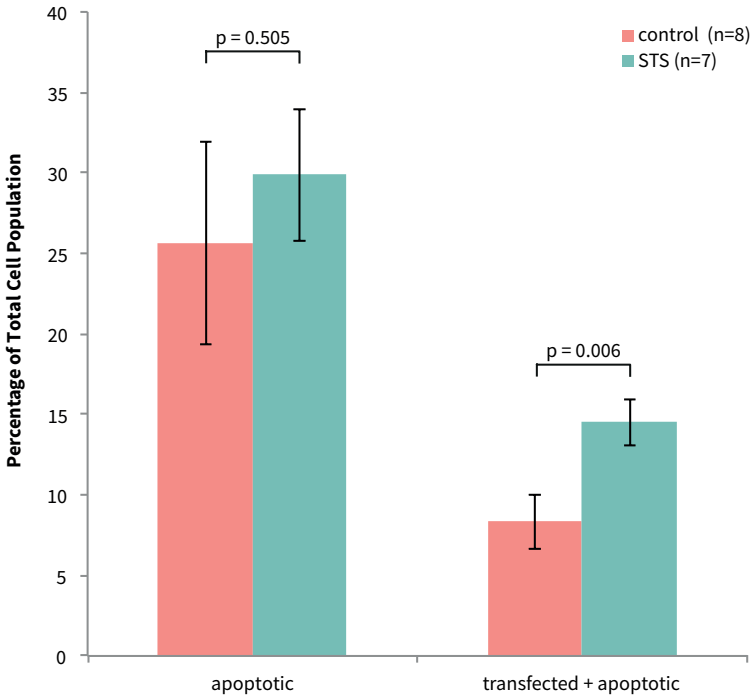

Figure 12: Comparison of apoptotic susceptibility of HeLa cells with suppressed SNCA with added staurosporine (STS) or without any staurosporine (control).

Figure 12 shows the cumulative outcome. On average approximately 26% (std = 6%) of the control showed apoptotic signs, whereas around 30% (std = 5%) of the cells treated with STS were apoptotic. A two sample Student's t-test indicates no significant differences between those two groups ($p = 0.505$).
From these apoptotic cells, approximately 8 % (std = 2%) were also transfected cells in the control group, compared to approximately 14% (std = 1%) in the group treated with STS. These differences are significant according to a two sample Student's t-test ($p = 0.006$).

3.4 DISCUSSION

With respect to the first part of this thesis, it should be noted, that SNCA is indeed in the CaGeDB database and marked as associated with PD. But the GO term annotations, while indicated a relationship to Ca^{2+} ion transport, do not specifically mention its relation to mitochondrial Ca^{2+} homeostasis. This is an example for the drawbacks of CaGeDB, which can be outdated because of the missing annotations directly from researchers, as discussed in Section 2.4.3.
The control experiment showed that both transfected and non-transfected cells were able to produce Ca^{2+} signals.
The main experiment showed that the apoptosis rate is approximately equal in the whole cell population comparing the control group with dishes incubated with STS. But in the subpopulation of cells with a suppressed SNCA expression significantly more cells died compared to cells with normal SNCA expression.
This strongly indicates that α-synuclein plays a role in the mitochondrial Ca^{2+} homeostasis, since staurosporine induces cell death via Ca^{2+} overload [55]. Therefore the protein is either involved in the suppression of the mitochondrial Ca^{2+} uptake or in the Ca^{2+} transport between mitochondria and endoplasmic reticulum.
The following pathway could explain the results: Staurosporine leads to a sustained increase in cytosolic Ca^{2+} which is beside other sites mostly buffered in the mitochondria. If α- synuclein controls the Ca^{2+} import into the cell, the non-transfected cells do not take up. In cells with non-disturbed SNCA expression, this Ca^{2+} is transported from the mitochondria to the endoplasmic reticulum, lowering the mitochondrial Ca^{2+} concentration under the apoptosis-inducing threshold. I, where it is transported to the endoplasmic reticulum in healthy cells. Both pathways lead to a prolonged elevated Ca^{2+}-level in mitochondria, starting the apoptosis pathway described in section 3.1.2.1.
Note that both functions of α-synuclein are not exclusive, in the meaning that it could be involved in the mitochondrial import of Ca^{2+} as well as its export.
Results by Calì et al. in [48] which indicate that mitochondrial Ca^{2+} uptake is independent of α-synuclein point toward the later option.

3.4.1 *Limitations*

The presented results could be biased because of a bleed-through of colors visible between RFP and the apoptosis marker. This effect was minimized by raising the signal to noise threshold during analysis, since the bleed through had rather low intensity.
There exist fluorphores which are used to directly measure the mitochondrial Ca^{2+} concentration, e.g., rhod-2. But all of them have the same emission spectra as the RFP which was used to label transfection. Additionally since this study was not focusing on the mitochon-

drial Ca^{2+} uptake, but rather on the Ca^{2+} exchange between mitochondria and the ER, this method wouldn't be as conclusive.

4

CONCLUSION

Ca^{2+} signals are an incredible versatile cellular mechanism. Exploring its genetic background gives a better understanding of many physiological and pathological processes. This thesis helped to advance both fronts. In the first part by the establishment of CaGeDB and in the second part by providing further evidence for the role of the gene SNCA in Ca^{2+} signaling.
The rapid development of both experimental and bioinformatical tools will surely increase the knowledge of Ca^{2+} signals and their effects in the coming years. Hopefully, the work in this thesis will serve as well as a small step to a deeper understanding of the function of Ca^{2+} in the cell.

A

APPENDIX

The following GO terms were used to query the gene databases.
In Table 2 all GO terms related to extracellular ON-reactions are shown. Therefore this terms describe cellular functions that control the influx of Ca^{2+} into the cell through channels in the plasma membrane.

Table 2: GO terms categorised as extracellular ON-reactions.

GO-ID	GO term
98703	calcium ion import across plasma membrane
1902606	regulation of large conductance calcium-activated potassium channel activity
1902608	positive regulation of large conductance calcium-activated potassium channel activity
1990035	calcium ion import into cell

Table 3 contains terms considered to be part of the intracellular ON-reactions, i.e., they increase the Ca^{2+} concentration in the cytosol by the release of Ca^{2+} from cell organelles, e.g., from the endoplasmic reticulum.

Table 3: GO terms categorized as intracellular ON-reactions.

GO-ID	GO term
1639	PLC activating G-protein coupled glutamate receptor activity
2115	store-operated calcium entry
5218	intracellular ligand-gated calcium channel activity
5219	ryanodine-sensitive calcium-release channel activity
5220	inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity
7223	Wnt signaling pathway, calcium modulating pathway
8377	light-induced release of internally sequestered calcium ion
8591	regulation of Wnt signaling pathway, calcium modulating pathway
10880	regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum
14808	release of sequestered calcium ion into cytosol by sarcoplasmic reticulum
15278	calcium-release channel activity

15279	store-operated calcium channel activity
31585	regulation of inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity
31586	negative regulation of inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity
31587	positive regulation of inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity
32237	activation of store-operated calcium channel activity
32471	negative regulation of endoplasmic reticulum calcium ion concentration
45812	negative regulation of Wnt signaling pathway, calcium modulating pathway
45813	positive regulation of Wnt signaling pathway, calcium modulating pathway
48763	calcium-induced calcium release activity
51209	release of sequestered calcium ion into cytosol
51279	regulation of release of sequestered calcium ion into cytosol
51280	negative regulation of release of sequestered calcium ion into cytosol
51281	positive regulation of release of sequestered calcium ion into cytosol
51482	positive regulation of cytosolic calcium ion concentration involved in phospholipase C-activating G-protein coupled signaling pathway
51562	negative regulation of mitochondrial calcium ion concentration
51565	negative regulation of smooth endoplasmic reticulum calcium ion concentration
60314	regulation of ryanodine-sensitive calcium-release channel activity
60315	negative regulation of ryanodine-sensitive calcium-release channel activity
60316	positive regulation of ryanodine-sensitive calcium-release channel activity
61454	Golgi calcium ion export
72345	NAADP-sensitive calcium-release channel activity
72346	cADPR-sensitive calcium-release channel activity
86094	positive regulation of ryanodine-sensitive calcium-release channel activity by adrenergic receptor signaling pathway involved in positive regulation of cardiac muscle contraction
1901339	regulation of store-operated calcium channel activity
1901340	negative regulation of store-operated calcium channel activity

1901341	positive regulation of store-operated calcium channel activity
1901472	regulation of Golgi calcium ion export
1903514	calcium ion transport from endoplasmic reticulum to cytosol
1990425	ryanodine receptor complex
2001256	regulation of store-operated calcium entry

Table 4 contains all GO terms describing processes increasing the intracellular Ca^{2+} concentration, without clarifying the exact location.

Table 4: GO terms categorised as ON-reactions at unspecified site.

GO-ID	GO term
5245	voltage-gated calcium channel activity
5246	calcium channel regulator activity
5262	calcium channel activity
5891	voltage-gated calcium channel complex
7204	positive regulation of cytosolic calcium ion concentration
7340	acrosome reaction
8086	light-activated voltage-gated calcium channel activity
8087	light-activated voltage-gated calcium channel complex
8331	high voltage-gated calcium channel activity
8332	low voltage-gated calcium channel activity
10522	regulation of calcium ion transport into cytosol
10523	negative regulation of calcium ion transport into cytosol
10524	positive regulation of calcium ion transport into cytosol
15275	stretch-activated, cation-selective, calcium channel activity
19855	calcium channel inhibitor activity
60072	large conductance calcium-activated potassium channel activity
60402	calcium ion transport into cytosol
60470	positive regulation of cytosolic calcium ion concentration involved in egg activation
61577	generation of L-type calcium current
70978	voltage-gated calcium channel complex assembly
86007	voltage-gated calcium channel activity involved in cardiac muscle cell action potential
86056	voltage-gated calcium channel activity involved in AV node cell action potential

86057	voltage-gated calcium channel activity involved in bundle of His cell action potential
86058	voltage-gated calcium channel activity involved in Purkinje myocyte cell action potential
86059	voltage-gated calcium channel activity involved SA node cell action potential
97364	stretch-activated, cation-selective, calcium channel activity involved in regulation of action potential
97365	stretch-activated, cation-selective, calcium channel activity involved in regulation of cardiac muscle cell action potential
97553	calcium ion transmembrane import into cytosol
1901385	regulation of voltage-gated calcium channel activity
1901386	negative regulation of voltage-gated calcium channel activity
1901387	positive regulation of voltage-gated calcium channel activity
1901841	regulation of high voltage-gated calcium channel activity
1901843	positive regulation of high voltage-gated calcium channel activity
1902225	negative regulation of acrosome reaction
1902514	regulation of generation of L-type calcium current
1902607	negative regulation of large conductance calcium-activated potassium channel activity
1902656	calcium ion import into cytosol
1990028	intermediate voltage-gated calcium channel activity
1990454	L-type voltage-gated calcium channel complex
2000344	positive regulation of acrosome reaction

GO terms related to extracellular OFF-reactions, i.e. cellular processes responsible for lowering the cytosolic Ca^{2+} level by transport through the plasma membrane, are shown in Table 5.

Table 5: GO terms categorised as extracellular OFF-reactions.

GO-ID	GO term
1990034	calcium ion export from cell

Table 6 contains terms considered to be part of the intracellular OFF-reactions, i.e., they increase the Ca^{2+} concentration in the cytosol by the uptake of Ca^{2+} by cell organelles, e.g., into the endoplasmic reticulum.

Table 6: GO terms categorized as intracellular OFF-reactions.

GO-ID	GO term
32470	positive regulation of endoplasmic reticulum calcium ion concentration
32865	ERMES complex
36444	calcium ion transmembrane import into mitochondrion
44233	ER-mitochondrion membrane contact site
51208	sequestering of calcium ion
51282	regulation of sequestering of calcium ion
51283	negative regulation of sequestering of calcium ion
51284	positive regulation of sequestering of calcium ion
51561	positive regulation of mitochondrial calcium ion concentration
51561	positive regulation of mitochondrial calcium ion concentration
51564	positive regulation of smooth endoplasmic reticulum calcium ion concentration
1902080	regulation of calcium ion import into sarcoplasmic reticulum
1902081	negative regulation of calcium ion import into sarcoplasmic reticulum
1902082	positive regulation of calcium ion import into sarcoplasmic reticulum
1903515	calcium ion transport from cytosol to endoplasmic reticulum
1990036	calcium ion import into sarcoplasmic reticulum
1990246	uniplex complex
1990629	phospholamban complex

Table 7 contains all GO terms describing processes decreasing the intracellular Ca^{2+} concentration, without clarifying the exact location.

Table 7: GO terms categorised as OFF-reactions at unspecified site.

GO-ID	GO term
5388	calcium-transporting ATPase activity
5432	calcium:sodium antiporter activity
8273	calcium, potassium:sodium antiporter activity
15368	calcium:cation antiporter activity
15369	calcium:proton antiporter activity
51481	negative regulation of cytosolic calcium ion concentration
86038	calcium:sodium antiporter activity involved in regulation of cardiac muscle cell membrane potential

86039	calcium-transporting ATPase activity involved in regulation of cardiac muscle cell membrane potential
90534	calcium ion-transporting ATPase complex
1901019	regulation of calcium ion transmembrane transporter activity
1901020	negative regulation of calcium ion transmembrane transporter activity
1901021	positive regulation of calcium ion transmembrane transporter activity
1901894	regulation of calcium-transporting ATPase activity
1901895	negative regulation of calcium-transporting ATPase activity
1901896	positive regulation of calcium-transporting ATPase activity
1903279	regulation of calcium:sodium antiporter activity
1903280	negative regulation of calcium:sodium antiporter activity
1903281	positive regulation of calcium:sodium antiporter activity

GO terms related to intracellular ON-OFF-reactions, i.e. cellular processes involving cell organelles where it is unclear if these increase or decrease cytosolic Ca^{2+} concentration, are shown in Table 8.

Table 8: GO terms categorised as intracellular ON-OFF-reactions.

GO-ID	GO term
6851	mitochondrial calcium ion transport
32468	Golgi calcium ion homeostasis
32469	endoplasmic reticulum calcium ion homeostasis
32472	Golgi calcium ion transport
51560	mitochondrial calcium ion homeostasis
51560	mitochondrial calcium ion homeostasis
51563	smooth endoplasmic reticulum calcium ion homeostasis
70296	sarcoplasmic reticulum calcium ion transport

Table 9 contains all GO terms describing processes decreasing or increasing the intracellular Ca^{2+} concentration, without clarifying the exact location.

Table 9: GO terms categorised as ON-OFF-reactions at unspecified site.

GO-ID	GO term
6816	calcium ion transport
6874	cellular calcium ion homeostasis
10617	circadian regulation of calcium ion oscillation

15085	calcium ion transmembrane transporter activity
34704	calcium channel complex
51480	cytosolic calcium ion homeostasis
51924	regulation of calcium ion transport
51926	negative regulation of calcium ion transport
51928	positive regulation of calcium ion transport
55074	calcium ion homeostasis
60401	cytosolic calcium ion transport
70509	calcium ion import
70588	calcium ion transmembrane transport
90279	regulation of calcium ion import
90280	positive regulation of calcium ion import
90281	negative regulation of calcium ion import
1901660	calcium ion export
1903169	regulation of calcium ion transmembrane transport
1903170	negative regulation of calcium ion transmembrane transport

The GO terms describing the signaling effects of Ca^{2+} are listed in Table 10.

Table 10: GO terms categorised as signaling functions.

GO-ID	GO term
4117	calmodulin-dependent cyclic-nucleotide phosphodiesterase activity
4198	calcium-dependent cysteine-type endopeptidase activity
4683	calmodulin-dependent protein kinase activity
4698	calcium-dependent protein kinase C activity
4723	calcium-dependent protein serine/threonine phosphatase activity
5227	calcium activated cation channel activity
5229	intracellular calcium activated chloride channel activity
5509	calcium ion binding
5513	detection of calcium ion
5516	calmodulin binding
5544	calcium-dependent phospholipid binding
5954	calcium- and calmodulin-dependent protein kinase complex
5955	calcineurin complex
8048	calcium sensitive guanylate cyclase activator activity

8294	calcium- and calmodulin-responsive adenylate cyclase activity
8427	calcium-dependent protein kinase inhibitor activity
8597	calcium-dependent protein serine/threonine phosphatase regulator activity
9931	calcium-dependent protein serine/threonine kinase activity
10857	calcium-dependent protein kinase activity
10858	calcium-dependent protein kinase regulator activity
10859	calcium-dependent cysteine-type endopeptidase inhibitor activity
10881	regulation of cardiac muscle contraction by regulation of the release of sequestered calcium ion
10882	regulation of cardiac muscle contraction by calcium ion signaling
14722	regulation of skeletal muscle contraction by calcium ion signaling
14723	regulation of skeletal muscle contraction by modulation of calcium ion sensitivity of myofibril
14809	regulation of skeletal muscle contraction by regulation of release of sequestered calcium ion
14810	positive regulation of skeletal muscle contraction by regulation of release of sequestered calcium ion
14811	negative regulation of skeletal muscle contraction by regulation of release of sequestered calcium ion
15269	calcium-activated potassium channel activity
16286	small conductance calcium-activated potassium channel activity
16339	calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules
16340	calcium-dependent cell-matrix adhesion
17156	calcium ion-dependent exocytosis
17158	regulation of calcium ion-dependent exocytosis
18025	calmodulin-lysine N-methyltransferase activity
19722	calcium-mediated signaling
21808	cytosolic calcium signaling involved in initiation of cell movement in glial-mediated radial cell migration
21845	neurotransmitter-mediated guidance of interneurons involved in substrate-independent cerebral cortex tangential migration
21945	positive regulation of cerebellar granule cell migration by calcium
22894	Intermediate conductance calcium-activated potassium channel activity

30172 troponin C binding
30899 calcium-dependent ATPase activity
32517 SOD1-calcineurin complex
32766 NHE3/E3KARP/ACTN4 complex
33173 calcineurin-NFAT signaling cascade
33192 calmodulin-dependent protein phosphatase activity
43006 activation of phospholipase A2 activity by calcium-mediated signaling
44548 S100 protein binding
45955 negative regulation of calcium ion-dependent exocytosis
45956 positive regulation of calcium ion-dependent exocytosis
46586 regulation of calcium-dependent cell-cell adhesion
46587 positive regulation of calcium-dependent cell-cell adhesion
46588 negative regulation of calcium-dependent cell-cell adhesion
47498 calcium-dependent phospholipase A2 activity
48101 calcium- and calmodulin-regulated 3',5'-cyclic-GMP phosphodiesterase activity
48306 calcium-dependent protein binding
48791 calcium ion-dependent exocytosis of neurotransmitter
50372 ubiquitin-calmodulin ligase activity
50429 calcium-dependent phospholipase C activity
51592 response to calcium ion
60471 cortical granule exocytosis
60472 positive regulation of cortical granule exocytosis by positive regulation of cytosolic calcium ion concentration
60478 acrosomal vesicle exocytosis
61400 positive regulation of transcription from RNA polymerase II promoter in response to calcium ion
61588 calcium activated phospholipid scrambling
61589 calcium activated phosphatidylserine scrambling
61590 calcium activated phosphatidylcholine scrambling
61591 calcium activated galactosylceramide scrambling
70884 regulation of calcineurin-NFAT signaling cascade
70885 negative regulation of calcineurin-NFAT signaling cascade
70886 positive regulation of calcineurin-NFAT signaling cascade
71277 cellular response to calcium ion
72732 cellular response to calcium ion starvation
97231 cell motility in response to calcium ion

98746	fast, calcium ion-dependent exocytosis of neurotransmitter
98747	slow, calcium ion-dependent exocytosis of neurotransmitter
1900621	regulation of transcription from RNA polymerase II promoter by calcium-mediated signaling
1900622	positive regulation of transcription from RNA polymerase II promoter by calcium-mediated signaling
1901197	positive regulation of calcium-mediated signaling involved in cellular response to calcium ion
1901876	regulation of calcium ion binding
1901877	negative regulation of calcium ion binding
1901878	positive regulation of calcium ion binding
1902938	regulation of intracellular calcium activated chloride channel activity
1902939	negative regulation of intracellular calcium activated chloride channel activity
1902940	positive regulation of intracellular calcium activated chloride channel activity
1903233	regulation of calcium ion-dependent exocytosis of neurotransmitter
1903234	negative regulation of calcium ion-dependent exocytosis of neurotransmitter
1903235	positive regulation of calcium ion-dependent exocytosis of neurotransmitter
1903610	regulation of calcium-dependent ATPase activity
1903611	negative regulation of calcium-dependent ATPase activity
1903612	positive regulation of calcium-dependent ATPase activity
1990092	calcium-dependent self proteolysis
1990504	dense core granule exocytosis

[1] M. J. Berridge et al. “Calcium signalling: dynamics, homeostasis and remodelling.” In: *Nature Reviews Molecular Cell Biology* 4.7 (July 2003), pp. 517–529.

[2] P. Uhlén et al. “Biochemistry of calcium oscillations.” In: *Biochemical and Biophysical Research Communications* 396.1 (May 2010), pp. 28–32.

[3] M. Whitaker. “Calcium at Fertilization and in Early Development.” In: *Physiological reviews* 86.1 (Jan. 2006), pp. 25–88.

[4] S. Orrenius et al. “Regulation of cell death: the calcium-apoptosis link.” In: *Nature Reviews Molecular Cell Biology* 4.7 (July 2003), pp. 552–565.

[5] K. D. Baker et al. “The role of intracellular calcium stores in synaptic plasticity and memory consolidation.” In: *Neuroscience and biobehavioral reviews* 37.7 (Aug. 2013), pp. 1211–1239.

[6] H. T. McMahon et al. “Membrane curvature in synaptic vesicle fusion and beyond.” In: *Cell* 140.5 (Mar. 2010), pp. 601–605.

[7] C. S. Haarmann et al. “Regulation of skeletal ryanodine receptors by dihydropyridine receptor II-III loop C-region peptides: relief of Mg2+ inhibition.” In: *Biochemical Journal* 387.Pt 2 (Apr. 2005), pp. 429–436.

[8] R. T. Rebbeck et al. “Skeletal muscle excitation-contraction coupling: who are the dancing partners?” In: *The international journal of biochemistry & cell biology* 48 (Mar. 2014), pp. 28–38.

[9] M. W. Berchtold et al. “Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease.” In: *Physiological reviews* 80.3 (2000), pp. 1215–1265.

[10] M. J. Berridge et al. “Calcium: Calcium signalling: dynamics, homeostasis and remodelling.” In: *Nature Reviews Molecular Cell Biology* 4.7 (July 2003), pp. 517–529.

[11] K. Mallilankaraman et al. “MICU1 Is an Essential Gatekeeper for MCU-Mediated Mitochondrial Ca2+ Uptake that Regulates Cell Survival.” In: *Cell* 151.3 (Oct. 2012), pp. 630–644.

[12] M. Murgia et al. “Controlling metabolism and cell death: at the heart of mitochondrial calcium signalling.” In: *Journal of molecular and cellular cardiology* 46.6 (June 2009), pp. 781–788.

[13] D. Chin et al. “Calmodulin: a prototypical calcium sensor.” In: *Trends in cell biology* 10.8 (Aug. 2000), pp. 322–328.

[14] E. Carafoli et al. “Generation, control, and processing of cellular calcium signals.” In: *Critical reviews in biochemistry and molecular biology* 36.2 (Apr. 2001), pp. 107–260.

[15] E. Carafoli. “Calcium signaling: a tale for all seasons.” In: *Proceedings of the National Academy of Sciences* 99.3 (Feb. 2002), pp. 1115–1122.

[16] M. J. Berridge. “Cell Signalling Biology: Module 1 - Introduction.” In: *Biochemical Journal* (Apr. 2012).

[17] F. Cunningham et al. “Ensembl 2015.” In: *Nucleic acids research* 43.Database issue (Jan. 2015), pp. D662–9.

[18] D. Maglott et al. “Entrez Gene: gene-centered information at NCBI.” In: *Nucleic acids research* 35.Database issue (Jan. 2007), pp. D26–31.

[19] C. SJ. *Cancer Genetics Web*. URL: http://www.cancer-genetics.org/index.htm.

[20] M. Ashburner et al. “Gene Ontology: tool for the unification of biology.” In: *Nature genetics* 25.1 (May 2000), pp. 25–29.

[21] N. Škunca et al. “Quality of computationally inferred gene ontology annotations.” In: *PLoS computational biology* 8.5 (May 2012), e1002533.

[22] McKusick.-Nathans Institute. of Genetic Medicine; Johns Hopkins University (Baltimore; MD). *Online Mendelian Inheritance in Man, OMIM®*. URL: http://omim.org/.

[23] A. P. Davis et al. “The Comparative Toxicogenomics Database’s 10th year anniversary: update 2015.” In: *Nucleic acids research* 43.Database issue (Jan. 2015), pp. D914–20.

[24] S. M. Bello et al. “Waiting for a Robust Disease Ontology: A Merger of OMIM and MeSH as a Practical Interim Solution.” In: *International Conference on Biomedical Ontology*. 2011, pp. 231–233.

[25] B.-C. Kim et al. “Tumor necrosis factor induces apoptosis in hepatoma cells by increasing Ca(2+) release from the endoplasmic reticulum and suppressing Bcl-2 expression.” In: *The Journal of biological chemistry* 277.35 (Aug. 2002), pp. 31381–31389.

[26] E. C. Winchester et al. “Association of the TNF-alpha-308 (G–>A) polymorphism with self-reported history of childhood asthma.” In: *Human genetics* 107.6 (Dec. 2000), pp. 591–596.

[27] J. A. Blake. “Ten quick tips for using the gene ontology.” In: *PLoS computational biology* 9.11 (2013), e1003343.

[28] À. Bayés et al. “Human post-mortem synapse proteome integrity screening for proteomic studies of postsynaptic complexes.” In: *Molecular Brain* 7.1 (2014), p. 635.

[29] L. du Plessis et al. “The what, where, how and why of gene ontology–a primer for bioinformaticians.” In: *Briefings in bioinformatics* 12.6 (Nov. 2011), pp. 723–735.

[30] L. P. Rowland et al. “Amyotrophic Lateral Sclerosis.” In: *New England Journal of Medicine* 344.22 (May 2001), pp. 1688–1700.

[31] B. Thomas et al. “Parkinson’s disease.” In: *Human molecular genetics* 16 Spec No. 2.R2 (Oct. 2007), R183–94.

[32] R. W. Jackman et al. “The molecular basis of skeletal muscle atrophy.” In: *American journal of physiology. Cell physiology* 287.4 (Oct. 2004), pp. C834–43.

[33] K. Furukawa et al. “Alteration in calcium channel properties is responsible for the neurotoxic action of a familial frontotemporal dementia tau mutation.” In: *Journal of Neurochemistry* 87.2 (2003), pp. 427–436.

[34] I. Bezprozvanny et al. “Neuronal calcium mishandling and the pathogenesis of Alzheimer’s disease.” In: *Trends in neurosciences* 31.9 (Sept. 2008), pp. 454–463.

[35] K. Kristensson et al. “Scrapie prions alter receptor-mediated calcium responses in cultured cells.” In: *Neurology* 43.11 (Nov. 1993), pp. 2335–2341.

[36] M. O. Zemaitaitis et al. “Transglutaminase-induced cross-linking of tau proteins in progressive supranuclear palsy.” In: *Journal of neuropathology and experimental neurology* 59.11 (Nov. 2000), pp. 983–989.

[37] D. Martino et al. “Immunopathogenic mechanisms in tourette syndrome: A critical review.” In: *Movement Disorders* 24.9 (July 2009), pp. 1267–1279.

[38] I. Bezprozvanny et al. “Deranged neuronal calcium signaling and Huntington disease.” In: *Biochemical and Biophysical Research Communications* 322.4 (Oct. 2004), pp. 1310–1317.

[39] A. A. Osman et al. “Wolframin expression induces novel ion channel activity in endoplasmic reticulum membranes and increases intracellular calcium.” In: *The Journal of biological chemistry* 278.52 (Dec. 2003), pp. 52755–52762.

[40] M. J. Khoury et al. “On the synthesis and interpretation of consistent but weak gene-disease associations in the era of genome-wide association studies.” In: *International journal of epidemiology* 36.2 (Apr. 2007), pp. 439–445.

[41] M. H. Polymeropoulos et al. “Mutation in the α-synuclein gene identified in families with Parkinson’s disease.” In: *Science (New York, N.Y.)* 276 (1997), pp. 2045–2047.

[42] M. G. Spillantini et al. “Alpha-synuclein in Lewy bodies.” In: *Nature* 388.6645 (Aug. 1997), pp. 839–840.

[43] A. Wood-Kaczmar et al. “PINK1 is necessary for long term survival and mitochondrial function in human dopaminergic neurons.” In: *PloS one* 3.6 (2008), e2455.

[44] E. Dolgin. “First therapy targeting Parkinson’s proteins enters clinical trials.” In: *Nature Medicine* 18.7 (July 2012), pp. 992–993.

[45] V. M. Nemani et al. “Increased Expression of α-Synuclein Reduces Neurotransmitter Release by Inhibiting Synaptic Vesicle Reclustering after Endocytosis.” In: *Neuron* 65.1 (Jan. 2010), pp. 66–79.

[46] J. Burré et al. “Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro.” In: *Science (New York, N.Y.)* 329.5999 (Sept. 2010), pp. 1663–1667.

[47] F. Kamp et al. “Inhibition of mitochondrial fusion by α-synuclein is rescued by PINK1, Parkin and DJ-1.” In: *The EMBO journal* 29.20 (Oct. 2010), pp. 3571–3589.

[48] T. Calì et al. “α-Synuclein controls mitochondrial calcium homeostasis by enhancing endoplasmic reticulum-mitochondria interactions.” In: *The Journal of biological chemistry* 287.22 (May 2012), pp. 17914–17929.

[49] J. R. Masters. “HeLa cells 50 years on: the good, the bad and the ugly.” In: *Nature reviews. Cancer* 2.4 (Apr. 2002), pp. 315–319.

[50] A. Elouahabi et al. “Formation and Intracellular Trafficking of Lipoplexes and Polyplexes.” In: *Molecular Therapy* 11.3 (Mar. 2005), pp. 336–347.

[51] E. E. Vaughan et al. “Intracellular trafficking of plasmids during transfection is mediated by microtubules.” In: *Molecular Therapy* 13.2 (Feb. 2006), pp. 422–428.

[52] K. R. Gee et al. “Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes.” In: *Cell Calcium* 27.2 (Feb. 2000), pp. 97–106.

[53] R. Yi et al. “Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs.” In: *RNA (New York, N.Y.)* 11.2 (Feb. 2005), pp. 220–226.

[54] A. Okuda et al. “ATP-induced calcium oscillations and change of P2Y subtypes with culture conditions in HeLa cells.” In: *Cell biochemistry and function* 21.1 (Mar. 2003), pp. 61–68.

[55] E. Norberg et al. “An increase in intracellular Ca2+ is required for the activation of mitochondrial calpain to release AIF during cell death.” In: *Cell Death and Differentiation* 15.12 (Sept. 2008), pp. 1857–1864.

[56] J. T. S. Su Ryeon Seo. “Calcium overload is essential for the acceleration of staurosporine-induced cell death following neuronal differentiation in PC12 cells.” In: *Experimental & Molecular Medicine* 41.4 (Apr. 2009), pp. 269–276.

[57] W. C. Earnshaw et al. “Mammalian caspases: structure, activation, substrates, and functions during apoptosis.” In: *Annual review of biochemistry* 68 (1999), pp. 383–424.

[58] L. Alvarez-Erviti et al. “Chaperone-mediated autophagy markers in Parkinson disease brains.” In: *Archives of neurology* 67.12 (Dec. 2010), pp. 1464–1472.

[59] P. Wyrsch et al. “Cytosolic Ca2+ shifts as early markers of cytotoxicity.” In: *Cell communication and signaling : CCS* 11.1 (2013), p. 11.

[60] C. A. Schneider et al. “NIH Image to ImageJ: 25 years of image analysis.” In: *Nature Methods* 9.7 (2012), pp. 671–675.