Small RNA dynamics in cholinergic systems

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Small RNA dynamics in cholinergic systems

Abstract

Science still is very much in the discovery stage when it comes to transcriptional interactions, be it the long known workings of transcription factors or the recently discovered subtle fine-tuning of expression by small RNA, including microRNAs and transfer RNA fragments.

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Contents

This is the dedication.

»Ever tried. Ever failed. No matter.
Try again. Fail again.
Fail better.«

Simon Beckett

Acknowledgments

Thanks are due, for every scientist is not only standing on the shoulders of giants, but also on those of very real persons, without whom this dissertation would not have been possible. consectetuer adipiscing elit. Morbi commodo, ipsum sed pharetra gravida, orci magna rhoncus neque, id pulvinar odio lorem non turpis. Nullam sit amet enim. Suspendisse id velit vitae ligula volutpat condimentum. Aliquam erat volutpat. Sed quis velit. Nulla facilisi. Nulla libero. Vivamus pharetra posuere sapien. Nam consectetuer. Sed aliquam, nunc eget euismod ullamcorper, lectus nunc ullamcorper orci, fermentum bibendum enim nibh eget ipsum. Donec porttitor ligula eu dolor. Maecenas vitae nulla consequat libero cursus venenatis. Nam magna enim, accumsan eu, blandit sed, blandit a, eros.

0 Introduction

0.1 Cholinergic Systems

Nary a process in the mammalian body can commence without participation of cholinergic systems. Acetylcholine (ACh) was chemically and pharmacologically described by Henry Dale more than 100 years ago[1]. A short time later, Otto Loewi published the first proof of signal transmission by small molecules: he transferred physiological solutions from electrically stimulated frog hearts to naive hearts and observed their reactions; the solution that provoked a parasympathetic response he proposed to contain a »vagus substance«[2]. Finally, in 1929, Henry Dale completed the picture by isolating acetylcholine from mammalian tissue and identifying it as the molecule responsible for the parasympathetic response[3]. Dale and Loewi's joint effort in »Discoveries in Chemical Transmission of Nerve Impulses« was rewarded with the Nobel Prize in Physiology or Medicine in 1936.

Although we have learned much about cholinergic systems in these past 100 years, our understanding of the mammalian nervous system still is fairly limited. Even when disregarding peripheral nervous systems, the complexity of cholinergic transmission is immense, and a myriad of functions have been attributed to cholinergic circuits in the central nervous system (CNS). Central nervous projections of cholinergic fibres were extensively mapped by M. Marsel Mesulam and others in the 1980s[4], with a majority of long projection neurons originating in one of the eight cholinergic nuclei, Ch1-Ch8. While many of these anatomical structures have been filled with meaning by associations with both rudimentary as well as higher brain functions, there are still as many cholinergic pathways whose function is entirely unclear (Figure 1, from my first manuscript[5]).

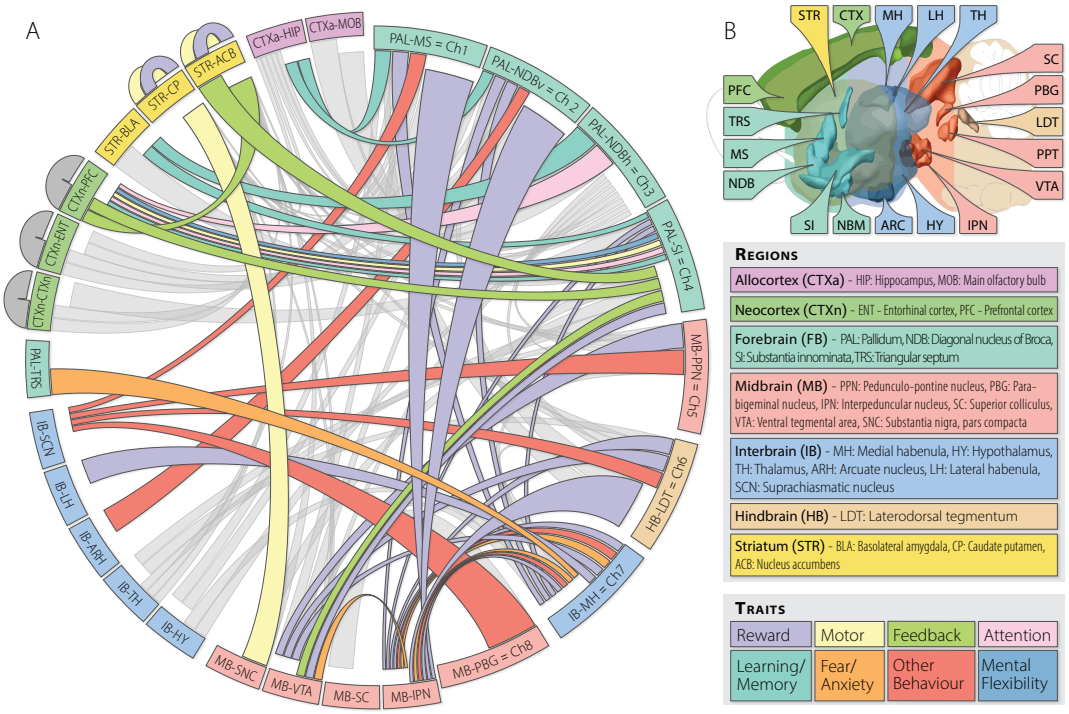

Figure 1: This is a figure that floats inline and here is its caption.

This holds particularly true for the only recently discovered cortical cholinergic interneurons, which, in comparison to their projecting counterparts, are very small and numerically vastly inferior to other neuron types in the cortex. Thus, their detection and analysis with current methods is challenging.

Disease? Subsection for disease?

0.1.1 Neurokines

In comparison to the widely studied cholinergic projection neurons originating in the basal forebrain (Ch1-Ch4) that are known to depend on a retrograde survival signal by means of neurotrophic growth factor (NGF), trophic influences on other cholinergic populations such as the cortical interneurons are unclear. NGF was described by Rita Levi-Montalcini in the 1950s as the first known instance of trophic peptides required for the survival of sympathetic ganglia[6], and the dependence of basal forebrain cholinergic neurons on retrograde NGF signalling was discovered in the 1980s[7].

A second group of trophic peptides with cholinergic implications are the so-called »neurokines«; the name results from the fact that this particular subgroup of cytokines has been associated with neuronal function in the central and peripheral nervous systems. Most prominently they include the ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), and interleukin 6 (IL-6), all of which coincidentally have been known under the acronym CDF. In the end of the 1980s, two groups of scientists (McManaman[8] and Rao[9]) independently identified proteins in extracts of muscle fibre that induced a differentiation of neurons towards a cholinergic type, and thus termed these proteins »choline acetyltransferase development factor« or »cholinergic differentiation factor« (both abbre-

viated CDF). Only later, through sequencing of the peptides, it became known that they had in fact discovered two distinct neurokines, LIF (Rao) and CNTF (McManaman, personal communication). IL-6, on the other hand, is abbreviated CDF for an entirely different reason: in this case it is short for »CTL (cytolytic T lymphocyte) differentiation factor«.

CNTF, LIF, and IL-6 convey their impact on neuronal activity through a partly redundant neurokine receptor pathway. There are two basic types of neurokine receptors: soluble and transmembrane. The primary receptors for CNTF (CNTFR) and IL-6 (IL6R) are soluble proteins that are secreted into the extracellular space and, upon binding of a neurokine, bind to transmembrane receptor dimers on the cell surface. These transmembrane receptors are the LIF-receptor (LIFR) and the »IL-6 signal transducer« IL6ST, also known as gp130. Every neurokine has its preferred constellation of soluble and transmembrane receptors: CNTF bind to the soluble CNTF receptor and a dimer consisting of one gp130 and one LIFR protein; IL-6 binds to the soluble IL6R and a dimer from two units of gp130; LIF does not usually bind a soluble receptor but rather binds immediately to a dimer comprising one of each gp130 and LIFR; however, there is significant redundancy and crosstalk between those systems [10,11].

All receptor constellations result in a main effect of activation of the JAK/STAT cascade. More specifically, neurokines can activate janus kinases (JAK) 1 and 2 or the homologous tyrosine kinase (TYK) 2, and successively »signal transducer and activator of transcription« (STAT) isoforms 1, 3, 5A, and 5B, which then convey a multitude of cellular effects (e.g. in immunity or differentiation) through transcriptional activation. The STAT cascade is inherently self-limiting in that it usually leads to expression of transcription factors that serve as repressors of the STAT genes (XXX). FIGURE STAT

0.2 Transcriptional Connectomics

No matter their location, cholinergic neurons are defined by their ability to synthesise ACh and release it to neighbouring cells to a certain effect. To fulfil this task, two particular proteins are essential: the choline acetyltransferase (ChAT) to synthesise ACh from choline and acetyl-Coenzyme A, and the vesicular acetylcholine transporter (vAChT, official gene symbol SLC18A3), which concentrates ACh in vesicles for later release. A notable genetic feature connects these two proteins beyond their functional association: the small *SLC18A3* gene (2 420 nucleobases) sits inside the first intron of the *CHAT* gene and thus is already included in its primary transcript, and is subject to the *CHAT* promoter. However, oftentimes the (mature) transcript levels of *CHAT* and *SLC18A3* mRNA seem to be independently regulated; from the perspective of the organism, the possibility of differential regulation between these two genes makes sense. Since *SLC18A3* does not possess its own promoter, this differential regulation has to be conveyed epigenetically.

This dissertation deals in large parts with approaches aiming to decipher these interactions; and while its primary topic revolves around cholinergic systems, the methods described in the following are designed to be applicable to the entirety of the genome/epigenome. Four particular types of cellular actors are subject of these methods and therefore will be briefly introduced: genes in the classical sense as the conveyors of cellular function by encoding for proteins; transcription factors, a subclass of protein coding genes that are able to regulate the expression of other genes; microRNAs (miRNAs), a class of small non-coding RNA that has been known for approximately two decades and is reasonably well described functionally and mechanistically; and transfer RNA fragments (tRFs), a second class of small non-coding RNA that has only recently been rediscovered and is significantly less well described regarding its functionality.

Where to put:

where goes this?

Distinguish neuronal connectomics from transcriptional connectomics

For the sake of simplicity, all descriptions of genomics and transcriptomics matters, genes, miRNAs, and tRFs in this dissertation are to be seen in the context of Homo sapiens, unless explicitly stated otherwise.

0.2.1 Transcription Factors

Transcription factors (TFs) were among the first intracellular regulatory mechanisms to be discovered (the earliest article referencing the term »transcription factor« in its title on PubMed was published in 1972). TFs commonly translocate from the cytosol into the nucleus upon activation (often by phosphorylation), where they bind specific DNA sequences that usually range in size from 6 to 12 nucleobases. The regions containing these binding sites (about 100 - 1 000 bases in size) determine the effect upon binding, which can be one of two main modes: either a promoter, leading to an increased activity of transcription in the downstream vicinity of the binding site, or a repressor, having the opposite effect.

There exists a vast body of knowledge on TF interactions with genes, mostly due to the long period of time since their discovery and the multitude of scientific publications, most often studying single TFs and their interactions with few genes, but cumulatively curated by several organisations. One of the currently largest curations of TF data, TRANSFAC, saw its original release in 1988. While these curation efforts can be extensive, they may present with serious bias towards particular TFs that might hold more scientific interest and thus are published far more frequently than others. Recently, comprehensive efforts have extended the available data significantly. Driven by the advent of RNA sequencing, computational approaches have become able to not only comprehensively predict TF-gene interactions, but to do so in a highly tissue-specific manner (see 1.2.3). The human body is estimated to express up to 2 600 distinct DNA-binding proteins, most of them presumed TFs[12], although other studies give lower estimates.

0.2.2 microRNAs

The first endogenous »small RNA with antisense complementarity« was described in 1993 [13], but miRNAs were only recognised as a distinct regulatory class of molecules in the early 2000s. They are typically between 18 and 22 nucleobase-long, single stranded RNA fragments, and their function is now largely undisputed: miRNAs serve as targeting molecules for a protein complex whose primary purpose is to repress translation of mRNA, and, in some cases, lead to mRNA degradation. The complex, therefore, is called »RNA-induced silencing complex« (RISC); central to its function is the family of Argonaute (Ago) proteins, which can bind the mature miRNA and orient it for interaction with its targets. Guidance of RISC to the target mRNA is generally mediated via sequence complementarity between miRNA and the targeted mRNA. Specifically, a »seed« region, usually bases 2-8 on the miRNA, is mainly responsible for the interaction; in case of perfect complementarity of this seed to the mRNA sequence, the interaction is considered »canonical«.

In early miRNA research, the 3' untranslated region (UTR) of the mRNA was believed to contain most miRNA binding sites due to its greater accessibility (i.e., the lack of active ribosomes); however, cumulative recent reports suggest that binding inside the coding region of the mRNA is a regular occurrence. The rules governing miRNA binding to target sequences show considerable flexibility; a recent study shows about 30% of analysed relationships to be of »non-canonical« nature. In those cases, seed pairing with the mRNA is often imperfect. To ameliorate this loss of stability, compensation occurs typically by a secondary complementary structure after a small gap of non-complementary bases, leading to a »bridge«-type constellation. This flexibility has implications in applications involving targeting algorithms; those that consider only the seed region are more prone to false negatives than models that consider, for instance, the free energy of the whole molecule (see 1.2.4).

cite

cite

FIGURE?

miRNAs, similar to coding genes, are transcribed from loci on the genome, many inside introns or even exons of coding genes [14]. The primary transcript (primary miRNA or pri-miRNA) typically contains a hairpin-like structure that usually results in a double-stranded molecule because of internal complementarity, and can contain up to six mature miRNAs. This hairpin structure is recognised by the DGCR8 protein (DiGeorge Syndrome Critical Region 8, in invertebrates called »Pasha«); the complex then associates with the RNA-cleaving protein »Drosha«, which removes bases on the opposite side of the hairpin, creating a miRNA precursor (or pre-miRNA), which is subsequently exported from the nucleus by the shuttle protein Exportin-5. In a final step in the cytosol, the RNAse »Dicer« removes the loop joining the 3' and 5' arms of the pre-miRNA, resulting in a duplex of mature miRNA, about 20 nucleotides long. Initially, it was thought to contain only one active miRNA, resulting in a designation of »miRNA*« for the complementary strand (commonly, the strand with lower expression). However, this notion has been disproven, and to reflect the possibility of both strands performing miRNA functions, nomenclature has changed to specify the arm of the

pre-miRNA from which the mature form originates (suffix »-3p« for the 3' arm, and »-5p« for the 5' arm).

miRNAs are organised and curated by means of a periodically updated web-based platform, miRBase[15]. For Homo sapiens, miRBase v21 contains 2 588 mature miRNAs from XXX precursors. Evolutionarily, the miRNA repertoire has grown from rodents to primates, resulting in a number of primate-specific miRNAs that may convey additional function. miRNA nomenclature is organised[16] in a way that assigns evolutionarily conserved miRNAs the same designation (number) in all species in which they are expressed. In their full names, a prefix stating the organism of origin is added; for example, hsa-miR-125b-5p (for Homo sapiens) and mmu-miR-125b-5p (for Mus musculus) share the same sequence and most of their functionalities. Disease?

miRNA genes, in the same way as protein coding genes, can also be subject to promoters and repressors, adding another layer of expression control by TFs. However, these TF-miRNA relationships are far less well described than common coding gene interactions, because miRNAs due to their shortness are not amenable to many standard gene expression assay forms. Estimation of the number of distinct targets of any one miRNA varies widely; however, it is generally accepted to not be less than several dozen targets per miRNA, and up to thousands of genes per miRNA (although that estimate might be overenthusiastic). Prediction?

0.2.3 Transfer RNA Fragments

Transfer RNA (tRNA) breakdown products have been known for decades, with first descriptions in the 1970s; back then, they were associated with a higher turnover of tRNA in cancer cells[17], and proposed as urine-based biomarkers for certain malignancies[18]. However, their genesis was attributed to random processes, and due to lacking molecular biology characterisation techniques, interest in those fragments quickly faded. It was not until recently that studies have shown tRNA to be a major source of stable expression of small noncoding RNA[19,20] in most mammalian tissues. Indeed, replicating the reports from the 1970s, tRNA breakdown products are the dominant form of small RNA in secreted fluids, such as urine and bile, and make up large parts of other bodily fluids as well[21]. They exist in two major forms: tRNA halves (tiRNAs), and the smaller tRNA fragments (tRFs). *from stroke paper* tiRNAs derive from either end of the tRNA, and are created by angiogenin cleavage at the anticodon loop[22,23]. Smaller fragments are derived from the 3' and 5' ends of the tRNA (3'-tRF/5'-tRF) or internal tRNA parts (i-tRF), respectively, and may incorporate into Ago protein complexes and act like miRNAs to suppress their targets[24,25].

However, there is considerable controversy about the generalisation of tRF functions, as distinct publications discover very different and sometimes opposing mechanisms of action for their respective fragments. An obvious assumption is the miRNA-like functionality, at least for those tRFs that are in the length range of miRNAs. There have been several instances of tRFs proven to act as

miRNA-like suppressors of translation in a RISC-associated manner[25], and of Dicer playing a large part in their biogenesis[19]. There are even instances of small RNA molecules previously mislabeled miRNAs that have been discovered to actually be tRNA-derived, such as miR-1280[26].

On the other hand, multiple groups have identified tRFs to function not in an antisense-complementary manner, but by homology aspects. A valine-derived tRF was found to regulate translation by competing with mRNA directly at the binding site at the initiation complex and thereby displacing the original mRNA, leading to its translational repression[27]. Others have found multiple classes of tRFs derived from glutamine, aspartate, glycine, and tyrosine tRNAs, that displace multiple oncogenic transcripts from an RNA-binding protein (YBX1), conveying tumor-suppressive activity[28]. Most counterintuitive is the recent finding of a tRF proven to bind to several ribosomal protein mRNAs and enhancing their translation, and, when specifically inhibited, leading to apoptosis in rapidly dividing cells[29].

There is no consistent nomenclature yet to describe and organise tRFs, which are by nature more heterogeneous than miRNAs; considering their biogenesis, one tRNA molecule can be the origin of several hundred distinct tRF molecules. Multiple approaches are common in current literature, most prominently tRFs are tied to the parent tRNA and the amino acid coded for by this tRNA. For example, the 22-nucleotide-long LeuCAG3′ tRF (meaning: a fragment of 22 bases starting at the 3' end of the leucine-carrying tRNA with anticodon »CAG«) was shown to play an important role in regulating ribosome biogenesis[29]. Since there is no repository of the likes of miRBase yet, this approach can be cumbersome for replication purposes, and explicit statement of the exact sequence of each fragment is a must in publication. In fact, since the aforementioned paper does not mention the sequence explicitly, there exist 6 distinct possibilities of fragments fitting this description. While manageable on this small scale, this system prohibits efficient analysis of larger sets of tRFs that cannot be individually controlled. For this reason, the approach of Loher and colleagues[30] might be preferable: they propose the generation of a "license plate" based on the sequence of the fragment directly, composed of the prefix »tRF«, the length of the fragment, and a custom oligonucleotide string encoding (e.g., »B3« stands for »AAAGT«). This way, tRF names are unique and unmistakably linked to the sequence, nomenclature is species-independent, and tRNA origin can be quickly determined by sequence lookup.

Disease?

? Levels of tRFs may be modulated even more rapidly than levels of miRs, since tRNA molecules are very abundant in the cell and generation of mature tRFs requires only enzymatic degradation of tRNA but no de-novo transcription of the molecule in the nucleus (citation).

0.2.4 Nested Multimodal Transcriptional Interactions - The Need for Connectomics

..., multiple levels of obstacles have to be overcome. The ultimate aim of any such approach is the generation of a robust model for the studied phenomenon; the theoretical and practical hurdles to

be surmounted to reach this goal are many. The more we know about the functioning of these intertwined systems, the more we understand how much there is still to learn.

For example, only recently it has become clear how complex transcriptional regulation by means of TFs really is, and, incidentally, the two systems studied foremost in this dissertation (nerve and immune cells) are the two most transcriptionally complex systems in any mammal. Through study of comprehensive genomic information of 394 tissue types in approximately 1 000 human primary cell, tissue, and culture samples (from the FANTOM5 consortium) it was estimated that the mean number of active TFs towards any given gene is highest in immune (12 TFs per gene) and nervous cells (10), and that any one TF in nervous and immune cells controls expression of a mean of 175 and 160 genes, respectively[34] (see also Section 1.2.3).

Similarly, it has been found that miRNAs, particularly in the nervous system, possess a much higher tissue specificity than coding genes, resulting in an expression landscape that varies widely between individual neuron types that are in close proximity in the brain. With the exception of single cell sequencing, no modern analysis method is capable of a resolution appropriate for accurate characterisation of these expression patterns, resulting in extinction of the signal of miRNAs that are not expressed consistently across cell types (similar to »housekeeping« genes) because of statistical interference. Very recent studies show that miRNA-gene co-expression networks are tightly linked to cell types in the nervous system, and that groups of miRs as functional modules associate with particular phenotypes in developmental and mature states[31]. This functional association with cell phenotype was found in quality comparable to the expression patterns of TFs, yet in quantity conveys smaller impact and thus is thought to be a fine-tuning mechanism, subtle and precise in purpose.

Another aspect of the tissue specificity of CNS-associated miRNAs is the high likelihood of underrepresentation for those very specifically expressed miRNAs. Adding to the problem is the experimental bias towards rodent models when it comes to thorough studies of the CNS, where human or other primate samples are a rarity compared to rats or mice. Assessments of the numbers of yet unknown novel primate- and tissue specific miRNAs estimate their magnitude in the thousands[32], resulting in an effective doubling of currently known miRNAs.

These high numbers of potentially interacting players present computational challenges: If estimating the number of expressed genes in a human cell at 20 000 (and the number of TFs at a low 1 000), this makes for an estimated minimum of 200 000 »real« interactions in the possible $C = \frac{1\,000!}{10!(1\,000-10)!} \cdot 20\,000$, which practically equals infinity; this is without accounting for different tissue types or cell states (e.g., differentiation or disease). Similarly, the amount of mature miRs (2 588 in miRBase v21) and their ability to target even more distinct transcripts than TFs with one single molecule present immense computational requirements for even listing all possible or actual relationships. An interaction table describing targeting of genes by miRNAs in one type of tissue has $2\,588 \cdot 20\,000 = 51\,760\,000$ individual fields.

Combining all aspects of transcriptional interaction presents additional challenges. A simple model

system to visualise (in only one type of cell) the interaction of TFs targeting genes, and of miRNAs targeting genes as well as TFs, contains about 20 000 genes (a subset of which of the size of about 2 000 are TFs), 2 588 mature miRNAs, and a total of $2\,588 \cdot 20\,000 + 2\,000 \cdot 20\,000 = 91\,760\,000$ potential interactions. In standard application scenarios, such as the generation of an interaction network around a group of genes (e.g., the cholinergic genes), the processing requirements grow linearly with each added interaction partner, and exponentially with every regulatory layer that is added.

example of standard interaction gene x miR x TF

Practically, this information has to be provided, gathered, and integrated, which further multiplies the amount of storage and processing power required. miRWalk 2.0, a collection of miRNA interaction data, has collected 12 of the most popular miRNA-targeting prediction datasets, each of which has their strengths and weaknesses (see 1.2.4). Experimentally validated interactions (e.g. as collected in DIANA TarBase or miRTarBase) are gold standard, but far from comprehensive and strictly speaking only relevant for the cellular context in which the experiment was originally performed; there are also different evidence qualities to be accounted for, depending on the type of experiment performed. Ideally, all of these data are still accessible when performing the analysis, so a database created for this purpose should be able to incorporate all this information without any data loss while still remaining feasible in terms of computation time as well as space and working memory requirements.

This dissertation will first describe the creation of such a database and what has been learned during its various stages, and then go on to apply the database to different biological problems from real world experiments, such as the cholinergic differentiation of male and female cultured cells, or the blood of stroke victims.

»Wir sehen in der Natur nie etwas als Einzelheit, sondern wir sehen alles in Verbindung mit etwas anderem, das vor ihm, neben ihm, hinter ihm, unter ihm und über ihm sich befindet.«

Johann Wolfgang von Goethe

1

miRNet: Creation of a Comprehensive Connectomics Database

The need for bioinformatical support in connectomics is immediately obvious from the sheer multitude of possible interactions between the participating factors. However, when I began working on this project (October 2015), there was no integrative database available for this purpose. Earlier that year, miRWalk 2.0 had been published, for the first time providing a relatively comprehensive source of predicted as well as experimentally validated miRNA targeting data[33] (see 0.2.2). One year later, Marbach's »regulatory circuits« were published[34], enabling analysis of comprehensive TF-gene relationships in 394 human tissues (see Section 0.2.1). These collections (as well as the data they were derived from) are the basis of the database further called *miRNet*, the development of which will be described in the following chapter.

Since a large part of the scientific progress of this dissertation deals with practical problems of multimodal connectomics, I will begin by describing the infrastructure that makes effective computation of these problems possible. After this technical description of database structure and creation, I will explain the types and organisation of its content. The remainder of the chapter will then deal with the application of this infrastructure to real-world problems in transcriptional connectomics, and the statistical approaches suited to this special case.

1.1 Implementation

For any biological question to be asked in a bioinformatics setting, the effectiveness of the computational query determines the practicality of the approach. Because resources (i.e., processing power, storage, and working memory) are limited, the database that is queried should be organised in a way that facilitates retrieval of the desired information without excess processing of useless information. In the simplified case of only miRNAs interacting with genes in one direction (miRNA → gene), this means retrieval of only those interactions relevant for the queried genes or miRNAs.

Traditional table-based approaches (also known as relational databases) such as SQL (»Structured Query Language«) cannot provide such an implementation, since individual entries for genes and miRNAs (rows and columns) have to be accessed in their entirety, whether there is a connection between gene and miRNA (1) or not (0). Additionally, adding layers to these interactions (e.g., distinct prediction algorithms, tissues, or the interaction between TFs and genes) require the addition of entire tables the same size as the database, which is detrimental to effective use of space; and more complex queries also necessitate the transfer of information between those distinct tables (in SQL typically via a JOIN command), which claims additional working memory and processing time. Overall, the so-called »many-to-many« organisation of data does not lend itself to representation in a relational database.

Figure to explain tables?

The actual performance is determined by the processing power of the machine it is running on and several structural properties, such as organisation, indexing, monotony, and of course the size of the database; therefore, an estimation of processing time for queries is bound to be inaccurate. However, processing times typically do not vary on the scale of orders of magnitude, and thus general estimations can be made. Well optimised SQL databases with a size of 5 to 10 GB on disk usually require tens of minutes if not hours to complete one single complex query[44]; *miRNet* in its current form takes up approximately 15 GB of storage. Since one analysis typically consists of several hundreds (and, in the case of permutation analyses, several hundreds of thousands) of these queries, processing times in SQL implementation are too long to be practically useful. (It seems important to note that, as of 2018, SQL also offers a graph-based organisation in addition to the traditional, relational layout. These two are separate systems, and not to be confused. The advantages of Neo4j as explained in the following should be seen from the perspective of 2015, when the database was established, and when there was no graph-based SQL implementation.)

1.1.1 Neo4j: A Graph-Based Infrastructure

To query and display biological data that are organised in a network-like structure (many-to-many), a database that lends itself to the efficient processing and storage of network data is optimal. »Neo4j« utilises a database structure that is built on the save and recall of data points in »nodes« and »edges«, which represent entities (nodes) and relationships between those entities (edges); both nodes and

edges can have any number of attributes and a unique property called »type«, typically used to describe the class of the entry (such as »gene« or »miRNA«). This database organisation replicates the network-like structure of the biological data studied. Theoretically, this makes the database more likely to be efficient in the setting of transcriptional interactions, an estimation that turned out to be true. Neo4j combines the network-like data structure with an efficient indexing system for quickly finding the entries queried for, and then »walks« along the edges of the nodes that have been found, thus only searching and returning the data that is relevant to the current query.

Fig DB structure

Depending on the input, these queries can also be rather large; however, the main pitfall of tabular databases such as SQL is circumvented: there is no need to process entire rows or columns of the table to make sure that the query is satisfied in its entirety. This is particularly useful in a setting of sparse information. For example: only 30 of the 2 588 miRNAs target a specific gene, which is common; a relational database, after finding the index of the queried gene, would have to search 2 588 fields for 1/0; the graph database, on the other hand, has to execute only 30 searches. In practice, even in the very first prototype implementations, this accelerated standard-case computations approximately thousand-fold, and was even able to accommodate advanced approaches in situations that were inaccessible in the tabular implementation.

1.1.2 High-throughput Database Generation

Java and R description? Where?

Neo4j provides several API possibilities in implementation. For the purpose of entering large amounts of data into the database at once, the Java implementation is superior to the other forms in that it provides a batch processing mode via its `BatchInserter` class. I thus wrote a custom Java program for the purpose of creating an initial state of the database from the largest set of data, the complete miRWalk 2.0 content with 12 algorithms and validated interactions. The downloaded data was organised in a plain text based file format, with one text file for each miRNA, totalling in size about 6 GB (for H. sapiens). The database was set up in a way that allows only one node for each individual miRNA and gene entered to avoid duplications, using the `createDeferredConstraint()`, `assertPropertyIsUnique()`, and `createDeferredSchemaIndex()` commands of the Neo4j Java package. This approach made sure to create only one node for each miRNA (type: MIR) and gene (type: GENE) in the data, which is essential for proper functioning of the database. Each of these nodes received several properties to store individual data, such as the various gene/miRNA identifiers, origin of data, and species.

Between those basic nodes, the batch insertion process created edges for each relationship that was found in the original data, assigning a type identifier to each edge detailing the origin of this interaction (type: name of the prediction algorithm or »VALIDATED« for experimental data). Thus, while the nodes for genes and miRNAs themselves are unique, an arbitrary number of relationships can exist between any two nodes, depending on how many interactions they share.

1.1.3 Maintenance and Quality Control

All additional datasets, such as the TF regulatory circuits or tRF targeting predictions, were entered into *miRNet* using the regular operation mode. Testing was also performed in regular operation, with manual as well as automated tests to assert the correct transfer of information from raw data to the graph database, and to avoid unpredictable behaviour. At times, conflicts had to be resolved manually, for instance when miRNA names conflicted between old »miRNA*« and new »3p/5p« notation; all manual edits are documented in the code.

Except for the rapid import of large amounts of data in creation of a database, the Java implementation of Neo4j does not offer many advantages over the native R implementation, »RNeo4j«. Thus, after creation and a short period of experimentation with graphical user interfaces, I abandoned the Java program in favour of the more flexible R programming. However, the entire Java-based code used in creation and maintenance is available in the code repository accompanying my first manuscript[5].

1.2 Materials

All materials used in the creation of *miRNet* have been acquired from resources that are non-commercial, web-available, and open-source (in the case of code).

1.2.1 Gene Annotation

Even though »regular« protein coding genes have been know for a long time, there is no consensus yet about their nomenclature and organisation. Complicated by newly discovered functions and properties of phylogenetic nature, the scientific representation of the human genome is in constant flux. Several large organisations strive to provide a robust annotation of the human gene catalog, but also in many cases contradict one another. There are three nomenclature systems that are of high importance in modern genomics:

- The traditional naming system of acronyms and fantasy-names (e.g. ChAT), also occasionally called »gene symbol«, is still widely popular because of its accessibility to humans, but is also not particularly robust because of a high amount of synonyms with high confusion potential and instances of genes without names having to carry unwieldy systematic names.
- The American Center for Biotechnology Information (NCBI), a branch of the National Institute of Health (NIH), curates and hosts a multitude of biological and medical data, and for the organisation of gene information uses its own systematic nomenclature termed »Entrez« ID. Entrez is a molecular biology database that integrates many aspects of biology and medicine in a gene-centered manner, and therefore Entrez IDs are useful to quickly connect a gene to its function, nucleotide sequence, or associated diseases. Entrez IDs are regular integers without additional characters.

- Akin to the NCBI effort, ENSEMBL is a project of the European Bioinformatics Institute (EBI) as part of the European Molecular Biology Laboratory (EMBL). Compared to the Entrez database, it is more focused on study and maintenance of the genome itself, and therefore has a more intricate nomenclature that allows for differentiation of, for example, genes and their various transcript isoforms (ENSEMBL IDs carry character prefixes for class identification, e.g., ENSG for genes, ENST for transcripts).

All of these are being used on a regular basis in many publications, and, often, they are used exclusively. As a result, the end user of the published data has to have access to all possible annotation forms, or, at least, a means to translate one into the other; often, this also introduces conflicts. For this reason, all ID types were entered into *miRNet* upon creation or during maintenance, for convenience and to minimise analysis time due to conflict resolution.

1.2.2 microRNA Annotation

miRBase provides a consistent annotation for miRNAs. Due to their relatively recent discovery, there still are major changes from version to version; the syntax, however, is stable. In addition to the miRNA »names« that are composed of species, the string »miR«, pre-miRNA designation number, and strand origin (not in all cases!), such as »hsa-miR-125b-5p«, miRBase provides IDs for pre-miRNA molecules (also called ancestors) termed »MIID«, and IDs for mature miRNA molecules termed "MIMAT". However, in practice, these are rarely used.

1.2.3 Transcription Factor Targeting

The FANTOM5 project has applied CAGE (5' cap analysis of gene expression) to a large number of human samples from diverse tissues to determine the accurate 5' ends of each transcript[35]. Knowledge of this fact enables accurate prediction of promoters likely to control a transcript's expression. Marbach and colleagues used this information in combination with detailed human gene expression data to derive a complex interaction network of TFs and genes (»regulatory circuits«), and in doing so aggregated samples with similar expression patterns and origins into 394 fictional tissues[34]. For every tissue, each TF was assigned transcriptional activities towards all genes that it supposedly targets (with the sum of all activities in any given tissue being 1); and the cumulative transcriptional activities towards any given gene correlate well with the actual gene expression in corresponding samples from an independent repository.

Even in its fifth iteration, FANTOM data is not entirely comprehensive, which came to my attention due to a cholinergic anomaly: the 5' CAGE peaks of the *CHAT* and *CHRNA7* (the nicotinic $\alpha 7$ receptor subunit) genes in raw FANTOM5 data do not pass the expression threshold, and therefore are not included in, e.g., Marbach's »regulatory circuits«. Both are critically important not only for neuronal cholinergic systems, but also for the non-neuronal aspect of immune processes. For instance, macrophages have been shown to produce ACh via ChAT , and the $\alpha 7$ homomeric ACh recep-

tor conveys direct immune suppression by expression on monocytes . Paradoxically, the CAGE peak of *SLC18A3*, which lies in the first intron of *CHAT*, crosses the threshold and therefore is included in the data. Unfortunately, I was not able to remedy these circumstances even upon personal communication with Daniel Marbach (author of »regulatory circuits«) and Hideya Kawaji of the FANTOM5 consortium, although the latter acknowledged the possibility of a gene annotation deficit leading to misattribution of the *CHAT* signal to *SLC18A3* due to the closeness of their 5' ends.

The entire collection of transcriptional activities in all tissues was downloaded from the project's web page[34], and neuronal and immune tissues were entered into *miRNet*. The collected data comprises XX neuronal tissues and XX immune cell tissues (Appendix A), and XX TF-gene relationships in total.

1.2.4 microRNA Interactions

The content of miRWalk 2.0 is freely available online[36]; however, there is no option of downloading the complete set. The targeting data thus was downloaded per miRNA with standard options for all 12 prediction algorithms (miRWalk, miRDB, PITA, MicroT4, miRMap, RNA22, miRanda, miRNAMap, RNAhybrid, miRBridge, PICTAR2, and TargetScan) in plain text format. For experimentally validated interactions, the main sources were DIANA TarBase[37] and miRTarBase[38], both of which offer complete download options. As of 2019, the 3.0 version of miRWalk allows complete species downloads; however, the developers have abandoned their third party algorithm plurality reducing the number of available alternatives from 12 to 4, which can be considered a significant disadvantage:

mention custom crawler?

While sequence complementarity, particularly of the »seed«-region, is the primary paradigm of miRNA-mRNA interaction, prediction algorithms vary widely in their implementation, general purpose, and approach to interaction prediction (for a comprehensive review of approaches and rules, see[39]). A large group of available options utilise sequence conservation aspects to increase candidate viability (such as miRanda, PicTar, TargetScan, and microT4). Others, such as RNA22 and PITA, utilise biophysical aspects such as free energy of binding or the accessibility of target sites due to secondary RNA structures as prediction arguments. All of these approaches have their up- and downsides, e.g. considering their general precision and sensitivity, or their adequate prediction of particular cases, such as multiple site targeting. Thus, it has been proposed to use a combination of complementary approaches instead of only one algorithm per analysis[40]. For this reason, I might have preferred the 2.0 version of miRWalk, even if 3.0 had been available at the time.

One advantage of the collection of all data in a quickly accessible database is the opportunity to compare the different approaches to target prediction. A statistical evaluation of the collected interaction data from miRWalk 2.0 showed vast differences in general prediction quantity (Table 1.1) as well as prediction accuracy and sensitivity when compared to the validated subset of data (Table 1.2). Since the ground truth is not known, this is an additional argument for the combination of multiple

algorithm	hit frequency
RNAHYBRID	71.62%
MIRMAP	19.90%
MIRWALK	19.74%
TARGETSCAN	16.33%
RNA22	12.34%
MICROT4	11.81%
MIRANDA	10.65%
PITA	4.90%
MIRDB	1.17%
MIRNAMAP	0.75%
PICTAR2	0.62%
MIRBRIDGE	0.15%

Table 1.1: Prediction algorithms ordered by the fraction of all possible interactions they predict as being real (positive rate). Different algorithms display a wide variation of hit rates in the entirety of predicted interactions between any miRNA and gene. Red: excluded from analysis.

algorithms instead of the use of a single set. Apart from RNAhybrid and miRBridge, all algorithms presented reasonable base hit frequencies and increases in the validated test set. Therefore, the remaining 10 algorithms were included in *miRNet* targeting data. For ease of use, an additional relationship type was created from the aggregated single algorithm hits of any miRNA→gene relationship, with the sum of algorithms predicting the interaction as a score variable. This yields a theoretical score range from 1 to 10. To account for experimentally validated interactions, each miRNA→gene relationship that was supported by strong evidence of interaction was modified by addition of 10.5 score points (a half point for quick identification of a validated relationship). The resulting optimised graph contains XX miRNA→gene targeting relationships with a distinct score distribution (Figure XX).

FIGURE: Histogram of score distributions?

The collected (human) data comprises XX miRNA→gene targeting predictions (all 12 algorithms) and XX experimentally validated interactions (XX with evidence type »strong«).

TFs not the only CHAT anomaly

1.2.5 Accumulation of microRNA Targeting

1.2.6 De-novo Prediction of tRF Targeting

Due to the recency of their (re-)discovery, no comprehensive interaction sources exist for transfer RNA fragments. There have been documented cases of miRNA-like behaviours of distinct RNA fragments [19,25], justifying an attempt to predict interactions in a comprehensive manner. Of the available options for nucleotide interaction prediction algorithms, TargetScan [41] seems particularly suited for this task because it provides the option of evaluating the evolutionary conservation of target sites in the putatively targeted genes, thereby providing an additional layer of security: The sequence of 3' UTRs is evolutionarily less stable than the coding part of genes; thus, high conservation of the bind-

algorithm	validated hit frequency	hit rate increase
PICTAR2	6.98%	1129.40%
MIRDB	9.80%	838.43%
MIRANDA	51.73%	485.94%
TARGETSCAN	70.63%	432.51%
MIRNAMAP	3.10%	410.95%
PITA	15.57%	317.20%
MICROT4	32.60%	276.10%
MIRMAP	53.86%	270.65%
MIRWALK	50.95%	258.15%
RNA22	22.51%	182.38%
RNAHYBRID	90.47%	126.32%
MIRBRIDGE	0.01%	0.00%

Table 1.2: Prediction algorithms ordered by their increase in true positive rate when considering only validated interactions. The hit rate increase when comparing experimentally validated interactions with the entire predicted data (Table 1.1) is also subject to strong variation. Hit rate increase is the increase of hit rate if only considering validated data as opposed to all predicted interactions. None of the studied algorithms unite a good precision (hit rate increase) and coverage (validated hit frequency).

ing site might indicate evolutionary pressure to keep up the interaction with the fragment, making an actual function of the interaction more likely. TargetScan also presents with reasonable sensitivity and specificity as confirmed by an independent group[42], and through an additional algorithm allows the attribution of a score based on the branch length (on the species tree) of conserved targeting[43].

miRNA-like behaviour implies the existence of a region on the tRF similar to a miRNA »seed«, and TargetScan also expects a seed as input to its targeting algorithm. Since there has been no definitive answer to the question as to where the seed region in tRFs might be, it is safest to assume nothing and explore all possibilities, i.e., simulate every possible seed position for interaction discovery. For this purpose, all discovered sequences of tRFs were chopped into 7-base pieces (7mers), which is the lenght of miRNA seeds, and statistically improbable enough to appear in the genome at random; the average length of a human 3' UTR is 800 bases, so the probability of finding any 7mer randomly in any one 3' UTR is $p = \frac{800}{4^7} = 0.049$.

Describe Targetscan process

1.3 Usage

1.3.1 Cypher Query Language

Neo4j uses a language (called »Cypher«) akin to SQL, which utilises keyphrases to issue commands, but combines it with a semi-graphical syntax to account for the graph-based layout of the data. In the following, I will describe its basic usage and the advantages it provides in the matter of transcriptional connectomics. The basic »finder« function (similar to SELECT in SQL) is called MATCH in Cypher, and, when combined with the semi-graphical syntax, can be used to identify nodes or more complex patterns in the database. The graphical syntax consists of two main building blocks

that represent the basic types of data inside the database: nodes as regular brackets »`( )`« and edges between nodes as a construct of hyphens and box brackets, that can also have a direction indicated by the greater sign »`( )-[ ]->( )`«. To specify the elements to be found, attributes of nodes and/or edges can be filtered by using curly brackets in the node definition, or the WHERE clause. To be returned, elements need to be assigned arbitrary variable names:

Listing 1.1: MATCH

```
MATCH (gene:GENE {species: 'HSA'})
WHERE gene.name = 'CHAT'
RETURN gene
```

Query 1.1 identifies a node (arbitrarily designated »gene«) with type GENE (indicated by the colon), with attributes »species« (HSA, i.e. H. sapiens) and »name« (CHAT), and returns the node with all its attributes. Since the nodes of type GENE are restrained, there can only be one gene of species H. sapiens with this name in the database, and thus, only one data point will be returned. The graphical syntax further allows for pattern matching of, for instance, miRNA→gene relationships:

Listing 1.2: Patterns

```
MATCH (mir:MIR)-[rel:TARGETS]->(gene:GENE {species: 'HSA'})
WHERE gene.name = 'CHAT'
RETURN mir, rel, gene
```

Query 1.2, similar to query 1.1, starts by identifying the node of species HSA with the name CHAT, and proceeds to look for miRNA→gene relationship edges arriving at this node; the relationships have to be of the type TARGETS (the pre-aggregated score-based accumulation of targeting). As soon as no further edges are found, the process terminates and returns all found miRNAs (»mir«), relationships (»rel«), and genes (»gene«) in discrete form, including all their attributes, such as the ENSG and Entrez IDs, the MIMAT IDs for all found miRNAs, or the score value of their targeting relationship. In this query, since there is a constraint on genes, the only gene returned is *CHAT*. However, Cypher is not limited to filtering on unique attributes; it allows for query and return of as many data points as are needed. For example, if one is interested in all miRNA→gene interactions in the cholinergic system, the query might look as follows:

Listing 1.3: Filtering

```
MATCH (mir:MIR)-[rel:TARGETS]->(gene:GENE {species: 'HSA'})
WHERE gene.name IN {cholinergic_genes}
RETURN mir, rel, gene
```

The effectiveness of graph-based databases becomes clear in this approach: Query 1.3 is processed starting at a user-defined filter, the list of cholinergic genes as an input (containing *CHAT*, *SLC18A3*, cholinergic receptor genes, acetylcholinesterase, etc). In a first step, all nodes are found that fulfil the criteria: type GENE, from species H. sapiens, that are in the list of names given. Since the gene nodes are indexed, this only requires milliseconds. Then, through the connection of edges to these nodes, it finds all miRNA nodes that have a miRNA→gene relationship towards any of the cholinergic genes. By using the gene nodes as starting point, the query can end as soon as no other edges fulfilling these criteria are found on any of the nodes. In comparison, to satisfy this query in a relational database, the rows representing these cholinergic genes would have to be assessed in their entirety, not only in those columns that represent an extant relationship, thus prolonging execution.

The database then returns all miRNA→gene relationships in this set, representing the network of cholinergic miRNA regulators, including all of their attributes. The advantages of graph-based data do not end there; say one wants to return only »master« regulators of cholinergic systems, defined as miRNAs that target at least 4 of the genes in the cholinergic set. In a relational database, this would have to be done post-hoc, by aggregation of relationships and removal of any results that do not exceed this threshold. This requires storage of the entire result in memory, and additional computational steps that can be very taxing depending on the size of the result table. In Cypher, this can be done during the query (code comments indicated by »//« explain single steps):

Listing 1.4: Two-stage Filtering

```
1 MATCH (gene:GENE {species: 'HSA'})
2 WHERE gene.name IN {cholinergic_genes}
3 WITH gene //the found genes are used as input for the second query
4 MATCH (mir:MIR)-[rel:TARGETS]->(gene)
5 WHERE count(rel) >= 4
6 RETURN mir, rel, gene
```

Query 1.4 essentially proceeds in the same way as query 1.3 in that it identifies the gene nodes filtered for and looks for the miRNAs connected to those nodes by TARGETS-type relationships; however, in the second step (which is performed per gene node as returned by the WITH clause), it returns only those patterns that have at least 4 incoming miRNA→gene relationships. Query 1.4 only requires little additional processing compared to query 1.3, and thus does not require nearly as much time as the post-hoc filtering required in a relational database query. This filtering can be applied in many stages, and in many forms, such as sums, averages, maximum and minimum, or other combinations of arithmetic and logical classifiers. Additionally, the patterns can be extended to represent complex relationships inside the graph. For instance, the following query 1.5 was used to find miRNAs that regulate any given gene in the database, and, simultaneously, affect TFs that are

involved in regulation of this same gene (this type of interaction is called feedforward loop, see also Section 4.9).

Listing 1.5: Feedforward Loop Identification

```
MATCH (gene:GENE) //find gene
WHERE gene.id = ID //by identifier (Entrez)
WITH gene //use as input for next step
MATCH (tf:GENE {species: 'HSA', tf:TRUE})-[rel]->(gene)
//find TFs targeting that gene
WHERE type(rel) IN {tissue_types} //TFs only from specific tissues
//for instance, CNS cell types (Appendix A)
WITH gene, rel, tf //use as input for next step
MATCH (gene)<-[rel_m1:TARGETS]-(mir:MIR {species:
    'HSA'})-[rel_m2:TARGETS]->(tf)
//find miRNAs that target both gene and TF
WHERE rel_m1.score > 5 AND rel_m2.score > 5
//filter by minimum cumulative score
RETURN gene, tf, rel, type(r) AS tissue, mir, rel_m1, rel_m2
```

This analysis can be done in real time on the whole genome and miRnome and only takes seconds for one iteration, a performance unimaginable in a relational database approach.

1.4 Statistical Approach to Transcriptional Connectomics

Permutation

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2.1 Background

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microRNA Dynamics in Cholinergic Differentiation of Human Neuronal Cells

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3.1 Background? - Intro?

3.2 The Cellular Model

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Dynamics Between Small and Large RNA in the Blood of Stroke Victims

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5 Discussion

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5.1 Methods

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Conclusion

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Transcription Factor Regulatory Circuits - Tissue Types