Summary of Professional Accomplishments

Regulation of gene expression at RNA level in the brain and pathologies of the central nervous system

Dr. Monika Piwecka

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Poznań 2024

1. NAME

Monika Piwecka

Maiden name: **Nowak**

2. DIPLOMAS, DEGREES

Ph.D. in Chemistry, Specialization: Biochemistry, Institute of Bioorganic Chemistry PAS, Poznan, Poland
Year of award: 2012
Title „*Therapeutic application of nucleic acids*"
Supervisor: Prof. Dr hab. Jan Barciszewski

M.S. in Biotechnology, Faculty of Biology, Adam Mickiewicz University, Poznan, Poland
Year of award: 2005
Title „*Inhibition of Tobacco Mosaic Virus with a catalytic RNA*"
Supervisor: Prof. Dr hab. Jan Barciszewski

B.S. in Biotechnology, Faculty of Biology, Adam Mickiewicz University, Poznan, Poland
Year of award: 2003
Title "*The role of telomeres and telomerase in the process of neoplastic transformation*"
Supervisor: Dr Przemysław Nuc

3. INFORMATION ON EMPLOYMENT IN RESEARCH INSTITUTES

11/2019 – up to now **Head of the Department of Non-coding RNAs**

Institute of Bioorganic Chemistry PAS in Poznań

02/2015 – 10/2019 **Postdoctoral Associate (**Post-doc)

Berlin Institute for Medical Systems Biology, Max Delbrück Center for Molecular Medicine, Helmholtz Association, Berlin, Germany

02/2012 – 01/2015 **Research Associate**

Group of Epigenetics, Department of RNA Biology, Institute of Bioorganic Chemistry PAS in Poznań

10/2005 – 01/2012 **PhD Candidate**

Dept. of tRNA Biochemistry, Institute of Bioorganic Chemistry PAS in Poznań

4. DESCRIPTION OF THE ACHIEVEMENTS, set out in art. 219 section 1 point 2 of the Act of 20 July 2018 - The Law on Higher Education and Science (Journal of Laws of 2021, item 478, as amended).

4.1. TITLE OF THE SCIENTIFIC ACHIEVEMENT

Regulation of gene expression at RNA level in the brain and pathologies of the central nervous system

4.2. A SERIES OF SCIENTIFIC ARTICLES ENCLOSED TO THE SCIENTIFIC ACHIEVEMENT

P1
Comprehensive analysis of microRNA expression profile in malignant glioma tissues.

Piwecka M*, Rolle K*, Belter A, Barciszewska AM, Żywicki M, Michalak M, Nowak S, Naskręt-Barciszewska MZ, Barciszewski J#. Molecular Oncology 2015 Aug; 9(7):1324-40.

IF 2014 = 5.367; MNiSW 2015 = 40; MNiSW 2021 =140; Cyt=78

My contribution: formulation of the research problem and the concept of the work, selection of the research methodology, planning and carrying out experiments: obtaining and preparing RNA material for RNA microarrays and RNA-seq libraries, meta-analysis of literature data, comparison of own results with literature data, analysis and interpretation of the obtained data, preparing figures and tables for the manuscript, writing the first version of the manuscript in its entirety, revising the manuscript, editing the final version of the manuscript (with the participation of the other co-authors).

P2
Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function.

Piwecka M*, Glažar P*, Hernandez-Miranda LR*, Memczak S, Wolf SA, Rybak-Wolf A, Filipchyk A, Klironomos F, Cerda Jara CA, Fenske P, Trimbuch T, Zywitza V, Plass M, Schreyer L, Ayoub S, Kocks C, Kühn R, Rosenmund C, Birchmeier C, Rajewsky N#. Science 2017 Sep 22;357(6357).

IF 2016 = 37.205; MNiSW 2016 = 50; MNiSW 2021 = 200; Cyt=881

My contribution: formulation of the research problem and the concept of the work – in cooperation with the corresponding author; planning and performing the experiments: CRISPR/Cas9 (design, cloning, testing of constructs), design of genetically mutated Cdr1as KO mouse line, development and implementation of genotyping procedure, expansion of mouse colony, planning of phenotyping, transcriptomic analyses (all total RNA-seq, poly(A)-selected RNA-seq, small RNA RNA-seq, preparation of libraries, sequencing and participation in the analysis of results, interpretation of results), supervision of electrophysiological analyses, conducting all qRT-PCR and TaqMan assay validation reactions, preparation of smRNA FISH and Nanostring validation

analyses, conducting part of the behavioral experiments, coordination of all behavioral experiments, coordination of scientific collaborations, selection of research methodology, analysis and interpretation of all obtained data, writing part of the first version of the manuscript (results and discussion), drafting figures for the manuscript, coordination and/or drawing the final figures for the manuscript, editing and revising the final version of the manuscript (with the participation of the other co-authors).

P3

Single-Molecule Fluorescence In Situ Hybridization (FISH) of Circular RNA CDR1as.

Kocks C, Boltengagen A, **Piwecka M**, Rybak-Wolf A, Rajewsky N#. Methods in Molecular Biology 2018;1724:77-96.

IF= n.d. (book series); MNiSW: n.d. (book series); Cyt=27

My contribution: planning and carrying out part of the experiments (stimulation of P19 cell differentiation towards neurons, fixation and preparation for RNA detection – results in Figure 2 and 3), analysis and interpretation of the obtained smRNA FISH data, participation in writing and editing the manuscript (with the participation of the other co-authors).

P4

miR-218 affects the ECM composition and cell biomechanical properties of glioblastoma cells.

Grabowska M*, Kuczyński K*, **Piwecka M**, Rabiasz A, Zemła J, Głodowicz P, Wawrzyniak D, Lekka M, Rolle K#. Journal of Cellular and Molecular Medicine 2022, 26(14):3913-3930.

IF 2021 = 5.295; MNiSW 2021 = 100; Cyt=4

My contribution: participation in formulating the research hypothesis and the concept of the work, planning and carrying out experiments using miRNA mimics, developing and selecting the research methodology, especially in the part of the work concerning glioma cell cultures, analysis and interpretation of the obtained data, editing figures for the manuscript, editing the final version of the manuscript (with the participation of the other co-authors).

P5

Single-cell and spatial transcriptomics: deciphering brain complexity in health and disease.

Piwecka M, Rajewsky N, Rybak-Wolf A#. Nature Reviews Neurology 2023, 19(6):346-362. https://www.nature.com/articles/s41582-023-00809-y

IF 2021 = 44.711; MNiSW 2021 = 200; Cyt =45

My contribution: formulation of the topic and scope of the review paper, literature study covering >200 original research papers, preparation of figures for the manuscript (Fig. 1-4), leading participation in writing the first and subsequent versions of the manuscript, editing and revising the final version of the manuscript (with the participation of other co-authors).

The Applicant's name is in bold font.
[#]corresponding author;
[*]authors who equally contributed to the work;
Cyt – number of citations.

n.d. – no data.

Scientometric data on the habilitation achievement was provided based on *Web of Science Core Collection*, record from 16/08/2024.

Total *Impact Factor* from the year preceding the publication = **92.578**

Total number of MNiSW points from the year preceding the publication = **390**

Total number of MNiSW points according to the newest list published 05/01/2024 = **640**

Total number of citations= **1035**

Statements confirming the habilitation candidate's contribution to the work are included in **Appendix No. 5**. Copies of the above publications are included in **Appendix No. 6**.

4.3. DISCUSSION of THE SCIENTIFIC ACHIEVEMENT

The research discussed below, which constitutes my scientific achievement entitled "Regulation of gene expression at the RNA level in the brain and pathologies of the central nervous system", was carried out in the years 2013-2023 at the Institute of Bioorganic Chemistry of the Polish Academy of Sciences (IBCH PAS) in Poznań and at the Max Delbrück Center for Molecular Medicine (MDC) in Berlin. My scientific achievement is a collection of five multi-authored publications, three of which present the results of original research, one is a chapter of a book which presents a protocol supported by the original results, and one is an extensive literature study and a review paper. I am the first author of three of these works. The selection of publications for the scientific achievement was dictated by the impact of these works on the development of the field and the scientific community, as well as on my personal scientific development.

Files containing the publications included to my scientific achievement in this proposal have been attached to the electronic version of the application in PDF format.

A. INTRODUCTION

Regulation of gene expression in cells of the nervous system

The mammalian nervous system comprises several thousands of cell types and subtypes that must develop in harmony and collaborate in order to establish functional circuits. The brain must also retain a high degree of flexibility (so-called neuroplasticity) to allow organismal adaptation to the environment and is a fundamental contributor to adaptive functioning. This is achieved through the implementation of gene expression and regulation programs, that are highly specialized in neural

cells. Historically, most research on RNA has focused on the open reading frames (ORFs) in mRNAs because of their protein-coding ability. It is now recognized however that the 5′ and 3′ untranslated regions (UTRs) of mRNAs exert a prominent regulatory role in transcript localization, stability and translation, among others by providing *cis*-elements that recruit RNA binding proteins and non-coding RNAs (ncRNAs).

Neurons comprise one of the most diverse subclasses of all the cell types [Zeng, 2022]. The exceptional diversity among neuronal subclasses together with their structural complexity and unique physiology, coupled with their long lifespan encompassing the lifetime of the organism, likely underlies the exceptional regulatory strategies that these cells have evolved.

In the past 10-15 years, it has become increasingly recognized that neural cells possess and imply **multilevel regulatory strategies that direct neural-specific RNA isoform processing and translation**. A number of neuronal mRNAs undergo alternative splicing and alternative exon selection to generate isoforms, among others, in response to neuronal stimulation. Alternative splicing is especially prevalent in the nervous system, which is reflected in the highest number of tissue-enriched exons, and the fundamental role of AS has been shown in neural development and in the establishment and function of neuronal networks [Yeo et al., 2004; Barbosa-Morais et al. 2012; Raj & Blencowe, 2015; Vuong et al., 2016]. AS and overall neuronal splicing programs are coordinated by the action of RNA binding proteins (RBPs) which are specific to neuronal cells (e.g. Rbfox, Nova, Ptbp families) and can modulate hundreds of splicing decisions throughout the genome [Raj & Blencowe, 2015]. The importance of AS and splicing choices in the brain is emphasized by the link between splicing changes and neurological disorders that have been recognized, among others, in autism spectrum disorders, spinal muscular atrophy, amyotrophic lateral sclerosis, ataxias, dementia, Huntington's disease, neuropsychiatric disorders (e.g. schizophrenia) and intellectual disability [Licatalosi DD & Darnell, 2006; Nik & Browman, 2019; Nussbacher et al., 2019]. It is worth mentioning that many of the aberrant splicing events in neurological diseases, especially neurodegenerative diseases, are directly related to mutations in genes encoding specific RBPs or other pathological events in the functioning of RNA-binding proteins in specific neuronal subtypes, e.g. their aggregation, altered cellular localization, or sequestration by abnormal RNAs [Nussbacher et al., 2019]. Consequently, disruption of the function of a single RNA-binding protein can result in an altered splicing program for many genes and transcripts, hundreds or even thousands of them, which is increasingly recognized as a phenomenon typical of neurological diseases.

In relation to alternative splicing in neural cells, the phenomenon called **backsplicing** (BS) should be mentioned, as it is the main source of **circular RNAs** (**circRNAs**). CircRNAs are atypical splicing isoforms that are particularly abundant in neurons and the nervous system compared to other cell types and tissues [Westholm et al., 2014; Rybak-Wolf et al., 2015, You et al., 2015]. Although circRNAs are most often generated from protein-coding genes and as a result of circularization of several exons, they very rarely contain an open reading frame and are classified

as non-coding RNAs. A more detailed characterization of this group of transcripts is included in the next section of the "Introduction" about non-coding RNAs.

The 3' ends of protein-coding transcripts undergo alternative cleavage and polyadenylation (APA), with most genes having multiple functional polyadenylation sites. Genomic and transcriptomic studies, including single-cell RNA sequencing analyses, demonstrate that **neurons have by far the longest 3' UTRs of all cell types** [Miura et al., 2012; Smibert et al., 2012; Agarwal et al., 2021]. Moreover, among different types of neuronal populations, differential patterns of spatial distribution of mRNA are observed in different neuronal compartments depending on the APA isoform and in response to neuronal activity [Tushev et al., 2018]. Isoforms with longer APA are characterized by greater stability and longer half-lives in the cell, and such more stable mRNAs are transported to the neural processes [Tushev et al., 2018].

Neurons, cells with long processes and polarized morphology, must have mechanisms that allow for the control of protein synthesis in different, sometimes distant from the cell body, cell compartments. Importantly, these mechanisms must be extremely efficient and temporally coordinated, giving the neurons the ability to respond to stimuli. Important mechanisms regulating the formation of the subcellular proteome in neurons are (1) **sorting of mRNAs to appropriate cellular compartments** and (2) **local protein translation**. It has been shown that different mRNA subsets are present in the dendrites and axons of neuronal cells [Cajigas et al., 2012]. Efforts to characterize mRNA transport have shown that depolarization of the cell membrane in neurons leads to an increase in the amount of certain mRNA in dendrites, e.g. mRNAs encoding calcium/calmodulin-dependent protein kinase II alpha (αCaMKII), BDNF or β-actin [Yoon et al., 2016]. Local translation in neurons is nowadays intensively studied and well-recognized phenomenon [Holt et al., 2019] which stimulated the research on other structurally-complex cells, including glial cells. Local translation in astroglia, oligodendrocytes and microglia is a process that regulates basic functions of these cells, e.g. efficient phagocytosis by peripheral microglial processes [Vasek et al., 2023]. Another interesting phenomenon that has been described recently in reference to neural cells is **alternative translation**, a process that depends on the differential usage of translation initiation sites within one individual transcript. Similarly to alternative splicing, alternative translation diversifies proteome and allows for greater biological complexity. Dougherty lab showed that the choice of translation initiation site might be activity-dependent in neurons [Sapkota et al., 2019].

Above mentioned examples do not exhaust the list of regulatory processes that work at the RNA level to diversify gene expression programs in neural cells. The other important layers of gene expression regulation involve also **RNA editing**, posttranscriptional adenosine-to-inosine modifications that is more and more recognized as cell-type specific in regard to different neuronal and glial populations [Cuddleston et al., 2022], other **RNA modifications**, e.g. the most abundant within mRNA N6-methyladenosine (m6A) [Mathoux et al., 2021], and regulation involving **regulatory non-coding RNAs**. Regulatory non-coding RNAs, like protein-coding mRNAs, also

undergo post-transcriptional processing, modifications, and localize to subcellular compartments, e.g. dendrites, which collectively influences their biological activity and specificity.

Non-coding RNAs in neural cells

Knowledge about **non-coding RNAs** (**ncRNAs**) has been developing very dynamically in the last 20 years. In eukaryotic cells, the canonical non-coding RNAs have been known for decades and comprise of ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), nucleolar RNA (snoRNA), which have been joined by new classes of small non-coding RNAs, long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), enhancer-derived RNAs (eRNAs), a long list of antisense RNAs, Y RNAs, and others. Current estimates indicate that over 80% of the human genome is pervasively transcribed, which proves that the number of ncRNAs significantly exceeds the number of protein-coding mRNAs, which are generated from less than 5% of our genome. **Brain-specific regulatory ncRNAs add an additional layer of regulatory complexity and fine-tune the gene output in neural cells.**

MicroRNAs (**miRNAs**) are 22-23 nucleotide-long non-coding RNAs that direct posttranscriptional repression of mRNA targets in diverse eukaryotic lineages. They are negative modulators of gene expression that fine-tune numerous biological processes. The discovery of miRNAs in 1993 [Lee *et al*., 1993; Wightman *et al*., 1993] and subsequent advances in understanding the biology of small RNAs have redefined the biological landscape, significantly changing the longstanding dogmas regarding the regulation of gene expression. It has been 30 years since the discovery of miRNAs, and the last 20 years have been largely devoted to exploring their biogenesis, mechanisms of action, including the recruited proteins and entire protein complexes in which miRNAs act, and finally to attempts to determine the target mRNAs and profile regulatory miRNAs in various organisms and tissues.

In animals, there are two main regulatory mechanisms of target transcript repression by miRNAs [Bartel, 2018], i.e. (1) translational repression and (2) induction of mRNA destabilization by deadenylation, decapping, and the subsequent mRNA decay (Fig. 1A). Less often, miRNA directs slicing of the target transcript and its endonucleolytic cleavage, which in this case is performed by Arganaute 2, Ago2 (Fig. 1A). **miRNAs form complex networks of interactions**, where multiple miRNAs can bind to 3'UTR and suppress one transcript (Fig. 1B), and one miRNA can be involved in the regulation of multiple mRNAs (Fig. 1C). Binding of miRNAs to closely spaced target sites may therefore contribute to cooperative repression of a target mRNA [Gebert & MacRae, 2019]. The majority of mature miRNAs are localized and act in the cytoplasm, however, some miRNAs were found to be predominantly localized to the nucleus. The nuclear function of miRNAs is still debated and remains elusive. Particular subsets of miRNAs can be also sorted into the extracellular space in exosomes (Fig. 1A), e.g. circulating miRNAs that are found in plasma and serum. The discovery of exosomal miRNAs led to the hypothesis that they might contribute to intercellular signaling and serve as biomarkers of human diseases.
miRNAs are grouped into families on the basis of the similarity of their ***seed*** sequence i.e. nucleotides 2–8 counting from the 5′ end of miRNA, which is primarily responsible for miRNA

targeting of mRNAs. In animal cells, miRNAs act via binding to 3' untranslated regions (3' UTRs) of mRNA and this interaction, assisted by Argonaut (AGO) and TNRC6 proteins, mediates mRNA decay and translational repression. miRNAs loaded into AGO are the core components of the RNA-induced silencing complex (RISC). miRNA biogenesis pathway has been fairly well-studied in multiple model organisms. They are transcribed from miRNA genes mainly by RNA polymerase II and the transcriptional units, called primary miRNA transcripts (pri-miRNAs), can consist of a single miRNA or cluster of often related and sometimes unrelated miRNAs (i.e. miRNAs from different families). miRNAs can be also derived from introns or lncRNAs [Bartel, 2018].
miRNAs exhibit tissue-specific expression patterns and are increasingly reported to have cell-type-specific expression patterns [Juzenas et al, 2017; Nowakowski et al., 2018; de Rie et al., 2017]. Perturbation of the miRNA maturation process or even a single miRNA locus in an individual cell lineage may result in serious physiological consequences and defects [Bartel, 2018]. That, for example, applies to **microRNA-7** (**miR-7**) which is the top one highly expressed microRNA in the mammalian pituitary gland [Zacharjasz et al., 2024]. Genetic ablation of one out of three *loci* for miR-7 in mice resulted in a profound phenotype reflected in the hypopituitarism and subsequent hypogonadism and infertility in both female and male mice [Ahmed at al.; 2017]. Further analyses of these mutant mice (*mir-7a2* knockout, KO) have implied an absolutely crucial role of miR-7 for gonadotrope and lactotrope cell function in the production of their respective hormones in the pituitary gland [Ahmed at al.; 2017; LaPierre et al., 2021]. More generally, *miR-7a2* knockout study showcased **the powerful role of a single miRNA transcript and its indispensability for the physiological processes,** in this case, associated with fertility.

In addition to pituitary cells, miR-7 is highly expressed in pancreatic β cells, where it is involved in the regulation of transcripts encoding proteins related to insulin secretion [Latreille et al., 2014], and in the hypothalamus, particularly high in the arcuate nucleus (ARC) and paraventricular nucleus (PVN) where it is associated with the regulation of genes related to melanocortin circuits [LaPierre et al., 2022]. Although at lower levels, miR-7 is also widely expressed in the other than hypothalamus brain regions. In the mouse brain, it is detectable at early developmental stages at embryonic day 12.5 (E12.5) [Pollock et al., 2014], and its expression has been linked, among others, to the repression of *Pax6* mRNA in dopaminergic neurons in the forebrain [de Chevigny et al., 2012]. The biogenesis of miR-7a has been shown to differ depending on whether it occurs in neurons or non-neuronal cells, and the two RNA-binding proteins that are important for miR-7 processing on top of classical miRNA biogenesis factors are Musashi homolog 2 (MSI2) and HuR protein [Choudhury et al., 2014]. Mature miR-7 is also tightly controlled post-transcriptionally in the mammalian brain and that property seems to be essential for the proper functioning of excitatory neurons, which I will discuss in the next stages of this dissertation during the discussion of my own results.

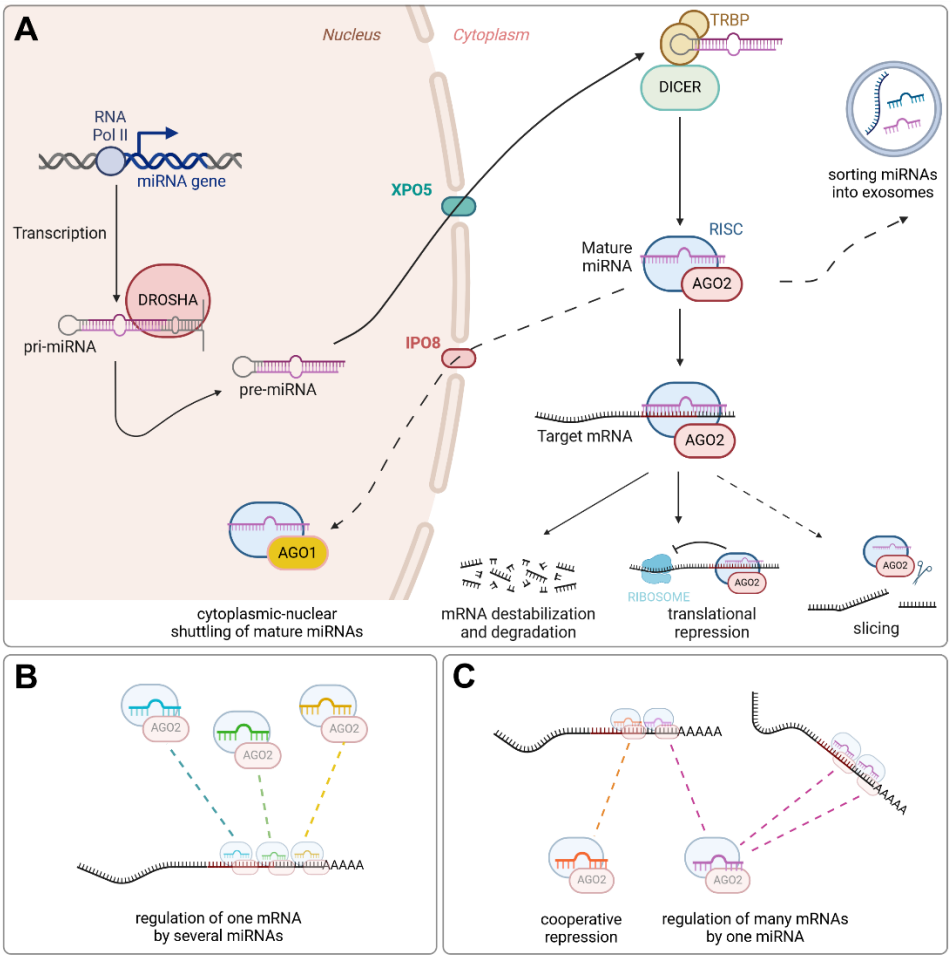

Figure 1.

Processing, maturation and regulatory functions of canonical miRNAs in animal cells. A. Biogenesis and function of a canonical miRNA. miRNA genes are transcribed into primary miRNA (pri-miRNA) transcripts and undergo multistep biogenesis. First, pri-miRNA is processed into precursor miRNA (pre-miRNA) in the nucleus. Exportin 5 (XPO5) recognizes pre-miRNA and transports it to the cytoplasm. In the cytoplasm, pre-miRNA is further processed into mature miRNAs and loaded into a silencing complex RISC. The main regulatory function of miRNA loaded into the silencing complex is to bind to target mRNA, typically within 3'UTR, and to provoke repression of a target mRNA. The most common types of mRNA repression in animal cells mediated by miRNA loaded into the silencing complex are mRNA decay and translational repression. miRNA-directed slicing (dashed line on the scheme) is rare in animal cells. Some mature miRNAs are shuttled back to the nucleus by importin 8 (IPO8), where Ago1 plays a dominant role in recruiting miRNAs. Some miRNAs are released from cells into the circulation bound to proteins, in microvesicles and in small extracellular vesicles, including exosomes. These miRNAs are proposed to form a novel mode of cell-to-cell communication. **B**. One mRNA can be targeted and regulated by many different miRNAs. **C**. One miRNA can target numerous different mRNAs. Binding of miRNAs to closely spaced target sites on mRNA may contribute to cooperative and more substantial repression of a target mRNA. Schemes A-C and above description is reproduced from the publication **P6** [Zacharjasz et al., 2024].

Early studies of miRNA expression during mammalian brain development suggested their function may be important for neural cell specification and dendritic spine development [Smirnowa et al.,

2005; Schratt et al., 2006]. The importance of miRNAs as a class of ncRNAs in the early brain development, proper neurogenesis and the survival of postnatal neurons has been demonstrated in multiple conditional KOs (cKOs) of *Dicer*, which is one of the main miRNA biogenesis factors. Many of these *Dicer* cKO in brain-specific lineages in mouse models e.g. under the control of *Wnt1-Cre*, *Nex-Cre* or *CaMKII-α-Cre* promoters were found lethal in embryonic or early postnatal stages with profoundly malformed brains [Radhakrishnan & Alwin Prem Anand, 2016]. Also, genetic ablation of *loci* coding brain-specific miRNAs resulted in severe neural phenotypes many times accompanied by premature death. For example, double-mutant mice with deletion of *microRNA-9-2* and *microRNA-9-3* exhibited severe growth retardation, had smaller cerebral hemispheres and olfactory bulbs, malformed lateral ventricles and died within one week postnatal [Shibata et al., 2011]. Overall, miR-9 is being known as an essential miRNA for proper telencephalic development and involved in controlling neural progenitor proliferation and differentiation [Radhakrishnan & Alwin Prem Anand, 2016].

Other small ncRNAs identified in neurons are **Piwi-interacting RNAs**, **piRNAs**. They are slightly longer than miRNAs (about 30 nucleotides), mainly found in nucleus, and their best-known function is related to transposon silencing in germ cells. Functional studies in invertebrates have shown that piRNAs may be involved in the processes of neuronal development and neuroplasticity [Rajasethupathy et al., 2012; Kim et al., 2018], while the biological significance of piRNAs in vertebrate somatic cells, including neurons, remains controversial [Tosar et al., 2018].

Circular RNAs

Circular RNA (**circRNA**) were first identified in the mid-1970s when it was discovered that plant pathogens, viroids, are infectious circular, single-stranded RNA molecules [Sanger et al., 1976]. In the following twenty years since this observation, there have been reports of detection of **endogenous circRNAs** in the cytoplasm of eukaryotic cells, e.g. in HeLa [Hsu et al., 1979], but the scientific community regarded them as an incomprehensible oddity or an artifactual splicing errors [Cocquerelle et al., 1993]. Only in 2012-2013, owing to the more common use of high-throughput RNA sequencing (RNA-seq) techniques, in particular total RNA-seq with RNase R treatment, and new bioinformatic algorithms dedicated to the identification of non-linear transcripts, it became clear that circular RNAs are a fairly common phenomenon in biology. Thousands of circRNAs have been identified in eukaryotes, including fungi, protists, plants, invertebrates, fish, and mammals. Additionally, RNA-seq analyses have shown that circRNA expression profiles are cell-, tissue- and developmental stage-specifically expressed [Memczak et al., 2013; Salzman et al., 2012]. It also became clear that circRNAs are much more stable than classical linear RNAs [Jeck et al., 2013; Enuka et al., 2016].

Most circRNAs are transcribed, similarly to mRNAs, from protein-coding genes. Most circRNAs contain one or more covalently closed exons and are created in the process of backsplicing, which has already been mentioned. Thus, from one protein-coding gene – in addition to one dominant or several mRNA isoforms – circular RNAs are generated, also in one dominant or several different isoforms. Circular RNAs have no ends, no cap, or poly(A) tail, but most of them are exported to

the cytoplasm. Internal retention of introns can lead to the production of circRNAs that contain sequences derived from both exons and introns (these are the so-called exon-intron circRNAs) [Li et al., 2015]. There are also known circular RNAs derived exclusively from introns, lariats (lassa structures), which are not completely degraded, but are only deprived of the 3' end, the so-called circular intronic RNA (ciRNA) [Zhang et al., 2013]. Both exon-intron circRNAs and ciRNAs are usually accumulated in the cell nucleus, in contrast to the vast majority of **exonic circRNAs**, which are located in the cytoplasm. The process of circRNA export from the cell nucleus to the cytoplasm is only partially understood.

Two seminal works on **circRNA in regard to the nervous system** were published at the beginning of 2015 [Rybak-Wolf et al., 2015; You et al., 2015] and both of them were inspiring and constituted very good high-throughput resources that helped develop my ideas and hypotheses in circRNAs research that is described below in "DISCUSSION OF THE RESULTS" section. These two independent studies showed that circRNAs are expressed much higher in the brain than in other organs and tissues in humans and rodents. The exceptionally high number of different circRNA molecules detected in the brain was confirmed by other studies researching and sequencing circRNA in the same or other mammalian species across different tissues, e.g. in macaque [Ji et al., 2019]. Moreover, circRNA profiles have been shown to be different across different brain regions, and in many cases, these profiles were different than for linear transcripts derived from the same genes. circRNAs have been shown to be dynamically expressed within CNS, oftentimes independent of linear transcript dynamics, i.e. showing upregulation or downregulation patterns over the maturation of the brain and during in vitro neuronal differentiation as well as primary neuronal culture maturation. Rybak-Wolf et al. found hundreds of circRNAs that are expressed several times higher than their canonical/linear isoform and found that the ratio of circular to linear expression was the highest in the cerebellum. They speculated, that the observed enrichment of circRNA expression in the cerebellum might be associated with high circRNA expression in neurons as the cerebellum has a higher density of neuronal cells in comparison to other brain regions. Also other studies demonstrated later that circRNAs are the major isoforms of transcripts derived from many neural genes in the human brain and that circRNAs are overall enriched in the cerebellum among different brain regions studied [e.g. Gokool et al., 2020]. Interestingly, it was noticed that among *loci* that are transcribed to circRNAs in the brain, there is an enrichment of host genes encoding proteins with synapse-related functions, and the highest peak in the global circRNA upregulation during brain development has been observed at postnatal day 10 in the mouse brain, i.e. around the onset of synaptogenesis [You et al., 2015]. Further, there is a subset of circRNAs that are highly concentrated in synaptosomes and some circRNAs were observed to change localization upon depolarization of neurons [Rybak-Wolf et al., 2015; You et al., 2015]. All of these facts indicate that circRNA likely play a functional role in neurons, yet that function or these functions for many years remained elusive. That is partially due to the fact that circRNA research is still a pretty "young" field. Another important caveat is that circRNA research is complicated because exonic circRNAs share the majority of their sequence with mRNA isoform. The only unique and distinctive sequence that can unambiguously serve for their identification is the so-

called *backsplice junction* (also: *head-to-tail* junction). Therefore, circRNA research oftentimes requires an adaptation of well-known protocols in molecular biology specifically ”tailored” for circRNA detection, identification and analyses. These novel circRNA-tailored methods have been emerging only in recent years and are still being optimized.

Of note, there is little study of circRNAs in glial cells. CircRNAs have been identified in recent time as being cell type-specifically expressed while analyzing the populations of astrocytes, microglia and oligodendrocytes derived from the human brain, specifically from the temporal lobe cortex of adult individuals undergoing surgery related to epilepsy [Curry-Hyde et al., 2020].

Our understanding of small and long ncRNAs, including circRNAs, in neurons and glia is still very incomplete and awaits a more detailed mechanistic and functional characterization.

B. RESEARCH OBJECTIVES

The main goal of my research was (and is up to date) to understand **how non-coding RNAs function, what molecular roles they play and how they influence the regulation of gene expression in cells of the central nervous system in health and disease**.

Among **the specific goals** of the presented scientific achievement, I distinguish:

(1) Identifying deregulated miRNAs in malignant gliomas, the most devastating tumors of the human brain,

(2) Determining the association of reduced miR-218 levels in malignant gliomas with the tumorigenesis process,

(3) Understanding the molecular functions of circular RNAs (circRNAs) in the mammalian brain, as exemplified by the highly expressed circRNA *Cdr1as* transcript enriched in the nervous system and using a mouse model,

(4) Determining the importance of the interaction of miRNA miR-7 with the non-coding transcript circRNA *Cdr1as* for brain function,

(5) Understanding the heterogeneity of transcriptomes in single cells from the nervous system in the context of CNS in health and disease in humans and mammalian model organisms.

The above-mentioned research goals have been built on the hypotheses put forward based on my previous research and literature analyses. The main research hypotheses will be presented in the next part of this dissertation, as an introduction to the discussed results.

The main and specific goals have been approached through studies of non-coding RNAs, both at the level of the entire transcriptome and more detailed analyses of single, selected RNA molecules in cell lines (primary and standard, immortalized), tissues from patients and in a mouse model. In my work, I used a wide range of methods and techniques of molecular biology, cell biology and systems biology to discover the undiscovered as fully as possible.

C. DISCUSSION OF THE RESULTS

microRNAs in glial tumors – from miRNA expression profiling to identification and validation of potential therapeutic targets

With the progress of high-throughput miRNA profiling methods, such as miRNA microarrays and small RNA sequencing, there has been an increasing number of works reporting miRNA catalogs in various types of healthy tissues and pathological conditions, in various organisms and at various stages of development. Unique miRNA signatures in hereditary, metabolic, infectious and neoplastic diseases have added a new dimension to the study of their pathogenesis and have highlighted the potential of miRNAs as reliable biomarkers and potential molecular targets in therapies. Concerning cancer research, it has been noted that a certain group of miRNAs is highly, aberrantly expressed in biopsies and specimens derived from patients and that increased expression of these miRNAs promotes cancer cell resistance to apoptosis and enhances their proliferation and invasiveness by repressing tumor suppressor genes. Such microRNAs have been termed "oncogenic miRNAs" or "oncomiRs". The term “oncomiR” was coined by Scott M. Hammond's lab in a work in which they reported upregulation and the potent oncogenic effect of the *miR-17~92* cluster, which is amplified in B-cell lymphomas [He et al., 2005]. This seminal study indicated that miRNAs could modulate tumor formation and function as oncogenes, implicating the miR-17-92 cluster as a potential human oncomiR. On the other hand, reduced levels or inhibition of some subsets of miRNA have been observed in multiple cancers, which under homeostatic conditions participate in the repression of mRNAs encoding oncogenes. This subgroup of miRNAs has been termed "suppressor miRNAs".

With the advancement of miRNA research, these short regulatory RNAs have begun to emerge as interesting therapeutic targets in cancer. It has been assumed that inhibition of one oncogenic miRNA or its transcriptional repression could lead to the repression and/or suppress the translation of many (dozens or even hundreds) "unwanted" mRNAs, i.e. miRNA targets, which contribute to the growth and invasion of cancer cells. The first work investigating the role of miRNAs in **glioblastoma multiforme** (**GBM**), the most aggressive of all human brain tumors, was a publication from Kenneth S. Kosik's laboratory on miR-21 in glioblastoma cells [Chen et al., 2005]. In this work the authors demonstrated that miR-21 levels are significantly increased in glioblastoma tissues, in cultured GBM cells derived from patients, and in six commercially available glioma cell lines compared to non-neoplastic fetal and adult brain tissues and compared to cultured glial cells. That study provided the foundation for subsequent studies focusing on miRNA analysis in glial brain tumors. Also in 2005, the first paper reporting the results of high-throughput miRNA profiling (microarrays) in a group of nine primary GBM has been published [Ciafrè et al., 2005]. This work showed that, in addition to miR-21, other miRNAs are upregulated in malignant gliomas, most notably miR-10b and miR-130a, while among miRNAs with reduced expression levels (potential suppressor miRNAs) have been noted miR-128a and brain-specific miRNAs from the *miR-181* family (miR-181a, miR-181b, miR-181c).

Based on the above emerging knowledge, in my research I put forward **a hypothesis** that **deregulated miRNAs in gliomas may constitute molecular targets that can be used to design future anticancer therapies in the treatment of malignant gliomas**.

The approach aimed at verifying the above hypothesis has been implemented in relation to malignant gliomas within the project "*Anti-miRNA as potential therapeutics in the treatment of human brain tumors*" (**PR5**, a project implemented under the Innovative Economy Operational Program, 2009-2014), of which I was a co-author and co-investigator. The two main components of the project included: (1) **selecting candidate miRNAs deregulated in malignant gliomas as potential molecular targets for designing therapies directed at the treatment of glial tumors**, (2) developing ribozymes as therapeutic tools to reduce the level of oncogenic miRNAs in glioma tumor cells.

The implementation of the aim #(1) in the first place has been based on gathering the data on the deregulated miRNAs in gliomas form the published studies. At the same time, in cooperation with clinicians from the Department and Clinic of Neurosurgery and Neurotraumatology in Poznań, we decided to conduct the research and identify miRNA profiles in malignant gliomas based on samples collected from Polish patients suffering from GBM. The justification that was the fact that one of the main characteristic features of GBM is heterogeneity of these tumors, i.e.it has been known that there is a very large molecular and genetic diversity, including gene expression profiles, between different patients, which is the basis of the so-called ***inter****tumor* heterogeneity. In addition, glial tumors are also diverse in terms of the cellular composition within individual tumors, and this feature is referred to as ***intra****tumor* heterogeneity. Heterogeneity of malignant gliomas is one of hallmarks of these tumors that complicates the treatment, which has remained ineffective for many years.

The results of miRNA profiling in malignant gliomas of grade III and IV from Polish patients suffering from anaplastic astrocytoma and glioblastoma multiforme, respectively, were published in 2015 in *Molecular Oncology* (FEBS Press) in the work entitled "*Comprehensive analysis of microRNA expression profile in malignant glioma tissues*" (**P1**). In the manuscript P1, based on **miRNA measurements preformed on miRNA microarrays and deep sequencing of small RNAs** using SOLiD technology, we presented the lists of deregulated miRNAs in the above types of malignant gliomas compared to samples isolated from healthy brains (postmortem samples). In addition, we performed miRNA measurements in tissues from the periphery of the tumors. In total, we analyzed 16 gliomas, 4 peripheries and compared them to 4 datasets from healthy controls (differential miRNA expression analysis). In addition, we performed a **meta-analysis** of miRNA profiling results in GBM tissues available in the literature (as of 2105: 17 published papers and datasets on this topic), we also combined it and discussed with our own results. The obtained results allowed us to select a set of miRNAs that were most frequently deregulated in gliomas, **propose a list of 35 most interesting potential therapeutic targets among miRNAs** (*Table 4* in the paper **P1**) and 30 new miRNA biomarkers in glioblastoma multiforme. Moreover, we identified miRNAs associated with the progression of glioma from grade III to the most malignant grade IV (GBM).

Among them, there were 14 miRNAs with increased expression levels in GBM compared to grade III astrocytomas and 11 miRNAs with decreased expression levels with increasing malignancy (*Table 2* in the paper **P1**), and all 25 deregulated miRNAs met stringent statistical criteria and cut-off (p-value <0.05, FC>3; FC - fold change). The atlas of deregulated miRNAs presented in the paper P1 is a comprehensive dataset on miRNAs in grade III and IV malignant gliomas, supported by meta-analysis and a useful resource and reference for other researchers dealing with miRNAs in gliomas. The number of citations of this paper according to the *Web of Science* database is 78 (as of 13/08/2024). More importantly, P1 work inspired subsequent studies on designing molecular tools for silencing the expression of precursor and mature miRNAs in GBM cells (**P13**), research on the sequence and structure of human miRNAs (**P14** and **P16**), and mechanistic and functional studies of selected miRNA candidates (**P4**, described below).

Publication **P4** is a conceptual continuation of work **P1**, and its genesis is related to the analysis of miRNA profiles in gliomas and to earlier studies conducted in the laboratory led by Prof. Jan Barciszewski with my participation on the protein tenascin C in GBM (publications **P18** and **P20**). Tenascin C (TNC), encoded by the *TNC* gene, is a glycosylated extracellular matrix (ECM) protein that is an important factor in development, including the development of the nervous system, among others involved in the regulation of proliferation of the precursors in oligodendrocyte and astrocyte lineages. TNC is also highly overexpressed in some types of cancers and associated with the invasiveness of malignant glioma cells [Hirata et al., 2009; Midwood & Orend, 2009; own publications: **P17**, **P18** and **P20**] .

During analysis of a subset of miRNAs that are downregulated in grade IV malignant gliomas and their target mRNAs, I concluded that some of these interactions may be impaired in glioma cells, which may affect the increased expression of genes encoding ECM proteins, including *TNC*, and transmembrane proteins involved in adhesion, e.g. syndecans. This **hypothesis** was developed based on bioinformatic predictions, our own results and literature data on the expression levels of selected genes, miRNAs and proteins in gliomas. Using tools such as PicTar, TargetScan and miRanda, I selected miR-218 and miR-495 as miRNAs that may potentially participate in the regulation of several genes encoding ECM proteins, including tenascin C, which we previously studied. In the course of pilot studies, I tested the effect of miR-218 supplementation on glioma cells and TNC levels by transfecting the so-called miR-218 mimics (modified short RNAs corresponding to the miR-218 sequence). In particular, I studied the effect of supplementation of the suppressor miR-218 on the culture of glioma cell lines (invasiveness test in *xCELLigence Real-Time Cell Analysis* system, migration tests, proliferation tests) and the production of ECM proteins, including tenascin C (Western blot). Since the production of ECM proteins in monolayer, adherent cultures of immortalized GBM cell lines is negligible, I introduced 3D cultures of glioma lines to my research, so-called hanging drop cultures, in which some cell lines (e.g. U118) organize into mini-tumors. The Master's student that I was supervising back to the time, Alicja Rabiasz, participated in the work on the optimization of transfection conditions and the development of 3D cultures from glioma lines. The analyses of ECM protein levels after miR-218 transfection were carried out by another Master's student, Małgorzata Grabowska. After my departure for a

postdoctoral fellowship in 2015, both students, under the supervision of Dr. Katarzyna Rolle, continued the work on the role of miR-218 in the control of ECM protein levels in glioma. The results of this project have been described in a manuscript published in 2021 in the *Journal of Cellular and Molecular Medicine* (**P4**).

In the publication **P4** we demonstrated that the overexpression of miR- 218 reduces the mRNA and protein expression levels of TNC and syndecan 2 (SDC-2), and subsequently influences biomechanical properties of GBM cells, in particular, impairs the migration potential and enhances their adhesive properties, and causes enhanced cell stiffens. Using dual luciferase assay, we showed that both *TNC* and *SDC2* mRNAs are direct targets of miR-218. One of the advanced observations in this study is the impact of miR- 218 on the mechanical properties of the cells i.e. direct changes in cell stiffness as measured with atomic force microscopy (AFM) technology. Overall, we concluded that the deregulation miR-218 in both primary and secondary GBMs is accompanied by upregulation of its targets, namely TNC and SDC-2, that in turn has an impact on ECM remodeling and enhanced migration potential of glioma cells. This study has provided an increased understanding of **GBM mechanobiology**, particularly in relation to **ncRNAs that are involved in the regulation of factors directly modulating mechanobiological properties** of glioma cells. Second thing, we confirmed that miR-218 is a potent tumor suppressor in glioma with a large impact on the ECM, and potentially, a good candidate for a therapy that will be directed at modulating the mechanical microenvironment of brain tumors. High tumor tissue stiffness is not only limited to malignant gliomas and miR-218 has been recognized to be downregulated also in other cancer types (as discussed in our article, here **P4**). Dense, abnormal ECM and high tumor stiffness are also features that limit the therapeutic efficacy of nanoparticles, and overall, can reduce cancer treatment efficacy [Zhang et al., 2024]. Strategies for modulating the mechanical microenvironment including disruption of ECM formation, can have beneficial effects for cancer patients and have important clinical implications for future therapies [Lampi & Reinhart-King, 2018].

circRNA Cdr1as – loss-of-function studies in mice and determination of cell specificity in the central nervous system

My inspiration for research on circular RNA (circular RNA, circRNA) was a series of publications that appeared in 2013 [Memczak et al., 2013; Hansesn et al., 2013; Jeck et al., 2013; Salzman et al., 2013]. Back then it became apparent that the genomes of many organisms, from nematodes to humans, generate RNA molecules with a circular structure during transcription, without any recognized biological function. At that time, the biological functions of circular RNA were a subject of speculation.

The aforementioned publications included (1) the first comprehensive characterization of circRNAs as a class of transcripts, and (2) a more detailed description of one of them, the circRNA CDR1as (*Cerebellar degeneration-related protein 1 antisense RNA*). A brief summary of my conclusions from this pioneering research: circular RNAs are expressed in a tissue-specific and developmental stage-dependent manner. The **circRNA *Cdr1as*** is enriched in neural tissues

[Hansen et al., 2011] and is one of the most highly expressed non-coding transcripts in the brain. It also has a very unique feature – in its sequence it has multiple and evolutionarily conserved repeats that correspond to the miR-7 binding site, within its *seed* sequence (nucleotides in positions 2-7 at the 5' end of miRNA, which are responsible for binding the target RNA), and in addition, in the sequence of human CDR1as there is a single binding site for miR-671-5p. The miR-671 binding site within CDR1as has a different characteristics compared to miR-7, i.e. the predicted binding site is almost fully complementary to the miR-671 and extends beyond the *seed* sequence. Jorgen Kjems's team from Aarhus University (Denmark) showed in 2011 that such specificity of miR-671 binding to CDR1as causes the cleavage of the circular transcript into a linear version by targeted binding of Ago2 and activation of its catalytic activity, the so-called *slicing* (i.e. initiating RNA hydrolysis/cleavage). Both miR-617 and miR-7 are expressed in the brain. Moreover, there have been reports published on miR-7 in brain development. Specifically, there has been evidence for target mRNAs regulated by miR-7 in the brain during fetal cortical development and in dopaminergic neurons (mouse studies, Pollock et al., 2014; de Chevigny et al., 2012). Such characteristics motivated me to put forward **the main research hypothesis**: **Cdr1as may serve regulatory functions for miR-7 and thus for mRNAs regulated by miR-7 itself, which will affect the regulation of gene expression and will have an impact on the physiology of cells in the mammalian brain.**

In order to validate the above-mentioned hypothesis, I prepared a plan and undertook a series of experiments that can be grouped into three main research threads: (1) analysis of miR-7 binding to Cdr1as in vivo in the brain, (2) characterization of Cdr1as expression in different tissues, different regions and cell types in the brain, (3) analysis of Cdr1as loss-of-function. The research model was mice and primary cells (cortical neurons) derived from mice. These results are described in the publication **P2**. Additionally, as an outcome of these studies we released a protocol for single-molecule detection and quantification of circRNA Cdr1as in cells by application of single-molecule RNA fluorescence in situ hybridization (smRNA FISH) and fluorescence microscopy (publication **P3**). I will discuss each of these three research strands separately, providing my motivation and/or partial hypothesis and the premises leading to these hypotheses.

(1) Analysis of miR-7 and miR-671 binding to *Cdr1as in vivo* in the brain – **first hypothesis**: the binding of miR-7 and miR-671 to *Cdr1as* RNA *in vivo* determines the regulatory functions of this long, circular and non-coding transcript.

Rationale: Human CDR1as contains over 70 evolutionarily conserved potential miR-7 seed binding sites and one highly complementary potential miR-671 binding site; both microRNAs and circRNA are expressed in brain tissue. The pairing of miR-671 to Cdr1as has been confirmed *in vitro* [Hansen et al., 2011]. My studies to test this hypothesis involved the use of existing high-throughput HITS-CLIP data, i.e., high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation. In particular, using raw (and recently published) HITS-CLIP data of the Ago2 protein in mouse brain [Moore et al., 2105] and a similar data set obtained from the human brain [Boudreau et al., 2014], together with Dr. Andrei Filipchyk (then a PhD student), we performed the detection and analysis of so-called chimeric reads. Andrei Filipchyk, a

bioinformatician, was one of the pioneers of the analysis of AGO CLIP data in *C. elegans* with the aim of developing new solutions for the identification of such chimeric reads, which allow for the unambiguous detection of specific miRNA binding sites to target transcripts and, at the same time, for the identification of individual miRNAs bound to individual transcripts [Grosswendt, Filipchyk et al., 2014]. The results obtained from human and mouse brains unequivocally supported the hypothesis that Cdr1as binds both miR-7 and miR-671 *in vivo* in the mammalian brain. We showed that among all miR-7-binding target transcripts detected in Ago2 HITS-CLIP data from mouse brain, the circular RNA Cdr1as is at the top of the ranking list, i.e., it binds the highest amount of miR-7 at different sites pairing with the miR-7 *seed* region. The total number of reads of *Cdr1as*-miR-7 chimeras was 127 and confirmed the direct interaction with miR-7 at several dozen different positions within *Cdr1as* (detailed data are presented in Fig. 1A and Supplementary Table S1 of publication **P2**). We also found one predominant binding site for miR-671 to be occupied by miR-671, and multiple chimeric reads supporting that (>50).

(2) Characterization of Cdr1as expression in different tissues, different regions and cell types in the brain.

To better understand the molecular functions of *Cdr1as*, it was crucial for me to find out about the spatial expression profile and cell specificity of this non-coding transcript in the brain. For this purpose, I performed a series of qRT-PCR experiments and measurements of circRNA *Cdr1as* expression in equal tissues collected from mice and, more specifically, from different brain regions. Then, extensive analyses of *Cdr1as* detection in brain sections of wild-type C57BL6/N mice were performed via *in situ* RNA hybridization with simultaneous immunostaining of protein markers of different types and subtypes of neuronal and glial cells.

The *Cdr1as locus* is located on the X chromosome. In the initial stages of my work, I established that there are no differences in the expression level of Cdr1as between male and female individuals in the mouse brain (wild-type mice, C57BL/6N), neither in the whole brain nor in individual regions, such as the forebrain (without the olfactory bulb), olfactory bulb, cerebellum, brainstem (*Supplementary Figure 7* in the publication **P2**). This was important because in the further stages I could focus on the analyses of one sex (in my work – male individuals), without duplicating all the results for both sexes. I also checked other tissues of the nervous system (spinal cord), neuroendocrine system (pituitary gland) and other organs taken from the mouse strain (lung, skeletal muscle, heart muscle, spleen) for *Cdr1as* expression. I performed similar studies to assess the expression levels of mature miR-7a-5p, its precursor (pre-miR-7a-1), and mature miR-671-5p. Collectively, these results showed that all the studied RNAs were present in the neural tissues, with Cdr1as having enormous enrichment in the brain without detectable expression or close to background expression in other tissues/organs. Within the brain, Cdr1as is most highly expressed in the cerebellum (exceeding the expression levels of well-known housekeepers e.g. *Gapdh* mRNA), around 4x higher in cerebellum as compared to cortex, hippocampus, and olfactory bulb (*Supplementary Fig. 7* in publication **P2**).

RNA in situ hybridization using a probe specific to Cdr1as and specifically, to its head-to-tail junction, confirmed the observations made with qRT-PCR measurements. Additionally, by overlapping circRNA *Cdr1as* signal with protein markers of neurons and glial cells, we were able to conclude that Cdr1as is predominantly expressed in excitatory neurons, to a lesser extent in inhibitory neurons, and absent in oligodendrocytes and astrocytes. **Single-molecule RNA FISH** (smRNA FISH) in primary cortical neurons revealed Cdr1as expression in both soma and neurites, indicating a possible functional role of Cdr1as in different subcellular localizations.

Experiments with visualization of circRNA Cdr1as at the single-molecule resolution using Stellaris probes (smRNA FISH) inspired us to publish a protocol that we used and were many times approached by other researchers with technical questions about it (publication **P3,** published as Chapter 7 in *Methods in Molecular Biology*, Springer). In that paper, we introduced the technical challenges with respect to imaging RNA at the single-molecule resolution, solutions to overcome them, and provided step-by-step protocol throughout the whole procedure, staring from probe design to finishing on spot quantification. We also provided a detailed description of how to culture cells and fix them prior to smRNA FISH. We provided numerous technical tips (17 technical notes) and recommended the software that turned out to be very useful e.g. for RNA spot quantification. The protocol is supported by our own data from HEK 293, HeLa cells and P19-derived neurons.

Worth mentioning, that in a recent publication by Cledi Cerda-Jara et al. (publication **P7**), we used that protocol for the detection and quantification of *Cdr1as* and lncRNA *Cyrano* in a bigger batch of primary cortical neurons, showing that Cdr1as is on average detected in ~300 copies/cell (hoverer the range is very wide, from few dozens to 800 copies per cell), and around 53% of these molecules can be found in neuronal somas, and the rest 47% is found in neurites and axons. Furthermore, we colocalized the circRNA *Cdr1as* signal with pre- and postsynaptic protein markers and measured the distances between protein punctate signals and RNA spots.

Another part of the characterization of the *Cdr1as locus* was the investigation of putative **Cdr1 mRNA** transcription, which is annotated on the sense DNA strand opposite and partially overlapping with *Cdr1as*. We used multiple experimental techniques as well as searched throughout strand-specific RNA sequencing datasets and proteomic datasets, but we failed to detect any evidence for transcription of the strand opposite to *Cdr1as* in mouse brains, specific mouse brain regions, or any other mouse or human tissue analyzed.

(3) Loss-of-function studies of *Cdr1as*.

In parallel to experiments and analyses presented above in points 1-2, I sought to design and perform a CRISPR-Cas9 experiment in murine P19 cell line in order to investigate if I can use that system for targeted deletion of the entire *Cdr1as locus* from the mouse genome. **The hypothesis** was that if I can efficiently generate *Cdr1as* knockout cell lines, then I can follow up with generating genetically modified mice with a deletion of *Cdr1as locus* and study *Cdr1as* loss-of-function at the organismal level.

With the CRISPR-Cas9 experiments in P19 cells and using two different guide RNAs designed to target close to PAM sequences within Cdr1as splice sites, I have learned that my design of the knockout experiment was successful. On top of multiple P19 clonal lines with smaller or bigger indel mutations within Cdr1as splice sites, I obtained clones with a full deletion of Cdr1as exon. Of note, the work with P19 lines and neuronal differentiation protocols was a basis for the implementation of smFISH protocol for the detection and quantification of Cdr1as molecules in neurons (publication **P3**).

We generated *Cdr1as* deletion mutant mice by pronuclear microinjection of Cas9 mRNA and the same two *Cdr1as*-specific guide RNAs which has been tested previously in P19 lines into mouse zygotes and *in vitro* fertilization of hormonally stimulated, pseudopregnant C7BL6/N female mice. As an outcome I obtained 5 founder animals with mutations within or in the proximity of *Cdr1as* splice sites, and one of them had the deletion of the entire *Cdr1as* exon (founder M2). I designed the methodology for testing the mutations and genotyping of mice (published in **P2**) which I am using along with many researchers with whom I shared Cdr1as knockout mouse line up to now. Further, I expanded the colony by breeding M2 founder (generated on the pure C57BL6/N background) with wild-type females on the same genetic background, which at the same time gave me the opportunity to investigate heterozygous Cdr1as +/- females (males are hemizygotes, *Cdr1as* -/Y). *Cdr1as* KO mice showed normal development and appeared healthy, without any gross differences in the brain anatomy as compared to wild-type littermates (+/Y). *Cdr1as* level was reduced by ~50% in heterozygotes (*Cdr1as* +/-) relative to the WT level in the mouse brain.

In the first place, I conducted (what I call) a **molecular phenotypic analysis** of *Cdr1as* KO mouse. I did rRNA-depleted total RNA sequencing, poly(A)-selected RNA-seq, and small RNA sequencing that all yielded interesting and unexpected results. Comparing miRNA expression levels from our sequencing data in WT and *Cdr1as* KO animals, I noticed that miR-7 was clearly downregulated in all analyzed brain regions (hippocampus, cerebellum, cerebral cortex, olfactory bulb; Fig. 2 and statistics in Table S2, publication **P2**). More specifically, both miR-7a-5p and miR-7b-5p, which share the same seed sequences but slightly differ in the mature miRNA sequence (position 10), were downregulated. The sequencing results have been confirmed with other analytical methods (Northern blot, TaqMan assay, qRT-PCR, Nanostring), which also additionally allowed us to conclude that **the reduction of mature miR-7 in the brains of *Cdr1as* KO mice** is not due to reduced transcription of these miRNAs, but **occurs post-transcriptionally** at the level of mature miRNA molecules. In contrast to the reduced expression of miR-7, **the expression of miR-671-5p in *Cdr1as* KO animals was increased** in the cerebellum, cerebral cortex and olfactory bulb. Several hundred other miRNAs identified in the mouse brains were not changed, except for one brain region (cerebral cortex), where we noted a reduction in the expression level of several miRNAs originating from two families and three miRNA clusters, *miR-200c/141*, *miR-200a/200b/429* and *miR-182/183/96*.

RNA-seq results showed that decreased levels of miR-7 in the brain of *Cdr1as* KO mice were accompanied by increased levels of mRNA molecules that are targets regulated by miR-7.

Additionally, we found that in the examined brain regions of *Cdr1as* KO mice, in addition to changes in miR-7 and miR-671, transcripts encoded by the **immediate early genes** (**IEG**) were deregulated, and interestingly, these were different IEGs in different brain structures. These results have been also confirmed with other analytical methods, not only at the level of IEGs mRNA, but also at the level of their protein products (e.g. for cFOS and EGR1). Worth mentioning, some of these genes, including the aforementioned *Fos* and *Egr1*, are also miR-7 targets.

Knowing that miR-7 is efficiently downregulated in the absence of *Cdr1as*, we concluded that *Cdr1as* might stabilize miR-7 through its direct binding. We also suspected that in the absence of *Cdr1as*, miR-7 might be destabilized by lncRNA *Cyrano*. *Cyrano* is the second top RNA target of miR-7 in the brain as revealed with our chimeric read measurements from Ago2 HITS CLIP data. It has a highly complementary binding site for miR-7, and that interaction was speculated to initiate the decay of mature miR-7 by its tailing and trimming [Ulitsky et al., 2013; Ameres et al., 2010]. Soon after our publication of the results described above (**P2**), our speculations about *Cyrano* were confirmed by David Bartel lab. In a study devoted to analysis of *Cyrano* lncRNA function, they showed that indeed Cyrano is involved in miR-7 destabilization in a process called target-mediated microRNA degradation, TDMD [Kleaveland et al., 2018].

The importance of microRNA miR-7 interaction with non-coding transcript Cdr1as in mammalian brain

In my research on Cdr1as-miR-7 interaction in the brain, I was interested in finding out if these molecular perturbations that we observed in the mouse brain, but not any other tissue of Cdr1as KO mice, are somehow reflected in overall physiology and if they affect the behavior of mutant mice. For this purpose, I designed a series of experiments to test if the lack of *Cdr1as* and subsequent disrupted transcriptome profiles are important for the individual's health and wellbeing. In the first place, in collaboration with the excellent experts in electrophysiology (Rosenmund lab, Charite) we prepared so-called autaptic hippocampal neuronal cultures from mutant animal brains and corresponding littermate controls to measure the basic electrophysiological properties of neurons. Along with many unaffected properties of *Cdr1as* knockout neurons, we noted that **spontaneous synaptic vesicle release was strongly increased** in the KO neurons, with more than a doubling of mEPSCs (miniature excitatory postsynaptic currents) frequency. The amplitude of mEPSC remained unaffected. That observation unequivocally **validated our hypothesis that Cdr1as is functional regulatory RNA, which through a complex network of interactions with miRNA and indirectly their mRNA targets serve a function in neurons**.

Second, we have performed a battery of behavioral tests under the supervision of an experienced behaviorist, Dr. Susanne Wolf. Me and Susanne, we performed, among others, the sociability test, novel object recognition test, open field test, elevated plus maze, Morris water maze, sucrose preference test, prepulse inhibition, etc., We measured many parameters related to memory, motivation, anhedonia, anxiety, exploratory and social behavior. Many of these parameters were normal or slightly impaired, with the exception of prepulse inhibition (PPI). Both male and female Cdr1as KO mutants had significantly enhanced responses in the PPI test. A disinhibition effect in

PPI is oftentimes referred as to the so-called **PPI deficit**. This led us to another important conclusion that animals with *Cdr1as* KO mutation exhibit a behavioral phenotype associated with neuropsychiatric disorders. PPI is a measure of the sensorimotor gating. This is a property of the brain that allows it to filter out unnecessary information, so that the body does not overreact to all stimuli. This function is impaired, for example, in the brains of people suffering from schizophrenia. It is also impaired in the brain of mice lacking *Cdr1as.*

To summarize my work on *Cdr1as* in here presented habilitation application: the experiments performed by me and co-authors and the obtained results have brought many new, interesting and groundbreaking discoveries on circRNA *Cdr1as*. It is the first functionally described circular RNA molecule. This function turns out to be important for excitatory neurons at the molecular and physiological level, and consequently is important for the proper functioning of the brain, as shown in behavioral studies. ***Cdr1as*-miR7-miR-671** and **lncRNA *Cyrano*** constitute a unique **network of regulatory non-coding RNAs** that affects the molecular life of neurons. Importantly, although the publication **P2** is very extensive (five main figures, following 18 figures in the supplement, 7 tables), it has not fully explained the complex interactions between the above-mentioned regulatory RNAs. In particular, the connections between the direct effects of these molecules on neuronal physiology and the link with the behavioral phenotype are certainly much more complex and remain an ongoing subject of research. The insight into these processes gained by me and the co-authors of the publications **P2** and **P3** can be summarized in the proposed model (**Fig. 2**). However, this model is constantly evolving with the inflow of new knowledge. The latest version of the model can be found in the publication **P7**. Another open question is whether the network of interdependent RNAs operating in excitatory neurons (Cdr1as-miR7-miR-671 and lncRNA Cyrano) functions in the same way in other cell types in which they are present, e.g. in the pituitary gland cells.

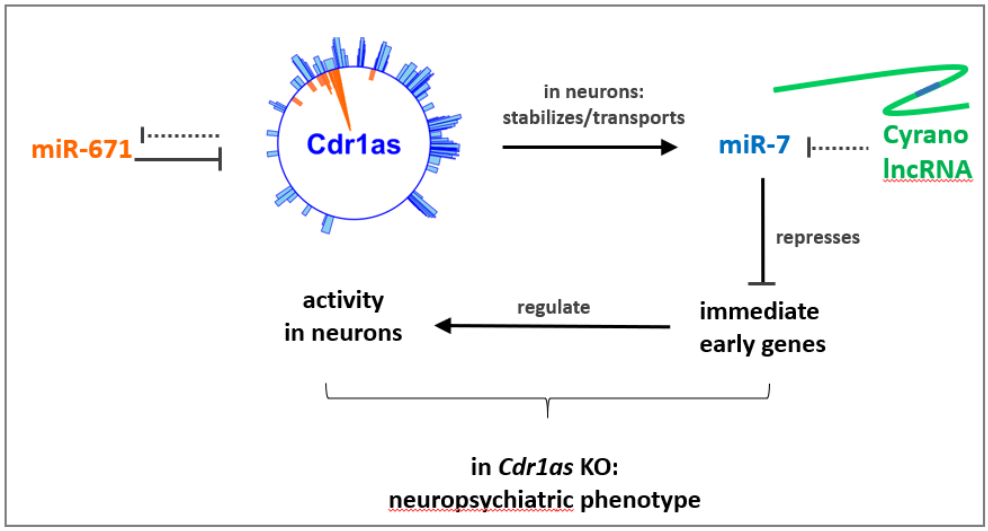

Figure 2.
Model of the interactions of *Cdr1as* with miR-7 and miR-671 and lncRNA *Cyrano* and their influence on neuronal activity as discussed in the publication P2.

How has single-cell RNA profiling advanced our knowledge of the brain and neurological disorders?

For several years now, we have been living in the era of systems biology and -omics studies, which are expanding from transcriptomics, genomics, and proteomics to include high-throughput studies of genome-wide chromatin accessibility, epigenetic marks (epigenomics), and endogenous metabolites (metabolomics). All of these analytical approaches are currently used to (1) comprehensively and systematically identify and analyze genetic and/or other molecular profiles in the human body and other living organisms, (2) and to delve into pathological processes underlying incurable human diseases. New transcriptomic technologies, such as single-cell transcriptomics and spatial transcriptomics, provide comprehensive insight into gene expression programs and RNA biodiversity between different cell populations and sometimes even within a single cell population depending on its localization within a tissue and communication with other cells.

My fascination with the transcriptomic heterogeneity of brain cells dates back to 2015-2016, when the first works on **single-cell RNA sequencing** (**scRNA-seq**) from the somatosensory cortex and hippocampus [Zeisel et al., 2015] and the visual cortex of the mouse brain [Tasic et al., 2016] were published. Since then, I have been constantly following the progress in this type of research and the development of single-cell transcriptome sequencing technologies, also in relation to my own results. I have summarized this knowledge and thoughts on modern transcriptomic techniques in relation to progress in understanding neurological diseases and designing new therapies in the

review paper **P5**, entitled "*Single-cell and spatial transcriptomics: deciphering brain complexity in health and disease*" published in 2023 in *Nature Reviews Neurology*.

In the paper **P5** we discussed how single-cell transcriptomics and ST have evolved from their beginnings (around 2009-2010) to 2023. In particular, we have tried to summarize the impact of these studies on the contemporary understanding of molecular processes occurring in brain cells and pathomechanisms underlying neurological disorders. The core of the review is a discussion of important "milestones", i.e. pioneering publications, whose authors, basing on the results of scRNA-seq and/or ST to study mouse or human brains and dedicated computational methods, tried and very often provided new data on molecular processes in different types and subtypes of neuronal and/or glial cells. We focused on three areas where we believed these new technologies provided particularly useful insights: (1) selective neuronal vulnerability, (2) neuroimmune dysfunction, and (3) cell-type-specific treatment response. In reference to the selective susceptibility of neurons to pathological processes, scRNA-seq and snRNA-seq (single-nucleus RNA sequencing) technologies give hope for understanding which nerve cells are more vulnerable to pathogenic processes and why. This applies to neurodegenerative diseases (e.g. Alzheimer's disease, Parkinson's disease) where these studies are the most advanced, but also neuropsychiatric disorders (schizophrenia, bipolar disease), or neurodevelopmental disorders (e.g. autism spectrum disorder). In the part referring to neuroimmune dysfunction, we focused on new reports on the participation of glial cells, especially microglia and astrocytes in pathology associated with neurodegeneration (e.g. in multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis), in brain tumors and in the development of neurological symptoms during SARS-Cov2 virus infection (so-called neuro-COVID). In the section entitled "*Drug-sensitive cell types*" we focus on studies aimed at optimizing drug response monitoring protocols and treatment methods based on new single-cell RNA sequencing technologies. Such cell type-specific drug sensitivity screening approaches with the aim of developing personalized therapies are being tested, for example, in glioma. For details, please see the publication **P5**.

In the publication **P5**, we reviewed >200 original research papers that analyzed the above-mentioned subjects. We also introduced and discussed the other issues e.g. related to the methodology and bioinformatic analysis of data obtained with the modern sequencing technologies. The evolution of high-throughput scRNA-seq and ST, both experimentally and computationally, has taken place at an incredible pace over the last few years. This has resulted in these technologies becoming increasingly accessible, increasingly used, increasingly improved, and providing insight into molecular processes in single cells or single cell nuclei in an increasingly complex way. To facilitate the orientation in these technologies by potential new users, we have included a list of the most commonly used computational tools for single-cell sequencing analysis, a table comparing different methods of single-cell RNA sequencing, and (in the supplement) a brief description of how to obtain live cells from the brain tissues so that they are of good quality for RNA studies. Furthermore, in an extensive part of the publication **P5,** we discussed the current limitations of these methods and indicated future directions of single-cell and spatially-resolved RNA sequencing technologies in relation to the CNS and neurological diseases.

D. THE MOST IMPORTANT ACHIEVEMENTS OF THE PRESENTED RESEARCH

1. Sequencing of miRNAs from different stages of malignancy (III and IV) of malignant gliomas.
2. Compilation of knowledge on de-regulated miRNAs in GBM, emerging from our own research and other studies (meta-analysis).
3. Determination of miRNAs that are most frequently de-regulated in glioma progression from stage III to stage IV of malignancy.
4. Determination of the role of miR-218 in modulation of expression of genes encoding ECM proteins in glioma.
5. Determination of the role of miR-218 and its target mRNAs (and their protein products) in regulating the mechanobiological properties of glioma cells.
6. Describing the role of miR-218 and its target mRNAs along with their protein products in regulating the mechanobiological properties of glioma cells.
7. Identification of miR-7 and miR-671 binding to Cdr1as *in vivo* in mouse and human brain.
8. Development of a strategy for deletion of the *Cdr1as locus* from the mouse genome and generation of a mutant mouse model lacking *Cdr1as locus*.
9. Determination of molecular changes in various tissues of *Cdr1as* knockout mice, including detailed characterization of the mouse brain and its different regions.
10. Determination of the significance of *Cdr1as* loss-of-function on neuronal physiology and animal behavior.
11. Description of the unique non-coding RNA network functioning in excitatory neurons of the mammalian brain: circRNA Cdr1as – miR-7 – miR-671 – lncRNA Cyrano.
12. Development and validation of a protocol for the fine identification and quantification of circRNA *Cdr1as* at the single-molecule resolution in cells using smRNA FISH and microscopy.
13. Summary and discussion of single-cell RNA sequencing and spatial transcriptomics technologies and their contribution during the pioneering years of their development to the advancement of neurobiology and neurological disease research.

E. SUMMARY AND PERSPECTIVES

Human brain is the most complex system in nature, containing billions of highly interconnected cells that form networks to control physical and cognitive function. Neurons are among the most

compartmentalized and interactive of all cell types. For several years now, neurobiologists have been increasingly drawing attention to the fact that glial cells play an important role in the homeostasis of the central nervous system and neurological disorders, which is partially due to their ability to acquire a wide range of activation states. At the molecular level, both neurons and glial cells are characterized by **multilevel regulation of gene expression programs** in order to provide their specialized functions e.g. by maintaining and modifying the proteome in axons and and dendrites, to enable local information processing and rapid response to stimuli. The importance and influence of **regulatory non-coding RNAs** on gene expression programs in neurons and glia is a relatively new research area that has been developing very intensively in recent years. Almost every week, new discoveries reveal the regulatory role that ncRNAs play in many biological processes. More generally, these studies increasingly demonstrate that, in both model organisms and humans, complexity is not a function of the number of protein-coding genes, but results from the ability to use combinations of genetic programs and controlling their spatial and temporal regulation during development, aging, and disease via regulatory RNAs.

In his biography the pioneering neuroscientist *Santiago Ramón y Cajal* penned: "*It is commonplace fact that scientific discoveries are a function of the methods used*". In my research, I constantly try to expand the scope of my research techniques, follow the latest scientific reports and design my experiments so that I can effectively participate in the advances of molecular biology, in particular in **RNA biology** and **the biology of neurons and glial cells**.

My ***scientific achievement*** presented in this dissertation is a series of 5 scientific publications. The experience gathered during that work allowed me to create an interdisciplinary scope and skills covering molecular biology, cell biology and systems biology methods, including advanced transcriptomic technologies, which are used by me and my team to explore the secrets of nerve and glial cells. In addition, the publications from this series have gained widespread recognition in the scientific community, as evidenced by the number of citations of these publications (>1000 in total). Without a doubt, a first-author publication in the prestigious journal *Science* is a distinctive achievement. In addition to several hundred citations to date, it has also been widely commented on in the world's scientific and non-scientific media, including news outlets such as *The Scientist* (https://www.the-scientist.com/first-in-vivo-function-found-for-animal-circular-rna-31102), GEN: *Genetic Engineering and Biotechnology News* (https://www.genengnews.com/news/loss-of-circular-rna-throws-brain-for-a-loop-2/), the *Phys.org* portal (https://phys.org/news/2017-08-circular-rna-linked-brain-function.html), the *Technology Networks* website (https://www.technologynetworks.com/tn/news/circular-rna-linked-to-brain-function-291126), etc. I was glad to find out that prominent scientists have considered this work to be groundbreaking in research on regulatory RNAs in neurons and representing significant progress in the field, e.g. Prof. Julia Salzman (Stanford University) and Prof. Sebastian Kedener (Brandeis University) expressed such statements in a commentary to the publication in *The Scientist*. Another commentary to the publication **P2** from *Science* has also appeared in the form of *Research Highlight* in *Nature Reviews Neuroscience* [Yates, 2017].

The series of 5 publications that are presented here for evaluation also have had an impact on the current and future scientific work of myself and other research teams. In the Department of Non-coding RNAs at IBCH PAS, under my supervision, we conduct the research projects aimed at identifying the function of selected circular RNAs that localize to synaptic areas in neurons. We also study circRNA-protein interactions, we create new protocols for studying selected properties of circRNAs in reference to improve capturing signal from circRNA without co-detection of linear mRNAs arising from the same host genes. In addition, we are keenly interested in discovering the **cell specificity of circRNAs and miRNAs**, especially in different cell types and cell subtypes from the brain, its distinct areas and the pituitary gland. In our laboratory, we also conduct advanced studies on the role of Cdr1as and miR-7 interaction in secretory cells of the pituitary gland.

Work on miRNA in relation to human brain tumors (included in manuscripts **P1**, **P4**) has been continued in the Department of Molecular Neurooncology at IBCH PAS under the supervision of Dr. Katarzyne Rolle, with whom I remain in contact. In my Department, we have initiated a project (pilot phase) aimed at understanding the cell specificity of selected miRNAs and circRNAs in glioblastoma multiforme (preliminary results of the project were included in the bachelor's thesis of student Maria Gwit, carried out under my supervision).

The work on the role of *Cdr1as*-miR-7 interactions in neurons has been continued by two research teams, one from MDC in Berlin, the other from the University of Eastern Finland in Kuopio (Finland). Both projects were conducted in collaboration with me and both projects used *Cdr1as* knockout mice as a model (or one of the models analyzed). Both works have been published this year in prestigious scientific journals (papers **P7** and **P8**) and served as a basis for obtaining a PhD diploma by the first authors of these studies. Both projects were conducted independently and focused on different aspects and goals, but in the final version many of the obtained results were complementary, contributing to the extension of our understanding of how the network of non-coding RNAs, at the center of which is the circRNA *Cdr1as*, functions during neuronal activation and during ischemic stroke. Other scientists, inspired by the gaps in the full understanding of *Cdr1as*-miR-7 interactions in different types of neurons, are also making efforts that result in interesting discoveries, e.g. regarding the localization of *Cdr1as* in the synaptic compartments of neurons from the medial prefrontal cortex and participation in fear extinction memory [Zajaczkowski et al., 2023]. It is worth adding that *Cdr1as* is also actively explored in regard to cancer. These studies suggest that the role of *Cdr1as* in melanoma cells may not be related to the regulation of miR-7 and its target transcripts, but rather to the regulation of IGF2BP3 protein availability [Hanniford et al., 2020] and that Cdr1as is not present in many human cancer types but is found in stromal cells in the tissue adjacent to tumor [Kirsiansen et al., 2020]. Currently, *Cdr1as* knockout mouse model is the subject of research in my laboratory and several other laboratories around the world (from Finland, Denmark, the Netherlands, USA), and I hope that the studies on this model will result in many more discoveries that will bring us more detailed insights into the role of regulatory non-coding RNA networks in the brain, tumors, and their periphery.

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5. OVERVIEW OF OTHER SCIENTIFIC ACHIEVEMENTS

(Presentation of significant scientific activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions)

My scientific achievements has been obtained in two research institutes:

- Institute of Bioorganic Chemistry of the Polish Academy of Sciences in Poznań, where I completed my doctoral studies, obtained my doctoral degree in 2012, and then continued working as a research assistant until January 2015. During my doctoral studies, I was awarded a supervisory grant, received a prestigious scholarship from the President of the Polish Academy of Sciences, and was honored with a scholarship by the Voivodeship Labor

Office as part of the "Scholarship support for doctoral students in fields considered strategic from the point of view of the development of Wielkopolska". Currently, since November 2019, I have been heading the Non-coding RNA Department at ICHB PAN.

- Berlin Institute for Medical Systems Biology (BIMSB), Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany, where I completed a postdoctoral internship in 2015-2019.

5.1. LIST OF OTHER PUBLICATIONS (that are not included in point 4)

Scientific publications in journals listed in the Journal Citation Reports (JCR) database

P6
Micromanaging the neuroendocrine system - a review on miR-7 and the other physiologically relevant miRNAs in the hypothalamic-pituitary axis.

Zacharjasz J*, Sztachera M*, Smuszkiewicz M, **Piwecka M**#. *FEBS Letters* 2024 Jul; 598(13):1557-1575. doi: 10.1002/1873-3468.14948.
https://febs.onlinelibrary.wiley.com/doi/10.1002/1873-3468.14948
IF 2022 = 3.5; MNiSW 2023 = 140; MNiSW 2024 = 100; Cyt=0

P7
miR-7 controls glutamatergic transmission and neuronal connectivity in a Cdr1as-dependent manner.

Cerda-Jara CA, Kim S, Thomas G, Farsi Z, Zolotarov G, Georgii E, Woehler A, **Piwecka M**, Rajewsky N#. *EMBO Reports* 2024; 2024 Jun 3. doi: 10.1038/s44319-024-00168-9. Online ahead of print. https://www.embopress.org/doi/full/10.1038/s44319-024-00168-9
IF 2021 = 9.071; MNiSW 2023 = 140; MNiSW 2024 = 140; Cyt=0

P8
ciRS-7 and miR-7 regulate ischemia-induced neuronal death via glutamatergic signaling.

Scoyni F#, Sitnikova V, Giudice L, Korhonen P, Trevisan DM, Hernandez de Sande A, Gomez-Budia M, Giniatullina R, Ugidos IF, Dhungana H, Pistono C, Korvenlaita N, Välimäki NN, Kangas SM, Hiltunen AE, Gribchenko E, Kaikkonen-Määttä MU, Koistinaho J, Ylä-Herttuala S, Hinttala R, Venø MT, Su J, Stoffel M, Schaefer A, Rajewsky N, Kjems J, LaPierre MP, **Piwecka M**, Jolkkonen J, Giniatullin R, Hansen TB, Malm T#. *Cell Reports* 2024; 43(3):113862. https://doi.org/10.1016/j.celrep.2024.113862
IF 2023 = 8.8; MNiSW 2023 = 200; MNiSW 2024 = 200; Cyt=1

P9
Editorial: RNA at a breaking point? Cytoplasmic cleavage and other post-transcriptional RNA processing in neurodevelopment and disease.

Piwecka M[#], Luisier R, Andreassi C. *Frontiers in Molecular Neuroscie*nce 2023; 16:1214853. https://www.frontiersin.org/articles/10.3389/fnmol.2023.1214853/full, eCollection 2023.
IF 2022 = 5.639; MNiSW 2021 = 140; MNiSW 2024 = 140; Cyt=0

P10
RNA regulation in brain function and disease 2022 (NeuroRNA): A conference report.

Piwecka M[#], Fiszer A, Rolle K, Olejniczak M. *Frontiers in Molecular Neuroscience* 2023, 16:1133209. https://www.frontiersin.org/articles/10.3389/fnmol.2023.1133209/full
IF 2022 = 5.639; MNiSW 2021 = 140; MNiSW 2024 = 140; Cyt=0

P11
Analyses of circRNA Expression throughout the Light-Dark Cycle Reveal a Strong Regulation of Cdr1as, Associated with Light Entrainment in the SCN.

Ivanov A[#], Mattei D, Radscheit K, Compagnion AC, Pett JP, Herzel H, Paolicelli RC, **Piwecka M**, Meyer U, Beule D. *International Journal of Molecular Sciences* 2022, 23(20):12347. https://www.mdpi.com/1422-0067/23/20/12347
IF 2021 =5.6; MNiSW 2021 = 140; MNiSW 2024 = 140; Cyt=4

P12
RNA-protein interactomes as invaluable resources to study RNA viruses: Insights from SARS CoV-2 studies.

Koliński M, Kałużna E, **Piwecka M**[#]. *Wiley Interdisciplinary Reviews: RNA* 2022, 13(6):e1727. https://wires.onlinelibrary.wiley.com/doi/10.1002/wrna.1727
IF 2021 = 9.349; MNiSW 2021 = 140; MNiSW 2024 = 140; Cyt=3

P13
Inhibition of miR-21 in glioma cells using catalytic nucleic acids.

Belter A*, Rolle K*, **Piwecka M***, Fedoruk-Wyszomirska A, Naskręt-Barciszewska MZ, Barciszewski J[#]. *Scientific Reports* 2016, 6:24516. https://www.nature.com/articles/srep24516
IF 2015 = 5.228; MNiSW 2015 = 40; MNiSW 2024 = 140; Cyt=26

P14
The Sequence and Structure Determine the Function of Mature Human miRNAs.

Rolle K*, **Piwecka M***, Belter A*, Wawrzyniak D, Jeleniewicz J, Barciszewska MZ, Barciszewski J[#]. *PLoS One* 2016; 11(3):e0151246. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0151246
IF 2015 = 3.234; MNiSW 2015 = 40; MNiSW 2024 = 100; Cyt=36

P15
Hyperosmia, ectrodactyly, mild intellectual disability, and other defects in a male patient with an X-linked partial microduplication and overexpression of the KAL1 gene.

Sowińska-Seidler A, **Piwecka M**, Olech E, Socha M, Latos-Bieleńska A, Jamsheer A[#]. *Journal of Applied Genetics* 2015; 56(2):177-84. https://link.springer.com/article/10.1007%2Fs13353-014-0252-7
IF 2014 = 1.67; MNiSW 2014 = 20; MNiSW 2024 = 140; Cyt=9

P16
Mature miRNAs form secondary structure, which suggests their function beyond RISC.

Belter A, Gudanis D, Rolle K, **Piwecka M**, Gdaniec Z, Naskręt-Barciszewska MZ, Barciszewski J[#]. *PLoS One* 2014; 9(11):e113848.
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0113848
IF 2013 = 3.534; MNiSW 2013 = 40; MNiSW 2024 = 100; Cyt = 37

P17
Nucleic acid-based technologies in therapy of malignant gliomas.

Piwecka M, Rolle K, Wyszko E, Żukiel R, Nowak S, Barciszewska MZ, Barciszewski J[#]. *Current Pharmaceutical Biotechnology* 2011; 12(11):1805-22. http://www.eurekaselect.com/75835/article
IF 2010 = 3.455; MNiSW 2010 = 20; MNiSW 2024 = 100; Cyt=12

P18
Promising human brain tumors therapy with interference RNA intervention (iRNAi).

Rolle K, Nowak S, Wyszko E, **Nowak M**, Zukiel R, Piestrzeniewicz R, Gawronska I, Barciszewska MZ, Barciszewski J[#]. *Cancer Biology & Therapy* 2010; 9(5):396-406. https://www.tandfonline.com/doi/pdf/10.4161/cbt.9.5.10958
IF 2009 = 2.305; MNiSW 2009 = 27; MNiSW 2024 = 100; Cyt=45

P19
A new and efficient method for inhibition of RNA viruses by DNA interference.

Nowak M, Wyszko E, Fedoruk-Wyszomirska A, Pospieszny H, Barciszewska MZ, Barciszewski J. *FEBS Journal* 2009; 276(16):4372-80.
https://febs.onlinelibrary.wiley.com/doi/abs/10.1111/j.1742-4658.2009.07145.x
IF 2008 = 3.321; MNiSW 2008 = 20; MNiSW 2024 = 100; Cyt=6

P20
A multivariate analysis of patients with brain tumors treated with ATN-RNA.

Wyszko E, Rolle K, Nowak S, Zukiel R, **Nowak M**, Piestrzeniewicz R, Gawrońska I, Barciszewska MZ, Barciszewski J. *Acta Poloniae Pharmaceutica* 2008; 65(6):677-84. http://www.ptfarm.pl/pub/File/Acta_Poloniae/2008/6/677.pdf
IF 2007 = 0.234; MNiSW 2007 = 4; MNiSW 2024 = 100; Cyt=27

P21
Leadzyme formed in vivo interferes with tobacco mosaic virus infection in Nicotiana tabacum.

Wyszko E, **Nowak M**, Pospieszny H, Szymanski M, Pas J, Barciszewska MZ, Barciszewski J. *FEBS Journal* 2006; 273(22):5022-31. https://febs.onlinelibrary.wiley.com/doi/abs/10.1111/j.1742-4658.2006.05497.x
IF 2005 = 3.304; MNiSW 2005 = 24; MNiSW 2024 = 100; Cyt=3

The Applicant's name is in bold font.
[#]corresponding author;
[*]authors who equally contributed to the work;
Cyt – number of citations.

Scientometric data on the habilitation achievement was provided based on *Web of Science Core Collection*, record from 16/08/2024.

Total *Impact Factor* from the year preceding the publication = **73.883**

Total number of MNiSW points from the year preceding the publication = **1275**

Total number of MNiSW points according to the newest list published 05/01/2024 = **1980**

Total number of citations = **209**

Additional information about the habilitation candidate's **publications in journals other than those included in the JCR database** can be found in Appendix 4 (*List of scientific achievements*, p. 6).

5.2. LIST OF PATENTS

I am a co-author of two international patents:

- European Patent, Patent No. EP2978847 B1; Date of publication: 15.02.2017.

HAMMERHEAD RIBOZYMES TARGETING MIR-21

Inventors: NASKRET-BARCISZEWSKA, Miroslawa (PL); BELTER, Agnieszka (PL); ROLLE, Katarzyna (PL); **PIWECKA, Monika** (PL); SOSINSKA, Patrycja (PL); FEDORUK-WYSZOMIRSKA, Agnieszka (PL).

- United States Patent, Patent No.: US 8,404,660 B2; Date of Patent: Mar. 26, 2013.

METHOD OF OBTAINING OF 4-N-FURFURYLCYTOSINE AND/OR ITS DERIVATIVES, AN ANTI-AGING COMPOSITION AND USE OF 4-N-FURFURYLCYTOSINE AND/OR ITS DERIVATIVES IN THE MANUFACTURE OF ANTI-AGING COMPOSITION

Inventors: Jan Barclszewskl, Poznan (PL); Wojciech T. Markiewicz, Poznan (PL); Eliza Wyszko, Poznan (PL); Maria Markiewicz, Poznan (PL); **Monika Nowak, Kostrzyn (PL)**; Katarzyna Rolle, Kamionki (PL); Ewelina Adamska, Kamien Pomorski (PL); Marcin K. Chmielewski, Poznan (PL).

5.3. LIST OF SCIENTIFIC COLLABORATIONS

Below I present a list of past and current collaborations with scientists and/or research groups from Poland and abroad.

PAST:

Prof. Henryk Pospieszny - Institute of Plant Protection, national research Institute, Poznan, Poland.

Prof. Wojciech T. Markiewicz – IBCH PAS, Poznan, Poland.

Dr. Anna Maria Barciszewska, Dr. Rafał Piestrzeniewicz, Prof. Ryszard Żukiel, Prof. Stanisław Nowak – Department and Clinic of Neurosurgery and Neurotraumatology, Medical University of Karol Marcinkowski in Poznań, Poland.

Dr. Thorsten Trimbuch, Dr. Pascal Fenske, Prof. Christian Rosenmund - Department of Neurophysiology, NeuroCure Cluster of Excellence, Charité-Universitätsmedizin, Berlin, Germany.

Dr. Ralf Kühn - Transgenic Core Facility, Max Delbrück Center for Molecular Medicine, Berlin, Geramny.

Dr. Luis R. Hernandez-Miranda, Prof. Carmen Birchmeier - Laboratory for Developmental Biology and Signal Transduction, Max Delbrück Center for Molecular Medicine, Berlin, Germany.

Dr. Flavia Syconi, Prof. Tarja Malm - Neuroinflammation Research Group, University of Eastern Finland (UEF), Kuopio, Finland.

CURRENTLY:

Dr. Michał Szcześniak – Institute of Human Biology and Evolution, Adam Mickiewicz University in Poznan, Poland.

Dr. Agnieszka Rybak-Wolf – Organoid Core Facility, Max Delbrück Center for Molecular Medicine, Berlin, Germany.

Dr. Remigiusz Serwa - The International Institute of Molecular Mechanisms and Machines Polish Academy of Sciences, Warsaw, Poland.

Dr. Luiza Stanaszek - NeuroRepair Department, Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland.

Dr. Andranik Ivanov, **Prof. Dieter Beule** - The Core Unit Bioinformatics (CUBI), Berlin Institute of Health (BIH), Charité–Universitätsmedizin Berlin, Berlin, Germany.

Dr. Anne-Claire Compagnion, Prof. Rosa Chiara Paolicelli - Department of Biomedical Sciences, University of Lausanne, Lausanne, Switzerland.

Dr. Fanny Langlet - Department of Biomedical Sciences, University of Lausanne, Lausanne, Switzerland**.**

Dr. Cledi A. Cerda Jara, **Prof. Nikolaus Rajewsky** - MDC, Berlin, Niemcy.

Dr med. Norbert Wąsik, Prof. Włodzimierz Liebert - Department and Clinic of Neurosurgery and Neurotraumatology, Medical University of Karol Marcinkowski in Poznań, Poland.

Dr Krzysztof Brzeziński, Prof. IBCH PAS in Poznan, Poland.

Dr Katarzyna Rolle, Prof. IBCH PAS in Poznan, Poland.

6. INFORMATION ON TEACHING, ORGANIZATIONAL AND SCIENCE PROMOTION ACHIEVEMENTS

6.1. TEACHING ACHIEVEMENTS

- In the years 2022-2024 – member of the committee conducting mid-term evaluations of doctoral students from the Poznań Doctoral School of Institutes of the Polish Academy of Sciences (PSD IPAN).
- Lectures and seminars for PhD students:
  - 04-05.2021 – two lectures for PhD students from the Institute of Human Genetics PAS in Poznań within "*Epigenetics*" series. Title of the lectures: "*Circular RNAs (circRNAs) – functional significance and methodologies for their identification*", "*PIWI-interacting RNAs (piRNAs) - functions in genome stability and transmission of epigenetic information*", Poznań, Poland.
  - 01.2024 –a lecture for PhD students from the Institute of Human Genetics PAS in Poznań within "*Genetics of Human Development*" series. Title: "*microRNAs in development*", Poznań, Poland.
  - 03-04.2024 – two seminars for PhD students from PSD PAN on the subject of spatial RNA sequencing. Title: "*(A few words about) Spatial Transcriptomics*", Poznań, Poland.
- Detailed information on the **scientific supervision** provided by the habilitation candidate during the preparation of **doctoral theses** (n=3), **Master's theses** (n=2), **Bachelor's theses** (n=2) and student internships (n=15) can be found in **Appendix no. 4** (*List of scientific achievements*, pp. 16-18).

6.2. ORGANIZATIONAL AND SCIENCE POPULARIZATION ACHIEVEMENTS

- Workshops for high school students from a biology and chemistry class of STO School in Szczecinek, 31.03.2023, Poznań, Poland, **organizer**.
- Scientific editing of the Polish edition of the book entitled "The Autobiography of a Transgender Scientist" written by Ben Baress, 2021-2022; Publisher: Scientific Publishing Center of the Institute of Bioorganic Chemistry of the Polish Academy of Sciences, ISBN 978-83-7712-029-3, **scientific editor**.
- Lecture at the Brain Week in Poznań 2022 during the international Brain Awareness Week, title: "*Brain cell heterogeneity*", 14/02/2022, Poznań, Poland, **presenter**.
- Preparation of an information brochure for foreigners undertaking or planning to undertake scientific work in Poznań, a project under the NAWA "*Welcome to Poland*" program, 2021, **co-organizer and co-author**.
- Presentation at the Smart Development Forum, Open Stage: Scientists of the Future - a Prelude to Innovation, Life Science, title of the talk: "*The Devil is in the Details - What Do Circular RNAs Do in the Brain*?", 27/09/2021, Toruń, Poland, **forum participant and presenter**.
- Lecture for the Poznań doctoral student community within the series "*Science in Poznan*" (pl. *Nauka po poznańsku*), title: "*Choose your postdoc wisely and have fun doing science! A brief story of my research path and some tips*.", 27/05/2021, online, **meeting leader** and **lecturer**.
- Long Night of Science in Berlin, 3x in 2017, 2018, 2019, **organizer and performer**.
- 5th edition of Open Lectures within the series *Meetings of Young People with Science in Poznań*, 12.04.2014, Poznań, organizer: Student Science Club Biosfera; function: **member of the Evaluating Committee**.
- Scientific support and preparation of an artistic installation entitled "*Cosmic Garden*", the author: Zbigniew Oksiuta, 2nd edition of Mediations Biennale, National Museum in Poznań, 11.09.-30.10.2010; **preparation of the installation**.
- Workshops for junior high school students as part of the educational project "*e-School - My Greater Poland*", 2012; **conducting workshops**.
- Poznań Night of Science - presentation "*See what genes look like*" and organization of workshops on DNA isolation, Poznań Night of Science, 25.09.2009, **organizer, performer**.

6.3. OTHER IMPORTANT ORGANIZATIONAL ACHIEVEMENTS

- Since November 2019, I have been leading the Department of Non-coding RNAs in IBCH PAS in Poznań;

- I was a co-organizer of the international **NeuroRNA Conference** "***RNA regulation in Brain Function and Disease***" (September 28-30, 2022) and two local scientific conferences in Poland. Detailed information on participation in the organizing and scientific committees of national and international conferences can be found in **Appendix No. 4** (*List of scientific achievements*, p. 12);
- I am a co-organizer of the *RNA Salon Poznań* lecture series as part of the RNA Society, 2020-present.

7. OTHER INFORMATION ON PROFESSIONAL CAREER

Detailed information about my scientific activity can be found in Appendix no. 4 (*List of scientific achievements*), **including**:

- a full list of published articles in scientific journals,
- a list of published conference presentations,
- a list of lectures and conference presentations (as a main presenting author and as a co-author);
- information on participation in the organizing and scientific committees of national or international conferences,
- information on participation in research projects as principal investigator and co-investigator;
- information on membership in international or national organizations and scientific societies;
- a list of courses and workshops completed in Poland and abroad;
- information on peer-review roles in scientific papers,
- information on participation in European programs;
- information on membership in editorial committees and scientific councils of journals;
- information on participation in teams assessing applications for research funding;
- awards and distinctions.

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MONIKA
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26.08.2024 16:06:40 [GMT+2]
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podpisem zaufanym