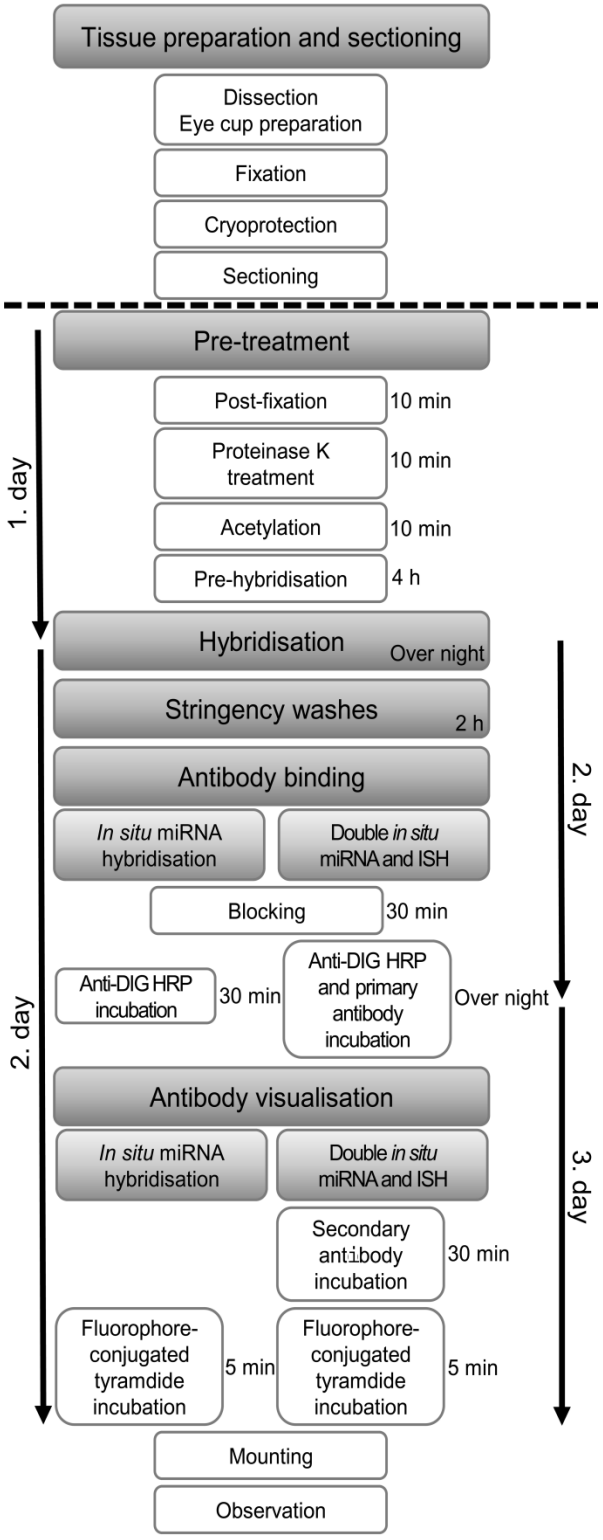
Background. Recently, non-coding RNAs especially microRNAs are deeply researched as crucial factors of the complex gene regulation network involved in generating retinal cell diversity (Sundermeier & Palczewski 2012; Xiang 2013). Now it is well accepted that retinal development, aging and some disorders have been connected to abnormal miRNAs expression such as diabetic retinopathy, retinitis pigmentosa, retinoblastoma and age-related macular degeneration (Andreeva and Cooper 2014). It is also important to know that studies on Dicer ablation animals demonstrated selective loss of miRNAs caused developmental changes. In conditional Dicer retinas the early born cell types such as retinal pigment epithelial cells (RPCs) were increased and prolonged, while late RPC markers including Sox9 and Ascl1 were not expressed and late-born cell types including rod and Müller cells were not generated (Gao 2010; Georgi & Reh 2010). Although structural development of the vertebrate retina has long been researched, knowledge on miRNA aspects is still very limited. Therefore ***in our original application we aimed at to describe (i) the small RNA profile of postnatal retina*** in Wistar WU albino rat by Ion-Torrent PGM sequencing technology, (ii) ***develop a new and reliable in situ hybridisation technology for miRNAs in retinal tissue*** that is suitable for combination with immunocytochemistry. This powerful technology provides the tool for characterisation of specific miRNAs in well-known retinal cell types during development. Further, (iii) ***two specific miRNAs (mir-9 and mir-23)*** were studied in detail regarding the time course of their expression, comparing images from insitu hibridasation, sequencing results and qPCR detection and (iv) ***their relation to possible target appearance*** during development.

MicroRNAs of the retina. Small RNA library construction from pooled retinal samples (N=3, in each age-group) were carried out according to the Ion Total RNA-Seq Kit v2 protocol (Revision E) with slight modification. Enzymatic reaction was performed in half reaction volume, while cleaning procedures were executed with the accurate final volume according to the protocol. Ion Torrent Suite Platform was used to trim the raw sequence data and remove any residual sequencing adapter fragments that remained on the 5' or 3' ends. Reads were mapped to the non-coding RNAs from ENSEMBL [Rnor_6.0 (GCA_000001895.4)] using TMAP algorithm. These aligned BAM (Binary Alignment Map) files were further processed in Galaxy Web-based platform (Afgan, 2018) via Cufflinks, Cuffmerge and Cuffdiff (Version 2.2.1.3) application. The expression level of individual transcripts was calculated using the number of reads per kilobase of the exon model per million mapped reads (RPKM) method. Pearson's correlation algorithms were used to assess the correlation between read counts per miRNA of biological replicates. Further ascertainment of the most informative miRNAs were based on statistical analysis in R Studio Software environment. DIANA miRPath 3.0 were used for miRNA target and pathway analysis [34]. Experimentally validated miRNA targets were identified using DIANA-TargetScan and the Genes Union option (an a posteriori method) was used for pathway analysis. The integrated Fischer's exact test followed by FDR adjustment were used for statistical analysis. Enriched pathways from the KEGG database were exported from the tool along with the corresponding FDR-corrected P values. ***The gene expression profiling data have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE159168.*** The publication of these detailed results are in progress. The expression profile of miRNAs could provide indication of their potential functions during development. We observed that although there was no obvious difference in the total number of miRNAs detected in retina during development process, the expression level of different miRNAs in retina was very dynamic over stages. There are some miRNAs that show constant high abundance during the development process such as mir-19, mir-101; mir-181, mir-183, mir-124 and let-7. While there are miRNAs that appear as most abundant only in the early stages such as mir-20a, mir-206, mir-133, mir-466, mir-1247 or mir-3582, others are characteristic with high abundance at later stages or increasing with development for example mir-29, mir-96, mir-125, mir-344 or mir-664. Validation of the sequencing results was done by using TaqMan® MicroRNA Assays. Isolated RNA from six retinas in each age-group were reverse-transcribed with TaqMan MicroRNA Reverse Transcription Kit. Negative controls (without template) were also established. Relative quantification was performed by the 2-ΔΔCT method and P21 was used as reference value. ***The most abundant miRNAs at each developmental***

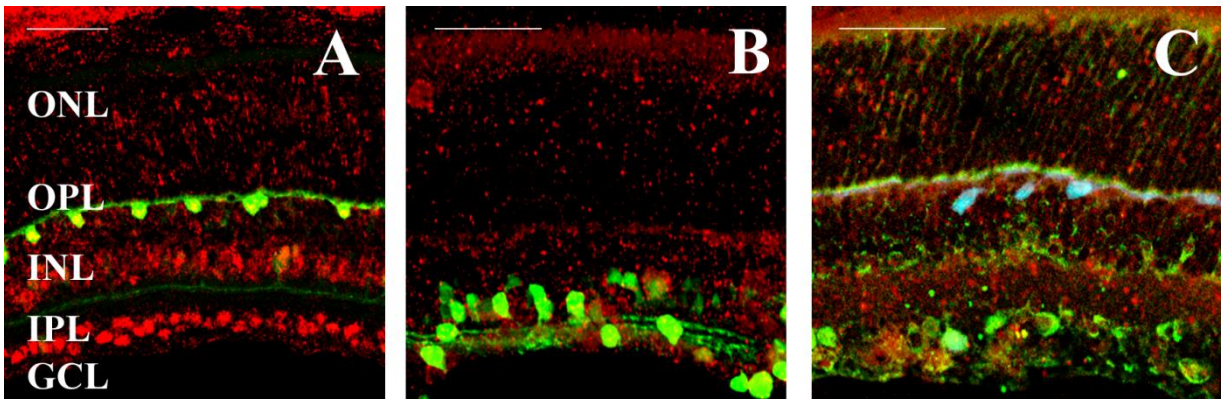
stage are collected in Table 1. These data could provide new insight into the postnatal retinogenesis and could lead to identification of new-potential miRNA candidates.

Development of a novel miRNA *in situ* hybridisation technology. In one of published paper we suggested the following protocol



This protocol was needed to standardise staining intensity and it is suitable for revealing microRNAs that are present in average or low quantity in the retinal cells. The unique aspects of this protocol lies

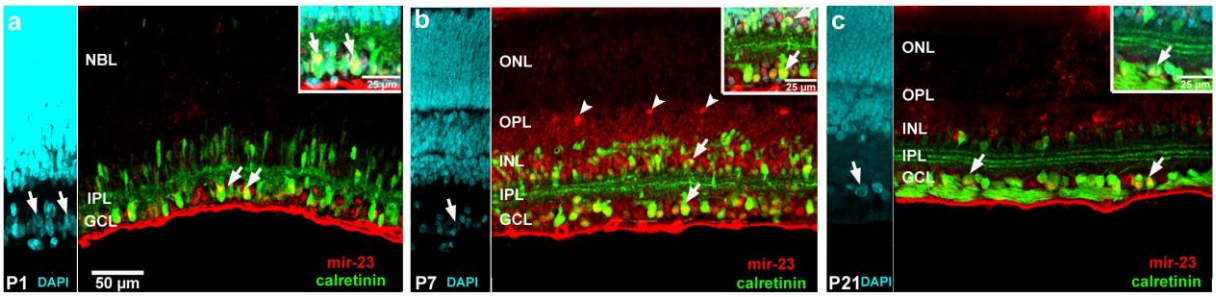
in the following points: (i) the concentration and duration of ***proteinase K treatment***, (ii) ***hybridisation temperature*** of microRNA probes and (iii) ***temperature of stringency washes***. Further improvements made it possible to combine our *in situ* hybridisation protocol with double-label immunofluorescence allowing for the simultaneous detection of microRNAs with high sensitivity and a neuronal cell marker and/or a synaptic marker protein. Thus the regulatory microRNA-s can be localised in an identified cell type along with its potential target protein. An example is hown below.



Co-detection of microRNAs (mir-9, mir-23), neuron-specific marker protein (calbindin D28k or calretinin) and synaptic marker protein (syntaxin-3), respectively. Abbreviation: GCL – ganglion cell layer; IPL – inner plexiform layer; INL – inner nuclear layer; OPL – outer plexiform layer; ONL – outer nuclear layer). (**A**) *In situ* hybridization detection of mir-9 (red – Cy3) showed specific co-labelling in all horizontal cells (green – Alexa Fluor 488; calbindin D28k, marked with arrows), while only a few amacrine cells were double labelled for mir-9 and calbindin. Cells in the GCL were labelled invariably for mir-9 only. (**B**) Dual ISH-IHC of mir-23 (red – Cy3) and calretinin (green – Alexa Fluor488) was detected mainly in the amacrine cells and presumed ganglion cell bodies in the GCL. (C) A representative image showing the *in situ* hybridisation signal (red: mir9 - Cy3) combined with double immunocytochemical labelling (blue: calbindin - Alexa Fluor405 and green: syntaxin-3 - Alexa Fluor 488)

With this methodology at hand we started to analyse the possible role of specific miRNAs in retinal cell development. MicroRNAs have a tissue-specific expression manner and contribute to the regulation of many physiological processes in the retina, including development and maturation. During retinal development, miRNAs are important key regulators in a variety of cellular processes and have a unique spatiotemporal expression pattern.

Role of mir-23 regulation in glutamic acid synthase (67kDa) profile development. We employed different publicly available miRNA-target interactions platforms to determinate experimentally validated miRNAs, which are potentially GAD1 specific (Miranda [mir-216a, mir-216b, mir-493]; mirTarbase, Miranda-[mir-23], Miranda [mir-542, mir-495]). This study aims to gain a deeper understanding of the molecular regulatory networks that contribute to the final expression pattern formation of the major GABA synthetizing enzyme GAD67 during the first three weeks of the postnatal retinal development. Postnatal expression patterns of GAD1 related miRNAs (mir-216a, mir-216b, mir-493; mir-23, mir-542, mir-495) were identified from the above mentioned datbases. Subsequently, we revealed these miRNAs and GAD1 mRNA postnatal expression patterns by qPCR measurements. Comparing the results of GAD1 qPCR with tendencies of its targeting miRNAs, we could classify these miRNAs into two groups: the first group with similar patterns of GAD1 expression (both mir-23 and mir-495 are lower at most examined time points compared to P21) and the second group with non-matching expression patterns (mir-542, mir-216a, mir-216b, mir-493. Consequently, we choose mir-23 for further analysis by in situ hybridization (ISH) in our investigation. The presence of mir-23 was low before P7, where horizontal cells showed remarkable GAD67 fluorescence. Strong labelling occurs in the GCL. To confirm that mir-23 signals belong to specific cell types, co-detection of mir-23 and calretinin was executed. Mir-23 showed co-labelling in some cells in the GCL (see below a-c) and the horizontal cells show mir-23 positivity at P7 (b).

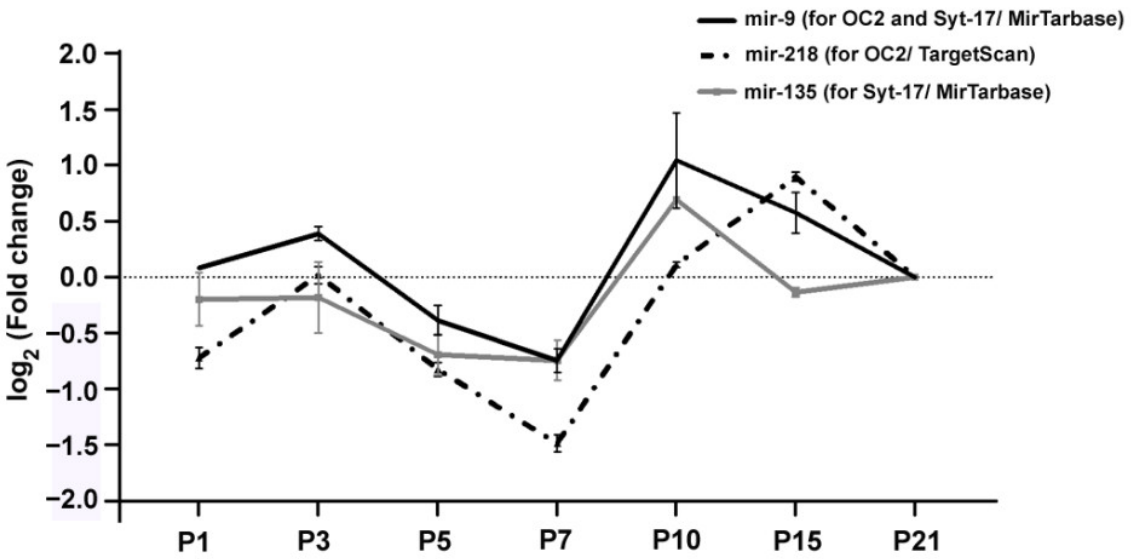


Representative images of mir-23 (red: TSA Cy 3) co-detection with calretinin (green: Alexa Fluor-488) at the indicated postnatal days (mir-23 with calretinin: (a)-P1; (b)-P7; (c)-P21). Inserted images demonstrate the overlap of ISH-dual IHC. Arrows indicated co-localizations in cells in the GCL and the INL, while non-colocalized cells (horizontal cells) were demonstrated by arrowheads. Scale bar: 50 µm. GCL - ganglion cell layer; IPL - inner plexiform layer; NBL - neuro blast layer; INL - inner nuclear layer; OPL - outer plexiform layer; ONL - outer nuclear layer.

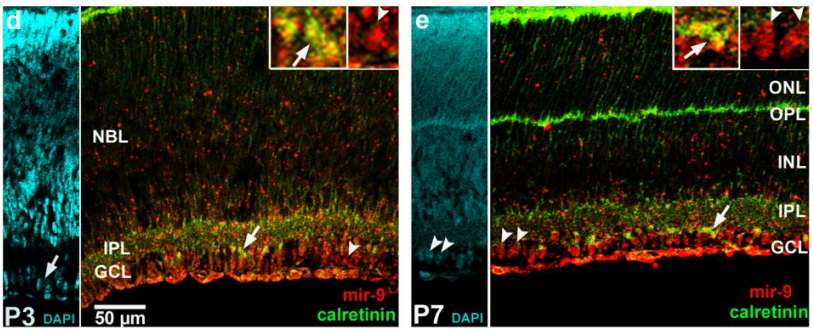
GAD67 is expressed in the retina during development, especially in the IPL and the GCL, and to a lesser extent, in the (OPL) and INL. Before P7, GAD67 expression was less prominent and some cells in the GCL appeared double-labelled (Figure 5 a1-c4), while at P7 both markers could be detected in horizontal cells. After P7, the GAD67 staining intensity gradually increased and showed elevated levels both at P10 and P15, then decreased by P21 in the examined retinas. In the last two timepoints, at P15 and P21, only a few amacrine cells were double labelled in the INL. At P10 and P21 we found double labelled presumed ganglion cells in the GCL. We comcluded that the regulatory function of miRNAs are vitally important for controlling molecular pathways of developmental processes (Hackler et al. 2010; La Torre et al. 2013). Previous microarray and in situ examinations revealed the spatiotemporal occurrence of several miRNAs in the retina and through their combined expression, miRNA-dependent changes have been described during sequential actions of retinal development. MicroRNAs contribute to the regulation of molecular processes on varying levels: for one, they contribute not only at posttranscriptional level (e.g. mRNA degradation, repression of translation; Bagga et al. 2016) where they could target several mRNA, and the same mRNA could be targeted by multiple miRNAs. Furthermore, they can also regulate expression at the level of transcription, where they mediate the activation or inhibition of the target gene transcription (Hahimoto et al. 2013; Huang et al. 2012). ***Our results highlight the significance of the transcriptional regulatory role of mir-23 expression through the induction of GAD1 transcription during postnatal retina development***. Our study focusing on this special miRNA regulatory mechanism shed some light on the shaping of the inhibitory network of the retina and stresses the need for continued investigation for a better understanding of the miRNA regulation of these process since these are of eminent importance in synaptogenesis and synapse specification.

Possible roles of mir-9 in postnatal retinal development. For similar reasons, another target of our investigation was mir-9. La Torre et al. (2010) described that mir-9 is a key regulator factor which takes part in the competence change of retinal progenitors during development, and shows increasing expression levels between the embryonic and postnatal periods. They found that the expression of three microRNAs (mir-125, let-7, mir-9) increased in progenitors during development, while through overexpression in normal retinas, these microRNAs cause acceleration in development (Arora et al. 2010). MicroRNA array results from ciliary epithelial stem cells in developing and adult retinas have shown that mir-9 is highly expressed in P4 retinas [9]. By molecular analysis, mir-9 was identified as a Müller glia-specific miRNA in the postnatal retina and detected in the middle part of the inner nuclear layer (INL), with an increasing level from P11 to adulthood [33,34]. Although prior studies have thoroughly investigated the role of mir-9 in embryonic retinal neurogenesis, little research has been conducted to reveal its quantity and distribution during postnatal development. Therefore we decided to gain deeper insight into molecular regulation networks during postnatal retinal development by characterizing mir-9's spatial and temporal expression with its potential two targets. We used the user-friendly and widely annotated miRNA-target interaction platforms

miRTarBase and TargetScan to reveal the experimentally validated targets of mir-9. For further analysis, OneCut2, a transcriptional factor and synaptotagmin-17, known as a synaptic physiology-related regulator, was selected. For better understanding the expression of these target proteins, we also searched for miRNAs (mir-135, mir-218) which had an effect on their expression in databases and from the literature. ***We decided to examine mir-9 with 3 methods parallelly: sequencing, qPCR and in situ hybridisation.*** We performed a miRNA-Seq study to get deeper insight into the gene expression regulation at the miRNA level of retinal late-born cell development (GSE159168). From this study, we took postnatal expression results for mir-9 and two other (OneCut2- and synaptotagmin-17-related) miRNAs (mir-135-synaptotagmin-17, mir-218-OneCut2). The result of this comparison is presented below:

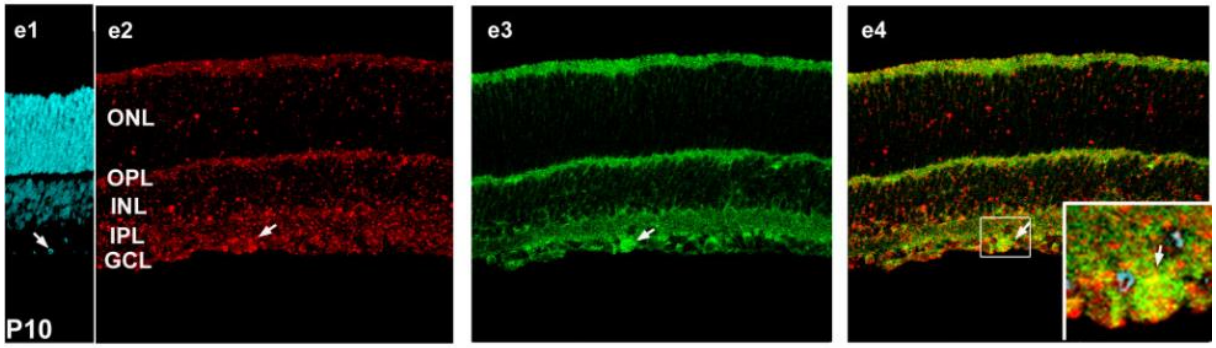


While we were expecting mir-9 to appear in certain cell types during development and this expectation was fully met (for example in several calretinin-positive amacrine cells, see below), there was no clear correlation between the potential target proteins and mir-9 in time.



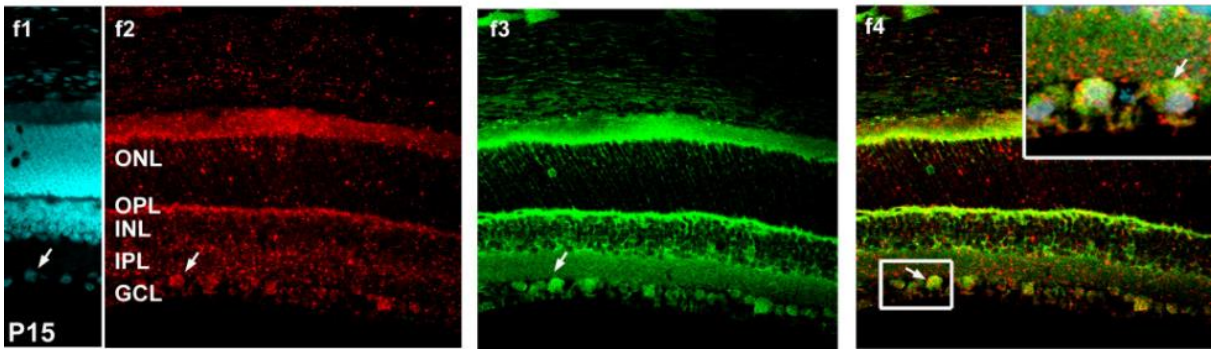
Calretinin and mir-9 postive amacrine cells at P3 and P7 (arrows). Single mir-9 labelled cells are marked with arrowheads.

One of the predicted target proteins, synaptotagmin 17, appeared int he retina as early as P1 and became quite strongly expressen by P10, only a fraction of these cells showed mir-9 positivity at the same time. We expected a strong correlation in time but our expectations were not met neither in this case, nor in case of Onecut protein. ***Syt 17 and mir-9 was colocalized in some large ganglion cells*** (see below). The other cells types showed either mir-9 or syt17 postivity at this stage of development.



A large ganglion cell (arrow) colocalizing mir-9 (red) and syt-17 (green)

A much stronger (but not 100%) correlation was seen in case of Onecut 2. Again, ganglion cells seemed most prominently expressing both markers.



Onecut 2 and mir-9 expressing ganglion cell (arrow) at P15 in the retina.

The analysis of the cellular co-localization of mir-9 with One-Cut2 and synaptotagmin-17 suggests that they are also strongly co-expressed, mainly in ganglion cells and less intensively in amacrine and horizontal cells. Furthermore, our results show that mir-9, mir-218 and mir-135 could act on these targets in a stage-dependent manner. A transcriptional regulatory role of mir-9 in case of OneCut2 and synaptotagmin-17 in the postnatal development of the rat retina can definitely be postulated. However the exact mexchanism is still to be determined.

Experiments still in progress. We are int he middleof investigating the effects of miR antagonists (antagomirs) in retinal pattern formation and synaptogenesis. Antagomir-9 was injected into the vitrous of 2-day-old, and 9 day old rats, just below the peak expression of mir-9. We shall investigate the effects of these ttreatments after 1 and 3 month survival. The 1 month survival samples have already been collected, we are waiting for the other grouop to start the experiments.

Other studies related to the wider context of retina morphogenesis, aging and diseases. As a grantee of in a previos round of support from the granting organization we have finished up and published several papers due to the continuous support. We have shown the protective role of a PARP nhibitor during ypoxia/reoxygenation-Induced retinal injury and the age-related alterations of cell-type specific marker proteins in albino Wistar rat retina. Besides, we attempted to lay down a foundation of a model in studying long-term effrect of neuropeptides on natural aging of the retina. The papers resulted from these continuing efforts are listed in our report.

Difficulties during the project. While my group tried to maintain a steady pace of work, I have to note here that one of my key collegue became a two-time mother during this period. She was away from the lab for over 2 years as she is away omn aternity leave today. Also, the COVID situation hindered us in 2020 and 2021, since the delivery of some consummables and experimental animals were delayed sometimes.

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Table 1. The top 20 most abundant miRNAs (RPMM) at each developmental stage.

P5	P7	P10	P15	P21
Mir376c	Mir376c	Mir106a	Mir106a	Mir376c
Mir106a	Mir106a	Mir376c	Mir376c	Mir106a
Mir206	Mir206	Mir206	Mir206	Mir206
Mir19a	Mir377	Mir377	Mir124-1	Mir124-1
Mir141	Mir124-1	Mir124-1	Mir129-1	Mir129-1
Mir143	Mir19a	Mir129-1	Mir377	Mir377
Mirlet7c-2	Mir143	Mir19a	Mir19a	Mir320a
Mir377	Mir129-1	let7c-2	Mir34b	Mir19a
Mir124-1	Mir320a	Mir141	let7c-2	Mir3571
Mir152	Mir152	Mir103a1	Mir298	Mir100
Mir129-1	Mirlet7c-2	Mir34b	Mir18a	let7c-2
Mir320a	Mir141	Mir18a	Mir141	Mir3570
Mir100	Mir-344b-2	Mir298	Mir103a1	Mir18a
Mir103a1	Mir103a1	Mir152	Mir144	Mir144
Mir3570	Mir100	Mir34a	Mir320a	Mirlet7b
Mir18a	Mir3570	Mir29b1	Mir222	Mir29b1
Mir34b	Mir18a	Mir143	Mir34a	Mir103a1
Mir298	Mir222	Mir320a	Mir29b1	Mir222
Mir344b-2	Mir29b1	Mir222	Mir152	Mir141