Reading the Early Signs

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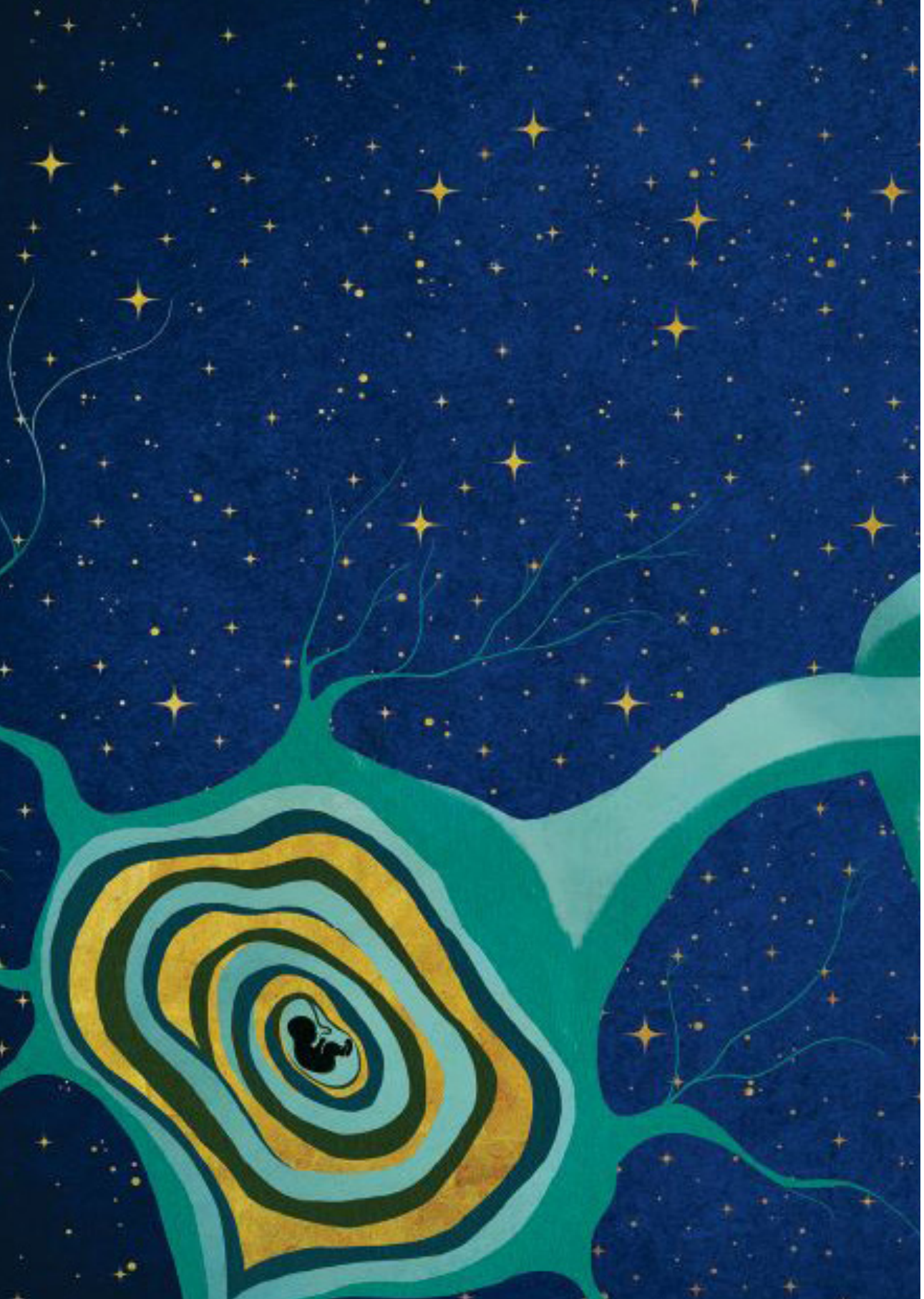
2020

document version
Publisher's PDF, also known as Version of record



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GENERAL DISCUSSION AND FUTURE DIRECTIONS

When Prof. Dr. Huda Zoghbi and her team in 1999 discovered that mutations in *MECP2* cause classical RTT, researchers in the field were convinced that this was the major key to finally understand the mechanisms behind RTT.[1] However, this task was more complicated than anticipated. Due to the regulatory interactions of MeCP2, mutations in *MECP2* affect hundreds of other genes, many different pathways, and different cell types in the human body.[2] Furthermore, as we know today, RTT is not only about the simple presence or absence of MeCP2, it is rather an alteration of a very defined dosing during the appropriate time points of development in specific brain regions.[3–5] Today, more than 20 years after the identification of *MECP2*-mutations being responsible for classical RTT, researchers are still trying to reach the initial goal. One hurdle in better understanding RTT and identifying therapeutic targets is the lack of representative disease models. Mouse models do not fully represent human pathology, partly caused by species differences. *Post mortem* tissues are providing valuable insights, but are not widely available and do not provide the opportunity to study the development of the disease course. New opportunities were given by the introduction of induced pluripotent stem cells (iPSC). IPSC technology is of special interest to those neurological disorders that are polygenic, caused by unknown genetic changes; hence they can only be properly studied in human tissue with patient genetic background. While RTT is a monogenetic disorder, MeCP2's regulatory functions are broad and depend on many other genetic and epigenetic factors, therefore making RTT also a good candidate for iPSC-based approaches. Here we used RTT patient *post mortem* tissue and iPSC-based models to provide insight into changes occurring during early neural development.

In **Chapter 2** we aimed to confirm findings of previous hypotheses, suggesting that changed Potassium-Chloride-Co-Transporter 2 (KCC2) levels result in affected chloride homeostasis and underlie the phenotype of neuronal maturation delay. Therefore, we studied KCC2 levels in different brain regions in human *post mortem* tissue from RTT patients and age-matched controls.[6–8] Interestingly, we were able to verify a decrease of *KCC2* transcript levels in all tested RTT samples and observed a trend towards reduced KCC2 protein levels. Looking more in-depth, we showed that the KCC2a isoform specifically seems to be affected, warranting further study.

To develop a patient iPSC-based *in vitro* RTT model that disregards the patient genetic background potentially contributing to cellular phenotypes, we presented a new method to generate isogenic controls in **Chapter 3**. We show that MecP2 is already expressed at the iPSC stage. We developed an easily implementable method to generate an isogenic control for X-chromosomal linked disorders. However, not all patient fibroblast lines allowed robust generation of iPSC lines presenting X-chromosomal inactivation of both the healthy and mutated allele.

To further identify whether our iPSC-based model represents the specific RTT phenotype, in **Chapter 4** we generated iPSC-derived neuronal cultures from RTT and control iPSCs and studied early synchronous network activity known to be associated with KCC2 expression in the brain.[9,10] Here, we identified a delay in activity appearance and a reduced frequency of synchronous activity in RTT cultures. The neuronal differentiation protocol allowed the study of network development. However, this protocol represents not all network behaviour existing in the brain, nor includes other factors, such glia components that earlier showed to affect RTT phenotypes. Future studies could benefit from recently presented differentiation assays to study more complex neuron-glia interactions likely involved in RTT phenotypes.

To investigate at which time point developmental impairment can be measured, in **Chapter 5** we performed mass spectrometry on developing iPSC-derived neuroepithelial stem (NES) cells from RTT patients and isogenic controls**.** We showed that already three days after neuronal induction, MeCP2 deficiency affects expression of genes involved in different cellular pathways. The identified alterations were associated with cellular pathways which are known to be altered in RTT patient cells including 'cell-cell adhesion', 'actin cytoskeleton organization' and 'neuronal stem cell population maintenance' but also morphological alterations typical for RTT neurons, such as 'dendrite morphogenesis', 'axon guidance' and 'positive regulation of synapse assembly'. To further select key mechanisms, further studies would benefit from larger sample sizes and the inclusion of RTT patient lines harbouring other genetic defects. Nevertheless, these results could point to earlier suggestions that AKT-mTOR pathway dysregulation is involved. Together, based on findings in *in vitro* human model systems and *in post mortem human tissue*, e.g. changes associated with mechanisms involved in the maturation of cellular and neuronal network development, suggest that brain abnormalities take place much earlier than clinical symptoms indicate. Our findings provide insight into altered basic mechanisms being involved in neuronal development, which potentially lead to the typical RTT phenotype. Better insight into these pathways in combination with early RTT diagnostics by genetic testing could therefore be highly beneficial to support neonatal brain development in patients.

THE MODEL

The iPSC based Rett Model

To investigate RTT, a multitude of *in vitro* studies was performed based on patient-derived induced pluripotent stem cell (iPSC) models.[11–14] The advantages of iPSCs are their human origin, which abolishes translational issues and their patient-specific genetic background. Furthermore, research on iPSCs involves the opportunity to investigate cellular changes during early developmental stages, even before the onset of functional and clinical phenotypes.[11,15,16] As MeCP2 influences the expression of hundreds of genes, investigations of alterations require precise measurements and reliable controls to find multifaceted interactions and complex disease pathways.[2,17] *MECP2* is located on the X-Chromosome, therefore female RTT patients show mosaicism of affected and unaffected cells depending on the random X-chromosomal inactivation.[18,19] This mosaicism provides the opportunity to generate isogenic controls as described in Chapter 3, which gives the possibility to detect even minor alterations in the corresponding diseased line that are usually covered by sample variability.[20] However, the investigation of pure disease lines corresponds more to a phenotype observed in male RTT patients. To depict the *in vivo* situation of female patients, this model gives the possibility to investigate phenotypes in controlled mixtures of affected and unaffected cells. This is of special interest as mosaicism in RTT patients does have an effect on disease severity.[21], Although, we did not study this aspect, it could be interesting e.g. to draw conclusions about thresholds needed to provoke certain phenotypes.[22] In conclusion, we successfully generated RTT and isogenic control iPSC lines. However, we still encountered challenges in controlling x-chromosomal skewing making this method challenging for some cell lines.

Heavily skewed fibroblast lines made a selection for cells having either an X-chromosomal inactivation of healthy or mutated allele complicated. When we started our studies in 2014, several studies already investigated iPSC-derived RTT neurons. However, due to X-chromosomal skewing, they used a mixed population of affected and unaffected cells or reported issues in generating iPSC RTT lines expressing mutated *MECP2*.[12,23] Isogenic controls were mostly generated by chance as viral reprogramming approaches usually lead to X-chromosomal reactivation (XCR) in iPSCs followed by a random inactivation pattern. Cloning of iPSC lines was further challenged by the lack of tools to perform single cell passaging in iPSCs. In chapter 3, we represented a new approach to generate RTT lines and isogenic controls by sorting fibroblasts followed by an episomal reprogramming approach, not inducing XCR. This approach was thought to present an easier and less cost intensive strategy to study RTT and control cells with the same genetic background. Nevertheless, based on initial X-chromosomal skewing of fibroblast lines, cloning and identification of both, RTT and isogenic control fibroblasts appeared challenging. Single cell seeding actions were dependent on the survival rate of the seeded cells in combination with the ratio of affected and unaffected cells. A heavily skewed fibroblast line therefore required several passaging and expansion steps before enough RTT and isogenic control fibroblasts were generated for reprogramming. Besides X-chromosomal skewing, age and expansion potential of a patient line are crucial for the successful cloning. One patient line used in chapter 4, showed 95-98% of the fibroblasts expressing mutated *MECP2,* but failed in generating isogenic controls suitable for reprogramming within the timeframe. Which is why we needed to eventually use additional healthy controls in that specific chapter. Nevertheless, if fibroblast lines are not senescent, all lines could potentially be created via the presented method, providing an easily implementable method to generate isogenic controls for X-chromosomal linked disorders.

The iPSC research field evolved fast in recent years, also improving culture methods to maintain iPSC products in a pluripotent state. Previous culture methods did not allow single cell passing, as that would push pluripotent stem cells towards apoptosis or spontaneous differentiation. However, newly commercially developed stem cell media and matrices, such as laminin-521, do support ESC and iPSC-based culture better. This includes single cell passaging of iPSC lines, which more recently became a robust procedure, making cloning activities possible at the iPSC level. Nowadays, instead of selecting fibroblasts for specific X-chromosomal states, iPSC cultures could undergo single cell selection. This would overcome issues with aging fibroblasts and ensure the establishment of proliferating cell lines.[24] Nevertheless, this will still not overcome the issues of heavily skewed lines completely. Altogether, improved cultivation conditions have made iPSC-based studies more flexible and have reduced some labour-intensive steps in protocols, which provides new opportunities to generate disease models.

Likewise, the field of gene editing including CRISPR/Cas9 technologies advanced quickly in recent years. This also provided new ways to modify genes and correct mutations. As shown recently, the usage of CRISPR/Cas9 technology allows researches not only to introduce a common *MECP2* mutation (*MECP2*^{R270X}) into a human control iPSC line, but also to correct this mutation with a similar CRISPR/Cas9 approach.[25] This technique, therefore, opens new possibilities with regards to sample availability, as now *MECP2* mutations can be corrected in RTT iPSC lines, but also introduced into control iPSC lines. This could additionally help to compare phenotypes of different *MECP2* mutations and give insight into how the patient genetic background contributes to this.[26] Nevertheless, CRISPR/Cas9 also requires knowledge in viral transfection of iPSC lines, which due to their high proliferation rate can be challenging. Altogether, it showed to be a powerful

method for the generation of isogenic controls from RTT patients and will evolve further in the coming years.

The method described in chapter 3 has its limitations regarding the cloning of fibroblasts that have a limited time window to proliferate and are heavily skewed. Nevertheless, we present a system to generate isogenic lines that is easily implementable in labs not having advanced molecular biology and gene editing tools at hand. Furthermore, by using episomal vectors to reprogram we were able to maintain a part of the epigenome that might be of importance to the cellular phenotypes. Specifically for X-linked genetic disorders as RTT, our model is sufficient and easy to implement to generate RTT iPSC lines and isogenic controls.

The neuronal model and its limitation

Together with the rapid development of the iPSC field, the number of differentiation protocols increased within the last years. Differentiations into neuronal cell types specifically benefited from this development. Many different protocols are currently available to differentiate iPSCs into different types of neurons and are more or less suitable based on the scientific question. The choice of differentiation protocol is based on what aspect in the *in vivo* situation needs modelling. In Chapter 4 and 5 we investigated cortical neurons, the most affected neuronal cell type in RTT, by making use of an in-house established protocol that was invented to generate a mixed population of excitatory and inhibitory neurons.[27–30] However, this protocol can only reproduce parts of the *in vivo* situation as it does not represent different neuron-neuron and neuron-glia interactions.

MeCP2 is not exclusively expressed in neurons.[31–33] Only a few years ago, researchers explored the expression of MeCP2 in glia cells. Even though they showed that the expression levels in glia are much lower compared to neurons, they found that lack of functional MeCP2 in glial cell types contributes to the RTT phenotype.[34,35] As astrocytes and other glia cells are predominantly involved in neuronal development, these changes and their neuro-glia interactions, likely contribute to developmental changes in neuronal cells.[36–38] This was also indicated by a study from Williams *et al.*, who co-cultured iPSC-derived astrocytes from RTT patients together with wild type (WT) mouse neurons and observed a neuronal phenotype similar to MeCP2 lacking neurons.[39] Together with other studies, these results point towards an impaired support of neurons and neuronal development due to reduced secretion of growth factors or by disturbed neurotransmitter homeostasis from MeCP2 lacking astrocytes.[31,40,41] Additionally, the contribution of other glial cells, namely oligodendrocytes and microglia,

has been discussed. Oligodendrocyte contribution is mainly proposed due to the phenotypic alterations in RTT patient's brain tissue and reduction of white matter.[42–44] The lack of MeCP2 is known to alter protein expression in these glia cells, which most likely leads to an impaired myelination of neurons.[45] This was further supported by findings using conditional MeCP2 knockdown in mouse oligodendrocytes.[46] These mice displayed milder but similar symptoms to other RTT mouse models, which supported the assumption of oligodendrocytes playing at least a minor role with regards to a disease phenotype. Besides astrocytes and oligodendrocytes, microglia were shown to express MeCP2 as well. Here, researchers suspect inflammatory processes and impaired synaptic pruning to play a role in RTT.[47–49] Taking the contribution of all three glia cell types into consideration, our *in vitro* model used in chapter 4 and 5 depicts a limited picture of the disease mechanisms. Future studies would benefit from including other cell types such as different glial cells to their experiments. However, these different cell types should also not be investigated in monocultures. To properly study, how MeCP2 lacking glia cells influence neuronal development, i.e. neuron-glia interactions, we suggest further studies in more complex co-culture systems.

In recent years, co-culture models became important to study cell-cell interactions and provide a better insight into brain physiology. To understand how different cell types affect each other, different co-culture models have been invented. The most basic approach is the combination of two different cell types as e.g. neurons and astrocytes. As described above, this has already been used to investigate the influence of MeCP2 lacking astrocytes on WT neurons.[39] While the co-culturing of two cell types might still be a feasible approach, this model is still highly artificial, lacking the input of other cell types. A possible approach to consider several different cell types could be the differentiation of brain organoids, also called cerebral spheres or spheroids.[50] The increasing complexity of iPSC-derived neuronal models including more than two cell types is highly limited in regular 2D cell culture. Due to oversimplification of actual physiological conditions, cell growth and cell-cell interactions are impaired.[51–53] However, organoids allow cells to develop in a three-dimensional (3D) approach, which is more similar to the *in vivo* conditions.[54] In several studies, neurons showed more complex branching and more closely resembled primary neurons when grown in 3D.[53,55] Also support of glia cells seems to be increased in these cultures.[56,57] For this reason, 3D-cultured neurons can be kept in culture for a prolonged period of time, overcoming rather early apoptosis as seen in 2D, which often occurs after cultivation periods of more than 50-60 days.[58] This prolonged cultivation time leads to neurons presented with significantly more synapses, spine-like structures and increased network connectivity more similar to primary human neurons.[53,55,59] To display a full picture of neurodevelopmental impairments in

RTT, organoid-cultured neurons are of a specific benefit, as they could not only be investigated for a longer time period but also present interactions with other cell types. The use of 3D organoid cultures should, therefore, be considered for future studies. Nevertheless, in comparison to our *in vitro* model, 3D organoids are complex and scientific conclusions require elaborative analysis.

Studying co-cultured neuronal cells to fully understand disease mechanisms behind RTT became an achievable approach due to the organoid 3D culture systems. However, organoid differentiation protocols have their drawbacks in reproducibility and analysis comes with challenges. The composition of organoids can often not clearly be revealed due to the occasional development of different brain areas, caused by size differences and non-standardized starting populations. Due to a development of mixed cell populations, cell-cell interactions can also have major impact on phenotypes in organoids.[60–62] This makes it so far challenging to generate identical organoids for comparisons between patient and control cells. Even though studies show similar RNA expression profiles between entire organoids, alterations due to the development of different brain areas within the 3D structure can be identified.[63] Especially for electrophysiological measurements, this can be an issue. As early oscillations described in Chapter 4, are known to be different depending on the investigated brain area, a comparison of two independent organoids requires specific markers to determine the analysed location.[64] So, analysing neuronal activities in 3D structures, comparing individual organoids and batch effects are still problematic. However, as the field of organoid culturing is evolving quickly, protocols to generate and analyse more defined and comparable organoids become slowly available.[65–68] Therefore, organoids can play a major role in the investigation of RTT in near future.

The investigation of single cell types, so far, still remains the gold standard to develop human *in vitro* disease models. Even though, new protocols to generate reproducible organoids are becoming available, alterations in single cell types should be studied upfront. Especially, as in RTT hundreds of target genes are affected by the lack of MeCP2, cell type specific pathways need to be identified before cell-cell interactions can properly be analysed.[2] This does not only apply to a specific time point of neuronal maturation but needs to be investigated throughout development of neuronal cells, as RTT and its symptoms are associated with different development time points.[69–71] Therefore, our approach used in chapter 4 and 5 of this thesis, depicts a rather simple but highly conclusive model, which gives insight into specific alterations in cellular pathways during defined developmental stages. To be able to understand the complexity of RTT, we suggest the investigation of neurons and glia (e.g. astrocytes, oligodendrocytes and

microglia) in mono-cultures. With proteomic approaches and Gene Ontology (GO) term analysis as described in chapter 5, insights into altered pathways in all of these different cell types in parallel might already show a certain degree of overlap and therefore, allowing to hypothesise how different cell types in RTT affect each other. This will help to further analyse potential co-culture systems and draw proper conclusions. The use of cortical neuron cultures to investigate RTT might therefore not be a comprehensive approach. However, it builds the basis for more complex experimental setups and co-culture systems.

CELLULAR AND MOLECULAR MECHANISMS UNDERLYING RTT

KCC2 level changes underlying delayed neuronal network development

In this thesis, we found decreased KCC2 levels in post-mortem brain tissue of RTT patients, delayed appearance of synchronous activity and reduced frequency in spiking behaviour of developing neurons in iPSC-based cultures. These findings are in line with earlier studies investigating KCC2 levels in human tissue and iPSC-based *in vitro* models.[7,11,72,73] In chapter 2, we showed that KCC2 levels are decreased within the cortex and hippocampus of RTT patients. This decrease was observed in all brain areas. As KCC2 plays a major role in chloride homeostasis, we suggested that functional impairments observed in RTT neurons are caused by an insufficient reduction of intracellular chloride during development. Our findings in Chapter 4, supported this hypothesis, as we observed the impaired appearance of synchronous activity in RTT neurons which is responsible for the GABAergic shift and associated with KCC2 expression levels.[9] We further suggested, that reduced neuronal activity leads to an impaired KCC2 expression, which subsequently causes disturbed chloride homeostasis and an insufficient GABAergic shift. As this process is essential for synaptogenesis, our hypothesis explains the neuronal phenotype in RTT, showing reduced dendritic spines and synapses.[11,74,75] The prevailing theory about the cellular changes leading to the clinical presentation, describes that decreased implementation of glutamatergic synapses and an incomplete GABAergic shift, consequently leading to E/I imbalances underlying the RTT phenotype.[76] Therefore, our findings support earlier hypotheses that chloride homeostasis and network developmental changes contribute to clinical presentation of RTT.

When we investigated the two existing isoforms of KCC2, namely KCC2a and KCC2b, we showed that in particular KCC2a was significantly lower expressed in RTT patients.[77] This was surprising, as KCC2a is not the dominant isoform in the cortex. Nevertheless, KCC2a is associated with the development of functional respiration in mammals.[78,79] As one main symptom in RTT is the disturbance of respiratory mechanisms causing

severe breathing issues, our results suggest KCC2a involvement behind this symptom.[80] To confirm this hypothesis, further validation is required. Therefore, we recommend the investigation of additional brain areas responsible for respiration such as the brain stem and more specifically the pons and medulla oblongata.[78,81] This might give new insight about respiratory impairments in RTT. However, as we also observed a reduction in KCC2b levels, we suggest re-evaluating our findings and include additional patient samples. Even though, our results did not reach statistical significance, an increased sample size could improve statistical power. As our findings indicate a reduction in KCC2 levels throughout all brain regions, it is likely that also KCC2b levels are altered.

Molecular changes start at early developmental stages

Already at the very early stages of neuronal stem cell development, we were capable of identifying protein level alterations. We already found major changes in proteins being involved in metabolic processes but also cell death and apoptosis such as BCL2 Interacting Protein 3 (BNIP3) or NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) only three days after neuronal induction.[82–84] During further differentiation of iPSCs towards neuronal stem (NES) cells, the number of proteins being altered increased, suggesting a progressive manifestation of RTT. Interestingly, we also identified alterations in proteins responsible for proper neuronal development. GO term analysis revealed downregulation of different pathways such as dendrite morphogenesis, axon guidance, negative regulation of neuron differentiation, central nervous system development, forebrain development and nervous system development. Partly, alterations in these processes can be seen in mature RTT neurons and are describes as the typical RTT phenotype.[43,85,86] Therefore, we suggest that the underlying mechanisms of RTT do affect not only mature neurons but influence early neuronal development already at NES cell stage. Consequently, this does also affect potential diagnostic approaches as early interventions can be beneficial for brain development.[87,88] Further validation of the early affected proteins and affected pathways will give better insight into disease progression and can be useful on the overall approach, identification of potential treatment strategies.

Dysregulated Akt/mTOR pathway

Based on our findings in chapter 2, 4 and 5, we strongly suggest the involvement of a fundamental pathway on the RTT phenotype, affecting early neurodevelopment and synaptogenesis. Previous research showed that one basic mechanism important in RTT could be the protein kinase B/ mammalian target of rapamycin (Akt/mTOR) pathway.[89–91] The Akt/mTOR pathway has an important role in many developmental mechanisms and is involved in protein synthesis and regulation of protein levels. Through

the activation of Akt/mTOR, S6 ribosomal protein is phosphorylated, leading to protein synthesis.[92,93] As Akt/mTOR is involved in synaptic protein translation, it plays a major role in the development of neuronal circuits. This pathway is further known to support brain development, synaptogenesis and to play a major role in long term potentiation (LTP).[94] Furthermore, it is involved in cytoskeletal reorganisation including soma sizes and dendritic outgrowth. Interestingly, we observed altered protein expression in NES cells from RTT patients (chapter 5) and also identified an effect on pathways involving cytoskeletal development. If the Akt/mTOR pathway is underlying these alterations, this needs further investigations. However, it could explain the immature neuronal phenotype with impaired dendritic growth observed in chapter 4 and the delayed synchronous activity. Previous studies in RTT already suggested that the activation of Akt/mTOR is decreased and that this has an impact on synaptic and neurodevelopmental protein translation.[90,95] Furthermore, it has also been shown that the down regulation of the Akt/mTOR pathway leads to a reduced expression of KCC2 in the hypothalamus of rats, suggesting a contribution of Akt/mTOR to the immature phenotype of RTT neurons.[96] This hypothesis is further supported by our results from chapter 5. Even though this is further discussed later in this chapter, it should be mentioned that in chapter 5 we identified additional proteins being altered in RTT samples, which are associated with other neuronal disorders such as lissencephaly and cortical dysplasia.[97,98] Interestingly, studies have also suggested that Akt/mTOR might play a role in these disorders, supporting our hypothesis of an essential pathway being affected during early neuronal development in RTT.[99,100] As Akt/mTOR can be regulated in two directions, it can either cause the overexpression of synaptic proteins and the increase neurite outgrowth or reduce translation of synaptic proteins and decrease neuronal growth. Therefore, interaction with MeCP2 could potentially explain the contrary phenotypes observed in RTT and *MECP2* Duplication Syndrome (Fig. 1).

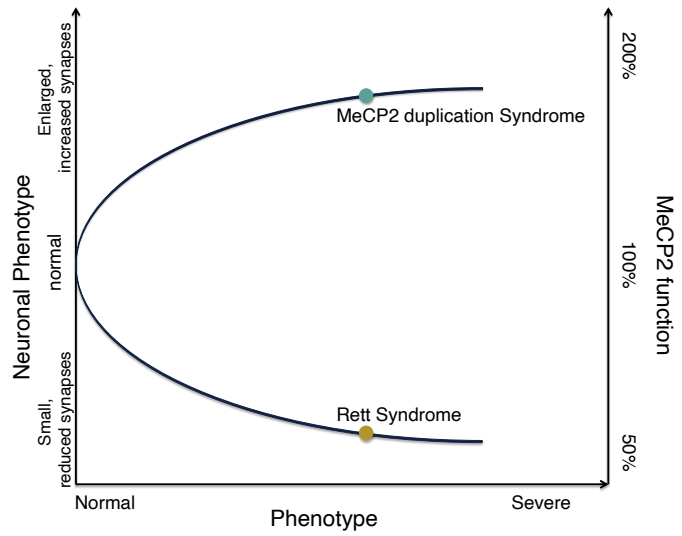

Figure 1. Phenotypic severity based on MeCP2 dosage (Adjusted figure from Chao & Zoghbi, 2012)[22]

Further input on the association of basic pathways with RTT comes from studies investigating more upstream targets of the Akt/mTOR pathway (Fig. 2). One of the first pathways involved in neuronal development and studied intensively is the BDNF pathway. The neuroprotective effect, as well as the importance of BDNF in neurodevelopment and synaptogenesis, was intensively investigated in fundamental studies but also in the context of different disorders.[101–104] Lack of BDNF is associated with several neurologic conditions such as schizophrenia, Alzheimer's, autism spectrum disorder and also RTT.[105–107] In 2006, studies indicated that BDNF concentration is reduced in *Mecp2* knock out mice and that overexpression of BDNF leads to rescue of RTT phenotype in mice including alterations of neuronal firing rates.[105] Interestingly, changed BDNF expression has also been linked to atypical forms of RTT caused by mutations in *CDKL5*.[108] However, the regulatory mechanism of MeCP2 on BDNF is still under discussion, as MeCP2 actually represses BDNF expression by binding to the promoter IV of the *BDNF* gene.[106,109,110] Furthermore, BDNF administration to patients proved rather inappropriate, due to BDNFs inability to pass the blood-brain barrier.[105,111,112] Nevertheless, research groups are still investigating possibilities to deliver BDNF to the brain or improve the impact of present BDNF.[113,114]

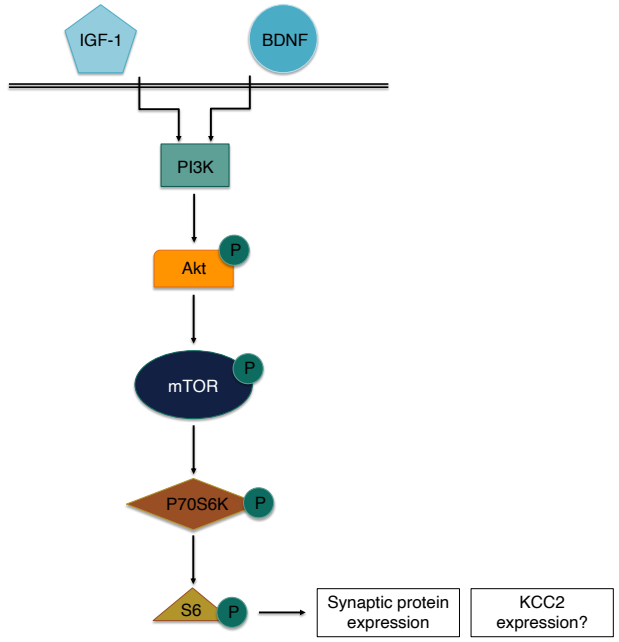

Figure 2. Influence of BDNF and IGF-1 on KCC2 expression via Akt/mTOR. Essential pathways for neurodevelopment associated with RTT.

Another intensively studied upstream target of Akt/mTOR and an important growth factor for neuronal development is insulin-like growth factor 1 (IGF-1).[115,116] Unlike BDNF, IGF1 is capable of passing the blood-brain barrier which makes it suitable as a potential candidate for clinical research. Several clinical studies were performed successfully on RTT patients, reporting a positive outcome and a beneficial effect of IGF-1 on Symptoms such as anxiety, breathing abnormalities and social behaviour.[117,118] Recently, results of a clinical phase II study on 82 children and adolescents with RTT using the IGF-1 analogon Trofinetide were published, indicating a statistical improvement of disease symptoms.[119] Based on these results, another phase III study was planned for the end of 2019 to begin. IGF-1 as a potential target for RTT therapy implements a more general pathway being involved in the pathology of RTT. Interestingly, we find the link to IGF-1 involvement also back in our results. Proteins being altered in RTT samples in proteome analysis in chapter 5 e.g. BNIP3 are known to be regulated by IGF-1.[84,120] As this specific protein is only altered three days after neuronal induction, it suggests that IGF-1 treatment could have a major impact already on early development. This hypothesis, as well as the involvement of Akt/mTOR pathway on the neuronal development, would need further investigation during early development, for example in RTT NES cells. By this approach

the importance of Akt/mTOR for proper development could be estimated and potential other pathways identified.

DIFFERENT MUTATIONS LEAD TO COMPARABLE CLINICAL PHENOTYPES

To dissect the molecular changes underlying RTT and to gain insight into mechanisms that underlie clinical phenotypes, we chose studying classical RTT caused by the lack of functional MeCP2.[1] However, other mutations also lead to similar clinical presentations and could involve the same molecular pathway changes. By increasing sample size, next to more MECP2 deficient lines, one could consider including iPSC lines with different mutations. By comparing also lines with different mutations overlapping alterations might be identified, which potentially suggest pathways that have a major impact on disease phenotypes. This will help to gather insight into mechanisms underlying RTT but may also help understanding other diseases and should, therefore, be considered for further research.

MECP2 Duplication Syndrome

In 2005, a few years after the identification of *MECP2* as responsible gene for classical RTT, a similar RTT phenotype was observed in patient expressing high levels of functional MeCP2.[5] Interestingly, patients with this phenotype do not show a loss of function mutation, but a duplication of *MECP2* in the genome. These patients, affected by *MECP2 Duplication Syndrome* (MDS), reveal signs of intellectual impairment, seizures, autism-like behaviour and anxiety similar to classical RTT patients.[121,122] However, cellular phenotypes are almost reversed, meaning that patients with MDS show enlarged neurons with higher dendritic branching level and increased numbers of synapses. The identification of MDS suggests that MeCP2 requires a critical dosage to support proper cellular and network development (Fig. 1). Therefore, MeCP2 seems to be involved in complex processes, tightly balancing the developmental processes of neuronal cells.[123] As we showed in chapter 5, lack of functional MeCP2 already affects protein expression early on in neuronal development, which most likely affects the neuronal phenotype observed in RTT. Even though patients with MDS display a reversed pathology compared to RTT, it is most likely that MDS does not simply reverse protein alterations. However, as MDS phenotypes are also associated with certain developmental stages, including developmental regression, it is likely that RTT and MSD share fundamental developmental pathway alterations.[124] A comparison to iPSC-derived neuronal stem cells from MDS patients could, therefore, be of great interest to understand basic developmental mechanisms and the contribution of MeCP2. By comparing classical RTT to MDS, we could gain a better understanding of the main pathways sensitive to alterations in the MeCP2 dosage and might lead to the identification of new treatment targets.

FOXG1 and CDKL5-associated syndromes

Mutations in *FOXG1* and *CDKL5* genes are known to cause atypical forms of RTT.[125–127] These forms of RTT usually have their symptomatic onset prenatally but can also show a developmental regression stage.[128] However, symptoms in classical and atypical RTT do show clear similarities, such as epilepsy, stereotypic hand movements and loss of speech. As earlier studies suggested, all three genes function as transcription factors, might influence gene expression of similar genes and therefore might influence similar pathways.[129,130] However, the exact overlap between classical and atypical RTT has not been identified yet. With the establishment of next-generation sequencing within the last years, it is expected that more studies will soon focus on potential links.[131–133] In addition to *FOXG1* and *CDKL5*, different studies have already identified more than 80 genes that are associated with phenotypes similar to RTT as Vidal and colleagues recently reviewed.[134] As some of these genes were already associated with other disorders, such as Pitt-Hopkins syndrome, Angelman syndrome and other epileptic encephalopathies, the overlap of phenotypes indicates coherence between neurodevelopmental disorders and RTT.[135–137]

Similarities to other neurodevelopmental disorders

In chapter 5 we identified altered proteins levels in RTT NES cells, which are also associated with other neuronal diseases, namely epilepsy, Niemann-Pick disease, mental retardation, cortical dysplasia, lissencephaly, neurotonia and axonal neuropathy. As we found these alterations already in NES cells we assume, that a potential link to those disorders is associated with early neuronal development. Interestingly, most of these disorders are associated with the mTOR pathway, which supports our hypothesis that RTT patients suffer from impairments in fundamental pathways needed during early development.[138,139] In the last years, the Akt/mTOR pathway has already been associated with other neurodevelopmental disorders such as e.g. Tuberous Sclerosis (TSC). In TSC patients, Akt/mTOR activation is increased leading to elevated cell size and sprouted, elongated neurites. Downregulation of the Akt/mTOR pathway by applying rapamycin rescues this phenotype.[94,140,141] This two-sided regulation of Akt/mTOR might, therefore, be a potential explanation why MDS patients show similar symptoms while developing opposite cellular phenotypes of RTT patients (Fig. 3). Nevertheless, to link general pathways to RTT and other neurodevelopmental disorders, we need to fully understand alterations underlying cellular development in RTT first. We suggest that, based on our findings, investigations of early alterations could be expanded by including other neurodevelopmental disorders. This approach could therefore not only give insight

into RTT but could also lead to a better understanding of general neurodevelopmental disorders.

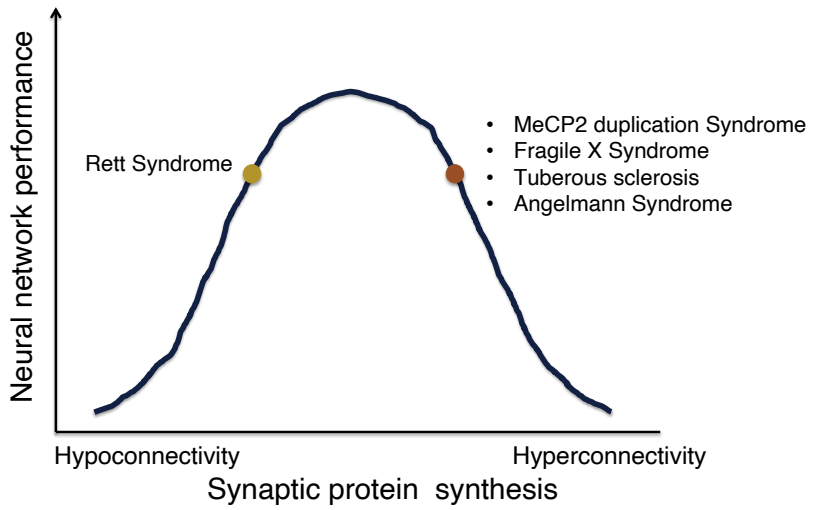

Figure 3. Protein Synthesis affected in different directions in other diseases indicate a dysregulation of mTOR and translational processes (Figure adjusted from Kelleher &Bear)[4]

CLINICAL IMPLICATIONS

With our proteomic approach in Chapter 5, we assumed that RTT has its molecular onset during early gestation. During this developmental period, diagnostics and treatment possibilities are rather restricted. However, it is still unclear whether molecular changes seen in *in vitro* models display the situation in patients. Nevertheless, studies suggest behavioural alterations of children with RTT already during the first month after birth, although their symptoms become distinct between 6 to 18 months.[142,143] As the brain still goes through a postnatal developmental phase and is highly plastic, early treatment might be beneficial.[144–146] By the early application of potential treatments such as NKCC1 inhibitor Bumetanide, intraneuronal chloride levels could be shifted to appropriate levels, thereby reducing the appearance of seizures.[147,148] Also, the early support of Akt/mTOR pathway activation could lead to a beneficial outcome. Therefore, the additional synaptic protein translation early in life could have a positive effect on synaptogenesis and support neuronal network development.[90,149]

To benefit from early treatment approaches, diagnostics need to be performed as

soon as possible. Diagnostics for RTT have often been delayed due to the lack of experience and variability of displayed symptoms.[150] This issue needs to be addressed by appropriate training and guidelines for paediatrics and therapists. But also, a broader genetic screening could help to identify *MECP2* or other RTT related mutations early in life. The identification of RTT at an early time point together with early treatment, supporting proper brain development, can therefore, be a key approach in RTT therapy and perhaps other neurodevelopmental disorders.

FINAL CONCLUSION AND FURTHER DIRECTIONS

Our approach was to get insight into the cellular and molecular changes in the brains of RTT patients, a devastating neurodevelopmental disorder, leading to severe symptoms manifesting at infancy. As we confirmed alterations of KCC2 levels in post mortem brain tissue of patients in chapter 2, we were interested in whether RTT causes molecular alterations in neuronal precursors. Our proteomic study indicates that RTT neurons are impaired during early development and suggest an involvement of the Akt/mTOR pathway. Other studies involving potential RTT treatment options, describing phenotypes of MDS as well as other neurological disorders (e.g. TSC), point to a bidirectional regulation of synaptic development underlying early-onset. As our results still lack clear evidence to prove the early involvement of Akt/mTOR, our findings need further validation. Future research is also needed to link classical and atypical RTT, MDS and other neurodevelopmental disorders.

For further validations, we suggest investigating a broader spectrum of patient material. Due to the difficulties we were facing regarding the generation of an iPSC *in vitro* RTT model, our assumptions are based on only two patient cell lines. Even though the use of isogenic controls does reinforce the results presented in this thesis, the inclusion of additional cell lines is required. Therefore, we suggest using our *in vitro* model described in chapter 3 to ensure the usage of isogenic controls, but include additional patient material. To further support our hypothesis, parallel investigations of cell lines from patients with MeCP2, CDKL5 or FOXG1 and other disorders such as Fragile-X or Angelman syndrome would be beneficial to identify a potential link in altered neurodevelopment. As our *in vitro* approach can be used specifically for X-chromosomal disorders, generation of disease lines and isogenic controls with other *MECP2* or *CDKL5* mutations, as well as X-linked mutations of other diseases as Fragile-X, can be generated. Additionally CRISPR/Cas9 gene editing approaches could be used to extend the number of diseased and control lines. All generated cell lines could then be analysed during early neuronal development at the NES cell stage and at a mature neuronal cell stage to study effects on functional behaviour. To confirm Akt/mTOR involvement, specific activators would be needed to

increase Akt/mTOR activity.[151] In addition, cell lines from MDS patients as well as e.g. TSC patients could be included in this study to investigate bidirectional alterations caused by Akt/mTOR pathway modulations. In conclusion, future studies would benefit from including additional RTT lines as well as samples from other disorders to gain insight into basic mechanisms underlying early developmental impairments.

Further knowledge needs to be gathered regarding KCC2 expression levels. As specifically KCC2a levels were altered in RTT patients based on our findings in chapter 4, we suggest to further investigate not only cortical neurons but also include samples from brain areas in which KCC2a is the dominant isoform.[78] Evaluation of KCC2b levels should also be further investigated in additional patient cell lines. This could be combined with functional analysis, by calcium imaging as described in chapter 4 or by using a Multi Electrode Array (MEA) approach, which would allow a high content analysis of cells. In combination with drug applications e.g. Akt/mTOR activators, this approach could generate a new understanding of chloride homeostasis alterations, impairments in GABAergic shift and E/I balance changes.

Finally, we suggest investigating the contribution of glia cells on the underdeveloped neuronal phenotype in RTT. Besides basic co-culture models, the investigation of more complex models, such as organoids will generate the possibility to observe neuronal maturation in a more physiological context.[51,60] In line with the fast evolving field, we might be soon studying early brain development in uniformly generated organoids as prevailing model system.

Basic laboratory studies might not directly lead to new treatment strategies. However, due to the early onset of developmental alterations, reading these early signs suggests early diagnostics and the earliest possible start of treatment in patients. With our *in vitro* approaches and the identification and understanding of pathways associated with RTT, we want to support the overall goal of RTT research. Even though, we did not provide a potential treatment with our research yet, we are confident that future approaches can build upon our findings and provide valuable insight and targeting options for further validations. We hope that this, together with the rapidly progressing field of RTT research, will finally lead to better treatment approaches for RTT patients.

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