Reading the Early Signs

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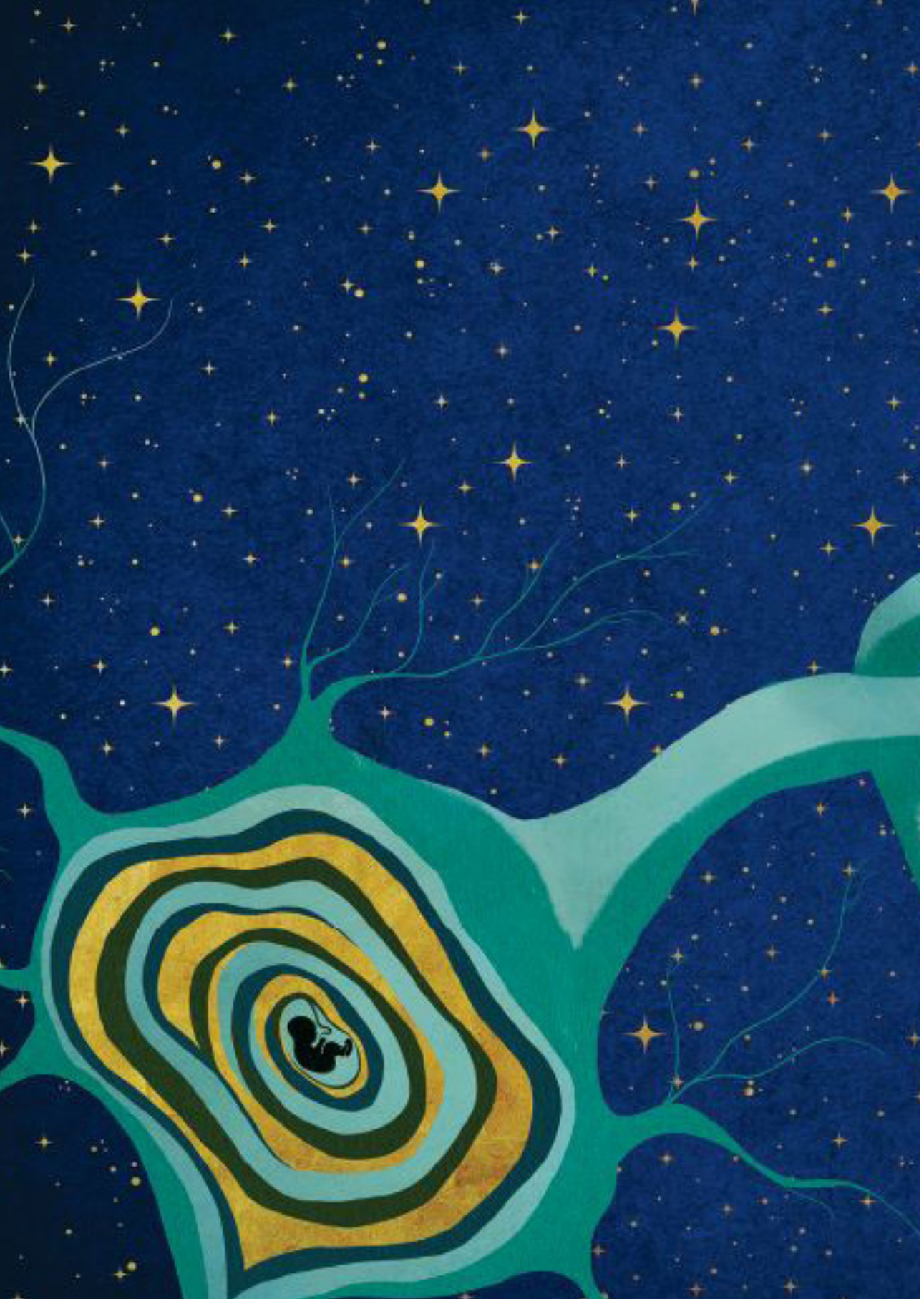

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INTRODUCTION

INTRODUCTION

Rett Syndrome (RTT) is a devastating neurodevelopmental disorder mainly affecting girls. Classical RTT is caused by various mutations within the Methyl-CpG-Binding-Protein 2 gene (MECP2) which is located on the X-chromosome.[1] With an occurrence of 1:10,000-15,000 it is the second most common reason for mental impairment in young women. After a relatively normal development, symptoms usually occur at 6 to 18 months. Besides mental retardation and autism-like behavior, patients suffer from stereotypic hand movements and loss of so far achieved abilities, such as walking and other motor functions or speech.[2] Furthermore, RTT patients often experience severe seizures, breathing abnormalities, and reduced growth. Even though a vast amount of research was done on RTT, the exact disease mechanisms remain unclear. There is currently no cure available and symptomatic treatments are often not successful. Studies on mouse models showed that MeCP2 replacement can reverse RTT and that this leads to improved cognitive behavior, less seizures and enhancement of breathing.[3] However, to translate these studies to humans, suitable human disease models are needed. Induced pluripotent stem cells (iPSCs) could be appropriate to use. By reprogramming patient-specific somatic cells into pluripotent stem cells, one can generate any cell type of interest, and study human cellular development from early to late developmental stages.[4] This makes iPSC technology an interesting tool especially for the study of developmental disorders.[5]

In this thesis, we aimed to develop an iPSC-based RTT disease model to investigate early disease mechanisms and to gain insight into the cellular pathways which are impaired in MeCP2 lacking cells. To this end, we studied molecular alterations subsequently followed by functional impairments in RTT iPSC-derived neuronal cells. Our overall goal was to get a better understanding of the mechanisms behind RTT which will help, eventually, to identify novel treatment approaches.

RETT SYNDROME

What is Rett Syndrome

Rett Syndrome (RTT) is a neurodevelopmental disorder. It mainly affects girls with a prevalence of 1:10,000-15,000.[1] Classical RTT symptom usually appear after a relatively normal development at an age of 6-18 months. Usually, affected girls do not reach developmental landmarks anymore and fall into a developmental regression. They unlearn the so far achieved abilities, such as motor abilities and language competencies.[2] Additionally, very common stereotypic hand movements such as wringing and washing movements occur. Girls affected have difficulties holding eye contact and often start

to show first epileptic episodes early in life. Also, irregular breathing and issues with sleeping can be observed.[6,7] At a later stage, affected girls often show reduced growth combined with scoliosis, dystonia, and or spasticity. Usually, classical RTT can be divided into 4 stages, of which the age of onset can vary based on severity (Table 1).

Table 1. The four stages of Rett Syndrome

Stage	*Age*	*Duration*	*Symptoms*
I. Stagnation	6-18 month	Weeks to month	Delay of development
II. Rapid regression	1-3 years	Up to 1 year	Loss of acquired skills, stereotypic hand movement, breathing abnormalities, seizures
III. Plateau period	2-10 years	Years to decades	Interest in surrounding and eye-pointing, seizures
IV. Motor deterioration	>10	Decades	Physical disability, scoliosis, wheelchair dependency

RTT is a disease that is not necessarily fatal. Depending on the severity of the symptoms and the care for patients, female RTT patients can reach seniority. The oldest woman reported suffering from RTT died at the age of 79.[8] However, the majority of patients lose communication ability, mobility and eventually become wheelchair-bound which complicates care-taking and makes these patients vulnerable to secondary infections.[9,10] Therefore, improvements based on a better understanding of the disease progression are urgently needed, to increase the patient's independence and life quality.

History of Rett Syndrome

Dr. Andreas Rett first described RTT after he observed two little girls sitting next to each other in the waiting room of his doctor's office in Vienna, Austria, in the year 1954.[11] Both girls showed the same stereotypic hand movements and were smaller than healthy girls their age. He studied the medical files of both girls and discovered that these two and an additional 6 female patients in his office showed high similarities. Therefore, Dr. Rett decided to search for similar patients within and around Austria. His search resulted in the report of 22 cases in the Journal *Wiener Medizinische Wochenschrift* in 1966. Here, he was the first to describe a Syndrome -later known as RTT- which was specified as the presence of stereotypic hand movements, impairment of intellectual development, brain atrophy, and hyperammonemia.[11] As Dr. Rett published his study only in the German language, researchers and medical doctors' awareness of the syndrome remained limited.

Only in 1983, RTT gained attention by a publication of Dr. Bengt Hagberg. In this article he described a cohort of 35 patients from his doctor's office in Sweden, France, and

Portugal.[12] Since then, more researchers started focusing on RTT, leading to reports of 1,250 new cases in 1987. More than 50 years later, the number of reported RTT cases increased up to 350,000 worldwide, but the biological mechanisms behind RTT remained unclear. At present, a treatment is still not available. Hundreds of researchers and neurologists are still investigating RTT hoping to provide new options for patients and families.

Classical and Atypical RTT

In 1999 Prof. Dr. Zoghbi and her team were the first to identify the genetic cause of classical RTT.[1] They showed that mutations in the Methyl-CpG-binding protein 2 gene (*MECP2*), located on the X-chromosome, were responsible for the development of the RTT phenotype. In their study, they performed a systematic gene screening analysis to identify several *de novo* mutations causing RTT.[1] Nevertheless, not all RTT patients showed *MECP2* mutations. Besides the finding that lack of functional MeCP2 leads to classical RTT, additional mutations in other genes were discovered only a few years later. Cyclin-dependent kinase-like 5 (*CDKL5*) and Forkhead box protein G1 (*FOXG1*) mutations were identified to cause an atypical or congenital variant of RTT.[13–17] The *CDKL5* mutation, also located on the X-chromosome, was identified to cause early-onset seizures in patients that in some cases can already appear immediately after birth. Therefore it is also known as early-onset seizure type of RTT.[13–15] The congenital variant which is caused by a mutation in the *FOXG1* gene, located on chromosome 14, is characterized by symptoms of classical RTT. In contrast to the delayed onset in classical RTT, the congenital form shows a much earlier onset with symptoms appearing within the first months of life.[16,18,19] Although, mutations in *CDKL5* and *FOXG1* are mentioned as atypical RTT, researchers more often consider them as separate syndromes due to the different patient conditions and different disease pathways involved.

MECP2

MeCP2 is a multifunctional protein and is known to play a key role in gene regulation. While older studies refer to MeCP2 as gene repressor, newer findings showed that MeCP2 is also able to activate gene expression. Whether MeCP2 has a repressing or activating effect is based on the methylation state of the target gene. Although MeCP2 binds to CpG islands which are methylated at the 5-carbon end of Cysteine (5mCG), it is also known to bind non-CG methylations (5mCH).[20] These bindings seem to result in repression of the target gene. MeCP2 binding to different methylation groups can cause gene activation. Oxidation of 5mCH causes genes to get hydroxymethylated (5hmC) which upon MeCP2 binding is suggested to increase expression of target genes.[21,22] Besides its ability to bind to methylated genes, MeCP2 is also involved in chromatin modulations.[23,24]

These modulations also affect gene expression and are therefore additional regulatory mechanisms. Interestingly, expression levels of MeCP2 differ throughout different tissues. One of the highest expression levels can be measured in brain tissue, specifically within the neuronal population.[25,26] Here, MeCP2 regulates especially long genes, known to be involved in neuronal maturation and brain development.[27] Therefore, alterations of expression levels of these genes might have a major impact on brain development and can cause severe neurological symptoms. To understand mechanisms underlying RTT, MeCP2's primary and downstream target genes need identification to evaluate their contribution to the typical RTT phenotype.

PATHOLOGY

Post mortem tissue analysis

Post mortem tissue studies provide insight into the neuropathology of RTT at a specific developmental time point. The majority of donated brain tissues comes from adolescent or adult patients, depicting a disease state later in the disease course. Studies on *post mortem* brain tissue from patients showed that RTT brains are on average smaller than healthy control brains.[28,29] Interestingly, these studies also revealed that the decrease in volume is not caused by atrophy or neuronal loss, but by a reduction in neuronal soma size and an increase in neuronal packing density.[30] This indicates that RTT is not a neurodegenerative disorder but rather is caused by impaired neurodevelopment. In 1995 Armstrong *et al.* showed that besides the reduced soma size, dendritic branching of pyramidal neurons in RTT brains was decreased. They identified these alterations in combination with a decrease in spine density in different cortical areas, indicating a reduction in synapses.[31,32] Region-specific alterations were discussed based on new research into neurochemical alterations .[33] Altogether, these investigations on RTT *post mortem* brain tissue and neuronal morphology, pointed towards a disturbed neuronal maturation, indicating that RTT brains remain underdeveloped.[31,34] Therefore, RTT was classified as a neurodevelopmental disorder.[35] Nevertheless, brain tissue of RTT patients could only depict a static picture, leaving the question unanswered how the neuronal phenotype of RTT is developed. Other disease models are needed to confirm underlying pathways and to perform functional and developmental studies.

Mouse models

To perform functional and behavioral studies, mouse models are generally powerful. The first RTT mouse model was already established in 2001.[36–38] These *Mecp2*-null mice showed similar symptoms as patients with RTT. Interestingly, affected male mice displayed a more accurate phenotype with regards to disease onset and severity of RTT, in comparison to the much milder affected female mice. Therefore, male mice

became the preferred model to perform RTT research studies. Besides their typical clinical phenotype showing ataxia, breathing abnormalities, and stereotypic behavior, these mice also present typical RTT cellular alterations described for human tissue, such as decreased soma size, reduced dendrite growth, and a reduction in excitatory synapses and neurotransmission.[37,39] Besides the *Mecp2*-null model, researchers created mouse models harboring known *Mecp2* mutations. As of today, mice carrying premature stop codons within *Mecp2*, as the 308X model, or different missense mutations such as T158M are very commonly used.[38,40,41] A milestone in mouse-based RTT research, was the investigation of Guy and colleagues in 2007, studying re-expression of *Mecp2* in adolescent mice. Interestingly, they showed a major decrease of symptoms after recovering *Mecp2* expression which indicated that MeCP2 plays a major role not only during brain development but also for neuronal maintenance and adult neurogenesis.[3] However, the most significant output of this study was that recovery of *Mecp2* expression rescued RTT phenotype and 'reversed' RTT in mice. To investigate this further and to identify the function of MeCP2 in different cells, researchers generated conditional knockout mice using Cre-recombination systems.[42,43] Especially, due to the severe seizures occurring in RTT patients, researchers focused on inhibition and excitation alterations.[43–45] The investigation by Chao and colleagues in 2010 displayed the major role of inhibitory γ-aminobutric acid (GABA)ergic neurons. The authors showed that conditional knockout of GABAergic neurons was sufficient to cause a characteristic RTT phenotype, including repetitive behavior, impaired motor skills, and learning as well as severe neuronal circuit dysfunction.[43] This indicates the importance of the GABAergic system within the RTT disease mechanism. Also, other studies described a key role for GABA. It was shown that depending on the specific brain area, MeCP2-lacking mice show disinhibition due to reduced GABAergic innervation pointing towards a disturbance in the balance of excitation and inhibition (E/I balance).[45,46] These models are still broadly used to understand neuronal phenotypes in RTT and to perform functional analysis studies.[47,48]

Besides neuronal subtypes, conditional knockouts were also used to target non-neuronal cells. In 2011 Lioy and colleagues showed that specifically the induction of normal *MECP2* expression in MeCP2 deficient astrocytes resulted in a reduction of symptoms and a partial rescue of the phenotype.[49] These results implied that MeCP2 is not exclusively influencing neurons, but also impact glial cells.[50] Glial cells support neuronal development by for example expressing insulin-like growth factor 1 (IGF-1).[51,52] Interestingly, studies showed that IGF-1 levels in RTT mice are significantly decreased and that treatment with IGF-1 is capable of restoring the neuronal phenotypes.[53,54] These results were successfully translated to humans, leading to a placebo-controlled, double-blinded phase II study, which showed that IGF-1 has beneficial effects on symptoms in RTT patients.[55]

Since the establishment of RTT mouse models, almost two decades ago, we gained a lot of insight into disease mechanisms and pathways. However, it is still unclear whether the findings in RTT mouse models can be properly translated towards humans. Due to the very mild phenotype of female mice it is likely that mice have compensatory mechanisms. These could interfere with potential treatment efforts. Also, the use of solely male mice, not depicting the physiological alterations observed in the main population of patients, might neglect gender-specific pharmacological aspects.[56–58] Consequently, mouse models are very beneficial with regards to functional studies but are not capable of representing this physiological situation-specific for females or human patients.

Current hypotheses on disease mechanisms

During the last decades, research on RTT yielded insight into new disease mechanisms. The field was changed by the identification of *MECP2* mutations as the cause for the classical RTT variant, and by the reclassification of RTT as a neurodevelopmental disorder.[59] As neurons in mice but also in human RTT brain tissue were reported to be immature and showed a decrease in the neuronal maturation phenotype, lack of MeCP2 was associated with neuronal maturation deficits.[60,61] This hypothesis was supported by a decrease in gene expression of specifically long genes, responsible for neuronal maturation.[27] The immature state of the neurons also explains the morphological alterations in synapse reduction, impaired neuronal plasticity and disturbed E/I balance.[45,46] This, negatively influences the development of neuronal circuits and probably causes severe seizures due to insufficient inhibition of GABAergic neurons.[62] Consequently, RTT brains can be described as overall underdeveloped and immature, with reduced functional connectivity and impaired networks. Treatments with IGF-1 were shown to have a beneficial effect and can restore these immature neuronal phenotypes.[53–55] Therefore, current research focuses on the possibility to restore neuronal maturation. However, it is not fully understood at which developmental stage brain maturation starts to deviate which is crucial for knowing when to trigger the correct maturation process.

BRAIN DEVELOPMENT

Network

Brain development is a very precisely timed and complex process (Fig. 1). After the differentiation into the different germ layers, the neuroectoderm forms the neural tube which develops into the forebrain (telencephanlon and diencephalon), midbrain (mesencephalon), hindbrain (rhombencephalon) and spinal cord.[63,64] Only few weeks postconceptional, neural stem cell proliferation starts in the ventricular zone and subventricular zone of the telencephalon.[65,66] Subsequently, neuronal progenitors migrate

towards the cortical plate, where the cortical layers are formed.[66,67] Approximately at postconceptional week 18 gliogenesis begins. Due to epigenetic modifications and cytokine induction in neural stem cells, gliogenesis is activated and developmental cell fate switches.[68] The development of glial cells is essential to support synaptogenesis which starts at about the same time as gliogenesis. Synaptogenesis takes place roughly until 15 years of age with a peak time at approximately 2 years.[69,70] During this peak time synaptic pruning, the elimination of unnecessary synapses, starts occurring, which is needed for functional neuronal network development, neuroplasticity, and learning.[71–73] As all these developmental processes are well-orchestrated, small divergences can have severe effects on overall brain development.

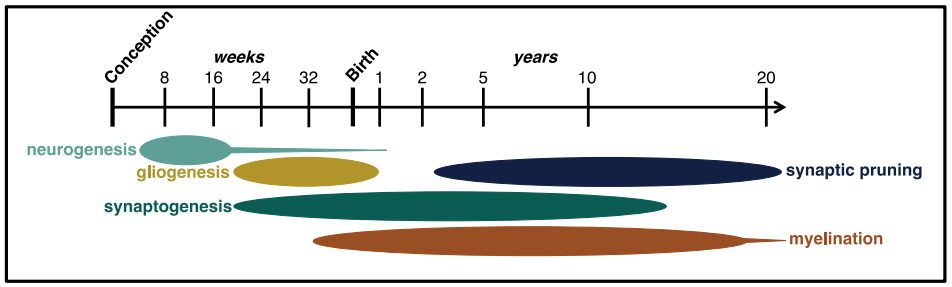

Figure 1. Timeline of cell development in the human brain (modified from Semple et al.)[74]

Cellular

The maturation of neurons and functional circuits on a cellular level is complex and follows strict directions. Cortical neurons are generated via asymmetric division of radial glial cells in the subventricular zone (SVZ). From there they migrate to the required brain area of the cortex and form the cortical layers in an inside-out structure.[75] Only when neurons reach their final destination, synapses are built, i.e. synaptogenesis starts, to form networks triggered by activity. Cells that are connected start to build clusters of synchronized activity which strengthens their connections and increases the number of synapses.[76] During human brain development, it is shown that one of the first functional neurotransmitters supporting synaptogenesis is GABA. Even though GABA acts as an inhibitory neurotransmitter in mature brains, it has an excitatory effect in immature brains.[77] Therefore, GABAergic synapses are mainly responsible for a specific electrophysiological pattern that can be observed in developing mammalian brains. (Fig. 2).[78–80] This activity pattern triggers the implementation of new synapses and is therefore essential for synaptogenesis. These synchronous appearing signals

have been extensively investigated in the hippocampus and cortex of mice, where neuronal networks show giant depolarizing potentials (GDPs). GDPs and GDP-like spontaneous synchronous activity (SSA) are observed in different brain areas to build functional connections and play a major role in brain patterning and the generation and maturation of functional networks.[80,81] Specifically, GDP-mediated activity triggers the connectivity and the development of glutamatergic synapses.[82] Small alterations within the duration of GDP appearance or GDP frequency have been shown to have a major impact on proper brain development and can lead to consequential damage to circuits. This leads to severe seizures which are also seen in RTT patients.[83,84]

During adolescence, neurons keep part of their plasticity via N-Methyl-D-Aspartate (NMDA) induced Long Term Potentiation (LTP) or Long Term Depression (LTD). This allows them to integrate new receptors, to build and to erase synapses and to form memory, all important for learning ability and flexibility of the mammalian brain up to adolescence.[85] All these processes rely on properly formed networks. Therefore, the process of network development follows strict rules and is very crucial for the entire early brain development.

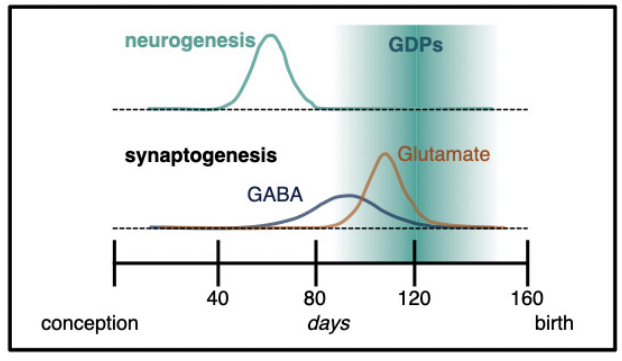

Figure 2. GDP appearance time line during primate pre-natal development (modified from Khazipov et al., 2001)[79]

KCC2/NKCC1

The excitatory effect of GABA in the immature brain is very important for proper brain development which is driven by hypertonic, intracellular chloride levels. High chloride levels are maintained by sodium–potassium–chloride cotransporters1 (NKCC1) which are mainly responsible for chloride uptake and the accumulation of chloride in the cytoplasm. When GABA is released and binds to $GABA_A$ receptors, the binding results in

the opening of chloride-channels.[81,86,87] The intracellular chloride then follows the osmotic pressure towards the extracellular space and the neuron depolarizes.

Repetitive and synchronous depolarization of neurons, as seen in GDPs, has an important impact on the developing neurons. On one hand, the activity triggers the building of new, glutamatergic synapses that take over the excitatory activity of neurons. On the other hand, it activates the expression of potassium-chloride co-transporter2 (KCC2), a neuron-specific chloride transporter which reduces intracellular chloride. This subsequently causes the switch of GABA from an excitatory to an inhibitory neurotransmitter (Fig. 3).

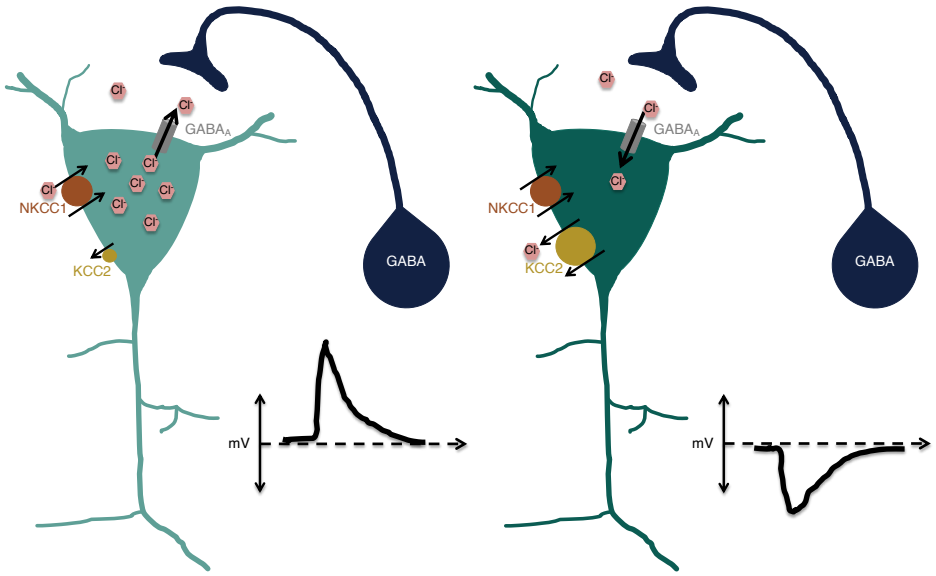

Figure 3. GABAergic shift via increased KCC2 expression (modified from Ben-Ari, 2014)[88] *Immature neuron (left) with high intracellular chloride levels due to low KCC2/NKCC1 ratio. Mature neuron (right) with low intracellular chloride levels due to increased KCC2 expression.*

The appearance of GDPs causing the GABAergic switch occurs during a very specific time period during development. In mice, it is observed 5-7 days *postnatal*, while in primates and humans GDPs occur during the second half of gestation (Fig. 2).[79,89] The timing of this event is very crucial and has a major impact on normal brain development. Studies on mouse models have shown that alterations in GDP appearance is strongly associated with neurodevelopmental disorders or autism spectrum disorders.[90]

Furthermore, changes in the frequency of GDPs can lead to altered neuronal circuits, disinhibition, and therefore severe seizures.[83,84,91,92] Therefore, the appearance of SSAs is of specific interest when studying neurodevelopmental disorders, as the timing of circuit development is essential and has a major impact on disease phenotypes.

THE DISEASE MODEL

Disease Models

Currently, there is no treatment for RTT available. Despite a lot of research within the last decades, no cure has been found yet and the success of symptomatic treatments is moderate. One reason for this is the lack of clearly identified disease mechanisms. As MeCP2 is involved in the regulation of hundreds of genes, several important pathways are affected and the major key players of RTT remain unclear. As investigations on patient material are limited due to the low incidence, it is of importance to develop a suitable new disease model that represents the mechanisms of interest. Dependent on the research question, disease models need to mimic the physiological situation in humans. Therefore, the use of a human model system can be highly beneficial. To investigate underlying mechanisms, specific cell types need to be investigated, preferably over different stages of development. Especially, the investigation of RTT which requires insight into the developmental processes is a challenging task. However, newly developed model systems are capable to address these issues.

Disadvantages of Traditional Models

Important basic findings in RTT, such as the immaturity of neurons or the reduced brain weight, were revealed using *post mortem* brain tissue.[93–95] Nevertheless, *in vivo* RTT models or *post mortem* tissue of patients, have their downsides when it comes to physiological or functional analysis. Due to *post mortem* delay, long-time storage, or thaw-freeze cycles, the available tissue can show signs of quality loss and protein degradation. This makes protein analyses difficult. Furthermore, due to limited availability, the generation of proper study layouts, generating statistically relevant results, can be challenging. This applies even more to rare diseases as RTT. Besides limited availability, *post mortem* tissue studies only supply insight into a progressed disease stage. As RTT is a neurodevelopmental disorder, disease onset is likely to be early in development. Studies performed on *post mortem* tissue do not cover these developmental stages and can therefore only provide a static picture of the disorder in a final state.

To overcome these obstacles and investigate development, a very commonly used model is a mouse model. Mice have been used for molecular biological assays but also for functional tests and behavioral studies. Mouse studies provided interesting and

important insight into RTT, as for instance the reversibility of RTT after recovering MeCP2 expression and the contribution of glial cells to the neuronal phenotype.[3,96,97] Different types of RTT mice were developed and major knowledge about RTT is based on mouse model investigations. However, these models do not reflect the physiological situation in humans. Besides the ethical issue of using animal models, researchers become more critical about the outcome of studies based on mouse models as they often fail in translation to human trials.[98,99] Furthermore, it is has been shown that mutations in MeCP2 affect mice and humans differently. A robust RTT phenotype is only observed in male mice carrying a MeCP2 mutation or in female mice homozygously affected. Heterozygous female mice carrying only MECP2 mutations in one X-chromosome, similar to patients, only show a very late disease onset and are often only mildly affected.[36,37,100] Consequently, it is suggested that mice have a compensatory mechanism that can cover the mosaic lack of MeCP2.[101] Due to this obstacle, the use of mouse models can give important insight about developmental aspects, however, it does not reflect mechanisms observed in patients with RTT properly.

Induced Pluripotent Stem Cells

A big step towards overcoming existing obstacles with disease models was made in 2006, when Yamanaka and colleagues showed that it is possible to reprogram somatic cells into a pluripotent state.[4] Till then, pluripotent stem cells, i.e. embryonic stem cells (ESCs) were harvested from embryos at the blastocyst developmental stage (Fig. 4A). Cells from the inner cell mass were collected and expanded *in vitro* and could be stored until further use and differentiated into the desired cell lineage. As this paradigm raised ethical concerns, the usage of ESCs was strictly regulated and limited to certain countries in the European Union.[102]

The new technique, Yamanaka *et al.* introduced in 2006, used small samples of somatic cells from patients and induced overexpression of defined pluripotency genes, the so-called Yamanaka factors.[4] These transcription factors could be overexpressed by different approaches such as viral transduction or plasmid transfection. This eventually caused the expression of downstream target genes and subsequently reprogrammed the somatic patient-derived cells into ESC-like cells (Fig. 4B). The generated iPSCs showed the expression of pluripotency markers similar to ESCs and could be differentiated towards all three germ layers.[103] The investigation of Shinya Yamanaka and his colleagues marked a new era, giving researchers the opportunity to use iPSCs to establish disease and human-specific *in vitro* models. Due to the minimally invasive procedure to gather somatic cells (e.g. skin biopsy, blood, urine samples), sample collection from any person is possible. This also includes samples from children, suffering from developmental disorders

such as RTT. The patient-derived iPSCs are therefore disease- and patient-specific and reflect specific phenotypes.[104] Besides the investigation of treatment opportunities and drug screenings, iPSCs can be used for disease modeling and investigations of disease mechanisms. Especially, for polygenic disorders or disorders with an unknown genetic cause, iPSCs are a good model system. Due to the unlimited source of iPSCs, different cell types can be generated from the same patient. This allows investigation of disease pathways by different approaches, without additional invasive procedures for the patients. As the differentiation can follow similar stages as shown during *in vivo* development, iPSC research is specifically applicable to investigate developmental processes.[105] Therefore, an iPSC-based *in vitro* model can be monitored over time, giving proper insight into developmental time windows as well as alterations in developmental mechanisms. Taken together, the iPSC-based *in vitro* model can address obstacles in traditional RTT disease models, making it an important new tool for the field and the development of therapeutic approaches.

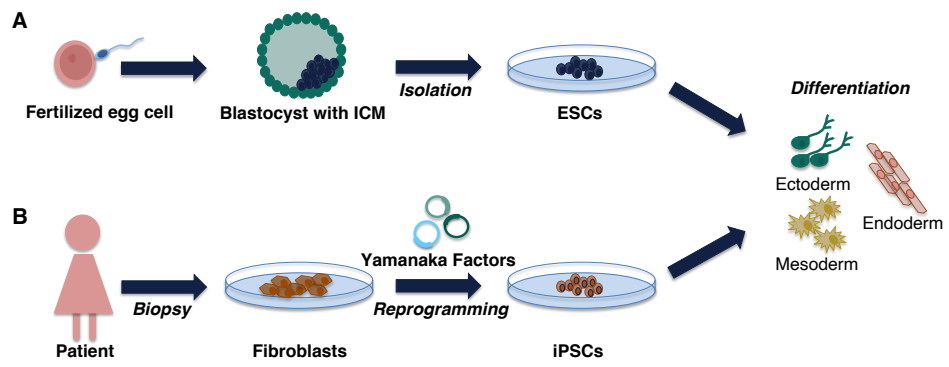

Figure 4. Comparison of ESCs and iPSCs. *Isolation of ESCs from inner cell mass of blastocyst (A) and generation of iPSCs from patient-derived fibroblasts by transcription of Yamanaka factors (B). Both cell types ESCs and iPSCs are pluripotent and can differentiate towards all three germ layers.*

IPSC RESEARCH ON RETT

Current Research

Since human iPSCs were established, several research groups have used iPSC technology to investigate RTT. In 2010, Marchetto and her colleagues were the first to develop an iPSC-derived RTT model.[104] With this model, they were able to reproduce alterations in RTT neuron morphology, in line with earlier findings in mice and *post mortem* tissue. They reported differences in dendritic length, soma size and the number of synapses, similar

to the known RTT phenotype. A maturation deficiency was confirmed in RTT patient's iPSC-derived neurons.[106–109]

This newly established model led to the investigation of specific pathways involved in RTT. Importantly, iPSC-based RTT models also showed a contribution of the IGF-1 pathway during development in RTT.[110] This study, among others, indicated a contribution of pathways involved in brain growth and development.[54,55,110,111] Consequently, researchers got specifically interested in the development of neurons and synaptogenesis. Especially, the balance of excitation and inhibition (E/I) was studied intensively in the last years.[62] In several studies, researchers found impaired synaptic connectivity and circuit alterations.[45,62,112] Processes like circuit- and network-development usually take place during early embryonic developmental stages. Therefore, these studies underline the importance of early development in RTT not only for a better understanding but also for treatment strategies. Taken together, research using iPSC models for RTT, confirmed previous findings based on mouse models or human *post mortem* tissue. Nevertheless, it additionally offers further opportunities investigating early developmental mechanisms in a human genetic background. Therefore, iPSC-derived *in vitro* models are of great benefit to understand disease mechanisms and to give answers with regards to disease onset and appearance of phenotypes.

Innovations in research

The Perfect Control

To perform reliable and high-quality research, proper controls are needed, such as age or gender-matched controls. However, matched controls still present differences due to individual genetic variations. Studying RTT on cellular level offers an approach to overcome this obstacle. Due to random X-chromosomal inactivation, cells of female patients are randomly affected by MeCP2 deficiency. By using a simple sorting approach, pure populations of diseased cells and isogenic controls can be generated. This approach has already been used in several iPSC-based studies to generate both, RTT and isogenic control neurons.[104,107,108] Although, the generation of isogenic controls was not always successful. For some missense mutations as T158M, in which still part of the truncated protein is expressed, researchers repetitively failed to generate both, patient and isogenic control lines.[107,108] Whether this is due to difficulties in identifying affected cell lines, expressing only truncated MeCP2, or by X-chromosomal skewing of those lines, is not clear. Especially, for skewed cells, sorting affected and unaffected cells can be a solution. However, depending on severity of skewing this approach can be challenging. Specifically, heavily skewed cell lines might only give rise to one certain cell type and therefore requires multiple rounds of sorting. Nevertheless, the use of isogenic

controls, specifically to study RTT, is very beneficial. As MeCP2 deficiency affects the expression of hundreds of genes, the identification of major pathways can be hidden by genetic variability. A comparison of RTT cell lines with isogenic controls allows excluding this variability and leads to a clearer and more defined outcome.

Disease Onset

The symptomatic onset of RTT is usually between 6 and 18 months of age. However, as RTT is a neurodevelopmental disorder, alterations are suggested to be present before symptomatic onset.[35] Phenotypic alterations caused by impaired brain development can be observed already before the first symptoms appear. Although stem cells are affected by a lack of MeCP2, the major disease mechanisms to cause the altered development of RTT neurons could not be identified yet.[109] More recently, early development of RTT neurons started to gain attention. By the identification of altered chloride homeostasis, together with alterations in E/I balance, researchers started to investigate the functional onset of RTT during early neuronal development.[62,113,114] However, identification of major mechanisms that are required to study further treatment options is still missing. Especially, critical time windows during brain development, before symptomatic onset, when brain plasticity is still high, need further attention. Treatments during these periods could potentially support brain development, delay the symptomatic onset or have a beneficial effect on disease progression. Therefore, studies investigating early neuronal development are required to understand how RTT affects early neuronal development and how the neuronal maturation can be supported.

AIMS AND GOALS

To advance current treatment strategies for RTT patients, we need a better understanding of the disease mechanisms. Different studies in *post mortem* tissues, rodent, and iPSC models, carrying *Mecp2* mutations, indicate morphologically and network changes starting at early developmental stages, considering changes in dendritic branching, impaired synaptic connectivity, and circuit changes. Key regulators in neural growth and development, such as IGF1 and KCC2, have been suggested to play a role in pathomechanisms. However, as MeCP2 is already present at an early stem cell stage, modulating many genes, it is expected that many other pathways during early neuronal development are involved. The major aim of this thesis is to understand early mechanisms that lead to cellular changes and clinical phenotypes. We hypothesize that the identification of those pathways gives new information regarding alterations in neuronal development occurring in RTT. Therefore, we plan to develop new iPSC models that allow us to examine these early developmental stages and to investigate the development of RTT neurons by different approaches.

Chapter 2:
It has been hypothesized that network changes underlying clinical phenotypes, e.g., seizures, are associated with affected neuronal development and changed KCC2 levels. KCC2 is essential for a functional E/I balance, further strengthening the importance of ion channel dysfunction in RTT patient brains. However, although earlier studies showed that KCC2 expression levels are decreased in the motor cortex and cerebellar tissue of RTT patients, we currently lack insight into alterations in other brain areas. In this chapter, we test expression levels of *KCC2* in different cortical areas and hippocampus from RTT patients and age-matched controls. We further investigate alterations of different isoforms of *KCC2*. In particular, we study *KCC2a* and *KCC2b* expression levels based on quantitative reverse transcription polymerase chain reaction (qPCR) analysis. By this approach, we want to further examine KCC2 alterations previously observed, to gather a better understanding regarding imbalanced E/I and impaired circuit development in RTT.

Chapter 3:
Earlier iPSC studies represented important features of the human disease, e.g. morphology, functionality, and impaired development. As MeCP2 affects multiple pathways, our aim, to study molecular pathways involved in network dysregulation at early development stages, requires RTT patient iPSC-based model system with isogenic controls. Additionally, it needs iPSCs generated via a non-integrative reprogramming method to retain the genome and ensure observed alterations are caused exclusively by *MECP2* mutations. In this chapter, describe an iPSC-based model system, involving RTT cells and isogenic controls from the same individual.

Chapter 4:
To confirm our model is representing previously observed RTT phenotype, we investigate KCC2 expression changes and impaired functionality of developing iPSC-derived RTT neurons. We differentiate iPSCs into cortical neuronal cultures that contain both inhibitory and excitatory neurons. We hypothesize that reduced levels of KCC2 are associated with a delay in SSA occurrence. Therefore, we analyse synchronous activity in RTT neurons and healthy controls over time by calcium imaging approach and identify alterations in the time point of occurrence, frequency of activity, and number of active cells.

Chapter 5:
Earlier objectives were aimed at creating a new iPSC-based model, representing typical features of RTT disease and involving isogenic controls. In this chapter, we aim to use this model as a base to study early alterations in neuronal stem cells. Here, we study protein

expression changes at early neuronal stem cell stages to identify the first pathways being affected in neuronal development in RTT. We hope that by comparing RTT and isogenic control neuronal stem cells, to identify proteins affected by the lack of MeCP2 and to gain new insights into underlying basic mechanisms involved in RTT.

Overall, with this thesis, we want to fill the gaps of knowledge regarding early developmental processes altered in RTT and identify what leads to the typically observed RTT phenotype in neurons. Our aim is to gather necessary insights to understand the causal relationship between mutations in *MECP2* and the devastating symptoms patients suffer from. By studying the early signs of RTT, we want to identify these coherences during early development and therefore present pathways which might be used as potential therapeutic target.

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