Pharmacotreatment of a mouse model of Rett syndrome with the radical scavenger Trolox: Detailed assessment of potential merits *in vitro* and *in vivo*

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Oliwia Alicja Janc

from

Chorzów, Poland

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Thesis Committee

Prof. Dr. Michael Müller
Universitätsmedizin Göttingen, Georg-August-Universität Göttingen,
Zentrum für Physiologie und Pathophysiologie, Institut für Neuro- und Sinnesphysiologie

Dr. Dr. Oliver Schlüter
European Neuroscience Institute Göttingen,
Molekulare Neurobiologie

Prof. Dr. Thomas Dresbach
Universitätsmedizin Göttingen, Georg-August-Universität Göttingen,
Zentrum für Anatomie, Institut für Anatomie und Embryologie

Examination Board

Prof. Dr. Dr. Hannelore Ehrenreich
Max-Planck-Institut für Experimentelle Medizin Göttingen,
Klinische Neurowissenschaften

Dr. Camin Dean
European Neuroscience Institute Göttingen,
Trans-synaptic Signaling

Prof. Dr. Tobias Moser
Universitätsmedizin Göttingen, Georg-August-Universität Göttingen,
Klinik für Hals-Nasen-Ohrenheilkunde

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By prevailing over all obstacles and distractions, one may unfailingly arrive at his chosen goal or destination.

Christopher Columbus

Declaration

I hereby declare that my doctoral thesis "**Pharmacotreatment of a mouse model of Rett syndrome with the radical scavenger Trolox: Detailed assessment of potential merits *in vitro* and *in vivo***" has been written independly with no other sources and aids than quoted. This thesis has not been submitted elsewhere for any academic degree.

Göttingen, February 2015

OLIWIA JANC

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List of abbreviations

ACSF	Artificial cerebrospinal fluid
ATP	Adenosinetriphosphate
Bdnf	Brain derived neurotropic factor
BMI	Body Mass Index
BRM	SWI/SNF-related protein
CA	Cornu ammonis
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CDKL5	Cyclin-dependent kinase-like 5
CN^-	Cyanide
CO_2	Carbon dioxide
CpG	Cytosine-phosphate-guanine
DC	Direct current
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
e.g.	Exempli gratia
FAD	Flavin adenine dinucleotide
fEPSP	Field excitatory postsynaptic potential
HDAC	Histone deacetylases
HSD	Hypoxia induced spreading depression
I/O	Input-Output
i.p.	Intraperitoneal
LTP	Long-term potentiation
MBD	Methyl-CpG-binding domain
MECP2 (human)	Methyl CpG binding protein 2 protein
MeCP2 (mouse)	Methyl CpG binding protein 2 protein
MECP2 (human)	Methyl CpG binding protein 2 encoding gene
Mecp2 (mouse)	Methyl CpG binding protein 2 encoding gene
$MgSO_4$	Magnesium sulfate
N_2	Nitrogen

NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotid
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Sodium phosphate
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate receptor
P	Postnatal
p	phosphorylated
PBS	Phosphate buffered saline
PFUAs	Polyunsaturated fatty acids
PPF	Paired Pulse Facilitation
Rh123	Rhodamine 123
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Sin3A	Paired amphipathic helix protein Sin3a
SLEs	Seizure-like events
STD	Standard deviation
STP	Short-term potentiation
st.	*Stratum*
TRD	Transcription repression domain
WT	Wildtype
3`	3-phosphate-end
4 – AP	4 aminopyridine
5`	5-phosphate-end

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Summary

Rett syndrome is a severe neurodevelopmental disorder, with an incidence of ~1/1000 female births, in which most patients carry mutations in the methyl-CpG binding protein 2 gene. No cure exists so far. Rett girls are born after a normal pregnancy and have an apparently normal development throughout the first 6-18 months of life. Subsequently, they start to show symptoms including severe cognitive impairment, stereotypic hand movements, loss of already learned skills, epilepsy, intermittent systemic hypoxia, breathing disturbances and impaired mitochondrial function. It has been reported that a subunit of complex III of the mitochondrial respiratory chain is among the potentially dysregulated genes, the inner mitochondrial membrane is leaking protons, and also brain ATP levels seem to be affected. In the present work, it could be clearly shown by FAD/NADH autofluorescence imaging that increased oxidative burden appears already after the first postnatal week in the hippocampus of Rett mice. Furthermore, it has been confirmed that the hippocampal synaptic plasticity and the susceptibility to hypoxia are impaired. To evaluate whether free radical scavengers are capable of improving neuronal and mitochondrial dysfunction, Trolox – a water soluble vitamin E derivative – was applied to acute brain slices *in vitro*. Also chronic *in vivo* treatment was performed by bidaily intraperitoneal injections of Trolox. *In vitro* experiments verified that Trolox dampens neuronal hyperexcitability, reinstates synaptic plasticity, ameliorates cellular redox balance, and improves hypoxia tolerance in *Mecp2*$^{-/y}$ hippocampus. Adverse side effects of Trolox on mitochondrial function and seizure susceptibility can be excluded. In contrast, chronic *in vivo* Trolox-treatment did not show any beneficial outcome on body weight and/or size, motor function and learning, exploratory behavior, breathing function, mitochondria, or neuronal network function. The blood glucose level, the hypoxia tolerance as well as the short-term potentiation were significantly improved in *Mecp2*$^{-/y}$ mice by low dose Trolox treatment. Taken together, these findings demonstrate that the scavenger treatment *in vitro* is very promising for the treatment of various aspects of the neuronal dysfunction in Rett syndrome. However, the *in vivo* study identifies the

route of drug administration and frequent animal handling as critical issues to be thoughtfully considered in study design.

1. Introduction

1.1. Classic Rett syndrome

Rett Syndrome, first described by the Austrian pediatrician Andreas Rett in 1966, is classified as a progressive neurodevelopmental disorder (International Statistical Classification of Diseases and Related Health Problems (ICD): F84.2). It affects almost exclusively girls and is estimated to occur with an incidence of ~1/10.000 live female births among all racial and ethnic groups worldwide (Hagberg, 1985; Leonard et al., 1997).

The disease progression of classic Rett syndrome, including the age of onset and especially the severity of symptoms, varies from girl to girl. Rett syndrome is characterized by an initial normal development and growth followed by a gradual appearance of mental and physical symptoms. As the syndrome progresses, the child loses the purposeful use of the hands and the ability to speak. Early symptoms mainly include problems with crawling or walking and diminished eye contact. The loss of functional use of the hands is followed by distinctive and compulsive hand movements such as wringing and washing. Symptoms include severe cognitive and mental disabilities – Rett girls often show autism-like behaviors -, slowed growth and seizures. Yet, other pronounced symptoms are cardiac abnormalities and very pronounced breathing difficulties, while awake, such as hyperventilation, long-lasting apneas and air swallowing (Shahbazian and Zoghbi, 2001).

Four stages of Rett syndrome have been characterized as described in detail below (Weaving et al., 2005).

Stage I, the early onset, typically starts between 6 and 18 months of age. Slowing down of the child's development may not necessarily be noticed. The child begins to show less eye contact and has reduced interest in the environment. Hand-wringing and decreased head growth may occur.

Stage II, the rapid destructive stage, usually begins between ages 1 and 4 and can last for weeks or months. Its onset can be rapid or gradual as the child loses purposeful hand skills and the language. Characteristic hand movements such as wringing, washing and clapping, as well as repetitive movements of the hands to the mouth often start during this stage. Breathing irregularities such as episodes of apnea and hyperventilation start to occur. Some girls also display autistic-like symptoms such as loss of social interaction and communication. Walking and initiating motor movements can be difficult. Slowed head growth is usually noticed during this stage.

Stage III, the plateau or pseudo-stationary stage, usually begins between ages 2 and 10 and can last for years. Apraxia, motor problems, and seizures are pronounced during this stage. Many girls remain in this stage for most of their lives.

Stage IV, the late motor deterioration stage, can last for years or even decades. Prominent features include reduced mobility, scoliosis, rigidity, spasticity, and increased muscle tone. Repetitive hand movements may decrease and eye gaze usually improves.

So far, there is no cure for Rett syndrome. Currently available treatments of the disorder are only symptomatic and supportive. Constant medication is needed to control breathing disturbances, motor difficulties, and anticonvulsant drugs should be administered to suppress seizures. Physical therapy can prolong mobility and some girls may need nutritional programs to help them maintain an adequate weight.

1.2. Genetic background - *MECP2* mutations in classic Rett syndrome

Since physicians can clinically diagnose Rett syndrome only by observing signs and symptoms and evaluating the child's physical and neurological status, a genetic test has been developed to complement the clinical diagnosis. This involves screening for

mutations in the human methyl CpG (cytosine-phosphate-guanine) binding 2 protein encoding gene (*MECP2*) (Buyse et al., 2000).

Mutations in exons 3-4 of the *MECP2* coding region have been found in 95% of classic Rett patients (Ravn et al., 2005). The *MECP2* gene resides on the X chromosome and codes for the human methyl-CpG-binding protein 2 (MECP2), which was shown to bind selectively to symmetrical 5`-methylated cystones within a single CpG dinucleotide (Lewis et al., 1992).

In 70%-90% of sporadic cases and approximately 50% of familial cases, it has been shown that mutations in the *MECP2* gene are the primary cause of Rett syndrome (Shahbazian and Zoghbi, 2001). Almost 70% of reported mutations result from C→T transition *de novo* mutations at one of the eight different 5`-CG.3`(CpG) hotspots within the *MECP2* gene (Lee et al., 2001). These include four missense and four nonsense mutations. Missense mutations have been reported to lead to milder forms of Rett syndrome compared to nonsense mutations which cause more severe phenotypes (Wan et al., 1999).

Due to the random chromosome X-inactivation pattern, girls with Rett syndrome exhibit on the tissue level a mosaic of “healthy” cells with normal MECP2 activity and “diseased” cells without MECP2 activity. In classic Rett syndrome, the X-inactivation pattern is close to 50:50 (Weaving et al., 2003). Yet, skewed X-inactivation has been implicated in easing the severity of specific X-linked mental retardation disorders due to preferred inactivation of the X chromosome that contains the mutant allele (Plenge et al., 2002). This explains the high variability in the phenotypes of Rett girls.

1.2.1. MeCP2 protein – Function and Expression

The human *MECP2* gene and the mouse ortholog *Mecp2* reside on the X chromosome (Xq28) and are composed of four exons (Quaderi et al., 1994). The encoded protein MeCP2 was first identified as a protein capable of binding to

methylated DNA and to interact with a high specificity and affinity to symmetrically methylated CpG dinucleotides (Lewis et al., 1992). CpG dinucleotides are most prominent in the heterochromatic regions of chromosomes as well as in the promoter regions of many genes. The mechanism of methylation of cytosine residues of CpG dinucleotides is important to guarantee gene silencing. The epigenetic mechanism of transcriptional repression has a high importance in X-inactivation and imprinting (Jeppesen and Turner, 1993; Pedone et al., 1999).

MeCP2 is a protein that couples deoxyribonucleic acid methylation to the silencing machinery. In mice and humans, alternative splicing of the *Mecp2/MECP2* gene, produces two different isoforms of the protein, MeCPE1 and MeCPE2 that bind symmetrically methylated CpG dinucleotides (Meehan et al., 1989; Lewis et al., 1992). Both MeCP2 isoforms are very similar and share the same functional domains (Zachariah and Rastegar, 2012). Yet, the MeCP2E1 is considered to be the major isoform in the brain (Dragich et al., 2007).

The human MECP2 polypeptide consists of a methyl-CpG-binding domain (MBD), a transcriptional repression domain (TRD) and the nuclear localization signal (NLS). The MBD consists of 85 amino acids and resides at the N-terminal end of the protein, where DNA can bind (Fig. 1).

Figure 1: Schematic representation of the human methyl-CpG-binding protein
The human MECP2 includes a MBD, the TRD, and the NLS. S80 and S421 indicate potential serine phosphorylation sites (modified according to Guy et al., 2011).

Nan and colleagues have shown that MeCP2 represses transcription via binding to the methylated CpG dinucleotides and a following recruitment of the co-repressor Sin3A and histone deacetylase (HDAC) (Fig. 2) (Nan et al. 1998). Yet, also a

correlation between MeCP2 and RNA polymerase II binding sites, which suggests that MeCP2 often binds to transcriptionally active promoters, has been shown (Fig. 2). Chahrour and colleagues have suggested that MeCP2 can function as a transcriptional activator. Thus, it appears that MeCP2 may be more correctly referred to as a transcriptional modulator rather than a transcriptional repressor (Chahrour et al., 2008). In addition to transcriptional regulation, MeCP2 has also been confirmed to be involved in alternative splicing (Meehan et al., 1992).

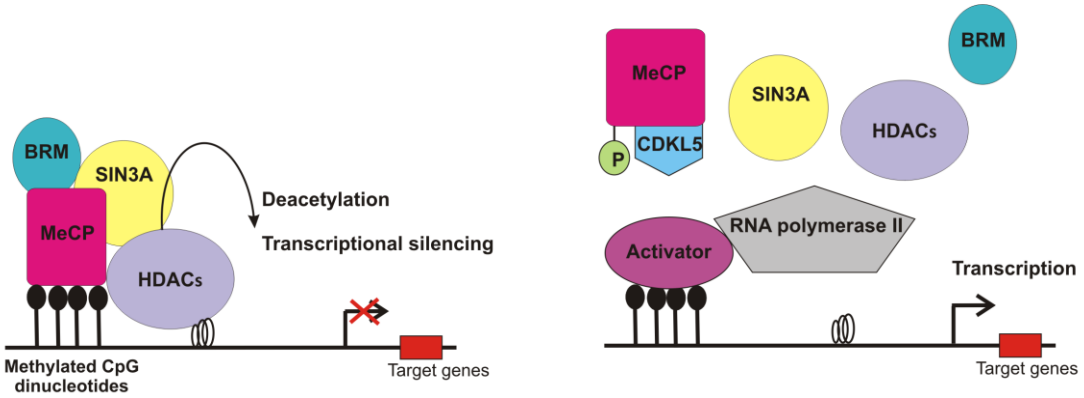

Figure 2: MeCP2 and its role in the regulation of transcription

Transcription is suppressed in promoter regions containing methylated CpGs that are bound by MeCP2 protein. MeCP2 binds methylated DNA and recruits chromatin-remodeling complexes that contain SIN3A (a transcriptional co-repressor), BRM (a SWI/SNF-related chromatin-remodeling protein) and histone deacetylases (HDACs). This leads to chromatin condensation owing to histone deacetylation, which results in a limited accessibility of the transcriptional machinery to promoter regions. When MeCP2 is not bound to methylated DNA (right panel), the complex that usually contains MeCP2, BRM, SIN3A and HDACs is not recruited. This lack of MeCP2 binding to DNA could be due to the activity of CDKL5 (cyclin-dependent kinase-like 5), which is thought to bind and contribute to the phosphorylation of MeCP2, resulting in the inability of MeCP2 to bind its methylated binding site (modified according to Bienvenu and Chelly, 2006).

According to the brain-specific phenotype associated with *MECP2* dysfunction, MeCP2 is believed to regulate genes involved in neuronal maturation and in maintaining synaptic plasticity. Several neuronal-related genes have been observed to be dysregulated in the brains of *Mecp2* mutant mice (Pelka et al., 2006; Smrt et

al., 2007; Chahrour et al., 2008). Therefore, it is interesting, that the brain derived neurotrophic factor (Bdnf), which has been shown to be a direct target of MeCP2, plays a major role in neuronal development and plasticity as well as the phenotypic overlap between mice lacking *Bdnf* or *Mecp2* (Chen et al., 2003; Chang et al., 2006). The highest expression of MeCP2 is in the brain, rather in neurons than in glia, and it increases progressively during human postnatal brain development (Balmer et al., 2003). As confirmed by immunohistochemical staining, the onset of increased MeCP2 expression is correlated with the state of maturation of different brain regions and cell types. This led to the suggestion that MeCP2 may play an important role in neuronal maturation (Smrt et al., 2007; Shahbazian et al., 2002; Balmer et al., 2003). These findings are consistent with the well known features of Rett syndrome such as an apparently normal early development and a reduced brain size with decreased dendritic branching complexity (Shahbazian et al., 2002). However, findings, that in the *Mecp2*$^{-/y}$ *mice* the late-onset neurological defects seem to be reversible by reexpression of MeCP2, revealed that MeCP2 plays also a role in the maintenance of neuronal function (Guy et al., 2007).

1.2.2. *MECP2* mutations in males

Male patients with mild *MECP2* mutations are able to survive the neonatal period but develop severe mental retardation as well as motor abnormalities (Hoffbuhr et al., 2001).

According to Ramocki and coworkers, they can be categorized into four main groups. In the first group, males with an extra X chromosome, known as Klinefelter syndrome (47, XXY) or somatic mosaicism, harboring a classic Rett syndrome mutation, phenotypically show classic Rett syndrome. The second group includes karyotypically normal males (46, XY) with *MECP2* mutations that cause classic Rett syndrome in females; these males show a severe congenital encephalopathy with early death. In the third group, males with *MECP2* mutations that have not been identified in females with Rett syndrome show a variable phenotype of mental retardation with spasticity and other features. The fourth group of male patients has

been reported to show an increased dosage of the *MECP2* gene due to duplication, the so-called *MECP2* duplication syndrome. These patients are characterized by almost the same symptoms like in classic Rett syndrome. Most males suffer from moderate to severe intellectual disability, have weak muscle tone in infancy, feeding difficulties, poor or absent speech, seizures, and/or spasticity. They have a delayed development of motor skills such as sitting and walking. Some lose their previously acquired skills (Ramocki et al., 2010).

1.3. Mouse model of Rett syndrome

In this study, *Mecp2*$^{tm\text{-}1\text{-}1Bird}$ mice, lacking exons 3 and 4 of the *Mecp2* gene were used; indicated as *Mecp2*$^{-/y}$ or male Rett mice (Fig. 3). These mice are bred on a C57/BL6 background and were generated originally using Cre-loxP technology (Guy et al., 2001). Male Rett mice with a null mutation show hindlimb-clasping, irregular breathing, decreased mobility, stunted body and head growth, microcephaly, and death within 6-10 weeks. Neuronal cell bodies and nuclei are reduced in the hippocampus, cortex and cerebellum (Chen et al., 2001; Guy et al., 2001). As shown by our group, mitochondria and cellular redox balance seem also to be impaired (Großer et al., 2012). In addition to this *Mecp2* knockout mouse, a variety of other Rett mouse models exists, most of which show markedly milder phenotypes (Shahbazian et al., 2002; Katz et al., 2009; Wegener et al., 2014).

Even though heterozygous female Rett mice (*Mecp2*$^{+/-}$) initially show no symptoms, they develop a variable onset of symptoms later in life. Typically, they develop hind-limb clasping and breathing abnormalities between 3-9 months of age (Guy et al., 2001).

Figure 3: *Mecp2*$^{-/y}$ mouse model of Rett syndrome

Mecp2$^{-/y}$ mice are not distinguishable from their WT littermates until postnatal day (P) ~20. During adolescence *Mecp2*$^{-/y}$ develop symptoms such as reduced body weight- and size, motor problems like hind-limb clasping, and highly irregular respiration.

1.4. Mitochondrial dysfunction, oxidative stress and its modulation of cellular physiology in Rett syndrome

Oxidative stress and abnormal mitochondrial metabolism seem to have a common origin in neurodegenerative and neurodevelopmental disorders associated with mental retardation and intellectual disability. There are strong indications that free radicals play a role in cerebral ischemia-reperfusion injury, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Down syndrome, fragile X syndrome, autism and Rett syndrome (Valenti et al.,2014; Zweier et al., 1987; Uttara et al., 2009; Gandhi and Abramov, 2012; Perluigi and Butterfield, 2012). In particular, the central nervous system is highly vulnerable to free radical damage, due to the brain's high oxygen consumption rate. The fundamental hypothesis of a disturbed redox balance is that cumulative oxidative damage over time may contribute to the progressive nature of these disorders.

1.4.1. Mitochondrial modulation of cellular processes

Mitochondria are the main source of the energy required for the maintenance and restoration of ion gradients (Duchen, 2000). Presynaptic terminals typically contain a couple of mitochondria. Neuronal stimulation of mature hippocampal neurons leads to a rapid mitochondrial accumulation in the proximity of dendritic spines (Li et al., 2004) and causes redistribution and enhancement of their activity in synapses (Miller and Sheetz, 2004). Hence, mitochondria-mediated changes in dendritic spine densities, shape and formation are suspected to be involved in neuronal and synaptic dysfunction and to contribute to neurological disorders (Yuste and Bonhoeffer, 2001).

Also neurotransmitters and neurotrophic factors control mitochondrial properties by influencing neuronal energy metabolism and dendritic and axonal motility (Liu and Shio, 2008; Mattson et al., 2008). Additionally, energy provided by mitochondria is essential for transmitting stimuli and signals and therefore crucial for neurogenesis and functioning of brain cells and neuronal networks (Valenti et al., 2014).

Disturbed function of the respiratory chain complexes can trigger increased reactive oxygen species (ROS) production, leading to oxidative stress (Raha and Robinson, 2000). Moreover, oxidative stress can be generated by an imbalance between ROS production and their scavenging by the cellular antioxidant system consisting of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase, and radical scavenging compounds, such as the vitamins C and E (Gandhi and Abramov, 2012).

1.4.2. Mitochondrial, synaptic and respiratory dysfunction in Rett syndrome

Mitochondrial dysfunctions, alterations in ROS homeostasis and redox regulation appear to be involved in Rett syndrome pathogenesis (Müller and Can, 2014). Muscle and frontal lobe biopsies of Rett patients revealed swollen mitochondria with vacuolization, granular inclusions and membranous changes (Eeg-Olofsson et

al., 1990; Dotti et al., 1993). Changes of mitochondrial structure have also been confirmed for cortical and hippocampal mitochondria of *Mecp2*$^{-/y}$ mice (Belichenko et al., 2009). Also a decrease in succinate-cytochrome *c* reductase and cytochrome *c* oxidase activity of muscle and frontal cortex biopsies of Rett patients have been shown (Heilstedt et al., 2002; Gibson et al., 2010). Indeed, subunits of complexes I and III of the respiratory chain seem to be indirectly controlled by *MeCP2* (Kriaucionis et al., 2006).

More evidence for oxidative burden in Rett syndrome has also been found in hippocampal slices of *Mecp2*$^{-/y}$ mice (Großer et al., 2012). Oxidative stress markers such as lipid peroxidation as well as redox imbalance have been reported to be increased in blood samples of Rett patients (Sierra et al., 2001; De Felice et al., 2011; De Felice et al., 2012). Since mitochondria are a major source of superoxide and because ROS output is directly linked to mitochondrial activity (Boveris and Chance, 1973) an increased basal mitochondrial respiration may lead to intensified ROS formation and consequently an imbalance in cellular redox status. In fact, blood samples of Rett patients revealed a reduced activity of the scavenging enzyme superoxide dismutase (Sierra et al., 2001) as well as decreased vitamin E levels (Formichi et al., 1998). Moreover, a recent study showed that brain redox changes could be detected already prior to symptoms onset (De Felice et al., 2014).

Long-term potentiation (LTP) is a form of synaptic plasticity, which is considered to underlie long-term memory formation. Impairments in LTP induction and/or maintenance have been correlated with general learning and memory deficits and alterations in synaptic plasticity. Electrophysiological studies of mouse models of Rett syndrome revealed impairments in long-term synaptic plasticity (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002; Pelka et al., 2006). Reduced LTP could be observed in hippocampal slices of *Mecp2*$^{-/y}$ mice (Asaka et al., 2006). Also cortical slices of *Mecp2*$^{308/y}$ mice, a mouse line expressing a truncated MeCP2 protein, showed diminished LTP induction suggesting that deficits in synaptic plasticity result from MeCP2 dysfunction (Moretti et al., 2006). Interestingly, even paired pulse facilitation (PPF), a well known test of short-term plasticity, is already

impaired in MeCP2 loss of function mutants (Asaka et al., 2006; Moretti et al., 2006; Fischer et al., 2009). In line with these findings, it has been also demonstrated that MeCP2 plays a fundamental role in synaptic homeostasis (Blackman et al., 2012).

It is known that the sudden death in Rett patients may arise as a consequence of cardiac dysfunction and breathing disturbances. In particular, the awake state is characterized by phases of irregular breathing and each of which goes along with a drop in the arterial O_2 level (< 60 mmHg) (Julu et al., 2001). This repeated hypoxia may lead to major changes of structure, function, and dynamics of mitochondria and because Rett patients run through several intermittent hypoxic episodes, this may aggravate the condition of already disturbed mitochondria.

1.5. Pharmacological tools to ameliorate oxidative burden

In neurodegenerative diseases most studies that focused on antioxidant therapies, have been done by administration of vitamin E, vitamin C, and coenzyme Q_{10}. Vitamin E was shown for example to reduce amyloid ß deposition and to improve cognition in an Alzheimer's disease mouse model (Conte et al., 2004). In another Alzheimer's disease mouse study, the administration of vitamin C was found to dissolve toxic protein aggregates (Cheng et al., 2011). Additionally, the administration of coenzyme Q_{10}, has been shown to protect MPTP-treated mice, a mouse model for parkinsonism, from dopaminergic neuronal loss and also attenuated α-synuclein aggregation (Shults et al., 1999). However, a benefit for the use of vitamin E and/or vitamin C in either Alzheimer's disease or Parkinson's disease from large randomized controlled trials could not be verified so far.

At present, there are no effective treatments to improve cognitive function, although numerous researchers aim to find new therapeutic treatments for various neurodevelopmental disorders. The strong evidence on the involvement of mitochondrial dysfunction and the resulting oxidative stress in the pathogenesis of Rett syndrome, leads to the conclusion that improvement of mitochondrial function can be an attractive strategy to improve clinical phenotypes.

In neurodevelopmental disorders such as fragile X syndrome, Down syndrome and Rett syndrome, studies regarding a diet rich of antioxidants, have been already intensively tested. Pathophysiological hallmarks such as free radical overproduction, oxidative stress and also behavior and learning deficits in a fragile X syndrome mouse model, were reversed by the pharmacological treatment with α-tocopherol, an antioxidant/radical scavenger (Osakada et al., 2003; de Diego-Otero et al., 2009). In case of Down syndrome, coenzyme Q_{10}, acetyl-l-carnitine, α-lipoic acid, α-tocopherol, and ascorbic acid, all strategies that support mitochondrial functions and reduce oxidative stress, have been intensely investigated for treatment of this disease (Lott et al., 2011; Pagano and Castello, 2012). Unfortunately, in clinical trials, the administration of standard antioxidant supplements has generally failed to produce satisfactory therapeutic effects (Lott et al., 2011; Lott et al., 2012).

In contrast, in Rett syndrome, the modulation of oxidative stress seems to be a promising approach in the potential therapeutic strategy to reverse some of the typical Rett symptoms. The administration of ω-3 polyunsaturated fatty acids (PFUAs) has been shown to significantly reduce the levels of several oxidative stress biomarkers in the blood of Rett patients. Moreover, a significant reduction in the clinical severity of symptoms such as motor-related signs, nonverbal communication deficits, and breathing abnormalities, has been observed (De Felice et al., 2012; Maffei et al., 2014).

1.6. Challenge to develop new therapies in Rett syndrome

Even though there is a strong indication of mitochondrial dysfunction in Rett syndrome, the detailed cause and origin of defective mitochondria remain still unclear. In particular, this includes the question how a loss of function of MeCP2 could be molecularly connected to a modification of mitochondrial function. Hence, further investigations need to be done to discover how alterations in the mitochondrial energy metabolism are involved in the pathogenesis of Rett syndrome. Furthermore, the very cause and consequences of oxidative stress in Rett syndrome need to be clarified.

Indeed, preclinical and clinical evidence supports the idea that pharmaceutical strategies aimed at improving mitochondrial function and reducing oxidative stress, may bear the potential of improving the quality of life of Rett patients.

2. Aim of this Thesis

1) First of all, the present thesis aimed to clarify if mitochondrial metabolism changes occur already at early postnatal stages and may be therefore a potential primary cause for the disease progression in Rett syndrome.

2) Vitamin E levels have been shown to be decreased in the blood serum of Rett patients (Formichi et al., 1998), therefore supplementation with vitamin E and/or its derivatives might be a potential pharmacotherapeutical approach. Hence, the next major task was to address, in detail, the potential merit of Trolox in acute hippocampal tissue slices of adult *Mecp2*$^{-/y}$ mice and WT. The main focus was on a potential improvement of synaptic function and plasticity, hypoxia tolerance, and mitochondrial function in the isolated hippocampal tissue of already symptomatic animals.

3) The concluding section of this thesis represents a preclinical trial. In detail, it was investigated whether *in vivo* treatment (intraperitoneal injections) would confirm the *in vitro* results regarding synaptic function and plasticity, hypoxia tolerance, and mitochondrial function. Moreover, potential changes in systemic parameters, behavioral and motor function after chronic radical scavenger injections were evaluated to get a full picture of the treatment efficacy of Trolox.

3. Materials and Methods

All experimental procedures were performed in accordance with the local regulations and were approved for the *in vitro* experiments, "Anoxia und neuronale Netzwerke (AZ: T13-08)", by the office for animal welfare of the Universitätsmedizin Göttingen. The *in vivo* experiments, „Behandlung eines Mausmodells für das Rett Syndrom mit Radikalfängern (AZ: 33.9-42502-04-12/0944)", were authorized by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit.

3.1. Solutions and pharmacological compounds

For the experimental procedures all chemicals were obtained from Sigma-Aldrich Chemie GmbH, unless stated otherwise.

During the preparation of acute slices and during the electrophysiological and optical recordings artificial cerebrospinal fluid (ACSF) was used as the bath solution. ACSF was composed of (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 1.2 $CaCl_2$, 1.2 $MgSO_4$, and 10 glucose. ACSF was constantly aerated with carbogen, a 95%O_2 and 5% CO_2 mixture to adjust the pH to 7.4 and to ensure proper tissue oxygenation. The composition of ACSF was based on the cerebrospinal fluid.

To inhibit the enzyme cytochrome c oxidase in the fourth complex of the electron transport chain, cyanide (CN^-, sodium salt) was dissolved as an aqueous 1 M stock solution and stored at -20°C. FCCP (carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone, Tocris Bioscience) was used as an uncoupling agent that dissipates the proton gradient across the inner mitochondrial membrane thereby causing maximal mitochondrial depolarization. This compound was dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock and stored at 4°C. Rhodamine 123 (Rh123), used as a measure of mitochondrial membrane polarization, was also dissolved as 10 mM stock, and stored at 4°C. All final DMSO concentrations were ≤ 0.02%. To evoke seizure-like events (SLEs) the convulsant 4-aminopyridine (4-AP) was dissolved as an aqueous 100 mM solution.

The water-soluble vitamin E derivative Trolox ((+/-)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Fig. 4) was directly added to the ACSF in the desired final concentration of 1 mM. All working dilutions were prepared immediately before use. The injection solution of Trolox was dissolved in phosphate buffered saline (PBS) and was prepared on a weekly basis.

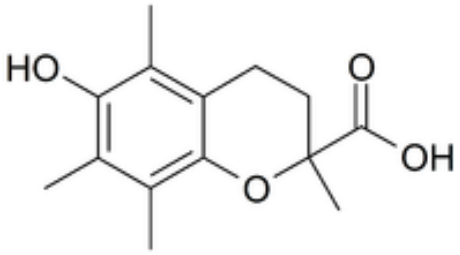

Figure 4: Chemical structure of Trolox

Trolox is a water-soluble analog of vitamin E and is used to reduce oxidative stress or damage.

3.2. Mouse model of Rett syndrome

Mice, lacking the MECP2 gene (B6.129P2(C)-Mecp2$^{tm\text{-}1\text{-}1Bird}$), were used as a Rett mouse model (Guy et al., 2001). Heterozygous female mice were obtained from Jackson Laboratories, Bar Harbor, ME. Mice were bred in the central animal facility of the University of Göttingen with C57BL/6J wild type (WT) males to generate heterozygous females (*Mecp2*$^{+/-}$), hemizygous males (*Mecp2*$^{-/y}$) and WT mice of either gender. Electrophysiological and optical recordings were performed on acute tissue slices obtained from hemizygous juvenile male mice between P7-10 or adult males ~P46. For all behavioral tests, mice were used around P45 ± 3. Animals were kept at a 12 h light-dark cycle and had free access to food pellets and water *ad libitum*. The genotyping of newborn mice was performed by technicians on tail biopsy material by using polymerase chain reaction. All Trolox-treated mice were genotyped twice for verification.

3.3. Systemic Trolox treatment – Drug application

Starting on P10-11, *Mecp2*$^{-/y}$ as well as WT mice were treated with Trolox, at concentrations of 10 and 40 mg/kg bodyweight, or PBS by performing intraperitoneal injections (i.p) every 48 h. Mice were therefore weighted and injected according to their body weight. The injection volume was limited to the allowed maximum for mice of 10 µl/g. The entire treatment (Fig. 5), the subsequent experiments and the analyses were performed as a blinded study.

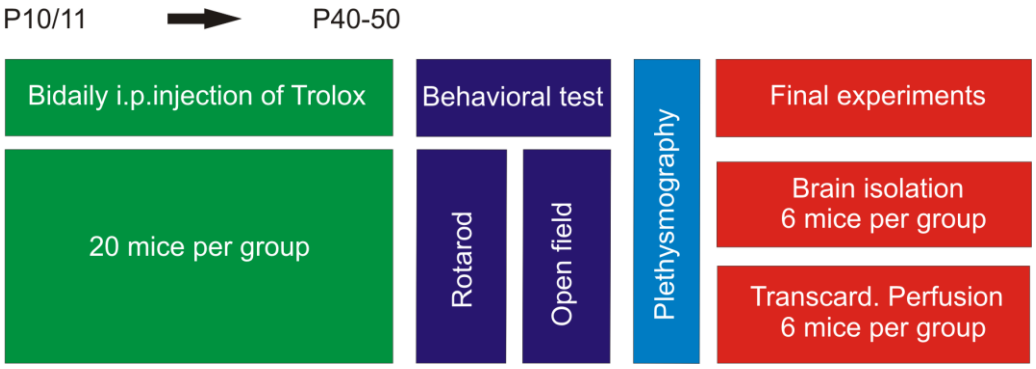

Figure 5: Schematic overview of the Trolox *in vivo* application

Mice were injected every 48 h for ~40 days. This was followed by behavioral tests like rotarod and open field. The breathing pattern was measured directly before the isolation of the brain and final experiments were performed.

3.4. Motor function & behavior

It is known that in general, *Mecp2* knockout mice display a noticeable difference in their behavior compared to the background strain. To evaluate whether Trolox treatment may rescue the strong behavioral and motoric phenotype in Rett mice, different tests were performed. To screen for general motor deficits the rotarod test was used (Fig. 6). To test the exploration behavior the open field was performed (Fig.7). The respective test area was cleaned thoroughly with 70% ethanol, after each mouse has been tested.

The rotarod test is used to evaluate motor skill, motor learning, coordination and balance and has been proven to be a good paradigm for the screening of drugs which could modulate motor coordination (Dunham and Miya, 1957). Here, the

rotarod system for mice (Ugo Basile, Comerio VA, Italy) with rotating rods, with a non-skid surface, was used (Fig. 6). Mice were placed on a rotating drum (3 cm diameter) and the time that each animal was able to maintain its balance on the rod was measured as latency to fall. During the test the rotation speed was continuously accelerated from 5 to 50 rpm over a period of 5 min (acceleration mode). Mice were tested on three consecutive days, in which the first day served to adapt the mice to the rotarod device. The second and third day were considered as the days the mice learned to stay on the rotarod and to improve their skills.

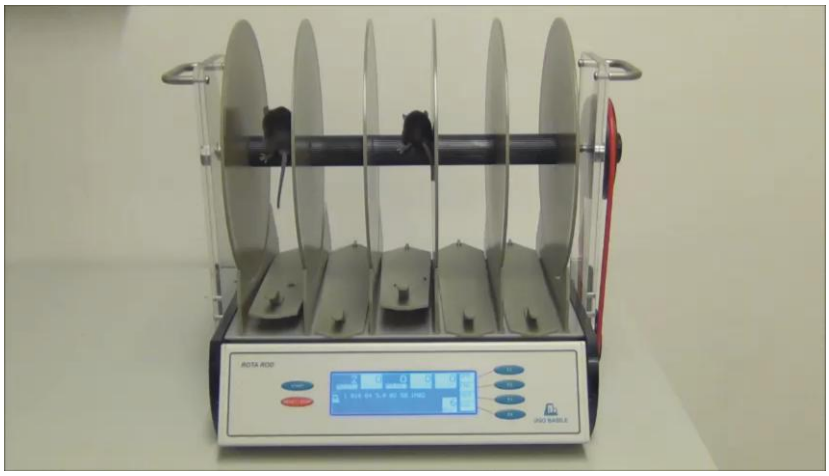

Figure 6: Rotarod – testing for motor function and learning

The rotarod apparatus can be used to assess motor coordination and learning. Mice were placed on the rotating drum and the time that passed before the mice fell down was recorded. All rotarod experiments were performed in an acceleration mode (5 rpm to 50 rpm in 5 min).

General locomotor activity and anxiety-like behavior of mice were evaluated in a square open field arena of 45×45 cm (Fig. 7A). Mice were tested once in the arena for 5 min, and their motility was monitored online by a grid of 2 frames of 16 infrared beams each covering the arena and tracked by using ActiTrack v2.7.13 software (Panlab, Harvard Apparatus). The open field was divided in 3 different areas, periphery, corner and center by a virtual grid, defined in the tracking software (Fig. 7B). The horizontal activity of the animals, their total distance traveled, the

relative time spent in the different areas, as well as the relative resting time were recorded.

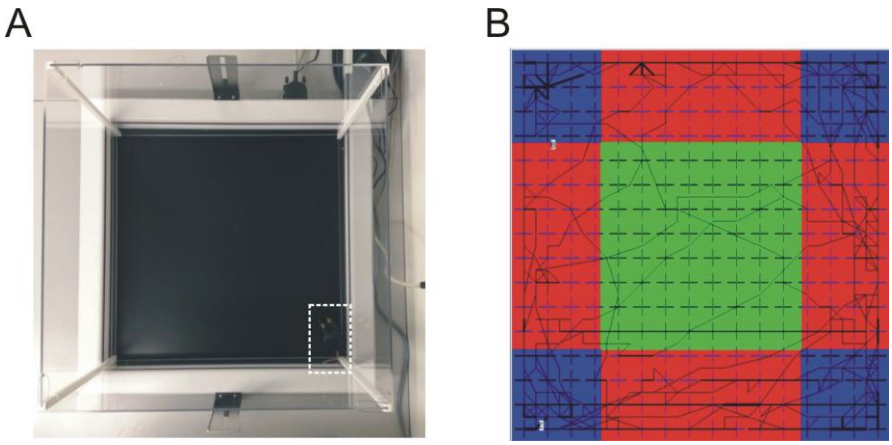

Figure 7: The open field – revealing exploration behavior

The open field test can be used to test activity, anxiety-like behavior, and drug effects on motor and exploration behavior. (**A**) Open Field while running an experiment (**B**) Original tracking trace, with the 3 distinct zones, red = periphery; green = center; blue = corner.

3.5. Unrestrained whole-body plethymography

The regularity of breathing was measured by the unrestrained whole-body plethysmography (Drorbaugh and Fenn, 1955) in collaboration with Prof. Swen Hülsmann (Klinik für Anästhesiologie), who assisted in the detailed respiration analyses since he has a long lasting expertise in plethysmograpic research. Mice were placed in a chamber and allowed to breathe naturally, unrestrained and untethered (Fig. 8A). The system measures the tiny airflow that is exchanged in and out of the entire chamber due to the animal's respiration. Recordings were performed by the software Ponemah v5 (Data Sciences International, St. Paul, Minnesota). Data were automatically analyzed by the threshold search event detection method of Clampfit 10.3 (Molecular Devices, Sunnyvale, CA). Breathing frequencies were calculated as the reciprocal of the averaged inspiratory interval. Furthermore, the irregularity score was determined as the normalized difference

between a pair of subsequent breathing cycles (Barthe and Clarac, 1997; Telgkamp et al., 2002; Wegener et al., 2014).

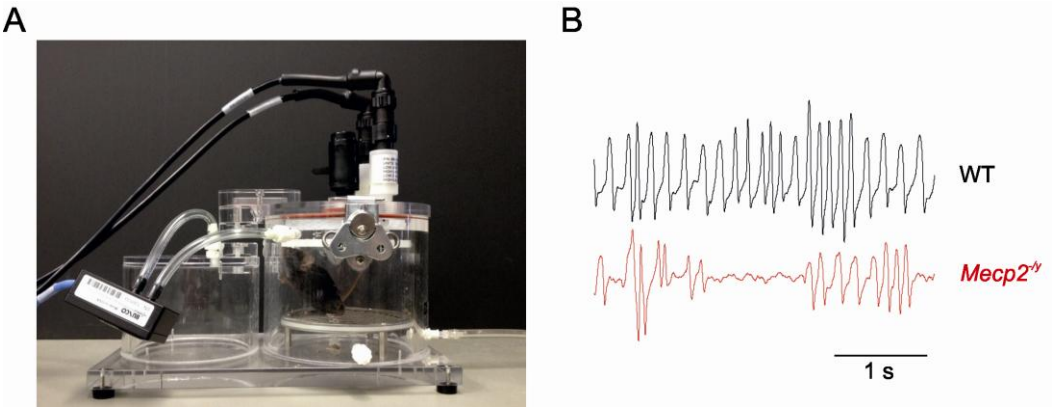

Figure 8: Unrestrained whole-body plethysmography – a measure of breathing

(A) The unrestrained whole-body plethysmography chamber enables to monitor physiologic respiration parameters. (B) Sample traces display a time segment of ~4 s of a healthy WT mouse and a *Mecp2*$^{-/y}$ mouse with clear apnea.

3.6. Preparation of acute brain slices

Mice were anesthetized with ether in an exsiccator and decapitated with a surgical scissor. The brain was quickly but gently removed from the skull and placed in chilled ACSF for 1-2 min. Coronal brain sections of 400 µm were cut using a vibroslicer (Campden Instruments, 752M Vibroslice). Whole brain slices were separated in the sagittal midline and depending on the experiments they were either directly transferred to an interface recording chamber or to a separate submersion-style storage chamber. Slices were left subsequently undisturbed for at least 90 min, to recover from the slicing procedure before experiments were started.

3.7. Blood parameters – Hematocrit and blood glucose level

The hematocrit was measured from blood samples obtained directly after decapitation from the neck of the respective mouse. It represents the volume percentage of the corpuscular fraction in the blood. The packed cell volume can be determined by centrifuging blood in standardized hematocrit capillaries (Brand

GmbH) at 12000 RPM for 5 min (Mikro 220; Hettich Zentrifugen). Blood glucose levels of each mouse were obtained by using a blood glucose meter (Contour®; Bayer Diabetes care).

3.8. Electrophysiology

Electrophysiological recordings were performed in an Oslo style interface recording chamber. For the assessment of synaptic plasticity and seizure activity slices were kept at a temperature of 31-32°C whereas for hypoxia experiments the temperature was increased to 35-36°C. To make sure that the slices stay viable and yield stable responses over the recording time, they were constantly aerated with carbogen at a flow rate of 400 ml/min and supplied with oxygenated and prewarmed ACSF at a flow rate of 3-4 ml/min. Extracellular recording electrodes were pulled from thin-walled borosilicate glass (GC150TF-10, Harvard Apparatus) on a horizontal electrode puller (Model P-97, Sutter Instruments). They were filled with ACSF, and their tips were trimmed to a resistance of ~5 MΩ. The stimulation electrode consisted of a bare stainless steel microwire (50 µm diameter, AM-Systems) soldered to a golden pin, which was connected to the photoelectric stimulus isolation unit (PSIU-6; Grass Instruments). Electrophysiological data were recorded with a locally constructed extracellular DC potential amplifier, constructed by the department's electronics workshop. Data were sampled using an Axon Instruments Digitizer 1322A and PClamp 9.2 (Molecular Devices, Sunnyvale, CA) and analyzed by Clampfit 9.2 software (Molecular Devices, Sunnyvale, CA). Field excitatory postsynaptic potentials (fEPSPs) were elicited by 0.1 ms unipolar stimuli (S88 stimulator with PSIU6 stimulus isolation units, Grass Instruments).

All electrophysiological extracellular recordings were performed in the Cornu ammonis (CA) 1 or 3 area of the hippocampus. Synaptic strength of Schaffer collateral/CA1 synapses was analyzed by using input/output (I/O) curves. Theses I/O curves were recorded by varying stimulus intensity from 10 µA to 150 µA in 10 µA increments. For basal synaptic transmission, fEPSP amplitudes were normalized to the fiber volley to abolish differences between the individual slices and variations in

electrode positioning. The detailed assessment of different types of synaptic plasticity was based on paired-pulse facilitation (PPF) and long-term potentiation (LTP) protocols. For PPF, stimulus intensity was set to the obtained half-maximum response and the interstimulus duration was varied in between 25 and 200 ms in 25 ms increments. Stable LTP was induced by high-frequency stimulation; 100 Hz delivered in three trains of 1 s duration each and separated by 5 min intervals. Potentiated responses were then followed for an additional hour. The temperature was set to 31-32°C, to prevent triggering of hypoxic spreading depression by high frequently stimulation.

Hypoxia-induced spreading depression (HSD) was triggered by switching the interface chamber's gas supply from carbogen to nitrogen (95% N_2 and 5% CO_2). O_2 withdrawal induced HSD within 2-3 min and O_2 was resubmitted 30 s after the onset of HSD. This ensured full recovery of the slices, but at the same time also allowed to fully develop its nadir.

Seizure-like events (SLEs) were monitored in *st. pyramidale* of the CA3 region as this region is more prone to induction of such discharges than the CA1 subfield. Frequently recurring SLEs representing the epileptic discharges were induced by application of the K^+-channel blocker 4-aminopyridine (4-AP), 100 µM for 15 min.

3.9. Optical recordings

For all optical recordings, analyses were performed in the st. radiatum, since synaptic function and HSD were assessed in this layer as well. A computer-controlled digital imaging system, composed of a polychromatic xenon-light source (Polychrome II, Till Photonics) and a sensitive charge-coupled device (CCD) SensiCam camera Imago QE (Till Photonics) attached to an upright epifluorescence microscope (Axiotech or Axioskop I) was used to image the flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH) autofluorescence as well as mitochondrial membrane potential (ψm). Acute hippocampal slices were kept in a

submersion-style chamber (30-33°C) and optical recordings were performed by using a 40x water immersion objective (Zeiss Achroplan, 0.8 NA).

FAD and NADH autofluorescence was monitored side by side by alternating the excitation at 360 nm (NADH) and 445 nm (FAD). The frame rate was 5 s and the exposure times were set to 70 ms (NADH) and 40 ms (FAD). The autofluorescence was recorded using a 450 nm beam-splitter and a 510/80 nm bandpass filter (Duchen and Biscoe, 1992; Foster et al., 2006; Gerich et al., 2006) (Fig. 9).

For ψm recordings (Emaus et al., 1986; Duchen, 1999) slices were bulk loaded with Rh123 (5 µM, 15 min) in a miniaturized staining chamber (Funke et al., 2007; Großer et al., 2012). Rh123 was used in quenching mode and therefore depolarization of mitochondria was indicated by an increase in Rh123 fluorescence (Emaus et al., 1986). Rh123 was excited at 480 nm with a frame rate of 5 s and an exposure time of 5 ms. The emission was recorded using a 505 nm beam-splitter and a 535/35 nm bandpass filter.

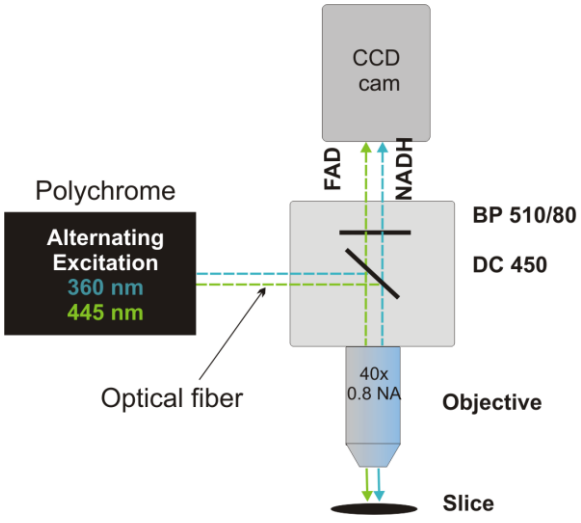

Figure 9: Monitoring NADH and FAD

NADH and FAD autofluorescence was recorded simultaneously from the same slice by alternating the excitation at 360 nm (NADH) and 445 nm (FAD).

3.10. Statistics

3-6 slices from at least 5 different brains of each genotype and treatment group were used for electrophysiological or optical recordings. For all behavioral experiments at least 10 mice of each group were tested. All numerical values are represented as mean ± standard deviation; the number of experiments (n) refers to the number of slices or mice analyzed. Significance of the observed changes was tested by SigmaStat 3.5 (Systat Software GmbH) one way ANOVA and a significance level of P=0.05. In the diagrams, statistically significant changes are indicated by asterisks (* P<0.05; ** P<0.01; *** P<0.001), and refer, if not mentioned otherwise, to differences between *Mecp2*$^{-/y}$ and the respective WT group.

4. Results

In the present thesis, the aim was to verify whether mitochondrial metabolism changes in *Mecp2*$^{-/y}$ mice occur already at early postnatal stages and therefore may be a primary cause of Rett syndrome. Moreover, synaptic function and plasticity, neuronal excitability, hypoxia susceptibility, mitochondrial function as well as behavior and motor function of *Mecp2*$^{-/y}$ mice were assessed. The potential benefit of a scavenger treatment was evaluated in the presence of Trolox by bath application (*in vitro*) and by a chronic systemic Trolox treatment (*in vivo*).

4.1. Higher oxidative burden already at early neonatal stages

Fluorescence recordings have the advantage to diagnose the status quo of tissue metabolism. One can use these applications to acquire functional information of the tissue's redox status. Since the mitochondrial metabolic coenzymes FAD and NADH, are autofluorescent, changes in the redox status can be monitored very elegantly without the use of further fluorophores (Duchen and Biscoe, 1992; Foster et al., 2006). The fluorescence signals of these endogenous fluorophores can be used as indicators of tissue metabolism in injuries, especially due to hypoxia which may occur for example during apneas in Rett patients.

In previous studies of our laboratory, a shift towards higher oxidation was detected in hippocampal slices of adult male Rett mice (Großer et al., 2012). Hence, the question arose, whether these mitochondrial changes already occur in early developmental stages. Therefore, dynamic recordings of FAD and NADH levels in acute hippocampal slices of neonatal mice (P7-10) were performed by monitoring tissue autofluorescence. An altered baseline FAD/NADH ratio was, indeed, already detectable in neonates (Table 1; Fig. 10A). In the *st. radiatum* of early neonatal *Mecp2*$^{-/y}$ slices the FAD/NADH ratio was increased by an average of 5.5% as compared to WT slices (Table 1). Moreover, pharmacological inhibition of the mitochondrial respiration by CN^-, an inhibitor of the enzyme cytochrome c oxidase in the fourth complex of the electron transport chain, increased NADH fluorescence,

decreased FAD fluorescence, and therefore decreased the FAD/NADH ratio. However, significant differences in the CN^--evoked responses (100 µM or 1 mM, 3 min) in the recorded autofluorescence among the genotypes were not observed. Although in the presence of CN^- the FAD/NADH ratio still tended to be slightly more oxidized in *Mecp2*$^{-/y}$ *st. radiatum* as compared to WT slices (Table1; Fig.10A).

	WT	***Mecp2*$^{-/y}$**
ACSF	2.57 ± 0.17; *n* = 16	2.71 ± 0.18; *n* = 26
100 µM CN^-	2.28 ± 0.18; *n* = 16	2.4 ± 0.19; *n* = 26
1 mM CN^-	2.12 ± 0.18; *n* = 16	2.22 ± 0.19; *n* = 26

Table 1: Overview of basal and CN^- mediated changes in mitochondrial metabolism analyzed by the FAD/NADH ratio

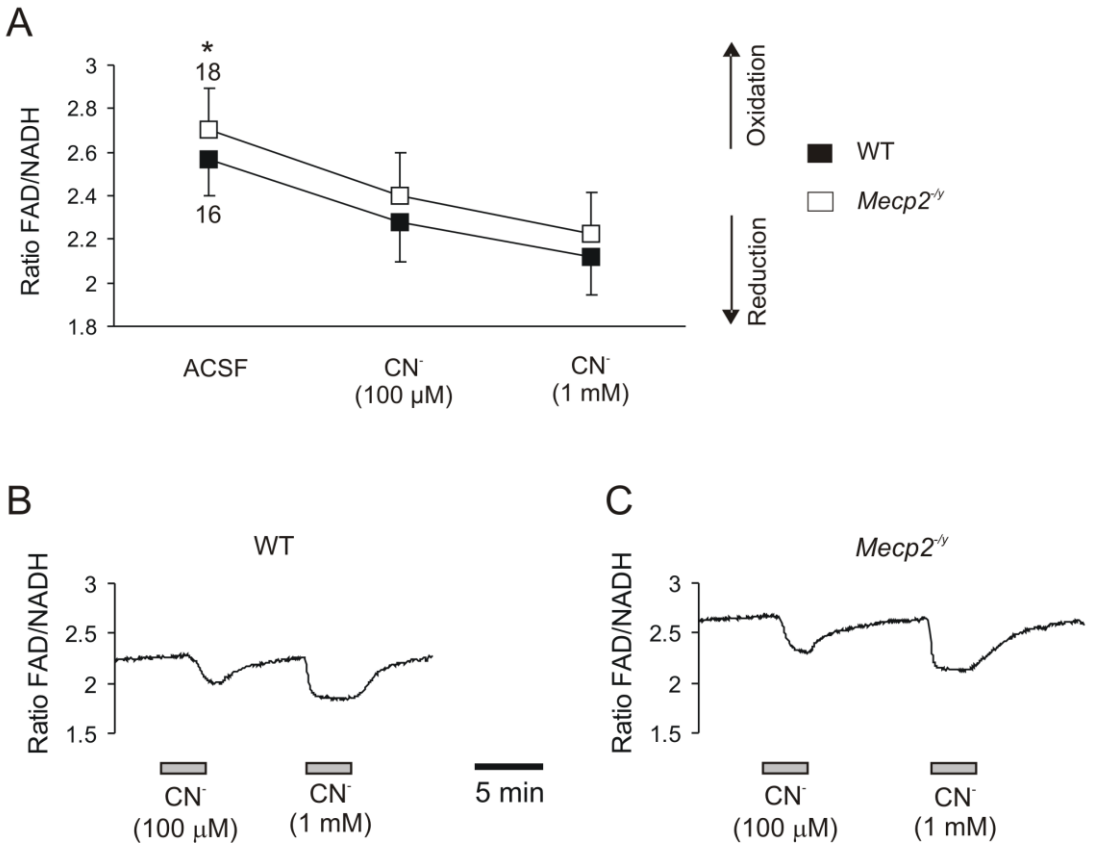

Figure 10: Imaging of tissue autofluorescence confirms a shift towards oxidation in neonatal *Mecp2*$^{-/y}$ hippocampus

NADH and FAD autofluorescence was recorded from hippocampal slices by alternating the excitation at 360 nm (NADH) and 445 nm (FAD). (**A**) Ratiometric analysis of FAD and NADH autofluorescence allows a quantitative comparison of *Mecp2*$^{-/y}$ and WT slices. Already first alterations in FAD/NADH ratio were evident in slices from neonatal mice (P7–10). This confirms an early onset of disturbed mitochondrial function. Mitochondrial poisoning by 100 µM or 1 mM CN^- unequivocally abolished these baseline differences. The error bars indicate standard deviations of the plotted averages. (**B,C**) Sample traces from *Mecp2*$^{-/y}$ and WT of the resulting FAD/NADH ratio recorded from neonatal mice. Time scaling is identical for both panels.

4.2. Acute Trolox treatment

To evaluate the potential outcome of a treatment of MeCP2-deficient neuronal networks with free radical scavengers, acute hippocampal tissue slices of adult *Mecp2*$^{-/y}$ and WT mice were incubated with 1 mM Trolox for at least 3 h (range 3–5 h). The detailed effects on synaptic function and synaptic plasticity, neuronal excitability, hypoxia susceptibility as well as mitochondrial function were then assessed in the continued presence of Trolox. To screen for a potential reversal of typical Rett syndrome symptoms, all experiments were performed at an age, at which *Mecp2*$^{-/y}$ mice have already developed clear phenotypic symptoms, i.e., around P45–50.

4.2.1. Modulation of neuronal excitability and synaptic function

4.2.1.1. Trolox treatment improves the hypoxia tolerance

Findings from our group previously reported that MeCP2-deficient mouse hippocampal tissue shows an increased susceptibility to hypoxia i.e., the onset of the response to severe hypoxia was significantly hastened in *Mecp2*$^{-/y}$ hippocampal slices compared to WT slices. Hence, MeCP2-deficient neurons tolerated only a shorter duration of O_2 shortage before neuronal membrane potentials collapsed (Fischer et al., 2009; Kron and Müller, 2010).

In the present thesis, it could be confirmed that untreated *Mecp2*$^{-/y}$ slices generated HSD upon O_2 withdrawal significantly earlier (~28%) as compared to WT slices (Table 2; Fig. 11B). In contrast to this, the amplitude and duration of the HSD-associated extracellular DC potential shift did not differ among genotypes (Table 2; Fig. 11B). To screen whether the *in vitro* Trolox treatment would modulate the hypoxia tolerance in *Mecp2*$^{-/y}$ slices, HSD was induced upon Trolox bath application. Interestingly, Trolox treatment (Trolox recirculated in the interface chamber) clearly delayed the onset to HSD in *Mecp2*$^{-/y}$ slices but did not postpone the occurrence of HSD in WT slices (Table 2; Fig. 11B).

	Treatment group	WT	*Mecp2*$^{-/y}$
Time to onset [s]	ACSF	146.77 ± 47; *n* = 37	106.15 ± 28; *n* = 32
	1 mM Trolox	133.14 ± 32; *n* = 37	147.21 ± 39; *n* = 36
Amplitude [mV]	ACSF	16.43 ± 3; *n* = 37	17.85 ± 4; *n* = 32
	1 mM Trolox	15.76 ± 3; *n* = 37	15.43 ± 3; *n* = 36
Duration [s]	ACSF	66.29 ± 13; *n* = 37	68.38 ± 18; *n* = 32
	1 mM Trolox	67.44 ± 15; *n* = 37	71.97 ± 21; *n* = 36

Table 2: Overview of the characteristic parameters of the HSD-associated DC potential shift such as time to onset, amplitude and duration with and without Trolox application

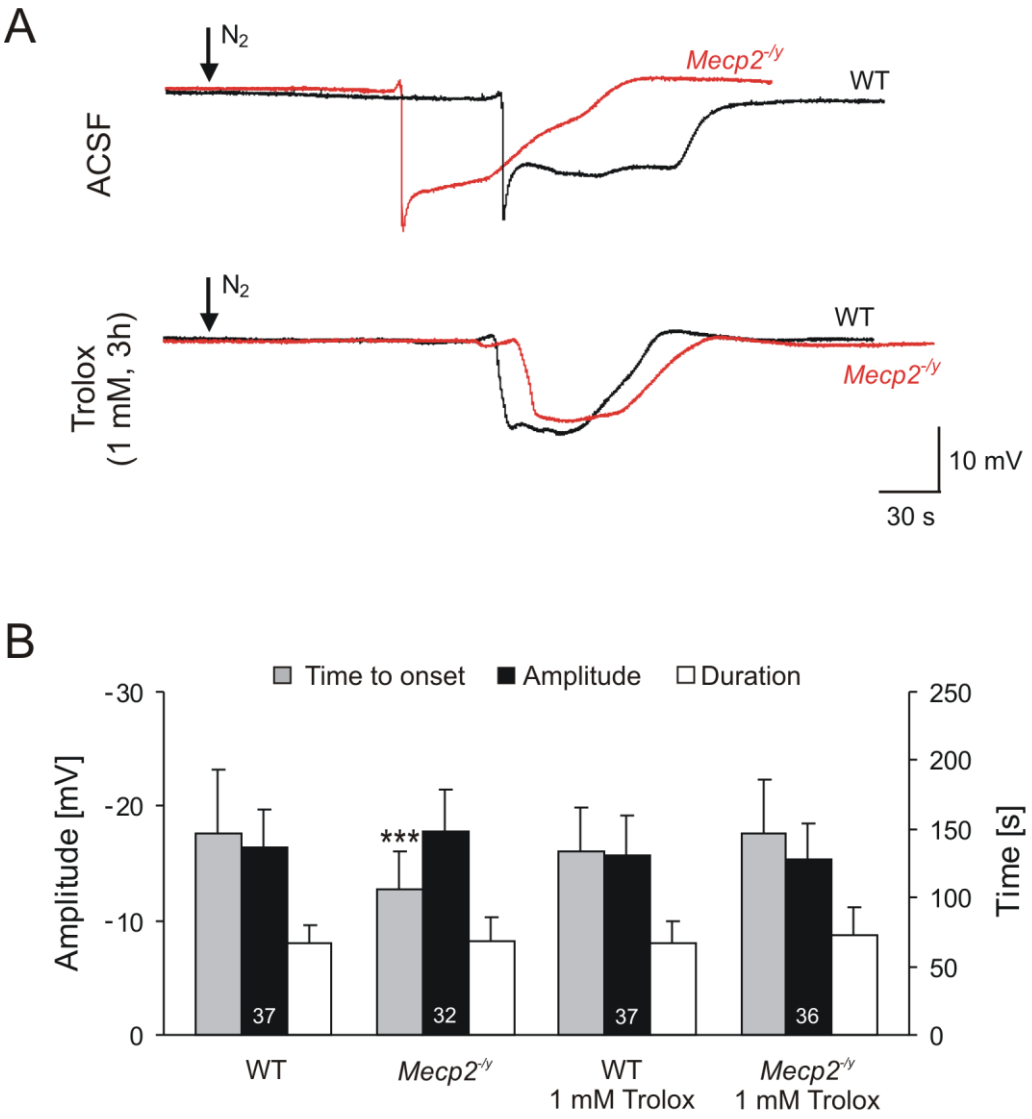

Figure 11: Trolox improves the hypoxia tolerance of *Mecp2*$^{-/y}$ hippocampus

(**A**) Sample traces from HSD recordings of *Mecp2*$^{-/y}$ and WT slices. Arrows indicate the start of severe hypoxia (O_2.withdrawal). The reoxygenation of slices was resubmitted 30 s upon HSD onset. The response to severe hypoxia was significantly hastened in *Mecp2*$^{-/y}$ hippocampal slices as compared to WT slices. However, incubation of slices with Trolox selectively postponed HSD onset in adult *Mecp2*$^{-/y}$ slices to WT level. (**B**) Characteristic parameters of the HSD-associated DC potential shift such as amplitude of the negative DC shift, the time to onset and the duration revealed that Trolox postponed the onset of HSD in *Mecp2*$^{-/y}$ slices, but did not affect the other parameters.

4.2.1.2. Input-output curve and paired pulse facilitation

Several studies showed that synaptic plasticity is impaired in Rett mice (Asaka et al., 2006; Moretti et al., 2006; Guy et al., 2007; Fischer et al., 2009). Hence, the impact of Trolox on various types of synaptic modulation was analyzed. To assess basal synaptic function and synaptic short-term plasticity, recordings of evoked fEPSPs were performed in the *st. radiatum* of the CA1 subfield of the hippocampus (Fig. 12).

For basal synaptic transmission, fEPSP amplitudes were normalized to the fiber volley to abolish differences between the individual slices and variations in electrode positioning. Under control conditions, *Mecp2*$^{-/y}$ slices (*n* = 37) showed significantly (~44%) higher fEPSP/fiber volley ratios than WT slices (*n* = 50) at all stimulation intensities tested (Fig. 12A), which indicates an increased postsynaptic responsiveness and neuronal hyperexcitability. Trolox treatment of slices abolished the genotypic differences in fEPSP/fiber volley ratios, by decreasing the responses in *Mecp2*$^{-/y}$ slices (*n* = 43) to those levels observed in untreated and treated WT slices. In WT slices, Trolox did not induce any significant changes in the fEPSP/fiber volley ratio (*n* = 44).

Synaptic short-term plasticity was assessed as PPF based on twin-pulse stimulation (Fig. 12B). Stimulation intensity was adjusted to evoke half-maximum response amplitudes and the interpulse interval was varied stepwise between 25 and 200 ms. While this potentiated the amplitude of the 2nd fEPSP in WT slices to 184.0 ± 34.4% (*n* = 52) of control, *Mecp2*$^{-/y}$ slices showed a significantly less pronounced fEPSP facilitation to only 165.9 ± 35.7% (*n* = 35) for the shortest interpulse interval tested (25 ms, Fig. 12B). Trolox treatment abolished this moderate genotypic difference in short-term plasticity at the 25 ms interval, but otherwise did not induce any significant changes in *Mecp2*$^{-/y}$ (*n* = 39) and WT slices (*n* = 47).

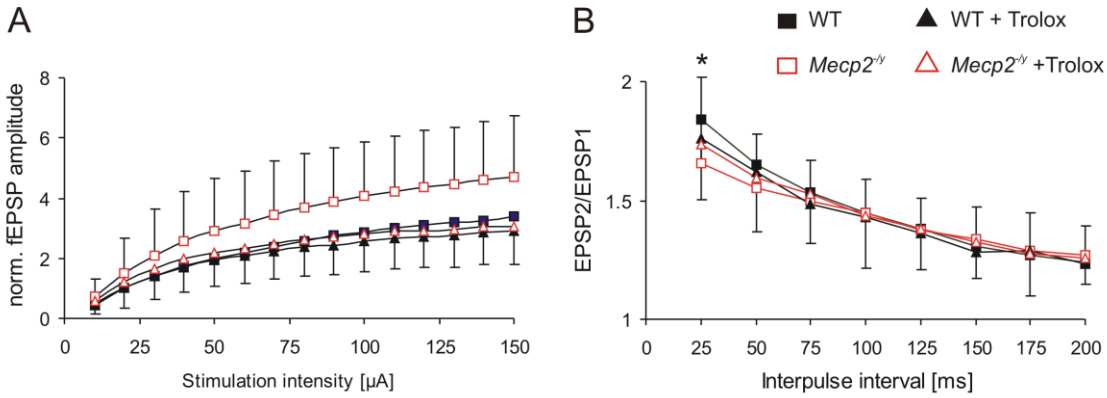

Figure 12: Trolox dampens neuronal hyperexcitability

(**A**) A significantly increased excitability in *Mecp2*$^{-/y}$ slices was found as compared to WT at all stimulation intensities. Trolox abolished this genotypic difference. The plotted fEPSP amplitudes are normalized to the fiber volley of the respective slice. (**B**) PPF, a measure of short-term plasticity, was elicited over a range of interpulse intervals. The degree of PPF in hippocampal slices from *Mecp2*$^{-/y}$ mice was lower than that in WT slices for the shortest interpulse-interval tested. Upon Trolox treatment this genotypic minor difference was no longer detectable. For clarity standard deviations are only included for untreated *Mecp2*$^{-/y}$ and Trolox treated *Mecp2*$^{-/y}$slices.

4.2.1.3 LTP is rescued by *in vitro* application of Trolox

LTP in the CA1 region of the hippocampus is an activity-dependent long lasting increase in the activity of neurotransmission that requires, among others, *N*-Methyl-D-aspartic acid (NMDA) receptor activation, and is thought to represent a cellular mechanism underlying some forms of learning and memory (Bliss and Collingridge, 1993; Lynch, 2004). Hence, alterations in the excitatory synaptic plasticity and strength could be considered as one cause contributing to the cognitive and functional deficits seen in Rett patients and has been also already shown by several other groups to be impaired in Rett mouse models (Asaka et al., 2006; Moretti et al., 2006). Based on this knowledge, the potential modulation of short-term potentiation (STP) and LTP by Trolox was assessed in this study.

Extracellular field potential recordings were performed in the CA1 of hippocampal slices with or without Trolox in the bath solution for the duration of experiments.

STP and LTP were induced by high-frequency stimulation (Fig. 13). The fEPSPs, taken after a 10-minute baseline, were significantly different between WT and *Mecp2*$^{-/y}$ slices. In detail, STP in *Mecp2*$^{-/y}$ was significantly reduced (Table 3; Fig. 13B) and 1h after LTP induction (range 50–60 min), fEPSPs were still potentiated in WT slices, but showed a less intense degree of LTP in *Mecp2*$^{-/y}$ (Table 3; Fig. 13B). Trolox, however, improved the extent of both, STP and LTP in *Mecp2*$^{-/y}$ slices which was represented by a stable LTP even 1 h after tetanic stimulation (Table 3; Fig. 13B,C). Moreover, a stimulating effect of Trolox on WT slices was not observed. Instead, the extent of STP slightly declined and LTP showed a tendency of being less pronounced in the presence of Trolox than in untreated WT slices (Table 3; Fig. 13B,C).

	Treatment group	**WT**	***Mecp2*$^{-/y}$**
STP [%]	ACSF	238.2± 62; *n* = 12	158.7 ± 38; *n* = 11
	1 mM Trolox	172.4 ± 54; *n* = 9	199.3 ± 35; *n* = 10
LTP [%]	ACSF	179.4 ± 31; *n* = 12	143.3 ± 19; *n* = 11
	1 mM Trolox	155.8 ± 33,; *n* = 9	181.1 ± 32; *n* = 10

Table 3: Effect of *in vitro* Trolox application on short and long-term synaptic plasticity

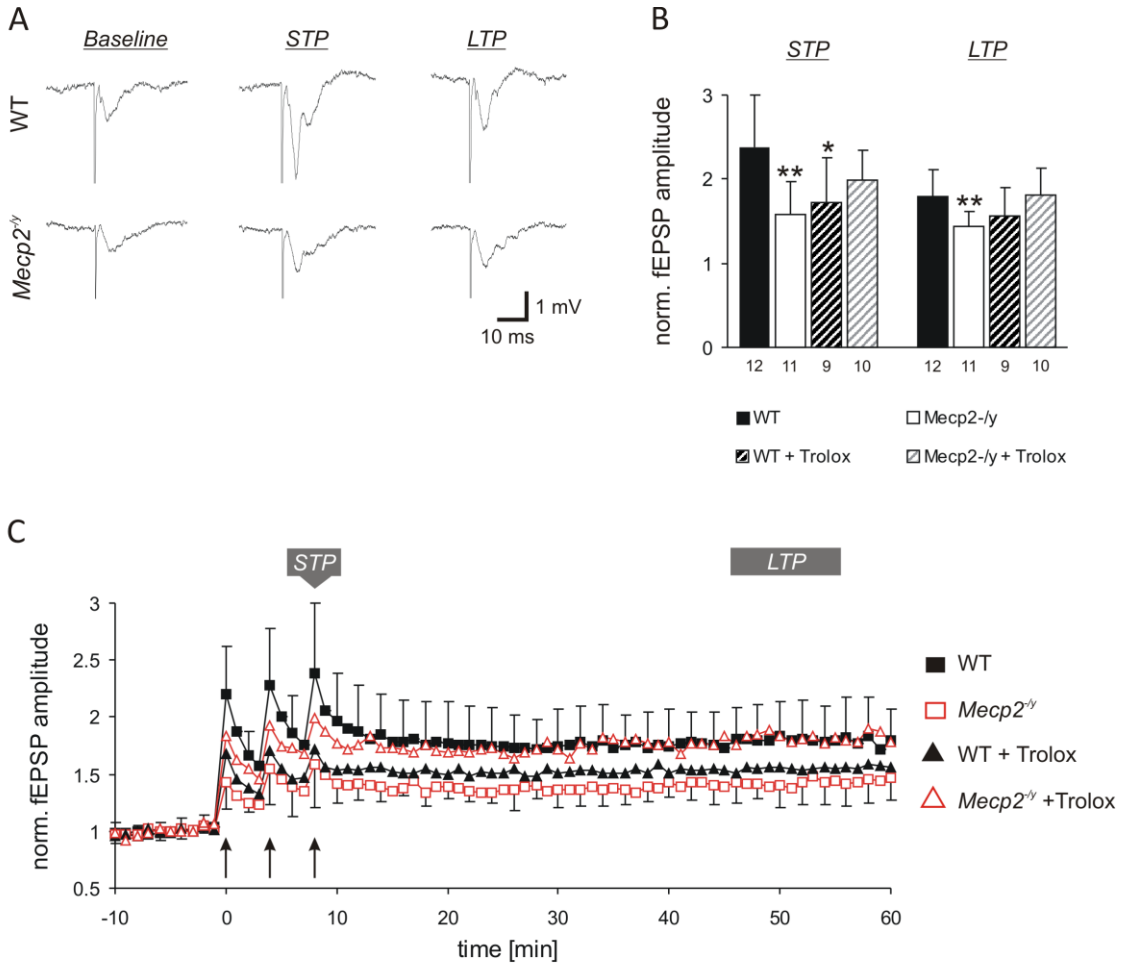

Figure 13: Improved STP and LTP after Trolox application

(**A**) Sample fEPSPs recorded from *Mecp2*$^{-/y}$ and WT slices in ACSF under baseline conditions, immediately after the 3rd high-frequency stimulation (STP) and 1 h after inducing potentiation (LTP). Please note the stimulus artifacts are truncated. For clarity, error bars are plotted for every second data point of WT and *Mecp2*$^{-/y}$ slices only. (**B**) STP and LTP were significantly impaired in *Mecp2*$^{-/y}$ compared to WT slices. Trolox, however, improved STP and LTP in *Mecp2*$^{-/y}$ slices to levels seen in untreated WT slices. In WT, Trolox dampened the extent of LTP to conditions typically found in untreated *Mecp2*$^{-/y}$ slices. The number of slices analyzed is indicated below each bar (**C**) LTP was induced by three consecutive trains of 100 Hz stimuli, lasting 1 s each (see arrow marks).

4.2.2. Anticonvulsive potential of Trolox

Rett patients show an increased incidence of epileptic seizures (Hagberg et al., 1983; Steffenburg et al., 2001) and increased neuronal excitability is also evident in MeCP2-deficient mice (Medrihan et al., 2008; Fischer et al., 2009; Calfa et al., 2011; Toloe et al., 2014). Since Trolox successfully dampened neuronal hyperexcitability in *Mecp2*$^{-/y}$ slices, the potential anticonvulsive effect of Trolox was tested. Seizure activity was provoked by the K^+-channel inhibitor 4-AP (Rutecki et al., 1987) and the resulting SLEs were recorded extracellularly in *st. pyramidale* of the CA3 subfield, as hyperexcitability in Rett mouse hippocampus arises particularly in this region (Calfa et al., 2011).

Extracellular recordings in CA3 from slices bathed in 4-AP (100 µM, 35 min) triggered SLEs which long-lasting discharges, which were analyzed in detail for the last 5 min of each experiment (Table 4; Fig. 14B). SLEs arose within similar times to onset in WT and *Mecp2*$^{-/y}$ slices. Trolox treatment (1 mM, 3–5 h) showed a tendency to postpone the onset of SLEs in WT slices only but the frequency of discharges was not significantly affected (Table 4; Fig. 14B). The duration of the individual SLEs was variable and showed only a tendency to decrease upon Trolox treatment in both WT and *Mecp2*$^{-/y}$ slices (Table 4; Fig. 14C). It should be noted, that in some experiments SLEs could only be evoked in some of the slices obtained from a particular mouse.

	Treatment group	WT	*Mecp2*$^{-/y}$
Time to onset [s]	ACSF	555.7 ± 124.3; *n* = 11	554.1 ± 136.2; *n* = 12
	1 mM Trolox	730.2 ± 253.8; *n* = 11	567.8 ± 104.8; *n* = 9
Frequency [SLEs/5 min]	ACSF	135.5 ± 87.3; *n* = 11	105.3 ± 77.8; *n* = 12
	1 mM Trolox	109.2 ± 52.4 ; *n* = 11	99.3 ± 56.6; *n* = 9
SLEs Duration [ms]	ACSF	361.5 ± 254.9 ; *n* = 11	428.6 ± 192.7; *n* = 12
	1 mM Trolox	278.2 ± 176.2; *n* = 11	293.1 ± 187.1; *n* = 9

Table 4: Detailed listing of the characteristic features of SLEs shift such as time to onset, frequency and duration with and without Trolox application

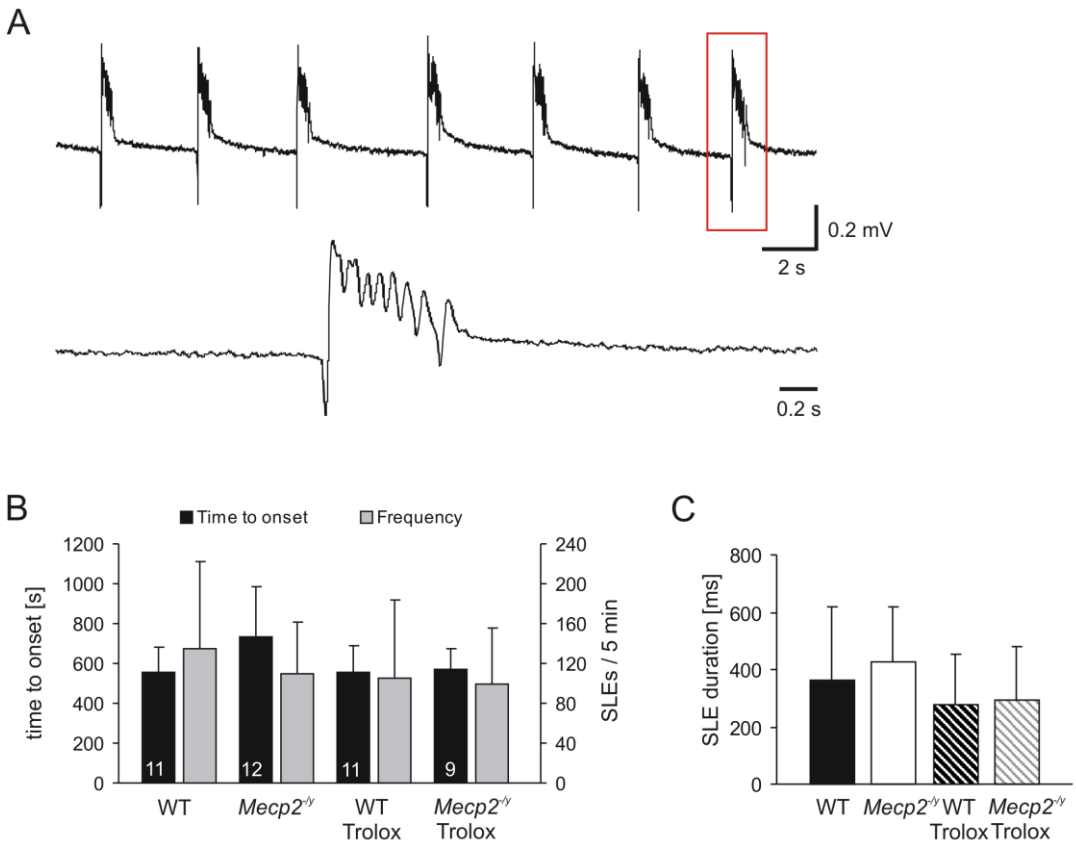

Figure 14: Trolox only shows a general tendency to dampen seizure susceptibility

(**A**) Sample traces of SLEs recorded from an untreated *Mecp2*$^{-/y}$ slice in CA3 *st. pyramidale*. The lower trace shows a SLE (red box) at a stretched time scale. (**B**) The time to onset and the SLE frequency, plotted as the number of discharges within the analyzed time interval of 5 min, did not differ among *Mecp2*$^{-/y}$ and WT slices under control conditions and Trolox application. Trolox statistically tended to postpone the onset of discharges in WT slices as compared to untreated slices. (**C**) Significant genotypic differences in the duration of the individual SLEs were not observed. Yet, Trolox tended to reduce the duration of the individual discharges in *Mecp2*$^{-/y}$ and WT slices.

4.2.4. Modulation of mitochondrial function

To check whether Trolox modulates mitochondrial function in adult Rett mice tissue, mitochondrial metabolism was assessed by imaging FAD and NADH autofluorescence (Duchen and Biscoe, 1992; Foster et al., 2006) and mitochondrial membrane potential ($\Delta\psi_m$) by recording Rh123 fluorescence (Emaus et al., 1986; Duchen, 1999). These imaging experiments were performed on the tissue level in *st. radiatum* of the CA1 subfield of acute adult tissue slices (Fig. 15A), as also synaptic function was analyzed in this layer.

Under baseline conditions the ratio of FAD/NADH autofluorescence was slightly increased in *Mecp2*$^{-/y}$ hippocampus, which indicates an intensified basal mitochondrial respiration. The FAD/NADH ratio was increased slightly by an average of 10.9% in *Mecp2*$^{-/y}$ slices as compared to WT (Table 5; Fig. 15A,B). Trolox did not mediate any statistically significant changes in these metabolic parameters of either WT or *Mecp2*$^{-/y}$ slices. Furthermore, also the influence of pharmacological inhibition of the respiratory chain by low and high doses of CN^- (100 µM, 1 mM) was examined. The application of CN^- for 3 min caused a dose-dependent decrease in FAD/NADH ratio which did not differ between WT and *Mecp2*$^{-/y}$ slices (Table 5; Fig. 15C). Trolox, however, significantly dampened the inhibitory effects of both low and high CN^- concentrations in *Mecp2*$^{-/y}$ slices. No such effects were evident in WT slices (Fig. 15C). These findings suggest that Trolox may reduce the susceptibility of mitochondrial metabolism against anoxia.

	Treatment group	WT	*Mecp2*$^{-/y}$
ACSF [ratio]	ACSF	1.92 ± 0.1; *n* = 19	2.09 ± 0.2; *n* = 17
	1 mM Trolox	2.02 ± 0.3; *n* = 15	2.11 ± 0.3; *n* = 19
100 µM CN^- [ratio]	ACSF	1.53 ± 0.1; *n* = 19	1.63 ± 0.2; *n* = 17
	1 mM Trolox	1.55 ± 0.1; *n* = 15	1.71 ± 0.2; *n* = 19
1 mM CN^- [ratio]	ACSF	1.27 ± 0.1; *n* = 19	1.35 ± 0.2; *n* = 17
	1 mM Trolox	1.29 ± 0.1; *n* = 15	1.44 ± 0.3; *n* = 19

Table 5: Summary of basal and CN^--mediated changes in mitochondrial metabolism with and without Trolox application, analyzed by the FAD/NADH ratio

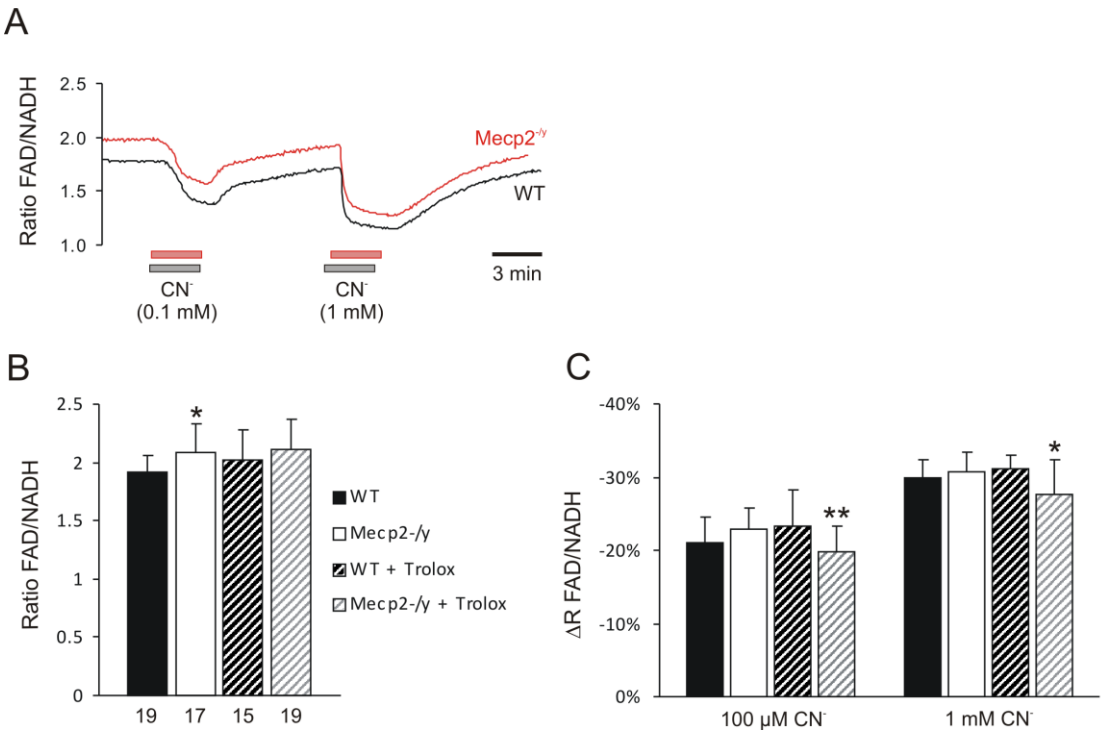

Figure 15: Trolox only slightly affects mitochondrial metabolism

(**A**) Sample traces of the decreases in FAD/NADH ratio induced by CN^- in untreated WT and *Mecp2*$^{-/y}$ slices recorded in CA1 *st. radiatum*. Gray and red bars indicate the time points and duration of drug application to the WT and *Mecp2*$^{-/y}$ slice, respectively. (**B**) Analyses of *Mecp2*$^{-/y}$ slices revealed an increased FAD/NADH baseline ratio, indicating intensified mitochondrial respiration. Application of Trolox did not affect basal respiration. Bar shading and patterns are identical for panels B and C. (**C**) Mitochondrial challenge by CN^- (3 min) inhibits the respiratory chain, and thereby decreases the FAD/NADH ratio. Trolox slightly dampened these effects of CN^- in *Mecp2*$^{-/y}$ slices but not in WT.

In addition, earlier experiments of our group revealed a partly depolarized $\Delta\psi_m$ in adult *Mecp2*$^{-/y}$ hippocampal slices (Großer et al., 2012). Corresponding Rh123 recordings of mitochondrial $\Delta\psi_m$ changes were performed. Less intense $\Delta\psi_m$ responses in *Mecp2*$^{-/y}$ slices upon mitochondrial uncoupling by 5 µM FCCP than in WT slices (Table 6; Fig. 16B,C), and thus a less negative $\Delta\psi_m$ could be confirmed in this thesis. Upon Trolox treatment, both WT and *Mecp2*$^{-/y}$ slices tended to show slightly more intense Rh123 responses to FCCP. Therefore, the genotypic difference became smaller and was no longer statistically significant (Table 6; Figure 16C).

Cyanide-mediated inhibition of mitochondrial respiration evoked marked increases in Rh123 fluorescence, indicating strong mitochondrial depolarization (Fig. 16D). Rh123 changes were normalized to the complete mitochondrial depolarization induced by FCCP. Genotypic differences were, however, not observed in the responses of *Mecp2*$^{-/y}$ and WT slices to low mitochondrial challenge and high CN^- doses (Fig. 16D). Trolox did not modulate the extent of the CN^--induced mitochondrial depolarization. Only in the case of high CN^- concentrations, the Rh123 responses tended to be slightly higher in Trolox-treated *Mecp2*$^{-/y}$ slices.

	Treatment group	**WT**	***Mecp2*$^{-/y}$**
normalized ΔF Rh123 to FCCP	ACSF	0.61 ± 0.20; *n* = 20	0.47 ± 0.19; *n* = 20
	1 mM Trolox	0.67 ± 0.23; *n* = 15	0.56 ± 0.19; *n* = 17

Table 6: Rh123 responses triggered by the mitochondrial uncoupler FCCP normalized to baseline

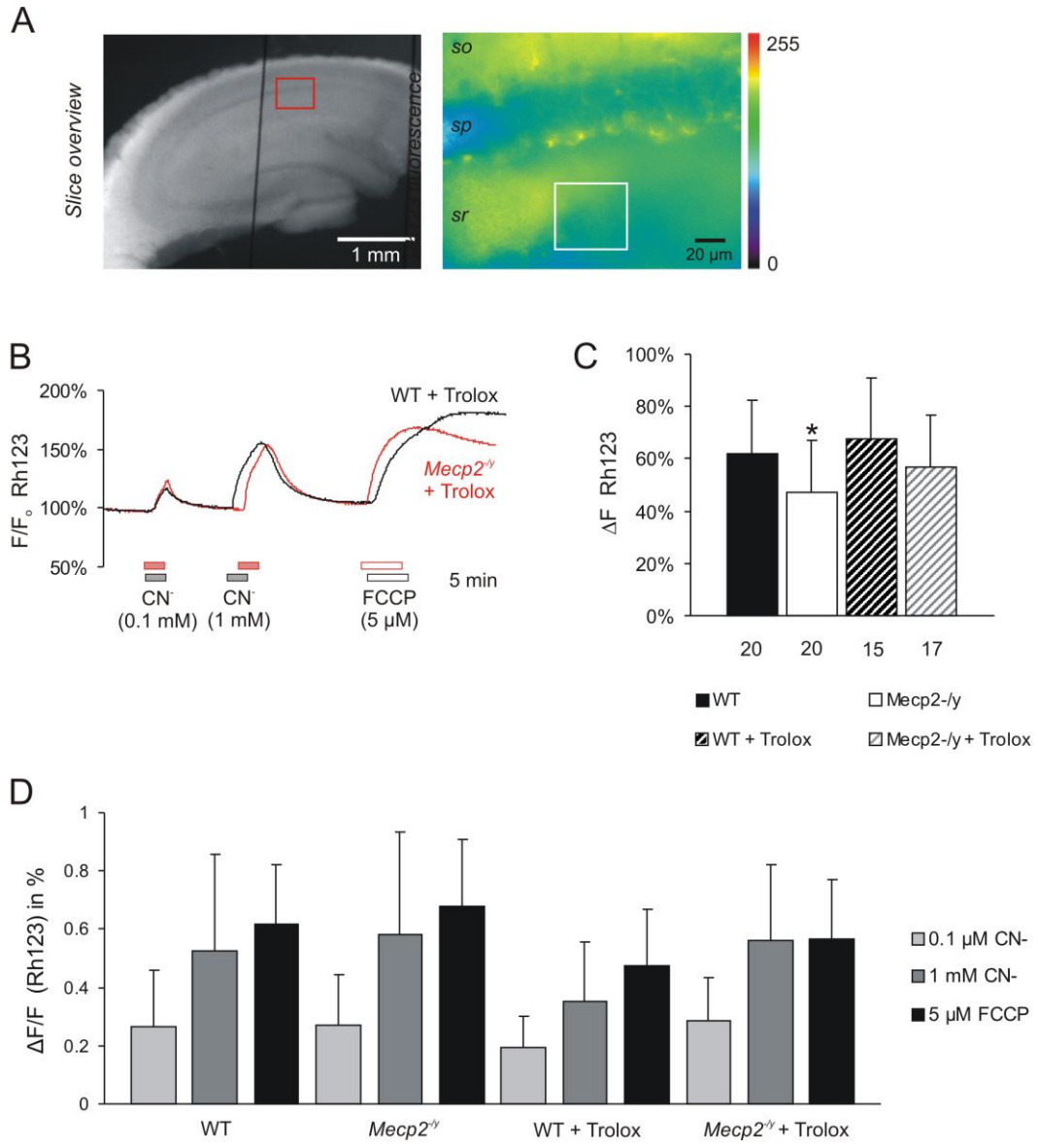

Figure 16: No effects of Trolox on mitochondrial metabolism and function

(**A**) Overview of a Rh123-labeled slice viewed under transillumination. The zoomed field of view (red box) used for the Rh123 recordings. A typical region of interest (white box) is shown under 485 nm excitation. (**B**) Superimposed sample traces of the relative Rh123 fluorescence changes (F/F_o) evoked by CN^- and FCCP in a Trolox-treated WT and *Mecp2*$^{-/y}$ slice. Drug treatment of the WT and *Mecp2*$^{-/y}$ slice is indicated by gray and red bars, respectively. (**C**) Rh123 responses to FCCP mediated uncoupling, as referred to baseline conditions. Note that *Mecp2*$^{-/y}$ slices show less intense Rh123 responses. Trolox application, however, diminished this difference among the two genotypes. (**D**) Summary of CN^--evoked and FCCP-mediated increases in Rh123 fluorescence as referred to baseline.

4.3. Systemic Trolox treatment

To evaluate the potential benefit of an *in vivo* treatment on the phenotype of *Mecp2*$^{-/y}$ mice and therefore also on MeCP2-deficient neuronal networks with the free radical scavenger Trolox, WT and *Mecp2*$^{-/y}$ mice were treated with either PBS, 10 mg/kg or 40 mg/kg of Trolox by i.p. injections. Injections were performed every second day and the entire study was blind until the last mouse had been measured and analyzed. Injections were conducted every second day to minimize the risk of peritonitis, embolism, adhesions, infections and to reduce stress. Mice tolerated the injections well and became less nervous with time. From time to time, the abdomen of a random chosen mouse was opened and inspected for changes in the intraperitoneal cavity. No obvious injuries could be ever observed.

As clearly early changes in the mitochondrial function have been seen in Rett mice, a resulting pharmacotherapeutical treatment should start already at early postnatal and presymptomatic stages. Mice were, therefore, injected from day 10/11 after birth. To be able to screen for a potential reversal of typical Rett syndrome symptoms, also in the systemically treated mice treatment was continued up to P45–50. Furthermore, to get a full picture of the treatment outcome, a multitude of parameters such as effects of the treatment on synaptic function and plasticity, neuronal excitability, hypoxia susceptibility as well as mitochondrial function was then assessed in the isolated tissue of these mice. These studies were performed as in the previous *in vitro* experiments. Also motor performance and exploration behavior were tested to evaluate potential beneficial outcome of the treatment.

4.3.1. Systemic parameters

Blood glucose level, hematocrit, Body Mass Index (BMI) and the body weight over injection time were assessed to define the efficacy of the *in vivo* Trolox treatment also on systemic parameters.

Increased glucose utilization in Rett patients confirmed alterations in glucose metabolism (Villemagne et al., 2002). In *Mecp2*$^{-/y}$ mice, a loss of MeCP2 function has been shown to increase insulin levels, decrease insulin signaling and therefore to result in a dissociation of insulin levels and the appropriate metabolic effects of insulin on glucose regulation (Pitcher et al., 2013). In the present study, the blood glucose level in *Mecp2*$^{-/y}$ mice, treated with PBS only, was significantly lower by about 30% as compared to the WT. Injection of *Mecp2*$^{-/y}$ mice with either low or high dose of Trolox increased the blood glucose to levels seen in the respective WT mice (Table 7; Fig. 17A), i.e., rescued this systemic parameter.

To test for a systemic adaptation of *Mecp2*$^{-/y}$ mice to the repeated apnea-related systemic hypoxic episodes, the hematocrit was determined. It was measured from blood samples collected during dissection, and was significantly higher in all *Mecp2*$^{-/y}$ treatment groups compared to WT mice. This suggests that *Mecp2*$^{-/y}$ mice, even after receiving chronic Trolox treatment, still underwent repeated systemic hypoxic episodes during their life (Table 7; Fig. 17B).

The BMI is a measure of the relative weight based on an individual's body size and does not consider body tissue composition. Trolox treatment did not evoke any changes in the BMI among the different treatment groups of both genotypes, but revealed, as expected from the reduced growth of *Mecp2*$^{-/y}$ mice, a significant lower BMI in all *Mecp2*$^{-/y}$ mice compared to WT mice (Table 7; Fig. 17C). The growth of mice was followed in detail as body weight had to be determined every second day for Trolox injection dosing.

In general, *Mecp2*$^{-/y}$ mice appeared normal at birth but started to develop significantly reduced body weights by around 3 weeks of age (Fig. 17D). The final body weight (~P46) of *Mecp2*$^{-/y}$ mice was about half of the weight of WT mice.

	Treatment group	WT	*Mecp2*$^{-/y}$
Blood glucose [mmol/l]	PBS only	14.1 ± 2.4; *n* = 17	10.1 ± 3.3; *n* = 16
	10 mg/kg Trolox	12.7 ± 2.3; *n* = 16	11.8 ± 2.0; *n* = 16
	40 mg/kg Trolox	12.2 ± 2.0; *n* = 21	12.2 ± 4.3; *n* = 15
Hematocrit [%]			
	PBS only	43.5 ± 2.5; *n* = 17	47.9 ± 3.6; *n* = 16
	10 mg/kg Trolox	42.3 ± 3.3; *n* = 16	47.5 ± 3.3; *n* = 19
	40 mg/kg Trolox	42.6 ± 3.3; *n* = 22	47.8 ± 3.6; *n* = 14
BMI [g/mm^2]			
	PBS only	0.34 ± 0.02; *n* = 17	0.29 ± 0.03; *n* = 15
	10 mg/kg Trolox	0.34 ± 0.02; *n* = 16	0.29 ± 0.03; *n* = 18
	40 mg/kg Trolox	0.34 ± 0.03; *n* = 22	0.29 ± 0.02; *n* = 13

Table 7: Comparison of systemic parameters such as blood glucose, hematocrit and BMI obtained from *in vivo* treated mice

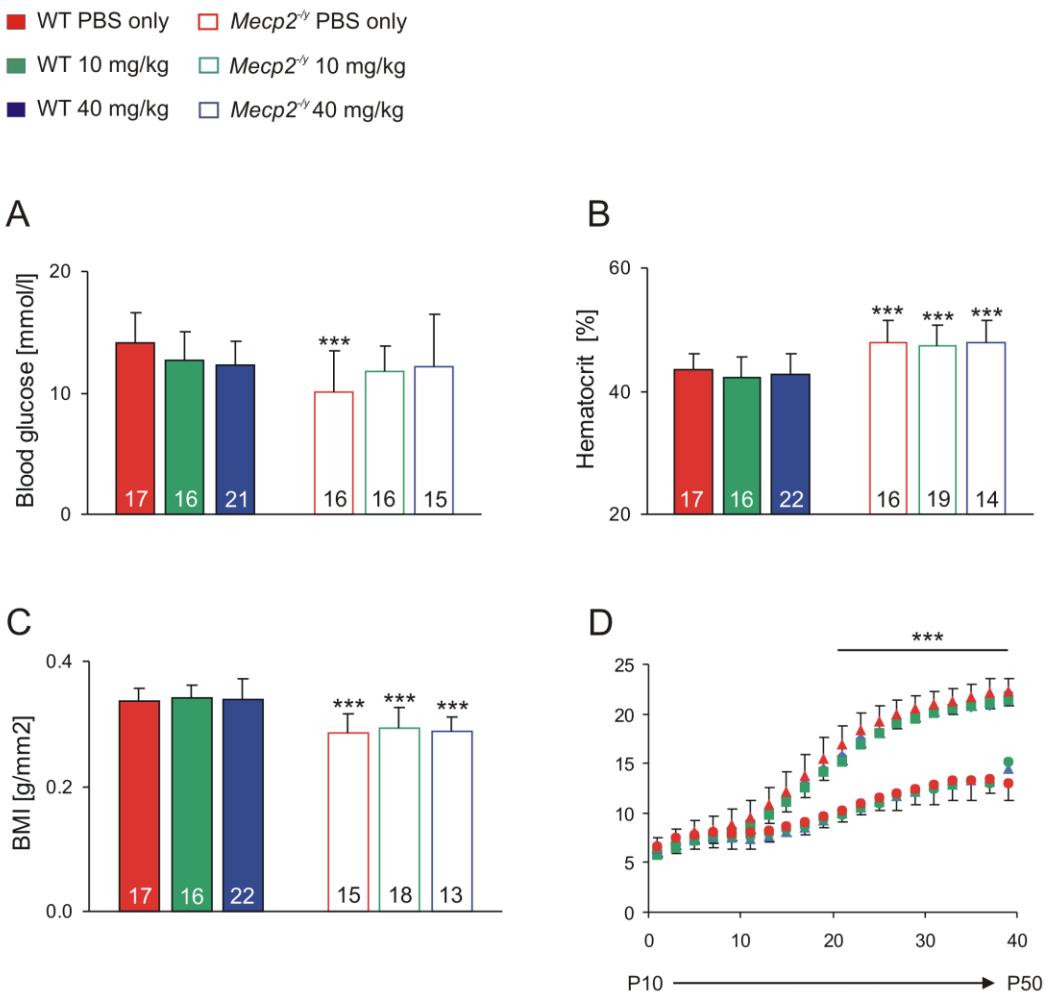

Figure 17: Systemic parameters do not reveal general improvements by *in vivo* Trolox treatment

(**A**) Blood glucose level of *Mecp2*$^{-/y}$ mice was significantly decreased in the PBS only group, but was rescued upon Trolox treatment. (**B**) The hematocrit value was significantly increased in all *Mecp2*$^{-/y}$ mice indepent of the treatment received. (**C**) The BMI was significantly decreased in all *Mecp2*$^{-/y}$ mice. (**D**) *Mecp2*$^{-/y}$ mice appeared normal at birth but showed a significantly reduced body weight by around 3 weeks of age.

4.3.2. Motor and exploration behavior

4.3.2.1. Motor dysfunction persists in *Mecp2*$^{-/y}$ mice after Trolox treatment

Rett patients as well as Rett mice show various and complex motor impairments. To determine whether Trolox treatment has any positive effect on motor performance and motor learning the accelerating rotarod, a standard paradigm widely used to evaluate the motor coordination, was run. The rotarod test was performed on 3 consecutive days, in which the first day was considered to be the habituation day. Motor learning is expected to occur between day 2 (trial day 1) and day 3 (trial day 2) and revealed an average increase in running time of ~15 % in WT mice regardless of the treatment (Table 8; Fig. 18). All *Mecp2*$^{-/y}$ mice groups showed a poor performance on the rotarod with a significantly decreased latency to fall off the rod compared to the respective WT group. Between day 2 (trial day 1) and day 3 (trial day 2) *Mecp2*$^{-/y}$ mice increased their skills by an average of 6 % in the PBS and low dose Trolox treated group, whereas the high dose Trolox treated group even showed a 20 % decrease in motor performance (Table 8; Fig. 18).

	Treatment group	WT	*Mecp2*$^{-/y}$
Latency to fall 1st [s]	PBS only	147.5 ± 55.2; *n* = 17	66.5 ± 39.4; *n* = 17
	10 mg/kg Trolox	157 ± 54.9; *n* = 15	67.6 ± 45.3; *n* = 22
	40 mg/kg Trolox	113.9 ± 68.5; *n* = 20	75.6 ± 30.6; *n* = 14
Latency to fall 1st [s]			
	PBS only	162.2 ± 48.2; *n* = 17	76.2 ± 50.2; *n* = 17
	10 mg/kg Trolox	170.3 ± 47.7; *n* = 15	92.6 ± 45.4; *n* = 22
	40 mg/kg Trolox	153.9 ± 66.2; *n* = 20	99.5 ± 55.6; *n* = 14
Latency to fall 2nd [s]			
	PBS only	185.7 ± 49.9; *n* = 17	80.5 ± 52.9; *n* = 17
	10 mg/kg Trolox	198.3 ± 50; *n* = 15	98.9 ± 49; *n* = 22
	40 mg/kg Trolox	175.6 ± 67.3; *n* = 20	81.4 ± 28.4; *n* = 14

Table 8: Comparison of Rotarod performance by the latency

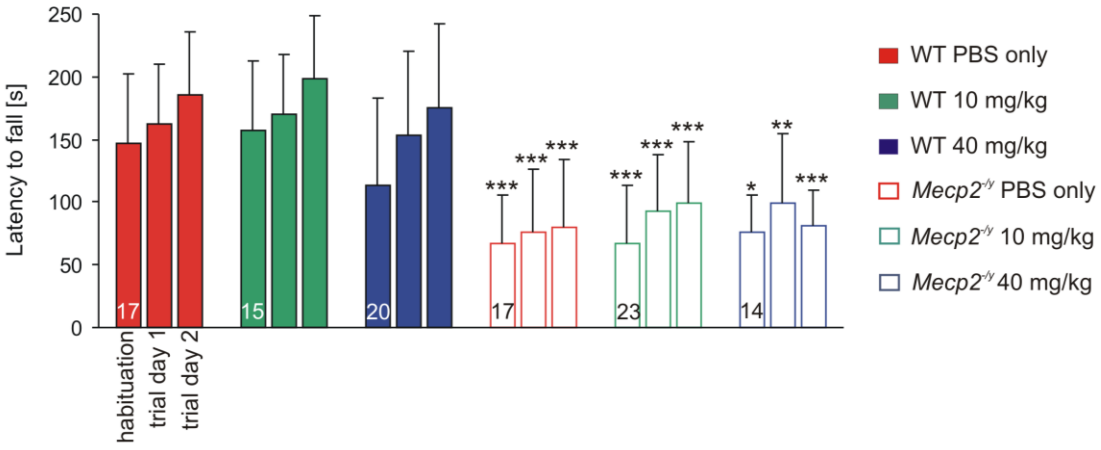

Figure 18: *Mecp2*$^{-/y}$ mice show a decline in motor skills

The latency to fall was recorded as a measure of motor performance. Comparison of the latency to fall revealed the significant difference in the motor performance between *Mecp2*$^{-/y}$ and WT mice but not in between the different treatment groups. All groups of mice, except the high dose *Mecp2*$^{-/y}$ group, improved with training and were thus able to learn the task.

4.3.2.2. *Mecp2*$^{-/y}$ mice display a lower activity but have a normal exploration behavior after treatment with Trolox

Since the hippocampus has been reported to be crucially involved in exploration (O'Keefe and Dostrovsky, 1971), exploratory behavior and locomotor activity of *Mecp2*$^{-/y}$ mice and respective WT mice was determined in an open field arena. The open field test paradigm mimicked the natural conflict in mice between exploring a novel environment and avoiding an illuminated open area. No obvious differences in the relative time spent within the 3 zones could be detected between *Mecp2*$^{-/y}$ and the respective WT mice. Based on this finding, it seems unlikely that a reduced exploratory activity is due to higher anxiety levels in *Mecp2*$^{-/y}$ mice (Table 9; Fig. 19A). Overall, *Mecp2*$^{-/y}$ mice, independent of the received treatment, showed lower activity as measured by the total distance traveled as well as a lower mean velocity. In part, this may be a consequence of motoric deficits of these mice (Table 9; Fig. 19B,C). Also the fact that *Mecp2*$^{-/y}$ showed longer resting times suggests motor impairments (Table 9; Fig. 19D).

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	Treatment group	WT	*Mecp2*$^{-/y}$
Periphery [s]	PBS only	39.3 ± 7.9; *n* = 13	42.9 ± 17.2; *n*= 16
	10 mg/kg Trolox	32.4 ± 9.2; *n* = 16	42.6 ± 9; *n*= 15
	40 mg/kg Trolox	35.7 ± 8.7; *n* = 19	40.1 ± 10.1; *n*= 14
Center [s]			
	PBS only	7.6 ± 4.2; *n* = 13	8.7 ± 12.9; *n* = 16
	10 mg/kg Trolox	6.9 ± 3.9; *n* = 16	7.3 ± 7.5; *n* = 15
	40 mg/kg Trolox	7.3 ± 4.5; *n* = 19	6.9 ± 5.6; *n* = 14
Corner [s]			
	PBS only	53.2 ± 10.4; *n* = 13	48.4 ± 17.9; *n* = 16
	10 mg/kg Trolox	60.5 ± 9; *n* = 16	50.7 ± 13.5; *n* = 15
	40 mg/kg Trolox	56.9 ± 11; *n* = 19	53 ± 12.2; *n* = 14
Distance [m]			
	PBS only	17.4 ± 3.6; *n* = 13	12.6 ± 5.2; *n* = 16
	10 mg/kg Trolox	17.9 ± 5; *n* = 16	11.8 ± 3.9; *n* = 15
	40 mg/kg Trolox	14.8 ± 4.9; *n* = 19	12.6 ± 3.7; *n* = 14
Mean velocity [cm/s]			
	PBS only	5.8 ± 1.2; *n* = 13	4.2 ± 1.7; *n* = 16
	10 mg/kg Trolox	5.9 ± 1.7; *n* = 16	3.9 ± 1.3; *n* = 15
	40 mg/kg Trolox	4.9 ± 1.6; *n* = 19	4.2 ± 1.2; *n* = 14
Resting time [%]			
	PBS only	25.5 ± 8.4; *n*= 13	38.3 ± 18.9; *n*= 16
	10 mg/kg Trolox	23.9 ± 8.3; *n*= 16	38.5 ± 14.2; *n*= 15
	40 mg/kg Trolox	32.2 ± 13.7; *n*= 19	37.3 ± 13.3; *n*= 14

Table 9: Effect of Trolox treatment on the mobility in the open field

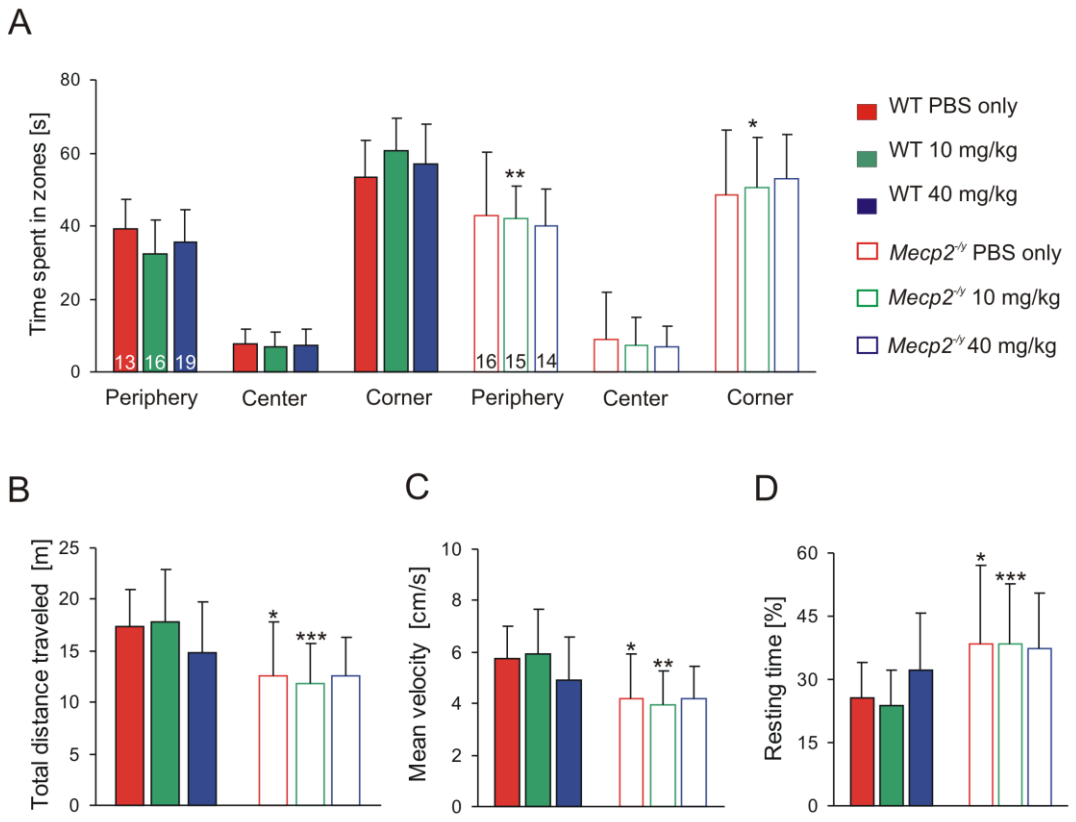

Figure 19: *Mecp2*$^{-/y}$ mice are less active in the open field arena

General locomotor activity levels and anxiety in *in vivo* treated *Mecp2*$^{-/y}$ and WT mice was tested after ~35-40 days of treatment. (**A**) *Mecp2*$^{-/y}$ and WT males did not differ in their exploration behavior. *Mecp2*$^{-/y}$ mice, independent of the treatment, visited the different zones in the same way as WT mice. Only the low dose treated Rett mice spent significantly longer times in the periphery and the corners. (**B**) All treated *Mecp2*$^{-/y}$ mice groups traveled shorter distances compared to WT mice. (**C**) Furthermore, *Mecp2*$^{-/y}$ mice traveled at a significantly lower speed compared to WT mice. (**D**) Moreover, *Mecp2*$^{-/y}$ mice showed significant longer resting times than the respective WT groups.

4.3.3. Breathing pattern is not affected by *in vivo* Trolox treatment

Rett patients undergo different severe forms of breathing disturbances including breath holding, air swallowing, hyperventilation and apnea. Also in various Rett mice a corresponding irregular breathing pattern has been confirmed (Julu et al., 2001; Viemari et al., 2005; Katz et al., 2009).

To evaluate the breathing pattern of Trolox treated mice, *in vivo* plethysmoraphy of unrestrained and conscious mice was performed. As expected, breathing irregularities such as breathing arrests lasting > 1 s, breaths per min and the irregularity score significantly differed among *Mecp2*$^{-/y}$ mice and WT mice. While breathing arrests (apneas) occurred more often in *Mecp2*$^{-/y}$ mice compared to WT (Table 10; Fig. 20B), the breathing frequency was significantly reduced in *Mecp2*$^{-/y}$ mice (Table 10; Fig. 20C). Also the irregularity score, i.e., the variability in duration of respiratory cycles, was significantly higher in *Mecp2*$^{-/y}$ mice than in WT mice (Table 10; Fig. 20D). Any changes or noticeably improvements in the breathing pattern of *Mecp2*$^{-/y}$ mice among the different treatment groups could, however, not be observed.

	Treatment group	WT	*Mecp2*$^{-/y}$
Breathing arrests > 1 s	PBS only	0.6 ± 1; *n* = 15	8.3 ± 9.7; *n* = 16
	10 mg/kg Trolox	0.2 ± 0.4; *n* = 15	9 ± 8.7; *n* = 15
	40 mg/kg Trolox	0.2 ± 0.7; *n* = 18	11.7 ± 7.8; *n* = 14
Breaths per min			
	PBS only	399.7 ± 37.4; *n* = 15	315.9 ± 70; *n* = 16
	10 mg/kg Trolox	432.1 ± 55.4; *n* = 15	283.6 ± 53.9; *n* = 15
	40 mg/kg Trolox	423.9 ± 50.5; *n* = 18	274.1 ± 63.3; *n* = 14
Irregularity score			
	PBS only	0.4 ± 0.1; *n* = 15	0.55 ± 0.2; *n* = 16
	10 mg/kg Trolox	0.3 ± 0.04; *n* = 15	0.53 ± 0.2; *n* = 15
	40 mg/kg Trolox	0.3 ± 0.1; *n* = 18	0.59 ± 0.1; *n* = 14

Table 10: Major respiratory parameters as determined by whole-body plethysmography

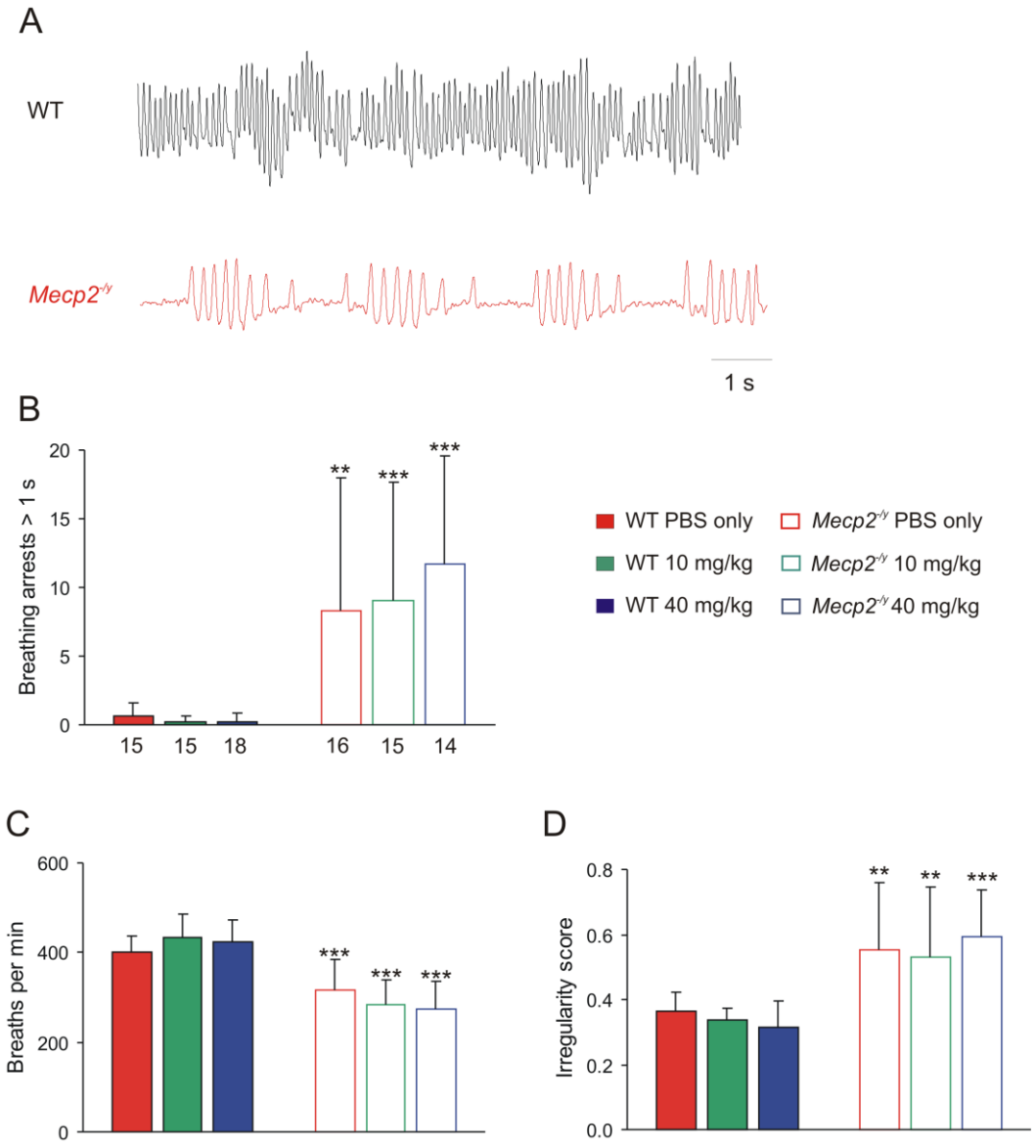

Figure 20: Breathing pattern of treated *Mecp2*$^{-/y}$ and WT mice

(**A**) Sample traces of the breathing pattern typically seen during whole-body plethysmography in the WT and *Mecp2*$^{-/y}$ PBS only group. Breathing arrests in *Mecp2*$^{-/y}$ mice are clearly visible. (**B**) The number of breathing arrests exceeding 1 s is significantly increased in all treatment groups of *Mecp2*$^{-/y}$ mice compared to WT mice. (**C**) The breathing frequency was significantly decreased in all *Mecp2*$^{-/y}$ mice compared to WT mice. (**D**) The irregularity score was significantly increased in all *Mecp2*$^{-/y}$ mice compared to WT mice. For all parameters, significant differences could be observed among genotypes but not in between the different treatment groups of each genotype.

4.3.4. Modulation of neuronal excitability and synaptic function

4.3.4.1. Increased hypoxia tolerance by low dose Trolox treatment of *Mecp2*$^{-/y}$ mice

In the *in vitro* trials, a single, acute application of Trolox improved the hypoxia tolerance of *Mecp2*$^{-/y}$ hippocampal slices. To screen for differences in the hypoxia susceptibility between injected *Mecp2*$^{-/y}$ and WT mice, HSD was triggered by O_2 withdrawal in hippocampal tissue, isolated from these mice. Surprisingly, comparing genotypes revealed that the characteristic HSD parameters such as amplitude, duration, and time to onset, determined for *Mecp2*$^{-/y}$ did not differ significantly from those obtained in WT mice. This is in contrast to the untreated mice studied earlier. Only in hippocampal slices of the low dose Trolox-treated *Mecp2*$^{-/y}$ group, the time to onset of HSD was significantly increased as compared to the respective WT group as well as compared to the *Mecp2*$^{-/y}$ PBS only group (Table 11; Fig. 21). HSD amplitude and duration were unchanged among the genotypes and treatment groups (Table 11). To check, if the loss of genotypic differences is due to handling, seasonal or breeding reasons, HSD was again triggered in slices from untreated *Mecp2*$^{-/y}$ and WT mice (n= 4; each) and revealed again hastened onset of HSD by ~30% in slices from *Mecp2*$^{-/y}$ mice as seen previously (Janc and Müller, 2014).

	Treatment group	WT	*Mecp2*$^{-/y}$
Time to onset [s]	PBS only	144.2 ± 32.9; *n* = 27	138.5 ± 24.3; *n* = 28
	10 mg/kg Trolox	127.9 ± 35.9; *n* = 27	173.6 ± 58.9; *n* = 26
	40 mg/kg Trolox	153.5 ± 45.6; *n* = 25	150.37 ± 46.4; *n* = 21
Amplitude [mV]			
	PBS only	14.9 ± 2.8; *n* = 27	15.7 ± 3.5; *n* = 28
	10 mg/kg Trolox	14.7 ± 3.9; *n* = 27	14.4 ± 3.5; *n* = 26
	40 mg/kg Trolox	14.5 ± 2.6; *n* = 25	14.1 ± 3.9; *n* = 21
Duration [s]			
	PBS only	69.5 ± 12.7; *n* = 27	138.5 ± 24.3; *n* = 28
	10 mg/kg Trolox	63.3 ± 13.3; *n* = 27	64 ± 9.8; *n* = 26
	40 mg/kg Trolox	70.8 ± 20.4; *n* = 25	67.4 ± 11.1; *n* = 21

Table 11: Overview of the characteristic parameters of the HSD-associated DC potential shift such as time to onset, amplitude and duration from *in vivo* treated mice

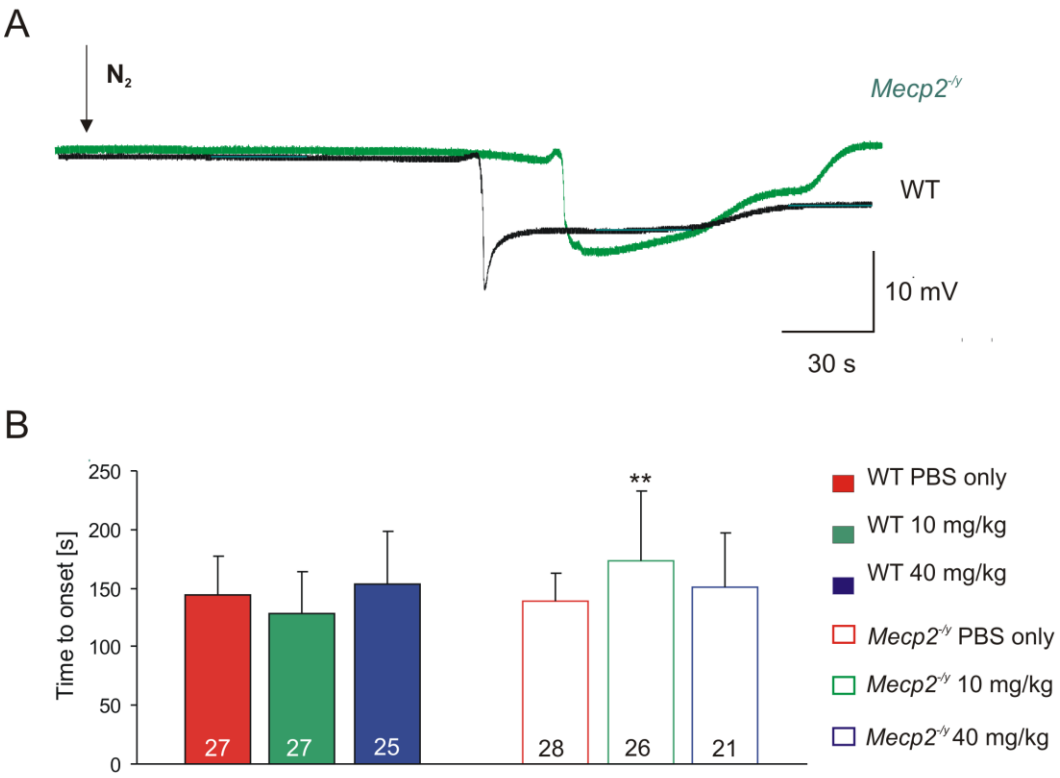

Figure 21: Low dose Trolox treatment improves hypoxia tolerance in slices of *Mecp2*$^{-/y}$

Sample traces from extracellular DC potential recordings in CA1 *st. radiatum* of *Mecp2*$^{-/y}$ and WT slices. Arrows indicate the time point of extracellular O_2 withdrawal. The reoxygenation of slices was started 30 s upon HSD onset. **B**) Contrary to what was seen earlier in untreated mice, the response to severe hypoxia did not longer differ in *Mecp2*$^{-/y}$ hippocampal slices as compared to WT slices. Even the PBS only group did not any reveal differences in HSD onset. Nevertheless, the low dose Trolox treatment significantly postponed the onset of HSD in *Mecp2*$^{-/y}$ slices as compared to the respective treated WT group but did not affect the other parameters of the HSD-associated DC potential shift such as amplitude of the negative DC shift.

4.3.4.2. No obvious changes in basal synaptic transmission

To test for treatment related changes in the general synaptic function, the I/O curves of the Schaffer collateral/CA1 synapses as well as the degree of synaptic short-term plasticity was assessed. Recordings of evoked fEPSPs were again performed in the *st. radiatum* of the CA1 subfield of the hippocampus. For basal synaptic transmission, fEPSP amplitudes were normalized to the fiber volley to cancel out differences between the individual slices and variations in electrode positioning. However, no significant difference in shape or amplitude ratio could be observed between *Mecp2*$^{-/y}$ and WT mice (*n* = 16-24 slices) (Fig. 22), which suggests an unaltered basal synaptic transmission. Earlier observed differences in untreated mice were also not found upon chronic treatment in the PBS only groups.

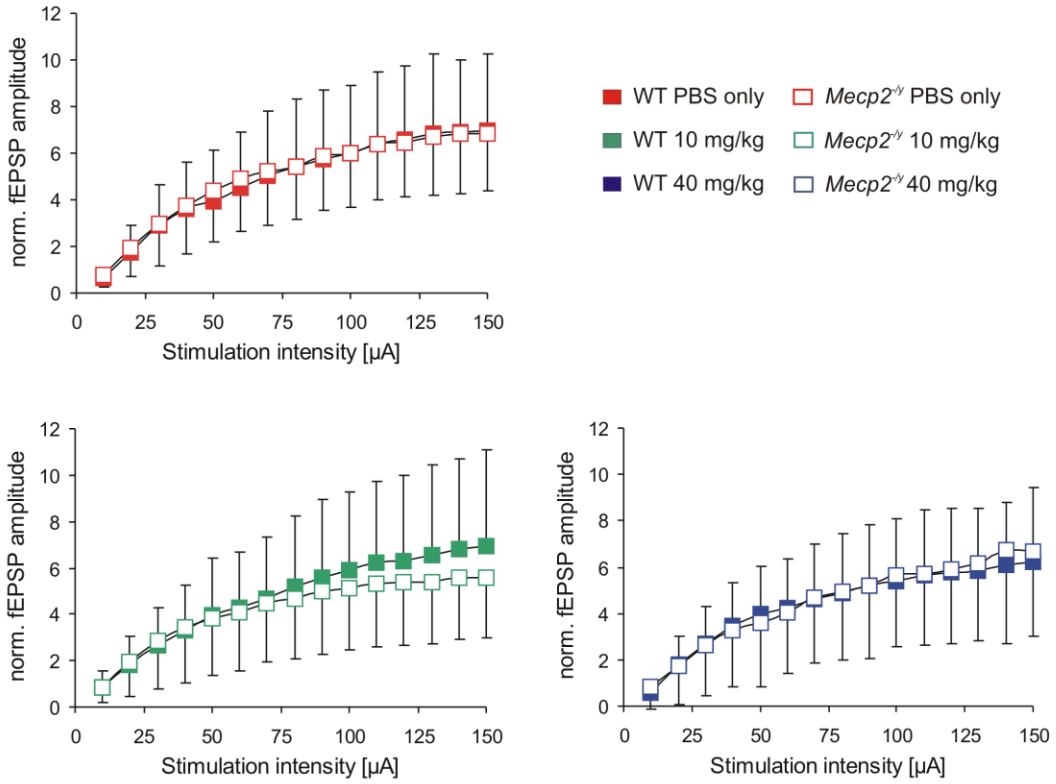

Figure 22: No obvious effects on neuronal excitability by *in vivo* Trolox treatment

Acute hippocampal tissue slices of injected *Mecp2*$^{-/y}$ and WT were used to assess neuronal excitability and basal synaptic function. I/O curves were recorded by a stepwise increase in the stimulation intensity from 10–150 µA**.** Genotypic differences and/or differences among the different treatment groups in neuronal excitability were not observed.

4.3.4.3. No alterations in paired-pulse facilitation in *Mecp2*$^{-/y}$ and WT mice

PPF is a presynaptic form of short-term synaptic plasticity resulting from enhanced residual calcium in the presynaptic terminal release in fEPSP response to two stimuli delivered at short interpulse intervals. To quantify the effects of *in vivo* Trolox treatment on the short-term plasticity, PPF was evaluated in *Mecp2*$^{-/y}$ and WT slices (Fig. 23). PPF was measured in response to 8 different interpulse intervals ranging from 25 to 200 ms in 25 ms increments. In all treatment groups, PPF recordings from *Mecp2*$^{-/y}$ and WT slice were indistinguishable at all intervals (*n* = 16-24 slices) (Fig. 23).

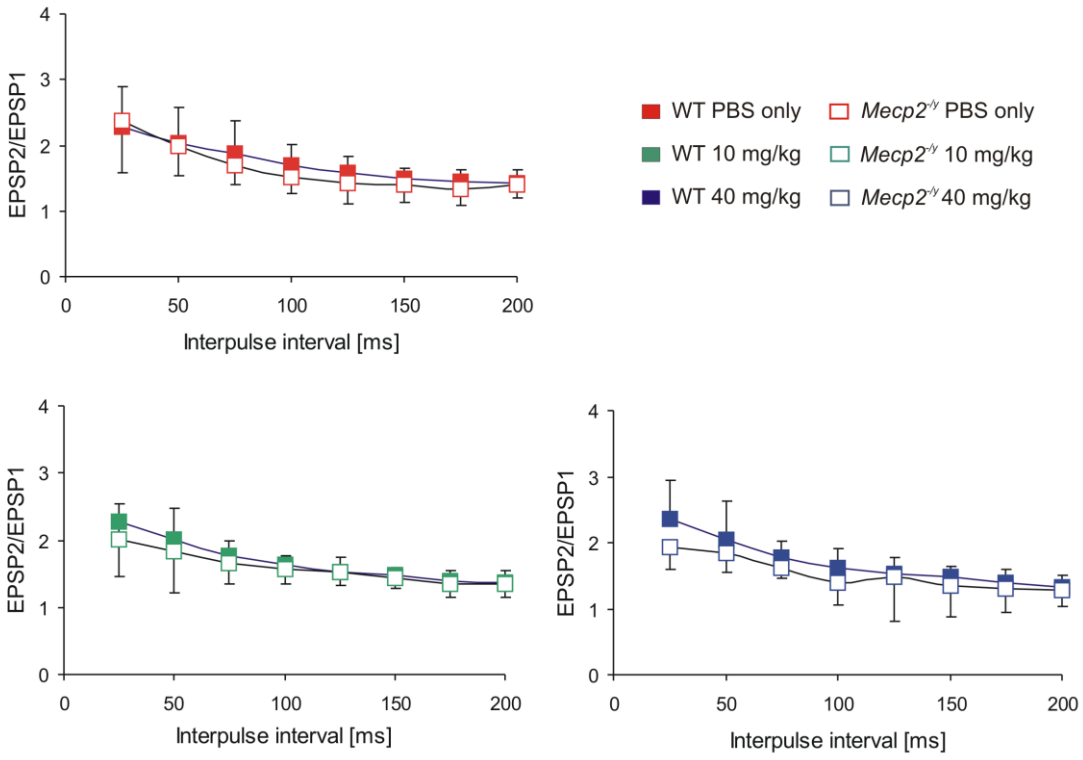

Figure 23: Short-term plasticity is not modulated by Trolox treatment

PPF was quantified as a paradigm for synaptic short-term plasticity in *Mecp2*$^{-/y}$ and WT mice. This twin-pulse stimulation was performed at various interstimulus intervals (25–200 ms) and quantified as the ratio of the second fEPSP to the first fEPSP amplitude. No significant changes among genotypes and the different treatment groups could be observed.

4.3.4.4. Improvement in *Mecp2*$^{-/y}$ hippocampal long-term synaptic plasticity by low dose Trolox treatment

Synaptic long-tem plasticity was induced in acute hippocampal slices obtained from the treated *Mecp2*$^{-/y}$ and WT mice and a potential modulation of STP and LTP by chronic Trolox-treatment was assessed. As described earlier, STP and LTP were induced by 3 trains of high-frequency stimulation. Induction of LTP led to a robust potentiation of the fEPSP amplitudes in slices from *Mecp2*$^{-/y}$ and WT mice regardless the treatment received. Nevertheless, slices from *Mecp2*$^{-/y}$ mice, treated with PBS only or high dose Trolox tended to have an impaired STP but the same characteristic LTP when compared to the respective WT group. In contrast, treatment with the low dose of Trolox significantly improved the STP and also tended to induce a more pronounced LTP (Table 12; Fig. 24C).

	Treatment group	**WT**	***Mecp2*$^{-/y}$**
STP	PBS only	1.75 ± 0.42; *n* = 14	1.56 ± 0.51; *n* = 11
	10 mg/kg Trolox	1.65 ± 0.36; *n* = 12	2.03 ± 0.53; *n* = 15
	40 mg/kg Trolox	1.72 ± 0.34; *n* = 15	1.59 ± 0.35; *n* = 12
LTP			
	PBS only	1.56 ± 0.41; *n* = 14	1.55 ± 0.36; *n* = 11
	10 mg/kg Trolox	1.59 ± 0.35; *n* = 12	1.72 ± 0.35; *n* = 15
	40 mg/kg Trolox	1.57 ± 0.28; *n* = 15	1.66 ± 0.25; *n* = 12

Table 12: Comparison of STP and LTP of mice which received *in vivo* treatment

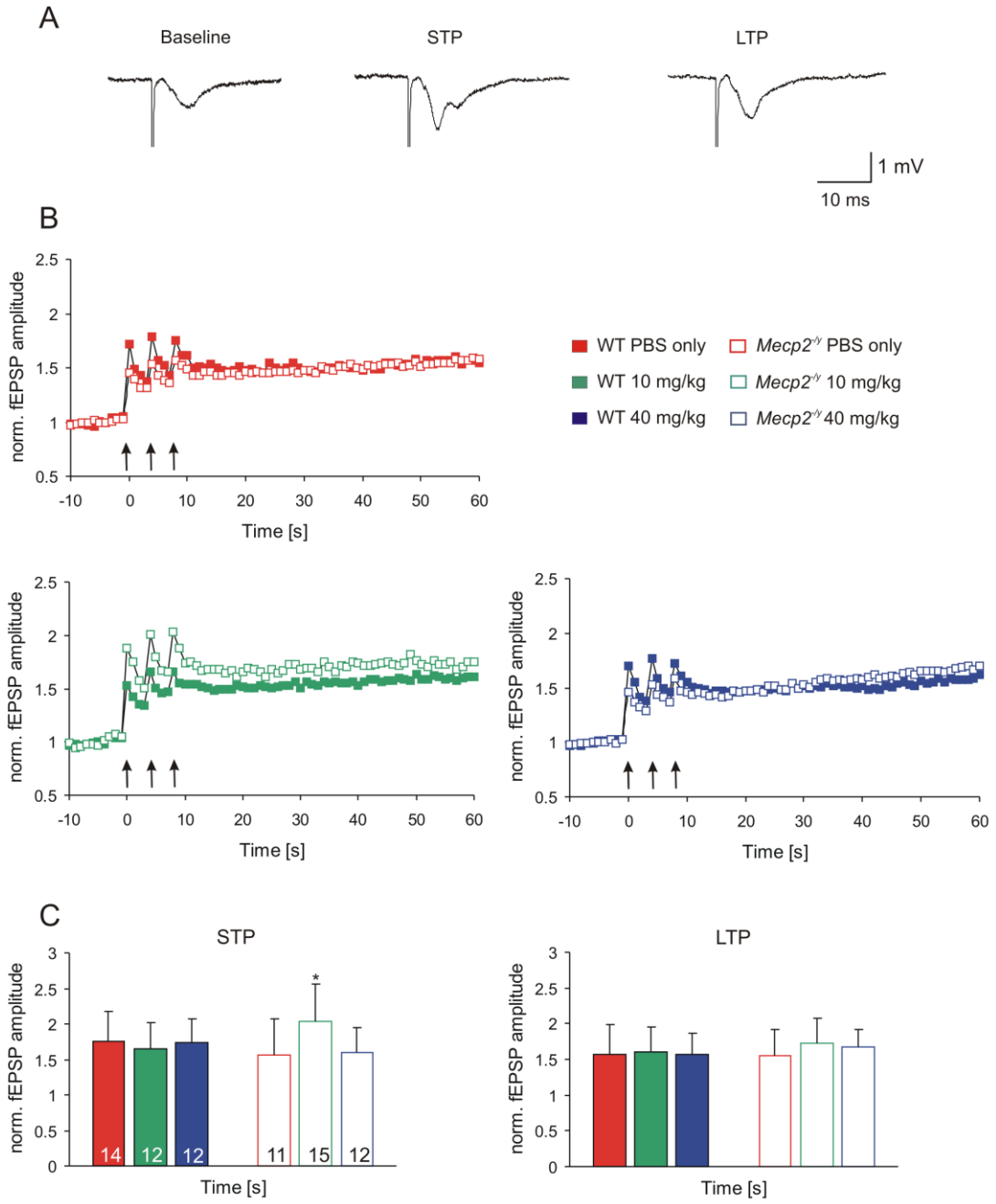

Figure 24: Unaltered STP and LTP in *Mecp2*$^{-/y}$ and WT mice after receiving chronic Trolox or PBS-treatment

(**A**) Sample traces of fEPSPs recorded for PBS treated WT mice under baseline conditions, immediately after the 3rd high-frequency stimulation (STP) and 1 h after inducing potentiation (LTP). Stimulus artifacts are truncated. (**B**) STP and LTP were no longer impaired in all treated *Mecp2*$^{-/y}$ slices as compared to WT slices. For clarity, error bars are not included. LTP was induced by three consecutive trains of 100 Hz stimuli, lasting 1 s each (see arrow marks). (**C**) Comparison of the extent STP and LTP induced in the different groups revealed that STP improved in the low dose treated *Mecp2*$^{-/y}$ mice. Further differences among genotypes and/or treatment groups were not observed.

4.3.5. Optical recordings of mitochondrial metabolism

Rett syndrome has clinical similarities to other disorders associated with mitochondrial dysfunction (Pieczenik and Neustadt, 2007) and previous findings indicated abnormalities in mitochondrial metabolism of *Mecp2*$^{-/y}$ mice (Großer et al., 2012). To assess whether *in vivo* Trolox-treatment had an effect on mitochondrial metabolism, FAD/NADH autofluorescence recordings were performed. The basal mitochondrial metabolism, analyzed by FAD/NADH ratio, was not altered between *Mecp2*$^{-/y}$ and WT mice. Furthermore, pharmacological inhibition of the mitochondrial respiration by CN^- also did not reveal differences in the autofluorescence responses among genotypes. Moreover, *in vivo* treatment with Trolox did not mediate any changes in mitochondrial metabolism (Table 13; Fig. 25). Therefore, at least negative effects of chronic treatment on mitochondria can be excluded.

	Treatment group	**WT**	***Mecp2*$^{-/y}$**
ACSF [ratio]	PBS only	0.66 ± 0.13; *n* = 20	0.66 ± 0.13; *n* = 23
	10 mg/kg Trolox	0.59 ± 0.09; *n* = 25	0.67 ± 0.09; *n* = 24
	40 mg/kg Trolox	0.69 ± 0.11; *n* = 23	0.67 ± 0.13; *n* = 20
100 µM CN^- [ratio]			
	PBS only	0.57 ± 0.1; *n* = 20	0.57 ± 0.10; *n* = 23
	10 mg/kg Trolox	0.52 ± 0.7; *n* = 25	0.56 ± 0.05; *n* = 24
	40 mg/kg Trolox	0.59 ± 0.07; *n* = 23	0.58 ± 0.11; *n* = 20
1 mM CN^- [ratio]			
	PBS only	0.50 ± 0.10; *n* = 20	0.5 ± 0.12; *n* = 23
	10 mg/kg Trolox	0.45 ± 0.06; *n* = 25	0.48 ± 0.05; *n* = 24
	40 mg/kg Trolox	0.52 ± 0.07; *n* = 23	0.52 ± 0.13; *n* = 20

Table 13: FAD/NADH baseline ratio and effects of challenging mitochondria with CN^- after *in vivo* treatment

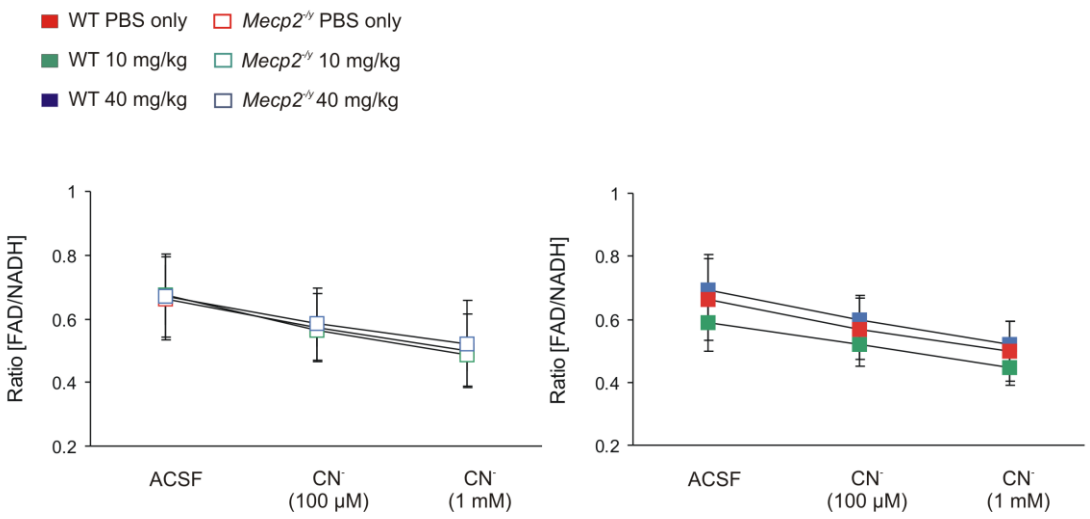

Figure 25: Mitochondria are not affected in Trolox-treated mice

NADH and FAD autofluorescence was recorded from hippocampal slices by alternating the excitation at 360 nm (NADH) and 445 nm (FAD). Differences in the basal mitochondrial respiration or differing responses to chemical mitochondrial challenging by CN^- were not obvious in slices from adult treated *Mecp2*$^{-/y}$ and WT mice. Therefore, negative effects of Trolox on mitochondria can be clearly excluded.

4.4. Survival of Trolox-treated mice

In total, 152 mice (75 *Mecp2*$^{-/y}$ and 77 WT mice) received a blinded PBS or Trolox treatment. During that treatment period, 8 *Mecp2*$^{-/y}$ mice died before they reached an age of 40 days (~10%). In detail, mice died between age P15 and P38. In comparison, the survival rate of untreated *Mecp2*$^{-/y}$ mice in our Rett mouse colony was nearly the same and therefore comparable with the findings in treated mice. A negative impact of the chronic treatment procedure (i.p. injections and frequent handling) on the survival can be excluded. Nevertheless, four mice that were treated with 10 mg/kg of Trolox and 4 mice with the 40 mg/kg of Trolox died. None of *Mecp2*$^{-/y}$ mice that received PBS died. WT mice did not die until being used for experiments.

5. Discussion

5.1. Early mitochondrial dysfunction and increased oxidative burden

Previously, our group has detected an increased oxidative burden in adult *Mecp2*$^{-/y}$ hippocampal tissue (Großer et al., 2012). Therefore, the question arose whether the underlying dysfunction in mitochondrial metabolism is a primary or secondary cause in Rett syndrome. In the present thesis, the analyses of mitochondrial metabolism in P7-10 *Mecp2*$^{-/y}$ mice revealed that in neonatal *Mecp2*$^{-/y}$ hippocampal slices the ratio of FAD/NADH tissue autofluorescence was already shifted towards more oxidized conditions. This suggests that mitochondrial dysfunction is an early and primary event in Rett syndrome (Großer et al., 2012). FAD/NADH changes may be explained by an increased turn-over rate of oxidative phosphorylation, which would lead to an intensified oxidation of the two reduction equivalents. Indeed, a proton leak across the inner mitochondrial membrane was assumed for this Rett mouse model, and increased respiratory rates and O_2 consumption were also detected in isolated mitochondria obtained from brain tissue (Kriaucionis et al., 2006; Menzfeld, 2014). Hence, the performed autofluorescence analyses of hippocampal slices are in line with those findings from isolated mouse brain mitochondria, and they clearly confirm a neonatal onset of mitochondrial impairment in Rett syndrome.

5.2. Antioxidant therapy – current status

The central nervous system, especially the brain, is one of the organs with a very high susceptibility to oxidative damage. Large amounts of ROS are produced, because of the high metabolic rates of neurons and to a lesser degree also in glial cells. Disruption of the cellular redox balance may have severe consequences (Uttara et al., 2009). In fact, free radicals or oxidative damage are widely considered to contribute to a various number of human neurologic diseases and neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease (Jenner, 2003), cancer (Reuter et al., 2010), cerebral stroke (Chan, 1996; Rodrigo et al., 2013) and diabetes (Maritim et al., 2003).

An antioxidant is considered *"any substance that delays, prevents or removes oxidative damage to a target molecule"* (Halliwell and Gutteridge, 1995; Halliwell, 2007). Accordingly, therapeutic approaches aiming to prevent, delay or ameliorate an oxidative burden by free radical scavengers seem to be a potential and promising approach.

Melatonin treatment for example reduced the lesion size in a rat focal brain ischemia study (Cuzzocrea et al., 2000) and suppressed epileptic discharges of posttraumatic epilepsy in rats (Kabuto et al., 1998). Coenzyme Q_{10} has been shown to prevent even ischemic brain lesions in rabbits (Grieb et al., 1997). The potential value of antioxidant treatment has not only been confirmed in animal models, moreover there were numerous clinical trials conducted and approved for routine clinical application that confirmed antioxidant treatment as an effective therapy. Patients with acute stroke (Nakase et al., 2011) or Alzheimer's disease (Gutzmann and Hadler, 1998) that were treated with antioxidants showed an improved outcome. Nevertheless, one should also mention, that a number of human clinical studies failed although rodent studies seemed to be promising.

Yet, the detailed function and effectiveness of antioxidants is not well known and the results of clinical studies are in part contradictory. Reasons for heterogeneous results may be the difficulty of optimal dose-finding and the challenge to first decipher in detail the individual oxidative status occurring in the different diseases conditions. Furthermore, an early identification of the disease and therefore, an early start of antioxidant treatment would be crucial to achieve clinical benefits.

5.3. Antioxidant treatment in Rett syndrome

Several studies have shown an improvement of certain clinical features of Rett syndrome (Chapleau et al., 2013). Especially, those pharmacotherapeutical treatments that aim to prevent oxidative stress by improving the cellular redox balance have been reported to be beneficial. In female Rett mice for example it has been found that alterations in the vascular and endothelial system were reversed by

administration of curcumin, a compound with antioxidant and anti-inflammatory properties (Panighini et al., 2013). Moreover, antioxidant treatment in Rett syndrome patients confirmed that oral supplementation with ω-3 PUFAs successfully improved the motor function, the non-verbal communication and the regularity of breathing (De Felice et al., 2012; Maffei et al., 2014).

5.4. Vitamin E – The scavenger of choice

Vitamins like vitamin A, C and E are the best studied antioxidants. They are widely considered to be generally safe and essential for normal development and maintenance. Beyond that, one more important aspect is that vitamin E deficiency can lead to severe neurologic dysfunctions. The primary symptoms hereby include cerebellar ataxia, dysarthria and mental retardation (Brigelius-Flohé and Traber, 1999). Vitamin E, in particular, was extensively studied in several neurologic disorders.

In transgenic amyotrophic lateral sclerosis mice, application of vitamin E led to a delayed onset of clinical symptoms (Gurney et al., 1996). In apoE$^{-/-}$ mice the supplementation of vitamin E significantly reduced isoprostane generation, which is formed *in vivo* from the free radical-catalyzed peroxidation of essential fatty acids (Pratico et al., 1998). Moreover, apoE$^{-/-}$ mice treated with vitamin E showed a significantly improved behavioral performance, which was associated with preservation of the dendritic structure. In addition, vitamin E-treated mice showed near normal levels of both lipid peroxidation and total glutathione (reduced and oxidized), whereas untreated apoE$^{-/-}$ mice had increased levels of lipid peroxidation and total glutathione (Veinbergs et al., 2000). Furthermore it could be shown that early supplementation of vitamin E reduced amyloid deposition and plaque formation in a mouse model of Alzheimer's disease (Sung et al., 2004). Taken together, there are various successful rodent studies which confirmed the positive outcome of vitamin E treatment. On this basis, clinical trials were the next logical step in the fight against the deleterious consequences of oxidative injury in different neurological diseases.

In human studies, supplementation of vitamin E reduced seizure frequency in children with refractory epilepsy (Ogunmekan and Hwang, 1989). In a small clinical trial of patients with Huntington disease, treatment with vitamin E showed some clinical benefits (Peyser et al., 1995). Moreover, in a clinical trial of patients with Alzheimer's disease, treatment with vitamin E successfully delayed some markers of the disease progression (Sano et al., 1997).

Based on the fact, that similar symptoms occur in vitamin E deficiency (Brigelius-Flohé and Traber, 1999) as well as low vitamin E levels were observed in Rett patients (Formichi et al., 1998), the main focus of this thesis was to analyze to what degree radical scavenging is able to rescue neuronal and mitochondrial function in the MeCP2-deficient mouse hippocampal network and/or to improve the physical constitution of *Mecp2*$^{-/y}$ mice.

Hence, the radical scavenger of choice was a vitamin E derivative based on the high scavenging efficiency of this class of compounds, and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) in particular was selected due to its water solubility. Vitamin E and its derivatives are chain-breaking antioxidants that prevent the propagation of free radicals in membranes and in plasma lipoproteins. Radicals are scavenged by vitamin E at ~1.000- times faster kinetics than by PUFAs (Buettner, 1993; Traber and Stevens, 2011; Alberto et al., 2013). Moreover, antioxidants are also able to react with singlet oxygen as well as superoxide, and therefore decrease the cell endogenous H_2O_2 formation (Brigelius-Flohé and Traber, 1999; Peus et al., 2001). In addition, vitamin E is not degraded in the scavenging process but is rather recycled and continuously restored by other antioxidants e.g. vitamin C, to maintain the antioxidant function (Buettner, 1993; Traber and Stevens, 2011).

5.5. Trolox treatment *in vitro* vs. *in vivo* – Synaptic plasticity

5.5.1. Short -and long-term plasticity

Acute *in vitro* 3–5h Trolox treatment clearly dampened neuronal hyperexcitability, improved synaptic plasticity, and increased the tolerance to severe hypoxia in acute isolated hippocampal tissue of adult and already symptomatic *Mecp2*$^{-/y}$ mice. Indeed, already in an earlier imaging study from our laboratory, Trolox has been shown to be able to decrease the elevated redox baselines in *Mecp2*$^{-/y}$ hippocampal slice cultures and to dampen the exaggerated redox responses to oxidant challenge in neonatal and hence presymptomatic mice (Großer et al., 2012).

Now, it could be shown that *in vitro* application of Trolox improved basal synaptic function by decreasing selectively neuronal hyperexcitability in *Mecp2*$^{-/y}$ without affecting WT slices. How exactly the modulation of the neuronal network function by changes in cellular redox balance occurs is not predictable, especially in regard to the various number of ion-channels and transmitter receptors that are modulated to different degrees and even respond oppositely to redox modulation. For example oxidant challenge blocks NMDA and $GABA_A$ receptors (Aizenman et al., 1989; Sah et al., 2002) but activates voltage-gated Na^+-channels and ryanodine receptors (Hammarström and Gage, 2000; Hidalgo et al., 2004; Gerich et al., 2009). But, independent of its underlying mechanism, the normalization of neuronal excitability by Trolox in *Mecp2*$^{-/y}$ slices may be of importance in view of the seizure susceptibility associated with Rett syndrome. *In vivo* Trolox treatment, however, did not reveal any differences between *Mecp2*$^{-/y}$ and WT mice regarding the shape of their I/O curves, which indicates that basal synaptic transmission is not altered.

In hippocampal slices of untreated *Mecp2*$^{-/y}$ mice, short- and long-term synaptic plasticity was significantly impaired compared to those obtained from WT mice. These findings are in line with reports of synaptic deficits in previous studies conducted on *Mecp2* null mice (Asaka et al., 2006; Moretti et al., 2006). Interestingly, Trolox treatment in slices improved various aspects of synaptic

plasticity. Since cognition is severely impaired in Rett syndrome, this may be an important finding. Although, PPF was not primarily affected, genotypic differences among *Mecp2*$^{-/y}$ and WT slices were no longer present upon acute Trolox treatment. Moreover, STP was improved and LTP was fully restored to its normal level by the acute *in vitro* application of Trolox in *Mecp2*$^{-/y}$ slices. It seems that in particular postsynaptic structures were modulated by the radical scavenger treatment, as especially long-term plasticity was improved. LTP induction at Schaffer collateral/CA1 synapses is mostly NMDA receptor dependent (Bliss and Collingridge, 1993). More oxidized conditions in *Mecp2*$^{-/y}$ hippocampus may partially lead to the inactivation or upregulation of the oxidation-sensitive NMDA receptors (Aizenman et al., 1989; Betzen et al., 2009) and therefore, contribute to the less stable LTP seen in Rett mouse hippocampus (Asaka et al., 2006; Moretti et al., 2006; Guy et al., 2007). It is also possible that disturbed LTP is a consequence of an altered NMDA receptor expression pattern or their particular NMDA receptor subunit composition within synapses (Maliszewska-Cyna et al., 2010). In conclusion, the Trolox-mediated normalization of redox balance may have restored normal NMDA receptor function and therefore LTP.

In vitro application of Trolox in WT slices showed no effects on basal synaptic function, but dampened STP and also depressed LTP. This finding may be explained by the strict dependence of LTP on exact cellular redox balance. It has been shown that a certain ROS level is necessary for signal transduction cascades during normal physiological processes (Serrano and Klann, 2004). How important a well-balanced ROS level is, has been shown by overexpression of extracellular superoxide dimutase 3 or superoxide scavenger administration, both of which revealed impairments in hippocampal LTP (Klann et al., 1998; Thiels et al., 2000). One more important finding is, that oxidative stress is able to influence the LTP maintenance without affecting STP or PPF (Pellmar et al., 1991). Hence, ROS are not only involved in LTP impairment but contribute to normal LTP and are therefore essential for synaptic plasticity (Knapp and Klann, 2002; Massaad and Klann, 2011). Based on the obtained *in vitro* data, a well-balanced cellular redox equilibrium and an optimized dosage of redox-modulators such as radical scavengers are very important. Here, only a single

concentration of Trolox was applied that led to an improved LTP in *Mecp2*$^{-/y}$ slices, but to a partial depression in WT. Accordingly, a more sensitive titration of redox conditions may be required to ensure that LTP improves in *Mecp2*$^{-/y}$ slices without dampening synaptic plasticity in WT.

In the present study, the genotypic differences between *Mecp2*$^{-/y}$ and WT mice were evident in uninjected mice but became less pronounced in chronically treated mice which received PBS only or the high dose of Trolox. Interestingly, by comparing data from slices of untreated WT and PBS injected WT mice, the STP and LTP in slices from PBS injected mice are significantly lower (STP 238.2 ± 62.1% vs. 175.5 ± 42.3%; LTP 179.3 ± 31% vs. 156.7 ± 41.6%; *n*= 9-15). This might be explained by previous findings from *in vitro* and *in vivo* electrophysiological studies that indicate stress and stress hormones as one potential reason for impaired LTP in the hippocampus (Kim and Yoon, 1998; Kim and Diamond, 2002). Hence, chronic i.p. injections could certainly be considered as a chronic stressor (Ryabinin et al., 1999) and therefore, may lead to stress hormone release and thus impair the LTP in slices of injected WT mice. In slices of *Mecp2*$^{-/y}$ mice, LTP is already impaired to a degree that it might not be further disturbed by stress hormones. This could explain the absence of genotypic differences in injected mice. Another explanation could be that bidaily handling of mice had a positive effect on synaptic plasticity of *Mecp2*$^{-/y}$ mice, as it has been reported in several “enriched environment” studies (van Praag et al., 1999; Rampon et al., 2000).

Nevertheless, also *in vivo* Trolox treatment revealed promising results on synaptic long-term plasticity. Low dose Trolox treated *Mecp2*$^{-/y}$ mice showed a significantly improved STP as compared to the respective WT group and the other *Mecp2*$^{-/y}$ groups. In contrast to the clear effects of the low dose Trolox treatment on hippocampal synaptic plasticity the chronic high dose *in vivo* treatment by repeated injections of Trolox did not change or improve synaptic plasticity in slices obtained from *Mecp2*$^{-/y}$ or WT mice, which may result from a probably too high concentration that might mediate negative effects.

5.5.2. Hypoxia induced spreading depression

Acute *in vitro* Trolox application abolished the increased susceptibility of *Mecp2*$^{-/y}$ hippocampus to O_2 withdrawal i.e., Trolox reverted in *Mecp2*$^{-/y}$ slices the onset of HSD to conditions seen in WT slices, whereas HSD in WT slices was not altered by Trolox. Also here, another protective effect that was mediated by Trolox occurred only in *Mecp2*$^{-/y}$ slices. It is known that treatments decreasing neuronal excitability postpone the onset of spreading depression while increased excitability favors its occurrence (Somjen, 2001). Following from this, the improvement of HSD in *Mecp2*$^{-/y}$ slices by Trolox may be a result of the selective dampening of neuronal excitability. Moreover, increased ROS levels (Grinberg et al., 2012), changes in thiol redox balance (Hepp et al., 2005; Hepp and Müller, 2008) and mitochondrial inhibition (Gerich et al., 2006) critically modulate the induction threshold of spreading depression. These results lead to the suggestion that a stabilized redox balance or an improved mitochondrial anoxia tolerance may be partly responsible for the postponement of HSD, seen in *in vitro* Trolox-treated *Mecp2*$^{-/y}$ slices.

The PBS control group, in *in vivo* Trolox-treated mice, did not show differences in hypoxia susceptibility among genotypes, but the treatment with the low dose of Trolox decreased the hypoxia susceptibility in *Mecp2*$^{-/y}$ slices significantly as compared to the respective treated WT. Interestingly, this has not been seen with the high dose of Trolox, which may again point out to the importance of proper antioxidant dosing. In view of the highly irregular breathing and the associated intermittent systemic hypoxia in Rett syndrome (Julu et al., 2001; Stettner et al., 2008; Katz et al., 2009), the Trolox-mediated increase in hypoxia tolerance is clearly of potential merit, as it may prevent additional complications especially in anoxia vulnerable neuronal networks such as the hippocampus and cortex.

5.6. Mitochondria are unaffected by Trolox treatment

Mitochondria are the primary cellular source of ROS (Boveris and Chance, 1973; Adam-Vizi, 2005) and mitochondrial alterations in Rett syndrome underlie the increased oxidative burden and altered cellular redox homeostasis (Großer et al., 2012). However, mitochondria are also potential targets for oxidative damage themselves, and may respond with morphological and functional changes including altered intracellular trafficking (Petronilli et al., 1994; Gerich et al., 2009; Qi et al., 2011; Lenaz and Genova, 2012).

Therefore, it was also assessed whether Trolox may modulate mitochondrial function directly. However, a noticeable improvement of mitochondrial function was not observed. The genotypic baseline differences in FAD/NADH ratio, a measure for the metabolic activity of mitochondria, were not significantly affected by acute Trolox administration. During mitochondrial challenging with 1 mM CN^-, only a small effect on the anoxic drop in FAD/NADH ratio was mediated by Trolox, which may suggest a slightly increased anoxia tolerance of mitochondrial respiration. Yet, no corresponding effects on $\Delta\psi_m$ were observed. Furthermore, slightly increased Rh123 responses in both WT and *Mecp2*$^{-/y}$ slices during FCCP uncoupling, or the tendency of increased Rh123 responses to high CN^- concentrations in *Mecp2*$^{-/y}$ slices may suggest some improvement of $\Delta\psi_m$.

These findings revealed that the protective effects of vitamin E reported for rat liver mitochondria, i.e., partial normalization of the increased state 3 and state 4 respiration upon acute lipid peroxidation (Ham and Liebler, 1997), do not necessarily apply to the disturbed hippocampal mitochondria of Rett mice. Nevertheless, regarding the obtained data it can at least be confirmed that Trolox treatment did not lead to any negative side effects on mitochondrial function in WT and especially in *Mecp2*$^{-/y}$ slices.

In contrast, after chronic i.p. administration of Trolox, any impairment in mitochondrial metabolism and FAD/NADH ratio could be no more observed.

Surprisingly, also the PBS control group did not show any differences between *Mecp2*$^{-/y}$ and WT mice. It should be noted here that the FAD/NADH recordings for acute and chronic treatment were performed on two different setups with different light sources. This may explain the differences seen in the FAD/NADH baseline conditions. Also stress, resulting from bidaily injection, might have had an impact on mitochondrial metabolism, which could mask the genotypic differences, since it has been shown that most of the primary mediators of stress responses exert numerous effects on mitochondrial metabolism and ROS generation and may even induce apoptosis (Manoli et al., 2007).

5.7. No *in vitro* effect of Trolox on seizure susceptibility

Even though acute Trolox treatment reduced neuronal excitability in *Mecp2*$^{-/y}$ slices, an obvious reduction in seizure susceptibility was not observed. Nevertheless, the onset of SLEs tended to be postponed only in WT slices and the duration of the individual SLEs tended to be decreased by Trolox in both WT and *Mecp2*$^{-/y}$ slices. Although a decrease in the SLE duration was observed in both genotypes, it may be of some profit by dampening the severity of seizures once such abnormal discharges are triggered. But it should be also considered, however, that the K+-channel inhibitor 4-AP is rather a strong convulsive stimulus. Nevertheless, a pronounced seizure susceptibility is associated with Rett syndrome, and it even constitutes a potential cause for sudden death (Hagberg et al., 1983; Steffenburg et al., 2001). Therefore, it is an important finding that the Trolox-mediated normalization of synaptic plasticity in *Mecp2*$^{-/y}$ hippocampus is not associated with negative side effects such as increased neuronal excitability and/or increased seizure susceptibility. The seizure susceptibility has not been assessed in slices of the chronically injected mice because of the following reasons: The results of untreated mice did not reveal a higher epileptic activity in slices of *Mecp2*$^{-/y}$ mice as compared to WT and therefore, there was no need to test this in slices from injected mice. Also I/O curves of injected mice did not differ among genotypes and no obvious signs of hyperexcitability were seen. Moreover, due to the restricted number of mice that

were allowed to use for the *in vivo* study, the main focus was to screen for improvements in those experiments that showed already promising results *in vitro*.

5.8. *In vivo* Trolox treatment - influences on systemic parameters

Systemic treatment of mice with Trolox up to ~P50 did change neither the progression of Rett-symptoms nor the systemic parameters, such as the body weight or the hematocrit. *Mecp2*$^{-/y}$ mice were not distinguishable from their WT littermates until around P20. Around this time mice were separated from their foster mothers and started to develop significant differences in their body weight, which is in line with earlier studies on untreated Rett mice (Guy et al., 2001).

The hematocrit of *Mecp2*$^{-/y}$ mice was in all treatment groups elevated as compared to WT mice. This can be explained by the fact that severe, intermittent hypoxia arising from unstable breathing increases the hematocrit as an adaptation to chronic oxygen shortage. Such adaptation to hypoxia has been consistently reported to increase the hematocrit and is one of the mechanisms contributing to the increased tolerance to hypoxia (Burton et al., 1969). Also in this aspect, treatment with Trolox did not mediate any improvement.

Analyses of the blood glucose content revealed a lower level in *Mecp2*$^{-/y}$ mice in comparison to WT. Nevertheless, the blood glucose level of the low dose Trolox treated group could be restored to WT level. Previously, it has been shown that, indeed, *Mecp2* null mice are associated with metabolic abnormalities including hyperinsulinemia (Pitcher et al., 2013). Such high levels of insulin could well lead to a depression of blood glucose content. As neurons rely on the energy derived from glucose, this may potentially contribute to disturbed neuronal network function in Rett syndrome.

5.9. *In vivo* treatment – breathing disturbances

One of the most prominent symptoms in Rett syndrome is a remarkable breathing abnormality. Most Rett patients and *Mecp2* mutant mouse models suffer from breath holding, aerophagia, hyperventilation and recurrent apneas (Julu et al., 2001; Stettner et al., 2008; Katz et al., 2009; De Felice et al., 2010; Ramirez et al., 2013).

Each episode of irregular breathing evokes a significant drop in arterial oxygen saturation, which is rapidly restored only after normal ventilation resumes. Thus, these repetitive changes of oxygen saturation may cause damage to the sensitive neuronal tissue (Love, 1999). Though, different mechanisms are involved, such damage is mainly linked to the formation of ROS. ROS are highly reactive molecules and are considered to play an important role in the development of cardiovascular diseases (Sugamura and Keaney, 2011) and moreover to mediate neuronal damage (Uttara et al., 2009).

In Rett patients, untreated episodes of hypoxia may lead to cumulative redox challenge and therefore, play a role in the onset and/or progression of cardiovascular and neuronal complications. This led to the question, if antioxidant treatment could improve the breathing of *Mecp2*$^{-/y}$ mice, by opposing and/or dampening the oxidative challenge. In line with other studies, *Mecp2*$^{-/y}$ mice displayed a highly irregular breathing pattern (Viemari et al., 2005; Stettner et al., 2008; Katz et al., 2009). Treatment with Trolox did not reveal any beneficial outcome on the regularity of breathing nor the frequency of observed apneas, but also did not deteriorate the breathing further. The number and the intensity of the severe apneas affecting *Mecp2*$^{-/y}$ mice were not changed after chronic Trolox treatment, but it has also to be mentioned that there is a great variability between individual *Mecp2*$^{-/y}$ mice. Some Rett mice displayed milder breathing disturbances with only 1 apnea over the evaluated time interval whereas others showed 35 apneas. Thus, the outcome of the Trolox treatment could also be masked by mice with a very pronounced respiratory phenotype.

5.10. *In vivo* Trolox treatment and its outcome on motor and exploration behavior

Another clinical feature of Rett syndrome is that Rett girls lose already acquired motor skills such as purposeful hand use and walking. Analysis of the impact of MeCP2 deficiency on motor and behavior skills has previously been conducted on *Mecp2*$^{308/y}$ mice, which display an obvious milder phenotype (Shahbazian et al., 2002; Moretti et al., 2005).

In the present study, *Mecp2*$^{-/y}$ mice showed a clear impairment in motor performance and motor learning with significant differences in the time spent on the rotarod among all *Mecp2*$^{-/y}$ treatment groups compared to the WT groups. These findings clearly show that antioxidant treatment was not able to restore or improve motor function. The poor performance on the rotarod can be most likely excluded to be a result of muscle weakness, as almost all *Mecp2*$^{-/y}$ mice were still able to climb around in their cages. Also, normal muscle strength has been shown by the wire suspension test, in which mice were forced to grasp a wire and hang from it on their forepaws. The ability of *Mecp2*$^{-/y}$ mice to grasp the wire was comparable to WT mice (Santos et al., 2007). However, intact motor coordination is necessary for a good performance in the rotarod test. The limb coordination deficiency that is indicated by the hind limb clasping is apparently present in *Mecp2*$^{-/y}$ mice. Regarding therefore the obtained data in this thesis, it seems that the poor performance on the rotarod is due to severely affected hind limbs.

Rett patients as well as *Mecp2*$^{-/y}$ mice show a decreased activity and hypoactivity (Guy et al., 2001). Also in the present study, *Mecp2*$^{-/y}$ mice traveled significantly shorter distances, which in the open field test became evident as a decline in their mean velocity and as prolonged resting times. Distinct differences in the times spent in the zones: periphery, center and corner could not be observed, although the low dose Trolox treated *Mecp2*$^{-/y}$ group tended to spend more time in the periphery and corner when compared to the respective WT. Taken together, *Mecp2*$^{-/y}$ mice showed an overall considerably lower locomotor activity in the open field compared to WT

mice. Furthermore, one could suggest that even though *Mecp2*$^{-/y}$ mice seem to habituate to the open field environment to some extent, based on their decreased locomotor activity over time they may have maintained a higher level of overall anxiety than WT mice (Paylor et al., 1998).

5.11. Résumé of systemic Trolox treatment

In contrast to the clear effects of acute Trolox application on hippocampal synaptic transmission *in vitro*, the chronic treatment of mice with Trolox did not change the progression of characteristic Rett syndrome symptoms. This leads to the suggestion that Trolox may not hold promise in treating the full range of symptoms of Rett mice. Yet, one has to bear in mind that *Mecp2*$^{-/y}$ male mice are very severely affected. Therefore, it remains to be tested whether Trolox would be more valuable in heterozygous females, which exhibit a much milder and more stable phenotype. Furthermore, assessment of the full pharmacotherapeutic potential of Trolox to improve the phenotype of Rett mice *in vivo* requires adequate specific outcome assays, especially defined tests for the blood brain barrier permeability of this compound. Also, the potential influence on frequent animal handling and drug injections have to be taken into account. In particular, injections can lead to a high stress response and may change or mask the outcome of possible positive results.

In the present study, Trolox was administered via i.p. injections. It is possible that other administration routes would be more effective and potentially could improve or rescue some of the symptoms. Also, alternatives such as the use of minipumps or oral feeding should be considered, though injections are the only way to ensure the animal, indeed, received the desired concentration of the drug. The use of minipumps underlies certain limitations as they are not feasible for mice at early postnatal stages. Oral feeding could also just start ~P20, otherwise the mother would have to be fed, so that the administered drug could be forwarded via the mother's milk to the suckling pups. Yet in that case, a tight control of the exact drug concentration taken up by the pups would be almost impossible.

5.12. Issues in therapeutic treatment strategies

Although, there is strong evidence that early mitochondrial dysfunction and early oxidative stress occur in *Mecp2*$^{-/y}$ mice, further evaluation whether oxidative stress is really the primary cause of the disease, needs to be performed. It is important to know if oxidative damage is a direct initial critical factor or just a byproduct of other disturbed upstream pathways. Thus, if oxidative injury is only a consequence of overall failing cellular cascades in Rett syndrome, it may be too late for an antioxidant treatment. In addition, oxidative stress may be only one of different processes in disease development and progression. The mechanistic insights into other diseases showed that in ischemic cell death oxidative damage goes along with many other deleterious processes such as inflammation and excitotoxicity. Hence, to achieve successful therapy, compounds which act on multiple targets have to be developed.

Even though all *Mecp2*$^{-/y}$ mice have the same genetic background, there is a high variance in the phenotype. Some mice show an almost normal body weight, do not display obvious motor problems and appear quite healthy as compared to other *Mecp2*$^{-/y}$ mice. Differences in the phenotype are also very prominent in Rett girls. It may be that a poor outcome of the supplementation of antioxidants is due to the fact that mice and/or patients do not equally benefit from an antioxidant treatment. The degree and true course of oxidative stress might also vary between individuals. Because of this heterozygous population, the outcome of antioxidant treatment may differ considerably among the treated individuals. Some may clearly benefit from such treatment whereas others might not. The resulting variability might than be interpreted as a less satisfactory result. Defined biochemical analysis would be required, to ensure treatment of only those individuals with a strong redox imbalance. Hence, patients without clear biochemical evidence of increased oxidative stress could be excluded from clinical trials.

In summary, careful recruitment criteria have to be established to determine who is especially suited for which kind of study. Furthermore, for the planning of a clinical

trial, it has to be certain that the antioxidant is, indeed, able to reduce oxidative injury *in vivo*. Therefore, one has to consider different points (Firuzi et al., 2011).

(1) The bioavailability should be high enough, to ensure that reliable amounts of the drug can reach the target. Indeed, when a medication is administered, its bioavailability generally decreases or may vary from patient to patient. Bioavailability is one of the crucial tools in pharmacokinetics, and must be considered when calculating the exact dosage.

(2) The time and duration of supplementation plays an important role for the success of a clinical trial. Most patients are likely to suffer already for a long time from the consequences of oxidative stress. Clearly, in most cases, a short-time antioxidant therapy cannot compensate or reverse for those long-term existing effects and damages. In addition, in most cases the therapy starts too late in the disease progression to show beneficial effects. Moreover, the optimal duration of treatment has to match the very type of the disease. While, long-term treatment is needed for cancer prevention, long-term therapy may not always be feasible, as long-term treatment could lower ROS formation to a level that may be detrimental for cells as ROS are essential signaling molecules (Finkel, 2011).

(3) The use of insufficient doses of vitamin E, could be another explanation for the lack of obvious positive results. Therefore, a dose-ranging study in which different doses of a compound are tested against each other to clarify which dose works best, should be run first. In addition, it has to be excluded that the reaction products, arising from the interaction of antioxidant and ROS, do not lead to potentially harmful downstream products.

(4) In some diseases it might be not easy to successfully overcome oxidative stress. If the balance in the cell is already intensely disturbed, the amount of antioxidants that would be necessary to equilibrate the redox imbalance may be too high and the required concentrations could be even toxic.

(5) Target specificity is another point that has to be considered. The administration of antioxidants mediates systemic effects but oxidative damage may be restricted to one specific organ or tissue.

(6) In most studies, only a single antioxidant compound is used, but to achieve the optimal outcome, one should consider a combination of different antioxidants, especially as vitamins work synergistically. It has been shown that vitamin E is particularly more efficient when administered together with vitamin C (Robinson et al., 2006).

(7) One of the biggest challenges is the limited knowledge of the blood brain barrier permeability for antioxidants when applied systemically. Therefore, for any novel planned antioxidant treatment it should be mandatory that this compound is well able to cross the blood brain barrier after systemic administration.

In summary, it is challenging to identify “the” best working antioxidant, but it should be desirable to develop new strategies by combining the existing knowledge of the properties of antioxidant compounds on novel drug approaches for Rett syndrome and other disorders related to mitochondrial dysfunction.

6. Conclusions and future perspectives

In conclusion, the obtained data suggest that oxidative stress plays a key role in the mechanisms of some of the characteristic neurological features in Rett syndrome. The present thesis confirmed a reduction in neuronal hyperexcitability, improvement of synaptic short-term plasticity, and restoration of synaptic long-term potentiation in *Mecp2*$^{-/y}$ mice following the incubation of acute hippocampal slices with the free radical scavenger Trolox (Janc and Müller, 2014). This concept was also supported by a previous pilot study in Rett patients at an early stage of the disease using ω-3 PUFAs (De Felice et al., 2012).

Like the *in vitro* promising results, *in vivo* Trolox treatment also turned out to be an effective pharmacological therapy in blood glucose level, hypoxia tolerance and short-term plasticity. Based on the these positive findings upon treatment, the low dose treatment of Trolox should be preferred and optimized for further experiments that may either slow down the disease progression or reverse the typical symptoms in male Rett mice.

Further experiments regarding the survival of *in vivo* Trolox-treated mice have to be performed. The expected lifespan *of Mecp2*$^{-/y}$ mice is about 50-60 days. To evaluate whether Trolox treatment leads to a prolongation of the lifespan, mice shall be injected over a longer period of time (~100 days) or as long as they survive.

Moreover, for future studies, and especially for long-term treatment, other application routes of Trolox such as self feeding or forced oral supplementation have to be considered as frequent i.p. injections seem to have a strong impact on the outcome of antioxidant treatment. Therefore, the design of the study has to be optimized.

To test whether hippocampus-dependent learning may improve by Trolox treatment, the Y-maze test has been already started, but so far only a small number of mice was tested to draw a conclusion.

Although, MeCP2 expression in heterozygous female mice leads to inconsistent symptoms and highly variable phenotypes, to get a full picture of the Trolox therapy, also female mice have to be included in the scavenger treatment. Possibly, it is only in those less severely affected female mice, that some degree of improvement could be achieved by antioxidant treatment.

After all, redox imbalance is only one potential reason of the several symptoms associated with Rett syndrome and it may potentially challenge neuronal function of MeCP2-deficient neuronal networks.

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# equally contributing authors

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