ENHANCED HYPOXIA SUSCEPTIBILITY IN HIPPOCAMPAL SLICES FROM A MOUSE MODEL OF RETT SYNDROME

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Running Title: Hypoxia susceptibility in RETT syndrome

37 text pages including 2 tables, 10 figures

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ABSTRACT

Rett syndrome is a neurodevelopmental disorder caused by mutations in the X-chromosomal MECP2 gene encoding for the transcriptional regulator methyl CpG binding protein 2 (MeCP2). Rett patients suffer from episodic respiratory irregularities and reduced arterial oxygen levels. To elucidate whether such intermittent hypoxic episodes induce adaptation/preconditioning of the hypoxia-vulnerable hippocampal network, we analyzed its responses to severe hypoxia in adult Rett mice. The occurrence of hypoxia-induced spreading depression (HSD) – an experimental model for ischemic stroke – was hastened in Mecp2−/y males. The extracellular K⁺ rise during HSD was attenuated in Mecp2−/y males and the input resistance of CA1 pyramidal neurons decreased less before HSD onset. CA1 pyramidal neurons were smaller and more densely packed, but the cell swelling during HSD was unaffected. The intrinsic optical signal and the propagation of HSD were similar among the different genotypes. Basal synaptic function was intact, but Mecp2−/y males showed reduced paired-pulse facilitation, higher field potential/fiber volley ratios but no increased seizure susceptibility. Synaptic failure during hypoxia was complete in all genotypes and the final degree of posthypoxic synaptic recovery indistinguishable. Cellular ATP content was normal in Mecp2−/y males, but their hematocrit was increased as was HIF-1α expression throughout the brain. This is the first study showing that in Rett syndrome, the susceptibility of telencephalic neuronal networks to hypoxia is increased; the underlying molecular mechanisms apparently involve disturbed K⁺ channel function. Such an increase in hypoxia susceptibility may potentially contribute to the vulnerability of male Rett patients who are either not viable or severely disabled.

Key words: synaptopathy, network dysfunction, anoxia, light scattering, potassium channel
INTRODUCTION

Rett syndrome is a genetic, X-chromosome linked neurodevelopmental disorder caused by mutations in the MECP2 gene (Amir et al. 1999). It almost exclusively affects girls, starting within their first 6-18 months. This apparent gender preference arises, because Rett boys are usually not viable; if born they are severely disabled and die prematurely. Rett syndrome primarily targets the brain. An initially normal development is followed by loss of motor capabilities and cognitive function. Other characteristics are stereotyped hand movements, spasticity, breathing disturbances, epileptic seizures, and autistic behavior (Hagberg et al. 1983; Steffenburg et al. 2001). The MECP2 gene affected in Rett patients encodes for a transcriptional regulator, methyl CpG binding protein 2 (MeCP2), which is involved in long term gene silencing (Bienvenu and Chelly 2006). Target genes being controlled include BDNF, the transcription factor DLX5 (Bienvenu and Chelly 2006), the modulator of Na⁺/K⁺ ATPase phospholemman (Deng et al. 2007), and a subunit of mitochondrial respiratory complex III (Kriaucionis et al. 2006). Since MeCP2 is required for the formation and maintenance of functional synapses and neuronal networks (Zoghbi 2003), Rett syndrome is considered a synaptopathy. At birth MeCP2 is highly expressed in those networks being vital at birth, i.e. respiratory, circulatory, and cardiac control. In the neocortex, hippocampus and cerebellum it is expressed later in life when these networks mature (Shahbazian et al. 2002; Zoghbi 2003). Interestingly, MeCP2 is abundant in mature neurons, but not expressed in glia (Jung et al. 2003; Shahbazian et al. 2002).

Different Rett mouse models are available carrying MECP2 knockout (KO) mutations (Guy et al. 2001) or MECP2 truncations (Shahbazian et al. 2002). They provide accumulating evidence that MeCP2 deficiency affects synaptic function and plasticity. A shift of excitation/inhibition causes cortical hyperexcitability and epileptic seizures (Glaze 2005), and among the affected molecular targets are a modulator of the Na⁺/K⁺ ATPase (Deng et al. 2007) and the GABA_A receptor β3
subunit (Samaco et al. 2005). Also a reduced expression level of the GABA$_A$ receptor $\alpha_2$ and $\alpha_4$ subunits has been reported (Medrihan et al. 2008). In these genetic mouse models, the typical signs of Rett syndrome are, however, milder than in the patients.

Rett patients are threatened by sudden death resulting from cardiac dysregulation and/or breathing disturbances (Julu et al. 2001; Kerr et al. 1997). In the awake state phases of irregular, inadequate breathing occur, paralleled by drops in arterial O$_2$ levels below 60 mm Hg (Julu et al. 2001). They apparently reflect over-excitability of post-inspiratory neurons resulting in prolonged expiratory intervals and apneas lasting up to a minute (Stettner et al. 2007; Stettner et al. 2008). To which extent these apneas and intermittent hypoxic episodes affect neural function is largely unclear. Hippocampal, neocortical, and cerebellar circuits are among the most hypoxia/ischemia vulnerable parts of our brain (Pulsinelli et al. 1982; Schmidt-Kastner and Freund 1991). Accordingly, they may be potentially impaired by such repeated metabolic compromise.

We therefore screened for changes in synaptic function, and in a multiparametric approach we analyzed the hippocampal responses to severe acute hypoxia, systematically comparing both transgenic males and females with wildtype mice. As hypoxic network response the hypoxia-induced spreading depression (HSD) was chosen, a concerted massive depolarization of neurons and glial cells that is critically dependent on intact neuronal networks, and whose ignition is affected by either kind of excitatory/inhibitory imbalance (Müller 2000; Müller and Somjen 2000; Somjen 2001). Multiple electrophysiological parameters of HSD were analyzed, complemented by an analysis of the associated intrinsic optical signals that report the propagation velocity of HSD and the severity of brain tissue invasion (Aitken et al. 1999; Andrew et al. 1999; Müller and Somjen 1999). Also, hypoxia-related synaptic failure and recovery were elucidated.
MATERIALS AND METHODS

Preparation

As a Rett model mice lacking the MECP2 gene [B6.129P2(C)-Mecp2<sup>tm1-1Bird</sup> (Guy et al. 2001)] were used. Heterozygous female mice were obtained from Jackson Laboratories, Bar Harbor, ME and bred with wildtype (WT) males (C57BL/6J) to generate heterozygous females (Mecp2<sup>+/−</sup>), hemizygous males (Mecp2<sup>−/−</sup>) and WT mice of either sex. Ether anesthetized mice were decapitated, the brain was rapidly removed from the skull and placed in chilled artificial cerebrospinal fluid (ACSF) for 1-2 minutes. At the same time blood samples were taken for hematocrit analysis. For later HIF-1α level analysis brain tissue samples from different regions were isolated and immediately frozen in liquid nitrogen. Acute neocortical/hippocampal tissue slices (400 µm thick transverse slices) were cut from the forebrain using a vibroslicer (Campden Instruments, 752M Vibroslice). The slices were then separated in the sagittal midline, transferred to an Oslo style interface recording chamber and left undisturbed for at least 90 min. The recording chamber was kept at a temperature of 35-36 °C, continuously aerated with 95% O₂ - 5% CO₂ (400 ml/min), and perfused with oxygenated ACSF (3-4 ml/min). The ACSF contained (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 1.2 CaCl₂, 1.2 MgSO₄, and 10 Dextrose; aerated with 95% O₂ - 5% CO₂ to adjust pH to 7.4.

Morphometric cell analysis

Mice were deeply anaesthetized with isoflurane (Abbott). Once respiration was severely depressed, final gasping was visible, the animal failed to respond to noxious pinch of tail or toe and the heart stopped beating, the chest was opened ventrally and the animals were perfused transcardially, first with phosphate-buffered saline (PBS, 100 ml) to remove blood and subsequently with 150 ml PBS containing 4% paraformaldehyde (PFA, Riedel de Haen). The isolated brain was postfixd (>48h) in 4% PFA containing 30% saccharose (NeoLab), before
consecutive coronal sections (20 μm) were cut using a freezing microtome (Reichert and Jung). Sections were placed on microscope slides, air-dried, incubated for 4 h in propanol-ethanol (1:1), rinsed in a descending ethanol series (99.9%, 96%, 90%, 70%), and washed in demineralized water. Slides were then incubated in thionin solution (1.25 mg/ml) for 1-2 min; excessive thionin was washed out by water and an ascending ethanol series (70%, 90%, 96%, 99.9%). Sections were cleared in terpineol-xylol 1:1 (Sigma-Aldrich) and xylol (Roth) and coverslipped with Depex (Serva). Morphometric analysis was performed with a digital microscope (Coolscope, Nikon) equipped with geometric cell analysis software (NIS-Elements AR 2.1, Nikon).

**HIF-1α Western blot**

Brain tissue samples were rapidly homogenized in a buffer containing 50 mM Tris·HCl, 1% SDS (sodium dodecyl sulfate), 1 mM sodium-orthovanadate, 2 mmol/l EDTA, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Proteins were quantified using the DC-protein assay (Biorad). Protein (200 μg) in 1x Laemmli SDS sample buffer were boiled for 5 min and after centrifugation loaded onto a 10 % SDS-PAGE gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The filters were blocked with 5% nonfat milk, and then incubated with antibodies directed against HIF-1α (Abcam) and β-actin (Sigma-Aldrich).

**Hypoxia-protocol and electrical recordings**

Severe hypoxia was induced by switching the recording chamber’s gas supply from carbogen (95% O₂-5% CO₂) to 95% N₂-5% CO₂ (carbogen aeration of the ACSF was continued), and it triggered HSD within a few minutes. O₂ was resubmitted 20 s after the onset of HSD, within that time the extracellular DC potential shift had reached its nadir. Extracellular recording electrodes were made from thin-walled borosilicate glass (GC150TF-10, Harvard Apparatus), filled with ACSF, and the tips were trimmed to a resistance of ~5 MΩ. Sharp microelectrodes for current-
clamp recordings were made from thick-walled glass (GC 150F-10, Harvard Apparatus), filled with 2 M K-acetate + 5 mM KCl + 10 mM Hepes ((N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)); pH 7.4. Their tips were beveled to a final resistance of ~80 MΩ.

Field excitatory postsynaptic potentials (fEPSPs) were elicited by 0.1 ms unipolar stimuli (Grass S88 stimulator with PSIU6 stimulus isolation units, Grass Instruments), delivered via steel microwire electrodes (50 µm diameter, AM-Systems). Orthodromic responses were elicited by stimulation of Schaffer collaterals and recorded in st. radiatum of the CA1 region with a locally constructed extracellular DC potential amplifier as described earlier (Hepp et al. 2005). All electrophysiological data were sampled using an Axon Instruments Digitizer 1322A and PClamp 9.2 software (Molecular Devices). Fiber volleys were analyzed from representative traces only, which showed clear fiber volleys and high signal to noise ratios. Synaptic failure during hypoxia and posthypoxic recovery were analyzed in continuous DC potential recordings, eliciting fEPSPs every 20 s (Hepp and Müller 2008).

Current-clamp recordings from CA1 neurons were performed with an intracellular recording amplifier (SEC-05L, NPI Instruments, Tamm, Germany) (Hepp et al. 2005). Bridge balance and electrode-capacitance compensation were continuously controlled. Only CA1 neurons with a stable membrane potential of at least -55 mV were accepted. Their input resistance was probed every 10 s by a hyperpolarizing current (400-600 pA amplitude, 200 ms duration) and measured at the steady state level of the voltage deflections, averaging 5 successive current pulses.

Changes in the extracellular K⁺ concentration ([K⁺]o) were recorded using double barreled K⁺ selective microelectrodes of the twisted type and a differential electrometer amplifier (FD 223, World Precision Instruments) as described in detail earlier (Hepp and Müller 2008). Two
borosilicate glass capillaries - one with and the other without a filament (GC150-15 and GC100F-15, Harvard Apparatus) - were cut in halves, glued together with epoxy glue and pulled on a vertical puller (Narishige PE-2). The designated ion-selective barrel was silanized by HMDS vapors (hexamethyldisilazane, 98%, Fluka), filled with the Potassium Ionophore I (Cocktail A, Fluka 60031) and backfilled with 150 mM KCl + 10 mM HEPES, pH 7.4. The reference barrel contained 150 mM NaCl + 10 mM HEPES, pH 7.4 (Hepp and Müller 2008; Müller and Somjen 2000). Electrode resistances of the reference and the ion-selective barrel were 10-20 MΩ and 180-250 MΩ, respectively. K⁺-selective electrodes were calibrated by detecting their responses in standard solutions (0, 1, 2, 5, 10, 20, 50, and 100 mM K⁺). Their average slopes were 52.7±4.3 mV/decade and their detection limits averaged 0.87±0.62 mM K⁺ (n=26). [K⁺]₀ were calculated directly from the electrode responses using the slope of the calibration.

Cell swelling during HSD was quantified using TMA (tetramethylammonium)-selective microelectrodes, determining changes in the extracellular background concentration (1.5 mM) of the cell impermeant marker TMA ([TMA]₀) according to the indicator-dilution technique (Hansen and Olsen 1980; Nicholson and Phillips 1981). The TMA-selective barrel was filled with the Corning 477317 K⁺ ion exchanger (IE190, World Precision Instruments) and backfilled with 150 mM TMA-Cl + 10 mM HEPES, pH 7.4. The reference barrel contained 150 mM NaCl + 10 mM HEPES, pH 7.4 (Müller 2000; Müller and Somjen 1999). TMA-selective electrodes were calibrated by detecting their responses in standard solutions (0, 0.1, 0.5, 1, 5, 10, 50 and 100 mM TMA solutions). Their average slopes were 59.5±5.1 mV/decade and their detection limits averaged 0.35±0.41 mM TMA (n=31). Changes in relative interstitial volume (ISV) were normalized to control conditions according to the formula (Dietzel et al. 1980; Müller 2000):

$$\Delta ISV(\%) = 100 \times \left(\frac{[TMA]_{\text{baseline}}}{[TMA]_{\text{hypoxia}}} - 1\right)$$
Optical recordings

The intrinsic optical signal (IOS) of HSD - an increase in light scattering - was monitored with a Polychrome II imaging system (Till Photonics, Gräfelfing, Germany) and a sensitive CCD camera (Imago QE, PCO Imaging, Kelheim, Germany) as described earlier (Gerich et al. 2006; Hepp and Müller 2008). Interfaced hippocampal slices were illuminated (white light) at an angle of ~45°, viewed with a 5x objective (Epiplan, Zeiss), and images were taken every 2 s (15 ms exposure time). Hypoxia-induced changes in tissue reflectance were visualized by offline image subtraction and normalized to pre-hypoxia baseline reflectance (Müller and Somjen 1999). They are displayed in a 256 gray-scale mode covering a range of ±20 % brightness changes and were quantified in a small rectangular region of interest in CA1 st. radiatum close to the recording electrode. The propagation velocity of HSD was calculated from the wave front progression of the reflectance increase. The invaded area was determined by counting those pixels whose brightness had increased by ≥5 % (Gerich et al. 2006). Image analysis was performed with Tillvision 4.0 (Till Photonics, Gräfelfing, Germany) and MetaMorph Offline 6.1 (Universal Imaging Corporation).

Determination of cellular ATP levels

Cellular ATP content was determined from acute hippocampal tissue slices (hippocampal formation detached from neocortex) as reported earlier (Foster et al. 2006; Gerich et al. 2006). Slices were transferred in perchloric acid (8%) and homogenized by sonication. ATP levels were quantified based on a coupled reaction of glucose-6P-dehydrogenase and hexokinase, by monitoring spectrophotometrically the formation of NADPH2 (Foster et al. 2006; Lamprecht and Trautschold 1974), and they were normalized to the protein content of the given sample determined by Bradford protein assay.
Statistics

Since the experiments did not last longer than ~1 hour, we used up to four slices from each brain. To ensure independence of observations, each experimental series was performed on at least 4 different animals of each genotype. All numerical values are represented as mean ± standard deviation; the number of experiments (n) refers to the number of slices investigated. Due to the different ages of male (38-60 days) and female mice (5-14 month) used, cross gender comparisons were not performed anywhere in the study. Significance of the observed changes (wildtype male versus Mecp2⁻ʸ male or wildtype female versus Mecp2⁺⁻ female) was tested using a two-tailed, unpaired Student’s t-test and a significance level of P=5 %. In the case of significant changes, the P value is reported and in the diagrams significant changes are indicated by asterisks (* P<0.05; ** P<0.01).
RESULTS

In acute hippocampal slices, severe hypoxia triggers within a few minutes hypoxia-induced spreading depression (HSD). It is characterized by a near complete depolarization of neurons and glial cells that is synchronized in neighboring cells, severely disturbed ionic distribution and a negative shift in the extracellular DC potential (Hansen 1985; Müller and Somjen 2000; Nicholson and Kraig 1981; Somjen 2001). Synaptic function and axonal conduction are blocked and once triggered, HSD slowly spreads out from its ignition site (Müller and Somjen 1999; Somjen 2001). To screen for differences in the hypoxia-susceptibility of wildtype (WT) and Rett mice, HSD was followed both electrically and optically (Fig. 1, Table 1). Hemizygous males (Mecp2\(^{-/}\)) die already around postnatal day 60, whereas heterozygous females (Mecp2\(^{+/}\)) develop symptoms only after several months. Therefore, the age of the males (38-60 days) and females (5-14 month old) used differed.

In WT males HSD occurred within 170±54 s of severe hypoxia, the sudden, negative DC shift (ΔVo) had an amplitude of -15.6±2.5 mV and measured at the half amplitude level it lasted 51±14 s (n=14). WT females generated similar HSD episodes (for details see Table 1, Fig. 1A). Comparing WT and Rett mice showed that the characteristic HSD parameters - amplitude, duration, and time to onset - determined for Mecp2\(^{+/}\) females did not differ significantly from those obtained in WT females. In Mecp2\(^{-/}\) males the onset of HSD was, however, significantly hastened, on average by 24 %, as compared to WT males (P=0.0179). HSD amplitude and duration were unchanged, neither did the time course or profile of the DC potential shifts clearly differ among the different genotypes (Fig. 1A, Table 1).

In addition to the electrophysiological features of HSD the optical signs – an increase in light scattering referred to as intrinsic optical signal (IOS) – was monitored as well. The IOS coincides
with the electrophysiological signs of HSD and is detectable as an increase in light reflectance of a given slice (Aitken et al. 1999; Andrew et al. 1999; Basarsky et al. 1998; Müller and Somjen 1999). In all Mecp2 genotypes the reflectance increase was most pronounced in the dendritic layers (st. radiatum, st. oriens) but relatively spared st. pyramidale. It invaded preferentially CA1 and dentate gyrus but did not spread into the CA3 subfield (Fig. 1B). As indicated by the reflectance increase, neocortical areas and parts of the diencephalon/basal ganglia also underwent HSD (Fig. 1B); nevertheless, we focused our detailed analysis on the hippocampus. Quantifying the dynamic changes in light reflectance in a small rectangular region close to the recording electrode (CA1, st. radiatum) yielded comparable increases in light reflectance for WT and Mecp2\(^{-/y}\) males as well as WT and Mecp2\(^{+/y}\) females. Also the time course of the IOS was similar (Fig. 1B, C; Table 1). The relative area, i.e. those parts of the total hippocampal area invaded by the IOS, (on average 54-56%) was indistinguishable among the different genotypes. The propagation velocity of HSD, determined from the spreading wave front of the IOS, was similar in WT and Mecp2\(^{-/y}\) males; neither did it differ among WT and Mecp2\(^{+/y}\) females (Fig. 1D).

**Ionic disturbance and repetitive hypoxia**

To identify possible mechanisms underlying the hastened onset of HSD in Mecp2\(^{-/y}\) males, we quantified the changes in \([K^+]_o\), because extracellular K\(^+\) accumulation is a key event in the generation and propagation of HSD (Grafstein 1956; Kager et al. 2002; Van Harreveld 1978). The \([K^+]_o\) was monitored continuously with ion-selective microelectrodes, and HSD was induced repeatedly to elucidate whether WT and Mecp2\(^{-/y}\) males respond differently to repeated hypoxic treatment. During hypoxia the \([K^+]_o\) shows a characteristic profile, consisting of an initial increase before HSD onset (threshold), followed by a massive rise as HSD is ignited (peak) and a transient undershoot of the prehypoxic baseline (undershoot) during posthypoxic recovery (Fig. 2A). In WT males, soon after O\(_2\) withdrawal \([K^+]_o\) started to increase at a rate of
5.8±1.6 mM/min, reaching a level of 9.3±4.9 mM before HSD was ignited. With the occurrence of the DC potential deflection, \([K^+]_o\) rapidly rose to its peak level of 73.7±17.5 mM. Upon reoxygenation \([K^+]_o\) recovered and transiently undershot its prehypoxic baseline, reaching a nadir of 2.1±0.5 mM (n=13, Fig. 2A). Repeated hypoxic treatment (20 min recovery in between HSD episodes) did not reveal any significant changes in the characteristic electrophysiological signs of HSD, i.e. time to HSD onset, amplitude and duration of the DC shift (Fig. 2A, B). In \(Mecp2^{-/-}\) males the initial rate of the \(K^+\) increase (5.6±1.2 mM/min) and the \(K^+\) level reached before HSD onset (8.2±3.6 mM) corresponded to the changes observed in WT males, the \(K^+\) peak level at the height of HSD was, however, significantly reduced, averaging 52.3±29.5 mM (n=12; \(P=0.0358\)). The posthypoxic undershoot of the \(K^+\) baseline (2.1±0.5 mM) was again indistinguishable from WT males (Fig. 2A). Inducing a second HSD again showed a significantly decreased \(K^+\) peak level at the height of HSD (\(P=0.0431\); Fig. 2C); during the third HSD episode no differences were observed.

The IOS responses remained unchanged when the hypoxic treatment was repeated. Comparing the reflectance increase in WT (n=13) and \(Mecp2^{-/-}\) (n=12) males did not reveal any differences in its intensity or time course, neither did the propagation velocity of HSD or the degree of tissue invasion show any signs of adaptation or differences among the genotypes (Fig. 3).

**Intracellular recordings during HSD**

In search for the cause of the reduced \(K^+\) peak level during HSD in \(Mecp2^{-/-}\) males, we performed intracellular recordings from CA1 pyramidal neurons in acute slices. Their resting membrane potential (point ‘a’ in Fig. 4A) and input resistances in WT (-68.3±10.3 mV; 25.3±8.3 M\(\Omega\), n=14) and \(Mecp2^{-/-}\) males (-66.2±11.6 mV; 27.7±9.4 M\(\Omega\), n=18) under control conditions were similar, but their responses to severe hypoxia showed some differences. Early during hypoxia, CA1
pyramidal neurons of WT males hyperpolarized by -3.2±4.3 mV and their input resistance decreased by 43.0±16.2% (point ‘b’ in Fig. 4A). The initial hyperpolarization then turned into a slow depolarization. At a threshold potential of -44.8±12.8 mV (point ‘c’ in Fig. 4A) the sudden depolarization occurred driving the membrane potential to -18.4±4.8 mV (point ‘d’ in Fig. 4A) and then more slowly to a final peak of -10.7±6.7 mV (point ‘e’ in Fig. 4A). During reoxygenation cell impalement usually became unstable (arrow mark in Fig. 4A) and then was lost – due to severe cell swelling. Accordingly, in most slices the posthypoxic recovery could not be followed completely and putative differences in that phase could not be elucidated. In Mecp2<sup>-/-</sup> males the initial hyperpolarization was comparable, but the decrease in input resistance (-27.9±15.4%) was significantly less pronounced than in WT males (P=0.0290). The threshold potential, the final depolarization reached, and the massive decrease in input resistance corresponded to those in WT males (Fig. 4). Since a similar massive depolarization was found in CA1 neurons of WT and Mecp2<sup>-/-</sup> males, incomplete neuronal depolarizations can not be the cause for the reduced K<sup>+</sup> peak level observed during HSD in Mecp2<sup>-/-</sup> males.

**Differences in ISV and cell swelling during HSD,**

Another reason for the reduced K<sup>+</sup> peak level and the reduced DC potential amplitudes associated with HSD in Mecp2<sup>-/-</sup> males might be a wider interstitial space due to smaller neuronal size and/or a different packing density of neuronal elements. To elucidate this possibility the neuronal size and packing were analyzed and cell volume changes during HSD monitored.

Rett mice have been reported previously to have a reduced brain size – about 13% for the Rett mouse model used (Belichenko et al. 2008). Morphometric analysis of thionin stained cortico-hippocampal sections (20-50 µm thickness, 3 slices for each genotype) of age-matched mice show an average reduction in the area of the hippocampal formation in Mecp2<sup>-/-</sup> males by 17.7%
(Fig. 5A), extending earlier studies that have found that Mecp2 deficiency reduces neuronal sizes in the CA2 subfield by 15-25% (Chen et al. 2001). Analyzing the soma size of CA1 pyramidal neurons we obtained clear differences between WT (152±29 µm²) and Mecp2<sup>-/y</sup> males (86±19 µm², n=300 neurons each, P<0.0001), amounting to an average reduction in neuronal size by 43% (Fig. 5B). Cell counting in an 80 µm long section of st. pyramidale yielded an average of 24.3±1.7 and 41.7±6.2 neurons in WT and Mecp2<sup>-/y</sup> males, respectively, i.e. a 71.6% higher neuronal packing density in Mecp2<sup>-/y</sup> males (n=10 slices each, P<0.0001). The width of the cell layer did not differ among WT (50.1±6.4 µm) and Mecp2<sup>-/y</sup> males (47.9±6.4 µm).

We next asked whether the smaller neuronal size and higher packing density in Mecp2<sup>-/y</sup> males may affect the degree of cell swelling during HSD and used TMA-selective microelectrodes (Hansen and Olsen 1980; Nicholson and Phillips 1981) to monitor the restriction of the interstitial volume (ISV). Since such recordings require a constant TMA background level, 1.5 mM TMA-chloride was added to the ACSF (Müller 2000; Müller and Somjen 1999). Cell swelling already started before HSD onset, increasing [TMA]<sub>o</sub> to 1.75 mM, which corresponds to a restriction in ISV by 12.6±10.7 % (Fig. 6A, B). The maximum degree of cell swelling occurred at the height of HSD, decreasing ISV by 50.3±13.7 % (n=19) and 50.2±12.3% (n=12) for WT and Mecp2<sup>-/y</sup> males, respectively. Upon reoxygenation [TMA]<sub>o</sub> slowly recovered and transiently undershot its prehypoxic baseline – obviously due to over-regulation of cell volume during posthypoxic recovery and a possible contribution of TMA loss from the interstitial space into the bathing medium (Fig. 6A). The undershoot indicates a transient widening of ISV by 20.8±18.2% (n=19) and 18.8±19.6% (n=12) for WT and Mecp2<sup>-/y</sup> males, respectively. Accordingly, despite differential neuron size and packing density, cell swelling during HSD does not differ among WT and Mecp2<sup>-/y</sup> males.
Synaptic function, plasticity and hypoxic failure

Another factor facilitating HSD ignition in Mecp2\textsuperscript{2/y} males might be an increased excitability of the hippocampal network. Disturbed GABAergic inhibition was reported for this Rett mouse model in medullary neurons, based on decreased expression of α2 and α4 subunits of the GABA\textsubscript{A} receptor (Medrihan et al. 2008), and diminished basal inhibitory rhythmic activity was found in the hippocampal CA3 subfield (Zhang et al. 2008). Therefore, synaptic function was assessed by monitoring orthodromically evoked field potentials (fEPSPs) in st. radiatum of the CA1 region under our experimental conditions (interface recording chamber, ~35.5°C). Input-output curves recorded for stimulation intensities of 10-150 µA (unipolar stimuli) did not significantly differ among the genotypes (Fig. 7A). Half maximum amplitude responses of WT and Mecp2\textsuperscript{2/y} males were obtained with 40-50 µA stimuli, half maximum amplitude responses of WT and Mecp2\textsuperscript{+/-} females were obtained with 30-40 µA stimuli (Fig. 7A). Absolute fEPSP amplitudes were equal among the different genotypes (data not shown). Normalizing the fEPSP amplitudes to the fiber volley did not show any differences for WT and Mecp2\textsuperscript{2/y} females, but yielded a significantly increased ratio for Mecp2\textsuperscript{2/y} than WT males, indicating increased postsynaptic excitability in Mecp2\textsuperscript{2/y} males (P=0.0314; Fig. 7B). Yet obvious signs of pronounced hyperexcitability, i.e. multiple population spikes, were not observed on a regular basis.

As a paradigm for synaptic short-term plasticity we quantified paired-pulse facilitation at various interpulse intervals (25-200 ms). With stimulation intensities adjusted to yield ~50% response amplitudes, marked paired-pulse potentiation was observed. At an interpulse interval of 25 ms it averaged 209.8±58.1% for WT males, but was significantly less pronounced in Mecp2\textsuperscript{2/y} males, 165.9±25.2% (n=14 each; P≤0.0362; Fig. 7C, D). In WT and heterozygous females no differences were observed (WT 168.2±33.8%, n=24; Mecp2\textsuperscript{+/-} 177.8±41.0%, n=14; Fig. 7C, D).
Furthermore, we monitored the loss of synaptic function during hypoxia, to decide whether an altered time course of synaptic failure may contribute to the increased HSD susceptibility of \( Mecp2^{-/-} \) males. Severe hypoxia blocks synaptic function (fEPSPs) within ~2 min (Hansen et al. 1982), as indicated by the inability to elicit evoked responses. We observed a 50% reduction in fEPSP amplitudes within ~80s of hypoxia and it was followed by a complete block of synapses; the time course and extent of synaptic failure were indistinguishable among the different genotypes (Fig. 8A). Yet some differences became evident during the posthypoxic recovery of synaptic function. Reoxygenation was started 20 s after HSD onset and it restored synaptic function within 7-8 minutes (Fig. 8B). Synaptic recovery tended to be slightly delayed in WT females which showed 50% synaptic response amplitudes after 280 s of reoxygenation, whereas \( Mecp2^{+/+} \) females regained 50% responses after 220 s of reoxygenation. Both WT and \( Mecp2^{-/-} \) males regained 50% fEPSPs responses after 220 s of reoxygenation (Fig. 8B). Post-hypoxic potentiation, a transient increase of fEPSP amplitudes immediately after synaptic recovery (Crepel et al. 1993; Frenguelli 1997; Gozlan et al. 1994), was observed in WT males only, averaging 121.7±27.8% of control (n=13, P≤0.0390; Fig. 8B). The final degree of synaptic recovery reached after 20 min of reoxygenation was, however, indistinguishable among the different genotypes; fEPSPs stabilized at ~80% of their respective control amplitudes (Fig. 8B).

**Seizure susceptibility**

In Rett syndrome neuronal networks seem functionally disturbed by impaired inhibition and increased excitation, resulting in hyperexcitability (Medrihan et al. 2008; Zhang et al. 2008). The increased ratio of fEPSP/fiber volley observed in \( Mecp2^{-/-} \) males (Fig. 7B) suggests such increased excitability also for the hippocampal network. We therefore asked whether seizure susceptibility is increased and applied stimuli of different severity: low Mg\(^{2+}\) solutions to potentiate NMDA receptors (Anderson et al. 1986; Mody et al. 1987), low Mg\(^{2+}\) solutions plus
bicuculline to also diminish GABAergic inhibition (Borck and Jefferys 1999), and 4-
aminopyridine (4-AP) to block K⁺ channel-mediated inhibition (Rutecki et al. 1987). DC
potentials were recorded in st. radiatum of the CA1 subfield and the temperature was lowered to
33°C to prevent spontaneous spreading depression episodes (Mody et al. 1987).

Lowering [Mg²⁺]₀ to 0.25 mM induced spontaneous burst discharges with amplitudes of
~0.2 mV, but evoked fEPSPs did not show any obvious signs of hyperexcitability (Fig. 9, Table
2). Combining low Mg²⁺ solution with 10 µM bicuculline induced pronounced interictal spikes of
~1.5 mV amplitudes and ~0.5 s duration, and the evoked fEPSPs showed multiple population
spikes, indicating hyperexcitability (Fig. 9). Application of 100 µM 4-AP induced massive
seizure-like events which were characterized by an initial fast negative deflection by up to 2 mV
followed by pronounced bursting activity, but were early terminated and typically lasted only
~1.5-2 s. In the presence of 4-AP evoked fEPSPs also showed multiple population spikes (Fig. 9).
Quantitative analysis of the different types of spontaneous and seizure-like activity (for summary
see Table 2) did not reveal any significant differences for slices obtained from WT and MeCP2⁻/⁻
males. Accordingly an increased seizure susceptibility and/or severe hyperexcitability in MeCP2⁻/⁻
males can also be excluded to underlie the increased hypoxia-susceptibility.

**Blood and tissue analyses**
As a test for systemic adaptation of MeCP2⁻/⁻ males to the repeated apnea-related systemic
hypoxic episodes, we determined the hematocrit, tissue levels of hypoxia-inducible factor (HIF)
1α, and cellular ATP content. The hematocrit – determined from blood samples collected during
dissection – was significantly higher in MeCP2⁻/⁻ (49.8±2.3, n=8) than in WT males (44.1±1.4,
n=7, P=0.0001; Fig. 10A), which suggests that during their life these MeCP2⁻/⁻ males experienced
repeated systemic hypoxic episodes. Analysis of brain tissue samples isolated from neocortex,
cerebellum, lower brainstem and hippocampus consistently showed a higher HIF-1α expression level in \( \text{Mecp2}\) (n=4) than in WT males (n=3; Fig. 10B). Since HIF-1α expression increases in a transient manner within minutes of systemic hypoxia (Jewell et al. 2001; Stroka et al. 2001), the underlying cause for the observed increase in HIF-1α expression is obviously the global ischemic condition inherent to the isolation of the brain tissue during dissection. Nevertheless this proves that in addition to hippocampus also the other brain regions of \( \text{Mecp2}\) males are more sensitive to hypoxia/ischemia.

To decide, whether tissue ATP content may be altered under control conditions or affected differently by metabolic compromise, ATP levels were determined in acute tissue slices (isolated hippocampal formation only). Significant differences were, however, not detected; slices from WT males contained 10.2±3.8 nmol ATP/mg protein (n=6) and those from Mecp2 males 13.8±7.1 nmol ATP/mg protein (n=8). Metabolic challenge by 100 µM CN\(^-\) (25 min) did not cause significant alterations – neither in WT nor \( \text{Mecp2}\) males (Fig. 10C). Therefore, a lower ATP content or accelerated ATP consumption can be excluded to hasten the onset of HSD in \( \text{Mecp2}\) males.
DISCUSSION

Rett syndrome impairs cortical function and due to severe breathing disturbances and frequent apneas it is associated with intermittent episodes of systemic hypoxia. In the present study we analyzed in a Rett mouse model whether the systemic hypoxic episodes experienced by the animals disturb the function of the highly anoxia/ischemia vulnerable hippocampal formation or modulate its responses to acute severe hypoxia. The increased hematocrit of Mecp2<sup>−/−</sup> males confirms a systemic adaptation to the recurrent hypoxic episodes, but evidence of preconditioning of the hippocampal formation was not found. Rather, we observed impaired synaptic short-term plasticity in Mecp2<sup>−/−</sup> males and an increased susceptibility of the hippocampus to acute severe hypoxia. Our study is the first report on an increased hypoxia-susceptibility of telencephalic neuronal networks in Rett syndrome. In multiparametric analyses we obtained first evidence that disturbed K<sup>+</sup> channel function is among the underlying molecular mechanisms.

HSD onset was hastened, as reported by the accelerated occurrence of the DC potential shift in Mecp2<sup>−/−</sup> males. This clearly indicates a reduced hypoxia tolerance, as in these mice the neurons and glial cells lost their membrane potentials within a shorter period of hypoxia as compared to WT males. Once ignited though, the neuronal hypoxic depolarizations underlying the generation of HSD were complete in both WT and Mecp2<sup>−/−</sup> males. Neither did WT and Mecp2<sup>−/−</sup> males react differently when HSD was induced repeatedly. The invasion of the hippocampal formation by HSD, its propagation velocity and the intensity of the reflectance increase did not differ among the WT and Rett mice. Cross gender comparison was not performed, as the age of males (38-60 days) and females (5-14 month) used differed.

Despite the hastened onset of HSD in Mecp2<sup>−/−</sup> males, the time course of synaptic failure during hypoxia and the final degree of posthypoxic synaptic recovery did not differ. This suggests that
the earlier HSD onset in \( \text{Mecp}^{2/y} \) males does not culminate in more pronounced neuronal damage, at least not with the short duration of hypoxia applied and the time span of posthypoxic recovery analyzed. Whether long-term neuronal damage differs cannot be investigated in acute slices, but rather would have required \textit{in vivo} stroke-models to be able to quantify neuronal damage several days after the insult.

So far, there are only single reports on increased hypoxic responses in Rett syndrome. Respiratory responses to hypoxia (5 min, 8% \( O_2 \)), i.e. the initial increase in minute ventilation as well as the respiratory depression following hypoxia, were more pronounced in \( \text{Mecp}^{2/y} \) and \( \text{Mecp}^{2/+} \) females than in WT mice (Bissonnette and Knopp 2006). In cultured cerebellar granule cells cell death upon hypoxia (95% \( N_2/5\% \text{ CO}_2 \)) and NMDA/AMPA-mediated excitotoxicity was more pronounced, as short insults not threatening WT cells already were deleterious in \( \text{Mecp}^{2/y} \) cells (Russell et al. 2007). Activation of caspase-3 and mitochondrial release of apoptosis inducing factor were intensified (Russell et al. 2007), suggesting that mitochondria may be more vulnerable in \( \text{Mecp}^{2/y} \) males.

Other mechanisms that might increase the hypoxia vulnerability are a decreased \( \text{Na}^+/\text{K}^+ \) ATPase activity as found in neocortical neurons of \( \text{MECP2}\)-null mice and Rett patients (Deng et al. 1999) and reduced brain ATP levels reported for \( \text{MECP2}\)-null mice (Saywell et al. 2006). In hippocampal slices from \( \text{Mecp}^{2/y} \) males we found, however, normal cellular ATP levels that did not react differently to moderate chemical anoxia. Also the resting membrane potentials of CA1 pyramidal neurons in WT and \( \text{Mecp}^{2/y} \) males did not differ. Accordingly, such changes are unlikely to hasten the onset of HSD in \( \text{Mecp}^{2/y} \) males.
In general, conditions increasing neuronal excitability hasten HSD onset while those reducing excitability postpone it (Aitken et al. 1991; Müller 2000; Müller and Somjen 2000). Hyperexcitability arising from reduced inhibition and giant evoked potentials were reported for neocortical networks of Rett patients (Glaze 2005), which explains why Rett patients suffer from epileptic seizures (Steffenburg et al. 2001). GABA_A receptor α2, α4 and β3 subunit expression is reduced in the brain of MECP2 deficient mice (Medrihan et al. 2008; Samaco et al. 2005), basal inhibitory rhythmic activity is diminished (Zhang et al. 2008), and increased mouse brain glutamine levels suggest changes in glutamate release and recycling (Viola et al. 2007). Such molecular disturbances potentially induce hyperexcitability and indeed we observed a slightly increased ratio of EPSP/fiber volley amplitudes in Mecp2^−/− males, which corresponds to observations on Mecp2^308/0^ males with a truncated MECP2 gene (Moretti et al. 2006). Signs of pronounced hyperexcitability such as multiple population spikes or markedly enhanced fEPSPs amplitudes were, however, not found. Also our epilepsy tests confirm that the seizure susceptibility in Mecp2^−/− males is not noticeably increased.

Basal synaptic function, i.e. the synaptic response to single pulse stimulation, was intact in Mecp2^−/− males and Mecp2^+/− females, which extends earlier observations restricted to Mecp2^−/− males only (Asaka et al. 2006). Paired-pulse facilitation was impaired in Mecp2^−/− males but not in Mecp2^+/− females. Accordingly, in female Rett mice the X-chromosomal inactivation pattern seems sufficient to guarantee synaptic function, at least on the level of field potential recordings. The degree to which paired-pulse facilitation was suppressed in Mecp2^−/− males matches earlier studies which were performed on the same mouse model and/or mice carrying a truncated MEPC2 gene and which also found hippocampal long-term potentiation to be impaired (Asaka et al. 2006; Guy et al. 2001; Moretti et al. 2006). Paired-pulse facilitation is of presynaptic origin (Kuhnt and Voronin 1994), whereas early long-term potentiation is induced postsynaptically.
(Malenka and Bear 2004). Accordingly, in the hippocampal formation MeCP2 deficiency apparently affects both, pre- and postsynaptic targets.

As a possible reason for the increased hypoxia susceptibility we suggest reduced $K^+$ flux during hypoxia. Intracellular recordings from CA1 pyramidal neurons reported a less intense decrease in input resistance early during hypoxia in $Mecp2^{-/y}$ males. In this early phase of hypoxia $K^+$ channels are activated (Hansen et al. 1982; Müller and Somjen 2000), and both $K_{ATP}$ and BK-type $K_{Ca}$ channels are considered to mediate the initial hyperpolarization (Erdemli et al. 1998; Fujimura et al. 1997; Zawar and Neumcke 2000). We therefore propose that in $Mecp2^{-/y}$ males the function and/or expression level of these channel types may be impaired. The unequivocal identification of the very $K^+$ channel affected and the type of modulation imposed will require in-depth single-channel characterization complemented by immunohistological studies. In line with these thoughts of altered $K^+$ channel function we found the $[K^+]_o$ peak level at the height of HSD to be attenuated in $Mecp2^{-/y}$ males, which cannot be explained by incomplete hypoxic depolarizations of the single neurons.

Other critical parameters are the tissue morphology and the interstitial volume. In Rett patient brain sections cortical and subcortical neurons are of smaller size and more densely packed (Bauman et al. 1995). Others observed a mild loss of cortical pyramidal neurons as well as reduced complexity of dendritic arborizations and spine densities (Belichenko et al. 1994). The number of presynaptic terminals seems unchanged in $MECP2$ deficient neurons though (Nelson et al. 2006). Furthermore, delayed neocortical maturation and growth were assumed to underlie the reduced cortical thickening (Fukuda et al. 2005). Each of these changes may have contributed to the smaller dimension of the hippocampal formation. Assuming that the ISV might be restricted as well could easily explain the hastened HSD onset (Chebabo et al. 1995), because
extracellular $K^+$ and glutamate could accumulate to critical levels within a shorter duration of hypoxia. A closer proximity of hippocampal neurons may also intensify ephaptic interactions (Francis et al. 2003), facilitating the generation of HSD and seizures. Our morphometric analyses revealed a smaller size and denser packing of CA1 pyramidal neurons in $Mecp2^{-/+}$ males, and the TMA-recordings verify that at least the relative degree of cell swelling during HSD does not differ in WT and $Mecp2^{-/+}$ males. This excludes less intense cell swelling and ISV restriction as the underlying cause for the reduced $K^+$ peak levels observed during HSD. Whether the absolute interstitial volume fraction differs cannot be judged on the basis of our data. This would rather require pulsed iontophoretic application of TMA and detailed analysis of diffusion profiles (Nicholson 1991; Nicholson and Phillips 1981).

Oxidative stress, which is associated with hypoxia/ischemia, also seems augmented in Rett syndrome. Quantification of antioxidant enzyme activities in red blood cells of Rett patients shows a decreased activity of superoxide dismutase, whereas plasma malondialdehyde, a marker for lipid peroxidation, is increased (Sierra et al. 2001). Accordingly, reactive oxygen and nitrogen species could also contribute to the neuronal dysfunction of Rett syndrome. As we have reported earlier for hippocampal slices, such changes in cellular redox status may modulate the susceptibility of neuronal networks to hypoxia (Gerich et al. 2006; Hepp et al. 2005) as well as the outcome (Hepp and Müller 2008). Whatever the very molecular mechanisms of the accelerated HSD onset may be, they are likely to be of neuronal origin, as only hippocampal neurons, but not glial cells express MeCP2 (Jung et al. 2003).

**Concluding remarks**

In conclusion, preconditioning of Rett mice, as may be expected from the intermittent systemic hypoxic episodes they experience, does not seem to take place. Instead, in the case of acute
metabolic compromise, the hypoxia tolerance of Mecp2+/y males is diminished. As evident from the hastened onset of HSD, their hippocampal network failed to tolerate the same duration of severe hypoxia as WT mice or Mecp2+/− females. The loss of neuronal function occurred earlier and the neurological outcome of metabolic compromise, e.g. stroke, reduced perfusion, brain concussion/edema, might be worse under the condition of MeCP2 deficiency. The increased HIF-1α expression levels found in Mecp2+/y males throughout the brain strongly suggest that this increased hypoxia susceptibility is not restricted to the hippocampus. In cortical/hippocampal networks it may well contribute to the cognitive dysfunction and mental disabilities of Rett patients. In medullary control circuits of cardio-respiratory control such an increased susceptibility to hypoxia could be life-threatening – especially in view of the respiratory disturbances and the concomitant decreases in arterial oxygen levels experienced by Rett patients. Furthermore, the increased hypoxia sensitivity could potentially contribute to the vulnerability of male Rett patients, who are either not viable or if they survive until birth, show more severe disabilities than Rett girls.

ACKNOWLEDGEMENTS

We are grateful to Prof. Dr. George. G. Somjen for a critical reading of the manuscript and to Prof. Dr. Irmelin Probst for her help with the determination of ATP levels. This study was supported by the DFG Research Center Molecular Physiology of the Brain (CMPB) and by Göttingen University (Ausstattungsmitte Juniorprofessur).


Table 1: Summary of the electrical and optical parameters of hypoxia-induced spreading depression (HSD) determined for the different mouse genotypes.

<table>
<thead>
<tr>
<th></th>
<th>Δt  (s)</th>
<th>ΔV₀ (mV)</th>
<th>t1/2  (s)</th>
<th>reflectance increase (%)</th>
<th>invaded area (%)</th>
<th>velocity [mm/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>170.0±53.9</td>
<td>-15.6±2.5</td>
<td>50.9±13.9</td>
<td>19.0±9.0</td>
<td>53.8±9.6</td>
<td>6.2±2.5</td>
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<tr>
<td></td>
<td>(n=14)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>Mecp2⁻/⁻</strong></td>
<td>128.9±28.3</td>
<td>-15.3±2.4</td>
<td>43.0±7.1</td>
<td>22.9±10.9</td>
<td>55.3±14.9</td>
<td>6.2±1.8</td>
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<tr>
<td></td>
<td>(n=14) **</td>
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<td></td>
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<tr>
<td><strong>WT female</strong></td>
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<tr>
<td></td>
<td>164.5±48.4</td>
<td>-16.3±3.5</td>
<td>52.9±15.1</td>
<td>24.4±10.0</td>
<td>55.6±14.0</td>
<td>5.7±1.7</td>
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<td>(n=24)</td>
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<tr>
<td><strong>Mecp2⁺/-</strong></td>
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<tr>
<td></td>
<td>162.3±29.6</td>
<td>-15.9±3.1</td>
<td>48.2±6.6</td>
<td>27.9±10.0</td>
<td>56.3±10.5</td>
<td>4.6±1.3</td>
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<tr>
<td></td>
<td>(n=15)</td>
<td></td>
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</tbody>
</table>

Data represent mean ± standard deviation and the number of experiments (n) is indicated; Δt, time to HSD onset; ΔV₀, amplitude of the extracellular DC potential shift; t1/2, HSD duration measured at the half-amplitude level. Significant changes are marked by asterisks (** P < 0.01).
# Table 2: Summary of drug evoked spontaneous activity and seizure-like activity.

<table>
<thead>
<tr>
<th>Type of activity</th>
<th>low Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>low Mg&lt;sup&gt;2+&lt;/sup&gt; + bicuculline</th>
<th>4-AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>transport</td>
<td>WT male</td>
<td>WT male</td>
<td></td>
</tr>
<tr>
<td>spontaneous</td>
<td>42.9% (3/7 slices)</td>
<td>50.0% (3/6 slices)</td>
<td></td>
</tr>
<tr>
<td>bursts</td>
<td>7.7±2.3</td>
<td>8.4 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>frequency</td>
<td>0.53±0.47</td>
<td>0.10±0.04</td>
<td></td>
</tr>
<tr>
<td>interictal</td>
<td>85.7% (6/7 slices)</td>
<td>60.0% (6/10 slices)</td>
<td></td>
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<tr>
<td>spikes</td>
<td>17.8±6.2</td>
<td>10.3±6.9</td>
<td></td>
</tr>
<tr>
<td>onset after</td>
<td>11.6±2.6</td>
<td>11.8±3.1</td>
<td></td>
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<tr>
<td>(min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>seizure-like</td>
<td>100% (8/8 slices)</td>
<td>100% (11/11 slices)</td>
<td></td>
</tr>
<tr>
<td>events</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Listed is the type of spontaneous or seizure activity occurring in response to the respective drug treatment (0.25 mM Mg<sup>2+</sup>, 10 µM bicuculline, 100 µM 4-AP) in WT and Mecp2<sup>−/−</sup> males. Drugs were administered for at least 20 min before the spontaneous/seizure activity was analyzed.
FIGURE LEGENDS

Figure 1: The susceptibility to hypoxia-induced spreading depression (HSD) is increased in Mecp2⁻/⁺ males.

A) Statistical comparison of the characteristic parameters of HSD (amplitude of the extracellular DC-potential shift plotted in mV; time to HSD onset and HSD duration plotted in seconds) determined for wildtype (WT) and Rett mice. The profile of the DC potential shifts (recorded in st. radiatum of CA1) does not markedly differ, but in Mecp2⁻/⁺ males HSD onset is hastened by ~24 % as compared to WT males. In the DC potential recordings the arrow indicates the time point of oxygen withdrawal, reoxygenation was started 20 s after the onset of the sudden negative DC shift. Asterisks mark statistically significant changes (** P<0.01) and the number of trials is reported at the bottom of the bars.

B) The intrinsic optical signal (IOS) associated with HSD is an increase in tissue reflectance which clearly identifies those slice regions invaded by HSD. The displayed images show the maximum spread of the IOS in the respective slices, and the IOS did not noticeably differ among the tested genotypes. Note that the neocortex and parts of the diencephalon/basal ganglia underwent HSD as well. Images are subtraction images and the changes in light reflectance are coded in 256 gray levels.

C) The time course of the averaged reflectance changes does not show any pronounced differences for WT and KO mice. The intensity of the IOS is highest in Mecp2⁺/⁻ females and lowest in WT males, but it does not differ among gender-matched WT and Rett mice. Averaging of the IOS traces required their alignment with respect to HSD onset. Therefore, in the plotted profiles the increase in tissue reflectance occurs simultaneously for all genotypes, which does not reflect the true time to HSD onset.

D) The propagation velocity of HSD does not differ among the different genotypes.
**Figure 2:** Extracellular K⁺ accumulation at the height of HSD is less severe in Mecp2⁻/⁻ males.

A) Recordings of [K⁺]₀ and extracellular DC potential (ΔV₀) during severe hypoxia show that HSD can be induced repeatedly in WT and Mecp2⁻/⁻ males.

B) Statistical comparison of the electrical signs of HSD in WT and Mecp2⁻/⁻ males does not reveal any significant adaptation to such repeated hypoxic treatment.

C) The peak K⁺ level reached at the height of HSD was, however, less pronounced in Mecp2⁻/⁻ than in WT males, suggesting a possible impairment of K⁺ fluxes in Rett syndrome.

**Figure 3:** Inducing HSD repeatedly does not intensify its propagation velocity or final spread.

A) As already seen for the electrical signs of HSD, the intensity and duration of the IOS does not adapt to repeated HSD either.

B) The propagation velocity of HSD and the relative invasion of the hippocampal formation did not change when up to three HSD episodes were elicited.

**Figure 4:** Sharp electrode recordings suggest less intense activation of K⁺ channels early during hypoxia in Mecp2⁻/⁻ males.

A) Intracellular sharp electrode (current-clamp) recordings from CA1 pyramidal neurons confirm a near complete loss of membrane potential in the course of the hypoxic depolarizations in both WT and Mecp2⁻/⁻ males. DC potentials were recorded in st. radiatum nearby the impaled neuron to verify the occurrence of HSD. The entire posthypoxic recovery could only be recorded in a few of the analyzed slices (e.g. the WT male trace shown). Due to severe cell swelling at the height of HSD, sharp electrode recordings usually became unstable (see arrow mark in lower trace) and cell impalement was lost before the recovery was complete. The letters indicate resting membrane potential (a), initial hypoxic hyperpolarization (b), threshold potential at which the
sudden depolarization occurs (c), peak of the sudden depolarization (d) and absolute peak reached at the height of HSD (e).

**B)** Summary of the membrane potential and input resistance changes of CA1 pyramidal neurons during the different stages of the hypoxic response. Resting membrane potential (a) and input resistance are similar in WT and \( \text{Mecp}^{2-\text{y}} \) males. Neither do the threshold potential at which the sudden depolarization occurs (c), the levels of the massive depolarizations (d, e) or the associated decreases in input resistance differ. Yet the input resistance decrease during the initial hyperpolarization (b) is less pronounced in \( \text{Mecp}^{2-\text{y}} \) than in WT males, which strongly suggests a reduced \( K^+ \) channel activity early during hypoxia. The analyzed characteristic points (letters a-e) are identified in the current-clamp trace of panel A.

**Figure 5:** Morphometric cell analysis indicates reduced neuronal size and increased packing density in \( \text{Mecp}^{2-\text{y}} \) males.

**A)** Thionin (Nissl) stained cortico-hippocampal sections of WT and \( \text{Mecp}^{2-\text{y}} \) males (postnatal day 50) viewed at 2x magnification (50 µm thick sections). Quantification of the area of the hippocampal formation (pixel count in the digital images; 3 slices/brains of each genotype) revealed an average reduction in the size of the hippocampal formation of \( \text{Mecp}^{2-\text{y}} \) males by 17.7%.

**B)** The pyramidal cell layer (\emph{st. pyramidale}) of the CA1 subfield shown at 40x magnification (20 µm thick sections) suggests a reduced size and higher packing density of pyramidal cell somata in \( \text{Mecp}^{2-\text{y}} \) males.

**Figure 6:** MeCP2 deficiency does not affect the degree of cell swelling during hypoxia and HSD.

**A)** Sample traces of the DC-potential shift and the restriction in interstitial volume (ISV) – measured as an increase in [TMA]o. At the height of HSD the [TMA]o is almost doubled,
indicating a restriction of ISV by ~50%. A background concentration of 1.5 mM TMA-chloride was added to the ACSF; recordings were performed in *st. radiatum* of the CA1 subfield.

**B)** Statistical comparison of the $[\text{TMA}]_0$ reached at the time of HSD ignition (threshold), the height of HSD (peak), and upon reoxygenation (undershoot) does not reveal any differences in the HSD-related cell volume changes of WT and *Mecp2*<sup>-/-</sup> males. The right hand diagram shows the relative changes in ISV as calculated from the monitored $[\text{TMA}]_0$.

**Figure 7:** MeCP2 deficiency leaves basal synaptic function intact, but reduces synaptic plasticity.

**A)** Input-output curves of orthodromically evoked fEPSPs (*st. radiatum*, CA1) do not differ among the tested genotypes. Plotted are the averaged fEPSPs amplitudes. For clarity, error bars (representing standard deviations) are shown for *Mecp2*<sup>-/-</sup> males (n=14) and wildtype (WT) females only (n=24), but were omitted for WT males (n=14) and *Mecp2*<sup>+/−</sup> females (n=15).

**B)** Normalizing the fEPSP amplitudes to the fiber volley reports an increased ratio in *Mecp2*<sup>-/-</sup> males as compared to WT males, but no differences for *Mecp2*<sup>+/−</sup> and WT females. For this type of analysis fEPSPs evoked by stimuli of 120-150 µA were selected. Asterisks mark significant changes (* P< 0.05); the number of trials is reported.

**C)** Twin-pulse stimulation (25 ms interpulse interval) revealed reduced paired-pulse facilitation in *Mecp2*<sup>-/-</sup> males but not *Mecp2*<sup>+/−</sup> females. Plotted are the averages of four consecutive stimuli. Stimulation intensity was adjusted to elicit half-maximum responses.

**D)** Summarizing the twin-pulse stimulation performed at various interstimulus intervals confirms that short-term plasticity in *Mecp2*<sup>-/-</sup> males is reduced in almost the entire range tested. Note that no changes in short-term plasticity were found in WT and *Mecp2*<sup>+/−</sup> females. Plotted are the normalized fEPSP amplitudes (fEPSP2/fEPSP1); number of observations according to panel A.
**Figure 8:** Synaptic failure during severe hypoxia and synaptic recovery after reoxygenation.

A) Upon oxygen withdrawal (time=0, arrow mark) synaptic function ceased within 2 minutes in all genotypes. Plotted are the normalized, averaged amplitudes of orthodromically evoked fEPSPs (elicited every 20 s). Zero amplitudes were not quite reached, because automated data analysis was configured to detect the nadir of the fEPSPs and thus upon synaptic failure detected noise peaks within the traces. For clarity, error bars were omitted (WT males n=13, Mecp2<sup>+/y</sup> males n=14, WT females n=24, Mecp2<sup>+/</sup> females n=15).

B) Recovery of synaptic function after HSD and reoxygenation (time=0) typically occurred within 6-7 min, and was somewhat slower for WT females. The final level of recovery was, however, indistinguishable for all genotypes. A transient post-anoxic potentiation of synaptic responses occurred in WT males only. Error bars are shown for WT males and WT females only. Asterisks mark significant differences between WT males and Mecp2<sup>+/y</sup> males.

**Figure 9:** Burst discharges and seizure-like activity are not facilitated in Mecp2<sup>+/y</sup> males.

DC potential recordings showing spontaneous and seizure-like activity evoked by 20 min treatment with low Mg<sup>2+</sup> (0.25 mM), low Mg<sup>2+</sup> plus bicuculline (10 µM), and 4-AP (100 µM) in Mecp2<sup>+/y</sup> males. Lowering [Mg<sup>2+</sup>]<sub>o</sub> elicited spontaneous bursts resembling synchronized EPSPs with amplitudes of ~0.5 mV. Low [Mg<sup>2+</sup>]<sub>o</sub> plus bicuculline induced interictal spikes (~ 2 mV amplitudes) followed by multiple population spikes. Most severe discharges were triggered by 4-AP. They resembled seizure-like events and included several population spikes, but were early terminated and lasted no longer than ~2 s. Orthodromically-evoked fEPSPs (150 µA stimuli) obtained upon drug treatment from the same slices are displayed on the right hand side. All recordings were performed in *st. radiatum* of the CA1 subfield.
**Figure 10:** Systemic adaptation proves the occurrence of repetitive hypoxic episodes in *Mecp2*<sup>−/−</sup> males.

**A)** Blood analyses show an increased hematocrit in *Mecp2*<sup>−/−</sup> males, confirming that these animals experienced several systemic hypoxic episodes.

**B)** Western blots indicate increased tissue levels of hypoxia-inducible factor (HIF)-1α in all brain regions tested, suggesting that the increased hypoxia-susceptibility of *Mecp2*<sup>−/−</sup> males is not restricted to the hippocampal formation (Nc neocortex; Ce cerebellum; Hi hippocampus, Bs brainstem).

**C)** The cellular ATP content analyzed in acute hippocampal tissue slices (isolated hippocampal formation) did not significantly differ among WT and *Mecp2*<sup>−/−</sup> males. Also, upon moderate metabolic compromise - induced by 100 µM CN<sup>−</sup> (25 min administration) - no differences could be observed.
Figure 1
Figure 2
Figure 3

A

WT male

Mecp2⁻⁻

Normalized reflectance

1 min

B

Propag velocity [mm/mm]

Norm. invaded area

WT male Mecp2⁻⁻
Figure 4
Figure 6
Figure 8

Panel A: Graph showing the normalized IEPSP amplitude over time in response to hypoxia. The graph compares WT male, Mecp2 (-/y), WT female, and Mecp2 (+/+) conditions.

Panel B: Graph showing the normalized IEPSP amplitude over time in response to reoxygenation. The graph compares WT male, Mecp2 (-/y), WT female, and Mecp2 (+/+) conditions.