Altered responses of MeCP2-deficient mouse brainstem
to severe hypoxia

Miriam Kron\textsuperscript{1,2,*}, Jasper L. Zimmermann\textsuperscript{1}, Mathias Dutschmann\textsuperscript{1,2,**}, Frank Funke\textsuperscript{1}, and Michael Müller\textsuperscript{1,2}

\textsuperscript{1} DFG Research Center Molecular Physiology of the Brain (CMPB),
Zentrum für Physiologie und Pathophysiologie,
Abteilung Neuro- und Sinnesphysiologie,

\textsuperscript{2} Bernstein Center for Computational Neuroscience,
Georg-August-Universität Göttingen,
Göttingen, Germany

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* Present address: Department of Neurosciences, Case Western Reserve University,
Cleveland OH, 44106-4975, USA

** Present address: Institute of Membrane and Systems Biology
University of Leeds, Leeds LS2 9JT, UK

Correspondence to: Prof. Dr. Michael Müller
Zentrum Physiologie und Pathophysiologie
Universität Göttingen
Humboldtallee 23, D-37073 Göttingen, Germany
Phone: +49-551-39-22933,
Fax: +49-551-39-19650
Email: mmuelle7@gwdg.de

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Abstract

Rett syndrome (RTT) patients suffer from respiratory arrhythmias with frequent apneas causing intermittent hypoxia. In a RTT mouse model (methyl-CpG-binding protein 2-deficient mice; \textit{Mecp2}\(^{\text{+/}}\)) we recently discovered an enhanced hippocampal susceptibility to hypoxia and hypoxia-induced spreading depression (HSD). Here, we investigated whether this also applies to infant \textit{Mecp2}\(^{\text{+/}}\) brainstem, which could become life-threatening due to failure of cardiorespiratory control. HSD most reliably occurred in the nucleus of the solitary tract (nTS) and the spinal trigeminal nucleus (Sp5). HSD susceptibility of the \textit{Mecp2}\(^{\text{+/}}\) nTS and Sp5 was increased upon 8 mM K\(^+-\)-mediated conditioning. \(\text{5-HT}_{1\text{A}}\) receptor stimulation (8-OH-DPAT) postponed HSD by up to 40%, mediating genotype-independent protection. The deleterious impact of HSD on \textit{in vitro} respiration became obvious in rhythmically-active slices where HSD propagation into the preBötzinger complex (preBötC) immediately arrested the respiratory rhythm. Compared to wildtype, the \textit{Mecp2}\(^{\text{+/}}\) preBötC was invaded less frequently by HSD, but if so, HSD occurred earlier. Upon reoxygenation, \textit{in vitro} rhythms reappeared with increased frequency, which was less pronounced in \textit{Mecp2}\(^{\text{+/}}\) slices. 8-OH-DPAT increased respiratory frequency but failed to postpone HSD in the preBötC. Repetitive hypoxia facilitated posthypoxic recovery only if HSD occurred. In 57% of \textit{Mecp2}\(^{\text{+/}}\) slices, however, HSD spared the preBötC. While this occasionally promoted residual hypoxic respiratory activity ("gasping"), it also prolonged the posthypoxic recovery, and thus the absence of central inspiratory drive, which \textit{in vivo} would lengthen respiratory arrest. In view of the breathing disorder in RTT, the increased hypoxia susceptibility of MeCP2-deficient brainstem potentially contributes to life-threatening disturbances of cardiorespiratory control.
Introduction

Rett syndrome (RTT) is a heritable neurodevelopmental disorder caused by spontaneous (germ line) mutations in the MECP2-gene (Amir et al. 1999) which encodes the transcriptional modulator methyl-CpG-binding protein 2 (MeCP2) (Chahrour et al. 2008). Newborn patients initially appear healthy and achieve regular developmental milestones. After 6-18 months of age, however, motor capabilities, language and cognitive function are regressing, and the clinical picture of RTT with motor dysfunction, spasticity, seizures and cognitive impairment emerges (Chahrour and Zoghbi 2007; Hagberg et al. 1983; Percy 2002; Steffenburg et al. 2001).

The majority of RTT patients also suffers from severe cardiorespiratory symptoms which are believed to account for most sudden premature fatalities (Katz et al. 2009; Ogier and Katz 2008; Stettner et al. 2008). The respiratory disturbances in RTT are complex, yet the most striking features are frequent breath-holds and apneas, which can lead to potentially life-threatening systemic hypoxia. Various studies in MeCP2−/− mice revealed that in addition to disturbed resting ventilation, respiratory-related reflexes are exaggerated (Roux et al. 2008; Voituron et al. 2009) and lack distinct forms of reflex plasticity (Stettner et al. 2007). There is growing evidence that these deficits arise from a progressively developing neurochemical dysfunction and an imbalance of neuronal excitation and inhibition skewed towards hyperexcitability in cardiorespiratory ponto-medullary brainstem areas (Abdala et al. 2010; Kline et al. 2010; Medrihan et al. 2008; Stettner et al. 2007; Taneja et al. 2009).
Our previous analyses in a mouse model for RTT (*Mecp2*<sup>−/−</sup> mice) revealed clear signs of systemic adaptation to the intermittent hypoxic episodes, such as an increased hematocrit and elevated HIF-1α expression levels throughout the brain (Fischer et al. 2009). The impact of frequent systemic hypoxia on neuronal network activity per se and its potential contribution to the disease progression in RTT, however, still remain to be defined in more detail. Recent studies from our lab revealed that the hippocampus of symptomatic, adult *Mecp2*<sup>−/−</sup> mice is more susceptible to metabolic arrest (Fischer et al. 2009; Kron and Müller 2010). During severe hypoxia, *Mecp2*<sup>−/−</sup> hippocampal neurons lose their membrane potentials, i.e. their function earlier than WT neurons, ultimately resulting in a hastened onset of hypoxia-induced spreading depression (HSD) (Fischer et al. 2009). This potentially arises from disturbed intracellular Ca<sup>2+</sup>-homeostasis which prevents sufficient protective K<sup>+</sup> channel activation and thus attenuates neuronal hyperpolarization early during anoxia (Kron and Müller 2010).

We recently found that spreading depression, which is detectable as a sudden propagating negative deflection in the extracellular DC potential and arises from a massive synchronized neuronal and glial depolarization as well as severe derangements of ionic distribution [for review see: (Somjen 2001)], can also occur in rat brainstem, especially at infant stages (Funke et al. 2009); a finding which extended earlier observations from *in vivo* recordings (Richter et al. 2010, 2008; Richter et al. 2003). Since the *Mecp2*<sup>−/−</sup> hippocampus was found to be more susceptible to hypoxia and HSD, and HIF-1α expression was increased also in *Mecp2*<sup>−/−</sup> brainstem (Fischer et al. 2009), we now asked whether the generation of HSD episodes might be facilitated in brainstem as well.
HSD-induced failure of brainstem areas associated with autonomic control would disrupt, or at least severely impair cardiorespiratory control, which – depending on the onset and duration of neuronal depolarization – could be fatal.

The nucleus of the solitary tract (nTS) is the principal terminal field of various cardiorespiratory sensory afferents [for review see (Kubin et al. 2006)] and plays a crucial role in the timing of the sensory evoked inspiratory/expiratory phase transition (Hering–Breuer reflex) (Wasserman et al. 2002; Wasserman et al. 2000). Therefore, neuronal excitation and inhibition have to be balanced accurately, and a shift towards hyperexcitability or reduced excitability can cause apnea and apneusis, respectively (Wasserman et al. 2002). It was previously demonstrated that the Hering-Breuer reflex in adult Mecp2<sup>−/−</sup> mice shows hyperexcitability and a lack of habituation (Stettner et al. 2007). Furthermore, parts of the adult Mecp2<sup>−/−</sup> nTS do exhibit hyperexcitability at the cellular level (Kline et al. 2010) which could favor the occurrence of HSD. Once HSD propagates into the nTS, Hering-Breuer reflex control would be disturbed and could contribute or further exaggerate fluctuations in the inspiratory/expiratory phase transition as a hallmark of the breathing disorder observed in RTT (Abdala et al. 2010; Stettner et al. 2007; Stettner et al. 2008).

Moreover, loss of neuronal/synaptic function during HSD within the ventral respiratory column (VRC) and the preBötzinger complex (preBötC) as the primary respiratory pattern generator would be expected to cause cessation of central inspiratory drive and thus,
arrest of breathing. In fact, in rat brainstem we did confirm the propagation of HSD into the nTS and occasionally the VRC (Funke et al. 2009). Others obtained proof that the occurrence of spreading depression in brainstem in vivo may indeed cause the cessation of spontaneous breathing (Richter et al. 2003). Accordingly, an enhanced hypoxia susceptibility of the Mecp2<sup>+/y</sup> brainstem could be part of a deleterious vicious circle, by both being a consequence of apneic episodes in RTT, but also favoring their incidence.

In the present study we therefore investigated the HSD susceptibility of standard (non-rhythmic) and rhythmically-active Mecp2<sup>+/y</sup> brainstem slices. First, we monitored the intrinsic optical signal associated with HSD to define the most preferred ignition site and propagation path of HSD in WT and Mecp2<sup>+/y</sup> mouse brainstem. These recordings were complemented by electrophysiological analyzes to determine genotype-dependent differences of HSD parameters in the nTS, Sp5 and preBötC. To define the very consequences of frequent hypoxic episodes, as occurring in RTT, on brainstem circuits involved in cardiorespiratory control, we adapted the rhythmically-active slice preparation (Ramirez et al. 1996; Smith et al. 1991) to interface-chamber recording conditions, and performed in-depth analyzes of the effects of (repeated) hypoxia and HSD episodes on in vitro respiratory rhythmogenesis in both WT and Mecp2<sup>+/y</sup> brainstem. Since MeCP2 deficiency is associated with reduced brain serotonin levels (Hilaire et al. 2010; Ide et al. 2005; Isoda et al. 2010; Katz et al. 2009; Lekman et al. 1989; Santos et al. 2010; Viemari et al. 2005), and 5-HT<sub>1A</sub> receptor activation attenuates spreading depression (Krüger et al. 1999), stabilizes respiration (Dutschmann et al. 2009; Lalley et al. 1994; Richter et al. 2003; Stettner et al. 2008), and reduces apneas and periodic breathing episodes in
MeCP2-deficient mice *in vivo* (Abdala et al. 2010) as well as in a RTT patient (Andaku et al. 2005), we furthermore examined the effects of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT.
Material and Methods

Preparation

As an animal model for RTT, mice carrying a mutant MECP2 gene [B6.129P2(C)-Mecp\textsuperscript{2tm-1-Bird}] were used (Guy et al. 2001). Since the infant brainstem is most likely to generate HSD (Funke et al. 2009; Richter et al. 2003), we focused our analyses on that developmental stage. All experiments were performed in accordance with local regulations and the ethical guidelines for the care and use of laboratory animals (U.S. National Institutes of Health).

Brainstem slices were prepared as reported for rats in detail earlier (Funke et al. 2007; Funke et al. 2009). In brief, infant (postnatal day 7-15) male Mecp\textsuperscript{2+/y} (wildtype, WT) and Mecp\textsuperscript{2-y} mice were decapitated under deep ether anesthesia. The brain was removed from the skull, the brainstem dissected and placed into ice-cold artificial cerebrospinal fluid (ACSF) equilibrated to pH 7.4 with carbogen (95% O\textsubscript{2}/5% CO\textsubscript{2}). ACSF contained in mM: NaCl 130, KCl 3.5, CaCl\textsubscript{2} 1.2, MgSO\textsubscript{4} 1.2, NaH\textsubscript{2}PO\textsubscript{4} 1.25, NaHCO\textsubscript{3} 24, glucose 10 (all chemicals obtained from Sigma-Aldrich). Brainstems were then glued onto agar blocks and 400 µm thick slices were cut using a vibroslicer (752M, Campden Instruments). The slices were transferred to an Oslo-style interface recording chamber (35-36\textdegree C) and allowed to recover for ~90 min. The interface-chamber was aerated continuously with carbogen (400 ml/min), and perfused with oxygenated ACSF (~5 ml/min).

For preparation of rhythmically-active slices (Ramirez et al. 1996; Smith et al. 1991), the glucose concentration of the ACSF was raised to 30 mM; brainstems were glued onto agar
blocks rostral side up (cutting direction was from ventral to dorsal; the rostrocaudal axis was tilted by 25 degrees away from the blade) and serial sections were cut until the rostral border of the PreBötzinger complex (preBötC) appeared. Then, a single 500-600 µm thick slice was cut and allowed to recover at room temperature for ~30 min in oxygenated, 30 mM glucose-containing ACSF. For recordings, the slice was transferred to the interface-chamber (temperature ~30°C) and the extracellular K⁺ concentration was raised to 8 mM, a standard maneuver to maintain rhythmicity (Funke et al. 2007; Ramirez et al. 1996). Upon such treatment, inspiratory-related mass activity could be recorded reliably from the preBötC. After obtaining stable rhythmic activity the temperature was raised slowly to 35.5 ± 0.5°C and the extracellular glucose concentration was lowered to 10 mM to create those near-physiological conditions in which brain tissue slices are most likely to generate HSD. Rhythmic activity was monitored continuously during these procedures and only those slices maintaining stable rhythmic activity were used for subsequent recordings. When slices were directly transferred to the interface-chamber in ACSF containing 10 mM glucose and 8 mM K⁺ at 35.5°C, stable rhythmic activity was obtained rarely.

Hypoxia-protocol and electrical recordings

Severe hypoxia was induced by switching the recording chamber’s gas supply from carbogen to 95% N₂/5% CO₂; oxygenation of ACSF was continued. Such treatment induced HSD within a few minutes. To ensure reversibility of the hypoxia-induced changes, oxygen was resupplied 20 s after the HSD-associated DC potential deflection had reached its nadir. Only those slices were accepted which generated a well pronounced HSD, i.e. a sudden negative DC potential shift by at least 10 mV amplitude or – in rhythmically-active
slices – a sudden DC potential shift instantly abolishing the respiratory rhythm. If HSD did not occur within 5 min of hypoxia, oxygen was resupplied. Extracellular recording electrodes were made from thick-walled borosilicate glass (GC150F-10, Harvard Apparatus) and filled with ACSF. DC potentials were monitored with a custom-made extracellular DC potential amplifier and sampled at 100 Hz using an Axon Instruments Digitizer 1440A and PClamp 10.2 software (Molecular Devices). In rhythmically-active slices, HSD-related DC potential shifts and respiratory-related mass activity were recorded with a single electrode (~1 MΩ, 50–100 µm outer diameter) connected to a field potential amplifier (Ext 10C, NPI). The DC-coupled signal was amplified 100x. To isolate the respiratory activity, the AC-coupled signal was amplified 10,000x, low-pass filtered at 3 kHz cut-off frequency and rectified/integrated at a time constant of $\tau=200$ ms (see Fig. 4A). Both signals were then digitized at 2 kHz. Respiratory frequency was analyzed in 5-10 min segments during normoxic control conditions and after posthypoxic recovery. Recovery of the respiratory rhythm was measured as the time period between reoxygenation and occurrence of the 1st and 10th (posthypoxic) respiratory burst.

*Intrinsic optical signals*

The intrinsic optical signal (IOS) associated with spreading depression constitutes an increase in light scattering that can be followed by monitoring light reflectance at the tissue surface (Aitken et al. 1999; Andrew et al. 1999; Müller and Somjen 1999). The IOS was monitored using a computer-controlled imaging system (Polychrome II, TILL Photonics) and a sensitive CCD camera (Imago QE, PCO Imaging) as described earlier (Fischer et al. 2009; Funke et al. 2009; Gerich et al. 2006; Hepp and Müller 2008). Brainstem slices were
illuminated with white light at an angle of 40-45°. Images were acquired at 2 s intervals (15 ms exposure) using a 5x, 0.13NA objective (Epiplan, Zeiss). The hypoxia- and HSD-related reflectance changes were visualized by offline image subtraction and displayed in a 256 level gray-scale mode covering a range of ±10% brightness changes. Images were processed with TILLvisION 4.0 (TILL Photonics) and MetaMorph Offline 6.1 (Molecular Devices).

Data analysis and statistics

All data are presented as means ± standard deviation and are grouped into WT and Mecp2<sup>−/−</sup> experiments. The number of observations (n) refers to the number of slices analyzed. In the case of repeated hypoxia or drug treatment, experimental parameters were normalized to the first HSD recorded in each slice under control conditions (defined as 100%). Statistical comparisons of (normalized) HSD and recovery parameters between genotypes were performed with an unpaired two-tailed Student’s t-test (Microsoft Excel). For multiple comparisons (i.e. HSD and recovery parameters with/without 8-OH-DPAT between the genotypes) we used one-way ANOVA or one-way repeated measures ANOVA followed by Tukey’s post-hoc test (Sigma Stat, v 3.5, Systat Software, Inc.). Since 8-OH-DPAT treatment was started immediately after recovery from the 1<sup>st</sup> hypoxia, the post-hypoxic respiratory frequencies of those slices were not included in the control group. Moreover, our recordings involved several optical and electrophysiological parameters. Therefore, if a parameter was unstable in the recording (e.g. respiratory rhythm disappeared before induction of the 2<sup>nd</sup> HSD), then the other stable parameters were still analyzed, varying the numbers of observations (n).
Results

Based on earlier work from our lab demonstrating an enhanced susceptibility of the 
Mecp2<sup>-/-</sup> hippocampus to hypoxia, we are now interested whether this also applies to the 
Mecp2<sup>-/-</sup> brainstem. To address this issue we analyzed genotype-dependent differences of 
hypoxic responses, including the properties of HSD, in acute WT and Mecp2<sup>-/-</sup> brainstem 
slices. The occurrence and propagation of HSD was monitored by recording both the 
extracellular DC potential and the intrinsic optical signal (IOS). Furthermore, in view of the 
respiratory disturbances in RTT we compared the effects of severe hypoxia and HSD on in 
vitro respiratory rhythmogenesis using the rhythmically-active slice.

Hypoxic spreading depression occurs in WT and Mecp2<sup>-/-</sup> mouse brainstem

Initial experiments in normal (3.5 mM K<sup>+</sup> containing) ACSF confirmed that infant WT and 
Mecp2<sup>-/-</sup> mouse brainstem reliably generate HSD as observed earlier in rat brainstem 
(Funke et al. 2009). Monitoring of the IOS revealed that in both genotypes HSD 
consistently occurred in the nTS, which also was the preferred ignition site (WT 58.3%, 
Mecp2<sup>-/-</sup> 44.4% of slices). The subsequent propagation of HSD into the other brainstem 
regions seemed more pronounced in WT than Mecp2<sup>-/-</sup> slices. Specifically, the spinal 
trigeminal nucleus (Sp5) was invaded in 91.7% of WT, but only in 56% of Mecp2<sup>-/-</sup> slices. 
Similarly, the ventral respiratory column (VRC) and the inferior olive (IO) were invaded in 
66.7% and 17% of WT slices, but only in 22.2% and 11.1% of slices derived from Mecp2<sup>-/-</sup> 
mice. Based on that spatiotemporal pattern, the following electrophysiological analyses of 
HSD were focused on the nTS and Sp5.
In the nTS, the characteristic negative deflection of the extracellular DC potential showed an average amplitude of \(-22.0 \pm 3.7\) mV in WT (n=14) and \(-20.8 \pm 2.8\) mV in \(\text{Mecp}^{2-/-}\) (n=11). The time to HSD onset did not differ between genotypes (WT: 63.8 ± 14.6 s; \(\text{Mecp}^{2-/-}\): 69.1 ± 17.0 s), whereas the duration at half maximum amplitude was significantly shorter in \(\text{Mecp}^{2-/-}\) (WT: 54.7 ± 11.3 s; \(\text{Mecp}^{2-/-}\): 43.0 ± 4.7 s, p<0.01, unpaired t-test, Fig. 1A, B). In the Sp5 HSD showed a similar time to onset, but a slightly reduced DC potential amplitude and/or duration as compared to the nTS (unpaired t-test); differences among the genotypes were not observed (Fig. 1A, B). A detailed overview of the HSD parameters recorded under the various experimental conditions is presented in Table 1.

Hypoxia was repeated in a subset of slices after 20 min of recovery. In the nTS and Sp5 of both genotypes the 2nd HSD showed similar DC potential amplitudes and times to onset; the halfwidth durations tended to increase, but reached the level of significance in WT slices only (p<0.05 paired t-test; Fig. 1C, Table 1). To confirm that HSD can be induced repeatedly under our experimental conditions [as previously shown for rat brainstem and rat/mouse hippocampus (Fischer et al. 2009; Funke et al. 2009; Müller and Somjen 1998)] also a 3rd HSD was induced on occasion - without performing further analyses though.

Increasing extracellular K⁺ levels reveals an enhanced HSD susceptibility of \(\text{Mecp}^{2-/-}\) brainstem

In the next set of experiments we asked whether an increased tissue excitability and metabolic demand might uncover subtle differences in HSD susceptibility between the genotypes already at infant developmental stages. Therefore, similar to the rhythmically-
active slice preparation described below, the extracellular potassium concentration ([K⁺]₀) was raised to 8 mM. Compared with control conditions, such K⁺-mediated conditioning caused a general hastening of HSD onset in the nTS and Sp5 of both genotypes, and these effects were especially pronounced in MeCP2⁻/⁻ slices. In detail, HSD occurred within 42.8 ± 5.4 s in WT (n=10), and even 29% earlier, within 30.4 ± 5.8 s (n=12), in MeCP2⁻/⁻ nTS (p<0.001, unpaired t-test); a somewhat less pronounced further hastening of HSD onset by 22% was observed in MeCP2⁻/⁻ Sp5 (WT 47.0 ± 9.0 s, n=11; MeCP2⁻/⁻ 36.9 ± 5.9 s, n=12; p<0.01, unpaired t-test). The DC potential amplitudes and halfwidth durations of the DC potential shifts were comparable between the two genotypes (Fig. 1D, Table 1). A subsequent 2nd hypoxic episode in the presence of 8 mM K⁺ elicited HSDs with similar properties compared to the 1st HSDs in both genotypes (Table 1).

5-HT₁A receptor stimulation postpones HSD onset

It was shown that 5-HT₁A receptor stimulation counteracts spreading depression in cortical slices (Krüger et al. 1999) and also stabilizes respiration in vitro and in vivo (Dutschmann et al. 2009; Lalley et al. 1994; Richter et al. 2003; Stettner et al. 2008). Furthermore, MeCP2 deficiency is associated with reduced serotonin levels (Hilaire et al. 2010; Ide et al. 2005; Isoda et al. 2010; Katz et al. 2009; Lekman et al. 1989; Santos et al. 2010; Viemari et al. 2005). Therefore, the 5-HT₁A receptor agonist 8-OH-DPAT (aqueous 50 mM stock solution; 50 µM final concentration) was applied to WT and MeCP2⁻/⁻ brainstem slices for 20 min before a 2nd HSD was induced to define a potential merit of 5-HT₁A receptor-mediated signaling. With normal (3.5 mM) [K⁺]₀, such treatment postponed the onset of the 2nd HSD by 18.7 ± 7.7% in the nTS of WT slices (p<0.01, paired t-test, n=7) and by 22.8 ±
12.2% in the Mecp2\(^{-/y}\) nTS (p<0.01, paired t-test, n=5, Fig. 2A, B), which in both genotypes was significantly different from a 2\(^{nd}\) HSD induced in control slices without prior 8-OH-DPAT treatment (p<0.001, one-way ANOVA). HSD amplitudes and halfwidth durations were not affected (Fig. 2B). In the Sp5 the 8-OH-DPAT-mediated postponement of HSD was less intense, but still reached the level of significance (WT +9.4 ± 4.4 %, n=7; Mecp2\(^{-/y}\) +11.1 ± 14.9%, n=7; p<0.05, one-way ANOVA, Fig. 2B).

In a second set of experiments the effects of 8-OH-DPAT were examined in the presence of 8 mM extracellular K\(^+\). Compared to a 2\(^{nd}\) HSD induced in control slices in the presence of 8 mM K\(^+\), this treatment postponed the onset of HSD by 30.2 ± 14.2% in WT (n=6) and by 39.7 ± 25.7% in the Mecp2\(^{-/y}\) nTS (n=6; p<0.05, one-way ANOVA); HSD amplitudes and halfwidth durations were not affected (Fig. 2C). Similar effects were also observed for the Sp5, where 8-OH-DPAT postponed HSD onset by 33.9 ± 25.3% (n=8) in WT and 35.3 ± 21.2% (n=8) in Mecp2\(^{-/y}\) (p<0.05, one-way ANOVA; Fig. 2C).

The preBötC is invaded less frequently by HSD in rhythmically-active Mecp2\(^{-/y}\) brainstem slices

To define whether severe hypoxia differentially affects respiratory rhythmogenesis in the two genotypes, we took advantage of the rhythmically-active slice. This preparation was adapted successfully to interface-chamber recording conditions (see Materials and Methods) which allows for the simultaneous recording of extracellular DC potential and preBötC mass activity at near-physiological temperature (35.5°C); maintaining rhythmicity of this slice required to increase [K\(^+\)]\(_o\) to 8 mM (Funke et al. 2007; Ramirez et al. 1996).
During normoxic control conditions, the *in vitro* respiratory frequencies were similar in WT (0.30 ± 0.21 Hz, n=25) and *Mecp2*<sup>-/y</sup> brainstem slices (0.32 ± 0.24 Hz, n=29).

Since our initial experiments showed that the VRC is less frequently invaded by HSD episodes in *Mecp2*<sup>-/y</sup> brainstem, the IOS was analyzed also in the rhythmically-active slice which, due to the elevated [K]<sub>i</sub> (8 mM), can be expected to exhibit a higher metabolic rate, increased excitability and a facilitated ignition of HSD (Funke et al. 2009). Overall, upon oxygen withdrawal, the IOS showed a wide-spread increase in tissue reflectance with sometimes multifocal origin which then invaded large parts of the brainstem slice (Fig. 3). The Sp5 was identified as the preferred ignition site of HSD (WT 83.3%, but only 47.6% of *Mecp2*<sup>-/y</sup> slices). Subsequently, Sp5 and nTS were invaded by HSD to a comparable extent in either genotype (WT, Sp5 95.8%, nTS 87.5%; *Mecp2*<sup>-/y</sup>, Sp5 90%, nTS 81%). Yet, the IO and the preBötC were less frequently affected in *Mecp2*<sup>-/y</sup> (23.8% and 42.9%) as compared to WT slices (50.0% and 75.0%, respectively, Fig. 3), as already seen in normal [K]<sub>i</sub>.

**HSD onset is hastened in the *Mecp2*<sup>-/y</sup> preBötC, but posthypoxic recovery appears normal**

If HSD invaded the preBötC, the characteristic DC potential shift occurred within 58.8 ± 17.2 s of hypoxia in rhythmically-active WT slices (n=21), but significantly earlier, by 20%, in *Mecp2*<sup>-/y</sup> slices (47.1 ± 15.4 s, n=16, p<0.05, unpaired t-test, Fig. 4B, C). The average DC potential amplitudes (WT: -8.9 ± 4.2 mV; *Mecp2*<sup>-/y</sup>: -11.6 ± 5.1 mV) and halfwidth durations (WT: 32.1 ± 6.6 s; *Mecp2*<sup>-/y</sup>: 34.9 ± 9.5 s) were comparable between the genotypes. Simultaneous monitoring of the extracellular DC potential and respiratory-
related mass activity (see Fig. 4A for schematic amplifier configurations) revealed that the propagation of HSD into the preBötC consistently caused a sudden and complete arrest of respiratory rhythmogenesis (see Fig. 4D, middle panels). The hastened onset of HSD in the \textit{Mecp2\(^{-/}\)} preBötC strongly suggests that the \textit{Mecp2\(^{-/}\)} respiratory network is more vulnerable to hypoxia-induced loss of function.

In both genotypes, respiratory rhythmogenesis reappeared within a few minutes upon reoxygenation, gaining a clearly increased respiratory frequency (Fig. 4D, 5C). In detail, recovery times defined as the time period between reoxygenation and occurrence of the 1\(^{st}\) and 10\(^{th}\) respiratory burst averaged 146.4 ± 55.1 s and 229.1 ± 68.8 s in WT slices (n=20). Recovery was similar in \textit{Mecp2\(^{-/}\)} slices (160.3 ± 55.6 s and 242.3 ± 87.4 s; n=16). The posthypoxic increase in respiratory frequency, however, was more pronounced in WT than in \textit{Mecp2\(^{-/}\)} slices (by 99.1 ± 67.0%, p<0.001 vs. prehypoxic control frequency, repeated measures ANOVA, and by 60.7 ± 43.0%, n=11 and n=5, respectively, Fig. 4D, 5A, C).

\textit{The occurrence of HSD may facilitate posthypoxic recovery}

To define the impact of repeated hypoxic episodes, as they do occur in RTT, on \textit{in vitro} respiratory rhythmogenesis, a 2\(^{nd}\) HSD was induced in some rhythmically-active slices after 20 min of recovery. In WT slices the onset of the 2\(^{nd}\) HSD was significantly accelerated by 19.1 ± 20% (n=14, p<0.01, paired t-test) as compared to the 1\(^{st}\) HSD. In \textit{Mecp2\(^{-/}\)} slices the time to onset of the 2\(^{nd}\) HSD was variable, but on average did not significantly differ from the 1\(^{st}\) HSD (n=8). The amplitude of the DC potential shifts tended
to increase (WT: by 81.1 ± 155%; Mecp2<sup>−/−</sup>: by 24.2 ± 28.3%, Fig. 5A, B), but halfwidth durations were unchanged. The pronounced increase with large standard deviations of the HSD amplitudes in WT results from the fact that the 1<sup>st</sup> HSD sometimes gave rise to only a small DC potential shift, whereas the 2<sup>nd</sup> hypoxic episode triggered a full-blown HSD (see Fig. 5A). Nevertheless, in the rhythmically-active slices also such moderate DC potential shifts were rated as “real” HSD, as long as a sudden arrest of respiratory rhythm occurred (see also below).

The posthypoxic recovery time - defined as the 10<sup>th</sup> respiratory burst upon reoxygenation - after the 2<sup>nd</sup> HSD was shortened consistently (by 28.0 ± 21.1% in WT, p<0.01 vs. 1<sup>st</sup> recovery, paired t-test, n=11) and by 32.2 ± 21.4% in Mecp2<sup>−/−</sup> slices (n=8, p<0.05, Fig. 5A, C). This was accompanied by a further increase of in vitro respiratory frequency in WT (by 124.3 ± 113.2%, p<0.001, repeated measures ANOVA), and a similar tendency in Mecp2<sup>−/−</sup> slices (by 97.8 ± 76.3% as normalized to prehypoxic control conditions, Fig. 5A, C). Accordingly, repeated hypoxia may induce compensatory short-term facilitation and/or hypoxic preconditioning in rhythmically-active slices, and these mechanisms also remain functional in the Mecp2<sup>−/−</sup> brainstem, but seem less pronounced.

5-HT<sub>1A</sub> receptor signaling in the preBötC mediates protective effects, partially independent of genotypes

We next examined whether the effects of 8-OH-DPAT on HSD also apply to rhythmically-active slices and in vitro respiration. 8-OH-DPAT (50 µM) was applied for 20 min as soon as rhythmic activity had reappeared after the 1<sup>st</sup> HSD, and it tended to counteract the
hastening of the 2\textsuperscript{nd} HSD observed in untreated WT slices (time to onset hastened by only 5.8 ± 6.2\%, n=7); in the Mecp2\textsuperscript{-/y} preBötC, the time to onset of the 2\textsuperscript{nd} HSD was comparable to control slices (n=8, Fig. 6A, B). 8-OH-DPAT moderately increased the 2\textsuperscript{nd} HSD amplitude compared to the 1\textsuperscript{st} HSD, which was more pronounced in WT slices (by 26.9 ± 17.5\% in WT, p<0.01 vs. 1\textsuperscript{st} HSD, paired t-test; by 21.5 ± 36.3\% in Mecp2\textsuperscript{-/y}). However, compared to the 2\textsuperscript{nd} HSD in untreated slices, 8-OH-DPAT did not exert any significant effects on the DC potential amplitudes in either genotype (one-way ANOVA). The halfwidth duration became significantly decreased in the preBötC of both genotypes (by 34.2 ± 22.5\% in WT, p<0.01, paired t-test; by 32.3 ± 27.2\% in Mecp2\textsuperscript{-/y} slices, p<0.05, paired t-test, Fig. 6A, B). Compared to the halfwidth ratios in untreated slices we found a significant difference only in WT slices (p<0.01, one-way ANOVA). These results may suggest that the Mecp2\textsuperscript{-/y} brainstem is somewhat less responsive to 8-OH-DPAT.

On reoxygenation, 8-OH-DPAT treated slices displayed a similar shortening of the posthypoxic recovery time as observed in untreated slices. Specifically, respiratory activity reappeared by 37.0 ± 19.0\% earlier in WT slices (p<0.01, paired t-test) and by 34.4 ± 25.1\% earlier in Mecp2\textsuperscript{-/y} slices (p<0.05), as compared to the 10\textsuperscript{th} burst of the 1\textsuperscript{st} recovery (Fig. 6A, C). However, 8-OH-DPAT treatment significantly enhanced the posthypoxic respiratory frequency in WT slices by 325.6 ± 162.7\%, (p<0.001, repeated measures ANOVA) and, somewhat less, by 240.2 ± 196.4\%, in Mecp2\textsuperscript{-/y} slices (p<0.001). This frequency increase persisted even after wash-out of 8-OH-DPAT after the 2\textsuperscript{nd} HSD (WT: +340.8 ± 186.4\%, p<0.001, repeated measures ANOVA; Mecp2\textsuperscript{-/y}: +308.5 ± 195.5\%, p<0.001, Fig. 6A, C).
Absence of HSD allows for a residual “gasping-like” hypoxic respiratory activity, but prevents posthypoxic shortening of the recovery time

As already indicated by the spatiotemporal profile of the IOS, electrical recordings confirmed that the preBötC was spared by the 1\textsuperscript{st} HSD despite 5 min of hypoxia in 4 WT and 16 MeCP2\textsuperscript{-/y} slices. During such hypoxia without HSD we often observed a residual hypoxic respiratory, “gasping-like” activity in both genotypes, which was abolished immediately on reoxygenation (see Fig. 7A). The posthypoxic recovery times (MeCP2\textsuperscript{-/y}: 1\textsuperscript{st} burst, 160.2 ± 64.5 s; 10\textsuperscript{th} burst, 222.7 ± 93.3 s; WT: 1\textsuperscript{st} burst, 176.9 ± 81.9 s; 10\textsuperscript{th} burst, 242.5 ± 136.1 s) were similar to those observed in slices generating the 1\textsuperscript{st} HSD and also the pronounced increase in respiratory frequency occurred (MeCP2\textsuperscript{-/y}: +97.5 ± 95.3%; WT: +51.7 ± 40.6%). However, in all investigated WT slices, and in 10 out of 16 MeCP2\textsuperscript{-/y} slices, the 2\textsuperscript{nd} (and also a 3\textsuperscript{rd}) hypoxic episode succeeded to induce HSD, with similar (recovery) properties as observed above (data not shown).

In contrast, in the remaining 6 MeCP2\textsuperscript{-/y} brainstem slices, 3 episodes of severe hypoxia (lasting 5 min each) failed to induce HSD within the preBötC. In these slices a progressive posthypoxic increase of respiratory frequency occurred, by 66.3 ± 59.0% after the 1\textsuperscript{st} hypoxia and by 97.7 ± 55.0% during the 2\textsuperscript{nd} recovery (p<0.01, repeated measures ANOVA). Also the recovery time was somewhat shorter as compared to those slices generating a HSD (1\textsuperscript{st} burst 127.5 ± 58.5 s; 10\textsuperscript{th} burst 182.4 ± 79.0 s). Yet, without the occurrence of HSD, repeated hypoxia did not accelerate the posthypoxic recovery, but instead, progressively prolonged the posthypoxic respiratory arrest (2\textsuperscript{nd} hypoxia:
prolongation by 58.1 ± 41.3%, 3\textsuperscript{rd} hypoxia: by 108.6 ± 85.1%, p<0.05, repeated measures ANOVA, Fig. 7A, B).
Discussion

In the present study, we show complex responses of different brainstem regions involved in cardiorespiratory control to severe hypoxia and HSD as well as their alterations associated with MeCP2 deficiency. A major focus was on the effects of severe hypoxia on 

in vitro respiratory rhythmogenesis. Here, for the first time, we adapted the rhythmically-active brainstem slice to interface-chamber conditions, which allows for optical and electrophysiological recordings of hypoxic responses at almost physiological temperature.

HSD occurs in the MeCP2<sup>−/−</sup> brainstem, but its propagation seems restricted

For a long time, brainstem has been considered comparably resistant to the occurrence of spreading depression (Bures et al. 1974; Somjen et al. 1992). Yet, more recently, under certain experimental conditions especially infant rat brainstem was confirmed to generate spreading depression episodes (Funke et al. 2009; Richter et al. 2010, 2008; Richter et al. 2003). Here, we show that even without prior conditioning, severe hypoxia triggers HSD also in the brainstem of mice, consistently affecting the nTS and Sp5 in both genotypes. The preferred ignition of HSD in the nTS is somewhat different from rats, where the Sp5 was the primary ignition site of HSD (Funke et al. 2009). It therefore seems that in mouse brainstem the nTS has the lowest induction threshold for the generation of HSD, and interestingly, this preferred HSD ignition site is the same in WT and MeCP2<sup>−/−</sup> brainstem slices.

Once ignited, HSD propagated into other brainstem areas, yet its final spread seemed genotype dependent, because in MeCP2<sup>−/−</sup> slices the Sp5, VRC and IO were less frequently
invaded. The propagation of HSD is critically modulated by the interstitial volume [ISV, (Chebabo et al. 1995; Huang et al. 1996)], which in turn is affected by cell density, soma size, or dendritic complexity. Interestingly, in addition to reduced brain weight and volume of cortex, hippocampus or cerebellum (Belichenko et al. 2008), various regions of the adult MeCP2 mutant brain, including brainstem, exhibit an altered cell density, reduced dendritic branching and/or smaller neurons (Fischer et al. 2009; Fukuda et al. 2005; Jentarra et al. 2010; Mironov et al. 2009; Taneja et al. 2009) all of which are supposed to affect the ISV. Since brainstem matures earlier than forebrain, these changes may already be established in the infant MeCP2<sup>−/−</sup> brainstem and potentially hamper HSD propagation. Indeed, the MeCP2<sup>−/−</sup> preBötC shows a decreased neuronal packing in organotypic cultures already after 14 days <i>in vitro</i>, becoming more pronounced with time (Mironov et al. 2009). Thus, these alterations in the MeCP2<sup>−/−</sup> brainstem could explain the less frequent propagation of HSD into the MeCP2<sup>−/−</sup> VRC and preBötC.

**HSD susceptibility of the infant MeCP2<sup>−/−</sup> brainstem**

Recently we reported an increased hypoxia susceptibility of the adult MeCP2<sup>−/−</sup> hippocampus being evident as a hastened onset of HSD (Fischer et al. 2009). Here we show that the HSD susceptibility of the infant MeCP2<sup>−/−</sup> brainstem seems normal, at least without further conditioning stressors. However, compared to the hippocampus which is highly vulnerable to metabolic compromise (Pulsinelli et al. 1982; Schmidt-Kastner and Freund 1991), brainstem networks controlling vital functions are relatively hypoxia-tolerant especially at infant stages (Ballanyi et al. 1992). Therefore, only subtle changes in basal hypoxia susceptibility of the infant MeCP2<sup>−/−</sup> brainstem may not be detectable at rest.
However, when hypoxia coincides with additional stimuli causing an increased metabolic demand, such as elevated $[K^+]_o$, the onset of HSD in the infant $Mecp2^{−/y}$ nTS, Sp5 and preBötC are hastened. Consequently also the loss-of-function within important (cardiorespiratory) sensory relays and the main respiratory pattern generator occur earlier.

Hyperexcitability critically promotes HSD generation, and signs of hyperexcitability are found in various regions of the $Mecp2^{−/y}$ brainstem at later developmental stages, including the nTS (Kline et al. 2010; Stettner et al. 2007; Taneja et al. 2009). Whereas it is unknown at which developmental stage hyperexcitability develops in the $Mecp2^{−/y}$ nTS, decreased GABAergic inhibition already emerges around postnatal day 7 within the ventrolateral medulla (Medrihan et al. 2008). Such early imbalance of inhibition and excitation may well contribute to the enhanced HSD susceptibility of infant $Mecp2^{−/y}$ brainstem, and since the nTS is the preferred ignition site of HSD in the $Mecp2^{−/y}$ brainstem, a shift towards neuronal/synaptic hyperexcitability might be especially pronounced in that very nucleus.

In addition, the fact that HSD onset is also hastened within the Sp5, may suggest a shift to excitation also in this sensory relay. The Sp5 relays the trigemino-cardiac/diving reflex (McCulloch 2005; McCulloch and Panneton 1997), a powerful protective reflex causing breath-hold and bradycardia by activation of postinspiratory neurons (Dutschmann and Paton 2002). In $Mecp2^{−/y}$ mice fluctuations of postinspiratory activity causes breathing arrhythmia and breath-hold in situ (Abdala et al. 2010; Stettner et al. 2007) and in vivo
(Voituron et al. 2010). Thus, it is not unlikely that hyperexcitability in the $\text{Mecp}^2\gamma$ Sp5 could also decrease the threshold of trigemino-postinspiratory breath-holds.

Moreover, recent findings that young, obviously presymptomatic $\text{Mecp}^2\gamma$ mice exhibit erratic breathing/reflex responses when challenged by hypoxia or hypercapnia (Voituron et al. 2009) further support the view that MeCP2 deficiency causes subtle changes of respiratory network function already before the clinical respiratory phenotype of RTT emerges.

HSD disrupts the normal hypoxic response

So far, the immediate impact of (repeated) HSD episodes on respiratory rhythmogenesis has not been analyzed, even though the occurrence of spreading depression in brainstem seems capable of arresting spontaneous breathing in anesthetized rats (Richter et al. 2003). In the present study such correlated analyses became possible for the first time, as we succeeded to adapt the rhythmically-active brainstem slice to interface-chamber recording conditions.

Hypoxia triggers various forms of short- and long-term respiratory plasticity. The acute hypoxic response is an initial increase in respiratory frequency (Cross and Oppe 1952; Dick and Coles 2000; Lawson and Long 1983; Powell et al. 1998; Richter et al. 1991), which is largely preserved in rhythmically-active slices (Blitz and Ramirez 2002; Ramirez et al. 1998; Telgkamp and Ramirez 1999). Depending on the duration and severity of hypoxia, this initial frequency increase is followed by a secondary respiratory depression,
and if hypoxia persists, finally leads to central apnea (Ramirez et al. 1998; Richter et al. 1991). Our experiments extend these findings, since for the first time we show that within only 1-2 min of severe hypoxia HSD may propagate into the preBötC and cause a sudden and complete arrest of respiratory activity already during/after the initial frequency increase (Figs. 5A, 6A, but compare Fig. 7A). Since that kind of hypoxic apnea is caused by a massive and synchronized neuronal depolarization, it prevents any resuscitation mechanism of the in vitro respiratory network (gasping). As such it represents a new pathophysiological mechanism which deserves further detailed analyses and the verification of similar mechanisms under intact network conditions.

Interestingly, in the Mecp2⁻/⁻ brainstem, the propagating HSD more frequently spared the preBötC, which then allowed the network to reconfigure and generate gasping-like activity during hypoxia (see Fig. 7). In view of the severe respiratory disturbances in RTT and the associated systemic hypoxic episodes, such occasional sparing of the VRC by HSD seems clearly beneficial.

Repeated hypoxia increases respiratory frequency, but facilitation of posthypoxic recovery depends on HSD

Frequent apneas are a key feature of respiratory disturbances in RTT, and cause intermittent systemic hypoxia. Simulating such repeated hypoxia in vitro revealed complex network adaptations even on the level of the rhythmically-active slice. In both genotypes the occurrence of HSD completely abolished respiratory-related rhythmic activity, which
resumed upon reoxygenation and stabilized at a markedly increased respiratory frequency. Repeated episodes of hypoxia further augmented the respiratory frequency.

In addition to the transient initial increase in respiratory frequency, repeated hypoxia also elicits long-lasting changes in respiratory activity, increasing both phrenic nerve discharge amplitude and frequency (Baker-Herman et al. 2004; Baker and Mitchell 2000). Changes in discharge amplitude are ascribed to plasticity of phrenic motor neurons (Fuller et al. 2000). In contrast, changes in discharge frequency seem to arise from plasticity at the level of the central respiratory rhythm generator; a transient increase in respiratory frequency follows a single anoxic episode, but long-lasting increases occur only after repetitive anoxia (Blitz and Ramirez 2002). These findings are in line with our results, showing pronounced increases in respiratory frequency after severe hypoxia, regardless of the occurrence of HSD. Accordingly, both studies favor the view that hypoxia induces frequency-associated plasticity on the level of the rhythmically-active slice. Since respiratory frequency increases also occurred in MeCP2<sup>/−</sup> slices, such mechanisms seem largely intact in the MeCP2<sup>/−</sup> preBötC.

An interesting aspect is that only the repeated occurrence of HSD within the preBötC shortened the posthypoxic recovery time, suggesting that HSD mediates some preconditioning within the brainstem, similar to observations in cortex (Kawahara et al. 1997; Kobayashi et al. 1995). In contrast, repeated hypoxia without the occurrence of HSD, although it allowed for gasping-like activity, rather prolonged the posthypoxic recovery time.
5-HT$_{1A}$ receptor activation mediates partial hypoxic protection and potentiates the posthypoxic increase in respiratory frequency

Activation of 5-HT$_{1A}$ receptors can attenuate spreading depression (Krüger et al. 1999), stabilizes respiration (Dutschmann et al. 2009; Lalley et al. 1994; Richter et al. 2003; Stettner et al. 2008), and was shown recently to correct the breathing phenotype of MeCP2-deficient mice in vivo (Abdala et al. 2010) as well as in a RTT patient (Andaku et al. 2005). We therefore examined the effects of 8-OH-DPAT on HSD and the in vitro respiratory rhythm. In normal [K$^+$]$_o$, 8-OH-DPAT postponed the onset of HSD in the nTS and Sp5 of both genotypes, thereby increasing the hypoxic time window which, if oxygen is restored in time, might preserve neurons from undergoing HSD. Since 5-HT$_{1A}$ receptor activation inhibits adenylylcyclase and reduces cytosolic cAMP-levels, it can be assumed that the protective postponement of HSD by 8-OH-DPAT in the nTS and Sp5 results from a decrease in neuronal excitability. An interesting aspect is that 8-OH-DPAT also postponed the onset of HSD in the presence of increased extracellular K$^+$ levels and even tended to be more potent under these conditions.

Surprisingly, 8-OH-DPAT mediated postponement of HSD was not obvious in the preBötC of rhythmically-active slices; instead 8-OH-DPAT shortened HSD episodes in both genotypes, which is in line with earlier reports in rat cortex (Krüger et al. 1999). Also, 8-OH-DPAT markedly augmented respiratory frequency, similar to in situ and in vivo reports (Manzke et al. 2009). This augmentation might arise from a functional reorganization of the isolated and reduced respiratory network caused by disinhibition of glycinergic synapses,
especially in the preBötC (Manzke et al. 2009) where glycinergic neurons are numerous (Tanaka et al. 2003; Winter et al. 2009) and express 5-HT$_{1A}$ receptors (Manzke et al. 2009). In this respect, it can be expected that 8-OH-DPAT inhibits these glycinergic neurons, and thereby causes a net increase in excitability resulting in an increased respiratory frequency. Since increases in excitability also promote the occurrence of HSD, this may explain why 8-OH-DPAT did not postpone HSD onset in the preBötC.

**Concluding remarks**

In contrast to adult $Mecp2^{−/−}$ hippocampus, the hypoxia susceptibility of infant $Mecp2^{−/−}$ brainstem was increased only when hypoxia coincided with additional challenges. Propagation of HSD into the preBötC was confirmed to abolish respiratory rhythmogenesis, i.e. to arrest breathing. Despite this deleterious effect, the occurrence of HSD also facilitated the recovery of rhythmogenesis upon reoxygenation, whereas its consistent absence during repeated hypoxia - especially in $Mecp2^{−/−}$ preBötC - prolonged the posthypoxic recovery. 5-HT$_{1A}$ receptor stimulation mediated a partial and genotype-independent protection against HSD and stabilized/increased the *in vitro* respiratory rhythm, suggesting that 5-HT$_{1A}$ receptor-mediated signaling seems largely intact in $Mecp2^{−/−}$ brainstem. Therefore, well-targeted stimulation of these receptors may provide a potential pharmacotherapeutical concept to ameliorate both the irregular breathing pattern as well as the consequences of the intermittent hypoxic episodes associated with RTT.
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References


**Figure Legends**

**Figure 1:** Infant mouse brainstem reliably generates HSD during severe hypoxia.

A) Severe hypoxia (oxygen withdrawal) reliably triggered HSD episodes in the nTS and Sp5 of both WT and *Mecp2*<sup>−/−</sup> brainstem slices.

B) Statistical comparison of the characteristic HSD parameters (Δt, time to HSD onset; ΔV, amplitude of the extracellular DC potential shift; t½, HSD duration at half maximum amplitude) did not reveal marked differences between the genotypes, except for a slightly shortened HSD duration in *Mecp2*<sup>−/−</sup> nTS. For exact definition of the HSD parameters see panel A. Plotted are the averaged parameters; error bars report standard deviations. Statistically significant changes in this and the following figures are indicated by asterisks (* p<0.05; ** p<0.01; *** p<0.001) and the number of trials (n) is indicated at the bottom of the bars.

C) In both genotypes HSD could be induced repeatedly. Marked changes in the HSD parameters were not observed; only in WT slices the duration of the 2<sup>nd</sup> HSD increased slightly. Plotted are the parameters of the 2<sup>nd</sup> HSD, normalized to the 1<sup>st</sup> HSD from continuous recordings in the respective brainstem region of the analyzed slices.

D) Increased extracellular K<sup>+</sup> levels hasten the onset of HSD and unveil an enhanced hypoxia susceptibility of the *Mecp2*<sup>−/−</sup> nTS and Sp5.

**Figure 2:** Stimulation of 5-HT<sub>1A</sub> receptors postpones the onset of HSD.

A) Incubation of slices with 8-OH-DPAT (50 µM, 20 min) postpones HSD onset in both genotypes. The displayed traces were recorded in the nTS.
B) Statistical comparison of the HSD parameters after 8-OH-DPAT treatment (normal [K$^+$]$_o$) shows a comparable postponement of HSD for both genotypes. Plotted are the normalized parameters referred to the 1$^{st}$ HSD recorded under pretreatment control conditions in the respective brainstem region. Asterisks indicate significant changes as compared to the 2$^{nd}$ HSD induced in control slices without drug treatment.

C) In the presence of increased [K$^+$]$_o$ 8-OH-DPAT still postponed HSD onset in the nTS and the Sp5 of both genotypes, and its efficacy even tended to be somewhat improved as compared to normal [K$^+$]$_o$.

Figure 3: In Mecp2$^{-/y}$ slices, the VRC is less frequently invaded by HSD.

Monitoring of the intrinsic optical signal (IOS) visualizes the ignition and propagation of HSD within the tissue slices. Tissue areas undergoing HSD show an increased tissue reflectance (shown as brightening). Whereas in WT slices HSD showed a near-complete invasion of the entire hemi-slice, the propagation of HSD was more restricted in Mecp2$^{-/y}$ brainstem. Especially the more ventral and ventrolateral aspects of the slice – including the VRC – were less frequently invaded as compared to WT slices; instead large areas of the Mecp2$^{-/y}$ slice darkened, indicating cell swelling. The displayed images are subtraction images (range of ±10% reflectance changes) recorded from rhythmically-active slices in 8 mM K$^+$ containing ACSF. The position of the recording electrode and some anatomical landmarks are indicated (nTS, nucleus of the solitary tract; Sp5, spinal trigeminal nucleus; VRC, ventral respiratory column). Numbers report the elapsed time after occurrence of the first IOS changes for each image pair (t=0). The last image pair was taken after near-complete posthypoxic recovery of the IOS.
**Figure 4:** Propagation of HSD into the preBötC instantly abolishes *in vitro* respiratory rhythmogenesis.

A) To define the consequences of HSD on respiratory network activity, the rhythmically-active slice was adapted to interface-chamber recording conditions. The HSD-associated negative DC-potential deflection and respiratory-related mass activity were simultaneously recorded with a single extracellular electrode (see the indicated recording site in a thionin-stained brainstem slice). The compound signal was split in a DC (HSD-related extracellular DC potential shift) and an AC component (respiratory-related mass activity), the latter of which was rectified and integrated.

B) In rhythmically-active slices (8 mM K⁺ containing ACSF), HSD onset is hastened in the *Mecp2<sup>−/−</sup>* preBötC.

C) Statistical comparison of the absolute HSD parameters between the genotypes confirms that HSD occurs ~20 % earlier in the preBötC of *Mecp2<sup>−/−</sup>* as compared to WT slices, whereas the other parameters are not significantly affected.

D) Monitoring of respiratory-related mass activity within the preBötC confirmed that in both genotypes propagation of HSD into the preBötC immediately arrests *in vitro* rhythmogenesis. Note that absolutely no residual respiratory activity during hypoxia could be observed. Upon reoxygenation, respiratory-related mass activity recovered, stabilizing at a markedly higher respiratory frequency than before oxygen withdrawal.
Figure 5: Repeated occurrence of HSD facilitates the posthypoxic recovery of respiratory mass activity.

A) During the early hypoxic response, shortly before HSD, respiratory activity increases (arrow mark 1), but is immediately abolished as soon as HSD occurs (arrow mark 2). Respiratory-related activity recovered within 2-4 minutes after HSD and reoxygenation, a process which was hastened if HSD was induced repeatedly. For comparison among genotypes the time span between reoxygenation and occurrence of the 1st and 10th respiratory burst were analyzed.

B) Summary of the normalized parameters of the 2nd HSD as referred to the 1st HSD. In the preBötC of WT slices the 2nd HSD showed a slightly, but significantly hastened onset; no significant differences were observed in the Mecp2<sup>−/−</sup> preBötC.

C) Interestingly, such repeated HSD facilitated the posthypoxic recovery of respiratory mass activity. In both genotypes, the posthypoxic recovery (10th burst) was shortened significantly. Furthermore, in both genotypes, respiratory mass activity stabilized at clearly increased frequencies after the 1st and even more so after the 2nd HSD.

Figure 6: 8-OH-DPAT treatment shortens HSD duration and potentiates posthypoxic respiratory frequencies in both genotypes.

A) Sample recordings of the extracellular DC potential and respiratory-related activity showing the clear increase in respiratory frequency upon 8-OH-DPAT treatment. Note that the frequency increase was more pronounced in WT than in Mecp2<sup>−/−</sup> slices and persisted upon drug wash-out after the 2nd HSD. The bottom traces show sections of the recording (indicated by the boxes) at a stretched time scaling.
B) Statistical comparison shows that 8-OH-DPAT treatment shortened the duration of HSD in both genotypes. In addition, the amplitude of the DC potential shift slightly increased in WT slices.

C) Statistical summary of the 8-OH-DPAT effects on the respiratory frequency and posthypoxic recovery time.

Figure 7: Shortening of posthypoxic recovery requires the occurrence of HSD.

A) In those slices in which HSD did not invade the preBötC (despite 5 min-lasting hypoxia), a residual respiratory activity (“gasing-like activity”) was observed occasionally in both genotypes. Upon reoxygenation it did, however, immediately vanish and respiratory activity then recovered within 3-4 min, also stabilizing at an increased frequency. Against expectation, in slices in which repeated episodes of hypoxia did not induce HSD in the preBötC, the facilitation of the posthypoxic recovery failed. It therefore seems that such “preconditioning” requires the presence of HSD and that hypoxia itself is not sufficient. Instead, repeated hypoxia in the absence of HSD even prolonged the posthypoxic recovery in Mecp2^−/− slices.

B) Statistical comparison of the posthypoxic frequency increase and the prolonged posthypoxic recovery time in the preBötC of Mecp2^−/− slices.
Table 1: Summary of the characteristic HSD parameters recorded in the nTS and Sp5 of WT and Mecp2\(^{-/+}\) slices under various experimental conditions.

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Values are means ± standard deviation.
Fig 2

A

WT

Mecp2^−/−

1st HSD (CSF)

(6-OH-DPAT)

onset

hypoxia

onset

hypoxia

2nd HSD

(B

B

2nd HSD (8-OH-DPAT)

nTS

Sp5

WT

Mecp2^−/−

normalized HSD parameter (%)

nTS

Sp5

normalized HSD parameter (%)

nTS

Sp5

*** p < 0.001

** p < 0.01

* p < 0.05

0 100 150 0 100 150

Δt ΔV t 1/3 Δt ΔV t 1/3

Δt ΔV t 1/3 Δt ΔV t 1/3

Δt ΔV t 1/3 Δt ΔV t 1/3

Δt ΔV t 1/3 Δt ΔV t 1/3

Δt ΔV t 1/3 Δt ΔV t 1/3
Fig. 7

A

hypoxia

"gasping"

reox

reox

B

![Bar chart showing normalized recovery parameters](chart.png)

- Posthypoxic frequency increase (after 1st and 2nd hypoxia, normalized to control)
- Posthypoxic increase of recovery time (after 2nd and 3rd hypoxia, normalized to 1st hypoxia)