Altered responses of MeCP2-deficient mouse brain stem to severe hypoxia

Miriam Kron,1,2 Jasper L. Zimmermann,1 Mathias Dutschmann,1,2 Frank Funke,1 and Michael Müller1,2

1Deutsche Forschungsgemeinschaft Research Center for Molecular Physiology of the Brain, Zentrum für Physiologie und Pathophysiologie, Abteilung Neuro- und Sinnesphysiologie, and 2Bernstein Center for Computational Neuroscience, Georg-August-Universität Göttingen, Göttingen, Germany

Submitted 27 September 2010; accepted in final form 1 April 2011

Kron M, Zimmermann JL, Dutschmann M, Funke F, Müller M. Altered responses of MeCP2-deficient mouse brain stem to severe hypoxia. J Neurophysiol 105: 3067–3079, 2011. First published April 6, 2011; doi:10.1152/jn.00822.2010.—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; Mecp2−/−) we recently discovered an enhanced hippocampal susceptibility to hypoxia and hypoxia-induced spreading depression (HSD). In the present study we investigated whether this also applies to infant Mecp2−/− brain stem, 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 Mecp2−/− NTS and Sp5 was increased on 8 mM K+ -mediated conditioning. 5-HT1A receptor stimulation with 8-hydroxy-2-(di-propylamino)tetralin (8-OH-DPAT) postponed HSD by up to 40%, mediating genotype-independent protection. The deleterious impact of HSD on in vitro respiration became obvious in rhythmically active slices, where HSD propagation into the pre-Bötzinger complex (pre-BötC) immediately arrested the respiratory rhythm. Compared with wild-type, the Mecp2−/− pre-BötC was invaded less frequently by HSD, but if so, HSD occurred earlier. On reoxygenation, in vitro rhythms reappeared with increased frequency, which was less pronounced in Mecp2−/− slices. 8-OH-DPAT increased respiratory frequency but failed to postpone HSD in the pre-BötC. Repetitive hypoxia facilitated posthypoxic recovery only if HSD occurred. In 57% of Mecp2−/− slices, however, HSD spared the pre-BötC. Although this occasionally promoted residual hypoxic respiratory activity (“gasping”), it also prolonged the posthypoxic recovery, and thus the absence of central inspiratory drive, which in vivo would lengthen respiratory arrest. In view of the breathing disorders in RTTs, the increased hypoxia susceptibility of MeCP2-deficient brain stem potentially contributes to life-threatening disturbances of cardiorespiratory control.

brain tissue slice; hypoxia/anoxia; respiration; Rett syndrome; spreading depression; methyl-CpG-binding protein 2

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). Newborns patients initially appear healthy and achieve regular developmental milestones. After 6–18 mo 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 suffer 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. 2008a). 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 have 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 toward hyperexcitability in cardiorespiratory pontomedullary brain stem 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−/− mice) revealed clear signs of systemic adaptation to the intermittent hypoxic episodes, such as an increased hematocrit and elevated hypoxia-inducible factor (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 laboratory revealed that the hippocampus of symptomatic adult Mecp2−/− mice is more susceptible to metabolic arrest (Fischer et al. 2009; Kron and Müller 2010). During severe hypoxia, Mecp2−/− hippocampal neurons lose their membrane potentials, i.e., their function, earlier than wild-type (WT) neurons, ultimately resulting in a hastened onset of hypoxia-induced spreading depression (HSD) (Fischer et al. 2009). This potentially arises from disturbed intracellular Ca2+ homeostasis, which prevents sufficient protective K+ 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 brain stem, especially at infant stages (Funke et al. 2009), a finding that extended earlier observations from in vivo recordings (Richter F et al. 2003, 2008, 2010). Because the Mecp2−/− hippocampus was found to be more susceptible to hypoxia and HSD, and HIF-1α expres-
HSD-induced failure of brain stem 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. 2000, 2002). Therefore, neuronal excitation and inhibition have to be balanced accurately, and a shift toward hyperexcitability or reduced excitability can cause apnea or apneusis, respectively (Wasserman et al. 2002). It was previously demonstrated that the Hering-Breuer reflex in adult MeCP2-/- mice shows hyperexcitability and a lack of habituation (Stettner et al. 2007). Furthermore, parts of the adult MeCP2-/- 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 to 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, 2008a).

Moreover, loss of neuronal/synaptic function during HSD within the ventral respiratory column (VRC) and the pre-Bötzinger complex (pre-BöC) 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 brain stem 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 brain stem in vivo may indeed cause the cessation of spontaneous breathing (Richter F et al. 2003). Accordingly, an enhanced hypoxia susceptibility of the MeCP2-/- brain stem could be part of a deleterious vicious circle by 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 (nonrhythmic) and rhythmically active MeCP2-/- brain stem 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-/- mouse brain stem. These recordings were complemented by electrophysiological analyses to determine genotype-dependent differences of HSD parameters in the NTS, spinal trigeminal nucleus (Sp5), and pre-BöC. To define the very consequences of frequent hypoxic episodes, as occurring in RTT, on brain stem circuits involved in cardiorespiratory control, we adapted the rhythmically active slice preparation (Ramirez et al. 1996; Smith et al. 1991), with such treatment, inspiratory-related mass activity could be recorded reliably from the pre-BöC. After stable rhythmic activity was obtained, 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 that generated a well-proounced 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 record-
ing 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- to 100-μm outer diameter) connected to a field potential amplifier (Ext 10C; NPI). The DC-coupled signal was amplified 100×. To isolate the respiratory activity, the AC-coupled signal was amplified 10,000×, low-pass filtered at 3-kHz cutoff frequency, and rectified/integrated at a time constant τ = 200 ms (see Fig. 4A). Both signals were then digitized at 2 kHz. Respiratory frequency was analyzed in 5- to 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 1998), a 3rd HSD was induced on occasion, although without performing further analyses.

RESULTS
Following earlier work from our laboratory demonstrating an enhanced susceptibility of the MeCP2+/- hippocampus to hypoxia, we were next interested in whether this also applies to the MeCP2+/- brain stem. To address this issue, we analyzed genotype-dependent differences of hypoxic responses, including the properties of HSD, in acute WT and MeCP2+/- brain stem slices. The occurrence and propagation of HSD were monitored by recording both the extracellular DC potential and the 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+/- mouse brain stem. Initial experiments in normal (3.5 mM K+ containing) ACSF confirmed that infant WT and MeCP2+/- mouse brain stem reliably generate HSD as observed earlier in rat brain stem (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% of slices; MeCP2+/-: 44.4% of slices). The subsequent propagation of HSD into the other brain stem regions seemed more pronounced in WT than in MeCP2+/- slices. Specifically, the Sp5 was invaded in 91.7% of WT slices but only in 56% of MeCP2+/- slices. Similarly, the 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 MeCP2+/- slices. On the basis of 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 ± 3.7 mV in WT (n = 14) and −20.8 ± 2.8 mV in MeCP2+/- (n = 11). The time to HSD onset did not differ between genotypes (WT: 63.8 ± 14.6 s; MeCP2+/-: 69.1 ± 17.0 s), whereas the duration at half-maximum amplitude was significantly shorter in MeCP2+/- (WT: 54.7 ± 11.3 s; MeCP2+/-: 43.0 ± 4.7 s; P < 0.01, unpaired t-test; Fig. 1, A and B). In the Sp5, HSD showed a similar time to onset but a slightly reduced DC potential amplitude and/or duration compared with the NTS (unpaired t-test); differences among the genotypes were not observed (Fig. 1, A and 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 half-width 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 brain stem and rat/mouse hippocampus (Fischer et al. 2009; Funke et al. 2009; Müller and Somjen 1998)], a 3rd HSD was induced on occasion, although without performing further analyses.

Increasing extracellular K+ levels reveals an enhanced HSD susceptibility of MeCP2+/- brain stem. 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 K+ concentration ([K+]o) 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 half-width 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⁺/H11001 elicited HSDs with similar properties compared with the 1st HSDs in both genotypes (Table 1). It was shown previously 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 DW et al. 2003; Stettner et al. 2008b). 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; 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⁻⁻ brain stem 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⁺]o, 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⁻⁻ NTS (P < 0.01, paired t-test, n = 5; Fig. 2, A and B), which in both genotypes was significantly different from a 2nd HSD induced in control slices without prior 8-OH-DPAT treatment (P < 0.001, 1-way ANOVA). HSD amplitudes and half-width durations were not affected (Fig. 2B).

In a second set of experiments the effects of 8-OH-DPAT were examined in the presence of 8 mM [K⁺]o. Compared with a 2nd HSD induced in control slices in the presence of 8 mM

---

Fig. 1. Infant mouse brain stem reliably generates hypoxia-induced spreading depression (HSD) during severe hypoxia. A: severe hypoxia (oxygen withdrawal) reliably triggered HSD episodes in the nucleus of the solitary tract (NTS) and spinal trigeminal nucleus (Sp5) of both wild-type (WT) and methyl-CpG-binding protein 2-deficient mouse (Mecp2⁻⁻) brain stem 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⁻⁻ NTS. For exact definition of the HSD parameters, see A. Plotted are the averaged parameters. C: in both genotypes HSD could be induced repeatedly. Marked changes in the HSD parameters were not observed; the duration of the 2nd HSD increased slightly only in WT slices. Plotted are the parameters of the 2nd HSD, normalized to the 1st HSD from continuous recordings in the respective brain stem region of the analyzed slices. D: increased extracellular K⁺ levels ([K⁺]o) hasten the onset of HSD and unveil an enhanced hypoxia susceptibility of the Mecp2⁻⁻ NTS and Sp5. Values are means ± SD; the number of trials (n) is indicated at the bottom of each bar. *P < 0.05; **P < 0.01; ***P < 0.001.
Table 1. Summary of the characteristic HSD parameters recorded in the NTS and Sp5 of WT and MeCP2<sup>−/−</sup> slices under various experimental conditions

<table>
<thead>
<tr>
<th>HSD Parameters</th>
<th>AC SF (3.5 mM K&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>AC SF (8 mM K&lt;sup&gt;+&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>MeCP2&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>NTS (1st HSD)</td>
<td>n</td>
<td>14</td>
</tr>
<tr>
<td>Δt, s</td>
<td>63.8 ± 14.6</td>
<td>69.1 ± 17.0</td>
</tr>
<tr>
<td>ΔV, mV</td>
<td>-22.0 ± 3.7</td>
<td>-20.8 ± 2.8</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;, s</td>
<td>54.7 ± 11.3</td>
<td>43.0 ± 4.7</td>
</tr>
<tr>
<td>NTS (2nd HSD)</td>
<td>n</td>
<td>7</td>
</tr>
<tr>
<td>Δt, s</td>
<td>65.6 ± 17.1</td>
<td>71.8 ± 17.9</td>
</tr>
<tr>
<td>ΔV, mV</td>
<td>-22.3 ± 3.0</td>
<td>-21.1 ± 2.3</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;, s</td>
<td>60.1 ± 13.6</td>
<td>45.7 ± 6.3</td>
</tr>
<tr>
<td>Sp5 (1st HSD)</td>
<td>n</td>
<td>10</td>
</tr>
<tr>
<td>Δt, s</td>
<td>67.5 ± 18.1</td>
<td>75.3 ± 19.2</td>
</tr>
<tr>
<td>ΔV, mV</td>
<td>-17.7 ± 5.0</td>
<td>-16.5 ± 2.3</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;, s</td>
<td>41.7 ± 9.0</td>
<td>38.0 ± 6.2</td>
</tr>
<tr>
<td>Sp5 (2nd HSD)</td>
<td>n</td>
<td>6</td>
</tr>
<tr>
<td>Δt, s</td>
<td>66.3 ± 12.0</td>
<td>73.8 ± 18.7</td>
</tr>
<tr>
<td>ΔV, mV</td>
<td>-18.5 ± 4.6</td>
<td>-16.1 ± 3.5</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;, s</td>
<td>51.4 ± 10.3</td>
<td>41.0 ± 2.0</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of observations. Hypoxia-induced spreading depression (HSD) parameters were recorded in the nucleus of the solitary tract (NTS) and spinal trigeminal nucleus (Sp5) of wild-type (WT) and methyl-CpG-binding protein 2-deficient mouse (MeCP2<sup>−/−</sup>) brain stem slices. AC SF, artificial cerebrospinal fluid; Δt, time to HSD onset; ΔV, amplitude of the extracellular DC potential shift; t<sub>1/2</sub>, HSD duration at half-maximum amplitude.

[K<sup>+</sup>]<sub>i</sub>, 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<sup>−/−</sup> NTS (n = 6; P < 0.05, 1-way ANOVA); HSD amplitudes and half-width 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 by 35.3 ± 21.2% (n = 8) in MeCP2<sup>−/−</sup> (P < 0.05, 1-way ANOVA; Fig. 2C).

The pre-BötC is invaded less frequently by HSD in rhythmically active MeCP2<sup>−/−</sup> brain stem 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 pre-BötC mass activity near-physiological temperature (35.5°C); maintaining rhythmicity of this slice required us to increase [K<sup>+</sup>]<sub>i</sub> 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>−/−</sup> brain stem 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>−/−</sup> brain stem, the IOS was also analyzed in the rhythmically active slice, which, due to the elevated [K<sup>+</sup>]<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, on oxygen withdrawal, the IOS showed a widespread increase in tissue reflectance with sometimes multifocal origin which then invaded large parts of the brain stem slice (Fig. 3). The Sp5 was identified as the preferred ignition site of HSD (83.3%, of WT but only 47.6% of MeCP2<sup>−/−</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>−/−</sup>: Sp5 90%, NTS 81%). However, the IO and pre-BötC were less frequently affected in MeCP2<sup>−/−</sup> (23.8 and 42.9%, respectively) compared with WT slices (50.0 and 75.0%, respectively; Fig. 3), as already shown in normal [K<sup>+</sup>]<sub>i</sub>.

HSD onset is hastened in the MeCP2<sup>−/−</sup> pre-BötC, but posthypoxic recovery appears normal. If HSD invaded the pre-BötC, 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>−/−</sup> slices (47.1 ± 15.4 s, n = 16; P < 0.05, unpaired t-test; Fig. 4, B and C). The average DC potential amplitudes (WT: -8.9 ± 4.2 mV; MeCP2<sup>−/−</sup>: -11.6 ± 5.1 mV) and half-width durations (WT: 32.1 ± 6.6 s; MeCP2<sup>−/−</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 pre-BötC consistently caused a sudden and complete arrest of respiratory rhythmogenesis (see Fig. 4D, middle). The hastened onset of HSD in the MeCP2<sup>−/−</sup> pre-BötC strongly suggests that the MeCP2<sup>−/−</sup> respiratory network is more vulnerable to hypoxia-induced loss of function.

In both genotypes respiratory rhythmogenesis reappeared within a few minutes on reoxygenation, gaining a clearly increased respiratory frequency (Figs. 4D and 5C). In detail, recovery times, defined as the time period between reoxygenation and occurrence of the 1st and 10th respiratory burst, averaged 146.4 ± 55.1 and 229.1 ± 68.8 s in WT slices (n = 20). Recovery was similar in MeCP2<sup>−/−</sup> slices, averaging 160.3 ± 55.6 and 242.3 ± 87.4 s (n = 16). The posthypoxic increase in respiratory frequency, however, was more pronounced in WT than in MeCP2<sup>−/−</sup> slices (WT by 99.1 ± 67.0%, n = 11; P < 0.001 vs. prehypoxic control frequency, repeated-measures ANOVA, MeCP2<sup>−/−</sup> by 60.7 ± 43.0%, n = 5; Figs. 4D and 5, A and C).

Occurrence of HSD may facilitate posthypoxic recovery. To define the impact of repeated hypoxic episodes, which do occur in RTT, on in vitro respiratory rhythmogenesis, we induced a 2nd HSD in some rhythmically active slices after 20 min of recovery. In WT slices, the onset of the 2nd HSD was significantly accelerated by 19.1 ± 20% (n = 14; P < 0.01, paired t-test) compared with the 1st HSD. In MeCP2<sup>−/−</sup> slices, the time to onset of the 2nd HSD was variable but on average did not significantly differ from that of the 1st 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. 5, A and B), but half-width durations were unchanged. The pronounced increase with large standard deviations of the HSD amplitudes in WT results from the fact that the 1st HSD sometimes gave rise to only a small DC potential shift, whereas the 2nd 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 10th respiratory burst upon reoxygenation, after the 2nd HSD was shortened consistently, by 28.0 ± 21.1% in WT (n = 11; P < 0.01 vs. 1st recovery, paired t-test) and by 32.2 ± 21.4% in Mecp2−/− slices (n = 8; P < 0.05; repeated-measures ANOVA). 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−/− slices (by 97.8 ± 76.3% as normalized to prehypoxic control conditions; Fig. 5, A and 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−/− brain stem but seem less pronounced.

5-HT1A receptor signaling in the pre-BötC 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 1st HSD, and it tended to counteract the hastening of the 2nd HSD observed in untreated WT slices (time to onset hastened by only 5.8 ± 6.2%, n = 7); in the Mecp2−/− pre-BötC, the time to onset of the 2nd HSD was comparable to that in control slices (n = 8; Fig. 6, A and B). 8-OH-DPAT moderately increased the 2nd HSD amplitude.

Fig. 2. Stimulation of 5-HT1A receptors postpones the onset of HSD. A: incubation of slices with 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT; 50 μM, 20 min) postpones HSD onset in both genotypes. The displayed traces were recorded in the NTS. ACSF, artificial cerebrospinal fluid. 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 1st HSD recorded under pretreatment control conditions in the respective brain stem region. 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 compared with normal [K+]o. *P < 0.05; **P < 0.01; ***P < 0.001 vs. 2nd HSD induced in control slices without drug treatment.
compared with the 1st HSD, which was more pronounced in WT slices (by 26.9 ± 17.5% in WT; P < 0.01 vs. 1st HSD, paired t-test; by 21.5 ± 36.3% in MeCP2-/-). However, compared with the 2nd HSD in untreated slices, 8-OH-DPAT did not exert any significant effects on the DC potential amplitudes (1-way ANOVA). The half-width duration became significantly decreased in the pre-BötC of both genotypes (by 34.2 ± 22.5% in WT; P < 0.01, paired t-test; by 32.3 ± 27.2% in MeCP2-/- slices; P < 0.05, paired t-test; Fig. 6, A and B). Compared with the half-width ratios in untreated slices, we found a significant difference only in WT slices (P < 0.01, 1-way ANOVA). These results may suggest that the MeCP2-/- brain stem is somewhat less responsive to 8-OH-DPAT.

On reoxygenation, 8-OH-DPAT treated slices displayed a shortening of the posthypoxic recovery time similar to that observed in untreated slices. Specifically, respiratory activity reappeared 37.0 ± 19.0% earlier in WT slices (P < 0.01, paired t-test) and 34.4 ± 25.1% earlier in MeCP2-/- slices (P < 0.05) compared with the 10th burst of the 1st recovery (Fig. 6, A and 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-/- slices (P < 0.001). This frequency increase persisted even after washout of 8-OH-DPAT after the 2nd HSD (WT: +340.8 ± 186.4%; P < 0.001, repeated-measures ANOVA; MeCP2-/-: +308.5 ± 195.5%; P < 0.001; Fig. 6, A and 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 pre-BötC was spared by the 1st HSD despite 5 min of hypoxia in 4 out of 25 WT and 16 out of 32 MeCP2-/- 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-/-: 1st burst, 160.2 ± 64.5 s; 10th burst, 222.7 ± 93.3 s; WT: 1st burst, 176.9 ± 81.9 s; 10th burst, 242.5 ± 136.1 s) were similar to those observed in slices generating the 1st HSD, and the pronounced increase in respiratory frequency also occurred (MeCP2-/-: +97.5 ± 95.3%; WT: +51.7 ± 40.6%). However, in all investigated WT slices and in 10 of 16 MeCP2-/- slices, the 2nd (and also a 3rd) hypoxic episode succeeded to induce HSD, with properties and a recovery similar to those observed above (data not shown).

In contrast, in the remaining six MeCP2-/- brain stem slices, three episodes of severe hypoxia (lasting 5 min each) failed to induce HSD within the pre-BötC. In these slices a progressive posthypoxic increase of respiratory frequency occurred, by 66.3 ± 59.0% after the 1st hypoxia and by 97.7 ± 55.0% (Fig. 3). In MeCP2-/- slices, the ventral respiratory column (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 HSD showed a near-complete invasion of the entire hemislice in WT slices, the propagation of HSD was more restricted in MeCP2-/- brain stem. Especially, the more ventral and ventrolateral aspects of the slice, including the VRC, were less frequently invaded compared with WT slices; instead, large areas of the MeCP2-/- 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 anatomic landmarks are indicated. 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.
during the 2nd recovery ($P < 0.01$, repeated-measures ANOVA). Also, the recovery time was somewhat shorter compared with that of those slices generating a HSD (1st burst: 127.5 ± 58.5 s; 10th 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 (2nd hypoxia: prolongation by 58.1 ± 41.3%; 3rd hypoxia: by 108.6 ± 85.1%; $P < 0.05$, repeated-measures ANOVA; Fig. 7, A and B).

**DISCUSSION**

In the present study we have shown complex responses of different brain stem regions involved in cardiorespiratory control to severe hypoxia and HSD, as well as their alterations associated with MeCP2 deficiency. A major focus was placed on the effects of severe hypoxia on in vitro respiratory rhythmogenesis. In this study, for the first time, we adapted the rhythmically active brain stem slice to interface-chamber conditions, which allows for optical and electrophysiological recordings of hypoxic responses at almost physiological temperature.

HSD occurs in the MeCP2−/− brain stem, but its propagation seems restricted. For a long time, brain stem 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 infant rat brain
stem was confirmed to generate spreading depression episodes (Funke et al. 2009; Richter F et al. 2003, 2008, 2010). In this study we have shown that even without prior conditioning, severe hypoxia also triggers HSD in the brain stem 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 brain stem 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> brain stem slices.

Once ignited, HSD propagated into other brain stem 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 brain stem, 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 brain stem matures earlier the NTS is the preferred ignition site of HSD and even more so after the 2nd HSD. *P < 0.05; **P < 0.01; ***P < 0.001. ctrl, Control.

HSD susceptibility of the infant Mecp2<sup>−/−</sup> brain stem. 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). In this study we have shown that the HSD susceptibility of the infant Mecp2<sup>−/−</sup> brain stem seems normal, at least without further conditioning stressors. However, compared with the hippocampus, which is highly vulnerable to metabolic compromise (Pulsinelli et al. 1982; Schmidt-Kastner and Freund 1991), brain stem 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> brain stem may not be detectable at rest.

However, when hypoxia coincides with additional stimuli, causing an increased metabolic demand such as elevated [K<sup>+</sup>]<sub>es</sub>, the onset of HSD in the infant Mecp2<sup>−/−</sup> NTS, Sp5 and pre-BötC is hastened. Consequently, the loss-of-function within important (cardiorespiratory) sensory relays and the main respiratory pattern generator also occurs earlier.

Hypereexcitability critically promotes HSD generation, and signs of hypereexcitability are found in various regions of the Mecp2<sup>−/−</sup> brain stem at later developmental stages, including the NTS (Kline et al. 2010; Stettner et al. 2007; Taneja et al. 2009). Although it is unknown at which developmental stage hypereexcitability develops in the Mecp2<sup>−/−</sup> NTS, decreased GABAergic inhibition 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<sup>−/−</sup> brain stem, and since the NTS is the preferred ignition site of HSD
in the MeCP2−/− brain stem, a shift toward 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 in this sensory relay, as well. 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−/− 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 MeCP2−/− Sp5 could also decrease the threshold of trigemino-postinspiratory breath-holds. Moreover, recent findings that young, obviously presymptomatic, MeCP2−/− 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, although the occurrence of spreading depression in brain stem seems capable of arresting spontaneous breathing in anesthetized rats (Richter F et al. 2003). In the present study such correlated analyses became possible for the first time as we succeeded in adapting the rhythmically active brain stem 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 Oppie 1952; Dick and Coles 2000; Lawson and Long 1983; Powell et al. 1998; Richter DW 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 DW et al. 1991). Our experiments extend these findings, since for the first time we have shown that within only 1–2 min of severe hypoxia, HSD may propagate into the pre-BötC and cause a sudden and complete arrest of respiratory activity already during/after the initial frequency increase (Figs. 5A and 6A; compare with 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 (gaspig). As such, it represents a new pathophysiological mechanism that deserves further detailed analyses and the verification of similar mechanisms under intact network conditions.

Interestingly, in the MeCP2−/− brain stem, the propagating HSD more frequently spared the pre-BötC, 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 on 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> pre-BötC.

An interesting aspect is that only the repeated occurrence of HSD within the pre-BötC shortened the posthypoxic recovery time, suggesting that HSD mediates some preconditioning within the brain stem, 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<sub>1A</sub> receptor activation mediates partial hypoxic protection and potentiates the posthypoxic increase in respiratory frequency. Activation of 5-HT<sub>1A</sub> receptors can attenuate spreading depression (Krüger et al. 1999), stabilize respiration (Dutschmann et al. 2009; Lalley et al. 1994; Richter DW et al. 2003; Stettner et al. 2008b), and was recently shown 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<sup>+</sup>]<sub>i</sub>, 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<sub>1A</sub> receptor activation inhibits adenylyl cyclase 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 [K<sup>+</sup>]<sub>i</sub> and even tended to be more potent under these conditions.

Surprisingly, 8-OH-DPAT-mediated postponement of HSD was not obvious in the pre-BötC 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 respira-
tory 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 pre-BöC (Manzke et al. 2009), where glycinergic neurons are numerous (Tanaka et al. 2003; Winter et al. 2009) and express 5-HT1A 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 pre-BöC.

Conclusion. In contrast to adult MeCP2−/− hippocampus, the hypoxia susceptibility of infant MeCP2−/− brain stem was increased only when hypoxia coincided with additional challenges. Propagation of HSD into the pre-BöC was confirmed to abolish respiratory rhythmmogenesis, i.e., to arrest breathing. Despite this deleterious effect, the occurrence of HSD also facilitated the recovery of rhythmmogenesis on reoxygenation, whereas its consistent absence during repeated hypoxia, especially in MeCP2−/− pre-BöC, prolonged the posthypoxic recovery. 5-HT1A receptor stimulation mediated a partial and genotype-independent protection against HSD and stabilized/increased the in vitro respiratory rhythm, suggesting that 5-HT1A receptor-mediated signaling seems largely intact in MeCP2−/− brain stem. Therefore, well-targeted stimulation of these receptors may provide a potential pharmacotherapeutical concept to ameliorate both the irregular breathing pattern and the consequences of the intermittent hypoxic episodes associated with RTT.

ACKNOWLEDGMENTS

We thank Belinda Hildebrandt for expert technical assistance.
Present address of M. Kron: Department of Neurosciences, Case Western Reserve University, Cleveland OH 44106-4975.
Present address of M. Dutschmann: Institute of Membrane and Systems Biology, University of Leeds, Leeds LS2 9JT, United Kingdom.

GRANTS

This study was supported by the Deutsche Forschungsgemeinschaft Research Center for Molecular Physiology of the Brain, the German Ministry for Education and Research via the Bernstein Center for Computational Neuroscience Göttingen under Grant 01GQ0432, and the University Medical Center Göttingen (Ausstattungsmittel Juniorprofessur). Furthermore, Gö4med dissertation stipend of the University Medical Center Göttingen was awarded to J. L. Zimmermann.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

Isoda K, Morimoto M, Matsui F, Hasegawa T, Tozawa T, Morikoa S, Chiyonobu T, Nishimura A, Yoshimoto K, Hosoi H. Postnatal changes in...


