Early Defects of GABAergic Synapses in the Brain Stem of a MeCP2 Mouse Model of Rett Syndrome

L. Medrihan, E. Tantalaki, G. Aramuni, V. Sargsyan, I. Dudanova, M. Missler, and W. Zhang

Abstract

Early defects of GABAergic synapses in the brain stem of a MeCP2 mouse model of Rett syndrome. J Neurophysiol 99: 112–121, 2008. First published November 21, 2007; doi:10.1152/jn.00826.2007. Rett syndrome is a neurodevelopmental disorder caused by mutations in the transcriptional repressor methyl-CpG-binding protein 2 (MeCP2) and represents the leading genetic cause for mental retardation in girls. MeCP2-mutant mice have been generated to study the molecular mechanisms of the disease. It was suggested that an imbalance between excitatory and inhibitory neurotransmission is responsible for the behavioral abnormalities, although it remained largely unclear which synaptic components are affected and how cellular impairments relate to the time course of the disease. Here, we report that MeCP2 KO mice present an imbalance between inhibitory and excitatory synaptic transmission in the ventrolateral medulla already at postnatal day 7. Focusing on the inhibitory synaptic transmission we show that GABAergic, but not glycineergic, synaptic transmission is strongly depressed in MeCP2 KO mice. These alterations are presumably due to both decreased presynaptic γ-aminobutyric acid (GABA) release with reduced levels of the vesicular inhibitory transmitter transporter and reduced levels of postsynaptic GABA_A-receptor subunits α2 and α4. Our data indicate that in the MeCP2 –/y mice specific synaptic molecules and signaling pathways are impaired in the brain stem during early postnatal development. These observations mandate the search for more refined diagnostic tools and may provide a rationale for the timing of future therapeutic interventions in Rett patients.

Introduction

Rett syndrome [RTT, Online Mendelian Inheritance in Man (OMIM) database entry #312750] is an X-linked debilitating neurodevelopmental disorder leading to mental retardation in females (Amir et al. 1999). RTT patients are supposed to achieve normal developmental milestones until 6–18 mo of age when they begin to regress (Hanafeld et al. 1986; Kozinetz et al. 1993). Subsequently, patients develop cognitive and motor symptoms, including severe ventilation problems (Hanefeld et al. 1986) that may lead to lethal apnea (Kerr and Burford 2001).

Mutations in the transcriptional repressor methyl-CpG-binding protein 2 (MeCP2) account for the majority of RTT cases (Kozinetz et al. 1993). Therefore MeCP2-mutant mice have been generated to study the cellular and molecular mechanisms of Rett syndrome (Amir et al. 1999; Chen et al. 2001; Guy et al. 2001). Similar to patients, the MeCP2-mutant –/y mice are apparently normal until about 5 wk of age, when they begin to exhibit abnormal motor and social behavior, including respiratory irregularities (Stettner et al. 2007; Viemari et al. 2005). Since morphological alterations in brain architecture are subtle in MeCP2 mutants (Chen et al. 2001), most studies have focused on functional impairments to explain their profound behavioral abnormalities. Previous electrophysiological studies of adult mutant MeCP2 mice demonstrated an enhanced excitatory neurotransmission (Moretti et al. 2006) and reduced synaptic plasticity in the hippocampus (Asaka et al. 2006; Moretti et al. 2006). In addition, it was shown that deletion of MeCP2 may result in a reduction of excitatory activity and an increase of inhibitory release in neocortical neurons (Dani et al. 2005). Furthermore, deletion of MeCP2 has been shown to decrease whereas overexpression of MeCP2 enhanced the excitatory synaptic transmission in autaptic hippocampus neurons (Chao et al. 2007). Although these studies found opposite effects of MeCP2 on excitatory versus inhibitory neurotransmission, it appears likely that an imbalance of synaptic activity is a crucial aspect of the phenotype. However, with respect to the underlying mechanisms and putative strategies for therapeutic interventions, important questions remain to be answered: Which synaptic components are the primary targets affected by MeCP2 mutations? How do cellular impairments relate to the time course of clinical symptoms? These aspects are important because clinical research has begun to emphasize the problems of young RTT patients (Einspieler et al. 2005a,b; Nomura 2005; Trevarthen and Daniel 2005). Consistently, studies on young MeCP2-deficient mice revealed that more subtle behavioral alterations may be present early (Picker et al. 2006) and that abnormalities of younger mice are resembling symptoms of RTT girls (Santos et al. 2007).

Here, we have addressed the open questions by investigating the ventrolateral medulla of early postnatal MeCP2-deficient mice, using a combined electrophysiological, biochemical, and histological analysis of this brain stem model system for synapse maturation (Missler et al. 2003; Varoquaux et al. 2006; Zhang et al. 2005). We found that MeCP2 KO mice present a depression of GABAergic but not glycineric inhibitory neurotransmission and this depression is due to a decrease in GABAergic release and an alteration of the subunit composition of postsynaptic γ-aminobutyric acid type A (GABA_A) receptors. Our data suggest that specific molecular and cellular impairments are already present early postnatally, supporting the underlying mechanisms and putative strategies for therapeutic interventions, important questions remain to be answered: Which synaptic components are the primary targets affected by MeCP2 mutations? How do cellular impairments relate to the time course of clinical symptoms? These aspects are important because clinical research has begun to emphasize the problems of young RTT patients (Einspieler et al. 2005a,b; Nomura 2005; Trevarthen and Daniel 2005). Consistently, studies on young MeCP2-deficient mice revealed that more subtle behavioral alterations may be present early (Picker et al. 2006) and that abnormalities of younger mice are resembling symptoms of RTT girls (Santos et al. 2007).

Here, we have addressed the open questions by investigating the ventrolateral medulla of early postnatal MeCP2-deficient mice, using a combined electrophysiological, biochemical, and histological analysis of this brain stem model system for synapse maturation (Missler et al. 2003; Varoquaux et al. 2006; Zhang et al. 2005). We found that MeCP2 KO mice present a depression of GABAergic but not glycineric inhibitory neurotransmission and this depression is due to a decrease in GABAergic release and an alteration of the subunit composition of postsynaptic γ-aminobutyric acid type A (GABA_A) receptors. Our data suggest that specific molecular and cellular impairments are already present early postnatally, supporting the underlying mechanisms and putative strategies for therapeutic interventions, important questions remain to be answered: Which synaptic components are the primary targets affected by MeCP2 mutations? How do cellular impairments relate to the time course of clinical symptoms? These aspects are important because clinical research has begun to emphasize the problems of young RTT patients (Einspieler et al. 2005a,b; Nomura 2005; Trevarthen and Daniel 2005). Consistently, studies on young MeCP2-deficient mice revealed that more subtle behavioral alterations may be present early (Picker et al. 2006) and that abnormalities of younger mice are resembling symptoms of RTT girls (Santos et al. 2007).

Here, we have addressed the open questions by investigating the ventrolateral medulla of early postnatal MeCP2-deficient mice, using a combined electrophysiological, biochemical, and histological analysis of this brain stem model system for synapse maturation (Missler et al. 2003; Varoquaux et al. 2006; Zhang et al. 2005). We found that MeCP2 KO mice present a depression of GABAergic but not glycineric inhibitory neurotransmission and this depression is due to a decrease in GABAergic release and an alteration of the subunit composition of postsynaptic γ-aminobutyric acid type A (GABA_A) receptors. Our data suggest that specific molecular and cellular impairments are already present early postnatally, supporting the underlying mechanisms and putative strategies for therapeutic interventions, important questions remain to be answered: Which synaptic components are the primary targets affected by MeCP2 mutations? How do cellular impairments relate to the time course of clinical symptoms? These aspects are important because clinical research has begun to emphasize the problems of young RTT patients (Einspieler et al. 2005a,b; Nomura 2005; Trevarthen and Daniel 2005). Consistently, studies on young MeCP2-deficient mice revealed that more subtle behavioral alterations may be present early (Picker et al. 2006) and that abnormalities of younger mice are resembling symptoms of RTT girls (Santos et al. 2007).
the idea of early abnormalities in the development of MeCP2-mutant animals and RTT syndrome patients.

**METHODS**

**Animals**

Experiments were performed on mice using the strain [B6.129P2-C-Mecp2 tm1.1Bird] as a mouse model for Rett syndrome (Guy et al. 2001). The mice were obtained commercially from The Jackson Laboratory (Bar Harbor, ME) and maintained on a C57BL/6J background. Hemizygous mutant MeCP2 males were generated by crossing heterozygous knockout (KO) females with C57BL/6J wild-type (WT) males. All experiments were performed on hemizygous (-/-) MeCP2 males and their sex- and age-matched littermate controls (+/y). We used (+/-) MeCP2 females because heterozygous (-/-) MeCP2 female mice have a heterogeneous phenotype as a result of the X-inactivation profile of the X-linked MeCP2 gene. Genotyping was performed as previously described (Stettner et al. 2007), essentially following The Jackson Laboratory protocols (forward primer CreF: 5'-GAC CGT ACA CCA AAA TTT GCC TG-3'; reverse primer CreR: 5'-TTA CGT ATA TTC TGG CAG CGA TC-3'). All experiments were done according to institutional regulations of animal welfare.

**Ventilation recordings**

Ventilation patterns were recorded by whole body plethysmography, for which unaesthetized postnatal day (P) 7 WT and their KO littermates were placed in a 50-ml closed chamber connected to a differential pressure transducer (CD15 Carrier Demodulator, Valdiyne Engineering, Northridge, CA). The analog signal of ventilation-related changes of air pressure was amplified and digitized using an A/D converter (DigiData 1322A, Molecular Devices, Sunnyvale, CA) and analyzed using commercially available pClamp 9.2 software (Molecular Devices).

**Electrophysiological recordings**

All electrophysiological analyses were performed on brain stem neurons of mice whose genotype was unknown to the experimenter. Acute coronal brain stem slices (200 μM) containing the ventrolateral medulla from P7 littermate mice were used for whole cell recordings (Ritter and Zhang 2000; Varoqueaux et al. 2006; Zhang et al. 2005). We recorded from neurons localized in the rostroventrolateral medulla (RVLM) (cf. inset of Fig. 3D) (Franklin and Paxinos 1997), in an area containing respiratory and other neurons (Smith et al. 1991). However, no further characterization of these neurons was performed to test whether these were inspiratory or other respiratory neurons.

The bath solution in all experiments consisted of (in mM) 118 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1 NaH₂PO₄, and 5 glucose (pH 7.4), aerated with 95% O₂-5% CO₂ and kept at 32°C. The pipette solution for all experiments contained (in mM): 140 KCl, 1 CaCl₂, 10 EGTA, 2 MgCl₂, 4 Na₂ATP, 0.5 Na₂GTP, and 10 HEPES (pH 7.3). Spontaneous GABA- and glycinegic inhibitory postsynaptic currents (IPSCs) were recorded at a Cl⁻ reversal potential of about 0 mV in 10 μM 6-cyano-7-nitroquinoline-2,3-dione (CNQX) and 40 μM 2-amino-5-phosphonomovaleric acid (APV). Miniature GABA- and glycinegic PSCs (mIPSCs) were recorded as described earlier, but in the presence of 0.5 μM tetrodotoxin (TTX). Spontaneous glutamatergic excitatory postsynaptic currents (EPSCs) were recorded in the presence of 1 μM strychnine and 1 μM bicuculline. Signals with amplitudes at least twofold above the background noise were selected.

In some experiments (cf. Fig. 4) the drug was directly applied in close proximity to neurons by glass pipettes filled with muscimol (5 mM) or glycine (5 mM) dissolved in the above-described bath solution. To minimize the variation between experiments, we kept tip size of the pipette, pressure (0.5 mbar), and time (500 ms) constant for all experiments. In addition, the distances between pipette tips and the cell were monitored using a camera with a liquid crystal display device and were also kept constant between different experiments.

The remaining variation between experiments was random in nature and was not specifically related to the genotype of the tested animals, especially because the experimenter was unaware of the genotype. To elicit a hypertonic response (Rosenmund and Stevens 1996), sucrose (300 mM) was applied in the perfusion flow for 2 s. In all tested animals, there were no significant differences in the noise levels between different genotypes. Patches with a serial resistance of >10 MΩ, a membrane resistance of <0.2 GΩ, or leak currents of >200 pA were excluded. The membrane currents were filtered by a four-pole Bessel filter at a corner frequency of 2 kHz and digitized at a sampling rate of 5 kHz using the DigiData 1322A interface (Molecular Devices).

**Kinetics and data analysis**

mIPSCs decay was fitted by double-exponential equations of the form I(t) = Afast exp(-t/τfast) + Aslow exp(-t/τslow), where I(t) is the amplitude of mIPSCs at time t, Afast and Aslow are the amplitudes of the fast and slow decay components, and τfast and τslow are their respective decay time constants (Jonas et al. 1998; Nabekura et al. 2004). mIPSCs were considered to have a monoexponential decay when the relative contribution of one of the exponential distributions was <1%. Thus the decision was made whether a single mIPSC decayed with a single or dual component was completely objective. GABA-mediated miniature postsynaptic current (mGSPC) decay was fitted to a monoexponential function. All data are expressed as mean ± SE. P values represent the results of two-tailed unpaired Student’s t-tests, with or without Welch’s correction, depending on the distribution of the data (tested with a Kolmogorov–Smirnov test). Data acquisition and analysis were done using commercially available software: pClamp 9.0 and AxFraph 4.6 (Molecular Devices), MiniAnalysis (Synaptosoft, Decatur, GA), and Prism 4 (GraphPad Software, San Diego, CA).

**Biochemical procedures**

Brain stem samples from P7 WT and their KO littermates were homogenized with a glass Teflon homogenizer at setting 2,000 rpm (10 strokes) in a buffer containing 1 mM EDTA, 20 mM HEPES (pH 7.4), 0.1 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. The homogenates were placed in ice-cold lysis buffer (1:1 RIPA with 1% NP40), incubated at 4°C for 2 h, and the supernatant was centrifuged at 14,000 rpm at 4°C for 30 min. The protein concentration was determined by Lowry assay using the total protein kit from Sigma with bovine albumin serum (Sigma–Aldrich, St. Louis, MO) as a standard. A 30-μg sample was resuspended in 3 × loading buffer (62.5 mM Tris-HCl, 20% glycerol, 6% SDS, 0.01% Bromphenol Blue, 10% β-mercaptoethanol), boiled at 100°C for 5 min, and separated by 10% SDS-PAGE. Proteins were transferred to Hybond nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) and probed with antibodies against the following proteins: GABARα1β1, GABARα2, GABARβ1, GABARβ3, and GlyR (Abcam, Cambridge, UK); GABARα1β1 (Abcam, Cambridge, UK), and Hsp70 (Santa Cruz Biotechnology, Santa Cruz, CA); GABAB2, GABARα3, and GlyR (Abcam, Cambridge, UK), and probed with antibodies against the following proteins: GABAB1, GABAB2, and GlyR (Alexis Biochemicals, San Diego, CA). Proteins were detected by AceGlows chemiluminescence detection reagents and imaging system Chemi-Smart 5000 (Peqlab, Erlangen, Germany). The quantitative analysis of immunoblots was done using Bio-1D software (Vilbert-Lourmat, Torcy, France), and included the results from tissue samples of at least four (as indicated in Fig. 6) independent mice in each group.
**Immunofluorescence staining**

Brains from three P7 MeCP2 KO mice and three WT littermates were removed and immersion fixed overnight in 4% paraformaldehyde, cryoprotected in sucrose gradient, and frozen on dry ice. Serial medulla coronal sections (14 μm) were collected on slides (cf. Fig. 3D). Before staining slices were washed three times with phosphate-buffered saline. After blocking, sections were stained for vesicular inhibitory amino acid transporter (VIAAT; 1:1,000, anti-rabbit; Synaptic Systems). Sections from immunofluorescence staining were visualized by confocal laser scanning microscopy (Zeiss LSM 510 META). The region of the ventrolateral medulla was recognized based on the appearance of nucleus ambiguous and inferior olive (cf. Fig. 3D). Images (1,024 × 1,024 pixel) were recorded at a zoom factor of 5, using a ×40 oil-immersion objective. The gain and offset were held constant across all images to allow for intensity comparisons. For quantification, one image per mouse was chosen and VIAAT puncta were manually counted with the experimenter being blind with respect to the genotype. Regions for quantifications were chosen using the same criteria as for electrophysiology.

**RT-PCR**

Total RNA was isolated with RNAzol B (WAK-Chemie, Steinbach, Germany) from the brain stem of P7 WT mice and their KO littermates. The brain stems from six animals for each genotype were pooled to obtain enough material for RNA isolation. The RNA was reverse-transcribed with the GeneAmp Gold RNA PCR Core Kit (Applied Biosystems, Foster City, CA), using Oligo-dT primer. All the gene-specific primers were purchased from Qiagen (QuantiTect Primer Assay 200, Qiagen, Hilden, Germany; available on-line at http://www1.qiagen.com/products/pcr/quantitect/primerassays.aspx). Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). All reactions were performed in duplicate and the data are from at least three experiments. Signals were analyzed by ABI Prism Sequence Detection software (Applied Biosystems). All reactions were performed in duplicate and the data are from at least three experiments. Signals were analyzed by ABI Prism Sequence Detection software (Applied Biosystems). All reactions were performed in duplicate and the data are from at least three experiments.

**RESULTS**

Excitatory–inhibitory imbalance appears early in the postnatal development of the brain stem respiratory network of MeCP2 KO mice

To investigate the early cellular and molecular dysfunctions that may lead to an important complication in Rett syndrome patients, i.e., ventilation problems that may cause lethal apnea (Hanefeld et al. 1986; Kerr and Burford 2001), we studied neurotransmission in the ventrolateral medulla, an area of the reticular brain stem formation that contains pre-Bötzinger complex (preBötC), a neuronal network responsible for generating the respiratory rhythm (PBC; cf. Richter and Spyer 2001). Normal synaptic transmission and development have been extensively characterized in this brain stem model (Ritter and Zhang 2000) and various defects of synaptic function have been successfully studied in this system (Missler et al. 2003; Varoquaux et al. 2006; Zhang et al. 2005). Since adult MeCP2-deficient mice show respiratory problems (Stettner et al. 2007; Viemari et al. 2005) and we were interested in the cellular defects leading to these symptoms, we first determined a time point when the behavioral symptoms are not yet fully present in mutant mice but the underlying neuronal network already shows abnormalities. At postnatal day 7 (P7), no major difference could be observed in ventilation activity between the hemizygous MeCP2 males (KO) compared with their sex- and age-matched littermate controls (WT) (Fig. 1, A and B). However, patch-clamp recordings of the overall activity in acute brain stem slices revealed a markedly depressed amplitude (WT: 73.5 ± 0.9 pA; KO: 46 ± 0.8 pA; P < 0.001) and frequency (WT: 6.8 ± 0.1 Hz; KO: 3.3 ± 0.1 Hz; P < 0.001) of spontaneous inhibitory synaptic currents (sIPSCs) in MeCP2-deficient mice at P7 (Fig. 1, C–E). In contrast, both the amplitude (WT: 25.2 ± 0.3 pA; KO: 34.3 ± 0.3 pA; P < 0.001) and frequency (WT: 5.5 ± 0.2 Hz; KO: 6.9 ± 0.3 Hz; P < 0.001) of spontaneous excitatory activity (sEPSCs) were slightly increased in MeCP2 KO mice compared with their littermate controls of the same age (Fig. 1, F–H). These results show a specific imbalance in the network activity of ventrolateral medulla in MeCP2-deficient mice, thereby confirming previous data from other brain regions but demonstrating for the first time that these cellular impairments are already present at a very early stage of CNS development.

**GABAergic, but not glycinergic, inhibitory synaptic transmission is depressed in the postnatal ventrolateral medulla of MeCP2 KO mice**

To directly examine the GABAergic synaptic transmission, we pharmacologically isolated GABA-mediated miniature postsynaptic currents (mIPSCs) in the presence of TTX to block action potential–driven release and 1 μM strychnine to suppress the glycinergic component (Fig. 2F). Quantification demonstrated that the average frequency of mGPSC...
FIG. 1. Deletion of methyl-CpG-binding protein 2 (MeCP2) gene causes excitatory–inhibitory imbalance in the brain stem respiratory network. A and B: representative ventilation traces (A) and averaged ventilation frequencies (B) of MeCP2-mutant mice (knockout, KO) and their littermate controls (wild-type WT), measured by whole body plethysmography at postnatal day (P) 7. C: representative recordings of spontaneous, pharmacologically isolated [50 μM 6-cyano-7-nitroquinoloxaline-2,3-dione (CNQX) and 20 μM 2-amino-5-phosphonovaleric acid (APV)] inhibitory postsynaptic currents (sIPSCs) from neurons in the ventrolateral medulla at P7. D: averaged amplitude (D) and frequency (E) of sIPSCs in KO and WT neurons. F: representative recordings of spontaneous, pharmacologically isolated (1 μM strychnine and 1 μM bicuculline) excitatory postsynaptic currents (sEPSC) from neurons in the hypoglossus nucleus at P7. G: averaged amplitude (G) and frequency (H) of sEPSCs in KO and WT neurons. Data shown represent means ± SE. Numbers within the bar graphs indicate the number of neurons/mice tested for each genotype.

Presynaptic components of GABAergic synapses are affected in the postnatal ventrolateral medulla of MeCP2 KO mice

The reduction in frequency of miniature GABAergic synaptic events in the MeCP2 KO mice (Fig. 2) may suggest that presynaptic target molecules of the MeCP2-dependent gene regulation exist at these synapses. To independently confirm the presynaptic impairment of GABAergic terminals and to test whether molecules involved in the Ca2+-independent steps of exocytosis are among the putative MeCP2 targets, we evoked charges of miniature GABAergic events (mGSCs) by application of 300 μM sucrose (Rosenmund and Stevens 1996). As evident from representative recordings (Fig. 3A), the frequency of mGSCs induced by the hyperosmolar solution was about threefold higher in neurons of WT mice than that of their MeCP2-deficient littermates (Fig. 3B). Since this difference reflects changes in the readily releasable pool of synaptic vesicles, we screened mRNA levels of a number of synaptic vesicle proteins in quantitative RT-PCR experiments. We found that the mRNA for the vesicular inhibitory transmitter transporter, VIAAT, was significantly lower in the MeCP2 KO mice, whereas levels of molecules present in both types of synaptic terminals (synapsin, synaptotagmin, synaptophysin, synaptobrevin) were unchanged (Table 2). To confirm this observation at the protein level, we performed immunofluorescence staining against VIAAT essentially as described (Varoquaux et al. 2006), using slices containing the ventrolateral medulla of MeCP2 KO mice and their WT littermate controls at P7 (Fig. 3, C–E). Quantification revealed that the area density of VIAAT-positive punctae in the ventrolateral medulla was reduced in MeCP2 KO mice (8.6 ± 0.5 per 100 μm²) compared with that in WT controls (14.9 ± 0.6 per 100 μm²; Fig. 3E), with no differences detectable in the background intensity between genotypes. Since GABA and glycine can be coreleased from the same vesicles (Jonas et al. 1998; Wojcik et al. 2006) and glycineric release was found largely intact (Fig. 2), these data indicate the density of GABAergic synaptic vesicles and/or terminals is specifically reduced in the ventrolateral medulla of MeCP2-deficient mice.

Impaired function and subunit composition of postsynaptic GABA_A receptors in MeCP2 KO mice

In addition to the presynaptic defect described earlier, our observation of reduced amplitudes of miniature inhibitory synaptic transmission in the ventrolateral medulla (Fig. 1A) also raised the possibility of a postsynaptic defect, for example, in the expression of functional receptors. To analyze this aspect of the MeCP2 phenotype, we compared the responses of GABA_A and glycine receptors to extracellular pressure ejec-
tion of the corresponding receptor agonists in the presence of 0.5 μM TTX, 10 μM CNQX, 40 μM APV, and 1 μM strychnine (Fig. 4, A and B) or 1 μM bicuculline (Fig. 4, C and D), respectively. Postsynaptic responses to the GABA_A-receptor agonist muscimol (5 mM) were decreased from 4.9 ± 0.9 nA in WT to 2.2 ± 0.9 nA in mutant neurons from ventrolateral medulla (P < 0.05; Fig. 4B), suggesting an impairment of functional GABA_A receptors. In contrast, the response to glycine (5 mM) was almost identical in both genotypes (WT: 2.7 ± 0.5 nA; KO: 2.5 ± 0.4 nA; Fig. 4D). Interestingly, the absence of changes in glycine-mediated activity might explain the lack of an overt respiratory phenotype because the glycine receptor is essential for respiratory rhythm generation at this developmental age (Richter and Spyer 2001).

Since GABA_A receptors that mediate phasic synaptic inhibition predominantly contain a γ2 subunit in association with one of the α1, α2, or α3 subunits (α1/2/3β2/3γ2), the α subunit present determines the deactivation kinetics of the receptor currents (Farrant and Nusser 2005). Faster decay times as observed here in MeCP2-mutant mice (Fig. 2, I and J) could therefore be explained, for example, by an increased expression of GABA_A receptors that contain α1 subunits (McClellan and Twyman 1999; Vicini et al. 1998). To test this possibility, we used a specific GABA_A-receptor agonist, zolpidem, which shows a high affinity for the GABA_A 1 subunit. In the presence of functional GABA_A receptors containing the α1 subunit, zolpidem prolongs the decay time of miniature events (Perrais and Ropert 1999). Consistent with these studies, addition of 1 μM zolpidem induced a 30% increase in the decay

FIG. 2. GABAergic but not glycinergic miniature postsynaptic currents are decreased in MeCP2 knockout neurons. A: representative recordings of pharmacologically isolated [50 μM CNQX, 20 μM APV, and 0.5 μM tetrodotoxin (TTX)] miniature inhibitory postsynaptic currents (mIPSCs) in neurons from the ventrolateral medulla at P7. B and C: averaged mean frequency (B) and cumulative amplitude (C) of mIPSCs in KO and WT neurons. D: prototypical averaged GABAergic, glycinergic, and mixed mIPSC can be characterized by one (GABAergic with τ_slow, glycinergic with τ_fast) or 2 (mixed) exponential functions, respectively. E: number of GABAergic, glycinergic, and mixed events in WT and KO neurons. F: representative recordings of pharmacologically isolated (50 μM CNQX, 20 μM APV, 0.5 μM TTX, and 1 μM strychnine) miniature GABAergic postsynaptic currents (mGAPSCs) in brain stem mutant and WT neurons. G and H: averaged frequency (G) and cumulative amplitude (H) of mGAPSCs in mutant and WT neurons. I: representative mGAPSCs (averaged from one trace each) from KO and WT mice. J: averaged decay time of mGAPSCs in KO and WT mice, determined with a monoexponential fit as shown in D. Data shown represent means ± SE. Numbers within the bar graphs indicate the number of neurons/mice tested for each genotype.
time of mGPSC in both WT and MeCP2-deficient mice (Fig. 5). Although zolpidem did not cause a change in frequency or amplitude (data not shown), the results indicate that an increased expression of postsynaptic GABA<sub>A</sub> α1 subunit is unlikely to be responsible for the shortening of the decay time in MeCP2-mutant mice.

Since pharmacological means to distinguish between different GABA<sub>A</sub>-receptor subunit compositions are very limited, we directly compared the protein levels of different GABA<sub>A</sub>-receptor subunits and glycine receptors in mutant and WT mice. Quantitative immunoblots of brain stem lysates showed that the relative level of the GABA<sub>A</sub> α2 subunit was decreased by >30% (P < 0.01), whereas the levels of GABA<sub>A</sub> α1, α3, α4, and β3 subunits, and of glycine receptors, were not significantly different between the MeCP2-mutant and the control mice (Fig. 6, A and B). Since other antibodies against additional GABA<sub>A</sub>-receptor subunits were not suitable for analysis of early postnatal brain stem material, their amounts could not be tested at the protein level. However, because MeCP2 is a transcriptional repressor, putative changes in the expression of GABA<sub>A</sub>-receptor subunits should be detectable at the mRNA level in the MeCP2-deficient mice. Using quantitative RT-PCR, we probed for all GABA<sub>A</sub> α, β, and γ subunits as well as glycine receptors subunits. Consistent with immunoblotting experiments, we found a significant decrease in the mRNA of the GABA<sub>A</sub> α2 subunits, whereas the levels of almost all other subunits tested were unchanged (Table 2). Although the two approaches were not identical with respect to the α4 subunit (reduced in mRNA, Table 2; unchanged at protein level, Fig. 6), the shorter decay time of mGPSCs measured in MeCP2-mutant mice (Fig. 2, I and J) can be sufficiently explained by the reduction in GABA<sub>A</sub>-receptor α2 subunits (mRNA, Table 2; protein level, Fig. 6) because this subunit normally confers slower kinetics to GABA<sub>A</sub> receptors (McClellan and Twyman 1999).

**TABLE 1. Kinetics of GABAergic minicurrents in WT and MeCP2 KO neurons**

<table>
<thead>
<tr>
<th></th>
<th>Rise Time (10–90%), ms</th>
<th>Decay Time, ms</th>
<th>Half-Width, ms</th>
<th>Area, pA-ms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>2.6 ± 0.4</td>
<td>26.8 ± 1.8</td>
<td>14.5 ± 1.3</td>
<td>700.5 ± 75.1</td>
</tr>
<tr>
<td><strong>KO</strong></td>
<td>1.8 ± 0.3</td>
<td>20.7 ± 1.2*</td>
<td>11.9 ± 0.8</td>
<td>566.2 ± 88.8</td>
</tr>
</tbody>
</table>

Values are means ± SE, obtained from 11 WT mice and 9 KO mice, respectively. Decay was fitted by a single-exponential function. *P < 0.05 (two-tailed Student’s t-test).

**DISCUSSION**

Rett syndrome appears to be a disorder of synaptic development (Johnston et al. 2001; Zoghbi 2003). This hypothesis is based on the developmentally regulated expression pattern of MeCP2 in different brain regions (Cohen et al. 2003; Mullaney et al. 2004; Shahbazian et al. 2002). In the central nervous systems of humans and mice, the expression of MeCP2 starts during embryonic development and reaches peak levels during synaptogenesis at an early postnatal age (Mullaney et al. 2004). In contrast, both RTT patients and MeCP2-mutant mice apparently show a period of undisturbed postnatal development
before clinical symptoms start to develop (Amir et al. 1999). However, recent studies questioned this hypothesis by pointing out the early appearance of symptoms in both RTT patients (Einspieler et al. 2005a,b; Nomura 2005; Trevarthen and Daniel 2005) and MeCP2-deficient mice (Picker et al. 2006; Santos et al. 2007). To reconcile these observations and to understand the cellular mechanisms that provide a basis for evidence-based therapeutic intervention, it is important to analyze the early events that lead to such a protracted developmental disorder. In the present study, we explored early molecular and cellular impairments that may lead to a life-threatening symptom by investigating the ventrolateral medulla of MeCP2-deficient mice at postnatal day 7 (P7). Such a

### TABLE 2. mRNA levels of synaptic proteins in WT and MeCP2 KO mice

<table>
<thead>
<tr>
<th>mRNA</th>
<th>WT</th>
<th>KO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA α1</td>
<td>0.47 ± 0.18</td>
<td>0.29 ± 0.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>GABA α2</td>
<td>0.65 ± 0.12</td>
<td>0.20 ± 0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GABA α3</td>
<td>0.13 ± 0.06</td>
<td>0.03 ± 0.01</td>
<td>n.s.</td>
</tr>
<tr>
<td>GABA α4</td>
<td>0.24 ± 0.05</td>
<td>0.05 ± 0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GABA α5</td>
<td>1.10 ± 0.39</td>
<td>0.62 ± 0.09</td>
<td>n.s.</td>
</tr>
<tr>
<td>GABA β1</td>
<td>0.82 ± 0.08</td>
<td>0.01 ± 0.003</td>
<td>n.s.</td>
</tr>
<tr>
<td>GABA β2</td>
<td>0.48 ± 0.19</td>
<td>0.13 ± 0.02</td>
<td>n.s.</td>
</tr>
<tr>
<td>GABA β3</td>
<td>0.56 ± 0.22</td>
<td>0.26 ± 0.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>GABA γ1</td>
<td>0.04 ± 0.01</td>
<td>0.003 ± 0.003</td>
<td>n.s.</td>
</tr>
<tr>
<td>GABA γ2</td>
<td>1.65 ± 0.67</td>
<td>0.45 ± 0.37</td>
<td>n.s.</td>
</tr>
<tr>
<td>GABA γ3</td>
<td>0.13 ± 0.06</td>
<td>0.06 ± 0.02</td>
<td>n.s.</td>
</tr>
<tr>
<td>GlyR α1</td>
<td>0.46 ± 0.33</td>
<td>0.19 ± 0.10</td>
<td>n.s.</td>
</tr>
<tr>
<td>GlyR α2</td>
<td>0.73 ± 0.35</td>
<td>0.34 ± 0.16</td>
<td>n.s.</td>
</tr>
<tr>
<td>Synaptotagmin</td>
<td>1.64 ± 0.81</td>
<td>1.26 ± 0.45</td>
<td>n.s.</td>
</tr>
<tr>
<td>Synapsin</td>
<td>1.70 ± 1.15</td>
<td>2.56 ± 1.65</td>
<td>n.s.</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>2.89 ± 2.07</td>
<td>2.95 ± 2.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>Synaptobrevin</td>
<td>0.37 ± 0.29</td>
<td>0.29 ± 0.14</td>
<td>n.s.</td>
</tr>
<tr>
<td>VIAAT</td>
<td>5.93 ± 1.36</td>
<td>2.18 ± 0.61</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Gephyrin</td>
<td>1.77 ± 0.66</td>
<td>0.81 ± 0.27</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Values are means ± SE and represent percentage of β-actin levels; n = 6 mice were used for each genotype. Relative mRNA levels were quantified by an RT-PCR strategy. Significance level was tested by a two-tailed Student’s t-test; n.s., not significant.

FIG. 4. The GABA-dependent but not the glycine-mediated postsynaptic receptor response is reduced in MeCP2 knockout mice. A and B: GABA-mediated postsynaptic currents. Sample traces (A) and averaged amplitude (B) of postsynaptic responses to pressure-applied GABA agonist muscimol (5 mM) in KO and littermate WT neurons in the ventrolateral medulla at P7. C and D: glycine-mediated postsynaptic currents. Sample traces (C) and averaged amplitude (D) of responses to pressure-applied glycine (5 mM) in KO and WT neurons. Data shown represent means ± SE. Numbers within the bar graphs indicate the number of neurons/mice tested for each genotype.

FIG. 5. GABAA-receptor subunit α1 pharmacology is not altered in absence of MeCP2. A: representative traces of mGPSCs before and after zolpidem (1 μM) application in WT and KO neurons from the ventrolateral medulla at P7. B: averaged mGPSC currents from WT KO neurons before (black) and after (gray) application of 1 μM zolpidem. C: averaged decay prolongation induced by zolpidem in WT and KO neurons fails to detect differences in GABAA-α1-mediated responses between genotypes. Data shown represent means ± SE. Numbers within the bar graphs indicate the number of mice/neurons tested for each genotype.

FIG. 6. Protein levels of the GABAα2-receptor subunit are specifically decreased in MeCP2 knockout mice. A: representative immunoblots of GABAα2-receptor subunits α1, α2, α3, α4, and β3, glycine receptor (GlyR), and heat shock protein Hsp70 as input control. B: quantitative analysis of the protein levels of GABAα2 and glycine receptor subunits in brain stem lysates of littermate control (open bars, WT) and MeCP2-deficient mice (closed bars, KO) at P7. Data shown represent means ± SE. Numbers within the bar graphs indicate the number of mice used for lysates.
strategy appears prudent because, first, RTT patients suffer from severe ventilation dysfunction (Hanefeld et al. 1986) that can lead to sudden lethal apnea (Kerr and Burford 2001) and, second, the neuronal network for respiratory control is fully functional because the formation and maturation of synapses in this brain stem area are more advanced than in other brain regions at this age (Richter and Spyer 2001). It thus appeared likely that the first signs for MeCP2-related network disturbance would be present in the brain stem respiratory network.

The experiments in the present study were performed on unidentified neurons within the ventrolateral medulla that contains both respiratory and nonrespiratory neurons. It is quite likely that the results of the present study do apply for a respiratory rhythm-generating network. More detailed work will be required to ascertain whether the present findings apply to the cellular mechanisms of the respiratory rhythm generation. Nevertheless, our data demonstrate that inhibitory and excitatory network activity in the ventrolateral medulla is already altered in early postnatal mutant mice (Fig. 1), although no obvious impairment in the overall ventilation activity was evident at this age (Viemari et al. 2005). The absence of overt respiratory problems may be surprising with respect to such a strong cellular phenotype, although the respiratory network is known to be one of the most robust networks due to the redundant assembly of its connectivity that confers stability against disturbances (Feldman and Del Negro 2006; Richter and Spyer 2001). Consistent with this stability, our previous studies of neurexin triple KO mice (Missler et al. 2003; Varoqueaux et al. 2006) and neuroligin triple KO mice (Missler et al. 2003; Varoqueaux et al. 2006) demonstrated that a reduction of inhibitory network activity of ≥50% was necessary to cause first visible irregularities in the resting ventilation activity, and only a reduction of >75% caused a life-threatening failure of ventilation. Therefore a moderate reduction of <40% of inhibitory network activity as reported in the present study of MeCP2-deficient mice is not expected to change resting ventilation activity but may have an effect on the adaptability of the respiratory network during stressful activities. Indeed, the resting ventilation activity is strongly impaired in adult MeCP2-deficient mice (Stettner et al. 2007; Viemari et al. 2005), at least partly due to impaired synaptic function (Stettner et al. 2007).

Published data on impairments of synaptic transmission in MeCP2-deficient mice are controversial with respect to the components affected: in the hippocampus, loss of MeCP2 caused enhanced excitatory transmission (Moretti et al. 2006), and reduced paired-pulse facilitation and long-term potentiation (Asaka et al. 2006; Moretti et al. 2006), whereas Dani et al. (2005) showed that MeCP2 KO mice present a reduction of excitatory activity and an increase of spontaneous but not miniature IPSC in neocortical layer 5 neurons. Additionally, in hippocampal cultures from MeCP2 KO mice, the frequency of miniature EPSCs is decreased (Nelson et al. 2006), with no effect on either the frequency or the amplitude of mIPSCs (Nelson et al. 2006). Although the divergent results may reflect the different brain regions and ages investigated, these studies uniformly emphasized the general importance of the balance between inhibitory and excitatory synaptic transmission for the etiology of RTT, and is confirmed by our current findings in the brain stem (Fig. 1). Extending the previously published data, we now show that it is specifically the GABA A receptor that is responsible for the glycine-mediated component of inhibitory synaptic transmission that is significantly compromised in MeCP2 mutants (Fig. 2). Furthermore, we demonstrate here for the first time that the impaired GABAergic synaptic transmission is due to a reduction of specific presynaptic (Fig. 3, Table 2) and postsynaptic components (Figs. 4 and 6, Table 2) in MeCP2 KO mice. Our findings of reduced levels of VIAAT and GABA A receptor subunits in the MeCP2-mutant mice suggest a role for MeCP2 in the regulation of genes important for the formation, function, and maintenance of synapses. These data are in line with recently published data that MeCP2 might play a key role in regulating glutamatergic synapse formation in early postnatal development (Chao et al. 2007). Furthermore, our current results are consistent with the demonstration that brain-derived neurotrophic factor (BDNF) is a target of MeCP2 transcriptional repression (Chang et al. 2006) because BDNF promotes GABAergic synaptogenesis (Carrasco et al. 2007; Marty et al. 2000; Yamada et al. 2002), and the BDNF receptors appear to be abundant in the ventrolateral medulla of neonatal mice (Thoby-Brisson et al. 2003).

The growing brain and its numerous synapses undergo tremendous changes in their molecular composition during postnatal development. The maturation of synapses depends on switches of distinct subunits of many receptor and channel classes; e.g., in the thalamus and cortex of rats the GABA A receptor subunits undergo a change from α2 (or α3) to α1 during postnatal development (Bosman et al. 2002; Fritschi et al. 1994). Here, we found that in MeCP2-deficient mice the expression of the α2 subunit of GABA A receptors in the ventrolateral medulla was significantly decreased, as shown independently by immunoblotting and quantitative RT-PCR experiments (Fig. 6, Table 2). GABA A receptors generally consist of two α and two β subunits and one γ subunit (Baumann et al. 2001; Farrant and Nusser 2005). The α2 subunit is very abundant in early postnatal ages in many brain regions (Laurie et al. 1992; Liu and Wong-Riley 2004) and is subsequently downregulated in the adult. However, in regions like hippocampus, neocortex, or midbrain, postnatal α2 expression does not decrease and is maintained at the same level in adult life (Laurie et al. 1992; Liu and Wong-Riley 2004). Interestingly, changes in GABA A receptor subunit composition can be correlated with an alteration of binding kinetics and allosteric properties of the GABA A receptor (Okada et al. 2000). The decrease in expression of the GABA A α2 subunit observed in our study (Fig. 6, Table 2) could explain the changes in the decay time of mGPSCs (Fig. 4), whereas the alternative explanation of a changed expression of the GABA A α1 subunit was not validated (Figs. 5 and 6, Table 2). Our results on altered GABA A receptor composition in MeCP2 knockout mice are consistent with decreased benzodiazepine receptor binding in brains of RTT patients (Yamashita et al. 1998). However, another recent study reported a downregulation in the GABA A β3 subunit expression levels in both RTT patients and two strains of MeCP2-deficient mice (Samaco et al. 2005), which was not altered in our study. Our observation of a changed expression of the α2 subunit of the GABA A receptor in MeCP2 KO mice may be relevant to symptoms in RTT patients: the α2-containing GABA A receptors are expressed in synapses located mainly on the axon initial segment (Brunig et al. 2002; Cruz et al. 2003) of the reticular activating system, limbic system, amygdale, and hippocampus (Rudolph
and Mohler 2000), where it mediates the anxiolytic action of benzodiazepines (Low et al. 2000). These data suggest that α2-containing GABA receptors are crucially involved in processing of emotional stimuli (Rudolph and Mohler 2004) and its downregulation, as reported here, could provide an explanation of the autism-like symptoms of Rett syndrome patients.

Unlike brain diseases caused by sudden lesions, the clinical symptoms of developmental disorders result from complex interactions among many brain regions, networks, and proteins. It is therefore mandatory to study the initial events of the maintenance of inhibitory and excitatory synaptic balance at a very early developmental stage. If those changes observed in a mouse model also apply to human RTT patients, a very early diagnosis and therapeutic intervention might be essential to halt or reverse the inexorable course of the disease.

ACKNOWLEDGMENTS

We thank C. Hüneke and S. Gerke for excellent technical assistance and R. Fairless for critical reading of the manuscript.

GRANTS

This work was supported by Deutsche Forschungsgemeinschaft (DFG) grants through the DFG Research Center for Molecular Physiology of Brain to W. Zhang and M. Missler and Lichtenberg Stipends of the State of Lower-Saxony to L. Medrihan and I. Dudaonova.

REFERENCES


Carrasco MA, Castro P, Sepulveda FJ, Tapia JC, Gatica K, Davis MI, Cohen DR, Matarazzo V, Palmer AM, Tu Y, Jeon OH, Pevsner J, Ronnett Chen RZ, Akbarian S, Tudor M, Jaenisch R. Proper maturation of GABAA synaptic transmission and for the natal MeCP2 knockout mice demonstrated for the first time proteins. It is therefore mandatory to study the initial events of interactions among many brain regions, networks, and proteins. It is therefore mandatory to study the initial events of

Mol Cell Neurosci


Downloaded from http://jn.physiology.org/ by 10.220.33.1 on May 20, 2017


