Neurobiology of Disease 48 (2012) 102–114

Contents lists available at SciVerse ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Oxidative burden and mitochondrial dysfunction in a mouse model of Rett syndrome

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ARTICLE INFO

Article history:
Received 10 November 2011
Revised 22 May 2012
Accepted 22 June 2012
Available online 30 June 2012

Keywords:
Oxidative stress
Redox imbalance
Optical imaging
roGFP
Reactive oxygen species (ROS)
Microarray
Autofluorescence
Radical scavenger
Neurodevelopmental disorder
Synaptopathy

ABSTRACT

Rett syndrome is an X chromosome-linked neurodevelopmental disorder associated with cognitive impairment, motor dysfunction and breathing irregularities causing intermittent hypoxia. Evidence for impaired mitochondrial function is also accumulating. A subunit of complex III is among the potentially dys-regulated genes, the inner mitochondrial membrane is leaking protons, brain ATP levels seem reduced, and Rett patient blood samples confirm increased oxidative damage. We therefore screened for mitochondrial dysfunction and impaired redox balance. In hippocampal slices of a Rett mouse model (*Mecp2*$^{-/y}$) we detected an increased FAD/NADH baseline-ratio indicating intensified oxidization. Cyanide-induced anoxia caused similar decreases in FAD/NADH ratio and mitochondrial membrane potential in both genotypes, but *Mecp2*$^{-/y}$ mitochondria seemed less polarized. Quantifying cytosolic redox balance with the genetically-encoded optical probe roGFP1 confirmed more oxidized baseline conditions, a more vulnerable redox-balance, and more intense responses of *Mecp2*$^{-/y}$ hippocampus to oxidative challenge and mitochondrial impairment. Trolox treatment improved the redox baseline of *Mecp2*$^{-/y}$ hippocampus and dampened its exaggerated responses to oxidative challenge. Microarray analysis of the hippocampal CA1 subfield did not detect alterations of key mitochondrial enzymes or scavenging systems. Yet, quantitative PCR confirmed a moderate upregulation of superoxide dismutase 1 in *Mecp2*$^{-/y}$ hippocampus, which might be a compensatory response to the increased oxidative burden. Since several receptors and ion-channels are redox-modulated, the mitochondrial and redox changes which already manifest in neonates could contribute to the hyperexcitability and diminished synaptic plasticity in MeCP2 deficiency. Therefore, targeting cellular redox balance might qualify as a potential pharmacotherapeutic approach to improve neuronal network function in Rett syndrome.

Introduction

Rett syndrome is a neurodevelopmental disorder that arises from spontaneous mutations in the X-chromosomal *MECP2* gene, encoding the transcriptional regulator methyl CpG binding protein 2 (MeCP2) (Amir et al., 1999; Chahrour et al., 2008). Such mutations are usually lethal or result in very severe disabilities in boys (Villard, 2007). Female patients show normal development for the first 6–16 months of their lives, then developmental stagnation and regression occur which are characterized by severe breathing problems, motor dysfunction, loss of speech, autistic features, cognitive impairment and epilepsy (Chahrour and Zoghbi, 2007; Hagberg et al., 1983; Percy, 2002; Steffenburg et al., 2001). About 25% of Rett syndrome patients are threatened by sudden death arising from a severe failure of cardio-respiratory control (Julu et al., 2001; Kerr et al., 1997).

Based on the genetic cause of this disorder, a variety of mouse models for Rett syndrome has been developed, which carry either large deletions, truncations or nonsense mutations in the *MECP2* gene (Brendel et al., 2011; Guy et al., 2001; Shahbazian et al., 2002). Rett patients and mouse models suffer from highly irregular breathing with frequent episodes of apneas (breath holds), which results in marked drops of arterial oxygen (O_2) saturation, i.e., intermittent systemic hypoxia (Julu et al., 2001; Katz et al., 2009; Stettner et al., 2008). Such repetitive hypoxic stimuli could potentially induce

Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; ACSF, artificial cerebrospinal fluid; ATZ, 3-amino-1,2,4-triazole; BSO, buthionine sulphoximine; CA1, *cornu ammonis*, subfield 1; CN^-, cyanide; DEDTC, diethyldithiocarbamic acid; DIV, days in vitro; DMSO, dimethyl sulfoxide; DPI, diphenyleneiodonium; DTT, 1,4-dithio-DL-threitol; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; FCS, fetal calf serum; FDR, false discovery rate; H_2O_2, hydrogen peroxide; L-NMMA, N^G-methyl-L-arginine acetate; MeCP2, methyl CpG binding protein 2; *Mecp2*, methyl CpG binding protein 2 encoding gene; *Mecp2*$^{-/y}$, *MeCP2 deficient male mouse*; NO, nitric oxide; Rh123, rhodamine 123; roGFP1, reduction–oxidation sensitive green fluorescent protein 1; ROS, reactive oxygen species; SH, sulfhydryl; SOD, superoxide dismutase; *st.*, *stratum*; WT, wildtype.

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doi:10.1016/j.nbd.2012.06.007

preconditioning and/or neuronal adaptation to intermittent hypoxia, but we and also others rather found an increased hypoxia susceptibility of the hippocampus and brainstem of MeCP2-deficient mice (Fischer et al., 2009; Kron and Müller, 2010; Kron et al., 2011; Mironov et al., 2009).

Elucidating the detailed underlying molecular mechanisms for the increased hypoxia susceptibility in hippocampal CA1 neurons, we detected a dysfunction of K^+ channels and a disturbed cellular Ca^{2+} homeostasis (Fischer et al., 2009; Kron and Müller, 2010). However, mitochondrial impairment could also potentially contribute to the increased hypoxia susceptibility. Mitochondria are the primary cellular consumer of O_2, and besides providing ATP they are involved in Ca^{2+} homeostasis and constitute a major source of reactive oxygen species (ROS). Hence, O_2 shortage arising from respiratory irregularities may directly affect mitochondrial function and cellular energy supply. Indeed, in Rett patients, fluctuations in blood lactate levels were observed (Haas et al., 1995), which may be a consequence of the respiratory irregularities.

There are very clear indications for morphological alterations of mitochondria in Rett syndrome. Muscle and frontal lobe biopsies of Rett patients revealed swollen mitochondria with vacuolization, granular inclusions and membraneous changes (Cornford et al., 1994; Eeg-Olofsson et al., 1990; Ruch et al., 1989). Such ultrastructural changes have also been confirmed for cortical and hippocampal mitochondria of *Mecp2*$^{-/y}$ mice (Belichenko et al., 2009). Furthermore, there is convincing evidence for a disturbed function of the mitochondria. Biochemical analyses on muscle and frontal cortex biopsies of Rett patients showed lower levels especially of cytochrome c oxidase and succinate cytochrome c reductase (Coker and Melnyk, 1991; Gibson et al., 2010). Also, a subunit of complex III of the respiratory chain seems to be among those genes being controlled by MeCP2 and a proton leak across the inner mitochondrial membrane has been proposed (Kriaucionis et al., 2006). In consequence, mitochondrial respiration would be less efficient, leading to reduced brain ATP levels as detected in a magnetic resonance study on MeCP2-deficient mice (Saywell et al., 2006).

Since mitochondria are a major source of superoxide and ROS output is directly linked to mitochondrial activity (Boveris and Chance, 1973), an increased basal mitochondrial respiration should lead to intensified ROS formation and consequently an imbalance in cellular redox status. There is indeed supportive evidence for such an oxidative burden in Rett syndrome. Blood samples of patients revealed a reduced activity of the scavenging enzyme superoxide dismutase (SOD) (Sierra et al., 2001) as well as decreased vitamin E levels (Formichi et al., 1998). Furthermore, the increased plasma and intraerythrocyte free iron levels, plasma protein carbonyl levels, plasma malondialdehyde levels, and F2-isoprostanes indicate an intensified lipid and protein oxidation in Rett syndrome (De Felice et al., 2009; Sierra et al., 2001).

Even though such mitochondrial defects could contribute to and potentially worsen the neuronal dysfunction in Rett syndrome, detailed studies on mitochondrial function and especially cytosolic redox changes are lacking so far. In the present study, we therefore assessed mitochondrial function in acute and organotypic hippocampal tissue slices from neonatal as well as adult *Mecp2*$^{-/y}$ mice. Mitochondrial metabolism and polarization were evaluated by optical recordings of cellular NADH and FAD autofluorescence and rhodamine 123 (Rh123) fluorescence, respectively. Furthermore, for the first time, we obtained direct evidence for an increased oxidative burden in Rett syndrome by taking advantage of the genetically-engineered optical redox probe roGFP1 (reduction–oxidation sensitive green fluorescent protein 1). Dynamic and semi-quantitative monitoring of cytosolic redox changes and intracellular ROS formation unequivocally confirmed an increased oxidative burden. A more vulnerable cellular redox balance was detected for baseline conditions as well as in response to oxidative challenge and mitochondrial inhibition. A genetic microarray screen did not indicate a differential expression of key mitochondrial or scavenging enzymes. Yet, quantitative PCR confirmed a moderate upregulation of SOD1 in *Mecp2*$^{-/y}$ hippocampus.

Material and methods

Preparation

As a mouse model for Rett syndrome we chose mice lacking the *MECP2* gene [B6.129P2(C)-*Mecp*$^{2tm-1-1Bird}$ (Guy et al., 2001)]. All experiments were performed on isolated brain tissue; the animal dissection procedures and anesthesia were in accordance with local regulations and approved by the Office of Animal Welfare of the University of Göttingen. Acute hippocampal tissue slices were prepared from either adult (>p45) or neonatal (p7–10) male mice as described in detail earlier (Fischer et al., 2009; Kron and Müller, 2010). In brief, ether anesthetized mice were decapitated, the brain was rapidly isolated and incubated in ice-cold artificial cerebrospinal fluid (ACSF) for 1–2 min. ACSF contained (in mM) 130 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 1.2 $CaCl_2$, 1.2 $MgSO_4$, and 10 dextrose; aerated with carbogen (95% O_2–5% CO_2) to adjust the pH to 7.4 (chemicals, unless stated otherwise, were obtained from Sigma-Aldrich). Transverse cortico-hippocampal slices of 400 µm thickness were cut using a vibroslicer (752 M Vibroslice, Campden Instruments). Slices were separated along the sagittal midline, the hippocampal formations were isolated, transferred to a storage chamber (room temperature, constantly aerated with carbogen) and allowed to rest for at least 90 min.

Organotypic hippocampal slice cultures were prepared from neonatal male mice (usually p5–6, range 2–12 days old) according to the Stoppini method (Funke et al., 2011; Stoppini et al., 1991). Upon dissection, the isolated hippocampi were cut into 350 µm-thick slices using a McIlwain tissue chopper (Stoelting Co.). The slices were then transferred onto the porous membranes of culture plates (Transwell Permeable Support, Corning), and incubated under interface conditions (37 °C, 5% CO_2-atmosphere). Minimum essential medium (Invitrogen) also contained 5% FCS (fetal calf serum), 5 mg/ml glucose, 0.2 mg/ml $NaHCO_3$, 0.1 mg/ml transferrin (Calbiochem), 30 mg/ml L-glutamine, 20 µl/ml B-27 50× supplement (Invitrogen), and 100 µg/ml penicillin–streptomycin (Biochrom). Every 2–3 days 50% of the medium was refreshed, and slice cultures were taken into the experiments within 5–10 days in vitro (DIV). The B-27 supplement used contains antioxidants. Therefore, in a few explicitly stated trials we used B-27 supplement without antioxidants, to elucidate its potential modulatory effect on baseline redox balance and the cellular responses to acute oxidizing stimuli.

Transfection procedures

The pEGFP-N1/roGFP1 plasmid vector expressing roGFP1 was kindly provided by S. J. Remington, University of Oregon, USA. On DIV 1–4 the slice cultures were transiently transfected with this vector using lipofectamine 2000 (Invitrogen). A volume of 370 µl transfection solution (OptiMEM, Invitrogen) was complemented with 0.4% lipofectamine and 1.1 µg DNA, and 30–50 µl of this solution was topically applied onto each slice. The transfection efficiency was rather low (<1%) but still a number of cells per slice showed sufficient roGFP1 expression levels within 48 h after transfection.

Solutions

DPI (diphenyleneiodonium, Tocris), FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, Tocris), indomethacin (Tocris), Rh123 and rotenone were dissolved in dimethyl sulfoxide (DMSO) as 10–50 mM stocks, and kept refrigerated (4 °C); final DMSO concentrations were ≤0.05%. Antimycin A was dissolved in absolute ethanol (20 mM stock) and kept in the freezer. Allopurinol, ascorbic acid, DEDTC (diethyldithiocarbamic acid), DTT (1,4-dithio-DL-threitol, Fluka), H_2O_2 (hydrogen peroxide, 30% aqueous stock solution) and Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] were directly added to the respective solutions in the final concentrations. Cyanide (CN^-, sodium salt) and 3-amino-1,2,4-triazole (ATZ) were dissolved