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Epigenetic modifications of Dexras 1 along the nNOS pathway in an animal model of multiple sclerosis

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ABSTRACT

The development of multiple sclerosis, a major neurodegenerative disease, is due to both genetic and environmental factors that might trigger aberrant epigenetic changes of the genome. In this study, we analysed global DNA methylation in the brain of mice upon induction of experimental autoimmune encephalomyelitis (EAE), and the effect of environmental enrichment (EE). We demonstrate that global DNA methylation decreased in the striatum, but not in the cortex, of EAE mice compared to healthy controls, in particular in neuronal nitric oxide synthase (nNOS)-positive interneurons of this brain area. Also, in the striatum but again not in the cortex, decreased DNA methylation of the nNOS downstream effector, dexamethasone-induced Ras protein 1 (Dexras 1), was observed in EAE mice, and was paralleled by an increase in its mRNA.
Interestingly, EE was able to revert EAE effects on mRNA expression and DNA methylation levels of Dexras 1 and reduced gene expression of nNOS and 5-lipoxygenase (Alox5). Conversely, interleukin-1β (IL-1β) gene expression was found up-regulated in EAE mice compared to controls and was not affected by EE. Taken together, these data demonstrate an unprecedented epigenetic modulation of nNOS-signaling in the pathogenesis of multiple sclerosis, and show that EE can specifically revert EAE effects on Dexras 1 along this pathway.
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1. Introduction

Multiple sclerosis (MS) is a common disorder of the central nervous system (CNS), characterized by inflammation, demyelination, and axonal degeneration (Noseworthy et al., 2000). Recently, it has been shown that basal ganglia are vulnerable to the MS-associated neurodegeneration (Tao et al., 2009; Audoin et al., 2010; Batista et al., 2012). Specifically in experimental autoimmune encephalomyelitis (EAE), one of the best known rodent models of MS, the nucleus striatum is one of the most affected brain region (Pitt et al., 2003; Centonze et al., 2009). Both genetic background and environmental factors represent critical contributors to MS aetiology (Ramagopalan et al., 2008). Genome-wide association studies have identified more than 20 *loci* linked to MS susceptibility (De Jager et al., 2009), and accumulated evidence has pointed to a possible involvement of multiple genes in disease onset and progression. Among these, genes of immune or inflammatory response, of oxidative stress and metabolic pathways, as well as markers of CNS functionality have been proposed so far (Tajouri et al., 2007).

Environment may, through epigenetic mechanisms, further influence gene function, thus affecting disease progression. For instance, it should be recalled that in different rodent models of neurodegenerative diseases, environmental enrichment (EE) protocols have been shown to delay appearance and mitigate progression of neurological deficits (Rossi et al., 2009). Notably, the beneficial effect of exercise on disease severity in EAE was associated with the rescue of striatal dendritic spine density and synaptic transmission abnormalities (Rossi et al., 2009), described to represent early alterations in this reliable model of MS (Centonze et al., 2007, 2009).

Abbreviations: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; EE, environmental enrichment; nNOS, neuronal nitric oxide synthase; Dexras 1, dexamethasone-induced Ras protein 1; Alox5, 5-lipoxygenase; IL-1β, interleukin-1β; CNS, central nervous system; NO, nitric oxide; SAM, *S*-adenosylmethionine; MOG, myelin oligodendrocyte glycoprotein; DARPP32, dopamine and cyclic AMP-regulated phosphoprotein 32 kDa; 5-MeC, 5-methylcytosine; SE, standard environment; β-ACT, β-actin.

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Table 1
Primers used for PCR amplification and DNA methylation.

Human gene	Forward primer (5′ → 3′)	Reverse primer (3′ → 5′)
nNOS	agcacgctggaaacggggtg	ctgcccacacagcgagaggc
Dexras1	atctcagccaagaagaacagc	atgtacatgaggtcgctgtgc
Alox5	cggggcaaaaaggcctcaggttt	ggtggggcccgcatagttgg
IL-1β	acggaccccaaaagatgaagggct	gggaacgtcacacaccagcagg
β-ACT	tgttaccaactgggacga	gtctcaaacatgatctgggtc
nNOS M	acgtttataaattagtcggttggac	aaaactaccaacttcccctacg
nNOS U	tgtttataaattagttggttggatgt	taaaaactaccaacttcccctaca
Dexras 1 M	taaaggtatagttaggatttttgcg	actaaaatacgtcacgtcccg
Dexras 1 U	aaaggtatagttaggatttttgtgg	aactaaaatacatcacatcccacc
Alox5 M	gttaagaagttggtgggttgttac	taccttatccaacaaatacttctcg
Alox5 U	gttaagaagttggtgggttgttat	ccttatccaacaaatacttctcact
IL-1β M	ttttagtttaagtataagaggcga	acacattcgcaaatatatcatcgta
IL-1β U	ttttagtttaagtataaggaggtga	acacattcacaaatatatcatcata
β-ACT NoCpG	ggtattgttgataggatgtagaagga	tctaaatactaaaattccccttaaacc

Epigenetic mechanisms can evoke transient changes in gene expression that involve chemical modifications (*e.g.*, DNA methylation and histone modifications), without affecting DNA sequence. Thus, gene expression is a somewhat transient process, and how long it actually lasts depends on the specific context (Jaenisch and Bird, 2003). Environment and heritable factors certainly contribute to individual vulnerability to disease development, although it remains largely unexplored how they mutually interact. Recently, molecular research has paved the way to understand how these factors may facilitate MS progression, pointing indeed to a major role for epigenetic regulation (Kürtüncü and Tüzün, 2008). For instance, in the context of MS it has been recently suggested that epigenetic drugs could be used to treat patients (Mangano et al., 2014). In line with this, epigenomic changes in genes affecting oligodendrocyte susceptibility to damage have been detected in pathology-free areas of MS-affected brains (Huynh et al., 2014).

Also epidemiological studies have suggested a role for environmental factors (geographic location, month of birth, nutritional status, and smoking) in MS development (Ebers, 2008), but evidence for the underlying epigenetic mechanisms is still missing.

Excessive production of nitric oxide (NO) has been implicated in neuronal cell death and demyelination in different CNS diseases, including MS. In line with this, neuronal nitric oxide synthase (nNOS) has been proposed to be directly involved in MS pathophysiology (Liñares et al., 2006), where it might drive neuronal cell death and demyelination *via* NO signaling (Gao et al., 2010).

Dexamethasone-induced Ras protein 1 (Dexras 1), a downstream effector of nNOS signaling, is a brain-enriched member of the Ras subfamily of guanosine triphosphatases (Li et al., 2008; Shen et al., 2008), whose expression has been found to be altered in EAE rats (Gao et al., 2010). On this basis, we analysed Dexras 1 DNA methylation. We also investigated DNA methylation of the genes encoding for additional players in MS, such as 5-lipoxygenase (Alox5) and interleukin-1β (IL-1β). Indeed, the expression of Alox5, the gene that encodes for a key-enzyme in the biosynthesis of pro-inflammatory leukotrienes such as 5-lipoxygenase, increases in EAE brains (Whitney et al., 2001), as well as in blood samples from relapsing-remitting MS patients (Arthur et al., 2008). Addictionally, also genes encoding for cytokines are candidates to study disease susceptibility, due to the autoimmune nature of MS. Among them, a distinct role for IL-1 family (and for IL-1β in particular) has been clearly documented in MS (Hooper-van Veen et al., 2003).

Against this background, here we sought to: *i*) analyze DNA methylation upon MS, both globally and in selected brain regions of EAE mice: striatum, an area critically affected by MS (Centonze et al., 2009; Hasan et al., 2009; Hannoun et al., 2012) and highly responsive to the effects on environmental stimulations in EAE (Rossi et al., 2009); and cortex, that can be considered an unaffected control where EE does not induce a reduction of synaptic defects (Rossi et al., 2009; Centonze et al., 2007); and *ii*) investigate how EE could impact on chromatin remodelling in the striata of EAE and control mice. To this end we studied global DNA methylation and epigenetic regulation of the selected MS-related genes, nNOS, Dexras 1, Alox5 and IL-1β.

2. Materials and methods

2.1. Chemicals

All chemicals were of the purest analytical grade. Pertussin toxin and Fluoromount were from Sigma Chemical (St. Louis, MO, USA). *S*-Adenosyl-*L*-[methyl-^{3}H] methionine ([^{3}H]-SAM, 63 Ci/mmol) was from Amersham Biosciences (Buckinghamshire, UK), SssI methylases and non-radioactive *S*-adenosylmethionine (SAM) from New England Biolabs (Ipswich, MA, USA). RNeasy extraction kit, QuantiTect Reverse Transcription Kit and QuantiFast Multiplex PCR Kit from Qiagen (Crawley, UK). Myelin oligodendrocyte glycoprotein (MOG, 35–55; > 85% purity) was from Espikem (Florence, Italy). *Mycobacterium tuberculosis* (strain H37Ra) was from Difco (Lawrence, KS, USA). Rabbit anti-dopamine and cyclic AMP-regulated phosphoprotein 32 kDa (DARPP32; #Ab-10518) antibody was from Millipore, mouse monoclonal antibody against 5-methylcytosine (5-MeC; #MAb-006-100) was from Diagenode (Liège, Belgium), and rabbit anti-neuronal nitric oxide synthase (nNOS; #SAB4502010) antibody was from Sigma. Cy2 and Cy3-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA, USA).

2.2. EAE induction and clinical evaluation

EAE was induced in 6–8 weeks C57/BL6 female mice (Jackson Laboratory), as previously described (Grasselli et al., 2013). Briefly, 200 μg MOG was administrated by three sub-cutaneous injections of incomplete Freund's adjuvant (100 μl each), containing 8 mg/ml *Mycobacterium tuberculosis* (strain H37Ra). The three injections were made under deep anesthesia in the sacral area and in the lateral thoracic areas in the same

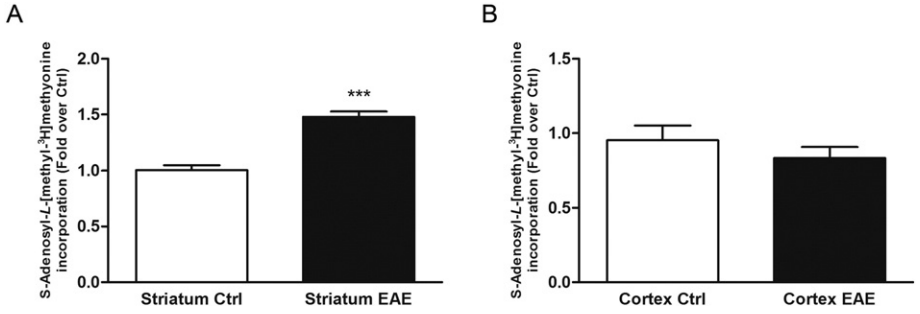

Fig. 1. Methylation levels of genomic DNA (A) in the striatum and (B) in the cortex of Ctrl and EAE mice in SE. Methylation levels of genomic DNA were measured by methyl-accepting assay with CpG methylase SssI, in the presence of *S*-adenosyl-L-[methyl-^{3}H]-methyonine (see Materials and Methods for details). Higher levels of [^{3}H] methyl group incorporated into DNA indicated lower level of genomic DNA methylation. Values are expressed as mean ± standard error of the mean of 8–11 mice. Student's *t*-test. ***$p < 0.001$ *versus* Ctrl.

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