Journal of Neuroimmunology 262 (2013) 11–18

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

Complexity of trophic factor signaling in experimental autoimmune encephalomyelitis: Differential expression of neurotrophic and gliotrophic factors

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ARTICLE INFO

Article history:
Received 18 December 2012
Received in revised form 22 March 2013
Accepted 17 May 2013

Keywords:
Experimental autoimmune encephalomyelitis
Multiple sclerosis
BDNF and TrkB tyrosine kinase receptor
Neuregulin1
Glatiramer acetate

ABSTRACT

Soluble factors that promote survival and differentiation of glia and neurons during development are likely to play key roles in neurodegeneration and demyelinating diseases such as multiple sclerosis (MS) and have the potential to be important therapeutic targets. We examined the effect of TrkB signaling and the expression patterns of neurotrophic and gliotrophic factors in the mouse brain in MOG-induced experimental allergic encephalomyelitis (EAE). With induction of mild disease, TrkB heterozygous mice were more severely affected compared to their wild type littermates. However, with more potent disease induction, TrkB heterozygotes fared similar to their wild type littermates, suggesting complex modulatory roles for TrkB signaling. One possible explanation for this difference is that the expression patterns of neurotrophic factors correlate with disease severity in individual mice with mild disease, but not in more severe disease. With the less potent induction in C57BL/6 mice, we found that BDNF was consistently increased at EAE onset, while the soluble gliotrophic factor neuregulin (NRG1) was increased only in the chronic phase of the disease. Treatment of these animals with glatiramer acetate (GA) to decrease disease severity resulted in lower levels of both BDNF and NRG1 expression in some mice at 35 days after immunization compared to those in untreated EAE mice, but had no direct effect on these factors in the absence of EAE. Our results suggest a complex interplay between neurotrophic and gliotrophic factors in EAE that is dependent on disease stage and severity. While signaling by BDNF through TrkB is protective in mild disease, this effect was not seen in more severe disease. The late induction of NRG1 in the chronic stage of disease could also worsen disease severity through its known ability to activate microglial, inflammatory pathways. While complex, these studies begin to define underlying axoglial trophic activities that are likely involved in both disease pathogenesis and repair.

1. Introduction

Based on the efficacy of immunomodulatory therapies in patients with MS, much of the research and thinking about pathogenesis and treatment have traditionally focused on suppressing or modulating the immune system. One of the most successful therapeutic strategies in combating this disorder is a mixture of basic peptides, randomly synthesized at specific molar ratios, called glatiramer acetate (GA) (Ragheb et al., 2001; Kieseier and Stuve, 2011; Lalive et al., 2011; Racke and Lovett-Racke, 2011). Recent reports suggest that in addition to its immunoregulatory anti-inflammatory effects, GA may also have neuroprotective effects and that this protection may be conferred through the local expression of neurotrophic factors such as BDNF in the brain (Aharoni et al., 2005). BDNF is involved in neuronal survival, differentiation, and function, as well as glial development in the central nervous system (CNS) (Chao, 2003). BDNF and its receptor truncated TrkB tyrosine kinase receptor (gp145trkB) were found in lesions of MS patients (Stadelmann et al., 2002) and also in EAE (Aharoni et al., 2005; De Santi et al., 2009; Colombo et al., 2012). The therapeutic effect of GA was reported previously to have neuroprotective effects with the potential to modulate BDNF expression in the diseased brain (Aharoni et al., 2005). The most recent report from Lee et al. (2012) indicates that the CNS damage is increased in the early stages of the disease when BDNF is lacking, but there was no significant effect in later stages of the disease.

Recent studies have rekindled the notion that neurological dysfunction in patients with MS relates best to the degree of neuronal (axonal) and glial damage (Loeb, 2007; Ilieva et al., 2009; Amor et al., 2010; Derfuss et al., 2010; Siffrin et al., 2010). Therefore, to understand the pathogenesis of MS and to develop biologically targeted therapeutics, a greater understanding of axoglial biology is needed in the context of inflammatory modulation. In addition to neurotrophic factors, such as BDNF, central to axoglial biology are protein regulatory factors that signal in the opposite direction, from neurons to glia, and are referred to as "gliotrophic" factors. One of these is called neuregulin derived from the neuregulin1 gene (NRG1) that is important for peripheral

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http://dx.doi.org/10.1016/j.jneuroim.2013.05.012

and central nervous system development (Loeb, 2007; Hohlfeld, 2008; De Santi et al., 2009, 2011; Linker et al., 2009; Nagahara and Tuszynski, 2011). Despite their importance in development, little is known of the interrelated roles of neurotrophic and gliotrophic factors in neurodegeneration and demyelinating diseases, such as MS (Loeb and Fischbach, 1997; Loeb, 2007).

Our recent work suggests that NRG1 is tightly regulated by neurotrophic factors, including BDNF and GDNF, both at the transcriptional and post-translational levels (Loeb and Fischbach, 1997; Loeb et al., 2002; Esper and Loeb, 2004; Loeb, 2007; Ma et al., 2011). NRG1 has been shown to be critical for peripheral nerve development, myelination, and more recently, spinal cord microglial activation in a model of chronic pain (Falls, 2003; Esper et al., 2006; Calvo et al., 2010). The *NRG1* gene has also been implicated as a susceptibility factor in families with schizophrenia (Mei and Xiong, 2008; Buonanno, 2010). The *NRG1* gene carries out these important activities through a large repertoire of both secreted (type I) and membrane-bound (type III) forms that are highly expressed in brain and spinal cord neurons (Meyer et al., 1997; Loeb et al., 1999). Evidence from a number of studies suggests a positive feedback loop between neuronal NRG1 and glial- and muscle-derived, neurotrophic factors (Loeb and Fischbach, 1997; Loeb et al., 2002; Falls, 2003; Esper and Loeb, 2004; Esper et al., 2006; Ma et al., 2011). To date, this important reciprocal signaling pathway has not been explored in MS or the EAE mouse model where marked inflammation, demyelination and glial activation are observed.

EAE is an inflammatory autoimmune disease of the CNS that serves as a useful animal model for testing treatment strategies for MS (Lisak and Behan, 1975; Steinman and Zamvil, 2006). The disease is inducible in the susceptible mouse strain C57BL/6 by immunization with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (Papenfuss et al., 2004; O'Neill et al., 2006). Here, we examined the expression patterns of neurotrophic and gliotrophic factors in the mouse brain in MOG-induced EAE in C57BL/6 mice as well as the effects of reduced BDNF signaling in mice heterozygous for the BDNF receptor, TrkB. We also examined the effect of GA treatment. We found a complex pattern of gliotrophic and neurotrophic gene expression that varied with disease severity and could be suppressed by GA treatment. Understanding the changes of gliotrophic- and neurotrophic-factors as a function of disease severity will be important to optimize neuroprotective treatment strategies in MS.

2. Materials and methods

2.1. Mice

Adult female C57BL/6 mice (JAX stock 000664), TrkB heterozygous (TrkB+/−, 5 generations of backcrossing into C57BL/6 background; JAX stock 002544) (Klein et al., 1993) and age-matched female wild type littermates were purchased from Jackson Laboratories (Bar Harbor, ME). A second series of experiments utilized TrkB+/− mice with 7 generations of backcrossing into C57BL/6 background (JAX stock 003098), also from Jackson Laboratories. Wild type littermates for 5 and 7 generations of TrkB heterozygous (JAX stock 002544 and 003098) were also from Jackson Laboratories.

2.2. Induction and evaluation of EAE

C57BL/6, TrkB+/− and TrkB+/+ (wt littermates) mice (5 generations of backcrossing, JAX stock 002544) were immunized subcutaneously on day zero in four sites over the back (left and right shoulders, left and right flanks) with 200 μg of $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) (Princeton Biomolecules, Langhorne, PA) emulsified in a volume of 100 μl of complete Freund's adjuvant (CFA) containing 1 mg/ml of M. tuberculosis (M. Tbc) (CFA, Difco Laboratories, Detroit, MI, USA) (Papenfuss et al., 2004; O'Neill et al., 2006). On days 0 and 2 post-immunization, 200 ng of pertussis toxin (PT) (List Biological, Campbell, CA) was administered intraperitoneally (i.p.) in 0.2 ml of PBS (Sigma, St. Louis, Minnesota). Mice were observed daily for clinical signs and scored as follows: limp tail or waddling gait with decreased tail tonicity +1, waddling gait +2, partial hind limb paralysis +3, full hind limb paralysis +4, and death +5.

In some of experiments (Fig. 1C and D), to induce more severe disease, TrkB+/− and TrkB+/+ (wt littermates) mice (7 generations of backcrossing, JAX stock 003098) were injected as above except that M. Tbc was increased to 5 mg/ml in CFA and PT was increased to 300 ng on days 0 and 2 post-immunization.

2.3. GA treatment

GA (Copaxone, Copolymer 1) from batch 242900110 with an average Mr of 7 kDa, was obtained from Teva Pharmaceutical Industries. GA treatment was administered by consecutive daily i.p. injections (1.5 mg per mouse per day) at different stages of disease: 1) at EAE induction (day 0), 8 injections daily (suppression); or 2) after appearance of first clinical sign, 8 injections daily (treatment) (Aharoni et al., 2008).

2.4. RNA isolation and real-time quantitative RT-PCR (qPCR)

Mice designated for qPCR were anesthetized (ketamine 80 mg/kg, i.p. and xylazine 40 mg/kg, i.p.) and then had intracardiac perfusion with PBS (0.1 m, pH 7.4). Perfused mouse brains were dissected and rapidly frozen and stored at the minus 80 °C. RNA was extracted using the Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) (Rakhade et al., 2005). Quantification of RNA was carried out using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The quality of RNA was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using an RNA 6000 Nano chip kit, RNA ladder and Agilent analysis software (Agilent Technologies). All samples had RIN (RNA integrity number) values above 8.0 and 260/280 ratios near 2.0.

The expression of mouse types I and III NRG1, BDNF and GDNF were each measured relative to GAPDH using Taqman Assays-On-Demand primers (Applied Biosystems, Foster City, CA, U.S.A.). 1.5 μg of total RNA was used in a 20 μl reverse transcription synthesis reaction primed with oligo-dT primers (Superscript First Strand Synthesis System, Invitrogen, Carlsbad, CA, U.S.A.). PCR was performed in triplicate using 1× Taqman Universal PCR master mix (Applied Biosystems) with the DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, MA, U.S.A.) utilizing the following primers and Taqman probes: type I NRG1: Mm00626552_m1; type III NRG1: Mm01212129_m1; BDNF: Mm01334047_m1; GDNF: Mm00599849_m1 and GAPDH: Mm99999915_g1 (Song et al., 2012). Cycle threshold (Ct) values were calculated using Opticon monitor software, with the threshold set at 40 standard deviations above background. The relative expression was calculated by normalizing the expression of individual genes to GAPDH and using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Statistical analysis

A nonparametric ANOVA with Tukey's post hoc test was performed for disease severity: 1) between groups of wt (TrkB+/+) and TrkB+/− mice (Table 1), and, 2) groups of control and GA-suppression as well as GA-treatment mice (Table 2).

A parametric statistical test (test for association/correlation using cor.test {stats} software) was performed between gene expression and disease severity to analyze the correlation and p value in TrkB+/+ and TrkB+/− mice (Fig. 2).