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Research Paper

Expression of brain-derived neurotrophic factor in astrocytes - Beneficial effects of glatiramer acetate in the R6/2 and YAC128 mouse models of Huntington's disease

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ABSTRACT

Glatiramer acetate (GA) is a FDA-approved drug which is licensed for the treatment of relapsing-remitting multiple sclerosis and which may exert neuroprotective effects via brain-derived neurotrophic factor (BDNF). In this study, we investigate effects of GA on BDNF expression especially in astrocytes *in vitro* and *in vivo* in brains of R6/2 and YAC128 transgenic mouse models of Huntington's disease (HD) where a pathogenic role of astroglial cells has recently been shown. We show that GA increases the expression of functionally active BDNF in astrocyte culture and in astrocytes of GA treated HD mice. In the brains of these mice, GA decreases neurodegeneration and restores BDNF levels. The beneficial effect of GA in R6/2 mice also comprises reduced weight loss and prolonged life span and, for both models, also improved motor performance. Further studies with this safe and effective drug in HD are warranted.

1. Introduction

Since the identification of the mutation relevant for Huntington's disease (HD) in 1993, the understanding of its pathophysiology and molecular biology has significantly improved. Mechanisms of tissue damage in HD comprise excitotoxicity, mitochondrial damage, free radicals and possibly also inflammatory mechanisms including microglia activation (Ellrichmann et al., 2013; Orr et al., 2008; Panov et al., 2002; Zadori et al., 2012). It is important to note that similar destructive components common in neurodegenerative diseases have also been identified in autoimmune diseases such as multiple sclerosis (MS) (Bjartmar and Trapp, 2001; Meyer et al., 2001; Perry and Anthony, 1999). Based on all these mechanisms, different treatment approaches were designed and analyzed, but have failed in HD therapy so far. Up to now, HD lasts to be an incurable neurodegenerative disease.

Interestingly, several studies reveal a down regulation of BDNF in the CNS of HD patients and in its mouse models. In particular, the transport of BDNF secretory vesicles from the cortex to the striatum may be impaired (Gauthier et al., 2004; Zuccato and Cattaneo, 2007). A recent study further postulated a postsynaptic BDNF signaling defect in HD involving the high and low affinity BDNF receptors tyrosine-related kinase B receptors and p75 neurotrophin receptor (Plotkin et al., 2014). As yet it is not clear whether this is a secondary phenomenon implied in the chain of neurodegenerative events. Well in line with these observations, a lack of BDNF may lead to a higher susceptibility of neurons to cell death not only in HD, but also in other neurodegenerative diseases (Mogi et al., 1999; Narisawa-Saito et al., 1996; Parain et al., 1999). The restoration of BDNF thus constitutes a promising therapeutic target, which may ameliorate or decelerate the disease progress. Indeed, overexpression of BDNF in the forebrain of HD transgenic mice improves motor function and reduces neuronal loss (Xie et al., 2010).

Astrocytes were recently shown to be very important in the pathophysiology of HD (Tong et al., 2014), in particular for maintaining a balanced energy metabolism or controlling glutamate levels in the brain (Acuna et al., 2013; Kettenmann and Verkhratsky, 2011). Additionally, astrocytes may play a role via secretion of trophic factors. Amongst these are glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) (Wiese et al., 2012).

Based on the altered BDNF expression in HD and the known effects of glatiramer acetate (GA) on BDNF production (see below), we explore

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the potential of GA in two different transgenic murine models of HD. GA is a FDA approved drug for the treatment of relapsing remitting MS. Up to now, the exact mechanisms of action are not fully understood. As an immunomodulator in MS, GA induces a shift from the dominant pro-inflammatory T_H1 response to a more anti-inflammatory T_H2 response (Aharoni et al., 1997). GA-reactive T cells are then entering the CNS secreting anti-inflammatory cytokines (Aharoni et al., 1997; Miller et al., 1998). Besides these immunomodulatory mechanisms of action, GA may also exert neuroprotective effects via the neurotrophin BDNF (Linker et al., 2010). These effects are summarized in the term "protective autoimmunity" as coined by Moalem et al. (Moalem et al., 2000). In particular, GA increases BDNF expression in T cells (Kerschensteiner et al., 1999; Stadelmann et al., 2002) and thus may lead to increased neuroprotection in models of MS (Aharoni et al., 2005a, 2005b) or Alzheimer's disease (Butovsky et al., 2006). In the setting of an altered blood brain barrier, GA may also exert effects directly in the CNS (Liu et al., 2007), which raised the question on the potential direct target cells in the brain. However, GA did not directly protect neurons from inflammatory cell death in *in vitro* or EAE models (Herrmann et al., 2010) thus shifting the focus of interest towards effects of GA on glial cells (From et al., 2014).

As models of HD, we explore the R6/2 and YAC128 transgenic mouse models. These mouse strains are well-characterized animal models mimicking many histopathological aspects of HD (Mangiarini et al., 1996; Stack et al., 2005). We show that GA treatment improves motor performance in R6/2 and YAC128 transgenic mice, diminishes progressive weight loss and prolongs life span in R6/2 mice. Neurodegeneration is decreased in both mouse models. GA restores BDNF levels in the brain and induces functionally active BDNF expression in astrocytes *in vitro* as well as *in vivo* in the CNS of HD mice.

2. Materials and methods

2.1. Primary mesencephalic astrocyte cultures

Astrocytes were prepared from the cortices of one day old C57BL/6 pups. Meninges were removed and the cortices were dissected and diced into pieces. After incubation with 0.1% trypsin at 37 °C for 20 min, cells were washed twice with medium (DMEM GlutaMAX, Lifesciences) containing 10% endotoxin-free foetal calf serum. Cell suspensions were centrifuged and the pellets were re-suspended in culture medium (DMEM GlutaMAX, 10% endotoxin-free foetal calf serum, 100 units penicillin/streptomycin). Pellets were then seeded on 25 cm^2 culture flasks (BD Primaria, BD Biosciences). Culture media were refreshed three times per week. After seven days of incubation, cultures were shaken on an orbital incubator shaker over night to remove the adherent microglia. Supernatants were removed, adherent astrocytes trypsinized, centrifuged and washed in culture media. Obtained astrocytes were plated at a density of 1.25×10^6 cells.

On day 14 *in vitro*, cells were stimulated for six hours (h) with 50 μg/ml GA or remained untreated as controls. Stimulation time was based on time-course studies (Herrmann et al., 2010). After six h, medium was discharged, astrocytes washed and fresh medium without supplements added. Astrocyte conditioned medium (ACM) was collected 24 h later, cell debris removed and frozen at −80 °C.

2.2. Preparation of motoneurons and motoneuron survival assay

To prove a neurotrophic/neuroprotective effect of BDNF, we established a motoneuron survival assay with astrocyte conditioned medium (ACM) after pre-stimulation with GA and a neutralizing anti-BDNF-antibody (Abcam) was added to certain conditions.

Spinal cord of E12.5 mouse embryos was isolated and transferred to HBSS (Conrad et al., 2011). Dorsal root ganglia (DRG) were removed carefully and the lumbar parts were collected in an Eppendorf reaction tube. Motoneurons were enriched by lectin-based purification. Cell numbers were counted using a Neubauer counting chamber and an appropriate number of cells was plated on laminin coated coverslips.

For motoneuron survival, cells received 1 μg/ml BDNF as positive or medium only as negative control. Astrocyte conditioned medium (ACM) was pre-stimulated with GA. After 48 h cells were stained with a caspase-3 antibody (1:200, Abcam) to detect apoptotic motoneurons. Ten representative pictures per well were taken and the percentage of caspase-3 positive cells reference to whole number of cells was evaluated by a blinded observer ($n = 3$ independent experiments).

2.3. RT-PCR

Total mRNA was isolated from the cortex area in middle brain slices (Bregma −0.82 mm to Bregma −1.18 mm). The following set of oligonucleotides (Applied Biosystems, California, USA) was used for expression analysis in a TaqMan assay:

BDNF: *sense*: *5′GGG CCG GAT GCT TCC TT 3′ antisense*: *5′GCA ACC GAA GTATGA AAT AAC CAT AG 3′ probe*: *5′ TTC CAC CAG GTG AGA AGA GTG ATG ACC AT 3′*.

β-Actin: *TaqMan® Gene Expression Assay Mm00607939_s1*.

Results were normalized to β-actin.

2.4. ELISA

Primary mesencephalic astrocyte cultures were prepared as described above. On day 14, wild-type derived cells were stimulated for 6 h with 10 μg/ml, 25 μg/ml, 100 μg/ml GA or remained unstimulated.

Immediately before measurement, aliquots were brought to room temperature and analyzed for BDNF with sandwich ELISA kit (R&D Systems) according to manufacturers' protocols.

96 well plates were coated with capture antibody diluted in PBS buffer and incubated at room temperature. Aspiration and washing for three times followed. Residual buffer was removed on absorbent paper. Blocking by 10% BSA for one hour at room temperature reduced unspecific binding. An anti-BDNF antibody was added as second antibody. The specificity of the ELISA was validated by cell culture supernatants of BDNF heterozygous animals versus wild-type mice ($n = 4$) stimulated with or without GA (data not shown).

2.5. Histochemistry and immunohistochemistry

Histochemistry and immunohistochemistry were performed with paraffin embedded brain tissue (5 μm) or TissueTek embedded frozen tissue (10 μm). Brain sections (Bregma 0.14 mm to Bregma 1.10 mm) were subjected to cresyl violet staining to assess neuronal degeneration or NeuN (1:200, Chemicon, Temecula, USA) labeling neurons. Mutant htt aggregates were detected by using a mouse anti-ubiquitin antibody (1:1000; Chemicon, Temecula, USA). Biotinylated secondary antibodies, followed by the avidin-biotin-peroxidase complex (Vector Laboratories, California, USA) were used according to the manufacturers' instructions. Sections were developed with diaminobenzidine tetrahydrochloride (Merck, Darmstadt, Germany) solved in 0.003% hydrogen peroxide and PBS, counterstained with hemalaun and coverslipped in Entellan (Merck, Darmstadt, Germany). BDNF was labeled with a rabbit anti-BDNF antibody (1:100; Millipore, Temecula, USA) and astrocytes with a mouse anti-glial fibrillary acidic protein antibody (1:800; Millipore, Temecula, USA). An appropriate secondary antibody was added, labeled either with Alexa488 or with Alexa647 (1:1000, Invitrogen, Frankfurt, Germany *later* Lifetechnologies Carlsbad, California/USA) and slides were coverslipped with ProLong Gold mounting medium (Invitrogen, Frankfurt, Germany *later* Lifetechnologies Carlsbad, California/USA).

For light microscopy (BX51 Olympus, Hamburg, Germany), standardized 2 mm frontal brain slices were prepared using a brain slicer. Fixed areas in the medial and lateral striatum and motor cortex M1 layer ventral and rostral within Bregma 0.14 mm–1.10 mm were analyzed. Immunohistochemistry was analyzed by light microscopy,