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Neurobiology of Disease

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Neurobiology of Disease 28 (2007) 143–153

Reduced CXCL12/CXCR4 results in impaired learning and is downregulated in a mouse model of Alzheimer disease

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Received 1 March 2007; revised 9 June 2007; accepted 1 July 2007
Available online 10 July 2007

Alzheimer disease (AD) is characterized by the presence of plaques and tangles in parallel with progressive cognitive decline. The underlying cause of the cognitive decline is unknown. The purpose of this study was to identify factors that could affect learning and memory using the Tg2576 mouse model of AD. Un-biased GeneChip analysis at the time-point coinciding with the onset of behavioral deficits but prior to plaque deposition revealed that Tg2576 show altered gene expression for a number of molecules including the chemokine CXCL12. We show that this chemokine's mRNA, protein and receptor are downregulated in this mouse model coinciding with cognitive deficits. Furthermore, we demonstrate that CXCL12 levels are decreased in AD patients as compared to controls. To determine if CXCL12 might be related to impaired learning and memory, we chronically treated young non-transgenic mice with an antagonist to the CXCL12 receptor to simulate the reduction seen in transgenic animals. Treated animals showed selectively impaired learning and memory suggesting a potential role for this chemokine in cognitive functioning.
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Keywords: Chemokine; CXCL12; AMD3100; Tg2576; Learning; Inflammation; Cognition; Brain; AD; GeneChip

Introduction

AD is a chronic neurodegenerative disorder marked by progressive memory deterioration. Neuropathologically, two primary features characterize the disease; plaques composed of aggregated amyloid beta (Aβ) peptides and neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau protein. These lesions are closely linked with chronic inflammation and synaptic/neuronal dysfunction.

The amyloid cascade hypothesis states that Aβ triggers AD and that neuropathological events occur as a consequence of Aβ accumulation (Hardy and Allsop, 1991; Selkoe, 1996). Furthermore, the hypothesis postulates that Aβ or a consequence of its accumulation is responsible for the cognitive decline in AD. However, plaque pathology is a poor correlate of the degree of memory impairment (Braak and Braak, 1997; Crystal et al., 1988; Polvikoski et al., 2001). Apart from plaques, soluble Aβ including oligomers such as the recently identified Aβ12mer, dubbed Aβ56*, may play a role in memory impairment. Evidence shows a robust correlation between soluble Aβ load and severity of cognitive impairment in AD (McLean et al., 1999; Wang et al., 1999). To date, it remains unknown whether Aβ affects cognition directly through altered synaptic plasticity or may also involve a secondary effect of Aβ such as oxidative stress or inflammation.

Learning and memory are thought to depend on changes in synaptic efficacy in key brain structures including the hippocampus. Long-term potentiation (LTP) has been extensively shown as a model of use-dependent enhancement in synaptic efficacy (Bliss and Collingridge, 1993). Deficits in LTP as a result of Aβ-soluble oligomeric species has been documented in rats *in vivo* (Walsh et al., 2002) as well as in hippocampal slices (Wang et al., 2002). Interestingly, it was found that these LTP deficits induced by Aβ can be prevented by the addition of minocycline, a blocker of microglia activation (Wang et al., 2004), suggesting that a secondary amyloid-dependent mechanism may contribute to the cognitive decline in AD.

The purpose of the current study was to use the Tg2576 model to identify novel factors that might affect cognitive performance. An un-biased screen at the time-point coinciding with the onset of behavioral deficits in Tg2576 mice identified the inflammatory marker CXCL12 as a potential candidate for the behavioral deficits found in the transgenic mice. Importantly, we find that CXCL12 levels are also decreased in AD patients as compared to non-demented controls further supporting a role for this chemokine in cognitive functioning. We focused on CXCL12 for three reasons: this chemokine has been previously shown to modulate neuronal firing and neuron/glia communication (Bezzi et al., 2001; Meucci et al., 1998), there is a characterized pharmacological agent available

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Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2007.07.001

with which we can mimic decreased CXCL12 function (Fricker et al., 2006; Rosenkilde et al., 2004) and lastly, we and others have previously shown that inflammatory responses in AD correlate with cognitive decline (Bajetto et al., 2001; Luterman et al., 2000; Parachikova et al., 2006). We treated young non-transgenic mice with a CXCL12 receptor antagonist and show that this results in learning and memory deficits. Hence, this study has identified a novel pathway mediated via CXCL12 that directly affects learning and memory and may in part be responsible for the dementia component of AD.

Materials and methods

Mice

The study used Tg2576 transgenic and age-matched C57Bl6/SJL non-transgenic mice ages 5 to 6, 12 and 17 months for gene expression and protein analysis ($n=5$–6/group). Mice were obtained from Jackson Labs and a colony established. We also used aged 24-month-old triple transgenic (3×Tg-AD) and age-matched non-transgenic mice ($n=6$/group) for CXCL12 protein analysis (3×Tg-AD mice were kindly provided by the laboratory of Frank LaFerla, UCI) (Oddo et al., 2003). Young, 3-month-old C57Bl6/SJL ($n=9$/group) were used for the antagonist study and were obtained from Jackson Labs. All animal housing and procedures were performed in accordance with the guidelines established by the University of California, Irvine.

mRNA expression level analysis

Brains were extracted from 5- to 6-month-old Tg2576 and non-transgenic mice ($n=6$/group) and the hippocampus microdissected and stored at −80 °C. RNA was isolated from the frozen hippocampus by the guanidium thiocyanate method (Chomczynski and Sacchi, 1987) with TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Total RNA was purified using RNeasy quick spin columns (Qiagen, Valencia, CA) and quantified using a UV spectrophotometer. RNA quality was assessed by Bioanalyzer on DNA 500 chips (Agilent Technologies, Santa Clara, CA) ensuring high RNA quality (28S/18S ratio of >1.5).

Affymetrix GeneChip

Gene expression analysis of transgenic and non-transgenic mice was achieved using the Affymetrix U74Av2 GeneChip array (Affymetrix Inc., Santa Clara, CA) containing 12,488 probe sets. Gene expression changes in the hippocampus of 5- to 6-month-old TG2576 transgenic mice were determined as compared to age-matched non-transgenic littermates ($n=6$/group). Briefly, high-quality RNA samples (28S/18S ratio of >1.5) were hybridized to high-density oligonucleotide GeneChip arrays. Hybridization was performed with 5 μg of mRNA pooled from two animals onto a single Affymetrix GeneChip with three chips per condition. Labeling, hybridization, and scanning followed the Affymetrix GeneChip Expression Analysis Technical Manual (http://affymetrix.com). Statistical analysis was performed using GC RMA with criteria of $P<0.05$ and a greater than 1.5-fold change in gene expression.

Quantitative real-time polymerase chain reaction (RT–PCR)

Validation of the CXCL12 mRNA changes found in the GeneChip analysis between 5- and 6-month-old Tg2576 and age-matched non-transgenic controls was achieved via RT–PCR performed on the individual samples of the animals used in the GeneChip study ($n=6$/condition). We used the CXCL12 primers 5′GCAGACTGTGTTGGGTGAGA3′ and 5′CATCTATCCTCCCCACGAGA3′. In addition to CXCL12, we performed RT–PCR for synaptotagmin IV in 5- to 6-month-old Tg2576 and age-matched controls. We used the synaptotagmin IV primers 5′GGCCTCGTCTTCACTGTCTC3′ and 5′CCATTGAGGTCTCGCTTCTC3′. 18S was used as control with 5′AACGAGACTCTCGGCATGCTAA3′ and 5′CCGGACATCTAAGGGCATCA3′. Real-time PCR was performed on 100 ng of total RNA using the One-Step iQ SYBR Green supermix (BioRad, Hercules, CA). Threshold cycle (Ct) values were calculated with MyiQ software (BioRad, Hercules, CA), and the quantitative fold changes in CXCL12 mRNA were determined as relative to 18S mRNA levels. Data analysis used Student's T test statistics.

Protein level analysis

6-, 12- and 17-month-old TG2576 and 24-month-old 3×Tg-AD mice and age-matched non-transgenic controls were used for protein analysis ($n=5$–6/group). We also analyzed CXCL12 and CXCR4 protein levels in human AD and age-matched control subjects ($n=4$/group). The hippocampus was microdissected from the brain and homogenized in T-PER buffer (Pierce, Rockford, IL) in the presence of a complete protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN) and centrifuged at 100,000×g for 1 h at 4 °C. Supernatants were collected as the detergent-soluble fraction. Protein quantification was performed using the bicinchoninic acid (BCA) assay (BioRad, Hercules, CA).

CXCL12 ELISA

CXCL12 protein levels were determined in 6- and 17-month-old TG2576 as well as 24-month-old 3×Tg-AD and age-matched non-transgenic mice using quantitative sandwich ELISA (enzyme-linked immunosorbent assay) technique ($n=6$/group). Mouse CXCL12 Immunoassay using pre-coated plates was obtained from R&D Systems (Minneapolis, MN). Briefly, standards and hippocampus samples were added in duplicate to the pre-coated plate and incubated at 37 °C for 2 h. Plate was subsequently washed to remove unbound substances and next an enzyme-linked polyclonal antibody specific for mouse CXCL12 was added to the wells. Plate was then washed again followed by the addition of a substrate solution and color develops in proportion to the amount of chemokine bound. The plates were read at 450 nm on a Molecular Dynamics (Sunnyvale, CA) plate reader. Results were normalized to protein concentrations. Data analysis used Student's T test statistics.

Western blot

CXCR4 protein levels were examined in 6-month-old Tg2576 and non-transgenic controls ($n=5$/group). 10 μg of total protein was resolved by SDS–PAGE and electrophoretically transferred onto nitrocellulose membrane. Membranes were blocked in 5% milk, washed, then incubated with the rabbit polyclonal CXCR4 primary antibody (1:1000; overnight at 4 °C; Abcam, Cambridge, MA). On the following day, the membrane was washed and incubated with HRP-conjugated goat anti-rabbit secondary (1:1000; 1 h; BioRad, Hercules, CA). Membranes were visualized using enhanced chemiluminescence (Pierce, Rockford, IL). Sub-

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