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

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Abstract

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.

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 (A β) 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 A β triggers AD and that neuropathological events occur as a consequence of A β accumulation (Hardy and Allsop, 1991; Selkoe, 1996). Furthermore, the hypothesis postulates that A β 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 A β including oligomers such as the recently identified

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A β 12mer, dubbed A β 56*, may play a role in memory impairment. Evidence shows a robust correlation between soluble A β load and severity of cognitive impairment in AD (McLean et al., 1999; Wang et al., 1999). To date, it remains unknown whether A β affects cognition directly through altered synaptic plasticity or may also involve a secondary effect of A β 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 A β 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 A β 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 timepoint 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 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; Lutermaier et al., 2000). 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 $\times$ Tg-AD) and age-matched non-transgenic mice (n=6/group) for CXCL12 protein analysis (3 $\times$ 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 5ug of mRNA pooled from 2 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- to 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 100ng 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 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 $100,000 \times g$ 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 immunoassay) 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 hours. 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 T test statistics.

Western blot—CXCR4 protein levels were examined in 6 month old Tg2576 and non-transgenic controls (n=5/group). 10 micrograms 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; 1hr; BioRad, Hercules, CA). Membranes were visualized using enhanced chemiluminescence (Pierce, Rockford, IL). Subsequently, membranes were stripped and re-probed with a monoclonal β -actin antibody (Sigma, St. Louis, MO) as a loading control. Quantification of CXCR4 protein levels was performed using Scion Image Software (NIH). Three measurements were taken for each band and CXCR4 protein levels were obtained relative to β -actin across all cases. Data analysis used Student T test statistics.

CXCL12 and CXCR4 protein levels were determined in human AD and age-matched non-demented subjects (n=4/group) using Western blot analysis as described previously. Briefly, we used 20ug of hippocampal homogenates for CXCL12, CXCR4 and GAPDH Western blots. Specifically, we employed the monoclonal CXCL12 primary antibody (1:1000, R&D Systems, Minneapolis, MN), the rabbit polyclonal CXCR4 primary antibody (1:500, Abcam, Cambridge, MA), and the rabbit polyclonal GAPDH primary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate secondary antibodies were used. Again, three measurements were taken for each band and CXCR4 as well as CXCL12 protein levels were obtained relative to GAPDH across all cases. Data analysis used Student T test statistics.

A β ₄₀ and A β ₄₂ ELISA—A β ₄₀ and A β ₄₂ levels were determined in 6 and 12 month old Tg2576 mice (n=6/group) using an in-house amyloid sandwich ELISA, a sensitive method for determining the levels of soluble and insoluble A β . Detergent-soluble and insoluble fractions were used to detect A β ₄₀ and A β ₄₂ using T-PER Extraction Buffer (Pierce Biotechnology, Rockford, IL, USA) and 70% formic acid (FA) respectively. The FA fractions were diluted 1:20 in neutralization buffer (1 M Tris base and 0.5 M Na₂HPO₄) prior to loading. MaxiSorp immunoplates (Nunc, Naperville, IL) were coated with the monoclonal A β 20.1 antibody at a concentration of 25 μ g/ml in coating buffer (0.1 M NaCO₃ buffer, pH 9.6) and blocked using 3% BSA. Standards of both A β ₄₀ and A β ₄₂ were prepared in antigen capture buffer (20 mM NaH₂PO₄, 2 mM EDTA, 0.4 M NaCl, 0.5 g of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 1% BSA, pH 7.0) and loaded onto the ELISA plates in duplicate. Hippocampus samples were also loaded in duplicate and incubated overnight at 4°C. The plates were subsequently washed and probed with either HRP-conjugated anti-A β ₃₅₋₄₀ (MM32–13.1.1) or anti-A β ₃₅₋₄₂ (MM40–21.3.4) overnight at 4°C. 3,3',5,5'-Tetramethylbenzidine was used as the chromagen, and the reaction was stopped by 30% O-phosphoric acid and read at 450 nm on a Molecular Dynamics (Sunnyvale, CA) plate reader. Results were normalized to protein concentrations. Data analysis used Student T test statistics.

Immunofluorescent staining

6 month old Tg2576 and age-matched non-transgenic mice (n=6) were sacrificed and their brains were fixed in 4% paraformaldehyde for 48 hours. Tissue blocks were sectioned at 50 μ m using a vibratome. Fluorescent double-label immunohistochemistry for CXCL12 and either microglia or GFAP employed a biotin conjugated SDF1 α (CXCL12) antibody (Abcam, Cambridge, MA) and the polyclonal rabbit antibody IBA1 (Wako, Richmond, VA) for microglia or the polyclonal rabbit antibody GFAP (Dako, Glostrup, Denmark) for astrocytes. Hippocampal sections from non-transgenic and Tg2576 transgenic mice were washed in PBS, blocked with 5% normal goat and horse serum for one hour, and incubated overnight at 4°C with either CXCL12 (1:200) and IBA1 (1:600) or CXCL12 (1:200) and GFAP (1:10'000). On the following day, sections were washed and incubated in secondary goat anti-rabbit Alexa 555 antibody for either IBA1 or GFAP (Molecular Probes) and streptavidin-conjugated (SA-) Alexa 488 for CXCL12 (Molecular Probes) (1:200; 1hr). Following three washes in Tris, sections were mounted and coverslipped with Fluoromount-G (Southern Biotechnology).

Controls were performed to rule out nonspecific immunostaining by eliminating the primary or secondary antibody. Controls were negative. Immunofluorescent labeling was also performed for the CXCR4 receptor in hippocampal sections of non-transgenic and Tg2576 transgenic mice (n=6/group). Staining employed the rabbit polyclonal antibody to CXCR4 (Abcam, Cambridge, MA) at 1:200. Secondary antibody used was goat anti rabbit Alexa 488. Staining procedures followed the same design as outlined above. Again, controls were negative.

***In Vivo* AMD 3100 Treatment**

We treated 3 month old non-transgenic mice with either AMD3100 or PBS (N=9/group). AMD3100 octahydrochloride hydrate (1,1'-[1,4-Phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride) is a specific CXCR4 antagonist (Fricker et al., 2006; Rosenkilde et al., 2004), which has been previously used in mice (Jiao et al., 2006; Matthys et al., 2001; McCandless et al., 2006) and has been shown to have efficacy on mouse CXCR4 in the CNS (Rubin et al., 2003).

AMD3100 was delivered via subcutaneous osmotic pumps (Alzet, Palo Alto, CA). Pumps were loaded with 30 mg/ml AMD3100 (Sigma, St. Louis, MO) in sterile PBS or sterile PBS alone according to the manufacturer's instructions. Drug or PBS was delivered for a period of 4 weeks at a constant rate of 0.25µl/h. Amount and duration of antagonist treatment were based on (Rubin et al., 2003).

Behavioral Study

Learning and memory of AMD3100 and PBS treated animals was assessed using a battery of behavioral tasks 3 weeks following treatment onset. The behavioral tasks included novel object/place/context, T-maze and inhibitory avoidance.

Object/Place/Context recognition—These tasks are based on the spontaneous tendency of rodents to explore a novel object more often than a familiar object (Ennaceur and Delacour, 1988) and have been found to not be dependent on the amygdala (Moses et al., 2005).

Perirhinal cortex lesions and studies of neuronal activation and responses in rats suggest that it is cortical and not hippocampal neurons that are involved in the object recognition task (Aggleton et al., 1997; Wan et al., 1999). For the novel object task, mice were familiarized with an empty open field for a period of 10 minutes. On the following day, mice were subjected to a 5 minute exploration session in the same context with two identical objects (Object A; e.g. two identical balls or two identical dice) placed in symmetrical locations in the open field. 90 minutes and 24 hours later, animals were subjected to a 3 minute retention phase test where they were exposed to one Object A and also to a novel object, Object B (for the 90 min time point) and Object C (for the 24 h time point) placed in the same, symmetrical locations in the open field.

A different version of the novelty task requires mice to recognize that an object is placed in a new location (Ennaceur et al., 1997; Save et al., 1992). This task is primarily dependent upon hippocampus (Mumby et al., 2002). For the novel place paradigm, mice were again placed in the open field with two identical objects (Object D), different from the objects used for the novel object task, for 5 minutes. 90 minutes later, animals were subjected to a 3 minute retention phase test where they were exposed to Objects D again but with one of the object having been moved from its original location.

Another version of the novelty-preference paradigm requires mice to remember an object encountered in a particular context (Dix and Aggleton, 1999). This memory task has also been

shown to be hippocampus dependent (Mumby et al., 2002). The novel context task required the mice to be familiarized with a second context. Mice were placed in a second open field in a different room for 10 minutes. Mice were presented with two identical objects in context 1 (Object E) and then presented with two different identical objects in context 2 (Object F). For the 90 min retention phase test, animals were placed in context 1 with one Object E and one object from context 2 (Object F).

The time spent exploring the familiar object and the novel object were calculated where exploration equals touching the object with nose or paws, or sniffing within 1.5 cm of the object. Time spent with the novel object as compared to time spent with both objects was used as memory index.

T maze—The T-maze is a primarily hippocampus dependent spontaneous alternation task (T-CAT) that measures exploratory activity (Dember and Fowler, 1958; Dember and Fowler, 1959). The T-maze protocol used in the study was adapted from (Lalonde and Strazielle, 2005). Animals were tested in a T-maze containing a central starting arm and two side arms, 30 cm in length and 10 cm in width, surrounded by 20 cm high walls. On the initial trial, the animal was placed in the central arm with the right arm blocked closed. After entering the left arm (entire body criterion), the mouse was kept inside for 2 min by closing the barrier. The mouse was then removed from the maze and then quickly placed back in the starting arm for a free-choice trial. The starting arm was varied between animals. On the following 4 days, the same procedure was adopted, except that the blocked arm on the initial trial was changed alternatively from right to left. Also, the cues surrounding the maze were changed on a daily basis in order to ensure the animals view the maze as “new” every day. We obtained an alternation ratio by recording if the animal made the correct choice (i.e. alternate to the initial trial) or incorrect choice (i.e. same as initial trial) on each day.

Passive Inhibitory Avoidance (IA)—The inhibitory avoidance task is used in mice to assess primarily amygdala-dependent learning (Blanchard and Blanchard, 1972; Holahan and White, 2002; Phillips and LeDoux, 1992). IA consisted of a training session followed by testing 1.5 and 24 hours post training. During the training session, a mouse was placed in a lightened chamber and when the mouse crossed to the dark compartment, it received a mild footshock (0.15 mA/1 s). During testing, the mouse is placed again in the light compartment and the latency to cross over to the dark compartment is measured. This latency measure was used as an index of passive fear avoidance.

Statistical analysis of the behavior data

Analysis of behavioral data from all of the above described tasks employed unpaired Student T test to compared between AMD3100 treated and untreated mice.

Results

Gene expression changes in Tg2576 mice at the timepoint of cognitive decline

We aimed to identify gene expression changes in Tg2576 mice at the timepoint coinciding with behavioral deficits (Jacobsen et al., 2006; Westerman et al., 2002). Unbiased microarray analysis revealed an altered gene expression profile for a number of mRNA sequences in 6 month old Tg2576 mice compared to age-matched non-transgenic controls (Table 1). Of particular interest, we found that APP mice have increased gene expression levels of cyclin-dependent kinase 5 (cdk5), a key molecule leading to tau phosphorylation. Besides cdk5, Tg2576 mice exhibited increased mRNA levels for synaptotagmin IV, protein kinase C β 1, 3-phosphoinositide dependent protein kinase 1 and the gamma-aminobutyric acid receptor β 2 and decreased mRNA levels for the proteasome 28 subunit α .

In addition to these, the mRNA screen identified CXCL12 as being a potential candidate for the behavioral deficits found in the Tg2576 mouse model of AD. CXCL12 is a molecule that has been shown to regulate neuronal excitability and synaptic transmission (Bezzi et al., 2001; Innocenti et al., 2000; Kang et al., 1998). The gene expression analysis revealed that CXCL12 levels were decreased by 1.8 fold in APP mice compared to non-transgenic controls at this timepoint, which coincides with the onset of behavioral deficits. This decrease in CXCL12 mRNA levels was confirmed using quantitative RT-PCR ($P<0.01$), and we further validated the GeneChip data with RT-PCR analysis of synaptotagmin IV ($P<0.01$), which decreased 1.75 fold in accordance with the GeneChip data (Figure 1A).

CXCL12 protein levels are concurrently decreased in Tg2576 mice

To determine if the decrease in CXCL12 at the mRNA level corresponded to decreased protein, we examined CXCL12 protein levels in the hippocampus of 6 and 17 month old Tg2576 mice and age-matched non-transgenic controls. Concurrent with mRNA levels, we found that CXCL12 protein levels are significantly decreased in APP mice compared to age-matched non-transgenic mice at both 6 and 17 months of age ($P<0.05$, Figure 1B), as measured by sandwich ELISA. We were additionally able to demonstrate that this decrease in CXCL12 is not restricted to the Tg2576 mouse model. We found that CXCL12 protein levels are decreased in 24-month old triple transgenic mice compared to non-transgenic controls ($P<0.01$, Figure 1B). The sole receptor for the CXCL12 chemokine is CXCR4. Examining the expression level of this receptor, we found that CXCR4 protein levels are concurrently decreased in 6 month old Tg2576 mice compared to age-matched non-transgenic controls ($P<0.01$, Figure 1C, D).

CXCL12 levels are found decreased in AD patients

In order to determine the relevance of this chemokine in AD, we examined CXCL12 protein levels in AD patients and age-matched non-demented controls. Western blot analysis revealed that CXCL12 levels are significantly decreased with AD ($P<0.05$, Figure 1E, F) in concurrence with the findings using the Tg2576 mouse model of the disease. We additionally examined the levels of CXCR4 in AD and control cases and found that while CXCR4 levels decrease in Tg2576 mice, the levels in AD patients are significantly increased as compared to controls ($P<0.01$, Figure 1E, F). These findings of decreased CXCL12 in AD patients suggest that CXCL12 is integrally involved in the disease process. The increase in CXCR4 with AD may be indicative of compensatory up-regulation of the CXCL12 receptor.

CXCL12 is associated with astrocytes

In order to determine which cell type express this chemokine, we immunostained for CXCL12 and a marker for either microglial cells or astrocytes in 6 month old non-transgenic and Tg2576 mice. Our findings revealed that CXCL12 co-localizes with astrocytes (Figure 2A-C), while we find no co-localization between CXCL12 and microglial cells (Figure 2D-F).

CXCR4 is expressed in brain regions critical for learning and memory

We localized CXCR4 expression to the hippocampus with little to no expression in the amygdala in accordance with previous *in situ* gene expression data (Magdaleno et al., 2006). Furthermore, immunofluorescent labeling for CXCR4 confirmed the western blot findings of decreased CXCR4 protein levels in the hippocampus of Tg2576 transgenic mice as compared to non-transgenic controls (Figure 1G, H). These findings suggest that CXCL12 signaling occurs primarily in areas of the brain important for learning and memory, and also brain areas which are impacted in AD.

A β levels are low in 6 month old Tg2576 mice compared to 12 month old animals

To show that transgenic mice at 6 months of age exhibit low levels of plaque/insoluble A β , we compared 6 month old transgenic animals to 12 month old Tg2576 mice. We found that compared to 12 month old mice, 6 month old Tg2576 mice exhibited 10000-fold less insoluble A β levels ($P<0.01$, Figure 2J). We also sought to quantify soluble A β levels at these ages and found that while A β_{42} remains unaltered, A β_{40} levels were significantly lower in 6 month old as compared to 12 month old transgenic mice ($P<0.05$, Figure 2I). In summary, compared to 12 month old transgenic mice, Tg2576 at 6 months exhibit little soluble A β pathology in the absence of insoluble plaque deposits. This does not exclude that oligomers were present in the soluble pool.

CXCR4 antagonist impaired primarily hippocampus and cortex dependent memory while sparing amygdala dependent memory

We identified decreased levels of the chemokine CXCL12 as being potentially responsible for the behavioral deficits observed in Tg2576 mice as they occurred at ages with cognitive impairments. Furthermore, this chemokine has been previously shown to modulate synaptic transmission and information processing in the CNS (Bezzi et al., 2001; Meucci et al., 1998) while we have found that altered inflammatory responses early on in AD correlate with cognitive decline (Parachikova et al., 2006). If the changes in CXCL12/CXCR4 in the Tg2576 were responsible for the early cognitive impairments in these animals, then mimicking CXCL12/CXCR4 downregulation in young non-transgenic, non-impaired mice should cause similar cognitive deficits. Therefore, we sought to determine if the reduced CXCL12/CXCR4 signaling could be directly responsible for learning and memory deficits. Towards this goal, we treated young (three-month old) non-transgenic mice with the CXCL12 receptor antagonist, AMD3100 for a period of three weeks. AMD3100 is a specific CXCR4 antagonist that inhibits the binding and function of CXCL12 with high affinity and potency (Fricker et al., 2006; Rosenkilde et al., 2004). After the 3 week treatment with AMD3100, we performed a battery of behavioral tasks, testing learning and memory performance on mainly cortical, hippocampal and amygdala dependent tasks (Aggleton et al., 1997; Blanchard and Blanchard, 1972; Dember and Fowler, 1958; Dember and Fowler, 1959; Holahan and White, 2002; Mumby et al., 2002; Phillips and LeDoux, 1992; Wan et al., 1999).

AMD3100 treatment had no visible negative effects on the animals' health. Animals appeared healthy and no change in weight was observed at the end of treatment.

Treatment of young, non-transgenic mice with the CXCR4 antagonist for a period of 3 weeks affected their ability to perform on mainly cortex dependent memory tasks. On the novel object task, on both 1.5 and 24 hour retention tests, we found that treated mice performed significantly worse than untreated controls and spent less time with the novel object ($P<0.01$, Figure 3A, B). Tests for novel place and context also showed that at the 1.5 hour retention test, AMD3100 treated mice performed at chance level as compared to untreated controls, which performed at 70% ($P<0.01$) and 60% ($P<0.05$) respectively (Figure 3C, D).

We also found that 3 weeks of AMD3100 lead to a significant abolishment of primarily hippocampus dependent memory as measured via T maze. Treated animals performed at chance level and were significantly different from untreated controls, which performed at 80% ($P<0.01$, Figure 4A). When examining performance over time, we find that treated animals do actually learn; they performed at 60% on days 4 and 5 compared to 40% on days 1 through 3 (Figure 4B).

The effects of AMD3100 treatment on the mainly amygdala dependent task, inhibitory avoidance, were rather different from the cortex and hippocampal task findings. Treated

animals performed similar to untreated controls on both 1.5 and 24 hour retention tests (Figure 4C, D). These findings suggest that amygdala dependent learning and memory are not affected by AMD3100 treatment. Importantly, when we examined the expression level of the CXCL12 receptor CXCR4, we found little to no expression in the amygdala while the expression in the hippocampus was high (Figure 2G).

Discussion

The current study provides the first evidence that CXCL12/CXCR4 signaling can contribute significantly to learning and memory and may be critically involved in AD. We show that decreased levels of the chemokine CXCL12 and its receptor CXCR4 correlate with the timepoint of behavioral deficits in Tg2576 mice. CXCL12 levels are additionally found to be decreased in AD patients as compared to non-demented controls, suggesting that this chemokine may play a role in AD pathogenesis. We have then been able to directly link CXCL12/CXCR4 and cognitive functioning by demonstrating that systemic administration of a CXCR4 antagonist results in impaired learning and memory in young non-transgenic mice.

Despite 10 years of studying the Tg2576 mouse model of AD (Hsiao et al., 1996), the exact cause of the cognitive decline has not been elucidated. Previous research using this mouse model has primarily focused on plaque pathology, but behavioral deficits are shown to occur months earlier (Jacobsen et al., 2006; Westerman et al., 2002). Therefore, in the current study, we used an un-biased approach to identify the gene expression changes present at the time of cognitive deficits. We have identified several factors including cdk5, synaptotagmin IV, protein kinase C β 1, 3-phosphoinositide dependent protein kinase 1, gamma-aminobutyric acid receptor β 2 and proteasome 28 subunit α as being significantly altered at the gene expression level in 6 month old Tg2576 mice as compared to age-matched non-transgenic controls. Cdk5 is a putative tau kinase, which has been linked to tau phosphorylation (Baumann et al., 1993; Flaherty et al., 2000). However, in the Tg2576 mice at this age, there is no report of tau phosphorylation. Aberrant tau phosphorylation has been linked to memory deficits (Arendt et al., 1995; Liu et al., 2003). Also, cdk5 functions as an essential modulator of synaptic transmission through the phosphorylation of a numerous synaptic proteins (Cheung et al., 2006). Another factor that may affect synaptic transmission is the synaptic marker synaptotagmin IV (SYT IV). Tg2576 mice at 6 months show increased mRNA levels for this marker compared to age-matched non-transgenic controls. Synaptic markers decrease in clinically end stage AD patients but early AD has been associated with increased levels of presynaptic markers (Mukaetova-Ladinska et al., 2000). This increase in synaptic markers early in AD and at the onset of behavioral deficits in the Tg2576 model of the disease may reflect increased synaptic plasticity. Interestingly, SYT IV knockout mice exhibit deficits in fine motor coordination and hippocampus-dependent memory (Ferguson et al., 2000). Notably this is another glia protein which has been shown to contribute to hippocampal-dependent memory like CXCL12 and it does so by regulating glia glutamate release through local calcium signals (Zhang et al., 2004). In the Tg2576 mice, however, the mRNA levels of SYT IV are increased, while CXCL12 levels are decreased. This could suggest a compensation mechanism in calcium dependent glia to neuron communication, or increases in SYT IV levels could themselves affect cognition.

Even though these transcriptional changes or other post-transcriptional modifications could contribute to the behavioral deficits in Tg2576 mice, in the current manuscript, we have decided to focus on CXCL12 (SDF-1) as a potential candidate underlying the learning and memory deficits. We found decreased mRNA and protein levels of CXCL12, which is a chemokine involved in neuron-glia communication and thus may affect memory via altered communication in the CNS. We also show that the receptor for CXCL12, CXCR4 is concurrently decreased at the protein level in 6 month old transgenic mice compared to age-

matched non-transgenic controls. Chemokines are small chemoattractant molecules playing a key role in inflammation and immunity (Feng, 2000; Murdoch and Finn, 2000; Rossi and Zlotnik, 2000). In addition to their role in neuroinflammation, chemokines, such as CXCL12, have been shown to participate in neuronal signaling (Asensio and Campbell, 1999; Limatola et al., 2000; Meucci et al., 1998; Zheng et al., 1999). The mechanism by which CXCL12 has been proposed to regulate neuronal excitability is by enhancing glutamate release from astrocytes (Bezzi et al., 2001). Glutamate release from astrocytes has been implicated in modulating the firing activity in neuronal circuits (Araque et al., 1998; Newman and Zahs, 1998), inducing plastic changes of synaptic transmission (Kang et al., 1998) and mediating neuronal synchrony through the activation of NMDA receptors (Fellin et al., 2004). We speculate that decreased levels of CXCL12 might interfere with proper neuronal signaling and may therefore negatively affect memory. We additionally have provided evidence for CXCL12 co-localizing with astrocytes and not with microglial cells. Hence, our findings of decreased CXCL12 at the timepoint of behavioral deficits in Tg2576 mice suggest that it may be an A β -mediated (or APP related) secondary mechanism that underlies the behavioral deficits in these transgenic mice. In order to establish if CXCL12/CXCR4 directly affects learning and memory and to mimic the behavioral deficits observed in Tg2576 mice, we treated young non-transgenic mice with the CXCR4 antagonist, AMD3100. We show that 3 weeks of AMD3100 treatment results in impaired learning and memory.

The current study thus suggests that the behavioral deficits in Tg2576 mice are mediated, at least in part, through a secondary effect of A β on CXCL12 signaling. In addition, we demonstrate that the lack of CXCL12/CXCR4 leads to impaired memory further supporting a key role of this chemokine and its receptor in learning and memory. In conclusion, we have identified a potential new pathway that is in part responsible for aspects of cognitive functioning and can thus represent a novel target in disorders affecting learning and memory.

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Figure 1.

CXCL12/CXCR4 levels (A - E). (A) Quantitative RT-PCR confirmation of GeneChip findings showing a 1.8 fold decreased CXCL12 mRNA levels and a 1.75 fold increase in synaptotagmin IV mRNA in 6 month old Tg2576 transgenic compared to non-transgenic mice ($P < 0.01$) ($n = 6$ /group). (B) ELISA data for CXCL12 protein levels in 6 and 17 month old Tg2576 ($n = 6$) as well as 24 month old triple transgenic ($n = 6$) (3xTg-AD) compared to age-matched non-transgenic mice. CXCL12 protein levels are significantly decreased in transgenic compared to non-transgenic mice. * indicates $P < 0.05$ and ** indicates $P < 0.01$. (C) Western blot for the CXCL12 receptor CXCR4 and β-actin loading control in 6 month old Tg2576 and age-matched non-transgenic mice ($n = 5$ /group). (D) CXCR4 quantification obtained as the CXCR4/β-actin ratio revealed that Tg2576 transgenic mice at 6 months of age have significantly decreased CXCR4 protein levels as compared to age-matched non-transgenic controls ($P < 0.01$). (E)

Western blot for CXCL12, CXCR4, and GAPDH loading control in AD and control subjects (n=4/group). CXCL12 protein levels obtained as the CXCL12/GAPDH ratio are significantly increased ($P<0.05$) in AD patients as compared to age-matched controls. In contrast, CXCR4 levels obtained as the CXCR4/GAPDH ratio for both the CXCR4 monomer and dimer combined are significantly decreased ($P<0.01$) in AD patients as compared to age-matched controls.

Figure 2. CXCL12/CXCR4 immunostaining (A-H) and amyloid load analysis (I, J). Immunofluorescent staining for CXCL12 (B, E) and the astrocyte marker GFAP (A) or the microglial marker IBA1 (D) in non-transgenic and Tg2576 transgenic mice (n=6/group) revealed that CXCL12 co-localized with GFAP (D) and not with IBA1 in the hippocampus (F). Images taken at 20X magnification. (G, H) Immunofluorescent staining for the CXCL12 receptor, CXCR4, in non-transgenic and Tg2576 transgenic mice (n=6/group). Images taken at 40X magnification. (G) Non-transgenic mice exhibit high expression of CXCR4 in the hippocampus and little to no expression in the amygdala (data not shown). (H) Tg2576 mice show little CXCR4 expression in the hippocampus and also no staining in the amygdala. The decreased expression

of CXCR4 in transgenic as compared to non-transgenic mice validates the western findings. Amyloid load analysis is depicted in **(I, J)**. **(I)** Soluble $A\beta_{40}$ and $A\beta_{42}$ levels in 6 and 12 month old Tg2576 mice. There is no difference in $A\beta_{42}$ levels, while $A\beta_{40}$ levels are significantly higher in 12 month old transgenic mice ($P<0.05$). **(J)** Insoluble $A\beta_{40}$ and $A\beta_{42}$ levels in 6 and 12 month old Tg2576 mice. Both $A\beta_{40}$ and $A\beta_{42}$ levels are significantly increased by 10000 fold in 12 month old compared to 6 month Tg2576 mice.

Figure 3.

Behavioral analysis of primarily cortex dependent memory in AMD3100 and PBS treated young non-transgenic mice (n=9). Novel object recognition is depicted in (A, B). Treatment with the CXCR4 antagonist AMD3100 resulted in memory deficits at the 1.5 hour retention (A) and the 24 hour retention (B) tests. Treated animals performed at chance level while PBS controls spent 60-70% with the novel object. Tests for novel place (C) and context (D) confirm the primarily cortex dependent memory deficit in AMD3100 treated animals as compared to PBS treated controls. AMD3100 treated mice performed at chance level. * indicates $P<0.05$ and ** indicates $P<0.01$.

Figure 4.

Behavioral analysis of primarily hippocampus (A,B) and amygdala (C,D) dependent memory in AMD3100 and PBS treated young non-transgenic mice. (A) T-maze performance showed that AMD3100 treated animals performed significantly worse than PBS treated mice on spontaneous alterations. Drug treatment resulted in performance at chance level while controls had an 80% alteration rate over the course of 5 days of testing. (B) T-maze performance over time showed that while AMD3100 mice performed at chance level on average, they so learn the task over the 5 days of testing. (C,D) Testing of primarily amygdala dependent memory via inhibitory avoidance (IA). AMD3100 treated mice performed the same as PBS treated animals on both IA 1.5 (C) and the 24 hour (D) retention tests.

Table 1**Significantly changed mRNA sequences in 6 month Tg compared to 6 month nonTg**

Significantly changed mRNA sequences in the hippocampus of 6 month old Tg2576 compared to age-matched non-transgenic mice (n=6/group). GeneChip analysis was performed on hippocampal samples of transgenic and non-transgenic mice. Data was analyzed using GC-RMA criteria of $P < 0.05$ and fold change > 1.5 , where P stands for significance and fold change represent the gene expression change between transgenic and non-transgenic. Highlighted are sequences of particular interest in relation to cognitive dysfunction.

GC RMA		Affymetrix ^b	
P	fold change ^a	sequence annotation	id
4.06E-03	2.14	exostoses (multiple) 1	102811_at
3.69E-02	2.13	ELAV -like 4 (Hu antigen D)	92361_at
1.56E-02	2.10	special AT-rich sequence binding protein 1	99665_at
2.24E-03	2.06	v-maf AS42 oncogene homolog	92354_at
4.06E-03	2.02	crystallin, lamda 1	161779_r_at
6.86E-03	2.02	hypothetical protein LOC544836	161688_r_at
3.04E-02	2.01	myeloid ecotropic viral integration site-related gene 1	97988_at
3.54E-02	2.00	nuclear receptor subfamily 4, group A, member 2	92249_g_at
6.17E-03	1.98	methyl-CpG binding domain protein 4	103285_at
5.07E-03	1.98	LIM domain binding 2	93682_at
4.62E-03	1.96	F-box and WD-40 domain protein 7	93667_at
2.36E-02	1.93	forkhead box P1	96183_at
7.24E-03	1.90	zinc finger protein 312	92229_at
1.18E-02	1.88	synaptotagmin IV	160190_at
1.47E-02	1.68	protein tyrosine phosphatase, receptor type, D	93896_at
4.24E-02	1.67	casein kinase II, alpha 2, polypeptide	98245_at
3.06E-02	1.66	protein kinase C, beta 1	99511_at
1.96E-03	1.62	acidic nuclear phosphoprotein 32 family, member A	93372_at
2.53E-02	1.61	ST3 beta-galactoside alpha-2,3-sialyltransferase 5	98596_s_at
1.22E-02	1.61	LIM domain only 4	98122_at
4.65E-02	1.58	3-phosphoinositide dependent protein kinase-1	102063_at
2.44E-02	1.57	CBFA2T1 identified gene homolog (human)	92935_at
2.96E-02	1.54	cyclin-dependent kinase 5, (p35) 1	102664_at
1.60E-02	1.53	zinc and ring finger 1	95157_at
1.37E-02	1.52	neurogranin	96273_at
4.80E-02	1.52	gamma-aminobutyric acid receptor, beta 2	101138_at
1.12E-02	1.52	nuclear receptor subfamily 1, group D, member 1	98507_at
2.34E-02	1.51	EF hand domain containing 2	160100_at
4.45E-02	-1.55	hippocalcin	99944_at
4.82E-02	-1.55	proteasome (prosome, macropain) 28 alpha	101510_at
4.87E-02	-1.58	enoyl coenzyme A hydratase 1, peroxisomal	93754_at
4.08E-02	-1.66	kinesin 2	97984_i_at
3.09E-02	-1.75	tumor protein D52-like 1	101446_at
2.21E-02	-1.76	WNK lysine deficient protein kinase 1	161270_i_at
3.43E-02	-1.77	copine V1	93151_at
3.55E-02	-1.79	chemokine (C-X-C motif) ligand 12	160511_at
2.62E-02	-1.82	activating signal cointegrator 1 complex subunit 1	92375_at
4.16E-02	-1.87	folate receptor 1 (adult)	93785_at
3.21E-02	-1.96	carbonic anhydrase 12	103905_at
4.68E-02	-2.12	histidyl-tRNA synthetase	92580_at
7.59E-03	-2.15	huntingtin-associated protein 1	102323_at
1.16E-02	-2.24	calmodulin-like 4	96203_at
3.15E-02	-2.44	neuronatin	97520_s_at

^a Fold change in transgenic as compared to non-transgenic.

^b Reference sequence annotation and probe set id from U74Av2 GeneChip array.