Published in final edited form as:

Brain Res. 2010 March 8; 1318: 11–22. doi:10.1016/j.brainres.2009.12.091.

Sex effects of Interleukin-6 deficiency on neuroinflammation in aged C57Bl/6 mice

VM Miller^{1,2,#}, DA Lawrence^{1,2}, GA Coccaro², TK Mondal¹, K Andrews¹, A Dreiem^{1,2}, and RF Seegal^{1,2}

¹ Biggs Laboratory, Wadsworth Center, New York State Department of Health, Empire State Plaza, Albany, NY 12201, USA

² School of Public Health, University at Albany, State University of New York, 1400 Washington Avenue, Albany NY 12222, USA

Abstract

High levels of Interleukin-6 (IL-6) are associated with an increased risk of dementia in the elderly and can increase neuroinflammation in mice. Dementia is more frequent in females, and IL-6 is regulated by estrogen, suggesting elevated IL-6 levels may contribute to neuroinflammation and dementia particularly in women. Therefore we hypothesized that IL-6 deficient ($-/-$) female mice would have lower aging-related neuroinflammation than wild type (WT). We quantified neuroinflammatory markers which are affected by aging, and regulated by both estrogen and IL-6; glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), interferon gamma ($\text{IFN}\gamma$), lipid peroxidation (MDA), and synaptic density (SNAP25) and in IL-6 $-/-$ and WT C57Bl/6 mice. To determine age effects we used mid-age (18 months) and old-age (24 months) mice, and to determine region specific effects we used the hippocampus which is impaired in dementia and the cerebellum which is unimpaired in dementia. Unexpectedly, there were no effects of IL-6 deficiency on GFAP, MDA or SNAP25 levels in females, but IL-6 deficiency was associated with lower cerebellar MBP ($p < 0.05$) levels. Interestingly, the old-aged IL-6 $-/-$ males had higher GFAP and MDA levels ($p < 0.05$) in both the hippocampus and cerebellum, in addition to a greater body weight than WT. We suggest that IL-6 is important for promoting myelin synthesis in aged females, and that drugs which inhibit the synthesis of IL-6 in males may inadvertently affect fatty acid metabolism and augment aging-related neuroinflammation.

Keywords

Age; sex; neuroinflammation; interleukin-6; hippocampus; cerebellum; glial fibrillary acidic protein; myelin basic protein; lipid peroxidation

1. Introduction

Elevated levels of Interleukin-6 (IL-6) are associated with an increased risk of dementia and decreased hippocampal volume in older people [Angelopoulos *et al.* 2008; Huberman *et al.* 1995; Wada-Isoe *et al.* 2004; Marsland *et al.* 2008; Ravaglia *et al.* 2007]. Some epidemiological

#Corresponding Author: Veronica M Miller, vmiller@wadsworth.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

studies suggest that the use of anti-inflammatory agents can decrease risk of dementia in the elderly, however other studies suggest there is no beneficial effect of the use of anti-inflammatory agents to decrease dementia risk in older people [Wyss-Coray 2006; Breitner *et al.* 2009; Arvanitakis *et al.* 2008]. Therefore it is important to determine if lowering the levels of cytokines associated with dementia, such as IL-6 can reduce aging-related inflammation in dementia related brain regions such as the hippocampus. In mice the over-expression of IL-6 is associated with higher levels of neuroinflammation [Bellinger *et al.* 1995; Steffensen *et al.* 1994; Di Santo *et al.* 1996]. Conversely, IL-6 deficient ($^{-/-}$) mice have improved cognition; attributed to improved hippocampal function, suggesting that lowering IL-6 levels may have beneficial anti-inflammatory and cognitive effects [Braidia *et al.* 2004].

However, IL-6 is not only associated with hippocampal function, it is also a sex-specific risk factor for immune diseases, contributing to an increased risk of auto-immune disease in women, and liver disease in men [Bouman *et al.* 2004; Lleo *et al.* 2008; Hootman *et al.* 2002; Balschun *et al.* 2004]. IL-6 synthesis is inhibited by estrogen, and women have twice the risk of developing dementias such as Alzheimer's disease than men and suggesting elevated IL-6 levels may particularly contribute to dementia risk in women [Gao *et al.* 1998; Kurebayashi *et al.* 1997; Liu *et al.* 2005]. In aged mice, lower levels of estrogen are associated with greater hippocampal astrocyte density and lower synaptic density in females compared with males [Mouton *et al.* 2002; Berchtold *et al.* 2008; Liu, Liu, and Bodenner 2005]. Therefore, we suggest that lower IL-6 levels may reduce aging-related neuroinflammation particularly in females. Whether sex alters the effects of IL-6 deficiency on the brain is unclear, because previous studies on the neurological effects of IL-6 deficiency were predominantly conducted in males [Bellinger, Madamba, Campbell, and Siggins 1995; Braidia, Sacerdote, Panerai, Bianchi, Aloisi, Iosue, and Sala 2004].

The aging-process is associated with elevated astrocyte density, oxidative stress, decreased synaptic plasticity, and decreased white matter volume and protein density [Godbout and Johnson 2009; Mouton, Long, Lei, Howard, Jucker, Calhoun, and Ingram 2002; Resnick *et al.* 2003; Morrison and Hof 1997]. Interestingly, astrocytic, synaptic, and myelin basic proteins are also regulated by IL-6 and estrogen [Martinez 2006; Jung-Testas *et al.* 1994; Behl *et al.* 1997; Zhang *et al.* 2006; Nakanishi *et al.* 2007]. Therefore if IL-6 deficiency can lower aging-related inflammation in aged females, it may particularly affect the expression of astrocytic, synaptic or myelin associated proteins. To address our hypothesis and determine if IL-6 deficiency can lower aging-related inflammation in aged females in particular, we quantified markers directed against; glial-fibrillary acidic protein (GFAP), lipid peroxidation (MDA), IFN γ ; a cytokine regulated by IL-6, myelin-basic protein (MBP) and SNAP25, a synaptic protein in mid age (18 month) and old age (24 months) IL-6 $^{-/-}$ and WT C57Bl/6 mice [Andorfer *et al.* 2005]. To determine if IL-6 deficiency effects were region specific we included the hippocampal formation which is affected in Alzheimer's type dementia, and the cerebellum which is unaffected in dementia [Thal *et al.* 2002]. In summary this paper describes the age, sex and regional effects of IL-6 deficiency on neuroinflammation in C57Bl/6 mice.

2 Results

2.1 Gross Morphological changes

In the 24 month mice, ANOVA revealed there was a main effect of sex ($p=0.000$) but not strain ($p=0.45$) on body weight. Subsequent analysis with Bonferroni correction revealed the 24 month IL-6 $^{-/-}$ males were significantly heavier (43.5 g) than the 24 month WT males (36.18 g) ($p=0.045$) but there were no differences in body weight between the 24 month WT (37.5g) and IL-6 $^{-/-}$ (37.7g) females (Figure 1a). In the 18 month mice ANOVA revealed a main effect of sex; males were significantly heavier than females ($p=0.05$), but no main effects of strain on body weight (Figure 1b).

2.2 Hippocampus GFAP density

In the 24 month mice, ANOVA revealed a main effect of sex on GFAP levels; females had higher GFAP levels than males ($p=0.006$), but there was no main effect of strain on GFAP levels ($p=0.182$). Bonferroni correction for intra-group comparisons revealed higher GFAP levels in the hippocampus of WT females compared with WT males ($p=0.045$). The IL-6^{-/-} males had higher GFAP levels than WT males ($p=0.001$), but there were no differences in GFAP levels between the IL-6^{-/-} and WT females ($p=0.361$) (Figure 2b). Photomicrographs of hippocampal tissue sections immunostained with the anti-GFAP antibody are shown in figure 2a. There was stronger GFAP immunoreactivity in the hippocampus of the aged WT females compared with WT males (Figure 2c).

In the 18 month mice ANOVA determined a main effect of sex on GFAP levels; males had higher GFAP levels than females ($p=0.010$), but there was no main effect of strain on GFAP levels ($p=0.845$) (Supplemental Figure 1a).

2.3 Cerebellum GFAP density

Within the 24 month mice, ANOVA determined no main effect of strain (0.189) or sex (0.814) on GFAP levels. Bonferroni analysis revealed higher GFAP levels in the WT females compared with the WT males ($p=0.001$). The IL-6^{-/-} males also had higher levels of GFAP than the WT males ($p=0.05$) but there were no differences in GFAP levels between the IL-6^{-/-} and WT females (Figure 3b). Photomicrographs taken of cerebellum tissue sections illustrate that the WT 24 month females had the most prominent GFAP immunoreactivity (Figure 3c).

In the 18 month mice, ANOVA revealed no effect of strain ($p=0.283$) or sex ($p=0.136$) on cerebellum GFAP levels. Further analysis with Bonferroni correction found no differences in GFAP levels between the WT males and females ($p=0.827$) or between the IL-6^{-/-} and WT male or female mice (data not shown).

2.4 MDA levels in Hippocampus and Cerebellum

The TBA assay was determined MDA production as a measure of lipid peroxidation in the 24 month mice. In the hippocampus ANOVA determined no effect of sex ($p=0.770$) or strain ($p=0.379$) on MDA levels. Bonferroni analysis revealed that the male IL-6^{-/-} mice had higher MDA levels in the hippocampus than WT males ($p=0.015$) and female IL-6^{-/-} mice ($p=0.015$) (Figure 4a). In the cerebellum ANOVA revealed no main effect of sex ($p=0.822$) or strain on MBP levels ($p=0.593$). There were no intra-group differences in MDA levels revealed in the cerebellum using Bonferroni analysis (Figure 4b).

2.5 IFN γ in Hippocampus and Cerebellum

In the hippocampus of the 24 month mice ANOVA revealed no main effect of sex ($p=0.142$) or strain ($p=0.225$) on IFN γ levels. Bonferroni analysis revealed a trend towards lower IFN γ in the IL-6^{-/-} males compared with WT males ($p=0.061$) (Fig 5a). In the cerebellum ANOVA revealed no main effect of sex ($p=0.763$) or strain ($p=0.0671$) on IFN γ levels. Bonferroni analysis revealed that IL-6^{-/-} females had higher IFN γ levels in the cerebellum compared with WT females ($p=0.024$) (Figure 5b).

2.6 MBP in the Cerebellum

Myelinated axons in the cerebellum tissue sections were stained with luxol fast blue (LFB) a histological stain for white matter. The WT females showed less rarefaction of the white matter than the WT males and IL-6^{-/-} mice (Figure 6a). In the cerebellum ANOVA revealed no main effect of sex ($p=0.134$) or strain (0.175) on MBP levels. Bonferroni analysis found a significant sex by strain interaction ($p=0.015$). There were higher MBP levels in the WT females compared

with the IL-6^{-/-} females (p=0.039), WT males (p=0.038) and IL-6^{-/-} males (p=0.044) (Figure 6b).

To determine if the elevation in MBP in the WT females at 24 months was associated with dominant isoform of MBP, we compared the composition of the 24 kDa, 22 kDa, 21.5 kDa, 18 kDa and 14 kDa protein isoforms of MBP between the groups. ANOVA determined no effect of sex, strain or sex by strain on MBP isoform composition. Bonferroni analysis determined a higher expression of the 24kDa isoform in the WT females compared with WT males (0.015), IL-6^{-/-} females (0.009) and IL-6^{-/-} males (0.062) (Figure 6c). Additionally, in the 18 month mice using ANOVA we found no differences in MBP levels between the WT males and IL-6^{-/-} males, nor a difference between WT females and IL-6^{-/-} females (data not shown).

2.7 SNAP25 in the Hippocampus and Cerebellum

In the tissue sections stained with the anti-SNAP25 antibody, there were bands of synaptic rich tissue stained adjacent to the hippocampal CA1–4 neuronal fields and cells of the dentate gyrus (Figure 7a). In the 24 month mice ANOVA determined a main effect of sex on SNAP25 expression; males had higher SNAP25 levels than females (p=0.048). There was no main effect of strain on SNAP25 levels (p=0.598). Bonferroni revealed no significant sex by strain interactions on SNAP25 expression (Figure 7bc). In the 18 month mice ANOVA revealed a main effect of sex on SNAP25 levels: females had higher SNAP25 levels than males (p=0.017), but there were no main effects of strain on SNAP25 levels (p=0.860) (Supplemental Figure 1b).

2.7. Serum levels of Thyroxine and Estradiol

A commercially available thyroxine ELISA kit was used to determine serum levels of thyroxine in the 18 month mice (n=3 per group). We found no effects of sex or strain on thyroxine levels (Table 1). A commercially available estradiol kit was used to determine serum levels of estradiol in the aged and IL-6^{-/-} mice. ANOVA and Bonferroni analysis determined no effects of sex or strain on estradiol levels at 18 months (Table 1).

Discussion

In some agreement with our hypothesis we found that IL-6 deficiency was associated with sex-specific effects on neuroinflammation in aged mice. Unexpectedly, there were no effects of IL-6 deficiency on GFAP, or lipid peroxidation levels in the aged females, but the old-aged IL-6^{-/-} males had higher GFAP and lipid peroxidation levels in the hippocampus compared with WT males. The old-aged IL-6^{-/-} males also had a higher body weight than aged WT males. There is a report of increased somatic adipogenesis, obesity and fatty acid metabolism in IL-6 deficient mice [Wallenius *et al.* 2002]. Interestingly, increased fatty acid metabolism and decreased glucose sensitivity are reportedly associated with elevated astrocytic density in the agouti viable yellow mice (a model of obesity) and in rat models of diabetes mellitus [Pan *et al.* 2008; Lebed *et al.* 2008]. Therefore we suggest that the unexpectedly “pro-inflammatory” effects of IL-6 deficiency in the aged male actually reflects metabolic abnormalities induced by IL-6 deficiency, latterly associated with increased body weight and higher levels of GFAP and lipid peroxidation in the brain. There were lower levels of IFN γ in the aged IL-6^{-/-} males which may appear to confound the increased lipid peroxidation and GFAP levels found therein. However, lower IFN γ levels are found in obese individuals; suggesting obesity in the old-aged IL-6^{-/-} male mice may likely have contributed to central metabolic responses manifest as an increase in lipid peroxidation and GFAP levels [Fontana *et al.* 2007]. Furthermore, there is evidence in the literature to suggest that IL-6 may be particularly important for metabolic regulation in males as compared with females; IL-6 levels are associated with altered insulin

sensitivity in men but not women [Fernandez-Real *et al.* 2001]. In addition, the fact that the effects of IL-6 deficiency in the aged males were not specific to the hippocampus and was also found in the cerebellum suggests a global rather than a centralized effect of IL-6 deficiency in the males.

In the literature there are conflicting reports of both improved and impaired cognition in young IL-6^{-/-} males [Bellinger, Madamba, Campbell, and Siggins 1995; Braida, Sacerdote, Panerai, Bianchi, Aloisi, Iosue, and Sala 2004]. In the aged IL-6^{-/-} males, in spite of a noticeable elevation in hippocampal GFAP and lipid peroxidation levels, there were no differences in SNAP25 levels. The lack of an effect of IL-6 deficiency on hippocampal SNAP25 levels implies cognition, or synaptic plasticity may not have been substantially affected, however, cognitive impairment may be associated with central metabolic insufficiency associated with elevated GFAP and lipid peroxidation. Diabetes and dislipidemia are known to increase the risk of vascular dementia in older people, in addition to causing cognitive impairment in mice [Kloppenborg *et al.* 2008; Biessels and Gispen 2005]. Thus, neurobehavioral assays would be useful to determine if there are cognitive effects of increased GFAP, lipid peroxidation, and obesity in the aged IL-6^{-/-} males rather than females.

Contrary to our hypothesis, there were no differences in GFAP or MDA levels in either the hippocampus or cerebellum of the aged IL-6^{-/-} and WT females. In accordance with other studies which found age and sex effects on hippocampal synaptophysin expression in aged C57Bl/6 mice, we found sex and age effects on hippocampal SNAP25 and also GFAP levels [Benice *et al.* 2006]. At 18 months the females had higher hippocampal SNAP25 levels than the males, whereas at 24 months, the males had higher SNAP25 levels than the females. In addition, at 18 months the males had higher GFAP levels than 18 month females, but at 24 months the females had higher GFAP levels than the males. The literature suggests that lower estrogen synthesis with age contributes to elevated astrocytic density and synaptic loss in females compared with males. For example estrogen treatment to aged and ovariectomized 24 month female C57Bl/6 mice can lower inflammation in the hippocampus [Mouton, Long, Lei, Howard, Jucker, Calhoun, and Ingram 2002; Lei *et al.* 2003]. However, unlike the aforementioned anti-inflammatory effect of estrogen treatment, we found that IL-6 deficiency was insufficient to decrease hippocampal inflammation in the aged females.

Interestingly, in the cerebellum we found that IL-6 deficiency was associated with lower MBP levels in the old-aged females but not males. In females, higher myelin levels are attributed to a greater level of myelin protein turnover [Li *et al.* 2006; Yang *et al.* 2008]. Interestingly elevated myelin turnover is not solely dependent on estrogen, sex differences in myelin turnover persists even after gonadectomy in rats [Li, Penderis, Zhao, Schumacher, and Franklin 2006]. IL-6 treatment directly increases oligodendrocyte myelin synthesis and promotes the differentiation of embryonic stem cells into myelin expressing oligodendrocytes [Zhang, Izrael, Ainbinder, Ben-Simchon, Chebath, and Revel 2006; Valerio *et al.* 2002]. The aged WT females had higher MBP levels than the IL-6^{-/-} aged females which we suggest indicates that IL-6, rather than estrogen per se is particularly important for white matter synthesis. Furthermore, treatment with IL-6 may promote myelin synthesis in older females with demyelinating diseases.

There was a striking similarity in the levels of GFAP and MDA in both the hippocampus and cerebellum between the aged IL-6^{-/-} males and WT females. It could be argued that the aged IL-6^{-/-} male mice acquired a phenotype akin to the aged WT females. Potentially such a phenotypic shift may involve altered estrogen levels in the IL-6^{-/-} males. Estrogen synthesized via the aromatization of testosterone in adipose tissues of the obese old-aged IL-6^{-/-} males is a plausible source of estrogen in the males. However, there are reports of no differences in serum estrogen, testosterone or dehydroxytestosterone levels between IL-6^{-/-} and WT males

[Morales-Montor *et al.* 2001]. In addition we found no differences in estradiol levels at 18 months between the IL-6^{-/-} and WT mice. In fact, estrogen deficiency rather than increased estrogen has been associated with obesity in male mice; for example aromatase knock-out (ARKO) males become obese compared with WT males [Misso *et al.* 2003]. We suggest that a metabolic abnormality in the IL-6^{-/-} males is a more likely cause of increased body weight, GFAP density and lipid peroxidation.

IL-6 deficiency also affects hypothalamic pituitary axis activity and it is associated with a two fold increase in circulating cortisol levels, not to mention obesity and altered metabolism [Gomez *et al.* 2006]. Interestingly, estrogen treatment to both ARKO and IL-6^{-/-} mice lowers body weight in females but not males, suggesting that estrogen may be protective against metabolic changes induced by IL-6 deficiency in females but not males [Misso, Murata, Boon, Jones, Britt, and Simpson 2003; Wallenius, Wallenius, Ahren, Rudling, Carlsten, Dickson, Ohlsson, and Jansson 2002]. Estrogen treatment can protect against insulin resistance and glucose intolerance, therefore we suggest that estrogen synthesis mid life may have protected the IL-6^{-/-} females from the metabolic effects of IL-6 deficiency in later life [Riant *et al.* 2009].

In summary, we found higher levels of hippocampal and cerebellar GFAP, in addition to elevated body weight in old-aged IL-6^{-/-} males compared with WT males. IL-6 deficiency did not affect aging-related inflammation in the aged females, but did lower MBP levels in the cerebellum. Therefore we suggest IL-6 is important for promoting myelin synthesis in aged females, and that drugs which inhibit the synthesis of IL-6 in males may affect metabolism and augment aging-related neuroinflammation.

4. Methods

4.1 Mice

Male and female C57Bl/6 mice were obtained from Jackson laboratories. IL-6^{-/-} mice were constructed on a C57Bl/6 background. Mice were housed in clear plexiglas cages (29 × 18 × 12.5 cm) with stainless steel wire lids in a temperature (21–23°C) controlled room, and maintained in a pathogen-restricted environment, on a 12:12 h light:dark cycle (lights on at 7:00am). Mice were sacrificed at 18 and 24 months of age. All procedures were IACUC approved, and all experiments were conducted in a blinded fashion.

4.2 Tissue Preparation

Mice were sacrificed by CO₂ anesthesia followed by decapitation. Brains were immediately removed and bisected. The left hemisphere was frozen at -80°C and cryo-micro-dissected on a rostra-caudal gradient into 350µm thick sections. Hippocampal tissue punches were taken at Bregma +1.7mm, and cerebellar white matter punches at Bregma -2.3mm. Micro-punches were suspended in 100µl of lysis buffer (containing 1% NP40 and protease inhibitors) and immediately sonicated on ice. Samples were then retained for western blotting, ELISAs and measurement of lipid peroxidation using the thiobarbituric acid (TBA) assay. The right hemisphere of each animal was immersion-fixed in 4% paraformaldehyde for 24 hr at 4°C. Fixed tissue was dissected into 2mm tissue blocks on a rostra-caudal gradient using a Zivic Miller mouse brain mold. Then tissue blocks were fixed for another 2 hrs at room temperature (RT) then processed to paraffin using a Tissue-Tek paraffin embedding machine [22]. For immunohistochemistry (IHC) n=6 animals per group were used. For ELISAs, Western Blots and MDA assays n=3 animals per group were used.

4.3 Histology and IHC

Sections (6 μ m) were cut from paraffin embedded tissue blocks, using a rotary microtome and mounted onto charged slides. At least four sections for each region of interest, per antibody/histology stain were used per animal. At least five individual animals were used per group. Histological stains were haemotoxylin and eosin (H&E), Luxol fast blue (LFB), Nissl and Toluidine Blue. Prior to staining, tissue sections were cleared in two xylene baths for 5 min, then 10 min. Sections were then rehydrated through graded alcohols; 100%, 95%, 70%, 50% to distilled water. Briefly, sections were incubated in a 0.1% LFB solution (0.1g LFB, 100 ml 95% alcohol, 0.5 ml glacial acetic acid) at 56°C overnight, sections were then rinsed in 95% alcohol, then distilled water, followed by differentiation with a lithium carbonate solution (0.05 g lithium carbonate in 100 ml water) and 70% alcohol. Sections were then counterstained with Cresyl Fast Violet (CFV); 0.1 g CFV, 100 ml distilled water and 0.5 ml glacial acetic acid, then rinsed in distilled water prior to rehydration through graded alcohols, clearance with xylene and mounting with DPX (Sigma) a commercially available mountant.

For IHC, sections were deparaffinized and rehydrated as described. For antigen retrieval, sections were pretreated in citrate buffer pH 6 and micro-waved for 10 min at full power. To quench endogenous peroxidase activity, sections were immersed in a 0.3 % H₂O₂ solution for 15 min. To block non-specific antigen binding, sections were pre-incubated for 30 min with the appropriate blocking serum (horse or goat) from the Vector ABC kit (Vector Labs, Burlingame, CA). Antigen specificity was demonstrated by using antibodies on tissue sections known to contain the epitope of interest. Antibodies (polyclonal or monoclonal) were suspended in PBS and 0.1% BSA, incubated for 1 hr RT. Primary antibodies were commercially available; anti-mouse SNAP25 (Millipore 1:500), anti-mouse GFAP identified astrocytes (Calbiochem, San Diego, CA; 1:500). Nickle DAB was used to visualize antibodies. Sections were then dehydrated through graded alcohols; 50, 70, 95, 100%, to xylene prior to being cover-slipped with DPX [17].

4.4 Digital in-Image Acquisition

For immunohistochemistry regions of interest were identified through comparison with a stereotaxic mouse brain atlas [22]. Digital photo-micrographs were generated using a Nikon (50i) microscope using 10x, 20x and 60x oil immersion lens coupled to a camera with image grabber software. For analysis of western blots, Image J (NIH) software was used. Regions of equivalent areas were selected and optical density of the immunostained band (GFAP, MBP or SNAP25) was measured in Image J. In the same lane the density of B-actin; a house-keeping protein was also calculated. Three samples from each group were run on each gel per antibody, and the gels performed in triplicate. The data was entered into SPSS and the density of GFAP;MBP or SNAP25 was normalized to β -actin per lane.

4.5 SNAP25, MBP and GFAP by Immunoblotting

Hippocampal and cerebellum homogenates were diluted with one-half volume of sample buffer containing 10% (w/v) sodium dodecyl sulphate (SDS), 30% (v/v) glycerol, 2% (v/v) 2-mercapto-ethanol, and 0.25% (w/v) bromophenol blue, and Tris Buffered Saline with Tween (TBST) (pH 7.2). Each gel contained one sample per group and a low-range molecular weight marker, and individual gels run per region of interest in triplicate. 10 μ g protein per sample was loaded per well, into Pierce precise protein gels. SDS-PAGE was performed with 4–20 % agarose gels for 65 mins at 120 mV, after which the gels were washed in transfer buffer and electroblotted using a semi-dry transfer apparatus onto Immobilon-P transfer membranes (Millipore Corporation, MA). The nonspecific sites on membrane were blocked by incubating with 5% fish gelatin in TBST overnight at 4°C. The membranes were then incubated overnight with antibodies to SNAP25 (mouse monoclonal 1/1000 Millipore), GFAP (mouse monoclonal 1:250 Upstate), anti-myelin basic protein (MBP) (mouse monoclonal 1:500 Millipore), or Beta-

Actin (Sigma 1:20,000) in blocking buffer, at 4°C for 24 hr. The membranes were further washed with TBST and incubated with biotinylated goat-anti-mouse IgG (1:10,000; Pierce Biotechnology Inc., Rockford, IL) for 1 hr at RT. The membranes were washed with TBST and incubated with streptavidin conjugated with HRP (1:5,000 in blocking buffer; Pierce Biotechnology) for 1 hr RT. The blot was developed by incubating with Super Signal/ECL chemiluminescent substrate (Pierce Biotechnology) for 5–60 sec and was photographed with a LAS-1000plus camera (Fuji).

4.6 Hippocampal GFAP by ELISA

The initial analysis of western blots stained with the GFAP antibody revealed that GFAP levels were much lower in the hippocampus than in the cerebellum and for this reason a more sensitive assay was used to detect GFAP in the hippocampus. A commercially available sandwich ELISA was used to detect GFAP (Biovendor). A commercially available ELISA kit (R&D biosystems) was used to detect IFN γ in the hippocampus and cerebellum of the 24 month WT and IL-6^{-/-} mice. Plates were pre-coated with a capture antibody (GFAP/IFN γ) to which 50 μ l of the assay dilutant and 50 μ l of either the tissue homogenate or the GFAP standards supplied with the kits were added. Samples were incubated for 2 hr RT and washed with PBST (PBS + 0.05% Tween 20). 100 μ l of the detection antibody (IFN γ /GFAP-conjugated with biotin) was added and the plate incubated for 1 hr RT. Unbound antibody was removed by washing the plates with PBST; 100 μ l of HRP-conjugated streptavidin was added and the plate incubated at 45 min RT. Plates were washed in PBST and the substrate solution for HRP (TMB) was added. Plates were protected from light and incubated for 15 min after which 100 μ l of the stop solution was added. A micro-plate reader was used to measure optical density (OD) per well at 450 nm with correction set to 540nm. The standard curve for GFAP/IFN γ supplied with the kit was then used to quantify GFAP/IFN γ expression per well. Samples contained pooled tissues from animals per group which were then analyzed in triplicate.

4.7 Sera Thyroxine and Estradiol by ELISA

Commercially available competitive ELISA assays were used to determine serum levels of estradiol and thyroxine in the 18 month IL-6 deficient mice. Three mice, from each of the IL-6^{-/-} male and female, 18 month mice were used per assay to determine if age and sex related changes in circulating hormones were related to neuroinflammatory changes. Briefly, 25 μ l or 50 μ l of sera and standard control samples to generate the standard curve were added to the thyroxine (R&D Biosystems) or estradiol (BioVendor) kits as appropriate according to the manufacturer's instructions, and samples were loaded in triplicate. Samples were incubated with the HRP-conjugated estradiol or thyroxine standard supplied with the kit, at 1 hour room temperature. The ELISA plates were then washed six times using wash buffer, then further incubated with a HRP-TMZ reagent for 15 min. After the substrate color was developed an acidic stop solution provided with the kit was added to each well, and then the mean absorbance of the solution per well was read using a spectrophotometric plate reader at 450 nm within 15 min. The concentration of estradiol and thyroxine per well was generated by comparing values per sample with a standard curve generated with standards provided with the kit. In addition control samples spiked with estradiol or thyroxine standards were used as positive controls in the assays.

4.8 Determination of Malondialdehyde (MDA) Levels

There was a significant effect of IL-6 on GFAP and MBP levels at 24 months therefore we quantified MDA as an index of lipid peroxidation to ascertain if there were oxidative stress changes also apparent at this age, using the TBA assay with derivative spectroscopy [3]. In brief, pooled samples from each group and brain region (50 μ g/ml) were incubated with TBA (20 mM) in the presence of trichloroacetic acid (3.1%) and butylated hydroxytoluene (2.5 mM)

for 45 min at 70°C. The samples were cooled on ice and extracted with 100 µl n-butanol. The butanol phase was collected and absorbance scans (400–650 nm) recorded in a Beckman DU-600 spectrophotometer, scan speed 600 nm/min. The scans were analyzed with NWLSS™ Derivative Application to obtain 3rd derivatives. MDA levels were determined by comparing the peak heights at 528 nm to a calibration curve obtained from hydrolysis of 1,1,3,3-tetraethoxypropane (0.15 – 0 µg/ml). Samples contained pooled tissues from n = 6 animals per group and pooled samples were then analyzed in triplicate.

4.8 Statistical Analysis

Data from all analysis was entered into SPSS v17 which was then used to calculate statistically significant differences in mean data values between groups. ANOVA was used to determine main effects of sex, strain and age. ANOVA with Bonferroni correction was then used to determine intra-group differences in parameters of interest. Significance was set at $p < 0.05$. All analyses in this study were performed in a blinded fashion and data uncoded only after experimental analysis was completed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge assistance from the Biochemistry and Advanced Light Microscopy cores at the Wadsworth Center. This research was supported by NIEHS ES014675 and ES015688 to RF Seegal.

Reference List

- Andorfer C, Acker CM, Kress Y, Hof PR, Duff K, Davies P. Cell-Cycle Reentry and Cell Death in Transgenic Mice Expressing Nonmutant Human Tau Isoforms. *J Neurosci* 2005;25:5446–5454. [PubMed: 15930395]
- Angelopoulos P, Agouridaki H, Vaiopoulos H, et al. Cytokines in Alzheimer's disease and vascular dementia. *Int J Neurosci* 2008;118:1659–1672. [PubMed: 18937113]
- Arvanitakis Z, Grodstein F, Bienias JL, et al. Relation of NSAIDs to incident AD, change in cognitive function, and AD pathology. *Neurology* 2008;70:2219–2225. [PubMed: 18519870]
- Balschun D, Wetzel W, del Rey A, et al. Interleukin-6: a cytokine to forget. *FASEB J* 2004;04–1625fje.
- Behl C, Skutella T, LezoualcFÖH F, et al. Neuroprotection against Oxidative Stress by Estrogens: Structure-Activity Relationship. *Molecular Pharmacology* 1997;51:535–541. [PubMed: 9106616]
- Bellinger FP, Madamba SG, Campbell IL, Siggins GR. Reduced long-term potentiation in the dentate gyrus of transgenic mice with cerebral overexpression of interleukin-6. *Neuroscience Letters* 1995;198:95–98. [PubMed: 8592650]
- Benice TS, Rizk A, Kohama S, Pfankuch T, Raber J. Sex-differences in age-related cognitive decline in C57BL/6J mice associated with increased brain microtubule-associated protein 2 and synaptophysin immunoreactivity. *Neuroscience* 2006;137:413–423. [PubMed: 16330151]
- Berchtold NC, Cribbs DH, Coleman PD, et al. Gene expression changes in the course of normal brain aging are sexually dimorphic. *Proceedings of the National Academy of Sciences* 2008;105:15605–15610.
- Biessels GJ, Gispen WH. The impact of diabetes on cognition: What can be learned from rodent models? *Neurobiology of Aging* 2005;26:36–41. [PubMed: 16223548]
- Bouman A, Schipper M, Heineman MJ, Faas MM. Gender difference in the non-specific and specific immune response in humans. *Am J Reprod Immunol* 2004;52:19–26. [PubMed: 15214938]
- Braida D, Sacerdote P, Panerai AE, et al. Cognitive function in young and adult IL (interleukin)-6 deficient mice. *Behav Brain Res* 2004;153:423–429. [PubMed: 15265638]

- Breitner JC, Haneuse SJ, Walker R, et al. Risk of dementia and AD with prior exposure to NSAIDs in an elderly community-based cohort. *Neurology* 2009;72:1899–1905. [PubMed: 19386997]
- Di Santo E, Alonzi T, Fattori E, et al. Overexpression of interleukin-6 in the central nervous system of transgenic mice increases central but not systemic proinflammatory cytokine production. *Brain Research* 1996;740:239–244. [PubMed: 8973820]
- Fernandez-Real JM, Vayreda M, Richart C, et al. Circulating interleukin 6 levels, blood pressure, and insulin sensitivity in apparently healthy men and women. *J Clin Endocrinol Metab* 2001;86:1154–1159. [PubMed: 11238501]
- Fontana L, Eagon JC, Colonna M, Klein S. Impaired mononuclear cell immune function in extreme obesity is corrected by weight loss. *Rejuvenation Res* 2007;10:41–46. [PubMed: 17378751]
- Gao S, Hendrie HC, Hall KS, Hui S. The relationships between age, sex, and the incidence of dementia and Alzheimer disease: a meta-analysis. *Arch Gen Psychiatry* 1998;55:809–815. [PubMed: 9736007]
- Godbout JP, Johnson RW. Age and Neuroinflammation: A Lifetime of Psychoneuroimmune Consequences. *Immunology and Allergy Clinics of North America* 2009;29:321–337. [PubMed: 19389585]
- Gomez CR, Goral J, Ramirez L, Kopf M, Kovacs EJ. Aberrant acute-phase response in aged interleukin-6 knockout mice 1. *Shock* 2006;25:581–585. [PubMed: 16721265]
- Hootman JM, Snizek JE, Helmick CG. Women and arthritis: burden, impact and prevention programs. *J Womens Health Gend Based Med* 2002;11:407–416. [PubMed: 12173574]
- Huberman M, Sredni B, Stern L, Kott E, Shalit F. IL-2 and IL-6 secretion in dementia: correlation with type and severity of disease. *J Neurol Sci* 1995;130:161–164. [PubMed: 8586980]
- Jung-Testas I, Schumacher M, Robel P, Baulieu EE. Actions of steroid hormones- and growth factors on glial cells of the central and peripheral nervous system. *Journal Steroid Biochem Mol Biol* 1994;48:45–54.
- Kloppenborg RP, van den Berg E, Kappelle LJ, Biessels GJ. Diabetes and other vascular risk factors for dementia: Which factor matters most? A systematic review. *European Journal of Pharmacology* 2008;585:97–108. [PubMed: 18395201]
- Kurebayashi S, Miyashita Y, Hirose T, Kasayama S, Akira S, Kishimoto T. Characterization of mechanisms of interleukin-6 gene repression by estrogen receptor. *J Steroid Biochem Mol Biol* 1997;60:7–11.
- Lebed YV, Orlovsky MA, Nikonenko AG, Ushakova GA, Skibo GG. Early reaction of astroglial cells in rat hippocampus to streptozotocin-induced diabetes. *Neuroscience Letters* 2008;444:181–185. [PubMed: 18708122]
- Lei DL, Long JM, Hengemihle J, et al. Effects of estrogen and raloxifene on neuroglia number and morphology in the hippocampus of aged female mice. *Neuroscience* 2003;121:659–666. [PubMed: 14568026]
- Li WW, Penderis J, Zhao C, Schumacher M, Franklin RJM. Females remyelinate more efficiently than males following demyelination in the aged but not young adult CNS. *Experimental Neurology* 2006;202:250–254. [PubMed: 16797535]
- Liu H, Liu K, Bodenner DL. Estrogen receptor inhibits interleukin-6 gene expression by disruption of nuclear factor [kappa]B transactivation. *Cytokine* 2005;31:251–257. [PubMed: 16043358]
- Lleo A, Battezzati PM, Selmi C, Gershwin ME, Podda M. Is autoimmunity a matter of sex? 10. *Autoimmun Rev* 2008;7:626–630. [PubMed: 18603021]
- Marsland AL, Gianaros PJ, Abramowitch SM, Manuck SB, Hariri AR. Interleukin-6 covaries inversely with hippocampal grey matter volume in middle-aged adults. *Biol Psychiatry* 2008;64:484–490. [PubMed: 18514163]
- Martinez FG. Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity in the medial amygdala subnuclei across the estrous cycle and in castrated and treated female rats. 2006
- Misso ML, Murata Y, Boon WC, Jones MEE, Britt KL, Simpson ER. Cellular and Molecular Characterization of the Adipose Phenotype of the Aromatase-Deficient Mouse. *Endocrinology* 2003;144:1474–1480. [PubMed: 12639931]
- Morales-Montor J, Baig S, Mitchell R, Deway K, Hallal-Calleros C, Damian RT. Immunoendocrine Interactions During Chronic Cysticercosis Determine Male Mouse Feminization: Role of IL-6. *J Immunol* 2001;167:4527–4533. [PubMed: 11591780]

- Morrison JH, Hof PR. Life and Death of Neurons in the Aging Brain. *Science* 1997;278:412–419. [PubMed: 9334292]
- Mouton PR, Long JM, Lei DL, et al. Age and gender effects on microglia and astrocyte numbers in brains of mice. *Brain Research* 2002;956:30–35. [PubMed: 12426043]
- Nakanishi M, Niidome T, Matsuda S, Akaike A, Kihara T, Sugimoto H. Microglia-derived interleukin-6 and leukaemia inhibitory factor promote astrocytic differentiation of neural stem/progenitor cells. *European Journal of Neuroscience* 2007;25:649–658. [PubMed: 17328769]
- Pan W, Hsueh H, He Y, et al. Astrocyte Leptin Receptor (ObR) and Leptin Transport in Adult-Onset Obese Mice 247. *Endocrinology* 2008;149:2798–2806. [PubMed: 18292187]
- Ravaglia G, Forti P, Maioli F, et al. Blood inflammatory markers and risk of dementia: The Conselice Study of Brain Aging. *Neurobiology of Aging* 2007;28:1810–1820. [PubMed: 17011077]
- Resnick SM, Pham DL, Kraut MA, Zonderman AB, Davatzikos C. Longitudinal Magnetic Resonance Imaging Studies of Older Adults: A Shrinking Brain. *J Neurosci* 2003;23:3295–3301. [PubMed: 12716936]
- Riant E, Waget A, Cogo H, Arnal JF, Burcelin R, Gourdy P. Estrogens Protect against High-Fat Diet-Induced Insulin Resistance and Glucose Intolerance in Mice. *Endocrinology* 2009;150:2109–2117. [PubMed: 19164473]
- Steffensen SC, Campbell IL, Henriksen SJ. Site-specific hippocampal pathophysiology due to cerebral overexpression of interleukin-6 in transgenic mice. *Brain Research* 1994;652:149–153. [PubMed: 7953712]
- Thal DR, Rub U, Orantes M, Braak H. Phases of A β -deposition in the human brain and its relevance for the development of AD. *Neurology* 2002;58:1791–1800. [PubMed: 12084879]
- Valerio A, Ferrario M, Dreano M, Garotta G, Spano P, Pizzi M. Soluble Interleukin-6 (IL-6) Receptor/IL-6 Fusion Protein Enhances in Vitro Differentiation of Purified Rat Oligodendroglial Lineage Cells. *Molecular and Cellular Neuroscience* 2002;21:602–615. [PubMed: 12504593]
- Wada-Isoe K, Wakutani Y, Urakami K, Nakashima K. Elevated interleukin-6 levels in cerebrospinal fluid of vascular dementia patients. *Acta Neurol Scand* 2004;110:124–127. [PubMed: 15242421]
- Wallenius V, Wallenius K, Ahren B, et al. Interleukin-6-deficient mice develop mature-onset obesity. *Nat Med* 2002;8:75–79. [PubMed: 11786910]
- Wyss-Coray T. Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nat Med* 2006;12:1005–1015. [PubMed: 16960575]
- Yang S, Li C, Zhang W, Wang W, Tang Y. Sex differences in the white matter and myelinated nerve fibers of Long-Evans rats. *Brain Research* 2008;1216:16–23. [PubMed: 18486116]
- Zhang PL, Izrael M, Ainsbinder E, Ben-Simchon L, Chebath J, Revel M. Increased myelinating capacity of embryonic stem cell derived oligodendrocyte precursors after treatment by interleukin-6/soluble interleukin-6 receptor fusion protein. *Molecular and Cellular Neuroscience* 2006;31:387–398. [PubMed: 16325417]

a

b

Figure 1.

The mean body weights per group of the WT and IL-6^{-/-} male and female 18 and 24 month aged animals at time of sacrifice. There were $\geq n=6$ mice per group. * $p<0.05$. Significance was calculated using ANOVA with Bonferroni correction.

Figure 2.

a) Illustration shows the location of the hippocampus at Bregma +1.77mm. b) Bar chart shows the absorbance of GFAP, measured using an ELISA kit. In the hippocampus Bonferroni analysis revealed higher GFAP levels in the WT females compared with WT males. There were also higher GFAP levels in the IL6^{-/-} males compared to the WT males. * $p < 0.05$. Bars represent mean $\pm$ SEM. c) The panel of photomicrographs shows tissue sections of the hippocampus stained with an anti-GFAP antibody. The first two photomicrographs are low magnification images showing the hippocampus of a WT male and female mouse. The next four photomicrographs show higher magnification images of anti-GFAP stained sections from the WT and IL-6^{-/-} mice. The arrows point to the sparse astrocytic density in the WT males compared with WT females. Bar is 70 μ m.

Figure 3.

a) Illustration shows inferior cerebellar nuclei at Bregma -2.3 mm. b) The bar chart shows GFAP levels in the cerebellum. Using Bonferroni analysis there were higher GFAP levels in the WT females compared with WT males in the cerebellum. There were also higher GFAP levels in the IL-6^{-/-} males compared to the WT males. * $p < 0.05$. Bars represent mean $\pm$ SEM. c) The panel of photomicrographs shows tissue sections of the cerebellum stained with an anti-GFAP antibody. The first photomicrograph shows the regional variation in GFAP density in the cerebellum. The cerebellar white matter had very dense immunoreactivity to the anti-GFAP antibody, while the purkinje and granular fields had sparse immunoreactivity. The next four photomicrographs show higher magnification images of anti-GFAP stained sections from the WT and IL-6^{-/-} mice. The arrows point to the sparser astrocytic density in the WT males compared with the IL-6^{-/-} males. Bar is $70\mu\text{m}$.

Figure 4.

The mean levels of MDA are shown in a) the hippocampus and b) the cerebellum. In the hippocampus Bonferonni analysis revealed IL-6^{-/-} males and WT females had higher MDA levels than the WT males. In the cerebellum the IL-6^{-/-} females had higher MDA than WT females. *p<0.05. Bars represent mean ± SEM.

a

b

Figure 5.

Bar chart shows the mean IFN γ levels in a) the hippocampus and b) the cerebellum of IL-6^{-/-} and WT mice. In the hippocampus there were lower levels of IFN γ in the IL-6^{-/-} males compared with WT males. In the cerebellum there were significantly lower levels of IFN γ in the IL-6^{-/-} males compared with WT males and significantly higher levels of IFN γ in the IL-6^{-/-} females compared with the WT females the cerebellum. * $p < 0.05$. Bars represent mean $\pm$ SEM.

Figure 6.

a) The first photomicrograph shows a low magnification image of a cerebellum tissue section stained with LFP. Adjacent there is a panel of four higher magnification images of sections from the equivalent level in the cerebellum of WT and IL-6^{-/-} male and female mice are shown in the adjacent panel of photomicrographs. The arrow in the WT male shows the rarefaction of myelin, as compared to denser fibers in the WT females. The star highlights the denser myelinated fibers in the female WT cerebellum compared with the other groups. b) The bar chart MBP density in the WT and the IL-6^{-/-} mice. Bonferroni analysis revealed higher MBP levels in the WT females compared with IL-6^{-/-} females, WT males and IL-6^{-/-} males. *p<0.05. Bars represent mean ± SEM. The antibody directed against MBP identified five isoforms of the MBP. Figure c) shows the isoform composition within each group. Bonferroni analysis revealed significantly more 24kDa MBP isoform in the MBP of aged WT females compared with aged IL-6^{-/-} females, IL-6^{-/-} males and WT males.

Figure 7.

a) The panel of photomicrographs show sections containing the hippocampal CA-3 and CA-1 fields stained with an anti-SNAP25 antibody visualized with nickel-DAB (blue-black). As can be seen synaptic rich processes extending from the neurons in the CA-3-CA-1 fields are clearly stained with the anti-SNAP25 antibody in WT and IL-6^{-/-} male and female mice. There appears to be sharper more distinctive staining in the IL-6^{-/-} male mice in particular. The larger image at the bottom of the panel shows the clear synaptic rich processes extending out of neurons in the CA-1 field of an IL-6^{-/-} mouse. b) This figure shows representative lanes from gels run with hippocampal homogenates of the WT and IL-6^{-/-} male and female mice. Gels were probed with a SNAP25 antibody and normalized to β-actin within lanes. 1; WT male 2; WT female 3; IL-6^{-/-} male 4; IL-6^{-/-} female. c) The bar chart shows the density of SNAP25 in AU in the hippocampus of the aged WT and IL-6^{-/-} mice. There was no difference in SNAP25 levels between the groups. *p<0.05. Bars represent mean ±SEM.

Table 1

shows the mean sera thyroxine levels and SEM in the 18 month WT and IL-6^{-/-} male and female mice. ANOVA revealed no main effects of sex or strain on thyroxine or estradiol levels.

	WT M	IL-6 ^{-/-} M	WT F	IL-6 ^{-/-} F
Thyroxine ug/dl	1.22 (+/-0.14)	0.76 (+/- 0.1)	1.38 (+/- 0.17)	0.73 (+/- 0.19)
Estradiol pg/dl	82 (+/- 4.37)	98.66 (+/-) 31.8	79 (+/- 0.33)	99 (+/- 15.17)