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ORIGINAL PAPER

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# Pattern of interleukin-6 receptor complex immunoreactivity between cortical regions of rapid autopsy normal and Alzheimer's disease brain

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**Abstract** Involvement of the interleukin-6 receptor complex (IL-6RC) in neuroregulatory and immunological processes of the brain and particularly in Alzheimer's disease (AD) has been hypothesized. The functionally active IL-6RC consists of the cytokine IL-6, which acts through the ligand binding IL-6R and the signal transducing gp130. Using a new immunocytochemical protocol on rapid autopsy cryostat brain sections we studied the expression of the IL-6RC in Braak IV-V staged AD patients compared to normal age-matched controls (HC) across five different cortical regions. Inter-rater reliability of the method was high. The "baseline" expression in normal human brain was determined for IL-6, IL-6R and gp130 in all cortical regions. In normal tissue IL-6 expression was lower in parietal cortex. Higher IL-6R expression was shown in frontal, occipital and parietal cortex, lower expression in temporal cortex and cerebellum. In AD IL-6 expression levels were generally increased in parietal cortex and decreased in occipital cortex compared to controls. IL-6R expression levels were strongly increased in AD frontal and occipital cortex and decreased in temporal cortex and cerebellum. Our findings indicate an altered cortical immunoreactivity pattern of the functional IL-6RC in AD supporting the hypothesis of a disease-related role of IL-6 in AD pathophysiology.

**Key words** Alzheimer's disease · interleukin-6 receptor · cytokine receptors · gp130 · immunocytochemistry · rapid autopsy

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## Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine that modulates multiple effects on the immune, hematopoietic and the central nervous systems (CNS) (Heinrich et al. 1990, 1998; Hirano et al. 1997; Van Snick et al. 1990). IL-6 has been reported to serve as an important mediator of neuroregulatory and inflammatory processes in the CNS (Kishimoto et al. 1992; Vandenabeele et al. 1991). In particular, IL-6 may play a role in controlling neuroimmune responses and neuronal protection and growth (Campbell et al. 1993; Gruol et al. 1997; Hama et al. 1989, 1991), and may serve as an early marker of tissue damage in the brain (Gruol et al. 1997).

IL-6 exerts its biological action on target cells through the IL-6 receptor complex (IL-6RC), composed of IL-6, the IL-6 binding IL-6 receptor (IL-6R, gp-80 or α-chain) and the signal transducing glycoprotein 130 (gp130 or β-chain) (Hibi et al. 1996; Taga et al. 1989). Initiation of IL-6 signaling occurs, when IL-6 binds to IL-6R, forming an IL-6/IL-6R heterodimer which can associate with gp130, leading to the activation of various signaling pathways (e. g. Janus kinases/signal transducers and activators of transcription, MAP [microtubule associated proteins] kinases and subsequent gene activation (Taga et al. 1997).

Recent studies have shown that components of IL-6RC are related to changes in AD and may be a potential biomarker for AD when measured in CSF (Hampel et al. 1999). In the brain of AD patients and elderly control subjects microglia was demonstrated to be a brain endogenous source of IL-6. Significant, dose-dependent increases in the production of IL-6 were observed after exposure to pre-aggregated amyloid-β peptide [1–42] (Lue et al. 2001) and IL-6 protein expression was observed in the brain of AD patients, but not in age-matched controls (Bauer et al. 1991; Hüll et al. 1995; Strauss et al. 1992). These studies demonstrated that amyloid in senile plaques surrounded by activated microglia and astrocytes show enhanced IL-6 immunoreactivity.

The current study examined brain-region dependent differences in the expresssion (i. e. cellular content) of the components of IL-6RC, including IL-6, IL-6R and gp130 between AD patients and healthy controls, using post-mortem brain rapid-autopsy brain tissue.

## Material and methods

### Tissue

Frozen post-mortem human brain samples of five different cortical regions (frontal-, temporal-, parietal-, occipital cortex and cerebellum) obtained from five AD cases and five normal age-matched controls by rapid brain autopsies (Hulette et al. 1997) were supplied by the Kathleen Price Bryan Brain Bank, Bryan Alzheimer's Disease Research Center, Duke University Medical Center, Durham, USA, and the Tissue Repository, Institute for Brain Aging and Dementia, University of California, Irvine, CA, USA. Postmortem delays ranged between 1–6 h. Both groups were screened and matched for the apoE-genotype to exclude a possible effect of the apoE-polymorphism on IL-6RC expression. Descriptive patient characteristics are shown in Table 1. The mean age of the healthy controls was 77 years (SD = 7.81) and of the AD patients 75.2 years (12.07), with no statistically significant group difference (p = 0.79).

The specimen were stored at –80 °C until they were sectioned at –25 °C into 10 µm thick sections using a cryostat (SLEE, Mainz, Germany). Tissue sections were mounted on precoated glass slides, air-dried and stored at –80 °C until use. Glass slides were precoated by immersion in a solution of 0.3 % gelatin, 0.05 % chrome (III) alum and 0.0001 % thymol in distilled water.

### Antibodies

To detect GFAP, 3 µg/mL anti-GFAP IgG (DAKO, Hamburg, Germany) was used, to identify microglia, anti-leucocyte common antigen (LCA) (DAKO) was used at a concentration of 9 µg/mL and neurofilament staining was performed with a working concentration of 10 µg/mL. Anti-IL-6 IgG (R&D systems) was used at a working concentration of 20 µg/mL, IL-6R IgG (Santa Cruz Biotechnologies) at a concentration of 4 µg/mL and anti-gp130 IgG (Santa Cruz Biotechnologies) at a concentration of 4 µg/mL.

Specificity for IL-6, IL-6R and gp130 was controlled by preabsorbing the antibodies with their respective antigens prior to the immunohistochemical procedure, resulting in complete blockage of the specific signals. IL-6 peptide was purchased from R&D systems, IL-6R as well as gp130 was purchased from Santa Cruz Biotechnologies.

### Immunocytochemistry

#### Indirect peroxidase method

Sections were thawed at room temperature and fixed in acetone (100 %) at –20 °C for 10 min. In order to inhibit the endogenous peroxidase, the tissue was treated with 1 % (v/v) perhydrole/phosphate-buffered saline (PBS, pH = 7.4) for 10 min at room temperature. After washing in PBS, non-specific binding was blocked for 30 min using 10 % (v/v) normal goat serum (DAKO) in PBS. The tissue sections were then incubated for 1 h at RT with the primary antibody in PBS at previously determined optimal concentrations. The sections were carefully washed in PBS followed by an incubation with the secondary antibody, horseradish-peroxidase (HRP)-labeled goat anti-mouse or anti-rabbit IgG (DAKO) for 30 min. After rinsing in PBS, the antigen-antibody complex was visualized using 0.5 mg 3,3'-diaminobenzidine (DAB) (SIGMA, Munich, Germany) in 1 ml PBS and 1 µl Perhydrole. Development was terminated after 5 min. Cell nuclei were shortly counterstained with Mayer's Haemalum. Afterwards, the slides were rinsed in running water and dehydrated in increasing alcohol con-

**Table 1** Patient characteristics are shown including cases, age, gender, diagnostic category, apoE-genotype and post mortem delay

| Patient Number | Diagnosis | Age | Gender (M = male F = female) | Diagnostic Staging | Post-mortem delay [h] | APOE genotype |
|---|---|---|---|---|---|---|
| 1 | HC | 85 | M | Normal Brain | 2:00 | ε3/3 |
| 2 | HC | 66 | M | Normal Brain | 1:05 | ε3/3 |
| 3 | HC | 82 | M | Normal Brain | 3:15 | ε3/3 |
| 4 | HC | 72 | F | Normal Brain | 3:00 | ε3/4 |
| 5 | HC | 80 | F | Normal Brain | 1:10 | ε3/3 |
| 6 | AD | 90 | F | Braak V | 2:20 | ε3/3 |
| 7 | AD | 64 | F | Braak V | 3:36 | ε3/3 |
| 8 | AD | 83 | M | Braak IV | 1:15 | ε3/4 |
| 9 | AD | 62 | M | Braak V | 1:30 | ε3/3 |
| 10 | AD | 77 | F | Braak V | 4.05 | ε3/3 |

centrations, followed by xylene (Merck KG aA, Darmstadt, Germany). Finally, the sections were mounted in Eukitt (O. Kindler GmbH, Freiburg, Germany).

#### Microscopy and analysis of immunoreactivity

The tissue samples were investigated using a Zeiss Axiophot 2 microscope (Carl Zeiss Jena, Germany) with a magnification of 400x. Semiquantitative analysis (Table 2) between different brain regions, as well as between AD tissue and control samples was performed by two blinded (diagnosis) and independent raters. According to the previously published protocol (Morris et al. 1998) we used five categories to grade the number of positive cells and intensity of staining: 0 = no reactivity, 1 = rare or occasional weakly positive cells, 2 = few scattered positive cells, 3 = many widespread intermediately strongly positive cells and 4 = numerous intensely positive cells. Assessment of inter-rater reliability showed that ratings from two blinded (diagnosis) and independent investigators were identical for 16 of 20 samples (Table 2). Cohen's kappa (Cohen 1960) was 0.71 with a standard deviation of 0.128, indicating excellent inter-rater reliability of the rating method. Kappa was significantly different from zero at $p < 0.01$.

### Statistical analysis

Two independent investigators blinded to diagnoses rated immunoreactivities in 20 randomly selected samples to assess inter-rater reliability of the used scale. Prior to sample selection, the whole data set was divided into four clusters of IL-6, IL-6R, gp130 and negative control stainings, respectively. From the negative control cluster only one sample was randomly drawn, from each of the three other clusters six samples and finally one sample from the whole data set. Data were not stratified according to brain region or diagnosis. Degree of inter-rater agreement was assessed using Cohen's kappa (Cohen 1960) and significance of deviation of kappa from zero was assessed using the approximation of its standard deviation provided by Cohen (1960). Because of the small sample sizes differences in marker expression were assessed visually by transformation into error bar diagrams. To support the visual impression, differences in marker expression between AD patients and controls were assessed within each brain region using the non-parametric Mann-Whitney U-test. To control for Typ I error accumulation of multiple comparisons, the level of significance was Bonferroni corrected for the number of comparisons within each cluster of markers (five pairwise comparisons for five brain regions), resulting in a value of $p < 0.001$. A two-independent samples t-test (two-tailed) was used to test for differences in age between both groups.

**Table 2** Agreement (bold) and disagreement (italic) between two independent investigators rating immunoreactivities from 20 randomly selected samples on a semiquantitative rating scale according to Morris and Esiri (1998)

| | Rater 1 | | | | | |
|---|---|---|---|---|---|---|
| Values | 0 | 1 | 2 | 3 | 4 | A |
| Rater 2 | | | | | | |
| 0 | **1** | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | **2** | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | **1** | 0 | 0 | 0 |
| 3 | 0 | *1* | 0 | **4** | 0 | 0 |
| 4 | 0 | 0 | *2* | 0 | **8** | 0 |
| A | 0 | 0 | 0 | 0 | *1* | 0 |

*0* no immunoreactivity; *1* rare or occasional weakly positive cells; *2* few scattered positive cells; *3* many widespread intermediately strongly positive cells; *4* numerous intensely positive cells; *A* artifact

## Results

### Cytokine and cytokine receptor expression

The distribution of the cytokine expression was shown for specific morphological cell types of the brain: astrocytes (Fig. 1A), microglia (Fig. 1C), and endothelial cells (Fig. 1B). The results are summarized in Table 3.

### Interleukin-6

IL-6 expression was frequently distributed in white matter. IL-6 was generally seen in astroglia (Fig. 1A), microglia (Fig. 1C), neurons (Fig. 1D) and endothelial cells (Fig. 1B). In particular, expression in large star-shaped astrocyte-like cells as well as in microglia cells with an irregular shape was visible (Fig. 2). In addition, a strong reaction was shown in endothelial cells, which were often surrounded by IL-6-expressing astrocytes.

### The interleukin-6 receptor

The membrane-bound interleukin-6 receptor expression tended to be slightly less expressed than IL-6 and was primarily seen in astroglia and endothelial cells, which were commonly distributed in white matter; however, expression was reduced in microglia and neurons. Immunoreactivity was extended into the periphery of the astrocytic processes (Fig. 3A–D).

### Glycoprotein 130

The membrane-bound gp130 was shown in astroglia, microglia, neurons and endothelial cells. In general, compared to IL-6 and IL-6R, the number of gp130 reactive cells was increased. Gp130 expression was generally and predominantly distributed in white matter astrocytes, microglia and endothelial cells (Fig. 4A–D). Distinct astrocytes and microglia with particularly short processes were shown.

### Regional pattern of expression in healthy control subjects

IL-6 generally showed a uniform expression in all investigated regions. Only the parietal cortex generally showed a lower expression on most cells. The membrane-bound IL-6 receptor expression was generally seen in all brain regions. A fairly intense expression was particularly shown in the temporal and parietal cortex and cerebellum, whereas uniformly low expression was seen in the frontal and occipital cortex.

The membrane-bound gp130 consistently showed

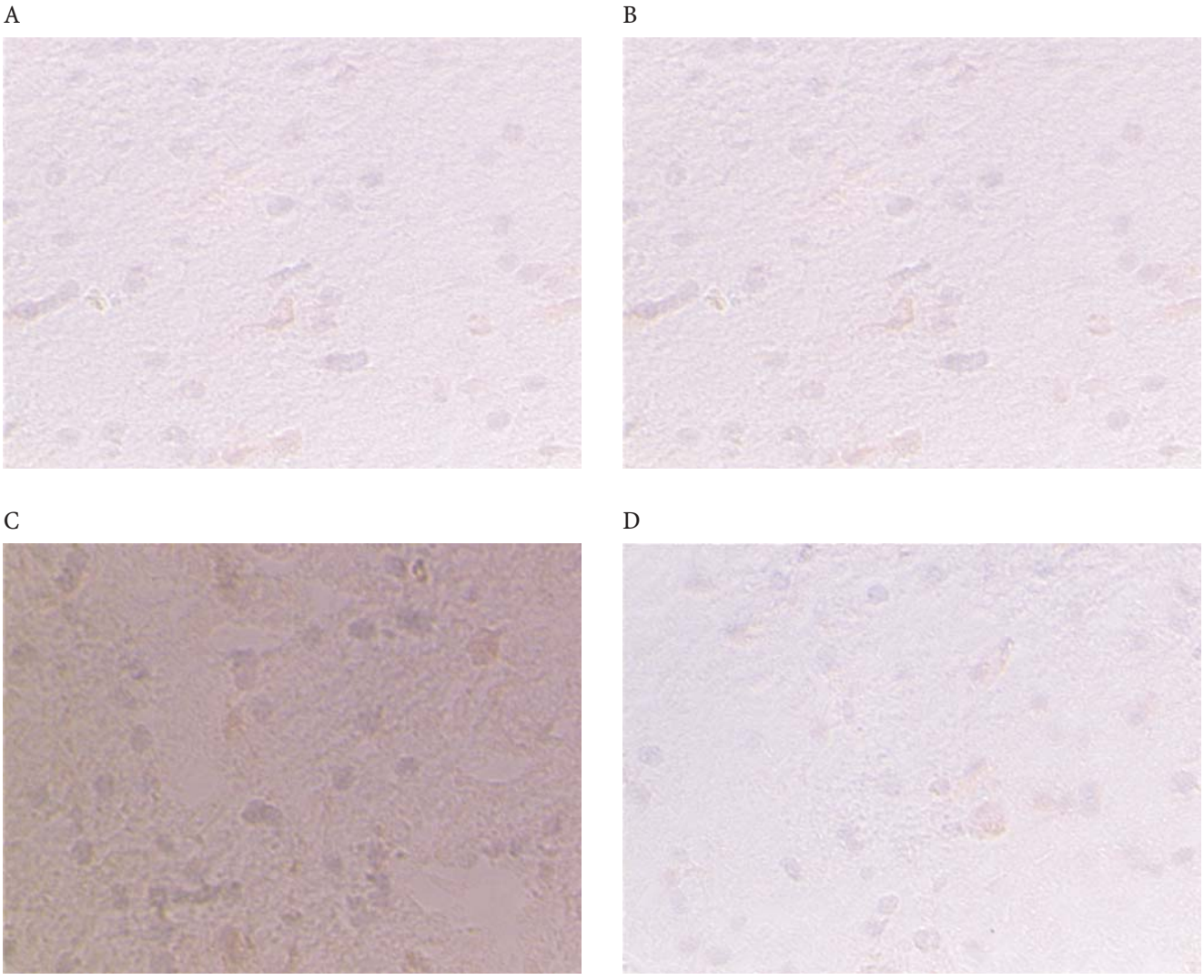


**Fig. 1** Immunohistochemical IL-6 staining on astroglia (**A**), endothelium (**B**), microglia (**C**) and neurons (**D**) (Frontal cortex, AD patient)

strong expression in all brain regions with no prominent differences.

### Regional pattern of expression in AD patients

IL-6 expression was generally shown in all investigated brain regions. There tended to be a lower expression level in occipital cortex compared to other regions. The membrane-bound IL-6R was strongly expressed in frontal, parietal and occipital cortex. Lower expression levels and fewer expressing cells were seen in temporal cortex and in cerebellum.

### Comparison between healthy controls and AD patients

In general, comparison between HC and AD cases revealed prominent differences, showing a consistent expression pattern of IL-6 and the membrane-bound IL-6R. The membrane-bound gp130, however, was very strongly but uniformly expressed in both groups.

In particular, IL-6 expression levels were generally increased in AD parietal cortex and decreased in occipital cortex compared to HC (Fig. 5A). IL-6R expression levels were generally and strongly increased in AD frontal and occipital cortex and decreased in temporal cortex and cerebellum compared to HC (Fig. 5B).

Using the Mann-Whitney-U test the observed differences were statistically significant at $p < 0.008$. Expression of gp130 was not different between groups.

## Discussion

The aim of the current study was to examine cell-type specific expression and regional differences in the occurrence of the components of the IL6RC including IL6, IL6R and gp130. A major finding was that IL-6 expres-

**Table 3** Relative immunoreactivities were rated on a semiquantitative scale of 0–4, depending on numbers of positive cells and their intensity of staining (Morris and Esiris, 1998)

| | AD patient | | | | HC controls | | | |
|---|---|---|---|---|---|---|---|---|
| Brain Region | Case | IL-6 | IL-6R | gp130 | Case | IL-6 | IL-6R | gp130 |
| Frontal cortex | AD patient 1 | 3 | 3 | 4 | HC control 1 | 4 | 1 | 4 |
| | AD patient 2 | 3 | 4 | 4 | HC control 2 | 4 | 1 | 3 |
| | AD patient 3 | 4 | 4 | 3 | HC control 3 | 4 | 1 | 4 |
| | AD patient 4 | 3 | 3 | 4 | HC control 4 | 3 | 1 | 3 |
| | AD patient 5 | 3 | 3 | 4 | HC control 5 | 3 | 1 | 3 |
| | NC | 0 | 0 | 0 | NC | 0 | 0 | 0 |
| Temporal cortex | AD patient 1 | 4 | 2 | 4 | HC control 1 | 4 | 4 | 4 |
| | AD patient 2 | 3 | 1 | 4 | HC control 2 | 4 | 3 | 3 |
| | AD patient 3 | 3 | 2 | 3 | HC control 3 | 4 | 4 | 4 |
| | AD patient 4 | 3 | 2 | 3 | HC control 4 | 3 | 4 | 3 |
| | AD patient 5 | 3 | 1 | 3 | HC control 5 | 3 | 3 | 3 |
| | NC | 0 | 0 | 0 | NC | 0 | 0 | 0 |
| Occipital cortex | AD patient 1 | 2 | 3 | 4 | HC control 1 | 4 | 1 | 4 |
| | AD patient 2 | 2 | 3 | 4 | HC control 2 | 3 | 2 | 3 |
| | AD patient 3 | 2 | 4 | 3 | HC control 3 | 3 | 1 | 4 |
| | AD patient 4 | 1 | 3 | 3 | HC control 4 | 3 | 1 | 3 |
| | AD patient 5 | 1 | 3 | 3 | HC control 5 | 3 | 1 | 4 |
| | NC | 0 | 0 | 0 | NC | 0 | 0 | 0 |
| Parietal cortex | AD patient 1 | 3 | 3 | 4 | HC control 1 | 1 | 4 | 3 |
| | AD patient 2 | 3 | 3 | 4 | HC control 2 | 2 | 3 | 3 |
| | AD patient 3 | 4 | 3 | 4 | HC control 3 | 1 | 3 | 3 |
| | AD patient 4 | 3 | 3 | 3 | HC control 4 | 1 | 3 | 3 |
| | AD patient 5 | 3 | 4 | 3 | HC control 5 | 1 | 3 | 3 |
| | NC | 0 | 0 | 0 | NC | 0 | 0 | 0 |
| Cerebellum | AD patient 1 | 3 | 1 | 3 | HC control 1 | 3 | 3 | 3 |
| | AD patient 2 | 3 | 1 | 3 | HC control 2 | 3 | 3 | 3 |
| | AD patient 3 | 3 | 1 | 3 | HC control 3 | 3 | 4 | 4 |
| | AD patient 4 | 3 | 1 | 3 | HC control 4 | 3 | 3 | 4 |
| | AD patient 5 | 3 | 2 | 3 | HC control 5 | 3 | 3 | 3 |
| | NC | 0 | 0 | 0 | NC | 0 | 0 | 0 |

*0* no immunoreactivity; *1* rare or occasional weakly positive cells; *2* few scattered positive cells; *3* many widespread intermediately strongly positive cells; *4* numerous intensely positive cells; *A* artifact

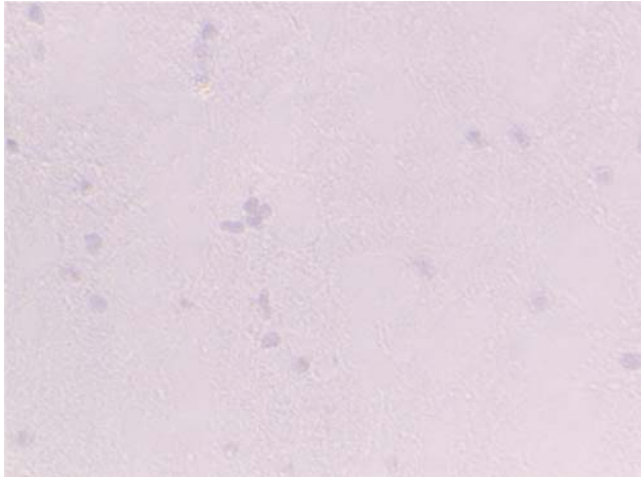

**Fig. 2** Control for the quality of immunohistochemistry. HE staining and DAB staining procedure with an isotype-specific antibody. The neuropil and glia cells are not specifically stained. (Frontal cortex, AD patient)

sion levels were generally increased in AD parietal cortex and decreased in occipital cortex when compared to HC brains. IL-6R expression levels were generally and strongly increased in AD frontal and occipital cortex and decreased in temporal cortex and cerebellum. In contrast, no group differences were found in gp130. To our knowledge, this is the first report on IL-6RC protein expression in human control and AD frozen and rapid-autopsied brain tissue.

The brain region specific expression of IL-6 protein in AD patients has been investigated previously (Bauer et al. 1991; Hüll et al. 1995; Strauss et al. 1992), demonstrating IL-6 positive staining in plaques of AD patients in contrast to age-matched control samples (Bauer et al. 1991; Strauss et al. 1992). The presence of IL-6 was shown to a large extent in early diffuse plaques and less concentrated in neuritic plaques (Hüll et al. 1996). Hüll and colleagues (Hüll et al. 1995) suggested that IL-6 is involved in the transformation from diffuse into neuritic plaques. However, there was no information regarding the expressing cells. In a related study, IL-6 immunoreactivity was shown in cortical and hippocampal senile

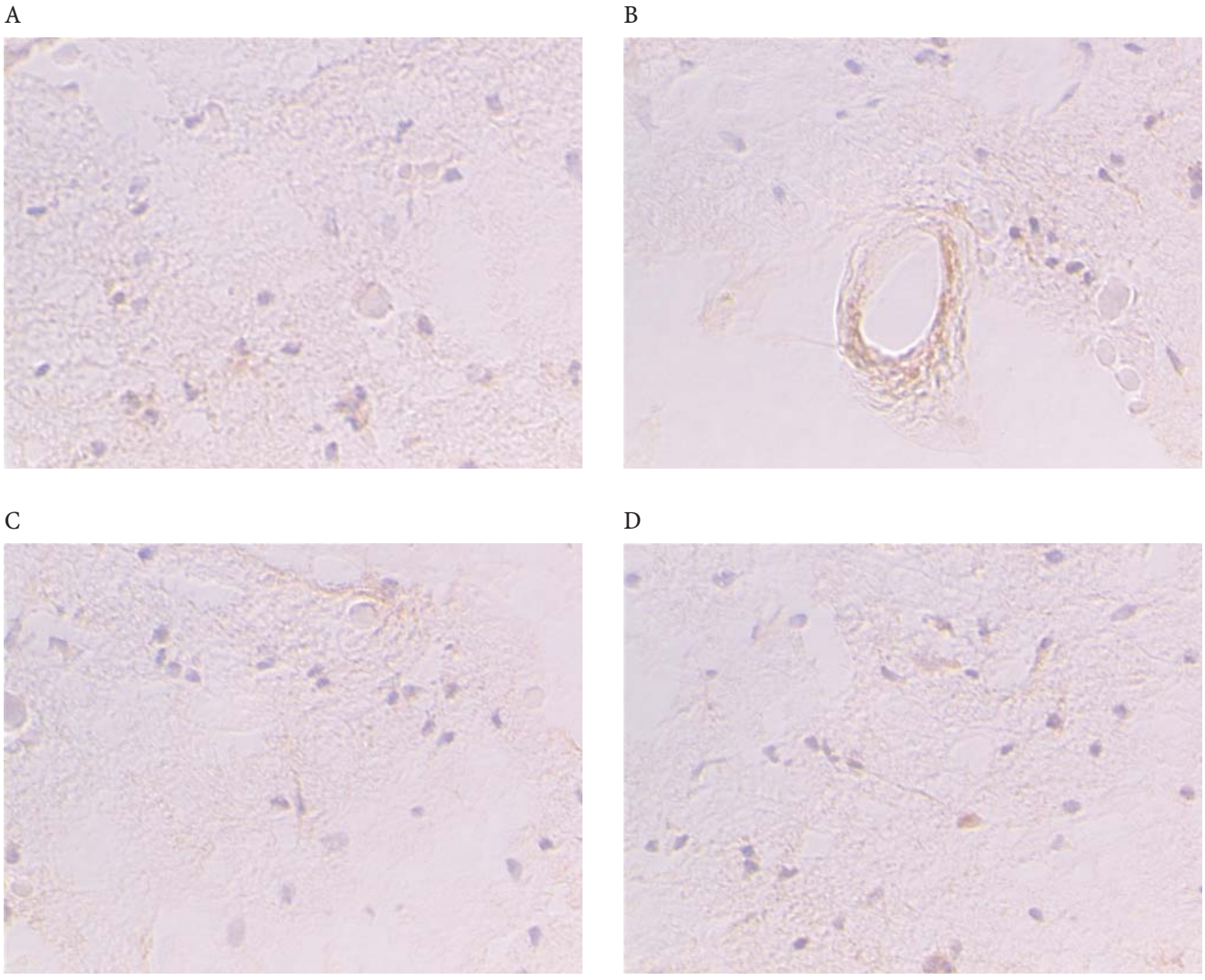


**Fig. 3** Immunohistochemical IL-6R staining on astroglia (**A**), endothelium (**B**), microglia (**C**) and neurons (**D**) (Frontal cortex, AD patient)

plaques, again without consideration of specific cell types (Strauss et al. 1992). All previous studies used formalin-fixed tissue with a large range of post mortem delays, which is less sensitive for easily degradable proteins compared to our rapid-autopsy cryostat sections (Scheloske et al. 1999). These immunocytochemistry protocols varied remarkably between studies. These methodological differences may account for differences in sensitivity and expression between the reported studies. Furthermore, our semiquantitative data of unchanged IL-6 levels in temporal cortex are not in agreement with those of Wood and colleagues (Wood et al. 1993) who reported increased IL-6 concentrations in AD temporal cortex homogenates compared to controls. This discrepancy may be attributable to the stage of AD in the samples studied or by methodological differences, since this group used ELISA assays on brain tissue homogenates, which, in contrast to body fluids (such as serum or CSF), generally show very limited reliability and stability of results. On the molecular level, only one pilot study (Zarow et al. 1996) showed increased IL-6 m-RNA expression in AD hippocampus and area 22 compared to controls. In conclusion, only a few studies on IL-6 expression in human CNS have been reported and the mechanism underlying the complex pattern of changes in the expression of the different components of the IL6RC in AD need to be elucidated in further studies.

Astrocytes and microglia have been identified as cellular sources of IL-6 in the CNS. In culture, murine and rat astrocytes have been shown to produce IL-6 after stimulation with IL-1β (Frei et al. 1988; Sawada et al. 1992). Rapid-autopsy human elderly AD and control microglia was demonstrated to be a brain endogenous source of IL-6. Significant, dose-dependent increases in the production of IL-6 were observed after exposure to pre-aggregated amyloid-β peptide [1–42] (Lue et al. 2001). Therefore, another aim of our study was to characterize IL-6 expression in the different CNS cell types. Across brain regions, IL-6 was detectable in astroglia, microglia, neurons and endothelial cells. These findings

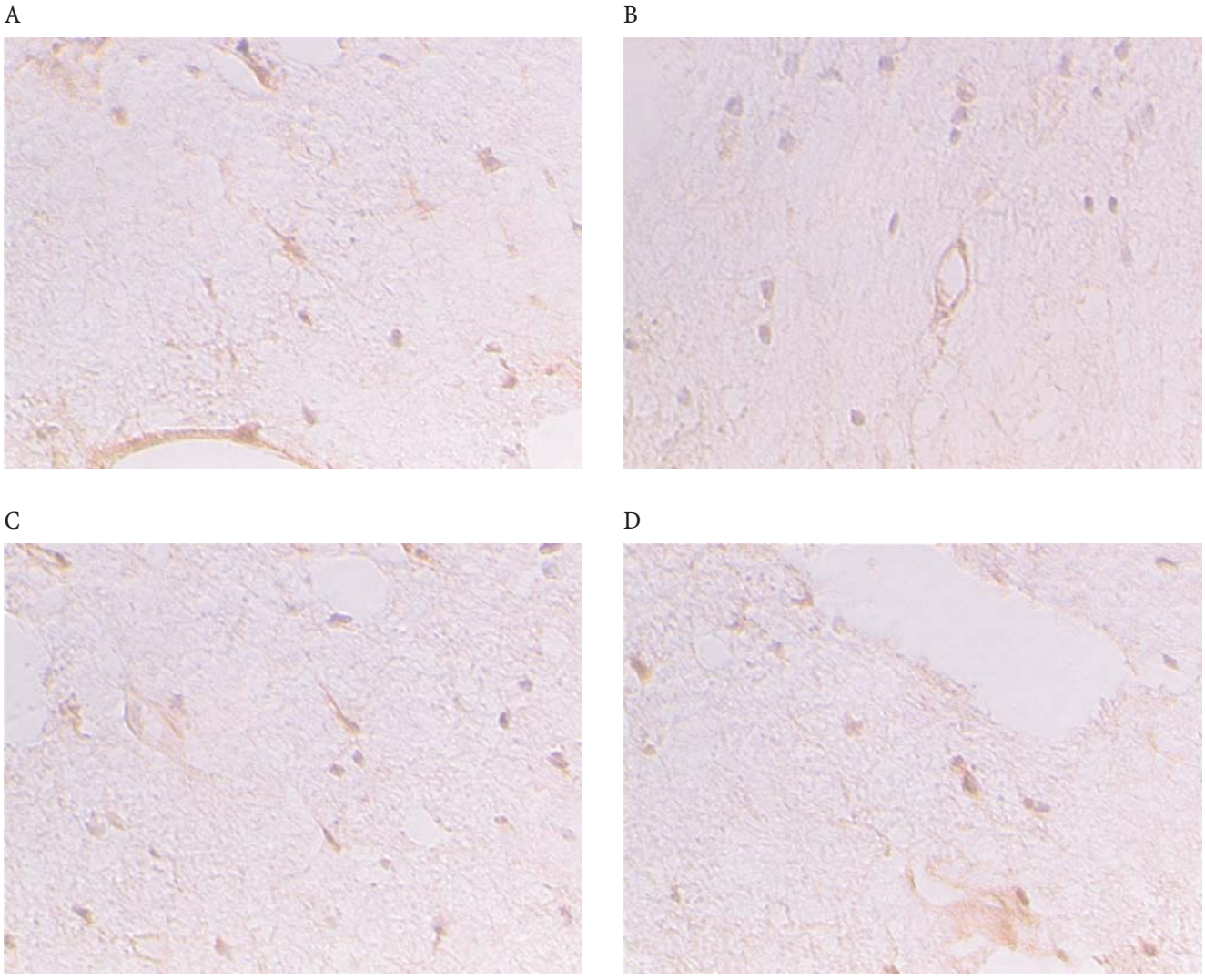


**Fig. 4** Immunohistochemical Gp 130 staining on astroglia (**A**), endothelium (**B**), microglia (**C**) and neurons (**D**) (Frontal cortex, AD patient)

are in agreement with our pilot study using formalin-fixed brain tissue (Haslinger et al. 1998). There, we showed all components of IL-6, as well as IL-6R in astroglia and endothelial cells, and to a lesser extent in microglia or neurons, whereas gp130 immunoreactivity was equally seen in astroglia, microglia, neurons and endothelial cells. Samples of freshly frozen material, then used for methodological comparison, however, consistently presented higher and more reliable staining intensities (Haslinger et al. 1999). These methodological issues may account for differences in expression or even lack of expression, as reported in earlier pilot studies.

In conclusion, we suggest that in studies of IL-6 expression and function, the complete interacting IL-6RC should be analyzed. To test for possible differences in IL-6RC expression in control patients compared to neurodegenerative disorders, further systematic studies of IL-6RC expression in AD are warranted since relatively limited numbers of brain samples and patients have been investigated so far. For a robust statistical analysis of quantitative data, sample size should be increased. To gain further insight into a possible immunoregulatory, immunological or inflammatory role of the IL-6RC in AD neurodegeneration, testing of potential correlations with typical neuropathological hallmarks, like formation of amyloid plaques, total amyloid load, stages of plaque formation, neurofibrillary tangles or stages of synaptic degeneration should be performed.

Based on accumulating evidence the NIA Biological Markers Working group, as part of the Alzheimer Disease Neuroimaging Initiative has suggested the S/L-6RC as a feasible core biological marker candidate for Alzheimer disease. Evaluation in large-scale multicenter validation trials was recommended (Frank et al. 2003).

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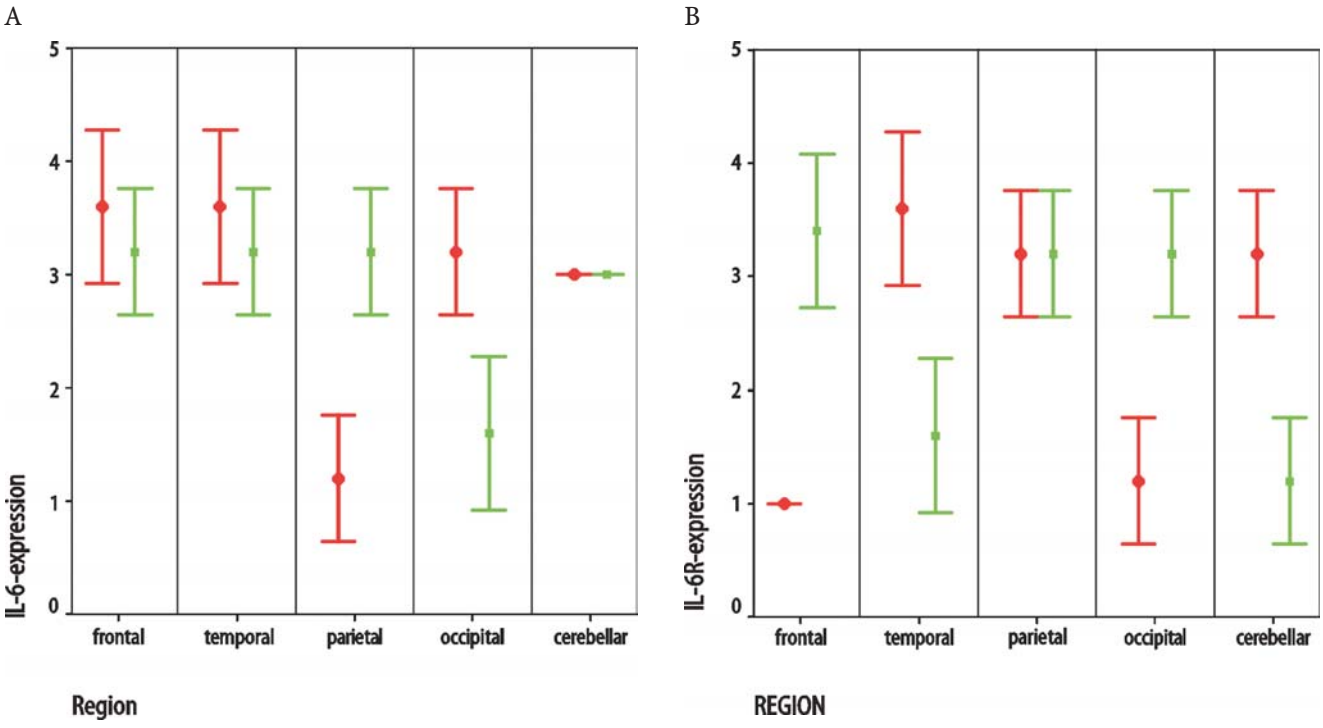


**Fig. 5** **A** Error bars, indicating mean and 95 %-confidence interval for the expression of IL-6 in frontal, temporal, parietal and occipital cortex and in cerebellum of AD patients (green bars) and controls (red bars). **B** Error bars, indicating mean and 95 %-confidence interval for the expression of IL-6R in frontal, temporal, parietal and occipital cortex and in cerebellum of AD patients (green bars) and controls (red bars)

collaboration. APOE genotypes were graciously supplied by the laboratory of Ann M. Saunders, Ph. D.

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