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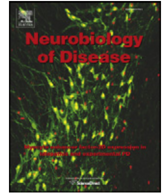

Cytokine profiling in the prefrontal cortex of Parkinson's Disease and Multiple System Atrophy patients

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ABSTRACT

Parkinson's Disease (PD) and Multiple System Atrophy (MSA) are neurodegenerative diseases characterized neuropathologically by alpha-synuclein accumulation in brain cells. This accumulation is hypothesized to contribute to constitutive neuroinflammation, and to participate in the neurodegeneration. Cytokines, which are the main inflammatory signalling molecules, have been identified in blood and cerebrospinal fluid of PD patients, but studies investigating the human brain levels are scarce. It is documented that neurotrophins, necessary for survival of brain cells and known to interact with cytokines, are altered in the basal ganglia of PD patients. In regards to MSA, no major study has investigated brain cytokine or neurotrophin protein expression.
Here, we measured protein levels of 18 cytokines (IL-2, 4–8, 10, 12, 13, 17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1α and 1β, TNF-α) and 5 neurotrophins (BDNF, GDNF, bFGF, PDGF-BB, VEGF) in the dorsomedial prefrontal cortex in brains of MSA and PD patients and control subjects. We found altered expression of IL-2, IL-13, and G-CSF, but no differences in neurotrophin levels. Further, in MSA patients we identified increased mRNA levels of GSK3β that is involved in neuroinflammatory pathways. Lastly, we identified increased expression of the neurodegenerative marker S100B, but not CRP, in PD and MSA patients, indicating local rather than systemic inflammation. Supporting this, in both diseases we observed increased MHC class II^{+} and $CD45^{+}$ positive cells, and low numbers of infiltrating $CD3^{+}$ cells. In conclusion, we identified neuroinflammatory responses in PD and MSA which seems more widespread in the brain than neurotrophic changes.

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1. Introduction

Parkinson's disease (PD) and Multiple System Atrophy (MSA) are both neurodegenerative diseases that are neuropathologically characterized by accumulation of alpha-synuclein (α-syn). In PD, α-syn primarily accumulates in neurons in Lewy body (LB) formations whereas in MSA the protein also accumulates into glial cytoplasmic inclusions (GCIs) in oligodendrocytes (Kim et al., 2014). MSA and PD post-mortem studies have revealed dopaminergic neuronal loss in the order of 35–45% in the substantia nigra pars compacta (SNpc) (Pakkenberg et al., 1991; Salvesen et al., 2015) which can be considered the main epicentre of the disease (Braak and Braak, 2000). Moreover, MSA results in neuronal loss in several other regions besides the SNpc, suggesting a more widespread neurodegeneration (Salvesen et al., 2015; Salvesen et al., 2017). In PD, α-syn accumulation is evident in brain areas distant to the basal ganglia (as summarized by Dickson et al., 2009). Though, the extent of neuronal loss in PD has not yet been fully quantified as in MSA. As MSA progresses patients may experience decreased cognitive functions (Kawai et al., 2008; Stankovic et al., 2014). This is likely due to the extensive neuronal loss observed in frontal cortical areas (Salvesen et al., 2017). In contrast, PD patients show no loss of neurons in the frontal cortex (Pedersen et al., 2005) despite comparable cognitive impairments observed in PD (Yang et al., 2016).

In PD and MSA, neuroinflammation is manifested in the brain by increased numbers of activated microglia (Gerhard et al., 2003; Gerhard et al., 2006; Stefanova et al., 2007) and increased microglial and astrocytic numbers (Salvesen et al., 2015; Salvesen et al., 2017). This constitutive neuroinflammation, which is likely due to accumulation of α-syn, is thought to contribute to the atrophic events occurring in both PD and

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MSA (Stefanova et al., 2007; Tansey and Goldberg, 2010; Vieira et al., 2015). Furthermore, *SNCA*, the gene for α-syn, is also expressed in different cells of the immune system, e.g., T cells, and in microglia (Austin et al., 2006; Shin et al., 2000). Activated microglia can either take up a pro-inflammatory, or an anti-inflammatory phenotype. Depending on their phenotypic activation, microglia release specific cytokines, which are the key signalling molecules in inflammation (Tang and Le, 2016). Both up- and downregulation of cytokine expression has been detected in blood, cerebrospinal fluid (CSF) (Kaufman et al., 2013; Mogi et al., 1996; Mogi et al., 1994b; Stemberger et al., 2011), the SNpc, and the striatum of the brain in PD and MSA patients (Mogi et al., 1994a; Mogi et al., 1996; Mogi et al., 1994b; Mogi et al., 2001; Mogi et al., 1999). However, no extensive study has investigated cytokine expression in cortical areas.

Neuroinflammation interacts with neurotrophin functions (Song et al., 2013). Neurotrophins are proliferating factors supporting cell survival and are secreted by different cells in the brain including neurons, and have been found to be altered in the basal ganglia in brains of PD patients (Mogi et al., 2001; Mogi et al., 1999). So far, only minor studies on neurotrophin expression in MSA brains have been conducted (Kawamoto et al., 2000; Ubhi et al., 2010).

The aim of this study was to perform an extensive comparative investigation of cytokine and neurotrophin protein levels in brains of MSA and PD patients in comparison with normal controls (NCs). We wanted to investigate an area distant to the epicentre of the diseases, i.e., the basal ganglia. Hence, we investigated the dorsomedial prefrontal cortex (dmPFC) of the brain as this area is still subjected to degenerative events in MSA, and at least affected by increased neuroinflammation in PD. We expected that this strategy would provide us with a better indication of the extension of neuroinflammatory processes in the brain. We applied a combination of enzyme-linked immunosorbent assays (ELISAs), multiplex assays, and Western Blotting (WB) along with reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR) for assessment of protein and gene expression levels, respectively. In order to identify the plausible source of the cytokines in question, our observations are strengthened by single and double immunostainings on slices from the same areas in a subsample of the investigated brains.

2. Materials and methods

2.1. Human brain tissue

Samples from 67 human brains were included in this study. The brains had been donated to from the Brain Bank at Bispebjerg-Frederiksberg Hospital (University Hospital of Copenhagen, DNK; approved by the Danish Data Protection Agency, j.no. BFH-2017-001, I-Suite no. 05190), the Netherlands Brain Bank (Netherlands Institute for Neuroscience, NLD), and the Harvard Brain Tissue Resource Center (Harvard Medical School Teaching Hospital, USA). Demographic and autopsy-related information is summarized in Table 1. Full information for all subjects is provided in Supplementary Table 1. All brains have undergone pathological examinations to verify diagnosis through the presence of LBs and GCIs. All brain samples have been collected, and handled in accordance with Danish ethical standards of the Brain Bank and the Danish Health and Medicine Authorities. Samples have been stored at −80 °C prior to handling. This project has been approved by the regional ethical committee of Region Hovedstaden (DNK), journal no. H-16025210. Informed consent was obtained from all donors. All experiments have been performed in accordance with the Declaration of Helsinki.

2.2. Protein extraction

Approximately 50 mg of each brain tissue sample was transferred to 500 μl Tissue Extraction Reagent II (Invitrogen, USA; #FNN0081) containing 1% (v/v) Protease Inhibitor Cocktail (Sigma-Aldrich, USA; #P8340; mixture hereafter known as “buffer”) in MagNA Lyser Green Beads tubes (Roche Life Science, DEU; #03358941001). Samples were disrupted and homogenized twice on a MagNA Lyser instrument (Roche Life Science; #03358976001) for 25 s at 6 k RPM followed by cooling on a frozen MagNA Lyser Rotor Cooling Block (Roche Life Science; #03359085001) for 90 s. Samples were spun for 1 min at 10k ×*g* at 4 °C to reduce foam. Supernatants were transferred to Eppendorf tubes at spun for 20 min at 16k ×*g* at room temperature (RT). Samples were kept on ice while the concentration was determined.

2.3. Bradford protein assay

Samples were diluted 1:4 in buffer. 140 μg bovine serum albumin (BSA; Sigma-Aldrich; #A4503-100G) was dissolved in 100 μl buffer (w/v) and serially diluted 1:2 in buffer producing a standard curve ranging 0.088–1.4 mg/ml. 5 μl diluted samples and standards were added in duplicate on a 96-well polypropylene plate (PP; Thermo Fisher Scientific, USA; #269620). 250 μl Bradford Reagent (Sigma-Aldrich; #B6916) equilibrated to RT was added to each well followed by incubation for 5 min at RT. The plates were read at 620 nm on a Multiskan FC Microplate Photometer (Thermo Fisher Scientific; #51119000). Intra-assay coefficient of variability (CV) was calculated to 10.9% (n = 42, data not shown). A fraction of the samples were diluted to 2 mg/ml in buffer for multiplex analyses, remaining sample was aliquoted for WB and ELISA. All aliquots were stored at −80 °C.

2.4. Multiplex assays

Levels of 21 cytokine and neurotrophin proteins were measured following a multiplexing strategy using the Bio-Plex Pro Human Cytokine 17-plex Assay (BIO-RAD, USA; #M5000031YV) which included interleukin (IL)-1beta (β), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (INF)-gamma (γ), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein (MIP) 1β, and tumor necrosis factor (TNF)-α. Additional singleplex targets were added: basic fibroblast growth factor (bFGF; BIO-RAD; #171B5016M); MIP1α (BIO-RAD; #171B5022M); platelet-derived growth factor (PDGF)-BB (BIO-RAD; #171B5024M); and vascular endothelial growth factor (VEGF; BIO-RAD; #171B5027M). The assays were run on a Bio-Plex 200 System (Luminex xMAP Technology, BIO-RAD; #171000201) following the manufacturer's instructions. A Bio-Plex Pro Wash Station was used for washing steps (BIO-RAD; #30034376). Samples were diluted 1:2 in sample diluent included in the kit. Samples had not been thawed prior to analysis.

Table 1
Demographic data of cohort. Data for age, RNA Integrity Number (RIN), and post-mortem interval (PMI) were analysed using Welch's weighted ANOVA. A χ^2 test was used to test sex ratios. M: male; F: female; NC: normal control; PD: Parkinson's Disease; MSA: Multiple System Atrophy; BBH: Brain Bank at Bispebjerg Hospital; NBB: Netherlands Brain Bank; HV: Harvard Brain Bank. Age, RIN, and PMI data are shown as mean ± standard deviation.

Patient group	Origin	n	Sex (M/F)	Age (years)	RIN	PMI (h)
NC	7 BBH, 10 NBB	17	7/10	76.1 ± 10.7	5.2 ± 1.4	21.8 ± 20.2
PD	11 BBH, 20 HV	31	21/10	79.0 ± 7.5	5.7 ± 2.4	26.2 ± 17.6
MSA	BBH	19	6/13	64.9 ± 6.5	5.1 ± 2.0	44.2 ± 22.8
p-Value			0.030	<0.001	0.104	<0.001

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