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Research article

Pathological histone acetylation in Parkinson's disease: Neuroprotection and inhibition of microglial activation through SIRT 2 inhibition

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ABSTRACT

Parkinson's disease (PD) is associated with degeneration of nigrostriatal neurons due to intracytoplasmic inclusions composed predominantly of a synaptic protein called α-synuclein. Accumulations of α-synuclein are thought to 'mask' acetylation sites on histone proteins, inhibiting the action of histone acetyltransferase (HAT) enzymes in their equilibrium with histone deacetylases (HDACs), thus deregulating the dynamic control of gene transcription. It is therefore hypothesised that the misbalance in the actions of HATs/HDACs in neurodegeneration can be rectified with the use of HDAC inhibitors, limiting the deregulation of transcription and aiding neuronal homeostasis and neuroprotection in disorders such as PD. Here we quantify histone acetylation in the Substantia Nigra pars compacta (SNpc) in the brains of control, early and late stage PD cases to determine if histone acetylation is a function of disease progression. PD development is associated with Braak-dependent increases in histone acetylation. Concurrently, we show that as expected disease progression is associated with reduced markers of dopaminergic neurons and increased markers of activated microglia. We go on to demonstrate that *in vitro,* degenerating dopaminergic neurons exhibit histone hypoacetylation whereas activated microglia exhibit histone hyperacetylation. This suggests that the disease-dependent increase in histone acetylation observed in human PD cases is likely a combination of the contributions of both degenerating dopaminergic neurons and infiltrating activated microglia. The HDAC SIRT 2 has become increasingly implicated as a novel target for mediation of neuroprotection in PD: the neuronal and microglial specific effects of its inhibition however remain unclear. We demonstrate that SIRT 2 expression in the SNpc of PD brains remains relatively unchanged from controls and that SIRT 2 inhibition, via AGK2 treatment of neuronal and microglial cultures, results in neuroprotection of dopaminergic neurons and reduced activation of microglial cells. Taken together, here we demonstrate that histone acetylation is disease-dependently altered in PD, likely due the effects of dopaminergic neurodegeneration and microglial infiltration; yet SIRT 2 remains relatively unaltered with disease. Given the stable nature of SIRT 2 expression with disease and the effects of SIRT 2 inhibitor treatment on degenerating dopaminergic neurons and activated microglia detected *in vitro*, SIRT 2 inhibitors warrant further investigation as potential therapeutics for the treatment of the PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, and the most prevalent movement disorder, presenting clinically as cardinal symptoms of rigidity, tremor, and bradykinesia [11,26]. These primary motor symptoms are the result of degeneration of dopaminergic nigrostriatal pathways, thought to be due to intracytoplasmic protein inclusions in dopaminergic neurons within the Substantia Nigra pars compacta (SNpc), known as Lewy bodies and Lewy neurites, composed predominantly of a synaptic protein called α-synuclein (αSyn) [11,48]. Dopaminergic SNpc neurodegeneration in PD is also associated with activation of the brain's innate immune response, with an activation of resident microglia in the brain [11,26]. Recruitment of activated microglia to the SNpc in PD therefore leads to an

Abbreviations: BCL2, B-cell lymphoma 2; BDNF, brain derived neurotrophic factor; COX2, cyclooxygenase 2; GDNF, glial derived neurotrophic factor; HAT, histone acetyltransferase; HDAC, histone deacetylase; HLA-DPα1, human leukocyte antigen DPα1; IL1β, Interleukin 1β; IL6, Interleukin 6; iNOS, inducible nitric oxide synthase; LCM, laser capture microdissection; LPS, lipopolysaccharide; NO, nitric oxide; PD, Parkinson's disease; SIRT 2, Silent Information Regulator 2; SNpc, Substantia Nigra pars compacta; TH, tyrosine hydroxylase; TNFα, tumour necrosis factor α; αSyn, αSynuclein

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upregulation and secretion of proinflammatory cytokines, such as Tumour Necrosis Factor α (TNFα), Interleukin 6 (IL6) and Interleukin 1β (IL1β), and activation of inducible Nitric Oxide Synthase (iNOS) resulting in production of nitric oxide (NO) [23], which in turn are thought to exacerbate degeneration of dopaminergic SNpc neurons [11]. In recent years epigenetic mechanisms such as DNA methylation and histone remodelling through acetylation have also become implicated in PD pathogenesis [2], and as such have become increasingly studied in PD pathogenesis.

In healthy cells there is a tightly controlled equilibrium between the effects of histone acetyltransferases (HATs) and histone deacetylases (HDACs) enabling histone (de)acetylation and the dynamic control of gene transcription [13,45]. In healthy neurons this results in appropriate regulation of gene expression and subsequently facilitates appropriate neuronal homeostasis [45]. In neurodegenerative disease however, there is known to be an imbalance between the activities of HATs and HDACs in favour of histone deacetylation, thought to be pathogenic in disease progression [13,43,45]. This misbalance in neurodegeneration was first noted in both an *in vitro* model of cortical neuronal cell death induced by activation of amyloid precursor protein signalling, a hallmark of Alzheimer's disease, and in an *in vivo* model of amyotrophic lateral sclerosis: the G86R mutant SOD-1 mice displaying motor neuron degeneration [43]. More specific to PD, we demonstrated recently that intracellular protein accumulation in a ubiquitin proteasome inhibitor rat model of PD results in histone hypoacetylation [21]. Likewise αSyn accumulation itself has been shown to promote histone H3 hypoacetylation as ascertained from overexpression studies in SH-SY5Y cells as well as in an *in vivo* αSyn transgenic drosophila model, thought to be achieved through αSyn 'masking' acetylation sites on histone proteins [31]. From these findings then it is hypothesised that the misbalance in the activities of HATs/HDACs could be rectified with the use of HDAC inhibitors (HDACIs) to reduce the extent of cell death in the nigrostriatal pathways in PD [10,13,19,22,29]. For example, inhibitions of HDACs 1 and 2 [9], and 6 [27,38], as well as broader inhibitors of entire HDAC classes such as I and IIa [20,21,36], and IIb [50], have all been demonstrated recently to be neuroprotective in models of PD. Notably, inhibition of the class III HDAC, Silent Information Regulator 2 (SIRT 2), has become increasingly implicated as a novel target for mediation of neuroprotection in PD [8,12,14,16,17,32,34,52]. For example, Outeiro et al. [34] have previously demonstrated that AGK2, a potent inhibitor of SIRT 2 dose dependently protects dopaminergic neurons from death in a transgenic αSyn overexpressing drosophila models of PD.

Although the neuroprotective phenotype of HDAC selective, such as SIRT 2, inhibitors have been demonstrated *in vivo* in animal models of PD, thus far pathogenic histone hypoacetylation and transcriptional dysfunction in the nigrostriatal of PD is yet to be confirmed. The acetylation level of histones within degenerating regions of the Parkinsonian brain must therefore be quantified and compared with age matched control subjects to confirm this hypothesis in the human disease and rationalise the use of HDACIs for the treatment of PD. Additionally, although it is thought that pathogenic histone hypoacetylation is in part due to the 'masking' effects of αSyn aggregates toward histone proteins, it remains unanswered whether the expression levels of HDACs in the brain are affected in PD. This is crucial as without confirming the maintenance of HDAC expression levels in the Parkinsonian brain, the use of HDACIs for therapeutic use in PD cannot be rationalised. Therefore here, for the first time, we quantify histone acetylation levels in the SNpc, the area known to predominantly degenerate with PD development, in brain tissue from both early (Braak stage 3/4 [3]) and late (Braak stage 6 [3]) stage PD cases, as well as age matched controls, to determine if histone acetylation is indeed a function of PD development. Additionally, given the implication of the use of HDAC (such as SIRT 2) inhibitors for neuroprotection in PD, we quantify HDAC expression in the SNpc of these same cases, to determine if the expression level of these enzymes changes with PD development. Furthermore, we seek to validate the neuroprotective and anti-inflammatory potential of SIRT 2 inhibition with the use of cell culture models of dopaminergic neurodegeneration and microglial activation, respectively, highlighting this HDAC as a potential therapeutic target for the treatment of PD.

2. Materials and methods

2.1. Human brain tissue

Human brain tissue samples (PD and aged-matched controls) were obtained from the Parkinson's UK Tissue Bank at Imperial College London, and all experiments using the tissue samples were previously approved by the PUKTB's Ethical Review Panel. Tissue from 10 control cases (4♂: 6♀, 82.1 ± 1.9 years), 8 early PD cases (Braak stage 3.5 ± 0.2, 4♂: 4♀, 79.3 ± 1.8 years), and 12 late PD cases (Braak stage 6 ± 0, 4♂: 4♀, 79.3 ± 1.8 years) were included for study, and data presented represents all cases studied. Cases were selected based upon the Tissue Bank's availability of snap frozen tissue from the central region of the SNpc, in Braak stage 3-4, and Braak stage 6 PD cases, and healthy age matched controls. The suitability of tissue and sampling of the central SNpc was determined and performed by an experienced Tissue Bank technician at time of sample retrieval. Supplementary Table 1 summarises the cases used, including case by case age at death, cause of death, post-mortem delay, and for PD cases, age at disease onset, disease duration, Braak stage, and any PD medication taken in life.

2.2. Cell cultures

The rat mesencephalic dopaminergic 1RB3A$_{N27}$ (N27) cell line possesses both biochemical and physiological properties of dopaminergic neurons [1], making it an ideal candidate cell line for the modelling of the Parkinsonian dopaminergic neuronal cell death *in vitro*. Prior to experimentation, N27 expression of NeuN and TH were confirmed by western blot analysis (see below for methods), confirming the dopaminergic neuronal phenotype of this cell line (supplementary Fig. 1). N27 cells (Millipore, UK) (up to passage number 45) were maintained in RPMI 1640 medium (Sigma, UK) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50U/ml Penicillin and 50 μg/ml Streptomycin (all Gibco, UK) (complete N27 medium), in a humidified incubator temperature controlled at 37 °C and with 5% CO_2 ventilation. For experimentation, neurons were seeded into 96 well plates (Corning, UK) at a density of 10 × 10^3 cells/well, and left for 24 h to allow neurons to readopt their natural morphology. On the day of experiments cell medium was removed and replaced with fresh complete N27 medium. For induction of neurodegeneration, N27 cells were treated with lactacystin (Enzo Life Sciences, UK) (0.75 μM in distilled phosphate buffered saline (DPBS) (Sigma, UK)) for 24 h as this was shown to produce suitable robust sub-maximal levels of cytotoxicity (data not shown). For SIRT 2 inhibitor treatment, N27 cells were pre-treated with AGK2 (Tocris, UK) (in DPBS) for 24 h as this timepoint was shown produce significant hyperacetylation (supplementary Fig. 2).

The mouse microglial (N9) cell line stably retains microglial phenotypic cell surface markers, and most importantly are stringently activated upon treatment with LPS [41] making them an ideal cell line for the study of microglial activation *in vitro*. Prior to experimentation, N9 expression of Iba-1 was confirmed by western blot analysis (see below for methods), confirming the microglial phenotype of this cell line (supplementary Fig. 1). N9 cells (a kind gift from Dr Deanne Taylor) (up to passage number 45) were maintained in Dulbecco's Modified Eagle's Medium (Sigma, UK) supplemented with 5% foetal calf serum, 4 mM L-glutamine, 50U/ml Penicillin and 50 μg/ml Streptomycin (complete N9 medium), in a humidified incubator temperature controlled at 37 °C and with 5% CO_2 ventilation. For experimentation, microglia were