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# Tyrosine hydroxylase and Lewy body molecules immunoreactivity in the SNC neurons of an AS/AGU mutantrat

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## ABSTRACT

**The AS/AGU rat has a recessive single point mutation in the gene coding for the gamma isoform of protein kinase C (PKC-γ) resulting in a failure to release dopamine in the striatum and impaired movement including a staggering gait, difficulty in initiating movement and a slight whole body tremor. This study examined the levels tyrosine hydroxylase, ubiquitin and parkin in individual SNC cell bodies. There was no evidence of a reduction in tyrosine hydroxylase levels although levels of ubiquitin and parkin were elevated in the cytoplasm. The findings support the hypothesis that the initial bar to dopamine availability in the striatum is reduced release, with substantia nigra cell death being a later phenomenon.**

**Keywords:** PKC-Gamma; Tyrosine Hydroxylase; Ubiquitin; Parkin

## 1. INTRODUCTION

The AS/AGU rat originated as a recessive mutation (*agu*) in a closed colony of Albino Swiss (AS) rats. The mutation is in the gene coding for the gamma isoform of protein kinase C [1]. The rats are characterized by a movement impairments including rigidity of the hind limbs, a staggering gait, a tendency to fall over every few steps, a slight whole body tremor and difficulty in initiating movements [2,3] by progressive dysfunction of the nigro-striatal dopaminergic (DA) and raphe-striatal serotonergic (5-HT) systems. The chief defect in both systems is a failure to release transmitter within the striatum under normal physiological conditions. Thus, extracellular dopamine levels in the mutant (measured using microdialysis with HPLC-ECD in conscious animals) are only 10% - 20% of control levels [4] and 5-HT is similarly reduced [5]. There is also a marked depletion in utilization of 2-deoxy-glucose in the substantia nigra pars compacta, subthalamic nucleus and ventrolateral thalamus [6]. At later ages, there is loss of aminergic cell bodies [7].

Clinical conditions such as Parkinson's disease (PD) are characterized by widespread loss of dopaminergic cell bodies and terminals, but this can only be determined post mortem. Death is usually many years after the onset of symptoms. There is, therefore, little indication of the mechanism of cell loss or of the cellular changes taking place prior to that. In animal models of PD, the death of dopaminergic neurons following MPP+ or MPTP administration has been variously attributed to necrosis [8] to apoptosis [9-11] and to autophagy [12]. Furthermore, because the toxic agents used to produce models of PD act rapidly (e.g. MPTP/MPP+, 6-OH-DA), for reviews see [13] Schwarting and Huston, 1996; [14] Flint Beal, 2001, they are unlikely to give insight into any chronic stage during which function might potentially be retrievable.

Commonly used experimental procedures leading to death of SNC neurons (e.g. treatment with 6-hydroxydopamine or MPTP administration), [14] do not exhibit cell inclusions akin to Lewy bodies [15,16] although rotenone treatment is an exception [17]. Similarly, inclusions do not occur in the AS/AGU rat. However, it is unclear if molecules associated with Lewy bodies, such as ubiquitin, alpha-synuclein or parkin, are elevated.

The AS/AGU rat therefore presents an opportunity to examine dopaminergic cell bodies and terminals in a naturally occurring rat model which combines striatal dopamine dysfunction with motor disturbance. This study was undertaken to measure levels of the Lewy body associated proteins ubiquitin and parkin in the nigral cell bodies of the mutant and to compare them with control (unaffected) animals, individual cells of the two strains. Animals aged twelve months were used, as all mutants are reliably symptomatic at this age.

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## 2. MATERIALS AND METHODS

A comparison of tyrosine hydroxylase, ubiquitin and parkin levels in individual midbrain cell bodies of AS and AS/AGU rats using a quantitative fluorescent ICC technique.

Six male AS rats and six male AS/AGU mutants aged twelve months were killed by carbon dioxide euthanasia followed by intracardiac perfusions of 100 ml mammalian Ringer solution and 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and stored in 4% paraformaldehyde in phosphate buffer overnight before routine dehydration, embedding in paraffin wax at 57˚C and serial sectioning at 7 μm on a Spencer 820 microtome. Representative sections from the ribbons were collected and stained with 1% aqueous toluidine blue and examined so that matching pairs of unstained sections (AS and AS/AGU) could be taken from the central substantia nigra pars compacta of each animal. These corresponded approximately to coronal sections at –5.3 mm relative to bregma [18]. All sections were processed, examined and quantified in pairs from this stage onwards.

Sections were stretched in a mounting bath for 1 - 3 minutes at 40˚C and mounted on APES (3-aminopropyltriethoxysilane) coated slides, dried overnight at 37˚C and then at 56˚C for two hours. The sections were deparaffinized and rehydrated before undergoing a heat-mediated antigen retrieval technique [19] in boiling 0.01 M sodium citrate buffer (pH 6.0) and then at 120˚C for 1 min in a Prestige stainless steel pressure cooker [20]. After this, sections were rinsed in distilled water followed by 0.01 M phosphate buffered saline (PBS, 5 min) and treated with 1% normal goat serum (NGS, Sigma-Aldrich G9023, UK) in PBS with 3% Triton X-100 for 60 minutes to reduce non-specific background staining.

1) For fluorescent staining for tyrosine hydroxylase, sections were initially incubated for 24 h in a humidity chamber at 4˚C with the primary antibody (Monoclonal mouse anti-tyrosine hydroxylase, MAB 5280, Chemicon Europe Ltd.) at a concentration of 1:500, diluted in blocking serum (1% NGS in PBS with 0.3% Triton X-100). After 3 × 5 min washes in PBS, sections were incubated in a humidity chamber for 24 hours at 4˚C with a fluorescent antibody (Rhodamine Red-X goat-anti-mouse, 115-295-146, Jackson Immunoresearch) at a dilution of 1:100. Slides were covered during this (and all subsequent processes) to protect from bleaching. Sections were mounted with glass coverslips using Vectashield (H-1400, UK) after rinsing in distilled water (3 × 5 min), and fluorescent quantification was carried out the same day;

2) For fluorescent staining for ubiquitin (polyclonal rabbit anti-ubiquitin Z0458, DAKO, Cambridge, UK), sections were incubated in a humidity chamber for 24 h at 4˚C with a mixture of two primary antibodies (anti-tyrosine hydroxylase and anti-ubiquitin) diluted at 1:500 in the blocking serum. After rinsing in PBS (3 × 5 min), the sections were incubated in a humidity chamber for 24 h at 4˚C with two fluorescent secondary antibodies with Fluorescein (goat-anti-mouse 115-095-116, Jackson Immunoresearch) for TH and Rhodamine (goat-anti-rabbit, Jackson Immunoresearch) for ubiquitin, at dilutions of 1: 100 in PBS for both. Slides were covered to give protection from bleaching in this step and the rest of the procedure. The sections were then mounted with glass coverslips using Vectashield (H-1400, UK) after rinsing in distilled water (3 × 5 min);

3) For fluorescent staining for parkin, the primary anti-parkin antibody (anti-parkin I-126 raised in rabbit) was a gift from Professor Poul Henning Jensen, Institute of Medical Biochemistry, bldg. 170, University of Aarhus, DK-8000 Aarhus, Denmark [21]. Double staining procedures were identical to those for ubiquitin (see above).

Quantitative measurements of the immunofluorescence of individual cells was carried out on a Zeiss Axioskop using Zeiss Axiovision (version 4.8) software to perform densitometric analysis of fluorescence levels (Zeiss Axioskop with HBO 110 illuminator and Rhodamine filter set emission 575 - 640 nm, Germany). Image capture was carried out with a Zeiss AxiocamMRc.

For all animals, a minimum of 50 cells with visible nucleoli from each of three regions (rostral, central and caudal substantia nigra pars compacta) were highlighted from the captured file and the fluorescence measured densitometrically. In each case, readings were taken from unstained regions also to give a background fluorescence value, which was then subtracted from the display value. Values were compared between AS and AS/AGU pairs using a paired *t*-test.

## 3. RESULTS

### Levels of Tyrosine Hydroxylase (TH), Ubiquitin and Parkin

Double labeling showed that 90% of cells in the area of the substantia nigra pars compacta stained positively for TH. This figure is made up of 89% which stained both for TH and ubiquitin and 1% which were ubiquitin-negative. A further 10% of cells were ubiquitin-positive and TH-negative. The frequencies obtained by double labeling for TH and parkin were identical to those of TH and ubiquitin.

Fluorescent measurements were not made on double-labeled sections, but on adjacent sections where one was stained for TH and the adjacent one for either ubiquitin or parkin (see **Figure 1**). **Table 1** shows that the fluorescence levels for all three molecules were at similar levels throughout the substantia nigra when this was divided into rostral, central and caudal thirds. However, TH lev-

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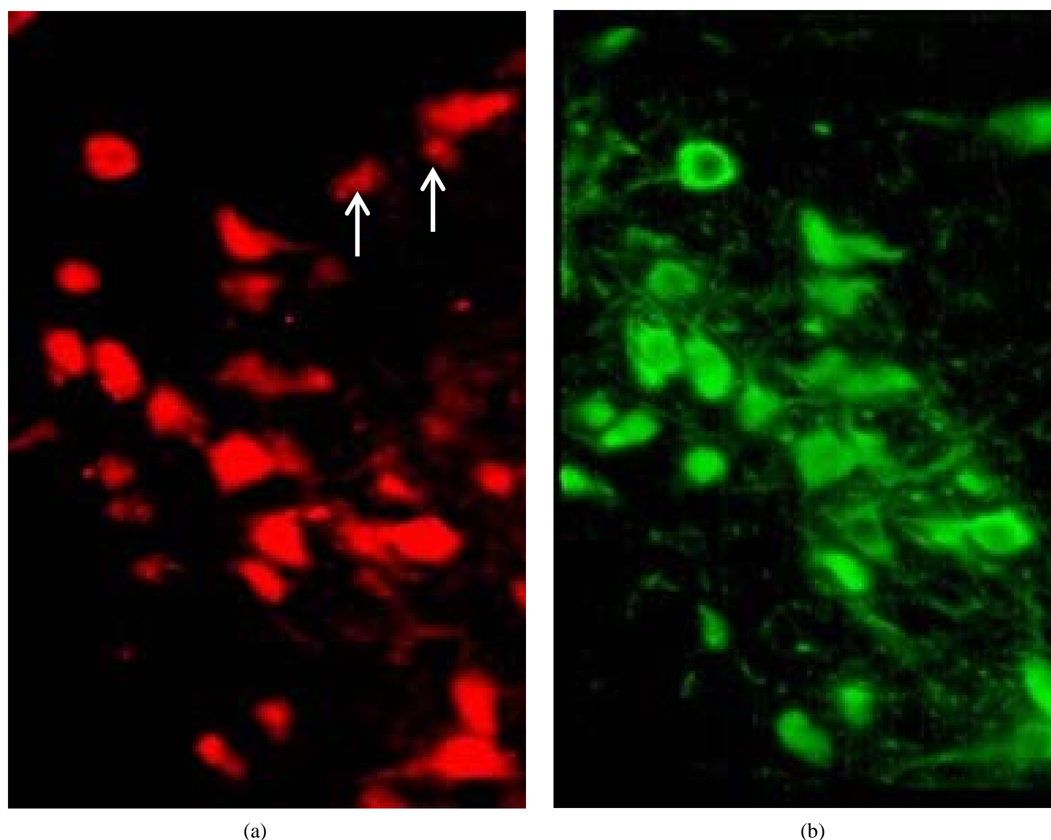
els were some 40% higher in AS/AGU mutants than in AS controls. Similarly, ubiquitin was 36% higher, and parkin 47% higher in the mutant rat SNC cells. These strain differences were highly significant.



(a) (b)

**Figure 1.** Ubiquitin (Ub) and TH immunostained cells of AS/AGU substantia nigra. (a) Ubcells (red) in (a), white arrows indicate examples of ubiquitin-positive cells which are non-dopaminergic and which were omitted from the count; (b) TH cells (green) (×500).

**Table 1.** Levels of tyrosine hydroxylase, ubiquitin and parkin in dopaminergic cells of the rostral, central and caudal substantia nigra pars compacta (SNC) of AS and AS/AGU rats aged 12 months (units are mean densitometric units ± SEM).

| | | AS | AS/AGU | |
|---|---|---|---|---|
| | Rostral SNC | 1856 ± 78 | 2732 ± 94 | <0.001 |
| **Tyrosine hydroxylase** | Central SNC | 1994 ± 20 | 2511 ± 113 | <0.01 |
| | Caudal SNC | 1769 ± 93 | 2573 ± 118 | <0.001 |
| | Rostral SNC | 772 ± 70 | 1165 ± 118 | <0.01 |
| **Ubiquitin** | Central SNC | 710.6 ± 84 | 1000 ± 98 | <0.001 |
| | Caudal SNC | 618 ± 20 | 1220 ± 113 | <0.01 |
| | Rostral SNC | 151 ± 6 | 239 ± 7 | <0.001 |
| **Parkin** | Central SNC | 169 ± 32 | 247 ± 19 | <0.01 |
| | Caudal SNC | 133 ± 8 | 228 ± 6 | <0.001 |

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## 4. DISCUSSION

Nigral dopaminergic neurons in the AS/AGU rat are known to release very little dopamine in the striatum—with extracellular levels only 10% - 20% of normal [4]. However, there is no evidence that they lack the ability to synthesise dopamine—in fact whole tissue micropunches of the midbrain and striatum have shown that dopamine levels remain normal until six months or more [22]. The present experiment demonstrates that TH levels in the cell bodies of the SNC are actually elevated in the mutant (**Table 1**). One possible conclusion is that, whilst striatal release of dopamine is dysfunctional, the cell bodies of DA neurons are capable of normal physiological responses.

Whole tissue dopamine levels in micropunch samples from the dorsal and lateral caudate-putamen analysed by HPLC-ECD are known to be reduced by some 30% - 40% in the AS/AGU mutant rat compared to the AS control between 6 and 12 months of age [23]. Similar reductions of dopamine levels in the dorsal striatum have been seen in the weaver mouse [24], in post-mortem Parkinson's disease patients [25] and in living patients with the disorder [26,27]. MPTP exposure can also greatly reduce striatal dopamine [28,29]. By contrast, extracellular levels of dopamine in the striatum as measured in microdialysis samples from conscious, freely-moving AS/AGU rats are reduced by 80% - 90% [4]. This leaves the possibility that dopamine is present in striatal terminals in reasonable amounts, but is not releasable under normal physiological conditions.

Levels of ubiquitin and parkin were elevated in nigral cell bodies of the AS/AGU mutant compared to the AS parent strain. In idiopathic Parkinson's disease, levels of ubiquitin and parkin (which contains a ubiquitin-like homology domain at its N-terminus and may be involved in the recognition of the substrates and the subsequent degrading of mis-folded proteins) are elevated and the proteins incorporated into cell inclusions [30-39]. Such inclusions have not been found in laboratory models of Parkinson's disease such as those produced through 6-OH-dopamine or MPTP toxicity [15,16], though they are present in rotenone-induced degeneration in rat [17].

It is unclear what these elevations signify. Dysfunction of the ubiquitin-proteasome system (UPS) has been implicated in Parkinson's disease and several other neurodegenerative disorders, and rats treated with proteasome inhibitors demonstrate neurodegeneration of midbrain cell groups [40,41]. Mutations in the gene responsible for parkin synthesis have been implicated in early onset forms of Parkinson's disease [42,43].

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