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Age- and Strain-related Changes in the Mutant Albino Swiss/ Anatomy Glasgow University Rats: A comparative Study of Lipofuscin and Calbindin D-28k Levels in Cerebellar Purkinje Cells

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Abstract

Background A spontaneous recessive mutation in the gene coding for protein kinase gamma has created a new rat strain called Albino Swiss/ Anatomy Glasgow University (AS/AGU). It characterized by disordered locomotion due to impaired dopamine release from certain areas of the brain and eventual neurodegeneration. The mutant rats also have a shorter life span.

Objective The present study investigates Lipofuscin level as an indicator of age and calbindin D-28k level as an indicator of neuronal integrity in cerebellar Purkinje cells of the mutant AS/AGU strain in comparison to the normal AS strain.

Methods Eighteen rats (9 AS and 9 AS/AGU) were selected to study the Lipofuscin and calbindin in relation to age and strain using multiple histological techniques including fluorescent microscopy.

Results Fluorescent microscopical study has shown early age related Lipofuscin accumulation in the AS/AGU rats compared to the normal strain. Moreover, Calbindin D-28k showed age related increase in both strains but marginally significant decline in the AS/AGU strain.

Conclusions Our study presents AS/AGU as an animal model of early aging in addition to its value as a model of neurodegeneration.

Keywords Aging, Albino Swiss Rat, Calbindin D-28k, Fluorescent Microscopy, Lipofuscin.

List of abbreviation: LF = Lipofuscin, CNS = central nervous system, AS = Albino Swiss, AS/AGU = Albino Swiss/Anatomy Glasgow University, ICC = immunocytochemistry, FM = Fluorescent Microscopy, PBS = Phosphate Buffer Saline solution, PKC-γ = Phosphate Kinase C - gamma.

Introduction

Lipofuscin (LF) is a brown pigment that accumulates with age in postmitotic cells and cells with low mitotic activity [1,2], but not in actively proliferating cells except where division has been inhibited [3,4]. While LF accumulates in a time dependent linear fashion, ceroid accumulation is fast and age-independent [7].

Electron microscopy shows LF as dense material bounded by a single layer membrane (typical for lysosomes) often containing vacuoles [8]. LF is formed predominantly by un-categorized proteins and lipids with traces of carbohydrates and metals [6,8,9]. It auto-fluoresces in a narrow wavelength range in intact cells (440-460 nm) but in a wider range when extracted [8,10]; while the source of fluorescence in LF is unknown, several studies have managed to produce similar auto-fluorescent material from limited combinations of molecules [11,12].

The mechanism of LF formation is unclear, but most theories assume dysfunction of lysosomal

mechanisms in which there is a mismatch between formation and degradation, and LF forms from residual macromolecular and cellular components [8,13-17]. One of the factors which may precipitate lysosomal dysfunction, is the formation of excessive reactive oxygen species which leads to mitochondrial disorganization and proteosomal accumulation of non-degradable LF [8,18,19]. It is noteworthy that LF still exhibits lysosomal enzyme activity [9].

Calbindin D-28k is one of the intracellular calcium binding proteins that regulate the critical intracellular level of calcium ion for normal cellular functions [20] and it may be altered by cell injury [21]. Many studies have shown that calcium binding proteins have neuroprotective effects in acute brain injury [22], in Parkinson's disease and after calcium induced toxicity [23]. Age related changes of Calbindin D-28k are therefore a potential field of study for many researchers in both health and disease conditions. Calbindin D-28k is highly expressed in most central nervous system (CNS) neurons in both humans and animals including the Purkinje cells of the cerebellum.

The Albino Swiss/Albino Glasgow University (AS/AGU) rat has a recessive mutation in the gene ***agu*** coding for PKC-gamma [24] which leads to disordered locomotion [25,26] and an inability to release dopamine and serotonin in the striatum [27]. Mutant rats also reach a lower body weight than the parent AS strain, and have a shorter life span. They may therefore be useful as a spontaneous model of accelerated aging and neuronal injury. Since LF accumulation is inversely related to remaining life span [28] and Calbindin D-28k changes might have effects on vulnerability to neuronal dysfunction, this present study was carried out to examine LF deposits and Calbindin D-28k levels in the brain of AS/AGU rat and compare them with the AS control rat, using Purkinje cells of the cerebellum as a large and easily defined population. Purkinje cells are a class of GABAergic neurons located in the cerebellum of human and other mammalian brains including rats [29,30]. They are characterized by large number of dendritic spines and by their large size, which make them easily identifiable. They are found within the Purkinje layer in the cerebellum and aligned linearly stacked one in front of the other [31]. Purkinje cells send inhibitory projections to the deep cerebellar nuclei, and constitute the sole output of all motor coordination in the cerebellar cortex [32,33].

This study aimed to investigate changes in the AS/AGU rat strain to demonstrate early aging and state of neuroprotection.

Methods

A total of 18 male rats were used in this study and fixed with 4% paraformaldehyde by cardiac perfusion post-mortem. The CO_2 method used to sacrifice the animals was followed and according to the ethical standards employed by the University of Glasgow. These rats included six animals aged 6 months (3 AS and 3 AS/GU) and twelve animals aged 12 months (6 AS and 6 AS/AGU). In each case, the brain was extracted from its fixative and cut into three parts: forebrain, midbrain and cerebellum as shown in Fig. 1.

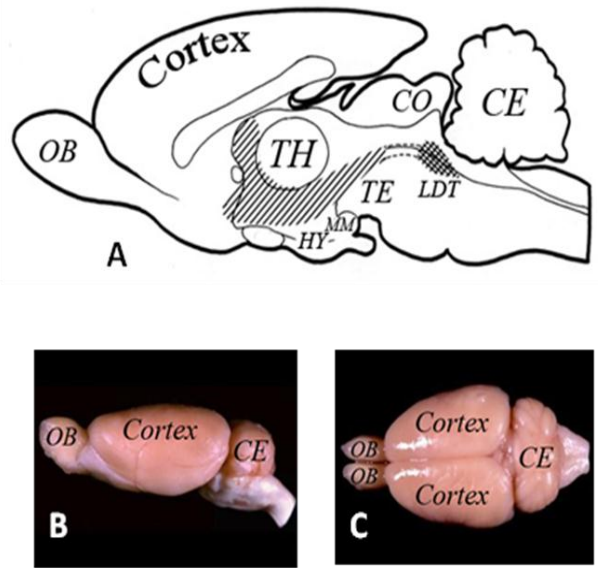

Fig. 1. Gross Anatomy of rat brain showing (A) sagittal section with major brain areas illustrated (B) lateral view and (C) axial top view of the rat brain. CO = colliculi, HY = hypothalamus, LDT = laterodorsal tegmental nucleus, MM = mammillary bodies, TH =

thalamus, TE = tegmentum.

For fluorescent microscopy and immunocytochemistry (ICC), these parts were put in a tissue processer for 24 hours to prepare them for paraffin embedding. After paraffin embedding, cerebellar blocks were selected and cut using a Jung Biocut-2035 rotatory microtome and the slides were standardized to 8 micron thickness for consistency in measurement and for ordinary visualization. The sections were fixed to APES-coated slides and kept overnight to dry.

Fluorescent Microscopy (FM) and Immunocytochemistry (ICC)

A total of 12 cerebellar blocks (6 AS and 6 AS/AGU) from two age groups (half at 6 month age and half at 12 month age) were chosen for LF and Calbindin D-28k measurement. Two widely separated sections (8 micron thickness) from each cerebellar block were selected for LF measurement and another two were used for Calbindin D-28k measurement.

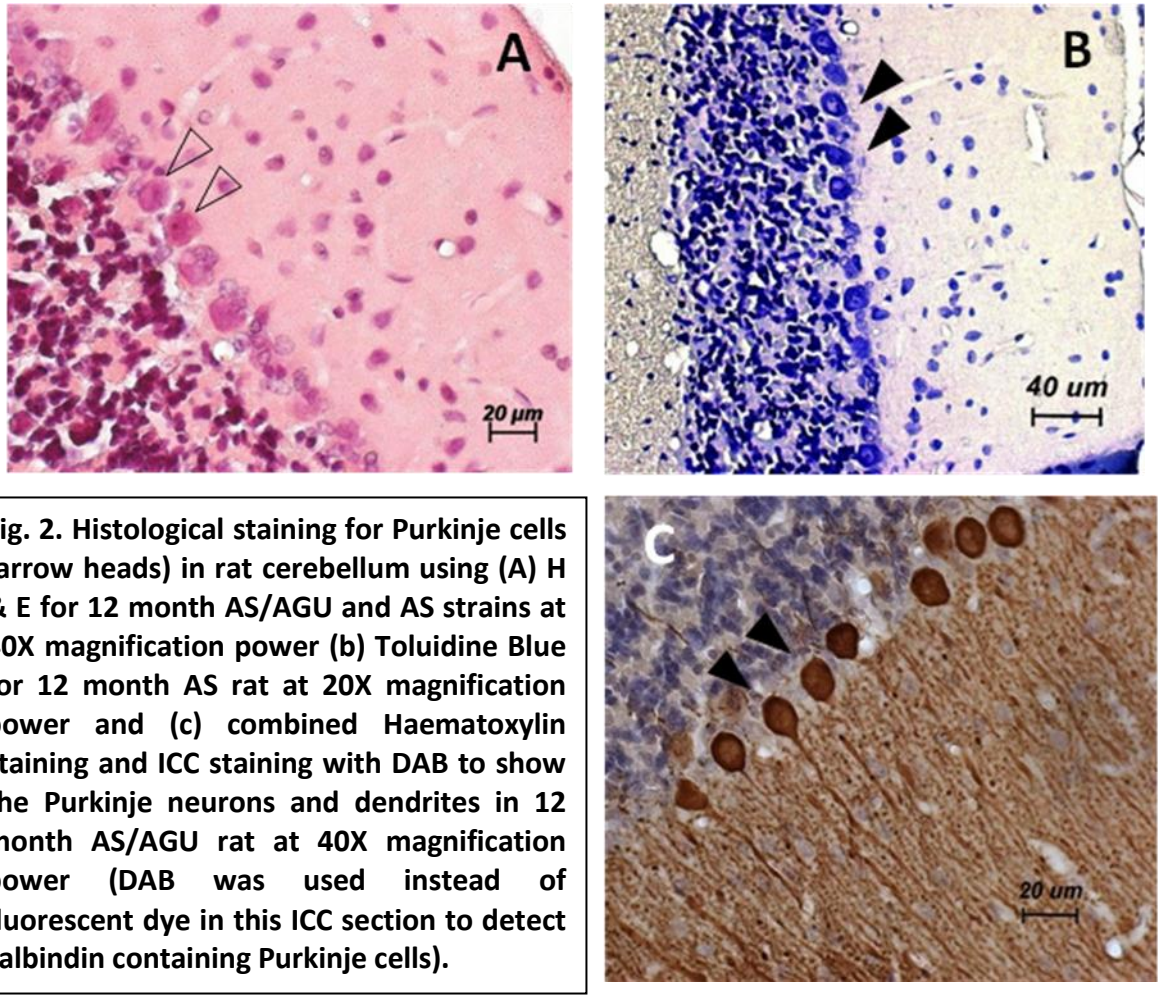

Fig. 2. Histological staining for Purkinje cells (arrow heads) in rat cerebellum using (A) H & E for 12 month AS/AGU and AS strains at 40X magnification power (b) Toluidine Blue for 12 month AS rat at 20X magnification power and (c) combined Haematoxylin staining and ICC staining with DAB to show the Purkinje neurons and dendrites in 12 month AS/AGU rat at 40X magnification power (DAB was used instead of fluorescent dye in this ICC section to detect calbindin containing Purkinje cells).

The LF sections were deparaffinised in Histo-Clear solution for 15 minutes and mounted. The ICC slides for calbindin, were deparaffinised and boiled for 30 minutes in citrate buffer (pH=6) to unbind the cellular proteins (a critical step in ICC), then they were rinsed three times using 0.2M Phosphate Buffer Saline solution (PBS).

Blocking serum (1% normal goat serum in 0.3% Triton/PBS) was prepared and added on the slides to prevent nonspecific binding of calbindin specific primary antibody.

Then, the primary antibody against Calbindin D-28k (Swant® monoclonal Ab CB300) was used at a dilution of 1:500 in the blocking serum to continue blocking during primary

antibody application.

Next day, a fluorescent secondary antibody (at 1:100 dilutions in PBS) was added after three rinses in 0.2M PBS and kept overnight.

Finally, slides were washed three times using 0.2M PBS and mounted with hard fluorescent mounting material (VectaShield© Hard set™ H-1400).

Both LF and ICC slides were measured spectrophotometrically using a LEITZ LABRORLUX Fluorescent Microscope.

The fluorescent microscope parameters were standardized to 650 volts of the UV power source and an objective lens with 40X magnification power was used in all fluorescent microscope measurements.

Each slide was examined and 50 Purkinje cells in row with an obvious nucleus were selected for measurement providing that the appearance of the nucleus indicated that we were roughly in the centre of the Purkinje cell.

Measurement of fluorescence (autofluorscence in LF slides and fluorescent dye in ICC slides) were recorded in tables designed for this purpose. In addition, five background fluorescence measurements were taken and their mean value was subtracted from the cells' records to eliminate the effect of background emission resulting from nonspecific binding of fluorescent antibody or other tissue emission.

Statistical Analysis of Variance (ANOVA) was carried out using Statistica© software package (version 8) to compare the AS and AS/AGU rats in both age groups.

Results

Lipofuscin Results

Under fluorescent microscope, LF showed two kinds or arrangements: polar and dispersed aggregations (Fig. 3).

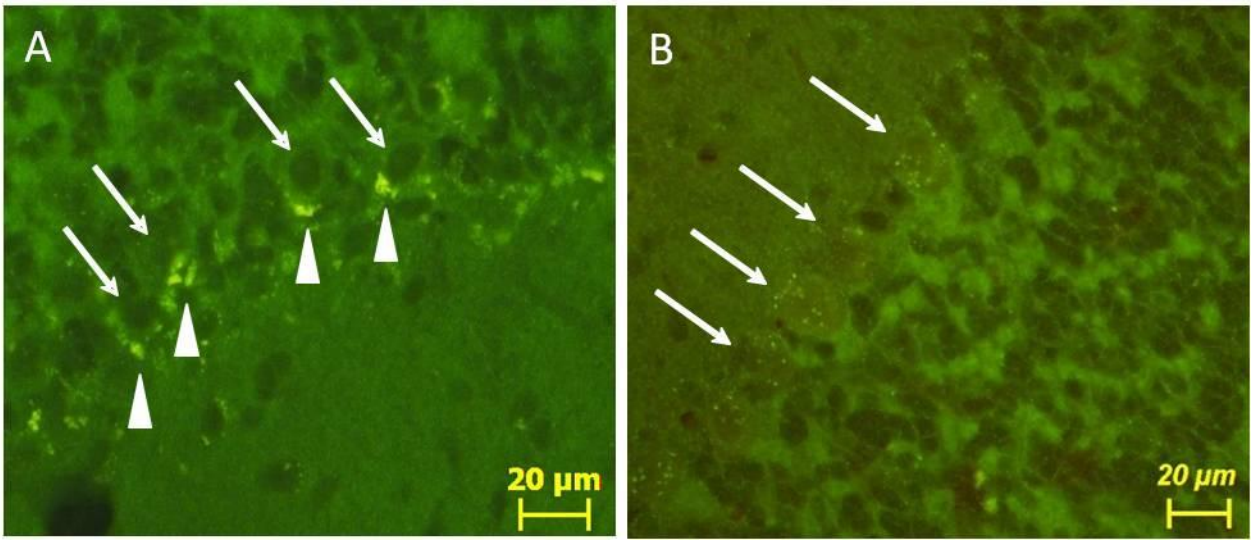

Fig. 3. Age difference and Patterns of Lipofuscin accumulations in Purkinje cells using fluorescent microscope (green filter). (A) 6 month aged Albino Swiss rat showing scanty and dispersed distribution of Lipofuscin in Purkinje cells (arrows). (B) Twelve months Albino Swiss/Albino Glasgow University rat showing Purkinje cells (arrows) with great amount of Lipofuscin accumulation in polar distribution (arrow heads), (40X).

LF was seen through a wide range of wavelengths since it was even clear through the red filter of the fluorescent microscope.

More importantly, analysis of LF auto-fluorescence showed an age related increase (Fig. 4).

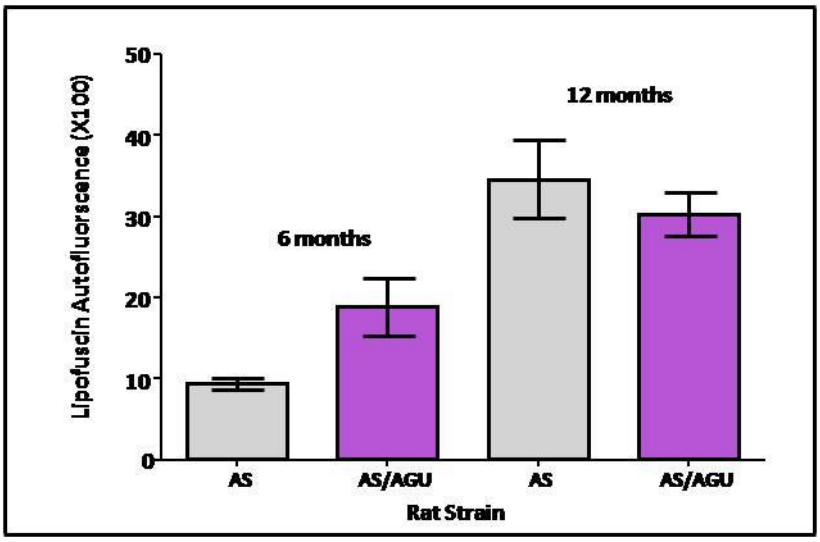

Fig. 4. Age- and Strain-related changes in autofluorescence levels of Lipofuscin measured spectrophotometrically. There is a clear trend toward an age-related increase of Lipofuscin and strain difference in young animals. Each column in the graph represents a mean value of 3 animals in the study with standard error.

There was a significant increase (*P* = 0.006) in LF accumulation in the AS strain with ageing (Table 1). The AS/AGU strain showed a twofold increment of LF at the 12 months age compared with 6 months age however, the statistical significance was only marginal (*P* = 0.061).

Table 1. Age and strain statistical comparisons of Lipofuscin autofluorescent results.

Group 1	Group 2	P value
9.4	34.6	0.006*
9.4	18.9	0.056‡
18.9	30.2	0.061‖
34.6	30.2	0.471‼

* = AS 6month vs. AS 12month, ‡ = AS 6month vs. AS/AGU 6month, ‖ = AS/AGU 6month vs. AS/AGU 12month, ‼ = AS 12month vs. AS/AGU 12month.

The cut off significant values for the analysis of variance are: significant level $P < 0.05$, marginal or weak significant level $0.10 > P > 0.05$, not significant $P > 0.1$

Comparative Lipofuscin analysis between AS and AS/AGU strains has demonstrated interesting figures (Table 1 and Figure 4). The level of LF was very close and statistically not different at age 12 month between the two strains; while 6 month AS/AGU rats showed approximately double the autofluorscence of AS rats. The small sample size has contributed to the marginal significant values.

Calbindin D-28k Results

The immunecytochemistry and fluorescent microscopy investigations revealed an overall increase of Calbindin D-28k at the 12 months age compared with 6 months age in both strains (AS and AS/AGU) (Fig. 5 and 6).

There was a highly significant increase of Calbindin D-28k between age 6 months and 12 months in the AS rat strain (*P* = 0.001) with an almost 10-fold increase (Table 2). However, the statistical evidence was weaker in the AS/AGU strain with *P* value of (0.093). There was no significant difference between strains at either age (Table 2).

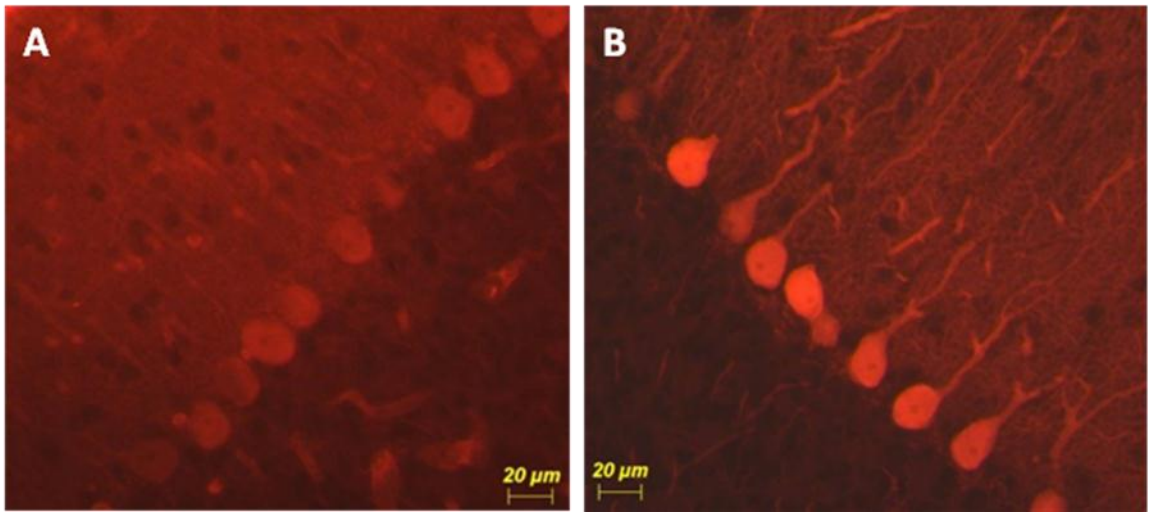

Fig. 5. Age- and strain-related differences in Calbindin D-28K levels using immunocytochemistry and fluorescent microscop (red filter). (A) six months aged Albino Swiss rat showing low immunoreactivity of Purkinje cells (faint color and no dendrites were visible). (B) Twelve months Albino Swiss/Albino Glasgow University rat showing high calbindin immunoreactive cells (40X).

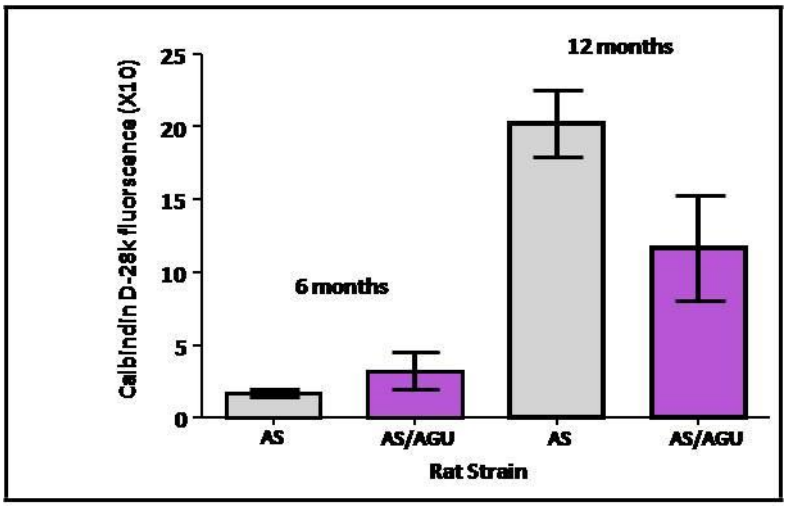

Fig. 6. Age and strain related changes of Calbindin D-28k levels in cerebellar Purkinje cells. Overall trend is an increase with age in both strains and in between 6 month age till 12 month age. The levels were relatively similar in the two strains. Each column in the graph represents a mean value of 3 animals in the study with its standard error

Discussion

Lipofuscin measurements

The age related increase in Lipofuscin is consistent with all previous LF studies in which LF has been reported to increase as a function of age (its alternative name is age pigment) [34-36]. Moreover the marginally significant difference between 6 and 12 months old AS/AGU rats could be explained by either a slow rate of accumulation in the AS/AGU strain in comparison to AS strain or the AS/AGU strain at 6 month having an already high intracellular LF level and the LF concentrations are levelled off at age 12 months. The first possibility has been excluded since Lipofuscin levels were similar at 12 month age.

Table 2. Age- and Strain-related changes in Purkinje cell Calbindin D-28k levels measured using immunocytochemistry and fluorescent microscopy.

Group 1	Group 2	P value
1.7	20.2	0.001*
1.7	3.7	0.303ǂ
3.7	11.7	0.093‖
20.2	11.7	0.116‼

* = AS 6month vs. AS 12month, ǂ = AS 6month vs. AS/AGU 6month, ‖ = AS/AGU 6month vs. AS/AGU 12month, ‼ = AS 12month vs. AS/AGU 12month

The overall trend is an increase with age and no strain difference. The cut off significant values of the analysis of variance are: significant level ($p < 0.05$), marginal or weak significant level ($0.10 > p > 0.05$), not significant level ($p > 0.1$) and $t > 2.77$.

If there is a true slow accumulation of Lipofuscin in AS/AGU strain, LF levels would be lower than in the AS strain at 12 month age which is not a finding of this study. Furthermore, we would expect a lower LF level at age 6 months in the AS/AGU strain because of slow LF accumulation before 6 months of age, which is again not obtained in results of this study.

Therefore, there was tendency to accept the second possibility since LF level was statistically similar at age 12 month in both strains indicating that they reach similar adult LF level at the same age (12 months).

Additional supportive evidence is the relatively significant increase of Lipofuscin level in AS/AGU strains at 6 months age inferring earlier LF accumulation in the mutant strain. Since LF is related to aging, one simple explanation is that AS/AGU rats are aging faster than AS rats.

The onset of dopaminergic neuronal loss at 6 month [37] and an early onset of motor and behavioral changes in this mutant strain is additional evidence [38].

Then, the AS/AGU LF levels would reach similar levels at age 12 months to their counterpart rats from AS strain. This could be simply because the Purkinje cells were not the target cells for ***agu*** mutation effects and the case in other brain regions may be different. Campbell et al [31,37] has pointed out a substantial loss of extracellular dopamine at age 3 month and expected that these changes already started at the early weeks of life. Consequently, the eventual cell death is at the end of a long pathway of cellular injury that may involve free radical or neurotoxin formation.

This might support a common cell death and aging pathway theory that claims involvement of oxidative stress and dysfunctional mitochondria and lysosomes, both of them are involved in LF formation [8]. Consequently, LF accumulation, whether it is a major step or by-product of the common cell death and aging pathway, is probably a suitable aging indicator and could be useful in further studies.

Calbindin D-28k measurements

Calbindin D-28K results are consistent with the findings of Amenta et al [20] who used radioimmunoassay to measure intracellular Calbindin level and showed that Calbindin D-28k increases from birth till old age in rats (12 months).

The insufficient statistical evidence of age-related difference in AS/AGU rats, implies that there is no or slow rise of Calbindin D-28k level in AS/AGU rats. However, there was no statistical evidence that AS rats have higher Calbindin D-28K level than AS/AGU strain at age 6 months and age 12 months (Table 2).

An older age group (16 months or 18 months) and a larger sample size are necessary to decide whether the Calbindin D-28k curve of AS/AGU is different from that of AS strain. Providing that many studies showed the old age group (24 -27 months) has the lowest Calbindin D-28k level, we tend to believe that

12 month age groups will reveal no statistical difference between both strains. One of the weaknesses of this study is the small sample size and further large-scale study is vital to fortify the results of this study and to fully differentiate between actual and confounding data.

This study did not investigate the Calbindin D-28k level at old age group (18-24 months) but Amenta et al [20] argued that after 12 month Calbindin D-28k starts to decline to reach its lowest level at 24 months. Reaching the lowest level will make the cell vulnerable to intracellular Calcium ion rises and probably neurodegenerative cell injury and death [20,39].

Intracellular calcium ion rise is thought to be the final step in aging and in some neurodegenerative processes [20]. Similarly Iacopino et al [40] pointed out the same trend in mice, where the maximum Calbindin D-28k level is reached at age 4-8 weeks and starts to decline gradually till old age (120 weeks) reaching its birth level again. Lacopino et al [40] supported their experiment by measuring Calbindin D-28k gene expression which shows a 3-4 fold rise in the first week and a steady state level at age 4-8 weeks before its regression to its birth level at 120 weeks of age.

However, Dutar et al [41] argued that Calbindin reactive cells are decreasing in number between age 4 months (young) and 24-27 months (old). They provided no data about the adult age and they estimate the Calbindin level using staining intensity. Similarly, another two studies [42,43] have shown the same age related decrease in Calbindin immunoreactive cells in human cerebral and hamster cerebellar cortices respectively.

Moreover selective age related decreases of Calbindin D-28k were reported in basal forebrain cholinergic neurons of human by Wu et al [39] and Geula et al [21]. They put this decrease as a cause of the selective vulnerability of basal forebrain cholinergic neurons for degenerative changes in old aged people and Alzheimer diseased patients.

It is worth noting that some studies have found no or little changes in Calbindin D- 28k with age. In studies on rat CNS [44], on Gerbil duodenal neurons [45] and on the cochlear nucleus of impaired hearing mice [46], the number of calbindin reactive cells remains unchanged with age. However, these studies either chose other nervous system area or other animal models, hence comparison of the current results with these studies would be most probably biased especially there is still a possibility of selective neuronal changes in the study animal as in case of selective cholinergic neuronal loss in basal forebrain of human with age [21,39].

In conclusion, the results clearly showed an age related pattern of increase in both age pigment (LF) and the neuroprotective Calbindin D-28k between the ages of 6 months and 12 months. The strain specific difference in Lipofuscin accumulation indicates that AS/AGU rats aged faster than their counterpart AS rats.

Nevertheless, more extensive investigation and large scale studies are required to fully establish this hypothesis; the AS/AGU strain is therefore a potential model for early aging investigations. With respect to Calbindin D-28k, the age related rise is not consistent with all previous studies; however, the statistical and immunocytochemical proof in this increase cannot be questioned.

The logical reason for this discrepancy is the different techniques and animal ages used in these studies. However, this study did not claim any changes between strains or beyond the 12 month age of AS and AS/AGU strains. Further study with larger sample size and wider age range is recommended to establish the calbindin age- and strain-related differences between AS/AGU mutant rats and control group (AS rats).

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Author contribution

This study was conducted in the Department of Anatomy at the University of Glasgow. The author has the main role in dissection of the preserved samples, histological staining, measurements using the immunocytochemical and fluorescent techniques, recording the data, analysis of the results and preparation of the discussion.

Conflict of interest

The author declares that there was no conflict of interest in this study as the researcher has any personal or financial relations with the organization funding the research. The research was undertaken as a master project for which the funding organization required no benefits apart from addition to the scientific literature in the topic studied.

The researcher declares that this study will bring him no personal benefits whether direct or indirect.

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