Cell numbers in the dorsal and median raphe nuclei of AS and AS/AGU rats

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Abstract

The AS/AGU rat is a spontaneous recessive mutation derived from a closed colony of Albino Swiss (AS) rats. This mutation generates a stop codon within the coding region of the PKC-gamma gene. The rats exhibit locomotor dysfunction, which is progressive with age. The nigrostriatal system has already been shown to be affected by this mutation that a) there is marked (80-90%) reduction in extracellular dopamine in the dorsal caudate- putamen as revealed by in vivo microdialysis and HPLC-ECD and b) there is a loss of tyrosine hydroxylase immunoreactive cells in the substantia nigra pars compacta Because PKC-gamma may be involved in the packaging or release of vesicles, and because other aminergic neurons may also be involved in basal ganglia disorders, we investigated the raphe-striatal serotonergic system in these mutants and the parent AS strain. Animals aged 12-15 months (6 pairs) were killed by administration of sodium pentobarbitone and were perfused with Ringer's solution + Lignocaine followed by 4% paraformaldehyde. 30 m sections were cut, rostral to caudal, in a one in five series. The collected sections were processed for immunostaining using the Avidin-Biotin-Peroxidase Complex (ABC) technique. All sections of dorsal raphe and median raphe nuclei were counted using the light microscope and also by unbiased Sterological methods using "Stereologer". In each case, AS/AGU rats possessed an average of 20-25% fewer cells in the dorsal raphe than AS control rats (p<0.05); cell count in the median raphe were unaffected.

Key words: AS/AGU rat raphe-striatal   serotonergic system.

Introduction

The cell bodies of most serotonergic neurons are located within the brainstem [1,2,3]. Those in the hindbrain project down to the spinal cord whilst another group in the midbrain projects rostrally [2]. It is the group in the midbrain which was looked at in this experiment.

The area of the midbrain called the raphe nucleus can be divided into dorsal raphe and median raphe nuclei (DRN and MRN, respectively) and it is within these that the serotonergic neurons are concentrated [4,1,2,5], being most abundant in the dorsal raphe nucleus.

The cells of the dorsal and median raphe nuclei provide the vast majority of axonal processes containing serotonin (5-hydroxy tryptamine, 5-HT) that innervate the forebrain of the rat [4,6,2]. However, the pattern of termination of 5-HT fibres to the forebrain differs between the DRN and MRN. Thus, whereas 5-HT cells within the DRN mainly innervate brain structures related to motor activity such as the basal ganglia, those in the MRN project preferentially,

though not exclusively, to limbic regions such as the medial septum and hippocampus [7,4,8,6,2,9].

In addition, the axons of 5-HT cells of the MRN are thicker and have larger varicosities compared to serotonergic cells in the DRN [10,11]. The two nuclei also show differences in their sensitivity to neurotoxins [12,13], agonists at somatodendritic 5-HT1A autoreceptors [14,15,16,17] and in their activation as a response to aversive stimuli [18,19]. However, conflicting studies exist which have found no difference in the response to neurotoxins [20] or the sensitivity to 5-HT1A agonists or selective 5-HT reuptake inhibitors [21].

As described in the Introduction, Parkinson's disease was initially defined as a loss of both dopaminergic and serotonergic neurons, with particular loss of large 5-HT cells in the dorsal raphe found post-mortem [22]. Other related syndromes (e.g. MSA, PSNP) show cell loss in multiple aminergic systems [23].

The purpose of this experiment, therefore, was to see if AS/AGU mutants had reduced 5-HT neurons as well as reduced DA neurons as previously reported [24].

Both midbrain raphe nuclei, the dorsal raphe nucleus and median raphe nucleus were examined. The reasons for choosing these two nuclei is to see:

A. whether the serotonergic system is affected at all in the AS/AGU rat and

B. whether the damage is general or specific to neurons projecting to the basal ganglia.

So numbers of serotonergic cells have been counted in both nuclei in AS and AS/AGU rats.

The numbers of cells in particular brain regions or nuclei is a fundamental piece of information and cell counts have been carried out in many brain areas in many different species and using many different methods. Serotonergic neurons in the brain and the raphe nuclei in particular have been counted in rat [25,26,27,28,29,30], cat [31], monkey [32] and in human [33,23].

Quantitive methods for analyzing the morphology of the brain have undergone a revolution in the past fifteen years. The unique elements of this revolution include systematic random sampling, measuring three-dimensional quantities such as total neuronal number and volume, and using unbiased methods to estimate these three-dimensional quantities [34,35,36,37,38,39,40,41]. One of these unbiased methods is the Cavalieri principle, which enables the determination of the total volume of the brain structure of interest. Another is the optical disector method, which enables the determination of the number of neurons in a sub-volume of the brain structure. The product of the total volume and the number of neurons in a sub-volume (i.e., the neuronal density, or the Nv) by these methods yields an unbiased estimate of the total number of neurons.

Such studies of total numbers permit reliable comparisons to be made between species, between normal and diseased human conditions, and between control and experimentally injured animals [42,43,41,44]. Total neuronal numbers are also important for theoretical approaches such as the computer modeling of brain function.

Data on total neuronal numbers within the rat raphe nuclei [25,26,27,28,29,30,31,32,33] as well as counts involving human control and PD subjects [23] have not been made using unbiased stereological methods. Therefore, analysis of the DRN and MRN of AS and AS/AGU rats were done by:

A. using conventional cell counts in an interrupted series of sections in order to compare findings with previous authors.

B. using unbiased Cavalieri and optical disector methods.

Materials and Methods

Animals

Six male AS and six male AS/AGU rats were used, aged 12 months.

Immunocytochemistry (ICC)

In order to ensure that 5-HT cells were counted, these cells were identified by ABC immunocytochemistry, a technique first employed [45].

The rats were deeply anaesthetized with an overdose of Sodium pentobarbitone BP (Vet) (Rhone-Merieux, Spire Greencentre, Harlow, Essex, 60mg/1ml). They were then perfused through the left ventricle with 100ml Ringer's solution containing the vasodilator Lignocaine, followed by 500ml 4% Paraformaldehyde (2944744 BDH) in 0.1M phosphate buffer. The brains were removed and post-fixed in the perfusion fixative for 3 hours. They were then washed three times for 10 minutes in PBS and immersed in 7% Sucrose (10274, BDH) and Sodium azide (S2002, SIGMA) in PBS before storage overnight at 4 ^{o}C.

The brains were embedded in tissue-tek (4583, Miles) frozen in liquid-nitrogen and then cut at 30 μm thickness on a cryostat at −20 ^{o}C (Leica, Jung Frigocut 2800E). Coronal sections were taken in a rostral to caudal direction and collected serially in bottles containing 0.1M phosphate buffer (PB). The sections were processed for 5-HT immunostaining.

Immunocytochemistry on cryostat sections

The sections were rinsed in PBS and incubated initially in Goat anti-serum in 0.3 Triton X-100 (30632, BDH) for one hour. This is used to bind charged proteins within the tissue and allow enhanced penetration of primary antibody. This was followed by incubation with the Primary antibody, at an optimal dilution in PBS containing 0.3% Triton X-100.

The primary antibody was Rabbit anti-Serotonin (Z02597, AFFINITI) at a dilution of 1:10,000, which reliably labels serotonergic neurons in the midbrain. Initially, different dilutions of antibodies were tested under the same conditions in order to determine the optimum antibody concentrations.

PBS was used to dilute the antibodies and for all washes. Triton X-100 increased the penetration of antibodies [46]. Incubation was performed in a humidified chamber at room temperature overnight.

The sites of polyclonal antibody-antigen reaction were visualized by the application of biotinylated donkey anti-rabbit immunoglobulin (IG) (RPN 1004, AMERSHAM) at a dilution of 1:250 for one hour at room temperature, followed by Avidin-Biotin-Peroxidase complex (20 l of solution A and 20 l of solution B in 1ml of PBS Vector ABC elite kit) which binds to the secondary antibody as the Avidin portion of the complex contains 4 binding sites specific to biotin. Finally, 5-HT-immunoreactivity was visualised using 3,3'-diaminobenzidine (DAB). This reacts with the peroxidase portion of the ABC complex to form a brown reaction product after 3-5 minutes. As DAB is a carcinogen, care was taken to ensure that this part of the experimental protocol was carried out in a fume cupboard, using disposable laboratory equipment.

Cell Counting techniques

Conventional counts

The rostral-caudal length of the DRN/MRN is approximately 3mm thus yielding c. 100 x 30 m thick coronal section. Every fifth section was collected, stained and counted using a microscope with a 10x eyepiece fitted with a graticule. All stained cells were counted throughout the dorsal and median raphe nuclei, yielding a value of the average number of 5-HT-ir cells per 30 μm section.

Stereology

The optical disector technique [47] was used to estimate 5-HT cell numbers in DRN & MRN. Total raphe nuclei volumes were estimated using the Cavalieri Principle [48]. Slides used to estimate the number of cells were also used to estimate raphe nuclei volume to avoid any requirement for correction factors due to tissue shrinkage. A computer using AutoCADlt97 software (Autodesk lnc, San Rafael, CA) and a digitising Tablet was used to estimate the surface area of selected sections (every fifteenth section). The total surface area of these selected sections is designated *SA*. The thickness of each section is known (h), as is the distance between the sections (d), and total raphe nuclei volume (tv) can be calculated by tv = SA x h x d. The numerical density was estimated using an Olympus BX50 microscope fitted with a motorized stage (Prior Scientific Instruments, Cambridge) and *Stereologer* software (Systems Planning Analysis, Alexandria, VA).

The absolute number of cells in each nucleus was then calculated by multiplying its volume by the cells numerical density.

Results

To ensure optimal cell staining, it was first necessary to determine the appropriate concentration of primary antibody required. The concentration of primary antibody that resulted in clearly visible serotonergic cells was 1:10,000 and this was used throughout the successive ICC experiments. At this concentration, serotonergic cells within the dorsal and median raphe nuclei stained positively and could easily be identified (Fig. 1). A clear distinction could readily be seen between the cells in the median raphe nucleus and those contained in the larger, dorsal raphe nucleus. Cells clusters were carefully examined at high magnification with repeated re-foucusing until the number of cells forming the cluster could be determined.
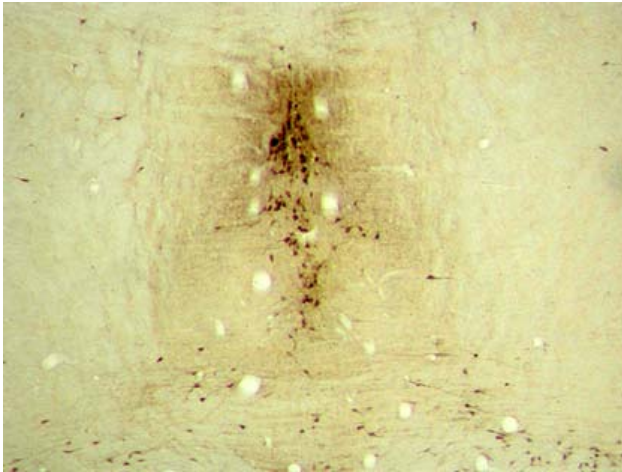
There was no difference in raphe nucleus size between two group of animals (AS and AS/AGU), nor any obvious difference in cell size or shape.

Figure 1: *Coronal section of the midbrain showing both dorsal (top) and median (bottom) raphe nuclei. This section is approximately –7.8mm relative to bregma. Magnification (x25)*
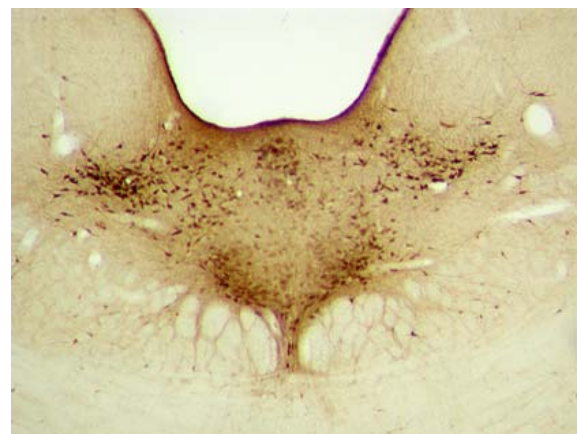
Figure 2: *Higher magnification of Fig. 1 shows the dorsal raphe nucleus after serotonin immunohistochemistry. (x65)*
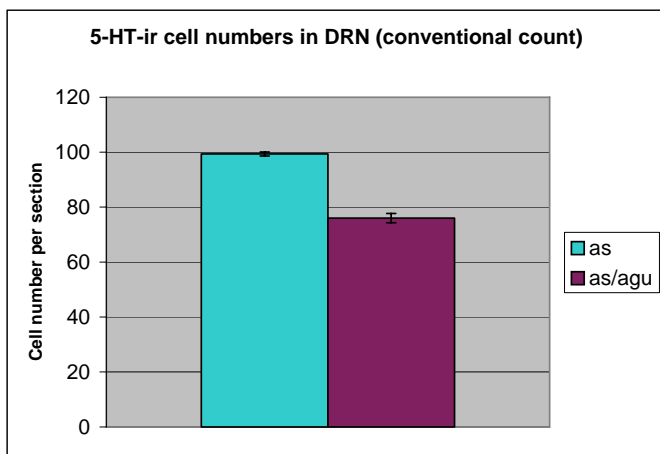
pictorially in Fig. 4. The data show a significant difference between the AS (control) and AS/AGU (mutant) rats, with mutants possessing c. 23% fewer 5-HT-ir cells in the dorsal raphe than controls (p<0.001).

Nevertheless, all serotonergic cells could be easily counted in both regions.

Figure 3: Higher magnification of fig 1 shows the median raphe nucleus after serotonin immunohistochemistry. (x65)

There appeared to be slight regional differences in cell morphology in the two raphe nuclei. Cells of the DRN were more densely stained, tended to have a triangular shape and their dendritic processes were highly visible (Fig 2). In contrast, cell bodies contained in the MRN were rounder, smaller and paler and the neuronal projections were less visible.

Conventional Cell counts

The number of serotonergic cell bodies within the DRN and MRN of AS and AS/AGU rats can be seen in fig 4 and fig 5 respectively. A student t-test for independent samples was performed, using the Minitab statistics package. The results are summarized in Table 1 and shown

Table 1: *The average number of 5-HT immunoreactive cells per 30 m section in the dorsal raphe nucleus of control (AS) and mutant (AS/AGU) rats.*

Animal	AS	AS/AGU
1	98	82
2	97.7	77.7
3	98.7	75.4
4	100	74
5	99.7	76
6	102	71
Average	99.35	76.02
SEM	0.65	1.50

$t = 14.22$ $\quad$ $df = 10$ $\quad$ $P < 0.001$

Figure 4: The average number of 5-HT immunoreactive cells per 30 m section in the dorsal raphe nucleus of control (AS) and mutant (AS/AGU) male rats aged 12 months (n = 6 per group). Counting was made by conventional means.
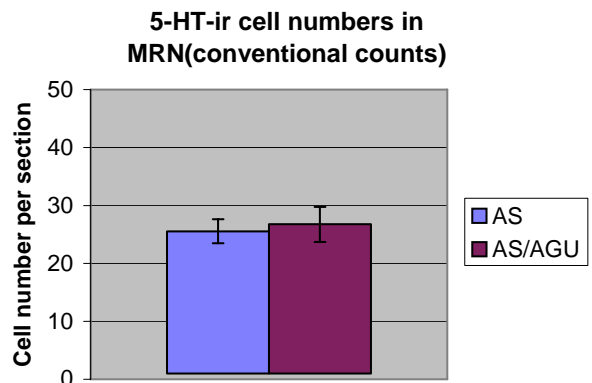
Figure 5: the average number of 5-HT-immunoreactive cells per 30 m section in the median raphe nucleus of control (AS) and mutant (AS/AGU) male rats aged 12 months (n= 6 per group). Counting was made by conventional means

By contrast, Table 2 and Fig. 5 show that there was no significant difference between AS and AS/AGU rats in the numbers of serotonergic cells in the median raphe.

Cell count (using computerised stereology)

Estimation of the total volume of each raphe nucleus (Tv):

This was calculated according to the Cavalieri principle by multiplying three factors:

1. Surface area of selected sections (Sa)
2. The thickness of each section   (H)
3. The distance between sections   (D).
4.

The surface area was measured by a computer with Auto CAD1t97 software and digitising Tablet.
So the total volume (Tv) of each raphe nucleus is calculated as:

$$Tv = Sa \times H \times D$$

Estimation of the numerical density (Nv) of 5-HT-ir cells:

Pilot studies were performed to determine appropriate measurement factors, which will reduce a coefficient of error (CE) to less than 0.10 (Gundersen and Jensen, 1987). Then the full study of calculating the Nv was achieved automatically using an Olympus BX50 microscope fitted with a motorized stage and *stereologer* software.

Table 2: *The average number of 5-HT immunoreactive cells per 30 m section in the median raphe nucleus of control (AS) and mutant (AS/AGU) rats.*

| Animal	AS	AS/AGU
1	30	25
2	22	19.82
3	23	28.78
4	25	32
5	26	25
6	25	24
Average	25	25.9
SEM	1.23	1.9
T = 0.33	P> 0.75	DF= 8

Total neuronal number in dorsal and median raphe nuclei (Tn):

The absolute number of cells in each nucleus was calculated by multiplying the cell density by the volume
$$Tn = Nv \times Tv$$

The total number of serotonergic cells in the dorsal raphe nucleus of the two groups is shown in Table 3. stereological methods.

Table 3. **The total number of 5-HT immunoreactive cells in the dorsal raphe nucleus of control (AS) and mutant (AS/AGU) rats, as measured by computerised**

Animal	AS	AS/AGU
1	8562	7196
2	8312	6850
3	8697	6611
4	8854	6484
5	9062	6991
6	8780	6749
Average	8711	6813
SEM	114.9	115.4
t = 12.77	df = 10	P<0.001
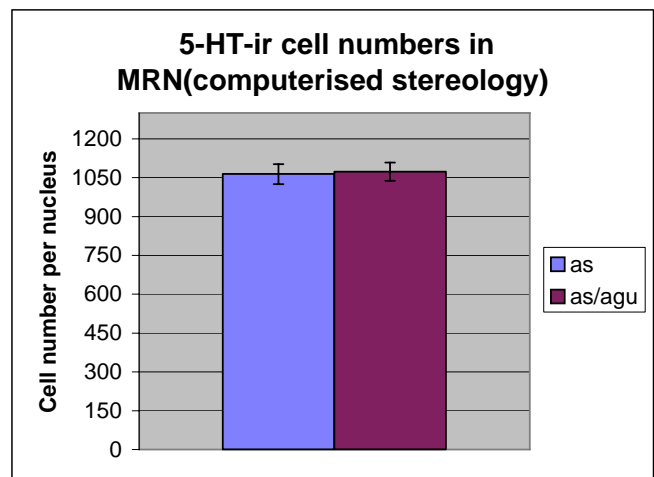
Figure 6: *The total numbers of 5-HT-immunoreactive cells per dorsal raphe nucleus in AS and AS/AGU male rats aged 12 months (n = 6 per group).*
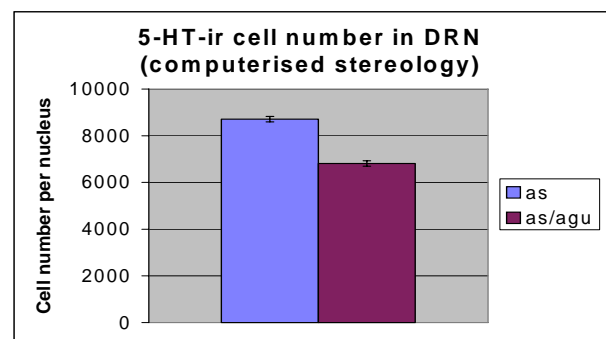
Figure 7: *The total number of 5-HT-immunoreactive cells per median raphe nucleus in control (AS) and mutant (AS/AGU) male rats aged 12 months (n = 6 per group). Counts were obtained by computerised stereological means.*

Table 4. *The total number of 5-HT immunoreactive cells in the median raphe nucleus of AS (control) and AS/AGU (mutant) rats as measured by computerised stereological methods.*

Animal	AS	AS/AGU
1	1022	1215
2	998	1017
3	1076	1002
4	1086	1042
5	1221	1053
6	985	1111
Average	1064	1073
SEM	38.8	35.3

$t = 0.18 \quad df = 10 \quad P < 0.86$

Counts were obtained by computerised stereological means.

From Table 3 and Fig. 6, it is apparent that there is a very significant difference in the number of 5-HT-ir cells in the dorsal raphe nucleus of AS (control) and AS/AGU (mutant) rats. Mutants possess 22% fewer serotonergic cells than controls.

By contrast, we can see from Table 4 and Fig. 7 that there are no differences between AS (control) and AS/AGU (mutant) rats with respect to the numbers of 5-HT-ir cells in the median raphe nucleus.

Discussion

Two points require initial discussion. Firstly, are total cell numbers comparable to those found in previous studies and, secondly, are there differences in cell numbers between control and mutant animals?

Cell numbers in the midbrain have been counted using conventional methods in different species. Descarries, Lemay, Doucet, Berger reported that the total population of serotonergic cells in the DRN of adult rat was 11,428 [29]. In cat DRN the number was estimated to be 24,257 [31]. More recent work has indicated that there are 11,500 serotonergic neurons in the DRN of adult rat while there are 1100 serotonergic neurons in the MRN (49). Cell numbers in primates may be more equal in the two nuclei. Thus, 5-HT-ir cell counts in the DRN and MRN of squirrel monkeys showed that the numbers were 8,269 and 7,034 respectively (32).

In the present study the total numbers of serotonergic neurons were analysed using unbiased computerised sterological software and Cavalieri principle [47,48 respectively]. The data obtained by unbiased methods in the present study showed that serotonergic neuron numbers were 8,711 for the DRN and 1064 for the MRN of AS animals. These numbers are slightly lower than those reported with conventional methods [29] but this may be accounted for by age and strain difference as well as the different methodology employed.

To answer the second question, this study shows that there are fewer 5-HT-ir cells in the DRN of the mutant rat compared to the AS control rats. The reduction is approximately 23% when the total numbers were obtained using unbiased methods and 22% when counted by more conventional means. By contrast with the DRN, counts of the MRN serotonergic cells showed no difference between strains when measured either by conventional counts or by unbiased methods.

The extent of 5-HT cell loss in the mutant rat is less than found previously for DA-ergic cells (C. 40%) in 1 year old animals (49), but is consistent with a general depletion of aminergic cells.

The reduction of 5-HT cells in DRN along with 40% loss in DA-ergic cells in the mutant rat strongly suggests that the AS/AGU rat is a valuable model for studying neurodegenerative processes. Cell reductions of this magnitude have been reported in many human conditions related to movement disorders.

The change in cell numbers is likely to affect the target regions to which these cells project. The DRN is the principal source of striatal and cortical serotonin and axons from the DRN project mainly to the caudate-putamen, amygdala and substantia nigra pars compacta [4,6]. There are also projections to the thalamus [4] and to the locus coeruleus [6]. In contrast, the MRN was found to send projections mainly to the hippocampus and the anterior hypothalamus [4], and also the mammillary body [50]. Additionally, there was no evidence of axonal projection from the MRN to the striatum or amygdala [50] or the substantia nigra (6). Moreover, lesions of the DRN were found to cause much greater decreases of serotonin in the striatum than lesions of the MRN [51].

The reduction in cell numbers in the mutant DRN leads to hypothesis that this will affect levels of 5-HT in the DRN and the striatum.

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Cell numbers in the dorsal and median raphe nuclei…….