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Changes in the number of neurons in the mesencephalic and motor nuclei of the trigeminal nerve in the ageing mouse brain

R. R. STURROCK

Department of Anatomy, University of Dundee, Dundee DD1 4HN, Scotland

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INTRODUCTION

One of the characteristic features of ageing is a reduction in motor activity (Strong *et al.* 1980; Jänicke, Schultze & Coper, 1983). As the latter authors pointed out, it is very difficult to determine to what extent the muscle wasting associated with old age is due to inactivity or vice versa. As well as loss of muscle fibres the contributory effects of joint stiffness and loss of tendon elasticity must be taken into account. At present it is also not known whether a progressive loss of motor neurons occurs in the ageing central nervous system and whether any such loss is the result or the cause of muscle wasting.

There is evidence that between one and two years of age mice lose about 20% of motor neurons from the spinal cord (Wright & Spink, 1959) and that many neurons in the brainstem and spinal cord of the ageing mouse show a loss of dendrites and dendritic spines (Machado-Salas, Scheibel & Scheibel, 1977). So far no study has investigated the effects of ageing on the number of both sensory and motor neurons in the same system. The trigeminal system is an ideal model for such a study since the mesencephalic nucleus, which receives proprioceptive input from the muscles of mastication, connects directly with the motor nucleus (Cajal, 1911; Dacey, 1982) and is unique in that the primary sensory neurons are located within the central nervous system.

Although the neurons of the mesencephalic nucleus are widely scattered throughout the upper pons and midbrain their characteristic appearance enables accurate counts to be carried out (Sivanandasingham & Warwick, 1976). The motor nucleus of the trigeminal is a much more discrete structure containing typical motor neurons and is equally amenable to accurate numerical analysis.

The number of neurons in the mesencephalic and motor nuclei was estimated in ASH/TO strain mice at intervals between 6 and 31 months of age using the same material as that used in a recent investigation into the effects of ageing on the locus caeruleus (Sturrock & Rao, 1985). The ASH/TO strain has a mean lifespan of 22 months (Sturrock, 1985) and the oldest animals examined had survived well beyond this.

MATERIALS AND METHODS

Under Sagatal anaesthesia male ASH/TO mice were killed by perfusion fixation with Bouin's solution after the vascular system had been flushed out with physiological saline at 37 °C. Three mice were killed at 6, 9, 12, 15, 22, 25, 28 and 31 months

of age. After completion of perfusion the animals were left overnight at 4 °C after which the brains were removed and bisected in the mid-sagittal plane, dehydrated and embedded in paraffin wax.

The left half of each brain was serially sectioned at 6 μm in the coronal plane and stained with haematoxylin and eosin. The right half was serially sectioned at 6 μm in the parasagittal plane and stained with Lapham's stain (Lapham, Johnstone & Brundjar, 1964).

The parasagittal sections were used for the neuronal counts. The main group of mesencephalic neurons lay adjacent to, and in some cases scattered among, the neurons of the locus caeruleus. This group of cells was identified and the sections medial to it were scanned until no mesencephalic neuron could be found in ten successive sections. Each section was scanned in a caudorostral direction from the IVth ventricle caudally to the anterior border of the superior colliculus rostrally. When the most medially situated mesencephalic neuron had been identified, every fifth section lateral to the section containing it was carefully scanned at $\times 250$ magnification and each mesencephalic neuron containing a nucleus or nuclear fragment was recorded, until at the lateral side of the nucleus ten successive sections which contained no mesencephalic neurons had been scanned.

The trigeminal motor nucleus was identified by first finding the motor root of the trigeminal. In the most lateral sections containing the root of the nerve no motor neurons were present. The first section containing a motor neuron was identified and every fifth section medial to this was scanned at $\times 250$ and every motor neuron containing a nucleus or nuclear fragment was recorded until the medial border of the nucleus was reached. The motor nucleus is a discrete group of neurons, in contrast to the widely scattered neurons of the mesencephalic nucleus, and the medial border is easily identifiable.

Nuclei are rarely spherical and elliptical nuclei may be orientated randomly or with their long axis in a particular plane. An example of the latter is the nuclei of developing glia cells in the spinal cord whose long axes lie parallel to the fibre tracts within which they lie (Sturrock, 1983). Nuclear diameters are measured to enable correction factors to be applied to take into account the fact that many nuclei lie in more than one section. From this point of view the important diameter is the depth diameter rather than the length or breadth diameter. The simpler method of finding the 'depth' diameter of nuclei is to measure the diameter in the plane at right angles to that in which the section in which counts were made was cut. In the case of sagittal sections the depth diameter corresponds to the left to right diameter in coronal sections. The left to right diameter of 50 nuclei from each nucleus was measured in each set of coronal sections using a calibrated micrometer eyepiece at $\times 1000$ under oil immersion. In each section the eyepiece micrometer was lined up precisely at right angles to the vertical cut edge of the hemisected coronal section. This method ensured that any orientation bias would be accounted for. Three sets of coronal sections were used at each age, the only exception to this was in one of the sets of 6 months old sections in which the cerebellum and brainstem had not been sectioned in the coronal set. Therefore at 6 months the mean diameters were estimated from only 2 sets of coronal sections. The mean diameter was corrected using the formula described by Abercrombie (1946), as recommended by Smolen, Wright & Cunningham (1983). The total number of neurons in each nucleus in each animal was estimated by multiplying the total number of neurons counted by the total number of sections containing that nucleus, dividing by the number of sections in which

neurons were counted and applying Abercrombie's (1946) correction formula to the result.

The results of the neuron counts and nuclear diameter estimations at the different ages were subjected to analyses of variance. Linear regression analyses were performed between the number of neurons in each nucleus; between the diameters of neuronal nuclei in each nucleus; and between neuronal number and neuronal nuclear diameter in the same nucleus.

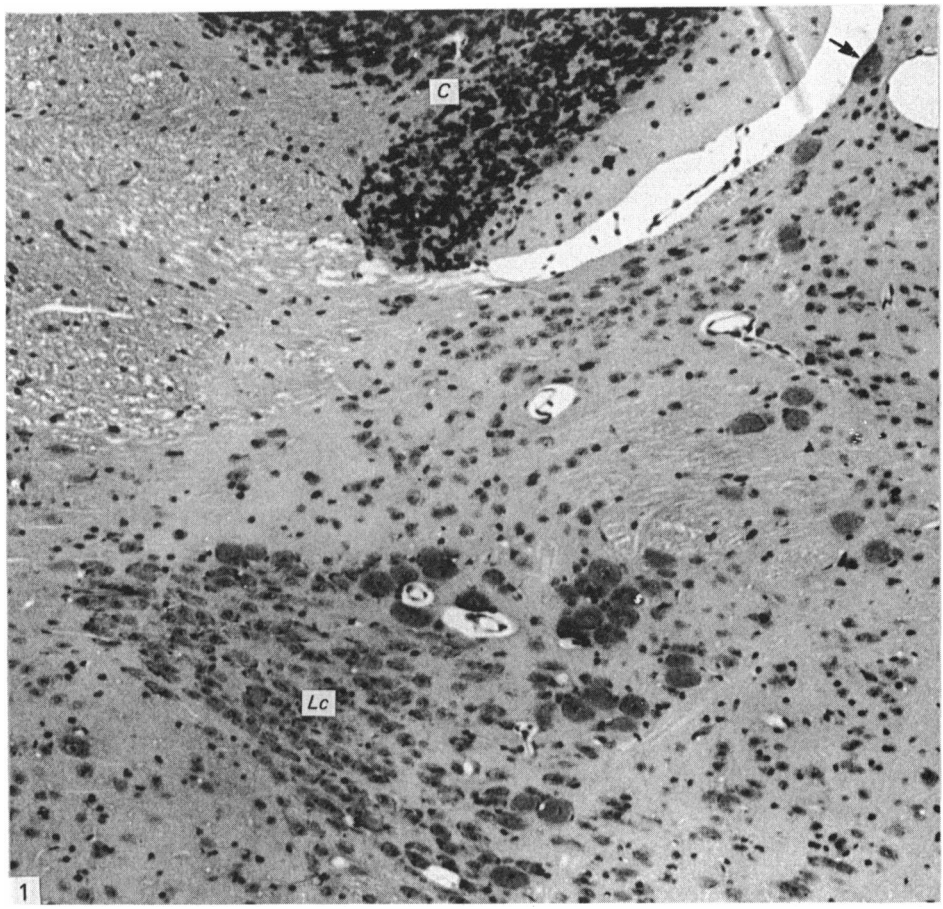
RESULTS

The main group of neurons in the mesencephalic nucleus of the trigeminal nerve was found in close proximity to the locus caeruleus (Fig. 1). In the most medial sections the mesencephalic nucleus neurons lay dorsal to the locus caeruleus but as one moved laterally the neurons were found rostral to the locus caeruleus. Other neurons of the mesencephalic nucleus were scattered throughout the midbrain either singly or in groups. There was no constant pattern of scattering and the position of the scattered neurons varied between animals of the same age as well as between age groups. Very often a few of these scattered neurons lay immediately beneath the pia-glial membrane (Fig. 1) or adjacent to blood vessels but the majority appeared to be randomly situated. Two types of neuron could be recognised in the mesencephalic nucleus. The more common type had a large perikaryon which was oval in both sagittal and coronal sections (Fig. 3, 5). Nissl substance was present peripherally and varied in quantity. The second type (Fig. 4) was smaller and the perikaryon was circular in cross section in both sagittal and coronal sections. Nissl substance was found peripherally and was usually more obvious than in the larger neurons and the cytoplasm of the smaller neurons was usually slightly paler in Lapham-stained material. Examination of serial sections confirmed that the smaller neurons were not simply larger neurons cut in a different plane. One gained the impression that small neurons were more common amongst the scattered neurons in the midbrain than in the main group. The nuclei of both types of neuron had a similar diameter and no attempt was made to carry out separate counts.

From 12 months of age onwards lipofuscin was present in the cytoplasm. In material fixed in Bouin's solution and stained with Lapham's stain lipofuscin appears as a bright, shining, light blue material and cannot be mistaken for Nissl substance. This is not obvious in black-and-white illustrations. Lipofuscin increased in amount with age and by 31 months (Fig. 5) quite large aggregations were present in many mesencephalic trigeminal neurons. A degenerating mesencephalic trigeminal neuron was found in one 31 month brain (Fig. 6).

In contrast to the mesencephalic nucleus the motor nucleus of the trigeminal nerve was a well circumscribed nucleus which was easily identified (Fig. 2). Neurons of this nucleus had the characteristic appearance of motor neurons with many coarse clumps of Nissl substance (Fig. 7). Lipofuscin was very rarely found in motor neurons of the trigeminal nerve even at 31 months of age. Increasing age led to a decrease in the amount of coarsely staining Nissl substance (contrast Figs. 7 and 8) in most, but not all, neurons.

The number of neurons in each nucleus, the mean diameter of the neuronal nuclei and the ratio of motor neurons to mesencephalic neurons are shown in Table 1. The number of mesencephalic neurons showed little variation between 6 and 25 months but decreased at 28 and 31 months. An analysis of variance showed that there was a significant variation in neuron number in the mesencephalic nucleus between 6 and



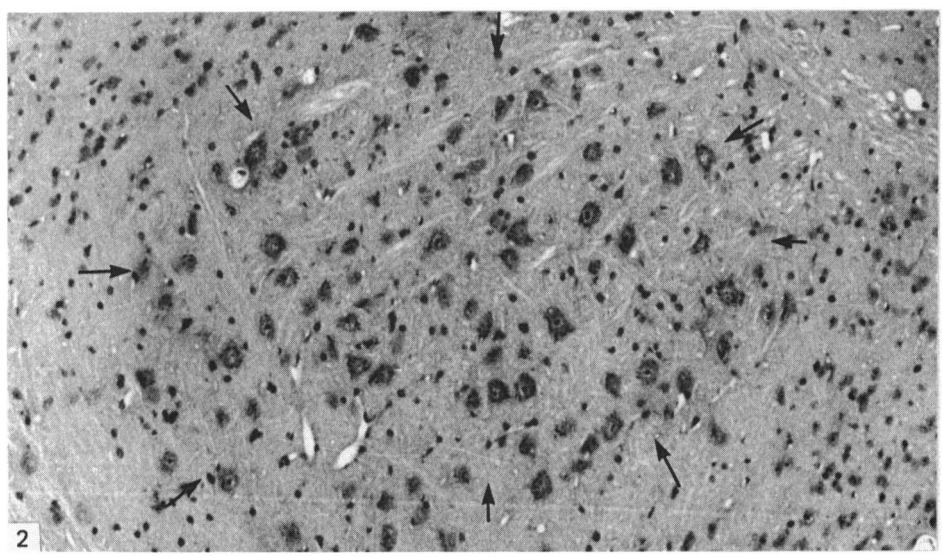


Fig. 1. Parasagittal section stained with Lapham's stain, × 150. Neurons of the mesencephalic nucleus lie in groups above the locus caeruleus (*Lc*) and scattered into the midbrain. Note the neuron lying beneath the pia glial membrane (arrow). *C*, Cerebellum. 6 months old mouse.

Fig. 2. Parasagittal section stained with Lapham's stain, × 150. The motor nucleus of the trigeminal is easily identifiable within the arrows. 6 months old mouse.

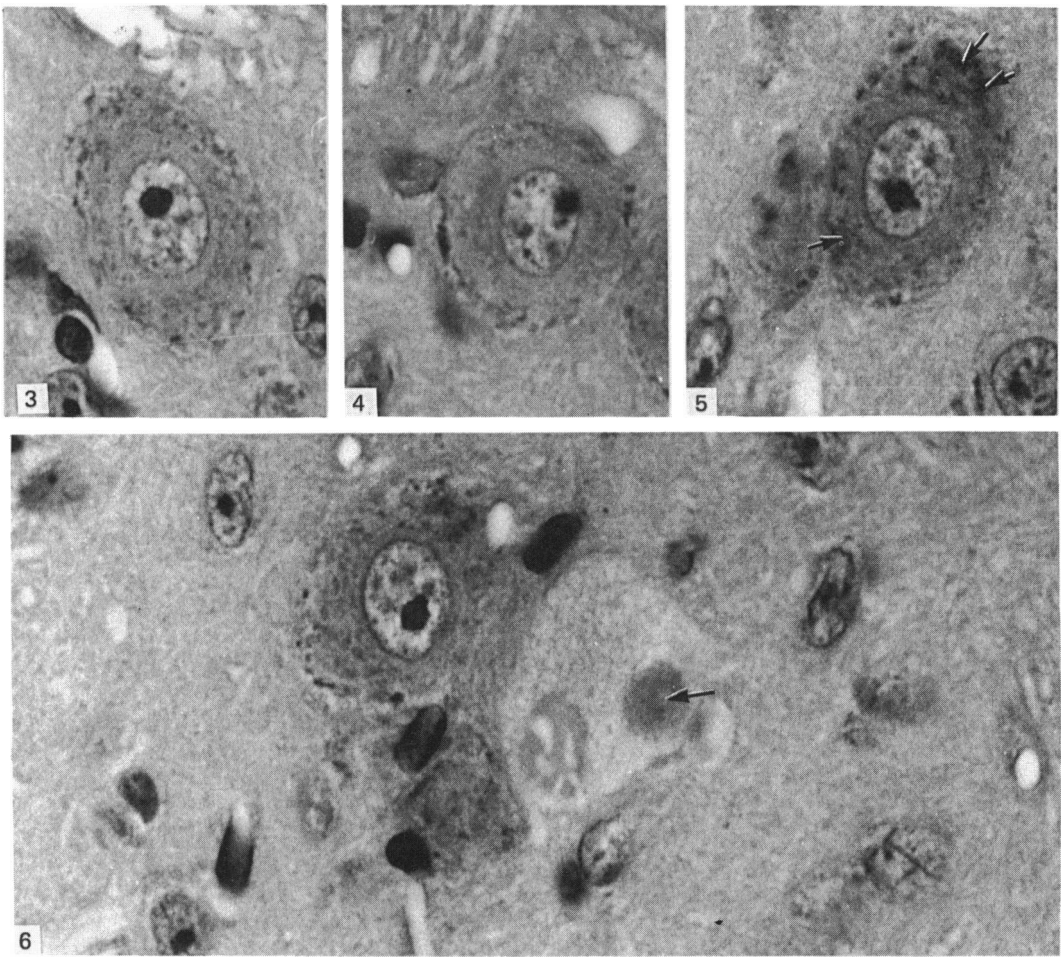


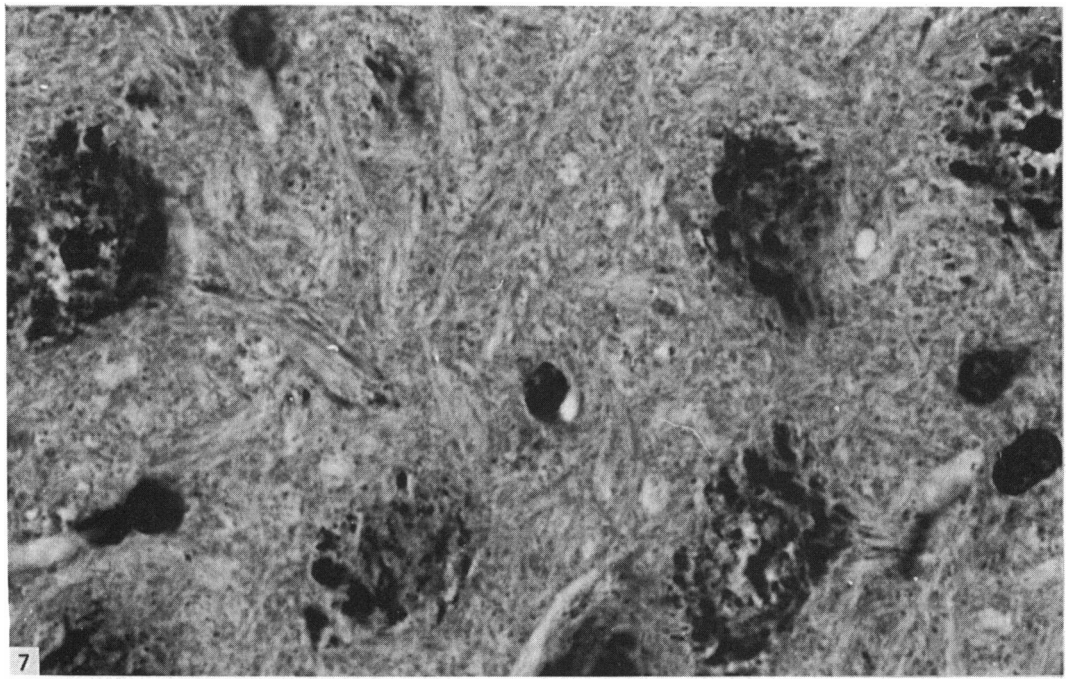
Fig. 3. Coronal section, H & E, ×1000. This shows a larger oval cell of the mesencephalic nucleus. Nissl substance is sparsely scattered in a thick band peripherally. 6 months old mouse.

Fig. 4. Coronal section, H & E, ×1000. Smaller neuron of the mesencephalic nucleus. The Nissl substance is sparse but arranged in a narrower band which makes it appear more prominent than in the larger neurons. 6 months old mouse.

Fig. 5. Parasagittal section, Lapham's stain, ×1000. The dark cytoplasmic inclusions indicated by the arrows stain bright, light blue in the sections and are lipofuscin. 28 months old mouse.

Fig. 6. Parasagittal section, Lapham's stain, ×1000. Degenerating mesencephalic neuron at 31 months of age. The lighter dense body was a green structure (? degenerating nucleus) whereas the other (arrow) stained reddish-purple (? Hirano body).

31 months ($F(7,16) = 4{\cdot}07$, $P < 0{\cdot}01$). The mean number of neurons in the motor nucleus was least at 31 months but the variation in neuron number between 6 and 31 months was not statistically significant ($F(7,16) = 1{\cdot}39$, $P > 0{\cdot}05$) although the number of neurons in each 31 month motor nucleus was lower than in any other nucleus at any other age. In both nuclei the neuronal nuclear diameter increased late in life and this increase was statistically significant (mesencephalic nucleus, $F(7,15) = 16{\cdot}30$, $P < 0{\cdot}001$; motor nucleus, $F(7,15) = 6{\cdot}30$, $P < 0{\cdot}01$). There was no statistically significant variation in the ratio of the number of motor neurons to mesencephalic neurons ($F(7,16) = 0{\cdot}69$, $P > 0{\cdot}05$).



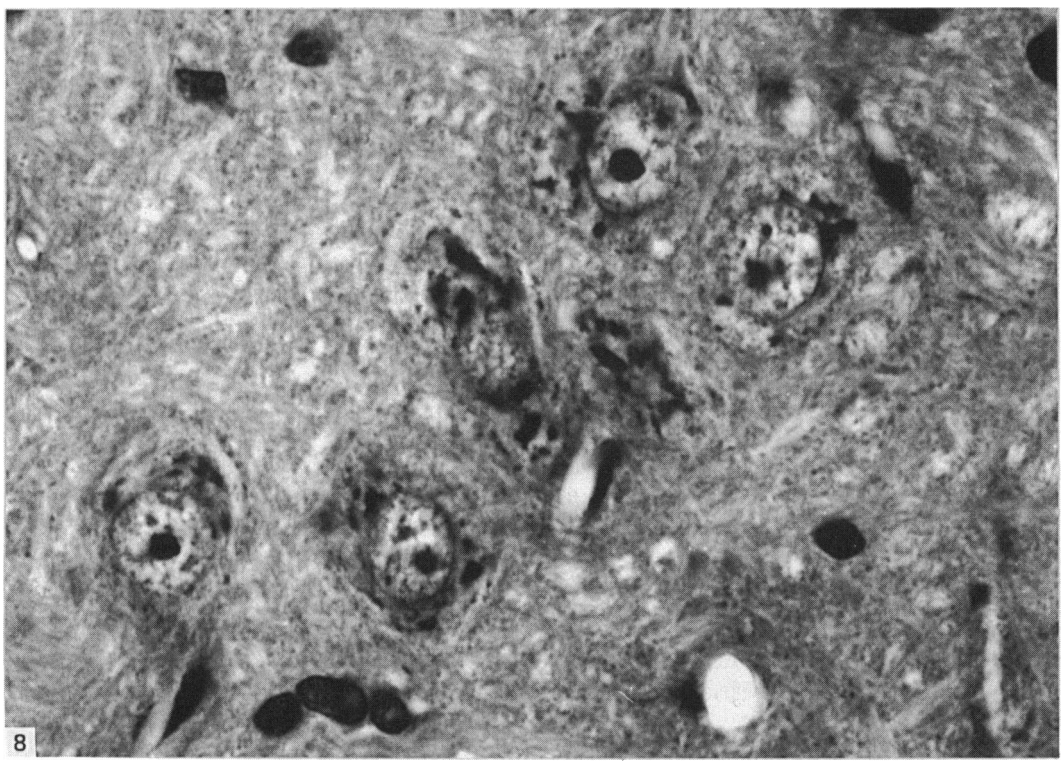


Fig. 7. Parasagittal section, Lapham's stain, × 1000. Neurons of the trigeminal motor nucleus at 6 months of age. Note the darkly staining abundant Nissl substance.

Fig. 8. Parasagittal section, Lapham's stain, × 1000. The neurons of the trigeminal motor nucleus at 31 months of age contain less Nissl substance than those in Figure 7.

Table 1. *Number of neurons and neuronal nuclear diameter (+SEM) in the mesencephalic and motor nuclei of the trigeminal nerve and the motor to mesencephalic neuron ratio*

	Mesencephalic nucleus		Motor nucleus		
Age in months	Number of neurons	Mean diameter (μm)	Number of neurons	Mean diameter (μm)	Ratio
6	415±7	11·2±0·5	601±6	13·4±0·1	1·45±0·04
9	425±14	11·0±0·4	607±17	13·4±0·6	1·43±0·07
12	405±9	10·9±0·1	602±45	13·4±0·2	1·49±0·12
15	410±20	10·3±0·1	620±32	13·0±0·2	1·52±0·11
22	433±15	10·3±0·4	633±42	13·2±0·7	1·45±0·06
25	411±17	12·3±0·7	617±18	13·0±0·7	1·50±0·06
28	336±34	13·7±0·03	581±58	15·2±0·1	1·76±0·26
31	336±22	13·7±0·2	504±21	16·1±0·2	1·51±0·13

Linear regression analyses showed a significant negative correlation between the changes in the number of neurons and nuclear diameter with age in both the mesencephalic nucleus ($r = -0{\cdot}9031$, $P < 0{\cdot}01$) and motor nucleus ($r = -0{\cdot}9063$, $P < 0{\cdot}01$). There were also statistically significant correlations between the number of neurons in the mesencephalic nucleus and in the motor nucleus at different ages ($r = 0{\cdot}8286$, $P < 0{\cdot}05$) and between the mean nuclear diameters of neurons in the mesencephalic and motor nuclei at different ages ($r = 0{\cdot}9348$, $P < 0{\cdot}001$).

DISCUSSION

In quantitative histological studies of neuron number either cell bodies, nuclei or nucleoli may be counted. It would have been practical to estimate mesencephalic nucleus neuronal number by counting cell bodies since the cell bodies are large, easily identified, and above all, regularly shaped. The irregular cell bodies of the motor nucleus, however, are much less satisfactory for counting purposes. Konigsmark (1970) recommended counting nucleoli but mesencephalic neurons occasionally contain more than one nucleolus. There are also problems of split nucleoli and nucleoli which have been pushed or rolled out of the nucleus during sectioning. Coggeshall & Chung (1984) have recently developed an empirical method for dealing with split nucleoli, which was considered for use in the present investigation, but they found that pushed or rolled nucleoli were absent from their material. In our material pushed or rolled nucleoli were not uncommon, probably due to the different fixatives used. For these reasons the nuclei were selected for counting. Counts were carried out directly by visual inspection instead of using the photographic technique employed in the study of the locus caeruleus (Sturrock & Rao, 1985) because there were fewer cells in each nucleus and the cells were less tightly packed than the neurons of the locus caeruleus. A further reason for using direct microscopy was the very wide anatomical scatter of neurons in the mesencephalic nucleus.

Although two different types of neuron were present in the mesencephalic nucleus there was no evidence of a bimodal distribution of nuclear diameter in any set of sections. In their comprehensive quantitative study of the mesencephalic nucleus Sivanandasingham & Warwick (1976) estimated that the rat mesencephalic nucleus contained 551 neurons, the cat nucleus contained 574 neurons and the slow loris

nucleus contained 483 neurons. Foster (1973) reported that there were 579 neurons in the rat mesencephalic nucleus. The mean value of 417 neurons estimated in the present study of the mouse between 6 and 25 months compares favourably with these other studies but differs substantially from the 717 neurons reported in the adult mouse by Hinrichsen & Larramendi (1969) who counted mesencephalic neuron number in one rat, one cat and three mice ranging in age from newborn to 35 days. They estimated that there were 850 neurons in the adult mouse motor nucleus. The difference between these results and those of the present study are probably largely due to the failure to apply a correction factor to the counts. If Abercrombie's (1946) correction is applied to the data of Hinrichsen & Larramendi (1969) the neuron numbers in the mesencephalic and motor nuclei become 418 and 451 respectively. The former now compares remarkably closely with the results of the present study. The difference in the motor nucleus number is probably partly due to the difficulty the authors noted in identifying the rostral and caudal extent of the motor nucleus in coronal sections. The counts were also carried out at a low magnification ($\times 100$). Hinrichsen & Larramendi's results for the rat and cat were also substantially higher than those of Sivanandasingham & Warwick (1976) which the latter authors also attributed to a failure to correct for split nuclei. It is also possible that different strains of mice have different numbers of neurons in their trigeminal nuclei as is the case with the adjacent locus caeruleus (Berger *et al.* 1979; Touret, Valatx & Jouvet, 1982; Sturrock & Rao, 1985).

In their morphometric investigation of the rat motor nucleus Howard, Scales & Lynch (1980) concentrated on separating neurons from the motor nucleus into α and γ motor neurons and did not give the total number of neurons in the nucleus. The division into two groups was estimated from the size of the soma. Nuclear diameter was not measured. In the present study there was no evidence of a bimodal distribution of nuclear diameter in the motor nucleus and Hinrichsen & Larramendi (1969) noted that axon diameter in the mouse trigeminal motor root was unimodal.

The difference between lipofuscin accumulation in mesencephalic nucleus neurons and the scarcity of lipofuscin in motor nucleus neurons was quite marked. Glees & Hasan (1976), in their comprehensive review of lipofuscin, note that motor neurons of the spinal cord accumulate lipofuscin early but that motor neurons in the cranial nerve nuclei vary, both in the age at which lipofuscin first appears and in the quantity eventually present. The nuclei of the trigeminal, abducens, trochlear and oculomotor nerves contain much less lipofuscin than either spinal motor neurons or motor neurons of other cranial nerves. Wilcox (1959) showed that lipofuscin appeared in guinea-pig mesencephalic nucleus neurons at 650 days and in the trigeminal motor nucleus at three years. Brizzee, Ordy & Kaack (1974) found that mesencephalic nucleus neurons in the monkey brain contained more lipofuscin than trigeminal motor nucleus neurons but, unlike the mouse, there was a moderate to large accumulation of lipofuscin in trigeminal motor neurons in the very old monkey brain. In contrast to these findings, and to the present observations, Few & Getty (1967) noted that in the dog spinal cord motor neurons were more pigmented than dorsal root ganglion neurons, whilst in the pig, lipofuscin accumulation was similar in both groups.

Loss of Nissl substance as a consequence of ageing is a well known phenomenon (Marinesco, 1909; Andrew, 1936, 1971; Brizzee, Klara & Johnson, 1975) and a loss of neuronal RNA with increasing age has been confirmed by microspectrophotometric (Mann & Yates, 1974) and morphometric methods (Hinds & McNelly, 1979).

Such a loss, however, is usually associated with a marked accumulation of lipofuscin (Mann & Yates, 1974; Brizzee *et al.* 1975; Miquel, Johnson & Cervós-Navarro, 1983; Brizzee *et al.* 1983) which was not the case in the trigeminal motor nucleus of the mouse. Cammermeyer (1963) observed that loss of Nissl substance was a very prominent feature of ageing in the trigeminal mesencephalic nucleus of the rabbit and chinchilla. This was not evident in the mouse, probably because Nissl substance was never a prominent cytoplasmic component.

The increase in nuclear diameter of neurons in both nuclei examined was unexpected since most quantitative histological studies of normal ageing have found no change in nuclear diameter with age (e.g. Keithley & Feldman, 1979; Curcio & Coleman, 1982; Peters, Feldman & Vaughan, 1983; Curcio, McNelly & Hinds, 1985) although Hinds & McNelly (1977) reported an increase in nuclear diameter in mitral cells of the rat olfactory bulb up to 27 months of age, followed by a decrease thereafter. Cammermeyer (1963) noted a significant decrease in nuclear diameter of granule cell neurons in the cerebellum of ageing rabbits and chinchilla. Mann *et al.* (1981) also reported a significant decrease in nuclear diameter of pyramidal cells from the temporal cortex in Alzheimer's disease. Volume changes always raise the question of artefactual swelling or shrinkage of tissue due either to technical variation or the effects of ageing but in the present study this seems unlikely since measurements of glial (Sturrock, 1985) and neuronal nuclear diameter (Sturrock & Rao, 1985) using the same sets of sections, technique, and apparatus showed no significant variation with age. In particular, the absence of any change in neuronal nuclear diameter in the locus caeruleus (Sturrock & Rao, 1985), neurons of which intermingle with those of the mesencephalic nucleus, and which also loses neurons in old age, indicates that the increase in nuclear diameter found in the present study is not an artefact.

The increase in nuclear diameter probably reflects a significant change in metabolism of the neuron. The statistically significant negative correlation between nuclear diameter and decrease in neuron number might indicate an increase in vulnerability of the neuron, taking into account that the increase in nuclear diameter precedes the decrease in neuronal number.

The loss of neurons from the mesencephalic nucleus was statistically significant but proportionally about half that from the adjacent locus caeruleus (Sturrock & Rao, 1985), being around 20% in contrast to the 40% loss from the locus caeruleus over the same time scale. Although the analysis of variance was not significant in the case of the motor nucleus all the other statistical evidence points to the reduction in the number of neurons at 31 months being the beginning of neuronal fallout.

It has been demonstrated by the HRP technique that trigeminal mesencephalic neurons make synapses with many trigeminal motor neurons (Dacey, 1982). Some mesencephalic neurons, especially the more rostral may be associated with the oculomotor, trochlear and trigeminal nuclei (Warwick, 1964; Foster, 1973). Nevertheless the majority of mesencephalic neurons connect directly with trigeminal motor neurons and the ratio does remain constant from 6 to 31 months at 1:1·5 or 2:3.

In conclusion, in the very old mouse brain there is a significant decrease in neuronal number in the mesencephalic nucleus of the trigeminal nerve between 25 and 31 months of age. The loss of neurons is preceded and accompanied by an increase in neuronal nuclear diameter. An increase in nuclear diameter is found later in the motor nucleus and there is tentative evidence that this may also precede a loss of neurons from the motor nucleus. If this is the case the loss of motor neurons could

be the result of transneuronal degeneration. Since signs of ageing first appear in the mesencephalic nucleus it appears that loss of input from the periphery precedes any loss of motor neurons.

SUMMARY

The number of neurons and neuronal nuclear diameter was estimated in the mesencephalic and motor nuclei of the mouse trigeminal nerve at 6, 9, 12, 15, 22, 25, 28 and 31 months of age. Analyses of variance showed that neuronal number decreased significantly ($P < 0{\cdot}01$) in the mesencephalic nucleus between 25 and 31 months, but, although lowest at 31 months of age in the motor nucleus, the decrease in neuronal number in the motor nucleus was not statistically significant.

Neuronal nuclear diameter increased significantly in both nuclei between 25 and 31 months, being obvious first in the mesencephalic nucleus.

The main histological features of ageing were a marked accumulation of lipofuscin granules in neurons of the mesencephalic nucleus and a loss of Nissl substance from the motor neurons of the trigeminal motor nucleus.

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