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Neurons in the mouse anterior commissure. A light microscopic, electron microscopic and autoradiographic study

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INTRODUCTION

While studying neuroglia in the corpus callosum of the adult slow loris Ling & Ahmed (1974) noted a small number of neurons in the corpus callosum which they suggested had probably become trapped during migration to the cortex. The results of a developmental study of the mouse corpus callosum (Sturrock, 1976*b*) appeared to confirm this, as the percentage of neurons fell very rapidly between birth and five days postnatum, suggesting that a large proportion of the neurons present at birth had migrated to the cortex.

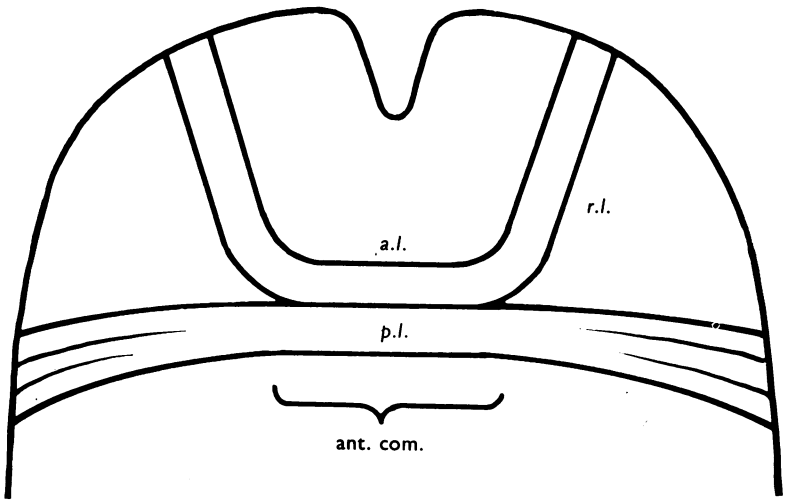
Ling & Ahmed (1974) demonstrated the presence of synapses in association with the isolated neurons, but queried whether these cells had any functional significance. During a quantitative study of myelination in both limbs of the anterior commissure (Sturrock, 1976*a*) a few synapses were observed in each limb which appeared morphologically to be axo-dendritic synapses. The present study is an attempt to find out how many neurons are present in each limb of the anterior commissure of the adult mouse brain, and at what ages these neurons are produced. As very few neurons are present a detailed electron microscopic study would be excessively time-consuming, but the observations on the ultrastructure of the few neurons found in ultrathin sections will be presented.

MATERIALS AND METHODS

Total neuron counts

Five mice aged 19 weeks postnatum were anaesthetized with ether, their vascular systems were flushed out with physiological saline at 37 °C, and perfusion–fixation was commenced with a solution of 1 % paraformaldehyde and 1·25 % glutaraldehyde in phosphate buffer at pH 7·4 for 5 minutes, followed by 4 % paraformaldehyde and 5 % glutaraldehyde in phosphate buffer at pH 7·4 for 15 minutes. After perfusion the complete animals were placed in polythene bags at 4 °C in a refrigerator. After 2 hours the animals were removed from the bags, their skulls opened, and the brains removed, dehydrated, embedded in paraffin wax, and serially sectioned at 6 μm in the sagittal plane. The sections were stained with the PFG stain of Lapham, Johnstone & Brunjar (1964).

The series was examined to find the sections at either end of the anterior commissure where the two limbs began to diverge (Fig. 1). The sections between them containing the anterior commissure were counted, and 15 sections were photographed at equal



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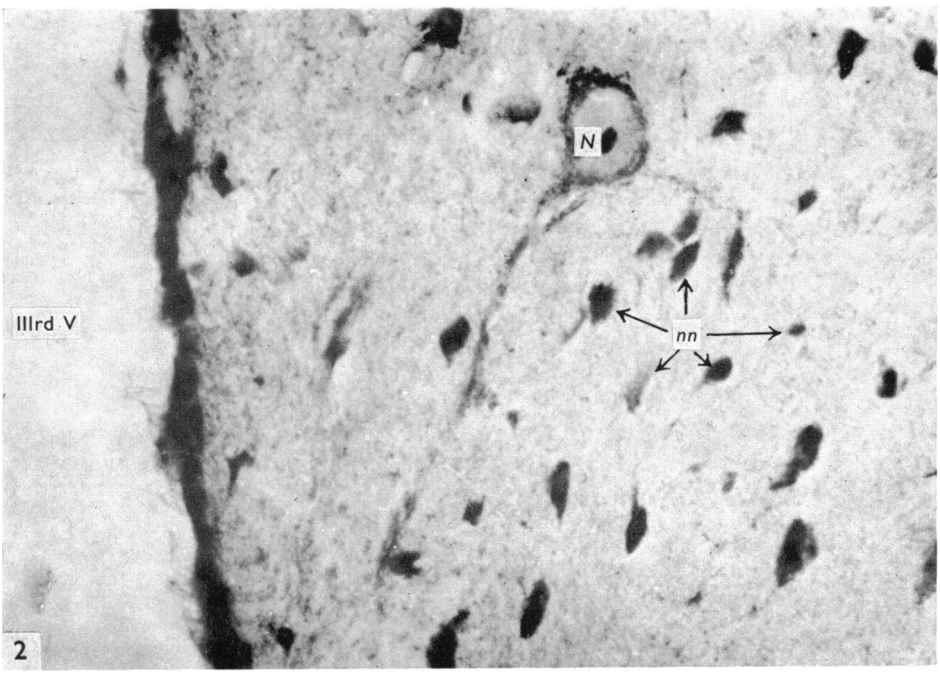

Fig. 1. Sketch of an idealized horizontal section of a mouse brain showing the part of the anterior commissure (ant. com.) examined, and the anterior (*a.l.*) and posterior (*p.l.*) limbs, diverging. The anterior limb turns rostrally to form the rostral (*r.l.*) part of the anterior limb.

Fig. 2. Neuron (*N*) lying in the posterior limb of the anterior commissure. This is a sagittal section from the midline area with the IIIrd ventricle (IIIrd V) on the left of the picture. The section is similar to that shown in Fig. 5. Note the large nucleus with a prominent nucleolus, two fairly well defined neuronal processes (probably dendrites) and the much smaller neuroglial nuclei. (*nn*). Lapham's stain. ×800.

distances along the commissure. The micrographs were printed at a total magnification of ×300. All nuclei and nuclear fragments in each limb, excluding those of endothelial cells, were counted on each micrograph, and the mean number of nuclei per section in each limb was multiplied by the total number of sections containing the anterior commissure to obtain the crude total in each limb. The corrected total was estimated by applying Abercrombie's (1946) formula to the crude total.

Each section containing part of the anterior commissure was examined at ×400 magnification and every neuron lying completely within each limb of the commissure was counted. The percentage of neurons was calculated by dividing the total number of neurons by the estimated total number of cells.

Autoradiography

Female mice were left overnight in separate cages with a male. The following morning conception was confirmed by the presence of a copulation plug. The morning after this was taken as the end of the first day of gestation, and each pregnant mouse received one injection with 10 μc/gm body weight of [^{3}H]thymidine by the intraperitoneal route on a different post-conceptional day. The first mouse was injected on the 11th day of gestation and the others on the 12th, 13th, 14th, 15th, 16th and 18th day of gestation respectively. The mice were allowed to have their litters and one mouse from each litter was killed at 22 days postnatum by perfusion–fixation with Bouin's solution after the vascular system had been flushed out with physiological saline. At the end of perfusion the parietal and occipital bones were removed and the heads were immersed in Bouin's solution for 4 hours, then the brains were removed from the skulls and placed in Bouin's solution for a further 24 hours before processing.

The brains were serially sectioned at 6 μm in the coronal plane, pre-stained with haematoxylin and eosin and all the sections were autoradiographed by dipping in Ilford K_2 emulsion (Rogers, 1967). The slides were exposed for 28 days before developing for 10–11 minutes in a freshly prepared solution of Kodak D19 developer and post-staining with haematoxylin and eosin.

The sections were examined with a Leitz microscope using dark-ground illumination. Neuronal nuclei with 25 grains or more overlying them were considered heavily labelled, and only such heavily labelled neurons were recorded.

Electron microscopy

Five 19 week old mice were killed by perfusion–fixation with two solutions of mixed aldehydes as described above. After cessation of perfusion the animals were left overnight in sealed polythene bags at 4 °C. The following morning the brains were removed and blocks containing the anterior commissure were obtained by careful microdissection. These blocks were rinsed in 0·1 M cacodylate buffer for 1 hour, post-fixed in 1 % phosphate buffered osmium tetroxide, dehydrated and embedded in Spurr's resin. Ultrathin sections for electron microscopy were cut using a Reichert ultramicrotome and stained with uranyl acetate and lead citrate.

Sections were scanned in an attempt to find neurons which lay within the anterior commissure, and when a neuron was found it was photographed. The anterior and

posterior limbs of the commissure were identified in each animal, and photographed at regular intervals across each limb at × 10000 magnification. Ten photographs were taken of each limb in each animal. These micrographs were examined and the number of synaptic boutons was counted.

Adjacent 1 μm sections were cut, stained with 1% toluidine blue, and the cross sectional areas of each commissural limb, in each animal, measured with a planimeter.

Estimation of number of boutons

As few boutons were observed (and indeed, in some sets of micrographs no boutons were present) it was decided to consider all five sets of micrographs of each limb together. The cross sectional area of each limb in each animal was measured from the adjacent toluidine blue stained semithin sections, using a planimeter. The mean cross sectional area of each limb was estimated. The estimated number of boutons per mean cross sectional area was found by multiplying the observed number by the mean cross sectional area and dividing by the area actually examined. The estimated total number of boutons in the whole anterior commissure, from the points at each end at which the two limbs begin to diverge, was found as follows. The mean diameter of a bouton was found by direct measurement to be 0·5 μm. The number of boutons per complete cross section could be corrected using Abercrombie's (1946) correction where the corrected total (C) is found by the formula $E \times t/(d+t)$, where E is the estimated total, t is section thickness, and d is the mean diameter of the boutons. The total number of boutons in the anterior commissure was found by multiplying the corrected number of boutons by the number of sections containing the whole commissure, which was obtained by the formula N (number of sections) $= l/t$, where l is the length of the commissure, and t is section thickness. The

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Fig. 3. This shows a neuron in the anterior limb in a 1 μm toluidine blue stained, sagittal section. Note the irregular nucleus and prominent nucleolus. × 2000.

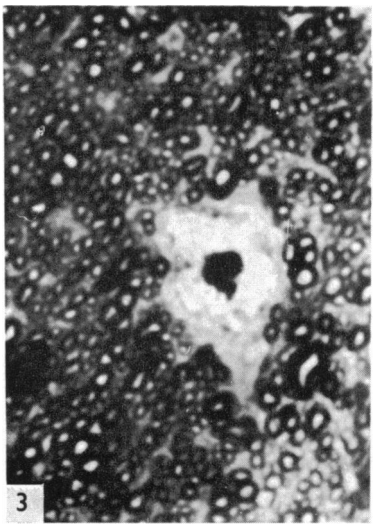
Fig. 4. Heavily labelled (⩾25 grains) neuron in the anterior commissure of a mouse injected with tritiated thymidine 14 days post-conception. H & E. × 2000.

Fig. 5. Sagittal section of the anterior commissure. The anterior limb (*a.l.*) is more darkly stained than the posterior (*p.l.*). The IIIrd ventricle lies posteriorly. The darkly staining neurons of the nucleus triangularis septi (*NTS*) can be seen above and partly surrounding the anterior limb. Lapham's stain. × 100.

Fig. 6. Sagittal section of the anterior commissure in the region of the bed nucleus of the anterior commissure (BN) which can be seen lying postero-superiorly and intimately related to the palely staining posterior limb (*p.l.*). Lapham's stain. × 100.

Fig. 7. 1 μm toluidine blue stained sagittal section of the anterior limb. The myelinated axons are more tightly packed than in the posterior limb (see Fig. 8), and the border of the commissure is clearly demarcated by rows of unmyelinated axons lying parallel to it. This is taken from the region of the nucleus triangularis septi (cf. Fig. 5). × 2000.

Fig. 8. 1 μm toluidine blue stained sagittal section of the posterior limb in the region of the bed nucleus (BN), part of which is visible on the right of the picture. The border of the posterior limb (*p.l.*) is not clearly defined, and appears to merge imperceptibly with the bed nucleus. × 2000.



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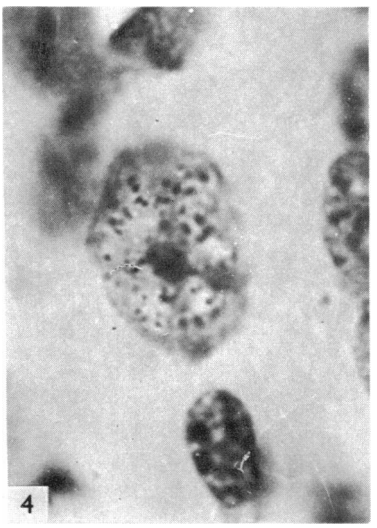

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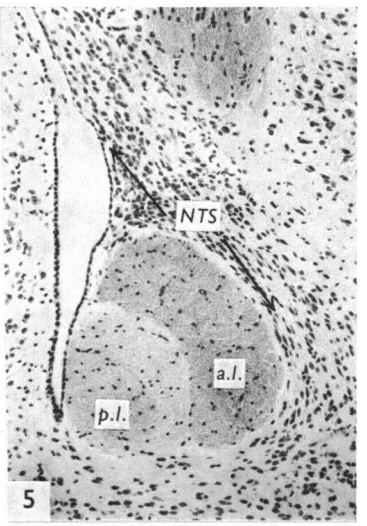

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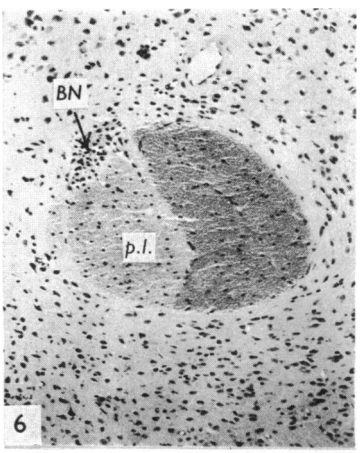

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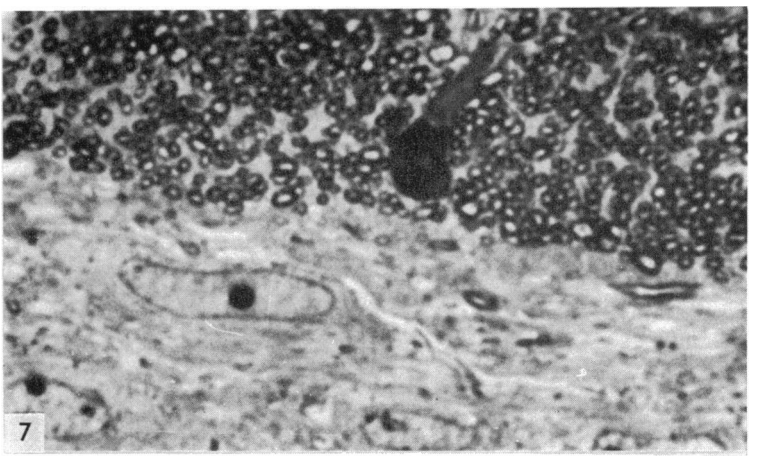

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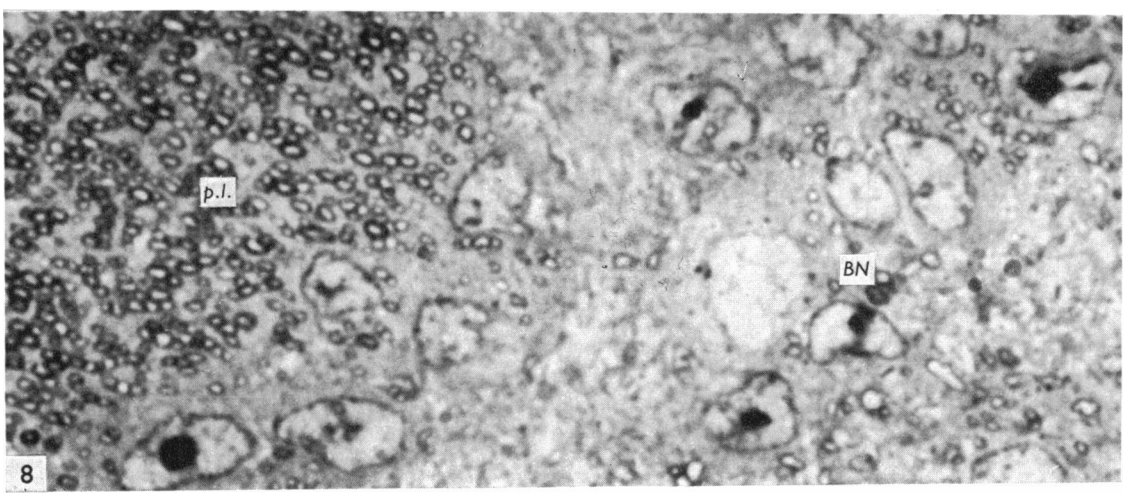

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complete formula for the total number of boutons in the whole commissure was therefore,

$$E \times \frac{t}{(d+t)} \times \frac{l}{t} = \frac{E \times l}{(d+t)}.$$

E had already been calculated, the length l was 2000 μm, (measured from 1 μm longitudinal sections stained with 1 % toluidine blue) and d was 0·5 μm. The thickness of the ultrathin sections t, was very small in relation to d, and for all practical purposes could be ignored, giving the modified formula $(E \times l)/d$.

RESULTS

Total neuron count

Neurons were easily recognized in light microscopic sagittal sections both in 6 μm sections (Fig. 2) and in semithin sections (Fig. 3). Their characteristic features were large nuclei (10–12 μm diameter), a prominent nucleolus, and occasionally Nissl granules. Rarely the plane of section coincided with part of the dendritic tree (Fig. 2).

There were 37·0 ± 2·7 neurons in the anterior limb and 70·4 ± 19·3 neurons in the posterior limb. The large S.E.M. in the number of neurons in the posterior limb was due to one animal, in which 147 neurons were found. If this one animal was excluded the mean number of neurons would be 51·3 ± 3·1. Perhaps significantly, the posterior limb containing the large number of neurons also had a larger than usual total cell number, 18848, in contrast to the mean value of 15520 for the other four animals. Neurons appeared to be randomly arranged as regards their position within a sagittal section. When the number of neurons per limb per slide was examined, however, a pattern emerged. The largest numbers of neurons in the anterior limb occurred in the sections in which nucleus triangularis septi appeared (Fig. 5), while the largest numbers of neurons in the posterior limb occurred in the sections in which the bed nucleus of the anterior commissure (Fig. 6) appeared. Neurons were present in both limbs in other areas, with around 1–2 neurons per 18 sections (i.e. per slide), except in the areas of these nuclei. 5–7 neurons per slide were present in the anterior limb in the region of the nucleus triangularis septi, and 5–10 neurons were present in the posterior limb in the region of the bed nuclei.

Occasionally neurons situated peripherally in the commissure were observed whose processes seemed to extend towards the septal nuclei, and neurons of the septal

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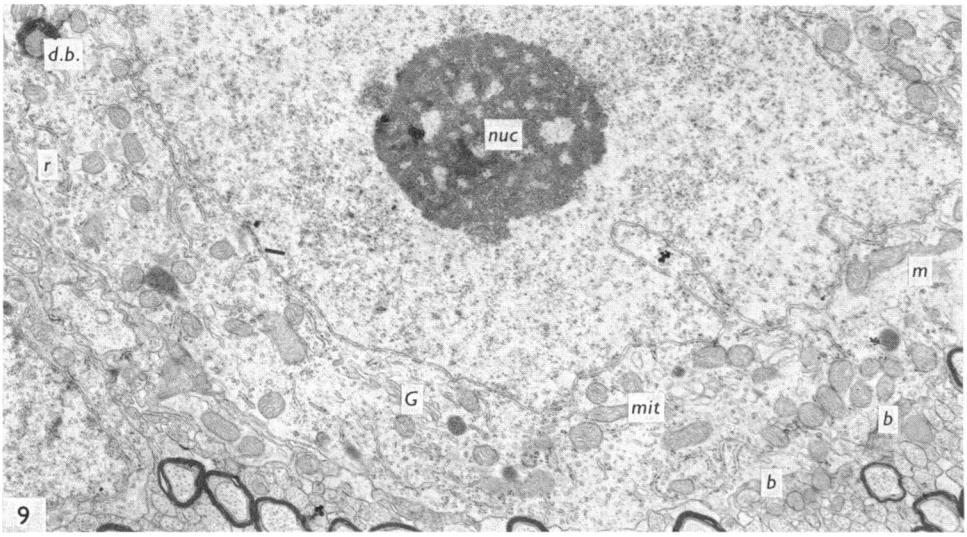
Fig. 9. Electron micrograph of posterior limb neuron. This neuron has a pale, irregularly shaped nucleus with a prominent nucleolus (*nuc*). The pale cytoplasm contains numerous mitochondria (*mit*), a Golgi apparatus (*G*), dense bodies (*d.b.*), strands of rough endoplasmic reticulum (*r*), and microtubules (*m*). Boutons (*b*) can be seen in contact with the perikaryon. × 10000.

Fig. 10. This shows a posterior limb neuron which is smaller than that in Fig. 9, and which has a relatively sparse cytoplasm. A few mitochondria are present in close proximity to the only bouton (*b*) in contact with soma. Free ribosomes are numerous, and a Golgi complex (*G*) is present. × 15750.

Fig. 11. This shows part of the neuron in Fig. 9 at higher magnification. Note the dense bodies (*d.b.*), the numerous mitochondria (*mit*) and the synaptic boutons (arrows). × 25000.



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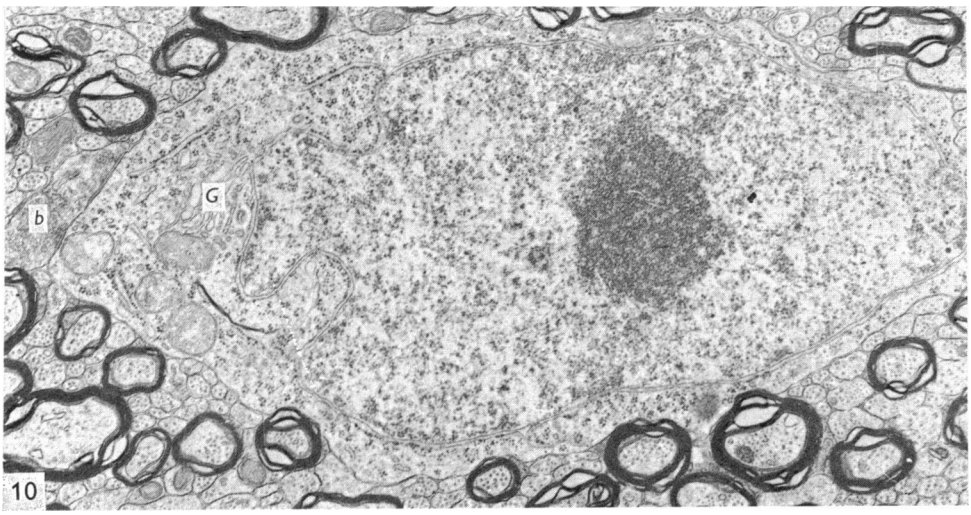

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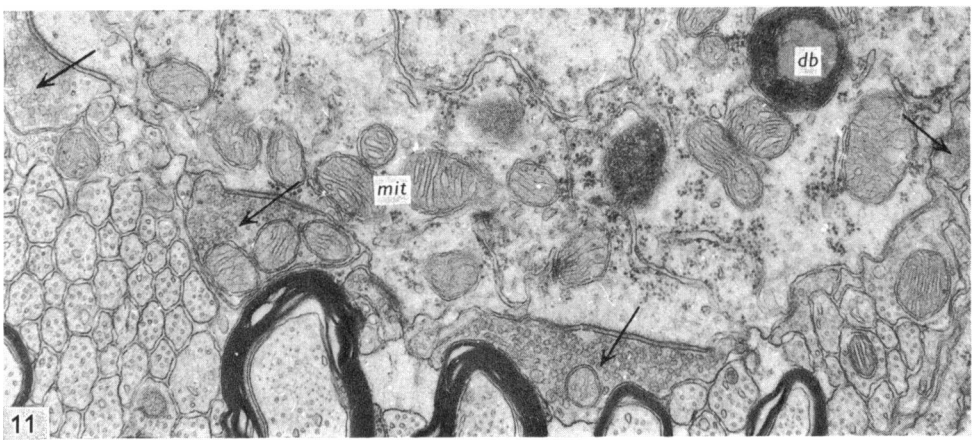

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nuclei were observed with processes apparently entering the commissure. In both paraffin and semithin sections it was apparent that the relationship of these nuclei to the two limbs differed (Figs. 5–8). The border of the anterior limb adjacent to the nucleus triangularis septi was distinct (Figs. 5, 7) and clearly demarcated from the nucleus by parallel rows of unmyelinated axons (Fig. 7), whereas the border of the posterior limb was indistinct (Figs. 6, 8), and it was difficult to be completely certain where the posterior limb ended and the bed nucleus began (Fig. 8), probably because of axons leaving the posterior limb to end in the bed nucleus (van Alphen, 1969).

The total number of cells in the anterior limb was 22890 ± 350 and the total number in the posterior limb was 16190 ± 470. The percentage of neurons in the anterior limb was $0{\cdot}16 \pm 0{\cdot}01$ and in the posterior limb, $0{\cdot}42 \pm 0{\cdot}09$ %.

Autoradiography

No labelled neurons were found in the anterior commissure of the mouse injected at 11 days of gestation, nor in the mice injected at 16 and 18 days of gestation. Heavily labelled (25 grains or more) neurons were found in the commissures of mice injected at 12, 13, 14 and 15 days of gestation (23 heavily labelled nuclei in the 12 day animal; 11 in the 13 day animal; 35 in the 14 day animal; and 5 in the 15 day animal). Labelled neurons were identified by their size and the presence of a prominent nucleolus (Fig. 4). The timing of the appearance of labelled neurons in the commissure coincided with the appearance of heavily labelled neurons in adjacent septal nuclei, particularly neurons in the bed nuclei of the anterior commissure and the nucleus triangularis septi.

Electron microscopy

As neurons make up only 0·1–0·4 % of the total cell population of the anterior commissure they were rarely observed in electron microscopic sections (there are on average only 107 neurons in 2000 μm of anterior commissure). Because of this the following observations have necessarily been based on a small number of neurons. Neurons were easily recognized because of their large size in comparison with neuroglia. Neuronal nuclei were irregularly ovoid (Figs. 9, 10), with numerous indentations of the surface membrane. A nucleolus was prominent (Fig. 9), usually lying centrally within the homogeneously dispersed nucleoplasm. The cytoplasm was pale and contained a variable number of organelles (compare Figs. 9, 10). Mitochondria were usually plentiful (Fig. 9) and strands of rough endoplasmic reticulum were scattered throughout the cytoplasm. Rosettes of free ribosomes (Fig. 11) and

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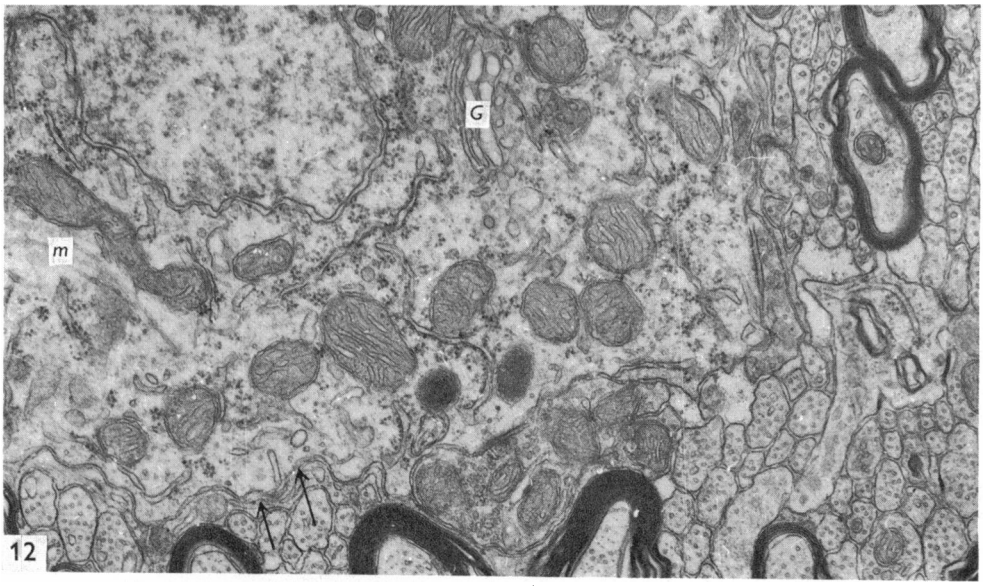
Fig. 12. Neuron. Note the Golgi apparatus (*G*), the microtubules (*m*) and the astrocytic process (arrows) intervening between the neuronal perikarya and unmyelinated axons. × 25000.

Fig. 13. This figure also shows an astrocytic process (arrows) intervening between some unmyelinated axons and the perikaryon, but it can be clearly seen that other unmyelinated axons are in direct contact with the perikaryon. × 40000.

Fig. 14. Synapse (*s*) between a bouton and the perikaryon of a commissural neuron. × 40000.

Fig. 15. Synaptic bouton (*b*) in the posterior limb which is not in the vicinity of any commissural neuron. × 40000.



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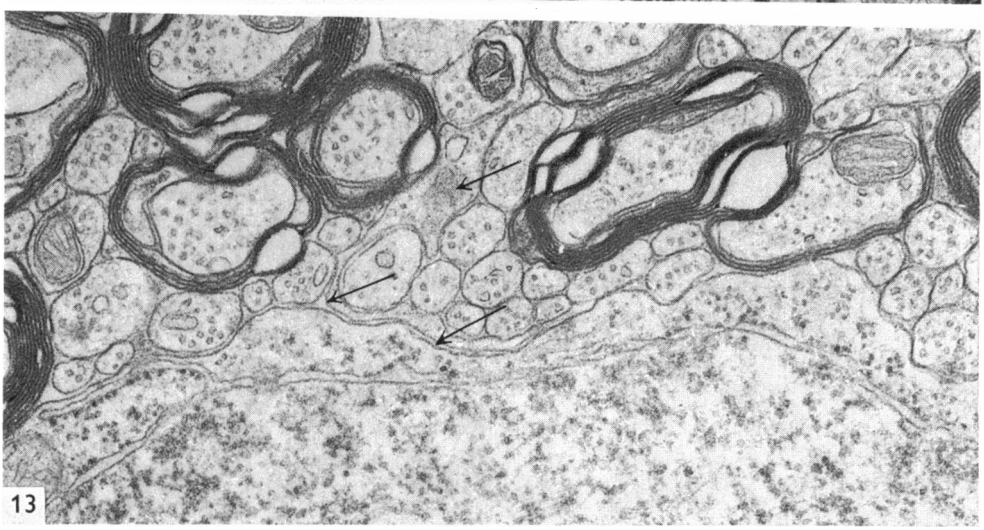

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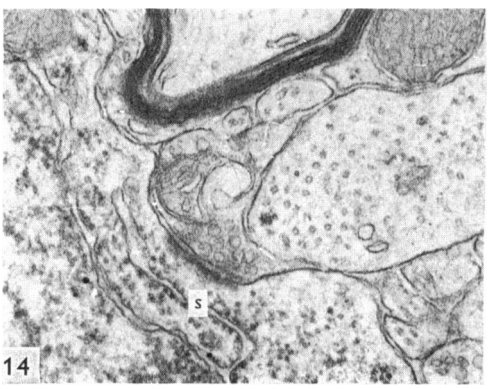

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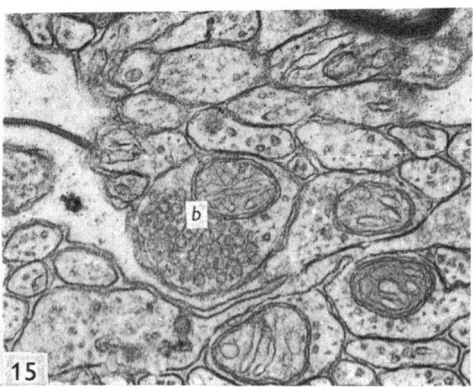

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a Golgi apparatus were also present (Figs. 10, 12). Microtubules were a prominent feature and dense bodies, presumably lysosomes, were present in the perikaryon (Figs. 9, 11 and 12). The number of synaptic boutons (Figs. 9–13) around the neuronal soma varied, and it seemed as if there was a relationship between the number of synaptic boutons and the number of mitochondria (contrast Figs. 9, 10). Occasionally unmyelinated axons were separated from the neuronal perikaryon by astrocytic processes (Figs. 12, 13), but this was by no means always the case (Fig. 13).

These neurons appeared ultrastructurally similar to the neurons in the bed nuclei of the anterior commissure and the nucleus triangularis septi, both in their morphology and in the variation in the number of axo-somatic synapses.

Synaptic boutons (Fig. 15) were extremely rare except in contact with neuronal perikarya. In the 100 micrographs (approx. 32000 μm^2 area) examined only 17 boutons were observed, 8 in the anterior limbs and 9 in the posterior limbs. The estimated total number of boutons per limb, however, was 1362900 in the anterior limb, and 866300 in the posterior limb. If these synapses are equally distributed between the axons in the commissure then each axon would synapse three or four times as it traversed the commissure.

DISCUSSION

The neurons in the anterior commissure are produced early in development, the largest number undergoing their final division on the fourteenth day of gestation when the commissure is just beginning to be visible in haematoxylin and eosin sections. Although autoradiography is uninformative regarding the site of production, or the time of migration, of the labelled neurons, it seems unlikely that they would use the anterior commissure as a migratory pathway, and much more likely that they are surrounded by axons as the commissure develops. The method of acquisition of neurons in the corpus callosum is different, as it seems almost certain that the callosal neurons are migratory neurons which remain in the corpus callosum (Ling & Ahmed, 1974; Sturrock, 1976*b*). Despite these differences the percentage of neurons in each tract is similar (0·1–0·4 %). Considering the large number of neurons migrating through the corpus callosum, and the large number of neurons which could conceivably be enclosed by axons of the anterior commissure, it is perhaps remarkable that so few neurons are found in these tracts. At the moment it is not known whether the formation of synapses plays a part in ensuring that the neurons remain within the commissure, or whether these synapses occur secondarily; nor is it known whether there are other neurons which fail to achieve synaptic contacts and die during development, but these are possibilities which cannot be ignored.

If these neurons in the anterior commissure are present purely by chance, having been separated from their nuclei by axons of the commissure during development, it would seem unlikely that they would be functional. Axosomatic synapses might develop as a result of random contact of commissural axons with partially differentiated neurons enclosed within the commissure, but this seems extremely unlikely in view of the remarkable specificity of neuronal connexions over long distances within the central nervous system. The presence of axosomatic synapses, therefore, probably means that these neurons are functional. Gurdjian (1925), Valverde (1963) and van

Alphen (1969) have shown that a small part of the posterior limb distributes to the bed nuclei of the anterior commissure. The displacement of a few neurons from the bed nucleus into the posterior limb of the commissure would probably not affect the function of these neurons. Likewise the neurons in the anterior limb are probably derived from, and connect with, the nucleus triangularis septi which, like most septal nuclei, is involved in olfactory circuits, although anatomically the relationship between the nucleus triangularis septi and the anterior limb is less close than that between the two bed nuclei and the posterior limb. The pattern of distribution of neurons in each limb tends to support these explanations.

The presence of neurons within the anterior commissure probably permits a large number of *en passant* synapses to occur between commissural axons and the dendrites and perikarya of these neurons, thus allowing connexions to occur between the septal nuclei and other areas of the brain without reducing the number of axons traversing the commissure. Although the number of neurons is very small it should not be forgotten that, even taking into account the large number of possibilities for error in calculation of the number of synapses, there are probably of the order of 10^6 synapses in each limb. It is quite likely that many of these synapses, particularly in the posterior limb, occur between axons of the commissure and dendrites of neurons whose perikarya lie outside the commissure. The neurons whose perikarya lie within the commissure may only play a secondary role, even in the *en passant* connections between the commissural axons and the septal nuclei, but it seems unlikely that such a large number of synapses would be present if these connexions had no significant functional part to play, nor does it seem likely that the number and distribution of commissural neurons would occur so constantly in different animals purely by random enclosure of neurons by growing axons.

SUMMARY

Both limbs of the anterior commissure of the mouse brain were examined to find the number, distribution, times of origin and structure of the neurons present, and also the number of synapses within the commissure. Neurons form between 0·1 and 0·4% of the total cell population and are produced between the twelfth and fourteenth days of gestation.

It seems likely that the neurons within the anterior commissure are derived from adjacent septal nuclei, with the bed nuclei of the anterior commissure mainly contributing to the posterior limb and the nucleus triangularis septi mainly contributing to the anterior limb. The neurons are almost certainly functional, and distribute to the nuclei from which they are derived. There are probably also other connexions between these nuclei and both limbs of the anterior commissure through dendrites from the septal nuclei which ramify throughout the commissure. The large number of synapses scattered throughout the anterior commissure suggests that the neurons within the commissure, and dendrites entering it, may contribute substantially to the pathways between the anterior and posterior limbs and the septal nuclei without diminishing the number of axons in the commissure.

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