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CORRELATING STRUCTURE WITH FUNCTION IN DEVELOPMENTAL NEUROLOGY USING STEREOLOGICAL AND CONFOCAL MICROSCOPICAL TECHNIQUES

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Abstract. Highly efficient stereological methods can be employed to obtain global estimates of quantities such as mean particle volume, surface and number. In developmental neurology the monitoring of neural connectivity is of prime interest. A possibility of achieving this aim in a single animal over a developmental time window is offered by confocal scanning light microscopy.

INTRODUCTION

The majority of morphometric studies in the field of embryology involve the rearing and sacrificing of cohorts of animals at set developmental stages. Measurements can then be made on the group and then mean values used to assess normal development and for comparing the effects of various treatments on embryogenesis. In stereological studies this is an approach where unbiased measures of feature volume, surface, length and number can be obtained from standard histological material, provided that a correct sampling scheme has been employed. For a review of these methods see Gundersen (1986).

There are some features that are excusively three dimensional and these include spatial distribution, connectivity, size distributions and real feature shape (De Hoff, 1983). In studying the nervous system the number of information processing units (neurons) and their connectivity are sure to be of interest. While the estimation of total cell number (Pakkenberg and Gundersen, 1988) is now very efficient, connectivity poses much more severe problems.

It is possible to envisage average measures of connectivity such as mean dendrite length per neuron and mean number of synapses per neuron. However to measure these parameters stereologically requires that the animals be sacrificed. One

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of the most interesting questions in developmental neurobiology today is to gain insight into the mechanism of selective stabilisation hypothesised by Changeau and Danchin (1976). For such a study it is necessary to follow the detailed connectivity of groups of neurons throughout their development. Such an approach requires a method of probing the brain in three dimensions which is non-destructive, ie a vital microscope.

The advent of confocal scanning light microscopy provides the opportunity to examine the internal structure of an individual animal many times because sectioning is done 'optically' (Wilson, 1989). The main proviso is that the feature of interest should fluoresce or reflect. The main future of this technology appears to be in immuno-epi-fluorescent microscopy (Amos *et al.*, 1987) although for developmental work there is much yet to be done in finding and improving non-toxic vital labels.

In this paper we will demonstrate preliminary results from a study of developing rat neocortex using stereological techniques. We will also discuss the impact of confocal scanning light microscopy in the study of connectivity patterns in developing nervous tissue.

MATERIALS AND METHODS

A. Perinatal rat study: an example of the estimation of mean parameters from cohorts of developing animals.

1. *Histology*

Seven groups of six animals, consisting of 3 pairs of littermates, at developmental ages 17, 19 and 21 day post coital and 5, 10 and 20 days post natal, were sacrificed under ether anaesthesia. Their brains were immediately dissected out, weighed and then immersed in 2% paraformaldehyde with 2.5% glutaraldehyde in phosphate buffer. For each animal this was performed in under 5 minutes. Each brain remained in fixative for 48 hours and was then dehydrated in serial alcohols. The brains were then embedded in Historesin. After polymerisation the blocks were serially sectioned coronally on a Historange microtome, using a Ralph knife, at a sectioning depth of 2 microns. The microtome was calibrated according to Evans and Howard (1989).

A uniform random sampling scheme was employed, after the design of Pakkenberg and Gundersen (1988) so that for each developmental stage on average 10 sections from the serial stack were sampled. With each section thus selected its immediately preceding neighbour section was also collected. The sections were initially floated onto water and then dried onto glass slides for 24 hours.

Prenatal brains were stained with Safranin and toluidine blue (Martino-Partido, 1985) while post natal brains were stained with Haematoxylin and eosin. Both stains were assumed to stain all cells present and as a control the neonatal cohort was split into two groups of 3, one set receiving the Safranin and Toluidine blue with the others being stained with Haematoxylin and eosin. No significant difference was seen qualitatively or quantitatively between the groups.

2. Stereological methods

a. Neocortical volume estimation

Each of the 10 or so reference sections from each brain were projected onto a uniform grid of points with a Zeiss (Jena) histological slide projector. The number of points falling within neocortex was counted and the Cavalieri estimator used to calculate the neocortical volume after the method described by Pakkenberg and Gundersen (1988). Necortex was arbitrarily distinguished from archicortex by projecting a line normal to the pial surface from the bottom of the rhinal fissure to the underlying white matter.

b. Cell counting methods

A double projection light microscope was built to facilitate the simultaneous viewing of two adjacent sections. A cold light source was used to project the two images downwards onto a flat work surface and viewing took place in a darkened room. The 'disector' principle (Sterio, 1984) was applied. Counting was performed in the manner of Pakkenberg and Gundersen (1988). Systematic random fields were sampled on the reference plane with an unbiased 2-D counting frame (Gundersen, 1977). Cell nucleus profiles sampled on the reference plane but not appearing on the adjacent 'look up' plane were deemed to have their 'tops' in the volume described by the product of the area of the counting frame and the distance between sections, according to the 'disector', 3-D counting rule. Sampling over uniform random disectors leads to an unbiased estimate of the numerical density of particles, in this case cell nuclei.

Prior to 5 days postnatal it was not possible to distinguish between neurons and glial cells, using the standard cell body stains described. After 5 days postnatal it was possible to make this distinction.

c. Total cell population estimation

The product of the total reference volume, from the Cavalieri estimator, and the numerical density gives an unbiased estimate of total number. Error estimation of these estimates is described in Pakkenberg and Gundersen (1988).

B. Study on intact specimens of the parasitic worm *Grillotia erinaceus*: an example of a possible future model for the monitoring of a developing neural connectivity pattern within one animal.

1. Histological methods

Conventional immuno-epifluorescence studies of parasite neuroanatomy usually require the specimen to be completely flattened before useful images can be obtained. This distorts the spatial arrangement of the elements of the nervous system and makes the examination of neuronal connectivity impossible. In this study intact worms were stained for the presence of serotonin (5-HT) reactive neurons according to the following protocol:

1. Specimens were fixed with 4.0%, paraformaldehyde in 0.2M P. B. S. buffered for 4 hours at room temperature. The fixative also contained 0.1% Triton X, 1% bovine serum albumin and 0.04% sodium azide.

2. After fixation, the specimens were washed overnight with 0.2M P. B. S. which also contained the above specified quantities of Triton X, B. S. A. and sodium

azide.

3. Specimens were incubated with the primary antibody, anti 5HT (rabbit), at its recommended working concentration using 0.2M P. B. S. containing 1% B. S. A. and 0.04% sodium azide for 12 hours at room temperature.

4. The worms were washed for 1 hour in the solution shown in 2, minus the Triton X.

5. Incubation in the secondary biotinylated antibody, donkey anti-rabbit, at 1:200 dilution for 1 hour at room temperature, (dilute to the required dilution with 0.2M P. B. S. containing 1% B. S. A.)

6. Repeat step 4.

7. Incubation with fluorescent antibody in a dark container at 1:100 dilution at room temperature (1 hour).

8. Repeat step 4.

9. Storage of material in 0.2M P. B. S. at 4C in the dark.

2. Imaging

Whole mount specimens were examined in a conventional epifluorescence Nikon Optiphot light microscope. They were also examined in an identical microscope attached to a BioRad Lasersharp MRC SOM500 confocal scanning light microscope.

Through-focal series of optical sections traversing the whole thickness of the nervous system of the animal were collected viewed in projection after Amos *et al.* (1987).

RESULTS

A. Rat neocortical development.

The results for total neocortical cell number and volume are shown in Fig. 1.

B. Confocal laser scanning epifluorescent imaging of intact specimens of metacestodes of *Grillotia erinaceus.*

Immunofluorescence within the worm was restricted to nerve cell bodies and processes. Unflattened intact specimens were examined in a Lasersharp MRC500 system attached to a Nikon Optiphot microscope using a ×20 NA 0.75 air objective through supported coverslip. The blastocyst demonstrates an interconnected circumferential net of fine calibre nerve fibres and these can be seen to be connected to the scolex. This provides a cellular basis for the directional spread of activating impulses from blastocyst to scolex in the dormant worm (McKerr and Allen, 1986).

A centralised nervous system exists within the anterior scolex only. Here it lies deep within the parenchymal tissue. High contrast through-focal series of optical sections were collected throughout the depth of the scolex by confocal scanning light microscopy. These could be studied in stereo and provide a wealth of detail about the spatial arrangement of neurons within the central ganglion of the scolex.

We plan to extend this work to include a quantitative study of spatial distribution after Baddeley *et al.* (1988). We will also investigate the presence of

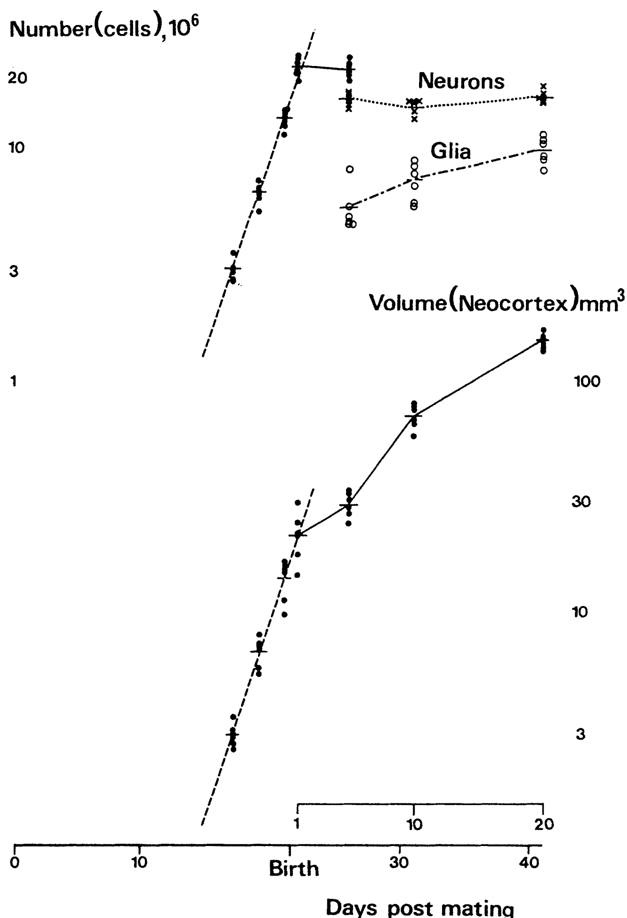

Fig. 1. On the lower curve the mean volume of the neocortex for six animals is given at different developmental ages. The upper curve shows for the same animals the mean total number of neocortical cells. After birth these are broken down into separate counts for neurons and glial cells. The vertical axis for both plots is logarithmic.

other transmitter substances.

DISCUSSION

Stereological methods are very efficient, the combination of the disector with a Cavalieri volume estimate yielding a stable estimate of total neocortical cell number in under half a day's total work. Furthermore the results give us some genuine insight into some aspects of neocortical development. From day 5 postnatal the total number of neurons in the neocortex appears to be constant. During prenatal development the cells appear to occupy a constant volume. However when the neuronal population becomes fixed the volume of the cortex continues to grow,

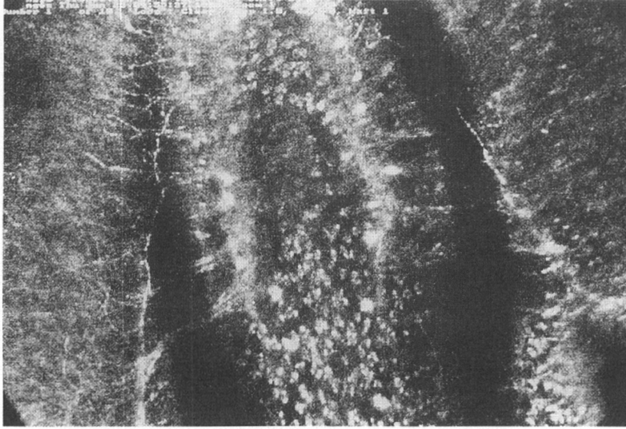

Fig. 2. A projection of a through-focal series of 10 optical sections of the central ganglion in the scolex of a metacestode of *Grillotia erinaceus* by confocal scanning light microscopy. Fluorescing neurons are stained for 5HT according to the protocol described in the text. Magnification ×200.

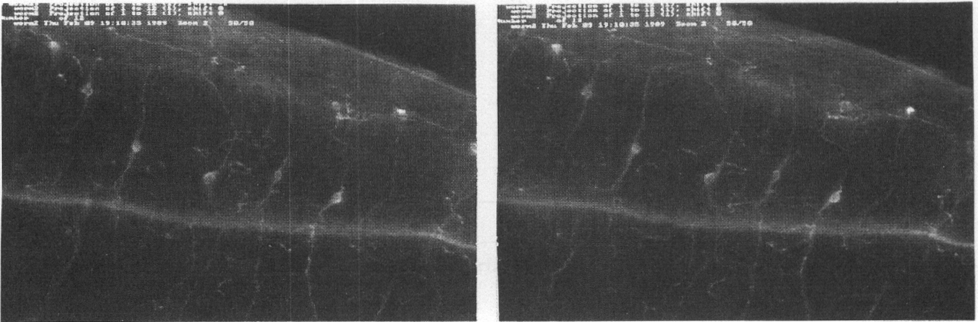

Fig. 3. A stereo-pair of a neural network in the bladder wall of a metacestode of *Grillotia erinaceus* formed from a through-focal series of 12 optical sections on a BioRad MRC500 CSLM. Staining regime as in Fig. 2. Magnification through a×3 stereo viewer approximately ×300.

accompanied by an increase in the number of glial cells. This would indicate that the functional requirements of the glial cells, which perform 'housekeeping' duties for the neuronal population, are related to volume. Furthermore it must indicate that the neurons, though not increasing in number, are probably developing neurites and establishing contact for a considerable period postnatally. We are at present engaged in extending this research to include an electron microscopic study to estimate the total length of dendrite in the rat neocortex at different developmental stages. By knowing also the total number of neurons we will obtain an estimate of the mean dendritic length per neuron, which we envisage will be a measurement of interest in developmental neurology. However it must be stated that the statistical mean estimates outlined above give no insight into the specific connectivity of the system under investigation, nor to the way the micro-circuitary changes during

development.

Confocal scanning light microscopy affords us the ability of examining the same animal a number of times. In our preliminary studies with simple Platyhelminth worms we have shown that it is possible to image the complete extent of the nervous system without the need to physically section these animals. The Avidin-Biotin staining regime requires fixed tissue and we are now concentrating on methods of non-toxic vital immuno-staining. If successful that should permit us to monitor the development of neuronal spatial arrangements as for example in Baddeley *et al.* (1988), where a 3-D K-function has been applied to the study of the spatial distribution of osteocyte lacunae in skull bone. It was shown that osteocytes were more uniformly distributed in 3-D than random, as defined by a 3-D Poisson field.

Furthermore it should be possible to study specific connectivity patterns of neurons over time using confocal scanning light microscopy. This, combined with spatial distribution data, may afford insight into the phenomenon of epigenetic cell death (Changeau and Danchin, 1976). With such information we would have a model which could be perturbed under experimental control to study the effect of various bio-active compounds or toxins on the development of specific neuronal connectivity.

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