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COMPUTER ASSISTED IMAGE ANALYSIS OF REGENERATING NERVE TERMINALS IN THE PERIPHERAL AND CENTRAL NERVOUS SYSTEMS

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ABSTRACT

Computer assisted image analysis (IA) was used to measure the extent of nerve terminal arborization (terminal density) in three different parts of the nervous system. Model experiments, using mouse irides, showed that IA and biochemical analysis techniques gave very similar results. It was also shown that IA had very good reproducibility, but that results should be interpreted in relation to an adequate control rather than in absolute terms. Studies of 5-HT nerve terminals in cerebral cortex and substance P containing terminals in spinal cord showed that important information could be made available through IA where biochemical analysis was not feasible. IA can thus be an important tool in studies of regenerating nerve terminals in regions where biochemical methods do not have sufficient morphological resolution.

Keywords: Image analysis, regeneration, monoamines, substance P, ganglioside.

INTRODUCTION

The mechanisms underlying the regrowth of damaged nerve terminals are of great biological and medical interest. To investigate these mechanisms it is necessary to find a sensitive, reliable, and quantitative technique to measure the extent of nerve terminal arborization with minimal loss of morphological information. The most common approach is to use a microdissection procedure followed by biochemical assays for neuron specific markers, usually a transmitter substance or the specific uptake of radioactively labelled transmitter (Sachs and Jonsson, 1973). The disadvantages are mainly the loss of morphological information and the distinct possibility of not detecting a regrowth in a small volume of the dissected tissue.

A computer assisted image analysis technique that can determine the density of nerve terminals visualized by transmitter specific histochemical procedures will be described. The Falck-Hillarp method for visualization of monoamine transmitters (Falck et al., 1962) and the indirect immunohistochemical fluorescence method (Coons, 1958) have been used in this work due to the good specificity and high signal-to-noise ratio they allow. It has been shown (Einarsson et al., 1975) that the relative amount of transmitter present in dense nerve terminal networks can be measured by quantitative microfluorimetry using a photo multiplier tube to measure the emitted fluorescence. In the present experiments this relatively time consuming technique (cf. Schipper and Tilders, 1982) was not used since the aim was to rapidly find the density of a sparse nerve terminal network rather than the amount of fluorescence from each terminal.

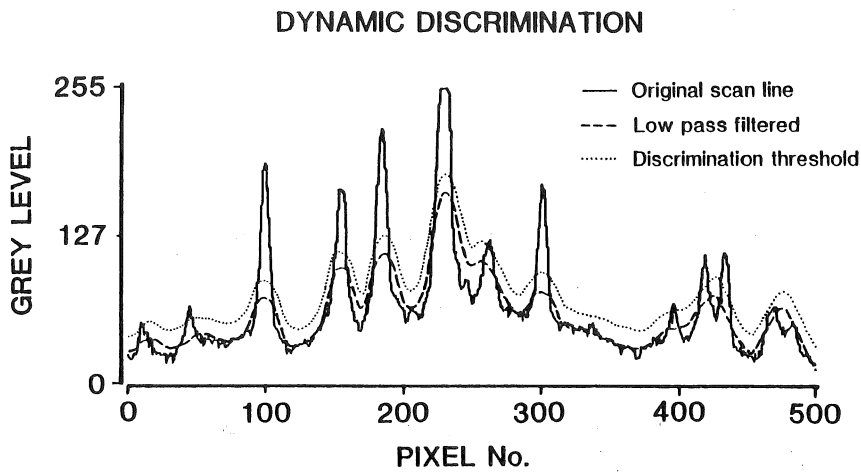

Figure 1. Schematic illustration of the dynamic discrimination procedure. The grey levels across a digitized iris image are represented by the "Original scan line", the low pass filtered image (scanned along the same line) was used as background in the segmentation procedure. The peaks correspond to fluorescent fibers. The discrimination threshold was chosen interactively.

MATERIALS & METHODS

The histological specimens were investigated in a fluorescence microscope in epi-illumination (25 X objective) or in transmitted light using a dark field condenser (10 X objective). The higher magnification was used for localized terminal networks, the lower magnification for diffuse networks. A silicon intensified target (SIT) video camera was mounted on the microscope, allowing a large area of the histological specimen to be analyzed, even at very low signal levels. The SIT camera was connected to an IBAS2 image array processor, allowing a rapid A/D conversion (744x512 or 512x512 8 bit pixels) of the image to be analyzed, which is an important point since the fluorophores are degraded by the excitation light. The image could then be further treated in the IBAS, irrelevant image elements (e.g. cracks and cell bodies) was removed and, in some cases, the reference area used in percentage measurements was defined. The image was then low pass filtered and the filtered image used as background in an interactive gray scale segmentation procedure to select specifically fluorescent elements (dynamic discrimination, Figure 1). The resulting binary image was median filtered to remove noise elements. The nerve terminal density was then estimated by measuring the per cent of the image area covered by the remaining pixels. In some experiments the binary image elements were skeletonized to one pixel thickness before the measurement.

The uptake of ^{3}H labelled noradrenaline (NA) was measured according to Lidbrink and Jonsson (1973). The irides were dissected out, stretched and dried on glass slides and reacted with gaseous formaldehyde (see Sachs and Jonsson, 1973). The immunohistochemical procedure has been described earlier (Jonsson et al., 1984). Liquid chromatography with electrochemical detection (Keller et al., 1976) was used to determine the total amount of NA in the irides.

RESULTS

Peripheral Nervous System

Mouse irides were used as a model system to test the reproducibility, reliability, and stability of the technique. The NA terminals in the iris were visualized by the Falck-Hillarp histofluorescence method, and investigated in transmitted light with a 10 X objective, giving a measuring field of appr. 500 x 700 micrometers. Repeated measurements of the same irides gave almost identical results (18.8 ± .84; 18.9 ± .1.2; 19.1 ± .75; n = 5). These measurements were performed with the same setting of the dark field condenser. Some variation of absolute values due to differences in the illumination could be seen, relative values did not change, however. It is therefore essential to measure controls and experimental groups in the same session.

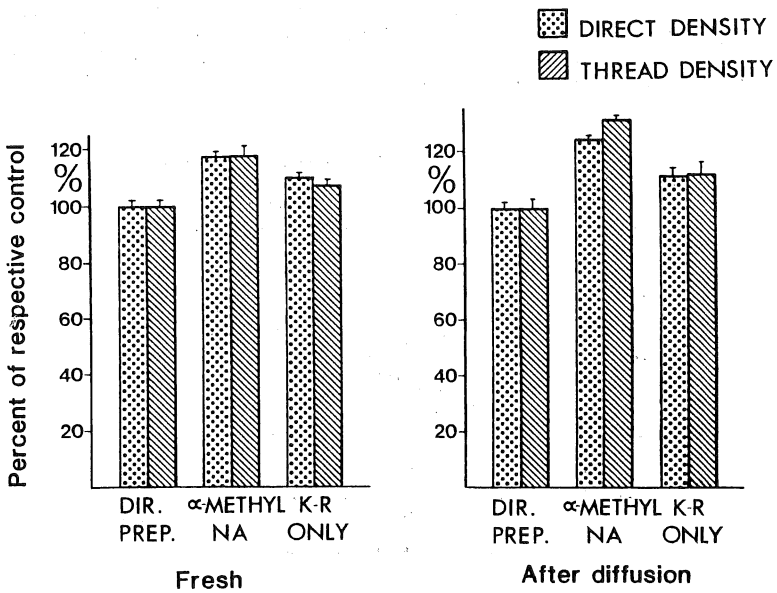

Figure 2. Area % values in per cent of control after incubation in 5 x 10^{-6} M α-methyl-NA and in Krebs-Ringer buffer only. The measurements were repeated after storage of the irides in ambient temperature and humidity for 2 weeks, conditions that cause a diffusion of fluorophore from the nerve terminals. Values are expressed as mean ± s.e.m.; n = 5.

When the irides were loaded with α-methyl-NA to increase the fluorescence intensity of the NA fibres, a slight increase of the area % values could be seen (Figure 2), this increase seemed to be mostly due to a filling of the intervaricose segments with fluorophore. However, the incubation procedure itself (30 min in 37 °C Krebs-Ringer solution) increased the measured area % with appr. 8% of untreated control (Figure 2), showing the necessity of using incubated control irides in incubation experiments. The stability of the method was further tested by storing the irides in ambient temperature and humidity for two weeks, thus allowing the fluorophore to partly diffuse out of the fibres. There was an increase of the area % values after diffusion

but there was little difference when compared to a diffused control. The influence on the area % measurements of diffusion of fluorophore out of the nerve, or an artefactual enlargement of strongly fluorescent objects, due to light scatter in the section or "blooming" in the video camera, was much lessened by a reduction of all identified objects in the binary image to one pixel thickness. This procedure allowed a faster throughput of images, since the monoamine containing mast cells of the irides did not have to be deleted from the image after being reduced to one pixel width. A study on the regrowth of NA nerve terminals in mouse irides after chemical lesioning with the selective NA neurotoxin 6-OH- dopamine (6-OH-DA) showed that the nerve terminal density measured with the image analysis procedure agreed very well with the NA neuron membrane area estimated by selective ^{3}H-NA uptake (Figure 3). There were only minor differences (<10% of control) between the nerve terminal density measured directly or after skeletonization ("Thread Density" in Figure 3). One important finding was that the endogenous levels of NA (measured by liquid chromatography with electrochemical detection) recovered considerably slower than the nerve terminal density (see Figure 3), showing that low levels of transmitter does not affect the ability of the procedure to properly detect the terminal network.

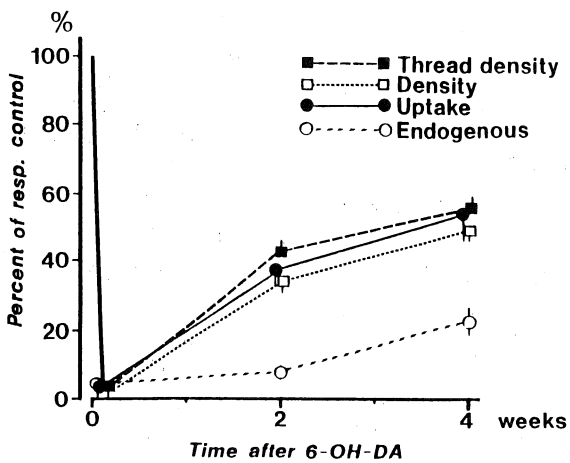

Figure 3. The effect of sympathectomy with 6-OH-DA on mouse irides. Density = nerve density measured by image analysis. Thread density = nerve density after reduction of the image elements to one pixel width. Uptake = neuronal uptake of ^{3}H-NA. Endogenous = NA content of whole iris, measured by liquid chromatography with electrochemical detection.

Central Nervous System

The regeneration of 5-hydroxytryptamine (5-HT) containing neurons in anatomically well defined regions of the central nervous system (CNS) after neonatal lesions with the selective neurotoxin 5,7-HT was also studied using IA. Indirect immunohistochemistry was used to visualize the 5-HT terminals. The possible regrowth stimulating effect of the monosialoganglioside GM1 was investigated. In piriform cortex and the posterior basal amygdaloid nucleus there were no effects on the nerve terminal density. In the frontal and occipital cortices, hippocampus, and the anterior horn of the spinal cord, the nerve terminal density was reduced by 66%-84%. The endogenous 5-HT levels

in these regions were determined and the correlation between these levels and the nerve terminal density values was high (r=0.996). The effects of GM1 treatment was a two- to threefold increase of the terminal density in the latter four regions, compared to the 5,7-HT treated control.

The substance P (SP) containing nerve terminals in the superficial layers of the dorsal horn was investigated after neonatal capsaicin treatment, alone and followed by GM1. The SP was visualized by indirect immunohistochemistry. Capsaicin, the pungent factor of red pepper, causes degeneration of primary sensory neurons involved in mediation of chemogenic pain, among these are SP neurons terminating in the dorsal horn. The superficial layers of the dorsal horn was defined as the reference area in the area % measurements. The dorsomedial part of the lateral funiculus (adjacent to the dorsal horn), which also contains SP terminals, was investigated separately. It was found that two months after the capsaicin treatment the SP terminal density in the dorsal horn was reduced by 54% of control, while the animals that had recieved GM1 after the capsaicin treatment had a reduction of the SP terminal density of only 18% (Figure 4). In the lateral funiculus there was no discernible effect of GM1 on the regrowth.

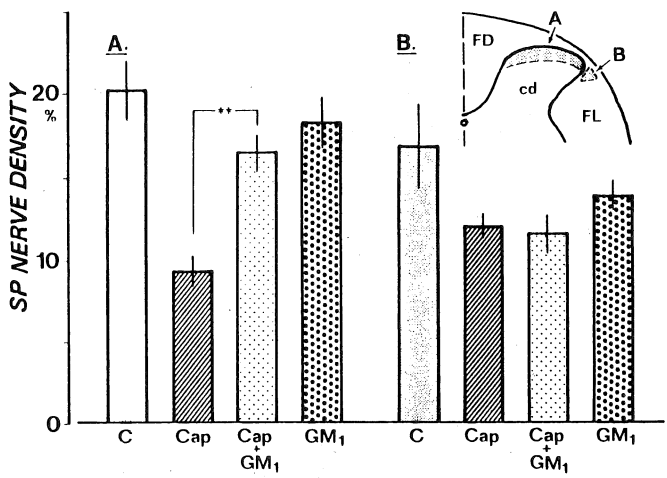

Figure 4. Effect of GM1 on the capsaicin (Cap) induced reduction of SP nerve terminal density in the lumbar spinal cord of rats, two months after neonatal Cap treatment (50 mg/kg s.c.). Starting 1 h after Cap, GM1 (20 mg/kg) was injected daily for 12 days. A (superficial layers of the dorsal horn) and B (dorsomedial part of the lateral funiculus) in the inset indicates the location of the terminal fields investigated. Each bar represents the mean ± s.e.m. of 4-6 determinations. FD = funiculus dorsalis; FL = funiculus lateralis; cd = dorsal horn. ** = $0.001 < p < 0.01$ (Students t-test).

DISCUSSION

These results indicate that in homogenously innervated and easily dissected tissues like the iris, where there are no alternative uptake mechanisms, IA and ^{3}H-NA uptake are equally useful methods. In the CNS, however,

there are often ambiguous results using the transmitter uptake paradigm, due to uptake into other neurons or glia. The interpretation of results is often further complicated by the imprecision of the microdissection procedures. There are also many neuronal systems that do not have a selective uptake mechanism and where there still are no biochemical assays sensitive enough to allow measurements of transmitter levels.

From the two examples of applications in the CNS it is clear that the preserved morphology allowing the exact determination of the region to be investigated is of great importance in this type of experiments. The available biochemical evidence confirms the reliability of the method. It should be noted, however, that the most reliable results will be those that are expressed as relative to an adequate control (cf. Einarsson et al., 1975). It is also important to find a grey level segmentation procedure that can compensate for the uneven distribution of the excitation light caused by the dark field condenser, if the selected magnification does not allow the use of epi-illumination. Image analysis is thus a sensitive, reliable, and very useful instrument in the analysis of the density of transmitter identified axon terminals, especially in sparsely and/or inhomogenously innervated regions.

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