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IMAGE ANALYSIS AND THE DETERMINATION OF CODISTRIBUTION AND COEXISTENCE OF NEUROACTIVE SUBSTANCES IN NERVE TERMINAL POPULATIONS

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ABSTRACT

The section method of determining coexistence in nerve terminal populations using automatic image analysis and the occlusion principle is described. By means of the dilatation function of the IBAS image analyzer an index has been developed for the description of the density and/or eveness of nerve terminal networks. Furthermore, by means of a logical operation "and" codistribution of densely packed nerve terminals can be determined based on the field area of the overlap image automatically determined in the image analysis. This procedure has been used in the analysis of codistribution of transmitter identified nerve terminals in the median eminence.

Key-words: Codistribution, coexistence, image analysis, nerve terminals.

INTRODUCTION

Two quantitative methods for the evaluation of the entity of coexistence of neuroactive substances in nerve terminal populations have been introduced using the occlusion priciple of Agnati et al. (1982). The methods are the synaptosomal method and the section method (Fuxe et al. 1985). In this paper we will also describe an index for evaluation of the density and/or eveness of nerve terminal distribution based on automatic image analysis procedures.

The synaptosomal method to quantitate coexistence

This method is based on studies on suspensions of purified synaptosomal fractions, which are fixed in 2 % formaldehyde dissolved in phosphate buffered saline. The staining procedure is in this case the indirect FITC immunofluorescence method. Evaluation of coexistence in the synaptosomal method is made on smears of the synaptosomal preparation. A systematic sampling was performed on the immunoreactive profiles. By a TV camera highly sensitive to fluorescence light, the fluorescence image could be directly transfered into the image memory of the IBAS image analyzer. The number of immunoreactive profiles within the sampled image could be directly obtained by means of the measuring programs in the IBAS image analyzer.

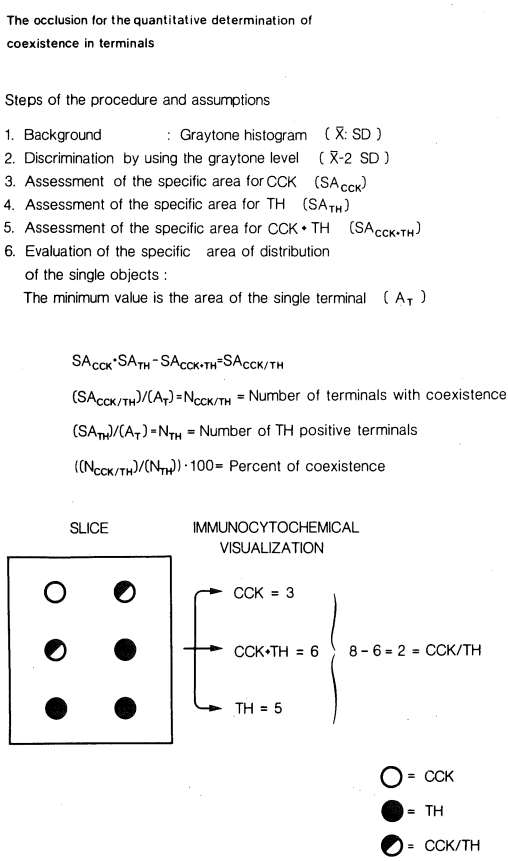

Fig. 1. Schematic illustration of the procedures used to determine the coexistence in nerve terminal networks present in sections by means of the occlusion method. SA= the size of the specific area evaluated by means of the IBAS image analyzer. The specific area has been obtained for TH, CCK and TH+CCK-like immunoreactivity. A_T = area of the single terminal obtained by means of the histogram of the single objects present in the image. The principles of the occlusion method is also indicated in the lower part of the figure.

The section method to quantitate coexistence in nerve terminals

The indirect immunoperoxidase procedure has been used to demonstrate the A and B immunoreactive profiles. The principle procedure is shown in Fig. 1. Thus, the occlusion principle for the determination of coexistence is used. This method is based on analysis of three adjacent sections stained in a random way with antisera against A and B and with both antisera together. All evaluations of coexistence could then be calculated by subtracting from the sum of the number of immunoreactive nerve terminals

demonstrated with the antiserum A alone and antiserum B alone, the number of immunoreactive nerve terminals demonstrated with the combined use of antisera A and B. Thus, coexisting terminals can be demonstrated both with antiserum A alone and antiserum B alone as well as with the combined use of the two antisera. This method is used on a statistical basis and it therefore becomes possible to determine the entity of coexistence in the nerve terminal populations.

As seen in Fig. 1 it is possible to perform the section method by means of automatic image analysis using the IBAS image analyzer. As seen the background can be defined by initiating the program for the grey tone histogram. All sections can then be discriminated in the same way by always using the mean grey tone level of the background minus two standard deviations. The same areas are analyzed in three thin adjacent sections and in this example the specific area (field area; SA) of tyrosine hydroxylase (TH) immunoreactivity alone, cholecystokinin (CCK) immunoreactivity alone and the immunoreactive area obtained when staining with both antisera together could be determined by the use of the identification and measuring programs. The area of the single terminals was determined by analyzing the histogram of the diameter of the various single objects forming the specific field area in the regions analyzed in the respective sections. The minimum value in the histogram was assumed to represent the diameter of the single terminal (1.5 μm). The number of coexisting terminals could then be obtained by dividing the area (field area, SA) of TH and CCK immunoreactivity with that value of the area of the immunoreactive objects which is based on the value of the minimum diameter (see above). The per cent of coexistence can be obtained by expressing the number of terminals in which coexistence is present in per cent of the number of e.g. TH immunoreactive terminals. It is also possible to determine the entity of coexistence in per cent simply by taking the ratio (field area A + field area B - field area AB) divided by field area A multiplied by 100. Recently it has also been possible to show that similar results or better results on coexistence can be obtained by an interactive discrimination procedure, by which the discriminated image is made similar to the original image of the transmitter-identified profiles. The results of a quantitative analysis of PNMT/NPY coexistence in the dorsal motor nucleus of the vagus of the rat medulla oblongata is shown in Table 1.

Table 1

STUDIES ON COEXISTENCE IN THE NPY AND PNMT IMMUNOREACTIVE NERVE TERMINALS IN THE MEDIAL SUBNUCLEUS OF NTS AND IN THE DORSAL MOTOR NUCLEUS OF THE VAGUS USING THE OCCLUSION PRINCIPLE

WKY rats (16 weeks old). Formula for coexistence (%) $\frac{FA(NPY) + FA(PNMT) - FA(NPY \& PNMT)}{FA(NPY)} \times 100$

	NPY terminals		PNMT terminals		NPY & PNMT terminals		Coexistence (see formula above)
	Mean Area $\bar{A}$ μ^2	Field Area FA(NPY) μ^2	Mean Area $\bar{A}$ μ^2	Field Area FA(PNMT) μ^2	Mean Area $\bar{A}$ μ^2	Field Area FA(NPY+PNMT) μ^2	%
Animal group							
WKY	4.40 ±0.07	2160 ±399	4.52 ±0.03	1220 ±239	4.44 ±0.01	1660 ±429	82 ±5

Means ± s.e.m. are shown above.

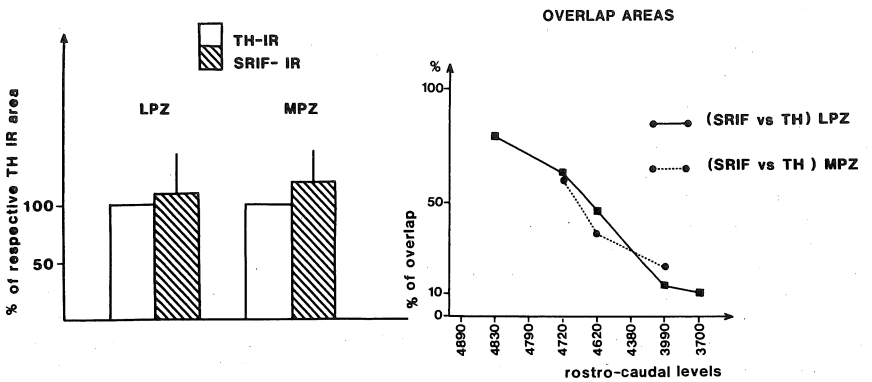

Fig. 2. Morphometrical analysis of codistribution of TH and somatostatin immunoreactive areas in the MPZ and the LPZ in the median eminence of the male rat by means of automatic image analysis using an IBAS-II image analyzer. The analysis has been performed on adjacent 30 µm thick coronal vibratome sections as illustrated in figs. 3-5 at various rostrocaudal levels of the hypothalamus. The analysis has been performed on binary images, which exclusively demonstrate the specific immunoreactivities within the external layer of the median eminence. An interactive discrimination procedure was used. By means of a logic function, which can perform an "and" operation, the binary images of the TH and SRIF like immunoreactive areas in the MPZ and the LPZ have been compared and the overlap region determined. In the figure the per cent of overlap is shown at the various rostrocaudal levels expressed in per cent of the respective TH immunoreactive region. To the left the overall mean SRIF immunoreactive areas are shown in % of the respective TH immunoreactive area. Means +/- SEM.

Development of an index to describe density and/or evenness of nerve terminal networks

By means of the dilatation function using the octagonal shape in the automatic image analysis an evaluation of the eveness and/or density of the nerve terminal distribution of A and B immunoreactive profiles can be obtained. The dilatation procedure can be repeated many times and at each step the size of the protein is increased by 1 pixel at the boundary. The higher the degree of eveness of the terminal distribution the higher will be the ratio of the dilated field area and the original field area (e.g. FA_A^D/FA_A). Thus, when dilated areas overlap the increase of the dilated field area will be reduced. When a substantial overlap exists after dilatation, immunoreactive profiles are clustered and thus an uneven distribution is present.

Determination of codistribution of densely packed terminals by automatic image analysis

Automatic image analysis has also been found very useful in the analysis of codistribution of A and B immunoreactive nerve terminal networks which are densely packed. Densely packed networks of transmitter-identified nerve terminals are often found in the external layer of

the median eminence controlling neuroendocrine function. This area has therefore often been analyzed with regard to codistribution of transmitter-identified nerve terminals such as tyrosine hydroxylase and luteinizing hormone releasing hormone (LHRH) immunoreactive nerve terminal networks (Calza et al. 1983) and somatostatin (SRIF) and TH immunoreactive nerve terminals (Fuxe et al., 1985a,b) (Fig. 2). Image analysis has been performed on thin adjacent coronal sections from the mediobasal hypothalamus, using the indirect immunoperoxidase procedure to demonstrate e.g. the TH and LHRH immunoreactive areas of aggregates of terminals. The analysis was performed on binary images following the discrimination procedures described above. By means of identification and measuring programs the shape factor and area of TH and LHRH immunoreactivity could be determined in the external layer of the median eminence. Thereafter, the two images showing TH and LHRH immunoreactivity were compared with one another by means of the "Boolop" function which automatically determines and visualizes the overlap area of the TH and LHRH immunoreactive nerve terminal populations. In Fig. 2 it is shown that in the case of TH and SRIF immunoreactive terminals a codistribution exists predominantly in the rostral and middle parts of the rat median eminence.

In this way it was possible to determine the per cent degree of codistribution by calculating the ratio of the field area for the overlap image ($FA_{TH+LHRH}$) and the mean field area of the TH and LHRH immunoreactive aggregates of terminals ($FA_{TH+}FA_{LHRH}/2$). By this type of procedure it has been possible to demonstrate the existence of aggregates of rostrocaudal strips of chemically well defined nerve terminal systems in the median eminence. The view on the neuroanatomical organization of the median eminence must therefore be revised giving a new understanding of the regulation of neuroendocrine function. Finally, it must be emphasized that all the present methods are designed to be used when performing relative quantitative comparisons with a control mean value. Thus, the sampling regime is biased in favour of detecting larger objects and the disector must be used in order to obtain an unbiased estimation of number and sizes of arbitrary particles (Sterio 1984).

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