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STEREOLOGICAL STUDIES OF TMB-REACTED TERMINALS AFTER AXONAL TRANSPORT OF WGA-HRP - A POSSIBILITY OF CHARACTERIZING DIFFERENT BOUTON POPULATIONS

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ABSTRACT

Anterograde transport of lectin-conjugated horseradish peroxidase followed by incubation with tetramethylbenzidine was employed to label the external afferent fibers and their terminals in several somatosensory and reticular nuclei of the cat. Bouton profiles containing the typical needle-shaped reaction products were analyzed stereologically and matched against randomly chosen unlabelled boutons. In the thalamus the external axon terminals from the lateral cervical nucleus, the dorsal column nuclei and the spinal cord were very similar and in comparison with the unlabelled boutons were very large, with loosely packed large synaptic vesicles and a high mitochondrial content. In the lateral reticular nucleus and the lateral cervical nucleus, where the labelled spinal terminals also formed characteristic populations, the boutons from the lumbar spinal cord were larger than those from the cervical cord. The relative number of labelled terminals was calculated in the different regions and found to be generally larger than had previously been assessed from degeneration experiments.

Key-words: Bouton, LCN, LRN, thalamus, tracing, ultrastructure.

INTRODUCTION

Orthograde transport of native or wheat germ conjugated horseradish peroxidase (HRP and WGA-HRP, respectively) followed by reaction with tetramethylbenzidine (TMB) is a suitable technique (Sakumoto et al. 1980) for studying the fine structure of selected bouton populations, provided the tissue processing is performed at a pH not below 6.0. Often the typical reaction products only cover a small area, while the rest of the terminals appear unchanged by the histochemical processing. This gives a good opportunity to make quantitative studies of synaptic vesicles, mitochondria and other features of the labelled bouton.

In the present study this method was applied to the boutons from ascending fibers to several somatosensory and reticular relay nuclei, such as the afferents from the lateral cervical nucleus (LCN) and the dorsal column nuclei (DCN) to the ventrobasal thalamus (VB), and the spinal afferents to the thalamic central lateral nucleus (CL), the lateral reticular nucleus (LRN) and LCN.

EXPERIMENTAL PROCEDURE

The tracer, which consisted of WGA-HRP (15-25%), was delivered into different parts of the central nervous system of adult cats by pressure injections through micropipettes with an outer tip diameter of about 15 µm. The VB investigation comprised six animals - three with LCN and two with DCN injections; for the CL, LRN and LCN studies one, five and five animals, respectively, were used, with injections into the cervical or lumbar enlargements. After a survival time of 2 (VB study), 3 (LRN, LCN, and CL studies, cervical injections) or 5 days (LRN and LCN studies, lumbar injections), the animals were perfused transcardially with 2 liters of Tyrode's solution (pH 7.4, 38°C) followed by 4 liters of 2% glutaraldehyde and 1% formaldehyde in 0.1 M phosphate buffer (pH 7.4, 38°C). The perfusion was terminated with 2 liters of the phosphate buffer at 4°C. The parts of the brain or spinal cord containing the VB, CL, LRN or LCN were cut transversely with a Vibratome into sections 50-70 µm thick. Following a short rinse in 0.1 M citric acid-ammonium acetate buffer (pH 6.0), the sections were incubated at room temperature with TMB at pH 6.0 and after osmification they were flat-embedded in Epon between acetate foils and polymerized for 48 h at 60°C. After light microscopic examination they were attached to Epon blocks and labelled areas were trimmed for ultrathin sectioning. The ultrathin sections were collected on one-hole grids. Every second grid was stained with uranyl acetate and lead citrate, and the remaining grids were analyzed uncontrasted in the electron microscope. The injected part of the brain and spinal cord was cut transversely into 40-µm-thick frozen sections, which were incubated and examined for the greatest longitudinal and transverse spread of the extracellular label.

Labelled bouton profiles from each of the investigated regions were analyzed quantitatively. In VB 270 labelled terminals (150 after LCN injections and 120 after DCN injections), in CL 27, in LRN 60 and in LCN 293 terminals were studied. The terminals were photographed at a magnification of 7 000-14 000. For a comparative analysis one unlabelled bouton profile was randomly selected from each of the micrographs. For each selected profile the total number of synaptic vesicles was counted on a projection screen at a magnification of 36 000 to 72 000 x and with the use of a scale lupe (giving an additional magnification of 10 x) the longest and the shortest diameter of five randomly selected vesicles were measured. The area of the bouton profiles and of peroxidase-positive inclusions and mitochondria within each bouton were measured by means of a graphic pen-data tablet device (MOP, Kontron). Reaction products were excluded from the bouton area when the vesicle and mitochondrial densities were calculated.

In all regions except CL and LRN after lumbar injections, the volume densities of labelled and unlabelled boutons were calculated, using the point-counting principle. Randomly taken micrographs from vibratome sections showing labelled structures in the light microscope were analyzed in a stereological device at a magnification of 22 500 x. The point-sampling screen had 168 points, 20 mm apart and evenly distributed in a hexagonal pattern.

RESULTS

In VB the external axon terminals from LCN and DCN, were very similar and differed from the unlabelled terminals (Table 1). Compared with the unlabelled terminals, the LCN and DCN boutons were much larger, and had larger, loosely packed synaptic vesicles and a higher mitochondrial

Table 1. Differences between labelled and unlabelled thalamic bouton profiles (mean values).

Injected area		LCN	LCN	LCN	DCN	DCN	Spinal cord
				Animal no.			
		1	2	3	4	5	6
Size (μm^2)	labelled	3.0^{**}	2.8^{**}	2.9^{**}	3.1^{**}	3.0^{**}	3.6^{***}
	unlabelled	0.5	0.5	0.6	0.6	0.3	0.6
Vesicle density ($/\mu m^2$)	labelled	55^{**}	49^{**}	54^{**}	29^{**}	31^{**}	52^{***}
	unlabelled	127	115	123	81	113	137
Diameter of vesicles (nm)	labelled	54^{**}	54^{**}	55^{**}	52^{**}	55^{**}	52^{***}
	unlabelled	45	47	44	48	48	45
Volume density of mitochondria (%)	labelled	11^{**}	13^{**}	14^{**}	15^{**}	14^{**}	13
	unlabelled	8	6	4	7	4	11

LCN = lateral cervical nucleus, DCN = dorsal column nuclei
LRN = lateral reticular nucleus, VB = ventrobasal thalamus

* indicates that the values differed significantly, with $p<0.05$, using a paired-sample t test
** with $p<0.01$
*** with $p<0.001$.
The differences between sizes of labelled and unlabelled bouton profiles were still significant when the bouton size was reduced by the area of reaction products.

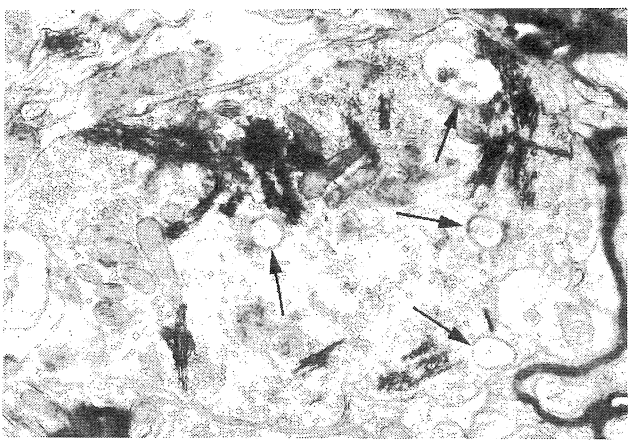

Fig. 1. 20 000X. Large labelled bouton from the spinothalamic tract. The aggregates of needleformed reaction products are easily recognizable. The bouton is in synaptic contact with several thin dendritic profiles presumably spines (arrows).

Table 2. Differences between labelled and unlabelled bouton profiles in the LRN and the LCN (mean values).

	LRN		LCN	
	Labelled	Unlabelled	Labelled	Unlabelled
Size (μm)	2.5	1.1**	2.2	1.1**
Volume density of mitochondria (%)	10.6	15.0*	11.4	13.0
Vesicle density (no./μm^2)	54	94***	36	67**

Abbreviations and *, **, *** see Table 1.

Table 3. Differences in mean profile size between boutons labelled from the cervical and lumbar spinal cord.

	Injected part of the spinal cord	
Investigated region	Cervical	Lumbar
LRN	2.47	3.41*
LCN	1.97	2.64

Abbreviations and ** see Table 1.

Table 4. Volume densities and relative number of boutons (mean values).

Type of terminal	Volume densities			Relative number (%)
	Labelled terminals	Unlabelled terminals	Relative labelled/ all terminals (%)	
LCN terminals in VB	0.6	27.3	2.2	0.9
DCN "	3.6	14.8	19.6	7.4
Spinal terminals in LRN (cervical injections)	1.4	6.9	16.9	11.2
Spinal terminals in LCN (cervical injections)	5.9	10.2	36.6	27.3
Spinal terminals in LCN (lumbar injections)	5.3	10.8	32.9	21.2

Abbreviations see Table 1.

content. Also in the other thalamic nucleus, CL, the labelled spinal afferent boutons (Fig. 1) displayed the same characteristics with regard to size, mitochondrial volume and the size and number of synaptic vesicles. In the other two nuclei, LRN in the medulla oblongata and LCN in the upper part of the spinal cord, the external spinal afferents also appeared larger and showed fewer synaptic vesicles (Table 2) than the unlabelled terminals. No other significant differences were found between labelled and unlabelled terminals in these regions. On comparison between labelled spinal boutons from the cervical and lumbar cord, the former were found to be smaller than the latter (Table 3). The relative numbers of labelled boutons in the different regions were estimated by a two-step calculation. First the volume fraction of peroxidase-labelled terminals in relation to the total volume fraction of boutons was calculated (Table 4). The relative number could then be estimated, assuming that the boutons were approximately spherical and dividing the relative volume density of labelled boutons by $\sqrt{B/b}$, where B is the mean area of the labelled bouton profiles and b that of the unlabelled ones (cf. Tables 1 and 2).

DISCUSSION

Tracing with WGA-HRP followed by TMB-incubation seems to provide a material which is well suited for stereological studies of the fine structure of different extrinsic bouton populations. Although further investigations are required to determine whether the terminals are altered outside the space where the reaction products are deposited, it is quite clear that the changes are less pronounced than after orthograde degeneration. Compared with autoradiography the present technique seems technically less complicated and much faster. As the label is situated within the structure of interest, no statistical consideration is needed to decide whether the structure is marked or not.

In the thalamus the terminals investigated belong to the three most important somatosensory systems in higher animals, the spino-cervico--thalamic tract, the dorsal column-medial lemniscus system and the spino-thalamic tract. As demonstrated, the boutons of the three systems are closely similar with respect to size and density, size of synaptic vesicles and volume density of mitochondria. This similarity, which was also evident in the formation of the postsynaptic contacts, is in accordance with the organization in other specific relay nuclei of the thalamus, such as the lateral and medial geniculate nuclei (Jones and Powell 1969, Morest 1971).

Somewhat unexpected in the present study was the finding in LRN and LCN of a difference in size between boutons from neurones in the cervical and lumbar spinal cord. When the animal variation was taken in account this difference was found to be statistically significant in the LRN ($P<0.05$) but not in the LCN. Although the figures must be considered with great caution (the lumbar LRN material is based on only 10 boutons from one animal), they might indicate that nerve cells with longer axons have larger terminals.

The relative number of labelled boutons found in this study was several times greater than the number of boutons calculated from degeneration studies in LRN (Mizuno et al. 1975) and LCN (Westman 1969). When, however, the number of labelled boutons is compared with the number obtained by assessing quantitatively the number of boutons disappearing after a spinal deafferentation (Griph and Westman 1977), the accordance is much better. This is also the case in VB, where estimations of the number of boutons have been based on comparative studies of normal and degenerating terminals (Ralston 1969, Ralston and Herman 1969). There are

several possible explanations for the larger number of labelled than of degenerating boutons. One is the process of degeneration, which might proceed at different rates in different boutons, which would mean that some terminals would already be indistinguishable as boutons when others are showing the first indubitable signs of degeneration. Another is the obvious shrinkage of dark degenerating boutons which will diminish the probability of getting a profile of the degenerating structure in the ultrathin section. In both cases, however, the presently employed method gives a truer estimation of the relative number of boutons belonging to a particular population of external afferents.

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