Microscopy techniques and the study of synapses

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Microscope techniques have been largely underestimated as powerful tools for the study of cell function and with some exceptions mostly restricted to descriptive studies. The development of new techniques and methods as well as the development of more powerful image analysis software in the last decade finally has provided the conditions to use microscopy in much more extensive and powerful ways. The development of new imaging software has also resuscitated the interest in "old" techniques such as Golgi staining methods that have suddenly come back to the spot light due to the possibility to be combined with image analysis tools. Microscopy is no longer the way to only get a "pretty picture" to illustrate our papers, but also a very powerful tool to study brain function. We propose here different strategies and methodologies that can be applied to the study of synaptic development and function using fluorescence, brightfield and electron microscopy. The use of these techniques allows the study of parameters such as the expression of synaptic proteins, number and morphology of dendritic spines, and number, type and structure of synapses.

Keywords: ultrastructure; immunofluorescence; brightfield microscopy; Golgi staining; stereology; spines; tree shrew, human postmortem.

1. Introduction

Normal synaptic connections, number and efficacy are crucial for proper brain function. The development of microscopy techniques or strategies for the quantification of synapses, or of dendritic spines (which receive the majority of synapses) is important for the study of brain function in normal development, adulthood and in diseases of the brain. In the present chapter we will describe methods and strategies for the study of synaptic connections that can be used in a variety of species. The methods that will be presented in the following pages can be used not only in well perfused animal brain samples, but can also be successfully applied to well preserved post-mortem human brain tissue. In the particular case of post-mortem human samples, a crucial factor is the post-mortem interval before preservation that especially can affect the quality of the tissue for the performance of ultrastructural studies. Regardless of the species, the study of synapses can be challenging because of their intrinsic complexity and plasticity which requires multiple strategies for the study of different aspects of the dynamics and functionality of these structures. When studying synaptic development or synaptic pathology several questions need always to be addressed: Which proteins are expressed in the synapse? Are there changes in the expression of these proteins? Are there changes in synaptic connections, morphology or number? Many of these questions can be answered using a combination of microscopic techniques as we will present in the following paragraphs.

To illustrate the usefulness of the methodology proposed we have selected a series of examples based on studies performed in our laboratory. We have intentionally chosen examples based on studies in different species to demonstrate the versatility of the methods described here.

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2. Using fluorescence and brighfield microscopy for the study of synapses

2.1 Study of the expression of synaptic proteins: immunofluorescence techniques.

In this section we present practical examples of the use of fluorescence techniques for the detection of synaptic molecules. The cases presented are part of our study of the expression of synaptophysin (a presynaptic protein) during development (figures 1A-C) and in the adult (figures 1D-F) mammalian brain. In both cases double immunofluorescence was used to study the expression of synaptophysin in the different compartments of the striatum. The mammalian striatum presents two anatomically distinct compartments: the patch and the matrix which differ from each other in several ways including neurochemical composition, neuronal organization, developmental schedule and connectivity [1-8].

Tissue preservation: Rats were perfused using a saline solution followed by a cold solution of 4% paraformaldehyde in phosphate buffer 0.1 M (PB). Tree shrew brains prepared in the same manner were kindly provided by Dr. Thomas Norton (UAB). Brains were then postfixed in the same fixative for a minimum of 24 hours. After that the tissue was cryoprotected in a solution of 30% sucrose in PB and frozen using dry ice. Free floating 30 μm coronal sections were obtained using a cryostat and processed using standard immunofluorescence techniques.

Immunofluorescence and optical density values have been previously used for the study of synaptic proteins [9, 10]. Using appropriate image analysis software, mean intensity values for the area of interest are obtained and converted to optical density values using the following formula:

OPTICAL DENSITY= - log [(intensity-background / maximum reflection – background]

-Intensity: in a fluorescent sample the intensity value obtained using image analysis software is a measurement of the emission of light of a given wavelength by the fluorophore present in the sample.

-Background: in a fluorescent sample the background is the residual fluorescence produced by autofluorescence of the tissue or other components of the preparation.

-Maximum reflection or maximum reflected light is the intensity value that will be obtained by measuring a fluorophore capable of emitting the maximum of reflected light at a given wavelength. In a practical case the maximum reflected light will be measured in a standardized slide containing the same fluorophore than our samples and capable of producing high and (as much as possible) homogeneous intensity values.

Analysis of optical density is a useful tool for the study of changes in synaptic protein expression during development or due to a pathological state. The accuracy of this methodology has been very recently improved by the introduction of antibodies coupled with fluorophores that can be excited to emit in near infrared wavelengths (700-800 nm) that produce images with lower autofluorescence [11, 12].

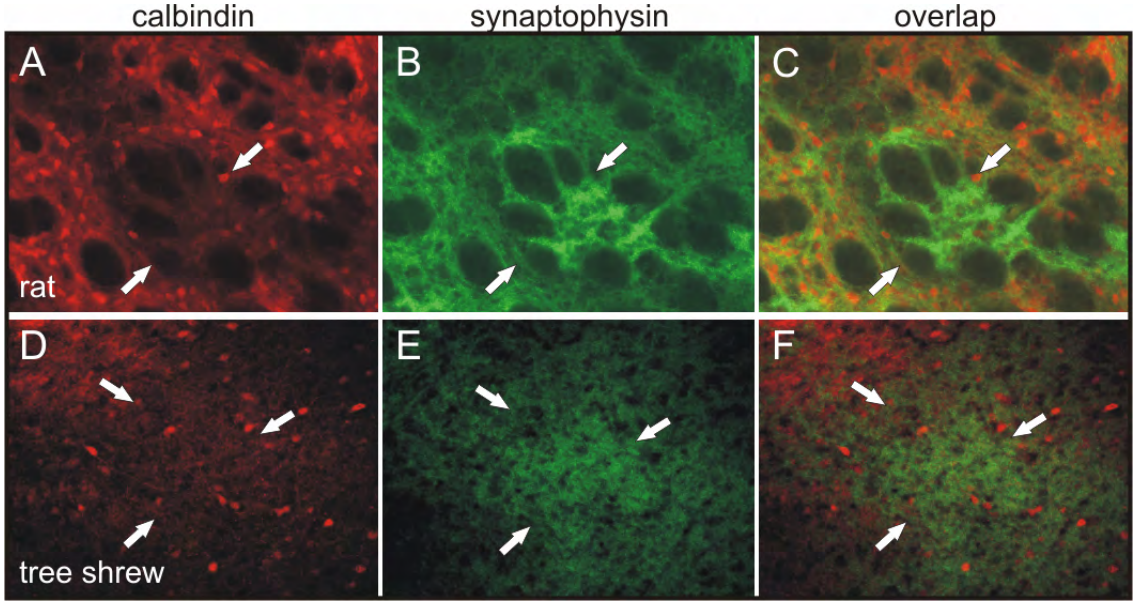

Figure 1: A-B) Double immunofluorescence for calbindin (A) and synaptophysin (B) at postnatal day 7 of the rat striatum. D-E) Double immunofluorescence for calbindin (D) and synaptophysin (E) in the striatum (caudate nucleus) of an adult tree shrew (Tupaia glis). Images C and F are overlaps of the calbindin and synaptophysin stainings in the rat and tree shrew respectively. Calbindin is used as a marker to discern between two distinct structures of the striatum denominated the patch and matrix compartments. White arrows in all the images indicate the patch compartment that is largely devoid of calbindin staining (A, D), and that in both cases presents a high expression of the presynaptic protein synaptophysin (note the punctate appearance of the staining in B, E) corresponding to the presence of synaptophysin in synaptic terminals. Magnification in all figures is 20X

2.2 Study of dendritic spine number and morphology: Golgi techniques and brightfield microscopy.

In spite of having been introduced more than 100 years ago by Camillo Golgi, Golgi staining is still a very useful and widely use technique that allows to reveal the fine structure of neuronal processes, both in the normal and in the pathological nervous system. Several variations of the original Golgi procedure have been developed and used in brain tissue preserved in different manners, obtaining variable results [13-15]. One of this variations is named Golgi-Cox [15, 16], and is based on the elimination of the silver nitrate of the original procedure, and the addition of mercury chloride to the chromation solution [17]. The Golgi-Cox staining procedure can be applied to brain tissue from experimental animals and human brain samples including long-term stored human tissue [18]. Examples of the use of this technique in rat and human brain tissue are shown below (figure 2). Small variations of this technique can be used to produce reliable and reproducible staining of neurons (figure 2A) and neuronal processes such as dendrites and dendritic spines (figures 2A-B).

The ability to obtain clear images of dendritic spines allows the use of this technique for morphological and quantitative studies of these cellular structures that are one of the major players in synaptic transmission. Using appropriate image analysis software, parameters such as number of dendritic spines, size and morphology can be easily analyzed, and are very useful for the study of normal synaptic development as well as pathological conditions.

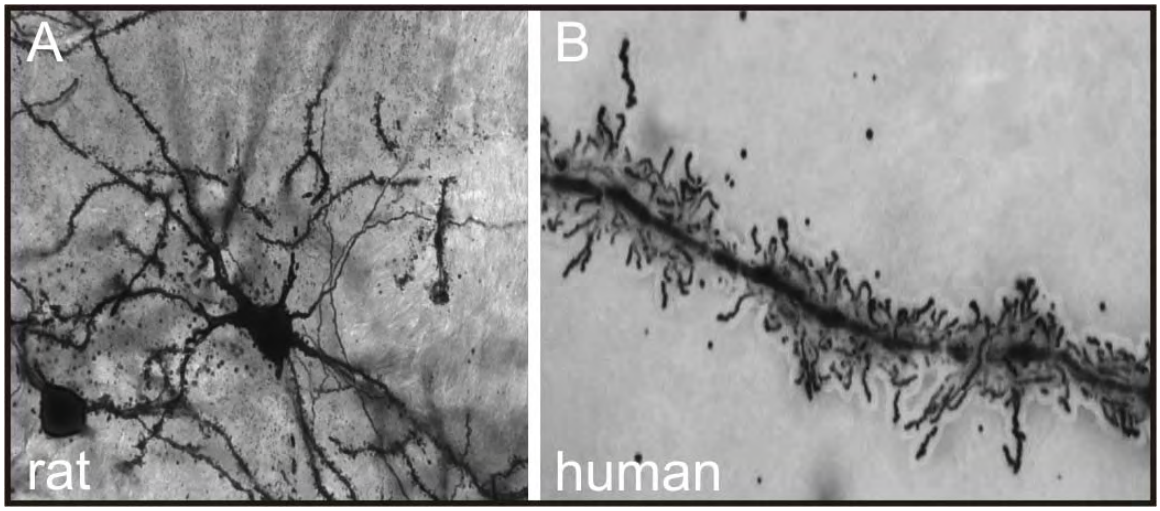

Figure 2: Golgi-Cox staining of a medium spiny neuron of the adult rat striatum (**A**) and detail of the dendritic spines of a dendrite of a pyramidal cell in the human prefrontal cortex (**B**). Both images illustrate how the combination of brightfield microscopy and Golgi staining is an useful tool to study morphology and number of dendritic spines. Magnification is 20X in A and 100X in B.

3. Ultrastructural study of the synapses: stereologycal techniques

3.1 Tissue preparation for electron microscopy

Electron microscopy techniques require a careful preservation of the tissue to avoid artefacts that can alter the ultrastructure of the tissue. For animal tissue samples the use of perfusion techniques is recommended for a better preservation of the tissue. In the case of human samples it is recommended that the post-mortem interval does not exceed 8 hours from time of death to fixation and in addition the tissue should be re-dissected in smaller pieces (1 cm thick is recommended) to improve penetrability of the fixative. Appropriate fixatives for electron microscopy studies generally require a combination of paraformaldehyde and glutaraldehyde in buffered solutions. The preferred fixative in our laboratory is a cold solution of 4% paraformaldehyde and 1% glutaraldehyde in filtered 0.1 M PB (for more specific methodology for tissue preservation see [19, 20]).

Tissue appropriately preserved is normally sliced in a vibratome obtaining sections of 40µm that can be immediately embedded for electron microscopy or can be processed for immunocytochemistry prior to embedding (figure 3A). In the example presented here, vibratome sections of the human striatum were immunostained with an antibody against calbindin to discern between the patch and matrix compartments (figure 3A). After immunocytochemistry sections were flat embedded for electron microscopy (for embedding techniques see [19, 20]). Once the sections are embedded they can be viewed with the light microscope and regions of interest can be selected, cut out and mounted on embedding capsules (figure 3A).

3.2 Electron microscopy stereological analysis of the synapses

For stereological studies the strategy for ultramicrotome sectioning requires obtaining serial ultrathin (90 nm thick) sections that will be photographed with the electron microscope (for details see figure 3B). To perform synaptic counts the method used is based on the dissector stereological technique [21].

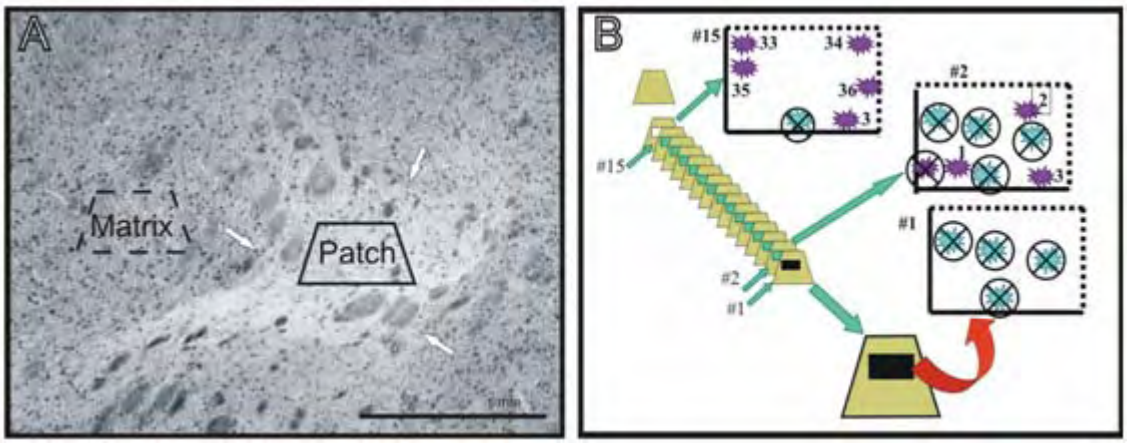

Figure 3: Sample selection for electron microscopy studies of synapses. **A)** Brightfield microscope image of a calbindin-immunostained vibratomed section through the human striatum. Calbindin is used as a marker of the approximate boundaries between patch (almost devoid of calbindin) and matrix (immunoreactive for calbindin) striatal compartments. The trapezoids depicted in this image represent the area selected for the stereological analysis as illustrated in B. White arrows indicate the approximate boundaries of a patch compartment . **B)** Schematic representation of the application of stereologycal methods for the study of synapses at the electron microscope level: a ribbon of 15 serial ultrathin sections (taken from the patch or the matrix, see figure A), represented by trapezoids, is shown. The black rectangle shown in section #1 represents the area that will be photographed for analysis (this area corresponds with a photomontage of 3 micrographs). The same region is photographed in the rest of the ribbon (green rectangles in trapezoids). For synaptic counts, those synapses that appear in the first section (turquoise profiles with an X drawn through them) are not counted but are followed throughout the ribbon to avoid counting them later on by mistake. Synapses appearing in sections #2 to #15 (purple profiles) that do not cross exclusion lines (represented in the figure as solid black lines) are labeled and identified by symmetry (symmetric or asymmetric synapses) and postsynaptic target (spine, dendrite, or axon). Figure 3A is modified from figure 2 of reference [19].

Briefly, one area within the trapezoid of the first section of a ribbon of 10 to 15 sections is selected at random and photographed at 10000X (3 to 6 photomicrographs are taken to form a photomontage with an area large enough for the study of synapses). Then, photomicrographs of the exact same area are taken from the rest of the sections of the ribbon (see figure 3B for details). The criteria for identifying a synapse are parallel pre-and post-synaptic membranes with a discernable synaptic cleft, a postsynaptic density, and synaptic vesicles at the synapse in the presynaptic terminal (see as examples figures 4A and B). All synapses combined and the various subtypes of synapses, such as asymmetric axospinous, asymmetric axodendritic, symmetric axospinous and symmetric axodendritic can be thus identified and counted.

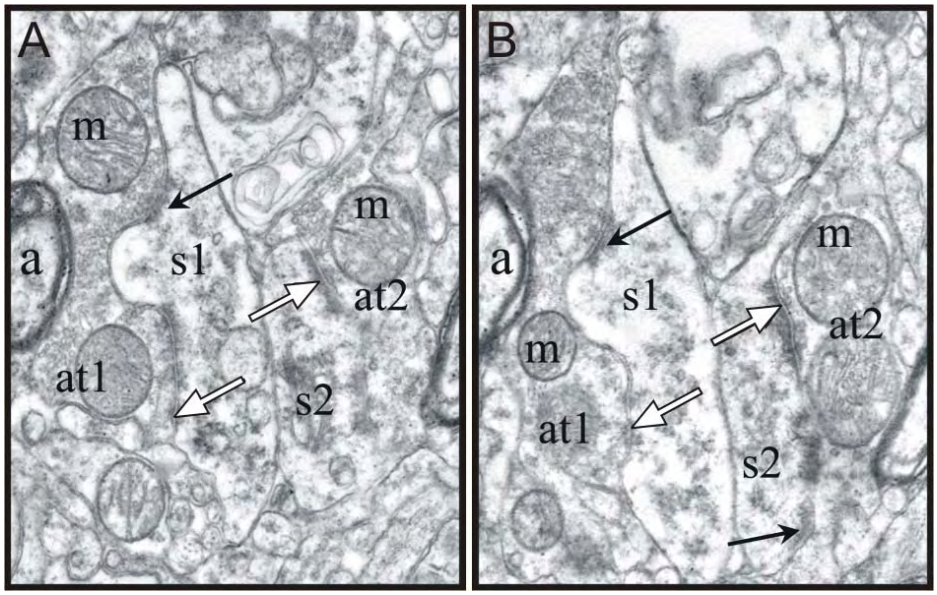

Figure 4: A-B) Study of synapses at the electron microscope: Images are taken of the same synapses from 2 ultrathin sections (270nm apart) in a ribbon of serial sections through the human striatum (caudate nucleus) using the method described in figure 3B. These micrographs show that the ultrastructural preservation is adequate to determine synapse morphology. Two spines (s1 and s2) receive asymmetric axospinous synapses (white outlined arrows) from different axon terminals (at1 and at2). Black arrows indicate active zones in each synapse. (a): myelinated axon; (m): mitochondria. Magnification in both figures is 10000X. Modified from figure 1 of reference [20]

Stereological analysis of synapses at the electron microscope provides valuable information about the number of synapses as well as accurate classification of the subtypes of synapses. This is a very powerful tool for the study of normal synaptic development, adult synaptic distribution and for the study of synapses in the diseased brain.

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References

[1] Graybiel AM., Ragsdale Jr. CW (1978) Histochemically distinct compartments in the striatum of human, monkey and cat demonstrated by acetylcholinesterase staining. Proc Natl Acad Sci USA **75**: 5723-5726.

[2] Holt DJ., Graybiel AM., Saper CB (1997) Neurochemical architecture of the human striatum. J Comp Neurol. **34**: 1-25.

[3] Prensa L., Gimenez-Amaya JM., Parent A (1999) Chemical heterogeneity of the striosomal compartment in the human striatum. J Comp Neurol. **413**: 603-618.

[4] Kubota Y., Kawaguchi Y (1993) Spatial distributions of chemically identified intrinsic neurons in relation of patch and matrix compartments of rat neostriatum. J Comp Neurol **332**: 499-513.

[5] Van der Kooy D., Fishell G (1987) Neuronal birthdate underlies the development of striatal compartments. Brain Res. **401**: 155-161.

[6] Liu FC., Graybiel AM (1992) Heterogeneous development of calbindin D-28K expression in the striatal matrix. J Comp Neurol **320**: 304-322.

[7] Gerfen CR., Baimbridge KG, Miller J (1985) The neostriatal mosaic: compartmental distribution of calcium-binding protein and parvalbumin in the basal ganglia of the rat and monkey. Proc Natl Acad Sci USA **82**: 8780-8784.

[8] Ragsdale Jr CW. Graybiel AM (1991) Compartmental organization of the thalamo-striatal connection in the cat. J Comp Neurol **311**: 134-167.
[9] Mossberg K., Arvidsson U., Ulfhake B (1990) Computerized quantification of immunofluorescence-labeled axon terminals and analysis of co-localization of neurochemicals in axon terminals with a confocal scanning laser microscope. J Histochem Cytochem **38 (2)**: 179-790.
[10] Sytnyk V., Leshchynsk I., Nikonenko AG., Schachner M (2006) NCAM promotes assembly and activity-dependent remodelling of the postsynaptic signalling complex. JCB **174 (7)**: 1071-1085.
[11] Hawes JJ., Bruncell DH., Wynick D., Zachariou V., Picciotto MR (2005) Gal-R1 but not Gal-R2 or Gal-R3 levels are regulated by galanin signalling in the locus coeruleus through a cyclic AMP-dependent mechanism. J Neurochem **93**: 1168-1176.
[12] Noorbakhsh F., Vergnolle N., McArthur JC., Silva C., Vodjgani M., Andrade-Gordon P., Ollenbergh MD., Power C (2005) Proteinase-activated receptor-2 induction by neuroinflammation prevents neuronal death during HIV infection. J Immunol **174 (11)**: 7320-7329.
[13] Ramon y Cajal S., de Castro F (1933) Elementos de tecnica micrografica del sistema nervioso. Salvat (Eds). Barcelona (Spain). Reprinted in 1972 by "Tipografia Artistica" Madrid (Spain).
[14] Kopsch F (1896) Erfahrungen uber die Verwendung des Formaldehyds bei der Chromsilber-Impragnation. Anat Anz **11**: 727.
[15] Cox W (1891) Impragnation des centralen Nervensystems mit Quecksilbersalzen. Arch Mikr Anat **37**: 16-21.
[16] Ramon-Moliner E (1970) The Golgi-Cox technique, In: Nauta WJH., Ebbesson SOE. (Eds.) "Contemporary research methods in neuroanatomy". pp: 106-131. Springer, NY.
[17] Glaser EM., van der Loos H (1981) Analysis of thick brain sections by obverse-reverse computer microscopy: application of a new, high clarity Golgi-Nissl stain. J Neurosci Methods **4**: 117-125.
[18] Melendez-Ferro M., Perez-Costas E. (2006) Golgi staining of long-term stored human brain tissue. FENS FORUM. FENS Abstr. Vol **3**, A059.20.
[19] Roberts RC., Knickman JK. (2002) Ultrastructural organization of the patch matrix compartments in the human striatum. J Comp Neurol **452**: 128-138.
[20] Roberts RC., Roche JK., Conley RR (2005) Synaptic differences in the postmortem striatum of subjects with schizophrenia: a stereological ultrastructural analysis. Synapse **56**: 185-197.
[21] Geinisman Y., Gundersen HJ., van der Zee E., West MJ (1996) Unbiased stereological estimation of the total number of synapses in a brain region. J. Neurocytol. **25 (12)**: 805-819.