Histol Histopathol (2002) 17: 799-803

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Location of Zinc and ^{65}Zn in spinal ganglia of the rat

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Summary. Following the works of Velazquez et al. (1999), Jo-Seung et al. (2000), Wang et al. (2001), Danscher et al. (2001) and the criteria of Zinc-containing neurons established by Frederickson et al.(2000), we have found the presence and localisation of Zinc in the neurons of the dorsal root ganglia of Wistar rat, by using Timm's thecnique and by studying the autoradiographic uptake of ^{65}Zn.

The agreement between the results of both techniques allows us to classify these spinal ganglion neurons as Zinc-containing neurons and also, to confirm some of the results of Velazquez et al. (1999)

Key words: Spinal ganglia, Zinc, ^{65}Zn, Histochemistry, Autoradiography

Introduction

The presence of Zinc in the CNS has been known for a long time, thanks to the studies of Maske (1955), Fleischauer and Hortsman (1957), Timm (1958a,b), Durbin et al. (1957), Millar et al. (1961) and V.Euler (1962), among many others.

Already in the 80's of the last century, Frederickson, Howell, Kasarskis and their collaborators, determined the inter-relations of Zinc in the nervous tissue. The monograph entitled The neurobiology of Zinc (1984) is a masterpiece that summarised the bases of metal-C.N.S. relations.

On the other hand, Danscher and his collaborators (1984, 1994, 1996, 1997), improved the histochemical methods for the detection of the metal, introducing the method of Zinc selenide, which confirms the results obtained with the intravital technique of ditizon and with Timm`s sulphide method.

In this period, Dreosti (1984) relates the function of Zinc in the CNS to glutamate activity. Later, Pérez Clausell and Danscher (1985, 1986), located Zinc in the synaptic vesicles of the telencephalic buttons of the rat.

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Recently, Frederickson and his collaborators (1989, 2000) have defined the term: Zinc-containing neurons, while Danscher and his collaborators (Jo-Seung et al. 2000) have defined the term Zinc-enriched terminals.

In this way, it has been possible to define a family of neurons with their connection pathways in the CNS., found in a more generalised way than was thought at first and which were defined as a subgroup of the glutaminergic neurons.

In using these criteria Velazquez et al. (1999) give a sensorial significance to the Zinc-containing neurons, due to their location in the dorsal horn of the spinal cord, shown by the retrograde transport of Zinc-selenite and the expression of MT-III.

Jo-Seung et al. (2000), Wang et al. (2001), Danscher et al. (2001) confirm the presence of Zinc-enriched terminals by means of immunochemistry and autometallography in the spinal cord. It was mainly found in sheets I, III & IV, being moderately positive in sheets V & VI and scarcely positive in sheet II.

We have always proposed the study of Zinc location with the compared use of Timm`s histochemical technique and ^{65}Zn autoradiographic uptake (Vera-Gil 1974, Vera-Gil and Pérez-Castejón 1994). From this point of view, based on the evidence of the presence of Zinc in the spinal cord, we decided to study Zinc location and ^{65}Zn uptake in the ganglia of the dorsal root of the spinal cord.

Materials and methods

Twenty-four young adult Wistar rats weighing an average of 250 gr were used. They were divided into 2 groups.

The first group, consisting of 12 animals, was used to carry out the histochemical technique for location of heavy metals described by Timm (1958a,b) but following de protocol of the Bargmann's school. The animals were submitted to thiopental anaesthesia and they were perfused through the heart with sulphidric alcohol. After this fixation, samples were extracted, paraffin embedded and sectioned at a thickness of 6 μm. Developing was carried out in a laboratory with an average temperature of 21 °C. and soft illumination. Developing was interrupted when reactive changed into

a silver reflectance and control inspection (samples of pancreas, small intestine and Ammon horn) showed the expected reaction in the tissues. Then, a normal process of washing in hot water was done. Eventually, the slides were counterstained with hematoxylin-eosin (HE).

The second group, also consisting of 12 animals, was injected intraperitoneally with 0.5 ml of a saline solution of $^{65}ZnCl2$, named ZAS (supplied by Nuclear Ibérica), with a calibrated activity of 835 microCuries. The animals were killed on the 6^{Th} postinjection day. Samples of spinal ganglia from cervical and thoracic regions were used for autoradiography at conventional histological level. Immediate fixation was done in a Carnoy solution, embedding in paraffin and sectioning at 6 micron thick. An autoradiographic method was carried out by dipping in Ilford K5 emulsion, 50% dissolved in double distilled water. Exposure was in black plastic boxes with a drying system, inside a refrigerator at 4 °C for 21 days. Kodak D19 was used as developer and Hypam as fixer.

Controls of both histochemical and autoradiographical techniques were made. Regarding the latter, fading and blackening controls were carried out in order to discard chemography. The background index was also controlled.

Results

Timm's histochemical technique has shown a high perikarya positivity in all the neuron populations of the spinal ganglia, as it is shown in a general view in Fig. 1a,b. Scarce histochemical positivity signals could be seen in the fibres of the segmentary root.

Higher magnifications showed a high histochemical positivity in the perikarya of the ganglia neurons, this fact allows us to relate the location of the metal with the rough endoplasmic reticulum (RER) structure (Fig. 2a).

^{65}Zn autoradiography, showed a well-defined uptake of the radionuclide with a high distribution in the perikarya of the ganglia neurons (Fig. 1b,c). There was also a lower uptake in certain nuclear elements, a fact already known in ^{65}Zn relations, and certain images suggesting the uptake in fibres of the dorsal root (Fig. 2c).

We believe that the use of overdose of ^{65}Zn and the particular characteristics of radionuclide emission has been the cause that the unavoidable background of these autoradiographs was higher than expected.

Discussion

The agreement of results of the two techniques, histochemical positivity and ^{65}Zn autoradiographic uptake, are a good evidence of the location of Zinc in the perikarya of the neurons of the spinal ganglia. Not only the location of histochemical positivity but also the autoradiographic uptake, seem to relate the metal to the RER of these neurons, although the need to be sure leads us to point out the idea of a further confirmation by EM.

What seems to be doubtless is the reliability of Timm`s technique for detecting Zinc because of the continuous agreement of the results and those of the other techniques, more specific and modern, such as the ones developed by Danscher and his research team.

Without Danscher's studies, we would have always doubted about what bivalent, heavy metal with electric balance on its more exterior layer, reacts with the sulphide radical, making it able to be developed with silver. About Timm's technique, we can learn from Danscher (1984b) that: there is "*an in situ transformation of metal cations (either electrovalently bound or free ions) to their respective sulphides*". Danscher adds about the comparison between Dithizone, Timm and Selenium techniques that: "*In conclusion, the three methods demonstrate the same metal in the neuropil and the choice of technique should be based on criteria other than selectively for a specific metal*".

Up to this point, it would be interesting to take into account Haug's comments about the staining of the neuronal perikarya and, to also remember his warnings about how easy it is to lose this staining during the fixation and embedding. To avoid this risk, we were always very watchful while Timm's process was carried out.

On the other hand, it is obvious that ^{65}Zn autoradiographic uptake detects Zinc. It is true that always in a lower amount than with histochemical techniques, which detects all the present Zinc. ^{65}Zn autoradiographic uptake only detects the radionuclide, which has been incorporated into the body. This, obviously, is always a part of and never the total present Zinc.

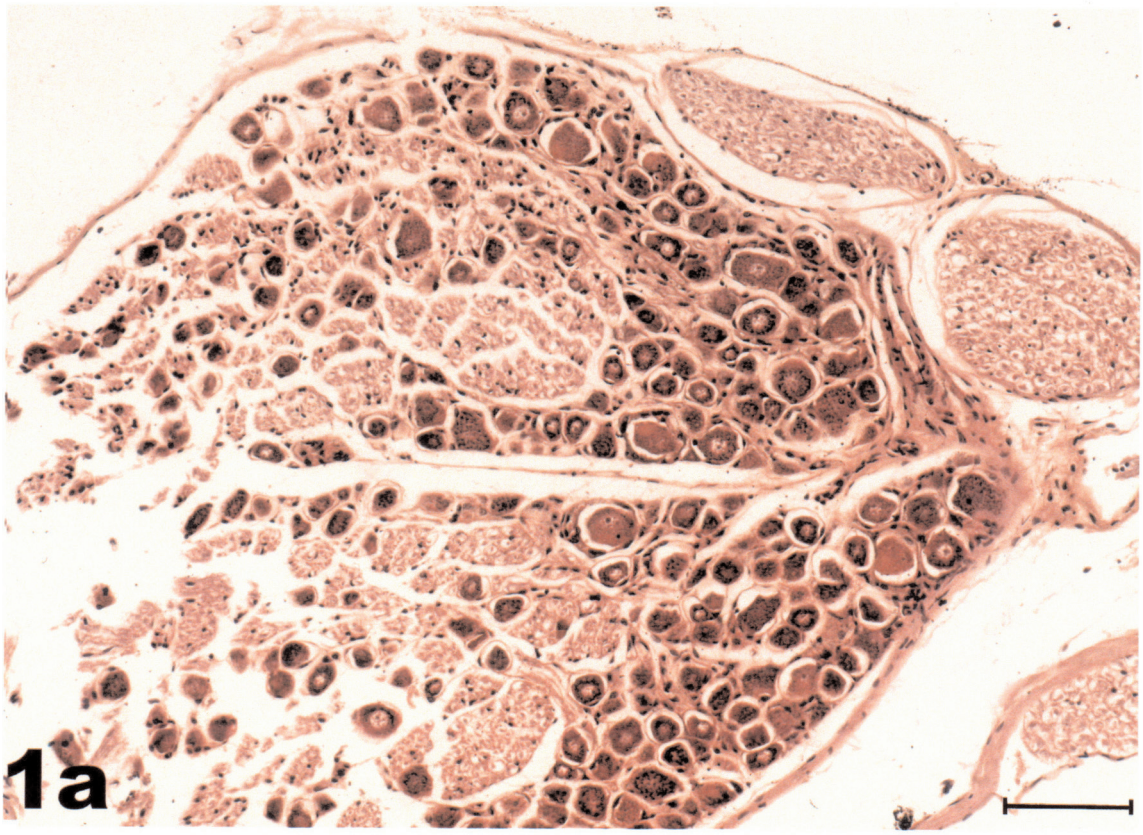
About the suitability of ^{65}Zn for this kind of experience, we call to the reference of Kasarskis (1984) and Crawford and Harris (1984).

With regards to the technical particularities of the radionuclide emission and its consequences in the autoradiographs, we call to our reference (1994) in which this subject was deeply studied.

It may now be added that, using an overdose of ^{65}Zn, using autoradiographs at histological conventional level, with 6 microns of tissue thickness and using a correct emulsion layer to these circumstances, it is obvious that we will obtain more background than desirable and it is possible that sometimes, very located light artefacts may occur and may affect the quality of the preparation. This will have to be taken into account when autoradiographs are analysed

However autoradiographers are used to this and they know that the autoradiographic technique in some circumstances of concentration and with some type of radionuclide emission, is not a clean technique. Furthermore, if it were clean, we would suspect the incidence of negative chemography. Nevertheless, our autoradiographs are within acceptable limits of background.

After this, we can confirm that spinal ganglia neurons are Zinc-containing neurons after the definition



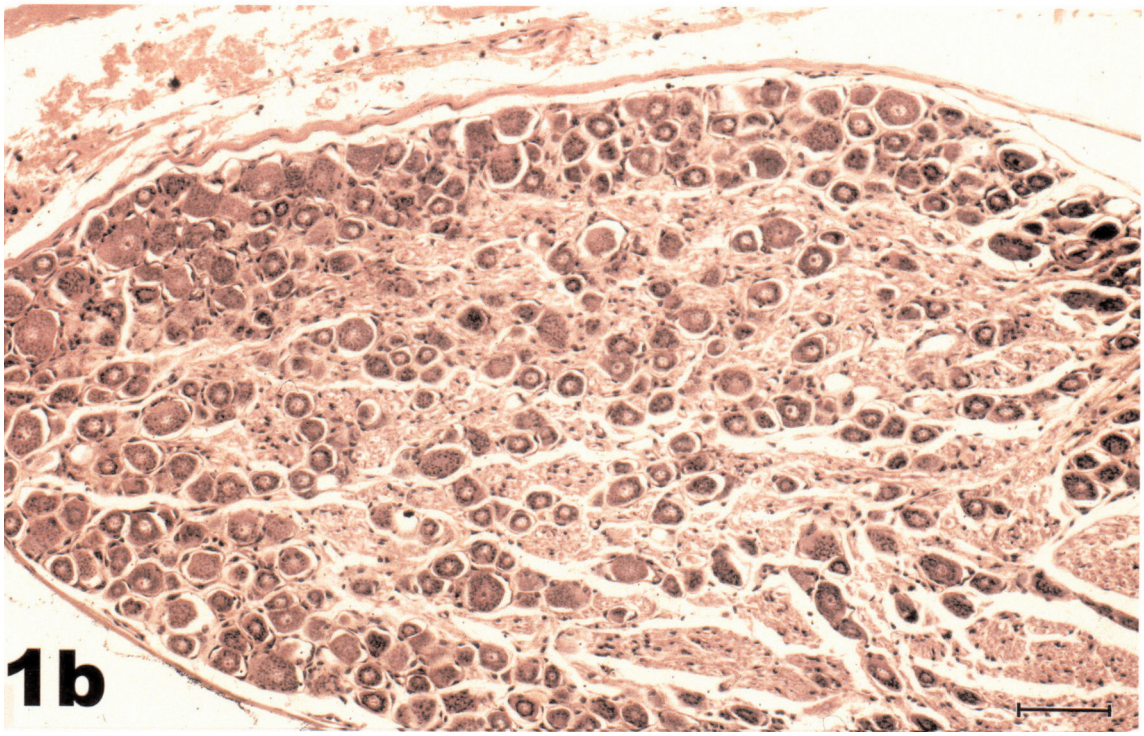
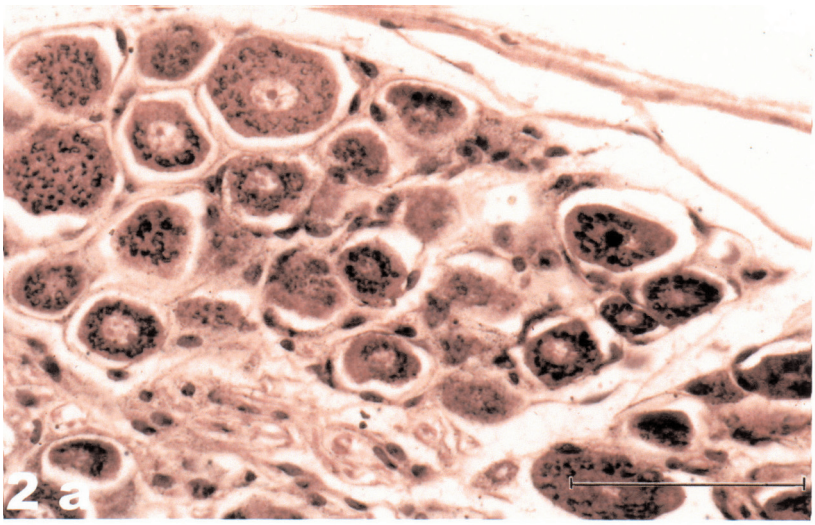
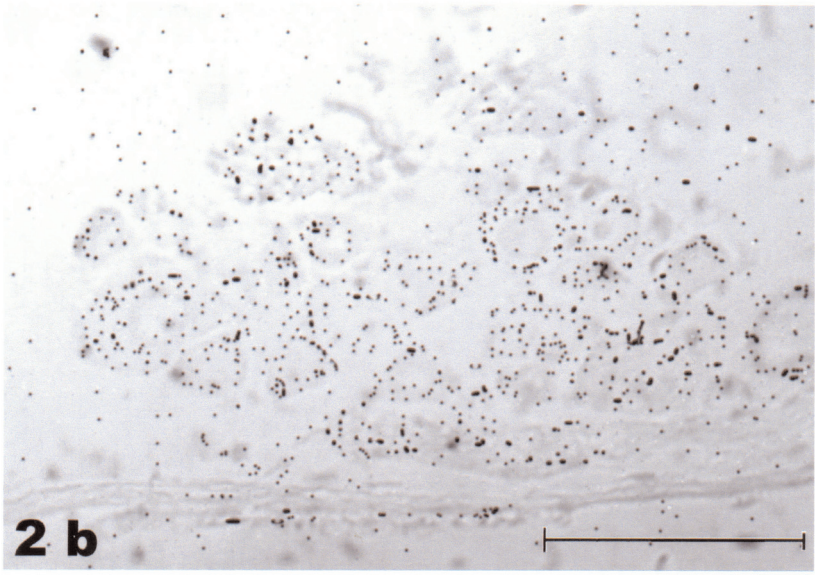


Fig. 1a,b. Timm's technique counterstained with hematoxylin-eosin. (HE). General view of spinal ganglia, showing a high positivity in the neuron perikarya. To a lesser degree, there are also some positivity signals in the nerve fibres of the spinal root. Scale bar: 0.1 μm.





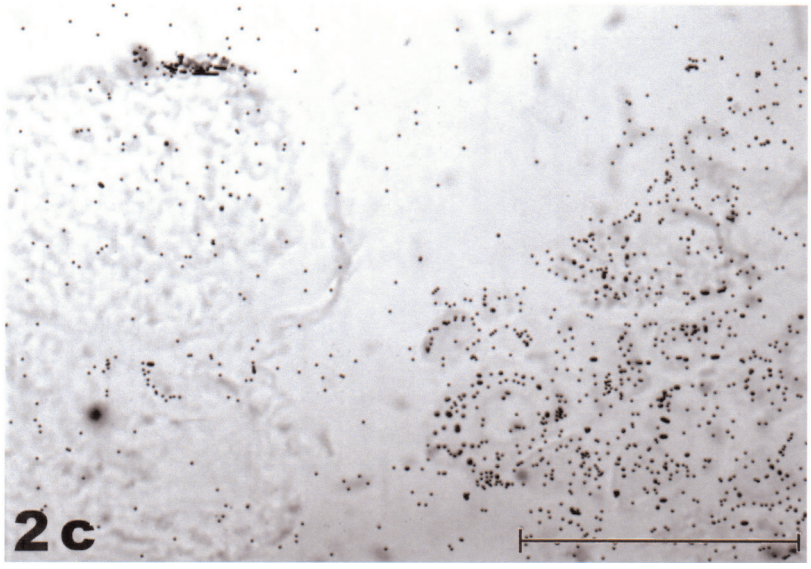


Fig. 2. a. Timm's technique counterstained with H.E. Higher magnification shows the histochemical positivity in the neuron perikarya. Scale bar 0.1 μm. **b and c**. ^{65}Zn autoradiography, showing a high uptake in the same location that is shown by the histochemical technique, including certain traces of fibrillar uptake (**c**). Scale bar 0.1 μm.

established by Frederickson et al.(1989, 2000). It also seems clear that metal location that has been shown in the present work is fundamentally in relation to the perikarya of these neurons, without discarding its presence in the dorsal root fibres.

This is compatible with the zinc enriched terminals described by, Jo-Seung et al. (2000), Wang et al. (2001) and Danscher et al. (2001) in spinal cord, because one of the origins of those terminals could be ganglia neurons.

Anyway, this could confirm some of the results and functional valuations of Velazquez et al. (1999)

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Accepted April 4, 2002