

⁶⁵Zn uptake in the rat cerebellum and brainstem

A. Vera-Gil, M.C. Pérez-Castejón, M. Lahoz, J. Aisa, M.P. Recreo, P. Serrano and N. Pes

Department of Human Anatomy and Histology, Faculty of Medicine, University of Zaragoza, Zaragoza, Spain

Summary. We have studied the autoradiographic uptake of ⁶⁵Zn in the cerebellum and brainstem of the rat, contrasting these results with Timm's positivity in these structures.

Both, autoradiographic uptake and histochemical positivity, have demonstrated Zinc in a location that could be accepted as in climbing fibres and glomeruli of the cerebellum cortex, and also in brainstem neurons that project their axons to the cerebellum cortex, suggesting a circuit where zinc may act as a neuromodulator.

Key words: Brainstem, Cerebellum, ⁶⁵Zn, Zinc, Autoradiography, Histochemistry

Introduction

The presence of zinc in the rat cerebellum and the uptake of ⁶⁵Zn in this area has been known since the 1960s (Euler 1962; Fujii 1967; Hassler and Soremark, 1968).

Later, in the eighties, analytical studies on the determination of this metal showed that the cerebellum presents with the second highest amount of Zinc in the Central Nervous System (CNS) (Kishi et al., 1982; Crawford and Harris, 1984; Dreosti, 1984; Dvergsten, 1984; Janghorbani and Young, 1984; Farkas et al., 1988).

More recently, studies from Amano and Enomoto (1998) and Takeda et al. (1995, 1999, 2000 a-c) established the basis on ⁶⁵Zn uptake in the CNS, and improved our knowledge of ⁶⁵Zn uptake in the cerebellum.

Our group studied the location of ⁶⁵Zn in the cerebellum by means of autoradiography (Vera-Gil et al., 1990; Vera-Gil and Pérez Castejón, 1994) and suggested the relationship between this metal and the system of climbing fibres.

In the present study, we have related our autoradiographic studies on ⁶⁵Zn uptake to studies

performed by means of Timm's technique to investigate the meaning of the presence of zinc in the cerebellum and brainstem.

Materials and Methods

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

The first group, consisting of 12 animals, was injected intraperitoneally with 0.5 ml of a saline solution of ⁶⁵ZnCl₂, named Z.A.S. (supplied by Nuclear Ibérica) with a calibrated activity of 500 microCuries.

The animals were sacrificed on the 6th postinjection day. Samples of the cerebellum and brainstem were used for autoradiography at conventional histological level. Immediate fixation was in a Carnoy solution and they were embedded in paraffin and sectioned at a thickness of 6 microns. The autoradiographic method was carried out by dipping in 50% Ilford K5 emulsion, 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.

The second group, also consisting of 12 animals, was used to carry out the histochemical technique for location of heavy metals described by Timm (1958a,b) but following the protocol of the Bargmann school. The animals were subjected to thiopental anaesthesia and they were perfused through the heart with sulphidric alcohol. After this fixation, samples of the cerebellum and brainstem were extracted, paraffin embedded and sectioned at a thickness of 6 microns. Developing was carried out in a laboratory with an average temperature of 21 °C and soft illumination. Developing was interrupted when the reaction changed into a silver reflectance and control inspection (samples of pancreas, small intestine and Ammon horn) showed the expected reaction in those tissues. Then the normal process of washing in hot water was performed. Eventually, the slides were counterstained with hematoxylin-eosin.

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

was also controlled.

Results

The autoradiographic uptake of ⁶⁵Zn in the cerebellum cortex presented with a three level distribution (Fig. 1a). In the granular layer, there were evident impacts in the granular cells as well as in the neuropilum (Fig. 2b).

In the layer of the Purkinje cells, impacts defined the silhouette of the soma of these cells (Fig. 1a, b) and displayed through the molecular layer, where they spread together with the dendritic prolongation of these cells (Fig. 1a).

The images in the cerebellum cortex obtained by means of Timm's technique contrasted with hematoxylin-eosin corroborated the autoradiographic description. At this level, the detail of the histochemical positivity in the granular layer (Fig. 2a) could be observed with the described double distribution in the granular cells and in the neuropilum. With regard to the neuropilum, images suggesting the outline of some mossy terminals were seen.

The histochemical positivity fully agreed with the autoradiographic uptake in the Purkinje cell layer, but showed more intensity, as usual (Fig. 1c).

The role of zinc in the CNS has long been related to the excitatory activity of Glutamate. Frederickson (1989) and Frederickson et al. (2000) defined the term ZEN (zinc enriched neurons) to describe a subpopulation of Glutamatergic neurons distributed all along the CNS and containing zinc. From the studies of Pérez-Clausel and Danscher (1985, 1986) and Danscher et al. (1997, 2001) we have learned the synaptic role of Zinc in what they called ZET (zinc enriched terminals). Following this hypothesis on the location of ⁶⁵Zn in the climbing fibres (Vera-Gil et al., 1990), we analysed the uptake and localisation in the lower olive of the brainstem, and observed histochemical positivity in its perikaryon, as it can be observed in Fig. 3a,b. This fact was also demonstrated by the autoradiographic uptake in some neurons in the same brainstem location (Fig. 3c). In addition, we considered additional evidence for the presence of fibrillar tracts in the surroundings of the restiforme body, which were clearly marked by ⁶⁵Zn. (Fig. 3d)

Discussion

We should like to start by discussing the suitability of our techniques. We selected the techniques used from a wide range of techniques that can be used to study the presence and location of zinc, because they are well proven, exact and resolute enough to obtain results in conventional histology at the level of optic microscopy. In addition, they are backed by five decades of research, which has permitted the improvement of the techniques, learning to differentiate between the adequate result and the artifact. This is the reason why they work perfectly if

they are properly applied. In fact, more recent and sophisticated techniques have confirmed the results. At the same time, we chose these techniques because our group has thirty years experience with them without error.

With regard to the choice of our autoradiographic methodology, we followed the criteria of Kristov (1970) on the biological half-life of the radionuclid. We used a single intraperitoneal dose, and we also took into account the criteria of Takeda et al. (1995) as well as our own experiences (Vera-Gil et al., 1990; Vera-Gil and Perez Castejón, 1994).

Iford K5 emulsion is ideal for the type of emission of the radioisotope and for high resolution in optic microscopy. In addition, the distance from the source to the impact is not significant at this degree of magnification.

We realise that in the histochemical field, the techniques of silver selenide and autometalography, which were widely used by Danscher (1996) may provide excellent results, especially by means of electron microscopy. However, by means of optic microscopy, a well performed Timm technique with the modifications of the Bargman school is very useful in expert hands, as Haugh (1984) and Danscher (1984) established and as we have demonstrated in several tissues apart from the nervous tissue (Pérez Castejón et al., 1991; Vera-Gil et al., 1991). But always backed by ⁶⁵Zn uptake studies, in order to resolve the unspecificity of Timm's technique.

With regard to our results, firstly we would like to underline the evidence which associates zinc with a part of the system of climbing fibres. Both, the autoradiographic uptake and the histochemical positivity, detected some neurons whose cell bodies were located in the lower olive of the brainstem and whose fibres spread through the restiforme body as far as the periphery of the Purkinje neurons and their dendritic tree.

This is the classical system of excitatory cerebellous afference, which has always been considered as being mediated by aspartate. However, not all the fibres of this system have been demonstrated to be zinc positive. Our autoradiographic study shows relatively selective positive areas, especially in the brainstem and in the restiforme body, although they are more generalised in the cerebellum cortex. In addition, the results by means of Timm's method agree with this.

Now, we must consider that while Timm's staining could react with all free zinc ions or electrovalently-bound ones (Danscher, 1984), only a part of the total zinc present in the structures (just the radioactive one) is detected by the autoradiographic technique, but both, free and electrovalently bound ions ⁶⁵Zn. This is the reason that we may follow the pathway of ⁶⁵Zn and in this way explain our findings.

Is the positivity around the Purkinje cells due to something else, apart from the neurons and tracts identified and some climbing fibres? The answer is beyond the aim of this study, and should be addressed by means of electron microscopy.

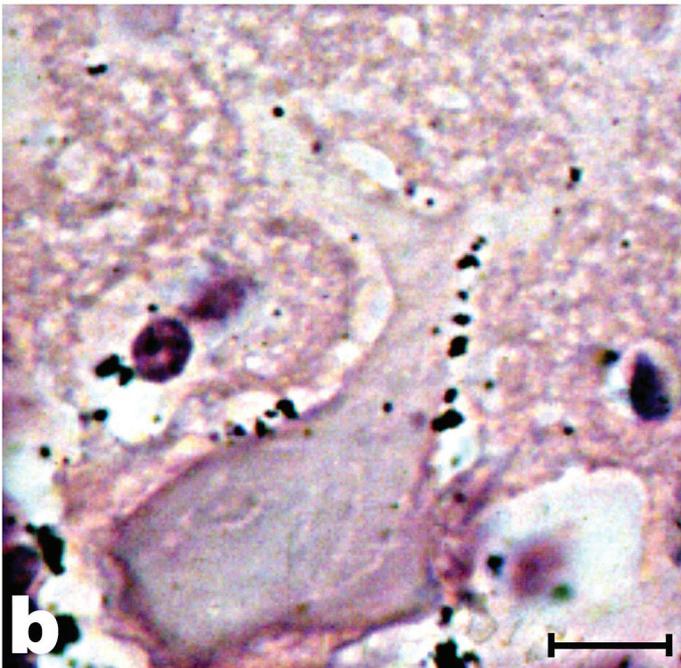
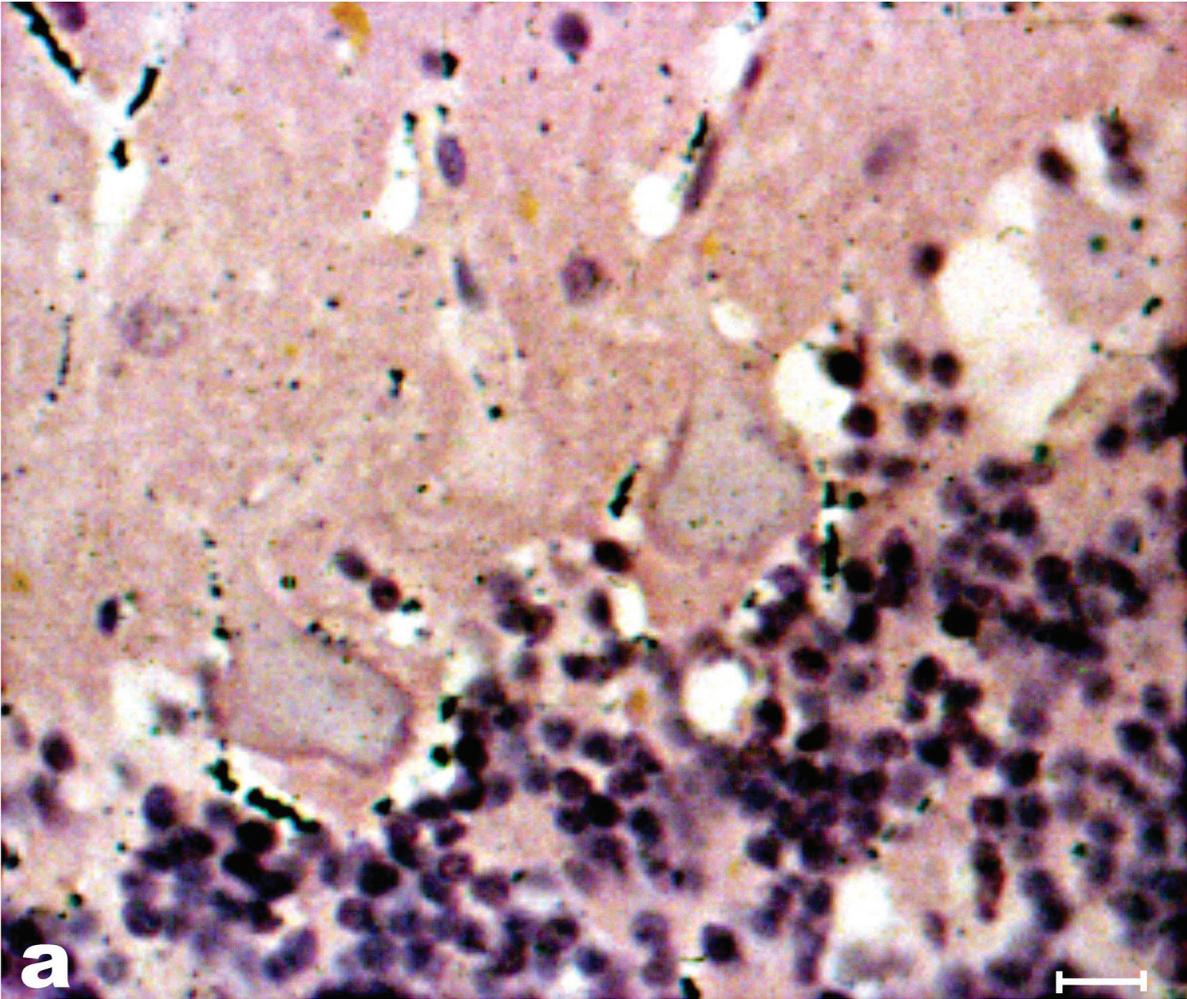


Fig. 1. **a.** Autoradiography with ^{65}Zn in the cerebellum cortex, showing intense uptake in the surroundings of the Purkinje cells, continuing along their dendritic tree, as well as impacts in the neuropilum of the granular layer. **b.** Detail of the ^{65}Zn uptake around a Purkinje cell. **c.** Positivity of Timm's technique around a Purkinje cell. Scale bar: 10 μm .

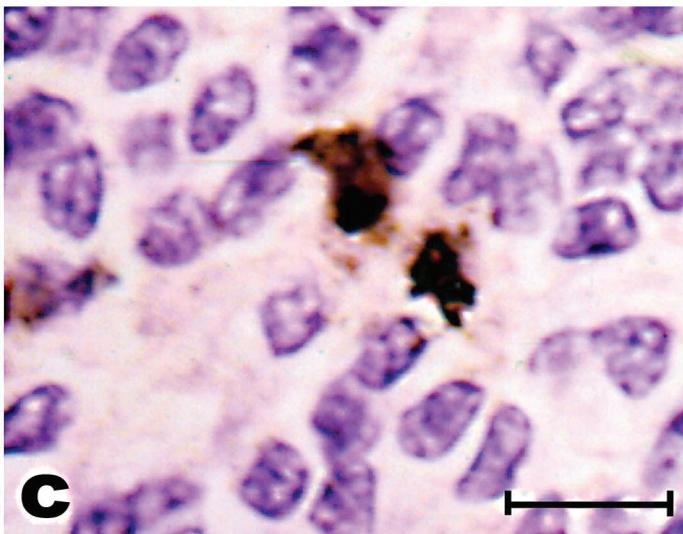
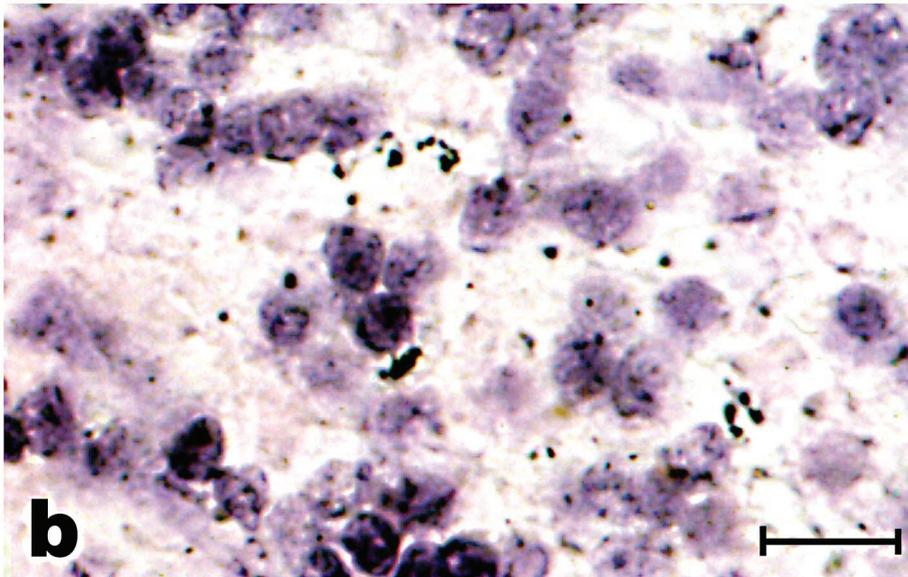
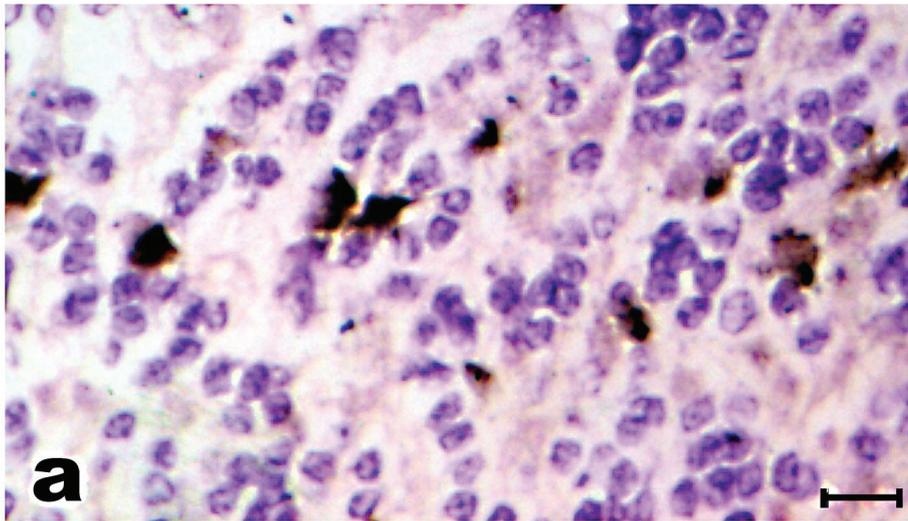


Fig. 2. **a.** Positivity of Timm's technique in the granular layer. **b.** Autoradiographic uptake of ⁶⁵Zn in the granular layer. **c.** Detail of Timm positivity, showing an image that suggests the outline of the terminal of a mossy fibre. Scale bar: 10 μ m.

⁶⁵Zn in cerebellum and brainstem

The autoradiographic uptake and Timm's positivity in the granular layer may agree with the relationship with Glutamate, which is the neurotransmitter used by the granular cells. However, the location in the neuropilum of this cerebellum layer, which has been confirmed by both techniques, is surprising (Fig. 2a-c). this could be explained as a participation of the granular terminals in the cerebellum glomerulae, but the images from Timm show frequent silhouettes that seem to outline terminals of mossy fibres. Is zinc related with the GABAergic function and the inhibitory phenomena?

According to Baraldi et al. (1994) and in the last studies from Jo-Seung et al. (2000), Danscher et al. (2001) and Wang et al. (2001a,b, 2002), this could be possible in the spinal medulla and also in the mouse cerebellar cortex too. In this territory, Wang et al. (2002) describes location of zinc in Golgi cells and in basket cells, according our findings this is perfectly possible because Golgi cells also give endings to glomeruli. With respect to the basket cells, also some of our autoradiographic grains and histochemical positivity could be due to that.

In conclusion, we believe that a subpopulation of

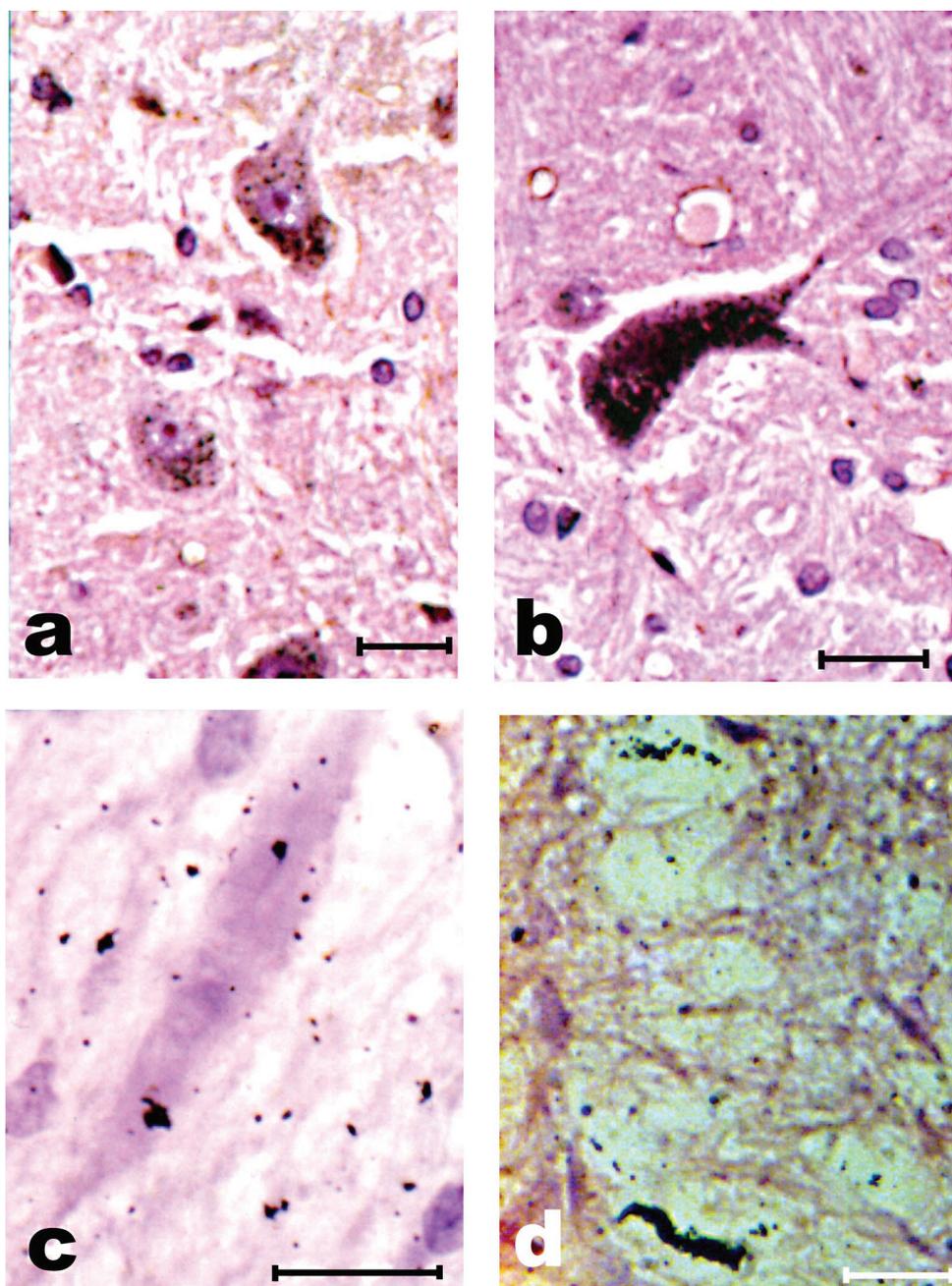


Fig. 3. a,b. Different aspects of Timm's positivity in the perikaryon of brainstem neurons in the bulbar olive. c. Autoradiographic uptake of ⁶⁵Zn in the same location. d. Autoradiographic uptake of ⁶⁵Zn in fibres in the restiforme body. Scale bar: 10 μm.

brainstem neurons that project towards the cerebellum cortex, uptakes, transports and releases zinc. These neurons can match the definition of Z.E.N by Frederickson (1989). But also, the recent papers mentioned above, show some evidence that support a different role for zinc in some areas of the CNS. Our findings of uptake and location of zinc in the granular layer also supported that. In consequence, in the cerebellum, we believe that zinc may act as a neuromodulator of both excitatory or inhibitory processes.

References

- Amano R., Oishi S. and Enomoto S. (1998). Regional uptake behaviors or radioactive Rb, Zn, Se, Mn, Sc and Zr tracers in brain of young and aged C57BL/6N mice. Inabisi'98. 5th internet World Congress on Biomedical Sciences at McMaster University. Canada.
- Baraldi M., Caselgrandi E. and Santi M. (1984) Effect of zinc on specific binding of GABA to rat brain membranes. In: The neurobiology of zinc. Frederickson C.J., Howell G.A. and Kasarskis E.J. (eds). Alan R. Liss inc. New York, pp 59-71.
- Crawford I.L. and Harris N.F. (1984). Distribution and accumulation of Zinc in whole brain and subcellular fractions of hippocampal homogenates. In The neurobiology of Zinc. Frederickson C.J., Howell G.A. and Kasarskis E.J. (eds). Alan R. Liss inc. New York, pp 157-171.
- Dansch G. (1984). Do the Timm sulphide silver method and the selenium method demonstrate Zinc in the brain? In: The neurobiology of Zinc. Frederickson C.J., Howell G.A. and Kasarskis E.J. (eds). Alan R. Liss Inc. New York. pp 273-287.
- Dansch G. (1996). The autometallographic zinc-sulphide method. A new approach involving in vivo creation of nanometer-sized zinc sulphide crystal lattices in zinc-enriched synaptic and secretory vesicles. *Histochem. J.* 28, 361-373.
- Dansch G., Juhl S., Stolenberg M., Krunderup B., Schroder H.D. and Andreassen A. (1997). autometallographic silver enhancement of zinc sulphide crystals created in cryostat sections from human brain biopsies: a new technique that makes it feasible to demonstrate zinc ions in tissue sections from biopsies and early autopsy material. *J. Histochem. Cytochem.* 45, 1503-1510.
- Dansch G., Jo S.M., Varea E., Wang Z., Cole T.B. and Schroder H.D. (2001). Inhibitory zinc-enriched terminals in mouse spinal cord. *Neuroscience* 105, 941-947.
- Dreosti I.E. (1984). Zinc in the central nervous system: The emerging interactions. In: The neurobiology of Zinc (B). Frederickson C.J., Howell G.A. and Kasarskis E.J. (eds). Alan R. Liss inc. New York. pp 1-26.
- Dvergsten Ch.L. (1984). Retarded synaptogenesis and differentiation of cerebellar neurons in zinc-deficient rats. In: The neurobiology of Zinc (B). Frederickson C.J., Howell G.A. and Kasarskis E.J. (eds). Alan R. Liss inc. New York. pp 17-31.
- Euler C. (von). (1962). On the significance of the high zinc content in the hippocampal formation. In: *Physiologie de l'hippocampe*. Passouant P. (ed) Centre National de la Recherche Scientifique. Paris. pp 135-146.
- Farkas I., Szerdahelyi P. and Kasa P. (1988). An indirect method for quantitation of cellular zinc content of Timm-stained cerebellar samples by energy dispersive X-ray microanalysis. *Histochemistry* 89, 493-497.
- Frederickson C.J. (1989). Neurobiology of Zinc and zinc-containing neurons. *Int. Rev. Neurobiol.* 31, 145-238.
- Frederickson C.J., Shu-Sang W., Silva D., Frederickson Cathy J. and Thompson R.B. (2000). Importance of zinc in the central nervous system: The zinc-containing neurons. *J. Nutr.* 130 (5 Suppl), 1471-1483.
- Fujii M. (1967). Distribution of ⁶⁵Zn in mice brain. I.- Topographic peculiarity of the distribution of ⁶⁵Zn in the brain of intact mice. *Bull. Yamaguchi Med. Sch.* 14, 97-104.
- Hassler O. and Soremark R. (1968). Accumulation of zinc in mouse brain. *Arch. Neurol.* 19, 117-120.
- Haug F-M.S. (1984). Sulphide silver stainable (Timm stainable) fiber systems in the brain. In: The neurobiology of Zinc. Frederickson C.J., Howell G.A. and Kasarskis E.J. (eds). Alan R. Liss inc. New York. pp 213-228.
- Janghorbani M. and Young V. (1984). Applications of stable isotope tracers to trace metal metabolic studies in man. In: The neurobiology of Zinc. Frederickson C.J., Howell G.A. and Kasarskis E.J. (eds). Alan R. Liss Inc. New York. pp 343-360.
- Jo-Seung M., Danscher G., Schroder H.D., Won-Moo H. and Cole T.B. (2000). Zinc-enriched (ZEN) terminals in mouse spinal cord: Immunohistochemistry and autometallography. *Brain Res.* 870, 163-169.
- Kishi R., Ikeda T., Miyake H., Uchino E., Tsuzuki T. and Inoue K. (1982). Regional distribution of lead, zinc, iron and copper in suckling and adult rat brains. *Brain Res.* 251, 180-182.
- Khristov K.H.D. (1970). Behaviour of Zn-⁶⁵ in the rat body. In: Toxicology of radioactive substances. Vol 5, Zn⁶⁵. Letaret A.A. and Kurlyandskaya E.B. (eds). Pergamon Publish. Co. Oxford. pp 12-35.
- Perez Castejon M.C., Barral M.J., Vera Gil A., Perez Castejon M.J. and Lahoz M. (1991). Presence and possible function of Zn in the hyaline cartilage. *Histol. Histopathol.* 6, 123-126.
- Perez-Clausell J. and Danscher G. (1985). Intravesicular localisation of zinc in rat telencephalic buttons. A histochemical study. *Brain Res.* 337, 91-98.
- Perez-Clausell J. and Danscher G. (1986). Release of zinc sulphide accumulations into synaptic clefts after in vivo injection of sodium sulphide. *Brain Res.* 362, 358-361.
- Takeda A. (2000). Movement of zinc and its functional significance in the brain. *Brain Res. Rev.* 34, 137-148.
- Takeda A., Sawashita J. and Okada S. (1995). Biological half-lives of zinc and manganese in rat brain. *Brain Res.* 695, 53-58.
- Takeda A., Hanajima T., Ijio H., Ishige A., Iizuka S., Okada S. and Oku N. (1999). Release of zinc from the brain of EL (epilepsy) mice during seizure induction. *Brain Res.* 828, 174-178.
- Takeda A., Takefuta S., Okada S. and Oku N. (2000). Relationship between brain zinc and transient learning impairment of adult rats fed zinc-deficient diet. *Brain Res.* 859, 352-357.
- Takeda A., Suzuki M., Okada S. and Oku N. (2000). ⁶⁵Zn localization in rat brain after intracerebroventricular injection of ⁶⁵Zn-histidine. *Brain Res.* 863, 241-244.
- Timm F. (1958a). Zür histochemie des Ammonhorngebietes. *Z. Zellforsch.* 48, 548-555.
- Timm F. (1958b). Zür histochemie des schwermetalle. Das sulfid-silberverfahren. *Dtsch. Z. Ges. Gerichtl. Med.* 46, 706-711.
- Vera-Gil A., Pérez-Castejón M.C., Barral M.J., Pérez-Castejón M.J., Lahoz M.J. and Pinilla M.J. (1990). The location of Zn in cerebellum and its possible meaning. *An. Anat.* 36, 341-343.

⁶⁵Zn in cerebellum and brainstem

- Vera Gil A., Perez Castejon M.C., Barral M.J., Recreo P. and Perez Castejon M.J. (1991). Location of Zn in the testicle of the rat. *Acta Anat*, 141, 70-73.
- Vera-Gil A. and Perez-Castejón M.C. (1994). ⁶⁵Zn in studies of the neurobiology of Zinc. *Histol. Histopathol.* 9, 413-420.
- Wang Z., Danscher G., Mook J.S., Shi Y. and Schroder H.D. (2001a). Retrograde tracing of zinc-enriched (ZEN) neuronal somata in rat spinal cord. *Brain Res.* 900, 80-87.
- Wang Z., Li J-I., Dahlstrom A. and Danscher G. (2001b). Zinc-enriched GABAergic terminals in mouse spinal cord. *Brain Res.* 921, 165-172.
- Wang Z., Li J-I., Danscher G., Kuk Kim Y., Dahlstrom A. and Mook J.S (2002). Inhibitory zinc-enriched terminals in the mouse cerebellum:double-immunohistochemistry for zinc transporter 3 and glutamate decarboxilase. *Neurosci. Lett.* 321, 37-40.

Accepted January 2, 2003