J. Phys. D: Appl. Phys. **29** (1996) 259–262. Printed in the UK

Synchrotron spectromicroscopy of cobalt accumulation in granule cells, glial cells and GABAergic neurons

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Received 27 July 1995

Abstract. Recent neurobiophysics experiments based on synchrotron spectromicroscopy indicated that aluminium is selectively accumulated by GABAergic neurons and glial cells rather than by granule cells. Does a similar cell specificity occur in the accumulation of other metals? We provide experimental evidence to the contrary: cobalt is found in granule cells at least with equal probability as in glial cells and GABAergic neurons. This result also confirms that neurobiophysics studies based on surface physics techniques have reached a stage of maturity.

Experimental methods in surface physics are being applied with increasing success to biophysics; one of the most relevant examples is the use of synchrotron radiation photoelectron spectromicroscopy to study the accumulation of metals in neurophysics [1, 2]. A series of recent experiments first suggested [1] and then confirmed [2] a peculiar property of metal accumulation caused by exposure to aluminium of rat cerebellar primary cultures. In cultures with mostly granule cells, aluminium was only found [1] in a small subset of cells morphologically identifiable as GABAergic neurons or glial cells. Conversely, the majority of cells in cultures consisting of GABAergic neurons or glial cells did accumulate Al [2].

Does a similar cell selectivity occur for cobalt uptake? We tried to answer this question by a series of comparative experiments on the accumulation of cobalt by cultures primarily consisting of granule cells, glial cells or GABAergic neurons.

The accumulation of cobalt is well known to occur in granule cells in the presence of neurotransmitters such as kainate. Extending the study to the aforementioned other types of neural cells, we found that they play no special role with respect to granule cells [3]. On the other hand, we found differences in the spatial localization of cobalt among these types of cells.

The experimental probe used to identify the spatial distribution of accumulated cobalt was the XSEM (x-ray secondary electron emission microscopy) version of photoelectron spectromicroscopy with synchrotron radiation. This technique, discussed in detail in the literature [4], has been quite extensively used since 1992 for experiments of this type, concerning the accumulation not only of Al and Co but also of Ni, Fe, Cr, Cu and Zn [1, 2, 5, 6].

The XSEM technique measures the local x-ray absorption by detecting the secondary photoelectron yield versus the photon energy [4]. The synchrotron-radiation photons were produced by the Wisconsin storage ring Aladdin and filtered by a 10 m toroidal grating monochromator. Lateral resolution in measuring x-ray absorption is achieved by processing the secondary photoelectrons with a magnifying electrostatic optics system before detection [4]. This apparatus can produce either photoelectron yield micrographs at fixed photon energy, or yield against photon energy spectra from specific microscopic areas.

An XSEM micrograph [4] reveals the specimen micromorphology; an XSEM spectrum reveals instead the chemical composition of the corresponding microscopic area—each element produces characteristic absorption edges.

Note that XSEM is surface sensitive [4]. In fact, the

0022-3727/96/010259+04$19.50

secondary photoelectrons originate from a depth of about 50–100 Å, thus probing a surface layer that includes the cell membrane (30 Å thick) and the outermost portion of the cytoplasm. We tested the actual penetration of cobalt inside the cells' cytoplasm by performing XSEM experiments on cells that had been decapped as explained later.

The specimen preparation was identical to that described in the literature [1, 2]. In short, primary cultures enriched in granule cells, GABAergic neurons and glial cells were obtained by enzymatic and mechanical dissociation of eight-day-old rat cerebellum. Cerebella was sliced, suspended in Krebs–Ringer solution and incubated with trypsin, centrifuged, resuspended, treated with DNAse and finely triturated. Dissociated cells were recovered by centrifugation and resuspended in Basal Eagle's Medium with Eagle's salts containing foetal calf serum. The cells were plated and allowed to grow on poly-L-lysine treated gold-coated stainless steel plates.

Primary cerebellar granule neuron-enriched cultures were prepared from postnatal rats. Granule cells are the most common type of neurons in the central nervous system, and they grow as a well organized network of neuronal processes when cultured on flat substrates. They utilize the excitatory neurotransmitter glutamate.

Primary cerebellar GABAergic neuron-enriched and glial cell-enriched cultures were also prepared from postnatal rats. GABAergic neurons are neural cells that use gamma amino butyric acid (GABA) as neurotransmitter and generate inhibitory synapses. In our experiments they were identified by their ability to accumulate [^{3}H]GABA and immunocytochemically using anti-GABA antibodies [7]. Morphological and cytochemical analysis of specific cell markers (calbindin) suggested that the cells of such a culture may represent a population or subpopulation of Purkinje cells [7].

Glial cells are again neural cells, but they are not neurons, i.e. they do not transmit nerve pulses. They provide mechanical and chemical support for the neurons of the central nervous system. The glia cultures were obtained with the method described by Wilkin *et al* [8] and immunocytochemically identified using [9] anti-GFAP (glial fibrillary acid protein) antibodies; they are mainly populated by large flat polygonal cells, the subpopulation of astrocytes known as type 1 [8].

At the end of the growth period, all types of cultures were washed and exposed for 20 min to a 5 mM $CoCl_2$ (Fluka) solution in uptake buffer [10]. Then, the granule and GABAergic neuron cultures were again carefully washed to remove all the non-uptaken cobalt.

The cells in some of the glia cultures, after exposure and washing, were decapped with the novel approach described previously [11]. In short, the excess uptake buffer was drawn; then each specimen was covered with a filter membrane soaked with CS buffer (100 mM PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid) laboratory reagent gade from Sigma), 5 mM EGTA (ethylene glycol-bis-(b-aminoethyl ether)-N,N,N′,N′-tetraacetic acid laboratory reagent grade from Sigma). The contact with the cells' top lasted 5 min, after which the filter membrane was removed. This peeled off the portion of cell membrane adhering to the filter membrane. The membrane removal was tested by fluorescence detection of anti-GFAP antibodies [9] which cannot reach the cytoplasm unless at least part of the membrane has been removed.

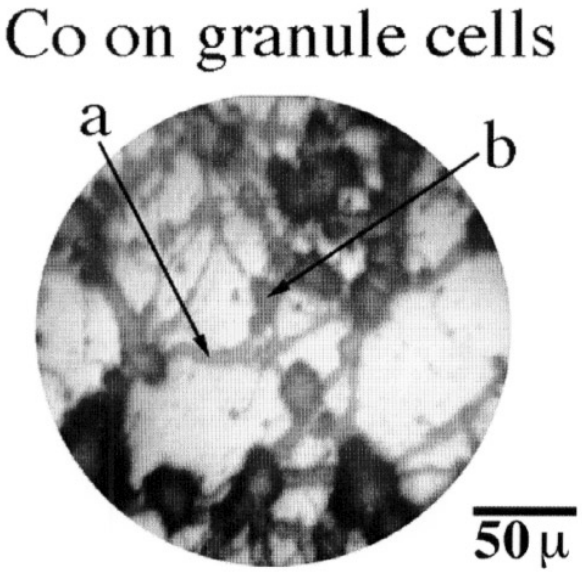

Figure 1. XSEM micrograph of a portion of a granule cell culture. The labels a and b refer to the microscopic areas where the corresponding spectra of figure 2 were acquired.

Finally, all types of specimens were fixed with 4% paraformaldehyde (Merck) in phosphate buffer saline solution and dehydrated.

The presence of cobalt was revealed by observing the Co 2p x-ray absorption edge in the XSEM spectra. Figures 1 and 2 present typical results representative of granule cell cultures; figure 1 shows the XSEM micrograph of a portion of one such specimen, and figure 2 the spectra from 10×10 μm^2 areas placed at locations a and b in figure 1. The upper curve of figure 2 is the spectrum of a dry $CoCl_2$ droplet from the same exposure solution of the cell culture. The clearly visible Co 2p edge provides a reference for the other spectra. Although weaker, the Co signal is clearly visible both in area a and in area b curves.

Note that the observation of Co in granule cells is *per se* an interesting result, since it was obtained in the absence of neurotransmitters. To the best of our knowledge, this is the first evidence of Co accumulation in resting-state granule cells. Our success is most likely due to the higher sensitivity of our experimental method with respect to those conventionally used in neurobiology, such as silver staining [3].

Figures 3 and 4 present similar data for a decapped glial cell specimen; Co is visible only in the area b spectrum. Results concerning a GABAergic (Purkinje) neuron culture are presented in figures 5 and 6. Note the presence of Co in cell body and neurite areas.

Each set of data is representative of a much larger body of consistent results from different specimens of each type of culture: approximately 10^2 spectra each for granule and glial cell cultures, and approximately 50 spectra for

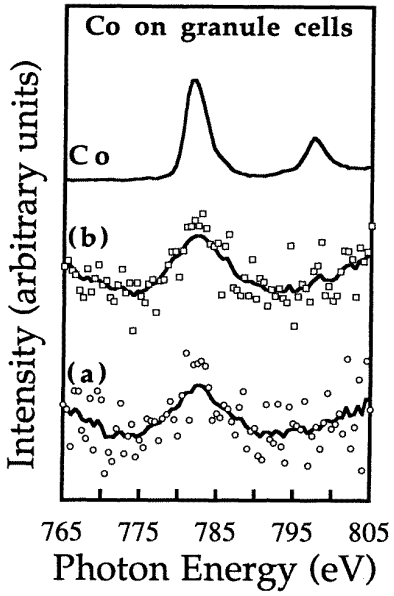

Figure 2. XSEM (x-ray absorption) spectra taken in the two 10×10 μm^2 areas marked as a and b in figure 1, in the photon energy spectral range of the Co 2p x-ray absorption edge. The upper curve is the spectrum of a dry $CoCl_2$ droplet.

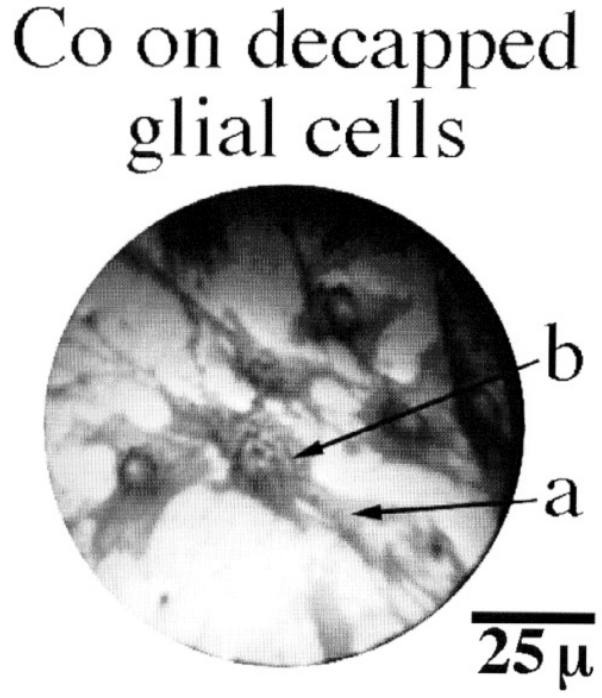

Figure 3. XSEM microimage of a portion of a decapped glial cell culture.

GABAergic neuron cultures.

No cobalt was ever found on the substrate areas (data not shown).

We will address the two issues of this study of cobalt accumulation: cell specificity and spatial distribution. We found no evidence that non-granule cells play a special

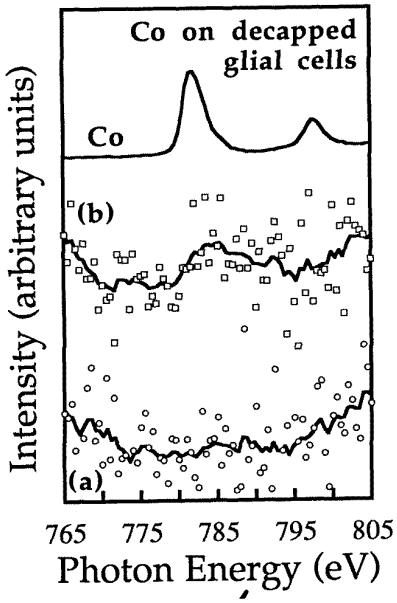

Figure 4. XSEM reference spectrum, and two spectra taken in the areas marked as a and b in figure 3.

role. In the case of aluminium accumulation, there was an extreme case of selective uptake by non-granule cells. In the case of cobalt, comparing the Co accumulation in granule cell cultures, GABAergic neuron cultures and glial cell cultures, we found that the accumulation does not have higher probability to occur in non-granule cells than in granule cells. Preliminary results on Ni accumulation indicate a similar lack of specificity.

As to the spatial distribution of the accumulated Co, we did find some significant differences between different types of cells. All of the approximately 10^2 spectra obtained from granule cell cultures show Co in neurite areas, and never on the cell bodies. On the contrary, no such confinement to neurites is seen for GABAergic neurons—see figure 6, spectrum b, which is consistent with all other spectra on this type of culture. Also consistent with all other spectra is the fact that no clear evidence of Co is found in the debris (area a), that is well known to contaminate these types of cultures. This indicates that the Co presence is the result of active uptake by live cells.

In the case of decapped glial cells, Co was found in several spots throughout the cell structure, without selective localization and without correlation to the cell architecture. We believe that the Co signal spots correspond to areas of removed membrane, since their size is similar to that of the cell membrane holes observed with fluorescence microscopy in GFAP-stained specimens.

No cobalt was ever observed on undercapped glial cells (data not shown). This result indicates that Co is uptaken and stored inside the cytoplasm of glial cells, and not on their cell membranes or in membrane proteins, XSEM being a surface sensitive technique.

In conclusion, we found no evidence of selectivity of cobalt accumulation by a specific type of neural cells. Cell specificity, like for Al [1, 2], can now be ruled out by our results. This statement was specifically supported

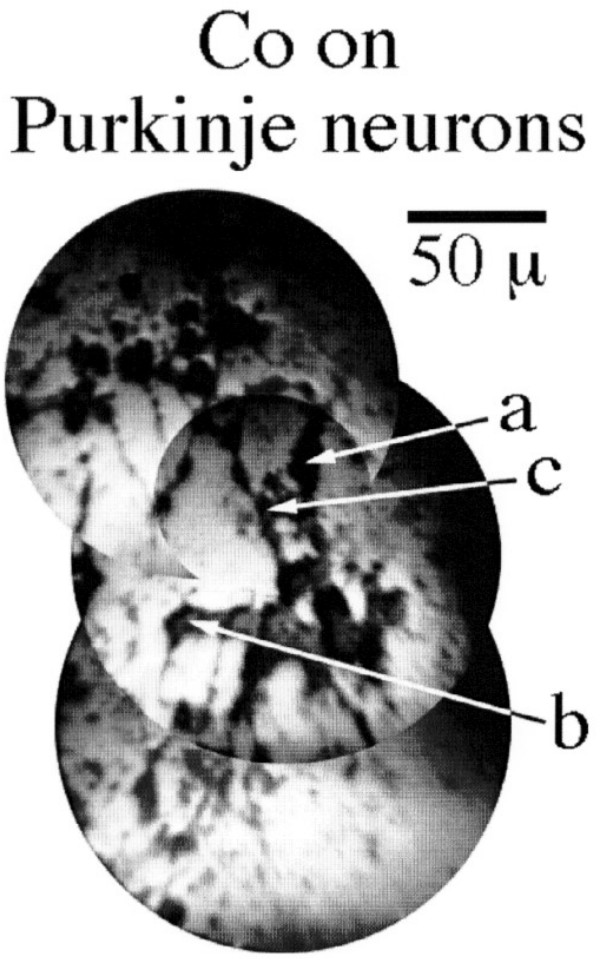

Figure 5. XSEM patchwork microimage of a portion of a GABAergic neuron enriched culture.

by our comparative studies of GABAergic neuron, glial and granule cell cultures. We also found characteristic Co localization for each type of cell: in granule neurons cobalt is always present in neurites and absent in cell bodies. In GABAergic neurons it is accumulated both in cell bodies and neurite structures. In glial cells we find cobalt only in the decapped areas, indicating Co uptake in the cytoplasm and not in the membrane region.

We believe that these results may pave the way for novel applications of synchrotron spectromicroscopy. Such applications may extend well beyond the neurotoxicology presented here, and involve environmental analysis, as well as other areas of medicine and biology.

Acknowledgments

This work was supported by: Fonds National Suisse de la Recherche Scientifique; Consiglio Nazionale delle Ricerche; Progetto Finalizzato 'Invecchiamento' (to DM); Ecole Polytechnique Fédérale de Lausanne, and performed at the Wisconsin Synchotron Radiation Center (supported by the NSF). The first author (GDS) was supported by the EU within the 'Biotechnology' program. We thank Roger Hansen, Mario Capozi, Sandro Rinaldi, Bruno Nicolini for their expert help.

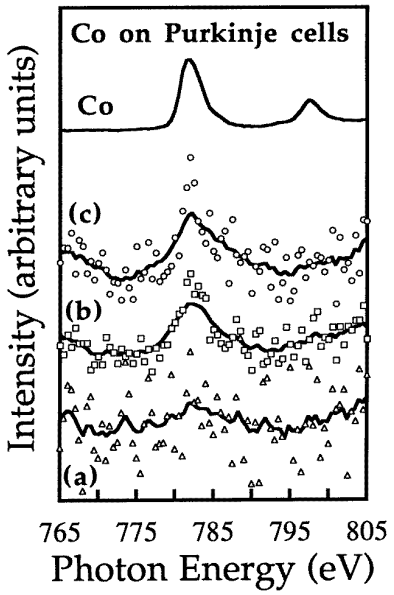

Figure 6. XSEM reference spectrum, and three spectra taken in the correspondingly marked areas in figure 5.

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