CELLULAR LOCALIZATION OF 2-[³H]DEOXY-D-GLUCOSE FROM PARAFFIN-EMBEDDED BRAINS¹

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Abstract

Results of experiments in which regional neuronal activity is revealed by a 2-[³H]deoxy-D-glucose (³H-2-DG)-paraffin section-emulsion autoradiography method are described. The trigeminal pathway of freely behaving mice was activated differentially by selective patterns of whisker removal. One hour after injection of concentrated ³H-2-DG, the animals were perfused systemically with a periodate/lysine/paraformaldehyde mixture (McLean, I. W., and P. K. Nakane (1974) *J. Histochem. Cytochem.* 22: 1077-1083), the brains were embedded in paraffin, and serial sections were taken and coated with emulsion for autoradiography. Diffusion of the isotope out of the tissue was assessed visually and by liquid scintillation counting. While substantial loss of ³H isotope into the embedding fluids (about 95%) was found, the scintillation counts and the autoradiograms showed good fixation of the isotope *in situ*, no evidence of isotope movement into the emulsion, and no gradients of diffusion in the sectioned material.

Patterns of regional labeling were similar to those reported from brains prepared by conventional 2-[¹⁴C]deoxy-D-glucose (¹⁴C-2-DG) autoradiography; for instance, auditory and vestibular pathways in the brainstem were heavily and specifically labeled. Trigeminal structures associated with the intact (stimulated) whiskers were labeled relatively heavily, indicating that label uptake is specific with respect to neuronal activity. In the cortex, the patterns of label corresponded directly and precisely to those barrels known to receive inputs from the intact whiskers. Distribution of silver grains in the cortex and in the brainstem was correlated directly with neuronal profiles, including processes, some of which were identified by means of a Nissl counterstain. Clearly, this approach offers considerable technical advantages, in particular, the ease with which the histological material is prepared. The resolution of the autoradiograms and the quality of the histology are excellent.

Sokoloff and co-workers first demonstrated that radioactive 2-deoxy-D-glucose (2-DG) could be used to localize regional functional activity in the nervous system (Kennedy et al., 1975). This powerful method is now widely used for studies of brain function. Since it is well known that many standard tissue-processing methods will extract carbohydrates, including 2-DG and 2-DG-6-phosphate, the standard protocol for processing 2-[¹⁴C]deoxy-D-glucose (¹⁴C-2-DG)-labeled brains (Sokoloff et al., 1977) is to freeze them quickly, section them in a cryostat, and use the rapidly dried sections to expose x-ray film. Although systemic perfusion with aldehydes improves the

quality of frozen cryostat-sectioned material with little isotope loss (Collins, 1978; Des Rosiers and Descarries, 1978), a cryostat is not a convenient device from which to obtain the long series of sections which are necessary for the analysis of activity in large structures or system pathways. Recently, several groups have demonstrated good isotope localization in tissues processed for plastic embedding and emulsion autoradiography (e.g., Basinger et al., 1979; Des Rosiers and Descarries, 1978; Sejnowski et al., 1980). Advantages of the approach are: The isotope cannot diffuse out of the plastic into the emulsion; 2-[³H]deoxy-D-glucose (³H-2-DG) can be used to obtain higher resolution autoradiograms; the histological quality is excellent; and in addition, the method is suitable for electron microscopy. The approach has worked particularly well in small isolated tissues, such as the retina. However, plastic embedding is impractical especially when many serial sections from larger brains are needed routinely (but see Des Rosiers and Descarries, 1978). Thus, we wanted to find a technique which would allow us to cut serial sections through an entire brain conven-

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iently, would be compatible with emulsion autoradiography, and would have the increased resolution available with tritium.

The results of experiments in which ^3H -2-DG was used to label various parts of the brain are reported in this paper. The animals were perfused systemically with the fixative designed by McLean and Nakane (1974) to stabilize carbohydrates *in situ*, and the tissue was processed using standard paraffin embedding and emulsion autoradiography. The resultant ^3H -2-DG labeling corresponded to areas of known functional activity, including neuronal cell bodies. For this demonstration, we have chosen the mouse somatosensory system because of the known one-to-one correspondence between the whiskers on the face and the barrels in layer IV of the contralateral somatosensory cortex (SmI).³ Our previous ^{14}C -2-DG studies with frozen sections and film autoradiography were used as "controls" (Durham and Woolsey, 1978).

Materials and Methods

Over twenty adult Swiss Webster mice of either sex were used. Under Nembutal anesthesia, various combinations of vibrissae were cut off. The next day, 1 or 2 mCi of concentrated ^3H -2-DG (New England Nuclear; specific activity = 8.3 Ci/mmol) was resuspended in saline and injected into a tail vein. The mice explored an empty cage for 60 min as described previously (Durham and Woolsey, 1978). Animals then were deeply anesthetized with sodium pentobarbital (50 mg/kg) and perfused transcardially with saline followed by a fixative designed by McLean and Nakane (1974) to stabilize carbohydrates (0.01 M periodate, 0.075 M lysine, and 2% paraformaldehyde in 0.037 M phosphate buffer). After perfusion for 3 min, the brains were removed intact, immersed in 30 ml of 50% ethanol overnight, and subsequently dehydrated in graded alcohols (70%, 4 hr; 95%, 4 hr; butanol I, 1 hr; and butanol II, overnight). Then brains either were blocked in various planes or left intact, after which, they were embedded in Paraplast. Serial sections were cut at 15 μm and mounted onto slides with albumin. Following overnight de-paraffinization in fresh xylene, the sections were hydrated through graded alcohols to distilled water, dried overnight at 37°C, and dipped in NTB-2 emulsion (Kodak) diluted 1:1 with distilled water. Dipped slides were dried for 2 hr in the dark and then stored in light-tight boxes at 5°C for 12 to 20 weeks. After exposure, the sections were developed for 2 min in D-19 (Kodak) at 16°C, fixed, dehydrated, and coverslipped with DPX mountant. Sections were examined under bright- and dark-field illumination and photographed. Silver grains over selected unstained sections were counted using a

small computer microscope system (Woolsey and Dierker, 1978). The coverslips then were removed, and the sections were stained through the emulsion with 0.12% thionin and coverslipped again.

The question of bulk isotope loss during embedding was assessed in the experimental animals by measuring ^3H label in embedding fluids and liver and brain samples for all animals. Since the point of this type of experiment is to demonstrate differential distribution of isotope in the brain, liver was used also as a tissue in which label might be assumed to be evenly distributed. To address this question directly, two control mice with intact vibrissae were treated in a manner otherwise identical to the experimental animals. Each mouse received an injection of 1 mCi of ^3H -2-DG. After perfusion, weighed pieces of brain and liver were carried through the same embedding protocol as for the brains except that pieces were removed after each step to assay the amount of label in the tissue. Weighed samples were dissolved in Soluene (Packard Instrument Co.), neutralized with glacial acetic acid, and counted by liquid scintillation after the addition of fluor (3a70, Research Products International). Tritium levels also were measured from aliquots of the embedding liquids. The ratio of the amount of tritium in the brain or liver to that in the embedding liquid was calculated for each step in the protocol as an index of fixation of the isotope.

Results

The results from the animals prepared for this study are qualitatively similar. We will present, in detail, the findings in one animal which received an injection of 2 mCi of isotope.

Under low magnification with dark-field illumination, the appearance of the developed, emulsion-coated sections is qualitatively similar to that of x-ray film images from ^{14}C -2-DG-labeled frozen sections (Durham and Woolsey, 1978). Figure 1 shows photomicrographs of a section through the medulla at the level of the lateral apertures. The Nissl-stained section in Figure 1a shows the cytoarchitectonic details that permit nuclear boundaries to be recognized easily.

Several areas in the emulsion over this section show higher silver grain densities than the rest of the tissue. In normal, behaving animals, the auditory and vestibular nuclei characteristically appear as areas of increased activity in autoradiograms from frozen sections (Schwartz and Sharp, 1978). In the unstained dark-field view (Fig. 1b), both the lateral vestibular and dorsal cochlear nuclei are covered densely with silver grains. Under bright-field illumination at higher magnification (Fig. 1c), the same gross pattern of silver grain density is evident. The most striking observation, however, is the dense clusters of grains covering cell somata (discussed below). Of interest here is the sharp drop in grain density at the edges of the section and the absence of gross artifacts that would suggest bulk diffusion of the label into the emulsion. The ventricular space, for example, contains only background levels of labeling. Thus, (i) the patterns of labeling correspond to those known from the standard method of 2-DG autoradiography; (ii) there is differential labeling of areas known to have different

³ Vibrissae are arranged in five rows labeled A to E, dorsal to ventral. Vibrissae within a row are numbered sequentially beginning with the largest and most caudal ones. Four whiskers straddle the caudal ends of the rows and are labeled α to δ , dorsal to ventral. The contralateral cortical barrels are arranged in five rows labeled A to E, posterolateral to anteromedial. Barrels within a row are numbered sequentially beginning with the most posteromedial ones. Four barrels straddle the rows and are labeled α to δ . A similar anatomical and functional arrangement of the ascending pathways exists—namely the ventrobasal thalamus, contralaterally, and of importance here, the principal sensory and spinal nuclei of V, ipsilaterally.

Figure 1. Photomicrographs of a coronal 15- μm paraffin section through the medulla at the level of the lateral apertures. *a*, Nissl-stained section showing cytoarchitectonic details, including nuclear boundaries. *CP*, choroid plexus; *DCN*, dorsal cochlear nucleus; *LCN*, lateral cerebellar nucleus; *LVN*, lateral vestibular nucleus; *NG*, nucleus gigantocellularis; *NTS*, nucleus of the solitary tract. Nomenclature follows Sidman et al., 1971. *b*, Dark-field illumination of unstained section showing heavily labeled areas (e.g., *DCN*, *LVN*). *c*, Bright-field photomontage of the area outlined in *b*. At this higher magnification, dense clusters of grains can be seen over cell somata in *DCN*, *LVN*, *NTS*, and *NG*. The ventricle (*) contains only background levels of grain density. Arrows indicate labeled cells near the tissue surface, evidence that fixed isotope does not leave the tissue during embedding.

levels of activity during similar experiments; and (iii) there is no evidence for diffusion of label *into* the emulsion after the sections were dipped.

Patterns of silver grain density specifically related to the experimental stimulus were observed. Figure 2 shows a section at the level of the spinal trigeminal nucleus,

pars caudalis. Only the vibrissae in rows *B* and *D* remained on the left face of the animal. Two bands of increased silver grain density can be seen in the spinal trigeminal nucleus which was identified from the Nissl stain (Fig. 2*a*). The segmented organization of the vibrissae representations within the brainstem trigeminal nu-

clei cannot be seen with Nissl staining. However, comparison of the autoradiographic section with a similar section prepared with the succinic dehydrogenase (SDH) histochemical method provides indirect evidence that the portions of the nucleus appropriate to the stimulus are more heavily labeled (D. Durham, T. A. Woolsey, and L. Kruger, unpublished data; Belford and Killackey, 1979). In sections containing the same representation on the right side, one central band of increased silver grain density could be seen, corresponding to the single row of vibrissae (*row C*) which was left intact on the right face of the animal. Thus, in this portion of the trigeminal pathway, a correlation exists between the amount of label observed and the sensory stimulus. Figure 2c shows a higher magnification bright-field view of the section through the spinal trigeminal representation in which labeled cell bodies are evident. Note also that the grain density falls to background levels at the section boundary.

To assess the possibility of gross diffusion of label from the tissue during processing, computer grain counts over selected sections were generated on a small computer microscope system (Woolsey and Dierker, 1978). Counts from the section in Figure 2 are shown in Figure 3. Raw binned data (Fig. 3a) reveal no substantial decline in grain density in the radial direction, i.e., from deep to superficial in the brain, as might be expected if the label were not fixed in the tissue. By subtracting out successively higher bin values (Fig. 3, *b* to *d*), patterns of the heaviest labeling within the section are emphasized.

A direct correlation between cytoarchitecture and in-

creases in silver grain density related to the stimulus is best seen in tangential sections of the barrel region of the somatosensory cortex (SmI). Figure 4b is a dark-field photomicrograph of an unstained tangential section through layer IV of the barrel region in the right hemisphere, corresponding to the brainstem shown in Figures 2 and 3. Two bands of increased silver grain density are evident, and later comparison with the underlying pattern of stained cell bodies verified that the increased activity is related directly to the appropriate *row B* and *D* barrels. In Figure 5b, a dark-field photomicrograph of a similar section through the left hemisphere shows only one band of high grain density over *row C*, corresponding to the presence of only *row C* vibrissae on the right face. These differences in grain density extend above and below the appropriate barrels in adjacent sections of supra- and infragranular cortical layers. Both the layer IV and columnar patterns with the ^3H -2-DG emulsion method confirm our earlier results with ^{14}C -2-DG frozen section autoradiography using the same stimulus paradigm (Durham and Woolsey, 1978).

At low magnification, the boundaries between areas of low and high grain density were sharper than those seen in x-ray films, suggesting that the new method offered better resolution. Examination of cortical sections containing the stimulated barrels indeed revealed that, in addition to an increase in grain density in the neuropil, dense clusters of silver grains could be seen over individual cell somata. Figures 4c and 5c show higher magnification bright-field views of the cortical barrels with many labeled cells and densely labeled neuropil. Thus, (*i*) the

Figure 2. Photomicrographs of a coronal 15- μm paraffin section through the spinal trigeminal nucleus, pars caudalis (*SVc*). *a*, Nissl-stained view showing cytoarchitectonic landmarks. *CB*, cerebellum; *CC*, central canal; *CST*, corticospinal tract; *MCN*, medial cuneate nucleus; *SVc*, spinal trigeminal nucleus, pars caudalis; *, fourth ventricle. *b*, Dark-field illumination of unstained section. Note that the ventricle is relatively free of label, while several other areas show intense label (e.g., *MCN*, two bands in *SVc*). *c*, Bright-field photomontage of the area outlined in *b*. Note again the absence of label in the ventricle and the labeled cell somata in *MCN* and *SVc*. Heavily labeled cells and neuropil in *SVc* correspond to the representation of intact stimulated vibrissal rows *B* and *D*.

Figure 3. Binned computer grain counts of unstained section shown in Figure 2. Grains are represented as *dots* in which the size is proportional to the silver grain area in fields $20\ \mu\text{m}$ on a side ($400\ \mu\text{m}^2$). *a*, Raw counts, including background. *b*, Count data from which background values have been subtracted. Note the well defined section edge (*arrows*). Several areas show increased label (e.g., *MCN*, *SVc*). *c* and *d*, Count data from which successively higher background values have been subtracted. Note the absence of grains in the ventricle (*) and that areas of increased grain density appear more prominent. Representations in *SVc* of two intact rows of vibrissae (*B* and *D*) are labeled. *MCN*, medial cuneate nucleus; *SVc*, spinal trigeminal nucleus, pars caudalis.

Figure 4. Photomicrographs of a $15\text{-}\mu\text{m}$ tangential paraffin section through layer IV of SmI of the right hemisphere corresponding to the brainstem representation in Figures 2 and 3. Barrel rows (*A* through *D*) are indicated. *a*, Nissl staining allows cytoarchitectonic visualization of barrel boundaries. Section orientation with respect to the animal is shown at *left*. *a*, anterior; *l*, lateral; *m*, medial; *p*, posterior. *b*, In this dark-field unstained view, the two prominent bands of heavy label correspond to barrel rows *B* and *D*. *c*, Bright-field photomicrograph of the area outlined in *b*. Heavily labeled cell bodies and neuropil are localized to barrel rows *B* and *D*.

results with ^3H emulsion autoradiography confirm our previous findings with ^{14}C film autoradiography on the effects of acute vibrissae removal in the mouse somatosensory system; in particular, the label is associated with

the appropriate cytoarchitectonic markers—the barrels and (ii) the use of ^3H isotope with paraffin embedding and emulsion autoradiography greatly increases the resolution of the 2-DG technique. Most importantly, both

Figure 5. Photomicrographs of a 15- μ m tangential paraffin section through layer IV of the left SmI. Barrel rows (C through E) are indicated. *a*, Nissl-stained view shows cytoarchitectonic definition of barrel rows. Section orientation is as indicated in Figure 4. *b*, Dark-field unstained view shows one band of heavy label in row C, corresponding to row C vibrissae left intact on the animal's right face. *c*, Bright-field view of the area outlined in *b*. Heavily labeled cell somata and neuropil are seen in row C.

cell bodies and the surrounding neuropil have increased 2-DG uptake during increased functional activity.

The freely moving stimulus paradigm used here is not specific just for the somatosensory system. We examined other areas of the brain, known from our own and others' previous ^{14}C film studies to be active metabolically in behaving animals, for evidence of increased activity in single cells. Figure 6 shows bright-field photomicrographs of several sections, demonstrating specific label of various intensities over different cell somata. Many labeled cell bodies can be seen. A particularly striking example of single labeled cells is the nucleus gigantocellularis in the medulla, shown in Figures 1*c* and 6*b*. Here, cell bodies and the proximal portions of some processes are clearly heavily labeled. These could be followed through adjacent sections in the series. Thus, in addition to the system under investigation, using emulsion autoradiography, we find (i) high levels of functional activity in single neurons in areas that correlate with early results using frozen sections and x-ray film and (ii) some neurons not only have intensely labeled somata but heavily labeled processes as well.

The results from all of our isotope measurements in both brain and liver indicate that there was 6 times as much label per gm of tissue in the brain as in the liver.

We observed a substantial loss of isotope from both tissues during embedding, usually at least 95% of that present after perfusion. Changes in the ratio of isotope in tissue to that in its embedding liquid, however, are compatible with the idea that 2-DG and 2-DG-6-phosphate are fixed. Early in embedding, this ratio is small and similar in both liver and brain (in 50% alcohol, 0.12), indicating that unfixed isotope is diffusing out of the tissue. This ratio increases with later embedding steps such that, by butanol II, the average ratio is 17 to 50, an increase of over two orders of magnitude. Thus, more isotope remains in the tissue than diffuses out and so must be fixed. These measurements are an independent means of confirming the results evident in the autoradiograms, e.g., that label has been fixed specifically in the tissue. In fact, it may well be that the embedding procedure improves the quality of the results by removing non-fixed label prior to autoradiography, i.e., improving the signal-to-noise ratio.

Discussion

The 2-DG method has been shown in a number of applications to be extremely useful in determining qualitative and quantitative levels of functional activity in discrete areas of the nervous system (Hubel et al., 1978;

Figure 6. Higher magnification photomicrographs of selected stained sections to show labeled cell somata. $\blacklozenge$ indicates heavily labeled somata, $\blacktriangleright$ lightly labeled somata, $\diamond$ unlabeled cells, and $*$ the ventricle. *a*, Cells in the dorsal cochlear nucleus (Fig. 1). Note the absence of label in ventricle and cells labeled with different intensities. *b*, Cells in the nucleus gigantocellularis (Fig. 1). Note that, in heavily labeled cells, proximal processes are also labeled. Several lightly labeled and unlabeled cells are also shown. *c*, Cells in the C1 barrel in the left hemisphere (Fig. 5). Again note that different cells are labeled to different degrees.

Collins, 1978; Stewart et al., 1979; Lippe et al., 1980). Because the label is water soluble, however, it has been necessary to process the tissue to avoid diffusion of the isotope, i.e., cryostat frozen sectioning and x-ray film autoradiography, at the expense of good histology and high resolution. The periodate/lysine/paraformaldehyde

fixative⁴ was devised by McLean and Nakane to bind carbohydrates, theoretically by first oxidizing them with periodate to form aldehydes and then cross-linking the aldehydes with lysine. The paraformaldehyde stabilizes proteins and lipids, probably by polymerization (McLean and Nakane, 1974). The following evidence suggests that we have been successful in fixing the 2-DG-6-phosphate and/or 2-DG in our tissue sections: (i) Analysis of embedding liquids and tissue samples shows an increase in the ratio of isotope in tissue to that in embedding liquid at later steps in the protocol. (ii) The boundaries between high and low grain densities at the edges of the sections and within sections are sharp. (iii) There are no patterns of grain density to suggest preferential leaching of the isotope from the surface of the tissue (see "Results"). Heavily labeled cells can be seen in some sections near the brain surface (e.g., Fig. 1*c*). (iv) The presence of clearly labeled cell bodies in areas of high activity is perhaps the strongest evidence that the isotope is fixed within the tissue. (v) With the same stimulus paradigm, similar zonal distribution patterns of label result using either emulsion or film autoradiography.

The use of conventional sectioning methods is an important practical advantage of this 2-DG protocol. Paraffin sections are easy to cut and handle, are of good histological quality, and do not require access to a cryostat. On a microcurie basis, ^3H -2-DG is less expensive than ^{14}C -2-DG, but it should be noted that comparatively high doses of isotope (2 mCi/30-gm mouse or ~\$130/mouse) were used here, mainly to shorten the exposure time of the emulsion-coated sections.⁵ In other experiments, we have processed brains from animals given the conventional low dose of ^{14}C -2-DG (15 $\mu\text{Ci}/100\text{ gm}$) following the above protocol and used the paraffin sections to expose x-ray film. Exposure time is somewhat longer than for thicker frozen sections, but the resolution is at least as good as with fresh frozen tissue (D. Durham, T. A. Woolsey, and L. Kruger, unpublished data). One advantage of emulsion versus film autoradiography is that the silver grains are directly adjacent to the tissue, facilitating the matching of labeled areas with cytoarchitectonics. Exposure time using film is much shorter, but, due to the increased grain size of the film and the separation between the sections and the film emulsion, there is a concomitant loss of resolution.

Our data show that, with the protocol described, the resolution of the 2-DG technique is increased to the single cell level. Labeling of single cells has been reported previously with emulsion autoradiography but only using thin plastic sections (Basinger et al., 1979; Des Rosiers and Descarries, 1978; Sejnowski et al., 1980) or cryostat sections cut in the dark and caught on emulsion-coated

⁴ It is possible that other fixatives could yield satisfactory results, but we have not yet evaluated this issue systematically. The important point here is that, in our hands, the McLean and Nakane (1974) protocol works.

⁵ At these doses, the cost of ^3H -2-DG is considerable, particularly for larger laboratory animals, such as rats, cats, and monkeys. Therefore, this approach is of value principally in studies where the actual locations of active cells, and perhaps their relative activity, need to be determined precisely. The detailed information that can be obtained from a single experiment is, of course, enormous.

blank slides (Sharp, 1976; Wagner et al., 1979). In our experiments, we have labeled both cell bodies and, in some cases, at least proximal dendritic processes (Figs. 1c and 6b). We also have examples of some cell bodies which are labeled more densely than others (Fig. 6), suggesting the capacity with this technique to measure differences in activity between single cells. Increases in metabolic activity are not confined to somata, however; the neuropil around labeled cell bodies and in other areas also shows a higher grain density. These increases have been verified photographically and quantitatively with computer grain counts.

The significant isotope loss measured during embedding is distressing at first glance, but the appearance of the autoradiograms and the increasing tissue to embedding fluid isotope ratio show that some label is being fixed specifically in the tissue. Since fixation involves a number of chemical reactions at a number of reaction sites and since there is no *a priori* reason to expect that all available carbohydrate moieties, especially 2-DG and 2-DG-6-phosphate, should be fixed, a high dose of ^3H -2-DG may increase the probability that labeled, rather than unlabeled, glucose molecules are fixed. It is also likely that a longer exposure to the perfusate would increase fixation of sugars. Studies are now in progress to address this possibility. Alternately, the isotope loss in tissue processing may actually serve the useful purpose of improving the differentiation between active neurons and surrounding structures.

In these studies, we have modified the 2-DG technique for routine paraffin embedding and emulsion autoradiography. This has many practical consequences for tissue processing and data analysis. The resolution obtained with ^3H -2-DG is superior to the extent of intensely labeling individual neurons. Although isotope cost remains a concern, in many instances, this may be offset by the increased information returned and the shorter time necessary to recover the data. With these improvements, it is now possible to use the 2-DG method to analyze functional activity with great precision in discrete areas, including populations of individual neurons, throughout the nervous system.

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