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[^{3}H]-2-Deoxyglucose autoradiography in a molluscan nervous system

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We have used [^{3}H]2-deoxyglucose autoradiography to correlate the labeling of individual neurons with electrical activity within the central nervous system of a terrestrial mollusc, *Limax maximus*. In an electrically quiescent control preparation where a single neuron is impaled with a glass microelectrode but not stimulated, several somata are uniformly labeled at 3–5 times background. In preparations where a single cell is impaled and stimulated, one or more somata are heavily labeled with [^{3}H]2-deoxyglucose at 10–50 times tissue background. This technique may be useful for surveying metabolically active neurons during spontaneous and driven electrical activity.

Regionally specific uptake of radioactively labeled 2-deoxyglucose (2-DG) is used to identify areas of functional activity within the vertebrate[8] and invertebrate[2,6,7] central nervous system. Quantitative analysis of 2-DG uptake allows determination of relative levels of metabolic activity within the brain[8]. These studies show labeling of large areas of brain in response to selective stimulation compared with unstimulated controls. Recently, cellular resolution of 2-DG labeling has been achieved with [^{3}H]2-DG in cultured mouse neurons[4], goldfish retina[1] and ganglia of the terrestrial mollusc *Limax maximus*[6,7]. In the present communication, we correlate [^{3}H]2-DG labeling in Limax with electrical activation of single nerve cells and with general levels of electrical activity in isolated buccal ganglia.

Laboratory reared slugs were anesthesized by cold. Buccal ganglia were removed and pinned to a layer of transparent resin (Sylgard, Dow Chemical). Dissection and experimentation were carried out in cold saline with increased Mg^{2+} (5 × normal) and decreased Ca^{2+} (0.07 × normal) to depress chemical synaptic transmission (56 mM Na^+; 4.2 mM K^+; 0.5 mM Ca^{2+}; 23 mM Mg^{2+}; 104.5 mM Cl^-; 0.2 mM $H_2PO_4^-$; 2.5 mM HCO_3^-; 5 mM dextrose). Buccal ganglion output was recorded with suction electrodes on peripheral nerve roots. Under trans-illumination, individual nerve cells were impaled with glass microelectrodes (filled with 4 M potassium acetate, resistance

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30–80 MΩ). After penetrating a neuron, saline was replaced with 100 μCi [^{3}H]2-DG/ml saline (New England Nuclear NET 549, sp. act. 40 Ci/mmol). Depolarizing current pulses were then delivered through the microelectrode using a bridge circuit in the amplifier and the impaled neuron driven at 1–2 action potentials/second for 1 h or less. Intracellular and nerve root activity were monitored continuously and filmed directly from an oscilloscope. In a control experiment, a neuron was impaled and incubated as above in [^{3}H]2-DG for 1 h, but not driven with intracellular current injection.

After incubation in [^{3}H]2-DG, ganglia were washed in 5 changes of non-radioactive saline over 30 min. Ganglia were then freeze-substituted in anhydrous acetone at —70 °C for 48 h or acetone dehydrated at 4 °C overnight. These techniques replace water within the tissue with fluid in which 2-DG and 2-DG-6-phosphate are not soluble, thus limiting diffusion of the labeled sugar. Both freeze-substitution and acetone dehydration are sufficient to resolve [^{3}H]2-DG within individual nerve cells[6,7]. Ganglia were embedded in plastic (Araldite or Spurrs's Medium), sectioned at 2–5 μm, and sections floated onto microscope slides on anhydrous glycerol which was subsequently evaporated. Microscope slides were then dipped in Nuclear Emulsion (Kodak NTB-2), exposed for 2–4 weeks at 4 °C, developed (Kodak D-19 for 6 min at 16 °C), and counterstained with 1% toluidine blue or 1% methylene blue. All slides were examined with light- and dark-field illumination, and numbers of silver grains over tissue and emulsion were counted. Cells were considered labeled if grain density was at least 3 × tissue background, and if label could be followed through several serial sections. Resolution was better than 1 μm[7].

In all preparations examined in this study, some, but not all, neuronal somata are labeled. Cell nuclei are less heavily labeled than cell cytoplasm. Thus, [^{3}H]2-DG specifically labels neurons within the central nervous system of Limax (Table I).

The pattern of labeling from stimulated ganglia should be viewed in comparison with the pattern of labeling from an unstimulated, control ganglion (C1 in Table I). In this preparation, a buccal ganglion neuron was impaled but not driven. Very low

TABLE I

Preparation	*Peripheral nerve root activity*	*Total number of cells labeled**	*Mean labeling above background***
F6***	from impaled cell only	1	10 ×
F8***	high spontaneous activity on several roots	8	7 cells: 3–5 × 1 cell: 40 ×
F9***	no recorded activity	5	10–50 ×
C1§	very low spontaneous activity (1–2 spikes over an hour)	9	3–5 ×

* A labeled cell satisfies the following criteria: (1) the density of silver grains over the cytoplasm is at least 3 × tissue background; and (2) the cell body can be traced with label through several serial sections.

** Determined by counting silver grains over cytoplasm and comparing with tissue neuropil background.

*** A single cell was impaled and driven to spike at 1–2 spikes/sec for 1 h or less.

§ A single cell was impaled but not driven.

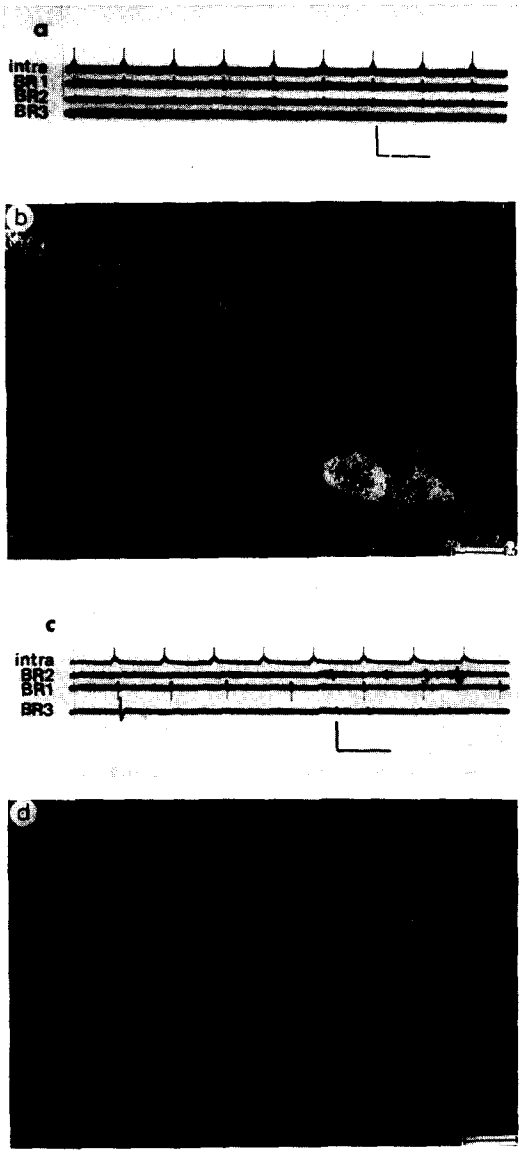

Fig. 1. a: record of intracellular and peripheral nerve root activity during [^{3}H]2-DG incubation. Abbreviations: intra, intracellular record with action potential driven by depolarizing current injection; BR 1, 2 and 3, suction electrode record of buccal nerve roots 1, 2 and 3, respectively. Scale, 100 mV (intracellular trace only); 1 sec. b: dark-field autoradiograph of section of ganglion in 1a, after freeze-substitution. Only one cell body is labeled with [^{3}H]2-DG. Scale, 50 μm. c: record of intracellular and peripheral nerve root activity during [^{3}H]2-DG incubation for another preparation. Abbreviations: intra, intracellular record with action potentials driven by depolarizing current injection; BR 2, 1 and 3, suction electrode records of buccal nerve roots 2, 1 and 3, respectively. Scale, 100 mV (intracellular trace only); 1 sec. d: dark-field autoradiograph of section of ganglion in 1c after freeze-substitution. Several cell bodies are labeled with [^{3}H]2-DG, one very heavily. Scale, 75 μm.

spontaneous peripheral root activity was recorded. Tissue is labeled at about 10 × emulsion background, and 9 cell somata are lightly labeled with [^{3}H]2-DG at about 3–5 × tissue background. Many somata are labeled below the average labeling of the surrounding tissue.

In one experimental preparation (F6 in Table I), where a single neuron was stimulated intracellularly and where peripheral root activity indicated only that cell active (Fig. 1a), only a single cell body is labeled (Fig. 1b). The cytoplasm of this soma is labeled about 10 × more heavily than tissue background. Reconstruction of the ganglion from serial sections places this labeled soma in the same area which had been impaled during the experimental procedure. While it is likely that the labeled cell is the previously stimulated cell, absolute identification of this soma is not possible.

More than one cell was sometimes active within the buccal ganglion, even under conditions designed to reduce chemical synaptic transmission. In such cases, more than one cell body is labeled with [^{3}H]2-DG. For the preparation shown in Fig. 1d (F8 in Table I), a single cell was impaled and driven as described. Although suction electrode recordings indicate electrical activity in a number of neurons, the impaled cell does not have a discernable peripheral process in the roots recorded (Fig. 1c). In autoradiographs from this preparation, 8 cells are labeled: 7 are lightly labeled (about 3–5 × tissue background) and one is heavily labeled (Fig. 1d) (about 40 × tissue background). In another preparation (F9 in Table I), a single cell was impaled and driven, but neither its peripheral process or any other peripheral activity was detected. Five cells are labeled about equally heavily (10–50 × tissue background).

These data indicate that [^{3}H]2-DG labels neuronal cell cytoplasm in buccal ganglia of Limax. Even without driving an impaled neuron, and under conditions which should decrease chemical synaptic transmission, lightly labeled cells were seen in preparation C1. Such labeling could be the result of spontaneous activity from electrically or still chemically coupled interneurons. In preparation F9, a stimulated preparation for which little or no peripheral activity could be recorded with suction electrodes, [^{3}H]2-DG heavily labels a number of neurons uniformly. Spontaneous activity or driven activity from the impaled neuron in interneurons may be responsible for such multiple cell labeling. In addition, metabolic activity in nerve cells not associated with action potentials or sub-threshold electrical activity may account for some of the observed labeling in these and all other preparations.

In experiments where a single cell is driven intracellularly and peripheral activity is recorded, either only one cell is labeled (preparation F6) or one cell is much more heavily labeled than others (preparation F8). In spite of attempts to localize labeled cells by reconstruction of ganglia from serial sections, unequivocal identification of a labeled cell as one which had been impaled and driven will require simultaneous intracellular labeling with another marker, such as the dye Lucifer yellow[10].

In the present experiments, the relative degree of labeling of cells within a preparation was determined by comparing silver grain density above a labeled cell with that above adjacent tissue. However, it was not possible to compare absolute levels of labeling of cells between different preparations. Levels of detected labeling with [^{3}H]2-DG may vary due to differences in intrinsic metabolic rates between prepa-

rations or uncontrolled differences in experimental conditions such as temperature and levels of oxygenation. Furthermore, a variety of differences in the preparation of autoradiograms could contribute to the variations in the intensity of labeling. Examples include freeze-substitution versus acetone dehydration, apposition of tissue to emulsion, and length of exposure and development time.

Buccal ganglia of Limax contain neurons involved in generating feeding motor program[3,5]. The experiments presented here raise the possibility that anatomical analysis of neuronal circuits underlying such behaviors might be possible with [^{3}H]2-DG techniques. However, the labeling of metabolically or spontaneously electrically active neurons may lead to erroneous identification of cells as part of a behavioral circuit. Nonetheless, [^{3}H]2-deoxyglucose autoradiography should prove useful in systems such as Limax, where metabolically active cells located initially with [^{3}H]2-DG can be studied electrophysiologically using intracellular recording techniques.

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