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Mapping of odor-related neuronal activity using a fluorescent derivative of glucose

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Abstract

Activity labeling was applied to the olfactory systems of the terrestrial slug *Limax valentianus* using 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), a fluorescent derivative of glucose. 2-NBDG was incorporated into cultured *Limax* olfactory interneurons, and this was partially blocked by the presence of a high concentration of glucose in the medium, indicating that a part of the uptake of 2-NBDG is mediated by glucose transporters. Next, in order to map odor-related neuronal activity in the primary olfactory center, tentacular ganglion, we injected 2-NBDG into the body cavities of slugs and exposed them to odors or clean air (control). In the odor-stimulated animals, the cell mass region was strongly stained. The digit-like extensions and the neuropil region were also stained in some animals. The control animals showed no staining. The neurons in the cell mass are thought to be involved in generating oscillating activities in the tentacular ganglion, and their activation may imply modulation of oscillatory activity during odor processing. Our results show that 2-NBDG is useful for mapping neuronal activity in vivo.

Keywords: 2-NBDG; Mollusk; Olfaction; Tentacular ganglion; Oscillation

Activity labeling has been widely used for visualizing neurons activated during sensory processing in both vertebrates and invertebrates. Radioisotope-labeled 2-deoxy-D-glucose (2-DG) has been used to detect active cells in which glucose uptake is enhanced [3,15,20]. The 2-DG method has a good spatial resolution and relatively high sensitivity [21]. The major shortcoming of this method is that autoradiography is used for its detection, which requires sectioning of the specimen and exposure to a film or emulsion for up to a few weeks. To overcome these shortcomings, fluorescent derivatives of glucose have been synthesized. One of these derivatives, 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) [25], is taken up by glucose transporters but after phosphorylation in the cell, it is not catabolized and consequently accumulates in the cytoplasm, just in the same way as 2-DG. This technique allows us to detect glucose uptake with a fluorescence microscope immediately after the experiments without further time-consuming processing. 2-NBDG has been used to monitor living *Escherichia coli* [25], and to characterize glucose uptake in hippocampal neurons [19] and in cerebellar Purkinje cells [12] and glial cells [2,17], pancreatic β-cells [24] and in erythrocytes [22].

Activity labeling is useful for identifying neurons involved in sensory processing. The tentacular ganglion (TG) of the land slug *Limax* is the first relay center of olfactory information, and several types of neurons have been described from morphological analyses [10,11]. The TG shows oscillatory activity, like the higher order center, the procerebral lobe [6], and other olfactory centers of both vertebrates [1,5,14] and invertebrates [16]. Olfactory information is believed to be processed not only by afferent neurons but also by local interneurons that produce or modulate oscillatory activities. Our previous studies show that the TG of *Limax* comprises several distinct populations of neurons. However, it is not clear which neurons are activated by olfactory stimuli. In the present study, we used 2-NBDG to visualize neurons in the TG of *Limax valentianus* to answer this question. This is the first report of 2-NBDG application to study sensory coding.

Using cultured *Limax* procerebral neurons, we first examined whether 2-NBDG is incorporated into the cytoplasm of neurons, and whether glucose, which competitively blocks glu-

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cose transporter-mediated uptake of 2-NBDG, can block this incorporation in cultured *Limax* neurons. Culture of procerebral neurons was prepared according to the method described previously [23]. Briefly, the procerebral lobe was isolated from the rest of the cerebral ganglion, digested with protease type IX (Sigma), and cultured on a glass-bottomed dish. The neurons were cultured in standard *Limax* saline which contained (in mM) NaCl 70, KCl 2, $CaCl_2$ 4.9, $MgCl_2$ 4.7, D-glucose 5, HEPES 5, pH 7.6. On the next day, the cells were loaded with 200 μM 2-NBDG dissolved in a modified saline solution which contained either 0 mM or 20–30 mM D-glucose for 30 min at 20 °C. The cells were then washed with standard saline solution for 10 min, and observed with a fluorescence microscope (Olympus, IX70) equipped with a filter set (Olympus, U-MIB/Fluo) and a 40× objective (NA = 0.9). The images were acquired using a cooled CCD camera (Hamamatsu Photonics, Orca-ER). Uptake of 2-NBDG was evaluated by the fluorescence intensity at the soma of the neurons that clearly extended neurites. The background level of fluorescence was subtracted.

The cells incubated with 2-NBDG under the glucose-free condition showed stronger fluorescence than those under the high glucose condition (Fig. 1A–D). Fluorescence intensities at the soma (in arbitrary units, based on the output of the camera) were 37.1 ± 2.0 for the glucose-free condition (mean ± S.E.M., $N = 52$) and 25.9 ± 1.4 for the 20–30 mM glucose condition ($N = 67$), and these values were significantly different (*t*-test, $P < 0.001$; Fig. 1E). This indicates that 2-NBDG uptake was partially blocked by D-glucose, presumably by competition at glucose transporters.

We then injected 2-NBDG into the body cavity of *Limax* and analyzed if any neurons in the TG would be labeled by odor stimulus. Immediately before stimulation with an odorant, 10 mM 2-NBDG dissolved in glucose-free saline was injected into the body of *Limax* at 50 μl/g body weight. The slug was then placed on the wall of a 500 ml glass flask which contained approximately 50 ml of water for moisturization and ventilated with an air pump. The slug did not contact the water. The airflow into the flask was 40 ml/s. For the odor-stimulated slugs, a 100 ml glass flask containing one of the odor sources, 1 ml of 10^{-6} (v/v) 2-ethyl-3-methoxypyrazine (EMOP), 1 ml of 10^{-2} or 10^{-4} (v/v) amyl acetate (AA), or 200 g of mashed carrot, was inserted into the air flow. For the control slugs, a 100 ml glass flask containing 1 ml of distilled water was placed instead. After 4 h, the animal was anesthetized by injection of isotonic Mg buffer ($MgCl_2$ 57.6, HEPES 5, pH 7.6) and the tentacles were dissected. The fluorescence was observed from the ventral side using a confocal laser scanning microscope (Zeiss, LSM510) with a 488 nm Ar/Kr laser and a 20× objective (NA = 0.5). Stacks of optical sections were combined and the background level was subtracted.

In odor-stimulated animals, fluorescence was observed in the cell mass (CM), the digits, the neuropil of the TG and the procerebral lobe in the cerebral ganglion (Fig. 2). Among these regions in the TG, the digits and the cell masses are cell body rich regions [10]. The strongest fluorescence was observed in the CM, and this was consistent among all the odor-stimulated animals with only one exception in a slug stimulated with 10^{-4} AA. Only weak staining was observed in the CM of the control animals (Fig. 2I and J). Staining was found in 34 out of 48 odor-stimulated tentacles. Among these 34 stained tentacles, 20 tentacles showed staining in both sides of the CM (Fig. 2B–H). The digits were stained only when other regions were also stained strongly, with only one exception in a slug stimulated with 10^{-2} AA. The neuropil of the TG was also broadly stained in some preparations. Many small ganglion cell-like cells were observed in the digits (Fig. 2K) and the cell mass (Fig. 2L). Gamma cell-like large neurons were sometimes stained (Fig. 2K). The fiber bundles leaving the TG from the CM and entering the tentacular nerve were also observed in some preparations (Fig. 2L). We could see some fibers running beneath the sensory epithelium in the tip region of the digits (Fig. 2M). In some animals stimulated with odors, the procerebral lobe was also stained (Fig. 2N), while no staining was observed in the procerebral lobe of the control animals (Fig. 2O). Table 1 summarizes the occurrence of staining in the digits, the CM, the neuropil of the TG and the procerebral lobe.

Only weak or no staining was observed in the slugs stimulated with a low concentration (10^{-4}) of AA (Table 1). Although this concentration of AA can produce a much greater electro-

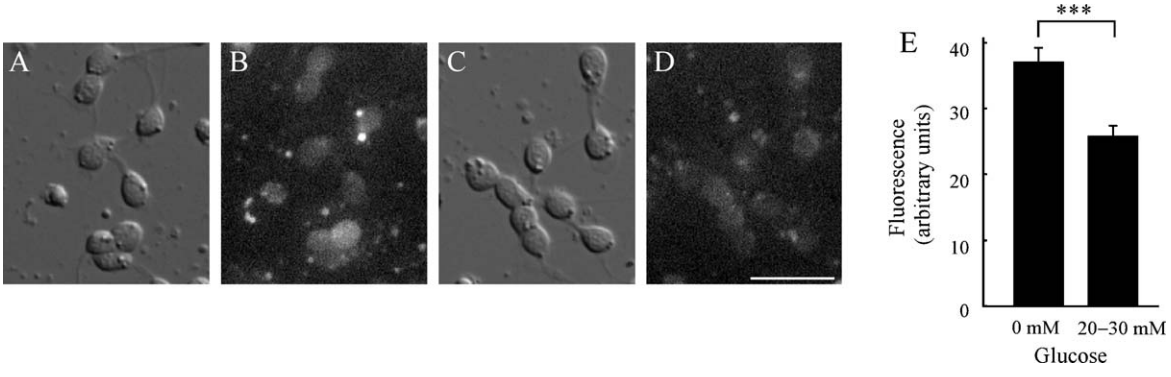

Fig. 1. Uptake of 2-NBDG in cultured procerebral neurons. Nomarski (A) and fluorescence (B) images after incubation with NBDG in glucose-free solution. Nomarski (C) and fluorescence (D) images after incubation with 2-NBDG in 30 mM glucose solution. Scale bar is 20 μm. (E) Summary of fluorescence intensities in the cell bodies incubated with 2-NBDG in glucose-free and high (20–30 mM) glucose solutions. The cells under these two conditions showed significantly different levels of fluorescence (*t*-test, $^{***}P < 0.001$).