2-Deoxyglucose Labelling of the Infrared Sensory System in the Rattlesnake, *Crotalus viridis*

EDWARD R. GRUBERG, ERIC A. NEWMAN, AND PETER H. HARTLINE
Biology Department, Temple University, Philadelphia, Pennsylvania 19122 (E.R.G.), Eye Research Institute of Retina Foundation, Boston, Massachusetts 02114 (E.A.N., P.H.H.)

ABSTRACT

Infrared (IR) responsive nuclei in the rattlesnake *Crotalus viridis* were identified by using ¹⁴C-2-deoxyglucose (2DG) and autoradiography. Following 2DG intracardial injection, the IR-sensitive pit organ was stimulated periodically with an IR stimulus for 5 hours. The nucleus of the lateral descending trigeminal tract (LTTD, the primary IR sensory nucleus) was labelled heavily with 2DG. Labelling was bilateral, but somewhat heavier ipsilaterally to the stimulated pit organ. The nucleus reticularis caloris (RC, the secondary nucleus of the IR system) was lightly labelled ipsilaterally. The middle laminae of the contralateral optic tectum (which contain IR-responsive units) were distinctly labelled; the corresponding layers of the ipsilateral tectum were lightly labelled. A subcerebellar nucleus not known to be part of the IR system was heavily labelled bilaterally. No consistent labelling was found in the diencephalon or telencephalon.

Since units in the LTTD do not respond to stimulation of the contralateral pit yet the LTTD is labelled with 2DG when there is contralateral pit stimulation, several controls were carried out. Unilateral injection of ³H-proline into LTTD revealed no projection to the contralateral LTTD. In a monocularly, visually stimulated animal with both pits occluded, the LTTD still showed heavy but equal 2DG labelling bilaterally. In addition, the outer layers of the contralateral optic tectum were heavily labelled. No 2DG labelling of the LTTD was obtained when branches of the trigeminal nerve innervating the LTTD were previously cut.

These results suggest that much of the 2DG labelling in the LTTD is due to spontaneous ongoing activity from the pit organ rather than from IR evoked activity. Using single-unit recording, almost all LTTD units were found to have high spontaneous firing rates. IR stimulation increases the firing rate above spontaneous levels. When the afferent trigeminal nerves were cut, LTTD cell activity was almost totally silenced.

Key words: 2-deoxyglucose, infrared stimulation, rattlesnake, autoradiography

The infrared sense of rattlesnakes and other pit vipers is specialized in detection and localization of remote infrared sources such as the snake's warm-blooded prey (Noble and Schmidt, '37). Evidently, a part of the generalized somatic sensory system has evolved into this specialized sense. Thus, the pit viper provides an opportunity to examine ways in which thermoreceptors, sense organs, and the brain are modified to accommodate a new sensory function.

In this paper we address the problem of identifying the central pathways by which infrared sensory information is distributed in the brain. The infrared-sensitive pit organ is innervated by the trigeminal nerve. Molenaar ('74) described a trigeminal nucleus that was found only in infrared-sensing snakes, the nucleus of the lateral descending trigeminal tract (LTTD). Schroeder and Loop ('76) showed that the LTTD receives afferent input from the ipsilateral pit organ. Horseradish peroxidase (HRP) studies (Gruberg

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et al., '79; Newman et al., '80; Kishida et al., '82) and silver
dergination studies (Stanford et al., '81) showed that in
rattlesnakes the LTTD projects to a nucleus in the ipsilat-
eral lateral reticular formation, the nucleus reticularis ca-
oris (RC). Following their LTTD lesions, Stanford et al.,
('81) identified a region of degeneration dorsal and anterior
to the RC, beneath the cerebellum which they suggested
might be part of the infrared sensory pathway. They provi-
sionally called this region the lateral tegmental nucleus
(LT). Neurons in the LTTD (Terashima and Goris, '77; Stan-
ford and Hartline, '78, '84) and in the RC (Newman et al.,
'80) are responsive to infrared stimulation.

The central layers of the optic tectum, which contain
many infrared sensitive neurons (Goris and Terashima, '73;
Hartline, '74; Kass et al., '78) receive input from the RC, as
was shown by HRP methods (Gruberg et al., '79; Newman
et al., '80). There have been no reports of infrared respons-
ive regions in the thalamus, cerebellum, or forebrain from
either anatomical or electrophysiological studies.

In the present investigation, we studied the infrared path-
way using the uptake of 14C-labelled 2-deoxyglucose (2DG)
as a marker for neural metabolic activity and hence of
inferred electrical activity. Our chief objectives were (1) to
confirm or extend knowledge of the infrared pathway be-
tween LTTD and optic tectum; (2) to look for evidence of
infrared driven activity in the LT of Stanford et al., ('81) and
(3) to search for other brain regions where infrared driven
activity might be present, such as the thalamus and fore-
brain. A preliminary summary of this work has been re-
ported (Gruberg et al., '82).

METHODS

We used Southern Pacific rattlesnakes, Crotalus viridis,
weighing 200–250 gm.

2DG labelling

Animals were anesthetized by chilling them in crushed
ice. The heart was then exposed, and 25 or 50 µCi of 14C-2-
deoxyglucose (2DG, New England Nuclear), dissolved in 0.2
ml saline, was injected into the ventricle. The larger amount
decreased the exposure time needed to produce adequate
labelling. Xylocaine was applied to the incision and the
wound was taped closed. The animal was restrained in a
prone position by taping it to a board and was warmed to
room temperature (20–22°C).

Following the 2DG injection the animal was stimulated
unilaterally with either an infrared or visual stimulus (see
below) for 5 hours. At the end of this period the snake was
decapitated and its head was cooled in an ice bath. The
brain was quickly removed and frozen by immersion in 2
methyl-butane chilled by liquid nitrogen.

The brain was processed for autoradiography by a freeze-
drying technique. The frozen brain was freeze-dried for 2
days at −40°C at a pressure less than 0.01 torr. The tem-
perature was raised gradually to −20°C over an additional
2-day period and then raised to room temperature in 2–3
hours while the brain was continuously under vacuum. The
brain was then fixed over the fumes of paraformaldehyde
for 1.5 hours at −4°C and vacuum embedded in paraffin in
sections were cut at 12 µm and placed on dry, uncoated
slides which were then warmed to 37°C to promote
flattening.

Clean microscope slides were coated with a thin layer of
Kodak NTB-2 nuclear track emulsion. Emulsion-coated
slides were abutted against the slides containing the la-
belled sections and held together with binder clips (similar
to the method of Buchner et al., '79). These slide pairs
were exposed in the dark for 2–6 months at 4°C. Following this
exposure, the slides coated with emulsion were developed
using standard techniques for autoradiography (Cowen et al.,
'72).

One advantage of this freeze-drying technique over the
standard cryostat sectioning technique is that once the
brain is frozen, there is negligible spread of 2DG due to
thawing. When cryostat sections are thawed on warm slides
on the other hand, loss of resolution due to spreading can
occur. In addition, in the method described here, the sec-
tions themselves are available for histological study after
the emulsion-coated slides have been developed.

Stimulation during 2DG labelling

IR stimuli. One pit organ was exposed intermittently to
a warm black surface subtending an area 100° in diameter.
This warm surface was occluded by rotating thermoneu-
tral cardboard disc containing pie-section cutouts, produc-
ing a moving stimulus that swept across the pit’s field of
“view,” exposing most receptors of the pit for 0.5 seconds
every 2.0 seconds. We chose a moving border of moderate
radiation contrast that swept across almost the entire field
of view of the pit organ in order to maximize stimulation of
infrared responsive neurons within the brain (see Discus-
sion). The infrared stimulus produced an energy flux of 5.6
mW/cm² at the pit organ. During infrared stimulation, the
contralateral pit organ and both eyes were covered with
aluminum foil blinder held in place with an opaque mix-
ture of lampblack and Vaseline (petroleum jelly). Stimula-
tion took place in a quiet, darkened room.

Visual stimuli. One eye was stimulated with a white
rotating disc (rotational period 6 seconds) containing black,
irregularly spaced pie sections. The disc subtended a visual
angle of 120°. The contralateral eye and both pit organs
were covered with aluminum foil. Stimulation occurred in
a quiet, normally lighted room.

LTTD deafferentation

In one experiment the trigeminal nerve branches project-
ing from the pit organ to the LTTD were severed on one
side of the head. A snake was anesthetized with Metofane
(Methoxyflurane) anesthesia and the superficial and deep
branches of the superior maxillary trigeminal nerve were
severed just distal to the Gasserian ganglion. The oph-
thalmic branch was cut as it exited from the pit organ.

Labelled proline injection

The efferent projections of the LTTD were traced by au-
roradiography. Following electrophysiological localization
of the nucleus, 25 µCi of 3H-proline was injected into the
nucleus. The animal was maintained for 2 days at 20°C
and then anesthetized and decapitated. The brain was fixed
overnight in a solution containing 1 ml 37% formaldehyde,
1 ml glacial acetic acid, and 18 ml 80% ethanol. It was then
dehydrated, cleared in cedarwood oil, and embedded in par-
affin. Fifteen-micron serial sections were cut and coated
with Kodak NTB-2 emulsion. The slides were exposed for
21 days and developed by the method of Cowan et al., ('72).
2DG LABELLING OF RATTLESNAKE IR SYSTEM

Electrophysiology

In an experiment that explored the origin of spontaneous activity in the LTTD, one snake was prepared by cutting the ophthalmic branch of the trigeminal nerve at the pit organ. The superficial and deep superior maxillary branches were then pulled gently outwards through a surface incision, by using a loop of silk thread. This allowed electrophysiological recordings to be made from the same site in the LTTD both before and after the trigeminal branches were cut. Single- and multiunit activity was recorded through metal microelectrodes plated with platinum black (Dowben and Rose, '53). The electrophysiological preparation has been described previously (Newman et al., '80).

In an experiment designed to verify the effectiveness of the infrared stimulation regime that was used for 2DG uptake experiments, single-unit activity was recorded from the deeper layers of the optic tectum by using micropipettes filled with 3 M NaCl.

RESULTS

IR stimulation

The pattern of 2DG labelling following unilateral infrared stimulation was similar for all three snakes used. The LTTD nucleus was heavily labelled. As shown in Figure 1, the labelling was bilateral but was somewhat heavier ipsilaterally to the stimulated pit organ. Light labelling of the ipsilateral RC nucleus was seen in two of the three brains (Fig. 2). The middle layers of the contralateral optic tectum were well labelled in all three brains. As illustrated in Figure 3, labelling was confined to a broad, definite band encompassing the intermediate layers and extending from the medial tectum to the lateral extreme of the tectum. There was sparse labelling in the equivalent layers of the ipsilateral tectum in two of the three brains. A discrete region at the level of the eighth nerve root just below the floor of the fourth ventricle was heavily labelled bilaterally in all three brains (Fig. 2). Although areas of the diencephalon were lightly labelled bilaterally, no area was consistently labelled unilaterally in either the diencephalon or telencephalon. Nor was labelling seen in the region of the hypothalamus (bilaterally).

Deafferented LTTD nucleus

We investigated the effect of cutting all three branches of the trigeminal nerve innervating the pit organ on one side of the head in a snake that had both pits and both eyes occluded during 2DG injection. Under these conditions, there was no 2DG labelling in the LTTD nucleus ipsilaterally to the cut nerve. But heavy labelling persisted in the contralateral LTTD (Fig. 7). The subcerebellar nucleus was labelled bilaterally in this snake, but neither the RC nucleus nor the tectum showed any labelling.

Unit activity in the LTTD

The results described above suggest that ongoing activity in the afferent fibers to the LTTD is sufficient to produce heavy 2DG labelling in unstimulated snakes. We obtained support for this supposition by recording single- and multiunit activity from the LTTD of a lightly anesthetized snake. Neurons of the LTTD have a high level of ongoing (unstimulated) activity, as shown by multiunit recordings made with large electrodes (Fig. 8A). Almost all LTTD single units we recorded had high firing rates (10–18 spikes per second) in the absence of infrared stimulation. In several units we measured ongoing activity and then stimulated the ipsilateral pit organ with an infrared stimulus similar to that used in the 2DG experiments (0.5-second stationary flashes repeated every 2 seconds having an intensity of 4.8 mW/cm² at the pit organ). During each infrared flash, the activity of single units increased above the unstimulated level, while in the inter-flash intervals, activity was depressed below the normal unstimulated level.
Fig. 1. Autoradiogram of transverse section of the medulla at the level of the LTTD after 2DG injection and unilateral IR stimulation of the right pit. Note that the right LTTD is more heavily labelled than the left LTTD. Scale bar in this and subsequent Figures is 1 mm unless otherwise noted.

Fig. 2. Autoradiogram of transverse section of the medulla at the level of the RC nucleus. Same brain as in Figure 1. The right RC is labelled lightly (arrow). Additional bilateral labelling is seen in the reticular region and in a subcerebellar nucleus (arrowheads) just below the surface of the IV ventricle.

Fig. 3. Autoradiogram of transverse section of midbrain. Same brain as in Figure 1. The intermediate layers of the left (ventrolateral) tectum are well labelled.

Fig. 4. Autoradiogram of transverse section of medulla at the level of the LTTD showing $^3$H-proline injection site. Inset: Autoradiogram of ventrolateral region of transverse section of medulla at the level of the RC nucleus. Same brain as in main figure. Labelling is around the large, sparsely distributed cells of the RC. Scale bar = 206 μm.
Fig. 5. Autoradiogram of transverse section of medulla at the level of the LTTD after 2DG injection and monocular visual stimulation but without IR stimulation to pit. In this case there is equal labelling of both LTTD nuclei.

Fig. 6. Autoradiogram of transverse section of midbrain. Same brain as in Figure 5. The outer layers of the tectal lobe contralateral to the stimulated eye are labelled but the intermediate layers are virtually unlabelled. Compare to Figure 3.

Fig. 7. Autoradiogram of transverse section of medulla at the level of the LTTD after cutting the branches of the trigeminal nerve innervating the left pit. Both pits and both eyes were occluded during 2DG uptake. The deafferented LTTD shows no labelling.

Fig. 8. Electrical activity from the LTTD nucleus recorded with a large-tipped microelectrode. A. Ongoing (unstimulated) activity from the normally innervated LTTD nucleus. B. Ongoing activity (only one active unit) recorded from the opposite LTTD nucleus, deafferented 24 hours previously. Records A and B were made with the same electrode within minutes of each other. The activity patterns in A and B are typical of those recorded from the two LTTD nuclei in many penetrations.
The average firing rate of LTTD units (averaged over both "on" and "off" phases for at least 80 seconds) was greater for the stimulated than for the unstimulated case. For single units, the increase in average activity ranged from 10% to 300%.

We tested whether deafferentation reduced the spontaneous activity of LTTD units as might be inferred from our 2DG experiment. Large-tipped multiunit metal electrodes were used to monitor LTTD activity before (Fig. 8A) and after (Fig. 8B) we cut the two major nerve branch afferents to the LTTD. The ongoing firing of LTTD units ceased almost completely when the afferents were cut. This decreased activity level remained when we retested the animal the next day. The LTTD was completely silent except for rare units which we could not drive with infrared stimuli and which fired in bursts at very regular rates of approximately 3 bursts per second (Fig. 8B). At most, only one or two of these units were encountered per penetration of our large, multiunit electrodes.

Unit activity in the optic tectum

Since infrared-stimulated 2DG labelling in the RC nucleus and in the optic tectum was relatively light, we optimized our infrared stimulus, using single-unit activity recorded from the optic tectum to assay stimulus efficacy. A moderate strength, periodic infrared stimulus (0.5-second every 2 seconds. 4.8 mW/cm² at the pit organ) was found to generate a high level of activity in infrared tectal units when averaged over many cycles as it did in the previously described LTTD experiments. In the absence of infrared stimulation there was little activity in most tectal infrared units. This contrasts with the situation in the LTTD.

DISCUSSION

The pattern of infrared-stimulated 2DG labelling that we found confirms that the LTTD, the RC nucleus and the middle layers of the optic tectum are all part of the infrared sensory pathway of the rattlesnake. These structures are known to contain many neurons that are responsive to infrared stimulation. Though we used a stimulus regime known to activate infrared units effectively, 2DG uptake in the tectum, RC, and LTTD that can be attributed to infrared stimulation was relatively modest.

We found that the LTTD ipsilateral to the stimulated pit organ was more heavily labelled than the contralateral LTTD in all three of our infrared-stimulated snakes. However, the contralateral LTTD was heavily labelled, and the contrast between the two sides was not dramatic. Labelling in the contralateral LTTD was not due to inadvertent infrared stimulation of the opposite pit, because the pit was completely occluded with an aluminum foil blinder sealed with opaque petroleum jelly.

The heavy bilateral labelling of the LTTD was the most surprising finding of our 2DG experiments. Our control experiments showed that the trigeminal fibers innervating the LTTD on the unstimulated side are responsible for activating LTTD neurons even in the absence of specific infrared stimulation. Cutting the afferents to the LTTD silences its neurons and eliminates 2DG uptake.

In retrospect, the bilateral labelling of the LTTD is in consonance with other known properties of this nucleus and its sensory input. Primary fibers projecting from the pit organ to the LTTD have a high level of unstimulated activity (Bullock and DIECKE, '56). The LTTD itself was known to have much ongoing activity in the absence of stimulation (Stanford and Hartline, '84). In addition, the LTTD may have a high level of metabolic activity, as suggested by the high density of capillaries present in the nucleus (Messier et al., '81).

In a study similar to ours of the rattlesnake infrared system, Auker et al. ('83), found heavy 2DG labelling only in the LTTD ipsilateral to a pit that was stimulated with intermittent infrared radiation. However, the opposite pit was excised. Thus, the effect of infrared stimulation of the first pit and of removing the ongoing afferent input to the opposite LTTD were combined. We believe that most of the labelling that they found in the LTTD ipsilateral to the stimulated pit was not due to infrared stimulation, but to activity in the nucleus, driven by ongoing activity in the uncut afferents.

The RC nucleus, which receives a direct projection from the LTTD, was labelled unilaterally, but only lightly in our experiments. RC neurons respond robustly to infrared stimulation of the type used in this study (Newman et al., '80). Thus, there must be only weak 2DG uptake associated with the firing of RC cells. The paucity of labelling may be accounted for by the relatively small number of large neurons in the RC (Newman et al., '80) which have relatively small total surface area and thus, a low metabolic activity rate per unit of tissue volume (Schwartz et al., '79). Auker et al. ('83) did not report 2DG uptake by the RC.

The intermediate laminae of the tectal lobe contralateral to the stimulated pit were well labelled following infrared stimulation. This labelling pattern confirms previous electrophysiological results (Kass et al., '78; Newman and Hartline, '81) which demonstrate that infrared-responsive neurons are localized in these tectal laminae. The 2DG labelling pattern contrasts with the one observed following visual stimulation where only the superficial layers are labelled. These layers are known to receive afferent input from the eye. The low density of labelling of the intermediate tectal layers ipsilateral to the stimulated pit could be due to infrared activity in this region driven by tectal commissural fibers. There has been one report of tectal cells that could be evoked by stimulation of either pit (Goris and Terashima, '73).

Auker et al. ('83) found no tectal labelling with 2DG after infrared stimulation for either a 2 Hz or 0.1 Hz stimulus frequency. They concluded that tectal IR neurons, although activated by infrared stimulation, do not take up much 2DG and suggested that this could be accounted for if 2DG transport is largely determined by synaptic activity. The difference between our results and theirs may be due to the fact that they used significantly shorter stimulation times than we did (1.25 hours compared to 5 hours) as Auker et al. ('83) pointed out. However, there are three other differences between our stimulus regime and that used by Auker et al. First, the stimulus frequency that we used was 0.5 Hz, providing time for recovery between stimuli and reducing the effect of response decrement with repetition (such as Auker et al. found for their 2-Hz stimulus); our stimulus was repeated five times as often as the stimulus in the 0.1 Hz regime of Auker et al. Second, we chose a broad-field stimulus with a warm-cold border that swept most of the pit's field of view every 2 seconds. Hartline et al. ('78) emphasized that a moving stimulus that sweeps through a tectal unit's receptive field is particularly effective in evoking responses. Finally, the fact that the stimulus swept most of the pit's field of view meant that most infrared responsive cells in the entire tectum were equally and
strongly stimulated. Auker et al. used a small-field stationary stimulus (a heat lamp at 30 inches, which subtends less than 10°). Stimulation from such a source probably does not generate excitation over as large a tectal area as did our broad-field moving stimulus. For all of these reasons, it is likely that our stimulus regime evoked more intense and more broadly spread activation of infrared responsive neurons than did the regime used by Auker et al.

In our study, we found heavy 2DG labelling of the superficial layers of the tectum contralateral to an eye that was stimulated with a pattern of moving white and black stripes for 5 hours. We also found labelling in several other known visual structures in the thalamus and midbrain. The density of labelling in the visual layer of the tectum was substantially greater than was infrared-related labelling in the deeper tectum. Auker and Carpenter ('77) and Auker et al. ('83) found an even greater difference between infrared and visual stimulation (no infrared-related label detectable). They suggested that the difference should be attributed to differences in synaptic activity evoked by the different sensory stimuli rather than to differences in spike-related 2DG transport. It is difficult to interpret our data as supporting or detracting from their suggestion. Equivalence of stimulation across modalities is virtually impossible to attain. While some infrared cells may be more strongly activated by a chosen infrared stimulus regime than are some visual cells by a visual regime, there is no way of knowing how many cells in either system are activated by their respective stimuli. There is a much higher density of cells in the superficial (predominantly visual) layers of the tectum than in the deeper layers that contain infrared sensitive cells (Kass et al., '78); deep layer cells tend to be large and sparse. This would tend to make labelling caused by visual stimulation appear much more dramatic than labelling caused by infrared stimulation.

One of the purposes of this study was to search for brain regions other than the LTTD, the RC, and the tectum that might also contain infrared responsive neurons. Besides the LTTD, the only prominently labelled structure in our 2DG experiments was a subcerebellar nucleus, which was labelled bilaterally. This nucleus was labelled equally well when the snake was stimulated with infrared or visual stimuli, or not stimulated at all. Thus, this structure is probably not a part of the infrared sensory system, but rather takes up 2DG in the absence of known specific sensory stimulation.

After lesion of the LTTD, Stanford et al. ('81) traced degenerating fibers to an ipsilateral field that they designated the lateral tegmental nucleus (LT). LT is located just rostral of the principal sensory nucleus of the trigeminal nerve, and ventral and lateral to the cerebellum. The lateral tegmental nucleus may be the same region between the trigeminal nerve root and the posterior limit of the tectum that we identified by autoradiography following injection of labelled proline into the LTTD. We found no region in this vicinity that took up 2DG in our infrared stimulation experiments. However, it is possible that in both the degeneration experiments of Stanford et al. ('81) and in our proline experiments, the same structure outside of the LTTD was inadvertently involved. The lack of 2DG label would in this case be the expected result. Lack of 2DG label does not rule out the possibility that the LT is part of the infrared system, however, especially since in known infrared nuclei, stimulus-related labelling was light.

We were unable to identify any infrared-responsive structures in either the diencephalon or telencephalon that were consistently labelled asymmetrically with 2DG. Nonetheless, we have identified electrophysiologically two structures in these regions—a rostral portion of the dorsalventricular ridge, and a region in the ventrolateral thalamus (most likely the nucleus rotundus)—which contain infrared-responsive cells (unpublished observations). We are uncertain whether our negative 2DG results in these areas are due to a weak correlation between electrical activity and 2DG uptake in Crotalus or due to limitations of our technique, such as suboptimal stimulation of neurons in these regions. An effective stimulus regime for activating tectal neurons may not necessarily activate forebrain neurons well.

In summary, using the 2DG technique combined with unilateral infrared stimulation we found heavy labelling of the LTTD bilaterally but somewhat heavier labelling ipsilaterally. Much of the LTTD labelling appears to be due to spontaneous activity and not from specific infrared stimulation. We also found light labelling of the ipsilateral RC nucleus, significant labelling of the middle layers of the contralateral tectum, and light labelling of the middle layers of the ipsilateral tectum.

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