Dark Neurons in the Mouse Brain: An Investigation into the Possible Significance of Their Variable Appearance within a Day and Their Relation to Negatively Charged Cell Coats

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Summary. This study aims to investigate the occurrence and nature of dark neurons in the central nervous system under physiological conditions. Mouse brain tissues were perfusion-fixed with paraformaldehyde or glutaraldehyde at 4 h intervals during one day (3:00, 7:00, 11:00, 15:00, 19:00, 23:00). Paraffin sections were stained with the cationic colloidal iron method, and counterstained with nuclear fast red or carbol-thionin.

The dark neurons were readily distinguishable as their shrunken cell bodies stained densely with nuclear fast red or thionin. Some of the dark cells were coated with perineuronal sulfated proteoglycans; this coat, which formed a smoothly extended meshwork in light cells, presented spicule-like forms in the dark cells.

The occurrence of dark cells in the retrosplenial cortex varied by the time of day: the incidence of the dark neurons was low (10-15%) at 11:00, 15:00 and 23:00, while it was significantly high (50-60%) at 3:00 and 19:00. Previous authors have ascribed the occurrence of dark neurons either to artifacts due to inappropriate fixation or to pathological damage. However, the present study strongly suggests that this type of neuron occurs under physiological conditions as reversible changes, and vary over a day, showing distinct peaks. These peaks occurred coincidentally while the mice were awake. Such morphological changes may be involved in the neuronal activation and exhaustion. Our view is consistent with the hypothesis (TEWARI and BOURNE, 1963) that the neurons take such dark profiles at certain stages of neurosecretion.

In these studies, some nerve cells, including those with the perineuronal surface coats, had profiles of dark neurons, whose condensed cell bodies were intensely stained with nuclear fast red and carbol-thionin (MURAKAMI et al., 1995a, b).

Several pathological conditions, such as hypoxic ischemia (BROWN and BRIERLEY, 1968), epileptic damage (SLOVITER, 1983), severe hypoglycemia (AGARDH et al., 1980; AUER et al., 1985), deafferentation of vestibular nuclei (JOHNSON, 1975), and cryogenic lesion (LØBERG and TORVIK, 1993) have been reported to cause brain cell damage characterized by the appearance of dark neurons. However, TEWARI and BOURNE (1963) suggested that the occurrence of dark neurons might be concerned with the physiological secretory activity of the cells. The present study shows that the population of dark cells in the mouse retrosplenial cortex varies by the time of day, and demonstrates that a part of the dark neurons possess distinctly negatively charged surface coats, as stained with our cationic colloidal iron.

MATERIALS AND METHODS

Male ICR mice (10 week-old) were used in this study. They were kept on ordinary light (6:00–18:00) and dark (18:00–6:00) cycles with free access to water and feeding. Under ether anesthesia, each animal was perfused through the cannulated left ventricle with physiological saline and successively with 4% paraformaldehyde or 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The brain tissue blocks (1-2 mm thick) were excised, immersed in the same fixative for 8 h at room temperature, and processed for paraffin sectioning. Then, the sections were incubated
in cationic colloidal iron sol with pH values of 1.0-1.5 (MURAKAMI et al., 1986), treated with 1% K₃[Fe(CN)]₆ in 0.1 N HCl for Prussian blue reaction, and counterstained with nuclear fast red or carbol-thionin. Some sections were stained solely with carbol-thionin for Nissl preparation. Stained specimens were observed with a photomicroscope (BX50, Olympus).

To examine DNA fragmentation, the nick end labeling method (GAVRIELI et al., 1992; NITATORI et al., 1995) was applied. Briefly, the sections were pretreated with 20 μg/ml proteinase K (Boehringer-Mannheim) in 0.01 M tris buffer, pH 7.4, for 15 min at room temperature. After treatment with 2% H₂O₂ aqueous solution, they were incubated with 300 U/ml terminal deoxynucleotidyl transferase (TdT) (Gibco BRL) and 40 nmol/ml biotinylated 16′-2′-dUTP (Boehringer-Mannheim) in TdT buffer (Gibco BRL) for 1 h at 37°C. Then, the sections were blocked with 1% bovine serum albumin, and treated with peroxidase-conjugated streptavidin (Vector). Labeled sites were visualized with a diaminobenzidine reaction.

Cell count study

Eighteen male mice were divided into six groups. Mice of each group were killed at 3:00, 7:00, 11:00, 15:00, 19:00 or 23:00. Twenty serial sections as shown in Figure 1 were cut from one block and stained as above; four sections from the serial sections were used for the cell count study. Every left retrosplenic cortex was photographed (Fig. 1). In layers III-V, the populations of all nerve cells, dark cells and neurons with negatively charged surface coats and dark cells with such coats were counted. Each cell population ratio to all neurons was calculated; the mean ratio in each animal group was calculated with standard deviation.

RESULTS

Perfusion-fixation well preserved the delicate cellular and intercellular structures in the mouse brain. Neurons with negatively charged surface coats were confirmed again, particularly in the cerebral cortex, hippocampus, intracerebellar nucleus, and certain other areas, as shown in our previous studies (MURAKAMI, 1994; MURAKAMI et al., 1994b). The surface coats were stained distinctly and intensely with cationic colloidal iron even at pH 1.0-1.5, and formed a fine meshwork around the nerve cell bodies and roots of the processes (Figs. 2, 2 lower inset, 3 center and 3 left).

Among the usual, lightly stained neurons were recognized dark neurons in the cerebral cortex, hippocampus, cerebellar cortex, intracerebellar nucleus, and certain other areas. Their cell bodies, including nucleus were densely, i.e. intensely stained with nuclear fast red and carbol-thionin (Figs. 2 upper inset, 3 right, 4a, b, 5b). The dark neurons revealed shrunken cell bodies with condensed cytoplasm. The sizes of the cell bodies were reduced to two thirds or one half the length of the light cells. When dark neurons possessed negatively charged surface coats, their surface coats appeared as spicules studded around the cell body (Figs. 2 upper inset, 3 right, 5b inset).

Sections whose adjacent sections contained many dark neurons in the cerebral cortex, hippocampus and intracerebellar nuclei showed negative labeling of the DNA nick end in the same regions.

Cell count study

Under ordinary light and dark cycles, mice were sleeping or resting at the experimented times of 7:00, 11:00 and 15:00, and eating and playing at 3:00 and
In the retrosplenial cortex as shown in Figure 1, the mean population ratio of dark cells, nerve cells with negatively-charged surface coats and dark cells with such coats of each animal group are summarized in Table 1. The present cell count study of the retrosplenial cortex showed that, at 3:00 and 19:00, the dark cells occurred in abundance (50-60% of the total nerve cells) (Figs. 4a, b, 5b, 6, Table 1). At 11:00, 15:00 and 23:00, the population of dark cells decreased (10-15%) (Figs. 4c, d, 5a, 6, Table 1).

In the retrosplenial cortex, the nerve cells which possessed negatively charged surface coats occurred at a frequency of 7-13%, which was almost constant during the one day of experimentation (Table 1). While not all, many nerve cells which had negatively charged surface coats showed a dark neuron profile at 3:00 and 19:00. The occurrence of dark neurons possessing a negatively charged surface coat varied concomitant with the rise and fall of the total population of dark cells.

![Fig. 2. Neurons with negatively charged surface coats, as seen in the hippocampal subiculum. Glutaraldehyde fixed, and cationic colloidal iron (pH 1.5) plus nuclear fast red stained sections. Main figure. A typical light neuron. Note the surface coats covering the cell body (arrowheads) and process (P, double arrowhead). Lower inset. A similar light cell showing a fine meshwork of surface coat form (arrowheads). Upper inset. A typical dark neuron with a negatively charged coat. Note the spicule-like structure of the surface coat (small arrowheads). X 1,300, insets: x 1,300](image1)

![Fig. 3. Neurons with negatively charged surface coats, as seen in the cerebral cortex. Glutaraldehyde fixed, and cationic colloidal iron (pH 1.5) plus carbol-thionin stained sections. Main two figures, the Left hand one showing a high power view of the Central one. The surface coat covers the nerve cell body (arrowheads) and the process (double arrowhead). Note the fine meshwork of the surface coat (arrow). Right figure. A dark neuron with a negatively charged coat. Note the condensed cytoplasm and shrunken cell body and process; also note the spicule-like formation of the surface coat (small arrowheads). Center: x 500, left and right figures: x 1,300](image2)
DISCUSSION

In previous studies, the dark neurons have been suggested to occur as artifacts due to inadequate fixation (Cammermeyer, 1961; Stensaas et al., 1972) or as pathological damage such as hypoxic ischemia (Brown and Brierley, 1968), deafferentation of vestibular nuclei (Johnson, 1975), insulin-induced severe hypoglycemia (Agardh et al., 1980; Auer et al., 1985), epileptic stimulation (Sloviter, 1983), cryogenic lesion (Løberg and Torvik, 1993), or repeated anodal polarization (Islam et al., 1994). In the present study, however, all mice were maintained under similar physiological conditions, and fixation was carefully performed using a perfusion method. In our previous experiments, cationic colloidal iron reaction to the perineuronal negatively charged surface coats could not be detected when fixation was inadequate or delayed (Murakami et al., 1993a). In all specimens of the present study, such delicate perineuronal surface coats were clearly recognized.

Fig. 4  Retrosplenial cortex at different times of a day. Paraformaldehyde fixed and Nissl stained sample. a and b. At 3:00 am, most neurons exhibit a dark profile; dark neurons (arrows in b) have a condensed cytoplasm stained densely with thionin. c and d. At 11:00 am, there are many light neurons, whose plump cell somata is lightly stained (arrowheads in d), except a few dark cells (arrows in c). a, c: x260, b, d: x1,000
The specimen preparations in this study, including fixation and specimen excision, were performed under the same conditions and in uniform manner. Inspite of this, the population of dark cells showed significant differences by the time of day. This result is difficult to ascribe to insufficient fixation or incidental pathologic damages. Negative labeling of nick end in the dark neurons suggested that such dark profiles may not represent apoptosis.

TEWARI and BOURNE (1963) showed the possible appearance of dark neurons under physiological conditions, and suggested that this dark profile may reflect a certain stage of neurosecretion. The present study complemented our recent study of the rat brain (MURAKAMI and OHTSUKA, 1996) in showing that dark neurons appeared under usual or normal conditions in healthy animals. The present study further showed that the frequency of their occurrence has two peaks, as our count in a determined region of the retrosplenial cortex indicated. These peaks were coincident with the waking or feeding times of the mice. In our concomitant experiments in mice, dark neurons

![Fig. 5](image-url)  
**Fig. 5.** Examples used in the cell count study. Paraformaldehyde fixed, and cationic colloidal iron (pH 1.5) plus nuclear fast red stained sections. a. At 11:00 am, most neurons show light and plump cell somata, coated (large arrows) and uncoated (small arrows). b. At 3:00 am, many densely stained dark cells, either coated (large arrowheads) or uncoated (small arrowheads), have shrunken somata. a and b: ×260, both insets: ×1,000

![Fig. 6](image-url)  
**Fig. 6.** Dark cell population in the retrosplenial cortex. Vertical axis indicates percent ratio of dark cells/all nerve cells (● mean, bar standard deviation). Horizontal axis represents time over a one-day-period.
were observed in the third week after birth, but before that no dark neurons were recognizable (MURAKAMI et al., 1995b). These postnatal findings are consistent with the previous observation by EBELS (1975).

The retrosplenial cortex has many corticocortical and thalamic connections (REEF et al., 1994). It is suggested that this cortical area is involved in cognitive functions (SARTER and MARKOWITSCH, 1985). This area with such functions is supposed to be activated during waking time. It seems thus reasonable to propose that dark neurons might be caused by the excessive activation, or exhaustion of nerve cells.

It is interesting that the negatively charged surface coat of dark cells showed an appearance of spicules. Our previous electron microscopic studies indicate that the surface coats exist in the perineuronal space, except for the synaptic sites (MURAKAMI, 1994; MURAKAMI et al., 1994a). The present spicule, like profile of the coat may be explained as the result of cell shrinkage and traction from immobile synaptic sites. The negatively charged surface coats and shrinkage may act as electrical and spatial insulators for keeping the neurons from further excessive excitation.

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### Table 1. Populations of dark cells, neurons with negatively charged surface coats and dark neurons with such surface coats

<table>
<thead>
<tr>
<th>Time</th>
<th>Dark cells</th>
<th>Cells with negatively charged surface coats</th>
<th>Dark cells with negatively charged surface coats</th>
<th>Behavior of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>3:00</td>
<td>57.4±24.8</td>
<td>8.5±3.4</td>
<td>6.4±4.2</td>
<td>Awake, eating</td>
</tr>
<tr>
<td>7:00</td>
<td>30.4±16.1</td>
<td>7.8±2.9</td>
<td>2.6±2.2</td>
<td>Sleeping</td>
</tr>
<tr>
<td>11:00</td>
<td>10.6±2.6</td>
<td>7.4±1.3</td>
<td>0.3±0.4</td>
<td>Sleeping</td>
</tr>
<tr>
<td>15:00</td>
<td>13.7±13.3</td>
<td>12.5±6.5</td>
<td>0.9±0.7</td>
<td>Sleeping</td>
</tr>
<tr>
<td>19:00</td>
<td>56.1±18.9</td>
<td>7.9±3.0</td>
<td>4.9±3.8</td>
<td>Awake, eating</td>
</tr>
<tr>
<td>23:00</td>
<td>15.0±10.8</td>
<td>9.8±2.4</td>
<td>0.4±0.7</td>
<td>Awake</td>
</tr>
</tbody>
</table>

% (mean±SD)

REFERENCES


