GABAergic neurons comprise a major cell type in rodent visual relay nuclei: an immunocytochemical study of pretectal and accessory optic nuclei*

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Summary. The enzyme glutamic acid decarboxylase (GAD) has been localized in sections of rodent brains (gerbil, rat) using conventional immunocytochemical techniques. Our findings demonstrate that large numbers of GAD-positive neurons and axon terminals (puncta) are present in the visual relay nuclei of the pretectum and the accessory optic system. The areas of highest density of these neurons are in the nucleus of the optic tract (NOT) of the pretectum, the dorsal and lateral terminal accessory optic nuclei (DTN, LTN), the ventral and dorsal subdivisions of the medial terminal accessory optic nucleus (MTNv, MTNd), and the interstitial nucleus of the posterior fibers of the superior fasciculus (inSFp). The findings indicate that 27% of the NOT neurons are GAD-positive and that these neurons are distributed over all of the NOT except the most superficial portion of the NOT caudally. The GAD-positive neurons of the NOT are statistically smaller (65.9 μm²) than the total population of neurons of the NOT (84.3 μm²) but are otherwise indistinguishable in shape from the total neuron population. The other visual relay nuclei that have been analyzed (DTN, LTN, MTNv, MTNd, inSFp) are similar in that from 21% to 31% of their neurons are GAD-positive; these neurons are smaller in diameter and are more spherical than the total populations of neurons. The data further show that a large proportion of the neurons in these visual relay nuclei are contacted by GAD-positive axon terminals. It is estimated that approximately one-half of the neurons of the NOT and the terminal accessory optic nuclei receive a strong GABAergic input and have been called “GAD-recipient neurons”. Further, the morphology of the GAD-positive neurons combined with their similar distribution to the GAD-recipient neurons suggest that many of these neurons are acting as GABAergic, local circuit neurons. On the other hand, the large number of GAD-positive neurons in the NOT and MTN (20-30%) in relation to estimates of projection neurons (75%) presents the possibility that some may in fact be projection neurons. The overall findings provide morphological evidence which supports the general conclusion that GABAergic neurons play a significant role in modulating the output of the visually related NOT and terminal accessory optic nuclei.

Key words: GABAergic neurons – Accessory optic nuclei – Pretectal nuclei – Gerbil – Rat – Visual system

Introduction

Previous studies have established the presence of GABAergic, local circuit neurons within the visual thalamic nuclei and the visual cortex of mammals (Ribak 1978; Sterling and Davis 1980; McDonald et al. 1981; Hendrickson et al. 1983; Ohara et al. 1983; Somogyi et al. 1983; Fitzpatrick et al. 1984; Montero and Singer 1984; Penny et al. 1984). The present study now directs attention to another portion of the visual system which is located along the mesencephalic junction and includes the nucleus of the optic tract (NOT) (Scalia 1972), the dorsal, lateral and medial terminal accessory optic nuclei (DTN, LTN, MTN) (Hayhow et al. 1960), and the associated interstitial nucleus of the posterior bundle of the superior fasciculus (inSFp) (Giolli et al. 1984, 1985).
In rodents, these nuclei are abundantly supplied with retinal afferents through which they receive information related to the speed and direction of visual surround movement (see Simpson et al. 1979). In addition, these nuclei are extensively interconnected (Blanks et al. 1982; Holstege and Collewijn 1982; Giolli et al. 1984) and are found to have numerous projections to precolulomotor and precerebellar nuclei which are concerned with the control of eye movements (Takeda and Maekawa 1976; Hoffmann et al. 1976; Terasawa et al. 1979; Weber and Harting 1980; Holstege, and Collewijn 1982; Robertson 1983; Giolli et al. 1984, 1985).

Mackawa and Simpson (1972, 1973) have presented electrophysiological evidence in the rabbit for an inhibitory projection that originates in the MTN and acts upon the NOT. The neurotransmitters involved in this projection are unknown. On the other hand, the recent studies showing the presence of substantial numbers of GABAergic neurons within the NOT of the opossum, rabbit and cat (Ottersen and Storm-Mathisen 1984; Penny et al. 1984; Weber and Chen 1984) and the MTN of the rabbit (Ottersen and Storm-Mathisen 1984; Penny et al. 1984) increases the possibility that this inhibition may be mediated by GABA. In the present report, we have examined the distribution and morphology of GAD-immunoreactive neurons within pretectal and terminal accessory optic nuclei of rodents (gerbil and rat) as stained immunocytochemically for glutamic acid decarboxylase (GAD), the synthesizing enzyme for the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). As will be shown, a substantial number of GAD-positive neurons was found within the NOT and the terminal accessory optic nuclei. It is likely that these GABAergic neurons (local circuit and/or projection neurons) play a major role in modulating the output of this interrelated group of visual relay nuclei.

### Material and methods

Ten adult and four juvenile gerbils (*Meriones unguiculatus*) as well as one adult rat were deeply anesthetized with sodium pentobarbital (Nembutal: 50 mg/kg, I.P.) and perfused transcardially with 4% paraformaldehyde in ice cold sodium phosphate buffer (0.15M, pH 7.4). Immediately after perfusion, the brains were removed from the skull, placed in fixative with 25% sucrose as a cryoprotectant, and stored overnight at 4°C. Frozen sections were cut in the traverse plane on a sliding microtome at 40 μm. Every fifth section was used for immunocytochemical staining (see below) and adjacent sections were Nissl-stained with cresyl violet.

Immunocytochemical localization of GAD was performed according to a double-bridge modification (Ordronneau et al. 1981) of the procedure described by Oertel et al. (1981a, b). The gerbil and rat brains were treated in the same manner. Sections were first incubated overnight at 4°C in a blocking medium ([0.1M D.L.-lysine + 10% normal rabbit serum (NRS)] in 0.05M Tris buffered saline (TBS), pH 7.6 and were then transferred to sheep anti-GAD serum (1:2000 in TBS + 1% NRS) for 18 to 24 h at 4°C. After several washes in TBS, the sections were incubated in biotinylated rabbit anti-sheep immunoglobulin G followed by several buffer washes and then incubated in avidin-biotin HRP complex (ABC). The biotinylated antibody and ABC were supplied in kit form by Vector Laboratories (Burlingame, CA). After reincubation in both the biotinylated antibody and the ABC, the sections were incubated in a solution containing 0.6% diaminobenzidine hydrochloride and 0.02% hydrogen peroxide in TBS. After rinsing, mounting and drying, the reaction was intensified by exposing the sections to dilute (0.005%) osmium tetroxide or to osmium thiocarbohydrazide-osmium according to Willingham and Rutherford (1984).

The number of GAD-positive somata and of GAD-recipient neurons (identified by the presence of puncta on somal surfaces), relative to the total number of Nissl-stained neurons in the adjacent sections, was determined in each of the pretectal and terminal accessory optic nuclei as illustrated in Figs. 1A–D. In addition, somal area and shape factor of the GAD-positive neurons were measured (Table 1). Nissl-stained somata were counted in a region of each of the nuclei delimited by a grid reticule and the GAD-positive and GAD-recipient neurons were counted in the same region of the adjacent GAD-immunoreacted sections. Virtually every neuron in the analyzed brain regions had GAD-positive puncta adjacent to its soma. The number ranged from one to 30. However, given this variation and the technical problems of accurately assessing the number of puncta, we have arbitrarily defined a GAD-recipient neuron as one having seven or more GAD-positive puncta on its somal surface. The numbers of GAD-positive and GAD-recipient neurons were counted bilaterally in one 25 day old gerbil brain and in six adult gerbil brains, and the results are expressed as a percentage of the total number of Nissl-stained neurons. The area and shape measurements were performed by tracing the somata on a digitizing tablet connected to a Bioquant Morphometrics System supplied by R & M Biometrics (Nashville, TN). Shape factors were calculated by this system and were expressed as a fraction (4π × Area/Perimeter^2). Defined in this manner, shape factor ranged between 0 and 1, with 1 representing a perfect circle and factors less than 1 representing progressively greater degrees of ellipses (see Table 1).

### Results

The present immunocytochemical study uses sheep anti-GAD serum (Oertel et al. 1981a, b) to examine the distribution of GABAergic neurons and terminals within visual relay nuclei of the mesodiencephalic junction. The distribution of GAD-positive somata within single sections is plotted in Figs. 1A–D for thalamic, pretectal and midbrain nuclei at four representative brain levels from an adult gerbil brain. This pattern of distribution is typical of what has been seen in all the rodent brains studied. Although GAD-positive somata are located in all nuclei, the greatest concentrations of these neurons are found within the nucleus of the optic tract (NOT), the dorsal (DTN) and lateral (LTN) terminal accessory optic nuclei, and the ventral (MTNv) and dorsal
Fig. 1A–D. The density and distribution of GAD-immunoreactive neurons in the nucleus of the optic tract and the terminal accessory optic nuclei, as well as in other thalamic, pretectal and midbrain nuclei, have been charted on the left side in each of the four transverse sections from an adult gerbil brain (each dot = two GAD-positive cells). The density and distribution of GAD-recipient neurons (neurons that show seven or more puncta on their somal surfaces) are mapped for the nucleus of the optic tract and the terminal accessory optic nuclei on the right side in each of the sections (each dot = two GAD-recipient cells). Note that GAD-recipient neurons have not been mapped for other nuclei. Each of the transverse sections (A–D) is composed of half sections of brain which have been connected at the midline.
Fig. 2. A shows the GAD-positive neurons in the rostral portion of the nucleus of the optic tract (NOT) in a transverse section of an adult gerbil brain. The arrangement of some of the GAD-positive neurons in rows between fiber bundles of the optic tract is apparent. The insert at the lower right in A is from the rectangular area. This insert demonstrates several intensely stained GAD-positive neurons (arrows). B shows the GAD-positive neurons in a transverse section through the caudal part of the NOT and the dorsal terminal accessory optic nucleus (DTN) of the same gerbil brain as A. As with the rostral NOT (A), large, intensely stained NOT neurons are present. The heavy GAD staining of the DTN (neurons and puncta) is evident.

(MTNd) subdivisions of the medial terminal accessory optic nucleus. Another related population of GAD-positive neurons is found within the interstitial nucleus of the posterior fibers of the superior fasciculus (inSFp).

The distribution and density of GAD-recipient neurons in the nucleus of the optic tract and the terminal accessory optic nuclei are also illustrated in Figs. 1A–D. Again, the distribution pattern serves as a model for all of the rodent brains. The term “GAD-recipient” denotes the presence of seven or more GAD-positive puncta (axon terminals) on each soma. These plotings (Figs. 1A–D) demonstrate that many neurons in these visual relay nuclei receive relatively heavy GABAergic input. Each of these visual relay nuclei is discussed individually below.
Fig. 3. A illustrates GAD-positive neurons within nuclei of the ventral midbrain tegmentum in a transverse section of an adult gerbil brain. GAD-positive neurons are seen in the dorsal and ventral subdivisions of the medial terminal accessory optic nucleus (MTNd, MTNv) and in the interstitial nucleus of the posterior fibers of the superior fasciculus (inSFp). GAD-positive neurons are also present in the pars compacta and pars reticulata of the substantia nigra. The rectangular areas in (A) show details of the MTN (B) and in SFp (C). Arrows in B and C depict representative GAD-positive neurons, and GAD-positive puncta in B are seen to cover the somata of GAD-positive neurons and to outline GAD-negative neurons.
GAD-positive somata

1. Nucleus of the optic tract (NOT). All five recognized pretectal nuclei of rodents (Scalia 1972) contain GAD-positive neurons, but the greatest density occurs within the NOT (Figs. 1A–D) where approximately 27% of the neurons are GAD-positive (see Table 1). In the rostral NOT, the GAD-positive neurons are distributed rather evenly throughout the nucleus (Figs. 1A and 2A) with many being tightly clustered into rows between bundles of optic tract axons (Figs. 2A). In the caudal NOT, GAD-positive neurons are present only in the deeper part of the nucleus (Figs. 1B–D and 2B).

The GAD-positive neurons, like all NOT neurons, are multipolar and have elliptical to round shapes with an overall shape factor of 0.71 (Table 1). On the other hand, the GAD-positive neurons of the NOT are smaller (mean area: 65.9 \( \mu \text{m}^2 \)) than the total NOT cell population (84.3 \( \mu \text{m}^2 \)) (Table 1; Fig. 4). Thus, the GAD-positive neurons of the NOT are 22% smaller than the total NOT cell population. This value is in agreement with comparable measurements of GAD-positive neurons in the NOT of the opossum, rabbit (Penny et al. 1984) and cat (Weber and Chen 1984).

2. Dorsal and lateral terminal accessory optic nuclei (DTN & LTN). As with the rat (Hayhow et al. 1960), the gerbil has a small, but discrete DTN which is located at the surface of the pretectum between the superior colliculus and the medial geniculate nucleus (Fig. 1A). Approximately 28% of the DTN neurons are GAD-positive. Their somal area averages 46.6 \( \mu \text{m}^2 \) compared with an average of 58.3 \( \mu \text{m}^2 \) for the total population of DTN neurons (Table 1; Fig. 4).

The GAD-positive neurons are multipolar, elliptical to round in shape, and more spherical than the total population of DTN neurons (see Table 1).

The LTN of the gerbil and rat is small. It lies dorsal to the cerebral peduncle, ventral to the medial geniculate nucleus, and lateral to the peripenduncular nucleus (Figs. 1B, D). About 21% of the LTN neurons are GAD-positive, and these have an average somal area which is smaller (45.7 \( \mu \text{m}^2 \)) than the total cell population (58.7 \( \mu \text{m}^2 \)) (Table 1; Fig. 4). The GAD-positive neurons of the LTN are also more spherical than the total LTN neuron population (Table 1).

3. Medial terminal accessory optic nucleus (MTN). This nucleus lies medial to the substantia nigra and cerebral peduncle and lateral to the nuclei of the ventral tegmental area of Tsai (1925) (Figs. 1B–D.) It is divisible into a ventral (MTNv) and a dorsal (MTNd) subdivision on the basis of anatomical (Giolli et al. 1968; Gregory and Giolli 1985) and electrophysiological data (Hamasaki and Marg 1962; Hill and Marg 1963). GAD-positive somata are found in both nuclear subdivisions (Fig. 1B–D and 3A) in about equal proportions (MTNv: 28.5%; MTNd: 30.5%).

### Table 1

| Nucleus | Areas | Shape factors | % |
|---------|-------|---------------|---|
| NOT     | 84.3 ± 28.8 | 65.9 ± 25.4** | 0.71 | 0.71 | 27% |
| MTNv    | 61.1 ± 24.8 | 43.9 ± 13.4*  | 0.72 | 0.78* | 30% |
| LTN     | 58.7 ± 16.8 | 45.7 ± 9.9*   | 0.75 | 0.85* | 21% |

** The percentages of GAD-positive neurons were obtained by counting equivalent regions of each nucleus or nuclear subdivision in adjacent Nissl and GAD-stained sections.

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SHAPE FACTOR = \( \frac{4\pi \times \text{AREA}}{\text{PERIMETER}^2} \)

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Golgi studies have revealed that the majority of the MTN neurons are small with somata that are bipolar or multipolar in shape (Giolli et al. 1968; Gregory and Giolli 1985). GAD-positive somata fall equally into these two shape categories, yet they have smaller areas than the total cell population (Table 1). For example, the mean somal area of GAD-positive neurons in the MTNv is 43.9 um² which contrasts with an average of 61.1 um² for the total MTNv cell population (Table 1; Fig. 4). The neuronal size in the MTNd is somewhat greater than in the MTNv, and a few large multipolar GAD-positive soma (ca 130 um²) are among the largest neurons found in the MTNv. The shape factor of the GAD-positive neurons of the MTNv and the MTNd are virtually equal, and on the average they are more rounded than the total population of neurons (see Table 1).

The present immunocytochemical technique labels not only neuronal somata but also other profiles which have been determined from electron microscopic studies to be the axon terminals (puncta) of GABAergic neurons (see Discussion). For technical reasons we have defined a “GAD-recipient neuron” as one which shows seven or more puncta on its soma. Of extreme importance to the present analysis is the fact that GAD-recipient neurons are found in high density in the visual relay nuclei (Figs 1A–D). In fact, about fifty-seven percent of the NOT neurons, and between 48–56% of the neurons of the terminal accessory optic nuclei and the inSFp, have seven or more puncta on their somal surfaces. The GAD-recipient neurons are evenly distributed throughout most of the NOT and in all of the terminal accessory optic nuclei and inSFp, as is the case for the GAD-positive neurons. This study has not permitted the analysis of possible GAD-positive puncta contacting the distal dendrites of neurons of the NOT and terminal accessory optic nuclei. However, puncta are seen which appear unassociated with somal surfaces and which may, at least in part, represent a system of GAD-positive, axo-dendritic contacts.

Discussion

Glutamic acid decarboxylase (GAD), the synthetizing enzyme for the inhibitory neurotransmitter
GABA, is localized within a large number of neurons in the nucleus of the optic tract (NOT), and within each of the nuclear components of the accessory optic system (i.e. the DTN, LTN, MTNv/d and insFp). The comparatively high concentration of GAD-positive somata (21–30%) distinguishes each of these visual relay nuclei from adjacent thalamic, pretectal and midbrain tegmental nuclei. Furthermore, the GAD-positive neurons in each nucleus are more spherical and statistically smaller than the GAD-negative neurons present.

These five visual relay nuclei contain unusually high proportions (about 50%) of GAD-recipient neurons compared to adjacent pretectal and midbrain tegmental nuclei. In the present study, we have defined a GAD-recipient neuron as one which has seven or more puncta surrounding the soma. It is likely that these neurons are GAD-recipient because most puncta adjacent to somata in other brain regions have been shown to form axosomatic synapses in electron microscopic studies (see Ribak et al. 1981). Although a small proportion of these puncta identified with the light microscope could also be profiles of GAD-positive somata and/or dendrites, many are probably synaptic axon terminals. The criterion of seven puncta per soma is quite arbitrary, particularly given the fact that virtually all neurons in these visual relay nuclei contain at least one puncta. The choice of seven puncta per soma provides a working definition for the term “GAD-recipient neuron” and in practice should be interpreted as designating a neuron which receives a strong (versus a weak) GABAergic input onto its soma.

The question can be asked as to whether the GAD-positive neurons of the NOT and terminal accessory optic nuclei are all local circuit neurons or if some are, in fact, projection neurons. We can only speculate on this issue, and we point to several observations which suggest that the GAD-positive neurons of the NOT are signifi-
cantly larger than those in the accessory optic nuclei (Table 1; Fig. 4). This finding is probably due to the fact that the general population of neurons in the NOT is, on the average, larger than that in the accessory optic nuclei. Given the observation that between 21% and 30% of the total neurons in the NOT and terminal accessory optic nuclei are GAD-positive and the findings that about 75% of the rat MTN neurons project to the NOT (Blanks et al. 1982) while a similar percentage of the rat and rabbit NOT neurons project to brain stem nuclei (Takeda and Maekawa 1976; Holstege and Collewijn 1982; Robertson, personal communication), it is likely that some of the neurons of these visual/oculomotor-related nuclei are projection neurons. Furthermore, Ottersen and Storm-Mathisen (1984) have demonstrated with an antiserum to GABA that a majority of NOT neurons are GABAergic and that many GABAergic fibers course among the fibers of the brachium of the superior colliculus and the posterior commissure. It is, therefore, likely that some of the GABAergic NOT neurons project upon somata of preoculomotor and precerebellar brain stem nuclei.

Finally, what can be said about the role of GABA in the functional interaction of these visual relay nuclei? It is difficult to explain the functional significance of the larger number of interconnections between the NOT and terminal accessory optic nuclei. Anatomical studies by Weber and Harting (1980) in tree shrew and by Holstege and Collewijn (1982) in rabbit have shown a major NOT projection to the MTN. In parallel, there is a strong reciprocal projection from the MTN to the NOT (Berson and Graybiel 1980; Blanks et al. 1982). The latter projection is probably responsible for the inhibition of visual information through the retino-NOT-olivocerebellar pathway following electrical stimulation of the MTN (Maekawa and Simpson 1972, 1973). The relatively large number of GAD-positive MTN neurons and “GAD-recipient NOT neurons” suggests that this inhibition is mediated by GABA. Such inhibition may be based on one or both of the following circuits: 1) GABAergic projection neurons of the MTN terminate on the output neurons of the NOT, or 2) GABAergic local circuit neurons of the NOT are activated as a result of an excitatory input from the MTN to provide feedforward inhibition. Either mechanism would produce a greater tuning of the well described speed- and direction-selective properties of the NOT and the related accessory optic nuclear neurons (see Simpson et al. 1979) as has been postulated for the functional role of the reci-
procal connections between these nuclei (Blanks et al. 1982; Holstege and Collewijn 1982; Giolli et al. 1984, 1985).

Abbreviations to figures

A Cerebral aqueduct
CP Posterior commissure
DK Nucleus of Darkschewitsch
DMN Deep mesencephalic nucleus
DTN Dorsal terminal nucleus, accessory optic system
HITr Habenulointerpeduncular tract
IGL Intergeniculate leaflet
INC Interstitial nucleus of Cajal
insFp Interstitial nucleus, superior fasciculus, posterior fibers
LGNd Dorsal lateral geniculate nucleus
LGNv Ventral posterior nucleus
LP Lateral posterior nucleus
MB Mammillary body
MGN Medial geniculate nucleus
ML Medial lemniscus
MTNd Medial terminal nucleus, dorsal subdivision, accessory optic system
MTNv Medial terminal nucleus, ventral subdivision, accessory optic system
NOT Nucleus of the optic tract
NPCC Nucleus of posterior commissure
OP Optic tract
PA Anterior pretectal nucleus
PAG Periaqueductal gray
ppb Nucleus parabrachial pigmented
PC Cerebral peduncle
PM Medial pretectal nucleus
pn Nucleus paraniigralis
PO Pretectal olivary nucleus
pp Posterior pretectal nucleus
PPN Peripeduncular nucleus
RNm Magnocellular division, red nucleus
RNp Parvocellular division, red nucleus
SC Superior colliculus
SGP Stratum griseum profundus, superior colliculus
SGS Stratum griseum superficiale, superior colliculus
SGM Stratum griseum medium, superior colliculus
SNc Substantia nigra, pars compacta
SNr Substantia nigra, pars reticulata
SO Stratum opticum, superior colliculus
VB Ventrobasal complex
ZI Zona incerta
SN Oculomotor nerve, root fibers
3V Third ventricle

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