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Septal neurons containing glutamic acid decarboxylase immunoreactivity project to the hippocampal region in the rat brain*

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Summary. Injections of the fluorescent dyes Fast Blue or Granular Blue into either the hippocampus (volume approximately 50 nl) or the entorhinal area (100–150 nl) resulted in labeling by retrograde axonal transport of cells in the diagonal band of Broca (dbB) and the medial septum (MS). A large number (approximately 30%) of these cells contained glutamic acid decarboxylase (GAD)-like immunoreactivity, as determined by combined retrograde fluorescent tracing and GAD-immunohistochemistry. Not all GAD positive cells in the dbB and MS were labeled by fluorochromes in a single experiment. The GAD-stained and fluorochrome-containing cells were present at all rostro-caudal levels of the septum and appeared not to belong to any single morphological class of cells. Double staining experiments showed that the GAD-positive cells did not contain acetylcholinesterase reaction product. These findings provide evidence that a significant portion of the septo-hippocampal projection may utilize gamma-aminobutyric acid as a neurotransmitter.

Key words: Hippocampus – Septum – GAD – Immunocytochemistry – GABA

Cells in the diagonal band of Broca (dbB) and the medial septal nucleus (MS) project to all parts of the hippocampal region, including the entorhinal area (EA) (Alonso and Köhler 1982; Crutcher et al. 1981; Swanson and Cowan 1979; Wyss et al. 1979). Previous studies have provided evidence that the septo-hippocampal pathway may utilize acetylcholine (Ach) as a neurotransmitter (Oderfeld-Nowak et al. 1974; Ropert et al. 1980; Sethy et al. 1973). Recent studies combining retrograde transport of horseradish peroxidase (HRP) and acetylcholinesterase (AChE) histochemistry suggest that part of the septo-hippocampal projection may utilize a neurotransmitter other than ACh (Alonso and Köhler 1982; Mesulam and Van Hoesen 1976). In a search for possible neurochemical candidates for this projection we (Köhler and Chan-Palay 1983) have found that the dbB and MS are rich in neurons immunoreactive for glutamic acid decarboxylase (GAD), the enzyme responsible for the synthesis of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). In the present study we provide evidence that some of the septal neurons that project to the hippocampal region, including the EA, contain GAD-immunoreactivity.

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Materials and methods

Male Sprague-Dawley rats (weight 200 g; Anticimex, Stockholm, Sweden) were anesthetized with phenobarbital (Mebumal; 60 mg·kg^{-1}) and injected stereotaxically with Granular Blue (GB; 5% in distilled water) and Fast Blue (FB; 1% in distilled water) into different parts of the hippocampal region. Each injection ranged in volume from 50 nl (the hippocampal formation) to 150 nl (the entorhinal area). Three to five days after the fluorochrome injections the rats were re-anesthetized and injected intraventricularly with an inhibitor of fast axonal transport (colchicine; Sigma Chemical Co., St. Louis, U.S.A.; 120 µg in 20 µl) (Hökfelt and Dahlström 1971), 24 to 48 h before sacrifice.

For immunohistochemistry the brains were fixed transcardially by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Coronal sections (10–30 µm) were cut in a cryostat (Dittes, Heidelberg, FRG) and collected onto gelatin-albumin coated glass slides. The sections were incubated with an antibody against purified GAD (12) diluted 1:1000 in phosphate buffered saline (PBS) containing 0.3% Triton X-100 for 24 to 72 h. Subsequently, they were washed and incubated in FITC conjugated goat-antirabbit IgG (Cappel, diluted 1:200 in 0.3% Triton X-100) for 45 min, washed and cover-slipped in glycerol-PBS.

Analysis of neurons containing fluorochromes and GAD-immunoreactivity was made in a Leitz Ortholux fluorescence microscope under different filter systems as previously described (Köhler and Steinbusch 1982). To investigate the possibility that acetylcholinesterase resides in GAD positive cells the GAD stained sections were photographed, washed carefully and restained for the visualization of AChE, according to the method of Geneser-Jensen and Blackstad (1971).

Results

The cases reported here had restricted injections of FB into the septal part of the hippocampal formation, the medial (M) EA and the lateral (L) EA, respectively. The hippocam-

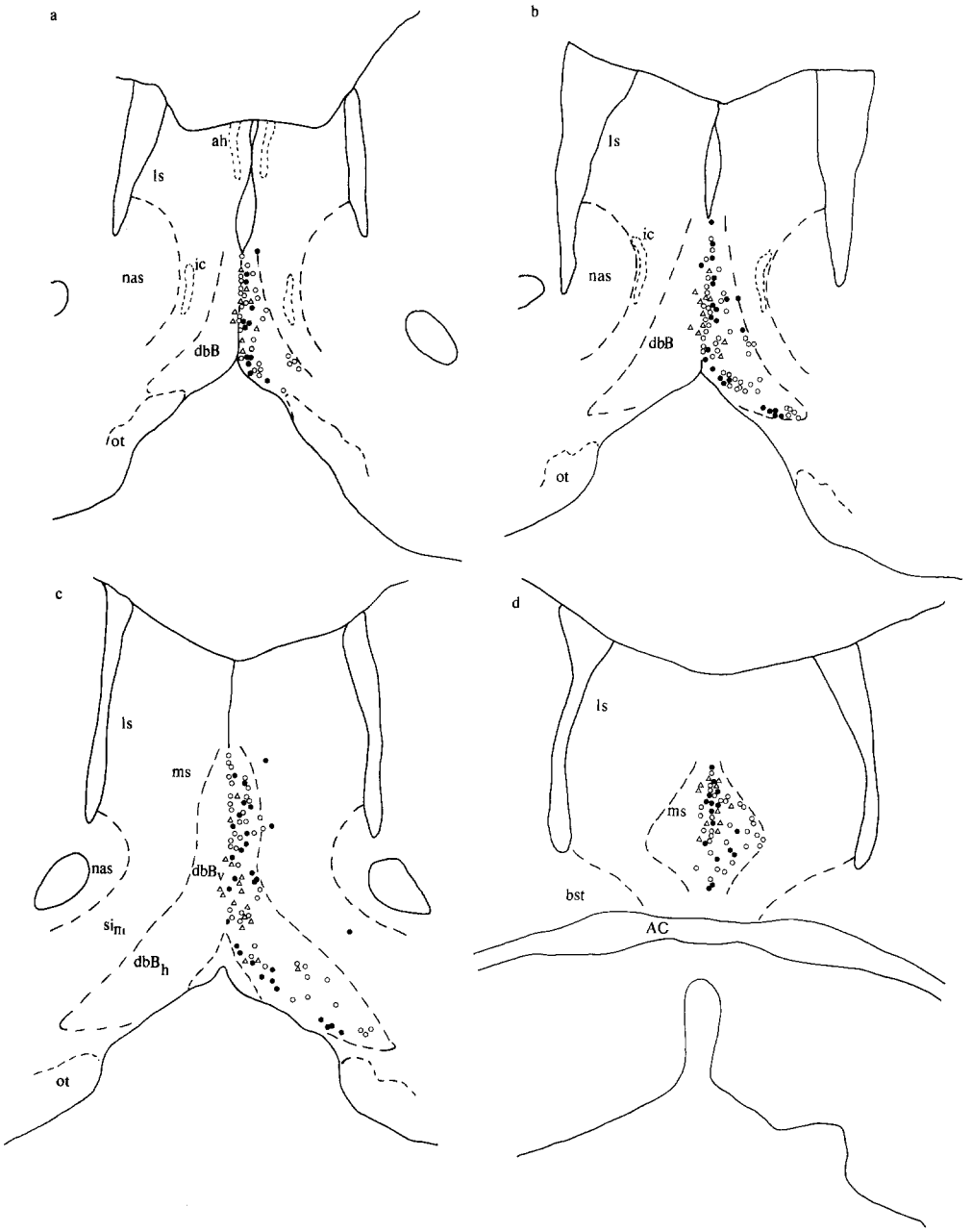

Fig. 1. a–d Line drawing of coronal sections through the septum showing the position of cells in the septum containing GAD-immunoreactivity (○), FB after injections into the medial entorhinal area (●) and cells containing the fluorochrome and the GAD immunoreactivity (△) at four rostro-caudal levels. The most rostral level is shown in (**a**) and the most caudal section is shown in (**d**). Abbreviations: *ah* anterior hippocampal rudiment; *bst* bed nucleus striae terminalis; *dbB* diagonal band of Broca (*v* and *h* vertical and horizontal limb, respectively); *ic* island of Calleja; *ls* lateral septum; *ms* medial septum; *nas* nucleus accumbens septi; *ot* olfactory tubercle; *sim* rostro-medial extension of substantia innominata; *AC* commissurae anterior

pal injection resulted in spread of the dye only into the overlying cingulate cortex. The MEA and the LEA injections overlapped only marginally and there was little invasion by the dye into adjacent areas such as the parasubiculum and the piriform cortex, respectively. Each of the EA injections extended approximately 2 mm along the dorsoventral axis of the structure.

All injections resulted in labeling by retrograde axonal transport of neurons in the dbB and the MS in a pattern similar to that reported previously (Fig. 1) (Alonso and Köhler 1982; Wyss et al. 1979). The distribution of retrogradely labeled cells in these areas overlapped to a great extent with the distribution of AChE (Alonso and Köhler 1982; Harkmark et al. 1975) and GAD (Köhler and Chan-Palay 1983) stained neurons in these parts of the septum. Examination of the same section under different filter systems revealed that a significant number (approximately 30% of the population as estimated by semi-quantitative methods involving direct counting of cells in tissue) of FB labeled cells contained GAD-immunoreactivity (Figs. 1, 2). However, far from all GAD positive cells were retrogradely labeled by FB (or GB) in any of the single experiments. The GAD-stained cells found to project to different parts of the hippocampal region were present in all portions of

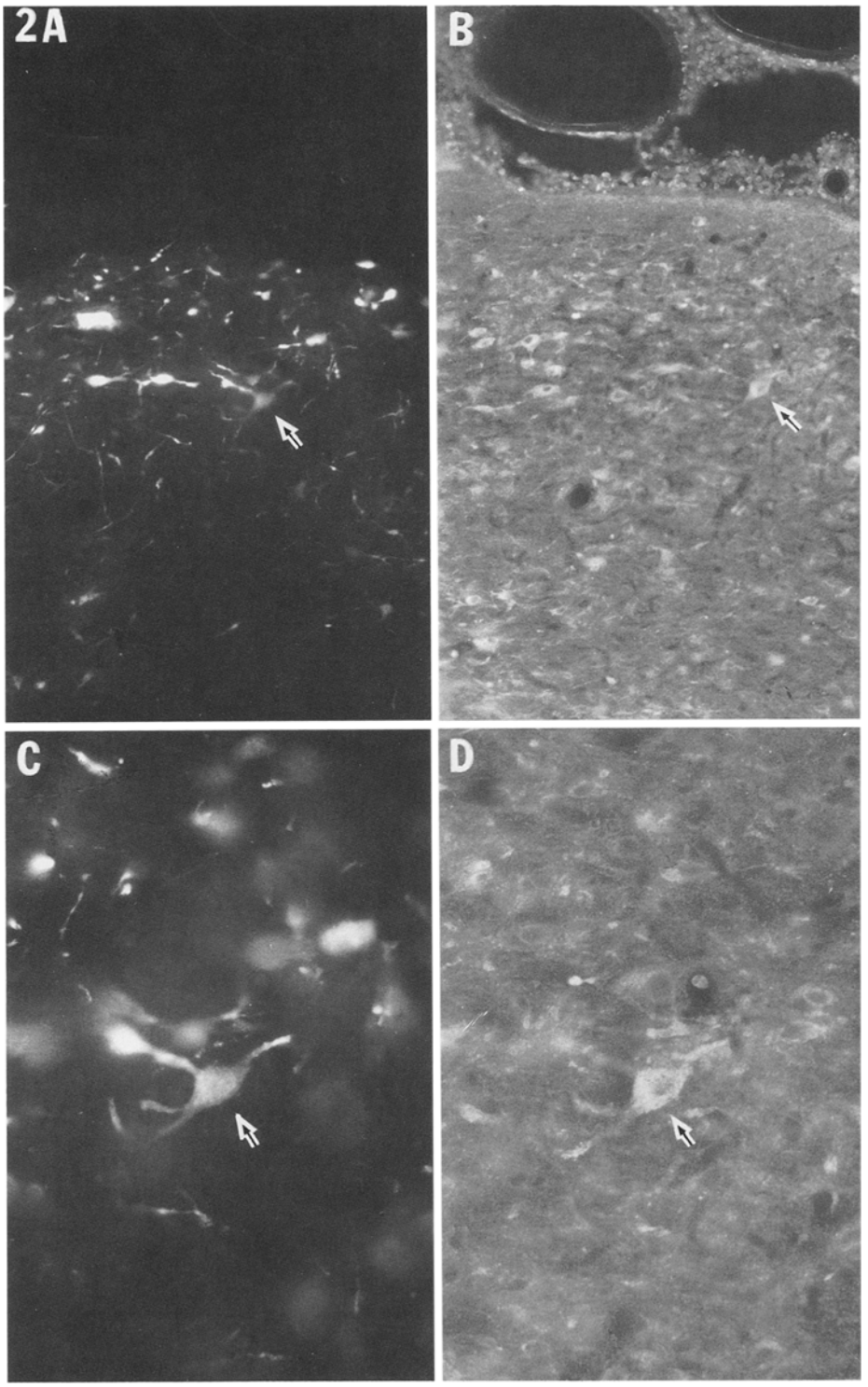

Fig. 2. A–C Photomicrographs to illustrate examples of a fluorochrome labeled septal neuron (**A**, **C**) which have demonstrable GAD immunoreactivity as well (**B**, **C**) ×130 (A, B); ×520 (C, D).

the dbB and at all rostro-caudal levels of this structure, although there were fewer double-labeled cells at the most caudal parts of this structure. The double-labeled cells dominated in the dbB, but at caudal levels many such cells were found also in the MS. When the same sections containing GAD-immunoreactivity were restained for AChE, it was clear that the GAD positive neurons in the dbB and MS did not contain the AChE reaction product, indicating that a relation with this transmitter system system was not likely.

The morphology of the GAD positive cells that project to the hippocampal region seemed not to belong to any single class of cells on the basis of their morphology. Thus, small (10–15 μm) round cells located along the midline, as well as medium (approximately 25 μm) and large (25–35 μm) multipolar cells in the vertical and horizontal limbs of the dbB contribute to this projection. The lateral septum, medial part of the substantia innominata, or the anterior hippocampal rudiment did not contain retrogradely labeled cells, although all these areas are known to

be rich in GAD positive cells (Köhler and Chan-Palay 1983).

Discussion

The present study has shown the existence of a projection from cells located in the diagonal band of Broca and the medial septum that contain immunoreactivity for GAD, the enzyme responsible for the synthesis of gamma-aminobutyric acid, to different parts of the hippocampal region in the rat brain. Previous studies, based on combined HRP and AChE histochemistry on the same tissue section have suggested that the septal projection to the hippocampus (Mesulam and Van Hoesen 1976) and the EA (Alonso and Köhler 1982) may contain a non-cholinergic component in addition to the more well known cholinergic innervation. This non-cholinergic component of the septo-hippocampal system could be identical to the GABAergic projection shown in the present study, since we did not detect significant amounts of AChE in the GAD-stained neurons. However, we cannot rule out the possibility that the colchicine treatment necessary to visualize the GAD neurons suppressed the AchE staining of these cells. It still remains to be shown, however, the GAD is not co-localized in choline acetyltransferase containing cells of the dbB. Our present findings partly disagree with findings from earlier biochemical studies (Fonnum and Walaas 1978) reporting no reduction in GABA markers after surgical removal of hippocampal afferents in the rat. One explanation for this discrepancy may be that the GAD positive septo-hippocampal projection constitutes a relatively small input to the hippocampus, and, in addition, it is masked in the biochemical studies by the prominent intrinsic GABAergic systems present in the hippocampus.

Although the origin of the septo-hippocampal projection has been localized to the dbB and the MS, its exact termination remains to be estabished. Preliminary studies by using transections of the dorsal and the ventral projections from the septum to the hippocampus suggest that part of the septal GAD-projection forms a diffuse innervation of the hilus and subicular cortex. Further studies are needed, however, to clarify the exact terminal distribution of these and other GABAergic afferents of extra-hippocampal origin.

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