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# Electron Microscopical Observation of GABA Terminals in the Rat Anterior Cingulate Cortex

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**Abstract:** The rat anterior cingulate cortex was examined by electronmicroscopic immunohistochemistry using anti-GABA antibodies. With respect to the synaptic interrelations, 689 synapses involving GABA profiles were analyzed. A high incidence of contacts between two GABA elements was worthy to note. The percentages of GABA-GABA contacts were about 25%, that is, 7% in axodendritic, 4% in axosomatic and 13% in axoaxonic synapses. Most of GABA terminals made synaptic contacts of symmetric type. The partners in GABA-GABA contacts furthermore formed contacts with other nonGABA elements to organize a large synaptic complex. Triadic arrangements of GABA terminals to nonGABA axodendritic (spine) synapses were also examined. The results suggest that intrinsic GABA neurons organize a network of GABA local circuits, which may have an inhibitory role in the integration of convergent inputs in the anterior cingulate cortex.

Key words: γ-aminobutyric acid, immunohistochemistry, synaptology, symmetric synapse, intrinsic GABA circuit, anterior cingulate cortex

## INTRODUCTION

It is well known that γ-aminobutyric acid (GABA) functions as an inhibitory neurotransmitter in the central nervous system[4,9,17,22]. Evidence for the presence of GABA neurons in the cerebral cortex has been obtained by immunohistochemistry for glutamic acid decarboxylase (GAD), a biosynthetic enzyme of GABA[11,18,21]. However, GAD immunohistochemistry seemed to be unsuitable to visualize somas and dendrites of GABA neurons without pretreatment by colchicine, an inhibitor of axonal transport[1,10,11]. Recently, GABA neurons have been clearly demonstrated without colchicine pretreatment, using anti-GABA antibodies[26]. In the present experiment, the same antibodies were employed for the examination of synaptic arrangements of GABA profiles in order to clarify the role of intrinsic GABA neurons in the anterior cingulate cortex.

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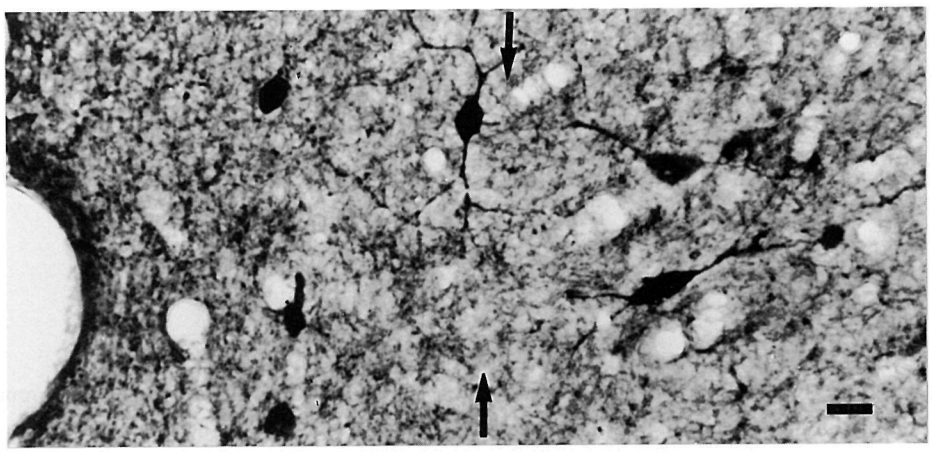

Fig. 1 GABA immunostained neurons are clearly demonstrated in the ACd. Numerous grains are regarded as GABA terminals in the neuropil. The left margin is the pial surface. Arrows indicate the border between layer I and II. Scale bar = 10 $\mu$m.

## MATERIALS AND METHODS

After anesthesia, twelve male Sprague-Dawley rats weighing 200-250 g were perfused with saline through the aorta for 2 min and subsequently followed with a mixed aldehyde solution (4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M PB at pH 7.4) for 15 min. Excised brains were postfixed overnight in a fresh fixative and stored in 15% sucrose PB at 4℃ for several days. Coronal sections were made on a cryostat: 16 $\mu$m in thickness for light microscopy (LM) and 48 $\mu$m for electron microscopy (EM). Free-floating sections were processed for immunohistochemistry by ABC method. Antisera to GABA (offered from Dr. I. Nagatu, Fujita Health University) were used at dilution of 1 : 10,000 in 0.1M PBS. Incubation time lasted for 2 days at 4℃ (for LM) and for 7-10 days (for EM). After washing in 0.1M PBS, sections were incubated in biotinylated IgG (1 : 1,000 in PBS) for 2 hs and then in avidin-biotin complex (1 : 1,000 in PBS) for 2 hs at room temperature. Reaction products were visualized in a solution containing 0.8% nickel ammonium sulfate, 0.01% diaminobenzidine (DAB) and 0.005% $H_2O_2$. Sections for LM were mounted on gelatine coated glass slides. Sections for EM were postfixed in 1% osmium tetroxide for 1 h, dehydrated in a graded ethanol series and flat-embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by a H-500 electron microscope.

## RESULTS

At the level of LM, GABA immunoreactive neurons were evenly and sparsely distributed in all layers of the cerebral cortex. However, the ratio of GABA neurons to the total cells counted in each layer of the mesocortex clearly differed from layer to layer. In layer I where only a few small neurons were located, the ratio was 80%. These cells displayed processes extending in parallel with the pial surface (Fig. 1), corresponding to horizontal cells originally mentioned by Cajal in Golgi preparations[5,15]. In layers II /III where a moderate number of GABA neurons were intermingled with pyrami-

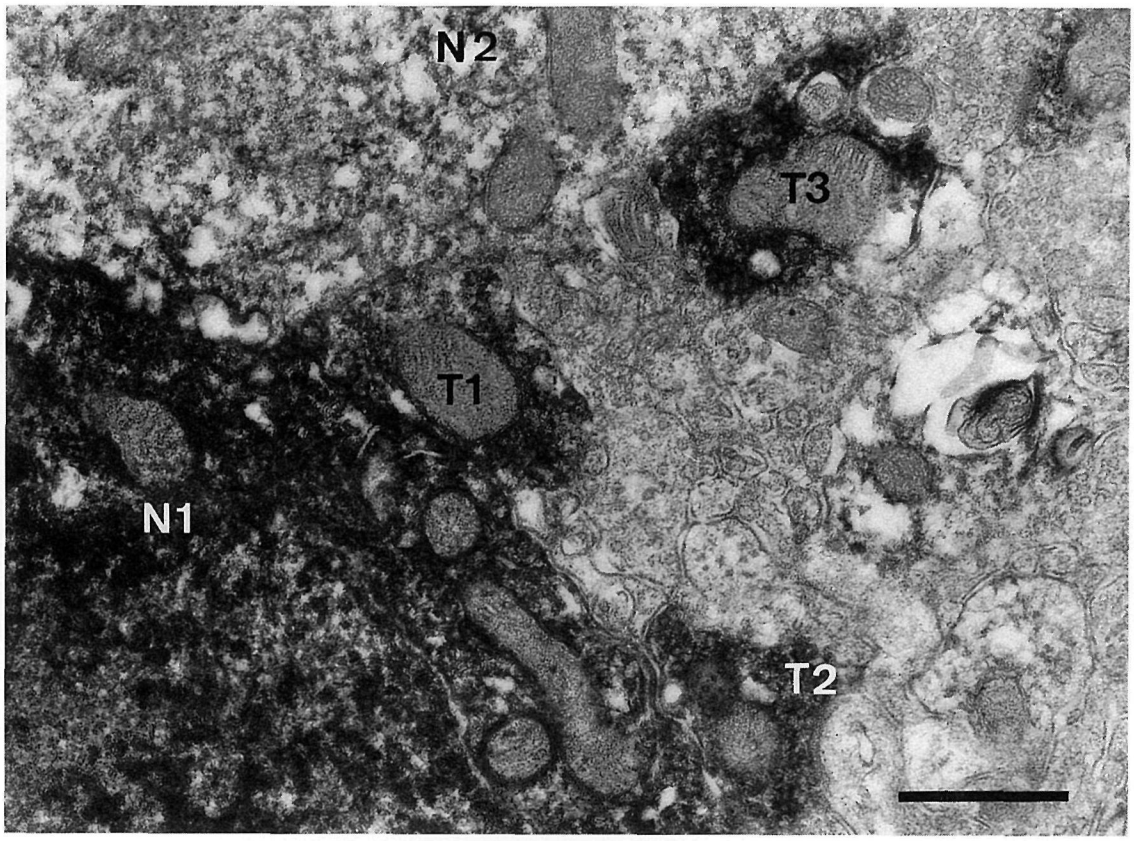


Fig. 2 Adhesion of GABA and non GABA neurons (N1, 2) indicating axosomatic contacts of symmetric type with GABA terminals (T1-3). Scale bar = 0.5$\mu$m.

dal neurons, the ratio dropped to 15%. The GABA neurons in layers II/III were mainly of medium-sized stellate or pleomorphic type extending their processes in all directions[11]. In layers V/VI where a fair number of GABA neurons and numerous pyramidal neurons were squeezed in a narrow space, the ratio was only 3%. None of pyramidal cells were essentially immunoreactive. Small dot-like GABA profiles were spread heavily in layer I and moderately in layers II/III (Fig. 1). They were regarded as terminals of intrinsic GABA neurons.

In the ventral and dorsal anterior cingulate cortex, GABA immunopositive elements were carefully examined by EM. These profiles consisted of somas, dendrites, unmyelinated axon syringes and terminals. Occasionally, small myelinated fibers encircled by 3-5 myelin lamellas also indicated immunodeposits as observed previously in rats and monkeys[16]. In general, reaction products were associated with cytoplasmic matrix filling around mitochondria, microtubules, pleomorphic synaptic vesicles and other cell organelles and also with nucleoplasmic matrix. The matrix of mitochondria had never revealed deposits but synaptic vesicles were often faintly covered with deposits (Fig. 2-4). Detailed identification of GABA profiles were rather difficult because of electron dense deposits and poor tissue preservation in the immunostained materials.

A number of GABA profiles made synaptic contacts with GABA and nonGABA neuronal elements. Somas of GABA neurons usually re-

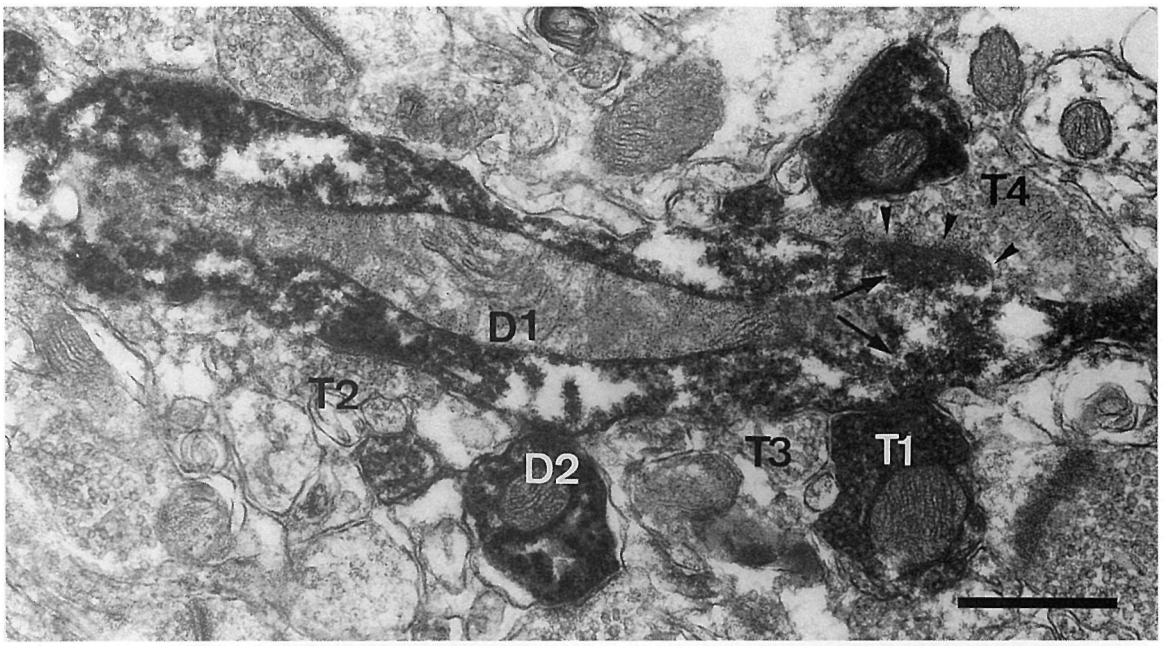


Fig. 3 A GABA dendrite (D1) containing a huge mitochondria and vesicles (arrows) forms synaptic contacts with one GABA terminal (T1) and three nonGABA terminals (T2, 3 and 4). A dendrodendritic contact (D1, 2) and a synapse of asymmetric type (arrow heads) are seen.

Table I. The percentages of various synaptic patterns analyzed in 689 contacts involving GABA neuronal elements. See the text.

| GABA-GABA contacts | | nonGABA-GABA contacts | |
|---|---|---|---|
| axodendritic | 7.0% | axodendritic | 24.0% |
| axosomatic | 4.0% | axosomatic | 6.0% |
| axoaxonic | 13.0% | axoaxonic | 17.0% |
| dendrosomatic | 0.7% | AIS-axonic | 3.0% |
| somatosomatic | 0.3% | dendroaxonic | 12.0% |
| | | somatoaxonic | 8.0% |
| unidentified | 4.7% | somatosomatic | 0.3% |

ceived several terminals making axosomatic contacts of symmetric type. In single section examinations, about two fifths of them were composed of GABA terminals. Somas of GABA neurons occasionally displayed somatosomatic contacts with nonGABA neurons on a wide surface (Fig. 2). Dendrites of GABA neurons received much more terminals than the somas. They were often surrounded by both GABA and nonGABA terminals (Fig. 3). In general, nonGABA terminals formed synapses of asymmetric type, while GABA terminals formed those of symmetric type. Occasionally vesicular structures were noted in GABA dendrites (Fig. 3). However, it need more data to determine whether they were real synaptic vesicles or the traversed tubular structures.

Most of GABA terminals containing pleo-

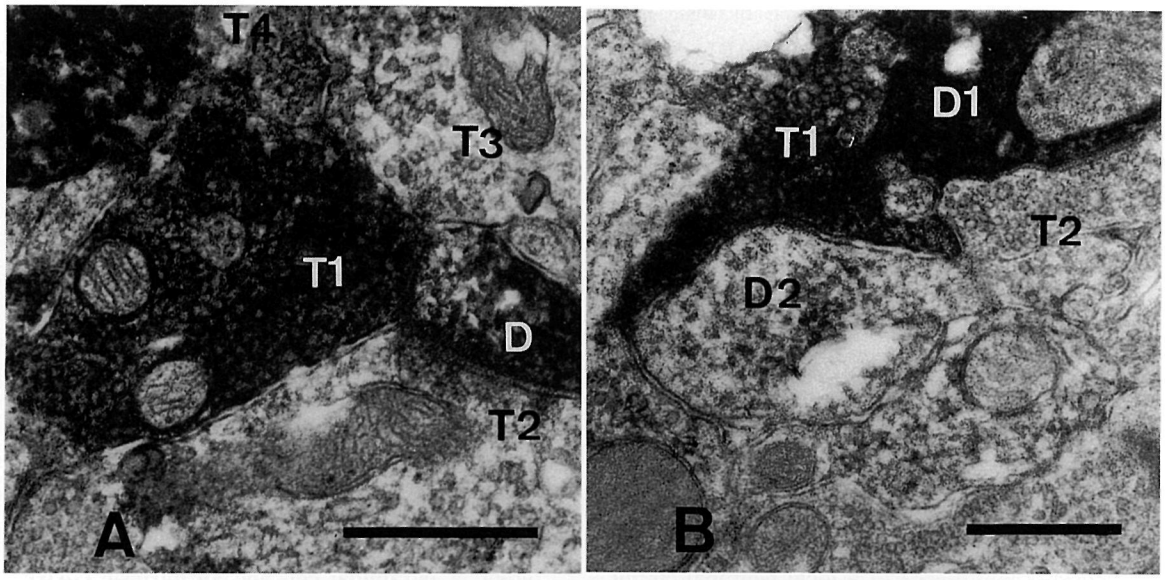


Fig. 4 Large synaptic complexes. A: An electron dense GABA terminal (T1) forms a synaptic contact with a GABA dendrite (D), which in turn forms a synaptic contact of asymmetric type with a nonGABA terminal (T2). Other nonGABA terminals (T3 and T4) were adjacent to T1. B: A dense GABA terminal (T1) forms contacts of symmetric type with a GABA dendrite (D 1) and a nonGABA one (D2). D1 forms another contact of asymmetric type with a nonGABA terminal (T2).

morphic vesicles made contacts of symmetric type with GABA and nonGABA elements. Large synaptic complexes composed of two GABA elements forming an axodendritic synapse and further adhering to the other neuronal elements were occasinally noted (Fig. 4A, B). Triadic arrangement of a GABA terminal to an axodendritic (spine) synapse was also observed. Although the membrane specialization was hardly confirmed at the site of adhesion, these arrangements were regarded as particular patterns of synaptic contacts. A few GABA terminals made synaptic contacts with typical pyramidal cells at the sites of somas and apical dendrites extending vertically toward the pial surface as well as the axon initial segments (AIS).

With respect to the synaptic interrelation, 689 contacts including GABA profiles were analyzed and the results were summarized in Table 1. It was especially worthy to note a high incidence of contacts between two GABA elements. The percentages of GABA-GABA contacts were 7% in axodendritic, 4% in axosomatic, 13% in axoaxonic, 0.7% in dendrosomatic and 0.3% in somatosomatic synapses. Namely about 25% of GABA profiles made contacts with GABA elements. Even though GABA neurons were rather sparse in layers II/III and layers V/VI, GABA neuronal elements were apt to form contacts each other, probably organizing a network of GABA local circuits in the anterior cingulate cortex.

## DISCUSSION

Most available data on morphology of pyramidal and nonpyramidal neurons in the cerebral cortex have been obtained by Golgi impregnation method[5,8]. Nonpyramidal cells of various types are likely intrinsic cells having

well-ramified short axons. Some intrinsic non-pyramidal cells are regarded as GABA neurons in the cerebral cortex on the basis of Golgi preparations and immunohistochemistry[10,11,14,18,21,26]. Particularly, chandelier cells and large basket cells are the most possible candidates for GABA neurons in comparison with the other intrinsic cells. These cells have locally well-ramified terminations known as axonal candles or pericellular nests forming a characteristic plexus[11,18]. Such specific terminations might be involved in the formation of a large synaptic complex including GABA-GABA contacts reported in this study.

A high incidence of GABA-GABA contacts suggests the presence of a local neuronal circuit or an inhibitory network composed of intrinsic GABA neurons. However, there is a possibility that contacts between two GABA profiles are partly related with the inhibition of the inhibitory neurons or "disinhibition" as stated by the previous authors[2,9]. This network may subserve to modulate the primary afferents to a particular site or a restricted cortical column, and also to relay the information from the site into a whole pyramidal cell group functioning as a unit. Since there are numerous convergent afferent fibers derived from the cortical and subcortical regions related to the limbic system[5,7,10] as well as monoaminergic fibers derived from the brain stem[12], intrinsic GABA systems might be involved in the integration of convergent inputs and the modulation of the output from pyramidal cells[3,6,13,24].

Axoaxonic contacts in the cerebral cortex have been first described by Westrum[25]. Those contacts were regarded as inhibitory in function and a more powerful "stop-valve" arrangement on the AIS than that on the soma of the pyramidal cell. In axoaxonic contacts between axonal candles of chandelier cells and AIS of pyramidal cells, the direction of impulse proceeding was evident, because only the axonal candles indicated synaptic vesicles as presynaptic sites[7,18,23]. In this report, both terminals forming axoaxonic contacts contain synaptic vesicles. At the apposition site, the synaptic membrane specialization was obscure. Nevertheless, we considered that these contacts may imply reciprocal transmission or additive one. It is possible that GABA released from a terminal may modulate the transmission of the other GABA or non-GABA terminals through the presynaptic association[19], and GABA neurons could inhibit their own release through presynaptic autoreceptors[16]. The triadic arrangement has been considered as a particular pattern of synapses among three elements: a thalamocortical afferent, a pyramidal cell and an intrinsic GABA neuron[13], and portly a dopaminergic fiber and an axodendritic synapse in the cingulate cortex[12]. At the site of triads, the released GABA as an inhibitory transmitter might interact with the presynaptic receptors and modulate the excitatory transmitter of primary afferents[12,14,17,19,24].

Concerning reciprocal synapses, Rall and coworkers have mentioned the features of dendrodendritic synapses in the olfactory bulb[20]. Thereafter, Carlton and Hayes described reciprocal synapses between GABA dendrites containing small vesicles and axon terminals in the spinal cord in serial sections[2]. In such a GABA dendrite small synaptic vesicles had been apparently demonstrated as an aggregation near the junctional membrane. Dendrites containing some vesicles in our observation were still remained uncertain because of poor tissue preservation of the immunostained materials. However, somatosomatic and dendrosomatic contacts between two GABA profiles or between GABA and non-GABA elements may induce an electrophysiological influence at the narrow adhesion gap. To confirm the synaptic relationships in the anterior cingulate cortex, further experiments will be necessary.

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〈和文抄録〉

# ラットの前帯状野における GABA 免疫陽性終末の電顕的観察

今本喜久子

滋賀医科大学解剖学第一講座

抗 GABA 抗体を用いて前帯状野を電顕免疫組織化学により観察した．シナプス部の相互関係に注目し，GABA 陽性部が関与した689個のシナプスを分析すると，特に GABA 成分同志の接着が多い事が注目された．GABA-GABA 接着は約25%あり，そのうち７%は軸索終末—樹状突起，４%は軸索終末—胞体，そして13%は軸索終末—軸索終末の接着であった．GABA 終末の大部分は対称性のシナプスをなしていた．GABA 成分同志が接着をなすものは更に他の nonGABA 成分とも接着し大きなシナプス複合体を作ることもあった．GABA 終末が non-GABA の軸索終末—樹状突起シナプスと三つ組関係をなすものも観察された．これらの結果は，内在性の GABA 陽性神経細胞が互いに接着して局所的な GABA 神経回路網を形成し，前帯状野に多方面から集中する情報の統合上で抑制的調節の役割を持つことを示唆した．