ULTRASTRUCTURAL STUDY OF GAP JUNCTIONS BETWEEN DENDRITES OF PARVALBUMIN-CONTAINING GABAERGIC NEURONS IN VARIOUS NEOCORTICAL AREAS OF THE ADULT RAT

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Abstract—Parvalbumin (PV)-containing GABAergic neurons in the hippocampus form dual networks linked by both dendrodendritic gap junctions and mutual inhibitory synapses. Recent physiological studies have demonstrated similar functional connectivity among cortical GABAergic neurons, but the corresponding structures have not been fully analyzed at the electron microscopic level. In this study we examined detailed ultrastructural features of gap junctions between PV neurons in the mature neocortex. Light microscopic observations and confocal laser scanning microscopy revealed frequent dendrodendritic contacts between PV neurons. Electron microscopic analysis provided direct morphological evidence for the existence of gap junctions between 22 pairs of PV-immunoreactive dendrites in the visual, auditory, and somatosensory cortices. Their ultrastructural features that were characteristic of immunolabeled profiles were consistent with the general structure of gap junctions. In one case a gap junction coexisted with a dendrodendritic chemical synapse, making a mixed synapse. Importantly, we also encountered a gap junction between PV positive and negative, presumptive non-principal cell-derived, dendrites. Quantitative analysis was made in 16 pairs of PV positive dendrites forming gap junctions in the infragranular layers of the somatosensory cortex. Diameters of these dendrites ranged from 0.3 to 2.7 μm, suggesting diverse locations of gap junctions along the proximal–distal axis of dendritic trees, but the majority (81%) were less than 1 μm. The mean size of gap junctions along apposing membranes was 0.22±0.09 μm. By using this size, the theoretical value of a junctional conductance was estimated to be 2.1–5.3 nS. Dendrites of PV neurons in the infragranular layers of the somatosensory cortex were reconstructed light microscopically and the sites of contacts with other PV neurons were mapped. Although these contacts do not necessarily imply gap junctional coupling, their number (5.3±2.3 per cell, n = 11) suggested the degree of connectivity of less than 10% from single PV neurons with others. Sholl analysis revealed that only 38% of their dendrites occurred within 200 μm from the soma.

The present study demonstrated detailed ultrastructural features of gap junctions between mature cortical PV neurons. These features will facilitate not only identification of gap junctions in various labeled neurons but also analysis of their functional aspects by enabling theoretical estimate of their junctional conductances. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: interneuron, dendritic network, neocortex, electron microscopy, confocal laser scanning microscopy, synchronization.

Gap junctions are specialized sites for intercellular communication. They are composed of transmembrane channel proteins, connexins (Cxs), which connect the cytoplasm of participating cells and couple them electrically. One effect of such coupling is the facilitation of synchronous activities. Unambiguous demonstration of gap junctions requires morphological analysis at the electron microscopic level. In mammalian brains, previous ultrastructural studies have established the existence of gap junctions between certain types of neurons (Brightman and Reese, 1969; Sotelo and Palay, 1970; Sloper, 1972; Sotelo and Linán, 1972; Sotelo et al., 1974; Sloper and Powell, 1978; Kosaka, 1983a, 1983b; Kosaka and Hama, 1985; Katsumaru et al., 1988b; Kita et al., 1990; Fukuda and Kosaka, 2000a; for review, see Sotelo and Korn, 1978). Although the exact functional roles of neuronal gap junctions remain unknown except in the brain stem nuclei (Korn et al., 1973; Linán et al., 1974), recent physiological techniques have proven electrical coupling among GABAergic interneurons in the neocortex and suggested a role in the synchronization of cortical activities (Galarreta and Hestrin, 1999, 2001; Gibson et al., 1999; Beierlein et al., 2000; Tamás et al., 2000). We have previously shown that a population of hippocampal GABAergic neurons containing a calcium-binding protein parvalbumin (PV) forms a dense network of dendrites that are linked to one another by gap junctions (Katsumaru et al., 1988b; Fukuda and Kosaka, 2000a). The same neuronal population is also connected by mutual axosomatic synapses (Sik et al., 1995; Fukuda et al., 1996; Cobb et al., 1997). We concluded that hippocampal PV immunoreactive (PV-ir) neurons form dual networks, one by mutual inhibitory chemical synapses and the other through dendrodendritic gap junctions. Mutual inhibitory networks of GABAergic neurons have been assigned a role in the generation of oscillatory activity in the γ frequency range (Whittington et al., 1995; Traub et al., 1996), while gap junctions have been reported to be important for the maintenance of synchrony (Yang and Michelson, 2001; Traub et al., 2001). As PV-ir neurons target perisomatic domain of thousands of principal neurons and possibly
regulate the timing of their spike generation (Kosaka et al.,
1987a; Buhl et al., 1994a; Cobb et al., 1995; Sik et al.,
1995), periodic firing patterns of mutually connected PV-ir
neurons may underlie the coordination of rhythmic popu-
lation activities in the hippocampus.

Oscillations and phase synchrony have also been ob-
served in the neocortex and it has been proposed that they
are related to cognitive processes (Gray, 1999; Singer
1999). Recent physiological findings suggest that neocor-
tical PV-ir neurons form dual networks similar to those in
the hippocampus. Indeed, our preliminary analysis of adult
brains (Fukuda and Kosaka, 2000b) and observations by
others in postnatal developing brains (Tamás et al., 2000)
demonstrated ultrastructural profiles of gap junctions be-
tween neocortical PV-ir neurons. Since there is a transient
postnatal increase in mRNA expression for neuron-specific
Cx 36 (Belluardo et al., 2000) just during the period when
most of physiological slice experiments on electrical cou-
pling have been performed (Galarreta and Hestrin, 1999,
2001; Gibson et al., 1999; Beierlein et al., 2000; Tamás et
al., 2000; Amitai et al., 2002), it is of great importance to
identify unambiguous ultrastructural profiles of gap junc-
tions in the mature neocortex and describe their accurate
morphological features in detail. Moreover, based on the
quantitative data on the size of morphologically identified
gap junctions, it will also become possible to calculate
theoretical value of junctional conductance between PV-ir
neurons and compare it with physiologically inferred val-
ues in both immature and adult (Galarreta and Hestrin,
2002) neocortex.

**EXPERIMENTAL PROCEDURES**

Seven male Wistar rats (200–300 g, 8–12 weeks old, purchased
from SLC, Shizuoka, Japan) were used in accordance with the
Guidance for Animal Welfare in Kyushu University, and efforts
were made to minimize both the suffering and the number of
animals used. Under deep anesthesia induced with sodium pen-
tobarbital (10 mg/100 g body weight, i.p.) the animals were per-
fused quickly through the ascending aorta with 300 ml of fixative
A containing 2.5% glutaraldehyde and 2% paraformaldehyde in
0.1 M phosphate buffer (pH 7.2) or fixative B containing 4%
paraformaldehyde, 0.1% glutaraldehyde, and 0.2% picric acid in
the same buffer at room temperature. Specimens fixed with solu-
tions A and B were processed in the same manner as described
previously (Fukuda and Kosaka, 2000a), many PV/GAD double immu-
noelectrophoretic electrophoretic procedures for immunocytochemistry,
munostaining results were visualized with NeuroLucida (MicroBright-
Field, Colchester, VT, USA), and electron microscopy (EM) were
the same as described previously (Fukuda and Kosaka, 2000a)
except for the incubation time with primary antibodies, which in
the present study was 2 weeks at room temperature for both LM and
CLSM and 4 days at 10 °C for EM. The primary antibodies used
were rabbit polyclonal antibody against rat PV (gift from Dr. C. W.
Heizmann, dilution 1:5000; Kági et al., 1987) and sheep polyclonal
antibody S3 (gift from Dr. W. H. Oertel, 1:2000; Oertel et al., 1981)
that recognizes both isoforms of glutamic acid decarboxylase
(GAD), GAD65 and GAD67. PV-immunoreactivity was visualized
in EM by using ABC kit (Vector Laboratories, Burlingame, USA)
with 3.3’ diaminobenzidine tetrahydrochloride as a chromogen.
For double-immunofluorescent labeling, PV was visualized with
fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:
100; Jackson Immunoresearch, West Grove, USA), and GAD
with biotinylated donkey anti-goat IgG (1:500; Jackson Immu-
noResearch) followed by rhodamine-X-conjugated streptavidin (1:
500; Jackson Immunoresearch). CLSM was performed using a
TCS confocal microscope system (Leica) equipped with a kryp-
ton–argon ion laser and mounted on a light microscope (DMRE,
Leica). Serial ultrathin sections were stained lightly with uranyl
acetate and lead citrate and examined in an H-7100 (Hitachi,
Tokyo, Japan) or EM10C (Zeiss, Oberkochen, Germany) electron
microscope. Data on the size of gap junctions were normalized,
stratified into four groups around the mean value, and the degree
of fitness for the normal distribution was assessed by chi-square
test with a significance level of 0.05.

**RESULTS**

**Light microscopic observations**

General morphological features of PV-ir structures in the
somatosensory, auditory, and visual cortices were consis-
tent with previous observations (Celio and Heizmann,
1981; Kosaka et al., 1987b; Stichel et al., 1987; Celio,
1990; Hendry and Jones, 1991; van Brederode et al.,
1991; Kawaguchi, 1995; DeFelipe, 1997; Kawaguchi and
Kubota, 1997, 1998). PV-ir somata were distributed
throughout these cortical areas and located in all layers
except layer 1 (Fig. 1a). They were multipolar in shape,
extending dendrites in all directions (Figs. 1d, 2b). Den-
drites were of the smooth type lacking spines in most
cases, but some showed sparsely spiny appearances. The
most proximal parts of the dendrites were usually 2 to 4 µm
in diameter and their diameters decreased gradually to-
ward the distal endings.

Numerous PV-ir boutons were distributed in layers 2 to
6, some surrounding somatic profiles while others were
scattered throughout the neuropil (Fig. 1b). As in observa-
tions in the hippocampus (Fukuda et al., 1996, 1998;
Fukuda and Kosaka, 2000a), many PV/GAD double immu-
noreactive boutons were located on somata and proximal
dendrites of not only GAD-negative, presumptive excita-
tory pyramidal or spiny stellate neurons but also PV-ir
neurons (Fig. 1b, c). In electron microscopic observations,
we confirmed that PV-ir axon terminals formed symmetri-
cal synapses with somata and dendrites of both PV-la-
beled and unlabeled neurons (data not shown). These
observations suggest the possibility that neocortical PV-ir
neurons form a network of mutual inhibitory connections
just as in the hippocampus.

**Dendrodendritic contacts**

In conventional immunocytochemical procedures, the per-
meation of antibodies from the section surfaces into their
depth parts was rather limited in the neocortex, and the
network structure of PV-ir dendrites was not as apparent
as expected. In order to facilitate the permeation of anti-
odies we prolonged the incubation of sections with pri-
mary antibodies up to 2 weeks, and this permitted visual-
ization of a dense plexus of PV-ir dendrites extending in
various directions (Figs. 1d, 2a, b). When these dendrites
Fig. 1. CLSM images showing morphological characteristics of neocortical PV-ir neurons. (a) Low power micrograph of PV-ir neurons taken from a coronal section of the primary visual cortex. Labeled somata are distributed throughout cortical layers from II/III to VI. One PV-ir neuron in layer VI (arrow) is enlarged in b and its dendrite is shown in d–f. (b, c) Double immunostaining with antibodies against PV (b) and GAD (c). Note multiple PV/GAD double immunoreactive boutons (open arrowheads) abutting on somata and a proximal dendrite of PV neurons. Unstained somata of pyramidal cells (p) are also surrounded by double immunoreactive boutons. (d–f) A long dendrite originating from the PV-ir soma (arrow), which is also shown in a and b, runs obliquely toward the lower left and makes contact with a vertical dendrite (open arrow). The contacting dendrites are enlarged in both the projected image taken from several confocal images (e) and single confocal image (f), the latter strongly suggesting the direct contact between the crossing dendrites. Note also in d that many PV-ir somata extend dendrites in all directions but that vertical dendrites have a tendency to be bundled together. Scale bars = 100 μm (a, d); 10 μm (b, c, e, f).
were examined with CLSM, they appeared to make direct contacts with one another (Fig. 1e, f). Such contacts were ubiquitous in all areas of the neocortex examined, both in the supragranular and infragranular layers. These contact sites were found between dendrites of various orientations. Some crossed at fairly wide angles (Fig. 1e) while others ran almost parallel to each other, making side-to-side contact (Fig. 2c). When such side-to-side contacts occurred between vertically oriented dendrites, their parent somata had a tendency to be aligned vertically (Fig. 2a, arrows indicating somata of cells 1 and 2) suggesting that these contacting neurons belonged to the same columnar structure. Dendritic contacts were made also between cells whose somata were separated up to 300 to 400 μm horizontally.

Electron microscopic observations of gap junctions

It is essentially important to confirm that apparent contact sites seen at the light microscopic level really form gap junctions. Therefore, PV-ir structures in the neocortex were examined with correlated light and electron microscopy (Fig. 4) as well as with usual immunoelectron microscopy (Figs. 3, 5, 6, 7). Ultrastructural identification of gap junctions depended critically on two factors: one was a good preservation of tissue, particularly of unit membranes, and the other was the appropriate angle for observing the apposing membranes which make gap junctions. This is because gap junctions consist of disc-like assemblies of membrane-spanning proteins (connexons) and can be detected in transmission electron microscopy.

Fig. 2. Contacts between two parallel dendrites originating from two vertically arranged PV neurons in layer VI of the primary somatosensory cortex viewed in a coronal section. (a) One PV neuron (1) gives rise to an ascending dendrite (filled arrowheads) toward layer V while another PV neuron (2), the soma of which (white arrow) is out of focus in this micrograph, elongates a descending dendrite (white arrowheads), both come close to each other at the position indicated by an asterisk. (b) Enlargement of cell 1 showing that it gives rise to dendrites in all directions (filled arrowheads), one of which ascends and makes contacts with one of descending dendrites (white arrowheads) from cell 2. (c) Further enlargement of the contact site. Spatial relationship of two dendrites (filled and white arrowheads) were fully examined with a high-resolution objective and found that they run parallel and make contacts with each other. Scale bars = 100 μm (a); 20 μm (b); 5 μm (c).
only when cross-sectional profiles of apposing membranes are viewed (Sotelo and Korn, 1978). Therefore, it was essential to tilt the sample stage to find an appropriate angle where the laminar structure of unit membrane was best discernible on both sides of the junctions. At this angle, gap junctions between unlabeled profiles such as in conventional EM were visible as heptalaminar structures composed of alternating four electron-dense lines and three electron-lucent spaces, the former corresponding to the outer and inner leaflets of apposing plasma membranes whereas the central clear space being the gap between the leaflets (2, 6), and a central gap (4). A similar heptalaminar profile could be detected in a junctional site located deep in a flat embedded, 40 μm-thick section where immunoreactivity of the contacting dendrites became very weak (Fig. 3a–c); the same dendrites were confirmed to be PV positive in the more superficial locations by exploring consecutive ultra-thin sections (data not shown). In most instances, however, profiles of gap junctions between immunolabeled dendrites were slightly modified. A single straight or slightly curved line was clearly recognizable at the center of the junction, which was sandwiched by electron-lucent spaces and further by inner leaflets of individual membranes, resulting in a pentalaminar appearance (Figs. 3e, 4d, f, 5b, d, f). This pattern is thought to be caused by the immunocytochemical procedures that might unavoidably occlude the central gap and make it rather difficult to distinguish two apposing outer leaflets of the junctional membranes. This interpretation is supported by tracing continuity of unit membranes from surrounding non-junctional part to the contact site (arrowheads in Figs. 3e, 4d, 5f, 6b, e, h). Importantly, the central line was not uniform but displayed some periodicity. In favorable sections, the central line showed alternating highly electron-dense and moderately dense patterns, each having a periodicity of approximately 8 nm (Fig. 3f). This reminded us of a known structure of

Fig. 3. Electron micrographs showing typical ultrastructures of gap junctions seen in almost immunolabeling-free (a–c) and apparently labeled (d–f) profiles in the infragranular layers of the somatosensory cortex. (a) Two dendrites (D) make direct contact with each other. Immunoreactivity is hardly visible in this position located deep in the 40 μm-thick section. (b) Enlargement of the contact site in a. Plasma membranes of the two dendrites (m1, m2) come close to each other and make gap junction at the site demarcated by arrowheads. Semi-dense material indicated by dots is characteristic of neuronal gap junctions. Note ordinary extracellular space (ex) that ends at the edge of gap junction. (c) Enlargement of the area shown by a bracket in b, demonstrating heptalaminar appearance of gap junction consisting of inner (1, 7) and outer (3, 5) leaflets of plasma membranes, spaces between the leaflets (2, 6), and a central gap (4). (d) Two PV-ir dendrites (D) make contact with each other. (e) Enlargement of the framed area in d. As in b, two plasma membranes (m1, m2) form gap junction at a site demarcated by arrowheads. Continuity of membranes from non-junctional part to the edge of gap junction is clearly seen at the left border of the junction, where outer leaflets of apposing membranes are fused and visible as a distinct central line. Semi-dense material is shown by dots. (f) Further enlargement of the central line in e at the left border of the junction (arrowhead). Outer leaflets (3, 5) and a central gap are fused to make the line (3 + 4 + 5), but close view reveals alternating highly electron-dense (black arrows) and moderately dense (white arrows) patterns along the line. Scale bars=0.5 μm (a, d); 0.1 μm (b, e); 0.01 μm (c, f).
Fig. 4. Micrographs of correlated light and electron microscopy showing PV-ir dendrites that form gap junctions in layer VI of the secondary auditory cortex. (a) Light micrograph of two PV-ir dendrites; one descending obliquely while the other runs horizontally in a coronal section. Both were found in EM to make gap junctions with other dendrites at the contact sites 1 and 2, respectively. (b) Low power electron micrograph showing the oblique dendrite and its surrounding structures. All immunoreactive elements in b correspond to the labeled profiles in a. (c) Enlargement of the contact site 1. The oblique dendrite in b is demonstrated here as horizontal one, which makes direct contact with a cross-sectional profile of another PV-ir dendrite. Note many synaptic terminals (asterisks) on these dendrites. (d) Further enlargement of the contact site in c, demonstrating a gap junction formed between two PV-ir dendrites. Note the close apposition of the plasma membranes of the two contacting cells as demarcated by arrows. Trilaminar profiles of unit membranes at the surface of presynaptic terminals (asterisks) can be used as a standard for analyzing membranous structures. (e) Electron micrograph of the contact site 2 in a showing direct contact between the horizontal dendrite and a cross-sectional profile of another PV-ir dendrite. (f) Enlargement of the contact site 2 exhibiting profile of gap junction as demarcated by arrows. Scale bars—10 μm (a); 5 μm (b); 1 μm (c, e); 0.1 μm (d, f).
Fig. 5. Electron micrographs showing gap junctions between PV-ir dendrites (D) in various cortical areas. Contact sites in layer VI of the primary somatosensory cortex (a), layer VI of the primary visual cortex (c), and at the border between layer III and IV in the secondary auditory cortex (e) are enlarged in b, d, f, respectively, and gap junctions are demarcated by arrows. Profiles in c and d are from a tangential section whereas others from coronal sections. Note presynaptic terminals (asterisks) and a trilaminar structure of the unit membrane (arrowhead in b). Scale bars = 1 μm (a, c, e); 0.1 μm (b, d, f).
gap junctions in which connexons are arrayed two-dimensionally with a mean interparticle distance of 8–9 nm (Makowski et al., 1977; Unwin and Zampighi, 1980). Similar periodic profiles of spot contacts bridging the apposing membranes have already been demonstrated clearly in neuronal gap junctions in conventional, unlabeled specimens (Sotelo and Palay, 1970; Sloper, 1972). Therefore, the detailed structure of the central line shown here was thought to reflect the ultrastructure of gap junctions, although it was not determined which of the alternating patterns corresponded to connexons. Another important feature of immunolabeled gap junctions was that its width was compatible with the known size of neuronal gap junctions seen in unlabeled profiles (Fukuda and Kosaka, 2000a). This could be confirmed most easily and reliably by using trilaminar profiles of unit membranes in the surrounding tissue as a standard of membrane thickness (Figs. 3e, 4d, f, 5b, d, f, 6b, e, h). Also it should be taken into consideration that the most outer lines of the five layers, the inner leaflets of individual plasma membranes, were often studded with reaction products inside the cytoplasm and appeared thicker than their original size. Semi-dense material specific to neuronal gap junction (Sotelo and Korn, 1978) was occasionally seen (Fig. 3b, e) but often rather indistinguishable from reaction products. Simple apposition of membranes lacking any specialized structures sometimes resembled gap junctions when such membranes were viewed obliquely, but at an appropriate angle they could be clearly distinguished from gap junctions by both their much larger total width and the larger intercellular spaces between the outer leaflets of apposing membranes.

Unambiguous ultrastructural profiles of gap junctions could be observed at 22 contact sites of PV-ir dendrites in the somatosensory (n=16), auditory (n=4) and visual (n=2) cortices (Figs. 3, 4, 5, 6, 7). As observations were carried out mainly in the infragranular layers, 21 out of 22 identified gap junctions were located there. In one case, a gap junction was found at deep layer III or upper layer IV (Fig. 5e, f). Correlated light and electron microscopic images as well as observations in consecutive ultrathin sections suggested that gap junctions were formed both be-
Fig. 7. Gap junction between PV-positive and negative dendrites. (a) Two dendrites, one is PV-ir and the other PV negative, make direct contact with each other. (b) Enlargement of the contact site in a. Typical heptalaminar appearance of gap junction is clearly seen. Note the central gap displaying periodic bridging structures and semi-dense material undercoating the junctional membranes. (c–e) Exclusion of the possible false negative labeling of one of the coupled dendrites. Two contacting dendrites shown in a were traced in consecutive ultrathin sections toward the surface of the 40 μm-thick section and reconstructed in e, where arrow indicates the site of gap junction whereas the superficial position is seen on the left side. Profile in the most superficial part shown in c demonstrates that permeation of antibodies at this position is sufficient to label many PV-positive elements (arrowheads) surrounding the traced dendrites (PV+ and PV−). Framed area in c is enlarged in d, showing that one of the gap junction-forming dendrites consistently lacks PV immunoreactivity even in the section surface. Scale bars—0.5 μm (a, d, e); 50 nm (b); 1 μm (e).
between crossing dendrites (Figs. 4, 5a, e) and between parallel dendrites (Fig. 5c), just as seen in light microscopic observations. For instance, in Fig. 5c serial ultrathin sections taken from a tangential section of layer VI in the primary visual cortex revealed a gap junction formed between cross-sectional profiles of two dendrites that appeared to run in a common orientation perpendicular to the plane of the micrograph. The contact of this type, another example of which was shown in our previous report (Fig. 3A in Fukuda and Kosaka, 2000b), possibly corresponds to the side-to-side contacts between those dendrites as seen in Fig. 2C.

In one case a gap junction coexisted with a dendrodendritic chemical synapse in the somatosensory infragranular layers (Fig. 6f–h). Synaptic vesicles occurred in a small cluster close to the junctional membrane of one side. The synaptic contact was of a symmetrical type. These features were common to dendrodendritic synapses between non-pyramidal cell dendrites in the monkey neocortex (Sloper and Powell, 1978a) and those between hippocampal PV-ir neurons (Fukuda and Kosaka, 2000a).

We encountered a gap junction that was formed between PV positive and negative dendrites (Fig. 7a, b). In order to exclude the possibility that the apparent lack of immunolabeling might be false negative due to its location deep in the specimen, the coupled dendrites were traced in serial ultrathin sections toward the cut surface of the 40 μm-thick section (Fig. 7e). Even near the cut surface where many PV-ir profiles surrounded the traced dendrites (Fig. 7c), one of these gap junction-forming dendrites remained to be PV negative (Fig. 7d). The PV negative dendrite did not show spiny appearance (Fig. 7e) and it received several asymmetrical synaptic contacts on its shaft (Fig. 7a, d).

Diameters of dendrites forming gap junctions were measured in all 23 pairs examined in the present study (Fig. 8a). Most of the dendrites making gap junctions were small to medium in size, 0.5 to 1.5 μm. Moreover, at least one of the paired dendrites in 22 out of 23 cases had a diameter smaller than 1 μm. On the other hand, such a pair as was composed of two thin (<0.5 μm) dendrites was found in only two cases. These results suggest that gap junctional coupling was not so frequent between two thin dendrites nor between two thick dendrites. In 16 pairs in the infragranular layers of the somatosensory cortex (Fig. 8b), dendritic thickness ranged from 0.3 to 2.7 μm, suggesting diverse locations of gap junctions along the proximal–distal axis of dendritic trees, but the majority (81%) was less than 1 μm (mean±S.D., 0.78±0.42 μm).

In order to estimate the theoretical value of a junctional conductance, the sizes of gap junctions (n=16) were measured along apposing membranes in the infragranular layers of the somatosensory cortex (Fig. 8c). Profiles of individual gap junctions were observed in one to six serial ultrathin sections, and the maximum length for each gap junction was determined by observing these serial sections. The size of gap junction ranged from 0.08 to 0.42 μm (Figs. 6b, e, 8c). The histogram showed a rather symmetrical distribution around the average size (0.22±0.09 μm, mean±S.D.), and fitness for the normal distribution was not denied in chi-square test (χ=3.64; degree of freedom=1).

**Light microscopic reconstructions of PV-ir dendrites**

To know the average number of gap junctions a single PV neuron forms with others will be essential for understanding functional significance of gap junctions in the network activities of PV neurons. Therefore dendrites arising from PV neurons in the infragranular layers of the somatosensory cortex were reconstructed from serial sections by using a computer-assisted tracing system. Sites of contact between PV-ir dendrites were carefully identified through a high-resolution objective and marked in the reconstructed traces (Fig. 9a). Although these contacts do not necessarily imply gap junctional coupling, this analysis could provide a rough estimation of maximum number of couplings per neuron. The number of light microscopically identified...
contacts in a single PV neuron was found to be $5.3 \pm 2.3$ per cell ($n=11$). Considering that the identification of contact sites using light microscopy originally contains errors of counting non-junctional sites, it would be reasonable to infer from the present value that the connectivity of gap junctional coupling between cortical PV-ir neurons appears to be in the range that does not exceed 10 direct connections with others. Finally, degree of the extension of dendrites was quantitatively analyzed by Sholl method (Fig. 9b, c). In the infragranular layers of the somatosensory cortex, dendritic segments classified by the distance from the soma showed a distribution rather symmetrical around the moderate value (200–280 μm), and 38% of the dendrites occurred within 200 μm of the soma, which was in sharp contrast with previous data on layer IV fast spiking cells (Amital et al., 2002).

**DISCUSSION**

The present study provides direct ultrastructural evidence for the existence of gap junctions between dendrites of PV-containing GABAergic neurons in various neocortical areas of the adult rat. Their structural features were fully described and confirmed to be consistent with known profiles of gap junctions. The size of strictly identified gap junctions was measured for the estimation of theoretical value of conductance across a single gap junction. Light microscopic reconstructions of
PV-ir neurons were made to know the degree of their connectivity.

**Methodological considerations**

Gap junctions are ultrastructurally defined structures and therefore identifiable most reliably by electron microscopic analysis (Brightman and Reese, 1969; Sotelo and Korn, 1978). Identification of gap junctions with immunoelectron microscopy was performed in several steps: first, we adjusted the appropriate angle for viewing the junctions, second we visualized cross-sectional profiles of apposing membranes that exhibited the pentalaminar appearance typical for immunolabeled specimens, and finally we determined whether the total width of the junctional complex was consistent with the known size of gap junctions.

As the structural integrity of the tissues was a prerequisite for identifying gap junctions, we used a fixative containing a high concentration of glutaraldehyde for the immunoelectron microscopy (Kosaka et al., 1986). This enabled us to visualize membranous structures clearly and identify gap junctions correctly. However, fair preservation of the tissue inevitably reduced the permeation of antibodies into sections in EM, leading to the labeling of only short dendritic segments exposed to the section surface. Therefore, it was difficult to trace dendrites from the sites of ultrastructurally identified gap junctions all the way back to their parent somata. Because of this limitation, it could not be determined directly how gap junctions are distributed three dimensionally along the dendritic trees.

**Gap junctions in the neocortex**

The ultrastructural features of gap junctions that we observed in immunolabeled specimens are consistent with those in previous electron microscopic studies performed in unlabeled specimens from the sensory-motor cortex of the adult monkey (Sloper, 1972; Sloper and Powell, 1978b). In these early studies gap junctions were found exclusively between cytologically identified non-pyramidal neurons, in most cases between dendrites and occasionally between a dendrite and a soma. Although we did not encounter dendrosomatic gap junctions but rather observed profiles of simple apposition of somatic and dendritic membranes, we could not exclude the existence of dendrosomatic gap junctions. Our failure of detecting them might reflect their less frequent occurrence as compared to gap junctions between dendrites.

Sloper and Powell (1978b) described that most of the dendrites making gap junctions were of small to medium size, 0.5 to 1.5 μm, and our data corroborated their observations. We further compared the diameters of coupled dendrites, and this analysis indicated that gap junctions were not frequently located at the position either very close to or far away from somata. However, in most of physiological experiments identifying electrical coupling in the neocortex, cell recordings have been made from pairs of neurons which were located very close to each other (Galarreta and Hestrin, 1999, 2001, 2002; Gibson et al., 1999; Beierlein et al., 2000; Tamás et al., 2000). As a high percentage of electrical coupling has been observed in such pairs, our data of infrequent occurrence of gap junctions between two thick dendrites do not appear to support those physiological findings. One possible explanation is that closely located pairs may form gap junctions at positions which are at some distance away from at least one of the paired somata. Our present data on less frequent occurrence of gap junctions between two thin (<0.5 μm) dendrites may reflect physiological findings that electrical coupling was not observed between pairs located more than 200 μm apart (Amitai et al., 2002). However, Sholl analysis revealed marked difference in the dendritic morphology between layer IV fast spiking, most presumably PV-ir neurons (Amitai et al., 2002) and infragranular PV neurons (Fig. 9b, c). It was reported that 80–90% of the dendrites occurred within 200 μm from the soma in the former, whereas only about 40% of dendrites occurred within 200 μm in the latter. Our preliminary observations suggest further variability in dendritic structures of PV-ir neurons across areas, layers, and species. Future morphological investigations will reveal whether some of light microscopically detectable contact sites at distal dendrites (Fig. 9a) may correspond to gap junctions or only proximal dendrites form gap junctions even in larger neurons.

Another specialized form of dendrodendritic contact, the chemical synapse between dendrites of non-pyramidal neurons, was previously described in the monkey neocortex (Sloper, 1972; Sloper and Powell, 1978a). In the present study we encountered a profile of mixed synapse consisting of a gap junction and a dendrodendritic chemical synapse. We observed similar structures between PV-ir dendrites in the hippocampus (Fukuda and Kosaka, 2000a). Occurrence of synaptic vesicles in a small cluster close to junctional membrane is common to profiles in all these studies and suggests some specific functional properties of these synapses. It is also an important issue whether presynaptic dendrites have active properties and transmit signals like axons or only local excitatory inputs can activate dendrodendritic synapses.

The gap junctional coupling between PV positive and negative dendrites was newly identified in the present study. Structural features of the negative dendrite such as aspiny appearance and some asymmetrical synaptic inputs on its shaft strongly suggest that the dendrite originated from a PV negative non-principal neuron. Physiological studies in slice experiments have shown that electrical coupling is observed mainly between cortical interneurons of the same group (Galarreta and Hestrin, 1999; Gibson et al., 1999; Beierlein et al., 2000). However, coupling between fast-spiking cell (presumptive PV neuron) and low-threshold spiking cell was also recorded with a probability of about 10% (Gibson et al., 1999; Beierlein et al., 2000). It cannot be determined whether the gap junction-forming, PV negative neuron we observed belongs to a low-threshold spiking cell, a fast-spiking cell of a different group lacking PV immunoreactivity, or another type. At least it can be said that gap junctional couplings between morphologically different cell types persist in the adult neocortex. The incidence of such coupling and characterization of
the PV negative population should be systematically explored in future morphological studies.

Direct cell coupling has been suggested between not only PV neurons but many other populations (Gibson et al., 1999; Schmitz et al., 2001; Landsman et al., 2002) including those in human neocortex (del Rio and DeFelipe, 1997). Ultrastructural examination will facilitate identification of their nature as specific junctional structures.

**Estimation of junctional conductance**

We measured the length of gap junctions along the central line and obtained a value of 0.22±0.09 μm. Profiles of these gap junctions were detectable in 1 to 6 of serial ultrathin sections (each 65–70 nm thick), and in each case the total thickness corresponded well to the maximum length observed in such series. Therefore the two-dimensional structure of gap junctions we observed was thought to be of the usual plaque type, just as has been demonstrated in freeze-fracture studies of Cx 36-labeled neuronal gap junctions in the rat brain (Rash et al., 2000, 2001). We estimated the number of connexons in a single gap junction by hypothesizing its round shape with a diameter of 0.22 μm and by applying available data on connexon density in neuronal gap junctions (4000–10,000 connexons/μm; Tuttle et al., 1986; Baldridge et al., 1987). The obtained value, 150–380 connexons per single gap junction, was compatible with the number (average, 247 connexons per gap junction) counted in Cx 36-labeled plaques in the inferior olive of adult rat (Rash et al., 2000), where the size of gap junctions appears similar to that in cortical PV-ir neurons (Sotelo et al., 1974; Rash et al., 2000, 2001). Then this number was multiplied by unitary conductance (14 pS) of a single connexon composed of Cx 36 (Teubner et al., 2000). We conclude that the theoretical value of conductance of a single gap junction between PV-ir dendrites in the infragranular layers of the somatosensory cortex would be 2.1–5.3 nS on average.

This value is larger than that inferred from electrophysiological recordings in slices from both immature (0.7–1.6 nS on average; Galarreta and Hestrin, 1999; Gibson et al., 1999) and adult (0.2 nS; Galarreta and Hestrin, 2002) neocortex. There are two issues which should be considered for explaining these discrepancies. First, our estimation is that of theoretical maximal value under the ideal condition where all connexons are open. One important character of gap junctions is that their gating is regulated by many factors such as intracellular pH, calcium concentration, and temperature. Therefore smaller values in physiological recordings might reflect intracellular conditions which will close part of connexon channels. An estimation of the goldfish Mauthner cell gap junctions even suggested that only 0.6% of connexons are open at any time during electrical transmission (Tuttle et al., 1986). Another issue is that calculation of junctional conductance from physiological recordings is usually based on a simple two-cell model, which ignores electrotonic length along dendrites. In this model gap junctions are regarded as somatostematic contacts. If actual location of gap junctions is taken into consideration, the junctional conductance calculated from physiological recordings might become higher. In this respect, the smaller value of physiological estimation in mature cortical slice than in immature one may be attributed to more remote positions of gap junctions from the soma in adult brains. Considering that both the gating and distance factors will probably be involved in physiological recordings, our estimation appears to be compatible with previous data, and such a morphological approach can provide some basic parameters for further analysis and modeling.

**Dual networks of cortical PV neurons**

Ultrastructural findings in the present study clearly indicate that dendrites of PV neurons in the neocortex of the adult rat are connected through gap junctions. We also confirmed profiles of PV-ir boutons contacting on PV-ir neurons by double-labeled CLSM (Fig. 1b, c). In the cat visual cortex, basket cells that are most probably PV-ir have been shown previously to establish frequent axosomatic contacts with other surrounding PV-ir neurons (Kisvárday et al., 1993). Ultrastructural profiles of synaptic contacts between PV-ir axon terminals and PV-ir somata have also been identified in the neocortex (Williams et al., 1992; our present observations). These morphological data indicate that cortical PV neurons form dual networks as in the hippocampus.

**Functional implications**

PV-ir neurons of both hippocampus (Kosaka et al., 1987a; Katsumaru et al., 1988a; Sik et al., 1995) and neocortex (DeFelipe et al., 1989; van Beredenode et al., 1991; Williams et al., 1992; Kawaguchi, 1995; Kawaguchi and Kubota, 1998) include populations of so-called somatic inhibitory interneurons, basket cells and chandelier cells, which target the perisomatic domain of a large number of principal neurons (Buhl et al., 1994a,b; Halasy et al., 1996) and possibly regulate the timing of spike generation in these targets (Cobb et al., 1995). Physiological evidence suggests that a synaptic connected network of hippocampal GABAergic interneurons may be critically involved in generating synchronous oscillatory activities (Soltész and Deschênes, 1993; Michelson and Wong, 1994; Bragin et al., 1995; Whittington et al., 1995; Ylinen et al., 1995; Traub et al., 1996). On the other hand, roles of gap junctions in synchronous firing have been demonstrated in simultaneous double-recordings in pairs of cortical GABAergic neurons that are linked by electrical synapses (Galarreta and Hestrin, 1999, 2001; Gibson et al., 1999; Beierlein et al., 2000; Tamás et al., 2000). Simulation and electrophysiological evidence further indicated that gap junctions between interneurons (presumably dendritic) can enhance the synchrony of γ oscillations in spatially extended interneuron networks (Traub et al., 2001). These data support the idea that the dual networks of PV-ir GABAergic neurons constitute at least part of the mechanism responsible for the generation of rhythmic synchronous activities. Electrophysiological studies on Cx 36 knockout mice corroborate this idea. In slices of these animals, the electrical coupling is absent, the synchrony of
rhythmic inhibitory potentials is weaker and more spatially restricted than in normal animals (Deans et al., 2001; Hormuzdi et al., 2001). Recent study on in vivo activities further suggested that Cx 36 interneuronal gap junctions selectively contribute to γ oscillation (Buhl et al., 2003). However, it appears that there still remain many unknown factors that are involved in the naturally occurring synchronous activities including those in humans. Efforts should be continued to accumulate further basic morphological as well as physiological data before obtaining an overview of these intriguing phenomena in the brain.

Conclusion

PV-immunoreactive GABAergic neurons in the neocortex of the adult rat form dual networks connected by both gap junctions and mutual chemical synapses. The structural features of these networks are consistent with previous physiological findings, but exact functional roles of these dual networks need to be more firmly established by combined anatomical and physiological approaches such as clarifying the precise spatial relationship between the gap junction-coupled network and the columnar organization in the neocortex.

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