GABAergic Cell Subtypes and their Synaptic Connections in Rat Frontal Cortex

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Physiological, morphological and immunohistochemical characteristics of non-pyramidal cells in frontal cortex of young rats were studied *in vitro* by whole-cell recording and biocytin injection. Several groups of GABAergic non-pyramidal cells were identified: (i) parvalbumin fast-spiking (FS) cells with low input resistances and spikes of short duration, including extended plexus (basket) cells and chandelier cells. These cells showed abrupt episodes of non-adapting repetitive discharges; (ii) late-spiking (LS) cells exhibiting slowly developing ramp depolarizations, including neurogliaform cells; (iii) the remaining groups contained both burst-spiking (BS) or regular-spiking (RS) non-pyramidal (NP) cells. BSNP cells exhibited bursting activity (two or more spikes on slow depolarizing humps) from hyperpolarized potentials. Both these physiological types corresponded to a range of morphologies: (i) somatostatin-containing Martinotti cells with ascending axonal arbors to layer I (some were also positive for calbindin D_{28k}); (ii) VIP-containing double bouquet cells with descending axonal arbors as well as arcade cells (these included small cells immunoreactive for CCK or calretinin). Each subtype of cells made GABAergic synapses onto relatively specific portions of cortical cells, but similar domains were innervated by multiple classes of GABA cells.

Introduction

In pyramidal cells of the rat frontal cortex, callosal fiber stimulation induces excitatory potentials followed by two types of inhibitory postsynaptic potentials (IPSPs) with different time courses (Kawaguchi, 1992): an early one mediated through $GABA_A$ receptors and a late one via $GABA_B$ receptors that is dependent on extracellular potassium concentration (Connors *et al.*, 1988; McCormick, 1989). Since both types of IPSPs are also suppressed by antagonists for excitatory amino acids, these IPSPs are conveyed indirectly via intrinsic GABAergic cells excited by callosal fibers. The neocortex contains pyramidal and non-pyramidal cells (Peters and Jones, 1984; DeFelipe and Fariñas, 1992). Because pyramidal cells are excitatory and have been assumed to be glutamatergic (McCormick, 1992; Thomson and Deuchars, 1994; Hendry, 1996), cortical cells inducing GABAergic IPSPs are thought to be non-pyramidal cells (Ribak, 1978; Houser *et al.*, 1983; Thomson *et al.*, 1996; for review, Somogyi, 1989; Jones, 1993).

The neocortical output neurons, pyramidal cells, are apparently organized into laminae according to their projection sites (Jones 1984a; White, 1989). Pyramidal cells projecting to the ipsi/contralateral cortex, brainstem/spinal cord or thalamus are clustered in layers II/III, V or VI respectively. Although there are several types of pyramidal cells with different projection sites, they take relatively uniform shapes with apical and basal dendrites, and primary axons descending toward the white matter (Feldman, 1984; DeFelipe and Fariñas, 1992). In contrast, non-pyramidal cells show morphological diversity of dendritic and axonal arborization patterns as revealed by Golgi staining (Ramón y Cajal, 1911; Jones, 1975; Fairén *et al.*, 1984; Peters and

Saint Marie, 1984). These non-pyramidal cells are considered to be GABAergic inhibitory interneurons, suppressing and modulating cortical cell activity. The structural heterogeneity of cortical GABA cells may reflect functional differences, but its significance remains to be elucidated.

To understand the possible functional role of each type of GABA cell better, it is necessary to examine a number of physiological, chemical and morphological characteristics. How does each type of GABA cell fire spikes in response to excitatory input (Connors and Gutnick, 1990)? This may reflect non-linear transformations and the production of time delays in information processing through local circuits. Which types and portions of cortical cells does each type of GABA cell inhibit (Somogyi, 1989)? This would indicate which interneuron subtypes are involved in laminar or columnar inhibition (DeFelipe and Fariñas, 1992). What kind of neuroactive substances does each subtype express other than GABA (DeFelipe, 1993)? Some GABAergic cells express peptides such as somatostatin (Hendry *et al.*, 1984; Somogyi *et al.*, 1984) which further depresses the excitability of cortical cells (Wang *et al.*, 1989; Schweitzer *et al.*, 1993). GABA itself produces inhibition with different time courses depending on which receptor subtypes are expressed in the postsynaptic membrane (Connors *et al.*, 1988; McCormick, 1989; Benardo *et al.*, 1994; Kang *et al.*, 1994). GABA cells may therefore produce several types of postsynaptic responses with different transduction mechanisms and time course.

Recently, we have been investigating the firing response to depolarizing current or excitatory input, axon arborization pattern and synaptic connectivity, and co-expression of neuroactive substances in non-pyramidal cells considered to be GABAergic. On the basis of this characterization, cortical GABAergic interneurons of rat frontal cortex have been found to be heterogeneous and composed of several distinct classes, like GABAergic interneurons in other forebrain areas (Somogyi, 1994; Kawaguchi *et al.*, 1995; Freund and Buzsáki, 1996; Kawaguchi, 1997). We summarize our observations here in an preliminary attempt to establish general organizing principles of local circuits in the neocortex.

Materials and Methods

Immunohistochemical Methods for Co-localization of Two Antigens

Immunohistochemical methods were described in detail elsewhere (Kubota *et al.*, 1994; Kubota and Kawaguchi, 1997). Briefly, Wistar rats were perfused with a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB). Co-localization of pairs of substances was investigated using double immunofluorescence with cryostat sections (4–8 μm thickness). Morphological differences between cell types were studied using a combination of the double immunofluorescence and immunoperoxidase
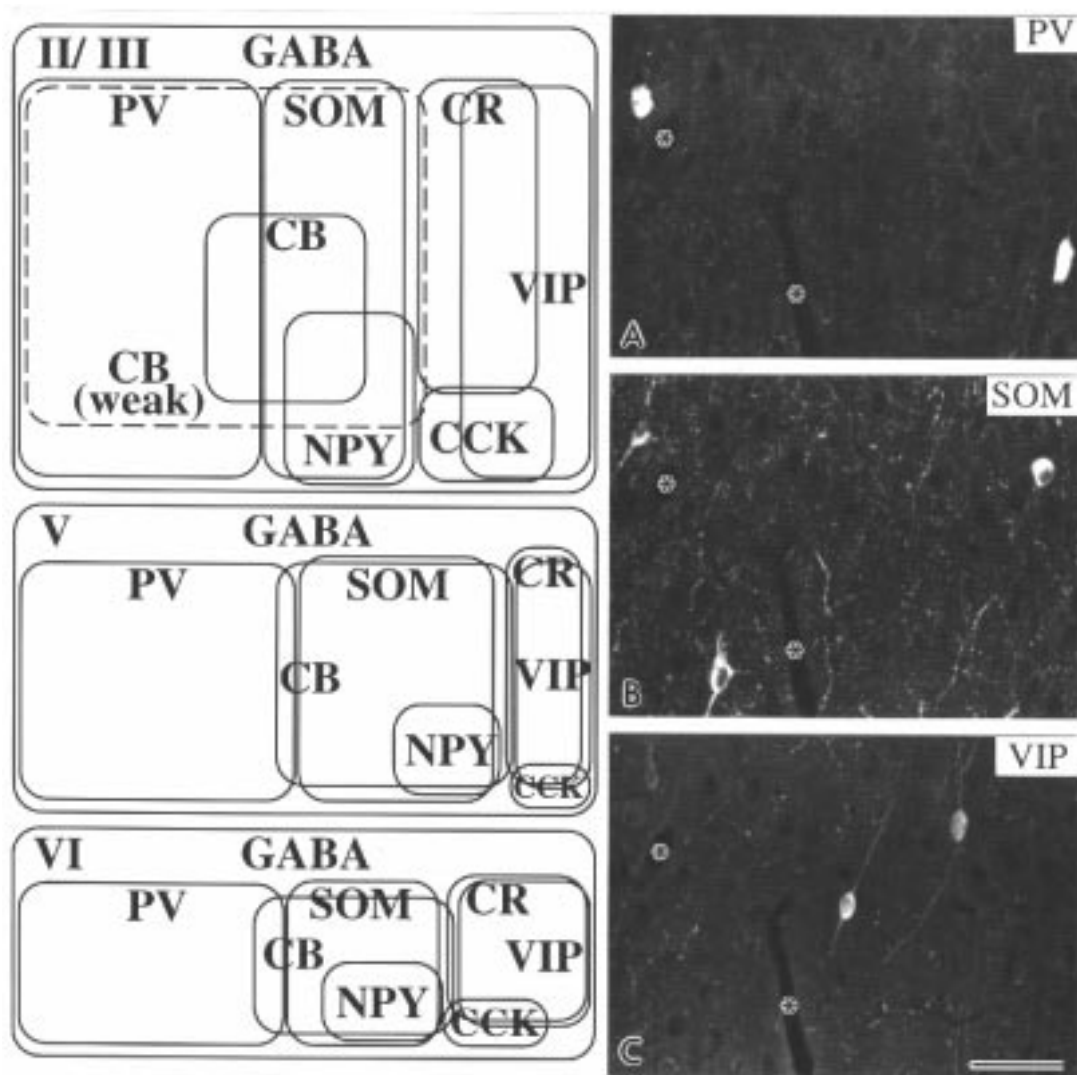
Figure 1. Immunohistochemically classified GABA cells in rat frontal cortex. (Left) Shared chemical composition of GABA cells. CB, calbindin D_{28k}; CCK, cholecystokinin; CR, calretinin; NPY, neuropeptide Y; PV, parvalbumin; SOM, somatostatin; VIP, vasoactive intestinal polypeptide. In addition to pyramidal cells of layers II/III, calbindin D_{28k} is expressed either weakly or strongly in GABA cells immunoreactive for somatostatin or parvalbumin. In layers V and VI, calbindin D_{28k} is strongly expressed mainly in somatostatin cells. The relative number of cells immunoreactive for a substance is proportional to the size of the box. (Right) Three chemical classes of GABA cells stained by triple immunofluorescence method using DTAF for parvalbunin (*A*), TRITC for somatostatin (*B*) and AMCA for VIP (*C*). Parvalbumin (PV), somatostatin (SOM) and VIP-immunoreactive cells are single-labeled cells. Asterisks show capillary as landmarks. Scale bar, 50 μm.

method with vibratome sections (50 μm). For the double immuno-fluorescence method, sections were incubated simultaneously with two primary antibodies and then with a combination of two secondary antiserums conjugated with different fluorescent dyes (TRITC, DTAF or AMCA) (Chemicon).

Slice Preparation and Whole-cell Recording
Physiological methods have been described in detail elsewhere (Kawaguchi, 1995). Briefly, 200 μm thick sections of rat frontal cortex (18–22 days postnatal) were cut in a plane oblique to the horizontal and put into a solution (in mM: NaCl 124.0, KCl 3.0, $CaCl_2$ 2.4, $MgCl_2$ 1.2, $NaHCO_3$ 26.0, NaH_2PO_4 1.0, glucose 10.0) bubbled with a mixture of 95% O_2 and 5% CO_2. Cells were recorded from frontal cortex in a whole-cell current-clamp mode at 30°C using 40× water immersion objective. Electrode solution consisted of (in mM) potassium methylsulfate 120, EGTA 0.6, $MgCl_2$ 2.0, ATP 4.0, GTP 0.3, HEPES 10, biocytin 20. Recordings were made by conventional bridge-balance and capacitance neutralization techniques using an intracellular amplifier (Axoclamp-2B).

Fluorescence Immunohistochemistry for Recorded Cells
Immunohistochemistry for recorded cells was described in detail elsewhere (Kawaguchi and Kubota, 1993, 1996). Briefly, the slices were fixed with 4% paraformaldehyde and 0.2% picric acid overnight at 4°C, followed by incubation in PB containing 10% and 20% sucrose. The tissue was next frozen with dry ice and thawed. The slices were then incubated overnight at room temperature with primary antibodies, followed by incubation in a mixture of DTAF- or AMCA-conjugated secondary antibodies for 4 h, and Texas Red–avidin for 90 min. Cross-reactivity of the secondary antibodies was not observed. After fluorescence observations, the slices were reacted with avidin–biotin–peroxidase complex and DAB. The dendrites, axonal processes and somata of DAB-labeled neurons were drawn using a camera lucida.

Postembedding GABA Immunohistochemistry for Ultrathin Sections
Postembedding GABA immunohistochemistry has been described in detail elsewhere (Kawaguchi and Kubota, 1996). After fixation with 4%
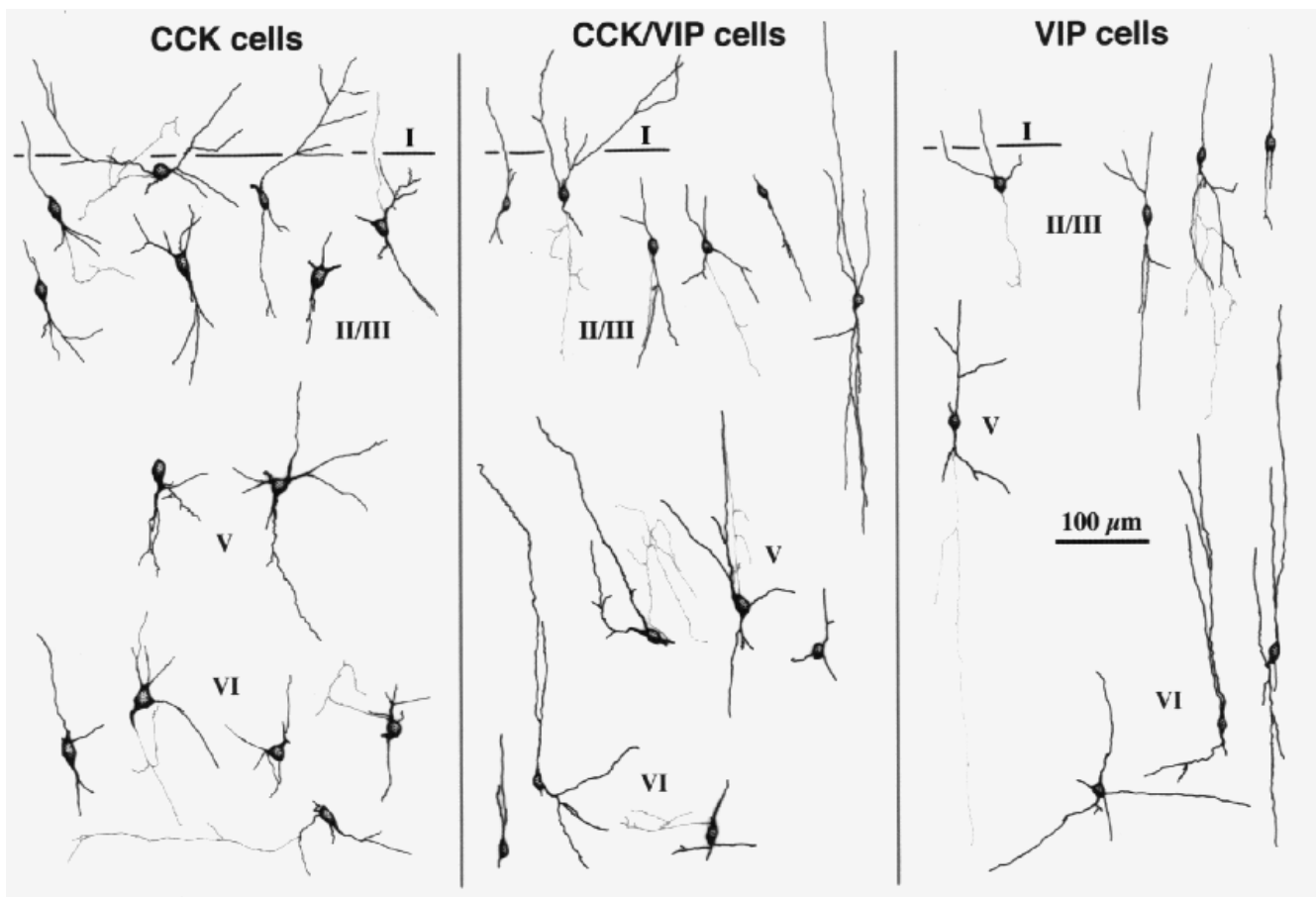
Figure 2. Drawings of CCK- and/or VIP-positive cells of the rat frontal cortex. In layers II/III, CCK single-labeled cells are larger than CCK/VIP double-labeled or VIP single-labeled cells, and are multipolar or bitufted. The other two types are both small, and are bipolar and bitufted. Modified from Kubota and Kawaguchi (1997).

paraformaldehyde, 2.5% glutaraldehyde and 0.2% picric acid overnight at 4°C, the slices were incubated in PB containing 10% sucrose and 20% sucrose, followed by freeze–thawing using liquid nitrogen. Then the slices were incubated with avidin–biotin–peroxidase complex, stained with DAB, osmicated, dehydrated and embedded in Epon. After camera lucida reconstruction, stained cells were serially sectioned with an ultramicrotome. Ultrathin sections were incubated with antiserum for GABA overnight. The ultrathin sections were incubated with colloidal gold-conjugated anti-rabbit IgG.

Results

Cortical GABA cells are Divided into Distinct Chemical Classes on the Basis of Calcium-binding Protein and Neuropeptide Content

Many kinds of neuropeptides and calcium-binding proteins have been shown to be expressed by neocortical cells. This chemical heterogeneity is much more diverse among non-pyramidal cells than pyramidal cells (DeFelipe, 1993). We examined the diversity of GABAergic neurons in rat frontal cortex by double immunohistochemical methods (Fig. 1) (Kawaguchi and Kubota, 1993; Kubota et al., 1994; Kubota and Kawaguchi, 1997).

Parvalbumin-, somatostatin- and vasoactive intestinal polypeptide (VIP)-immunoreactive cells were positive for GABA and not immunoreactive for each other in both layers II/III and layers V/VI. (See also Hendry et al., 1984; Somogyi et al., 1984; Celio, 1986; Kosaka et al., 1987; Demeulemeester et al., 1988.)

Most neuropeptide Y (NPY) cells showed somatostatin immunoreactivity (71% in layers II/III, 90% in layers V) and one-third of the somatostatin cells were positive for NPY. Although cholecystokinin (CCK) mRNA and pro-CCK immunoreactivity have been detected in the somata of both pyramidal and non-pyramidal cells (Burgunder and Young, 1990; Morino et al., 1994), CCK-immunoreactive somata are of non-pyramidal type in the neocortex (Peters et al., 1983; Morino et al., 1994). CCK-immunoreactive cells were also positive for GABA, and negative for parvalbumin and somatostatin. Small CCK cells demonstrated VIP immunoreactivity, whereas large CCK cells lacked expression of VIP (Fig. 2). (See also Hendry et al., 1984; Somogyi et al., 1984; Kosaka et al., 1987; Demeulemeester et al., 1988,1991.)

Another calcium-binding protein, calbindin D_{28k}, was expressed in some GABA cells in addition to layer II/III pyramidal cells. Most parvalbumin cells in layers II/III were either weakly (80%) or strongly (11%) immunoreactive for calbindin D_{28k}. In layers V/VI, few parvalbumin cells (6%) were immunoreactive for calbindin D_{28k}. Most somatostatin cells in layers II/III were either weakly (50%) or strongly (35%) immunoreactive for calbindin D_{28k}. In layers V/VI, most somatostatin cells were strongly immunoreactive for calbindin D_{28k} (85% in layers II/III, 92% in layers V/VI). (See also Demeulemeester et al., 1988; Hendry et al., 1989; van Brederode, 1990; DeFelipe and Jones, 1992; Rogers, 1992.)

Cells immunoreactive for another calcium-binding protein, calretinin, were negative for parvalbumin and somatostatin.
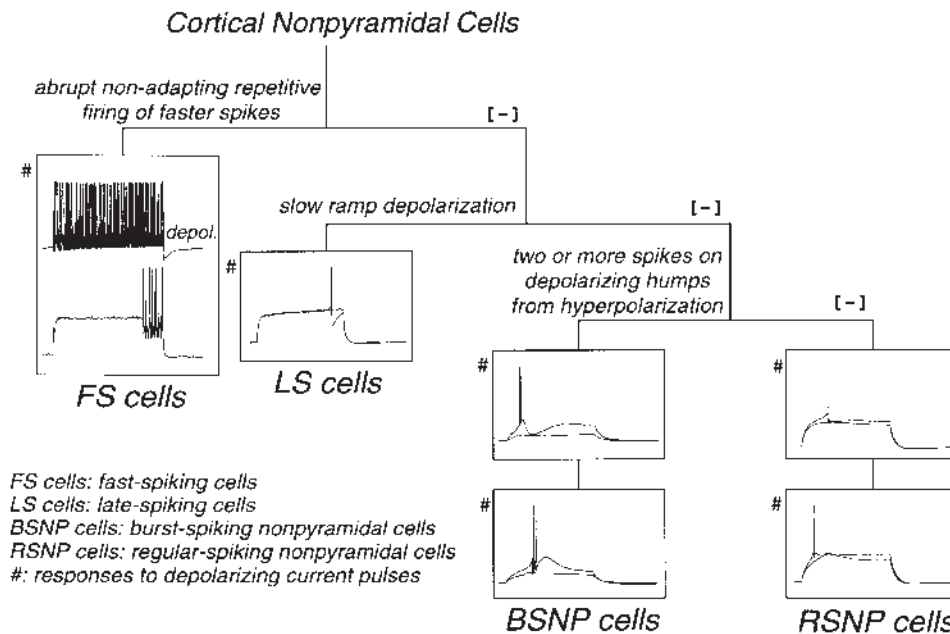
Figure 3. Neocortical nonpyramidal cells can be divided into four subgroups by their firing patterns. FS cells have lower input resistances and show an abrupt episode of nonadapting repetitive discharges of short-duration action potentials above some current threshold intensity, and continuous repetitive firing is easily achieved when combined with constant depolarizations (depol.). LS cells exhibit slowly developing ramp depolarizations near threshold. BSNP cells fire two or more spikes on slow depolarizing humps from hyperpolarized potentials. RSNP cells can not be categorized into the above three subgroups. In some RSNP cells depolarizing pulses from a hyperpolarized state induce depolarizing humps with single spikes (lower traces of RSNP cells).

Most of the calretinin cells were positive for VIP (71% in layer II/III, 94% in layers V/VI), but a few cells in layers II/III were immunoreactive for one or the other only (29% of calretinin cells, 43% of VIP cells). (See also Rogers, 1992; Vickers *et al.*, 1993; Condé *et al.*, 1994.)

These observations show that cortical GABAergic cells can be classified into at least four chemically distinct subgroups: (i) parvalbumin cells; (ii) somatostatin cells (most of them also containing calbindin D_{28k}) including NPY cells; (iii) VIP cells (some of them also containing calretinin or CCK); and (iv) large CCK cells.

### Cortical GABA Cells Are Heterogeneous in Firing Response Pattern to Depolarizations.

The above results indicate a chemical heterogeneity of calcium-binding proteins and neuropeptides among cortical GABAergic cells. Physiological properties may also be heterogeneous among GABA cells in the frontal cortex. Intrinsic biophysical properties of a neuron determine how it integrates inputs and the nature with which it generates a response to an input. In keeping with this principle, neocortical cells show variation in their intrinsic electrophysiological properties that lead them to respond differently to applied stimuli (McCormick *et al.*, 1985; Amitai and Connors, 1995; Deuchars and Thomson, 1995a; Hestrin and Armstrong, 1996; Thomson *et al.*, 1996; Zhou and Hablitz, 1996).

To reveal physiological heterogeneity among GABA cells, physiological properties of morphologically and chemically identified non-pyramidal cells in layer V of the frontal cortex were investigated *in vitro* using whole-cell current-clamp recording combined with intracellular staining and immuno-histochemistry. Because layer V pyramidal cells are large, we thought that it would be easier to obtain a large sample of non-pyramidal cells if recordings were preferentially directed at smaller cells with a non-pyramidal appearance. In the initial

series of experiments, two subgroups of layer V non-pyramidal cells were characterized by their different firing modes (Kawaguchi, 1993; Kawaguchi and Kubota, 1993). Fast-spiking cells (FS cells) had exceptionally brief action potentials and fast, non-adapting patterns of firing (McCormick *et al.*, 1985). Low-threshold spike cells (LTS cells) generated low-threshold spikes from hyperpolarized potentials in response to depolarization (Foehring *et al.*, 1991; Kawaguchi, 1993).

We now reclassify LTS cells as burst-spiking non-pyramidal (BSNP) cells (Kawaguchi and Kubota, 1996). BSNP cells are non-pyramidal cells which fire two or more spikes on slow depolarizing humps from hyperpolarized potentials. BSNP cells had input resistances higher than FS cells. FS and BSNP cells in layer V were also distinct in morphology and immuno-histochemical characteristics: FS cells had axonal arborizations that were denser near their somata, and they expressed the calcium-binding protein parvalbumin, whereas BSNP cells had more vertical axonal arborizations, extending up to layer I, and exhibited calbindin D_{28k} immunoreactivity. Both types of non-pyramidal cells were assumed to be GABAergic (Kawaguchi and Kubota, 1993). This group of FS cells therefore included morphologically extended plexus cells (Fairén *et al.*, 1984; DeFelipe, 1993). BSNP cells with mainly ascending axonal arbors in deep layers corresponded to Martinotti cells described in Golgi studies (Fairén *et al.*, 1984). These observations suggest that GABAergic non-pyramidal cells in neocortex can be divided into several functional groups on the basis of different firing modes, axonal distributions, and chemical properties.

To confirm the existence of two functional types of GABA cells in the upper layers, physiological and morphological properties of layer II/III non-pyramidal cells were investigated. However, the following two observations in layers II/III contradicted our expectation: (i) physiological classes other than FS cells and BSNP cells were seen; (ii) some physiological classes were not so well correlated with morphological types. In
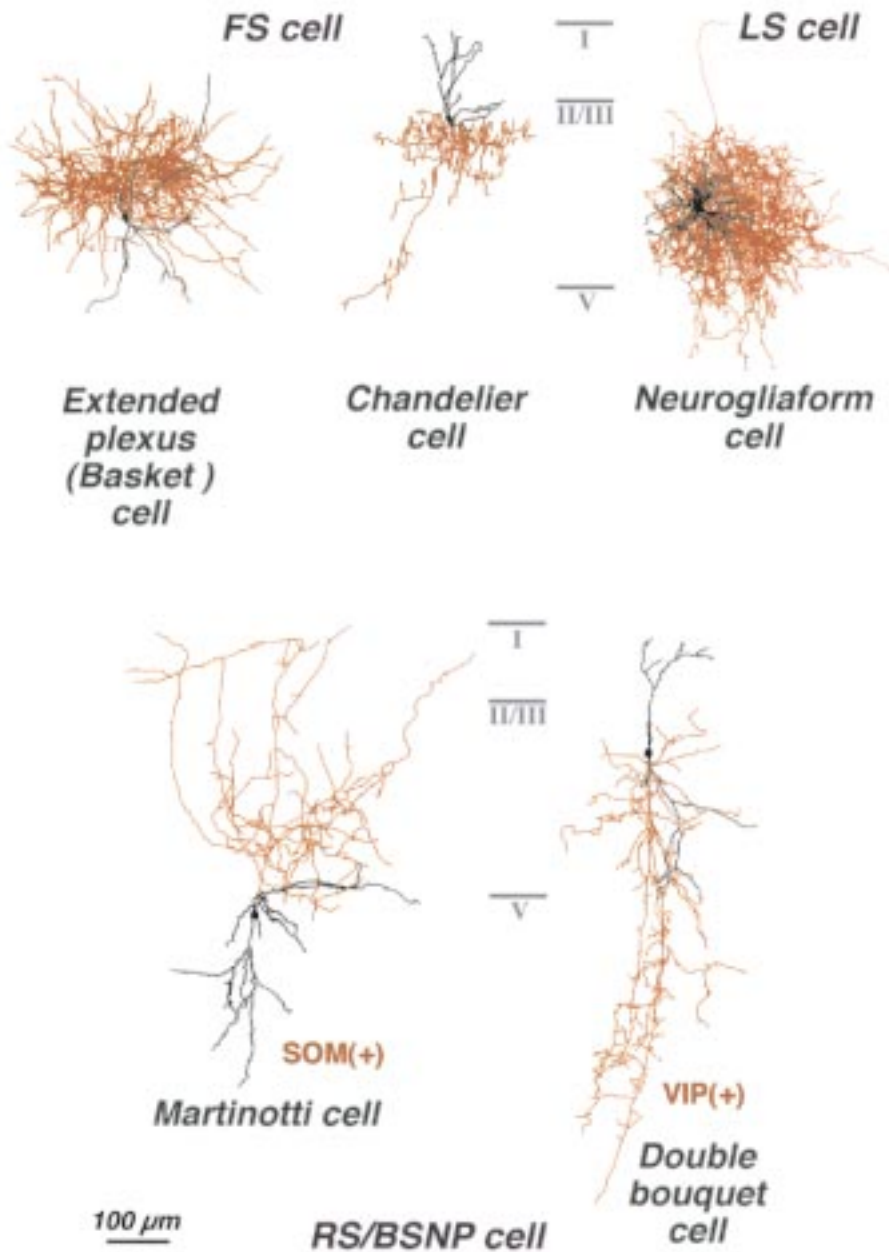
Figure 4. Drawings of GABA cell subtypes of the rat frontal cortex assembled from reconstructions of intracellularly labeled neurons characterized physiologically and immunohistochemically. The somata and dendrites are shown in black, and the axons in red. Roman numerals correspond to the cortical layers. SOM(+) and VIP(+), immunoreactive for somatostatin and VIP respectively.

addition to FS and BSNP cells, late-spiking (LS) cells and regular-spiking non-pyramidal (RSNP) cells were found in layers II/III. LS cells exhibited slowly developing ramp depolarizations to near threshold. RSNP cells could not be categorized into the above three subgroups. In some RSNP cells, depolarizing pulses from a hyperpolarized state induced a depolarizing hump with single spike (Fig. 3). Later it was confirmed that these four physiological subtypes also exist in layer V.

Morphologically, FS cells included extended plexus cells and chandelier cells with axon 'cartridges' bearing synaptic boutons (Somogyi, 1977; Peters, 1984a). LS cells included neurogliaform cells, which are multipolar cells with a dendritic field of

100–200 μm and an axonal arbor twice as wide as that of their dendritic field (Jones, 1984b). In contrast, BSNP cells and RSNP cells were not so well correlated with morphological types. For example, some bitufted cells with narrow descending axonal arbors, namely double bouquet cells (Valverde, 1978; Fairén et al., 1984; Somogyi and Cowey, 1984), as well as some bipolar cells with narrow descending arbors (bipolar cells) (Peters, 1984b), belonged to the RSNP class. Other double bouquet cells also belonged to the BSNP group. Some Martinotti cells were regular-spiking, but other Martinotti cells were burst-spiking.

To summarize, non-pyramidal cells in the rat frontal cortex can be divided into three main physiological classes with regard

Group	Firing patterns	Neurochemical substances	Morphological types	Synaptic targets
I	FS lower Ri, faster spike abrupt non-adapting firings	Parvalbumin	Extended plexus (Basket) Chandelier	Thick dendrites Somata Initial segments
II	LS slow ramp depo		Neurogliaform	Dendrites Somata
III	RSNP	a Somatostatin Calbindin D28k (layer V)	Martinotti	Thin dendritic branches
	BSNP two or more spikes on hump from hyper	b VIP Calretinin	Double bouquet Arcade	Dendrites Somata

Figure 5. Tentative categorization of GABAergic nonpyramidal cells in neocortex. GABAergic cells in the cortex are divided into three main physiological classes with regard to their firing patterns in response to depolarizing current pulses: (I) FS cells, (II) LS cells and (III) RS/BSNP cells. Parvalbumin is expressed in FS cells including (1) extended plexus (basket) cells innervating somata and dendrites and (2) chandelier cells making synapses on axon initial segments. Immunoreactivity for parvalbumin or peptides has not been detected in LS cells which include neurogliaform cells innervating dendrites and somata. Somatostatin and VIP are exhibited in RS/BSNP cells. Somatostatin is expressed in Martinotti cells with ascending axonal arbors up to layer I, which innervate thin dendritic branches. Layer V Martinotti cells are also positive for calbindin D_{28k}. VIP is expressed in (1) double bouquet cells which innervate dendrites and somata and (2) arcade cells forming axonal arcades. Some of the VIP cells are also immunoreactive for calretinin.

to their firing patterns in response to depolarizing current pulses. (i) FS cells have lower input resistances than the other types of cells and show abrupt episodes of non-adapting repetitive discharges of short-duration action potentials. (ii) LS cells exhibit slowly developing ramp depolarizations to near threshold. (iii) The remaining cells include BSNP and RSNP cells. BSNP cells fire two or more spikes on slow depolarizing humps from hyperpolarized potentials. FS and LS cells correspond to some known morphological types respectively, whereas BSNP cells and RSNP cells do not correlate well with morphological types.

There are two types of pyramidal cells, regular-spiking (RS) and intrinsically bursting (IB) (Connors and Gutnick, 1990; Mason and Larkman, 1990). While RS cells generate a single spike to a just-threshold stimulus, the minimal response of the IB subgroup of pyramidal cells is usually a high-frequency cluster of action potentials: a burst. These two types differ in layer distribution and morphology (Amitai and Connors, 1995). However, among the same class of pyramidal cells, for example, the corticospinal cells, some show bursts, but others do not (Tseng and Prince, 1993). Burst firing of spikes in non-pyramidal cells may be also heterogeneous within the same class.

Each Morphological Subtype of Cortical GABA Cell Has Distinct Chemical and Physiological Characteristics

To understand the cellular organization of non-pyramidal cells, immunohistochemical characterization of physiologically identified cells using antibodies for calcium-binding proteins was very useful in our initial series of experiments in layer V. Parvalbumin was expressed in extended plexus cells with FS characteristics, whereas calbindin D_{28k} was seen in Martinotti cells with BS characteristics. Further chemical characterization of physiological subtypes in both layers II/III and V might be useful.

In layers II/III, calbindin D_{28k} was expressed in many types of cells, including pyramidal cells and parvalbumin cells as described above. Therefore, in addition to parvalbumin,

peptides such as somatostatin and VIP were used for further chemical characterization since these peptides are exclusively expressed in GABAergic non-pyramidal cells in both layers II/III and V (Fig. 1) (Connor and Peters, 1984; Peters *et al.*, 1987; Demeulemeester *et al.*, 1988; Rogers, 1992; Kubota *et al.* 1994). Parvalbumin-containing FS cells were found to be negative for somatostatin and VIP. Extended plexus cells with FS characteristics were positive for parvalbumin. LS neurogliaform cells were negative for parvalbumin, somatostatin and VIP.

Somatostatin-positive cells included both RSNP and BSNP cells. Somatostatin cells had main ascending axons to layer I and more ascending collaterals than descending and horizontal ones. This group included Martinotti cells in deep layers (Fairén *et al.*, 1984; Wahle, 1993). RSNP and BSNP cells containing somatostatin in layer V were also positive for calbindin D_{28k}. VIP cells showed RS and BS characteristics, and were composed of distinct morphological subtypes. One group was cells with long descending main axons and more descending collaterals. These included bipolar cells (Peters, 1984b) and double bouquet cells (Somogyi and Cowey, 1984). Among 31 morphologically identified VIP cells, two cells were considered to be small basket cells (Fairén *et al.*, 1984) whose axonal branches made multiple contacts preferentially onto cell bodies and proximal dendrites. Another VIP cell type was the arcade cells forming axonal arcades (Jones, 1975; Peters and Saint Marie, 1984).

To summarize, parvalbumin is expressed in FS extended plexus cells, whereas peptide-containing GABAergic cells in the neocortex are mostly of RSNP and BSNP type. While double bouquet cells with descending axonal arbors express VIP, Martinotti cells with ascending axonal arbors to layer I contain somatostatin (Fig. 4).

Each Domain on Cortical Cell Surfaces Is Innervated by Specific but Multiple Classes of GABAergic Cells

The above results suggest that the neocortex contains several types of axonal arbors characterized by the presence of parvalbumin or different neuropeptides, which arise from different classes of non-pyramidal cells. From light microscopic observations using Nomarski optics, some boutons of FS cells positive for parvalbumin were found to surround cell somata (Kawaguchi and Kubota, 1993; Kawaguchi, 1995). Some of the branches of a few VIP cells made multiple axonal contacts preferentially onto cell bodies and proximal dendrites of other cells (Kawaguchi and Kubota, 1996). These observations suggest that the somata of cortical cells may be innervated by several groups of GABAergic cells.

Heterogeneous GABAergic input onto somata is also suggested by electron microscopic observations of synaptic terminals on cortical somata and by immunohistochemical findings. Peters and Harriman (1990, 1992) found that at least three different types of axon terminals forming symmetric synapses with the cell bodies. Some GABAergic terminals on somata contain calcium-binding proteins parvalbumin (Hendry *et al.*, 1989; van Brederode *et al.*, 1990; Akil and Lewis, 1992; Williams *et al.*, 1992; Lund and Lewis, 1993; Czeiger and White, 1997) or calretinin (Gabbott and Bacon, 1996; Gabbott *et al.*, 1997; Meskenaite, 1997), and some express neuropeptides CCK (Hendry *et al.*, 1983a; Freund *et al.*, 1986; Meyer and Wahle, 1988; Oeth and Lewis, 1993) or VIP (Connor and Peters, 1984; Peters *et al.*, 1987; Hajós *et al.*, 1988; Peters 1990).

FS cells also included non-pyramidal cells with vertical rows of axonal boutons: the chandelier cells (Kawaguchi, 1995). The synapses made by chandelier cells are symmetrical, GABA-
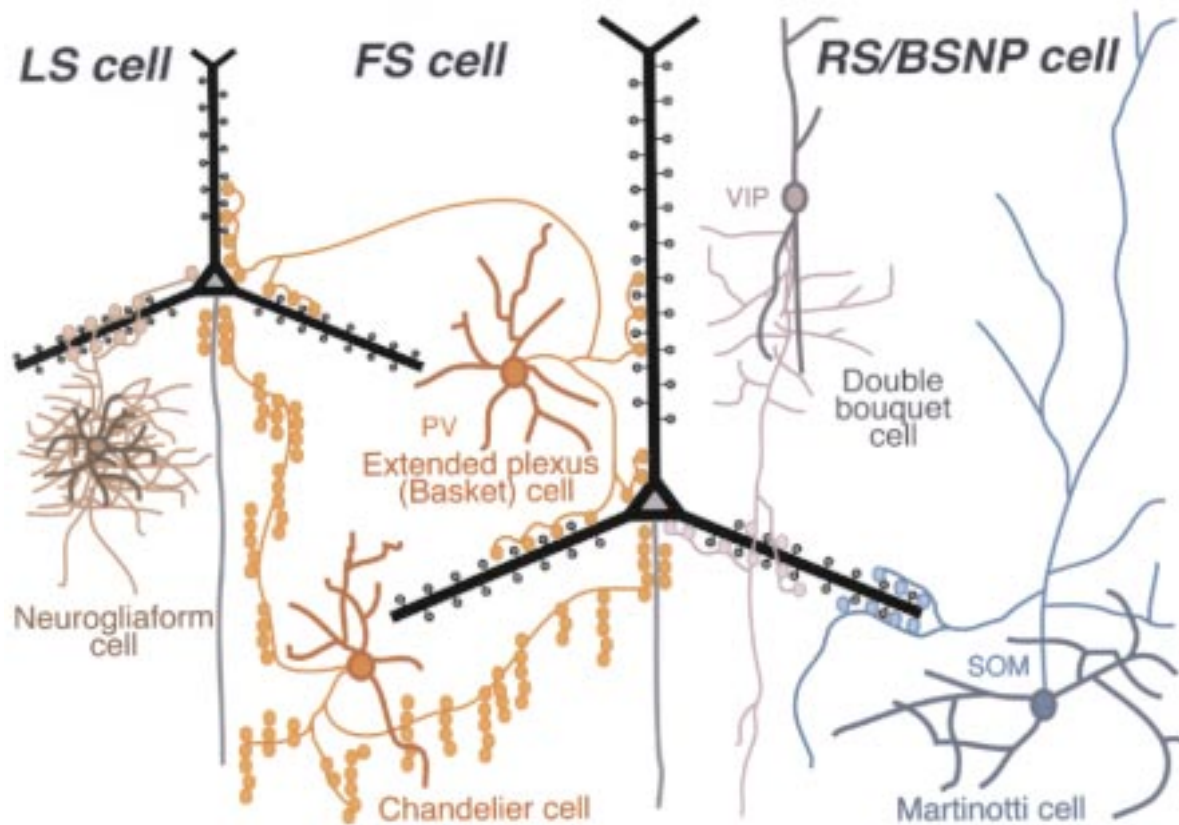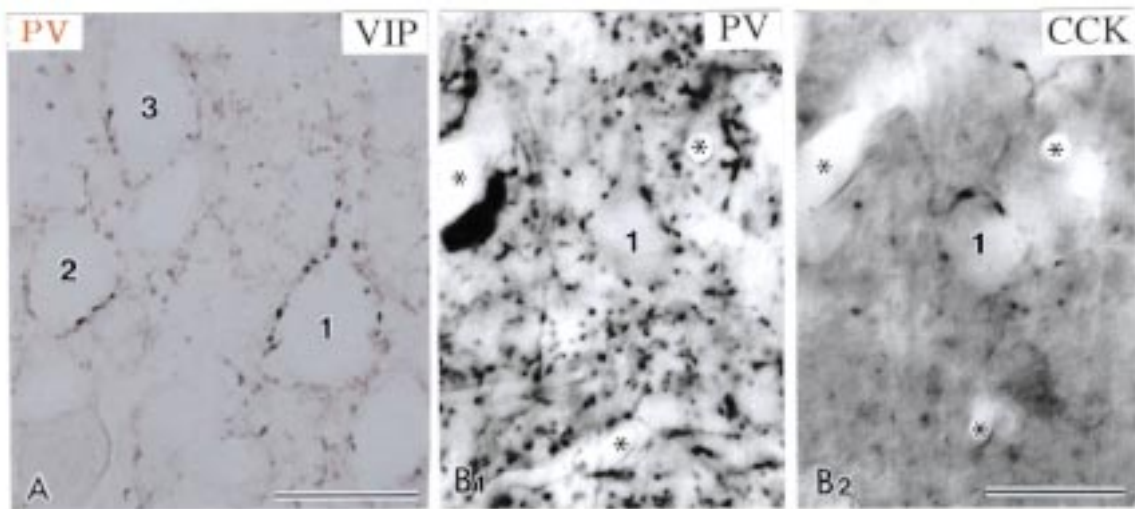
Figure 6. (A) Parvalbumin- and VIP-immunoreactive boutons were visualized by Tris-(4-aminophenyl)methane (red) and nickel diaminobenzidine (black) reactions respectively. They are on the same cell somata (cells 1–3). (B) Immunohistochemical staining of parvalbumin (PV) (B1) and CCK (B2) in the rat frontal cortex. B1 and B2 are paired serial sections. Numbered cell (cell 1) is the same cell in the paired sections. Parvalbumin- and CCK-immunoreactive boutons coexist on the same perikarya. Asterisks indicate blood vessels as landmarks for pairing. Scale bars, 20 μm.

Figure 7. Schematic view of known distributions of axon terminals of GABAergic cell subtypes on surfaces of pyramidal cells in rat frontal cortex. FS cell, fast-spiking cell; LS cell, late-spiking cell; RS/BSNP cell, regular-spiking or burst-spiking nonpyramidal cell; PV, parvalbumin; SOM, somatostatin; VIP, vasoactive intestinal polypeptide.

immunoreactive and almost exclusively on axons (Somogyi, 1977; Somogyi *et al.*, 1982; Freund *et al.*, 1983; DeFelipe *et al.*, 1989; Buhl *et al.*, 1994). FS chandelier cells are axo-axonic cells which are likely to be crucial in modulating the generation of action potentials of cortical cells (Stuart and Sakmann, 1994). The number of axo-axonic synapses differs among projection subtypes of pyramidal cells (Fariñas and DeFelipe, 1991b), suggesting multiple levels of specificity. The GABA$_A$ receptor subunit α2 is expressed to a greater extent on the axon initial segment than the somata and dendrites (Nusser *et al.*, 1996), which also implies that axo-axonic GABAergic synapses may express distinct inhibitory currents and mechanisms of modulation. Vertical arrays of axonal boutons of chandelier cells have been labeled immunohistochemically by parvalbumin and corticotropin-releasing factor, but some axo-axonic boutons are immunoreactive for neither of these (Lewis and Lund, 1990; DeFelipe and Fariñas, 1992). Axo-axonic synapses may thus also be heterogeneous in chemical characteristics, suggesting there are a variety of chandelier cells, including examples which are not of the parvalbumin FS type (Kawaguchi, 1995).

Morphological identification of somatostatin and VIP cells suggests that the neocortex contains two types of vertical axonal arbors characterized by the presence of different neuropeptides which arise from different classes of non-pyramidal cells. Somatostatin-immunoreactive terminals have been shown to form synapses with dendritic spines or the shafts of small or medium-sized dendrites (Hendry *et al.*, 1983b; De Lima and Morrison, 1989). VIP-positive axon terminals form symmetrical synapses with the shafts of small or medium-sized dendrites or the somata (Peters *et al.*, 1987; Hajós *et al.*, 1988; Peters 1990). The vertical axonal arbors of cells physiologically identified as RSNP or BSNP cells were investigated under the electron microscope (Kawaguchi and Kubota, 1996). The axon collaterals of RSNP and BSNP cells, both those with descending and those with ascending axonal arbors, were immunoreactive for GABA. Preliminary observations showed that non-pyramidal cells with descending axonal arbors made symmetrical synapses onto dendrites, and a few onto somata; and non-pyramidal cells with ascending axonal arbors made symmetrical synapses onto thin dendritic branches including spine necks. An LS neurogliaform cell made GABA-immunoreactive symmetrical synapses onto dendrites and a few onto somata (Y. Kawaguchi and Y. Kubota, 1996, unpublished observation). In human cerebral cortex, neurogliaform cells innervate the distal dendrites of pyramidal cells (Kisvárday *et al.*, 1990).

The above results suggest that the somata or dendrites of cortical cells are innervated by boutons arising from different classes of GABAergic cells as characterized by their firing patterns and the presence of parvalbumin or peptides (Fig. 5). Cortical cell bodies are surrounded by parvalbumin-, CCK- and VIP-immunoreactive boutons as described above (Hendry *et al.*, 1983a; Peters *et al.*, 1987; Freund *et al.*, 1986; Hajós *et al.*, 1988; Hendry *et al.*, 1989; Peters 1990; Akil and Lewis, 1992). Mirror image immunostaining of two adjacent sections or double-colored immunohistochemistry of the same sections with nickel diaminobenzidine (black) and Tris-(4-aminophenyl)methane (red) reactions for parvalbumin and CCK or VIP antibodies easily identified cell bodies surrounded by both parvalbumin- and CCK- or VIP-immunoreactive boutons (Fig. 6). This observation suggests that parvalbumin- and peptide (CCK and/or VIP)-immunoreactive boutons coexist on some cortical cell bodies.

To summarize, each subtype of non-pyramidal cell innervates

relatively specific domains of cortical cells with GABA-containing synapses (Fig. 7). The somata of neocortical cells are innervated by subgroups of cells containing parvalbumin or neuropeptides. The same areas on dendrites may also be innervated by multiple types of GABAergic terminals from separate populations of non-pyramidal cells with distinct physiological and chemical properties. Thus, in some cases cortical cells are regulated by convergent GABAergic inputs from separate sources.

Discussion

Each GABAergic cell subtype is assumed to make axon terminals on relatively specific surfaces of cortical cells (Somogyi, 1989; Jones, 1993). In addition to which subcellular domain is innervated, it is also important to know whether each GABA cell subtype innervates cortical cells uniformly or specifically. There may be GABA cells specialized to inhibit specific cell types in cortical circuits. Recently a systematic analysis with double immunostaining techniques identified specialized interneurons that innervate specific subsets of interneurons in the hippocampus (interneuron-selective cells) (Freund and Buzsáki, 1996). Hippocampal GABAergic interneurons that selectively innervate other interneurons are immunoreactive for calretinin and/or VIP (Acsády *et al.*, 1996a,b; Gulyás *et al.*, 1996). These interneuron-selective cells are proposed to have a role in synchronizing inhibitory cells (Freund and Buzsáki, 1996). In the cortex, there is also a GABAergic cell group immunoreactive for VIP and/or calretinin (Fig. 1), including double bouquet cells and arcade cells (Fig. 5). These cells may make GABAergic synapses onto specific cell subtypes in the cortex. The axons of cortical calretinin-immunoreactive cells indeed form synapses with pyramidal cells in deeper layers, but with GABAergic neurons in the superficial layers (Meskenaite, 1997), including parvalbumin- or calbindin D$_{28k}$-immunoreactive cells (Gabbott and Bacon, 1996).

Electron microscopic observations with postembedding GABA immunohistochemistry revealed that axon terminals of all identified non-pyramidal cell subtypes contain GABA. Using paired intracellular recording, FS cells as well as other physiological classes of non-pyramidal cells indeed elicit fast IPSPs in pyramidal cells, probably via GABA$_A$ receptors (Deuchars and Thomson, 1995a; Thomson *et al.*, 1996). Each GABA cell subtype differs in firing mode, axonal arborization pattern, postsynaptic target, co-localized transmitters and perhaps postsynaptic cell type. Furthermore, IPSPs induced by these GABAergic cells may also be different among cell subtypes. Recent paired recordings between identified inhibitory non-pyramidal cells and pyramidal cells show that FS cells elicit shorter-duration IPSPs than other physiological classes of non-pyramidal cells (Thomson *et al.*, 1996). GABA$_A$ and GABA$_B$ IPSPs are separately induced by stimulation at particular locations, and are considered to be mediated by distinct classes of inhibitory neurons (Benardo *et al.*, 1994; Kang *et al.*, 1994). These findings suggest that different types of GABA receptors are expressed on postsynaptic sites of terminals of GABA cell subtypes.

Each type of GABA cell displays specific patterns of axonal arborizations in the cortex. Excitatory pyramidal cells also have extensive arborizations of local axon collaterals (Gilbert and Wiesel, 1979, 1983; Martin and Whitteridge, 1984). These collaterals of pyramidal cells innervate non-pyramidal cells as well as other pyramidal cells (Thomson and Deuchars, 1994). Some of the GABA cell subtypes take characteristic dendritic

forms and may be innervated by specialized afferent fibers. Burst firing non-pyramidal cells in layers V and VI are excited by regular spiking pyramidal cells whose apical dendrites do not reach layer I, implying they are corticothalamic neurons (Deuchars and Thomson, 1995b). Callosal pyramidal cells receive a greater number of axosomatic and axoaxonic synapses than corticothalamic cells (Fariñas and DeFelipe, 1991a,b). There may be specialized connections between pyramidal cell subtypes and GABA cell subtypes.

Both pyramidal and non-pyramidal cells are considered to be heterogeneous in firing patterns (Connors and Gutnick, 1990; Amitai and Connors, 1995). Firing patterns of each cortical cell subtype are influenced by the membrane potential level regulated by intrinsic conductances and tonically released transmitters (modulators), as well as phasic excitation by glutamatergic inputs and inhibition via GABAergic inputs. Glutamate and GABA receptor subtypes may be differentially expressed in cortical cell subtypes. Excitatory postsynaptic currents of the non-NMDA type in non-pyramidal cells exhibit faster decay time constants (Hestrin, 1993) and higher calcium permeability (Jonas et al., 1994) than those of pyramidal cells. The mRNA expression of GluR2 (GluR-B) is significantly low in FS cells with higher calcium permeability (Jonas et al., 1994). Most of GluR1-expressing/GluR2-undetectable non-pyramidal cells posess parvalbumin, whereas the majority of GluR1-/GluR2-expressing non-pyramidal cells is intensely positive for calbindin D_{28k} (Kondo et al., 1997). The decay time constant of $GABA_A$ receptor-mediated inhibitory postsynaptic currents is slower in FS cells than in pyramidal cells (Xiang et al., 1996). Physiological and pharmacological properties of excitatory and inhibitory inputs may also vary even among GABA cell subtypes.

Thus, the cortex is equipped with multiple GABAergic inhibitory systems. This may correspond to multiple roles of GABAergic inhibition necessary for cortical information processing (Sillito, 1984). Rapid changes in cortical representation maps are known to occur in sensory and motor areas in which afferent activities are deprived or modulated (Gilbert, 1993; Jones, 1993). $GABA_A$ receptor-mediated inhibition may be involved in the rapid shift of topographic representations (Jacobs and Donoghue, 1991) and in determination of the spacing of columns in developing cortex (Hensch and Stryker, 1996). Some subtypes of GABA cells with columnar or laminar (horizontal) axonal arbors may be related to these expansions and reductions of receptive fields. Slow, rhythmic membrane potential oscillations occur spontaneously in cortical neurons (Metherate and Ashe, 1993; Steriade et al., 1993). A faster subthreshold oscillation of membrane potentials corresponding to the gamma rhythm is observed only in the depolarized state and can trigger action potentials (Cowan and Wilson, 1994). This oscillation is attributed to the action of rhythmic IPSPs, and may be generated by networks of mutually inhibitory GABAergic interneurons (Jefferys et al., 1996; see also Gray and McCormick, 1996). Thus, multiple GABA cell subtypes with different synaptic connections and firing patterns may be involved in regulating the representation patterns of cortical surfaces spatially and the synchronized firing of cortical cells temporally.

Notes

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