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# Increased $GABA_A$ Receptor Binding in Superficial Layers of Cingulate Cortex in Schizophrenics

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**Recent investigations of postmortem brain from schizophrenic patients have revealed reduced numbers of neurons in several different corticolimbic brain regions. In the prefrontal and anterior cingulate cortices, more specific decreases in the numbers of interneurons, but not pyramidal cells, have been reported to occur preferentially in layer II. Based on this latter finding, a loss of inhibitory basket cells leading to a compensatory upregulation of the $GABA_A$ receptor has been hypothesized to occur in schizophrenic patients and to be a contributory factor in the pathophysiology of this disorder. We now report the results of a high-resolution quantitation of $GABA_A$ receptor binding in anterior cingulate cortex of postmortem specimens from normal and schizophrenic cases. The results indicate a preferential increase in bicuculline-sensitive $^3$H-muscimol binding on neuronal cell bodies of layers II and III, but not layers V and VI, of the schizophrenic cases. There was no difference in the size of neurons in any of the layers examined when the control and schizophrenic groups were compared. The neuropil of layer I also showed significantly greater $GABA_A$ binding in schizophrenics. The differences seen in the schizophrenic group did not appear to be the result of exposure to antipsychotic medication because one patient who was medication naive and a second who had received minimal exposure to antipsychotic drugs also showed elevated $GABA_A$ receptor binding. Since information processing depends on corticocortical integration in outer layers I–III, a disturbance of inhibitory activity in these superficial layers of limbic cortex may contribute to the defective associative function seen in schizophrenia.**

A renewed interest in whether schizophrenia involves a process of neuronal degeneration has resulted in several investigations that report evidence of volume shrinkage (Bogerts et al., 1985; Brown et al., 1986), neuronal loss (Benes et al., 1986; Bogerts et al., 1986; Falkai and Bogerts, 1986; Falkai et al., 1988; Jeste and Lohr, 1988), and a variety of cytoarchitectural disturbances (Kovelman and Scheibel, 1984; Jakob and Beckmann, 1986; Benes and Bird, 1987; Benes et al., 1987) in corticolimbic brain regions of chronically psychotic individuals. More recently, we have sought to understand these nonspecific alterations in relation to more detailed features of the intrinsic circuitry of the anterior cingulate cortex, a key component in the integration of emotional experiences with higher cognitive functions (Papez, 1937). Since an earlier study showing lower densities of neurons in several different cortical regions (Benes et al., 1986) did not distinguish large-projection neurons (pyramidal cells) from neurons involved in local circuits (interneurons), a subsequent investigation differentially counted these latter two cell types. The results showed preferential losses of interneurons in both the prefrontal and anterior cingulate regions of chronically psychotic patients, although the differences were most striking in layer II of both areas (Benes et al., 1991). Because the lower numbers of interneurons occurred in most layers of the anterior cingulate cortex, it seemed plausible that a preferential loss of inhibitory basket cells that have a broad distribution among the cortical layers (Jones and Hendry, 1984) might explain these results. Based on this interpretation, it was hypothesized that a loss of basket neurons would be accompanied by a compensatory upregulation of the $GABA_A$ receptor in schizophrenic patients (Benes et al., 1989).

In order to test the hypothesis that there might be more high-affinity GABA receptor binding in anterior cingulate cortex of chronically psychotic individuals, we have employed a nuclear-track, coverslip-emulsion techniqiue (Young and Kuhar, 1979) to obtain a sufficiently high degree of spatial resolution to permit quantitative estimates of receptor binding on individual neurons (Herkenham, 1988). We herein report the use of this method for the localization of bicuculline-sensitive $^3$H-muscimol ($^3$H-MUS; $GABA_A$) binding in anterior cingulate cortex of postmortem specimens from both normal and schizophrenic individuals.

## Materials and Methods

*Postmortem specimens.* Postmortem specimens of anterior cingulate cortex from control ($N = 8$) and schizophrenic ($N = 6$) subjects were obtained through the Human Brain Tissue Resource Center at McLean Hospital. The diagnosis of schizophrenia was made by retrospective chart review and applying the criteria of Feighner et al. (1972). Postmortem brains from both normal and schizophrenic cases were examined by a neuropathologist and were excluded from the study if there was evidence of intracranial disease, particularly Alzheimer's disease, cerebrovascular disease, or alcohol abuse. The age, postmortem interval (hours), and exposure to neuroleptic medication, expressed as the chlorpromazine-equivalent dose, were recorded for each case.

*Tissue handling.* The specimens were removed at autopsy and transported on ice prior to immersion fixation in ice-cold 0.1% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1.5 hr. They were then placed in ice-cold 30% sucrose in 0.1 M phosphate buffer, pH 7.4, overnight. After

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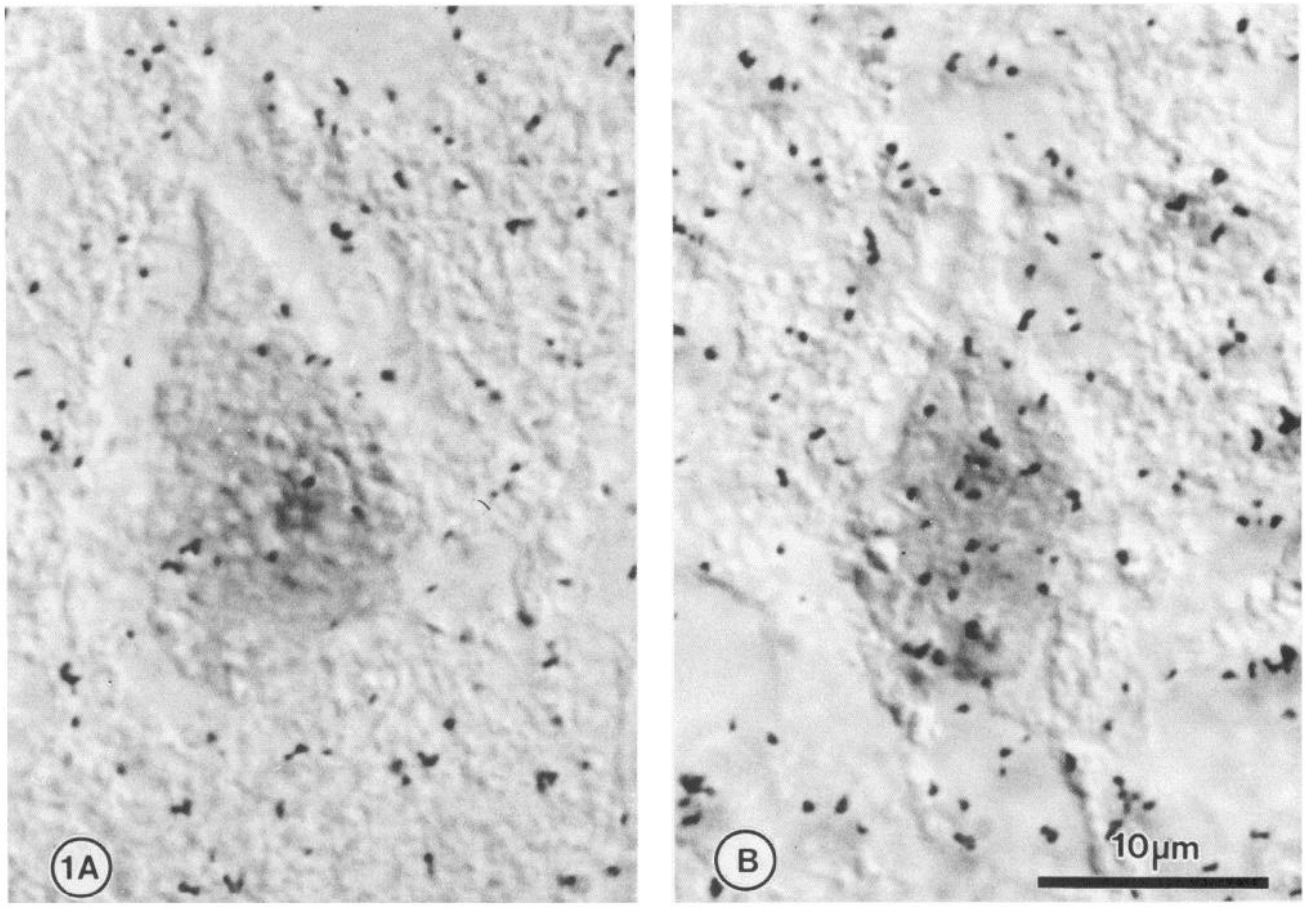


*Figure 1.* Composite light photomicrographs of pyramidal neurons with autoradiographic grains representing bicuculline-sensitive $^3$H-MUS binding ($GABA_A$ receptor) in layer II of anterior cingulate cortex. *A,* A normal control. *B,* A schizophrenic case. The pyramidal cells were photographed using Nomarski interference contrast illumination. At a different level of focus, autoradiographic grains in the nuclear-track emulsion were photographed in register with their corresponding cell. It was from such fields that grain distribution and density were determined with a user-interactive, automated image processing system. See Methods and Results sections for details.

sinking in sucrose, the tissue blocks were frozen on dry ice in Tissue-Tek O.T.C. Compound (Miles, Elkhart, IN) imbedding media and stored at −70°C. Tissue sections were cut at a thickness of 10 μm on a cryostat at a temperature of −20°C, thaw mounted on gelatin-coated glass slides, and stored at −20°C for 24 hr. $GABA_A$ receptors were labeled according to a modification (Benes et al., 1989) of a technique described by Unnerstall et al. (1981) and Young and Kuhar (1979). The sections were preincubated in 0.31 M Tris-HCl buffer, pH 7.4, at room temperature for 20 min and were then transferred to a solution containing 5 nM $^3$H-muscimol ($^3$H-MUS; specific activity, 25.8 Ci/mmol; DuPont/New England Nuclear) in Tris-HCl buffer at room temperature for 40 min. Nonspecific binding was determined in serial sections by incubating the tissue in tritiated ligand with 100 μM (+)-bicuculline methiodide (BIC). The tissue sections were washed and immersed in cold distilled $H_2O$, rapidly dried in a stream of air, and stored at 4°C. Acid-cleaned coverslips (no. 00, Corning Glass Works, Pittsburgh, PA) coated with Kodak NTB-3 nuclear emulsion (diluted 1:1 with distilled water) were apposed and glued to the slide-mounted sections under darkroom conditions. After exposure for 6 weeks, the latent images were developed with Dektol for 4 min at 19°C, washed in distilled water for 15 sec, fixed in Kodak rapid fixer (without hardener) for 4 min, and rinsed with water for 30 min, and the tissue sections were stained with thionin.

*Microscopic analyses.* The autoradiograms were viewed with a Leitz Diaplan microscope interfaced with a Bioquant MEG IV Image Analysis system (R and M Biometrics, Nashville, TN) via a Dage CCD-71 video camera. A 100× oil immersion objective was used to obtain an image magnification of 3800× on the video monitor that had a sufficiently high resolution to provide clear visualization of neuronal cell bodies and single autoradiographic grains. The methods for sampling and quantitation were based on the morphometric principles described by Weibel (1979) and were performed under strictly "blind" conditions to eliminate observer bias. The numbers of grains in subregions of neuropil or on individual neurons were determined for each control and schizophrenic case with a user-interactive, computer-assisted program as previously described (Benes et al., 1989). For individual cell bodies, the first 15 neurons identified by their shape and thionin-positive cytoplasm that were encountered in layers II, III, V, and VI were traced using a Houston Hipad digitizing board. The microscopic image was refocused to visualize autoradiographic grains, and those present within the exact boundaries of the traced cell were automatically counted. Each automated grain count was manually corrected for overlapping grains (Benes et al., 1989). For neuropil, a 200 $\mu m^2$ field was aligned over the thionin-stained section to eliminate identifiable cell bodies or blood vessels from the field. The numbers of grains in such fields were automatically determined and manually corrected as described above for individual neurons. The data were expressed either as the numbers of grains per cell or per 200 $\mu m^2$ of neuropil, and a mean and a standard error of the mean (SEM) were obtained for the various layers in each of the normal and schizophrenic cases. Sampling of neuronal cell bodies in layer I was not performed because this lamina contains few neurons that are very small in size, making it difficult to obtain reliable data. Sampling of both neuronal cell bodies and neuropil was also not conducted in layer IV because the light thionin staining and relatively thin sectioning required for these analyses made it difficult to identify this lamina reliably in the cingulate region, where it is present in a rudimentary form.

Specific $GABA_A$ binding was determined by subtracting the number of grains for $^3$H-MUS plus BIC from those for $^3$H-MUS alone for each individual normal or schizophrenic case, and a mean and SEM were generated for each group. A Student's *t* test was used to assess the significance of differences between the control and schizophrenic groups.

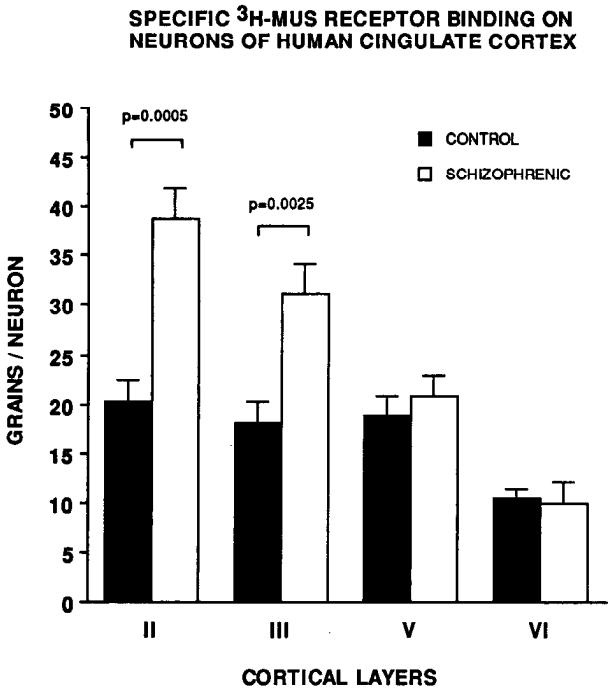


*Figure 2.* A bar graph showing the laminar distribution of bicuculline-sensitive $^3$H-MUS binding ($GABA_A$) on individual neurons in anterior cingulate cortex of postmortem brain. The data for the $GABA_A$ receptor were obtained by subtracting the number of grains for $^3$H-MUS plus BIC from the number of grains for $^3$H-MUS alone. The *horizontal bracket above the bars* indicates the *p* value of significance for differences between the control ($N = 8$) and schizophrenic ($N = 6$) groups. The *roman numerals below the graph* indicate the individual cortical layers. The schizophrenics show significantly more binding in layers II and III, but not layers V and VI. See Methods and Results sections for details.

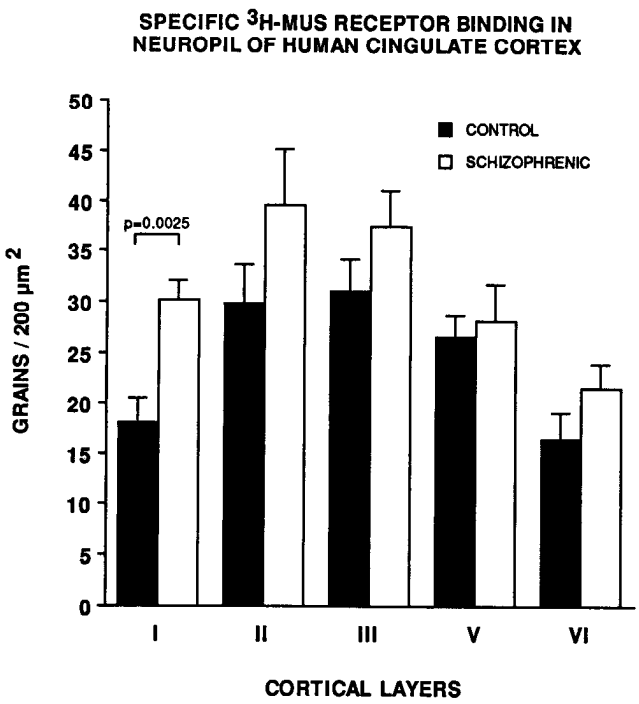


*Figure 3.* A bar graph showing the laminar distribution of bicuculline-sensitive $^3$H-MUS binding in neuropil regions of anterior cingulate cortex of postmortem brain. The data for the $GABA_A$ receptor were obtained by subtracting the number of grains for $^3$H-MUS binding plus BIC from the number of grains for $^3$H-MUS alone. The *horizontal bracket above the bars* indicates the *p* value of significance for the difference between the control ($N = 8$) and schizophrenic ($N = 6$) groups. The *roman numerals below the graph* indicate the individual cortical layers. The schizophrenics show significantly more binding in layer I only. See Methods and Results sections for details.

## Results

Visual inspection of the thionin-stained autoradiogram assemblies revealed good grain development and preservation of morphological detail in both the control and schizophrenic specimens (Fig. 1*A,B*). The results of the grain quantitation revealed that the amount of nonspecific $^3$H-MUS binding on individual neurons (range, 8–10.5 grains/neuron) and in neuropil (range, 15–17 grains/200 $\mu m^2$) remaining after the addition of BIC was remarkably similar across the various layers of both the control and schizophrenics groups. The amount of $^3$H-MUS binding was greater for the schizophrenic group for neuronal cell bodies in layers II and III, but not layers V and VI, while in neuropil, it was higher for the schizophrenics in layer I only. When the specific $^3$H-MUS receptor binding on individual neurons of the schizophrenic group was determined, it was found to be 84% higher in layer II and 74% higher in layer III when compared to normal controls (Fig. 2); however, no differences in specific $^3$H-MUS binding occurred on neurons in the deeper layers V and VI (Fig. 2). For neuropil, specific $^3$H-MUS binding was significantly higher in layer I, while it was only slightly, but not significantly, higher in layers II and III and no differences were observed in layers V and VI (Fig. 3). There were no differences in the size of neurons in any of the layers examined (Fig. 4).

The postmortem interval was similar for the control and schizophrenic groups (11.7 ± 6.6 and 11.1 ± 7.6 hr, respectively), while the average age (76.6 ± 16 and 43.5 ± 6.1 years, respectively) was lower for the schizophrenic patients. Age effects, however, do not account for the differences in specific $^3$H-MUS receptor binding between the two groups because both young (32.8 ± 6.1 years) and old (70.5 ± 4.9 years) patients showed increased receptor sites (36.4 ± 7.8 and 41.1 ± 6.2 grains/neuron, respectively) as compared with normal controls (20.2 ± 2.2 grains/neuron) for layer II neurons. Moreover, the youngest control (aged 35 years) also showed low specific $^3$H-MUS receptor binding (24.4 grains/neuron). Neuroleptic exposure also did not seem to be responsible for the current findings since two schizophrenic patients, one who had not received any medication and the other with minimal exposure, both showed elevated $GABA_A$ binding (45.4 and 38.2 grains/neuron, respectively).

## Discussion

The results of this study have demonstrated that an increase of bicuculline-sensitive $^3$H-MUS binding to the $GABA_A$ receptor for neuronal cell bodies and neuropil occurs preferentially in superficial layers of the anterior cingulate cortex of schizophrenic patients. These findings are consistent with previous investigations in which increased $^3$H-MUS binding (Hanada et al., 1987), decreased glutamate decarboxylase activity (Bird et al., 1979), and decreased GABA uptake (Simpson et al., 1989) were reported in the prefrontal cortex of schizophrenic brain, although small effects and large variances have plagued such studies and discouraged interest in the findings. In contrast, the high-resolution technique employed here has effectively eliminated unwanted "noise" from the analyses and thereby permitted the detection of a large effect with small variance for the increased

$GABA_A$ receptor density in upper cortical layers of schizophrenics. Abnormalities of the GABA system in schizophrenia have long been suspected (Roberts, 1972), although it is not clear whether similar deficiencies of this neurotransmitter system occur in other brain areas as well (Cross et al., 1979; Perry et al., 1979).

Previous studies of the anterior cingulate cortex in schizophrenic brain have revealed a variety of abnormalities in the superficial layers including (1) decreased numbers of neurons (Benes et al., 1986) that occur preferentially for small interneurons (Benes et al., 1991), (2) decreased size of neuronal clusters in layer II (Benes and Bird, 1987), and (3) increased numbers of neurofilament 200K–immunoreactive vertical axons in layers II and IIIa (Benes et al., 1987). Since the superficial cortical layers are primarily involved in processing incoming activity from other cortical areas (Jones, 1984; Marin-Padilla, 1984), the increased $GABA_A$ receptor binding in layers I, II, and III observed here in the schizophrenic group provides further evidence for a defect in corticocortical information processing in the cingulate region of schizophrenic brain. It is well known that the cortex develops in an "inside-out" fashion with layer II being the last cell-rich lamina to appear (Poliakov, 1965; Marin-Padilla, 1970a; Sidman and Rakic, 1973). At birth, basket cells of this layer and, to a lesser extent, layer III, have not as yet differentiated in humans (Marin-Padilla, 1970b). Since birth complications, particularly prolonged labor, occur in a high percentage of schizophrenics (Jacobsen and Kinney, 1975; Parnas et al., 1982), it is tempting to speculate that a perinatal insult could result in a failure of basket cells to form in cortical layers II and III of schizophrenics (Benes, in press). A loss of these GABAergic inhibitory cells (Jones and Hendry, 1984) during early ontogeny would be expected to give rise to a compensatory increase of $GABA_A$ receptor binding in superficial layers II and III in the cingulate cortex of adult brain as noted in the schizophrenic cases in this study. Since layer I is one of the first laminae to appear during cortical ontogenesis and it ordinarily contains few neuronal cell bodies, the increased $GABA_A$ receptor binding found in neuropil of this layer probably reflects a loss of basket cells in layers II and III. Presumably, the $GABA_A$ binding in layer I is localized to distal portions of pyramidal cell dendrites.

The importance of GABAergic inhibition for the normal functioning of the CNS is well established (Jones, 1987). Thus, a loss of GABAergic interneurons would have an important impact on the activity of intrinsic circuits of the cingulate cortex (refer to Fig. 5*A–C*). In the visual cortex, bicuculline-induced interruption of GABAergic transmission results in disturbances of directional and orientational selectivity of simple or complex visual neurons (Sillito, 1984). A similar defect in associative cortical processing could contribute to the loss of the "central filter" that has been postulated to give rise to the overinclusive thinking, bombardment by external stimuli, and a loss of a focal direction to attention seen in schizophrenia (Detre and Jarecki, 1971). Lesions of the anterior cingulate cortex result in neglect of surrounding objects in cats (Kennard, 1955), monkeys (Glees et al., 1950), and humans (Laplane et al., 1981). Consistent with this, recent cerebral blood flow studies have demonstrated a robust activation of the anterior cingulate cortex in human subjects performing a Stroop attentional paradigm (Pardo et al., 1988), although the attentional deficit seen in schizophrenia may involve abnormalities of this region that occur preferentially in the left hemisphere (Posner et al., 1988). It is noteworthy that

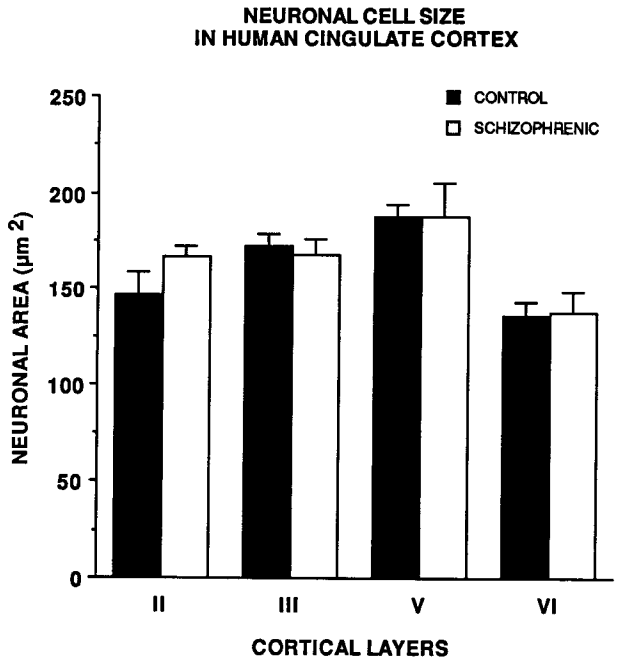


*Figure 4.* A bar graph showing the average size of neurons in individual layers of the anterior cingulate cortex of postmortem brain. The area of individual neurons in layers II, III, V, and VI shows no difference between the control ($N = 8$) and schizophrenic ($N = 6$) groups.

attentional mechanisms probably occur in parallel with emotional responses (Mesulam and Geschwind, 1978) and that the latter are also disturbed in schizophrenia. Since the anterior cingulate region probably plays a central role in the integration of affect at the cortical level (Papez, 1937), this region can be implicated in at least two of the core features of schizophrenia. Thus, alterations of GABAergic activity in the intrinsic circuits of the anterior cingulate cortex could contribute to the abnormalities of both affective experience and attention that are observed in schizophrenic patients.

It is not clear whether a loss of GABAergic activity by itself would be sufficient to account for the core symptoms of schizophrenia. A loss of GABAergic activity occurring in combination with another abnormality, such as an increase in the numbers of incoming associative fibers to this region (Benes et al., 1987), could produce significant shifts in the input–output relationship of the cingulate region with its various termination sites in associative cortex and limbic system (Fig. 5*C*). Since associative fibers probably employ glutamate as a neurotransmitter (Monaghan and Cotman, 1985; Conti et al., 1988), excess numbers of such fibers could predispose a region to an excitotoxic injury (Rothman and Olney, 1986) in response to an hypoxic perinatal insult that might not otherwise cause damage to the immature brain. Injury to GABAergic interneurons has been shown to occur in response to kainate-induced lesions in adult animals (Zhang et al., 1990). While kainate is not a potent neurotoxin in the immature brain (Coyle, 1983), ibotenate does produce excitotoxic injury, possibly via NMDA receptor sites, in young animals (Steiner et al., 1984) and could theoretically give rise to GABA neuron loss in upper cortical layers where NMDA receptor sites are most abundant (Monaghan and Cotman, 1985). It is pertinent to note that during the first 7 d of postnatal life, there is a robust loss of neurons that occurs preferentially in layers II and III of medial cortical regions of rat brain (Ferrer

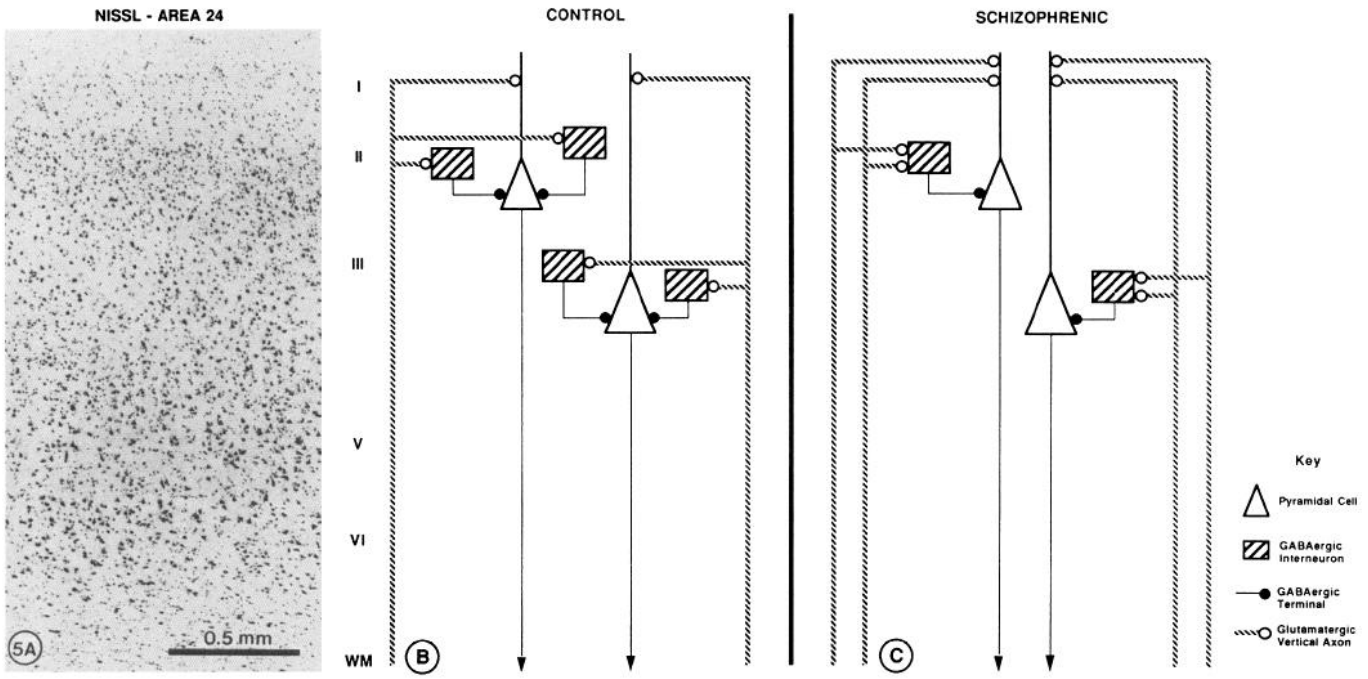


*Figure 5.* A photomicrograph and model circuits depicting the various differences in the anterior cingulate cortex of schizophrenic patients. *A,* A Nissl-stained low-power photomicrograph of the cingulate cortex in human brain showing a characteristic six-layered organization. *Layers I–III* are predominantly involved in corticocortical processing, while those in the deeper layers are preferentially involved in processing activity from subcortical regions. *B,* A schematic diagram showing an intrinsic circuit in the cingulate cortex of normal individuals. There are pyramidal neurons in *layers II* and *III,* and each receives inputs from *two* inhibitory GABAergic interneurons. There are also *two* vertical associative fibers from other regions that enter the cortex and travel vertically toward layer I, where they form excitatory synapses with distal portions of the pyramidal cell dendrite. These associative fibers also give off collateral branches that form excitatory connections with the inhibitory interneurons. *C,* A diagram similar to that in *B* but representing the changes that may have occurred in the circuits of schizophrenic patients. The two pyramidal cells each receive inhibitory inputs from only *one* GABAergic interneuron. As suggested by a previous study, schizophrenics may have increased numbers of vertical associative fibers (Benes et al., 1987) so that the diagram shows *four* vertical associative fibers synapsing on pyramidal cell dendrites in *layer I.* Since the data in the present study are consistent with a loss of GABAergic interneurons, the schizophrenic circuit has only one inhibitory interneuron forming connections with each of the pyramidal cells in *layers II* and *III.* With increased numbers of excitatory associative inputs, but decreased inhibitory interneurons, the outflow of pyramidal neuron activity from the cingulate region toward the prefrontal cortex, hippocampal formation, and other areas with which it connects would be altered in schizophrenics.

et al., 1990). Thus, it is also possible that normal regressive changes during ontogenesis might potentiate an excitotoxic process and contribute to the losses of interneurons (Benes et al., in press) and increased $GABA_A$ receptor binding found in superficial layers of the cingulate region in adult schizophrenic brain.

Recent investigations have suggested that dopaminergic afferents may terminate on GABAergic neurons in the corpus striatum and nucleus accumbens (Onteniente et al., 1987). If a similar pattern of connectivity occurs in the cortex, then the decreased numbers of inhibitory basket cells postulated to occur in the cingulate region of schizophrenics could result in a relative hyperinnervation of individual GABAergic follower cells by the mesocortical dopamine projection. Thus, the original dopamine hypothesis of schizophrenia in which excess dopaminergic activity was suggested to be a cause of psychosis (Kety and Matthysse, 1972) is consistent with the above model. Since dopamine afferents may exert an inhibitory effect on GABAergic cells (Marco et al., 1978), a relative increase of these fibers with respect to individual basket cells would tend to exacerbate a deficiency in GABA neurotransmission, while conversely, blockade of dopamine receptors would tend to release GABA interneurons to fire more effectively. The therapeutic efficacy of antipsychotic medications in treating schizophrenia could potentially be understood in the context of the present findings and model concerning abnormal circuitry in the cingulate region of schizophrenics.

In conclusion, using a high-resolution autoradiographic technique, the present study has demonstrated markedly upregulated $GABA_A$ receptor binding on individual neurons and in neuropil of superfical layers of the cingulate cortex of schizophrenic patients. These data provide support for an earlier suggestion that some of the interneurons found to be lacking in chronically psychotic patients may be inhibitory basket cells. The fact that these differences in $GABA_A$ receptor binding in the schizophrenics studied here were found most strikingly in upper layers of limbic cortex is consistent with the idea that disturbances in associative information processing related to emotional behavior occur in schizophrenia and may arise from perturbations of early brain development (Murray and Lewis, 1987; Weinberger, 1987). Taken together, the present findings may eventually contribute to the further understanding of certain observations concerning the etiology, symptomatology, and perhaps even the treatment of schizophrenia.

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