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ABNORMAL CHOLECYSTOKININ mRNA LEVELS IN ENTORHINAL CORTEX OF SCHIZOPHRENICS

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Summary—Limbic cortical regions, including anterior cingulate cortex (ACC), prefrontal cortex (PFC) and entorhinal cortex (ERC), have been implicated in the neuropathology of schizophrenia. Glutamate projection neurons connect these limbic cortical regions to each other, as well as to the terminal fields of the striatal/accumbens dopamine neurons. Subsets of these glutamate projection neurons, and of the GABA interneurons in cortex, contain the neuropeptide cholecystokinin (CCK). In an effort to study the limbic cortical glutamate projection neurons and GABA interneurons in schizophrenia, we have measured CCK mRNA with *in situ* hybridization histochemistry in post-mortem samples of dorsolateral (DL)PFC, ACC and ERC of seven schizophrenics, nine non-psychotic suicides and seven normal controls. CCK mRNA is decreased in ERC (especially layers iii–vi) and subiculum in schizophrenics relative to controls. Cellular analysis indicates that there is a decrease in density of CCK mRNA in labelled neurons. In so far as ERC CCK mRNA is not reduced in rats treated chronically with haloperidol, this decrease in schizophrenics does not appear to be related to neuroleptic treatment. In contrast, in DLPFC, where schizophrenics do not differ from normals, the suicide victims have elevated CCK mRNA (especially in layers v and vi), and increased cellular density of CCK mRNA, relative to both normals and schizophrenics. These results lend further support for the involvement of ERC and hippocampus in schizophrenia, suggesting that neurons that utilize CCK may be particularly important. Similarly, an increase in CCK mRNA levels in the PFC of suicides adds to a growing body of evidence implicating this structure in this pathological state. In so far as CCK is co-localized with GABA or glutamate in cortical neurons, both of these neuronal populations need to be studied further in schizophrenia and suicide. © 1997 Elsevier Science Ltd.

Introduction

No discrete neuroanatomical locus of pathology has been found to account for schizophrenia. An alternative approach to elucidation of the neuropathology of schizophrenia has been the search for a "network" of abnormal neural circuits (e.g. Weinberger, 1991).

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One such network involves "limbic" cortex, which both receives dopamine innervation (Hökfelt et al., 1974) and in turn sends projections to striatum and/or nucleus accumbens, modulating dopamine function in these structures (Selemon & Goldman-Rakic, 1985; Yeterian & Van Hoesen, 1978; Baleydier & Mauguière, 1980; Newman & Winans, 1980; Sørensen & Witter, 1983). Limbic cortical regions, including DLPFC, ACC and ERC, are reciprocally connected (Selemon & Goldman-Rakic, 1988; Baleydier & Mauguière, 1980; Swanson & Köhler, 1986; Insausti et al., 1987a; Goldman-Rakic et al., 1984) and may modulate the same striatal subregions (Yeterian & Van Hoesen, 1978). Furthermore, aberrations in this "network" have been implicated by morphological, neurochemical and functional studies in schizophrenia (e.g. DLPFC: Akbarian et al., 1993a,1995; Fey, 1951; Weinberger et al., 1986; ACC: Benes et al., 1986, 1992a,b; Kawasaki et al., 1993; ERC: Falkai et al., 1988; Wolf et al., 1995; Friston et al., 1992).

Moreover, perhaps the site of the most convergent and replicable findings for neuropathology in schizophrenia has been the mesial temporal lobe (e.g. Brown et al., 1986; Jakob & Beckmann, 1986; Colter et al., 1987; DeLisi et al., 1988; Suddath et al., 1989; Casanova et al., 1990; Altshuler et al., 1990; see Hyde & Weinberger, 1990 for review). In addition to ERC, there is considerable evidence for neuropathology in hippocampus (e.g. Falkai & Bogerts, 1986; Bogerts, 1984; Bogerts et al., 1985, 1990; Suddath et al., 1990; Okada et al., 1991; Arnold et al., 1991; Akbarian et al., 1993b), especially in the hippocampal outflow from subiculum to nucleus accumbens (for reviews see Gray et al., 1991; Weinberger & Lipska, 1995) in schizophrenia. The hippocampus and ERC have extensive reciprocal interconnections (e.g.Blackstad, 1956; Van Hoesen & Pandya, 1975; Rosene & Van Hoesen, 1977). The ERC acts as the "gateway" in and out of hippocampus, through which multimodal sensory inputs are funnelled for integration into motivational, affective, attentional and cognitive functions (e.g. as proposed by Bogerts, 1989). Indeed, a great deal of complex processing of hippocampal input and output has been shown to occur in ERC (Jones, 1993). Pathology of these sites may explain some of the memory and cognitive problems in schizophrenia.

Some of the interneurons within these three limbic cortical regions (Hendry et al., 1984; Köhler, 1986), and projection neurons connecting them with each other as well as with their striatal/accumbens targets (Meyer et al., 1982a and Meyer et al., 1982b; Burgunder & Young, 1990), contain the neuromodulator CCK. CCK was initially studied in schizophrenic brains because of its co-localization with dopamine in nigrostriatal and mesolimbic neurons (Hökfelt et al., 1980). However, findings of decreased CCK levels in the mesial temporal lobe and frontal cortex of schizophrenics (Ferrier et al., 1983; Davidson et al., 1994), although not always replicated (Kleinman et al., 1983), and decreased binding to CCK receptors in hippocampus, parahippocampal gyrus and frontal cortex of schizophrenics (Farmery et al., 1985; Ferrier et al., 1985; Kerwin et al., 1992), could equally well reflect deficits in cortical circuitry. Indeed, a more recent report of reduced mRNA for CCK in soma in temporal and frontal cortex, found with *in situ* hybridization histochemistry (Virgo et al. 1995), implicates cortical interneurons or efferent projections rather than dopamine afferents. We have attempted here to extend this strategy to the study of ACC, DLPFC and ERC in schizophrenia, relative to a non-psychotic suicide control group and normal controls, with *in situ* hybridization histochemistry for CCK mRNA.

Methods

Postmortem human brain samples

Postmortem brains from the Neuropathology Section, Clinical Brain Disorders Branch, IRP, NIMH, of the NIMH Neuroscience Center at St. Elizabeths Hospital, were donated by the families of the deceased after autopsies were performed by the Office of the Medical Examiner (Washington, D.C.). Coronal blocks (1.5 cm thick) were rapidly frozen at autopsy by immersion in a mixture of isopentane and dry ice and stored at −70 C until sectioning. Studies were performed on 14 μ thick coronal sections, sectioned by cryostat, thaw-mounted and dried on gelatin-coated slides and stored with desiccant at −70 C until *in situ* hybridization. Sections were taken from a level of Brodmann's area 24 corresponding to that designated as 24a by Vogt et al. (1995) for ACC, Brodmann's area 9, just anterior to the genu of the corpus callosum for DLPFC, and an intermediate ERC level corresponding to 28I (as defined by Saunders & Rosene, 1988), where characteristic laminar features of ERC become most prominent.

Patient cohorts included: normal controls (no history of psychiatric illness or neuroleptic exposure from medical examiner's records), schizophrenics (psychiatric diagnosis determined by independent review of the medical records by two psychiatrists), and non-psychotic suicides (exclusion of symptoms suggesting a previous psychotic disorder), for which the demographics are presented in Table 1. Age and post-mortem interval (PMI) were similar between these groups ($F_{2,20} = 0.27$, $p = 0.76$; and $F_{2,20} = 1.01$, $p = 0.38$; respectively). No subject had a history of neurological illness, abnormal gross or microscopic brain pathology, or measurable levels of drugs of abuse or neuroleptics in blood and urine toxicology. Information on the symptoms of psychosis, defect (negative) symptoms, and past neuroleptic history were obtained from the medical records of the schizophrenics. ERC data for one normal control (male, black, 51 years, PMI = 10 hours) were excluded from the analysis because the ERC block sectioned from this brain was not taken from the same rostral:caudal plane as that of the others.

Neuroleptic-treated rat brain samples

To control for the effects of chronic neuroleptic treatment, samples of ERC and substantia nigra were examined from rats treated chronically with haloperidol decanoate. Male Sprague-Dawley rats (Zivic Miller), initially weighing 140–160 g, were housed in groups of two with free access to food and water, a 12 h light dark cycle, and constant tem-

Table 1
Demographics of Patient Cohorts for Brain Samples Used

Group	N	Gender	Race	Age	PMI
Normal controls	7	2F,5M	1C, 1I, 5AA	51.1 ± 11.1	27.8 ± 14.8
Schizophrenics	7	5F,2M	3C, 4AA	57.3 ± 15.0	21.4 ± 8.0
Suicides	9	2F,7M	7C, 1O, 1AA	57.2 ± 23.9	36.1 ± 29.3

Groups are defined in methods. Values are mean ± SD, in years for age and in hours for postmortem interval (PMI). F = female, M = male, C = Caucasian, O = Oriental, I = Indonesian, AA = African American. For the schizophrenic group, the mean ± SD for duration of illness were 30 ± 16.3 years.

perature (25 °C). A group of 17 rats received haloperidol decanoate (McNeil Pharmaceuticals, i.m. 28.5–42.25 mg/kg every three weeks [the equivalent of 1.0 mg/kg/day] for 36 weeks as previously described (Egan et al., 1994). A control group (n = 7) received comparable injections of vehicle (provided by McNeil Pharmaceuticals). The rats were monitored for 28 weeks after the final injection, at which time the brain haloperidol levels averaged 61.5 ng/mg tissue. The rats were then killed by intracardiac perfusion with phosphate buffered saline at 4 °C while under deep anesthesia. Their brains were quickly removed, blocked and frozen in powdered dry ice, and stored at −70 °C. Coronal sections from the midbrain block containing substantia nigra and ERC (20 μ thick) were sectioned by cryostat and thaw-mounted and dried onto gelatin-subbed slides.

In situ hybridization histochemistry

An oligonucleotide probe (48 bases in length) (synthesized in Lab of Cell Biology, NIMH, and obtained from W. S. Young, III), complementary to bases 315–362 of rat preprocholecystokinin mRNA (Deschenes et al., 1984) was labelled, using terminal deoxynucleotidyl transferase and deoxyadenosine [alpha-[^{35}S]-thio] triphosphate, at the 3′ end, to a specific activity of $5-10 \times 10^5$ cpm/μl, according to the method described by Young (1992). This probe has previously been shown to label human CCK mRNA (Rance & Young, 1991). A 48-base "randomer" probe (purchased from Dupont New England Nuclear, Boston) was similarly labelled to serve as a control probe.

In human slides, hybridization was carried out according to the method described by Chesselet et al. (1987), with modifications as described by Frohna et al. (1993), except that the heparin hybridization buffer (200 μl, with 4×10^6 cpm of labelled probe added, pipetted onto each slide), and coverslipping of slides with parafilm, were as described by Young (1992), and slides were incubated at 37 °C overnight. Washes the following day were performed as described by Frohna et al. (1993). A few control sections were treated with the labelled randomer probe, or were pretreated with RNase as in Virgo et al. (1995), to evaluate the specificity of the CCK mRNA probe. In addition, some sections were treated with concentrations of labelled CCK mRNA probe that ranged from 3 to 150% of the probe used for the experimental slides, to establish that the concentration used was adequate for the levels of mRNA in these sections. A simpler hybridization method, as described by Young (1992), was used for the rat slides.

X-omat (Kodak, Rochester, NY) film was apposed to the slides and ^{14}C standards for 10–12 days and then developed. Autoradiographs were digitized with a flat-bed optical scanner. Quantitation of optical densities from the autoradiographs, interpolated along the ^{14}C standard curve, and converted to dpm/mm^2 (Miller, 1991), was done by an observer blind as to psychiatric diagnosis, using an image analysis program (NIH IMAGE, Rasband, NIH) on a Macintosh computer. Samples were measured from human ACC layers ii/iii, v and vi, PFC layers i/ii, iii/iv, v and vi, and layers ii, iii, v and vi from the ERC (this scheme is from Saunders & Rosene [1988] and differs from Lorente de Nó [1933] in that iv is the lamina dessicans and iii corresponds to iii + iv of Lorente de Nó [1993]). Hippocampal samples from dentate and subiculum were also taken from the human ERC section. Samples

were taken bilaterally from substantia nigra (vehicle: n = 7, drug: n = 14) and ERC (vehicle: n = 6, drug: n = 17) in rat midbrain sections.

Subsequent to film exposure to the slides, the human slides were dipped in photographic emulsion, allowed to expose for six weeks, developed, thionine-stained, and cover-slipped, for cellular image analysis. Cellular image analysis from the emulsion-dipped slides was done in human ERC layers ii and vi and in human DLPFC layer vi by an observer blind to psychiatric diagnosis. Through a Zeiss microscope connected by a CCD camera (Sierra Scientific) to the Macintosh, samples were located in these layers at 10 × magnification in light-field, and then visualized at 40 × magnification in dark-field. From random samples of neurons (approximately 30 per layer per slide), the area and number of silver grains were measured for each neuron, again using NIH IMAGE, and the average density of silver grains/background area was subtracted from the density for each neuron. Neurons with a density of silver grains/area greater than four times background level were considered to be CCK mRNA-positive neurons.

Statistical analysis was by two-way ANOVA (for group and region) using Statview (Abacus Concepts, Berkeley, CA) plus *post-hoc* group contrasts by ANOVA for ACC, DLPFC and ERC, and *post-hoc* *t*-tests for individual regions, for the human autoradiographs. Because of earlier findings of reduced CCK levels in temporal and frontal cortex of schizophrenics (Ferrier et al., 1983; Davidson et al., 1994) and of reduced mRNA for CCK in schizophrenic cortex (Virgo et al., 1995) one-tailed *t*-tests were used for these *post-hoc* tests for a predicted decrease in schizophrenics relative to normal controls and to suicides, while, in the absence of evidence implicating CCK in suicides, two-tailed *t*-tests were used as *post-hoc* tests of differences between normals and suicides. Accordingly, for the cellular analysis from the human DLPFC and ERC sections, one-tailed *t*-tests were also used for comparisons between schizophrenics and normal controls in ERC, and two-tailed *t*-tests were used for comparisons between suicides and the other two groups in DLPFC. For the human data, correlations between CCK mRNA levels and age and PMI were also calculated (with one-tailed tests). Two-way ANCOVAs (group by region with age or PMI as the covariate) were also calculated using Statistica (Statsoft, Tulsa, OK, U.S.A.). The data from the rat autoradiographs were analysed by one-way ANOVA. Statistical significance was set at $p \leqslant .05$.

Results

No section labelled with randomer probe or pretreated with RNase exhibited any labelling. In the saturation curve for the CCK mRNA probe, density of label was in the plateau of the curve for concentrations from 25 to 150% of that used on experimental slides. Clear laminar patterns of label were evident in human ACC, DLPFC and ERC sections. Representative images from these regions are illustrated in Figures 1–3.

The two-way ANOVA for all three groups and all regions yielded a statistically significant group effect ($F_{2,12} = 2.99$, $p \leqslant 0.05$). Hence, separate two-way ANOVAs and *post hoc* group contrasts were also run for ACC, DLPFC and ERC. In ACC there was not a significant group effect ($F_{2,2} = 0.05$, $p = 0.95$), so no further analyses were performed. These results are presented in Figure 4.

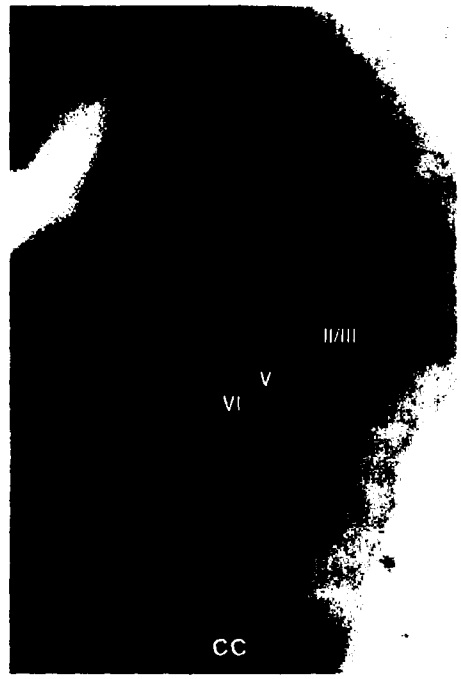

Figure 1. Autoradiograph from human anterior cingulate cortex after exposure for 12 days to tissue hybridized with a probe for cholecystokinin mRNA. Layers ii/iii, v and vi are indicated.

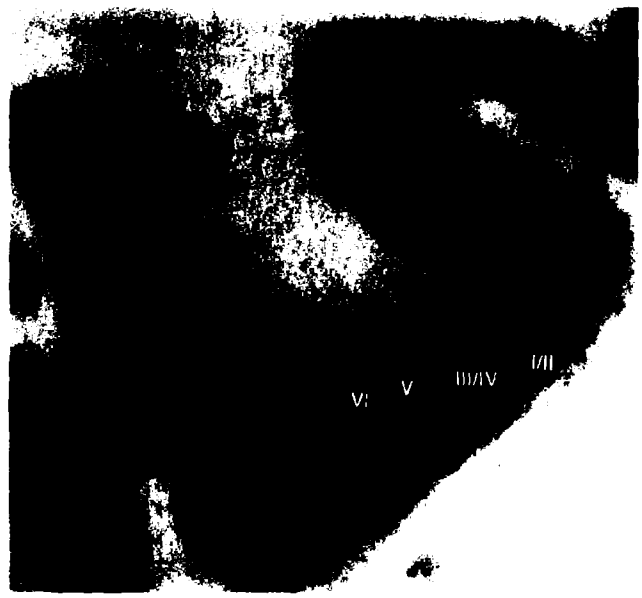

Figure 2. Autoradiograph from human dorsolateral prefrontal cortex after exposure for 10 days to tissue hybridized with a probe for cholecystokinin mRNA. Layers i/ii, iii/iv, v and vi are indicated.

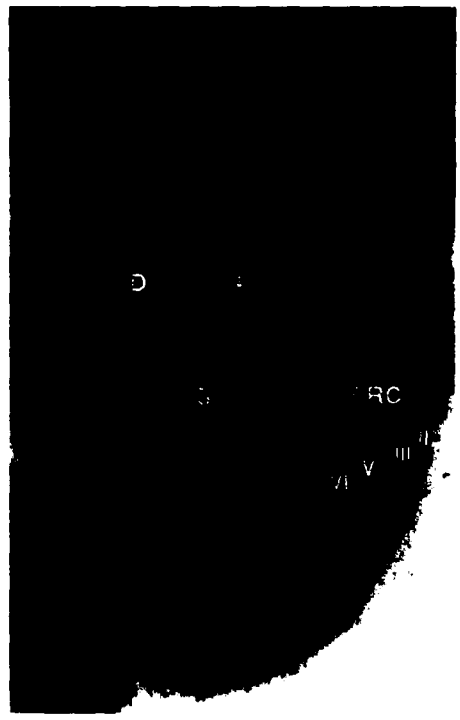

Figure 3. Autoradiograph from human entorhinal cortex after exposure for 10 days to tissue hybridized with a probe for cholecystokinin mRNA. Fascia dentata (D), subiculum (S), and entorhinal cortex layers ii, iii, v and vi are indicated.

Human ERC

In ERC there was an overall group effect ($F_{2,5}=4.28$, $p=0.02$). Normal controls and suicides did not differ in ERC CCK mRNA ($F_{1,5}=0.70$, $p=0.40$). Schizophrenics had reduced ERC CCK mRNA relative to both normal controls ($F_{1,5}=10.32$, $p=0.002$) and suicides ($F_{1,5}=4.87$, $p=0.03$). *Post-hoc* group comparisons by layers indicated that there were significant differences from normal controls in subiculum ($t=1.96$, 11 df, $p=0.04$), and ERC layers iii ($t=2.27$, 11 df, $p=0.02$), v ($t=3.48$, 11 df, $p=0.003$) and vi ($t=4.18$, 11 df, $p=0.001$), and from suicides in subiculum ($t=1.72$, 14 df, $p=0.05$), and ERC layers v ($t=1.93$, 14 df, $p=0.04$) and vi ($t=2.34$, 14 df, $p=0.02$). These results are presented in Figure 5.

All of the schizophrenics had evidence of hallucinations and delusions, but only three of them had a formal thought disorder. Similarly, only three of the seven had clear-cut evidence in their charts of defect symptoms. Both those schizophrenics with ($F_{1,5}=8.36$, $p=0.006$) and without ($F_{1,5}=3.89$, $p=0.05$) defect symptoms, and those with ($F_{1,3}=5.08$, $p=0.03$) and without ($F_{1,3}=6.43$, $p=0.01$) thought disorder had decreased ERC CCK mRNA relative to normal controls.

Across all subjects, there were no significant correlations between age and ERC CCK mRNA, but there was a significant positive correlation between PMI and CCK mRNA

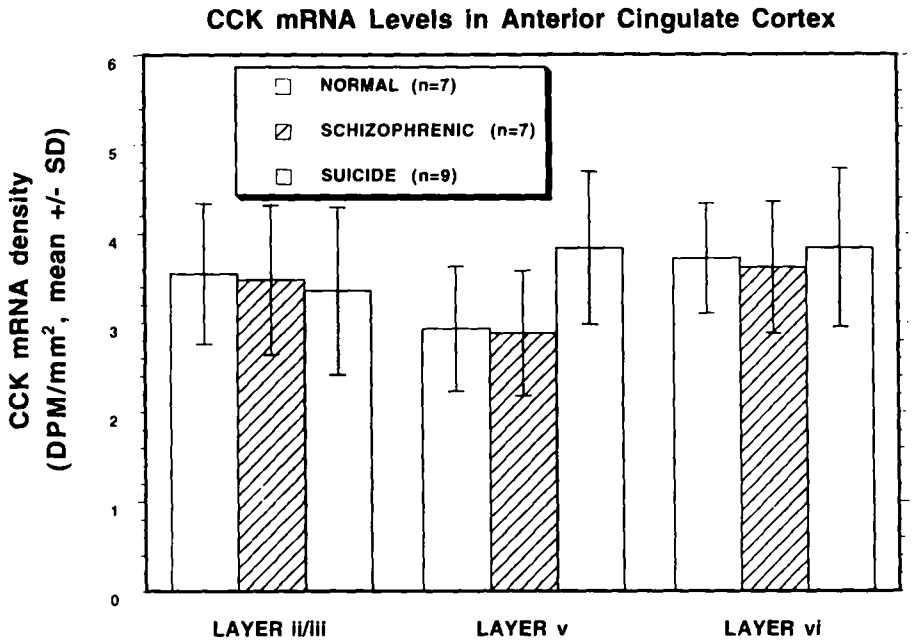

Figure 4. Comparison of CCK mRNA levels in anterior cingulate cortex between normals, schizophrenics and suicides. There was no statistically significant group effect ($F_{2,2} = 0.05$, $p = 0.95$).

in ERC layer vi ($r = 0.61$, 20 df, $p < 0.005$). The two-way ANCOVA for ERC between schizophrenics and normal controls remained statistically significant with age as the covariate ($F_{1,5} = 8.61$; $p = 0.005$) and with PMI as the covariate ($F_{1,2} = 10.46$, $p = 0.002$).

We selected an ERC layer in which schizophrenics did not differ from normal controls, layer ii, and one in which the difference was highly significant, layer vi, to evaluate differences between schizophrenics and normal controls in the densities of silver grains/area of CCK mRNA-labelled neurons. One slide from a normal control had uniform high background label, which made an evaluation impossible, and was discarded from the analysis. In layer ii, there were no statistically significant differences between normal controls and schizophrenics in silver grain density of CCK mRNA-positive cells or in percent of sampled cells that were positive for CCK mRNA. In layer vi CCK mRNA-positive neurons there was a statistically significant reduction in density of silver grains/area in schizophrenics relative to normals ($t = 1.77$, 10 df, $p \leqslant 0.05$), but no difference in proportions of CCK mRNA-positive neurons. These results are presented in Figures 6 and 7.

Human DLPFC

In DLPFC there was a significant group effect ($F_{2,3} = 8.98$, $p = 0.0003$). Normal controls did not differ from schizophrenics in DLPFC CCK mRNA ($F_{1,3} = 0.01$, $p = 0.94$). However, suicides had significantly elevated DLPFC CCK mRNA relative to both normals ($F_{1,3} = 12.42$, $p = 0.001$) and schizophrenics ($F_{1,3} = 12.42$, $p = 0.001$). *Post-hoc* comparisons by layers indicated that these differences reached statistical significance in layer vi ($t = 3.20$,

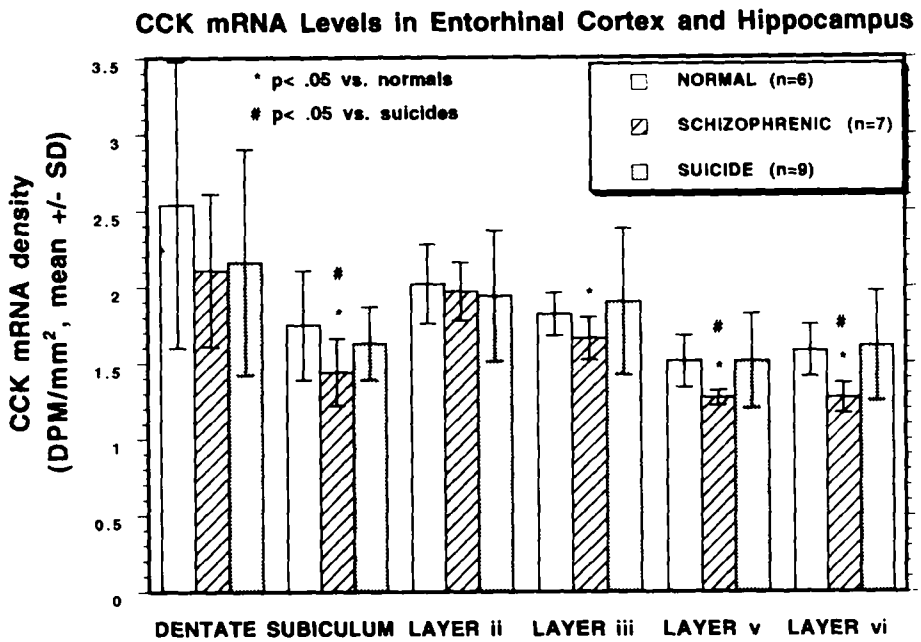

Figure 5. Comparison of CCK mRNA levels in entorhinal cortical section between normals, schizophrenics and suicides. There was a statistically significant group effect ($F_{2,\cdot}=4.28$, $p=0.02$). Only schizophrenics differed from other groups ($F_{1,\cdot}=10.32$, $p=0.002$ relative to normals; $F_{1,\cdot}=4.87$, $p=0.03$ relative to suicides).

14 df, $p=0.006$) and approached significance in layers v ($t=1.73$, 14 df, $p=0.10$) and i,ii ($t=1.69$, 14 df, $p=0.12$), between suicides and normals, and were significant for layers v ($t=2.20$, 14 df, $p=0.04$) and vi ($t=2.80$, 14 df, $p=0.014$) and approached significance in layers iii/iv ($t=1.74$, 14 df, $p=0.10$), between suicides and schizophrenics. These results are presented in Figure 8. Because of our finding that DLPFC CCK mRNA was elevated in suicides, the difference between schizophrenics and normals was re-evaluated after exclusion of the one schizophrenic who died by suicide, and still failed to attain statistical significance ($F_{1,3}=0.09$, $p=0.77$).

Neither those schizohrenics with ($F_{1,3}=1.34$, $p=0.26$) nor those without ($F_{1,3}=1.18$, $p=0.28$) defect symptoms had decreased DLPFC CCK mRNA relative to normal controls. Neither those with ($F_{1,3}=1.59$, $p=0.22$) nor those without thought disorder ($F_{1,3}=1.01$, $p=0.32$) had a DLPFC CCK mRNA reduction relative to normal controls. Schizophrenics without thought disorder had lower DLPFC CCK mRNA than those with thought disorder ($F_{1,3}=4.40$, $p<0.05$) though they did not differ from normal controls.

Across all subjects, there were no significant correlations between PMI and DLPFC CCK mRNA, but there was a significant inverse correlation between age and DLPFC CCK mRNA ($r=-0.45$, 21 df, $p<0.025$) in layers iii/iv. The two-way ANCOVA for DLPFC between normal controls and suicides remained statistically significant with age as the covariate ($F_{1,3}=18.94$; $p<0.0001$) and with PMI as the covariate ($F_{1,3}=20.47$; $p<0.0001$).

Cellular analyses from layer vi of DLPFC indicated that suicides had greater density of CCK mRNA label in CCK mRNA-positive cells relative to normal controls ($t=2.15$, 14

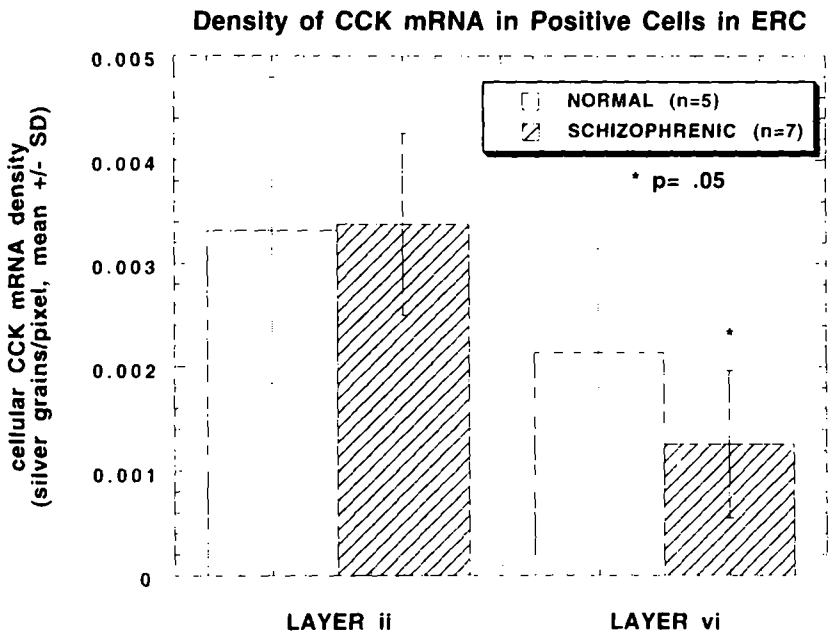

Figure 6. Comparison of cellular density of CCK mRNA in positive cells (density ⩾4 times background), from which background density has been subtracted, in entorhinal cortex layers ii and vi, between normals and schizophrenics.

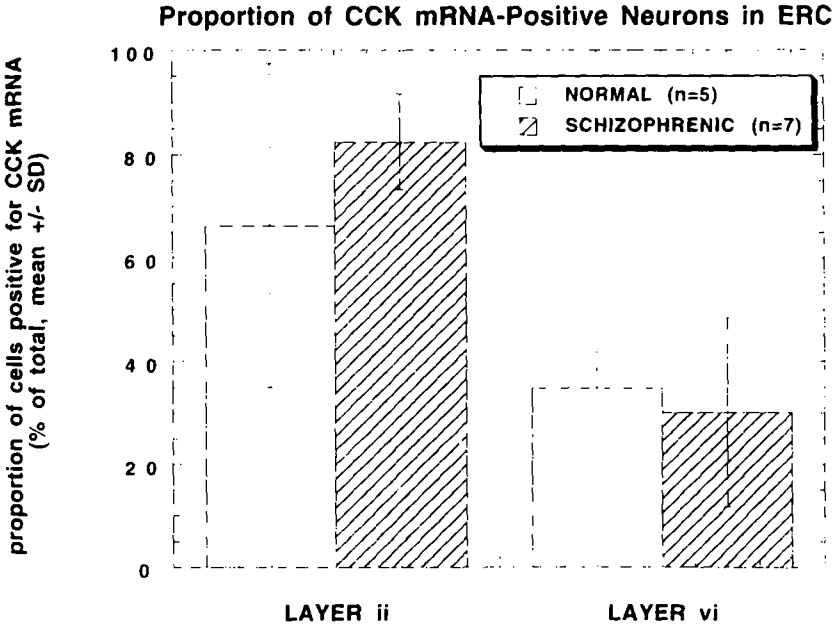

Figure 7. Comparison of proportion of CCK mRNA-positive cells (density ⩾4 times background) among all cells sampled, in entorhinal cortex layers ii and vi, between normals and schizophrenics.

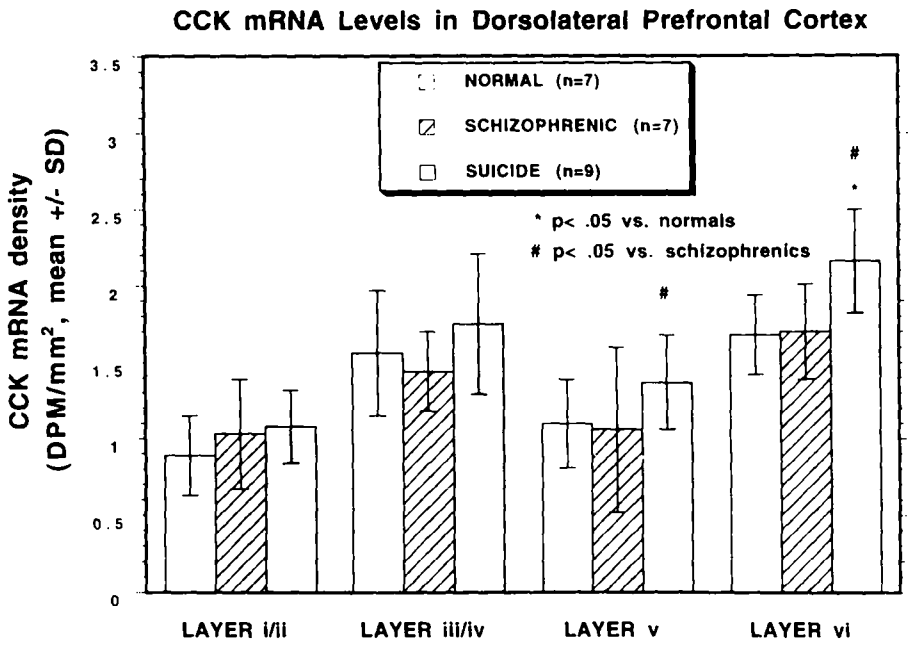

Figure 8. Comparison of CCK mRNA levels in dorsolateral prefrontal cortex between normals, schizophrenics and suicides. There was a statistically significant group effect ($F_{2,}$ = 8.98, p = 0.0003). Only suicides differed from other groups ($F_{1,}$ = 12.42, p = 0.001 relative to normals; $F_{1,}$ = 12.42, p = 0.001 relative to schizophrenics).

df, p = 0.05), whereas proportions of CCK mRNA positive cells did not differ (t = .49, 14 df, p = 0.64). Schizophrenics did not differ significantly from normal controls in either density of silver grains/area in CCK mRNA-positive cells (t = 0.47, 12 df, p = 0.64) or proportion of cells that were positive for CCK mRNA (t = 0.92, 12 df, p = 0.38). These results are portrayed in Figures 9 and 10.

Neuroleptic-treated rats

Chronic neuroleptic treatment in rats produced a 34% elevation in nigral CCK mRNA ($F_{1,19}$ = 7.12, p = 0.02). ERC CCK mRNA from the same neuroleptic-treated rats was unaffected ($F_{1,21}$ = 0.24, p = 0.63). These results are presented in Figure 11.

Discussion

Our results in schizophrenia confirm and extend the findings of Virgo et al. (1995), of reduced temporal cortical CCK mRNA in schizophrenia. These results further implicate the mesial temporal lobe (see above) in schizophrenia. These reductions occur in ERC (layers iii–vi), and subiculum, but not ACC or DLPFC. The finding from the cellular analysis is consistent with the possibility that this decrease is due to reduced density of CCK mRNA in CCK mRNA-expressing neurons. Another possibility, which was not addressed by this sampling strategy, suggested by the findings of Falkai et al. (1988) and

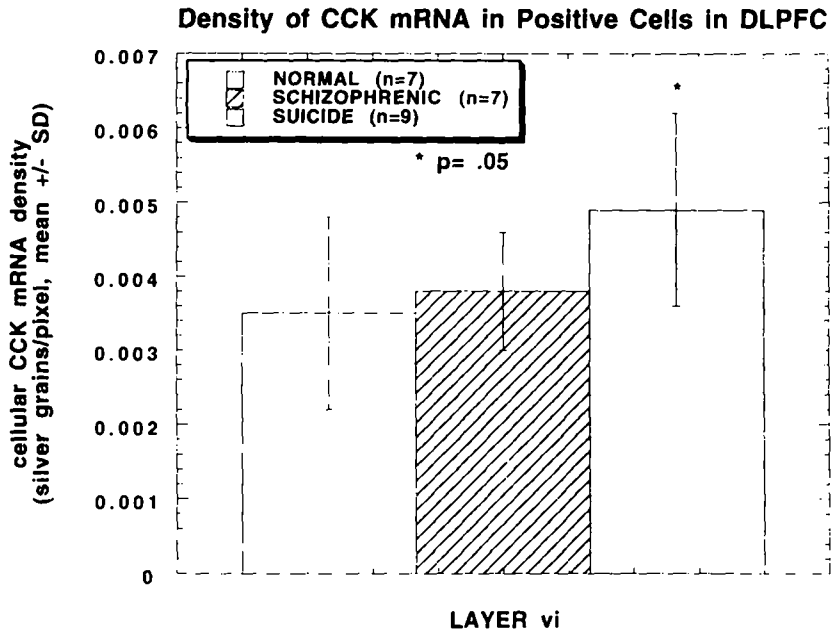

Figure 9. Comparison of cellular density of CCK mRNA in positive cells (density ≤ 4 times background), from which background density has been subtracted, in dorsolateral prefrontal cortex layer vi, between normals, schizophrenics and suicides.

Krimer et al. (1995), is that decreased neuronal counts and/or density, respectively, in ERC might contribute to this reduction.

Thus, it may be that there is some selectivity for ERC, relative to ACC and DLPFC, in the CCK mRNA decrease in schizophrenia. Indeed, the contribution of DLPFC pathology to schizophrenia might be via its connections (or mis-connections) with ERC/hippocampus. Consistent with this possibility are the observations that ERC appears to "gate PFC influence on nucleus accumbens" (O'Donnell & Grace, 1995), that there is apparently not a direct projection in primate from DLPFC to nucleus accumbens (Selemon & Goldman-Rakic, 1985), and that CCK innervation of nucleus accumbens appears to derive principally from subiculum (Kresse et al., 1995) and not from PFC. Moreover, the correlation between hippocampal size and DLPFC hypofunction in schizophrenics performing the Wisconsin Card Sort (Weinberger et al., 1992) suggests that temporal lobe neuropathology might be responsible for DLPFC functional deficits in schizophrenia.

A question that remains to be answered is which CCK-containing neurons in ERC and hippocampus might be the affected population. Two obvious candidates are GABA interneurons in cortical layers ii, iii, v and vi and hippocampus, where CCK and GABA have been shown to be co-localized (Somogyi et al., 1984) and glutamate projections neurons from cortex to striatum (McGeer et al., 1977; Meyer et al., 1982b), from ERC to hippocampus (Storm-Mathisen, 1977; Fredens et al., 1984), other limbic cortical sites (Meyer et al., 1982a), and nucleus accumbens (Christie et al., 1987), and from subiculum to nucleus accumbens (Walaas & Fonnum, 1979).

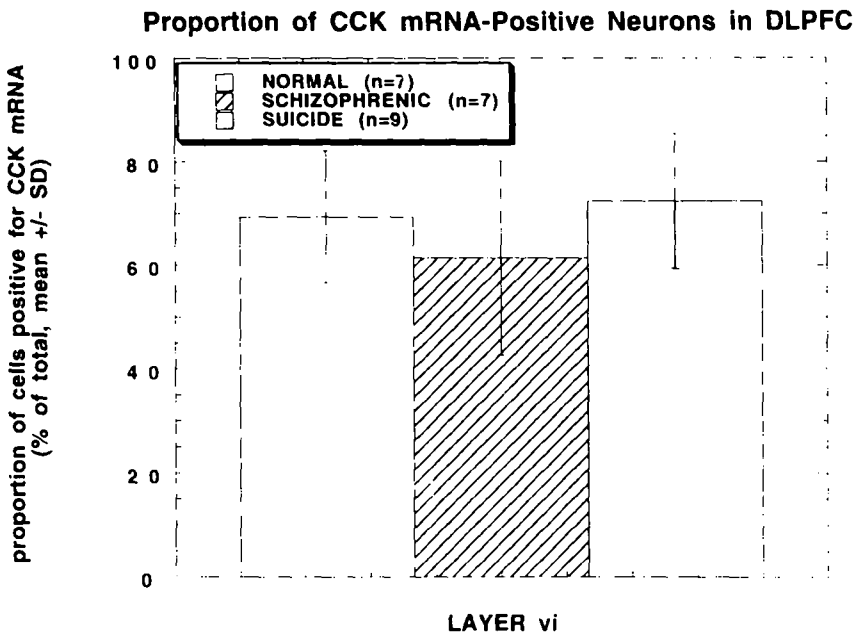

Figure 10. Comparison of proportion of CCK mRNA in positive cells (density ≥ 4 times background) among all cells sampled, in dorsolateral prefrontal cortex layer vi, between normals, schizophrenics and suicides.

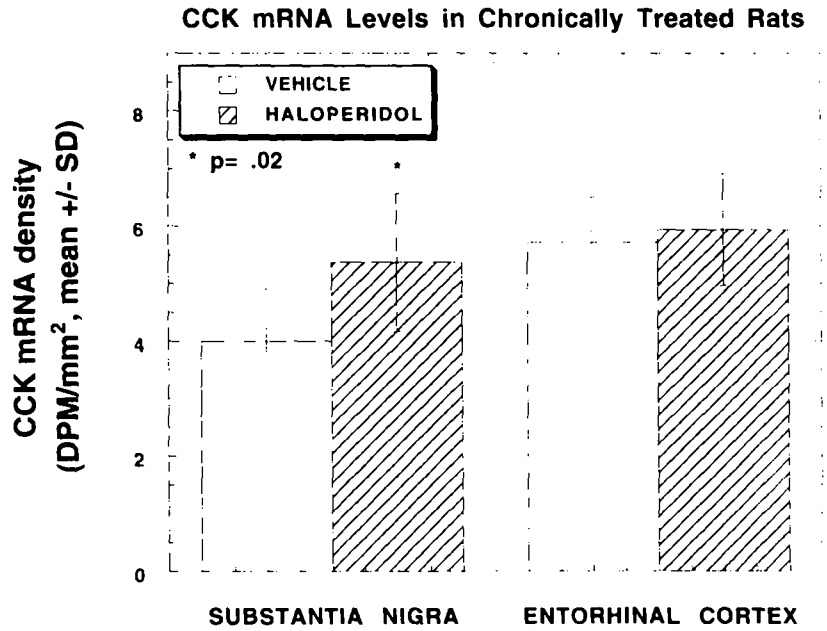

Figure 11. Comparison of CCK mRNA levels in substantia nigra and entorhinal cortex of rats treated chronically with haloperidol or vehicle.

GABA deficits in schizophrenics have been reported in frontal and temporal cortical glutamate decarboxylase (GAD) activity (Sherman et al., 1991a), GAD mRNA in DLPFC (Akbarian et al., 1995), temporal cortical synaptosomal GABA release (Sherman et al., 1991b), GABA uptake sites in amygdala and hippocampus (Simpson et al., 1989; Reynolds et al., 1990) and hippocampal and cortical benzodiazepine receptor binding (Squires et al., 1993). Receptor upregulation (which could be secondary to a presynaptic deficit) has also been reported in schizophrenics, for $GABA_A$ receptors in PFC and caudate (Hanada et al., 1987), and ACC (Benes et al., 1992a) and hippocampus (Vincent et al., 1995) and for cortical benzodiazepine receptors (Kiuchi et al., 1989).

Glutamate deficits have also been suggested in schizophrenia (see Deutsch et al., 1989; Olney & Farber, 1995 for reviews). There have been recent reports of reduced levels of glutamate and NAALADase, and increased levels of the glutamate antagonist NAAG, in PFC and hippocampus (Tsai et al., 1995), reduced temporal cortical synaptosomal glutamate release (Sherman et al., 1991b) and decreases in dendritic spines on glutamate cortico-cortical pyramidal neurons (Garey et al., 1995; Glantz & Lewis, 1995). Abnormalities in glutamate receptors in schizophrenics include those for mRNA for non-NMDA receptors in hippocampus, and AMPA-A and -β-B subunits of the AMPA receptor in hippocampus (Collinge & Curtis, 1991; Harrison et al., 1991; Eastwood et al., 1995). Binding to kainate receptors in hippocampus and the parahippocampal gyrus, DLPFC and orbitofrontal cortex (Kerwin et al., 1990; Nishikawa et al., 1983; Toru et al., 1988; Deakin et al., 1989), to NMDA receptors in putamen and the orbital gyrus (Matsunaga et al., 1995; Kornhuber et al., 1989), to AMPA receptors in sulcal ERC (Noga et al., 1994) and to glutamate uptake sites in orbitofrontal cortex (Deakin et al., 1989) have also been shown to be affected in schizophrenia.

One possible contribution of a cortical CCK deficit to abnormal cortical function might derive from its neurotrophic and neuroprotective properties (e.g. Roberts, 1990). CCK-positive subplate cells appear to serve a trophic function in the establishment of cortical connectivity (Chun et al., 1987). CCK has been shown to protect neurons against the neurotoxic effects of glutamate and NMDA (Akaike et al., 1991; Tamura et al., 1992). Alterations in primate limbic cortical CCK into adulthood have been suggested to play a role in late maturational aspects of limbic cortical circuitry (Oeth & Lewis, 1993).

A cortical CCK reduction in schizophrenia suggests that a CCK agonist might offer pharmacotherapeutic benefit in schizophrenia, a notion supported by a number of non-blinded trials with the CCK agonist ceruletide and CCK (Itoh et al., 1982; Moroji et al., 1982; Albus et al., 1984; Yamagami et al., 1986; Nair et al., 1982; Bloom et al., 1983), as well as single-blind and double-blind studies of ceruletide and CCK (van Ree et al., 1984; van Ree et al., 1987; Jenkins, 1984; Nair et al., 1984). Other studies, most of which were double-blind, have found CCK or ceruletide to be ineffective or of minimal efficacy (Peselow et al., 1987; Hommer et al., 1984; Mattes et al., 1985; Albus et al., 1986; Tamminga et al., 1986; Itoh et al., 1986; Lotstra et al., 1984; Boza & Rotondo, 1985). Possible explanations for these results include preferential transport of CCK-8 out of, rather than into, CSF (Passaro et al., 1982) and rapid enzymatic degradation of CCK-8 (Deschodt-Lanckman & Bui, 1981). Finally, there are not yet available agonists specific for CCK receptor subtypes. The advent of more specific CCK receptor agonists, or those that enter the brain more

readily, resist degradation, or have a preferential effect in the regions where the CCK cortical neurons affected in schizophrenia synapse, might yet prove to be beneficial for schizophrenics.

Cortical CCK deficits (e.g. Ferrier et al., 1983) may not involve midbrain neurons co-localizing dopamine and CCK (Hökfelt et al., 1980; Seroogy et al., 1989) as originally proposed. Indeed, it now appears that primate ventral tegmental area (Oeth & Lewis, 1992; Sirinathsinghji et al., 1992) and nigral (Palacios et al., 1989) dopamine neurons may not even contain CCK. While the levels were not quantified, Schalling et al. (1990) reported that there was more CCK mRNA in substantia nigra pars compacta in schizophrenics (visible signal in five of five) than in normals (signal in only two of five), an effect that could be secondary to neuroleptic treatment (see above). A more quantitative study found no difference in density of CCK mRNA in substantia nigra between drug-free schizophrenics and normal controls (J. Meador-Woodruff, personal communication). Still, a role for a cortical CCK deficit in schizophrenia is not only compatible with, but could provide a basis for dopamine hyperfunction in schizophrenia, via the cortico-striatal and accumbens projections.

Apparently contradictory reports of both facilitatory and antagonistic effects of CCK on dopamine function appear to depend upon a number of factors, including an inverted U dose-response function (e.g. Kádár et al., 1981), differences between striatum and nucleus accumbens (e.g. Vickroy & Bianchi, 1989), differences between rostral and caudal divisions (e.g. Studler et al., 1985 & Studler et al., 1986) and core and shell (e.g. Ladurelle et al., 1994) of nucleus accumbens, and differences between effects at type A and B CCK receptors (e.g. Marshall et al., 1991). Ultimately, what is most important is the effect of CCK on neurons that are post-synaptic to the dopamine neurons, especially since the bulk of CCK receptors in striatum are on intrinsic neurons, not afferents to striatum (Hays et al., 1981). Indeed, hippocampal and dopamine inputs have been shown to converge onto the same neurons in nucleus accumbens (Totterdell & Smith, 1986); moreover, those hippocampal neurons are CCK-receptive (Totterdell & Smith, 1989), thus may relay ERC CCK effects as well as their own. In addition, there are a series of excitatory glutamate links from ERC to accumbens that may relay effects of ERC CCK (Storm-Mathisen & Iversen, 1979; Walaas & Fonnum, 1979; Taxt & Storm-Mathisen, 1984). The "bottom line" is that CCK has excitatory effects on hippocampal pyramidal neurons (Dodd & Kelly, 1981) and cortical neurons (Delphs & Dichter, 1985) and consistently opposes dopamine's inhibitory effects on neurons in frontal cortex (Chiodo & Bunney, 1983) and nucleus accumbens (Yang & Mogenson, 1984; White & Wang, 1984; Wang & Hu, 1986; Yim & Mogenson, 1991; Liang et al., 1991). Thus, the functional consequence of hypofunction in corticostriatal CCK would be to duplicate the effects of mesolimbic dopamine hyperfunction, perhaps to some extent even when dopamine receptors are blocked by neuroleptics.

Since Virgo et al. (1995) noted that there was a greater reduction in CCK mRNA in frontal cortex of patients with predominantly reality distortion deficits, and that one patient with predominantly negative symptoms had a temporal but not frontal deficit, we explored the possibility that differences in symptomatology might explain this discrepancy. Our finding that patients without thought disorder had lower DLPFC CCK mRNA than those with thought disorder is in the opposite direction to the difference reported by Virgo et al.

(1995). Of course both our results and those of Virgo et al. (1995), must be interpreted with caution because of the very small number of patients studied.

The apparently greater magnitude of CCK mRNA reduction found by Virgo et al. (1995), (47-83%) compared to ours (20% in ERC layer vi) may be due to their use of optical density values relative to our use of a calibration curve, and/or their subtraction of RNase-insensitive levels from their samples. Still, one possible discrepancy between Virgo et al. (1995) and our study is our lack of a finding in schizophrenia in DLPFC. This could be because the PFC examined by Virgo et al. (1995), was Brodmann's area 10, whereas we examined DLPFC (Brodmann's area 9). There is other evidence that there may be some neuroanatomical specificity for this deficit. Some studies of schizophrenics have failed to find deficits in CCK levels in (nonspecified) cortex (Perry et al., 1981), hippocampus, nucleus accumbens and striatum (Kleinman et al., 1983) and "frontal" cortex (Ferrier et al., 1983; Ferrier et al., 1985). It should be noted with respect to these negative findings that the CCK measured in these regions includes that in intrinsic neurons (Adams & Fisher, 1990; Greenwood et al., 1981) and terminals of afferents from midbrain (Hökfelt et al., 1980). However, a recent study (Takahashi et al., 1995) has similarly failed to find changes in CCK mRNA at the level of film autoradiography resolution in a sample that included Brodmann's area 9, although they did report a difference at the cellular level, which we failed to find.

While we cannot exclude the possibility that low levels of ERC CCK mRNA are secondary to neuroleptic treatment in schizophrenics, the lack of an effect on ERC CCK mRNA in rats treated chronically with neuroleptics argues against that interpretation. Moreover, when chronic neuroleptics affected CCK mRNA in rats, they caused an increase in substantia nigra, not a decrease, as we found in schizophrenics in the ERC. The finding in rat substantia nigra is consistent with a report by Radke et al. (1989) that nigral levels of CCK were elevated after eight months of haloperidol treatment in rats. This effect appears to require chronic exposure, as a briefer regimen (19 days) did not affect rat nigral CCK mRNA (Cottingham et al., 1990). Levels of CCK in other regions (striatum, nucleus accumbens, olfactory tubercle) have been shown to be unaffected by chronic neuroleptics in rats (Gysling & Beinfeld, 1984). A transient reduction in rat cortical CCK levels after two weeks of neuroleptic treatment has been reported, but levels had normalized by four weeks of treatment (Frey, 1983). Nevertheless, future examination of ERC CCK mRNA in schizophrenics in comparison to neuroleptic-treated, non-schizophrenic controls should help to address this issue.

The intention of the inclusion of non-psychotic suicides in this study was to serve as a psychiatric control group for the schizophrenics, and the lack of an effect on ERC CCK mRNA in this group lends some specificity to the finding in ERC in schizophrenics. The finding of elevated DLPFC CCK mRNA in this group is consistent with other evidence that DLPFC function is abnormal in suicides. Elevations in binding to serotonin-2 and β-adrenergic receptors (Mann et al., 1986) and reduced serotonin reuptake sites (Arango et al., 1995) have been found in DLPFC in suicide victims. Reductions in both binding to serotonin-2 receptors and GAD activity after prefrontal cortical kainate lesions (Leysen et al., 1983) suggest that serotonin-2 receptors are located on GABA interneurons in PFC, which are found in cortical layers ii, iiia, iv and vi (Fagg & Foster, 1983; Jones & Hendry,

1986), and some of which, as noted above, contain CCK. Since serotonin appears to be excitatory at cortical serotonin-2 receptors (Sheldon & Aghajanian, 1991), upregulation of neuromodulators in those neurons might be a consequence of those receptor alterations. Alternatively, or in addition to alterations in DLPFC interneurons, pathology in the CCK-containing glutamate projection neurons in layers v and vi of DLPFC might be involved in the aberrant CCK mRNA in those layers in suicides.

In conclusion, our finding of reduced CCK mRNA levels in ERC and hippocampus in schizophrenia adds to the growing body of literature implicating a CCK deficit in the pathophysiology of schizophrenia. It also contributes to the shift in focus from the involvement of CCK co-localized with dopamine in midbrain neurons to CCK in cortical interneurons and/or efferent projections. There appears to be some neuroanatomical specificity to this reduction, as it was not found in ACC or DLPFC. Within the mesial temporal lobe the deficit was found in subiculum and in ERC layers iii–vi, and appears to be due to the reduced density of mRNA in CCK mRNA-positive neurons. These data also lend additional support to the increasing evidence implicating ERC and hippocampus in the neuropathology of schizophrenia. In so far as there is involvement of ERC layers iii–vi and subiculum in this deficit, it suggests that outflow from ERC and hippocampus, including projections to nucleus accumbens, ACC and DLPFC may be affected in schizophrenia, and that abnormalities in nucleus accumbens, ACC and DLPFC could be secondary to reduced glutamate innervation involving CCK-containing neurons. A companion study using adjacent sections (Wolf et al., 1995) has also found a decrease in neurotensin receptors in ERC layer ii, reflecting input to ERC, suggesting that the ERC in schizophrenics could be "isolated" by abnormal input and output. We are continuing to attempt to more specifically characterize this reduction, by studying a larger population, in a more extended rostral to caudal sampling of ERC levels, by including a non-schizophrenic neuroleptic-treated control group, by double-labelling for markers of both CCK and GABA or glutamate neurons, and by examining glutamate receptor function in the striatal, accumbens, hippocampal and cortical target sites of ERC efferents in schizophrenia. This knowledge may elucidate some of the pathophysiology of, and/or lead to new treatments for, schizophrenia.

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