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Analysis of expression of cholecystokinin in dopamine cells in the ventral mesencephalon of several species and in humans with schizophrenia

(neuropeptide/coexistence/substantia nigra/mental illness/neuroleptics)

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ABSTRACT **The ventral mesencephalons of hamster, guinea pig, cat, monkey, and several humans with and without the diagnosis of schizophrenia were analyzed with *in situ* hybridization and immunohistochemistry. Extensive codistribution of cholecystokinin mRNA and tyrosine hydroxylase [L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] mRNA was observed in cats and monkeys as well as in all five human subjects with the diagnosis of schizophrenia and in two out of five control brains. Double labeling revealed coexistence of the two markers in cat, monkey, and human. No cholecystokinin mRNA or cholecystokinin peptide was detected in the substantia nigra/ventral tegmental area of the hamster or guinea pig, even after acute and chronic neuroleptic treatment.**

The ventral mesencephalon (VMC) contains a dense population of dopaminergic neurons (1), which in humans have long been associated with disorders such as Parkinson's disease (2) and schizophrenia (3–5). The peptide cholecystokinin (CCK) (6), a putative transmitter/modulator widely distributed in the brain (see ref. 7), is found in many neurons in the VMC in the rat (8–16, 41). It coexists in the VMC with tyrosine hydroxylase [TyrOHase; tyrosine 3-monooxygenase, L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2], the rate-limiting enzyme in the catecholamine synthesis, showing that dopamine (DA) and CCK coexist (8, 9, 13, 16, 42) and may be released from the same nerve endings in certain forebrain areas. In fact, there are numerous studies in experimental animals showing different types of interactions between DA and CCK (see ref. 7).

Several lines of evidence suggest a role for DA in the pathogenesis of schizophrenia (see ref. 5), one being that the action of neuroleptic drugs seems to be mediated via DA-receptor blockade (17). Moreover, it has been pointed out that CCK exerts neuroleptic-like effects (18–21). It should therefore be of interest to analyze a possible role also for the coexisting peptide CCK in schizophrenia (22). In fact, several trials using CCK or its analogue, the decapeptide ceruletide (23), have been done; some open studies have reported symptomatic improvements, but several double-blind experiments failed to do so (24). Nevertheless, these studies raise the question whether or not CCK-like peptides are present in DA neurons in the human brain as well as in other species. So far only a preliminary note on the study of an infant brain with immunohistochemistry would support the notion of CCK-like immunoreactivity (LI) also in human DA neurons (8), whereas a recent *in situ* hybridization study, done on human brains from subjects without any known mental disease, raised the possibility of a species difference in that human substantia nigra DA neurons did not express CCK-LI (25).

In the present paper, ongoing research is summarized, which aims at establishing whether or not DA neurons in the VMC of several species including normal humans and patients with the diagnosis of schizophrenia contain a CCK-like peptide. We have used immunohistochemistry (26) with antisera raised to CCK octapeptide and to TyrOHase as a marker for DA neurons. In addition, *in situ* hybridization (27, 28) was employed by using synthetic oligonucleotide probes complementary to regions of CCK mRNA (29, 30) and TyrOHase mRNA (31, 32) that are highly conserved between human and rat. Using this strategy, we could successfully visualize CCK mRNA in species such as hamster, guinea pig, cat, and monkey in which CCK or TyrOHase have not yet been cloned. Some of the results of the present study have been reported in preliminary form (33, 34).

MATERIALS AND METHODS

Animals. Ten male hamsters and 10 male guinea pigs, 2 cats, 1 green monkey (New York University Medical Center), and 3 gray monkeys (*Macaca fasciculata*) (Statens Bakterio-Logiska Laboratorium, Stockholm) were used. Colchicine (Sigma) was given intraventricularly to 2 hamsters (60 μg in 20 μl of 0.9% NaCl), 2 guinea pigs (60 μg in 20 μl of 0.9% NaCl), 1 cat (200 μg in 200 μl of 0.9% NaCl; Lyon), and to the green monkey (500 μg in 500 μl of 0.9% NaCl; New York University Medical Center). Two guinea pigs were treated for 3 days with daily i.p. doses of haloperidol (Haldol) (total, 60 mg) and flupentixol (Fluanoxol) (total, 40 mg). In addition, one hamster and two guinea pigs were treated for 6 weeks with an oil-emulsion preparation of flupentixol (Fluanxol Depot). Guinea pigs were given 40 mg per 2 weeks, and hamsters were given 10 mg per 2 weeks.

Human Subjects. A total of 10 human VMCs was analyzed and divided into two groups: one with the diagnosis of schizophrenia and one with no such diagnosis. Groups were

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Abbreviations: CCK, cholecystokinin; DA, dopamine; LI, -like immunoreactivity; SNC, substantia nigra, zona compacta; TyrOHase, tyrosine hydroxylase; VMC, ventral mesencephalon; VTA, ventral tegmental area.

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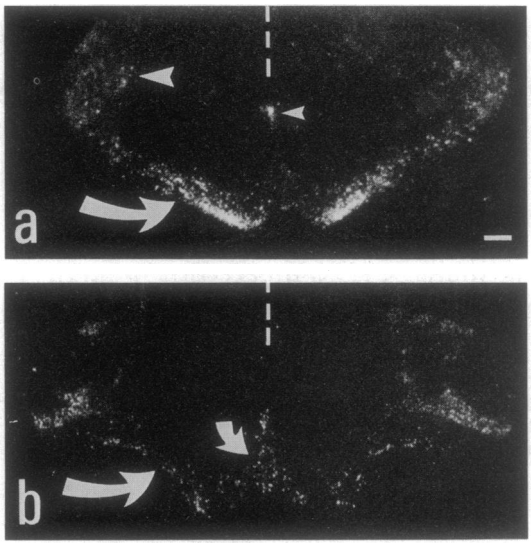

FIG. 1. Autoradiographs (film) of two levels (*a*, rostral; *b*, caudal) of VMC of cat showing CCK mRNA in SNC (big arrows) and in VTA (small arrow). Note signal in the medial geniculate body (big arrowhead) and in the periaqueductal central gray (small arrowhead). Dashed lines indicate midlines. (Bar = 1 mm.)

matched according to sex (three males and two females in each group), age (schizophrenics 36–83 years and controls 34–80 years), and postmortem time (schizophrenics 6.5–23.75 hr and controls 6–28.5 hr). Diagnosis of schizophrenia had been made in the United States (one case) and Germany (four cases). Control brains were from Germany and Belgium. All patients with the diagnosis of schizophrenia were treated with neuroleptic drugs for a substantial period of time; none of the controls received this type of drug.

Preparation of Probes. Oligonucleotide probes were synthesized on an Applied Biosystems DNA synthesizer and purified using Oligonucleotide Purification Cartridge (both Applied Biosystems). One oligonucleotide complementary to mRNA coding for amino acids 89–103 of human CCK (30), a region with 100% homology to the rat, one oligonucleotide complementary to amino acids 40–52 of human TyrOHase (32) with 98% homology to the rat, as well as one probe complementary to amino acids 1–16 of human TyrOHase were used. The purified oligonucleotides were labeled with deoxyadenosine [α-[^{35}S]thio]triphosphate at the 3′ end to a specific activity of 2–4 $\times$ 10^9 cpm/μg.

***In Situ* Hybridization.** The VMC was rapidly dissected out on frozen ice and cut at 14-μm thickness in a cryostat, thawed onto slides pretreated with poly-(L-lysine) at 100 μg/ml, and processed for *in situ* hybridization (28)—i.e., incubated at 42°C for 15–18 hr with 10^6 cpm of the labeled probe per 100 μl of a solution containing 50% (vol/vol) formamide/4× SSC (1× SSC is 0.15 M sodium chloride/0.015 sodium citrate, pH 7)/1× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/1% Sarcosyl/0.02 M $NaPO_4$, pH 7.0/10% (wt/vol) dextran sulfate/yeast tRNA at 250 μg/ml/salmon sperm DNA at 500 μg/ml/200 mM dithiothreitol, rinsed in 1× SSC at 55°C for 1 hr, dried, and exposed to Amersham Hyperfilm-βmax, developed, and fixed. Some sections were dipped in NTB2 nuclear track emulsion (Kodak), developed, fixed, and analyzed in a Nikon Mikrophot-FX microscope.

Immunohistochemistry. VMCs from formalin/picric acid perfusion-fixed cat, monkey, hamster, and guinea pig were cut in a cryostat and mounted onto chrom alum/gelatin-coated glass slides and processed directly for indirect immunofluorescence (26, 35). Sections were incubated with rabbit antisera to TyrOHase (36) or CCK (37) and/or a mouse monoclonal anti-CCK antibody (see ref. 35), rinsed, incubated with proper fluorescein isothiocyanate and/or tetramethylrhodamine isothiocyanate-conjugated secondary antibodies, examined in a Nikon Microphot-FX microscope, and photographed. To visualize both TyrOHase- and CCK-LI in the same section, an elution-restaining procedure was used in the monkey (38), whereas double labeling techniques were used in the cat (39). Some sections from human VMC were first processed for *in situ* hybridization (see above) and then for immunohistochemistry (see above).

RESULTS

In the *cat*, a strong CCK mRNA labeling was seen in the ventral tegmental area (VTA) and in the zona compacta of the substantia nigra (SNC) at all levels, and particularly dense

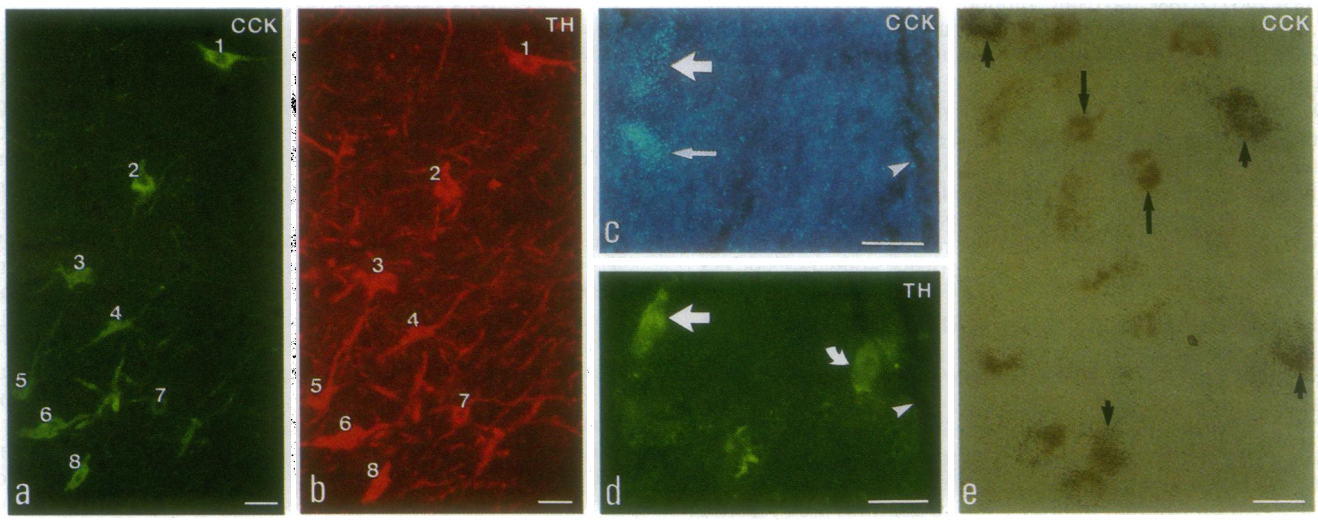

FIG. 2. Color micrographs (*a*, *b*, and *d*) and autoradiographs (*c* and *e*) of cat (*a* and *b*) and human VMC from a patient with diagnosis of schizophrenia (*c–e*), showing CCK-LI (*a*), TyrOHase-LI (TH) (*b* and *d*), and CCK mRNA (*c* and *e*). (*a* and *b*) All CCK-positive cells contain TyrOHase-LI (double staining of the same section of the medial SNC). (*c* and *d*) The same section from the medial VMC of human brain showing CCK mRNA (*c*) and TyrOHase-LI (*d*). One cell (thick arrow) contains CCK mRNA and TyrOHase-LI, a second cell (thin arrow) contains only CCK mRNA, and a third one (curved arrow) contains only TyrOHase-LI. Arrowheads point to the oculomotor nerve. (*e*) Numerous cells in the SNC exhibit both CCK mRNA and melanin pigment (short arrows). At least two pigmented cells (long arrows) without accumulation of grains can be seen. (Bar = 50 μm.)

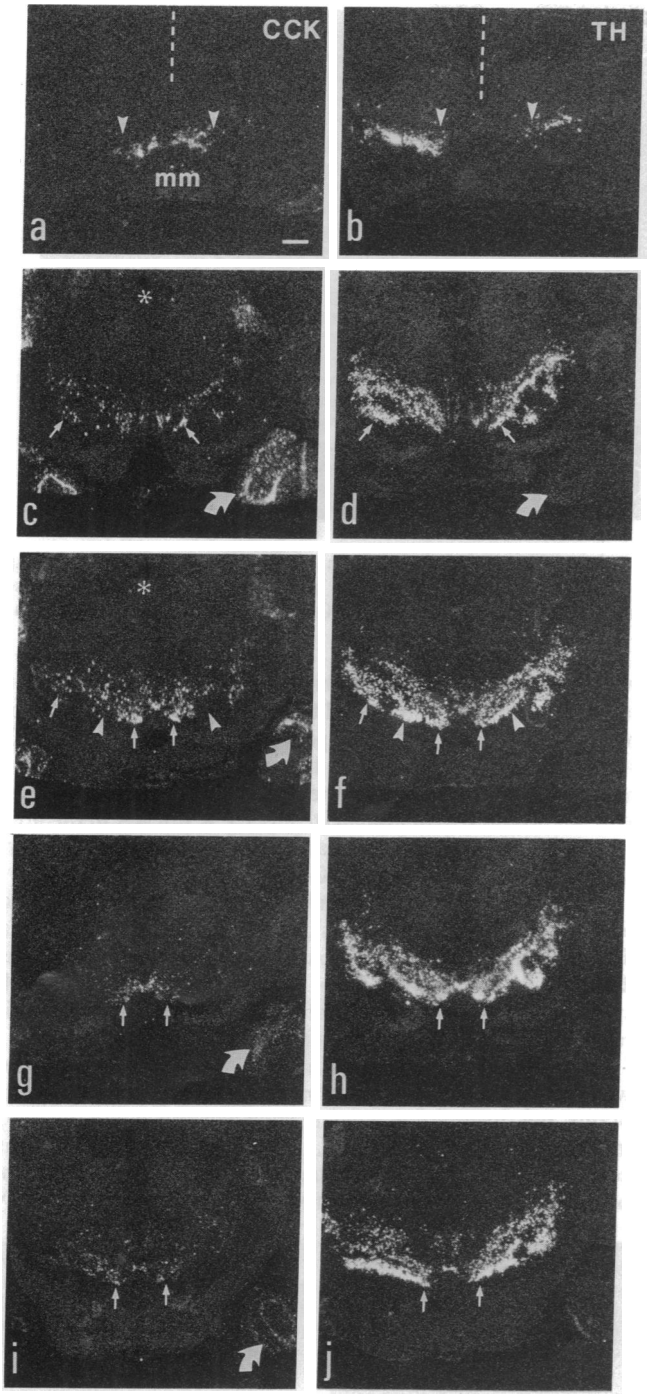

FIG. 3. Autoradiographs of pairs of adjacent sections from five different levels of the monkey VMC, showing CCK mRNA (*a*, *c*, *e*, *g*, and *i*) and TyrOHase (TH) mRNA (*b*, *d*, *f*, *h*, and *j*). CCK and TyrOHase messages overlap at all levels (arrows), except most rostrally (*a* and *b*), where CCK mRNA is in the midline area overlying the mammillary body (mm) (between arrowheads), whereas TyrOHase mRNA is found in the wings of the SNC (lateral to arrowheads) (*a* and *b*). Arrowheads in *e* and *f* point to areas containing TyrOHase mRNA without visible CCK signal. Curved arrows (*c*, *e*, *g*, and *i*) point to cortical area that contains CCK but not TyrOHase mRNA. Asterisks in *c* and *e* are just below aqueduct. (Bar = 1 mm.)

accumulations of cells appeared in the medial aspects (Fig. 1). Positive cells were also observed in the medial geniculate body (Fig. 1) and in the midline of the ventral periaqueductal gray (Fig. 1*a*). The hybridization signal for TyrOHase mRNA was very weak but exhibited a strong overlap in the SNC and

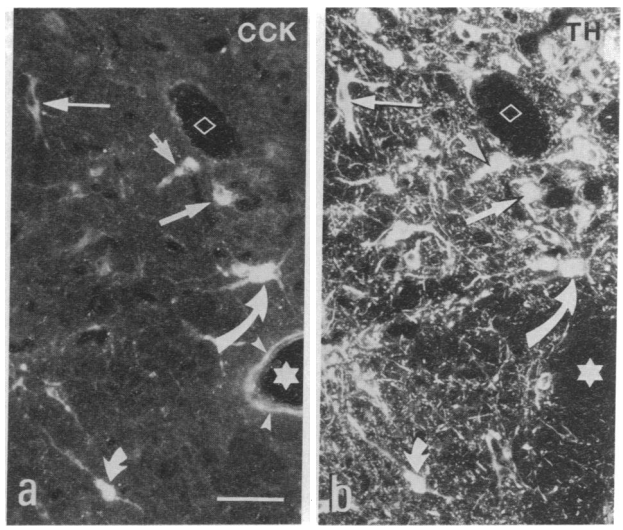

FIG. 4. Immunoflourescence micrographs of a section of the monkey VMC (midline area) (double staining) showing that virtually all CCK-positive cells are also TyrOHase (TH) immunoreactive (compare arrows and arrowheads). There are many more TyrOHase positive cells, however. Stars and open diamonds indicate blood vessels. Small arrowheads in *a* point to unspecific staining around a blood vessel. (Bar = 50 μm.)

VTA (data not shown). The immunohistochemical double-staining procedures revealed a high degree of coexistence between CCK- and TyrOHase-LI (Fig. 2 *a* and *b*).

In the *monkey* CCK mRNA-positive cells were observed throughout the VMC (Fig. 3 *a*, *c*, *e*, *g*, and *i*). At rostral levels the reaction seemed confined to the midline area overlying the mammillary body. Here no hybridization with the TyrOHase probe was seen; such hybridization, however, was strong in the SNC (Fig. 3*b*). At all other levels there was a good overlap between the two messages (Fig. 3 *c–j*). However, as compared with CCK mRNA, there were more and stronger labeled TyrOHase mRNA-positive cells. In addition, CKK mRNA was observed in adjacent cortical areas (Fig. 3 *c*, *e*, *g*, and *i*). Immunohistochemical double-staining experiments revealed moderate numbers of CCK-positive cells, especially in the midline areas, and most of these cells were TyrOHase positive (Fig. 4).

In the *human brain*, a consistent hybridization signal was seen with the CCK probe in all patients with the diagnosis of schizophrenia, and a particularly strong signal was observed in the youngest patient investigated (Figs. 2 *c* and *e* and 5*a*). Here CCK mRNA-positive cells could be seen in the entire SNC at the levels studied. In the age-matched control brain a strong CCK signal was seen only in the midline area of the VMC (the paranigral area). In the other control human brains a weak, but still distinct, CCK mRNA signal was observed in the SNC in two of four cases (data not shown). TyrOHase mRNA was strong and distinct in all human brains studied; there was no apparent difference between control and disease groups. No attempts were made to quantify the *in situ* hybridization reaction. At the cellular level most CCK mRNA-positive cells could be seen to also contain melanin pigment (Fig. 2*e*), although there were also pigment-containing cells without grain accumulations and CCK signal in cells without apparent pigment. In single cases it was possible to "stain" CCK mRNA autoradiographs with TyrOHase antibodies, revealing coexistence of CCK mRNA and TyrOHase-LI (cf. Fig. 2 *c* and *d*).

In the *guinea pig* (Fig. 6 *a* and *b*) and *hamster* (Fig. 6 *c* and *d*) no hybridization was observed with the CCK probe in the VMC (Fig. 6 *a* and *c*), not even after chronic or subchronic neuroleptic treatment. In contrast, a strong signal was seen

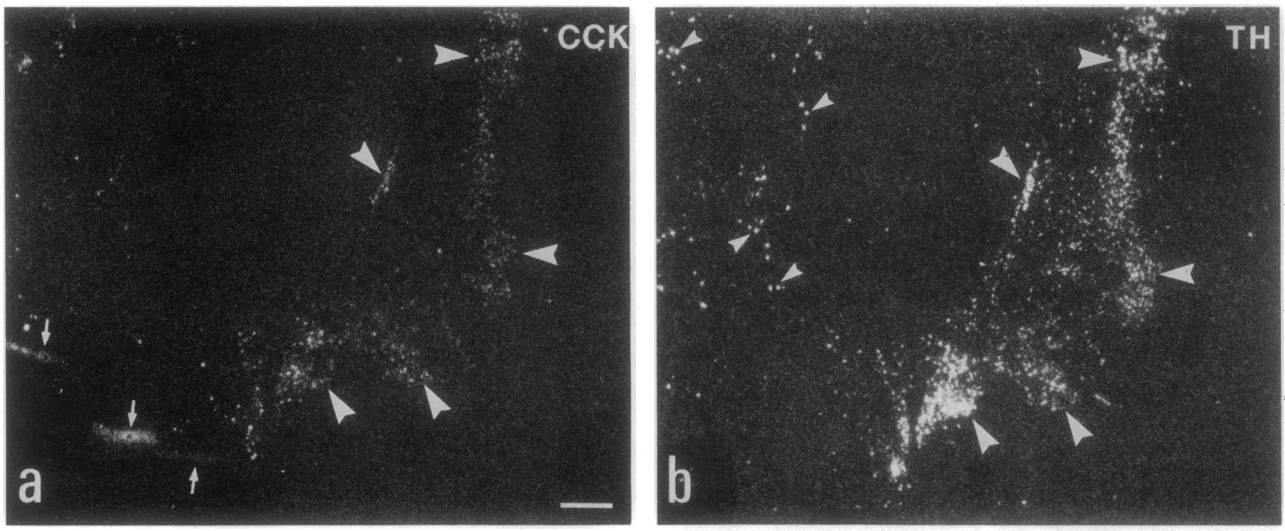

FIG. 5. Autoradiographs of two adjacent sections of human VMC of a patient with the diagnosis of schizophrenia, showing good overlap between the distribution of CCK mRNA (*a*) and TyrOHase (TH) mRNA (*b*) positive cells (big arrowheads) in the SNC. However, TyrOHase mRNA-positive cells are more numerous and more intensively labeled. Small arrowheads in *b* point to TyrOHase mRNA-positive cells in the midline area. Small arrows in *a* point to artefacts. (Bar = 1 mm.)

with the TyrOHase probe (Fig. 6 *b* and *d*) in SNC and VTA both of drug-treated and control animals. Cortex and hippocampus showed, however, a strong CCK mRNA signal in both species (Fig. 6 *a* and *c*). Immunohistochemical analysis of colchicine-treated hamster and guinea pig using CCK anti-serum did not stain any cells in SNC or VTA (data not shown).

DISCUSSION

Our results indicate that CCK can be expressed in DA neurons not only in rat (8–16, 41, 42) but also in cat, monkey, and human, suggesting that this type of coexistence occurs in the VMC of several species. However, we were unable to demonstrate this peptide or its mRNA in the corresponding neurons in the hamster and guinea pig, although CCK mRNA was found in great abundance in some other brain areas, strongly suggesting that our probe hybridizes with CCK mRNA of these two species. Also, in the immunohistochemical analysis of hamster and guinea pig the results were negative in spite of colchicine treatment, a drug known to arrest axonal transport and to increase cell body levels of many compounds, including peptides such as CCK (9, 35). After chronic or subchronic treatment of guinea pigs and hamsters with large doses of neuroleptics (see below), it was also not possible to demonstrate any CCK mRNA in the area where DA cell bodies are located. Thus, true species difference with regard to DA–CCK coexistence may exist.

In two other species, cat and monkey, CCK mRNA could be demonstrated in numerous cell bodies in the VMC with a distinct topographical distribution; to a large extent, this distribution overlapped with TyrOHase mRNA. In both species, immunohistochemical double-staining provided direct evidence for colocalization of CCK and TyrOHase.

In human, in agreement with Palacios and coworkers (25), we encountered CCK mRNA in the paranigral area in normal brains, but, in addition, a clear signal was present in the SNC in only two of five controls. However, CCK mRNA was consistently seen in the substantia nigra area of all brains of patients with the diagnosis of schizophrenia. A particularly

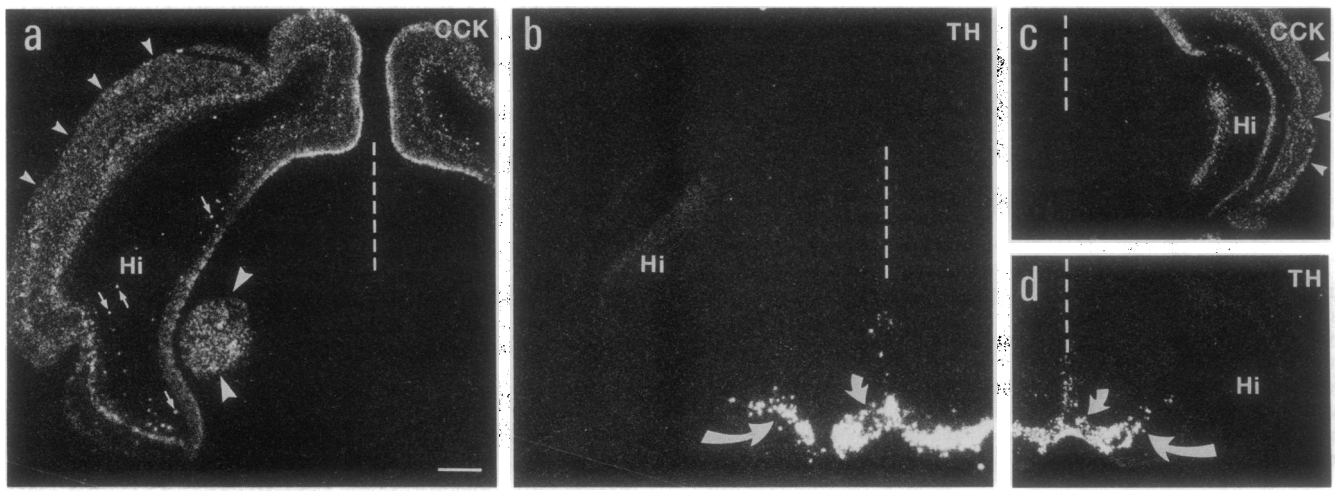

FIG. 6. Autoradiographs of frontal sections of guinea pig (*a* and *b*) and hamster (*c* and *d*) mesencephalon and cortex. A strong TyrOHase (TH) mRNA message can be seen in SNC (big arrows) and VTA (small arrows) of both guinea pig (*b*) and hamster (*d*), but no corresponding signal is seen for CCK mRNA (*a* and *c*). Note strong message for CCK in cortex (arrowheads) and hippocampus (Hi) of both species (*a* and *c*). Small arrows in *a* point to single CCK mRNA-positive cells in the hippocampus. Arrowheads in *a* point to medial geniculate body; dashed lines indicate midlines. (Bar = 1 mm.)

strong and distinct reaction was observed in the brain of a young, chronic schizophrenic male, where the entire zona compacta was labeled with the CCK probe. However, the signal was always weaker than that for TyrOHase mRNA and seemed to be expressed in fewer cells. The presence of CCK mRNA in DA cells in this brain could be established in two ways: by CCK *in situ* hybridization combined with TyrOHase immunohistochemistry and by the well-known fact that the human nigral DA neurons contain melanin pigment. Whereas the combination of immunohistochemistry and *in situ* hybridization (cf. ref. 15) was capricious and successful only in single cases, the association of CCK mRNA autoradiographic grains with pigment could be easily established in numerous cell bodies.

In summary, human nigral DA neurons have the capacity to express CCK mRNA, and perhaps CCK peptide, especially in schizophrenic patients who have been treated with neuroleptic drugs and, to a lesser extent, also in human brains without this diagnosis. Presence of CCK in human nigral neurons would be compatible with the reported decrease in CCK levels in the substantia nigra of parkinsonian brains (40). It is an interesting question whether or not neuroleptic treatment *per se* increases CCK mRNA levels and will need to be answered by studying patients receiving neuroleptic treatment for disorders other than psychosis. It is therefore premature to speculate about the significance of CCK mRNA in DA neurons in relation to schizophrenia. A more detailed analysis of levels of CCK mRNA (and CCK peptide), the exact localization of CCK mRNA-positive cells within the VMC of the human brain, and their relation to DA neurons, especially in the paranigral area both in normal and in diseased brains are first needed.

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