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## Co-localization of corticotropin-releasing hormone with glutamate decarboxylase and calcium-binding proteins in infant rat neocortical interneurons

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### Abstract

Corticotropin releasing hormone (CRH) has been localized to interneurons of the mammalian cerebral cortex, but these neurons have not been fully characterized. The present study determined the extent of colocalization of CRH with glutamate decarboxylase (GAD) and calcium-binding proteins in the infant rat neocortex using immunocytochemistry. CRH-immunoreactive (ir) neurons were classified into two major groups. The first group was larger and consisted of densely CRH-immunostained small bipolar cells, predominantly localized to layers II and III. The second group of CRH-ir cells was lightly labeled and included multipolar neurons mainly found in deep cortical layers. Co-localization studies indicated that the vast majority of CRH-ir neurons, including both bipolar and multipolar types, was co-immunolabeled for GAD-65 and GAD-67. Most multipolar, but only some bipolar, CRH-ir neurons also contained parvalbumin, while CRH-ir neurons rarely contained calbindin or calretinin. These results indicate that virtually all CRH-ir neurons in the rat cerebral cortex are GABA-ergic. Furthermore, since parvalbumin is expressed by cortical basket and chandelier cells, the colocalization of CRH and parvalbumin suggests that some cortical CRH-ir neurons may belong to these two cell types.

### Keywords

Neuropeptides; Parvalbumin; GABA; Cerebral cortex; Basket cells; Chandelier cells

### Introduction

Corticotropin-releasing hormone (CRH)-containing neurons in the adult rodent and primate cerebral cortex have been studied with immunocytochemical methods and identified as nonpyramidal cells based on morphological criteria (Merchanthaler 1984; Sakanaka et al. 1987; Lewis et al. 1989; Lewis and Lund 1990). Most commonly, CRH-immunoreactive (ir) cells are located in the supragranular layers and have a small, vertically oriented oval soma with bipolar dendrites. The features of this CRH-containing neuron are similar to those for cortical bipolar cells (Peters 1984). Another reported type of CRH-ir neuron is a round or triangular-shaped soma in the primate neocortex (Lewis et al. 1989, Lewis and Lund 1990). This latter group of CRH-ir neurons was found in layers II and IV across all neocortical areas, but with regional differences in frequency. In several neocortical association areas, many CRH-ir axon cartridges were found below the somata of pyramidal cells, and it has

been suggested that these CRH-ir multipolar cells represented chandelier cells (Lewis et al. 1989).

Most cortical interneurons are GABAergic, but these cells show a considerable heterogeneity in their morphology, circuitry, and neurochemical characteristics (Feldman and Peters 1978; Ribak 1978; DeFelipe et al. 1985, 1986; Seguela et al. 1985; Yan et al. 1992; DeFelipe 1993). Calcium-binding proteins, such as calbindin, parvalbumin, and calretinin, are excellent markers for defining subpopulations of cortical GABAergic neurons (Celio 1986; 1990; DeFelipe et al. 1989; Hendry et al. 1989; Hendry and Jones 1991; DeFelipe and Jones 1992; Roger 1992; DeFelipe 1993; Cao et al. 1996; Yan et al. 1996; Gabbott et al. 1997; Miettinen et al. 1997). For example, calbindin and calretinin are found in many bipolar and some multipolar cells, whereas parvalbumin is expressed mostly by multipolar neurons with varied sizes, including basket and chandelier cells (Celio 1986, 1990; DeFelipe et al. 1989; Williams et al. 1992). To better characterize the type of interneuron containing CRH in the mammalian cerebral cortex, we examined the morphology and co-localization of CRH with two isoforms of glutamic-acid decarboxylase (GAD), GAD-65 and GAD-67, and three calcium-binding proteins, calbindin, calretinin, and parvalbumin, in the rat neocortex. Immature rats in the second postnatal week were studied, because this age group shows the greatest expression of CRH receptor-1 (Avishai-Eliner et al. 1996) and, perhaps, of CRH itself (Yan et al. 1998).

## Materials and methods

### Animals and tissue preparation

Rats ( $n=30$ ) were offspring of timed-pregnancy Sprague-Dawley-derived dams (Zivic-Miller, Zelienople, Penn., USA). Dams ( $n=6$ ) were caged individually in NIH-approved animal facilities and kept on a 12-h light/dark cycle with unlimited access to lab chow and water. Delivery was verified at 12-h intervals and the date of birth was considered day 0 (Yi and Baram 1994). Twenty-four 10- to 14-day-old pups were perfused under relatively stress-free conditions, consisting of at least 24 h without any disturbance, and the pups were deeply anesthetized using pentobarbital (100 mg/kg, intraperitoneally), within 45 s of initial handling. This precaution was taken to avoid potential stress-induced secretion of CRH from cortical neurons prior to perfusion (Baram et al. 1997; Yan et al. 1998). Anesthetized pups ( $n=24$ ) were then brought to the laboratory for perfusion, including a vascular saline rinse followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Six young adult rats (2-months-old) were also perfused.

Brains were left in situ overnight at 4°C and dissected from the skull. They were post-fixed in the perfusion solution for 2 h, transferred to PBS, and immersed in 30% cold sucrose for cryoprotection. The cerebrum was cut coronally at 40  $\mu$ m with a cryostat; alternate sections were collected in tissue-culture wells in cold PBS and subjected to immunocytochemistry. The other sections were stained with cresyl violet for orientation purposes.

### CRH immunocytochemistry

Free-floating sections were immunostained for CRH using an avidin-biotin (ABC) method as described elsewhere (Yan et al. 1998). Briefly, following several washes with PBS, sections were treated with 0.05% hydrogen peroxide for quenching endogenous peroxidase activity, then preincubated in PBS, containing 5% normal goat serum and 1.5% bovine serum albumin, to block nonspecific reactivity (PBS+). Sections were further incubated in a solution of rabbit CRH antibody (final dilution 1:9000) in PBS+ containing 0.3% Triton-X-100 overnight at room temperature. After several rinses with PBS, sections were transferred to a solution containing 1% biotinylated universal IgG with Triton-X-100 for 1 h

at room temperature followed by 1-h incubation in 1% ABC solution (Vectastain, Vector Laboratories, Burlingame, Calif., USA). The immunoreaction product was visualized using 0.005% hydrogen peroxide and 0.05% 3,3-diaminobenzidine (DAB). The rabbit antiserum directed against rat/human CRH, a generous gift from Dr. W. W. Vale (Salk Inst., La Jolla, Calif., USA), does not cross-react with other neuropeptides, such as somatostatin, neuropeptide Y, endorphin, enkephalin, vasoactive intestinal polypeptide (VIP), or vasopressin (Vale et al. 1983).

### Double-labeling procedures

A modification of a previously described dual-chromogen procedure (Levey et al. 1986) was used for concurrent immunolabeling of CRH and other markers (Yan et al. 1998). Briefly, sections were first immunostained for CRH as described above, yielding a homogenous brown reaction product within the cytoplasm. Following this reaction for the localization of CRH, sections were washed with PBS, preincubated in buffer with 5% normal goat (for GAD-67 and calretinin) or horse (for GAD-65, calbindin, parvalbumin) sera for 1 h, after which the sections were incubated overnight with agitation at 4°C in a PBS solution containing 5% of the appropriate serum and one of the following primary antibodies. A monoclonal mouse antibody against GAD-65 (Boehringer Mannheim, Indianapolis, Ind., USA) was diluted at 1:2000, and a polyclonal rabbit anti-GAD-67 serum (Chemicon Inc., Temecula, Calif., USA) was diluted at 1:5000. Monoclonal mouse antisera directed against parvalbumin and calbindin were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) and were both diluted at 1:8000. The polyclonal rabbit anti-calretinin serum (Chemicon, Temecula, Calif., USA) was diluted at 1:5000. Following incubation with the primary antibodies, sections were washed and incubated with 1% goat anti-rabbit (for GAD-67 and calretinin) or horse anti-mouse (for the other markers) IgGs for 1 h, followed by 1% ABC solution for another hour. After this step, sections were processed through several changes of 0.02 M phosphate buffer, pH 6.5, to lower the pH and ionic strength. This process permitted visualization of immunoreaction products of the second group of primary antibodies using benzidine dihydrochloride (BDHC), leading to the formation of granular blue-black deposits.

### Morphometric analysis

To verify the extent of co-localization of CRH with each of the other neurochemicals in individual cortical neurons, we counted neurons in sections that had been immunostained for two markers. However, in order to exclude the possibility that the DAB deposits for the first marker might be masked by a heavy BDHC reaction for the second marker (particularly in the case of CRH and calretinin double immunostaining), we initially used an additional control method. After visualization of the first antigen, selected sections were mounted on slides and coverslipped with phosphate buffer. Immunoreactive somata (DAB reaction) and landmarks, such as blood vessels, were plotted with the aid of a drawing tube. Following labeling for the second antigen, the same sections were used to re-plot the cells demonstrated by BDHC on the original maps. The profiles showing an overlap of the two chromogens were considered to be double-labeled neurons. Among the markers examined in the present study, no difference was found between the data obtained through the two-counting methods in regard to the proportion of double-labeled cells. Cell counts were performed in two sections at 4–5 mm behind bregma (see Paxinos and Watson 1986) from each of three animals and within the cortical region that covered the occipital, parietal, and temporal areas using a light microscope equipped with a reticule grid. The sections were scanned vertically across the entire cortical depth using 20–100× primary objectives, and CRH-ir neurons were examined and recorded based on their morphology and staining features (single or double labeled). The percentage of CRH-ir neurons that were co-labeled for each marker was determined, without attempting to obtain their numbers per unit area.

## Results

### Distribution and morphology of CRH-ir neurons

Sections immunolabeled for CRH were examined throughout the immature neocortex, including the frontal, parietal, temporal, occipital, and cingulate regions. CRH-ir neurons were found throughout the depth of the cortex in all of the examined areas. The laminar distribution pattern and frequency of CRH-ir neurons were similar for these cortical areas. Thus, CRH-ir neurons were mainly located in layers II/III (Fig. 1A, B), with smaller numbers in the other cortical layers (Fig. 1A, C, D). No CRH-ir cells were found in the white matter (Fig. 1A, D).

Based on morphological features and staining intensity, CRH-ir neurons were classified into two major groups. The first group had small (major somal diameter less than 15  $\mu\text{m}$ ), oval or fusiform somata, which were strongly immunostained for CRH. The appearance of ascending and descending dendrites that originated at the somal poles and oriented perpendicular to the pial surface indicated that they were either bipolar or bitufted cells (Feldman and Peters 1978; Peters 1984). These dendrites were aspiny and gave rise to only a few branches 100–500  $\mu\text{m}$  from the soma. These branches were oriented roughly parallel to the main dendrites (Fig. 1E). The ascending dendrites were directed toward the pial surface and branched profusely in layer I (Fig. 1B). The descending dendrites transversed several cortical layers, occasionally reaching the white matter (Fig. 1E). This CRH-ir cell type was found in all cortical layers, but was most frequent in the supragranular layers (Fig. 1B, C, E).

The second group of CRH-ir cells had a similar or slightly larger cell body with a diameter of 15–20  $\mu\text{m}$  (Fig. 1F–H). The cell bodies were round, triangular, or multipolar, and gave rise to more than two dendrites. In many cases, these cells were immunostained more lightly than the cells in the first group (Figs. 1D, 2F–H). The number of the multipolar cells comprised less than 20% of the total population of CRH-ir cells in the neocortex, according to a count of more than 1500 CRH-ir somata (Table 1). These multipolar cells were more frequently encountered in layers V and VIa and were sparse in the supragranular layers (Fig. 1B–H). The dendrites of these cells were relatively short, lacked spines, and did not exhibit a preferential orientation.

CRH-ir processes were detected throughout the cortical mantle, including layer I and the white matter, though their frequency varied. CRH-ir processes were most common in layers I and II and consisted of the dendritic branches of immunolabeled somata in layers II and III (Fig. 1B). Layers V and VIa, b contained sparse labeled processes, some of which were found to be branches of dendrites originating from labeled cell bodies in layers II–IV. The cartridge-like axonal profiles of chandelier cells, previously described for CRH-ir axons in the monkey neocortex (Lewis et al. 1989; Lewis and Lund 1990), were not observed in any cortical regions of the young rats. Also, basket-cell axon terminals, which are found apposed to adult pyramidal cell bodies, were not immunolabeled for CRH in the immature rat neocortex.

CRH-ir neurons in the neocortex of adult rats were not as numerous as those in immature rats. Particularly, the multipolar neurons noted in the deep cortical layers of the immature rat were almost undetectable in the adult. Therefore, an age-related difference may exist for both the frequency and distribution pattern of CRH-ir neurons.

### Co-localization of CRH with GAD and calcium-binding proteins

Since the immature “stress-free” rat brain showed greater numbers of CRH-ir neurons, the co-localization study was only conducted for stress-free pups. The co-localization of CRH with GAD in a single neuron was easily recognized due to the simultaneous presence of a

brown, homogeneous DAB reaction product for CRH and blue granular deposits for other markers (Fig. 2A–C). The great majority of CRH-ir cells was also immunostained with GAD-67, and a smaller percentage co-localized GAD-65. In both cases, bipolar CRH-ir cells were more likely to contain GAD than multipolar CRH-ir cells (Table 1).

The co-localization analysis of CRH and parvalbumin demonstrated a different pattern from that of CRH and the GABA-synthesizing enzyme (Fig. 2D–F). Multipolar CRH-ir cells were frequently double-labeled for parvalbumin. The labeling of dendrites with CRH was variable. In extreme cases, it led to the bipolar identification of cells that were actually multipolar, as revealed with parvalbumin immunostaining (Fig. 2F). In general, CRH-ir bipolar cells in layers II–IV were not parvalbumin-ir (Fig. 2D, E; Table 1). The quantitative analysis of more than 300 CRH-ir cells showed that 90% of the multipolar CRH-ir cells and 10% of the bipolar CRH-ir cells were immunolabeled for parvalbumin (Table 1; Fig. 2D–F).

In sections processed for the colocalization of CRH and calbindin, cells immunoreactive for only calbindin were found in all layers of the cortex (Fig. 2G, H). Those in layers II–IV were more numerous and smaller than those in deeper cortical layers (Fig. 2G). They often had small bipolar cell bodies, similar to those of CRH-ir neurons. Other calbindin-ir cells in the deeper layers were multipolar, resembling CRH-ir multipolar cells. However, of the 264 CRH-ir bipolar and multipolar cells counted in this analysis, only a few were immunostained for calbindin (Fig. 2G, H; Table 1).

Calretinin-ir cells were found in all cortical layers and in the white matter. Their morphology was variable, as previously described for the rat cerebral cortex (Gabbott et al. 1997; Miettinen et al. 1997). In particular, calretinin-ir bipolar cells were a major cell type, located mainly in the supragranular layers with a peak distribution in layer III (Fig. 2I). Large multipolar neurons were also immunolabeled for calretinin, most frequently in layers III–V. However, based on 252 analyzed cells, neither the bipolar nor the multipolar CRH-ir neurons were immunolabeled for calretinin (Fig. 2I–K; Table 1).

## Discussion

### Technical considerations

This study demonstrated abundant CRH-ir interneurons in the immature rat neocortex compared with previous studies and our own data indicating only a limited number of CRH-ir cells in adult rats (Olschowka et al. 1982; Merchenthaler 1984; Sakanaka et al. 1987). Several reasons may account for the discrepancy in the prevalence and distribution of CRH-ir neurons in the rodent's neocortex at different ages and in different experiments. For example, even with the use of an axonal transport blocker, colchicine, the number of neocortical CRH-ir neurons described in previous studies was lower than that in the present study (Olschowka et al. 1982; Sakanaka et al. 1987). It is possible that the efficacy of our CRH antibody is one of the factors that may account for the difference between experiments. The CRH antiserum used in this study has been shown to be exceptionally potent and specific for CRH (Vale et al. 1983), enhancing signal-noise ratio and increasing sensitivity. The age of the rat may also be a factor (see above). However, an important factor that may have contributed to the improved CRH-immunoreactivity in the current study may be the attention paid to preventing the potential stress-induced release of CRH from somata prior to perfusion.

CRH is a stress hormone, whose levels in several brain regions are reported to be altered by stress, such as handling (Kalin et al. 1994; Hatafski et al. 1998). In fact, secretion of CRH from CRH-ir neurons in the hypothalamus occurs within minutes after the onset of a stressful stimulus in both adult (Rivier et al. 1983; Lightman and Harbuz 1993; Herman and



Cullinan 1997) and immature rats (Yi and Baram 1994). Rapid release of CRH after handling/injection stress has been demonstrated in the hypothalamus (Yi and Baram 1994). Thus, stress-induced release of CRH from cortical neurons, or axonal transport away from the soma, may diminish the intensity of a CRH-ir signal. Therefore, using rats free of disturbance and handling stress may account for the relatively high abundance and strong signal of CRH-ir cells in this study.

### **Cortical CRH-ir cells are GABAergic interneurons**

In the present study, the cell bodies and to various extents the dendritic arbors of CRH-ir cortical neurons were immunolabeled. This staining facilitated the classification of these neurons into two groups. The first group, bipolar cells, had dendrites that were labeled for considerably long distances and were aspiny. These morphological characteristics indicate that these CRH-containing cells are non-pyramidal neurons (Feldman and Peters 1978). The second group, multipolar cells, had mainly proximal dendritic immunolabeling. These dendrites were radially oriented and were aspiny, suggestive of an origin from local circuit neurons. Thus, all CRH-ir cells in the rat cerebral cortex are non-pyramidal cells.

Additional data from double-labeling immunocytochemistry revealed that the vast majority of CRH-ir neurons were immunostained for one of the GABA-synthesizing enzyme isoforms, GAD-65 or GAD-67. This fact, coupled with the morphological features discussed above, suggests that CRH-containing neurons of the rat neocortex represent a subpopulation of GABAergic interneurons.

A single previous study in the adult cat visual cortex reported that GAD-ir and calbindin-ir neurons did not contain CRH (Demeulemeester et al. 1988). However, the discrepancy in the GAD/CRH co-localization data between their study and the present one may be due to either technical aspects, such as the specificity and sensitivity of the antibodies, or a species difference.

### **Cortical CRH-ir cells represent multiple interneuron types**

Several interneuron populations have been defined in the cerebral cortex based on cell morphology, dendritic-arbor orientation, and postsynaptic targets (Feldman and Peters 1978; Seguela et al. 1985). The major class of neocortical CRH-ir neurons is bipolar, including bitufted neurons, whose primary dendrites branch soon after they leave the soma. Co-localization data of the present study clearly indicate that only a subpopulation of bipolar cells expresses CRH. In particular, CRH-ir bipolar neurons are virtually all small, whereas calretinin-ir bipolar cells are relatively large, as revealed in the same double-labeled sections. Thus, the present study provides additional data indicating a neurochemical heterogeneity of cortical bipolar cells. It needs to be noted that cortical neurons expressing other neuropeptides, particularly VIP, are often bipolar (Sims et al. 1980; Connor and Peters 1984; Morrison 1988; Bayraktar et al. 1997), and whether CRH is colocalized with these neurochemicals remains to be determined.

Our results also show that CRH-ir neurons in the rat neocortex are not confined to the bipolar type. For example, GABAergic basket cells are known to form synapses with the somata and proximal dendrites of principal neurons, and GABAergic chandelier cells form synapses with axon initial segments of principal neurons (Peters et al. 1982; Ribak and Seress 1983). Both types of these interneurons are characteristically labeled for parvalbumin (DeFelipe et al. 1989; Ribak et al. 1990; Hendry et al. 1989; Williams et al. 1992; Cao et al. 1996; Gabbott et al. 1997). In the current study, a large percentage of multipolar CRH-ir neurons (91.5%) was also immunostained for parvalbumin. This finding suggests that the CRH and parvalbumin co-expressing neurons might be basket and chandelier cells. Our

recent study in the immature rat hippocampal formation confirmed the presence of both basket and chandelier CRH-ir cells in this region at the light and electron microscopic levels (Yan et al. 1998). Two previous studies in the monkey neocortex also indicated that cortical chandelier cells may contain CRH (Lewis et al. 1989; Lewis and Lund 1990). It should be noted that, in the present investigation, the characteristic structures formed by axon terminals of basket and chandelier neurons (the basket and cartridge formations apposed to pyramidal somata and their axon initial segments, respectively) were not observed in the immature rat neocortex. The reasons for a lack of these immunolabeled terminal boutons could be due to a developmental immaturity of, and/or a low level of CRH in, axon terminals.

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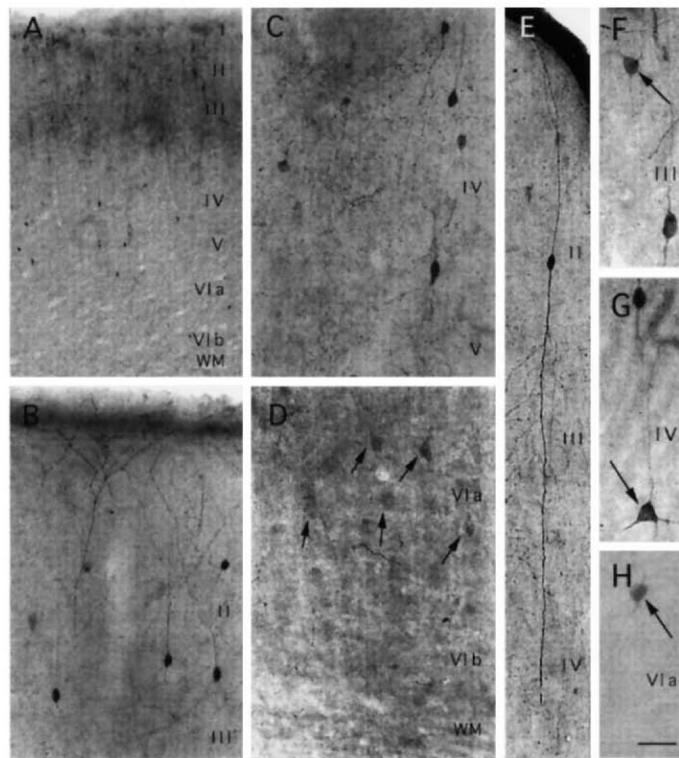
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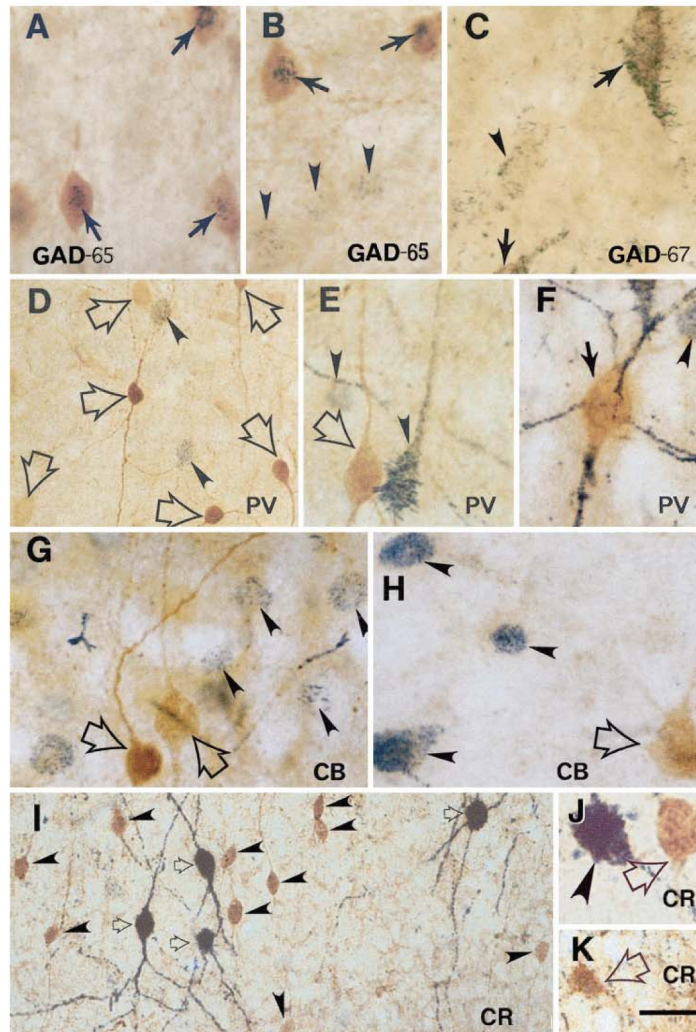
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**Figure 1.**

Fig. 1A–H Microphotographs of the parietal cortex of a 13-day-old rat showing the laminar distribution and morphology of CRH-immunoreactive (*-ir*) cell bodies and processes. **A** CRH-ir cells and processes were located predominantly in layers II/III and less frequently in layers IV and V. Layers I and VIa, b contained a few labeled somata, and no cells were found in the white matter (WM).

**B–D** High-magnification photographs showing the detailed distribution of CRH-ir somata and processes in different cortical layers. Note that labeled somata in layers II–IV were often densely stained (**B, C**), whereas those (*short arrows*) in layers V and VIa were more lightly labeled (**D**). **E** A typical layer-II bipolar cell and its ascending and descending dendrites. **F–H** Morphological details of CRH-ir multipolar cells (*long arrows*) in different cortical layers. Those in layers II–IV (**F, G**) were more darkly labeled than those in the deeper layers (**H**). *Scale bar* 100  $\mu\text{m}$  for **A**, 50  $\mu\text{m}$  for **B–E**, and 20  $\mu\text{m}$  for **F–H**



**Figure 2.**

Fig. 2A–K Co-localization of CRH with other interneuronal markers in the neocortex of an 11-day-old rat. CRH-immunoreactivity (CRH-ir) appeared orange, while that for glutamic-acid decarboxylase (*GAD*) -65 (A, B), *GAD*-67 (C), parvalbumin (*PV*) (D–F), calbindin (*CB*) (G, H), and calretinin (*CR*) appeared as blue, granular deposits. A–C Neurons (arrows) containing both CRH and *GAD* in layers II, III, and IV, respectively. D, E Parvalbumin-ir was not detected in most bipolar CRH-ir neurons, while, in F, multipolar cells contained parvalbumin. CRH and calbindin rarely co-localize in either bipolar (G) or multipolar (H) neurons. CRH and calretinin were not co-localized in either bipolar (I, J) or multipolar (K) cells. Double-labeled neurons are indicated with *solid arrows*. Single labeled CRH-ir cells are indicated with *open arrows*. Neurons labeled for *GAD* or calcium-binding proteins, but not for CRH, are indicated with *arrowheads*. Scale bar 50  $\mu\text{m}$  for D, I, 20  $\mu\text{m}$  for K, and 10  $\mu\text{m}$  for the other panels

**Table 1**

Quantitative analysis of morphological classification and neurochemical co-localization of CRH-immunoreactive neurons in the immature rat neocortex

Co-expressed neurochemical marker	Bipolar cells	Multipolar cells	Total
GAD-65	272 in 291 (93.5%)	66 in 73 (90.4%)	338/364(92.9%)
GAD-67	308 in 313 (98.4%)	77 in 81 (95.0%)	385/394 (97.7%)
Parvalbumin	29 in 251 (11.6%)	54 in 59 (91.5%)	83/310 (26.8%)
Calbindin	2 in 201 (1.0%)	1 in 43 (2.3%)	3/244 (1.2%)
Calretinin	0 in 206 (0.0%)	0 in 38 (0.0%)	0/244 (0%)
Total	1262 (81.1%)	294 (18.9%)	