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ORIGINAL ARTICLE

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Distribution of various neurochemicals within the zona incerta: an immunocytochemical and histochemical study

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Abstract To gain insight into the cellular organisation of the zona incerta, we have examined the chemoarchitectonic properties of this "uncertain zone". The brains of Sprague-Dawley rats and common cats were processed for immunocytochemistry or NADPH-diaphorase histochemistry using standard methods. For the immunocytochemistry, antibodies to γ-aminobutyric acid (GABA), glutamic acid decarboxylase (GAD), parvalbumin, calbindin, tyrosine hydroxylase, somatostatin, serotonin and glutamate were used. Two general patterns of distribution in the zona incerta were seen. First, labelled cells were restricted largely to one of the cytoarchitectonically defined sectors of the zona incerta. For instance, GABA, GAD and parvalbumin-immunoreactive cells were found principally within the ventral sector, NADPH-diaphorase and glutamate-immunoreactive cells within the dorsal sector and tyrosine hydroxylase- and somatostatin-immunoreactive cells within the rostral sector. Second, labelled cells were scattered somewhat across all incertal sectors, with no clear region of concentration. This pattern included the calbindin- and serotonin-immunoreactive cell groups. These results indicate that the zona incerta is made up of many neurochemically distinct cell groups, some of which respect the well-defined cytoarchitectonic boundaries of the nucleus, whilst others do not. This rich neurochemical diversity in the zona incerta suggests that this nucleus may have differential effects on the different structures that it projects to.

Key words Cat · Rat · Immunocytochemistry · NADPH-diaphorase · Thalamus

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Introduction

The zona incerta, considered by many to be a rostral extension of the midbrain reticular formation (Romanowski et al. 1985; Wagner et al. 1995), is a small collection of cells derived embryologically from the ventral thalamus (see Jones 1985). For such a small nucleus, the zona incerta has been implicated in a wide range of key brain functions, including nociceptive and somatosensory processing (Nicolelis et al. 1992, 1995; Lechner et al. 1993), locomotion (see Mogenson et al. 1985), socio-sexual behaviour (see Edwards and Maillard 1988), feeding and drinking (see Gonzalez-Lima et al. 1993), and arousal and attention (Shammah-Lagnado et al. 1985; Berry et al. 1986; Hermanson et al. 1995; but see Jurkowlaniec et al. 1990). These wide-ranging functions are matched by the extensive neural connections described for the zona incerta. For instance, reciprocal connections have been reported with the cerebral cortex, diencephalon, basal ganglia, brainstem, basal forebrain and the spinal cord (see Ricardo 1981; Roger and Cadusseau 1985; Romanowski et al. 1985; Shammah-Lagnado et al. 1985; Lin et al. 1990, 1997; Wagner et al. 1995).

Many previous studies have shown the zona incerta to be a heterogenous structure, made up of several distinct cytoarchitectonic sectors. Although the number of these sectors reported varies from author to author, more recent studies have argued for four sectors in the rat (rostral, dorsal, ventral, caudal; Kim et al. 1992; Nicolelis et al. 1992) and five sectors in the cat (rostral, dorsal, ventral, caudal, dorsolateral: May et al. 1997). To some extent, these cytoarchitectonic sectors have distinct patterns of connections and immunocytochemical character (Nicolelis et al. 1992, 1995; May et al. 1997).

The zona incerta is known to contain several neurochemical substances (neurotransmitters, neuromodulators, enzymes, and proteins), including GABA/GAD (Oertel et al. 1982; Ottersen and Storm-Mathison 1984; Ficalora and Mize 1989; Kim et al. 1992; Nicolelis et al. 1992, 1995; see also Nagai et al. 1983 and Araki et al. 1984, who used an antibody to GABA-transaminase),

parvalbumin (Celio 1990; Converas et al. 1991; Williams et al. 1994; Nicolelis et al. 1992, 1995), calbindin (Celio 1990; Williams et al. 1994; Hazrati et al. 1995; Nicolelis et al. 1995), tyrosine hydroxylase (Oertel et al. 1982; Chan-Palay et al. 1984; Sar 1984), somatostatin (Vincent et al. 1985; Finley et al. 1981; Jones 1985; Leroux et al. 1988), serotonin (Bosler et al. 1984), NADPH-diaphorase or nitric oxide synthase (Vincent and Kimura 1992), glutamate (van der Pol 1986; Beitz 1989; Border and Mihailoff 1991) and various others (see Kohler et al. 1984; Shiosaka et al. 1985). In all of these cases, except perhaps for GABA, tyrosine hydroxylase and parvalbumin, their distribution and relationship to the cytoarchitectonic sectors within the zona incerta is not entirely clear.

To understand better the neurochemical organisation of the zona incerta, we have examined the distribution of each of the above-mentioned neurochemical substances using immunocytochemistry and histochemistry. In particular, we wanted to determine if any substance(s) characterise(s) all incertal cells, or whether different substances characterise different pockets or groups of incertal cells. In other words, does any substance localise preferentially to cells in any particular sector(s) or subsector(s)? The results generated would provide a better insight into whether there is neurochemical diversity within the zona incerta, thus placing this nucleus in a position to exert differential effects on the structures it projects to.

We have used rats and cats in this study since aspects of incertal organisation are known already for these species (see Nicolelis et al. 1992, 1995; May et al. 1997), thereby forming a basis for the results we generate here. Further, these species belong to very distinct orders of mammals (cat: carnivore; rat: rodent) and marked differences in the distribution of substances within the zona incerta would challenge seriously any ideas of very generalised functions for this nucleus.

Materials and methods

Subjects

Sprague-Dawley rats (n=15) and common cats (n=15) of either sec were used. Animals were anaesthetised after an intraperitoneal injection of sodium pentobarbitone (60 mg/ml) and perfused transcardially with, alternatively: (1) phosphate-buffered saline (PBS; pH 7.4; 0.1 M) followed by 4% buffered formaldehyde (for GAD, parvalbumin, calbindin, tyrosine hydroxylase, somatostatin and serotonin antibodies); (2) PBS followed by 4% buffered formaldehyde with the addition of 0.5% glutaraldehyde (for GABA antibody); (3) cacodylate buffer (pH 7.4; 0.1 M) followed by 2.5% cacodylate buffered glutaraldehyde (for glutamate antibody). Five rats and five cats were perfused with each fixative method. Brains were removed, blocked, immersed in the same fixative for 20–30 min, and then placed in PBS with the addition of 20% sucrose until the block sank. They were then sectioned coronally on a freezing microtome at a thickness of either 30 or 50 μm. Every section was collected in PBS, washed in PBS with the addition of 0.5% Triton (Sigma, Mo., USA) for 20 min (except for sections immunostained for GAD that were not washed in PBS/Triton), and then processed for either immunocytochemistry or NADPH-diaphorase histochemistry as described below.

Immunocytochemistry

Sections were immersed in a solution of 3% H_2O_2 (in 50% ethanol) for 10 min and then in a solution of 10% normal horse serum (in PBS) for about 20 min. Alternate sections were then incubated in one of the following antibodies: anti-GABA (Sigma, Mo., USA; 1:500), anti-GAD (Chemicon, Calif., USA; 1:1,000), anti-parvalbumin (Sigma: 1:1,000), anti-calbindin D28k (Sigma; 1:200), anti-tyrosine hydroxylase (Sigma, 1:1,000), anti-somatostatin (Chemicon; 1:200), anti-serotonin (Chemicon; 1:200), and anti-glutamate (Chemicon; 1:500) for 48 h at 4° C. The sections were then incubated with either biotinylated anti-rabbit (for GABA, GAD, serotonin, glutamate antibodies), biotinylated anti-rat (for somatostatin antibody), or biotinylated anti-mouse (for parvalbumin, calbindin, tyrosine hydroxylase antibodies) (IgG, Sigma; 1:200) for 2 h at room temperature. Finally sections were incubated in the avidin-biotin-peroxidase complex (Sigma; 1:125) for 2 h at room temperature. The bound peroxidase molecule was visualized using a nickel intensified-3,3 diaminobenzidine (DAB: Sigma) solution (Clemence and Mitrofanis 1992). In between each incubation, the sections were washed several times with PBS. Each of the antibodies, together with the avidin-biotin-peroxidase complex, were diluted with PBS with the addition of 0.5% bovine serum albumin. Sections were mounted on chrom-alum gelatinised slides, dried overnight, dehydrated in ascending alcohols, cleared in Histoclear and coverslipped with DPX. Some sections were counterstained with neutral red prior to the alcohol dehydration and coverslipping. For control experiments, the antibodies were replaced by PBS with the addition of 0.5% bovine serum albumin and then reacted as above. Control sections were immunonegative.

NADPH-diaphorase histochemistry

Sections were processed using the protocol described previously (Mitrofanis 1989). Briefly, tissue was prepared as above, and the sections were incubated in PBS containing 0.2% Triton, 15 mM malic acid, 1 mM cobalt chloride, 0.5 mM β-NADPH, and 0.2 mM nitroblue tetrazolium. All chemicals were purchased from Sigma. Sections were mounted on gelatinised slides and coverslipped as above.

Analysis

For the generation of the incertal maps, coronal sections were drawn with reference to the atlases of Paxinos and Watson (1986) in rats and Berman and Jones (1982) in cats and the distributions of labelled cells were plotted with use of a microscope fitted with a camera lucida. Drawings and plots were then scanned onto a computer graphics programme and the schematic diagrams shown in Figs. 1–10 were constructed. In this analysis, plots were made from either 30 μm or 50 μm sections. To gain an insight into the percentage that each group of labelled (immunocytochemically or NADPH-diaphorase) cells made up of the total number, the following analysis was undertaken. In the same sections, the number of labelled (e.g. GAD, GABA, glutamate, NADPH-diaphorase) and Nissl-stained cells within each sector of the zona incerta was counted; from these counts, a percentage of the total number for each group of labelled cells was generated (the number of Nissl-stained and labelled cells in each sector made up 100%, and the number of labelled cells made up the percentage thereof). For each group of labelled cells, counts were pooled from between 5 to 10 sections. Sections were at least 300 μm apart, thus eliminating any double counting of cells. In this analysis, the 30 μm sections, as against the 50 μm sections, proved the better option. This was because the thinner 30 μm sections did not have any non-labelled cells located preferentially in the middle of the section (as did the 50 μm sections), suggesting that the antibody (or diaphorase staining) penetrated the full thickness of the section (see Mitrofanis 1992). If the antibody (or diaphorase staining) did not penetrate the full thickness of the sections, then there would be fewer labelled cells in the section and more Nissl-stained cells, since the Nissl stain would label all the cells, regardless of the section thickness (30 or 50 μm). Thus, in the 30 μm sections, all the cells that contained the targeted antigen (or NADPH-diaphorase) were more likely to be labelled, making the counts and subsequent calculations all the more accurate.

Abbreviations for figures *CL* Central lateral nucleus, *CM* central medial nucleus, *cp* cerebral peduncle, *cZI* caudal sector zona incerta, *DAB* 3,3-diaminobenzidine tetrahydrochloride, *dLGN* dorsal lateral geniculate nucleus, *dZI* dorsal sector zona incerta, *dlZI* dorsolateral sector of zona incerta, *EPN* entopeduncular nucleus, *f* fasciculus retroflexus, *GABA* γ-aminobutyric acid, *GAD* glutamic acid decarboxylase, *Hb* habenula, *ic* internal capsule, *LH* lateral hypothalamus, *LP* lateral posterior nucleus, *MD* mediodorsal nucleus, *MGC* medial geniculate complex, *ml* medial lemniscus, *mt* mammilothalamic tract, *NADPH* nicotinamide adenine dinucleotide phosphate, *ot* optic tract, *PBS* phosphate-buffered saline, *Pf* parafascicular nucleus, *PGN* perigeniculate nucleus, *Po* posterior thalamic nucleus, *pc* posterior commissure, *Pt* pretectum, *R* thalamic reticular nucleus, *rZI* rostral sector zona incerta, *SN* substantia nigra, *Sub* subthalamic nucleus, *VB* ventrobasal complex, *vLGN* ventral lateral geniculate nucleus, *VM* ventromedial thalamic nucleus, *VPL* ventral posterior lateral nucleus, *VPM* ventral posterior medial nucleus, *vZI* ventral sector zona incerta

Results

In this study, the rat zone incerta was subdivided into four sectors: rostral, dorsal, ventral and caudal (Kim et al. 1992; Nicolelis et al. 1992, 1995). In the cat zona incerta, five sectors were identified, namely the rostral, dorsal, dorsolateral, ventral and caudal sectors (May et al. 1997). In both rat and cat, the incertal sectors were distinguished by their distinct cytoarchitecture and, to some extent, the cell-free borders between them (see Nicolelis et al. 1992, 1995; May et al. 1997). Figure 1 shows schematic diagrams of coronal forebrain sections taken from three different levels of the rostrocaudal axis of the rat and cat. The shaded areas in each diagram incidate the regions of the zona incerta depicted in the maps shown in Figs. 2–10.

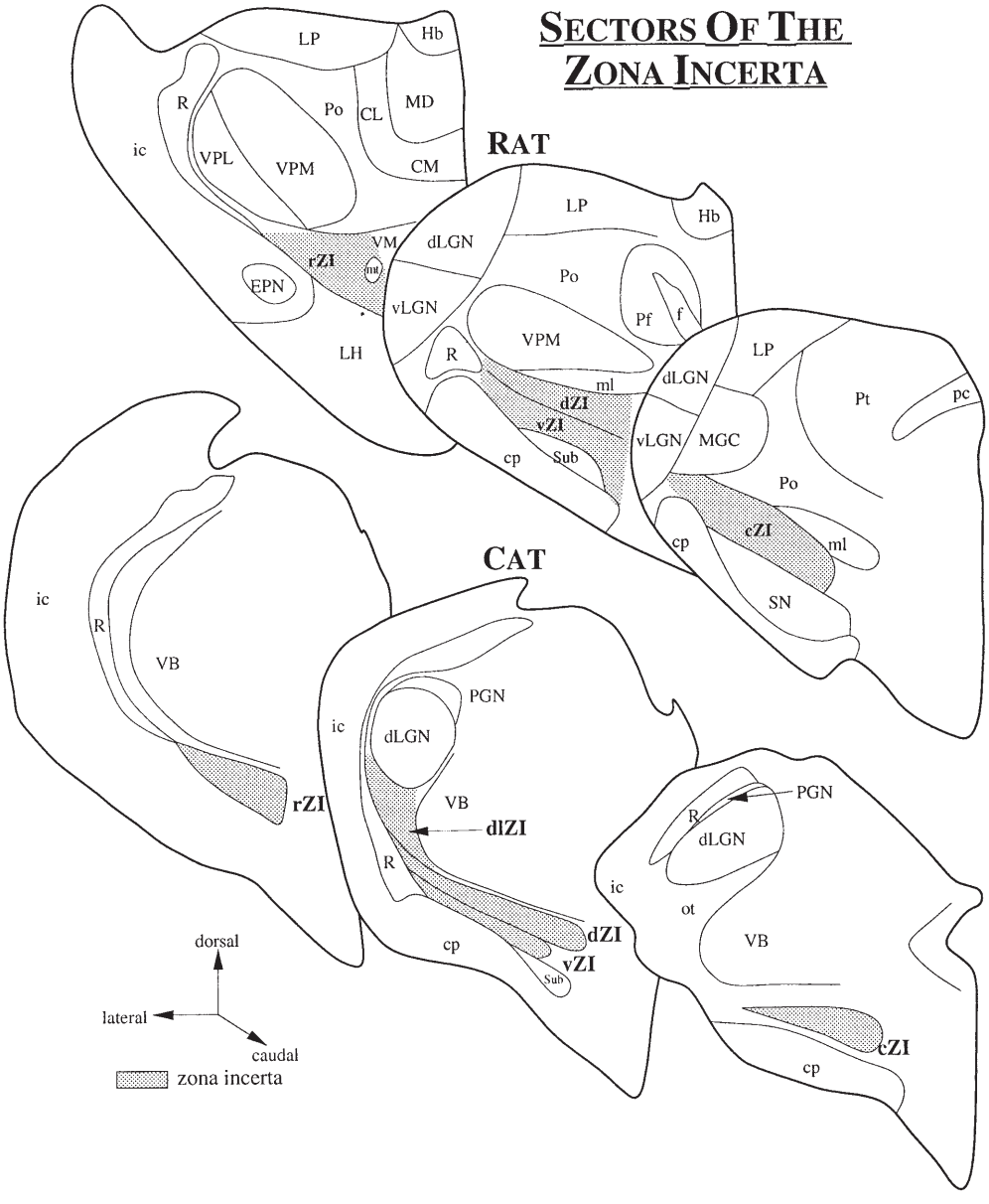

Fig. 1 Schematic diagrams of coronal sections taken from three different levels of the rostrocaudal axis in rats and cats. The *shaded areas* in each diagram indicate the regions of the zona incerta depicted in **C** and **D** of Figs. 2–10. Sections were drawn with reference to Paxinos and Watson (1986) for rats and Berman and Jones (1982) for cats

For each of the antibodies used, together with the NADPH-diaphorase staining, labelling was confirmed to neuronal profiles. In no instance, did we see labelling among classical glial-like profiles, such as star-shaped astrocytes and ramified microglia.

In the section that follows, the cellular distribution of each substance within the zona incerta of both species will be considered separately.

GABA

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the central nervous system and GAD (glutamic acid decarboxylase) is the enzyme that synthesises GABA (see Nieuwenhuys 1985). In this series of experiments the distribution of GABAergic cells in the rat and cat zona incerta was examined with the use of an antibody GABA itself (Fig. 2) and with an antibody to GAD (Fig. 3). Both antibodies yielded very similar patterns of immunolabelling within the zona incerta. Two distinct types of cells were immunoreactive to GABA and to GAD in the zona incerta of rats and cats. One type had large and ovoid somata (unfilled arrows Figs. 2A, B, 3A, B), whilst the other had smaller and quite elongated somata (filled arrows Figs. 2A, B, 3A, B). In general, the GABA and GAD immunostaining in incertal cells were appreciably weaker in cats, than in rats. This feature was seen in all cases examined, even when antibody concentrations were increased. Further, and as a rule, the GAD and GABA immunoreactivity seen in the incertal cells of cats, as well as rats, were not as conspicuous (i.e. intense) in the same sections as was the immunoreactivity in other areas of the forebrain, for example, within the cells of the thalamic reticular nucleus, the interneurons of the dorsal lateral geniculate nucleus and the non-pyramidal cells of the neocortex. The distribution of GABA- and of GAD-immunoreactive cells in the zona incerta of both species are shown schematically in Figs. 2C, D, and 3C, D. In both species, the majority of immunoreactive cells were located within the ventral sector. Fewer cells immunoreactive for these antigens were located elsewhere in the nucleus. In the histograms shown in Figs. 2E, F, 3E, F, counts of GABA and GAD immunoreactive cells in the zona incerta indicated that these cells formed 80–85% of the total number of cells in the ventral sector, about 20–25% of cells in the dorsal sector and about 10% of cells in the caudal sector in both rat and cat. In the dorsolateral sector of cats, no GABA or GAD-immunoreactive cells were ever seen (see Clemence and Mitrofanis 1992).

Parvalbumin

Parvalbumin is a protein that binds calcium with a high affinity. It is regarded as a “transport/buffer” protein because it acts either in calcium transport or as an intra-cellular calcium buffer. Although found in quite distinct groups of neurones in the brain, the precise function of parvalbumin is not clear (see Celio 1990). Parvalbumin immunoreactivity in the rat and cat zona incerta was seen in cells with either large ovoid somata (unfilled arrows, Fig.l 4A, B) or small elongated somata (filled arrows, Figs. 4A, B). The distribution of parvalbumin-immunoreactive incertal cells in both species was very uneven across the nucleus. In both series, most immunoreactive cells were seen within the ventral sector and very few in other sectors. Indeed, 95–100% of the cells in the ventral sector were immunoreactive to parvalbumin in both rats and cats (see Fig. 4E, F). In the rostral, dorsal and caudal sectors, parvalbumin-immunoreactive cells formed between 5 to 15% of the total population of cells, whilst in the dorsolateral sector of cats, about 65% of cells expressed parvalbumin (see Clemence and Mitrofanis 1992).

Calbindin

Calbindin D28-k, together with parvalbumin, is a calcium-binding protein. For the most part, calbindin and parvalbumin are localised to distinct sets of neurones in the brain. As with parvalbumin, the precise function of calbindin is not known (see Celio 1990). Calbindin-immunoreactive cells in the rat and cat zona incerta had small rounded, sometimes ovoid somata (Fig. 5A, B). In both species, these immunoreactive cells were scattered somewhat across all sectors (Fig. 5C, D). In the histograms shown in Fig. 5E, F, the calbindin-immunoreactive cells formed between 5 and 10% of the total number of cells in the rostral, dorsal, dorsolateral and ventral sectors, whilst forming 20–25% of the cells in the caudal sector of the nucleus.

Tyrosine hydroxylase

Tyrosine hydroxylase is the rate-limiting enzyme in catecholamine production (Nieuwenhuys 1985). In the diencephalon, and hence zona incerta, it is considered a marker for dopaminergic cells (see Eaton et al. 1994). Tyrosine hydroxylase immunoreactivity in the zona incerta of rats and cats was very similar. Immunoreactivity was seen in cells with small somata and extensively labelled dendritic processes (Fig. 6A, B). These immunolabelled cells were found exclusively within the rostral sector of the zona incerta (Fig. 6C, D). In both species, tyrosine hydroxylase immunoreactive cells formed about 15% of the total number of cells within that sector (Fig. 6E, F). It should be noted that these cells lay adjacent to a large densely populated group of tyrosine hydroxylase immunoreactive cells in the lateral hypothalamus. This group of cells is the classically designed A13 dopaminergic cell group (see Wagner et al. 1995).

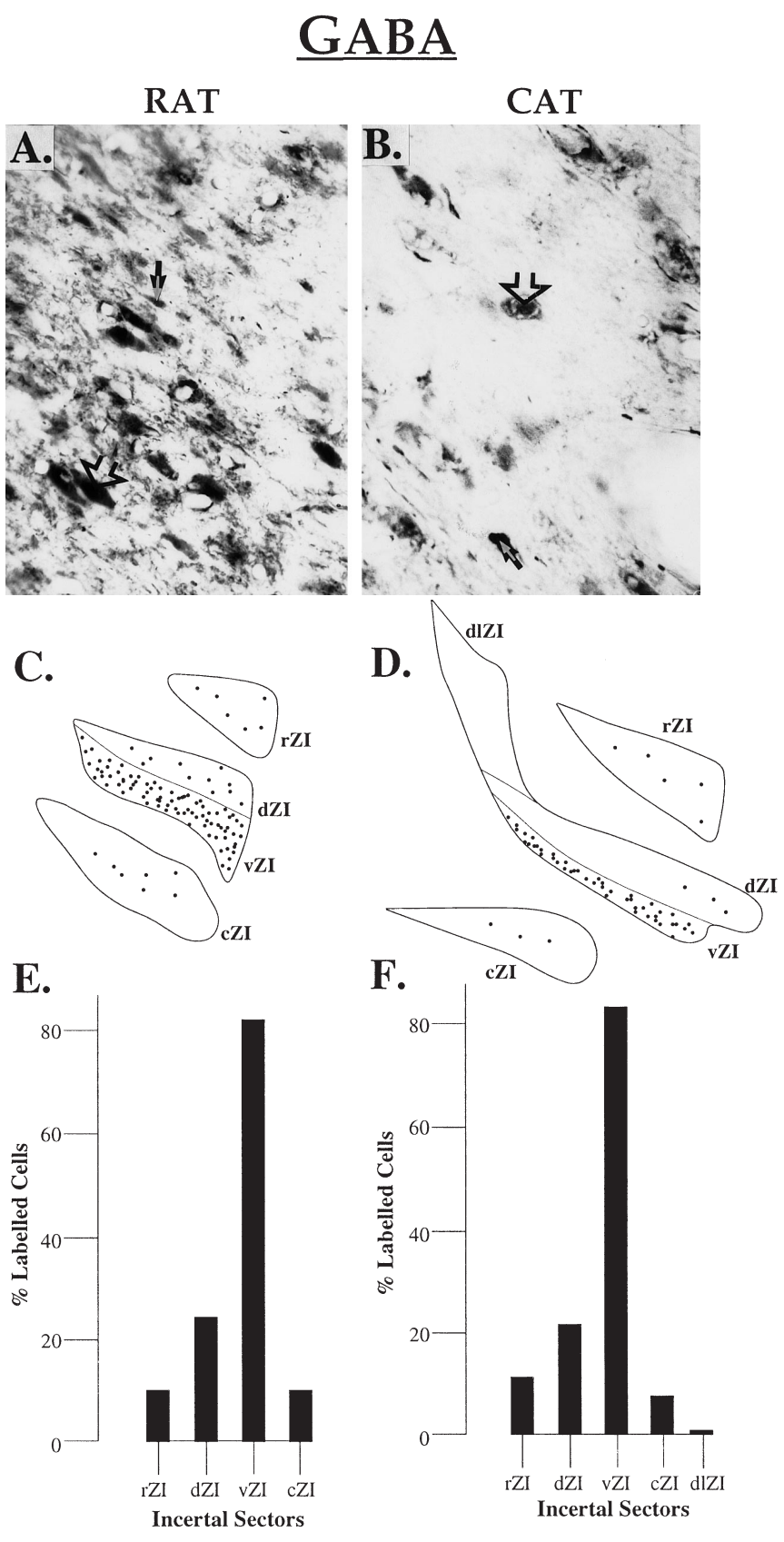

Fig. 2 GABA immunoreactivity in the zona incerta of rats (**A, C, E**) and cats (**B, D, F**). **A**, **B** Photomicrographs of GABA-immunoreactive cells in the ventral sector of the zona incerta (×500); *unfilled arrows* indicate larger pale immunostained cells (probably projection cells), whilst *filled arrows* indicate smaller cells (probably interneurones). Sections were counterstained with neutral red. GABA immunoreactivity, particularly in the cat, was quite weak among incertal cells, much weaker than among thalamic reticular cells and nonpyramidal cells of the neocortex in the same sections. **C**, **D** Schematic diagrams of the distribution of GABA-immunoreactive cells across the zona incerta. Each *black circle* represents one labelled cell. Maps can be orientated with respect to shaded areas in Fig. 1. In both species, most GABA-immunoreactive cells were found in the ventral sector of zona incerta. **E**, **F** Histograms showing the percentage that the GABA-immunoreactive cells made up of the total number of cells in each particular sector (see Materials and methods for details). The total number of cells was determined after Nissl-counterstaining the immunostained sections. In the ventral sector, GABA-immunoreactive cells formed the largest percentage of the total number of cells; in the other sectors, a much smaller percentage was recorded

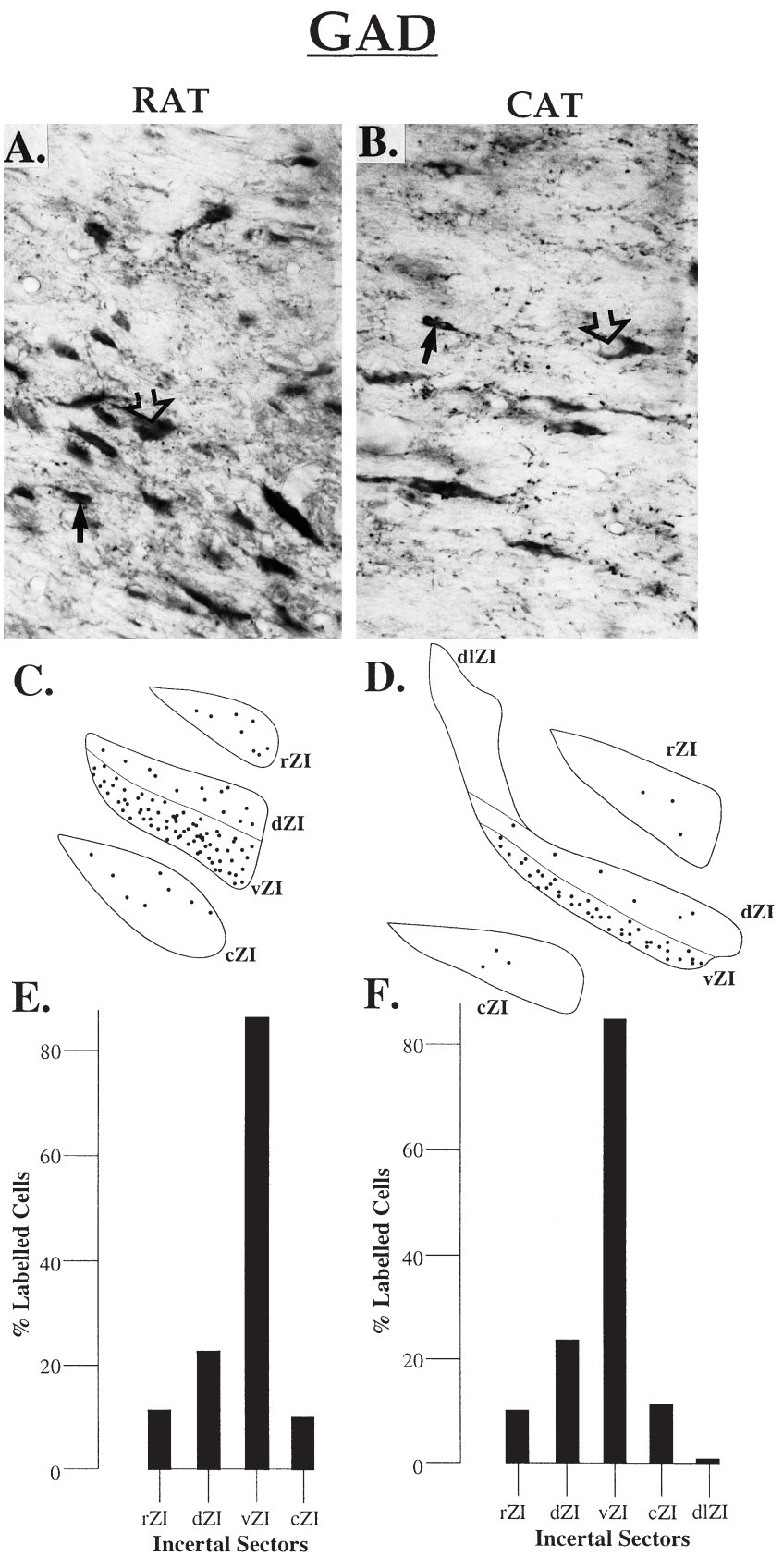

Fig. 3 GAD immunoreactivity in the zona incerta of rats (**A, C, E**) and cats (**B, D, F**). **A, B** Photomicrographs of GAD-immunoreactive cells in the ventral sector of zona incerta (×500); *unfilled arrows* indicate larger immunostained cells (probably projection cells), whilst *filled arrows* indicate smaller cells (probably interneurones). Sections were counterstained with neutral red. As with GABA immunoreactivity, GAD immunoreactivity, particularly in the cat, was quite weak among incertal cells, much weaker than among thalamic reticular cells and non-pyramidal cells of the neocortex in the same sections. **C, D** Schematic diagrams of the distribution of GAD-immunoreactive cells across the zona incerta. Each *black circle* represents one labelled cell. Maps can be orientated with respect to shaded areas in Fig. 1. In both species, most GAD-immunoreactive cells were found in the ventral sector of zona incerta. **E, F** Histograms showing the percentage that the GAD-immunoreactive cells made up of the total number of cells in each particular sector (see Materials and methods for details). The total number of cells was determined after Nissl-counterstaining the immunostained sections. In the ventral sector, GAD-immunoreactive cells formed the largest percentage of the total number of cells; in the other sectors, a much smaller percentage was recorded

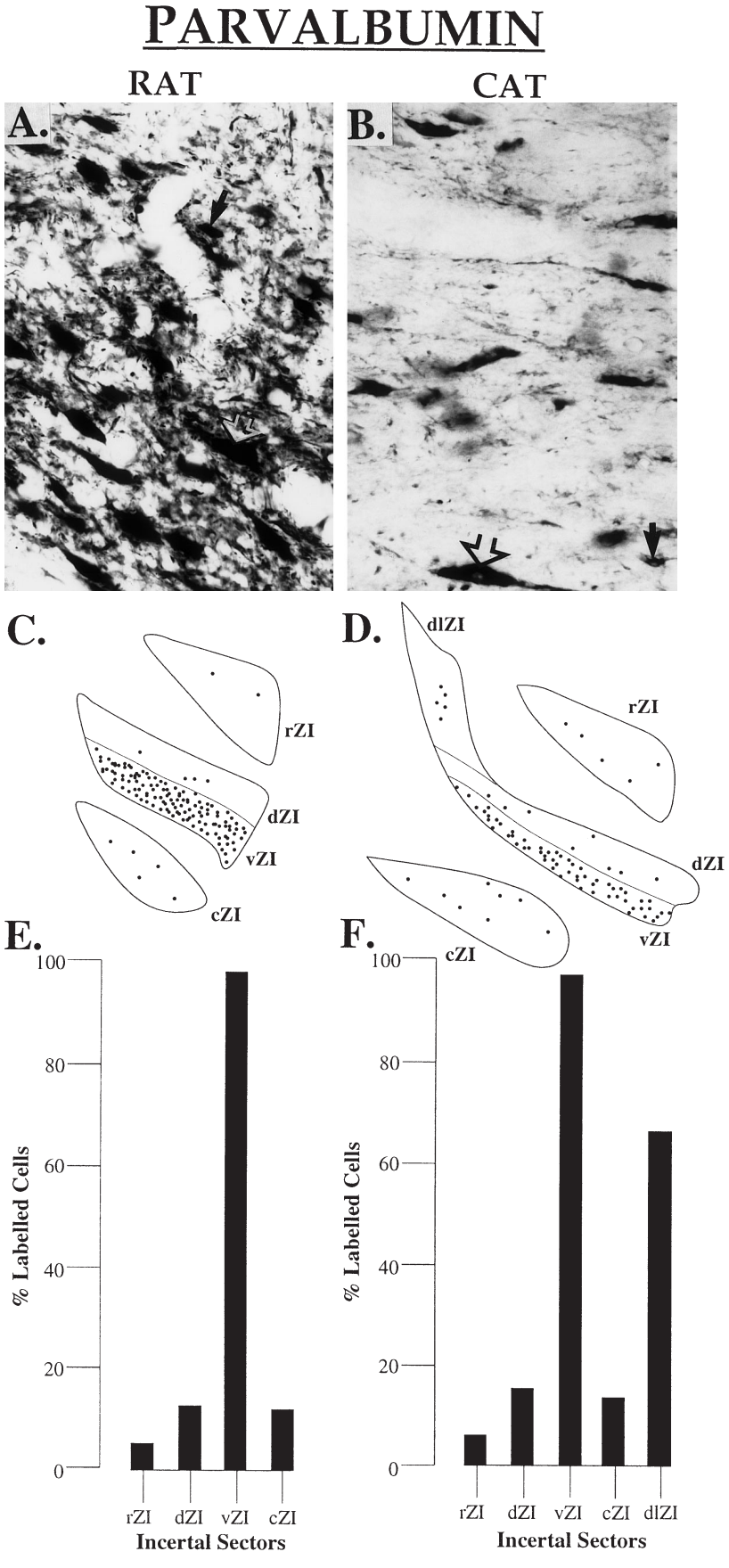

Fig. 4 Parvalbumin immunoreactivity in the zona incerta of rats (**A, C, E**) and cats (**B, D, F**). **A, B** Photomicrographs of parvalbumin-immunoreactive cells in ventral sector of the zona incerta (×500); *unfilled arrows* indicate larger immunostained cells (probably projection cells), whilst *filled arrows* indicate smaller cells (probably interneurones). Sections were counterstained with neutral red. **C, D** Schematic diagrams of the distribution of parvalbumin-immunoreactive cells across the zona incerta. Each *black circle* represents one labelled cell. Maps can be orientated with respect to shaded areas in Fig. 1. In both species, most parvalbumin-immunoreactive cells were found in the ventral sector of the zona incerta (as with the GABA- and GAD-immunoreactive cells). **E, F** Histograms showing the percentage that the parvalbumin-immunoreactive cells made up of the total number of cells in each particular sector (see Materials and methods for details). The total number of cells was determined after Nissl-counterstaining the immunostained sections. In the ventral sector, parvalbumin-immunoreactive cells formed the largest percentage of the total number of cells; in the other sectors, a smaller percentage was recorded

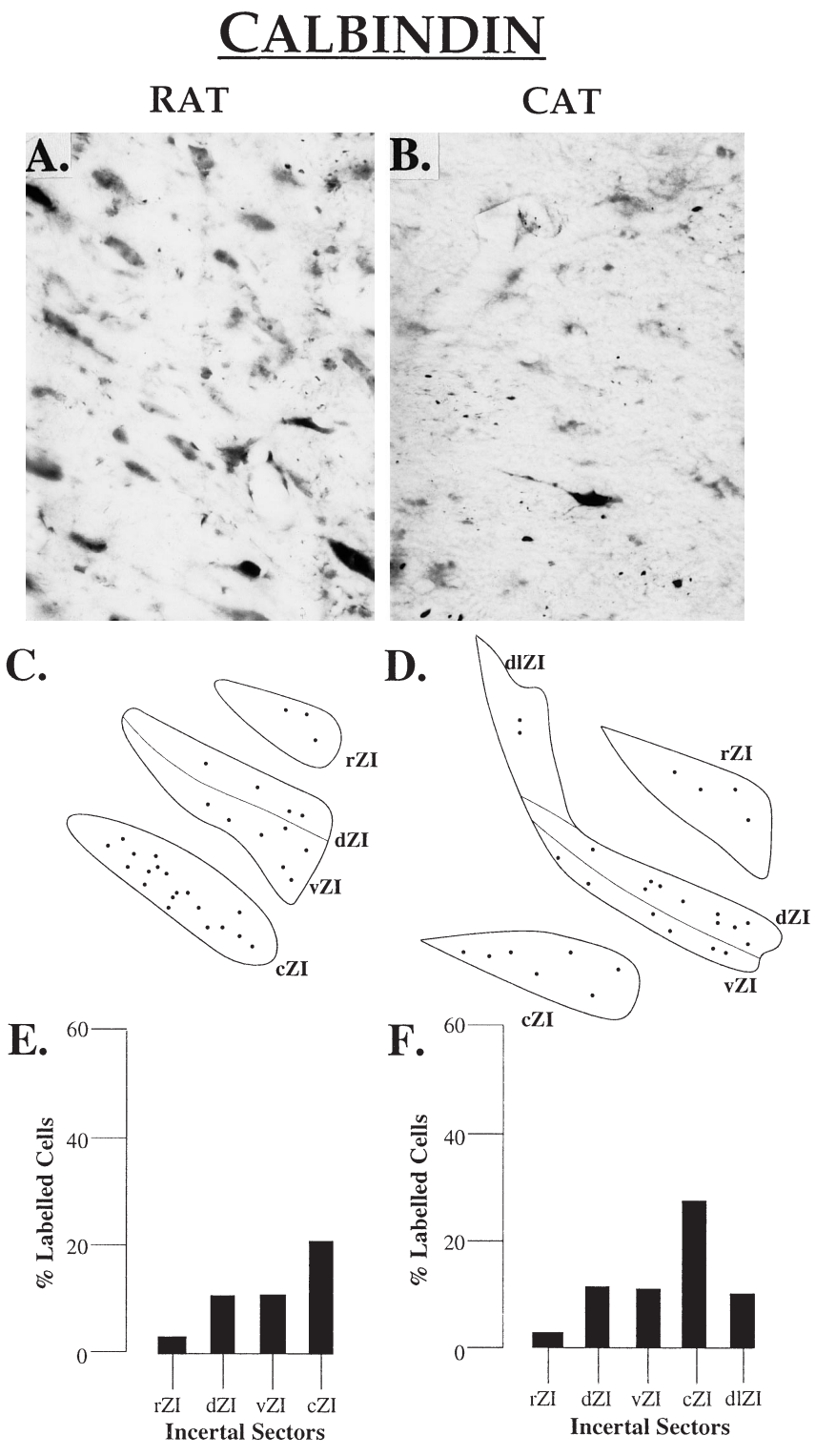

Fig. 5 Calbindin immunoreactivity in the zona incerta of rats (**A, C, E**) and cats (**B, D, F**). **A, B** Photomicrographs of calbindin-immunoreactive cells in the zona incerta (×500). These sections were counterstained with neutral red. **C, D** Schematic diagrams of the distribution of calbindin-immunoreactive cells across the zona incerta. Each *black circle* represents one labelled cell. Maps can be orientated with respect to shaded areas in Fig. 1. In both species, calbindin-immunoreactive cells were found scattered across all incertal sectors, with no readily apparent zone of concentration. **E, F** Histograms showing the percentage that the calbindin-immunoreactive cells made up of the total number of cells in each particular sector (see Materials and methods for details). The total number of cells was determined after Nissl-counterstaining the immunostained sections. In the caudal sector, calbindin-immunoreactive cells formed a slightly larger percentage of the total number of cells than in the other sectors

Somatostatin

The peptide hormone somatostatin is found among cells in many regions of the central nervous system. It is thought to be involved in several processes, including neuromodulation for neurotransmitters at the synapse (Vidal and Zieglgansberger 1989), as well as acting as a neurotransmitter itself (Finley et al. 1981). Somatostatin immunoreactivity in the rat and cat zona incerta was seen in small cells with ovoid somata (Fig. 7A, B). In both species, these cells were found mostly within the rostral sector of the zona incerta (Fig. 7C, D). In rats, labelled cells were seen also in the lateral regions of the dorsal and ventral sectors, whilst in cats, they were seen form-

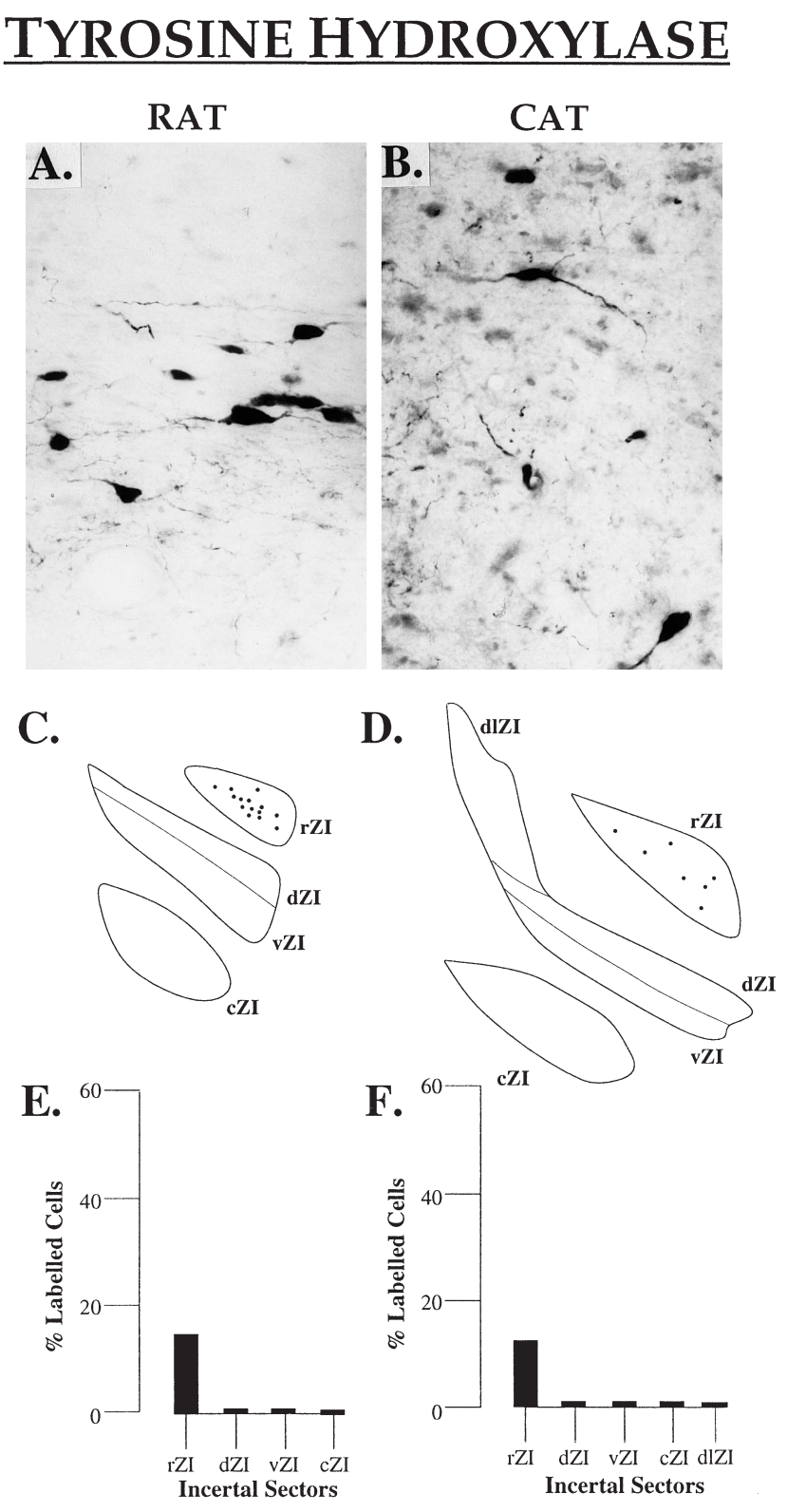

Fig. 6 Tyrosine hydroxylase immunoreactivity in the zona incerta of rats (**A, C, E**) and cats (**B, D, F**). **A, B** Photomicrographs of tyrosine hydroxylase-immunoreactive cells in the rostral sector of the zona incerta (×500). Section in **B** was counterstained lightly with neutral red. **C, D** Schematic diagrams of the distribution of tyrosine hydroxylase-immunoreactive cells across the zona incerta. Each *black circle* represents one labelled cell. Maps can be orientated with respect to shaded areas in Fig. 1. In both species, tyrosine hydroxylase-immunoreactive cells were limited to the rostral sector. **E, F** Histograms showing the percentage that the tyrosine hydroxylase-immunoreactive cells made up of the total number of cells in each particular sector (see Materials and methods for details). The total number of cells was determined after Nissl counterstaining the immunostained sections. In the rostral sector, tyrosine hydroxylase-immunoreactive cells formed about 15% of the total number of cells in that sector

ing a thin zone in the ventral sector (Fig. 7C, D). The histograms shown in Fig. 7E, F, the somatostatin-immunoreactive cells in rats and cats formed about 35% of the total number of cells in the rostral sector, 25% of cells in ventral sector, and 15% of cells in the dorsal sector. In the dorsolateral sector of cats, about 10% of cells were somatostatin-immunoreactive. There were no somatostatin-immunoreactive cells ever seen in the caudal sector of either species (Fig. 7C–F).

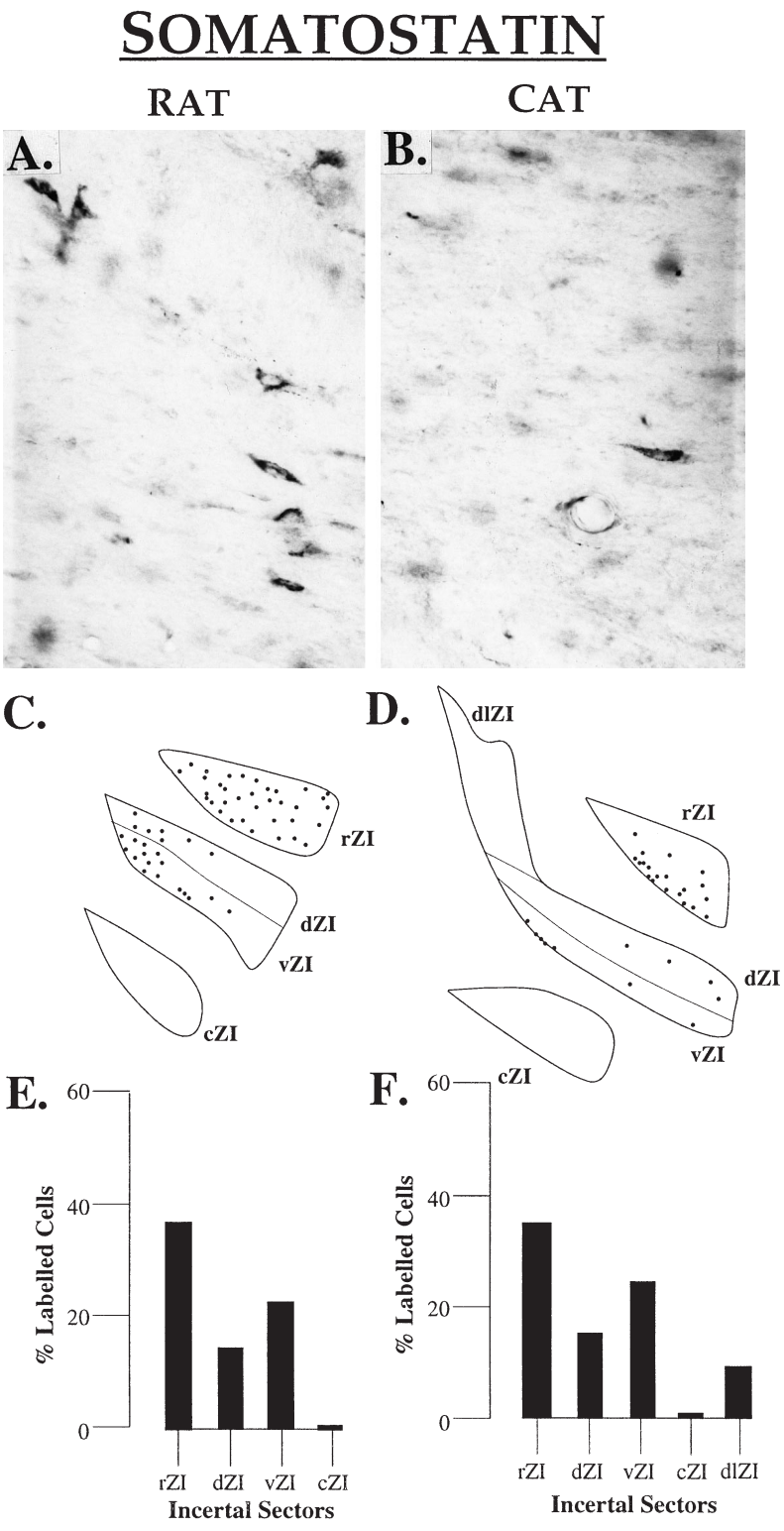

Fig. 7 Somatostatin immunoreactivity in the zona incerta of rats (**A, C, E**) and cats (**B, D, F**). **A, B** Photomicrographs of somatostatin-immunoreactive cells in the rostral sector of the zona incerta (×500). Sections were counterstained lightly with neutral red. **C, D** Schematic diagrams of the distribution of somatostatin-immunoreactive cells across the zona incerta. Each *black circle* represents one labelled cell. Maps can be orientated with respect to shaded areas in Fig. 1. In both species, somatostatin-immunoreactive cells were found mainly in the rostral sector. **E, F** are histograms showing the percentage that the somatostatin-immunoreactive cells made up of the total number of cells in each particular sector (see Materials and methods for details). The total number of cells was determined after Nissl-counterstaining the immunostained sections. In the rostral sector, somatostatin-immunoreactive cells formed the largest percentage of the total number of cells; in the other sectors, a smaller percentage was recorded

Serotonin

Serotonin is a neurotransmitter that is thought to be involved in a variety of neuroendocrine processes, such as the modulation of prolactin and gonadotrophin secretion (Bosler et al. 1984). Further, serotonin has also been shown to influence the activity and excitability levels of thalamic and cortical neurones, thereby modulating thalamocortical activity (see McCormick 1992). In rats, serotonin immunoreactivity in the zona incerta was seen in cells with large rounded somata and finely labelled dendrites (Fig. 8A). These cells were found scattered across

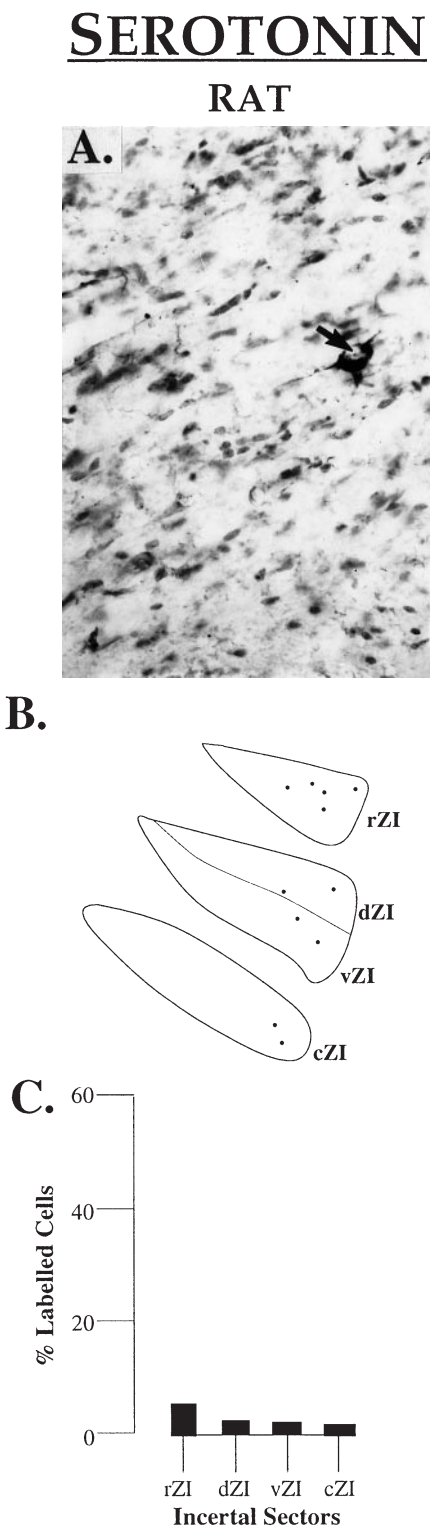

Fig. 8A–C Serotonin immunoreactivity in the zona incerta of rats. **A** Photomicrograph of a serotonin-immunoreactive cell (*arrow*) in the rostral sector of the zona incerta (×500). Sections was counterstained lightly with neutral red. The darkly immunostained structures to the left are blood vessels. **B** Schematic diagram of the distribution of serotonin-immunoreactive cells across the zona incerta. Each *black circle* represents one labelled cell. Maps can be orientated with respect to shaded areas in Fig. 1. Serotonin-immunoreactive cells were found scattered across all incertal sectors, with no readily apparent zone of concentration. **C** Histogram showing the percentage that the serotonin-immunoreactive cells made up of the total number of cells in each particular sector (see Materials and methods for details). The total number of cells was determined after Nissl-counterstaining the immunostained sections. In the rostral sector, serotonin-immunoreactive cells formed a slightly larger percentage of the total number of cells than in the other sectors. Note that serotonin-immunoreactive cells were not seen in the cat zona incerta

all incertal sectors, with no particular zone of concentration (Fig. 8B). Figure 8C shows that the serotonin-immunoreactive cells formed about 2% of the total number of cells in the dorsal ventral and caudal sectors, whilst forming 5% of cells in the rostral sector. In cats, in contrast to rats, no serotonin-immunoreactive cells were seen in the zona incerta, even though many immunoreactive cells were seen in the raphe nuclei of the brainstem of this species.

NADPH-diaphorase

Recently, nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase has been reported to be a nitric oxide synthase that produces the gas nitric oxide (Hope et al. 1991; Dawson et al. 1991). In rats, NADPH-diaphorase in the zona incerta was seen in cells with ovoid or triangular somata with finely dendrites (Fig. 9A). In all cases examined, these NADPH-dipahorase cells were concentrated within the dorsal sector of the zona incerta (Fig. 9B). Fewer labelled cells were seen in the other incertal sectors. The histogram in Fig. 9C shows that the NADPH-diaphorase cells formed about 40% of the total number of cells in the dorsal sector, 15% of cells in the rostral sector and less than 5% of the total in the other sectors. In cats, in contrast to rats, no NADPH-diaphorase cells were seen in the zona incerta, even though many strongly labelled cells were seen in the striatum, neocortex and brainstem of this species.

Glutamate

Glutamate is thought to be the major excitatory neurotransmitter in the central nervous system (see Hanson and Krogsgaard-Larson 1990). In both rats and cats, glutamate-immunoreactive cells in the zona incerta had large ovoid somata (Fig. 10A, B) and were found in all incertal sectors, but with a slight concentration in the dorsal sector (Fig. 10C, D). The histograms in Fig. 10E, F show that the glutamate-immunoreactive cells formed about 40% of the total number in the dorsal sector, whilst forming 15% of the cells in the other sectors. In the dorsolateral sector of cats, about 5% of cells were glutamate-immunoreactive.

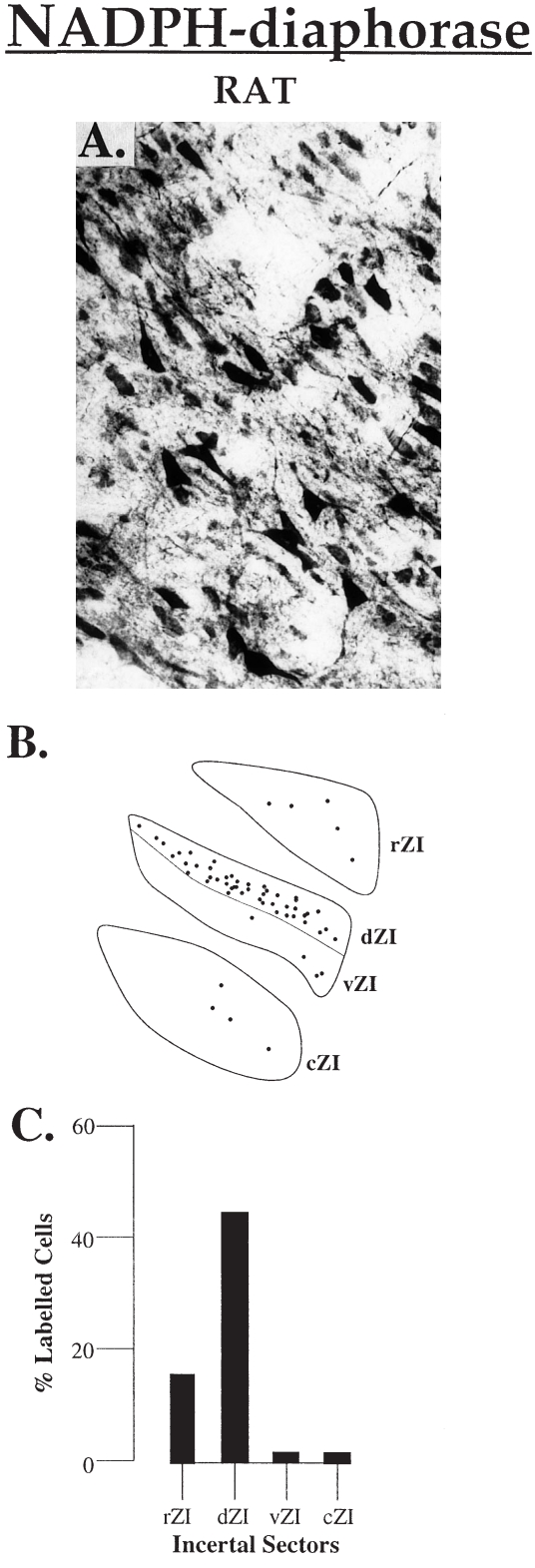

Fig. 9A–C NADPH-diaphorase reactivity in the zona incerta of rats. **A** Photomicrograph of NADPH-diaphorase cells in the dorsal sector of the zona incerta (×500). Section was counterstained lightly with neutral red. **B** Schematic diagram of the distribution of NADPH-diaphorase cells across the zona incerta. Each *black circle* represents one labelled cell. Maps can be orientated with respect to shaded areas in Fig. 1. Most NADPH-diaphorase cells were found in the dorsal sector. **C** Histogram showing the percentage that the NADPH-diaphorase cells made up of the total number of cells in each particular sector (see Materials and methods for details). The total number of cells was determined after Nissl-counterstaining the immunostained sections. In the dorsal sector, NADPH-diaphorase cells formed a larger percentage of the total number of cells than in the other sectors. Note that NADPH-diaphorase cells were not seen in the cat zona incerta

Discussion

This study has shown that cells of the zona incerta are characterised by a large number of distinct substances, making this nucleus one of the most neurochemically diverse cell groups in the greater thalamus. For the most part, the cell groups characterised by each of these substances had a rather distinct signature distribution across the zona incerta, which raised some important questions concerning incertal organisation, together with the possible areas of projection of these neurochemically particular cell groups. Each of these issues will be discussed below. Firstly, a comparison of our results with those of previous studies will be given.

Comparison with previous studies

Previous studies have described the presence within zona incerta of all the substances examined in this study. That is, GABA/GAD (Nagai et al. 1983; Araki et al. 1984; Kim et al. 1992; Nicolelis et al. 1992, 1995), parvalbumin (Celio 1990; Converas et al. 1991; Williams et al. 1994; Nicolelis et al. 1992, 1995), calbindin (Celio 1990; Williams et al. 1994; Hazrati et al. 1995; Nicolelis et al. 1995), tyrosine hydroxylase (Oertel et al. 1982; Chan-Palay et al. 1984; Sar 1984; Wagner et al. 1995), somatostatin (Vincent et al. 1985; Finley et al. 1981; Jones 1985; Leroux et al. 1988), serotonin (Bosler et al. 1984), NADPH-diaphorase (Vincent and Kimura 1992) and glutamate (van der Pol 1986; Beitz 1989; Border and Mihailoff 1991). In this study, we extend these previous findings of providing the distributions of cells containing each substance examined and their relative percentages in each incertal sector; except for GABA/GAD (Kim et al. 1992; Nicolelis et al. 1992, 1995), tyrosine hydroxylase (Wagner et al. 1995) and parvalbumin (Nicolelis et al. 1995), no previous study has examined these particular issues.

Regarding our distributions of the tyrosine hydroxylase- and parvalbumin-immunoreactive cells within the zona incerta, our results match closely those of previous studies. These studies also report on a concentration of tyrosine hydroxylase-immunoreactive cells in the rostral sector (see Wagner et al. 1995) and a concentration of parvalbumin-immunoreactive cells in the ventral sector (see Celio 1990; Nicolelis et al. 1992, 1995). Regarding our distribution of the GABAergic cells in the zona incerta, our results match those of some studies, but not of others. Some studies have indicated that GABA- and/or GAD-immunoreactivity characterises all or most cells in

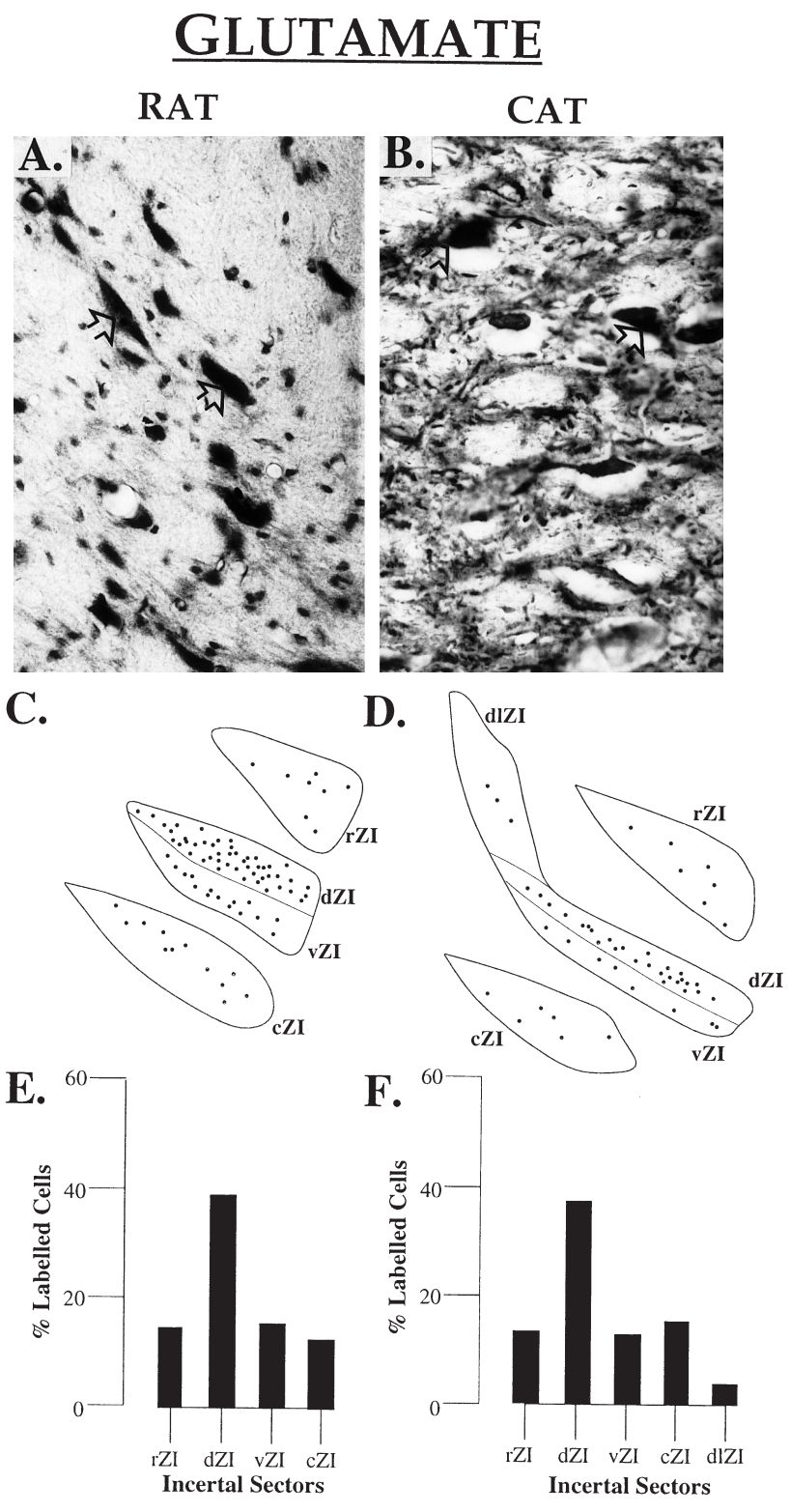

Fig. 10 Glutamate immunoreactivity in zona incerta of rats (**A, C, E**) and cats (**B, D, F**). **A, B** Photomicrographs of glutamate-immunoreactive cells in the dorsal sector of zona incerta (*unfilled arrows*). ×500. Section in **A** was counterstained lightly with neutral red. **C, D** Schematic diagrams of the distribution of glutamate-immunoreactive cells across the zona incerta. Each *black circle* represents one labelled cell. Maps can be orientated with respect to shaded areas in Fig. 1. In both species, glutamate-immunoreactive cells were found mainly in the dorsal sector. **E, F** Histograms showing the percentage that the glutamate-immunoreactive cells made up of the total number of cells in each particular sector (see Materials and methods for details). The total number of cells was determined after Nissl-counterstaining the immunostained sections. In the dorsal sector, glutamate-immunoreactive cells formed the largest percentage of the total number of cells; in the other sectors, a smaller percentage was recorded

all sectors of the zona incerta in rats (Oertel et al. 1982; Nicolelis et al. 1992, 1995; but see Nagai et al. 1983; Araki et al. 1984). Other studies in rats, however, have reported that GABA- and/or GAD-immunoreactive cells of the zona incerta are limited largely to the ventral sector and that even within this sector, not all cells are immunoreactive to GABA or to GAD (Ficalora and Mize 1989; Kim et al. 1992; see also Araki et al. 1984). Our results here in rats and cats support strongly the latter findings, by reporting that most GABAergic cells lie in the ventral sector and very few elsewhere. Thus, it appears that a GABAergic projection from the zona incerta

may not be as widespread across the central neuroaxis as thought previously (see Nicolelis et al. 1992, 1995).

Comparison of rat and cat zona incerta

For the most part, the zona incerta in rats and cats were organised in a similar fashion. Each had comparable cellular distributions of the different substances examined and each had the same incertal sector organisations. There were however some differences. First, in terms of neurochemical make up, the most notable species difference was the localisation and distribution of NADPH-diaphorase and serotonin within the zona incerta. Whereas the rat zona incerta had quite distinct NADPH-diaphorase and serotonin-immunoreactive cell populations, the cat zona incerta had no cells that contained either of these substances. Second, in terms of sector organisation, a clear species difference lay in the fact that the cat zona incerta contained an extra sector – the dorsolateral sector. This sector has only recently been described and was initially referred to as the "inner small-celled region". On the basis of immunochemical labelling, this region was suggested tentatively to be part of the zona incerta rather than the adjacent thalamic reticular nucleus (Clemence and Mitrofanis 1992). Since then, more immunocytochemical (Crabtree and Kind 1993; Vaccaro and Mitrofanis 1996, 1997) and connectional (Crabtree 1992; Vaccaro and Mitrofanis 1996, 1997; May et al. 1997) evidence has accumulated linking this area to the zona incerta. In this study, we have used the perhaps more appropriate name of "dorsolateral sector" (May et al. 1997), rather than inner small-celled region (Clemens and Mitrofanis 1992), since the former is more precise in terms of incertal topography.

In summary, although there are some differences in the sector organisation and localisation of neurochemical substances between the rat and cat zona incerta, this area of the thalamus is very similar in both species. The reasons for these species differences are not clear, although with gathering knowledge of incertal function in different species, the reasons for these differences may become more readily apparent.

Patterns of distribution

Although neurochemically distinct cells had, for the most part, rather different distributions within the zona incerta, one could identify two general patterns. First, labelled cells were restricted largely to one of the cytoarchitectonically defined sectors of the zona incerta. For instance, GABA-, GAD- and parvalbumin-immunoreactive cells were restricted to the ventral sector, NADPH-diaphorase and glutamate-immunoreactive cells were limited to the dorsal sector and tyrosine hydroxylase- and somatostatin-immunoreactive cells were found largely within the rostral sector. Second, labelled cells were scattered somewhat across all incertal sectors, with no clear area of concentration. This pattern of distribution included the calbindin- and serotonin-immunoreactive cell groups. Thus, these results indicate that some of these neurochemically distinct cell groups respect the well-defined cytoarchitectonic boundaries of the nucleus, whilst others clearly do not. The precise functional significance of these findings is not clear, although it does suggest that some types of incertal cells (those limited to distinct sectors) may have a more specific function than others (those scattered across sectors).

The histograms in Figs. 2–10 suggest that there may well be an overlap of expression, or co-localisation of some substances in the incertal cells. The best example of this feature is seen within the ventral sector. Here, there are sub-populations of GABA-, GAD-, somatostatin- serotonin- and glutamate-immunoreactive cells that all must express parvalbumin also, since approximately 100% of the cells in the ventral sector are parvalbumin-immunoreactive (see Results). Thus, there may be four populations (GABAergic, somatostatinergic, serotonergic, glutamatergic) of parvalbumin-immunoreactive cells in the ventral sector, although this remains to be shown conclusively (e.g. some parvalbumin-immunoreactive cells may also contain two or three of the above-mentioned substances). Further, in the dorsal sector about 40% of cells are NADPH-diaphorase and glutamate-immunoreactive; there could be some co-localisation of these substances within individual cells of this sector.

Thus, our results here indicate that there is much neurochemical diversity within the zona incerta and of the nine substances screened in this study, not one characterised all the incertal cells. It should be noted that this pattern is very different from the pattern seen in the thalamic reticular nucleus, the other major nucleus of the ventral thalamus. In the reticular nucleus, all cells are GABA- and parvalbumin-immunoreactive, for example (see Clemence and Mitrofanis 1992). This suggests that even though the zona incerta and the reticular nucleus have similar developmental origins, they have striking differences in their neurochemical composition and organisation, together with their recently described differences in their connections (see Lin et al. 1990, 1997).

Possible areas of projection

One of the striking features of the zona incerta lies in its diverse and widespread projection patterns across the neuroaxis, from the neocortex to the spinal cord (see Ricardo 1981; Roger and Cadusseau 1985; Romanowski et al. 1985; Shammah-Lagnado et al. 1985; Lin et al. 1990, 1997). Our findings here indicate that there is a rich mixture of neurochemically distinct cells within the zona incerta and it is possible that all, or many, of these distinct cell groups have particular areas of projection. Indeed, previous studies have shown that the superior colliculus (Ficalora and Mize 1989; Kim et al. 1990) and neocortex (Lin et al. 1990; Nicolelis et al. 1992, 1995) receive a GABAergic, whilst the lateral hypothalamus and preop-

tic area receive a dopaminergic projection (Wagner et al. 1995). Thus, this feature suggests that the zona incerta may not "blanket" the neuroaxis with the same type of neurochemical projection (for example, GABAergic), but rather send distinct neurochemical projections to particular structures. Further, there is evidence indicating that the zona incerta sends two different types of projection to the same structure, as has been shown by recent studies on the incertal projections to the superior colliculus; both GABAergic (Ficalora and Mize 1989; Kim et al. 1990) and glutamatergic (Beitz 1989) incertal cells have been reported to project to this structure. It remains to be determined where the other neurochemically distinct cell groups described in this study project to. It could turn out that each of these cell groups has a distinct area of projection.

It is of course possible that not all these neurochemically distinct cells have projections outside the zona incerta. There may be some cells that are interneurons and have all their processes restricted to the zona incerta itself (Ma et al. 1992). For instance, in the case of the parvalbumin-, GABA- and GAD-immunoreactive cells, the larger ovoid cells could be projection cells, whilst the smaller elongated cells may be interneurons (see Results).

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