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Relationship of neuronal vulnerability and calcium binding protein immunoreactivity in ischemia

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Summary. The relationship between neuronal calcium binding protein content (calbindin D_{28K}: CaBP and parvalbumin: PV) and vulnerability to ischemia was studied in different regions of the rat brain using the four vessel occlusion model of complete forebrain ischemia. The areas studied, i.e. the hippocampal formation, neocortex, neostriatum and reticular thalamic nucleus (RTN), show a characteristic pattern of CaBP and PV distribution, and are involved in ischemic damage to different degrees. In the hippocampal formation CaBP is present in dentate granule cells and in a subpopulation of the CA1 pyramidal cells, the latter being the most and the former the least vulnerable to ischemia. Non-pyramidal cells containing CaBP in these regions survive ischemia, whereas PV-containing non-pyramidal cells in the CA1 region are occasionally lost. Hilar somatostatin-containing cells and CA3 pyramidal cells contain neither PV nor CaBP. Nevertheless, the latter are resistant to ischemia and the former is the first population of cells that undergoes degeneration. Supragranular pyramidal neurons containing CaBP are the most vulnerable cell group in the sensory neocortex. In the RTN the degenerating neurons contain both PV and CaBP. In the neostriatum, ischemic damage involves both CaBP-positive and negative medium spiny neurons, although the degeneration always starts in the dorsolateral neostriatum containing relatively few CaBP-positive cells. The giant cholinergic interneurons of the striatum contain neither CaBP nor PV, and they are the most resistant cell type in this area. These examples suggest the lack of a consistent and systematic relationship between neuronal CaBP or PV content and ischemic vulnerability. It appears that some populations of cells containing CaBP or PV are more predisposed to ischemic cell death than neurons lacking these proteins. These neurons may express high levels of calcium binding proteins because their normal activity may involve a high rate of calcium uptake and/or intraneuronal release.

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Introduction

Ischemic death of the selectively vulnerable populations of neurons is thought to be preceded by a pathological accumulation of intracellular calcium (Andine et al. 1988; DeLeo et al. 1987; Deshpande et al. 1987; Van Reempts et al. 1986), although other authors interpret this process as a secondary phenomenon accompanying other irreversible mechanisms of damage (Dienel 1984; Hossmann et al. 1983; Sakamoto et al. 1986; Siesjo 1981; Yanagihara and McCall 1982). Calcium is likely to enter the neurons via different voltage dependent and neurotransmitter receptor-associated ion channels, most notably via the N-Methyl-D-Aspartate (NMDA) receptor-operated channel (Goldberg et al. 1987; Hamon and Heineman 1986; Mayer et al. 1987; Simon et al. 1984), but intracellular calcium pools may also be mobilized. Areas containing high concentrations of NMDA receptors (Monaghan and Cotman 1985) largely coincide with regions most vulnerable to ischemia (Crain et al. 1988; Jorgensen and Diemer 1982; Kirino et al. 1984; Pulsinelli et al. 1982; Smith et al. 1984), suggesting that NMDA receptor activation may be involved in the pathological process. However, the protective effect of NMDA-antagonists is the subject of current debate (Block and Pulsinelli 1987; Buchan and Pulsinelli 1988; Gill et al. 1987; Jarvis et al. 1988; Simon et al. 1984; Swan et al. 1988). According to the calcium hypothesis, the pathological level of intracellular calcium activates biochemical processes leading to enzymatic breakdown of proteins and lipids, to malfunctioning of mitochondria, energy failure and ultimately to cell death (Baudry et al. 1981; Siesjo 1981; Strosznajder 1980; for rev. see Siesjo and Bengtsson 1989). Therefore, it is reasonable to assume that neurons, which have a greater capacity to buffer

intracellular calcium, would be more resistant to ischemic damage.

One mechanism by which neurons could effectively control intracellular calcium concentrations is by the synthesis of large amounts of specific calcium binding proteins. Two such proteins, parvalbumin (PV) and calbindin D_{28K} (CaBP), are known to have a high capacity to buffer intracellular calcium in the brain (Baimbridge and Miller 1982; Heizmann 1984; Morrissey et al. 1978), and have a widespread distribution in different areas including those effected in ischemia (Baimbridge and Miller 1982; Baimbridge et al. 1982; Celio and Heizmann 1981; Gerfen et al. 1985; Mudrick and Baimbridge 1989; Sloviter 1989). A correlation between vulnerability and calcium binding protein content has been suggested for a model of ischemia (Wasterlain et al. 1988), and for some populations of neurons in the perforant path stimulation model of epilepsy (Sloviter 1989). Furthermore, intracellular calcium chelation was found to protect neurons from functional damage caused by prolonged stimulation of afferent pathways (Scharfman and Schwartzkroin 1989). CaBP has also been shown to protect against glutamate induced excitotoxicity in hippocampal neuronal cultures (Baimbridge and Kao 1988). In the present study we examined further whether there is any relationship between neuronal calcium binding protein content (CaBP and PV) and vulnerability to ischemia. The hippocampal formation, neocortex, neostriatum and reticular thalamic nucleus (RTN), have been selected for this study, because they all show a characteristic pattern of immunoreactivity for CaBP and PV, and are involved in ischemic degeneration to different extents.

Material and methods

Animals and surgical procedures

The experiments were carried out on 28 adult Wistar rats (body weight 250–350 g, Charles River). Animals of the first group ($n = 11$) were anesthetised with halothane (1–2% vaporised in air) and forebrain ischemia was induced by four vessel occlusion (4VO, for 30 min) according to Pulsinelli and Brierley (1979), while EEG. evoked potentials (EP, stimulating the perforant path), oxygen tension and temperature were recorded from the hippocampus with a chamber-type microelectrode (Freund et al. 1989). In the second group ($n = 17$) animals were treated in the same way as those in the first, except that Equithesin (chlornembutal, 3 ml/kg, Cowey and Perry 1979) was used as an anaesthetic.

Preparation of animals has been described in detail earlier in Freund et al. (1989). The animals were anesthetised with Equithesin (3 ml/kg) for surgery. The vertebral arteries were exposed bilaterally by drilling the alar foramina, then cauterized and split under direct visual control (Todd et al. 1986). One day later the animals were anesthetised again by 1–2% halothane vaporised in air and administered via a nose mask (group 1), or by Equithesin (group 2), and the head of the animals was fixed in a stereotaxic frame throughout the recording session. Holes (approx. 2 mm diameter) were drilled above the right hippocampus and angular bundle. A bipolar stimulating electrode was placed into the angular bundle at coordinates AP = –7 mm from the bregma, L = 4.5 mm, V = 3.5 mm below dura. A miniaturized thin-film electrode probe and recording device (Prohaska 1987) was used to record simultaneously potential (POT), oxygen tension (O_2) and temperature (T) from the hippocampus, as described earlier (Freund et al. 1989).

Accurate positioning of the probe was confirmed by stimulating the perforant path with single pulses (0.2 msec, at 0.1 Hz, intensity: 10–50 V) while recording field potentials characteristic of the different regions of the hippocampal formation (Buzsáki et al. 1986). Following 30 min baseline recording of electrical activity, oxygen tension and temperature, the carotid arteries were occluded bilaterally by pulling a silicon tube placed around the arteries. Indications of the completeness of the forebrain ischemia were the flattening of the EEG, the disappearance of the evoked potential, and 4–6° C fall of the local temperature. Body temperature was maintained at 37° C using a rectal temperature probe connected to a Homeothermic Blanket Control Unit (Harvard Apparatus). The duration of ischemia was 30 min. Some animals (2 in group 1 and 2 in group 2) died from respiratory failure during occlusion. Animals (2 in group 1 and 2 in group 2) which did not have a flat EEG, or in which the evoked potential did not disappear completely, were excluded from further studies because of incomplete forebrain ischemia.

Perfusion and processing of tissue sections. Six to seven days after the ischemia session the animals were deeply anesthetised and perfused through the heart first with saline (2 min) followed by a fixative containing 0.1% glutaraldehyde, 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH, 7.4), for 30 min. Brains were removed from the skull, and postfixed in the same fixative for 1–3 hr. Blocks of the dorsal hippocampus and overlying cortex, mesencephalon, septum, neostriatum and thalamus were dissected, immersed into 10 and then 20% sucrose (in 0.1 M PB) until they sank, and freeze-thawed in liquid nitrogen before sections were cut at 60 μm on a Vibratome. Alternate sections were processed for silver-impregnation and for immunocytochemistry using antibodies against parvalbumin and calbindin D_{28K}.

Silver impregnation. The procedure of Gallyas et al. (1980) has been used. Briefly this involves 2 × 5 min in the pretreating solution (2% NaOH and 2.5% NH_4OH, 10 min in the impregnating solution (0–0.8% NaOH, 2.5% NH_4OH, 0.5% $AgNO_3$), 3 × 5 min in washing solution (0.5% Na_2CO_3 and 0.01% NH_4NO_3 in 30% ethanol), 1 min in developing solution (0.4–0.6% formaldehyde and 0.01% citric acid in 10% ethanol, pH, 5.0–5.5), and 3 × 10 min washes in 0.5% acetic acid. The sections were then mounted on gelatine coated slides, dehydrated and covered with a neutral medium (XAM).

Immunocytochemistry. The following procedures were carried out at room temperature with constant agitation unless otherwise stated. The sections were washed in 0.1 M sodium phosphate buffer (pH, 7.4) and then in 0.05 M Tris buffered saline (TBS, pH, 7.4).

Fig. 1A–D. Sections from the hippocampus of normal and ischemic animals immunostained for calbindin D_{28K}(CaBP). **A** Pattern of CaBP-immunoreactivity in the CA1 region of a normal animal. Stratum radiatum contains strongly immunoreactive non-pyramidal neurons (arrows). The majority of pyramidal cells are also weakly immunoreactive (e.g. arrowheads). The immunostained apical dendrites of these pyramidal cells form a dense plexus in stratum radiatum. **B** CaBP-immunoreactivity in the CA1 region seven days after ischemia. The majority of pyramidal cells are degenerating, and have lost their immunoreactivity for CaBP. The strongly immunoreactive interneurons in stratum radiatum are apparently intact (arrows). **C** Higher magnification micrograph from the same section seen in **B** showing normal interneurons (open arrows). **D** CaBP-immunoreactivity in the CA3 region seven days after ischemia. None of the pyramidal cells (asterisk) are damaged, and the interneurons (arrows) in stratum radiatum appear normal. The mossy fibres (dense band) are also intact, and stain very strongly for CaBP. Scales: **A, B, D** 100 μm; **C** 20 μm

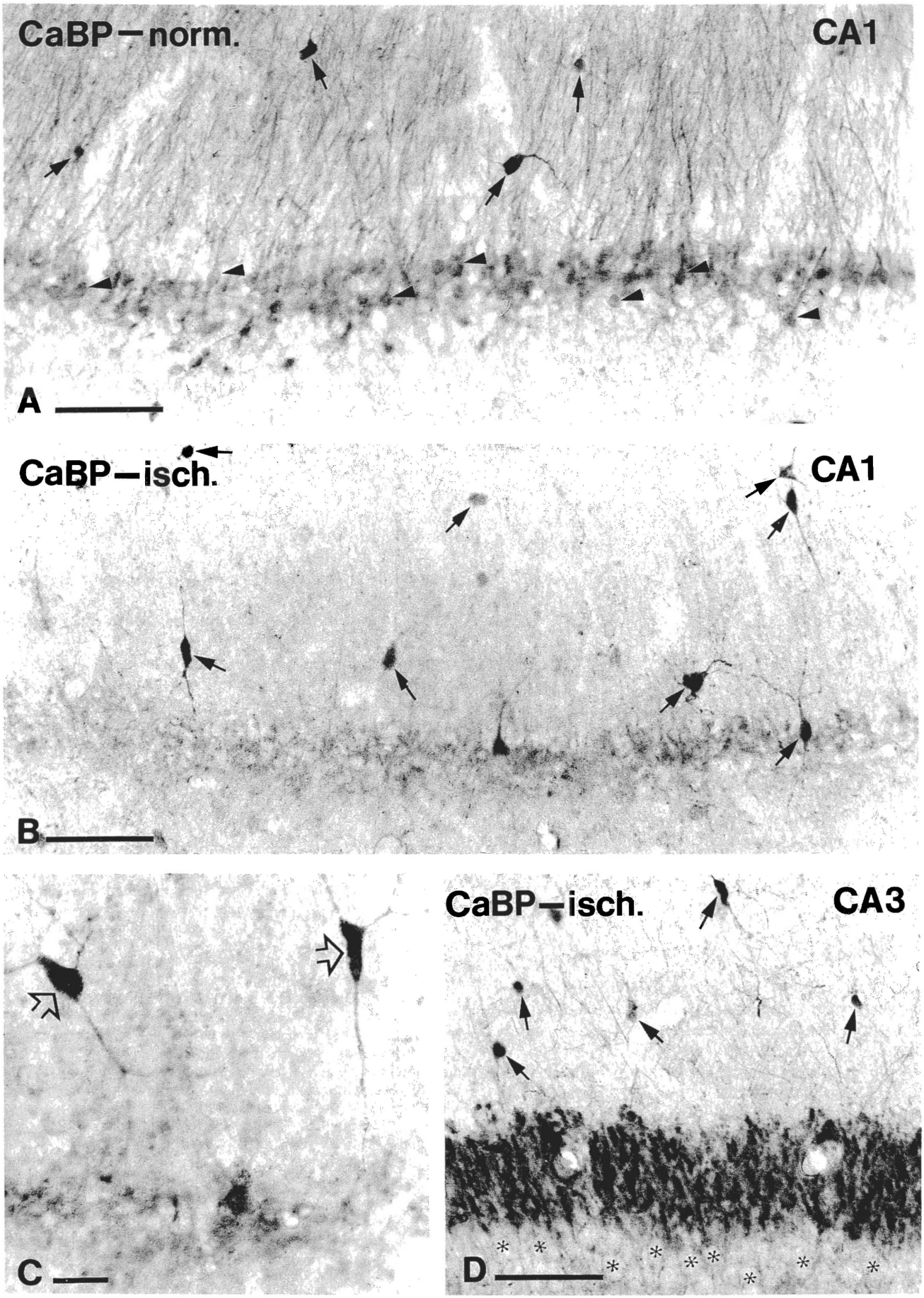

Fig. 1

They were transferred into a blocking solution of 20% normal goat serum (NGS) for 30 min. Two primary sera were used. Antiserum (Code No. R 202) to monkey cerebellar calcium binding protein (calbindin D_{28K}, CaBP) was raised in rabbit, and it was shown to detect a single band of 28 Kd protein in both one and two dimensional gel electrophoresis immunoblots. Immunoprecipitation of rat cerebellar soluble proteins with this antibody, followed by PAGE also yields a single 28 Kd band (Buchan and Baimbridge, 1988). Antiserum (Code No. R 301) to rat muscle parvalbumin was also raised in rabbit, and tested for specificity in the same way as CaBP (Baimbridge et al. 1982; Sloviter 1989). Sections were treated with the primary sera (at a dilution of 1:2000 for CaBP and 1:1000 for PV) for 2 days at 4° C, and then with goat anti-rabbit IgG (ICN) diluted 1:50, for 4–6 h, followed by rabbit PAP (Dakopatts) diluted 1:100, overnight at 4° C. The sections were washed for 3 × 30 min between the different steps. All washes and antibody dilutions were done in TBS containing 1% NGS and 0.5% Triton X 100. Diaminobenzidine-tetra HCl (DAB, SIGMA, 0.05% in Tris buffer) was used as a chromogen for the immunoperoxidase reaction, H_2O_2 (0.01% concentration) was added after 25 min preincubation, and the sections were incubated in this solution for 2–5 min. The sections were washed and mounted on gelatine coated slides, dehydrated, and covered in XAM neutral medium for light microscopy.

The specificity of the method was tested by incubating the sections in normal rabbit serum (Dakopatts, 1:100) in the place of the primary antisera. No specific staining was observed either in somata or axons in these sections, only a faint uniform background staining was seen in all cell bodies on the surface of the sections.

Results

The four examined regions of the rat brain – the hippocampus, neocortex, the reticular nucleus of the thalamus and the neostriatum – are known to contain neurons selectively vulnerable to ischemia (Blomqvist and Wieloch 1985; Ito et al. 1975; Petito and Pulsinelli 1984; Pulsinelli et al. 1982a, b; Smith et al. 1984). They also have characteristic patterns of immunoreactivity for calcium binding proteins calbindin D_{28K} and parvalbumin (see below). Thus, these regions appear to be ideal for studying the possible relationship between calcium binding protein content and ischemic vulnerability.

Hippocampal formation

Normal distribution of calbindin D_{28K} and parvalbumin. The most intense immunoreactivity for CaBP was localised in a subpopulation of interneurons, which mainly occur in strata radiatum and oriens of CA1–3 (Fig. 1). Their dendritic trees were also stained, which can be appreciated especially in CA3. Granule cells, as well as their dendrites in stratum moleculare and axons projecting to CA3 were also strongly immunoreactive for this calcium binding protein. Hilar neurons were mostly negative. Pyramidal cells in CA3 were also negative for CaBP, the non-pyramidal cells were the only stained neurons in this region (Fig. 1D). The majority of pyramidal cells in the CA1 region, mainly those located in the "superficial layer" of stratum pyramidale, were also immunoreactive for CaBP. Their apical dendrites form a dense network in strata radiatum and lacunosum moleculare intermingled with the more strongly immunostained dendrites and somata of nonpyramidal cells (Fig. 1A). The CA2 region appeared to be a transitional zone between CA1 and CA3 containing both strongly stained and negative pyramidal cells. In contrast, PV was present selectively in a subpopulation of non-pyramidal cells in all regions, none of the principal cells showed any degree of immunoreactivity. Somata of the PV-immunostained non-pyramidal cells occured exclusively in the principal cell layers, in stratum oriens and less frequently in the hilus of the dentate gyrus. The dendrites of these cells were found to span all layers. Immunoreactive axon terminals were restricted to the perisomatic region of the principal neurons.

Pattern of ischemic cell death. In agreement with earlier studies the most vulnerable neurons were the hilar neurons (mostly the somatostatin-containing cells, Johansen et al. 1987) and the CA1 pyramidal cells, as shown by their argyrophilia in silver impregnated sections (Crain et al. 1988). The variability in the degree of damage was substantial even among identically treated animals that showed similar changes in physiological parameters (EEG, evoked potentials, brain temperature, oxygen tension changes) during and after ischemia (for details see Freund et al. 1990). Pyramidal cells of the CA3 region were rarely affected and the granule cells appeared to be the most resistant.

The majority of CaBP-immunoreactive non-pyramidal cells in strata radiatum and oriens of CA1–3 survived ischemia (Fig. 1B–D), even if pyramidal cells in the same area of CA1 were completely degenerated. In the degenerated areas of the CA1 region the dendritic arbors of non-pyramidal cells became conspicuous, since the dense network of CaBP-immunoreactive apical dendrites disappeared. The somata of CA1 pyramidal cells were

Fig. 2A–E. Sections from the hippocampus of normal and ischemic animals immunostained for parvalbumin (PV). **A** Silver impregnated section from the hippocampus seven days after ischemia. The neurons of stratum pyramidale in the CA1 region are shrunken, and show strong argyrophilia from the border region with CA3 (open arrow) for approximately 1 mm (broken line). **B** PV-immunostained section from the same hippocampus, 120 μm away from the silver impregnated section shown in **A**. The CA3 region (left from the open arrow), and the other end of CA1 contains a large number of PV-immunoreactive interneurons with varicose dendrites passing through stratum radiatum. However, the area indicated by broken line contains only a very few PV-immunoreactive neurons. **C** Pattern of PV-immunoreactivity in the CA1 region of a normal animal. The PV-positive interneurons (curved arrows) have long varicose dendrites extending into strata radiatum and oriens, where they intermingle with PV-stained axons (arrowheads). Asterisks label PV-negative pyramidal cell somata surrounded by PV-immunoreactive axon terminals. **D, E** PV-immunostained sections from the CA1 and CA3 regions of an ischemic animal. The pyramidal cells and a PV-positive cell body (curved arrow) are shrunken in the CA1 area, but terminal labelling is still visible in stratum pyramidale. The varicose dendrites disappeared from strata radiatum and oriens, only axons (arrowheads) remained. In the CA3 region (**E**) pyramidal neurons (asterisks) as well as PV-positive interneurons (curved arrows) and their terminals around the pyramidal cell somata are intact. Scales: **A, B** 200 μm, **C–E** 20 μm

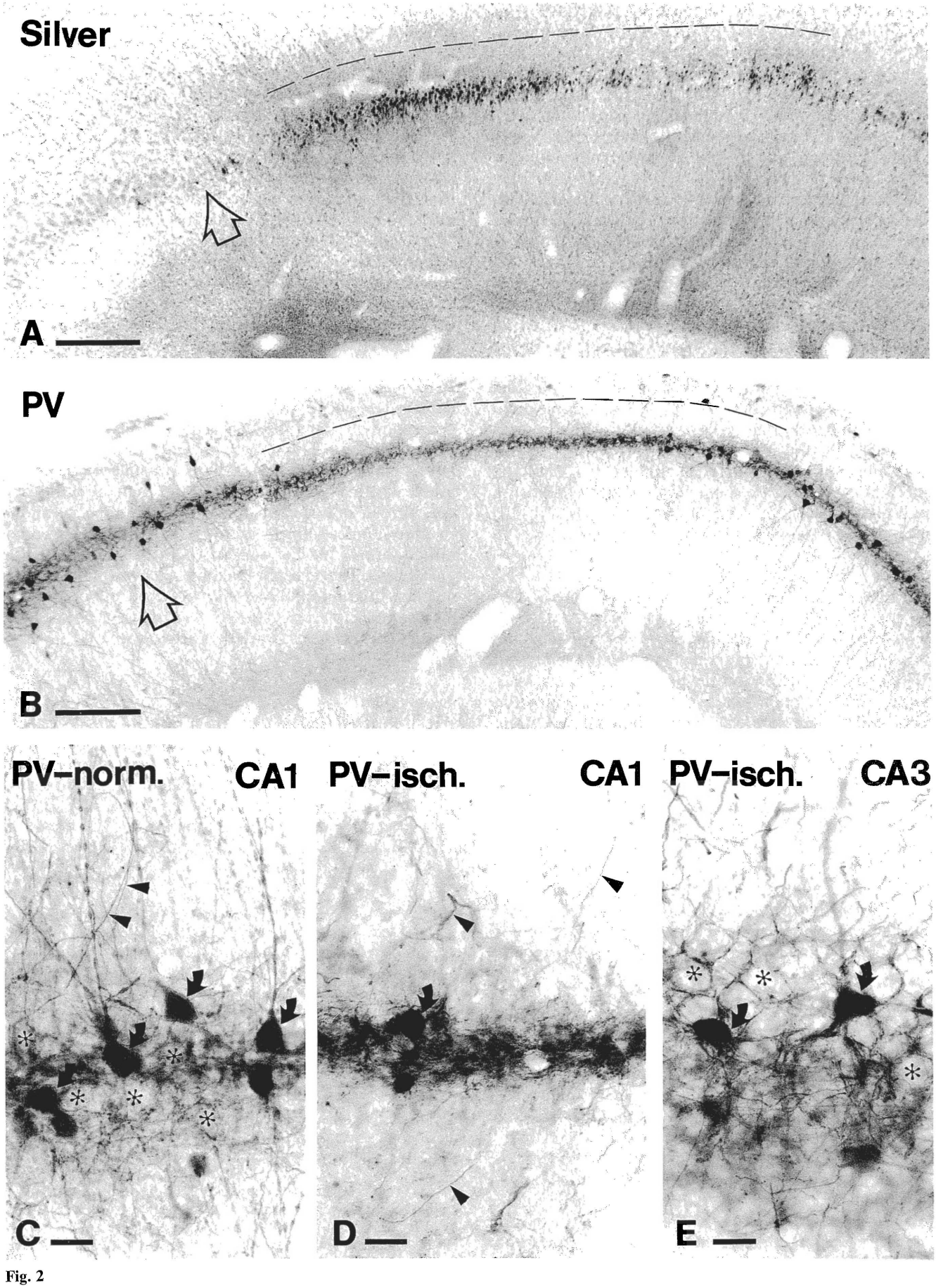

Fig. 2

shrunken and lost their immunoreactivity to CaBP (Fig. 1B, C), whereas the CA3 pyramidal cells and granule cells retained a normal appearance. The granule cells retained their normal level of CaBP immunoreactivity both in their somata and in their axons (the mossy fibres) innervating the CA3 pyramidal neurons (Fig. 1D). CaBP-positive reactive astrocytes were occasionally seen in strata radiatum and pyramidale in severely damaged fields of the CA1 region.

PV-immunoreactive non-pyramidal cells also survived ischemia in all regions of the hippocampal formation. However, in some segments of the CA1 region of 3 animals a complete loss of pyramidal cells was accompanied by a partial loss of PV-positive non-pyramidal cells (Fig. 2B, C). This was best seen in stratum radiatum, which normally contains a dense array of PV-positive varicose dendrites arranged parallel to the apical dendrites. Occasionally the density of such dendrites was reduced in areas where most of the pyramidal cells had degenerated (Fig. 2A, B, D). PV-positive main axons in strata radiatum and oriens and varicose axonal processes around shrunken somata in stratum pyramidale could still be seen in large numbers (Fig. 2D). The pattern of PV-immunoreactivity in the somata, dendrites and axon terminals of CA3 non-pyramidal cells (Fig. 2E) and in the dentate gyrus remained unchanged.

Neocortex

Normal distribution of calbindin D_{28K} and parvalbumin. Only sensory areas were examined in detail in the present study (areas 3b, 17, 18), but the pattern of immunoreactivity for both calcium binding proteins was consistent over a wide range of areas. Non-pyramidal cells were strongly immunoreactive for CaBP in layers II–VI, with long segments of dendrites visualized by the immunostaining. In addition, the majority of pyramidal cells in the supragranular layers also showed immunoreactivity for CaBP, although the intensity of the staining was lighter than that in the non-pyramidal cells (Fig. 3A). Similar to the hippocampus, PV immunoreactivity was present selectively in a subpopulation of non-pyramidal cells in layers II–VI, which had relatively well-stained dendrites and axon terminals. The latter were surrounding somata, proximal dendrites and axon initial segments of pyramidal cells.

Pattern of ischemic cell death. The neocortex was more resistant to ischemia than the hippocampus, as assessed by silver impregnation of degenerating neurons. Degenerating (i.e. shrunken and argyrophilic) neurons were scattered in layers II–III, and less frequently in layers V and VI (Fig. 3B). They had a triangular or pyramidal shape, and occasionally, impregnated apical and basal dendrites. Axonal degeneration was also visualized by silver impregnation, which showed large numbers of argyrophilic boutons in layers II, III and V, which are the known target layers of axon collaterals originating from supragranular pyramidal neurons. The frequency and distribution of CaBP- and PV-immunoreactive non-pyramidal cells and their processes remained qualitatively unchanged.

Reticular thalamic nucleus (RTN)

Normal distribution of calbindin D_{28K} and parvalbumin. Most if not all neurons in the RTN were found to be strongly immunoreactive for PV, whereas the adjacent thalamic nuclei (the ventrolateral and ventroposterior nuclei) contained only PV-immunoreactive axon trunks (Fig. 3C). CaBP-immunoreactivity in this region was more difficult to evaluate, since there were many nuclei containing neurons that showed a gradual shift of immunoreactivity from negative to faintly positive. In the RTN neurons a weak CaBP-like immunoreactivity was always detectable.

Pattern of ischemic cell death. The RTN was consistently damaged in all animals which showed a massive degeneration in the hippocampus. The majority of the neurons in the RTN in these animals were argyrophilic, but not shrunken (Fig. 3D). Thus, it could not be established whether they were only damaged reversibly, or they were in an earlier phase of irreversible degeneration. In cresyl violet-stained sections the neurons appeared rather normal, but the number of glial cells increased in the nucleus. The adjacent thalamic nuclei contained large numbers of argyrophilic axonal processes, but degenerating cell bodies were not found. An increase in the density of glial cells was seen in these nuclei as well.

Neostriatum

Normal distribution of calbindin D_{28K} and parvalbumin. In agreement with Gerfen et al. (1985), we found that the majority of medium size neurons in the striatal matrix compartment was positive for CaBP (Fig. 4A). In the dorsolateral region the CaBP immunoreactivity was relatively weaker, but numerous positive cells of apparently

Fig. 3. A Light micrograph of a section from the occipital neocortex (area 18b) of a normal animal immunostained for calbindin D_{28K} (CaBP). A subpopulation of non-pyramidal neurons (arrows) in layers II–VI show strong immunoreactivity, whereas pyramidal cells are faintly immunoreactive (arrowheads) in layers II–III. **B** silver impregnated section of the neocortex (same area as in **A**), from an animal subjected to ischemia by 4 vessel occlusion for 30 min. Shrunken, argyrophilic cell bodies (arrows) are present in layers II–III, and terminal degeneration (argyrophilia) is seen in layers II–III and V, corresponding to the known local projection of pyramidal cell axon collaterals. **C** PV-immunostained section from the thalamus of a normal rat. Most if not all neurons are immunoreactive for parvalbumin in the reticular thalamic nucleus (NRT), whereas only axons are stained in the ventrolateral thalamic nucleus (NVT). **D** Silver impregnated section of the thalamus from an animal subjected to ischemia by 4 vessel occlusion for 30 min. The majority of the neurons in NRT show strong argyrophilia. Terminal degeneration is seen in most nuclei of the thalamus. Scales: **A, B** 200 μm; **C, D** 500 μm

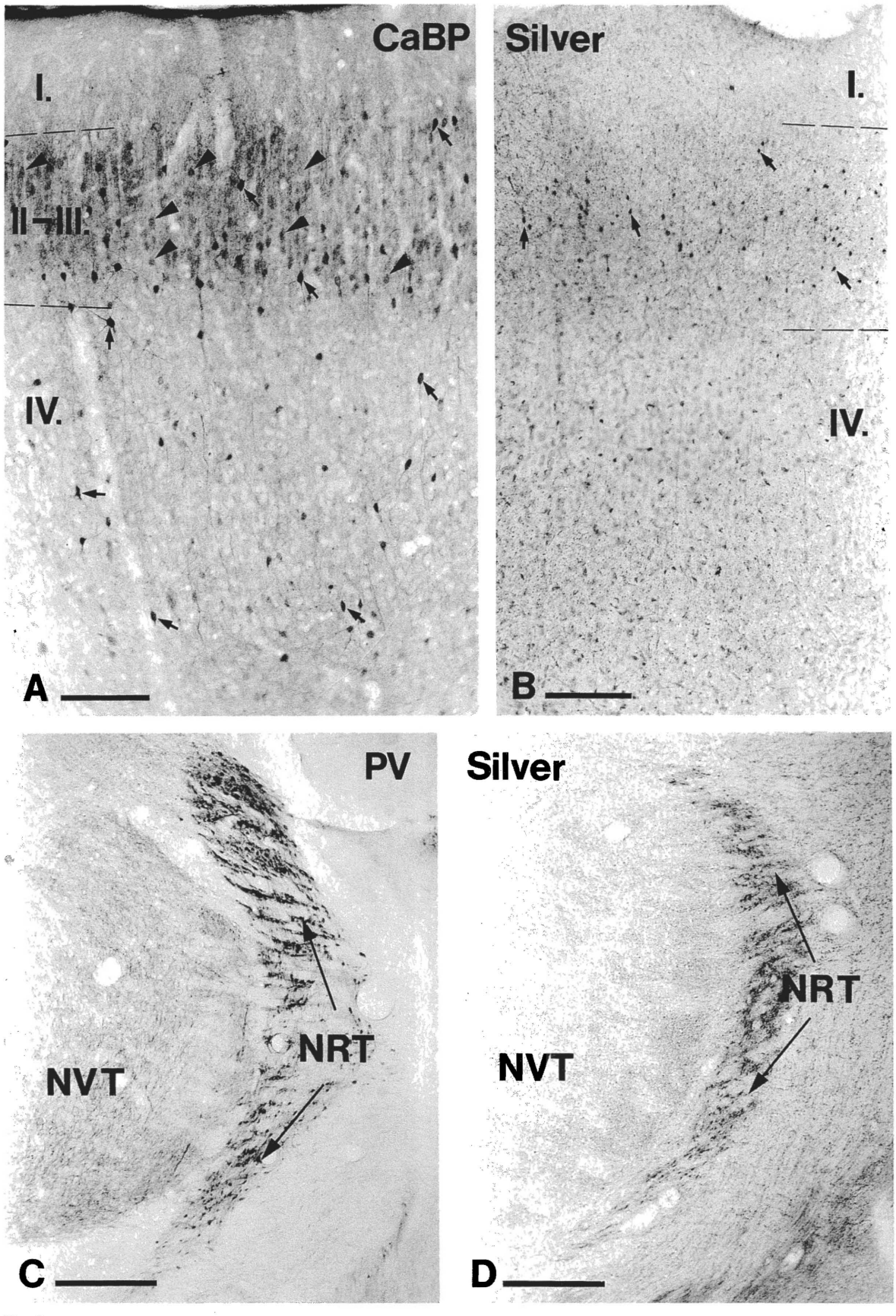

Fig. 3

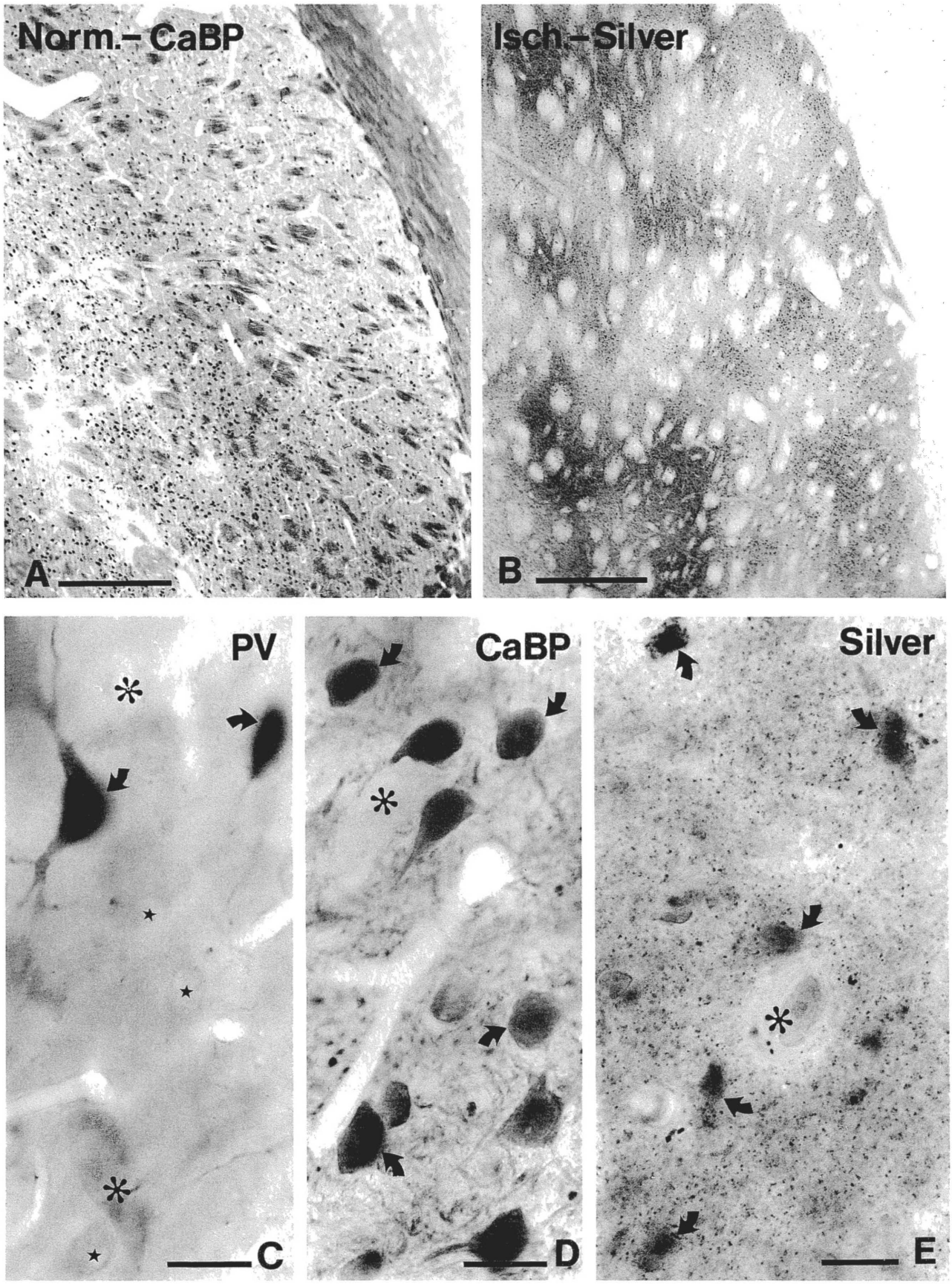

Fig. 4

the same type could be seen. All positive neurons had darkly stained somata and faint processes, in contrast to the non-pyramidal cells of the hippocampus and neocortex, which showed strong staining of their dendritic trees. Immunoreactivity for PV was detected in a subpopulation of medium sized neurons with smooth dendrites scattered in all regions of the neostriatum. They occurred with a higher frequency in the lateral quadrant, had long dendrites which were heavily stained and occasionally, stained axonal processes were evident. The giant cells represent a characteristic cell type of the neostriatum. They can be distinguished from the rest of the striatal neurons, since their soma diameter (25–35 μm in) is at least two times that of the medium size neurons (10–15 μm). Most if not all of these cells are cholinergic and are immunoreactive for choline acetyltransferase (ChAT) in the neostriatum (Bolam et al. 1984; Sofroniew et al. 1982). In the present study the large neurons showing the characteristic features of giant cholinergic cells (e.g. lobulated nucleus, large amount of endoplasmic reticulum) were not immunoreactive for either CaBP or PV (Fig. 4C, D).

Pattern of ischemic cell death. The neostriatum, especially its dorsolateral quadrant, showed advanced degeneration and argyrophilia in all animals which had severe hippocampal damage. Several degenerated neurons had already disappeared from the striatum after a survival time of 6–7 days used in this study. The remaining shrunken and argyrophilic neurons were found in patches intermingled with areas that contained apparently healthy cells (Fig. 4B). No correlation could be found in alternate sections stained for CaBP between patches of degeneration and areas rich in CaBP-immunoreactive neurons, except for the dorsolateral striatum. This region contains relatively fewer CaBP cells, and it is subject to ischemic degeneration more often than other striatal areas. The distribution and frequency of PV-immunoreactive neurons appeared unchanged. The most remarkable finding in the neostriatum was that the giant cholinergic cells (which do not contain either CaBP or PV) selectively survived in the neostriatum, even in areas, where all other cell types disappeared (Fig. 4E).

Fig. 4A–E. Relationship of neuronal calcium binding protein content and vulnerability to ischemia in the neostriatum. **A** CaBP-immunostained section cut from the medial and dorsolateral area of the neostriatum from a normal animal. The CaBP-positive medium size neurons form irregular patches, which are more sparse in the dorsolateral region. **B** The same area of the neostriatum is shown here as in **A**, but from an ischemic animal. Silver impregnation reveals numerous shrunken, argyrophilic neurons forming irregular patches. The dorsolateral part is affected to the same extent as the medial. **C** PV-immunostained section from the normal neostriatum showing medium size smooth dendritic neurons (curved arrows) strongly immunoreactive for parvalbumin. The medium size spiny neurons (stars) and giant cells (asterisk) are always negative for PV. One of the giant cells (below) is stained slightly stronger than the other negative cells, but this is only because this cell is right on the surface of the section, where background staining is stronger. **D** CaBP-immunostained section from the normal neostriatum showing medium size neurons (curved arrows) strongly immunoreactive for CaBP. The giant cells (asterisk) are always negative for this calcium binding protein as well. **E** Silver impregnated section from the dorsolateral neostriatum of an ischemic animal. Only a few shrunken, argyrophilic neurons of the medium size category (curved arrows) are still visible. The cell type which selectively survives ischemia in the neostriatum is the giant cell (asterisk). Scales: **A, B** 500 μm; **C–E** 20 μm

Discussion

No consistent relationship was found between calcium binding protein content and vulnerability to ischemia, in the four brain areas selected in this study. Two of these areas, the hippocampus and the neostriatum, are known to be the most vulnerable in all animal models of global forebrain ischemia (Blomqvist and Wieloch 1985; Crain et al. 1988; Francis and Pulsinelli 1982; Ito et al. 1975; Kirino 1982; Kirino et al. 1984; Petito and Pulsinelli 1984; Pulsinelli et al. 1982a, b; Schmidt-Kastner and Hossmann 1988; Schmidt-Kastner et al. 1989; Smith et al. 1984), whereas the pattern of damage in the neocortex and the reticular thalamic nucleus in ischemia is less predictable. The distribution of CaBP- and PV-immunoreactive cell types in these structures is well known, thus they provide an opportunity for studying the relationship between the patterns of ischemic damage and calcium binding protein content of neurons.

The hippocampal formation

Two major populations of principal cells contain CaBP in this region, the dentate granule cells, and the CA1 pyramidal cells (Baimbridge et al. 1982; Sloviter 1989). The former group is the most resistant and the latter the most vulnerable to ischemia. The CaBP-positive non-pyramidal cells in all regions and the CA3 pyramidal cells which do not contain CaBP are equally resistant (Mudrick and Baimbridge 1989).

Non-pyramidal cells immunoreactive for PV (Katsumaru et al. 1988; Kosaka et al. 1987; Sloviter 1989) are also remarkably resistant to ischemia, as also found recently in the gerbil (Nitsch et al. 1989) and in a long term survival experiment in the rat (Mudrick and Baimbridge 1989). However, in the present study 3 animals showed a partial loss of PV-immunoreactivity in patches of the CA1 region, where most if not all pyramidal cells were also damaged. In earlier studies GABAergic non-pyramidal cells were reported to be remarkably resistant in all regions of the hippocampal formation (Johansen and Diemer 1986; Schlander et al. 1988). This difference may be due to a longer ischemic period leading to damage of some interneurons in the present study. In a recent study Johansen et al. (1989) also found a small reduction in the number of GAD immunoreactive neurons in the hippocampus, which is likely to be due to the loss of PV-positive neurons in the most severely affected areas. It could be argued that these PV-positive cells only stopped parvalbumin synthesis, but they have remained alive. Our observation that the surviving PV-positive

cells in these areas were shrunken and occasionally had swollen dendrites suggests that the missing PV-containing cells in CA1 have in fact degenerated rather than merely stopped synthetising PV. The hilar somatostatin cells, which are the first to degenerate after ischemia (Johansen et al. 1987b), do not contain either CaBP or PV.

It should be noted here that protein synthesis never recovers in the CA1 region after ischemia, and is impaired in the resistant regions as well for up to 12 hours (Thilmann et al. 1986). However, if a persistently impaired synthesis of calcium binding proteins contributed to ischemic cell death then one would expect that neurons requiring calcium binding proteins for the regulation of intracellular calcium levels would suffer the most. Yet the interneurons containing either CaBP or PV and the strongly CaBP-positive dentate granule cells are among the most resistant cell types. We can conclude that in the hippocampal formation the presence or absence of calcium binding proteins is not a reliable predictor of vulnerability to ischemia.

Neocortex

The majority of supragranular pyramidal cells contain CaBP, and this is the population of pyramidal cells most frequently degenerating after ischemia in the sensory areas examined, especially in the somatosensory cortex, as also shown by Crain et al. (1988) in the gerbil. The density and distribution of non-pyramidal cells containing CaBP or PV (Celio and Heizman 1981) appeared qualitatively unchanged, none of the shrunken argyrophilic neurons had dendritic arbors similar to that of non-pyramidal cells (Crain et al. 1988). The distribution of terminal degeneration, i.e. in layers II–III and V, also suggests that the supragranular pyramidal cells having collateral projections to these layers (Nauta et al. 1973) are affected.

Reticular thalamic nucleus

Most if not all neurons in the RTN were strongly immunoreactive for PV and stained faintly for CaBP, and became argyrophilic after ischemia. However, the neurons did not appear shrunken, only their nuclei showed strong argyrophilia. This suggests that the RTN neurons are in an early phase of irreversible degeneration (Smith et al. 1984; Ross and Duhaime 1989), or they may be reversibly damaged and show only a transient argyrophilia. This question should be studied in long term survival experiments.

Neostriatum

No consistent relationship was found between ischemic vulnerability and calcium binding protein content in the neostriatum. CaBP is present in medium spiny neurons only of the matrix compartment (Gerfen et al. 1985), but this cell type was found to degenerate in large areas which had to include both patch and matrix compartments (Crain et al. 1988; Francis and Pulsinelli 1982; Ito et al. 1975; Pulsinelli et al. 1982a). Interestingly, striatal damage always begins in the dorsolateral quadrant of the nucleus (Crain et al. 1988; Ito et al. 1975; Pulsinelli et al. 1982a), which is relatively poor in CaBP-positive neurons (Gerfen et al. 1985). The giant cholinergic neurons, which contain neither PV nor CaBP (Gerfen et al. 1985; and this study), appear to be the most resistant, as also shown biochemically (Francis and Pulsinelli 1982).

Conclusions

Although, calcium influx is thought to be an important pathophysiological factor in ischemic neuronal degeneration (Andine et al. 1988; Deshpande et al. 1987; Dienel 1984; Siesjo 1981; Siesjo and Bengtsson 1989), the buffering of intracellular calcium by CaBP and PV appears to be insufficient to protect against cell death. In fact a large proportion of neurons in some areas which do contain one or both of these calcium binding proteins are particularly predisposed to ischemic cell death. It could be speculated that these neurons normally require calcium binding proteins for the strict regulation of intracellular calcium levels in connection with neurotransmitter receptors, second messenger systems or different mechanisms of neuronal plasticity, which employ calcium in their normal operations (Andine et al. 1988; Blaxter et al. 1986; Lynch and Baudry 1984; Monaghan and Cotman 1985; Rothman and Olney 1986; Siesjo and Bengtsson 1989). Such neurons are likely to be more sensitive to disturbances of intracellular calcium homeostasis caused by ischemia, which may lead to permanent malfunctioning of one or more of these mechanisms, and ultimately to cell death. The types and sensity of different excitatory amino acid receptors on different cell types may correlate with the degree of calcium overload in these neurons after ischemia. The relationship of the receptor type and distribution to neuronal calcium binding protein content and ischemic vulnerability should be the subject of future studies.

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