# THE DISTRIBUTION OF

# CAT-301 IMMUNOREACTIVITY

# IN THE CEREBRAL CORTEX

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Submitted to the University of Edinburgh
for the degree of M.D.
1992

Research performed in the Section of Neuroanatomy
Yale University School of Medicine

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# DECLARATION

I declare that this thesis has been composed by myself and that the work on which it is based was my own, with the exception of the generation of the Cat-301 antibody, which was performed by one of my collaborators.

**Signed:**

# ABSTRACT

The distribution of the monoclonal antibody Cat-301 was examined in the cerebral cortex of macaque monkeys. Throughout the cerebral cortex, Cat-301 labelled the soma and proximal dendrites of a restricted population of neurones. The distribution was uniform within cytoarchitecturally defined areas (or subareas) but varied between them, with respect to the density of labelled neurones, the intensity of their immunoreactivity, their morphology, and their laminar distribution.

Large numbers of intensely immunopositive neurones were evident in motor related areas in the frontal lobe, somatosensory areas in the parietal lobe, and areas specialised for the analysis of visual motion in the parietal and occipital lobes. The heavily labelled areas are known to be interconnected, and the Cat-301 positive cells within them were concentrated in the laminae from which their cortico-cortical connections arise. Given the critical role of somatosensory and visuospatial information in the execution of somatic and ocular movements, the heavily labelled areas may be regarded as sharing a broadly motor function.

The timing of the expression of the antigen recognised by Cat-301 during development, its peri-synaptic localisation and its biochemical characteristics suggest that it may play a role in the stabilisation of

synaptic connections. Cat-301 may label networks of areas with a similar functional specialisation because the antigen plays such a role in relation to the specific interconnections that exist between them.

# ACKNOWLEDGEMENTS

Professor Patricia Goldman-Rakic for her help, encouragement and expert advice, and for giving me the opportunity to work in her department.

Dr Susan Hockfield for her enthusiasm, her advice, and for providing generous quantities of the Cat-301 antibody.

Mary Pappy and JoAnn Coburn for expert technical assistance with the histology.

Joseph Musco for invaluable technical advice on the preparation of the figures.

The Saint Andrew's Society of the State of New York for their award of a scholarship supporting my work at Yale University.

Professor J. S. Kelly for the assistance and advice he provided in his role as supervisor of this thesis.

# ABBREVIATIONS

| | |
|---|---|
| AMOG | Adhesion molecule on glia |
| CNS | Central nervous system |
| CTB | Cytotactin binding proteoglycan |
| DAB | Diaminobenzidine |
| DEAC | Diethylaminoethylcellulose |
| DHMEM | Dulbecco's modified essential medium |
| DIC | Differential interference contrast |
| FEF | Frontal eye fields |
| GABA | γ-amino butyric acid |
| GABAergic | Containing γ-amino butyric acid |
| GAD | Glutamic acid decarboxylase |
| HAT | Hypoxanthine aminopteridine thymidine |
| HPRT | Hypoxanthine phosphoribosyl transferase |
| HRP | Horseradishperoxidase |
| $H_2O_2$ | Hydrogen peroxide |
| IgG | Immunoglobulin type G |
| IgM | Immunoglobulin type M |
| IPS | Intraparietal sulcus |
| KD | Kilodalton |
| LAMP | Limbic system associated membrane protein |
| L-CAM | Liver cell adhesion molecule |
| LGN | Lateral Geniculate Nucleus |
| LHRH | Luteinising hormone-releasing hormone |
| LIP | Lateral intraparietal area |

| | |
|---|---|
| μ | micron |
| MST | Medial superior temporal area |
| MT | Middle Temporal area |
| N-CAM | Neural cell adhesion molecule |
| Ng-CAM | Neural-glial cell adhesion molecule |
| NILE | Nerve growth factor-inducible large external glycoprotein |
| PEG | Polyethylene glycol |
| PBS | Phosphate buffered saline |
| PG | Posterior parietal area lateral to caudal part of IPS |
| POa | Posterior parietal area in ventral bank of IPS |
| POa(i/e) | Internal/External division of POa |
| SAM | Substrate adhesion molecule |
| SD | Standard deviation |
| SMA | Supplementary motor area |
| VIP | Ventral intraparietal area |
| VLa | Ventral lateral anterior nucleus |
| VLp | Ventral lateral posterior nucleus |
| VPL | Ventral posterior lateral nucleus |
| VPM | Ventral posterior medial nucleus |
| V1 | Primary visual cortex |
| V2 | Secondary visual cortex |
| 6D | dorsal part of area 6 |
| $6V_u$ | upper portion of the ventral part of area 6 |
| $6V_L$ | lower portion of the ventral part of area 6 |
| 7ip | Portion of area 7 in the ventral bank of the IPS |

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# Chapter 1:

# INTRODUCTION

## 1.1 APPROACHES TO STUDYING CELLS IN THE NERVOUS SYSTEM

The cells which constitute the nervous system have been studied in a number of ways, and probably the earliest approach employed was to examine their morphology. After light microscopy permitted the first description of nerve cells by Ehrenberg (1833), tissue staining techniques, notably that employed by Golgi (1878), began to reveal a variety of detailed morphologies which were subsequently catalogued in remarkable detail by Cajal (1911). More recently, equally striking images of neurones and their processes have been produced with the intracellular injection of dyes such as horseradish peroxidase (HRP) or Lucifer Yellow (Gilbert and Wiesel, 1979).

Morphology remains an extremely useful form of classification, and is still the principal means of defining neuronal types in a variety of regions, including the cerebral cortex (Peters and Jones, 1984). However, subsequently the advent of neurochemistry and electrophyisiology has allowed neurones to be studied from quite different perspectives. Since 1933, when Dale categorised autonomic nerve fibres as either adrenergic or cholinergic, neurones throughout

the nervous system have been characterised by their one or more neurotransmitters (Cooper et al, 1978; Campbell, 1987). For example, non-pyramidal neurones in the cerebral cortex contain gamma butyric acid (GABA), and may be subdivided with respect to the peptide transmitters they may also contain (Jones, 1986b). Electrophysiological recording has developed from the pioneering work of Hodgkin and Huxley in the squid giant axon (Hodgkin and Huxley, 1952), and been applied to virtually every neural region, most famously in the visual cortex, where Hubel and Wiesel (1962) characterised cells in terms of their responses to visual stimuli.

The development of these more recent techniques was significant, less because they offered greater sophistication or resolution than the morphological approach, but rather because they offered a qualitatively different perspective, providing access to new categories of information.

## 1.2 STUDYING NEURAL CELLS IN TERMS OF SURFACE MOLECULES

A relatively novel means of studying neurones involves their characterisation in terms of the surface molecules which reside on their plasma membranes. This type of approach has proved immensely fruitful in the immune system, where morphologically

similar cells can thus be differentiated into classes (and further subclasses) with distinct functional characteristics. For example, there is little to distinguish T and B lymphocytes morphologically, but their respective surface antigenic profiles are quite differerent, as are their respective functions (Roitt, 1982) The identification of cell surface antigens has also lead to great advances in the understanding of the development of different types of leucocytes from pluripotent stem cells. At each stage of differentiation, the cells carry particular combinations of specific molecules on their surface membranes, and the identification of these allows one to trace the lineage of different cell types (Foon, 1982).

Research in molecular biology and genetics suggests that the phenotype of a given cell at least partly reflects a particular pattern of gene expression. Each distinct neuronal phenotype may thus be associated with a specific constellation of cellular proteins, some of which are likey to be found in the surface membrane. By detecting such molecules, it may be possible to recognise distinct neuronal phenotypes with a sensitivity which is beyond the resolution of morphological, neurochemical or electrophysiological techniques.

The study of surface molecules may thus provide information on the functional characteristics of neurones, their lineage, and might permit the resolution of distinct neuronal phenotypes.

## 1.3 IDENTIFYING SURFACE MOLECULES - ANTIBODIES AND LECTINS

Antibodies seem to offer an ideal means of detecting surface molecules. The N-terminal binding sites of immunoglobulins confer an extremely high level of specificity, such that high affinity binding is effectively restricted to a particular 'epitope' with the complementary molecular conformation. A given epitope is often unique to a single antigen, so that the antibody which binds it will also be specific for the antigen concerned.

Lectins are plant proteins which bind to specific monosaccharides at the ends of oligosaccharide chains (White et al, 1978), including those which constitute the carbohydrate portion of cell surface glycoproteins. They can thus be used to recognise surface molcules in a similar way to immunoglobulins, although antibodies have been far more extensively used to this end.

## 1.4 PRINCIPLES OF IMMUNOCYTOCHEMISTRY

Immunoglobulins can be conjugated with enzymes, flourescent molecules or radioactive substances which can act as markers for the sites of antibody binding in a given tissuse. Agents commonly used

for this purpose are horseradishperoxidase (HRP), fluorescein and rhodamine.

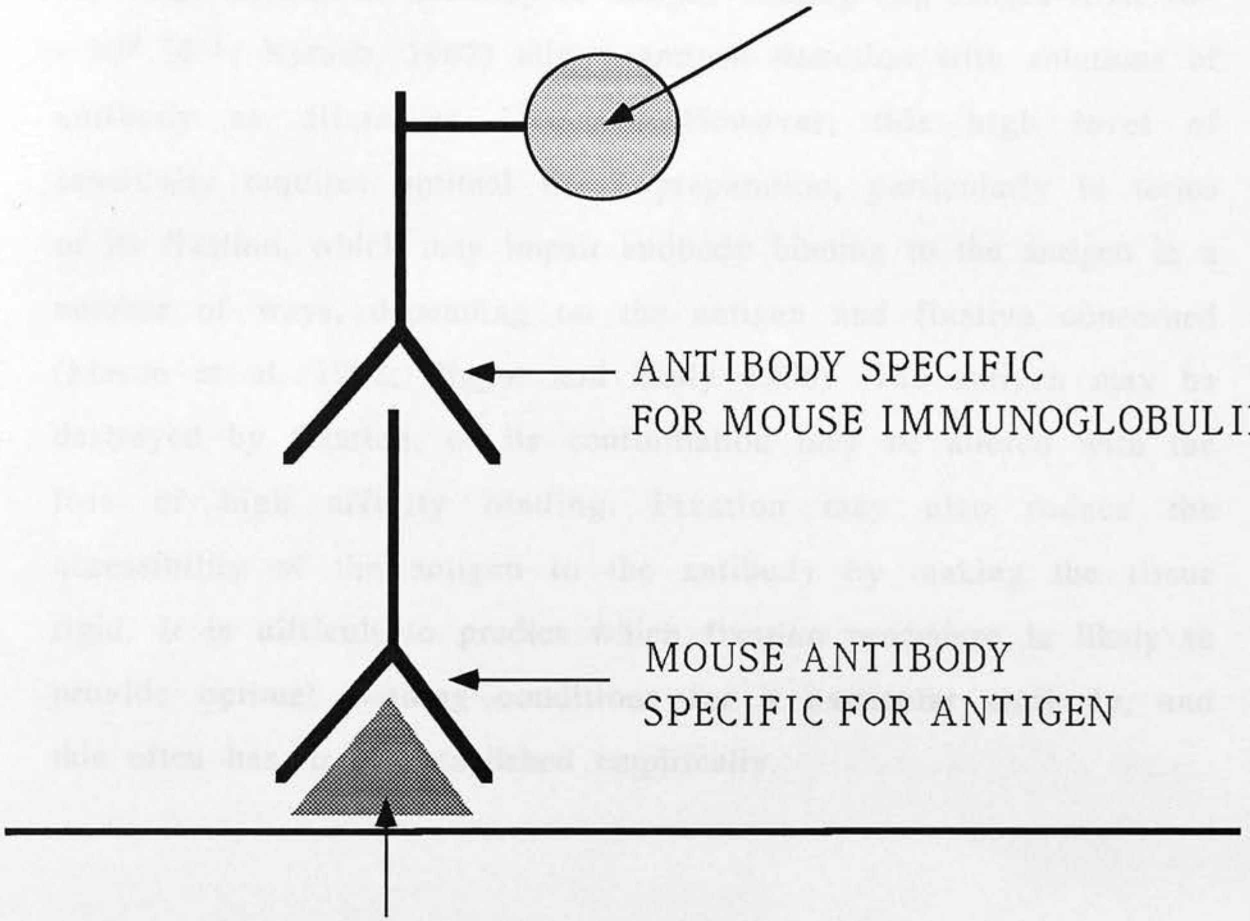
With immunofluorescent labelling, the tissue morphology cannot be visualised at the same time as its labelled elements, making precise identification of the sites of antibody binding difficult. The fluorescence fades with examination and with time, limiting the period which can be spent viewing the material and precluding the reassessment of sections at later dates (Mason et al, 1982).

Immunocytochemistry with HRP overcomes these difficulties, although it is methodologically more complicated. It can be performed in a number of ways, with the indirect immunoperoxidase technique one of the most popular and effective methods (Sternberger, 1986). A first antibody, typically raised in a mouse, binds to the antigen of interest, and is in turn bound by a second anti-murine antibody conjugated with HRP (Fig. 1). When hydrogen peroxide ($H_2O_2$), is added it is bound by the enzyme, which is oxidised to a more active form. This oxidation product can be reduced by amines which produce coloured compounds when they are oxidised. The oxidation of diaminobenzidine (DAB) produces an intensely brown reaction product which forms a polymer that is highly insoluble and unlikely to diffuse from its site of formation (Sternberger, 1986), thus providing a stable marker which remains localised to the point of antibody binding.

Immunoperoxidase labelling can be visualised with light micoscopy, and the surrounding unstained tissue can be seen at the same time, allowing accurate localisation of the sites of antibody binding. As the reaction product can be chelated with osmium tetroxide, it can also be visualised with electron microscopy.

**FIGURE 1. The indirect immunoperoxidase technique.**

A murine antibody specific for the antigen (triangle) binds to it on the cell surface. A second antibody specific for murine immunoglobulin and pre-conjugated with the enzyme HRP binds to the first antibody, thus indirectly linking the antigen with the HRP. With the application of $H_2O_2$ and DAB, the HRP catalyses the production of an insoluble reaction product which acts as a stable marker of the antigen's location in the tissue.


HORSERADISH PEROXIDASE
ANTIBODY SPECIFIC
FOR MOUSE IMMUNOGLOBULIN
MOUSE ANTIBODY
SPECIFIC FOR ANTIGEN
ANTIGEN ON CELL SURFACE

## 1.5 SENSITIVITY OF IMMUNOCYTOCHEMISTRY

The high affinity of antibody to antigen binding ($K_a$ ranges from $10^5$ - $10^9$ $M^{-1}$: Karush, 1962) allows antigen detection with solutions of antibody as dilute as 1/10,000. However, this high level of sensitivity requires optimal tissue preparation, particularly in terms of its fixation, which may impair antibody binding to the antigen in a number of ways, depending on the antigen and fixative concerned (Mason et al, 1982; Piggot and Kelly 1986). The antigen may be destroyed by fixation, or its conformation may be altered with the loss of high affinity binding. Fixation may also reduce the accessibility of the antigen to the antibody by making the tissue rigid. It is difficult to predict which fixation procedure is likely to provide optimal staining conditions for a particular antibody, and this often has to be established empirically.

## 1.6 SPECIFICITY OF IMMUNOCYTOCHEMISTRY

While antibodies posess a remarkably high degree of specificity, until relatively recently it was difficult to obtain pure solutions of single immunoglobulins for use in immunocytochemistry. Antibodies are usually generated by immunising an experimental animal with antigenic material, but it is often difficult to obtain a highly purified

preparation of the antigen of interest. Even when this is available, the resulting sera almost always contain a 'polyclonal' mixture of immunoglobulins with different specificities. When these are applied immunocytochemically, there may consequently be 'background' labelling of antigens other than that of the antigen of interest. Fortunately, this problem can largely be avoided with the use of 'monoclonal' antibodies (Chapter 1.7).

However, even with monoclonal antibodies, one cannot assume that the observed labelling reflects specific binding to a single antigen, as the epitope concerned may be common to more than one molecule. For example, the monoclonal VC1.1 binds to four molecules of 100, 140, 170 and 700KD, respectively, in the synaptosomal membrane fraction of cat visual cortex (Arimatsu et al, 1987). This can be investigated by separating the tissue proteins with electrophoresis and identifying the number of species of distinct molecular weight bound by the antibody, the method used in the example above.

## 1.7 MONOCLONAL ANTIBODIES

In 1975, Kohler and Milstein devised a means of isolating single antibody-producing cells from animals immunised with preparations containing antigens of interest. Splenic lymphocytes are mixed with myeloma cells deficient in hypoxanthine phosphoribosyl transferase

(HPRT), and fused to form hybrids which thus acquire the lymphocyte's capacity for antibody synthesis and the tumour cell's limitless capacity for division. The hybrid cells are selected out by growth on a medium which will not support cells deficient in HPRT, then separated by dilution, with each dividing to form a clone of identical progeny which produce 'monoclonal' antibodies of a single specificity. Those producing antibodies of interest can be identified by screening their immunoglobulins against tissue containing the relevant antigens, using conventional immunocytochemical techniques.

Hybridoma technology thus allows the generation of pure preparations of monospecific antibodies from specimens of tissue which may contain a vast number of antigenic species. Furthermore, limitless quantities of antibody are available as long as the relevant hybridoma line is maintained. This can be achieved using tissue culture technology, or by injecting hybidoma cells into the peritoneal cavity, producing peritoneal tumours whose antibodies can be harvested from the peritoneal fluid.

# Chapter 2:

# IMMUNOCYTOCHEMISTRY AND NEURAL SURFACE MOLECULES

## 2.1 IMMUNOCYTOCHEMISTRY IN NEURAL TISSUE

Immunocytochemistry has a well established place in the study of the nervous system and has provided a wealth of data, particularly in the field of neurochemistry, where immunocytochemical studies have played a major role in the characterisation of the neurotransmitter systems (Hockfelt et al, 1978; Jones, 1986b). Much of this work has dealt with the distribution of intracellular molecules in nervous tissue, such as components of the cytoskeleton, neurotransmitters, or enzymes. However, more recently there has been increasing interest in applying these techniques to the study of molecules which lie on the neuronal surface.

In the following review of the literature, the surface molecules that have been examined have been grouped together in terms of the broad functions in which they may participate, and the cell types on which they are found.

## 2.2 SURFACE MOLECULES AND EARLY NEURAL DEVELOPMENT

In 1977, Brackenbury et al raised antibodies against embryonic retinal cells and showed that they powerfully inhibited the aggregation of retinal cells that normally occurs *in vitro*. They identified an antigen which, when pre-absorbed to the antibodies, potently neutralised their action. This molecule was purified and characterised as a 140KD glycoprotein (Thiery et al, 1977). As the antibodies against it had specifically blocked cellular adhesion, it was suggested that it served to mediate the adhesive process, and was termed the neural cell adhesion molecule (N-CAM). Other cell adhesion molecules (CAMs) have subsequently been identified in a similar way.

N-CAM seems to be expressed at very early stages of development. In the chick embryo, the cells of the ectoderm are N-CAM positive, and at the time of neural induction, those in the region of the presumptive neural plate and groove become more immunoreactive, while those in the surrounding somatic ectoderm lose their N-CAM immunoreactivity (Thiery et al, 1982). Other CAMs can also be identified on ectodermal cells at this stage. Immunoreactivity to the liver cell adhesion molecule (L-CAM), which is structurally distinct from N-CAM, increases on the cells in the somatic ectoderm and decreases on those in the presumptive neural plate, thus being

reciprocally distributed to N-CAM immunoreactivity (Edelman et al, 1983). Conversely, immunoreactivity to N-Cadherin, an integral membrane protein of 127 KD which shares structural motifs with N-CAM (Shirayoshi et al, 1986; Lander, 1989), disappears from the notocord but is expressed on the developing neural groove and tube (Hatta et al, 1986; 1987).

In addition to their effects on neuronal adhesion *in vitro*, antibodies to N-CAM perturb neural development when administered *in vivo* (Fraser et al, 1984). N-CAM's adhesive function can be demonstrated more directly by transfecting cells with the N-CAM gene. The transfected cells then express the N-CAM on their surfaces and acquire the ability to aggregate (Edelman et al, 1987). N-CAM binds neurones which express it together in a homophilic manner, with molecules on the surface of each cell linking to form a dimer (Rutishauser, 1982). However, recent studies of retinal cell aggregation suggest that binding to a neuronal surface heparan sulphate proteoglycan may also be necessary for N-CAM's adhesive effect (Cole and Glaser, 1986), and a proteoglycan binding site has been identified on the N-CAM molecule (Cole and Akeson, 1989).

## 2.3 SURFACE MOLECULES AND NEURONAL MIGRATION

### 2.3a. Migration of cells from the neural crest

The development of the peripheral nervous system involves the migration of cells from the embryonic neural crest to form the presumptive sensory and sympathetic ganglia. During migration, the cells can be selectively labelled with antibodies to integrin (Duband et al, 1986; Krotoski, 1986), a protein comprising two transmembrane polypeptides (alpha) and (beta) (Lander, 1989). Integrin preferentially binds to fibronectin and laminin, two glycoproteins found in the extracellular matrix and on other cell surfaces (Sanes, 1989). In the cranial region of the embryo, the neural crest cells migrate between blocks of mesoderm termed somites, and their migratory paths are strongly labelled with antibodies against both fibronectin (Sanes, 1983) and laminin (Duband and Thiery, 1987). Moreover, antibodies specific for integrin inhibit cranial neural crest cell migration (Bronner-Fraser, 1986). Neural crest cell migration in this region may thus involve binding between integrin and fibronectin or laminin.

In the trunk region, rather than migrating between the somites, the neural crest cells pass through the anterior part of the portion of the somites known as the sclerotome (Sanes, 1989). Just before migration begins, the anterior parts of the sclerotomes become strongly

immunoreactive to antibodies specific for cytotactin, an extracellular matrix protein comprised of three polypeptides with molecular weights of 190, 200 and 220KD (Grumet et al 1985; Mackie et al, 1988). Meanwhile, the expression of its specific ligand, a 280KD extracellular matrix proteoglycan termed cytotactin binding proteoglycan (CTB), decreases in the anterior sclerotome, but persists in its posterior part (Tan et al, 1987). These changes occur in the absence of neural crest cells (suggesting that they are not induced by crest cell migration), and cytotactin inhibits the migration of neural crest cells *in vitro* (Tan et al, 1987). Thus, in the embryonic trunk, the migration of neural crest cells may be modulated by cytotactin.

### 2.3b. Migration of neurones along glial processes

After their generation, the presumptive external granular cells of the cerebellum migrate into their mature positions in the cerebellar cortex by adhering to and moving along the processes of Bergmann glial cells (Rakic, 1985). In mice and rats this occurs shortly after birth. A similar mechanisim is used by migrating cells in the developing cerebral cortex and hippocampus (Rakic, 1972; Hatten, 1990). Surface molecules have been implicated in mediating the neurone-glial interaction this process entails.

In the developing cerebellum, antibodies against cytotactin selectively label the molecular layer and the Bergmann glial

processes, and the application of such antibodies *in vitro* arrests granule cell migration in the molecular layer (Chuong et al, 1987). Cytotactin is synthesised by glial cells (Hoffman et al, 1988), whereas neural-glial cell adhesion molecule (Ng-CAM) is synthesised by neurones, and expressed on external granular cells and their parallel fibres in the molecular layer (Rathjen and Schachner, 1984). When its distribution is examined with the electron microscope, Ng-CAM immunoreactivity is localised between parallel fibres, rather than at points of neurone-glial apposition (Persohn and Schachner, 1987). Although antibodies specific for Ng-CAM arrest granule cell migration in the external granular layer in cerebellar slice preparations (Hoffman et al, 1988), they fail to block granule cell-glial binding *per se* (Stitt and Hatten, 1989). Cerebellar Ng-CAM expression also persists beyond the period of granule cell migration (Antioniceck, 1987). Ng-CAM may thus influence granule cell movement through an effect on neurone to neurone, rather than neurone-glial adhesion.

Astrotactin is a 100KD neuronal surface protein which is expressed by cerebellar neurones during their period of migration (Hatten, 1990). Antibodies against it inhibit adhesion between cerebellar neurones and glia *in vitro* (Edmondson et al, 1988), but their effect is blocked if they are pre-incubated with normal cerebellar neurones. The *Weaver* mutant is a strain of mouse in which there is a characterisitic failure of cerebellar neuronal migration. *Weaver* neurones are unable to neutralise the inhibitory effect of antibodies

to astrotactin on cerebellar neurone-glial adhesion. Moreover, biochemical analysis indicates that the mutant neurones are 95% deficient in astrotactin (Edmondson et al, 1988).

Another candidate for mediating cerebellar neurone-glial adhesion is AMOG (Adhesion Molecule On Glia), a 50KD surface protein which is detectable on glia at the time of granule cell migration, and shares a carbohydrate epitope with Ng-CAM (Antoniceck, 1987). Electron microscopy reveals that AMOG immunoreactivity is restricted to Bergmann glial processes, and AMOG-specific antibodies inhibit granule cell migration *in vitro* (Antoniceck, 1987).

Finally, the monoclonal antibody Rat-401 appears to recognise a 200KD antigen which is selectively expressed on radial glia, which are thought to mediate neuronal migration in the cerebral cortex. The expression is limited to the period in which cells in the ventricular zone of the neural tube proliferate and migrate to their adult positions. Rat-401 immunoreactivity then disappears and there is no labelling of cells in the mature rat brain (Hockfield and McKay, 1985)

## 2.4 SURFACE MOLECULES AND AXONAL GROWTH

In 1963 Sperry suggested that growing axons might successfully locate their appropriate synaptic targets among the enormous number of potential sites within the developing brain by 'recognising' chemicals specific to particular neurones. This 'chemoaffinity' hypothesis has subsequently been modified to the extent that the chemicals involved are thought to be expressed on surface membranes, and believed to exert their influence along the path of the growing axon, as well as its destination.

Surface molecules may influence the initial outgrowth of axons as they extend beyond the cell body, and several of these have been mentioned in the context of their putative roles at earlier developmental stages. N-CAM and N-cadherin are both expressed on the surfaces of neurones during periods of initial axonal extension, and antibodies specific to these glycoproteins inhibit axon outgrowth *in vitro* (Edelman, 1988; Takeichi, 1988). Both have relatively uniform distributions throughout the nervous system, suggesting that they may generally facilitate axonal growth rather than influence the particular routes axons take.

Axonal extension *in vitro* is promoted if the substrata over which they grow is coated with the extracellular matrix glycoprotein laminin (Rogers et al, 1983). Antibodies specific for laminin inhibit

neurite outgrowth (Edgar et al, 1988), and laminin immunoreactivity has a relatively restricted distribution in the developing nervous system. Thus, it delineates the path which trigeminal sensory axons follow through the mesenchyme to their peripheral targets (Rogers et al, 1986), and is expressed along the trajectories of axons in the ventral longitudinal fasiculus of the rat just before they invade (Letourneau et al, 1989), ahead of advancing retinal axons in the optic nerve (McLoon et al, 1988), and in developing muscle as motor axons approach (Chiu and Sanes, 1984). *In vivo*, laminin exists in a complex with heparan sulphate proteoglycan (Lander et al, 1985), which may influence its binding properties (Sanes, 1989). It binds to integrin (Chapter 2.3a), which is expressed on the surfaces of growing axons, and antibodies to integrin block axon extension on laminin-coated substrata (Bozyczko et al, 1986). Integrin also binds to fibronectin (Chapter 2.3a), which, like laminin, can act as an effective substrate for axonal outgrowth *in vitro* (Hatten et al, 1982).

The particular route which an axon follows as it grows inside a fibre tract may be critically influenced by its selective attachment to other axons which are already present within the tract. This process of 'fasciculation' can result in its extension along specific pre-existing axons and thus lead to its growth along a particular path. The 'labeled pathway hypothesis' proposes that fasciculation entails the recognition of specific molecules on the surfaces of pre-existing axons by the growing axon (Goodman et al, 1982).

In vertebrates, immunocytochemistry with antibodies specific to the glycoproteins 8D9, G4, and F11, indicates that each of these molecules is concentrated on fasciculating axons during development. These molecules promote the extension of neurites on axons *in vitro* (Langeur and Lemmon, 1987; Chang et al, 1987) an effect blocked by the antibodies against them (Chang et al, 1987). 8D9 and G4 are structurally similar to Ng-CAM, whereas F11 appears to be an unrelated species (Rathjen et al, 1987) Monoclonal antibodies RB-8 and TAG-1 are differentially distributed within fibre tracts, RB-89 recognising a 125KD integral membrane protein, while TAG-1 binds to a 135KD glycoprotein. Niether molecule has yet been shown to have an effect on fasciculation (Schwob and Gottlieb, 1988; Dodd et al, 1988).

In the nervous system of the leech, the monoclonal antibodies Lan 3-2 and Lan 4-2 recognise distinct glycoproteins which are selectively expressed on nociceptive mechanosensory axons (McKay et al, 1985). Axons which express the antigens are grouped together in fascicles within pathways termed connectives, and all the axons in such fascicles are immunopositive. During development, the Lan 3-2 glycoprotein is expressed as the axons grow into the connectives, and Lan 3-2 immunoreactivity appears on their terminal filopodia (McKay et al, 1985).

In the developing nervous system of the grasshopper, two glycoproteins, Fascilin I and II, are transiently expressed on axonal

surfaces in restricted locations within fibre tracts, during the period when these are negotiated by growing axons. Fascilin I is expressed along commissural pathways, whereas Fascilin II appears in longitudinal tracts (Bastiani et al, 1987). Another glycoprotein, Fascilin III, is expressed on axons within commissural connectives in the developing nervous system in drosophila (Patel et al, 1987). Deletion of the gene for Fascilin III results in only minor effects on axonal growth, with axons largely contacting their appropriate targets. Although this might suggest that it does not play a major role in axonal navigation, it has also been interpreted as indicating that multiple surface antigens are involved (Harrelson and Goodman, 1988).

## 2.5 SURFACE MOLECULES AND THE RECOGNITION OF AXONAL TARGETS

Once a growing axon has succesfully negotiated the appropriate route to its target region, it is still faced with the problem of locating and contacting specific neurones among the relatively large number within that region. A number of surface molecules have been implicated in this process.

The retino-tectal projection in many vertebrates is inverted such that axons from the dorsal half of the retina synapse on cells in the

lateral half of the tectum, and vice versa. In 1986, Constantine-Paton et al generated the JONES antibody against embryonic retinal cells. It is distributed along a dorso-ventral gradient on ganglion cells in the developing rat retina, and recognises a cell surface ganglioside. JONES immunoreactivity is also localised to regions of optic axon growth and the target regions of the optic axons (Medez-Otero, 1986). Its expression coincides with the arrival of retinal axons in the tectum, and persists throughout the period that the retino-tectal map is laid down.

The TOP glycoprotein has a similar distribution in the developing retino-tectal system of the chick. Antibodies specific for TOP have a dorso-ventral gradient of distribution in the retina, and a latero-medial gradient in the tectum, the level of TOP expression in a given part of the retina thus matching that in its tectal target zone (Trisler et al, 1986). Expression of the antigen coincides with the period of retino-tectal synapse formation, such that the distribution gradient is established when the initial axon-target interactions occur (Trisler et al, 1988). In the presence of TOP-specific antibodies, retinal growth cones persist beyond the usual period, and synapse formation is inhibited, suggesting that the antigen may facilitate retino-tectal synaptogenesis (Trisler et al, 1986).

The basal lamina of neuromuscular synapses is selectively labelled by antibodies specific for a heparan sulphate proteoglycan (Anderson and Famborough, 1983), a laminin-like glycoprotein termed JS1

(Sanes and Chui, 1983), and a protein termed Agrin (Resit et al, 1987). These antigens are expressed in the absence of motor neurones, suggesting that they are not induced by presynaptic elements, and JS1 is adhesive towards neurones (Hunter et al, 1988). A chondroitin sulphate proteoglycan, TAP1, is concentrated at the synapses of the *Torpedo* electric organ, which resemble those at the neuromuscular junction (Carlson and Wright, 1987). There are thus a number of molecules which are selectively expressed on the post-synaptic surface of the neuromuscular junction, and some are known to appear at a time when they could interact with ingrowing axons.

In the somatosensory cerebral cortex of the mouse, neurones are organised in 'barrel' fields, each of which receives thalamic afferents that convey inputs from a single snout whisker. During development, the presumptive boundaries of these fields are selectively delineated by antibodies to cytotactin and its ligand, cytotactin binding proteoglycan (CTB), before the corresponding cytoarchitectural features have emerged (Crossin et al, 1989). They are also labelled by lentil lectin, conconavilin A, and peanut and wheatgerm agglutinin (Cooper and Stiendler, 1986), but this may reflect the binding of these lectins to cytotactin or CTB (Crossin et al, 1989). It has been hypothesised that such surface antigens play a role in guiding thalamic axons towards their targets in the centres of each barrel field (Stiendler et al, 1990). However, disruption of afferent activity by removing whiskers in early post-natal life prevents the development of immunoreactivity in the barrel pattern, suggesting

that antigen expression depends on the *reception* of normal thalamic inputs, rather than being a primary event. Cytotactin may instead influence the migration of the cortical neurones which constitute the barrels and the extension of their processes within the developing barrel fields.

## 2.6 SURFACE MOLECULES EXPRESSED ON SPECIFIC NEURONAL CLASSES

### 2.6a. Basic cell types

Neurones can be distinguished from other cells by identifying surface antigens which are differentially distributed on neurones and non-neuronal cells. Neurones are bound by tetanus toxin and antibodies against the antigen Thy-1, while Schwann cells are selectively labelled by antibodies specific for the antigen Ran-1, and fibroblasts are bound by antibodies to Thy-1, but not by tetanus toxin (Fields et al, 1978). Barnstable generated a series of monoclonal antibodies against retinal cells and examined their distribution. The antibodies RET-P1, RET-P2 and RET-P3 labelled only neural (photoreceptor) cells, whereas RET-G1, RET-G2 and RET-G3 were specific for glial cells, both in the retina and in other parts of the CNS (Barnstable, 1980).

Surface antigens can also serve to distinguish central and peripheral neurones. Monoclonal antibody 38/D7, an IgM, is generated by immunising mice with rat dorsal root ganglia cells. It selectively labels neurones in embryonic and adult dorsal root ganglia, sympathetic ganglia and the myenteric plexus, but does not bind to any cells in the rat CNS (Vulliamy et al, 1981). It also binds to chromaffin-like cells from the adrenal medulla and phaechromocytoma (adrenal tumour) cells, but not tumours of CNS origin. The antigen is restricted to the neuronal cell body and appears to be a protein (Vulliamy et al, 1981). In a companion study, Cohen and Selvendran (1981) generated the monoclonal A4, an IgM, against cerebellar cells. In contrast to 38/D7, it labels neurones throughout the rat CNS, but does not recognise cells in sensory or autonomic ganglia, or in peripheral nervous system tumours. The antigen is expressed on all parts of the neuronal surface and appears at embryonic day 10, its levels increasing thirty fold by birth (Cohen and Selvendran, 1981).

The characterisation of fundamental cell types by their surface antigens has been employed by Raff and his colleagues to study the lineage of glial cells in the rat optic nerve. In cell culture, two forms of astrocyte (type 1 and type 2) can be recognised in terms of morphology and growth characteristics, and these are differentially immunoreactive to the monoclonal A2B5, which binds to a specific surface ganglioside. Type 2 astrocytes are labelled by A2B5, whereas type 1 astrocytes are not (Raff et al, 1983). Both types are recognised

by antibodies against glial fibrillary acidic protein (GFAP), which distinguishes them from oligodendrocytes, which are GFAP-negative, but immunoreactive to A2B5. These antigenic profiles allow morphologically similar immature cells to be distinguished *in vitro*, facilitating examination of their respective routes of differentiation. Oligodendrocytes and type 2 astrocytes have thus been found to be derived from A2B5-positive cells (Raff et al, 1983), and quantitative analysis suggests that they arise from a common stem cell. Type I astrocytes appear to arise from a separate stem cell which is A2B5-negative. It has also been possible to demonstrate that the differentiation pathway followed by the oligodendrocyte / type 2 astrocyte stem cell can be dramatically influenced by the content of the surrounding culture medium (Raff et al, 1983).

### 2.6b. Subclasses of neurones

While the surface antigens mentioned above can serve to distinguish broad classes of cell, such as central or peripheral neurones, or neurones and glia, other surface molecules are distributed on subgroups of neurones within such classes.

Monoclonal antibodies VC1.1 and VC5.1 are derived from immunising mice with homogenates of cat visual cortex. Both recognise similar, though not identical, populations of neurones in the cerebral cortex, hippocampus, cerebellum, retina, superior colliculus and spinal cord (Arimatsu et al, 1987). Immunoblot analysis indicates that VC1.1

binds to four molecules of 100, 140, 170 and 700KD, respectively, which are concentrated in the synaptosomal membrane fraction. The 100KD antigen may correspond to the myelin associated glycoprotein (MAG), while the 140KD and the 170KD antigens are labelled with 5D12, an antibody against N-CAM polypeptides (Barnstable and Naegele, 1989). MAG and N-CAM are known to share common amino acid sequences (Lander, 1989), suggesting that the smaller molecules recognised by VC1.1 may belong to a larger family of related surface molecules. Monoclonal VC5.1 recognises a 97KD and a 150KD protein in the soluble cytoplasmic fraction (Arimatsu et al, 1987).

In the primary visual cortex (area 17) of the cat, VC1.1 and VC5.1 label the same subset of neurones (Arimatsu et al, 1987). Immunoreactive cells are concentrated in lamina IV, with lesser numbers in layers V and VI, and almost all are non-pyramidal (Naegele et al, 1988). Under the electron microscope, VC1.1 immunoreactivity is restricted to the neuronal soma and proximal dendrites, and has a discontinuous surface distribution. Immunoreactive product lies between the membranes of pre and postsynaptic neurones, but is excluded from regions of synaptic contact. Labelling is evident around both asymmetrical and symmetrical synapses (Naegele et al, 1988).

Comparison of the distribution of VC1.1 labelling with that seen with antibodies to GABA indicates that all the neurones recognised by VC1.1 are GABAergic, constituting around 35% of all the GABAergic

neurones in area 17 (Naegele et al, 1988). The VC1.1-positive subset has a different laminar distribution to the VC1.1-negative GABAergic neurones, being more concentrated in layer IV and the deeper cortical laminae, and they have a larger somatic diameter (Naegele et al, 1988).

The subset of neurones labelled by VC1.1 overlaps with groups of visual cortical cells characterised by other surface molecules. VVA, a plant lectin which binds to N-acetyl Galactosamine-containing glycolipids and glycoproteins, also labels non-pyramidal neurones, recognising approximately 70% of all the GABAergic cells in area 17 (Naegele et al, 1987). At the ultrastructural level, VVA immunoreactivity has a similar periodic surface distribution to VC1.1, being evident around, but not within, synapses. All the VVA-positive neurones in layer IV are also VC1.1-positive, whereas in other laminae most of the VVA-positive cells are VC1.1-negative (Naegele et al, 1987). The combination of intracellular injections with Lucifer Yellow and VVA immunocytochemistry reveals that VVA-positive neurones include Basket and Neuragliaform cells, but not Chandelier or sparsely spinous cells or star pyramids (Naegele et al, 1987).

### 2.6c. Functionally related neurones

Some antibodies recognise antigens which are expressed by neurones which seem to share a common functional specialisation. The leech nervous system contains a relatively small number of neurones, many of which have been individually characterised in terms of their position, connections and physiology. It is thus well suited to the study of surface antigen distribution, as this can be correlated with a relatively detailed knowledge of the functional characteristics of the system's neurones. Zipser and McKay (1981) generated 40 monoclonal antibodies that labelled different subsets of neurones in the leech nervous system. Although in most cases, it was difficult to discern a clear functional link between the neurones within each labelled group, a number of antibodies recognised neurones with similar properties. Lan 3-1 recognised cells which play a role in reproductive behaviour, Lan 3-5 labelled neurones which act as cutaneous pressure sensors, and Lan 3-2 and Lan 4-2 both bound to nociceptive mechanosensory neurones. However, these antibodies also labelled other neurones of unknown function, so whether the antigens they recognised are absolutely functionally specific is unclear.

In the vertebrate nervous sytem, antibodies against the antigens SSEA-3 and SSEA-4 recognise specific carbohydrate epitopes found on glycolipids (Kannagi et al, 1983). In the rat, they selectively label

neurones in the dorsal root ganglia which have central processes that terminate in laminae III and IV of the spinal cord (Dodd et al, 1984). Anatomical and physiological data indicate that the principal inputs to these laminae are low threshold cutaneous afferents (Brown, 1981), suggesting that the sensory neurones which convey such information may selectively express SSEA-3 and SSEA-4.

Tor-23 is a monoclonal antibody generated using synaptosomes from *Torpedo* electric organ. Its recognises a 175KD polypeptide which is expressed on the soma and proximal dendrites of central neurones, appears early in development and remains throughout adult life. Tor-23 immunoreactivity is evident in numerous central regions, and in certain areas almost all the neurones are Tor-23-positive. These latter include the ventral horn of the spinal cord, the deep cerebellar nuclei, the reticular and vestibular nuclei, the cochlear nuclei, the supraoptic and paraventricular hypothalamic nuclei and the CA2 region of the hippocampus. Labelled neurones are slightly less common in the motor nucleus of the trigeminal nerve and the Nucleus Ambiguus, while in the primary motor and somatosensory cortices they constitute around 5% and 7% of the total number of neurones, respectively.

Many of these regions share motor-related functions. However, some, such as the auditory and hypothalamic nuclei, do not readily fall into this category, while structures associated with a motor specialisation, like the neostriatum, the ventral thalamic nuclei and several motor

cranial nuclei contain very few immunoreactive cells. The antigen recognised by Tor-23 may thus be common to a particular subset of neurones with motor functions, but may also be expressed on non-motor cells. Alternatively, the immunpositive neurones might share some as yet unrecognised characterisitic.

One of the most remarkable examples of a surface molecule which may be expressed by functionally related neurones is the limbic system-associated membrane protein (LAMP). In 1984, Levitt generated a monoclonal antibody, which was originally termed 2G9, using hippocampal cell membranes as the immunogen. In the rat CNS, neurones intensely labelled with anti-LAMP antibodies are evident in the medial prefrontal cortex, hippocampus and parahippocampal cortex, septum, nucleus accumbens, amygdala, hypothalamus, anterior and mediodorsal thalamic nuclei, nucleus tractus solitarius, dorsal motor vagal nucleus, periaqueductal grey and superior colliculus. This pattern of distribution suggests that the antigen is expressed in areas commonly regarded as 'limbic'. As there is no precise definition of what constitutes the limbic system, the argument as to whether all the LAMP-immunoreactive areas are part of it is difficult to resolve. In any event, many of them are interconnected and implicated in functions popularly associated with the term 'limbic'.

In the mature brain, the LAMP is expressed on the neuronal soma and proximal dendrites, but during development it also appears on

growing axons and growth cones (Keller and Levitt, 1989). The axonal expression of LAMP is transient, appearing at embryonic days 15-19, then disappearing around the third post-natal week (Horton and Levitt, 1988). Immunoreactivity also appears on regenerating processes after mature adult axons are mechanically injured (Keller and Levitt, 1989). Somato-dendritic LAMP expression is evident in presumptive cortical, thalamic and septal neurones approximately 24-36 hours after their final mitosis, and persists throughout adulthood. In the developing cerebral cortex, neurones become immunoreactive before the cytoarchitectural differentiation of cortical areas and the appearance of neurotransmitters (Horton and Levitt, 1988).

LAMP appears to be an integral membrane glycoprotein of 64-68KD and the epitope probably lies in the peptide part of the molecule (Zacco et al, 1990). LAMP immunoreactivty has a patchy surface distribution on neurones in the cerebral cortex, and is restricted to the post-synaptic cell membrane. Ultrastructural analysis indicates that this is not usually associated with regions of synaptic contact, but is occasionally evident in the post-synaptic density (Zacco et al, 1990). The gene for the antigen has been cloned and the polypeptide has been partially sequenced. There is no evidence of homology with other known surface molecules (Pennypacker et al, 1989).

The possible role of LAMP has been investigated by examining the formation of connections between two regions rich in LAMP-positive

neurones *in vitro*. Explants of cholinergic cells from the developing septum will normally extend axons into explants of hippocampal cells and form synapses with them, as they do *in vivo*. In the presence of antibodies against LAMP, the septal axons grow to the same extent, but pass *around* the hippocampal explant rather than growing into it (Keller et al 1990). This has lead to the suggestion that LAMP may serve as a target recognition molecule, facilitating the formation of connections between limbic areas, and perhaps maintaining them in the adult brain (Keller and Levitt, 1989a).

## 2.7 SUMMARY

Surface molecules appear to play a major role in the development of the nervous system. They may mediate cell:cell interactions which are central to the formation of the neural tube and may play a critical role in the process of neuronal migration The initial extension of growing axons and the particular routes they subsequently follow seem to depend on surface molecules, and surface molecules in prospective target regions may influence the process of synaptogenesis. Broad classes of neural cells may be distinguished by their differential expression of specific surface molecules, and such differences can be used to differentiate morphologically similar cell types during development and facilitate investigation of their

lineage. Surface molecules can also serve to characterise subtypes within broad neuronal classes.

One of the most intruiging findings to emerge from the study of neuronal surface molecules is that certain antigens may be selectively expressed on neurones which appear to be functionally related. One of the first antigens to be associated with groups of functionally related neurones was that recognised by the monoclonal antibody Cat-301. This particular antibody is the focus of Chapter 3.

# Chapter 3:

# THE MONOCLONAL ANTIBODY CAT-301

## 3.1 THE GENERATION OF CAT-301

In 1982, McKay and Hockfield generated 800 hybridoma cell lines from the immunisation of mice with unfixed specimens of feline spinal cord. Forty-seven of these produced antibodies which labelled sections of cat spinal cord, and five lines whose antibodies produced particularly intense neuronal staining were selected for cloning. The distribution of their monoclonals, termed Cat-101, -201, -301, -401 and -501, respectively, was examined throughout the central nervous system. All labelled a variety of neurones or neuronal processes in numerous regions, but the staining pattern of Cat-301 was particularly interesting.

## 3.2 INITIAL STUDIES OF THE DISTRIBUTION OF CAT-301

Cat-301, a type G immunoglobulin (IgG), binds to motor and other neurones in the spinal cord, and medium and large neurones in the

cranial nerve nuclei, incling both motor and sensory divisions of the trigeminal nucleus. Large neurones in the reticular formation are Cat-301 positive, while in the cerebellum labelled neurones are numerous in the deep nuclei, but less frequent in the cortex, where the Purkije cells are unlabeled, but the relatively rare Lugaro cell is immunopositive (McKay and Hockfield, 1982). Cat-301 positive neurones are also evident in a regularly-spaced pattern in the caudate nucleus, and are present in certain thalamic nuclei and the cerebral cortex (Hockfield et al, 1983).

In all these regions, despite the diversity of labelled cell types, immunoreactivity is consistently restricted to the soma and proximal dendrites, with an absence of label on the axon or distal dendrites (McKay and Hockfield, 1982). Considering the CNS as a whole, it is difficult to identify a single feature common to the large variety of neurones which are immunopositive. However, clues towards a functional similarity emerge when the distribution of Cat-301 immunoreactivity is subjected to detailed examination in the central visual system.

## 3.3 CAT-301 IMMUNOREACTIVITY IN THE CENTRAL VISUAL SYSTEM

### 3.3a. Segregated visual 'streams'

An accumulation of anatomical, electrophysiological and metabolic data, and information from the effects of discrete lesions in animals and humans, has led to the concept that different types of visual information are channeled along separate 'streams' within the visual system, from the retinal ganglion cells, through the lateral geniculate nucleus (LGN) and the primary visual cortex (V1) to the numerous extrastriate cortical areas. There appear to be (at least) two principal pathways, one mainly concerned with the analysis of motion and three-dimensional spatial relationships, and the other with the analysis of colour and form (Fig. 2).

The differential channeling of information begins in the retina, where there are two major types of ganglion cell, termed Pα and Pβ. In the primate, Pα cells project to neurones in the magnocellular layers of the LGN (Leventhal et al, 1981), which in turn project to layers IVcα and VI of the primary visual cortex (Hubel and Wiesel, 1972). Within V1, layer IVa is connected to layer IVb (Lund, 1988). Neurones in IVb project to the thick 'stripes', demarcated by cytochrome oxidase histochemistry, in the secondary visual cortex (V2; Livingstone and Hubel, 1987), which are in turn connected to the middle temporal

area (MT, V5; Shipp and Zeki, 1985). Neurones in laminae IVb and VI of V1 also project to area MT directly (Lund et al, 1975). MT projects to the ventral intraparietal area (VIP; Maunsell and Van Essen, 1983), and to the medial superior temporal area (MST; Ungerleider and Desimone, 1986), which itself projects to area PG (Neal et al, 1987; Cavada and Goldman-Rakic 1989a) (Fig. 2). Both areas VIP and PG lie in the posterior parietal cortex and project to the frontal eye fields (areas 8a and 45) in the prefrontal cortex (not shown in Fig. 2), although the connections from VIP are relatively stronger (Barbas and Mesulam, 1981; Andersen et al, 1985; Cavada and Goldman-Rakic, 1989b) The frontal eye fields receive additional projections from areas MT and MST (Ungerleider and Desimone, 1986; Heurta et al, 1987).

Pβ cells in the retina project to neurones in the parvocellular laminae of the LGN (Leventhal et al, 1981), which send axons to layer IVcβ of V1 (Hubel and Wiesel, 1972), which in turn projects to layers II and III (Lund and Boothe, 1975; Fig. 2) Cells in these laminae project to the thin and intermediate cytochrome oxidase 'stripes' in V2 (Livingstone and Hubel, 1984; Shipp and Zeki, 1985; De Yoe and Van Essen, 1985), which are connected to area V4, in the anterior bank of the lunate sulcus (Shipp and Zeki, 1985; De Yoe and Van Essen, 1985). V4 sends projections to the inferior temporal lobe (Desimone et al, 1980; Fig. 2).

The neurones which occupy the areas comprising these anatomically segregated pathways display correspondingly different physiological characteristics. In the 'magnocellular' stream, Pα retinal cells, and the magnocellular geniculate neurones to which they project, respond rapidly and briefly to visual stimuli, are sensitive to contrast (but not to colour) and have relatively large receptive fields (Dreher et al, 1976; Schiller and Malpeli, 1978). Neurones in layer IVb of V1, the thick stripes of V2, and in area MT are selectively responsive to the direction of stimulus movement, to binocular disparity and the orientation of stimulus contours (Dow, 1974; Zeki, 1974, 1980; Maunsell and Van Essen, 1983b, Shipp and Zeki, 1985; De Yoe and Van Essen, 1985; Hubel and Livingstone, 1987), while those in MST are selective for the direction of stimulus motion and can be driven by complex movements such as rotation (Saito et al, 1986; Tanaka et al, 1986). In the posterior bank of the intraparietal sulcus (which includes VIP), neurones are activated by eye movements (Andersen et al, 1987) and can drive eye movements when stimulated (Shibutani et al, 1984), while in area PG, cells respond to complex visual movements (Sakata et al, 1985; Motter et al, 1987; Steinmetz et al, 1987). Stimulation in the frontal eye fields elicits saccadic eye movements (Bruce et al, 1985) and the neurones respond to visual stimuli (Bruce and Goldberg, 1984).

In the 'parvocellular' stream, Pβ cells in the retina, and their targets in the parvocellular laminae of the LGN, respond in an opponent fashion to particular wavelengths of light, display sluggish and

sustained responses to visual stimuli, and have relatively small receptive fields (Dreher et al, 1976; Schiller and Malpeli, 1978). Neurones in layers II and III of V1, the thin and intermediate stripes of V2, and in V4 are selective for the wavelength of a stimulus, but are not responsive to the direction of its movement. They may also show orientation selectivity and selectivity for binocular disparity (Dow, 1974; Zeki, 1983; Livingstone and Hubel, 1984; Shipp and Zeki, 1985; De Yoe and Van Essen, 1985; Hubel and Livingstone, 1987; Felleman and Van Essen, 1987). Cells in inferior temporal cortex are selective for complex visual patterns, such as faces or hands (Perret et al, 1982).

These electrophysiological findings have been complemented by studies using $^{14}C$-labelled 2-deoxy-glucose to indicate the degree of metabolic activation in the laminae of V1 in response to different visual stimuli. High contrast stimuli produce increased metabolic activity in layers IVa, IVb and VI (Tootell et al, 1988a), whereas colour-varying stimuli fail to activate IVcα and IVb (Tootell et al, 1988b), and stimuli with a high spatial frequency increase 2-deoxy-glucose uptake in layer IVcβ (Tootell et al, 1988c).

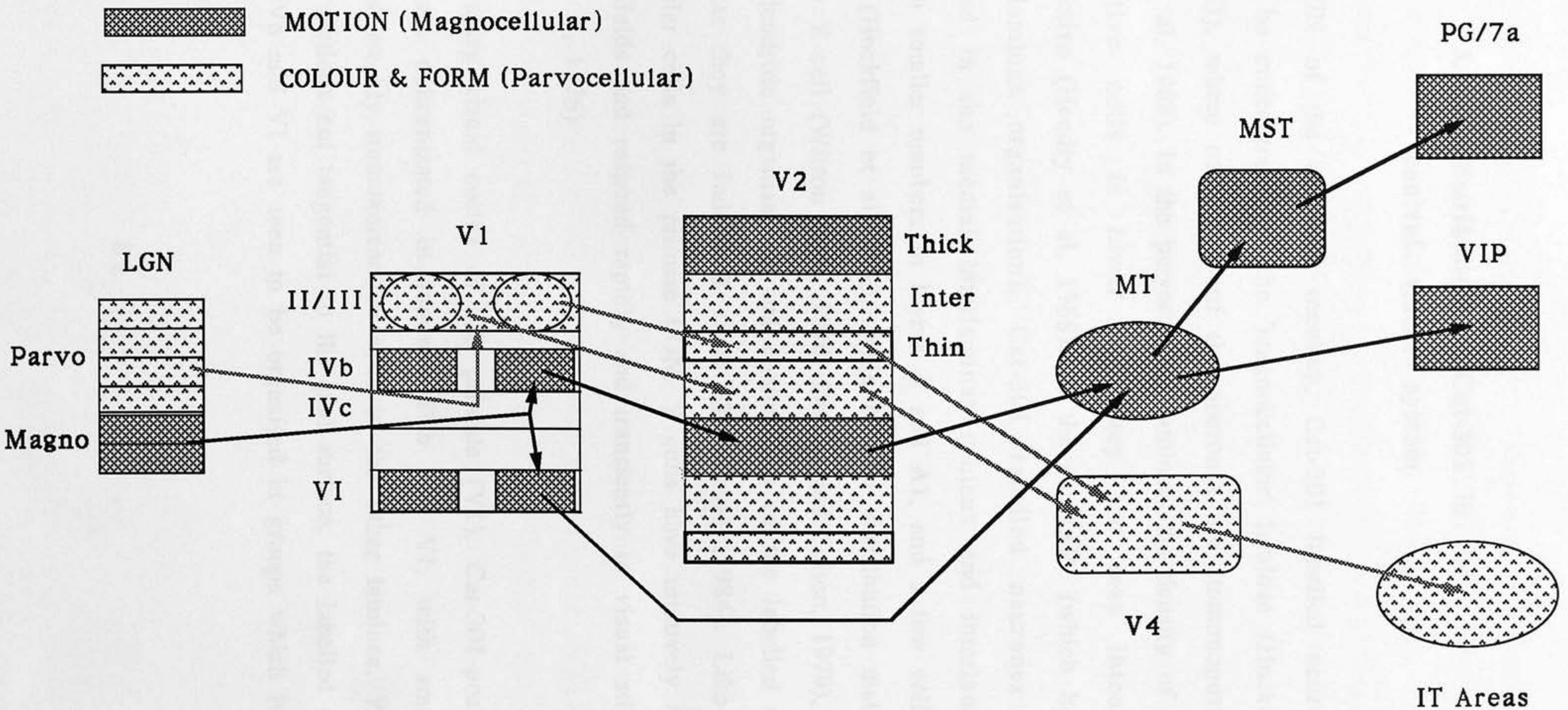
Finally, experimental lesions of area MT or MST in primates selectively impair the tracking of moving stimuli (Newsome and Wurtz, 1988), and pathological lesions in the equivalent areas in man may perturb the perception of moving objects but leave that of stationary objects intact (Zihl et al, 1983). Conversely, what are

thought to be selective lesions of V4 and inferior temporal cortex in primates impair hue discrimination (Heywood and Cowey, 1987; Heywood et al, 1988), while human subjects with ventro-medial occipito-temporal lesions may experience achromatopsia (Heywood et al, 1987).

**FIGURE 2. Simplified diagram of the central visual system, showing the magnocellular and parvocellular 'streams'.**

The magnocellular pathway (dark shading) is mainly concerned with the analysis of visual motion, while its parvocellular counterpart (lighter shading) is principally concerned with colour and form. The two streams are largely segregated, involving separate areas or different subdivisions of the same area. The connections between each area are indicated by arrows. The 'motion' pathway passes from the deep 'magnocellular' layers of the LGN (from which it takes its name), to layers IVb (via layer IVc) and VI of the striate cortex (V1). Neurones in layer IVb project to the thick cytochrome oxidase 'stripes' in the secondary visual area, V2, which in turn project to area MT, in the posterior bank of the superior temporal sulcus. MT also receives direct inputs from layer VI of V1, and projects to area MST, immediately medial to it, and to VIP, which lies in the posterior bank of the intraparietal sulcus. MST projects to area PG, which lies lateral to VIP in the posterior parietal cortex.

The parvocellular stream passes from the laminae of the same name in the LGN to layers II and III of V1, and thence to the thin cytochrome oxidase 'stripes' and 'interstripes' of V2. These regions project to V4, which lies between the lunate and superior temporal sulci and sends projections to the inferior temporal cortex. The two streams thus diverge, towards the parietal and temporal lobes, respectively.


MOTION (Magnocellular)
COLOUR & FORM (Parvocellular)
LGN
Parvo
Magno
V1
II/III
IVb
IVc
VI
V2
Thick
Inter
Thin
MT
MST
PG/7a
VIP
V4
IT Areas

### 3.3b. Distribution of Cat-301 in the central visual system

In the LGN of the macaque monkey, Cat-301 labelled neurones appear to be concentrated in the 'magnocellular' laminae (Hockfield et al, 1983), where over 70% of the neurones are immunopositive (Hendry et al, 1988). In the parvocellular laminae, the density of Cat-301 positive cells is lower and they are less intensely immunoreactive (Hendry et al, 1988). In the cat LGN (which has a diferent laminar organisation), Cat-301 labelled neurones are concentrated in the medial interlaminar nucleus and interlaminar zones, with smaller numbers in layers A and A1, and a few cells in lamina C (Hockfield et al, 1983). This laminar distribution matches that of the Y cell (Wilson et al, 1976; Dreher and Sefton, 1979), and the size, dendritic organisation, and connections of the labelled cells suggest that they are indeed Y cells (Sur et al ,1984). Like the magnocellular cells in the primate LGN, Y cells have relatively large receptive fields and respond rapidly and transiently to visual stimuli (Wilson et al, 1976)

In the primary visual cortex of the primate (V1), Cat-301-positive neurones are concentrated in laminae IVb and VI, with smaller densities of weakly immunoreactive cells in the other laminae. When viewed in sections cut tangential to the pial surface, the labelled cells in layers IVb and VI are seen to be organised in groups which lie in

vertical register with the centres of 'blobs' demarcated by cytochrome oxidase histochemistry in laminae II-III, and the centres of ocular dominance columns (Hendry et al, 1983). Most of the labelled cells in layer IVb have a non-pyramidal morphology (a minority being pyramidal), while those in layer VI comprise a mixture of pyramidal and non-pyramidal neurones. Estimates from cell counting indicate that approximately 10% of the neurones in the macaque striate cortex are Cat-301 positive, while the corresponding figure in that of the cat is 19% (Hendry et al, 1988).

The apparent selectivity of Cat-301 for neurones in subregions of the LGN and V1 which are part of the magnocellular stream lead to its application in 'extrastriate' cortical areas which receive projections from these regions. In the secondary visual cortex (V2), Cat-301 labelling co-localises with the thick 'stripes' of cytochrome oxidase activity which lie normal to the pial surface, although a few weakly labelled cells are also seen in the thin and interstripe regions (DeYoe et al, 1986; Hendry et al, 1988). The immunopositive cells are concentrated in layers IIIb and V (Hendry et al, 1988). In area MT, large numbers of intensely Cat-301 positive neurones are evident in laminae IIIb, V and VI, while in area MST, labelled cells are distributed in a bilaminar pattern (DeYoe et al, 1986). In contrast, in V4 there are relatively few cells immunoreactive to Cat-301, and they are weakly labelled and diffusely distributed across the cortical layers (De Yoe et al, 1986).

These observations suggest that Cat-301 might selectively recognise neurones in the magnocellular visual stream, raising the possibility that neurones with a similar functional specialisation, in an interconnected pathway, might share a particular surface antigen.

## 3.4 CAT-301 IMMUNOREACTIVITY OUTWITH THE VISUAL SYSTEM

While its distribution in the central visual system has attracted considerable attention, the pattern of Cat-301 immunoreactivity has also been examined in detail in other parts of the thalamus and cerebral cortex. Cat-301 positive neurones are densely concentrated in the thalamic nuclei VPL, VPM, VLa and VLp, which relay motor and somatosensory information to and from the cortex. The labelled cells are exclusively large neurones that project to the cortex, and double labelling with Cat-301 and GABA-specific antibodies suggests that they are not GABAergic (Hendry et al, 1988).

In the cortex itself, high densities of strongly immunoreactive neurones are evident in the primary motor and somatosensory areas (areas 4 and 1-2), which are interconnected with the heavily labelled thalamic nuclei (above). The labelled cells comprise both pyramidal and non-pyramidal neurones and are mainly concentrated in layers III and V. Cell counting indicates that they constitute approximately

10% of the total number of neurones in these areas (Hendry et al, 1988). Retrograde labelling with HRP demonstrates that Cat-301 positive neurones in these areas project to the spinal cord, another region that contains dense concentrations of neurones immunoreactive to Cat-301 (McKay and Hockfield, 1982; Kalb and Hockfield, 1988).

Thus, while Cat-301 may selectively label the magnocellular stream in the central visual system, it does not seem to be specific for such areas, as it also labels somato-motor regions of the thalamus and cortex. However, in both cases, the Cat-301 positive neurones are concentrated in areas with a common functional specialisation, and which are interconnected, suggesting that these features may characterise groups of neurones immunoreactive to Cat-301.

### 3.5 THE ANTIGEN RECOGNISED BY CAT-301

Cat-301 recognises neurones throughout the CNS of man (Hockfield et al, 1990), macaque monkeys, cats (Hockfield et al, 1983) and hamsters (Kalb and Hockfield, 1988). Labelled cells are relatively sparse and weakly immunoreactive in guinea pigs and bush babies (McGuire, unpublished observations), while in rats they are evident in the hippocampus but not in the cerebral neocortex (Hendry et al, 1988). The proportion of neurones which are immunoreactive to Cat-

301 varies between regions. Over 70% of those in the magnocellular laminae of the macaque LGN are Cat-301 positive (Hendry et al, 1988), while in V1, area 4 and areas 1-2 in the cerebral cortex, the corresponding figure is approximately 10% (Hendry et al, 1988). The proportions in less heavily labelled regions have yet to quantified, but are likely to be considerably lower. There is no region in which all of the neurones have been found to be Cat-301 positive, suggesting that the antigen is usually expressed on subsets of cells within an area. However, within a subset virtually all the neurones may express the antigen, as around 97% of hamster sciatic motor neurones are Cat-301 positive (Kalb and Hockfield, 1988).

The distribution of Cat-301 immunoreactivity on the neuronal surface seems to be constant across species, across regions of the CNS, and across different cell types (McKay and Hockfield, 1982; Hendry et al, 1988; Kalb and Hockfield, 1988). Immunoreactivity is consistently restricted to the soma and the proximal dendrites, and this also applies during CNS development (Chapter 3.6). At the light microscopic level, it forms a lattice-like pattern on the neuronal surface, while under the electron microscope, labelled material appears to be localised to the extracellular matrix, surrounding axonal boutons (Hockfield and McKay, 1983). Immunoreactive material is conspicuously excluded from the regions of synaptic contact, thus producing the reticular pattern of labelling seen with light microscopy (Fig. 3). Small granules of immunoreactivity are also evident in association with the rough endoplasmic reticulum near the

post-synaptic membrane, suggesting that the antigen is synthesised in the post-synaptic neurones (Hockfield and McKay, 1983).

The antigen can be extracted from neuronal membranes without detergent, indicating that it is not an integral membrane protein, and has been purified using diethylaminoethyl cellulose (DEAE) chromatography and Sepharose gel filtration in urea buffer (Zaremba et al, 1989). The molecular weight estimated from gel filtration is approximately 680 KD and the antigen is sensitive to chondroitin sulphatase ABC, suggesting that it is a proteoglycan (Zaremba et al, 1989). The antigen has been extracted from nervous tissue in the cat, the guinea pig, the hamster (Zaremba et al, 1989), and man (Hockfield et al, 1990). Following the injection of Cat-301 into the subarachnoid space of live animals, the same surface pattern of neuronal labelling is evident as on neurones in fixed tissue, confirming the antigen's extracellular location (Zaremba et al, 1989).

The monoclonal antibody Cat-304 is an immunoglobulin which was generated independently from Cat-301, but which has an identical distribution in the cat striate cortex. Double labelling experiments indicate that Cat-304 recognises the same neurones and has the same punctate distribution on the somata and proximal dendrites (Guimaraes et al, 1988). Immunoprecipitation experiments suggest that the two antibodies recognise the same 680 KD antigen. However, Cat-304 also binds to a smaller 50 KD peptide which is not recognised by Cat-301 (Zaremba et al, 1990), and while they have the same

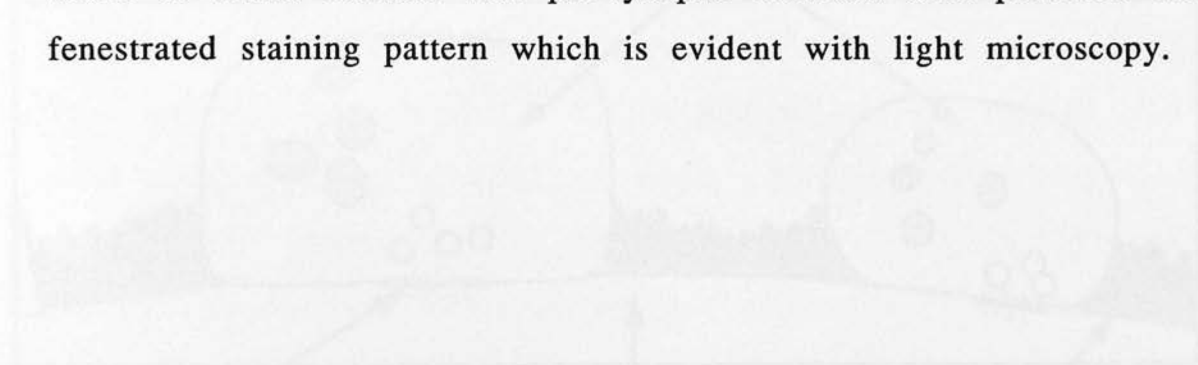
distribution in the cat striate cortex, Cat-301 labels neurones in the guinea pig and the hamster, whereas Cat-304 does not (Guimaraes et al, 1988). Cat-304 may thus recognise the same antigen as Cat-301, but at a separate epitope on the same molecule. The Cat-304 epitope is presumably present on the 50 KD peptide, but absent from the 680 KD proteoglycan in certain species. In theory, it is possible that the epitope recognised by Cat-301 is carried by more than one molecule, and thus that the distribution of Cat-301 immunoreactivity reflects that of more than one antigen. However, the fact that a different epitope has an identical distribution in striate cortex, and is present on the same molecule, suggests that this is unlikely.

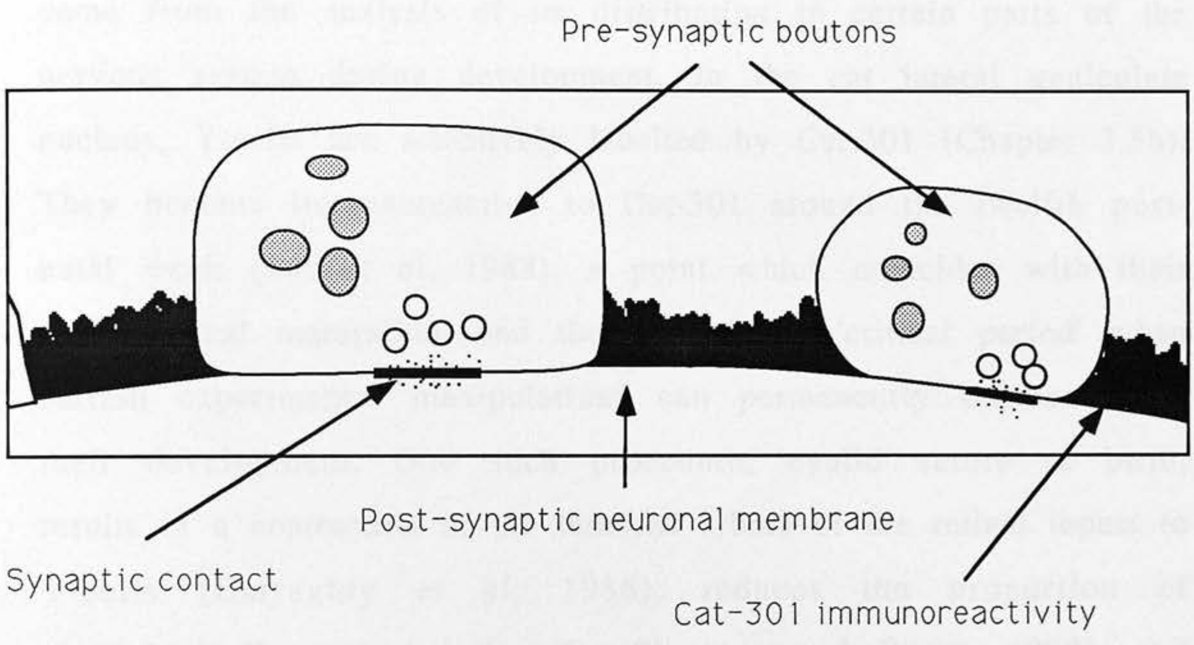
Another antibody whose distribution overlaps with that of Cat-301 is the monoclonal VC1.1 (Chapter 2.6b). In the macaque striate cortex, and in a number of other regions, three populations of neurones can be defined in terms of their immunoreactivity to these antibodies: one containing neurones which are VC1.1 and Cat-301-positive, one with neurones which are labelled by VC1.1 alone, and one in which cells are Cat-301-positive but VC1.1-negative (Zaremba et al, 1989). VC1.1 immunoreactivity, like that to Cat-301, is restricted to the neuronal soma and proximal dendrites and has a similar peri-synaptic distribution at the ultrastructural level. Moreover, the largest of the four species bound by VC1.1 has a molecular weight of approximately 700KD (Chapter 2.6b), which is comparable with that of the 680KD proteoglycan recognised by Cat-301. Cat-301 immunoprecipitates contain VC1.1 immunoreactivity and vice versa,

suggesting that the two antibodies may label different epitopes on a common antigen, or different antigens of similar size.

**FIGURE 3. Ultrastructural distribution of Cat-301 immunoreactivity.**

Immunoreactive product (black) is evident on the external surface of the post-synaptic neuronal membrane, but is excluded from regions where it makes contact with pre-synaptic boutons. This produces the fenestrated staining pattern which is evident with light microscopy.




Pre-synaptic boutons
Post-synaptic neuronal membrane
Synaptic contact
Cat-301 immunoreactivity

## 3.6 CAT-301 IMMUNOREACTIVITY IN THE DEVELOPING NERVOUS SYSTEM

Further clues to the role of the antigen recognised by Cat-301 have come from the analysis of its distribution in certain parts of the nervous system during development. In the cat lateral geniculate nucleus, Y-cells are selectively labelled by Cat-301 (Chapter 3.3b). They become immunoreactive to Cat-301 around the twelfth post-natal week (Sur et al, 1988), a point which coincides with their physiological maturation, and the end of the 'critical period' when certain experimental manipulations can permanently interfere with their development. One such procedure, eyelid suture at birth, results in a contraction of the terminal arbors of the retinal inputs to Y-cells (Garraghty et al, 1986), reduces the proportion of physiologically recorded Y-cells (Sherman and Spear, 1982), and produce a similar reduction in the number of Cat-301 positive cells (Sur et al, 1988). Suture after the critical period has no effect on the proportion of Y-cells (Sherman and Spear, 1982), or on the number cells which are Cat-301 positive (Sur et al, 1988). Expression of the antigen may thus be dependent on the reception of afferent activity during a circumscribed developmental period.

In the cat primary visual cortex (area 17), neuronal Cat-301 immunoreactivity is first detectable around the third post-natal week, reaching adult levels by twelve weeks (MacAvoy et al, 1985).

The organisation of the cortex into ocular dominance columns can be influenced by the same experimental manipulations that can affect development of Y cells, but only if these are performed within twelve weeks of birth (Hubel and Wiesel, 1970). The expression of the mature pattern of Cat-301 immunoreactivity thus approximately coincides with the end of this critical period.

Analogus findings have emerged from the analysis of the expression of Cat-301 immunoreactivity in the developing hamster spinal cord. Hamster motor neurones become immunoreactive to Cat-301 between postnatal days 7 and 14, but immunoreactivity fails to develop if a sciatic nerve crush or a thoracic hemicordotomy is performed before this period. These procedures have no effect on the numbers of Cat-301 positive neurones when performed in adults (Kalb and Hockfield, 1988). There may therefore be a critical period in motor neurone development, with the expression of Cat-301 immunoreactivity dependent on the presence of afferent activity in the dorsal roots or descending supraspinal tracts during this period. Prior to birth, the number of synapses on spinal motor neurones greatly exceeds that in the adult, and during the early postnatal period, the number of synapses declines dramatically (Conradi and Ronnevi, 1975). The development of Cat-301 immunoreactivity seems to occur after this process of synaptic elimination has begun to tail off (Hockfield et al, 1983).

Analysis of the development of Cat-301 immunoreactivity thus indicates that the antigen appears at a relatively late stage, at the end of critical periods in which synaptic connections have been sorted and arranged into the adult pattern. This suggests that the antigen serves some function *after* the mature configuration of connections has been achieved.

# Chapter 4:

# AIMS OF THE STUDY

The macaque monkey is a non-human primate, and two species of this genus, *Maccaca mulatta* and *Macacca fascicularis*, have been used extensively in biological research, especially in studies which involve the cerebral cortex. Although considerably smaller than that of man, the cortex of the macaque is comprised of cytoarchitectonic subdivisions which approximately correspond to those recognised in the human brain. Similarly, while its surface is less convoluted, the major sulci and gyri of the human cortex are also represented in that of the macaque, and in both species the cortex comprises six laminae which lie parallel to the pial surface.

The cortex in both the frontal and the parietal lobes of the cerebral hemispheres has long been recognised as cytoarchitecturally heterogeneous (Brodmann 1909; Walker, 1940; Von Bonin and Bailey, 1947; Pandya and Seltzer, 1982), and more recently the anatomical subdivisions within these lobes have been shown to have their own distinct connections and functional specialisations (Goldman-Rakic, 1987a; Andersen, 1987; Cavada and Goldman-Rakic, 1989a). Moreover, the two lobes are linked by strong and specific reciprocal projections which appear to connect areas which share similar functions (Barbas and Mesulam, 1981, 1985; Petrides and

Pandya, 1984; Jones, 1986; Matelli et al, 1986; Cavada and Goldman-Rakic, 1989b). For example, the posterior bank of the intraparietal sulcus (IPS) and area 8a in the prefrontal cortex are both implicated in the control of eye movements, and are heavily interconnected (Shibutani et al, 1984; Bruce, 1985; Andersen et al, 1985, 1987). Similarly, the anterior bank of the IPS is reciprocally connected to the premotor cortex, and both areas share a somato-motor specialisation (Chapman, et al, 1984; Petrides and Pandya, 1984; Passingham, 1988).

As discussed in Chapter 3.3B, the frontal and parietal lobes also contain areas which have specific connections with the visual 'motion' pathway, including the posterior bank of the intraparietal sulcus (IPS), (which contains area VIP), area PG, and the frontal eye fields. The neurones in these regions display electrophysiological characteristics consistent with a role in the analysis of visual motion or visuo-spatial performance, and these areas may represent rostral extensions of the visual 'motion' pathway (Van Essen, 1985; Zeki and Shipp, 1988).

The frontal and parietal lobes thus seem to contain functionally related areas which are interconnected, and some of these may form part of the visual 'motion' pathway. The apparent selectivity of Cat-301 for neurones in areas with such features, including those in the 'motion' pathway, suggests that it might provide a powerful means for investigating the functional organisation of the frontal and

parietal cortex. At the same time, our growing knowledge of the functional organisation of the cortex may shed light on the significance of neuronal immunoreactivty to Cat-301. This study was designed to examine the pattern of Cat-301 immunoreactivity in the cerebral cortex and neostriatum of the macaque monkey, with particular reference to the frontal and parietal lobes.

# Chapter 5:

# METHODS

The methodology pertaining to the generation of Cat-301 has been included in the appendix, as this work was principally carried out by my colleagues.

## 5.1 PERFUSION OF CASES

Under sterile conditions, adult *Macaca fascicularis* were deeply anaesthetised with intravenous sodium pentobarbital and underwent thoractomy. A catheter was inserted into the left cardiac ventricle and the animal was perfused with a series of solutions using a peristaltic pump, which ensured a constant pressure and rate of flow. Perfusion began with approximately 1 litre of 0.9% NaCl buffered with 0.1M sodium phosphate (37°C; pH 7.4), followed by approximately 1 litre of 4% paraformaldehyde, or 4% paraformaldehyde with 0.1%/ 0.8% glutaraldehyde (depending on the case), at 37°C in 0.1M phosphate buffer (Table 1). These fixatives had previously been found to produce optimal immunostaining when used in other cases.

Immediately after administration of the fixative, the brain was perfused with a graded series of sucrose solutions (500ml each of 5%, 10%, 20% and 30%) in phosphate buffer, at 4°C. The skull was exposed and carefully cut from around the fixed brain with bone clipppers. The brain was removed from the cranial fossae and photographed, then blocks of cerebral cortex were excised in the frontal plane and placed in 30% sucrose solution at 4°C for 12-48 hours. Tissue that was not for immediate sectioning was frozen in liquid isopentane at -75°C and stored in a freezer. In certain cases selected lobes, rather than the entire cortex, were processed (Table 1).

## Table 1

### Perfusion of cases

| Case number | Perfusion date | Fixative | Comments |
|---|---|---|---|
| 1 | 18/09/87 | 0.1% glutaraldehyde 4% paraformaldehyde | Frontal lobes unavailable |
| 2 | 17/10/87 | 0.8% glutaraldehyde 4% paraformaldehyde | - |
| 3 | 20/10/87 | 4% paraformaldehyde | - |
| 4 | 27/10/87 | 0.1% glutaraldehyde 4% paraformaldehyde | - |
| 5 | 22/01/88 | 4% paraformaldehyde | Parietal lobes only |
| 6 | 19/02/88 | 4% paraformaldehyde | - |
| 7 | 21/03/88 | 4% paraformaldehyde | Parietal lobes only |
| 8 | 30/03/88 | 4% paraformaldehyde | Frontal lobes only |
| 9 | 06/04/88 | 4% paraformaldehyde | Frontal lobes only |

## 5.2 HISTOLOGY AND IMMUNOCYTOCHEMISTRY

Blocks of tissue were sectioned coronally in a cryostat at 30μ, or on a freezing microtome at 40μ. Sections were collected in series and processed while free-floating in individual wells, rather than on slides, as this was associated with a stronger immunoreactive signal. They were washed twice in 0.1M phosphate buffer, then incubated in full-strength supernatant from Cat-301 hybrid cell lines for 18 hours at room temperature. 1% Triton X-100 and 0.2% sodium azide were added to the supernatant, to increase antibody tissue penetration and to retard bacterial growth, respectively. After two further washes in 0.1M phosphate buffer, sections were incubated in HRP-conjugated rabbit anti-mouse IgG (Cappel), diluted to a concentration of 1/500 in tissue culture medium, with 1% Triton X-100 and 0.2% sodium azide. After 4 hrs at room temperature, they were washed twice again in 0.1M phosphate buffer. Peroxidase activity was then visualised following incubation in a solution of 0.2% diaminobenzidine (DAB) and 0.002% $H_2O_2$ for 15-20 minutes. 0.2% $NiSO_4$ was added to the solution to intensify the reaction product. Sections were washed in buffer and mounted onto slides from 0.25M phosphate buffer, dehydrated through a graded series of alcohols (70%, 90%, 95% and 100%) into xylene and then coverslipped.

Every fourth section of each series was processed for Cat-301 immunoreactivity. Parallel series of adjacent sections were collected

and stained with thionin, to demonstrate the cytoarchitecture, and the Gallyas method (Gallyas, 1979) to demonstrate the myeloarchitecture. Each batch of sections processed for Cat-301 immunocytochemistry was accompanied by sections from cat spinal cord or monkey primary visual cortex, which were processed in an identical fashion and served as positive controls. Other sections from the frontal and parietal cortex were incubated in normal mouse serum rather than Cat-301, and served as negative controls.

## 5.3 DISTRIBUTION OF CAT-301 IMMUNOREACTIVITY IN RELATION TO CYTOARCHITECTONIC AREAS

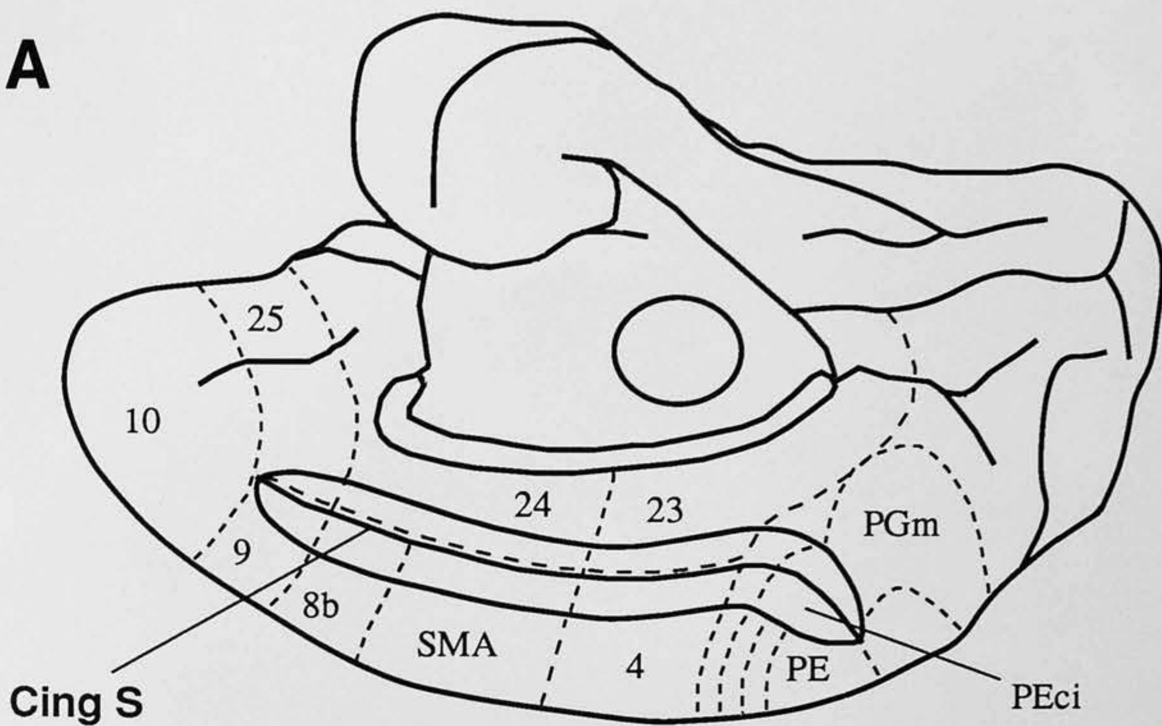
In order to make valid observations about the distribution of Cat-301 immunoreactivity with respect to different cortical areas, the accurate identification of each area was of critical importance. Many of the cytoarchitectural distinctions between the areas are subtle, particularly those between neighbours in the same region. The cytoarchitectural features used to characterise the areas examined and differentiate them from eachother are detailed below. They were derived from the descriptions of other authors, and modified by the experience of myself and my colleagues in the laboratory.

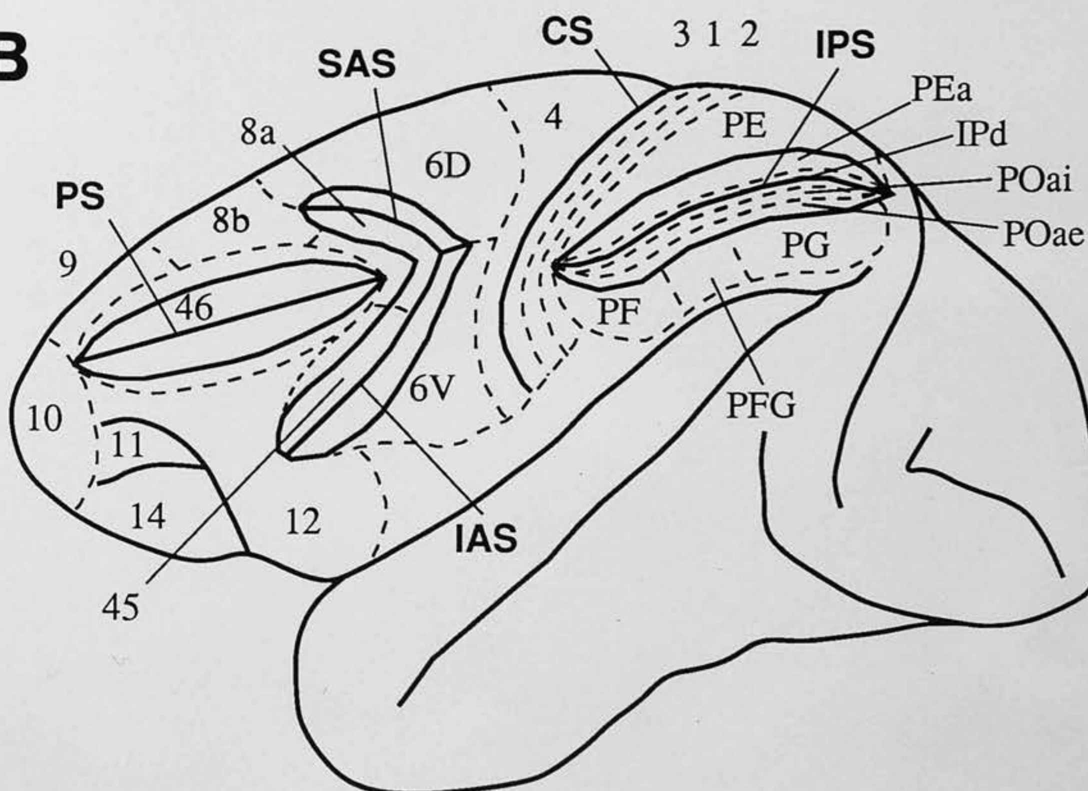
Detailed descriptions are provided for the areas within the frontal and parietal lobes, the regions which were the focus of the study. The

term 'frontal lobe' is used in its most inclusive sense, that is, comprising the prefrontal, premotor and motor cortices, and also the anterior cingulate cortex, on the lobe's medial surface. Similarly, the 'parietal lobe' is taken to include the posterior cingulate and medial parietal areas, as well as the somatosensory and posterior parietal cortices. The cytoarchitecture of the occipital cortex and the neostriatum have not been described as the distribution of Cat-301 immunoreactivity in these regions was examined in rather less detail.

**FIGURE 4. Cytoarchitectural areas of the cerebral cortex in the macaque monkey.**

The areas of the cortex have been defined according to the classifications of Walker (1940), Powell and Mountcastle (1959) and Pandya and Seltzer (1982). The subdivisions of area 6 are based on common features in the descriptions of Von Bonin and Bailey (1947) and Barbas and Pandya (1987). **A:** Medial surface. **B:** Lateral surface. Certain sulci have been opened to expose the areas which occupy their banks. Cytoarchitectural borders are delineated with stippled lines. Cing S: Cingulate sulcus. PS: Principal sulcus. SAS: Superior arcuate sulcus. IAS: Inferior arcuate sulcus. CS: Central sulcus. IPS: Intraparietal sulcus.


A
25
10
24
23
PGm
9
8b
SMA
4
PE
PEci
Cing S
3 1 2


B
CS
IPS
SAS
PEa
4
PE
IPd
8a
6D
POai
PS
8b
PG
POae
9
46
PF
6V
PFG
10
11
14
12
IAS
45

### *Prefrontal cortex*

The cortex in the most rostral part of the frontal lobe was divided according to the cytoarchitectonic parcellation of Walker (1940; Fig. 4). Area 10 has a well-developed layer IV, but a meagre layer VI, and occupies the lobe's rostral pole. The superior part of the dorsolateral surface contains areas 9 and 8b, which extend onto the lobe's medial surface, as far as the cingulate sulcus. The layer III neurones in area 8b are quite large, while those in area 9 are small. Both areas lack the prominent layer IV and columnar appearance of area 46, which lies in the banks of the principal sulcus and the adjacent parts of the lobe's dorsolateral surface. Area 12 lies inferior to area 46, extending from the dorsolateral onto the inferior surface, as far as the lateral orbital sulcus. Layers V and VI in area 12 are characteristically thick and poorly-differentiated. Caudally, areas 8a and 45 lie mainly in the anterior banks of the superior and inferior arcuate sulci, respectively. Area 8a has a thin granular layer and medium sized pyramidal cells in layer V, while area 45 is similar, but has larger pyramidal cells in layers III and V. Areas 8a and 45 together approximately correspond to the frontal eye fields as defined by low threshold elicitation of eye movements (Bruce et al., 1985).

On the inferior surface, area 11 lies between the rostral parts of the lateral and medial orbital sulci, with area 13 between their caudal

parts. Area 11 has small evenly-distributed cells in layer III and a well-defined layer IV. The cells in area 13 are more loosely arranged and layer IV is relatively faint. Area 14 lies medial to the medial orbital sulcus, immediately superior to the olfactory tract, and is characterized by a very broad layer I, but narrow fifth and sixth layers. On the medial surface, the rostral part is occupied by area 10. Areas 24 and 25 lie caudal to this and are described below (Cingulate cortex).

### *Premotor cortex*

The premotor cortex (Brodmann's area 6; Fig. 4) lies in the frontal lobe between the posterior banks of the arcuate sulcus and the precentral gyrus, extending onto the medial surface as far as the cingulate sulcus. Most cytoarchitectural classifications recognise distinctions between the cortex on either side of the arcuate spur (e.g. Von Bonin and Bailey, 1947, Barbas and Pandya, 1987), which I termed dorsal (6D) and ventral (6V) area 6, respectively. The dorsal division lacks the thin granular layer (IV) which can be discerned in its ventral counterpart (Barbas and Pandya, 1987). Each division has been further subdivided in terms of cytoarchitecture (Von Bonin and Bailey, 1947), cyto- and myeloarchitecture (Barbas and Pandya, 1987) and cytochrome oxidase histochemistry (Matelli et al., 1985), but the relationship between these different parcellations is unclear. The portion of area 6 on the medial frontal surface corresponds to

the supplementary motor area (SMA or MII), and has similar cytoarchitecture to 6D, but its pyramidal cells in layers III and V are more prominent (Barbas and Pandya, 1987).

### *Motor and Somatosensory cortex*

Primary motor cortex (Brodmann's area 4) lies in the caudalmost part of the frontal lobe, between area 6 and the fundus of the central sulcus (Fig. 4), and is characterised by the prominent pyramidal cells of Betz in layer V (Brodmann, 1909). Primary somatosensory cortex comprises three cytoarchitectural areas in the rostral part of the parietal lobe. Area 3 mainly occupies the posterior bank of the central sulcus. Its cortex is strikingly thinner than that of area 4, and layers II, III and IV are virtually fused and densely packed with small cells. Area 1 lies in the adjacent part of the post-central gyrus, contains pyramidal cells in layer III, and layers II, III and IV are more readily differentiated. Area 2 contains more pyramidal cells in layers III and V than area 1 and lies immediately caudal to it (Powell and Mountcastle, 1959).

### *Cingulate Cortex*

Cingulate cortex occupies the region between the cingulate sulcus and the corpus callosum, on the medial aspect of the frontal and parietal

lobes (Fig. 4). Walker's areas 25 and 24 occupy its rostral part, caudal to area 10 and medial to the dorsolateral prefrontal and premotor cortices. Area 25 has a poorly-developed layer IV and layers V and VI are narrow and blended. Area 24 is similar, but unlike area 25, it lacks a discernible layer IV. Pandya et al (1981) have described an additional medial area (area 32, not shown) which has slightly different cytoarchitecture to area 25 and occupies a region approximately corresponding to its upper part. In the posterior cingulate cortex, area 23 (Vogt et al., 1987) lies caudal to area 24 and medial to area 4. It has a distinct layer IV and more conspicuous layer IIIc pyramidal cells than area 24.

### *Posterior parietal cortex*

Posterior parietal cortex lies caudal to somatosensory cortex and encompasses the superior and inferior parietal lobules (on either side of the intraparietal sulcus), and the cortex on the medial surface of the parietal lobe (Fig. 4). The cytoarchitectonic classification employed was based on that of Pandya and Seltzer (1982). The superior parietal lobule is occupied by area PE (Brodmann's area 5), and extends as far as the cingulate sulcus on the medial surface. It has a broad third layer, a columnar appearance and a modest layer VI. Area PEa lies immediately lateral to PE in the dorsal bank of the intraparietal sulcus (Fig. 5). It appears less columnar than PE but layer VI is more clearly defined. The fundus of the sulcus is occupied

by area IPd (Seltzer and Pandya, 1986; Fig. 5), which has a paucity of cells in layer V, sharpening the definition of layers IV and VI.

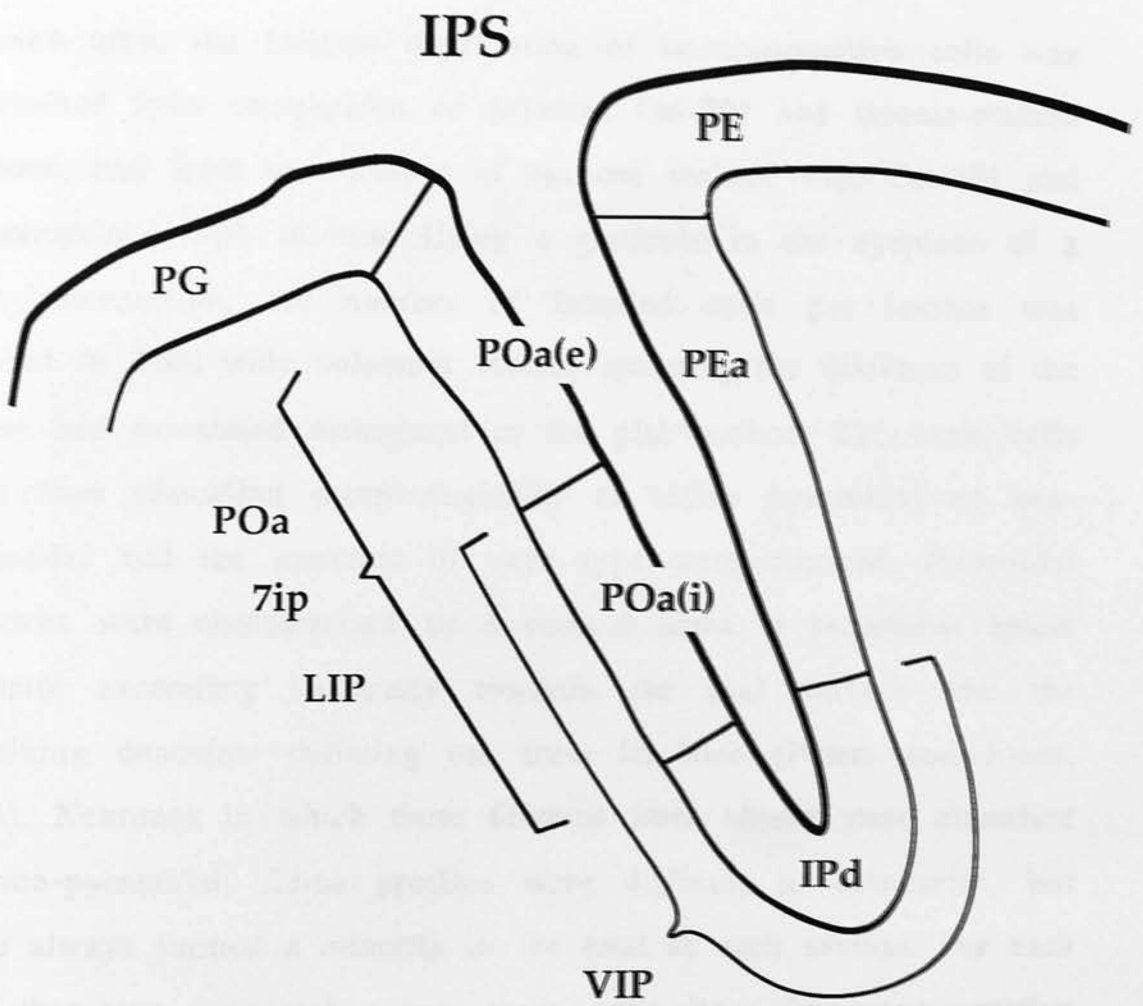
The ventral bank of the sulcus contains area POa. This area has also been termed 7ip (the intraparietal portion of Brodmann's area 7; Cavada and Goldman-Rakic, 1989a) and the lateral intraparietal area (LIP; Andersen et al., 1985). POa has internal and external subdivisions, POa(i) and POa(e), which lie in its inner and outer parts, respectively (Fig. 5). Layers IV and V in POa(i) blend together, serving to differentiate it from IPd. POa(i) has very similar cytoarchitecture to POa(e), but is more heavily myelinated (Seltzer and Pandya, 1983, Fig. 7B). The fundus and lower ventral bank contain a projection zone from visual area MT, known as the ventral intraparietal area (area VIP; Maunsell and Van Essen, 1983; Fig. 5). This zone has lightly- and heavily-myelinated parts which appear to lie within IPd and POa(i), respectively (Ungerleider and Desimone, 1986). In our material, POa(i) was more heavily-myelinated than IPd caudal to the level of the rostral end of the post-central sulcus, but there was little difference between them rostrally. The differentially-myelinated caudal parts of POa(i) and IPd may thus correspond to the two parts of VIP.

The inferior parietal lobule (Brodmann's area 7) has three subdivisions, areas PF, PFG, and PG (from rostral to caudal), which lie immediately lateral to POa(e). PF and PG correspond to areas 7b and 7a of Vogt and Vogt (1919). All these areas have a columnar

appearance and large pyramidal cells in layer IIIc. Moving from PF to PG, there is a progressive increase in cellularity and the laminae are more clearly defined. On the medial surface of the parietal lobe, area PEc(i) occupies the banks of the caudal end of the cingulate sulcus, while area PGm (7m of Von Bonin and Bailey and of Cavada and Goldman-Rakic) lies inferior to this. PEc(i) has few pyramidal cells in layer IIIc, while PGm has a columnar appearance like PG. In both areas, the cell density in the infragranular layers exceeds that in layer III.

**FIGURE 5. Classification of the cortical areas in the banks of the intraparietal sulcus.**

Pandya and Seltzer divide the cortex of the intraparietal sulcus into four areas, in terms of cyto- and myeloarchitecture. The cortex in the dorsal bank is termed PEa, that in the fundus is called IPd, and area POa corresponds to the cortex in the ventral bank. POa has internal and external subdivisions, termed POa(i) and POa(e), respectively. The cortex in the ventral bank has also been referred to as area 7ip (the intraparietal portion of Brodmann's area 7) by Cavada and Goldman-Rakic, and the lateral intraparietal area (LIP) by Andersen. The ventral intraparietal area (VIP) was described by Maunsell and Van Essen as a projection zone of visual area MT, and approximately corresponds to areas IPd and POa(i) combined.


IPS
PE
PG
POa(e)
PEa
POa
7ip
POa(i)
LIP
IPd
VIP

## 5.4 ANALYSIS OF THE LAMINAR DISTRIBUTION OF CAT-301 POSITIVE NEURONES

In each area, the laminar distribution of immunopositive cells was determined from comparison of adjacent Cat-301 and thionin-stained sections, and from examination of sections stained with Cat-301 and counterstained with thionin. Using a graticule in the eyepiece of a light microscope, the number of labelled cells per lamina was counted in 250μ-wide columnar sectors spanning the thickness of the cortex and orientated orthogonal to the pial surface. The same cells were then classified morphologically as either pyramidal or non-pyramidal and the numbers of each type were counted. Pyramidal neurones were characterised by a conical soma, a prominent apical dendrite ascending vertically towards the pial surface and the remaining dendrites radiating out from its base (Peters and Jones, 1984). Neurones in which these features were absent were classified as non-pyramidal. Some profiles were difficult to categorise, but these always formed a minority of the total in each section. For each area that was examined, counts were made from five representative sections from at least two cases which displayed staining of optimal quality in that region. The figures were then pooled to obtain mean values.

## 5.5 MORPHOLOGICAL SUBCLASSIFICATION OF CAT-301 POSITIVE NON-PYRAMIDAL NEURONES

In areas where neurones were intensely labelled, it was possible to divide the non-pyramidal cells into four basic morphological categories, based on the descriptions of Peters and Jones (1984). In areas with weakly immunoreactive neurones, the low level of dendritic labelling made this kind of analysis impracticable. Neurones with numerous dendrites emerging from all parts of the soma were described as multipolar. Neurones with two principal dendrites extending vertically from either pole of the cell body were classed as bipolar, while those with two vertical dendrites which bifurcated close to the cell body were described as bitufted. When a neurone displayed two dendrites emerging horizontally from opposite poles of the soma it was described as a horizontal.

## 5.6 CELL COUNTING

In selected areas, the number of nuclei in Cat-301 positive neurones was compared with the total number of neuronal nuclei, using sections which had been processed for Cat-301 immunoreactivty and counter-stained with thionin. This permitted estimation of the proportion of all the cortical neurones which were labelled by the antibody. Neuronal nuclei were identified by their large size, pallor, oval shape, prominent nucleolus, smooth nucleoplasm and association with cytoplasmic Nissl staining. Glial nuclei were characterised by their smaller size, darker appearance, irregular shape, less prominent nucleoli, the presence of clumps of heterochromatin and the absence of surrounding cytoplasmic staining (Ling et al., 1973; Vaughan, 1984). Some small neuronal nuclei were difficult to differentiate from those of glia, but these constituted a small proportion of the total in each area. Tissue for this analysis was cut at 20μ in order to maximize tissue penetration by the antibody.

Counts were made with a light microscope linked to a computerised plotting system (Fig. 6). A graticule in the microscope eyepiece delineated the boundaries of the zone to be counted and a camera lucida attachment allowed the position of a 'mouse' (plotter) on a computerised graphics tablet to be seen in relation to the field of view. Each neuronal nucleus with a visible nucleolus was registered on a computer by clicking the mouse over its profile, producing a

corresponding point on its monitor screen. This helped to ensure that every neuronal nucleus was counted and that the same profile was not counted twice. Nuclei were viewed at a final magnification of x625, and counts were made within 80μ-wide columnar sectors orthogonal to the pial surface. Mean values for each of the areas examined were obtained from five representative sections from two cases which displayed staining of optimal quality in the areas concerned. The antibody appeared to have fully penetrated the sections as it was possible to trace labelled processes from one surface to the other with no variation in the strength of immunoreactivty. The Abercrombie correction factor were used to calculate the final figures (Abercrombie, 1946). This compensates for the overestimation that results from the counting of profiles which are only partially within the field of view.

**Abercrombie's Equation:**

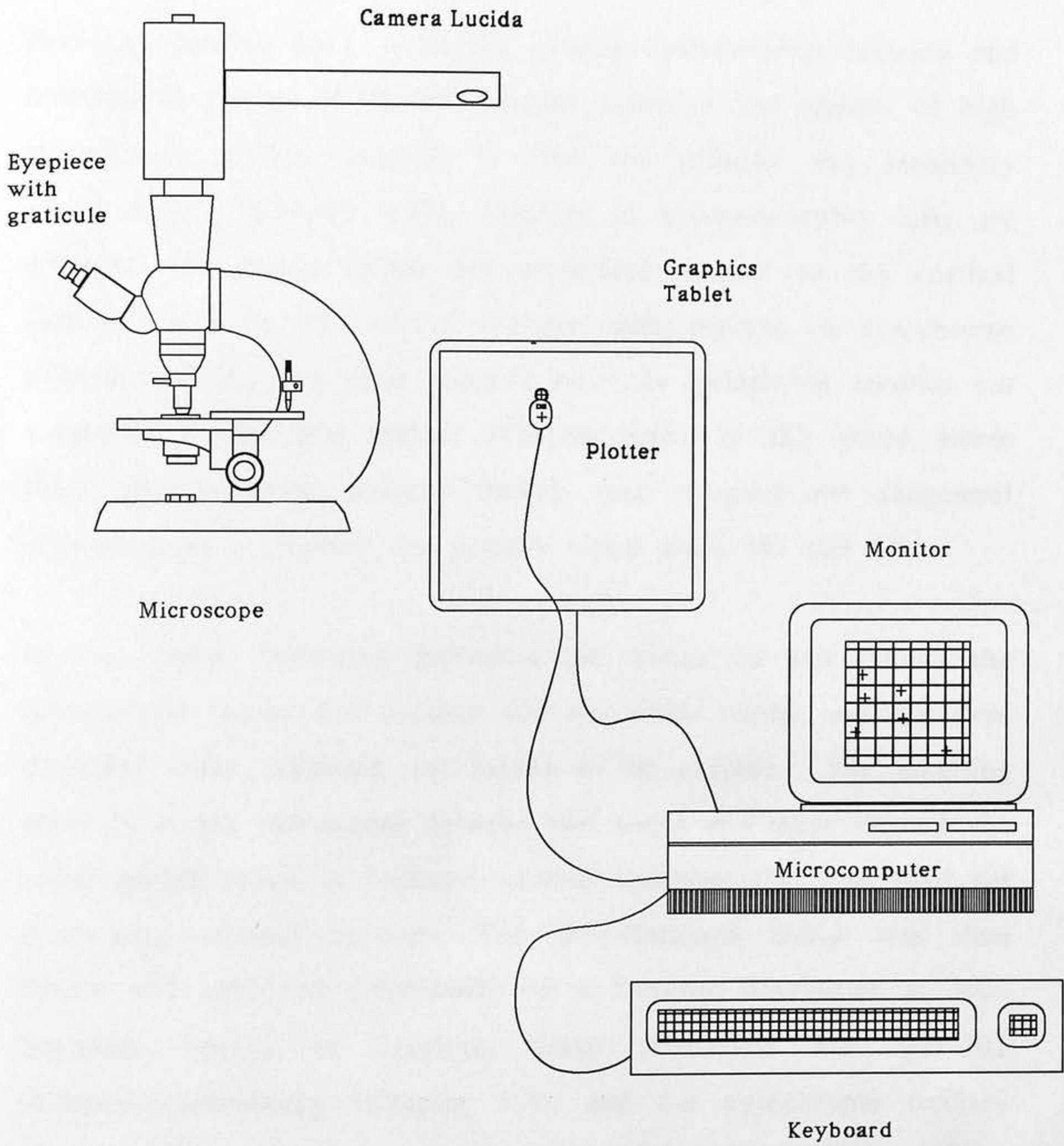
$$\text{Number} = \text{Count} \times \frac{\text{Section Thickness}}{(\text{Section Thickness} + \text{Height of measured structure})}$$

In this study the nucleolus was the counted structure. The nucleolar diameter was estimated using a microscope linked to video-enhanced differential interference contrast (DIC) optics, able to produce a final magnification of x 2,500 (Williams and Rakic, 1988). Mean nucleolar diameters were calculated from 10 neurones labelled with Cat-301 and 10 unlabeled neurones, giving figures of 3.94μ (SD=1.0) and 2.56μ (SD=0.3), respectively. Inserting these values into Abercrombie's equation, with a section thickness of 20μ resulted in correction factors of 0.835 for the labelled cell nucleoli and 0.885 for their unlabeled counterparts. The difference between the respective nucleolar diameters probably reflects the labelling of relatively large pyramidal cells by Cat-301, as the mean nuclear area of immunopositive pyramidal neurones was greater than that of the unlabeled pyramidal neurones, while there was no such difference between labelled and unlabeled neurones with non-pyramidal morphology.

The significance of the differences between the proportions of neurones which were Cat-301 positive in selected areas was estimated with the non-parametric Mann-Whitney U-test.

**FIGURE 6. Equipment used for neuronal counting.**

Counts were made within a zone delineated by a graticule in the eyepiece of the light microscope. The camera lucida attachment allowed the position of the 'mouse' (plotter) on the graphics tablet. to be seen in relation to the field of view. The position of each neuronal nucleus with a visible nucleolus was plotted on the computer by clicking the mouse over its profile, producing a corresponding point on the monitor screen. The latter helped ensure that every visible nucleolus was counted and that the same profile was not counted twice.


Camera Lucida
Eyepiece
with
graticule
Graphics
Tablet
Plotter
Monitor
Microscope
Microcomputer
Keyboard

## 5.7 FLATTENING OF POSTERIOR PARIETAL CORTEX

Previous studies have identified a close relationship between the location of groups of Cat-301-positive neurones and regions of high cytochrome oxidase activity in both the primary and secondary visual cortex (Chapter 3.3b). Clusters of immunopositive cells are arranged in groups which are orientated normal to the cortical laminae, and lie in vertical register with regions of cytochrome oxidase activity, but these features are only evident in sections cut tangential to the pial surface (Chapter 3.3b). In this study, tissue from the posterior parietal cortex was selected for tangential sectioning, as it contains two putative visual areas, PG and VIP.

In two cases, following perfusion the cortex in and around the intraparietal sulcus was excised and the white matter and pia were dissected away, allowing the sulcus to be unfolded. The resulting sheet of cortex was placed between two slides and taken through the same graded series of buffered sucrose solutions that was used for processing coronal sections. The thus-flattened tissue was then frozen and sectioned tangentially on a freezing microtome at 30μ. Separate series of sections were processed for Cat-301 immunocytochemistry (Chapter 5.3), and for cytochrome oxidase histochemistry using the protocol of Wong-Riley (Wong-Riley, 1979).

# Chapter 6:

# RESULTS

## 6.1 CORRELATION OF THE DISTRIBUTION OF CAT-301 IMMUNOREACTIVITY WITH CYTOARCHITECTURE AND MYELOARCHITECTURE

Throughout the cortex of the frontal, parietal and occipital lobes, the distribution of Cat-301-positive neurones was constant within each area (Figs. 8, 10). The only exceptions to this were in certain areas with recognised cytoarchitectural subdivisions, where the distribution was constant within each subdivision but varied between them. For example, the pattern of Cat-301 labelling within area POa in the parietal lobe varied markedly between its internal and external subdivisions (Fig. 7). Changes in the distribution of immunoreactivity invariably occurred at areal (or subareal) borders. In some cases these were pronounced, as at the border between POa(i) and POa(e) (Fig. 7) or between the SMA and area 24 (Fig. 8). In others, the same pattern of immunoreactivty was shared by neighbouring areas and changes were not discernible, e.g. at the boundaries between areas 1 and 2 or areas PEa and IPd (Fig. 10).

The distribution of Cat-301 immunoreactivity also varied with the cortical myeloarchitecture, and was constant within a given myeloarchitectural zone. In certain cases, changes in the distribution

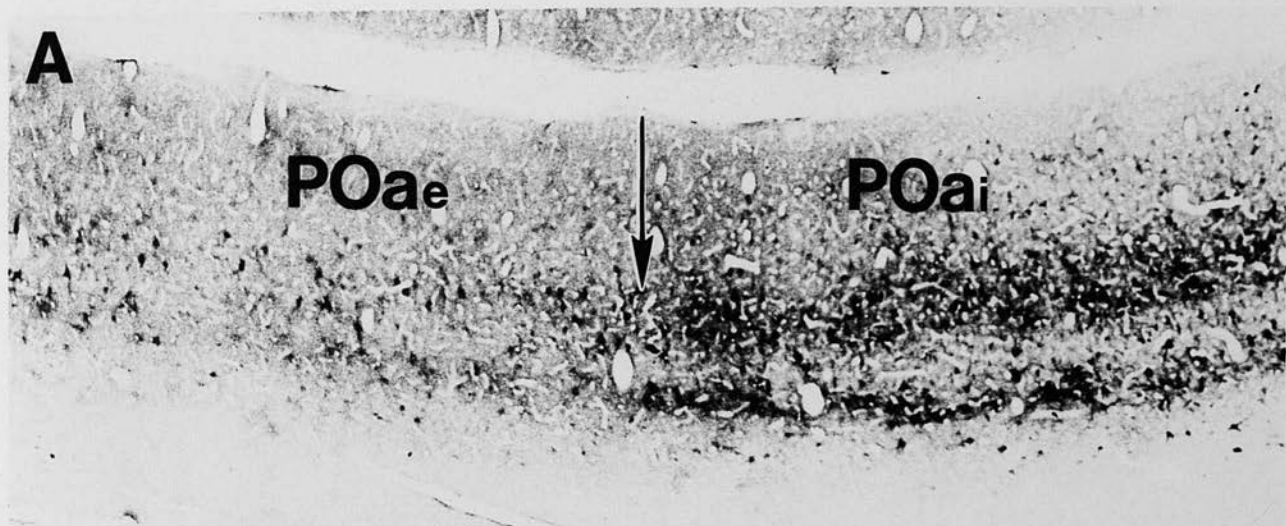
of labelling between areas were more readily associated with clear variations in myeloarchitecture than relatively subtle cytoarchitectural differences. In the ventral premotor cortex (6V), there was a clear difference in the distribution of immunoreactivity in its upper and lower parts (Fig. 11) which corresponded to a marked difference in their respective degrees of myelination, the upper having a more prominent outer band of Baillarger and vertical plexus. Similarly, the remarkable difference in labelling between the subdivisions of area POa matched their contrasting myeloarchitectural characteristics (Fig. 7).

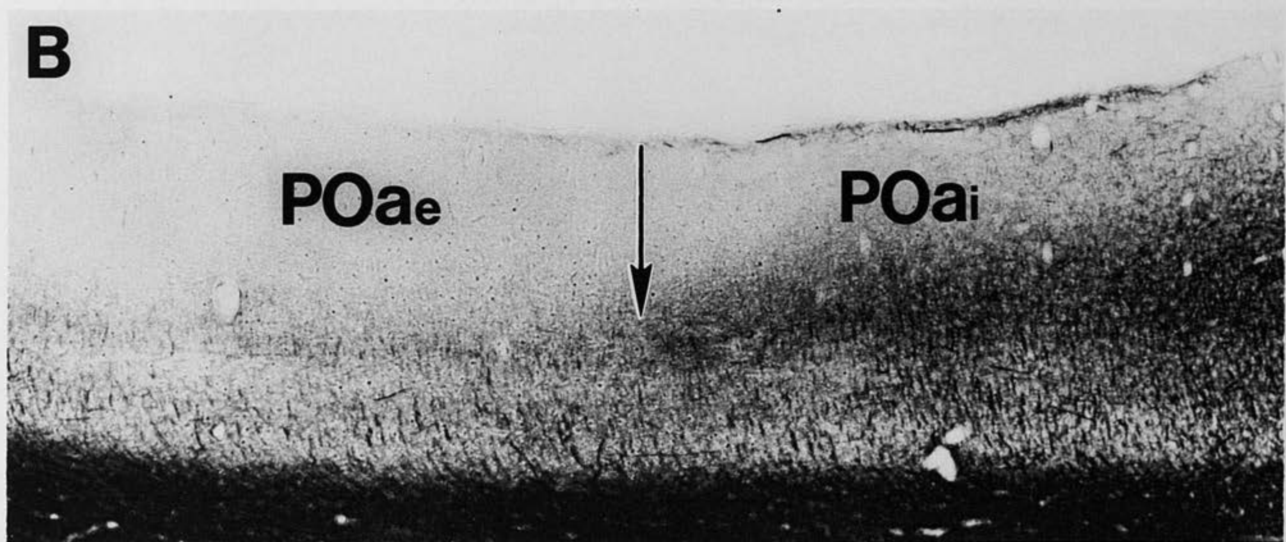
Prominent myelin staining was evident in the premotor and motor cortex, in areas 1 and 2, and in areas PE, PEa, Ipd and POa(i) in the posterior parietal cortex, and, in general, areas which were heavily labelled with Cat-301 were also heavily myelinated. However, some heavily myelinated areas, such as area 12 and area 3, were only weakly or moderately immunoreactive to Cat-301, and there was no simple relationship between the extent of an area's myelination and the distribution of Cat-301 immunoreactivity.

**FIGURE 7. Distribution of Cat-301-positive neurones at the border between the two parts of area POa, POa(e) and POa(i).**

**A:** Cat-301-positive neurones are concentrated in layers IIIc and V in both parts, but are more intensely immunoreactive and more numerous in POa(i). The abrupt change in their distribution (marked by an arrow) occurs at the POa(e)-POa(i) border, which is best seen in myelin-stained material. Magnification x36.

**B:** Myeloarchitecture of areas POa(e) and POa(i) in a section close, but not adjacent to that in A. POa(i) is more heavily myelinated than POa(e): it has a prominent external band of Baillarger whereas that in POa(e) is indistinct. The border between the two subareas is marked with an arrow. Magnification x36.


A
POae
POai


B
POae
POai

## 6.2 DISTRIBUTION OF CAT-301 IMMUNOREACTIVITY IN DIFFERENT CORTICAL REGIONS

The distribution of Cat-301 immunoreactivity was examined in the frontal, parietal temporal and occipital cortices. The results for each of the regions are presented in the same order as the descriptions of their respective cytoarchitectures in the Methods section.

### *Prefrontal cortex*

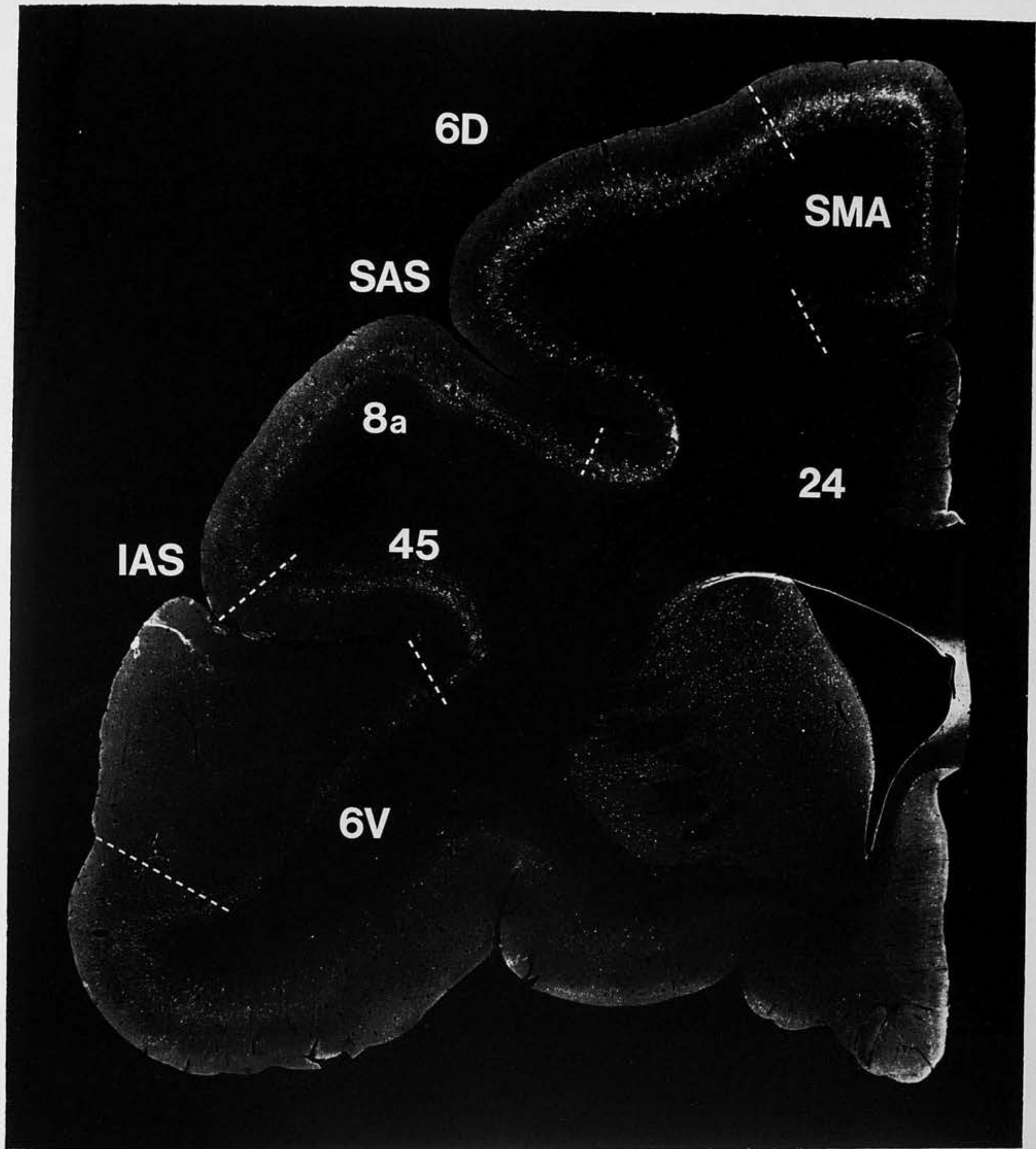
The most intensely immunoreactive neurones in prefrontal cortex were found in areas 8a, 8b and 45, (Figs. 8, 11). In these areas, Cat-301 positive cells were most concentrated in the lower part (b) of layer III, with smaller numbers in layer V and in layer VI. Layers II, upper layer III (IIIa) and IV contained few immunopositive cells, and there were none in layer I (Table 2). In layer IIIb, and to a lesser extent in layer V, there were also continuous bands of faint staining which were not associated with neuronal profiles. The approximate proportions of labelled cells with non-pyramidal and pyramidal morphology in these areas were very similar, ranging from 78% and 22% in area 8b, to 80% and 20% in areas 8a and 45, respectively (Table 2).

The remaining areas on the dorsolateral prefrontal surface contained neurones which were weakly labelled by Cat-301 (Figs. 8, 11), although the intensity of immunoreactivity in areas 9 and 12 was slightly greater than that in areas 10 and 46. In contrast to areas 8a, 8b and 45, in all of these areas, labelled cells were diffusely distributed over a zone extending from layer II to layer VI, with no cells visible in layer I (Fig. 9D). In addition, the proportion of immunoreactive cells with non-pyramidal morphology in these areas was relatively greater, ranging from 93% in area 46 to 97% in areas 10 and 12, while the proportion which were pyramidal was correspondingly lower, at 3-7% (Table 2).

On the inferior prefrontal surface, neurones in areas 11, 13 and 14, were also weakly immunoreactive (Fig. 11) and were diffusely distributed across the laminae below and including layer II (Fig. 9C, Table 2). Approximately 97-98% of the labelled cells in these areas were non-pyramidal while 2-3% were pyramidal (Table 2). In all of the prefrontal areas containing weakly-immunoreactive neurones (i.e. all those except areas 8a, 8b and 45), the Cat-301-positive cells were present in smaller numbers than in the areas with neurones which were strongly-immunoreactive (areas 8a, 8b and 45; Fig. 9).

**FIGURE 8. Distribution of Cat-301-positive neurones in caudal frontal cortex.**

Coronal section through the superior and inferior arcuate sulci (SAS and IAS), just posterior to the caudal end of the principal sulcus. Cytoarchitectural borders are marked with stippled lines. In the SMA and areas 6D, 8a and 45, Cat-301-positive neurones are concentrated in the lower part of layer III and in layer V. Cat-301-positive neurones in area 6V (this section shows only the lower part; the upper part has a different distribution) and in area 24 are present in smaller numbers, are more diffusely distributed and are relatively weakly immunoreactive. The section also includes the neostriatum, and Cat-301 labelled neurones are evident in the caudate nucleus and the putamen. These cells often appear to ring circular zones which contain relatively few labelled neurones. Examples of this are just visible in the dorso-medial part of the caudate. Magnification x8.

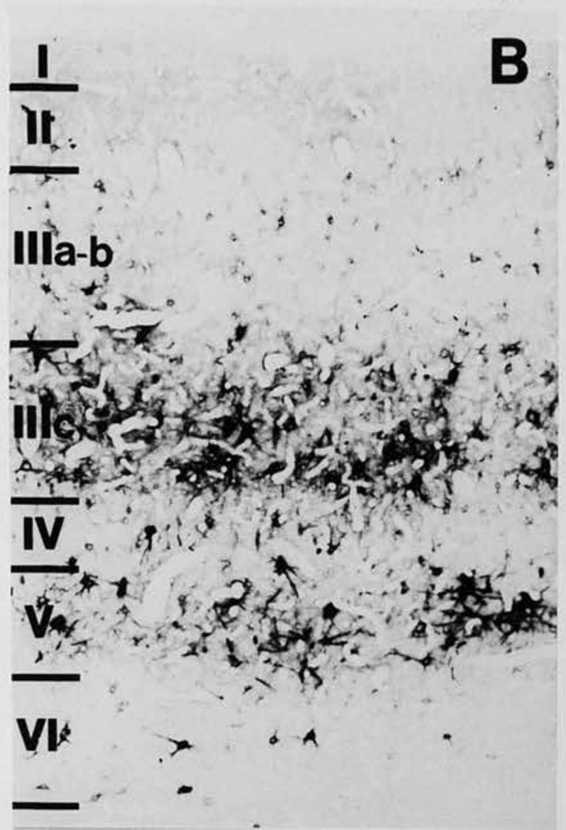
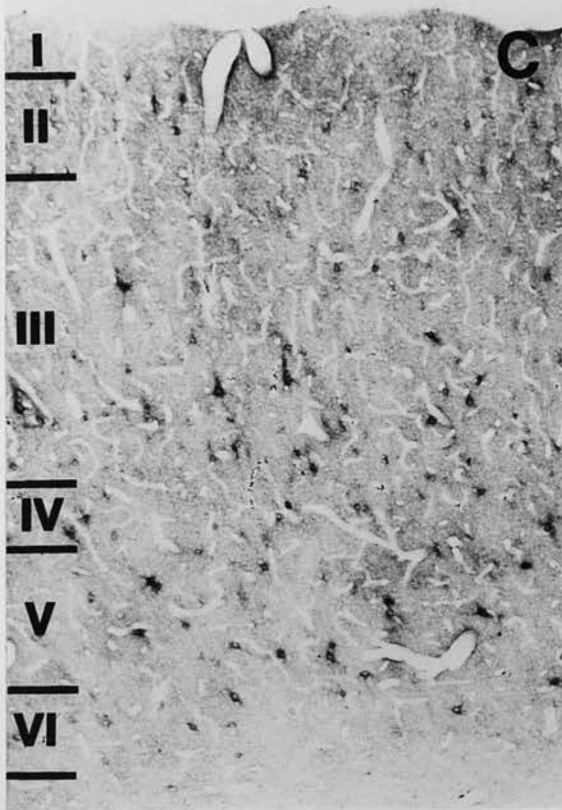

6D
SMA
SAS
8a
24
IAS
45
6V

| Table 2 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Laminar distribution and morphology of Cat-301 positive neurones in frontal cortical areas** | | | | | | | | | |
| **Area** | **% of labelled neurones in each lamina** | | | | | | | **Morphology (%)** | |
| | **I** | **II** | **IIIa** | **IIIb** | **IV** | **V** | **VI** | **Py*** | **Non-Py** |
| 4 | 0 | 3 | 14 | 35 | 0 | 34 | 14 | 32 | 68 |
| SMA | 0 | 4 | 11 | 41 | 3 | 21 | 20 | 22 | 78 |
| 6D | 0 | 4 | 12 | 41 | 4 | 25 | 14 | 28 | 72 |
| 6VU | 0 | 3 | 10 | 42 | 2 | 22 | 21 | 25 | 75 |
| 45 | 0 | 6 | 12 | 45 | 2 | 21 | 14 | 20 | 80 |
| 8a | 0 | 4 | 11 | 43 | 4 | 22 | 16 | 20 | 80 |
| 8b | 0 | 4 | 10 | 41 | 3 | 24 | 18 | 22 | 78 |
| 6VL | 0 | + | + | + | + | + | + | 4 | 96 |
| 46 | 0 | + | + | + | + | + | + | 7 | 93 |
| 9 | 0 | + | + | + | + | + | + | 4 | 96 |
| 10 | 0 | + | + | + | + | + | + | 3 | 97 |
| 11 | 0 | + | + | + | + | + | + | 3 | 97 |
| 12 | 0 | + | + | + | + | + | + | 3 | 97 |
| 13 | 0 | + | + | + | + | + | + | 2 | 98 |
| 14 | 0 | + | + | + | + | + | + | 3 | 97 |

6VU = upper portion of 6V, 6VL = lower portion of 6V. * Py = Pyramidal; Non-Py = Non-pyramidal. + = Labelled neurones diffusely distributed across laminae II-VI

**FIGURE 9. Laminar distribution of Cat-301-positive neurones in frontal and parietal areas.**

Two distinct distribution patterns are evident: in the caudal frontal areas and throughout parietal cortex (with the exception of area 3), immunoreactive neurones are concentrated in the lower part of layer III and in layer V. **A:** Area 6D. **B:** Area PEa. In the rostral frontal cortex, in area 23 and in the lower part of area 6V, Cat-301-positive neurones are diffusely distributed across layers II-VI and only a relatively small proportion have pyramidal morphology. **C:** Area 11. **D:** Area 12. Magnification x50.

A
I
II
IIIa
IIIb
IV
V
VI

B
I
II
IIIa-b
IIIc
IV
V
VI

C
I
II
III
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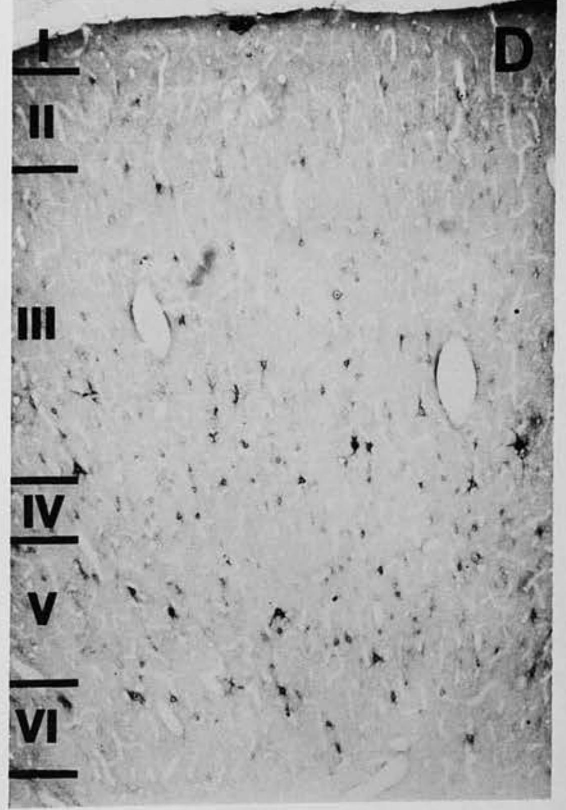
VI

D
I
II
III
IV
V
VI

***Premotor cortex***

The supplementary motor area (SMA), the dorsal division of area 6 (6D) and the upper part of ventral area 6 (6Vu) contained large numbers of neurones which were intensely immunoreactive to Cat-301 (Figs. 8, 11). In these areas, labelled neurones were most concentrated in the lower part (b) of layer III, with smaller numbers in layer V and in layer VI. Layers II, upper III (IIIa) and IV contained few immunopositive cells, and there were none in layer I (Fig. 9A; Table 2). As in the caudal prefrontal areas, diffuse bands of staining were seen across layers IIIb and V, with that in the layer IIIb being particularly prominent (Fig. 9A). Across these areas, the approximate proportions of labelled neurones with non-pyramidal and pyramidal morphology ranged from 72%-78% and 22%-28%, respectively (Table 2). In contrast, in the lower part of the ventral premotor area (6VL) there were relatively few Cat-301-positive cells, and these were weakly immunoreactive (Figs. 8, 11). Their distribution differed from that of the other premotor areas in that they were spread diffusely over a zone extending from layer II to layer VI, with no cells evident in layer I. Approximately 96% of these cells were non-pyramidal while 4% were pyramidal (Table 2).

### *Motor cortex*

There were large numbers of neurones in area 4 which were intensely immunoreactive to Cat-301 (Fig. 11). They were most concentrated in layers IIIb and V, with similar densities in each layer. Smaller numbers were found in layers IIIa and VI, with fewer cells in layer II. Layers I and IV seemed to be devoid of labelled cells. Diffuse bands of staining were seen across layers IIIb and V. Approximately 68% of the labelled neurones had non-pyramidal morphology while 32% were pyramidal (Table 2). The giant cells of Betz were among the pyramidal cells recognised by the antibody.

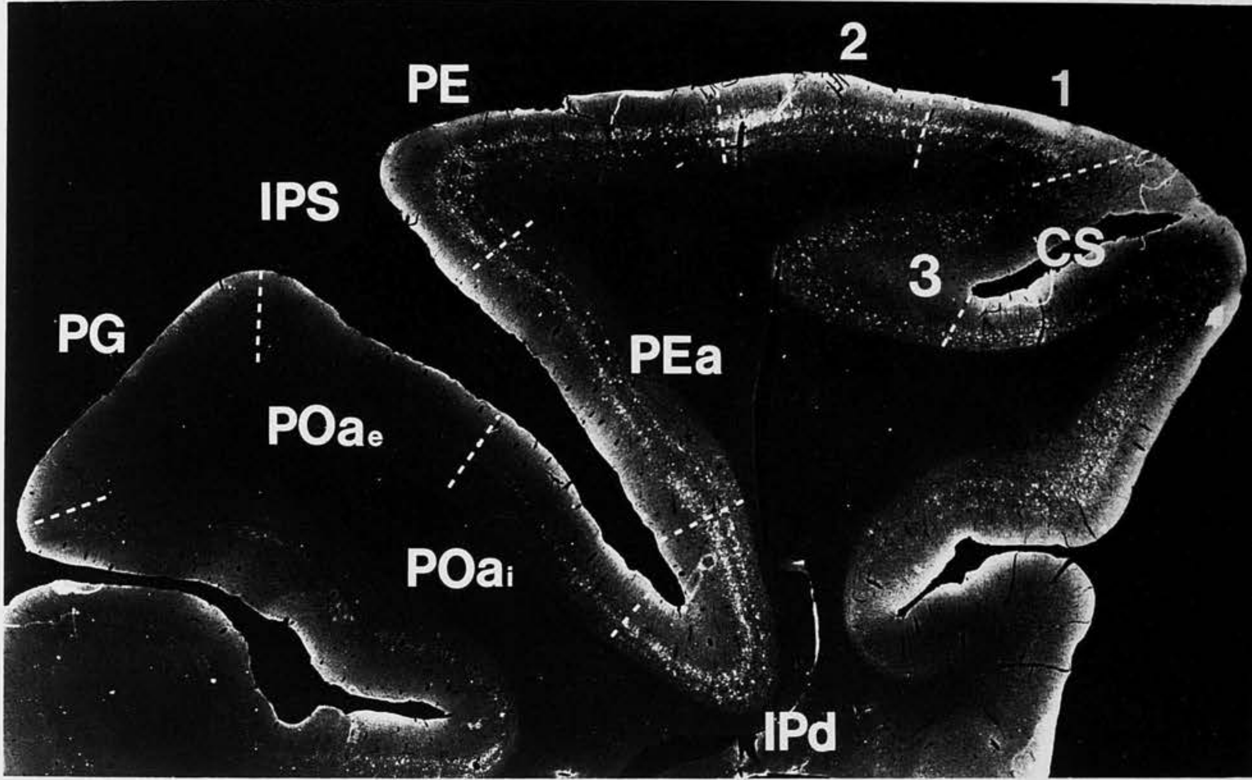
### *Somatosensory cortex*

Areas 1 and 2 contained large numbers of neurones which were intensely immunoreactive to Cat-301 (Figs. 10, 11). They were most concentrated in the lower part (b) of layer III, with smaller numbers in layer V and in layer VI. Layer II, the upper part (a) of layer III and layer IV contained few immunopositive cells, and none were seen in layer I. Bands of diffuse staining were visible across layer IIIb and, to a lesser extent, layer V. Approximately 72% of the labelled neurones in area 1 had non-pyramidal morphology while 28% were pyramidal (Table 3). The corresponding figures for area 2 were 69% and 31%, respectively. In contrast to its neighbours, area 3

contained relatively few Cat-301-positive neurones and these were only moderately immunoreactive (Figs. 10, 11). They were mainly found in layers IV and VI, with smaller numbers in layers II, III and V, and none in layer I. About 88% of these neurones were non-pyramidal and 12% were pyramidal (Table 3)

**FIGURE 10. Distribution of Cat-301-positive neurones in somatosensory and posterior parietal cortex.**

Photomicrograph of a coronal section through the intraparietal sulcus (IPS) and the caudal part of the central sulcus (CS). The approximate positions of cytoarchitectural borders are marked with stippled lines. In areas 1,2 ,PE, PEa, IPd, and POa(i), dense bands of Cat-301-positive neurones can be seen in the lower part of layer III and in layer V. Immunopositive neurones in POa(e) and PG have a similar laminar distribution, but are present in fewer numbers and are less strongly immunoreactive. In area 3 Cat-301-positive neurones mainly occupy layers IV and VI. Magnification x7.


2
PE
1
IPS
CS
3
PG
PEa
POae
POai
IPd

## Table 3

**Laminar distribution and morphology of cat-301 positive neurones in primary somatosensory areas**

| | % of labelled neurones in each lamina | | | | | | | Morphology (%) | |
|---|---|---|---|---|---|---|---|---|---|
| **Area** | **I** | **II** | **IIIa** | **IIIb** | **IV** | **V** | **VI** | **Py*** | **Non-Py** |
| 1 | 0 | 6 | 10 | 48 | 2 | 20 | 14 | 28 | 72 |
| 2 | 0 | 3 | 13 | 49 | 2 | 20 | 13 | 31 | 69 |
| 3 | Not quantified | | | | | | | 12 | 88 |

* Py = Pyramidal; Non-Py = Non-pyramidal

### *Cingulate Cortex*

The neurones in areas 25, 24 and 23 recognised by Cat-301 were weakly immunoreactive and diffusely distributed across layers II-VI (Figs. 8, 9D, 10, 11). In area 25, approximately 96% of the labelled neurones were non-pyramidal and 4% were pyramidal, while in areas 23 and 24, the corresponding figures were 97% and 3% (Table 4).

| Table 4 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Laminar distribution and morphology of Cat-301 positive neurones in cingulate cortical areas** | | | | | | | | | |
| | % of labelled neurones in each lamina | | | | | | | Morphology (%) | |
| Area | I | II | IIIa | IIIb | IV | V | VI | Py* | Non-Py |
| 23 | 0 | + | + | + | + | + | + | 3 | 97 |
| 24 | 0 | + | + | + | + | + | + | 3 | 97 |
| 25 | 0 | + | + | + | + | + | + | 4 | 96 |

+ = Labelled neurones diffusely distributed across laminae II-VI

* Py = Pyramidal; Non-Py = Non-Pyramidal

### *Posterior parietal cortex*

Large numbers of intensely-labelled neurones were present in the superior parietal lobule (area PE), and in the dorsal bank (PEa), fundus (IPd) and lower part of the ventral bank (POa(i)) of the intraparietal sulcus (Figs. 7, 10, 11). In these areas, labelled cells were most concentrated in the lower part (IIIc) of layer III and then in layer V (both Va and Vb), with smaller numbers in layers VI, the upper layers of III (IIIa, and IIIb) and very few in layers II and IV. No labelled cells were visible in layer I. Those in layers IIIc and V lay in bands of diffuse staining, with that in IIIc being particularly prominent (Fig. 9B). The approximate proportions of the Cat-301-positive cells in these areas which were non-pyramidal and pyramidal ranged from 64%-72% and 28%-36%, respectively, depending on the area concerned (Table 5).

On the medial parietal surface, labelled neurones in areas PEc(i) and PGm displayed a moderate intensity of immunoreactivity (Fig. 11), but their laminar distribution was very similar to that of the intensely-labelled neurones in the areas described above, with most being concentrated in layers IIIc and V. The respective proportions of labelled cells with non-pyramidal and pyramidal morphology were approximately 68% and 32% in PEc(i), and 70% and 30% in PGm (Table 5). Areas PF, PFG and PG, in the inferior parietal lobule, and POa(e), in the upper ventral bank of the intraparietal sulcus,

contained few Cat-301-positive neurones and these were weakly immunoreactive (Figs. 7, 10, 11). As in the other posterior parietal areas, labelled cells were mainly found in layers IIIc and V, with lesser numbers in layers VI, IIIa, and IIIb and very few in layers II and IV. Similarly, approximately 56%-69% of the immunopositive neurones had non-pyramidal morphology and 31%-44% were pyramidal, depending on the area concerned (Table 5).

Tangential sections of flattened cortex from the areas in the banks of the intraparietal sulcus (PEa, IPd, POa(i), POa(e)) and the adjacent caudal inferior parietal lobule (PG) (Fig. 5), were examined after processing for Cat-301 immunocytochemistry and for cytochrome oxidase histochemistry. There was no evidence of arrangements of Cat-301 positive neurones orientated orthogonal to the pial surface in any of these areas. This was true for all of the cortical laminae. Similarly, it was not possible to discern any regular pattern to the distribution of cytochrome oxidase activity in the tangential sections through the same regions.

## Table 5

**Laminar distribution and morphology of Cat-301 positive neurones in posterior parietal areas**

| | % of labelled neurones in each lamina | | | | | | | Morphology (%) | |
|---|---|---|---|---|---|---|---|---|---|
| Area | I | II | IIIab | IIIc | IV | V | VI | Py* | Non-Py |
| PE(5) | 0 | 3 | 13 | 45 | 1 | 24 | 14 | 36 | 64 |
| PEa | 0 | 4 | 13 | 43 | 2 | 27 | 11 | 28 | 72 |
| PEc(i) | 0 | 2 | 6 | 42 | 3 | 34 | 13 | 32 | 68 |
| IPd | 0 | 3 | 14 | 49 | 3 | 20 | 11 | 31 | 69 |
| POa(i) | 0 | 2 | 5 | 51 | 2 | 31 | 9 | 36 | 64 |
| POa(e) | 0 | 0 | 5 | 53 | 1 | 32 | 9 | 44 | 56 |
| PF(7b) | 0 | 1 | 4 | 67 | 4 | 19 | 5 | 37 | 63 |
| PFG | 0 | 3 | 8 | 60 | 3 | 21 | 5 | 41 | 59 |
| PG(7a) | 0 | 2 | 5 | 42 | 2 | 32 | 17 | 31 | 69 |
| PGm | 0 | 1 | 4 | 61 | 0 | 29 | 5 | 30 | 70 |

* Py = Pyramidal; Non-Py = Non-Pyramidal

*Occipital cortex*

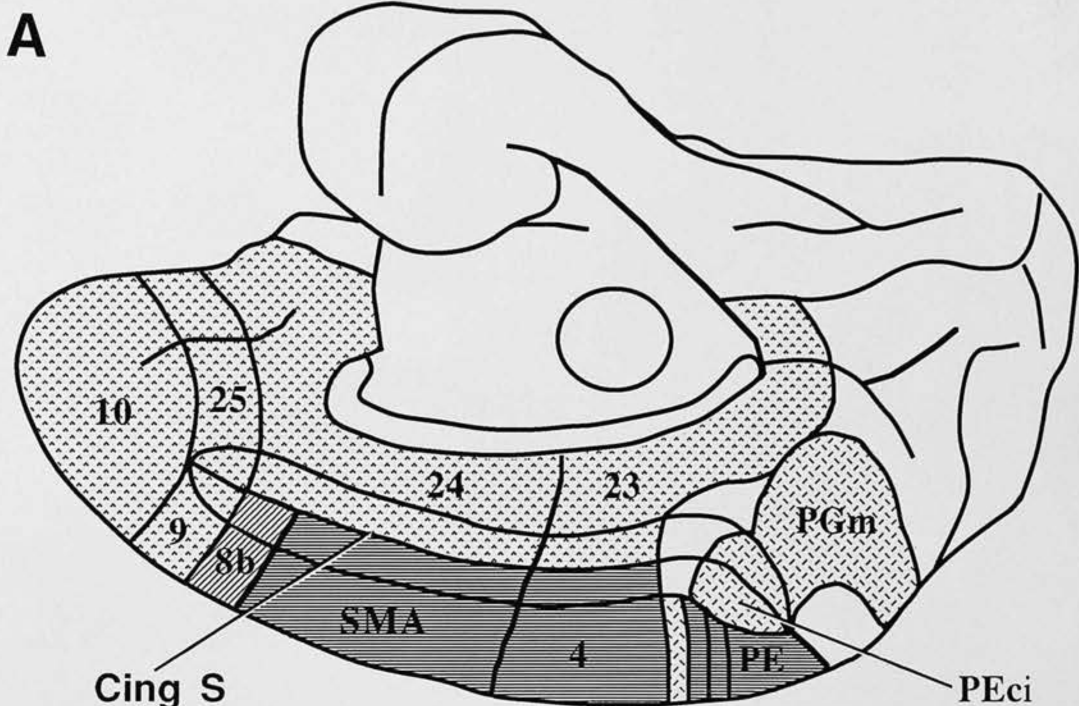
In the striate cortex (V1), large numbers of intensely immunoreactive neurones were concentrated in layers IVb and VI, with lower densities in layers II and III. In the secondary visual area (V2), Cat-301 positive neurones were strongly immunoreactive and concentrated in layers IIIb and V.

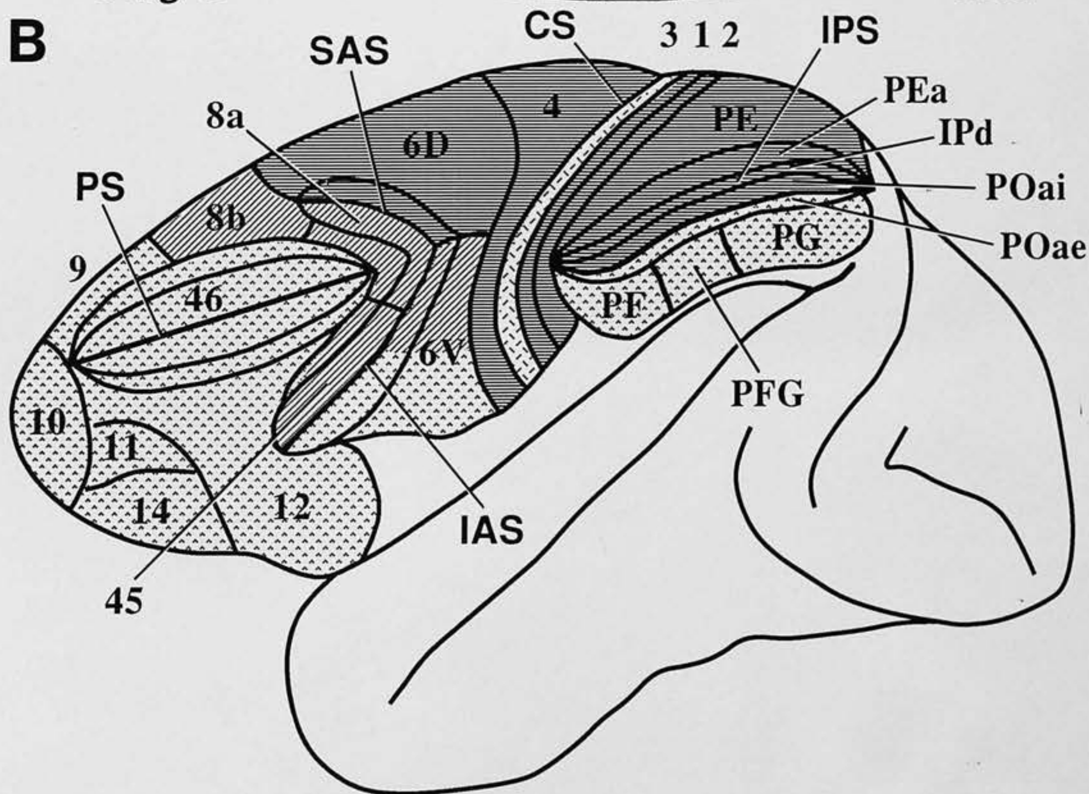
## 6.3 DISTRIBUTION OF CAT-301 IMMUNOREACTIVTY IN THE NEOSTRIATUM

The caudate nucleus and the putamen contained numerous cells which were intensely labelled with Cat-301 (Fig. 8). Labelled cells were also evident in the globus pallidus, but were present in smaller numbers. The immunopositive neurones in the caudate and putamen appeared to share the same basic morphology, and were all of similar size. They often seemed to ring circular or elliptical zones which contained a relatively low density of labelled cells, as can just be discerned in Figure 8.

**FIGURE 11. Variation in the distribution of Cat-301 positive neurones across cerebral cortical areas.**

**A:** Medial surface. **B:** Lateral surface. Large numbers of intensely immunoreactive neurones are present in premotor (areas 6D), supplementary motor (SMA) and motor cortex (area 4), areas 1 and 2 of somatosensory cortex, the superior parietal lobule (area PE) and the dorsal bank (PEa), fundus (IPd) and inner ventral bank (POa(i)) of the intraparietal sulcus (darkest, horizontal shading). Slightly fewer Cat-301-positive cells, with a slightly lower level of immunoreactivity are found in areas 8b, 8a and 45, immediately rostral to the premotor cortex, and in the upper part of 6V (dark diagonal shading). Moderate numbers of neurones with a modest degree of immunoreactivity are evident in the medial parietal areas PEc(i) and PGm, and in area 3 of somatosensory cortex (crossed pattern). Small numbers of weakly-immunoreactive neurones are present in the prefrontal cortex rostral to areas 8 and 45, in the lower part of ventral premotor cortex (6V), in area 23, in the superficial ventral bank of the IPS (POa(e)) and in the adjacent inferior parietal lobule (PF, PFG and PG). Cing S: Cingulate sulcus. PS: Principal sulcus. SAS: Superior arcuate sulcus. IAS: Inferior arcuate sulcus. CS: Central sulcus. IPS: Intraparietal sulcus.


A
10
25
24
23
9
8b
SMA
4
PE
PGm
Cing S
PEci


B
CS
3 1 2
IPS
SAS
4
PE
PEa
8a
6D
IPd
PS
8b
POai
9
46
PG
POae
PF
6V
PFG
10
11
14
12
IAS
45

## 6.4 MORPHOLOGY OF CORTICAL NEURONES IMMUNOREACTIVE TO CAT-301

In all the cortical areas which were examined, Cat-301 labelled a mixture of pyramidal (Figs. 12, 13) and non-pyramidal neurones (Figs. 13-17). Their relative proportions varied considerably, but two major types of distribution emerged, depending on the laminar pattern of immunoreactivity in the area concerned. In areas where the labelled cells were concentrated in a predominantly bilaminar pattern (in the lower part of layer III and in layer V), considerably more of the Cat-301 positive neurones were pyramidal than in the areas where the cells were diffusely distributed across the laminae. When the proportions of labelled cells with pyramidal and non-pyramidal morphology from all of the frontal and parietal areas with the bilaminar type of distribution were grouped together, the mean proportion with pyramidal morphology was 30.2%, while 69.8% were non-pyramidal. In such areas almost all pyramidal immunoreactive neurones were found in layers III and V (Figs. 9A, B), where they were densely packed together. Those with non-pyramidal morphology were much more evenly distributed, but were still most concentrated in the lower part of layer III and in layer V (Figs. 9C, D).

When the areas where the Cat-301-positive neurones were diffusely distributed across layers II-VI were considered together, the mean proportion of labelled cells with pyramidal morphology was 3.6%,

with 96.4% being non-pyramidal. Those with pyramidal morphology were usually present on their own and were most frequently seen in layer III, while those with non-pyramidal morphology were evenly distributed across laminae II-VI. Both the pyramidal and non-pyramidal neurones appeared to be smaller in size and present in much smaller numbers than their counterparts in areas which showed the bilaminar type of distribution.

In areas where the neurones were intensely labelled, it was possible to classify their morphology in more detail. Among the pyramidal cells, there was a variation in size, but all the labelled neurones shared the same basic morphology. In contrast, four broad morphological categories were evident among the non-pyramidal cells. In the caudal prefrontal areas (8a, 8b and 45) and in premotor cortex, multipolar (Figs. 14, 15) and bitufted (Fig. 16) morphologies predominated, and cells of this type were found in all the immunoreactive laminae. Bipolar (Fig. 17B) and horizontal cells (Fig. 17A) were present in much smaller numbers, and were largely restricted to layers II and III, and to layer VI, respectively. In posterior parietal cortex, multipolar and bitufted morphologies were again the most prevalent, and could be seen in all the immunoreactive laminae. Bipolar and horizontal cells were relatively uncommon, with the former mainly being found in layer III and the latter usually lying in layer VI. A more precise classification was precluded by the restriction of surface immunoreactivity to the soma

and proximal dendrites, rendering the axon and most of the dendritic tree invisible.

## 6.5 SURFACE LOCALISATION OF CAT-301 IMMUNOREACTIVITY

In all regions of the cerebral cortex, Cat-301 bound to the somata and proximal dendrites of neurones, and did not stain their distal dendrites or axons, or label glial cells (Figs. 12-17). The same applied to immunoreactive cells in the caudate nucleus and putamen. In the cortical areas, the surface distribution of immunoreactivity was constant, regardless of a given neurone's laminar position, or whether it displayed pyramidal (Figs. 12, 13) or non-pyramidal (Figs. 13-17) morphology. There was no clear variation with neuronal size, but on some large pyramidal cells, immunoreactivity seemed to extend along relatively more of the dendritic shaft than on smaller neurones, particularly on the apical dendrite (Fig. 12). Viewed under magnification at high power, the Cat-301 immunoreactivity appeared to form a reticular pattern on the neuronal surface (Fig. 12).

**FIGURE 12. Cortical pyramidal neurone labelled with Cat-301**

There is a prominent apical dendrite with smaller dendrites emerging from the base of the cell body. Cat-301 immunoreactivity is restricted to the soma and proximal dendrites, although in this example it extends along a considerable length of the apical process. The axon is not labelled. The reticular pattern formed by the exclusion of immunoreactive product from regions of contact with presynaptic boutons can be seen on the cell surface. Magnification x750.

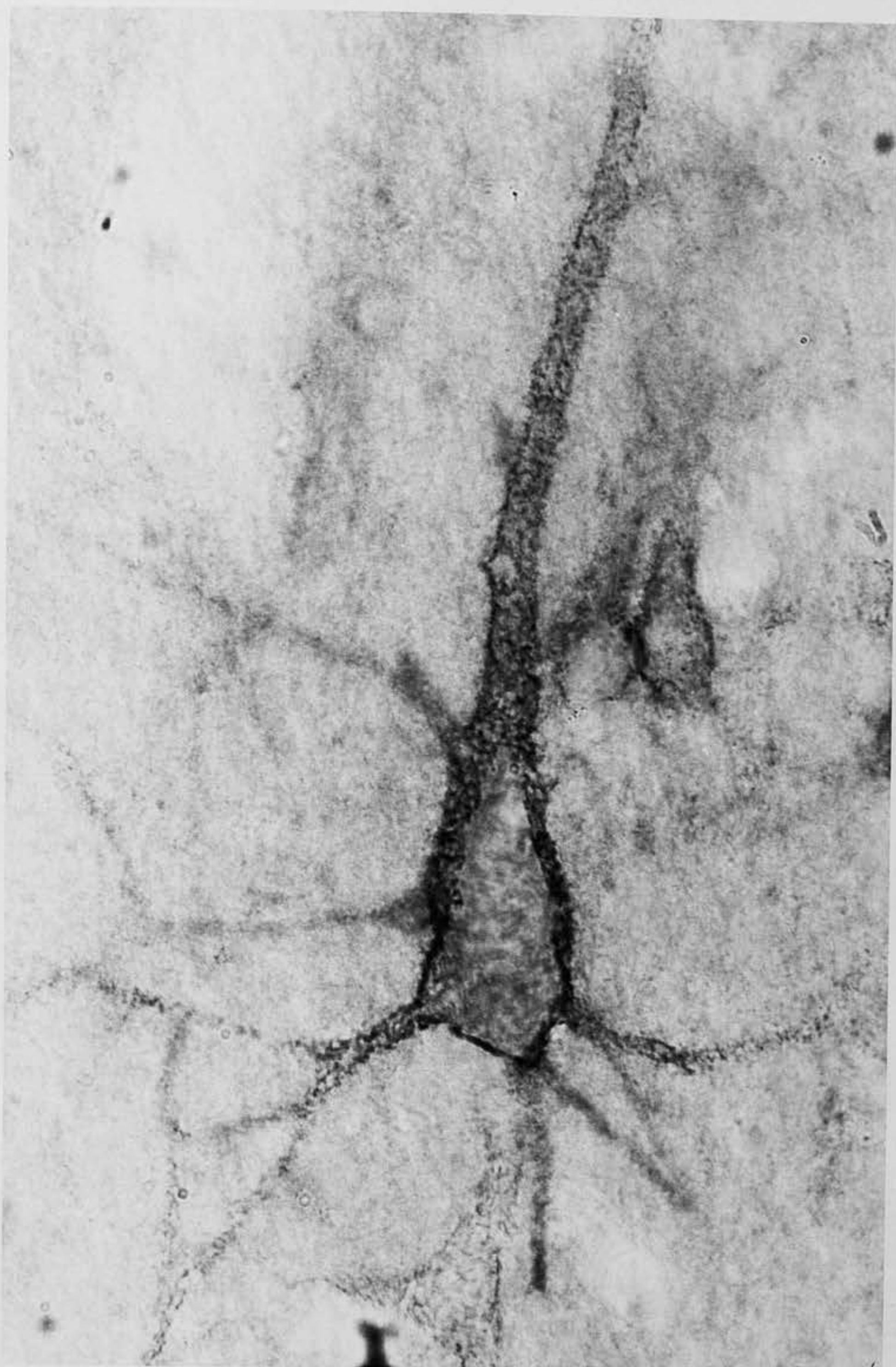


**FIGURE 13. Mixed group of pyramidal and non-pyramidal cortical neurones labelled with Cat-301.**

The neurone on the left has a conical soma with dendrites emerging from each corner of its base and a thick apical dendrite (shortened by the plane of section), indicating a pyramidal morphology. The dendrites of the central neurone branch out from each of its two poles, consistent with a bitufted morphology. The neurone on the right has an irregular cell body with dendrites extending in several directions, indicating a multipolar morphology. Magnification x400.

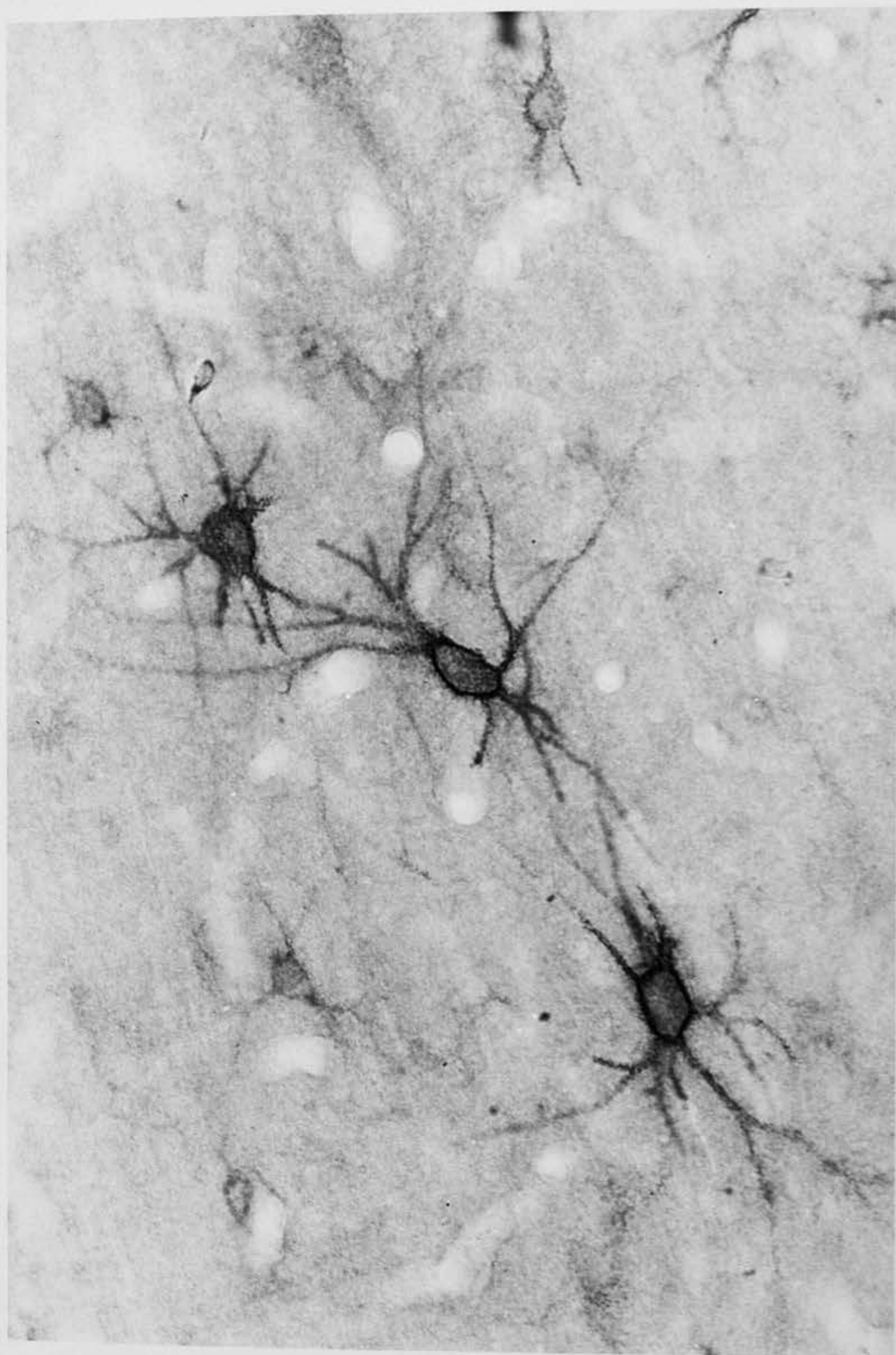


**Figure 14. Multipolar cortical neurone labelled with Cat-301.**

Labelled dendrites radiate in several directions from all parts of an irregularly-shaped soma, indicating a multipolar morphology. Labelling on the apical and basal dendrites appears to extend more distally than on the others, but this is probably an effect of the sectioning of processes which project out of the plane of section. Magnification x800.

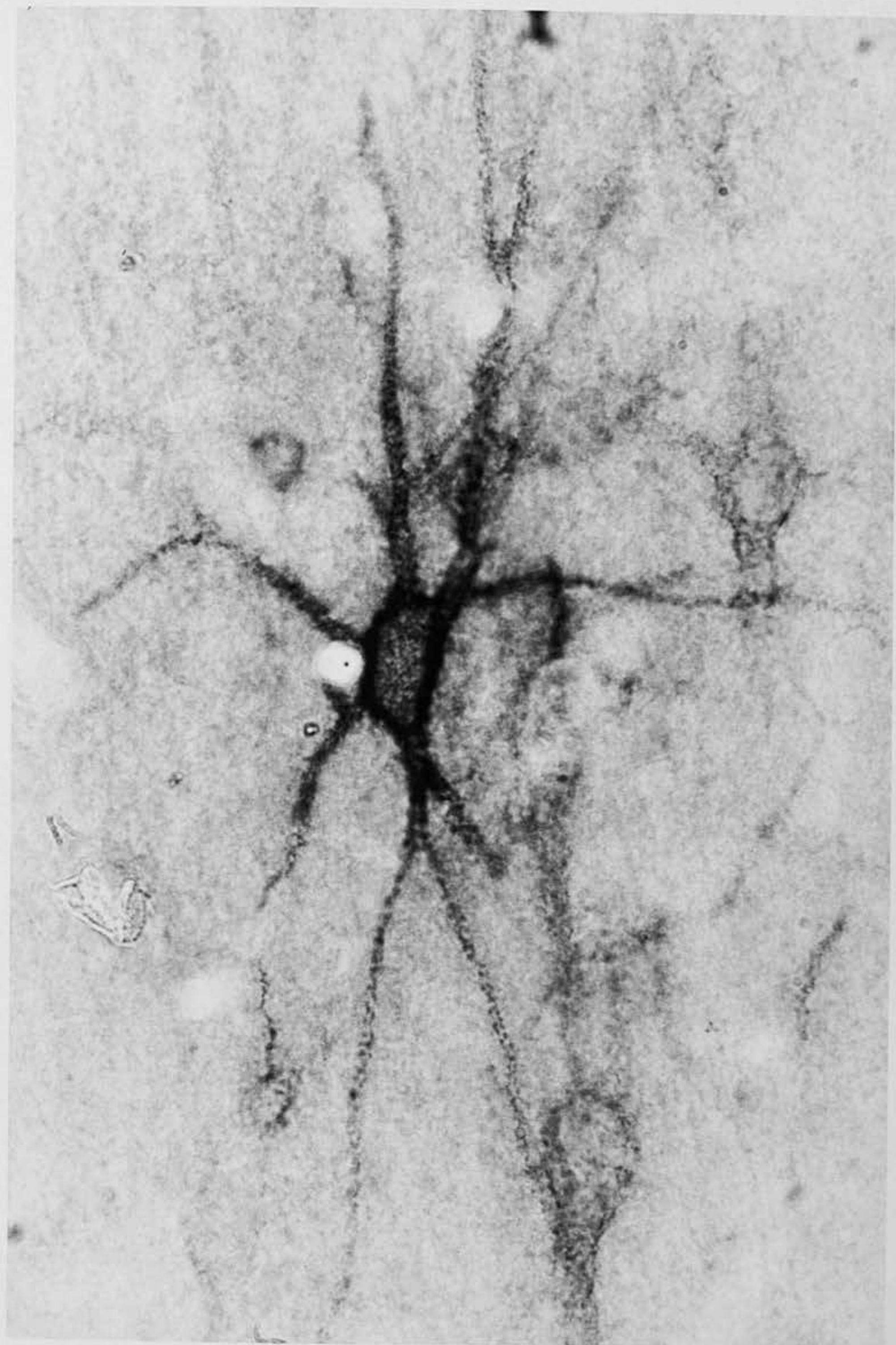


**FIGURE 15. Multipolar cortical neurone labelled with Cat-301.**

Dendrites labelled with Cat-301 extend from several points of the soma, some of them passing out of the plane of section. Magnification x800.

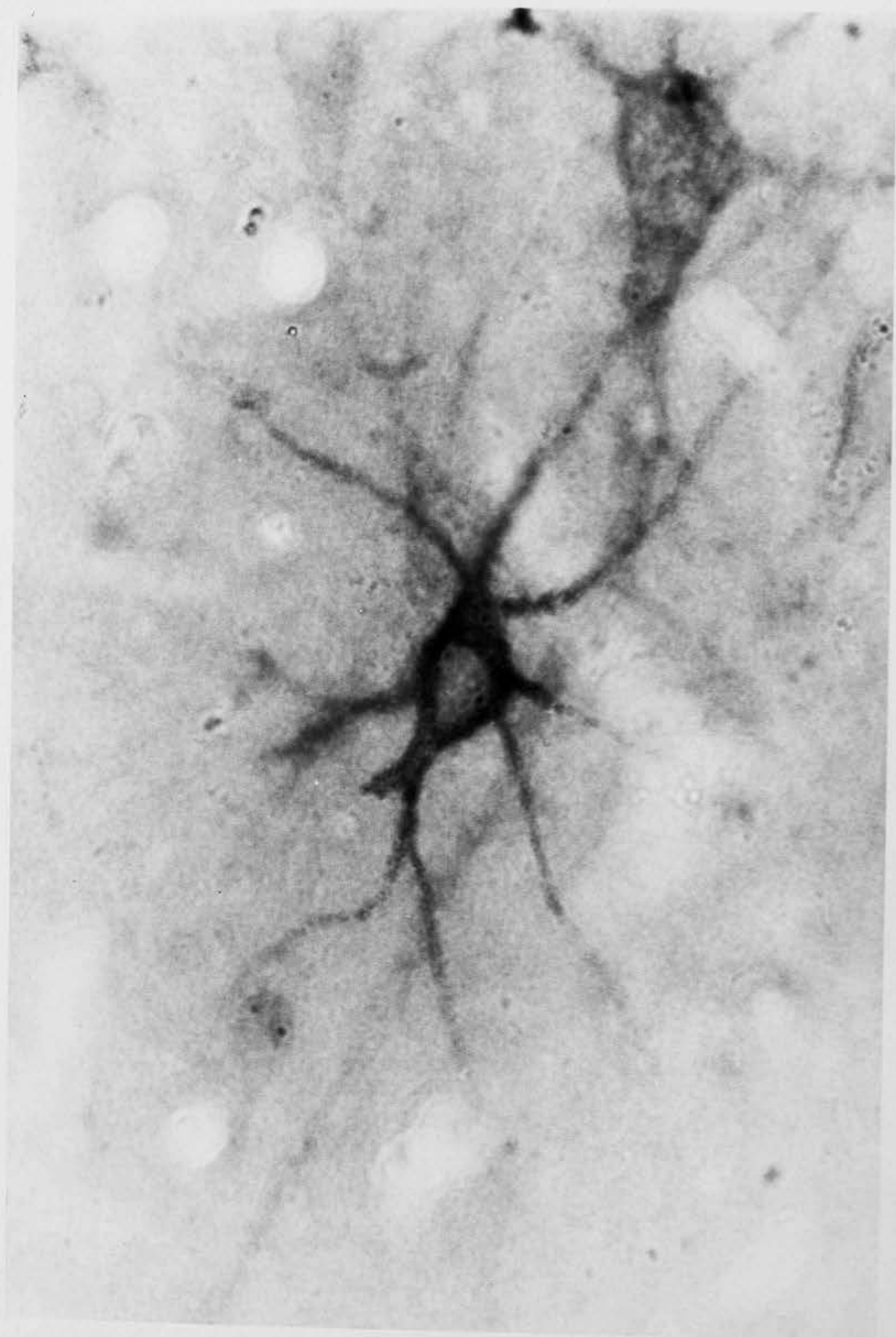


**FIGURE 16. Bitufted cortical neurone labelled with Cat-301.**

The proximal parts of this cell's dendrites have been labelled with Cat-301, demonstrating a pair of apical processes and another pair arising from the cell base, one of which has branched into two. The cell thus has a basically bitufted morphology, although visualisation of its distal processes with another staining process might permit a more detailed classification. The reticular nature of the labelling is evident on the dendrites and to a lesser extent on the soma. Magnification x840.

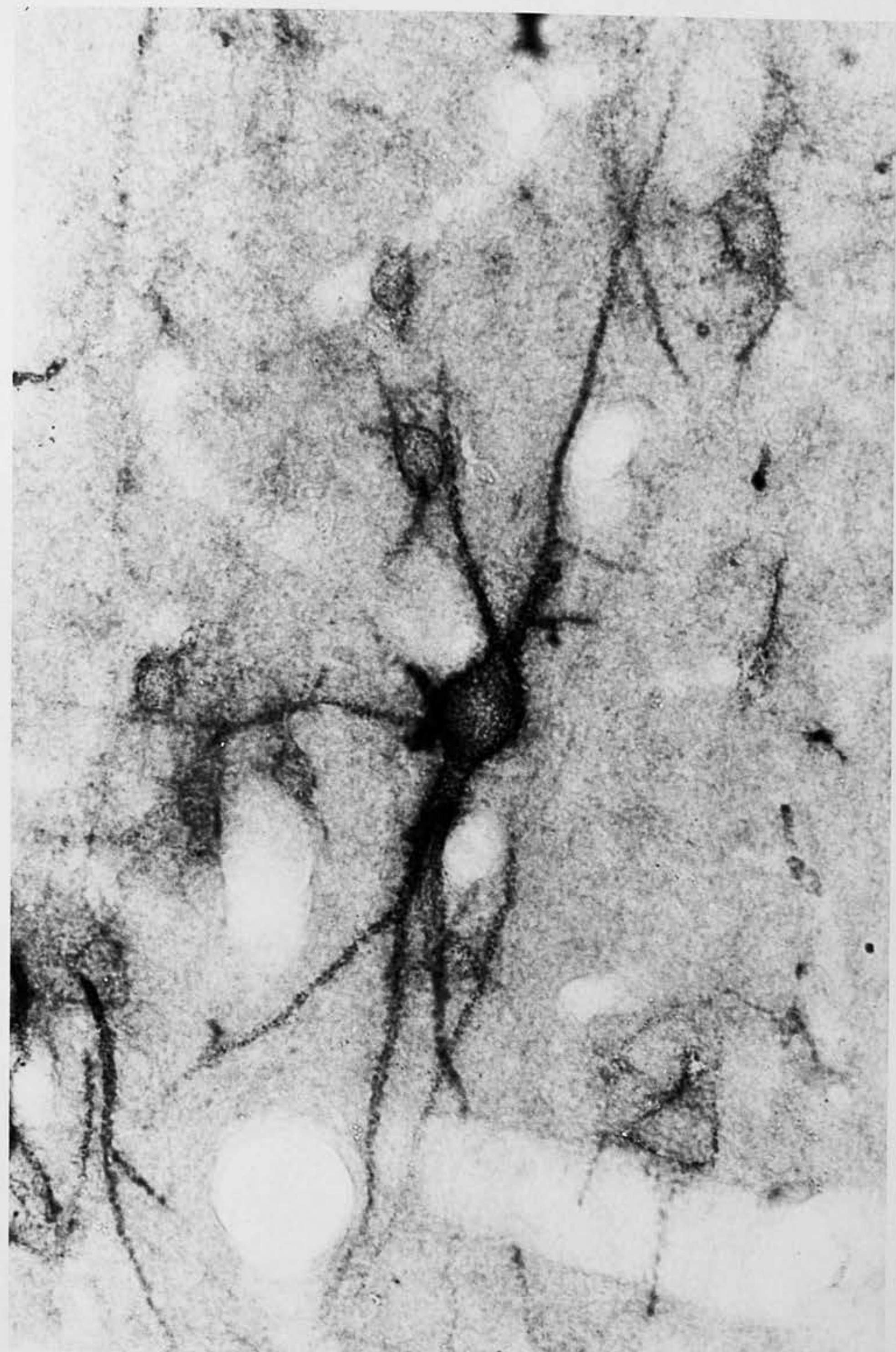
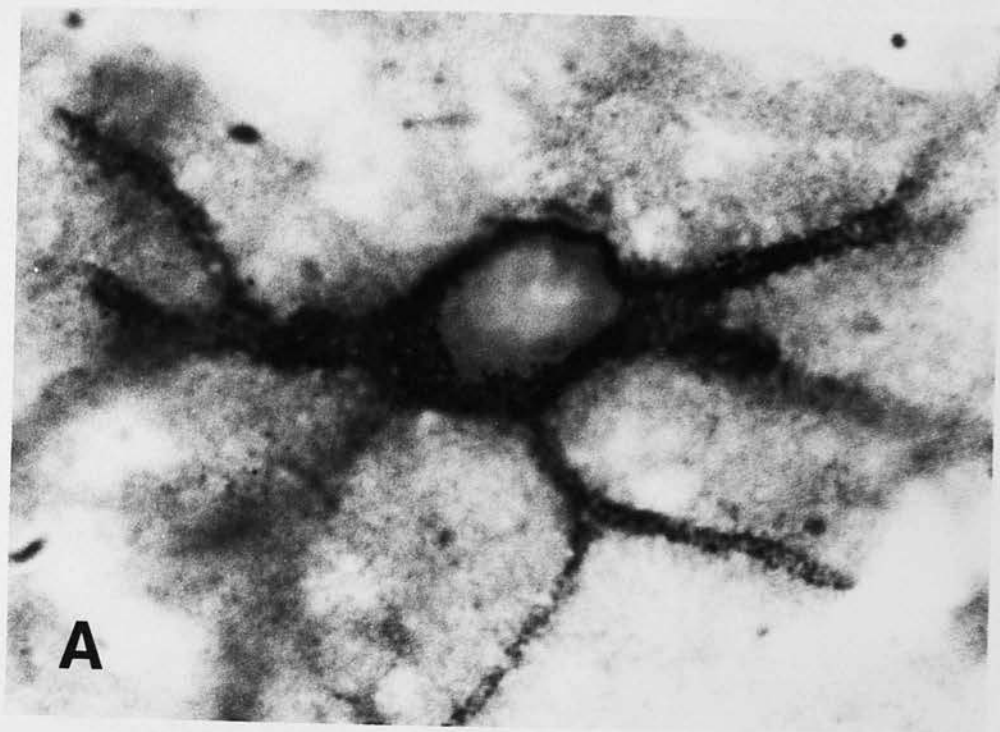


**FIGURE 17. Horizontal and bipolar cortical neurones labelled with Cat-301.**

**A.** Horizontal non-pyramidal cortical neurone, magnification x1200. Cat-301 labelled dendrites arise from each of the two poles of a cell orientated parallel to the cortical surface. There is also a process which descends in the vertical plane, but the predominant orientation of the dendrites is horizontal.

**B.** Bipolar cortical non-pyramidal neurone, x 1200. This neurone has an ovoid soma with single dendrites arising from each pole. Both dendrites branch close to the soma and are vertically orientated.


A

B

## 6.6 PROPORTION OF CORTICAL NEURONES LABELLED BY CAT-301

The proportion of cortical neurones which were immunoreactive to Cat-301 was quantified in a number of posterior parietal areas (Tables 6-9). The posterior parietal cortex was selected because there was a wide range in the intensity of labelling in its constituent areas, while the laminar distribution of immunoreactivity and the proportions of labelled pyramidal and non-pyramidal cells in each area were similar (Table 5). The latter helped control for the possibility that differences in the proportions of neurones labelled were a reflection of different distribution patterns of immunoreactivity.

In areas POa(e) and PG, which contained weakly-immunoreactive neurones, approximately 3.0% and 3.9% of the cortical neurones were labelled, respectively (Table 10). In PEa and POa(i), which contained intensely immunoreactive neurones, the respective proportions of cortical neurones labelled with Cat-301 were approximately 14.4% and 13.2% (Table 10). There thus appeared to be a correlation between the intensity of the immunoreactivity in a given area and the proportion of its neurones which were Cat-301-positive. The differences between the proportions of immunopositive cells in the heavily and lightly labelled areas were highly significant (Mann-Whitney U-Test; Table 11). It is unlikely that these differences

merely reflect a greater difficulty in identifying immunopositive neurones in areas where they are lightly labelled, as extremely low levels of immunoreactivity were detectable at the magnification which was used for the cell counting (x125).

| Table 6 | | | | | |
|---|---|---|---|---|---|
| **Counts of Cat-301 positive neurones in area PEa*** | | | | | |
| Sample | No. cells Cat-301 +ve | After Abercrombie correction | No. cells thionin-stained | After Abercrombie correction | % cells Cat-301 +ve |
| 1 | 10 | 8.35 | 53 | 46.9 | 17.8 |
| 2 | 7 | 5.85 | 46 | 40.71 | 14.4 |
| 3 | 6 | 5.01 | 47 | 41.6 | 12.0 |
| 4 | 8 | 6.68 | 51 | 45.14 | 14.8 |
| 5 | 7 | 5.85 | 49 | 43.36 | 13.5 |

* Counts made from 80μ wide columns orientated normal to the pial surface

| Table 7 | | | | | |
|---|---|---|---|---|---|
| **Counts of Cat-301 positive neurones in area POa(i)*** | | | | | |
| Sample | No. cells Cat-301 +ve | After Abercrombie correction | No. cells thionin-stained | After Abercrombie correction | % cells Cat-301 +ve |
| 1 | 10 | 8.35 | 82 | 72.57 | 11.46 |
| 2 | 7 | 5.85 | 67 | 59.3 | 9.82 |
| 3 | 7 | 5.85 | 50 | 44.25 | 13.16 |
| 4 | 14 | 7.51 | 68 | 60.18 | 19.34 |
| 5 | 9 | 7.52 | 68 | 60.18 | 12.44 |

* Counts made from 80μ wide columns orientated normal to the pial surface

## Table 8

**Counts of Cat-301 positive neurones in area POa(e)***

| Sample | No. cells Cat-301 +ve | After Abercrombie correction | No. cells thionin-stained | After Abercrombie correction | % cells Cat-301 +ve |
|---|---|---|---|---|---|
| 1 | 1 | 0.84 | 73 | 64.6 | 1.29 |
| 2 | 2 | 1.67 | 64 | 56.64 | 2.94 |
| 3 | 3 | 2.50 | 59 | 52.22 | 4.78 |
| 4 | 2 | 1.67 | 62 | 54.87 | 3.03 |
| 5 | 2 | 1.67 | 61 | 53.98 | 3.07 |

## Table 9

**Counts of Cat-301 positive neurones in area PG***

| Sample | No. cells Cat-301 +ve | After Abercrombie correction | No. cells thionin-stained | After Abercrombie correction | % cells Cat-301 +ve |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 66 | 58.41 | 0 |
| 2 | 3 | 2.5 | 52 | 46.02 | 5.43 |
| 3 | 2 | 1.67 | 60 | 53.1 | 3.13 |
| 4 | 5 | 4.18 | 81 | 71.68 | 5.8 |
| 5 | 3 | 2.5 | 70 | 61.95 | 4.03 |
| 6 | 5 | 4.18 | 77 | 68.14 | 6.1 |
| 7 | 2 | 1.67 | 64 | 56.64 | 2.94 |

* Counts made from 80µ wide columns orientated normal to the pial surface

## Table 10

**Proportion of Cat-301 positive neurones in selected posterior parietal areas**

| Area | Mean No..of Cat-301 +ve Neurones* | Mean Total No. of Neurones* | % Neurones Cat-301 positive | Intensity of labelling |
|---|---|---|---|---|
| PEa | 6.3 | 43.5 | 14.44 | Strong |
| POa(i) | 7.8 | 59.3 | 13.24 | Strong |
| POa(e) | 1.7 | 56.5 | 3.02 | Weak |
| PG | 2.3 | 59.4 | 3.92 | Weak |

*Means of counts from samples detailed in Tables 6-9.

## Table 11

**Significance of differences between the proportions of Cat-301 positive neurones in strongly and weakly immunoreactive areas***

| Areas compared | | 2-tailed p value |
|---|---|---|
| PEa | POa(e) | 0.0090 |
| PEa | PG | 0.0045 |
| POa(i) | POa(e) | 0.0090 |
| POa(i) | PG | 0.0045 |

* Mann-Whitney U-Test

# Chapter 7:

# DISCUSSION

## 7.1 REGIONAL DIFFERENCES IN THE DISTRIBUTION OF CAT-301 IMMUNOREACTIVITY IN THE CEREBRAL CORTEX

Throughout the cerebral cortex, the distribution of Cat-301 immunoreactivity varied between cytoarchitectonic areas, was constant within each area, and changes in its distribution occurred at areal borders (Figs. 7, 8, 10). These observations are consistent with the findings of previous studies which examined the distribution in the occipital and temporal lobes (Hockfield et al, 1983; De Yoe et al, 1986). Thus, whatever the functional significance of Cat-301 immunoreactivity, its cortical distribution clearly respects areal boundaries. This supports the notion that the cytoarchitectural distinctions between cortical areas, some of which were made decades ago (Brodmann, 1909; Walker, 1940; Powell and Mountcastle, 1959; Pandya and Seltzer, 1982), reflect genuine differences in the functional organisation of the cortex.

This point is of particular interest with regard to more recent cytoarchitectural classifications which have yet to be widely accepted. In area POa in the posterior parietal cortex, the distribution of Cat-301 immunoreactivity corresponded to the subdivisions POa(i) and POa(e) which have been described by Seltzer and Pandya (1980;

Fig. 7). Similarly, in the ventral premotor area (6V) the pattern of labelling varied between the upper and lower parts, which approximately correspond to areas 6Va and 6Vb of Barbas and Pandya (1987; Fig. 11) In both these examples, neighbouring areas with rather similar cytoarchitecture displayed strikingly different levels of Cat-301 immunoreactivity, suggesting that antibodies such as Cat-301 can reveal distinctions between areas which are difficult to discern in Nissl-stained material. Such area may thus differ with respect to certain functions to a greater extent than might be expected from their cytoarchitectural features.

## 7.2 PATTERNS OF CAT-301 LABELLING WITHIN AREAS

When the areas that constitute the frontal and parietal lobes are considered together, two major distribution patterns of Cat-301 immunoreactivity emerge. In prefrontal cortex (other than areas 8a, 8b and 45), the lower part of ventral premotor cortex (area 6V), and in cingulate cortex (areas 23, 24 and 25) Cat-301-positive cells were evenly distributed across layers II-VI, were weakly-immunoreactive and present in relatively small numbers (Fig. 9). The vast majority (93-97%) of these cells were non-pyramidal. In contrast, in areas 8a, 8b and 45, and in premotor (other than the lower part of 6V), supplementary motor, motor, somatosensory (excluding area 3) and posterior parietal cortex, Cat-301-positive neurones were

concentrated in the lower part of layer III and in layer V, and a greater proportion of them (20-31%) were pyramidal (Figs. 9A, 9B). Moreover, apart from posterior parietal cortex (where their numbers and intensity of immunoreactivity varied), the Cat-301-positive cells in areas with this pattern of labelling were present in large numbers and were intensely immunoreactive. Thus, a high intensity of Cat-301 immunoreactivity tended to be associated with a population of labelled cells which had a quite different morphological compostition, density and laminar distribution to that seen in areas with weakly immunoreactive neurones.

The distribution of Cat-301-positive cells in the caudal frontal areas and in most of the parietal cortex resembles that of neurones which send ipsilateral (associational) and contralateral (callosal) cortico-cortical projections from these regions. Both these cell types are mainly found in the deep part of layer III, with lesser numbers in layers V and VI (Jacobson and Trojanowski, 1977; Schwartz and Goldman-Rakic, 1984; Andersen et al., 1985, Barbas and Mesulam, 1985; Barbas and Pandya, 1987). Tables 2, 3 and 5 show that most Cat-301-positive cells occupied the lower portion of layer III, with V and then VI being the next most populous layers. Moreover, retrograde axonal tracing with HRP demonstrates that Cat-301 positive neurones in area V2, which have a similar laminar distribution, send their axons to another cortical area, MT (De Yoe, unpublished observations). However, the Cat-301-positive cells in the heavily labelled laminae in frontal and parietal cortex comprise a

mixture of pyramidal and non-pyramidal neurones, whereas associational and callosal neurones are almost exclusively pyramidal (Schwartz and Goldman-Rakic, 1984; Jones, 1984). Nevertheless, the non-pyramidal cells recognised by Cat-301 might participate in local circuits with pyramidal cells with cortico-cortical connections.

It is interesting to speculate whether the Cat-301 positive pyramidal cells in these laminae have ipsi- or contralateral projections. However, this cannot be reliably determined from their laminar position, as associational and callosal neurones can be intermixed in the same laminae (Schwartz and Goldman-Rakic, 1984). The combination of Cat-301 immunocytochemistry with the retrograde tracing of the cortico-cortical projections from such neurones could be used to address this question. This approach has been used in other areas, and indicates that at least some of the Cat-301 positive cells in V2 have associational projections (De Yoe, unpublished observations). In areas with the diffuse pattern of immunoreactivity, the striking preponderance of non-pyramidal cells (93-97% of the total) and their relatively even laminar distribution, suggest that the Cat-301 positive neurones in such areas are unlikely to have cortico-cortical projections, and probably participate in local circuits.

The laminar distribution of immunoreactivity in area 3 was unique among the frontal and parietal areas, in that its labelled cells were concentrated in layers IV and VI. A similar distribution is evident in primary visual cortex, where Cat-301 positive neurones mainly

occupy layers IV and VI (Hockfield et al., 1983), in contrast to the extrastriate visual areas (V2, MT and MST), where they are concentrated in layers III and V (De Yoe et al, 1986). Both area 3 and the striate cortex are primary sensory areas which receive thalamic information and convey this to a series of cortical areas for further processing (Jones et al., 1978; Van Essen, 1985), and in both cases the heavily labelled laminae correspond to those where the thalamic afferents terminate. Their similar laminar distributions of Cat-301 positive cells may reflect these functional similarities

In the caudal frontal areas, in addition to neuronal Cat-301 immunoreactivity, there were continuous bands of faint staining in certain laminae which were not clearly associated with neuronal profiles. These bands were invariably evident in layers IIIb and V, the laminae with the most intense neuronal labelling. A similar staining pattern with Cat-301 has been observed in the laminae containing strongly immunopositive neurones in the primary visual cortex, and ultrastructural analysis suggests that much of this immunoreactivty is not associated with cellular processes (Hockfield, unpublished observations). Its significance remains unclear.

Previous work has demonstrated that in the primary (V1) and secondary (V2) visual cortices, Cat-301 positive neurones are arranged in groups which are orientated normal to the pial surface, and lie in vertical register with regions of cytochrome oxidase activity (Hendry et al, 1984, 1988). The absence of any evidence of

similar organisational features among labelled cells in posterior parietal cortex, suggests that such arrangements are not universal among cortical areas containing Cat-301 positive neurones. Nor do such arrangements seem to be a typical feature of cortical areas with a visual specialisation, as the areas examined included two putative visual areas (PG and VIP).

Similarly, there was no evidence of orthogonally orientated regions of cytochrome oxidase activity in the posterior parietal areas, a finding consistent with that of a previous study (Tootell et al, 1985). The identification of orthogonal arrangements of labelled cells or histochemical activity in tangential material can be notoriously difficult, as it is often contingent on the sections passing through single cortical laminae. While negative findings from such studies must thus be treated with some caution, the present results raise the possiblity that orthogonally orientated arrangements of Cat-301 neurones are restricted to areas with corresponding patterns of cytochrome oxidase activity.

## 7.3 FUNCTIONAL CORRELATIONS OF THE DISTRIBUTION OF CAT-301 IMMUNOREACTIVITY IN THE FRONTAL AND PARIETAL LOBES

Within the frontal lobe, there were striking differences between the areas in its rostral and caudal portions, in terms of the intensity of immunoreactivty of the Cat-301 positive neurones and their laminar distribution (Fig. 11), suggesting that there may be some fundamental distinction between them. In this context, the caudal portion is taken to comprise areas 4, 6, 8 and 45, while the areas anterior to these are included in the rostral part (Fig. 11). The importance of motor cortex (area 4) in somatic motor function is well known. The various subdivisions of premotor cortex (area 6) seem to play a role in the programming and control of complex somatic movements (Weinrich et al, 1984; Goldberg, 1985; Kurata and Tanji, 1986; Rizolatti et al, 1987; Passingham, 1988; Rizolatti and Gentilucci, 1988), while areas 8a and 45 contain the frontal eye fields, which participate in the control of eye movements (Bruce et al., 1985; Bruce, 1988). All of these areas project into the cortico-pyramidal tract whereas the rostral frontal areas do not (Toyoshima and Sakai, 1982). Moreover, the rostral frontal areas are characterised by a prominent (granular) layer IV and lack large pyramidal cells, while their caudal neighbours possess large pyramidal cells but have a granular layer which is less prominent or absent (Walker, 1940). In short, a major difference between the caudal and rostral frontal areas may be that the caudal have direct or explicit motor functions,

whereas the rostral play a more indirect role in motor control (Goldman-Rakic, 1987b). This basic functional distinction might be reflected in their markedly different distributions and intensities of Cat-301 immunoreactivity.

In this respect, the lower part of area 6V is unusual, as it lies in the premotor cortex in the caudal part of the frontal lobe, yet displays the diffuse laminar distribution of weakly-immunoreactive neurones seen in the rostral areas. While physiological studies indicate that the ventral portion of area 6 clearly has motor functions, most of the recordings appear to have been made in the upper, and not the lower, part of this division (Weinrich et al, 1984; Godschalk et al, 1985; Kurata and Tanji, 1986; Rizolatti et al, 1987; Rizolatti and Gentilucci, 1988). Moreover, anatomical evidence indicates that the lower part of 6V differs from the rest of the premotor areas in that it projects to the brain stem but not to the spinal cord (Toyoshima and Sakai, 1982), and that it lacks the strong projections which its neighbours receive from area 4 (Matelli et al, 1986). This portion of 6V, which appears to correspond to area 6Vb of Barbas and Pandya (1987), may thus be functionally distinct from the rest of the premotor cortex.

In parietal cortex large numbers of intensely-immunoreactive neurones were found in primary somatosensory cortex (areas 1 and 2), the superior parietal lobule (PE) and the dorsal bank (PEa), fundus (IPd) and lower half of the ventral bank (POa(i)) of the

intraparietal sulcus (Figs. 10, 11). Neurones in areas 1,2, PE and PEa are primarily driven by somatosensory stimuli (Mountcastle and Powell, 1959; Sakata et al, 1973; Chapman et al, 1984, Kalaska et al, 1983). Areas IPd and POa(i) contain a zone termed VIP (Maunsell and Van Essen, 1983; Ungerleider and Desimone, 1986; Fig. 5), which receives a major input from MT, an area concerned with the analysis of visual motion (Van Essen et al, 1981). Neurones in POa(i) are responsive to visual stimuli, changes in eye position and saccadic eye movements (Andersen et al., 1987), and their stimulation elicits saccades (Shibutani, et al, 1984). The heavily labelled parietal areas may thus be divided into two functional categories, those with a broadly somatosensory function (1, 2, PE, and PEa) and those which may participate in the analysis of visual motion (IPd and POa(i)). However, Cat-301 may not heavily label *all* parietal areas with somatosensory or visuo-spatial functions, since area 3 and PF, whose neurones respond to somatosensory stimuli (Robinson and Burton, 1980; Jones, 1986), and area PG, which contains cells receptive to complex visual motion (Sakata et al, 1985; Motter et al, 1987), were relatively weakly labelled.

When the heavily labelled areas in the frontal and parietal cortex are considered together, one common feature is that they all have direct connections with the spinal cord or medulla oblongata (Murray and Coulter, 1981; Biber et al, 1978; Toyoshima and Sakai, 1982). Thus, when injections of HRP are made in the spinal cord, retrogradely labelled neurones are concentrated in the frontal and parietal cortex,

in a constellation of areas which is remarkably similar to that of the areas strongly immunoreactive to Cat-301 (Toyoshima and Sakai, 1982). Conversely, after the same procedure, the areas in frontal and parietal cortex with small numbers of weakly-immunoreactive neurones do not contain retrogradely labelled neurones. This is also true of areas in temporal cortex, which show a similar diffuse distribution of neurones which are weakly immunoreactive to Cat-301 (Hendry et al, 1988). Previous work has shown that retrogradely-labelled cortico-spinal neurones in layer V of motor and somatosensory cortex are Cat-301-positive (Hendry et al, 1988) and that the spinal cord and medulla are rich in Cat-301-positive neurones (Hockfield and McKay, 1983; Kalb and Hockfield, 1988). The pattern of labelling in the frontal and parietal lobes is thus consistent with the observation that Cat-301 often labels neurones in areas which are interconnected (Hendry et al, 1988). This is discussed further below, in the context of cortico-cortical connections.

Although variability of staining with the Gallyas technique made precise correlation difficult, all of the frontal and parietal areas which were heavily labelled with Cat-301 were heavily myelinated. However, some areas which were only weakly labelled (e.g. areas 3 and 12), were also well myelinated, indicating that there was no simple relationship between these two variables. Nevertheless, the visual areas in the occipital and temporal lobes which are known to be heavily labelled with Cat-301 (V1, V2, MT and MST) are also well myelinated (Tootell et al, 1983; Ungerleider and Desimone, 1986; Van

Essen et al, 1981), suggesting that this might be a general, but not exclusive, feature of cortical areas which are strongly immunoreactive to Cat-301.

Each of the heavily labelled areas in the frontal lobe has major connections with one or more heavily labelled areas in the parietal lobe, and vice versa. The motor cortex is interconnected with areas 1 and 2 (Jones et al, 1978), the motor cortex and area 6 have reciprocal connections with areas PE and PEa (Petrides and Pandya, 1984), and the frontal eye fields (areas 8a and 45) are interconnected with POa(i) (Barbas and Mesulam, 1981, Andersen et al, 1985; Heurta et al, 1987; Barbas, 1988). (The precise frontal connections of IPd are unknown.) However, these areas also have connections with weakly labelled areas. For example, the superior division of ventral premotor cortex (6V) has major connections with PF in the inferior parietal lobule (Matelli et al, 1986), and the frontal eye fields are reciprocally connected with POa(e) (Barbas and Pandya, 1981; Andersen et alΠ, 1985; Barbas, 1988). Nevertheless, previous work has shown that thalamic nuclei containing intensely-labelled neurones are exclusively interconnected with heavily-labelled cortical areas (Hendry et al, 1988) and that Cat-301-positive cells in the central visual system are concentrated in areas known to be interconnected (Sur et al, 1984; MacAvoy et al, 1986; De Yoe et al, 1986; Hendry et al, 1984; 1988).

The laminar distribution of Cat-301-positive neurones in the heavily-labelled frontal and parietal areas is similar to that of the

cortico-cortical neurones which project between them, and Cat-301 positive neurones in V2 project directly to MT, another area that contains many Cat-301-positive cells (Chapter 7.2). Projections from posterior parietal to prefrontal cortex mainly *terminate* in layers I and IV (Goldman-Rakic, 1987a), while those from somatosensory to motor and premotor cortex, and vice versa, terminate in layer IV, and to a lesser extent in layers I-III (Jones, 1986). It therefore appears that axons which pass from the frontal to parietal areas, and vice versa, largely contact laminae *other* than those where the Cat-301-positive cells are most concentrated (layers III and V). Cortico-cortical projections from Cat-301-positive neurones thus seem unlikely to directly contact Cat-301-positive processes or cell bodies in other areas. They might instead terminate on cells which are not recognised by Cat-301, or on the unlabeled distal dendrites of immunoreactive neurones which extend into the recipient layers.

The difference in the distribution and intensity of immunoreactivity between area 3, where moderately immunoreactive cells were concentrated in laminae IV and VI, and the other primary somatosensory areas (areas 1 and 2), which showed heavy labelling in layers III and V, might also be related to fronto-parietal interconnectivity. Area 3 projects to other areas in the parietal lobe, but conspicuously lacks the interconnections with motor cortex and the supplementary motor area that are features of areas 1 and 2 (Jones, 1986).

To summarise, Cat-301 seems to heavily label frontal areas with somato-motor and oculo-motor functions and parietal areas which process somatosensory or visuo-spatial information. These frontal and parietal areas are known to be interconnected and to project to the spinal cord or brain stem, regions which are also heavily labelled with Cat-301. The Cat-301-positive neurones in these frontal and parietal areas are concentrated in the laminae (III and V) from which their cortico-cortical and descending projections arise. As somatosensory and visuo-spatial information may be critical for somatic and ocular movements, these areas can be considered to share a broadly motor specialisation. Together with structures outwith the cerebral cortex, such as the spinal cord, they may constitute an interconnected network of areas devoted to motor functions.

## 7.4 CAT-301 AND THE MAGNOCELLULAR PATHWAY

In the central visual system there appear to be two segregated 'streams' of areas mainly specialized for the analysis of form and color, and of motion, termed the parvocellular and magnocellular pathways, respectively (Chapter 3.3a; Fig. 2). The distribution of Cat-301 immunoreactivity in this system has previously aroused considerable interest because it appears to be localised to neurones

in the magnocellular pathway. Thus, Cat-301 heavily labels neurones in the magnocellular layers of the LGN (Hockfield et al, 1983; Hendry et al, 1984), layers IVb and VI of striate cortex (Hendry et al, 1984), the thick cytochrome oxidase stripes in V2 (De Yoe et al, 1985; Hendry et al, 1988), and in MT and MST (De Yoe et al, 1986). Based on their connections and physiological characteristics, putative rostral extensions of this pathway include a zone in the fundus and deep half of the ventral bank of the intraparietal sulcus (area VIP, Fig. 5), area PG and the frontal eye fields (Chapter 3.3a).

The heavy density and strong immunoreactivity of Cat-301-positive neurones in areas IPd and POa(i) (which probably include VIP; Fig. 5) and in areas 8a and 45 (the frontal eye fields) is consistent with the notion that Cat-301 heavily labels areas specialized for the analysis of visual motion. However, the low levels of immunoreactivity in area PG suggest that this may not be true for all areas with this functional specialisation. Recently, it has been suggested that the concept of entirely segregated functional visual streams may be an oversimplification (Martin, 1988), as the two are interconnected at several levels (De Yoe and Van Essen, 1988; Zeki and Shipp, 1988), and in certain areas, including PG, seem to converge (Perret et al, 1985; Bayliss et al, 1987; Neal et al, 1988; Cavada and Goldman-Rakic 1989a). A given area may thus participate in more than one type of visual analysis, and the presence of neurones responsive to visual motion (such as PG) does not necessarily indicate that a given area is part of a pathway absolutely specialised for this function.

Given the heavy labelling of many areas in frontal and parietal cortex which do not seem to play a role in visual processing, Cat-301 cannot simply be regarded as a marker for areas specialized for visual motion. This view arose from initial studies of its distribution, which focussed on purely visual regions (Hockfield et al, 1983). Nevertheless, a major purpose of the analysis of visual motion is presumably to direct eye movements. Indeed, stimulation of the frontal eye fields or POa(i) elicits saccades (Bruce et al, 1985; Shibutani et al, 1984) and lesions of MT or MST disrupt the normal tracking of visual targets (Newsome and Wurtz, 1988). Thus, just as parietal somatosensory areas may provide the motor and premotor areas with sensory information crucial for the control of somatic movements, areas in the magnocellular pathway may supply POa(i) and the frontal eye fields with data for regulating ocular movements. The selective labelling of the visual 'motion' areas can therefore be viewed as a reflection of their role in the control of eye movements, which is consistent with the notion that Cat-301 recognises areas with a broadly motor specialisation (Chapter 7.3).

## 7.5 THE POPULATION OF CORTICAL NEURONES IMMUNOREACTIVE TO CAT-301

As in all other regions that have been analysed (Hockfield and McKay, 1983; Sur et al, 1984; MacAvoy et al, 1986), each area of the cerebral cortex examined in this study contained a sub-population of neurones that was immunoreactive to Cat-301. The size of this population in cortical areas (3.0-14.4% of the total neuronal population) is relatively small compared to that in the LGN, where 70-85% of the cells in the magnocellular layers are Cat-301 positive (Hendry et al, 1988; Sur et al, 1988), or the ventral spinal cord, where virtually all sciatic motor neurones are labelled by the antibody (Kalb and Hockfield, 1988). The significance of the restricted nature of the cortical population is unclear, but it seems likely that neurones which are morphologically homogeneous (e.g. pyramidal cells) may be heterogeneous with respect to other characteristics. Cat-301 presumably recognises an antigen which is expressed by a subset of cortical neurones which share an as yet unresolved functional or anatomical property.

Within the cerebral cortex, the proportion of neurones which were Cat-301 positive appeared to vary between areas, with respect to the intensity of Cat-301 labelling. In posterior parietal cortex, approximately 3.0% and 3.9% of the neurones in weakly labelled areas ($POa_{(e)}$ and PG) were Cat-301-positive, while in intensely labelled areas ($PE_a$ and $POa_{(i)}$) the corresponding figures were 13.2%

and 14.4%. This relationship is also evident in the LGN, where 70-85% of the cells in the intensely immunoreactive magnocellular laminae are Cat-301 positive, compared to fewer than 40% in the less intensely labelled parvocellular layers (Hendry et al, 1988; Sur et al, 1988). While it is often assumed that the density of neurones labelled with an antibody varies directly with the intensity of their immunoreactivity, this relationship does not seem to have been directly examined for other surface molecules.

Hendry et al (1988) estimated the proportion of Cat-301-positive cells in areas 4, 1-2 and V1, which were all intensely immunoreactive, as 10.3%, 10.4% and 10.1%, respectively. These estimates are broadly comparable with those from the intensely immunoreactive areas examined in this study (13.2% and 14.4%). The differences between them may reflect the different concentrations of primary antibody, and the different counting methodologies that were employed in the two studies. Interestingly, the size of the population of Cat-301 positive neurones (3.9-14.4%) is of a similar magnitude to those identified with antibodies to other surface antigens. In the rat, Tor-23 labels <1-7% of cortical neurones, depending on the area concerned (Stephenson and Kushner, 1988), while the monoclonal VC1.1 recognises 5-10% of neurones in area 17 of the cat (Naegele et al, 1987).

Just as the number of immunopositive neurones seems to vary with the level of expression of the Cat-301 antigen, so may the neuronal

composition of the labelled population. Cortical areas containing intensely immunoreactive neurones invariably displayed a bilaminar distribution of labelled cells, while a diffuse pattern was usually evident in those containing weakly immunoreactive neurones. Moreover, the bilaminar pattern was associated with a quite different ratio of pyramidal: non-pyramidal morphologies among the labelled neurones than its diffuse counterpart, and with a higher density of labelled neurones (Chapter 7.2). Thus, not only the intensity of immunoreactivity and neuronal density, but also the composition and laminar distribution of the labelled population, may vary with the level of expression of the Cat-301 antigen.

Cat-301 recognised both pyramidal and non-pyramidal neurones, and was not selective for any particular cell size within these morphological categories. Similarly, although they were often concentrated in certain laminae, immunoreactive neurones were evident in all of the cortical layers (with the exception of layer I). More detailed analysis indicated that most of the immunopositive non-pyramidal cells were bitufted or multipolar (Figs. 14-16). A number of non-pyramidal types (as defined by Golgi staining) can display these basic morphologies (Peters and Jones, 1984), and without the ability to visualise the axon and the bulk of the dendritic tree, it is not possible to identify these neurones in more precise morphological terms.

## 7.6 THE ANTIGEN RECOGNISED BY CAT-301

In all the regions of the cerebral cortex examined, and in the neostriatum, Cat-301 immunoreactivity was restricted to the surface of neuronal somata and proximal dendrites. The same distribution has been found on neurones in the spinal cord, brain stem, cerebellum and thalamus (Hockfield et al, 1983), and in other regions of the cerebral cortex (De Yoe et al, 1986; Hendry et al, 1988), and it is the same in rodents, cats, non-human primates (Hockfield et al, 1983) and man (Hockfield et al, 1990). The recognised antigen thus seems to be distributed in a remarkably constant pattern, despite the quite different morphological, neurochemical and functional characteristics of the labelled cells, and their range of locations within the central nervous system. This suggests that it might be involved in some function which is common to a wide diversity of neuronal types.

The absence of Cat-301 labelling on distal dendrites and axons could be attributed to difficulties in detecting the small amounts of antigen that might be expected to be found on processes of a relatively narrow calibre, rather than to a true absence of the antigen. However, raising the antibody titre is known to increase the intensity of somatic labelling without an appreciable effect on the size of the stained dendritic arbor (Hockfield and McGuire, unpublished observations), suggesting that the distinction between the

immunoreactivity of the proximal and distal dendrites is qualitative rather than quantitative.

A similar surface distribution to that seen with Cat-301 is evident with surface antigens which are recognised by other antibodies. The monoclonal VC1.1 labels the soma and proximal dendrites of neurones in area 17 and other regions of the cat CNS (Naegele et al, 1988), while Tor-23 has the same surface distribution on neurones throughout the CNS of the rat (Stephenson and Kushner, 1988). The monoclonal antibody which recognises the LAMP protein also binds to the soma and proximal dendrites of central neurones in the rat, although during development it also labels axons (Zacco et al, 1990). The prevalence of this particular surface distribution among a number of different antigens suggests that it may reflect a relatively general organisational feature of central neurones. The association between the antigens recognised by Cat-301 and by VC1.1 and synaptic boutons (below), and the observation that GABAergic terminals on pyramidal and non-pyramidal cortical neurones also have a somatic-proximal dendritic distribution (Houser et al, 1984), raises the possibility that it might be related to the presence of certain types of afferents, such as inhibitory inputs, on this part of the neuronal surface. This could be investigated by combining Cat-301 immunocytochemistry with that for GABA, or with an electron microscopic assessment of whether the synapses concerned were symmetrical or asymmetrical.

The surface distribution of Cat-301 immunoreactivity also resembles that of other antigens at the ultrastructural level. Immunoreactivity to VC1.1, Tor-23 and the antibody to LAMP forms a punctate pattern reminiscent of that seen with Cat-301 (Naegele et al, 1988; Stephenson and Kushner, 1988; Zacco et al, 1990). Moreover, VC1.1 has a similar peri-synaptic localisation, being excluded from synapses (Naegele et al, 1988). However, each of these antibodies labels quite different neuronal populations, and biochemical analyses of the molecular species that they recognise indicate that they are distinct antigens. Thus, while that recognised by Cat-301 is a 680 KD extracellular matrix proteoglycan, Tor-23 binds to a protein of 175 KD (Stephenson and Kushner, 1988) and LAMP is an integral membrane protein of 64-68 KD (Zacco et al, 1990). Nevertheless, there may be some overlap between the antigens bound by Cat-301 and VC1.1. In a number of regions, a proportion of the Cat-301 positive cells are VC1.1 positive, and vice versa, and one of the four species recognised by VC1.1 has a similar molecular weight (700KD) to the antigen recognised by Cat-301 (Zaremba et al, 1989).

Components of the extracellular matrix are thought to play important roles in neural development (Alberts et al, 1983; Sanes, 1983) and many of the implicated molecules have been characterised as proteoglycans. A number of proteoglycans appear to be selectively associated with regions of synaptic contact. Proteoglycans co-aggregate with acetylcholine receptors during the formation of frog neuromuscular synapses (Anderson and Fambrough, 1983) and

acetylcholinesterase is anchored to the extracellular matrix of the neuromuscular junction by a proteoglycan (Vigny et al, 1983). Heparan sulphate proteoglycan is concentrated in the basal lamina of neuromuscular synapses (Anderson and Fambrough, 1983) and TAP1, a chondroitin sulphate proteoglycan, is found at the synapse of the Torpedo electric organ (Carlson and Wright, 1987). The cytotactin binding proteoglycan (CTB) is thought to play a role in the formation of the thalamo-cortical 'barrel' fields in the mouse cerebral cortex (Crossin et al, 1989). The 473 proteoglycan may serve a similar function in the same region, and also demarcates the boundaries of developing nuclei in other parts of the CNS at a time when their synaptic connections are being formed (Steindler et al, 1990).

Proteoglycans may also influence the adhesion of neurones to one another or to adjacent surfaces, by complexing with known adhesion molecules, or by acting as receptors for them. Heparan sulphate proteoglycan forms a complex with laminin, and may thereby influence its functions in axonal growth and neuronal migration (Lander, 1985). Similarly, Heparan sulphate proteoglycan binding to N-CAM may be a prerequisite for its role in cellular adhesion (Cole and Glaser, 1989). Cytotactin binding proteoglycan (CTB) is a specific receptor for the adhesion molecule cytotactin.

Precedents for the functions which the Cat-301 antigen might perform can be sought from a review of the existing literature on neural surface molecules (Chapter 2). Surface molecules have been

implicated in mediating cell:cell adhesion during early development, subsequent neuronal migration, the growth of axons along particular routes, and the recognition of synaptic targets once an axon has reached its projection zone. The Cat-301 antigen is not expressed until relatively late in development, in the early post-natal period, and its appearance coincides with the ending of critical periods and the acquisition of physiological maturity (MacAvoy et al, 1985; Sur et al, 1988; Kalb and Hockfield, 1988). It then continues to be expressed throughout adult life. This indicates that the antigen is unlikely to play a role in the cellular adhesion, neuronal migration and axonal extension that characterise the early stages of neural development, but is more likely to serve some function once critical developmental periods have been successfully negotiated and the mature pattern of connections has been achieved.

Cat-301 immunoreactivity surrounds presynaptic boutons on the post-synaptic membrane, but is conspicuously excluded from the synapses themselves (Hockfield and McKay, 1983). Its absence from the synaptic cleft suggests that the antigen is unlikely to play a role in the process of synaptic transmission, and its peri-terminal distribution raises the possibility of a more structural function. A characteristic feature of areas which contain high densities of Cat-301 positive neurones is that they are linked together by strong and specific interconnections (Hockfield et al, 1983; Hendry et al, 1988; this study). The Cat-301 antigen might thus serve some function in relation to the formation or maintenance of these connections.

Interference with afferent activity during critical periods in the development of the LGN and the spinal cord seems to prevent both the development of the normal pattern of connections and the expression of the Cat-301 antigen, but has no effect in the adult (Sur et al, 1988; Kalb and Hockfield, 1988). Expression therefore appears to depend on the reception of appropriate afferent activity during such periods, and the development of the normal mature pattern of afferent connections. The ending of these critical periods signals the demise of the potential for synaptic plasticity and the expression of Cat-301 immunoreactivity at this time raises the possibility that the antigen might contribute to this process, perhaps by stabilising synapses in their adult positions. It may continue to do this throughout adult life, helping to maintain connections in the normal mature pattern. However, in the adult, sectioning of supraspinal inputs and crushing the sciatic nerve has no effect on the expression of the Cat-301 antigen by spinal motor neurones (Kalb and Hockfield, 1988).

# CONCLUSION

This work indicates that in the cerebral cortex, neurones intensely labelled with Cat-301 appear to be concentrated in areas concerned with broadly motor functions The same is true in the thalamus, where Cat-301 positive cells are concentrated in the nuclei which process somato-motor and visuo-spatial information (Hendry et al, 1988). Cat-301 labelling is also prominent in the basal ganglia and on spinal motor neurones. The areas of the central nervous system that are strongly immunoreactive to Cat-301 are characteristically interconnected, and in the cerebral cortex immunopositive cells are concentrated in the laminae from which cortical interconnections arise. The timing of the expression of the antigen recognised by Cat-301, the dependence of this expression on activity during critical developmental periods, its ultrastructural distribution and biochemical characteristics suggest that it could serve to stabilise synaptic connections. Its expression in motor related areas may thus be more a reflection of their being interconnected than of its having a specificity for neurones of a particular physiological class.

Current models of cortical organisation, based on anatomical and physiological data from primates and the results of cerebral blood flow studies in man, invoke the concept of parallel networks of areas distributed across cortical and subcortical regions (Roland and Friburg, 1985; Alexander et al, 1986; Goldman-Rakic, 1988; Posner et

al, 1988). The central features of such networks are that each is comprised of several cortical and subcortical areas which are interconnected and together form the neural substrate for a particular function. For example, the frontal eye fields, the cortex in the ventral bank of the intraparietal sulcus, the body of the caudate nucleus, the ventro-lateral substantia nigra and the ventral anterior thalamic nucleus are thought to constitute a distributed network devoted to oculomotor functions (Alexander et al, 1986). The constellation of cortical and subcortical areas heavily labelled by Cat-301 seem to be interconnected and to share a common functional specialisation, and might thus be viewed as an example of such a network, serving broadly motor functions.

The distribution of Cat-301 immunoreactivity has now been examined in most regions of the CNS, and while extending this analysis to further areas may provide additional information, focussing attention on the antigen itself may prove more rewarding. Determining the amino acid sequence would allow one to make predictions about its molecular conformation, possible binding sites and its relationship with the post-synaptic membrane, and might reveal sequences homologous to other molecules with established functions. Such homologies would not be unexpected, given the similarity between the antigen and that recognised by the monoclonal VC1.1 (Chapter 7.6), and the prevalence of common structural motifs among other neuronal surface molecules (Chapter 2). Examining the distribution of the antigen's mRNA could establish

whether the post-natal appearance of the antigen directly reflects the expression of it's gene, or whether the latter actually occurs at an earlier stage, and there is some post-translational antigenic modification. Cloning of the gene and its transfection into cell lines might allow the factors which control its expression, and the effects of inducing and supressing expression on the development of synaptic connections to be investigated *in vitro*. As with other surface molecules (Chapter 2), important clues to the role of the antigen might emerge from the effects of using Cat-301 to experimentally perturb the antigen's function. Sequencing of the antigen and the cloning of its gene are currently in progress (Hockfield, Personal Communication).

Future studies of the distribution of neuronal surface molecules are likely to reveal further associations between specific antigens and particular neural functions. As in the case of Cat-301, elucidating the significance of such molecules will require the concurrent investigation of their biochemistry and molecular genetics. A rather different approach is to regard neuronal surface molecules essentially as markers, and concentrate on using them to examine the lineage of neural cells, rather than pursuing the significance of the antigens themselves. Although this approach is not suitable for the Cat-301 antigen, which is expressed post-natally, it has already provided valuable insights into the development of glia from their precursor cells (Raff et al, 1983), and may prove to be a powerful means of studying the analogus process in neurones.

The application of immunocytochemistry to the study of neural surface antigens, like that of previously novel approaches to the investigation of the nervous system, has generated much interest and the hope that it may deliver answers to questions that could not be resolved with pre-existing techniques. However, as in the case of more established techniques, while this approach may itself provide new insights into the understanding of the nervous system, it is likely to be most valuable when allied to complementary data from other fields. This is well illustrated in the case of Cat-301, where the interpretation of the immunocytochemical data is greatly facilitated by knowledge of the relevant anatomy, physiology and biochemistry. Thus, the significance of the advent of the study of surface antigens for neuroscience is not that it provides a greater sophistication or resolution than existing techniques, but rather that it offers a qualitatively different perspective which can complement those derived from other approaches.

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# Appendix:

## GENERATION OF THE MONOCLONAL ANTIBODY CAT-301

### A.1 IMMUNISATION OF MICE WITH CAT SPINAL CORD

Cats were perfused intravascularly with 4% paraformaldehyde and the grey matter was dissected out from the cervical spinal cord, homogenised in phosphate buffered saline (PBS) and suspended in an equal volume of Freund's adjuvant. Mice were given three successive intraperitoneal injections of 50mg of this material over a period of seven weeks, followed by an intravenous injection of 20mg of unfixed cervical grey matter, which had been homogenised and boiled in 100µl of Laemmli loading buffer and diluted 1:5 with phosphate buffered saline.

### A.2 FUSION OF SPLENIC LYMPHOCYTES WITH MYELOMA CELLS

Three days after the last injection, the mice were killed by cervical dislocation and their spleens were removed via an abdominal

incision. Splenic cells were expressed from the capsule, dissociated with sterile forceps, then centrifuged with tissue culture media at 1000 r.p.m. for 5 minutes, separating the lymphocytes from the erythrocytes. Myeloma cells from the NS-1 line, which were deficient in hypoxanthine phosphoribosyl transferase (HPRT), and had been grown in Dulbecco's modified essential medium (DMEM), were collected while at the log-growth phase, centrifuged and resuspended in DMEM. The myeloma cells and lymphocytes were combined in a ratio of approximately 1:5 and centrifuged 3 times at 1000 r.p.m. for 5 minutes, with supernatant being removed and fresh medium added on each occasion. The cells were agitated with 50% polyethelyne glycol (PEG) for one minute, with increasing volumes of phosphate buffered saline added over the next 4 minutes. (PEG eliminates the forces which normally cause cells to repel each other and promotes fusion of their plasma membranes.) After further centrifugation for 4 minutes at 800 r.p.m., the cells were gently dissociated, and plated at a low dilution in HAT (hypoxanthine, aminopterin and thymidine) medium. This medium will not support the unfused myeloma cells, which lack the enzyme HPRT, and the unfused lymphocytes can only survive *in vitro* for a few days. In contrast, the hybrid cells, which are HPRT-positive by dint of their lymphocytic component, and have the tumour cell's capacity for unlimited division, can survive indefinitely.

## A.3 SCREENING OF CLONES FOR ANTIBODIES OF INTEREST

After 10-14 days, 50µ sections of 4%-fixed cat spinal cord were dehydrated into xylene, rehydrated and incubated with the supernatant from the hybrid cell colonies with 2% Triton X-100 for 12 hours. The sections were washed in PBS, incubated with rabbit anti-mouse antibodies conjugated with HRP (Cappel) for 2 hours, washed again, then processed for the visualisation with 3, 3'-diaminobenzidine. Accompanying control sections were incubated with PBS alone, an antibody against a leech neuronal antigen (negative control) and an antibody that binds to α-tubulin (positive control). From the 800 hybridoma lines generated by the fusion, 47 produced antibodies which labelled the spinal cord. The line which produced Cat-301 was one of five which were associated with intense neuronal labelling, and was grown in soft agar with HAT medium, producing colonies which were rescreened for antibody production after 3-5 days. This process was repeated for positive clones, then the cells were stored at -70$^{0}$C. High titres of Cat-301 were obtained by injecting the cells into the peritoneal cavity of mice, producing tumours which secreted the antibody into the ascitic fluid.