

**Ph.D. Dissertation**

**The dynamics of astroglial cytoskeleton and effects  
of gonadal steroids**

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## 1.1. Összefoglaló

(„Az asztroglia sejtvezérlésének dinamikája és a gonadális szteroidok hatása”)

A központi idegrendszer sejtjeit két csoportra oszthatjuk: az idegsejtekre, vagy neuronokra és a gliasejtekre, vagy neuroglia. Az idegsejtektől eltérően a glia az érett, kifejezett idegrendszerben is képes alak- és helyzetváltoztatásokra, zsugorodásra ill. megnagyobbodásra stb. Nyilvánvaló, hogy a sejt strukturális változásainak követéséhez a gliális sejtvezérlés (cytoskeleton) gyors adaptációs készsége szükséges.

Jelen munkánkban az asztrociták sejtvezérlésének változásaira összpontosítottunk. Az oligodendrocitákkal ellentétben az asztrociták jellegzetes, intermediér filamentumokból álló vezérlési rendszere nemcsak a sejtmagot övező citoplazmában, hanem a nyúlványokban is megfigyelhető. Az asztrociták által szintetizált legfőbb struktúrfehérje az ún. savanyú gliális rostos fehérje (GFAP – glial fibrillary acidic protein).

Megfigyelték, hogy agyi sérülésekre a lézió körüli asztrociták megnagyobbodással reagálnak és a citoplazmában felhalmozzák a fibrillaris fehérjét. Ezzel a szöveti hézagot töltik ki, ill. az elpusztult neuronok helyét foglalják el. Ílymódon a megnövekedett GFAP-immunreaktivitás a “reaktív gliózis” kórjelzésére használható. Az agyi sérülésre adott helyi, lokális glia-válaszon túlmenően megfogalmazódott a hipotézis, amely szerint a sérült agyrégió projekciós területén is várhatóak változások. Ezen jelenség leírására a “távoli asztroglia-válasz” (“RAR – remote astroglial response”) fogalmát vezették be.

Kísérleteinkkel a következőkre kerestünk választ:

- Az asztrociták reakciójakor a proliferatio, vagy a sejtek és rostok megnagyobbodása következtében fellépő hipertrófia bír-e elsődleges jelentőséggel?
- A távoli asztroglia-választ (RAR-t) szinapszisok pusztulása váltja-e ki?
- A GFAP-immunreaktivitásban észlelt módosulások a fehérjeszintézis változását jelzik-e, vagy az immunreaktivitás a fehérjemolekula módosulását, konformációs változását jelzi?
- A gonadális szteroidok befolyásolják-e a glia-reakció mértékét?

Amint az a modellként alkalmazott genikulo-kortikális rendszerben megfigyelhető,

- a távoli asztroglia-válasz (RAR) kiváltásában a szinapszisok pusztulása volt a meghatározó. Az asztrociták sejtteste és nyúlványai megnagyobbodtak és a

szinaptikus degeneráció irányába mozdultak el. Párhuzamos biokémiai kísérletek tanúsága szerint a RAR által érintett asztrocitákban az össz-GFAP mennyiségi növekedése volt megfigyelhető, ami fokozott fehérje-szintézist bizonyít.

Gonadális hormonok asztrociták sejtvezára gyakorolt hatásának vizsgálatakor megfogalmazódott a kérdés, hogy vajon létezik-e olyan agyrégió az “endokrin agyterületeken” kívül, ahol nemi dimorfizmus fedezhető fel. Befolyásolja-e az asztrociták sejtvezát ill. a GFAP-immunreakcióval nyomonkövethető asztrogliaválaszt a hormonális állapotok különbözősége? Ezen kísérleti sorozathoz az interpeduncularis nucleust (IPN) választottuk, mint egy magas GFAP-immunpozitivitással bíró, de szteroid-receptorokat nem tartalmazó agyterületet.

Hím és nőstény patkányok agyát összehasonlítva jelentős GFAP-beli különbség észlelhető az interpeduncularis nucleusban – tehát egy olyan magban, amelyik nem vesz részt hormonális folyamatok szabályozásában.

- Hímekben a gonadális szteroidok megvonása jelentősen csökkentette a GFAP-immunreaktivitást, míg helyettesítő hormonkezelés enyhítette a csökkenés mértékét. Nőstények esetében a GFAP-immunreaktivitás a nemi ciklussal összefüggő változásokat mutat: a legalacsonyabb szint ösztrospanban tapasztalható. Adataink arra utalnak, hogy gonadális szteroidok elengedhetetlenül szükségesek a normál GFAP-immunreaktivitás fenntartásához, és ilyen minőségükben az asztrociták sejtvezának aktiválóként szerepelnek. Ezen szteroidok megvonása csökkentette a távoli asztrogliaválasz intenzitását, a csökkenés jelentősebb mértékű volt nőstényekben, mint hímekben. MAP2, Tau és dystrophin immunreaktivitásában vizsgálataink nem mutattak nemi eltérést.

A szexuális szteroidok korábban feltételezettnél nagyobb területen kifejtett hatásáról tanúskodik az a tény, hogy az állat hormonszintje befolyásolja az asztrociták sejtvezát olyan agyrégióban is, ahol nem bizonyítottak ezen hormonok számára receptorokat.

Végezetül, a távoli asztrogliaválasz fellépése rámutat arra a tényre, hogy egy lokális agyi lézióban, a sérült idegsejteken kívül ezek projektios területei is érintettek, tehát holisztikus jelenségről van szó. Ennek értelmében a másodlagosan megjelenő szinaptikus degenerációt az asztrociták sejtvezának hipertrofiája követi. Ezen sejtmeagnagyobbodás mértéke nemi hormonokkal befolyásolható.

Mindezek tükrében elképzelhető, hogy jelen eredményekből kiindulva az asztrociták reakciójának káros hatásai csökkenthetőek a nemi szteroidok szintjének

változtatásával pl. agyi ödéma, fokális epilepszia és neurodegeneratív megbetegedések esetében.

## 1.2. Summary

The cellular units of the central nervous system are the nerve cells (neurons) and the glia cells (neuroglia) – called summarizingly neural cells. Unlike nerve cells, glia undergo shape alterations, regression or hypertrophy, migration, etc. also in the mature central nervous system. Therefore, it is conceivable that glial cytoskeleton has to adapt rapidly to structural changes of the cell.

In the present work we concentrated on changes in the cytoskeleton of astrocytes. In contrast to oligodendrocytes, astrocytes contain a characteristic system of intermediate filaments in both the perinuclear cytoplasm and processes. A major structural protein synthesized by astrocytes is the glial fibrillary acidic protein (GFAP).

It has been observed that astrocytes react to brain injuries with hypertrophy in the surrounding of the lesion and with the accumulation of cytoplasmic fibrillary material in order to fill the space of the tissue discontinuity or replace spatially the lost neurons. Thus, an increase in GFAP-immunoreactivity might be indicative for “reactive gliosis”. Beside the local reaction to a neural damage, a hypothesis was advanced concerning the effects of astroglial reaction also in the projection areas of the injured neurons. These areas might be at considerable distance from the site of the lesion therefore, we introduced the term “remote astroglial response (RAR)”.

In a group of experiments we addressed the question

- whether proliferation or hypertrophy of the cell is the leading feature of the astrocyte reaction,
- whether or not RAR is coupled to synaptic degeneration,
- whether changes in the GFAP-IR reflect changes in the synthesis of the protein or indicate conformational changes of the molecule that alter immunoreactivity, and
- whether steroids can alter glial reaction.

As revealed in the geniculo-cortical system used as a model,

- remote astroglial response was triggered by synaptic degeneration. The astrocyte cell bodies and large processes hypertrophized and moved slightly closer to



the site of synaptic degeneration. Parallel biochemical studies suggested that the increase in GFAP-IR during RAR was due to a net increase in the amount of this protein in the affected astrocytes. This phenomenon was a consequence of an enhanced GFAP synthesis.

To study the effect of gonadal steroids on astroglial cytoskeleton we were interested to find out if there is a region outside the “endocrine brain” where astroglial cytoskeleton shows a sexual dimorphism and if different hormonal states can alter astroglial cytoskeleton and its reaction as revealed by GFAP-immunoreactivity (-IR). For this set of experiments the interpeduncular nucleus (IPN) was chosen displaying a high GFAP-IR but not been described having steroid receptors.

Comparing male and female rat brains, a sexual dimorphism for GFAP could be pointed out in the interpeduncular nucleus – a brain region that is not involved in hormonal regulatory mechanisms.

- In males, the deprivation of testicular sex-steroid hormones caused a drastic fall in GFAP-immunoreactivity, while hormone substitution diminished the decrease. In females, the intensity of GFAP-immunostaining was sexual cycle-dependent with the lowest intensity reaction during estrus. This suggests that gonadal sexual hormones are essential in maintaining a high GFAP-IR, thus they can be regarded as astrocyte cytoskeleton-activating factors. Deprivation of gonadal steroids suppressed the remote astroglial response. The suppressive effect was more pronounced in females than in males. Immunoreactivity of MAP2, tau-protein and dystrophin didn't alter in our experiments.

The fact that the cytoskeleton of astrocytes in areas lacking steroid hormone receptors responded to alterations in the hormonal state of the animal, argues for a more widespread effect of gonadal steroids in the brain than earlier believed.

Finally, the phenomenon of RAR highlights the involvement of the projection area of lesioned neurons in the impairment caused to the brain by a focal lesion, thus it should be regarded as a holistic phenomenon. Accordingly, where secondary synaptic degeneration occurs, astrocytes react with a cytoskeletal hypertrophy. This hypertrophy could be influenced by gonadal hormones.

The main perspective of the present findings is the possible manipulation of gonadal steroid levels to reduce the detrimental effects of the astrocyte reaction, primarily in cerebral edema, focal epilepsy and neurodegenerative disorders.

## 2. Introduction

The cellular units of the central nervous system, the nerve cells (neurons) and the glia cells (neuroglia) called summarizingly neural cells, have structurally and functionally much in common with cells of other tissues. As far as nuclear material and cytoplasmic organelles are concerned, beside the highly specific synaptic terminals no principal differences can be encountered as compared to most cells of the organism. What makes neural cells structurally unique is their connectivity, i.e. the synapses, the myriads of determined contact sites between remote nerve cells established by an intricate system of processes often of considerable length. Glia cells appear to be also interconnected in a "glial reticulum" (Held, 1909). The extensive elongation of cells in the mature central nervous system - some neurons have a span from the hindlimb to the medulla - brings about the development of a special metabolism and related structure. The bulk of the metabolic machinery is concentrated in the perinuclear cytoplasm termed by the synonyms as perikaryon, soma or cell body, whereas remote neuronal parts are supplied by a special transport of materials, even of organelles, along the processes. If we consider the main functional features that characterize nerve cells, i.e. the capability of impulse generation, conduction and transmission, we may conceive the essential significance of cell shape including arborization pattern within the central nervous system. The development of this admirably complex organ can be described as the growth, pathfinding, and synapse formation of nerve cell processes arising from their cell bodies. During these events, a cytoplasmic apparatus growing out from the cell body together with the processes is responsible for stabilization and material transport. This is the neural cytoskeleton, an analogue of cytoskeleton in other cells which, however, adapting to the special demands of the extensively arborized neural cells undertakes additional functions.

### 2.1. The cytoskeleton in cell biology

If a cell is deprived of its cell membrane, either by sonication or enzymatic digestion, it still retains its original shape for a considerable period of time. This is only partly due to the colloidal consistency of the cytoplasm. The decisive element in stabilizing the cell and maintaining its form, is a cytoplasmic network of filaments and tubules coined by the summarizing term cytoskeleton.

Fibrillar elements of the cytoplasm have been described from the nineteenth century onwards, mainly in connection with the introduction of metal (chromium, silver, gold) salts in histological staining technology (see Pannese, 1994). It was soon realized that in addition to various intracytoplasmic reticular systems (Golgi-apparatus, rough and smooth endoplasmic reticulum), a fibrillary material exists in the cytoplasm which enmeshes the interior of the cell, surrounds the nucleus and establishes contacts with the inner aspect of the cell membrane.

The first hypotheses about the role of intracytoplasmic fibrillary material were put forward in connection with the study of muscular tissues since muscle cells were among the first shown to contain an abundance of intracytoplasmic fibrils. Quite understandably, these fibrils were interpreted as means of cell motility. Indeed, fibrils of the muscle cells turned out to be contractile and, as identified a few decades later, to contain a major 42 kDa protein, actin (Stossel, 1984; Holmes *et al.*, 1990). On analogy of the muscle cell, similar fibrils occurring in other cells were all thought to be actin fibrils subserving the contraction of the cell or, in the case of mobile cells within a complex organism, the amoeboid movement across vessel walls or along the loose connective tissue spaces.

The advent of electron microscopic investigations from the mid-1950ties brought about a rapid expansion of knowledge of cell structure. This resulted in a better understanding of the structure and composition of intracytoplasmic fibrillary material. The resolution of the electron microscope revealed that this material comprised different types of intracytoplasmic filaments and tubules which are unbranched and occur in the cytoplasm often in a remarkable orderliness.

### 2.1.1. Filaments of the cytoskeleton

On the basis of their thickness, three classes of filaments were distinguished: the thin (6-8 nm), the intermediate (10 nm), and the thick (above 10 nm) filaments.

❖ Thin filaments (microfilaments) were shown to contain the protein actin. In skeletal muscle fibers and in cardiac muscle cells contractile filaments were demonstrated to occur in organized, thick bundles constituting the myofibrils. In the skeletal muscle their association with the thick myosin filaments is regular so as to give a periodic structure which together with a level periodicity of neighboring fibrils results in the cross-striation of this type of muscular tissue. In smooth muscle cells contractile filaments are less organized therefore, no cross-striation can be observed. A number of non-muscular cells

contain thin, contractile filaments. There are claims that all cells contain at least some contractile filaments; actin was biochemically found in a variety of tissues. Although actin may be present in different types of cells, there are still some non-muscular cell types where actin has certainly a distinguished role. In addition to free cells such as macrophages, the myoepithelial cells, the microvilli of the enteric epithelial cells, nerve cells, etc. contain a substantial amount of actin and correspondingly microscopically demonstrable microfilaments (Stossel, 1984; Holmes *et al.*, 1990).

❖ Intermediate filaments occur mostly in epithelial cells, cells of connective tissues, particularly fibrocytes, fibroblasts, and macrophages, and in astrocytes of the nervous system. Intermediate filaments, but not neurofilaments are built up from 53-58 kDa proteins and tend to occur in thick bundles. Bundles form within the cytoplasm a network-like system. The best known intermediate filament proteins are keratin proteins, desmin, vimentin, and glial fibrillary acidic protein (GFAP). However, these proteins are present in the intermediate filaments with some degree of selectivity. For example, desmin occurs in muscle cells whereas keratin is typical for epithelial, particularly epidermal cells, vimentin for mesenchymal cells and immature glia, whereas GFAP is specific for mature astrocytes. It is generally believed that intermediate filaments are the cytoplasmic elements which stabilize the cell.

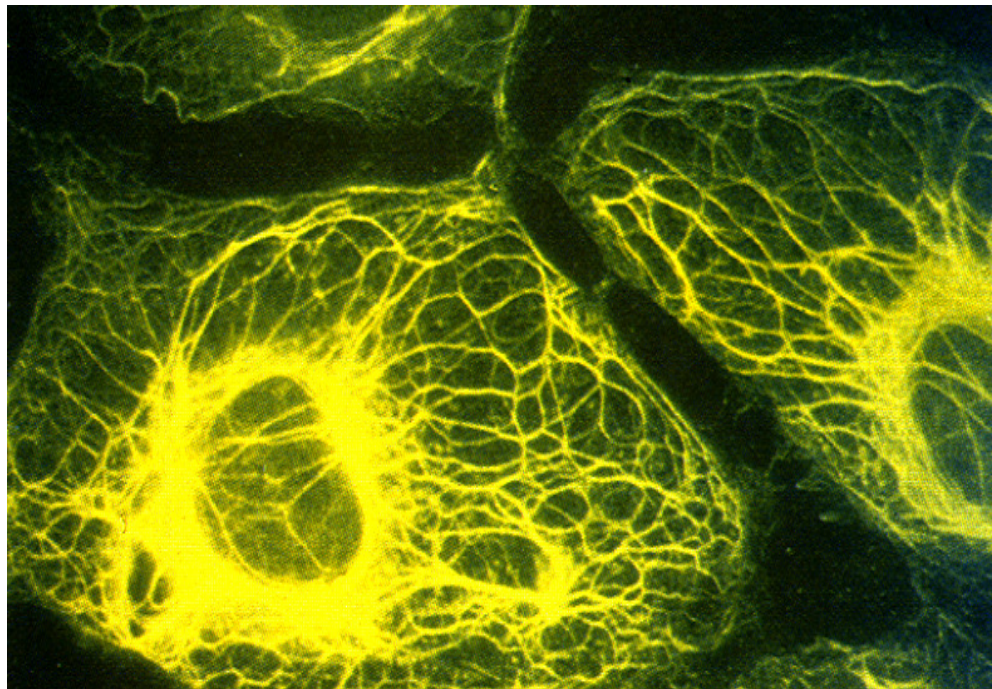


Fig. 1  
The fluorescence microscopic picture of the cytoskeleton in epithelial cell culture. x3000

❖ Thick filaments are mainly found in muscle cells where the configuration of myosin produces 15-18 nm thick filaments with strong birefringence and high electron density. Myosin in other cells although present, does not produce thick filaments.

### 2.1.2. Tubules of the cytoskeleton

In addition to filaments, a substantial fraction of intracytoplasmic fibrillary elements were described as 24-26 nm diameter tubules, termed microtubules. Microtubules have a fundamental role in cell division (mitotic spindle - centrioles composed of nine sets of triplet microtubules) and they are the major structural components of cilia being essential for their motion. They have a 18-20 nm electron lucent lumen surrounded by a wall composed of 5-6 nm globular particles making up the tubulin protofilaments. As verified biochemically, the major protein of these particles is tubulin (Dustin, 1984), a 110-120 kDa protein which is a dimer consisting of two subunits: the 55 kDa alpha and the 60 kDa beta-tubulin. Filaments are polarized by having a "plus" or growing end and a "minus" or almost stable end. The two subunits can dynamically bind and dissociate, so that protofilaments of microtubules are rapidly built up or disintegrated within the cell. Usually 13 of these protofilaments are helically arranged to form the wall of the cylinder. Substances such as colchicine, vinca alkaloids (vincristine, vinblastine), excess calcium can disaggregate microtubules including those of the mitotic spindle apparatus into alpha/beta-heterodimers, which is the basis of a cytostatic effect of the above substances (Amos and Klug, 1974). On the other hand, under favorable conditions disaggregated alpha/beta-heterodimers readily unite to form shorter or longer microtubules. The phenomenon of microtubule subunit aggregation and disaggregation can be reproduced also *in vitro*. Thus microtubules are one of the most dynamically changing structures of the cytoplasm. At their outer aspect, microtubules are contacted by specific microtubule-associated proteins (MAPs) whose number is estimated to over a dozen (Vallee and Bloom, 1984; Hirokawa, 1994; Matus *et al.*, 1982).

The function of microtubules is best understood in the nervous system where they are intimately associated with material transport along the processes. Accordingly, microtubules are believed to be instrumental in forwarding molecules on their surface by the so-called sliding mechanism which may carry with the help of MAPs macromolecules rolling them on the surface of the tubule. It also appears that microtubules, MAPs and the

mechanochemical motor proteins kinesin and dynein have a role in placing and stabilizing organelles such as mitochondria within the extensively branched neural cells. Less clear is the role of microtubules at other sites. Mitotic spindle microtubules are known to be essential for the dividing cell to pass metaphase and to separate the homologue chromosomes into the opposite poles of the dividing cell.

## 2.2. The neural cytoskeleton

The neural cytoskeleton is extremely elaborate. While the nerve cells (neurons) abound in both filamentous and tubular components, in glia cells the filamentous material dominates.

### 2.2.1. The neuronal (nerve cell) cytoskeleton

The main features of the neuronal cytoskeleton are the abundance and orderliness of its elements. This is particularly conspicuous in the neuronal processes (axon and dendrites) where cytoskeletal elements form parallel arrays found almost along the entire length of the process. Silver impregnation is the most suitable stain to demonstrate the arrays of cytoskeletal elements. Nineteenth-century histologists were much impressed by the so-called neurofibrils seen after silver impregnation to such an extent that they held neurofibrils as means of neuronal impulse conduction (Schultze, 1871; Apáthy, 1897). Due to the low resolution of silver stains even by light microscopic standards (deposited silver salts considerably thicken the stained fibrils), the hypothesis of neuronal continuity has been formulated claiming that neurofibrils traverse uninterrupted from one nerve cell to the other (Apáthy, 1897). This hypothesis was disproved by the neuron-principle clearly demonstrating that nerve cells together with their axonal and dendritic arbors are structural and functional units, moreover, that at the contact sites of neurons no continuity exists but a special set of structures form synapses where the impulse is transmitted from one neuron to the other (Robertson, 1953; Sjöstrand, 1953; Palade and Palay, 1954). As viewed with the electron microscope, the terminal portions of processes appeared to be devoid of cytoskeletal elements but a number of findings suggest that this may not be the case because under proper conditions of fixation synaptic axon terminals could be shown to contain various formations of microtubules (Hajós *et al*, 1979). Moreover, actin was

biochemically demonstrated in fractions of isolated nerve endings (Hirokawa *et al.*, 1989). At any rate, cytoskeletal elements were found to terminate in the presynaptic ending and not to pass through the synaptic cleft.

#### 2.2.1.1. Neuronal filamentous cytoskeleton

For the cytoskeletal intermediate filaments in the neuron, the term neurofilament has been introduced. In the neuronal cell body neurofilaments are apparently solitary and irregularly arranged but electron microscopy of serial ultrathin sections as well as immunocytochemistry with antibodies raised against neurofilament proteins shows a loose perinuclear network of neurofilaments.

Towards the origin of processes, particularly that of the axon, neurofilaments tend to converge so that in the process they run parallel to the longitudinal axis of the process. In cross-sections of axons they appear scattered.

Neurofilaments are composed of a family of specific proteins with three major components ("triplet proteins") having molecular weights of 70, 130-150, and 200 kDa, respectively. In the cytoplasm of the cell body these filaments are difficult to demonstrate because they occur as thin, 8-12 nm thick, poorly electron-dense solitary strands mostly in the neighborhood of structures having a high electron-density. In the processes, particularly in axons, neurofilaments are readily seen to form thick bundles. With high-resolution electron microscopy they appear to consist of an outer dense layer about 3 nm thick and a lighter core.

The exact function of neurofilaments is largely enigmatic. They have been envisaged as passive structures stabilizing the axon and regulating axonal diameter (Steinert *et al.*, 1984; Stewart, 1993).

Whether or not actin has a similar role in neurons than in other cells, remains to be answered. True, that in neurons a different type of actin is present than in muscle. Under certain circumstances nerve cells were shown to withdraw synaptic axon terminals as means of synaptic plasticity (Sotelo and Palay, 1971) and transected axons were demonstrated to grow by sprouting which involves a kind of pathfinding by trial and error. It appears that nervous system actin is involved in the stabilization of certain membrane proteins and the maintenance of domains. Locomotion of nerve cells has not been described as yet in the mature central nervous system, whereas glial cells may, within limits, change their position (Hajós *et al.*, 1993).

### 2.2.1.2. Neural microtubules

The microtubules are typical features of neuronal processes but are less abundantly found in the glial cells. In neuronal processes they form neatly parallel arrays so that the term "neurotubules" has been proposed to distinguish neural microtubules from other microtubules. This distinction is not justified since neural microtubules differ neither in structure nor in molecular composition from microtubules of other cells. Still their regular appearance is unique to the neural cells. Particularly axons abound in microtubules. In fact, there are no axons without microtubules. The original hypothesis of Weiss and Mayr (1971) that microtubules are as long as the axon has soon been seriously challenged (Nadelhaft, 1974; Tsukita and Ishikawa, 1981). Measurements in serial sections of long axons in a number of species suggest that axonal microtubules may be 400  $\mu\text{m}$  long. The bundle of microtubules would thus consist of such units. In cross-sections of large neuronal processes, bundles appear to be irregularly located but within a bundle there is an even distance between microtubules with frequently occurring cross-bridges.

It is interesting to note that the axonal microtubules are uniformly oriented with their minus ends towards the cell body and plus ends towards the axon terminal (Burton and Paige, 1981). By contrast, in dendrites microtubules are oriented in both directions (Baas *et al.*, 1988; Burton, 1988). This suggests an oriented growth and probably transport function of microtubules in the axons but not in dendrites. Moreover, spectacular differences were demonstrated in the set of microtubule-associated proteins (MAPs) between dendritic and axonal microtubules (Burgoyne, 1991). The most important difference, relevant also to the present work is that the protein MAP2 is found only associated with dendritic microtubules, while the microtubule-associated protein Tau is exclusively axonal. This enabled antibodies to be raised as specific axonal and dendritic markers.

The main function attributed to neuronal microtubules is their participation in axonal transport (see Grafstein and Forman, 1980 for a review; Gelfand and Bershadsky, 1991). There are claims that they also contribute to the maintenance of some membrane properties.



### 2.2.2. The glial cytoskeleton

Classes of glia cells greatly differ in the abundance and type of their cytoskeletal elements. What is common in them is that the cytoskeleton in the glia appears to be more of the stabilizing than of the functional nature. However, unlike nerve cells, glia undergo shape alterations, regression or hypertrophy, migration, etc. also in the mature central nervous system therefore, it is conceivable that glial cytoskeleton has to adapt rapidly to structural changes of the cell. This is indeed the case, and as compared to neurons which have a dynamic (microtubules) and a less-dynamic (neurofilament) fraction of the cytoskeleton, glial cytoskeleton shows as a whole a high degree of responsiveness to structural and functional alterations of the cells containing them.

#### 2.2.2.1. The cytoskeleton of oligodendrocytes

In oligodendrocytes the cytoskeleton is represented almost exclusively by microtubules. A few scattered microfilaments (most probably actin-type) are found in the perinuclear region. Here microtubules are also scarce, randomly oriented and difficult to notice under the electron microscope due to the high electron density of the ground cytoplasm of these cells. Towards the origin of processes the number of microtubules increases, they take a more regular course and within the processes microtubules run lengthwise in parallel bundles. As a matter of fact, it is difficult to differentiate between oligodendrocyte processes and dendrites: both contain a similar amount and arrangement of microtubules. It is only the presence or absence of synapses which helps identification.

#### 2.2.2.2. The cytoskeleton of astrocytes

Astrocytes have a few irregularly scattered microfilaments in the perinuclear region but in contrast to oligodendrocytes, they contain a characteristic system of intermediate filaments in both the perinuclear cytoplasm and along the processes.

The system of filaments has been recognized in the 19th century as the "glial fibrils" of astrocytes. Silver stains proved to be particularly useful to demonstrate the glial fibrillary material. Based on these observations two classes of astrocytes were distinguished according to the amount of fibrillary material they contained. Those which were heavily loaded with fibrils were termed fibrillary astrocytes, whereas those containing less fibrils were called protoplasmic astrocytes. The latter were conspicuous because

astrocytes have a very lightly staining, empty-looking cytoplasm poor in organelles. So if fibrillary material did not fill it, the cytoplasm appeared "watery".

As revealed by electron microscopy (Mugnaini and Walberg, 1964), fibrils are made up of 8 nm intermediate filaments arranged in parallel bundles. The bundles are irregular in the perinuclear cytoplasm, but run along the astrocyte process lengthwise. They can be followed into the irregular outbulgings of the process in such a consistent and characteristic pattern that they are mostly referred to as "glial filaments". It could be clearly demonstrated that the light microscopically observed, silver stained fibrils corresponded to bundles of intermediate (glial) filaments. It has also been realized that these filaments react in a most dynamic fashion to any functional change of the astrocyte. They accumulate when the cell is activated and withdraw to a stable pattern in the resting state of the cell. This dynamism questions any rigid distinction between fibrous and protoplasmic forms nevertheless, in histopathology the two terms are still used to denote different states of astrocytes.

Radial glia, astrocytes, and perhaps other cells in the CNS synthesize structural proteins, such as intermediate filaments, at different times during cortical development. Intermediate filaments composed of vimentin are expressed in radial glia and immature glia and they are detectable by immunocytochemical localization as early as E11. Other intermediate filaments, such as those composed of glial fibrillary acidic protein (GFAP) become detectable at E18 in mouse cortex, and the radial glia and astrocytes remain strongly GFAP-positive until P7 to P14. Mature cortical astrocytes decrease their GFAP content below detectable levels, except for a few GFAP-positive astrocytes in layer 1, around blood vessels, and in the subcortical white matter. The transition from glial cell production of vimentin to synthesis of GFAP occurs around the time of birth. (For the time of appearance of different intermediate filament-proteins during neural development see the review from Fedoroff *et al.*, 1982-83.) It has to be noted that in colony cultures both vimentin and GFAP persist into fibrous or reactive astrocytes, the vimentin/GFAP-switch is not as pronounced. A remarkable vimentin-GFAP transition occurs at the time of myelination.

Biochemistry of the astrocyte-filaments (Quinlan and Franke, 1983) has shown that their major proteins vimentin and glial fibrillary acidic protein (GFAP) are members of the type III intermediate filament family. Vimentin is present in astrocytes, in their precursors and in reactive astrocytes (Dahl *et al.*, 1981; Yen and Fields, 1981; Fedoroff *et al.*, 1983). As the major intermediate filament protein in the developing nervous system of mammals

vimentin is the marker of immature glia (Bignami *et al.*, 1982), whereas GFAP is the main component of filaments of mature mammalian astrocytes (Bignami *et al.*, 1980). Astrocyte processes proved to lack microtubules, thus it can be concluded that the cytoskeleton of mature astrocytes is dominated by a specific type of GFAP-containing intermediate filament. GFAP is generally accepted as a specific marker of astroglia and astroglia-related cell types such as radial glia, Bergmann-glia of the cerebellum, Müller cells of the retina, and tanycytes of the mediobasal hypothalamus.

In mammals a typical vimentin-GFAP switch occurs with astroglial maturation (Dahl and Bignami, 1985) whose postnatal time is species-dependent (Kálmán, 1993). Vimentin is dominant in astroglia of adults in submammalian species only. It has to be noted that in cultures both vimentin and GFAP persists in fibrous or reactive astrocytes, the vimentin/GFAP-switch is not as pronounced owing to a slight dedifferentiation of cultured cells.

Although the stabilizing role of astrocytic glial filaments is not questioned, their influence on membrane structure has also been proposed. With freeze-fracture electron microscopy, cell membranes of astrocytes showed orthogonal assemblies of intramembrane particles (Landis and Reese, 1981). Orthogonal assemblies are paracrystalline arrays composed of 7-nm particles that can be viewed only in freeze-fracture preparations (Mugnaini, 1986). These arrays of intramembrane particles were seen close to filament bundles approaching the membrane therefore, some kind of influence of filaments on membrane structure has been postulated. Arrays are most numerous on the astrocytic endfeet facing and surrounding blood vessels and on the astrocytic processes facing the pia mater. They may function in cell adhesion and, more importantly, in transport of substances between astrocytes and blood or cerebrospinal fluid. This arrangement was found typical for the astrocyte membrane, but not for the oligodendroglia cell membrane.

Aging is associated with changes in brain morphology and function. Changes observed in the aged brain include a combination of region-specific degenerative and compensatory events directly next to areas showing little or no change at all (Collier and Coleman, 1991; Finch and Day, 1994; Barnes, 1994). One marker that shows global increase during normal aging is glial fibrillary acidic protein (GFAP) (Goss *et al.*, 1991; Laping *et al.*, 1994). Its increased immunoreactivity in reactive astrocytes is an index of neurodegeneration.

Astrocytes from different regions of the brain have diverse biochemical characteristics and may respond in different ways to a variety of injuries. Astrocytic swelling and hypertrophy-hyperplasia are two common reactions to injury. (Montgomery, 1994). The concept of astrocytic morphologic, biochemical, and functional heterogeneity is important to remember when attempting to understand the diverse functions of astrocytes and their reactions in disease (Hatten *et al.*, 1991; Wilkin *et al.*, 1990).

During brain development, transient partitions of glia and glycoconjugates (glycoproteins, glycolipids, and glycosaminoglycans) surround forming functional units (e.g. nuclear divisions, whisker-related barrels, and neostriatal striosomes). These partitions, which Laywell and Steindler (1991) think as boundaries, consist of dense aggregates of glial fibrillary acidic protein (GFAP)-positive radial glia, young astrocytes and their processes, and developmentally regulated glycoconjugates – as recognition molecules on membranes or within the extracellular matrix. When functional patterns were formed and appear to be stabilized, these boundaries are no longer detectable. It is postulated that developmental boundaries and wounds in the adult brain possess some of the same inhibitory molecular substrates for molecular expansion.

### 2.3. The glial reaction

Glia are usually classified as macroglia and microglia. The former comprise astroglia and oligodendroglia, the latter is a highly controversial class owing to its non-neural, i.e. mesenchymal origin. The fact that injuries or infections of the central nervous system elicit a proliferation and an immigration of microglia from blood-vessels, strengthens the argument that these are mononuclear phagocytes, members of the MNP system of the organism. In this capacity they can be regarded as activated mesenchymal cells of a systemic defense mechanism, which are delivered to the brain by the bloodstream. Opinions concur as to the mesenchymal nature of microglia but highly diverge concerning the presence of microglia in the resting brain. A number of findings suggest that microglia cells occur also in the non-reactive neural tissue (Cammermeyer, 1970), and that microglia activation is a biphasic phenomenon: the local microglia are activated first followed by the immigration of blood-born mesenchymal macrophages. There are, however, claims that all microglia cells are derived from outside the central nervous system. Because of this

controversy, to date the terms "reactive glia" and "gliosis" are used for the astrocytes responding to injuries of the nervous system, either mechanical or pathological.

### 2.3.1. The reaction of central nervous system astrocytes

It has been observed in the 20ties (see Kimelberg and Ransom, 1986) that astrocytes react to ionic damage with swelling, whereas upon the loss of neurons either due to mechanical injuries or degeneration, they undergo a hypertrophy in the surrounding of the lesion. This is manifested in an increase in size of the astrocyte cytoplasm but even more conspicuously, in an accumulation of cytoplasmic fibrillary material. In this respect there was no difference observed between protoplasmic and fibrous astrocytes (which has cast the first doubts on the validity of this classification). The reaction had a definite time course and resulted in the formation of an astrocytic scar. Accordingly, hypertrophic astrocyte processes filled in the space of the tissue discontinuity or replaced spatially the lost neurons. Upon stabilization of the tissue volume, the amount of filamentous material decreased to normal but hypertrophic astrocyte processes remained permanently as the space-filling elements replacing the missing neural tissue. A similar astrocytic reaction has been described after the focal loss of neurons in various neurodegenerative diseases (for references see Montgomery, 1994).

The nature and consequences of the astrocytic scar are not unequivocally interpreted. While some authors claim that this is the only way of tissue repair in the central nervous system, the predominant view is that the astroglial scar is an obstacle to nerve cell regeneration by forming a barrier against the growth of axons in the direction where pre-lesional conditions might be restituted (Bovolenta *et al.*, 1992; Silver, 1994; Ridet *et al.*, 1997). Furthermore, if formed at certain sensitive places such as the temporal lobe or hippocampus, glial scar may act as an epileptogenic focus (Tiffany-Castiglioni and Castiglioni, 1986).

Under the electron microscope, reactive astrocytes were seen to contain a substantially increased amount of glial intermediate filaments and the presence of several glycogen particles became apparent (Shimizu and Hamuro, 1958; Maxwell and Kruger, 1965). It should be noted that glycogen is believed to be present also in normal astrocytes but difficult to notice owing to its low concentration and/or its poor preservation with routine procedures (Pannese, 1994). Nevertheless, glycogen particles are seen in abundance in the

astroglia of the developing nervous system and in the abovementioned reactive situations, indicative of an enhanced metabolism.

A major breakthrough in the assessment of reactive astroglia and the glial reaction has been brought about by the raising of specific antibodies against the glial filament protein GFAP (for references see Dahl *et al.*, 1986). The immunohistochemical demonstration of GFAP proved to be a sensitive indicator of the functional state of astrocytes. This method has gained general acceptance not only in neurohistology but also in experimental and more recently in clinical neuropathology. The expression of GFAP is coupled to astrocyte activities and in various pathologic states including glial tumors this protein is of diagnostic value.

### 2.3.2. The reaction of peripheral astrocytes

Although the peripheral nervous system is beyond our present scope, it has to be noted that astrocytes of peripheral nerves react to trauma or neurodegenerative processes similarly to their central counterparts. In fact, it is the transection of a peripheral nerve - either experimental or traumatic - where the relationship of nerve fiber regeneration to glial scar formation is most intensively studied and where the axonal growth and sprouting of axons is experimentally best accessible.

### 2.3.3. The remote astroglial reaction (RAR)

An early observation concerning astroglial reaction was that experimental lesions of the cerebral hemisphere elicited first a local reaction (Bignami and Dahl, 1976) which consisted of a proliferation of astrocytes around the experimental stab-wound followed by their hypertrophy. Hypertrophy could be verified also by the demonstration of an increase in GFAP-immunoreactivity. With time, however, GFAP-immunoreactivity increased in the other hemisphere as well. Based on this observation, a hypothesis was advanced in our laboratory concerning the effects of astroglial reaction also in the projection areas of the injured neurons. These areas might be at considerable distance from the site of lesion therefore, we introduced the term "remote astroglial response (RAR)" to denote the phenomenon which we were able to prove by inducing anterograde (Wallerian) degeneration of neural pathways and observing astrocyte reaction along the degenerating axon (Hajós and Csillag, 1995). We supposed that when degeneration reaches the terminal

arbor of axons degenerated synapses will be removed and their spaces occupied by reactive astrocyte processes. Synapses were shown earlier to be approached by delicate astrocyte processes penetrating as close as the edge of the synaptic cleft (Hajós, 1980), therefore, the hypertrophy of local astroglial elements would explain RAR.

The concept of RAR means that astroglial reaction may be a sensitive indicator of loss of synapses in the termination area of a lesioned neuron.

#### 2.4. Factors affecting astroglial reaction

From the foregoing it appears that a better understanding and the influencing of the astroglial reaction would be of importance with respect to neural repair mechanisms and the control of metabolic equilibrium in the central nervous system. An extensive research carried out in these fields has brought about fundamental observations that defined the direction of further research. First, that the inhibition of proliferation with cytostatics did not block the local astrocyte reaction (Kálmán, personal communication). Second, that astrocytes react to steroid hormones, many of them having receptors on the astrocyte membrane (Shughrue *et al.*, 1997).

### 3. Aims and scope

In all situations described in Introduction the astrocyte cytoskeleton plays a key role. It is this structure which is destroyed and rebuilt during reactive cell transformations. It appears that astroglial hypertrophy is accompanied by a matching hypertrophy of its cytoskeleton which is a dynamically changing means of adaptation to form changes and which stabilizes the newly shaped processes. Therefore, the present work concentrates on three major aspects.

The first group of questions comprise the elucidation of

- (i) whether proliferation or hypertrophy of the cytoskeleton is the leading feature of the astrocyte reaction;
- (ii) whether or not RAR is coupled to synaptic degeneration;
- (iii) whether GFAP immunohistochemistry is a reliable method to follow astrocyte reactions;
- (iv) whether changes in GFAP-immunoreactivity reflect changes in the synthesis of the protein or indicate conformational changes of the molecule that alter immunoreactivity.

The second group of questions were posed to answer

- (i) if there exists a sexual dimorphism of the astrocyte reaction as revealed by GFAP immunoreactivity;
- (ii) if there is a region outside the "endocrine brain" where astroglial cytoskeleton shows a sexual dimorphism;
- (iii) if different hormonal states can alter astroglial cytoskeleton.

In the third group of questions we were interested to find out

- (i) whether steroids can suppress local glial reaction;
- (ii) whether steroids can suppress RAR.

Our ultimate goal was to examine whether sexual steroids may be used as agents suppressing detrimental reactions of the astroglial cytoskeleton.



## 4. Materials and Methods

The keeping of experimental animals and the performance of all experimental interferences including operations, treatments and the sacrifice of animals were in compliance with international animal protection standards and the Animal Protection Act of the Hungarian Government. Accordingly, the number of animals per cage, their feeding and environment was adjusted meeting all regulations. Treatments did not use painful materials and all operations were carried out in deep anaesthesia. Operated animals were separated in single cages for the postoperative period and carefully looked after.

### 4.1. Animals

For the experiments Wistar rats were used kept on 25°C temperature with 12 h dark-light periods. Rats were fed with free access to standard laboratory rat food and received water *ad libitum*. For the study of glial reaction males of 250g body weight were used, whereas hormonal influences were studied in both males and females of 250g body weight. In some experiments and treatments, interventions were carried out at certain times prior to study therefore, the determination of age was necessary. In these cases one male was put into the cage of 3-4 females. Vaginal smears were taken each morning and females having spermatozoa in their vaginal secretion were separated as pregnant. The day of diagnosing pregnancy was considered as embryonic day 1 (E1).

### 4.2. Treatments

In experiments directed to study glial reaction following neurodegeneration, colchicine and vinblastine were applied in sublethal doses. Colchicine was also used in some experiments to block axoplasmic transport.

In some experimental lesions to the brain the degeneration of neurons was induced by ibotenic acid (Sigma, 5-10 µl/animal) deposited to the lesion site through a micropipette introduced with a stereotaxic apparatus.

In studying the effect of hormonal states, testosterone enanthate (Sigma, 6 mg/kg body weight) was administered either in a single dose, or in a depot preparation, or in the form of chronic treatment.

### 4.3. Operations

All operations were carried out under deep anaesthesia with a combination of 90 mg Ketamine (SBH-Ketamin<sup>®</sup>, SelBruHa) and 10 mg Xylazine (Rometar<sup>®</sup>, Spofa) per kg body weight. In the case of minor interferences and for the operation of young animals, ether inhalation narcosis was applied.

Operations were carried out under semi-sterile conditions, i.e. instruments were sterilized but the wider surrounding not. In intracranial operations antibiotics were not applied but in the abdominal operations the laparotomy-cut was occasionally closed after the local application of antibiotics. However, neither intra- nor postoperative antibiotic treatment was found appreciably needed.

#### 4.3.1. Experimental Wallerian degeneration

Under deep Ketamine-Xylazine anaesthesia, the head was fixed in a stereotaxic apparatus. The scalp was cut in the mid-sagittal line from between the eyes to the occipital region. Skin was pulled away, and on the right side, the calvaria was exposed, the area between the median suture and the origin of the temporalis was cleaned from all soft tissues. Using the coordinates of Paxinos and Watson (1986) a hole was bored with an electric drill at -0.4 caudal and lateral from the Bregma. Optimal hole-diameter for a safe introduction of the electrode was 1.5 mm. The surface of the brain was then exposed by opening the dura and the arachnoid. After careful suppression of both diploe and dura bleedings, an electrode was inserted into a depth of 0.7 cm in order to introduce its tip into the lateral geniculate body (dorsal-lateral geniculate nucleus, DLG). The electrode was purchased from Radionics (RFG-4A Research Lesion Generator System<sup>®</sup>, Burlington, MA). The target territory was lesioned by electric coagulation at 15 V, 4 mA producing locally 70°C for 60 sec.

After the lesion the skin was sutured and the animals were returned into a single cage for recovery. Aseptic conditions were ensured for the entire procedure.

When lesioned animals were - after different survival periods - sacrificed, the site of the lesion was histologically checked.

#### 4.3.2. Gonadectomy

Gonadectomies were carried out in three different age-groups: newborn, young (50 g body weight) and adult (200-250 g body weight) rats were used. After the first series of operations it appeared that in pups ether narcosis produced a higher survival rate than Ketamine-Xylazine. Thus, in the following, gonadectomies of newborns were performed in ether narcosis, young and adult animals were anaesthetized by the above mentioned combination of Ketamine-Xylazine.

##### 4.3.2.1. Ovariectomy

The first ovariectomies were performed from median laparotomy thought to have the advantage that both ovaries can be removed with one incision. Surprisingly, intraoperative complications, heavy bleedings were so frequent and survival rates so poor that we decided on the paralumbar approach. The animal was turned to its side, and skin, muscle and fascia of the paralumbar region was cut through with a pair of scissors. The ovary was pulled out the tuba and supplying vessels ligated in one and the ovary was cut off. It is interesting to note that there was so little tendency of bleeding that the ovary could be cut off even without an underlying ligature. Nevertheless, not to put results at risk by an eventual late postoperative bleeding, we always applied a ligature prior to cutting through the ovarian suspension. The stump was then pushed back into the abdomen and the operation was terminated by a monolayer muscle-skin suture.

Using this technique, ovariectomy on one side could be carried out within one minute, so the other ovary could readily be removed within the same session.

##### 4.3.2.2. Castration

Testes were removed in ether anaesthesia through an incision on the scrotum. The common vaginal tunic was left intact. The spermatic cord was ligated and redundant adipose tissue was removed. Interestingly, unlike the ovarian artery heavy bleedings of the

testicular artery were quite frequent and difficult to manage so in rats castration often proved to be more problematic than ovariectomy.

#### 4.4. Histology

Both light and electron microscopic studies were carried out to follow the structural and ultrastructural alterations of astrocytes.

##### 4.4.1. Light microscopy

For routine histological examinations tissue blocks were fixed in 4-10% formaldehyde and embedded in paraffin. In some cases unfixed or fixed but not embedded tissue blocks were snap-frozen in liquid nitrogen and cut in a cryostat at  $-20^{\circ}\text{C}$ . From the paraffin embedded and frozen blocks 8 and 20  $\mu\text{m}$  thick coronal sections were cut, respectively. Staining procedures applied were haematoxylin-eosin, Nissl's cresyl violet, Luxol-Fast Blue (according to Klüver-Barrera) and the periodic-acid-Schiff (PAS) stainings.

In some cases, mainly for quick histological checking, vibratome sections were stained with haematoxylin-eosin and Nissl.

Occasionally, light microscopic examination was carried out in resin-embedded, toluidine blue-stained semithin sections (see below).

##### 4.4.2. Electron microscopy

As fixative for electron microscopy two different solutions were used depending on the purpose of further processing. For routine electron microscopy the Karnovsky-fixative (glutaraldehyde and paraformaldehyde 2% each) was used (Karnovsky, 1965). For immuno-electron microscopy a modified Zamboni-fixative was used which contained reduced or no glutaraldehyde according to the glutaraldehyde-sensitivity of the antigen, and picric acid. Both fixatives were prepared in 0.1 M phosphate buffer adjusted to pH 7.4. In all cases, however, the fixative was applied in the form of transcordial perfusion. As a second step of fixation immersion of the specimen into a 1-2 % osmium tetroxide solution in 0.1M phosphate buffer (pH 7.4) was used as a routine for 2 h.

Under deep Nembutal anaesthesia the chest was opened and a cannula was inserted through the left ventricle into the aorta. Prior to the fixative a short rinse of 0.9 % saline of body temperature was performed to wash out blood. 400 ml of the fixative was perfused under controlled pressure. Brains were removed from the skull and stored for a minimum of 2 h but possibly for overnight in the fixative. Small (2-4 mm<sup>3</sup>) tissue blocks of the studied region were excised and rinsed in changes of phosphate buffer for 2 h, then dehydrated in graded ethanol and propylene oxide. Blocks were embedded in Durcupan<sup>®</sup> (Fluka) polymerized in a 60°C oven for 48h. Ultrathin sections (silver to gold, cca 40 – 60nm thick) were cut with a Reichert ultramicrotome, picked up either on uncoated copper grids or on single-slot Formvar-coated grids. Sections were contrast-stained with lead citrate and aqueous uranyl acetate. In some cases uranyl acetate was administered added to the 70% ethanol phase of the dehydration series. Preparations were viewed and photographed in a JEOL 100 B electron microscope.

Occasionally, for light microscopic survey 1 µm semithin sections were cut from the resin-embedded blocks and stained with 1% toluidine blue containing 1% sodium tetraborate.

#### 4.5. Immunohistochemistry

Antibodies raised against GFAP, MAP2, Tau-protein, tubulin and dystrophin were used to demonstrate the localization of these proteins at light and electron microscopic levels.

For the demonstration of GFAP polyclonal (Dako) and monoclonal (Boehringer) antibodies were used in respective 1 : 2000 and 1 : 400 dilutions. Polyclonal MAP2 antibodies were obtained from Dr. V. Jancsik and used in 1 : 20.000 dilution. Antibodies against tubulin were kindly provided by Dr. P. Viklicky, and were diluted to 1 : 750. Polyclonal antibodies to dystrophin were raised in the laboratory of Dr. R. Mornet and applied to our preparations in 1 : 10 dilution.

Brains were fixed for immunohistochemistry by the transcardial perfusion of Zamboni's fixative (see 4.4.2). The amount of perfused fixative was 400 ml. After perfusion brains were removed from the skull, postfixated for another 2 h in the same fixative and rinsed in three changes of phosphate buffer (pH 7.4). Forty to sixty µm thick

sections were cut on a vibratome and immersed successively into 10%, 20% and 30% sucrose-phosphate buffer (pH 7.4) solution. Sections were kept in 30% sucrose for overnight. Saturation of the specimen with sucrose was indicated by the sinking of the sections to the bottom of the vessel in which it initially floated. This procedure was carried out to ensure a cryoprotection of neural tissue because to enhance penetration of the antibodies, sections were exposed to repeated freezing-thawing cycles using liquid nitrogen.

#### 4.5.1. Light microscopic immunohistochemistry

Forty to sixty  $\mu\text{m}$  Vibratome sections were cut in the coronal plane and were processed for immunohistochemistry free-floating. First they were treated for 10 min with 1% sodium borohydride to suppress endogenous peroxidase activity, then for 2 h with 10% normal rabbit serum (NRbS, Vector) or 5% bovine serum albumin (BSA, Sigma) to inhibit non-specific immunoreactivities, both treatments being performed at room temperature. Sections were proceeded with solutions containing Triton X 100 (Reanal, 0.5%). Following repeated rinses in 0.05 M Tris-buffered saline (TBS, pH 7.4, Trizma<sup>®</sup>, Sigma), sections were incubated with the specific antibodies (primary antiserum) at 4°C for 36 h, under continuous shaking. Incubation was terminated by rinsing in three changes of TBS. As second antibody, biotinylated mouse anti-rabbit (MAR) or biotinylated rabbit anti-mouse IgG (RAM) was used at a dilution of 1 : 100 for 5h, followed by the avidin-biotin-peroxidase complex (Vectastain<sup>®</sup> Elite ABC Kit, Vector, dilution 1 : 100). The immune complex was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma). Sections were mounted on glass slides and coverslipped.

Control incubations were carried out either with the omission of the primary antiserum the sections being processed with the peroxidase conjugate only, or with preabsorbed antisera. In these cases no immunostaining occurred.

#### 4.5.2. Electron microscopic immunocytochemistry

To obtain information about the localization of the antigen also at the fine structural level, some immunostained sections were processed for electron microscopy. This was performed in one of the three following ways:

(i) After the immunohistochemical reaction vibratome sections were rinsed in phosphate buffer and immersed into a 1 % osmium tetroxide solution for 1-2 h. Then they were dehydrated with graded ethanol and propylene oxide, and mounted on a coverslip one section on each. In this stage the reaction could be checked, if necessary photographed in the light microscope. Then a gelatin capsule filled with Durcupan<sup>®</sup> (Fluka) was placed upside down on top of the section. After a 36 h polymerization period of the resin in a 60°C oven specimens were let to cool down to room temperature and the coverslip was removed either with a blade or by snap-freezing it in liquid nitrogen which removes the glass from the resin.

(ii) Sections were processed as above but on the coverslip they were covered with a film of Durcupan<sup>®</sup> (Fluka) by dropping the resin on the surface. The resin film was polymerized in the oven, and then it was covered with an inverted resin-containing gelatin capsule. Resin in the capsule was polymerized in a subsequent 36-h period.

(iii) Sections were allowed to sink in the resin-containing capsule during polymerization.

Owing to the poor penetration of antibodies into the depth of the tissue, in spite of all penetration-enhancing practices it is only the 10-15  $\mu\text{m}$  surface zone of the section in which a reasonable degree of immunostaining can be expected. Therefore, ultrathin sections must be cut exactly tangentially from the surface of the 40-60  $\mu\text{m}$  thick vibratome section. This is possible only if the vibratome section is embedded perfectly flat. The various methods of embedding differ in time and in the safety of flattening the sections. Nevertheless it is the researcher's choice which to apply.

Serial ultrathin sections were mounted alternately on single-slot Formvar-coated nickel and copper grids. Sections mounted on nickel grids were reacted to reveal GFAP-immunoreactive glial elements by means of the postembedding immunogold technique (Somogyi et al., 1985).

From the surface zone of flat-embedded vibratome sections, tangential ultrathin sections were cut and studied either contrast stained or without contrast-staining. DAB immunoprecipitate has a typical granular appearance, whereas immunogold particles are also distinct from any other electron-dense structure. So in most cases contrast staining was applied without obscuring the distribution or visibility of immunostaining.

#### 4.6. Image analysis

The evaluation of an immune reaction, particularly in semiquantitative, or comparative terms has several pitfalls. The thickness of vibratome sections is uneven, values given are approximate with a 15-20 % scatter. Staining-intensity might vary with reactions. As seen from the procedures described in 4.5., the penetration of antisera and reagents has to be enhanced with additional procedures which further increase the variability of reaction intensity. Moreover, assessment based on microscopic inspection contains unavoidable subjective elements. Therefore, beyond the description of localization or the statement of presence or absence of staining any conclusion relevant to grading of immunoreaction-intensity has on this basis a limited value.

To overcome this difficulty, immunoreactions are often evaluated by surface densitometry (Rosenfeld and Kak, 1976) but even with this method difficulties emerge concerning the assessment of background stainings. Recent advances in computer technology, however, enabled an accurate measurement of reaction intensity using the automated evaluation of digitized microscopic images.

Immunohistochemical preparations were analyzed with an IBAS2 image analyzer (Kontron, Munich). Whole sections were scanned with a computer-controlled procedure (Schleicher *et al*, 1986). The microscopic images (12.5-fold primary magnification) were digitized into grey-level images (8-bit grey-level resolution) by use of a CCD-camera. In these images, GFAP-immunopositive structure were segmented by adaptive thresholding (Rosenfeld and Kak, 1976). This segmentation procedure does not operate with a constant grey value threshold valid for the entire section, but with a local threshold that is dependent on the grey value in the immediate surrounding. In order to eliminate low-frequency variations in grey value, and to determine a local grey value threshold, a highpass filter was used. Since the size of the square filter matrix is set to 10  $\mu\text{m}$ , smaller image elements like GFAP-immunoreactive elements are preserved and larger variations in background staining are eliminated and are set to a constant value of 128. From these images, binary images are generated and thresholding with a fixed level of 8 grey values below the background level of 128. Thus, actually detected grey value level range between 0 and 120. This threshold eliminates noise particles, is not a critical value to the segmentation procedure, and allows the correct detection of GFAP-immunoreactive structures.

The territory occupied by GFAP-immunoreactive structures in a unit area was expressed in % (areal fraction) measured by complete scanning with measuring fields 40 x



40  $\mu\text{m}$  in size. The scanning was performed with a computer-controlled, automatic scanning device. By this procedure the boundaries of the 40- $\mu\text{m}$  square fields were precisely joined. According to the x, y count of the measuring field, data were stored in a data matrix that represented the spatial distribution of GFAP-immunopositive elements in the specimen. The data matrix was plotted by subdividing the range of areal fraction values (0 % to 100 %) into 11 equidistant subranges, each of which is plotted in a distinct density pattern. Plots were projected onto corresponding Nissl-stained sections by a camera lucida attachment, and the different brain regions were manually outlined according to the atlases of Paxinos and Watson (1986) and Zilles (1985). The mean values of areal fractions of GFAP-immunoreactive structures in cytoarchitectonically outlined brain regions were retrieved and calculated from the original data matrix by transferring plots to a digitizer connected with the image analyzer. Outlined regional contours were traced which resulted in the above described retrieval of original data transformed into the mean value relevant to a region.

In some cases only surface densitometry was carried out. In this procedure, a reference structure was chosen on the surface of the vibratome section.

#### 4.7. Biochemical demonstration of GFAP

Tissue samples were either snap-frozen and stored in liquid nitrogen or processed immediately. Tissues were homogenized in 0.1 M Tris-buffer (pH 6.8, Trizma<sup>®</sup>, Sigma) supplemented with 1  $\mu\text{M}$  EGTA and 1 mM phenylmethyl sulphonyl fluoride. Cell debris and nuclei were removed by centrifugation at 500 g for 10 min. Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. Electrophoretic separation of the proteins on 7.5 % lithium dodecyl sulphate (LiDS) polyacrylamide gel was done in a Mighty Small II<sup>®</sup> electrophoretic device (Hoefer Scientific). Separated proteins were then electroblotted onto nitrocellulose membrane (Serva, pore size 0.2  $\mu\text{m}$ ). Coomassie Brilliant Blue staining was used to verify blotting efficiency. Monoclonal antibody clone G-A-5 (Boehringer) was used for the immunodetection of GFAP. Horseradish peroxidase-labeled goat anti-mouse serum (Human, Gödöllő) served as second antibody. The immunoperoxidase reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) as chromogen,

in combination with nickel sulphate. Staining intensities of blot stripes were evaluated by densitometry (Shandon Cello 3<sup>®</sup> densitometer, red filter). When submitting different amounts of protein to the same sample (protein content 3 to 30 µg) to the above procedure, a linear relationship was obtained between the amounts of protein and the densitometric values. Hence densitometric values reflected the amount of GFAP applied to the gel. This allowed the comparison of samples by loading adjacent lanes with an equal protein amount.

#### 4.8. Statistical evaluations

For statistical analyses the Student's t-test and the  $\chi^2$ -test were employed.