5. Findings of our group underlying Results

5.1. The choice of brain areas to study astroglial cytoskeleton

Astroglial cytoskeleton can be studied in tissue cultures of astrocytes (Juurlink *et al.,* 1981; Kalnins *et al.*, 1984) but such *in vitro* systems lack the natural neuronal environment whose connectivity is essential to see alterations resulted from glia-neuron interactions. Study in organotypic tissue cultures yields results more close to *in vivo* situations. Still our goal was to observe the reactions of the astroglial cytoskeleton *in situ*, within its natural environment. This, of course, required a model system in which the complexity of the brain structure does not obscure findings and where reactive astroglia is well-observable.

5.1.1. Mapping of GFAP immunoreactivity

Astrocytes are known to be fairly evenly distributed throughout the brain (Tower, 1988; Wree *et al.*, 1980). Their cytoskeletal apparatus may, however, differ in amount and extent. It is believed that in the white matter fibrous astrocytes, in the grey matter protoplasmic astrocytes occur (Privat and Rataboul, 1986). If so, a thorough mapping of GFAP immunoreactivity should disclose major differences between the immunostaining of the grey and the white matter.

Serial sections cut in the coronal plane through the entire brain and stained for glial fibrillary acidic protein- (GFAP) immunoreactivity did not support this claim. Computer plots of these preparations were carefully analyzed. These suggest that there was little or no GFAP-staining in the white matter as compared to the grey matter. On the other hand, in the grey matter some territories were also devoid of immunoprecipitate, while there were consistent differences in staining-intensities between GFAP-immunoreactive grey matter regions. In some areas the staining intensity significantly exceeded that of other regions. Further significant differences were observed in the intensities of GFAP-immunoreactivity between grey matter areas. Numeric values retrieved from the data matrix of plots were expressed as areal fractions for circumscribed brain areas (usually anatomical units) and are shown in Table 1.

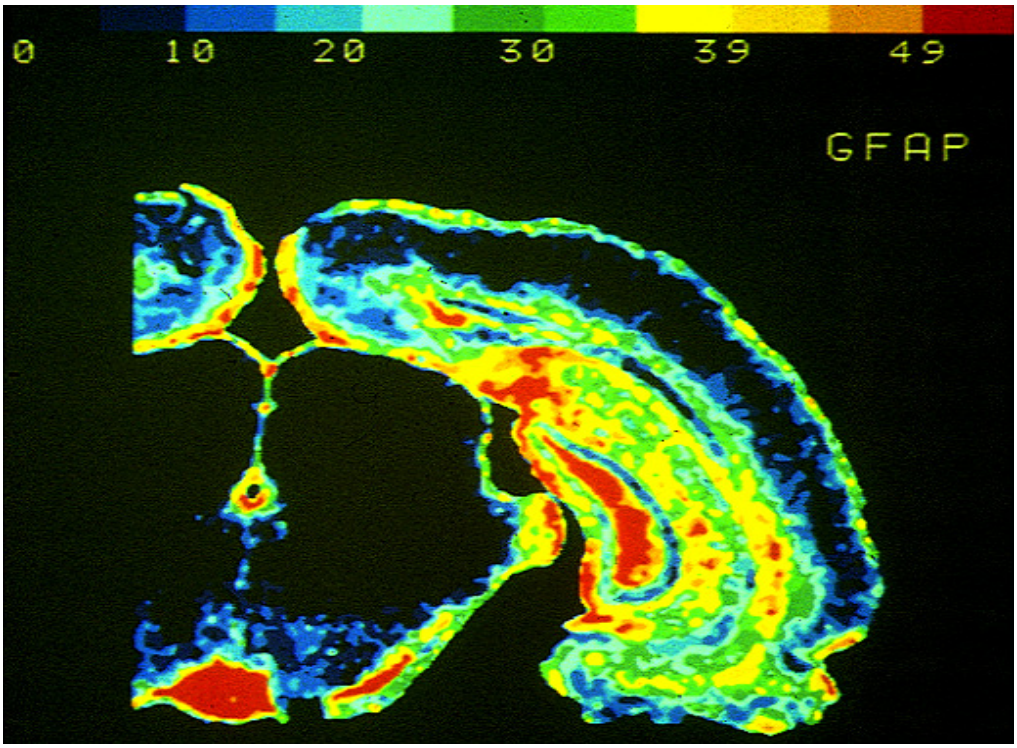
Brain region	mean AF	SE	Brain region	mean AF	SE
A	1.83	0.28	MG	0.91	0.20
AD	4.28	0.42	MM	0.65	0.18
Ald	1.02	0.15	MO/VO	1.78	0.27
Alp	2.39	0.29	MP	2.36	0.38
Alv	1.67	0.20	MPO	1.15	0.17
APT	0.84	0.19	MS	0.84	0.19
AO	1.17	0.18	o (CA1-3)	5.96	0.5
AV	1.79	0.29	Oc1	0.65	0.17
BFB	0.89	0.15	Oc2L	1.63	0.25
BSTL	0.90	0.24	Oc2M	1.68	0.25
Cg1	1.05	0.11	OPT	2.85	0.43
Cg2	1.79	0.28	opt	4.58	0.50
Cg3	0.77	0.21	OVLT	4.58	0.42
Cl	1.21	0.22	p (CA1-3)	3.25	0.36
cp	2.99	0.24	Par 1	0.82	0.18
CPu	0.78	0.15	Par2	0.82	0.19
CS	0.32	0.19	PaS	0.75	0.22
DLG	1.80	0.29	Pir	2.31	0.24
DPC	1.65	0.21	PO	0.38	0.17
E/OV	3.64	0.30	POP	3.46	0.49
Ent	0.83	0.22	PRh	2.18	0.41
eplm	2.65	0.23	PrS	1.56	0.31
FL	0.65	0.19	r (CA 1-2)	4.35	0.30
fo	1.95	0.30	RSA	1.98	0.30
Fr1	0.77	0.20	RSG	2.72	0.39
Fr2	0.98	0.19	Rt	2.18	0.34
Fr3	0.48	0.20	SHy	2.56	0.39
g	2.69	0.12	SI	1.45	0.32
gl	3.41	0.24	SNC	3.46	0.41
GP	3.23	0.54	SNR	5.70	0.44
gr	2.89	0.24	SO	4.80	0.45
h	5.86	0.09	st	4.72	0.52
HDB	0.59	0.20	Subdorsal	2.33	0.34
HL	1.14	0.19	Subventral	0.69	0.31
ic	1.12	0.29	Te1	2.01	0.19
IG	4.18	0.57	Te2	2.41	0.30
IL	0.87	0.20	Te3	2.39	0.31
IP	3.40	0.61	TT	2.46	0.37
LM	0.65	018	Tu	2.11	0.33
lm (CA1-2)	9.17	0.38	Vi	0.78	0.20
LO	0.89	0.19	VL	0.43	0.16
lo	2.49	0.43	VLG	3.40	0.45
LP	3.00	0.49	VLO	0.45	0.21
LPO	0.33	0.14	VP	1.16	0.23
LS	2.38	0.37	VTA	1.67	0.27
m	4.80	0.26	WM	3.78	0.58
MD	0.88	0.19	XO	3.40	0.56

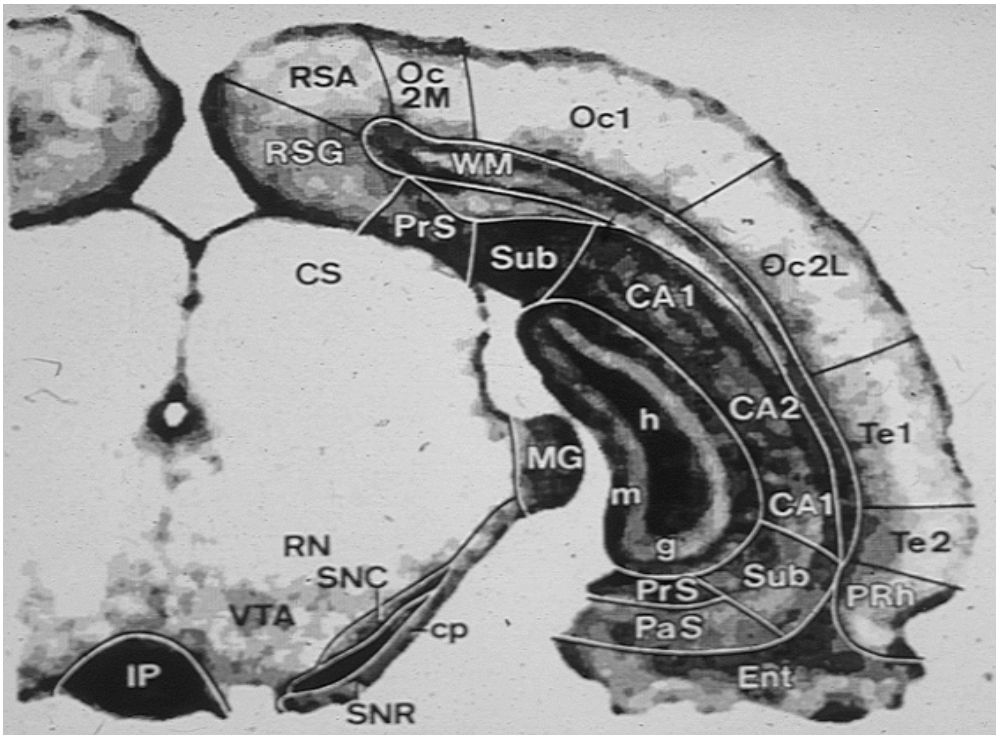
Table 1

Areal fraction (AF in %) of GFAP-IR structures in the rat brain. Mean values and standard deviations of the mean (SE) are calculated from three adult rat brains. For explanations of abbreviations of brain regions, see abbreviations list.

List of abbreviations

A	amygdala
AD	anterodorsal thalamic nucleus
Ald	agranular insular cortex, dorsal part
Alp	agranular insular cortex, posterior part
Alv	agranular insular cortex, ventral part
APT	anterior pretectal nucleus
AO	anterior olfactory cortex
AV	anteroventral thalamic nucleus
BFB	basal forebrain
BSTL	bed nucleus striae terminalis
Cg1-3	cingulate cortical areas
Cl	claustrum
cp	cerebral peduncule
CPu	caudate putamen
CS	superior colliculus
DLG	dorsal lateral geniculate body
DPC	dorsal peducular cortex
E/OV	subependymal layer of olfactory ventricle
Ent	entorhinal area
eplm	ext. plexiform and mitral layers of olf. bulb
FL	forelimb area
fo	fornix
Fr1-3	frontal neocortical areas
g	granular layer of dentate gyrus
gl	glomerular layer of olfactory bulb
GP	pallidum
gr	granular layer of olfactory bulb
h	hilus of dentate gyrus
HDB	horizontal limb of diagonal band
HL	hindlimb area
ic	internal capsule
IG	indusium griseum
IL	infralimbic cortex
IP	interpeduncular nucleus
LM	lateral mammillary body
lm (CA1-2)	lacunosum-molecular layer
LO	lateral orbital area
lo	lateral olfactory tract
LP	lateral posterior thalamic nucleus
LPO	lateral preoptic area
LS	lateral septum
m	molecular layer of dentate gyrus
MD	mediobasal thalamic nucleus
MG	medial geniculate body
MM	medial mammillary body, medial
MO/VO	medial and ventral orbital areas
MP	medial mammillary body, posterior
MPO	medial preoptic area
MS	medial septum
o (CA1-3)	oriens layer of CA 1-3
Oc1	occip. neocort. area 1 (prim. visual cortex)
Oc2L	occipital neocortical area 2, lateral part
Oc2M	occipital neocortical area 2, medial part
OPT	olivary pretectal nucleus
opt	optic tract
OVLT	organum vasculosum laminae terminalis
p (CA1-3)	pyramidal layer of CA 1-3
Par 1	parietal neocortical areas
PaS	parasubiculum
Pir	prepiriform cortex
PO	posterior thalamic nucleus
POP	periventricular preoptic area
PRh	perirhinal area
PrS	presubiculum
r (CA1-2)	radiatum layer of CA 1-3
RN	red nucleus
RSA	retrosplenial cortex, agranular part
RSG	retrosplenial cortex, granular part
Rt	recessus triangularis
SHy	septohypothalamic nucleus
SI	substantia innominata
SNC	substantia nigra, pars compacta
SNR	substantia nigra, pars reticularis
SO	supraoptic nuclues
st	stria terminalis
Sub	subiculum
Te1-3	temporal neocortical areas
TT	tenia tecta
Tu	olfactory tubercle
Vi	visceral cortex
VL	ventrolateral thalamic nucleus
VLG	ventral lateral geniculate body
VLO	ventrolateral orbital cortex
VP	ventral pallidum
VTA	ventral tegmental area
WM	white matter
XO	optic chiasm





Figs. 2, 3

The territorial distribution of GFAP-IR as revealed by computer plots converted to color-codes (Fig.2, see color-scale) or in black-and-white (Fig. 3), where regions are delineated. For abbreviations see list to Table 1.

Looking at the GFAP-plots, either color-coded or regionally delineated (Figs. 2, 3), the territorial distribution of immunostaining could be readily visualized. The most prominent regions of high GFAP-immunoreactivity were the hippocampus and dentate gyrus where the reaction followed the cytoarchitectonic layers (Fig. 3), the pallidum of the caudate nucleus, the habenulae and the so called midline structures (Fig. 4).

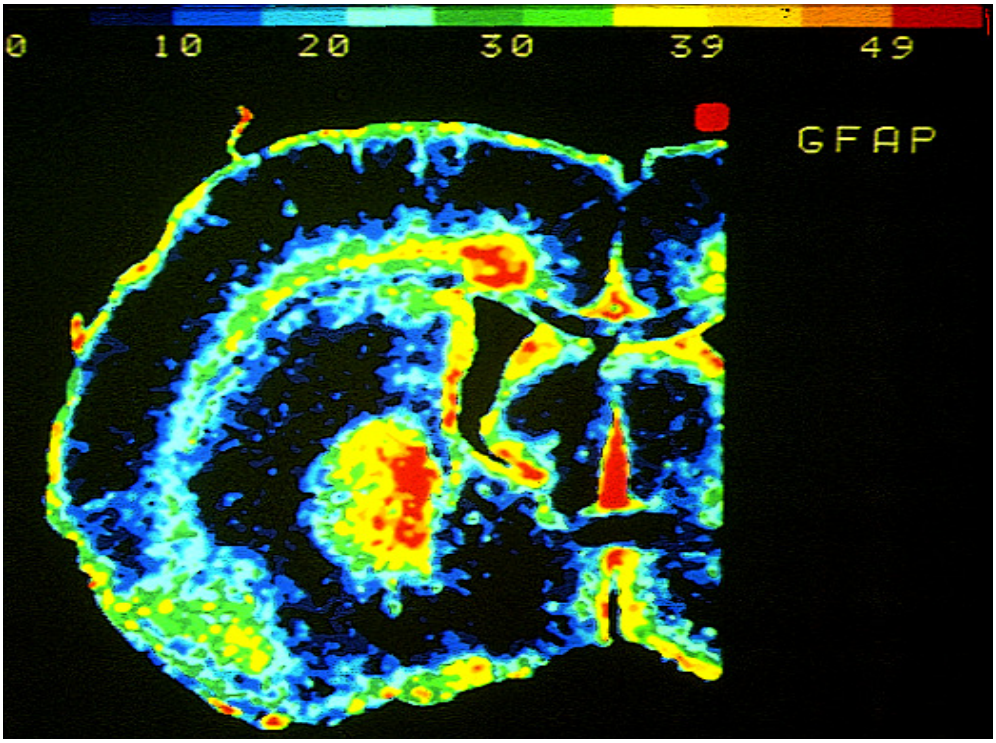


Fig. 4
A high GFAP-immunoreactivity is observed in the pallidum and the midline structures (septum, organum vasculosum laminae terminalis – OVLT). Note the lack of immunostaining in the middle layers of the neocortex.

The interpeduncular nucleus showed by far the highest GFAP-immunoreactivity (Fig.5). In the white matter there was little or no reaction visible except for the trunk of the corpus callosum (Fig. 6) where immunostaining occurred in parallel strips. It is noteworthy that a remarkable number of grey matter areas were found where little or no GFAP-immunostaining was encountered such as the neocortex, the pallidum and most of the hypothalamus (Hajós and Zilles, 1995). Particularly interesting was the GFAP-staining of the cerebral cortex. While in the archicortex (piriform cortex) a moderate but evenly distributed staining was seen, in the neocortex only layers I, II and VI were stained, the middle layers remaining consistently immunonegative (Fig. 4).

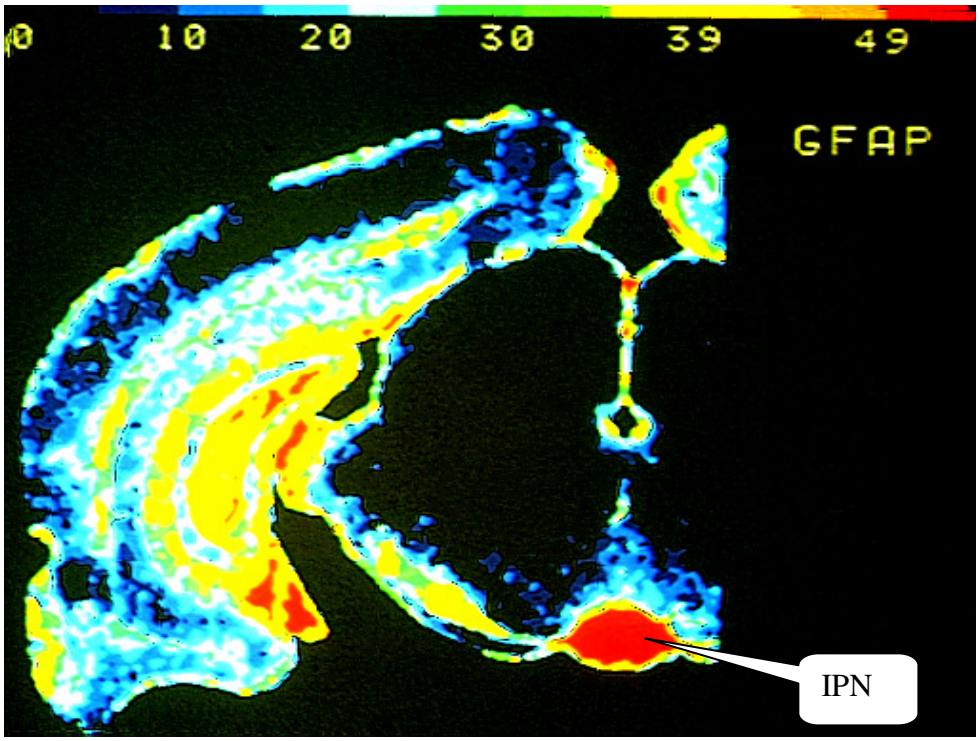


Fig. 5
Prominently intense immunostaining due to GFAP-IR in the interpeduncular nucleus (IPN).

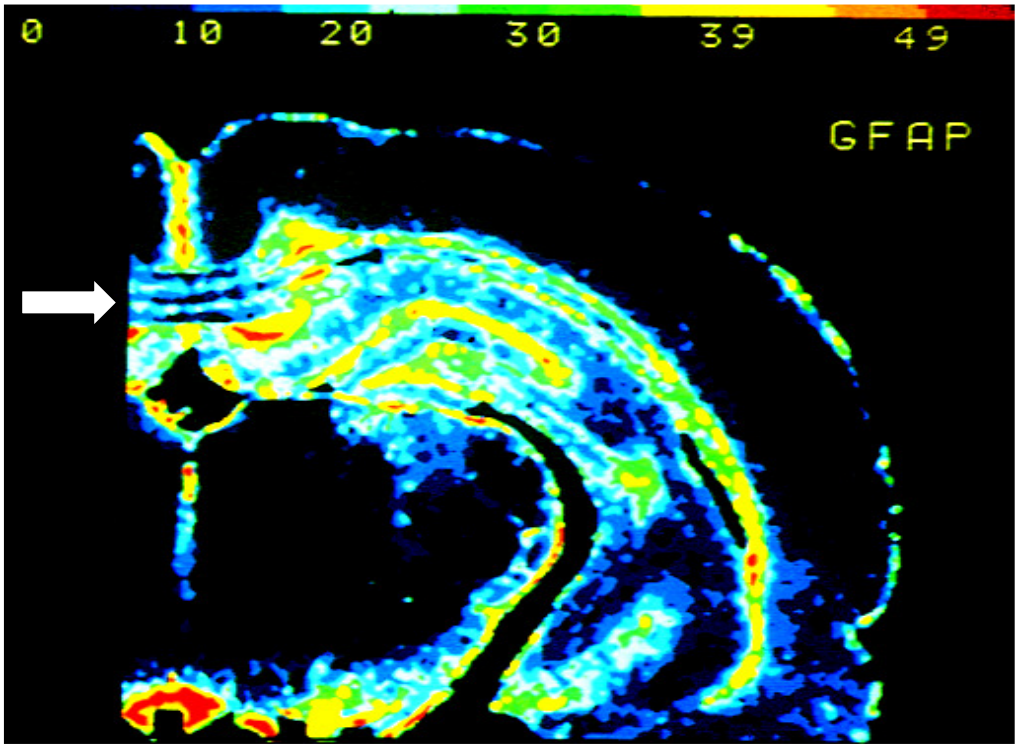


Fig. 6
The white matter is generally immunonegative with some exceptional areas such as the trunk of the corpus callosum where GFAP-IR occurs in parallel strips (arrow).

6. Results

Differences in the distribution of GFAP-immunoreactivity raised the question of a possible differential localization of astrocytes, in other words, if there is no lack or paucity of astrocytes at these sites? Astrocytes can be readily recognized in toluidine blue-stained sections of resin embedded neural tissue based on their characteristic nuclear structure (Fig. 7).

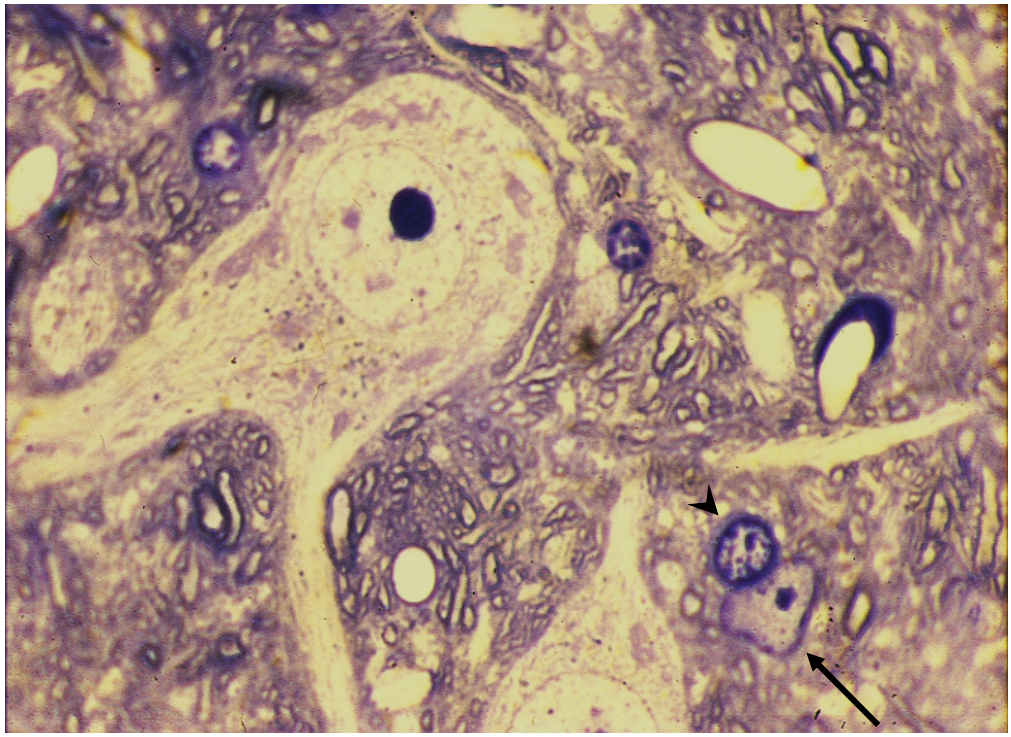


Fig. 7
In semithin, toluidine-blue-stained sections of resin-embedded neural tissue (midbrain) the basic cellular components of the CNS can be readily distinguished. The astrocytes (arrow) are distinct from oligodendrocytes (arrowhead) by their pale nucleus, prominent nucleolus and an accumulation of chromatin at the inner aspect of the nuclear envelope. x2000

Accordingly, we calculated astrocyte numbers per territory units in regions of low or no GFAP-immunoreactivity. For this we used large area semithin sections of resin-embedded brain tissue stained with 1% toluidine blue. In 25.000 μm^2 areas of various brain regions the number of astrocytes was counted under an eyepiece graticule. In the neocortex, where GFAP immunostaining showed extreme variations between layers, a total of 1635 astrocytes were counted. The entire width of the cortex was divided into three equidistant zones designated as external, middle, and internal. In terms of cortical cytoarchitectonics the external zone comprised layers I-IV, the middle zone layers V and external VI, and the inner zone internal VI. Within the measured territory 29.38%, 38.63%,

and 31.81% of astrocytes were found in the external, middle, and internal layers, respectively. These results suggested that there was no major difference in astrocyte number and packing density between regions of high and low staining-intensities (Hajós *et al.*, 1993).

Based on the above observations we selected two regions for further experimental studies: the occipital region of the neocortex and the interpeduncular nucleus of the midbrain.

6.1. Model I.: The geniculo-cortical system

The occipital cortex comprises the primary visual area (Oc1, Zilles, 1985) and the related associative regions. The afferents to this area originate from the caudal part of the thalamus, those of the primary visual area from the lateral geniculate body, and terminate in layers III-IV of the visual cortex. The geniculo-cortical pathway turned out to be an ideal model for our experiments because its neurons of origin are readily accessible for experimental lesionings and physiological stimulations. The projections of the geniculo-cortical system are strictly ipsilateral (Schober and Winkelmann, 1977), thus the contralateral side can be used as a natural control. An eventual increase in GFAP-immunoreactivity is in this system is confined to a circumscribed GFAP-immunonegative area and is thus well-detectable, even under circumstances when the lesion site exceeds the borders of the dorsal lateral nucleus of the lateral geniculate body.

After stereotaxic lesions of the lateral geniculate body (Fig. 8) a Wallerian degeneration was induced. The observation of astroglia in the target area confirmed earlier findings (Hajós *et al.*, 1990a) that under the circumstances of Wallerian degeneration, a remote astroglial response (RAR) occurs. Pilot experiments have also shown that RAR is coupled to a spectacular increase in GFAP-immunoreactivity.

As seen in sections incubated with antiserum against GFAP, immunostained astrocytes of the control side (contralateral to the lesion) can be seen only in the outer- and innermost cortical layers, while the middle layers are devoid of immunoprecipitate (Fig. 9). On the operated side all layers within a wedge-shaped area corresponding to the primary visual cortex were found to be intensely immunostained, i.e. the middle layers also contained evenly distributed, intensely immunostained astrocytes (Figs. 10, 11).

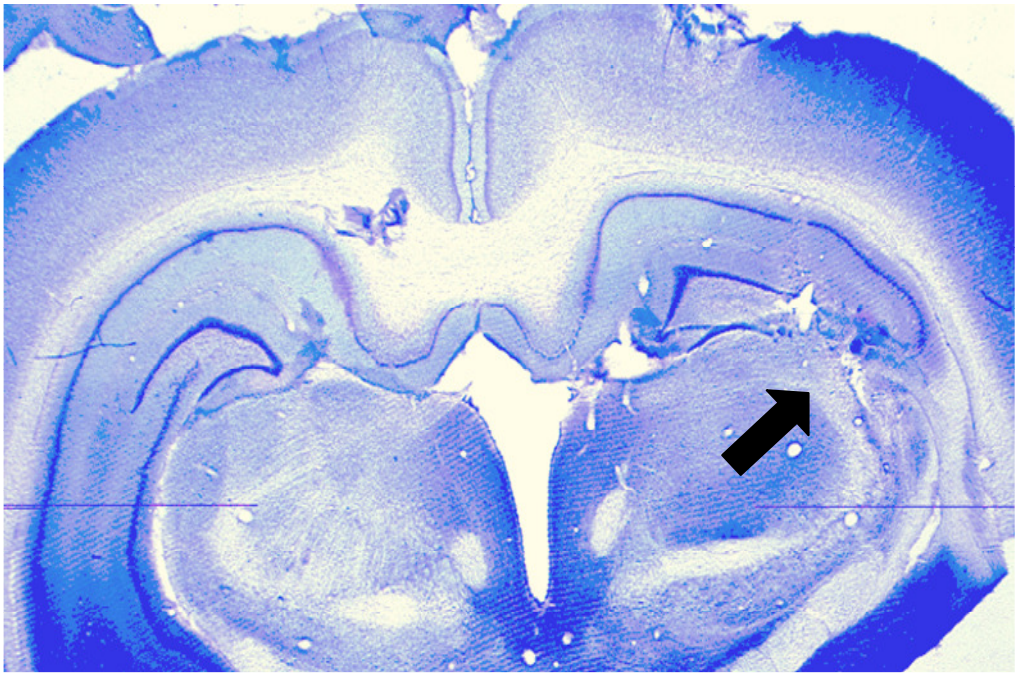


Fig. 8a

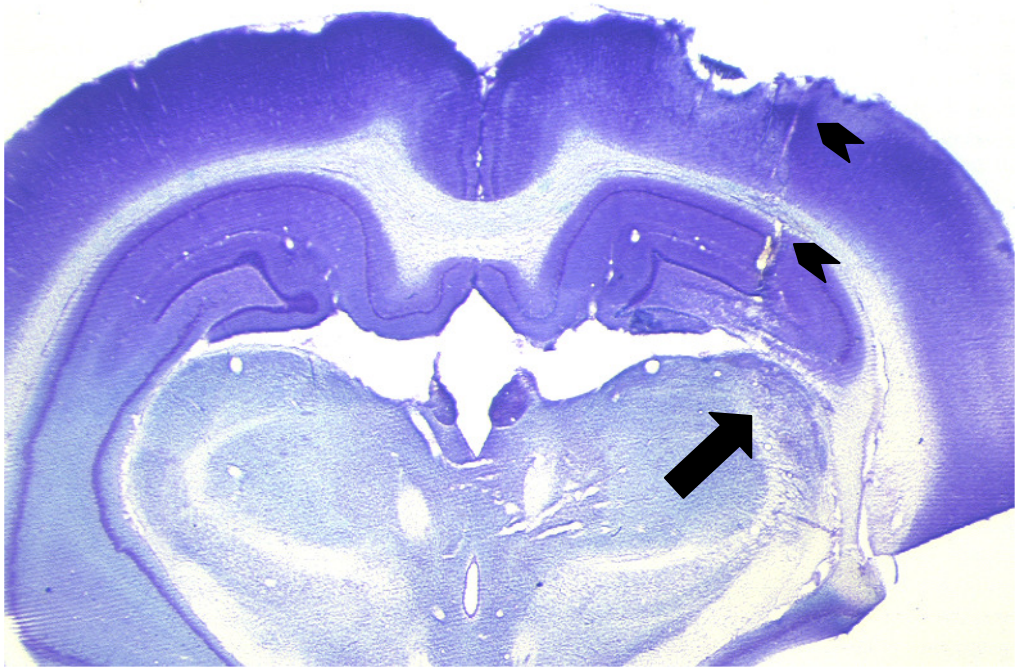


Fig. 8b

Fig. 8

Nissl-stained section showing the location of the lesion in the dorsal lateral geniculate nucleus (arrow). Degeneration is indicated by a darkening of the lesioned area as compared to the control side. In Fig. 8a the fresh lesion at the tip of the electrode is shown causing a local hemorrhage. In Fig. 8b the needle-track is seen (arrowhead), while the DLG is degenerated without visible mechanical damage. x14

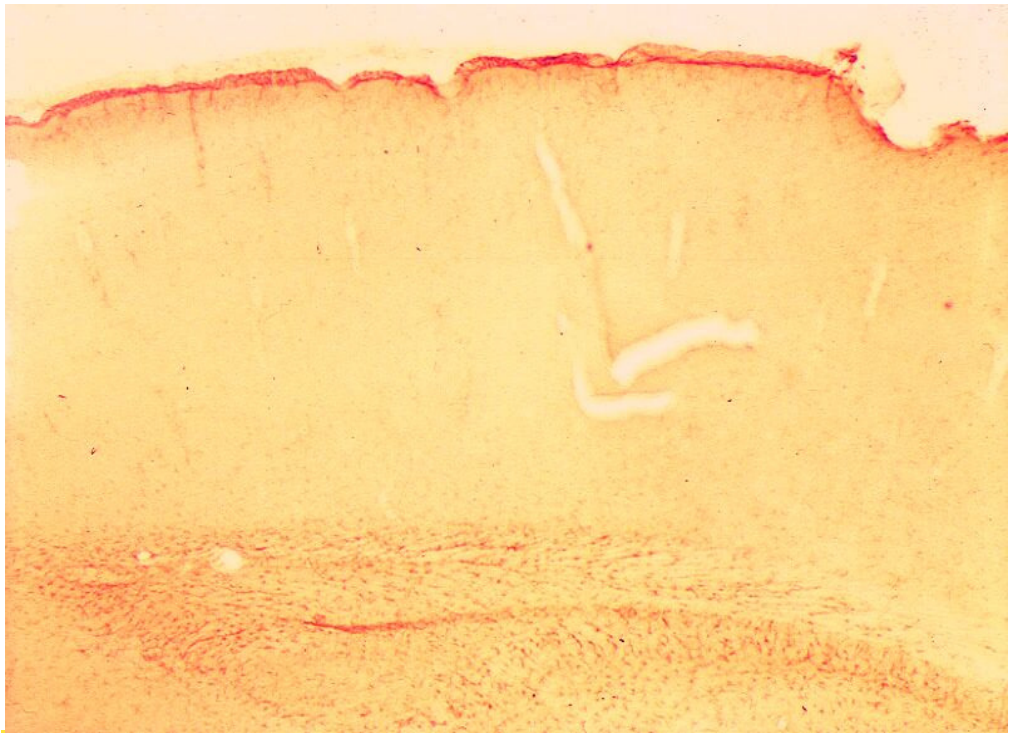


Fig. 9

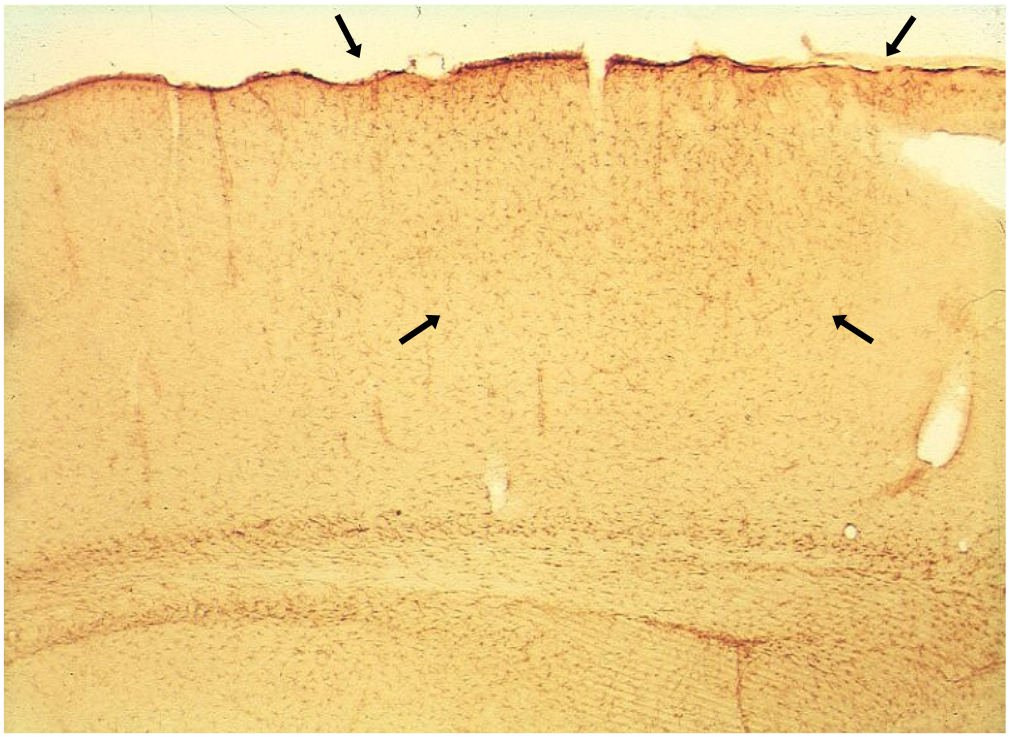


Fig. 10

Figs. 9, 10

After the lesioning of the dorsal geniculate nucleus the contralateral (control) visual cortex (Fig. 9) showed the usual GFAP-immunonegativity of its middle layers, while on the operated side a wedge-shaped area (between arrows) corresponding to the primary visual cortex exhibited the immunostaining of all cortical layers (Fig. 10). x120

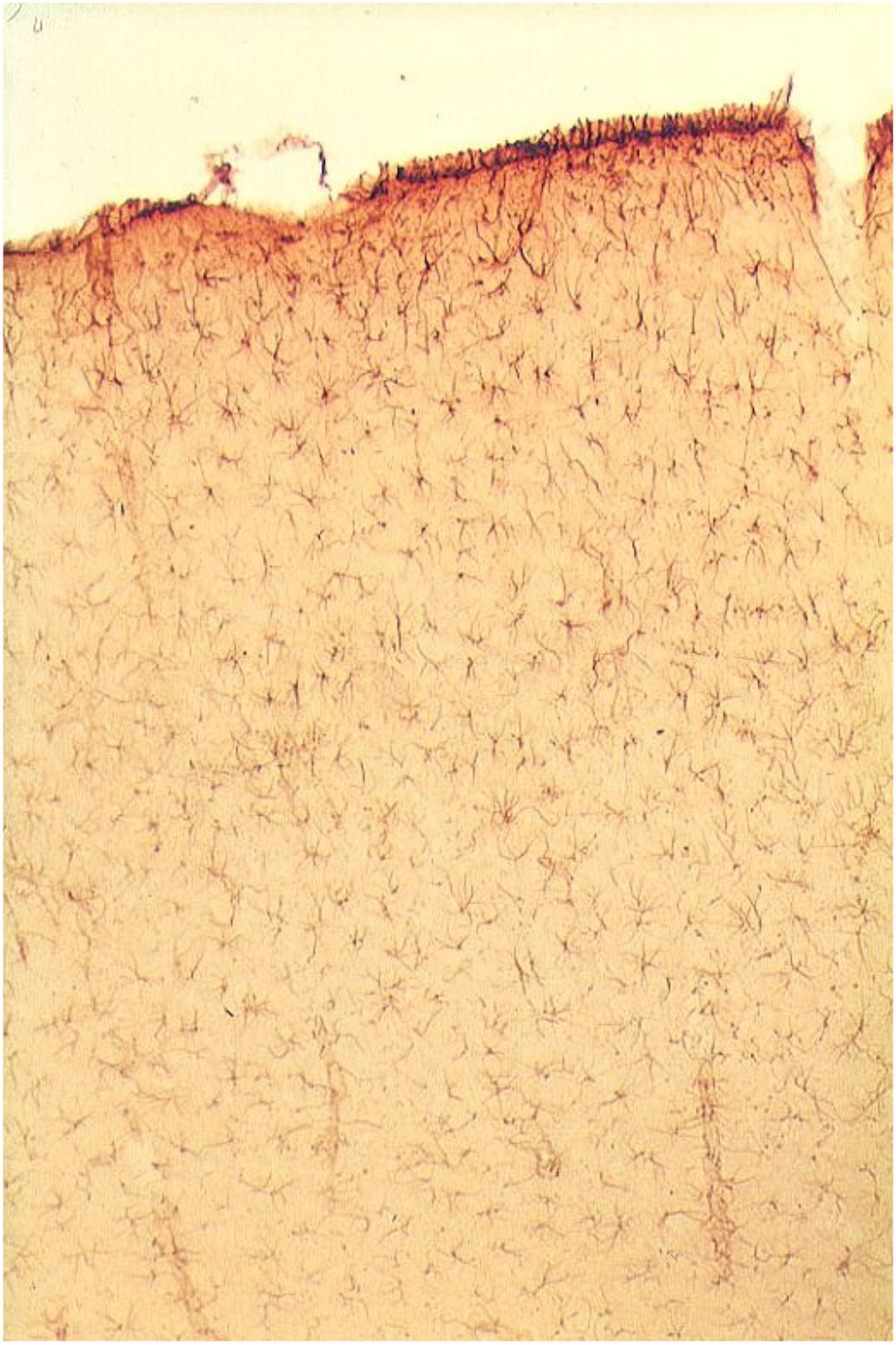


Fig. 11

Higher magnification of reactive glia in the visual cortex on the operated side showing that he wedge-shaped area appearing darker in Fig. 10 is due to reactive astrocytes distributed evenly throughout the width of the cortex. x300

This typical RAR was verified also with image analysis (Figs. 13, 14) and proved to have a time course specific for this system. Accordingly, the first signs of increase in GFAP-immunoreactivity could be detected on postoperative day 3, the peak intensity of the reaction was reached between days 7 and 14, after this time it declined so that three months after the lesion no reaction was observed in layers III-V of the occipital cortex, which corresponded to the pre-lesional situation, with the exception that around the major vessels of these layers the immunoreaction remained increased in patches (Fig. 12) even six months after the lesion.

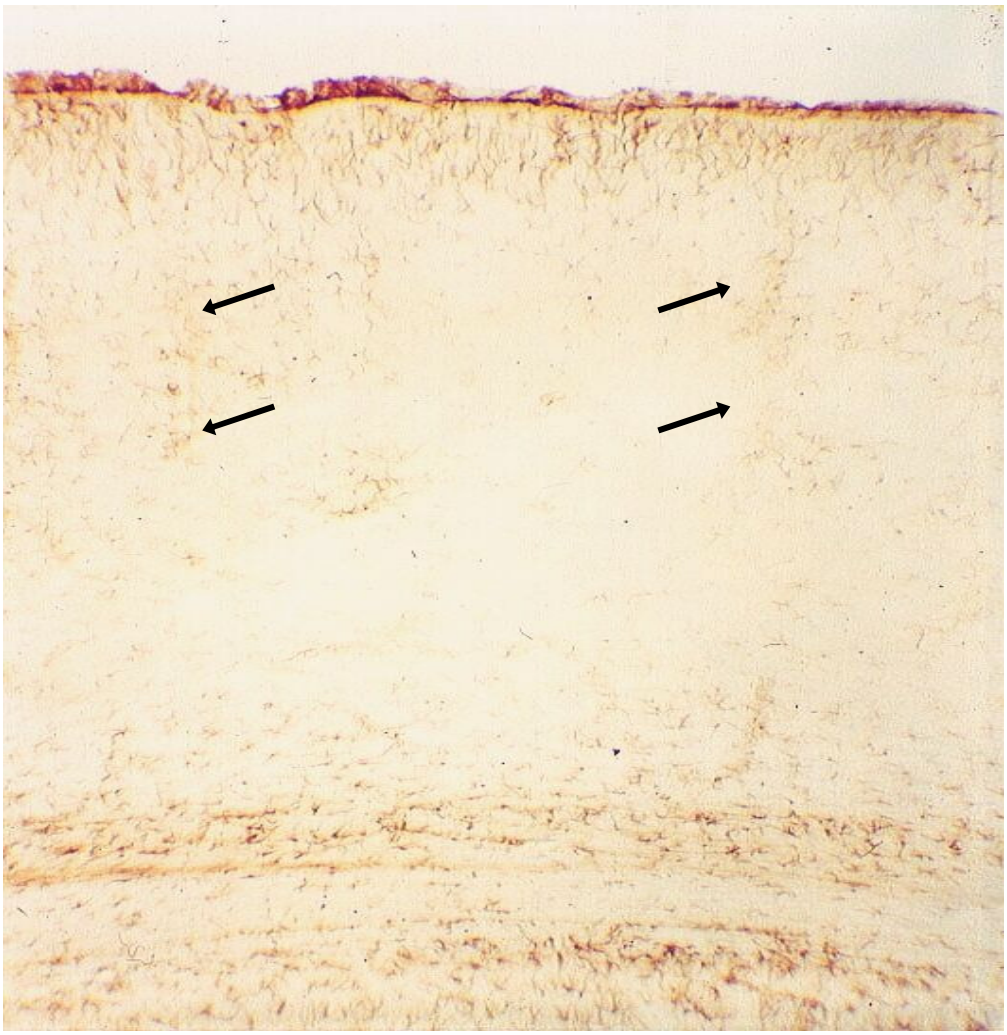


Fig. 12
Three months after CGL-lesion glial reaction disappeared from the middle layers of the ipsilateral visual cortex except patches (arrows) around major blood vessels. x160

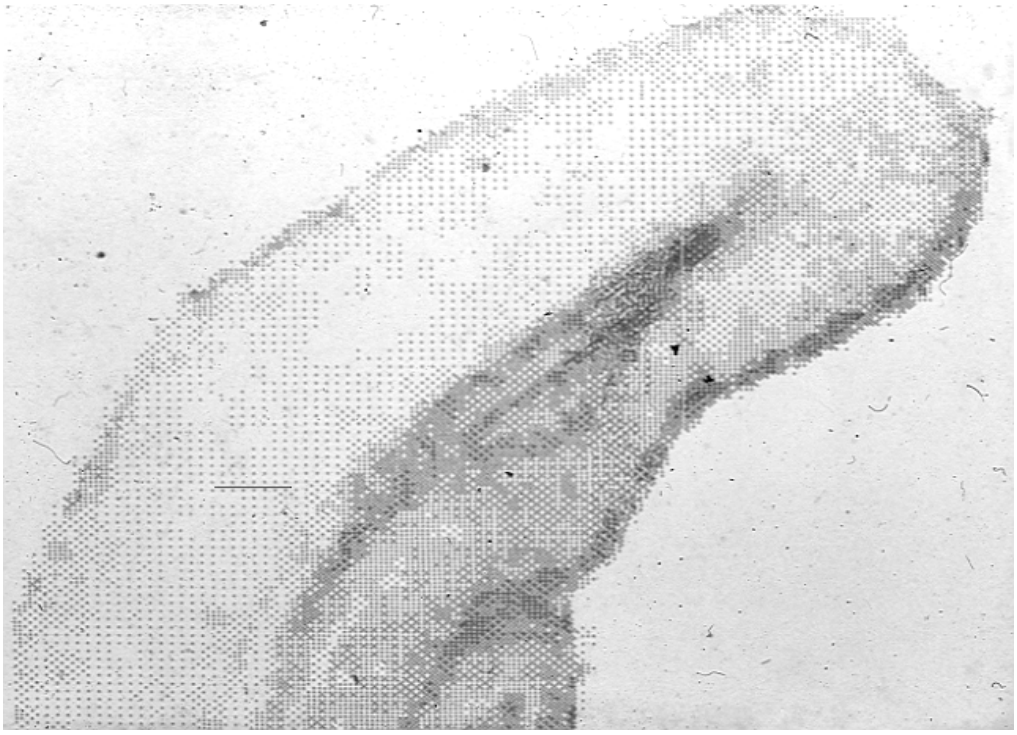


Fig. 13

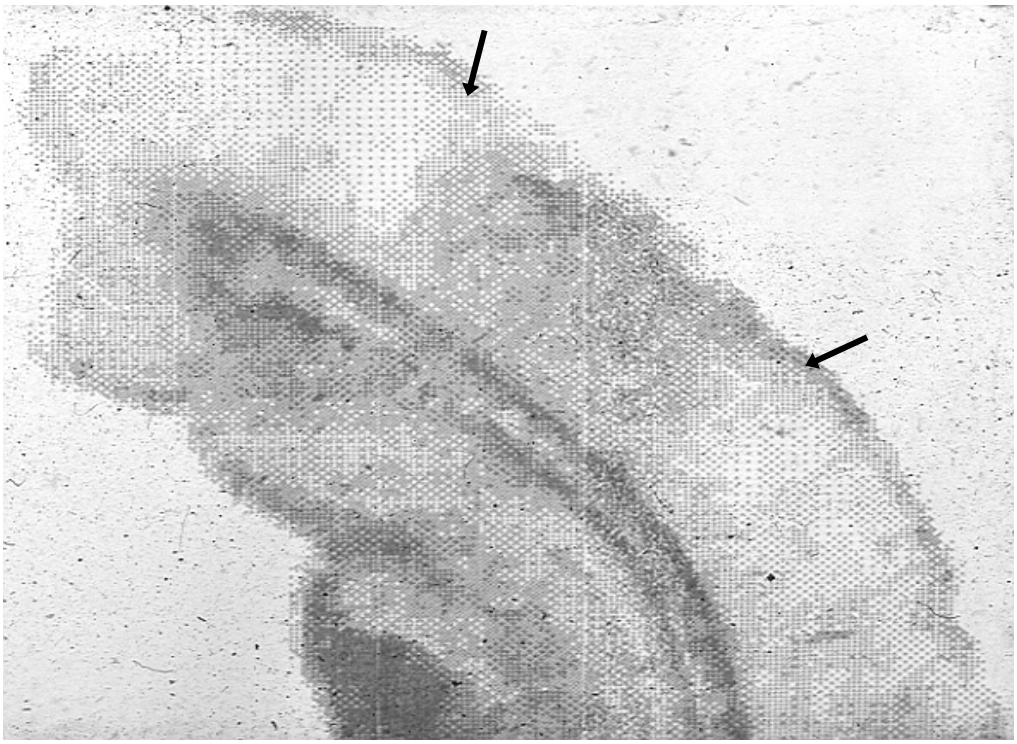


Fig. 14

Figs. 13, 14

Computer plots of the GFAP-IR in the visual cortex on the control (Fig. 13) and CGL-lesioned (Fig. 14) sides. Image analysis delineates the territory of activated glia (arrows).

6.2. Model II.: The interpeduncular nucleus

As a further model to study the effect of hormonal states on the astroglial cytoskeleton, in addition to the phenomenon of RAR in the geniculo-cortical system, the interpeduncular nucleus was selected (Fig. 15). This nucleus exhibits an outstandingly high GFAP-immunoreactivity and has no direct connections with brain centers involved in the regulation of endocrine functions. This is important when attempting to decide whether possible hormonal effects that may alter the astroglial cytoskeleton are mediated by hormone receptors contained by the astrocyte membrane (Wilkin *et al.*, 1990; Jung-Testas *et al.,* 1992) or act directly on intermediate filament protein(s).

Conclusions of 6.1.and 6.2. Extensive maps of GFAP-immunostaining throughout the brain have shown a highly uneven distribution of the immunoreaction between grey and white matters the latter being unexpectedly almost devoid of GFAP-immunostaining. In the grey matter, the immunostaining for GFAP showed extreme variations which was in sharp contrast to the even distribution of astrocytes.

Of the GFAP-immunonegative grey matter regions, the geniculo-cortical system was selected for experimental studies to induce and suppress remote astroglial response (RAR), whereas of the intensely immunopositive areas the midbrain interpeduncular nucleus was thought to be suitable to study the effect of hormonal states on an intact brain region.

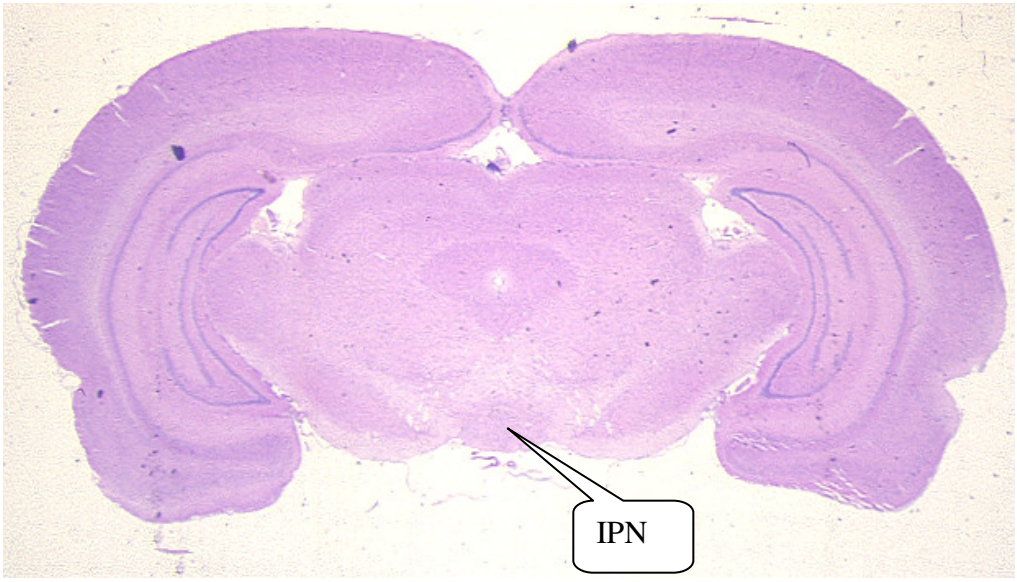


Fig. 15a

explanation: see overleaf

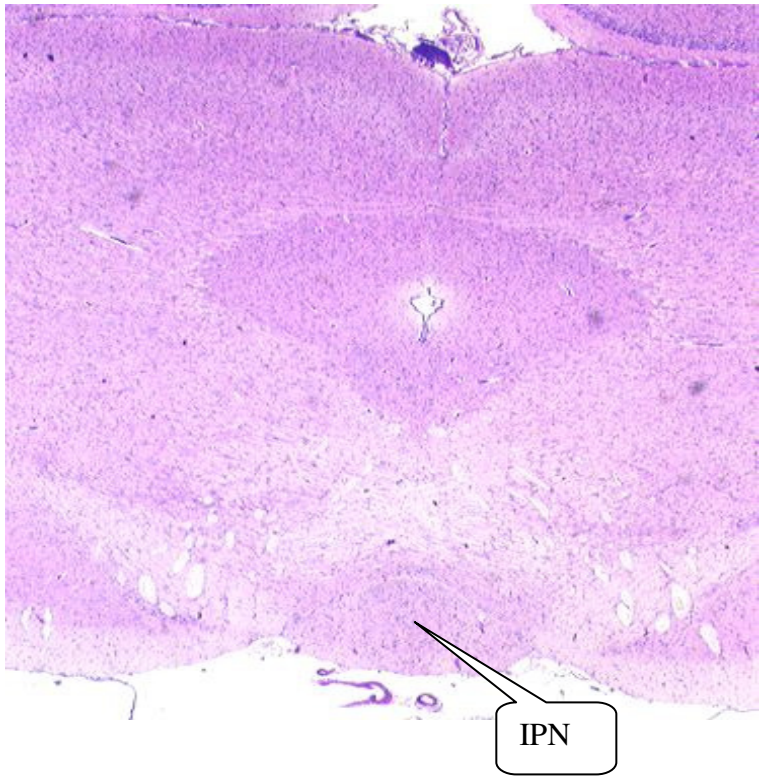


Fig. 15b

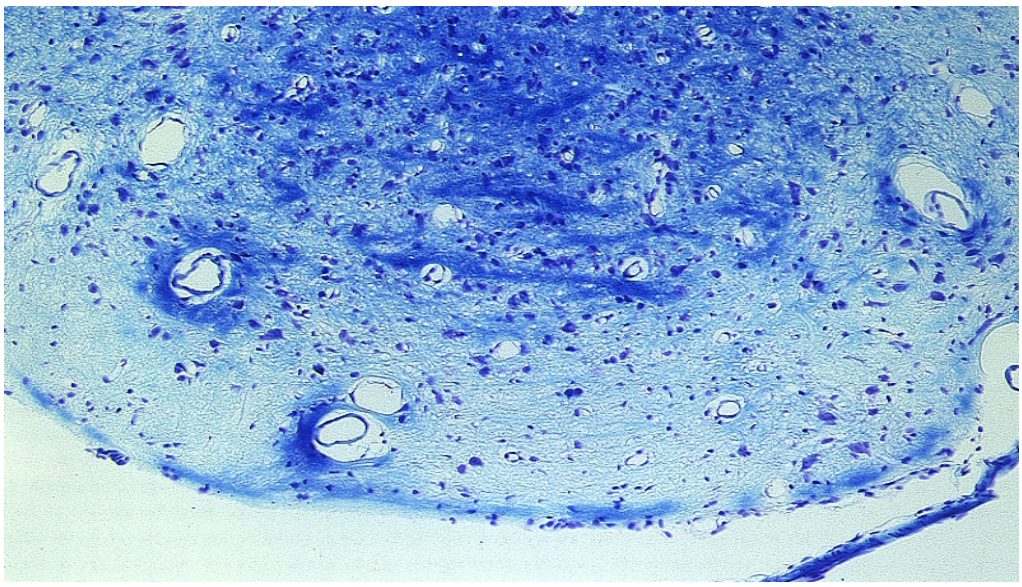


Fig. 15c

Fig. 15
The interpeduncular nucleus (IPN) as seen in a coronal section of the brain.

A) The nucleus is found at the depths of the interpeduncular fossa bordered by the cerebral peduncles. x10, stained with H-E

B) Observe the IPN at a higher magnification. x 60, stained with H-E

C) Under higher power the cells of the nucleus are stained purple, whereas myelinated fibers blue. It is evident that the IPN is densely permeated by transverse fibers. x125, stained with Luxol-Fast Blue

6.3. The astroglial reaction: proliferation or hypertrophy?

There exists a controversy in the assessment of the nature of the astroglial reaction. According to several claims, in this phenomenon a proliferation of astrocytes is involved, whereas other authors could not confirm the presence of astrocyte proliferation (Hajós *et al.*, 1993).

To decide this question under the circumstances of remote astroglial response (RAR), we checked carefully the visual cortex for mitoses and hypertrophic alterations in 16 animals with unilateral lesions of the lateral geniculate body. Six brains were examined 3 days after the lesion, 4-4 brains at 4 and 7 days, while the rest at 11 days. The search for mitotic figures, either glial or other (mesenchymal, inflammatory, endothelial, etc.) was carried out in 1 μm thick toluidine blue-stained sections of resin-embedded visual cortices ipsilateral to the lesion. Even with the most scrutinous survey no mitotic figures were encountered at either time interval after operation.

In semithin, toluidine blue-stained preparations astrocytes can be readily identified on the basis of their round or oval, lightly staining nuclei showing a characteristic accumulation of chromatin at the inner aspect of the nuclear envelope and an eccentrically located nucleolus (Fig. 7). Interneurons in the size-range of astrocytes were distinguished by their nuclear indentations and/or evenly distributed clumps of heterochromatin. Oligodendrocytes were well discernible having a compact, dark nucleus, and a small, darkly staining cytoplasm. Pyramidal neurons were evident due to their size, shape and orientation. Based on these identification criteria, visual cortex astrocytes were found spectacularly hypertrophic on the operated side (Figs. 16, 17). The cytoplasm was substantially enlarged showing the typical "watery" appearance described in low-power electron micrographs (Peters *et al.*, 1976). Also the processes became widened with numerous branches and appendages. In the enlarged cytoplasm mitochondria and bundles of cytoskeletal filaments could be perceived under oil immersion. The nucleus was also slightly enlarged but the distribution of chromatin seemed to be somewhat more even within the nucleoplasm and it formed a thinner contour line of the nuclear envelope as compared to the control. Even so, the nucleus of astrocytes displayed a more pronounced contour than that of neurons. In most hypertrophic astrocytes the nucleolus was also enlarged and centrally located. The peak of astroglial hypertrophy was observed on postoperative days 3-5.

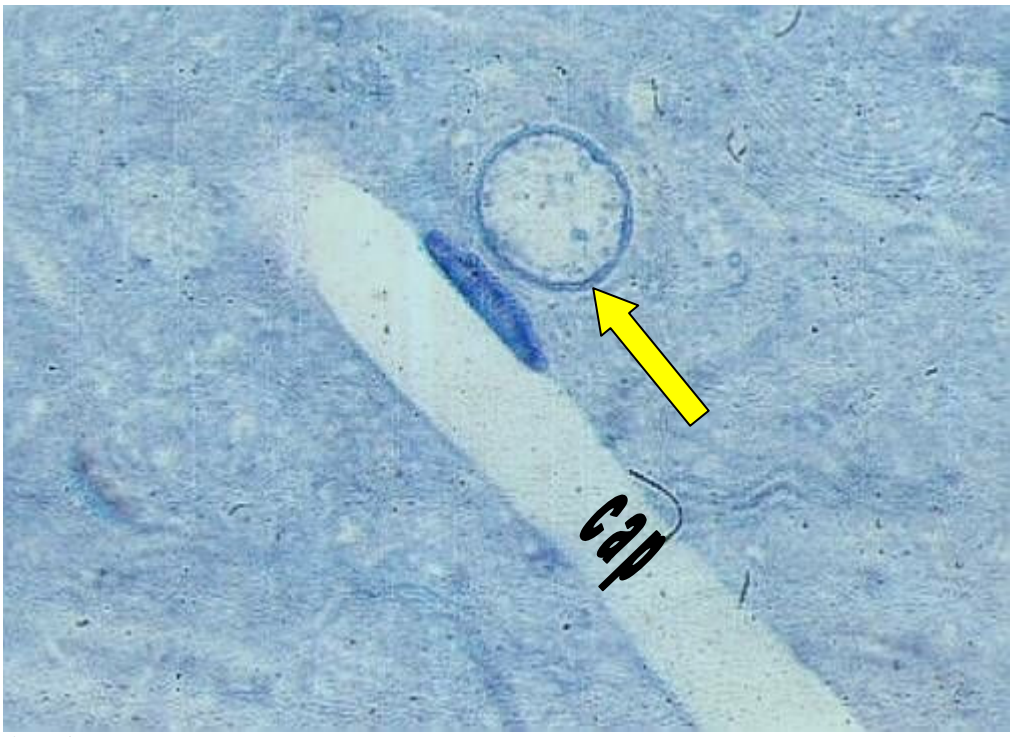


Fig. 16

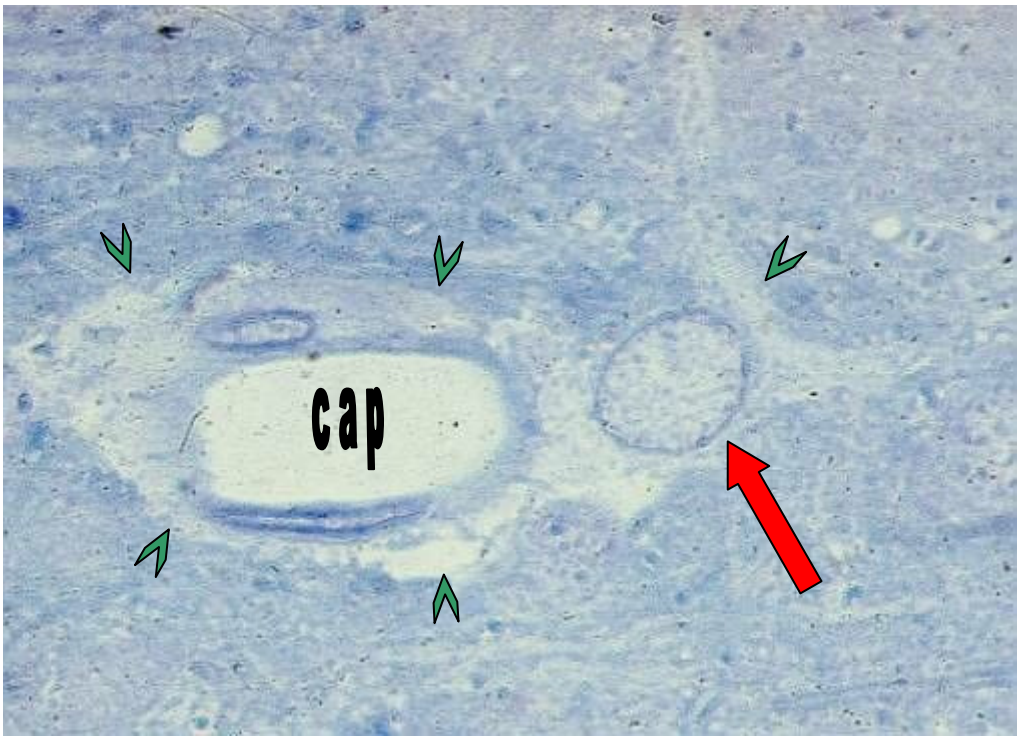


Fig. 17

Figs. 16, 17

Astrocyte in the visual cortex of the control side (Fig. 16) close to a blood vessel (cap). The cell is recognized on the basis of its typical nucleus (yellow arrow). Cytoplasm cannot be seen. On the operated side (Fig. 17) astrocytes are seen with pale nuclei (red arrow) and hypertrophic, empty-looking branched cytoplasm (arowheads) engulfing a capillary (cap). x250

When counting astrocyte numbers in the three equidistant cortical layers of the operated side, the outer layers contained 47,00 % of the astrocyte cell bodies, whereas 32,24 % and 19,70 % were present in the middle and internal layers, respectively. These figures, if compared to the values from the control side (Table 2) indicate a remarkable shift of astrocyte cell bodies towards the external cortical layers without any increase in their number within the cortical area where RAR occurred.

equidistant cortical zones	Intact (contralateral) side	Lesioned (ipsilateral) side
"external"	29.38 %	47.00%
"middle"	38.63 %	32.24%
"internal"	31.81%	19.70%

Table 2
Comparison of distribution of astrocytes in intact and RAR-activated visual cortices 3 days after CGL-lesion.

Conclusions of 6.3. Findings suggest that it is an astroglial hypertrophy rather than proliferation that may be the structural background of remote astroglial response (RAR). It is also apparent that RAR is accompanied by a limited migration of astrocytes towards the external layers.

6.4. RAR and synaptic degeneration

Wallerian (anterograde) degeneration that follows after an injury or loss of the neuronal cell body, spreads centrifugally along the axonal arbor. The terminal portions of axons end in highly specialized areas, the synapses, forming junction with another neuron and serve as the sites of impulse transmission. The final act of the anterograde degeneration is the degeneration of the synapses. This functionally most sensitive and important structure is believed to have a special microenvironment. The loss of a synapse is assumed to induce a complex chain of events to which the remote astroglial response (RAR) may also belong. From the foregoing it appears that astrocytes of the deafferented (CGL-lesioned) visual cortex migrate towards the external zone of the cortex which comprises layers I-IV. Peters *et al.* (1976) have shown that geniculo-cortical fibers

terminate in layers III-IV of the visual cortex. Thus it would appear that migration of astrocytes towards these layers pinpoints the trigger event of their hypertrophy: the degeneration of geniculo-cortical synapses. However, the slight degree of astrocyte migration cannot account for the massive increase of GFAP-immunoreactivity in the target area therefore, we supposed a substantial hypertrophy of local astrocyte processes as a major cause of the immunohistochemically observed alterations.

To test this assumption, we carried out an electron microscopic study of the visual cortex after the stereotaxic lesion of the lateral geniculate body.

Three days after operation swollen-like astrocyte processes were seen in the visual cortical layers III and IV to engulf synapses which were in the "dark" state of degeneration (Fig. 18).

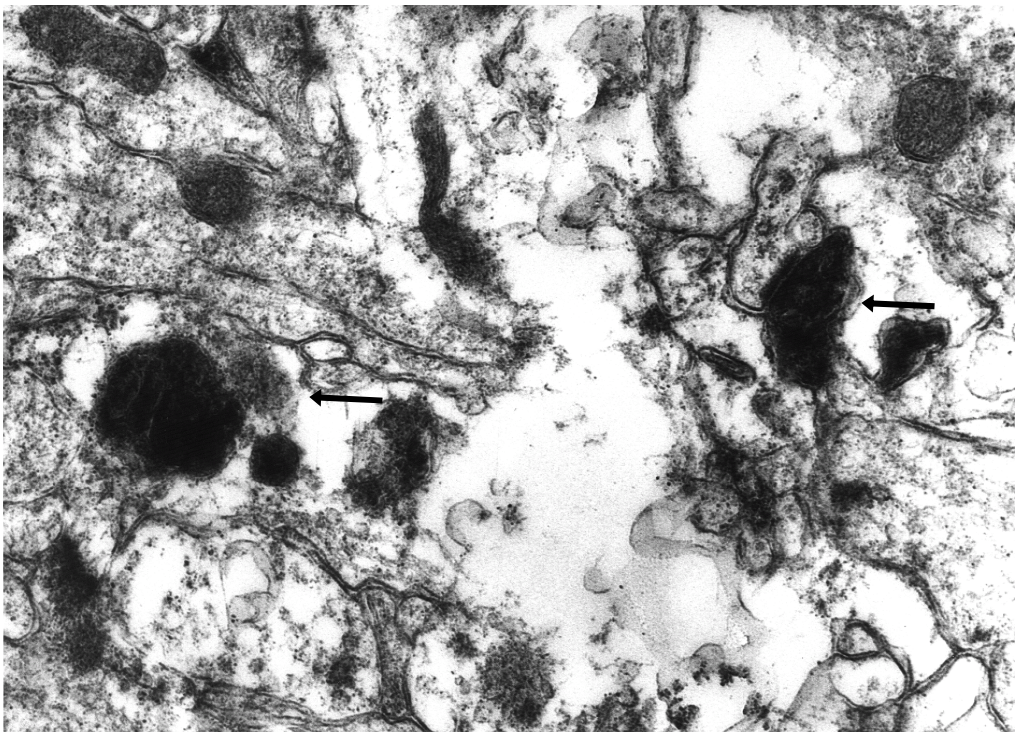


Fig. 18
From three days after CGL lesion, in the ipsilateral visual cortex axon terminals were seen in the “dark” stage of degeneration. In several cases the postsynaptic dendritic portion could still be recognized on the basis of the postsynaptic density (arrow). x18.000

At this time, synaptic cleft and postsynaptic elements such as the postsynaptic membrane and postsynaptic density could still often be recognized (Fig. 19). From postoperative day 5 onwards these became sporadic and mostly amorphous dark particles remained from the presynaptic axon terminal surrounded by large astroglial spaces. The

full detachment of the degenerating synapses from their postsynaptic sites was verified in serial sections.

Hypertrophic astrocyte processes were filled with bundles of glial intermediate filaments but the portions of processes that surrounded degenerating terminals were devoid of these filaments (Fig. 19). Perisynaptic astroglia looked swollen and contained glycogen granules as signs of local activation. By postoperative days 7-11, the appearance of degenerated synapses was similar to a cytolysome particle, and this, together with the surrounding hypertrophic astroglia gave the impression of an advanced stage of phagocytosis.

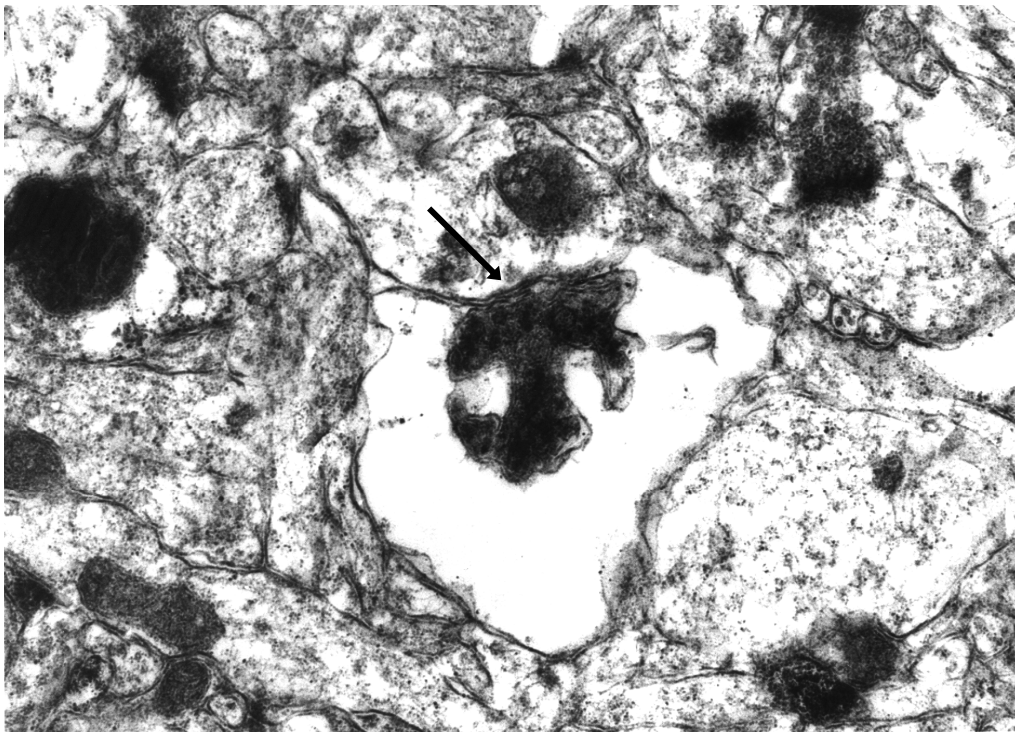


Fig. 19
In a more advanced stage of axonal degeneration the dark and shrunken axonal profile is detached from its postsynaptic element and is engulfed by hypertrophic glial processes. Note the absence of intermediate filaments in perisynaptic glia. x18.000

Conclusions of 6.4. As revealed by electron microscopy, the time and place of synaptic degeneration corresponded to the focus of astroglial hypertrophy. It is most likely therefore, that synaptic degeneration is the trigger event in the induction of remote astroglial response (RAR). Remarkable is the phagocyte-like behavior of astrocytes in RAR, a feature that has been argued in other situations.

6.5. The immunohistochemical monitoring of RAR

Based partly on data of the literature partly on own experiences, tubulin, microtubule-associated protein 2 (MAP2), dystrophin, and glial fibrillary acidic protein (GFAP) were selected as possible markers to monitor the appearance and time course of remote astroglial response (RAR).

To check specificity sections were incubated with preabsorbed antisera or with the peroxidase conjugate only. In these situations no staining occurred.

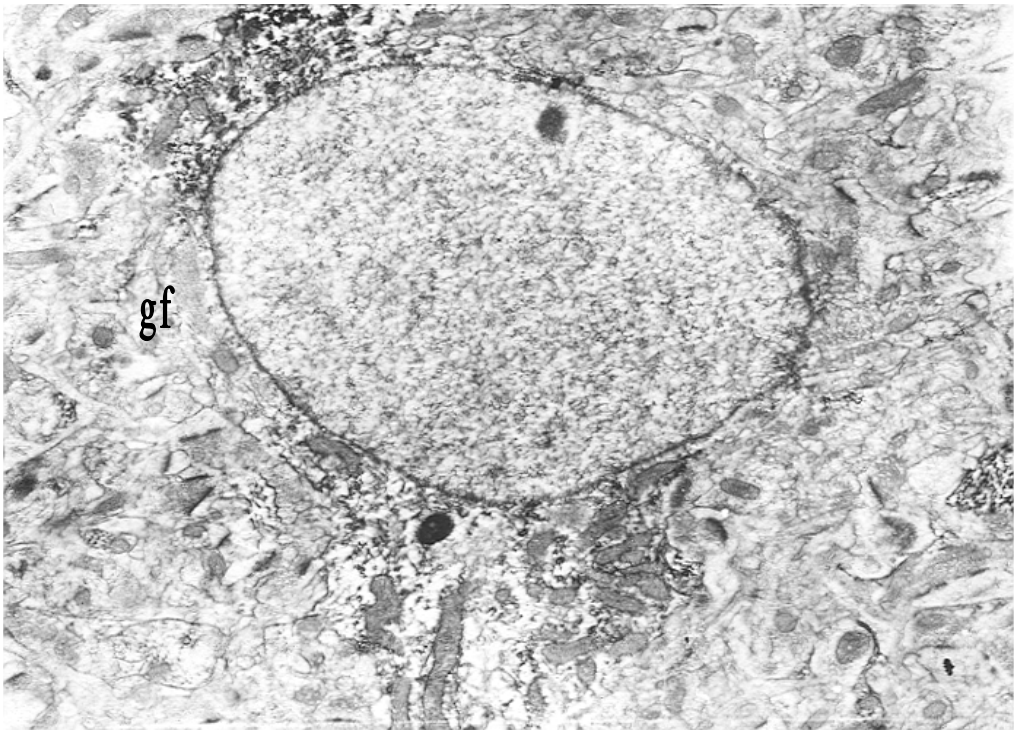


Fig. 20
Electron microscopic immunohistochemistry of tubulin shows a ribosomal localization of the immunoprecipitate in the visual cortex. Observe that the glial filament-bundles (gf) remain immunonegative. x6.000

6.5.1. Tubulin

Of tubulin isoforms the beta-III-tubulin was studied since it has been established that this beta isoform is of exclusively neuronal localization (Draberova et al., 1998). It is also known (Hajós and Gallatz, 1986) that antibodies against alpha-tubulin decorate astroglial cells. From our viewpoint, however, it was necessary to know whether alpha-tubulin-immunoreactivity is localized to cytoskeletal or other elements, the more so since

microtubules in astrocytes are scarce and seem not to account for the immunostaining of astrocytes.

Electron microscopic immunohistochemistry of tubulin in astrocytes at several sites including the visual cortex has clearly shown that reaction product binds to the granular endoplasmic reticulum and free cytoplasmic ribosomes (Fig. 20). It was also observed that glial intermediate filament bundles were spared by the immunoprecipitate due to beta-tubulin immunoreactivity.

6.5.2. MAP2 and Tau

Microtubule-associated protein 2 (MAP2) has been described as a dendritic, Tau as an axonal marker, hence both are believed to decorate the cytoskeleton of neurons (Jancsik *et al.*, 1996). Nevertheless, a few microtubules are present also in glial processes therefore we looked at the localization of these proteins also in astrocytes.

Immunostaining with antibodies raised against MAP2 and Tau proved to be exclusively neuronal. No reaction whatsoever was found in astrocytes.

6.5.3. Dystrophin

Although this protein has been originally described in skeletal muscle, recent studies have demonstrated its presence in various central nervous system structures such as certain types of synapses (postsynaptic densities), pericapillary astrocyte processes and also in some astrocyte cell bodies (Lidov *et al.*, 1990; Imamura and Ozawa, 1998). Thus it seemed worthwhile to study the localization of dystrophin at the electron microscopic level.

Electron microscopy of dystrophin immunoreactivity revealed that in the cell bodies and large processes of cortical astrocytes, immunoprecipitate due to dystrophin immunoreactivity labeled ribosomes, either membrane-associated or free, whereas bundles of glial intermediate filaments remained unstained (Fig. 21). Moreover, the astrocytic endfeet around cortical capillaries were consistently immunostained (Fig. 22).

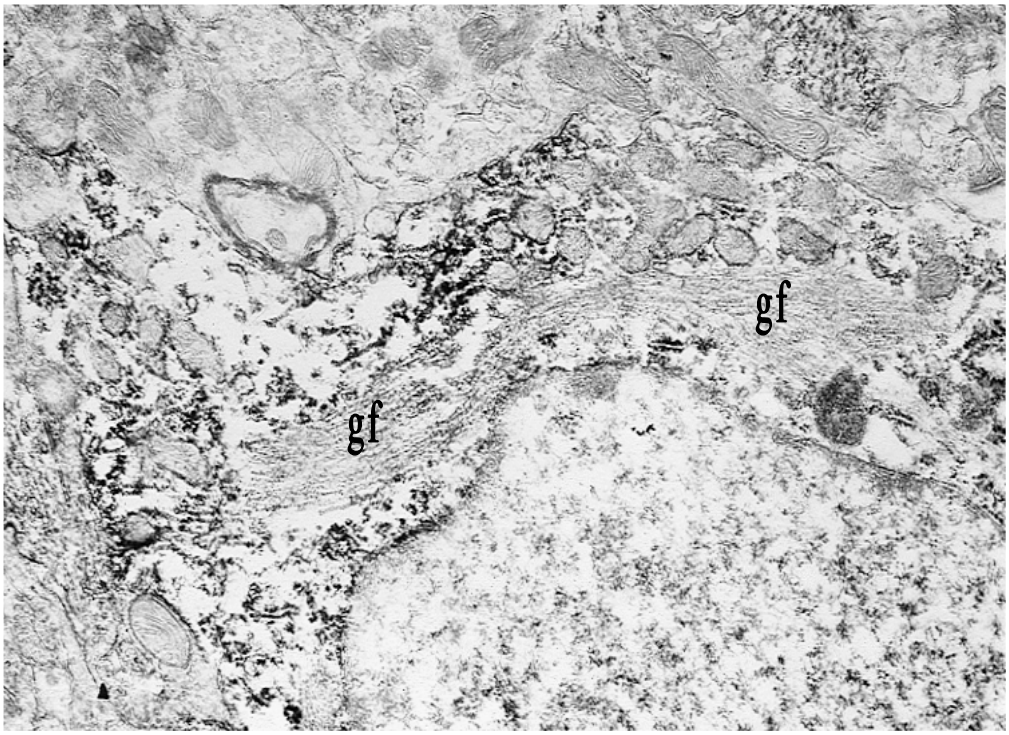


Fig. 21
Dystrophin immunolabelling consistently spears glial filament-bundles (gf) in the visual cortex, while it decorates ribosomes both free and membrane-bound. x12.000

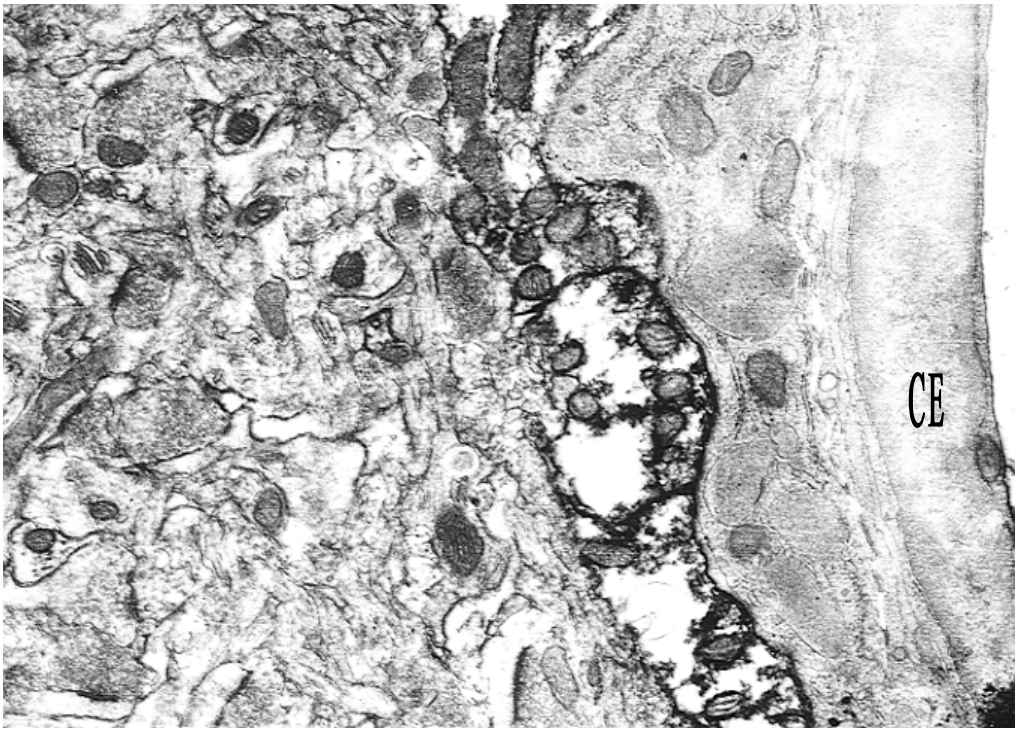


Fig. 22
Pericapillary astrocyte processes in the visual cortex stained by dystrophin-immunoreactivity. Reaction product is accumulated at the membranes and around mitochondria. CE = capillary endothelium. x12.000

6.5.4. GFAP

The classical astroglial marker glial fibrillary acidic protein (GFAP) was also investigated, not as if there existed any doubt concerning its astroglial localization but owing to the circumstance that immunostaining with antibodies, either polyclonal or monoclonal against GFAP give a full staining of the astrocyte. This is conflicting with the fact that glial intermediate filaments are found in circumscribed areas of the astrocyte cytoplasm. Furthermore both fibrous and protoplasmic astrocytes show an equally intense GFAP-immunostaining which raises the question of a cytosolic compartment of the protein (Patel *et al.*, 1985).

To this end, based on the observations of Hajós and Halasy (1998a) on the diffusibility of the DAB immunoprecipitate, we carried out the GFAP immunoreaction with two different methods: the pre-embedding immunostaining using DAB as chromogen, and the post-embedding immunostaining using immunogold for the visualization of the immune complex.

Our findings fully corroborated earlier observations. Using the pre-embedding method, immunoprecipitate in the astrocyte cell bodies and major processes was found to decorate glial intermediate filaments but heavy labeling was found around the filament bundles, occasionally filling entirely the astrocyte element (Fig. 23). On the other hand, post-embedding immunostaining with immunogold particles was confined to glial filament bundles either in astrocyte cell bodies and processes (Figs. 24, 25) or in perivascular astroglial endfeet. Perisynaptic glia lacking intermediate filaments was negative with both methods. From this we concluded that the overall staining of astrocytes after conventional pre-embedding immunohistochemistry, in addition to specific staining of glial cytoskeletal filaments causes a considerable diffusion of the immunoprecipitate.

Conclusions to 6.5. Among the possible candidates of immunohistochemical monitoring of the cytoskeletal reaction of astrocytes, glial fibrillary acidic protein (GFAP) may be the most suitable as being exclusively localized to the astroglial cytoskeleton.

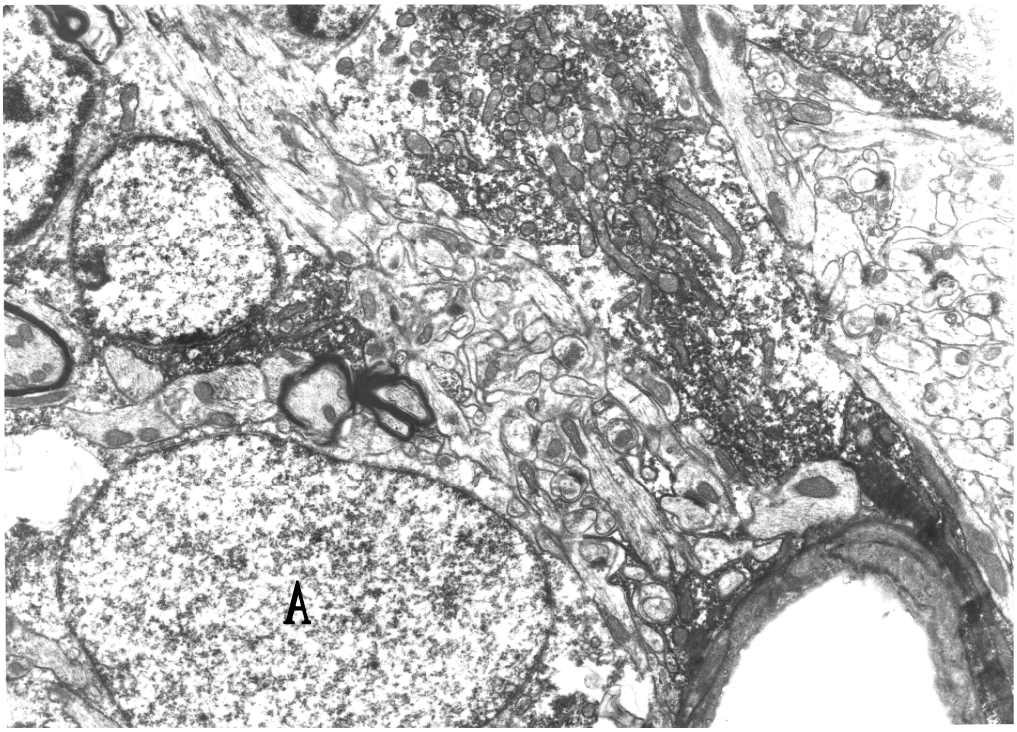


Fig. 23
x6.000

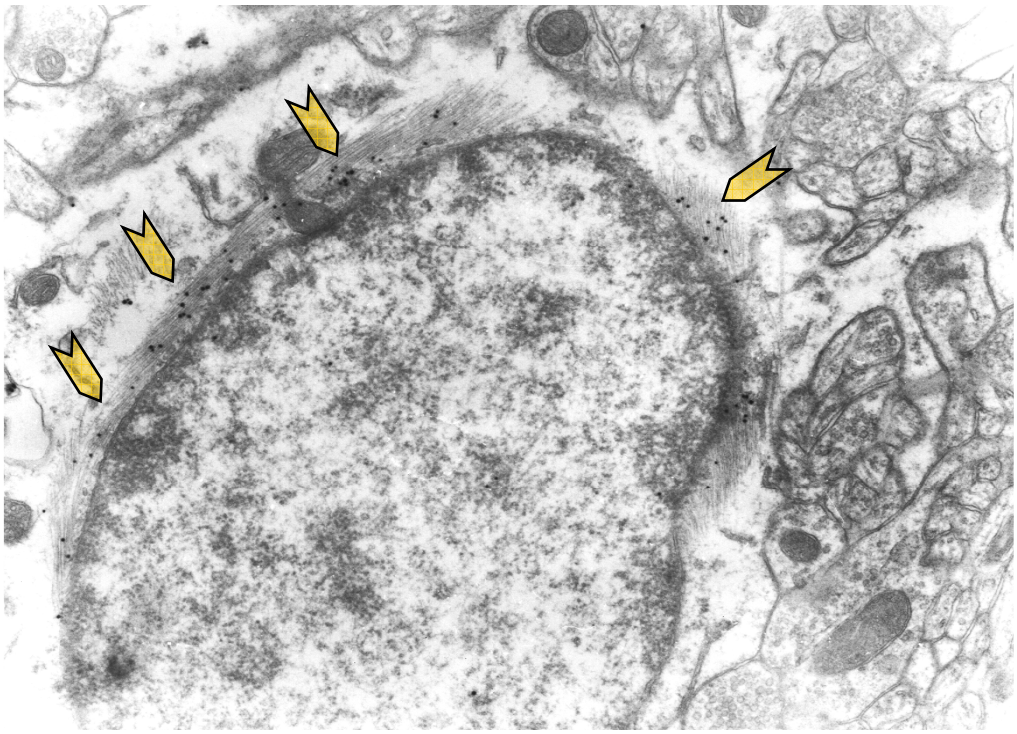


Fig. 24
x24.000

explanation: see overleaf

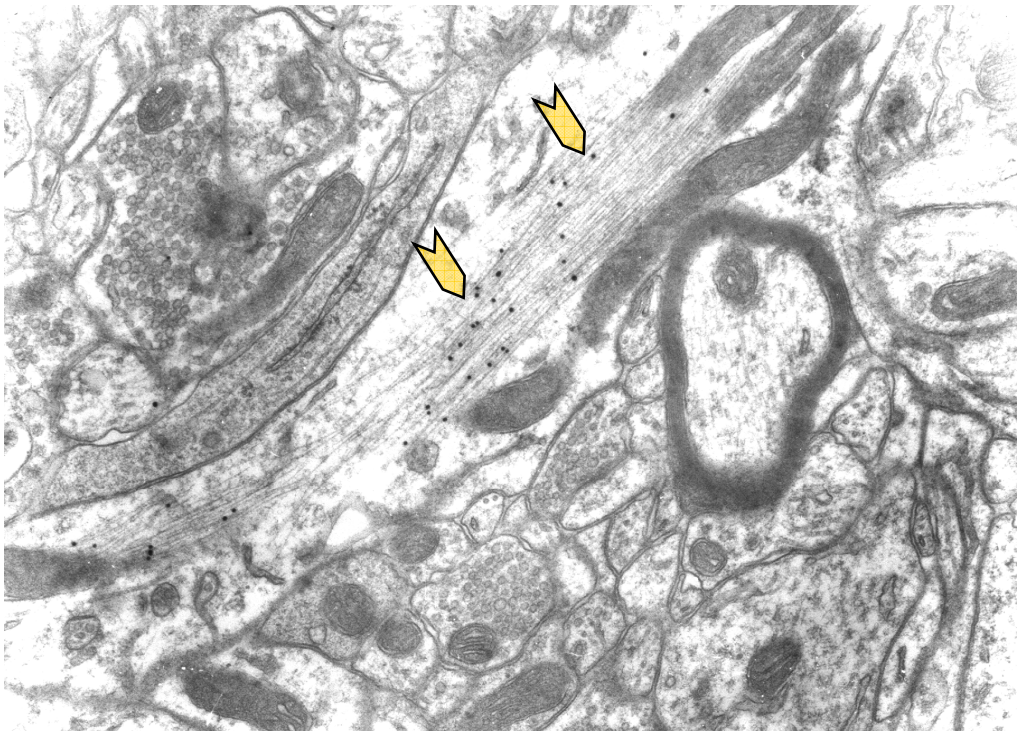


Fig. 25
x24.000

Figs. 23, 24, 25
The comparison of the localization of GFAP-immunoprecipitate as seen with the pre-embedding (Fig.23, cerebellum) and the post-embedding (Figs. 24, 25, hippocampus) procedures. With pre-embedding immunostaining precipitate fills diffusely astrocyte cell bodies (A) and major processes. The post-embedding method labels exclusively glial intermediate filaments (arrowheads).

6.6. GFAP-immunoreactivity as an indicator of RAR

As shown in 6.4., the trigger event in remote astroglial response (RAR) is the synaptic degeneration in the projection territory of affected nerve cell bodies. It was also demonstrated that in the experimental paradigm of anterograde (Wallerian) degeneration of the lesioned geniculo-cortical system, the time course of appearance and decline of glial fibrillary acidic protein- (GFAP-) immunoreaction coincided with the onset, progress and completion of synaptic degeneration, whereas by the structural reorganization and stabilization of the post-degeneration area GFAP-immunoreactivity returned to the pre-lesional level.

This would imply that the histochemical monitoring of GFAP-immunoreactivity, particularly when evaluated with computer-assisted image-analysis, is a reliable indicator

of RAR. Nevertheless, the lack of glial intermediate filaments in the hypertrophic perisynaptic glia remained still a question to be clarified.

To this end we carried out the GFAP-immunoreaction also at the electron microscopic level. It is well-established that the GFA protein which is a major constituent of the glial intermediate filaments may be present in the cytoplasm also in a soluble form (Patel *et al.*, 1985). We hoped therefore, that electron microscopy will reveal increased immunoreactivity also in the perisynaptic astrocyte processes where glial intermediate filaments were not encountered reflecting an increased immunoreactivity of the soluble GFAP-fraction. This, however, was not the case. While large- and medium-sized astrocyte processes were filled out with highly electron-dense immunoprecipitate, small astroglial elements including perisynaptic processes, were consistently negative. An even more restricted localization was obtained if the immunolabelling was carried out with immunogold particles (Fig. 26). This was in full accordance with the findings of Hajós and Halasy (1998a) suggesting that DAB precipitate may diffuse within the glial cells, and as indicated by the immunogold-method, the true source of GFAP-immunostaining are glial intermediate filaments.

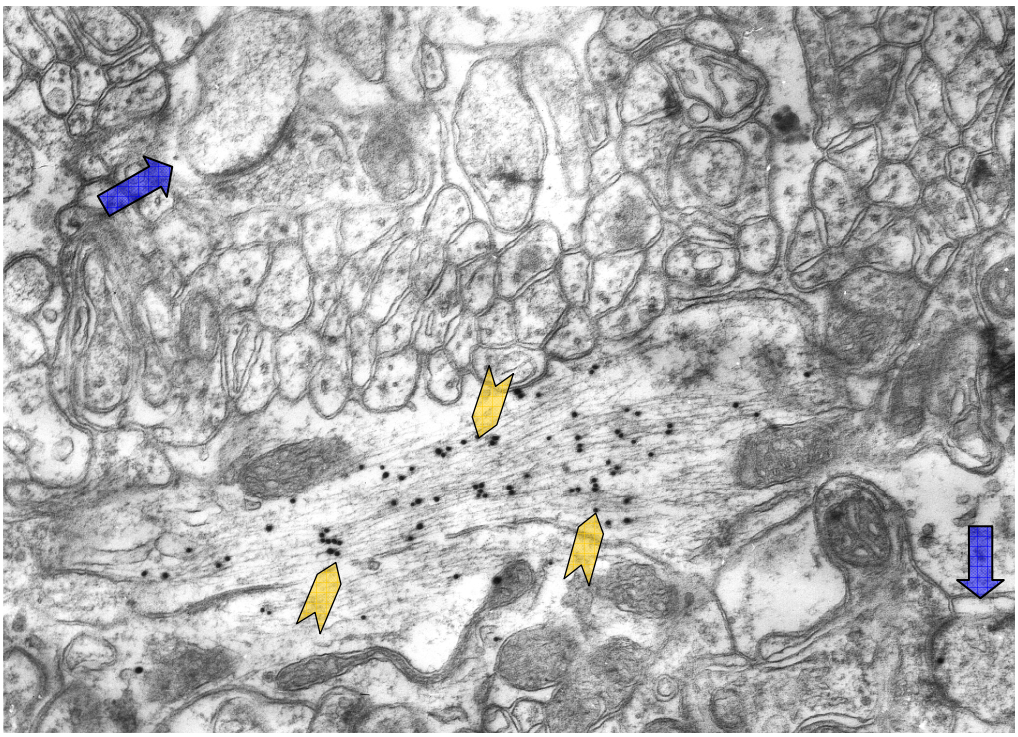


Fig. 26
Immunogold particles due to GFAP-immunoreactivity are seen to decorate glial filaments (arrowheads) in the hippocampus but no immunolabelling can be seen in the perisynaptic glia of a nearby synapse (arrows). x24.000

Conclusions to 6.6.. The glial fibrillary acidic protein- (GFAP-) immunoreaction is a reliable marker of remote astroglial response (RAR) but only at the level of astrocyte cell bodies, and large and medium astrocyte processes. The smallest astrocyte processes that approach synapses, react to synaptic degeneration with a volume-increase and glycogen deposition. These terminal processes do not contain glial filaments, not even under the conditions of RAR, therefore, we may regard the GFAP-immunoreaction as a purely cytoskeletal phenomenon and consequently its increase in RAR as a reflection of the hypertrophy of the cytoskeleton in response to the activation of astroglia.

6.7. GFAP-immunoreactivity and the net amount of GFAP

Since the increase in glial fibrillary acidic protein- (GFAP-) immunoreactivity is not necessarily the result of a net increase in the synthesis of the protein – it may result also from a conformational change within the GFAP molecule leading to the formation of new immunoreactive sites – we were interested to see whether remote astroglial response (RAR) is coupled to increased amounts of the GFA protein?

Comparison of relative GFAP-contents of the operated and intact visual cortices was carried out in lateral geniculate body-lesioned rats 7, 14, and 35 days after operation. Survival periods were chosen on the basis of the immunohistochemically observed time-course of RAR in this system. Values for each survival group are shown in Fig. 27 where the GFAP-content of the control was taken as 100 %.

Findings demonstrated that RAR manifested itself in a selective rise of GFAP-content and, in harmony with earlier immunohistochemical observations (Hajós and Csillag, 1995), the peak difference between lesioned and intact sides occurred on the second week after operation. It was also in good accordance with earlier immunohistochemical findings that after this period values rapidly declined but did not return to normal (Hajós and Csillag, 1995). The decline was parallel with the gradual cessation of astrocytic reaction accompanying the postlesional restructuration of the visual cortex. The phenomenon that values do not return to the prelesional values but remain slightly above normal is most likely due to perivascular reactive foci persisting even at 5 months after lateral geniculate body lesions (see Hajós and Csillag, 1995).

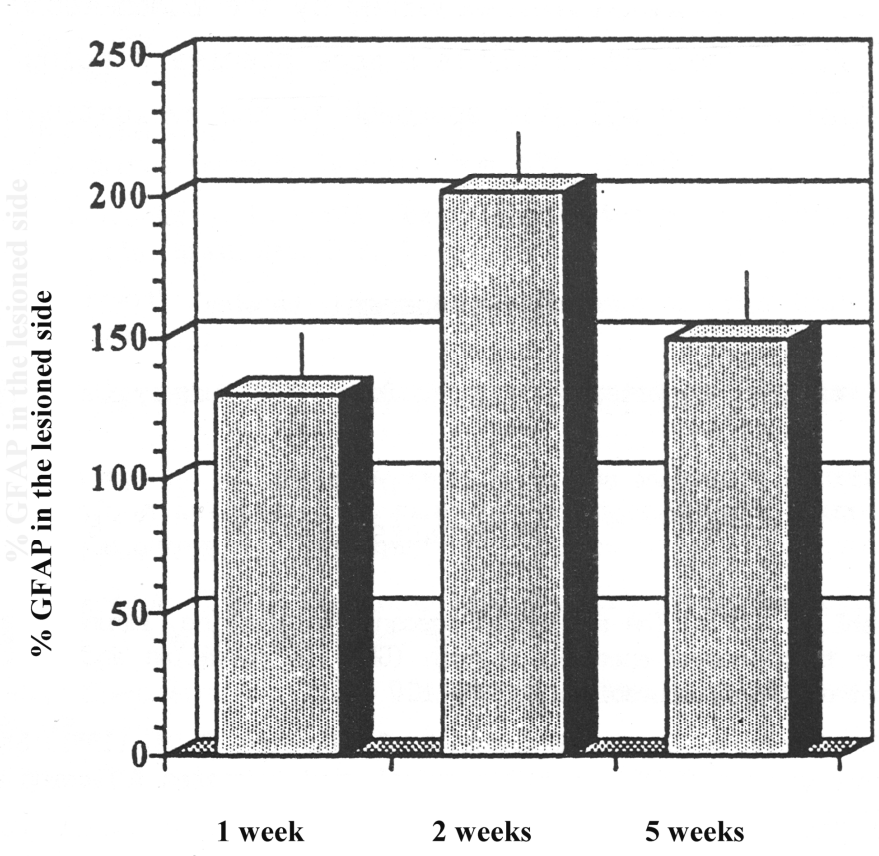


Fig. 27
Time course of RAR-induced GFAP level enhancement in the visual cortex ipsilateral to the CGL lesion. GFAP content of the intact sides were considered as 100%.

Conclusions to 6.7. There was a clear selective increase in the net amount of glial fibrillary acidic protein (GFAP) on the operated side as compared to the control. This indicates that within the general cellular response of the affected area, the increase in GFAP-synthesis is a leading phenomenon which accounts for the appearance in remote astroglial response (RAR) of GFAP-immunoreactivity at sites where it cannot be demonstrated in the intact cortex. It can be also concluded that in astrocytes negative for immunoreactive GFAP the rate of synthesis and the net amount of this protein is below the sensitivity threshold of immunohistochemistry.

6.8. Sexual dimorphism of GFAP-immunoreactivity

Glial fibrillary acidic protein (GFAP) –maps were prepared predominantly based on results obtained in males. Further experimental work was carried out in rats of either sex, and it was observed that in females the immunostaining was highly unpredictable. There

were some female brains in which immunostaining failed while in male brains incubated parallelly under identical conditions, with the same batches of antisera and other reactives, a marked reaction occurred. In other female brains, the GFAP-immunostaining showed similar distributions than in males, but the overall intensity of the reaction in females was mostly below that of the reaction in males. Furthermore, reaction intensities in females showed an extreme fluctuation from no staining at all to faint and moderate-intensity stainings. After several series of immunostainings we felt it suggestive that this was not a simple variability of reaction in our hands but a true sexual difference for GFAP. Indications of a sexual dimorphism of GFAP were found in the literature but mainly for the endocrine hypothalamus where such differences could be expected (Pfaff, 1979). The observation of this kind of sexual dimorphism in widespread extrahypothalamic locations was a new finding which deserved a more thorough investigation.

6.8.1. Sexual dimorphism of GFAP-immunoreactivity outside the "endocrine brain"

Since our observation concerned sexual dimorphism, an obvious assumption was that sexual hormones might be instrumental in causing the differences in GFAP-immunoreactivity between males and females. The mediobasal hypothalamus and its directly linked areas are known to contain cell groups which regulate through the synthesis of releasing or release-inhibiting hormones the peripheral endocrine system including the function of gonads. Accordingly, steroid receptors were described in these areas in both neurons and glia (Tobet and Fox, 1989; Jung-Testas *et al.*, 1991; Suárez *et al.*, 1991 and 1992; Langub and Watson, 1992). However, from our aspect not the endocrine regulation but rather a possible direct effect of sexual steroids on astroglial cytoskeleton was of interest, therefore, we carried out a series of studies on a sexually non-committed area, the midbrain interpeduncular nucleus. This nucleus has a GFAP-immunoreactivity among the highest in the brain and has – at least to our present knowledge – no direct involvement in the regulatory mechanisms of sexual hormone production.

The microscopic appearance of the interpeduncular nucleus (IPN) is shown in Fig.28. This is the largest unpaired midline nucleus in the brain situated at the depth of the interpeduncular fossa.

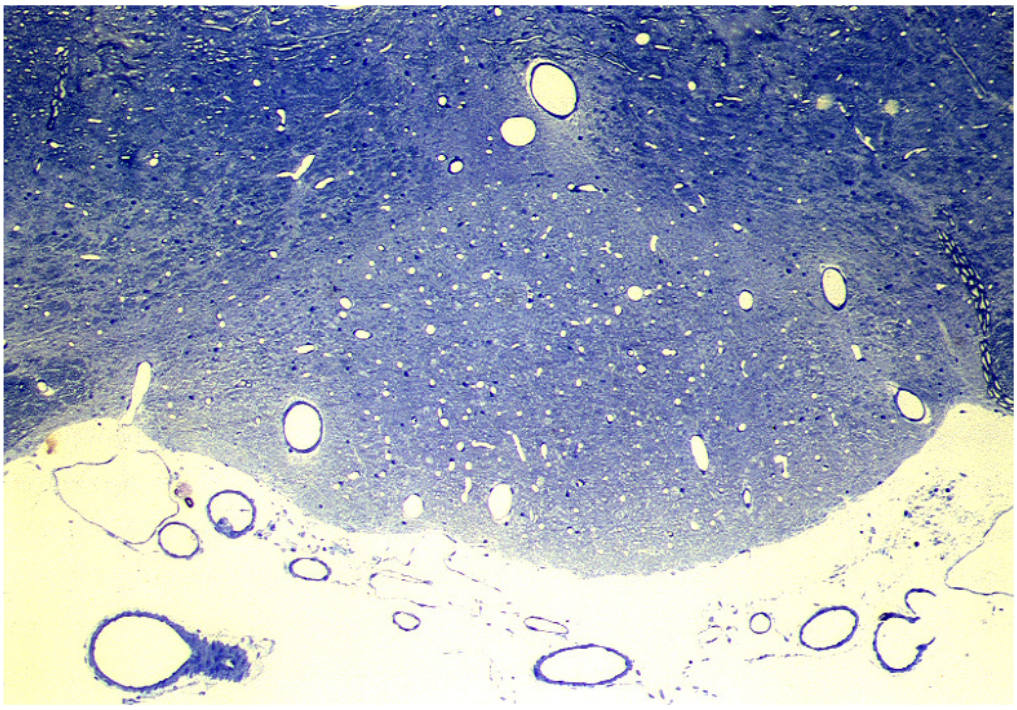


Fig. 28
A survey microphotograph stained with toluidine blue shows the extent of the interpeduncular nucleus at its mid portion. x100

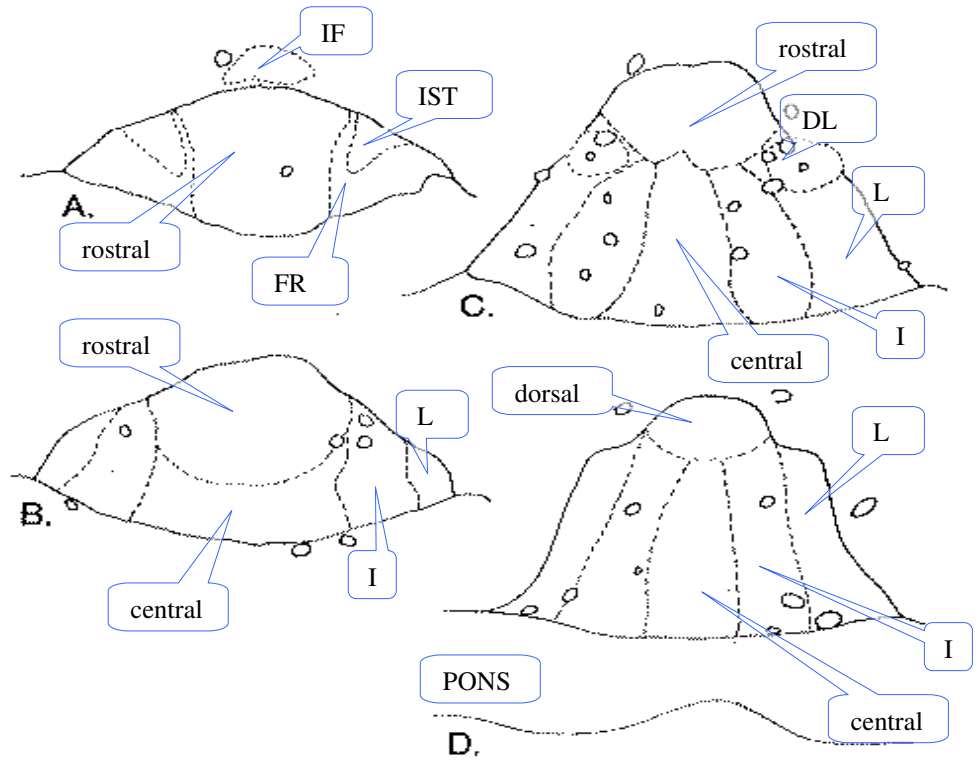


Fig. 29
The subdivisions of the interpeduncular nucleus (IPN) according to Hamill and Lenn (1984) from rostral (A) to caudal (D). Abbreviations: DL = dorsolateral subnucleus, FR = fasciculus retroflexus, I = intermediate subnucleus, IF = interfascicular nucleus, IST = interstitial subnucleus, L = lateral subnucleus

It has a rostro-caudal extent beginning caudal from the Bregma at the distance – 5.40 according to stereotaxic coordinates and extending caudally till the pons. Its division into subnuclei is not unequivocal (Hamill and Lenn, 1984; Fig. 29) but most descriptions distinguish the rostral, middle, lateral and caudal subnuclear groups. Within the interpeduncular nucleus, the distribution of GFAP-immunoreactivity although intense throughout, showed some variations. In the rostral part of the nucleus (distance from the Bregma – 5.80) immunoreactivity was evenly distributed (Fig. 30).

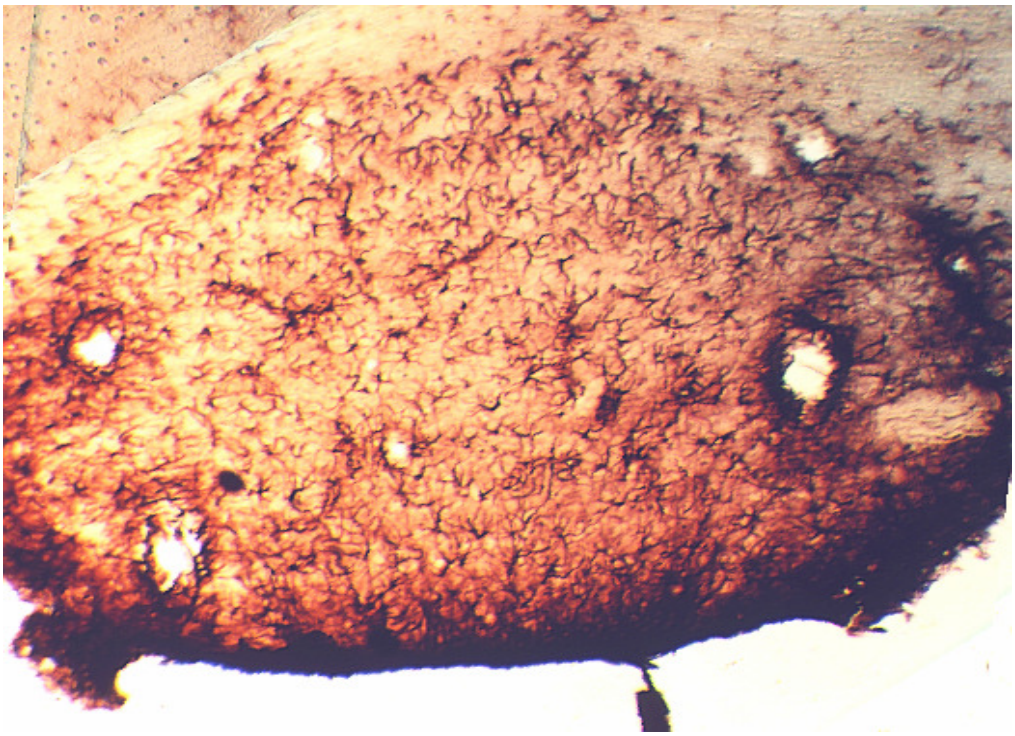


Fig. 30; x140

explanation: see overleaf

In the mid-portion of the nucleus (- 6.40), immunoreaction was even more intense at the periphery than in the core of the nucleus (Fig. 31). Viewed in the coronal plane, the more intensely reactive peripheral zone was bell-shaped and included the lateral, dorsolateral and dorsomedial subnuclei. The core corresponded to the intermediate and caudal subnuclei. The thin intensely stained line at the free ventral edge of the caudal subnucleus appeared to be artifactual at the edge of the vibratome section.

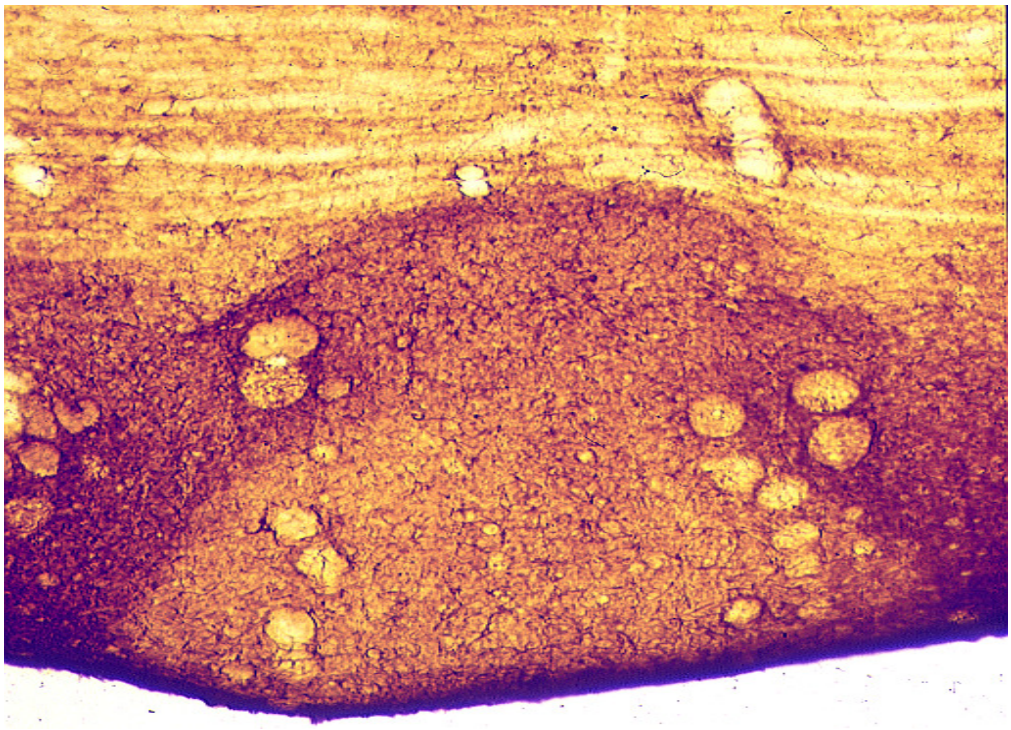


Fig. 31; x120

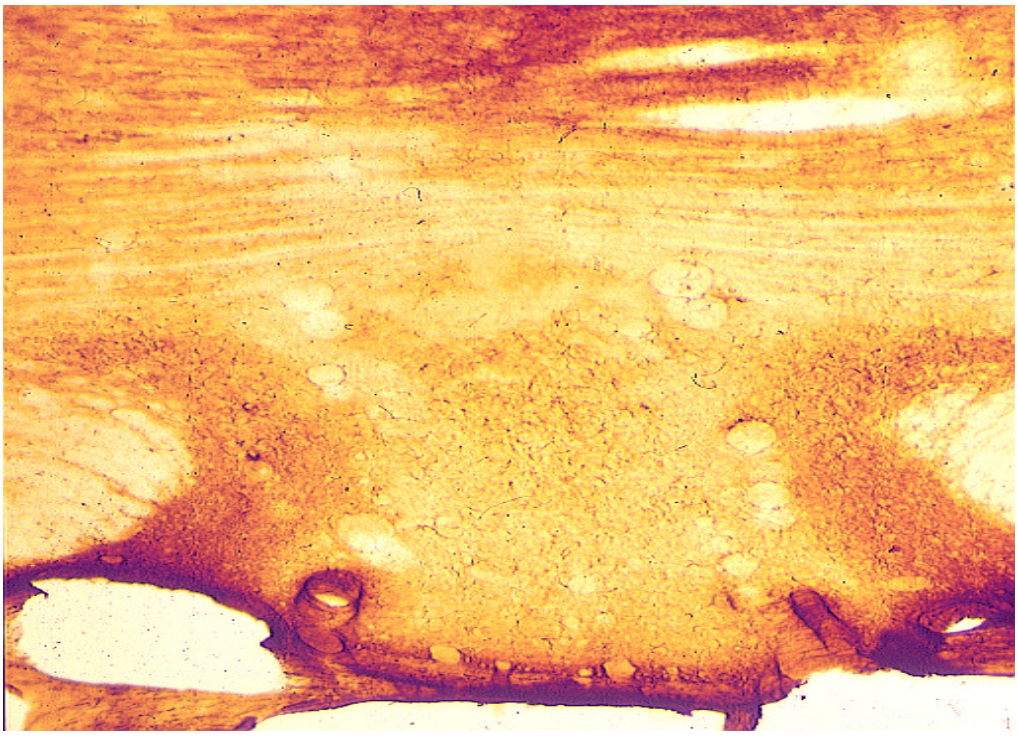


Fig. 32; x120

Figs. 30, 31, 32

The distribution of GFAP-IR at the rostral (Fig. 30), middle (Fig. 31) and caudal (Fig. 32) levels of the interpeduncular nucleus in the male rat. Rostrally the immunoreactivity is evenly distributed. In the mid-portion of the nucleus the dorsal and lateral subnuclei form a more intensely stained shell around the core of the nucleus. Caudally the lateral and dorsolateral subnuclei are very intensely stained, whereas dorsally the staining correspond to that of the core.

In sections between - 6.30 and - 6.72 (Fig. 32), the peripherally intense staining was only laterally observed, including the lateral and dorsolateral subnuclei. The dorsomedial subnucleus was less heavily labeled, as was the core area. The immunostaining of pericapillary astrocyte processes was marked throughout the entire interpeduncular nucleus.

In female rats a considerably lower intensity of immunostaining was observed as compared to similar-level sections from males. This applied to both core and periphery of the IPN. Unlike in males, however, in the females the intensity of GFAP-immunoreactivity exhibited wide individual variations. It is, however, necessary to note that the intensity-range of staining in females was below the main intensity observed in males. In terms of areal fraction values this meant 48 ± 36.07 (n=18) and 56 ± 9.63 (n=18), respectively.

The very high scatter of values in females, which reflects similarly extreme fluctuations of the visible GFAP staining intensities, suggests that these might be sexual cycle-related. This raised the necessity to investigate the reaction of astroglial cytoskeleton in various stages of the estrous cycle of females and the effects of gonadectomy in males and females on both RAR of the geniculo-cortical system and on the interpeduncular nucleus.

Conclusions of 6.8. Results indicate that there exists a sexual dimorphism of glial fibrillary acidic protein- (GFAP-) immunoreactivity. The general feature observed for this dimorphism was that throughout the entire brain the GFAP-immunoreaction was more intense in males than in females, whereas in females a wide-range fluctuation of the reaction occurred also in a non-endocrine brain region, the interpeduncular nucleus.

6.9. The astroglial cytoskeleton in different sex-hormonal states in the IPN

In males normally gonadal function and related hormonal states are stable, at least as far as pre- and postpubertal periods are concerned. (Circadian rhythms are more subtle variations than the ones we are currently dealing with.) In adult rats, therefore, castration that brings about a drastic fall in the levels of sex-steroids, can be expected to produce an altered hormonal environment also for astrocytes.

In females there is a natural fluctuation of sex-steroid levels during the estrous cycle. On the basis of previous experience we had ample reason to suppose that these natural fluctuations affect the astroglial cytoskeleton. Before we could proceed with the studies of effects of hormonal states on the astroglial reaction we had to learn about the behavior of astroglial cytoskeleton under natural fluctuations of sex-steroid levels that occur in the female.

6.9.1.Females

6.9.1.1. Ovarian cycle-related changes of GFAP in the IPN

Our findings demonstrated that the expression of glial fibrillary acidic protein – (GFAP-) immunoreactivity varied during the estrous cycle in a 'non-endocrine' brain region, i.e. the interpeduncular nucleus (IPN). Variations showed a trend similar to that observed in the rodent hypothalamus by Garcia-Segura *et al*. (1994a) and by Kohama *et al*. (1995) for GFAP and GFAP mRNA, respectively. This included a gradual increase till proestrus with a peak at late proestrus, than a significant fall in estrus.

In the females studied with routine histology, no changes were revealed in the number and distribution of astrocytes within the interpeduncular nucleus during the estrous cycle. In semithin sections astrocytes were particularly well recognizable on the basis of their nuclear structure (Privat and Rataboul, 1986, and Fig. 7). Also at this finer light microscopic level, astrocyte numbers and distributions proved to be constant during the estrous cycle.

In sections immunostained with antibodies against GFAP in different estrous cycle phases, minor differences were observed in the staining of the interpeduncular nucleus during metestrus and early proestrus but these differences were not significant. Since this was in full accordance with the results of Kohama *et al*. (1995) in the hypothalamus, we regarded the metestrus and early proestrus high-GFAP states an entity termed as metestrus-reaction after the longest estrous cycle phase. Any further distinction on the basis of immunostaining would have been unrealistic. In sharp contrast to the metestrus-reaction, in estrus the intensity of GFAP-immunostaining markedly declined.

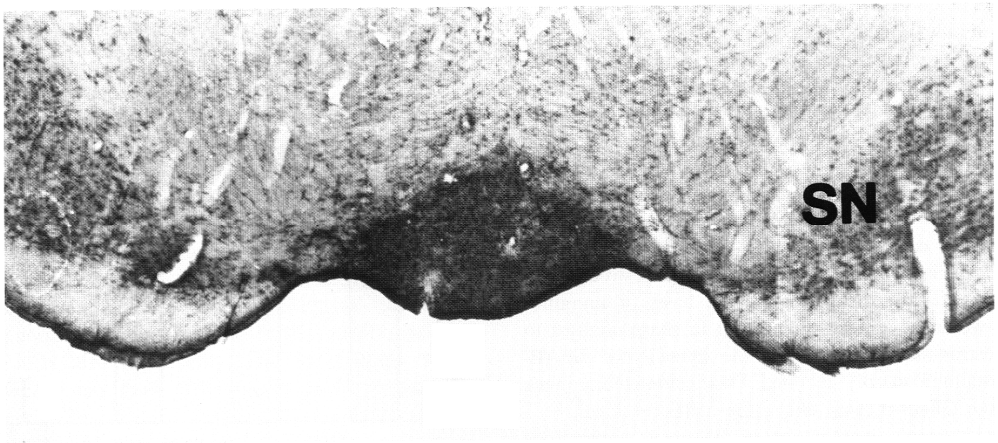


Fig. 33

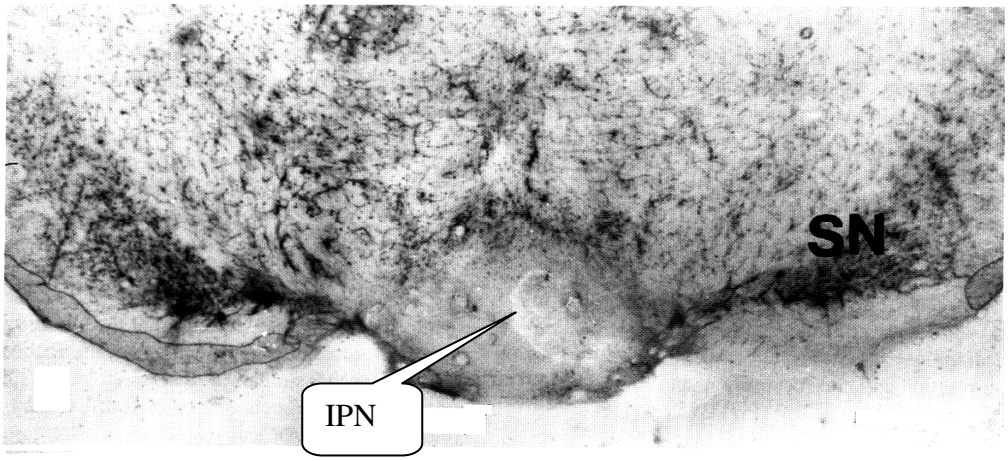


Fig. 34

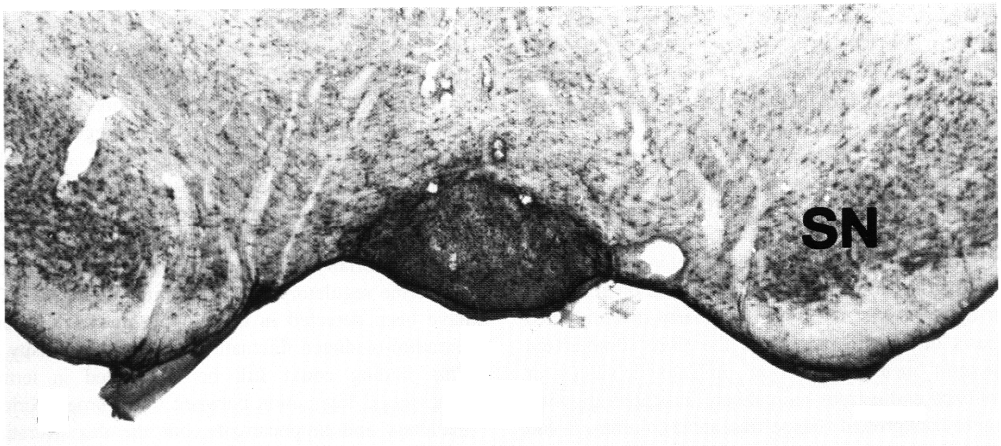


Fig. 35

Figs. 33-35

Coronal sections of the midbrain at 6.2 mm caudal to the Bregma. The interpeduncular nucleus (IPN) is seen in its largest cross-section. The substantia nigra (SN), what we used as a reference area, is also well distinguishable. In metestrus (Fig. 33), the IPN shows an intense GFAP-IR particularly pronounced in the mantle region of the nucleus. In estrus (Fig. 34), the nucleus is almost devoid of GFAP-IR, whereas in the neighboring regions a moderate immunostaining is present. After ovariectomy (Fig. 35), a metestrus-like intense reaction is visible. x60

The microscopic examination of metestrus preparations showed an even distribution of intensely GFAP-immunopositive astrocytes in the core region of the interpeduncular nucleus which was distinguishable in coronal sections from -6.04 an -6.30 (Fig. 33). Around the core, an even more intensely immunostained mantle was found consisting of astrocytes and astrocyte perivascular endfeet surrounding the arrays of capillaries found in this region.

In corresponding coronal sections cut from animals in estrus the GFAP reaction was conspicuously reduced in both core and mantle regions of the interpeduncular nucleus (Fig.34). It is noteworthy that pericapillary glia remained unaffected throughout the above cyclic changes of astrocytes in the neuropil of the interpeduncular nucleus. At the level of single astrocytes, immunoprecipitate due to GFAP decorated the cells and processes together with their elaborate ramifications in metestrus, whereas immunoprecipitate became fragmentary in estrus so that no cell outlines could be perceived (see Fig. 42).

Ovariectomy carried out 4 weeks before examination (n=6) produced a marked elevation of GFAP-immunoreactivity within the interpeduncular nucleus (Fig. 35). This applied not only for the overall intensity of immunostaining but also for a more extensive staining of astrocyte processes. One, ovariectomized animal was let to survive for 3 months but there was no difference in the intensity and extent of the GFAP-immunoreaction as compared to the 4-week survival.

Testosterone, administered to ovariectomized animals suppressed the gonadectomy-induced increase of GFAP-immunoreactivity in the IPN.

The observed alterations could be substantiated by computer assisted image analysis. For this purpose ovariectomized animals were used for reference in comparison to estrous animals because, as mentioned above, the other cycle-phases could not be clearly discriminated on the basis of GFAP-immunostaining and were therefore summarized under the term 'metestrus-reaction'. In Fig. 36 values for ovariectomized and estrous animals are indicated allowing for the optic background (tissue-free area; columns A and B) and for the tissue background (adjacent immunonegative tissue-area; columns C and D). Using either type of correction, the difference between estrous and ovariectomized interpeduncular nuclei is significant. The high scatter values after allowing for tissue background was due to the approximate timing of estrus on the basis of vaginal smears. The scatter of column D truly reflects that in some estrus-group animals the immunolabelling of the interpeduncular nucleus was lower than in the reference area (Fig.36).

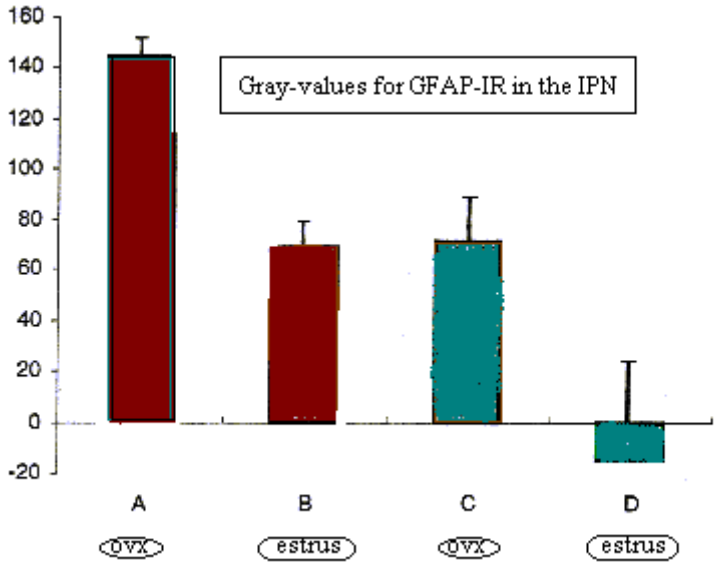


Fig. 36
Along axis Y, densitometric values of GFAP-IR in the interpeduncular nucleus (IPN) are indicated allowing for the optic background (A and B) and for the tissue background (C and D) for ovariectomized and estrous animals, respectively. In both comparisons a substantial decrease can be seen during estrus.

6.9.2. Males

In males, castration was thought to be the most suitable means to produce a drastic alteration in sex-hormonal state by suspending the production of gonadal steroids.

6.9.2.1. Effect of castration on GFAP in the IPN

Castrated animals (n=12) were allowed to survive for 2, 4, and 8 weeks, then the interpeduncular nucleus was immunostained for GFAP.

Findings have shown that GFAP-immunoreactivity was significantly reduced upon castration – irrespective the age at castration. Reduction was detectable after 2 weeks and became most pronounced after 4 weeks.

To assess reliably the castration-induced changes, great care was taken to compare identical pairs of sections from the control and operated animals. The vascular pattern and the transverse pontine fibers were used as identifying landmarks (see Figs. 37-40).

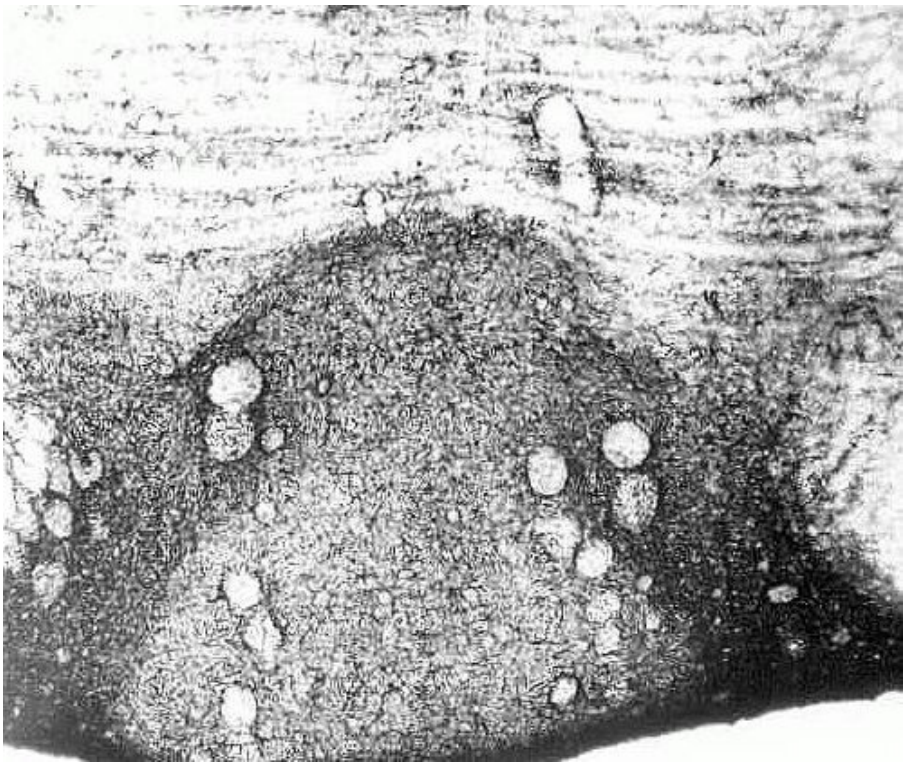


Fig. 37

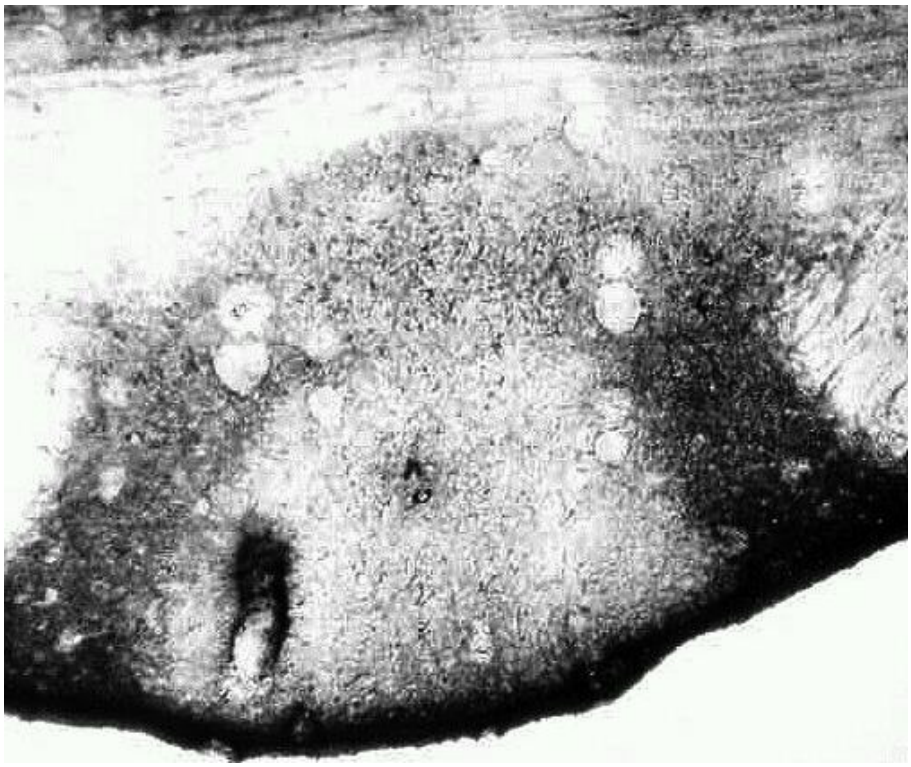


Fig. 38

Figs. 37, 38
In the mid-portion of the IPN, the central region of the nucleus shows a markedly reduced GFAP-IR upon castration (Fig. 38). The lateral subnuclei remain nearly unaltered. x120

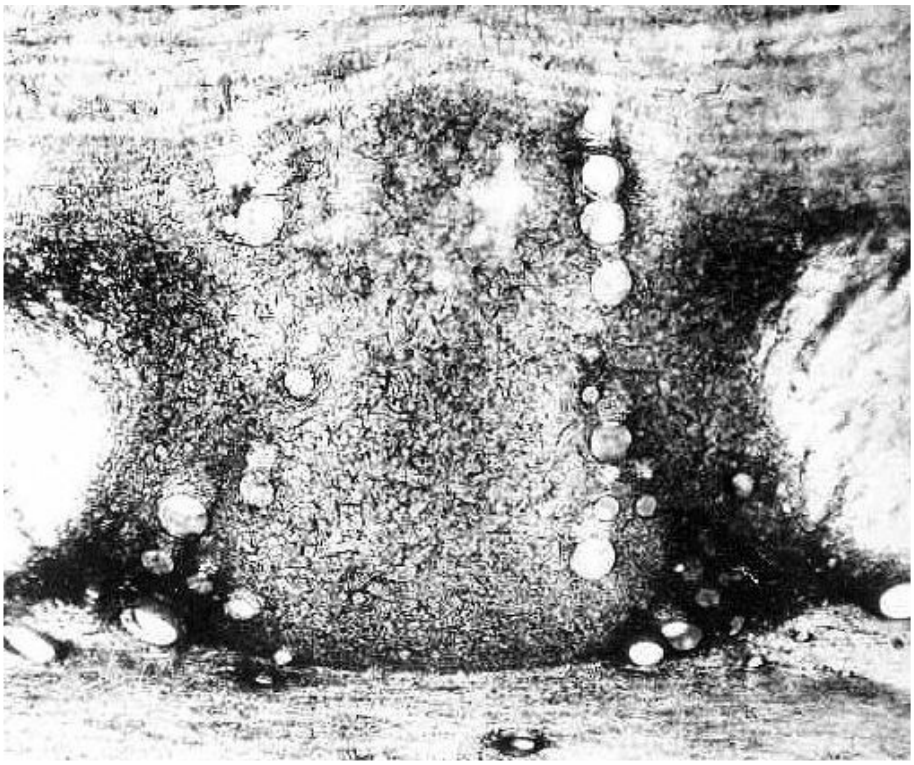


Fig. 39

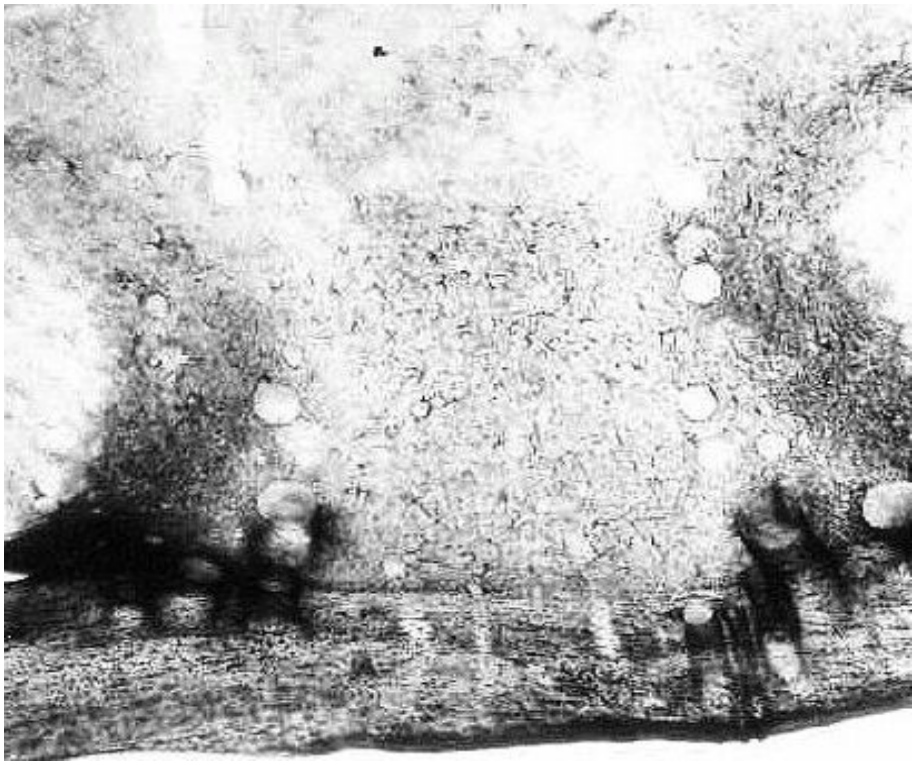


Fig. 40

Figs. 39, 40

At the caudal end of the IPN, castration (Fig. 40) also reduces GFAP-immunoreactivity in the central area. The staining of the lateral subnuclei is reduced to a lesser extent. x120

Fig. 41

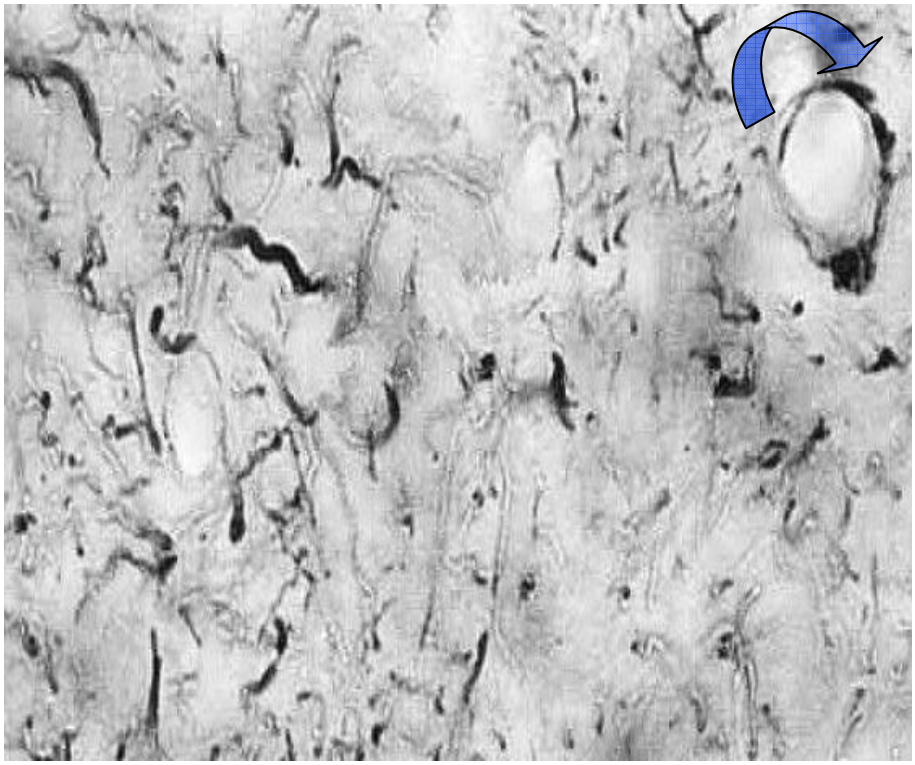


Fig. 42

Figs. 41, 42

Castration brings about a reduction of GFAP at the cellular level. In the control (Fig. 41) astrocyte processes show a thick, continuous branching which outlines whole astrocytes, while in the castrated animal (Fig. 42) GFAP-stained elements are delicate and fragmentary. No reduction is observed in the pericapillary astroglia (arrow). x900

In the rostral third of the nucleus (around -5.60), no difference was found between the reactions of control and castrated animals. In the middle and caudal thirds, the reduction of GFAP-immunoreactivity was conspicuous in the core region. The middle third (between -5.80 and -6.04, Fig. 37) could be identified on the basis of the free basal edge of the nucleus and the presence of several columns of capillary cross-sections indicative of microvessels permeating rostrocaudally the lateral subnuclei. In the caudal third (from -6.30 to -6.72, Fig. 39), the basal aspect of the interpeduncular nucleus was found to be underlain by the transverse pontine fibers, whereas capillary cross-sections were aligned in single columns. In the core area of both the middle and caudal thirds (Figs. 38, 40) revealed a reduced GFAP immunostaining also at the cellular level. While in the control interpeduncular nuclei, the full extent of the astrocytes was stained (Fig. 41), in the castrated animals their staining was fragmentary: the immunoprecipitate decorated short segments of processes (Fig. 42). The pericapillary astrocytic envelope was not affected by castration, irrespective of the intranuclear localization of the vessel.

In the peripheral area, the lateral and dorsolateral subnuclei showed no appreciable change of immunoreactivity, whereas in the dorsomedial subnucleus a slight decrease was observed.

Treatment with testosterone was carried out in castrated animals (n=4) from postoperative day 1. Testosterone treatment prevented the castration-induced decrease of GFAP-immunoreactivity in the IPN of male rats. If the treatment was started within 8 weeks after castration, it resulted in a substantial restitution of GFAP-immunoreactivity. Four months after castration the effect of testosterone was less pronounced but still detectable.

Conclusions to 6.9. Findings relevant to a 'non-endocrine' brain area (interpeduncular nucleus of the midbrain) suggest that in the female, fluctuations of glial fibrillary acidic protein- (GFAP-) immunoreactivity are due to cyclic changes of sex-hormones. GFAP-immunostaining was the most intense in late proestrus, whereas it decreased almost to zero in estrus. After ovariectomy a constantly high GFAP-immunostaining was obtained, although this increase could be suppressed by testosterone administration. In males, castration brought about a substantial reduction of GFAP-immunoreactivity. Testosterone treatment of castrated males prevented the decrease of immunoreactivity to a different degree – depending on the timing of hormone substitution. Our resultst are summarized in Hajós *et al.*, 2000, and Gerics *et al.*, 2001.

6.10. RAR and different gonadal hormonal states

Results described in the previous chapters point at an action of gonadal steroids on the astroglial cytoskeleton as indicated by hormone-dependent variations of glial fibrillary acidic protein- (GFAP-) immunoreactivity outside the 'endocrine brain'.

6.10.1. RAR and GFAP

6.10.1.1. RAR in males and females

When comparing the remote astroglial response (RAR), e.g. a lesion within the thalamo-cortical system in gonadally intact animals a slight difference between males and females could be observed. The sexual dimorphism of GFAP-immunoreactivity (-IR), as described in 6.8., applies not only to the “baseline” of GFAP-IR in the intact rat brain, but to the intensity of RAR as well. The overall reaction of the ipsilateral, lesioned visual cortex compared on both the 4^{th} and 12^{th} postlesional day was more intense in males than in females.

Gonadectomies were carried out to see if the development of RAR in the visual system can be influenced by a deprivation of gonadal hormones.

6.10.1.2. RAR in ovariectomized females

Having clarified the reasons underlying the wide-range fluctuations of the GFAP-immunoreactivity outside the 'endocrine brain' of intact females, and taking into consideration that within the areas involved in sex-steroid regulatory mechanisms this has been shown earlier (Garcia-Segura *et al.*, 1994a; Kohama *et al.*, 1995; Chowen *et al.*, 1995), we attempted to observe RAR of the geniculo-cortical system under altered hormonal conditions.

Owing to the fact that the onset of RAR in the geniculo-cortical system requires a minimum of 3 days, then its full development another 4 days, and the peak is observed only thereafter, the phases of the 4-day ovarian cycle of the rat are several times exceeded by the period needed for the full development of RAR. Thus the suspension of the sexual

cycle by ovariectomy appeared to be a feasible means to produce a sufficiently long period of altered sex-steroid environment.

Ovariectomy was performed 4 weeks prior to the lesioning of the lateral geniculate body. Animals were sacrificed 7 and 12 days after the stereotaxic lesion and the ipsi- and contralateral visual cortices were immunostained for GFAP.

As shown in Fig. 43 ovariectomy blocked the development of RAR in the visual cortex. The middle layers of the visual cortex were devoid of immunoprecipitate due to GFAP-immunoreactivity, while the outer- and innermost layers contained immunoprecipitate. This distribution pattern corresponds to the appearance of immunoreaction in the visual cortex of intact animals (Fig. 9). A similar distribution of the immunoreaction was observed on the side contralateral to the lesion.

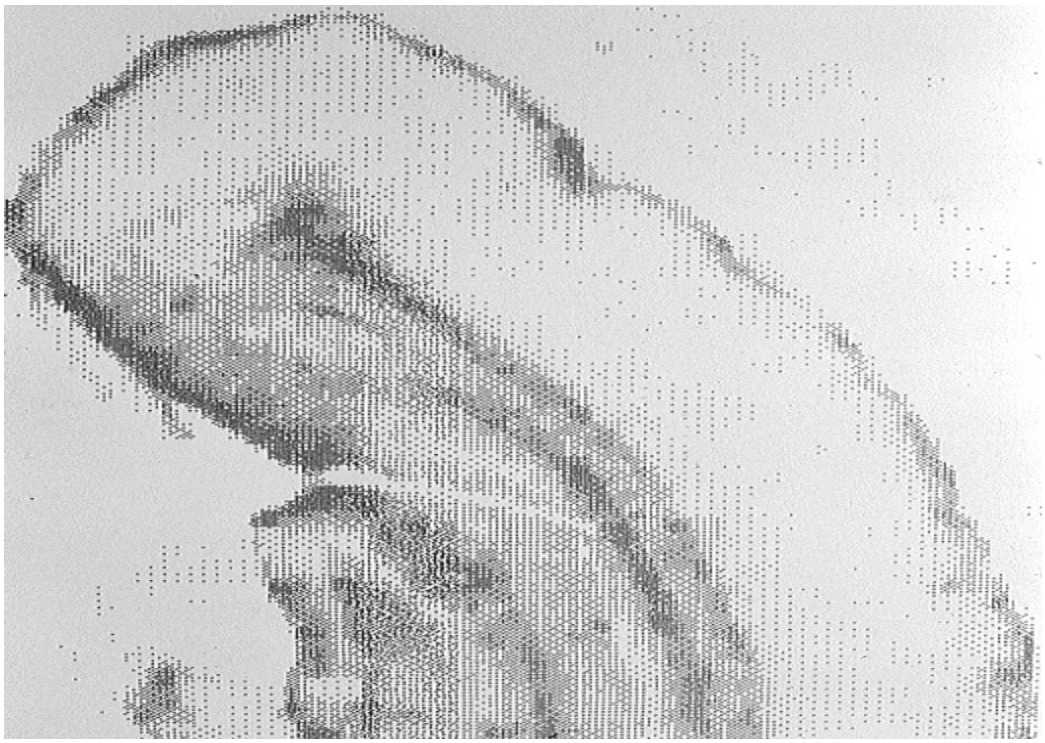


Fig. 43
Computer plot of the distribution of GFAP-IR in the visual cortex. If the lesioning of the CGL was carried out after ovariectomy, no sign of remote astroglial response (RAR) could be observed. (Compare this figure to Figs. 13, 14.)

6.10.1.3. RAR in castrated males

Our recent findings have shown (Gerics *et al.*, 1999, 2001) that castration reduced GFAP-immunoreactivity in the interpeduncular nucleus. This effect could be prevented by

testosterone-treatment. Therefore, it could be expected that castration may interfere also with the elevation of GFAP during RAR in the geniculo-cortical system.

The intensity of RAR in the geniculo-cortical system, as revealed by the GFAP-immunostaining was slightly reduced, and it faded away much earlier than in the control males. In intact males RAR was found to persist at least till day 19, whereas in castrated animals a rapid decline was observed. In castrated animals, at postlesional day 4 there was a RAR observed in the visual cortex comparable to that in the control. However, by day 16 almost no reaction was detected in the middle layers of the visual cortex.

It is noteworthy that in some cases where the size of the lesion exceeded the borders of the lateral geniculate body, a proportional extension of the RAR area occurred. Accordingly, not only the wedge-shaped primary visual cortex became prominent by its increased GFAP-immunostaining, but also in the neighbouring areas a moderate but appreciable staining of the middle cortical layers was present.

6.10.2. RAR and markers other than GFAP

In addition to GFAP, the immunoreactivities of beta-III-tubulin, microtubule associated protein 2 (MAP2), and dystrophin were also investigated in some animals for sexual dimorphism and under the conditions of RAR. In contrast to GFAP, tubulin, MAP2 and dystrophin did not exhibit any kind of sexual dimorphism. Nor did they participate in lesion-induced astroglial reactions including local or remote (RAR) responses.

As seen under the light microscope, with beta-III-tubulin and MAP2 mainly neurons were stained, while astroglial staining could be revealed either in regions where astrocyte processes are regularly arranged, or by using electron microscopy. No neuronal changes occurred in connection with the glial reaction or as a consequence of alterations in hormonal state.

Conclusions to 6.10. In females, ovariectomy blocked the development of remote astroglial response (RAR) in the geniculo-cortical system, as revealed by glial fibrillary acidic protein- (GFAP-) immunoreaction. In males, castration did not prevent the initial phase of this reaction but then it caused its rapid decline, so that at the time of peak reaction-intensity in the control, castrated animals showed a markedly reduced GFAP-immunostaining. The non-filament cytoskeletal proteins (MAP2) and the proteins tubulin and dystrophin did not participate in any kind of astroglial reaction.

7. Discussion

7.1. GFAP-IR

Astrocytes, as the name suggests, are process-bearing stellate or star-shaped cells distributed throughout the brain and spinal cord. In the light microscope the routine staining with haematoxylin and eosin shows astrocytes as pale-stained round or oval nuclei with almost no visible cytoplasm. To differentiate between astrocytes and small neurons the lobated nucleus of the latter can be used. The star-shaped appearance of astrocytes can be visualized only with special stains, impregnations or immunohistochemical demonstrations. Based on morphologic appearance and the content of intermediate-filaments astrocytes are divided into fibrous and protoplasmic, situated predominantly in the white and gray matter, respectively. The number of protoplasmic astrocytes shows also wide variations among different parts of the brain. The major component of glial fibrils (the amount of which serves for the distinction between fibrous and protoplasmic astrocytes), glial fibrillary acidic protein (GFAP), is specific for astrocytes in the CNS (Bignami *et al.*, 1972; Eng *et al.*, 1971). The classification of type 1 or type 2 astrocytes (Raff *et al.*, 1983) with additional marker proteins established *in vitro* cannot be routinely applied to glia in fixed brain tissue for technical reasons (Miller *et al*., 1986).

Glial fibrillary acidic protein (GFAP) has been shown to be an integral component of astrocyte filaments (Eng *et al*., 1971; Eng, 1985). Extensive studies of GFAP-immunoreactivity (GFAP-IR) have yielded information about the shape of individual astrocytes in various brain areas (Ikeda *et al*., 1980; de Vitry *et al*., 1981; Bascó *et al*., 1981; Suess and Pilska, 1981; Jessen and Mirsky, 1983; Bignami, 1984; Björklund *et al*., 1985; Hajós and Kálmán, 1989; Kálmán and Hajós, 1989). However, studies on the regional distribution of GFAP-IR are not numerous and are mainly focused on the cerebellum, lower brainstem and spinal cord (Bignami and Dahl, 1973; Schachner *et al*., 1977; Tapscott *et al*., 1981; Anderson *et al*., 1984; Bullon *et al*., 1984; D'Amelio *et al*., 1985; Liuzzi and Miller, 1987). Previous reports (Hajós and Kálmán, 1989; Kálmán and Hajós, 1989) produced an overview of the major aspects of the regional distribution of GFAP-IR in the rat brain. In contrast to the ubiquitous occurrence of astrocytes within the CNS, it has been found that GFAP-IR is unevenly distributed. Some brain areas being rich in astrocytes showed less intense GFAP. On the other hand, there were some prominent

sites where an accumulation of GFAP-IR cells could be seen, i.e. the most superficial part of the brain near the pia, periventricular zones, hippocampus and allocotrical areas, midbrain structures and globus pallidus showed the highest immunoreactivity.

The method of image analysis (Schleicher *et al.*, 1986) used in a study for mapping GFAP (Zilles *et al.*, 1991) has several advantages over visual evaluation of immunostained preparations. It not only expresses staining in quantitative terms, but measures areas of immunostained territories (areal fraction) independently of uncontrollable low variations in staining intensities and due to this it yields more accurate information when compared to simple densitometry. The findings show a wide variety of areal fractions throughout the brain indicating a highly uneven distribution of GFAP-immunoreactivity, which in most cases did not correspond to cytoarchitectonic units. However, there appeared to be an organizing principle underlying the observed distributional patterns.

A continuous system of relatively high areal fractions can be reconstructured connecting the so-called midline structures, beginning at the organum vasculosum laminae terminalis and the subfornical organ, continuing with the midline structures of the septum, thalamus and the periventricular hypothalamic zone, and reaching to the raphe, interpeduncular nucleus and periventricular structures of the mesencephalon. A mantle zone of medium areal fraction values is revealed beneath the external and internal surfaces of the brain. Within these areas, cells occurred with processes directed to the surface. We assume, therefore, that these intensely stained GFAP-immunoreactive astrocytes are derived from the former surface contact glia (Akers, 1977; Hajós and Bascó, 1984; Voigt, 1989).

It may be argued that areas of high areal fractions were detected also deeper in the brain without any apparent relationship to the adult surfaces. Viewed on an ontogenetic basis, however, these structures correspond to internalized surfaces of the embryonic brain in contrast to territories having developed by cell acquisition, proliferation or fiber ingrowth resulting in the local thickening of the neural tube wall. These findings suggest that under physiological conditions in the adult brain, astrocytes of high GFAP-immunoreactivity are mostly found close to inner and outer surfaces or former embryonic surfaces, whereas astrocytes located in areas having developed by the thickening of the neural tube show less or no GFAP-IR.

The presence of GFAP was biochemically shown also in the filament-independent, soluble cytoplasmic compartment of astrocytes (Patel *et al.*, 1985), hence there is no histological reason which explains the lack of immunoreactivity in some astrocytes. The wide range of variations in the distribution of GFAP-immunostained elements throughout the rat brain might be interpreted as GFAP-negative territories containing astrocytes with dormant immunoreactivity (Hajós and Zilles, 1995).

7.2. Sexual dimorphism of the cytoskeleton

Gonadal steroids are intricately involved in the development and function of sex-steroid-responsive structures in the CNS (Garcia-Segura *et al.*, 1996a). These steroids have organizational effect on the developing mammalian nervous system that persists as gender differences in adult neuronal structures. Synaptic density and organization, as well as neuropeptide and neurotransmitter synthesis and release are thus modulated.

The nervous system also synthesizes a variety of steroids from cholesterol (Hu *et al.*, 1987; Akwa *et al.,*1993; Kabbadj *et al.*, 1993). These endogenous steroids, the neurosteroids (Baulieu, 1981) are interacting with the circulating hormones. The complex interaction of progesterone and estradiol on hippocampal astroglia as described by Luquin *et al.*(1993).

Gonadal steroids, especially estrogens and aromatizable androgens, play an important role in neuronal development and circuit formation during the fetal and neonatal (so called „critical") periods (Arnolg and Gorski, 1984; Arai *et al.*, 1986; MacLusky and Naftolin, 1981; Matsumoto and Arai, 1980 and 1986). In the postpubertal animal, sex steroids modulate neurotransmitter and neuropeptide synthesis and secretion, receptor numbers, as well as nuclear volume (Commons and Yahr, 1984), dendritic morphology (DeVoogd and Nottenbohm, 1985; Kurz *et al.*, 1986), innervation patterns (DeVries *et al.*, 1984) and number of synaptic inputs (Luquin *et al.*, 1993; Naftolin *et al.*, 1993; Párcucz *et al.*, 1993; Pérez *et al.*, 1993). Most of these effects have been described in specific anatomical areas such as the ventromedial hypothalamic nucleus (Matsumoto and Arai, 1986), lateral septum (DeVries *et al.*, 1983; Miyakawa and Arai, 1987), hypothalamic arcuate nucleus (Garcia-Segura *et al.*, 1986; Párducz *et al.*, 1993), the preoptic area (Raisman and Field, 1973; Chen *et al.*, 1990), and the suprachiasmatic nucleus (Güldner, 1982), that are

involved in the control of reproductive or neuroendocrine events (Leedy *et al.*, 1987; Jordan *et al.,* 1989). Since these systems are clearly sexually dimorphic, it is generally thought that the structural differences in the CNS play an important role in the physiological differences seen between males and females.

In certain brain areas involved in steroid hormone feedback loops, e.g. the hypothalamus, the steroid hormone sensitivity of GFAP immunoreactivity expressed by astrocytes showed a clear sexual dimorphism. One of the rather surprising sexual dimorphisms in the adult brain is the synaptic patterning in some hypothalamic nuclei. In the arcuate nucleus males have twice the number of axosomatic and 50% more axodendritic spine synapses as females. The opposite pattern is observed in the immediately adjacent ventromedial nucleus (Mong *et al*., 1999). A similar situation has been reported by Párducz and Garcia-Segura in the hippocampus (1993). In the dentate gyrus they found a significantly higher number of mossy fiber synapses in males, whereas other types of nerve endings were not different between the two sexes.

Although much of the past effort devoted to understand how sex steroids modulate brain development and physiology was focused on neurons, recent evidence indicates that glial cells mediate some of the gonadal hormone actions in the CNS. Beside neurons, also glia play an important role in the metabolism of gonadal hormones (Jung-Testas *et al.*, 1991; Kabbadj *et al.*, 1993) and in the synthesis of endogenous steroids (Akwa *et al.*, 1993; Kabbadj *et al.*, 1993). Astrocytes are known to undergo changes in a number of experimental and pathological situations (for references see Lindsay, 1986). It has also been established that different hormonal states have an influence on astroglia mainly in the neurohormonal regulatory centers of the hypothalamus. At these sites, the astroglial marker GFAP has been shown to alter with changing hormone levels (Salm *et al*., 1985; Suarez *et al*., 1991; Chowen *et al*., 1995; Hawrylak *et al*., 1998).

In vitro studies have demonstrated the presence of receptors for progesterone, estrogen and androgen in glia (Jung-Testas *et al.*, 1991). Estrogen receptor protein and mRNA have been demonstrated in cultured astrocytes from several brain regions (Jung-Testas *et al*., 1992; Santagati *et al*., 1994; Murphy *et al*., 1998) and estrogen receptor immunoreactivity has been detected in astroglia from the arcuate nucleus and median eminence as the key centre for the integration of sex steroid effects on the control of pituitary secretion (Chowen-Breed *et al.*, 1989; Chowen *et al.*, 1993).

Steroids, such as estrogen, principally act via intracellular receptors that translocate to the nucleus after binding and alter gene transcription. Mong *et al*. (1999) postulated three possible scenarios for estrogen-induced changes in neuronal-astrocytic morphology in the neonate arcuate nucleus and ventromedial nucleus, all about possible influences on permissive factors for astrocyte differentiation and/or dendritic spine-formation. However, there has been no clear *in vivo* demonstration of any gonadal steroid receptors in hypothalamic astrocytes of neonatal rats (Garcia-Segura *et al*., 1996b).

The demonstration of hormonal influence on synaptic connectivity in cognitive areas of the brain, such as the cerebral cortex (Medosch and Diamond, 1982; Juraska, 1991) and the hippocampal formation (Meyer *et al.*, 1978; Woolley and McEwen, 1992; Párducz and Garcia-Segura, 1993; Shughrue and Merchenthaler, 2000) and reports of sexual dimorphic GFAP-IR in the cerebellum (Suárez *et al*., 1992; Day *et al*., 1998) takes the problem beyond the frontiers of neuroendocrine regulation. The experimental area investigated in our present studies, the interpeduncular nucleus (IPN) also lacks any close involvement in hormonal regulatory functions. The multilateral afferentation (from several midbrain and forebrain centers; for references see Marchand *et al*., 1980) of this relay station within the limbic system is neurochemically diverse: cholinergic terminations in the core of the nucleus, substance P-fibers at peripheral subnuclei (Contestabile *et al*., 1987 and Marchand *et al*., 1980, respectively). The regular internal organization of the termination fields of afferent pathways, and a differential localization of neurotransmitters within the IPN does, however, not always produce an overlap of cytoarchitectonic and neurochemical areas.

In view of the fact that no difference could be observed between males and females concerning size, distribution and packing density of astrocytes within the interpeduncular nucleus, our present findings indicate a sexual dimorphism caused by different levels of GFAP-IR in the intact male and female IPN. We could thus strengthen in a hormonally inactive brain area the argument that GFAP-IR is more intensely expressed in males than in females. Our findings also suggest that, at least in the IPN, this particular type of sexual dimorphism is genuine, i.e. not induced by synaptic or other reactive changes. The evenly intense immunostaining in the male has been shown to decrease upon castration without apparent structural reorganization of the nucleus (Hajós *et al*., 1999). Thus it is believed that in the IPN, the difference in GFAP-IR between males and females is a primary feature of astrocytes.

Our present findings demonstrate that in female rats the expression of GFAP-IR varies during the estrous cycle also in a "non-endocrine" brain region, i.e. the interpeduncular nucleus. Variations showed a trend similar to that observed in the rodent hypothalamus by Garcia-Segura *et al*. (1994), and by Kohama *et al* (1995) for GFAP and for GFAP mRNA, respectively: a gradual increase till proestrus with a peak at late proestrus, then a significant fall in estrus. Although GFAP-IR may not always reflect true amounts of the protein (McClendon and Bigner, 1994), the studies of Garcia-Segura *et al*. (1994) and of Kohama *et al*. (1995) together with our previous observations on the perisynaptic reactive glia (Hajós *et al*., 1996) suggest that in the present context a parallelism exists between three parameters, i.e. GFAP-IR, GFAP mRNA and the net amount of GFAP. Hence, any of them can be taken as an indicator to asses astrocyte response to hormonal states. This response seems to be more widespread than the hypothalamic endocrine regulatory centers. It is noteworthy that corticosteroids also proved to be effective in influencing GFAP expression (Nichols *et al*., 1990; Chou *et al*., 1990, 1991), therefore, this astroglial protein appears to be sensitive not only for gonadal steroids but also for other members of the steroid hormone family. Thus, our present findings taken together with the data available in the literature argue for a more generalized steroid hormone-dependence of astroglia throughout the brain. In females, due to the cyclic gonadal function, the investigation of this problem is more complicated than in males. Cyclic hormonal states in the female are accompanied by changes indicative of a regulation of GFAP synthesis and/or expression by estradiol.

Recent studies (see Lisman and Harris, 1993) have disclosed a considerable degree of synaptic plasticity in the adult CNS, associated with various functions – a phenomenon thought earlier to occur exclusively in developing and regenerating neural tissues. Synaptic plasticity in the normally functioning CNS implies not only function-related morphological changes of synapses (Geinisman *et al*., 1989) but also fluctuations in synaptic numbers and/or positions within a region (Chen and Hillman, 1992; Lisman and Harris, 1993; Anthes *et al*., 1993, Krizbai *et al.,*2000). Dynamic changes such as the simultaneous removal and generation of synapses can hardly be followed by morphological methods while the chemical and functional diversity of synapses hampers the biochemical assessment of plasticity.

The close interaction between synapses and astrocytes and the sensitivity of glia to sex steroids has been described (Garcia-Segura et al., 1994a; Párducz *et al*., 1996; Garcia-

Segura et al., 1996a; Melcangi *et al*., 1998) thus complex responses of the CNS to hormonal changes are evident. The question whether hormonal states affect primarily synapses which, in turn, would induce the reaction of astroglia, or astroglia are affected directly, is still open. The majority of sex differences in the rodent brain are caused by local conversions of testosterone to estrogen by the p450 aromatase enzyme (Naftolin *et al*., 1975; McEwen *et al*., 1977; Lephart, 1996) that is localized in neurons but not in astrocytes (Martini and Melcangi, 1991). Mong and McCarthy (1999) found, however, the glia of the neonatal arcuate nucleus responding to locally synthesized estrogen, rather than to testosterone.

In adult female rats, reversible modifications in glial ensheathing of arcuate neuronal somata result in transient disconnection of GABAergic synaptic inputs during the ovarian cycle (Párducz *et al.*, 1996). The decrease in number of GABA-immunoreactive synaptic profiles may be due in part to an effect of the hormone on the accumulation of the neurotransmitter in the synaptic terminals. Another possible explanation for the reduction in number of axosomatic synaptic profiles in estrogen-treated rats is the degenerative loss of synaptic terminals or a physiological disconnection of presynaptic boutons from neuronal somata (Párducz *et al.*, 1993). 17β-estradiol drives the estrus cycle and induces growth of astroglial processes which ensheath neuronal perikarya and cause the displacement of GABAergic synapses (Garcia-Segura *et al.*, 1994b).

Although in the hippocampus and arcuate nucleus a synaptic remodeling has been pointed out during the estrous-cycle with concomitant changes in GFAP (Woolley and McEwen, 1992; Luquin *et al*, 1993; Mong *et al*., 1999), this has not yet been confirmed in the IPN. Such an investigation, however, seems to be most feasible. The main input to the IPN, the largely cholinergic habenulo-interpeduncular tract terminates with characteristic crest-synapses in the core of the nucleus (Contestabile *et al*., 1987) where GFAP-IR has been shown to display the highest degree of sexual dimorphism and the most intense response for hormonal states (Hajós and Halasy, 1998b; Hajós *et al*., 1999; Gerics *et al.*, 1999, 2000 and 2001). GABAergic synapses have also been shown in this area (Contestabile and Fonnum, 1983; Pearson, 1988; Kawaja *et al*., 1991). Synapses with accumulations of large dense-core vesicles, on the other hand, were found at the periphery of the nucleus (Murray *et al*., 1979) where substance P (Artymyshyin and Murray, 1985; Contestabile *et al.*, 1987) and presumably other peptidergic afferent fibers project (Hamill *et al*. 1984, 1986). Electron microscopic studies in progress in our laboratory could not

demonstrate a fluctuation in synaptic numbers, even within the well-recognizable populations, that are associated with altered hormonal levels.

7.2.1. Gonadectomy and testosterone-treatment

There are not only regional differences in the CNS in the GFAP content of intact animals, but gonadectomy and steroid treatment differentially affect this astrocytic marker in hippocampus, cortex, septum, hypothalamus and other brain areas. Effects of gonadal steroids on astroglia shows prominent regional differences. Different effects of progesterone on hippocampal and hypothalamic astroglia have been reported by Luquin *et al.*(1993) and Garcia-Segura *et al.* (1994a), respectively. These findings suggest a heterogeneity in the response of these cells to gonadal hormones. The existence of astroglial subpopulations with various degrees of hormonal sensitivity should be considered. However, high responsiveness of astrocytes to a variety of steroids was shown in males and females, neonatal, as well as in young, adult and aged rats. (e.g. Garcia-Segura *et al*., 1986, 1988; Beyer *et al*., 1990; Torres-Aleman *et al*., 1992).

Day *et al*. (1990) described elevating effects of castration on GFAP-expression in male rat hippocampus. They registered a 20% increase in the GFAP immunoreactivity in the cortex, hippocampus and septum following castration of males (Day *et al*. 1993). Additionally, castration intensified the injury-induced synaptic reorganization of the hippocampus in response to deafferenting enthorhinal cortical lesions (Day *et al*., 1990) and the additive effect on increased GFAP mRNA was reported as well (Day *et al*. 1998). In contrast, gonadectomy in adult males resulted in decreased GFAP-IR in the hypothalamus (Chowen *et al*., 1995). In the dentate molecular layer of the hippocampus the number and area occupied by astrocytes (as marked by S-100 protein) showed no changes to hormonal manipulations. Nevertheless, castration caused a 50% increase in the surface area of GFAP positive fibrous processes of single astrocytes which was reversible by testosterone, dihydrotestosterone and estradiol (Day *et al*., 1993). A later study in the laboratory of Day proved a “protective” effect of testosterone – it reversed the age-related increase in GFAP in the male cerebellum (Day *et al*., 1998).

Legrand and Alonso (1998) tested effects of chronic administration of pregnenolone, the precursor of all steroid hormones on the age-related extension of astrocytic processes in

various brain areas. No effect was detected in young adult animals, whereas pregnenolone-injection in aged (2 year-old) rats showed a marked decrease of GFAP-immunoreactivity in the cortex, amygdala and thalamus without any significant change in the number of astrocytes, as indicated by immunostaining for the S100 protein.

Administration of testosterone to newborn females increased GFAP and its mRNA levels, the number of astroglial cell processes and the proportion of neuronal membrane covered by glia, while decreasing the final number of axo-somatic synapses to male levels (Garcia-Segura *et al.*, 1996a). Castration of newborn males resulted in the opposite outcome.

Chowen and colleagues (1995) reported significant effects of both the neonatal and adult sex steroid environments on GFAP mRNA levels in the arcuate nucleus and median eminence, but not in the lateral hypothalamic area. In the arcuate nucleus the GFAP-immunoreactivity and mRNA signal in intact females was decreased compared to intact males without any gender difference in the number of GFAP positive cells. Sex-related differences in the arcuate nucleus and median eminence probably reflect differences in the levels of expression per cell. Significant, but by administration of testosterone reversible reduction occurred after either neonatal or adult castration of male rats. Testosterone treatment resulted in either sex in a more dense GFAP mRNA-signal in both the arcuate nucleus and median eminence. As a response to testosterone treatment, an increase of the surface density of GFAP-immunoreactive profiles was recorded, without an effect on the number of GFAP-positive cells. High postpubertal levels of testosterone may compensate for the effect of low levels of hormone on astroglia during the prenatal period without having an additive effect on it.

Mong *et al*. (1999) found that testosterone exposure of neonatal rats induced a rapid and dramatic stellation-response of astrocytes in the arcuate nucleus with a reduction in the density of dendritic spines, but in contrast, the astrocytes in the ventromedial nucleus did not respond to hormonal manipulations. However, neurons of this later area had almost doubled the number of branches when exposed to testosterone compared with that in controls. These data of coordinated morphological plasticity in neurons and astrocytes in developing rat brain *in vivo* suggest that the degree of maturation and the differentiation of hypothalamic astrocytes are correlated with the ability of neurons to sprout branches or spines in response to steroid hormones. Testosterone seems to result in neuronal plasticity

in the form of changes in dendritic spine density – affecting thereby the sites of excitatory synapses.

The number of axosomatic synapses on arcuate neurons of the adult rat hypothalamus fluctuates following the sequence of increasing circulatory estradiol during the ovarian cycle. When GABAergic synaptic contacts were studied by Párducz *et al.* (1993), estradiol administration to ovariectomized rats resulted in a significant decrease in the number of immunoreactive synaptic profiles on perikaryal membranes. Furthermore the reduction of the percentage of somata covered by immunoreactive synapses was reported. The amount of glial ensheathing and the number of axosomatic synapses changed within the arcuate nucleus after treatment with estradiol (Párducz *et al.,* 1996). GFAP was increased in the afternoon of proestrus and morning of estrus. Also the synaptic remodeling in the olfactory bulb – an area with high synaptic plasticity and being important in the sexually differentiated behavioral and neuroendocrine functions – was recently reported to be hormone-dependent - it is modulated by estrogen (Krizbai *et al.,* 2000).

Our observations clearly indicate a differential distribution of GFAP-immunoreactivity within the IPN, which seems to correspond to the localization of cholinergic and peptidergic regions described by Contestabile *et al*. (1987). We demonstrated in male rats very intensely and intensely GFAP-immunostained zones at the periphery and in the core of the nucleus, respectively. Since GFAP-IR decreased upon castration mostly in the core region without apparent structural reorganization of the nucleus, it would be tempting to draw the conclusion that astrocytes of the cholinergic part of the IPN react specifically to the deprivation of testicular steroids. One should, however, also consider that the vascular architecture of the IPN favours a concentration of GFAP-immunoreactive perivascular glia at the lateral and dorsolateral subnuclei which did not react to castration. Thus, the selective reduction of immunostaining in the core could also be interpreted as an insensitivity of pericapillary glia to gonadal hormones in contrast to the sensitivity of astrocytes in the neuropil.

Simerly and Swanson (1986) reported about reversive effects of testosterone on the dramatic decrease of cholecystokinin in three sexually dimorphic forebrain nuclei following gonadectomy in male rats. In the rat hypothalamus, castration has been demonstrated to cause a reduction in both the immunoreactivity and levels of GFAP-related mRNA (Chowen *et al*., 1995) which also could partly be restituted by testosterone

treatment. Similarly, Day *et al*. (1998) proved a "protective" effect of testosterone in the cerebellum, a brain region not associated with gonadal steroid hormone regulation. In accordance to these findings our results show similar "protective" effects of testosterone outside the "endocrine brain". The drastic decrease in GFAP-IR in the IPN of castrated males compared to intact ones could be prevented if the testosterone-treatment was started within 8 weeks after castration. Four months after castration the restitution of GFAP-IR by testosterone-treatment was less pronounced but still detectable. To our present knowledge, the interpeduncular nucleus does not participate in the regulation of sex steroid levels (Shughrue *et al*., 1997). Therefore, our observations suggest that, at least in males, gonadal steroids are needed for normal GFAP expression also at sites outside the "endocrine brain". On the other hand, in females the cyclic rise and fall of estrogen levels is a natural phenomenon which is reflected in a decrease of GFAP in estrus. After ovariectomy the adrenocortical androgens are not any more suppressed by estrogen. The testosterone-like effect is predominant, hence the increase of GFAP-immunoreactivity.

7.3. Reactive gliosis, hypertrophy-hyperplasia, RAR

There is ample evidence for regarding astroglia as one of the functionally most responsive elements of the CNS (Hajós and Bascó, 1984). Basically, astrocytes are more resistant to several pathological stimuli than neurons (Duchen, 1984). Astroglial changes are widely recognized to be one of the earliest and most remarkable cellular responses following a wide variety of insults to the CNS. The injury might be either a traumatic, directly toxic or ischemic in its nature - the reactive gliosis at the sites of cellular necrosis has still a quite common basic pattern: swelling of astrocytes represents a more acute change, whereas hypertrophy-hyperplasia requires some time to develop. Many of the injuries that initiate astrocytic swelling may progress further to hypertrophy-hyperplasia with longer survival times such that both reactions may be seen in combination. Since the first demonstration of the post-traumatic and experimentally induced glial response called "reactive gliosis" (reviewed by Lindsay, 1986), a number of situations have been reported in which astroglia appeared to be involved in early and late CNS responses. Norenberg (1994) summarized different aspects of astrocytic responses to CNS injury.

GFAP synthesis can be triggered in astrocytes by influencing their neuronal environment. When "protoplasmic" astrocytes with low levels of pre-existing GFAP

change into reactive astrocytes, increases of GFAP are easily detected (Bignami and Dahl, 1976). However, reactive astrocytes with enhanced synthesis of GFAP must be separated from damaged or swollen astrocytes with enhanced labeling due to biochemical or "immunological" changes of GFAP molecules. In this respect, the time course of changes in GFAP-reactions after a defined insult should give further indication. Formation of reactive astrocytes with enhanced GFAP-labeling took in experiments of Schmidt-Kastner *et al*. (1993) about two days after deafferentation or direct tissue damage. Apparently, an interval is required for protein synthesis and transport of GFAP molecules into the processes. On the other hand, changes of GFAP staining due to biochemical or immunological events in glial damage or swelling would be expected at an early stage after pathological events, i.e. within hours after lesion or infarct. Glial swelling may later become a stimulus for transformation into reactive astrocytes. Graeber and Kreutzberg (1986) described in retrograde changes of the facial motor nucleus an initially diffuse staining pattern in the cell body and proximal processes of astrocytes evoked by the synthesis of soluble subunits of GFAP. At later stages, more defined and crisp staining of processes of stellate astrocytic figures were seen as newly formed GFAP molecules are incorporated into the cytoskeleton.

Owing to the high degree of connectivity in the CNS, nerve cell death necessarily brings about the degeneration of axons and synaptic terminals throughout the projection territory and vice versa, the transection of an axon effects the parent cell. Our findings provide experimental support to the assumption that GFAP immunoreactivity can be enhanced by means of a distant lesion affecting projections to the reactive area. The stimulus of this type of glial response is obviously the anterograde degeneration of axons and particularly synaptic terminals as it happened in the case of our experimental model, the geniculo-cortical system. Anterograde (Wallerian) degeneration is a process spreading along the axon distal to the neuronal injury and with time reaches the terminal field of the injured elements. The type of glial reaction resulting in remote areas differs essentially from what has been summarized under the term "reactive gliosis" which refers to a local response to injury and implies a surrounding glial proliferation.

A remote response can be evoked under experimental conditions by axotomy or destruction of the source nerve cell bodies. The destruction may be performed e.g. by the injection of some neurotoxic agents. Ben-Ari *et al*. (1979) injected kainic acid into the amygdala in order to develop pathological alterations in the hippocampus. Lesion of the rat

striatum by means of quinolinic acid injection (Block and Schwartz, 1994) caused a disinhibition in the target area which altered the local metabolic situation. The result of a transsynaptic change in astrocytes within the striatal projection areas was revealed by GFAP-immunostaining. Monoz-Mayor *et al.* (2000) destroyed the nucleus basalis as an experimental Alzheimer-model by injection of ibotenic or quisqualic acid. Studies of distant astroglial reaction by Niquet *et al*. (1994) showed that within fields which show cellular degeneration, glial cells proliferate *in situ* and do not migrate. This observation agrees with report from Hatton *et al*. (1993) which suggests that resident astrocytes do not migrate in adult brain in the absence of transplant-derived stimulus, even when they are stimulated by local injury. In contrast, astrocytes do not even proliferate in fields with exclusively axon-terminal degeneration, nevertheless they become hypertrophic in such fields.

The geniculo-cortical system offers a useful tool to study the remote astroglial response since the thalamo-cortical pathway represents a relatively simple one-source-one-target relationship. Further implications of remote astrocytic response may be relevant to the involvement of the CNS as a whole in any local destructive processes, depending on the extent of projections of the damaged area (Barret *et al*. 1981; Isacson *et al*, 1987).

Interruption of the geniculo-cortical pathway by lesioning of the dorsal lateral geniculate nucleus either by the injection of ibotenic acid (Hajós *et al*., 1990a) or electric coagulation (local heat spot), as in the current study, resulted in a measurable increase of GFAP immunoreactivity in the afferent area, the primary visual cortex. The nature of this type of glia activation, what can be termed according to Hajós and Csillag (1995) as remote astroglial response (RAR), appeared to be biphasic in time. The first phase in the target area, distant from the lesion was the engulfment and removal of degenerating terminals by perisynaptic glial processes; the second the GFAP response. The glial reaction may be induced by neuronal cell necrosis and/or the opening of the blood-brain barrier (Niquet *et al*., 1994). Presently, our interest was the second phase of remote astrocytic response: the increase in GFAP-immunoreactivity at a time when the phagocytosis of degenerated synapses had already been completed.

The rat visual cortex offered a favourable situation to document this phenomenon, as under normal conditions it almost completely lacked GFAP-immunoreactive astroglia (Bignami and Dahl, 1976; Kálmán and Hajós, 1989). On the operated side the appearance

of an intensive GFAP-immunoreaction in layers III, IV and V occurred from postoperative day 3, and reached a peak intensity between days 7 and 14. At this time the population of immunoreactive visual cortical astrocytes outlined the wedge-shaped projection area (primary visual cortex, OC1 of Zilles, 1985) of fibers emanating from the lesioned DLG. Increase in number of astrocytes in the external zone of the visual cortex with concomitant decrease in the middle, and particularly in the internal zones, represents a shift in the distribution of astrocytes. Layers III-IV where geniculo-cortical fibers terminate (Peters *et al*., 1976) contained indeed the bulk of degenerating synapses. The migratory translocation as part of the remote astroglial response seems to be triggered by the damage to the presynaptic terminals (Hajós *et al*., 1993). After two weeks GFAP-IR declined so that three months after the lesion no reaction was observed in layers III-V of the occipital cortex, except for some perivascular areas where a marked immunostaining persisted even six months after the lesion. The relatively late increase of GFAP-IR as compared to the peak time of terminal degeneration is in agreement with findings of Cortez *et al*. (1989), Janeczko (1989) and Niquet *et al*. (1994) and may represent the stabilization period of the cytoskeleton of newly formed glial processes adapted to the changed spatial distribution in a neuropil deprived of its main input. The temporary up-regulation of GFAP synthesis (as verified by immunoblot-findings of increase in net GFAP) coupled to the loss of afferent input in a particular region can be regarded as part of a space-sustaining glial hypertrophy necessary in the traumatized neuropil until it settles in a rearranged form. Having fulfilled their scavenger function, hypertrophic astroglia return to normal as a sign of cessation of a major rearrangement in the deafferented neuropil. In contrast to proliferations around a local brain injury as verified by Latov *et al*. (1979) and Janeczko (1989) or by the findings of Niquet *et al*. (1994) for a distant lesion in the hippocampus, in the geniculo-cortical system no cell division could be detected in the area affected by degeneration. Tetzlaff *et al*. (1988) have mentioned an observation with electron microscopic autoradiography demonstrating the lack of thymidine incorporation into astrocytes but a proliferation of microglia during retrograde reaction. This supports the view of Vaughn *et al*. (1970) that degenerating cell bodies are removed by the reactive microglia, while astroglia participate in the phagocytosis of degenerating synaptic boutons. In our experimental model, in the geniculo-cortical system, neither mitotic figures nor an increase in astrocyte numbers were encountered in parallel to the reactive enhancement of GFAP immunoreactivity. On the other hand, all signs of astroglial hypertrophy were observed apparently evoked by the massive degeneration of axons and synaptic terminals. Interpretation of our findings is

based on the fact that with routine histological methods astrocytes can be demonstrated evenly distributed in the visual cortex (Wree *et al*., 1980), i.e. also in the layers GFAP-immunonegative in the control. Thus the increase in GFAP of pre-existing glial elements may account for the results obtained, without the need to suppose a glial proliferation underlying the appearance or intensification of immunoreactivity. In addition to the well-established cytoplasmatic landmarks of astrocyte hypertrophy (see Barrett *et al*., 1981), we were able to demonstrate fine differences in nuclear structure between control and reactive astrocytes. These alterations can be interpreted as a transition from heterochromatic to euchromatic nuclear structure (Privat and Rataboul, 1986). In accordance with their cytoplasmic reaction, reactive astrocytes of the visual cortex had euchromatic-like nuclei. The thicker aggregation of chromatin against the nuclear envelope in the control may be indicative of a "resting" state.

It is important to note that neither other, non-cytoskeletal glial markers, nor neuronal markers reacted to experimental manipulations. Thus, it can be concluded that the cytoskeleton is the leading cellular element which defines glial reaction.

7.3.1. Glial response and gonadal hormones

Gonadal steroids and neurosteroids not only modulate physiological astroglial plasticity, but also affect astroglial responses in pathological conditions. Our findings in DLG-lesioned but sexually unaffected animals concerning the nature of remote astroglial response express a sexually dimorphic pattern. This seems to correlate to our observations in the interpeduncular nucleus, i.e. the GFAP-immunoreactivity was less pronounced in females as compared to males (Gerics *et al.*, 2001). This overall impression in the impaired visual cortex is based on observations at four and 14 days after lesioning. When observing the RAR at these two survival times, where the GFAP-IR occurred originally in layers III, IV and V and had its peak, respectively (Hajós and Csillag, 1995), degenerating presynaptic axon terminals occured (Hajós *et al.,* 1996).

In vivo studies of both normal and reactive astrocytes have shown them to constitute a heterogeneous cell population according to morphology, the expression of cell adhesion and/or substrate molecules (McKeon *et al*., 1991), and physiological properties (Qian *et al*., 1992), including their influence on synaptogenesis (Rouget *et al*., 1993). The close interaction between synapses and astrocytes and the sensitivity of glia to sex steroids has

been described (Garcia-Segura *et al.,* 1996a; Melcangi *et al.*, 1998; Gerics *et al.*, 1999), thus the reported differences are in agreement with our findings outside the "endocrine brain" in unlesioned animals.

Our present findings demonstrate that the possibility of influencing hormonally the remote versus local astroglial response shows essential differences. Orchidectomy had a less dramatic effect on RAR by a slight reduction and a shorter presence of GFAP-IR in the middle layers of the impaired visual cortex as compared to lesioned, but hormonally intact males. In contrast, ovariectomy blocked even the development of RAR in the geniculo-cortical system.

The effect of gonadectomies on RAR was in our experiments somewhat in contrast to the findings of Garcia-Segura and coworkers (1996a), but it has to be pointed out that those investigations refer to the site of primary brain damage. They found that castration of rats of both sexes increases the local proliferation of astrocytes after injury. In contrast, administration of high physiological levels of estradiol or progesterone to ovariectomized females, or administration of testosterone to castrated males, decreased the proliferation of astrocytes in the injured brain and also decreased the accumulation of hypertrophic GFAP-immunoreactive astrocytes in the proximity of the wound (Garcia-Estrada *et al.,* 1993).

Unlike Garcia-Segura *et al.* (1988), in the present study we did not observe any significant effect of the age at which the gonadectomy was performed. This might be because the interpeduncular nucleus and the visual cortex are not parts of the "endocrine brain". Another explanation might be the relatively high number of parameters changed when investigating the RAR (gonadal status, age at gonadectomy, time having elapsed between gonadectomy and lesion, survival time after lesion).

Concerning sites and mechanisms of hormone actions even within the "endocrine brain", conflicting opinions exist wheter genomic or non-genomic factors play a role in this phenomenon, since some genomic effects of steroids can be very rapid, i.e. within the range of 10 or 20 minutes (Mosher *et al.*, 1971). According to the working hypotheses of Kis *et al.* (1999) estradiol acts on the GABAergic system and the observed increase in activity is the consequence of the disinhibition of arcuate neurons.

7.3.2. The glial cytoskeleton as target of gonadal hormone action

It is assumed, that several complementary mechanisms underlie the genesis of the differences that exist between the sexes in synaptic connectivity and glial responses to injuries. One feasible mechanism is that gonadal steroids affect the formation and/or the elimination of synaptic contacts modulating thereby the area of neuronal membrane surface covered by glia, thus the number of synapses. The sexual dimorphism of GFAP-IR may reflect this difference as described by Garcia-Segura *et al.* (1994b). The geniculo-cortical system used in our studies as a model to investigate the phenomenon of RAR, produced similar results: an up-regulation of GFAP synthesis in the astroglial processes surrounding degenerating synapses has been shown (Hajós *et al*., 1996). Our findings in the IPN suggest, however, that in the interpeduncular nucleus this particular type of sexual dimorphism of the glial cytoskeleton is genuine, i.e. not induced by synaptic or reactive changes.

A second explanation might be the sexually different distribution of steroid-receptors. Estrogen-induced synaptic changes have been described e.g. by Pérez *et al.* (1993) in the arcuate nucleus when investigating the hypothalamic control of luteinizing hormone release. Madeira *et al.* (1991) demonstrated the complexity of the mechanisms underlying the genesis of sexually-dimorphic patterns in the hippocampal formation. Topographical differences in the number of synapses and in the distribution of hormonal receptors (as reviewed by Párducz and Garcia-Segura, 1993) suggest that both pre- and postsynaptic neurons expressing different steroid sensitivities most likely play a crucial role in the manifestation of sex differences in synaptic connectivity. It is important to note that synaptic function is not only affected by steroids from peripheral origin, but that nervous tissue also has the capacity to synthesize steroids (Baulieu, 1981). The glial cells are the primary site of the biosynthesis of neurosteroids (Hu *et al.*, 1987). Gonadal and neurosteroids may influence the brain function both as endocrine and autocrine/paracrine regulators. For instance astrocytes convert the neurosteroid pregnenolone into the gonadal hormone progesterone (Akwa *et al.*, 1993) which may then be released from astrocytes to neighbouring cells. Steroid receptors, however, have not been reported from areas outside the “endocrine brain” as the interpeduncular nucleus of the midbrain.

When the estradiol-effects on astroglia were studied *in vitro* by Torres-Aleman *et al.* (1992) an important feature was that a direct contact between neurons and glial cells is

necessary for the manifestation of the reversible and specific effect – the glial differentiation and proliferation. These results suggest that neuron cell surface molecules may be involved in the hormone-induced changes in the interaction between neuronal and glial membranes. Several cell adhesion molecules may participate. The expression of the so-called embryonic isoform of the neural cell surface molecule (PSA-N-CAM) by both neurons and glial cells persist in the adult rat in brain areas that maintain the capacity for neuro-glial plasticity, such as the hypothalamo-neurohypophysial system, the arcuate nucleus and the median eminence (Bonfanti *et al.*, 1992). The role of the adhesion molecule may be permissive rather than active.

A further component that may actively influence gender differences in synapses are the astrocytes being affected by gonadal steroids (Luquin *et al.*, 1993). Synaptic connectivity can be modulated by a change in the morphology of glial cells which results in the insertion of astrocytic processes between neuronal membranes (Theodosis and Poulain, 1993). The morphological modifications may influence neuronal connectivity since astrocytes extend their processes that cover the majority of the surface of neurons. Hence, modifications in the glial coverage of specific neurons may determine the number of sites available for the formation of synaptic contacts. Again, this mechanism is not described in the IPN, an area being not directly connected to brain centers involved in the regulation of endocrine functions.

Changes in synaptic morphology occur naturally throughout development and can also be modulated by the steroid environment. The underlying mechanism is thought to possibly involve a rearrangement of the cytoskeleton, that occurs mainly in cytoskeletal actin. (The protein actin is the major constituent of microfilaments.) Furthermore, alterations in intracellular calcium, which occur as a result of synaptic activity, may affect cytoskeletal actin, suggesting that synaptic activity may directly regulate synaptic morphology (Garcia-Segura *et al.*, 1994).

Since at the site of synaptic degeneration no filamental cytoskeletal elements are found, the changes in GFAP-IR reflect modifications of the adjacent cytoskeleton triggered by the degeneration. To get an insight, whether the effect of gonadal hormones is a direct cytoskeletal one or is mediated by the intranuclear change of transcription of the protein-synthesis *in situ* hybridizations are in preparation in our laboratory.

8. General conclusions

The present work provided experimental evidence for dynamic changes occurring in the astroglial cytoskeleton during the so-called remote astroglial response (RAR) induced by Wallerian degeneration of neurons.

While RAR was shown to be triggered by synaptic degeneration, only the cytoskeleton in the large processes and the cell body of astrocytes participated in this reaction. Perisynaptic processes exhibited a hypertrophy, whereas no astrocyte proliferation was observed.

It was demonstrated that in RAR, the astrocyte cell bodies and large processes hypertrophized and moved slightly closer to the site of synaptic degeneration

RAR could clearly be attributed to the filamentous GFAP-containing compartment of astrocytes, i.e. changes were detected where intermediate glial filament bundles were found. Hence GFAP-immunoreactivity proved to be a reliable marker of cytoskeletal changes associated with RAR.

GFAP-immunoreactivity maps of the brain showed wide intensity-variations, from massive immunoprecipitation to no reaction at all. The induction of the GFAP-immunoreaction at normally GFAP immunonegative sites appeared to be a suitable experimental model to study RAR-associated cytoskeletal changes.

Parallel biochemical studies suggested that the increase in GFAP-immunoreactivity 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.

Comparing male and female brains, a sexual dimorphism for GFAP could be pointed out. In males the immunoreaction was generally more intense than in females. To investigate into this phenomenon the interpeduncular nucleus (IPN), a strongly GFAP-immunopositive brain region that is not involved in hormonal regulatory mechanisms was selected as an experimental model.

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-immunoreactivity, thus they can be regarded as astrocyte cytoskeleton-activating factors.

Deprivation of gonadal steroids suppressed RAR. The suppressive effect was more pronounced in females than in males.

The fact that the cytoskeleton of astrocytes responded to alterations in the hormonal state of the animal even in areas, where steroid hormone receptors are not described, 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 possiblity that a blockage of gonadal steroid action may reduce the detrimental effects of the astrocyte reaction, primarily in cerebral edema, focal epilepsy and neurodegenerative disorders.

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