The dynamics of astroglial cytoskeleton and effects of gonadal steroids

Theses of the Ph.D.-Dissertation

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2000

The cellular elements of the central nervous system, the nerve cells or neurons and the glia cells or neuroglia function in a close interaction. In our present work we studied the function-dependent changes of the astrocytes, a characteristic type of neuroglia. Interest was focused on the astroglial cytoskeleton which consists predominantly of intermediate filaments and has a key role in the maintenance of structural stability of astrocytes.

Fibrillary elements of the cytoplasm have been described from the nineteenth century onwards. The first hypothesis about the role of intracytoplasmic fibrillary material were put forward in connection with the study of muscular tissues since muscle cells were among the first shown to contain them in abundance. Quite understandably, these fibrils were interpreted as means of cell motility. Electron microscopic investigations revealed that this material comprises different types of intracytoplasmic filaments and tubules which are unbranched and occur in the cytoplasm often in a remarkable orderliness. This is particularly conspicuous in the neuronal processes (axon and dendrites) where cytoskeletal elements form parallel arrays found almost along the entire length of the process.

Cytoskeletal filaments are so numerous **in the neuron** that the term neurofilament has been introduced to distinguish them from similar cytoskeletal filaments in other cells called the microfilaments. Neurofilaments are composed of a family of specific proteins with three major components ("triplet proteins") having molecular weights of 70, 130-150, and 200 kDa, respectively. The exact function of neurofilaments is largely enigmatic. They have been envisaged as passive structures stabilizing the axon and regulating axonal diameter.

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

hand have a few irregularly scattered microfilaments in the perinuclear region but in contrast to oligodendrocytes, they contain a characteristic system of intermediate filaments in both the perinuclear cytoplasm and the processes. A major structural protein synthesized by astrocytes is the **glial fibrillary acidic protein** (**GFAP**).

Astroglial cytoskeleton can be studied in tissue cultures of astrocytes 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. Astrocytes are known to be fairly evenly distributed throughout the brain. 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 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, significant differences were observed in the intensities of GFAP-immunoreactivity between grey matter areas: some territories were also devoid of immunoprecipitate, while there were consistent **differences in staining-intensities** between GFAP-immunoreactive **grey matter regions**.

These differences 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?

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 grey matter regions of high and low GFAP-staining-intensities.

GFAP-maps were prepared predominantly based on results obtained in males rats. 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 parallel 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. The observation of this kind of sexual dimorphism in widespread extrahypothalamic locations was a new finding which deserved a more thorough investigation.

One group of **questions** in the present work were posed to answer whether there is a region **outside the "endocrine brain"** where astroglial cytoskeleton shows **sexual dimorphism** and if different hormonal states can alter astroglial cytoskeleton and they reaction as revealed by GFAP-immunoreactivity (-IR).

As a model the **interpeduncular nucleus (IPN)** of the midbrain was selected. 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.

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. 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. 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.

In sections immunostained with antibodies against GFAP in different estrus 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 results 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.

The microscopic examination of metestrus preparations showed an even distribution of intensely GFAP-immunopositive astrocytes in the core region of the interpeduncular nucleus. 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. 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.

Ovariectomy carried out 4 weeks before examination produced a marked elevation of GFAP-immunoreactivity within the interpeduncular nucleus. This applied not only for the overall intensity of immunostaining but also for a more extensive staining of astrocyte processes. Upon longer survivals there was no difference in the intensity and extent of the GFAP-immunoreaction as compared to the 4-week survival. The observed alterations could be substantiated by computer assisted image analysis.

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. 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. In the core area of both the middle and caudal thirds of the interpeduncular nucleus, high-power micrographs revealed a reduced GFAP immunostaining also at the cellular level. While in the control interpeduncular nuclei the full extent of the astrocytes was stained, in the castrated animals their staining was fragmentary: the immunoprecipitate decorated short segments of processes. 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 from postoperative day 1. Testosterone treatment prevented the decrease of immunoreactivity. 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.

Thus, the comparison of male and female rat brains, revealed a sexual dimorphism for GFAP also in the interpeduncular nucleus – a brain region that is not involved in hormonal regulatory mechanisms. In males, the deprivation of testicular sex-steroid hormones caused a drastic fall in GFAP-immunoreactivity, while hormone substitution diminished the decrease. In females, the intensity of GFAP-immunostaining was sexual cycle-dependent with the lowest intensity reaction during estrus. This suggests that gonadal sexual hormones are essential in maintaining a high GFAP-IR, thus they can be regarded as astrocyte cytoskeleton-activating factors.

The system of cytoskeletal filaments has been recognized in the 19th century as the "glial fibrils" of astrocytes. It has also been realized that these filaments react in a most dynamic fashion to any functional change of the astrocyte. They accumulate when the cell is activated and withdraw to a stable pattern in the resting state of the cell. It has been observed that astrocytes react to **brain injuries** by hypertrophy in the surrounding of the lesion and accumulation of cytoplasmic fibrillary material in order to fill the space of the tissue discontinuity or replace spatially the lost neurons. Thus, an increase in GFAP-immunoreactivity might be indicative for “reactive gliosis”. Beside the local reaction to a neural damage a hypothesis was advanced concerning the effects of astroglial reaction also in the projection areas of the injured neurons. These areas might be at considerable distance from the site of the lesion therefore, we introduced the term **“remote astroglial response (RAR)”**.

In a group of experiments **we attempted to elucidate**, whether GFAP-immunohistochemistry is a reliable method to follow astrocyte reactions; whether or not RAR is coupled to synaptic degeneration; whether changes in the GFAP-IR reflect changes in the synthesis of the protein or indicate conformational changes of the molecule that alter immunoreactivity; and whether proliferation or hypertrophy of the cytoskeleton is the leading feature of the astrocyte reaction. Furthermore we asked the question whether steroids can alter glial reaction.

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 occipital cortex comprises the primary visual area and the related associative regions. The afferents to this area originate from the posterior thalamus, those of the primary visual area from the lateral geniculate body, and terminate in layers

III-IV of the visual cortex. The projections of the geniculo-cortical system are strictly ipsilateral, thus the contralateral side can be used as a natural control. An eventual increase in GFAP-immunoreactivity is in this system 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 a Wallerian degeneration was induced. The observation of astroglia in the target area confirmed earlier findings that under the circumstances of Wallerian degeneration, a remote astrocyte 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. 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. This typical RAR was verified also with image analysis and proved to have a time course specific for this system. Accordingly, the first signs of increase in GFAP-immunoreactivity could be detected on postoperational 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 even six months after the lesion.

The GFAP-immunoreaction is a reliable marker of 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. Consequently its increase in RAR as a reflection of the hypertrophy of the cytoskeleton in response to the activation of astroglia. There was a clear selective increase in the net amount of 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 of GFAP-immunoreactivity in RAR 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.

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. 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 (29.38%, 38.63%, and 31.81%, respectively) 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.

In females, ovariectomy blocked the development of RAR in the geniculo-cortical system, as revealed by GFAP-immunoreactivity. 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 ribosome-localized proteins (tubulin, dystrophin) do not participate in any kind of astroglial reaction.

Deprivation of gonadal steroids suppressed the remote astroglial response. The suppressive effect was more pronounced in females than in males. The fact that the cytoskeleton of astrocytes in areas where steroid hormone receptors are not described, responded to alterations in the hormonal state of the animal, argues for a more widespread effect of gonadal steroids in the brain than earlier believed.

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

The main perspective of the present findings is the possible use of gonadal steroids to reduce the adverse effects of the astrocyte reaction, primarily in cerebral edema, focal epilepsy and neurodegenerative disorders.