Study of myelination in the normal human hippocampus and in Down syndrome, as well as in the central nervous system of the mouse

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I. INTRODUCTION

Oligodendroglial cells and myelination

In the central nervous system oligodendroglial (OLG) cells form and maintain myelin sheets. Based on electron microscopic observations Mori and Leblond divided oligodendroglial cells to light, intermediate and dark types. The intermediate types form myelin sheets in the developing nervous system. The matured (dark) OLG cells form approximately 90% of all OLG cells in the adult central nervous system and play a role in maintanence of the myelin sheets. There are several proteins in the structure of myelin sheet. The proteolipid protein (PLP) and the myelin basic protein (MBP) form 80% of all protein sin the compacted myelin sheet. The PLP and MBP play essential role in the compaction of the myelin sheet, establishing the distance between individual myelin lamellae and formation of the major dense line.

Immunocytochemical studies have shown that MBP is found in both young non-myelinating and mature OLG cells that form myelin sheets. The MBP-immucocytochemistry is suitable to show the start of myelination, but also to detect demyelination. It is not known, whether the different types of OLG cells that can be differenciated in the electron microscopy express MBP activity or not. The MBP-immunocytochemistry is an excellent method to study myelination or demyelination in human tissue that is difficult to study with electron microscopy due to the postmortem changes. In addition to MBP-immunocytochemistry the modified Klüver-Barrera method is used, namely the Luxol fast Blue (LFB) method that is suitable to show elements containing high concentration of lipoproteins.

In the regulation of myelination process a number of surface factors play a role establishing connection between two neighbouring cells, such as the integrin β1, neuronal adhesion molecule (NCAM), notch, lingo-1 and contactin. In addition, there are mediators, such as the adenosin trifosfate (ATP), adenosin, leukaemia inhibitory factor (LIF) and growth factors such as the platelet derived growth factor (PDGF), fibroblast growth factor (FGF) that all exert an effect on the formation of myelin sheet during ontogenesis.

In addition to the above mentioned classic factors, polipeptides, such as the pituitary adenylate syclase activating peptide (PACAP) participate in the myelination process. PACAP is a polipeptide formed by 38 amino acids and belong to the vasoactive intestinal polipeptide family (VIP). Several studies have shown its role in neuroprotection. PACAP exerts its effect through PAC1, as well as VPAC1 and VCAP2 receptors. In PACAP-deficient (PACAP-KO) mice, the high mortality of animals is known. Among the central nervous deficits the late

differentiation of cerebellar granule cells, their high apoptotic affinity and significant delay in the regeneration of damaged axons have been noticed. There are very few data about the effect of PACAP on the myelination during ontogenesis. It is known that OLG precursors express receptors for PACAP. In addition, in *in vitro* cell cultures, PACAP enhances proliferation but delay differentiation of OLG cells.

Anatomy and connections of hippocampus

The hippocampus per se is part of the archicortical hippocampal formation

The hippocampus includes Ammon's horn and the dentate gyrus. The main neuronal type of Ammon's horn is the pyramidal cells. Based ont he morphology of pyramidal cells, Ammon's horn (Cornu Ammonis) can be devided to three subregions, CA1-3. Somata of pyramidal cells form the pyramidal layer, whereas apical dendrites locate in the strata radiatum and lacunosum moleculare. Basal dendrites of pyramidal cells locate in stratum oriens. Axons of pyramidal cells form several local branches but the efferent axons run toward their target in alveus. The main cell type of dentate gyrus is granule cells and their somata form the granule cell layer. Dendrites of granule cells locate in stratum moleculare of dentate gyrus, whereas the axons run into the hilus that is a layer below granule cells. Axons of granule cells (mossy fibers) terminate on hilar neurons as well as on the apical dendrites of CA3 pyramidal cells in the stratum radiatum. The zone where the axons of granule cell terminate in the stratum radiatum is called stratum lucidum. Axons of CA3 pyramidal cells establish connections through Schaffer's collaterals with the CA1 pyramidal cells.

The archicortical structure providing the major cortical input to hippocampus is the entorhinal cortex. The afferent pathway originating in layers II and III of entorhinal cortex is called perforant path, because it perforates the subiculum via its way to Ammon's horn and to the dentate gyrus. Axons of the perforant path establish connections with the most distal one-third of dendrites of granule cells in the molecular layer and with the terminal branches of pyramidal cell apical dendrites in the stratum lacunosum moleculare. The synapses of the perforant path on granule cells of dentate gyrus, the mossy fiber synapses on CA3 pyramidal cells and the synapses of Schaffer collaterals on CA1 pyramidal cells establish the major excitatory (glutamaterg) circuitry, the so called trisynaptic circuitry, of the hippocampus. One of the major efferent connections of the hippocampus runs to the deep layers (layers IV-V) of the entorhinal cortex. The others run to the subiculum and to lateral septal nuclei. The axons from the septum to the hippocampus and from the hippocampus to the septum are running in the fornix. In addition to septum, other subcortical structures (such as hypothalamus) are also

connected with hippocampus through the fornix. Two major types of medial septal nuclei establish separate connections with hippocampal neurons. The excitatory cholinerg fibers innervate all cell types in all layers, whereas the inhibitory GABAergic septal nuclei preferentially terminate oh inhibitory hippocampal cells.

Functional role of the hippocampal formation and involvement in disease

The wide cortical and subcortical afferent and efferent connections of the hippocampal formation as well as its one –way inner circuitry form the morphological basis for a specific function. The hippocampal formation plays an essential role in memory-related cognitive functions, memory and learning, influence memory formation, including space navigation, a specific type memory connected with navigation in space. The hippocampus plays a role in memory formation and possibly in transitional storage of memory in all mammals including humans. In humans, hippocampus plays an important role in verbal memory. In humans, the right hippocampus participate in visual memory -, whereas the left hippocampus in verbal memory formation. It has to be noted that to an intact declarative memory function, in addition to the intact hippocapus, the proper function of entorhinal, perirhinal and parahippocampal cortices is also necessary. Damage of hippocampus, subiculum and/or entorhinal cortex at both sides of the brain causes anterograde amnesic syndrome that cannot be influenced or corrected. Damages of individual parts of the hippocampal formation are charcteristic for memory related diseases such as Down's syndrome, epilepsy or Alzheimer's disease.

Development of the human hippocampus

Neurons and glial elements of the hippocampus are formed in the ventricular germinative zone and migrate to the final position. There is also a secondary germinal layer in the hippocampal formation which is the hilus of the dentate gyrus. Progenitors of granule cells migrate first from the ventricular zone to the hilus then proliferate again and migrate to the granule cell layer. In 15- week-old fetuses, the granular layer of dentate gyrus and the pyramidal layer of Ammon's horn are well recognizable. At the 22nd week, the ventricular zone is negligable and inside the hippocampal formation practically only the hilar region contains proliferating cells. After proliferation a long-lasting development of individual neurons follows. It is very difficult to characterize exactly, but the development of individual glutamatergic and GABAergic neurons (including their dendritic and axonal arbors) lasts until the 5-8th years. It is known that some types of memory formation (emotion-relatedmemories)

is present as early as the 3-5 months of age, but the characteristic, adult-like, long-lasting memory formation cannot be dated before the 5-8 years of age. It is very likely, that in addition to the development of individual neurons, development of myelination of the afferent and efferent pathways of the hippocampus influences memory formation.

It is known that myelination is essentially a postnatal phenomenon in the entire human cortex including the archicortex. It should be noted that development of the archicortex is faster than that of the neocortex therefore it is not surprising that myelination also starts earlier in the hippocampal formation than in the neocortex. A few MBP-immunostained axons can be shown in the subiculum, entorhinal cortex and CA1 area at birth. There are no data in the literature about the myelination of the human hippocampal formation, especially about the differences of the subregions inside the hippocampus.

Down syndrome

Down syndrome is caused by the trisomy of chromosome 21. It is frequent genetic disorder affecting one in every 700-800 birth. In addition to variable somatic developmental deficits, it always coincides with mental retardation. The IQ of the patients is between 20 and 80. In addition to mental retardation another tragic factor is the regularly appearing Alzheimer's diseases after the 30-35th years of Down syndrome patients.

The histopathology resulting in mental retardation is not known. In the literature, several authors concluded that shortly after birth the brain morphology of Down syndrome patients does not differ from the normal. The size, macroscopic morphology of the gyry and sulci are not differentiable from the normal, the histological structure of cerebrum and cerebellum, the neurotransmitter contents did not show significant differences. A few studies demonstrated minor morphological differences in the dendritic tree of individual neurons between Down syndrome and controls and a decrease in the cell proliferation in the cerebellar cortex was also shown.

In addition to cell proliferation and differentiation the volume of the brain is influenced by such factors as the myelination of the axons. Since myelination is a postnatal phenomenon and a descrease in the volume of the brain is detectable late after birth together with the fact that memory formation is a late postnatal phenomenon, we raised the question whether a decrease in myelin formation may play a role in the histopathology of the Down syndrome.

II. AIMS OF THE PRESENT STUDY

1. We wanted to establish the best histological method to describe myelination. The model that was used for this purpose was the corpus callosum (CC) in mice.
2. We wanted to study the effect of PACAP on postnatal myelination and therefore we have used normal and PACAP-KO mice strains.
3. We studied the time-course of myelination in the human hippocampal formation demonstrating the appearanace of MBP-positive axons and oligodendroglial cells.
4. We studied myelination in different parts of the human hippocampal formation in Down syndrome patiens compaired with that of controls.

III. MATERIAL AND METHODS

Animals

For the experiments we have used male control animals from the C57BL/6 mouse strain as well as PACAP-KO animals of the same age. The day of birth was considered as day 0. For light microscopic examination the animals were transcardially perfused with 4% buffered formaldehyde, the brain removed, embedded in paraffin and sectioned for 10 μm thick serial sections. For the electron microscopy the perfusion solution was a buffered 2.5 % glutaraldegyde and 4% paraformaldehyde solution. After perfusion, for electron microscopy small blocks of the corpus callosum was cut and embedded in Durcupan.

Human samples

The chromosomal diagnose of the Down syndrome and the following abortion was performed in the Department of Obstetrics and Gynecology of Pécs University. The proper documentation, with the consent of the patients was also done there. The autopsy was performed in the Department of Pathology except for the brain dissection that was done in the central Electron Microscopic Laboratory for detailed neuropathology. Hippocampi from control postnatal brains were donated by the Department of Pathology from authopsies where brain pathology was excluded. Brains of adult Down syndrome patients were also donated by the Pathology Department after the initial dissection. We have used 10 control fetal and prematurely born brains, 2 brains of children born at term

and 8 brains from different postnatal ages between birth and 17 years af age. Ten fetal and 10 postnatal Down's syndrome brains were used in this study. We got a few additional histological preparations of Down syndrome brains from the Department of Neuropathology of University of Vienna. By the use of brain tissue the regulations of Ministry of Health and the Declaration of Helsinki were followed. For light microscopy tissues were fixed in 4% paraformaldehyde solution. The tissue blocks were embedded in paraffin and sectioned for 10 μm. In addition to cresyl violet, Luxol Fast Blue staining mainly MBP-immunostaining was used for detection of myelination.

Luxol Fast Blue staining

Following deparaffination we used 0.1% solution of Luxol Fast Blue (LFB), followed by treatment with 0.05% litium-carbonate solution. After that sections were differentiated in 70% ethanol and cleared with xylene. For background staining we have used the classic cresyl-violet staining.

MBP-immunostaining

Sections were deparaffinated, rehydrated. Following antigen retrival (using microwave owen) sections were treated with 1% horse serum (Vector Burlingame , CA). The monoclonal mouse MBP-antibody (1:100, Novocastra, UK) was applied for 24 hours in room-temperature. Secundary anti-mouse antibody and avidine-biotin-peroxidase complex was applied (Vectastain, ABC Elite Kit, Burlingame, CA), followed by visualization with 3-3'diamino-benzidine (Sigma). For counterstaining we used the standard cresyl-violet stain.

Electron Microscopy

Tissue preparations of the mouse corpus callosum were treated with 1% osmium tetroxide followed by dehydration and embedding in Durcupan. Semithin and ultrathin sections were cut by Leica ultramicrotome. The semithin (1 micron thick) sections were stained with toluidine blue. The ultrathin sections were 50 nm thick, placed on copper mesh-grids and stained with uranyl acetate and lead cytrate. The sections were examined in JEOL 1200 EX-II electron microscope. Photographs were taken on negatives, and the developed negatives were scanned.

MBP-densitometry

In MBP-stained human tissue sections densitometry was applied using an AnalysSIS program on a Olympus BX 51 microscope.

IV. RESULTS

1. *Study of myelination in the corpus callosum (CC) of mice using light and electron microscopy*

On the 3rd postnatal day, large sized (13.3±1.73 μm) MBP-positive OLG cells with short thin processes were observed in the CC above the septum (septal level) and hippocampus (hippocampal level).

On the 5th day, the number of large sized (12.9±2.19 μm) MBP-positive OLG cells was increased in the CC below the fronto-parietal cortex, both at the septal and hippocampal level.

On the 7th day, large numbers of large (12.1±1.77 μm) MBP-positive OLG cells with multiple long cytoplasmic processes were visible both at the septal and hippocampal levels. In the vicinity of MBP-positive OLG cells, the first MBP-positive myelin sheets also appeared below and in layer VI. of the fronto-parietal cortex. The first MBP-positive OLG cells appeared below the cingulate cortex. LFB staining did not show myelination yet in the CC. In the electron microscope the intermediate-type OLG cells dominated in the CC at this age.

On the 10th day many large MBP-positive OLG cells with multiple cytoplasmic processes and a few immunopositive myelinated axons were found in the CC below the fronto-parietal cortex. The average diameter of the OLG cells was 12±1.68 μm that is still larger than the average size in adults. In addition to MBP-positive OLG cells there were also immunoreactive axons in the deep layers of cingulate cortex. In the electron microscope, intermediate-type and dark OLG cells were seen together with the first myelin sheets.

On the 14th day, the first LFB-stained myelinated axon-bundles appeared in the CC below the fronto-parietal cortex and a few were present in deep layers of cingulate cortex. In the whole extent of CC, large numbers of MBP-positive axons were obvious with a few small OLG cells (7.4±1.42 μm). There were large numbers of MBP-positive cells in cingulum and in the alveus. In the electron microscope both

intermediate- and dark-types OLG cells were present together with myelin sheets consisting of several lamellae.

On 28th day, LFB staining already showed a strong myelination in the CC. MBP-immunostaining revealed strong staining everywhere in the CC, in the cingulum and in the fimbria fornicis. In the electron microscope, most OLG cells appeared to be matured and myelin sheets were compacted.

2. *Study of myelination in PACAP-KO mice using*

On 3rd and 5th days MBP-positive OLG cells were already present in CC and in smaller numbers in the fimbria fornicis. There were no MBP-immunoreactive cells either in the neocortex or in hippocampus.

On 8th day the first MBP-positive myelinated axons appeared in the ventro-lateral part of CC, but their number was higher in PACAP-KO than in wild type mice. The first immunoreactive myelin sheets appeared in the layers V-VI of the cingulate cortex and in layer VI of the somatosensory cortex. In addition, strong myelination was detected in the CC and cingulate cortex of PACAP-KO mice as well as in the fimbria. There was still no reaction (neither OLG cells nor myelinated axons) inside the hippocampus either in PACAP-CO or in wild type mice.

On the 10th day, large numbers of MBP-immunopositive myelinated axons were seen in CC of PACA-KO mice together with large numbers of axons in several neocortical areas. The MBP-immunoreactive myelinated axons reached the most superficial layers of the cingulate cortex, but in all cortical territories the axons appeared to reach more superficial layers in PACAP-KO mice than in wild type. Myelination was strong in the fimbria, hippocampal stratum lacunosum-moleculare and alveus of PACAP-KO mice, weaker, but present in stratum oriens of CA1 and in strata pyramidale and radiatum of CA3 area. In contrast, wild type showed weak myelination in fimbria and a few axons in the alveus, and myelinated axons were seen neither in subregions of Ammon's horn nor in the dentate gyrus.

On the 21st day, the myelination appears to be adult like in all areas of CC in PACAP-KO mice. A similar progress in myelination was observed in the ventro-lateral part of the CC in wild type mice, wehereas the dorso-medial part of CC still displayed a few MBP-immunopositive fibers. There were more MBP-positive fibers in all layers of the neocortex as well as in the entorhinal cortex of PACAP-KO mice.

On the 44th day (young adult mice), the myelination reached adult-like level in the CC as well as in the neocortical areas, although there appears to be more MBP-immunoreactive axons in the cingulate, somatosensory and entorhinal cortex of PACAP-KO mice than in wild type.

On the 60th day, there were no further changes compared to the previous group. In both wild-type and PACAP-KO mice, the density of MBP-immunostaining was similar, except for the somatosensory cortex, where we have seen less fiber in wild-type animals than in PACAP-KO mice.

In order to control whether only the MBP amount has changed or the entire myelination is altered, we applied LFB staining and electron microscopy in 10 and 15 days old PACAP-KO and wild-type animals. With LFB staining we have seen more intensive staining in the CC of PACA-KO mice than in wild-type. In contrast to the wild-type, in the PACA-KO animals several large diameter myelinated axons were observed with the electron microscope in the CC at the 10th day. These results support that differences seen with MBP-immunostaining reflects differences in myelination.

3. *Myelination in the human hippocampus*

Myelination in the fetal and prenatal periods in controls

The first MBP-stained OLG cells appeared in the fimbria fornicis and in alveus at the 20-22nd fetal weeks. These cells were large displaying a few short cytoplasmic processes. In the following weeks their number increased and the first immunopositive cells appeared in stratum lacunosum-moleculare of CA1 area. On the 37th week, the number of MBP-immunoreactive OLG cells was larger everywhere in the hippocampal regions than in the previous weeks. At this age a few thin immunopositive myelinated fibers were also observed, therefore the beginning of axonal myelination in the fimbria or in the layers of the hippocampal subregions probably starts at or around the 37th week. The number of immunoreactive cells was increased at term both in the fimbria and alveus. In addition, there was a considerable number of MBP-positive OLG cells in stratum lacunosum-moleculare of CA1 area, together with the first similar cells in the same layer of the CA3 area. These data suggest that myelination of perforant path starts before birth between the 37-39th weeks, whereas afferents from septal and other subcortical areas may myelinate a few

weeks earlier. Since inside the pyramidal layers and close to pyramidal cells, myelination is negligable at birth it is very unlikely that hippocampal efferents myelinate at this age.

Myelination in the fetal and prenatal periods in Down syndrome

Similarly as in controls, the first MBP-immunoreactive OLG cells appeared in fimbria at the 20th fetal week, but no immunoreactive cells could be observed in the alveus, and CA1-3 areas of Ammon's horn either in 20 or in 22 weeks old Dwn syndrome fetuses. The number of MBP-immunoreactive cells was increased until the 37th week both in the fimbria and alveus, and the first immunopositive myelinated fibers also appeared in both of these regions. It should be noted, however, that the length and thickness of immunoreactive fibers was smaller than in controls. In contrast to controls, there were no MBP-positive OLG cells and fibers in the stratum lacunosum-moleculare of CA1 area.

Postnatal myelination in controls

During the first postnatal weeks myelination proceeded in stratum oriens and stratum lacunosum-moleculare. In contrast, the first MBP-positive OLG cells and a few thin immunoreactive fibers appeared in the pyramidal layer of Ammon's horn only at the 3rd month. In addition to CA3 area, the first myelinated fibers appeared in stratum radiatum of CA1 area, in the hilus and in the outer two-thirds of the molecular layer of the dentate gyrus.

At the 5th month, the number of immunoreactive fibers and OLG cells increased in all layers mentioned above. Interestingly, there are more immunoreactive cells and myelinated fibers in CA3 than in CA1 area. In the dentate gyrus, the number of MBP-positive OLG cells and fibers increased around 1 year of age in most layers of Ammon's horn. In the fimbria the density of myelination reached a plateau level, but the molecular layer and the hilus of dentate gyrus showed only low density. The difference in density could still be observed in the strata radiatum and pyramidale of CA3 and CA1 areas. In contrast, the stratum lacunosum-moleculare of CA1 area showed higher density than that of CA3. In the dentate gyrus the first myelinated axons of the molecular layer appeared in close vicinity of granule cells, suggesting that they do not belong to perforant path. In the second year, myelination in the stratum lacunosum-moleculare of CA1-3 areas reached the level that is seen in older

ages and in adults, whereas in other regions the density increased, but still not reached the adult level. At the 8th year practically in all sublayers the density of myelinated fibers reached the adult level, except for the CA1 area, where strata radiatum a pyramidale still expressed lower density than in adults. At the 11th year of age all layer displayed an adult like density of myelination except the hilus of the dentate gyrus. We don't have samples from young adults therefore we only assume that myelination further continues in puberty.

Postnatal myelination in Down syndrome

The number of MBP-immunoreactive OLG cells and myelinated fibers increased between birth and 2 months of age. The morphology of OLG cells was similar as in the control hippocampus at the same age. In the stratum lacunosum-moleculare of CA1-3 areas, less myelinated axons were present in Down syndrome than in control hippocampus. In contrast to controls, there were no MBP-positive cells in stratum pyramidale of CA3 of Down syndrome at this age. In the dentate gyrus the first MBP-positive OLG cells appeared in the molecular layer.

At the 5-6th month the first myelinated axons appeared in the hilus of the dentate gyrus and in the pyramidal layer of CA1-3 areas. The density of immunoreactive fibers was higher in the outer one-third of the molecular layer. In general, the length and thickness of immunoreactive myelinated fibers were smaller in Down syndrome than in the control hippocampus.

At the 8th and 11th months strong immunoreaction was seen in the fimbria fornicis and in alveus. The hippocampal layers showed lower density in all layers. When compared with controls the density of myelinated fibers was lower in Down syndrome.

At 2 years of age, similarly as in controls, the density of immunoreactive fibers was homogeneous and adult-like in stratum oriens of the CA1-3 areas. In strata radiatum and pyramidale of CA1-3 areas as well as in the hilus and molecular layer of dentate gyrus there were less immunoreactive fibers than in controls.

Between 8 and 11 years of age density of MBP immunoreaction reached a plateau level, characteristic for further age groups and adults, exept for the hilus of dentate gyrus where density was lower than in adults. Density of MBP-positive fibers were smaller in the hilus of Down syndrome than in controls and this difference persisted in adults as well.

Measurement of density of MBP-immunoreaction

The density of MBP-immunoreaction correlated with age in Down syndrome hippocampi (R=0.85) as well as in controls (R= 0.7). In stratum moleculare of the dentate gyrus a positive correlation was found both in Down syndrome (R=0.73) and control hippocampus (R=0.7). In contrast, analysis of immunodensity showed only a week correlation in the stratum lacunosum-moleculare of CA1-3 areas in Down syndrome (R=0.35) and controls (R=0.27).

V. DISCUSSION

1. Myelination in mice

In this work we have used three different methods to study myelination, the classic hittochemical method of LFB, MBP-immunocytochemistry and electron microscopy. We concluded that results obtained with the light microscopic MBP-immunocytochemistry correlare well with the results of electron microscopy. During development a few of the oligodendroglial cells and their fine cytoplasmic processes are MBP-immunopositive, although at that stage myelinated axons are not observed yet. Between the postnatal 3-10 days the average diameter of an OLG cell was found larger than in later age-groups, an observation that was previously mentioned in the literature. These MBP-immunoreactive OLG cells have 12-13.3 μm large diameter and correspond to the intermediate- type cells observed in the electron microscope. Previous electron microscopic studies suggested that intermediate-type OLG cells are capable to form myelin sheets. We can support this notion, because these cells already produce MBP and probably also other proteins found in myelin. In the electron microscope the first sign of myelination is the repsence of an axon embraced by OLG processes, or surrounded by a few lamellae of myelin sheet. In the light microscope MBP-immunostaining have shown the first very thin myelin at the same time and at the same location where electron microscopy could reveal. We conclude that MBP-immunostaining is sensitive enough to visualize very thin myelin sheets composed only by 2-3 lamellae around a single axon. In contrast, LFB staining is only positive when large number of myelin is present in myelinated axon boundles are present, but

not individual myelinated axons. Therefore, especially in developmental studies MBP-immunostaining is far superior to LFB staining.

With all three methods used differences could be observed in the time-course of myelination among the different parts of the brain. In the CC the first OLG cells and myelinated axons appeared below the somatosensory cortex followed by the myelination of the cingulate cortex. In general our results are in harmony with previous observations that sensory pathways are myelinated before the motor pathways and the associational connections are myelinated latest. It could also be observed that in archicortical regions myelination starts earlier than in neocortex. Myelination follows developmental pattern of the neocortical layers, because the first myelin sheets appeared in layers V-VI.

2. Myelination in PACAP- deficient *mice*

In this study we applied MBP-immunostaining to reveal differences in myelination between control and PACAP- deficient mouse strains. In PACAP-deficient mice myelination started significantly earlier in all brain region when compared with age-matched wild-type mice. In addition, the MBP-immunostaining was stronger in PACAP-deficient mice suggesting presence of a larger amount of myelin. In order to control this notion we used LFB staining for the comparison. In addition, we compared myelination in the same region of corpus callosum in the electron microscope. These studies concluded that in PACA-deficient mice the number and thickness of myelin sheets are larger that suggests that in wild-type mice PACAP (or any related chemical agent) inhibits myelin formation.

In general the myelination pattern among different brain areas was the same in PACAP-deficient and wild-type mice: sensory pathway myelinated earlier than motor pathways folowed by the associational areas. Among the layers of neocortex, deep-layers myelinated at first following the inside-out cell migrational and maturational pattern of cortical neurons.

Our results suggest that endogenous PACAP *in vivo* inhibits maturation of OLG cells and the result is a delay in the start and termination of myelination process in the mammalian brain. It is known, that certain protein components of the myelin sheet inhibits regeneration of the damaged axon. Therefore axonal growth and synapse formation should precede myelination. Since PACAP enhance synapse formation and the growth of dendrites it is reasonable to assume that diminished

myelination correlates with the above-mentioned effects. It may also be concluded that lack of PACAP decreases the plasticity of the neurons and neuronal connections in the central nervous system.

3. Myelination in the human hippocampal formation

Myelination starts in the human hippocampus at the 20th fetal week and lasts at least for 2 decades into early adolencence. In addition, myelination follows the maturation of the major afferent and efferent pathways that can be detected with examination of the fornix. The first MBP-immunoreactive OLG cells and myelinated axons appeared in the fimbia fornicis adjacent to Ammon's horn. The fornix includes afferent and efferent axons connecting the hippocampus with hypothalamic and basal forbrain nuclei. Hypothalamus and the septal nuclei are among the earliest developing brain structures. Myelination starts similarly early in the alveus that contains axons from septum, locus coeruleus, raphe nuclei and from the anterior thalamic nuclei which are all early developing brain regions. In the last trimester of pregnancy, myelinated axons appeared in stratum oriens of Ammon's horn, where among others the septal and amygdalar afferents terminate. In the stratum lacunosum-moleculare of the CA1 area the first myelinated axons appeared at the 37^{th} week. Earlier studies described the first synapses at the 15^{th} week in this region, suggesting that in the terminal field of perforant path the beginning of synapse formation is followed by the axonal myelination with an approximate 20 weeks delay. It is interesting to note, that perforant path also innevates the apical dendrites of granule cells of the dentate gyrus, but myelination in that termination field (molecular layer) starts only in the third postnatal month. We know, that axons of perforant path to Ammon' horn and dentate gyrus did not originate from the same neurons of the entorhinal cortex, but from adjacent layers (layers II and III). In addition, we can assume that a late development of the granule cells also influence myelination. The myelinated axons in the hilus of the dentate gyrus arrive through the fornix and probably mainly originate from the early differentiating basal forebrain cells. In contrast, the first myelinated axons in the hilar region appear only postnatally. In the hilus, the major cellular component, the mossy cells morphologically develop for almost a year postnatally, because their afferents, the granule cells, gradually increase the synaptic input to these cells. We may assume that the extended period of granule cell development (both axons and

dendrites) influence the establishment of synaptic connections and as a consequence the beginning of myelination of the afferent fibers.

There is strong afferent-efferent connection between septal nuclei and hippocampus. From the medial septal nuclei cholinergic and GABAergic neurons project to the hippocampus, including the hilus. The GABA-ergic axons are thick, and myelinated, therefore we may assume that with the time course of myelination in the hilar region, we follow the maturation of septo-hippocampal pathway although raphe nuclei and locus coeruleus also project to the hilus but with thinner fibers. Due to special innervation of hippocampal GABAergic cells through the GABAergic septal neurons the input will result in a strong stimulation of excitatory cells of hippocampus. Therefore the medial septum is called the pace-maker of hippocampus. Function of an axon bundle depend on the speed of conducting velocity, therefore it is plausible to assume that the gradual myelination of septo-hippocampal pathway influence the functional capacity of the hippocampus proper. Myelination starts about 3 month postnatally and lasts until the 5^{th} -8^{th} years that correlate with the functional capability if we consider memory formation as a main function of hippocampus. This correlates with the delayed development of memory formation lasting until school-age. Myelination is not equally proceeding in all sublayers of Ammon's horn and in those of the dentate gyrus. However, myelination probably continues into adulthood, but on histological sections using immunocytochemistry we cannot detect an increase in density after a certain point. MRI studies described an increase of the volume of hippocampus until adulthood that supports or hypothesis that myelination lasts until adulthood, since myelination can be one of the factor that causes an increase in volume.

4. Myelination of the hippocampus in Down syndrome

Our results suggest that myelination of the hippocampal formation is decreased in the Down syndrome patients. The OLG cells appear in both controls and Down syndome patients approximately at the same time myelination appears to be weaker in all layers. The most visible difference appears in the early postnatal period. The myelination pattern in the different subregions of the hippocampal formation do not show differences from controls. The progress in myelination appered to be similar in control and in Down syndome patients, but the number of OLG cells and the amount of MBP- immunostained axons were smaller in the trisomy 21. A decreased

myelination has previously been described in the gray and white matter of neocortex, in corpus callosum and in nucleus of caudate-putamen using the Klüver Barrera staining. In Down syndrome, previous studies noted a decrease in MBP and CNP-ase expression in cortical areas as well as in subcortical regions.

After the intensity of immunostaining reached a plateau level, further differences cannot be observed between myelination of the different hippocampal regions in controls and in Down syndrome, except for the hilar region. In the hilus of the dentate gyrus, the difference in myelination remains obvious even in adulthood, probably due to a mesh-like loose structure of the hilar myelinated axons. In Down syndrome, areas a decrease in the number of neuronal elements was described in several cortical, such as in the frontal, temporal, parietal, occipital cortices. Considering the size of the human brain, estimation of cell numbers is extremely difficult, but it can be assumed that smaller number of neurons project from subcortical areas to the hilus. In addition, it is also possible that the number of axons did not change, but myelination is retarded. In both cases, the decrease in functionally capable septo-hippocampal axons may well explain the mental retardation of Down syndrome patients, since memory formation should be retarded in both cases from the birth on.

VI. CONCLUSIONS

1. Our studies in mice provide evidence that light microscopic MBP-immunostaining and electron microscopy give identical results about maturation of OLG cells and myelin formation. In contrast, LFB staining is less sensitive. Sensory pathway myelinate before motor pathways and both precede the myelination of associational pathways. Myelination in the archicortex, including the associational area that belongs to the limbic system starts earlier than myelination in the neocortex. Inside the cortex, the first myelinated axons appear in deep layers corresponding to the developmental pattern of neocortex.
2. In PACAP-deficient mice myelination starts earlier than in wild-type. Myelinated axons appear to be shorter and thinner in wild-type than in age-matched PACAP-deficient mice. Our results suggest that endogeneous PACAP may inhibit myelination in the central nervous system during development.

3. In the human hippocampal formation myelination starts relatively early about half-time of pregnancy. However, the long lasting myelination that can be followed in histological preparations through several years until the 5^{th} -8^{th} years suggest together with extended neuronal development the long-lasting myelination which participate in the slow functional maturation (adult-like memory formation) of the hippocampal formation.
4. In Down syndrome, myelination of the hippocampal formation is significantly decreased during postnatal development. In some hippocampal region, such as the hilus of dentate gyrus the loose structure and low density of myelinated axons allow us to observe a difference between control and Down syndrome patients even in the adulthood between 40 and 60 years. A decreased myelination of the afferent pathways to the hippocampus in Down syndrome may result in an impaired memory formation and learning after birth. This may explains the mental retardation observed in trisomy 21.

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PUBLICATIONS RELATED TO DISSERTATION:

1. Vincze A, Mázló M, Seress L, Komoly S, Ábrahám H. A correlative light and electron microscopic study of postnatal myelination in the murine corpus callosum. Int. J. Dev. Neuroscience. 2008. 26:575-584. IF: 1.869

2. Ábrahám H, Vincze A, Jewgenow I, Veszprémi B, Kravják A, Gömöri E, Seress L. Myelination in the human hippocampal formation from midgestation to adulthood. Int. J. Dev. Neurosci. 2010. 28:401-410. IF: 1.938

3. Vincze A, Reglődi D, Helyes Zs, Hashimoto H, Shintai N, Ábrahám H. The role of endogenous PACAP on myelination in the rodent brain. Lessons from PACAP deficient mice. Int. J. Dev. Neurosci. 2011. 29 :923-935. IF: 1.938 (2010)

4. Ábrahám H, Vincze A, Veszprémi B, Kravják A, Gömöri E, Kovács GG, Seress L. Impaired myelination of the human hippocampal formation in Down-syndrome. 2011. doi:10.1016/j.ijdevneu.2011.11.005 IF: 1.938 (2010)

PUBLICATIONS NOT RELATED TO DISSERTATION:

1. Kasza G, Kollár L, Rőth E, Vincze A, Gömöri É. Histological examination of vascular lesions resulting from stent implantation in humans and in comparative experimental animal model. Acta. Biologica. Hungarica. 2011. (in press) IF: 0.793 (2010)

2. Vincze A, Kapás I, Molnár MJ, Kovacs GG. Clinicopathological variability in neurodegeneration with brain iron accumulation. Clinical Neurosci.. 2010. 63:129-35.

ABSTRACTS RELATED TO DISSERTATION:

1. Vincze A, Mária M, Seress L, Komoly S, Ábrahám H. Comparative study of myelination in the developing murine corpus callosum using immunohistochemistry, electron microscopy and conventional myelin staining. IBRO International Workshop, Debrecen, 2008. január 24-26. Abstract: Clinical Neurosci.. 2008. 61(S1): p. 69.

2. Vincze A, Veszprémi B, Kravják A, Gömöri É, Seress L, Ábrahám H. Myelination in the developing human hippocampal formation. MITT XII. Meeting, Budapest, 2009. january 22-24. Abstract: Front. Syst. Neurosci. doi: 10.3389 / conf.neuro. 01.2009.04.081

3. Vincze A, Kravják A, Veszprémi B, Seress L, Ábrahám H. Myelination of the human hippocampal formation in Down-syndrome. IBRO International Workshop, Pécs,

2010. january 21-23. Abstract: Front. Neurosci. doi: 10.3389/conf.fnins.2010.10.00018

4. Vincze A, Reglődi D, Helyes Zs, Hashimoto H, Shintani N, Ábrahám H. Myelination in the pituitary adenylate cyclase activating polipeptid (PACAP) deficient mice. MITT XIII. Meeting, Budapest, 2011. January 20-22. Abstract: Front. Syst. Neurosci. (in press)

5. Vincze A, Reglődi D, Helyes Zs, Hashimoto H, Shintani N, Ábrahám H. Az endogén PACAP szerepe a központi idegrendszer myelinizációjában PACAP-deficiens egérben végzett vizsgálatok alapján Conference of the Hungarian Anatomz Society, Pécs, 2011. 06. 20-22.

ABSTRACTS NOT RELATED TO DISSERTATION:

1. Vincze A, Budka H, Kovács GG. Correlation of taupathy and alpha-synucleinopathy with tissue lesioning in neurodegeneration with brain iron accumulation (NBIA). XVIIth International Congress of Neuropathology, Ausztria, Salzburg, 2010. September 11-15. Brain. Pathology. 20(S1): 45.

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