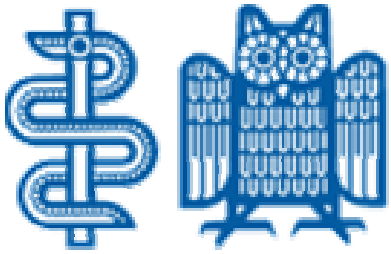
Division of Neurobiology, Department of Psychiatry
Saarland University Clinic, Homburg/Saar
Director: Prof. Dr. Thomas A. Bayer



Impact of the Traumatic Brain Injury on development of Alzheimer's disease.
An experimental study in APP transgenic mice.

Doctoral thesis
to be presented with the permission of the Medical Faculty
of the Saarland University
in conformity with the requirements for the degree of the Doctor of Medicine.

submitted by Jacek Szczygielski
born on 22.08.1978 in Łuków, Poland

Homburg, 2007

To my Wife, my Son

and my Parents

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LIST OF ABBREVIATIONS

Aβ	beta-amyloid
ABC	avidin–biotin complex
AD	Alzheimer's disease
ADRDA	Alzheimer's Disease and Related Disorders Association
ALS	amyotrophic lateral sclerosis
APH-1	anterior pharynx defective-1
APOE	apolipoprotein E- gene
apoE	apolipoprotein E
APP	amyloid precursor protein
APPswe	amyloid precursor protein bearing swedish mutation
APP-SL	amyloid precursor protein bearing Swedish and London mutations
BACE	beta-site-APP-cleaving enzyme
BB	beam balance test
C99APP	carboxy-99 fragment of APP
CA3	CA3 region of the hippocampus
CAA	cerebral amyloid angiopathy
CCA	caspase-cleaved APP fragments
CCI	controlled cortical impact
CHI	closed head injury
CNS	central nervous system
CSF	cerebrospinal fluid
CTF	carboxy-terminal fragment
DAB	3,3-Diaminobenzidine-tetrahydrochloride

DABCO	1,4-Diazabicyclo[2.2.2]octane
dAD	definite Alzheimer's disease
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
D-BB	change in beam balance test score
DF	degenerative fibers
DNA	deoxyribonucleic acid
D-NSS	change in neurological severity scale score
DP	dementia pugilistica
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
F	female
FCS	fetal calf serum
FGF	fibroblast growth factor
GAP	growth associated protein
GFAP	glial fibrillary acidic protein
Glu	glutamate
GSK	glycogen synthetase kinase
hAPP	human amyloid precursor protein
HSP	heat shock protein
ICD-10	International Statistical Classification of Diseases and Related Health Problems, tenth revision
IL1-β	interleukin-1-beta
i. p.	intraperitoneally
KPI	Kunitz protease inhibitor
LH	luteinising hormone
LOC	loss of consciousness

M	male
MAP	microtubule associated protein
mCHI	mild closed head injury
ML	medial lemniscus
MLF	medial longitudinal funiculus
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
mTBI	mild traumatic brain injury
MWM	Morris water maze
NFT	neurofibrillary tangle
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NSS	neurology severity scale
pAD	probable Alzheimer's disease
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDAPP	platelet-derived growth factor promoter expressing amyloid precursor protein
PDC	parkinsonism / dementia complex of Guam
PDGF-B	platelet-derived growth factor-B
PEN-2	presenilin enhancer-2
PEP	postencephalitis parkinsonism
PET	positron emission tomography
PHF	paired helical filament
PrP	prion protein
PS-1	presenilin-1
PSP	progressive supranuclear palsy

PBS	phosphate buffered saline
rmTBI	repetitive mild traumatic brain injury
sAPPα	secreted amyloid precursor protein alpha
S-GAG	sulphated glycosaminoglycane
SOD	superoxide dismutase
SPF	specific pathogen free
SPECT	single photon emission computed tomography
TAI	traumatic axonal injury
TBE	TRIS-boric acid-EDTA buffer
TBI	traumatic brain injury
Tg	transgenic
Thy-1-APP_{751}SL	Thy-1 promoter expressing 751-amyloid precursor protein bearing Swedish and London mutations
UV	ultraviolet
WHO	World Health Organization
WT	wild type
YAC	yeast artificial chromosome
XX	double chromosome X karyotype
XY	chromosome XY karyotype

1. SUMMARY

Traumatic Brain Injury (TBI) has been established in epidemiological studies to be a risk factor for subsequent development of Alzheimer's disease (AD). In some analyses, the risk of AD development was increased by head trauma only in male population. However, the women are more susceptible to develop AD in general, regardless of its background.

AD is a devastating brain disorder characterised by neurofibrillary tangles and Aβ amyloid plaques. Aβ is derived from the larger amyloid precursor protein (APP). Overexpressing of the human mutant APP in the brain of transgenic mice leads to age–dependent Aβ amyloid plaque formation. Previously published reports using APP transgenic mice in neurotrauma research provided conflicting results. Also no analysis of gender influence has been presented. Therefore, a new procedure for posttraumatic injury and possible impact on AD pathology in mice has been developed in the present thesis. The Closed Head Injury (CHI) model was adapted for repetitive mild- to-moderate TBI and applied to anaesthetised 3 month-old $APP_{751}SL$ transgenic or wild type (WT) mice of both sexes. The neurological assessment was performed before trauma and at regular intervals during the following 4 weeks. After this period the animals were sacrificed and their brains were analysed using Aβ40 ELISA, as well as standard histopathological and immunohistochemical methods.

Pre-injury assessment revealed poorer neurological performance in female APP(+) vs. WT animals, which, however, didn't impact the posttraumatic course and outcome. Histopathological sequelae of trauma didn't vary significantly between the mice of different gender and genotype. Also no differences in Aβ deposition could be observed among APP(+) mice. However, biochemical analysis in APP(+) animals revealed a trend towards increased Aβ40 content in female vs. male mice in sham, but not in TBI group. The latter result suggests that a gender- related difference in posttraumatic AD–risk may be potentially reproduced in animals. However, the number of factors makes the temporarily available transgenic murine models of Alzheimer's disease to be not an optimal background for neurotrauma studies. The role of these factors and results of current and previous similar studies have been critically discussed.

2. INTRODUCTION

2.1 Nosological position of Alzheimer's disease

Alzheimer's disease (AD) according to nosological criteria is the primary degenerative disease of the brain. The dementia developing in course of AD is classified in the International Statistical Classification of Diseases and Related Health Problems, tenth revision (ICD-10) among other mental and behavioural disorders (F 00_0-2,9).[2]

The World Health Organisation defines AD as 'a degenerative brain syndrome characterized by a progressive decline in memory, thinking, comprehension, calculation, language, learning capacity and judgement',[1] requiring, however, an differentiation from normal age-related decline in cognitive functions which is more gradual and leads to much milder disability.[183]

2.2 Clinical and histopathological features of Alzheimer's disease

2.2.1 Clinical manifestation

A variety of clinical features may be observed in the course of AD. They include gradual onset of dementia, progressive cognitive decline, memory loss associated with executive and other cognitive deficits, and impaired functional abilities.

According to Morris, AD can be described as insidiously developing debilitating process.[183] Normally, initial AD progresses for several years, before any medical attention is paid. The cardinal feature in early AD is memory loss which is represented by uncharacteristic forgetfulness and difficulties in aquisition and retreival of newly learned information. In contrast, highly learned material (from past years or rather decades) may be recalled without problems. Inintially, simple everyday activities (e.g. dressing, bathing and grooming, toileting) remain spared from impairment, but more complex tasks such as driving, operating appliances or balancing a checkbook may be performed less effecively. Language skills may be affected by word-finding difficulties, hesistancy of speech, and decreased verbal output. Personality changes at this stage are not prominent. Neurological and psychiatrical signs are usually absent. In general, an early stage AD patients appear healthy to casual medical inspection and usually remain self-dependently engaged in many activities of daily living that do not require high intelectual and memory effort.

As AD progresses, memory disability becomes profound at moderate stages of the disease. Rapid forgetting of new information and frequent inaccuracies in recall of established memories are main characteristics of this stage. The social function is

impaired by difficulties in recollection of relationship and identity of relatives or friends. Gradually a patient may become lost even in familiar surrondings, tolerating poorly any changes in his/her environment. Also judgement and problem solving skills decline further: all complex activities cannot be performed. Additionally, appropriate accomplishing of simple tasks including self-care activities requires help or supervision, so that patient gradually becomes fully dependent in daily living. Deterioration in language is expressed in circumlocutory or incomplete sentences and poor comprehension of speech and writing. At this stage some personality changes and psychiatric signs may become apparent: An affected person may present disruptive behaviours, agitation, restlessness, day-night disorientation with sleep disturbances, aggressive verbal or physical behaviour, suspiciousness and delusions (e.g. in false belief that misplaced item has been stolen) and hallucinations. Generalising, the moderate stage of AD can be characterised by gradual loss of independence from caregivers with retrieval from social function. A growing burden of behavioural and psychiatric disturbances may prompt institutionalisation of the AD patients.

In the severe stage of AD only memory fragments remain. Even closest persons cannot be properly identified and only emotional recognition may occur. Not only remnant social function, but also all semblance of the patient's personality eventually disappear. Language skills are usually sparse: extremely limited output (short phrases and repetition of words) and comprehension of only simplest spoken language can be present. Behavioural disturbances may be represented by screaming, however, with the progress of the AD even this primitive activity is vanishing. Characteristic for this stage are neurological disturbances, including motor complications (e.g. extrapyramidal dysfunction, generalised tonic-clonic seizures and falls), urinary and fecal incontinence and dysphagia. Terminal stage of AD is represented by bed-ridden, uncomprehending patients. Death usually results from one or more complications associated with chronic severe debilitation (pulmonary embolus, pneumonia, urosepsis, aspiration or inanition). Median duration of AD (from the onset to death) is 9-10 years, with a wide variability seen.[183]

2.2.2 Diagnosis and criteria for Alzheimer's disease

Despite numerous attempts to establish a reliable molecular or neuroradiological marker of 'definite Alzheimer's disease' (dAD) in living patients, such diagnosis still remains a combination of clinical picture of progressive dementia and post mortem confirmation basing on the histopatological analysis of brain tissue. Instead, more or less 'probable Alzheimer's disease' (pAD) may be diagnosed in individuals, basing mainly on clinical criteria, while laboratory and imaging techniques remain complementary. These criteria have been established by McKhann et al. and accepted by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) (see Table 2.1.).[173] Basing on the NINCD/ADRDA criteria three levels of diagnostic certaininty may be distinguished:

- *possible AD* for patients with non typical AD dementia or with coexistent potentially dementing disorder, that however is believed not to be primarily responsible for dementia;
- *probable AD* for patients with AD-like dementia and deficits in $\geq$ 2 cognitive areas and with no other diseases that could cause dementia;
- *definite AD* for patients, in whom clinical diagnosis has been confirmed by histopathological biopsy or autopsy studies of the brain.

Estabilishing of these criteria carried a few advantages. First, they enable a physician to diagnose and treat AD in certain patients with dementia before histopathological confirmation (most often resulting from autopsy study) is available. Second, due to them a standardisation of epidemiological analyses in AD may be performed. Third, these criteria create an excelent tool for clinical research as eligibility criteria for studies. Importantly, NINCD/ADRDA criteria for probable AD have documented reliability and validity (reaching app. 90% of neuropathological confirmations of clinically diagnosed probable AD).[183]

Table 2.1: NINCD/ADRDA criteria of diagnosis of probable Alzheimer's Disease.
Clinically probable Alzheimer's disease
Dementia established by examination and documented by objective testing
Deficits in two or more cognitive areas
Progressive worsening of memory and other cognitive functions
No disturbance in consciousness
Onset between 40 and 90 years of age
Absence of systemic disorders or other brain disease that could account for the progressive deficits in memory and cognition
Diagnosis supported by:
Progressive deficits in langauage (aphasia), motor skills (apraxia), and perception (agnosia)
Impaired activities of daily living and altered patterns of behaviour
Family history of similar disorders
Consistent laboratory results (e.g., cerebral atrophy on computed tomography)

2.2.3. The neuropathology of Alzheimer's disease

2.2.3.1. Macroscopic changes

Gross changes in AD involve not only the brain itself, but also meninges and cerebral vasculature. The leptomeniges over convexity and near the midline are often thickened, due to fibroblastic infiltration with collagen addition and deposition of amyloid (the amino acid sequence of which slightly differs from this of parenchymal deposits). Amyloid infiltration particularily affects the meningeal vessels, which often are involved in this process more prominently than parenchymal vessels.

The general brain atrophy is seen as decrease in average brain weight and shrinkage of the gyri. Gyral atrophy may be apparent in the frontotemporal areas, parietal lobe and on the ventral surface of the temporal lobe (involving in particular parahippocampal gyrus). In some cases this atrophy seems to affect all brain regions, but, in overall comparison occipital lobe is often spared. Also a cerebellar vermis may be shrunken; however, this phenomenon is atttributed to general malnutrition seen in advanced AD. The cranial nerves are usually spared (with exception of the olfactory bulb and tract that may also be atrophied).

In its typical form, AD changes in cross sections of the brain also are displayed mainly in cortex and hippocampus, while basal ganglia remain usually unaffected, save potential ischemic foci. The cortical ribbon is thinned and some atrophic changes may be seen in centrum semiovale. The demarcation between white matter and cortex is

however preserved. Variable degree of pigmentation loss may be seen in locus coeruleus and substantia nigra, the latter phenomenon being observed rather in Lewy body variant of AD.[270]

2.2.3.2 Microscopic lesions

2.2.3.2.1. Amyloid plaques and amyloid angiopathy

One of the typical pathological lesions described already in 1907 in the first reported case of AD are amyloid plaques. They use to be regarded as characteristic for AD, nevertheless, some specimens from cognitively normal, non-demented aged people have displayed AD-like abundance of plaques. The main component of the plaques, beta-amyloid is classically described as an extracellular, filamentous, congophilic protein, that is however chemically different from systemic amyloid. Plaques, also those found in one individual may vary significantly in their morphology. Basically, two forms of plaques may be distinguished:

So called diffuse plaques are characterised by absence of abnormal neurites. They contain nonstructured amyloid peptides and have only minute wisps of formed filamentous amyloid. They may be visualised using both silver staining as well as thioflavin-S staining techniqe.

The neuritic plaques, in turn, contain well- structured dense bundles of 9-nm, thioflavin-S-positive amyloid fibrils. However, their most characteristic components are dystrophic neurites. -Biochemical analysis of these neuronal fibers reveals content of APP, growth associated protein (GAP43- implying regenerative, not only degenerative processess involving the neurites), protein kinase C, tau protein, ubiquitin, brain spectrin, epidermal growth factor receptor (EGFR), neurofilaments, synaptophysin and chromogranin, and various neurotransmitters (including substance P and acetylcholine). The amyloid core of the plaques and peripheral fibrills are, in turn, highly immunoreactive with anti- Aβ antibodies. Additional components of both diffuse and neuritic plaqes are α1-antichimotripsin, protein kinase C, complement proteins, apolipoprotein E, fibroblast growth factor (FGF) and sulfated glycosaminoglycanes (S-GAG). The probably crucial element of the plaques and their surrondings are reactive astrocytes and microglial cells. In particular, microglial cells have been implicated in amyloidogenesis and neurodegeneration, as possibly taking part in amyloid production, formation of the mature neuritic plaques or phagocyting amyloid. Notably, microglial activation is observed not only in proximity of the plaques but is widespread in the cortex of AD-cases.[270]

2.2.3.2.2. Neurofibrillary tangles and neuropil threads

The second classical feature of AD is the presence of neurofibrillary tangles. In contrast to plaques, there is a little doubt on their pathological role in dementia, since *first* they are very uncommon in neocortices of mentally normal elderly and *second*, their presence has been confirmed in numerous disorders other than AD with core neurologic and / or psychiatric symptomatology (e.g. postencephalic Parkinson's disease, dementia pugilistica, hydrocephalus associated with mental retardation, subacute sclerotic panencephalitis). Nevertheless, some cases of AD represent so called plaque- only, or plaque- predominant form of AD, where NFTs are absent or very sparse, respectively.

Most AD cases display abundance of NFTs, localised mainly in entorhinal and hippocampal areas, as well as in neocortex, locus coeruleus and dorsal raphe. On the cytological level, presence of NFTs is characteristic for larger neurons, while small and medium ones remain spared. NFTs are located in their cytoplasm, presenting form of flame-shaped or globoid masses, with affinity for silver, Congo red and thioflavin S staining. In the entorhinal cortex and hippocampus, but only rarely in neocortex, remnants of the NFTs may be found in form of fiber masses without accompanying organelles of the neurones that have died.

The main ultrastructural component of the NFTs are paired helical filaments (PHFs), made of tau protein. Additional constituents of the NFTs, as may be stated by immunostainting, are: A-68 protein, casein kinase II, protease nexin I, fibroblast growth factor (FGF), microtubule- associated protein 5 (MAP-5), ubiquitin and Aβ.

The pathological role of NFTs results from disturbance of axoplasmic transport and intracellular organisation as well as from their neurotoxic properties. Neurotoxicity of NFTs is undebatable in respect to hippocampal and entorhinal neurones. However, in neocortex severe neuronal loss may be seen even in absence of NFTs, therefore an alternative cause of neuronal depletion must be considered. The dysfunction in intraneuronal transport and organisation is a result of formation of PHFs and NFTs. In this process a phosphorylation of tau protein takes place, which leads to instability of polymerised tubulin. In effect the dysfunction and loss of microtubules is to be observed.

Other pathological structures that are biochemically close to NFTs are neuropil threads. These relatively short, straight or curly fibres may be observed in the neuropil of neocortex as argentophilic and thioflavin positive structures. They present also anti-tau and anti-ubiquitin immunoreactivity. The characteristic compound of NTs is so called neuropil thread protein, largely homologous with pancreatic thread protein.

Ultramicroscopically they are built of PHFs, and some of them are encompassed by fine myelin sheath. The latter phenomenon resembles their axonal origin; however most of them are degenerated dendrites. Their pathogenesis is still a subject of discussion: both degenerative as well as abnormal fibre sprouting processes may be involved in their production. A strict relationship exists between number of NT and both NFT presence and clinical severity of the AD.[21,81,270]

2.2.3.3. Neuronal loss and synaptic alterations

Neuronal loss seen in AD displays certain preponderance to selected brain structures, although different grades of decreased neuronal density may be seen in almost all regions of the brain in most AD cases. The structures mostly affected are: hippocampi, enthorinal cortex, basal nucleus of Meynert, neocortex, locus caeruleus and the dorsal raphe. Of particular importance is neuronal loss in nucleus of Meynert and locus coeruleus, since neuronal depletion there affects in a widespread way the cholinergic and noradrenergic activity, respectively.[270]

2.2.4. Molecular background of Alzheimer's Disease

Amyloid precursor protein is a large transmembrane protein possessing a long extracellular N-terminal domain and a small intracellular cytoplasmic domain. A normal function of full-length APP and its soluble fragments applies to intra- and interneuronal signalling, synaptic transmission, neural growth, morphology and plasticity and learning and memory process (for exhaustive review see Turner et al.[277]). APP protein is produced by different types of the cells including endothelia, glia and neurones.[242] Due to posttranscriptional alternative splicing APP is produced in at least three different forms i.e. APP770, APP 771 and APP695, differing in length of peptide chain. APP695 is only form lacking so called Kunitz Protein Inhibitor (KPI) sequence (which function is currently not clear) and is thought to be a substrate for Aβ-generating variant of APP-processing. Notably, this form is the dominating product of APP gene expression in neurones.

Processing of APP occurs via different pathways, which are considered as non-amyloidogenic (without the generation of Aβ-peptides) or amyloidogenic (with the generation of Aβ-peptides) (reviewed by De Strooper and Annaert[51] and by Nunan ant Small[194]) (see Fig. 2.1.). The main physiological cleavage occurs at the beta-secretase site within the Aβ-domain, causing the release of a secreted form of APP (sAPPα), but also β- and γ-secretase cleavage can occur under certain physiological conditions.

Cleavage of APP within the Aβ domain (between amino acid 16 and 17) by α-secretase releases the large, soluble ectodomain (~100–120 kDa) of APP (sAPPα) and leads to the generation of a membrane-bound carboxy-terminal fragment (~10 kDa), named α-CTF or C83. Further cleavage of C83 by gamma-secretase results in the liberation of a secreted fragment called p3 (reviewed by Bayer et al.[14]).

The alternative pathway of APP secretion results in the cleavage of APP at β- and γ-cleavage sites, liberating secreted beta-cleaved APP (sAPPβ), as well as Aβ-peptides. Cleavage of APP by β-secretase releases the truncated ectodomain of APP (sAPPβ) and generates an amyloidogenic C-terminal fragment of ~12 kDa (C99, β-CTF)[246], which is further processed by gamma-secretase activity to generate Aβ peptides.

γ-cleavage occurs at different sites resulting in Aβ peptides of varying lengths (39–43 amino acids). The residual ~7 kDa fragment (γ-CTF) was recently identified[211] and seems to form a transcriptively active complex with the cofactors Fe65 and Tip60.[38] Four independent groups reported the identification of the candidate for β-secretase.[113,253,284,308] This enzyme is called BACE (β-site APP-cleaving enzyme) or Asp2. Besides the long known beta-secretase site, BACE alternatively cleaves also at position Glu11 in the Aβ sequence. Mice deficient in BACE1 are healthy, fertile and appear normal. BACE knockout mice, which are also hemizygous for APP, lack brain β-amyloid and β-secretase-cleaved APP C-terminal fragments (β-CTFs). These results provide validation of BACE1 as the major beta-secretase in vivo.[154] Notably, an efficient beta-secretase-like APP cleavage may be preformed also by proteins from caspase family,[80,141,295,313] which, in turn are activated in neurones and glia by different forms of CNS injury (reviewed by Yakovlev and Faden[305]), including also TBI.[18,30,127,209,306]

Cleavage of the beta-CTF is the final step in the generation of Aβ-peptides. The position of γ-secretase cleavage is variable, which leads to the generation of different Aβ peptides, of which Aβ40 (40 amino acids) and Aβ42 (42 amino acids) are the major components. Recently it was shown that nicastrin, a transmembrane glycoprotein, forms high molecular weight complexes with presenilin-1 and presenilin-2 and also binds to the C-terminal part of APP.[311] Besides nicastrin, further components of the gamma-secretase complex have recently been identified. APH-1 and PEN-2 encode multipass transmembrane proteins that interact strongly with presenilin and nicastrin.[61]

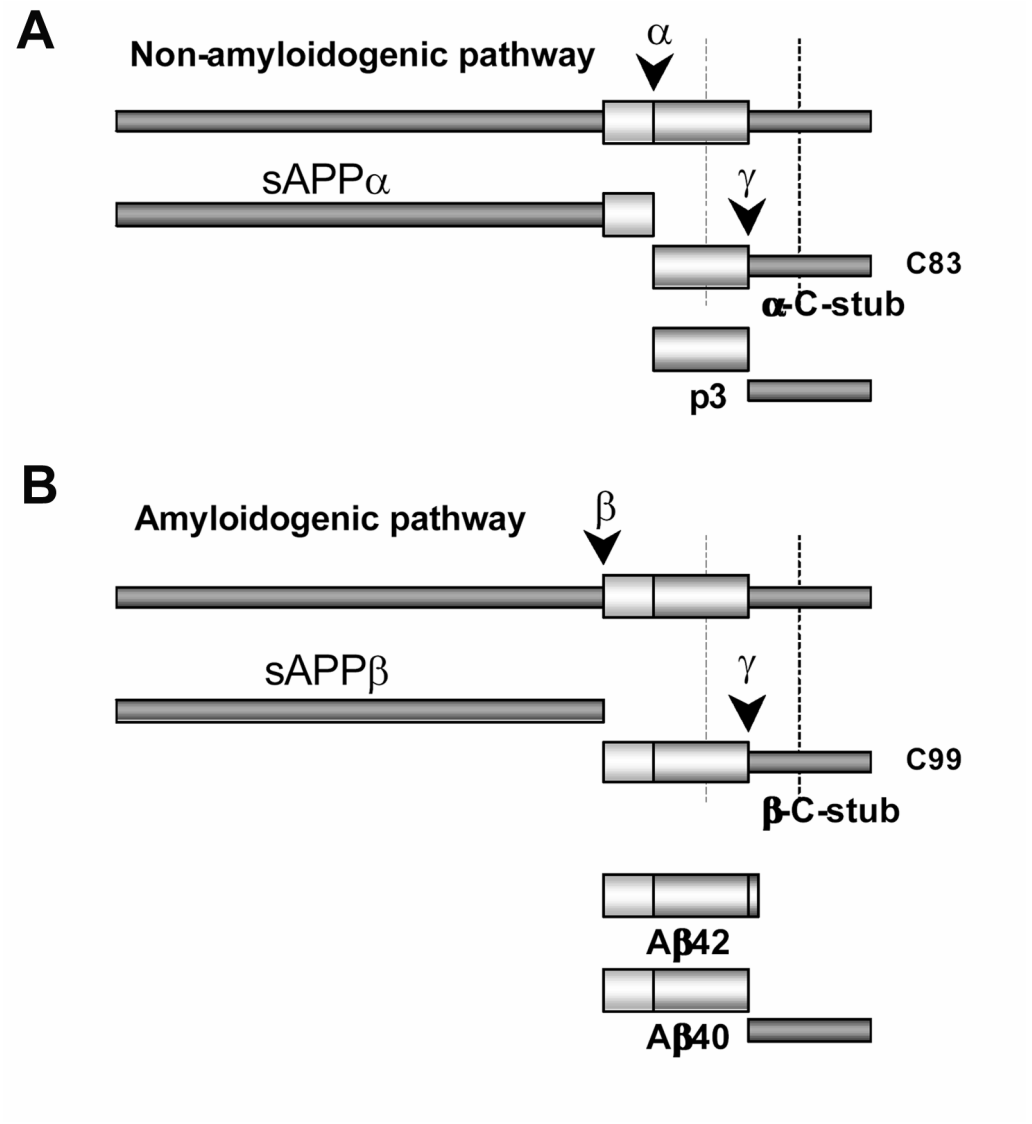


Fig. 2.1. Schematic representation of Amyloid Precursor Protein (APP) processing.

A: Non-amyloidogenic processing pathway, by alpha- and subsequent gamma-secretase cleavage precludes the formation of Aβ peptides.

B: Amyloidogenic processing by beta-secretase generates amyloidogenic c-terrminal fragments (C99). Further cleavage by gamma-secretase leads to generation of Aβ40 and Aβ42 peptides.

2.3 Interconnections between Traumatic Brain Injury and Alzheimer's disease

2.3.1 Epidemiological link

The epidemiological research reveals TBI to be a significant risk factor for AD. The first suggestions, that TBI may be the cause or risk factor for AD can be found in early case descriptions. Thus, Khaime Ts et al. described 3 cases of TBI preceding development of full blown AD with clinical diagnosis confirmed by autopsy.[128] Thereafter, Rudelli et al. reported on 38-year-old man, suffering from AD with severe TBI occurred 16 years earlier.[238] Following latter publication, numerous case-control studies were undertaken in order to estimate the odds ratio for this correlation. According to recent Fleminger's et al. meta-analysis 36 case-control studies were completed until 2001. Among 15 studies that passed through Fleminger's strict acceptance criteria, odds ratio for TBI as a risk factor of AD varied from 0.44 to 6.00. The meta-analyses of all those studies and enrolled subjects gave an odds ratio estimate of 1.58 (1.21 to 2.06 for confidence interval CI 95%).[59] This result deviates not far from this of Mortimer's re-analysis (EURODEM-study)[184] reporting an relative risk of 1.82 (95% confidence interval: 1.26-2.67). Both meta-studies revealed gender difference for TBI-related AD-risk (according to Fleminger et al., an odds ratio for male was of 2.26 while for females of 0.92). Thus, according to this result TBI may contribute to AD development only in males. Barring suspected sex-difference of bias, a biological protection from AD-producing effects of head trauma in women is assumed.[59,184]

APOE e4 is considered as a risk factor for both AD and for outcome of TBI. On this ground several case control studies attempted to extract impact of APOE polymorphism on TBI-related AD-risk. The results are inconsistent. Mayeux et al. reported, that risk of AD was increased 10- fold, when head injury was combined with APOE e4 presence, however, TBI alone did not increase this risk at all, comparing with twofold increase by APOE e4 separately.[169] In contrast, O'Meara et al.[197] referred, that impact of APOE genotype on epidemiological relations AD-TBI is not statistically significant. According to these results head injury and APOE e4 were separate risk factors for AD, with odds ratio of 2.1 and 4.1, respectively.

Apart from case control studies, reputedly more reliable cohort studies were published. Though Katzman et al.[125] and Metha et al.[177] report, that head injury is not a significant risk factor for AD, Schofield et al. found length of loss of consciousness (LOC) as a important variable (RR of AD was of 11.2 for LOC > 5 min, but no increase in risk was found for LOC < 5 min).[243] Most interesting results are reported by Nemetz et al.[191] Comparing AD general risk and time of eventual AD onset between the population

cohorts with and without head injury in the past, they stated that TBI increased the risk of early AD onset twice, and shortened the time to AD onset, while general risk of AD-occurrence wasn't increased. This suggests, that TBI may be rather accelerating, not causing factor for AD, acting only in people predisposed to develop AD.

2.3.2. AD- related pathophysiological sequele of TBI in human subjects and animal experiments.

2.3.2.1 Changes in APP immunoreactivity and expression as a posttraumatic reaction

Increase in immunoreactivity of APP has been widely demonstrated in various forms of CNS injury, including: ischemia (of different type: complete,[4,117,213,261,307] transient[121,274,289,310] and chronic[121]), toxic damage,[91,126,189,250-252,275,299] cholinergic deafferentiation of subcortical nuclei[19,150] and axotomy.[200] No doubt, TBI deserves a special place on this list. Thus, increased APP immunoreactivity after mechanical injury was first discovered by Otsuka et al. who used a needle stab injury model in rats. The authors reported APP presence in swollen axons (already 30 min postinjury) and glial cells (mostly astrocytes- 6 h after injury) close to the site of injury.[199] Later on, Lewen et al. stated APP deposition also in axons of ipsilateral subcortical white matter and thalamus of rats subjected to mild compression contusion trauma, accompanied by loss of neuronal body APP immunoreactivity.[146] Additionally, using the same model, some APP positive profiles were found in hippocampal fissure 24 h postinjury.[147] Subsequently, Pierce et al. examined different time points after fluid percussion injury in rats and observed that APP immunoreactivity was increased 1h (in thalamus) and 2h (in cortex and subcortical white matter) up to 2 weeks posttraumatically (mainly in swollen axons).[208] The detailed temporal and regional patterns of these alterations, regarding APP- immunoreactivity were described by Bramlett et al. who reported APP-reactive profiles in axons of striatum, hippocampus and external capsule as well as in the cell bodies of cortical and thalamic neurones. Notably, the APP-reactivity of striatal axons decreased dramatically between 24h and 3 days postinjury. Moreover in this latter report, the severity of trauma was found to be a significant factor directly correlated with the intensity of APP accumulation.[28] Newertheless, alternative rodent model of mild TBI (fluid percussion injury in mongolian gerbils) proved that already concussion-like trauma is able to produce early (6h posttrauma) and significant accumulation of axonal APP, which was correlated with cognitive function impairment (as revealed in open field and T-maze tests).[149]

Investigations of Van Den Heuvel et al. revealed that an increase of APP reactivity was found in pericarya of neurons, remote from trauma site (even in contralateral hemisphere). Based on the ovine focal brain trauma model increased APP immunoreactivity was observed in particular 2 h after trauma and was described as a diffuse, widespread staining present in neuronal cell bodies rather than in axons.[283] The more recent study of this group, in which paediatric head trauma in young lambs was analysed, revealed a similar pattern of APP immunostaining 2 h posttrauma.[281]

One of the reasonable explanations for this phenomenon was increased APP production. Indeed, ischemia,[4,102,129,134] axotomy[245] and chemical injury to cholinergic structures[292] result in surge of mRNA-APP or its translation. Similarly, TBI also followed this pattern. Alone pericarial localisation of APP accumulation, in an ovine head injury model was suggestive of APP's increased production.[283] This presumption was confirmed via revelation of the same APP mRNA distribution using an *in situ*–hybridisation technique.[281,282]

Further verification of this hypothesis has been provided by studies of TBI in rats.[24,45,118,161,186] In those experiments an increased expression of APP has been demonstrated in cerebral cortex and hippocampus ipsilaterally to the trauma. This effect seem to be short lasting, since APP quantity returned then to sham level after several days postinjury.[24,118,161] Additionally, a surge of APP expression was observed regarding isoforms APP751/770, while APP695 was rather decreased.[118,161] Moreover, gradual decrease in accumulation of APP in CA3 pericarya was accompanied by signs of apoptotic degeneration, linking it to death of the APP-producing neurons.[186] Based on the aforementioned data a hypothesis was formed, which stated that increase of APP expression is a part of cerebral acute phase response and that this fact is responsible for APP accumulation and later overproduction of its derivative i.e. Aβ, with consequent development of AD.[74,87,97]

However, this theory is still not free from several inconsistencies. In a model of transient ischemia, upregulation of HSP-genes occurred but this phenomenon didn't affect APP mRNA[3] and, in TBI-studies APP accumulation was not synchronised with APP overexpression, as it occurred long after or even before trauma.[118,161] Moreover, increased translation of APP in some circumstances was not able to result in accumulation of mature APP.[292] Finally, an isoform analysis for a shift in APP-mRNA level revealed that posttraumatic increase characterises APP751/770, which is preferentially expressed in glia, while the level of 'neuronal' mRNA-APP695 undergoes gradual decrease in damaged cortex.[118,161] Therefore (without minimising the

importance of APP upregulation), some accessory mechanisms responsible for the rapid posttraumatic increase in APP immunoreactivity need to be searched.

One may notice that the peak in detectable APP can be produced even without any increase of its total amount, if its local concentration increases. Since APP undergoes fast axonal transport,[36,135,182,254] any disturbance in this mechanism could result in such a phenomenon. For instance, immunoreactivity of APP was increased after intraventricular injection of colchicine, which is toxic to the axonal microtubules, necessary for the axonal transport to occur.[117]

Most importantly, head trauma is the best depicted cause of axonal impairment, described as traumatic axonal injury (TAI). According to current concept, this phenomenon is caused by indirect mechanisms and the so called primary axotomy, resulting from mechanical tearing of neurites by the trauma is rather ephemeral and marginal event[168,215]. Instead, an axolemma has been proposed as a prime site of injury and mechanical damage to its continuity results in loss of ionic homeostasis, with Ca^{2+} gradient disturbance as a main cause of subsequent changes.[64,73,164-166,168,206,207,217] Uncontrolled influx of Ca^{2+} into axoplasma promotes depolymerization and dearrangement of microtubules what results in impaired axonal transport.[167,217] This, in turn leads to accumulation of proteins, normally carried via anterograde trafficking in the portion of axon proximal to injury. Such a swelling ultimately causes interruption and disconnection of axons.[167,258]

APP is one of the proteins involved in this pathology. Its accumulation in mechanically traumatised axons has been presented in whole human nervous system, including spinal cord[48,226] and optic nerve,[227] not only in the brain. Regarding the latter one, axonal injury with APP accumulation has been observed even in the slightest forms of TBI[25,26] and in fatally injured persons its prevalence reached up to 92%.[78] Axonal APP accumulation was observed as early as 2h after head injury[172,249] and persisted even up to 99 days,[25] thus creating possible background for Aβ production for a prolonged period of time. The amount of axonal damage increases up to 10-15h posttraumatically,[172] while the size of swollen axons representing the mass of accumulated APP reached plateau at 85h, suggesting, that secondary waves of axonal swelling may occur as a result of secondary complications such as oedema and hypoxia.[297] Since TBI shares those features with other types of neurodamage (e.g. brain ischemia), axonal damage and APP accumulation are not strictly specific for mechanical impact to the brain.[137,193,196] Nevertheless, APP immunostaining remains at date the best method for visualisation of injured axons, both in experimental[28,107,208,263] and medical settings.[26,68,70,77,172,225,226,248,249]

Summarising, axonal pathology produces potentially a second source of APP, in addition to its perikaryal accumulation. However, basing on this evidence an important question needs to be raised: May subsequent Aβ production and depositon origin in any of the two types: 1) perikaryal and 2) axonal of posttraumatically accumulated APP?

2.3.2.2. Posttraumatic Aβ pathology in non-transgenic animals

The phenomenon of posttraumatic Aβ deposition has not been observed in the majority of non-transgenic animal studies. In some of them, i.e. in both adult and young ovine head trauma model, β-amyloid staining was not used as a research tool.[7,148,281-283] In rodent models however, efforts were undertaken in order to identify Aβ deposition in traumatised brain but most of them failed.[28,45,138,147,161,208,240] Contrasting results have been presented in studies of chronic TBI stage in rats. Here the fluid percussion injury of moderate or severe degree has been used. A moderate trauma resulted in Aβ accumulation that was observed in axonal bulbs and partially around them from 1 month to 1 year posttraumatically.[118] However, after severe brain trauma Aβ was not observed until 6 months postinjury and Aβ deposition was present in regions of perikaryal cytoplasm and apical dendrite of, especially, pyramidal neurones.[108]

In contrast to this lately occurring Aβ pathology formation, a pig model of rotational head injury developed quite rapid Aβ accumulation, manifested in axonal bulbs and (in form of diffuse deposits) around them already 3 days after trauma.[256] Similar results were presented also in a rabbit model of rotational acceleration trauma, where Aβ immunoreactivity emerged acutely i.e. increasing from 1 to 14 days posttrauma in pericaryal area and axonal compartment.[92] In a separate study using a pig model with 6- months' survival, deposits of Aβ were observed at all analysed time points (3, 6 days and 6 months after TBI); however, they didn't increase with time after trauma. More importantly, this study reported axonal co-accumulation of enzymatic factors that produce Aβ (BACE, PS-1 and caspase-3), as well as products of their activity (CCA). A general expression and activity of some of these factors (as presented with the aid of Western Blot and ELISA analysis) can undergo increase after trauma.[42] This surge however, is short–lasting (only up to 7 days in rat study).[24] Thus, local peak in concentration of proteins needed for Aβ generation is crucial for abnormal APP proteolysis. Since this co-accumulation includes APP, a reasonable final conclusion could be that APP, which is highly concentrated in traumatically injured axons, indeed provides an ample substrate for Aβ production and aggregation. As presumed, this mechanism is functional also in the chronic stage after TBI. It should be noted, that for

some reasons this statement seems to be valid only for fully disconnected axons, displaying the so called axonal bulb pathology.[42,256]

2.3.2.3. Posttraumatic Aβ pathology in human

At this point next question raises: Does human TBI follow 'rodent' (chronic, slow progressing) or 'porcine' (rapid persistent) pattern of posttraumatic Aβ pathology? Aβ deposition has been initially observed in the brains of patients, who suffered repetitive blows to the head, as seen in the so called dementia pugilistica or punch drunk syndrome.[6,46,228,231,273] Later post mortem studies revealed, that in about 30%–50% of subjects with severe TBI, diffuse Aβ deposits were spread in various cerebral structures.[112,229,230] Recent analysis of surgically resected temporal cortex from survivors of severe TBI brought similar results.[116] In autopsy studies this sequel was strongly dependent on APOE genotype (but not on another genetic AD background i.e. presenilin-1 gene polymorphism[317]) and present predominantly in APOE4 carriers.[106,157,192] The prevalence of Aβ deposition was significantly higher in TBI-subjects than among age- matched non- traumatised controls and this difference was observed for all (save age 61-70 years) decades of life.[230] Those data support the postulate that the observed amyloid deposits originated after traumatic insult and that they were no previous changes, present before injury and disclosed by co-occurrence. Interestingly, this deposition was observed relatively early (starting as early as few hours after trauma[116,230]) and when analysed by survival time longer than 1 month after TBI, the occurrence of Aβ deposition was not significantly different between TBI patients and age–matched controls.[157] Taken together, it seems that human posttraumatic pathology follows the pattern seen in porcine brain i.e. rapid Aβ accumulation.

Autopsy studies also confirmed Aβ generation from APP amassed in injured axons. Though previous pathological reports stated that localisation of posttraumatic plaques is not related to any other pathology, including axonal damage[86], more recent work presented Aβ immunoreactivity in terminal ends of disconnected human axons.[255] Finally, co-accumulation of BACE, PS-1 and proteins responsible for axonal transport (kinesin and glycogen synthase kinase GSK) has been also confirmed in patients with TAI.[43]

2.3.2.4. TBI in transgenic models of AD

Since the animal transgenic model of AD pathology is available, some efforts were undertaken in order to establish the pathophysiological background of AD- TBI association observed in humans. Using controlled cortical impact model a series of experiments was proceeded in PDAPP transgenic mice using different age and posttraumatic survival time (for details see Tab. 2.2.). PDAPP transgenic mouse is characterised by deposition of Aβ first at 6 months of age. Applying trauma before this age, both short and long term impact on general and Aβ pathology was described. First, using this paradigm, rapid, short lasting surge in Aβ level occurred, especially in hippocampus. However, trauma did not result in expected Aβ deposition[259] and, after long term outcome analysis, even substantial reduction of amyloid burden ratio (as assessed by planimetry performed on images of Aβ-immunostained brain slices) both in cortices and hippocampi in TBI group was reported.[187] Moreover, subsequent experiment with aged PDAPP mice resulted in TBI-related resolution of established Aβ deposits.[188] Notably, in all of those experiments, increased neuronal damage, especially in hippocampal structures has been noted in PDAPP injured mice when compared with traumatised WT littermates. This neuronal loss was accompanied by significant memory dysfunction (impaired memory retention) as presented using Morris Water Maze (MWM).[259] The newest analysis of Brody et al concludes that this impairment in PDAPP mice is caused by performance–deteriorating shift in spatial search strategy as well as by decrease in efficiency of use of given strategy; both effects being exacerbated by TBI.[29] Since the impact of genotype has not been observed among sham injured animals, authors raised a 'two-hit hypothesis'. According to this hypothesis β-amyloid per se is not neurotoxic in transgenic animals and accessory noxious factor (e.g. TBI) is necessary to reveal damaging effects of Aβ on neuronal structures.[259]

Interestingly, separate analysis of Aβ isomorphs in PDAPP and YAC-APP mouse showed the decrease in Aβ40 amyloid level, while more detailed study in PDAPP mice revealed that in fact there is early posttraumatic surge in both forms of Aβ (more significantly in hippocampus than cortex), after which more amyloidogenic Aβ42 not only returned to baseline but was actually decreased.[185,259] Regarding YAC-APP mouse, this model is characterised by overexpression of APP without spontaneous Aβ deposition.[34] Also TBI was not able to induce the latter phenomenon.[185]

An exceptional result was yielded from repetitive mild TBI (mTBI) experiment. Such a murine model has been developed[138] and used Tg2576-APP transgenic mice. Here, mTBI, when repeated twice, at remote time points gave increase in deposited Aβ that

was associated with cortical surge in level of Aβ in soluble and insoluble form, both Aβ40 and Aβ42. In contrast, single head trauma resulted in increase of only Aβ42 level with plaque formation of degree greater than in sham treated animals, but not so escalated as after repetitive trauma. Additional analysis revealed increase in oxidative stress marker levels, conferring this type of damage to be an important factor for posttraumatic AD development.[47,280]

Therefore, the results of TBI- studies in transgenic models of AD were conflicting when compared with sequele of human TBI. First, in none of the presented models a rapid Aβ deposition has been presented. This includes also repetitive mild head trauma, with slow progress of relatively late changes that makes it look similar in particular with progress of dementia pugilistica (see Section 2.3.2.3.). However, in comparison with other studies made on the same PDAPP mice the trend may be observed that greater impact of damage is correlated with reduced Aβ deposition or even resolution of already established plaques. This trend seems to be reversed in humans, since both the risk[212] and the posttraumatic Aβ deposition increased with head trauma severity.[69,230]

What is origin of these discrepancies? It may be postulated, that murine transgenic model of AD is just too remote from physiological APP metabolism in human. Thus, in transgenic animals APP carries highly amyloidogenic mutations and it is expressed at much higher level than the physiological one. This situation resembles some variants of familial AD and transgenic mouse models would be therefore comparable with them. Nevertheless, the confrontation with clinical data cannot here be made, since co-occurrence of APP mutation and head injury is rather rare and regarding this problem only some single notices were published.[230] Moreover, model of head trauma in APP mouse has been anticipated as simulation of pathology in general population of head trauma patients, not in the very selected group. Thus, other explanations are worthy to be found and necessary for eventual amelioration of this concept. Some of them are outlined in Section 5. (discussion).

Regarding this, the most reasonable approach has been presented by Abrahamson et al. In this study non- transgenic, so called APP knock-in murine construct has been used. In such animal, the sequence of mutated, highly amyloidogenic human APP is under control of own murine APP promoter, ensuring therefore physiological expression of APP gene. In this model, TBI resulted in early (3h posttrauma) raise of both Aβ40 and Aβ42. While at 72h posttrauma Aβ40 returned to baseline level, the ‘more aggressive’ Aβ42 remained elevated through whole posttraumatic period analysed (up to 7 days after TBI). In the same study, increased beta-secretase like

activity of caspases has been confirmed to be relevant cause of raise in Aβ level, which, notably, can be prevented by administration of caspase-3 inhibitor.[5]

Refference	type of transgenic animal/ TBI model	spontaneous apperiance of Aβ deposits	TBI induced in the age of	Posttraumatic timepoints analysed	Aβ deposition	Aβ level		APP immunoreactivity	neuronal damage	Motor deficit	Cognitive deficite
						Aβ40	Aβ42				
Murai H et al. 1998	APP-YAC mice	-	10-16 months	24h					ns	ns	
	CCI			7d	no	↓	ns	↑	ns	ns	ns
Smith DH et al. 1998	PDAPP mice	6 months	4 months	2h		↑	↑				
	CCI			6h		ns	↓				
				24h		ns	↓				
				3d		ns	ns				
				7d	ns	ns	ns		↑↑	ns	ns
				14d	ns				↑↑		
				2 months	ns						
Nakagawa Y et al. 1999	PDAPP mice	6 months	4 months	2 months					↑		
	CCI			5 months	↓				↑		
				8 months	↓				↑		
Nakagawa Y et al. 2000 [a]	PDAPP mice	6 months	22- 24 months	7d	ns			↓	↑		
	CCI			9 weeks	ns			↓	↑		
				16 weeks	↓↓			↓	ns		
Uryu K et al. 2002	Tg2576 mice	9 months	9 months	2d	ns / ns				ns / ns		
	single mTBI/repetitive mTBI [b]			9 weeks	ns / ns				ns / ns		
				16 weeks	↑ / ↑	ns / ↑	↑↑ / ↑↑ [c]		ns / ns	ns / ns	ns / ↑

CCI- Controled Cortical Impact; mTBI- mild Traumatic Brain Injury; ns- not significant statistically; no- no Aβ deposition; a- analysis ipsi- vs. contralateral hemisphere in TBI- transgenic animals; b- both single/repetitive mTBI results are presented; c- refers to soluble Aβ42 fraction; insoluble Aβ42 fraction was increased after repetitive but not single mTBI.

Table 2.2.. Selected experimental studies on traumatic brain injury using Alzheimer transgenic mice

2.4. Aim of the study

Basing on the aforementioned data the following problems were intended to be dissolved experimentally:

1. Regarding impact of TBI on Alzheimer's pathology development: The existing results of animal studies are not fully consistent with evidence from epidemiological or neuropathological analyses in humans.
   Therefore: Would it possible to simulate this impact more accurately in transgenic model of APP751SL mice?
2. Though some of the epidemiological studies report AD occurrence as increased after TBI in gender-dependent manner, up to date none of animal experiments analysed this issue.
   Thus: is there any difference between male and female animals in development of amyloid pathology in our posttraumatic AD model?

3. METHODS

3.1. Animals

Mice of both sexes, both transgenic $APP_{751}SL$ mice, as well as their wild–type (WT) littermates were used in this study. Breeding animals were obtained from and breeding line was established with permission of Aventis Pharma, Centre de Recherche de Paris. All animals were handled according to German guidelines for animal care.

Animals were bred and kept under specific pathogen free (SPF) conditions in Laboratory Animal Resources Facility, Department of Experimental Surgery, Saarland University. Animals were housed in groups of 1 to 4 per cage, in a 12h :12h dark-light cycle. Food and water were provided *at libitum.* Animals were kept until 12 weeks of age. After genotype of mice was assessed, animals were designated to one of the four genotype– and sex–matched groups (i.e. APP–female, APP–male, WT–female, WT–male). Each of the groups consisted of 10 animals, thereof 5 animals were randomly selected for repetitive head injury treatment, while remaining 5 for sham–injury treatment.

3.2. Techniques for nucleic acid analysis

3.2.1. Isolation of mouse tail DNA

A piece of mouse tail was put into lysis buffer (100 mM Tris/HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) including Proteinase K (300 µg/ml) and was incubated overnight at 55℃ in a thermomixer with continuous agitation. After a centrifugation step at 13.000 rpm at room temperature for 10 min, the supernatant was transferred into a new tube containing 500 µl 2-propanol. After a further centrifugation step, the supernatant was discarded and the pellet washed once with 500 µl 70% ethanol. After second centrifugation, the pellet was dried at 37℃ and resuspended in 70 µl distilled H_2O. For polymerase chain reaction an 1:20 solution was used (5 µl of resuspded DNA in 95 µl of distilled water)

3.2.2. Polymerase chain reaction

The polymerase chain reaction (PCR) is a method, which allows specific and exponential amplification of selected DNA sequences for molecular biological analysis. During this reaction, cyclic annealing and elongation of short oligonucleotides (primers) occurs, which flank the sequence of interest. DNA itself is a double-stranded chain of nucleotides, whereas primers are single-stranded and able to bind to complementary

sequences in another piece of single–stranded DNA. First, the target DNA must be denatured, unwound and separated by heating to 90–96℃. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the now single–stranded DNA. In the third step, DNA is synthesized by a polymerase, which results in two new helices, each composed of one of the original strands and its newly assembled complementary strand. For further amplification the process is just repeated, usually up to 35 times.

Solutions and material:

dNTP-mix (Invitrogen)	10 µM of dATP, dGTP, dCTP, dTTP
Taq DNA Polymerase 10 x PCR Buffer (Invitrogen)	200 mM Tris HCl (pH 8.4), 500 mM KCl, 50 mM $MgCl_2$
Taq-Polymerase (Invitrogen)	5 U/µl, storage at –20℃

PCRs were carried out in volume of 10 µl. Each reaction mix contained 1 µl of dNTPs, 1 µl of PCR-buffer, 5 pmol sense– and antisense primer, as well as 0.25 U/µl Taq-polymerase and 1 µl of 1:20 DNA solution. Finally, distilled water was added to total volume of 10 µl for each mix.

3.2.3. Genotyping of transgenic mice

The PCR was carried out in a volume of 10 µl in a PTC-200 Thermocycler (MJ Research).

Primer sequences:

APP-forward: 5'-GTA GCA GAG GAG GAA GAA GTG-3'

APP-reverse: 5'-CAT GAC CTG GGA CAT TCT C-3'

PCR-program:

94℃	5 min	
94℃	60 s	
55℃	60 s	35 Cycles
72℃	90 s	
72℃	5 min	

3.2.4. Agarose gelelectrophoresis

Agarose gelelectrophoresis is based on the principle of the mobility of charged particles in an electric field. Nucleic acids move during electrophoresis from the cathode to the anode. The velocity of the nucleic acids is both influenced by their molecular mass and their conformation (super–helical, double–stranded linear or single–stranded). In agarose gels, DNA fragments can be separated in a wide range, depending on the concentration of the agarose in the gel.

Solutions:

10 x TBE:	108 g Tris-HCl, 55 g boric acid, 40 ml 0.5 M EDTA ad 1000 ml with distilled H_2O
agarose loading buffer:	0.5 ml glycerol, 0.08 ml of 0.5 M EDTA, 0.002 g Bromphenolblue, 3 drops of 5 M NaOH, diluted with dist. H_2O ad 1ml
Ethidiumbromide	10 mg/ml in dist. H_2O

Procedure:

The 4 g agarose was dissolved in 200 ml of 0.5 x TBE and boiled in a microwave oven. After chilling to a temperature of approximately 50°C, 4 µl of ethidiumbromide-solution was added and the agarose was poured into a gel chamber. After polymerization, the samples were mixed with an equal volume of loading buffer and the electrophoresis was started at a voltage of 200 V, using 0.5 x TBE as running buffer. The dye ethidiumbromide attaches irreversible to the DNA and fluoresces when irradiated with UV-light. Comparison with a molecular weight standard yields information about the size of the DNA sample.

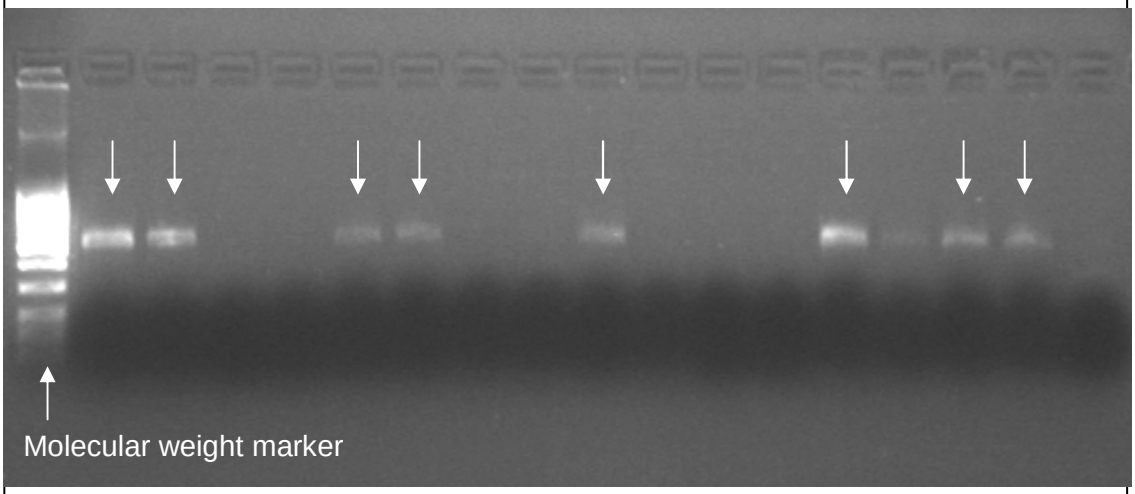


Fig. 3.1. Genotyping of APPSL751 mice, basing on PCR and gel electrophoresis.
By use of appropriate set of primers DNA amplification occurs only in samples from hAPP- transgenic animals. Electrophoresis and UV detection of DNA- ethidium bromide binding reveals hAPP-gene containing material as marked by fluorescent stripes (white arrows).

3.3. Repetitive Closed Head Injury (CHI) model

To induce brain trauma, a well established mouse model of closed head injury (CHI) was used (adapted from Chen et al.[44]). The principles of this model are based on transmission of weight drop energy to intact, non-trephined mouse skull. Using low injury severity (without causing skull fracture) concussive- like TBI without overt contusion or focal lesion can be achieved.[139,140] Preserved bones of the skull enable also repetitive applying of trauma. This aspect, together with the advantage of easy feasibility made CHI paradigm optimal for purposes of this study.

3.3.1. Head trauma device

The weight drop device (Laboratory Tools Workshop, Department of Pharmacology, School of Pharmacy, The Hebrew University of Jerusalem, Israel) consists of the bottom aluminium plate (A) with silicon pad (B) and of vertical Plexiglas tube (C), with lower opening closed with silicon cone (D). After manual positioning of the anaesthetised animal on the plate (head over the silicon pad B), the silicon tip was placed on its skull, targeting the injury site mark. Head trauma was applied by brass rod (E), free falling along the Plexiglas tube. Weight drop impact was transmitted on animal head via silicon cone. The energy of the trauma can be adjusted by using metal rod of different weight and by changing the height of its fall. For the purpose of this study, the following settings were used:

Brass rod weight: 50.5 g

Height of the fall: 12.5 cm

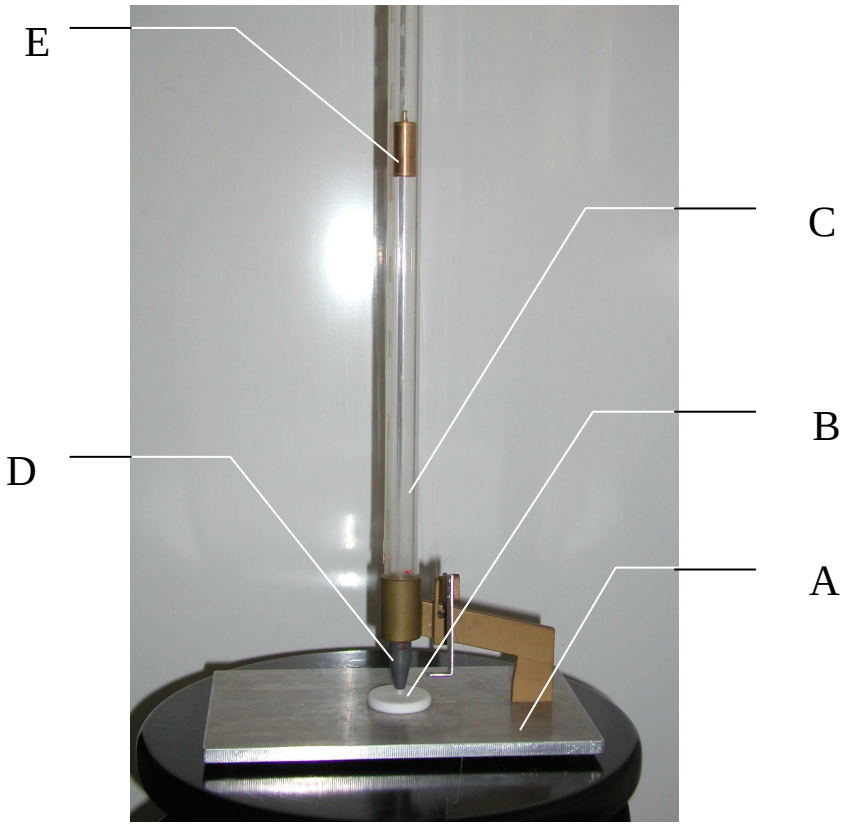


Fig. 3.2. Weight drop device. Description in text

3.3.2. Procedure

Surgical procedures were approved by the animal rights committee of the University of Saarland at Homburg/Saar, Germany.

Trauma was induced under pentobarbital anaesthesia (Narcoren 65 mg/kg i. p.), confirmed by loss of corneal and pinprick-withdrawal reflexes. The eyes were covered with an ointment (Bepanthen, Roche) to prevent noxious input due to drying of the cornea. The head fur was removed using hair trimmer. The animals were positioned on surgery tablet and scalp skin was disinfected with antiseptic (Octenisept Farblos, Schülke & Mayr). A longitudinal midline incision in the skin covering the skull was performed and the skin and subjacent soft tissues were retracted to expose the skull bone. The trauma site was determined using bregma and sagittal suture as landmarks and flagged with permanent waterproof marker. The head was manually fixed at the bottom plate of impact device.

Due to various time of response to anaesthesia, a standard interval of 60 min from administration of anaesthetic to weight drop impact (or sham treatment) was exercised.

After trauma, each mouse was placed again on the surgery tablet and head incision was closed using 6.0 polypropylene sutures (Prolene, Ethicon). Thereafter, animals returned to their home cages, and were kept under heating lamp until fully recovered from the anaesthesia (as evidenced by ambulation).

At 24h after the first TBI, animals from TBI–group were re anesthetized as described above, and were then subjected to a second TBI in the same location over the left parietal region.

Sham animals received identical treatment: on 2 consecutive days they were also anesthetized and placed on the base of impact device; the skull was exposed and the silicon tip was placed on the skull, however, without applying fall energy to silicon tip thus without brain injury. Finally, the skin incision was suture closed, thereby following exactly the surgical procedures of repetitive TBI.

3.4. Neurological assessment

24h before operation each animal has been pre-trained according to modified beam–balance (BB) test protocol. The beam–balance task involves placing the mouse on a suspended narrow wooden beam and rating of the animal's ability to balance (the maximum time limit was 60 s). The rating of beam–balance performance was from 0 to 5 (see Fig. 3.3. for BB rating). The trials were given to the animal until it was able to perform task correctly (with a note of 0) three times in a row. The pre-training performance index was calculated by simple adding of single rates. After CHI or sham operation the neurological status was evaluated at different times (from 3 h and up to 28 days) using a set of motor and behavioural tasks referred to as the Neurological Severity Score (NSS). This score was modified from Sabo et al.[239] and is outlined in Table 3.1.

Neurological Severity Score for head-injured mice

1.	Presence of monoparesis or hemiparesis	1
2.	Inability to walk on a 3 cm wide beam	1
3.	Inability to walk on a 2 cm wide beam	1
4.	Inability to walk on a 1 cm wide beam	1
5.	Inability to balance on a beam (0.5 cm wide)	1
6.	Inability to balance on a round stick (0.5 cm diameter)	1
7.	Failure to exit a 30 cm circle within 2 min	1
8.	Inability to walk straight	1
9.	Loss of startle reflex	1
10.	Loss of seeking behaviour	1
	Maximal total score	10

For each failed task the mouse received 1 point;
for tasks 2-4, 8-10 a gradated rating was permitted.

A maximal score was 10 (failure in all tasks) and a minimal was 0 (success in all tasks).

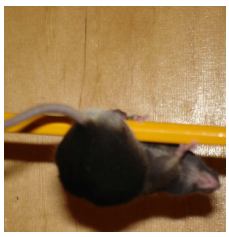
Table 3.1. Neurological Severity Score (NSS) scale. Adapted from Sabo et al. [239]

At the same time points posttraumatic beam–balance test was performed. Design and rating was analogical to the pre-training protocol. During every assessment each animal was given 3 trials and mean of their results was used for further analysis.

For analysis of temporal profile of neurological impairment changes in both NSS and BB were calculated. For given time point change in NSS (D-NSS) or in BB (D-BB) was obtained in following way: a result of previous measurement was subtracted from actual value.

0 steady posture on the beam

1 grasps the side of the beam and/or has unsteady movements



2 hugs the beam without falling

3 hangs on the beam without falling

4 hangs on the beam and falls off

5 does not attempt to balance

If the mouse attempts to perform the task with result 0-3, but falls off before end of scoring time (<60”), one point is added

Fig 3.3. Beam Balance Test scoring (modified after Scherbel et al. [241])

3.5. Techniques for histological analysis

3.5.1. Histopathological analysis

$APP_{751}SL$ and WT littermate male and female mice used in this study received sham injury or repetitive TBI, and the mice were killed 4 weeks after second surgery. Each experimental group (8 groups: APP–TBI, APP–sham, WT–TBI, WT–sham for both female and male mice) consisted of 5 animals (n = 5 for each group; in total n = 40)

After the study on living animals was concluded mice were humanely euthanized with an overdose of sodium pentobarbital (Narcoren, 200 mg/kg i.p.) and their brains were excised and dissected in coronal plane app. 3 mm anteriorly from the injury site. Both parts of the brain were snap- frozen in hexane chilled to –80℃ and stored in –80℃ until further processing.

Thereafter, 8 µm–thick frozen sections were cut in 3 series from posterior part of each brain.

Sections were mounted on microscope slides (Superfrost®, Carl Roth for histology and Superfrost®Plus, Carl Roth for immunostaining), dried overnight at room temperature and stored at –80℃ until staining.

The histology and location of the TBI site were examined by haematoxylin and eosin (H&E) as well as by Perls Prussian Blue iron staining, and the gross neuronal loss assessment was performed using the Nissl staining.

Alternate sections were examined by immunohistochemistry. Brain sections were immunostained with previously characterized monoclonal and polyclonal antibodies that are highly specific for: Aβ peptides (692), APP (23850) and cathepsin D. Sections adjacent to those immunostained for Aβ were stained with thioflavin S, specific for protein β-structure of fibrillary Aβ.

Light microscopic examination (Leica DM1000) and fluorescent microscopic examination for thioflavin S–stained slices (Olympus BX 51) were then performed on the sections.

3.5.1.1. H&E staining of cryosections

Solutions and Material:

Fixative:	4% phosphate buffered Formaldehyde (100 ml 40% Formaldehyde, Merck + 900 ml 0,1 M phosphate buffer); pH = 7
Differentiating solution:	acid alcohol: 0.1% HCl in 70% Ethanol (10 ml 37% HCl , Merck + 1 L 70% Ethanol)
Ehrlich's Haematoxylin staining solution	Hematoxylin (Merck) 6 g + Ethanol 300 mL + Aluminum potassium sulphate (Carl Roth) 45 g + Distilled water 300 mL + Glycerol 300 mL (Merck) + Glacial acetic acid (Merck) 30 mL; ripen after preparation
Eosin staining Solution:	Eosin G (Merck) 0.1 g + 100 mL distilled water
Quick-hardening mounting medium	Entellan (Merck)

Procedure

- 10 min fixation of tissue sections in phosphate buffered formaldehyde, pH = 7
- 2 x short rinsing in tap water
- short rinsing in distilled water
- 15 min staining in Harris Haematoxylin solution
- short differentiation in acid alcohol (0.1% HCl)
- 30 min bluing in running tap water
- short rinsing in distilled water
- 15 min staining in 0.1 % Eosin solution
- Dehydration of tissue sections in series of graded alcohols
  (1 x 1 min 70% Ethanol, 1 x 5 min 96% Ethanol, 2 x 5 min 100% Ethanol, 5 min Xylol)
- Embedding of tissue sections using a quick hardening mounting medium (Entellan, Merck)

3.5.1.2. Nissl staining of cryosections

Solutions and Material:

Nissl staining Solution:	Cresyl Violet (Certistain®, Merck) 0.1 g + 100 mL 0.2 acetic buffer
Rinsing buffer	0.2 M acetic buffer (3.28 g sodium acetate, Merck + 9.76 mL glacial acetic acid, Merck + distilled water ad 1000 mL)
Quick-hardening mounting medium	Entellan (Merck)

Procedure

- 10 min staining in Cresyl Violet solution
- short rinsing in 0.2 M acetic buffer (pH = 5)
- Dehydration of tissue sections in series of graded alcohols (1 x 1 min 70% Ethanol, 1 x 5 min 96% Ethanol, 2 x 5 min 100% Ethanol, 5 min Xylol)
- Embedding of tissue sections using a quick hardening mounting medium (Entellan, Merck)

3.5.1.3. Prussian Blue staining of cryosections

Solutions and Material:

Fixative:	4% phosphate buffered Formaldehyde (100 ml 40% Formaldehyde, Merck + 900 ml 0,1 M phosphate buffer); pH = 7
Staining solution- component A	1% hydrochloric acid (0.86 mL 32% HCl, Merck + 100 mL distilled water)
Staining solution- component B	2% Potassium hexacyanoferrate (II) trihydrate (2 g Potassium hexacyanoferrate (II) trihydrate, Merck + 100 mL distilled water); prepared immediately before use
Nuclear fast red staining Solution:	0.1 g nuclear fast red (Certistain®, Merck) dissolved in hot solution of 5 g aluminium sulphate hydrate (Merck) in 100 mL distilled water
Quick-hardening mounting medium	Entellan (Merck)

Procedure

- 10 min fixation of tissue sections in phosphate buffered formaldehyde, pH = 7
- 2 x short rinsing in tap water
- short rinsing in distilled water
- 60 min staining in mixture of equal volumetric parts of A and B staining solutions prepared immediately before use
- 10 min washing in distilled water
- 10 min counterstaining in 0.1% nuclear fast red
- short rinsing in distilled water
- Dehydration of tissue sections in series of graded alcohols
  (1 x 1 min 70% Ethanol, 1 x 5 min 96% Ethanol, 2 x 5 min 100% Ethanol, 5 min Xylol)
- Embedding of tissue sections using a quick hardening mounting medium (Entellan, Merck)

3.5.1.4. Thioflavin S–staining of cryosections

Solutions and Material:

Thioflavin S staining solution:	1 g Thioflavin S + 100 mL distilled water
DAPI staining solution	5 mg 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Merck) + 100 mL PBS
Mounting medium for fluoroscopy	0.5 mL Moviol 488 (Calbiochem) + 20 mg 1,4-Diazabicyclo[2.2.2]octane (DABCO, Merck)

Procedure

- 1 min hydration of tissue sections in 70 % Ethanol
- 2 x 1 min washing in distilled water
- 8 min staining in 1% Thioflavin solution
- 2 x 1 min washing in distilled water
- 4 min staining in 1% Thioflavin solution
- 2 x 1 min washing in 80% Ethanol
- 3 x 1 min washing in distilled water
- 1 min counterstaining in DAPI solution
- Embedding of tissue sections using a Moviol / DABCO mounting medium

3.5.2. Immunostainings on cryosections

Preparation of tissue sections and immunostaining using the ABC-Vectastain-Kit

To determine the localisation and expression pattern of different proteins in brain tissues of mice, the frozen tissue sections have been analysed with immunohistochemical detection methods. These methods make use of a covalent and irreversible binding between avidin, an egg white protein, and biotin, a vitamin. By establishing a biotin link through avidin, between the horseradish peroxidase enzyme and a secondary antibody reagent, enzyme location can be achieved at the site of primary antibody interaction within the specimen. Many biotin molecules can be incorporated by horseradish–peroxidase, without losing enzymatic activity. On the other hand biotin can also be conjugated to immunoglobulin. These biotin molecules can be joined via an avidin molecule by creating a complex of avidin and biotinylated enzyme, which can be attached to the biotinylated secondary antibody. Each avidin molecule harbors four biotin binding sites and there are two further binding sites for avidin on each biotin molecule. This complex reacts with hydrogenperoxide and an electron donor to the endproducts water and a dye molecule. 3,3-Diaminobenzidine-tetrahydrochloride (DAB) serves as an electron donor, which forms a brown, electron dense precipitate, which is insoluble in alcohol. The preparations made by this method can be dehydrated in an ascending row of alcohol solutions and conserved by embedding the stained tissue sections in mounting media. Due to using frozen, non-fixated tissue sections, antigen- retrival step (post fixation treatments) could be omitted.

Solutions and Material:

Fixative:	Acetone (Roth)
Methanol Blocking-Solution:	200 ml methanol + 2 ml 30% H_2O_2
Unspecific Blocking-Solution:	10% fetal calf serum (FCS) + 4% skim milk (Roth) in PBS
Primary Antibody:	10% FCS in PBS + varying concentrations of primary antibody (see Table 3.2.)
Secondary Antibody (DAKO):	10% FCS in PBS + 1:200 anti-mouse-IgG or anti-rabbit IgG
ABC-complex:	10% FCS + 1:100 Solution A + 1:100 Solution B (ABC-Kit, Vector Laboratories) in PBS. Solution has to be prepared 30 min before use. Storage at 4℃.

DAB-Stock-Solution:	10 mg 3,3-Diaminobenzidine-Tetrahydrochloride (DAB) (Sigma)/ml in 0.05 M Tris/HCl, pH 7.4. Solution has to be filtered and stored at –20°C.
DAB-Substrate-Solution:	5 ml 0.05 M Tris/HCl, pH 7.4 + 100 µl DAB Stock-Solution + 2.5 µl 30% H_2O_2 (Roth)

Quick-hardening mounting medium (Riedel–de Haen)
Vectastain ABC-Elite PK 6100 Standard (Vector Laboratories)
Harris' hematoxylin solution (Merck)

Procedure

- Fixation of tissue sections in pre-chilled (–20°C) Acetone (Carl Roth)
- Drying at room temperature for 2h
- 5 min washing in PBS
- incubation for 60 min in unspecific blocking-solution to saturate unspecific binding epitopes
- overnight-incubation with primary antibody at 4°C
- 15 min washing in PBS
- 60 min incubation with the respective secondary antibody at 37°C
- 15 min washing in PBS
- 30 min methanol blocking-solution to block endogenous peroxidase-activity
- 10 min washing in PBS
- 90 min incubation with ABC at 37°C
- 15 min washing in PBS
- Incubation with DAB substrate-solution. Staining takes place under microscopic control and is stopped by incubation in PBS
- 15 min washing in PBS
- Counterstaining in Harris' hematoxylin-solution
- Dehydration of tissue sections in series of graded alcohols
  (1 x 1 min 70% Ethanol, 1 x 5 min 95% Ethanol, 2 x 5 min 100% Ethanol, 2 x 5 min Xylol)
- Embedding of tissue sections using a quick hardening mounting medium (Corbit Balsam, Riedel–de Haen)

Table 3.2. Commercially available and provided antibodies used as primary antibodies

Protein (epitope)	Antibody	Clone	Dilution	Manufacturer
APP	23850	polyclonal	1:500	G.Multhaup (ZMBH)
Aβ	692	polyclonal	1:500	G.Multhaup (ZMBH)
GFAP	GFAP	polyclonal	1:1000	DAKO
Cathepsin D	Cathepsin D	polyclonal	1:500	DAKO

3.6. Protein content analysis

Analysis of β-amyloid content was performed in brain samples of APP-positive animals using enzyme–linked immunosorbent assay (ELISA)

The antigen, hAβ40 is detected by selective monoclonal anti-Aβ-antibodies, which interact with binding sites (epitopes) on the hAβ40. An N-terminal selective antibody is coated on the surface of a microtiter plate as capture antibody. A second, biotinylated antibody conjugate serves as the detection antibody in the assay.

The antibodies, in concert with the amyloid-peptide, form an antibody–amyloid–antibody complex (sandwich–complex). The complexed detection antibody is then indirectly coupled to an enzyme (horseradish peroxidase) through a biotin–streptavidin link. This peroxidase catalyzes the conversion of a substrate (Chromogen) into a coloured product; which is detected by the test–kit through means of photometry and correlates directly to the hAβ-concentration present in the sample. Measured values are quantified in correlation to synthetic peptide standards.

Procedure

For quantitation of Aβ40 brain levels, the both anterior and posterior part (remaining after sectioning for histological analysis) of each brain was used. The pieces of brain tissue were additionally divided into left and right part and fragments of respective hemispheres were collected in individual test tubes and weighed. Dissection was performed on dry ice (at ~ –70℃) and utmost care was given to prevent samples from thawing.

Extraction of samples was performed with phosphate-buffered saline (PBS) to measure brain Aβ40. The brain samples were homogenized (1/10, weight/volume) in 1 x PBS

(prepared with Milli-Q water and superpure grade reagents from Merck) by 12 strokes at 650 rpm with a CAT Potter Teflon pestle. The homogenate was centrifuged at 16,000 rpm for 30 min at 4℃.

Homogenate was aliquoted into fresh tubes (200 µl each aliquot), 8 µl of 1:25 stock solution of proteinase inhibitor (Complete®, Roche, Mannheim, Germany) was added to each probe and vortexed vigorously.

Thereafter, samples were incubated on ice for 30 minutes and centrifuged at 16 000 rpm, 4℃ for 30 min. Supernatant was aliquoted (50 µl), collected into fresh Eppendorf tubes, snap–frozen on dry–ice and stored at –80℃ for further processing.

For assessment of brain Aβ40 level, commercially available hAβ40 hs ELISA kit® (The Genetics Company, Zurich, Switzerland) was used according to protocol of the manufacturer.

3.7. Statistical analysis.

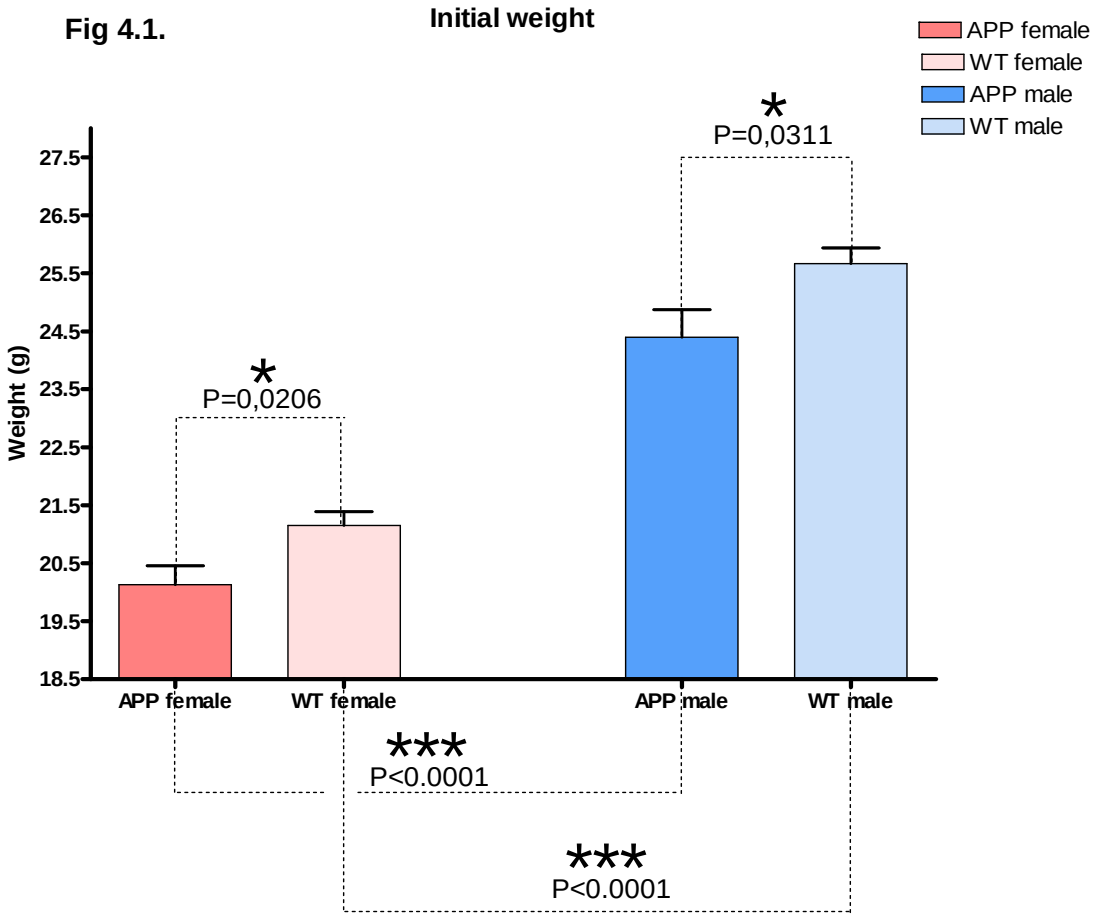
One–way ANOVA with Bonferroni's multiple comparison post–hoc test and Student t-test were used for statistical analysis of the numerical results of the study (weight, duration of anaesthesia, duration of apnoea, mortality, D-weight, NSS, D-NSS, BB, D-BB). All calculations were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego).

4. RESULTS AND ANALYSIS.

4.1. Initial weight

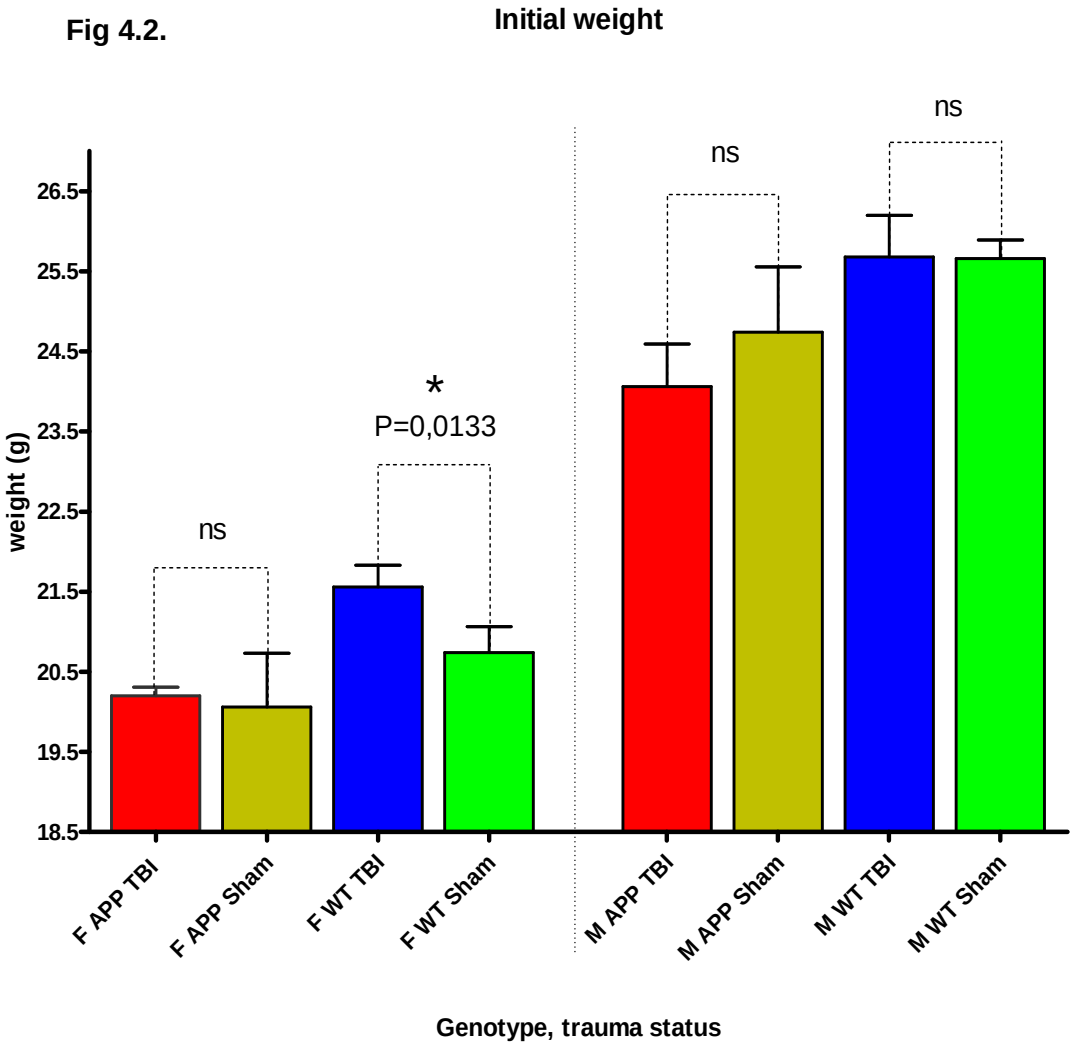
Measurements of body weight in groups of animals prior to surgery revealed significant sex– and genotype–related differences (Fig. 4.1.).

Fig 4.1.



Subsequent additional analysis for TBI/sham subgroups separately revealed unintended pre-selection in F WT group, caused by random selection (significant difference between TBI and sham subgroups) (Fig. 4.2.).

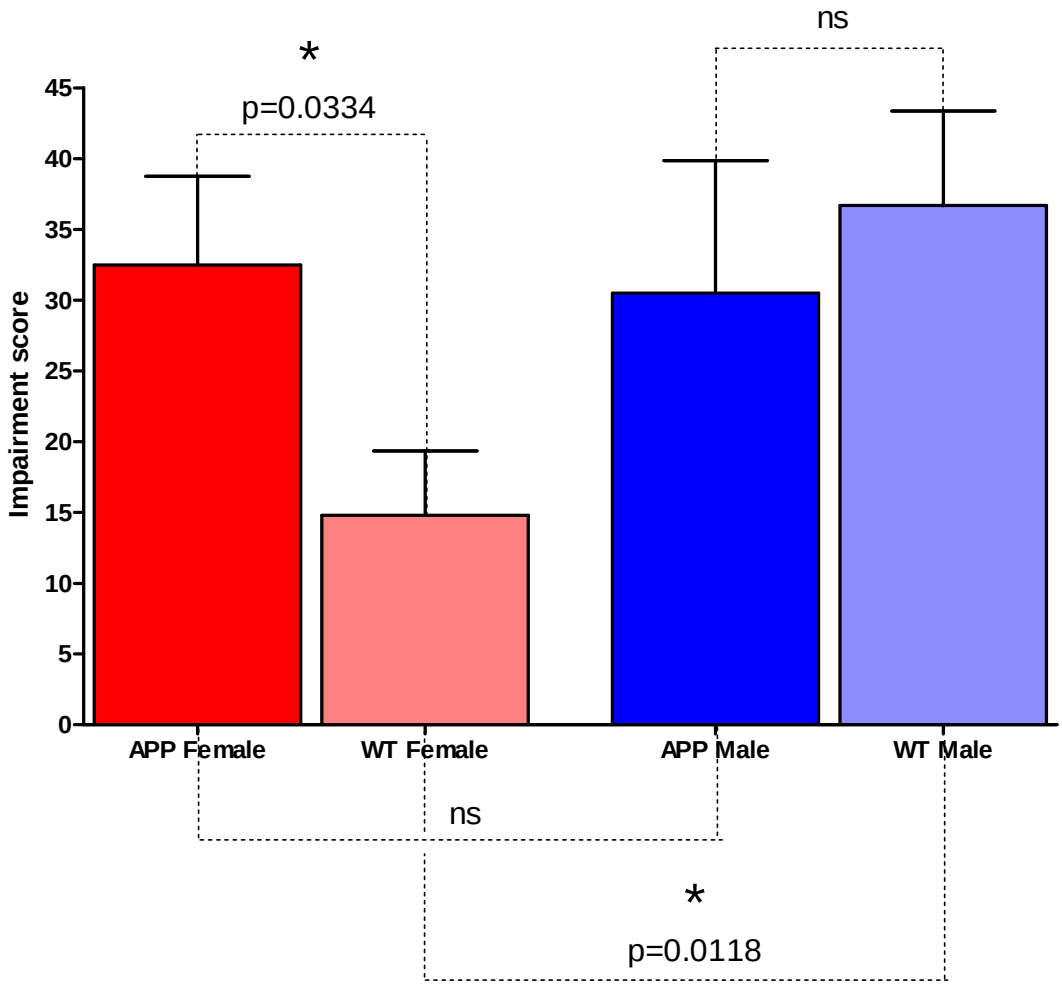
Fig 4.2.



4.2. Pre- training

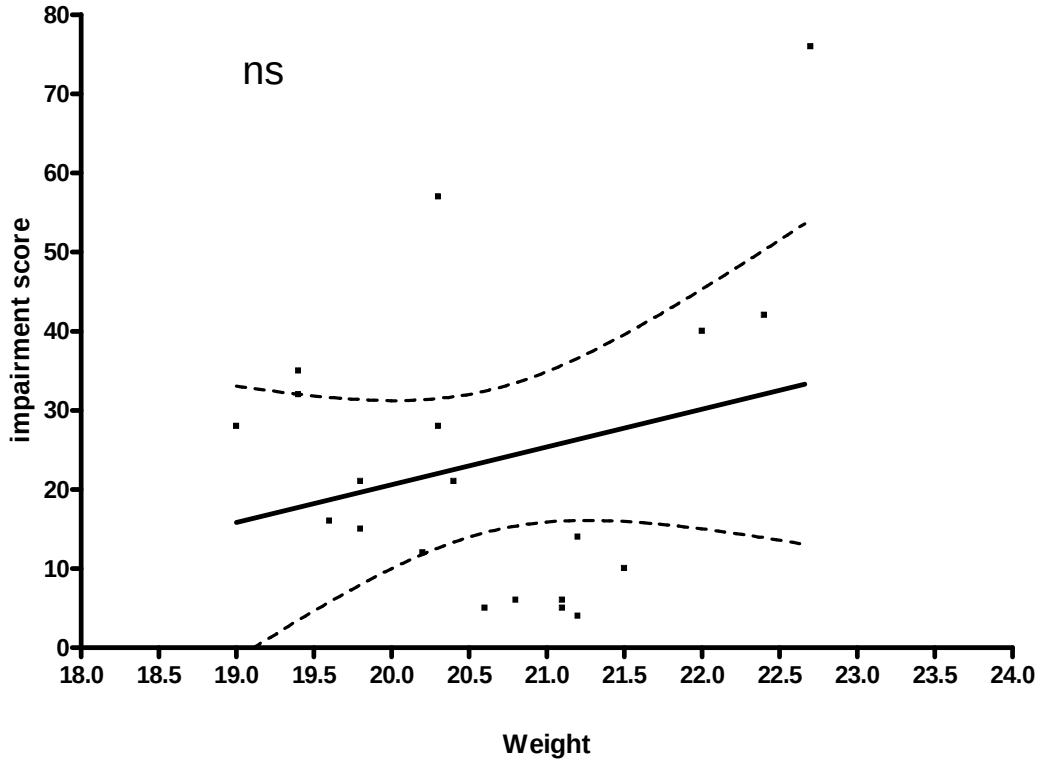
Neurological status assessment performed before any surgical intervention revealed significantly higher neurological impairment score in female but not male APP transgenic animals in comparison to their WT littermates. There was also a significant sex–related difference in test results between WT but not APP mice (Fig 4.3.).

Fig. 4.3. Impact of genotype and gender on neurological performance



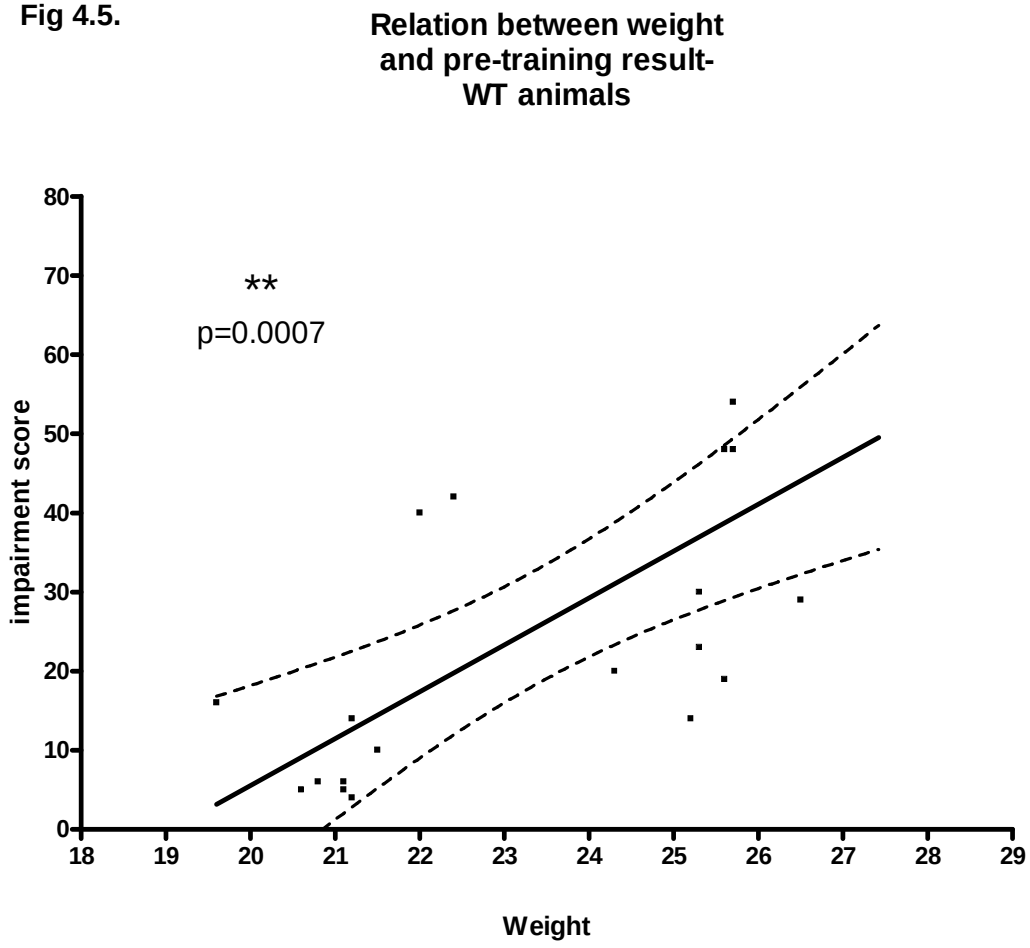
Since in physiological conditions, increased weight without substantial neurological impairment is correlated with poorer performance in balance–based tests, significant initial weight differences were regarded. In order to exclude this as potential source of a bias in interpretation, analysis of correlation between neurological performance and weight has been performed. There was no significant correlation between weight and neurological impairment in female animals (Fig 4.4.).

Fig 4.4. **Relation between weight and pre-training result- female animals**



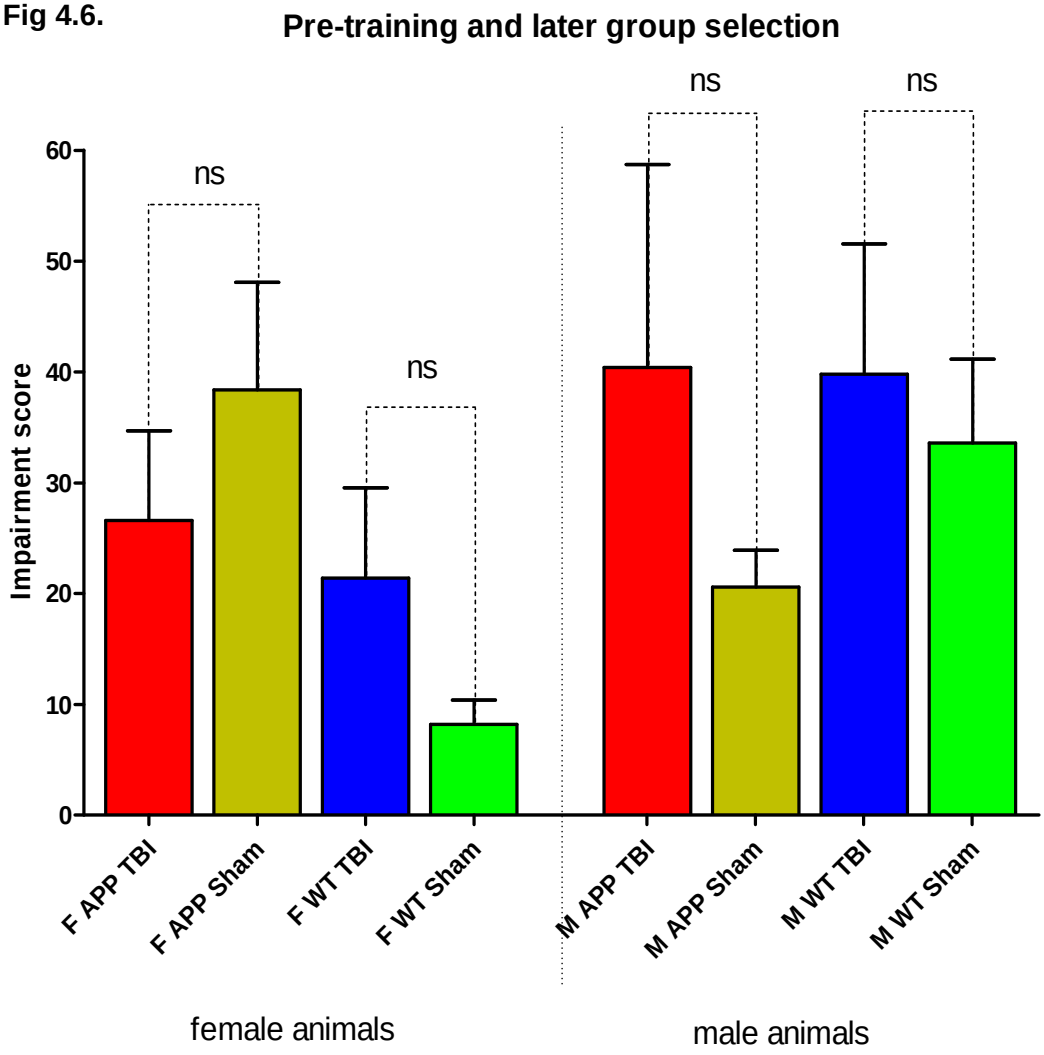
The same analysis however revealed that observed difference in neurological performance between M WT and F WT animals was strongly weight–dependent (Fig 4.5.).

Fig 4.5.



Subsequent additional analysis for TBI/sham subgroups separately revealed no pre-selection caused by random selection (no significant difference between TBI and sham subgroups) (Fig. 4.6.).

Fig 4.6.


Pre-training and later group selection
ns
ns
ns
ns
Impairment score
60
50
40
30
20
10
0
F APP TBI
F APP Sham
F WT TBI
F WT Sham
M APP TBI
M APP Sham
M WT TBI
M WT Sham
female animals
male animals

4.3. Mortality

The mortality analysis is presented in Table 4.1. All the animals that died during experiment before 28 day posttrauma were excluded from other analyses, therefore each experimental subgroup consisted ultimately of 5 animals.

Table 4. 1.

Mortality % (died animals/animals destined for group)	APP		WT	
	TBI	Sham	TBI	Sham
Female	**28,6%** (2/7)	**0%** (0/5)	**0%** (0/5)	**0%** (0/5)
Male	**37,5%** (3/8)	**37,5%** (3/8)	**16,7%** (1/6)	**0%** (0/5)

Table 4.2 presents detailed stratification of mortality regarding time and presumed cause of death.

Postoperative / posttraumatic seizures were occasionally observed throughout the further experiment among M APP animals. However, due to lack of continuous monitoring of animals for the whole experimental period (28 days) this phenomenon could not be assessed as to the pattern of occurrence, duration, and lethality.

Table 4.2.

Mode of death	No of animals	Subgroup specifications
Prolonged posttraumatic apnoea	4	2 F APP TBI 1 M APP TBI 1 M WT TBI
Perioperative (trauma, surgery– or anaesthesia–related?)	2	1 M APP TBI 1 M APP Sham
Delayed (>24 h after second surgery; seizure–related?)	3	1 M APP TBI 2 M APP Sham

The statistical analysis of mortality revealed a clear trend (p = 0,058) toward increased mortality among APP transgenic animals in general. Subsequent single factor analysis among separate two–factors–matched groups disclosed no additional statistical relationship.

4.4. Duration of anaesthesia

Analysis of anaesthesia time among different subgroups revealed single differences in sex–matched groups, of however inconsistent pattern (Fig 4.7. and 4.8.). No significant sex–related difference was found in duration of anaesthesia (Fig 4.9.).

Fig 4.7. Duration of anaesthesia in female animals

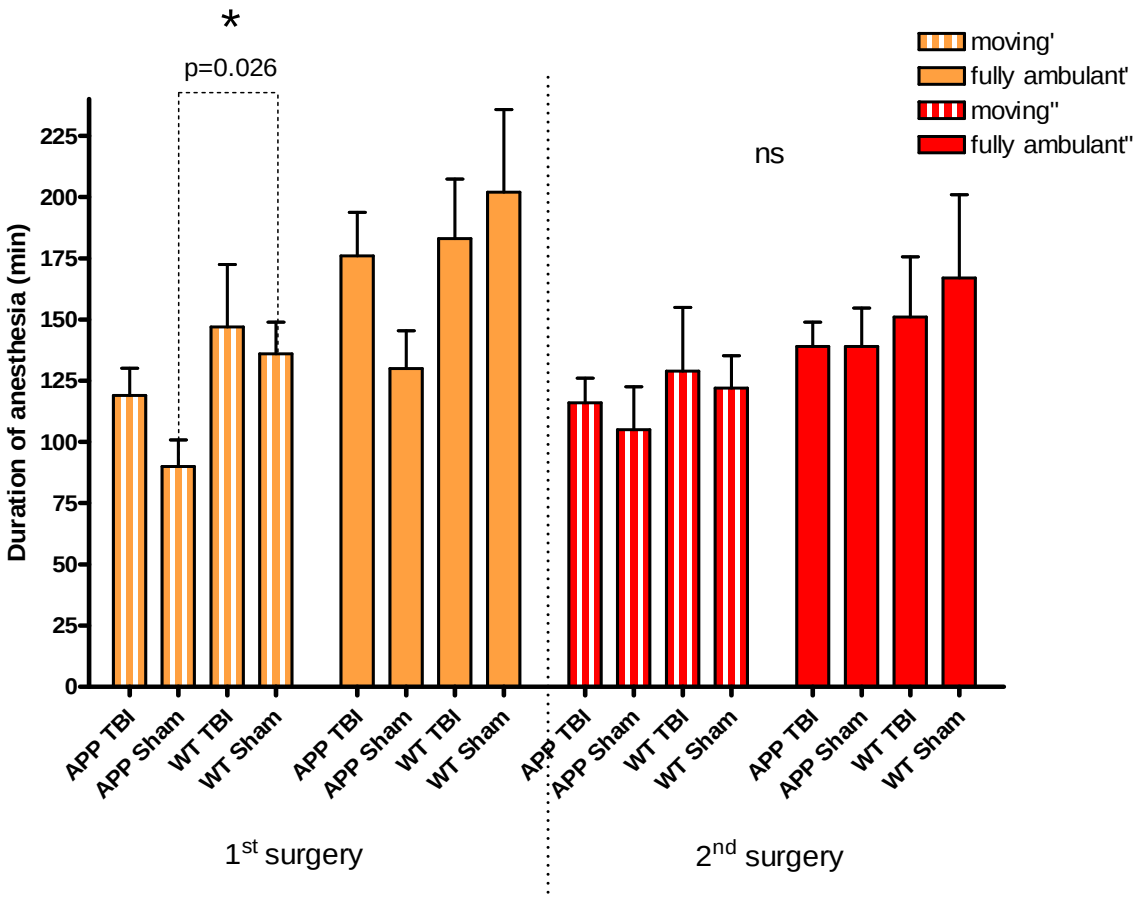


Fig 4.8. **Duration of anaesthesia in male animals**

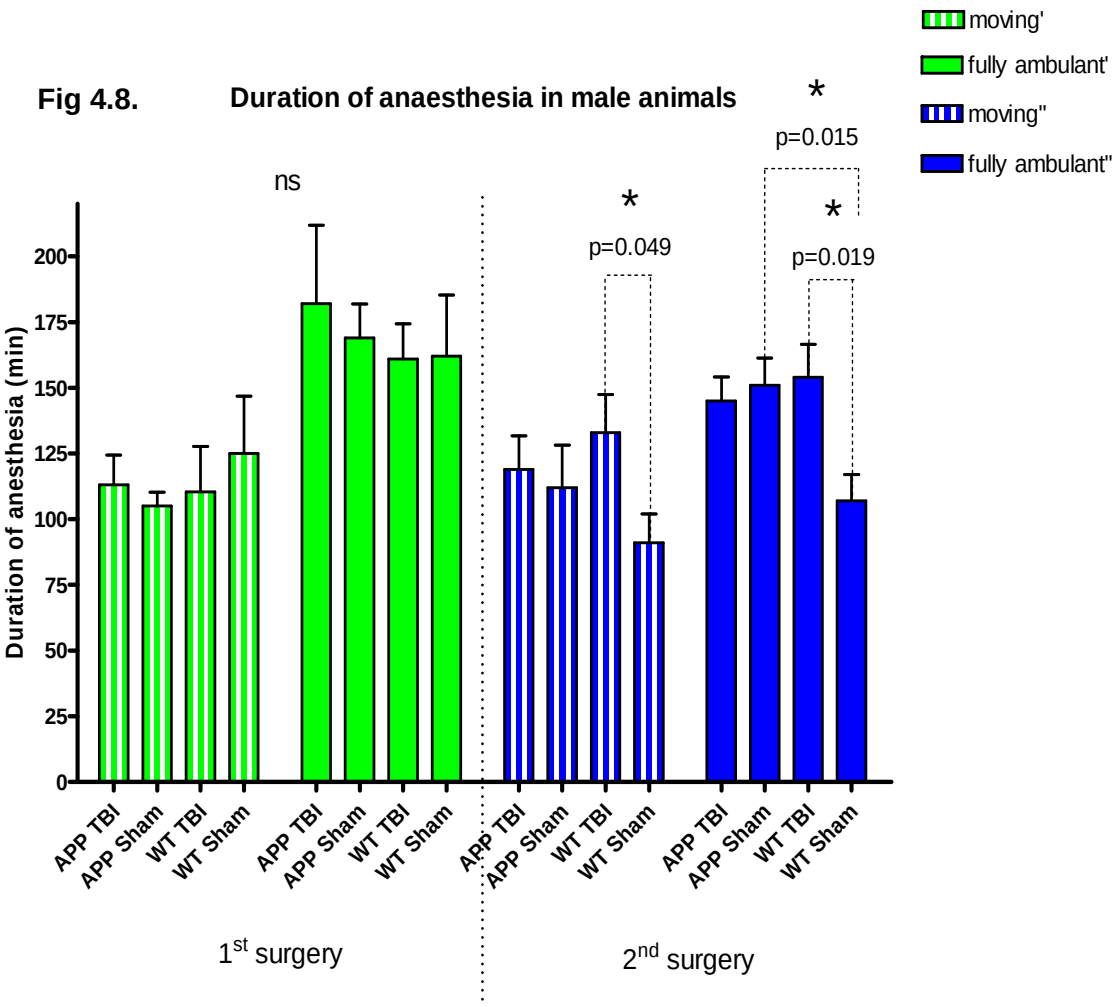
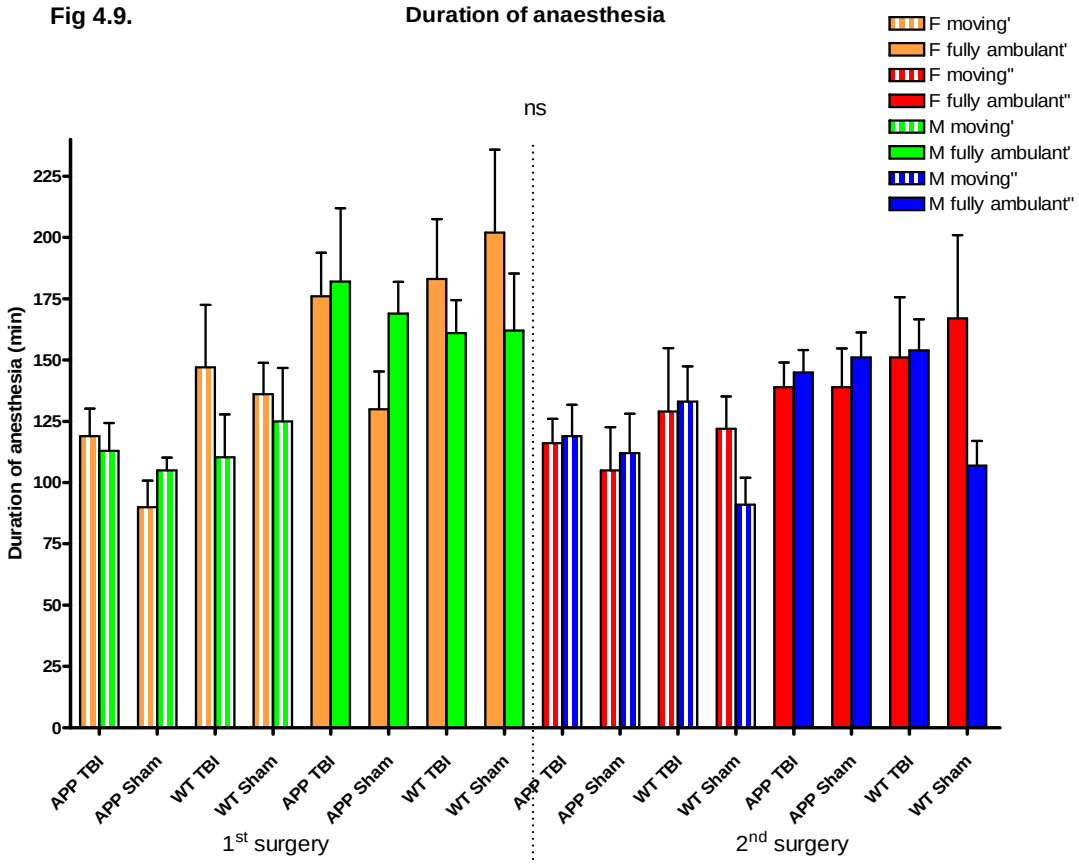


Fig 4.9.



4.5. Weight changes

During experimental period only single, elusive differences between experimental groups were found. Summarising, no consistent pattern of weight changes could be described

4.6. Neurological impairment

According to NSS and BB tests results trauma did not impact neurological performance among WT animals save NSS in female animals at time point 72h posttraumatically (Fig 4.10.B). There was also no impact among APP mice except BB in male animals at time point 28 d after second CHI (Fig 4.15.A).

The impact of genotype on outcome was limited to poorer NSS performance in TBI-subjected APP transgenic females at time point 4h" and 7d" when compared to WT ones (Fig 4.10.C). However, in BB test TBI APP females presented lower impairment index than WT at time point 6h' (Fig 4.14.C). Notably, neurological impairment was also present among sham injured APP animals (NSS, time points 4h" and 24h" for females

and 28d for males) in comparison to sham WT littermates of respective sex (Fig 4.10.D, 4.11.D).

Regarding gender influence, BB test revealed strongly significant difference among TBI APP mice at time point 24h' (Fig 4.16.A), and less though still significant in sham APP group at 14 d (Fig 4.16.B), both toward poorer performance in males. These results were however not confirmed by simultaneous NSS test (Fig 4.12.AB).

Additional analysis has been performed in genotype- matched, gender unsplit groups (genotype– and TBI-status–matched F and M [e.g. TBI WT F and TBI WT M] analysed as one group, composed of 10 animals). In that way, additional significance has been refined at 7d posttrauma among APP animals (TBI subjected mice performed worse) in NSS (Fig 4.13.A). Supplementary genotype effect has been here revealed at 4h" and 28d timepoints (worse performance after TBI among APP mice)(Fig 4.13.C). However, gender unsplit analysis of BB results disclosed no additional trauma or genotype effect (Fig 4.17.).

Summarising, significant differences in neurological performance were seen only at single post TBI / sham injury time points and they didn't follow any certain pattern of impairment. In particular, the points of significant difference that were present in NSS testing did not overlap those in BB test.

Fig 4.10. NSS scoring–female animals

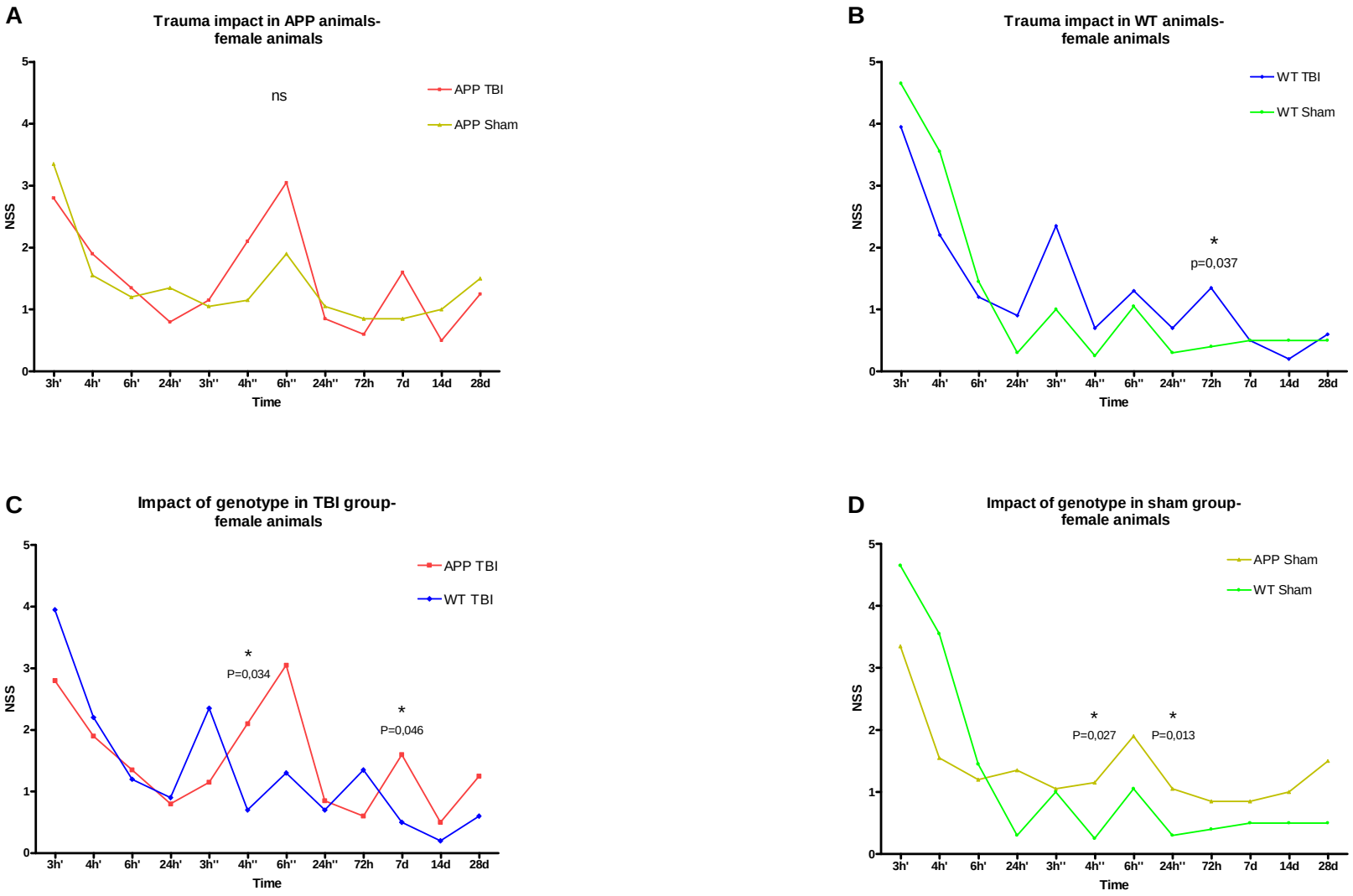


Fig 4.11. NSS scoring–male animals

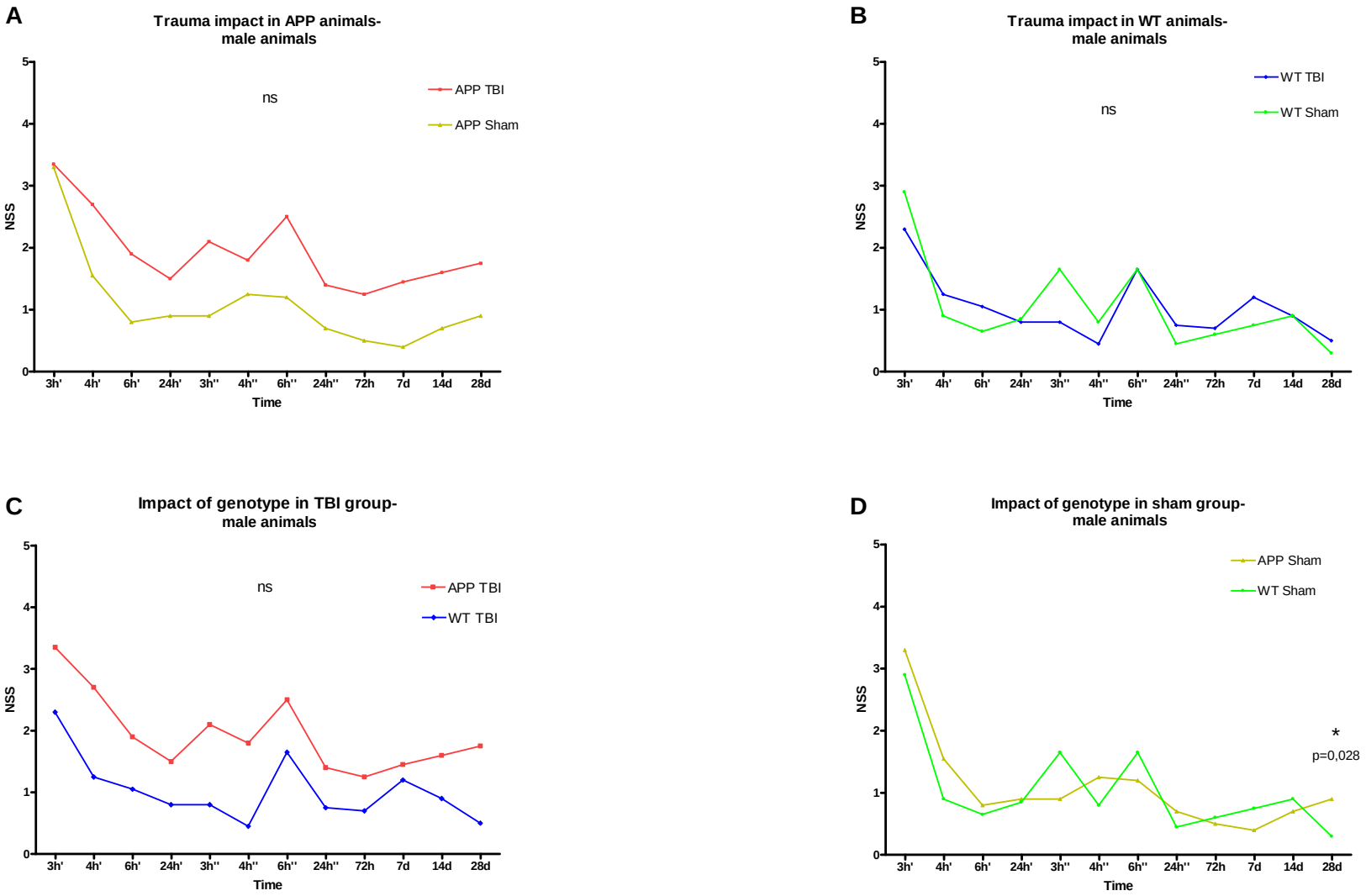


Fig 4.12. NSS scoring–female vs. male animals

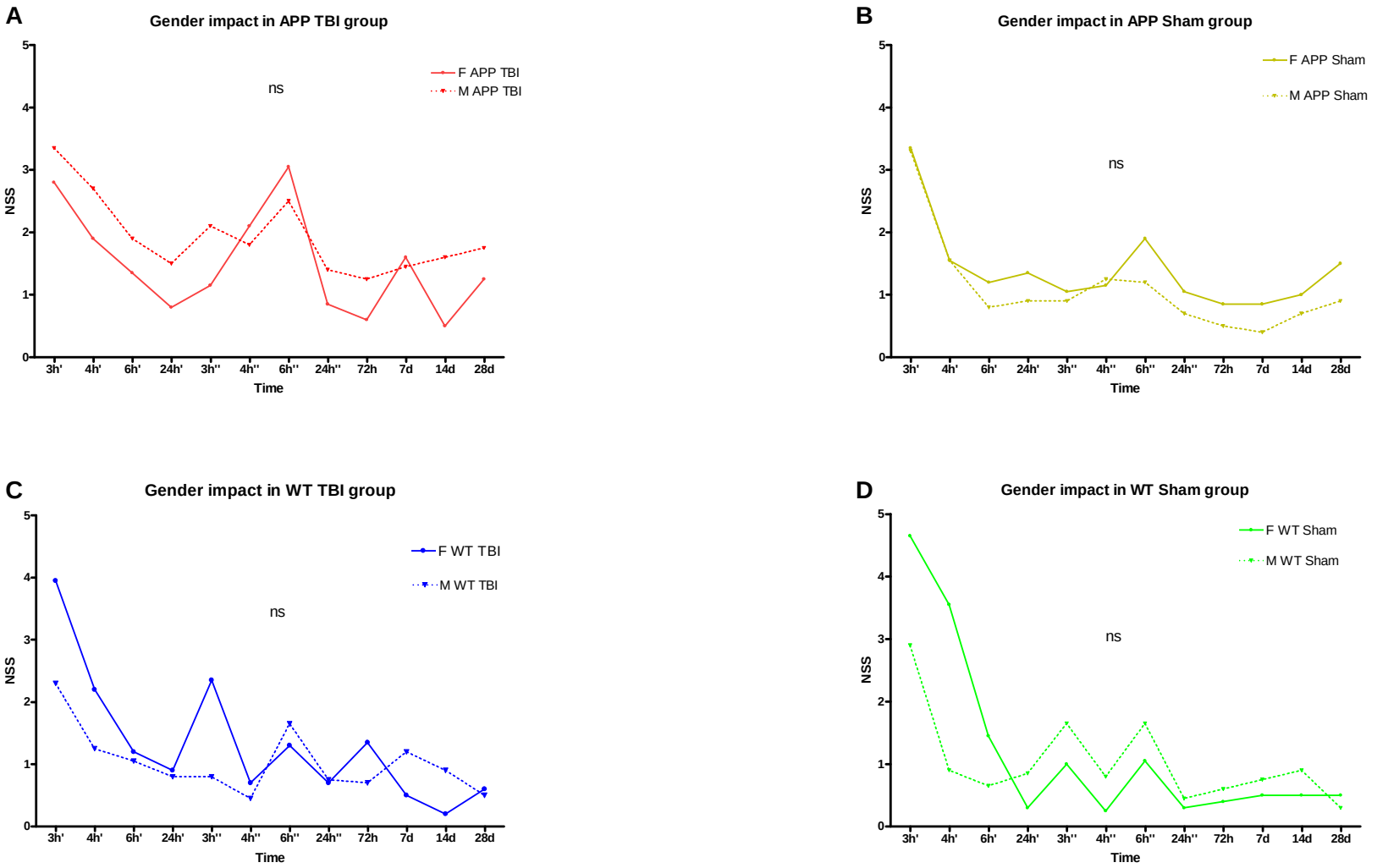


Fig 4.13. NNS scoring–APP vs. WT animals (gender unsplit)

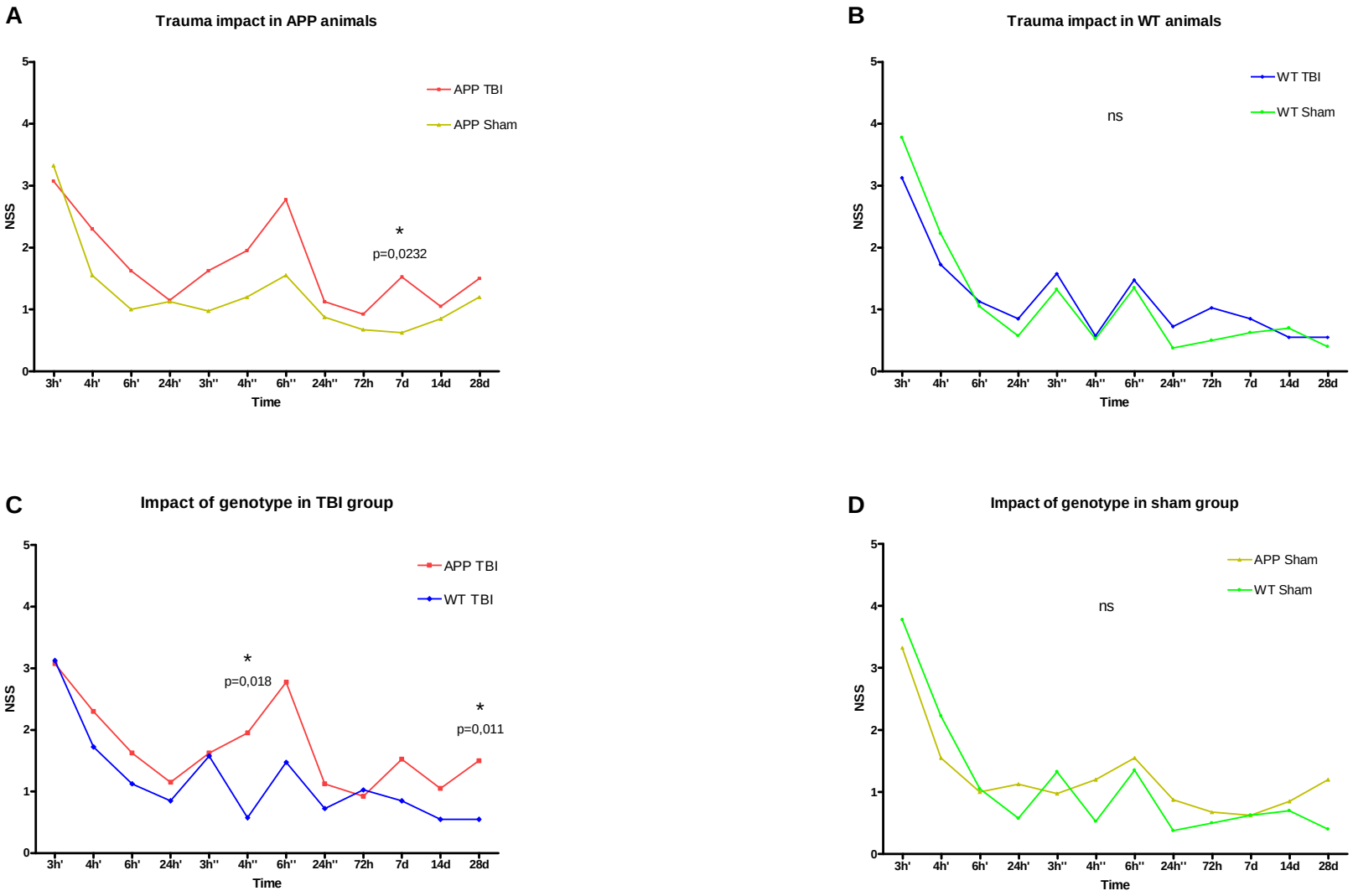


Fig 4.14. Beam Balance scoring–female animals

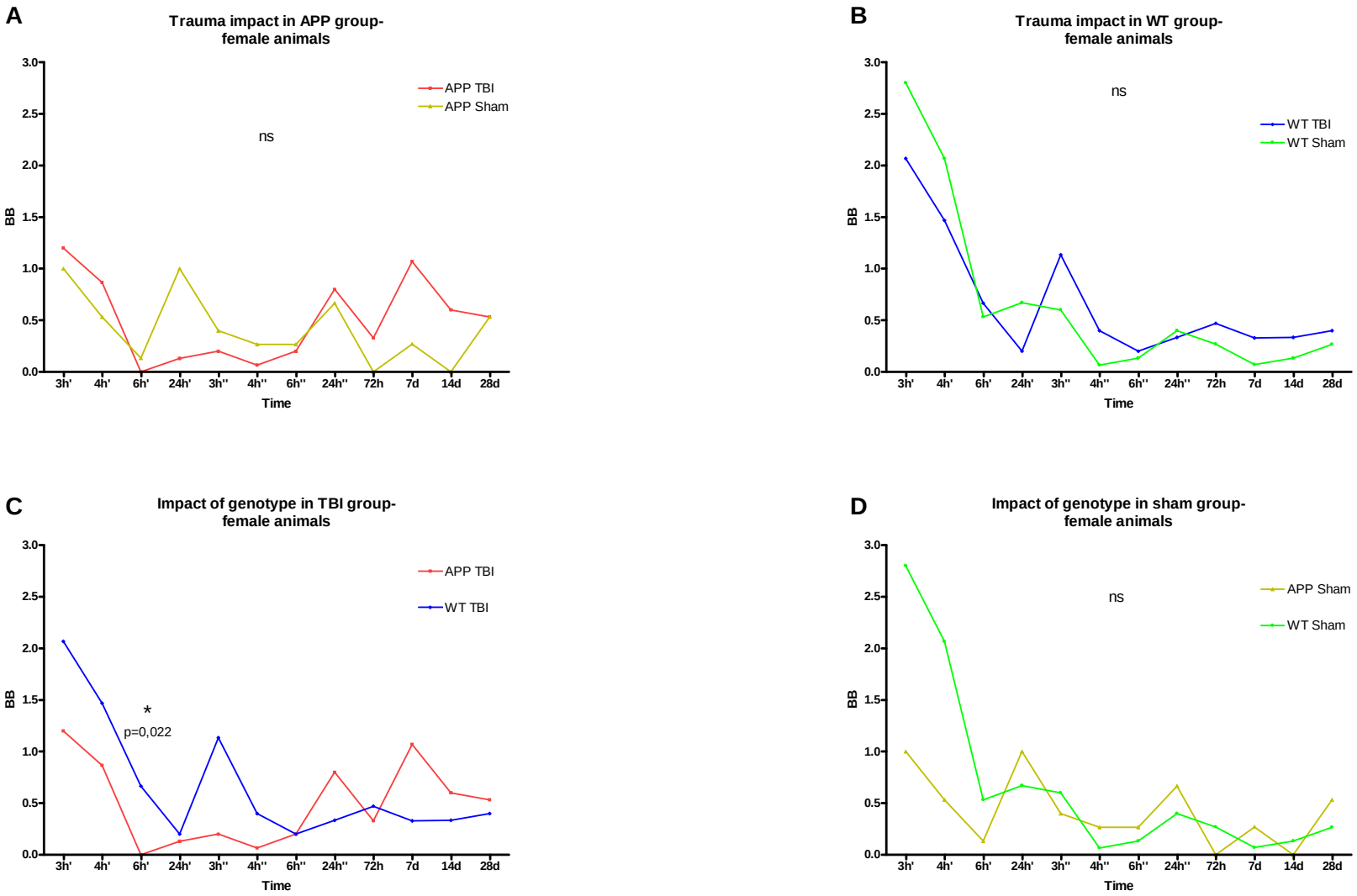


Fig 4.15. Beam Balance scoring–male animals

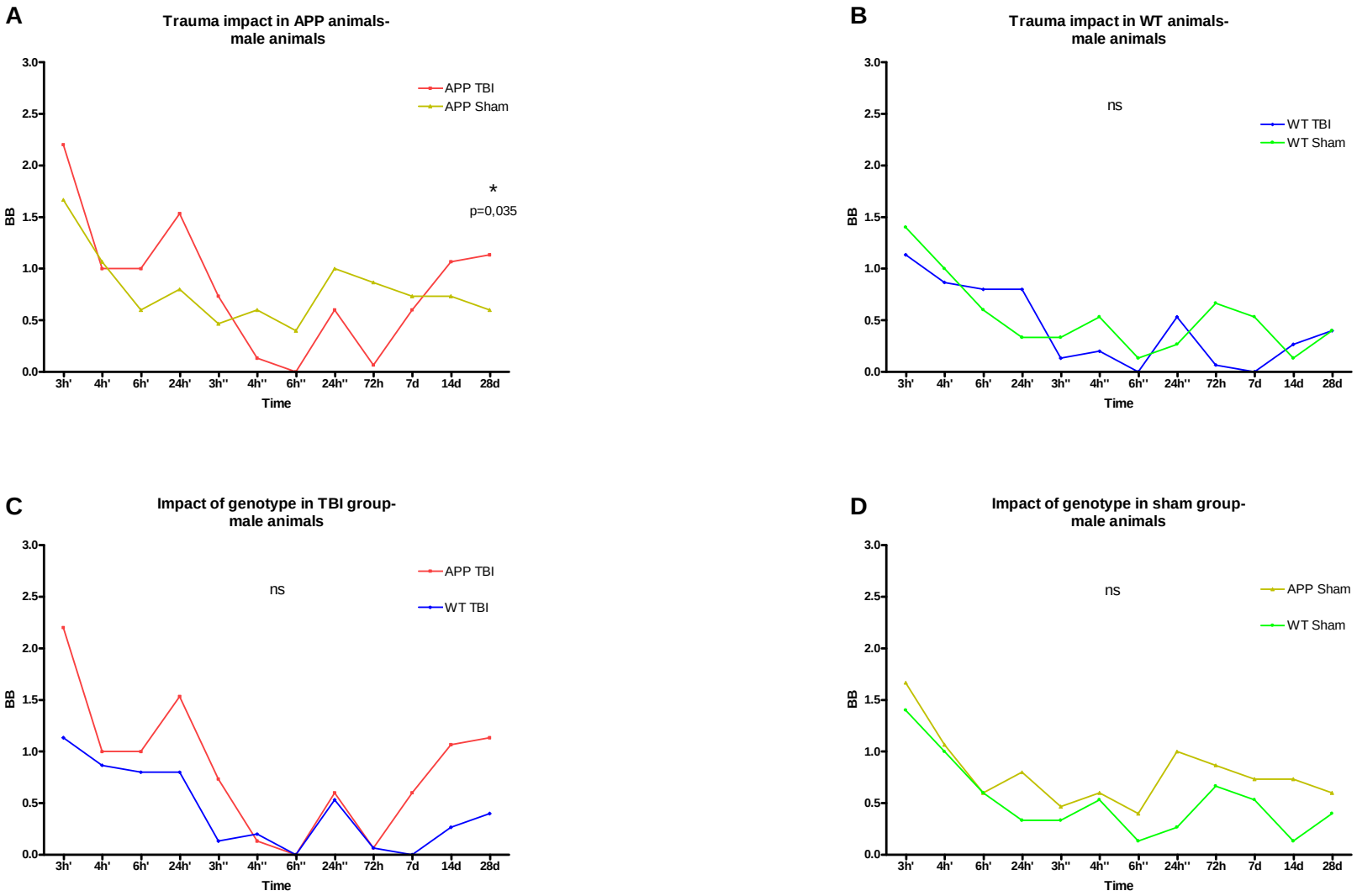


Fig 4.16. Beam Balance scoring–female vs. male animals

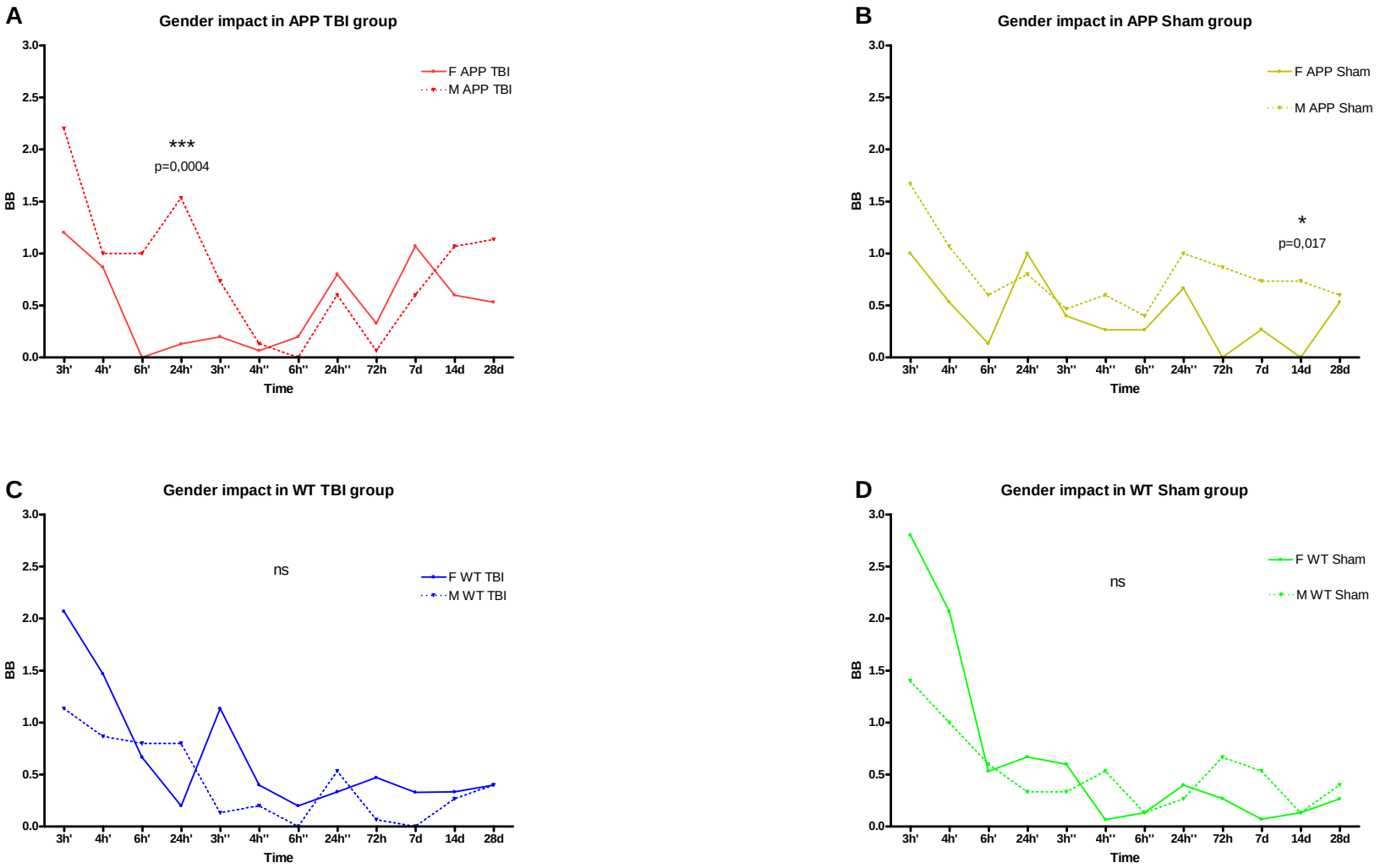
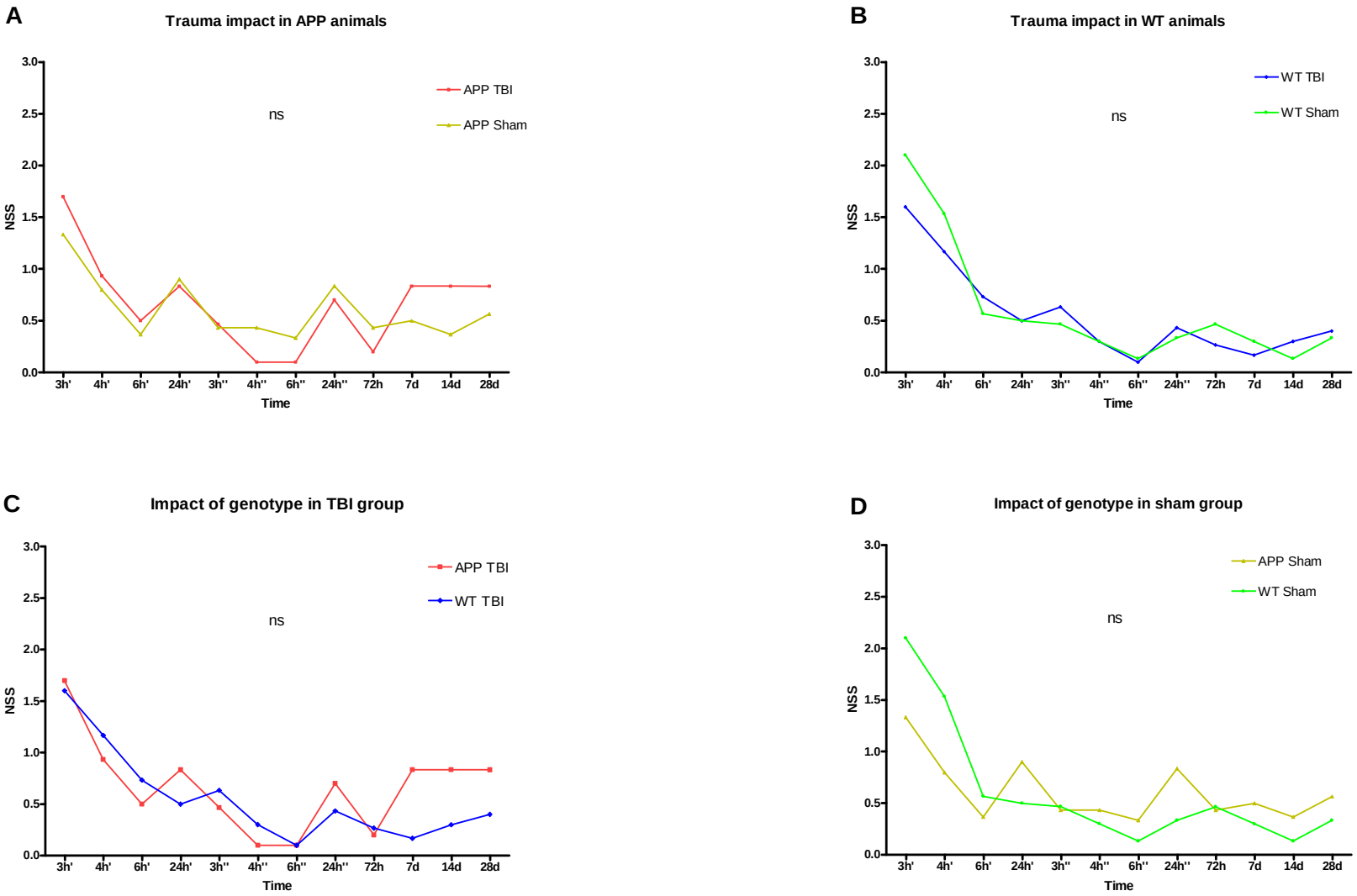


Fig 4.17. Beam Balance scoring–APP vs. WT animals (gender unsplit)



4.7. Post-surgery recovery profile

Beside of single points of significant difference, two potential tendencies could be outlined.

1. There was a shift in late recovery occurring first from 72h to 7d in female APP TBI animals, but as early as from 24h” to 72h in their WT littermates (Fig 4.18.C).

2. A similar shift could be observed also in WT TBI animals of different sex, where late recovery of females occurred from 24h” to 72h and from day 14 to 28, while correspondent recovery of male WT TBI mice emerged from 72h to 7 days posttraumatically (Fig 4.20.C). Notably, such a gender- related drift was not observed among TBI APP animals (Fig 4.20.A).

The abovementioned correlations were, however observed only in NSS results. No statistically significant differences could be outlined basing on BB test results (not shown).

Neither NSS nor BB analysis performed in gender unsplit groups revealed additional effect (not shown).

Fig 4.18. Temporal profile of NSS changes–female animals

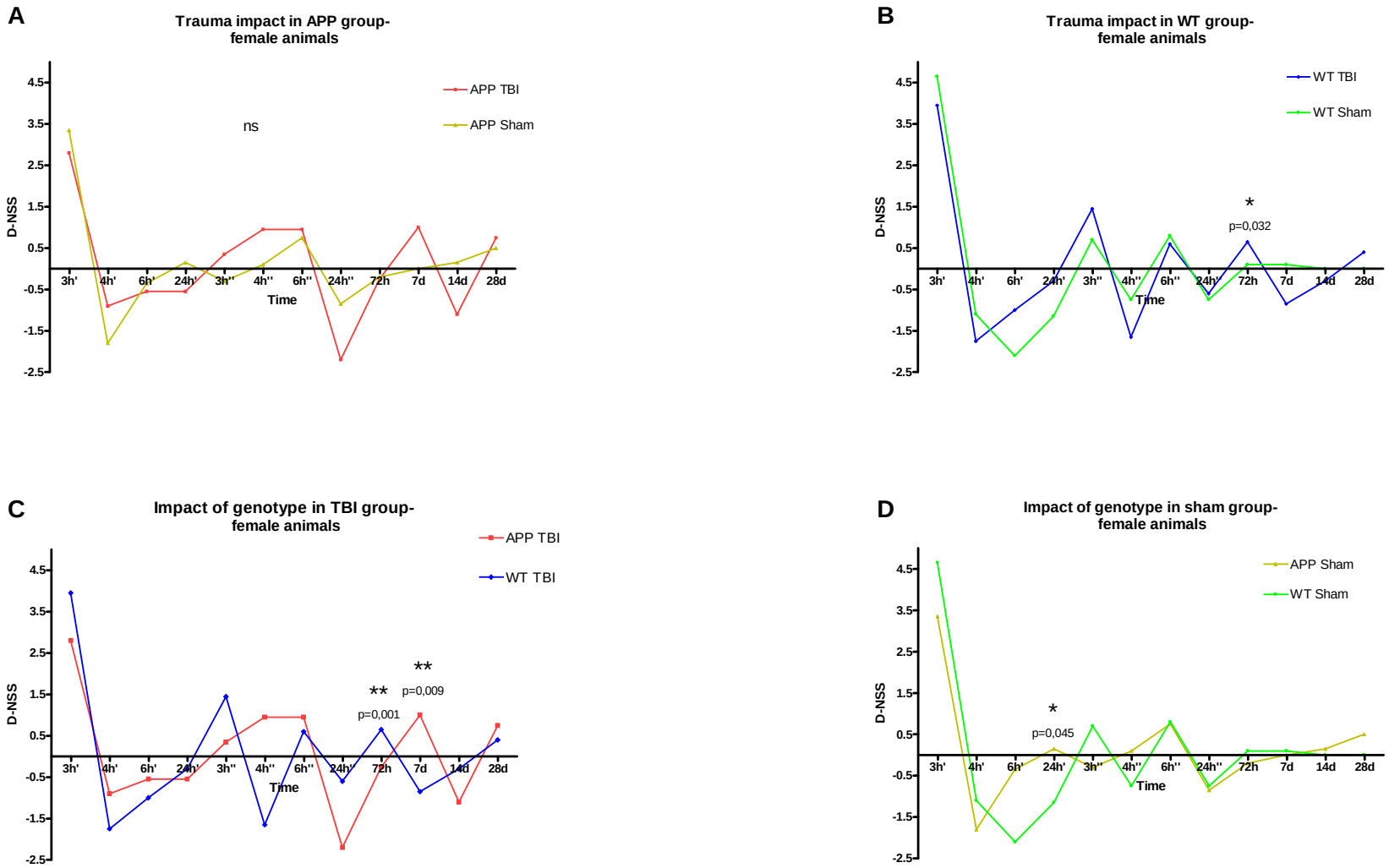


Fig 4.19. Temporal profile of NSS changes–male animals

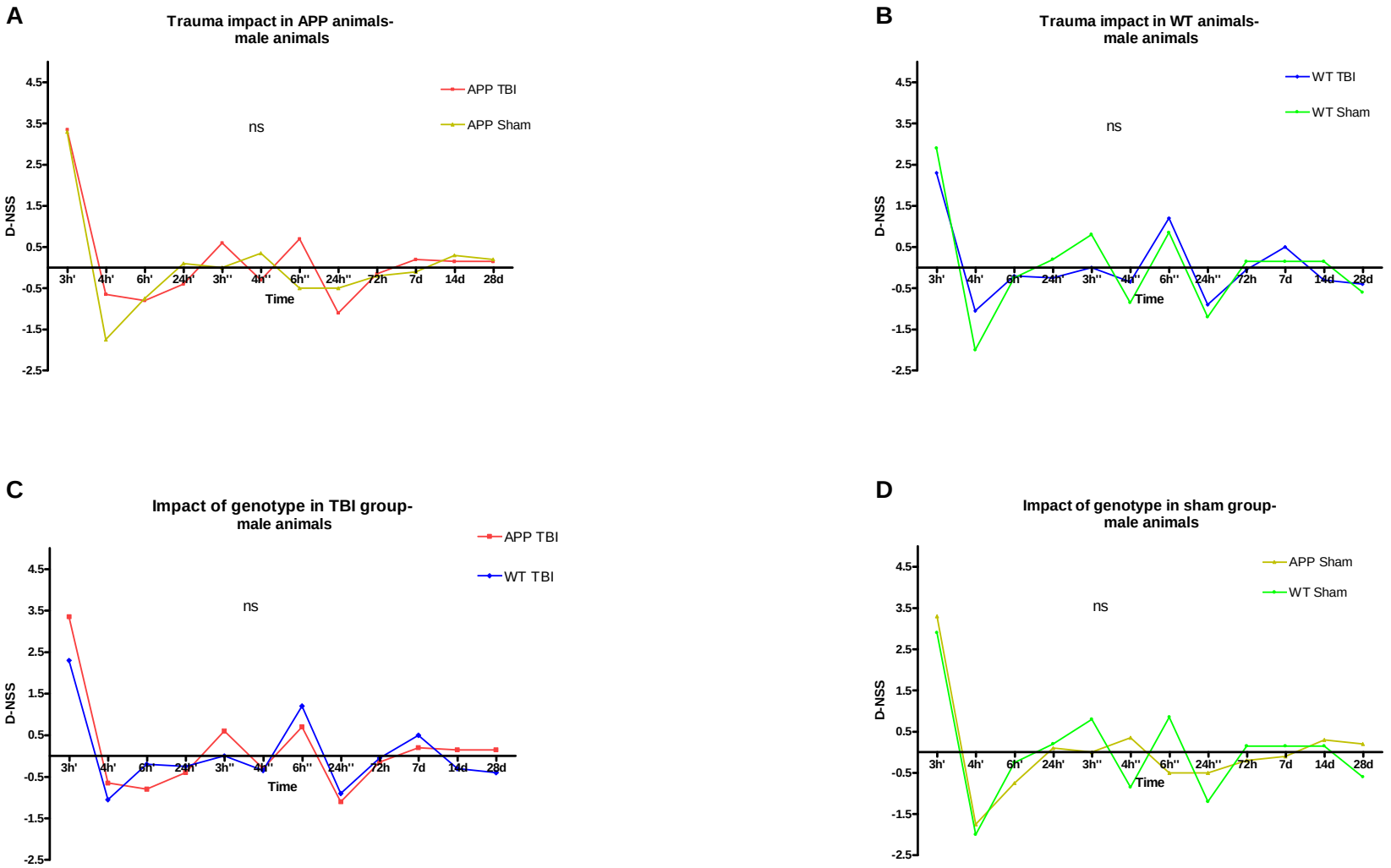
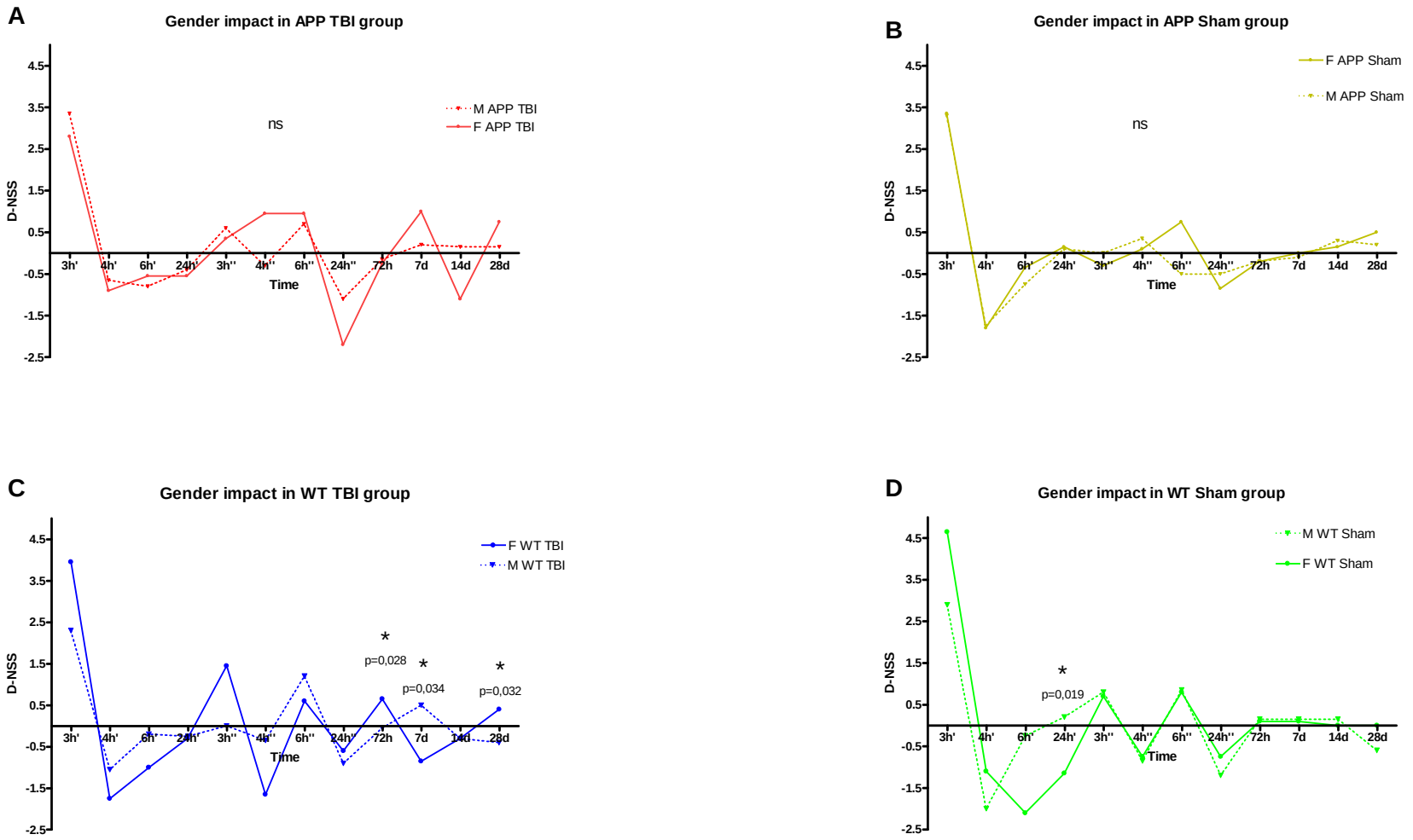


Fig 4.20. Temporal profile of NSS changes–female vs. male animals



4.8. Analysis of Aβ40 content

As Aβ40 content in WT animals as assessed by hAPP–ELISA was below the detection limit of the assay (previous, unpublished observation) this assay was performed only in tissue obtained from APP transgenic mice.

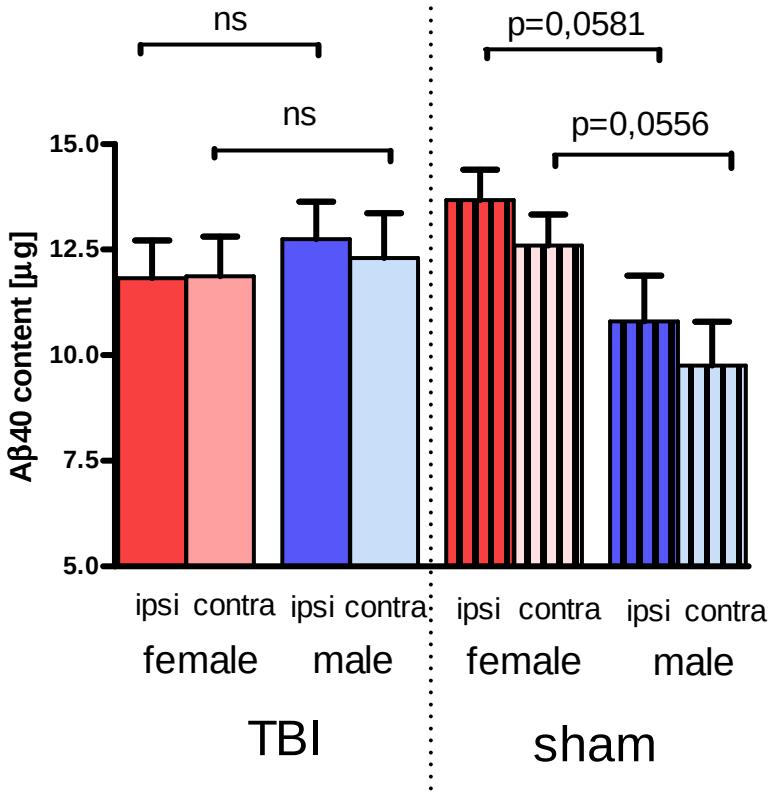
The results of this analysis are presented on the Fig. 4.21.

Comparison of gender–matched (male or female) and site–matched (hemisphere ipsi- or contralateral to CHI) samples revealed no significant differences in Aβ40 content between TBI and sham treatment groups (not outlined).

Regarding gender–related differences, in sham treatment group females had higher Aβ40 content for both ipsi- and contralateral hemisphere. This result, though nearly matching, failed however to reach the threshold of statistical significance.

Importantly, such a trend could not be revealed in animals subjected to TBI.

Fig 4.21. **Impact of TBI on Aβ40 content**



4.9. Histopathological assessment

4.9.1. Classical staining

No posttraumatic cortical cavitation could be found in both TBI– and sham–injured animals regardless of both sex and genotype.

Analysis of brain slides with H&E and cresyl violet staining revealed observable neuronal loss neither in cortical areas nor among hippocampal neurons on the site of trauma

Also no inflammatory infiltration could be distinguished.

Prussian Pearl staining revealed no overt iron deposition in meninges / cortex subjacent to CHI site.

4.9.2. GFAP immunostaining

There was no evidence of reactive gliosis as a response to TBI in both APP transgenic and WT animals. Also no increase in glial proliferation/activation could be noted in APP sham injured animals in respect to their WT littermates.

4.9.3. Cathepsin D immunostaining

There was no evidence of increased lysosomal activity as a response to TBI in both APP transgenic and WT animals. Also no increase in lysosomal activation could be noted in APP sham injured animals in respect to their WT littermates.

4.9.4. Aβ staining

Aβ deposition was detectable in the cerebral cortex and hippocampus in the APP_{751} SL mice at 4 months of age and thereafter earlier than previously reported. At the analysed time point (28d) the Aβ deposits were mostly solitary and infrequent in all groups of transgenic mice, whereas there were no Aβ deposits in any of the WT mice. These amyloid deposits were detectable in selected brain regions, i.e. in parietal cortical regions, dentate gyrus and in perihippocampal cortex, as well as in the hippocampus (Fig 4.22.).

Thioflavin staining of slides adjacent to those immunostained revealed overlapping fluorescence only for several deposits, what normally characterises dense amyloid plaques. Thus, observed deposits could be in most cases described as diffuse, non-mature plaques.

Notably, there was no increase in the Aβ burden in repetitive TBI mice relative to sham–treated APP mice as assessed semiquantitatively by simple count of single deposits.

4.9.5. APP staining

Immunostaining for APP revealed widespread immunoreactivity of pericaryal areas of neurones, clearly manifested in cortical and subcortical areas as well as in parahippocampal cortex, hippocampus, dentate gyrus, and some subcortical nuclei (amygdala).

No overt pattern of posttraumatic axonal pathology was found on APP stained slides.

Since adjacent slides in series were stained for Aβ and APP, a potential involvement of APP into diffuse plaque formation could be assessed. Indeed, areas occupied by plaques as identified by Aβ immunostaining displayed also increased APP immunoreactivity.

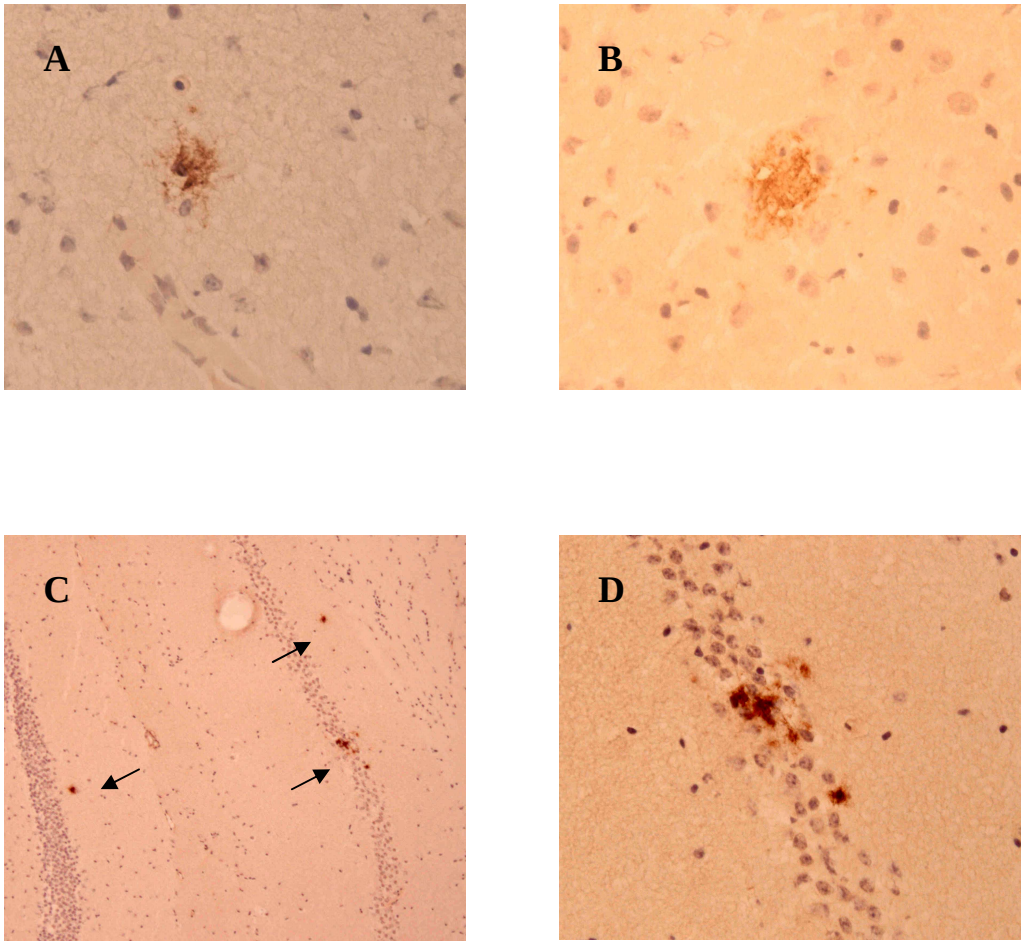


Fig 4.22.
a)-b) Diffuse Aβ deposits in cortical areas of a) sham injured and b) CHI subjected F APP mice.
c) dense plaques (arrows) located in hippocampus and parahippocampal structures of F APP sham animal.
d) the same plaques in hippocampus, greater magnification. Note intensively stained core of the plaques.
a)-d): immunostaining using 692 anti- Aβ antibody, a),b),d): magnification 1x100, c): magnification 1x40

5. DISCUSSION

5.1. Concepts of AD-TBI relationship

Regarding epidemiological evidence of impact of TBI on AD occurrence (see 2.3.1), one needs to ask, what are the mechanisms promoting development of amyloid pathology after trauma. Here at least two of them need to be outlined. First, increased amyloidogenesis may result from raised production of APP as a substrate for subsequent Aβ derivation. Second mechanism is a shift in APP processing toward amyloidogenic β-cleavage. Both events have been reported after TBI; however their pathophysiological background requires more precise insight. According to the concept of Gentleman et al., inflammatory posttraumatic reactions play a key role.[76] Microglial activation, mediated by interleukin-1 is the common point for AD and TBI: Increased levels of IL-1 and activated microglia were found in both TBI[105,268] and AD animal models[20] as well as in head-injured[100,104] and AD patients.[90,247] Interestingly, IL1 gene polymorphysm is modulating the risk of AD.[223] The effect of IL-1–mediated microglial activation explains in some aspect impact of APOE genotype on AD development and outcome in TBI.[13,156] More importantly, IL-1 is able to increase both production[60,82,202,309] and processing of APP.[35] This impact on APP metabolism was outlined also in TBI in rat[45] and in human study.[89] Thus, posttraumatic IL-1 potentially affects both mechanisms.

Another posttraumatic event is energetic dysfunction and free–radical–mediated oxidative stress. These phenomena are clearly observed in traumatised brain[12,124,278,285] and correlate well with the severity of the trauma in experimental settings.[269] In turn, also AD–affected brain, both in patients and hAPP transgenic animals displays increased free radical production and levels of oxidative stress markers, in particular oxidised lipids (reviewed by Practico[218]). The impact of free radicals on Aβ accumulation is mediated by increased Aβ production[203,265,266] and aggregation of available Aβ,[55] while raise in APP production seems to result from other mechanisms.[309] However, on the other hand, the observed overexpression of APP post TBI, is potentially able to increase vulnerability of neurons to oxidative stress.[94,162]
An oxidative stress-based link between TBI and AD has been tested directly by Uryu et al.: TBI has been presented to cause increased lipid peroxidation in Tg2576 mice. Increased oxidative stress in turn was associated with increased Aβ accumulation.[280]

Moreover, prolonged pre-treatment by the antioxidant vitamin E was able to reduce Aβ42 levels and MWM performance in TBI subjected Tg2576 mice.[47]
It is intriguing whether a raise in oxidative stress is the cause or the result of amyloidosis. Interestingly, coexistence of both options is quite possible, since elevated levels of oxidative stress markers have been reported in WT animals[219,278] and humans[15,285] subjected to TBI. Oxidative stress, in turn, promotes amyloid production[203] and aggregation.[178] On the other hand; Aβ is increasing oxidative damage, closing therefore this vicious circle (reviewed by Reddy[224] and Butterfield et Bush[33]) (See Fig. 5.1.).

A specific role in posttraumatic amyloidogenesis may be played also by apoptotic processes. Both AD and TBI are characterised by apoptotic cell death, related to the activation of caspase enzymes (reviewed by Raina et al.,[222] Cribbs et al.[50] and Liou et al.,[151] respectively). There is evidence that these enzymes are able to take part in the proteolytical cleavage of WT–APP, thereby promoting amyloid production.[50,80,295] Additionally, in relation to sweAPP they are able to play a direct beta-secretase role.[80] More specifically, caspases are able to perform proteolytical cleavage of APP accumulated after TBI in form of traumatic axonal swellings. This phenomenon is correlated with accumulation of Aβ in traumatic axonal bulbs. Such generation of Aβ from posttraumatically accumulated APP has been presented in animal studies[5,42,262] and confirmed in autopsy material[255] as well as in brains obtained ex vivo during neurotrauma surgery.[116] Thus, axonal APP and Aβ constitute the next potential link between AD and TBI.

Summarising, most of the molecular background of AD is reflected in both early and late posttraumatic phase. It means that posttraumatic AD may result from the perpetuation of inflammatory, oxidative, apoptotic and amyloidogenic processes seen after TBI. However, according to the author's concept, the crucial role in triggering and self-perpetuation of these events is played by Aβ itself. There is strong evidence that Aβ is accumulated early in human TBI precipitating to diffuse deposits.[77,106,116,229,230] Additionally, local supersaturation of Aβ concentration in axons is also possible, resulting from processing of locally accumulated APP[42,255,262] and, potentially, from direct disturbance in Aβ axonal transport.[122] In turn, Aβ is known to produce glial activation,[66,111,175,176] neuroinflammation (reviewed by Tuppo et Arias[276]), cause oxidative stress damage (reviewed by Reddy[224] and Butterfield et Bush[33]) and trigger

apoptotic death with caspase activation.[96,153,160] Thus, such a posttraumatic raise in Aβ levels and deposition may activate mechanisms responsible for its own production. Interestingly, an even more direct link can be reasoned since polymerisation of Aβ has been increased by amyloid itself, both in vitro (reviewed by Harper and Lansbury[98] as well as in transgenic animals after intracerebral infusion of human AD–brain extracts[123,290,291].

Thus, regardless, whether these early deposits are persisting or vanishing in prolonged posttraumatic course, already at this stage they may be able to trigger the abovementioned deleterious effects. Such secondary damage in turn is very likely to undergo self-propagation, even if the primary deposits are already dissolved (see Fig. 5.1.). Concluding, TBI may be regarded as incipient stage of subsequently apparent AD.

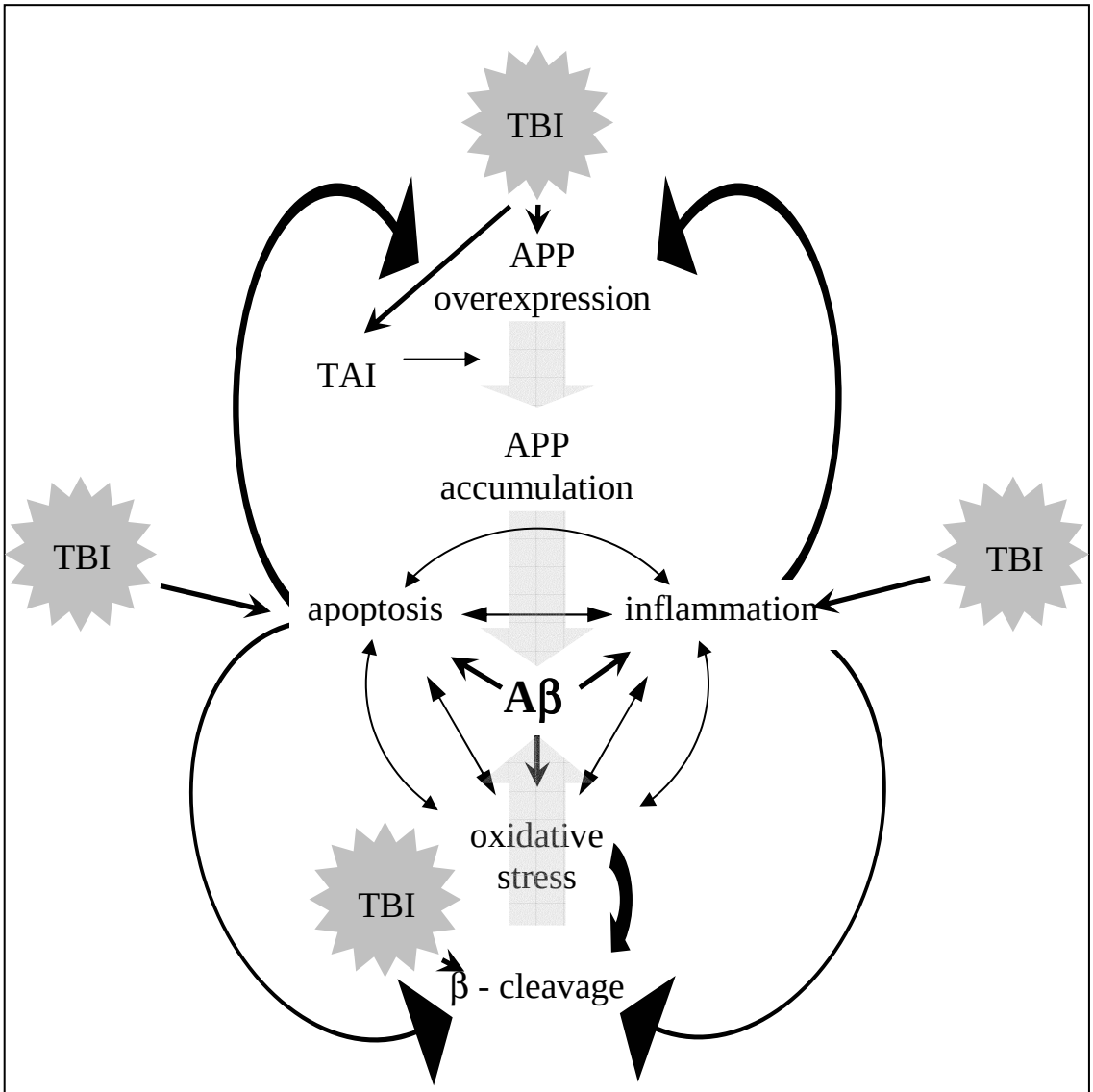


Fig. 5.1. Author's concept of role of TBI in amyloid pathology development.

Posttraumatic increase in both APP expression as well as in β - cleavage of APP leads to raise in Aβ. Note the central role of β - amyloid in 'small vicious circle' of deleterious processes, triggered also otherwise by TBI. These processes (oxidative stress, apoptosis and inflammation) are reciprocally propelling each other. They also increase Aβ production ('large vicious circle'). Note also potential direct impact of TBI on APP local concentration (APP accumulation) via TAI. Arrows present positive influence on given process, shaded arrows- amyloid production.

5.2. Validity of the model

The transgenic model of AD chosen for purpose of this study is described by far similarity to the human AD pathology, as by the presence of amyloid deposits with periplaque markers of glial activation and formation of degenerative fibers (DNs) with accompanying molecular flags of mitochondrial dysfunction and proapoptotic processes recapitulating human Aβ plaques with accompanying high expression of the hAPP transgene. The presence of the Swedish mutation leads to an increase in the C99 APP fragment / full–line–hAPP ratio.[23] Since relatively high levels of C99 APP-fragments were shown to correspond with early Aβ accumulation and deposition, in this transgenic model a presence of Aβ plaques was to be expected already at age of 6 months.[23] However, according to presented observation Aβ deposition may occur as early as at 4 months of age in this transgenic line. Nevertheless, such aberration from original results didn't create a great discrepancy, since the presence of the plaques was not uniform and rather an exception among animals; moreover the plaques observed were rather scarce, of diffuse form and present only in selected areas (hippocampal and cortical fields) previously reported as affected most early by Aβ deposition in different transgenic hAPP models.[23,109,120,179] Of greater importance is the conclusion, that appearing of Aβ- plaques at age chosen for posttraumatic analysis was the optimal validation for suitability of this animal model for the purposes of our study. *First*, it was a direct proof, additional to strong literature evidence[23] that the brain of Thy1-APP_{751}SL mouse gives a possibility for Aβ deposition, similar to human one. *Second*, on this background it was able to postulate that, if the factor of TBI (in given experimental settings) were able to impact significantly the plaque formation, this effect would be seen in our analysis of Aβ deposits pattern (this, however, was not the case).

In this study a well established model of Closed Head Injury (CHI) has been used.[44]
The main reason for this choice among other models was its basic characteristic: CHI is technically simple, easy to implement model and provides high reproducibility. Also lack of need for threpining of the skull and possibility of trauma parameter setting (height and weight of fall) was very convincing, for the great concern was preserving of intact cranial bones which was crucial for TBI repetition.[44,139,143,181] Considering that examination of the skull vertex in experimental animals after each trauma as well as by tissue harvesting revealed no bone fracture, we may state that reliability of provided results has not been hazarded by the factor of nonuniform damage to the skull and brain.[44,139,140,181]

The separate problem was the trauma severity and the mode of repetition. On the one hand, the results of human studies were respected, where higher TBI severity was correlated with greater risk of AD.[212] On the other hand, the abovementioned bias of inequality in trauma energy was impending, if using settings allowing fracture of cranial bones. Therefore the decision was to apply trauma load slightly under the threshold of skull breakage, as estimated during preliminary experiments with WT animals (data not presented). According to a further analysis of mortality, reaching up to 37,5% and of neurological deficit, where no consistent pattern of impairment has been seen, trauma severity in such a paradigm could be rated as mild to moderate.

Furthermore, as mentioned, intact skull enabled repetition of the trauma. Analysis of previous AD–TBI studies gave the conclusion that most experiments, using APP transgenic murine models subjected to single TBI (save double, APP–APOE transgenic mouse[99]) failed to reproduce posttraumatically increased Aβ pathology.[185,187,188,259] Therefore, some patterns were adopted from the single successful concept of Uryu et al., where repeated mild TBI repeated was used.[280] One important difference was the use of more intensive trauma. Of separate importance is the question, whether the chosen interval between two consecutive hits (24 hours) was adjusted properly, i.e. in the way providing their synergistic effect on the brain vulnerability. Again, this setting was taken after Uryu et al.[280] A recent study of the same group provided experimental data about postconcusional vulnerable state protracted to 5 days in regard to axonal and potential subcellular damage, confirming proper timing.[152]

Summarising, the experimental approach presented here differ from this of Uryu et al.,[280] in type of transgenic animal used (Thy1–APP_{751}SL vs. Tg2576 mouse), as well as in mode and severity of trauma (mild–to–moderate CHI vs. mild CCI).

The anaesthesia mode we used for our study (pentobarbital anaesthesia) is widely accepted in experimental neurotraumatology and has been previously used in research on head trauma in hAPP transgenic animals.[185,187,188,259,280] Pentobarbital is a short acting barbiturate, metabolised primarily in liver. Though use of pentobarbital has been justified by general use in neurotrauma research, some of its potential disadvantages need to be mentioned. First, it presents significant individual differences regarding especially time and profoundness of anaesthesia.[190] Second, pentobarbital-anaesthetised animals use to tend to prolonged posttraumatic apnoea, what resulted in dramatic increase in mortality in preliminary study. Thus, to avoid both of these hindrances a 1h–interval between administration of anaesthetic and trauma deliver has been set. This gave a reasonable and humane compromise between anaesthesia

depth and tendency to fatal apnoea. The third potential source of bias was pentobarbital–related impairment of thermoregulation. Pentobarbital anaesthesia produces dose-dependent brain hypothermia with decrease of brain temperature up to app. – 4–4,5℃ deviation from baseline with accompanying cor e hypothermia of lesser degree, so that normal brain–core temperature gradient is reversed. This phenomenon is commonly attributed to barbiturate–induced brain hypometabolism.[49,133]

Hypothermic condition (during or after TBI) has been reported to possess significant cerebroprotective properties. In experimental settings, various basic mechanisms of hypothermic protection at the cellular level have been analysed including reduction of glutamate surge and intraneuronal calcium mobilisation, diminishing microglial activation and proliferation, reduction of superoxide activity and direct impact on posttraumatic protein synthesis as well (for review, see Fritz et Bauer[62]). Most importantly, this spectrum is widely (if not completely) overlapping with the list of the factors, potentially contributing to posttraumatic surge of APP and Aβ production. Therefore it can be assumed that posttraumatic events leading to an increase in Aβ accumulation will be reduced in the given experimental setting due to pentobarbital-related hypothermia. In consequence, the differences (TBI vs. sham) related to APP-Aβ- metabolism in transgenic animals would be diminished to some degree, what sets one possible explanation for respectively indiscriminate result of microscopic and biochemical analysis. This hypothetical impact would however require further evaluation with multiple time– and target temperature point paradigm.

One potential advantage of pentobarbital–related hypothermia was the possible influence on sexual hormones regulation in female. Struton and Cohen presented that hypothermic condition suppresses pulsatile secrection of luteinizing hormone.[264] This may implicate unintentional interruption of hormonal cyclicity in female mice, with potential synchronisation of ovarian cycle.

However, this could equalise the impact of sexual hormones among female mice only in posttraumatic course: since hormonal status before the CHI was not checked and no modification of sexual hormone cycle was implemeted (by means of ovariectomy or hormone administration), a phase of oestrous cycle at the moment of trauma remained unknown. Taking into account that hormonal status at the TBI time may influence the later outcome[27,236,287] and that part of our results regards gender- difference, overwhelming of this disadvantage in further studies is warranted.

Additional advantage is the lack of barbiturate effects on APP metabolism, at least at transcription level,[201] while for other considered anaesthetics, potential impact on amyloidogenesis needed to be considered.[56,233]

5.3. Results of neurological testing

A particular attention is called by the results of the pre- training assessment, based on BB–test. The differences in neurological performances (APP vs. WT female animals and female vs. male WT animals, respectively) (Fig. 4.3.) present before any operative intervention has been performed, could be attributed to the following background:

- since heavier animals perform normally poorer in balance- based neurological assessments, the weight difference should be considered as the potentially most obvious reason for this difference;
- regardless of impact of weight on the performance, a virtual neurological impairment could be suspected.

Subsequent analysis of weight–performance correlation (Fig. 4.4. and 4.5.) allows to state that among WT animals, sex-related difference represents physiological weight–dependent phenomenon, while APP vs. WT female difference represents a virtual neurological impairment in APP female mice.

BBS test, including BB–pretraining is usually categorised as vestibulomotor test.[17,63,93,241] However, since this test included pre-surgery training, it can no longer be described as a purely motor test. In particular, the difference in pre-training scores presented here could result from both motor impairment and disturbed motor task acquisition (occasionally conceptualised as 'motor learning'). Therefore several reasons for observed differences need to be outlined.

First, the amyloid load could interfere with cerebral neuronal circuits, responsible for movement and balance performance. The list of relevant structures encompasses the association cortex, sensorimotor cortex, subcortical nuclei, cerebellum, and brainstem, as well as spinal cord neurons. Vestibulomotor function, in turn is described as mediated by activity of corticospinal neurons, nigrostriatal neurons, the nucleus accumbens, the basal ganglia, and the thalamus.[63,67] Facing this complexity, damage to any of these elements may result in disturbed BB–pre-training score. Previous reports present impaired behaviour of already young single Tg2576 APP transgenic and double PS1/APP transgenic mice in beam balance test.[11,130,131] The early onset of impairment in the balance test has been described as attributable to sensivity to Aβ deposition, soluble Aβ or overexpression of mutated hAPP.[11] Interestingly, King et al. reports vestibulomotor impairment in young Tg–APP mice in mixed gender group, but no separately for females or males. On the other hand, in the same study selective impairment of female animals was present in cognitive area (circular platform test and

error score for Morris Water Maze test).[131] In the results presented here, the impairment was observed only in BB–pre-training but not in posttraumatic BB performance, suggesting that in scoring of pre-surgery training the accent may be shifted from static (single performance) to dynamic ('motor skill learning') component of motor task acquisition. However, to refine this concept, both elements should be extracted by i.e. additional use of 'more purely' motor tests as reflex–based tests (as reviewed by Fujimoto et al.[63]).

One novel aspect is the possibility of neuronal dysfunction on the spinal cord level. The motor component of BB pre-training score could be impaired not only on cerebral, but also spinal level. The recent observation published in cooperative work of the author[303] clearly presents neuronal damage in spinal cord of PS1/APP transgenic mice. Since strong expression of the hAPP in Thy1–APP_{751}SL in spinal neurones was observed already at 3 months of age, it can be premised that both APP and Aβ overload could lead to neuronal dysfunction. However, in some analyses of sensimotor test results, impairment that appeared at the young age was not further progressing (in relation to WT animals) while Aβ concentration and deposition was clearly increased with the age.[11,131] Moreover, spinal intraneuronal Aβ depositon could be detected not before 8 months of age in Thy1–APP_{751}SL animals.[303] This suggests that impairment at spinal level might be caused solely by hAPP expression, or that even undetectable levels of intraneuronal hAβ were sufficient to cause neuronal / axonal dysfunction. The character of such impairment remains unknown. The change in behaviour (abnormal extension reflex in tail–suspended mouse), most probably of spinal origin, was observed not only in PS1/APP_{751}SL animals[303] but also in ApoE[271]– or tau[221]–transgenic animals. A disturbance in axonal transport is proposed as possible explanation.

The cited results prove also that more aggressive amyloidogenesis, as seen in double transgenic PS/APP mice is necessary for structural spinal damage (axonopathy with axonal spheroids) to occur. The localisation of axonal and neuronal pathology in spinal cord of transgenic animals, matches quite well the achieved result in BB–pre-training scores.[303] Neurons of the ventral horn are the final common path of the motor innervation and their dysfunction results in decreased strength of the respective muscle group. In turn, damage to dorsal column causes ataxia described as spinal or sensory.[67] Both signs potentially affect the performance in BB–pre-training testing.

Unfortunately, more specific assessments of neurological function have yet not been completed (Wirths O, personal communication).

Current observation is in accordance with clinical evidence, reporting motor impairment and ataxia in some AD patients.[288] In another study, poor physical function was associated with development of dementia, including AD, preceding its onset. The authors concluded that AD development impairs primarily balance and gait performance as 'more challenging brain function'.[294]

Second aspect is the ability or motivation to acquire balance task. This could be reduced due to affection of corticostriatal, cerebellar and / or limbic structures. There is rationale to suspect that behavioural changes interfered with BB–pre-training, since neophobia, anxiety as well as episodes of aggression and hyperactivity have been reported previously in APP transgenic animals, in particular in Thy-1 promoter driven–APP transgenic models.[110,179,180] As a background, APP expression and Aβ accumulation in structures responsible for managing emotional context of the task (hippocampus itself as well as firmbria, prefrontal cortex, amygdala and thalamus[9,67]) may be proposed.[179]

The problem of aquisition and retention of mobility task in BB test deserves more detailed analysis. The clear memory impairment in different strains of hAPP- transgenic mice is the obvious fact.[41,109,131,179,214,296] Of note, in 3 months old Tg2576 mice the interrelation between sensimotor task score (beam balance and wire grip tests) and memory retention in circular maze test has been reported.[10,131]

Most commonly, rotarod training paradigm has been used in various experiments.[40,79,132] Recently, this paradigm has been also tested in murine model of AD. 12 months old PS1/APP transgenic animals displayed impaired motor performance on rotarod.[58]

Interestingly, studies on learning in AD patients revealed opposite results. There is clear dissociation of implicit (motor) and explicit (verbal) learning in AD patients, since they presented no significant difference in motor task aquisition vs. age–matched controls.[57,101] The same statement was true also in apractic AD patients, who, though generally performing a given motor task worse, were not disabled in improvement of the same[119] or alternative motor skill.[298]

Regardless of the components of disclosed difference in BB–pre-training score (static i.e. performance vs. dynamic i.e. 'motor learning') more general question of its molecular background emerges. The impairment presented here may be linked to

amyloidogenic processes in APP animals. Theoretically, it may result from both structural damage (neuronal loss) since Aβ in its aggregated form possesses strong neurotoxic activities,[210,279] as well as from synaptic or neuronal dysfunction without loss of neural cells as related to Aβ potential to cause neuroinhibition and reduction of synaptic transmission (reviewed by Turner et al.[277]). It was unapplicable to assess the level of Aβ and stage of neuronal loss at pre-operative time point. However, basing on previous description of Thy1–APP_{751}SL transgenic model it should be premised, that at analysed point (12 weeks of age) APP mice were free of apparent Aβ deposition.[23] This presumption is granted further by the observation that in the same mice 4 weeks later (at time point of sacrificing) amyloid deposits, if present at all, were extremely scarce and rather diffuse in form, what is attributed to initial phase of accumulation of Aβ (at its low concentration). At this time point also no apparent neuronal loss could be described in sham animals, leading to further conclusion that neurological impairment in pre-injury analysis was caused rather by functional than structural disturbance.

Therefore, this result confirms the hypothesis, that neuronal trafficking is affected already by intracellular load of amyloid. This hypothesis shifts an emphasis from extracellularly deposited Aβ (in form of plaques) to its soluble intraneuronal fraction as main cause of AD signs and symptoms.[22,83,300-302] From a vantage point of a clinician, such a shift means that AD may be manifested long before any structural damage has occurred.[83] Moreover, results of intracellular Aβ accumulation may potentially be treated (by the mean of i.e. pharmacotherapy or immunotherapy) easier than established amyloid plaques.[32,195]

From a nosological point of view in turn, the current results augment recent evidence[83,84,300] that allows to incorporate β-amyloid–related disorders (thereunder AD) to a family of intraneuronal ‘proteinopathies’. Previous categorisation to distinct nosological group of neurodegenerative diseases with excusively extracellular protein accumulation[84] seems to be no more valid.

In posttraumatic course, at the timepoints analysed, no consistent pattern of neurological impairment could be detected in gender– and genotype–matched groups. Most probable cause here was the minor severity of trauma. It is commonly accepted to describe severity of experimental trauma according to neurological impairment achieved in early posttraumatic course. Most experiments in mice have defined trauma with no or with minute effects on neurological performance as mild.[52,138,267,316] Considering the results from repetitive mild trauma studies, no significant neurological

impairment has been noted. DeFord et al. reported two different grades of rTBI severity, stating 30% mortality in the group considered later as moderate trauma.[52] A similar mortality rate was observed in the current study. This, together with no neurological deficit, was convincing to describe the used paradigm as mild to moderate trauma. Nevertheless, in contrast to DeFord's et al. results in the non-survivor group no apparent skull fracture could be noted and mortality should be attributed to other factors (e.g. deleterious effect of anaesthesia).

Of special concern was the potential difference between APP and WT animals. The current study presented no such divergence. This result is concordant with previous works, where also no genotype impact on neurological performance has been noted. This regards both rmTBI[280] as well as more severe TBI experiments,[185,259] though different methods of neurological assessment were used (mouse composite neuroscore,[185,280] Rotarod test,[185] angle board score[185] or simply determining swimming speed in MWM test[259]). Considering, that Uryu et al.[280] and Smith et al.[259] (but not Murai et al.[185]) reported parallel memory impairment, it can be stated, that cognitive function is more fragile to TBI effects than neurological one also in hAPP transgenic animals. Such observation has been made previously in WT animal studies and in clinical settings (as discussed more specifically above).

The current results however may not be free from bias. One aspect is the quite early posttraumatic timepoint used. Fujimoto et al. suggested that to avoid interference anaesthesia effects, neurological assessment should not be regarded as conclusive before 7 days posttrauma.[63] Indeed some single point differences were observed early in sex– and genotype–matched animals. Thus, also due to their irregularity they should be regarded rather as post-operative noise.

Next question is the validity of the NSS score. NSS, analogous to composite neuroscore consists of more tasks, allowing to assessment of whole motor function basing both on strength / reflex tests as well as on vestibulomotor components.[63] It has been previously described and used in numerous studies on head trauma in rats and mice. The main advantage of this method is its relative simplicity, as well as the ability to analyse more than one aspect of neurological deterioration (as presented above). The disadvantages are: task rating made arbitrary by human observer and grossly stepwise assessment. These disadvantages were tried to be overcome here by *first* blind assessment of NSS tasks and *second* refinement of NSS scoring by use of gradual rating. However it still cannot be excluded that a more 'fine-grained' experimental tool (as e.g. Accelerod test) could be more advantageous here.

Nevertheless a previous report of NSS use in similar trauma settings (mild CHI)[316] allows to postulate that choosing of NSS was valid.

Another aspect is the sample size. To analyse probable gender effect, small groups of 5 sex–, genotype– and trauma status–matched animals has been established. However, the statistical power of analysis performed in such scarce clusters may be questioned. To address this, additional analysis in genotype– and TBI status–matched but gender–unsplit groups (of 10 animals each) was performed. This analysis indeed revealed few additional effects in NSS analysis (deleterious trauma effect at 7d in APP group and unfavourable APP–genotype impact on performance at 4h" and 28d posttrauma).
The additional aspect is the posttraumatic course. In hAPP animals it may be expected that both faster or more significant improvement (due to neurotrophic APP properties) as well as deleterious effects (due to increased Aβ concentration) may occur.[185] However, in the current study none of these effects was observed. It is possible that the bare NSS scores were not significantly increased, at least not enough to have an influence in a statistically significant manner.

A separate problem is the neurological assessment in sex-matched groups. Regarding posstraumatic performance, there have been only single differences between male and female animals noted. In particular, female APP animals strongly outperformed males in BB testing at 24h after first insult, while this effect has been observed neither in respective NSS scoring, nor in WT TBI animals, nor after second trauma. This result is somehow consistent with reports from studies with more severe CCI trauma, where female rats were better in motor tasks as early as 24h after TBI and thereafter up to 5 days.[286] In present study, however, such prolonged effect has not been recorded, potentially due to second injury applied, obscuring this gender difference or due to minor trauma severity with quick recovery.

More clearly there was a gender impact on delayed posttraumatic course among WT TBI animals. The D-NSS pattern here suggested a shift in posttraumatic course towards an earlier onset of recovery in female vs. male animals. The explanation for this effect could be based on the previous observations that female gender was associated with reduced contusion volume[27] and posttraumatic edema,[235] improved

cerebral blood flow[236] and more protracted course of cytoskeletal degeneration and rearrangement.[136] One may presume that these sex–related beneficial effects were influencing neurological recovery most significantly in the described time window (3-28d). Intriguing is the lack of such gender effect among APP TBI animals. Interference of intracellular amyloid burden with the gender–dimorphic recovery processes is one reasonable explanation.

One important aspect is the result of BB testing. There was a clear genotype–related difference among female animals in acquisition of BB–related task in pre-training (as discussed above). However, no significant difference in performing this task once learned was seen during the posttraumatic period (save time point 6h after first trauma. Here WT animals however performed worse, and in respect to the early posttraumatic point this effect may result rather from different anaesthesia recovery, than being a virtual impairment). This shows that retention of balance task, which was acquired before the trauma, was not disturbed by hAPP and Aβ production, or by the TBI paradigm used here. One may presume that frequent task repetition and its constant conditions could serve as preserving factors. Indeed, AD patients were able to gain and keep motor skill equally to healthy controls if performed under constant practice conditions.[53,54] According to Dick et al.,[53] performing a task in conditions similar to those of the original task learning may be described as hippocampus–independent. This fits well with the current BB–assessment situation and histological analysis as described in 4.10.1. and 4.10.4.

5.4. Biochemical analysis

Basing on the results of previous analyses of the Thy1–$APP_{751}SL$ transgenic model, the decision was made to include the measurement of only soluble Aβ40 level, since at the age designated to be the posttraumatic time point for tissue harvesting, no significant amount of cerebral Aβ42 type and no overt amyloid deposition has been reported. Moreover, the $Aβ_{x-42}/Aβ_{1-40}$ ratio remained constant in time though accruing Aβ deposition during ageing. For lateralised trauma, as in given experimental TBI, an inter-hemisphere difference needed to be considered, therefore separate concentrations of Aβ for contra- and ipsilateral hemispheres have been estimated. However, no statistically significant difference between contra- and ipsilateral hemispheres of respective experimental groups has been found, what is consistent

with previous observations of Uryu et al.[280] and Conte et al.,[47] where analyses in Tg2576 mice were performed at 8, 16 and 8 weeks posttrauma, respectively.
There was also no difference in $A\beta_{1-40}$ content and amyloid deposition between TBI-subjected and sham injured animals. One possibility is, that given TBI settings were just not sufficient to cause any change in APP metabolism and Aβ accumulation. Nevertheless, this result can be attributed also to the specific posttraumatic time point we have chosen for analysis (4 weeks). In one aspect it could be too early to expect any changes in Aβ level, as in the model of Philadelphian group significant changes in presence of deposited amyloid were not observed until 16 weeks of posttraumatic course, while Aβ ELISA measurement revealed no significant increase in $A\beta_{1-40}$ concentration even at this time.[47,280] On the other hand, this point can be also criticised as too remote, since Smith et al. presented posttraumatic surge of Aβ40 to be present in 2h but not at later time points after TBI.[259] Also in the recent study of Abrahamson et al., a raise in $A\beta_{1-40}$ level returned to the baseline as early as 24 h posttraumatically.[5] Moreover, Murai et al. presented in his experimental setting actually a decrease of Aβ40 level 7 days after TBI.[185]

Despite the mentioned studies are unintegrated in respect to animal AD–model used (PDAPP in studies of Smith et al. and Nakagawa et al.,[187,259] Tg2576 in Uryu's and Conte's group,[47,280] $APP^{NLh/NLh}$ in Abrahamson's et al. study[5] and APP-YAC in this of Murai et al.[185]), a certain concept may be already based on their results. It is possible, that analysis time point of our study is situated in a kind of 'equilibrium period', when posttraumatic Aβ levels and deposition are near to non-trauma baseline. This period would be preceded by rapid but short lasting surge in amyloid production and it would later proceed to late chronic amyloidogenesis with reappearance of elevated Aβ levels and deposition. Of course, during such subchronic AD-pathology respective pathophysiological processes will take place, however, they will be occult, in means of microscopic and biochemical Aβ analysis. A strong support for this concept can be found in studies of TBI–victims' brains. Both post-mortem studies and analysis of surgically excised fragments revealed increased Aβ deposition at early posttraumatic stage (up to 24h) while in long-term survivors of TBI (after 30 d post-TBI) no difference to age-matched controls could be revealed.[157] TBI as cause for AD, however, emerges again in epidemiological studies with multiannual follow–up[212] or retrospect.[59] Moreover, the study of Nakagawa et al. confirms the existence of posttraumatic

mechanisms that are able to dissolve already established deposits,[188] assumedly also those created acutely after TBI.

One important aspect, both in experimental and in autopsy studies is heterogeneity of Aβ, since apart Aβ40 also more amyloidogenic species of $Aβ_{1-42}$ and $Aβ_{x-42}$ exist. The two later forms seem to play a more crucial role in posttraumatic amyloid pathology. Interestingly, $Aβ_{x-42}$ level remained stable despite significant decrease in $Aβ_{1-40}$ in experiment of Murai et al.[185] Two further studies showed both Aβ40 and 42 levels to be increased early[259] and remotely[280] after single and repetitive mild TBI, respectively; in both studies however, surge in Aβ42 species was much more prominent. Finally, Abrahamson et al. in non-transgenic mouse model reported not only a rapid surge in Aβ42 (500% of the sham level) within 3h post TBI, but also secondary protracted elevation (to 150% of sham base) persisting up to 14 days. This double-phase raise was accompanied by transient increase in Aβ40, declining to sham levels already 72 after trauma.[5] Together with previous reports, it suggests a predominant role of $Aβ_{1-42}$ in posttraumatic AD pathology. This has been confirmed in autopsy studies, where $Aβ_{1-42}$ content in diffuse amyloid deposits was shifted toward increase in $Aβ_{1-42}$ species, comparing to cases of 'regular' AD.[75]
Regarding this, it would be reasonable to include Aβ42 analysis also in our study. However, one need to notice that animals used in cited experiments were of much more advanced age than in present study and significantly increased levels of more amyloidogenic Aβ species could be easily and credibly estimated. Thus, for reliable results of Aβ42 analysis more remote points for analysis need to be chosen or TBI needs to be applied in more aged animals. Also time points before and after discussed latency period of AD- pathology should be targeted. These problems will be the main focus by designing of subsequent studies. (Aβ42 levels below detection limit of the assay).

In current study a clear trend towards decreased levels of Aβ40 in male animals, compared to females in the sham group is presented. This difference however was not present in animals subjected to TBI. The former data is consistent with previous reports, that amyloid production, accumulation and deposition is more intensive and occurs earlier in female animals expressing hAPP in different transgenic models of AD, including Tg2576,[37,142] double transgenic APPswe/PS1 mice[293] and in the here used Thy1–APP_{751}SL strain.[244] Interestingly, a gender difference in Aβ plaque load has been observed first in aged animals (coincident with anestrus phase),[37] while increased

Aβ40 levels were reported already in young female animals, consistently to the current report. As an explanation Callahan et al. proposed a direct impact of estrogen on amyloid production, since cultured neuroblastoma cells, bearing mutated βAPPswe released more Aβ40 and Aβ42 after estrogen treatment.[37] Another aspect, outlined by Lee et al. is the gender difference in synaptic zinc release. Zinc ions in physiologically plausible conditions are able to precipitate soluble Aβ fraction in vitro.[31] In Tg2576 mice synaptic zinc concentration was increasing during ageing in female but not male mice, corresponding to amyloid burden. However, these results regarded aged animals and rather insoluble than soluble Aβ level was correlating with zinc concentration, suggesting that synaptic zinc is playing a main role in Aβ accumulation but not production.[142] In contrast, Schuessel et al. presented increased Aβ40 production in both 3– and 12 month–old female Thy-1 APP mice compared to males. This was associated with impaired Cu/Zn superoxide dismutase activity and increased levels of lipid peroxidation products as markers of oxidative stress in comparison to non-transgenic female age-matched animals. The similar genotype–related differences appeared in male animals first at 12 months of age. Thus, gender–dependent Aβ and C99 production causes impaired antioxidant activity and an increase in oxidative stress.[244]

The observations in animal studies are consistent with clinical epidemiological data, according to which female gender is affected by higher incidence and earlier onset of AD.[114,125]

There are several ideas trying to explain this gender effect on AD development. First, referring to the concept of brain reserve capacity as derivate of brain size, earlier onset of AD has been linked to antropometric gender differences in cerebral volume.[155] Second, other theories have been based on molecular interactions between sex steroid hormones and APP production and processing. In general, influence of estrogen is regarded as protective in AD. Addressing APP metabolism: estradiol reduces APP transcription via methylation of APP promoter,[158] shifts in adult mice posttranscriptional APP splicing toward APP695 form[272] and increases α-cleavage of APP as represented by sAPPα level.[159,304,314] Thus, clinically observed preponderance of female sex among AD subjects has been attributed to estrogen depletion in postmenopausal anestrus phase. Male subjects in turn will be protected by the impact of testosterone on amyloid pathology similar to that of estrogen.[85]

Nevertheless, this does not fit to present and previous observation in Thy1–APP_{751}SL mice, since Aβ levels were raised in females already at 3–4 months of age.[244] It is possible that the observed effect may be attributed to properties of selected hAPP transgenic constructs, in particular to the mutated hAPP sequence used. It has been presented that estrogen supplementation reduces Aβ derivation from APP in APPswe but not in PDAPP mice, harbouring the APP V717 mutation.[88,145] Summarising, in female Thy1–APP_{751}SL mice the stages associated with normal levels of circulating estrogen (i.e. development and young adulthood) will be associated with a more pronounced Aβ generation from APPswe.

While sex matched groups (TBI M vs. sham M and, separately TBI F vs. sham F) seemed to show no significant impact of trauma on Aβ level, the clear trend towards sex–related difference in this rate has been observed only in sham but not TBI APP animals. This implies that head trauma selectively impacted Aβ accumulation in male animals so that it reached the level seen in female animals, while in 'normal' condition (without accessory damage due to TBI) female Aβ rate outranks the male one. Interestingly, this observation finds a solid ground on epidemiological studies: Fleminger et al. reports positive odds ratio for TBI as an AD risk factor in general population and among males, but not in females.[59] The possible explanations for current results, as well as for this epidemiological evidence may be as following: *First,* in females certain protective mechanisms are present, ceasing or reducing those effects of post-TBI events which propel development of AD. These mechanisms were therefore absent or less effective in male subjects. *Second,* in female brain, APP and Aβ turnover is physiologically set on the high level that cannot be further significantly increased by TBI sequelae (this would also explain to some part greater susceptibility of females to AD, as discussed above). Thus, male APP–metabolism would be more vulnerable to TBI, as possessing greater freedom range for up-regulation.
A possible explanation may be found again on the field of oxidative stress. It has been presented that female Thy1–APP_{751}SL mice are burdened with less lipid peroxidation products than age matched transgenic males, though in the females antioxidative SOD-activity was decreased more than in males.[244] With regard to posttraumatic course, the levels of oxidative stress markers were more profoundly and persistently elevated in CSF of male TBI patients.[16,285] This suggests that antioxidative mechanisms in females are more efficient or supported by e.g. respective properties of sex hormones (reviewed by Roof et Hall[237]). Indeed, in female animals bearing

increased CuZn-SOD activity no protective effects on cortical damage could be seen after TBI, in contrast to male animals, though trauma impact was in general reduced by female gender.[115]

Taking together, for the results of the present study it may be hypothesised that antioxidant defence in Thy1–APP_{751}SL females is sufficient to resist Aβ–related oxidation, even if one compound of it i.e. CuZn-SOD function is impaired by Aβ load. Moreover, antioxidant activity remains efficient even in the face of additional posttraumatic raise of oxidative stress. These mechanisms could therefore stop the impetus of oxidative damage, generated by TBI itself. In male animals in turn, posttraumatic processes might lead to more pronounced, less restricted free radical damage, resulting potentially in raised amyloidogenesis.

Thus, the male brains would be more eager to succumb to vicious circle of free radical production and Aβ generation. As a result, in the relatively short posttraumatic period, they were able to equate the Aβ level to this seen in female animals.

To the best knowledge of the author, this is the first study attempting to compare impact of TBI on AD in APP transgenic animals of both sexes. Prior studies were performed using either sex-–uniform experimental groups (males or ovariectomised females), or the analysis was performed without regard to this factor. There are some obvious shortcomings of presented experimental settings in respect to the gender problem. In particular, the information about estrous cycle status at the time of TBI is lacking. Facing the data, describing the impact of hormonal status on the posttraumatic outcome[27] this creates a potential bias source. However, the general presence of circulating sex hormones has here a more clear impact than estrous cycle stage during traumatic insult.[287] Moreover, such settings approximate more closely the clinical ones, where no certain hormonal status can be imposed at the moment of trauma.

5.5. Histological analysis

In here presented study, no overt neuronal loss could be observed in traumatised animals, regardless of genotype and sex. Neuronal loss is a universal consequence of neurotrauma, as it has been reported both in experimental and clinical setting analysing TBI of different severity. Neuronal loss has also been precisely described in a murine CHI model.[44] However, regarding rmTBI mode, no significant depletion in neuronal number was observed in studies of DeFord et al.[52] and Uryu et al.[280] what is consistent with current observation.

Generally, TBI resulted in abundant neuronal loss in hAPP transgenic animals as comparing to their WT littermates. This phenomenon was attributed to neurotoxic properties of Aβ that, however, were uncovered first after additional damage (here TBI). This was based on the observation, that hAPP transgenic mice do not develop neuronal loss spontaneously, though manifold increase in Aβ concentration. Nevertheless, in the current study no such difference could be observed. This may be explained by the young age of animals used, in which Aβ levels were still only slightly elevated. This fits with the study of Murai et al., where cell loss in APP-YAC animals did not differ from WT after trauma.[185] Notably, APP–YAC animals are characterised by no spontaneous plaque deposition due to only slightly evevated level of Aβ.[34,204]

Analysis of the glial response to the trauma displayed no difference between the experimental groups. Astrocytic activation and gliosis are the common sequele of both TBI and AD[111,170,175,205] and have been seen also in respective animal models.[20,39,163,260] In a previous report on the mTBI paradigm, moderate gliosis has been detected, though not in prolonged posstraumatic observation. This regards both to WT and Tg2576 hAPP transgenic animals in repetitive mTBI mode.[280] The lack of apparent glial activation in the current study was in accordance with the abovementioned reports, since the time point chosen for analysis was quite remote from TBI. A separate problem is the potential impact of the posttraumatic glial activation on the development of AD pathology. Both main components of brain tissue: glia and neuronal cells are participating in APP production. However, the production of APP isoforms differs between these two cell populations. Alternative splicing of APP leads to the production of three major APP isoforms: APP_{770}, APP_{751} and APP_{695}. The two former isoforms contain the so-called Kunitz protease inhibitor sequence (KPI), what enables to identify them by generating KPI–specific antibodies. Using methods specifically targeted to separate APP isoforms and/or respective mRNA it has been stated, that in cell cultures neurons produce mainly KPI–lacking APP_{695}, while in astrocytes the proportion $APP_{770/751}/APP_{695}$ is reversed since they produce mainly KPI–containing longer isoforms.[234] Considering the general role of glia in AD pathology, glial cells may not only be an executor of toxic Aβ properties in and around the deposits. There is evidence that glial cells, in particular astrocytes participate in Aβ production.[144] Moreover, there is a clear shift in the concentration of APP isoforms among the age, since foetal brain contains predominantly mRNA APP_{695}, while in aging and, in particular AD–affected brain mRNA $APP_{770/751}$ is the dominant form.[220]

Interestingly Masumura et al. stated that the posttraumatic surge in general APP was due to the glial form, while neuronal APP expression was gradually decreasing.[161]
This raises a possibility, that posttraumatic increase in Aβ production and accumulation is dependent rather of glial than of neuronal APP overexpression.
However, Zhao et al. presented that APP transgenic mice with APP expression driven by the GFAP promoter produce no significant amounts of Aβ and that TBI was not able to increase amyloidogenesis in this model.[315] Also Harada et al. presented that glial cells from Alzheimer patients are lacking activity of BACE-1 enzyme.[95] Contrasting this with evidence suggesting glial Aβ generation (especially from *in vitro* studies), the question of whether astrocytes are producing Aβ posttraumatically needs to be further addressed. In particular it would be interesting to test, whether other cells, in particular neurones or microglia are able to derive Aβ from APP released from astrocytes.

An important posttraumatic aspect is APP immunoreactivity as a measure of potential posttraumatic APP accumulation. In the current study no difference in APP immunoreactivity has been detected between injured and sham–injured APP animals. In particular, no intraaxonal APP or formation of axonal bulbs could be observed. As mentioned above, APP overexpression and accumulation is regarded to play a causative role in posttraumatic AD development. The lack of raise in APP immunoreactivity could be an explanation, why no difference in Aβ deposition and level between trauma–subjected and sham animals was observed. However, immunohistochemistry is not an optimal method to assess APP concentration, thus no quantitative assessment could be here made.

An additional problem is again the time point of analysis. In previous experiments, WT animals presented an increased APP expression lasting for a few days.[24,118,149,161] However, TAI–associated APP accumulation has been observed between 28d to 56d after rmTBI.[138] Similarly, in human autopsy studies APP immunoreactivity has been raised early in neuronal somata[171] and was used to visualise TAI in a time window between 2h to app. 100 days after TBI.[25,172,249] Thus the selected timepoint in this study might be too remote to display APP overexpression. On the other hand, APP as a marker of axonal injury also could not be disclosed, since in the here used CHI model TAI is not the dominating form of the injury.[139,140,143] Thus, potentially 28 d was too late to disclose a posttraumatic increase in APP expression, while TAI–related APP immunoreactivity was too scarce due to the basic characteristics of the TBI model.

5.6. Feasibility of reproducing AD-TBI relationship in animal models: General considerations

The lack of success in achieving increased β-amyloid deposition in the presented experimental paradigm raises also some general question: Is it really feasible, to reproduce closely a human–like posttraumatic AD pathology in transgenic murine model?

To address this question, some possible hindrances, applying both to here presented as well as to previous experiments will be discussed.

5.6.1. Difference in regulation of APP expression

One aspect is the way of neuronal APP expression in physiological settings and in transgenic animals. As mentioned above, neurons are the predominant source of APP in transgenic brains. However, to obtain amyloid pathology resembling human one a high level of baseline APP overexpression is needed. Moreover, to achieve desirable levels of Aβ, usually a mutated, highly amyloidogenic form of hAPP is expressed. Thus, in transgenic animals both levels of hAPP and Aβ are much higher than may be seen in natural settings, including even those in patients with familial AD. Such conditions are required to overcome some obstacles related to use a mouse as a hAPP gene–carrier. Apart from differences in molecular environment (described in 4.6.2.) the opportunity for Aβ to accumulate and to be deposited is hindered also by short life span of mice. Aβ accumulation is to some degree a simple chemical reaction that is dependent to time and concentration of amyloid molecules as a substrate.[98] In human, amyloid pathology may take many years to decades to develop. To replicate this process in relatively short time (i.e. months) in murine models, the second predictor of the reaction i.e. Aβ concentration and, thus, APP expression must be increased manifold.[232]

To achieve such a high level of neuronal APP expression, gene constructs are required, in which the APP gene is driven by a strong promoter, such as a modified PDFG promoter (in PDAPP mice),[65] PrP prion promoter (in Tg2576 mice)[109] or Thy-1 promoter (in hAPP SL751, used here).[8] In that way, the regulation of APP expression is also not physiological, but dependent on the properties of the given promoter construct. Considering that baseline APP expression is set on an extremely high level one may expect, that its expression would not be further up-regulated in reaction to noxious factor e.g. TBI.

Since TBI is resulting in neuronal loss, it is clear that a number of APP producing units (i.e. neurones) are decreased posttraumatically. However, this loss needs to be

overcompensated for amyloid pathology to occur. Here, two mechanisms may be proposed: (i) increase in posttraumatic Aβ- derivation from available APP and (ii) overbalancing raise in APP expression in preserved neurons. The former mechanism is clearly seen as a surge in BACE-1 activity and activation of caspases as alternative way for amyloidogenic APP–processing. This phenomenon is probably present also in transgenic animals and it would explain a short lasting raise in posttraumatic level of Aβ.

Let's consider the latter mechanism (i.e. neuronal APP overexpression). Such activity may be lacking in transgenes, since APP expression is governed in the way attributable to the respective foreign promoters. Thus, even if any posttraumatic surge in neuronal APP expression takes place, it may not be as abundant as in the case of APP expression driven by its own promoter. Therefore overbalancing of neuronal loss by increase in APP production by single surviving neurons may be absent. This explanation fits well to the results of previous studies on TBI in hAPP murine models. Severe trauma resulted in profound neuronal loss (exacerbated by toxic properties of abundant Aβ) and eventual decrease in subsequent Aβ deposition. In turn, a mild trauma paradigm with no observed neuronal loss succeeded in increased Aβ production and deposition. In presented paradigm no neuronal loss could be detected and no change in Aβ deposition was observed. Apart from other explanations (see 5.5.), a reaching of a balance point between raise in Aβ production and neuronal loss could be also speculated.

Considering this disadvantage of contemporary AD models, the use of APP–promoter driven constructs is a reasonable approach. Such experiments have been recently performed, presenting early surge in both APP expression and Aβ level as measured biochemically. The price paid for physiological regulation of APP expression is the lack of full blown amyloid plaque pathology in such knock-in constructs. Reconciling of the advantages of both knock–in and transgenic animals seems to be a difficult task, but the goal of achieving an animal model of AD, almost ideal for neurotrauma research is very tempting.

5.6.2. Structure of white matter and axons

Apart from molecular features, a pivotal role may be played by some biomechanical characteristics of traumatic brain injury in different species.

The human brain, relatively to its size, demands disproportionally long axonal fibres in the white matter for its remote structures to be connected. Such a structural organisation makes human axons extraordinary vulnerable to mechanical damage.[258] Additionally, during trauma the anatomical constitution of human brain, head and neck promotes induction of inertial forces in numerous brain regions containing white matter.[72,198] Taken together, axons of the traumatic brain injury patients are mechanically the most vulnerable cerebral structures,[258] the damage of which (in form of traumatic or –when more extensive– diffuse axonal injury) may be observed in the mildest forms of head trauma, even without substantial injury to the neurons *per se.*[25,26]

On the contrary, the axons of lissencephalic small rodent brain are much shorter as the white matter amount is reduced according to some scaling laws.[312] In result, to achieve axonal damage by single insult, relatively stronger non-inertial mechanical impact is demanded since the anatomical constitution of these animals hampers usage of TAI–favouring angular forces.[257] However, this type of trauma correlates with increased neuronal damage. Thus, it seems that traumatic axonal injury of a similar degree with the one observed in humans cannot be achieved easily in small animal models, since the energy required for the trauma would cause drastic or even lethal neuronal damage.[71,174] Indeed, in small rodent models of axonal injury significant necrotic and neurodegenerative changes have been observed while axonal pathology was of a proportionally lesser degree. Moreover, axonal swellings were predominant in damage pattern while formation of axonal bulbs, which represents disconnection of neurites, was less extensive.[28,39,103,108,208,216]

Also a difference in timing for axonal bulb formation and disconnection may be of great importance for Aβ pathology. Those hallmarks of TAI have been observed in animal models of head trauma; however in smaller animals the sequence of events leading to axonal disruption was exceptionally fast.[167,215,216] The model of CHI used in presented study has not been utilised before in AD-relevant studies. Unfortunately, the tissue preparation (frozen slices) was not convenient in microscopic analysis of axonal pathology. Therefore relevant statements about presence or lack of TAI on amyloid

pathology development may not be formulated. Previous descriptions of CHI consider it as a rather focal model of TBI; therefore widespread TAI should not be expected. This is the next reasonable explanation for a lack of visible difference in Aβ deposition at the rather early posttraumatic 28d time point. The similar paradigm of trauma (force of trauma applied directly to closed skull) was established in mild TBI model of Laurer et al.[138] later used also in Uryu's et al. study.[280] These studies report a presence of delayed formation of TAI hallmarks (after 7 days posttrauma)[138] with an increase in Aβ deposition also occurring in a retarded manner.[280] This temporal congruence emphasise above discussed role of axonal injury on amyloid pathology development.

Summarizing, it is tempting to assume that the axonal length has a key role in rapid posttraumatic Aβ accumulation as all, save porcine animal models of TBI failed to produce this phenomenon. This regards also to transgenic mouse models, since in the single successful paradigm of Uryu et al. increased Aβ deposition was not observed until 9 weeks of posttraumatic course. However, further evidence is required for verification of this concept.

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PERSONAL DATA

Jacek Szczygielski

Lagerstraße 30

66424 Homburg / Saar

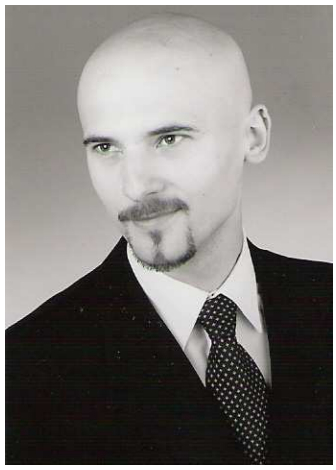
Germany

+49 176 65362615

neuro-jacek@graffiti.net

* 22.08.1978, Łuków / Poland

married



EDUCATION

10.1997 – 10.2003 Study at the Medical University Lublin

04.2002 – 10.2002
KAAD- Fellowship, study at the Saarland University, Medical Faculty, Department of Neurosurgery
Graduation with honours

09.1993 – 09.1997 Tadeusz – Kościuszko – Secondary School, Łuków / Poland
Final exam with honours

POSTGRADUATE EDUCATION

from 03.2004 MD- Study at the Medical Faculty of Saarland University
Topic: Head trauma as a risk factor of Alzheimer's disease development

10.2005 – 09.2006 MD–Study at the II Medical Faculty of Medical University Lublin
Topic: Influence of hypothermia on brain oedema development and epileptogenesis after traumatic brain injury in animal experiments.

10.2003 – 10.2004 Marie- Curie- Fellowship
Co- laborator of Division of Neurobiology of Saarland University Hospital
Research focus: Influence of head trauma on Alzheimer pathology development in APP – transgenic mice.

AWARDS

Award of Polish Minister of Health in academic year 2002/03
Awards of Polish Minister of National Education in school year 1996/97 und 1995/96

EXPERIENCE

from 07.2006	Resident physician, Department of Neurosurgery, Saarland University Hospital
10.2004 – 11.2005	First–year resident, University Hospital Nr. 4 in Lublin
08.2003 - 10.2003	Voluntary practical training in Department of Neurosurgery, Medical University Lublin
10.2000 – 08.2003	Voluntary participation on the clinical activities (on inpatient basis, including operative therapy) of the Department of Neurosurgery, Medical University Lublin

LANGUAGES & SKILLS

Polish (native language)
German (fluent, C1–exam)
English (fluent)
MS Windows, MS Office Tools, SPSS for Windows, GraphPad Prism, professional Web und MEDLINE search

LIST OF PUBLICATIONS

Wirths O, Weis J, **Szczygielski J**, Multhaup G, Bayer TA.
Axonopathy in an APP/PS1 transgenic mouse model of Alzheimer's disease.
Acta Neuropathol (Berl). 2006 Apr;111(4):312-9.
(Original paper)

Szczygielski J, Wirths O, Bayer TA, Steudel WI, Falkai P, Mautes A.
Traumatic brain injury: cause or risk of Alzheimer's disease? A review of experimental studies
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(Oral presentation)

Szczygielski J, Mautes A, Steudel WI, Falkai P, Bayer TA, Wirths O.
Traumatic brain injury: cause or risk of Alzheimer's disease? A review of experimental studies.
J Neural Transm. 2005 Nov;112(11):1547-64
(Review)

Szczygielski J, Wirths O, Schäfer S, Mautes A, Bayer TA.
The impact of repetitive mild- to- moderate Traumatic Brain Injury on development of Alzheimer's pathology. Results of an experimental study in APP_{751} SL transgenic mice.
8th EURON Ph.D. Students Days, Homburg / Saar 2004
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Schwerdtfeger K, **Szczygielski J**, Müller M, Mautes A, Steudel WI.
Vergrößert die Entzündungsreaktion nach schwerem Schädelhirntrauma den Sekundärschaden?
54. Jahrestagung der DGNC Saarbrücken 2003
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