UNIVERSITY OF TURKU

CHOLINERGIC SYSTEM IN SEQUELAE OF TRAUMATIC BRAIN INJURY

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ABSTRACT

Background: Traumatic brain injury (TBI) is one of the most significant causes of disability and lowered capacity. TBI cause also a considerable financial burden since the majority of patients are young at the time of injury. Though much scientific work has been conducted, the pathophysiological mechanisms behind the sequelae of TBI are still largely unknown. However, there is evidence emerging from experimental and clinical studies that the cholinergic system seems to be at least partly involved in the cognitive impairment associated with TBI. In the TBI aftermath, patients commonly experience problems with attention, initiative and processing speed, i.e. functions which are mainly regulated by the cholinergic system. Additionally, in particular there are indications that the structures containing acetylcholine-containing neurons are commonly injured in TBI. Furthermore, there is preliminary evidence that at least some TBI patients may benefit from cholinergic medication.

Aims of the study: Our aim was to utilize positron emission tomography (PET) and magnetic resonance imaging (MRI) to evaluate possible alterations in the cholinergic system after TBI. An additional goal was to clarify the association of these structural or functional changes to the patient's response to cholinergic medication. Patients with moderate-to-severe TBI were compared to healthy controls with PET using the [^{11}C]MP4A tracer. MP4A targets acetylcholinesterase (AChE), which is the pre- and post-synaptic acetylcholine degrading enzyme. The TBI patient group was divided into two depending on their response to rivastigmine (inhibitor of AChE) treatment. These patient groups were imaged with MP4A-PET at baseline (without medication) and after 4 weeks of rivastigmine therapy to compare differences in AChE activity. Cholinergic structures were also investigated with atlas-based MRI morphometry. It was also examined whether the atrophy rates of frontal cholinergic structures were associated with neuropsychological tests results. The subjects filled in a questionnaire to determine whether their smoking histories had any connection to the outcome of TBI.

Results: The AChE activity in TBI patients was clearly lowered in cortical regions when compared to controls. Most significantly, AChE activity was reduced in parieto- and occipital-cortices. A comparison of the two TBI patient groups in the primary time point scan showed evidence of lowered AChE activity in frontal cortical structures in rivastigmine responders. However, the inhibitory effect of rivastigmine on AChE activity was similar with patient groups when scanned during drug therapy and there was no longer any significant difference between groups in their AChE activities. MRI morphometry revealed that the higher the atrophy rate in frontal cortical structures, the poorer the performance in neuropsychological tests measuring attention. Smoking history was not associated with TBI outcome.

Conclusions: According to the results of this study, it appears that the cholinergic system is altered chronically after TBI. It also seems that these structural alterations and the consequential functional changes in the cholinergic system are connected to the response to cholinergic medication. Additionally, the atrophy rate of frontal cortical structures, which are mainly innervated by cholinergic neurons, appears to have correlation to neuropsychological performance concerning attention. There did not seem to be any link between smoking and TBI outcome.

Key words: traumatic brain injury, cholinergic system, acetylcholine, magnetic resonance imaging, positron emission tomography, atlas-based morphometry

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TIIVISTELMÄ

Tausta: Aivovammat ovat kansantaloudellisesti hyvin merkittävä potilasryhmä. Yli puolet sairastuvista on nuoria aikuisia, jonka vuoksi aivovammat ovatkin yksi merkittävimmistä alentuneen toimintakyvyn tai työkyvyn aiheuttajista. Mittavasta tutkimustyöstä huolimatta patofysiologiset mekanismit aivovamman jälkioireiden taustalla ovat vain osin tiedossa. Kuitenkin jo pitkältä aika väliltä on useista kokeellisista sekä kliinisistä tutkimuksista saatu näyttöä, että asetyylikoliini-välittäjäainejärjestelmän (kolinerginen järjestelmä) on ainakin osin kognitiivisten jälkioireiden taustalla. Tavallisimpia aivovamman jälkitilan oireita ovat keskittymisvaikeudet, aloitekyvyttömyys ja yleinen kognitiivista hitaus, jotka ovat toimintoja, joista kolinerginen järjestelmä pääosin huolehtii. Lisäksi on näyttöä siitä, että erityisesti kolinergisen järjestelmän rakenteet monesti vaurioituvat aivovamman seurauksena. Alustavaa näyttöä on myös siitä, että osa aivovamman potilaista hyötyy asetyylikoliini-järjestelmään vaikuttavasta lääkityksestä.
Tavoitteet: Tämän tutkimuksen tavoitteena on selvittää sekä positroniemissio-tomografia (PET) että magneettiresonanssi (MRI) kuvantamismenetelmin kolinergisen järjestelmän mahdollista vauriota aivovamman jälkitiloissa sekä mahdollisen vaurion yhteyttä vasteeseen asetyylikoliinitoimintaa lisäävälle rivastigiini lääkitykselle. Vähintään keskivaikean aivovamman saaneita potilaita verrattiin terveisiin verrokkeihin käyttämällä PET kuvantamisessa merkkiaineena [^{11}C]MP4A:ta, joka on asetyylikoliinia hajottavan entsyymin, asetyylikoliiniesteraasin (AChE), kohdeaine. Kahta aivovamma potilasryhmää, jotka oli jaettu ryhmiin asetyylikoliiniesteraasi-inhibiittori (rivastigmiini) lääkitysvasteen mukaan, verrattiin myös toisiinsa käyten edellä mainittua PET merkkiainetta. Tutkimuksen aikana tehtiin kuvaukset ilman lääkitystä sekä 4 viikon rivastigmiini lääkityksen jälkeen. Lisäksi MRI segmentaatio menetelmällä selvitettiin kolinergisten aivorakenteiden tilavuus muutosta aivovamman jälkeen ja tämän muutoksen yhteyttä neuropsykologisten testien tuloksiin. Kyselytutkimuksella selvitettiin mahdollista tupakoinnin yhteyttä aivovamman toipumistulokseen.
Tulokset: Aivovamma potilailla todettiin hyvin laaja-alaisesti, mutta erityisesti päälaki- ja takaraivolohkon alueella alentunut aivojen kuorikerroksen AChE aktiivisuus terveisiin verrokkeihin nähden. Kahden potilasryhmän välisessä vertailussa todettiin niillä potilailla, jotka olivat saaneet rivastigmiini lääkityksestä hyvän vasteen, alkuvaiheen kuvauksessa matalampi AChE aktiivisuus aivojen otsalohkojen kuorikerroksessa. Kuitenkin rivastigmiinin AChE aktiivisuutta alentava vaikutus oli ryhmien välillä samanlainen, eikä AChE aktiivisuudessa voitu osoittaa enää eroa lääkityksen kanssa tehdyssä kuvauksessa. MRI tilavuus analyysissa todettiin tilastollisesti merkitsevä korrelaatio valittujen otsalohkojen kuorikerroksen alueiden tilavuusmuutoksen sekä neuropsykologisten testien, jotka mittavat keskittymiskykyä, tulosten välillä. Kyselytutkimus ei tuonut esiin yhteyttä tupakointi historian ja aivovamman toipumistuloksen välillä.
Johtopäätökset: Tulosten perusteella voidaan todeta, että kolinergisessä järjestelmässä näyttää tapahtuvan pitkäaikaisia muutoksia aivovamman seurauksena. Nämä rakenteelliset ja toiminnalliset muutokset ovat myös yhteydessä vasteeseen kolinergiselle lääkitykselle. Myös otsalohkojen kuorikerroksen, kolinergisinä pidettyjen, rakenteiden surkastuminen vaikuttaa olevan yhteydessä selviytymiseen keskittymiskykyä vaativissa tehtävissä. Tupakoinnilla ei näytä olevan yhteyttä aivovammasta toipumiseen.

Avainsanat: traumaattinen aivovamma, kolinerginen järjestelmä, asetyylikoliini, magneetti-resonanssi-kuvantaminen, positroniemissiotomografia, atlaspohjainen volymetria

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Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
AChEI	acetylcholinesterase inhibitor
AD	Alzheimer disease
ANOVA	analysis of variance
BuChE	butyrylcholinesterase
CANTAB	Cambridge Neuropsychological Test Automated Battery
ChAT	choline acetyltransferase
CNS	central nervous system
CT	computer tomography
CTE	chronic traumatic encephalopathy
DAI	diffuse axonal injury
DTI	diffusion tensor imaging
DWI	diffusion weighted imaging
GCS	Glasgow coma scale
GFAP	glial fibrillary acidic protein
GOS-E	Glasgow outcome scale-extended
LOC	loss of consciousness
mAChR	muscarinic acetylcholine receptor
MALP-EM	Multi-Atlas Label Propagation with Expectation–Maximization based refinement
MAPER	Multi-Atlas Propagation with Enhanced Registration
MOT	motor screening task
^{11}C MP4A	[methyl-^{11}C] N-methylpiperidyl-4-acetate
MRI	magnetic resonance imaging
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NPV	negative predictive value
NSE	neuron-specific enolase
OR	odds ratio
PET	positron emission tomography

PTA   post-traumatic amnesia
ROI   region of interest
RVP   rapid visual information processing
S100B   astroglia S100 calcium-binding protein B
SPECT   single-photon emission computed tomography
SPM   Statistical Parametric Mapping
SRT   simple reaction time
SSRI   selective serotonin reuptake inhibitor
SWI   susceptibility weighted imaging
TAC   time-activity curve
TAI   traumatic axonal injury
TBI   traumatic brain injury
UCH-L1   C-terminal hydrolase-L1
vAChT   vesicular acetylcholine transporter
VBM   voxel-based morphometry

List of Original Publications

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV:

I   Östberg A, Virta J, Rinne J, Oikonen V, Luoto P, Någren K, Arponen E, Tenovuo O. Cholinergic dysfunction after traumatic brain injury – preliminary findings from a PET study. Neurology 2011; **76**:1046-50. doi: 10.1212/WNL.0b013e318211c1c4.

II   Östberg A, Virta J, Rinne J, Oikonen V, Luoto P, Någren K, Arponen E, Tenovuo O. Brain cholinergic function and response to rivastigmine in patients with chronic sequels of traumatic brain injury – a PET study. Journal of Head Trauma Rehabilitation 2018; **33**:25-32. doi: 10.1097/HTR.0000000000000279.

III Östberg A, Ledig C, Katila A, Maanpää HR, Posti J, Takala R, Tallus J, Glocker B, Rueckert D, Tenovuo O. Volume change in frontal cholinergic structures after TBI and cognitive outcome. Submitted

IV   Östberg A, Tenovuo O. Smoking and outcome of traumatic brain injury. Brain Injury 2014; **28**:155-160. doi: 10.3109/02699052.2013.860468.

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1   Introduction

Traumatic brain injury (TBI) is a disease spectrum of overwhelming complexity, it is said to be "the most complex disease of the most complex organ". TBI is defined as an alteration in brain function, or by other evidence of brain pathology, caused by an external force (Menon et al. 2010). It is a common cause of death and varying degrees of disability in a significant number of persons each year. Epidemiological numbers of TBI vary extensively, mainly due to methodological differences. However, one recent publication estimated the overall incidence in Europe as 262 per 100,000 inhabitants (Peeters et al. 2015).

TBI is a significant clinical problem with very few therapeutic interventions being successfully translated from the laboratory to the clinic so far. A survivor of TBI often suffers from cognitive symptoms. In fact, TBI patients commonly experience deficits in their cognitive functions to have a greater negative impact on their quality of life than other aspects of their injury (Hoofien et al. 2001). The mechanisms behind the cognitive disability are only partly understood despite much effort are being put both on animal models and on human clinical studies during the last decades. These studies have, however, revealed the possibility of a secondary injury and clarified some of the biological mechanisms altered in TBI, including oxidative stress, mitochondrial dysfunction, inflammation and neurotransmitters dysfunction (Unterberg et al. 2004, Corps et al. 2015, Loane et al. 2015). Our current understanding of the pathophysiology of TBI highlights the multi-factorial character of injury events and cascades associated with TBI. After the primary injury, there are many secondary events and injuries which all exert an impact on the final manifestation of the injury and on the outcome of the patients.

The association between TBI and the cholinergic system was already postulated decades ago. In 2003 Arciniegas proposed that TBI induced a dysregulation of the cholinergic system and believed it to be one of the significant underlying causes of the impairment of cognitive functions (Arciniegas 2003). It is clear that also many other neuroanatomical, neurotransmission-related, individual, psychological, social and environmental factors are involved in the development and maintenance of cognitive sequelae after TBI. However, there is an impressive body of evidence highlighting the important role of cholinergic dysfunction after TBI in the

development and maintenance of the cognitive impairment (Salmond et al. 2005, Shin et al. 2015).

Cholinergic nuclei in basal forebrain and brainstem have wide projections not only to frontal cortex but also to the medio-temporal region and thalamus (Mufson et al. 2003). These are important for attention, memory and information processing - functions commonly impaired after TBI (Salmond et al. 2006, Arciniegas 2003). Post-mortem studies in TBI patients have revealed the widespread presynaptic cholinergic dysfunction in cortex and damage, particularly in nucleus basalis of Meynert (Dewar et al. 1996, Murdoch et al. 2002). Furthermore, voxel-based morphometry (VBM) studies have revealed a selected reduction in grey matter density after TBI in structures which are innervated with cholinergic neurons: frontal and temporal cortices, basal forebrain, hippocampal formation and thalamus (Gale et al. 2005, Salmond et al. 2005). The grey matter density in the frontal and temporal regions are also correlated with attentional abilities (Gale et al. 2005). Additionally, there are several studies indicating that acetylcholinesterase inhibitors (AChEI) may be effective in relieving the cognitive symptoms after TBI, however the results from randomized controlled trials have been only moderate (Silver et al. 2006, Tenovuo et al. 2009). Nonetheless, there are indications that a subgroup of TBI patient do benefit from cholinergic medication.

Experimental, clinical and also post-mortem studies have introduced a cholinergic dysfunction after TBI (Ray et al. 2002, Shaw 2002, Murdoch et al. 2002). According to these studies, it seems that there is a short hypercholinergic state at the acute phase of injury which is followed with persistent hypocholinergic state. Overall, it seems that the cholinergic system undergoes remarkable change throughout the days to months after TBI. Cognitive function which are mainly mediated by cholinergic system as vigilance and attention are frequently compromised after TBI (Bentley et al. 2003, Semba 2000). These finding have conducted to the conception of association between cholinergic system and cognitive sequelae after TBI. Leaning on the assumption of hypocholinergic state of chronic TBI, acetylcholinesterase inhibitors that act to increase the amount of endogenous acetylcholine at the synaptic sites, could theoretically alleviate the cholinergic-mediated symptoms of chronic TBI.

Here we investigated changes in cholinergic structures and function after TBI. In addition, the association of these changes with the response of AChEIs was investigated. Atrophy of cholinergic structures involved in sustained attention and correlation to neuropsychological test measuring attention was examined. The connection between the smoking history and TBI outcome was also evaluated.

2 Review of Literature

2.1 Traumatic brain injury

2.1.1 Epidemiology

The global incidence of all-cause, all-severity TBI has been estimated at 939 cases per 100,000 people; thus, a huge number of people, as many as 69.0 million, in different countries of the world will suffer a TBI every year. The majority of all TBI cases are mild. One meta-analysis claimed that the incidence of mild TBI is approximately 740 cases per 100,000 people (total of 55.9 million people each year) and the incidence of severe TBI is approximately 73 cases per 100,000 people (total of 5.48 million people each year) (Dewan et al. 2018). The overall incidence of TBI is higher in males than in females as approximately two thirds of all TBI patients are male (Peeters et al. 2015). In a European population, it was estimated that age-adjusted mortality rate of TBI was 11.7 per 100 000 in 2012 and TBI was linked with 37% of all injury-related deaths in that year. From the same study, the mortality rate in Finland was 24.3 cases per 100 000 for males and 10.0 cases per 100 000 for females (Majdan et al. 2016). However, the Finnish study included also penetration injuries which increase the mortality numbers. There is a broad variation in the incidence values in different epidemiological studies investigating TBI; in some meta-analyses, the estimates of incidence and mortality are considerably lower than those previously presented (Peeters et al. 2015). Nevertheless, it seems that these variations are reflected mostly by differences in patient registries and social and healthcare systems in different countries rather than the actual incidences being different.

The patterns in TBI epidemiology appear to be changing. Epidemiological studies have indicated that the overall incidence of TBI in high income countries seems to be slightly declining but the incidence of TBI in elderly patients is increasing (Peeters et al. 2017). Nowadays the leading cause of TBI is falls, especially in the older age group (Fu et al. 2016, Khan et al. 2015). On the other hand, the incidence of TBI worldwide is increasing, mainly in low- and middle-income countries due to the increased number of motor vehicles and traffic accidents (Khan et al. 2015, Roozenbeek et al. 2013).

2.1.2   Pathophysiology

Traumatic brain injury is characterized as a complex and heterogeneous pathology. The principal mechanisms involved in TBI are classified as focal brain damage due to the contact injury resulting in contusion, laceration, intra- and/or extra-cerebral hemorrhage or diffuse brain damage due to acceleration/deceleration linear forces or a rotational force injury type. The pathological processes following the injury are very complex and the detailed mechanism leading to the functional impairment is not completely understood (Smith 2011).

2.1.2.1   Secondary injury mechanisms

The pathophysiology of TBI is delineated by two substantially different mechanisms: the primary insult (occurring at the moment of impact) and the secondary insult (representing subsequent pathological processes initiated in the minutes to days, even months, post-injury). Both intracranial and systemic insults (e.g. hypoxemia, hypotension, hyponatremia, hyperglycemia) may exacerbate the secondary damage (Chesnut et al. 1993, Unterberg et al. 2004). The secondary insults result from delayed neurochemical, metabolic and cellular events; their pathophysiological neurochemical mechanisms include mitochondrial dysfunction, excessive neurotransmitter release, disturbances in ionic homeostasis, acute inflammatory and an immune response (Corps et al. 2015), neuronal apoptosis and lipid degradation (Loane et al. 2015). These neurochemical events induce the production of toxic and proinflammatory molecules which disrupt the blood–brain barrier leading to the development of cerebral edema and additionally to impaired regulation of cerebral perfusion pressure and metabolism. These events are associated with an increase in intracranial pressure which can contribute to cerebral hypoxia and ischemia (Andriessen et al. 2010).
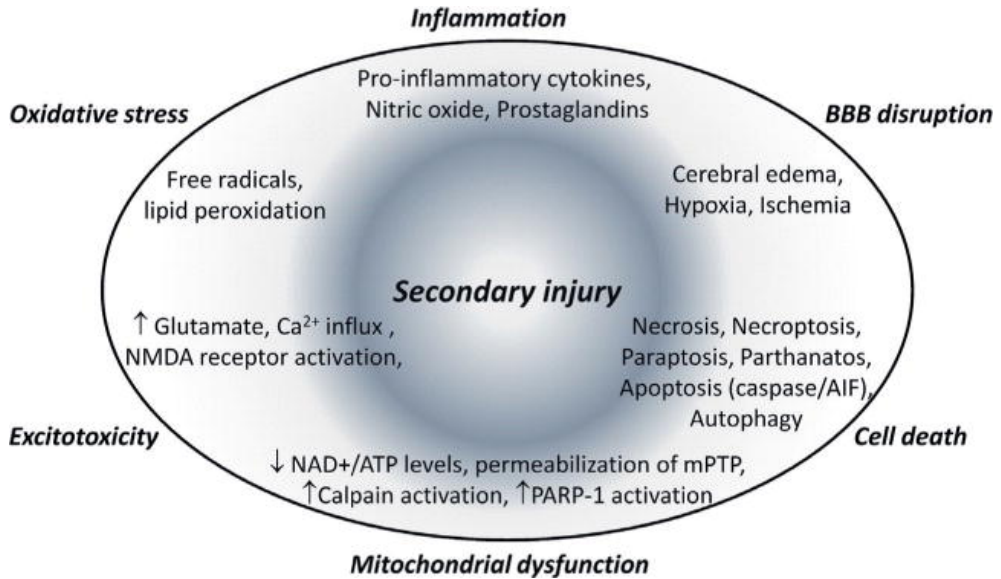
Figure 1. Secondary injury mechanisms after TBI. Reprinted from the Handbook of Clinical neurology, Vol 127, Loane DJ, Stoica BA, Faden AI, Neuroprotection for traumatic brain injury, Pages 343-366, Copyright 2015, with permission from Elsevier.

2.1.2.2   Diffuse axonal injury

Diffuse axonal injury (DAI) or traumatic axonal injury (TAI) occurs due to the transmission of rapid dynamic loads from the head to the brain and specifically to its neurons. DAI is known to be the result of the tearing of axons caused by simultaneous acceleration-deceleration and rotational forces occurring in the traumatic event (Zhang et al. 2006), but the fundamental cellular mechanisms are not yet fully understood (Montanino et al. 2018). Traumatic axonal injuries are associated with high velocity traumas. DAI can be diagnosed with MRI as both hemorrhagic and non-hemorrhagic lesions. Axonal damage may also be a secondary consequence of metabolic changes (Büki et al. 2006). The most widely used MRI grading system of DAI is based on the depth of the lesions: I cortical, II corpus callosum, and III brainstem (Gentry 1994). DAI displays both microcellular (Adams et al. 1989) and macrocellular characteristics, although the latter can be absent in mild cases. The prevalence of DAI detected with MRI depends on the sensitivity of the MRI sequences, selection of patients, and time between injury and scan and therefore values from different studies tend to vary (Vieira et al. 2016, Skandsen et al. 2010, Scheid et al. 2006).

DAI severity is a major factor determining outcome after TBI (Vieira et al. 2016). It is the most common cause of post-traumatic coma and a persistent neurovegetative state (Gennarelli 1993). DAI is also a common reason for a poor

outcome from TBI (Maas et al. 2008, Weiss et al. 2007). It is known that the load of DAI lesions is a prognostic factor for an unfavorable outcome (Gu et al. 2013, Marquez de la Plata et al. 2007, Maas et al. 2008, Weiss et al. 2007). A higher-grade DAI associates with a higher mortality rate and poorer functional outcome (Chelly, et al. 2011, Skandsen et al. 2010, van Eijck et al. 2018, Mannion et al. 2007). On the other hand, the prognostic value of a grade I DAI lesion is still unclear (Scheid et al. 2006, Pierallini et al. 2000). In clinical practice, the DAI grade cannot be considered alone as a reliable tool in the assessment of an individual's prognosis.

2.1.3 Evaluation of TBI and stratification of injury severity

The diagnosis of TBI is based on clinical findings of altered brain functions caused by the external force and abnormal imaging findings in MRI or CT compatible with the clinical findings (Menon et al. 2010). TBI is generally defined as a closed head injury as a result of acceleration/deceleration or impact forces and is differentiated into three categories: severe, moderate and mild. The accurate classification of TBI severity is crucially important when evaluating outcome, assessing the need for rehabilitation as well as in follow-up monitoring.

2.1.3.1 Evaluating severity in the acute phase of trauma

There are several classical parameters which are used to classify TBI severity in acute phase based on its clinical presentation, i.e. Glasgow coma scale (GCS), post-traumatic amnesia (PTA) and duration of loss of consciousness (LOC). The initial severity of TBI has been measured for decades with the GCS which measures the level of consciousness. It consists of three categories: eye opening, verbal response, and motor response. GCS has been the primary clinical variable for grading severity, especially in the initial state of the brain injury, to mild (GCS 13–15), moderate (GCS 9–12) or severe (GCS ≤ 8) (Teasdale et al. 1974, Teasdale et al. 1976). GCS has a clear association with outcome (Teasdale et al. 2014). Another important classification is the length of the PTA (Nakase Richardson et al. 2011). PTA is the interval from injury until the patient is orientated and can form new memories. TBI is classified by PTA as mild (≤ 24 hours), moderate (> 1 to < 7 days) or severe (> 7 days). PTA has been shown to be a robust and important independent predictor of TBI outcome (Walker et al. 2018, Brown et al. 2005).

2.1.3.2 Evaluation by outcome, Glasgow outcome scale – extended

There are multiple instruments to evaluate the recovery from TBI. These place different emphases on different aspects like quality of life, health related quality of

life or neurological and occupational outcome. There are also scales which measures community participation and neuropsychiatric symptoms (Tate 2010). The Glasgow outcome scale-extended (GOSE) is the most popular and most widely used scale to estimate the TBI patient's general functional ability and the outcome from TBI. What was initially a 5-point scale inquiry (Jennett et al. 1981) was extended to an 8-point scale to make it more objective and reliable (Wilson et al. 1998). GOSE has its limitations, it concentrates on the patient's physical functional ability and may be considered to be crude in the mild TBI group. Still, GOSE has proved to be a very valuable adjunct in the management of TBI patients and in clinical trials (Levin et al. 2001, Bagiella et al. 2010).

2.1.4   Imaging of TBI

2.1.4.1   Computer tomography

Imaging plays a crucial role in the evaluation, diagnosis and triage of patients with TBI. It also has a strong predictive value with respect to outcome. The initial imaging modality of choice is CT scanning, because it is widely available, and it is quick to perform. However, quite recent evidence suggests that up to one third of patients with mild TBI according to GCS with a normal CT scan upon presentation will demonstrate structural abnormalities in a later MR imaging (Yuh et al. 2013). As a result of CT's low sensitivity to detect the structural changes present in TBIs, MRI and its derivatives are the golden standard of imaging of mild TBI. In order to avoid unnecessary CT scans and exposure to radiation, there are guidelines for assessing the necessity of imaging the TBI patient. (Undén et al. 2013, Haydel et al. 2000, Mower et al. 2005).

2.1.4.2   Magnetic resonance imaging

Magnetic resonance imaging (MRI) is not usually the primary imaging modality in TBI evaluation, because it takes more time, patient must be able to lay still and requires more effort. In addition, MRI is generally less available, less sensitive to fractures and more expensive. MRI is superior to CT in its ability to detect intracranial structural abnormalities, but on the other hand in the acute phase, CT is superior at demonstrating surgically significant hemorrhages and in determining the age of the hemorrhage. MRI is far more sensitive at revealing the traumatic pathology especially in mild TBI, particularly in the detection of non-hemorrhagic contusion and DAI (Yuh et al. 2013, Tong et al. 2003). In the acute phase, MRI often plays a complementary role to CT and is most often applied in the acute phase in the evaluation of mild TBI when clinical findings and/or symptoms are not clarified by CT images. Subacute/chronic TBI is best assessed with MRI as this imaging modality

is more accurate in detecting micro-hemorrhages, parenchymal atrophy and white matter lesions (Wintermark et al. 2015, Mutch et al. 2016).

A joint workgroup of TBI experts has issued a recommendation for the MRI imaging protocol in TBI (Haacke et al. 2010). The recommended clinical protocol contains multiplanar axial and sagittal T1-weighted images (T1WI3D), axial T2-weighted images (T2WI), T2-FLAIR weighted, and susceptibility weighted (SWI) sequences, in addition to standard diffusion weighted imaging (DWI).

2.1.4.2.1   Susceptibility weighted imaging

Microbleeds which are a pathognomonic sign of traumatic axonal disruption after TBI can be seen in post-mortem investigations. Axonal disruption can also be indicated by the presence of micro-hemorrhages in the gradient echo or SWI sequences. Small deposits of hemosiderin in the injured structures are responsible for a low signal in the generated image. High-resolution SWI is the most sensitive of these techniques in detecting micro-hemorrhages (Scheid et al. 2007). It has been shown that there is an association between the number and volume of SWI lesions and an unfavorable clinical outcome (Beauchamp et al. 2013, Schaefer et al. 2004).

2.1.4.2.2   Diffusion tensor imaging

Diffusion tensor imaging (DTI) is an MRI imaging technique based on the discovery that diffusion of water molecules in cerebral white matter is directionally dependent, with the favored direction of diffusion being parallel to the direction of axonal tracts in the brain (Moseley et al. 1990). The discovery of DTI raised hopes that it could work as a biomarker of white matter pathology and in TBI especially as a marker of DAI. At the group level, DTI has been proven to be robust and to have prognostic value (Lipton et al. 2009, Yuh et al. 2014, Wallace et al. 2018, Newcombe et al. 2016), but currently there is still insufficient evidence to indicate that DTI could be used as a diagnostic tool in TBI in individual patients (Niogi 2010, Ware et al. 2017). It is important to be aware that alterations in DTI metrics are not specific to TBI and have also been observed in a wide variety of other CNS disorders (Fakhran et al. 2013). In summary, it is evident that there is a need for more studies and also the development of techniques capable of converting these advanced imaging methodologies into everyday use in the diagnosis and treatment of TBI.

2.1.4.2.3   Atlas-based regional morphometry

Structural segmentation of MRI offers a potential way to evaluate structural changes, to assess the brain volume loss following TBI and further to clarify the pattern of

atrophy (Bendlin et al. 2008, Warner et al. 2010). Several different atlas-based techniques have been introduced. Some of these techniques are manual or semi-automated but recently also a fully automated segmentation technique was described (Ledig et al. 2015). These techniques are purely for research purposes and methods are not applicable at the level of the individual patient. On a group level, a few of these methods have been proven to be robust and have demonstrated region-dependency and thus could be used as a predictor for unfavorable disease outcome (Ledig et al. 2017, Strangman et al. 2010). Other MRI based approaches for measuring volume changes are based on VBM (Ashburner et al. 2000), deformation-based morphometry (DBM) (Ashburner et al. 1999), or tensor-based morphometry (TBM) (Koikkalainen et al. 2011).

2.1.4.3 Positron emission tomography imaging

Positron emission tomography (PET) is a quantitative and non-invasive *in-vivo* imaging method, which utilizes short-lived radioactive positron-emitting isotopes to study a variety of metabolic, physiologic and functional processes. A wide variety of biological molecules can be labeled with radioactive isotopes (most commonly ^{11}C, ^{15}O and ^{18}F); the labeled molecules are called radioligands. These radioligands can be used to investigate a wide range of physiological processes such as receptor-ligand interactions and glucose metabolism.

The unstable isotopes are produced in a cyclotron. These unstable isotopes have one excess proton in their nucleus and when these radioligands are injected into body, the unstable nucleus undergoes positron decay, causing the nucleus to emit a positron and a neutrino (Turkington 2001). The positron travels only a short distance in tissue before it collides with an electron. In this collision, which is called an annihilation, the positron and electron are transformed into two photons travelling in opposite directions. The PET scanner detector can identify these two photons. Due to the assumption that photons travel in opposite directions, an imaginary line known as the line of response can be drawn between the detectors. Each line of response is registered by its location and angle of orientation in a table called a sinogram. The data in the sinogram is then reconstructed into PET images (Humm et al. 2003).

Positron emission imaging (PET), which involves tracer molecules tagged with radioisotopes, is widely used to image physiological processes. Most of the PET studies investigating TBI have focused on cerebral blood flow and metabolism, demonstrating TBI-associated decreases or increases in metabolism depending on the time frame from TBI (Bergsneider et al. 1997, Peskind et al. 2011, Komura et al. 2018). Fluorodeoxyglucose (FDG, a marker of glucose metabolism) has been the most extensively used tracer in these metabolism studies. It has been also observed that both the level and duration of decreases of glucose metabolism after TBI are associated with

unfavorable cognitive outcomes (Giza et al. 2001). In addition to these perfusion and metabolism studies, PET has been used in several small-scale studies investigating inflammation, amyloid accumulation and neurotransmitter changes after TBI. PET imaging has been claimed to be especially sensitive at revealing inflammation. Ligands which bind to activated microglia cells have been used to investigate inflammation after TBI in the chronic phase of disease (Ramlackhansingh et al. 2011, Wang et al. 2014). Tau and amyloid plaques are suggested to occur in chronic traumatic encephalopathy (CTE), a rare and controversial form of chronic TBI, and some recent studies have revealed the potential of PET radiotracers for targeting these pathological features (Hong et al. 2014, Mitsis et al. 2014).

Despite the numerous studies using PET imaging in TBI patients, it is still difficult to draw any definitive conclusions about its diagnostic value due to the differences in imaging protocols, subject populations, and multiple other factors that contribute to PET signals. As with other advanced imaging techniques of TBI, PET studies have examined only small numbers of patients and further validation will be needed before PET can be widely introduced into clinical practice. At present, high costs and poor availability are the main limitations concerning PET imaging. However, PET imaging does seem to hold some potential in TBI research.

2.1.5   Chemical biomarkers of TBI

The heterogeneity of TBI and the poor predictability of outcome, particularly in the mild TBI group, have highlighted the need for better tools for the evaluation of TBI. This explains the search for blood or cerebrospinal fluid biomarkers, which could be utilized for predicting outcome and help to guide clinical decision-making. One of the most popular approaches for identifying putative biomarkers in biological fluid from TBI patients has been to determine proteins abundant in brain cells. The four most widely studied protein biomarkers are: neurons neuron-specific enolase (NSE) (Böhmer et al. 2011) and Ubiquitin C-terminal hydrolase-L1 (UCH-L1) (Papa et al. 2016) and astroglia S100 calcium-binding protein B (S100B) and glial fibrillary acidic protein (GFAP) (Takala et al. 2016).

Biochemical biomarkers are regarded to have good potential in TBI diagnostics; it is hoped that they will provide easy-to-use tests in acute phase diagnostics for evaluating possible mild TBI. In the future, biomarkers may have a role in the acute phase treatment or in predicting the ultimate outcome of TBI or in estimating the need for more detailed follow up. However, at the moment there is no single biomarker or a combination of them which would have sufficient sensitivity and specificity to be useful in clinical practice (Posti et al. 2017, Mondello et al. 2018). It is possible that a single biomarker may not ever be sufficient to categorize the severity of the injury and the prediction of outcome. Combinations of multiple biomarkers may represent a

more fruitful approach in view of the recognized complexity of TBI (Shan et al. 2016). On the other hand, blood-based brain biomarkers hold the potential to predict the absence of intracranial injury and thus prevent unnecessary head CT scanning. Bazarian et. al studied combination of UCH-L1 and GFAP for detection of intracranial injury, the test had a sensitivity of 97.6% and a negative predictive value (NPV) of 99.6% (Bazarian et al. 2018). Furthermore, S100B has shown a considerable ability to predict the absence of CT pathology and neurosurgical intervention in a subgroup of mild TBI patients (Undén et al. 2010, Asadollahi et al. 2016). Consequently, Scandinavian guidelines for minimal or mild TBI recommend the assessment of S100B as an option for reducing unnecessary CT scans in a subgroup of mild TBIs with a low risk for intracranial complications (Undén et al. 2013).

2.1.6   Outcome of TBI

The assessment of TBI outcome is complex and difficult at the individual level. Single variables like age, GCS, PTA, pupil reactivity and CT scan findings have prognostic value in assessing the TBI outcome (Walker et al. 2018, Sherer et al. 2008). However, predictive statements should only be made by combining many other variables in a multivariate model. This has led to the development of prognostic models and CT based scoring systems. Prognostic factors seem to be partly different in mild TBI (Carroll et al. 2014) and therefore the predictive value of existing prognostic multivariable models in mild TBI is poor (Lingsma et al. 2015, Losoi et al. 2016)

2.1.6.1   Prognostic models

In the past decade, various prognostic models have been devised which combine different variables from an individual patient to predict his/her outcome. The two models which are the best validated and most generalizable are the International Mission on Prognosis and Analysis of Clinical trials in Traumatic brain injury database (IMPACT models) and the Corticosteroid Randomization After Significant Head Injury trial data (CRASH models) (Perel et al. 2008, Steyerberg et al. 2008, Majdan et al. 2014). Both of these models use clinical data and CT imaging data to predict outcome. These models have been externally evaluated to be valid instruments to quantify outcome of TBI (Roozenbeek et al. 2012). However, it seems that existing prognostic models are not suitable for application in the clinic in assessing the prognosis of an individual patient, at least in one with mild TBI (Carter et al. 2016, Silverberg et al. 2015). One large prospective study concerning prognostic factors of mild TBI found that psychological factors in combination with pre-injury mental health problems were the most important predictors for recovery at 6 months following mTBI (van der Naalt et al. 2017).

2.1.6.2   CT scoring systems

Nowadays there are many different CT scorings systems which can be used to evaluate TBI and predict its outcome, with the Marshall classification being the best known and most widely used (Marshall et al. 1991). Although it was not initially developed as an outcome prediction tool, it has been shown to do so quite well (Steyerberg et al. 2008). Later, the Rotterdam CT score was devised as a prognostic tool, evolving from the Marshall classification (Maas, Hukkelhoven et al. 2005). Quite recently, two new prognostic tools were introduced, with increments in the components and different emphasis on the precursors: Stockholm CT score (Nelson et al. 2010) and Helsinki CT score (Raj et al. 2014). These newer CT scoring systems have been demonstrated to be more accurate and reliable predictors of TBI outcome than the Marshall classification and the Rotterdam score system (Thelin et al. 2017, Moretti et al. 2012).

2.1.7   Cognitive symptoms

Cognitive deficits vary with the severity of injury. Generally, these include problems with attention, memory, processing speed and executive functioning and as well cognitive fatigue (McAllister 1992, Whyte et al. 1995, Hellawell et al. 1999, Wylie et al. 2017). These deficits are typically found in patients with DAI and particularly if there has been damage to frontotemporal regions (Salmond et al. 2005, Scheid et al. 2006). Prefrontal brain systems are vulnerable to diffuse and focal damage, especially in TBIs with flexion-extension and rotation forces, leading to problems in executive capacities such as planning, inhibition, organizing and reasoning. Cognitive deficits are a common consequence of TBI, even in cases with the lowest levels of injury severity. Cognitive problems are the leading cause of disability after a TBI, affecting 45-65% patients with moderate to severe injuries (Rabinowitz et al. 2014, Selassie et al. 2008). The relationship between TBI severity and cognitive sequelae is believed to be linear, with a longer duration of impaired consciousness predicting a greater extent of cognitive dysfunction (Dikmen et al. 1995).

Epidemiological research from various studies indicates that people with a previous history of TBI have a higher risk of developing dementia later in life as compared with those without such a history (Moretti et al. 2012). The mechanism behind this phenomenon is unknown; for example, it is unclear whether dementia is caused by TBI or if it is associated with some other factor such as APOE (Teasdale et al. 2005). Or, perhaps it is matter of diminished cognitive reserve or alternatively, TBI might hasten the ageing process (Moretti et al. 2012).

There is also a pathologically distinct disease, which is somewhat controversial, called chronic traumatic encephalopathy (CTE). CTE is associated with repetitive brain injury, which may have been as mild as concussions. This entity has been

especially linked to contact sports like American football. The clinical manifestation of CTE is variable, but the symptoms of this progressive disease include impaired memory and cognition, affective disorders and motor control problems (Sundman et al. 2015). The mechanism behind this rare entity is still a mystery, but one plausible explanation is that there is an accumulation of phosphorylated tau (McKee et al. 2016) possibly due to a dysfunctional lymphatic system (Sullan et al. 2018) or via some other mechanism (Mufson et al. 2018). This controversial entity can be still diagnosed only post-mortem.

2.1.8   Neuropsychiatric problems

Patients with TBI have an increased risk for developing mental health symptoms e.g. depression (Jorge et al. 2004), agitation (Bogner et al. 2015), impulsivity, and aggressive behavior (Dyer et al. 2006). The most common mood disorders encountered after TBI are depressive disorders and bipolar spectrum disorders (Jorge et al. 2018). The incidence of depression is 31 % at 3-months post injury rising to 53% at 12 months (Bombardier et al. 2010). The mechanisms behind post-TBI depression are unclear, but it is not connected to TBI severity (Osborn et al. 2014, Whelan Goodinson et al. 2010). There is evidence suggesting that the DAI lesion load is associated with psychiatric disorders, cognitive impairment, and poor functional outcome (Aldossary et al. 2018). The most significant risk factors for post-TBI psychiatric disorders are preinjury psychiatric disorders and substance use (Whelan Goodinson et al. 2010, Gould et al. 2011).

2.1.9   Neurotransmitter alterations after TBI

Beyond the actual structural injuries in the brain after the TBI, there are changes in neuronal communication caused by alterations in many neurotransmitter systems. These changes continue to develop into chronic stages, months even years after the injury, at the same time as the secondary injury and recovery process are ongoing (McGuire et al. 2018). It is crucial to understand how transmitter regulation systems have been disrupted and how these alterations progress in a temporal manner if one wishes to develop a treatment for the cognitive and behavioral problems suffered by TBI patients.

There is evidence that TBI pathology results in several changes to different neurotransmitter systems, e.g. glutamatergic and GABAergic systems, cholinergic system, catecholaminergic systems (epinephrine, norepinephrine and dopamine) and serotoninergic neurons (see Figure 2). Knowledge about these changes in neurotransmission after TBI has accumulated from experimental and clinical studies and as well from post-mortem studies. There are different alterations occurring in

these neurotransmitter systems in the acute and chronic phases of the disease. Information about acute changes in the glutamatergic transmission after TBI has accumulated from preclinical and clinical studies (Yamamoto et al. 1999, Katayama et al. 1990, Henry et al. 2010, Shohami et al. 2014); chronic phase alterations have been examined in experimental models (Shohami et al. 2014) and also post-mortem (van Landeghem et al. 2006). TBI induced alterations in the GABAergic and serotonin systems have been demonstrated only in experimental settings (Raible et al. 2012, Guerriero et al. 2015, Abe et al. 2016). In addition, although experiments involving the changes in the catecholamine system after TBI have been almost mostly pre-clinical (Jenkins et al. 2016, Levin et al. 1995), there are two clinical PET and single-photon emission computed tomography (SPECT) studies investigating dopamine receptor alterations after TBI (Donnemiller et al. 2000, Wagner et al. 2014).

In the acute phase of TBI, extracellular glutamate increases and GABAergic signaling changes due to disturbances in equilibrium at the receptor level. In contrast, in the chronic phase, there is sustained depression of glutamate signaling and chronic dysregulation of GABAergic tone via mechanisms that vary according to which brain region has been affected (Guerriero et al. 2015). The changes in the catecholaminergic system include an acute catecholamine flood, which is followed by chronic depletion of dopamine and chronic reductions in the numbers of dopamine type-2 receptors (Osier et al. 2016). Serotonin changes are characterized by sustained decreases in both transporter and receptor densities after TBI (Abe et al. 2016).
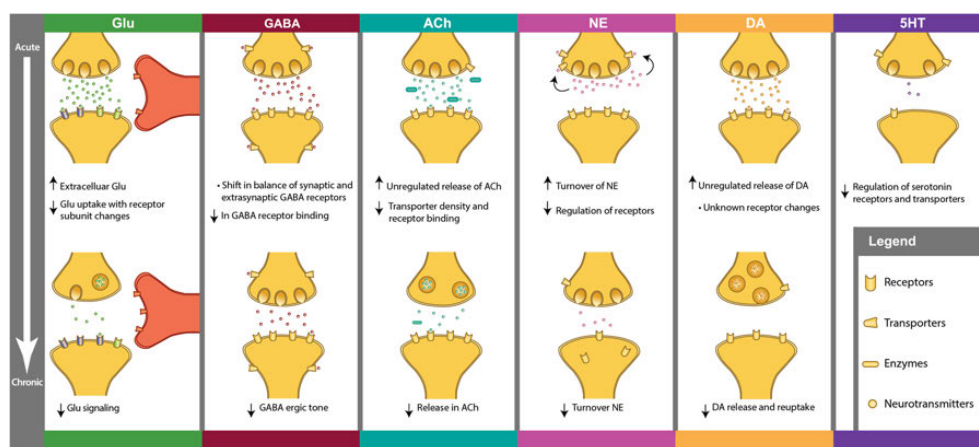

Figure 2. Summary of acute and chronic time-point changes in neurotransmitter systems after experimental TBI. (Glu=glutamate, GABA=gamma-aminobutyric acid, ACh=acetylcholine, NE=norepinephrine, DA=dopamine, 5HT=serotonin.) (McGuire, Ngwenya et al. 2018, Epub ahead of print) Reprinted with permission from Springer Nature. Copyright 2018.

2.1.9.1 Pharmacological treatment of neurobehavioral symptoms after TBI

The findings of altered neurotransmission post-TBI have formed the basis for a variety of approaches to treat the cognitive and psychiatric symptoms. The main strategy in these trials has been the administration of pharmacological agents, acting either as agonists or antagonists, on various neurotransmitter systems. Most of these trials have been pre-clinical, based on experimental models. Although the results from pre-clinical studies have often been promising, clinical studies have rarely been able to repeat the positive results (Osier et al. 2016, McGuire et al. 2018). Altogether, the level of evidence for pharmaceutical therapy of cognitive symptoms is weak. Despite a significant number of studies on drug treatment of neurobehavioral sequelae after TBI, the quality of evidence does not support any treatment recommendations (Warden et al. 2006). Additionally, there are no United States Food and Drug Administration (FDA) approved treatments for cognitive impairments due to TBI. The most extensively investigated medications have been methylphenidate (augments cerebral levels of dopamine and norepinephrine), bromocriptine (dopamine receptor agonist), amantadine (enhances dopaminergic neurotransmission) and selective serotonin reuptake inhibitors (SSRIs).

2.1.9.2 Methylphenidate

Methylphenidate augments synaptic levels of dopamine and norepinephrine via the promotion of their release. In the chronic phase of TBI, methylphenidate may improve attention, processing speed (Whyte et al. 2004, Willmott et al. 2009) and executive functioning. It has also been claimed to enhance cognitive rehabilitation. According to McDonald it is possible to achieve a greater improvement in cognitive functioning after TBI than with either treatment on its own. (McDonald et al. 2017).

2.1.9.3 Amantadine

The mechanism of action of amantadine is still unclear, but it appears to act as an N-methyl-D-aspartate (NMDA) antagonist, increasing glutamate signaling and an indirect dopamine receptor agonist, secondarily enhancing dopaminergic neurotransmission (Peeters et al. 2002). One open-label multi-center study (Whyte et al. 2005) and one double-blinded trial (Giacino et al. 2012) concluded that amantadine appeared to accelerate the rate of functional recovery during active rehabilitation in patients with post-traumatic disorders of consciousness. However, there are also several trials, which could not detect any efficacy of amantadine to hasten recovery and/or to relieve the cognitive symptoms post-TBI versus placebo (Schneider et al. 1999, Meythaler et al. 2002, Ghalaenovi et al. 2018). Hence, the proof

of evidence about amantadine's clinical efficacy is inconsistent and there are no general clinical recommendation for its administration.

2.1.9.4 Bromocriptine

Bromocriptine is a direct dopamine receptor agonist acting primarily on D2 receptors; it also seems to have activity with respect to specific executive function and attentional abilities (Arciniegas et al. 2006). In the chronic phase of complicated mild to severe TBI, bromocriptine may improve executive function (McDowell, Whyte et al. 1998), but it appears that bromocriptine does not improve memory (McDowell et al. 1998) or attention (Whyte et al. 2008).

2.1.9.5 Selective serotonin reuptake inhibitors

SSRIs block the reuptake of serotonin into presynaptic cells which leads to increased serotonergic activity in the synaptic cleft. Over 25 randomized controlled trials, open label, or case studies have evaluated the efficacy of SSRIs as a treatment for post-TBI depression and cognitive dysfunction, with varying results (Yue et al. 2017). SSRIs seem to exert some effects on post-TBI depression (Rapoport et al. 2010), but do not to restore deficits in attention, memory, or executive function (Baños et al. 2010, Meythaler et al. 2001). In conclusion, SSRIs are promising interventions for improving post-TBI depression, but there is insufficient proof of efficacy in combatting the associated cognitive symptoms (Yue et al. 2017).

2.2 Cholinergic system

2.2.1 Functional anatomy of the cholinergic system

In the central nervous system, the cholinergic system is composed of several different parts, with broad and complex connections. Two major centers of the acetylcholine (ACh)-containing neurons are located in the basal forebrain nucleus and in the brainstem (Mufson et al. 2003). The clear majority of the cholinergic input to neocortex and subcortical structures arises from neurons located in the basal forebrain (Woolf 1991). The frontal cortex has the highest cholinergic fiber density, followed by the occipital and parietal cortices. Cells in the basal forebrain (not exclusively cholinergic cells) are distributed rostro-caudally in four partially overlapping groups, with different projection structures: medial septal nucleus, the vertical limb of the diagonal band of Broca, the horizontal limb of the diagonal band of Broca and the nucleus basalis of Meynert (Coppola et al. 2018). The boundaries between basal forebrain structures are not well established, hence projection targets

of subregions have varied across studies. The nucleus basalis of Meynert is recognized for its large, multipolar cholinergic cells (Woolf 1991), providing the main cholinergic input to the entire neocortical mantle (Price et al. 1983). The septo-hippocampal pathway is made up of the basal forebrain medioseptal nucleus neurons which innervate the hippocampus via the fimbria-fornix bundle and the supracallosal striae targeting mainly the hippocampus proper and the entorhinal cortex, with fewer efferent tracks in perirhinal and postrhinal cortices (Kondo et al. 2016). The septo-hippocampal pathway is the main source of ACh in the hippocampus (Dannenberg et al. 2015). The vertical limb of the diagonal band of Broca sends extensive projections to the hippocampal formation, while the horizontal limb of the diagonal band of Broca projects to the olfactory bulb and frontal cortices (Mesulam et al. 1983). The olfactory bulb cholinergic innervation from basal forebrain nucleus apparently modulates the incoming olfactory information (Castillo et al. 1999). Basal forebrain nuclei are topographically organized so that the projection to medial targets are located medially and rostrally, whereas those projecting to more lateral targets are situated in more lateral and caudal parts (Zaborszky et al. 2015, Semba 2000).

The cholinergic nuclei of the brainstem and midbrain include the pedunculo-pontine nucleus, the dorsolateral tegmental nucleus, the medial habenular nucleus and the parabigeminal nucleus. The cholinergic neurons in pedunculopontine and laterodorsal tegmental nucleus have very extensive innervations i.e. to the cortical neurons, thalamus, hypothalamus, globus pallidus and forebrain cholinergic nuclei etc. (Kobayashi et al. 2002). The medial habenular nucleus projects to interpeduncular nucleus and the parabigeminal nucleus to the superior colliculus (Ren et al. 2011). Thalamus, especially its reticular nucleus, is an important target of basal forebrain cholinergic tracts. However, the majority of the cholinergic innervation comes from brain stem nuclei (Hallanger et al. 1987). In fact, 90 % of the brainstem projections to the thalamus are cholinergic (Bentivoglio et al. 1990). The reticular nucleus of thalamus anatomically covers the whole dorsal thalamus, separating it from the cerebral cortex, thus gating the information through the thalamus. Another thalamic nucleus, the mediodorsal nucleus, receives also innervation from the basal forebrain nucleus. This nucleus is thought to participate in limbic functions through its connections with the prefrontal cortex (Kalivas et al. 1999). The cholinergic innervation from the vicinity of the amygdala to the piriform cortex seems to modulate the neuronal output to the limbic and brainstem autonomic regions (Semba 2000). The basal forebrain innervation to the subputaminal nucleus is considered to participate in the neural modulation of speech (Simic et al. 1999).
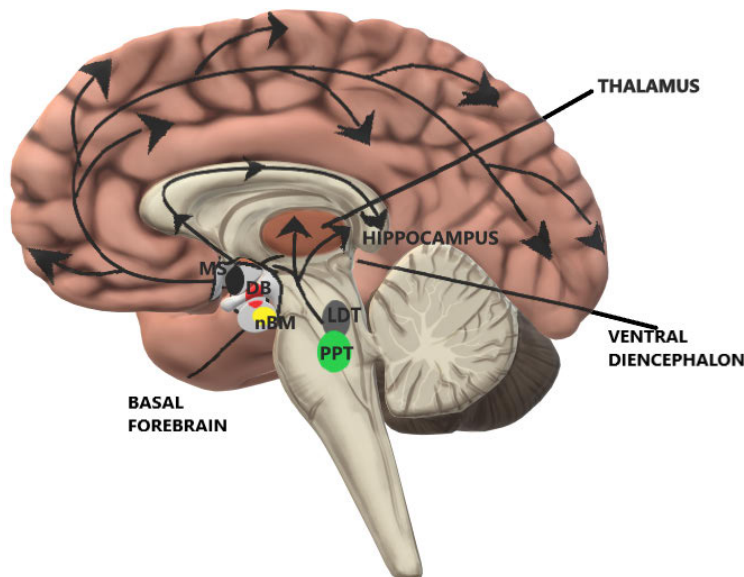
Figure 3. Major cholinergic projections. Two major centers: the basal forebrain cholinergic system and the brainstem cholinergic system. The basal forebrain cholinergic system includes the medial septal nucleus (MS), the vertical and the horizontal limbs of the diagonal band of Broca (DB), and the nucleus basalis of Meynert (nBM). The brainstem cholinergic system includes the pedunculopontine tegmental nucleus (PPT) and laterodorsal pontine tegmentum (LDT).

The neurotransmitter ACh is an ester of choline and acetic acid. The synthesis, release and degradation of ACh are regulated by presynaptic choline acetyltransferase (ChAT), the vesicular acetylcholine transporter (vAChT), and postsynaptic acetylcholinesterase (AChE) (Ma et al. 2018). ACh is synthesized from choline and acetyl CoA by the ChAT enzyme and stored in presynaptic vesicles. The vAChT is responsible for loading ACh into these vesicles. After its release into the synaptic cleft, ACh signals through two classes of receptors expressed on both neurons and glia, 1) muscarinic receptors which can be either excitatory or inhibitory, and 2) ionotropic (nicotinic) receptors which are excitatory. While nicotinic receptors belong to the superfamily of ligand-gated ion channels, all muscarinic receptors are coupled to G proteins, which accounts for their diversity of effects and longer latency of action. ACh signaling is considered to reflect a dynamic equilibrium between the rates of ACh production and packaging, interactions with activators and blockers of ACh receptors, and subsequent hydrolysis by AChE and butyrylcholinesterase (BChE) (Karczmar 1990). AChE directly regulates the extracellular concentration of ACh, making it a key component within the cholinergic synapse.

2.2.1.1   Muscarinic receptors

Muscarinic acetylcholine receptors (mAChR) are G-protein coupled receptors that are categorized into five classes (M1-M5) (Bonner et al. 1987). All muscarinic receptors share the general structural features of the G protein-coupled receptor superfamily, a single amino acid chain with seven transmembrane domains. mAChR are important in neurogenesis (Van Kampen et al. 2010) and long-term potentiation (Auerbach et al. 1996). Three of the subtypes, M1, M3, and M5, mediate excitatory functions, whereas subtypes M2 and M4 evoke inhibitory effects. M1, M3 and M5 activation upregulates AChE and downregulates the expressions of ChAT and vAChT. The M2 and M4 subtypes are auto-receptors; they are found presynaptically and they inhibit ACh release. The activation of these receptors is involved in the presynaptic feedback inhibition of ACh signaling.

2.2.1.2   Nicotinic receptors

Nicotinic acetylcholine receptors (nAChR) belong to the ligand-gated ion channel receptors and are composed of ligand binding subunits ($\alpha2$–$\alpha8$) and structural subunits ($\beta2$–$\beta4$) (Sargent 1993). The many possible combinations account for the large diversity of receptor subtypes with different physiological functions. Activation of nAChR leads to an influx of Na^+ and Ca^{2+} to the neuron. The degree of permeability of Ca^{2+} depends on the subunit composition of the nAChR. The nAChR containing the $\alpha7$ subunit has a much higher Ca^{2+} permeability than the $\alpha3$ or $\alpha4$ subunit containing receptors (Dani 2001). The ability of these receptors to regulate the intracellular Ca^{2+} level makes it an important mediator of learning and memory (Thomas et al. 2004). In addition, the enhanced Ca^{2+} permeability after activation of $\alpha7$ receptors means that they are a possible contributor to excitotoxicity since excessive influx of Ca^{2+} activated by glutamate release can lead to neuronal death.

2.2.2   Actions of acetylcholine in the CNS

In the CNS, ACh has various roles in the control of cortical functions. It appears that ACh acts more like a neuromodulator rather than as a direct excitatory neurotransmitter, altering the activity of neuronal networks and modifying their response to external and internal signals. In other words, the cholinergic innervations seem to modify and allow the fine control of the neuronal flow in the cortical networks (Ljubojevic et al. 2018, Bunzeck et al. 2016, Kimura 2000). This modulation seems to be partly under the control of the prefrontal cortex, which is capable of altering the cholinergic innervation of other cortical regions both by modulating the basal forebrain and by direct cortico-cortical connections (Nelson et al. 2005). ACh seems to function also extra-synaptically, thus producing possible

tonic effects on cortical target areas (Descarries et al. 1997). Through this mechanism, ACh alters neuronal excitability, influences neuronal transmission and induces synaptic plasticity (Gu 2002, Warburton et al. 2003).

It has also been postulated that cholinergic modulation may help CNS networks to separate significant sensory stimuli from the non-significant, such as improving the signal-to-noise ratio, perhaps making the relevant stimuli more salient by reducing background noise (Wenk 1997, Furey et al. 2000). Evidence from experimental as well as from human studies indicates that the cholinergic input from the basal forebrain promotes activity-dependent synaptic modifications in the visual, somatosensory and auditory cortex (Furey et al. 2000, Sachdev et al. 1998, Dimyan et al. 1999).

The cholinergic projections from the basal forebrain neurons are involved in both tonic and phasic activations of the cerebral cortex (Détári et al. 1999). ACh plays an important role in the cortical activation that accompanies the states of wakefulness and paradoxical sleep when the release of this neurotransmitter from the cerebral cortex occurs at the highest rates (Jones 1989). Basal forebrain neurons, which are the major source of ACh, may impair or facilitate the cortical response to sensory input and furthermore modulate the major frequencies of cortical activity across the different states of the sleep-waking cycle (Khateb et al. 1992). ACh's effects on plasticity may partly be associated with its role in sleep regulation (Steriade 2003). It has been proposed that cholinergic activity during the REM-sleep evokes the plastic changes required for the consolidation of memory (Grossberg 2017, Power 2004).

2.2.3 Cholinergic system, cognition and behavior

ACh has been associated with many cognitive functions, most notably sustained attention (Howe et al. 2017), arousal (Kasanuki et al. 2018), learning and memory (Blokland 1995) although it is not the only neurotransmitter that influences these functions. ACh levels are known to oscillate with circadian rhythms (Crouzier et al. 2006), increase with caffeine administration through its action as an adenosine receptor antagonist (Carter, A. J., O'Connor et al. 1995) and vary in a task-dependent manner that correlates with attentional demands (Parikh et al. 2007). ACh is also involved in plasticity and the processing of sensory information (Pinto et al. 2013). Septal cholinergic inputs can modulate different types of synaptic plasticity in hippocampus by coordinating presynaptic and postsynaptic activities (Gu et al. 2012). The cholinergic inputs to sensory cortices also cause receptive field reorganizations (Froemke et al. 2007) and mediate novel encoding of non-sensory information (Chubykin et al. 2013).

2.2.3.1   Attention

Ascending neuronal projection systems, particularly the cholinergic projections arising from basal forebrain and brainstem regions, have been proposed to contribute to attentional performance by modulating the processing of information in the fronto-parietal attentional network (Sarter et al. 2006). In addition, studies measuring ACh release using microdialysis, have revealed increases in cortical ACh release specifically in association with demands on attentional performances (Himmelheber et al. 2000, Arnold et al. 2002). It has also been observed that it is the degree of attentional effort rather than the attentional performance that predicts the increases in cortical ACh release (Kozak et al. 2006). Cortical cholinergic inputs, particularly those to prefrontal regions, have been suggested to mediate the detection of cues (Sarter et al. 2005). ACh release can be present in cue-evoked spikes in ACh receptor activity and in the slower increases in activity corresponding to attentional processes (Parikh et al. 2007). The conclusions emerging from rat lesion and microdialysis studies are that attentional functions are most strongly concentrated in the prefrontal and frontoparietal regions. Findings from human fMRI studies are at least partly in parallel with experimental studies as the results indicate that the cholinergic system is involved in both attention- and memory-related activity in frontal and parietal cortex (Rosier, Cornette et al. 1999, Bozzali et al. 2006). In addition, when human subjects with a lesion in the ventro-medial frontal cortex were evaluated, it was found that an injury in this structure most severely affected those tasks where sustained attention was demanded (Vaidya et al. 2015).

2.2.3.2   Vigilance and Arousal

Here the term vigilance is not applied to describe an attentional concept but instead refers to neurophysiological arousal. It is recognized that basal forebrain cholinergic neurons have a crucial role in stimulating and maintaining cortical activation during waking (Parikh et al. 2013, Hasselmo et al. 2011). In parallel to these findings, ACh concentration transients can occur rapidly at the timescale of a behavioral episode depending on the attentional need (Palma et al. 2012). Attention, i.e. the ability to detect, select and respond to salient stimuli, requires arousal. Orexin neurons which are found in the area of hypothalamus are known to be important for arousal and the maintenance of wakefulness (Robbins 1997). Furthermore, it seems that the ascending orexin projections to basal forebrain may be an important regulator of the activity of basal forebrain cholinergic neurons and correspondingly, cortical ACh release (Kilduff et al. 2001, Palma et al. 2012, Fadel et al. 2005).

2.2.3.3   Memory and learning

There are numerous pharmacological trials which have studied the effects of cholinergic antagonists on learning and memory performance (Smith 1988, Molchan et al. 1992). The muscarinic receptor antagonist, scopolamine, is the drug most widely used to induce amnesia in experimental studies. The first trials with this drug observed that scopolamine induced amnesia in young healthy subjects (Drachman et al. 1974). In addition, AChEIs, which enhance the availability of ACh in the synaptic cleft, were able to reverse the scopolamine-induced deficit, indicating that the cognitive deficit was cholinergic in its nature. Later, the effects of anti-cholinergic medications to impair encoding of new memories have been confirmed in several studies (Aigner et al. 1991, Ghoneim et al. 1977). In addition, impaired function of cholinergic neurons in the basal forebrain has been associated with memory impairment in neurodegenerative diseases (Schliebs et al. 2006). However, it seems that the role of the cholinergic system in memory and learning is more complex, as it has been shown that selective cholinergic lesions have little or no effect on memory (Parent, Marise B., Baxter 2004, Baxter, Bucci et al. 2013). It seems that ACh plays more of a modulatory role in memory function. It also appears that rather than the steady state level of ACh, it is the dynamic pattern of cholinergic activity which better reflects its involvement in memory processes (Kukolja et al. 2009, Hasselmo 2006). ACh's role seems to be diverse in different memory phases (encoding, consolidation and retrieval) as cholinergic stimulation has been claimed to enhance the neural activity associated with encoding but to reduce the neural activity associated with retrieval (Kukolja et al. 2009). This theory has been further supported by studies which have shown that the slow cholinergic tone during slow-wave sleep is essential for the consolidation of declarative memory (Gais et al. 2004, Grossberg 2017). Another aspect of ACh's role on learning is its impact on attention. Effective learning demands that the individual attends selectively to information while avoiding distractions. The cholinergic system is important for priority detection and attention (Paolone et al. 2013) - processes that are crucial for effective learning and memory (Hasselmo 2006).

2.2.3.4   Sleep-wake cycle

Although the details of sleep regulation are not yet fully understood, it seems that the basal forebrain and brainstem cholinergic systems have an important role in the regulation of sleep cycles through their cortical and thalamic projections (Steriade 2004, Khateb et al. 1992). ACh was one of the first postulated transmitters to play a key role in circadian rhythmicity (Rusak et al. 1990). Details of ACh's action on the sleep-wake cycle are far from clear, but they seem to be mediated at least partly by the orexin neuronal systems (Yamanaka et al. 2003). ACh is considered to be a

"transmitter of wakefulness", as its release is increased during wakefulness and motor activity and correspondingly decreased during sleep other than Rapid Eye Movement sleep (Power 2004, Sitaram et al. 1977). On the contrary, in a microdialysis study, ACh release was greater during REM sleep than during wakefulness indicating that conscious awareness does not depend exclusively on ACh modulation (Vazquez et al. 2001). It is known that during slow wave sleep, there is activation of the ACh-modulated laminar cortical circuits that carry out processes in conscious individuals of attentional modulation, decision-making and activity-dependent habituation and these slow wave sleep circuits interact with networks that control circadian rhythms and memory consolidation (Grossberg 2017).

2.2.4   Cholinergic system and TBI

Several studies and reviews have examined the pattern of the early injury-induced cerebral cholinergic excesses and late post-injury cerebral cholinergic deficits (Ray et al. 2002, Shaw 2002). Because of the well-known important role of ACh in various cognitive processes, the relationship between alterations in cholinergic neurotransmission and cognitive deficits after TBI has been investigated in many studies and discussed in numerous reviews (Arciniegas 2003, Salmond et al. 2005, Shin 2015). However, the knowledge of alterations in the cholinergic system after TBI derives mainly from studies conducted in experimental animals and very few of these results have been confirmed in human patients.

2.2.4.1   TBI and changes in acetylcholine receptors

2.2.4.1.1   mAChR

There is only one published human study, which showed no post-mortem alterations in muscarinic receptor binding in inferior temporal gyrus tissue after TBI (Bonner, Buckley et al. 1987). However, many animal studies have detected a reduction in the numbers of mAChR early after TBI, ranging from hours to weeks (Jiang et al. 1994, Donat et al. 2010, Sihver et al. 2001). Most of these studies used either a PET tracer or a radioactive ligand which is nonspecific with respect to the muscarinic receptor subtypes, but several studies seem to indicate that the reduction in mAChR density may be largely attributable to a decrease in the numbers of the M2 subtype (DeAngelis et al. 1994). Depending on which report is considered, the reduction of mAChR was most marked in brainstem, hippocampal areas (DeAngelis et al. 1994, Sihver et al. 2001, Lyeth et al. 1994) or in basal forebrain (Donat et al. 2010). Since it is known that presynaptic M2 receptors inhibit ACh release, this has given rise to the theory that there is a compensatory downregulation of inhibitory auto-receptors in

order to maintain excessive ACh release chronically. Nevertheless, it is far from clear whether these alterations in mAChRs are compensatory changes induced by injury or if they are direct consequences of the injury. Importantly, post-mortem study in human TBI patients detected no alterations in the binding properties of either mAChR or nAChR (Murdoch et al. 1998, Dewar et al. 1996).

2.2.4.1.2   nAChR

Although there are no human studies, many experimental trials have reported a reduction in the levels of nAChR after TBI (Donat et al. 2008, Verbois et al. 2002), especially the α7subtype nAChRs (Hoffmeister et al. 2011). Changes in these α7 receptors would have numerous impacts on the mechanisms behind the secondary injury as activation of the α7 receptors suppresses the release of inflammatory mediators from macrophages and microglia (Yu et al. 2003), and these receptors also exert an anti-apoptotic effect (Ren et al. 2005). The finding of alterations in nAChR levels after TBI have stimulated attempts to discover nAChR targeting pharmacological agents which would prevent these molecular-level changes and ultimately improve the behavioral outcomes.

2.2.4.2   Alterations in the ACh level and cholinergic enzymes after TBI

In experimental animals after TBI, there is an initial unregulated release of ACh accompanied by decreases in transporter density and receptor binding beginning as early as 1 h after the injury (Hayes et al. 1986, Saija et al. 1988, Dixon et al. 1995). In addition, the AChE level is elevated in basal forebrain during the following hours to days (Donat et al. 2010). However, in one study, the AChE level declined at acute time points after the injury in hippocampus, hypothalamus, and motor cortex (Donat et al. 2007). There is one human study where concentrations of ACh were also elevated in ventricular CSF acutely following a head injury (Haber et al. 1980). With the exception of this single study, findings of an acute surge of ACh after TBI originate only from work done in experimental animal models. It has been speculated that AChE activity is upregulated in the basal forebrain as a compensatory response to the acutely increased cholinergic neurotransmission. One human study indicated that a lowered cholinesterase level in serum was associated with TBI severity and poorer outcome (Zhang et al. 2015) supporting the theory that the increase in free ACh levels is harmful. However, animal models have indicated that an increased extracellular ACh concentration is followed by a general hypofunction of the cholinergic system as evidenced by the reduction in ACh's synthesis (Dixon et al. 1995) and release (Dixon et al. 1996) which are associated with changes in AChE activity.

ChAT and vAChT are critical for the function of presynaptic ACh release. ChAT is the ACh synthesizing enzyme and vAChT is responsible for loading ACh into the secretory vesicles. Animal studies have reported that ChAT activity decreases and there is vAChT downregulation acutely after TBI, followed by upregulation at the subacute and chronic time points (Donat et al. 2008, Pike et al. 1997). The decrease in ChAT activity in the acute phase has been shown to occur in dorsal hippocampus, frontal and temporal cortices, but at more chronic time points, the increase in activity was most distinct in the parietal cortex of rat (Gorman, Fu et al. 1996). On the other hand, the upregulation of vAChT occurring in the chronic phases has been most prominent in hippocampus (Ciallella et al. 1998, Shao et al. 1999) and cortex (Dixon et al. 1999).

Human post-mortem studies have been contradictory to animal models showing a decrease in ChAT activity after TBI (Dewar et al. 1996). ChAT activity was reported to be reduced in the inferior temporal gyrus, bilateral cingulate, inferior temporal, and posterior parietal regions (Murdoch et al. 1998). In the same TBI patients, levels of a presynaptic terminal marker, synaptophysin, were also reduced supporting the conclusion that cholinergic terminals had been acutely damaged after the head injury (Murdoch et al. 1998). In a histological analysis, neurons in the nucleus basalis of Meynert were significantly damaged in patients who had died after TBI, and furthermore in the damaged neurons, the intensity of ChAT immunoreactivity was decreased (Murdoch et al. 2002). The finding of the vulnerability of basal forebrain nuclei after TBI originated from experimental animals where it was observed that although the cholinergic nuclei of basalis forebrain were damaged, dopaminergic and noradrenergic innervations were left unaffected (Schmidt et al. 1995, Leonard et al. 1994) before actually being confirmed in human studies.

2.2.4.3 Structural cholinergic CNS changes after TBI

CNS cholinergic neurons and their projections are situated in regions which are particularly vulnerable in TBI because of the brain anatomy and injury biomechanics (Newcombe et al. 2016). For example, it has been claimed that local contusions are usually localized in ventral and polar frontal and anterior temporal structures where the brain is confined by the bony ridges of the inner skull (Gentry et al. 1988). Voxel-based morphometry (VBM) images of TBI patients with cognitive sequelae revealed a selective reduced grey matter density in the frontal and temporal cortices, basal forebrain, hippocampal formation, thalamus, (Gale et al. 2005, Salmond et al. 2005), regions which are innervated by cholinergic neurons. These investigators found also a strong correlation between the grey matter content in the frontal and temporal regions and the attention capabilities of the patients (Gale et al. 2005), a cognitive function mainly controlled by the cholinergic system. In addition, another VBM

study found evidence that atrophy was more regional selective rather than diffuse after a traumatic axonal injury (Warner et al. 2010). Thus, atrophy was most prominent in amygdala, hippocampus, thalamus, corpus callosum, putamen, precuneus, postcentral gyrus, paracentral lobule, and parietal and frontal cortices. A functional MRI study also revealed that the prefrontal activation required for a task demanding attention was altered in TBI patients in comparison with controls (McAllister et al. 1999). The actual brain structures which are altered, atrophied or dysfunctional have tended to vary in the published studies employing different imaging techniques, but generally, it has been found that the cholinergic structures are involved. In animal models of TBI, the anatomical regions most frequently damaged are also those regions which are heavily innervated by ACh projections in the normal brain: the hippocampal formation (Sihver et al. 2001, Chen et al. 2003) and the thalamus (Chen et al. 2003). A different aspect of the possible involvement of the cholinergic systems in the cognitive sequelae appearing after TBI is how this impact on CTE. Research investigating tau pathology has linked the cholinergic system also to CTE as p-tau containing neurofibrillary tangles within nucleus basalis of Meynert neurons have been identified as a pathological lesion in CTE inducing cholinergic neuronal dysfunction and possibly some involvement in the cognitive syndrome associated with CTE (Mufson et al. 2016).

2.2.5   Cigarette smoking and TBI

Nicotine evokes its action by binding to nAChR. Exposure to nicotinic agonists and tobacco smoke is known to increase the number of CNS nAChRs both in humans and animal studies (Flores et al. 1992) by as much as twofold (Lindstrom et al. 1996). Smokers have a widespread up-regulation of nAChR, probably related to a desensitization of these receptors after chronic exposure to nicotine (Mansvelder et al. 2002). The effects of nicotine are mainly excitatory as this alkaloid increases the release of many neurotransmitters including acetylcholine (Hatsukami et al. 1989). Nicotine enhances vigilance and rapid information processing during smoking deprivation. It seems to improve attention even in the absence of nicotine withdrawal and in non-smokers, although the results are somewhat inconsistent (Wignall et al. 2011, Warburton et al. 1994, Ahrens et al. 2015).

Animal studies have shown that experimental TBI causes a persistent decrease in CNS $\alpha7$ nAChR expression (Verbois et al. 2002, Donat et al. 2008). In an experiment performed in rats, the TBI-induced deficits in $\alpha7$ nAChR density could be reversed by long-term infusion of nicotine (Verbois et al. 2003) and a subsequent study from the same group showed also that nicotine administration could attenuate the cognitive impairment after TBI (Verbois et al. 2003) as had been claimed in an older rat study (Brown et al. 2000).

There are no human studies that have investigated possible alterations in the nAChR numbers after TBI and furthermore, the effects of nicotine on the cognitive sequel after TBI are unknown. There is one human study which has indirectly examined a possible connection between smoking and mild TBI outcome. It was found that non-smokers experienced a significantly greater improvement on measures of processing speed, visuospatial learning and memory, visuospatial skills, and global neurocognition as compared to smokers (Durazzo et al. 2013). Furthermore, both a greater lifetime duration of smoking and pack-years were related to a significantly poorer improvement in multiple domains. However, it is difficult to draw any firm conclusions from this study as it is known that smoking is intimately associated with many predictors of TBI outcome (substance use, age, education), which were only partly taken into account.

2.2.6   Cholinergic treatment after TBI

Reports on cholinergic medication after TBI mainly originate from experimental animal studies. The main research lines have focused on mAChR and nAChR targeting drugs and acetylcholine esterase inhibitors (AChEI). Scopolamine is one of the most widely investigated muscarinic receptor targeting drugs as it blocks these receptors and possibly reduces the impact of the acute phase surge in ACh. Animal studies have reported potential neuroprotective effects of scopolamine when used acutely after the injury (Lyeth et al. 1992). Nicotine is one of the nAChR targeting agents, which has been investigated in experimental TBI models. In addition, newer pharmacological agents with more selective affinities for the α7 nAChR have been used in various animal brain injury studies (Hijioka et al. 2012). These agents have shown neuroprotective properties and been able to attenuate the brain injury (Duris et al. 2011, Gatson et al. 2015).

There are no studies on the effects of nicotinic or muscarinic receptor targeting agents in human TBI. There are a few reports of human studies of cholinergic treatment after TBI involving AChEIs. A Cochrane review and the guidelines on the pharmacotherapy for chronic cognitive impairment in traumatic brain injury included cholinergic medications. However, in that review, only one trial on AChEIs was accepted and the level of evidence on the efficacy of AChEI against the cognitive impairment was estimated to be low (Dougall 2015). In fact, reports on treatment of cognitive sequel after TBI with cholinergic medication are mainly limited by small sample sizes, lack of randomization, with the outcomes being difficult to interpret due to the heterogeneity of the study populations. The evidence for the benefits of AChEIs after TBI is relatively weak, based on a few, mainly small, studies with somewhat conflicting findings.

2.2.6.1   Acetylcholinesterase inhibitors

2.2.6.1.1   Physostigmine

The efficacy of physostigmine in treating cognitive sequel after TBI has been studied in many animal experiments as well as in patients with TBI. Early studies with physostigmine reported improvements in memory (Goldberg et al. 1982), attention (Levin et al. 1986) and arousal (Weinberg et al. 1987) in TBI patients.

Physostigmine, however, has a very short half-life and a narrow therapeutic window. It has also significant systemic side effects, like bradycardia and hypotension, which limit its clinical usability and led to the search for newer AChEIs that have more CNS specific effects and due to its major side effects, physostigmine is no longer in clinical use.

2.2.6.1.2   Donepezil

Various studies have investigated the efficacy of donepezil on the cognitive sequelae of TBI; these include several small case series and case reports, as well as randomized controlled trials (Ballesteros et al. 2008). There are two randomized controlled, only one of which was placebo controlled (Zhang et al. 2004, Kang et al. 2001) see Table 1. Donepezil has been shown to improve vigilance and attention (Tenovuo 2005, Zhang et al. 2004), intelligence quotient (Whelan et al. 2000), MMSE (Kang et al. 2001), visual memory (Zhang et al. 2004, Morey et al. 2003) and verbal learning and memory (Trovato et al. 2006) in the chronic phase of TBI. However, the available evidence is not conclusive as the studies have been small and often associated with methodological limitations, in particular there is a lack of double-blind, randomized controlled trials.

2.2.6.1.3   Rivastigmine

In experimental TBI studies, rivastigmine has been shown to exert neuroprotective effects and improve spatial memory (Chen et al. 1998). However, clinical studies have shown only a very modest effect. There are two randomized, prospective, placebo-controlled studies investigating rivastigmine's efficacy and safety in TBI patients with cognitive sequelae (Tenovuo et al. 2009), one with double blinding (Silver et al. 2006) see Table1. Tenovuo et al. concluded that rivastigmine improved working memory and sustained attention when compared to placebo, although the results were scarcely significant. Silver et al. observed no difference between the placebo and rivastigmine although a subgroup analysis among moderate to severe

injured patients treated with rivastigmine did reveal some improvements in information processing, verbal learning and memory.

2.2.6.1.4   Galantamine

There are very few animal or human studies evaluating the possible efficacy of galantamine on the cognitive symptoms associated with TBI. Animal studies have suggested that galantamine may enhance the effect of rehabilitation (de la Tremblaye et al. 2017) and improve multiple modalities of memory (Zhao et al. 2018). Nonetheless, a placebo controlled, randomized study could not find any evidence that galantamine would be superior to placebo (McAllister et al. 2016) as presented in Table 1.

Table 1. Reported randomized controlled studies of acetylcholinesterase inhibitors in Human Traumatic Brain Injury.

Author	Design	N	Injury severity	Testing	Results
Rivastigmine					
Silver, et al. 2006	RDBPC	157	all severities	12 weeks	no difference to placebo; improvement in memory and reaction time in patients with poorer points at baseline
Tenovuo et al. 2009	RPCT	102	all severities	20 weeks	improvement in sustained attention
Donepezil					
Kang et al. 2001	RCT	36	mild to moderate	3 weeks	improvement in MMSE
Zhang et al. 2004	RPCT	20	all severities	10 weeks	increased short-term memory and sustained attention
Galantamine					
McAllister et al.	RDBPC	23	mild	12 weeks	no difference to placebo

(RDBPC= randomized, double blinded, placebo-controlled trial, RPCT=randomized, placebo-controlled trial, RCT=randomized controlled trial)

3   Aim of the Study

The neurobiology of cognitive impairments after TBI is poorly understood. Although the pathology is most certainly multifactorial, one of the strongest theories is that the cholinergic system is substantially involved in a chain of events. The principal aim of this thesis was to add to our knowledge in the challenging field of cognitive impairment after traumatic brain injury, by attempting to clarify the role of cholinergic systems in the cognitive sequelae of TBI and to reveal possible alterations in the cholinergic system after TBI.

The specific aims of this study were:
1.   To investigate with PET whether the cholinergic system of TBI patients in the chronic phase of the injury differs from that in healthy control patients. (Study 1, original article I)

2.   To evaluate with PET does the cholinergic system differ between those TBI patients who have responded to cholinergic medication with those who have not responded. (Study 1, original article II)

3.   To assess volume changes in brain cholinergic structures at 6 months after TBI and to examine whether the volume changes display any association with the patients' cognitive tests results. (Study 2, original article III)

4.   To determine whether smoking history is associated with the outcome of TBI. (Study 3, original article IV)

Outline of the work

This work consists of three separate Studies (1-3), on which the four Original articles (I-IV) are based. In the following text, the Studies are referred to with Arabic numbers (1-3) and Original articles with Roman numerals (I-IV).

Study 1 investigated TBI induced changes in the brain cholinergic system using PET by comparing chronic TBI patients with cognitive symptoms to healthy controls and evaluated the effect of treatment with an AChEI on the brain cholinergic system in different patient subgroups. Study 2 exploited MRI and an atlas-based

morphometry technique to examine possible regional selective, cholinergic regions atrophy after TBI and to assess whether there was any correlation between these measurements and the results of neuropsychological tests. Study 3 clarified with a retrospective questionnaire if the patient's smoking history or use of other nicotine-containing products exerted any effect on his/her TBI outcome.

4 Patients and Methods

4.1 Subjects

Study 1 (Original articles I-II)

TBI subject

The subjects were identified from a database from the Outpatient Clinic of the Department of Neurology, Turku University Central Hospital. The subjects had been evaluated and diagnosed with TBI in the Outpatient Clinic between 1993 and 2006. Initially, the database consisted of 1040 patients. The inclusion criteria were: 1) chronic cognitive sequelae of TBI with the presence of all four main symptoms: memory problems, fatigue, decreased initiation, and attentional deficits (in addition, the symptoms had only emerged after the trauma), 2) more than one year post-injury and the above-mentioned symptoms subjectively remained unchanged for at least three months before study entry, 3) mainly a diffuse traumatic injury mechanism without large focal traumatic brain lesions, according to either CT or MRI, 4) an earlier treatment trial with rivastigmine with a minimum duration of one week and a minimum daily dose of 3 mg, and 4) at least 18 years of age. The exclusion criteria were: 1) Other diseases of the CNS or psychiatric disorders requiring medication, 2) current use of centrally acting drugs or drugs known to affect the cholinergic system that cannot be safely interrupted for at least four weeks, 3) contraindication for PET or MRI imaging, 4) uncertainty about the TBI diagnosis or about the etiology of the above mentioned clinical symptoms, 5) contraindication to rivastigmine treatment or the appearance of an adverse event during an earlier treatment trial with rivastigmine. In all, 38 subjects from the TBI database fulfilled the inclusion and exclusion criteria. These individuals were contacted and 19 of them agreed to participate in these experiments and provided informed consent but two individuals discontinued the study and consequently the final study group consisted of 17 subjects.

In article II, the above mentioned TBI patients study group (n=17) were divided into two according to how the individuals had responded to rivastigmine with

respect to their cognitive problems after TBI. Ten of the group were responders, having shown a subjective treatment response to this central AChEI and seven of group were non-responders. The response to rivastigmine was evaluated systematically in all subjects using a 5-step Likert scale from considerable harm to considerable benefit. All non-responders reported neither harm nor benefit whereas the responders all reported either clear or considerable benefit, with a statistically significant difference between groups (p<.0002). The non-respondents had not experienced notable effects from at least a 3 mg daily dose of rivastigmine, while the respondents had displayed either a marked or a very marked improvement in their daily functioning. The response to treatment was also confirmed in an interview with the patient and his/her proxy. The respondents received a 4 weeks' rivastigmine wash-out period before study entry. The duration of rivastigmine treatment before the study had varied from some weeks to several years. A four weeks' wash-out period of rivastigmine was estimated to be long enough, as there is no evidence of prolonged effects on AChE activity and rivastigmine has a short pharmacokinetic half-life (Polinsky 1998). There was no control group included in this part of the study.

Healthy controls

The control group (n=12) was recruited from healthy volunteers matched for age and sex. The exclusion criteria were as follows; contraindication for PET or MRI imaging, current use of centrally acting drugs, previous head injuries with loss of consciousness, amnesia, or post-injury symptoms lasting for more than one week, or signs of brain remarkable pathology in MRI imaging. No presence or history of neurological or psychiatric disease.

Study 2 (Original article III)

The subject collection and imaging data acquirement were conducted in Turku University Hospital, Finland during the TBIcare project (http://www.tbicare.eu). Data collection took place between November 2011 and October 2013. Source data was adult patients, age ≥ 18 years, with clinically diagnosed mild, moderate or severe brain trauma. Exclusion criteria were blast-induced or penetrating mechanism causing the TBI, unable to live independently because of a brain disease or other medical cause before the injury, TBI or suspected TBI not needing cranial CT imaging, not speaking the native languages, or no consent obtained. Over the course of the project, a total of 141 subjects with mild to severe TBI have been scanned by MRI both in the acute stage of the injury (baseline) and during the chronic phase (follow-up) of the disease. A total of 120 subjects were processed for which both

baseline and follow-up images were available when the analysis was started. After visual review, six subjects were excluded due to low image quality or errors in the data description. Thus, the final study group consisted of 114 patients.

During the trial control group was also recruited (n=40). The control group consisted of patients with acute orthopedic nontrivial trauma without any signs of acute central nervous system involvement, previous central nervous system disease, or previous non-concussional TBI. Seventeen control patients were imaged by brain MRI in primary and control time points and therefore only these 17 controls could be included in these analyses.

Study 3 (Original article IV)

The study subjects were recruited from outpatients with a diagnosis of TBI (n=1,151) who had been evaluated and treated in the Department of Neurology, Turku University Central Hospital, between January 1993 and June 2006. Inclusion criteria were information about the severity of the injury, either as GCS at admission or PTA available in the medical records, and age ≥ 15 years at injury. Patients with uncertain TBI diagnosis or non-traumatic neurological disorders which would prevent the outcome evaluation of TBI were excluded. The severity of TBI was classified using the duration of PTA as mild (1 - 24 hours), moderate (1 - 7 days), severe (1 - 4 weeks), or very severe (> 4 weeks), and according to the GCS as mild (scores 13 - 15), moderate (9 - 12), or severe (3 - 8). Demographic data included age at the time of the injury and at the time of the survey, gender, education, severity of TBI, and outcome of TBI. The outcome of TBI was classified with the GOS-E. Out of the original group, 52 had died, 36 had an uncertain TBI diagnosis, in 12 subjects the medical records were missing, 25 were < 15 years at the time of the injury, and four patients had a non-traumatic neurological condition preventing the evaluation of the outcome. Therefore, the final study group consisted of 1022 subjects.

4.2   Study design

Study 1 (Original article I-II)

All the study participants including controls, were scanned with PET imaging using the tracer [methyl-^{11}C] N-methylpiperidyl-4-acetate (^{11}C-MP4A, a lipophilic acetylcholine analogue with good AChE specificity). Treatment with drugs with effects on the CNS was interrupted for 4 weeks before the scan. In the analyses, all subjects with TBI (n=17) were compared to controls (n=12).

Original article II

The study subjects were scanned twice, with and without rivastigmine medication. The primary time point scan was the same as used in original article I. In further analyses, TBI patient group were divided into two i.e. as responders and non-responders according to their response to rivastigmine. Four weeks before the time of the first PET (11C-MP4A) scan, none of the participants were taking any medication known to affect cholinergic neurotransmission. For a minimum of four weeks before the second PET scan, the participants were administered rivastigmine at a dose of 1.5 mg twice a day. The flowchart of Study 1 is presented in Figure 4. To exclude structural lesions, for anatomical reference and volumetric analyses, each individual was scanned with MRI (Philips Gyroscan Intera 1.5 T) before the first PET scan. Group differences between responders and non-responders were examined between both time point PET scans. It is important to note that the analyses included in original article II did not include a control group.
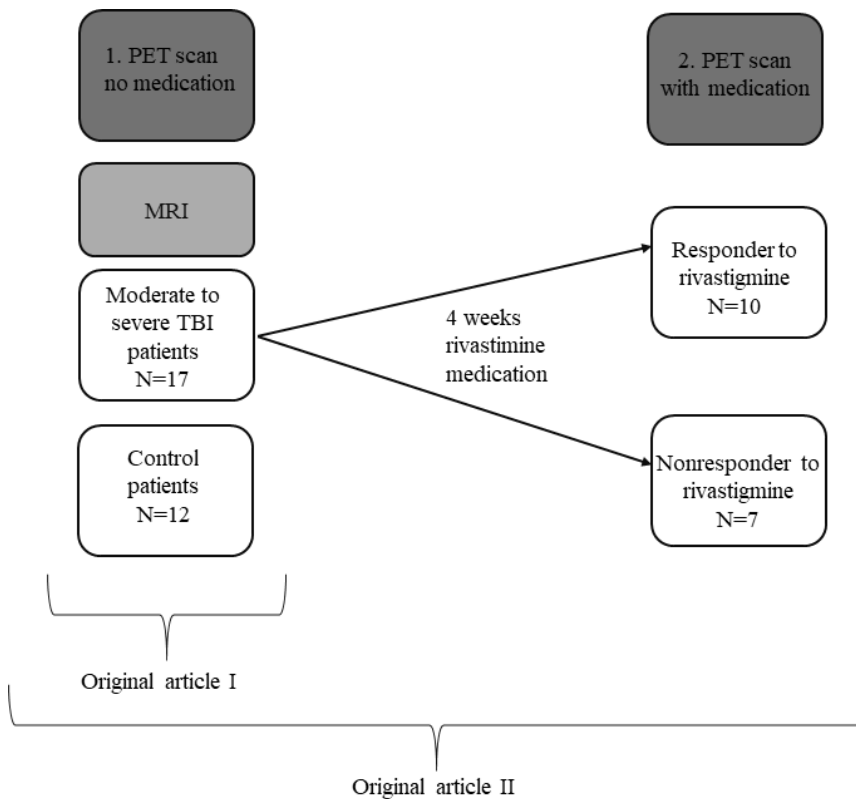

Figure 4. Flowchart of Study 1.

Study 2 (Original article III)

The TBIcare project was a prospective clinical observational study with structured data collection. All patients were treated according to the accepted, standardized, existing guidelines that are based on national and international recommendations. No new treatment interventions were evaluated during the data acquisition for this study.

Study participants were scanned with brain MRI at two time points: in the acute phase of the TBI and in the follow-up visit in the chronic phase of TBI. The follow-up visit was about 6 months after the primary injury. During the follow-up visit, also the neuropsychological tests in the Cambridge Neuropsychological Test Automated Battery (CANTAB) and an evaluation of outcome by GOSE were conducted. The control participants were imaged in the same manner at the primary point and after 6 months. This study focused on structural T1-weighted MR images, so that subtle volumetric changes could be assessed. Structural atrophy occurring between the acute and chronic disease stage was quantified with automated atlas-based segmentation of MRI (Ledig et al. 2015). In longitudinal analyses, our aim was to investigate the structural volume change on individual region of interests (ROIs) in cholinergic regions and as well as in larger anatomical structures. It was investigated whether the rates of structural atrophy correlated with the participants' responses in the neuropsychological tests measuring attention.

Study 3 (Original article IV)

A specially-designed questionnaire, including 10 closed questions, was sent by mail to the 1022 study subjects and 689 of them (67.4 %) replied, forming the final study group as shown in Figure 5. The questionnaire provided a subjective estimate of the functional outcome of TBI as well as assessing their smoking and drinking histories, smoking and alcohol effects and changes after the injury, and potential use and effects of AChEIs. Results concerning alcohol consumption, possible changes in alcohol and tobacco use after TBI were not reported in article IV. The patient groups were divided into subgroups, smokers and non-smokers, according to whether the participants were smokers at the time of injury. The duration of formal education was inquired as complementary background information.
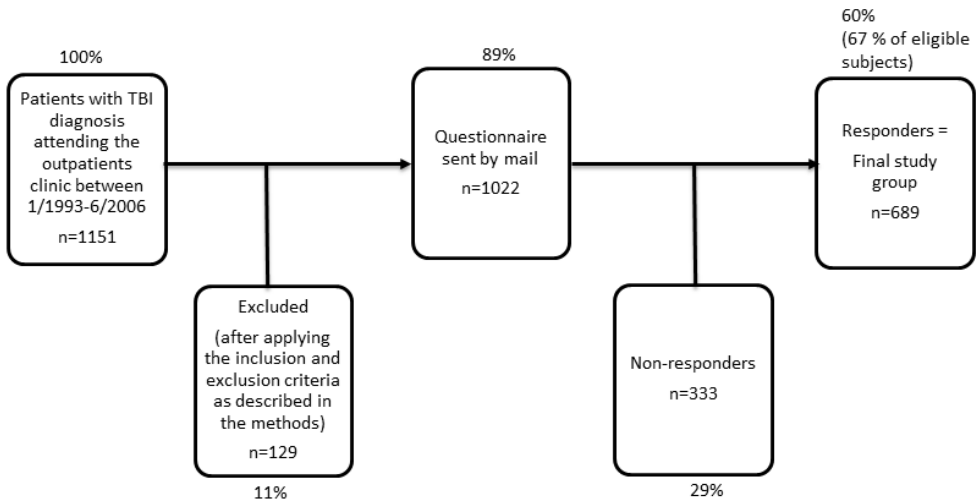
Figure 5. Flowchart of Study 3.

4.3   PET image acquisition and processing, tracer modelling

Study I (Original article I-II)

In these PET studies, we focused on the cholinergic neurotransmitter system; this was studied with [^{11}C]MP4A, which is a lipophilic acetylcholine analogue with high AChE specificity.

The radiochemical synthesis and quality control of ^{11}C-MP4A have been described in detail elsewhere (Kaasinen et al. 2002). The PET imaging was performed with a GE Advance PET scanner (General Electric Medical Systems, Milwaukee, WI). Cannulas were placed in an antecubital vein for ^{11}C-MP4A injection. At the start of the scan, ^{11}C-MP4A was injected intravenously as a steady bolus during 80 s, and the radioactivity was measured in the consecutive series of 22 frames with a total scan duration of 60 min.

All subjects were scanned in a Philips Gyroscan Intera 1.5 T CV Nova Dual scanner (Philips, Best, the Netherlands). Axial T2 and coronal FLAIR sequences were obtained to visualize the TBI lesion load and in controls, to exclude any brain pathology. A whole brain T1-weighted 3D data set was acquired in the transverse plane.

PET and MRIs were processed with in-house software (http://www.turkupetcentre.net) and Statistical Parametric Mapping version 2 (SPM2) (Wellcome Department of Cognitive Neurology, London, UK; http://www.fil.ion.ucl.ac.uk/spm) running on Matlab 6.5 (The MathWorks, Natick,

MA, USA). First, the images were movement corrected; from 26 frames frame 14 was used as the reference to which other frames were individually realigned, except for the first 10 frames where a summated image over the frames was used to calculate the transformation and the obtained parameters were applied to each frame separately. For the spatial normalization of PET images, a ligand-specific template for ^{11}C-MP4A was generated using MRI-aided procedures as described previously (Meyer et al. 1999). In conclusion, the individual MRIs were first co-registered to summated ^{11}C-MP4A images and then the MRIs were normalized to a T1-weighted MRI template in the MNI (Montreal Neurological Institute) Space. Using the acquired transformation parameters, ^{11}C-MP4A summated images were normalized and the mean of these normalized images was averaged with its mirror copy (left-right flipped image). This symmetrical mean ^{11}C-MP4A summated image was smoothed using an 8 mm Gaussian kernel, to create a ligand-specific template. Individual dynamic PET images were spatially normalized to the ligand-specific template with parameters estimated from summated ^{11}C-MP4A images in native space (Virta et al. 2011).

Co-registration of MR images to the summated PET image in native space was also written in the MNI space with the parameters estimated from the individual's summated PET image to the ligand-specific template. ROIs were defined from the average of these normalized MR images using Imadeus software (version 1.50, Forima Inc., Turku, Finland), and the location of ROIs was visually checked to match each normalized PET image. ROIs were delineated bilaterally on the frontal cortex (FCX), lateral temporal cortex (LTC), medial temporal lobe (MTL), inferior part of the parietal lobe (LPI), occipital cortex (OCC), posterior cingulate (PC) and putamen. The ROIs were then transferred to the corresponding planes in the normalized dynamic PET images and time-activity curves (TAC) for ROIs were calculated.

AChE activity was calculated utilizing the transport-limited reference tissue model for irreversible uptake, in which the k_3 rate constant represents the hydrolysis rate of the tracer in tissue, and thus reflects the local AChE activity. This model is based on the reference tissue compartment model, but here it is assumed that in the reference tissue, the tracer metabolism is fast, and therefore the tracer uptake is limited only by its transport into tissue. The putamen was used as reference tissue. The model was solved for ROI-TACs to calculate regional AChE activity, and for each image pixel separately to produce parametric images representing AChE activity.

4.4 Atlas based morphometry

Segmentation of structural MR images offers a potential way to gain a better evaluation of the disease progression of TBI and to obtain an accurate quantitative assessment of the structural changes occurring during and after TBI. Multi-atlas label

propagation is a commonly used class of automatic segmentation algorithms, in which semi-automatically or completely manually annotated atlases are individually aligned with the unsegmented target image (Heckemann et al. 2006). Atlas propagation techniques are based on the accurate registration of the atlas and unsegmented MR image to define the spatial transformation of the atlas labels into the target space. The presence of pathology complicates this approach as the target image differs from the atlas image. Several different techniques have been introduced to overcome this problem such as Multi-Atlas Propagation with Enhanced Registration (MAPER) (Heckemann et al. 2011), patch-based label fusion (Coupé et al. 2011) and state-of-the-art label fusion (Wang et al. 2013). Atlas-based segmentation can be further improved by including intensity information from the unseen image through a Gaussian mixture model (GMM). The resulting optimization problem is often solved using expectation–maximization (EM) (Lötjönen et al. 2010) or graph cuts (van der Lijn, den Heijer et al. 2008). However, in cases of TBI, these approaches have been shown to be inconsistent and there is a broad range of pathological changes which these techniques have not been able to handle adequately due to substantial and persistent errors of alignment.

The above-mentioned issues have been the impetus to develop an automated and robust segmentation method that would provide an accurate measurement of various brain structures in MR images in the presence of severe pathologies. One of the methods striving to achieve this goal combines several of the above-mentioned techniques. It incorporates the best features of state-of-the-art atlas-based segmentation tools into a new framework, Multi-Atlas Label Propagation with Expectation–Maximization based refinement (MALPEM), by building on MAPER while adding the benefits of joint label fusion and an intensity-based refinement using EM. Furthermore, to adjust this method to deal with abnormal brain configurations, one can apply a prior relaxation scheme that corrects anatomical atlas priors in regions where accurate alignment of the images is impossible due to missing brain tissue or severe deformation. (Ledig et al. 2015). This automated atlas-based MRI segmentation method has been proven to be robust in morphometric analysis of TBI cohorts (Ledig et al. 2017, Ledig et al. 2015). In addition, this method is able to stratify TBI patients with favorable outcomes from those experiencing non-favorable outcomes with almost 70% accuracy.

4.4.1   MR image acquisition and processing

Study 2 (Original article III)

In the MR images examined in this project, we focused on structural T1-weighted MR images, which allow an evaluation of volumetric changes. The analyses

concentrated on cholinergic system structures i.e. longitudinal changes in cholinergic structures after TBI were investigated with the above mentioned, fully automated, atlas-based MRI morphometry. The method is based on dependable segmentation of images into many anatomical regions, rather than tissue classes. Automated brain morphometry provides informative assessments based on either single time point MR images (structural volumes) or longitudinal MR images (structural atrophy). In this project, we focused on longitudinal changes i.e. structural atrophy.

All subjects were scanned with the Siemens Verio 3T system to acquire T1w MR images extracted from a Magnetization-Prepared Rapid Gradient-Echo (MPRAGE) sequence with the following parameters: TR 2300 ms, TE 2.98 ms, TI 900 ms, flip angle 9°, matrix size 256↦249↦176 and an isotropic voxel size of 1.0mm ↦ 1.0mm ↦ 1.0 mm, sagittal slices, using Prescan Normalizer, 2D distortion correction and a standard 12 channel head coil.

Brain segmentation analyses based on T1-weighted MR images were conducted in Imperial College London, UK. The method of volumetric analyses has been described previously (Ledig et al. 2015). First, the images were preprocessed correcting for intensity inhomogeneities with the N4 bias correction algorithm (Tustison et al. 2010). Images were then brain extracted using Pincram (Heckemann et al. 2015), an iterative, atlas-based method that was developed with a focus on robustness. Each image was then segmented individually using MALPEM (Ledig et al. 2015). Thirty manually annotated neuromorphometric brain atlases were employed as brain atlases; these were provided by Neuromorphometrics, Inc. under the terms of academic subscription (http://neuromorphometrics.com/). In MALPEM, the 30 manually annotated brain atlases were propagated to the image to be segmented based on transformations calculated with the robust registration approach, MAPER (Heckemann et al. 2011). The propagated atlases were subsequently fused into a consensus probabilistic prior estimate by a locally weighted fusion approach based on the Gaussian-weighted sum of squared distances (GSSD). The GSSD was calculated on images that were intensity normalized using a linear rescaling approach (Nyúl et al. 1999). The time-point specific probabilistic segmentation output and the intensity normalized, brain extracted images were then employed to perform the consistent longitudinal segmentation (MALP-EM4D) (Ledig et al. 2015). MALPEM4D is an approach that employs spatially and temporally varying coupling weights between time-points to obtain temporally consistent segmentation estimates. In the context of TBI, broad structural changes could be expected between both imaging time points. Thus, the weighted differential bias field correction procedure was used. All brain masks and segmentations results were visually reviewed to ensure reasonable accuracy, taking into consideration the pathology.

All the study subjects were analyzed cross-sectionally in the acute stage of the TBI and longitudinally employing the follow-up image acquired during the chronic state of TBI. The whole data set included 134 atlas labels. When right and left cortical and non-cortical corresponding regions were merged, the dataset included 63 anatomical structures which had symmetric counterparts in the opposite hemisphere, i.e. a total of 126 labels. The remaining eight unpaired structures are as follows: 3rd and 4th ventricle, brain stem, CSF, optic chiasma, cerebellar vermal lobules I–V, cerebellar vermal lobules VI–VII, cerebellar vermal lobules VIII–X. Thus, there were 71 structures identifiable with this method, but our analyses included only those regions that are known to be connected to the cholinergic system as well as to sustained attention (16 cortical structures and 4 non-cortical regions). The basal forebrain cholinergic innervation of the medial prefrontal cortex is crucial for cognitive performance and as a result, the analyses included the following: cholinergic nuclei in the basal forebrain region and brainstem, important cholinergic afferent non-cortical projections (ventral diencephalon, thalamus) and frontal cortical projections. We also included in the analyses 3 large sum-structures, in an attempt to estimate the association of diffuse atrophy with CANTAB results; these sum-structures were: cortical grey matter (CGM), cerebral white matter (CWM) and lateral ventricles. In the longitudinal analyses, structural volumes of all 114 subjects and 17 controls were extracted based on the respective MALP-EM4D segmentations. Atrophy rates were calculated using the logarithmic transform as $\Delta v(t^1, t^2) = 100\% \ln(vt^2/vt^1)$. It should be noted that the atrophy rate and the volume change are used here interchangeably, which means that a positive atrophy rate indicates an increase in volume.

Table 2. The structures subjected to longitudinal volume change analysis.

Cortical structures:	Non-cortical structures:	Sum structures:
1) Anterior cinculate gyrus	1) Thalamus	1) Cortical grey matter
2) Anterior orbital gyrus	2) Ventral diencephalon	2) Cerabral white matter
3) Frontal operculum	3) Basal forebrain	3) Lateral ventricles
4) Frontal pole	4) Brainstem	
5) Gyrus rectus		
6) Lateral orbital gyrus		
7) Medial frontal cortex		
8) Middle frontal gyrus		
9) Medial orbital gyrus		
10) Precentral gyrus medial segment		
11) Superior frontal gyrus medial segment		
12) Opercular part of the inferior frontal gyrus		
13) Orbital part of the inferior frontal gyrus		
14) Posterior orbital gyrus		
15) Superior frontal gyrus		
16) Supplementary motor cortex		

4.5   Neuropsychological tests

Study 2 (Original article III)

The neuropsychological test we applied was the widely used Cambridge Neuropsychological Test Automated Battery (CANTAB). CANTAB provides sensitive, explicit and objective measures of cognitive function, correlated to neural networks (Sahakian et al. 1988). The whole test battery includes tests of working memory, learning and executive function; visual, verbal and episodic memory; attention, information processing and reaction time; social and emotion recognition, decision making and response control. Our analyses included tests measuring sustained attention and processing speed: motor screening test, rapid visual processing and simple reaction time.

4.6 Questionnaire

Study 3 (Original article IV)

A questionnaire was designed to evaluate the association between smoking and TBI. It included 10 closed questions, which provided a subjective evaluation of the outcome of TBI as well as details of the participants' smoking and drinking histories, smoking and alcohol effects and changes after the injury, and use and effects of AChEIs. Education level was inquired as background information.

4.7 Statistical analyses

All the statistical analyses were conducted with SAS System for Windows (V9.1-4, SAS Inc., Cary, NC, USA).

Study 1 (Original articles I-II)

To test for the differences in demographic variables, Chi-Square tests were applied for categorical variables and t-test for continuous variables. One-way analysis of variance (ANOVA) was used in the comparison of regional AChE activity between the TBI subjects and control subjects. In region of interest (ROI) analyses, k3 values of the first scan, without medication were compared between the patient groups with ANOVA. Group comparisons of non-respondent and respondent groups with respect to the baseline scan and the scan while receiving rivastigmine therapy, were performed with analysis of variance of repeated measurements. P-values less than 0.05 were interpreted as statistically significant.

The statistical analyses of voxel-wise AChE activity were performed with SPM2. Prior to the statistical analyses, the parametric PET images were smoothed with a 14 mm FWHM Gaussian kernel. A binary mask including neocortical gray matter was applied for the SPM analyses. The cerebellum and striatum were excluded because in these regions, the tracer uptake is too rapid for the assumptions of the kinetic model to be met. The ROI mask was generated with a WFU PickAtlas Tool. After correction for multiple comparisons, group differences were tested with one-tailed t-contrasts, and cluster-level p-values below 0.05 were regarded as significant. The SPM results were localized with MSU Space Utility (http://www.mrc-cbu.cam.ac.uk/Imaging/Common/mnispace.shtml).

Study 2 (Original article III)

To test for the differences in demographic variables, Chi-Square tests were applied for categorical variables and Mann-Whitney U-test for continuous variables. The non-parametric Mann-Whitney U-test was used in the comparison of the volumetric changes in TBI patients and controls. Spearman's rank correlation coefficient was fitted to evaluate whether the atrophy rate in selected structures of the TBI subjects associated with CANTAB results. In further regression analyses, linear regression models were applied to evaluate whether the atrophy rate of selected structures could explain the results of CANTAB tests. Correlation analysis results includes correlation coefficients as Spearman rank correlation and R squares from linear correlation analysis. The F values are also partly presented as a part of correlation analysis results. F test tests the null hypothesis that all of the regression coefficients are equal to zero. The F value is the ratio of the mean regression sum of squares divided by the mean error sum of squares. A higher F-statistic means that the correlation model explains much more of the variation per parameter than there is error per remaining degree of freedom. F test can be considered as the overall significance of the regression model.

Study 3 (Original article IV)

Categorical variables (gender, education, smoking, PTA) differences were analysed with Chi-Square tests or, if necessary, Fisher's exact tests. Continuous variables (age, GCS, GOSE) were analysed with one-way ANOVA, and the Kruskal-Wallis test was applied to test the coherence of the analysis. Further analyses were done with logistic regression analyses. The odds ratios (OR) with 95 % confidence intervals (95 % CI) were also calculated. The level of significance was $p < 0.05$ in all analyses.

4.8 Ethical aspects

Study 1 (Original articles I-II)

Written informed consent was obtained from all patients participating in the study. The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland.

Study 2 (Original article III)

All study subjects gave their informed consent for participating in the study, and the study protocol was accepted by the Ethical Committee of the Hospital District of

Southwest Finland. Specifically, a written informed consent was obtained from all subjects, or in cases where the subject was unable to give the consent, from his/her proxy.

Study 3 (Original article IV)

An information sheet and a consent form were included in the posting, the latter to be returned with the questionnaire. The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland.

5 Results

5.1 Alteration in AChE activity after TBI (Study 1, Original article I)

Differences in AChE activity between TBI patients and healthy controls were characterized in the cross-sectional design. AChE activity was measured with PET using ^{11}C-MP4A. The subjects with TBI and controls did not differ from each other with regard to either gender or age. The injury severity of TBI subjects according to the duration of PTA was from moderate to severe. 17 TBI patients and 12 healthy controls were scanned with PET without rivastigmine medications in the first part of the study 1. In the SPM analyses, the AChE activity was significantly lower in subjects with TBI as compared to controls in several areas of the neocortex. The most remarkable lowering of AChE in TBI subjects in comparison with controls was noted in the parieto-occipital regions, as presented in the Figure 6.
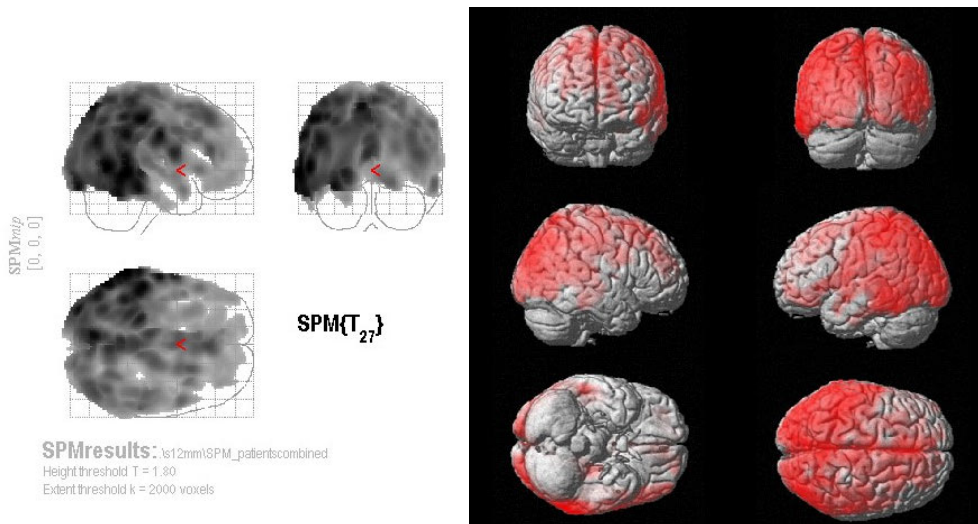

Figure 6. Brain regions where 11C-MP4A is lowered in TBI patients in comparison to to healthy controls. The red scale indicates the level of statistical significance of the difference in AChE activity (solid red indicates the most significant difference). Modified from original article I, figure 1.

In parallel to the SPM analyses, according to the ROI analysis, the AChE activity was broadly lower in subjects with TBI when compared to controls (-5.9 to -10.8 %, p = 0.053 to 0.004). The ROI analysis included 1) frontal cortex (including anterior cingulate and lateral frontal cortices), 2) posterior cingulate, 3) medial temporal cortex (including amygdala, hippocampus and parahippocampal regions), 4) lateral temporal cortex (including inferior, medial and superior temporal gyri), 5) inferior part of parietal lobe (including angular and supramarginal gyri), and 6) occipital cortex. AChE activity was significantly lower in frontal cortex (lateral frontal cortex, anterior cingulate), parietal inferior cortex, posterior cingulate, lateral temporal cortex and occipital cortex. The medial temporal cortex was the structure where the difference between controls and TBI subject was not statistically significant (p=0.053), but even in that structure, it was possible to discern a trend towards lower AChE activity as presented in Figure 7. The most remarkable differences AChE activity between controls and patients were noted in posterior cingulate (-10.5%) and occipital cortex (-10.8%).
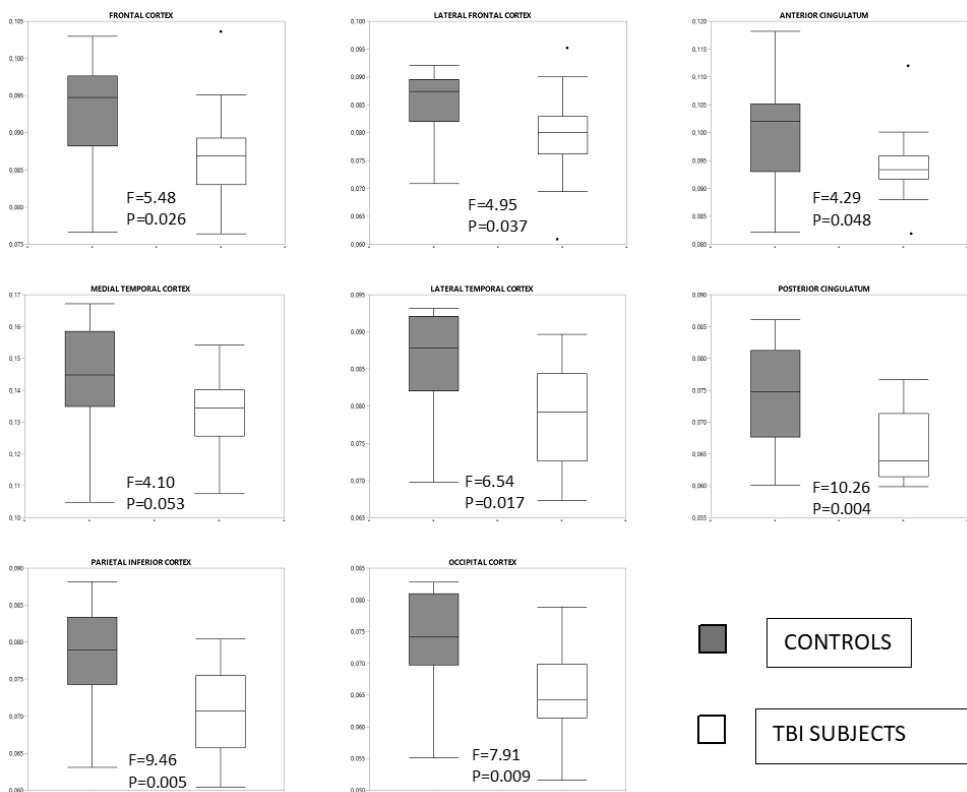

Figure 7. AChE activity in selected ROIs between controls and TBI subjects.

5.2 The association of AChE activity with the response to rivastigmine (Study 1, Original article II)

The difference of cholinergic function between the two TBI patient groups was assessed with ^{11}C-MP4A-PET, which reflects the activity of the AChE enzyme. The sub-division of patients was done according to their response to rivastigmine i.e. into responders (n=10) and non-responders (n=7). The moderate-to-severe TBI subjects were PET scanned twice: without medication and after a four-week treatment with rivastigmine 1.5 mg b.i.d. Differences of AChE activity were investigated at two time points, i.e. the primary point without medication and subsequently with medication. Patient groups did not differ with respect to either gender (p=0.63) or age (p=0.85) nor by injury primary severity (GCS p=0.42, PTA duration p=0.43).

At baseline, without medication, significantly lower AChE activity was detected bilaterally in the frontal cortex in responders in comparison with non-responders as shown in the Figure 8. ROI based analysis showed that the difference was most evident in the lateral frontal cortex (-9.4 %, p = 0.034) and anterior cingulate (-6.0 %, p = 0.049).
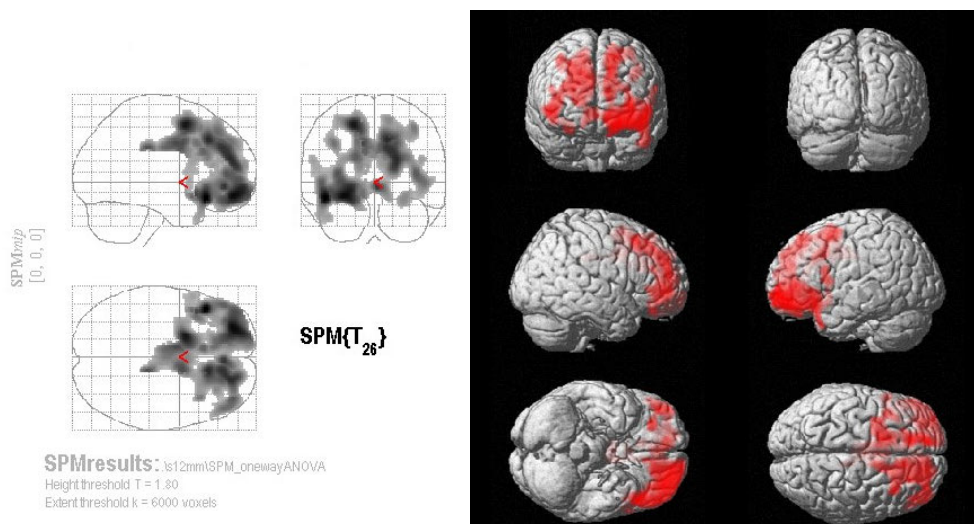

Figure 8. Statistical parametric mapping (SPM) analysis of the drug-naïve condition shows lower AChE activity in frontal structures in responders as compared to non-responders. Modified from original article II, figure 1.

Rivastigmine evoked a marked reduction of AChE activity throughout the cortex in both responders and non-responders as this drug inhibits AChE. In SPM analyses, AChE activity was lowered broadly in the neocortex as presented in Figure 9. ROI

analyses showed that in the responders, rivastigmine induced the most marked reduction in AChE activity in the anterior cingulate (-10.2 %, p = 0.021), lateral temporal (-11.6 %, p = 0.023), and occipital (-11.3 %, p=0.038) cortices. In the non-responders, the decline in AChE activity was most marked in the lateral frontal (-10.2 %, p = 0.043) and posterior cingulate (-9.8 %, p = 0.031) cortices.

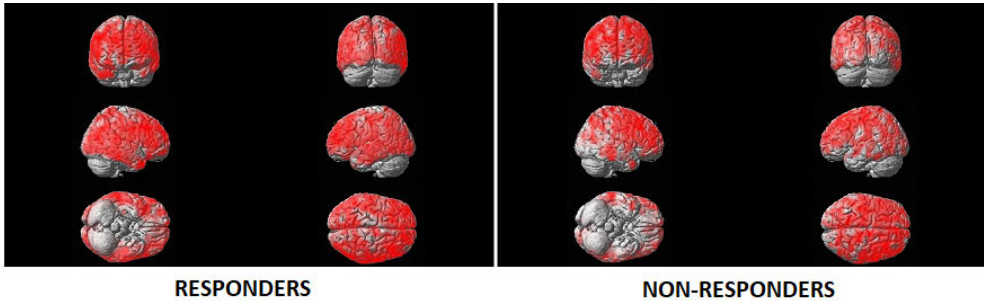**RESPONDERS**   **NON-RESPONDERS**

Figure 9. The SPM analysis of post-treatment scans of TBI subgroups reveals a significant lowering of the AChE activity widely in the neocortex after rivastigmine treatment as compared to each group's baseline.

The SPM or ROI analyses of post-treatment scans detected no significant difference in the level of ^{11}C-MP4A binding between the responders and non-responders. However, in ROI analyses, there was a trend towards a lower average AChE activity in responders versus the non-responders, but differences were not statistically significant; this was seen most distinctly in lateral frontal cortex (see Figure 10).
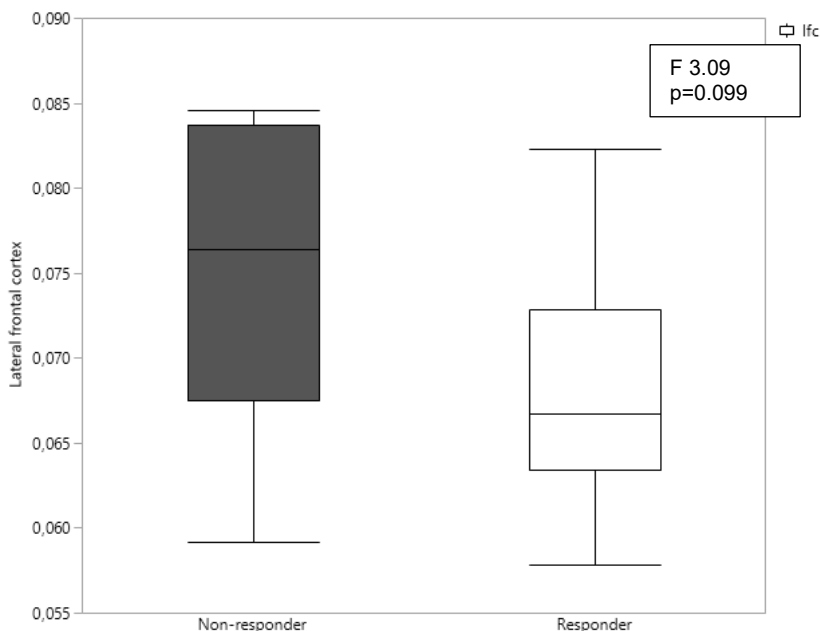
Figure 10. Lateral frontal cortex ^{11}C-MP4A activity of post-treatment PET scans between patient groups.

5.3 Atrophy rate of cholinergic structures and correlation between the atrophy rate and neuropsychological tests measuring attention (Study 2, Original article III)

The association between the atrophy in cholinergic brain structures after TBI and neuropsychological outcome was investigated with atlas-based morphometry and CANTAB tests. Analyses included mild to severe TBI patients (n= 120) and also a control group (n=12) which had been matched to the patients by age and gender. The analyses included 16 frontal cortical structures, 4 non-cortical structures and 3 sum-structures. When examining significant longitudinal volume changes, patient group atrophy rates were compared to the corresponding values in control patients. In 9 structures, atrophy rates did not differ significantly between patients and controls and these structures were excluded from further analyses. The excluded structures were various frontal cortical regions and from non-cortical structures, we omitted basal forebrain.

The possible association between atrophy rate and CANTAB results (MOT, SRT, RVP) were initially assessed with the Spearman correlation coefficient. This analysis included atrophy rates in 8 frontal cortical structures, 3 non-cortical structures and 3 sum-structures. The detected correlations were overall rather weak, but statistically

significant in five frontal cortical structures, all being non-cortical structures and sum-structures. The strongest correlation was observed between the simple reaction time and supplementary motor cortex atrophy rate (Spearman correlation -0.36, p<.0001).

Linear regression was used to investigate the correlation of changes in the longitudinal volumes of selected structures with the results from the CABTAB test. It was evaluated whether the atrophy rate of selected structures could predict the results of the CANTAB tests. Those structures which did not show any association according to the Spearman correlation coefficient were excluded, thus the data set included 5 frontal cortical structures brain stem, ventral diencephalon, thalamus and 3 sum-structures: cortical grey matter, cerebral white matter and lateral ventricles. Table 3 presents results from those anatomical structures which had a significant linear correlation with any of the CANTAB tests. The analyses were done with different group separations: with all GOSE classes/whole patient group and then after excluding GOSE 8. The exclusion of GOSE 8 (no disability after TBI) reduced the study population from 114 to 80, but also reduced the number of subjects which did not show any longitudinal volume change and therefore it improved the correlation.

Table 3. Relationship between atrophy rate and CANTAB results by linear regression. Modified from original article III, Table 5.

	CANTAB		Precentral gyrus medial segment	Superior frontal gyrus medial	Opercular part of the inferior frontal gyrus	Supplementary motor cortex	Thalamus	Cortical grey matter
ALL GOSE CLASSES	MOT	R sq	0.01	0.07	0.08	0.16	0.03	0.08
		p-value	0.0007	0.0035	0.0019	<.0001	0.0805	0.0019
	SRT	R sq	0.03	0.02	0.03	0.06	0.01	0.02
		p-value	0.0537	0.1322	0.0672	0.0078	0.2939	0.1548
	RVP	R sq	0.00	0.02	0.00	0.06	0.00	0.01
		p-value	0.4750	0.1182	0.4825	0.0121	0.3730	0.3210
GOSE 3-7	MOT	R sq	0.15	0.11	0.16	0.26	0.05	0.15
		p-value	0.0003	0.0024	0.0002	<.0001	0.0496	0.0005
	SRT	R sq	0.03	0.02	0.06	0.07	0.01	0.01
		p-value	0.1080	0.1968	0.0321	0.212	0.400	0.1974
	RVP	R sq	0.00	0.04	0.00	0.09	0.00	0.01
		p-value	0.4092	0.0983	0.2874	0.0107	0.1413	0.3276

The most significant correlation was found between supplementary motor cortex and MOT (All GOSE classes: R-square 0.16 p<.0001, GOSE 8 excluded: R square 0.26, p<.0001). Figure 11 shows linear regression scatter plots between frontal cortical structures and MOT (the figure shows only these structures which exhibited a statistically significant correlation), analyzed as either the whole patient group or as GOSE 3-7 group. Cortical grey matter was the only sum structure to display a significant correlation between post-traumatic diffuse atrophy and MOT (All GOSE classes: F-ratio=10.2, p=0.0019, GOSE 8 excluded: F-ratio=13.2, p=0.0005).
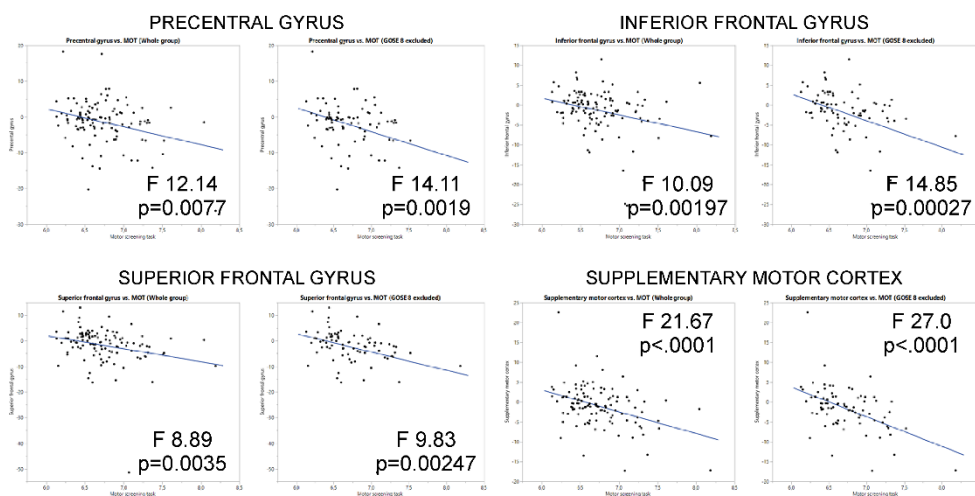

Figure 11. Linear regression between frontal cortical structure and the results in a motor screening task assessed either as the whole study group or as a subgroup analysis with GOSE 8 excluded.

5.4 The effect of smoking on TBI outcome (Study 3, Original article IV)

Data was collected from the all severity TBI cohort with a specially designed questionnaire. In all, 42.9 % of subjects were smokers at the time of TBI, the remaining 57.1 % were non-smokers. Smokers were more often men (p<0.001), younger at the time of injury (p<.0001) and had less education (p=0.008). Non-smokers and smokers did not exhibit any differences in their TBI severity (with GCS p=0.14 or with PTA p=0.06). Younger subjects had more severe injuries (both GCS and PTA p<.0001).

The univariate analysis investigated the effect of age at TBI, smoking, education and gender on the TBI outcome for each variable separately. Outcome did not differ between the sexes. In the analysis of demographic factors, it was observed that younger patients had more severe injuries, thus also the outcome differed

significantly according to age at TBI (p<.0001). No significant associations were found between education and outcome (by GOSE, p=0.76) but there was a significant difference in the outcome of TBI as assessed by GOSE between non-smokers and smokers (p=0.02).

Smoking is strongly associated with age, education and gender as was discerned in the demographic analyses. These factors may have also a relationship with TBI outcome and therefore in subsequent analyses, logistic regression was used to study if there was a possible independent association between smoking and TBI outcome. In our study material, the younger patients suffered more severe primary injuries and also smokers were younger than non-smokers, thus the PTA classes were analysed separately to control for the effect of these associations. Another possible way to manage this phenomenon would have been to exclude subjects aged under 55 years at the time of injury which would have reduced the sample size from 689 to 120. Figure 10 demonstrates the effect of exclusion of that portion of the subjects. After this limitation to the study group, the severity of the injury did not differ by age, but also there was no longer any difference detected between smokers and non-smokers in their outcome of TBI.
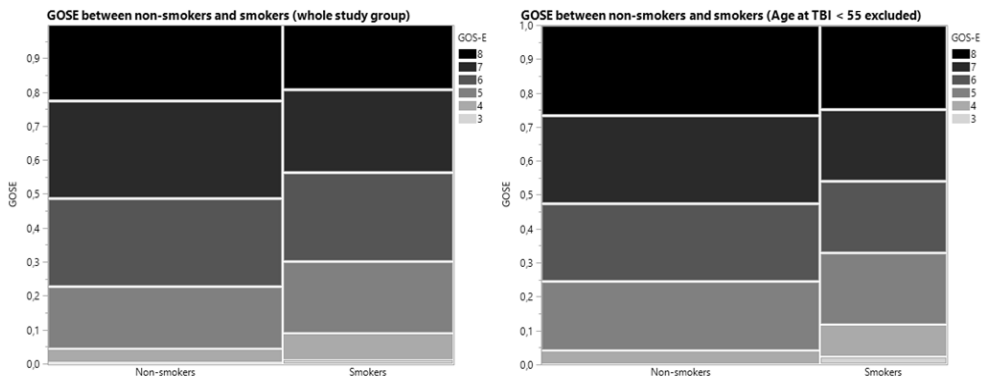

Figure 12. GOSE classes between non-smokers and smokers. Histogram 1. Whole group in the analyses. Histogram 2. Subjects aged <55 excluded as the TBI severity differed by age such that younger individuals had suffered more severe injuries.

In the multivariate analysis, age at TBI, education, gender, and smoking history were evaluated as predictors for TBI outcome. Education was classified into three categories: 0 to 9 years, 10 to 12 years, and 13 years or more. Age at the time of TBI was grouped into three classes: 18 – 25 years, 26 – 40 years, and 41 - 55 years; subjects who were ≤ 17 years or ≥ 56 years at the time of TBI were excluded from the logistic regression analysis as smoking is common in this latter age range. Logistic regression

analyses were performed to examine each predictor's relationship to the outcome, with and without adjustment for the effects of all of the other predictors.

Logistic regression analysis did not identify any significant difference in TBI outcome between smokers and non-smokers. Furthermore, age in this selected age group (from 17 to 55 years at the time of TBI) did not seem to have any association with outcome. Only in the moderate and severe injury classes as assessed by PTA, was there a weak trend that the younger subjects had a better recovery, and in only one analysis, i.e. between 41-55 and 18-25 age groups in the severe injury class was this difference statistically significant (p=0.003). Neither education nor gender exhibited any significant associations with outcome.

6 Discussion

6.1 AChE activity is altered chronically after TBI

We detected widely lowered cortical AChE activity in subjects with TBI as compared to controls. Our finding is in parallel to earlier findings from experimental and post-mortem studies which have demonstrated alterations in an important enzyme in the CNS cholinergic system after TBI (Murdoch et al. 1998, Dixon et al. 1996, Donat et al. 2008). Experimental TBI studies have shown that there is an unregulated surge of ACh in the acute phase of injury and a chronic decrease of cholinergic neurotransmission later in disease progression (Dixon et al. 1995, Dixon et al. 1996). Our finding of lowered AChE activity at the chronic TBI time point is in agreement with these reports. An experimental study has also claimed that AChE activity is increased in the acute phase after TBI (Donat et al. 2007). In addition, one human study observed that a reduced cholinesterase level in the acute phase was associated with TBI severity and poorer outcome (Zhang et al. 2015). These authors speculated that the increase in the activity of AChE in acute phase could be induced by an elevation in the ACh level and that the later changes in AChE activity might be a compensatory down regulation because of low ACh concentration in chronic TBI as experimental studies have suggested that there is a reduction in the level of ACh during the chronic phase of TBI.

The reason for the lowered level of AChE remains speculative and it is not possible to directly conclude the mechanism from ^{11}C-MP4A PET studies. Nevertheless, an injury to the cholinergic neurons may be a consequence of a direct insult to the central regions containing major cholinergic centres such as ventral forebrain and upper brainstem, which are known to be particularly vulnerable in TBI (Dekker et al. 1991). On the other hand, injury and degeneration of these especially cholinergic nuclei with wide cortical innervations, have been associated with TAI and the secondary injury (Murdoch et al. 2002, Hong et al. 2012). Thus, it is possible that cholinergic dysfunction and the lowered AChE level derive from neuronal loss or a functional deficit of neurons. Experimental studies have suggested that chronic reductions in cortical cholinergic function may be attributable to reduced synthesis of acetylcholine, altered release of acetylcholine due to changes in receptor binding

and signal transduction or actual injury to cholinergic projections (Leonard et al. 1994, Dixon et al. 1994).

Our finding of lowered cortical AChE activity in chronic TBI patients strengthens the theory of altered cholinergic communication after TBI, at least with the diffuse type of TBI. It seems that TBI induces cholinergic dysfunction in cortical regions which are likely to be involved in the cognitive sequelae of TBI. Earlier studies have revealed a relationship between post-traumatic cognitive problems and dysfunction or injury of cerebral cholinergic systems (Arciniegas et al. 2001, Salmond et al. 2005). This is interesting from a therapeutic standpoint, as cholinergic agents might be able to correct cognitive impairments produced by chronic cholinergic deficits. In fact, there are small case reports, case series, open-label studies, and a few randomized controlled studies investigating AChEI, but the results have been partly conflicting and the efficacy rather modest (Silver et al. 2006, Tenovuo et al. 2009).

6.2 AChE activity in the central nervous system and its response to cholinergic medication

Our study demonstrates that AChE activity was significantly lower bilaterally in the frontal cortex when TBI patients who had benefitted from rivastigmine were compared to those who had not benefitted from the drug. At present, there is no approved medication to combat the cognitive sequelae of TBI. Discovery of a chronic hypofunction in the cholinergic system after TBI have led to studies conducted with AChEIs in attempts to reverse these deficits. Several case series, open-label studied and some controlled blinded studies have investigated the effect of AChEIs on the cognitive symptoms associated with TBI (Ballesteros et al. 2008, Whelan et al. 2000, Tenovuo et al. 2009). These studies have been small-scale and the results rather disappointing, although rivastigmine has been claimed to improve working memory, sustained attention and processing speed chronic in TBI patients (Silver et al. 2006, Tenovuo 2005). The disappointing results emerging from the AChEI trials might be partly explained by that only a subgroup of TBI patients seems to benefit from AChEIs (Silver, Koumaras et al. 2006). The finding that responders to rivastigmine had lower frontal cholinergic activity strengthen this theory since we demonstrated that cholinergic activity, at least in the frontal region, is different among TBI patients according to their response to cholinergic medication. Furthermore, it has been suggested that frontal cholinergic innervations are involved in mediating attentional and vigilance demanding tasks (Kozak et al. 2006), and thus the altered frontal cholinergic activity among responders is another piece in the puzzle.

The reason for the lowered AChE activity in the frontal cortex of responders remains speculative. The lowered AChE activity may stem from the dysfunction of

these neurons, either in the form of neuronal loss or functional deficit. Still, it is not likely that the reduction in frontal AChE activity would represent the basis for the therapeutic effect of AChEIs. The severity of TBI between patient groups did not differ, but the outcome by GOSE between groups was only marginally insignificant (p=0.089). Thus, it is possible that there are some other factors which we were not able to take into account, but which were potentially exerting an effect on outcome. Our study included patients with mainly a diffuse type of brain injury, which should diminish the possibility of regional atrophy or differences in the atrophy rates between patient groups. However, it is possible that the atrophy of frontal structures may be partly involved in the diminished AChE activity in frontal region of responders to rivastigmine, as the frontal regions are highly innervated with cholinergic neurons. One could speculate that the main reason for the difficulties to identify treatment strategies for cognitive sequelae of TBI are related to TBI's highly heterogeneous pathophysiology.

AChEI treatment reduces AChE activity, confirming earlier PET studies conducted in patients with Alzheimer disease (AD) (Shinotoh et al. 2001). Here, AChE activity was lowered in both patient groups compared to the post-treatment scan. However, the reduction was rather slight (around 10%) probably due to the low dosage of rivastigmine as the extent of the inhibition has been clearly higher in earlier AD studies conducted with the same PET tracer (around 30%) using higher rivastigmine doses (Kaasinen et al. 2002). The level of inhibition was similar between the patient groups regardless of differences in the primary scan. Nevertheless, it was still possible to discern a trend towards lower AChE activity, especially in lateral frontal cortex of responders, as compared to non-responders; this is shown in **Figure 8**, although this difference was no longer statistically significant.

6.3 Atrophy in brain cholinergic structures after TBI is associated with the neuropsychological outcome

We utilized an atlas-based morphometry technique to reveal that atrophy in frontal cortical structures after TBI was correlated with the cognitive outcome of TBI. Our main goal was to determine whether atrophy in cholinergic structures would be particularly associated with cognitive outcome. Therefore, from the neuropsychological test pattern tests, we selected those evaluating sustained attention, a skill that is mainly monitored by the cholinergic system (Kuo et al. 2007). We did detect a correlation between CANTAB tests measuring sustained attention and atrophy rates in selected brain structures, especially with frontal cortical structures. This is consistent with an earlier study where subjects with a ventro-medial frontal injury coped poorly in a test measuring guiding of attention (Vaidya

et al. 2015). The most significant correlation was found between supplementary motor cortex and a motor screening task with a 22 % coefficient of determination and in the subgroup analysis excluding GOSE class 8, this value was elevated to 27 %. Thus, one could estimate that about one quarter of the variation in the results of the MOT test can be explained with atrophy rates in this specific frontal cortical structure. However, causality between these factors still remains to be proven.

The analyses included all frontal cortical structures in the data set, 16 structures; of these, atrophy rates in 4 cortical structures displayed a significant linear correlation with the MOT results. Furthermore, a non-cortical cholinergic structure, thalamus, exhibited a linear association with the MOT results, but only in the subgroup analysis excluding GOSE class 8. The volume change of cortical grey matter from sum structures representing volume changes in large brain structures, in other words diffuse atrophy, did show a clearly significant correlation with MOT results. However, most of the other correlations were insignificant. The study group consisted mainly of individuals with mild and very mild TBI, who do not reputedly display any remarkable brain atrophy and in whom the cognitive outcome is rather good, and this is likely to have an impact on our results. The findings that the correlations were higher in the subgroup analysis which excluded completely recovered subjects, favor this assumption. Inclusion of only moderate and severe injuries or analyses by injury classes separately, however, would have notably reduced the numbers of study subjects and substantially weakened the statistical power of the analysis.

Volume changes in lateral ventricle or cerebral white matter did not show any correlation to CANTAB tests, unlike the situation with the volume change in cortical grey matter. It has been stated that frontal cortical regions are especially crucial in attentional tasks (Vaidya 2015, Tobyne et al. 2017) and possibly the cortical grey matter volume is intrinsically associated with success in tasks requiring attention. Slightly unexpectedly, atrophy in non-cortical cholinergic structures was also not associated with CANTAB results. The cholinergic projections arising from basal forebrain and brainstem nuclei have been claimed to be involved in attentional performances (Sarter et al. 2006). However, with the method used here, it is not technically possible to measure volume changes in the levels of these small structures. We were forced to use larger sum-structures for these regions and therefore the negative result may partly derive from the limitations of our morphometric method. On the other hand, thalamus, which has a sufficiently large volume to measure with the method used, did reveal a weak, but statistically significant association with MOT.

In the linear regression analysis, only MOT from CANTAB tests displayed a linear regression to atrophy rates except in supplementary motor cortex, which also correlated significantly with SRT and RVP. One possible reason for this finding may

be that SRT and RVP do not solely measure attentional capabilities and consequently brain regions involved in these test performances are likely to be broader than frontal cortical structures and their cholinergic structures. In particular, SRT measures eye-hand coordination whereas RVP demands co-operation with visual cortices in the occipital region, which were not included in our analyses.

Earlier reports with VBM have shown region selective, but quite varying degrees of atrophy in the frontal and temporal cortices, basal forebrain, amygdala, hippocampus, thalamus, corpus callosum, putamen, and precuneus (Gale et al. 2005, Salmond et al. 2005, Warner et al. 2010, Bendlin et al. 2008, Kim et al. 2008, Newcombe et al. 2011). The regions with the most significant atrophy vary at least partly in the different studies, probably due heterogeneity in the study populations and TBI severity, but thalamus and frontal cortical structures invariably appear to be involved, as found in our study. Furthermore, Gale et al. also found a strong correlation between a reduced grey matter concentration in the frontal and temporal regions and lower scores in tests of attention, and Newcombe et al. noted that in different frontal structures, VBM abnormalities such as impaired decision-making were associated with performance specific cognitive domains. These reports are in agreement with our findings of an association between the change in frontal volume and the performance in attention demanding tasks. Apart from these studies, there are only few reports on the association between region-specific volume loss and neuropsychological outcome, instead, most of the studies in this field have investigated the neuropsychological outcome and its association with diffuse brain atrophy (Sidaros et al. 2009, Ding et al. 2008).

6.4 Smoking does not associate with outcome after TBI

The main goal of this study was to evaluate if a patient's smoking history would exhibit any association with the outcome from TBI according to GOSE. Data was collected retrospectively from subjects via a questionnaire. Interestingly, our study was not able to detect any association between smoking and TBI outcome. The presence of strong associations of smoking with many known or suggested predictors of outcome of TBI like lower education, younger age (Mushkudiani et al. 2007), social factors (unemployment) (Yue et al. 2018) and alcohol abuse (Tomko et al. 2017) can lead to biased results. We attempted to take these into account by using adjusted logistic regression to exclude other factors which could impact on TBI outcome. Additionally, in our study population, severe injuries were over-represented in younger subjects and also the smokers were younger. Ignoring these facts would have also led to biased results. Accordingly, we conducted further

analyses according to PTA subgroups, but this also clearly reduced the statistical power of the analyses and also partly invalidated the actual results.

Very few studies have examined the association between smoking and TBI. Two studies have reported that patients with a previous TBI are more frequently smokers than the average population (Ilie et al. 2015, Silva, Belanger et al. 2018), but this study investigated veterans and it is known that smoking among this population is more common than in the general population (Brown 2010). Unpublished data from our project showed that subjects with a poorer outcome more frequently stopped smoking after TBI (p=0.0028). There are two additional studies showing a relationship between TBI outcome and smoking. Silvan et al. stated that smoking was associated with a better functional outcome in comparison with non-smokers (Silva 2016), whereas Durazzo et al. suggested that the cognitive outcome in non-smokers was better than in smokers in a cohort with mild TBI (Durazzo et al. 2013). However, we were not able to detect any such associations.

6.5 Strengths and limitations

This is the first study which has investigated the cholinergic system in human TBI patients directly with PET. There are two reports of changes in the cholinergic system as examined with PET in TBI animal models (Cyr et al. 2015, Parent, Maxime, Bedard et al. 2012). In contrast, human PET studies investigating the cholinergic system have been mainly limited to AD research.

One major limitation concerning the PET studies (Original articles I-II) is the small number of participants, partly because it is a laborious and expensive technique and partly due to the need to expose the subjects to ionizing radiation. Additionally, the strict inclusion and exclusion criteria of the study partly accounted for the small number of study subjects. In studies involving radioactive isotopes, the number of subjects and scans should be kept as low as possible to obtain sufficient information about the hypothesis based on power calculations. As a result, one cannot rule out that a larger material would have revealed differences also in those brain structures which did not yield statistically significant results in the current analysis. Another limitation concerning the PET study is patient selection as the subjects were selected from outpatient clinic patients and therefore the mildest and most severe injuries may be underrepresented, and the results cannot be generalized to all patients with TBI.

One point that was taken into account when planning the rivastigmine PET study was the length of the "wash out" period as some of the responders to rivastigmine were still on the medication at the time of recruitment but they discontinued rivastigmine for four weeks during the study. The duration of rivastigmine treatment before the study recruitment varied from some weeks to

several years. Nonetheless, there is no evidence that rivastigmine would have long term effects on AChE activity (Polinsky 1998). On the contrary, it seems that the inhibitory effect of rivastigmine on AChE and BuChE activity only lasts for a maximum of 12 hours. Furthermore, there is no evidence of drug accumulation, which is consistent with the short pharmacokinetic half-life of rivastigmine (Polinsky 1998). Therefore, it was assumed that our 4-week washout period was long enough. On the other hand, there is no data about the duration of the time period needed by cholinesterase before its activity as determined by PET would return to normal.

The major shortcoming of the rivastigmine PET study is the lack of a systematic evaluation of efficacy by stringent cognitive testing. Additionally, the dose-response with rivastigmine was not studied and the assessment of the drug response lacked blinding. However, most of the patients had participated in an earlier double-blinded trial (Tenovuo et al. 2009), and the reappearance of the former effect was confirmed during this study.

Often with TBI, gross pathological changes in imaging data are observed and this is a challenge to atlas-based morphometry techniques as these approaches are restricted to the labelling of structures that are represented in the reference atlases. In our technique, we tried to take this into account in the methodology, but there is still the possibility for significant segmentation inaccuracies. However, segmentations were visually reviewed to rule out gross failures and this method has been proven earlier to be accurate and robust at the population level (Ledig et al. 2017, Ledig et al. 2015). Therefore, with this limitation in mind, the assumption was made that the anatomical structures had been segmented successfully in the entire study cohort.

Another limitation of the morphometry study is related to the timing of the control MRI, which took place on average 6 months after the primary injury. There are reports that brain atrophy after TBI continues until 11-12 months after the injury (MacKenzie et al. 2002, Brezova et al. 2014), but the most remarkable reduction in brain volume seems to occur between 3-6 months after TBI (Dennis et al. 2016). Based on these results, we assume that it should be possible to detect accurately significant atrophy at 6 months after injury, although it is acknowledged that the extent of volume loss may be even greater at later time points.

One limitation of the questionnaire study is patient selection as most of its subjects had been hospitalized in our referral area and this probably caused some over-representation of subjects who remained symptomatic. On the other hand, it is also possible that the most severe injuries were under-represented as the capability of these subjects to answer to questionnaire would undoubtedly have been impaired. Additionally, cognitive problems and the long time-gap between the TBI and the survey may have influenced the reliability of the results.

In the questionnaire study, we used GOSE as an indicator of outcome of TBI. However, our main goal was to measure cognitive outcome but GOSE does not directly measure cognitive outcome as it is a comprehensive functional ability instrument. Nonetheless, our study design did not make it possible to conduct a detailed neuropsychological assessment and the indicator used to assess the outcome may have been too insensitive to measure the more subtle alterations in certain targets.

6.6   Future directions

Knowledge in the field of pathophysiology, neurotransmission and diagnostics of TBI is growing rapidly, but the treatment is still challenging due to the multifactorial nature of the injury. Certainly, various environmental, anatomical, genetic, and psychological factors contribute to the outcome after TBI. However, multiple lines of clinical and experimental evidence demonstrate that TBI can produce acute and chronic changes in cholinergic systems; these alterations are probably at least partly involved in the development of the cognitive sequelae associated with TBI. It seems that there is a reasonable scientific foundation on which to base investigations and to seek therapies to reverse these neurochemical alterations. Medical therapies targeting the cholinergic system have succeeded to some extent in experimental studies, but in the future, improved studies are needed before these theories can be translated into actual clinical benefit and individualized treatments. The significant pathophysiological and biological variability of TBI represents the main barrier to the development of treatment strategies to combat the cognitive problems experienced by TBI patients. In the future, large randomized controlled studies will be needed to establish whether AChEIs are effective for improving cognition after a moderate or severe traumatic brain injury. One major challenge may be the requirement of identifying biologically distinct subpopulations of TBI subjects; these could provide important insights into tailoring individual treatment strategies. Another important question is the timing of the medication, as all current trials have included patients being treated at chronic time points although there are experimental studies indicating that the cholinergic dysfunction is initiated in the acute phase of the injury. Combining structural and functional or metabolic imaging in TBI patients is still not common, but it would increase the detection of injury in the clinical setting and also help in clarifying the pathophysiology of TBI. In the future, novel, advanced neuroimaging methods together with TBI specific biomarkers may be able to provide more direct evidence of the presence of cholinergic deficits among patients with TBI and finally lead to more individualized therapies and better outcomes.

7   Conclusions

Our aim was to clarify the role of the cholinergic system in mediating the cognitive impairment occurring after TBI. This multimodal brain imaging study revealed alterations in the cholinergic system in frontal cortical structures and changes in structural volumes after TBI and, furthermore, correlated them with the cognitive outcome after the TBI. Additionally, we found that the activity of the cortical cholinergic system function was altered broadly and chronically after TBI. Earlier clinical studies investigating alterations in the cholinergic systems after TBI have been based on post-mortem specimens and structural imaging investigations, providing only indirect evidence of changes in the cholinergic system. At present, this is the first human study which has exploited PET imaging to explicitly reveal the dysfunction in the cholinergic system after TBI. The conclusions based on the results presented in this thesis are as follows.

1.   Our PET study indicated that cortical acetylcholinesterase activity of TBI patients was extensively lowered in comparison to controls which reflects the hypo-cholinergic state in chronic TBI. This provides preliminary evidence that at least the diffuse type of TBI with cognitive sequelae is likely to induce a wide cholinergic impairment.

2.   We observed with PET imaging that acetylcholine activity was significantly lower in the frontal cortex in those patients with TBI who had benefitted from rivastigmine. Potentially the frontal cholinergic hypofunction is essential for the therapeutic effect of central AChE inhibitors, but the reason for the lowered AChE activity in the frontal cortex still remains hypothetical.

3.   MRI volumetric analysis indicated that more extensive atrophy in frontal cortical regions was associated with poorer performance in cognitive tests measuring attention. As the previous human studies have pointed that the cholinergic system is involved in the attentional activity in frontal structures, our finding strengthens the hypothesis that there is participation of the cholinergic system in the cognitive sequelae of TBI.

4. Smoking influences the acetylcholine system via nicotine and nicotinic cholinergic receptors. Previous studies have indicated that smoking changes the nAChRs density and increases the release of ACh. However, we could not find any association between smoking history and TBI outcome.

It is unlikely that deficits in cholinergic systems occur independently although this study concentrated selectively on the cholinergic system. Acetylcholine interacts with other neurotransmitters and their projections and structures are partly overlapping. However, it seems that only the changes in the function of the cholinergic system are consistent with the neuropsychological profile of TBI patients. Our study strengthens the theory that there is a dysfunction and structural damage in the cholinergic systems and that these are involved in the sequelae of TBI and also in the pathophysiological cascade encountered in TBI. In addition, this study supports the hypothesis that there is a hypo-cholinergic state present in the chronic phase after TBI. Finally, this project provides support for the association between cholinergic dysfunction and the cognitive impairment appearing after TBI. Since there is no recognized treatment at the moment to combat the cognitive impairment after TBI, it is crucial to search for possible therapeutic targets for this challenging condition; and cholinergic medication still represents one promising solution.

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Anna Östberg

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