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On the search for translational biomarkers of mild cognitive impairment

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Zusammenfassung

Die frühestmögliche Identifizierung von gefährdeten Patientengruppen und die rasche Einleitung einer Therapie gelten als wesentliche Schritte, um die Heilungschanzen von Demenz zu verbessern. In diesem Zusammenhang ist das Krankheitsbild “mild cognitive impairment” (MCI), welches als prodromales Stadium der Demenz diskutiert wird, von entscheidender Bedeutung. Während die kognitiven Defizite einiger MCI-Patienten nicht über den Bereich des physiologischen altersbedingten Gedächtnisverlusts hinausgehen, entwickeln andere Demenz. Die Frühidentifizierung von MCI-Patienten basiert jedoch nach wie vor auf unsensiblen klinischen Untersuchungen, während die Suche nach spezifischen Biomarkern bisher nicht erfolgreich war.

Indem ich ein Tiermodell von MCI etabliert habe, war ich nicht mit den Nachteilen von Humanstudien konfrontiert und konnte einen translationalen Biomarker für kognitive Defizite, myo-inositol (mIns), identifizieren.

Zu diesem Zweck habe ich das Abschneiden von männlichen BALBc-Mäusen, dem Modellorganismus, mit dem Abschneiden von C57BL/6N (B6N)-Mäusen, der Kontrollgruppe, in zwei etablierten Gedächtnistests, Water Cross Maze (WCM) und Morris Water Maze (MWM), verglichen. In der Tat bestätigten BALBc-Mäuse ihre Rolle als MCI-Modellorganismus, indem sie subtile kognitive Defizite in Form von erhöhten Fluchtlatenzen zeigten, ohne dass die Gedächtniskonsolidierung dauerhaft beeinträchtigt war.

Durch die Kombination der Verhaltenstestungen mit wiederholten *in-vivo* ^{1}H-MRS Messungen, durchgeführt mit einem 9.4T BRUKER MRI Scanner, ist es mir gelungen, kontinuierlich erhöhte mIns Levels in dem dorsalen Hippocampus (dHPC) von BALBc Mäusen als potentiellen Marker von kognitiven Defiziten zu identifizieren.

Die erhöhten mIns Levels in BALBc mice waren assoziiert mit verstärkter Glia Zell Aktivierung, nachweisbar durch erhöhte Werte von Mikroglia und Astroglia Markern in Western Blot Analysen aus Proben vom dHPC. Des Weiteren suchte ich nach zusätzlichen metabolischen Korrelaten der kognitiven Defizite mit Hilfe einer MassSpec-Analyse, welche auf spezifisch veränderte Astroglia Aktivität in BALBc Mäusen hinwies. Der Versuch, Verhaltensänderungen in BALBc Mäusen durch Gabe von Acetylcarnitin hervorzurufen, scheiterte jedoch.

Zusammenfassend ist dies die erste Studie, welche *in-vivo* den Zusammenhang zwischen Astroglia Aktivität und mIns Levels, sowie deren Rolle als potentieller Marker von kognitiven Defiziten aufzeigt.

Abstract

Identifying susceptible patients and initiating the treatment as early as possible is considered as an essential step towards better chances of cure of dementia. In this context, the concept of mild cognitive impairment (MCI), suspected as prodromal stage of dementia, is crucial. Whereas some MCI patients remain within the range of normal age-related memory loss, other progress into dementia. However, the identification of MCI patients is still based on unsensitive clinical examinations, while the search for specific biomarkers hasn't been successful so far.

By overcoming the shortcomings of human studies in the establishment of an animal model of MCI, I succeeded in identifying a translational biomarker of cognitive impairment, myo-inositol (mIns).

For this purpose, I compared the cognitive performance of male BALBc mice, the model organism, and C57BL/6N (B6N) mice, the control strain, in two established spatial memory tasks, Water Cross Maze (WCM) and Morris Water Maze (MWM). BALBc mice confirmed their role as model organism of MCI by showing subtle cognitive deficits in form of increased escape latencies without being consistently impaired in memory consolidation.

By combining behavioral testing with repeated in vivo ^{1}H-MRS-measurements using a 9.4T BRUKER MRI scanner, I was able to identify consistently elevated mIns levels in the dorsal hippocampus (dHPC) of BALBc mice as a potential marker of cognitive impairment at strain level.

The higher mIns levels in BALBc mice were associated with enhanced glial cell activation, reflected by increased markers of microglia and astroglia in Western Blot analysis using specimens from the dHPC.

On the search for additional metabolic correlates of cognitive impairment, MassSpec analysis revealed distinct metabolic profiles within both strains, supporting altered astroglia activity in BALBc mice. Our attempts to reembalance acetylcarnitine levels, however, failed to affect behavioral performance.

Taken together I was able to confirm, for the first time, mIns as marker of altered astroglia activity *in-vivo*, which may serve as marker of MCI.

Content

Abbreviations

^{1}H-MRS	Proton magnetic resonance spectroscopy
AD	Alzheimer's disease
ADP	adenosine diphosphate
AL	adjacent left quadrant
AR	adjacent right quadrant
ATP	adenosine triphosphate
CNS	central nervous system
CR	Charles River
Cr	creatine
CT	computed tomography
DBM	deformation-based morphometry
dHPC	dorsal Hippocampus
DNA	deoxyribonucleic acid
FOV	field of view
FWE	family-wise error
GFAP	glial fibrillary acidic protein
Glu	glutamate
GluR	glutamate receptor
GMV	grey matter volume
HPC	Hippocampus
Iba1	ionized calcium-binding adapter molecule 1
ICD	International Classification of Diseases
LAC	L-acetylcarnitine
LC-MS	liquid chromatography–mass spectrometry
LL	long-latency
LTP	long-term potentiation
MassSpec	mass spectrometry
MCI	mild cognitive impairment
mGluR	metabotropic glutamate receptor
mIns	myo-inositol
mPFC	medial prefrontal cortex

MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MWM	Morris Water Maze
NAA	N-acetylaspartate
NAAG	N-acetylaspartylglutamate
NAD	nicotinamide adenine dinucleotide
NE	north east
NMDA	N-methyl-D-aspartate
n.q.	not quantifiable
n.s.	not significant
NW	north west
OQ	opposite quadrant
Ppm	parts per million
PSD-95	postsynaptic density protein 95
SE	south east
SEM	standard error of the mean
SL	short-latency
SNP	single-nucleotide polymorphism
SW	south west
TCA	tricarboxylic acid
TE	echo time
Tph2	Tryptophan Hydroxylase-2
TQ	target quadrant
TR	repetition time
VHC	vehicle
vHPC	ventral Hippocampus
WB	Western Blot
WCM	Water Cross Maze

1. Introduction

1.1 Dementia and MCI

Dementia is a heterogonous syndrome with different neuropathological causes and multiple clinical manifestations and subtypes (e.g. vascular dementia, Lewy Body dementia, Alzheimer) (Elahi and Miller, 2017). However, all subtypes share the same essential features which define dementia as pathological syndrome. Demented patients show cognitive deficits which are strong enough to interfere with activities of daily life (Gale et al., 2018). According to the ICD-10 diagnosis criteria they lose their capability to "think, remember, learn, make decisions, and solve problems". In addition, they often show personality changes and emotional instability (Cipriani et al., 2015). The cognitive deficits mostly manifest themselves in increasing memory impairment. Nonetheless, other cognitive domains (e.g. language, executive functions) can be affected as well (Jones, 1997). Due to the increasing cognitive deficits, demented patients may lose their personal autonomy and depend on family and nursing support. These circumstances lead to an enormous psychological, medical, social and economic burden on patients with dementia and their relatives, but also on society at large (Prince et al., 2014). Today approximately 50 million people worldwide suffer from dementia (World Alzheimer report 2018). However, the incidence of dementia is expected to increase further. This is explained by an increasing number of susceptible people in most ageing "Western" societies, which live into old age (2015). In 2050 152 million people will be affected by this syndrome according to estimates (World Alzheimer report 2018). This development makes dementia one of the most demanding challenges for today's aging societies and health systems.

Dementia as a syndrome includes different disease pattern. Alzheimer`s disease (AD) is by far the most common subtype of dementia (Garre-Olmo, 2018), whereof it is also one of the most popular research topics. Nonetheless, treatment options remain limited.

At this point two classes of substances have been approved as therapeutic agents for AD: Cholinesterase-Inhibitors and Memantine, an uncompetitive NMDA receptor antagonist (Robinson and Keating (2006). Whereas both drug groups have moderate overall effect on the clinical symptoms, the progression of the disease stays unaffected (Briggs et al., 2016). Other potential, more causal, pharmaceutical treatments as monoclonal antibody or anti-amyloid therapy are not approved for clinical use yet, due to inconclusive results in clinical

studies (Briggs et al., 2016). This may be one of the reasons why so many older people refuse to see the doctor at the occurrence of first cognitive deficits. The fear of receiving the diagnosis of the "incurable disease" AD may be stronger than potential benefits of an earlier treatment.

A major drawback in the establishment of effective therapeutic means against AD is the late timepoint of diagnosis. Some studies suggest that first neuroanatomical changes occur years to decades before the first clinical symptoms (Braak and Braak, 1997), which makes it nearly impossible to identify patients in early prodromal phases through sole clinical examinations. More technical screening methods are also limited in their informative value without agreeing which pathological changes occur first in AD patients' brains (Mann and Hardy, 2013), let alone how to quantify essential features of the disease, e.g. synaptic and neuron loss, *in-vivo* (Hane et al., 2017).

Nonetheless, identifying susceptible patients as early as possible may be the "key for success" in the treatment of AD. Insights from previous studies revealed that starting the treatment in early preclinical phases is the most promising approach to attenuate the disease progression (Aisen et al., 2017; Weller and Budson, 2018). Therefore, the establishment of biomarkers in early prodromal phases could be an essential step for the generation of effective therapeutic options.

Regarding early intervention strategies the concept of MCI appears to be crucial. MCI is suspected as a prodromal stage of dementia (Petersen et al., 1999; Petersen, 2004). It includes everyone who is situated in an "intermediate stage of cognitive impairment", which often, but not always, progresses to dementia (Petersen et al., 2014; Roberts et al., 2014); fig. 1). In contrast to demented patients, patients suffering from MCI are still able to handle their daily routine. General intellectual and functional abilities are preserved and patients only show subtle cognitive deficits (Petersen, 2004).

Analogous to dementia, MCI is a heterogenous syndrome with diverse clinical manifestations related to cognitive decline (Roberts and Knopman, 2013). In the clinical setting, clinicians discriminate between different subtypes of MCI on basis of amnestic phenotypes. While patients of the amnestic subtype show memory impairments exclusively, patients with non-amnestic MCI additionally exhibit deficits in other cognitive domains such as language, executive functions or visuospatial abilities (Roberts and Knopman, 2013). There is only sparse evidence that each subtype is associated with one particular type of dementia, as e.g.

AD or could predict the further development of the disease (Busse et al., 2006; Yaffe et al., 2006; Jungwirth et al., 2012; Michaud et al., 2017).

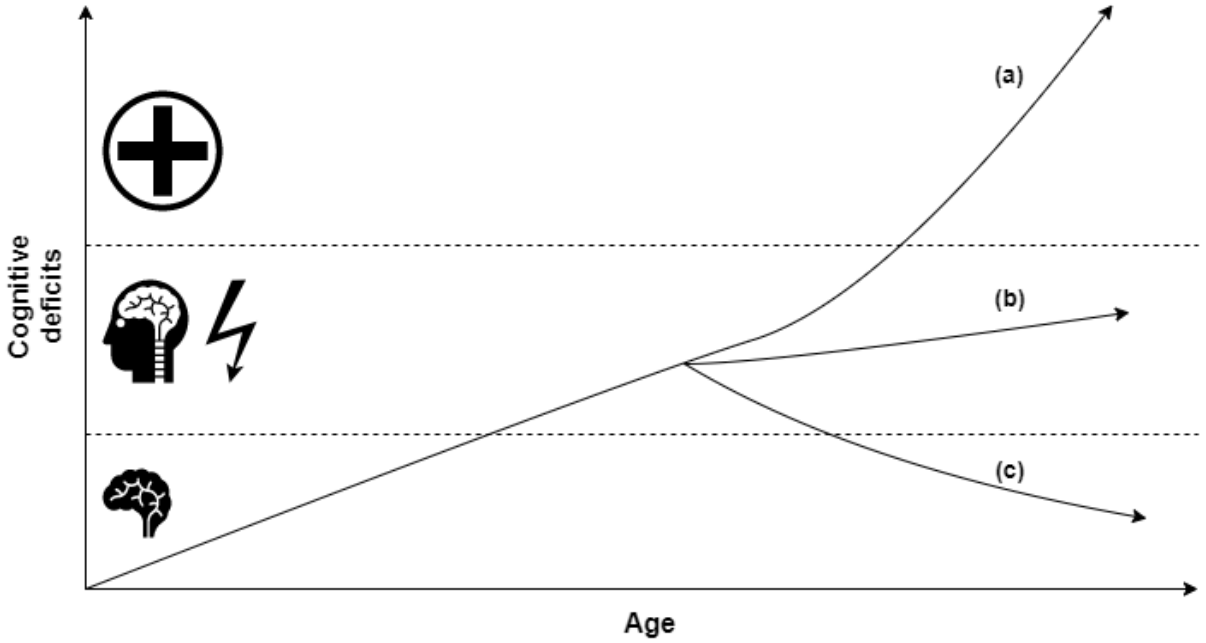

Figure 1: ***Variable clinical outcome of MCI.*** Mild cognitive impairment occurs with increasing age. Whereas **(a)** some patients develop dementia, cognition of others **(b)** remains stable within the range of MCI or **(c)** even improves (adapted from Canevelli et al., 2017).

So far, the establishment of specific biomarkers of MCI hasn't been successful (Giau et al., 2019). However, this could be a crucial step in future treatment of MCI. It can be reasonably assumed that biomarkers of MCI may allow to identify and treat susceptible patients earlier with more effective drugs.

Even if there is a broad body of clinical literature, the mechanisms behind MCI remain unknown. One reason might be that human studies are confronted with several limitations: To conduct longitudinal measurements within the same patient, human studies require long-lasting challenging follow-ups vulnerable to selection or performance bias (Akobeng, 2008). Moreover, the elemental neurobiological mechanisms which lead to e.g. metabolite alterations in humans are largely unknown (Patel and Ahmed, 2015) and more difficult to study than in animals. The human clinical populations are heterogenous in aetiology, clinical outcome and genetic background. Animal studies, on the other side, allow to assess these mechanisms in defined mouse strains with smaller genetic variability such as inbred mice (Keifer and Summers, 2016).

The establishment of an animal model of MCI could be a possible solution for the restraints

of human studies.

1.2 How to create an animal model

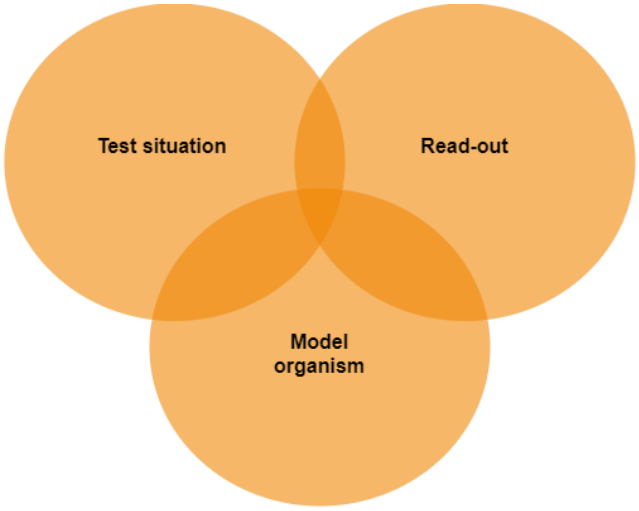

Figure 2: ***Components of an animal model.***

First, an animal model requires a model organism. Whereas non-human primates show the most resemblances to humans (Sallet et al., 2012; Sallet et al., 2013; Mars et al., 2016), rodents, in specific mice and rats, are the most frequently applied model organisms (Sedivy, 2009; Hånell and Marklund, 2014). The model organism is set as independent variable of the animal model (O'Leary and Cryan, 2013). It is possible to modulate the model organism by a wide range of manipulations. Drug withdrawal, stress and brain lesions or genetic modifications are only a few examples.

Second, an animal model is based on a test situation with defined behavioural readout. The readout is set as dependant variable of the animal model (O'Leary and Cryan, 2013). It can either refer to the syndrome as a whole or only to a single symptom of the disorder. If an animal model only imitates single symptoms of a psychiatric disorder, it is defined as endophenotype (Gass and Wotjak, 2013).

Major goal of animal models is to achieve results which can be translated into human studies. Therefore, animal models should fulfil different criteria which allow to estimate their translational validity (McKinney and Bunney, 1969). *Face validity* is the similarity of investigated symptoms in the animal model to the symptoms in the human modelled organism (Belzung and Lemoine, 2011). *Etiological validity* describes similarities in pathogenesis and *construct validity* refers to the theoretical rationale of the disease (Willner, 1986). In addition, *predictive validity* characterizes analogies in the effect of therapeutic interventions (Cryan and Holmes, 2005).

1.3 Towards an animal model of MCI

Cognition includes a variety of different processes which aim to create thoughts and followed by specific, prepared actions. By integrating multiple functions and processes, humans are able to control and plan their behaviour and thinking. Memory, executive functioning, attention, language and visual-spatial perception are the primary neurocognitive domains which form our line of thoughts and reactions (Keeler and Robbins, 2011; Al Dahhan et al., 2019). Cognition is achieved by connection of several regions of the neocortex (e.g. temporal, parietal and frontal lobe) with the subcortical brain (Keeler and Robbins, 2011). Pathological stated as injuries (Gorgoraptis et al., 2019) or numerous neuropathological disorders, e.g. attention deficit hyperactivity disorder, autism disorder, Alzheimer's disease, vascular dementia and Parkinson's disease (Al Dahhan et al., 2019) can cause cognitive impairment. In the context of MCI, cognitive deficits emerge with increasing age, whereby it is assumed that old, cognitively impaired people must use compensatory mechanisms to cope with different tasks and phenotypes are not always indicated. They need to invest more mental effort to handle the manyfold aspects of cognition compared to controls (Grady, 2012). If the "cognitive reserve" is exhausted, cognitive deficits can't be compensated anymore and come to light (Whalley et al., 2004; Tucker and Stern, 2011).

Forming memory is an essential neurocognitive domain which is impaired in most demented patients. Several behavioural tests, e.g. Morris Water Maze (Morris, 1984), Water Cross Maze (Kleinknecht et al., 2012), Novel object recognition test (Ennaceur and Delacour, 1988), Barnes Maze (Barnes, 1979), allow to assess memory deficits in rodents. Based on the literature, memory tasks focusing on spatial memory are the most promising approach to investigate cognition in an animal model of MCI. It has been shown that human patients affected by MCI show lower performances in spatial navigation tasks than age-matched controls (Lithfous et al., 2013), while it is better possible to predict the conversion rate from MCI to AD with a spatial memory task than with the widely used Mini-Mental State Examination (Chan et al., 2016). Moreover, impairment in spatial navigation is considered as one of the earliest symptoms of AD (Pai and Jacobs, 2004).

In general, spatial memory can be acquired by two different strategies: allothetic (place) learning or idiothetic (response) learning.

Place learning relies on allothetic (exteroceptive, external) cues (Morellini, 2013) and includes the hippocampus-dependent formation of a cognitive map (O'Keefe et al., 1975;

Kleinknecht et al., 2012). It is not based on the observer and its starting position, but on external cues around him. Thus, it is a more flexible strategy of spatial navigation. Induced lesions of the hippocampus can impair allothetic navigation in rodents (Kleinknecht et al., 2012).

In contrast response learning is an egocentric method of navigation which relies on body-centered representations (Lithfous et al., 2013)**.** Test subjects only remember the direction of possible body turns on their path to the goal location. Thus, idiothetic navigation is dependent on the starting position of the subject, but not on external visual cues. It is a very rigid, stimulus-response based navigation strategy and can be impaired by lesions of the basal ganglia (Packard et al., 1989; McDonald and White, 1994; Tzavos et al., 2004; Eichenbaum, 2017).

Since pathological changes in MCI and AD lead to deficits in spatial memory tasks, which are more pronounced in allocentric than in idiothetic navigation (Kalova et al., 2005; Hort et al., 2007; Laczo et al., 2009; Weniger et al., 2011; Vlcek and Laczo, 2014), spatial navigation tasks with a focus on allothetic (place) learning may be the most suited test situations for an animal model of MCI. This may be attributable to the large number of cell types e.g. place cells, grid cells, head direction cells and border cells , which interact in the assessment of allothetic spatial memory (O'Keefe, 1976; Hafting et al., 2005; Sargolini et al., 2006; Solstad et al., 2008).

The Water Cross Maze is a behavioural spatial navigation test, which allows to differentiate between animals that have applied response or place learning strategies. It is a task based on the classic Toleman Maze and, as unique selling point, enables to promote HPC-dependent place learning while excluding response learning strategies to a great extent (Essman and Jarvik, 1961; Kleinknecht et al., 2012). Moreover, the two main readouts of the WCM, accuracy and latency, make it possible to narrow down the nature of the cognitive deficits. Lower accuracy levels point to fundamental deficits in the consolidation and appliance of spatial memory. In contrast single anomalies in latency levels reveal deficits in spatial learning which can be caused by cognitive or non-cognitive deficits (motoric deficits, stress coping etc).

However, the WCM is a rather simple task which only requires basic skills of spatial navigation and memory. The walls of the maze restrict the movement directions of the animals. Thus, the animals are able to find the platform even if they only have a broad idea

of the platform position.

In contrast, the Morris Water Maze is a more challenging spatial navigation task. It is a large circular water pool with no internal cues. The position of the platform, which is invisible for rodents, isn't close to the walls or to the center. The animals need to form distinct spatial memory to find the platform's position continuously. Moreover, the duration of the trials in the MWM are nearly twice as long compared to the WCM, which may lead to increased stress load (Engelmann et al., 2006). Despite the increased difficulty level 60 % of intact dorsal hippocampus volume in rats is still sufficient to enable them to solve this spatial navigation task (Moser et al., 1995).

The performance of rodents in spatial memory paradigms depends critically on their external or internal motivation to solve the task. In an ideal test situation, all test subjects are equally motivated to solve the task (Hånell and Marklund, 2014). Otherwise their performance depends strongly on confounding factors, e.g. curiosity, instead on the originally considered abilities, e.g. memory capacity.

Depending on the spatial memory test, motivation of rodents can be "created" by different methods. In general, mice can be motivated in learning paradigms by either external reinforcement or by their internal "exploratory drive" (Wotjak, 2004). One way to achieve external reinforcement is to present a positive reward, e.g. food, when successfully completing the test situation. It is a common method to enhance motivated behaviour towards the positive reward e.g. by depriving food of the animals. This method is applied in most dry cross-maze tasks (e.g. T-maze) (Wenk, 2001). The appliance of aversive stimuli is another approach to motivate rodents with external reinforcement. In the WCM and MWM, the test situation per se represents the aversive stimuli. It is not necessary to use other external negative reinforcement strategies since rodents are motivated to escape from the water by their natural instincts. In other memory tests punishment of the test subjects by e.g. mild electric footshocks motivates the animals to perform the test paradigm.

Beside motivational aspects stress coping is another crucial factor in the performance of mice in spatial memory tasks. While performing spatial memory tasks test subjects have to leave their habituated environment and are often confronted with aversive stimuli. This leads normally to an increased stress load in the animals (Engelmann et al., 2006; Hånell and Marklund, 2014). Depending on their stress susceptibility and their stress coping strategies mice can handle these situations with varying degrees of success (Shea et al., 2015; Jung et

al., 2017). Strain-specific and interindividual differences in stress coping behaviour as well as in stress susceptibility need to be considered in analysis of spatial memory tasks. In the case of water-associated memory tasks floating is a frequently used passive stress coping strategy of stress-susceptible strains (Commons et al., 2017). Extensive floating leads to increased escape latencies in e.g. the MWM, which may be misunderstood as "impaired spatial memory". Thus, it is crucial to take the occurrence of floating behaviour into account while analyzing the performance of mice in water-associated spatial memory tasks, so that strain-specific differences in stress coping strategies can be considered.

1.4 BALBc: A model organism of MCI?

Although the establishment of an animal model of MCI could be a crucial step in gaining a better understanding of cognitive impairment, the search for a model organism of MCI has not been satisfying so far. Most animal models for dementia focus on model organisms with well-advanced cognitive deficits. These cognitive deficits are either obtained by destroying specific brain regions (e.g. the forebrain cholinergic nuclei) or by creating transgenic mice with pathological gene overexpression (e.g. ß-amyloid and presenilin-1) (Götz et al., 2018).

These most specific model organisms mimicking defined molecular mechanisms lead to a face validity of AD or other forms of dementia. However, the full complexity of the disease situation is not reflected. In line with this, human MCI patients show mild cognitive deficits which still allow them to handle their daily routine, exclusively (Petersen, 2004). Hence, the pathological changes which result in cognitive deficits should remain under a certain threshold, so that therapeutical interventions are still success promising. Therefore, the expectation of an ideal model organism of MCI includes cognitive deficits leading to an increased working memory while solving the memory tasks successfully.

Mice from the BALBc strain exhibit several behavioural characteristics, which evoke interest in cognitive research. They show performance deficits in the MWM task and need significantly more time to find the escape platform (Francis et al., 1995). BALBc mice show lower cognitive flexibility if revaluation of acquired spatial memory is necessary (Francis et al., 1995). Moreover, they are specifically susceptible to external stressors (Francis et al., 1995), maternal factors (Zaharia et al., 1996) and environmental factors (Thoeringer et al., 2010), which may lead to distinct cognitive deficits. In general, they are known as an anxious and stress-susceptible strain (Belzung and Le Pape, 1994; Belzung and Berton, 1997; Belzung

and Lemoine, 2011).

In contrast to BALBc mice, B6N mice are known as fast and effective learner in cognitive tasks (Upchurch and Wehner, 1988; Roullet and Lassalle, 1995). They are less susceptible to stress and robust in acquiring spatial memory (Brinks et al., 2007). Moreover, the influence of maternal or environmental factors is less pronounced compared to BALBc mice (Thoeringer et al., 2010).

All in all, comparing the performance of BALBc and B6N mice in two spatial memory tasks (WCM and MWM) is a promising approach to establish an animal model of MCI.

1.5 On the search for predictive biomarkers

Predictive biomarkers of MCI may help essentially in the identification of susceptible patients and therefore, in the development of effective therapeutic means against dementia. However, research in human patients did not result in specific biomarkers of MCI so far (Giau et al., 2019). Thus, it is a promising approach to use the potential of translational research by transmitting scientific findings acquired in animal models into insights about potential biomarkers in humans.

Neuroimaging is one of the most promising translational, non-invasive way to investigate animal models of neurodegenerative disorders. It allows to generate results in animal models and to compare them with analogous findings in humans. Commonly used neuroimaging methods are computed tomography (CT), measuring X-ray attenuation, and magnetic resonance imaging (MRI), using the varying magnetic properties of molecules. Moreover, functional imaging techniques as fMRI (functional MRI), PET (Positron-emission tomography) or SPECT (Single-photon emission computed tomography) refine the methodological spectrum of neuroimaging techniques. MRI is the most promising tool measuring cerebral volumetric changes in translational research on a global brain level while it creates images of the brain with high sensitivity in a radiation-free, non-invasive manner (Fulham, 2004).

It has been shown in previous studies that age-related cognitive decline in humans goes along with structural changes in the CNS, especially in the Hippocampus (Skullerud, 1985; Fjell and Walhovd, 2010; Callaert et al., 2014; Reichel et al., 2017). This can be explained by the key role of the dHPC in the formation of spatial memory (Pothuizen et al., 2004). In line with this hypothesis, cognitive impairment is associated with volume loss of the dorsal HPC

(Fjell and Walhovd, 2010; de Flores et al., 2015), whereas forming spatial memory leads to an increase of volume of the dorsal HPC (Maguire et al., 2000; Lerch et al., 2011). In the context of MCI, it has been shown in humans that volumetry measurements of the (dorsal) hippocampus make it possible to differentiate between MCI, AD and healthy subjects (Weiner et al., 2015).

Nonetheless, volumetry measurements of the dorsal hippocampus with MRI have a limited prognostic value. Mice are able to compensate the volume loss of the HPC until it passes a certain threshold and cognitive deficits emerge (Moser et al., 1995; Reichel et al., 2017). Thus, it is difficult to correlate volume loss with cognitive deficits in prodromal stages. Moreover, the occurrence of cognitive impairment is not only associated with the total volume loss, but also with the structural alterations' temporal dynamic (Wang et al., 2018). Finally, the underlying mechanisms of volumetric changes in the brain and the contribution of different cell types to volume loss or increase are not yet fully understood (Driemeyer et al., 2008). The lack of knowledge about the prognostic value and the underlying mechanisms of volumetric changes complicates making conclusive prognoses on cognitive decline based on brain imaging findings.

Hence, it is preferable to involve other translational methods than volumetry measurements with MRI to establish reliable biomarkers of MCI. One promising approach is the measurement of metabolite concentrations in brain regions which play a key role in cognitive processes, e.g. the dorsal HPC. By receiving insights about metabolite concentrations, it may be possible as well to draw conclusions on changes in brain cell populations based on their differing metabolic profile and therefore, on the morphological changes revealed in MRI measurements.

For this purpose, ^{1}H-MRS is a suitable method. While MRI allows to measure macroscopic structural abnormalities in the brain by the creation of detailed images, ^{1}H-MRS allows to obtain "*in vivo* biochemistry" (Jones and Waldman, 2004). It measures a spectrum of the concentration of several metabolites from either one single voxel or from various brain regions *in vivo*. ^{1}H-MRS is non-invasive and radiation free, which is an important advantage for translational research. So far, only metabolites with increased concentrations (N-Acetyl-Aspartate, choline, creatine, myo-inositol, glutamate and glutamine) can be reliably detected by the scanner. In humans the predictive value for progression from MCI to AD was higher when combining MRS and MRI than using MRI alone (Kantarci et al., 2009).

In most MRS studies with human MCI patients, two metabolites, myo-inositol (mIns) and N-Acetyl-Aspartate (NAA), showed significant changes in concentration compared to healthy adults, whereas most other measured metabolites remained consistent. MRS measurements in the hippocampus revealed that mIns levels were mostly elevated, while NAA levels were often reduced.

Table 1: ***MRS measurements in the HPC of human MCI patients.*** (n.s. = not significant; mIns = myo-inositol; NAA = N-Acetyl-Aspartate; Cr = Creatinine; Glu = Glutamate)

Study	**Subjects (MCI/control)**	**NAA/Cr**	**mIns/Cr**	**Other metabolites**
Ackl et al. (2005)	19/22	↓	n.s.	n.s.
Franczak et al. (2007)	5/5	↓ (left HPC)	↑ (mIns)	↑ (mI/NAA)
Zhang et al. (2009)	9/13	n.s.	↑	n.s.
Wang et al. (2009)	16/16	↓	↑	n.s.
Watanabe et al. (2010)	47/52	↓ (NAA)	n.s.	↓ (Cr in left HPC)
Foy et al. (2011)	21/38	n.s.	n.s.	↓ (Cr + PCr)
Rupsingh et al. (2011)	12/15	n.s.	n.s.	n.s.
Watanabe et al. (2012)	42/54	↓ (NAA)	n.s.	n.s.
Seo et al. (2012)	13/11	n.s.	n.s.	n.s.
Wang et al. (2012)	32/56	n.s.	↑ (left HPC)	n.s.
Targosz-Garniak et al. (2013)	41/35	↓ (left HPC)	n.s.	n.s.
Zhu et al. (2015)	52/34	↓ (left HPC)	n.s.	n.s.
Chen et al. (2016)	38/30	↓ (left HPC)	↑ (left HPC)	n.s.
Huang et al. (2017)	21/17	n.s.	n.s.	n.s.
Wong et al. (2020)	8/16	n.s.	n.s.	↓ (Glu in left HPC)

MIns is a cyclic molecule whose role in the CNS has not been clarified yet. It is predominantly intracellular and a main osmolyte in central nervous system tissue. Beside its role in the phosphoinostitol cycle to form intracellular signaling molecules, mIns mainly aroused interest due to its suspected function as marker of glial cell proliferation (Brand et al., 1993; Best et al., 2014). It has been shown that mIns levels and mIns uptake in glial cell are much higher compared to neurons, which supports the idea of mIns as marker of gliosis (Glanville et al.,

1989). However, mIns is also detectable in neurons (Fisher et al., 2002). It is even discussed as a marker of neuronal activity since electrical stimulation of the cortex was associated with decreased mIns levels in rats' and humans' brain (Xu et al., 2005; Rango et al., 2008; Best et al., 2014).

Whereas mIns is mainly regarded as a marker of glial cell proliferation, NAA is a well-recognized neuronal marker. NAA is predominantly localized in neurons and its concentration is more than 100 times greater in brain tissues compared to non-neuronal tissues (Miyake et al., 1981). There are several hypotheses about the functional role of NAA. On the one hand, NAA is suspected to be part of a trophic support mechanism for oligodendrocytes and to play a critical role in osmoregulation in the brain (Moffett et al., 2014). On the other hand, NAA may be crucially involved in neural processes through its role as precursor of N-acetylaspartylglutamate (NAAG), the highest most concentrated neuroactive peptide in the human brain (Tsai and Coyle, 1995; Neale et al., 2000). Emerging evidence indicates that NAAG exerts its influence on the brain either as neurotransmitter or as neuromodulator (Wroblewska et al., 1993). It is known that NAAG is primarily located in neurons, whereas glial cells only show small concentrations of NAAG (Neale et al., 2000). It activates NMDA receptors and is a selective agonist at the type 3 metabotropic glutamate receptor (mGluR3). By this mechanism NAAG influences several processes in the CNS, e.g. long-term potentiation and depression (Neale et al., 2000; Moffett et al., 2014).

In most MRS studies NAA and NAAG are not measured separately, but represented as a collective peak due to superposition of the spectra (Landim et al., 2016). Whereas NAAG only accounts for a minor part, NAA is responsible for 80-90 % of the peak (Pouwels and Frahm, 1997; Baslow and Guilfoyle, 2007).

The *in-vivo* MRS measurements might be related to cellular or metabolic correlates, for what reason I also perform western blotting and MassSpec analysis in my thesis.

Iba, glial fibrillary acetic protein (GFAP) and S100B are proteins, which allow to quantify the amount of glial cell activation. Thus, they may be suitable cellular marker of altered mIns content in the brain. Iba-1 is a calcium-binding protein, which is uniformly distributed in microglia (Ito et al., 1998; Ahmed et al., 2007). In contrast, GFAP is mostly localized in astrocytes, where it builds up the cytoskeletal framework of astrocytes (Mondello and Hayes, 2015). It is known as an explicit maker of astrocytes, which are part of the macroglia in the CNS (Zhang et al., 2019). S100B is released mainly from glial cells and suspected as a general

glial marker (Rothermundt et al., 2001; Schroeter and Steiner, 2009). It may influence the differentiation of neurons and glial cells.

Reduced neuronal activity may lead to decreased NAA/NAAG-levels in MRS measurements, reflected by reduced levels of the protein PSD-95. PSD-95 is highly concentrated in excitatory synapses (Hering and Sheng, 2001) and is suspected to play a crucial role in synaptic transmission at postsynaptic sites (Hering and Sheng, 2001; Kim and Sheng, 2004; Beique et al., 2006).

Moreover, elevated mIns levels may be correlated with an altered metabolic profile of the brain, which I aim to reveal by MassSpec analysis. Increased glial cell activation may diminish the influence of neurons' metabolic profile on brain metabolism, which is different than astrocytes' metabolic profile. Whereas neurons are predominantly oxidative, characterized by predominant energy production through the TCA cycle in mitochondria (Magistretti and Allaman, 2015), astrocytes mainly focus on glycolysis (Hamberger and Hyden, 1963; Belanger et al., 2011; Zhang et al., 2014; Magistretti and Allaman, 2015). The differing metabolic profiles have synergistic effects, while lactate, formed in astrocytes by glycolysis, can be used in neurons as energy source (Magistretti and Allaman, 2015).

L-acetylcarnitine (LAC) is a promising substance to manipulate brain energy metabolism pharmacologically. LAC enhances beta-oxidation in mitochondria and thereby improves brain energy supply (Nasca et al., 2018; Cherix et al., 2020). By treating BALBc mice with a chronic oral dose of LAC in my study, I aim to investigate the influence of this pharmacological intervention on cognitive as well as on metabolic function.

1.6 Aims of the study

The **first goal** of this study was the establishment of an animal model of MCI. To this end, I compared the behavioral performance of BALBc (potentially susceptible) and B6N (potentially resilient) mice in two established spatial memory tasks (WCM and MWM).

As a **second goal**, I wanted to combine the behavioral tasks with in vivo ^{1}H-MRS. In this way, I hoped to identify line differences, which would predict deficits in cognitive performance. Based on the literature, I focused my attention on mIns and NAA/NAAG levels as indicators of glial and neuronal substrates, respectively. To ensure the validity of the measurements, I embedded the behavioral testing in repeated ^{1}H-MRS measurements (i.e., before and after

learning).

As a **third goal**, I wanted to relate the in vivo measurements of mIns and NAA/NAAG to ex vivo measurements of glial (Iba for activated microglia, GFAP and S100B for astrocytes) and neuronal (PSD-95 as postsynaptic marker of excitatory synapses) markers in specimens from the dorsal hippocampus.

As a **fourth goal**, I searched for line differences in brain metabolism, using MassSpec analyses in samples from the dorsal hippocampus, and related them to glial and/or neuronal metabolic pathways. In case of alterations, I wanted to manipulate those selected metabolite levels by pharmacological means, e.g. L-acetylcarnitine, to study the impact on behavioral performance.

2. Material and methods

2.1 Animals

Test animals were either male C57BL/6NCrl (B6N, n = 44) or BALB/cAnNCrl (BALBc, n = 80) mice. All 124 animals were obtained from Charles River (Bad Sulzfeld, Germany) at an age of six weeks. The experiments were performed at an age between eight and 48 weeks (Exp. 2, T3). On their arrival at the MPI of Psychiatry, they were housed in groups of four under standard housing conditions (23°C ± 4°C and 50 % humidity ± 10 %) in individually ventilated cages (IVC; Tecniplast Green Line, Hohenpeißenberg, Germany) with ad libitum access for food (1314, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and water. The experiments were conducted in the light cycle of the animals (light on: 08:00). In Exp. 1 and 2, animals were transferred into single-house cages at least one week prior to the start of the experiment, so that the animals got used to the new environment.

All animal studies were in agreement with the government of Upper Bavarian (AZ: ROB-55.2-2532.Vet_02-17-22) and were conducted in accordance with the recommendations of the Federation for Laboratory Animal Science Associations and according to the European Community Council Directive 2010/63/EEC.

2.2 Water Cross Maze (WCM)

The WCM testing was performed as previously described in Kleinknecht et al. (2012) by applying the hippocampus-dependent place learning protocol.

The WCM consists of four different arms, each 50 cm long and 10 cm wide, which taken together form a cross. Before each trial, the arm opposing the starting arm was blocked with a wall of clear acryl plexiglas, so that the WCM took a T-shape. The squared escape platform (8 x 8cm) was placed at the end of either the N- or the S-arm 1 cm beneath the water surface, invisible for the mice. The computer in the SE corner and the tank on the opposing side were the only prominent landmarks in 309 cm x 357 cm x 283 cm squared-like experimental room.

At the beginning of every test day, the WCM was filled with fresh tap water of 22°C temperature up to a height of 11 cm. After performing half of the daily trials water was stirred to avoid any olfactory cues. Moreover, walls were cleaned and faeces was removed after every trial.

After transporting the animal from the adjacent holding room to the experimental room, the

experimenter gently inserted the test subject into the end of the starting arm facing the end wall. Each animal could swim freely for 30 sec searching for the platform whereas the experimenter remained motionless behind the starting arm. If a mouse couldn't find the platform within 30 sec, the experimenter gently guided it to the platform by indicating the path with a stick without touching the animals. After climbing on the platform, the mouse stayed there for further five seconds until the experimenter carried them back into their home cage by using a metal grid. While waiting for the start of the next trial, the animals had the possibility to warm up under infrared light in the adjacent holding room.

Occasionally animals showed floating behavior, characterized by "immobility, except for movements necessary to maintain balance" (Bachli et al., 2008). In this case, the experimenter "woke up" the animal by snapping its gloves.

The applied place learning protocol was characterized by the stable platform position which remained the same over the course of five days of training, whereas the starting position changed in a pseudorandomized order. To exclude any spatial bias, the platform was either constantly placed in the N- or the S-arm, divided equally between the animals. Each animal had to perform six trials per day, conducted in groups of six animals, over a course of five consecutive days.

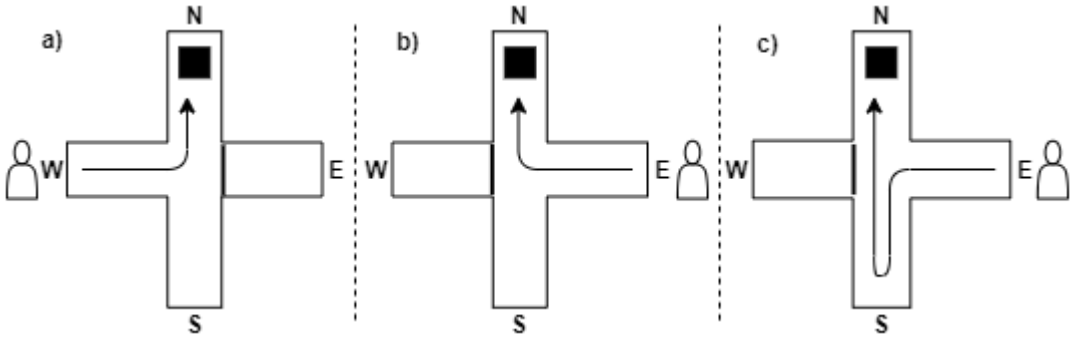

Figure 3: ***WCM training.*** The arm opposing the starting arm was blocked before each trial so that the WCM was transformed into a T-maze. **(a, b)** Whereas the escape platform (black quadrat) stayed at the same position over the course of training, the starting arm changed in a pseudorandomized order. **(c)** Presentation of an inaccurate trial, in which the animal found the platform after entering the opposite arm first.

The experimenter noted several parameters by hand, which were digitalized and analyzed afterwards. Latency described the average time of each animal until successfully climbing on the platform over six trials per day. If an animal didn't reach the platform within 30 sec, the

experimenter noted 31 sec as latency for this trial. Latency score summed up the latency over the course of five days. A trial was noted as accurate if an animal did not enter the arm opposing the target arm or went back into the starting arm. Accurate trials were scored as 1, inaccurate trials as 0. Accuracy defined the percentage portion of accurate trials per day. Accuracy score reflected the average accuracy over the complete training. An animal was called an accurate learner if it performed in at least 5 out of 6 trial per day accurately. In addition, the experimenter counted the incidence of floating behavior, which was summed up over the course of five days of training.

2.3 Morris Water Maze (MWM)

The test was performed in a 150 cm large round pool built of white synthetic. The pool was placed on a table so that the borders of the walls were around 110.5 cm above the floor. Testing took place in the 309 cm x 357 cm x 283 cm squared-like experimental room with spatial landmarks on the walls (rectangular, triangular and circular posters with different black/white patterns, fixed 169 cm above the floor). Except these landmarks, no other prominent cues were visible for the animals in the room, while the computer in the SW corner and the sink on the opposite site were outside of the animals' field of vision. Two spots lights were illuminating the room indirectly, resulting in a light intensity of 11.5 lux.

The pool was filled to a depth of 11cm with fresh tap water (22°C) at the beginning of the experiment on the first day. Water temperature was controlled at regular intervals while adding warm or cold water if necessary. The circular transparent escape platform (8 x 8cm) was situated in the NW corner of the pool, 35 cm away from the wall and 1 cm beneath the water surface. The platform remained at the same position for all trials, except for the probe trial.

Each mouse performed four trials per day within the first seven days of training. Every trial lasted for a maximum of 60 sec. If animals failed to find the platform within 60 sec, they were gently shown the way to the platform by indicating the path with a stick without touching the animals. Animals, which climbed successfully on the hidden platform, stayed there for further five seconds until the experimenter carried them back into their home cages by using a metal grid. After performing all four trials of the day, animals were brought back into the holding room, where they could warm up under infrared light if needed.

Each trial was tracked by video recording and analyzed with ANY-MAZE (version: 5.26;

Stoelting, Dublin, Ireland). “Latency” described the average time of each animal until successfully climbing on the platform over the course of four trials per day. If animals needed more than 60 seconds to climb on the platform, latency was noted as 61 seconds and the trial was marked as “failure”. The sum of all latencies over the period of seven days was summed up as “latency score”, whereas the average “swimming path length” was calculated over a course of four trials per day.

On Day 8, the platform was removed from the pool to perform the probe trial. Animals started the trial in the opposing quadrant of the initial target quadrant while their overall performance was analyzed by assessing the time and distance in each specific quadrant as main parameters.

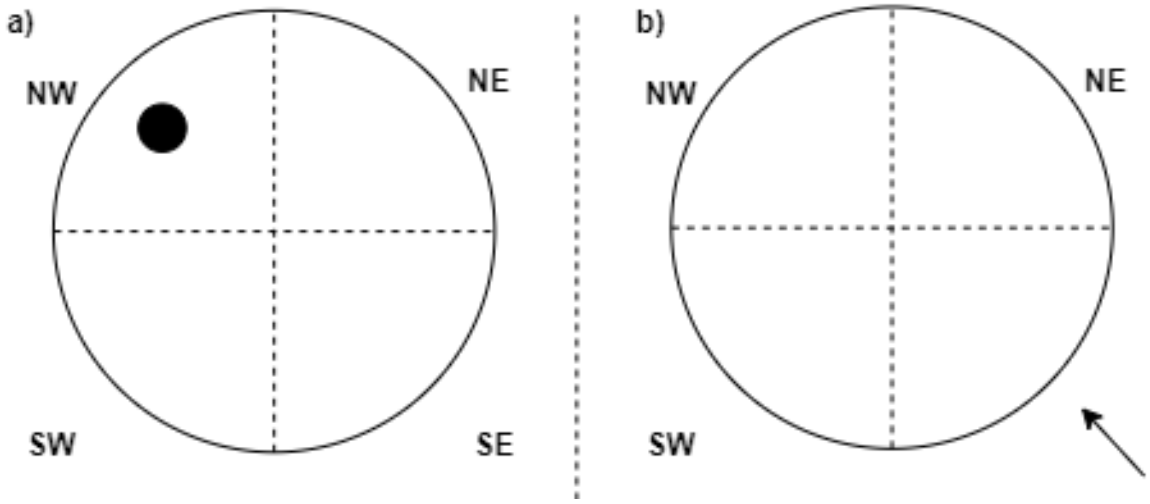

Figure 4: ***MWM training.*** **(a)** The platform was situated in the NW corner during spatial learning from day 1 until day 7. **(b)** In the probe trial on day 8, the platform was removed from the pool and the animal started the trial in the SE corner.

2.4 *In-vivo* Imaging

2.4.1 General procedure

The anaesthetic Isoflurane (Isofluran CP®, cp-pharma®, Burgdorf, Germany) was administered at a concentration of 2.0 % (air flow around 1.3 l/min) and kept stable at 1.5 - 2.0 % throughout the imaging procedures. Mice were put in abdominal position while monitoring the respiratory rate with a pressure pillow (adjusted to 80-120 bpm by the isoflurane concentration) and preventing ophthalmic damage with eye ointment (Bepanthen® Bayer AG, Leverkusen, Germany). The mice’s heads were fixed stereotactilly in order to exclude disturbing movements. While body temperature was measured

continuously with a rectal probe, it was kept stable with a heating pad (water bath, Haake S 5P, Thermo-Fisher Scientific, Waltham, United States). After the scanning procedure with 65-90 min total time of anaesthesia, mice were brought back into their home cages and monitored while waking up.

Scanning was performed with a 9.4T BRUKER Biospec 94/20 system using Paravision software (Paravision 6.0.1, Bruker, Ettlingen, Germany). Whereas a two channel transmit/receive cryo coil was used in the MRS measurements of Exp. 1, whole body coil for transmission and a room temperature 2x2 array surface coil for signal detection were applied in Exp. 2.

2.4.2 MRS acquisition and analysis

MRS localization was achieved with a 2D T2-weighted structural image with 25 slices (slice thickness 0.25 mm; 0.1 mm gap; image matrix 384 x 384 voxel; TR=328 ms; TE=3.2 ms; flip angle 30°; FOV=20x20 mm2). PRESS spectroscopy (TR=5000 ms; TE=16.5 ms; 128 averages; voxel size for the hippocampus 2.5 x 1.5 x 1.5 mm^3, for the prefrontal cortex 1.5 x 2 x 1 mm^3) was performed for the prefrontal cortex (Exp. 1), the right and left dorsal HPC (Exp. 1; Exp. 2) as well as the ventral HPC (Exp. 2).

LCModel 6.3 (s-provencher.com/lcmodel.shtml), including the 9.4T basis spectra, were used for metabolite analysis, with an analysis window ranging from 4.3 - 0.2 ppm. Eddy current were corrected by using the undistorted water signal acquired at the beginning of the sequence as reference. Metabolic concentrations were indicated as ratio to Creatine, the internal reference metabolite.

2.4.3 DBM acquisition and analysis

FLASH sequence with TR=34.1 ms; TE=6.25 ms; flip angle 10°; FOV=19.8 x 12.8 x 15.8 mm3; image size = 256 x 166 x 205, resulting in isotropic resolution of 77 µm, was applied to create structural 3D images.

Thereafter, post-processing was performed with SPM12. After converting the images to NIFTI format and multiplying the voxel size by the factor 10, images were adjusted to the template orientation (Hikishima template (Hikishima et al., 2017) for brain extraction; SPMmouse template for tissue segmentation). In a first step, GM and WM compartments were roughly estimated with the Hikishima template, followed by the creation of a whole brain mask (with filled ventricles) in native space. In a second step, brain extracted images were created out of

the whole brain mask and SPMmouse template was applied for the second segmentation step. After the generation of a DARTEL template and a study specific group template, the resulting images were smoothed using a FWHM kernel of 4 mm for later deformation-based morphometry (DBM) analysis. Global signal was calculated by using individual deformation fields for each anatomical image as well as for tissue compartments. Then, the last GM and WM DARTEL templates were summed up and holes of the lateral ventricles were filled with the MATLAB infill function. By binarization at a threshold > 0.3, the final brain mask was created, which was restricted to the midbrain to avoid most disturbing factors, e.g. tissue misclassification in the cerebellum. Due to this final midbrain mask, it was possible to calculate global values of GM, WM and CSF (summation of all voxel values of the respective normalized and modulated tissue segments), as well as the total intracranial volume (TIV; the sum of the global values of all three compartments).

The factors “strain” (BALBc or Bl6) and “training” (naïve or MWM-experienced), as well as TIV as covariate, were taken into account in the full factorial model for DBM analysis. Proportional scaling led to global normalization.

2.5 Western Blotting

RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% Sodium deoxycholate, 0.1% SDS 50 mM Tris (pH8.0)) supplemented with protease inhibitor (Merck Millipore, Darmstadt, Germany), benzonase (Merck Millipore), 5 mM DTT (Sigma Aldrich, Munich, Germany), and phosphatase inhibitor (Roche, Penzberg, Germany) inhibitor cocktail were added to the lysate, so that proteins were extracted.

Samples were separated and analyzed by capillary electrophoresis on Wes™ (ProteinSimple) using the 12–250 kDa cartridges. For immunodetection the following primary antibodies were used: anti-GFAP (1:100, Cell Signalling Technology, #80788), anti-S100B (1:100, Cell Signalling Technology, #9550), anti-IBA1 (1:100, FUJIFILM Wako Pure Chemical Corporation, 019-19741), anti-PSD95 (1:100, Cell Signalling Technology, #3450), anti-actin (1:300, Cell Signalling Technology, #4970). Expression levels were normalized to the intensity of Actin.

2.6 Liquid chromatography–mass spectrometry (LC-MS) based metabolite analysis

LC-MS analysis was performed with following equipment: the Agilent Technologies 1100 HPLC system (Agilent Technologies; Santa Clara, CA, USA) was connected to the Bruker Impact II TOF MS system (Bruker Corporation; Billerica, MA, USA). Both systems were controlled by Brukers Hystar 3.2 Software.

Samples were separated with two eluents, eluent A containing H2O with 20mM ammonium formate pH 3 and eluent B containing 100% ACN with 0.1% formic acid, were forming an eluent gradient, which was running with a flow rate of 500 µl*min^{-1} over a Waters XBridge 3 x 100 mm column with particles of 2.6 µm size and 200Å poor size from Waters (Waters Corporation, Milford, MA, USA) with a Waters XBridge guard column. Both columns were heated to 25°C in the LC oven.

For the extraction of plasma samples, 200 µl MeOH was added to 50 µl plasma. Thereafter, samples were shaked for 1h at 25 °C at the ThermoShaker and centrifuged for 10 min (at max). 200 µl supernatant was transferred into an Eppendorf tube. Samples were dried in a vacuum centrifuge.

For the extraction of tissue samples, tissue samples were weight and cold 80 % MeOH was added, concentrated according to the weight of the tissues (factor 30, e.g. 30 µl MeOH was added to 1 mg tissue sample). Tissue samples were grinded with a pistil and incubated in a shaker for 1.5 h at 4 °C. Samples were centrifuged for 10 min (at max). 200 µl supernatant was transferred into an Eppendorf tube. Samples were dried in a vacuum centrifuge.

Samples were solved in water and diluted 1:4 with ACN to a final concentration of 0.05 ug*ul^{-1} . Subsequently 2 µl were injected into the injection loop of the HPLC. For the first two minutes isocratic separation has been performed with 95% of eluent B. Subsequently, percentage of eluent B was decreased to 65% over a course of 23 min followed by a drop to 50 % in 1 min. Thereafter, percentage was maintained at 50 % for 3 min. As final step percentage of eluent B was reincreased to 95 %. And column was equilibrated for 26min.

For positive mode (ESI+), capillary voltage was set to 4000 V. The dry gas N2 was heated to a temperature of 220°C with a flow rate of 10 l*min^{-1}. The pressure of the nebulizer was maintained at 3.5 bar. The profile data was assessed by Brukers Compass 1.9 with a spectra rate of 1 Hz (full scan) and a mass range from 20-1300 m/z.

Internal calibration of the mass accuracy was performed by injecting sodium format clusters

prior to each run and adjusting the results with Brukers DataAnalysis 4.4 software. Thereafter, mzXML files were created out of the chromatograms by applying MSConvert from Proteowizard (ProteoWizard, Palo Alto, CA, USA).

MZmine2 (Pluskal et al., 2010) was used for data pre-processing. Chromatographic peaks were created after processing of converted mzXML files with the ADAP module (Chowdhury et al., 2009). Deisotoped peaklists were aligned according to retention time and exact mass, based on an inhouse database. For statistical analysis the final compound list was exported to CSV files.

2.7 Pharmacological treatment

The applied treatment protocol was similar to the protocol previously described in Cherix et al. (2020). L-Acetylcarnitine (LAC) (Sigma-Aldrich, Taufkirchen, Germany) was dissolved at a concentration of 0.3 % in the drinking water of the animals. Four animals, which lived together in one cage, shared one drinking bottle. The control group had free access to regular drinking water. The treatment started seven days before the beginning of the experiment and was continued until the end of the experiment. The amount of fluid intake was measured at regular intervals (2-3 days). If water ran short, new bottles were prepared.

2.8 Experiments

Experiment 1:

Eight weeks old single housed C57BL/6N (B6N, CR, n = 12) and BALBc (CR, n = 12) mice underwent place learning over the course of five days (six trials per day). Within 9 to 17 days after completing WCM training, mice were scanned (medial prefrontal cortex + bilateral dorsal hippocampus) with ^{1}H-MRS. Brains were collected immediately after scanning and stored at -80°C. Brain punches from the dorsal HPC were processed for GFAP, S100B and Iba1 by Western Blot (see figure 5).

Experiment 2:

A new cohort of C57BL/6N (B6N, CR, n = 12) and BALBc (CR, n = 24) adult male single housed mice were scanned with ^{1}H-MRS at an age of 9-10 weeks (MRS1; bilateral dorsal and ventral hippocampus) before WCM training. After scanning, they underwent WCM training at an age of 12-13 weeks (Training 1, T1), which was repeated two more times at an age of 29-30

weeks (T2) and 47-48 weeks (T3). Within two weeks after completing Training 3, mice underwent a second ^{1}H-MRS scan (MRS 2). Around one month after scanning, brains were collected and stored at -80 °C for subsequent WB and MassSpec analyses. Brain punches from the dorsal HPC were analyzed with WB as well as MassSpec (see figure 7).

Experiment 3:

Adult male group housed C57BL/6N (CR, n = 10) and BALBc (CR, n = 10) mice were trained in the Morris Water Maze to find a hidden platform over the course of seven days with four trials per day, followed by one last probe trial on Day 8 with the platform removed from the water. Thereafter, MRI measurements were performed and compared with results from naive controls of both strains, which remained undisturbed in their home-cages instead of performing MWM testing (see figure 9).

Experiment 4:

To observe potential beneficial effects by LAC treatment, I treated adult male BALBc (CR) mice with either in water dissolved LAC (n=12) or with tap water (VHC) (n=12). Seven days after the start of the treatment, the performance of BALBc mice treated with LAC was compared with the performance of water treated BALBc mice in the MWM. Within three days after performing MWM testing, BALBc mice were scanned by MRS measuring mIns content in the dHPC in both cohorts of mice. Brain specimens of the dHPC and plasma samples were collected within three weeks after scanning. LAC levels in plasma and dorsal HPC were measured with MassSpec analysis (see figure 13).

2.9 Statistical analysis

Data were analyzed and graphs were generated using GraphPad Prism 8.0 (San Diego, United States), presented as mean with or without individual data. Error bars are reflecting the standard error of the mean (SEM). The applied statistical tests are indicated in the text section or in the figure legends. Results were classified as significant if $p < 0.05$. If two groups have been compared, effect sizes (*Cohen's d)* were calculated in addition.

Statistical analysis of the *in-vivo* imaging data is described in the respective material and methods section.

3. Results

The data obtained is currently under review for publication in a peer-reviewed journal with the aim to publish the data.

3.1 BALBc mice show spatial learning deficits, which are associated with increased mIns levels

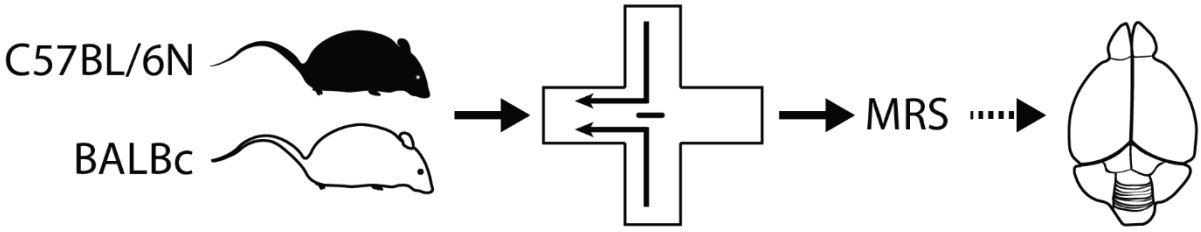

Figure 5: ***Experimental design Exp. 1.*** Single housed C57BL/6N (B6N, CR, n = 12) and BALBc (CR, n = 12) mice underwent place learning over the course of five days (six trials per day). Thereafter, mice were scanned (medial prefrontal cortex + bilateral dorsal hippocampus) with MRS. Brains were collected in the end and stored at -80°C. Brain punches from the dorsal HPC were processed for GFAP, S100B and Iba1 by Western Blot.

Comparing the performance of BALBc and B6N mice in the WCM paradigm, BALBc mice showed significantly higher escape latencies than B6N mice (factor *Strain*: $F_{1,110} = 115.9$, $p < 0.0001$; 2-way ANOVA for repeated measures; Fig. 1a). The sum of all escape latencies (30 trials) of both strains did basically not overlap ($t_{22} = 5.757$, $p < 0.0001$; Cohen's d: 2.2; Fig. 6b). However, strain differences in escape latencies were not reflected by differing levels of accuracy, as both strains showed similar accuracy levels over the course of five days (factor *Strain*: $F_{1,110} = 0.792$, $p = 0.376$; *Strain x Day* interaction: $F_{4,110} = 0.793$, $p = 0.532$; Fig. 6c), but also considering the averaged accuracy scores ($t_{22} = 0.690$, $p = 0.497$; Fig. 6d). On Day 5 of training 12/12 B6N (100%) and 9/12 BALBc mice (75%) were labelled as accurate learners which requires at least 5 out of 6 correct trials.

Increased floating behavior of BALBc mice has contributed most definitely to the strain differences in escape latencies, at least to a certain extent. Whereas most B6N mice avoided floating behavior, BALBc mice exhibited increased floating behavior over the complete course of training (Mann-Whitney u-test: $p = 0.0002$; Fig. 6d, f). However, if analyzed on an

interindividual level, high-floating as well as low-floating BALBc mice showed increased escape latencies (high-floating: 605 ± 39s; t_{16} = 11.48, p < 0.0001; low-floating: 352 ± 29s; t_{16} = 4.815, p = 0.0002; B6N: 207 ± 16s; Fig. 6g), as well as similar accuracy levels compared to B6N mice (statistics not shown; Fig. 6h).

^{1}H-MRS measurements of the dHPC and mPFC were performed after WCM training to assess potential strain differences in metabolite content. Whereas NAA+NAAG levels did not differ within both strains (t_{19} = 0.331, p = 0.744; Cohen's *d*: 0.1; Fig. 6i), mIns levels were significantly increased in the dHPC of BALBc mice (t_{19} = 2.997, p = 0.0074; Cohen's d: 1.2; Fig. 6j). Interestingly, high-floating, but not low-floating, BALBc mice had increased mIns content in the dHPC exclusively ($F_{2,18}$ = 6.482, p = 0.0076; Tukey's post hoc: B6N vs. BALBc_HF p = 0.0056; Fig. 6j).

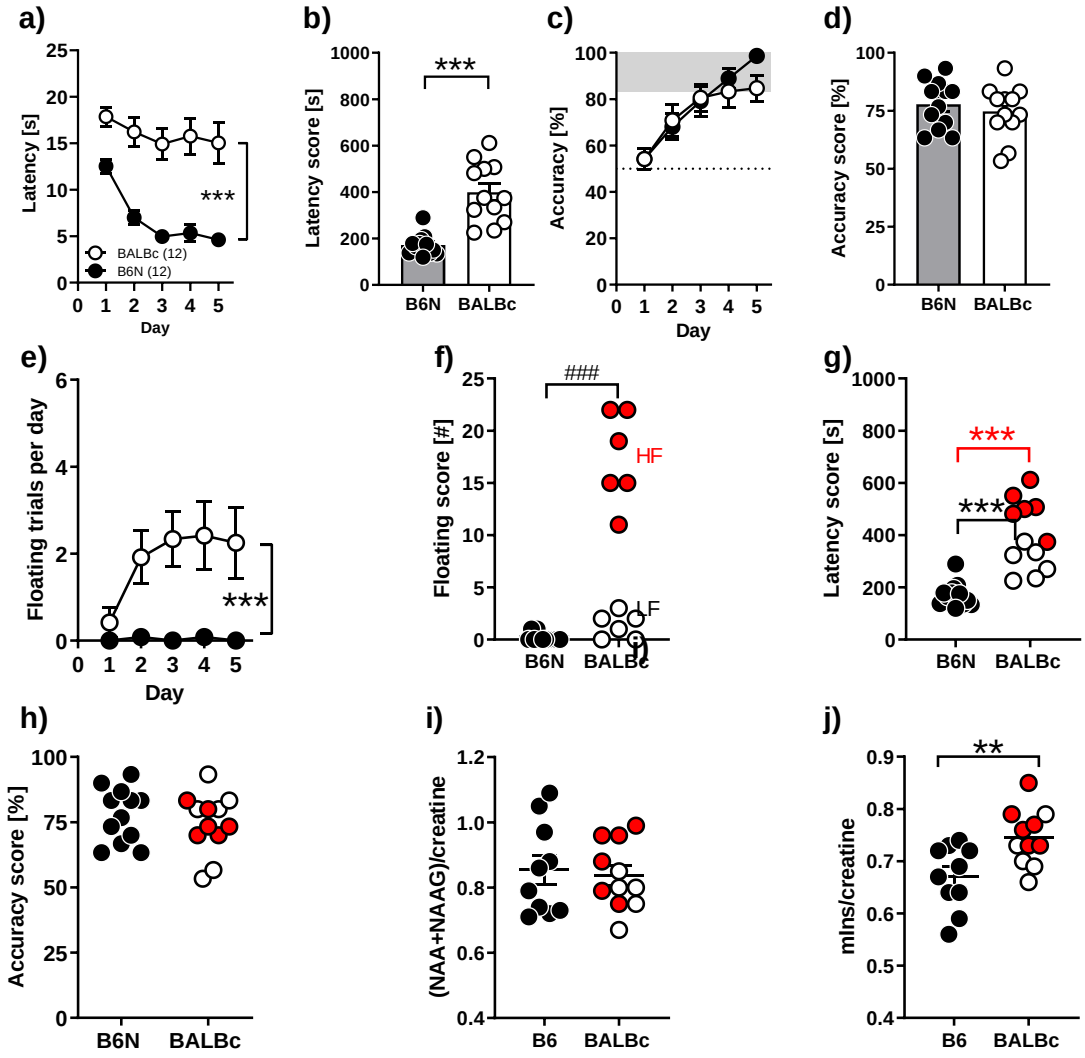

Figure 6: ***Performance deficits of BALBc mice in the WCM were associated with increased mIns-levels in the dHPC.*** BALBc mice (n=12) and B6N mice (n=12) performed WCM training over a course of 5 days. **(a, b)** Whereas BALBc mice exhibited increased escape latencies, **(c, d)** I couldn't observe any strain differences in accuracy levels.**(e)** BALBc mice developed increased floating behavior, **(f)** reflected by a bimodal group distribution. Both groups of BALBc mice, high and low floating mice, **(g)** showed increased escape latencies **(h)** and similar accuracy levels compared to B6N mice. After performing WCM training, both mice strains were scanned with *in-vivo* ^{1}H-MRS. **(i)** Whereas no strain differences has been found in NAA/NAAG content at level of the dHPC, **(j)** mIns levels were increased in the dHPC of BALBc mice. ** $p < 0.01$, *** $p < 0.0001$ (main effect of Strain in 2-way ANOVA, 1-way ANOVA followed by Tukey's post-hoc test (g) or t-test); ### $p < 0.001$ (Mann-Whitney U-test)

3.2 The increased mIns levels and the deficits in spatial learning are consistent over time

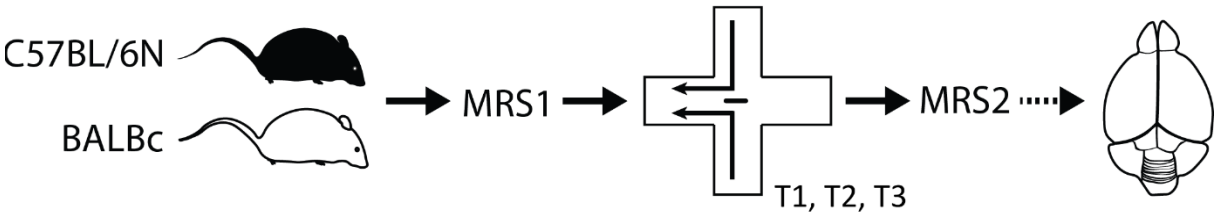

Figure 7: ***Experimental design Exp. 2.*** A new cohort of C57BL/6N (B6N, CR, n = 12) and BALBc (CR, n = 24) adult male single housed mice were scanned with ^{1}H-MRS (MRS1; bilateral dorsal and ventral hippocampus) before WCM training. After scanning, they underwent WCM training at an age of 12-13 weeks (Training 1, T1), which was repeated two more times at an age of 29-30 weeks (T2) and 47-48 weeks (T3). Within two weeks after completing T3, mice underwent a second ^{1}H-MRS scan (MRS 2). Thereafter, brains were collected and stored at -80 °C for subsequent WB and MassSpec analyses.

In line with the results of Exp. 1, BALBc mice needed significantly longer to swim to the hidden platform than B6N mice over the whole course of training, reflected by longer escape latencies in T1 (*Strain*: $F_{1,31} = 52.45$, $p < 0.0001$; Fig. 8a), but also in T2 (*Strain*: $F_{1,29} = 32.95$, $p < 0.0001$; Fig. 8a) and T3 (*Strain*: $F_{1,29} = 13.83$, $p = 0.0009$; Fig. 8a).

Nonetheless, BALBc mice particularly improved their spatial learning performance over the course of training, reflected in the pronounced decrease of escape latencies (*Strain x Training episode*: $F_{2,58} = 12.21$, $p < 0.0001$; Fig. 8b).

Whereas increased escape latencies of BALBc mice were not associated with lower accuracy levels in T1 (*Strain*: $F_{1,31} = 1.221$, $p = 0.277$; Fig. 8c), BALBc mice showed deficits in the consolidation of spatial memory in T2 in form of reduced accuracy levels (*Strain*: $F_{1,31} = 4.723$, $p = 0.038$; Fig. 8c). This distinction between both strains, however, was not consistent whereby accuracy levels of BALBc and B6N mice were not different in T3 (*Strain*: $F_{1,29} = 0.018$, $p = 0.895$; Fig. 8c) and no strain differences in accuracy levels were observed considering the whole course of training (*Strain*: $F_{1,29} = 2.001$, $p = 0.167$; *Strain x Training*: $F_{2,58} = 2.521$, $p = 0.0892$; Fig. 8d).

On a closer inspection, BALBc mice were divided into two subgroups based on their performance in T3, long-latency (LL) and short-latency (SL) mice. LL mice already showed significant higher escape latencies in T2 (*Training*: $F_{1,17} = 15.19$, $p = 0.0012$; Fig. 8h), pointing

to consistent deficits in spatial learning.

Remarkably, LL mice's deficits in spatial learning were associated with impaired spatial memory formation, reflected by reduced accuracy levels in T3 (*Training*: $F_{1,17}$ = 3.746, p = 0.07; T3: t_{17} = 2.289, p = 0.010, Cohen's *d*: 1.2), but not in the previous training sessions.

The behavioral testing has been enclosed by two ^{1}H-MRS measurements, which took place prior to the first training and after the third training. This time, the dHPC as well as the vHPC were defined as regions of interest.

In both ^{1}H-MRS measurements I observed elevated mIns content in the brain of BALBc mice. In younger naïve BALBc mice mIns levels were only increased in the dHPC (t_{31} = 2.451, p = 0.020; Cohen's *d*: 0.9; Fig. 8f), while in older (1 year), experienced BALBc mice, mIns levels were increased in the dHPC (t_{26} = 4.768, p < 0.0001 ; Cohen's *d*: 1.8; Fig. 8f) as well as in the vHPC (t_{28} = 4.499, p = 0.0001 ; Cohen's *d*: 1.6; Fig. 8f).

The grouping of BALBc mice into HL and LL mice was not mirrored by differences in mIns levels before (t_{14} = 1.139, p = 0.274) or after training (t_{15} = 0.552, p = 0.589; Fig. 8k). Thus, in contrast to Exp. 1, mIns levels were unrelated to interindividual behavioral anomalies within BALBc mice in Exp. 2.

Apart from varying mIns levels, the first MRS scan with younger mice did not reveal any other strain differences, while the second MRS scan with aged, experienced mice displayed increased NAA/NAAG levels in the vHPC of BALBc mice (t_{27} = 2.589, p = 0.015; Cohen's *d*: 0.9; Fig. 8e).

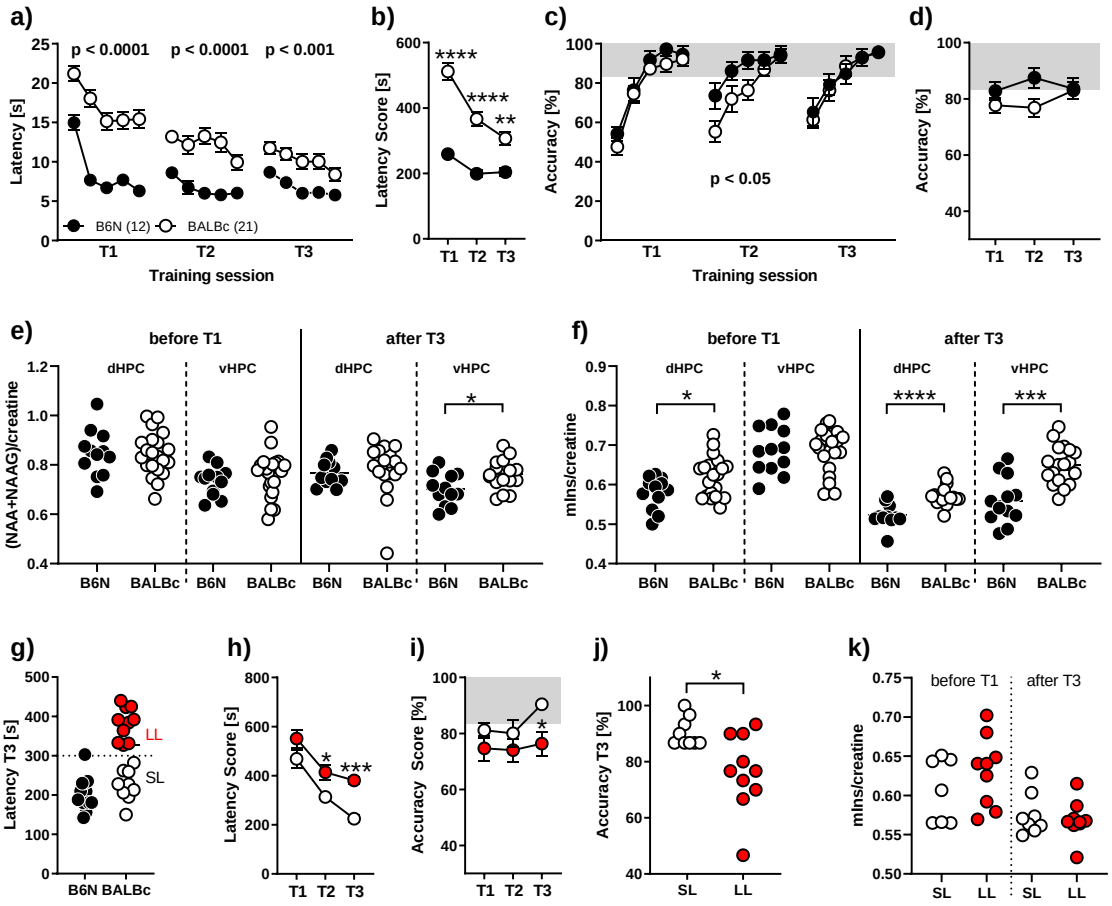

Figure 8: ***Deficits of BALBc mice in spatial learning, but not in memory consolidation were consistent with increasing age, associated with persistent increased mIns levels***. B6N (n=12) and BALBc mice (n=19) performed repeated WCM training at an age of 3 months (T1), 7 months (T2) and 12 months (T3). **(a, b)**, BALBc mice exhibited increased escape latencies in all three WCM trainings. **(c, d)** In contrast, accuracy levels of BALBc mice were reduced in T2 exclusively, whereas this strain difference was not consistent. **(e)** Before and after training, NAA/NAAG levels were not different between both strains as revealed by repeated ^{1}H-MRS measurements. **(f)** In contrast, BALBc mice showed increased mIns content before (dHPC) and after training (dHPC + vHPC). **(g)** At closer inspection BALBc mice were divided into two groups, long-latency (LL) and short-latency (SL) mice, by an arbitrary threshold of 300 sec in T3. **(h)** Both groups were different in escape latencies in T2 and T3, **(i, j)** as well as in accuracy levels in T3. **(k)** However, these differences were not reflected by varying mIns levels before or after training. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (2-way ANOVA followed by Tukey's post-hoc test or t-test)

3.3 BALBc mice are impaired in spatial learning, but not in the consolidation of spatial memory in the MWM

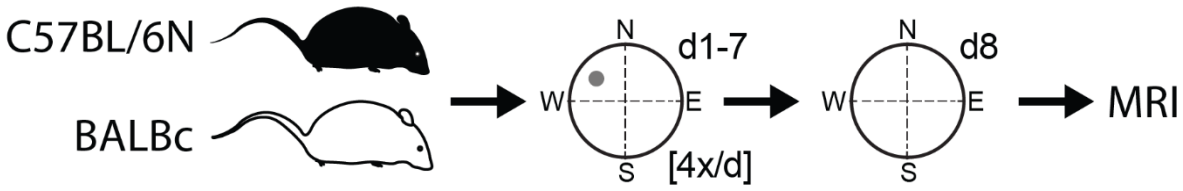

Figure 9: ***Experimental design Exp. 3.*** Adult male group housed C57BL/6N (CR, n = 10) and BALBc (CR, n = 10) mice were trained in the Morris Water Maze to find a hidden platform over the course of seven days with four trials per day, followed by one last probe trial on Day 8 with the platform removed from the water. Thereafter, MRI measurements were performed and compared with results from naive controls of both strains, which remained undisturbed in their home-cages instead of performing MWM testing.

Since the WCM may be not sensitive enough to reveal subtle deficits in memory consolidation as featured in an animal model of MCI, I additionally performed MWM testing, which may be a more challenging spatial memory task.

Nonetheless, both mice strains achieved to obtain spatial memory in the MWM, reflected by a clear preference for the target quadrant in BALBc ($F_{3,27}$ = 9.10, p = 0.0002), as well as in B6N mice ($F_{3,27}$ = 10.15, p = 0.0001; Fig. 10e) in the probe trial without significant line differences (t_{18} = 1.069, p = 0.2991; Fig. 10f,g). Moreover, both strains showed similar swimming path lengths while spatial learning within the first seven days of training (*Strain*: $F_{1,17}$ = 1.843, p = 0.1923; *Strain x Day*: $F_{6,102}$ = 1.939, p = 0.0817; Fig. 10d).

However, BALBc mice needed significant more time to reach the escape platform over the course of training (*Strain*: $F_{1,17}$ = 14.66, p = 0.0013; *Strain x Day*: $F_{6,102}$ = 2.302, p = 0.0396; Fig. 10a) and if the total latency scores were considered (t_{17} = 3.828, p = 0.0013; Fig. 3b; Cohen's *d*: 1.7). Furthermore, BALBc mice were unsuccessful in almost 50 % of the trails to find the platform within 60 sec (t_{17} = 2.846, p = 0.0112). Regarding the failure rate BALBc mice were divided into two groups, high and low failure mice. Interestingly, this bimodal distribution in failures was not associated with deficits in spatial memory consolidation, reflected by similar distance in the target quadrant during the probe trial (Fig. 10f).

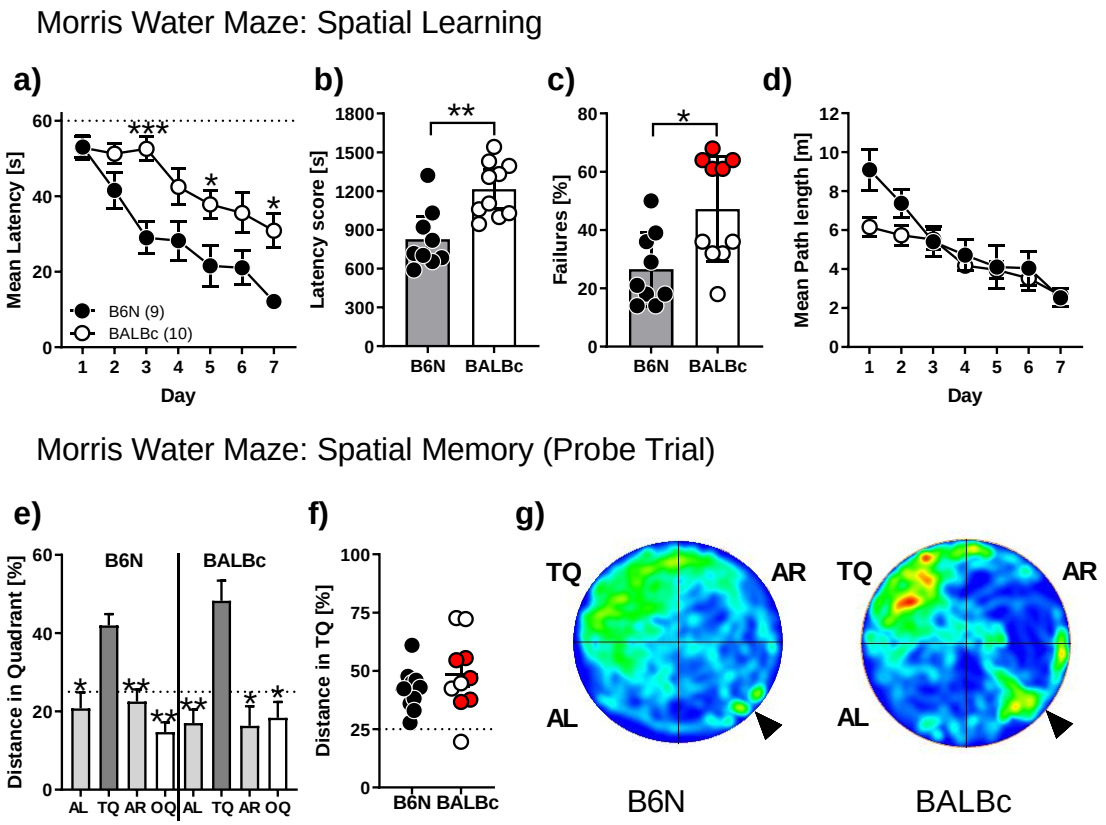

Figure 10: ***BALBc mice were impaired in spatial learning in the MWM, reflected by increased escape latencies, but not in spatial memory consolidation.*** BALBc (n = 10) and B6N mice (n = 10) underwent MWM training over a course of seven days with subsequent probe trial on Day 8. **(a, b)** Escape latencies of BALBc mice were increased throughout training, **(c)** which was associated with a higher percentage of failed trials, in which the animals didn't succeed in finding the platform within 60 sec. However, performance deficits were not associated with deficits in spatial memory consolidation , reflected by **(d)** similar path lenghts while spatial learning and **(e-g)** similar preference for the target quadrant in the probe trial at strain as well as at interindividual level. (AL – adjacent left quadrant, AR – adjacent right quadrant, OQ – opposite quadrant, TQ – target quadrant). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (1-way or 2-way ANOVAs followed by Tukey's post-hoc test or t-test). (a, b, c) Differences between BALBc and B6N mice (e) or non-target quadrant versus target quadrant.

3.4 BALBc mice's hippocampal CA3 region and dentate gyrus are reduced

To get a rough estimate of volumetric strain differences and the brain structures implemented in spatial learning of BALBc and B6N mice (cf. Lerch et al.,2011), I performed structural MRI scans after MWM training and compared the findings to naïve home cage controls.

Structural differences between both strains were observed in increased c1 tissue volume (white matter), c3 tissue volume (ventricular space) and total intracranial volume (TIV) in BALBc mice.

Comparing brain regions involved in spatial learning, DBM measurements revealed several volumetric strain differences. The volume of the nucleus accumbens, the endopiriform nucleus, the lateral amygdala and the CA1 region of the HPC were increased in BALBc mice compared to B6N mice. In contrast, the volume of the anterior commissure, the lateral ventricles, the third ventricle, the antero-medial and antero-ventral nuclei of the thalamus, the hippocampal CA3 region, the subiculum and the dentate gyrus were reduced in BALBc mice compared to B6N mice.

Due to its prominent role in cognition and the formation of memory (Moser et al., 1995; Kesner et al., 2004; Opitz, 2014), I will focus in my analysis on the structural strain differences in the hippocampal formation, namely on the reduced volume of the hippocampal CA3 region and the dentate gyrus (DG) in BALBc mice (FWE = 0.001).

MWM training was associated with a trend towards an increased volume in the CA1 region of the HPC in both strains, which, however, was not significant ($p < 0.001$ (uncorr.); FWE > 0.05). DBM measurements did not reveal any evidence for a strain x training interaction (stats not shown), wherefore training wasn't making a clear distinction between BALBc and B6N mice.

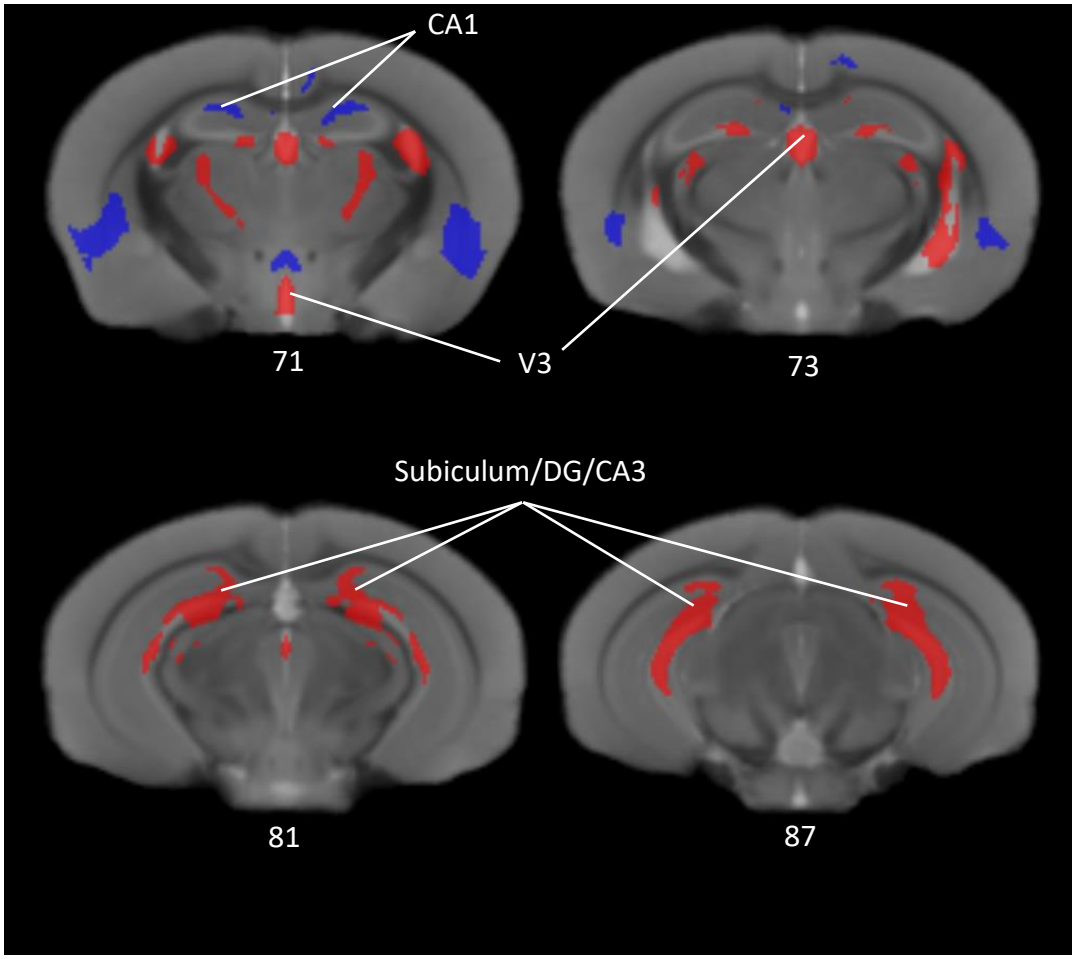

Figure 11: Strain differences in brain morphometry between BALBc and B6N mice. Numbers refer to plates from the Allen Mouse Brain Atlas. Volumetric strain differences are highlighted in red (B6N > BALBc) or blue (BALBc > B6N). CA1: CA1 region of the hippocampal formation; CA3: CA3 region of the hippocampal formation; DG: dentate gyrus; V3: third ventricle. Highlighted regions persist to show volume differences after using family-wise error (FWE) correction.

3.5 Increased mIns levels in BALBc mice are associated with enhanced glial cell activation

After performing behavioral and MRS testing in Exp. 1 and 2, brain samples were collected to search for molecular correlates of the observed strain differences. Therefore, brain probes from the dorsal hippocampus were analyzed for expression of microglia (Iba) and astrocyte markers (S100B and GFAP). In Exp. 1, BALBc mice showed significant higher levels of Iba (t_{15} = 2.294, p = 0.0367, Cohen's *d*: 1.0; Fig. 12), S100B ($U_{44,109}$ = 8, p = 0.0023; S100B was not quantifiable in 8/8 B6N, but only in 2/9 BALBc; chi^2 = 10.58, p = 0.0011) and GFAP (t_{15} = 2.784, p = 0.0139, Cohen's *d*: 1.3). Similar results have been obtained in Exp. 2, reflected in increased levels of Iba (t_{20} = 5.760, p < 0.0001, Cohen's *d*: 2.2), S100B (t_{20} = 15.39, p < 0.0001, Cohen's *d*: 6.2; S100B was not quantifiable in 4/11 B6N, but in 0/11 BALBc; chi^2 = 4.889, p = 0.0270) and GFAP (t_{20} = 6.803, p < 0.0001, Cohen's *d*: 2.8) in BALBc mice. Thus, performance deficits in the WCM and higher mIns levels in the dHPC were associated with increased glial cell activation.

In Exp. 2, levels of PSD95, a postsynaptic marker of excitatory neurons, was analyzed in addition, which did not reveal any further significant strain differences (t_{20} = 1.029, p = 0.316, Cohen's *d*: 0.4; data not shown).

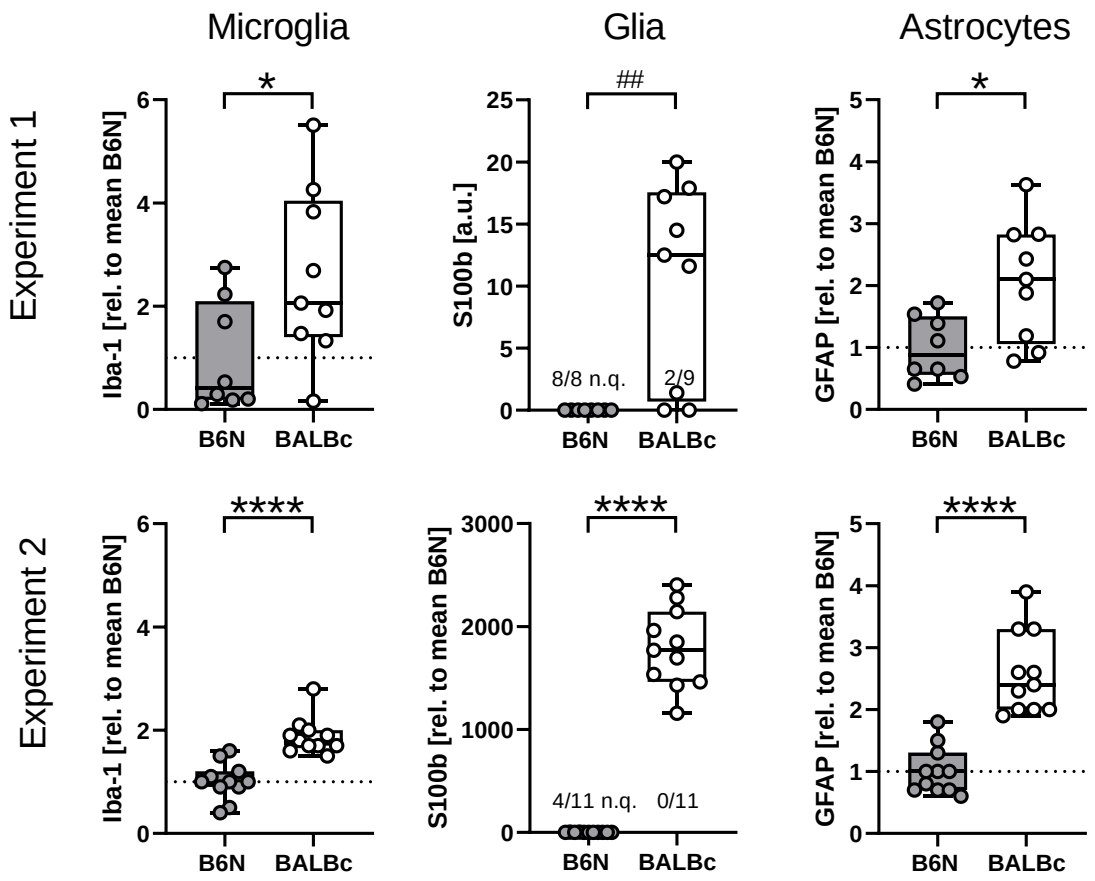

Figure 12: ***Glial markers were elevated in the dHPC of BALBc mice.*** After completing *in-vivo* MRS scans of Exp. 1 and Exp. 2 at an age of 2 vs. 12 months, brain specimens were analyzed by Western blotting. Measurements revealed increased levels of micro- (Iba) and astroglia (GFAP, S100B) markers in BALBc compared to B6N mice. Except for cases in which the signal was not quantifiable (n.q.), results were normalized to the mean of B6N. * $p < 0.05$, **** $p < 0.0001$ (t-test), ## $p < 0.01$ (Mann-Whitney U-test)

3.6 BALBc mice and B6N mice exert distinct metabolic profiles

By performing MassSpec analysis with brain specimens of the dHPC in Exp. 2, I was able to assess the complete metabolic profile of both mice strains and thereby, reveal characteristic strain differences.

The neurotransmitter acetylcholine as well as glutamine and lactate were reduced in BALBc mice. Moreover, BALBc and B6N mice were different in levels of the "carnitine family", whereof in particular reduced levels of the "mitochondrial booster" (Cherix et al., 2020) acetylcarnitine in BALBc mice may interfere with brain metabolism.

Comparing MassSpec *ex-vivo* with MRS *in-vivo* results from the only metabolite measured with both methods, glutamine, I observed similar strain differences indicating high reliability of both methods.

Table 2: ***Metabolomics.*** BALBc (n=15) and B6N mice (n=12) from Exp. 2 showed different metabolic profiles within the dHPC revealed by MassSpec analysis. Whereas cold colors reflect lower levels of the analyzed metabolites in BALBc mice compared to B6N mice, warm colors express the opposite (BALBc > B6N). The metabolites are aligned according to the effect sizes. (t-test, uncorr.)

Metabolite	B6N	BALBc	p-Value	Cohen's d
Hydroxyvaleryl-carnitine	**23668 ± 1626**	**13976 ± 524.8**	**<0.0001**	**2.3**
Pantothenic acid	**147895 ± 9769**	**88085 ± 6278**	**<0.0001**	**2**
nicotinamide	**2607895 ± 1 57403**	**1883355 ± 125118**	**0.0012**	**1.4**
Acetyl-Choline	**250888 ± 21131**	**171917 ± 11613**	**0.002**	**1.3**
glutamine	2643156 ± 151447	2179947 ± 99718	0.014	1
L-Lactic acid	733640 ± 49511	591062 ± 36271	0.0255	0.9
Acetyl-carnitine	919733 ± 85655	697228 ± 43003	0.0209	0.9
isoleucine	5735 ± 426.7	4583 ± 308.9	0.0343	0.8
Octadecenyl-carnitine	742996 ± 27345	866361 ± 44861	0.0425	0.3
NAD+_pos	103925±11772	137070 ± 10452	0.0453	0.8
Carnosine	**49676 ± 2794**	**62865 ± 3275**	**0.0065**	**1.1**
homoserine	**12687 ± 705.2**	**15930 ± 783.1**	**0.006**	**1.1**
3-phospho-serine	**5846 ± 734.1**	**8974 ± 489.6**	**0.0012**	**1.4**
Homocarnisine	**57848 ± 3155**	**80455 ± 3959**	**0.0002**	**1.6**
Threonine	**108391 ± 2672**	**133319 ± 3082**	**<0.0001**	**2.2**
Glutaryl-carnitine	**5077 ± 493.8**	**10616 ± 444.2**	**<0.0001**	**3.1**
^{1}H-MRS glutamine*	0.481 ± 0.016	0.432 ± 0.015	0.041	0.8

* re-analysis of the ^{1}H-MRS spectrograms obtained after completion of training in Experiment 2.

3.7 LAC treatment did not impact spatial learning or mIns levels in BALBc mice

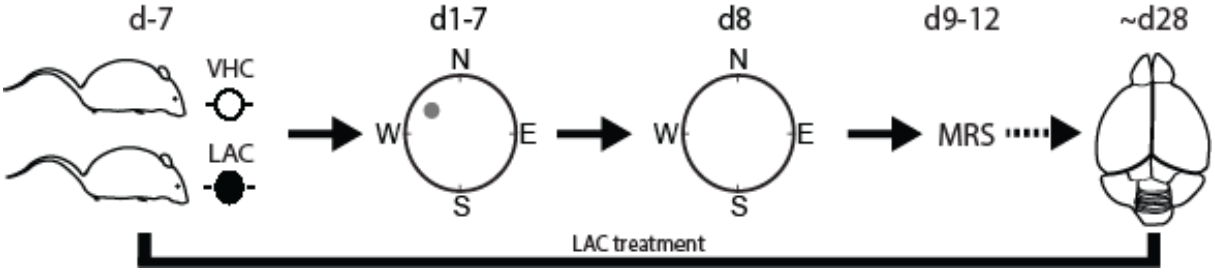

Figure 13: ***Experimental design Exp. 4.*** To observe potential beneficial effects by LAC treatment, I treated BALBc mice with either in water dissolved LAC (n=12) or with tap water (VHC) (n=12). Seven days after the start of the treatment, I compared the performance of BALBc mice treated with LAC with the performance of water treated BALBc mice in the MWM. After performing MWM testing BALBc mice were scanned by MRS measuring mIns content in the dHPC in both cohorts of mice. Brain specimen of the dHPC were collected and analyzed by MassSpec in addition.

MassSpec analysis revealed reduced acetylcarnitine levels in the brain of BALBc mice, the proposed model organism of MCI (Tab. 2). This is in line with results from previous studies, which assign acetylcarnitine a crucial role in brain metabolism (Nasca et al., 2018) as well as beneficial effects on cognition in humans (Montgomery et al., 2003). Therefore, I manipulated acetylcarnitine content in the brain and analyzed potential effects on spatial learning and memory as well as on mIns levels.

Whereas LAC treatment did not impact acetylcarnitine plasma levels (t_{22} = 0.3423, p = 0.7354, Cohen's *d* = 0.1 ; Fig. 14c), LAC-treated BALBc mice showed higher acetylcarnitine levels in the dHPC compared to controls (t_{22} = 3.647, p = 0.0014, Cohen's *d* = 1.5 ; Fig. 14d).

Both groups of BALBc mice improved their spatial learning performance over the course of the training, reflected by decreasing escape latencies ($F_{6, 132}$ = 4.204, p = 0.0007; Fig. 14a), without differences between treated and non-treated group (*Treatment:* $F_{1, 22}$ = 0.7958, p = 0.3820). Interestingly, both cohorts didn't succeed in consolidating their spatial memory, reflected by missing preference for the target quadrant in the probe trial in both cohorts of BALBc mice ($F_{3, 33}$ < 1.5, p > 0.2294; Fig. 14b).

After performing the MWM paradigm mice underwent ^{1}H-MRS to measure mIns levels within the dHPC. LAC treatment did not significantly impact mIns content in the brain, reflected by similar mIns levels in the dHPC in both cohorts (t_{19} = 1.452, p = 0.1672, Cohen's *d*

= 0.6; Fig. 14e).

Thus, LAC treatment could not reverse the cognitive deficits of BALBc mice and didn't affect the mIns levels.

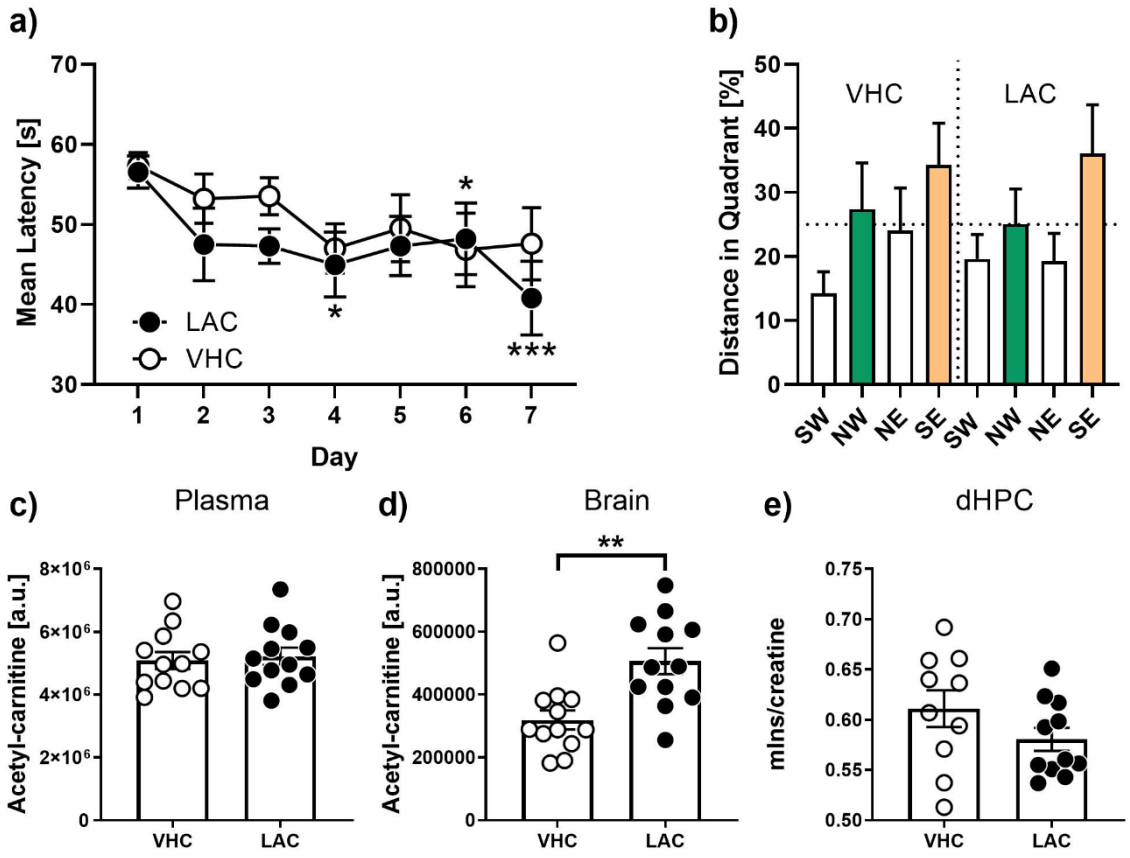

Figure 14: ***LAC treatment does not affect spatial learning or mIns levels in BALBc mice.*** LAC treated (n = 12) and VHC treated (n = 12) BALBc mice were trained in the MWM over the course of 7 days with 4 training trials per day, followed by a probe trial at day 8 whereby the hidden platform was removed from the NW quadrant. **(a**) Both cohorts showed decreasing escape latencies in the MWM over the course of training (2-way ANOVA followed by Dunnett's multiple comparisons). **(b)** Interestingly, both cohorts did not develop any preference for the target quadrant (NW) in the probe trial, which may point to deficits in spatial memory consolidation. **(c)** Whereas plasma acetyl-carnitine levels remained unaffected by the treatment, **(d)** LAC treated BALBc mice showed increased acetyl-carnitine content in the dHPC (t-test). **(e)** The LAC treatment did not result in altered mIns content in the dHPC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

4.1 BALBc mice – a model organism of MCI?

The first aim of my study was the establishment of an animal model of MCI. As previously explained MCI is suspected as a prodromal stage of dementia. The identification of susceptible patients in early prodromal phases may be a crucial step towards improved chances of cure of dementia. Since studies with human patients have not resulted in the desired success, the establishment of specific biomarkers of MCI (Giau et al., 2019), I aimed to exploit the potential of translational research by identifying biomarkers of cognitive impairment in an animal model of MCI. In doing so, I investigated three domains of the animal model. Whereas WCM (Essman and Jarvik, 1961; Kleinknecht et al., 2012) and MWM (Morris, 1984) as test situations with defined behavioral readouts are well-established in cognitive research, the potential of BALBc mice as model organism of MCI hasn't been investigated so far. In this context I observed several behavioral anomalies of BALBc mice, which were reflected in the performance in the applied test paradigms, WCM and MWM.

Compared to B6N mice, the most commonly used lab mice (Bryant, 2011), BALBc mice learned deficiently during the spatial learning training. Increased escape latencies in the WCM (fig. 6; fig. 8) as well as in the MWM (Fig. 10) were consistently detectable over the course of training in both test paradigms with different cohorts of animals.

In contrast to the clear deficits in spatial learning processes, the analysis of memory consolidation revealed ambiguous results. During the probe trial in the MWM, BALBc mice developed the same preference for the target quadrant as B6N mice (fig. 10). Moreover, both strains performed at the same level of accuracy in T1 and T3 of Exp. 2 (fig. 8). However, in Training 2 of Exp. 2, BALBc mice were less accurate in their choice of the target arm (fig. 8). Remarkably, in contrast to the results of Exp. 3, they failed to develop a preference for the target quadrant in the MWM in Exp. 4 (fig. 14). Thus, I observed deficits in memory consolidation in addition to impairments in spatial learning in some of the experiments. However, the deficits in spatial memory in BALBc mice were not consistent. These results are in line with previous studies, which also revealed significant performance deficits in spatial memory tasks in BALBc mice (Upchurch and Wehner, 1988; Francis et al., 1995; Zaharia et al., 1996).

The observed behavioral strain differences need to be discussed further. My data suggest deficits in different cognitive or non-cognitive domains. In the following, I will introduce

different hypothesis discussing the structural classification of the observed deficits.

(1) "BALBc mice are impaired in spatial memory"

First, the performance deficits in both cognitive tasks (fig. 6; fig. 8) may be caused by impairments in the acquisition and consolidation of spatial memory. In fact, I could not observe consistent deficits between spatial memory consolidation and retrieval in the behavioral performance of BALBc mice (fig. 6; fig. 8). The animals' age, however, needs to be considered. R26R mice, a model organism with particular longevity, show memory deficits in the WCM at an start age of 16 month, exclusively (Reichel et al., 2017). In another study, BALBc mice display cognitive decline at an age of 19-21 months old, whereas younger BALBc mice (3-5 months) perform better in cognitive tasks compared to aged controls (Matzel et al., 2008). In my study, BALBc mice were only tested until an age of twelve months, which may explain the missing consistent memory deficits.

Regarding BALBc mice as model organism of MCI, however, it is appropriate that consistent memory deficits are not present, while they start when MCI has progressed into dementia. Prior to the progression to dementia, MCI patients can show reversible and subtle memory deficits (Shimada et al., 2019; Thomas et al., 2020), while they may need to invest more mental effort to handle the cognitive load. More pronounced and consistent deficits may emerge with increasing age when the cognitive reserve may be exhausted (Soldan et al., 2017).

Moreover, the applied behavioral paradigms may not be sensitive enough to detect subtle deficits in spatial memory as featured in an animal model of MCI. This is in line with previous studies, that demonstrated that significant memory deficits in the MWM require pronounced neuronal cell loss. In case of rats, more than 40 % intact dorsal HPC is enough to prevent memory deficits in the MWM (Moser et al., 1995). An optimal test situation in an animal model of MCI would approach the animals' limits of feasibility, so that cognitively impaired animals start struggling to solve the task, whereas "healthy" animals still succeed.

Furthermore, the interindividual performance differences of BALBc mice during the consolidation of spatial memory need to be considered. In fact, I identified two subgroups of BALBc mice in Exp. 2 (fig. 8) as long and short latency mice. Long latency mice did not only show performance deficits, represented by longer escape latencies, but also significant deficits in the consolidation of spatial memory in form of decreased accuracy levels (fig. 8). Thus, deficits in spatial learning have been associated with memory impairment, at least in

long latency mice. Considering the clinical course of MCI (Roberts and Knopman, 2013), short latency mice may represent cognitive stable MCI patients, whereas cognitive impairment of long latency mice may have progressed into dementia with increasing age.

(2) "BALBc mice are impaired in non-amnestic cognitive domains"

The observed deficits in spatial learning in BALBc mice may also result from deficits in other cognitive domains than memory formation due to synaptic plasticity, e.g. executive function or attention. As previously described, MCI is a heterogeneous syndrome with multiple subtypes (Roberts and Knopman, 2013). Cognition includes a variety of processes which interact to form our line of thoughts (Keeler and Robbins, 2011). MCI can be caused by impairment in several cognitive domains, which is reflected in the variety of subtypes (Petersen, 2004; Mansbach et al., 2016). If non-amnestic cognitive deficits caused the observed performance deficits in BALBc mice, BALBc mice may serve as a model organism of non-amnestic MCI. Animal models of non-amnestic MCI are also in demand in translational research about dementia. Patients from both subtypes, amnestic and non-amnestic MCI, show a higher conversion rate to dementia than healthy individuals (Busse et al., 2006; Jungwirth et al., 2012; Petersen, 2016).

One non-amnestic cognitive domain, which disbalance may lead to performance deficits, is the ability to concentrate. In line with that, it may be possible that BALBc mice lack in attentional processes instead of memory deficits leading to obtained performance deficits (fig. 6; fig. 8; fig. 10). This hypothesis is supported by a study in rats that demonstrated that animals with known attentional deficits show increased escape latencies in the MWM (Anisman and McIntyre, 2002), while the administration of Methylphenidate, a therapeutic drug against attention deficit hyperactivity disorder, improves the performance of rats in the MWM (Salman et al., 2019).

The executive function, as another non-amnestic cognitive domain, is suspected to be impaired in BALBc mice, too. BALBc mice are known as a strain which shows lower cognitive flexibility if revaluation of acquired spatial memory is necessary (Francis et al., 1995). This may be caused by deficits in executive function. Executive function is a cognitive domain which is defined as "rapid generation of behavior in response to a new stimulus" (Talpos and Shoaib, 2015). Thus, executive function involves reversal learning as well as attentional flexibility. Reversal learning paradigms are the most frequently used protocols investigating cognitive flexibility in rodents (Tanila, 2018). However, cognitive flexibility is not exclusively

assigned to reversal learning, but also to “common” spatial memory tasks. Every new test situation is *per se* new to the animals and requires flexible adaptations of their behavioral output to the given situation, the applied task. Impairment in BALBc’s executive function and cognitive flexibility may require more time to handle the new test situation, resulting in the given performance deficits (fig. 6; fig. 8; fig. 10).

In context of cognitive deficits, a dysfunction of the serotonergic system, as a neurobiological particularity, may be essential. In fact, BALBc mice have a coding SNP in the gene coding for Tryptophan Hydroxylase-2 (Tph2), which results in reduced serotonin levels in the brain (Zhang et al., 2004). Tph2 is involved in the regulation of brain serotonin synthesis (Zhang et al., 2004), which is suspected to play a key role in mammalian cognition (Geldenhuys and Van der Schyf, 2011). Studies associated low extracellular serotonin content in the brain with impaired memory formation (Porter et al., 2005; Mendelsohn et al., 2009; Cowen and Sherwood, 2013), while MCI patients lack serotonin transporter availability (Smith et al., 2017). Moreover, Tph2 conditional knockout (Tph2 CKO) AD mice showed increased amyloid plaque generation (Xu et al., 2019), which may have led to frank memory impairment. Thus, as a hypothesis, the SNP in the gene coding for Tph2 in BALBc mice may be part of the underlying mechanisms resulting in the observed deficits in spatial memory tasks (fig. 6; fig. 8; fig. 10).

(3) “BALBc mice are impaired in non-cognitive domains”

Controversially, the literature involves evidence that BALBc mice are not cognitively impaired. The observed behavioral anomalies may result from deficits in non-cognitive domains, independent of MCI.

Motor function, as an example of a non-cognitive domain, can be deficient followed by increased latencies resulting in performance deficits in spatial memory tasks (Lindner, 1997). Moreover, it has been shown in previous studies that locomotor disturbances other than swimming speed, e.g. thigmotaxis swimming, also lead to increased escape latencies in the MWM (Cain et al., 1996; Saucier et al., 1996; Hölscher, 1999). In line with this hypothesis some studies revealed decreased swimming speed of BALBc compared to B6N mice (Klapdor and van der staay, 1996; Van Dam et al., 2006). However, increased floating as the main causal factor instead from deficits in motor functions was not discussed. Floating is suspected to be a passive stress coping strategy (Commons et al., 2017), which is used more often by BALBc mice than by other mouse strains, e.g. B6N, at least at similar water

temperatures as in my study (22 °C) (Bachli et al., 2008). Based on that, further measurements as swimming speed while excluding periods of immobility need to be evaluated and integrated in potential follow-up studies

Increased anxiety levels and stress susceptibility of BALBc mice may be other non-cognitive factors, which may contribute to the observed performance deficits in the spatial memory tasks (Belzung and Le Pape, 1994; Belzung and Berton, 1997; Chapillon and Debouzie, 2000; Belzung and Griebel, 2001). High levels of anxiety normally correlate with an increased individuals stress load (Frank et al., 2006; Sandi et al., 2008; Castro et al., 2012). Chronically increased stress load impairs memory functions as spatial learning (Hölscher, 1999) by inhibiting the induction of LTP which is crucial for forming memory (Diamond et al., 1990; Xu et al., 1997; Hölscher, 1999). Based on these mechanisms stressed rodents are impaired in memory consolidation (Xu et al., 2015), as well as in the acquisition and retrieval of spatial memory (Luine et al., 1994; de Quervain et al., 1998).

Reducing the stress load by e.g. handling the animals in advance improves the performance of stressed mice in spatial learning tasks (Hölscher, 1999), in particular in BALBc mice (Zaharia et al., 1996).

Thus, enhanced stress load may have an essential impact on the performance of BALBc mice in spatial memory tasks. Nonetheless, I couldn’t observe any significant correlation between an increased stress load, reflected by increased floating behavior, and the consolidation of spatial memory, reflected by similar accuracy levels in both strains (fig. 6; fig. 8). Moreover, floating as well as non-floating BALBc mice showed increased escape latencies (fig. 6). This contradicts the hypothesis that the observed spatial learning deficits are caused by intensified passive stress coping (floating) in response to an increased stress load.

To conclude, my data, together with the literature suggest BALBc mice as a model organism of MCI. The observed spatial learning deficits point to impairments in spatial memory or in non-amnestic cognitive domains (e.g. executive function), which may be caused, at level of genetics, by a SNP in the gene coding for Tph2.

Additionally possible, impairments in non-cognitive domains, however, need to be evaluated in further studies. Confounding, non-cognitive factors such as deficits in motoric function or stress coping may interfere in the analysis of an animal model of MCI.

4.2 Morphologic strain differences in the brain

By performing volumetric MRI scans after the mice completed MWM training and comparing the results with scans from naïve home-cage controls, I aimed to identify volumetric strain differences as well as morphologic changes of brain structures involved in spatial learning or memory.

One of the key structures in the formation of spatial memory is the hippocampus, which integrates information from hippocampal cells (e.g. place cells (O'Keefe, 1976)), parahippocampal/postrhinal cortices, as well as the entorhinal cortex (Lavenex and Amaral, 2000) and creates a "cognitive map" of the individual's environment (O'Keefe et al., 1975). The HPC can be divided into different anatomical subregions (Schultz and Engelhardt, 2014), whereof the DG, CA3 and CA1 region form a trisynaptic circuitry interacting as memory-encoding system (Kesner et al., 2004; Rolls, 2007, 2018). Afferent input from the entorhinal cortex (perforant path) enters the trisynaptic network to a large extent through the DG (van Groen et al., 2003). The DG projects via Mossy fibers to the CA3 region (Xiong et al., 2017), where the information can be integrated into the autoassociative network of the CA3 region (Mishra et al., 2016) or forwarded to the CA1 region via Schaeffer collaterals (Xiong et al., 2017). The CA1 region is the origin of the primary output of the HPC to the neocortex (Kesner et al., 2004), where long-time memory is stored (Maviel et al., 2004). Comparing the functional roles of the CA1 and CA3 region relating to spatial memory, the CA3 region is particularly involved in the initial acquisition of spatial memory (spatial learning) (Lassalle et al., 2000; Florian and Roullet, 2004), supported by the DG contributing to neuronal pattern separation (Leutgeb et al., 2007). Long-time retrieval, in contrast, may be performed by the CA1 region (Lee and Kesner, 2004). In line with the literature, BALBc mice had decreased volume in the CA3 region and the DG compared to B6N mice (fig. 11), which may have caused the observed spatial learning deficits in the MWM. In contrast, the retrieval of spatial memory in the probe trial wasn't disturbed (fig. 10), which is in line with the increased volume of the hippocampal CA1 region in BALBc mice (fig. 11).

The reduced volume of the CA3 region may be based on the high stress susceptibility of BALBc mice prior to spatial learning (Belzung and Berton, 1997; Belzung and Griebel, 2001). Vyas et al. have shown that chronic stress load can lead to hippocampal atrophy in the CA3 region. This may be caused by a dendritic remodeling in response to chronic stress exposure, which is characterized by shortening and debranching of apical dendrites (Watanabe et al.,

1992; Conrad et al., 1996; Sousa et al., 2000; Vyas et al., 2002; Dias et al., 2014). The morphological changes may be a sort of stress coping strategy, which result in a decreased hippocampal volume, mostly pronounced in the CA3 region (Vyas et al., 2002), and thereby, to the observed spatial learning deficits in BALBc mice.

Depending on the applied training regimen, spatial learning may lead to a volumetric increase in distinct brain structures. Whereas idiothetic (response) learning results in a volumetric increase in the striatum, allocentric (place) learning is associated with specific growth of the hippocampus (Lerch et al., 2011). In my study, MRI scans were performed shortly after MWM training, which promotes hippocampus-dependent allocentric learning strategies (Vorhees and Williams, 2014; Braun et al., 2015). In line with the literature, I could observe a trend towards an increased volume in the dorso-rostral HPC in response to spatial learning, which, however, failed to reach statistical significance after correction.
Comparing the effects of spatial learning at strain level, MWM training is not making a clear distinction between BALBc and B6N mice, reflected by the lack of significant interaction between the factors “strain” and “training”. This might relate to the fact that both mice strains consolidated the spatial memory, reflected by the similar preference for the target quadrant in the probe trial. Thus, the similar neuronal matrix may have been activated in both strains leading to analogous morphological changes.

4.3 Hippocampal mIns serves as marker of cognitive impairment at strain level, but not at interindividual level

By comparing mIns levels of BALBc and B6N mice, I aimed to establish mIns as marker of cognitive impairment. Indeed, naïve (fig. 6; fig. 8) as well as older, and swimming/handling-experienced (fig. 8f) BALBc mice had increased mIns content in the dHPC. This finding was a consistent strain-specific trait unaffected by increasing age or behavioural experience. My findings of increased mIns content in BALBc mice promote the translational potential as similar observations were made in human MCI (Franczak et al., 2007; Wang et al., 2009; Zhang et al., 2009) and AD patients (Miller et al., 1993; Kantarci, 2013; Voevodskaya et al., 2019). However, alterations of mIns levels in human MCI patients were not sensitive enough to predict the disease progression and to identify those MCI patients, which will suffer from dementia at later age (Kantarci et al., 2009; Zhang et al., 2015).

Within BALBc mice, mIns levels served as marker of spatial learning deficits at interindividual level, reflected by a significant correlation between floating behavior and mIns levels (Fig. 6k) in experiment 1. This correlation, however, was not robust over the course of the study while all BALBc mice, regardless of varying escape latencies, exhibited elevated mIns levels in Exp. 2 (Fig. 8k).

Taken together, mIns levels hold the potential as marker of cognitive impairment at strain level (BALBc vs B6N; fig. 6; fig. 8), however, the prognostic value of mIns levels at an interindividual basis may be limited (LL vs SL mice; fig. 8).

The reason why mIns levels failed to predict interindividual performance deficits in BALBc mice over the course of the study may be found in the missing genetic variability of inbred strains. The behavioral output of each animal is mainly formed by two differing factors: genetics and environment. Since inbred mice strains are genetic identical (isogenic), environmental factors must be the main factor for behavioral differences within inbred mice strains (Loos et al., 2015). Indeed, it has been shown that environmental factors, e.g. intrauterine position, can lead to intraindividual changes in behavior within inbred mice strains (Lathe, 2004), most likely through influencing the animals epigenetic machinery (Kaminsky et al., 2009; van Dongen et al., 2012). Epigenetics and other lead to differences in interindividual susceptibility to environmental factors, resulting in high or low “phenotypic robustness” of inbred strains (Queitsch et al., 2012). BALBc mice exhibit a high phenotypic robustness, while showing low interindividual variability in their behavior (Loos et al., 2015). This character trait may make it more difficult in BALBc mice than in other mice strains to assess predictive biomarkers on an interindividual level.

4.4 What are the underlying mechanisms of increased mIns levels and how may they lead to cognitive impairment?

As previously described, patients of MCI, cognitive impaired BALBc mice as well as AD patients exhibit enhanced level of mIns. Whereas a broad body of literature tries to understand the pathophysiology and the mechanisms underlying AD, little is known about MCI. In brief, multiple pathological processes such as amyloid deposition or neuronal loss may take place in the brains of MCI patients either simultaneously or separately, resulting in multifaceted cerebral dysfunction (for review: (Mufson et al., 2012)). Mechanisms trying to clear the overload of beta-amyloid include glial activity (Frautschy et al., 1992; Weldon et al.,

1998; Streit et al., 2004) and degrading processes such as autophagy (Nilsson et al., 2013). Increased levels of mIns were described resulting from enhanced glial activity (Brand et al., 1993; Best et al., 2014). The lack of MCIs patient samples due to the early, non-lethal onset, may explain the lack of knowledge about mechanisms behind cognitive decline and increased levels of mIns. In my study, however, I aimed to investigate the molecular signature in a model organism of MCI by using western blotting (fig. 12)

In a first step, I investigated if increased mIns content in the brain is associated with glial cell activation. Therefore, protein levels of Iba-1, GFAP and S100B, which are associated with glial cell activity (Ito et al., 1998; Rothermundt et al., 2001; Schroeter and Steiner, 2009; Zhang et al., 2019), were studied and found to be elevated in both cohorts (Exp. 1, Exp. 2) of BALBc mice (fig. 12). Enhanced glial activity is associated with neuroinflammation (Hernández-Rabaza et al., 2016) and amyloid deposition (Barger and Harmon, 1997; Edison et al., 2018), which could lead, like in progressing AD pathology, to neuronal loss (Lull and Block, 2010).

Based on previous findings, it is conceivable that the increased mIns content in the brain of BALBc mice is induced by elevated astrocytic, but not microglial activity. Whereas enhancing microglial activity didn't impact mIns levels (Yanez Lopez et al., 2019), inhibiting microglial activity, induced by the appliance of minocycline, resulted in increased, but not reduced mIns content in the brain (Khiat et al., 2010). In contrast, inducing astrocytic reactivity led to increased mIns content in the brain (Ligneul et al., 2019). In line with these results, protein levels of GFAP, an astrocytic marker, were constantly elevated in BALBc mice in my study (fig. 12). It need to be considered, however, that elevated GFAP levels can also be induced by an increased cell number instead of astrocytic reactivity.

In contrast to glial markers, however, neuronal markers as NAA/NAAG (MRS measurements; fig. 6; fig. 8) as well as PSD-95 in Western blotting, were predominantly not different between both mouse strains. Only at an age of 12 months BALBc mice showed slightly elevated NAA/NAAG-levels in the vHPC, but not in the dHPC (fig. 8), what couldn't be confirmed in protein content analysis.

Therefore, it is necessary to draw attention on the distinct functional roles of the ventral versus the dorsal HPC. Whereas the dHPC is primarily involved in cognition and memory, the vHPC is mostly associated with emotionality and anxiety (Bannerman et al., 2003; Fanselow and Dong, 2010; Reichel et al., 2017). As previously described, BALBc mice are known to be more anxious compared to other mouse strains (Belzung and Berton, 1997; Belzung and

Lemoine, 2011). Thus, in general higher emotional loads of anxiety and stress may be enhanced over the course of different experimental procedures, resulting in increased neuronal activity, neuronal growth, and therefore led to increased NAA/NAAG-levels in the vHPC of BALBc mice.

On the search for the underlying mechanisms of cognitive impairment, neuronal degeneration is mostly designated as one of the key processes in the pathogenesis of cognitive deficits (Jeong, 2017), supported by routinely decreased NAA/NAAG-levels in human MCI patients (Franczak et al., 2007; Wang et al., 2009; Watanabe et al., 2010). However, neuronal degeneration may have not yet started at very early stages of cognitive impairment, reflected by similar levels of neuronal markers in BALBc mice (fig. 6; fig. 8), whereas astrocytic activation may play a pioneer role, reflected by increased mIns levels and cellular glial markers in BALBc mice (fig. 6; fig. 8; fig. 12). My data is in line with few results from human studies which associate increased marker of astrocytic activation, e.g. mIns levels, with earlier stages of AD in humans, whereas changes of NAA/NAAG-levels as neuronal marker appear at later stages of AD (Kantarci et al., 2008; Voevodskaya et al., 2016). Moreover, alterations in mIns-levels in cognitively impaired humans (Kantarci et al., 2000; Catani et al., 2001; Huang et al., 2001) as well as in transgenic changed AD mice (APP/PS1) (Chen et al., 2009) can also occur in the absence of reduced NAA/NAAG-levels.

The mechanisms how glial activation may initiate the pathological processes leading to cognitive deficits or even AD, are not yet fully understood. According to the neuromodulation theory, activated glial cells initiate a pathological cascade, which promotes tau hyperphosphorylation in AD patients. To be more precise, it is postulated that AD pathogenesis starts with a neuroinflammatory reaction of glial cells on “damage signals”, which results in tau hyperphosphorylation and neuron damage (Maccioni et al., 2018b; Maccioni et al., 2018a). In particular, the suspected general glial marker S100B (Rothermundt et al., 2001; Schroeter and Steiner, 2009), elevated in both cohorts of BALBc mice (fig. 12), may exert negative influence on cognition. Transgenic mice overexpressing S100B were strongly susceptible to neuroinflammation (Craft et al., 2005) and showed analogous changes in the brain as mice with AD (Shapiro et al., 2010), whereas reduced S100B synthesis leads to reduced beta-amyloid plaque forming in the brain (Mori et al., 2010).

To conclude, increased mIns levels were in line with increased glial cell activation in BALBc mice at molecular level. Glial cell activation may have led to the observed deficits of BALBc

mice, whereas markers of neuronal degeneration were not detectable in this animal model of MCI.

4.5 BALBc mice show an altered metabolic profile which is particularly defined by deficits in astrocytic contribution to brain metabolism

Next to *in vivo* measurements of mIns and the molecular quantification of glial protein markers, other mechanisms may be involved in the process of cognitive deficits associated with MCI. By performing MassSpec analysis I was able to assess the complete metabolic profile of both mice strains (see table 2).

Acetylcholine, a neurotransmitter known to play a critical role in cognitive processes and memory function in the brain (Ferreira-Vieira et al., 2016), was reduced in the brain of BALBc mice. The cholinergic system modulates essential neural functions as acquisition, consolidation and retrieval of memory (Blokland et al., 1992; Boccia et al., 2003; Power et al., 2003). In line with this, MCI patients have a reduced cholinergic function (Shinotoh et al., 1999; Rinne et al., 2003; Schliebs and Arendt, 2011). Moreover, in the pathogenesis of AD, the degeneration of acetylcholinergic neurons in the nucleus basalis of Meynert is a crucial process leading to memory loss (Whitehouse et al., 1981; Whitehouse et al., 1982). Counteracting this dysfunction, the most common class of drugs in the treatment of AD are cholinesterase-inhibitors, which inhibit the breakdown, and therefore enhance levels of acetylcholine (Mufson et al., 2008). Accordingly, BALBc seem to be construct valid in the pathological processes underlying MCI.

With an interfered glucose metabolism, energy homeostasis including reduced lactate content seems to be disturbed in the brain of BALBc mice (table 2) as in AD and MCI patients (Arnold et al., 2018; Croteau et al., 2018; Weise et al., 2018). Essential for the allocation of ATP is the glycolysis with its catalyzing enzymes. Such enzymes as Phosphoglycerat-mutase, Enolase or Glyceraldehyde-3-phosphate dehydrogenase are described to be disturbed in the pathology of AD (for review: (Butterfield and Halliwell, 2019)).

Reduced lactate content in BALBc mice suggest glycolysis-disturbing processes as well. Whereas the main product of glycolysis, pyruvate, is primarily introduced into the TCA cycle in mitochondria, pyruvate can also be transformed into lactate. Instead of feeding the mitochondrial electron transport chain resulting in the most efficient ATP production, lactate is produced under anaerobic conditions, known as anaerobic glycolysis. However, specific cell

types such as astrocytes regularly transform pyruvate to lactate through aerobic glycolysis under regular conditions (Lovatt et al., 2007; Zhang et al., 2019), known as the Warburg effect (Warburg, 1956), whereas neurons' energy production is based on the TCA cycle in mitochondria (Herrero-Mendez et al., 2009; Magistretti and Allaman, 2015). Reasonably it can be assumed that the differing metabolic profiles of neurons and astrocytes have synergistic effects by partly transferring lactate from astrocytes to neurons (Wyss et al., 2011; Machler et al., 2016). Known as astrocyte–neuron lactate shuttle model several studies support this theory (for review (Magistretti and Allaman, 2018); fig. 15).

Restrictions in the availability of lactate for neurons e.g. by inhibiting astrocytic glycogenolysis (Gibbs et al., 2006) or the transfer of lactate from astrocytes to neurons (Suzuki et al., 2011; Tadi et al., 2015), can result in cognitive deficits. Interestingly, reduced lactate content has also been observed in homogenized brain probes from the double-transgenic amyloid precursor protein/presenilin 1 (APP/PS1) mouse model of AD, thereby associated with defective lactate transporters (Zhang et al., 2018). With this, my data support the hypothesis of a disrupted astrocytic metabolism or of a deficient astrocyte-neuron lactate transport. Reflected by reduced lactate content in brains of BALBc mice (table 2), these deficits may have led to the observed performance deficits in spatial memory in the model organism of MCI.

Next to the direct energy support of neurons, astrocytes' contribution includes other molecules in addition, e.g. the synthesis of glutamine (fig. 15). Glutamine is the immediate precursor of glutamate, the major excitatory neurotransmitter of the CNS (Tapiero et al., 2002), which plays a crucial role in synaptic plasticity and forming memory (Riedel et al., 2003). The synthesis of glutamine takes place in astrocytes exclusively (Schousboe et al., 2014). In the glutamate-glutamine cycle, glutamate is converted into glutamine, followed by a transport into neurons, where it is back-transformed into glutamate again (Walton and Dodd, 2007). This glutamate-glutamine cycle is crucial for neuronal plasticity and accomplished to a large extent in astrocytes (Rose et al., 2013). Interfering the glutamate-glutamine cycle by inhibiting the glutamine synthesis in astrocytes leads to significant memory deficits in chicks and rats (Gibbs et al., 1996; Gibbs and Hertz, 2005; Kant et al., 2014). Cognitively impaired BALBc mice, compared to B6N mice, have reduced glutamine content. Data revealed by MassSpec analysis (table 2) as well as in MRS measurements point to the potential influence of astrocytic metabolism on the observed performance deficits in

spatial memory tasks (fig. 6; fig. 8; fig. 10).

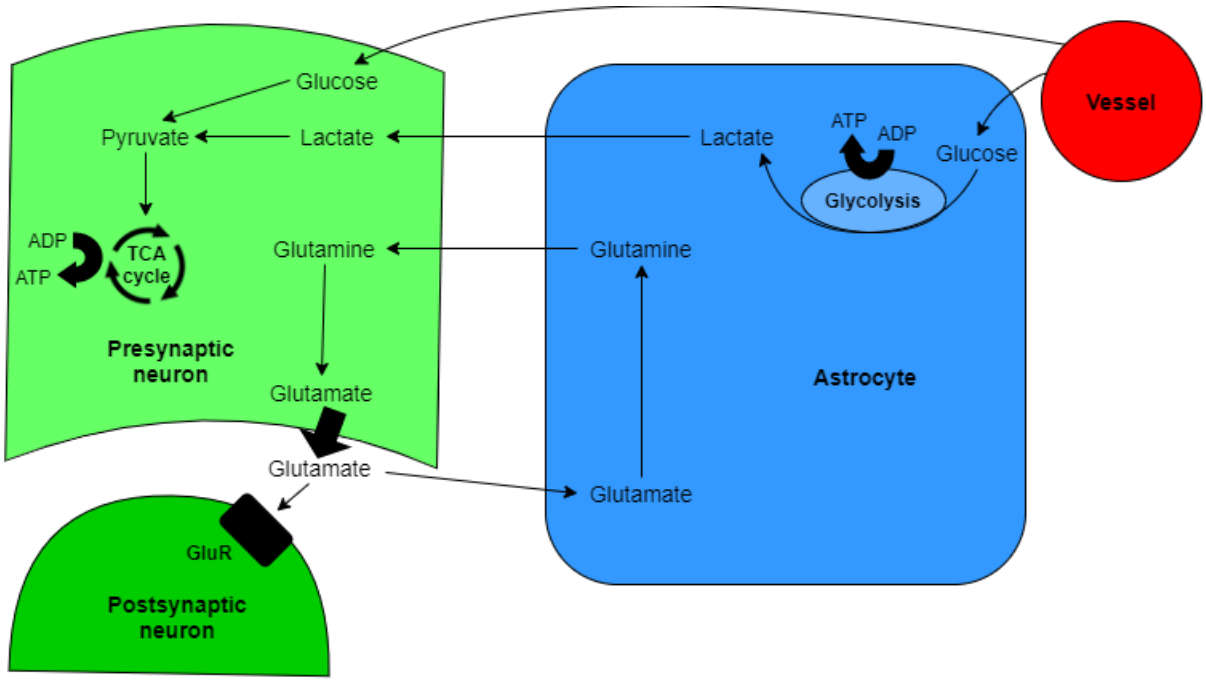

Figure 15: ***Neuron-astrocyte metabolic coupling.*** Neuronal energy generation is based on the availability of lactate, which is performed to a large extent in astrocytes by glycolysis. Glutamine synthesis, which is essential for glutamatergic transmission between neurons, takes place in astrocytes exclusively. GluR = glutamatergic receptor; TCA cycle = tricarboxylic acid cycle; ADP = adenosine diphosphate; ATP = adenosine triphosphate (adapted from Magistretti and Allaman, 2015).

4.6 L-acetylcarnitine (LAC) "doesn't cure" the behavioral phenotype of BALBc mice

On closer inspection, the MassSpec results revealed major differences in the content of several metabolites of the "carnitine family" within both strains (table 2). Of particular interest is the reduced content of acetylcarnitine in the hippocampus of BALBc mice. Plasma acylcarnitines are proposed to play a key role in brain metabolism (Ciavardelli et al., 2016) by providing high-energy acetylgroups to metabolic pathways (Pettegrew et al., 1995; Jones et al., 2010). Moreover, acetylcarnitine improves overall metabolic function by preserving mitochondrial membrane integrity (Aliev et al., 2009; Jones et al., 2010). With this, the "mitochondria-boosting supplement" (Cherix et al., 2020) is able to enhance beta-oxidation and energy metabolism in the brain (Nasca et al., 2018). In fact, mitochondrial dysfunction is suggested to play a major role in the pathogenesis of AD (Lustbader et al., 2004; Beal, 2005; Chen and Zhong, 2013), while preceding the rise of amyloid plaques and neurofibrillary

tangles in preclinical models of AD (Yao et al., 2009; Ciavardelli et al., 2016). Aging-dependent mitochondrial DNA mutations (Melov et al., 1995; Michikawa et al., 1999) may lead to an overproduction of mitochondrial reactive oxygen species, resulting in increased amyloid generation ("mitochondrial cascade hypothesis") (Swerdlow et al., 2014; Swerdlow, 2018). In line with mitochondrial dysfunction, studies found several mitochondrial enzymes, as pyruvate dehydrogenase, isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase and cytochrome C oxidase, impaired in AD (Bubber et al., 2005). Reduced systemic and brain acetylcarnitine content may contribute to the pathological mitochondrial processes taking place in the brains of cognitively impaired humans as well as in BALBc mice, the potential model organism of MCI.

Moreover, oral acetylcarnitine dose induces epigenetic mechanisms, leading to enhanced transcription of *grm2*, encoding for the mGlu2 receptor (Nasca et al., 2013). The mGlu2 receptor is known to modulate synaptic transmission in the hippocampus (Kew et al., 2002; Higgins et al., 2004). Several studies have shown that mGlu2 receptor agonists have beneficial effects on cognition in rodents as well as in humans (Moghaddam and Adams, 1998; Krystal et al., 2005; Lyon et al., 2011). On the other side, antagonists of the mGlu2 receptor were shown to be beneficial on memory processes as well, and, therewith, discussed as potential neuroenhancing treatment (Gregory et al., 2003; Higgins et al., 2004; Goeldner et al., 2013).

Accordingly, supplying acetylcarnitine may reduce deficits in brain metabolism or synaptic transmission, which is in line with beneficial effects on cognitively impaired patients (Montgomery et al., 2003). Moreover, correlating levels of plasma- and brain acylcarnitine hold the potential as pro- and diagnostic blood marker for cognitive deficits (Mapstone et al., 2014).

Even if BALBc mice showed reduced acetylcarnitine content in the brain (table 2), oral application of L-acetylcarnitine could not reverse the cognitive deficits and both groups performed at similar level in the MWM (fig. 14). The treatment did neither impact spatial learning (fig. 14a) nor memory consolidation (fig. 14b), reflected by similar escape latencies as well as no preference for the target quadrant during the probe trial. It did also not affect the mIns content in the brain, which would have promoted decreased glial cell activation in the treated cohort (fig. 14e).

The reason of the lack of success by l-acetylcarnitine treatment in my setting, however,

needs to be discussed further since the potential remains promising.

First, the treatment protocol lacked detectable impact on peripheral levels of LAC (fig. 14c), suggesting a defective way of application, whereof the treatment couldn't exert its beneficial effect. Other than peripheral level, however, I could detect significant increased levels of LAC in the dHPC compared to the non-treated controls (fig. 14d). Since I used a similar treatment protocol as described in previous studies with observed effects on behavior (Nasca et al., 2013; Cherix et al., 2020) and LAC arrived at its target region in my experiment, the missing efficacy must have other reasons than "wrong application" of the substance.

Second, LAC treatment may only exert subtle influence on cognition. Even if clinical investigations suggest a neuroenhancing function of LAC, many rodent studies focused on anti-depressional effects of the treatment, reflected by less immobility time in behavioral tasks (Nasca et al., 2013; Cherix et al., 2020), while there is only sparse evidence of diminishing cognitive deficits in rodents (Singh et al., 2018). The cognitive deficits of BALBc mice in the MWM test may have been already too pronounced and advanced, so that the treatment may have remained without effect. Appliance of the therapeutic intervention at earlier stages of cognitive impairment may have shown stronger effects. Alternatively, a treatment together with the simpler Water Cross maze as behavioral test situation may result in a better outcome, whereas the cognitive reserve of BALBc mice may have been too exhausted for an improvement in the complex MWM.

In addition, in a recent published study, the efficacy of the treatment depended on interindividual behavioral differences such as the degree of social avoidance (Cherix et al., 2020). In my study, however, BALBc mice weren't ranked according to differing behavioral phenotypes, which may have reduced the sensitivity of the analysis.

Third, LAC treatment may not correlate with mIns levels and its underlying mechanisms. Increased glial cell activation and defects in astrocytes' metabolism are potential reasons for altered behavior and changed levels of mIns in BALBc mice. LAC treatment, however, is mainly suspected to exert its effects by enhancing oxidative processes in mitochondria (Nasca et al., 2018), which may primary takes place in neurons (Magistretti and Allaman, 2018), whereas astrocytes metabolism may remain unaffected by the treatment. In line with this, LAC treatment had no effect onto levels of mIns (fig. 14e). Thus, other treatments, which mainly affect astrocytes metabolism instead of neurons metabolism, may be more suited as potential medication for BALBc mice.

5. Summary and outlook

In the first part of my study I compared the behavioral performance of BALBc (potentially susceptible) and B6N (potentially resilient) mice in two established spatial memory tasks, WCM and MWM. BALBc mice exhibited deficits in spatial learning, reflected by increased escape latencies, in both test paradigms, whereas my data didn't reveal consistent memory deficits in the potential model organism of MCI. In close to 1-year aged BALBc mice, however, deficits in spatial learning were directly related to memory impairment.

These findings support the face validity of BALBc mice as model organism of MCI, characterized by subtle, age-dependent cognitive deficits (Shimada et al., 2019; Thomas et al., 2020). Consistent memory deficits may occur in more challenging memory tasks as the Atlantis Platform (Buresova et al., 1985; Spooner et al., 1994) or the Novel object recognition task (Ennaceur and Delacour, 1988), and at older age.

Considering the varying cognitive decline of human MCI patients, it would be of interest to investigate the behavioral performance of BALBc mice with advancing age. Whereas some mice may stay in the non-pathological age-dependent range, others may expose a pathological cognitive decline above the norm. Moreover, further studies are necessary to investigate the potential of BALBc mice as model organism of early stages of AD. Therefore, it would be of interest to measure the amyloid and hyperphosphorylated tau protein load to support the etiological validity of the animal model.

In the second part of my study, I correlated the behavioral performance with *in vivo* ^{1}H-MRS measurements, focusing on mIns- and NAA/NAAG-levels. Similar to MCI patients, low performing BALBc mice showed consistently elevated mIns levels in the dHPC, which promotes mIns as a marker of spatial memory performance deficits at strain level. However, they failed to predict the further cognitive decline on an interindividual level, which may be attributed to a certain part to the missing genetic variability of inbred mice strains.

Molecular analyses were able to associate elevated mIns with enhanced glial cell activity by increased glial markers such as Iba, GFAP and S100B. Neuronal markers as NAA/NAAG (MRS) and PSD-95 (WB), however, were not altered in BALBc, compared to B6N mice. Glial cell activation in prodromal stages may initiate pathological processes, resulting in neuronal degeneration followed by profound cognitive dysfunctions in progressed stages (Maccioni et al., 2018b; Maccioni et al., 2018a).

A promising therapeutic approach in the treatment of BALBc mice would be the appliance of

lithium. Based on the “inositol-depletion hypothesis” (Harwood, 2005), lithium is suspected to induce its therapeutical effect by the inhibition of several enzymes involved in inositol metabolism (e.g. inositol-1(or 4)-monophosphatase)(Hallcher and Sherman, 1980) and thereby decrease the myo-inositol content in the brain (Allison and Stewart, 1971). In line with this hypothesis, several MRS studies revealed reduced mIns levels in humans (Moore et al., 1999; Davanzo et al., 2001) and rodents (Huang et al., 2000) after lithium treatment.

In addition to ^{1}H-MRS measurements, *in- vivo* DBM measurements revealed decreased volume of two structures crucially involved in the initial acquisition of spatial memory, the hippocampal CA3 region and the DG, which may have interfered with spatial learning in BALBc mice.

Next to *in vivo* imaging procedures, I investigated strain differences in hippocampal metabolism using *ex vivo* MassSpec analyses. In doing so, reduced levels of acetylcholine emphasize construct validity in the underlying mechanisms of MCI. While the cholinergic system modulates essential neural functions (Blokland et al., 1992; Boccia et al., 2003; Power et al., 2003), deficits are crucially involved in the pathogenesis of cognitive decline (Whitehouse et al., 1981; Whitehouse et al., 1982).

Moreover, reduced levels of glutamine and lactate, both mainly provided by astrocytes in the brain (Wyss et al., 2011; Schousboe et al., 2014; Machler et al., 2016), are in a line with disruptions in neuronal-glial metabolic coupling. Considering the increased levels of GFAP in WB analysis as well as the results of MassSpec analysis, my data points to altered metabolism in astrocytes, which may have led to elevated mIns content in the brain of BALBc mice. An ideal therapeutic application would enhance astrocytes’ metabolism, while decreasing glial activity.

A pharmacological intervention, enhancing mitochondrial metabolism, is acetylcarnitine (Aliev et al., 2009; Jones et al., 2010). While increasing levels of acetylcarnitine in MCI patients could increase their cognitive performance (Montgomery et al., 2003), BALBc mice were unaffected in its spatial memory performance.

Contrary to my previous experiments, BALBc mice were impaired in the acquisition of spatial memory in this experiment. Therefore, it may be interesting to repeat MWM testing in follow-up studies to investigate if these results are reproducible. If BALBc mice fail continuously to form spatial memory in the MWM task, they might be even more impaired in cognition than expected.

To conclude, my study promotes an animal model of MCI using BALBc mice as a model organism. Moreover, I could demonstrate that increased mIns levels are associated with performance deficits in spatial memory paradigms and suggesting altered glial cell activation, in particular astrocytic activation. Treatment using acetylcarnitine, however, remained without detectable effect.

The prevalence of dementia increases from year to year (Ferri et al., 2005), while treatment options remain limited. The establishment of prognostic marker in prodromal stages is suspected as crucial step towards effective therapies. However, little is known about prodromal marker of cognitive decline and its underlying mechanisms. Therefore, my data can contribute in a decisive manner to increase the knowledge about early stages of cognitive impairment. Nonetheless, many questions and concerns still need to be addressed prior to any clinical application.

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7. List of figures

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9. Contributions

The author contributed to the design of all experiments, performed and analyzed all the WCM (except for Training 3 of Exp. 2) and MWM testing (except for MWM testing of Exp. 4, which had been performed for the most part by Daniel E. Heinz). He also performed the pharmalogical treatment in Exp. 4 and analyzed all the data together with Dr. Carsten Wotjak.

Dr. Carsten Wotjak supervised and contributed to the design and analysis of all experiments.

Daniel E. Heinz performed most of the MWM testing of Exp. 4 and extracted the brains for subsequent analysis in Exp. 2 and 4.

Renata Cruz performed MRS scanning of Exp. 1 and Scan 1 of Exp. 2, as well as brain extraction of Exp. 1.

Tibor Stark performed *in-vivo* imaging in Exp. 3 and Exp. 4.

Suellen de Almeida Correa performed WCM training 3 of Exp.2 and Scan 2 of Exp. 2.

Dr. Frederik Dethloff performed all the MassSpec analysis.

Dr. Nils C. Gassen performed all the Western Blotting.

Dr. Michael Czisch supervised all the *in-vivo* scanning and analyzed the related data.

The data obtained is currently under review for publication in a peer-reviewed journal with the aim to publish the data.

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