Exploring molecular mechanisms of mouse hibernation for novel therapeutic angles in Alzheimer’s disease

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General Discussion
NOVEL INSIGHTS INTO EFFECTS OF TORPOR IN MICE ON THE BRAIN

The primary aim of this thesis was to unravel hibernation-derived mechanisms that would aid in finding new therapeutic angles in Alzheimer’s Disease (AD). To that extent I first focussed on characterizing the effects of torpor in mice on the brain (Chapter 2 and 3), as torpor in mice has long been largely overlooked and researchers have mainly focused on effects of torpor on the brain of seasonal hibernators, such as the ground squirrel, Syrian hamster and black bear (1-3). Yet, torpor in mice offered us a unique opportunity to study torpor associated effects on the brain in a standardized manner and in AD relevant disease models. As prior knowledge was limited, the data in this thesis led to a novel understanding of effects of torpor in mice, specifically on hippocampal synaptic plasticity, memory and protein dynamics, and on tau hyperphosphorylation, as will be discussed below.

Hippocampal synaptic plasticity alterations during torpor in mice

In chapter 2 we measured mouse hippocampal long-term potentiation (LTP) during arousal after torpor and during euthermia. We hypothesized that during arousal, a state of enhanced plasticity might occur to reactivate the brain. Indeed, we found that LTP was transiently increased during arousal when compared to euthermia. Unfortunately, we could not reliably measure LTP in slices obtained during torpor, as the core temperature during torpor is about 15 degrees lower (22°C) than during arousal and euthermia (37°C). Measuring LTP during torpor would be interesting as it would give more insight into torpor-associated shutdown of neuronal communication in mice. Slices from Syrian hamster in torpor shown reduced hippocampal LTP (4), which is consistent with neuronal shutdown during torpor. In addition, it was shown that LTP can readily be elicited above 20°C but not below 15°C (2), which would explain reduced LTP during torpor in seasonal hibernators, but not in mice which only reach body temperatures as low as 20 °C (5-15). Measuring LTP at the same temperature of 20 °C in slices from both torpid and euthermic animals might give more insight into a torpor-specific component of LTP reduction in mice. Another more elegant, yet challenging, option would be to perform in vivo local-field potential recordings of the hippocampus during the different phases of torpor (16).

Hippocampal synaptic protein dynamics during torpor in mice

Opposed to what is seen in seasonal hibernators, little structural dendritic or synaptic alterations were found during the different phases of torpor in mice in Chapter 2. We therefore chose to look into specific molecular alterations of the synapse, and were the first to perform mass-spectrometric analyses on hippocampal synaptosomes of
different phases of torpor. The strength of a mass spectrometric approach is that
dynamics of synapse-enriched proteins can be monitored for all measurable proteins
and not just for only a hand full of selected target proteins, resulting in an unbiased
view of functional synaptic protein alterations during torpor. We found that arousal
mainly affected postsynaptic and mitochondrial protein levels in the hippocampus,
with increased levels of plasticity proteins, and complex I and IV respectively.
This prompted us to investigate torpor-related plasticity mechanisms via arousal-
associated mitochondrial reactivation, and the relevance of this approach for the
treatment of AD (Chapter 4, 5; this is discussed below in the review: ‘Mitochondrial
targeting against Alzheimer’s disease: lessons from hibernation’). However, it would
be very interesting from a hibernation perspective to focus on hippocampal protein
alterations during entry into hibernation, the torpor phase, for example by com-
paring euthermia vs. pre-torpor and pre-torpor vs. torpor samples. This would give
insight into how the brains of hibernators prepare for and executes shut-down of
neuronal communication, a process important to prevent NMDA receptor-mediated
excitotoxicity that would otherwise occur during gradual cooling of the brain (17).
It is very well conceivable that these processes are differentially regulated in daily
torpor mice compared to seasonal hibernators, the latter going through longer and
deeper torpor bouts and more extreme hypothermia and -metabolism, and which
show extensive dendritic and synaptic retraction during torpor.

Daily torpor in mice offers us a unique model to study a natural state of in-
creased synaptic plasticity and cognitive enhancement, which might be relevant
for the AD field (discussed below) or other conditions with cognitive impairment
in humans, and to help understand the basic principles of maintaining healthy
synaptic plasticity.

Effects of torpor on hippocampal memory in mice
In this thesis we focussed on memory-enhancing effects of daily torpor during the
arousal phase (Chapter 2). As mentioned above, postsynaptic plasticity protein levels
and long-term potentiation were increased during arousal. These findings inspired
us to also study the effects of daily torpor on memory in mice. Memory in seasonal
hibernators has been explored mostly in the frame of memory retention over the
course of hibernation. Proper memory retention is important for hoarding hiberna-
tors to remember where they stored their food for restocking of fat reserves, and for
all hibernators to avoid inbreeding and to optimize summer behaviour based on pre-
vious experiences. However, studies on memory retention in seasonal hibernators
are ambiguous, with some species showing disrupted memory after hibernation and
others showing no impairments (18-20). We found that memory retention in mice
after daily torpor is unaffected when mice were trained 3 days prior to torpor induc-
tion. Yet, when torpor was induced shortly after training (6 h) memory retention was decreased compared to controls, likely because of torpor interfering with memory consolidation (data unpublished). Next, we asked whether the plasticity-enhancing effects of arousal might be beneficial effects for memory encoding and retrieval after torpor. In Chapter 2, using a fear conditioning (FC) paradigm that measures hippocampus dependent contextual memory, we found that mice that had undergone torpor, had increased memory formation and retrieval capacity during arousal compared to mice that did not go through torpor. This cognitive ‘enhancement’ could be a coincidental side effect of systems reactivation associated synaptic plasticity. It may also be important to maximize the animal’s adaptive response to a new post-torpor environment, offering an evolutionary advantage of crucial importance in animals that use torpor as an emergency strategy. However, it is important to note that the increased memory towards the context does not have to be favourable or an ‘enhancement’. The fear conditioning paradigm relies on anxiety behaviour, and is measured in freezing levels, with higher freezing denoting better memory towards a context, providing generalized fear is excluded. However, it is unclear at what point an anxiety-driven freezing response changes from a healthy response into pathological anxiety behaviour. Future experiments should focus on memory formation capacity during arousal using anxiety-independent memory paradigms, such as an object place recognition task which measures hippocampus dependent spatial memory. The rescue of fear conditioned memory in the APP/PS1 AD mouse model during arousal after a single torpor bout, as shown in the last figure of Chapter 2, might be more relevant as it increases memory towards homeostatic levels. Consequently, mechanisms underlying torpor-arousal related plasticity in mice may serve as targets relevant for novel therapeutic approaches in AD (discussed below).

**Tau hyperphosphorylation in the brain during torpor**

Tau hyperphosphorylation and consequent aggregate formation are closely linked to severe neurodegenerative tauopathies such as AD. Fascinatingly, hibernators (e.g. ground squirrel and black bear) show extensive, yet reversible, tau hyperphosphorylation during torpor bouts. Even though tau hyperphosphorylation in torpor has been studied for over two decades, research in daily torpor mice has only recently gained more attention, and little is known about tau hyperphosphorylation in the brain of these mice. In Chapter 3 we show that torpor leads to robust tau phosphorylation in the brain in mice expressing either native or human tau (htau). Importantly, tau phosphorylation and its somato-dendritic accumulation in torpid htau mice were completely reversible and even lower than baseline at 24h after torpor, suggesting that torpor prompts unique tau dephosphorylation and clearance mechanisms that
may be relevant for human tauopathies. Below are some considerations regarding this work and its contribution to the AD field.

We chose to study the effects of daily torpor in mice expressing human tau (htau). Studying these effects in mice is unique, as all previous research focussed on native tau in hibernating species. Since tauopathies do not naturally occur in these species, the effect of torpor on htau is more relevant in the context of identifying novel targets and treatment strategies against AD and other tauopathies. Apart from difference in the tau protein itself, species-specific differences in other proteins, such as key enzymes involved in (de)phosphorylation, proteolysis and protein degradation, could also play a role in why only humans develop AD-associated tau pathology (21, 22), and should therefore not be overlooked. It would be very challenging, if not impossible, to humanize a mouse with respect to all these proteins, and primate models would possibly be more suitable. Interestingly, several primates, including lemur species (23) and the pygmy slow loris (24) hibernate. This has sparked interest as it might give insight in why humans do not hibernate, and, on a more futuristic note, how we might change that (25). Interestingly, grey mouse lemurs which are facultative daily hibernators, also show age-associated neurodegeneration with similarities to human AD, i.e., massive brain atrophy, abundant amyloid plaques, tau pathology, loss of cholinergic neurons and correlated loss of memory and social interaction (26). Lemurs may therefore have the potential for translating AD-relevant hibernation mechanisms with a stronger translational potential towards humans than mice. However, the studies reporting age-associated neurodegeneration, did not report whether the lemurs in the laboratory colonies had undergone torpor-arousal cycles, common to animals living in the wild. As it is known that hibernation affects AD associated pathology in multiple species, it would be highly interesting to compare hibernating to non-hibernating aging lemurs.

An important limitation of our research is that we observed a 3x higher expression of human tau in the htau mice compared to native tau in mtau mice. Future research should aim to study effects of daily torpor on htau under more physiological expression levels. Also, we did not examine the potential interaction between tau hyperphosphorylation and pathogenic Aβ. Applying torpor in a mouse model that contains htau as well as (mutant) human APP might help to better understand this interaction.

In our study, we only assessed phosphorylation of a well-known AD phosphoepitope, AT8, which is known to mark aggregated tau in (pre) tangles (27). However, actual aggregation should also be assessed biochemically, for example by measuring phospho-epitopes in soluble vs. insoluble fractions. Whether aggregated tau or non-aggregated but hyperphosphorylated tau is the main pathological actor in AD remains unknown, but clinical trials with kinase inhibitors or phosphatase activa-
tors in patients with mild cognitive impairment or early AD show promising results (21, 22). Unfortunately, off-target effects may limit this strategy, and other more out-of-the-box targets may be disclosed by exploring torpor-associated tau hyperphosphorylation and dephosphorylation mechanisms. For instance, mass-spectrometric analyses during different phases of daily torpor in htau mice might be useful to unveil such targets.

Tau knockout mice were used in our study as negative controls, but interestingly also shed light on the role of tau in torpor itself. A previous study described tau to be a ‘master switch’ to escape NMDA-mediated hyperexcitability of neurons during torpor (17). Tau hyperphosphorylation normally leads to dissociation of the tau-microtubule interaction, which results in structural remodelling of the cytoskeleton. This remodelling impairs recruitment of NMDA receptors to the postsynaptic density, thereby interfering with excitatory synaptic transmission and synaptic plasticity. This is suggested by Arendt et al. (17) to be advantageous to animals entering into torpor, and possibly even a prerequisite for entering torpor via controlled shutdown of brain activity. In our experiments, all tau knockout mice entered torpor without differences in the onset, duration or extent of torpor (Chapter 3, figure S1), refuting – in mice – the idea that tau hyperphosphorylation is a prerequisite for torpor entry. However, we did not assess excitatory damage due to torpor entry in tau knockout mice, so it remains to be studied whether tau indeed protects the brain during torpor, even if it does not regulate torpor. An alternative explanation may be that the decrease in metabolism and body temperature during torpor in mice is less profound than those in seasonal hibernation and that mice therefore use different, tau-independent, strategies to trigger torpor entry and prevent neurotoxicity.

HIBERNATION-DERIVED MECHANISMS AGAINST ALZHEIMER’S DISEASE

In Chapter 2, we found increased synaptic plasticity during arousal after torpor in mice, which was paralleled by increased levels of mitochondrial complex I and IV proteins and rescued memory in an APP/PS1 mouse model of AD. This prompted us to test whether supporting mitochondrial bioenergetics via complex I/IV activation, using a SUL compound, would mimic these effects, and if this compound could reduce Aβ pathology in an APP/PS1 mice (Chapter 4). In a preliminary study, we also examined the effects of the SUL compound on memory and protein levels in mice expressing the AD risk factor ApoE4 (Chapter 5). Here I discuss the main findings and some limitations and considerations with regard to unravelling hibernation-derived mechanisms that are relevant for AD.
SUL-138 treatment in APP/PS1 mice

We chose to target mitochondrial complex I and IV function in AD relevant mouse models using the SUL-138 compound, as the levels of these proteins were prominently increased during arousal. We found that SUL-138 treatment increased hippocampal synaptic plasticity and memory in both wildtype and APP/PS1 mice, similar to what was seen during arousal after torpor in mice. This was paralleled by an increase in fatty acid metabolism proteins in both genotypes, and increased glycolysis and amino acid metabolism protein levels in APP/PS1 mice. Hibernation-derived mitochondrial targeting (discussed more extensively in the Review below) therefore appears to be a valuable new therapeutic angle in AD. Future experiments should focus on the exact mechanism of action of SUL-138, e.g. through mitochondrial respiration measurements to assess mitochondrial adaptation, on its therapeutic potential by initiating treatment in pre- vs post-symptomatic disease stages, and on demonstrating causality of potential mechanisms through which SUL-138 acts using interventions.

SUL-138 has many advantages, such as proper blood-brain-barrier crossing (28) and absence of detectable (mitochondrial) toxicity (data unpublished). In addition, SUL-138 does not induce decreased consciousness, which would be undesirable in AD treatment. The compound is therefore planned for a Phase I clinical trial, hopefully further paving the way for hibernation-derived mitochondrial targeting against AD.

SUL-238 treatment in APOE mice

In Chapter 4, we tested SUL-138 in a well-established mouse model of AD, the APP/PS1 mice. Disadvantage of this model is that it is based on familial AD mutations and consequently may primarily model familial AD. In Chapter 5, we therefore set out to test SUL-238 (the HCl-salt form of SUL-138) in a mouse model for sporadic AD. We chose to use human ApoE4 targeted replacement (TR) mice as the most prevalent genetic risk factor for late-onset AD is expression of the E4 isoform, over the more common E3 isoform of ApoE (29). We found that SUL-238 treatment increased the levels of mitochondrial glycolysis proteins in ApoE4 TR mice, similar to what was found in APP/PS1 mice, suggesting that mitochondria may be affected comparably. We did however not observe any memory deficit in the untreated ApoE4 mice at 6 months of age, possibly due to insufficient ageing or lack of other pathogenic factors. Proteomic analyses did reveal differences in protein regulation between E3 and E4 mice, involving postsynaptic density proteins and proteins involved in amino acid metabolism, suggesting isoform specific (dys)regulation of synaptic and mitochondrial processes. Further exacerbation of potential pathological effects of ApoE4 might lead to AD associated outcomes in these mice, which would make the model more suitable for AD modelling. A proper additional pathological stressor
could be administration of a high-fat western diet in these mice, as a high-fat diet constitutes a common health strain in the western world and is a risk factor in AD development (30, 31). Future experiments should focus on further delineating the use of ApoE4 mice in modelling AD, before conclusions on the effectiveness of SUL-238 on AD relevant parameters in this mouse model can be made.

LIMITATION OF MEASURING PROTEINS

Mass-spectrometry-based proteomics is an exploratory approach to identify proteins with altered expression levels, predict molecular and cellular pathways and build hypotheses about potential disease and treatment mechanisms. Ultimately, however, one would need targeted experimental intervention to demonstrate causal relations and proof that such mechanisms are actually involved. The work in this thesis is mostly exploratory, suggesting the involvement of mechanisms underlying the increased synaptic plasticity found during arousal and after SUL treatment. Future causality studies, e.g. on how synaptic plasticity is increased during arousal, how arousal from torpor reduces tau hyperphosphorylation, or how SUL-138 mimics arousal-associated enhanced synaptic plasticity and reduces Aβ plaques in APP/PS1 mice, are needed. For now, the proteomics data gave us clues on which we build novel hypotheses, such as a mitochondrial reactivation-driven increase of synaptic plasticity after SUL treatment, that are worth following up.

CONCLUDING REMARKS

Hibernation is an exceptional state, exerting numerous beneficial effects both peripherally and centrally, including neuroprotective effects, reversion of tauhyperphosphorylation, rapid and healthy de/re-activation of mitochondria and enhanced neuronal plasticity, all potentially relevant for the AD field. Unfortunately, humans do not hibernate. Many have therefore opted ways of inducing a state of hypometabolism and hypothermia in non-hibernators using ‘synthetic torpor’, generally focussed on deep space travel and organ transplant research (32, 33). These synthetic torpor inducers range from mitochondrial inhibition via H2S, central inhibition using A1 adenosine agonists controlling thermoregulation in the hypothalamus (34), to recently published stimulation of glutamatergic neurons in the hypothalamus (12, 35), which might also be possible via ultrasound stimulation (36), and are able to mimic both the hypothermic and hypometabolic state of torpor. However, concerns ranging from practical issues such as the absence of
potential hibernation specific protective mechanisms in non-hibernators (37), and ethical issues regarding lowered or absent consciousness during torpor (38) can only be lifted via identification and mimicking of hibernation-derived mechanisms that exert the same beneficial effects as observed in hibernation, and are translatable to non-hibernators without causing lowered consciousness. We opted to find beneficial hibernation-derived mechanisms by first studying the effects of torpor in mice on the brain. We found that torpor in mice induces reversible tau (hyper)phosphorylation, including reversible somato-dendritic accumulation of phosphorylated tau specific to human tau-expressing mice, making it a valuable model in finding new tau dephosphorylation and clearance mechanisms (Chapter 3). Interestingly, torpor also induced a state of increased synaptic plasticity during arousal, which was paralleled by increased levels of mitochondrial complex I and IV proteins (Chapter 2). This increased synaptic plasticity might potentially be valuable for treating AD, a notion supported by the rescued memory found in APP/PS1 mice after 1 torpor bout. We followed up on this work by mimicking arousal mitochondria, using the SUL-138 compound, which stimulates complex I/IV function, and found that this indeed led to similar effects on synaptic plasticity found during arousal from torpor (Chapter 4). In addition, it also reduced Aβ plaque size and numbers and led to a partial rescue of protein dysregulation in APP/PS1 mice. Finally, we tested the compound in another model based on the most prevalent risk factor of developing AD, ApoE4 expression. Though ApoE4 TR mice did not model AD-associated memory deficits, SUL-238 treatment, similar to what was found in APP/PS1 mice, increased mitochondrial proteins involved in glycolysis, suggesting that mitochondria may be affected in a similar manner (Chapter 5). As SUL-138/238 does not induce a state of decreased consciousness, which would be undesirable in AD treatment, and toxicity studies show absence of (mitochondrial) toxicity, it is a promising new compound that might be used against AD.

Hibernation-derived mitochondrial targeting appears to be a valuable novel therapeutic angle for AD. Though future experiments are needed to fully delineate the overlap between torpor and torpor derived mitochondrial activation by the SUL compound, to determine how the SUL compound reduces Aβ plaques and APP/PS1 protein dysregulation, and to determine the causality between mitochondrial (re)activation and enhanced synaptic plasticity during arousal and after SUL treatment, the work in this thesis represent the initial steps towards identification and use of hibernation-derived mechanisms against Alzheimer’s disease.
REFERENCES


Mitochondrial targeting against Alzheimer’s disease: lessons from hibernation

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ABSTRACT

Alzheimer’s disease (AD) is the most common cause of dementia worldwide and yet remains without effective cure. Amongst the many proposed causes of AD, the mitochondrial cascade hypothesis is getting increased attention. Evidence accumulates that mitochondrial dysfunction is a driving force behind synaptic dysfunction and cognitive decline in AD patients. However, therapies targeting mitochondria in AD have proven unsuccessful so far, and out-of-the-box options such as hibernation-derived mitochondrial mechanisms may provide valuable new insights. Hibernators uniquely and rapidly alternate between suppression and re-activation of mitochondria while maintaining sufficient energy supply and without ROS damage. Here, we briefly give an overview of mitochondrial dysfunction in AD, how it affects synaptic function and why mitochondrial targeting in AD remained unsuccessful so far. We then discuss mitochondria in hibernation and daily torpor in mice, covering current advances in hibernation-derived mitochondrial targeting strategies. We conclude with new ideas on how hibernation-derived dual mitochondrial targeting of both ATP and ROS pathways may boost mitochondrial health and induce local synaptic protein translation to increases synaptic function and plasticity. Further exploration of these mechanisms may provide more effective treatment options for AD in the future.
INTRODUCTION

Alzheimer’s disease (AD) is the most prevalent neurodegenerative disease worldwide and as the population ages, numbers are only expected to rise (1). So far, despite decades of research and efforts, no cure has been found for this devastating disease. Treatments targeting either of the two main pathological hallmarks of the AD brain, amyloid-beta (Aβ) and tau aggregation, have been mostly unsuccessful, driving the search for alternative treatable causative agents of AD. A growing body of evidence is supporting a causal role of mitochondrial dysfunction in AD (2-6). This has led to the mitochondrial cascade hypothesis of AD, proposing a progressive decrease in mitochondrial fitness as causative factor in AD. Up to now, small molecule compounds with antioxidant (i.e. ROS scavenging) properties, such as vitamin E and curcumin, are most studied as mitochondrial targeted treatments in AD (7-9). Whereas antioxidants show some promising results in in vitro and in mouse models of AD, they fail to restore cognitive function in clinical studies, possibly due to poor blood-brain-barrier permeability and the singular effect of ROS scavenging without improving mitochondria function itself (9, 10).

Remarkably, hibernating animals show exceptional brain plasticity when alternating between torpor (periods of hypothermia and hypometabolism) and arousal (periods when metabolism and body temperature return to normal)(11-19). Hypometabolism during torpor is caused by the active shutdown of mitochondria, which is rapidly reversed upon arousal without causing ROS damage. Depending on species and environmental conditions, seasonal hibernators such as ground squirrels and dormouse employ deep and long torpor bouts (1-3 weeks typically at ~5 °C) whereas daily hibernators such as Siberian hamster and mouse employ short (4-10 h at ~17 °C) torpor bouts (20, 21). Regulation of mitochondrial activity in hibernation coincides with extensive neuronal plasticity in many brain regions, ranging complete dendritic retraction and restoration in seasonal hibernators, to specific synaptic changes during daily torpor in mice(11, 22, 23). In addition, daily torpor in mice increases levels of mitochondrial complex-I and -IV proteins, proteins involved in synaptic plasticity, long-term potentiation in the hippocampus, and restores memory function in an AD mouse model (23). Interestingly, mimicking arousal-induced mitochondrial changes using the 6-chromanol SUL-138, which supports complex-I and -IV function while suppressing ROS production, was able to mirror these effects (24). This suggests that hibernation-associated mitochondrial regulation could be beneficial in treating AD-related synaptic and mitochondrial impairments.

In this review we briefly discuss mitochondrial deficiencies that lead to synaptic impairment in AD and mitochondrial targeted intervention strategies that
have so far proven insufficient in improving AD clinical outcome. Next, we discuss mitochondria in hibernation and how hibernation-derived mitochondrial targeting offers new and unique opportunities in the search for novel treatment strategies against AD, including current advances. Finally, we propose a model via which hibernation-associated mitochondrial activation could lead to increased synaptic plasticity relevant for the treatment of cognitive decline in AD.

MITOCHONDRIA IN AD

Mitochondrial dysfunction in AD: the mitochondrial cascade hypothesis
The mitochondrial cascade hypothesis, which originated in 2004 (25), is getting increasing attention and by now a large body of evidence implicates mitochondrial dysfunction as a fundamental causative mechanism in AD (6, 26). The hypothesis states that a progressive decrease in mitochondrial fitness, excess release of reactive oxygen species (ROS) and decreased ATP production are important contributors to pathological protein aggregation, decreased synaptic plasticity and cognitive decline in AD. Already over 40 years ago, electron microscopy and PET imaging revealed altered mitochondrial morphology and decreased glucose metabolism in AD brains (27), a parameter that appears superior to tau or Aβ pathology in predicting AD progression (28). Mitochondrial deficits, as reflected in increased oxidative stress (e.g. free radical production, lipid peroxidation, oxidative protein damage) (29) and altered calcium homeostasis (30), are observed in AD patients prior to histological and clinical abnormalities (31-33). Interestingly, caloric restriction, intermittent fasting and ketogenic diets that alter metabolic input to mitochondria have shown mild beneficial effects in animal models of neurodegenerative disorders and in human clinical trials (34, 35). Consequently, mitochondria should be seriously considered as potential pharmacotherapeutic targets in the treatment of AD.

Glucose dysmetabolism and oxidative stress: an ATP/ROS disbalance in AD
The brain is the highest energy demanding organ and therefore relies heavily on glucose (the predominant substrate for the human adult brain under physiological conditions) to efficiently produce ATP. The production of ATP occurs through glycolysis, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation system (OXPHOS) via the electron transport chain (ETC) (Fig 1.) (36). The impairment of glucose metabolism in AD brains is detrimental for ATP levels necessary for normal functioning of neurons (29, 37-40). A main component in the dysregulation of glu-
cose metabolism is oxidative stress caused by ROS, an inevitable, albeit normally well-handled, by-product of oxidative phosphorylation complex-I and -III. Mitochondrial dysfunction caused e.g., by aging accumulated damage or genetic defects, leads to increased ROS production, causing oxidative modification of enzymes involved in glycolysis and the TCA, reducing their efficiency and creating a negative feedback loop that further impairs mitochondrial function (29, 36, 41). In addition, many studies have shown reduced levels of all 5 complexes of the ETC in AD brains, leading to further impairment of ATP production (3, 42-46). Moreover, the efficiency of all 5 complexes is lowered in AD, with a particularly clear defect in cytochrome c, leading to increased ROS levels and further ATP deficiency (47).

Figure 1: Mitochondrial impairments in AD
The major energy source for the brain is glucose which is metabolized to ATP via glycolysis, pyruvate metabolism, the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation system (OXPHOS) via complex I-V in the electron transport chain. Mitochondria also produce ROS as by-products of activity in the OXPHOS system. Other, less prominent, inputs into the OXPHOS system are via fatty acid degradation and -oxidation (FAD & FAO) and amino acid metabolism. In AD, excess levels of ROS, probably due to aging-related inefficient proton motive force over complexes-I and -III, leads to oxidative damage to enzymes involved in glycolysis/pyruvate metabolism, the TCA cycle and the OXPHOS system, thus augmenting ATP deficits and ultimately leading to high ROS and low ATP levels found in AD.

Mitochondrial impairment leading to synaptic dysfunction in AD
Whether or not the mitochondrion represents a true causative agent from which AD originates, mitochondrial dysfunction plays an eminent role in synaptic impairment in AD (48). Synaptic dysfunction is an early pathological feature and correlates highly with memory loss in AD (49-51). Neurons heavily rely on healthy functioning mitochondria to meet the high energy demands for e.g., mobilizing synaptic vesicles, synaptic transmission, maintaining ion gradients for action potential generation and propagation and local mRNA translation for synapse function and synaptic plasticity (6, 48). Decreased ATP levels in AD brains can interfere with each of these
processes (52-55). In addition, increased ROS levels in AD brains can lead to impaired mitochondrial Ca\(^{2+}\) buffering which in turn leads to the loss of microtubule assembly dependent transport of synaptic vesicles and mitochondria, further lowering energy supply to synapses, additionally impairing synaptic transmission (56, 57).

**Mitochondrial targeted strategies in AD**

Drugs that effectively target Aβ or tau pathological protein aggregation have not lived up to their therapeutic promise in AD yet. In the context of the mitochondrial cascade hypothesis, therapeutics targeting mitochondria have been tested both in the preclinical and clinical setting. As such, antioxidants are obvious candidates to mitigate ROS damage (29). Unfortunately, treatment with antioxidants such as Vitamin E and C (8, 58, 59), and Ginkgo biloba (60, 61), a natural antioxidant extensively used in Chinese traditional medicine, have proven ineffective in improving clinical outcome or preventing AD progression and have therefore not entered the AD drug market. Another group of mitochondrial compounds that have been shown to counteract mitochondrial dysfunction and oxidative stress are phenylpropanoids (62-64). For example, Curcumin, a commonly known spice, showed promising results on oxidative damage and Aβ plaque burden in an APP/PS1 AD mouse model (65). However, positive clinical outcomes in humans remain absent, presumably due to low bioavailability (66, 67). Apart from relieving ROS levels, stimulating or protecting mitochondrial ATP production has been attempted using for example oxaloacetate (OOA) (68), an intermediate of the TCA cycle, or Nicotinamide adenine dinucleotide (NAD) (69), an intermediate common to several mitochondrial metabolic pathways, including glycolysis, TCA cycle, and oxidative phosphorylation, but clinical efficacies still remain to be studied (62). Interestingly, mild inhibition of complex I of the OXPHOS system, e.g., by the small molecule tricyclic pyrone compound CP2, has been suggested as potential treatment strategy against AD, with positive effects in the APP/PS1 mouse model of AD (70, 71), both in a pre- and post-symptomatic treatment approach. It is thought that the inhibition of complex I, while lowering ATP production, exerts its beneficial effects by triggering a mitochondria-mediated integrated stress response and due to lower ROS production via Complex I (69). However, clinical studies with non-specific complex I inhibitors such as metformin and resveratrol have been unsuccessful, possibly due to limited bioavailability and non-specificity (72, 73). Although the general consensus is that mitochondria should be seriously considered as targets against AD, issues with bioavailability, specificity, efficacy and side effects of mitochondrial targeting have hindered progression in this field beyond preclinical findings. Thus, novel strategies and out-of-the-box approaches are needed to overcome this hurdle.
MITOCHONDRIA IN HIBERNATION

Metabolic adaptation during hibernation

Hibernation is a natural phenomenon during which animals escape energetically challenging environmental conditions by entering torpor, a state of extreme hypometabolism and hypothermia. During torpor, hibernators show a drastic, up to 98%, reduction in metabolic rate (70). Though its exact molecular underpinnings are still poorly understood, torpor features the halt of glycolysis which is established by changing the levels and activities of several key enzymes involved in metabolic conversion during glycolysis, pyruvate metabolism and the TCA cycle, including glycogen phosphorylase, pyruvate dehydrogenase, phosphofructokinase, pyruvate kinase and citrate synthase (74-79). ATP production is further halted due to a reduction in the efficiency (but not levels) of complex-I & -II (80) of the OXPHOS and of the mitochondrial H₂S oxidizing enzyme sulphide: quinone oxidoreductase (SQR), which provides electrons to the ETC via Coenzyme Q (81, 82). The activities of these OXPHOS proteins are thought to be regulated though allosteric inhibition by oxaloacetate, an TCA cycle intermediate that accumulates due to decreased activity of TCA enzymes, and through posttranslational modifications by intramitochondrial kinases and deacetylases (83). In addition, reduced efficiency of H₂S oxidizing SQR and increased levels of H₂S synthesizing enzymes, in brain mainly Cystathionine beta-synthase (CBS), leads to an increase in H2S levels during torpor which inhibit of complex IV (cytochrome c oxidase) function of the OXPHOS system (84). The limited remaining metabolism during torpor shifts to fatty acid metabolism (75, 77, 85).

During arousal, reactivation of mitochondria occurs in a matter of hours. This reactivation initially leads to ‘non shivering thermogenesis’ via ATPase uncoupling in brown adipose tissue (BAT). Further rewarming is caused as a by-product of reinstated ATP production in other tissues such as skeletal muscle (‘shivering thermogenesis’). Lipids are the predominant fuel during the high-energy demanding arousal phase (77-79, 86), however other non-lipid fuels, such as carbohydrates and glycogen have also been shown to be recruited upon arousal in 13-lined ground squirrel (87). Even though gaps in our knowledge on mitochondrial adjustments during hibernation remain, the abovementioned mechanisms involved in healthy mitochondrial regulation might harbour new AD treatment options.

Defence mechanisms against ROS damage during hibernation

Interestingly, suppression and re-activation of mitochondrial function during torpor and arousal occurs while maintaining sufficient energy supply and without the occurrence of ROS damage normally seen during rewarming and reperfusion (79, 88). Excess substrate at - and reverse electron flux through - complex II during
ischemia-reperfusion is usually a significant source of electrons for ROS production at complex I (79, 80). Therefore protection against ROS production during arousal has been linked to the significant reduction in complex-I and -II function during torpor (89). In addition, brown adipose mitochondria, which uncouple respiration from ATP for non-shivering thermogenesis and release the energy of the proton gradient directly as heat, are also protected against ROS damage. This respiratory uncoupling of BAT mitochondria is due to relatively high levels of Uncoupling protein 1 (UCP1) which reduces proton motive force and is thought to alter the redox state of the respiratory chain, effectively reducing the production of ROS (90). Other putative oxidative stress protective strategies associated with hibernation are the increased levels of antioxidants such as ascorbate and glutathione during arousal (85, 91).

ROS damage preventing strategies observed during hibernation have been of particular interest for the ischemia-reperfusion field as in humans the overproduction of reactive oxygen species (ROS) upon reperfusion jeopardizes cellular integrity (92, 93). Further exploiting the mechanisms that hibernators use to maintain sufficient mitochondrial ATP production without ROS damage may thus also represent a promising therapeutic strategy in AD, given that mitochondrial dysfunction and subsequent ATP/ROS imbalance are prominent in the AD brain.

Figure 2: mitochondrial regulation during hibernation
During the torpor phase of hibernation, glycolysis is halted and remaining energy metabolism shifts to fatty acid metabolism (FAD and FAO). Enzymes involved in glycolysis, pyruvate metabolism and the TCA cycle are reduced and/or less efficient. Efficiencies of complex-I and -II of the OXPHOS system are drastically reduced through reduced input from the TCA cycle, and posttranslational modifications and allosteric hindrance by Oxaloacetate (OAA), an accumulated TCA intermediate, of these enzymes. Increased levels of H₂S due to decreased H₂S oxidation efficiency of SQR and increased production of H₂S by CBS, in turn inhibit complex IV function. ROS damage is prevented due to the downregulation of complex I which normally produces a large fraction of ROS in the OXPHOS system. In addition, UCP1 upregulation leads to uncoupling of the OXPHOS system, thereby altering its redox state and inhibiting ROS production. Finally, antioxidant levels e.g., ascorbate, are higher during hibernation, directly neutralizing ROS.
Mitochondria in daily torpor mice

Though long overlooked, laboratory mice are also capable of entering a hibernation state, particularly daily torpor (23, 94-98). They exert facultative daily torpor as a response to energetic challenges, such as low food availability or increased energy expenditure. Torpor in mice is generally induced using a fasting protocol, completely omitting food availability, or a work-for-food protocol, in which mice have so called high foraging-costs typically due to wheel running as requirement to access food (99, 100). Both ultimately lead to a negative energy balance that is compensated for by daily torpor cycles with body temperatures as low as 21 °C and a metabolic rate reduction up to 70%. It has been found that, like seasonal hibernators, the efficiency of complex-I and -II of the OXPHOS system and of ADP phosphorylation is reduced during daily torpor in Balb/c, CD1, and C57/6N mice (89). In addition, we observed robust hippocampal mitochondrial protein regulation during daily torpor in C57BL/6 mice (23). Notably, many of these mitochondrial proteins were part of electron transport chain complex-I and -IV, previously shown to be involved in mitochondrial activity regulation during hibernation (78-80, 83). The effects of arousal on mitochondrial protein levels are in line with a study showing that leptin, which is reduced during torpor and reinstated during arousal, normally regulates several OXPHOS proteins (96, 101). It would be interesting to additionally assess complex activity in daily torpor mice, to parallel data from previous studies on mitochondrial regulation during hibernation.

HIBERNATION-DERIVED MECHANISMS OF MITOCO ndrial Targeting: is It Relevant for the Treatment of AD?

Targeting UCP1

The significance of hibernation-derived mitochondrial activation in neuronal protection has been demonstrated in a study that compared iPSC-derived neurons from the hibernating arctic squirrel to iPSC-derived neurons from humans during cold exposure. They found that neurons derived from arctic squirrel exerted a cell autonomous protection against cold-induced stress which normally results in ROS overproduction, and lysosomal membrane permeabilization and consequential microtubule destruction. This neuronal protection seems to be conferred via torpor associated mitochondrial uncoupling (102), as mimicking this effect using the mitochondrial uncoupler BAM15, was able to protect against cold-induced stress in human iPSC-derived neurons. Transient overexpression of mitochondrial uncoupler proteins UCP1 or UCP2 likewise produced cold-stable neurites supporting
the protective effects of BAM15 indeed rely on mitochondrial uncoupling. BAM15 has also been tested in an C. elegans model of Alzheimer’s disease as Alzheimer’s disease could also benefit from this neuronal protection from ROS damage could also be relevant for AD. Indeed, it was able to relieve neurodegeneration and aging in this model, as it reduced abnormal shapes of mechanosensory neuronal cells, and maintained touch-response and short-term memory (103). However further studies are needed to substantiate causal effects of mitochondrial uncoupling on neuronal protection, and relevance for AD in other more complex AD models. The notion that mitochondrial uncoupling can protect neurons from ROS damage and devastating microtubule destabilization that normally occurs during cold-induced stress, is in line with the limited damage that occurs during extreme hypothermia in torpor which is paralleled by a strong upregulation of UCP1 in brown adipose tissue mitochondria and neuronal mitochondria during this phase (104).

**Targeting mitochondrial Ca\(^{2+}\) handling**

Alterations in mitochondrial calcium homeostasis during hibernation confers another benefit of hibernation that could be interesting for AD treatment. Intracellular calcium handling is important for regulating membrane excitability, signal transduction, neurotransmitter release, synaptic plasticity, cell cycle, cell migration and axon growth. It is regulated through plasma membrane channels and intracellular storage in endoplasmic reticulum, Golgi apparatus and mitochondria. Disrupted Ca\(^{2+}\) homeostasis, e.g., following NMDA receptor activation, leads to mitochondrial dysfunction and to rapidly increasing mitochondrial Ca\(^{2+}\) levels, resulting in increased mitochondrial membrane depolarization and excitotoxic cell death (30, 105-107). During hibernation the mitochondrial calcium uniporter (MCU) complex, important for transporting calcium into the mitochondrion, and Leucine Zipper and EF-Hand Containing Transmembrane Protein 1 (LETM1), which mediated calcium release from the mitochondrion, are upregulated in skeletal muscle in Daurian ground squirrels (108, 109). It is thought that regulation of mitochondrial calcium handling via these complexes helps hibernators to maintain intracellular Ca\(^{2+}\) homeostasis during the extremes of hibernation, for example in heart and skeletal muscle that otherwise would suffer from cytoplasmic Ca\(^{2+}\) overload during inactivity (108, 110). In addition, it has been shown that a high rate of mitochondrial Ca\(^{2+}\) uptake lowers the OXPHOS proton-motive force sufficiently to cause a transient reversal of the ATP synthase, which is necessary for ATP shutdown during torpor. Studies on Ca\(^{2+}\) homeostasis in brain tissue of hibernators are scarce, however one study reported reduced intracellular Ca\(^{2+}\) levels in ground squirrel cerebral synaptosomes during torpor, which is thought to increase tolerance against prolonged ischemia during hibernation (111). Calcium dyshomeostasis in AD leads to intracellular calcium
overload, resulting in neurodegeneration via its effects on mitochondrial- and synaptic dysfunction and affecting production and aggregation of Aβ peptides and tau hyperphosphorylation (112). Therefore, several studies suggested the potential of reducing mitochondrial Ca\(^{2+}\) uptake as potential treatment strategy against AD. MCU deletion improved mitochondrial function and prevented neurodegeneration in C. elegans expressing AD relevant mutations in their PSEN homolog, sel-12 (113). In addition, a knockdown of endogenous MCU rendered primary cultured neurons resistant to oxidative stress (114), and the MCU inhibitor Ru360 prevented mitochondrial Ca\(^{2+}\) uptake in mice in vivo after exposure to soluble Aβ (115). However, all these studies focused on inhibiting MCU activity, thereby solely preventing Ca\(^{2+}\) influx into the mitochondrion. Yet, hibernators upregulate both MCU and LETM1, stimulating both Ca\(^{2+}\) import and export, possibly to enhance overall calcium buffering capacity of the mitochondrion. Further studies should explore this dual targeting of mitochondrial calcium handling as a neuroprotective strategy in AD.

**Targeting H\(_2\)S**

H\(_2\)S is a gasotransmitter, like NO and CO, and regulates numerous cytoprotective and physiological functions through its anti-oxidative and anti-inflammatory actions (116). Interestingly, in 2005 researchers found that low doses of H\(_2\)S inhalation led to suspended animation (a torpor like state) in mice (117). Though questions remain regarding the low O\(_2\) levels used in this study (17.5 %, which is hypoxic) (88), it sparked interest in the role of H\(_2\)S in hibernation and it is now thought that H\(_2\)S plays a key role in suppressing mitochondrial activity during torpor (118). As previously mentioned, the level of H\(_2\)S is regulated through SQR and CBS, and H\(_2\)S inhibits Complex IV function through cytochrome c reduction. The role of H\(_2\)S in AD is also getting increasing attention over the years. AD patients show lower levels and reduced activity of the H\(_2\)S-producing enzyme CBS and lower levels of H\(_2\)S in the brain, which correlate to AD severity (119-121). Increasing H\(_2\)S levels as therapeutic strategy against AD has been extensively reviewed (122, 123). Indeed, increasing H\(_2\)S levels similar to what is seen during hibernation leads to relief of AD-related pathophysiologic outcomes in various animal and cell models of AD. For example, NaHS, an H\(_2\)S donor, ameliorated Aβ-induced damage in PC12 cells by reducing the loss of mitochondrial membrane potential and attenuating the increase of intracellular ROS (122). In addition, increasing H\(_2\)S levels in the brain of AD animal models via a donor or through a diet rich in taurine, cysteine, folate, B12 and betaine, has been shown beneficial against oxidative stress, neurodegeneration and cognitive impairment (123, 124). However, clinical translation of these results is hindered by the complex dosage regimen of an H\(_2\)S donor, requiring multiple high doses, and H\(_2\)S’s potential toxicity at such high doses (123). Currently, an H\(_2\)S-targeting
compound, the CBS activator s-adenosyl methionine (SAM), has entered a phase II clinical trial, studying its effects in patients with mild cognitive impairment. The results will hopefully shed more light on the potential of H₂S-based treatments in AD patients (125).

**Targeting complex-I or -II**

Recently, mRNA expression levels of OXPHOS subunits, primarily complex II, were shown to be decreased during torpor in 13-lined ground squirrels, while levels of TCA intermediates prior to complex II were simultaneously increased, suggesting that hibernation halts TCA input towards the OXPHOS through complex II inhibition (126). This is thought to prevent ROS formation during hibernation via blocking reverse electron transport from complex II to complex I. Mimicking this effect via blocking of complex II with dimethyl malonate (DMM) was able to rescue hypoxia damage in hypoxic human SH-SY5Y differentiated neurons and in vivo in ischemic stroke in mice (126). In addition to complex II inhibition, inhibition of Complex I, which is also downregulated during hibernation, has been suggested as potential strategy against AD as well, with positive effects in an APP/PS1 mouse model of AD (70, 127). However, as mentioned previously, clinical studies with non-specific complex I inhibitors, such as metformin and resveratrol were not successful (72, 73). Solely targeting ROS damage by mimicking torpid mitochondria, without stimulating ATP production, might therefore be a too unidirectional approach in the treatment of AD.

**Dual targeting: improving ATP/ROS balance**

The notion that laboratory mice are also capable of daily torpor (23, 94, 96-98, 128-130), showing bouts of torpor and arousal of several hours instead of days to weeks, offers the opportunity to explore hibernation in a standard animal model and in a relatively short timeframe, with AD mouse models readily available to study its relevance for AD. We found an upregulation of mitochondrial complex-I and -IV proteins during arousal after torpor in mice, which was paralleled by a significant increase in the levels of proteins involved in synaptic plasticity such as AMPAR/NMDA receptor subunits, CAMK2A and SHISA6/7 and in long-term potentiation and learning and memory (23). Interestingly, a single torpor bout was sufficient to rescue memory impairment in an APP/PS1 AD mouse model. These observations suggest that arousal-induced mitochondrial activation has the potential to enhance synaptic transmission and plasticity. Similar effects were observed when mimicking the arousal-associated mitochondrial state using SUL-138, a hibernation-inspired 6-chromanol derivative that preserves mitochondrial respiratory chain function by supporting complex-I and -IV function, thereby preventing ROS formation and stimulating ATP
production (24). Three months of oral administration of SUL-138 increased synaptic transmission and memory performance in both APP/PS1 and wildtype mice, similar to a single torpor/arousal cycle (24). Notably, improved memory in SUL-138-treated APP/PS1 mice was accompanied by a partial rescue of synaptic protein expression, and a significant upregulation of mitochondrial proteins involved in fatty acid degradation and oxidation and a substantial decrease in brain amyloid plaque load and size. Collectively, these data suggest that SUL-138 not only activates complex-I and -IV, but also alters metabolic input into the mitochondrion, possibly further enhancing ATP production while limiting ROS. However, direct measurements of mitochondrial respiration are needed to confirm this. The SUL-138-evoked long-term metabolic adaptations in mitochondria, and its effects on synaptic transmission and memory performance, illustrate that targeting mitochondrial bioenergetics might be a promising strategy to prevent cognitive impairment in AD. Interestingly, SUL-138 supports complex-I and -IV activity without affecting basal mitochondrial membrane potential or causing apparent mitochondrial toxicity (131). Moreover, hibernation-inspired 6-chromanols prevent organ damage in various preclinical models of conditions or diseases with impaired mitochondrial function, including whole body cooling (132), renal ischemia/reperfusion (131), COPD (133, 134) and diabetes (135). As SUL-138 passes the blood-brain-barrier, it would be a suitable candidate to also treat AD. In that regard it is important to further explore how dual hibernation derived mitochondrial targeting would be able to increase synaptic plasticity in AD.

Figure 3: Targeting ATP/ROS balance via mitochondrial stimulation with SUL-138
During arousal from torpor complex-I and -IV are upregulated, and, while ROS remains low, ATP levels are reinstated (orange arrows). The 6-chromanol SUL-138 mimics arousal mitochondria after daily torpor in mice by stimulating complex-I and -IV function, thereby reducing ROS levels and increasing ATP levels. SUL-138 affects complex IV function via reduction of cytochrome c. In addition, SUL-138 changes metabolic input towards the oxidative phosphorylation system (OXPHOS) to fatty acid degradation and -oxidation (FAD & FAO) (green arrows).
HIBERNATION-DERIVED MITOCHONDRIAL REACTIVATION AND SYNAPTIC PLASTICITY: A POSSIBLE LINK WITH REL-EVANCE FOR AD TREATMENT

As discussed, hibernation affects both mitochondrial function and synaptic plasticity, and it is interesting to speculate how the two may be linked. LTP and other fast acting postsynaptic plasticity processes are often facilitated by increased synaptic protein translation, a process that is impaired in neurodegenerative diseases such as AD (53, 136-138). We postulate that mitochondrial priming increases synaptic de novo protein translation of postsynaptic proteins, which in turn increases LTP and memory formation (Figure 4), and that mitochondrial dysfunction in AD directly impacts on synaptic function and cognition. Future studies should aim to substantiate this, e.g. via measuring translation in isolated torpor/arousal and SUL-138 treated synaptoneurosomes (139-141) as measure of synaptic local de novo protein translation, and by short term selective inhibition of complex-I / -IV activation during arousal or SUL-138 treatment, while assessing local translation, synaptic plasticity and memory.

CONCLUSION

The beneficial effects of dual targeting interventions, improving fitness of mitochondria not only via reducing ROS but also via stimulating ATP production, offers new opportunities in the search for novel AD treatment strategies. This adds to various studies showing advantageous mitochondrial mechanisms employed by hibernation with relevance for AD treatments but with limited clinical efficacy so far. How mitochondrial (re)activation enhances synaptic plasticity and reduces AD pathology in AD mice remains to be explored. We suggest enhanced synaptic plasticity, at least in part, leans on stimulation of local translation of synaptic plasticity proteins. Altogether, nature’s solution to maintain mitochondrial health in energetically unfavorable conditions may hold substantial promise for the future of AD treatment.
Figure 4: proposed model for beneficial effects of hibernation-derived mitochondrial activation on synaptic plasticity

Alzheimer’s disease mitochondria have a disbalance in ROS /ATP, with excess ROS production and reduced ATP levels. This leads to pathological secondary outcomes including inhibited local translation of synaptic plasticity proteins which depends heavily on sufficient ATP supply. This local translation is important for synaptic maturation, which is essential for LTP and memory, both impaired in AD (red arrows). Mitochondrial reactivation during arousal after torpor, offers a unique state in which ROS formation is inhibited and ATP production is reactivated. In daily torpor mice this arousal phase is characterized by an increase in complex-I and -IV levels (orange arrows). SUL-138 mimics this reactivation of arousal mitochondria by stimulating complex-I and -IV function, while preventing ROS formation (green arrows). Therefore, both torpor and torpor derived mitochondrial activation by SUL-138 can lead to enhanced local translation of synaptic plasticity proteins (e.g. AMPA/NMDA Receptor subunits, auxiliary subunits and neurofilaments), hence may lead to enhanced LTP and memory formation capacity.
Chapter 6.1 | Mitochondrial targeting against Alzheimer’s disease: lessons from hibernation

REFERENCES


