THE IMPACT OF DIET AND SLEEP ON SYNAPTIC AMPA RECEPTOR LEVELS IN THE HYPOTHALAMUS: IMPLICATIONS FOR METABOLIC HOMEOSTASIS

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Tübingen, den …… ……

Datum/Date Unterschrift/Signature

To my beloved parents – Changhua Li and Changming Liu,

Grandfather – Zhilun Li and Xiangshu Lei,

and

Yan Zhu

致我的父母：李长华和刘长明

外公李治伦, 外婆雷祥淑, 以及

爱人朱燕

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Abbreviations

ACS	Acyl-CoA synthetase
AgRP	agouti-related peptide
AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors
ANOVA	analysis of variance
ARC	arcuate nucleus
ASI	axon-spine interface
BBB	blood-brain barrier
BCA	bicinchonic acid assay
BDNF	brain-derived neurotrophic factor
CaMKII	calcium/calmodulin-dependent protein kinase II
CAMKK	Ca^{2+}/calmodulin-dependent protein kinase kinase
CART	cocaine and amphetamine-regulated transcripts
CNS	central nervous system
CP-AMPARs	Ca^{2+}-permeable AMPARs
EEG	electroencephalogram
ELISA	enzyme-linked immunosorbent assay
EMG	electromyography
EW	enforced wakefulness
FFA	free fatty acid
GABA	gamma-aminobutyric acid
GRIP1	glutamate receptor-interacting protein 1
HF1D	high-fat diet 1-day
HF3D	high-fat diet 3-day

HFD	high-fat diet
IL-6	interleukin-6
LH	lateral hypothalamus
LTD	long-term depression
LTP	long-term potentiation
MCH	melanin concentrating hormone
mEPSC	miniature excitatory post-synaptic current
MSHs	melanocyte stimulating hormones
NMDA	N-methyl-d-aspartate
NPY	neuropeptide Y
NREM	non-REM
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with tween 20
PDZ	postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1
PICK1	protein interacting with C kinase 1
PKA	protein kinase A
PKC	protein kinase C
POMC	pro-opiomelanocortin
PSA	polysialic acid
PSD-95	postsynaptic density-95
PVN	paraventricular nucleus
REM	rapid eye movement
SBEM	serial block-face scanning electron microscopy
SCN	suprachiasmatic nucleus

SD	sleep deprivation
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	standard error of mean
SFAs	saturated fatty acids
SHY	synapse homeostasi hypothesis
SNRIs	serotonin and norepinephrine reuptake inhibitors
SWS	slow wave activity
TARPs	transmembrane AMPA receptor regulatory proteins
TLR4	toll-like receptor 4
TMS	transcranial magnetic stimulation
TNF	tumor necrosis factor
TTX	tetrodotoxin

Abstract/Summary

The rhythm of sleep and wakefulness is a dynamic behavioral phenomenon present in virtually every animal species investigated to date. It is regulated by a fine-tuned network of sleep- and wake-promoting neurons in the brain. Broad evidence suggests that a consequence of staying awake is a progressive increase in synaptic strength – synaptic upscaling –, as the waking brain adapts to the changing environment primarily through synaptic changes. Enhanced synaptic strength, however, might soon become unsustainable, as stronger synapses consume more energy, take up more space, and require more supplies. It is known that sleep induces synaptic downscaling to re-establish synaptic homeostasis in the cortex and hippocampus. It is not clear, however, whether sleep-dependent changes in synaptic plasticity happen in other brain regions and, moreover, how changes in energy supply interact with the impact of sleep and wakefulness on synaptic homeostasis. This dissertation aimed to investigate net synaptic changes in the hypothalamus, the main center integrating metabolic and sleep-wake regulatory signals, resulting from disruptions of the sleep-wake rhythm and high-calorie food intake. It was hypothesized that sleep induces synaptic downscaling in the hypothalamus, whereas wakefulness and sleep deprivation are associated with upscaling. It was furthermore assumed that high-fat food intake leads to hypothalamus-specific synaptic upscaling.

In order to explore net changes in synaptic strength in the hypothalamus across the sleep-wake cycle and during sleep deprivation, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) that contain the subunits GluA1 and GluA2 were determined in hypothalamic and cortical synaptoneurosomes of rats (study I). Since wakefulness- and sleep-dependent up- and, respectively, downscaling has been previously described in the cortex, this region was chosen to verify our approach. It was found that GluA1-containing AMPARs both in hypothalamus and cortex are high during wakefulness and low during sleep, indicating net synaptic upscaling during wakefulness and downscaling during sleep. Moreover, sleep deprivation (extension of the wake period by six hours)

prevented synaptic downscaling in both structures. Furthermore, the impact of high-fat feeding on hypothalamic and cortical GluA1- and GluA2-containing AMPARs (study II) was investigated. Three days, but not one day of high-fat diet (HFD) decreased the levels of AMPAR GluA1 and GluA2 subunits, as well as GluA1 phosphorylation at Ser845, in the hypothalamus but not cortex. In a control experiment, the reversibility of HFD-induced changes was investigated by comparing the 3-day HFD with a 3-day HFD followed by four recovery days of normal chow. This experiment corroborated the suppressive effect of high-fat feeding on hypothalamic but not cortical AMPAR GluA1, GluA2 and GluA1 phosphorylation at Ser845, and indicated that the effects can be reversed by normal chow feeding. In both experiments, high-fat feeding stimulated energy intake and raised body weight as well as serum concentrations of insulin, leptin, free fatty acids and corticosterone. Only the 3-day HFD increased wakefulness assessed via video analysis in the six hours before tissue collection.

These results demonstrate (i) that in accordance with our hypothesis, wakefulness is associated with net synaptic upscaling in the hypothalamus and sleep induces respective synaptic downscaling; sleep deprivation interferes with the latter process and induces upscaling. (ii) Contrary to our expectations, short-term high-fat feeding downregulates rather than upregulates hypothalamic synaptic strength; this effect is rapidly reversible and does not extend to the cortex. These findings suggest that global downscaling processes accompany the initial phase of high-calorie intake to shift the overall hypothalamic activity balance and, possibly, to counteract anabolic drive and weight gain. Sleep in comparison to wakefulness or sleep deprivation likewise attenuates synaptic strength in the hypothalamus, corroborating the assumption that sleep exerts a normalizing effect on neuronal metabolic homeostasis that is thwarted by sleep deprivation. It will be important to investigate how long-term exposure to high-calorie food and the eventual development of obesity tip these synaptic scales, and how manipulations of sleep and wakefulness might be employed to recalibrate the hypothalamic regulation of energy homeostasis.

1. Introduction

Sleep is a phylogenetically highly conserved process that occurs in almost all known species, but its functions for the organism are not conclusively clear, the detrimental effects of sleep deprivation notwithstanding (Siegel 2009). Previous studies on the effects of sleep on memory indicate that sleep benefits the formation of memories of contents acquired during the previous wake phase (Born et al 2006, Rasch & Born 2013). One of the leading theories on this effect, the theory of synaptic homeostasis, suggests that sleep is essential for the regulation of synaptic strength in the central nervous system (CNS) (Cirelli & Tononi 2015, Tononi & Cirelli 2014). According to this theory, sleep induces a global reduction in synaptic strength that leaves only particularly relevant associations intact, so that in the subsequent wake phase new information can be stored. The main mechanism of synaptic scaling and plasticity is trafficking of postsynaptic glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) (Collingridge et al 2004). As AMPARs are delivered to excitatory synapses, synaptic strength increases, whereas synaptic depression is associated with their removal (Malenka & Bear 2004). Wakefulness is associated with extensive synaptic potentiation, whereas sleep, and in particular deep sleep, leads to a global decrease in the relative strength of synaptic connections. Thus, sleep leads to synaptic downscaling and reduces synaptic strength in the cortex and hippocampus (Vyazovskiy et al 2008).

The relevance of sleep for metabolic control has received increasing interest in recent years. Shortening or impairing sleep can adversely affect eating behavior and homeostatic metabolic control, for example, by increasing food intake and weakening glucose homeostasis (Schmid et al 2015). Therefore, it is important to study whether sleep-dependent changes in synaptic connections also occur in areas which are important for metabolic regulation. The hypothalamus, specifically its ventromedial and arcuate nuclei, plays a critical role in the regulation of eating behavior and energy balance (Abizaid & Horvath 2008). Many metabolic hormonal signals from the periphery of the body, such as the fat cell product leptin, act on the hypothalamus, and corresponding neurotransmission in hypothalamic circuits

mediate the effects of these hormones, thereby inhibiting or stimulating feeding behavior (Beck 2000, Klok et al 2007). These observations suggest that dietary manipulations could affect glutamatergic signaling and plasticity in the hypothalamus (Suyama et al 2016).

In this thesis, I investigate in study I whether synaptic downscaling during sleep as compared to wakefulness or sleep deprivation also occurs in the hypothalamus, a brain region most relevant for the regulation of appetite and energy balance. It is hypothesized that sleep-induced downscaling in hypothalamic circuits (i.e., a decrease in the number of receptors containing AMPARs) is a physiological mechanism that mediates a resetting influence of sleep on energy balance. In study II, I set out to assess global synaptic changes in the hypothalamus in response to dietary manipulations (i.e., short-term increase in calorie intake) to delineate the impact of dietary changes on hypothalamic synaptic homeostasis. In combination, both sets of studies are expected to elucidate how sleep and food intake dynamically regulate AMPAR-based neurotransmission in the hypothalamus as a key center of metabolic control.

1.1 AMPA receptors

AMPA receptors have been named after α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, a synthetic pharmacological ligand that exerts an agonistic effect on these receptors (Honore et al 1982). AMPARs are ionotropic transmembrane receptors for glutamate, which is the most abundant excitatory neurotransmitter system supporting fast synaptic transmission in the vertebrate CNS.

1.1.1 AMPARs structure and subunit composition

AMPARs are transmembrane protein complexes comprised of four distinct subunits, assembled as dimers-of-dimers to form hetero-tetrameric receptors (Hollmann & Heinemann 1994, Traynelis et al 2010, Zhao et al 2019). All AMPAR subunits contain approximately 900 amino acids and have a molecular weight of approximately 105 kDa. Figure 1 depicts the AMPAR subunit structure. The four AMPAR subunits exhibit functional diversity after different post-transcriptional editing and post-translational modifications. Although the extracellular and transmembrane regions of the AMPAR subunit are very similar, their intracellular tails are different (Shepherd & Huganir 2007). For instance, GluR1 and GluA4 have longer cytoplasmic tails, while GluR2 and GluR3 have shorter tails. GluA2 is the most important part of the AMPAR function. This subunit determines many of the major biophysical properties of natural receptors, including receptor kinetics, single channel conductance, Ca^{2+} permeability, and endogenous polyamine blocks (Shepherd & Huganir 2007). The vast majority of AMPARs in principal neurons contain the subunit GluR2, which prevents AMPAR from penetrating Ca^{2+} (Isaac et al 2007). In contrast, in the absence of GluA2, AMPARs are highly permeable to Ca^{2+} and not efficiently integrated into synapses (Grosskreutz et al 2010). Although receptors lacking GluR2 account for a small fraction of neurons (Carroll et al 1999a). However, it is also possible to cause an increase in the level of receptors lacking GluR2 by various manipulations such as long-term potentiation (LTP) stimuli or pharmacy treatment (Greger & Esteban 2007). The dimeric GluA1-GluA2 and GluA2-GluA3 receptors are the most common assemblies in the mammalian brain.

Although there are many approaches, such as immunoprecipitation with subunit-specific antibodies (Wenthold et al 1996) and single-cell genetics (Lu et al 2009), the subunit arrangement and molecular structure of a single native AMPAR are still unknown.

Each AMPARs subunit comprises four domains, an extracellular amino (N)-terminal domain, a ligand-binding domain, a transmembrane domain that contains a pore loop and an intracellular carboxyl (C)- terminal domain. The molecular structure is similar between different subunits (approximately 70% homology in amino acid residue identity) with conserved transmembrane and extracellular domains and diverse C-terminal intracellular tails (Chater & Goda 2014). The extracellular N-terminal domain has a large lobe comprising almost half of the subunit's amino acids but lacks a clear function role. The extracellular ligand-binding domain consists of two lobes which create a clamshell-like binding site for glutamate (Erreger et al 2004). The transmembrane domain is constructed out of three transmembrane segments (TM1, TM2 and TM4) and a "U" loop on TM2 to form the ion channel of the receptor. TM2 is different from the others as it forms an intracellular hairpin loop, thus not full traversing the cell membrane (Baude et al 1994). The intracellular C-terminal domain is the most functionally relevant region and contains multiple binding sites for intracellular proteins (i.e., the C-terminal domain is the most relevant to protein phosphorylation sites that contribute to trafficking of AMPARs, which is particularly important for the activity-dependent regulation of fast excitatory transmission in synapses). The C-terminal domain interacts with signaling proteins (i.e., kinases, phosphatases) and determines several aspects of AMPARs function including gating, trafficking, and stabilization at the synapse (Lu & Ziff 2005, Nicoll et al 2006, Terashima et al 2004, Tomita et al 2006). AMPARs are also capable of further modulating receptor properties by binding to transmembrane AMPAR regulatory proteins (TARPs), the first recognized auxiliary subunit involved in neurotransmitter-gated ion channels (Chen et al 2017, Kato et al 2010).

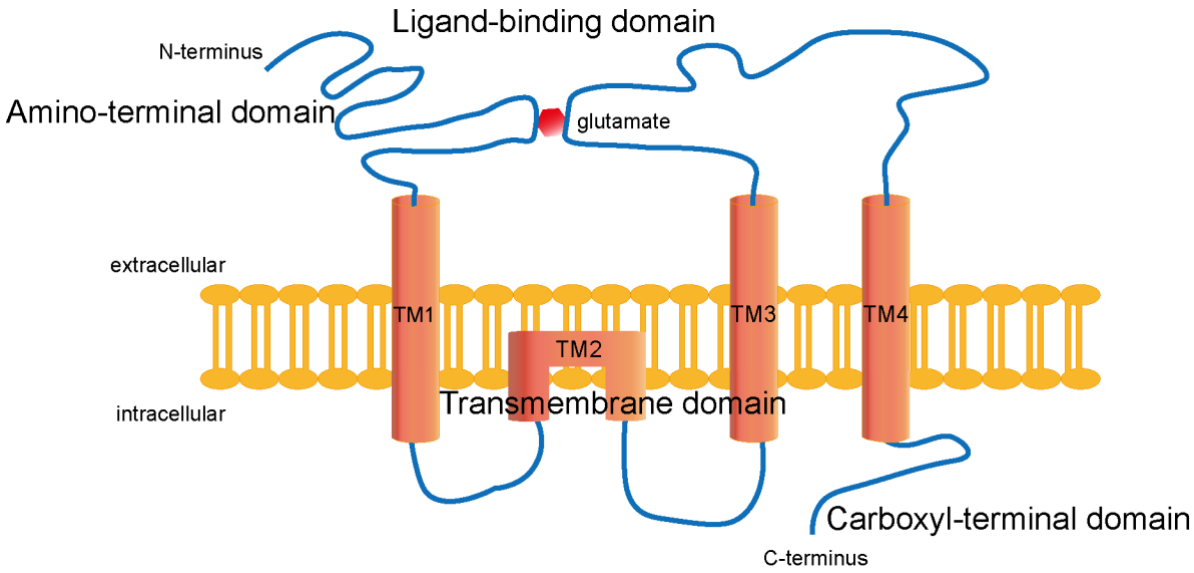

Fig. 1. Schematic representation of the structure of AMPAR subunits in the cell membrane. The four main domains (an extracellular N terminus domain, a ligand-binding domain, a transmembrane domain and an intracellular C-terminus) of the AMPA receptor are depicted. The N-terminal domain comprises almost half of the subunit's amino acids but lacks a clear functional role. The ligand-binding domain has two lobes which form a binding pocket for glutamate. The carboxyl-terminal domain contains interaction sites for post-translational regulation such as phosphorylation. Abbreviations: N, amino-terminus (NH2-terminus); C, carboxyl-terminus; TM1-4, transmembrane domains 1-4.

1.1.2 AMPAR trafficking and location

AMPARs widely expressed throughout the central nervous system (Belachew & Gallo 2004, Wisden & Seeburg 1993) in dependence of the brain region (Collingridge et al 2004, Dingledine et al 1999). The trafficking of AMPARs into and out of the synapse has been one of the most fascinating and intriguing areas of neuroscience over the past 30 years. As mentioned above, the main determinant of AMPARs trafficking is dependent on the AMPAR subunit composition; e.g., in cortex, GluA1 rather than GluA2 is the primary determinant during activity-dependent AMPAR exocytosis, while GluA2 is more dominant than GluA1 during endocytosis (Shepherd & Huganir 2007). This difference is mainly due to interactions of the intracellular C-terminal domains of AMPAR subunits with various signaling proteins, indicating that AMPAR trafficking is a result of the combination of multiple signaling proteins and their own composition (van der Sluijs & Hoogenraad 2011). Bidirectional

regulation of the number of synaptic AMPA receptors is the basis for two of the most well-studied phenomena of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) in the brain (Bredt & Nicoll 2003, Collingridge et al 2004, Malinow & Malenka 2002). In addition, AMPARs can cycle into and out of the synapse very quickly and dynamically (Lüscher et al 1999, Lüthi et al 1999, Malinow et al 2000, Osten et al 1998). The number of AMPARs is tightly regulated by basic cellular trafficking mechanisms such as Golgi-derived secretory transport, receptor exocytosis, lateral diffusion, endosomal recycling, and receptor degradation (Bredt & Nicoll 2003, Choquet & Triller 2003, Greger & Esteban 2007, Kessels & Malinow 2009, Sheng & Lee 2003, Shepherd & Huganir 2007).

AMPARs are biosynthesized and assembled as dimers-of-dimers in the endoplasmic reticulum and Golgi apparatus in the soma (and dendrites) and are delivered to the synaptic membrane (Figure 2) (van der Sluijs & Hoogenraad 2011). The delivery of AMPARs to the synapse requires kinesin-dependent vesicular trafficking on microtubule networks before insertion into the plasma membrane. Surface AMPARs are internalized into the synapse by endosomal sorting, like any other internalized membrane protein, before constitutive recycling back to the surface membrane (LTP) and degradation to the lysosomes (LTD) (Bredt & Nicoll 2003, Malinow & Malenka 2002) depending on the endocytic stimulus (Ehlers 2000). On the other hand, endocytosis of AMPARs occurs via a clathrin- (Glebov et al 2015) and dynamin-mediated pathway (Carroll et al 1999a, Montgomery et al 2005). Small Rab GTPases and their effectors are involved in the further endosomal trafficking (Novick et al 2006).

AMPARs

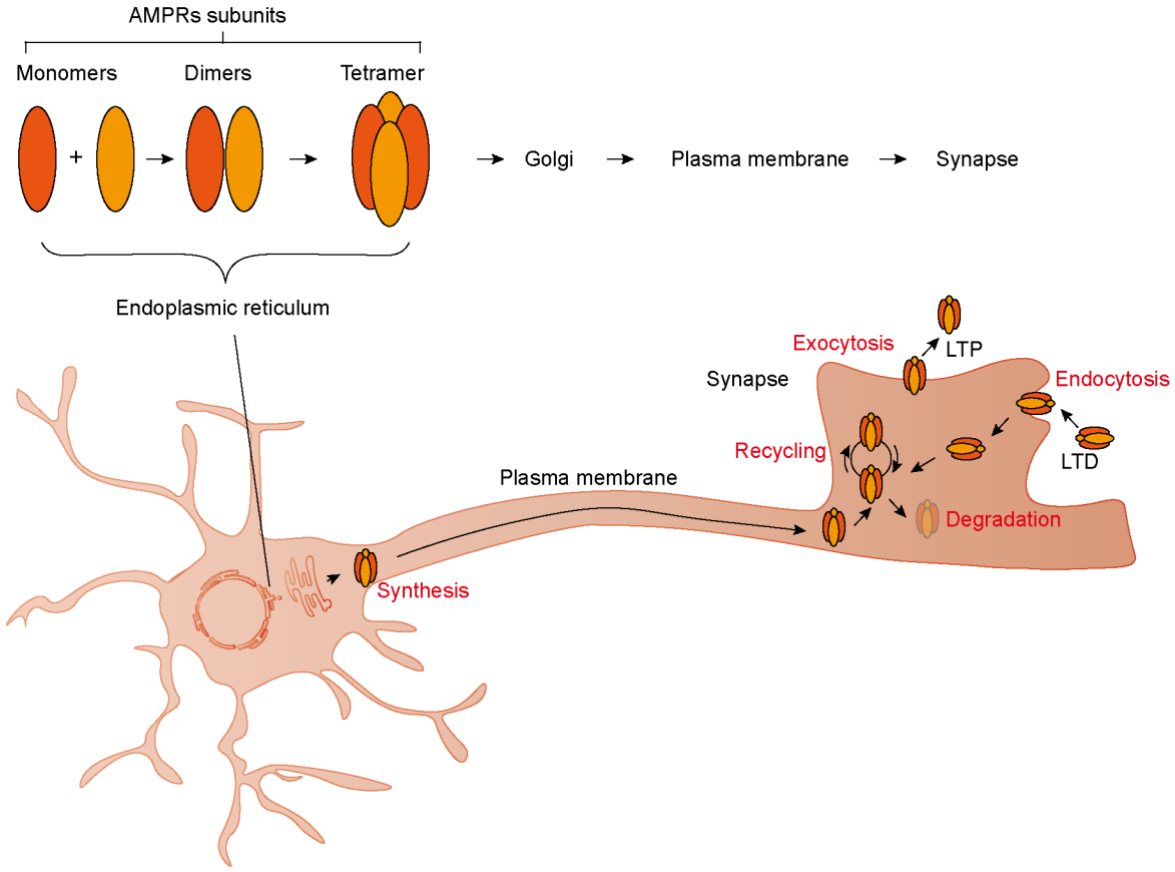

Fig. 2. Schematic diagram illustrating the trafficking of AMPARs during synaptic plasticity. AMPARs are assembled in the endoplasmic reticulum by the successive formation of dimers and tetramers. The receptors are transferred to the recycling endosomes and returned to the plasma membrane by exocytosis, followed by the induction of LTP. Conversely, LTD is induced by enhanced endocytosis at extra-synaptic sites or AMPARs diffusion away from the synapse.

The AMPAR expression profile has been identified by multiple methods at different stages of protein synthesis (e.g., at mRNA and at protein level), i.e., by receptor autoradiography (Monaghan et al 1984), in situ hybridization (Keinanen et al 1990) and immunohistochemistry (Petralia & Wenthold 1992). Moreover, AMPAR subunit specificity has been shown with the help of the two latter techniques. In the forebrain, including the hippocampus, cerebral neocortex and hypothalamus, co-immunoprecipitation experiments showed that the predominately expressed subunits are GluA1 and GluA2, with low levels of GluA3 and GluA4 (Bartanusz et al 1995, Derkach et al 2007, Geiger et al 1995, Isaac et al 2007, Tsuzuki et al 2001). At present, the study of the function of AMPAR first requires obtaining high-purity synaptoneurosomes, which are functional synaptic particle-containing AMPAR molecules in the brain and commonly used to study synaptic function (Wishart et al 2006).

The expression of AMPARs differs between developmental stages. GluA2 appears as early as on embryonic day 16 in rats, whereas other receptors are up-regulated during the later stages of development (Dravid et al 2010). The GluA2 subunit can also be selectively altered during synaptic plasticity as well as during CNS injury (e.g., global ischemia). These subunit composition changes will alter functional receptor properties (described in chapter 1.1.1).

1.1.3 AMPARs in synaptic plasticity

AMPARs are the main ionotropic glutamate receptors mediating fast excitatory synaptic transmission in the mammalian brain, and their regulated trafficking underlies activity-triggered changes in transmission (Anggono & Huganir 2012). LTP and LTD are the two most studied and prevalent models of synaptic plasticity (Malenka & Bear 2004). Protein phosphorylation and protein kinase play essential roles in the regulation of neuronal function (Greengard 2001) and synaptic plasticity in almost all cell types and have helped to elucidate many signaling pathways involved in LTP and LTD (Citri & Malenka 2008, Thomas & Huganir 2004). Accumulating evidence suggests that changes in AMPAR phosphorylation and AMPAR number are associated with LTP and LTD (Gomes et al 2003).

Phospho-specific antibodies to GluA1 phosphorylation have been detected at two sites, Ser845 or Ser831, indicating that both sites of GluA1 are phosphorylated when LTP is induced in the hippocampal CA1 region (Lee et al 2000). LTP diminished in mice after mutation of AMPAR subunit GluA1 on Ser845 or Ser831 (Lee et al 2000). *In vivo* studies showed that high-frequency stimulation reversibly increases the amount of GluA1- and GluA2-containing AMPARs in synaptoneurosomes (a fraction including the presynaptic and postsynaptic elements of the synapse) isolated from the hippocampal CA1 region (Heynen et al 2000). This reversible increase indicates that membrane fusion is required for LTP in the postsynaptic membrane. In cultured hippocampal neurons, induction of LTP by stimulating synaptic N-methyl-d-aspartate (NMDA) glutamatergic receptors with glycine is also accompanied by a rapid insertion of AMPARs in the surface of dendritic membranes (Lee et al 2000).

Ca^{2+} influx through NMDA receptors is critical for inducing LTP and leads to calcium/calmodulin-dependent protein kinase II (CaMKII) activation (Fukunaga et al 1993). Many studies support a key role for CaMKII in inducing LTP (Lisman et al 2002). Genetic deletion of CaMKII or postsynaptic injection of inhibitors blocks the induction of LTP (Malenka et al 1989, Malinow et al 1989, Silva et al 1992). Furthermore, intracellular perfusion of constitutively active CaMKII was found to enhance synaptic transmission (Lledo et al 1995), strongly suggesting direct effects of CaMKII in LTP induction. CaMKII directly phosphorylates GluA1 at Ser831 (Barria et al 1997a), which enhances single-channel of AMPAR conductance (Lee et al 2000, Whitlock et al 2006). Ser831 phosphorylation of GluA1 is a substrate for CaMKII, and increased levels of CaMKII and its phosphorylation at Thr286 are also associated with synaptic potentiation and LTP, both in vitro and *in vivo* (Lisman et al 2002). When active CaMKII was expressed together with GluA1, an increase in effective conductance of about 50% was observed (Lisman et al 2002).

Synaptic incorporation induced by CaMKII requires GluA1 phosphorylation on Ser845 by Protein kinase A (PKA) (Esteban et al 2003), which is another critical event in LTP. However, synaptic incorporation of GluA1 in hippocampal slices cannot be induced by PKA activation alone, which indicates that PKA and CaMKII act in parallel to induce the transmission of AMPARs. A nearly 40% potentiation of the peak amplitude of the whole-cell glutamate-gated current was induced by intracellular perfusion of PKA into GluA1-transfected HER 293 cells (Roche et al 1996) and this enhancement did not occur after the mutation of Ser845 to alanine.

The endocytosis of surface AMPARs is assumed to be important for the emergence of LTD (Beattie et al 2000, Lissin et al 1999). The first experiment to validate the role of AMPAR endocytosis in LTD relied on immunocytochemical data showing that the induction of LTD in hippocampal culture results in a concurrent decrease in the number of synapses containing surface AMPARs (Carroll et al 1999b). Furthermore, hippocampal LTD induced *in vivo* caused a reduction in the number of AMPAR in

synaptoneurosomes (Heynen et al 2000). However, many stimuli can react to AMPARs internalization and it is difficult to pinpoint the actual molecular pathway establishing LTD.

Regulation of the phosphorylation of AMPAR subunits is also important for LTD. LTD has been found to be associated with the dephosphorylation of postsynaptic protein kinase C (PKC) and PKA substrates (Hrabetova & Sacktor 1996, Kameyama et al 1998, Lee et al 2000). Dephosphorylation of the PKA substrate during LTD is particularly convincing because post-synaptic inhibition of PKA (or its replacement with intracellular anchoring proteins) results in a disruption of synaptic transmission that occludes LTD (Citri & Malenka 2008). Consistent with the role of PKA, LTD is involved in selective dephosphorylation of GluA1 at the Ser845 site, which is associated with endocytosis of AMPARs (Lee et al 2000). Moreover, knock-in mouse lacking both Ser845 and Ser831 exhibit defects in LTD (Lee et al 2000).

The interactions of GluA2 with several proteins are associated with LTD, and the phosphorylation of GluA2 is also a significant determinant of LTD. The phosphorylation of GluA2 at the site of Ser880 is located within the GluA2 C-terminal PDZ ligand [postsynaptic density-95 (PSD-95), discs large, zona occludens-1], which is responsible for binding to PDZ domain proteins. For example, since CRIP1 binding stabilizes GluA2 at the surface and protein interaction with C kinase 1 (PICK1) promotes receptor internalization, it is proposed that this differential binding to GluA2 at the phosphorylated site is the basis for removal of GluA2 during LTD (Palmer et al 2005). Another phosphorylation site of GluA2 at Thy876 is involved in the binding to the guanine-nucleotide exchange factor BRAG2, which activates the small GTPase Arf6 to internalize synaptic AMPA receptors upon LTD induction (Scholz et al 2010).

1.2 Sleep and synaptic homeostasis

Trafficking of postsynaptic glutamatergic AMPARs as described in the previous paragraphs is assumed to be a key mechanism of synaptic plasticity, but also synaptic scaling (Collingridge et al 2004). When changes in neuronal activity persist for a long time, synaptic strength and cell membrane excitability show compensatory changes that lead to renormalization in the network (Tononi & Cirelli 2014) such that the firing frequency of neurons is restored to a suitable working range. For example, when neural networks are silent for a long time, synaptic strength and membrane excitability in neurons will increase until the discharge frequency returns to normal. When neurons are in a highly active state for a long time, their synaptic strength and membrane excitability will decrease so the discharge frequency and k to the normal working range. Therefore, it is generally believed that these processes including *synaptic homeostatic scaling* ensures that electrical activity in neural circuits remains within a normal working range, thereby promoting the circuit stability.

The theory of synaptic homeostasis posits that sleep is essential for synaptic homeostasis in the CNS (Cirelli & Tononi 2015, Tononi & Cirelli 2014). According to this theory, sleep induces a global reduction in synaptic strength that leaves only particularly relevant associations intact, so that in the subsequent wake phase new information can be stored. Sleep is present in every species and vital for physiological processes such as cognitive (Born et al 2006, Sejnowski & Destexhe 2000), metabolic (Aldabal & Bahammam 2011) and immune (Besedovsky et al 2012, Kalin et al 2015) function. The theory proposes that the synaptic connections in many neural circuits increase during wakefulness (synaptic upscaling) and induce the need for sleep. Therefore, one function of sleep is to appropriately attenuate these enhanced synapses, returning the total synaptic strength of the network to the baseline. This effective and economic adjustment is necessary for proper neuronal functioning during subsequent wakefulness. Some scholars have compared it to a "reset button" (downscale) (Liu et al 2010, Tononi & Cirelli 2014, Vyazovskiy et al 2008). After a period of waking, the brain will reach a saturation point, and the stored energy and space will no longer provide synaptic activity. Meanwhile,

the demand for sleep is gradually increasing, so some researchers speculate that sleep is a favorable time period for synaptic downregulation and the restoration of synaptic transmission efficiency (Tononi & Cirelli 2007). Synapses decreases in number and size during sleep (Bushey et al 2011, Donlea et al 2009) as they prepare for a new round of learning-induced synaptic enhancement.

How does the high/low active state of neurons become positive or negative compensation? Genes and proteins expressed during electrical activity of neurons, especially those that play a role in regulating synaptic and membrane excitability, are reasonable candidates. A systematic comparison of expression of sleep and wake-related molecules was performed using mRNA differential analysis and cDNA microarray technology (Cirelli & Tononi 2000). To some extent, the two analytical methods are complementary, the former can detect changes in the expression of identify known genes, and the latter is mainly used to any known or unknown mRNA. In the experiment, the expression of about 10,000 genes in the cortex was analyzed. Only a few genes change with sleep and wakefulness; mRNA for 44 genes is highly expressed during wakefulness, and ten genes are highly expressed during sleep.

The primary mechanism underlying synaptic potentiation and depression is the trafficking of GluA1- and GluA2-containing AMPARs into and out of the synaptic membrane. Homeostatic adaptation of synaptic strength may therefore be achieved through an increase (upscaling) or decrease (downscaling) of functional AMPARs. Studies at the molecular level have verified this via western-blotting and a consistent conclusion is that sleep induces homeostatic scaling-down, i.e., a loss of synaptic AMPARs and AMPAR dephosphorylation (Diering et al 2014), which are considered major determinants of synaptic strength (Huganir & Nicoll 2013). In addition, Homer1a, an immediate early gene whose activity is involved in AMPARs downscaling (Chowdhury & Hell 2018), was recently shown to drive global downscaling during sleep in rodents whereas no homeostatic changes were observed in Homer1a-knockout neurons, indicating the importance of Homer1a in homeostatic synaptic plasticity (Diering et al 2017). Moreover, induction of LTD can dephosphorylate the serine 845 of AMPAR GluA1 subunit (Whitlock et al 2006), and sleep is also beneficial for dephosphorylation in the brain

(Cirelli & Tononi 1998). Vyazovskiy et al. (Vyazovskiy et al 2008) found that the number of AMPAR subunit GluA1 increased by 50% after 4-6 hours of waking, along with phosphorylated GluA1 and CamKII. However, these substances decreased simultaneously after sleep, indicating that dephosphorylation in the brain during sleep is one of the causes of synaptic downregulation.

Electrophysiological experiments have yielded evidence that supports this assumption. Currently, SWA (slow-wave activity) is widely used as a reliable electrophysiological indicator of sleep. Sustained awakening for 6 hours resulted in a 26.4% increase in SWA during subsequent sleep (Meerlo et al 2001). Human studies have shown that the slow component of SWA (0.5-2.5 Hz) increases during the first hour of sleep after excessive learning (Ferri et al 2008). It was further found that expression of synaptic enhancement markers increased with extended periods of exploration of the environment, with respective increases in SWA during sleep (Huber et al 2007). The slope of local field potentials (LFPs) is also typically used as a measure of synaptic strength. After wakefulness of 4 hours, the slope increased by an average of 22%, but after the same time spent sleeping, the slope decreased by about 20% (Vyazovskiy et al 2008). Additionally, brain metabolic rate is an external manifestation of synaptic strength. After a night of sleep, absolute cerebral blood flow was 18% lower than when the previous night was awaking (Tononi & Cirelli 2006). However, most of the current research is focused on cognitive brain region, whereas almost nothing is known about the corresponding mechanisms in the hypothalamus.

1.3 The hypothalamus in the regulation of energy balance and sleep

1.3.1 Hypothalamic regulation of energy balance and sleep/wake behavior

The hypothalamus is a highly plastic brain region that can quickly adapt to environmental changes (Dietrich & Horvath 2013). The hypothalamus controls energy homeostasis and many neuroendocrine functions in the body (Williams & Elmquist 2012). The key to this regulatory function is the central

melanocortin system (CMS) (Cone 1999), which contains neurons that express agouti-related peptide (AgRP), neuropeptide Y (NPY) or pro-opiomelanocortin (POMC). Elucidating the mechanism by which the melanocortin system regulates energy balance has become a hot topic in human and rodent research. These neurons are mainly expressed in the arcuate nucleus (ARC) of the hypothalamus (Luquet et al 2005). Another non-negligible component that affects energy homeostasis are the circulating levels of hormones, such as insulin and leptin, which activate or inhibit AgRP neurons or POMC neurons, thereby altering energy intake and consumption (Benoit et al 2002, Choudhury et al 2005, Cowley et al 2001, Ernst et al 2009). Melanocyte-stimulating hormones (MSHs) are released from the synaptic terminals of POMC neurons and acts on the melanocortin receptor (MC3R/MC4R) expressed by the paraventricular nucleus (PVN) (Vogt & Bruning 2013). Leptin is also a key factor involved in energy homeostasis. Leptin is a protein hormone secreted by white fat cells with functions in CNS and periphery. When body weight declines, fat cells secrete less leptin, and the leptin that is transmitted to the brain through the blood is also reduced, thereby causing an increase in energy intake. Conversely, an increase in leptin leads to a decrease in energy intake within a certain range of body weight (Benoit et al 2000).

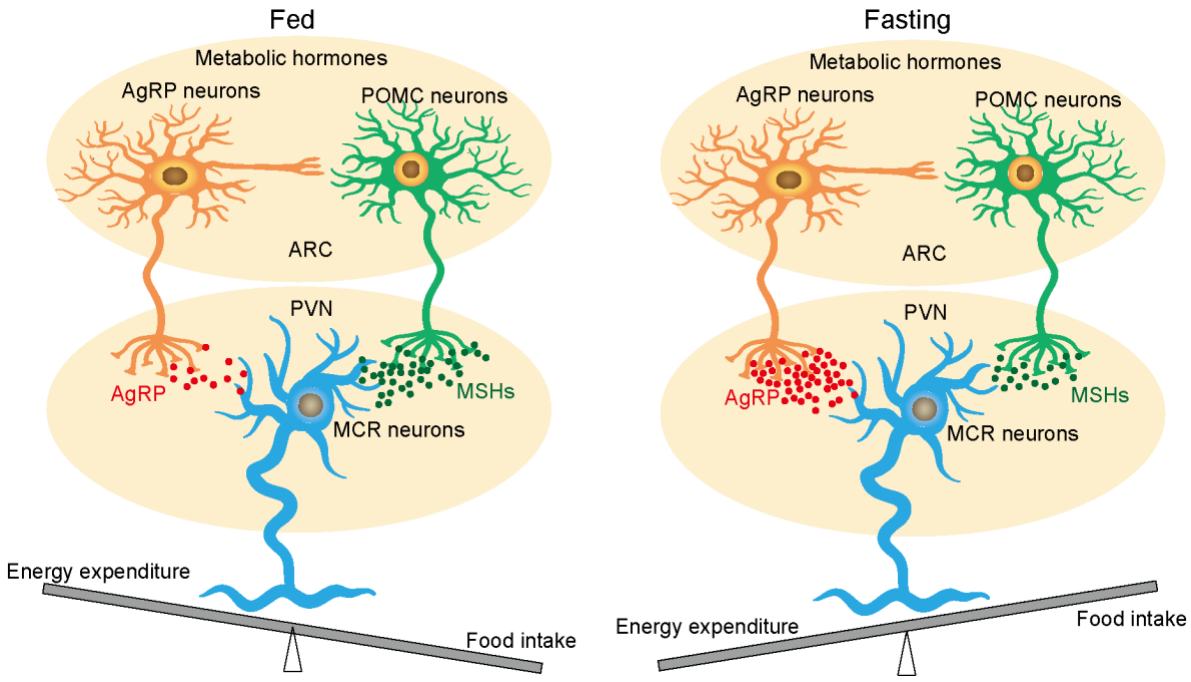

Fig. 3. Hypothalamic control of energy homeostasis. The hypothalamus senses and integrates feedback in the form of metabolic hormones, which directly act on neurons in the ARC of the hypothalamus to control energy homeostasis. During feeding, the expression of AgRP decreases, whereas POMC expression is increased, which triggers MCR signaling and cumulates in satiety and stimulation of energy expenditure. During fasting, AgRP levels increase and POMC levels decrease, resulting in decreased MCR signaling. Abbreviations: ARC, arcuate nucleus; PVN, paraventricular nucleus; AgRP, Agouti-related protein; POMC, Pro-opiomelanocortin; MCR, melanocortin receptor; MCHs, melanin-concentrating hormones. Modified from (Jais & Bruning 2017).

The hypothalamus contributes to the regulation of sleep by homeostatic mechanisms and the circadian rhythm, both of which are reflected in compensatory increases in the intensity and duration of sleep after insufficient sleep (Elbaz et al 2013). Sleep/wake regulation is a physiological process involving multiple systems with complex neuromodulation mechanisms. In almost all mammals, sleep and other circadian rhythms are controlled by a master oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Granados-Fuentes & Herzog 2013). This master oscillator in the SCN targets hormonal and neuronal circuits which in turn feedback on the master oscillator and affect the sleep/wake cycle (Elbaz et al 2013). Another important structure involved in sleep/wake regulation is the lateral hypothalamus, which is the exclusive source of the arousal-promoting peptides hypocretins I and II (also known as orexin A and orexin B) (Koyama et al 2002). A large number of excitatory

signals are sent from the neurons in the lateral hypothalamus to wake-promoting centers: adrenergic, histaminergic, dopaminergic, and cholinergic nuclei (Siegel 2004). The adrenergic and serotonergic neurons can inhibit the production of calcitonin. On the other hand, cholinergic neurons stimulate low creatinine neurons with positive feedback. Thus, the hypothalamus has a direct role in sleep-wake transitions (Siegel 2004). Contrary to the stimulation of the posterior hypothalamus, the nucleus located in the ventrolateral preoptic nucleus (VLPO) of the hypothalamus is a "sleep-generating" center (Kalia 2006). These neurons contain the inhibitory transmitters γ-aminobutyric acid (GABA) and galanin and projected to the arousal neurons of the hypothalamus and brainstem.

1.3.2 Interactions between sleep and metabolic control

The brain acts as the primary coordinator of behavioral and peripheral tissue function. The main function of the hypothalamus is the maintenance of energy homeostasis, including energy intake and expenditure (see section 1.3.1). While maintaining energy homeostasis, the hypothalamus is also critical for sleep and wake regulation, in addition to the brainstem and thalamic cortex systems. The sleep control systems in the hypothalamus and their interaction with circadian pacemakers in the suprachiasmatic nucleus (SCN) have been demonstrated (Mignot et al 2002). In addition, there is sufficient evidence for interactions between hypothalamic control of sleep and other functions such as food intake, metabolism, hormone release, and temperature regulation. For instance, sleep deprivation alters hormone release, increases body temperature, stimulates appetite, and activates the sympathetic nervous system (Rechtschaffen et al 1989, Spiegel et al 1999). Therefore, interactions between sleep control in the hypothalamus is tightly integrated with the homeostatic energy system.

Sleep has long been considered an energy-conservation process that is ubiquitous in the animal kingdom (Berger & Phillips 1995, Siegel 2005). However, historically, the energy-conservation mechanism of sleep has only been studied in terms of the degree of metabolic rate reduction during sleep compared to quiet waking, conceptually similar to numbness or hibernation. For instance,

mammals typically reduce the metabolic rate by 15-30% (Jung et al 2011, Rechtschaffen 1998) during sleep. For organisms such as humans with a sleep quota of 8 hours per day, this 8-hour metabolic rate reduction is equivalent to energy savings of only 5-9% per 24 hours. It is clear that modest daily energy savings are insufficient to explain the prevalence of sleep, especially when considering the costs of a sleep strategy such as increased predation risk and reduced foraging opportunities (Illius et al 2002).

According to the energy allocation hypothesis proposed in recent years, the sleep-wake cycle is actually a behavioral strategy to enhance dynamic energy allocation (Schmidt 2014, Schmidt et al 2017). Energy utilization is prioritized for waking efforts (i.e., alertness, foraging, and reproduction), whereas these wake-predominant processes are downregulated during sleep. Microarray studies show that the maximum activation of many genes is specific to either sleep or wakefulness (Cirelli et al 2004, Cirelli et al 2005, Mackiewicz et al 2007, Terao et al 2006). Of the genes whose expression is regulated according to the animal's behavioral state, about half of the genes increase during waking, and about half of the genes increase during sleep (Cirelli et al 2004, Cirelli et al 2005). More specifically, the sleep-related genes are involved in brain synthesis and intracellular transport (Mackiewicz et al 2007), while waking-related genes are mainly involved in excitatory neurotransmission, synaptic enhancement and memory (Schmidt 2014).

Looking at the relationship between energy and sleep from another perspective, the quality or duration of sleep directly affects energy intake and expenditure (Schmid et al 2015). In humans, energy expenditure after sleep deprivation is higher than after normal sleep (Jung et al 2011). Even this phenomenon is more pronounced in rodents. In rats, whole-day energy expenditure is 50% higher during the first 24 hours of total sleep deprivation compared to baseline (Everson et al 1989). These changes are inextricably linked to changes in appetite-related hormones (e.g., leptin and ghrelin). Both leptin and ghrelin are hormones produced by peripheral tissues that act on the corresponding hypothalamic nucleus to regulate energy homeostasis and appetite (Copinschi 2005). After sleep deprivation, leptin secretion is reduced, but the secretion of the appetite factor ghrelin is increased

(Schmid et al 2008). The combined result is increased hunger and increased appetite, especially for high-calorie food. There was a significant correlation between the increase in hunger and the ratio of ghrelin/leptin during sleep deprivation (Spiegel et al 2004).

1.3.3 Energy balance and behavioral state integration in the hypothalamus

Nearly a century of evidence suggests that the wakefulness is promoted by neurons in the posterior lateral hypothalamus and sleep by neurons in the preoptic area (Lin et al 1989). These identified behavioral state regulation system in the hypothalamus interact with circadian pacemakers in the SCN. Furthermore, interactions between sleep and other hypothalamic functions, such as food intake, metabolism, hormone release, and temperature regulation, are increasingly being explored (Rechtschaffen et al 1989, Spiegel et al 1999). Therefore, the behavioral state control system in the hypothalamus is tightly integrated with the homeostasis system.

A compelling hypothesis has been that the orexins (orexin-A and orexin-B, also called hypocretin-1 and hypocretin-2) are hypothalamic peptides with an important role in the regulation of sleep-wake cycle and related hypothalamic functions (Beuckmann & Yanagisawa 2002, Ohno & Sakurai 2008, Taheri et al 2002). The orexin system is closely related to sleep (Peyron et al 2000, Thannickal et al 2000). In rodents, orexin-A administration (via i.c.v. injection) increases wakefulness in a dose-dependent manner (Piper et al 2000), while pharmacological antagonism of the orexin receptor results in increased non-REM (NREM) and REM sleep and reduces wakefulness in animals and humans (Brisbare-Roch et al 2007). In addition, orexin neurons synthesize Fos protein (an indicator of neuronal activity) during wakefulness and the number of Fos-positive orexin-containing neurons correlates closely with the amount of wakefulness (Chemelli et al 1999, Estabrooke et al 2001). In contrast to the predominant role in wakefulness, orexins have an effect on neuroendocrine secretion. Orexin-deficiency might result in neuroendocrine abnormality, which might result in obesity (Sakurai 2005). Electrophysiological studies showed that peripheral humoral factors influenced the activity of orexin

neurons. The activity of isolated orexin neurons is decreased by glucose and leptin and increased by ghrelin (Yamanaka et al 2003). Accumulated evidence supports that feeding regulation as well as behavioral state control are segregated within the hypothalamus (Korotkova et al 2006).

In contrast to orexins, the melanin-concentrating hormone (MCH) neurons in hypothalamus are also involved in metabolism and sleep-wake behavior (Bittencourt et al 1992, Verret et al 2003). The MCH promotes sleep and is responsive to energy status. For instance, mice lacking MCH or MCH1 receptors tend to decrease their food intake with an increased metabolic rate (Marsh et al 2002, Shimada et al 1998). Moreover, recordings from identified MCH neurons across the sleep-wake cycle revealed that they are silent during wake and fire occasionally during NREM sleep but maximally during REM sleep (Gillin et al 1991). MCH neurons also release Nesfatin-1, a recently discovered satiety hormone produced in the hypothalamic nucleus (Oh et al 2006), and disruption of Nesfatin-1 signaling reduces REM sleep expression (Jego et al 2012).

In summary, the two vital physiological processes, energy balance and sleep-wake behavior, have to be coordinated under the tightly monitoring of hypothalamus. Both MCH and orexin neurons in the hypothalamus dominate multiple brain regions controlling metabolism, and sleep-wake behavior.

1.4 Diet-induced changes in hypothalamic synaptic plasticity

1.4.1 Acute effects of high-fat diets in the hypothalamus

As a main brain center of metabolic control, the hypothalamus rapidly responds to metabolic challenges. Substantial evidence indicates that rodents after exposure to a highly palatable calorically dense high-fat diet (HFD) last only 24 hours (Thaler et al 2012). During the initial 24-hour period after the HFD introduction, rodents undergo hyperphagia, an uncontrollable instinctual desire to eat, before the homeostatic mechanism prevails to restore energy intake to an isothermal level (Buckman et al 2015). However, this acute binge response to HFD exposure can last about 1 week (Benani et al 2012,

Butler et al 2001, Thaler et al 2012). After this acute phase, the calorie intake between mice and/or rats fed normal chow and high-fat diet tends to be equal (Dietrich & Horvath 2013).

A lot of research shows that acute HFD (1-3 days) could effectively induce hypothalamic inflammation (Thaler et al 2012) which is an important mechanism leading to insulin resistance in the hypothalamus (Jais & Bruning 2017). The inflammatory response was observed in mice one day after exposure to HFD (Waise et al 2015). The leading cause of this rapid effect is the activation of hypothalamic microglia, which is also the major glial cell type that mediates inflammatory processes in response to HFD (Andre et al 2017, Gao et al 2014), resulting in the production of various pro-inflammatory cytokines that ultimately cause neuronal apoptosis in arcuate nucleus (Thaler et al 2012). Besides, astrocytes in the hypothalamus are also crucial for maintaining neuronal energy supplies (Allaman et al 2011, Belanger et al 2011). Hypothalamic astrocytes express metabolic enzymes, receptors and transporters of many critical metabolic factors, and it has been reported that hypothalamic glial cells can sense changes in shuttle nutrients and hormones (Levin et al 2011). Moreover, another recent report examined the acute effects of HFD feeding on synaptic changes in ARC of the hypothalamus (Benani et al 2012). It revealed possible mechanisms associated with the synaptic remodeling in the ARC. Mice fed an HFD for less than one week specifically showed changes in the plasticity gene in ARC, but not in the paraventricular nucleus (PVN) and lateral hypothalamus (LH), which are also important for the regulation of energy balance. Excitatory enhancement of POMC cells was observed by *in vitro* electrophysiology accompanied by a decrease in inhibitory tone. This study identified polysialic acid (PSA) molecules, a glycan involved in synapse formation, that has been found to act as a mediator of synaptic changes induced by HFD (Benani et al 2012).

1.4.2 Chronic effects of high-fat diets in the hypothalamus

Chronic consumption of HFD feeding has been well documented for its ability to induce obesity and metabolic dysfunction. In humans, body mass index (BMI), an indicator of obesity, is negatively associated with performance at learning, memory and executive functioning (Cournot et al 2006, Elias et al 2003, Sabia et al 2009). Mice (or rats) fed a chronic HFD gain excessive weight over time and develop glucose intolerance, hyperinsulinemia, hyperleptinemia, hyperlipidemia and hypercholesterolemia, which mimic human metabolic alterations associated with obesity (Buettner et al 2007, Fraulob et al 2010, Gallou-Kabani et al 2007, Winzell & Ahren 2004).

HFD-induced obesity leads to deficits in learning and memory processes (Boitard et al 2014, Jeon et al 2012, Lu et al 2011, Sobesky et al 2014). Although design and cognitive outcomes cannot be directly compared between humans and rodents, the results of different experiments in both human and rodents indicate that obesity is associated with poor memory (Cournot et al 2006, Elias et al 2003, Elias et al 2005, Sabia et al 2009). One clear example is that chronic exposure (16 weeks) to a HFD significantly reduced alternation behavior (SAB) in mice (Almeida-Suhett et al 2017). Moreover, HFD consumption alters brain neurochemistry in a region-specific manner (Molteni et al 2004, Sharma et al 2013), and part of the reason for this change may be due to behavioral disorders induced by HFD. For example, chronic (3 months) HFD feeding alters striatal and midbrain marginal dopamine (DA) signaling in rodents (Davis et al 2008, Sharma & Fulton 2013). In addition to DA, the serotonin and norepinephrine reuptake inhibitors (SNRIs) also changed with dietary changes, and these changes were confirmed to be associated with obesity and behavioral deficits (Chudasama & Bhatt 2009). Behavioral tests such as open-field and pole tests, typically used to assess motor coordination in mice, demonstrated that mice fed a HFD for five weeks exhibited locomotor alterations and behavioral deficits (Brooks & Dunnett 2009, Fleming et al 2004). Last but not least, similar to behavioral deficits, long-term HFD also leads to the development of anxiety and anhedonia, which are the two core symptoms of depression observed in humans and rodents (Dutheil et al

2016). Current theories suggest that depression is usually triggered by stress and is associated with an increased inflammatory response (Muller 2014, Wager-Smith & Markou 2011). Increases in inflammatory cytokine levels in depression and chronic stress rodent models and in relation to the severity of depression have been widely reported (Dowlati et al 2010, Dunn & Swiergiel 2005, Howren et al 2009, Iwata et al 2013). In response to an HFD challenge, the hypothalamus responds to both acute and chronic HFD by producing proinflammatory cytokines [see review (Kalin et al 2015)]. Results from rodent and human studies indicate that the presence of inflammatory factors is positively associated with obesity and metabolic syndrome. Obesity-related inflammatory reactions occur not only in peripheral systems but also in the CNS, especially in the hypothalamus. The interactions between inflammation, hypothalamus and obesity have been extensively reviewed (Kalin et al 2015, Tang et al 2015). The hypothalamus contains a series of resident glial cells, including microglia, macrophages, and astrocytes, which are embedded in highly heterogeneous neuronal groups to control metabolic homeostasis.

The relationship between metabolic control and sleep is becoming a research hotspot. In particular, the current prevalence of metabolic disorders (such as obesity, type 2 diabetes, etc.) is increasing year by year. These metabolic disorders are closely related to unhealthy lifestyles such as sleep disturbances and circadian sleep desynchronization. Against this background, experimental work suggests that insufficient sleep stimulates changes in the activity of neuroendocrine systems, as directly manifested in increased appetite, and ultimately promotes increased energy intake. While previous research on the effects of dietary changes on non-hypothalamic AMPAR levels and function focused on brain's reward circuitry (Oginsky et al 2016, Peng et al 2015), there is a considerable knowledge gap about the role of the hypothalamus in the interaction between sleep and metabolic regulation; this thesis is intended to help fill this gap.

2. Aims and hypotheses

Because sleep is considered to play an essential role for brain plasticity, understanding the role of sleep at the molecular level is of utmost importance to gain insight into how the brain regulates fundamental processes such as metabolism and food intake. Epidemiological and pre-clinical studies show that impaired or inferior sleep promotes increases in energy intake and, on the long run, in body weight. These findings suggest that sleep is essential for metabolic control, but the neuronal mechanisms that establish this function of sleep are unknown.

The primary hypothesis of this thesis is that sleep contributes to metabolic control by downscaling synaptic strength in the hypothalamus, which is the main brain center integrating nutritional and hormonal signals and moreover a pivotal structure for sleep regulation. This question is addressed by investigating the expression of AMPA receptors that contain the subunits GluA1 and GluA2 in hypothalamic and cortical synaptoneurosomes of male rats (a) in dependence of sleep, wakefulness and sleep deprivation and (b) in response to high-fat feeding.

The specific hypotheses of this thesis are the following:

I) Sleep in comparison to wakefulness induces synaptic downscaling, i.e., a decrease in the number of GluA1- and GluA2-containing AMPARs in the hypothalamus.

II) Sleep deprivation prevents synaptic downscaling in the hypothalamus.

III) Short-term increases in calorie intake – high-fat feeding – increase the number of GluA1- and GluA2-containing AMPARs in the hypothalamus, but not in the cortex.

In sum, it is expected that high-calorie food intake increases, while sleep decreases markers of synaptic plasticity in the hypothalamus, reflecting the dynamic regulation of synaptic strength in dependence of metabolic challenges and sleep as a normalizing factor.

3. Materials and Methods

3.1 Overview over studies I and II

In study I, to investigate the net change in hypothalamic synaptic strength during the sleep-wake cycle (experiment IA), GluA1- and GluA2-containing AMPARs were measured in hypothalamic and cortical synaptoneurosomes of rats. Sleep/wake behavior was monitored via video recording. Since sleep is associated with a corresponding degradation in synaptic strength, we expect that this mechanism will be impaired if sleep is insufficient or shortened (experiment IB). In study II, we hypothesized that acute HFD impairs hypothalamic and cortical synaptic transmission in conjunction with changes in sleep/wakefulness behavior. In the main experiment (experiment IIA), we compared the effect of 1-day or 3-days of HFD with that of normal chow. In a control experiment (experiment IIB), we compared the effect of 3-day HFD with a that of HFD for 3 days followed by normal chow in the subsequent 4 days.

3.1.1 Design of study I

The design of this set of experiments is illustrated in Figure 4A-B. In the Experiment A, 12 rats were separated into two groups of six rats/group. The sleep/wake behavior of the rats in the sleep group was recorded from 07:00 to 13:00. The sleep behavior of the rats in the wake group was recorded from 19:00 to 01:00. In the Experiment B, 18 rats were separated into two groups of nine rats/group. The sleep behavior of the rats in both sleep group and sleep deprivation was recorded from 07:00 to 13:00. The sleep/wake behavior of the animals in the sleep group and the sleep deprivation group was recorded from 07:00 to 13:00, while the sleep behavior of the animals in the awake group from 19:00 to 01:00. The behavioral experiments were performed in a standard operant chamber (32.7 cm by 27.5 cm; height: 23.8 cm). Two of the four side walls were made of polycarbonate; the other two were made of aluminum (Figure 4C). During the experiment, the behavior was monitored using a video camera

placed in front of the animal (Logitech, Netherlands) and analyzed offline using ANY-maze software (Stoelting Europe, Dublin, Ireland). Light for image recording was provided by an infrared light source (Winger, Germany).

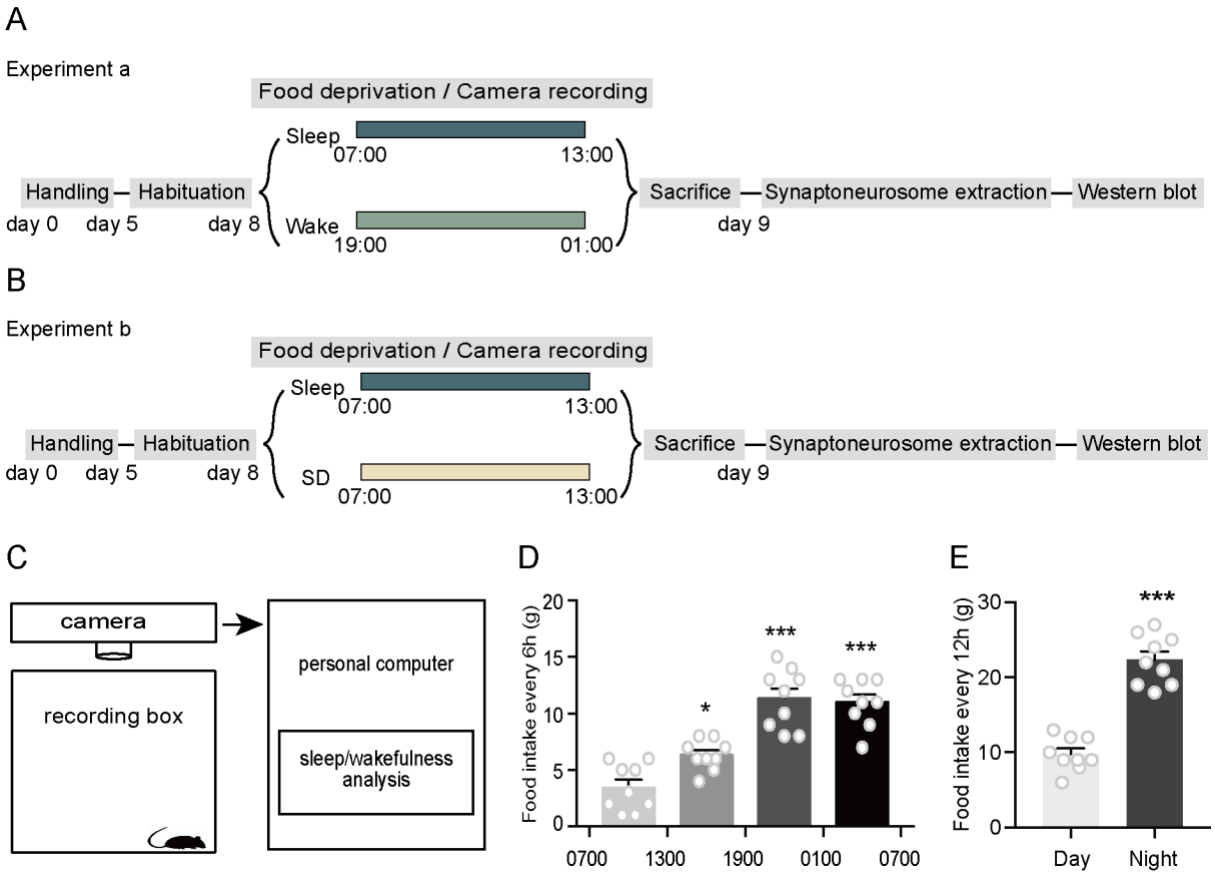

Fig. 4. Study design of Study I and food intake during 24-hour cycle. A,B. Experimental timelines of experiments in study I. **C**. A schematic overview of sleep/wakefulness recording system. **D,E**. Food intake were measured every six hours and respectively, twelve hours (Water and food were available *ad libitum*). SD: Sleep deprivation. Values are mean ± SEM (n = 6 rats/group); Mann-Whitney U test, * < 0.05, *** < 0.001.

In order to check for differences between the amounts of food consumed during the light and during the dark period, in pilot measurements we measured food intake during *ad libitum* feeding every 6 hours (starting at 07:00) when animals were in recording box for habituation. Food was weighed (g) and individual consumption in the 6 hours of interest were obtained by subtracting the weight measured at the start of the assessment period. Results showed that rats consumed more in the afternoon than in the morning and consumed the maximum of their food

immediately after lights went out (19:00) and maintained the food consumption over the dark period (Figure 4D). Food intake in the night period was significantly higher than in the light period (Figure 4E). Based on these results, in the actual experiments all animals were food-deprived in the six hours before sacrifice to ensure that inter-group differences in food intake would not interfere with the effect of sleep vs. wakefulness on synaptic plasticity.

3.1.2 Design of study II

After arrival at the central animal facility, all animals were fed 9% fat standard chow (Ssniff, V1534, Soest, Germany) and handed for seven days. Subsequently, food intake was assessed daily by weighing initial food supply and respective remains and animals were weighed. In the main experiment (experiment A), 18 rats were separated into three groups of six rats/group. The rats in control group were fed normal chow throughout the experiment, whereas after five baseline days of normal chow, the rats of the high-fat diet 1-day (HF1D) and the high-fat diet 3-days (HF3D) groups were fed high-fat diet containing 45% fat (D12451, Ssniff, Soest, Germany) for one day and, respectively, three days (Figure 5A). In Experiment B, twelve rats were divided into two groups of six rats/group that in general were submitted to the feeding schedule of the HF3D group of experiment A (five days of normal chow followed by three days of the respective high-fat diet). In contrast to the HF3D group of experiment B, the animals of the recovery control group (RControl) were afterward returned to normal chow feeding for four additional days (Figure 5B).

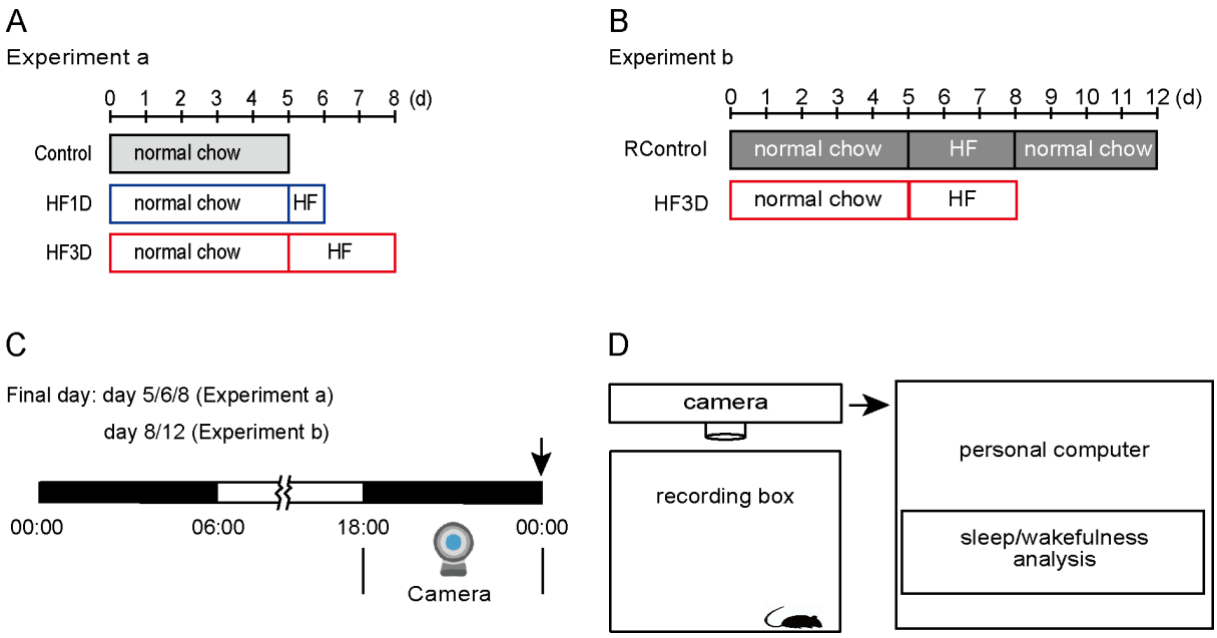

Fig. 5. Study design and feeding schedule of dietary intervention. A,B. Experimental timelines of study II spanning up to eight and twelve days, respectively. Five days of a normal-chow baseline were followed by one or three days of high-fat feeding (A), or subsequent return to normal chow (B), before tissue collection on the last day. **C**. Schedule of the final day of dietary intervention. **D**. A schematic overview of the sleep/wakefulness recording system.

3.2 Animals

Experiments were conducted in male Wistar rats aged 10-11 weeks and weighing 300-400 g (Janvier, Le Genest-Saint-Isle, France). Animals were housed and experiments were performed at controlled temperature (20 ± 2 °C) and humidity (55 ± 10%), and a controlled 12 h/12 h light/dark cycle with light onset at 6 a.m. Water and food were available *ad libitum*. Animals were routinely checked by laboratory staff. Failure to groom and/or loss of more than 20% body were set as criteria of potential sickness and thereby euthanasia.

All experimental procedures in this thesis were approved by the Regierungspräsidium Tübingen, Baden-Württemberg (Permission number: MPV 1/17) and performed in accordance with the European Directive 2010/63/EU for animal experiments.

3.3 Sleep/wakefulness assessment

Animals were videotaped for offline wakefulness/sleep analysis. Wakefulness and sleep were assessed using standard visual examination (Kelemen et al 2014): sleep was scored whenever the rat showed a typical sleep posture and stayed immobile for at least 10 s. If brief movements < 5 s interrupted sleep epochs, continuous sleep was scored. For the aim of the present experiments, we considered visual sleep scoring optimal because we did not aim at differentiating specific sleep stages. This visual scoring approach has been shown in previous rodent studies by our and other groups to consistently match conventional EEG/EMG-based scoring by more than 92% (Borquez et al 2014, Pack et al 2007, Sawangjit et al 2017, Van Twyver et al 1973).

3.4 Synaptoneurosome preparation

Since synaptic sites and receptors can provide valuable information about the molecular mechanisms involved in neuronal activity (Cohen et al 1977), it is important to extract protein components quickly and efficiently from synapses. Cortical and hypothalamic tissues were homogenized in a glass Teflon homogenizer in Syn-PER synaptic protein extraction reagent (Thermo Scientific, Rockford, USA) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Scientific). The samples were centrifuged at 1,200×g for 10 minutes at 4 °C to remove cell debris and save a sample of the supernatant (used as homogenate sample for analysis). The left supernatant was centrifuged at 15,000×g for 20 minutes at 4 °C. The supernatant containing cytosolic fractions was used for supernatant analysis and the pellets containing the synaptoneurosomes were resuspended in Syn-PER buffer. To prevent cross-contamination the supernatants from the last centrifugation step in the synaptoneurosomes isolation procedures were carefully removed (Figure 6). The protein concentration of the synaptoneurosome fraction was determined via bicinchoninic acid assay (BCA) kit (Thermo Scientific).

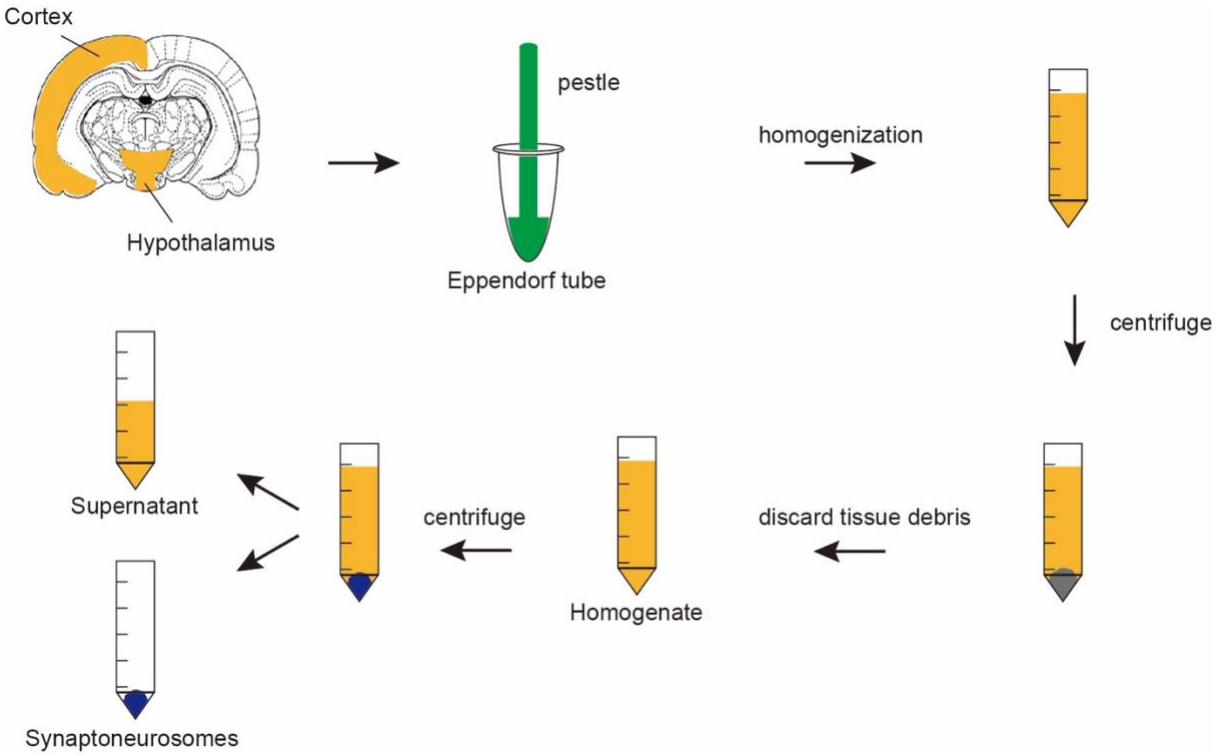

Fig. 6. Schematic illustration of protocol for isolation of synaptoneurosomes from rat brain. Methods were represented by the step-by-step synapse protein preparation from the brain tissue (hypothalamus and cortex) and isolation by differentiation centrifugation.

3.5 Western blot analysis of protein levels

Denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using the Mini-PROTEAN system (Bio-Rad, Germany) and discontinuous polyacrylamide gels. Separating gels were poured between two glass plates and overlaid with ethanol. After gels had polymerized for 20 min, the ethanol was removed, and the gel surface was carefully dried with lint-free paper. The stacking gel was poured on top of the separating gel. A comb was inserted, and the gel was allowed to polymerize for 20 min. In this study, an 8% separating gel was used, and the concentration of the overlaid stacking gel was 5%. Samples were heated for 10 min in 95 °C and centrifuged for 1 min. Equal amounts (30 µg) of samples from each animal were loaded on the gels and SDS-PAGE was performed using 1x SDS-running buffer (25 mM Tris base, 192 mM Glycine, 0.1% (w/v) SDS) for 1.5 h. Then the proteins were transferred onto a 0.45 µm-pore nitrocellulose membrane (Carl Roth, Karlsruhe, Germany) via a semi-

dry transfer system at 100 V for 1 hour. Membranes were first blocked for one hour at room temperature in freshly prepared 5% powdered non-fat milk (Carl Roth) in phosphate-buffered saline (PBS) and subsequently incubated with primary antibodies overnight with agitation at 4°C. Primary antibodies were diluted using blocking-buffer containing 0.1% Tween 20 (Carl Roth) as table 1. After several washes in phosphate-buffered saline supplemented with Tween 20 (PBST) for 1 hour, membranes were incubated in HRP-conjugated secondary antibody for 2 hours. HRP activity was detected using the chemiluminescence reagents provided with the ECL kit (Thermo Scientific).

Fluorescence images of the blots were obtained with a FUSION-FX7 imaging system (Vilber Lourmat, Marne La Vallée, France). Integrated (background-subtracted) signal intensity for each antibody band was quantified with ImageJ software, and then normalized with reference to the β-actin band, which was used as a loading control. To assess GluA1 phosphorylation, we first probed blots with anti-phospho-Ser845 or anti-phospho-Ser831 antibodies, stripped them and subsequently probed with anti-GluA1 antibody, which recognizes both phosphorylated and non-phosphorylated GluA1. We then determined the ratio of the signal obtained with the Ser845 or Ser831 bands and the GluA1 band.

Table 1. List of antibodies used for immunoblotting detection

	Antibody	Source	Dilution	Reference
Primary antibodies	GluA1	rabbit	1:3,000	Merck Millipore, Darmstadt, Germany
	GluA2	rabbit	1:1,000	Merck Millipore, Darmstadt, Germany
	phospho-Ser845	rabbit	1:3,000	Merck Millipore, Darmstadt, Germany
	phospho-Ser831	rabbit	1:750	Merck Millipore, Darmstadt, Germany
	β-actin	mouse	1:10,000	Abcam, Germany
	β-tubulin	rabbit	1:50,000	Biolegend, San Diego, CA, USA
	PSD-95	mouse	1:5,000	Biosciences, Heidelberg, Germany
Secondary antibodies	rabbit IgG	HRP conjugated	1:5,000	Merck Millipore, Darmstadt, Germany
	mouse IgG	HRP conjugated	1:4,000	Biolegend, San Diego, CA, USA

3.6 Sleep deprivation via gentle handling (Study I)

Gentle handling is widely used to induce the model of typical SD (Hagewoud et al 2010, Vecsey et al 2009, Yang et al 2014). In this study, eighteen animals were randomly assigned to two groups: control (undisturbed sleep group) and SD group (Figure 4B). The rats were kept awake with minimal disturbance by introducing novel objects (the novel objects included wooden blocks, small rubber balls, plastic, metallic, wooden, or paper boxes and tubes of different shape and color) into the cage, disturbing the bedding, tapping or shaking the cage and a gently touching the animals with soft brush when the rats appeared to drowsy. Whereas the rats were never disturbed when they were spontaneously awake. SD was carried out for six hours starting from the light onset. During the SD

period, rats were continuously monitored, while the control rats were left with undisturbed sleep during the same period. Due to that hypothalamus plays a key role in food intake, therefore, short-term of food deprivation for six hours in SD group is necessary to ensure comparability with the animals sleeping during the same period.

3.7 Measurement of metabolic parameters (Study II)

Blood samples were collected via cardiac puncture under anesthesia and immediately centrifuged at 3,000×g for 10 min to collect serum, followed by storage at -80 °C until further analysis.

Serum insulin was measured using the commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kits (Enzo Life Sciences Inc., Farmingdale, NY, USA). A monoclonal anti-insulin antibody immobilized on a microtiter plate was provided in the kit. Standards and samples containing insulin were added to the plate. The microplate was first incubated on a plate shaker for one hour at room temperature for one hour at ~500 rpm. The plate was washed with wash solution provided in the kit and subsequently incubated for one hour with a solution of biotinylated monoclonal anti-insulin antibody which bands to the insulin captured on the plate. To remove the unbound biotinylated antibody, the plate was washed and subsequently added the streptavidin conjugated to horseradish peroxidase. After 30 minutes incubation on a plate shaker at room temperature, stop solution was added and read at 450 nm using an LB 942-TriStar$_2$S microplate reader (Berthold, Bad Wildbad, Germany).

Leptin ELISA kits (Enzo Life Sciences Inc) is a solid-phase ELISA based on the sandwich principle. The microtiter wells were coated with a polyclonal anti-leptin antibody. An aliquot of serum sample containing endogenous leptin was incubated in the coated well with a specific anti-leptin antibody. A sandwich complex was formed. After one-hour incubation, the unbound material is washed off and streptavidin conjugated to horseradish peroxidase was added. A substrate solution was added followed by 30 minutes of incubation in the dark. That resulted in color development in proportion to the amount

of leptin bound in the initial step. Finally, color development was stopped via stop solution. The optical density was measured at 450 nm via a microplate reader (Berthold, Bad Wildbad, Germany).

The free fatty acid (FFA) concentration was detected in a 96-well plate using FFA quantification kit (Abcam, Germany). Serum samples were prediluted 10-fold after pretesting with prediluted with 5-fold and 100-fold. Standards and samples containing FFA were added to the plate and subsequently added 2 μL Acyl-CoA Synthetase (ACS) Reagent (provided in the kit) in each well. After incubating at 37 °C for 30 min, 50 μL reaction mixtures were pipetted into each well. After incubating for 30 minutes output were measured at OD 570 nm on a microplate reader (Berthold, Bad Wildbad, Germany).

Serum corticosterone was measured using the commercially available ELISA kits (Enzo Life Sciences Inc). A polyclonal antibody specific for corticosterone has been precoated. Standards and samples were added, and any corticosterone present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for corticosterone was added to the wells. After incubating for 30 min output were measured at OD 405 nm on a microplate reader (Berthold, Bad Wildbad, Germany).

3.8 Data analyses and statistics

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). In both study I and II, unpaired t-tests were used to compare time spent sleeping/awake between groups. Western ratio data the phosphorylated ratio of AMPAR subunit GluA1 were analyzed via Mann-Whitney U tests. ANOVAs used and Tukey's test for post-hoc multiple comparisons. Food intake during 24-hour cycle in study I was analyzed via Mann-Whitney U tests. In study II, two-way analyses of variance (ANOVA) were used to assess changes in energy intake and body weight gain between experimental groups. Data of blood parameters were analyzed via Mann-Whitney U tests. All

data are expressed as means ± SEM (errors are given as standard error of the mean, SEM). A p-value (p) < 0.05 was regarded as significant (*), p < 0.01 (**), p < 0.001 (***).

4 Results

4.1 Effects of sleep vs. spontaneous wakefulness on synaptic AMPA receptors in hypothalamus and cortex (Study IA)

4.1.1 Sleep/wakefulness and synaptoneurosome extraction

In order to investigate the effect of sleep vs. natural wakefulness on synaptic plasticity, experimental animals on a regular sleep-wake schedule were sacrificed at different times of day. I.e., one group of rats was sacrificed during light hours (around 13:00), i.e., after naturally occurring sleep, and another group of spontaneously awake rats was sacrificed in the dark phase (around 01:00). Results of immobility-defined sleep showed that animals in the wakefulness group remained asleep for 26.9% ± 3.64% of the first six hours of the dark phase. Animals in sleep group remained asleep for 71.5% ± 2.57% of the first six hours of the light period ($p < 0.001$, n=6 per group; Figure 7A).

Next, we set out to verify the efficiency of the synaptoneurosome extraction process. We compared hypothalamic and cortical homogenate, supernatant and synaptoneurosomes using Syn-PER Regent (according to the protocol described in section 3.4). To determine the specificity of the extraction process, we performed western blot analyses to detect individual synaptic proteins PSD-95 and non-synaptic Tubulin (see section 3.4). Figure 7 shows that PSD-95 enriched in hypothalamic synaptoneurosome supernatants to a greater degree than in homogenates and was almost undetectable in supernatant (Figure 7B). The levels of the non-synaptic protein tubulin were diminished in both hypothalamic synaptoneurosomes and supernatant as compared to their homogenates (Figure 7B). The same pattern was found in cortical samples: synaptic protein PSD-95 was increased in cortical synaptoneurosomes as compared to homogenates and supernatants prepared from the same animal (Figure 7C).

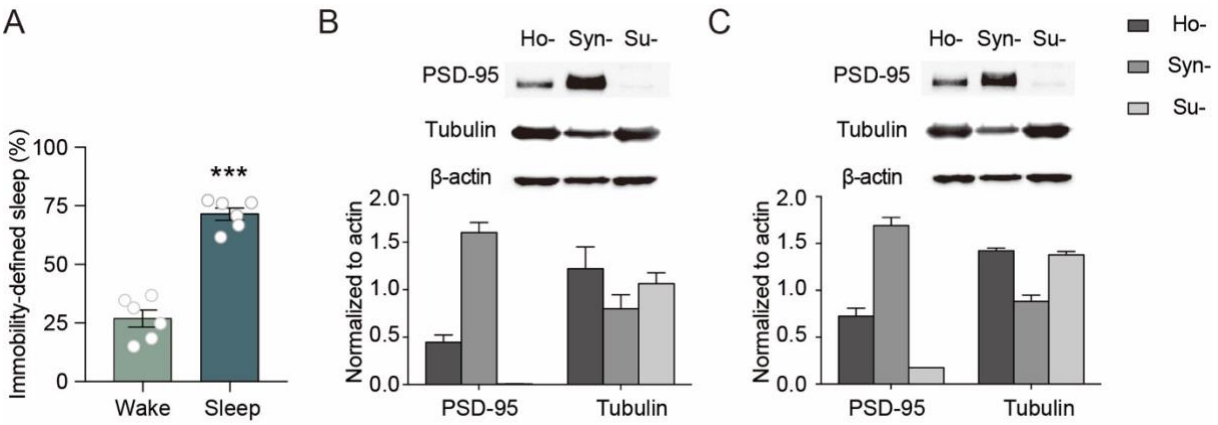

Fig. 7. Time spent awake during the final six hours before sacrificing and western blot profiles of synaptic protein prepared form hypothalamus and cortex. A. Percentage of time spent awake during the final six hours before sacrifice. **B,C.** Representative immunoblots depicting synaptic protein PSD-95 and non-synaptic protein Tubulin in the hypothalamus (B) and cortex (C) and quantitative analysis of protein levels in the hypothalamus and cortex, respectively.

4.1.2 AMPARs expression in hypothalamic and cortical synaptoneurosomes

As mentioned previously (in section 3.4), synaptoneurosomes are enriched with synaptic profiles and are ideal for detecting activity-dependent changes in glutamate receptor levels. We measured the protein levels of GluA1- and GluA2-containing AMPARs in the synaptoneurosomes in both hypothalamus and cortex. The entire hypothalamus and the left cortical hemisphere were sampled. As shown in figure 8, the total level of expression of hypothalamic AMPARs was lower when animals slept than stayed awake before sacrifice (Figure 8A-D). Compared with the wake group, the sleep group showed a nearly 50% decrease in total GluA1 levels in synaptoneurosomes (0.541 ± 0.0721, $p < 0.05$; Figure 8A), whereas the decrease in GluA2 did not reach significance ($p = 0.30$; Figure 8B). Compared with the awake rats, the sleep group also showed decreased absolute levels of GluA1 phosphorylation at Ser845 (0.644 ± 0.091, $p < 0.05$; Figure 8C), while the respective decrease in GluA1 phosphorylation at Ser831 shortly failed to reach significance ($p = 0.06$; Figure 8D).

We also examined the protein levels of GluA1- and GluA2-containing AMPARs in cortical synaptoneurosomes. Rats of the sleep as compared to the wake group showed a decrease in the

levels of cortical GluA1-and GluA2-containing AMPARs ($p < 0.05$; Figure 8E/F) and also in the absolute levels of GluA1 phosphorylated at Ser845 (Figure 8G), while signs of a respective decrease in GluA1 phosphorylation at Ser831 did not reach significance ($p=0.93$; Figure 8H).

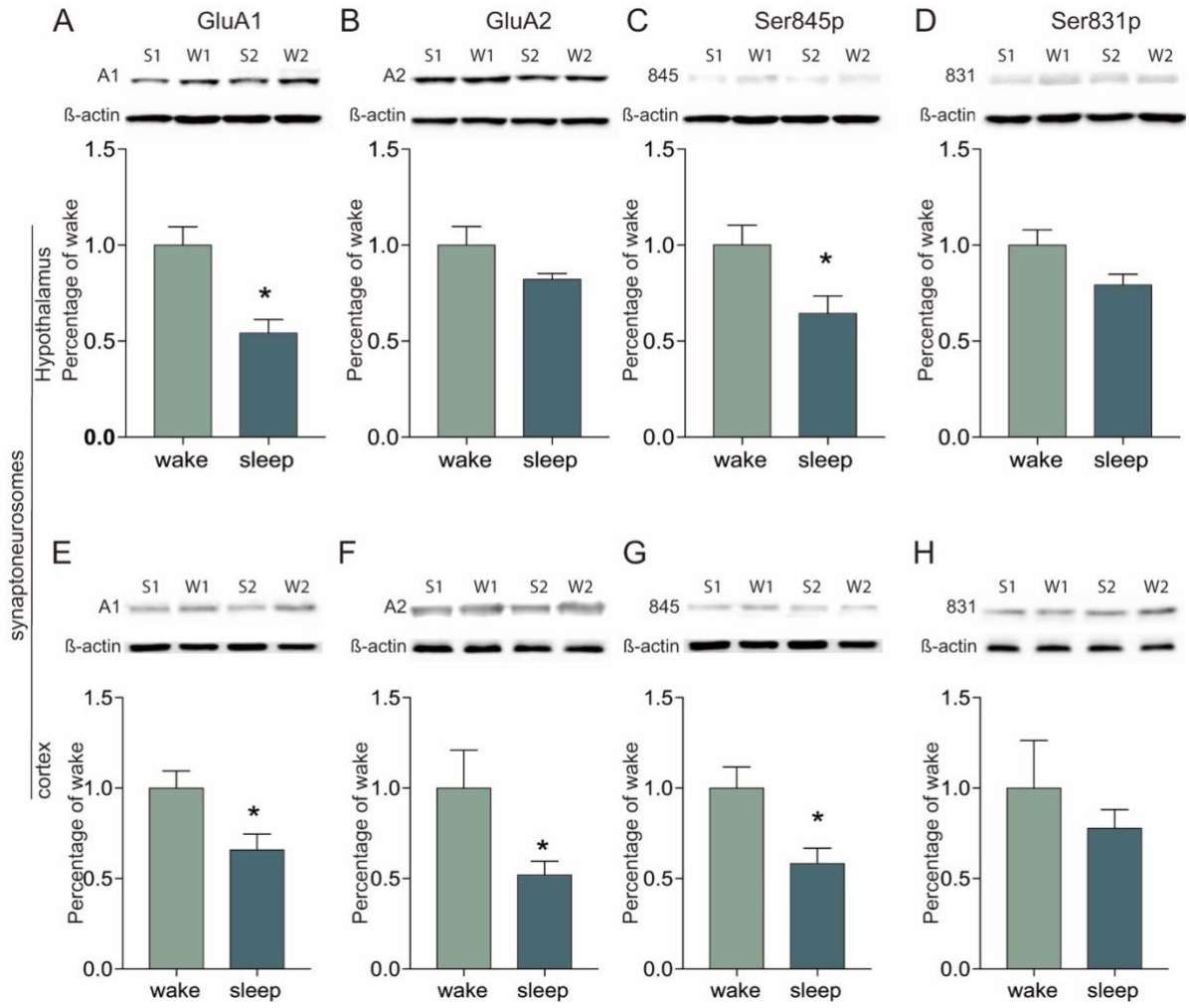

Fig. 8. AMPAR levels in hypothalamic and cortical synaptoneurosomes after wakefulness and sleep. **A-D**. Levels of GluA1- and GluA2-containing AMPARs as well as GluA1 phosphorylation at Ser845 and Ser831 determined in hypothalamus (A-D) and cortex (**E-H**). Values were compared to respective results obtained in the awake group set to 1.0. Representative immunoblots ("1" and "2" indicate two different representative samples) and quantification of the gels are presented. Values are mean ± SEM (n = 6 rats/group); * $p < 0.05$ (Mann-Whitney U test).

4.1.3 AMPARs expression in the hypothalamic and cortical supernatant

In order to obtain more comprehensive information about AMPAR expression in the brain, we also examined the levels of GluA1- and GluA2-containing AMPARs in supernatant. As

expected, no detectable differences between the sleep and wakefulness groups were observed in supernatant, both with regard to hypothalamic and cortical GluA1- and GluA2-containing AMPARs (Figure 9). These results indicate that the observed changes in expression levels of GluA1- and GluA2-containing AMPARs in synaptoneurosomes are specific for the synapses.

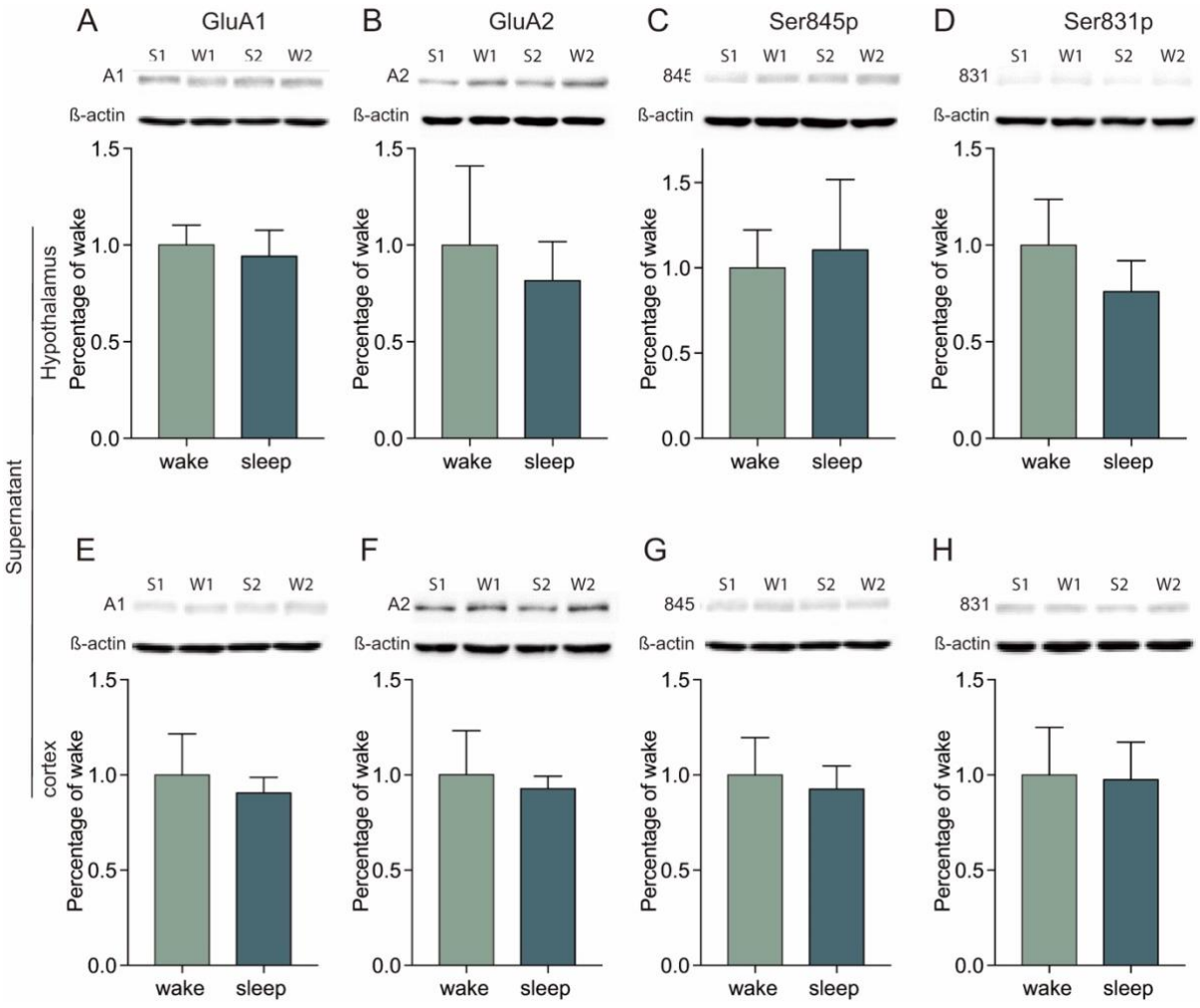

Fig. 9. AMPAR levels in the hypothalamic and cortical supernatants after wakefulness and sleep. A-D. Levels of GluA1- and GluA2-containing AMPARs as well as GluA1 phosphorylation at Ser845 and Ser831 were determined in the hypothalamus **(A-D)** and cortex (**E-H)**. Values were compared to respective results obtained in the awake group set to 1.0. Representative immunoblots ("1" and "2" indicate two different representative samples) and quantification of the gels are presented. Values are mean ± SEM (n = 6 rats/group); Mann-Whitney U test.

4.1.4 Relative levels of GluA1 phosphorylation at Ser845 and Ser831 in hypothalamus and cortex

Since phosphorylation of GluA1 is also important for regulation of AMPARs activity (Barria et al 1997b, Lee et al 2000), we examined the effects of sleep and wakefulness on two GluA1 phosphorylation sites: Ser845 and Ser831, two distinct phosphorylation sites implicated in the regulation of AMPA receptor expression. The levels of phosphorylation were determined by obtaining the ratio of phospho-GluA1 band intensity to total GluA1(e.g., Ser845/GluA1). Quantification analyses yielded no detectable differences in the relative levels of GluA1 phosphorylation between sleep and wakefulness in both hypothalamic and cortical synaptoneurosomes (all $p > 0.59$, Figure 10). Similar patterns of relative levels of GluA1 phosphorylation were found in both hypothalamic and cortical supernatants (all $p > 0.49$).

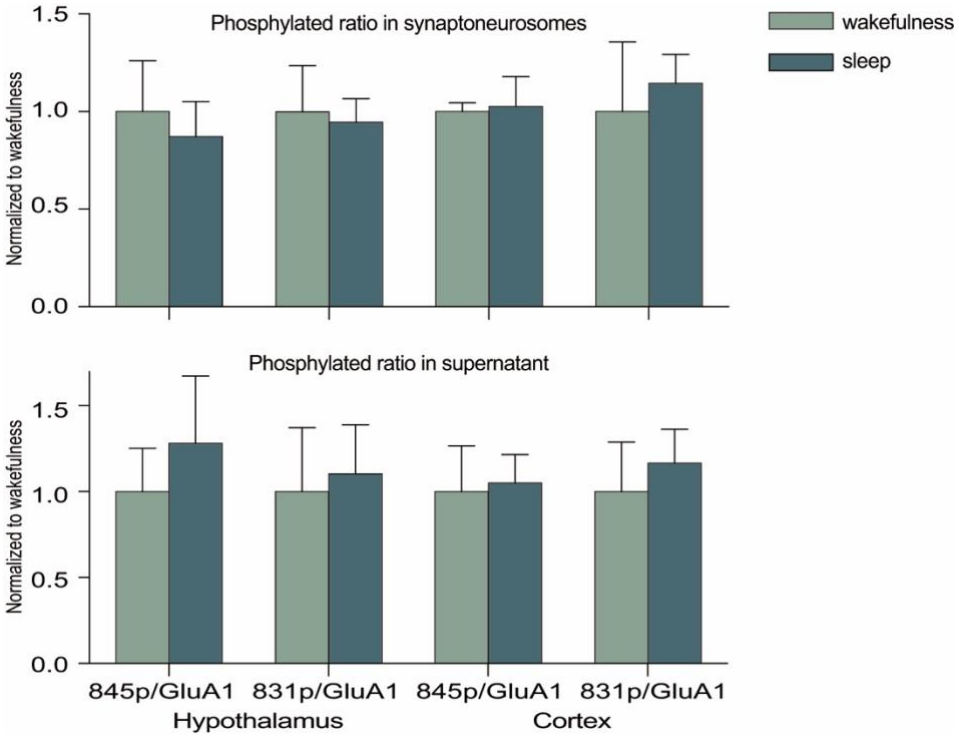

Fig. 10. Quantification of relative phosphorylated GluA1 at Ser845 or Ser831 in the cortex and hypothalamus. The ratios of Ser845p/GluA1 and Ser831p/GluA1 were determined for each sample and normalized to the wakefulness group.

4.2 Effects of forced sleep deprivation vs. sleep on synaptic plasticity in the hypothalamus and cortex (Study IB)

4.2.1 AMPAR expression in the hypothalamus and cortical synaptoneurosomes

In order to assess the effect of sleep deprivation on AMPAR expression, animals of sleep deprivation group were kept for awake for 6 hours during the light phase, i.e., when they would normally have slept, by gently handling that kept them busy and awake. To ensure that the animals in the sleep group slept most of the time, immobility-defined sleep was assessed via video recording. Results show that animals in the sleep group remained asleep group for 74.3% ± 2.94% of the first six hours of the light phase whereas as intended, animals in the SD group stayed awake (Figure 11A). Next, we verified the efficiency of the synaptoneurosome extraction process. The experimental results show that the tissue extraction process was effective (Figure 11B,C).

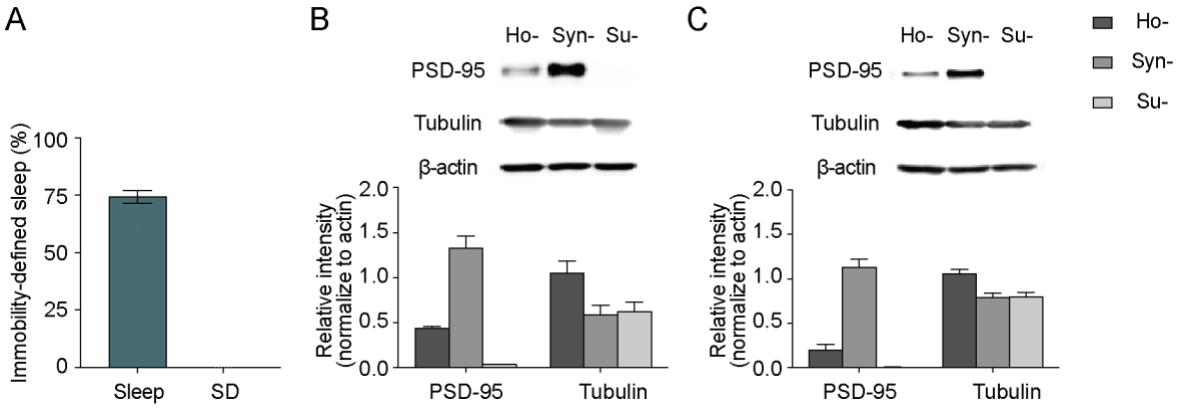

Fig. 11. Time spent awake during the final six hours before sacrificing and western blot profiles of synaptic protein prepared form hypothalamus and cortex. A. Percentage of time spent awake during the final six hours before sacrifice. **B,C.** Representative immunoblots depicting synaptic protein PSD-95 and non-synaptic protein Tubulin in the hypothalamus (B) and cortex (C) and quantitative analysis of protein levels in the hypothalamus and cortex, respectively.

In accordance with the methodology of the first part of study I, we used western blotting experiments to detect the expression levels of GluA1- and GluA2 containing AMPARs in

hypothalamic and cortical synaptoneurosomes. In the hypothalamic synaptoneurosomes, sleep-deprived in comparison to rats that slept before sacrifice showed increased expression levels of GluA1 (1.98 ± 0.294; p = 0.011; Figure 12A) and of the absolute levels of GluA1 phosphorylation at Ser845 (1.68 ± 0.194; p = 0.003; Figure 12C), while the respective increase in GluA1 phosphorylation at Ser831 did not reach significance (p = 0.077; Figure 12D). No detectable difference between groups was found for GluA2 (1.06 ± 0.079; p = 0.258; Figure 12B) in hypothalamic synaptoneurosomes.

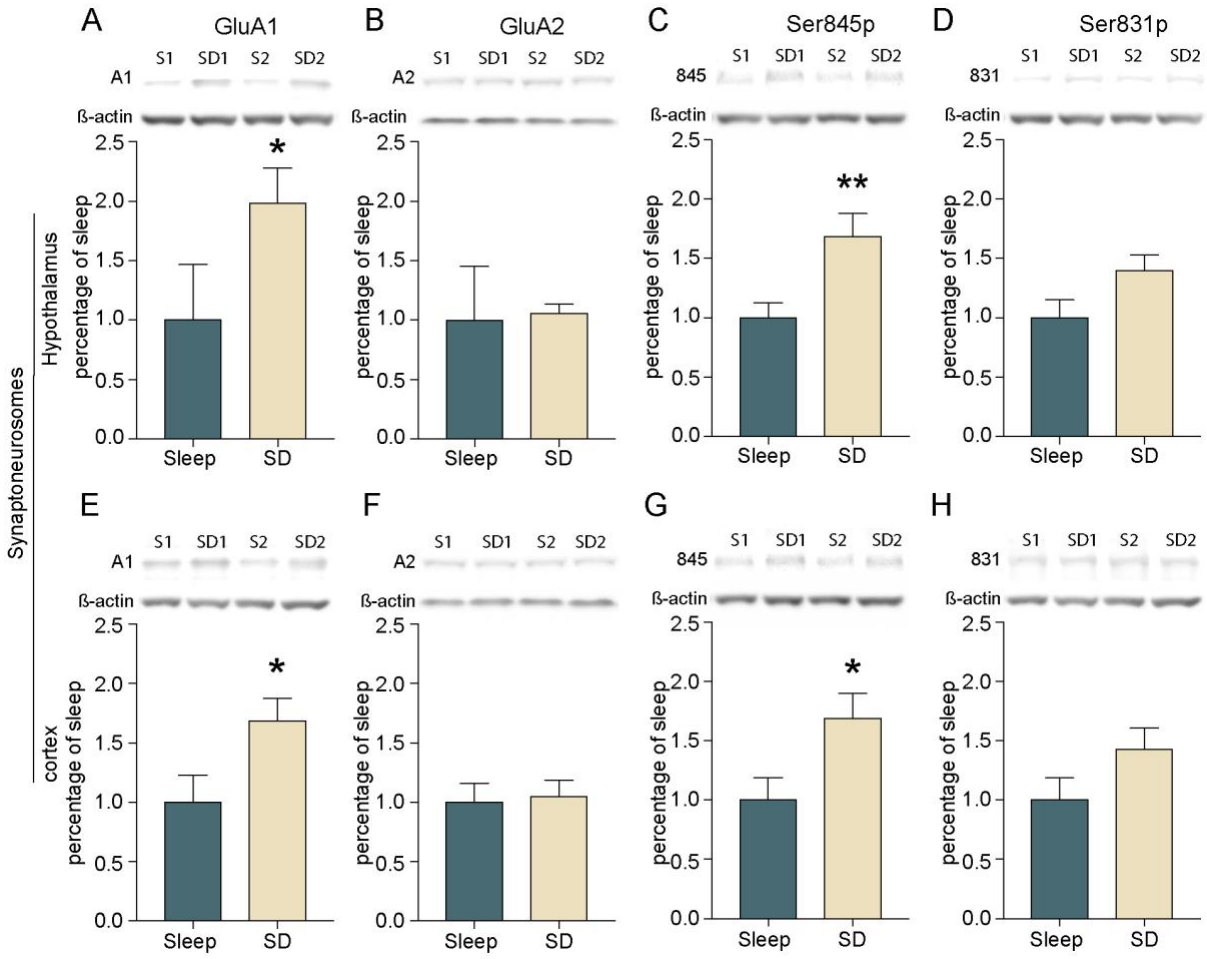

Fig. 12. AMPAR levels in the hypothalamic and cortical synaptoneurosomes after sleep deprivation and sleep. A-D. Levels of GluA1- and GluA2-containing AMPARs as well as GluA1 phosphorylation at Ser845 and Ser831 were determined in the hypothalamus (A-D) and cortex (**E-H).** Values were compared to respective results obtained in the sleep group set to 1.0. Representative immunoblots ("1" and "2" indicate two different representative samples) and quantification of the gels are presented. Values are mean ± SEM (n = 9 rats/group); Mann-Whitney U test, * < 0.05, ** < 0.01.

Similar patterns were found in cortical synaptoneurosomes. Sleep-deprived rats in comparison to rats of the sleep group showed increased levels of cortical GluA1-containing AMPARs (1.69 ± 0.193; p = 0.024; Figure 12E) and also increased absolute level of GluA1 phosphorylated at Ser845 (1.69 ± 0.212; p = 0.032; Figure 12G), while signs of an increase in GluA1 phosphorylation at Ser831 did not reach significance (p=0.114; Figure 12H). The total expression levels of GluA2 in cortical synaptoneurosomes were not affected (p = 0.258; Figure 12F).

4.2.2 AMPAR expression in hypothalamic and cortical supernatant

In order to obtain more complete and comprehensive results, we investigated whether SD also induce changes of AMPARs in supernatants. Supernatants of respective synaptoneurosomes isolation were sampled and we examined the levels of GluA1- and GluA2-containing AMPARs via western blotting. As expected, no significant differences were observed in supernatants between the sleep and SD group in the hypothalamus. No significant differences were observed in both levels of cortical GluA1-containing AMPARs and also the absolute levels of GluA1 phosphorylated at Ser845 and Ser831 (all p > 0.258; Figure 13A-D). Similar expression pattern levels in cortical supernatants were found (all p > 0.162; Figure 13E-H). These results provide more comprehensive evidence that the observed changes in the expression levels of AMPAR containing GluA1- and GluA2 are specific in synaptosome endosomes.

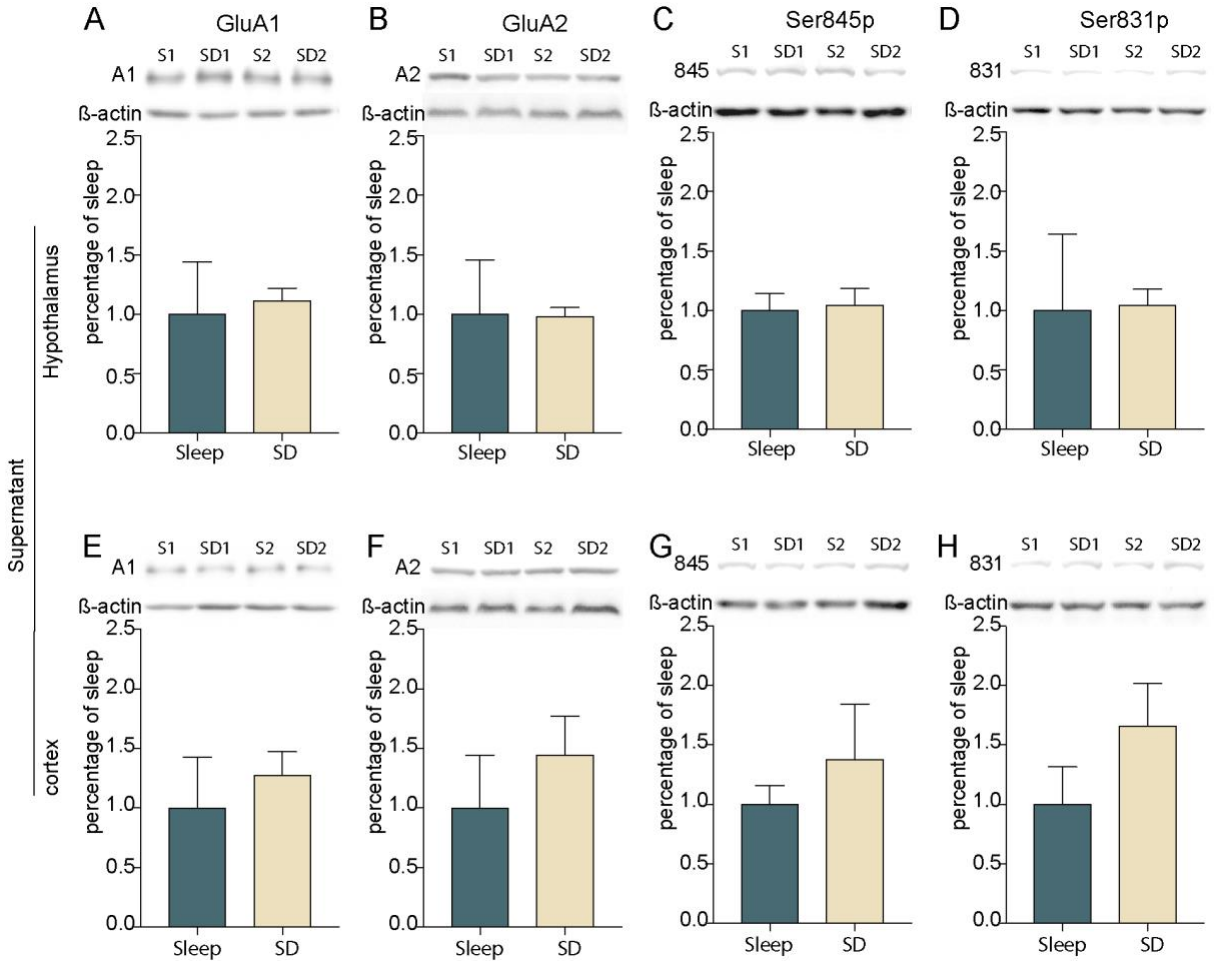

Fig. 13. AMPAR levels in the hypothalamic and cortical supernatants after sleep deprivation and sleep. A-D. Levels of GluA1- and GluA2-containing AMPARs as well as GluA1 phosphorylation at Ser845 and Ser831 were determined in the hypothalamus (A-D) and cortex (**E-H**). Values were compared to respective results obtained in the sleep group set to 1.0. Representative immunoblots ("1" and "2" indicate two different representative samples) and quantification of the gels are presented. Values are mean ± SEM (n = 9 rats/group); Mann-Whitney U test.

4.2.3 Relative levels of GluA1 phosphorylation at Ser845 and Ser831 in hypothalamus and cortex

We next examined the relative levels of phosphorylation by calculating the ratio of phospho-GluA1 band intensity to total GluA1. Quantification of relative phosphorylation showed that no significant changes in both hypothalamic and cortical synaptoneurosomes were found between sleep and SD group (all $p > 0.450$; Figure 14). Results of relative phosphorylated GluA1 in supernatants also do not indicate any detectable changes (all $p > 0.298$; Figure 14).

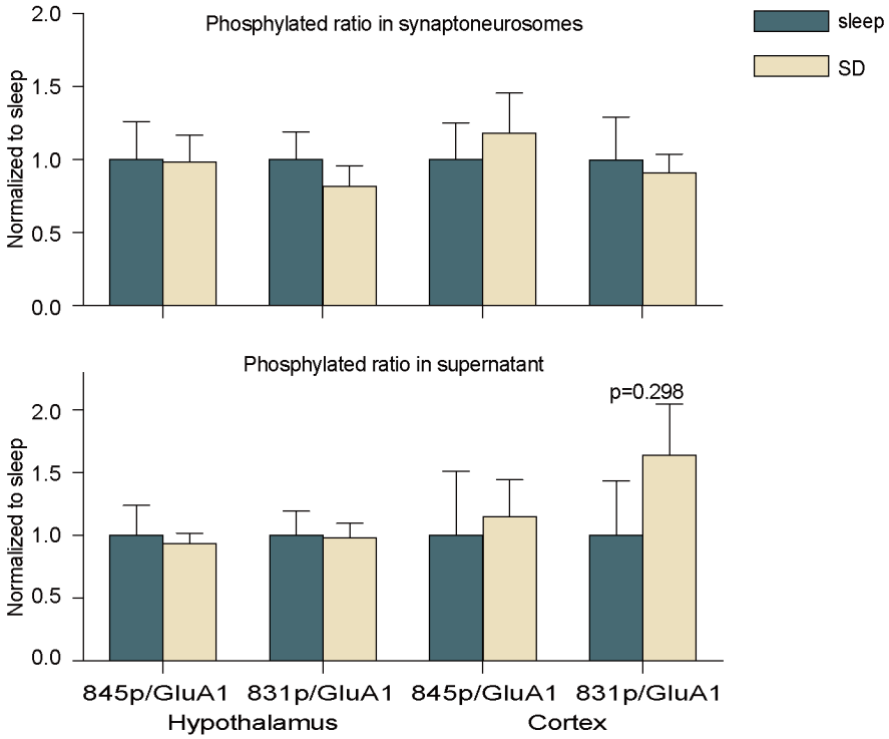

Fig. 14. Quantification of relative phosphorylated GluA1 at Ser845 or Ser831 in the cortex and hypothalamus. The ratios of Ser845p/GluA1 and Ser831p/GluA1 were determined for each sample and normalized to the sleep group.

4.3 Effects of short-term high-fat feeding on synaptic AMPA receptors in hypothalamus and cortex (Study II A/B)

In this study, two experiments were performed to test our hypothesis that acute high-fat diet (HFD) enhances hypothalamic but not cortical synaptic transmission in conjunction with changes in sleep/wakefulness behavior. Experiment A (the main experiment) compared the effect of 1-day (HF1D) or 3-day (HF3D) high-fat diets with a control group fed a normal chow. Experiment B compared the effect of HF3D with a control group also fed with HFD for 3 days but kept on normal chow in the subsequent 4 days. In both experiments, expressions of AMPAR in synaptoneurosomes in the hypothalamus and cortex were assessed to investigate synaptic strength via western blotting.

4.3.1 Energy intake and wakefulness/sleep

All three groups of rats in experiment A (control, HF1D and HF3D) consumed comparable amounts of normal chow during the five baseline days ($p > 0.14$; Figure 15A). After the switch to HFD, both high-fat groups increased their energy intake compared controls on day 6 ($F(2,6) = 17.9$), $p < 0.01$; $p > 0.99$ for comparison between HF1D and HF3D), and energy intake in the HF3D group remained elevated during the three days of HFD ($p < 0.05$ for comparison between days 8 and 5). In accordance with energy intake, body weight increased in both the HF1D and the HF3D groups after the switch to HFD on day 6 ($F (2,15) = 99.5$, $p < 0.001$; Figure 15C). A similar pattern was observed in experiment B. After comparable energy intake during baseline ($p > 0.28$; Figure 15C), energy intake increased in both groups during the three days of HFD ($p < 0.001$ for both), and in the recovery control group returned to baseline levels during the subsequent four recovery days ($p > 0.74$ for comparison with baseline). Changes in body weight followed those in energy intake, with comparable baseline values ($p > 0.20$), subsequent increases upon HFD ($p < 0.05$); and return to baseline levels in the recovery control group during recovery days ($p > 0.79$ for comparison between days 8-12 and days 2-4; Figure 15D).

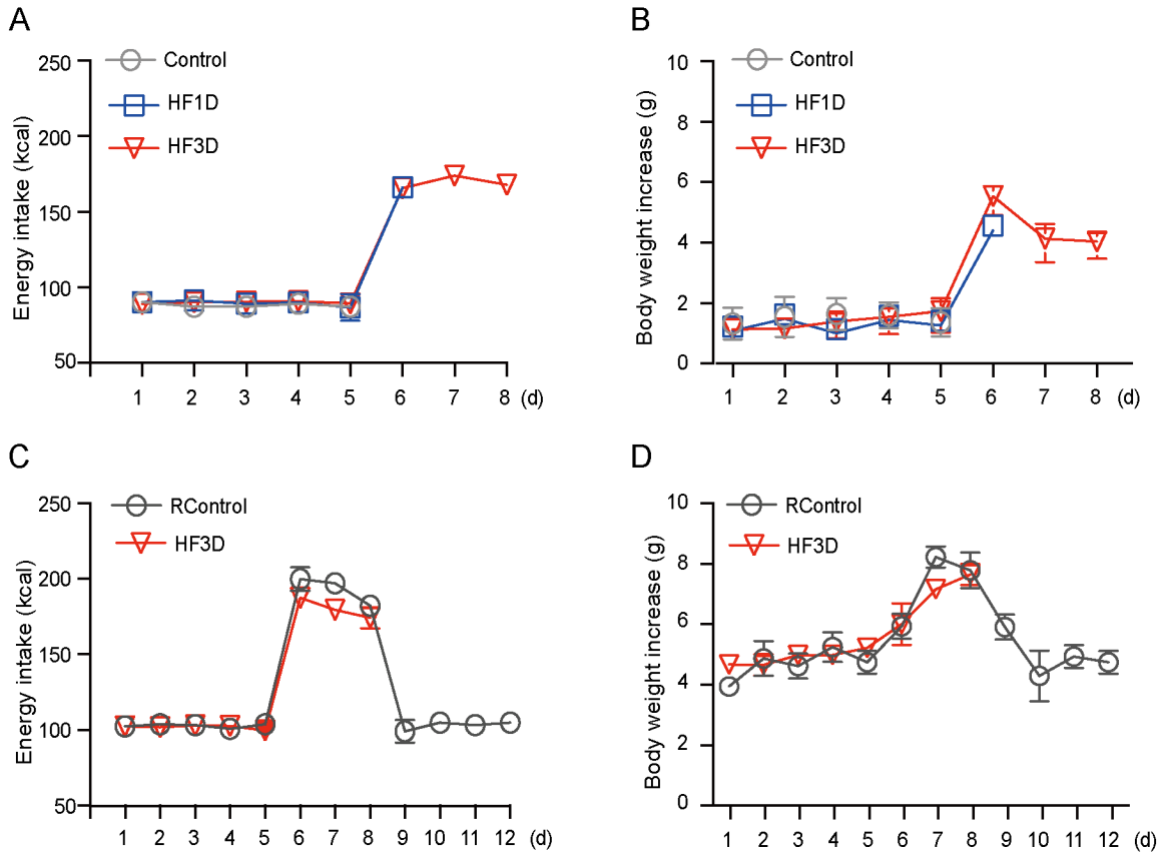

Fig. 15. Results of metabolic parameters. A, B. Energy intake and body weight gain on experimental days in experiment A which spanned up to eight days including five days of a normal-chow baseline and three days of high-fat feeding; **C, D.** Energy intake and body weight gain on experimental days in experiment B which spanned up to twelve days including five days of a normal-chow baseline and three days of high-fat feeding with subsequent reversal normal-chow.

4.3.2 Effects of short-term HFD on wakefulness/sleep

To investigate the potential impact of HFD on sleep/wake patterns, sleep and wakefulness were monitored during the six hours of dark period before tissue collection (Figure 16). In both experiments, three days of HFD increased the time spent awake in comparison to the respective control groups ($p < 0.04$, Figure 16A; $p < 0.001$, Figure 16B). In contrast, one day of HFD was not sufficient to increase wake time ($p=0.50$ vs. control, Figure 16A).

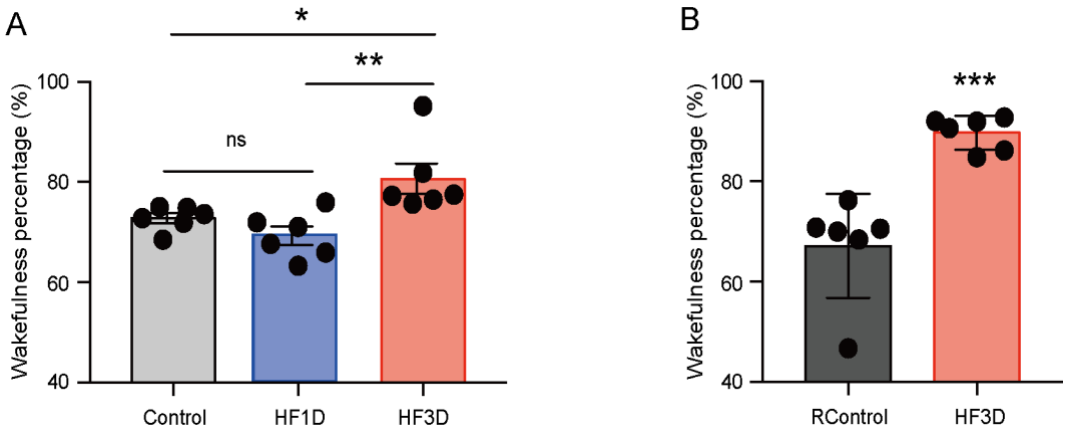

Fig. 16. Results of wakefulness/sleep assessments. A. Mean ± SEM percentage of time spent awake during the final six hours before sacrifice in experiment A; **B.** Mean ± SEM percentage of time spent awake during the final six hours before sacrifice in experiment B. Unpaired t-tests, *p < 0.05, **p < 0.01, ***p < 0.001, ns, non-significant.

4.3.3 AMPAR levels in hypothalamic and cortical synaptoneurosomes

In experiment A of this study, the impact of high-fat feeding and associated body weight increases on protein levels of hypothalamic GluA1- and GluA2-containing AMPARs in synaptoneurosomes was assessed. Three days, but not one day of high-fat feeding compared to normal-chow triggered marked reductions in AMPAR levels (Figures 17E-H and 17A-D, respectively), both regarding GluA1 (Figure 17E) and GluA2 (Figure 17F). The HF3D group also displayed decreased levels of GluA1 phosphorylated at Ser845 (Figure 17G), while the respective decrease in GluA1 phosphorylation at Ser831 did not reach significance (p = 0.24; Figure 17H). These results were robustly corroborated in experiment B where AMPAR levels after the three-day HFD were compared to findings in animals exposed to the three days of HFD and four subsequent days of regular chow (recovery control). Here, levels of both GluA1 and GluA2 AMPARs were reduced in the HF3D compared to the recovery control group (Figure 17I/J).

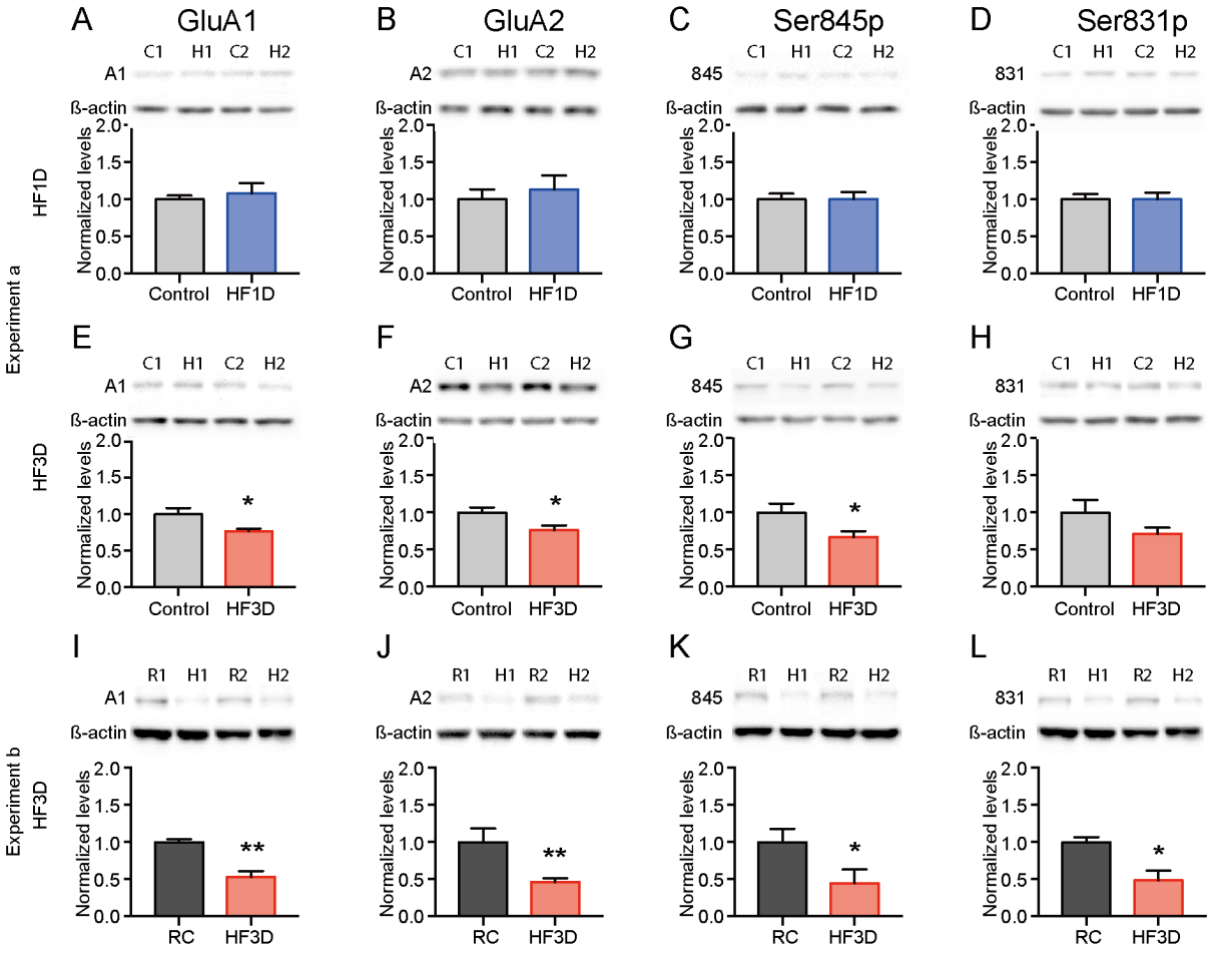

Fig. 17. Suppressive effect of high-fat feeding on AMPARs in the hypothalamic synaptoneurosomes. In part A, levels of GluA1- and GluA2-containing AMPARs as well as GluA1 phosphorylation at Ser845 and Ser831 were determined in the hypothalamus of rats after one day of high-fat feeding (HF1D; panels A-D) and three days of high-fat feeding (HF3D; panels E-H), each compared to respective results in rats on normal chow (Control). In part B, respective findings in the hypothalamus of animals after three days of high-fat feeding were compared to findings in rats after three days of HFD and four subsequent days of normal-chow feeding (Rcontrol/RC, recovery control; panels I-L). Representative immunoblots (C, Control; H, high-fat feeding; R, recovery control; "1" and "2" indicate two different representative samples) and quantification of the gels are presented. Values are mean ± SEM (n = 6 rats/group); Mann-Whitney U test, * < 0.05, ** < 0.05.

Next, markers of synaptic plasticity were assessed in the cortex of the animals exposed to high-fat feeding. This structure was chosen as a control region because in previous experiments, neither presynaptic glutamate release nor postsynaptic AMPARs on pyramidal neurons in orbitofrontal cortex changed in response to (albeit long-term) exposure to cafeteria diet (Thompson et al 2017). Consequently, a pattern emerged that was clearly distinct from the decrease in hypothalamic AMPARs:

in both experiments, three days of high-fat in comparison to normal-chow feeding appeared to increase rather than decrease levels of cortical GluA1 and GluA2 AMPAR subunits (Figure 18E/F and IJ) and also the level of GluA1 phosphorylated at Ser845 (Figure 18G/K) and Ser831 (Figure 18H/L). Significant or trendwise signs of three-day HFD-induced increases were restricted to GluA2 ($p < 0.03$; Figure 18F) and GluA1 phosphorylation at Ser845 ($p = 0.06$) in experiment 1. These findings robustly indicate that three-day high-fat feeding does not decrease markers of synaptic plasticity in cortex. Moreover, one day of high-fat feeding did not elicit any changes (all $p > 0.24$; Figure 18A-D).

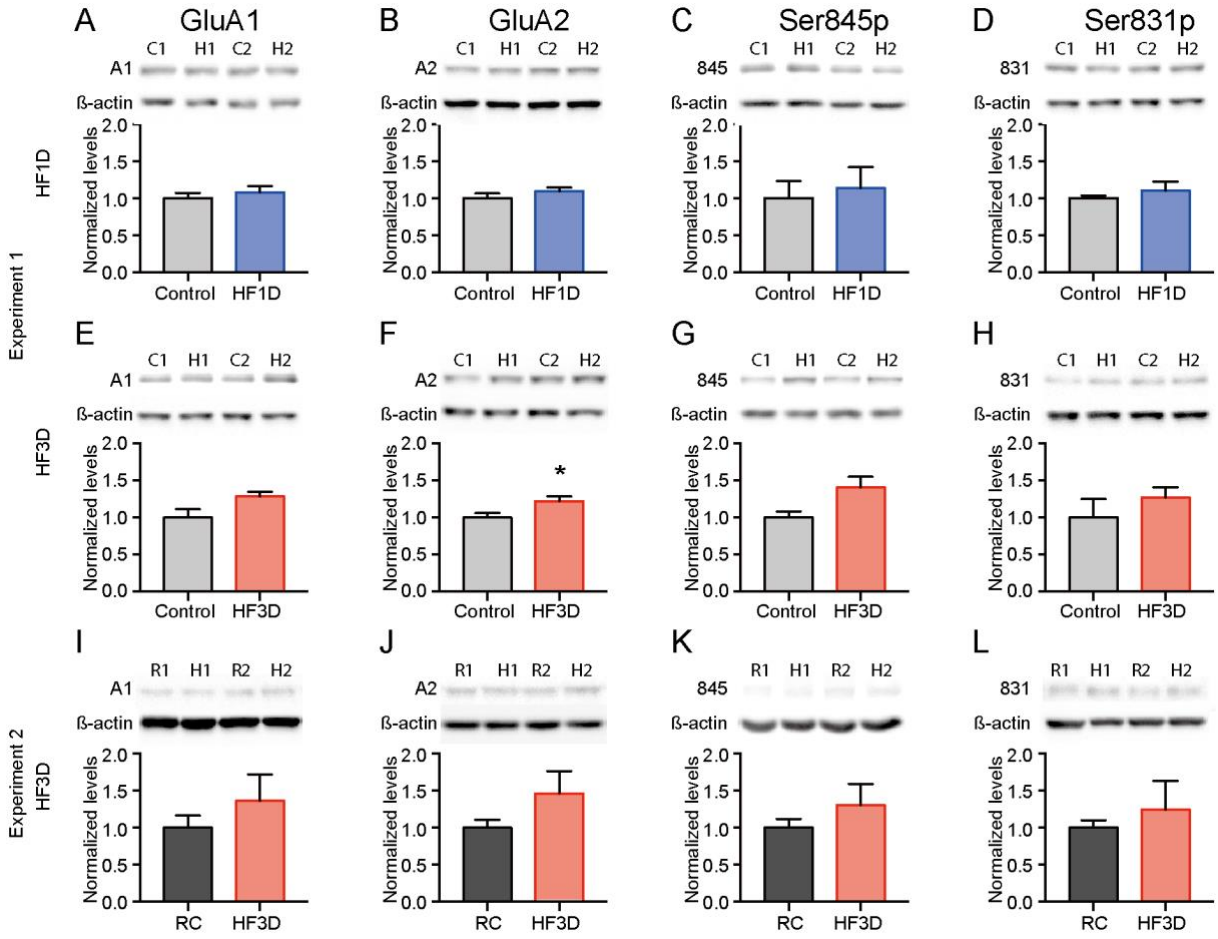

Fig. 18. Enhancing effect of high-fat feeding on AMPARs in cortical synaptoneurosomes. In part A, levels of GluA1- and GluA2-containing AMPARs as well as GluA1 phosphorylation at Ser845 and Ser831 were determined in the whole cortex of rats after one day of high-fat feeding (HF1D; panels A-D) and three days of high-fat feeding (HF3D; panels E-H), each compared to respective results in rats on normal chow (Control). In part B, respective findings in the cortex of animals after three days of high-fat feeding were compared to findings in rats after three days of HFD and four subsequent days of normal-chow feeding (Rcontrol/RC, recovery control; panels I-L). Representative immunoblots (C, Control; H, high-fat feeding; R, recovery control; "1" and "2" indicated two different representative samples) and quantification of the gels are shown. Values are mean ± SEM (n = 6 rats/group); Mann-Whitney U test, * < 0.05.

4.3.4 AMPAR levels in hypothalamic and cortical supernatant

The next question was if the observed changes are specific to synaptoneurosomes, or whether high-fat feeding affects the overall cellular levels of AMPARs. Towards this end, GluA1 and GluA2 content as well as GluA1 phosphorylation were measured in the supernatant fraction of our tissue preparation, which contains mainly the cytosolic components of the neurons. In contrast to the results in synaptoneurosomes, no diet-induced changes were detected (all $p > 0.31$; Figure 19).

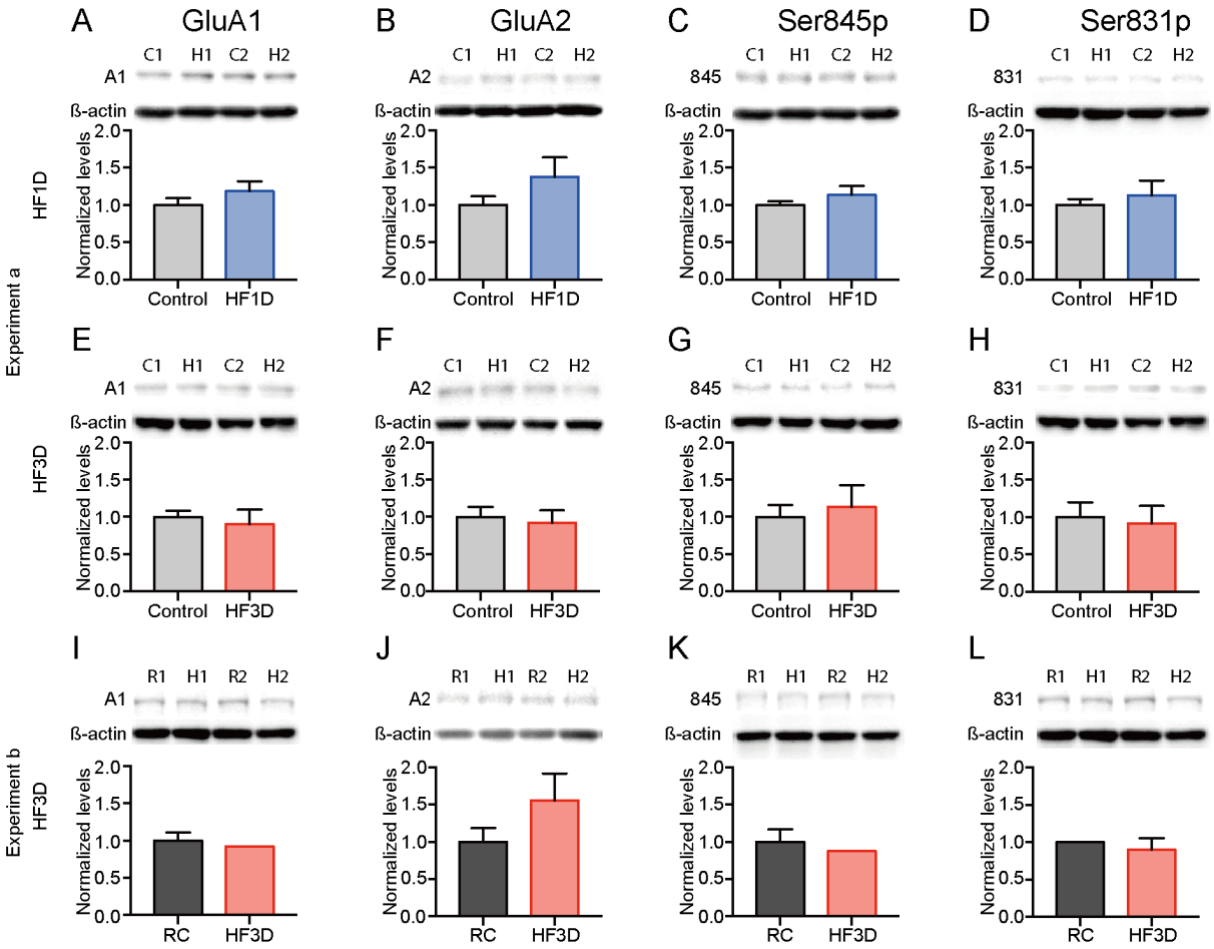

Fig. 19. Effects of three-day high-fat feeding on AMPAR levels in the hypothalamic supernatant. In part A, levels of GluA1- and GluA2-containing AMPARs as well as GluA1 phosphorylation at Ser845 and Ser831 were determined in the hypothalamus of rats after one day of high-fat feeding (HF1D; panels A-D) and three days of high-fat feeding (HF3D; panels E-H), each compared to respective results in rats on normal chow (Control). In part B, respective findings in the hypothalamus of animals after three days of high-fat feeding were compared to findings in rats after three days of HFD and four subsequent days of normal-chow feeding (RC, recovery control; panels I-L). Representative immunoblots (C, Control; H, high-fat feeding; R, recovery control; "1" and "2" indicate two different representative samples) and quantification of the gels are presented. Values are mean ± SEM (n = 6 rats/group).

Moreover, GluA1 and GluA2 content as well as GluA1 phosphorylation were measured in the supernatant fraction of cortical tissue preparation. In accordance with the findings in the hypothalamic supernatants, high-fat feeding did not affect AMPAR subunit levels or GluA1 phosphorylation in the supernatant of cortical fraction in both experiments (all $p > 0.09$; Figure 20).

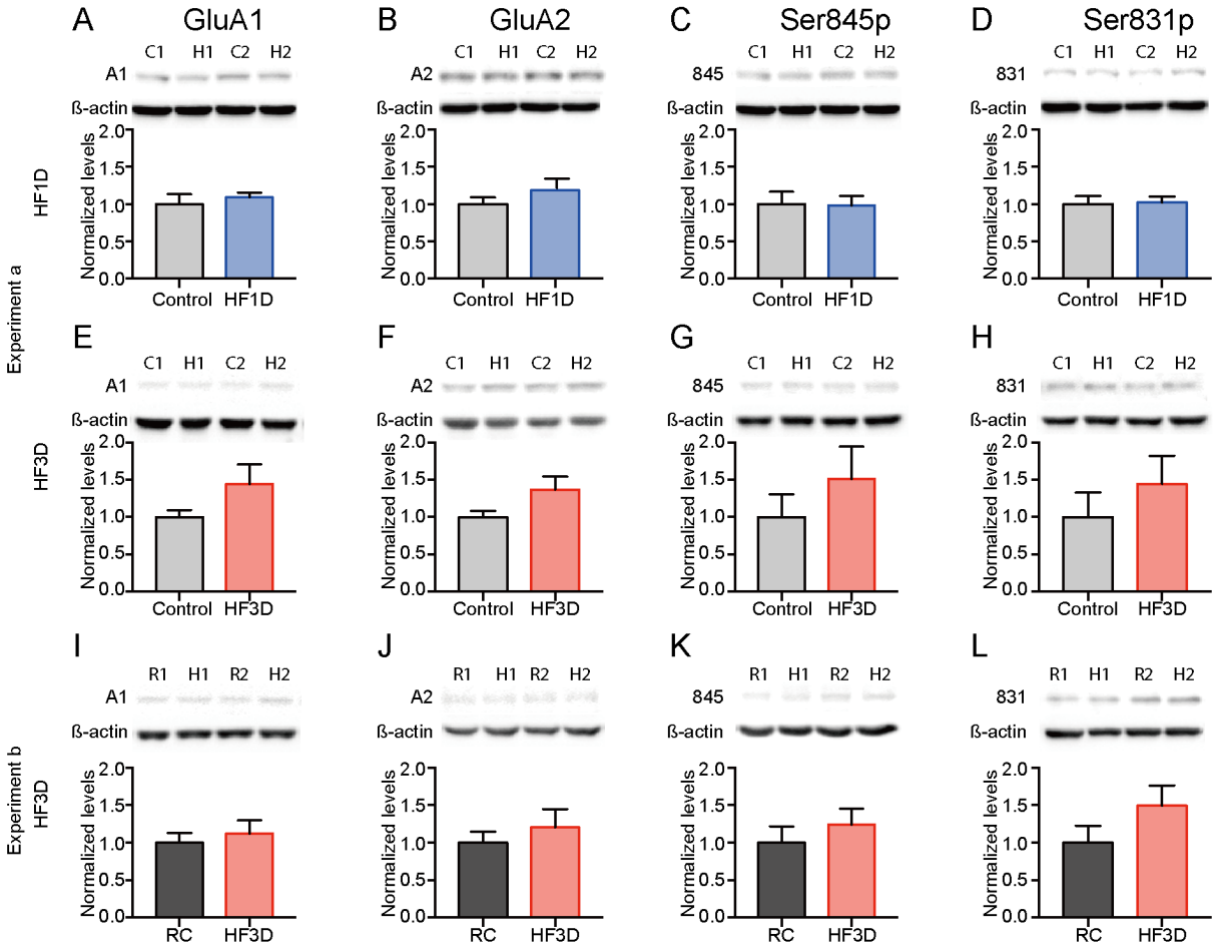

Fig. 20. Effects of three-day high-fat feeding on AMPAR levels in the cortical supernatant. In part A, levels of GluA1- and GluA2-containing AMPARs as well as GluA1 phosphorylation at Ser845 and Ser831 were determined in the whole cortex of rats after one day of high-fat feeding (HF1D; panels A-D) and three days of high-fat feeding (HF3D; panels E-H), each compared to respective results in rats on normal chow (Control). In part B, respective findings in the cortex of animals after three days of high-fat feeding were compared to findings in rats after three days of HFD and four subsequent days of normal-chow feeding (RC, recovery control; panels I-L). Representative immunoblots (C, Control; H, high-fat feeding; R, recovery control; "1" and "2" indicated two different representative samples) and quantification of the gels are shown. Values are mean ± SEM (n = 6 rats/group).

4.3.5 Changes in metabolic parameters after three days of high-fat feeding

In addition to synaptic changes in response to short-term high-fat diet, serum levels of insulin, leptin, FFA and corticosterone were measured in experiment A. High-fat in comparison to normal-chow feeding increased serum levels of insulin, leptin, FFA and corticosterone in both one and three-day high-fat rats when compared to control animals (Table 2). Insulin concentrations were higher still after three than one day of HFD, with a reversed pattern found for FFA.

Table 2. The effect of HFD on the serum insulin, leptin, free fatty acid and corticosterone concentrations in Wistar rats

	Control	HF1D	HF3D
Insulin (ng/mL)	1.18 ± 0.05	1.46 ± 0.05***	1.87 ± 0.01***,#
Leptin (ng/mL)	3.87 ± 0.31	5.44 ± 0.57*	6.34 ± 0.26**
FFA (mEq/L)	0.35 ± 0.11	0.57 ± 0.08**	0.41 ± 0.05***,#
Corticosterone	261.48 ± 38.60	489.57 ± 61.90*	583.34 ± 48.30**

Results are mean ± SEM (n = 6 rats/group); Wilcoxon signed rank test, * < 0.05, ** < 0.01, *** < 0.001 significant difference vs control group; # < 0.05 significant difference vs one day of high-fat feeding group.

5 Discussion

In this thesis, I present the results of studies on global synaptic changes in synapse-enriched biochemical fractions (synaptoneurosomes) of hypothalamus and cortex during different behavioral states (sleep/wakefulness) and in response to a metabolic challenge (high-fat feeding). The up- or down-regulation of AMPARs can be used as a molecular indicator of synaptic strength. In accordance with my first and second hypotheses, levels of GluA1-containing AMPARs in rat hypothalamus were found to be reduced during sleep as compared to spontaneous wakefulness and to be increased after sleep deprivation compared to sleep. These results provide direct evidence that sleep compared to wakefulness induces a net decrease in synaptic strength in hypothalamus and that sleep deprivation is associated with a net increase. Contrary to the third hypothesis, high-fat feeding induced a net decrease in hypothalamic AMPARs containing the subunits GluA1 and GluA2 as well as GluA1 phosphorylation at Ser845 and Ser831. However, cortical levels of GluA1 and GluA2 AMPARs and Ser845 showed a tendency to increase after three days of HFD. Thus, these experimental data show that sleep/wake regulation affect AMPAR homeostasis at the molecular level, and that these changes not only affect cortical but also hypothalamic structures. The results also show that the metabolic input affect AMPAR homeostasis in a brain region-specific manner. They point towards global changes in glutamatergic AMPAR signaling as a common denominator of changes in sleep/wake rhythms and metabolic input (Adamantidis & de Lecea 2008, Dash et al 2009), suggesting that they might also mediate the detrimental effects of sleep loss on metabolic functions.

5.1 GluA1- containing AMPARs are essential for synaptic scaling across wake/sleep cycle

The expression of AMPAR subunits reflecting the strength of synaptic connections depend on the wake state of the animal. Previous reports have revealed that wakefulness leads to a synaptic potentiation in the GluA1 protein levels in the cortex and hippocampus and that sleep reduces them

(Heynen et al 2000, Vyazovskiy et al 2008). Our results in the cortex are in line with this finding and indicate that synaptic renormalization by net weakening should occur during sleep, when animals are disconnected from environment. This renormalization is a complementary mechanism inasmuch as synaptic potentiation during wakefulness (while learning) is thought to cost energy and space and it may therefore be necessary to reduce synaptic strength and eliminate some weak memory during sleep (Cirelli & Tononi 2008, Feld & Born 2019). Therefore, sleep can facilitate the consolidation of new information, which refers to the strengthening of memory representations at the synaptic level (Born & Wilhelm 2012).

In addition to the findings in the cortex consistent with previous research, the new finding obtained in study I is a significant increase in the expression of AMPARs subunit GluA1 in the hypothalamus during spontaneous wakefulness or when the awake time was prolonged (SD group) as it often occurs in real life. Researchers currently assume that sleep deprivation may lead to mild activation of the hypothalamus (Meerlo et al 2002), which plays essential roles in a variety of brain functions, most notably energy metabolism (Abizaid & Horvath 2008, Dietrich & Horvath 2013) and homeostatic sleep/arousal regulation (McGinty & Szymusiak 2003, Szymusiak & McGinty 2008). There is growing evidence that short sleep periods are prone to trigger metabolic changes that can lead to metabolic diseases such as obesity, diabetes and cardiovascular disease (Chaput et al 2006, Gangwisch et al 2006, Gottlieb et al 2005). Synaptic plasticity of the hypothalamus is affected by various factors, such as metabolic hormones (Pinto et al 2004), which may further affect the selection of calorie-intensive foods, excessive food intake, and changes in energy expenditure.

Changes in GluA1 phosphorylation were also consistent with synaptic potentiation during wakefulness and with depression during sleep. Several studies show that the phosphorylation of GluA1 at the sites of Ser845 and Ser831 are both critically involved in synaptic transmission efficiency observed during LTP and LTD (Huganir & Nicoll 2013). Although phosphorylation of the GluA1 subunit on Ser845 or Ser831 enhances AMPARs function, it appears to do so through different biophysical mechanisms

and occurs completely independently (Banke et al 2000, Derkach et al 1999, Diering & Huganir 2018). Coupling of Ser845 between PKA and GluA1 during scaling up promote receptor insertion and synaptic potentiation that could enable multiplicative scaling up (Man et al 2007). Synaptic upscaling involves an increase in phosphorylation of GluA1 Ser845 (Diering et al 2014, Esteban et al 2003), but Ser831 phosphorylation does not appear to be necessary (Hayashi et al 2000).It is consistent with our findings that natural wakefulness or sleep deprivation, compared to sleep, showed an increase level of GluA1 phosphorylation at Ser845 but not Ser831. To the opposite, the dephosphorylation of Ser845 during LTD is thought to be important for endocytosis of GluA1-containing AMPARs from membrane (Beattie et al 2000, Lee et al 2000). Findings in cultured neurons indicate that decreased Ser845 phosphorylation during synaptic downscaling is not mediated by phosphatase activation, but rather by reduction of PKA-targeted synapses, thereby facilitating the dephosphorylation of GluA1 (Diering et al 2014). Therefore, our present finding indicates that LTD-induced internalization of AMPARs is regulated by Ser845 phosphorylation.

Furthermore, stronger synapses are larger and weaker ones smaller (Nishiyama & Yasuda 2015), indicating that there should be a selective mechanism of the global synaptic scaling across the wake/sleep cycle otherwise unselective strengthening would produce a system overflow (Rasch & Born 2013). A recent study in mice showed the ultrastructural evidence (i.e., the size of axon-spine interface) for synaptic scaling across wake/sleep, which suggests that the synaptic scaling is not uniform but selectively renormalization during sleep (de Vivo et al 2017). A key issue in synaptic scaling is the identification of initial prompts during activity alteration that trigger AMPAR recruitment. Calcium, a free extracellular ion, is essential to the induction of associative synaptic plasticity (Davis 2006). The dynamic regulation of GluA2-containing AMPARs enable these glutamate-gated channels to serve as signaling molecules presumably via calcium influx (Man 2011). Changes in GluA1 levels in both hypothalamus and cortex between wakefulness and sleep were not found at GluA2 levels, indicating that GluA1 and GluA2 play different roles in synaptic scaling.

5.2 Synaptic plasticity in the hypothalamus

Molecular indicators (AMPARs) of synaptic strength in cortex and the hypothalamus changed in a similar manner after spontaneous wakefulness in dark-phase (because rodents are nocturnal) and 6 h sleep deprivation during daytime. Our findings further confirm that sleep and wakefulness are global and are associated by neuromodulation in most forebrain structures including the hypothalamus. In addition, synaptic plasticity in the hypothalamus is affected by metabolic hormones such as ghrelin (signaling food deprivation) and leptin (signal satiety) (Pinto et al 2004). This process is thought to play an important role in energy homeostasis (Horvath 2005). Experimental studies using transgenic mice have shown that both the number of excitatory/inhibitory synapses and postsynaptic currents in leptin-deficient ($Lep^{ob/ob}$) mice are different from those in wild-type mice. However, when leptin was delivered systemically to $Lep^{ob/ob}$ mice, synaptic density quickly returned to baseline (Pinto et al 2004). Recently, an *in vitro* study using electrophysiology approaches found that food deprivation increases the frequency of action potentials in NPY/AgRP neurons through a mechanism that relies on AMPA receptor-mEPSC (miniature excitatory post-synaptic current) (Yang et al 2011). Moreover, the effects of food deprivation are mediated by elevated circulating ghrelin levels, which also have important implications for synaptology (Diano et al 2006). Similar to food deprivation, ghrelin-induced synaptic changes have similar mechanisms that are dependent on AMPK signaling pathway which could be activated by Ca^{2+}/calmodulin-dependent protein kinase kinase (CAMKK) (Hawley et al 2005, Woods et al 2005), thereby affecting glutamate release (Yang et al 2011).

5.3 Effects of short-term HFD feeding on synaptic AMPARs in the hypothalamus and cortex

Recent findings in mice indicate that the positive energy balance resulting from ten days of high-fat feeding leads to hypothalamic expression of calcium-permeable (i.e., GluA2-lacking) AMPARs in POMC neurons via the leptin-mTOR pathway (Suyama et al 2017). In related experiments, one day

as well as three days of HFD in mice induced an increase in markers of synaptic plasticity in the arcuate but not the paraventricular nucleus and lateral hypothalamus, along with increased glutamatergic input onto POMC neurons after three days of HFD, which were mediated by increased levels of cell-surface glycan polysialic acid molecule (PSA) (Benani et al 2012). The present result of general synaptic down-regulation of AMPARs after three days, but not one day of HFD may reflect a net effect of glutamatergic synaptic scaling in the hypothalamus, and therefore does not exclude concurrent local synaptic up-regulation in specific circuits. As a central hub of homeostatic autonomic and hormonal control, hypothalamic pathways regulate functions including hypothalamic-pituitary-adrenal stress axis activity, which itself interacts with body weight control (Incollingo Rodriguez et al 2015). Thus, the observed net synaptic down-regulation, which emerged in conjunction with a strong increase in corticosterone levels, might result from changes that occur in different neuronal networks and might even be of opposite polarity.

HFD-induced hypothalamic down-regulation also emerged in comparison to HFD-exposed control animals returned to normal chow. This indicates that the effect (1) is rapidly reversible by diet normalization, (2) is triggered by high-fat food per se rather than the associated elevation in body weight, which in absolute terms was still present after return to normal chow, and (3) does not depend on the novelty of high-fat feeding. In accordance, providing animals with a novel isocaloric control diet remained without effect on arcuate PSA levels in the study by Benani and colleagues (Benani et al 2012).

It was also observed that in the cortex, in contrast to hypothalamus, high-fat feeding in general did not affect or rather even increased AMPAR levels. Prolonged high-fat feeding and obesity are associated with decreased activation in dorsolateral and anterior prefrontal cortices (Val-Laillet et al 2011) and decreased spine density in the lateral orbitofrontal cortex (Thompson et al 2017), detrimental effects of HFD and obesity on cognitive function are generally well-known (Dye et al 2017, Greenwood & Winocur 1990, Heyward et al 2012, Molteni et al 2002). While previous research on non-hypothalamic

effects of dietary changes on AMPAR levels and function focused on the brain's reward circuitry (Oginsky et al 2016, Peng et al 2015), the pattern observed in our study suggests that the short-term synaptic impact of high-fat feeding in the cortex is limited, and might even imply an acute compensatory response of superordinate brain centers in the early stages of high-fat feeding.

Changes in the availability of nutrients like FFA in the body periphery and related neuronal and endocrine signals including insulin and leptin are constantly tracked by the brain, in particular hypothalamic ventromedial and arcuate nuclei (Gao & Horvath 2007, Levin et al 2011). Against this background, our results indicate a distinct, reversible pattern of net synaptic scaling in response to short-term high-fat feeding (Benani et al 2012, Suyama et al 2017) that precedes the manifestation of obesity and associated synaptic changes (Oginsky et al 2016, Thompson et al 2017) and may underlie global regulatory efforts to counteract diet-induced body weight gain.

5.4 Effects of short-term HFD feeding on sleep/wakefulness

Animals on HFD spent more time awake than control animals in the final six hours of three-, but not one-day HFD. Unchanged wakefulness at the end of the single day of high-fat feeding argues against the assumption of novelty as a wake-promoting factor. Longer periods of high-calorie feeding in rodents have been repeatedly, although not unanimously (Panagiotou et al 2018) observed to decrease wakefulness (Guan et al 2008, Luppi et al 2017, Perron et al 2015) possibly via increases in sleep-promoting cytokines like tumor necrosis factor (TNF) and interleukin-6 (IL-6) (Spath-Schwalbe et al 1998, Vgontzas et al 1997). In humans, increased fat and sugar intake for one day yielded signs of lighter sleep (St-Onge et al 2016), and fat intake was observed to be associated with decreased sleep duration (Grandner et al 2010). In conjunction with these findings, our results suggest that initial HFD-associated increases in wakefulness give way to more pronounced sleep once overweight and related sleep-regulatory changes become manifest. Wakefulness and sleep are associated with net increases and, respectively, decreases in synaptic strength in the cortex and hippocampus as reflected by changes

in synaptic AMPAR GluA1 and GluA2 subunits and GluA1 phosphorylation at Ser845 and Ser831 (Diering et al 2017, Vyazovskiy et al 2008) observations that gave rise to the 'synaptic homeostasis hypothesis' of net synaptic potentiation during wakefulness and depression during sleep (Tononi & Cirelli 2014). It is therefore tempting to speculate that the HFD diet-associated increases in wakefulness observed here contributed to signs of cortical synaptic up-regulation.

5.5 Convergent significance of sleep- and diet-induced hypothalamic synaptic changes?

Our results demonstrate that sleep is associated with net synaptic downscaling (a decrease in the number of receptors containing the AMPAR subunit GluA1) in the hypothalamus that is the central hub of homeostatic autonomic and hormonal control. This synaptic change during sleep might be a physiological mechanism that mediates a normalizing influence of sleep on eating behaviors. One thing to note is that this change in the hypothalamus is a result of a gradual accumulation over a few hours. Another important question is whether these synaptic changes in the hypothalamus affect hypothalamic neuronal activity, thereby modulating metabolic control. Related research suggests that this is the case. Synaptic plasticity of the hypothalamus is inherent in the neural circuits that control appetite (Dietrich & Horvath 2013), and a recognizable subset of neurons is involved in the regulation of feeding behavior. For instance, AgRP/POMC neurons in the hypothalamus are mandatory for the regulation of food intake (Gropp et al 2005, Luquet et al 2005). Therefore, we can speculate that these neurons in the hypothalamus may be involved in the supposed contribution of synaptic plasticity to the interplay between food intake and sleep/wake regulation. As shown in our experiments, reducing sleep or short-term sleep deprivation can weaken this mechanism via increasing hypothalamic synaptic strength. Our findings of study II indicate that short-term high-fat feeding in rats down-regulates hypothalamic but not cortical synaptic strength. This pattern might imply an acute compensatory response of superordinate brain center in the early stages of high-fat feeding. It may be because of this compensatory effect that the down-regulation effect of the high-fat diet is weakened and eventually

returns to the baseline after return to normal chow. It remains to be seen whether sleep potentiates this presumed compensatory effect because sleep/wake behavior was not manipulated in study II. Therefore, unraveling the interaction between sleep and diet in the control of hypothalamic synaptic plasticity will clearly necessitate more future experiments.

5.6 Limitations and outlook

The research contained in this thesis indicates that sleep induces a net downscaling of synaptic strength whereas wakefulness is associated with synaptic potentiation. Also, the present study is the first to show that acute HFD exposure induces changes on synaptic plasticity that depend on feeding duration and differ between brain regions via quantification of AMPAR number, indicating that short-term high-fat feeding in rats down-regulates hypothalamic but not cortical synaptic strength, and that this effect is rapidly reversible.

However, our investigation did not provide the evidence of which sleep stages or featured sleep parameters mainly contribute to the synaptic downscaling. In addition, due to the polysynaptic components that is hard to isolate, the functional state of synapses *in vivo* cannot be monitored via molecular assays performed in specific brain regions. Synaptoneurosomes cannot distinguish between where the receptors are located, such as between the surface and internal receptors, or between the presynaptic and postsynaptic pools. Nevertheless, quantification of AMPARs number is a common approach used to examine experience-induced modifications to synaptic organization. Measurements of the surface AMPARs is also crucial to reflect the synaptic changes as a function of sleep.

Furthermore, specific nuclear groups participating in this counter-regulation of metabolic information in the hypothalamus should be studied. Although our results do not allow insights into the rewiring of specific neuronal populations that fine-tune food intake control, our findings suggest that global scaling

processes accompany the initial phase of high-calorie intake to shift the overall hypothalamic activity balance and, possibly, to counteract anabolic drive and weight gain.

Last but not least, our study was not specifically designed to test the possible effects of the circadian rhythms because our measurements only measured the initial phase and were not performed within a 24-hour cycle under constant conditions. Therefore, we cannot rule out that in addition to the behavioral state, circadian rhythm time may also affect markers of synaptic strength.

In the future, it will be essential to extend this approach with electrophysiological studies, which can reflect variations in factors besides synaptic strength that can affect the neuronal excitability, offering more complementary evidence. For example, they might answer the question how slow-wave activity (SWA) relates to the impact of sleep/awake duration on synaptic scaling. So far, many studies have shown that specific learning tasks such as object exploration experiments induce an increase in SWA in cortex after restricted sleep or sleep deprivation (C Hanlon et al 2011). This will be another evidence supporting what we are currently discovering, that is, sleep will lead to a global decrease in hypothalamic circuits, which might be the physiological mechanism that mediates alterations of eating behaviors. It will also be necessary to go deeper into specific nuclei like the arcuate nucleus and to study homeostatic synaptic plasticity in these nuclei to obtain a more fine-grained picture of the relevance of synaptic scaling in different hypothalamic regions for the interplay between sleep/wake rhythms and food intake.

6. Publication

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