Slow Wave Sleep and Sleep Need Resolution Through L-type Voltage Gated Calcium Channel

（L-type 電位依存性カルシウムチャネルに通じた徐波睡眠と睡眠圧の解消）

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Abstract

Slow wave or non-rapid eye movement (NREM) sleep is tightly homeostatically regulated and essential for survival. Slow waves are observed in the electro-encephalogram (EEG) as oscillations in the delta (0.5-4 Hz) range. Slow wave activity is to date the best indicator for homeostatic sleep regulation; it is increased after prolonged waking and slowly reduced during NREM sleep. The precise mechanisms underlying sleep homeostasis and the generation of slow waves are unknown. Activity-dependent neuronal calcium influx has been hypothesized to play an important role in generating slow oscillations and might be involved downstream signaling that mediates sleep function. Dihydropyridine blockers of L-type voltage gated calcium channels (VGCCs) are in wide clinical use to treat hypertension and other cardiovascular disorders and are readily blood-brain-barrier (BBB) penetrant. We therefore wanted to investigate their potential effect on slow wave generation and homeostatic NREM sleep regulation. In-vivo two-photon imaging of cortical neurons showed larger spontaneous calcium transients in slow wave sleep compared to waking. Application of the dihydropyridine calcium blocker nicardipine significantly reduced cortical calcium transients, without affecting slow wave generation. Nicardipine also did not affect the slow wave activity over 24h following application. Time spent in slow wave sleep and episode duration were also not affected. We conclude that despite evidence that neuronal calcium influx may be involved in NREM sleep function, blocking calcium entry through L-type VGCCs does not interfere with slow wave generation or regulation.

Table of Contents

1. Introduction

1.1 Sleep physiology

We spend one third of our time sleeping. This behavior has been seen across all animals, and evolutionarily speaking, may not be beneficial especially in danger of the predators. Despite this risk of survival, without sleep, we are also at a risk of cognitive impairment, decrease productivity, metabolic syndromes and even death (Everson et al. 1989). The need to make up for loss of sleep only emphasizes how important this behavior is (Dinges et al. 2005). There is considerable amount of research in the function of sleep in theories of metabolic regulation, immune function, memory consolidation, memory protection and plasticity (Sharma and Kavuru, 2010, Beseovsky et al 2012, Ellenbogen et al. 2006).

We as humans sleep around 7-9 hours of sleep optimally, with four stages that include very shallow stage 1 sleep, deeper than stage 1 but still shallow stage 2 sleep, very deep stage 3 sleep, also known as non-rapid eye movement (NREM) sleep, and the fourth stage, rapid-eye movement (REM) sleep. Each sleep stage has very distinct brain wave pattern in that there is higher amplitude during NREM sleep than during REM sleep which is also accompanied by muscle atonia. Other animals such as apes, monkeys and sloths sleep 10-16 hours per day. Larger animals such as elephants sleep as little as 2 hours per day (Gravett et al. 2017). The characteristics of different sleep stages are conserved across many terrestrial mammals; although because of short duration in elephants, it is hard to catch REM sleep. There are some variations in the patterns of sleep architecture as well. We sleep at night, the dark cycle; however, some animals such as mice sleep during the day, the light cycle. Each sleep episode duration and also REM occurrences throughout the night change accordingly, REM appears every 90-120 minutes of sleep in humans and 10-15 minutes for mice possibly due to their fragmented sleep (Purves et al., 2001, Campbell and Tobler 1984).

There have been many animal models that helped us understand some sleep disorders. Genetically mutated orexin neurons in dogs and mice facilitated in drug discovery and development (Toth and Bhargava 2013, Kaneko et al. 2021). Caffeine consumption in rats showed how it inhibits adenosine, byproduct of ATP catabolism known to induce sleep, and in turn, prolonged wakefulness (Haulica et

al. 1973, Lazarus et al. 2011). Circadian clock-deficient mice not only showed the link between sleep arousal behavior, but also between hypertension and other metabolic syndromes (Doi, 2012). Despite vast amount of research in sleep, we still do not understand the fundamental questions of sleep: how do we sleep? How is sleep regulated? Why do we sleep?

1.2 How is sleep measured?

There are various guidelines in distinguishing sleep across all different animals. Sleep of *drosophila melanogaster*, for example, is discerned to arousal and quiescence (Hendricks et al. 2000, Shaw et al. 2000). In mammals, the stages mostly comprise of three states: wakefulness, NREM and REM sleep. As briefly mentioned above, NREM stages are characterized by larger amplitude in brain waves than during wakefulness and REM sleep. REM sleep, however, has both low amplitude and almost no muscle tone. There are several ways to measure the brain waves: electroencephalogram (EEG), and local field potential (LFP). The earliest recordings of EEG and LFP were performed by Caton (Caton, 1875). Hans Berger was the first to record EEG of humans. EEG has been widely accepted method of measuring the brain waves of the entire cortex; whereas LFP looks at more local group of neuronal firing. Inside the brain, neurons are exchanging with the extracellular space to exchange ions for resting potential and for action potentials. This process can be seen in a wave of pushing and pulling similar or opposite of ionic charges, which creates electrical potential that can be read as voltages. The summation of electrical potentials generated by millions of neurons is shown in the EEG recordings. The EEG recordings show different varieties of frequencies measured in cycles per second (Hertz): delta power (0.5-4.5Hz), theta power (4.5-8Hz), gamma power (25-149Hz), alpha power (8-12Hz) and so on. These different frequencies can reflect different states, for example during wakefulness, there can alpha power dominance during a rest, delta power dominance during NREM and theta power dominance during REM sleep. Thus, from different frequency dominance in a span of time, we can distinguish which state we are in. LFP shows much different waves in that they are recorded from a group of individual neurons, instead of the entire cortex. Thus, researchers can use this information to interpret data of integration of regional neuronal activities, or extract the unit firing of a specific subset of neurons in a specific region to analyze their behavior.

1.3 Regulation of sleep: hypotheses of circadian rhythm and sleep homeostasis

Sleep is thought to be regulated by two processes: circadian process and sleep homeostasis. Borbely introduced a model which constitutes of Process C for the circadian rhythm and Process S for the sleep homeostasis. Throughout the day, process S rises, which dissipates during the night through sleep, and through process C, the animals rise up, which all depend on not only the timing of sleep and wakefulness but also the daytime vigilance during the day for interactions in both processes (Borbely et al. , 1980, 1982). Given the relatively small number of genetic components, *drosophila* has long been studied through their genetic manipulation, which uncovered not only the mechanisms of sleep disorders but also the master genes for circadian rhythms (Hendricks et al. 2000). Circadian rhythms are rooted in our innate nature, which regulates our biological rhythms through regulation of transcriptomes, genes such as *Timekeeper* (Kloss et al 2001). In rats, later found in humans, the process S build up was observed through EEG recordings (Borbely et al., 1980, 1982). This process S is very well explained as a buildup of sleep pressure or sleep need and is modeled after the buildup of sleep need during wakefulness and its dissipation during slow wave sleep (SWS) during NREM, shown as higher slow wave activity (SWA) after sleep deprivation and rapid dissipation of SWA as subjects slept (Aeschbach et al. 1997).

SWS during NREM sleep is characterized by their high amplitude delta waves in EEG recording, and UP and DOWN waves in LFP recording, which is hypothesized to reflect sleep need dissipation (ref). It was found in LFP data that during SWS, these UP state of synchronous firing and DOWN state of synchronous silence are spatiotemporally correlated to unit firing of individual neurons (Destexhe et al. 1999). There have been studies that showed temporal relationship between delta waves and the firing pattern of depolarized UP state, and hyperpolarized DOWN states, further hypothesized by regulation by appropriate channels (Amzica and Steriade 1998, Consteras and Steriade, 1995). These correlations were exclusively shown in SWS and were not seen in wakefulness and REM sleep. However, the criteria of judging UP and DOWN states are convoluted, and the direct link between synchronous neuronal firing and sleep need is still missing.

1.4 Calcium is more than for bones.

We consume calcium in forms of milk, spinach, and some fish for its functions in building bones, regulating muscle contraction and normal blood clotting. Calcium is the fifth most abundant element in our body, of which 0.015% is widely distributed for various physiological roles.

The concentration gradient of calcium across the cellular membrane and across the storage is very huge. Concentration of calcium ion in the cytoplasm is kept at nano-micro-molar level while the extracellular and the calcium storage in the cell are in millimolar level. When there is a trigger to open the calcium channels, there is a passive calcium influx from the extracellular space or within the cellular storage through the channels, to respond to the trigger. However, when the stimulus stops, the calcium is actively pumped out of the cell in order to keep the large concentration gradient state. This is how calcium, as one of the second messengers, delivers a response to a stimulus.

Calcium was first found to be the second messenger through how cardiac muscles contracted in 1883 by Ringer who showed that the calcium in the medium where the cardiac muscle was stored is important for contraction (Ringer, 1883). However not until 1962, the excitation-contraction coupling of the skeletal muscle does not get demonstrated. Ebashi showed that only a miniscule amount of calcium in micro level is needed for contraction and that it is actively removed for relaxation in the skeletal muscle (Ebashi, 1962). In the cardiac muscle alone, it is highly regulated so that the duration, intensity, and speed are always kept according to what our body needs. The level of calcium should be kept at certain amount extracellularly and intracellularly at each heartbeat and can change according to certain movements that we make. For all of these different functions and different areas (extracellular, intracellular or both), there are several proteins that calcium responds to differently.

1.5 Calcium in the neuronal network

Calcium not only works as a second messenger, but it plays as a charge carrier as well in neurons. In the 19th century, Cajal reported that neurons are the core functional members of the nervous system. Neurons are comprised of several parts: the soma, the body that has the nucleus; the dendrites that receive and gather information from other neurons; axon which is wrapped in myelin sends information to other neurons through synapses. Neurons respond in an all or none principle, when the

trigger stimulates beyond certain threshold, the neurons respond in the frequency range. For an action potential to occur successfully, the membrane potential needs to surpass the threshold of excitation from its resting potential, where it would be depolarized upon stimulus and increase the membrane potential. At this threshold, voltage gated sodium channels or voltage gated calcium channels open, allowing for sodium to enter and make the electrochemical gradient positive. Sodium channels or calcium channels close and voltage gated potassium channels open to decrease the membrane potential, creating hyperpolarization and eventually the potassium channels close and the membrane potential stabilizes at resting potential, while sodium or calcium is actively pumped out of the cell.

The axon and soma have voltage-gated ion channels, NMDA receptors, AMPA receptors, and metabotropic glutamate receptors (mGluRs) to generate ad propagate electrical signals for action potential by ions such as sodium, potassium, and calcium. NMDA receptors are ionotropic glutamate receptors that are permeable for sodium, potassium, and calcium ions, and are important for synaptic strength. AMPA receptors are another ionotropic glutamate receptor permeable to cations just as NMDA receptors, and serve an important role for plasticity and synaptic transmission. mGluRs are G-protein coupled receptors are involved in learning, memory, perception of pain, which activate cascades of modification of other proteins leading to ion channels—where receptors in the ER induces calcium release. Voltage-gated calcium channels are the most responsible for calcium entry by depolarization of the neurons.

Because of calcium's dual role as second messenger and as a carrier ion for membrane potential alteration, it serves to change the voltage of the channels and also in the downstream of gene transcription. As mentioned before, at a certain voltage, the voltage gated calcium channels open and give rise to intracellular calcium concentration increase from nanomolar range to micromolar range (Wadel et al.,2007), triggering gene transcription, neurotransmitter release, and activation of calcium-dependent enzymes (Wheeler et al., 1994, Wheeler et al., 2008, Wheeler et al., 2012). These voltage gated calcium channels and calcium-permeable channels are tightly controlled since prolonged increased intracellular calcium levels can lead to apoptosis (Stanika et al., 2012). Many neurological

disorders, i.e. epilepsy, have been linked to dysregulation of cell signaling pathways that control these channel activities.

1.6 Introduction to voltage gated calcium channels

Voltage gated calcium channels are categorized to two major types: high voltage activated channels that open in response to large membrane depolarizations and low voltage activated channels that open to smaller voltage changes. Of the high voltage activated calcium channels, L-type has been extensively researched, since they carry major calcium currents for cardiac, smooth and skeletal muscles. In neurons, L-type channels are responsible for gene expression, integration of synaptic input and neurotransmitter release (Tsien et al., 1988, Bean, 1989, Flavell and Greenberg, 2008). Other high voltage gated calcium channels constitute of N -type, P/Q-type, and R-type, all responsible for neurotransmitter release and dendritic Ca transients. The initiation of synaptic transmission starts with presynaptic P/Q-, N-, and R- type calcium currents for tightly controlled regulation such as fast release of glutamate, acetylcholine, and GABA (Caterall, 2011). T-type channels are low voltage activated calcium channel, which is responsible for repetitive firing. Because T-type channels close inactivate faster than other channels, they are appropriate for burst firing of action potentials (Nowycky et al. 1985, Destexhe et al., 1998, Alvina et al. 2009). L-type channels are prominently found postsynaptically anchored via Shank protein (Tippens et al., 2008, Zhang et al.,2005). Bading et al reported that CaM kinase is the intermediary between the cell membrane and gene transcritption in the nucleus. It was found later that CREB activation was inhibited by L-type channel blockers (Eickelberg et al., 1999, Wheeler et al. 2006). There have been substantial amount of research invo.8 lving regulation and mechanism of gene transcription involved with L-type channel; however neuronal firing is not very well elucidated due to their variability in combinations of its channel subunits. Of the four isoforms of L-type channel, Cav1.2 and Cav1.3 at the spines and dendrites are involved in neuronal firing shape and signaling pathway for excitation-transcription pathway, known for its association in learning, memory, drug addiction and neuronal development (Jenkins et al. 2010, Di Biase et al. 2008, Zhang et al. 2006). Cav1.3 has been reported to be essential for fear memory consolidation (McKinney et al. 2008), and Cav1.2 has even more complex behavior for fear memory

in that acute pharmocological block inhibited thalamo-lateral amygdala longterm potentiation, and in turn inhibited fear memory acquisition (Langweiser et al., 2010), which was not seen in Cav1.2-deficient mice. Clinically, Cav1.2 gain-of-function is seen in Timothy syndrome, which also manifests to autism symptoms, possibly caused by activity-dependent dendrite retraction which is independent of the calcium entry through the mutant channel (Krey et al., 2013). Although not many, there have been many studies involving L-type voltage gated channel. However there seems to be no link between how these channels could play a role during sleep.

1.7 Relationship between voltage gated calcium channels and sleep

The relationship between sleep and voltage gated calcium channels was not discovered until Jahnsen and Llinas (Jahnsen and Llinas, 1982). From then on, Steriade group introduced an evidence of involvement of T-type channel during sleep and showed a direct link to sleep spindles and delta waves (Deschenes et al. 1984, Domich et al. 1986, Steriade et al. 1985, Bal et al., 1995). It was shown that abolition of thalamic Cav3.1 in mice decreased the total NREM sleep, and identified essential role in slow sleep oscillation of thalamocortical and nucleus reticularis thalami neurons (Lee J et al., 2004, Anderson et al. 2005, Hughes et al., 2004). Thus, it is widely accepted that voltage gated channels—at least T-type—play an essential role in SWS. Despite considerable research into the link between sleep and T-type channels, the link to L-type channels have been very limited.

1.8 Main Objective

One of very few research in L-type channels and sleep showed that sleep duration and REM sleep decreased in heterozygous Cacna1c KO mice and other voltage gated channel KO mice (Kumar et al. 2015, Tatsuki et al., 2016). It was also found during sleep, there is very high extracellular calcium concentration which is lower during awake state (Ding et al. 2016). Thus, one of widely accepted hypotheses involving voltage gated calcium channels and neuronal firing states that during SWS, this neuronal firing switches from ON and OFF states accordingly, whose main working component is fluctuation of intracellular calcium concentration through voltage gated calcium channels (Ode et al. 2017).

Aforementioned above, during SWS, these ON states of firing mostly are from cortical neurons, especially pyramidal cells (Evarts, Edward, 1964, Steriade et al. 2001), whose major voltage gated calcium channels are L-type. It is plausible to hypothesize that for these ON states of firing are mitigated through L-type calcium currents and that if these currents are inhibited, slow wave activity would be hindered, ensuing in higher need to resolve sleep pressure. Through acute pharmacological hindrance of L-type calcium currents in vivo, slow wave activity measured through EEG is expected to decrease and in turn result in rebound sleep.

2. Methodology

2.1 Animals

Experimental procedures were carried out in accordance with local and national regulations and after approval by the animal care and use committee of the University of Tsukuba. Eight- to 25-week-old C57BL/6J male mice (Jackson Laboratory) and GCamP6f; CamKIICre were used for sleep recording and imaging, respectively. Mice were singled housed after surgery in 12 hour light and 12 hour dark cycle with food and water ad libitum.

2.2 Surgeries

2.2.1 Electrode Implantation

Mice were anesthetized with isoflurane (3-4% for induction and 2-2.5% for maintenance) and and place in a stereotaxic frame (David Kopf Instruments). The body temperature was maintained at 34-35degC with a heating pad. The scalp was removed, and the skull surface cleaned with a scalpel blade. For sleep recording, two electroencephalogram (EEG) screw-electrodes were inserted into the skull: two wire-electrodes were implanted into the neck muscles bilaterally for electromyogram (EMG) recordings. Electrodes were connected to a standard 0.1” four-pin connector. This connector was fixed to the skull with cyanoacrylate and dental cement.

2.2.2 Intracerebroventricular (i.c.v.) Cannulae Implantation

A single guide cannula of 27G (RWD, U.S.A.) was inserted 1.9mm from the surface of the brain at a 10° angle. During the i.c.v. injection, injector cannulae of 33G (RWD, U.S.A.) were inserted through the guide cannula with 0.1mm from the tip of the guide cannula.

2.2.3 Optical Window Insertion

Optical windows were inserted in some animals after electrode and i.c.v. cannula insertion. A craniotomy was performed over motor and somatosensory (M1/S2) region of the cortex using a dental drill. A hooked needle was used to remove a circular (2mm) flap of skull, leaving the dura intact. A Lipidure-CM5206 (NOF corporation) treated circular glass coverslip was held against the brain surface and glued to the skull with ultraviolet light hardening glue. A metal headplate with a sufficient central opening was then secured to the skull with dental cement.

2.3 Pharmacology

The mice were injected i.c.v. with vehicle of 10% sorbitol (Sigma-Aldrich, U.S.A.), with 100nM of nicardipine (Sigma-Aldrich, U.S.A.) in vehicle solution. During the i.c.v. injection, injector cannula of 33G is inserted through the guide cannula with 0.1mm from the tip of the guide cannula. The solution was carried via PE tubing (RWD, U.S.A.) from 1ml syringe (Terumo, Japan).

2.4 Recording

EEG and EMG electrodes were connected to a low-noise amplifier and digitizer board from (Intan technologies) at sampling rate of 1kHz. Signals were recorded with open-source software provided by Intan. EEG and EMG were extracted and filtered using MATLAB (Mathworks). After the electrode implantation, the mice were given recovery and acclimatized to recording chambers for seven days, followed by 3 days of baseline, by two days with vehicle injection at ZT0, ZT12 and ZT4 after sleep deprivation (SlDep) and rest, by 2 days with nicardipine injection at ZT0, ZT12 and ZT4 after SlDep and rest.

As for EEG and EMG recording during imaging, the signals were band-pass filtered and amplified using an analog amplifier (MEG-5200, NIHON KODEN), which was digitized at 2000Hz (Digidata 1440A, Molecular Devices) and acquired using Clampex 10.3 (Molecular Devices). Each scan was digitized and recorded to match to the imaging data.

2.5 Imaging

The two-photon laser microscope used was Olympus FluoView FV300/FV1000. After the optical window surgery, mice were given 7 days to recover/habituate to the headplateand a spherical treadmill. The mice could undergo spontaneous wake, NREM and REM sleep states on the spherical threadmill during imaging. Imaging GCaMP6f expressing neurons was conducted in II/III layer, with excitation at 910nm with MaiTai Control (Spectra-Physics). For each session, 1400 frames were acquired at a rate of 440ms per scan, which took about 11 minutes. In total, there were 13 sessions: 3 sessions for baseline, 3 sessions after vehicle injection and 7 sessions after nicardipine injection.

2.6 Sleep Deprivation

The mice were deprived of sleep by gentle handling and cage change.

2.7 Analysis

2.7.1 Sleep Recording

EEG signals underwent fast fourier transformation and further analyses through a custom-made MATLAB-based calculations. The classifications of wakefulness, REM and NREM were based on delta (0.1-4Hz) power, theta (6-10Hz) power to delta power ratio, and the integral of EMG signals. The epoch length was set to 10s. Epochs with high EMG was scored as wakefulness. Epochs with low EMG and high delta power were scored as NREM, and those with very low EMG and high theta to delta power ratio were REM.

2.7.2 Imaging data

The scanned images were loaded to custom-made MATLAB program, where they were analyzed in a semi-automatic fashion. Extracted raw data were motion-corrected

(Pnevmatikakis and Giovannucci, 2017). Regions of interest (ROI) were manually drawn to match the soma of the neurons, and their calcium activity was detected as change in fluorescence over fluorescence traces (dF/F), following this function: $dF/F = (F_t-F_0)/F_0$, where F_0 is the baseline mean of 4 frames previous to the F_t frame. For comparison of the calcium activity among different conditions, the integral of dF/F over 0.5 threshold was calculated.

2.7.3 Statistical Analysis

Statistical analyses were performed in MATLAB and in IBM SPSS Statistics (for Windows, Version 25.0. Armonk, NY: IBM Corp.) for two tailed paired Student's T-test, and 2-way repeated measures ANOVA. Statistical significance was considered when $p < 0.05$.

3. Results

3.1 Calcium activity is increased during SWS.

Under baseline conditions, I distinguished how neuronal activity changes during wakefulness, NREM and REM sleep. My imaging data showed that calcium activity increased during NREM sleep, than other states (Fig.1).

3.2 Nicardipine successfully blocked calcium activity.

Despite many researchers reporting their success in delivering nifedipine, I could not manage to dissolve the drug into the vehicle solutions they used. Because the reported to be successful vehicle is a quite established one, I tried to find other calcium blockers with similar structure and size; I tried dissolving nimodipine and nicardipine. Again, I failed to dissolve these compounds under other conditions: sonification, slow addition of the drug to vehicle solution; none worked until I mimicked the nicardipine solution human patients are injected with.

My imaging data under baseline, vehicle-injected and nicardipine-injected conditions showed that after only after i.c.v. nicardipine injection, the calcium activity decreased substantially (Fig.4). The vehicle and nicardipine injections were scheduled similarly as before: before the fourth session and before the seventh session, respectively. In this representative data, there were total of 63 neurons. Because the number of spikes and mean amplitude could vary according to neuronal type; integral of calcium transients over a threshold of 0.5 dF/F was calculated and showed that 100minutes after

nicardipine injection, the calcium activity reduced. The recovery from these injections were quite quick. After 120min, the calcium activity started to recover (data not shown).

3.3 SWA after nicardipine injection did not change.

Established that nicardipine blocks the calcium activity, I measured the SWA after nicardipine injection at various time points: ZT0, ZT12 and ZT4 after sleep deprivation (Fig.5). For ZT0 injection, after vehicle injection, it seems that there was a normal buildup of the SWA in the first hour, however for nicardipine injection, the buildup was rather jagged, which could indicate that the SWA did not build up properly, but there was no significant difference. Because we believed that the buildup of SWA was not uniform when injected at ZT0, we conducted the injection at ZT12, when the buildup of SWA is usually not seen. However, there was no significant difference in the delta energy after injection at ZT12. We next sleep deprived the mice for four hours and injected them at the beginning of their recovery sleep, however we observed that there was no difference in either the buildup or the dissipation of SWA. This suggests that L-type calcium influx is not a major contributor to generation of slow waves or the sleep need resolution.

Since we first looked at the average of delta power found every 30 minutes, we next looked at the level of sleep episode to see if there was an impairment in sleep need resolution. From each sleep episode during the first 3 hours, the initial peak within the first 4 minutes and the end of delta power are compared. In all conditions of ZT0, ZT12 and ZT4 after sleep deprivation, the delta power decreased (Fig 5B). The differences between the peaks and ends of delta power across all conditions showed no significant difference. This shows that the sleep need resolution was achieved even when the L-type voltage gated channel is blocked (Fig 5C).

The peaks of delta power and the end of delta power from each sleep episode did not show any difference across all the different injection time points (Fig 5). When we compared the delta power difference between end and peak, there were no significant difference across all the injections. No changes in NREM duration or episode length were observed.

I observed that calcium activity was higher during SWS, than the other states. However, block of L-type calcium influx not only did not influence overall SWA, but also did not affect SWA at episode

level. Sleep as quantified by EEG and EMG recordings are not affected by nicardipine over vehicle in terms of SWS duration, bout duration, for injections at ZT0 and injections at ZT12 and after sleep deprivation (Fig 4 and Table 1).

4. Discussion

Previously reported sleep research exhibited that during sleep, there is less activity for downregulation and also there is more activity for memory consolidation (Niethard, et al, 2016, 2021; Steriade et al. 2001). However, my data showed that during NREM sleep, there is more calcium activity in cortical excitatory neurons, showing that NREM sleep is composed of active excitatory neurons, which could reflect the ON and OFF oscillations. Genetic manipulations of inhibiting calcium influx through voltage gated calcium channels have been proven difficult; thus, I opted for acute pharmacological intervention. Imaging data showed clear block of calcium activity within 100minutes after the i.c.v. nicardipine injection; however, sleep analysis showed that there was no change in sleep architecture, sleep bouts and duration, suggesting that other voltage gated calcium channels could play more essential role in regulating NREM sleep. SWA decay time constants showed that not only L-type calcium channel does not play a big role in NREM sleep, but also that P/Q type calcium channel plays an essential role.

A model proposed by Tatsuki et al. showed that NREM ON and OFF oscillations depend on neuronal calcium dynamics. Their previous studies reported that knockout models of voltage-gated calcium channels and of calcium-dependent potassium channels exhibited shorter NREM sleep times. There are 8 genes involved in the voltage gated potassium channels; of those, *Kcnn2* KO mice showed the shortest sleep of around 10 hours, which is 2 hours shorter than WT mice (Tatsuki et al.,2016). This shorter NREM time runs counter to expected homeostatic upregulation, expected after disturbed sleep need resolution. Direct measurement of cortical ON/OFF state generation or EEG spectral power in these mice was not systematically studied. It is therefore difficult to assess the precise mechanism for the shorter NREM sleep times. Pharmacological block of NMDA receptors also resulted in shorter NREM times, which have again explained with disruption of activity-dependent calcium influx. A number of studies have shown that systematic application of the NMDA receptor blocker ketamine

results in particularly intense SWA (Chauvette et al., 2011; Fontanini et al., 2003; Ahnaou et al, 2017). The obligatory link between neuronal calcium influx and slow wave generation therefore seems unlikely.

L-type calcium channels represent the bulk of postsynaptic VGCC in the cortex and an estimated 50% of action potential-driven postsynaptic calcium influx is mediated by L-type VGCC (Wu et al., 1999). Complete knockout of CACNAC the alpha subunit of L-type VGCC is lethal and therefore it was not included in the systematic analysis of VGCC knockouts and a direct comparison between the results of VGCC knockouts and our pharmacological manipulation are not possible. Nevertheless, we observed robust reduction of neuronal calcium influx after intracerebroventricular application of the L-type VGCC blocker nicardipine. Animals showed no overt behavioral phenotype under nicardipine treatment and still freely transitioned between waking and NREM sleep. EEG slow wave activity was unaffected.

It is possible that activity-dependent calcium influx is not directly responsible for slow wave generation, but links slow wave activity to downstream signaling that resolves sleep need. Under these circumstances a compensatory increase in slow wave activity would be expected. We were not able to measure a significant increase in slow wave activity after nicardipine application, either immediately, or with a certain delay. Downstream mechanisms involving gene transcription factors from the acute inhibition of calcium entry should be investigated. Since bulk of calcium influx is through LVGCC, calmodulin complex would not be activated to its full capacity. Calcium-calmodulin complex is involved in activating adenylyl cyclase which activates breakdown of ATP. ATP is broken further down to cAMP, then to AMP whose final byproduct is adenosine. Adenosine kinase inhibitor increases adenosine levels, which increase SWA in SWS (Radek et al., 2004). Other endogenous factor altering sleep is adenosine deaminase inhibitors, which increase adenosine levels by inhibiting the breakdwon

of adenosine, increasing sleep (Porkka-Heiskanen et al., 1997; Radek et al., 2004; Radulovacki et al., 1983). When there is not enough calcium level, it is plausible that the adenosine level would decrease, promoting wakefulness. CREB signaling has been associated with spatial learning and memory formation; without proper influx of calcium through L-type VGCC, these two cognitive functions could be affected (Moosmang et al. 2005). Adenylyl cyclase KO mice showed high concentration of activated CREB during SWS with significant decrease during REM (Luo et al. 2013). From our results, however calcium does not affect the SWA which supports that LVGCC is not one of the essential calcium channels that affect SWA ultimately. However, further investigations on the downstream mechanisms involving adenosine levels should be conducted.

There are other sources of calcium influx contribute to slow wave generation or sleep need resolution. Voltage gated calcium channels are further categorized to high voltage activated (HVA) channels and low voltage activated (LVA) channels. L-type VGCC is merely one type of channel among the HVA; calcium influx through other channels such as R-, P/Q-, N-, and T-type of LVA should be investigated. Involvement of T-type VGCC in sleep waves has been well known since its discovery in thalamic neurons (Deschenes et al. 1984). Calcium influx through T-type channels was exhibited to characterize high-frequency bursts of action potentials during SWS and to initialize UP states (Crunelli et al. 2014). However, some studies indicated that SWS of KO mice of T-type VGCC isoform in thalamus displayed no change possibly due to compensation by other T-type VGCC isoforms and cortical channels (Lee et al., 2004). Because of their presynaptic physiology coupling neuronal excitation to secretion of neurotransmitter, any form of ablation of other HVA channels not only affects the sleep architecture, but also motor control, which can manifest to seizures, ataxia (Fletcher et al., 2001; Wormuth et al., 2016). Manipulations of P/Q-type channel for sleep research have never been discussed. Because they play a prominent role in neurotransmitter release,

abnormal P/Q channel activity can lead to epilepsy, ataxia, and Alzheimer's disease (Ophoff et al., 1998; Mezler et al., 2012). Despite the medical attention, there has not been much development, because of their structural similarity to N-type voltage gated calcium channel and that most of peptide compounds do not pass the blood-brain barrier. *Drowsy* mice are heterozygous KO model which has one allele that still expresses P/Q type channel. Although the data is not shown, there were no abnormal behavioral conditions, which suggests that drowsiness could be the solely phenotype these mice have, but their aberrant genetic structure and following complications need to be further investigated. Albeit challenging, further experiments are needed to further clarify the roles of other sources of intracellular calcium.

Homeostatic regulation of sleep and wakefulness has been researched for many years. Increase of homeostatic sleep need during wakefulness and its dissipation during sleep has been reflected in EEG SWA (Borbely, 1982; Huber 2004). Considerable studies suggest that increased EEG SWA reflects cortical synronicity in the cortex, caused by hyperpolarization and depolarization (Steriade et al., 1993; Benington and Heller, 1995). There has not been direct evidence showing the connection between SWS and sleep need resolution, but there is compelling evidence that there is an increase in SWA when there is higher need for sleep (Aeshbach et al., 1997). There should be a variable sensed by group of cells regulating sleep homeostasis. This physiological variable is yet to be discovered but one of the potential signaling molecules, calcium, has long been proposed to actively participate in the control mechanism. With its udal roles of tightly controlling intracellular concentration with huge magnitude and downstream mechanism of transcriptomes, calcium has made many scientists postulate that it should be involved in crosstalk among the neurons to display such synchronous activity during SWS. My study exhibited acute pharmacological intervention in L-type voltage gated calcium channel did not show significant changes in SWA, whereas heterogenous P/Q type KO mice showed a phenotype of longer sleep, which could be an adaptive mechanism to genetic manipulation. From the series of results from my study, there are two fundamental questions left unanswered: a. is SWA a suitable measure of sleep need, and b. what is the physiological tracker? SWA could just an

epiphenomenon; there is a convenient spatiotemporal correlation with this measure, which involves not only calcium but multiple molecules to make up for this intricate process.

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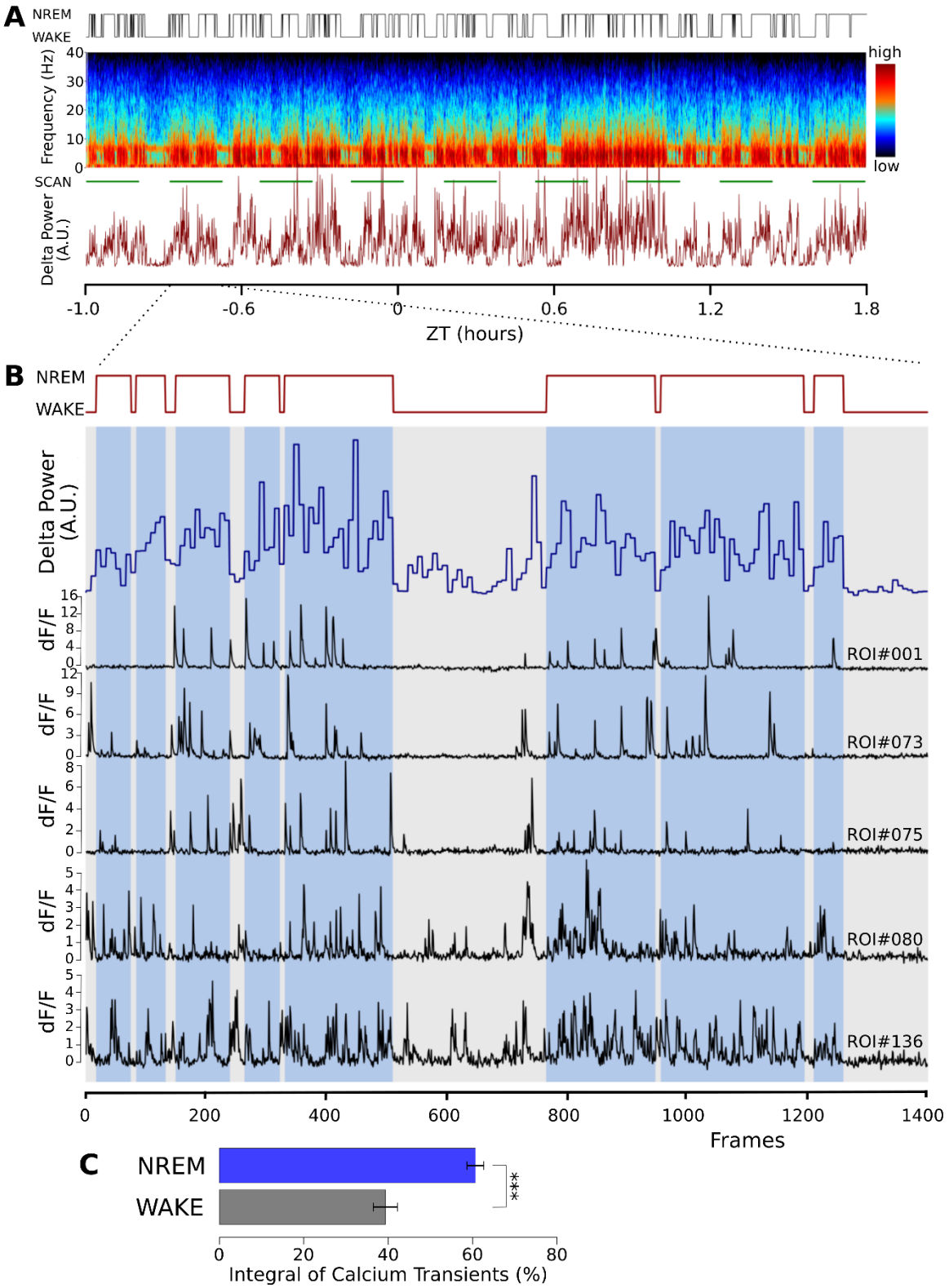
7. Figures

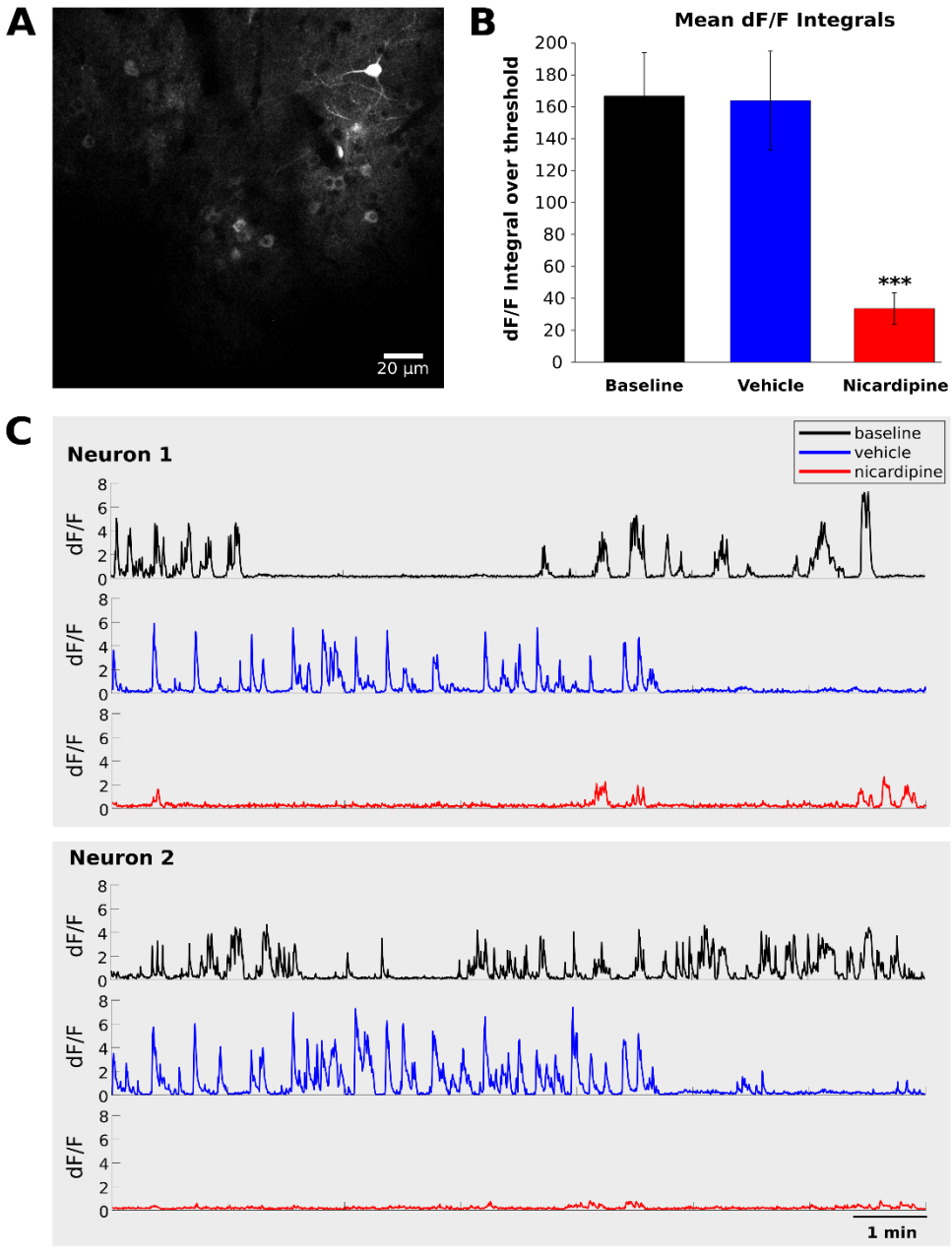
7.1 Somatic calcium activity is larger in excitatory neurons during natural SWS than wake.



Somatic calcium transients are larger in NREM sleep compared to waking in simultaneous EEG and EMG recordings and in-vivo two-photon GCaMP6f calcium imaging. A) Sample of one recording

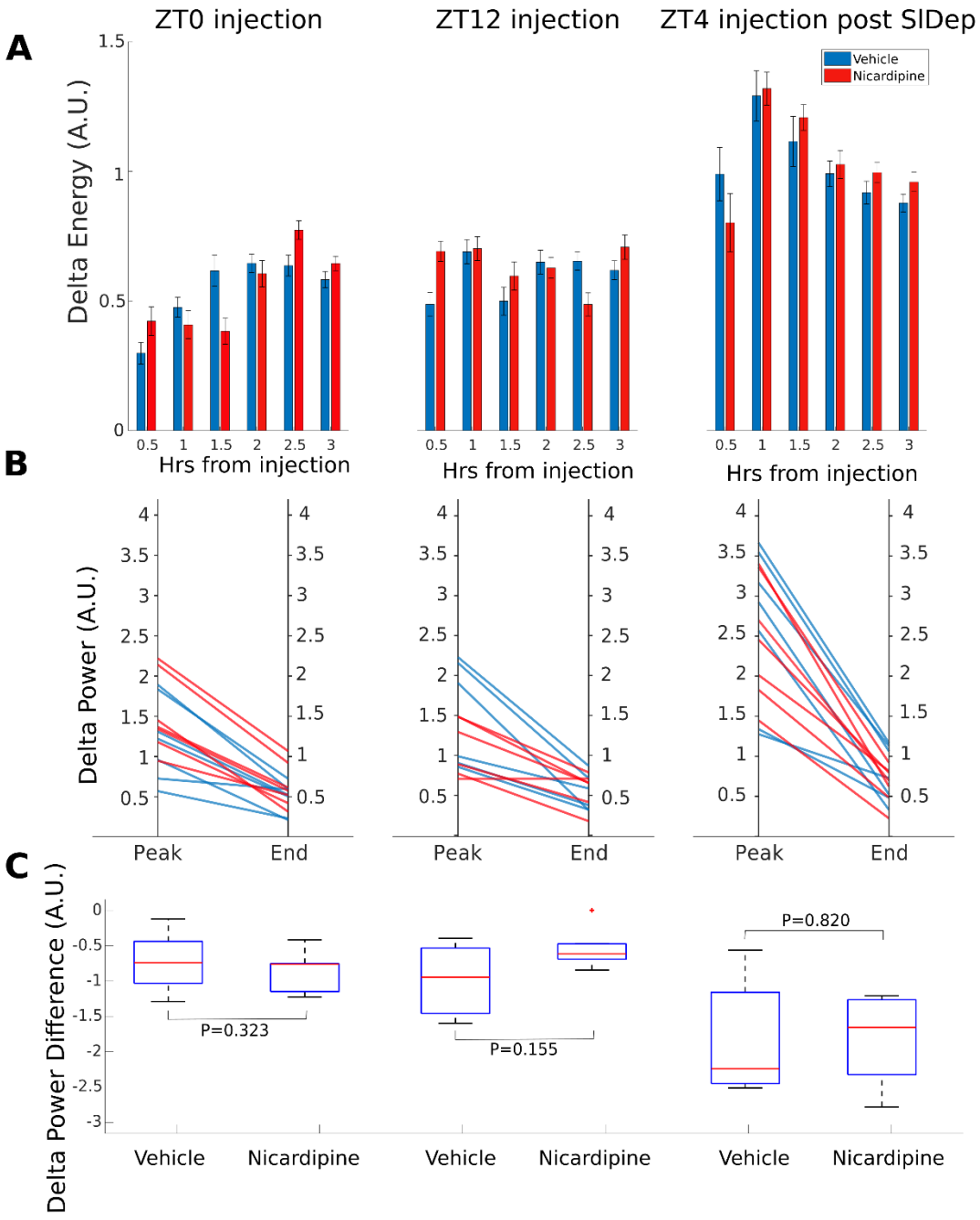
session. Top trace: hypnogram derived from the simultaneously recorded EEG and EMG. Middle graph: Pseudo-colored spectrogram of the EEG recording. The green line below indicates the time during which the imaging data was obtained (SCAN). The graph below shows power in the EEG delta band (0.5-4 Hz) over time. B) Enlarged time window from A (indicated by the dotted lines), representing one imaging episode of 1400 frames lasting 11 min. Top trace: Hypnogram, times in NREM sleep are marked with blue bars, showing two long wake episodes. Blue trace: EEG delta power in 4 s epochs. Black traces: sample of dF/F traces from five different regions of interest (ROI), drawn over somata of layer II/III excitatory neurons. Notice the decrease in activity during the wake episodes. C) Average data from a total of 100 neurons in 3 mice are shown (mean +/- SEM). The dF/F traces were thresholded at 0.5 for all ROIs (see Methods) and the integral over the threshold was calculated. Given the calcium fluorescence variability between ROIs, we calculated the activity in its percentage during NREM and its percentage during wakefulness, corrected by the relative durations of NREM and wakefulness. P values less than 0.001 are indicated with three asterisks.

7.2 Calcium activity decreased after i.c.v. injection of nicardipine.



Layer II/III neuron somatic calcium transients in in-vivo two-photon imaging of GCaMP6f under baseline conditions, after ICV vehicle injection and after ICV nicardipine injection. A) Representative grayscale image of a field of view from one experiment. B) Comparison of calcium signals under baseline conditions (black), after 40 min of ICV vehicle injection (blue) and after 100 min of ICV nicardipine injection. A threshold of 0.5 was applied to the dF/F signals (see Methods), which were then separately integrated over time for the three conditions. (N=3 animals; 63 neurons, 24 neurons, and 13 neurons), error bars are SEM. C) Representative dF/F traces over time of two neurons in the three conditions (black, baseline; blue after vehicle injection; red after nicardipine injection. P values less than 0.001 are indicated with three asterisks.

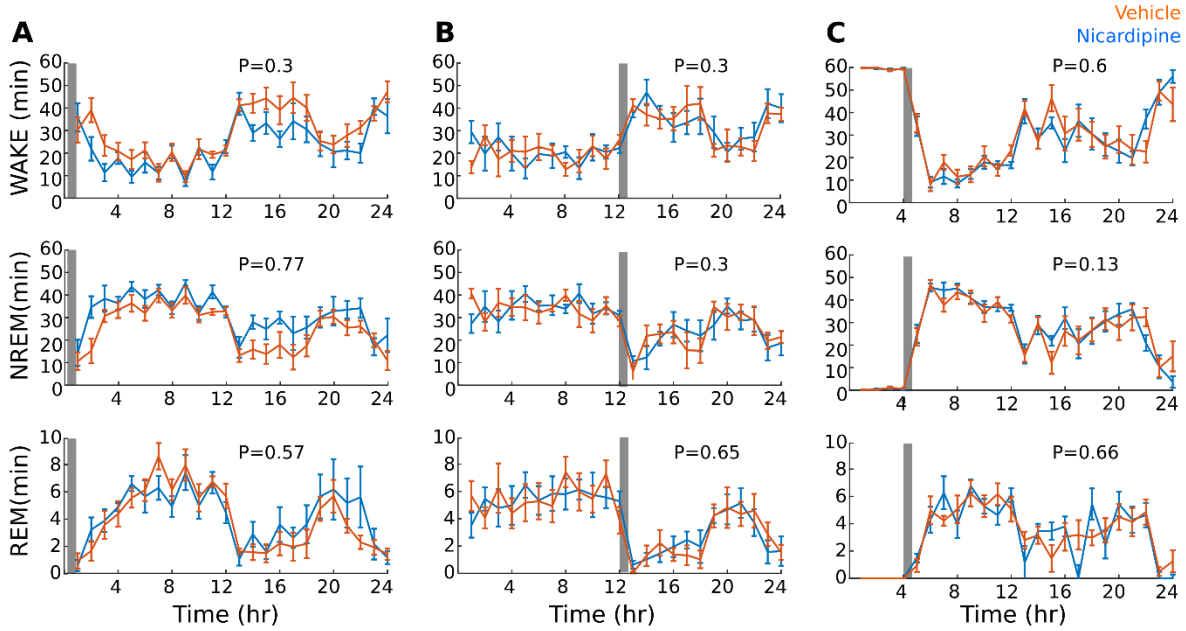
7.5 Delta energies of initial 3 hours of sleep from ZT0, ZT12 and ZT4 after sleep deprivation injected with vehicle and nicardipine.



The power of EEG oscillations in the delta (0.5-4 Hz) band during NREM sleep did not differ when the animals received ICV vehicle injections or ICV nicardipine injections. A) Power in the EEG delta band during NREM sleep is integrated over time to obtain delta energy. Delta energy production was measured for three hours, in three different conditions (at ZT0, at ZT12 and at ZT4 after 4 h of sleep deprivation (SlDep). Each animal was measured after vehicle injection (blue) and after nicardipine injection (red). Delta energy is plotted over time in 0.5-hour bins. Nicardipine did not affect EEG delta energy in any of the three conditions at any time point. B) The effectiveness of NREM sleep

episodes can be measured by the reduction in EEG delta power over the duration of the episode. We analyzed NREM sleep episodes longer than 5 min in the recordings from ZT0 ZT12 and ZT4 after sleep deprivation. Peak EEG delta power within the first three minutes is plotted on the left-hand side and the last epoch's delta power is plotted on the right-hand side. After both vehicle treatment (blue) and nicardipine treatment (red) EEG delta power was lower at the end of the episode compared to the beginning. C) Box plots (orange line, mean; blue box 25th percentile; black whiskers 90th percentile) of comparison between vehicle treatment and nicardipine treatment on the average reduction in delta power during NREM sleep episodes longer than 5 min. In all three conditions there was no significant difference between vehicle and nicardipine P-values were obtained with paired two-tailed Student's t-test (N=7).

7.6 No difference in the amounts of sleep and wake after vehicle and nicardipine injections



Plots of vigilance state duration in minutes per 1 h bin (top, wake; middle, NREM sleep; bottom, REM sleep) over 24 hours. Data is mean +/- SEM for N=7 animals. ZT0 to ZT12 is light (inactive) phase and ZT12 to ZT24 is dark (active) phase. Data from ICV vehicle treatment in orange, from ICV nicardipine treatment in blue. Treatment is indicated by gray vertical bar. A) Treatment time ZT0. B) Treatment time ZT12. C) Treatment time ZT4 after sleep deprivation from ZT0 to ZT4. P-values on top of graphs are from 2-way repeated measures ANOVA for treatment effect. There was no significant difference between treatment conditions for any vigilance state.

7.5 Table 1: Mean number of NREM episodes and mean NREM episode durations

	Number of NREM Episodes			NREM Episode Duration (min)		
	Vehicle	Nicardipine	P-value	Vehicle	Nicardipine	P-value
ZT0	116.143 (5.535)	109.857 (10.243)	0.552	19.357 (0.923)	18.301 (1.71)	0.3
ZT12	65.667 (3.221)	80 (14.507)	0.402	10.944 (0.537)	13.333 (2.418)	0.296
ZT4	100.714 (11.924)	77 (8.083)	0.051	16.786 (1.987)	12.83 (1.347)	0.07

The same NREM sleep episodes from Table 1 were selected in three different sleep conditions (ZT0, ZT12 and ZT4 after sleep deprivation). Number of NREM episodes and their durations were compared (N=7). Mean values and SEM values in parentheses are indicated. The p-values were obtained with paired two-tailed Student's t-test. This shows that the episode length and durations were not significantly different between vehicle and nicardipine conditions.