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The Effects of Neuroligin-2 Absence on Sleep Architecture and EEG Activity in Mice

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Résumé

Les troubles du sommeil sont comorbides avec la plupart des troubles psychiatriques, mais le lien entre ceux-ci n'est pas bien compris. Neuroligin-2 (NLGN2) est une molécule d'adhésion cellulaire qui joue un rôle dans la formation de synapses et la neurotransmission. En outre, NLGN2 a été associé à la schizophrénie, mais son implication dans le sommeil reste inexplorée. Dans le présent mémoire, l'effet de *Nlgn2* knockout (KO) sur l'architecture du sommeil et l'activité EEG chez la souris a été étudié.

Deux électrodes d'électroencéphalographie (EEG) ont été implantées au-dessus de l'hémisphère droit chez des souris pour enregistrer l'activité EEG pour quatre jours consécutifs. Les états de vigilance (éveil, sommeil paradoxal [REM], sommeil à ondes lentes [NREM]) ont été identifiés sur des époques de 4 s et la durée de l'état de vigilance a été calculée. L'analyse spectrale a été effectuée sur des époques sans artefact en utilisant la transformée rapide de Fourier.

Les souris *Nlgn2* KO ont montré plus d'éveil et moins de sommeil NREM et REM par rapport aux souris normales (WT). Les changements dans les durées d'éveil et de sommeil proviennent des altérations au le cours de la période de 12h obscurité, car les souris KO ont présenté une durée normale de sommeil/éveil pendant la période de lumière de 12h. L'activité spectrale a montré un effet de génotype significatif pour les fréquences rapides (25 à 50 Hz) pendant tous les états de vigilance, avec les souris KO ayant moins d'activité que les WT. Les souris KO ont aussi montré une activité spectrale accrue à 2-5 Hz pendant le sommeil NREM.

Ces données suggèrent que NLGN2 participe à la régulation de la durée du sommeil ainsi qu'à l'activité de l'EEG pendant l'éveil et le sommeil.

Mots clés: Neuroligine-2, états de vigilance, knock-out, architecture de sommeil-éveil, EEG, analyse spectrale

Abstract

Sleep disorders are comorbid with most psychiatric disorders, but the link between these is not well understood. Neuroligin-2 (NLGN2) is a cell adhesion molecule that plays roles in synapse formation and neurotransmission. Moreover, NLGN2 has been associated with schizophrenia, but its implication in sleep remains unexplored. In the present thesis, the effect of *Nlgn2* knockout (KO) on sleep architecture and EEG activity in mice has been investigated.

Two electroencephalography (EEG) electrodes were implanted above the right hemisphere in *Nlgn2* KO mice and littermates to record EEG for four consecutive days. Vigilance states (wakefulness, rapid eye movement sleep [REM sleep], non-REM sleep [NREM sleep]) were identified on 4 sec epochs and vigilance state duration was calculated. Spectral analysis was performed on epochs without artifact using a Fast Fourier Transform.

Nlgn2 KO mice showed more wakefulness and less NREM and REM sleep compared to wild-type (WT) mice. The changes in wakefulness and sleep durations originated from alterations during the 12h dark period because KO mice exhibited normal sleep/wakefulness duration during the 12h light period. The relative power spectra showed a significant genotype effect for fast frequencies (25-50Hz) during all vigilance states, with KO mice having less activity than littermates. The KO mice exhibited increased spectral activity at 2-5 Hz during NREM sleep. Moreover, abnormal burst of EEG activity was identified in the KO mice during wakefulness and REM sleep.

These data suggest that NLGN2 participates in the regulation of sleep duration as well as EEG activity during wakefulness and sleep.

Key words: Neuroligin-2, vigilance states, knockout, sleep-wake architecture, power spectra

Table of contents

List of Tables

List of Figures

Chapter 3. Methods

Figure 3.1 – A gel showing genotyping of *Nlgn2* knockout (KO) mice, and heterozygous (HET) and wild-type (WT) littermates. WT mice displayed bands at 582 bp that migrated less than KO mice which display bands at 565 bp. HET mice displayed two bands at 565 and 582 bp (but the WT bands in HET has been systematically weaker and higher migrating).

Figure 3.2 – Western blots showing NLGN2 and Actin stainings in brain extracts of *Nlgn2* KO mice, as well as WT and HET littermates. Green bands in the upper white rectangles represent NLGN2, and red bands in the bottom white rectangles represent actin.

Figure 3.3 – Schematic diagram of electroencephalographic (EEG) electrode locations. Three EEG electrodes were implanted on the right hemisphere; B= Bregma, L=Lambda, X_1= Anterior EEG electrode location, X_2= Posterior EEG electrode location, X_R= Reference electrode location, *= anchor screw location.

Figure 3.4 – Experimental design of the study. Brain activity of mice was continuously recorded for four consecutive days starting with light period. Sleep deprivation was performed on Recovery 1. Baseline 2, marked by red rectangle, was selectively investigated in this thesis.

Figure 3.5 – Representative 24-sec EEG and EMG traces for each vigilance states of WT mouse. (A) Wakefulness is characterized by low voltage fast frequency EEG activity with high EMG activity. (B) NREM sleep is characterized by high voltage slow frequency EEG activity with low EMG activity. (C) REM sleep is characterized by prevalent regular theta EEG activity and blunted EMG activity.

Figure 3.6 – Hypnogram of a wildtype mouse, subject# 850. Vigilance states of 24-h brain recording have been visually identified and hypnogram has been generated based on the visual identification. This hypnogram well illustrates the nocturnal rhythm of mice.

Chapter 4. Results

Figure 4.1 – (A) Duration of vigilance states during 24-h was measured in *Nlgn2* knockout (KO) mice, and heterozygous (HET) and wild-type (WT) littermates. A significant genotype effect was observed in all vigilance states. Different number of stars represents the significant differences assessed by simple effect analysis between indicated points (*: $p<0.05$, **: $p<0.01$; also for panels B and C). (B) Duration of vigilance states during the 12-h light period. There was no genotype effect found in all vigilance states. (C) Duration of vigilance states during the 12-h dark period. A significant genotype effect was found for all vigilance states.

Figure 4.2 – The time course of the duration of vigilance states for the full 24-h baseline recording. Genotypes were significantly different in the duration of all vigilance states and the hourly duration of all vigilance states were also significantly different; however, there was no interaction between the genotype and the time course.

Figure 4.3 – (A) Mean duration of vigilance state episodes during 24-h. *Nlgn2* KO mice showed longer mean duration of wake episodes than both HET and WT mice (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$; also for panels B and C). (B) Mean duration of vigilance state episodes during 12-h light period. While the mean duration of wake episodes of KO mice was significantly longer than WT mice, the mean duration of NREM sleep episodes of KO mice was longer than both WT and HET mice. (C) Mean duration of vigilance state episodes during 12-h dark period. KO mice showed longer mean duration of wake episodes than both HET and WT mice.

Figure 4.4 – The time course of the mean duration of vigilance state episodes for the full 24-h baseline recording. Genotypes were significantly different in the mean duration of wake episodes only, but the time course was significantly different in the mean duration of episodes of all vigilance states. These two variables, genotype and the time course, were interacting each other in NREM sleep. The significant interaction was observed between WT and KO mice at 2, 3, 4, 5, 6, 8, 10, and 14-h of the time course as represented by red symbols.

Figure 4.5 – (A) Total number of vigilance state episodes during 24-h baseline recording. KO mice had significantly less total number of wake episodes and NREM sleep episodes than WT and HET mice (*:$p<0.05$, **:$p<0.01$, ***:$p<0.001$; also for panels B and C). (B) Total number of vigilance state episodes during 12h light period. KO mice had significantly less total number of wake episodes than WT mice and had significantly less total number of NREM sleep episodes than WT and HET mice. (C) Total number of vigilance state episodes during 12 dark period. KO mice had significantly less total number of wake episodes and NREM sleep episodes than WT mice. The total number of REM sleep episodes of KO mice was significantly less than WT and HET mice.

Figure 4.6 – The time course of the total number of vigilance state episodes for the full 24-h baseline recording. Genotypes were significantly different in the total number of wake episodes and NREM sleep episodes, but the time course was significantly different in the total number of all vigilance state episodes. These two variables, genotype and the time course, were not interacting with each other significantly.

Figure 4.7 – Abnormal EEG activity. (A) Example of abnormal burst of EEG activity during wakefulness and REM sleep in KO mice in comparison to normal EEG activity in WT mice. The abnormal event was not observed during NREM sleep. (B) The abnormal events represented by green dots were frequently observed in KO mice and the range of the abnormal event duration was wider in KO mice than HET and WT mice.

Figure 4.8 – Mean duration and average number of abnormal EEG activity during the full 24-h, the 12-h light period, and the 12-h dark period. *Nlgn2* KO mice exhibited significantly more of abnormal EEG activity than WT and HET mice during all periods. The duration of abnormal EEG activity was also significantly longer in KO mice compared to both HET and WT mice in the full 24-h and the 12-h light period. During the 12-h dark period, KO mice exhibited significantly longer duration of the abnormal events than HET mice only.

Figure 4.9 – Occurrence of the abnormal events in vigilance states of *Nlgn2* KO mice. The abnormal events were mostly occurring during wakefulness and REM sleep.

Figure 4.10 – (A) Absolute spectral power between 1 and 50Hz in *Nlgn2* knockout (KO) mice and wild-type (WT) and heterozygous (HET) littermates for the three vigilance states calculated over the 24-h baseline recording. Differences between KO and WT mice are indicated by red symbols ($p < 0.05$, t-tests; same in B). In general, spectral power of KO mice was higher than WT and HET mice across most of frequencies (B) Relative spectral power between 1 and 50Hz in *Nlgn2* KO mice and littermates for the three vigilance states. In general, spectral power of KO mice at high frequency was lower than WT and HET mice.

Figure 4.11 – (A) 24-h dynamics of absolute delta (1-4Hz) activity in *Nlgn2* KO mice, and HET and WT littermates during wakefulness and NREM sleep. Significant genotype by interval interaction was found in both wakefulness and NREM sleep. Differences between KO and WT mice are indicated by red symbols [same in B]. In general, KO mice exhibited higher delta power at most of intervals compared to WT and HET mice during both wakefulness and NREM sleep (B) 24-h dynamics of relative delta activity in *Nlgn2* KO mice, and HET and WT littermates during wakefulness and NREM sleep. Significant genotype by interval interaction was found in both wakefulness and NREM sleep. During the 12-h light period, KO mice showed higher delta power compared to WT and HET mice in both wakefulness and NREM sleep. During the 12-h dark period. KO mice exhibited lower delta power in general compared to WT and HET mice in both wakefulness and NREM sleep.

Figure 4.12 – (A) 24-h dynamics of absolute sigma (10-13Hz) activity in *Nlgn2* KO mice, and HET and WT littermates during NREM sleep. Significant genotype by interval interaction was found in NREM sleep. Differences between KO and WT mice are indicated by red symbols (same in B). In general, KO mice exhibited higher sigma power across all intervals compared to WT and HET mice. (B) 24-h dynamics of relative delta activity in *Nlgn2* KO mice, and HET and WT littermates during NREM sleep. Significant genotype by interval interaction was not found for NREM sleep.

Chapter 5. Discussion

Figure 5.1 – The types of abnormal epileptiform activity that were observed from *NS-Pten* mutant mice during wakefulness. Short trains of repetitive spikes lasting less than 5 seconds were resembled to the abnormal EEG activity that was observed from *Nlgn2* KO mice. The figure has been taken from Ljungberg et al 2009.

Figure 5.2 – Summary diagram of the proposal. (A) Under normal conditions, GABARs are inserted at extrasynaptic site. The GABARs are diffusely distributed or clustered at synaptic or extrasynaptic sites by trapping laterally diffusing GABARs. NLGN2 has been suggested to drive postsynaptic assembly of $GABA_ARs$ containing $\alpha2/\gamma2$ or $\alpha1/\alpha2$ at perisomatic inhibitory synapses by interacting with gephyrin/collybistin or DGC/S-SCAM complexes respectively. (B) In the absence of NLGN2, postsynaptic cluster of gephyrin/collybistin complex is disrupted, possibly DGC/S-SCAM complex as well. Therefore, synaptic $GABA_ARs$ would diffuse out of the synaptic site and evenly distributed throughout the neuron. Then, extrasynaptic $GABA_ARs$ containing δ-subunit, $GABA_BRs$, and $GABA_CRs$ would move and settle into the less compacted

postsynaptic sites. It could be possible that NLGN2 regulates the subcellular localization of GABARs. This figure is influenced by one of figures from Bang, et al., 2013. DGC: Dystrophin-glycoprotein complex; S-SCAM: Synaptic scaffolding molecule; VGAT: Vesicuular gamma-aminobutyric acid transporter; NLGN: Neuroligin; NRXN: Neurexin; GABAR: Gamma-aminobutyric acid receptor.

Figure 5.3 – Representative traces of abnormal EEG activity. While wildtype mice (A) showed normal EEG signals, NLGN2 overexpressing mice (TgNL2.6; B) exhibited brief bursts of EEG activity during all of vigilance states. The abnormal EEG activity was consistently observed during wakefulness and REM sleep. This figure has been taken from Hines et al 2008.

List of Abbreviations

Acetylcholinesterase (AChE)

Alzheimer's disease (AD)

American Academy of Sleep Medicine (AASM)

Analyses of variance (ANOVAs)

Ascending Reticular Activating System (ARAS)

Autism spectrum disorders (ASDs)

Benzodiazepines (BZs)

Calcium (Ca^{2+})

Cell adhesion molecules (CAMs)

Deep sleep (N3)

Distilled water (dH_2O)

Dystrophin-glycoprotein complex (DGC)

Electroencephalography (EEG)

Electromyography (EMG)

Ephrin (Efn)

Ephrin receptor family (Eph)

Excitatory/inhibitory (E/I)

Extended ventrolateral preoptic nucleus (eVLPO)

Falling asleep (N1)

Functional Magnetic Resonance Imaging (fMRI)

GABA (Gamma-aminobutyric acid)

GABA receptors (GABARs)

Hertz (Hz)

Heterozygous (HET)

Hyperpolarization-activated cation current, h-current (I_h)

Immunoglobulin (Ig)

Knock-in (KI)

Knockout (KO)

Light sleep (N2)

Locus coeruleus (LC)

Low threshold, or transient Ca^{2+} current (I_T)

Miniature excitatory postsynaptic currents (mEPSCs)

Miniature inhibitory postsynaptic currents (mIPSCs)

N-Methyl-D-aspartic acid (NMDA)

Neurexins (NRXNs)

Neuroligins (NLGNs)

Neuronal subset (NS*)*

Non-rapid eye movement (NREM)

Obstructive sleep apnea (OSA)

Orexin (ORX)

Parkinson's disease (PD)

Postsynaptic density (PSD)

Postsynaptic density protein 95 (PSD 95)

PSD 95-DlgA-ZO1 (PDZ)

quantitative PCR (qPCR)

Rapid eye movement (REM)

Serotonin (5-HT)

Serotonin transporters (SERT)

Sleep deprivation (SD)

Slow wave activity (SWA)

Splicing site 4 (SS#4)

Splicing site A (SS#A)

Splicing site B (SS#B)

Standard error of the mean (SEM)

Synaptic homeostasis hypothesis (SHY)

Synaptic scaffolding protein (S-SCAM)

Tuberomammillary nucleus (TMN)

Ventrolateral preoptic nucleus (VLPO)

Wild-type (WT)

Zeitgeber time (ZT)

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Chapter 1: Introduction

Animal and human behavior is driven by biological and cognitive needs for their survival as supported by five major theories of motivation: intrinsic theory, drive reduction theory, arousal theory, psychoanalytic theory, and humanistic theory (Graham & Weiner, 1996; Gollwitzer, Delius, & Oettingen, 2000). Biological and cognitive needs/motives can be categorized into the innate and acquired drives respectively (Knoll, 2005). Unlike the acquired drives for attaining dispensable goals, the innate drives such as hunger, thirst, libido, and sleep are essential in the survival of individual and species (Knoll, 2005). Innate drives encourage organisms to consume nutrients and pass on their genes to the next generation, but the purpose of sleep is less intuitive. In the introduction, sleep will be well described in terms of its functions, electroencephalogram (EEG) signal phenotypes, and control mechanisms. Then, we will address how sleep disorders are comorbid with medical and psychiatric disorders and how postsynaptic cell adhesion molecules contribute to sleep regulation.

1.1 Sleep

Sleep is an innate drive that can be observed in all animal species from invertebrates to vertebrates (Tobler, 2000). During sleep, animals enter a quiescent state with greatly reduced responsiveness (Siegel J. , 2005); therefore, sleep had been considered earlier as a passive process that manifested in the decrease of sensory input (Bremer, 1935) or Ascending Reticular Activating System (ARAS) activity (Moruzzi & Magoun, 1949). The lesion studies by von Economo rejected the passive theory of sleep and showed that sleep was actively generated by the anterior hypothalamus (von Economo, 1930). Sleep is not simply a state of immobility and unalertness. When sleep is prevented, the amount of sleep loss can be recuperated by following sleep (Dinges, Rogers, & Baynard, 2005). Moreover, sleep comprises two main different stages

in mammals, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. These two stages oscillate throughout sleep (Aserinsky & Kleitman, 1953).

1.1.1. Functions of Sleep

Despite the fact that sleep is an essential part of animal functioning as prolong sleep deprivation is more fatal than starvation in animals (Rechtschaffen, 1998), there is no clear explanation for the functions of sleep.

From an evolutionary perspective, sleep has been adjusted to fit the ecological niche depending on the specie (Siegel, 2005; Siegel, 2009). The ecological hypothesis of sleep suggests that sleep can be shaped by factors like body size, diet, predation, and physical environment (Webb, 1974; Meddis, 1975). Mammals consume a lot of energy on maintaining body temperature and activities such as food consumption and predator avoidance, so sleep serves to conserve energy by reducing activity and body temperature (Webb, 1974). Small animals have higher surface area to volume ratio that radiates more heat per unit of mass; therefore, they require more energy to maintain a normal body temperature which make them prone to sleep more than large animals (Webb, 1974). The amount of sleep is further modified by the need for foraging requirements (Allison & Cicchetti, 1976; Elgar, Pagel, & Harvey, 1988; Siegel J. , 2005). Compared to carnivores, herbivores eat food poor in nutrients. In order to meet the energy requirement, herbivores spend more time in foraging/eating and sleep less than carnivores (Elgar, Pagel, & Harvey, 1988). Moreover, sleep helps animals to avoid danger during the time when they are most vulnerable (Meddis, 1975; Siegel J. , 2005). For such a reason, predators spend more time in sleep than preys. The ecological view of sleep is well supported by physical restoration hypothesis, but it contradicts some observations. Sleep is an ecologically

costly state that animals are exposed to external environmental threats, but it has survived throughout the course of evolution. Some animals such as migrating birds and dolphins require continuous vigilance, but they have developed alternating unihemispheric sleep to satisfy the need rather than eliminating sleep (Siegel J. , 2005). Moreover, there was no significant difference in energy consumption between wakefulness and sleep (Horne, 1980). Sleep may not have a functional role in physical restoration because muscle and other organs can recover from strain during quiet wakefulness.

Numerous studies have suggested a functional role of sleep in brain restoration as sleep deprivation causes cognitive impairments (Dinges, 2006). Sleep deprived subjects not only display decreased alertness towards monotonous tasks, but also show impairments in multitasking, task involving higher cognitive functions, and task requiring sustained attention (Lim & Dinges, 2008). Sleep may restore brain function by eliminating toxic factors accumulated during wakefulness or replenishing important resource. During sleep, interstitial space in the brain increases by about 60% that enhances the convective exchange between cerebrospinal fluid and interstitial fluid (XieL, et al., 2013); therefore, β-amyloid can be more effectively cleared out. Sleep may also replenish energy stores in the brain according to the energy hypothesis of sleep by Benington and Heller (Benington & Heller, 1995). During prolonged wakefulness, glycogen stores in astrocytes of the brain are depleted and extracellular adenosine accumulates in some brain regions including basal forebrain area which promotes sleep by inhibiting cholinergic neurons (Benington & Heller, 1995; Porkka-Heiskanen, Strecker, & McCarley, 2000). Sleep not only replenishes astrocytic glycogen level, but also decreases adenosine level in the cortex, basal forebrain, hypothalamus, and brainstem (Benington & Heller, 1995; Porkka-Heiskanen, Strecker, & McCarley, 2000). Moreover, multiple genes encoding

various enzymes and proteins are upregulated in the cerebral cortex and hypothalamus during sleep that might reconstruct multiple cellular components in preparation for the subsequent wakefulness (Mackiewicz, et al., 2007). The expression of *Homer1a* involved in neuroprotection is reliably affected by sleep loss but not affected by the corticosterone surge (Maret, et al., 2007; Mongrain, et al., 2010). During sleep, the overall level of HOMER1a decreased in the whole-forebrain while the post synaptic density (PSD) HOMER1a level increased (Diering, et al., 2017). The increased PSD HOMER1a level down scales excitatory synapses during sleep that might facilitate memory consolidation by improving signal-to-noise ratio (Diering, et al., 2017).

Additionally, a role of sleep in memory and learning has been demonstrated in a number of studies. In animals, sleep enhanced the performance of tasks learned during prior wakefulness by reactivating the task-related circuits primarily in hippocampus during NREM sleep (Born, Rasch, & Gais, 2006; Wilson & McNaughton, 1994). The reactivation during NREM sleep may consolidate memory traces of previous learning in the absence of interference from external information. By functional Magnetic Resonance Imaging (fMRI), the reactivation of the hippocampal areas that were activated during route learning in a virtual town during NREM sleep was confirmed in human (Peigneux, et al., 2004). Therefore, the role of sleep in brain functioning has been widely accepted.

1.1.2. EEG Signal Phenotypes

Continuous EEG recording has been performed to identify vigilance states as well as to quantify the quality of vigilance states (Aserinsky & Kleitman, 1953; Cooley & Tukey, 1965). Wakefulness is characterized by desynchronized low amplitude fast frequency EEG activity. When eyes are closed in the preparation for sleep, beta and theta activities (13-30 Hz and 4-8 Hz,

respectively) diminish and alpha activity (8-13 Hz) becomes dominant particularly in the occipital lobe. Under normal circumstances, sleep first appears in the form of NREM sleep. In humans, NREM sleep can be categorized into three stages according to the American Academy of Sleep Medicine (AASM) manual for the scoring of sleep and associated events: falling asleep (N1), light sleep (N2) and deep sleep (N3). During N1 stage of falling asleep, alpha activity diminishes and low amplitude mixed-frequency EEG activity appears. Theta activity can be prominently observed from the mixed-frequency EEG activity. Then, N1 stage progressively transit to N2 stage of light sleep. During N2 stage, specific EEG features can be observed such as spindles (12-15 Hz) and k-complexes, but there is no difference in the general brain activity between N1 and N2 stages. Finally, NREM sleep enters N3 stage of deep sleep which is characterized by delta (0.7-4.5 Hz) activity. In the course of sleep, REM sleep follows NREM sleep and these two states oscillate. REM sleep is also known as paradoxical sleep because its EEG activity is very similar to wakefulness (Jouvet, 1965). REM sleep can also be subdivided into tonic and phasic stages depending on saccadic eye movements (Sánchez-López & Escudero, 2011). During REM sleep, EMG become atonic and theta activity is prominently observed. Except humans and primates, most rodents exhibit single stage of NREM sleep that is generally equivalent to N3 stage (Lesku, Martinez-Gonzalez, & Rattenborg, 2009).

Visual inspection of the EEG reveals states of the brain such as wakefulness, sleep, and seizure, but it does not inform the activity of brain or the quality of vigilance states. To test how deep a subject is asleep, acoustic stimulus threshold for arousal was measured in the sleeping subject (Neckelmann & Ursin, 1993). A 1000 Hz sine tone increasing 1.5 dB per second for 45 seconds was turned on at different vigilance states and the latency to arousal was measured (Neckelmann & Ursin, 1993). N2 stage showed higher arousal threshold than N1 stage and REM

sleep (Neckelmann & Ursin, 1993). Moreover, the amount of delta activity in NREM sleep is positively correlated with the level of arousal threshold (Neckelmann & Ursin, 1993). Delta activity in NREM sleep has been used as an indicator of sleep intensity or pressure (Borbély, 1982; Pappenheimer, et al., 1975). Delta activity is not only observed in NREM sleep, but also observed in wakefulness. Delta activity in wakefulness was increased during the performance of a mental task such as a difficult mental calculation task or the Sternberg paradigm (Harmony, et al., 1996). The amount of delta activity in wakefulness was positively correlated with the difficulty of a mental task (Harmony, et al., 1996). A mental task requiring greater attention to internal processing may enhance the delta activity and delta activity in wakefulness can be used as a parameter for wake quality. Sleep spindle is a waxing and waning wave at 10-13Hz that occurs during N2 stage. It has been used as an index of memory consolidation because spindle density was increased after learning pairs of unrelated words (Gais, et al., 2002) or a maze task (Meier-Koll, et al., 1999). Moreover, sigma activity enhances sleep continuity in the presence of disturbing auditory stimulus (Dang-Vu, et al., 2010).

1.1.3. Models of Sleep Regulation

There are three major hypotheses on sleep control mechanism: two-process model, flip-flop switch model, and synaptic homeostasis hypothesis. These hypotheses construct the foundation of the research question in this study, so each model will be described in great details in following paragraphs.

The two-process model of sleep regulation suggests that there are two different processes, a homeostatic process S and a circadian process C, interacting together to regulate sleep propensity (Borbély, 1982). The pressure of process S accumulates in the course of wakefulness

and dissipates in the course of sleep (Borbély, 1982; Borbély & Achermann, 1999). Unlike process S, process C is independent from the duration of vigilance states (Borbély, 1982). It is regulated by the intrinsic pacemaker suprachiasmatic nuclei of the hypothalamus in mammals which modulates the timing of sleep and wakefulness (Borbély, 1982; Daan, Beersma, & Borbély, 1984). Process C sets up an upper and lower threshold on process S (Borbély, 1982; Daan, Beersma, & Borbély, 1984). When the pressure of process S accumulates and hits the upper threshold of process C, sleep is initiated (Borbély, 1982; Borbély & Achermann, 1999; Daan, Beersma, & Borbély, 1984). Sleep terminates as the pressure of process S dissipate to the lower threshold of process C (Borbély, 1982; Borbély & Achermann, 1999; Daan, Beersma, & Borbély, 1984). The two important concepts of the model are that process S and C are independent and sleep loss can be recovered by intensifying NREM sleep not necessary increasing NREM sleep duration (Borbély, 1982).

The Flip-flop switch model of sleep regulation well demonstrates the brain circuitry and neurotransmitters that regulate sleep and wakefulness [Figure 1.1] (Saper, Scammell, & Lu, 2005; Saper, Chou, & Scammell, 2001). Sleep inducing regions and wake inducing regions are mutually inhibited to generate abrupt transition between these two states (Saper, Chou, & Scammell, 2001; Saper, Scammell, & Lu, 2005). Wake inducing regions, locus coeruleus, tuberomammillary nucleus, and raphe nucleus, are monoaminergic (Aston-Jones & Bloom, 1981; Steininger, et al., 1999) and sleep inducing regions, ventrolateral preoptic nucleus (VLPO) and extended VLPO (eVLPO), are GABAergic [Gamma-aminobutyric acid] (Sherin, et al., 1996; Gaus, et al., 2002). Monoaminergic wake inducing regions can inhibit GABAergic sleep inducing regions by noradrenaline and serotonin (Gallopin, et al., 2000). Tuberomammillary neurons contain not only histamine, but also GABA which can inhibit VLPO (Vincent, Hökfelt,

& Wu, 1982). The flip flop switch is stabilized by orexin which reinforce the monoaminergic tone (Lee, Hassani, & Jones, 2005; Chemelli, Willie, & Sinton, 1999). Orexin may prevent unwanted transitions into sleep.

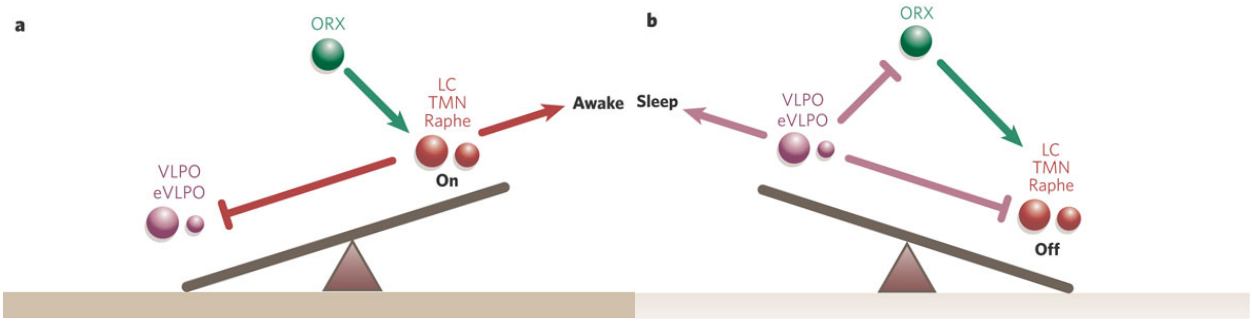

Figure 1.1 – Schematic diagram of the flip-flop switch model that represents a seesaw like effect of sleep and wakefulness. (a) During wakefulness, monoaminergic wake inducing regions (LC, TMN, Raphe), which are reinforced by orexin (ORX) neurons, inhibit GABAergic sleep inducing regions (VLPO, eVLPO). (b) During sleep, GABAergic sleep inhibits orexin neurons and wake inducing regions. The mutual inhibition between wake and sleep inducing regions induces abrupt transition. LC, locus coeruleus containing noradrenaline; TMN, tuberomammillary nucleus containing histamine; Raphe, dorsal and median raphe nuclei containing serotonin; eVLPO/VLPO, (extended) ventrolateral preoptic nucleus containing GABA (γ-aminobutyric acid) and galanin. This figure has been taken from Saper, et al. 2005.

The synaptic homeostasis hypothesis (SHY) is well compatible with the postulated functions of sleep such as memory consolidation and brain restoration. The hypothesis suggests that synaptic strength increases during wakefulness due to the occurrence of plastic processes and the synaptic strength decreases back to a basal level during sleep (Tononi & Cirelli, 2003). The downscaled synaptic strength is an energetically stable state and beneficial for memory consolidation and learning (Tononi & Cirelli, 2003). The high synaptic strength at the end of wakefulness synchronizes neurons which generate the high amplitude slow wave of early sleep (Esser, Hill, & Tononi, 2007). Delta activity which reflects the process S (Borbély, 1982) has been hypothesized to be positively correlated with the synaptic strength (Turrigiano & Nelson,

2004). However, the SHY remains controversial because Tononi and Cirelli have not identified the molecular mechanisms that alter synaptic strength as a function of sleep, and they have not excluded the role of other biological processes that occur during sleep (Frank, 2013).

1.1.4. GABAergic Transmission and Sleep

GABAergic transmission has been implicated in the physiology and pharmacology of sleep according to the development of sedative-hypnotic drugs for insomnia treatment (Winsky-Sommerer, 2009). Benzodiazepines (BZs) and Z-drugs are positive allosteric modulators that amplify the GABA effects (Winsky-Sommerer, 2009). These drugs bind to the interface between α- and γ-subunits of $GABA_A$ receptor called BZ-binding site (Sigel & Buhr, 1997). $GABA_A$ receptors containing γ-subunits are mainly localized synaptically due to the absence of δ-subunit (Sieghart, et al., 1999; Nusser, Sieghart, & Somogyi, 1998; Fritschy & Brünig, 2003) and mediate phasic inhibition (Mody, De Koninck, Otis, & Soltesz, 1994). BZs and Z-drugs decrease sleep onset latency and increase sleep continuity (Winsky-Sommerer, 2009). These drugs alter sleep architecture by promoting NREM sleep and suppressing REM sleep (Borbély & Achermann, 1991; Lemmer, 2007; Brunner, et al., 1991; Lader, 1992); moreover, they increase the spectral power of sigma activity and decrease the spectral power of delta activity (Lancel, 1999; Trachsel, et al., 1990; Brunner, et al., 1991).

THIP (gaboxadol), neuroactive steroids, tiagabine and alcohol are modulators of extrasynaptic $GABA_A$ receptors containing δ-subunits which mediate tonic inhibition (Nusser, Sieghart, & Somogyi, 1998; Fritschy & Brünig, 2003). The modulators enhance the total sleep time without affecting REM sleep (Winsky-Sommerer, 2009); moreover, they increase the spectral power of delta activity and decrease the spectral power of sigma activity (Faulhaber,

Steiger, & Lancel, 1997; Lankford, et al., 2008; Walsh, et al., 2005; Roth, Wright, & Walsh, 2006).

These observations imply that synaptic and extrasynaptic $GABA_A$ receptors regulate sleep differentially (Winsky-Sommerer, 2009). Synaptic $GABA_A$ receptors which mediate phasic inhibition might play a role in the induction/consolidation of NREM sleep and spindles, but they are also involved in the suppression of REM sleep and the slow-wave generation (Winsky-Sommerer, 2009). Extrasynaptic $GABA_A$ receptors which mediate tonic inhibition play a role in NREM sleep consolidation and EEG synchronization (Winsky-Sommerer, 2009). With the help of sedative-hypnotic drugs, it has been elucidated that $GABA_A$ receptors affect sleep differently depending on their synaptic localization; however, how phasic and tonic inhibitions modulate sleep needs to be understood

1.2. Comorbidity

Comorbidity is the presence of one or more additional disorders with a primary disease (Valderas, et al., 2009). The association among medical, psychiatric, and sleep disorders has been recognized by epidemiological studies (Dikeos & Georgantopoulos, 2011; Spiegelhalder, et al., 2013), but the link that causes the comorbidity is poorly understood. In this section, the detailed description of comorbid disorders will be stated to reveal associations with sleep.

1.2.1. Sleep Disorders

Sleep disorders such as insomnia, periodic limb movement disorder, and obstructive sleep apnea (OSA) affect the duration, timing, and quality of sleep; however, the detrimental effects of sleep impairment have been underrecognized (Hillman, Murphy, & Pezzullo, 2006). Direct and

indirect health costs of sleep disorders and of associated conditions in Australia in 2004 was about $7.5 billion which accounted for 3.5% of the total burden of diseases in Australia (Hillman, Murphy, & Pezzullo, 2006). OSA and insomnia are the most common sleep disorders affecting the general population, but about 75% of insomniacs are secondary to medical, psychiatric, circadian, or other sleep disorders (Hillman, Murphy, & Pezzullo, 2006). These two sleep disorders both result in sleep loss.

Insomnia is characterized by difficulty falling and/or staying asleep, early morning awakenings, and nonrestorative or poor quality sleep (Ohayon, 2002; Mendelson & et al, 2004; Roth, 2007). People who suffer with insomnia utilize greater health resources and show higher risk of developing medical and psychiatric comorbidities (Terzano, et al., 2004; Alkens & Rouse, 2005; Ohayon, 2002). The major medical comorbidities are heart disease, neurologic disease, and chronic pain (Taylor, et al., 2007; Terzano, et al., 2004; Ohayon, 2002). The major psychiatric comorbidities are depressive disorders, anxiety disorders, and psychotic disorders (Ohayon, 2002; Terzano, et al., 2004). Insomniacs show 8 to 10 times higher risk of developing major depressive disorder (Roberts, Shema, Kaplan, & Strawbridge, 2000) and sleep disturbance has been observed prior to the development of psychotic episodes in schizophrenics (Zarcone & Benson, 1997; Chemerinski, et al., 2002).

OSA is characterized by repetitive episodes of shallow or paused breathing during sleep due to partial or complete obstructions of the upper airway (Pinto, et al., 2016). OSA can cause frequent night awakening resulting in excessive daytime sleepiness, reduced productivity, and impaired quality of life which are similar to insomnia symptoms (Pinto, et al., 2016; Ohayon, 2002). The major comorbidities associated with OSA are insomnia, hypertension, obesity, and depression and these comorbid disorders become more prevalent in OSA patients with more

severe symptom (Luyster, Buysse, & Strollo, 2010; Pinto, et al., 2016). Insomnia could be a confounding factor that causes comorbidity in OSA patients.

1.2.2. Medical and Neurological Disorders

Chronic pain patients often complain about sleep disturbances, including difficulty falling and staying asleep as well as sleep interruption. Polysomnographic studies in painful medical conditions such as postoperative pain and rheumatic diseases have shown reduced sleep efficiency and altered sleep architecture (Onen, Onen, Courpron, & Dubray, 2005). Chronic pain patents exhibited an increase in wakefulness and N1 stage, and a decrease in N3 stage and REM sleep; moreover, alpha activity intrusions during sleep was also observed (Wittig, et al., 1982). While there are strong evidences that suggest the bidirectional pain-sleep relationship (Tang, Wright, & Salkovskis, 2007; Edwards, et al., 2008), the severity of pain does not indicate the severity of sleep disturbance (Smith, et al., 2000).

Neurological disorders are diseases of the central and peripheral nervous systems such as epilepsy, Alzheimer's disease (AD) and Parkinson's disease (PD), which are co-morbid with sleep disturbances. Epilepsy is a disorder characterized by recurring abnormal bursts of neural activity in the brain called seizure. People with epilepsy show sleep disturbances and abnormal sleep architecture which can be improved by epilepsy treatments (Derry & Duncan, 2013; Malow, Bowes, & Lin, 1997). Depending on the time of seizure occurrence, sleep is differentially affected (Bazil, Castro, & Walczak, 2000). While patients with nocturnal seizure showed reduced sleep efficiency and increased REM latency, patient with diurnal seizure exhibited reduced NREM and REM sleep after the seizure events (Bazil, Castro, & Walczak,

2000). Moreover, polysomnographic studies have demonstrated a significant decrease in N2, N3, and REM sleep of epilepsy patients (Bazil, Castro, & Walczak, 2000).

AD is the most common form of a primary dementia characterized by dramatically shrunken brain (von Strauss, et al., 1999). Polysomnographic studies in AD revealed increased REM latency, and decreased N2 stage and REM sleep (Kundermann, et al., 2011). PD is a movement disorder that is characterized by rigidity, resting tremor, bradykinesia, changes in speech, and impaired postural reflexes (Suzuki, et al., 2011). The degeneration of dopaminergic neurons in substantia nigra has been suggested to be the main cause of PD, but the involvement of the serotonergic, noradrenergic, and cholinergic systems cannot be neglected (Jellinger, 1991). The changes in monoaminergic and cholinergic systems can lead to sleep disturbances as revealed by polysomnographic sleep measure in drug-naïve PD patients (Joy, et al., 2014). They showed reduced sleep efficiency, increased sleep onset latency, prolonged N2 stage, and decreased REM sleep (Joy, et al., 2014). Both AD and PD patients show REM sleep abnormalities.

1.2.3. Psychiatric disorders

Depression and anxiety disorders are the main affective disorder that exhibit sleep disturbances. According to polysomnographic studies, patients with major depression showed decreased sleep efficiency and altered sleep architecture characterized by decreased total sleep time, increased sleep onset latency, decreased NREM sleep proportion, increased REM sleep proportion as well as decreased REM latency (Benca, et al., 1992). Patients with anxiety disorders exhibited decreased total sleep time, reduced sleep efficiency, and increased sleep

latency; however, there was no change in proportion of NREM and REM sleep (Benca, Obermeyer, Thisted, & Gillin, 1992).

Schizophrenia is a mental disorder characterized by a combination of positive [e.g. hallucinations, delusion], negative [e.g. apathy, lack of emotion], and cognitive symptoms [e.g. disorganized thoughts, memory problems] (Berman, et al., 1997). Polysomnographic studies in schizophrenia revealed decreased total sleep time, decreased sleep efficiency, disturbed sleep continuity, increased sleep onset latency as well as reduced sleep spindles; however, there was no change in proportion of NREM and REM sleep (Benca, et al., 1992; Wamsley, et al., 2012).

Autism spectrum disorders (ASDs) are neurodevelopmental disorders which exhibit impairments in social interaction. Since sleep disturbance has a negative impact on the quality of life, it is important to carry out polysomnographic studies in autism (Devnani & Hegde, 2015). ASD children showed an increase in the proportion of N1 stage and a decrease in total sleep time and REM sleep latency (Devnani & Hegde, 2015). REM sleep of the ASD children was more significantly altered as decreased REM quantity, increased undifferentiated/indeterminate sleep, and immature organization of eye movements were observed (Devnani & Hegde, 2015). The etiology of sleep disturbances in psychiatric disorders is not well understood, but sleep impairment has been suggested to cause a negative effect on the capacity to treat psychiatric patients.

The association among sleep, medical, and psychiatric disorders has been suggested, but the underlying link that causes the comorbidity is not well understood. In this thesis, the effect of NLGN2 on sleep has been evaluated that will give a clue on the link between comorbid disorders.

1.3. Cell Adhesion Molecules

Neurons communicate or transmit information through electrical or chemical synapses (Purves, et al., 2008). At electrical synapses, presynaptic and postsynaptic neurons are directly linked together by a gap junction which allows electric current to flow passively (Purves, et al., 2008). In contrast to electrical synapses, there is a space between presynaptic and postsynaptic neurons called synaptic cleft in chemical synapses which requires the secretion of neurotransmitters to induce cell-to-cell communication (Purves, et al., 2008). Chemical synapse is slower than electrical synapse, but it allows the amplification of signal as well as an additional level of signal regulation for the nervous system (Purves, et al., 2008). The synaptic cleft at chemical synapse is maintained by synaptic cell adhesion molecules (CAMs) which provide a mechanical link between pre- and post-synaptic terminals (Dalva, McClelland, & Kayser, 2007). There are several CAMs such as neurexins and neuroligins, Ephs and ephrins, immunoglobulin (Ig)-containing cell adhesion molecules and cadherins. These molecules have been suggested to do more than just bridging two neurons like regulating the formation, maturation, function and plasticity of synapses (Dalva, McClelland, & Kayser, 2007; Bang & Owczarek, 2013). Moreover, they share common protein domains which may allow them to coordinate synapse development in a similar way, but their unique motifs differentiate the function of each CAM and indicate the need of different CAMs to form a synapse. In this study, neuroligins will be specifically dealt with.

1.3.1. Neuroligins

Neuroligins (NLGNs) are postsynaptic cell adhesion molecules that play important roles in the establishment of functional synapses (Poulopoulos, et al., 2012; Südhof, 2008). While there

are only 4 NLGN genes found in rodents (Bolliger, et al., 2008), 5 NLGN genes have been identified in humans (Bolliger, et al., 2001). NLGN1 and 2 are expressed predominantly in the central nervous system, but NLGN2, 3 and 4 can be also detected in the peripheral nervous system (Suckow AT, et al., 2008; Philibert, et al., 2000). In humans, NLGN1 and 2 are only expressed in the brain (Bang & Owczarek, 2013).

1.3.1.1. Structure and Gene

NLGNs consist of a large extracellular catalytically inactive acetylcholinesterase (AChE) homologous domain which leads to the homo- or heterodimerization of NLGN (Poulopoulos, et al., 2012). The dimerization is required for surface trafficking of NLGNs (Poulopoulos, et al., 2012). Also, NLGNs have a short intracellular postsynaptic density protein 95 (PSD 95)-DlgA-ZO1 (PDZ)-binding domain that binds to postsynaptic components (Kurschner, et al., 1998). PDZ-binding domain of NLGNs does not only bind to postsynaptic scaffolding protein PSD95, but also bind to synaptic scaffolding protein (S-SCAM). S-SCAM is a postsynaptic scaffolding that interconnects NLGN with other CAMs such as β-cadherin and IgSF9b (Nishimura, et al., 2002; Woo, et al., 2013). While PSD 95 is only localized to excitatory synapses, S-SCAM is localized to both excitatory and inhibitory synapses. Proximal to the PDZ-binding domain of NLGN2 is the proline-rich region where collybistin and gephyrin binds (Nguyen, Horn, & Nicoll, 2016). The binding of the scaffolding protein gephyrin to NLGN2 activates collybistin (Kins, Betz, & Kirsch, 2000; Poulopoulos, et al., 2009). The activated collybistin tethers gephyrin to the postsynaptic membrane which recruits both glycine and $GABA_A$ receptors (Poulopoulos, et al., 2009). Gephyrin is only localized to inhibitory postsynaptic sites (TyagarajanSK & FritschyJM, 2014).

NLGNs undergo alternative splicing which determines their binding partners and regulates the expression selectivity toward excitatory or inhibitory synapses (Figure 1.2). Each of NLGN genes has a canonical splicing site A (SS#A) and NLGN1 has an additional splicing site B (SS#B). NLGN1 with SS#B selectively binds to only β-neurexin (NRXN) without splicing site 4 (SS#4), whereas NLGN1 without SS#B binds to α-NRXN without SS#4 and β-NRXN with or without SS#4 (Boucard, et al., 2005; Koehnke J, et al., 2010). The additional SS#B in NLGN1 favours the expression of NLGN1 toward excitatory/glutamatergic synapses, whereas NLGN1 without SS#B and NLGN2 are expressed predominantly at inhibitory/GABAergic synapses (Chih, Gollan, & Scheiffele, 2006). NLGN3 is a shared component in both excitatory and inhibitory synapses (Budreck & Scheiffele, 2007).

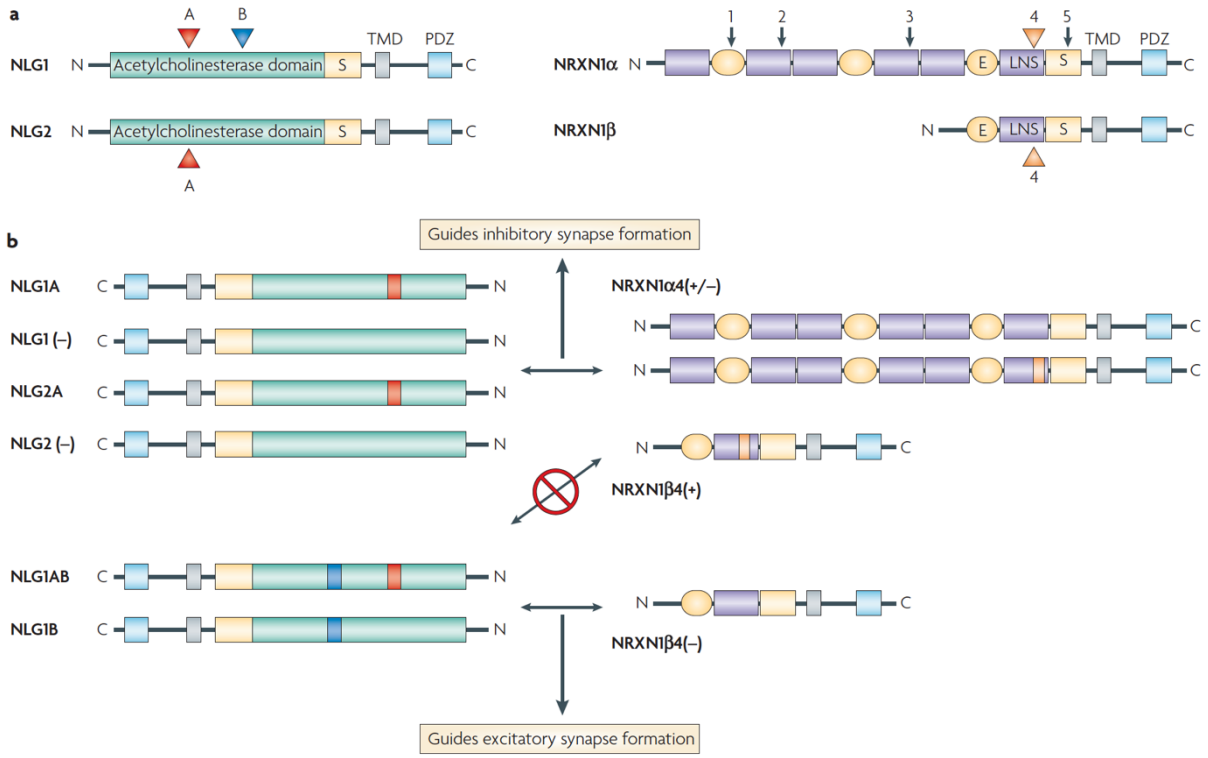

Figure 1.2 – Splice codes of neuroligins (NLGN) and neurexins (NRXN) determining binding partners and synapse formation. (a) In addition to the canonical splicing site A (red colored arrowhead) in *Nlgn* genes, *Nlgn1* has an extra splicing site B (blue colored arrowhead). *Nrxn*

genes have a canonical splicing site 4 and each *Nrxn* gene can be subdivided depending on the size of extracellular domain. *Nrxn* gene with long extracellular domain is classified as *Nrxnα* and *Nrxn* gene with short extracellular domain is classified as *Nrxnβ*. (b) *Nlgn* including splicing site B such as *Nlgn1AB* and *Nlgn1B* couples with *Nrxn1β* excluding splicing site 4 to guide excitatory synapse formation. *Nlgn* excluding splicing site B such as *Nlgn1A*, *Nlgn1(-), Nlgn2A,* and *Nlgn2(-)* couples with *Nrxn1α* independent to the presence of splicing site 4 as well as *Nrxn1β4(+)* to guide inhibitory synapse formation. This figure has been taken from Dalva, et al. 2007.

1.3.1.2. Functions

There are a number of *in vitro* studies suggesting a functional role of NLGNs in synaptogenesis. Overexpression and knockdown of NLGNs result in an increase and decrease of synapse number respectively as well as changes in dendritic spine density (Chih, Engelman, & Scheiffele, 2005). When neurons were co-cultured with NLGN expressing HEK29 cells, the *de novo* formation of morphological and functional presynaptic structure in contacting axons was triggered (Scheiffele, et al., 2000). However, *in vivo* studies have demonstrated a contradicting effect of NLGNs in synaptogenesis compared to *in vitro* studies. Mice lacking NLGN1-3 died shortly after birth not because of a reduced synapse number, but due to the impaired neurotransmission in brainstem respiratory centers (Varoqueaux, et al., 2006) . The insignificant change in synapse number of triple-KO mice left a controversial question whether NLGNs are involved in synapse formation or not.

On the other hand, both *in vitro* and *in vivo* studies have demonstrated the importance of NLGNs in synaptic maturation and function. Down-regulation of all three NLGN expression by RNA interference in cultured rat hippocampal neurons lowered the frequency of miniature inhibitory postsynaptic currents (mIPSCs), but did not cause any change in miniature excitatory postsynaptic currents (mEPSCs) (Chih, Engelman, & Scheiffele, 2005). In mice overexpressing

or ablated NLGN1, the frequency of mEPSCs was increased and decreased respectively without a significant change in the amplitude of mEPSCs (Schnell, et al., 2012; Kwon, et al., 2012). Spontaneous neural activity in prefrontal cortex of mice overexpressing NLGN2 was characterized by increased frequency of mIPSCs (Hines, Wu, Hines, & et al, 2008). In the case of *Nlgn2* KO mice, both frequency and amplitude of mIPSCs were decreased (Liang, et al., 2015). NLGNs have been suggested to regulate excitatory/inhibitory (E/I) balance in neurons (Prange, et al, 2004; Levinson, et al., 2005).

1.3.1.3. Association with Psychiatric Disorders

Mutations in the *Nlgn* genes have been linked to cognitive disorders such as schizophrenia and autism spectrum disorders (ASDs) (Bang & Owczarek, 2013). In autism patients, 13 different mutations in *Nlgn1* have been observed (7 point mutations, 2 distinct translocation events, and 4 different large-scale deletions), a single X-linked point mutation at R451C in *Nlgn3*, and 15 different mutations in *Nlgn4* (2 frameshifts, 5 missense mutations, 3 internal deletions, and 5 different copy-number variations) (Feng, et al., 2006; Yan, et al., 2008; Jamain & et al, 2003; Talebizadeh, et al., 2006; Marshall & et al, 2008; Südhof, 2008). In schizophrenia patients, 2 missense mutations at R215H and R621H in *Nlgn2* have been observed (Sun, et al., 2011).

One of the influential reviews on ASDs written by Dr. Rubenstein and Dr. Merzenich suggests that ASDs can be caused by an increased excitatory/inhibitory (E/I) balance which leads to hyper-excitability of cortical circuits in sensory, mnemonic, social and emotional systems (Rubenstein & Merzenich, 2003). Their postulate well matches with the frequent observation of reduced GABAergic signaling in the autistic brain (Cellot & Cherubini, 2014). Like ASDs, schizophrenia exhibits a change in E/I balance due to hypofunction of NMDA receptors on

inhibitory interneurons (Moghaddam & Javitt, 2012). The hypofunction of NMDA receptors results in the reduction of E/I balance on fast spiking interneurons which disinhibits glutamatergic neurons such as pyramidal cells in the neocortex and hippocampus (Moghaddam & Javitt, 2012). The disinhibition elevates the E/I ratio of glutamatergic neurons in cortical microcircuits, thereby increasing their firing rate (Yang, et al., 2016). Alterations in synapses such as spine morphology, synapse number and E/I balance have been associated with many other psychiatric and medical disorders ranging from retardation and autism to Alzheimer's disease and addiction (Van Spronsen & Hoogenraad, 2010; Eichler & Meier, 2008); therefore, change in NLGNs might play critical role in psychiatric disorders by regulating synaptogenesis, synaptic function, and E/I balance.

1.3.2. Effects on Sleep

While the effects of cell adhesion molecules (CAMs) on synapse and human diseases have been widely studied, the link between CAMs and sleep is poorly understood (O'Callaghan, Ballester Roig, & Mongrain, 2017). Dr. Mongrain has demonstrated the effect of CAMs such as NLGN1 and EphA4 on sleep. NLGN1 is predominantly expressed at excitatory synapses and determines the activity and synaptic localization of NMDA receptors (Barrow, et al., 2009). The activation of NMDA receptors regulates the cleavage of NLGN1 (Peixoto & et al, 2012). *Nlgn1* KO mice showed a decreased duration of wakefulness and the increased duration of NREM sleep (El Helou & et al, 2013). The quality of vigilance states of the KO mice was also altered (El Helou & et al, 2013). During wakefulness, KO mice exhibited a reduced activity in theta/alpha activity and they failed to show preference for social novelty (El Helou & et al, 2013). During sleep, KO mice showed an increase in delta activity (El Helou & et al, 2013; O'Callaghan,

Ballester Roig, & Mongrain, 2017). Moreover, NLGN1 is involved in sleep recovery process as Nlgn1 KO mice exhibited increased slow wave activity (SWA) rebound after sleep deprivation (El Helou & et al, 2013; Massart & et al, 2014).

EphrinA is a ligand that is bound by glycosylphosphatidylinositol linkage (Dalva, McClelland, & Kayser, 2007). The interaction between ephrinA and EphA4 tyrosine kinase receptor has been suggested to regulate the neuronal and neuroglial communications by manipulating the expression level of AMPA receptors (Murai & Pasquale, 2004; Fu, Hung, Fu, & et al, 2011). *EphA4* KO mice not only exhibited alterations in synaptic plasticity (Carmona, et al., 2009), but also showed changes in sleep. *EphA4* KO mice showed less REM sleep, but the duration of individual episodes of wake and NREM sleep was increased during the light period. Moreover, the 24-h rhythm of NREM sleep sigma activity was blunted (Freyburger & al, 2016).

Recently, Liu et al 2017 has shown a significant alteration of spectral power in $Nlgn3^{R451C}$ knock-in (KI) mice, an animal model of ASD. While the KI mice exhibited the normal sleep and wake architecture, the KI mice exhibited increased sigma and beta activity during wakefulness and decreased delta activity during NREM sleep (Liu, et al., 2017).

A number of studies have demonstrated an association of CAMs with psychiatric disorders and sleep disturbances; therefore, CAMs might play a critical role in the specific comorbidity between the two. The need for studying other CAMs is necessary to better understand their role in comorbid disorders as indicated in O'Callaghan et al., 2017.

Chapter 2: Objectives and hypotheses

2.1. Rationale and General Objective

People who suffer with sleep disorders have a higher risk of developing medical and psychiatric disorders, but the link that causes the comorbidities is not well understood (Dikeos & Georgantopoulos, 2011; Spiegelhalder, et al., 2013). Since medical and psychiatric disorders exhibit alteration in excitatory/inhibitory (E/I) balance, NLGNs can be suspected to play a key role in causing comorbidity (Prange, et al. 2004; Levinson, et al., 2005; Rubenstein & Merzenich, 2003; Moghaddam & Javitt, 2012; Van Spronsen & Hoogenraad, 2010; Eichler & Meier, 2008). NLGNs are postsynaptic cell adhesion molecules that regulate morphological and functional synaptic properties and their mutations have been linked to specific cognitive disorders such as autism and schizophrenia (Dalva, McClelland, & Kayser, 2007; Bang & Owczarek, 2013). Among several NLGN subtypes, NLGN2 is expressed predominantly at inhibitory synapses and the expression level of NLGN2 is inversely related to E/I balance (Bang & Owczarek, 2013). *Nlgn2* KO mice shows a decrease in both amplitude and frequency of mIPSCs (Liang, et al., 2015). The impaired inhibitory synaptic function is correlated with their behavior phenotypes such as anxiety, hypoalgesia, and ataxia (Blundell, et al., 2009). A recent study showed that the level of NLGN2 and α1-subunit of the $GABA_A$ receptor was increased on orexin neurons after 6-h deprivation on mice (Matsuki, et al., 2015). These changes have been implicated in increased sleep pressure (Matsuki, et al., 2015), but the specific effect of NLGN2 on sleep is not well understood. The general objective of this study is to evaluate the role of NLGN2 in sleep regulation by assessing changes in sleep architecture and EEG activity in mice lacking NLGN2.

2.2 Hypotheses

The brain circuitry that regulates sleep and wakefulness has been pictured in the flip-flop switch model of sleep regulation (Saper, Scammell, & Lu, 2005). GABAergic sleep inducing regions and monoaminergic wake inducing regions are mutually inhibited (Saper, Scammell, & Lu, 2005). Since *Nlgn2* KO mice exhibit impaired inhibitory synaptic function (Liang, et al., 2015), it is hypothesized that wake inducing regions will override sleep inducing regions in these mutant mice and that the KO mice will thus show more wakefulness and less sleep.

NLGN2 and gephyrin complex activates collybistin which tethers gephyrin to the postsynaptic membrane to recruits $GABA_A$ receptors at the synapse (Poulopoulos, et al., 2009). According to the development of sedative-hypnotic durgs for insomnia treatment, GABAergic transmission has been implicated in the physiology and pharmacology of sleep (Winsky-Sommerer, 2009). Synaptic $GABA_A$ receptor agonists have induced NREM sleep consolidation and REM sleep suppression; moreover, they increase the spectral power of sigma activity and decrease the spectral power of delta activity (Lancel, 1999; Trachsel, et al., 1990; Brunner, et al., 1991). Since the clustering of $GABA_A$ receptors at synaptic sites was abolished in *Nlgn2* KO mice (Poulopoulos, et al., 2009), the KO mice will exhibit the weakened-phasic inhibition and result in-increased spectral power of delta activity and decreased spectral power of sigma activity.

2.3 Specific contribution of the candidate

Throughout the study, I gained experience in three different fields: molecular study, animal study, and EEG phenotyping. In molecular study, I learned how to do genotyping and Western blotting. In animal study, I learned how to handle/sleep deprive mice and sacrifice mice. However, genotyping, Western blotting, sleep deprivation and mouse sacrificing were completed

with help from my colleagues. My main task in the project was phenotyping EEG signal by visually identifying vigilance states. Then, I marked abnormal EEG events throughout signals. After the quantification, I analyzed sleep-wake architecture and EEG activity. Based on the literature review, I have completed my thesis.

Chapter 3: Methods

3.1. Animals

3.1.1. Nlgn2 KO Mice

Mixed genetic background (B6;129-*Nlgn2*^{tm1Bros}/J) mice were purchased from Jackson Laboratories and bred on site by placing male and female heterozygous mice in a breeding cage to generate three genotypes: homozygous *Nlgn2* knockout (KO) mice, and heterozygous (HET) and wild-type (WT) littermates. *Nlgn2* KO mice were previously generated by homologous recombination (Varoqueaux, et al., 2006). A targeting vector containing a neomycin resistance cassette was electroporated into 192Sv-derived embryonic stem cells and disrupted *Nlgn2* exon sequences covering the translational start site and 380 base-pair of 5' coding sequence. Then, the targeted embryonic stem cells were transfected into C57BL/6 blastocysts. All the following experiments was approved by the Ethical Committee for Animal Experimentation of the Hôpital du Sacré-Coeur de Montréal.

3.1.2. Genotyping

In brief, mouse genomic DNA was isolated from ear tissue following overnight digestion at 56°C and 800rpm in 180µL ATL buffer and 20µL proteinase K using a DNA extraction kit from Qiagen. By using NanoDrop2000, it was confirmed that the amount of DNA was at least 10ng/µL. Then, PCR was performed with a master mix that comprised 8.18µL distilled water (dH_2O), 4.3µL 5XPCR buffer (KAPA), 2.5µL MgCl2 (25mM KAPA), 0.5µL dNTP (10mM), 1.2µL O29A primer (20µM, common forward: GTC TCA GTA AGC TTA TTT GAG AAG CCA A), 1.2µL 030A primer (20µM, wildtype reverse: CTC TGG GCC TTC TCA GGA CTG TAC), 1.6µL O31A primer (20µM, mutant reverse: GAG CGC GCG CGG CGG AGT TGT

TGA C), 0.12µL Taq DNA polymerase hotstart (5U/µL KAPA), and 2µL DNA (≥10ng of DNA). PCR conditions were as follows: the first cycle at 94°C for 3min, 30 cycles of 94°C, 66°C, and 72°C for 30sec each, and the last cycle at 72°C for 2 min. The PCR products were run in 2% agarose gels and DNA was visualized using a Syngen multi genius bio imaging system at 600ms EtBr/UV exposure (Figure 3.1). According to the information that is provided by Jackson Laboratories, the expected band sizes for WT, HET, and KO alleles are 582 bp, 565 and 582 bp, and 565 bp respectively.

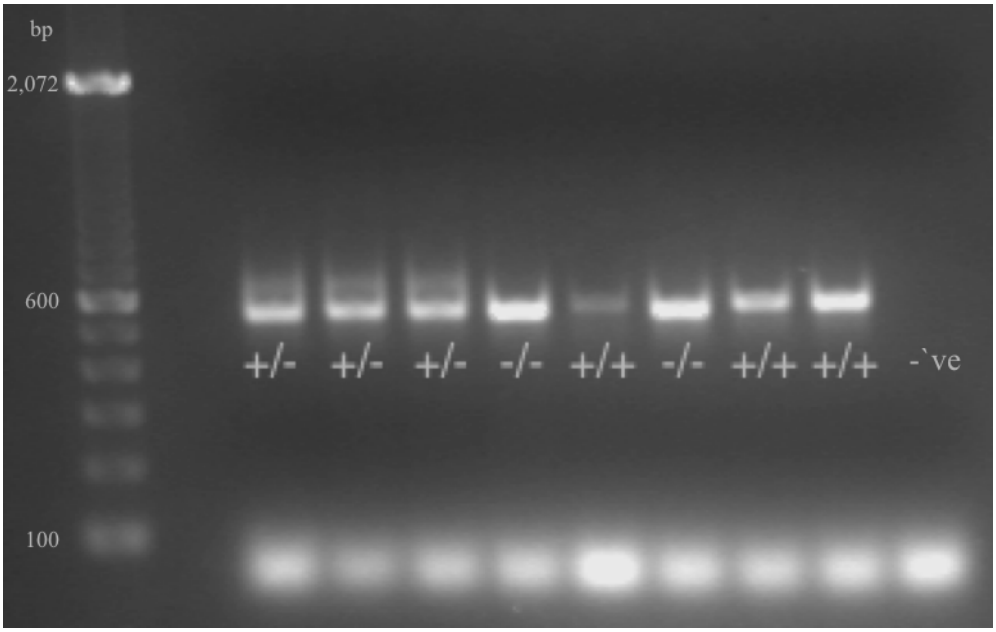

Figure 3.1 – A gel showing genotyping of *Nlgn2* knockout (KO) mice, and heterozygous (HET) and wild-type (WT) littermates. WT mice displayed bands at 582 bp that migrated less than KO mice which display bands at 565 bp. HET mice displayed two bands at 565 and 582 bp (but the WT bands in HET has been systematically weaker and higher migrating).

3.1.3. Protein Extraction and Western Blotting

In brief, mice whole brains, excluding cerebellum and olfactory bulb, were washed with cold PBS and homogenized in 2 mL RIPA lysis buffer (1 mL Hepes, 80 µL EDTA, 20 µL SDS

10%, 200 µL Igepal 10%, 100 µL sodium deoxycholate, 20 µL protease inhibitor, 20 µL phosphatase inhibitor, 560 µL dH_2O) using a rotor stator homogenizer. All the homogenization steps were carried out on ice. The homogenate was centrifuged at 8000 x g for 40min at 4°C and then the supernatant was collected. The total protein concentration was determined by DC protein assay with an EnSpire system (Perkin Elmer) to load an equal amount of protein. Before loading, proteins were denatured in laemmli buffer and β-mercaptoethanol (5min at 95ºC). The denatured protein was loaded onto 10% polyacrylamide gel and proteins were separated depending on molecular weight during electrophoresis at 150V for 85 min. Then separated proteins were transferred onto nitrocellulose membranes.

A snap I.D. 2.0 system (Millipore) has been employed for Western blotting. Blots were blocked with Odyssey blocking buffer to prevent non-specific binding of antibodies. Antibodies used were polyclonal rabbit anti-Neuroligin 2 (1:2000; SYSY; 129 203), mouse anti-actin (1:1000; FroggaBio; AM1829B), goat anti-rabbit (1:15000; Mandel Scientific; 926-32211) and goat anti-mouse (1:15000; Mandel Scientific; 926-68070). Membranes were revealed by Odyssey imaging system (Li-Cor Biosciences). Western blots confirmed the absence of NLGN2 in KO mice and the reduced protein level of NLGN2 in heterozygous compared to WT (Figure 3.2).

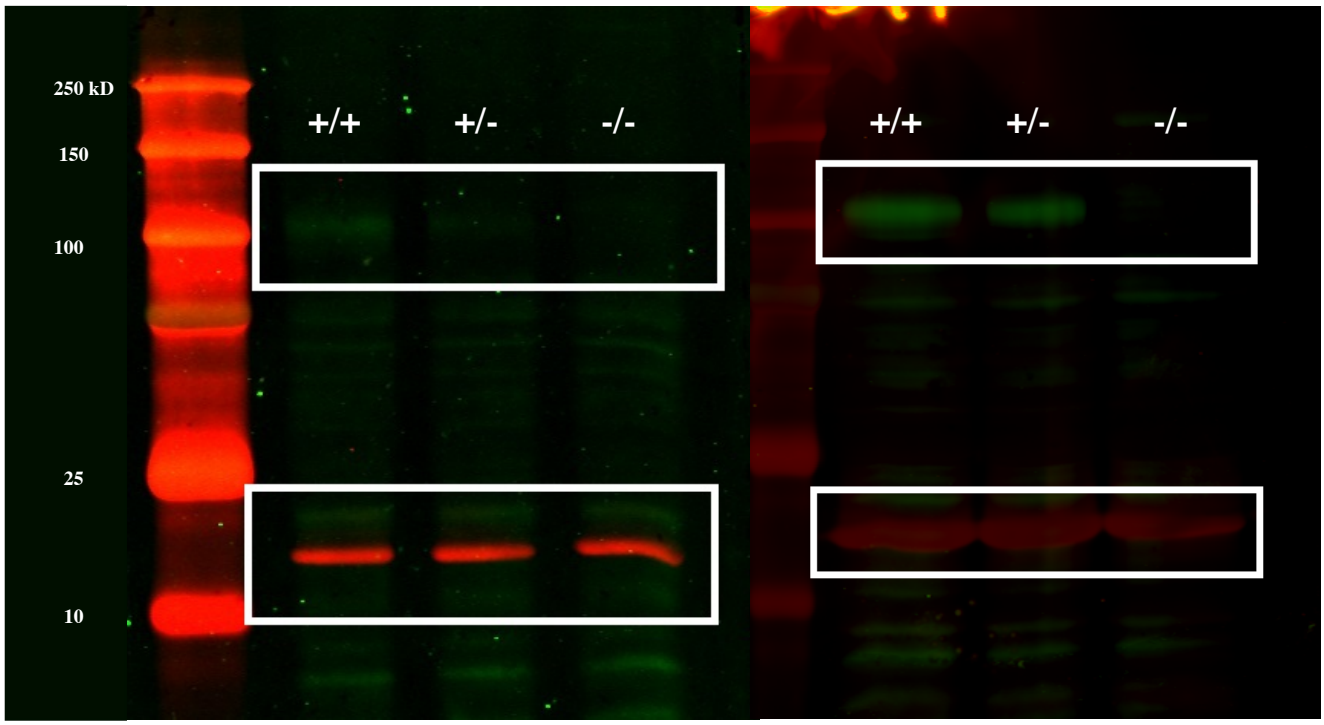

Figure 3.2 – Western blots showing NLGN2 and Actin stainings in brain extracts of *Nlgn2* KO mice, as well as WT and HET littermates. Green bands in the upper white rectangles represent NLGN2, and red bands in the bottom white rectangles represent actin.

3.1.4. Sex and Age

It has been suggested that sleep phenotype is different depending on sex, and many sex differences in sleep architecture and homeostasis are due to gonadal hormones (Manber & Armitage, 1999; Paul, et al., 2006). Therefore, male mice were only used in the experiment to reduce variability due to sex hormones. We have studied 14 wild-type (WT) mice, 14 heterozygous (HET) mice, and 13 *Nlgn2* knockout (KO) mice. At the time of the surgery (see below for surgery), WT mice were 65 ± 14 days old and 27.5 ± 2.2g; HET mice were 70 ± 12 days old and 27.3 ± 2.8g; and KO mice were 67 ± 14 days old and 24 ± 2.6g. Age was not significantly different between genotypes ($F_{(2,38)} = 0.3, \mathrm{p} = 0.68$), but weight was significantly different ($F_{(2,38)} = 7.6, \mathrm{p} = 0.002$). KO mice was significantly lighter than WT and HET mice

(p=0.0011; p=0.0023, respectively) while there was no significant difference between WT and HET mice (p=0.8).

3.2. Electrocorticographic/Electromyographic Electrode Implantation Surgery

The implantation surgery of electrodes for recording the electroencephalogram (EEG) and electromyogram (EMG) was performed as detailed previously (Franken, Malafosse, & Tafti, 1999; Mongrain, et al., 2010; Curie, et al., 2013; El Helou & et al, 2013). Briefly, when mice were between 9 and 10 weeks of age, EEG/EMG implantation surgery was performed under deep Ketamine/Xylazine anesthesia (120/10 mg/kg, intraperitoneal injection). Mice were placed in a stereotaxic frame and two gold-plated screws (diameter 1.1mm), which served as EEG electrodes, screwed through the skull over the right cerebral hemisphere (anterior: 1.7 mm lateral to midline, 1.5 mm anterior to bregma; posterior: 1.7 mm lateral to midline, 1.0 mm anterior to lambda) [Figure 3.3]. An additional screw, which served as a reference, was implanted on the right hemisphere (6 mm lateral to midline, 3 mm posterior to bregma). Three anchor screws were implanted on the left hemisphere. Two gold wires were inserted between neck muscles to monitor EMG activity. The EEG and EMG electrodes were soldered to a connector and secured on the skull by cementing the connector to the anchor screws. After four days of recovery, mice were connected to swivel contact, and the animals were habituated in the housing condition for a week before recording.

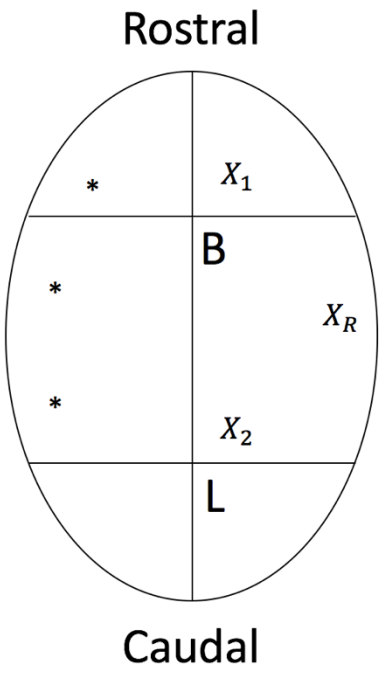

Figure 3.3 – Schematic diagram of electroencephalographic (EEG) electrode locations. Three EEG electrodes were implanted on the right hemisphere; B= Bregma, L=Lambda, X_1= Anterior EEG electrode location, X_2= Posterior EEG electrode location, X_R= Reference electrode location, *= anchor screw location.

3.3. Protocol: Housing Condition and 24-h Baseline Recording

After the surgery, mice recovered for 3 to 4 days and then each animal was housed in an individual cage under a 12-h light/12-h dark cycle at a temperature between 23 and 25 °C. The light is a Zeitgeber (meaning time giver) that entrains or synchronizes the 24-h biological rhythms; therefore, the onset of light will reset the circadian rhythm and is used as a reference for recording where Zeitgeber time 0 (ZT0) represents the onset of light within the 24-h light/dark cycle. The mice had free access to food and water and they were acclimated to cabling for a week before the experiment. Brain activity of mice was continuously recorded for four consecutive days starting with light period and 6 hours of sleep deprivation was performed on day 3. Sleep was disturbed by scratching a pipet on a floor. In this study, day 2 recording was

selectively investigated or analyzed to determine the effect of NLGN2 on normal sleep pattern (Figure 3.4).

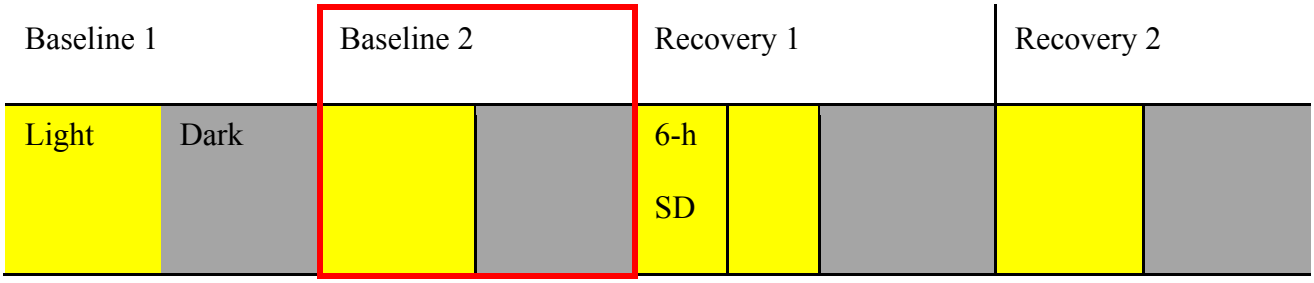

Figure 3.4 – Experimental design of the study. Brain activity of mice was continuously recorded for four consecutive days starting with light period. Sleep deprivation was performed on Recovery 1. Baseline 2, marked by red rectangle, was selectively investigated in this thesis.

3.4. EEG Recording

EEG/EMG was recorded continuously for 24-h starting with light period. Before the signals were sampled at 256 Hz, they were amplified (Lamont amplifiers) and filtered using the software Stellate Harmonie (Natus, San Carlos, CA). A bipolar montage was employed, so the signal from a channel represented the difference between two EEG electrodes.

The sampled signals were segmented in 4-second epochs to visually identify vigilance states based on the state dependent brain wave features as previously described in the section 1.1.2. (Franken, Malafosse, & Tafti, 1999)(Krueger, et al., 2008). EMG signal was also used to aid the identification of vigilance states. Low voltage fast frequency EEG activity with high EMG activity were the key features to identify wakefulness (Fig. 3.5A). High voltage slow frequency EEG activity with low EMG activity were the key features to identify NREM sleep (Fig. 3.5B). Prevalent regular theta EEG activity and blunted EMG activity were the key features to identify REM sleep (Fig. 3.5C). The scoring was performed blind to genotype. When segments of 4 seconds were screened, artifacts such as abnormal spikes, abnormal mixture of

frequencies, and transitions were identified and excluded from subsequent EEG spectral analysis (see below).

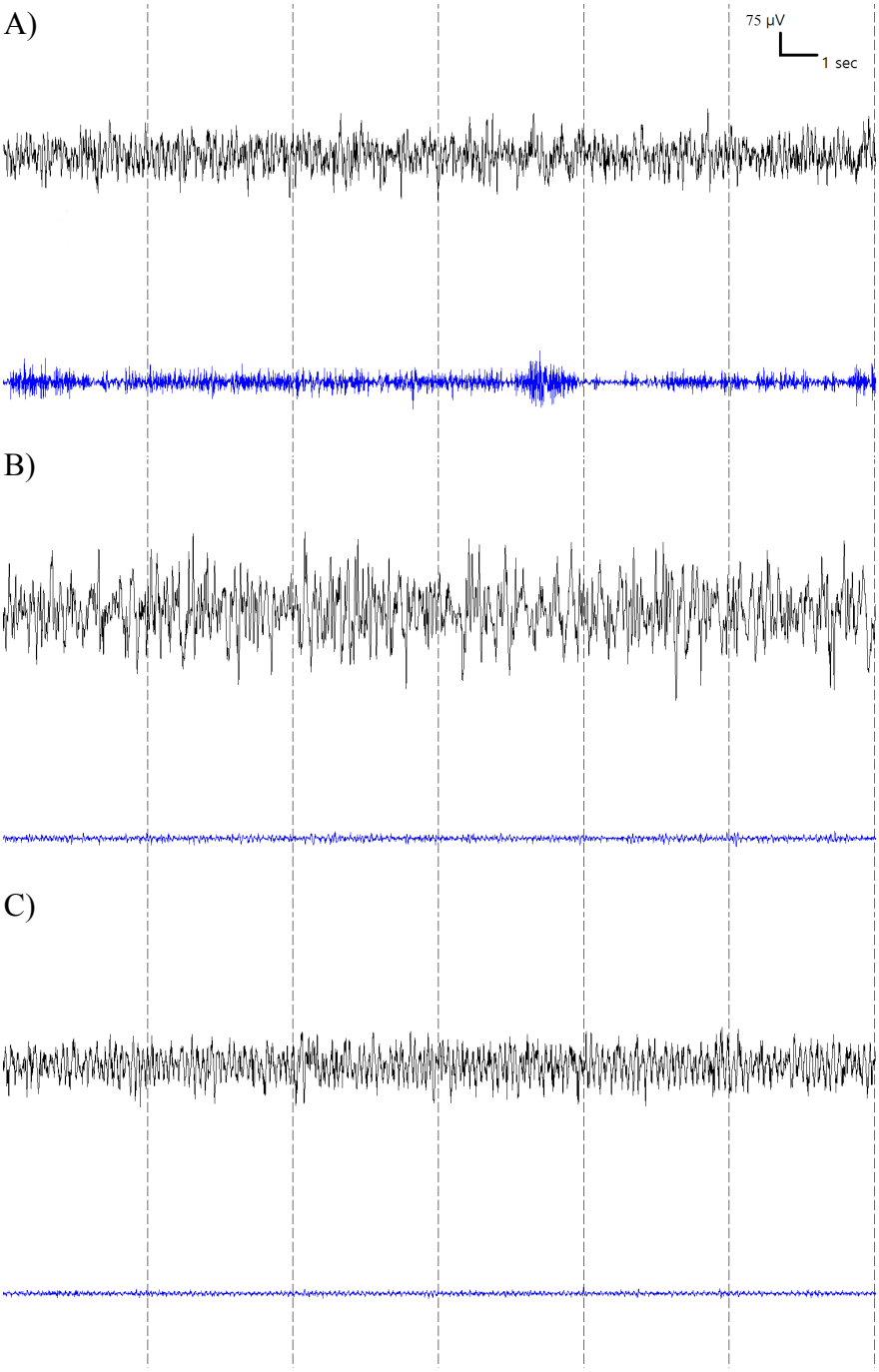

Figure 3.5 – Representative 24-sec EEG and EMG traces for each vigilance states of WT mouse. (A) Wakefulness is characterized by low voltage fast frequency EEG activity with high EMG

activity. (B) NREM sleep is characterized by high voltage slow frequency EEG activity with low EMG activity. (C) REM sleep is characterized by prevalent regular theta EEG activity and blunted EMG activity.

3.5. EEG Analysis

The visually identification of EEG served to quantify the sleep architecture (Figure 3.5) and this was further validated by spectral analysis as described below. More precisely, to observe changes in the total duration of vigilance states, and the consolidation/fragmentation of vigilance states, we computed the total duration of each vigilance state, the mean duration of vigilance state episodes, and the total number of vigilance state episodes during the full 24-h, the first 12-h (light period), the second 12-h (dark period), and per hour. The time course was calculated in reference to the light onset (= Zeitgeber time 0 [ZT0]) as mentioned above in the section 3.3.

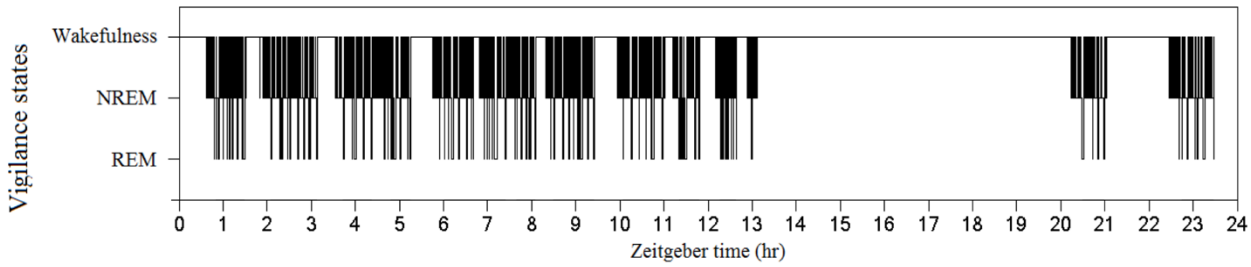

Figure 3.6 – Hypnogram of a wildtype mouse, subject# 850. Vigilance states of 24-h brain recording have been visually identified and hypnogram has been generated based on the visual identification. This hypnogram well illustrates the nocturnal rhythm of mice.

Spectral analysis was performed to investigate brain activity. The bipolar EEG signal was decomposed into its constituent frequency components by using fast Fourier transform. Fast Fourier transform was used under the assumption of stationary signal, so epochs with artifact were excluded from the spectral analysis. The EEG power density was calculated between 1 and

50 Hz (1 Hz resolution) during wakefulness, NREM, and REM sleep for the full 24 h. Two WT mice were removed from spectral analysis because of abnormal EEG signal. In addition, relative power density was computed: each animal's power density values were plotted in relative to the average power density of all frequency bins of all states of the mouse. This relative power spectra removed the variability of signal due to secondary factors such as altered cortical structure and surgical disruption which allowed us to clearly observe a state specific genotype effect. Nevertheless, it is important to analyze the absolute spectra to reveal the presence of the secondary factors and identify general changes in cortical network that could modulate EEG activity independent of state.

The time course of delta activity (1-4 Hz) has been further used to analyze the quality of sleep and wakefulness. The change in delta power was monitored for 18 intervals over 24 h during wakefulness and NREM sleep. The intervals were comprised of the same number of epochs of a state. Delta power was respectively averaged for 12 intervals in the light phase and 6 intervals in the dark phase for NREM sleep and vice versa for wakefulness. Relative delta power was computed with respect to the 24-h mean delta power of each animal, which allow us to better emphasize the dynamics of delta activity (El Helou & et al, 2013; Freyburger & al, 2016).

Spindle/sigma activity (10-13 Hz) has been suggested to consolidate memory as well as enhance sleep continuity in the presence of disturbing auditory stimulus (Gais, Molle, Helms, & Born, 2002; Meier-Koll, et al, 1999; Dang-Vu, et al., 2010). Sigma power during NREM sleep was also calculated for 18 intervals in a similar manner to delta activity and relative sigma power was also computed as done previously (Freyburger & al, 2016).

3.6. Abnormal EEG Activity Identification

During the visual inspection of EEG for vigilance state identification, occasional high amplitude bursts of EEG activity were observed. Since alteration in excitatory/inhibitory balance has been postulated as mechanism underlying epileptogenesis and seizure generation (Treiman, 2001), this abnormal EEG activity might be indicative of hypersynchronisation and/or epileptiform activity. The abnormal EEG events has been frequently observed during wakefulness and REM sleep. The number and duration of abnormal events were quantified by marking them according to the criteria as followed: the duration of events should last more than 1 second and their amplitude should be at least twice that of the background signal. When a break between two abnormal events was less than 0.5 second, these abnormal events were considered as one.

3.7. Statistical Analysis

Statistica has been used to compute statistical analysis. Vigilance state variables during the full 24-h, the 12-h light period, and the 12-h dark period and spectral power data were compared between genotypes by using one-way analyses of variance (ANOVAs). The vigilance state variables with significant genotype difference were decomposed by planned comparison. Despite the fact that spectral power data contained two variables such as frequency and genotype, we could not compute repeated-measure ANOVA because adjacent frequency bins are mathematically linked as a result of Fast Fourier computation. One third of spectral power data failed the Levene's test indicating an heterogeneity of variance between genotypes. To confirm the legitimacy of the use of parametric test, we performed nonparametric Mann-Whitney U tests on spectral power data. Results of parametric and nonparametric tests were similar and supported

the use of parametric test on spectral power data. Vigilance state variables per hour and Delta/Sigma activity per intervals were compared between genotypes as well as hours/intervals using two-way ANOVA with a repeated-measure design. Significance level for repeated-measure ANOVA was adjusted by Huynh-Feldt correction and the variables with significant difference were decomposed by planned comparisons. Standard error of the mean (SEM) was used as the variability parameter, and the threshold for statistical significance has been set to 0.05.

Chapter 4: Results

4.1. Sleep architecture

4.1.1. Duration of vigilance states

We measured the duration of vigilance states in *Nlgn2* KO mice, and HET and WT littermates to verify whether NLGN2 regulates the amount of vigilance states. During the full 24-h, *Nlgn2* KO mice, and HET and WT littermates were significantly different in the duration of wakefulness, NREM sleep and REM sleep ($Fig.\ 4.1A$ [$F_{(2,37)} = 4.9, \mathrm{p} = 0.013;\ F_{(2,37)} = 3.4, \mathrm{p} = 0.04; F_{(2,37)} = 4.3, \mathrm{p} = 0.02, \text{respectively}$]). KO mice were significantly more awake than WT mice ($\mathrm{p} = 0.003$), but there was no significant difference between WT and HET mice or HET and KO mice ($\mathrm{p} = 0.2;\ \mathrm{p} = 0.07, \text{respectively}$). Accordingly, KO mice spent less time in both NREM and REM sleep than WT mice ($\mathrm{p} = 0.014;\ \mathrm{p} = 0.007, \text{respectively}$), but there was no significant difference in NREM and REM sleep between WT and HET mice ($\mathrm{p} = 0.3;\ \mathrm{p} = 0.06, \text{respectively}$) or HET and KO mice ($\mathrm{p} = 0.1;\ \mathrm{p} = 0.3, \text{respectively}$). This implies a potential role of NLGN2 in sleep regulation.

The mouse is a nocturnal animal that is active during the night and sleep during the day. We examined the effect of NLGN2 on the nocturnal system by comparing the difference in the duration of vigilance states during the 12-h light period and the 12-h dark period among genotypes. During the 12-h light period, KO mice, and HET and WT littermates were not significantly different in the duration of wakefulness, NREM sleep and REM sleep ($Fig.\ 4.1B$ [$F_{(2,37)} = 1.2, \mathrm{p} = 0.3; F_{(2,37)} = 0.5, \mathrm{p} = 0.6; F_{(2,37)} = 2.4, \mathrm{p} = 0.1, \text{respectively}$]). During the 12 h dark period, KO mice, and HET and WT littermates were significantly different in the duration of wakefulness, NREM sleep and REM sleep ($Fig.\ 4.1C$ [$F_{(2,37)} = 7.3, \mathrm{p} = 0.002; F_{(2,37)} = 6.9, \mathrm{p} = 0.003; F_{(2,37)} = 6.4, \mathrm{p} = 0.004, \text{respectively}$]). KO mice were

significantly more awake than WT and HET mice ($p = 0.0013;\ p = 0.003$, respectively), but there was no significant difference between WT and HET mice ($p = 0.8$). KO mice spent less time in NREM sleep than WT and HET mice ($p = 0.002;\ p = 0.004$, respectively), but there was no significant difference between WT and HET mice ($p = 0.7$). KO mice spent less time in REM sleep than WT and HET mice ($p = 0.004;\ p = 0.003$, respectively), but there was no significant difference between WT and HET mice ($p = 1$). The significant increase and decrease in the respective duration of wakefulness and sleep of KO mice during the full 24 h was thus mostly due to the 12-h dark period.

Then, we measured the distribution of sleep and wakefulness over 24-h. A significant genotype effect was observed in wakefulness, NREM and REM sleep ($Fig.\ 4.2$ [$F_{(2,37)} = 4.9, p = 0.013; F_{(2,37)} = 3{,}4, p = 0.04; F_{(2,37)} = 4.3, p = 0.02$, respectively]). The amount of wakefulness, NREM and REM sleep per hour was also significantly different ($F_{(23,851)} = 51.3, p_{H-F\ adj.} < 0.001;\ F_{(23,851)} = 46.2, p_{H-F\ adj.} < 0.001;\ F_{(23,851)} = 51.7, p_{H-F\ adj.} < 0.001$, respectively). However, there was no interaction between genotype and the time course in wakefulness, NREM sleep, and REM sleep ($F_{(46,851)} = 1.3, p_{H-F\ adj.} = 0.2;\ F_{(46,851)} = 1.3, p_{H-F\ adj.} = 0.1;\ F_{(46,851)} = 1, p_{H-F\ adj.} = 0.5$, respectively), pointing to a global effect cumulated over multiple hours.

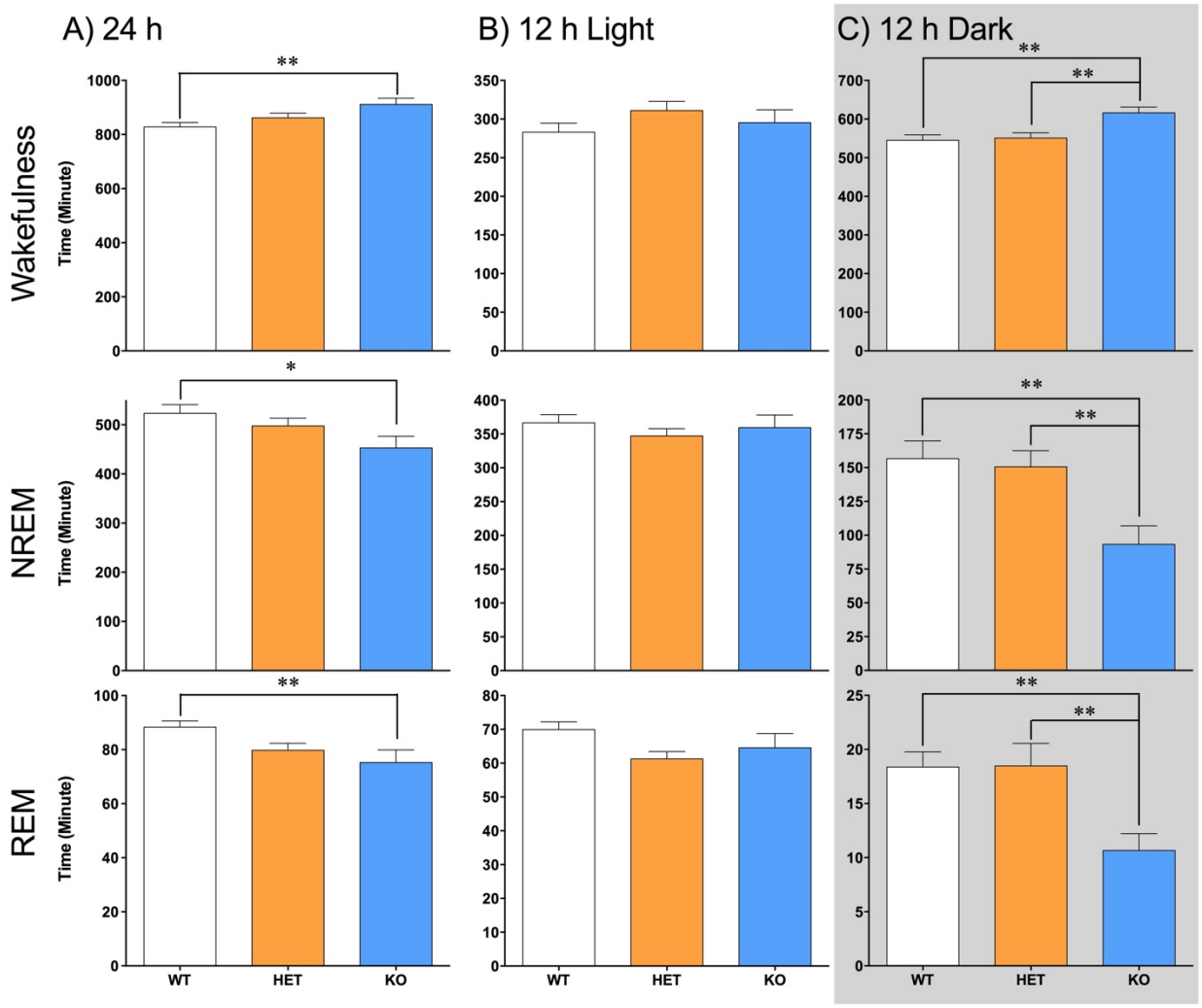

Figure 4.1—(A) Duration of vigilance states during 24-h was measured in *Nlgn2* knockout (KO) mice, and heterozygous (HET) and wild-type (WT) littermates. A significant genotype effect was observed in all vigilance states. Different number of stars represents the significant differences assessed by simple effect analysis between indicated points (*: $p<0.05$, **: $p<0.01$; also for panels B and C). (B) Duration of vigilance states during the 12-h light period. There was no genotype effect found in all vigilance states. (C) Duration of vigilance states during the 12-h dark period. A significant genotype effect was found for all vigilance states.

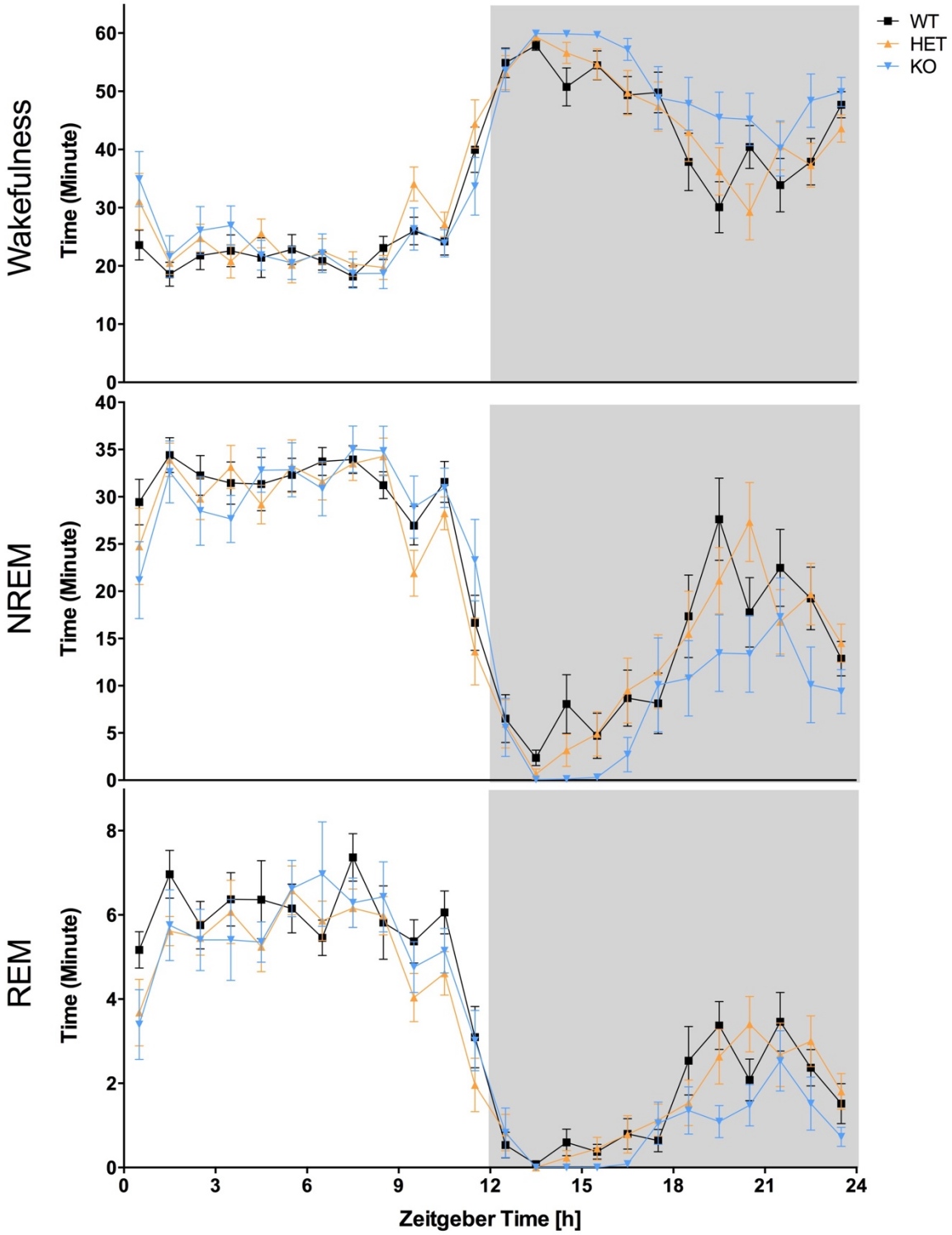

Figure 4.2—The time course of the duration of vigilance states for the full 24-h baseline recording. Genotypes were significantly different in the duration of all vigilance states and the hourly duration of all vigilance states were also significantly different; however, there was no interaction between the genotype and the time course.

4.1.2. Consolidation/Fragmentation of Vigilance States

Mean duration and total number of episodes were measured to explain the change in the duration of vigilance states that was observed in *Nlgn2* KO mice; moreover, this information provided us with the objective sleep quality such as consolidated or fragmented sleep.

During the full 24-h, *Nlgn2* KO mice, and HET and WT littermates were significantly different in the mean duration of wake episodes ($Fig.\,4.3A$ [$F_{(2,37)} = 13.9, \mathrm{p} = 0.00003$]), but not in the mean duration of NREM and REM sleep episodes ($F_{(2,37)} = 2.7, \mathrm{p} = 0.08; F_{(2,37)} = 0.2, \mathrm{p} = 0.8, \text{respectively}$). KO mice showed significantly longer mean duration of wake episodes than WT and HET mice ($\mathrm{p} = 0.00001;\ \mathrm{p} = 0.0004, \text{respectively}$) while WT and HET mice were not significantly different in the mean duration of wake episodes ($\mathrm{p} = 0.2$).

During the 12 h light period, KO mice, and HET and WT littermates were significantly different in the mean duration of wake and NREM sleep episodes ($Fig.\,4.3B$ [$F_{(2,37)} = 4.4, \mathrm{p} = 0.02$; $F_{(2,37)} = 4.1, \mathrm{p} = 0.03, \text{respectively}$]), but not in the mean duration of REM sleep episodes ($F_{(2,37)} = 0.5, \mathrm{p} = 0.6$). KO mice showed significantly longer mean duration of wake episode than WT mice ($\mathrm{p} = 0.006$), but there was no significant difference between WT and HET mice or HET and KO mice ($\mathrm{p} = 0.1;\ \mathrm{p} = 0.2, \text{respectively}$). KO mice showed significantly longer mean duration of NREM sleep episodes than WT and HET mice ($\mathrm{p} = 0.02;\ \mathrm{p} = 0.02, \text{respectively}$), but there was no significant difference between WT and HET mice ($\mathrm{p} = 1$).

During the 12 h dark period, KO mice, and HET and WT littermates were significantly different in the mean duration of wake episodes only ($Fig.\,4.3C$ [$F_{(2,37)} = 6.2, \mathrm{p} = 0.005$]), but not in the mean duration of NREM and REM sleep episodes ($F_{(2,37)} = 0.5, \mathrm{p} = 0.6; F_{(2,37)} = 0.3, \mathrm{p} = 0.7$, respectively). KO mice showed significantly longer mean duration of wakefulness

episodes than WT and HET mice ($p = 0.003;\ p = 0.006$, respectively), but there was no difference between WT and HET mice ($p = 0.7$).

Change in the mean duration of vigilance state episodes was measured over the 24-h time course. Genotypes were significantly different in the mean duration of wake episodes ($Fig.\ 4.4\ [F_{(2,37)} = 4.6, p = 0.02]$), but not in the mean duration of NREM and REM episodes ($F_{(2,37)} = 0.2, p = 0.8; F_{(2,37)} = 1.9, p = 0.2$, respectively). The mean duration of wake, NREM, and REM sleep episodes were significantly different over the 24-h time course ($F_{(23,851)} = 36.1, p_{H-F\ adj.} < 0.001;\ F_{(23,851)} = 21.3, p_{H-F\ adj.} < 0.001;\ F_{(23,851)} = 23.5, p_{H-F\ adj.} < 0.001$, respectively). Genotype and the time course interaction was not significant for the mean duration of wake and REM sleep episodes ($F_{(46,851)} = 1.02, p_{H-F\ adj.} = 0.4;\ F_{(46,851)} = 1.05, p_{H-F\ adj.} = 0.4$, respectively) but was significant for the mean duration of NREM sleep episodes ($F_{(46,851)} = 1.8, p_{H-F\ adj.} = 0.02$). The hourly mean duration of NREM sleep episodes was different between genotypes with longer bouts in KO mice compared to WT mice at 2, 3, 4, 5, 6, 8, 10, and 14 h of the time course ($p = 0.04;\ p = 0.002;\ p = 0.01;\ p = 0.04;\ p = 0.04; p = 0.03;\ p = 0.04;\ p = 0.04$, respectively).

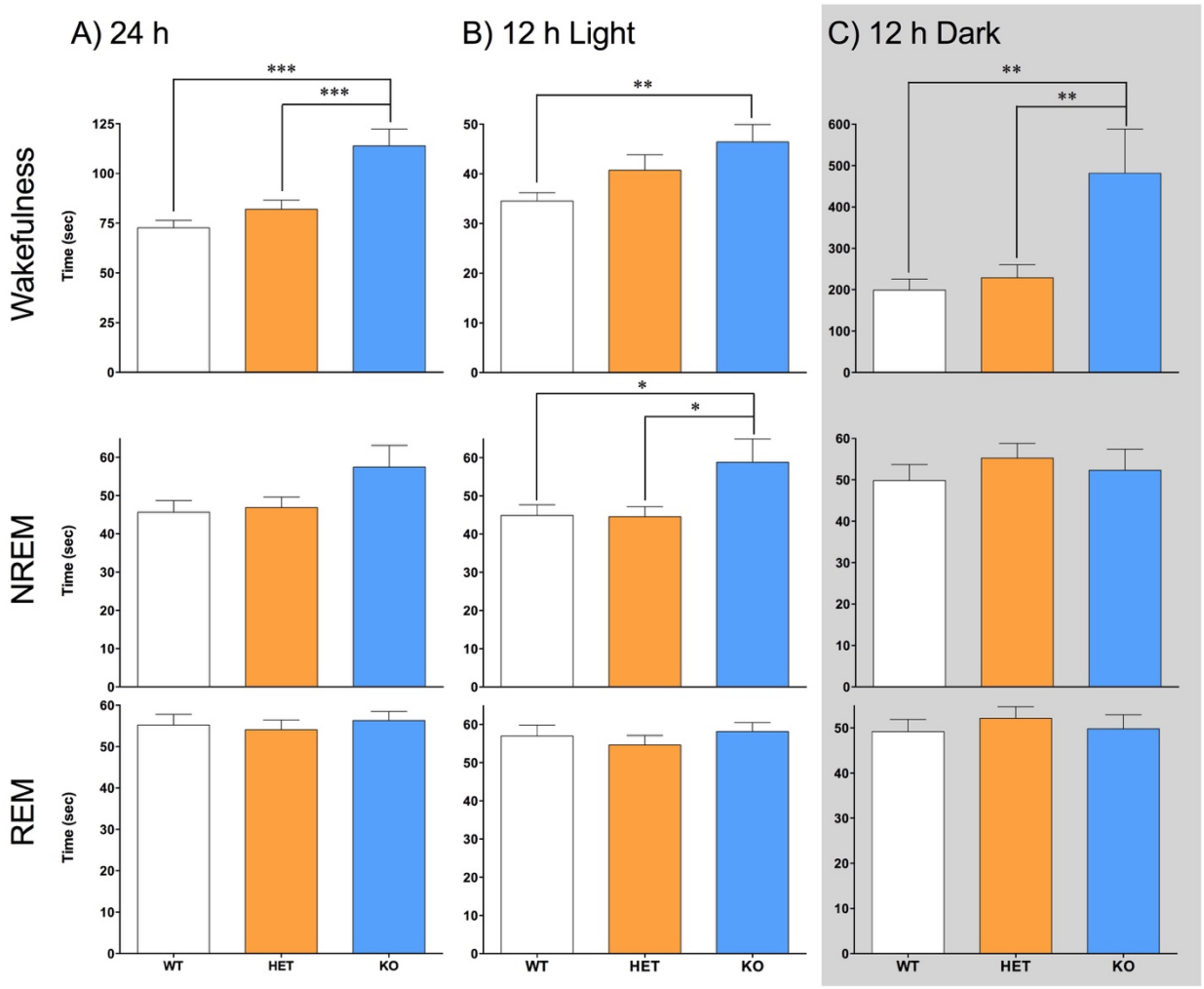

Figure 4.3—(A) Mean duration of vigilance state episodes during 24-h. *Nlgn2* KO mice showed longer mean duration of wake episodes than both HET and WT mice (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$; also for panels B and C). (B) Mean duration of vigilance state episodes during 12-h light period. While the mean duration of wake episodes of KO mice was significantly longer than WT mice, the mean duration of NREM sleep episodes of KO mice was longer than both WT and HET mice. (C) Mean duration of vigilance state episodes during 12-h dark period. KO mice showed longer mean duration of wake episodes than both HET and WT mice.

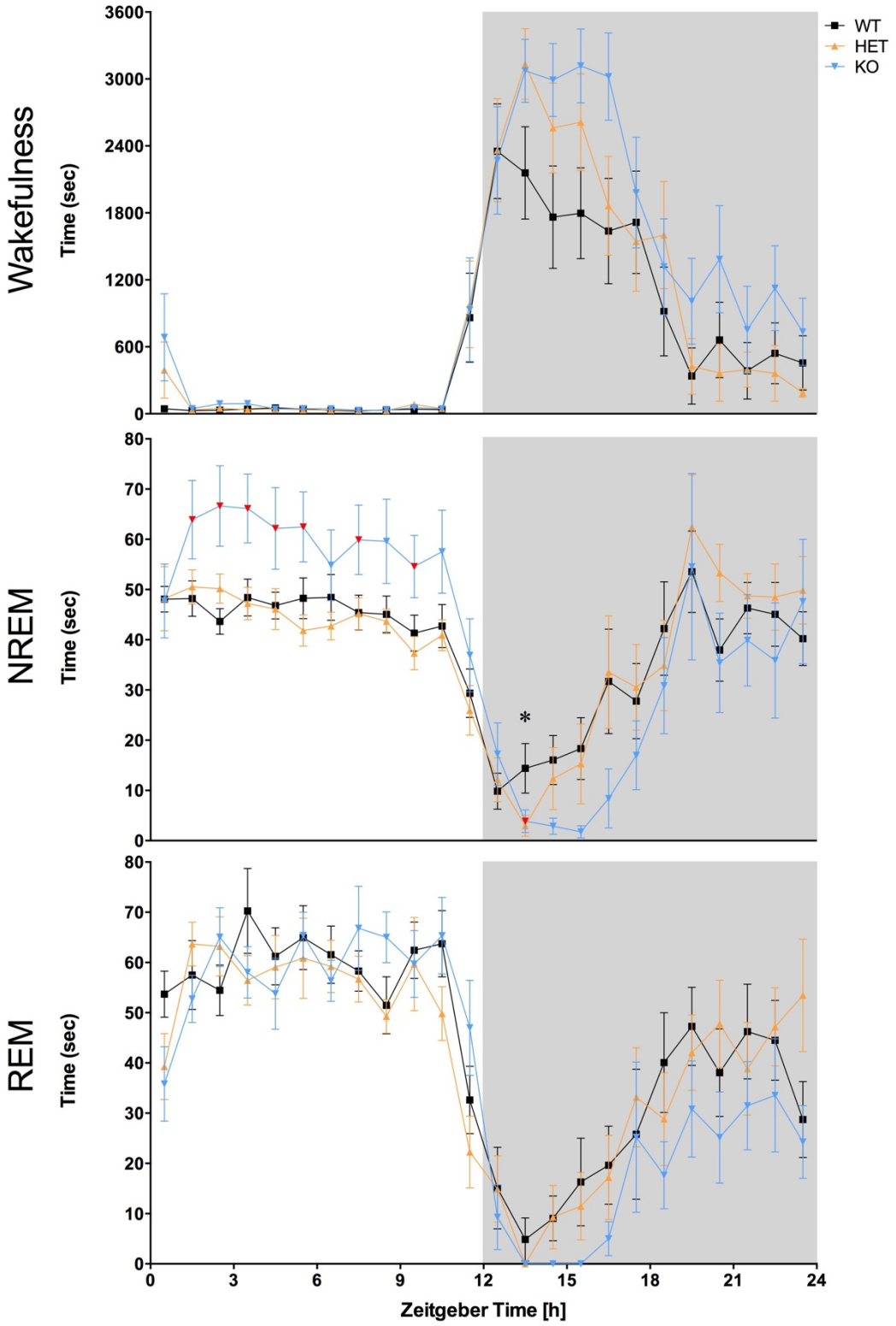

Figure 4.4—The time course of the mean duration of vigilance state episodes for the full 24-h baseline recording. Genotypes were significantly different in the mean duration of wake episodes

only, but the time course was significantly different in the mean duration of episodes of all vigilance states. These two variables, genotype and the time course, were interacting each other in NREM sleep. The significant interaction was observed between WT and KO mice at 2, 3, 4, 5, 6, 8, 10, and 14-h of the time course as represented by red symbols.

During the full 24-h, *Nlgn2* KO mice, and HET and WT littermates were significantly different in the total number of wake episodes and NREM episodes ($Fig.\ 4.5A$ [$F_{(2,37)} = 7.07, \mathrm{p} = 0.003;\ F_{(2,37)} = 7.4, \mathrm{p} = 0.002, \text{respectively}$]), but not in the total number of REM sleep episodes ($F_{(2,37)} = 1.3, \mathrm{p} = 0.3$). KO mice showed a significant decrease in the total number of wake episodes than WT and HET mice ($\mathrm{p} = 0.0007;\ \mathrm{p} = 0.014, \text{respectively}$) while WT and HET mice were not significantly different ($\mathrm{p} = 0.2$). KO mice showed a significant decrease in the total number of NREM episodes than WT and HET mice ($\mathrm{p} = 0.0006; \mathrm{p} = 0.011, \text{respectively}$), but there was no difference between WT and HET mice ($\mathrm{p} = 0.3$).

During the 12 h light period, KO mice, and HET and WT littermates were significantly different in the total number of wake and NREM sleep episodes ($Fig.\ 4.5B$ [$F_{(2,37)} = 3.6, \mathrm{p} = 0.04\ ; F_{(2,37)} = 3.9, \mathrm{p} = 0.03, \text{respectively}$]), but not in the total number of REM sleep episodes ($F_{(2,37)} = 0.4, \mathrm{p} = 0.7$). KO mice showed a significant decrease in the total number of wake episode than WT mice ($\mathrm{p} = 0.014$), but there was no significant difference between WT and HET mice or HET and KO mice ($\mathrm{p} = 0.6;\ \mathrm{p} = 0.052, \text{respectively}$). KO mice showed a significant decrease in the total number of NREM sleep episodes compared to WT and HET mice ($\mathrm{p} = 0.012;\ \mathrm{p} = 0.04, \text{respectively}$), but there was no significant difference between WT and HET mice ($\mathrm{p} = 0.6$).

During the 12 h dark period, KO mice, and HET and WT littermates were significantly different in the total number of wake, NREM and REM sleep episodes ($Fig.\ 4.5C$ [$F_{(2,37)} =$

$4.5, \mathrm{p} = 0.02;\ F_{(2,37)} = 4.5, \mathrm{p} = 0.02; F_{(2,37)} = 4.9, \mathrm{p} = 0.013$, respectively]). KO mice showed a significant decrease in the total number of wake episodes than WT mice ($\mathrm{p} = 0.005$), but there was no significant difference between WT and HET or HET and KO mice ($\mathrm{p} = 0.2;\ \mathrm{p} = 0.07$, respectively). KO mice showed a significant decrease in the total number of NREM episodes than WT mice ($\mathrm{p} = 0.005$), but there was no significant difference between WT and HET or HET and KO mice ($\mathrm{p} = 0.2;\ \mathrm{p} = 0.07$, respectively). The total number of REM sleep episodes of KO mice was significantly less than WT and HET mice ($\mathrm{p} = 0.006;\ \mathrm{p} = 0.02$, respectively), but there was no significant difference between WT and HET mice ($\mathrm{p} = 0.6$).

Change in the total number of episodes was measured over the 24-h time course. Genotypes were significantly different in the total number of wake, and NREM sleep episodes ($Fig.\ 4.6$ [$F_{(2,37)} = 7.1, \mathrm{p} = 0.003; F_{(2,37)} = 7.3, \mathrm{p} = 0.002$, respectively]), but not in the total number of REM sleep episodes ($F_{(2,37)} = 1.6, \mathrm{p} = 0.2$). The total number of wake, NREM and REM sleep episodes was significantly different over the 24-h time course ($F_{(23,851)} = 38.2, p_{H-F\ adj.} < 0.001;\ F_{(23,851)} = 39.8, p_{H-F\ adj.} < 0.001;\ F_{(23,851)} = 35, p_{H-F\ adj.} < 0.001$, respectively). However, there was no interaction between genotype and the time course ($F_{(46,851)} = 0.8, p_{H-F\ adj.} = 0.7;\ F_{(46,851)} = 0.9, p_{H-F\ adj.} = 0.7;\ F_{(46,851)} = 1, p_{H-F\ adj.} = 0.5$, respectively).

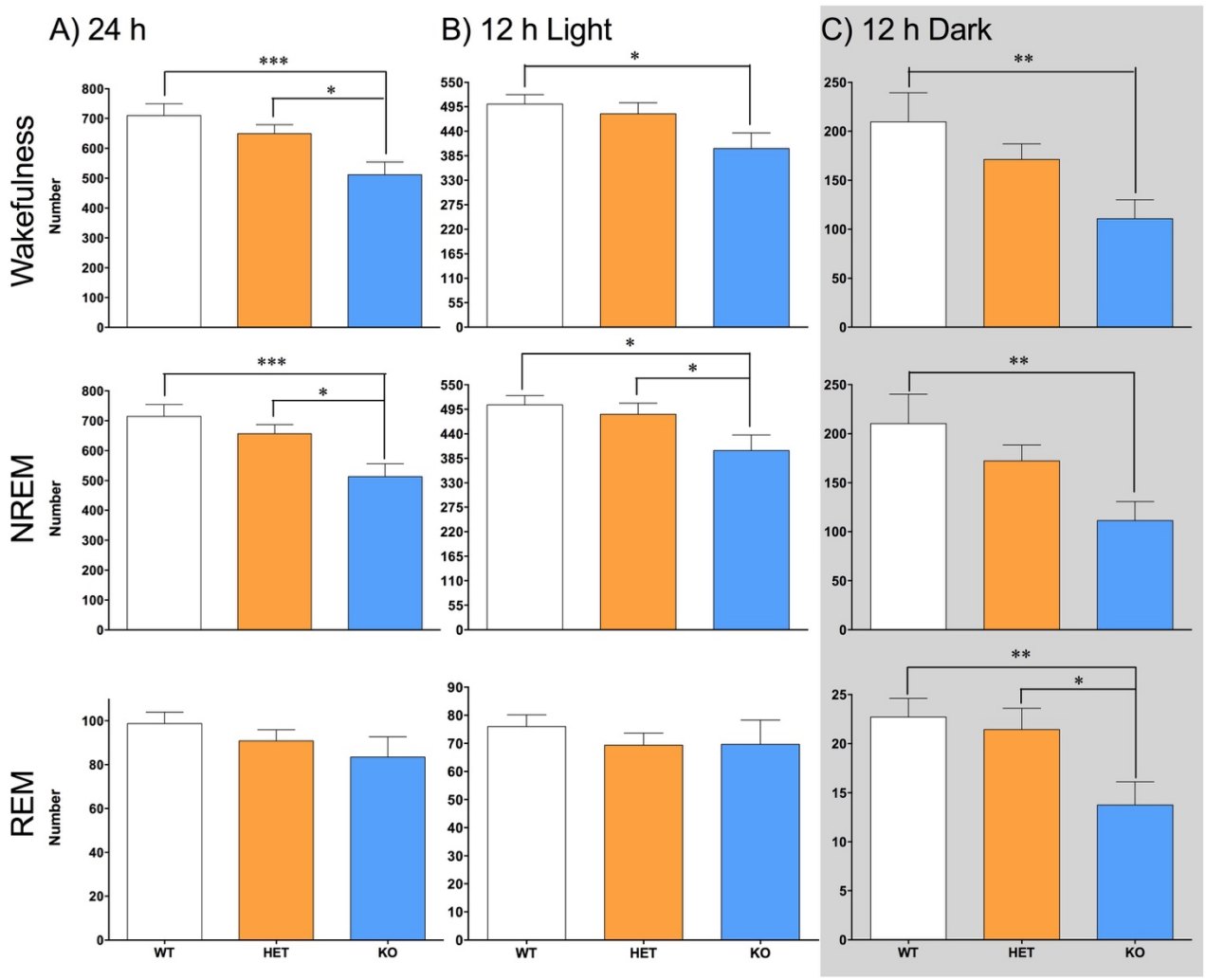

Figure 4.5—(A) Total number of vigilance state episodes during 24-h baseline recording. KO mice had significantly less total number of wake episodes and NREM sleep episodes than WT and HET mice (*:p<0.05, **:p<0.01, ***:p<0.001; also for panels B and C). (B) Total number of vigilance state episodes during 12h light period. KO mice had significantly less total number of wake episodes than WT mice and had significantly less total number of NREM sleep episodes than WT and HET mice. (C) Total number of vigilance state episodes during 12 dark period. KO mice had significantly less total number of wake episodes and NREM sleep episodes than WT mice. The total number of REM sleep episodes of KO mice was significantly less than WT and HET mice.

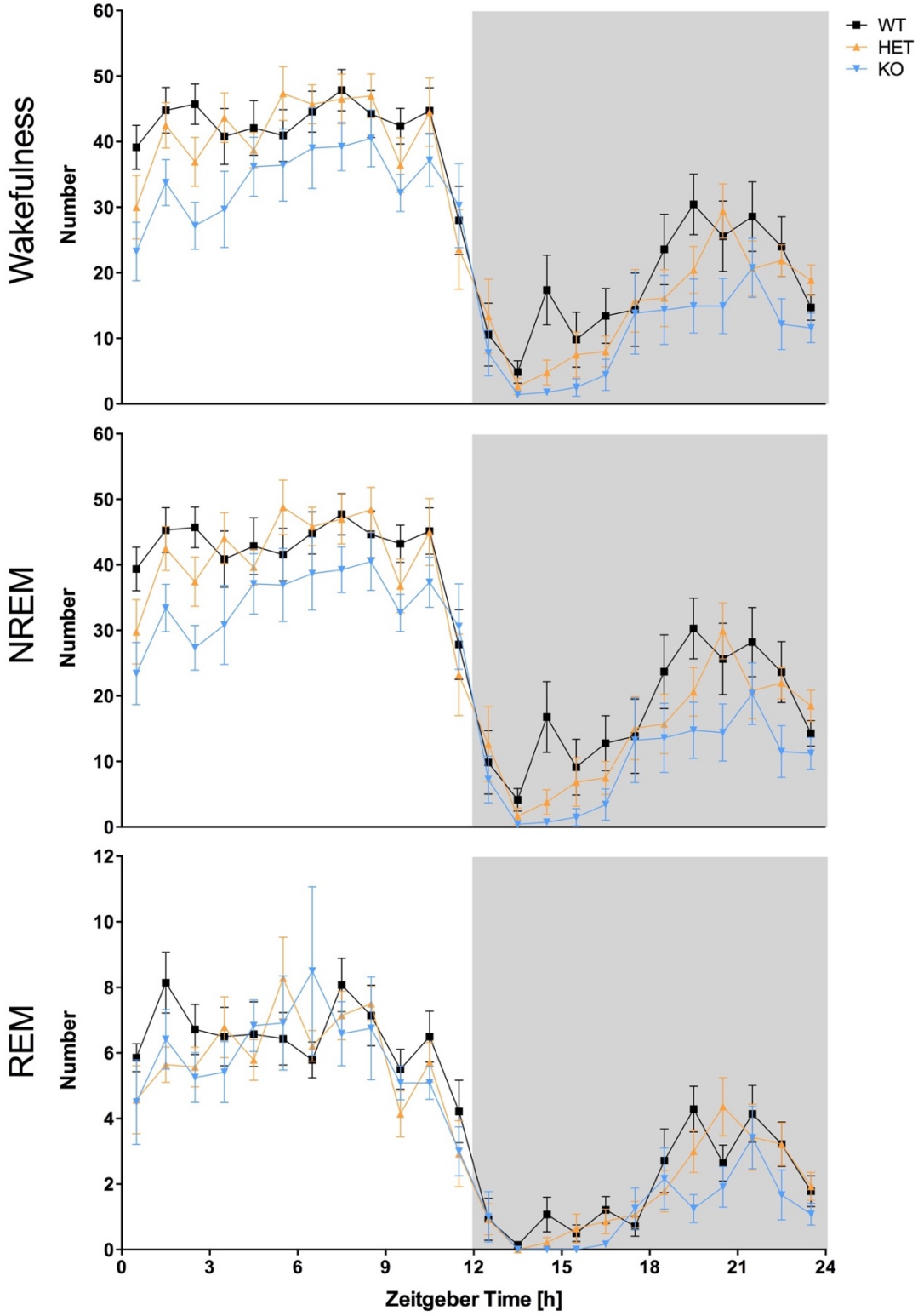

Figure 4.6—The time course of the total number of vigilance state episodes for the full 24-h baseline recording. Genotypes were significantly different in the total number of wake episodes and NREM sleep episodes, but the time course was significantly different in the total number of

all vigilance state episodes. These two variables, genotype and the time course, were not interacting with each other significantly.

While NREM sleep in *Nlgn2* KO mice was consolidated only during the 12-h light period, wakefulness was consolidated during the full 24-h, the 12-h light period, the 12-h dark period, and the 24-h time course. KO mice showed more consolidated sleep than WT mice during the 12 h light period when mice were mostly inactive.

4.2. Abnormal EEG Activity Event

Bursts of abnormal EEG activity were observed while identifying vigilance states in EEG signal (*Fig. 4.7A*). *Nlgn2* KO exhibited longer and frequent abnormal EEG event compared to HET and WT mice (*Fig. 4.7B*). Genotype effect was observed in the mean duration and number of abnormal events during the full 24-h, the 12-h light period, and the 12-h dark period (Fig. 4.8)[$Mean\ duration$: $F_{(2,20)} = 7.7, p = 0.003;\ F_{(2,20)} = 6.6, p = 0.006;\ F_{(2,20)} = 4.4, p = 0.03, respectively$]$\big[Number$: $F_{(2,37)} = 25.3, p < 0.001;\ F_{(2,37)} = 21.2, p = 0.000001;\ F_{(2,37)} = 22.8,\ p < 0.001, respectively\big]$. During the full 24-h, *Nlgn2* KO mice showed significantly more of abnormal EEG activity than WT and HET mice [$p < 0.001;\ p < 0.001, respectively$], but there was no significant difference between WT and HET mice [$p = 0.9$]. The mean duration of abnormal event was longer in KO mice compared to WT and HET mice [$p = 0.03; p = 0.0015, respectively$] whereas the duration was not significantly different between WT and HET mice. During the 12-h light period, *Nlgn2* KO mice showed significantly more of abnormal EEG activity than WT and HET mice [$p < 0.001; p < 0.001, respectively$], but there was no significant difference between WT and HET mice [$p = 0.9$]. The mean duration of abnormal event was longer in KO mice compared to WT and HET mice [$p = 0.02; p =$

$0.004, respectively$], but the duration was not significantly different between WT and HET mice [$p = 0.7$]. During the 12-h dark period, *Nlgn2* KO mice showed significantly more of abnormal EEG activity than WT and HET mice [$p < 0.000001; p < 0.000001, respectively$] whereas there was no significant difference between WT and HET mice [$p = 1$]. The duration of abnormal event was longer in KO mice compared to HET mice [$p = 0.02$], but there was no significant difference between WT and KO mice [$p = 0.06$] or WT and HET mice [$p = 1$].

Then, the occurrence of the abnormal events at different vigilance states was assessed in *Nlgn2* KO mice (Fig. 4.9). The number of abnormal events during wakefulness was significantly more than the number of abnormal events during both NREM sleep and REM sleep ($F_{(1,37)} = 69, p < 0.001;\ F_{(1,37)} = 60.4, p < 0.001, respectively$). The number of abnormal even during NREM sleep was significantly less than the number of both wakefulness and REM sleep ($F_{(1,37)} = 69, p < 0.001;\ F_{(1,37)} = 74.9, p < 0.001, respectively$). The abnormal EEG activity is frequently observed during wakefulness and REM sleep.

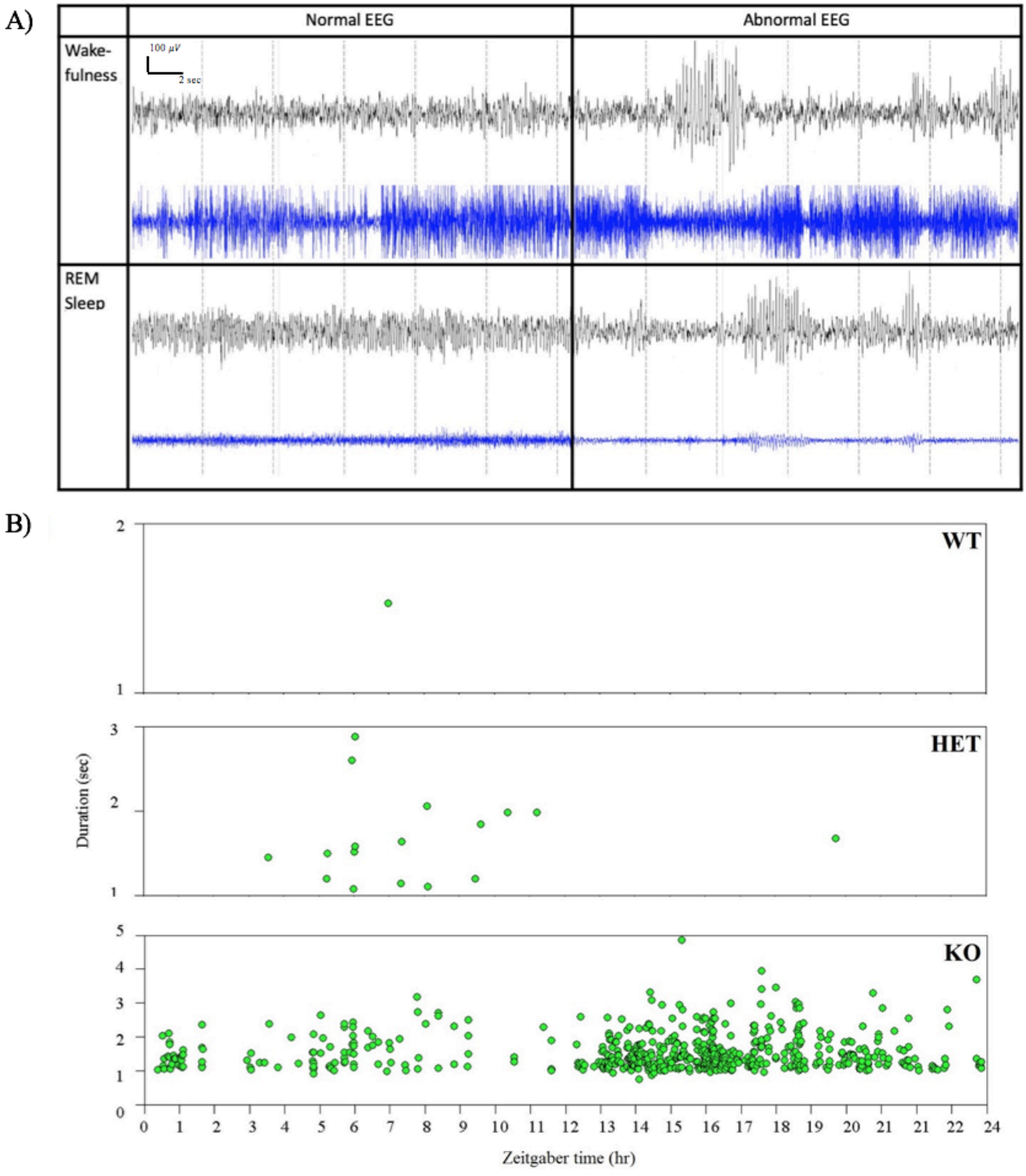

Figure 4.7 – Abnormal EEG activity. (A) Example of abnormal burst of EEG activity during wakefulness and REM sleep in KO mice in comparison to normal EEG activity in WT mice. The abnormal event was not observed during NREM sleep. (B) The abnormal events represented by green dots were frequently observed in KO mice and the range of the abnormal event duration was wider in KO mice than HET and WT mice.

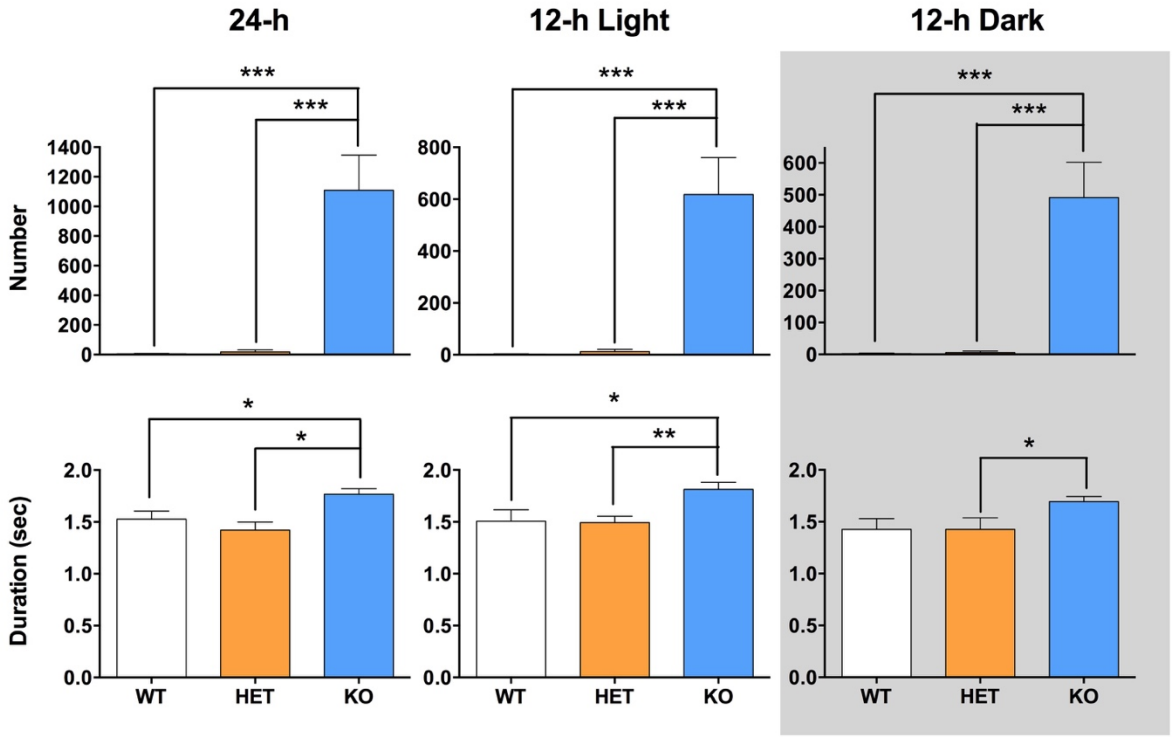

Figure 4.8 – Mean duration and average number of abnormal EEG activity during the full 24-h, the 12-h light period, and the 12-h dark period. *Nlgn2* KO mice exhibited significantly more of abnormal EEG activity than WT and HET mice during all periods. The duration of abnormal EEG activity was also significantly longer in KO mice compared to both HET and WT mice in the full 24-h and the 12-h light period. During the 12-h dark period, KO mice exhibited significantly longer duration of the abnormal events than HET mice only.

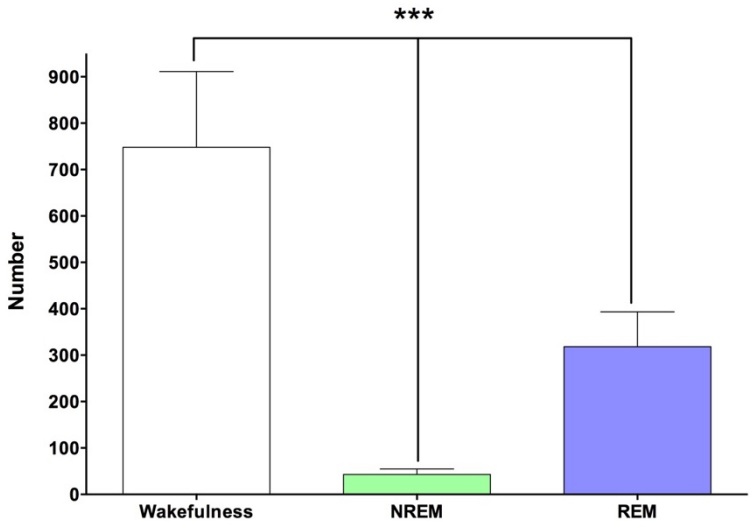

Figure 4.9 – Occurrence of the abnormal events in vigilance states of *Nlgn2* KO mice. The abnormal events were mostly occurring during wakefulness and REM sleep.

4.3. Power Spectra

Sleep architecture provides the information regarding brain state (wakefulness, NREM sleep, REM sleep), but no detailed about the brain activity. Brain activity can be better quantified by spectral analysis which decomposes a signal into its constituent frequency components. The spectral analysis was carried out on artifact-free epochs over the full 24-h in two different manners such as absolute and relative. The reason that both absolute and relative spectral analyses were carried out was to identify the involvement of secondary factors in the brain activity.

4.3.1. Spectral Power Density

Absolute power spectra revealed that *Nlgn2* KO mice, and HET and WT littermates were significantly different in power density of frequencies lower than 39, 34 and 25 in wakefulness, NREM sleep and REM sleep respectively (Fig. 4.10A). In wakefulness, KO mice showed a significant increase in power density at 1, 4, 5, 8-15, 21-23, 25, 26, 30, 34, 35 Hz compared to WT mice (refer to Table 4.1). In NREM sleep, KO mice showed a significant increase in power density at 1-33 Hz compared to WT mice (refer to Table 4.1). In REM sleep, KO mice showed a significant increase in power density at 3-5, 9-13, 16, 17-22 Hz compared to WT mice (refer to Table 4.1).

Relative power spectra normalized spectral activity of genotypes (Fig. 4.10B). In wakefulness, the *Nlgn2* KO mice showed a significant decrease in power density at 2, 3, 6, 7, 14-27, 41-49 Hz compared to the WT mice (refer to Table 4.2). In NREM sleep, the KO mice exhibited a significant increase in power density at 2-5 Hz and a significant decrease in power density at 11, 32-49 Hz compared to the WT mice (refer to Table 4.2). In REM sleep, KO mice

showed a significant increase in power density at 4 Hz and a significant decrease in power density at 6-8, 15, 20, 23-49 Hz (refer to Table 4.2).

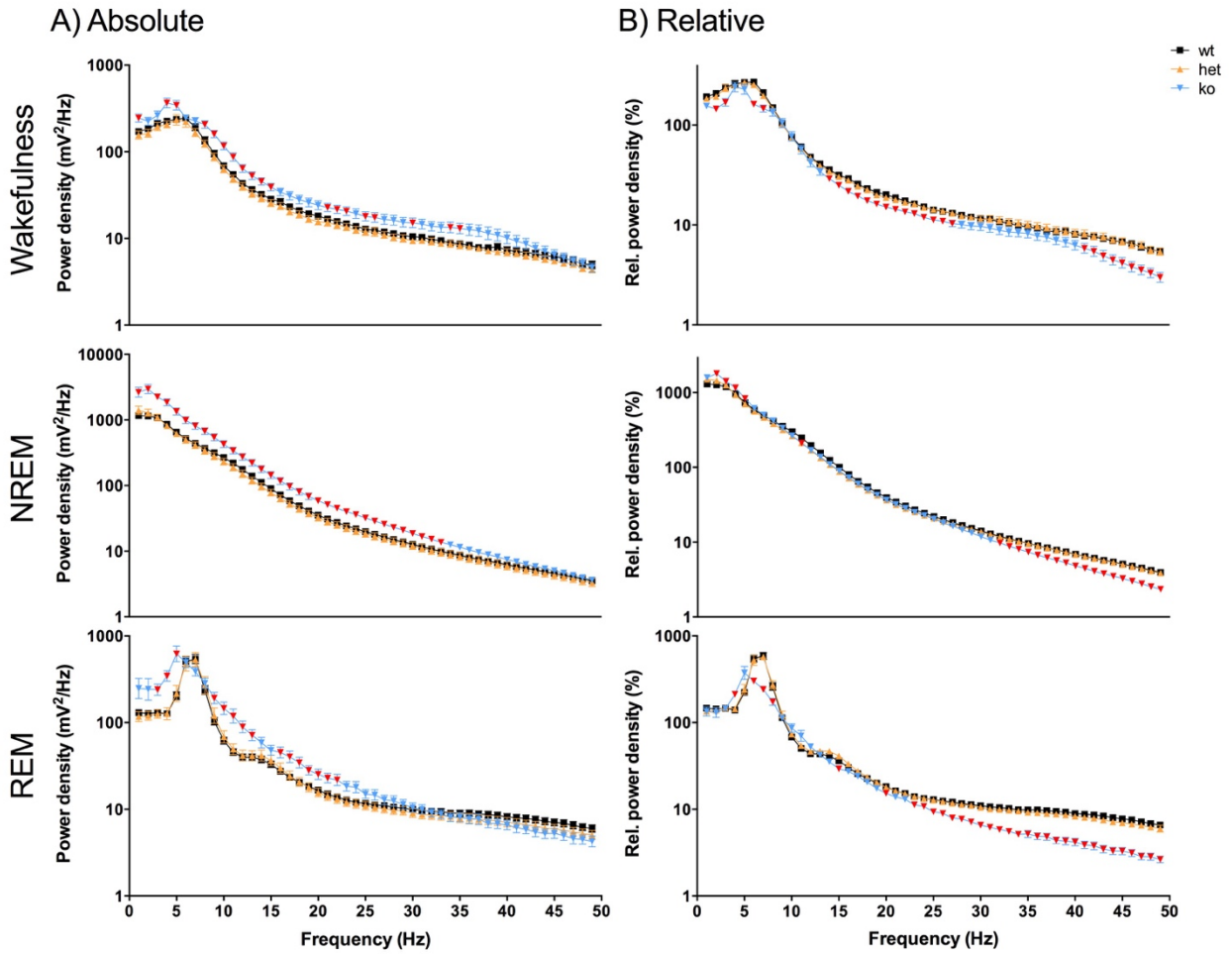

Figure 4.10—(A) Absolute spectral power between 1 and 50Hz in *Nlgn2* knockout (KO) mice and wild-type (WT) and heterozygous (HET) littermates for the three vigilance states calculated over the 24-h baseline recording. Differences between KO and WT mice are indicated by red symbols ($p < 0.05$, t-tests; same in B). In general, spectral power of KO mice was higher than WT and HET mice across most of frequencies (B) Relative spectral power between 1 and 50Hz in *Nlgn2* KO mice and littermates for the three vigilance states. In general, spectral power of KO mice at high frequency was lower than WT and HET mice.

Table 4.1 – Absolute spectral power statistical comparison between genotype. Frequency bins, that were significantly different by one-way ANOVA (marked by yellow shading), were subjected to t-test to compare the difference between WT and KO mice. Frequencies that exhibit significant difference between WT and KO mice are marked by orange shading.

Frequency bins (Hz)	Wake				NREM				REM			
	ANOVA		T-test		ANOVA		T-test		ANOVA		T-test	
	F value	P value	T value	P value	F value	P value	T value	P value	F value	P value	T value	P value
1-2	5.5	0.008	-2.4	0.02	6.7	0.003	-3.0	0.006	2.8	0.08	-	-
2-3	2.6	0.09	-	-	10.7	0.0002	-3.5	0.002	2.3	0.1	-	-
3-4	2.0	0.2	-	-	15.6	0.00002	-4.4	0.0002	7.2	0.002	-2.8	0.011
4-5	7.2	0.002	-2.6	0.02	16.3	0.00001	-4.2	0.0004	16.0	0.000012	-4.3	0.0003
5-6	3.3	0.05	-2.3	0.03	14.5	0.00003	-3.9	0.0007	7.6	0.002	-2.9	0.008
6-7	0.2	0.8	-	-	12.3	0.00009	-3.8	0.001	0.03	1.0	-	-
7-8	2.3	0.1	-	-	11.2	0.0002	-3.6	0.002	1.0	0.4	-	-
8-9	7.8	0.002	-2.8	0.01	11.0	0.0002	-3.6	0.002	0.2	0.8	-	-
9-10	11.7	0.00012	-3.6	0.002	10.0	0.0004	-3.3	0.004	3.4	0.05	-2.7	0.015
10-11	12.1	0.0001	-3.6	0.002	8.3	0.001	-2.8	0.01	8.0	0.0014	-3.2	0.004
11-12	9.5	0.0005	-3.0	0.006	7.1	0.003	-2.6	0.014	8.0	0.0014	-3.0	0.007
12-13	7.1	0.003	-2.8	0.011	7.4	0.002	-2.7	0.014	8.8	0.0008	-3.2	0.004
13-14	6.9	0.003	-2.7	0.014	8.0	0.0013	-2.7	0.012	5.6	0.008	-2.7	0.013
14-15	6.0	0.006	-2.5	0.02	7.6	0.002	-2.6	0.015	2.8	0.08	-	-
15-16	6.4	0.004	-2.5	0.02	7.9	0.0015	-2.7	0.013	2.1	0.1	-	-
16-17	3.8	0.03	-1.8	0.09	8.2	0.0012	-2.8	0.01	3.7	0.03	-2.4	0.02
17-18	4.8	0.014	-2.0	0.06	8.4	0.0011	-2.9	0.008	4.9	0.013	-2.5	0.02
18-19	4.2	0.02	-1.9	0.07	8.5	0.001003	-3.0	0.007	5.2	0.0105	-2.5	0.02
19-20	5.0	0.012	-2.0	0.06	8.9	0.0007	-3.1	0.005	4.9	0.013	-2.5	0.02
20-21	4.8	0.014	-1.9	0.07	8.9	0.0008	-3.2	0.005	4.6	0.02	-2.2	0.04
21-22	4.4	0.02	-2.1	0.05	9.1	0.0006	-3.2	0.004	5.0	0.012	-2.3	0.03
22-23	4.6	0.02	-2.1	0.04	9.8	0.0004	-3.4	0.003	4.4	0.02	-2.2	0.04
23-24	4.9	0.013	-2.2	0.04	9.0	0.0007	-3.3	0.003	3.9	0.03	-2.1	0.052
24-25	4.2	0.02	-1.9	0.07	9.7	0.0004	-3.5	0.002	3.6	0.04	-2.0	0.06
25-26	4.2	0.02	-2.1	0.05	9.0	0.0007	-3.5	0.002	2.2	0.1	-	-
26-27	3.5	0.04	-2.0	0.05	8.4	0.00104	-3.4	0.003	2.2	0.1	-	-
27-28	3.9	0.03	-1.9	0.07	7.7	0.002	-3.3	0.003	1.1	0.4	-	-
28-29	4.4	0.02	-1.9	0.07	7.0	0.003	-3.2	0.004	1.1	0.4	-	-
29-30	4.4	0.02	-2.1	0.051	6.3	0.005	-3.1	0.005	0.6	0.6	-	-
30-31	5.4	0.009	-2.1	0.05	5.5	0.008	-2.9	0.008	0.4	0.7	-	-
31-32	4.5	0.02	-2.0	0.06	4.8	0.014	-2.8	0.01	0.4	0.7	-	-
32-33	4.1	0.02	-2.0	0.06	4.6	0.02	-2.7	0.012	0.2	0.8	-	-
33-34	4.1	0.02	-1.9	0.07	3.6	0.04	-2.5	0.02	0.2	0.8	-	-
34-35	4.1	0.02	-2.1	0.05	3.2	0.053	-	-	0.3	0.8	-	-
35-36	4.5	0.02	-2.1	0.045	2.7	0.08	-	-	0.3	0.7	-	-
36-37	4.0	0.03	-2.0	0.06	2.4	0.1	-	-	0.5	0.6	-	-
37-38	4.2	0.02	-2.1	0.052	2.0	0.2	-	-	0.4	0.7	-	-
38-39	3.5	0.04	-1.7	0.1	1.7	0.2	-	-	1.0	0.4	-	-
39-40	3.2	0.053	-	-	1.5	0.2	-	-	0.8	0.4	-	-
40-41	2.5	0.1	-	-	1.1	0.3	-	-	0.8	0.5	-	-
41-42	1.9	0.2	-	-	1.1	0.4	-	-	1.1	0.3	-	-
42-43	1.7	0.2	-	-	0.8	0.5	-	-	1.7	0.2	-	-
43-44	0.9	0.4	-	-	0.7	0.5	-	-	1.8	0.2	-	-
44-45	0.7	0.5	-	-	0.5	0.6	-	-	1.9	0.2	-	-
45-46	0.6	0.5	-	-	0.4	0.7	-	-	1.4	0.3	-	-
46-47	0.6	0.6	-	-	0.3	0.7	-	-	1.8	0.2	-	-
47-48	0.2	0.9	-	-	0.2	0.8	-	-	1.9	0.2	-	-
48-49	0.4	0.7	-	-	0.2	0.8	-	-	1.9	0.2	-	-
49-50	0.3	0.7	-	-	0.1	0.9	-	-	1.8	0.2	-	-

Table 4.2 – Relative spectral power statistical data comparison between genotype. Frequency bins, that were significantly different by one-way ANOVA (marked by yellow shading), were subjected to t-test to compare the difference between WT and KO mice. Frequencies that exhibit significant difference between WT and KO mice are marked by orange shading.

Frequency bins (Hz)	Wake				NREM				REM			
	ANOVA		T-test		ANOVA		T-test		ANOVA		T-test	
	F value	P value	T value	P value	F value	P value	T value	P value	F value	P value	T value	P value
1-2	2.8	0.07	-	-	1.7	0.2	-	-	0.4	0.7	-	-
2-3	6.5	0.004	3.6	0.002	6.9	0.003	-3.8	0.001	0.3	0.7	-	-
3-4	4.8	0.014	3.1	0.006	4.0	0.03	-3.5	0.002	0.04	1.0	-	-
4-5	0.1	0.9	-	-	11.0	0.0002	-3.8	0.001	9.5	0.0005	-3.8	0.001
5-6	1.1	0.3	-	-	7.7	0.002	-2.8	0.011	3.2	0.054	-	-
6-7	12.0	0.0001	5.3	0.00002	1.7	0.2	-	-	5.6	0.008	4.3	0.0003
7-8	6.0	0.006	3.2	0.004	1.2	0.3	-	-	18.0	0.000004	5.8	0.000009
8-9	0.6	0.6	-	-	1.4	0.3	-	-	4.1	0.03	2.7	0.014
9-10	0.04	1.0	-	-	2.2	0.1	-	-	0.4	0.7	-	-
10-11	0.1	0.9	-	-	3.2	0.051	-	-	1.9	0.2	-	-
11-12	0.2	0.8	-	-	3.4	0.045	2.6	0.02	2.6	0.09	-	-
12-13	1.0	0.4	-	-	3.0	0.06	-	-	1.8	0.2	-	-
13-14	2.2	0.1	-	-	2.7	0.08	-	-	0.7	0.5	-	-
14-15	4.1	0.02	3.1	0.005	2.2	0.1	-	-	4.6	0.02	2.0	0.053
15-16	6.6	0.004	4.2	0.0004	2.3	0.1	-	-	6.5	0.004	3.3	0.003
16-17	8.7	0.0009	4.0	0.0005	1.8	0.2	-	-	2.3	0.1	-	-
17-18	8.5	0.001	5.4	0.00002	1.6	0.2	-	-	1.3	0.3	-	-
18-19	8.9	0.0007	4.7	0.0001	1.6	0.2	-	-	1.7	0.2	-	-
19-20	7.8	0.002	5.1	0.00004	1.0	0.4	-	-	2.8	0.1	-	-
20-21	7.5	0.002	5.0	0.00006	1.1	0.3	-	-	3.8	0.03	2.8	0.011
21-22	6.6	0.004	4.3	0.0003	1.1	0.3	-	-	3.0	0.06	-	-
22-23	5.7	0.007	4.0	0.0006	1.0	0.4	-	-	2.8	0.08	-	-
23-24	5.2	0.011	3.3	0.003	0.9	0.4	-	-	5.6	0.008	3.2	0.004
24-25	5.0	0.013	3.4	0.003	0.9	0.4	-	-	5.3	0.009	3.2	0.005
25-26	4.5	0.02	3.3	0.003	0.8	0.5	-	-	10.3	0.0003	5.1	0.00004
26-27	4.0	0.03	3.2	0.004	1.1	0.3	-	-	11.5	0.0001	5.4	0.00002
27-28	4.5	0.02	3.0	0.007	1.2	0.3	-	-	19.4	0.000002	7.3	<0.001
28-29	3.2	0.052	-	-	1.8	0.2	-	-	16.0	0.00001	6.0	0.000005
29-30	2.5	0.09	-	-	2.1	0.1	-	-	18.3	0.000004	6.4	0.000002
30-31	1.7	0.2	-	-	2.7	0.1	-	-	18.7	0.000003	6.8	0.000001
31-32	2.4	0.1	-	-	3.3	0.05002	-	-	24.6	<0.001	8.2	<0.001
32-33	2.4	0.1	-	-	3.6	0.04	3.1	0.0055	22.0	0.000001	7.2	<0.001
33-34	2.4	0.1	-	-	4.1	0.02	3.3	0.003	21.7	0.000001	6.9	0.000001
34-35	2.1	0.1	-	-	4.5	0.02	3.5	0.002	21.7	0.000001	7.4	<0.001
35-36	1.7	0.2	-	-	4.9	0.014	3.7	0.0012	19.5	0.000002	6.7	0.000001
36-37	1.9	0.2	-	-	5.4	0.01	3.9	0.0007	21.7	0.000001	7.4	<0.001
37-38	1.8	0.2	-	-	5.8	0.006	4.1	0.0005	21.0	0.000001	7.0	0.000001
38-39	2.1	0.1	-	-	6.1	0.0052	4.3	0.0003	23.8	<0.001	7.8	<0.001
39-40	2.7	0.1	-	-	6.5	0.004	4.4	0.0002	23.3	<0.001	7.3	<0.001
40-41	3.0	0.06	-	-	7.5	0.002	4.7	0.0001	21.9	0.000001	7.4	<0.001
41-42	3.9	0.03	2.3	0.03	7.7	0.002	4.9	0.00007	24.7	<0.001	7.7	<0.001
42-43	4.2	0.02	2.6	0.015	8.3	0.0011	5.1	0.00004	22.7	<0.001	7.5	<0.001
43-44	5.9	0.01	3.0	0.007	8.4	0.0011	5.0	0.00005	25.5	<0.001	8.4	<0.001
44-45	7.2	0.002	3.4	0.003	9.2	0.0006	5.4	0.00002	24.0	<0.001	8.2	<0.001
45-46	7.9	0.0014	3.8	0.0011	9.5	0.0005	5.5	0.00001	22.1	0.000001	8.0	<0.001
46-47	9.0	0.0007	4.0	0.0007	9.8	0.0004	5.7	0.00001	23.1	<0.001	8.4	<0.001
47-48	9.8	0.0004	4.0	0.0007	10.7	0.0002	5.9	0.000006	23.7	<0.001	8.3	<0.001
48-49	9.4	0.0005	4.2	0.0004	11.6	0.0001	6.3	0.000003	24.2	<0.001	8.6	<0.001
49-50	10.9	0.0002	4.4	0.0002	12.2	0.0001	6.4	0.000002	23.1	<0.001	8.6	<0.001

4.3.2. Delta Power

Delta activity has often been used as a marker for sleep pressure and sleep intensity (Borbély, 1982; Pappenheimer, et al., 1975); moreover, delta activity during wakefulness is correlated with the attention to internal processing (Harmony, et al., 1996). We examined the interaction between genotype and the time intervals of delta activity in wakefulness and NREM sleep.

In absolute delta activity, both genotype and the time intervals were significantly different in power density during wakefulness and NREM sleep ($Fig.\ 4.11A$ [$F_{(2,20)} = 7.3, \mathrm{p} = 0.004$; $F_{(17,340)} = 5.7, p_{H-F\ Adj.} < 0.001$; $F_{(2,35)} = 10.5, \mathrm{p} = 0.0003$; $F_{(17,595)} = 36$, $p_{H-F\ Adj.} < 0.001$, respectively]). However, the interaction between genotype and the time interval was only observed in wakefulness ($F_{(34,340)} = 2.5$, $p_{H-F\ adj.} = 0.002$), but not in NREM sleep ($F_{(34,595)} = 2.1$, $p_{H-F\ adj.} = 0.07$). The time interval was significantly different between genotypes with higher delta power in KO mice compared to WT mice at intervals 1-8, 11, 12 and 16 ($\mathrm{p} = 0.01$; $\mathrm{p} = 0.02$; $\mathrm{p} = 0.02$; $\mathrm{p} = 0.02$; $\mathrm{p} = 0.009$; $\mathrm{p} = 0.015$; $\mathrm{p} = 0.03$; $\mathrm{p} = 0.04$; $\mathrm{p} = 0.05$; $\mathrm{p} = 0.03$; $\mathrm{p} = 0.03$ respectively).

In relative delta activity, genotype was significantly different in NREM sleep only (Fig. 11B [$F_{(2,35)} = 3.3, \mathrm{p} = 0.05$]), not in wakefulness ($F_{(2,20)} = 1.4, \mathrm{p} = 0.3$). The time interval was significantly different in delta power during wakefulness and NREM sleep ($F_{(17,340)} = 7.3, p_{H-F\ Adj.} < 0.001$; $F_{(17,340)} = 80, p_{H-F\ Adj.} < 0.001$, respectively). However, these two independent variables, genotype and the time interval, exhibited interaction in both wakefulness and NREM sleep ($F_{(34,340)} = 1.9$, $p_{H-F\ adj.} = 0.007$; $F_{(34,595)} = 3.0$, $p_{H-F\ adj.} = 0.0002$, respectively). In wakefulness, the time interval was significantly different between genotypes with higher delta power at 1, 2, and 5 of the time intervals ($\mathrm{p} = 0.05$; $\mathrm{p} = 0.03$; $\mathrm{p} = 0.03$) and

lower delta power at 18 of the time intervals ($p = 0.02$) in KO mice compared to WT mice. In NREM sleep, the time interval was significantly different between genotypes with higher delta power at 2, 3, 4, and 8 of the time intervals ($p = 0.003$; $p = 0.0009$; $p = 0.0001$; $p = 0.004$, respectively) and lower delta power at 13 of the time intervals ($p = 0.05$).

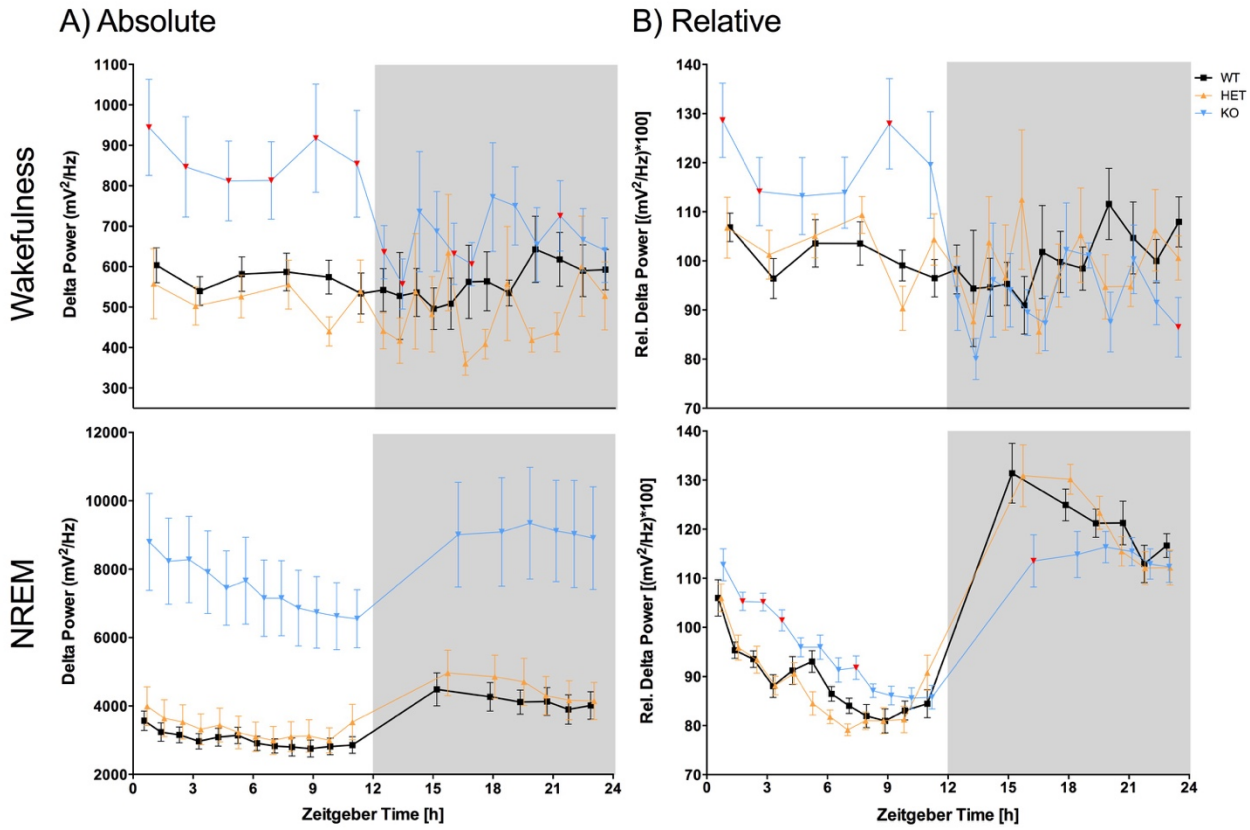

Figure 4.11—(A) 24-h dynamics of absolute delta (1-4Hz) activity in *Nlgn2* KO mice, and HET and WT littermates during wakefulness and NREM sleep. Significant genotype by interval interaction was found in both wakefulness and NREM sleep. Differences between KO and WT mice are indicated by red symbols [same in B]. In general, KO mice exhibited higher delta power at most of intervals compared to WT and HET mice during both wakefulness and NREM sleep (B) 24-h dynamics of relative delta activity in *Nlgn2* KO mice, and HET and WT littermates during wakefulness and NREM sleep. Significant genotype by interval interaction was found in both wakefulness and NREM sleep. During the 12-h light period, KO mice showed higher delta power compared to WT and HET mice in both wakefulness and NREM sleep. During the 12-h dark period. KO mice exhibited lower delta power in general compared to WT and HET mice in both wakefulness and NREM sleep.

4.3.3. Sigma power

Sigma activity has often used as a marker for memory consolidation and sleep continuity in the presence of disturbing auditory stimulus (Gais, Molle, Helms, & Born, 2002; Meier-Koll, et al., 1999). We examined the interaction between genotype and the time interval of sigma activity in NREM sleep.

In absolute sigma activity, both genotype and the time interval were significantly different in spectral activity ($Fig. 4.12A$ [$F_{(2,35)} = 8.1, \mathrm{p} = 0.0013;\ F_{(17,595)} = 3.9, p_{H-F\ Adj.} = 0.002$, respectively]). Moreover, a significant interaction between genotype and time interval was observed ($F_{(34,595)} = 2.1, p_{H-F\ Adj.} = 0.03$). The time interval was significantly different between genotypes with higher sigma power in KO mice compared to WT mice at all intervals (p=0.003; p=0.007; p=0.005; p=0.013; p=0.011; p=0.009; p=0.02; p=0.02; p=0.02; p=0.02; p=0.011, p=0.008; p=0.001; p=0.002; p=0.003; p=0.004; p=0.004; p=0.003, In order of interval on graph).

In relative sigma activity (Fig. 4.12B), genotype was not significantly different ($F_{(2,35)} = 1.8, \mathrm{p} = 0.2$), but the time interval was significantly different in spectral activity ($F_{(17,595)} = 5.8, p_{H-F\ Adj.} < 0.001$, respectively). The interaction between genotype and time interval was not significant ($F_{(34,595)} = 2.1, p_{H-F\ Adj.} = 0.06$). This indicated that NLGN2 absence does not significantly change the dynamics of sigma activity.

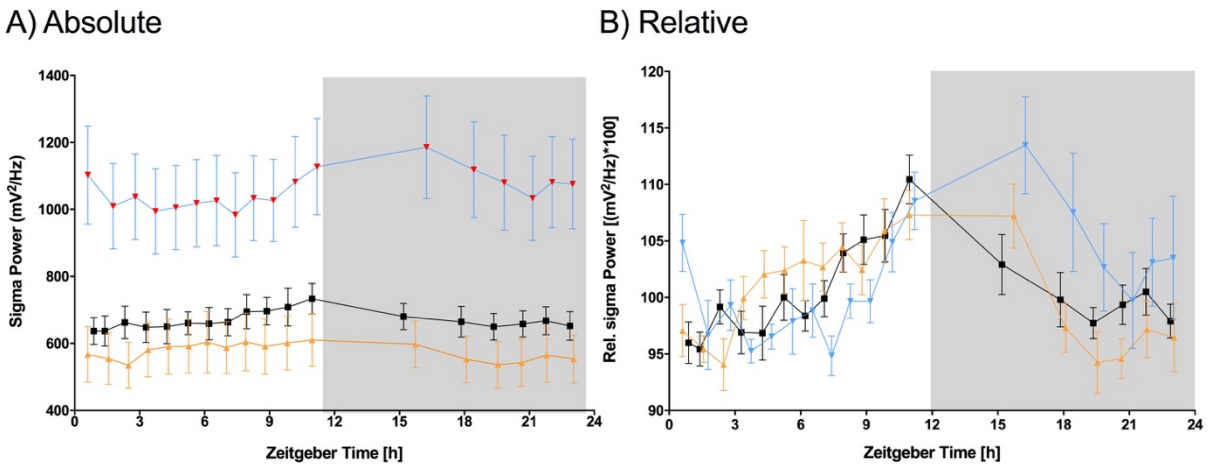

Figure 4.12 —(A) 24-h dynamics of absolute sigma (10-13Hz) activity in *Nlgn2* KO mice, and HET and WT littermates during NREM sleep. Significant genotype by interval interaction was found in NREM sleep. Differences between KO and WT mice are indicated by red symbols (same in B). In general, KO mice exhibited higher sigma power across all intervals compared to WT and HET mice. (B) 24-h dynamics of relative delta activity in *Nlgn2* KO mice, and HET and WT littermates during NREM sleep. Significant genotype by interval interaction was not found for NREM sleep.

Chapter 5: Discussion

5.1. Summary of the Results

5.1.1. Sleep Architecture

The thesis has demonstrated a role of NLGN2 in the regulation of sleep quality and quantity by assessing the effect of NLGN2 absence in mice. *Nlgn2* KO mice showed a significant increase in wakefulness and a significant decrease in both NREM and REM sleep during the total 24-h. These changes were attributable to the alteration in the duration of vigilance states during the 12-h dark period. Moreover, the KO mice exhibited a consolidation in NREM sleep during the 12-h light period and wakefulness during the full 24-h, the 12-h light period, and the 12-h light period.

The consolidation of vigilance states can partly explain the change in the duration of vigilance states. During the 12-h dark period, KO mice exhibited consolidated wakefulness while the total number of NREM and REM sleep episodes was decreased. The decreased total number of sleep episodes could result in the reduced proportion of sleep during the 12-h dark period and lead to the increase in the duration of wakefulness. During the 12-h light period, KO mice exhibited consolidated wakefulness and NREM sleep without changing the duration of vigilance states. This indicated that consolidation rearranged the occurrence of vigilance state episodes. During the full 24-h, KO mice exhibited the consolidated wakefulness and the reduced total number of NREM sleep episodes. The decreased total number of NREM sleep episodes would cause a decrease in the duration of NREM sleep which provides longer time to be awake. Interestingly, the duration of REM sleep of KO mice over 24-h was decreased even though there was no significant change in the mean duration or the total number of REM sleep episodes. However, the insignificant reduction in the total number of REM sleep episodes of KO mice during the full 24-h could have resulted from the significant decrease in the total number of REM

sleep episodes during the 12-h dark period and caused the significant reduction in the duration of REM sleep over 24-h.

This observation concurred with the hypothesis on sleep architecture in the thesis. The hypothesis has postulated that *Nlgn2* KO mice would show increased wakefulness and decreased sleep due to weakened sleep-inducing regions.

5.1.2. Abnormal EEG Activity

Nlgn2 KO mice exhibited abnormal burst of EEG activity which lasted 1~5 sec and the amplitude of the event was at least twice larger than the background level. The abnormal events were most frequently observed during wakefulness and least frequently observed during NREM sleep. Similar abnormal EEG activity was observed from an animal model of cortical dysplasia, neuronal subset (NS*)-Pten* mutant mice [Fig. 5.1] (Ljungberg, et al., 2009). *Pten* (phosphatase and tensin homolog on chromosome ten) gene is a tumor suppressor gene that has been suggested to regulate cell growth (Ljungberg, et al., 2009); moreover, specific deletion of *Pten* gene in brain neurons have caused ataxia and seizure (Backman, et al., 2001). (NS*)-Pten* mutant mice exhibited short trains of repetitive spikes lasting less than 5 seconds during wakefulness which was considered as an epileptiform type of activity (Ljungberg, et al., 2009); therefore, the abnormal bursts of EEG activity in *Nlgn2* KO mice can also be considered as an epileptiform type of activity.

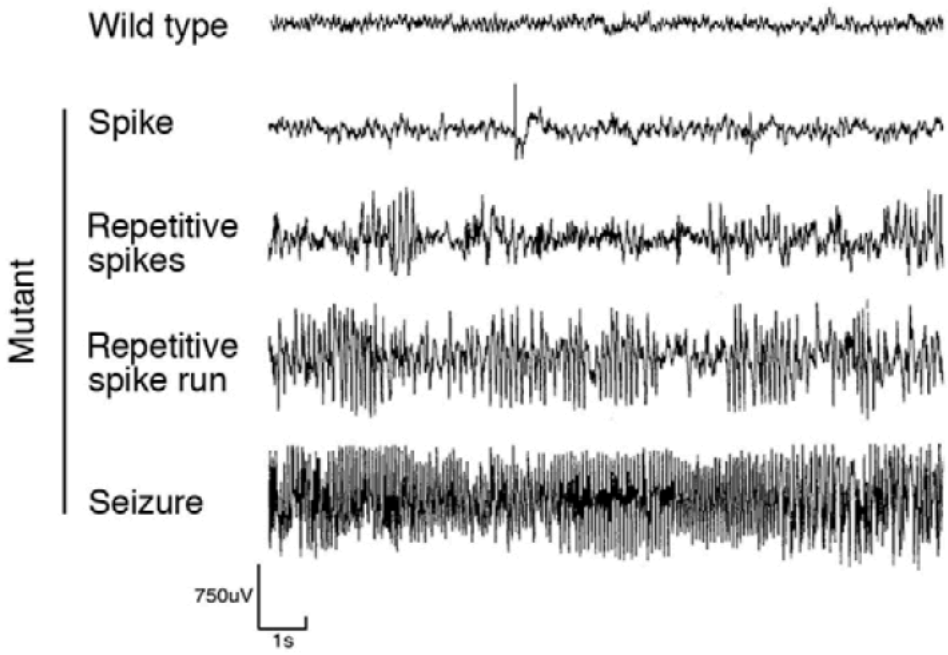

Figure 5.1 – The types of abnormal epileptiform activity that were observed from *NS-Pten* mutant mice during wakefulness. Short trains of repetitive spikes lasting less than 5 seconds were resembled to the abnormal EEG activity that was observed from *Nlgn2* KO mice. The figure has been taken from Ljungberg et al 2009.

5.1.3. Wakefulness and Sleep Quality

EEG activity was also altered in *Nlgn2* KO mice during all vigilance states. The absolute power spectra revealed a general increase in spectral activities of the KO mice compared to the WT mice during wakefulness, NREM sleep, and REM sleep. On the other hand, the relative power spectra revealed a general decrease in spectral activities of the KO mice compared to the WT mice during wakefulness and REM sleep. During NREM sleep, the relative spectral activities were increased at low frequency (<6 Hz) and decreased at fast frequency (>32 Hz) in the KO mice compared to the WT mice. The difference between the absolute power spectra and the relative power spectra could be due to bad electrode implantation surgery or changes in cortical structure of the KO mice. Dr. Mongrain's lab has been performing the surgery numerous

times, so it is unlikely that the difference was resulted from the surgery. Since the absolute EEG activities were affected by confounding factors, the relative EEG activities will be specifically described in delta and sigma power.

The time course of relative delta power was modified during wakefulness and NREM sleep. During wakefulness, the KO mice exhibited high delta power through the 12-h light period and lower delta power through the 12-h dark period. During NREM sleep, the KO mice showed higher delta power which dissipated at slower rate through the 12-h light period and lower delta power which accumulated at slower rate through the 12-h dark period. The time course of relative sigma power was not modified during NREM sleep.

This observation fits well with the hypothesis on EEG activity. The hypothesis has postulated that *Nlgn2* KO mice would exhibit increased EEG activity at low frequency (< 10 Hz) comprising delta activity and decreased EEG power at high frequency (> 10 Hz) comprising sigma activity during NREM sleep. because *Nlgn2* KO mice show decreased inhibitory transmission and opposite effects of $GABA_AR$ agonist are expected. According to the relative spectra power, the KO mice exhibited increased delta activity and decreased theta activity during NREM sleep.

5.2. Effects of NLGN2 on Inhibitory Transmission

Genetically modified animal models have established the inverse relationship between the level of NLGN2 and the E/I ratio (Liang, et al., 2015; Hines, Wu, Hines, & et al, 2008); however, the underlying mechanisms of NLGN2 modulation on inhibitory transmission are not well understood.

5.2.1. Subcellular Localization of GABA Receptors by NLGN2

$GABA_A$ receptors ($GABA_ARs$) are diffusely distributed or clustered at synaptic or extrasynaptic sites on neuronal cell surface (Jacob, Moss, & Jurd, 2008) by trapping laterally diffusing $GABA_ARs$ within plasma membrane (Jacob, et al., 2005; Thomas, et al., 2005; Bogdanov, et al., 2006). NLGN2 has been suggested to drive postsynaptic assembly of $GABA_ARs$ at perisomatic inhibitory synapses (Poulopoulos, et al., 2009; Fritschy, Panzanelli, & Tyagarajan, 2012).

NLGN2 interacts with the inhibitory scaffolding protein gephyrin to activates collybistin (Kins, Betz, & Kirsch, 2000; Poulopoulos, et al., 2009). The activated collybistin tethers more of gephyrins to the postsynaptic membrane which recruits both glycine and $GABA_ARs$ containing α2/γ2-subunits at synaptic sites (Jacob, Moss, & Jurd, 2008; Poulopoulos, et al., 2009). The NLGN2/gephyrin/collybistin complex was sufficient to induce the synaptic accumulation of $GABA_ARs$ (Poulopoulos, et al., 2009). In the absence of either NLGN2 or collybistin, the accumulation of gephyrins and $GABA_ARs$ containing α2/γ2-subunits at inhibitory synapses have been disrupted (Poulopoulos 2009). However, the postsynaptic localization of NLGN2 was not altered in the absence of collybistin, gephyrin, or receptors (Poulopoulos 2009). This suggested the role of NLGN2 in the initiation of postsynaptic differentiation at inhibitory synapses (Poulopoulos 2009).

NLGN2 also interact with the dystrophin-glycoprotein complex (DGC) via synaptic scaffolding molecule (S-SCAM) (Fritschy, Panzanelli, & Tyagarajan, 2012). In the absence of dystrophin, the accumulation of $GABA_ARs$ containing α1/α2-subunits at synaptic sites was partially disrupted without affecting the gephyrin accumulation (Kneussel, et al., 1999). The NLGN2/DGC/S-SCAM complex has been suggested to induce the accumulation of gephyrin-

independent $GABA_ARs$ containing α1-subunits at synaptic sites (Fritschy, Panzanelli, & Tyagarajan, 2012).

In the absence of NLGN2, synaptic clustering of gephyrin and $GABA_ARs$ at inhibitory synapses has been disrupted (Poulopoulos, et al., 2009). The disruption may influence the subcellular localization of other types of GABARs such as extrasynaptic $GABA_ARs$, $GABA_BRs$, and $GABA_CRs$. The γ2-subunit of $GABA_AR$ which is required for the synaptic clustering of $GABA_ARs$ (Essrich, et al., 1998) can induce internalization of $GABA_BR$s (Balasubramanian, et al., 2004); therefore, the interaction between the γ2-subunit and $GABA_BR$ could be the mechanism that prevents the expression of $GABA_BR$s at synaptic sites. Since $GABA_AR$ γ2 subunit puncta were reduced at perisomatic inhibitory synapses in the absence of NLGN2 (Poulopoulos, et al., 2009), the interaction between the γ2-subunit and $GABA_BR$ may no longer consider as the mechanism that prevent the $GABA_BR$ expression at synaptic sites. It could be speculated that the subcellular localization of GABAR subtypes might be lost in the absence of NLGN2 and that could lead to increase expression of extrasynaptic $GABA_ARs$, $GABA_BRs$, and $GABA_CRs$ and decrease expression of synaptic $GABA_ARs$ at synaptic sites.

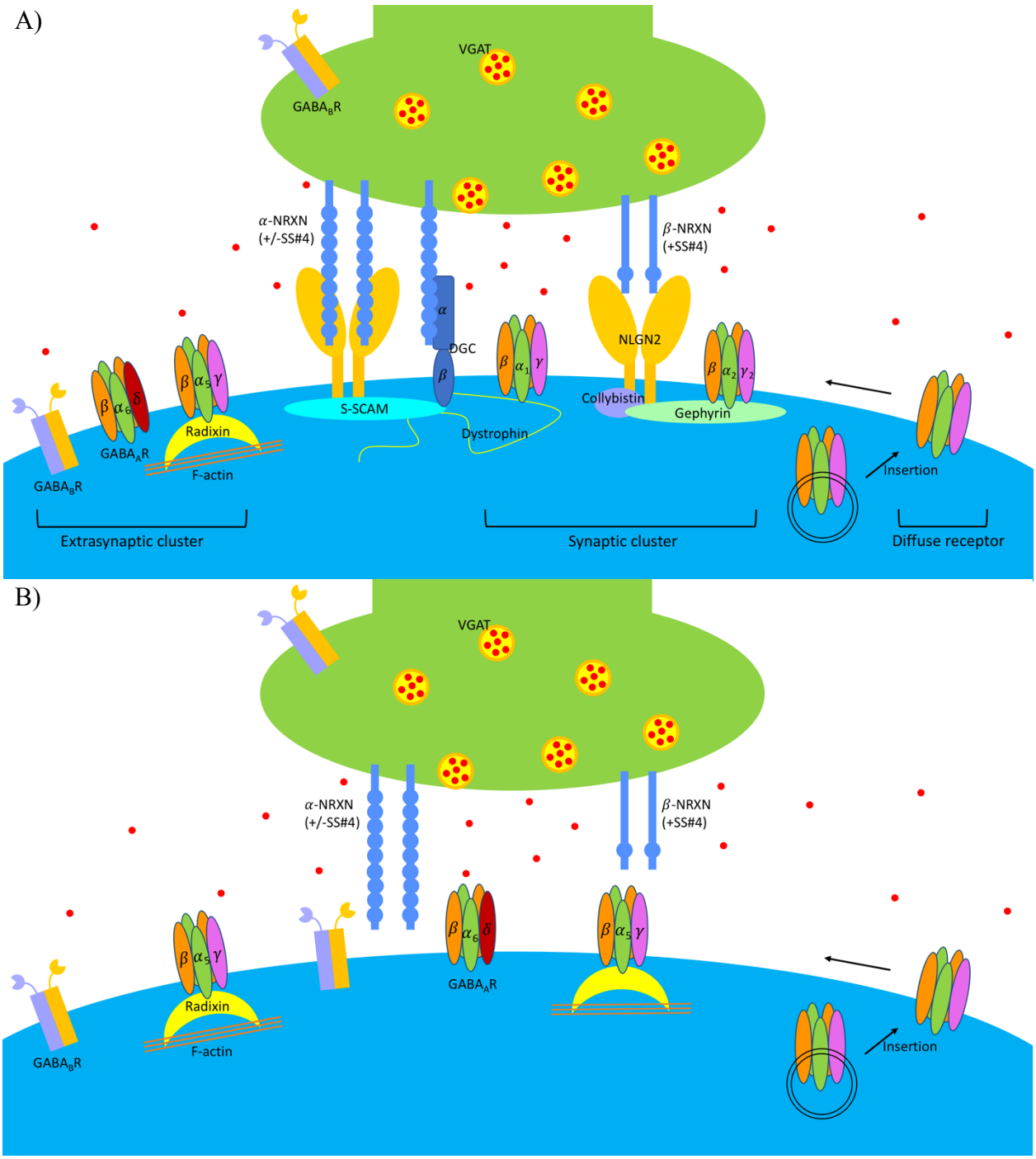

Figure 5.2 – Summary diagram of the proposal. (A) Under normal conditions, GABARs are inserted at extrasynaptic site. The GABARs are diffusely distributed or clustered at synaptic or extrasynaptic sites by trapping laterally diffusing GABARs. NLGN2 has been suggested to drive postsynaptic assembly of $GABA_AR$s containing α2/γ2 or α1/α2 at perisomatic inhibitory synapses by interacting with gephyrin/collybistin or DGC/S-SCAM complexes respectively. (B) In the absence of NLGN2, postsynaptic cluster of gephyrin/collybistin complex is disrupted, possibly DGC/S-SCAM complex as well. Therefore, synaptic $GABA_AR$s would diffuse out of the synaptic site and evenly distributed throughout the neuron. Then, extrasynaptic $GABA_AR$s

containing δ-subunit, $GABA_B$Rs, and $GABA_C$Rs would move and settle into the less compacted postsynaptic sites. It could be possible that NLGN2 regulates the subcellular localization of GABARs. This figure is influenced by one of figures from Bang, et al., 2013. DGC: Dystrophin-glycoprotein complex; S-SCAM: Synaptic scaffolding molecule; VGAT: Vesicuular gamma-aminobutyric acid transporter; NLGN: Neuroligin; NRXN: Neurexin; GABAR: Gamma-aminobutyric acid receptor.

5.2.2. *Effects of NLGN2 Absence in the Electrophysiology of Neurons*

Nlgn2 overexpressing mice showed a decrease in E/I ratio due to increased frequency of mIPSC (Hines, Wu, Hines, & et al, 2008). On the other hand, *Nlgn2* KO mice exhibited an increase in E/I ratio due to decreased amplitude and frequency of mIPSC (Liang, et al., 2015). However, mEPSC was not changed in both *Nlgn2* overexpressing and *Nlgn2* KO mice (Hines, et al, 2008; Hines, Wu, Hines, & et al, 2008). These changes could be attributable to the impaired subcellular localization of GABAR subtypes as it has been speculated in the thesis.

In the absence of NLGN2, extrasynaptic $GABA_ARs$, $GABA_BRs$, and $GABA_CRs$ might diffuse into synaptic sites in compensation for the loss of synaptic $GABA_ARs$ as it has been speculated in the thesis. Since synaptic $GABA_ARs$ containing γ-subunits (Sieghart, et al., 1999) mediate a transient phasic inhibition which is referred to inhibitory postsynaptic current (IPSC) (Mody, et al, 1994; Maconochie, et al, 1994), the down-regulation of synaptic $GABA_ARs$ would lower the amplitude of mIPSC. α-NRXN is a presynaptic cell adhesion molecule that interacts with NLGN2 (Dalva, McClelland, & Kayser, 2007; Bang & Owczarek, 2013). α-NRXN has been suggested to regulate presynaptic calcium channel conductivity without altering the expression level of N- and P/Q- type calcium channels (Missler, et al., 2003). In the absence of NLGN2, the expression of α-NRXN would be decreased in the presynaptic site. Reduced α-NRXN level would decrease the presynaptic calcium current and lower the frequency of mIPSC

(Missler, et al., 2003). Moreover, the disruption of postsynaptic clustering of $GABA_A$Rs in the absence of NLGN2 would increase silent inhibitory synapses and then lower the frequency of mIPSCs.

In the overexpression of NLGN2, there would be an increase in the interaction between α-NRXN and NLGN2. Increased level of NLGN2 does not necessary increase the overall level of α2/γ2-subunits because gephyrin-deficient mice was disrupted in synaptic clustering of $GABA_ARs$ containing α2/γ2-subunits without altering the overall level of these subunits (Kneussel, et al., 1999). Therefore, there would be no change in the expression level of synaptic $GABA_ARs$ and the amplitude of mIPSC would stay the same. The increased level of NLGN2 would promote the interaction with α-NRXNs. The increased level of α-NRXNs would increase the presynaptic calcium current (Missler, et al., 2003) and/or promotes presynaptic recruitment of synaptic vesicles (Craig, Graf, & Linhoff, 2006) to increase the frequency of mIPSC.

In both *Nlgn2* KO and overexpressing mice, there was no change in mEPSC. mEPSC can be regulated by extrasynaptic $GABA_ARs$ containing α5- and δ-subunits (Fritschy, et al., 1998; Brünig, et al, 2002; Sun, Sieghart, & Kapur, 2004; Mangan, et al., 2005) which mediate a persistent tonic inhibition (Semyanov, et al., 2004; Cavelier, et al., 2005). Extrasynaptic $GABA_ARs$ regulates the capability of excitatory inputs to generate action potentials (Pouille & Scanziani, 2001; Hamann, Rossi, & Attwell, 2002) by increasing membrane conductance and decreasing spatial and temporal integration of excitatory signal (Semyanow 2004, Cavelier 2005, pouille 2001); therefore, no change in the expression of extrasynaptic $GABA_ARs$ containing α5- and δ-subunits would be expected in both *Nlgn2* KO and overexpressing mice.

5.3. NLGN2 and Sleep

The role of NLGN2 in the regulation of inhibitory transmission has made it possible to hypothesize the involvement of NLGN2 in sleep regulation because a number of studies have indicated the association of inhibitory transmission in sleep.

5.3.1. NLGN2 and the Flip-Flop Switch Model

The flip-flop switch model implicates that two mutually inhibiting regions such as monoaminergic wake-inducing regions and GABAergic sleep-inducing regions regulate the generation of sleep and wakefulness (Saper, Scammell, & Lu, 2005). In this model, orexin works as a stabilizer which reinforce the monoaminergic tone to prevent unwanted transitions into sleep (Saper, Scammell, & Lu, 2005). NLGN2 might modulate the flip-flop switch model by regulating inhibitory transmission. Since *Nlgn2* KO mice exhibit reduced frequency and amplitude of mIPSC (Liang, et al., 2015), GABAergic output from sleep-inducing regions such as VLPO and eVLPO will be reduced and the balance between wakefulness and sleep will be shifted toward wakefulness. In the case of sleep deprivation, recovery sleep is prolonged and consolidated due to the increased sleep pressure over the prolonged wakefulness (Franken, et al., 1991; Elmenhorst, et al., 2008). When the molecular change in lateral hypothalamus was observed from sleep deprived mice, increased expression of NLGN2 and $GABA_AR$s containing α1-subunits was identified on orexin neurons (Matsuki, et al., 2015). The NLGN2/DGC/S-SCAM complex has been suggested to induce the accumulation of gephyrin-independent $GABA_ARs$ containing α1-subunits at synaptic sites (Fritschy, Panzanelli, & Tyagarajan, 2012), so the increased NLGN2 expression would have resulted in the increase in $GABA_AR$s containing α1-subunits. Matsuki, et al., 2015 also have shown the increase in the frequency and amplitude

of mIPSC in orexin neurons. The increased NLGN2 expression level would promote the interaction with α-NRXN. The increased interaction between α-NRXN and NLGN2 may have increased the presynaptic calcium current and resulted in the enhanced frequency of mIPSC in orexin neurons (Missler, et al., 2003). Matsuki, et al., 2015 have attributed the enhanced amplitude of mIPSC to sensitized $GABA_AR$ agonists in orexin neurons. When orexin neurons are susceptible to be inhibited, transitions into sleep will be favored. Therefore, the opposite consequence can be expected in *Nlgn2* KO mice. The absence of NLGN2 on orexin neurons will reduce transitions into sleep by decreasing inhibitory transmission. Thus, resulting in the consolidation of sleep in *Nlgn2* KO mice.

Also, NLGN2 has been suggested to interact with serotonin transporters (SERT) at cell bodies in midbrain and hippocampus (Ye, et al., 2016). The absence of NLGN2 leads to the reduction in SERT expression and function which impair the clearance of extracellular serotonin (5-HT) level (Ye, et al., 2016). The increased extracellular serotonin level in *Nlgn2* KO mice sufficiently stimulates $5-HT_{1A}$ autoreceptors on dorsal raphe 5-HT neurons which induce intrinsic inhibition to lower the excitability of the cells (Ye, et al., 2016). The effects of serotonin on VLPO/eVLPO are different depending on the types of VLPO/eVLPO. Serotonin inhibits type 1 VLPO/eVLPO whereas it excites type 2 VLPO/eVLPO (Rancillac, 2016) (Sangare, et al., 2016). In the absence of noradrenalin during sleep, serotonin activates type 2 neurons which inhibit inhibitory serotonin input to type 1 neurons (Rancillac, 2016) (Sangare, et al., 2016). In the presence of noradrenalin during wakefulness, both type 1 and type 2 are inhibited by noradrenaline, so serotonin can induce inhibitory effect on type 1 neurons (Rancillac, 2016). Therefore, the increased extracellular serotonin level in *Nlgn2* KO mice could mediate the consolidation of both NREM sleep and wakefulness.

Apart from the flip-flop switch model, dopamine system has been implicated in the regulation of sleep and wakefulness. Mice lacking the dopamine transporter gene or the dopamine D_2 receptor exhibited increased and decreased wakefulness respectively (Wisor, et al., 2001; Qu, et al., 2010); therefore, dopamine has been suggested to generate/consolidate wakefulness. Recently, NLGN2 has been suggested to mediate dopaminergic synaptic formation in the striatum by assembling presynaptic dopaminergic neurons and postsynaptic GABAergic neurons (Uchigashima, et al., 2016). In NLGN2 mediated dopaminergic synapses, dopamine receptors were not expressed at synaptic sites (Uchigashima, et al., 2016). D_2 receptors are found at synaptic sites inducing tonic activity (Vanderschuren, et al., 1999). The absence of D_2 receptors at NLGN2 mediated dopaminergic synapses may explain the role of NLGN2 in the subcellular localization of GABARs. After NLGN2 knockdown in striatum, GABAergic synapses were increased in compensation for the reduction of the heterologous dopaminergic synapses (Uchigashima, et al., 2016). The effects of NLGN2 mediated dopaminergic synapses on the sleep-wake cycle needs to be examined.

5.3.2. NLGN2 and Thalamocortical System

The thalamocortical system has been suggested to be involved in the generation of two distinct rhythms such as delta and spindle/sigma waves (Steriade & Deschênes, 1984). The thalamocortical system is comprised of corticothalamic cells, thalamocortical cells, and thalamic reticular cells (Khosravani & Zamponi, 2006). Among the compartments of the thalamocortical system, rhythmic bursts of action potentials in thalamocortical cells have been considered to play a key role in the generation of delta and spindle waves (McCormick & Bal, 1997). Thalamocortical cells generate two distinct states of action potential such as burst firing and

tonic single-spike activity depending on the activation of the T type Ca^{2+} channels that conduct low threshold, or transient (I_T), Ca^{2+} current (Jahnsen & Llinás, 1984a; Jahnsen & Llinás, 1984b). In order to generate rhythmic bursts of action potentials in thalamocortical neurons, inactivated T type Ca^{2+} channels should be removed by GABA-induced hyperpolarization (McCormick & Bal, 1997); therefore, NLGN2 might play a role in modulating thalamocortical activity. The involvement of NLGN2 in thalamocortical system will be addressed in greater details below.

5.3.2.1. Spindle Waves

Spindle waves have been suggested to be generated by the interaction between thalamocortical cells and thalamic reticular cells (McCormick & Bal, 1997) because removal of thalamic reticular nucleus abolished spindle waves in the thalamocortical neurons (Steriade, et al., 1987). Interestingly, the removed thalamic reticular nucleus exhibited synchronized spindle-like oscillations, so it has been considered as a pacemaker in the generation of spindle waves (Steriade, et al, 1987).

A burst of action potentials in a thalamic reticular neuron induces IPSP in a thalamocortical cell through $GABA_AR$ activation (McCormick & Bal, 1997). This fast IPSP removes inactivated T type Ca^{2+} channels and activates a hyperpolarization-activated cation current, h-current (I_h) (McCormick & Bal, 1997). The I_h depolarizes the thalamocortical cell and activates T type Ca^{2+} channels that mediate low-threshold Ca^{2+} current (I_T) (McCormick & Bal, 1997). I_T generates a low- threshold Ca^{2+} spike and results in a burst of action potentials (McCormick & Bal, 1997). The burst of action potentials in the thalamocortical cell generates a return barrage of EPSP in thalamic reticular cells (McCormick & Bal, 1997). The returned

barrage of EPSP can restart the cycle between thalamocoritcal cells and thalamic reticular cells (McCormick & Bal, 1997). In ferret geniculate slices, the complete cycle takes about 100-150 ms due to the duration of the $GABA_AR$-mediated fast IPSP in the thalamocortical neurons (80-130 ms) and the time required for the generation of a low-threshold Ca^{2+} spike in both thalamocortical and thalamic reticular cells (McCormick & Bal, 1997). According to the time required for the complete cycle, the frequency range of the oscillation can be expected at 6-10 Hz which is different from the spindle wave (10-13 Hz). This difference could be attributable to the choice of animal models. Unlike higher mammals, rodents exhibit spindle waves at 8-11 Hz (Blumenfeld, 2005). The rhythmic burst activity of thalamocortical cells conveys spindle rhythm to the cerebral cortex by sending barrages of EPSPs and cortical pyramidal cells generate occasional action potentials (Contreras & Steriade, 1996).

The IPSC in the thalamocortical neuron can be also mediated by $GABA_BRs$ (Huguenard & Prince, 1994; Bal, von Krosigk, & McCormick, 1995ab; Bal, 1995b; Sanchez-Vives, Bal, & McCormick, 1995). Since the $GABA_BRs$ are expressed at extrasynaptic sites and activated by GABA spillover, several thalamic reticular cells should discharge simultaneous to release enough GABA to generate the $GABA_BR$-mediated slow IPSP in the thalamocortical neurons (Sanchez-Vives, Bal, & McCormick, 1995; Scanziani, 2000). The $GABA_BR$-mediated slow IPSP removes greater number of inactivated T type Ca^{2+} channels and mediates pronounced low-threshold Ca^{2+} spikes compared to the $GABA_AR$-mediated slow IPSP (McCormick & Bal, 1997). The long duration of $GABA_BR$-mediated IPSP lowered the frequency range of the oscillation at 2-4 Hz (von Krosigk, Bal, & McCormick, 1993; McCormick & Bal, 1997). The loop between thalamocortical cells and thalamic reticular cells generates distinct rhythms depending on the activation of $GABA_AR$ or $GABA_BR$.

The interaction between thalamocortical cells and thalamic reticular cells explains the effect of $GABA_AR$ agonists and $GABA_BR$ antagonists on sleep to a certain extent. $GABA_AR$ agonists such as benzodiazepines and Z-drugs could have increased the sigma spectral power (Lancel, 1999; Trachsel, et al., 1990; Brunner, et al., 1991) by consolidating the $GABA_AR$-induced cyclical interaction between thalamocortical cells and thalamic reticular cells. $GABA_BR$ antagonists could have increased the light slow wave sleep (Juhász, et al., 1994) by preventing the $GABA_BR$-induced cyclical interaction.

In this thesis, it has been speculated that extrasynaptic clusters of $GABA_BRs$ and $GABA_CRs$ diffuse into synaptic sites due to the loss of postsynaptic assembly of $GABA_ARs$ at perisomatic inhibitory synapses in *Nlgn2* KO mice (refer to section 5.2). If $GABA_BRs$ and $GABA_ARs$ are expressed at synaptic and extrasynaptic sites respectively, simultaneous neuronal activity of thalamic reticular cells will be required for $GABA_AR$ activation not for $GABA_BR$ activation. This speculation may explain the reduced sigma spectral activity in *Nlgn2* KO mice during NREM sleep (Table 4.2, Figure 4.10B) by expecting opposite effects of $GABA_AR$ agonists and $GABA_BR$ antagonists.

5.3.2.2. Delta Waves

The generation of delta waves has been considered to originate from two distinct regions such as the cortex and thalamus because delta activity in the cortex was preserved after thalamectomy (Steriade, et al, 1993; Steriade, Nuñez, & Amzica, 1993ab). The synchronization in the cortex was disrupted when cortico-cortical interconnections were interfered by lidocaine infusion or transections (Amzica & Steriade, 1995a). However, neuronal substrates for delta waves in the cortex are not well understood.

The thalamus exhibits intrinsic rhythmic bursts of action potentials at 0.5-4 Hz during delta wave and spindle waves (McCarley, Benoit, & Barrionuevo, 1983; Roy, et al., 1984; Amzica & Steriade, 1995b). The rhythmic burst firing of thalamocortical cells is generated by the interaction between I_T and I_h (McCormick & Bal, 1997). The loop between thalamocortical cells and thalamic reticular cells which has been suggested to generate spindle wave might play a part in the generation of delta waves because the loop oscillates at 6-10 Hz (spindle wave) or 2-4 Hz (delta wave) depending on the activation of $GABA_ARs$ or $GABA_BRs$ respectively.

According to the speculation of this thesis, *Nlgn2* KO mice might exhibit synaptic $GABA_BR$ expression in compensation of synaptic $GABA_AR$ loss (refer to section 5.2). The change in the subcellular localization of GABARs will promote the spindle wave loop to oscillates at 2-4 Hz. This delta rhythm may have increased the delta activity as observed from *Nlgn2* KO mice during NREM sleep.

5.4. NLGN2 and Epileptiform Activity

Nlgn2 KO mice exhibited short trains of repetitive spikes lasting less than 5 seconds which was considered as an epileptiform type of activity (Ljungberg, et al., 2009). The epileptiform activity was frequently observed during wakefulness and REM sleep and least observed during NREM sleep. According to human studies, nocturnal seizure occurred mostly during N1 (23%) and N2 (68%) of NREM sleep (Herman, Walczak, & Bazil, 2001); therefore, the epileptiform activity is less likely to be observed during NREM sleep in mice because mice exhibit only one stage of NREM sleep which is resemble to N3.

During N2, epilepsy is most frequently occurring and the typical EEG feature of N2 is spindle wave. The alteration in the spindle wave loop has been suggested to induce epileptiform activity

resembling absence seizures (McCormick & Bal, 1997). In ferret geniculate slices, the pharmacological blockage of $GABA_ARs$ resulted in the transformation of spindle waves into epileptiform activity by disinhibiting perigeniculate neurons from one another (McCormick & Bal, 1997). Perigeniculate cells are considered as thalamic reticular cells (McCormick & Bal, 1997). The disinhibition of perigeniculate cells increased the intensity of its burst activity which sufficiently activated $GABA_BRs$ in thalamocortical neurons (Huguenard & Prince, 1994; Sanchez-Vives, Bal, & McCormick, 1995). In 5.3.2.2. Delta wave section, $GABA_BR$-medicated slow IPSC has been suggested to shift the oscillation of the spindle wave loop from 6-10 Hz to 2-4 Hz in ferret geniculate slices (McCormick & Bal, 1997). This slow oscillation may have consolidated the delta activity in *Nlgn2* KO mice, but the spike-and-wave discharge in absence seizure is characterized by the 3-4 Hz oscillation of the spindle wave loop (Steriade, Contreras, & Amzica, 1994).

During wakefulness and REM sleep, both thalamocortical cells and thalamic reticular cells exhibit tonic firing (McCormick & Bal, 1997). The reason that the tonic firing of thalamic reticular cells cannot induce a burst of action potentials in thalamocortical cells is the short duration of $GABA_AR$-mediated fast IPSP (McCormick & Bal, 1997). The fast IPSP cannot remove sufficient numbers of inactivated T type Ca^{2+} channels to generate a low-threshold Ca^{2+} spike; therefore, the burst activity of thalamic reticular cells is required to prolong the $GABA_AR$-mediated fast IPSP (McCormick & Bal, 1997). On the other hand, $GABA_BRs$ generate slow IPSP which de-inactivate enough T type Ca^{2+} channels to generate a low-threshold Ca^{2+} spike, but simultaneous neuronal activity of thalamic reticular cells is required (McCormick & Bal, 1997) because $GABA_BRs$ are expressed at extrasynaptic sites and they respond to GABA spillover (Scanziani, 2000).

In the section 5.2. of the thesis, it has been speculated that *Nlgn2* KO mice would exhibit an increase in $GABA_BR$ expression at synaptic sites in compensation for the loss of synaptic $GABA_ARs$. If $GABA_BRs$ are expressed at synaptic site, simultaneous neuronal activity of thalamic reticular cells is no longer required to activate the metabotropic receptors. The tonic firing of thalamic reticular cells during wakefulness and REM sleep would sufficiently generate the 3-4 Hz oscillation of the spindle wave loop which can result in epileptiform activity.

According to Hines et al 2008, *Nlgn2* overexpressing mice also exhibited epileptiform activity (Fig. 5.2). The epileptiform activity resembles to the characteristics of the abnormal EEG activity of *Nlgn2* KO mice that has been observed in this thesis. Hines et al 2008 has reported that brief bursts of EEG activity (<5 sec) at frequency of 6-8 Hz were frequently observed during wakefulness and REM sleep without visible signs of seizure. This abnormal EEG activity might be attributable to a change in inhibitory transmission. Overexpression of NLGN2 increases the frequency of mIPSC (Hines, Wu, Hines, & et al, 2008). The increased frequency of mIPSC might have prolonged the duration of fast IPSC and removed enough of inactivated T type Ca^{2+} channels to generate low-threshold Ca^{2+} spikes.

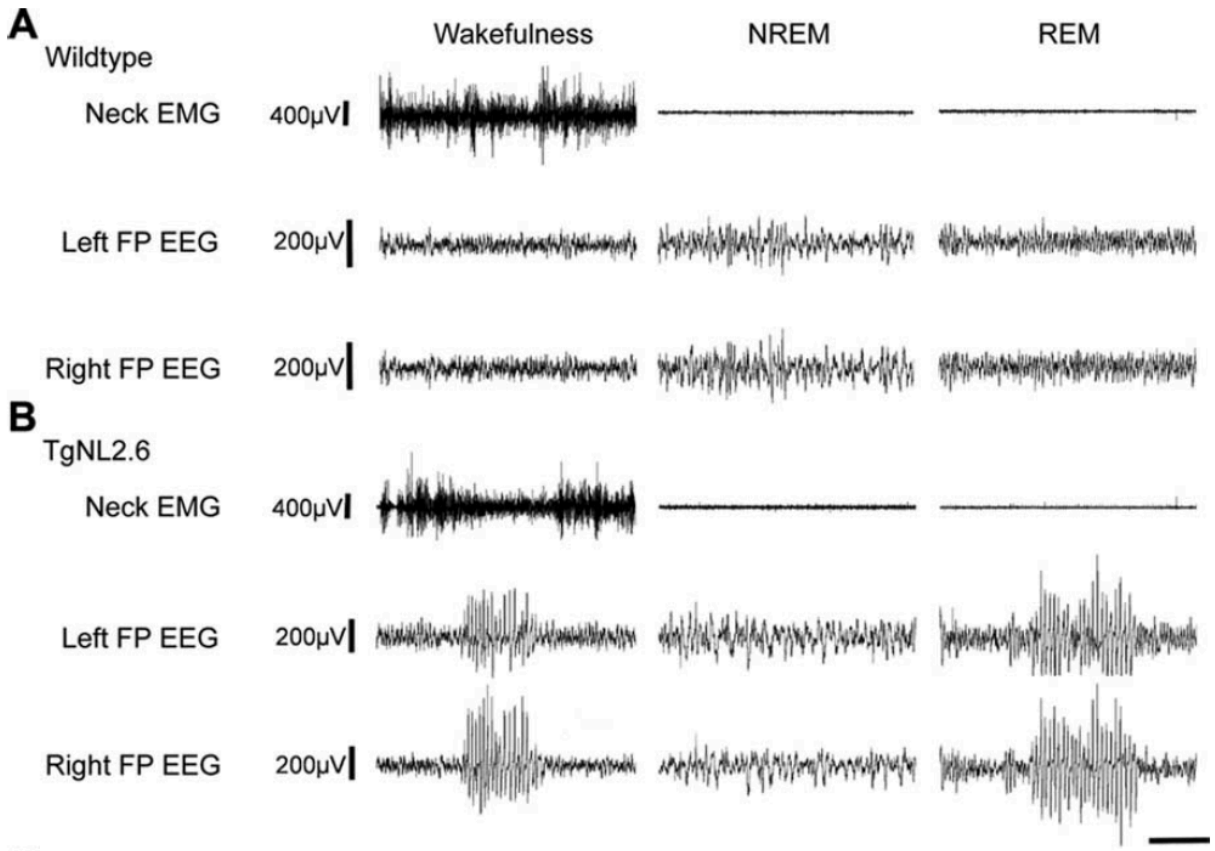

Figure 5.3 – Representative traces of abnormal EEG activity. While wildtype mice (A) showed normal EEG signals, *Nlgn2* overexpressing mice (TgNL2.6; B) exhibited brief bursts of EEG activity during all of vigilance states. The abnormal EEG activity was consistently observed during wakefulness and REM sleep. This figure has been taken from Hines et al 2008.

5.5. Limitations

The major limitation of the thesis comes from the use of full KO mice. Knockout mice have allowed researchers to investigate the functions of genes, but the change in phenotype after gene ablation could be attributable to "flanking gene effect" and/or "genetic background effect" (Eisener-Dorman, Lawrence, & Bolivar, 2009). "Franking gene effect" is caused by variable genes that are transported along with the "knocked out" gene (Eisener-Dorman, Lawrence, & Bolivar, 2009). "Genetic background effect" indicates the impurity of a primary genetic background after knocking out a gene of interest no matter how many times the KO mice are

backcrossed (Eisener-Dorman, Lawrence, & Bolivar, 2009). Moreover, developmental compensation can mask or distort the function of a gene (Picciotto & Wickman, 1998). In order to avoid these confounding effects, it is favorable to knockout a gene at a particular time period at a specific region by using Tet-Off system or conditional gene knockout techniques. Nevertheless, KO models have a value for a first screen of the involvement of a molecule.

For NLGN1, it has been suggested that the region of its expression is determined by splicing sites (Dalva, McClelland, & Kayser, 2007; Bang & Owczarek, 2013; El Helou & et al, 2013). NLGN1 including splicing site B is mostly expressed at excitatory synapses while NLGN1 excluding splicing site B is expressed at both excitatory and inhibitory synapses (Chih, Gollan, & Scheiffele, 2006). NLGN2s are primarily expressed at GABAergic synapses, but they can be also recruited at glutamatergic synapses (Graf, Zhang, Jin, Linhoff, & Craig, 2004). The expression of NLGN2 can be regulated by its splicing site A (Chih, Gollan, & Scheiffele, 2006). NLGN2 including splicing site A is preferentially expressed at GABAergic synapses whereas NLGN2 excluding splicing site A is found at both GABAergic and glutamatergic synapses (Chih, Gollan, & Scheiffele, 2006). The full *Nlgn2* KO model would have disrupted the expression of NLGN2 at both excitatory and inhibitory synapses, but the interpretation of the results of the thesis was focused on the NLGN2 effects on inhibitory transmission.

Lastly, the abnormal EEG activity of *Nlgn2* KO mice needs to be examined in greater detail to confidently classify the abnormal events as epileptiform activities. The frequency of the brief burst activity need to be measured to confirm if it falls in the range of 3-4 Hz. Then, behavioral observation is required to observe if the abnormal EEG activity induces behavioral change.

5.6. Perspectives

Sleep disorders have been considered as a serious economic burden because people who suffer with sleep disorders utilize more of medical resources and exhibit higher risk of developing comorbid medical or psychiatric disorders. Understanding the role of NLGN2 in sleep regulation will uncover, at least to some extent, the etiology of comorbidity as well as provide a potential therapeutic intervention for comorbid disorders.

There are several factors that need to be verified in this thesis. First, the postulated change in the subcellular localization of GABARs in *Nlgn2* KO mice need to be verified through double staining immunohistochemistry of *Nlgn2* KO brain tissues. The identification of change in the distribution of GABARs would help not only developing effective drugs in treating sleep disorders, but also understanding the underlying mechanisms of NLGN2 in sleep regulation. Second, behavioral tests need to be performed on the KO mice to evaluate the effect of NLGN2 on comorbidity. *Nlgn2* KO mice exhibited behavioral changes such as increased anxiety, hypoalgesia, and ataxia (Blundell, et al., 2009), but the association between the behavioral changes and sleep alteration is not well understood. By phenotyping the behavior of *Nlgn2* mice along with EEG monitoring, it would be possible to reveal the role of NLGN2 on linking behavioral changes and sleep alteration. Third, the abnormal EEG event needs to be examined in great detail as mentioned above. Fourth, mutations on the *Nlgn2* gene need to be observed in people who suffer with sleep, medical, or psychiatric disorders and a longitudinal cohort study on the subjects needs to be performed in order to examine the role of NLGN2 in comorbidity. Finally, the effects of NLGN2 on sleep recovery need to be assessed from 6-h sleep deprived mice.

5.7. Conclusion

The role of NLGN2 has been suggested in the regulation of synaptogenesis, synaptic function, and E/I balance (Bang & Owczarek, 2013; Dalva, McClelland, & Kayser, 2007), but its effect on sleep regulation has not been well understood. According to the flip-flop switch model (Saper, Scammell, & Lu, 2005) and development of sedative-hypnotic drugs for insomnia treatment (Winsky-Sommerer, 2009), inhibitory/GABAergic transmission has been implicated in sleep regulation; therefore, NLGN2 would play a significant role in sleep regulation by modulating inhibitory transmission. In the present thesis, it has been hypothesized that *Nlgn*2 KO mice would exhibit prolonged wakefulness and shortened sleep due to the weakened GABAergic sleep-inducing regions (Saper, Scammell, & Lu, 2005). The weakened inhibitory transmission in the KO mice would result in the opposite effects to $GABA_AR$ agonists which enhance the spectral power of sigma activity and lower the spectral power of delta activity during sleep (Lancel, 1999; Trachsel, et al., 1990; Brunner, et al., 1991).

The results of the present thesis have suggested the implication of NLGN2 in the regulation of sleep quality and quantity. *Nlgn*2 KO mice exhibited prolonged wakefulness and shortened sleep. Moreover, the KO mice showed increased delta activity and decreased theta activity during NREM sleep. These observations well concurred with the hypotheses. The effects of NLGN2 on sleep would imply the involvement of NLGNs in causing comorbid disorders because mutations on *Nlgn* genes have been identified from autism and schizophrenia.

Chapter 6: References

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