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# Sleep and Epilepsy Phenotypes in Rodent Models of Neurodevelopmental Disorders

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# Abstract

Globally an estimated 2-5% of children and adults have neurodevelopmental disorders (NDDs), which include autism spectrum disorder, intellectual disability, and attention deficit hyperactivity disorder. Genetic factors constitute a significant risk factor for NDDs, and genetic aetiologies underlie many of the more severe disorder cases. The rate of comorbidity between NDDs and epilepsy is high, with 20-30% of individuals with NDDs presenting with seizures, while sleep impairments are prevalent in an overwhelming 80% of NDD cases, further exacerbating cognitive deficits and the severity of epileptic activity. However, comprehensive long-term quantitative data on sleep and circadian disruptions in individuals with NDDs, especially in those with rare genetic disorders, is scarce, hindering the development of targeted novel therapeutic interventions. Moreover, epilepsy and NDDs result from disruptions in brain circuit formation and aberrant neuronal signalling and activity, although our understanding of the precise pathophysiological mechanisms associated with specific genetic mutations underlying NDDs remains limited.

Using EEG and pharmacological experiments, this thesis investigates and quantifies dysfunctional sleep and seizure activity in three rodent models of genetic NDDs and epilepsy: namely in rat lines of *Grin2b* haploinsufficiency (*Grin2b*<sup>+/-</sup>), *Nlgn3* disorder (*Nlgn3*<sup>-y</sup>), and *16p11.2* deletion syndrome (*16p11.2*<sup>+/-</sup>). Altered sleep physiology dynamics were observed in *Nlgn3*<sup>-y</sup> rats, while an unexpected male-specific increase in rapid eye movement (REM) sleep was found in *16p11.2*<sup>+/-</sup> animals. *Grin2b*<sup>+/-</sup> rats displayed REM sleep deficits and altered sleep physiology alongside an absence seizure phenotype. *Grin2b*<sup>+/-</sup> animals presented with spike and wave discharges in their EEG, a hallmark characteristic of absence epilepsy that correlates with seizures, thought to occur due to abnormal thalamocortical signalling. These seizures were blocked with systemic pharmacological interventions, which was replicated by drug application specifically to the reticular thalamic nucleus, suggesting an impairment of thalamocortical network activity in *Grin2b*<sup>+/-</sup> rats. Overall, findings from this thesis are consistent with clinical observations of dysfunctional sleep in NDDs and provide quantifiable evidence for specific sleep abnormalities in rare *GRIN2B*, *NLGN3* and *16p11.2* related disorder animal models. Furthermore, I report the presence of spontaneous absence seizures in a novel rat model of *GRIN2B* haploinsufficiency that are sensitive to pharmacological treatment. The sleep and seizure phenotypes, along with pharmacological findings from this thesis, may have translational utility if extended to the clinical setting.

# Lay summary

Neurodevelopmental disorders (NDDs) arise from both genetic and environmental factors and are relatively rare, affecting 2-5% of the population. NDDs include autism spectrum disorder (ASD), intellectual disability (ID), and attention deficit hyperactivity disorder (ADHD). These disorders can significantly impact on daily functioning by making social interaction harder, negatively affecting learning and problem-solving skills, and by causing difficulties with attention and impulse control, respectively. NDDs frequently occur alongside other neurological conditions, particularly epilepsy, which occurs in 20-30% of individuals with NDDs. Epilepsy is characterized by recurrent, unprovoked seizures, which are sudden bursts of abnormal electrical activity in the brain that can cause changes in behaviour, movements, or consciousness, leading to restrictions in daily activities. Sleep problems are also very common in people with NDDs and affect approximately 80% of children and adults with these disorders. Poor sleep can worsen learning, memory and problem-solving abilities, as well as increase seizure frequency and severity in individuals who also have epilepsy. Additionally, seizures and anti-seizure medications are known to further compromise sleep and influence sleep structure and quality. At the core of these disorders are abnormalities in how different parts of the brain connect and communicate with each other. However, understanding the precise ways in which such abnormalities contribute to NDDs, sleep problem and epilepsy has remained a challenge. A such, further research is needed to identify these mechanisms. This is also important for the development of effective therapies that could significantly improve the quality of life for individuals with NDDs and epilepsy.

This thesis investigates sleep impairment and epileptic activity in three genetically altered rat lines that model human genetic NDDs. These NDDs include *GRIN2B* and *NLGN3* related NDDs, which are caused by mutations in specific genes, as well as *16p11.2* deletion syndrome, which occurs when a section of a chromosome is missing. Since chromosomes are structures that contain many genes, this deletion leads to the loss of multiple genes, which contributes to NDDs. All three genetic NDDs are associated with ASD, ID, epilepsy and dysfunctional sleep to varying degrees. The rat lines modelling these disorders have specific genes and genome regions crucial for brain function and overall physiology selectively deleted. This genetic deletion prevents the production of essential proteins that are normally produced by the silenced genes, triggering pathological processes in the brain, which allow for the conditions observed in human disorders to be mimicked in these animals.

Electroencephalography (EEG) measures and records the electrical activity of the brain and is the gold standard technique for diagnosing and monitoring neurological conditions including epilepsy and sleep disorders. Using EEG methods adapted to rodents, this thesis quantifies sleep dysfunction and

seizure activity in each rat model. Altered sleep, was found in the model of *NLGN3* related NDD, while an unexpected male-specific increase in rapid eye movement (REM) sleep, a stage of sleep associated with memory formation and dreaming, was observed in the *16p11.2* deletion rat line. In the *GRIN2B* related NDD model, REM sleep was reduced, and seizures were observed. In addition, seizures in rats with deletion of *GRIN2B* gene were blocked by specific drugs used in the treatment epilepsy. Through surgical techniques, these drugs were then applied to distinct brain regions thought to cause seizure, and similarly this treatment resulted in seizure reduction.

The work presented in this thesis provides valuable insights into certain sleep abnormalities resultant from specific genetic mutations, which may be of clinical relevance. Furthermore, it describes the presence of spontaneous epileptic activity in a novel rat model of *GRIN2B* related NDD and offers a preliminary evaluation into some of the brain regions involved in generating seizures associated with this disorder.

# Acknowledgements

As I reach the end of this PhD thesis, I would like to express my heartfelt gratitude to those who have supported me throughout this journey. The successful completion of this work would not have been achievable without the invaluable guidance, encouragement, and support from my mentors, colleagues, friends, and family, each of whom has inspired me and helped me navigate the challenges along the way.

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# Declaration

This work was conducted at the Centre for Discovery Brain Sciences, within the School of Biomedical Sciences at the University of Edinburgh. I declare that all experimental procedures and data presented in this work are my own, with the following exceptions:

- Automated spike and wave discharge and sleep scoring algorithms used in my work were kindly provided by Dr Ingrid Buller-Peralta, University of Edinburgh, and Dr Alejandro Bassi and Dr Adrian Ocampo-Garces, Universidad de Chile.
- Verification of the sleep scoring algorithm was done by Ms Niamh McLaughlin, University of Edinburgh, and myself.
- Power analyses protocols were developed by Dr Melissa Fasol and analyses were conducted by Dr Melissa Fasol, Ms Ezrie Samuel, University of Edinburgh, and myself.
- Western blot data for the *Grin2b* haploinsufficiency model were generated by Dr Sarfaraz Nawaz, University of Edinburgh.

Data presented in Chapters 3 to 5 are available online as a preprint on *bioRxiv* under the title ‘Absence seizures and sleep abnormalities in a rat model of *GRIN2B* neurodevelopmental disorder’. This preprint represents the collaborative efforts between myself and Dr Melissa Fasol, Ms Niamh McLaughlin, Dr Sarfaraz Nawaz, Dr Mehmet Taskiran, Dr Ingrid Buller-Peralta, Dr Alejandro Bassi, Dr Adrian Ocampo-Garces, Dr Javier Escudero, Prof Peter C. Kind and Dr Alfredo Gonzalez-Sulser.

No part of the work contained in this thesis has been submitted for any other degree or professional qualification.

Signed: Katerina Damyanova Hristova

Date: 1<sup>st</sup> November 2024



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# List of abbreviations

|       |                                                                       |
|-------|-----------------------------------------------------------------------|
| ABD   | Agonist-binding domain                                                |
| ADHD  | Attention-deficit/hyperactivity disorder                              |
| AMAPR | $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor |
| ARAS  | Ascending reticular activating system                                 |
| ASD   | Autism spectrum disorder                                              |
| BMI   | Body mass index                                                       |
| CCK   | Cholecystokinin                                                       |
| CNV   | Copy number variant                                                   |
| CREB  | cAMP response element-binding protein                                 |
| CTD   | Carboxyl (C)-terminal domain                                          |
| DEE   | Developmental and epileptic encephalopathy                            |
| DRN   | Dorsal raphe nucleus                                                  |
| E(14) | Embryonic stage (14)                                                  |
| EE    | Epileptic encephalopathy                                              |
| EEG   | Electroencephalogram                                                  |
| EMG   | Electromyography                                                      |
| EPSC  | Excitatory post synaptic current                                      |
| EPSP  | Excitatory postsynaptic potential                                     |
| ERK1  | Extracellular signal-regulated kinase 1                               |
| GABA  | Gamma-aminobutyric acid                                               |
| ID    | Intellectual disability                                               |
| IP    | Intraperitoneal                                                       |
| IPSC  | Inhibitory post synaptic current                                      |
| iPSC  | Induced pluripotent stem cell                                         |
| LC    | Locus coeruleus                                                       |
| LDT   | Laterodorsal tegmental nucleus                                        |
| LFP   | Local field potential                                                 |
| LMM   | Linear mixed model                                                    |
| LPGi  | Lateral paragigantocellular nucleus                                   |
| LTD   | Long-term depression                                                  |
| LTP   | Long-term potentiation                                                |
| mEPSC | Miniature EPSC                                                        |

|       |                                    |
|-------|------------------------------------|
| mIPSC | Miniature IPSC                     |
| MnPO  | Median preoptic nucleus            |
| mPFC  | Medial prefrontal cortex           |
| MRN   | Medial raphe nucleus               |
| MS    | Medial septum                      |
| NDD   | Neurodevelopmental disorder        |
| NE    | Norepinephrine neurons             |
| NLGN3 | Neuroigin-3                        |
| NMDAR | N-methyl-D-aspartate receptor      |
| NREM  | Non-rapid eye movement             |
| nRT   | Reticular thalamic nucleus         |
| NRXN3 | Neurexin-3                         |
| NTD   | Amino (N)-terminal domain          |
| P(6)  | Postnatal day (6)                  |
| PAG   | Periaqueductal grey                |
| PFC   | Prefrontal cortex                  |
| PPT   | Pedunculopontine nucleus           |
| PRF   | Pontine reticular formation        |
| PSD95 | Postsynaptic density protein 95    |
| PTZ   | Postsynaptic density               |
| PV    | Parvalbumin                        |
| RBD   | REM sleep behaviour disorder       |
| REM   | Rapid eye movement                 |
| SCN   | The suprachiasmatic nucleus        |
| SLD   | Specific learning disorder         |
| SLD   | Sublaterodorsal nucleus            |
| SNP   | Single-nucleotide polymorphism     |
| SSR   | Somatostatin                       |
| SWD   | Spike-wave discharge               |
| TMD   | Transmembrane domain               |
| TMN   | Tuberomammillary nucleus           |
| TOAK  | Thousand and One Kinase 2          |
| UPR   | Cellular unfolded protein response |
| USV   | Ultrasonic vocalization            |
| VLPO  | Ventrolateral preoptic area        |
| VTA   | Ventral tegmental area             |



# Chapter 1

## Introduction

### 1.1 Neurodevelopmental disorders

#### 1.1.1 Historical perspective of neurodevelopmental disorders

The idea of disorders and disability can be traced as far back as antiquity, however it was not until the end of the 18<sup>th</sup> century, during the enlightenment period, when the scientific method and humanitarianism ideology were popularised that the notion of mental illness and dedicated study and treatment around it began. Phillipe Pinel (1745-1826), largely considered the father of modern psychiatry, was first to recognise that mental disabilities could be hereditary and physiological in nature and characterized mental illness as a psychiatric disorder. He developed four distinct categories of disorders of the nervous system - dementia, mania, melancholia and idiocy (the last term being a collective definition for the equivalent of modern day neurodevelopmental disorders, particularly intellectual disability) (Berrios & Porter, 1995; Morris-Rosendahl & Crocq, 2020). It was his students Jean-Étienne Esquirol (1772-1840) and Étienne Jean Georget (1795-1828), who then took Pinels theories further and introduced the concept of ‘developmental disorders’ in psychiatry (Berrios & Porter, 1995; Morris-Rosendahl & Crocq, 2020). Georget was first to describe ‘idiocy’ as a ‘lack of development of intellectual faculties’ and Esquirol adopted this notion stating that ‘idiocy’ was not an illness, but a condition in which intellectual faculties could not achieve sufficient development (Berrios & Porter, 1995; Esquirol, 1838; Georget, 1820).

Over the next century classification of psychopathology and mental disorders slowly developed and was initially aimed towards the regulation of treatment for the institutionalized mentally ill and the improvement of mental health policy (Grob, 1991). In 1918, these efforts culminated in the publication of the ‘*Statistical Manual for the Use of Institutions for the Insane*’ issued by the American Medico-Psychological Association (now the American Psychiatric Association). This volume was the first formal and standardized nomenclature of psychopathological conditions and included 22 diagnostic categories, mostly of psychotic conditions (Grob, 1991; Kawa & Giordano, 2012). It is considered the

predecessor of the ‘Diagnostic and Statistical Manual of Mental Disorders’ (DSM) series, the first of which ‘DSM-1’, was published post-World War II in 1952 (Kawa & Giordano, 2012). Three years earlier the sixth revision of the International Classification of Diseases (ICD), a parallel system published by the World Health Organisation included for the first-time mental disorders under the title ‘Mental, Psychoneurotic, and Personality Disorders’ (Clark et al., 2017). Thus, the post war decade marked a significant step in incorporating mental health conditions into an international classification system.

‘Developmental disorders’ were first included in DSM-3, comprising the category for autistic disorders (American Psychiatric Association, 1980), while ‘Neurodevelopmental disorders’ (NDDs) were first introduced in DSM-5, the most up to date and recent edition of the DSM series (American Psychiatric Association, 2013). In the latest 11<sup>th</sup> revision of the ICD, NDDs gained distinction by becoming an integral part of the title of the chapter on psychiatry ‘Mental, behavioural or neurodevelopmental disorders’ (World Health Organization, 2021). The classification of NDDs in ICD-11 does not diverge significantly from that in DSM-5, the gold standard manual for the assessment and diagnosis of mental disorders. Today, DSM-5 defines NDDs as a group of conditions with developmental period onset, resulting in deficits that produce impairments of personal, social, academic, or occupational function. There are seven classes of disorders categorised as NDDs and these include intellectual disability (ID), communication disorders, autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), neurodevelopmental motor disorders (including tic disorders), specific learning disorders (SLDs) and other NDDs (American Psychiatric Association, 2013).

The sections below describe in detail the prevalence, symptomatology, aetiology and pathophysiology of NDDs, and subsequently specifically focus on three genetic NDDs that are the focus of the present thesis.

### 1.1.2 Symptoms, prevalence and diagnosis of neurodevelopmental disorders

NDDs comprise a heterogeneous group of disorders resulting in complex health issues that often have lifelong implications. Characteristically, they manifest before the first 12 months of age and are frequently diagnosed prior to children beginning primary education (Gidziela et al., 2023). As a group, NDDs are thought to affect ~2–5% of children and roughly 120 million people worldwide are diagnosed with an NDD, which is associated with high rates of disability in young people and overall reduction in lifespan (Deciphering Developmental Disorders Study, 2017; Díaz-Caneja et al., 2021). In the United States, the incidence of NDDs has increased in children aged 3 to 17 from 12.84% to approximately 17% between 1999 and 2017. This latter figure corresponds to one in six children in this

age group, diagnosed with at least one NDD (Zablotsky et al., 2019), leading to impaired cognition, communication difficulties and poor psychomotor skills. Reports from 2001 indicate the economic burden of NDDs in the United States was \$81.5–167 billion per year (Szpir, 2006). Over the past two decades these values have climbed sharply and it is projected that economic costs for ASD alone will reach \$461 billion by 2025 in the United States (Leigh & Du, 2015). Moreover, in recent years it has become increasingly apparent and recognised that NDDs rarely present in isolation. Many individuals often present with more than one NDD and exhibit a range of co-occurring conditions, including mood and psychiatric disorders. Sleep, which is critical for normal brain development is also often significantly impaired. These population and monetary figures emphasize the need for further research into NDDs along with comprehensive assessment and personalized intervention strategies.

### *Autism spectrum disorder (ASD)*

ASD is defined by some degree of impairment in social interaction, communication problems, altered sensory processing, and restrictive and/ or repetitive behavioural activity patterns that can vary in their severity (American Psychiatric Association, 2013; Zeidan et al., 2022). The disorder was first reported in 1943, in 11 children with obsessive behaviours and impaired social interactions (Kanner, 1943). Diagnosis of ASD can be made as early as 18-24 months of age, when characteristic symptoms are distinguishable from typical development and from other developmental conditions (Zeidan et al., 2022). Typically, this involves behavioural observations, interviews and psychological questionnaires (Johnson & Myers, 2007). The prevalence of ASD is high, and it is estimated that 1 in 100 children worldwide has ASD (Zeidan et al., 2022). Epidemiological studies indicate a higher incidence of ASD diagnoses in males, and the DSM-5 maintains that ASD is diagnosed four times more often in males than in females (American Psychiatric Association, 2013). However, there is existing debate regarding the ASD male-to-female ratio of 4:1. Some studies suggest a male-to-female ratio closer to 3:1, citing the female protective effect, extreme male brain theory, and discrepancies between male and female ASD phenotypes leading to diagnostic gender bias (Loomes et al., 2017).

Comorbidity, in individuals with ASD is substantial, and an estimated 70% of ASD cases present with one additional cooccurring neurodevelopmental or psychiatric disorder, while 40% will present with at least two (Ivanović, 2021). Typically, these include developmental motor disorders, ADHD and SLDs. For example, in 30 to 50% of individuals with ASD, ADHD and neurodevelopmental motor disorders are also observed (Davis & Kollins, 2012). In addition, approximately 30% of individuals with ASD present with ID (Vaz et al., 2021), with the estimate being potentially greater (Thurm et al., 2019).

### *Intellectual disability (ID)*

ID is a lifelong condition, whereby individuals exhibit impairment in intellectual and adaptive functioning regarding conceptual, social, and practical domains in comparison to age, gender and

socioculturally matched peers (American Psychiatric Association, 2013). Diagnostic criteria are based on both clinical assessment and standardized intelligence quotient (IQ) test scores of less than 70 by the age of 18 (Mefford et al., 2012). Typically, this occurs during childhood, with more severe cases diagnosed earlier in infancy (Mefford et al., 2012). The incidence of ID is estimated to be between 0.05 and 1.55% and varies across different age groups, while the prevalence of severe ID is approximately 6 in 1000 live births (American Psychiatric Association, 2013; McKenzie et al., 2016). ID is diagnosed in both males and female individuals at an approximately similar rate with male-to-female ratios of 1.6:1 and 1.2:1 for mild and severe ID, respectively (American Psychiatric Association, 2013). In children and adults with borderline and mild ID, SLDs, communication and motor disorders are often identified (Deb et al., 2022). Around 20% of ID cases meet the criteria for an ADHD diagnosis (Perera et al., 2022) and as much as 40% meet criteria for an ASD diagnosis. Comorbidity between ID and ASD also presents a risk factor for the presence of additional NDDs (Cervantes & Matson, 2015).

#### *Attention-deficit/hyperactivity disorder (ADHD)*

ADHD diagnoses are based on the essential feature of persistent patterns of hyperactivity-impulsivity and/or inattention, which impede normal development and function (American Psychiatric Association, 2013). Individuals often exhibit a lack of persistence, difficulty maintaining focus, excessive motor activity (extreme fidgeting/ talkativeness), restlessness and impulsivity (Salari et al., 2023). Twice as many males are affected by ADHD when compared to females, except in inattentive ADHD for which the opposite is true. In addition, studies demonstrate that the characteristic features of ADHD change with age, with hyperactivity particularly becoming less evident in adults (American Psychiatric Association, 2013; Ramtekkar et al., 2010). Recent meta-analysis findings based on DSM-5 criterion determined the prevalence of ADHD to be 7.6% in children aged 3 to 12 and 5.6% in adolescents aged 12 to 18, while the figure for the adult population is lower, at 2.5% (American Psychiatric Association, 2013; Salari et al., 2023).

#### *Communication disorders, neurodevelopmental motor disorders and specific learning disorders (SLDs)*

Communication disorders, SLDs, and developmental motor disorders are among the most commonly encountered NDDs, with global prevalence rates estimated to be approximately 5–10% across all ages (Antolini & Colizzi, 2023; Deb et al., 2022).

SLDs, also known as developmental learning disorders (World Health Organization, 2021), are a group of disorders characterized by deficits in a domain of cognitive processing that manifest in difficulties with learning keystone academic skills, such as reading and reading comprehension, written expression and spelling, arithmetic calculation, and mathematical reasoning (American Psychiatric Association, 2013). Considered some of the most common NDDs, they affect learning in individuals with normal intellectual functioning (IQ scores above 70) and usually emerge during the first years of formal schooling, when taught academic skills are acquired and developed (American Psychiatric

Association, 2013). Diagnosis is only possible following the commencement of formal education and includes clinical interviews, psychometric tests and review of school report (Deb et al., 2022).

Communication disorders include language, speech and social (pragmatic) disorders that produce lasting difficulties in language acquisition and disrupted comprehension, expression and social interaction abilities of an individual. These can be a consequence of language and/or speech impairment; alternatively individuals may fail to understand the pragmatics of social cues (American Psychiatric Association, 2013; Deb et al., 2022). Manifestations of these disorder vary with age and in severity, but overall are diagnosed within the first few years of life, when communication abilities begin, and deficits become apparent. Vocabulary, variety of expression and grammatical complexity are reduced, and reluctance to engage in conversations is common (Deb et al., 2022; Palumbo et al., 2023). Communication disorders are diagnosed through review of developmental and clinical history, along with medical and psychiatric interviews (Deb et al., 2022; Palumbo et al., 2023).

Neurodevelopmental motor disorders are those defined by difficulties in acquiring motor and coordination skills (American Psychiatric Association, 2013; Deb et al., 2022). Examples include motor coordination difficulties whereby acquisition, and execution of coordinated motor skills are substantially below that of neurotypical individuals. Tic disorders, including Tourette's syndrome are characterized by sudden, rapid, recurrent, non-rhythmic motor movement or vocalization (Deb et al., 2022). Comprehensive medical and developmental history, physical examination and standardised test are used to for diagnosing motor NDDs (American Psychiatric Association, 2013; Deb et al., 2022). Up to 69% of children with a developmental coordination disorder have at least one other NDD, such as ASD, ADHD or a SLD (Visser, 2003).

Despite the high prevalence of these disorders and the significant economic impact associated, we have limited understanding of their causes. There is considerable biological complexity underlying NDDs, making it difficult to elucidate the precise mechanisms leading to them. However, what is known is that categorical neurodevelopmental (and neuropsychiatric) diagnoses are a consequence of abnormal or disrupted brain development and function. Furthermore, they share similar risk factors, as established from the 'developmental brain dysfunction' conceptual model first introduced by Morena-De-Luca and colleagues (Moreno-De-Luca et al., 2013).

## 1.2 Epilepsy in neurodevelopmental disorders

Epilepsy is characterized by the presence of spontaneous recurrent seizures, resulting from abnormal synchronised neuronal activity, and comprises a heterogeneous group of disorders with different aetiologies (Avanzini & Franceschetti, 2003; Balestrini et al., 2021). These include structural,

genetic, infectious, metabolic and immune factors, which can result in acquired structural brain lesions, inborn brain malformations, alterations in neuronal signalling, and/or defects in maturation and plasticity of neuronal networks (Balestrini et al., 2021). Epilepsy is a common disease, affecting roughly 1% of the global population. Epilepsy ranks as the fifth most common neurological condition globally, after stroke, migraine, dementia, and meningitis (American Psychiatric Association, 2013). Diagnoses of epilepsy are usually made following initial seizure presentation and formal electroencephalogram (EEG) recording. EEG, which measures electrical activity in the brain, is abnormal in individuals with epilepsy, and EEG abnormalities often include spikes, spike-waves and sharp waves (Benbadis et al., 2020; Stecker et al., 2016). The International League Against Epilepsy classes the disease into four main types based on how seizures spread through the brain, including generalised, focal, combined generalised and focal, and unknown epilepsy (where the epilepsy type is undetermined). In addition, where a cluster of seizure types, EEG abnormalities, and imaging features co-occur an ‘epilepsy syndrome’ diagnosis may be made, for example Dravet syndrome and Lennox-Gastaut syndrome (Scheffer et al., 2017a), which are frequently associated with genetic NDDs.

Epilepsy is a disorder separate from NDDs. There is good reason for this as many epilepsies have significantly different causes, symptoms, biomarkers and treatment possibilities (i.e. acquired epilepsies) to those in NDDs (American Psychiatric Association, 2013; Balestrini et al., 2021; Engel et al., 2013; Kobylarek et al., 2019; Riva et al., 2021). Yet epilepsy is highly comorbid with NDDs and an estimated 26% of individuals with an NDD report the presence of co-occurring seizures (American Psychiatric Association, 2013). This incidence rate is observed particularly in people with ASD, ID and ADHD, where over 30% of individuals with ASD, 25% of people with ID, and 15-20% of those with ADHD are estimated to have epilepsy (Akrouf Brizard et al., 2021; Keller et al., 2017). Amongst the complex syndromic NDDs that demonstrate highest seizure burden are the more frequently occurring genetic disorders with severe symptoms, which include Rett syndrome, Dravet syndrome, Fragile X and tuberous sclerosis, with epilepsy also common in trisomy 21 (Down syndrome) (Shankar & Walker, 2020).

The strong link between epilepsy and NDDs is particularly evident in a group of severe genetic conditions known as epileptic encephalopathies (EEs). In these disorders, epileptic activity exacerbates brain dysfunction beyond the underlying cause, with seizures directly contributing to developmental delays and cognitive impairment (McTague et al., 2016). However, for many syndromic NDDs currently classified as EEs, evidence supporting this process is limited. In fact, even when epileptic activity is controlled, developmental delays and cognitive impairments often persist (Howell et al., 2016). These cases are now termed developmental and epileptic encephalopathies (DEEs) and suggest that a primary underlying cause is responsible for brain maldevelopment or damage, leading to both NDD symptoms and epilepsy (Raga et al., 2021; Scheffer & Liao, 2020). This concept of shared origin of both epilepsy and NDDs stands especially true where genetic aetiologies are identified.

## 1.3 Causes and pathophysiology in neurodevelopmental disorders and epilepsy

Significant progress in neurobiology and genetics has revealed the causes of a subset of NDDs. A range of factors, which occur on their own or interact to produce a cumulative effect, have been shown to contribute to these disorders and include genetic mutations, immune disorders, infectious disease, physical trauma, and environmental factors. Research investigating NDDs of presumed non-genetic origin demonstrates that prenatal exposure to medications such as thalidomide and valproic acid, as well as exposure to drugs, alcohol, heavy metals like lead and mercury, certain pesticides, commercial flame retardants, and plastic compounds like bisphenol A, significantly increases the risk of ASD and other NDD (Lein, 2015). There are several hypotheses regarding how exposure to these toxic risk factors results in NDDs, and these include endocrine disruption, mitochondrial dysfunction, epigenetic modification, chemical perturbation of the microbiome (believed to influence brain function), and genetic mutations in proteins involved in relevant detoxification pathways (Lein, 2015). Maternal infection with vertically transmitted pathogens passed onto developing fetuses, including rubella, zika and cytomegalovirus amongst others, may result in growth abnormalities, epilepsy and NDDs in the foetus (Gordon-Lipkin et al., 2021, Nunes et al., 2021). In addition, maternal immune activation and in particular cytokine release also is seen to affect proliferation and differentiation of neural stem cells, disrupting signalling pathways (notably MARK/ERK) and the modulation of transmitter and synaptic processes in the brain, potentially leading to the development of NDDs such as ASD, ID and ADHD (M. B. Hall et al., 2023; K. Liu et al., 2023).

In 2010 the Deciphering Developmental Disorders study was established with the purpose of finding potential genetic determinants of diagnosed NDDs of unknown origins in children, using genomics. The project employed whole exome sequencing and looked at 13,000 children with severe NDDs, as well as their parents, and provided valuable insights into the genetic architecture of these conditions, which has significantly contributed to the field of clinical genetics (Firth & Wright, 2011). Since then, significant improvements have been made in the identification of risk genes associated with some NDDs, to reveal a robust genetic component underlying their pathophysiology. This is unsurprising as roughly 70% of coding genes hold functional dominance over brain development and functioning.

Over the past decade advances in DNA sequencing technologies and genomic analysis have greatly increased our understanding of genetic variants and how they contribute to the development of NDDs. Moreover, the implementation of sequencing technologies in the diagnostic flowchart of NDDs and epilepsy has dramatically increased the percentage of patients who receive a molecular diagnosis. Thus far, the mutational spectrum of NDDs and epilepsy includes many hundreds of candidate genes

and ranges from large chromosomal copy number variants (CNVs) to single-nucleotide polymorphisms (SNPs), related to neurodevelopmental pathways that are associated with synaptic function, transcriptional regulation, chromatin remodelling and neuronal differentiation and proliferation (Parenti et al., 2020). Amongst the most frequently observed and well documented are pathogenic mutations in *FMRI*, *TSC1* and *TSC2*, *NF1*, *UBE3A* and *MECP2* genes, responsible for Fragile X syndrome, tuberous sclerosis, neurofibromatosis, Angelman syndrome and Rett syndrome respectively (Betancur, 2011). Additionally, major genomic defects are usually detected in chromosome loci *1q21.1*, *16p11.2* and *22q11.2* and are associated with ASD, ID, epilepsy and schizophrenia. Collectively they account for 5-10% of ASD cases (C.-C. Jiang et al., 2022; Reale et al., 2021).

On a final note, a substantial amount of phenotypic variability is present even in individuals with overlapping genetic aetiologies, as evidenced by large genotype-phenotype correlation studies. This has prompted the notion that differences in clinical manifestations in NDD cases are polygenetic in nature and may also be heavily dependent on environmental risk factors (Parenti et al., 2020). For example, recent research has identified secondary disease-associated variants that differentiate the typical clinical presentation of individuals carrying a primary variant (Karaca et al., 2018; Pizzo et al., 2019; Posey et al., 2017; Schaaf et al., 2011). An additive effect of such mutational events, as well as the physical and functional interactions between affected genes, that is dosage sensitive, is thought to reflect the observed phenotypic spectrum diversity (Parenti et al., 2020).

### 1.3.1 Pathophysiology in genetic neurodevelopmental disorders and epilepsy

While the molecular and cellular mechanisms leading to NDDs and epilepsy are still not fully understood, some of the established pathophysiological processes broadly include those that impact on molecular regulatory mechanism and gene expression, and those that directly or indirectly affect synaptic transmission and consequently the formation and correct assembly of brain circuitry (Exposito-Alonso & Rico, 2022; Turner et al., 2021).

During development, proper formation and maturation of the nervous system depends on the appropriate regulation of gene expression and protein homeostasis (Kang et al., 2011). This precise regulation ensures that the complex sequence of developmental events unfolds in the correct order, however, any disruption in transcriptional or epigenetic control of gene expression can lead to NDDs and epilepsy. Unsurprisingly, the most commonly diagnosed severe NDDs and DEEs are those in which mutations target transcription factors, epigenetic regulators and genes encoding proteins responsible for global protein synthesis and degradation. To illustrate, developmental expression of numerous genes is impaired when CNVs or *de novo* SNPs are present in the *MECP2*, *FMRI* and *UBE3A* genes, which play important roles in transcriptional repression of genes, regulation of chromatic structuring, and

protein translation and degradation respectively (Amir et al., 1999; Buiting et al., 2016; Richter et al., 2015; Skene et al., 2010). The outcome of each is the downstream modification of numerous genes, particularly during embryonic and post-natal development, which collectively alters overall cellular and network function and results in the typical behavioural and clinical manifestations associated with severe Rett, Fragile X, and Angelman syndromes.

A substantial proportion of genes that confer risk of NDDs and epilepsy encode proteins that critically influence intrinsic cellular excitability, as well as synaptic transmission and synapse assembly, all of which are crucial mechanisms for neuronal signalling, the establishment of neural networks during development, and the plasticity processes underlying behaviour (Brunklaus & Lal, 2020; Kullmann, 2010; Südhof, 2018). Genetic NDDs in which these processes are disturbed are known as channelopathies and synaptopathies. In the former, pathological processes involve changes in the active properties of membrane ion channels, such as voltage gated sodium and potassium channels (i.e. those encoded by *SCN2A*, *SCN8A*, *KCNT1*), which leads to neuronal hypo- or hyperexcitability and a disordered brain state (Brunklaus & Lal, 2020; McTague et al., 2018). With synaptopathies, synaptic receptor functioning, as well as synapse formation and structural maintenance mechanisms are impaired. Disorder causing mutations have been identified in proteins expressed at both the pre- and post-synaptic terminal, for example in the genes *GABRG2*, *SYNGAP1*, *NLGN3* and *GRIN2B* related to severe and frequently comorbid NDDs and epilepsies (Jamain et al., 2003; Komulainen-Ebrahim et al., 2019; Platzer et al., 2017; Vlaskamp et al., 2019).

NDDs related to CNVs in chromosomal locus *16p11.2* and mutations in the latter two synaptic genes *NLGN3* and *GRIN2B* are the focus of the present thesis. Therefore, the following sections review the functional role of each gene, the clinical presentation in individuals with variants of these genes, and what is known about their underlying pathophysiological mechanisms.

## 1.4 Chromosomal CNVs and synaptic gene mutations in neurodevelopmental disorders and epilepsy

As discussed above the causal factors for many NDDs and epilepsies are complex and remain unknown in a large proportion of individual cases. However, abundant research has shown that a significant amount of these rare conditions are a result of chromosomal CNVs and sporadic *de novo* variants in single genes. The latter result in a ‘monogenic’ disorder aetiology (de Ligt et al., 2013). Such *de novo* variants are under strong negative selection, as the associated disorders are often severe, presenting with multiple clinical comorbidities and, in some cases, result in early mortality (Eyre-Walker & Keightley, 2007). Below, NDDs that are often comorbid with epilepsy, and associated with

single-gene mutations in *GRIN2B* and *NLGN3*, as well as the chromosomal copy number *16p11.2* deletion disorder, are discussed along with pathophysiological insights from animal and cellular models of each disorder.

#### 1.4.1 *16p11.2* deletion syndrome

In 2008, a study using a large patient database first linked CNVs in the *16p11.2* chromosomal region to ASD, occurring at a rate of 3 in 10,000 (Weiss et al., 2008). Since then, deletions in the *16p11.2* region have been identified as one of the most common known genetic aetiologies of ASD, with an estimated prevalence of 0.5-1% among individuals with the disorder (Chung et al., 2021; Fetit et al., 2020). In around 71% of cases, *16p11.2* deletions occur *de novo*, and these CNVs are seldom inherited (D'Angelo et al., 2016). Deleterious alterations in the *16p11.2* locus are associated with a diverse spectrum of neurodevelopmental phenotypes, including ID, ASD, ADHD and epilepsy (Chung et al., 2021; Fetit et al., 2020; Leone et al., 2024; Weiss et al., 2008). Speech and language impairment, as well as motor coordination difficulties are the most pervasive features amongst *16p11.2* deletion carriers and are present in over 70% and approximately 60% of patients respectively (Chung et al., 2021). As with many NNDs, sleep impairment is present in individuals with *16p11.2* deletion as quantified by several studies (B. A. Fernandez et al., 2010; Hanson et al., 2010; Kamara et al., 2023). Macrocephaly is found in 17% of individuals and typically high BMI or obesity emerge during childhood, with the latter conferring an additional risk factor in adulthood (Chung et al., 2021; Zufferey et al., 2012). Just over half of individuals with *16p11.2* deletions have structural abnormalities and other major medical issues (e.g. congenital heart disease) (Chung et al., 2021). Cerebro-cortical malformations include increased white matter volume and fibre density, accompanied by decreased fibre directional variability in the corpus callosum, internal and external capsules (Owen et al., 2014). Epilepsy is diagnosed in approximately a quarter of individuals with *16p11.2* deletions and seizures include focal, generalised tonic-clonic, absence and febrile seizures. Usually these emerge early in infancy and are mostly well controlled with conventional pharmacology (Fetit et al., 2020; Steinman et al., 2016; Zufferey et al., 2012). Of final note, *16p11.2* CNVs can result in either deletion (discussed here) or duplication. The two share clinical presentations but have mirroring BMI and head circumference phenotypes. *16p11.2* duplication constitutes an additional risk factor for schizophrenia and its thought that ASD and schizophrenia reflect dosage-dependent modifications in gene expression (Leone et al., 2024).

#### 1.4.1.1 *16p11.2* chromosomal region

The frequent and highly pathogenic microdeletion region of the *16p11.2* locus is approximately 600kb and defined by breakpoints BP4 to BP5 (Figure 1.1). This chromosomal section is flanked by highly homologous blocks of low copy repeats (Chung et al., 2021). Low copy repeats can act as substrates for non-allelic homologous recombination, resulting in genomic rearrangements that are in some cases pathogenic CNVs (Carvalho & Lupski, 2016). BP4 and BP5 contain non-coding RNAs and a total of 28 distinct genes that encode proteins, which are functionally important in specific molecular cascades (Figure 1.1) (Leone et al., 2024; Weiss et al., 2003). The majority of genes contained within this region are expressed in different areas of the brain and contribute to a wide range of signalling activities (for detailed review see Leone et al., 2024). Examples of critical proteins include TOAK (Thousand and One Kinase 2) and MAZ (MYC-associated zinc finger protein, a transcription factor) that are both encoded by extremely loss of function intolerant genes, the mutations of which contribute to severe NDDs independently of *16p11.2* variants. MAZ is also a category 2-risk gene for ASD in the SFARI GENE Scoring list (Figure 1.1) (Oliva-Teles et al., 2020; *SFARI Gene*, 2024). The transmembrane protein PPRT2 (proline-rich transmembrane protein type 2) located at the synapse, is associated with epileptic phenotypes (Padmanabha et al., 2024). It influences synapse formation during development and is a key component of neurotransmitter release machinery through its interactions with SNARE proteins and synaptotagmins (Valtorta et al., 2016). Similarly, *QPRT* (quinolinate phosphoribosyltransferase) encodes an essential enzyme involved in the breakdown of quinolinate, a strong excitotoxin endogenous to neurons. Increased levels of quinolinate have been associated with the onset of seizures (Figure 1.1) (Haslinger et al., 2018).

## Chromosome 16

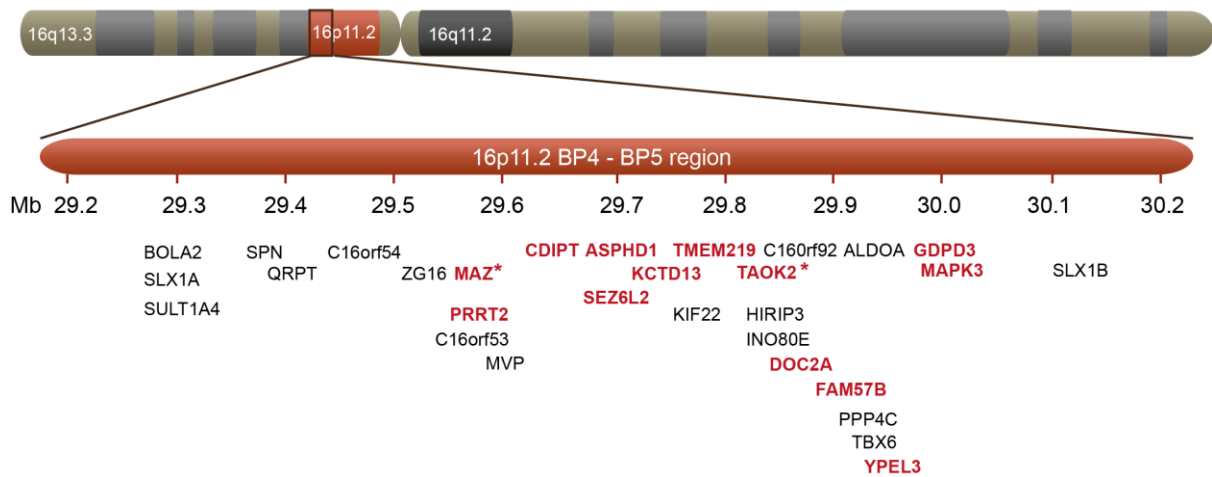


**Figure 1.1. The 16p11.2 deletion region.** The diagram illustrates the BP4-BP5 segment of chromosome 16p11.2 associated with deletion/ duplication CNVs. The region contains 28 protein coding genes and non-coding RNAs. Disorder associated genes are marked in red and genes highly intolerant to mutation are marked with an asterisk. Figure adapted from Oliva-Teles et al. (2020).

Of the 28 genes, *MAPK3*, *TOAK2*, *KCTD13* and *MVP* are clearly involved in the pathophysiology of *16p11.2* deletion (Figure 1.1) and crucially modulate neuroanatomical, synaptic and cognitive functions via their intracellular interactions. *MAPK3* encodes the enzyme ERK1 (extracellular signal-regulated kinase 1), a crucial protein kinase within the Ras-Raf-Mek-ERK signalling pathway (Leone et al., 2024). This molecular cascade controls gene expression and chromatin remodelling, and plays a pivotal role in diverse cellular functions, ranging from cell proliferation and survival to synaptic and behavioural plasticity (Fasano & Brambilla, 2011). Additionally, ERK1 interacts with the PI3K-AKT-mTORC1 pathway to regulate protein translation. *TAOK2* kinase is believed to inhibit protein translation and synaptic maturation, and interacts with multiple cytosolic and nuclear targets, including some kinases (e.g. p8 MAP) that may contribute to ERK1 signalling. Additional regulatory control appears to come from *MVP* (Major Vault Protein), which can act as a scaffold protein for both ERK1 and mTORC1 signalling. Finally, *KCTD13* functions upstream of ERK1 and therefore indirectly mediates ERK signalling (Leone et al., 2024).

### 1.4.1.2 Pathophysiology in *16p11.2* deletion

As with other NDDs, *16p11.2* deletion contributes to a highly heterogeneous clinical profile, due to CNV variability, and therefore a gene-dosage effect on 27 genes. Abundant research into the

neurophysiological and behavioural phenotypes in *16p11.2* deletion has come from three independently generated mouse models (Arbogast et al., 2016; Horev et al., 2011; Portmann et al., 2014).

Significant changes in neuronal excitability across different brain regions are observed in *16p11.2* deletion mice. For instance, reduced action potential frequency and deficits in N-methyl-D-aspartate receptor (NMDAR) - mediated transmission were found in mPFC pyramidal neurons (W. Wang et al., 2018). Reductions in excitability and membrane resistance have also been documented in iPSCs-derived forebrain cortical neurons (Deshpande et al., 2017), while in iPSCs-derived dopaminergic neurons a hyperexcitability phenotype was observed (Sundberg et al., 2021). Brain imaging has identified altered functional connectivity between the septum and amygdala, auditory cortex, hippocampal and prefrontal cortical regions of mice with *16p11.2* deletion (Openshaw et al., 2023). The prefrontal cortex (PFC) appears to be particularly affected by *16p11.2* copy number variation. Diminished connectivity of this area was observed in both human carriers of *16p11.2* deletion and in mice with the same genetic deficiency, in which reduced long-range functional synchronization and thalamo-prefrontal miswiring were also observed. Dendritic spine density in the PFC was reduced, although synaptic excitability of prefrontal pyramidal neurons in PFC was unaltered in these animals (Bertero et al., 2018). Furthermore, there is evidence pointing to potentially dysregulated inhibitory signalling. At hippocampal CA1 pyramidal cells, synaptic excitability is unchanged in *16p11.2* deletion mice (Arbogast et al., 2016). However, in a rat model of *16p11.2* deletion, CA1 somatostatin expressing interneurons were hyperexcitable at P21, and importantly the authors demonstrate that a subset of human inhibitory populations have enriched expression of risk factor transcripts associated with the *16p11.2 locus* (Y. Yang et al., 2023). In PFC and orbital cortex mRNA expression of calbindin is increased, while that of PV is decreased in both male and female mice. However, the authors also find a sex dependent decrease of region specific gamma-aminobutyric acid (GABA)-ergic gene expression in male mice only, suggesting an increased vulnerability to the CNV in males (Openshaw et al., 2023).

The degree of gene expression dysregulation in the CNV deletion animals is dependent on brain region and the striatum is more severely impacted (Arbogast et al., 2016). In the striatum, medium spiny neurons expressing dopamine receptor 2 were found to be significantly increased, while those expressing dopamine receptor 1 were decreased in neonatal mice (Portmann et al., 2014). These neuronal populations are important for motivation, attention and motor control and there is strong evidence for dysregulated dopaminergic signalling in ASD (Kosillo & Bateup, 2021). This dopamine receptor imbalance was also observed by Grimsson *et al.* in adult male *16p11.2* deletion mice, which again highlights a significant and peculiar sex-dependent variation associated with this CNV disorder, whereby some traits may only be expressed in males (Grissom et al., 2018).

Neuroanatomical alterations have been identified *ex vivo* and in *16p11.2* deletion mice, although structural phenotypes oppose what is observed in individuals with *16p11.2* CNVs. Increased

soma size, dendritic length and more elaborate dendritic arborization are observed in *16p11.2* locus deletion cortical organoids and iPSC-derived cell lines (Deshpande et al., 2017; Fetit et al., 2020; Sundberg et al., 2021; Urresti et al., 2021). In stark contrast to human deletion carriers, BMI is significantly lower in neonatal mice lacking the *16p11.2* locus (Arbogast et al., 2016; Horev et al., 2011; Portmann et al., 2014), and this can last into and throughout adulthood (Portmann et al., 2014). Reductions in brain size are seen during early post-natal development and in adulthood (Horev et al., 2011; Pucilowska et al., 2015). Compromised cortical architecture and decreased cortical thickness reminiscent of that observed in human carriers (Maillard et al., 2015), have been shown in *16p11.2* deletion mice and are likely resultant from enhanced cortical progenitor proliferation in the developing brain (Pucilowska et al., 2015). Furthermore, relative to decreased brain size, some regions are disproportionately increased or decreased in *16p11.2* deletion mouse models (Horev et al., 2011; Portmann et al., 2014; Pucilowska et al., 2018). Hearing, particularly in mouse pups with *16p11.2* deletion is significantly impaired (Portmann et al., 2014; M. Yang et al., 2015). Lastly, significant structural changes were identified in medial fibre tracts, particularly those near the striatum, which correlated to overexpression of genes involved in MAPK signalling in mice with *16p11.2* deletion. These observations were again specific to male mice only (V. J. Kumar et al., 2018).

Behaviourally, stereotypical behaviours are observed in *16p11.2* deletion mice along with hyperactivity, particularly in familial environments and during the dark phase of the 24-hour cycle (Angelakos et al., 2017; Arbogast et al., 2016; Horev et al., 2011; Portmann et al., 2014; M. Yang et al., 2015). One deletion mouse model was also found to have subtle motor impairment (Brunner et al., 2015). Several studies showed significant cognitive deficits in *16p11.2* deletion mice, including impairments in spatial memory and social preferences (Portmann et al., 2014; Pucilowska et al., 2015; W. Wang et al., 2018; M. Yang et al., 2015), visual learning and associative flexibility (M. Yang et al., 2015), as well as diminished hippocampal-dependent contextual memory (Tian et al., 2015). Reduced motivation and impaired reward learning are also observed and likely a result of dysregulated dopaminergic signalling (Grissom et al., 2018). *16p11.2* deletion mice also present with an anxiety phenotype (Pucilowska et al., 2015). During male-female interactions, male *16p11.2* deletion mice produce significantly fewer ultrasonic vocalizations (USVs), reflecting reduced responsive to social cues (M. Yang et al., 2015). Furthermore, in the same deletion model male pups had abnormal perinatal communication patterns, as measured by USVs (Agarwalla et al., 2020). Reduced body weight was also observed in two rat models of *16p11.2* deletion. Notably, animals from both rat lines presented with male specific social behaviour alterations and memory impairments (Martin Lorenzo et al., 2023). Lastly, hyperactive *16p11.2* deletion mice exhibit male-specific sleep deficits, while physiologically normal sleep was observed in females (Angelakos et al., 2017; H.-C. Lu et al., 2019).

Taken together, *16p11.2* deletions lead to widespread neurobiological alterations that contribute to network dysfunction and diverse behavioural phenotypes. Rodent models reveal disrupted gene

expression and molecular pathways across multiple brain regions. Importantly, these perturbations extend beyond the genes within the *16p11.2* locus, affecting broader gene networks and cellular processes throughout the brain. The prefrontal cortex shows reduced connectivity and thalamo-prefrontal miswiring, while the hippocampus exhibits altered excitatory-inhibitory balance. Striatal dysregulation of dopamine receptor-expressing neurons and compromised cortical architecture likely further impact on normal cognitive and motor functions. Notably, many alterations show male-specific effects, suggesting a greater impact of the *16p11.2* CNV on males. Ultimately, the rat and mouse *16p11.2* deletion lines discussed phenocopy many of the behavioural features observed in individuals with this CNV, including social, communication and memory impairments, and present an interesting model for future study.

#### 1.4.2 *NLGN3* related neurodevelopmental disorder

Mutations in *NLGN3* were first linked to ASD in 2003 by Jamain et al., who identified a missense mutation in two Swedish brothers with ASD and Asperger's syndrome (Jamain et al., 2003). Since then, *NLGN3* variants have been uncovered in individuals presenting with ASD and ID, with or without communication disorders and specific learning disorders (C Yuen et al., 2017; Iossifov et al., 2014; Mikhailov et al., 2014; Redin et al., 2014; Steinberg et al., 2012; Talebizadeh, 2005; Volaki et al., 2013; X. Xu et al., 2014; Yu et al., 2011). Epilepsy is rarely observed in *NLGN3* associated NDDs, although it was reported in one of the originally diagnosed brothers with ASD, who developed late onset tonic-clonic generalised seizures at the age of 19 (Jamain et al., 2003). Sleep deficits and abnormal sleep patterns are reported in roughly 80% of children and adults with ASD (Cohen et al., 2014) and while documentation of dysfunctional sleep in *NLGN3* specific ASDs is sparse, it was recently reported in three male cousins with ASD causing *NLGN3* variants (Quartier et al., 2019).

##### 1.4.2.1 Neuroligin-3

The X-linked gene *NLGN3* encodes the transmembrane postsynaptic cell adhesion protein neuroligin-3 (NLGN3), which regulates trans-synaptic signalling and is involved in the formation, organisation and remodelling of synapses (Ichtchenko et al., 1996; Uchigashima et al., 2021). In humans and primates there are five neuroligin genes in total (*NLGN1-NLGN5*), while in rodents there are only four (*Nlgn1-Nlgn4*) (Uchigashima et al., 2021). Despite their structural homology, the different neuroligin members have varied subcellular localization at inhibitory, excitatory, cholinergic and dopaminergic synapses (Figure 1.2) (Song et al., 1999; Takács et al., 2013; Uchigashima et al., 2016;

Varoqueaux et al., 2004). NLGN3 is expressed at the post synapse and is the only neuroligin found at both excitatory and inhibitory synapses, where it modulates synaptic functions (Budreck & Scheiffele, 2007). It predominantly interacts with presynaptic binding partner neurexin-3 (NRXN3, also associated with NDDs), a member of the neurexin family of cell adhesion proteins, to form complexes that span the synaptic junction (Dean & Dresbach, 2006; Südhof, 2008). Although synapse development and specification are strongly influenced by NLGNs, NLGN3 does not appear to influence transcriptional and translational regulators at the synapse (Parenti et al., 2020).

Structurally, NLGN3 is a single membrane spanning protein comprised of three distinct domains, including a hydrophobic (N)-terminal in the extracellular space containing a large cholinesterase-like domain, a highly conserved single transmembrane domain (TMD), and the intracellular (C)-terminal, which contains a short cytoplasmic domain with a postsynaptic density (PDZ) / gephyrin-binding motif for cis protein interaction in glutamatergic and GABAergic/ glycinergic synapses respectively (Ichtchenko et al., 1996; Uchigashima et al., 2021). At the (N)- terminal, the cholinesterase-like domain contains the interface for NLGN3-NLGN3/1/2 dimerization and an  $\alpha/\beta$ -hydrolase fold, which is non-catalytic and interacts with other adhesion molecules. Importantly binding with NRXN3 occurs here. This structure is highly conserved as it is present in over 70% of the different neuroligin members with which NLGN3 dimerizes (Uchigashima et al., 2021).

NLGN3 dimerization appears crucial for the formation and function of NLGN3-NRXN3 complexes (Figure 1.2). At the post synapse NLGN3 forms functional homodimers and heterodimers with NLGN1, which is expressed exclusively at excitatory synapses. The ability of NLGN3 to dimerize with NLGN2, which is found only at inhibitory synapses, is controversial and currently not entirely understood, although some anatomical, biochemical, and functional studies support the theory of heterodimer formation between the two, especially at hippocampal inhibitory inputs (Budreck & Scheiffele, 2007; Pouloupoulos et al., 2012). Notably, heterodimerization of NLGN3 with both dimer partners may serve to regulate excitatory and inhibitory synapses respectively, as activity-dependent proteolytic cleavage of NLGN1 and NLGN2 extracellular domains can only occur when either is bound with NLGN3. Cleavage via NLGN3 mechanisms is also present in homodimers (Bemben et al., 2019). Thus, NLGN3 specific heterodimerization may serve as a rapid method for modifying synaptic connections in response to neuronal activity.

At the synaptic junction, NLGN3-NRXN3 interactions are believed to modulate presynaptic functions in a retrograde manner and contribute to the alignment of presynaptic terminals with postsynaptic sites (Dean & Dresbach, 2006; Futai et al., 2007; Uchigashima et al., 2021). Additionally, there is significant evidence suggesting a role of NLGN3 in the regulation of synaptic transmission and plasticity processes. NLGN3 complexes are predominantly involved in modulating fast  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) transmission, although the adhesion

protein is thought to influence NMDAR-mediated transmission at excitatory synapses in hippocampal CA1 inhibitory interneurons, indicating synapse- and region-specific variability (Muellerleile et al., 2022; Polepalli et al., 2017; Uchigashima et al., 2021). These features are dependent on both extra and intracellular domains. Namely through trans-synaptic interactions, and within the intracellular domain the PDZ and gephyrin-binding interfaces are critical (Figure 1.2). In the context of AMPAR modulation, the NLGN3 PDZ motif interacts with postsynaptic density protein 95 (PSD95), a postsynaptic scaffolding protein, which anchors a range of molecular components including AMPARs, to the post synapse (Figure 1.2). Such NLGN3 PDZ-PSD95 interactions do not appear dependent on extracellular signalling (Dean & Dresbach, 2006; Uchigashima et al., 2021). The exact molecular mechanisms by which NLGN3 modulates AMPAR- and NMDAR-mediated excitatory transmission are not well understood, although post-translational modifications are thought to contribute to the observed synaptic specifications (Uchigashima et al., 2021).

Both *NLGN3* and *NRXN3* gene transcripts can generate alternatively spliced isoforms, with specific pairs typically only interacting with each other. There are four alternative splice variants of *Nlgn3* (*Nlgn3*(-), *Nlgn3A1*, *Nlgn3A2* and, *Nlgn3A1A2*) that have different levels of expression. For example, NLGN3(-) and NLGN3A2 are dominantly localised in hippocampal CA1 pyramidal cells (Uchigashima et al., 2020, 2021). The three main isoform types of NRXNs are alpha ( $\alpha$ NRXN-3), beta ( $\beta$ NRXN1-3), and the NRXN1-specific gamma ( $\gamma$ NRXN1). NLGN3 binds to alpha and beta NRXN3 isoforms with varying affinity in a calcium-dependent manner (Ichtchenko et al., 1996; Tabuchi & Südhof, 2002). It's believed that the isoform diversification is an important mechanism for synapse specification and formation and may play a part in the pathophysiological basis of ASD.

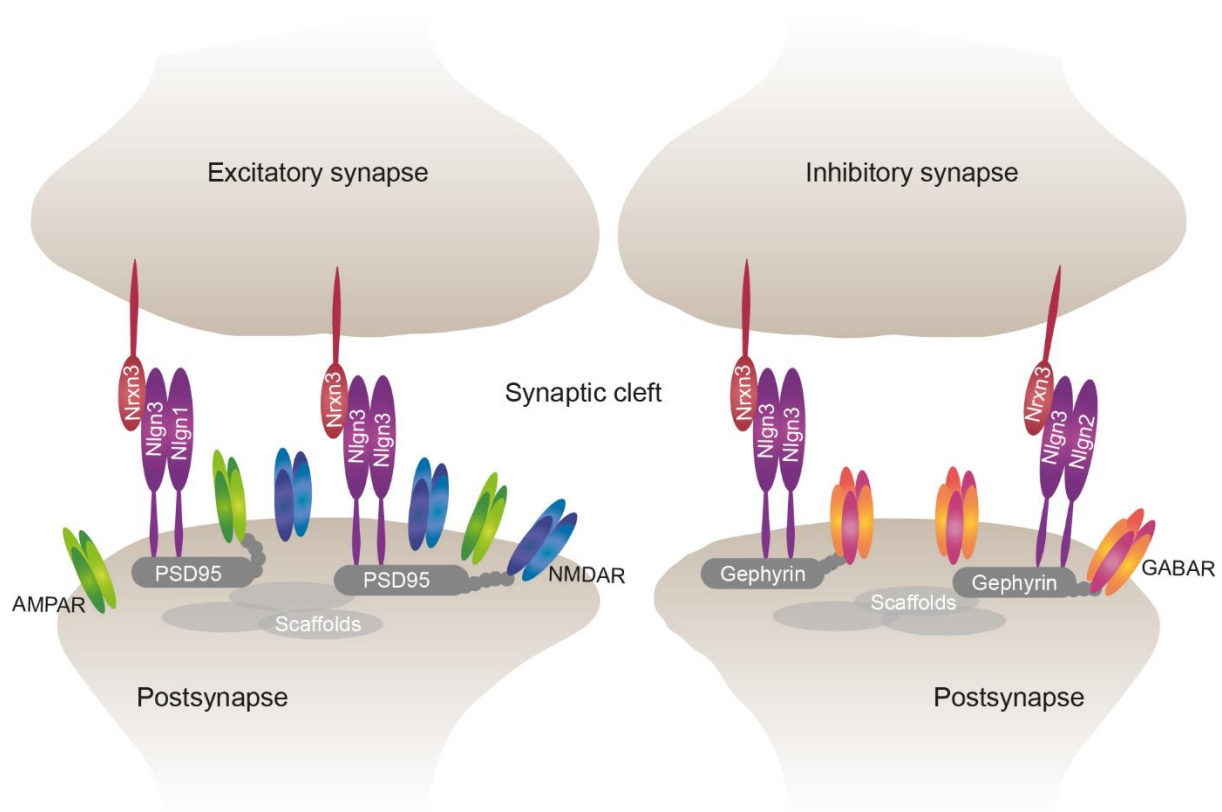


**Figure 1.2. Adhesion protein NLGN3 localises to excitatory and inhibitory synapses.** Dimerization of NLGN3 is required for NLGN-NRXN interaction. At the excitatory post synapse NLGN3 homodimerizes or heterodimerizes with NLGN1 to interact with NRXN3. The NLGN3 PTZ motif in the intracellular CTD interacts with PSD5, which anchors AMPAR and NMDAR receptors to the post synapse. NLGN3 modulates AMPAR- and NMDAR-mediated signalling, however the mechanisms for this are not well understood. At inhibitory synapses, NLGN3 forms either homodimers with itself or heterodimers with NLGN2, enabling interaction with NRXN3. Association with gephyrin, which clusters and stabilises GABA receptors at inhibitory synapses, occurs through the CTD gephyrin motif.

Until recently NLGNs were thought to exclusively interact with NRXNs, however, an emerging range of molecular partners have now been shown to bind NLGNs and compete with NRXNs at the N-terminal. For NLGN3, these include pre synaptically expressed type IIA receptor protein tyrosine phosphatase delta, and MAM domain-containing glycosylphosphatidylinositol anchor 1 and 2, which influence presynaptic differentiation and weakly block NLGN3-NRXN3 trans-synaptic interactions respectively (Elegheert et al., 2017; T. Yoshida et al., 2021). NLGN3 also binds with secreted protein acidic and rich in cysteine-like 1 (an extracellular matrix protein released from astrocytes), although it is not yet clear what effect the interaction contributes (Uchigashima et al., 2021).

#### 1.4.2.2 Spatiotemporal and cellular expression of *NLGN3*

Based on *NLGN3* mRNA studies in mice, *Nlgn3* expression begins around E12 throughout the central nervous system. During early postnatal development, expression levels are increased 2-3-fold and peak at postnatal weeks 2 to 3 in parallel with the onset of rapid synaptogenesis. These relatively high expression levels last into and throughout adulthood (Budreck & Scheiffele, 2007). The adhesion protein is detectable in neuronal and non-neuronal cell types, including astrocytes, myelinating oligodendrocytes and oligodendrocyte precursor cells, and expression levels are cell type specific (Proctor et al., 2015; Stogsdill et al., 2017; Uchigashima et al., 2021). For example, *NLGN3* expression is highest in oligodendrocyte precursor cells which form glutamatergic and GABAergic synapses with neurons in the hippocampus (Uchigashima et al., 2020). *NLGN3* is expressed globally, across the entire brain, with levels highest in the cortex, striatum, hippocampus and brainstem, and lowest in the cerebellum and thalamus (Uchigashima et al., 2021).

As with many synaptic receptors, *NLGN3* is expressed in an input dependent manner to facilitate circuit specific plasticity. For instance, in GABAergic neurons in the hippocampal CA1 region, inhibitory cannabinoid receptor CB1 or vesicular glutamate transporter (type 3) synapses selectively express *NLGN3*, while other major somatostatin or parvalbumin (PV) expressing synapses do not (Uchigashima et al., 2020). This is also observed in select subtypes of excitatory and inhibitory synapses in the cerebellum (Lai et al., 2021).

#### 1.4.2.3 Mutagenic spectrum and pathophysiology in *NLGN3* related neurodevelopmental disorder

The majority of *NLGN3* mutations identified in humans are missense, with deletion and frameshift variants also observed. The gene is relatively intolerant to missense changes and functional studies have revealed that the extracellular domain is a common site at which mutations occur (Quartier et al., 2019). A significant portion of variants alter protein structure (i.e. misfolded or unfolded protein), thereby impairing protein trafficking to the plasma membrane and leading to complete or near-complete functional loss of *NLGN3* expression at the postsynaptic membrane (De Jaco et al., 2010; Quartier et al., 2019). Paradoxically, functional studies on several missense variants identified in ASD and ID probands, including the originally discovered and most extensively researched R451C mutation, have observed both loss of function and gain of function phenotypes, whereby *NLGN3* is structurally compromised and retained in the endoplasmic reticulum, triggering the cellular unfolded protein

response (UPR) pathway (Jamain et al., 2003; Quartier et al., 2019). Cellular UPR is thought to participate in the pathogenicity of the mutation, through crosstalk with signalling pathways involved in energy and lipid metabolism, cell differentiation and immunity (Fujita et al., 2010; Hetz, 2012; Trobiani et al., 2018), while at the same time synaptic function of NLGN3 is lost. What is even more interesting, is that *NLGN3* variant gain and loss of function features may be circuit, cell or synapse type specific, indicating a context-dependent manner of pathophysiological NLGN3 expression.

As *NLGN3* is highly conserved between mammalian species, rodent models have offered significant insights into the role of NLGN3 in ASD and ID. Distinct abnormalities at the synapse are observed in several mouse and rat models with *Nlgn3* knockout and specific variant knock-in. Both complete knockout and conditional deletion of *Nlgn3* at P0 or P21 in mouse hippocampus do not affect excitatory synaptic transmission of CA1 pyramidal neurons (M. Jiang et al., 2017), which suggests a compensatory effect, likely from NLGN1, as reduced amplitude of AMPAR-mediated mEPSCs is observed in *Nlgn1/3* double knockout neurons, but not in *Nlgn1* or *Nlgn3* knockout neurons (Chanda et al., 2017). In stark contrast, the same neuronal population in mice harbouring the *Nlgn3*-R451C variant have increased AMPAR-mediated synaptic transmission and enhanced NMDAR-mediated LTP, which authors concluded is due to elevated expression of GluN2B-containing receptors (Etherton et al., 2011). Surface protein expression is reduced by 90% in this variant, and these data underscore the concordant gain of function effect of expressed mutant NLGN3 on synaptic properties and transmission. The role of NLGN3 on synaptic plasticity and remodelling is not well understood and few studies have evaluated this in detail. Thus far, research looking at LTD in constitutive *Nlgn3* knockout mice reports contrasting results, with either loss of metabotropic glutamate receptor-mediated LTD or no observed effect in cerebellar Purkinje cells (Martella et al., 2018; B. Zhang et al., 2015). LTD impairment is also observed at cortico-striatal excitatory synapses of *Nlgn3*-R451C knock-in mice, while as mentioned, LTP is enhanced at hippocampal excitatory synapses (Etherton et al., 2011; Martella et al., 2018).

NLGN3 has differential roles on postsynaptic specializations, with varying influence dependent on synapse type (excitatory or inhibitory), region, circuit and neuronal population. In CA1 in hippocampus, unlike in pyramidal cells discussed above, mouse PV expressing interneurons lacking NLGN3 have reduced NMDAR-mediated EPSCs and normal AMPAR-mediated synaptic transmission (Polepalli et al., 2017). Similarly, mice with the *Nlgn3*-R451C variant, showed enhanced excitatory transmission in hippocampus with no change in inhibitory signalling, however, increase in inhibitory synaptic transmission is observed in principal neurons in layer II/III somatosensory cortex. This signifies that the effect of *Nlgn3*-R451C mutation on excitatory and inhibitory transmission is different depending on the region or circuit involved (Etherton et al., 2011). A recent publication in *Nlgn3* knockout mice revealed increased neuronal excitability in the hippocampal CA2 region, characterized by enhanced AMPAR-mediated sEPSC frequency, which was due to strongly diminished cholecystokinin (CCK) expressing basket interneuron mediated inhibition, leading to altered network

oscillatory activity (Modi et al., 2019). Conversely Foldy *et al.*, showed that in *Nlgn3* knockout mice, inhibitory synaptic transmission is enhanced in hippocampal CA1 CCK basket cells, but not in PV interneurons, suggesting that in the absence of NLGN3, changes in GABAergic signalling may be region and cell specific. The CCK basket neurons phenotype was replicated in *Nlgn3*-R451C knock-in mice, which also had reduced inhibitory post synaptic current (IPSC) amplitude in PV expressing neurons (Földy et al., 2013). Furthermore, inhibitory synaptic transmission in the somatosensory cortex of these mutant mice is markedly enhanced, whereas *Nlgn3* knockout mice show no such increase, highlighting the distinct effects of this specific mutation compared to complete protein loss of the adhesion protein (Speed et al., 2015; Tabuchi et al., 2007). Notably, inhibitory potentiation and suppression may be dependent on NLGN3 isoforms, as is observed in the hippocampus (Uchigashima et al., 2020).

Altered cellular excitability and synaptic transmission is also observed outside of hippocampal and cortical circuitry. In striatal circuits both complete knockout and mutant *Nlgn3*-R451C knock-in mice selectively reduces miniature IPSC (mIPSC) frequency in D1-dopamine receptor-expressing neurons (Rothwell et al., 2014). Importantly, disrupted excitation and inhibition balance in striatal development and connectivity is known to contribute to abnormal reward circuitry and repetitive behaviours in ASD (Di Martino et al., 2011; Langen et al., 2014). Novelty-induced AMPAR-mediated synaptic plasticity at glutamatergic synapses onto dopamine neurons in the ventral tegmental area (VTA) is disrupted in *Nlgn3* knockout mice and mice with conditional *Nlgn3* knockdown in VTA dopaminergic cells. AMPAR trafficking and insertion was dysregulated in these mice, suggesting a crucial role for NLGN3 in this process (Bariselli et al., 2018). In the dorsal periaqueductal grey (PAG) of adult *Nlgn3* knockout rats, a midbrain region previously shown to control fear expression, neurons were found to be intrinsically hyperexcitable (Anstey et al., 2022). The basolateral amygdala is involved in social behaviour. Here, Hosie and colleagues found increased amplitude of mEPSCs, concordant with decreased IPSC amplitude in pyramidal-like neurons in *Nlgn3*-R451C (Hosie et al., 2018). Collectively, these studies demonstrate a likelihood for globally altered network activity in response to *Nlgn3* mutations.

Finally, work from Zhang and colleagues provided evidence that the overall phenotypes observed in *NLGN3* pathophysiology are likely also shaped by developmental plasticity and critically paced compensatory mechanisms. They showed that excitatory synaptic transmission at the calyx synapse, a synapse of the auditory system found in the medial nucleus of the trapezoid body, is not affected by germline *Nlgn3* knockout, but conditional re-expression of the gene later in development at P12 resulted in dramatically impaired synaptic transmission due to decrease in postsynaptic AMPARs. Interestingly, this result was recapitulated in *Nlgn3*-R451C knock-in mice, which had reduced mEPSC and postsynaptic AMPAR content at the calyx synapse, but not in *Nlgn3*-R704C knock-in mice.

Arguably, these mutations contribute near complete and 30% loss of NLGN3 expression respectively, and this could influence the discrepancy in observed phenotypes (B. Zhang et al., 2017).

*Nlgn3* mutations appear to predominantly affect synaptic transmission, and morphological features of dendrites do not appear to be influenced (Földy et al., 2013; Polepalli et al., 2017; Tabuchi et al., 2007), although some reports suggest there could be altered dendritic properties in specific regions. For instance, hippocampal pyramidal neurons from *Nlgn3*-R451C knock-in mice were shown to have significantly more dendritic branch points in the stratum radiatum, but not elsewhere in the hippocampus, while dendritic spine density remained unaltered (Etherton et al., 2011). Ultimately, research on *Nlgn3* mutations in rodent models reveals complex, region-specific effects on synaptic transmission that may contribute to ASD-related behaviours. *Nlgn3* knockout mice and mice with the *Nlgn3*-R451C variant exhibit altered neuronal excitability across the hippocampus and in various cortical and striatal circuits. These diverse changes in excitatory and inhibitory balance across brain regions suggest that *Nlgn3* mutations lead to widespread alterations in network activity, potentially driving the behavioural phenotypes associated with ASD.

A range of ASD-linked behavioural phenotypes, including social interaction and repetitive stereotypical activity have been identified in *Nlgn3* rodent models. *Nlgn3* knockout mice have been shown to exhibit impaired olfaction and social novelty preference, subtle reduction in brain size, as measured by MRI, and reduced USVs reminiscent of altered language acquisition and communication, which are core ASD phenotypes in humans (Modi et al., 2019; Radyushkin et al., 2009). Interestingly, these animals have slightly enhanced learning and memory compared to wildtypes. Hyperactivity and stereotypical repetitive behaviours, wall jumping and abnormal bias in directional rotation during locomotion are also observed (Radyushkin et al., 2009; Rothwell et al., 2014). Further evidence for network activity imbalance comes from hyperactive *Nlgn3* knockout mice with impaired social interaction and memory, which have reduced gamma and theta power in CA2 and CA3 hippocampal regions due to impaired inhibitory transmission in the CA2 area (Modi et al., 2019). Altered social interaction and memory was also found in *Nlgn3* knockout rats (Hamilton et al., 2014), which were shown to be hyperactive, and have altered sleep physiology and EEG power spectral features, most notably reductions in the beta (12-30 Hz) and gamma (30-50 Hz) range (Thomas et al., 2017). In a third recent study this knockout line exhibited altered fear expression, which consisted of reduced freezing and an increased ‘flight’ jump response (Anstey et al., 2022). Decreased freezing duration is also described in *Nlgn3* knockout mice (Radyushkin et al., 2009).

Many of these behavioural abnormalities are replicated in *Nlgn3*-R451C knock-in mice. Animals are hyperactive and display social interaction deficits (Etherton et al., 2011; Jaramillo et al., 2014; Tabuchi et al., 2007), as well as repetitive behaviours (Burrows et al., 2015; Rothwell et al., 2014). It was recently shown that impaired social behaviours correlated with reduced medial prefrontal cortex

(mPFC) activation and lower gamma band activity, and authors concluded that social abnormalities could be a result of disrupted functional encoding in mPFC during social interaction (W. Cao et al., 2018). Similarly to the knockout phenotype, spatial learning and memory is enhanced (Jaramillo et al., 2014; Tabuchi et al., 2007), while two independent studies observe an aggression phenotype in animals with this mutation (Burrows et al., 2015; Hosie et al., 2018). Sleep fragmentation with altered EEGs spectral power spectral profiles during sleep and wake is also documented in *Nlgn3*-R451C knock-in mice (J. J. Liu et al., 2017). Flexible responding and reaction time appears to be reduced in both R451C knock-in and germline knockout mouse lines (Norris et al., 2019).

Overall, pathogenic *NLGN3* mutation most commonly result in lack of synaptic protein expression, the effect of which is synapse, cell and network specific. Thus far rodent models have given insights into specific network and signalling alterations associated with *NLGN3* deficit, particularly those responsible for learning, memory and social behaviour. Irregular signalling and disrupted excitation and inhibition balance is observed in the hippocampus, cortex, amygdala, VTA and the periaqueductal grey. Moreover, behavioural abnormalities in rat and mouse lines have high face validity to the clinical presentation of *NLGN3* associated ASD, making them ideal for future investigation into aberrant circuit activity and NDD related phenotypes.

### 1.4.3 *GRIN2B* related neurodevelopmental disorder

*De novo* variants of the *GRIN2B* gene were first identified by O’Roak and colleagues over a decade ago in a proband of individuals with ASD (O’Roak et al., 2011). Since then, an abundant pool of pathological *GRIN2B* mutation have been found and the predicted diagnostic incidence of pathogenic *de novo GRIN2B* variants in NDD cases is estimated to be 5.91 per 100.000 births (Endele et al., 2010; Lemke, 2020; Lemke et al., 2014; Takasaki et al., 2016; Tarabeux et al., 2011). *GRIN2B* related NDDs affect both males and females and the overwhelming majority of affected individuals present with developmental delay and severe ID with or without ASD features (Platzer et al., 2017). Mutations in *GRIN2B* are also a cause of known syndromic NDDs including West and Lenox-Gastaut syndrome, characterized by a constellation of epileptic infantile spasms, abnormal EEG activity and ID (Lemke et al., 2014). Epilepsy is observed in approximately half of patients (52%) with a range of seizure types including focal, generalized (mostly tonic or tonic-clonic), epileptic spasms and absence seizures (Platzer et al., 2017). Seizure onset age in *GRIN2B* related NDDs is varied and ranges from around birth to nine years of age, and similarly different rates of seizure occurrence are observed, from multiple per day to a few seizures per year (Platzer et al., 2017). Other clinical presentations include movement disorders, loss of muscle tone, cortical visual impairment and developmental regression. Dysfunctional sleep is common amongst individuals with *GRIN2B* mutations, and parental reports

indicate at least 60% of patients struggle initiating and maintaining sleep, have breathing irregularities during sleep and suffer with daytime somnolence (Freunscht et al., 2013; Platzer et al., 2017; Simons Searchlight, 2021). MRI findings from *GRIN2B* patients include gross structural brain abnormalities, microcephaly, malformation of cortical development and generalised cerebral volume loss, which are also observed in other *GRIN*-related NDDs (Platzer et al., 2017).

#### 1.4.3.1 N-methyl-D-aspartate receptors

*GRIN2B* encodes the GluN2B subunit of NMDARs, which are tetrameric ligand-gated ionotropic glutamate receptors critical for normal development of the nervous system and synaptic plasticity (Hansen et al., 2021). NMDARs are composed of two obligate glycine or D-serine binding GluN1 subunits and either two GluN2 or GluN3 subunits, or a combination of both. GluN2 subunits bind glutamate, while GluN3 subunits bind glycine (Chatterton et al., 2002; Cull-Candy & Leszkiewicz, 2004; Hansen et al., 2021). NMDARs are sodium, potassium and calcium permeable and display unique properties that strongly impact developmental and long-term changes in cellular function. For one, they are coincidence detectors, activated only when voltage-dependent magnesium block is relieved from the channel pore, and co-agonist binding with GluN1 and GluN2/ 3 subunits is achieved. This typically only occurs when both pre- and postsynaptic cells are excited in unison (Cull-Candy & Leszkiewicz, 2004; Hansen et al., 2021), highlighting the activity-dependent role of these channels geared towards synaptic strengthening. Fundamental to this are two core properties of NMDARs – calcium permeability and the slow rise (~10 ms) and decay (> 100 ms) receptor kinetics that contribute to the generation of long-lasting excitatory post synaptic currents (EPSCs) (Cull-Candy & Leszkiewicz, 2004). These two features allow for significant calcium influx into the post-synapse, and therefore facilitate intracellular calcium signalling. In neurons, calcium can act as a second messenger that activates a cascade of intracellular events that modulate synaptic strengthening through long-term potentiation (LTP) or long-term depression (LTD) and regulate gene expression and cellular enzymatic activity (Hansen et al., 2021).

Each NMDAR subunit is made up of four separate modules (Figure 1.3) (Mayer, 2011; Paoletti et al., 2013; Traynelis et al., 2010a). These include the amino (N)-terminal domain (NTD), involved in allosteric regulation and subunit assembly, and the agonist-binding domain (ABD) formed of two discontinuous segments (S1 and S2) located in the extracellular space. The third module is the TMD, which is composed of three transmembrane helices (M1, M3 and M4) and a pore loop (M2), essential for ion channel selectivity, permeability, and regulation by voltage-dependent magnesium block. Finally, the carboxyl (C)-terminal domain (CTD), in the intracellular region, is involved in receptor trafficking, anchoring and coupling to signalling molecules (Figure 1.3) (Paoletti et al., 2013).

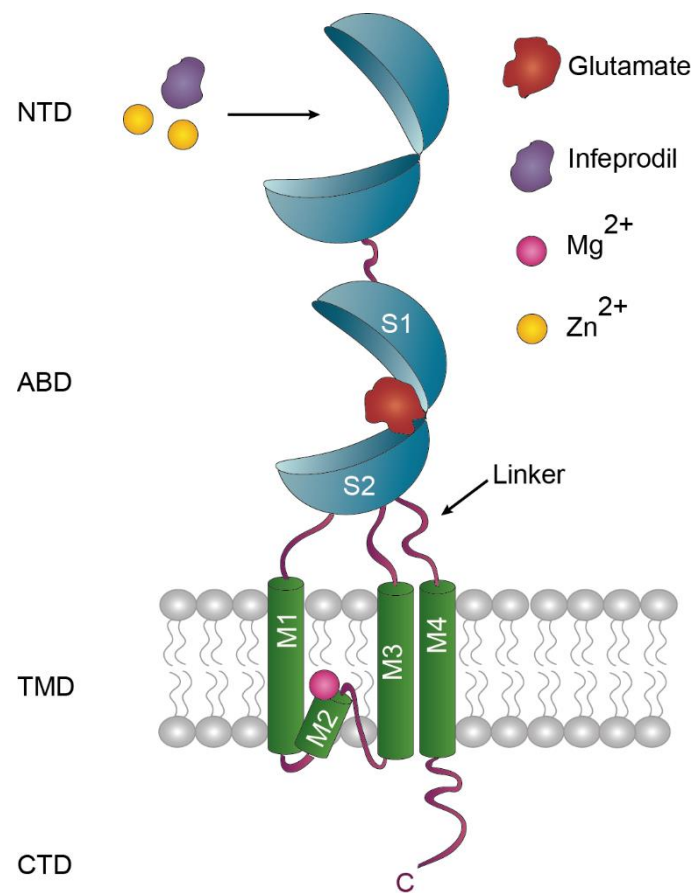


**Figure 1.3. GluN2B subunit structure.** The GluN2B subunit is modular and made of four distinct domains: the NTD, the ABD that binds to glutamate, the TMD containing the ion channel, and an intracellular CTD. The NTD and CTD are the most divergent regions. Interaction sites for selective antagonists ifenprodil and zinc are found in the NTD, and glutamate binding occurs within S1 and S2 ABD segments. The M2 unit forms the channel pore loop and is essential for channel ion selectivity, permeability, and regulation by voltage-dependent magnesium block. Schematic adapted from Paoletti et al. (2013).

Most native NMDARs appear to function as diheteromeric assemblies composed of two GluN1 subunits and two GluN2 subunits, of which there are four paralogs GluN2(A-D) that confer differential channel properties and activity dynamics including deactivation time courses, agonist potencies, open probabilities, single-channel conductances and sensitivities to magnesium and extracellular modulators (Cull-Candy & Leszkiewicz, 2004; Wyllie et al., 2013a; Yuan et al., 2009). In general, the deactivation time of diheteromeric GluN1/ GluN2 NMDARs can vary 50-fold depending on the GluN2 subunit composition, with the longest and shortest EPSCs observed in GluN2D and GluN2A respectively (Cull-Candy & Leszkiewicz, 2004; Paoletti et al., 2013). GluN2B and GluN2C show similar kinetics, with GluN2B diheteromeric receptors exhibiting slightly longer deactivation constants of around 400 ms (Yuan et al., 2009). Similarly, NMDARs composed of two GluN2B subunits require intermediate

glutamate potency for activation, with an  $EC_{50}$  value reported at 2.5  $\mu$ M concentration, and have an intermediate level of single-channel conductance of about 40 pS (Erreger et al., 2007; Vieira et al., 2020; Wyllie et al., 2013a). Finally, sensitivity to magnesium blockade and calcium permeability, which reduce channel activity and cation flow dynamics respectively, are high in GluN2B containing NMDARs when compared to GluN2C/ D containing receptors (Vieira et al., 2020; Wyllie et al., 2013a). The distinct differences in the latter three properties of the ion-conducting pore, are regulated by a single GluN2 residue in the M2 loop of the subunit TMD and are the key determinants of the relative influence of NMDAR subtypes on synaptic integration and plasticity (Retchless et al., 2012).

In addition to glycine-glutamate activation, NMDARs are further modulated by pharmacological compounds and endogenous extracellular protons, zinc ions and polyamines, with sensitivity to cations determined by subunit composition. GluN2B containing receptors are preferentially inhibited by protons, while extracellular polyamines are positive allosteric modulators of these receptors and specifically enhance their activity by stabilizing the NTD interface. In contrast, zinc ions antagonise GluN2B and GluN2A NMDARs, by interacting with the subunit zinc binding sites, which are also located in the NTD. GluN2B subunits display significantly lower affinity for zinc ( $IC_{50} \sim 2 \mu$ M) in comparison to GluN2A receptors ( $IC_{50} < 1 \mu$ M), and notably, are highly sensitive to the pharmacological compound ifenprodil (and its derivatives), which similarly act on the NTD to inhibit the receptor (Paoletti et al., 2013; Vergnano et al., 2014). This contrasting sensitivity to zinc and ifenprodil is typically used to differentiate between the GluN2B and GluN2A containing NMDARs (Paoletti et al., 2013). Finally, there are eight functional isoforms of the GluN1 subunit, and each variant additionally influences certain ion and ligand binding NMDAR properties, including zinc and proton inhibition and potentiation by polyamines (Cull-Candy & Leszkiewicz, 2004).

NMDARs co-expressing GluN3 are believed to predominate during early postnatal development, with certain reports suggesting they play a functional role in synaptic maturation (Henson et al., 2010; Paoletti et al., 2013). Mechanistically these receptors appear to act as regulatory modules, which moderate receptor activity by either reducing channel current flow or by altering active channel properties, such as rendering receptors impermeable to calcium ions and conferring resistance to magnesium block (Cavara et al., 2009; Chatterton et al., 2002; Pérez-Otaño et al., 2001).

#### 1.4.3.2 Spatiotemporal expression patterns and localization of GluN2B-NMDARs

NMDARs are distributed throughout the central nervous system with the GluN1 subunit expressed from embryonic stage 14 (E14) to adulthood. In contrast the spatiotemporal expression of the four GluN2 subunits, which confer the differing NMDAR properties discussed above, are significantly

different from each other (Figure 1.4). In rodents, during embryonic development, GluN2B expression is high and ubiquitous throughout the brain, while GluN2D is also present but restricted to caudal brain regions (Akazawa et al., 1994; Monyer et al., 1994; Paoletti et al., 2013; Vieira et al., 2020). Following birth, the first two postnatal weeks are marked with clear changes in expression and localization patterns of GluN2 subunits. Peak expression of GluN2B happens in the first postnatal week, after which it is progressively restricted to the forebrain (Figure 1.4) (Paoletti et al., 2013; Vieira et al., 2020). This restriction of GluN2B coincides with the start of GluN2A expression, which happens shortly after birth, at around postnatal day 6 (P6), and increases steadily until the subunit becomes abundant across the entire brain (Figure 1.4) (Myers et al., 2019; Sheng et al., 1994). The shift in expression patterns between these two subunits is known as the 2A/2B switch and it is strongly influenced by synaptic activity during critical developmental periods, when circuit formation and refinement is especially sensitive to experience. By the end of postnatal development (~P28 in rats), NMDAR composition in many structures, including the cortex and thalamus changes from predominantly GluN1/GluN2B to a combination of GluN1/GluN2B, GluN1/GluN2A and GluN1/GluN2A/GluN2B receptors (Myers et al., 2019). Lastly, GluN2C is expressed in the olfactory bulb and cerebellum, with expression beginning later in postnatal development at P10, while GluN2D expression drops markedly and is mostly confined to mid brain structures (i.e. the mesencephalon and diencephalon) (Figure 1.4) (Monyer et al., 1994; Paoletti et al., 2013; Vieira et al., 2020).



**Figure 1.4. The developmental profile and spatiotemporal expression patterns of GluN2 subunits in mice.** *GluN2B predominates in expression prenatally and in early postnatal development and governs NMDAR signalling activity. During this time GluN2D expression is mostly restricted to caudal regions. Expression of GluN2A begins at P6 and increases steadily, while GluN2B expression slowly shift to the forebrain. GluN2C expression starts at P10 and is restricted to the cerebellum and olfactory bulb. By P14, ample expression of all NMDAR subunits is achieved, and spatiotemporal patterns continue to shift until adult distribution is attained. In the adult brain GluN2B expression is restricted to the forebrain. Schematic adapted from Paoletti et al. (2013).*

In addition to developmental and regional expression dynamics, differential expression of NMDAR subunits is also observed in some distinct neuronal populations, such as in inhibitory cortical and hippocampal neurons which predominantly express GluN2C and GluN2D NMDARs (Monyer et al., 1994; Perszyk et al., 2016). Moreover, multiple receptor subtypes can be found in the same cell at different inputs, suggesting a finely tuned contribution of each node to a particular network. This scenario is observed in layer V pyramidal neurons, where in the same cell, GluN2B containing receptors primarily mediate intracortical synaptic inputs and GluN2A containing receptors mediate those arising from callosal inputs (S. S. Kumar & Huguenard, 2003). Similarly, in CA3 pyramidal neurons of the adult hippocampus, GluN2B subunits are present in dendrites that receive synapses from the entorhinal cortex, but not from the dentate gyrus (Fritschy et al., 1998). The above observations have prompted some authors to conclude that the preference for GluN2B NMDARs at specific inputs is due to their biophysical features. Namely, the slower receptor decay kinetics that contribute to more efficient excitatory postsynaptic potential (EPSP) summation, and therefore a more rapidly attained firing threshold in neurons, is presumed to allow cells to integrate information differentially depending on input origin (Cull-Candy & Leszkiewicz, 2004).

The subcellular localisation of NMDAR subunits varies and is also developmentally regulated. During early development GluN2B-containing NMDARs are distributed throughout dendrites and as synaptogenesis advances many receptors localise to the postsynapse, while some remain in extrasynaptic regions. Both synaptic and extrasynaptic NMDARs play a part in shaping the postsynaptic response (Cull-Candy & Leszkiewicz, 2004; Vieira et al., 2020). In the adult forebrain, GluN2B forms di and triheteromeric (GluN1/GluN2A/ GluN2B) assemblies at glutamatergic synapses, along with GluN1/GluN2A receptors that are predominantly located at most postsynaptic sites (Paoletti et al., 2013). The inverse is true for extrasynaptic receptors, which are overwhelmingly GluN2B expressing (Vieira et al., 2020). The physiological function of the latter localised NMDARs is not fully understood, although some evidence suggests they facilitate LTD, as well as glutamate-mediated extrasynaptic inhibition through coupling with calcium activated potassium channels (Specchio & Curatolo, 2021). A final note is that the synaptic NMDAR subunit composition can be dynamically modified throughout life in an experience dependent manner, enabling plasticity to occur (Kellermayer et al., 2018).

#### 1.4.3.3 Mutagenic spectrum and pathophysiology in *GRIN2B* related neurodevelopmental disorder

Typically, symptoms in individuals with *GRIN2B* related NDDs present within the first few years of life and molecular diagnostics have identified a large pool of likely pathogenic mutations attributed to the disorder. Mutations are most frequently located within or adjacent to the ABD and the

TMD, with a high proportion identified as missense and truncation/ haploinsufficiency variants (Figure 1.5) (Platzer et al., 2017). Functionally, variants appear to interfere with NMDAR gating and kinetic properties, sensitivity to glutamate and cations (magnesium, zinc and protons), calcium permeability and subunit trafficking to the plasma membrane (Kellner et al., 2021; S. Liu et al., 2017a; Platzer et al., 2017; X.-X. Xu & Luo, 2018). While the pathogenic profile of *GRIN2B* is still expanding, variants have thus far been shown to result in either gain or loss of function phenotypes, or alternatively contribute a dominant negative effect on NMDAR function by interfering with the function of wild-type GluN2B subunits (Lemke et al., 2014; Platzer et al., 2017).

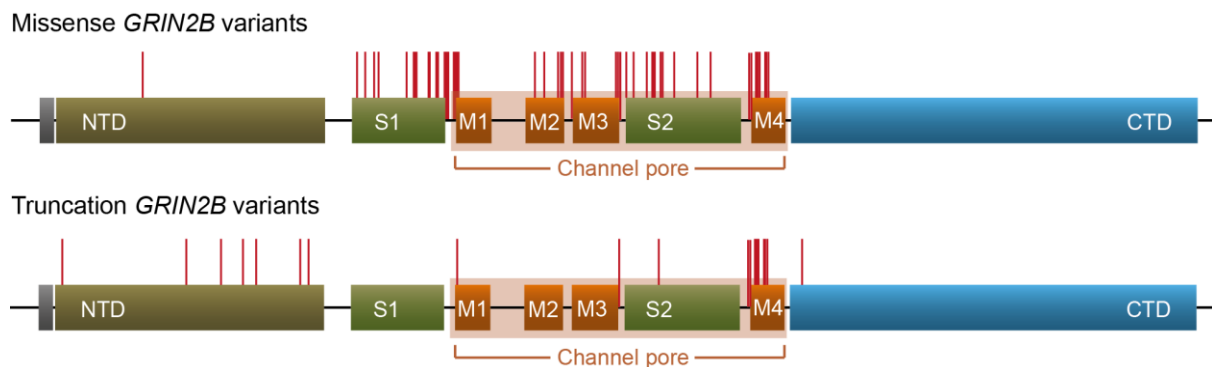


**Figure 1.5. De novo variants in *GRIN2B*.** Top: The majority of pathogenic *GRIN2B* variants missense mutations concentrate at the ABD and TMD. These areas show minimal benign missense variation in the general population. Only one (likely) pathogenic missense variant is found outside the clustered region in the NTD. Bottom: Location of pathogenic *GRIN2B* truncating variants located in the ABD, TMD and NTD. Red bars indicate pathogenic or likely pathogenic de novo variants. Figure adapted from Platzer et al. (2017) and Lemke et al. (2014).

Given *GRIN2B* NDDs are monogenic in nature, a variety of heterologous expression systems, as well as transgenic mouse and rat models have been utilised to study the behavioural phenotypes associated and the relevant pathophysiological mechanisms contributing to this disorder.

Beginning with neurodevelopment, the dominant spatiotemporal expression observed for GluN2B during embryonic and early postnatal periods implied that the subunit could be critically involved in multiple developmental processes, including neuronal differentiation and maturation, circuit formation and plasticity. Seminal evidence for this came from Kutsuwada et al., who showed that full knockout of GluN2B in mice resulted in perinatal lethality, with low body mass indices (BMIs), defects in the barrel cortex formation, and complete lack of suckling responses in neonatal pups (Kutsuwada et al., 1996). Interestingly, a homozygous truncating mutation in GluN2B CTD, impaired intracellular signalling but not channel properties and also resulted in lethality shortly after birth in mice, highlighting a fundamental recruitment of the subunit in molecular cascades (Sprengel et al., 1998).

Subsequent work by Wang and colleagues showed that one copy of GluN2B was sufficient for essential NMDAR function including synaptic plasticity and protein synthesis regulation via the subunits interaction with alpha calcium-calmodulin kinase II (CaMKII) and regulation of the mTOR pathway (C.-C. Wang et al., 2011). Premature postnatal replacement of GluN2B with GluN2A does not rescue plasticity related deficits, underscoring the crucial role of GluN2B-NMDAR specific signalling during early postnatal development. More recent studies looking at developing neurons in the prenatal brain found that insufficient expression of GluN2B interferes with both differentiation and migration processes. In the former, differentiation of induced pluripotent stem cell (iPSC) derived human forebrain neural progenitor cells was suppressed in response to *GRIN2B* haploinsufficiency (Bell et al., 2018). In the latter, impaired radial migration of cortical neurons from layer VI/ V to superficial layers was observed in rat pups following in utero knockdown of the *Grin2b* gene (H. Jiang et al., 2015). Notably, in both cases, the detrimental impact of *GRIN2B* haploinsufficiency appeared to be driven by overall developmental NMDAR hypofunction rather than by GluN2B specific cellular and molecular interactions. Furthermore, in a novel in utero patch clamp and imaging modality, Munz *et al.* were able to demonstrate the existence of transient cortical circuit motifs between E14 and 17 in Rbp4-Cre embryonic neurons, which are precursors to layer V pyramidal cells. It was also shown that in GluN2B heterozygous knockout mice these circuit assemblies were perturbed, with insufficient GluN2B expression evidently impairing the earliest steps in cortical development (Munz et al., 2023).

In early postnatal life, GluN2B-expressing NMDARs are critical for the functional maturation of excitatory synapses, with potentially opposing effects in pyramidal cells and interneurons. As excitatory synapses develop and mature, there is a crucial shift in glutamate receptors inserted into the plasma membrane. Typically, AMPAR-mediated neurotransmission is dominant at mature synapses, which have increased AMPA/NMDA current ratios (B. J. Hall et al., 2007). In mice with conditional knockout of GluN2B in hippocampus, AMPAR-EPSCs and miniature EPSC (mEPSC) frequency are increased in CA1 pyramidal neurons (Gray et al., 2011), while increased mEPSC amplitude was reported in dissociated cortical cultures from GluN2B knockout mice (B. J. Hall et al., 2007). These observations suggest that incorporation of AMPARs at developing synapses is inhibited by GluN2B signalling until strong correlated activity coupled with sufficient calcium entry can override inhibitory mechanisms (likely through LTP-related mechanisms), allowing for induction of synaptic maturation (Gray et al., 2011; B. J. Hall et al., 2007). In contrast, GluN2B signalling appears to have a pro-maturation effect on developing glutamatergic inputs in inhibitory cells. Hippocampal interneuron mEPSC frequencies were found to be significantly decreased in mice with GluN2B deletion restricted to GAD67-expressing neurons (Kelsch et al., 2014), and similar observations were made in mice with conditional GluN2B knockout in adult-born olfactory bulb inhibitory cells (Kelsch et al., 2012). Furthermore, mice lacking GluN2B in interneurons presented with severe seizures and died of status epilepticus, likely as a result of inadequate recruitment of GABAergic neurons, in which glutamatergic

synapses are insufficiently matured (Kelsch et al., 2014). The two opposing roles of GluN2B NMDARs, likely result in premature maturation of synapses in excitatory cells and maladaptive integration of interneurons into developing circuits, thereby perturbing the balance between excitation and inhibition balance and contributing to pathology.

In addition, numerous studies indicate that GluN2B regulates the growth, arborization and patterning of dendrites, as well as dendritic spine properties (Espinosa et al., 2009; Ewald et al., 2008). Dendritic morphology is a key determinant of synaptic connectivity, and structural defects could result in compromised circuit organisation, synaptic integration, and plasticity (Martínez-Cerdeño, 2017). It's believed that GluN2B-containing NMDARs may contribute to the formation and long-term stability of dendritic spines. In the hippocampus, decreased dendritic spine density has been observed in CA1 and CA3 subregions in a conditional GluN2B knockout mouse model with subunit deletion restricted to excitatory cells in the cortex and hippocampus and in cultured hippocampal neurons (Akashi et al., 2009; Brigman et al., 2010; M. J. Kim et al., 2005). Further evidence of impaired dendritic morphogenesis comes from research specifically looking at disease associated *GRIN2B* variants in cell cultures transfected with GluN2B pathological variants. In these studies, mutations that impair ligand-binding and CTD interactions, were shown to reduce receptor activation and subunit trafficking respectively, resulting in reduced dendritic spine density, dendrite length, complexity, and branching patterns in developing pyramidal and hippocampal neurons (Bahry et al., 2021; S. Liu et al., 2017a; Sceniak et al., 2019a). Notably, these variants appeared to contribute a dominant negative effect. In contrast, single cell analysis of dendrites from cortical layer IV spiny stellate cells of the barrel cortex showed normal dendritic morphology in homozygous and heterozygous knockout neurons, although patterning was irregular with ectopic barrelette targeting in the former (Espinosa et al., 2009). The observation that partial knockdown of the subunit resulting in just haploinsufficiency has more minor consequences on dendrite development, implies that morphological irregularities in dendrites could be dosage and/ or region specific. In sum, GluN2B-containing NMDARs likely play a critical role in the functional maturation of excitatory synapses during early postnatal life, exhibiting opposing effects on pyramidal cells and interneurons, through regulating AMPAR incorporation and dendritic growth properties, ultimately influencing synaptic connectivity, integration, and the balance between excitation and inhibition.

Loss of GluN2B impairs synaptic plasticity. Hippocampal CA1 LTD was near completely impaired in neonatal hand-reared mice with conventional homozygous knockout of GluN2B (Kutsuwada et al., 1996) and in adult mice with homozygous GluN2B deletion restricted to pyramidal neurons in the hippocampal CA1 region and cortex (Brigman et al., 2010). These results are recapitulated in a knock-in mouse line heterozygous for the *Grin2b*-C456Y variant, located within the ABD and first identified in a male with ASD and ID (O'Roak et al., 2011; Shin et al., 2020). Knock-in of the mutation reduced CA1 GluN2B-mediated EPSCs and significantly reduced NMDAR-dependent

LTD in CA1 and in layer I medial prefrontal cortex (mPFC) (Shin et al., 2020). A similar reduction in NMDAR-EPSCs and decreased GluN2B surface protein expression were observed in adult rats with the *Grin2b*-Trp373 NTD (amino-terminal binding portion) mutation, previously identified in an individual with ASD, ID and epilepsy (Platzer et al., 2017; X. Wang et al., 2022). In an earlier study, impaired LTP at hippocampal fimbrial-CA3 synapses was observed in mice with heterozygous GluN2B deletion (Ito et al., 1997). Thus, loss of GluN2B may disrupt synaptic plasticity by affecting LTP and LTD, leading to aberrant network activity and NDDs.

The neuro-morphological and plasticity impairments likely result in the cognitive deficits and behavioural abnormalities associated with *GRIN2B* related NDDs. Individuals with *GRIN2B* mutations present with variable symptoms, of which ASD, ID and epilepsy are most commonly observed and ASD and ID-like behaviours are replicated by several mouse lines with abnormal GluN2B expression. Brigman and colleagues identified impaired corticohippocampal-mediated learning and memory in mice, which do not express GluN2B in cortical and CA1 hippocampal pyramidal neurons. Mutant animals presented with a profile of selective deficits in spatial reference and working memory, as well as associative learning, which collectively relate to impaired network activity in ASD and ID cases (Brigman et al., 2010). These results are complementary to an earlier study that showed similar spatial learning and memory impairment, and visual discrimination deficits in mice with GluN2B knockout in principal neurons in the entire forebrain, and mice with GluN2B knockout in hippocampal excitatory cells only. In addition, animals from both models presented with hyperactivity and reduced anxiety phenotypes (von Engelhardt et al., 2008). ASD-like repetitive self-grooming, hypoactivity with brief anxiolytic-like behaviours were also observed in mice with conventional heterozygous knockout of *Grin2b* and in mice carrying one copy of the *Grin2b*-C456Y ABD variant. Interestingly, pups in the latter model also showed an increased anxiety phenotype, measured by ultrasonic vocalizations (USVs) upon mother separation (O’Roak et al., 2011; Shin et al., 2020). Mice in which perinatal expression of GluN2B subunits is replaced with GluN2A, recapitulate the behavioural phenotype of hyperlocomotion and altered sociability associated with schizophrenia (C.-C. Wang et al., 2011). The impaired learning and memory, abnormal activity patterns, anxiety phenotypes, and repetitive self-grooming in these models are likely consequences of the altered neuronal morphological and plasticity features associated with *Grin2b* mutations, leading to dysfunctional circuitry.

Few studies have successfully recaptured epileptic activity in models of *GRIN2B* related NDDs. Kelsche et al. reported severe seizures in 2–3-week-old mouse pups that lacked GluN2B in inhibitory neurons. Pups die following status epilepticus towards the end of the third postnatal week. Unfortunately, this phenotype was not further examined, although the authors conclude that the lack of synaptic maturation and recruitment of interneurons into developing circuits likely results in acute excitotoxicity (Kelsch et al., 2014). A recent publication did however find reduced threshold to pentylenetetrazol induced seizures in adult rats with the *Grin2b*-Trp373 NTD mutation, and these

animals also presented with NDD-like behaviours including a decreased tendency for social interaction, repetitive grooming and anxiety-like behaviours, as well as impaired spatial learning and memory (Platzer et al., 2017; X. Wang et al., 2022). As roughly half of individuals with *GRIN2B* variants present with epilepsy it will be important that future models are developed for studying the relevant underlying mechanisms, which could improve therapeutic outcomes.

In summary, loss of GluN2B likely contributes to dysfunctional circuit development, connectivity and signalling, by affecting neuronal differentiation and migration during embryonic development, and by altering synaptic plasticity and neuronal wiring properties. These changes result in compromised network function, which has been shown to manifest in a spectrum of disorder-associated abnormalities, including alterations in behaviour, activity, learning, and memory. The core epilepsy feature associated with *GRIN2B* related NDD, however, is seldom documented in mouse and rat models of the disorder. Furthermore, seizure activity in these models either results in lethality soon after seizures begin or fails to occur spontaneously. Nevertheless, these models provide evidence that *Grin2b* mutations in rodents can lead to seizure-generating neural circuitry, in addition to ASD and ID phenotypes. Studying network dysfunction contributing to *GRIN2B* related epilepsies is important, as over half of affected patients experience seizures. However, it is crucial that models that presents with reliable seizure phenotypes are developed, to allow for the study of underlying pathological mechanisms associated with epileptic activity in this disorder.

## 1.5 Sleep and Circadian Rhythms

Sleep is a process that has been evolutionarily preserved across species. Within the brain sleep facilitates neuroplasticity, learning and memory, and modulates consciousness. Furthermore, sleep plays a crucial role in maintaining physiological homeostasis, encompassing both neurological and systemic functions throughout the organism (Abel et al., 2013; Hobson & Pace-Schott, 2002; Vyazovskiy & Delogu, 2014). In 1953, groundbreaking research from Aserinsky and Kleitman showed that sleep is not a homogenous state, but rather a complex process comprising distinct substates, each characterized by unique physiological markers (Aserinsky & Kleitman, 1953). Since then, our understanding of the intricate mechanisms governing the development, generation and maintenance of sleep have dramatically expanded to reveal a complex interplay of genetic factors, neurotransmitter systems, and molecular signalling pathways (Hobson & Pace-Schott, 2002). Sleep has a progressive, cyclic architecture and is divided into two distinct stages known as non-rapid eye movement (NREM) sleep, associated with energy metabolism and information organization and transfer (learning and memory), and rapid eye movement (REM) sleep, which supports memory consolidation and is associated with

dreaming (Abel et al., 2013; Brown et al., 2012; Hobson & Pace-Schott, 2002; Vyazovskiy & Delogu, 2014).

Sleep structure is defined by the relative frequency and length of periods spent in NREM and REM phases, which differ between humans and rodents. In humans, sleep occurs during the night (dark phase) and NREM sleep takes up roughly 80% of total sleep time. NREM is further subdivided into lighter stages 1 and 2 and deep slow wave stages 3 and 4. Each sleep cycle is characterized by the progressive transitioning from lighter to deeper stages, with REM sleep initiated following stage 4 NREM. Notably, in normal sleep patterns, REM sleep is always preceded by NREM, never by wakefulness. Similarly to human sleep, rodents have both NREM and REM sleep, although there is debate regarding the substage division of rodent NREM. Furthermore, these animals are nocturnal, therefore predominantly sleep during the day (light phase), and have polyphasic sleep patterns, meaning they rapidly cycle through the two sleep states and wake both during the light hours and dark hours of the day. Despite this, the progression of sleep phases follows the highly conserved pattern from wake-NREM-REM-NREM or wake in both humans and rodent species (Brown et al., 2012).

NREM sleep, REM sleep, and wake in humans and rodents exhibit conserved electrophysiological signatures, characterized by distinct oscillatory patterns that fall within similar frequency ranges. In polysomnography studies, which investigate sleep physiology and distribution, delineation of the three states is achieved through the combination of EEG and electromyography (EMG) (and in some cases electrooculography for detection of eye movements). NREM sleep EEG is distinguishable for the presence of synchronous low frequency, high amplitude cortical oscillations (0.5-4 Hz), commonly referred to as delta waves with thalamocortical origin, alongside minimal muscle tone. It is also known as slow wave sleep for the above reasons. Coordinated activity during NREM sleep includes dominant slow oscillations (<1 Hz) generated in the cortex, hippocampal sharp-wave ripple events (100-250 Hz) and thalamocortical sleep spindles (12-15 Hz) (Brown et al., 2012; Hobson & Pace-Schott, 2002; Vyazovskiy & Delogu, 2014). The temporal frame for this coordinated activity is governed by the slow cortical rhythms, which synchronizes periods of activity (referred to as UP states) and silence (DOWN states) of cortical neurons in the brain (Sirota & Buzsáki, 2005). REM sleep is marked by high frequency, low amplitude EEG oscillations, resembling the desynchronized activity seen during periods of wakefulness, but with a notable increase in power within the theta frequency range (4-8 Hz). Unlike wake, REM sleep is also distinguished by the presence of rapid eye movements and a total loss of muscle tone. In rodents, wake can be subdivided into quiet and active wake. A predominance of theta oscillations is typically observed in active wake, while mixed-frequency EEG is characteristic of quiet wake (Brown et al., 2012).

### 1.5.1 Sleep homeostatic regulation and circadian rhythm

The regulation of sleep is explained through the two-process model, which posits that the timing, duration, and depth of sleep is determined by the continuous combined effect of sleep homeostatic mechanisms (Process S) and the circadian pacemaker (Process C). Sleep homeostasis refers to the mechanistic tracking of sleep-wake history, through factors that accumulate during wake periods (e.g. neuromodulators like nitric oxide and adenosine or increase in expression of genes during wake like *Homer1*) and decrease with sleep duration, while circadian rhythmicity defines the time of day at which sleep occurs (Borbély et al., 2016; Hobson & Pace-Schott, 2002).

The sleep homeostat represents the homeostatic drive for sleep. The process exhibits a non-linear trajectory and is characterized by a progressive accumulation during wakefulness and dissipation during sleep, reflecting the dynamics of sleep pressure (Borbély et al., 2016). At the neurophysiological level, during wake there is an accumulation of sleep-promoting substances, including nitric oxide and adenosine, which are two potent sleep homeostat modulators (Brown et al., 2012; Saper et al., 2005). Changes in synaptic strength and plasticity, as well as associated gene expression fluctuations further contribute to homeostatic sleep drive. The amplitude of delta slow wave activity during NREM sleep serves as a quantifiable marker for sleep pressure in both humans and rodents alike, and increases following extended periods of wake (Borbély et al., 2016). The sleep homeostat interacts dynamically with the circadian process to regulate the timing, duration, and structure of sleep-wake cycles. This interaction ensures that sleep propensity is highest when the circadian drive for wake is lowest. Typically, this is during the night phase in humans, while in rodents it is during the light phase (Brown et al., 2012; Hobson & Pace-Schott, 2002).

The suprachiasmatic nucleus (SCN), a bilateral structure located in the anterior hypothalamus, has been established as the central circadian oscillator in mammals, and functions to pace and structure sleep homeostasis to the light and dark cycles. The SCN receives direct environmental light cues through the retinohypothalamic pathway and subsequently coordinates various physiological and behavioural processes through neural and hormonal pathways. It modulates the secretion of neuroendocrine factors like cortisol and melatonin and regulates core body temperature fluctuations, the rhythms of which serve as robust markers of the circadian timing system (Brown et al., 2012; Franken & Dijk, 2024).

The molecular clock found within the SCN is crucial for circadian regulation and SCN rhythmicity. It consists of a transcription-translation feedback loop involving several key genes and proteins. The process is initiated when the transcription factors CLOCK and BMAL1 heterodimerize. CLOCK-BMAL1 complexes then bind the *Per* and *Cry* genes, thereby activating their transcription. Subsequent accumulation of *Per* and *Cry* proteins exert a negative feedback effect on CLOCK-BMAL1

complexes, effectively deactivating the transcription process. This cyclical process forms the core of the molecular clockwork that governs circadian rhythms. A distinct regulatory circuit involving the nuclear receptors ROR $\alpha$  and Rev-Erb $\alpha$  modulates the circadian expression of *BMAL1* (Partch et al., 2014; J. S. Takahashi, 2015). This auxiliary loop functions through the reciprocal actions of ROR $\alpha$  and Rev-Erb $\alpha$  on *BMAL1* transcription, with ROR $\alpha$  serving as a transcriptional activator and Rev-Erb $\alpha$  as a transcriptional repressor (Preitner et al., 2002). The interplay between these factors contributes to the robust oscillation of BMAL1 expression. A range of transcriptional modulators, which contribute supplementary influence on the molecular clock have been identified and are reviewed in Takahashi et al., 2015 and Partch et al., 2014.

### 1.5.2 Circuit control of sleep and state switching

In addition to homeostatic drive and circadian rhythms, network neuromodulatory nuclei across the brain influence the timing, duration, and transitions between different sleep states. Nuclei within the brainstem and hypothalamus, including the SCN, which exerts circadian influence, are critical for the regulation of sleep-wake cycles and the rapid transitions between NREM and REM sleep. These are reciprocally connected and mutually inhibit and disinhibit activity in a state dependent manner (Aston-Jones et al., 2001; Brown et al., 2012; Scammell et al., 2017).

Briefly, the ascending reticular activating system (ARAS) in the pons and midbrain promotes and regulates the wake state (Figure 1.6A). It comprises a network of fibres that ascends from nuclei in the reticular formation of the brainstem through the midbrain, where the projections diverge into two main pathways. The first dorsal pathway innervates the thalamus, while the second ventral network targets the basal forebrain, hypothalamus and cortex (Brown et al., 2012; Scammell et al., 2017). The ARAS consists of serotonergic neurons in the medial and dorsal raphe nuclei (MRN, DRN), dopaminergic neurons in the VTA, cholinergic neurons located in the pedunculopontine (PPT) and laterodorsal tegmental (LDT) nuclei, and noradrenergic neurons found in the locus coeruleus (LC). Additionally, orexinergic and histaminergic neurons are localised to the lateral and posterior hypothalamus, particularly in the tuberomammillary nucleus (TMN) (Figure 1.6A) (Brown et al., 2012; S.-H. Lee & Dan, 2012). In the dorsal pathway, PPT and LDT cholinergic projections target inhibitory and excitatory thalamic neurons, which relay signals throughout the cortex. Interestingly, while important for eliciting fast cortical rhythms and sleep spindles, the dorsal pathway is not essential for cortical activation and the sleep-wake cycle, but rather involved in modulating its activity patterns (Figure 1.6A). The ventral pathway of the ARAS is comprised of neuronal projections ascending from noradrenergic LC, serotonergic DRN and MRN, glutamatergic pontine reticular formation (PRF), and the dopaminergic PAG, which all synapse onto hypothalamic TMN histaminergic, glutamatergic and

orexin cells (Figure 1.6A). Orexin neurons project to the DRN, MRN, LC and TMN, and their activity is highest during wake. When these cells fire during sleep the likelihood of sleep to wake transitions increases (K. Takahashi et al., 2008). The caudal basal forebrain projects to and stimulates the cortex and is a major conjoining hub for afferents of the ascending pons and midbrain nuclei, which target glutamatergic, GABAergic and cholinergic basal forebrain neurons (Brown et al., 2012; S.-H. Lee & Dan, 2012; Scammell et al., 2017). Basal forebrain cholinergic neurons heavily innervate the cortex and excite pyramidal neurons thus supporting fast cortical activity during wake resulting in attentiveness, sensory processing, memory and plasticity (Figure 1.6A) (Scammell et al., 2017).

Critical for NREM sleep are GABAergic cells in the ventrolateral preoptic area (VLPO) and median preoptic nucleus (MnPO) (Figure 1.6B) (Brown et al., 2012; Scammell et al., 2017). The VLPO receives direct signals from the retina and indirect input from SCN. These specific inputs likely regulate circadian control of sleep to wake transitions (Aston-Jones et al., 2001). NREM promoting areas are reciprocally connected with ARAS nuclei, which constitutes a switch model, in which input interactions allow for rapid and stable transitions between sleep and wake states (Figure 1.6B). ARAS histaminergic, noradrenergic and serotonergic neurons strongly inhibit VLPO and MnPO during wake, while VLPO and MnPO innervate and suppress wake active orexin neurons, and the activity of basal forebrain, MRN, DRN and LC (Brown et al., 2012; Scammell et al., 2017). An inhibitory basal forebrain population is also NREM promoting and believed to directly inhibit cortical excitatory cells. In the cortex scattered NREM active interneurons that produce nitric oxide synthase, contribute to slow cortical synchronization via long-range, intracortical projections, together with input from thalamic nuclei (Figure 1.6B) (Crunelli et al., 2015; Scammell et al., 2017).

Nuclei in the pons and brainstem amongst other regions mediate REM sleep (Figure 1.6C) (Park & Weber, 2020; Scammell et al., 2017). The regulation of the transition between NREM and REM sleep involves the LC, DRN, PPT, LTD and PRF nuclei. In contrast, the lateral hypothalamus and preoptic midbrain regions regulate REM sleep dynamics (Scammell et al., 2017). Wake active PPT and LDT nuclei are also REM sleep promoting. PPT and LDT cholinergic and glutamatergic neurons fire to promote NREM-REM sleep transitions and activate GABAergic PRF and SLD neurons, which initiates downstream hippocampal theta rhythms and desynchronizes cortical activity (Figure 1.6C) (Boissard et al., 2002; Park & Weber, 2020). REM sleep muscle atonia is believed to arise from glutamatergic sublaterodorsal (SLD, located within the PRF) inputs onto brainstem medulla regions, which inhibit spinal cord motor neurons (Figure 1.6C) (Scammell et al., 2017). Noradrenergic neurons in the nearby LC, MRN, DRN and PAG suppress REM sleep via inhibition of SLD, PPT and LDT (Figure 1.6C). REM modulating regions are reciprocally connected, and some evidence suggests orexin signalling to LC and DRN further regulates the expression of REM sleep (Park & Weber, 2020; Scammell et al., 2017).

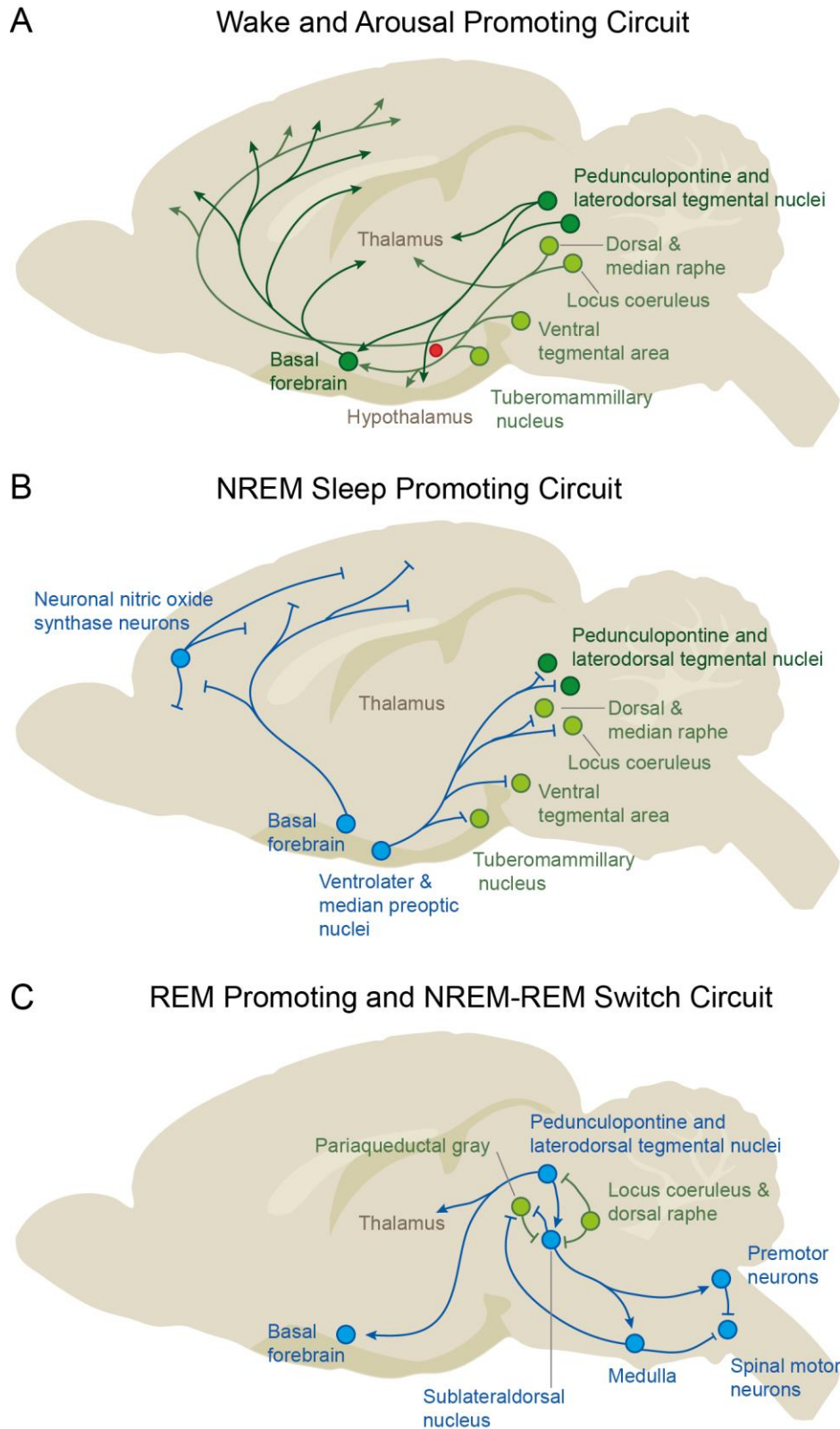


**Figure 1.6. Neuronal circuits of wake and sleep.** (A) Ascending reticular activating system promoting the wake state and associated fast cortical activity. In the rostral brainstem and caudal hypothalamus, monoaminergic neurons (light green) project directly to the cortex, thalamus and hypothalamus. These regions comprise serotonergic neurons from the dorsal and median raphe nuclei, noradrenergic neurons from the locus coeruleus, dopaminergic neurons from the ventral tegmental area, and

*histaminergic neurons from the tuberomammillary nucleus. Cholinergic projections (dark green) innervate the cortex, thalamus and hypothalamus and arise from pedunculopontine, laterodorsal tegmental and basal forebrain nuclei. Orexin neurons (red dot) in the hypothalamus are wake promoting and send diffuse projection to cortex, thalamus and all ARAS nuclei described above. (B) NREM sleep promoting pathways. Inhibitory neurons in medial and ventrolateral preoptic nuclei send GABAergic projection to inhibit caudal hypothalamus and brainstem wake promoting neurons (light and dark green). Sleep active GABAergic neurons in the basal forebrain project locally and to cortex and inhibit ARAS neurons. GABA and neuronal nitric oxide synthase NREM sleep promoting neurons are scattered across the cortex. Blue circles denote NREM sleep-promoting nuclei. (C) REM sleep promoting pathways. Sublaterodorsal nucleus glutamatergic neurons evoke REM muscle atonia by exciting GABAergic/glycinergic neurons in the ventromedial medulla and spinal cord that hyperpolarize motor neurons. Pedunculopontine and laterodorsal tegmental cholinergic neurons promote REM sleep and may help drive REM fast EEG activity. Periaqueductal gray GABAergic and monoaminergic locus coeruleus and raphe nuclei afferents inhibit sublaterodorsal nucleus during wake and NREM. During REM GABAergic sublaterodorsal tegmental nucleus and medulla neurons suppress activity in the ventrolateral periaqueductal grey area. REM promoting nuclei are shown in blue; REM suppressing nuclei are shown in green. Figure adapted from Scammell et al., (2017).*

Circadian flow and the regulation of sleep emerges from a complex interplay between environmental factors and neurotransmitter systems across various brain regions. In this section I overview the main proposed mechanism involved, although this is far from extensive and there are still many unknown fundamental principles, such as whether cortical signalling may have a top-down effect and support the ARAS. Furthermore, sleep and wake patterns change during early life, reflecting developing circuitry and the emergence of circadian rhythmicity.

### 1.5.3 Developmental trajectory of sleep

In humans and most mammals (including rodents), sleep and wake patterns undergo significant changes from birth to adolescence. At birth and the proceeding postnatal period, which varies by species, sleep is characterized by distinct, immature stages known as active and quiet sleep, which are precursors of the more mature REM and NREM states respectively. In the neonatal period, active sleep is predominant over quiet sleep (Cirelli & Tononi, 2015; Gorgoni et al., 2020).

Rodents are classified as altricial, born less developed than humans, and as such have offered valuable insights into the development of circadian rhythmicity, sleep-wake cycles and associated EEG oscillations in early life (Cirelli & Tononi, 2015; Gottlieb et al., 1977). In rat pups, active sleep during the first postnatal week is distinguished by frequent myoclonic twitches of individual muscles against a backdrop of muscle atonia. This state shares neurophysiological similarities with adult REM sleep, where specific pontine and medullary regions are involved in generating both muscle atonia and

twitches. These regions are active in both infant and adult sleep, supporting the notion that infant sleep is qualitatively comparable to adult sleep. In contrast to active sleep, quiet sleep is characterized by behavioural stillness and diminished muscle tone. Cortical maturation and synaptogenesis during the second postnatal week at P9 and P10 results in augmented EEG power across wake and infant sleep states, and initiates further differentiation of sleep. Specifically, following P12 EEG slow waves are visible, exclusively during quiet sleep, with power during this state rising to become consistently higher than that in active sleep and wake. Around the same time, active sleep twitches rapidly decline, and the sleep phase becomes more tonically activated. At this developmental stage NREM and REM become clearly identifiable. NREM thalamocortical sleep spindles emerge at P14 and sleep EEG patterns achieve adult-like characteristics by P18 (Cirelli & Tononi, 2015).

Little is known about the specific underlying network mechanisms and function of infant sleep (postnatal weeks one and two in rodents). Unlike in adult sleep, subcortical and cortical regions may function independently in early postnatal active and quiet sleep. While structured, early developmental cortical activity exhibits extended periods of electric silence, which reflect the presence of silent NMDAR synapses, reduced synaptic connectivity and scarce long-range intracortical and thalamocortical connections. Furthermore, it's likely that ascending pons, midbrain and basal forebrain projections to the cortex and thalamus are weak and underdeveloped (Cirelli & Tononi, 2015). For example, basal forebrain cholinergic axons only reach the supragranular cortical layers during the second postnatal week, with mature innervation achieved by the third and fourth postnatal weeks (Janiesch et al., 2011). Similarly, LC noradrenergic neurons, which are part of the ARAS, are silent during the first postnatal week (Nakamura et al., 1987). However, some basic mechanisms such as those that support active and REM sleep muscle atonia in the pons and medulla region, seem to be conserved between adults and perinatal infants (Karlsson et al., 2005).

The change in sleep features during the second postnatal week coincides with the time when animals engage in active exploration and importantly eye opening, stimulating circadian photic entrainment (this may begin as early as P6). During this time, rapid plasticity changes occur in the cortex, shifting from synaptic potentiation to a more nuanced balance between potentiation and depression for fine tuning cortical responses to external stimuli. These observations have led to the suggestion that immediately following birth the functional roles of sleep, and specifically active sleep, are more akin to those of wake (Cirelli & Tononi, 2015). Namely, to develop and refine cortical networks. Indeed, while not yet fully understood, perinatal sleep appears to contribute to the development of sensorimotor networks and functional connectivity (Del Rio-Bermudez et al., 2020; Del Rio-Bermudez & Blumberg, 2018). In particular, myoclonic twitches which occur abundantly and exclusively during active sleep elicit sensory feedback to the sensorimotor system. This initiates a cascade of neural activity across the developing sensorimotor system at ages when wake-related movements typically do not. Importantly, sensory feedback from twitches reliably triggers synchronized

neural oscillations (Del Rio-Bermudez et al., 2020; Del Rio-Bermudez & Blumberg, 2018), which are involved in neuronal differentiation, migration, apoptosis, and somatotopic mapping during neurodevelopment (Khazipov & Luhmann, 2006). In the context of active sleep, they are thought to contribute to activity-dependent development of sensorimotor networks and the development of long-range network connectivity. Consequently, any factors that significantly disrupt sleep will distort sensory processing during the earliest stages of life and could impair the normal development of local circuits and long-range connectivity, resulting in dysfunction such as that observed in NDDs (Del Rio-Bermudez et al., 2020).

Moreover, abnormal neural signalling in various brain regions both in infancy and adulthood could result in the overall dysregulation of sleep and circadian rhythms. The implications of these changes on sleep patterns and circadian cycles are addressed in the following section.

#### 1.5.4 Sleep and circadian dysfunction

Sleep emerges from the intricate interplay between the circadian rhythm and the accumulation of sleep pressure during wake. This complex state is orchestrated by a network of interconnected brain regions, utilizing various neurotransmitters and hormones to regulate the sleep-wake cycle. The resulting sleep and circadian timing systems are a sophisticated biological mechanism that balances multiple physiological processes to maintain optimal sleep patterns and overall health.

Adequate sleep and precisely timed circadian rhythms are fundamental to regulating metabolism, supporting immune function, and optimizing cognitive abilities such as learning and memory consolidation. Sleep, in particular, is vital for brain plasticity and information processing (Kaida et al., 2023). During sleep, the brain undergoes synaptic scaling, a process that selectively strengthens important neural connections while pruning less essential ones, thereby enhancing memory formation and recall. This synaptic homeostasis is critical for learning efficiency and cognitive flexibility in waking hours (Kaida et al., 2023; Klinzing et al., 2019). NREM and REM contribute differently to memory consolidation. During slow wave NREM, declarative memory (i.e. episodic memory) is consolidated and integrated into existing knowledge networks via neuronal ensembles in the cortex, hippocampus and thalamus. Sleep spindles and hippocampal slow wave ripples are critical for NREM memory consolidation. Sleep spindles promote synaptic plasticity, facilitate the consolidation of weakly encoded memories, and support hippocampal-cortical communication. Hippocampal ripples are associated with memory replay and can couple to spindles, beginning after spindle onset and ending before spindle offset. This mechanism allows for information transfer from the hippocampus to neocortical regions for long-term storage. The temporally coupled activity of cortical slow oscillations, thalamic sleep spindles and hippocampal ripples is believed to be a crucial

mechanism for effective memory consolidation and reorganization (Kaida et al., 2023; McDevitt et al., 2017). In contrast, REM sleep facilitates procedural and emotional memory processing (Kaida et al., 2023; Rho et al., 2023), and it has been suggested to influence memory modifications and their integration into existing knowledge (Kaida et al., 2023; Tamminen et al., 2017). Sleep and circadian rhythms also significantly influence mood stability and emotional regulation (Walker et al., 2020). At the cellular level, they facilitate not only synaptic scaling but also neurogenesis, and the clearance of metabolic waste from the brain. Furthermore, the timing of hormone release is intricately linked to these cycles, affecting various physiological functions (Foster, 2020). Motor performance, including coordination and reaction time, is also enhanced by proper sleep and circadian alignment (Klinzing et al., 2019). The far-reaching effects of sleep and circadian rhythms underscores their importance in nearly every aspect of human physiology and behaviour, making them critical factors in both physical and mental health.

Disruptions in any critical neurotransmitter pathway can have far-reaching consequences on the intricate sleep-wake cycle and circadian rhythms. Common sleep issues and dysfunction include sleep apnea, insomnia, difficulty falling asleep, frequent nighttime awakenings and fragmented sleep, as well as daytime somnolence. Furthermore, polysomnography work has identified sleep abnormalities in diverse patient groups in which specific sleep states are compromised or significantly reduced, effectively disrupting both quantitative and qualitative sleep features (Klinzing et al., 2019). For example, gross reductions in REM sleep and NREM spindle activity, as well as decreased NREM power have been observed in individuals with insomnia, Alzheimer's, NDDs and epilepsy (Lanigar & Bandyopadhyay, 2017; Robinson-Shelton & Malow, 2016; Y. Zhang et al., 2022). In particular, NREM power has been reported to be a critical qualitative marker in sleep disorders (G. Horváth et al., 2022). Spectral decomposition of NREM EEG into specific frequency ranges, including delta (1-5 Hz), theta (5-10 Hz), sigma (10-16 Hz), beta (16-30 Hz) and gamma (30-48 Hz), has demonstrated increases in delta in children, adults and rodents with the rare NDD, Angelman Syndrome (Ostrowski et al., 2021), while increased beta power, is considered a proxy for poor overall sleep and is often present in individuals with insomnia (Y. Shi et al., 2022).

Until recently, sleep and circadian abnormalities were mainly thought to be caused by secondary factors such as medication adverse effects, social or work environment issues, irregular light exposure, and general discomfort (Smith et al., 2000; Wirz-Justice, 2006; Wulff et al., 2010; Walker et al., 2020, Bruni et al., 2025) However, it is now widely believed that sleep and circadian dysfunctions are a result of common mechanistic pathways that are perturbed in many neurodegenerative, psychiatric and NDDs. In fact, there is a significant genetic and neuronal network level contribution to sleep impairment, such as dysregulation in clock gene expression (Yang et al., 2016) and abnormal activation of neurotransmitter systems (e.g. orexin) (Nevsimalova et al., 2005; Mieda et al., 2009; Omokawa et al., 2016). This is certainly the case with genetic NDDs and epilepsy, where sleep and circadian rhythm

is typically heavily compromised (Wulff et al., 2010; Walker et al., 2020; Bruni et al., 2025). Genetic and epigenetic associations between poor sleep and NDDs have been made for core clock genes including, *PER1*, *PER2* and *NPAS2* in individuals with ASD, ID and epilepsy (Nicholas et al., 2007; Yang et al., 2016; Bruni et al., 2025). Additionally, evidence from rodent studies further implicates irregularities in dopaminergic, nicotinic and cholinergic signalling systems, which are crucial modulators of sleep and circadian rhythm dynamics in NDDs (Wulff et al., 2010; Deutsch et al., 2010; Guo et al., 2018; Lu et al., 2018; Walker et al., 2020; Mariggio et al., 2021).

In terms of NDDs and epilepsy, establishing precise causal connections associated with impaired sleep or circadian disturbances has been challenging due to two main factors. First, there is significant variability in how these impairments manifest across individuals, even among those with similar diagnoses. The complexity of this relationship is further illustrated by the fact that patients who share a diagnosis, demographic characteristics, and even medication regimens can exhibit vastly different degrees of sleep or circadian rhythm disruption. Some individuals may experience severe disturbances, while others show no apparent disruption at all. And second, there is a scarcity of comprehensive, long-term quantitative data on sleep and circadian disruptions in individuals with NDDs with or without epilepsy (Barone et al., 2019), especially in those with rare monogenic disorders including *GRIN2B* and *NLGN3* related NDDs. This wide spectrum of outcomes complicates efforts to draw clear, universal connections between sleep and circadian issues, related compromised pathways and genetic influences. Therefore, further research is warranted into long-term sleep monitoring studies aimed at quantifying specific sleep abnormalities and their underlying pathophysiology, particularly in rare genetic NDDs. Such work could lead to more effective, personalized interventions and lead to potential new sleep biomarkers for interventions.

Overall, compromised sleep results in poor cognitive and motor abilities, as well as reduced immunity and health. The consequences of poor sleep are particularly detrimental to children and adults with NDDs, where reduced sleep quality further impairs learning, memory, cognition and mood. Moreover, sleep is intimately tied to the development and presentation of epileptic activity, further complicating patient outcomes where NDDs cooccur with seizures.

### 1.5.5 Association between sleep, epilepsy and neurodevelopmental disorders

There is complex interplay between sleep, epilepsy, and NDDs, which together have a profound negative impact on patient outcomes and quality of life. Sleep dysfunction is highly prevalent in children and adults with NDDs and individuals with epilepsy frequently experience some form of sleep impairment (Esbensen & Schwichtenberg, 2016; Stirling et al., 2023). In cases where NDDs are comorbid with epilepsy, sleep is highly likely to be perturbed. With genetic NDDs the neurobiological

mechanisms underlying cognitive impairment, sleep dysfunction and seizures may overlap or significantly influence each other (Malow, 2004).

The relationship between sleep and epilepsy is bidirectional, with each having the potential to modulate the other. It is well documented that sleep fragmentation lowers seizure thresholds, however, recent evidence suggests that sleep and wake time fluctuations are more strongly associated with increased seizure probability than sleep deprivation alone (Stirling et al., 2023). Seizures may themselves affect sleep, with this influence not being limited to the time of seizure onset but also the frequency of seizures. For example, temporal lobe focal to generalised seizures decrease REM sleep, particularly when occurring during sleep but also during daytime wake preceding sleep onset (Bazil et al., 2000). Furthermore, certain epileptic syndromes are characterized by seizures that occur around or exclusively in NREM sleep, and are associated with NREM processes and networks, such as fronto-hippocampal and corticothalamic circuits. To illustrate this, in mesial temporal lobe epilepsy, NREM hippocampal sharp wave-ripples evolve into epileptic spikes and pathological high frequency oscillations (80-500 Hz). Similarly, absence epilepsy and juvenile myoclonic epilepsy are associated with the progression of normal thalamocortical burst-firing sleep spindles during NREM, into bilateral spike-wave discharges (SWDs), the electrographic correlates of absence seizures. Perisylvian network epilepsies including idiopathic focal childhood epilepsies, EEs, and DEEs, exhibit an increase in interictal discharges characterized by EEG sharp waves or spikes. In these epilepsies interictal discharges occur between seizures and demonstrate a similar pattern of heightened activation during NREM sleep, especially at critical developmental periods (Malow, 2004; Roliz & Kothare, 2023). Conversely, they are suppressed during REM sleep, which underscores the complex interplay between sleep states, architecture and epileptic manifestations (Roliz & Kothare, 2023). Seizure patterns and preference for certain states is believed to be rooted in aberrant wiring across the brain and particularly, in NREM active pathways and maladaptive homeostatic compensatory mechanisms.

Sleep, epilepsy and NDDs are interconnected through a theoretical framework that emphasize the roles of synaptic and homeostatic plasticity, particularly during early development when high rates of plasticity occur (Roliz & Kothare, 2023). Sleep facilitates homeostatic downscaling of synaptic strength to a level that is optimal for associative plasticity during wake and therefore maintains neuronal excitation, inhibition and circuit level balance (Tononi & Cirelli, 2006). In mice, during wake, the net number of synapses increases, while during sleep it decreases with the total net number of synapses remaining constant (Maret et al., 2011). Therefore, any disruption of synaptic homeostasis processes may fail to suppress network hyperexcitability and result in epileptogenesis (Roliz & Kothare, 2023). This is a likely mechanism in genetic NDDs and epilepsy, where synaptic signalling and intracellular mechanisms are frequently compromised. It is speculated that NDD causing mutations could be responsible for the perturbation of sleep and its related networks in early developmental ages (Barone et al., 2019). In NDDs with epilepsy, maladaptive homeostatic plasticity and impaired neuronal

signalling, resultant from ion channel or transcriptional and translational abnormalities, may account for pathological epileptogenesis during neonatal periods. During this time, REM sleep, and to a lesser extent wakefulness, serves to stimulate synaptic connectivity (Del Rio-Bermudez et al., 2020). Subsequently, aberrant circuit organisation could impact on the timing of NREM sleep onset, as well as the amount and quality of NREM slow wave oscillations, critical for synaptic downscaling. This in turn can trigger epileptogenesis and enhance existing seizure phenotypes, leading to a severely disordered state. In such pathologies sleep and seizures could contribute to further worsening of the other and exacerbate comorbid cognitive, learning and memory deficits in individuals that also present with NDDs. Furthermore, elevated interictal epileptiform activity during sleep in early developmental stages, particularly in DEEs, is hypothesized to interfere with cortical organization and plasticity, potentially leading to irreversible neuropsychological deficits (Roliz & Kothare, 2023). However, the causal relationship between sleep disturbances and impaired developmental outcomes is not yet understood.

Ultimately, it remains unclear whether altered sleep architecture serves as an indicator of atypical cognitive development or if sleep abnormalities themselves contribute directly to neurodevelopmental aberrations (Roliz & Kothare, 2023). This ambiguity underscores the need for further research to elucidate the precise mechanisms linking sleep disturbances, epileptiform activity, and neurodevelopmental trajectories.

## 1.6 Hypothesis and Aims

This introduction has outlined causes and pathophysiological mechanisms related to core NDDs with and without epilepsy, specifically those of genetic origin. Additionally, I summarised sleep and circadian rhythm features, which are frequently and severely disrupted in individuals with NDDs. The overarching aim of this thesis is to therefore add to our understanding of specific sleep impairments, investigate pathological epileptic activity, and test if there is any relationship between sleep and seizures in three rare genetic NDDs with/ without epilepsy. Specifically, I address this in genetically engineered rat lines of *GRIN2B* and *NGLN3* related NDDs, and *16p11.2* deletion syndrome. Sleep disturbances in *GRIN2B* related NDDs remain unstudied and prior mouse and rat models of the disorder do not present with spontaneous seizures. In contrast, research on sleep and seizure phenotypes in rodent models of *16p11.2* deletion and *Ngn3* related NDDs has yielded valuable but inconsistent results, emphasizing the need for further standardized sleep assessments aimed towards uncovering robust and replicable phenotypes with clinical utility. The above considerations informed the choice for the three rat models used throughout this thesis. In **Chapter 2**, I describe the methodology used throughout this research, and below I outline the main hypothesis for **Chapters 3 to 5** and highlight the key findings of each.

**Chapter 3:** In this chapter, I hypothesised that rat models of *Grin2b* haploinsufficiency, *Nlgn3* knockout and *16p11.2* deletion will exhibit impaired and irregular sleep-wake physiology, similar to what is reported in patients with some of these disorders. I conduct polysomnography experiments through chronic wireless EEG experiments aimed at quantifying sleep phenotypes for each line and provide evidence of altered sleep patterns in *Grin2b* haploinsufficiency, *Nlgn3* knockout and *16p11.2* deletion rat models, as well as specific sleep state deficits in *Grin2b* haploinsufficiency rats.

**Chapter 4:** Epileptic activity is often observed in *16p11.2* deletion syndrome, while approximately half of individuals with *GRIN2B* related NDDs present with severe epilepsy, which in many cases worsens existing cognitive impairment (Platzer et al., 2017; Zufferey et al., 2012). Therefore, in Chapter 4, I addressed the hypothesis that *Grin2b* haploinsufficiency and *16p11.2* deletion rat lines would present with epileptic phenotypes reminiscent of those observed in patients. I find a robust absence epilepsy phenotype in rats with heterozygous *Grin2b* deletion. I further investigate the distribution of seizure occurrence with respect to sleep-wake patterns, as well as study seizure generation and propagation dynamics. Based off data from this chapter I suggests that *Grin2b* haploinsufficiency in rats likely results in perturbed thalamocortical network activity and discuss potential mechanisms that may reflect this.

**Chapter 5:** In this chapter I focus on potential translatability and attempt to pharmacologically rescue seizure activity in the *Grin2b* haploinsufficiency model using compounds that have either been shown to attenuate absence seizures in the clinic or inhibit NMDAR activity, associated with the function of *Grin2b*. I systemically administer drug compounds, successfully blocking absence seizures and subsequently investigate the likely brain areas that most likely contribute this effect by direct intracerebral treatment application to each region. Here, I provide evidence that absence seizure in rats with *Grin2b* haploinsufficiency are inhibited by pharmacological treatment and furthermore, that this effect could be replicated by targeting of specific thalamocortical circuit nuclei. These results could potentially have favourable outcomes if extended to individuals with *GRIN2B* related NDDs.



# Chapter 2

## Materials and Methods

### 2.1. Animals

#### 2.1.1. Animal husbandry

All animal procedures were undertaken in accordance with the University of Edinburgh animal welfare committee regulations and were performed in line with the United Kingdom Home Office guidelines under the 1986 Animals (Scientific Procedures) Act; Home Office project license – Brain Networks in Epilepsy and Neurodevelopmental Disorders (PP1538548). Pups from all rat models were bred in-house and weaned from their dams at P22. They were subsequently housed in mixed genotype cages with wild-type littermates (two to six rats per cage) with a 12-hour/12-hour light–dark cycle, and ad libitum access to water and food. Following surgery, animals were single housed.

#### 2.1.2. *16p11.2* deletion rats

*16p11.2* heterozygous deletion rats were created by CRISPR/Cas9 genome editing of Sprague-Dawley animals (Charles River Laboratory, Oncins, France) (Qiu et al., 2019). A deletion of 483,122 bp in chromosome 1, corresponding to the *16p11.2* homologous region of the rat genome, was obtained. Animals were bred inhouse at the Centre for Brain Discovery Sciences, University of Edinburgh and maintained on the Sprague-Dawley (Jackson Laboratories) strain. In text, *16p11.2* deletion and wild-type rats are referred to as *16p11.2*<sup>+/-</sup> and *16p11.2*<sup>+/+</sup> respectively.

### 2.1.3. *Nlgn3* knockout rats

*Nlgn3* knockout rats were generated on outbred Sprague-Dawley background by Horizon Discovery (SAGE Labs, St. Louis, Missouri, USA) (Hamilton et al., 2014). Zinc-finger nuclease mRNA was used to target *Nlgn3* exon 5, resulting in a 58 bp deletion and loss of *Nlgn3* in Sprague-Dawley rats. *Nlgn3* knockout animals are hereafter referred to as *Nlgn3*<sup>-/-</sup> and wild-type littermates as *Nlgn3*<sup>+/+</sup>. Animals were bred inhouse at the Centre for Brain Discovery Sciences, University of Edinburgh on the Sprague-Dawley (Jackson Laboratories) strain.

### 2.1.4. *Grin2b* haploinsufficiency rats

Long Evans *Grin2b* heterozygous knockout rats were generated by the Medical College of Wisconsin gene Editing Rat Resource Centre (Milwaukee, Wisconsin, USA) with support from the Simons Foundation Autism Research Initiative (LE-Grin2bem1Mcwi, RRID:RGD\_14394515). CRISPR/Cas9 technology was used on Long Evans rat embryos, to introduce a mutation in the *Grin2b* gene resulting in deletion of exon 3. *Grin2b* heterozygous knockouts are hereafter referred to as *Grin2b*<sup>+/-</sup>, wild-type littermates are *Grin2b*<sup>+/+</sup>. The line was bred and maintained inhouse at the Centre for Brain Discovery Sciences, University of Edinburgh on the Long Evans (Jackson Laboratories) strain. This methodology is detailed in a preprint that outlines the model and EEG phenotypes found in it (Hristova et al., 2024).

## 2.2. Genotyping

Ear notch tissue biopsies were collected from rat pups at the time of weaning and used for genotyping. Genotyping for each rat line was done by polymerase chain reaction, outsourced to Transnetyx (Cordova, TN, USA).

## 2.3. Western blot

Western blots for *Nlgn3* knockout rats were previously carried out under standard conditions on snap frozen whole brain (Hamilton et al., 2014), cortical and PAG tissue homogenates (Anstey et al., 2022). Western blots confirm the absence of NLGN3 protein in *Nlgn3*<sup>-/-</sup> rats. For the *Grin2b*

haploinsufficiency line, Western blots were performed on cortical and hippocampal homogenate and synaptosome fractions as outlined below.

Collection of Western blot data in this section were performed by Dr Sarfaraz Nawaz under the supervision of Prof Peter Kind. Adult 19-week-old *Grin2b*<sup>+/-</sup> (six male) and *Grin2b*<sup>+/+</sup> (one male, five female) rats were anaesthetised with isoflurane and decapitated. The hippocampus and somatosensory cortex from each hemisphere were quickly dissected in slushy PBS 1X, snap frozen and stored at -80 °C. For preparation of synaptosome fractions the discontinuous Percoll-density gradient (23% bottom, 10% middle, 3% uppermost) was made prior to homogenisation on the preparation day. The tissue was quickly thawed at 37 °C and homogenized in ice-cold homogenisation buffer (1x sucrose/EDTA, pH7.4) using 5-6 up-and-down strokes of a pre-chilled Teflon glass with a motorized homogenizer (Dunkley et al., 2008) followed by centrifugation at 2800 rpm for 10 min at 4°C. The supernatant was added gently on 3% Percoll-sucrose (Percoll, P1644, Sigma Aldrich, UK) and centrifuged at 20,000 rpm for 8 min at 4°C. The fraction between 23% and 10% was collected and re-suspended in HEPES-Buffered-Krebs (HBK; in mM: 118.5 NaCl, 4.7 KCl, 1.18 MgSO<sub>4</sub>, 10 Glucose, 1 Na<sub>2</sub>HPO<sub>4</sub>, 20 HEPES, pH 7.4 balanced with Trizma) followed by centrifugation at 13,000 rpm for 15 min at 4°C. The pellet (pure synaptosomes) was dissolved in RIPA buffer with protease inhibitor cocktail (5056489001, Roche, Switzerland) and phosphatase inhibitor cocktails II and III, (P5726 and P0044 respectively, Sigma Aldrich, United Kingdom). Protein concentration was determined with the MicroBCA Assay kit (Pierce BCA protein estimation kit, 23225, ThermoFisher Scientific, United Kingdom).

Approximately 15 µg of synaptosome protein was separated on a precast gradient gel (4-15% Mini-PROTEAN® TGX™ Precast Protein Gels, 4561086, BioRad, United Kingdom) and transferred to nitrocellulose membrane (Amersham™ Protran® Western Blotting Membrane, Nitrocellulose, GE10600002, Sigma Aldrich, United Kingdom) using a Bio-Rad transfer apparatus. Total proteins were stained with a reversible protein stain kit (Memcode 24580, Thermo Fisher Scientific, United Kingdom) according to the manufacturer's instructions. After removing the stain, membranes were blocked with 1:1 TBS1X: Odyssey Blocking Buffer (P/N-927-50003, LI-COR Biotech.) for an hour at room temperature, followed by overnight incubation with primary antibodies (NMDAR2A-1: 1000, #ab169873, Abcam; NMDAR2B- 1:1000, #610417, BD Biosciences; PSD95- 1:2000, #76115, Abcam; GluR1- 1:1000, #MAB2263, Millipore) at 4°C. Membranes were washed with TBST1X (0.1% Tween 20), and incubated for an hour at room temperature with secondary antibodies (IRDye 680RD Donkey anti-rabbit IgG- 1:5,000, #P/N 926-68073; IRDye 800CW Donkey anti-mouse IgG - 1:5,000, #P/N 926-32212, LI-COR Biotechnology, United Kingdom). Membranes were washed with TBST1X, dried and digitally scanned using an Odyssey M Imager, LI-COR, UK Ltd. Odyssey software, Licor Image Studio Lite (LICOR Biosciences, United Kingdom) was used to quantify individual bands. Data was normalised to respective total protein and normalised to wild-type levels.

## 2.4. *In vivo* electrophysiological methods

### 2.4.1. Surgical methods

In all surgical procedures adult 14 to 20-week-old rats were anaesthetised with isoflurane and mounted on a stereotaxic frame (David Kopf Instruments, United States). All animals received subcutaneous Rimadyl Small Animal Solution (5 mg/kg, Zoetis, United States) for analgesia and 2.5 ml of sterile 0.9% saline for rehydration. Procedures were performed under aseptic technique. Animals were left to recover for a minimum of 1 week prior to experiments commencing.

### 2.4.2. 14-channel skull surface EEG

To attain polysomnography recordings, 14-channel skull surface EEG grid coupled with EMG 2 channels were used. For surface EEG grid implantation, two holding screws for structural support (4 mm rostral,  $\pm 0.5$  mm lateral relative to bregma) and one ground screw over the cerebellum (-11.5 mm rostral, 0.5 mm lateral relative to bregma) (Yahata Neji, M1 Pan Head Stainless Steel Cross, RS Components, United Kingdom) were attached. 16-channel EEG surface grids with two integrated electromyogram (EMG) leads (Custom H16-Rat EEG16\_Functional-NeuroNexus, United States) were placed over the skull with the EEG surface grid plus-symbol reference point aligned over bregma. Silver paint was used for connecting the ground screw with the grid. The implant was fixed to the skull using dental cement (Simplex Rapid, Kemdent, United Kingdom). A 21 G sterile needle tip was used to guide EMG wires (2.5 cm in length with 0.5 cm stripped of insulation) into the neck muscle, after which the incision was sutured.

Fifteen 16–19-week-old male *Nlgn3*<sup>+/-</sup> and thirteen male *Nlgn3*<sup>+/-</sup> rats were implanted, recorded and used across sleep analysis. From the *16p11.2* deletion line, twelve 16–19-week-old *16p11.2*<sup>+/-</sup> (eight male, four female) and thirteen *16p11.2*<sup>+/-</sup> (five male, eight female) animals were implanted, recorded and used in sleep analysis. Fifteen 16–19-week-old *Grin2b*<sup>+/-</sup> (eight male, seven female) and nine *Grin2b*<sup>+/-</sup> (three male, six female) animals were recorded and used for analyses. Four *Grin2b*<sup>+/-</sup> and nine *Grin2b*<sup>+/-</sup> rats, descendants of one specific wild-type founder rat were excluded as they exhibited higher rates of absence seizures than descendants from other founders, which obfuscated genotype differences.

#### 2.4.3. 2-channel skull surface EEG

2-channel EEG recordings were used in systemic pharmacology experiments in *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> rats. A midline scalp incision was made, and bilateral screws were implanted to provide structural support (4 mm rostral, ± 0.5 mm lateral relative to bregma). Two ground screws were attached over the cerebellum (-11.5 mm rostral, ± 0.5 mm lateral relative to bregma), and two bilateral electrode screws were fixed over the primary somatosensory cortex (- 2.5 mm rostral, ± 4.5 lateral relative to bregma) (Yahata Neji, M1 Pan Head Stainless Steel Cross, RS Components). Electrode and ground screws were connected to an electronic interface board (EIB-16, Neuralynx) via silver wire soldered to each component (Teflon Coated Silver Wire, World Precision Instruments). The implant was fixed to the skull using dental cement (Simplex Rapid, Kemdent, United Kingdom) and the incision was sutured.

Twelve 21–26-week-old *Grin2b*<sup>+/-</sup> (ten male, two female) and five *Grin2b*<sup>+/+</sup> (one male, four female) animals were recorded and used in pharmacology experiments. An additional four *Grin2b*<sup>+/+</sup> rats were implanted and recorded but did not display SWDs during baseline recording days (see section 2.5.1 on *Systemic treatment administration*) and were therefore excluded from pharmacological experiments.

#### 2.4.4. Drug infusion cannula implantation with 2-channels EEG

Site specific drug infusion was conducted specifically on the *Grin2b* heterozygous deletion model. For drug infusion cannula implantation, two craniotomies were drilled bilaterally over either the somatosensory cortex (- 2.5 mm rostral, ± 5 lateral relative to bregma) or rostral reticular thalamic nucleus (nRT) (- 1.45 mm rostral, ± 2.2 lateral relative to bregma). The dura was gently removed from each craniotomy and a single 26 G sterile guide cannula (diameter = 0.46 mm, P1 Technologies) was slowly inserted to corresponding ventral coordinates for somatosensory cortex (-1 mm ventral from the brain surface) and rostral nRT (-5 mm ventral from the brain surface). Guide cannulas were cemented in place using UV activated cement (3M Relyx Unicem 2 Automix, Henry Schein) and dummy cannulas were placed into guides to ensure guides remained contaminant and blockage free. EEG and EMG implant components were then implanted as described in section 2.4.3 (*2-Channel skull surface EEG*).

Four *Grin2b*<sup>+/-</sup> rats with bilateral somatosensory cortex cannulas and four *Grin2b*<sup>+/-</sup> rats with bilateral nRT cannulas were included in the final drug infusion analysis. An additional four *Grin2b*<sup>+/-</sup> and ten *Grin2b*<sup>+/+</sup> rats were implanted with cannulas (two and five per location respectively) and recorded, but excluded from final drug infusion analysis as they did not display SWDs during experimental baseline recording days detailed in section 2.5.2 (*Site-specific treatment administration*).

#### 2.4.5. Multisite depth electrode EEG

Multisite local field potential (LFP) electrodes were implanted in *Grin2b*<sup>+/-</sup> rats to investigate SWD propagation dynamics. Six bilateral craniotomies were drilled over the somatosensory cortex (-2.5 mm rostral, ± 5 lateral relative to bregma), nRT (-1.45 mm rostral, ± 2.2 lateral relative to bregma) and substantia nigra (-5.5 mm rostral, ±2.1 lateral relative to bregma), and the dura was gently removed. Paired twisted LFP electrodes (made in-house, diameter = 50.8 µm, Teflon insulated stainless steel, A-M systems) were placed in guide cannulas (polar fused silica tubing, diameter = 0.32 mm, Sigma-Aldrich) to avoid bending upon insertion resulting in off-target implantation. Electrodes were slowly inserted to target the somatosensory cortex, nRT and substantia nigra (-1 mm, -5 mm and -7.5 mm ventral from the brain surface respectively). Electrodes were fixed in place using UV activated cement (3M Relyx Unicem 2 Automix, Henry Schein). Two ground screws were attached over the cerebellum (-11.5 mm rostral, ± 0.5 mm lateral relative to bregma) and connected to an electronic interface board (EIB-16, Neuralynx) via silver wire soldered to each component (Teflon Coated Silver Wire, World Precision Instruments). LFP electrodes were then attached to the electronic interface board (EIB-16, Neuralynx). The implant was fixed to the skull with dental cement (Simplex Rapid, Kemdent, United Kingdom) and the incision was sutured. A total of six *Grin2b*<sup>+/-</sup> animals were recorded and analysed.

#### 2.4.6. Recording methods

Prior to all recording procedures rats were habituated to the recording room for at least 24 hours. Animals were briefly anaesthetised with isoflurane to connect implants to wireless amplifiers. In polysomnography experiment rats were chronically recorded for 72 to 96 hours, while in systemic pharmacology and drug infusion experiments short-term recordings, approximately 4 hours in length, were taken. For depth electrode LFP recordings, I performed 24-hour recordings, however, analysis was conducted on a select 2 hours only between 11:00 and 13:00 o'clock. SWDs in *Grin2b*<sup>+/-</sup> animals occurred at similar rates throughout the 24-hours, and therefore the 2 hours from 11:00 to 13:00 o'clock were chosen for this and for pharmacology experiments detailed below (section 2.5). The TaiNi wireless multichannel recording system (Tainitec, United Kingdom) (Z. Jiang et al., 2017) was used for all recordings, which were made at a sampling rate of 250.4 Hz. I was blind to animal genotype.

## 2.5. Pharmacology experiments

### 2.5.1. Systemic treatment administration

For pharmacology experiments, time-matched 2-hour periods from 11:00 to 13:00 o'clock across treatment days, were used to analyse the effects of drug treatments on SWDs. Treatment was administered 30 minutes prior to the start of the recording and analysed period (at 10:30 am). Total recording durations for each experimental day did not exceed 4 hours. Drugs were dissolved in 1 ml of sterile 0.9% saline and rats received treatment based on individual rat weight. Each animal received either intraperitoneal (IP) ethosuximide (100 mg/kg, Merck, Germany), memantine (5 mg/kg, Enzo Life Sciences, United States), or saline as vehicle control on separate individual treatment days, between baseline and washout days, with 24 hours between each experimental day (baseline, drug and washout) (Figure 2.1A and B). The half-lives of ethosuximide and memantine have been reported to be 10-16 hours and less than 4 hours respectively in rats, suggesting that a 24-hour washout is sufficient to record a new baseline period for another treatment (Beconi et al., 2011; Löscher, 2007). Treatment order (including saline) was randomized and systematically weighted so that each possible permutation of treatment order occurred across an equal number of animals to minimize the risk of synergistic effects between drugs. SWD number for each treatment, per individual rat, was normalized to its baseline SWDs and data is presented as a percentage of the animal's baseline seizures. These percentages are then compared statistically across the population. I was blinded to treatment and genotype.

### 2.5.2. Site-specific treatment administration

To examine the critical structures mediating SWDs in the *Grin2b* haploinsufficiency model I infused pharmacological compounds into the somatosensory cortex and nRT (Figure 2.1A), two major hubs of the thalamocortical loop thought to be involved in SWD activity (Crunelli et al., 2020). Drugs were prepared and administered as described above (section 2.5.1). (Figure 2.1A and B). A total volume of 1 µl was infused for each treatment, with 0.5 µl administered per hemisphere using an infusion cannula connected by polyethylene tubing to a 1 µl Hamilton syringe. Injected volume (0.5 µl) and flow rate (10 nl/s) were controlled by a precision pump (World Precision Instruments, Hertfordshire, United Kingdom). During treatment administration rats were put under mild isoflurane anaesthesia, maintained between 1.5-2.5%, and the procedure was performed under a microscope to ensure infusion was effectively achieved. Drug infusions, under anaesthesia did not exceed 10 minutes (including brief anaesthetic induction). Treatment was administered 30 minutes prior to the start of the recording/analysed period (at 10:30 am) and a 1-hour period between 11:00 to 12:00 o'clock across days, was

used to analyse the effects of pharmacological treatments on SWDs (Figure 2.1B). Based off the pharmacokinetic two-compartment model the half-life for isoflurane elimination from the brain of rabbits is 26 minutes (Wyrwicz et al., 1987). Since the rat metabolism is slightly faster than that of rabbits (Subcommittee on Laboratory Animal Nutrition et al., 1995), and isoflurane exposure during infusions was short and at low concentrations, I reasoned that at 30 minutes most of the isoflurane is eliminated from the rat brain, while the following 1-hour period would still allow for observation of treatment effects. Treatment randomisation and SWD normalisation followed the same methods as for systemic administration (section 2.5.1). I was blinded to treatment and genotype.

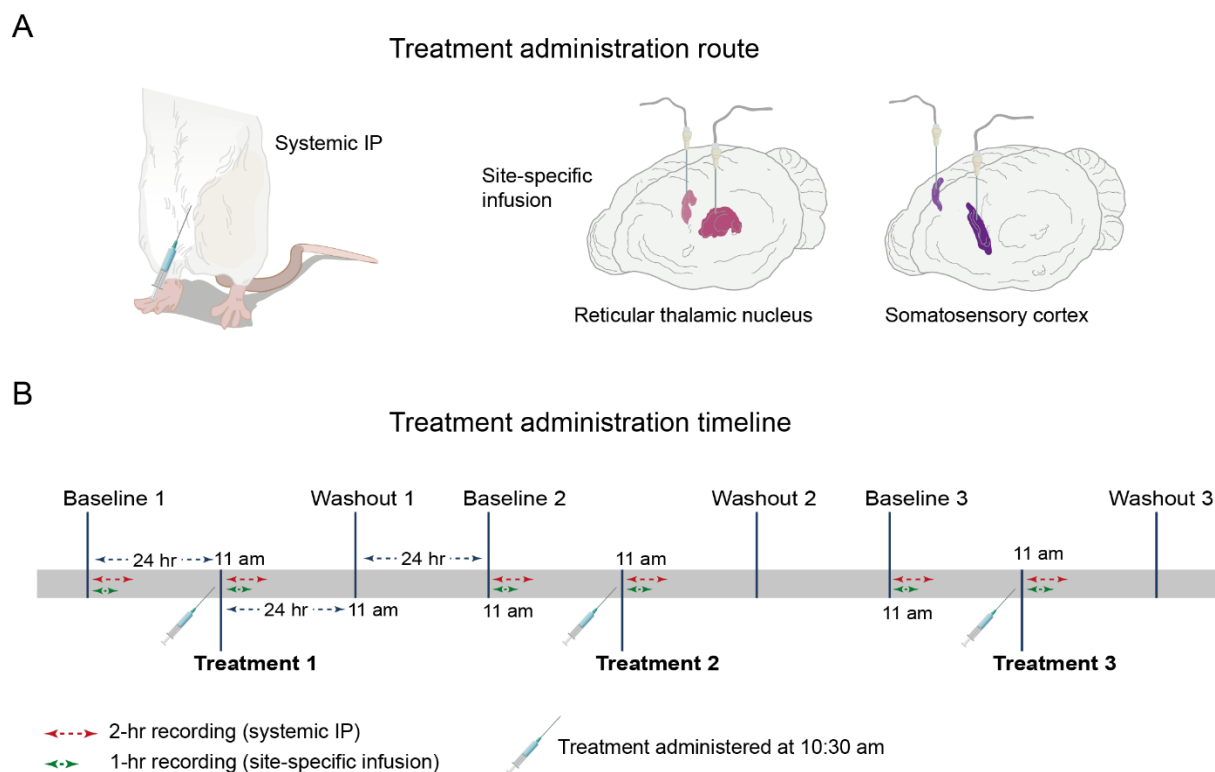


**Figure 2.1. Acute administration of anti-seizure drugs.** (A) Schematics for drug administration routes in pharmacology experiment. Anti-seizure drugs were given via systemic IP injection or in a separate set of experiments via site-specific infusion to the nRT or somatosensory cortex. (B) Diagram of pharmacology experiment timeline. Drugs were administered at 10:30 am, half an hour prior to recordings commencing. 2-hour recordings (red dotted line) were taken following systemic IP treatment, and 1-hour recordings (green dotted line) were taken after site-specific infusion of drugs.

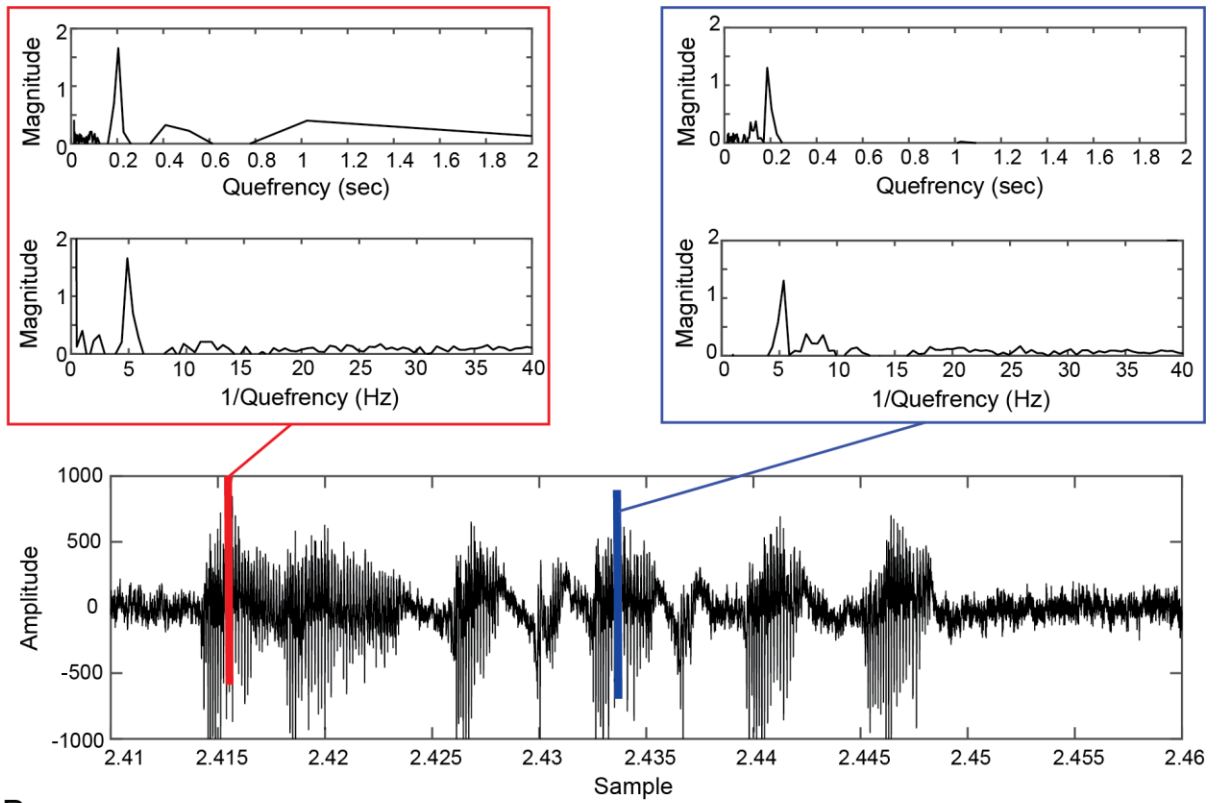
## 2.6. Spike and wave discharge detection

SWDs were characterized by periodic high-amplitude oscillations between 5 and 10 Hz (Meeren et al., 2002), which coincided with a spontaneous arrest of animal movement. I first utilized a previously published automatic SWD detector algorithm to quantify SWDs (Buller-Peralta et al., 2022; Katsanevaki et al., 2024), which uses identification of harmonic peaks at 5-10 Hz in the power spectra to label seizure periods. The code used for analysis is available at <https://github.com/Gonzalez-Sulser-Team/SWD-Automatic-Identification>.

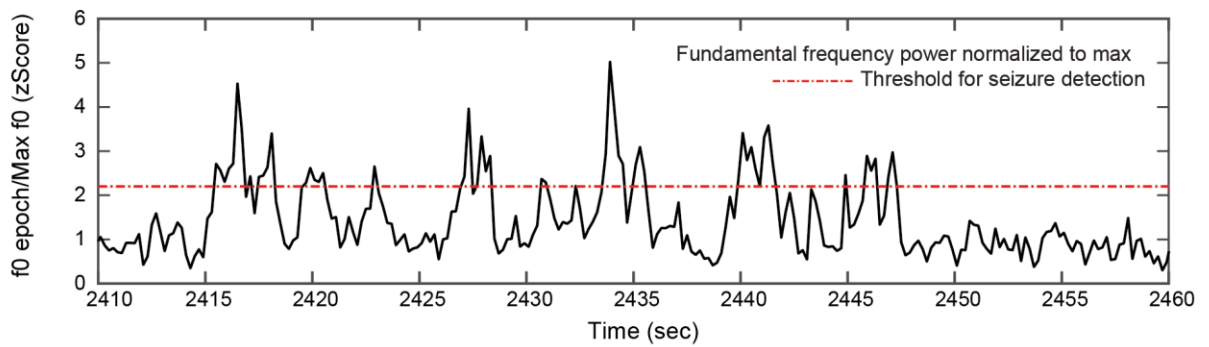
Briefly, spectral analysis of visually identified SWDs in rats recently showed that SWDs behave as high-energy echoes of a fundamental frequency ( $f_0$ ) in the 5-10 Hz theta band, resonating in multiple periodic harmonics across the frequency spectrum. This periodic waveform enables automatic SWD detection using cepstral analysis (Childers et al., 1977), which searches for high-amplitude peaks in specific frequency bands. When applied the automated SWD seizure detection algorithm works by deconvolving the raw EEG signal using a Fast Fourier Transform (number of tapers = 5), following which a logarithmic function is applied to obtain the magnitude of each signal frequency and identify harmonics. The resulting signal is treated as semi-periodic, and the inverse Fast Fourier Transform is applied to attain the cepstrum, which highlights periodic features of the original signal in the pseudo-time domain. This transformation allows for identification of the peak cepstral power of the fundamental frequency ( $f_0$ ) as a spike within this domain (Figure 2.2A). Cepstrum for the entire EEG recording is obtained in 0.2 sec sliding windows, and peak power cepstrum values within the SWD 5-10 Hz frequency range are identified and normalized by their absolute maximum. The resulting vector is transformed into z-scores to standardize potential power differences between recordings that may distort the identification of SWD thresholds. A threshold of  $\geq 2.2 \times 10^{-5}$  standard deviation is established by comparing the values of visually scored SWDs with other high-magnitude noise that may lead to false positives (Figure 2.2B). Time windows of 0.2 seconds are marked as seizures when the z-scored peak cepstral power in the theta band meets or exceeds the established standard deviation threshold. Finally, identified events which were  $< 0.8$  seconds in length are discarded. Sequential events with  $< 1$  second in between are counted as one event (Figure 2.2C).

In all seizure related experiments, the automated SWD seizure detection algorithms were applied on voltage traces from the EEG screw and grid electrode leads positioned roughly over the somatosensory cortex, as visual assessment indicated that these channels frequently displayed high-amplitude SWDs across the animals. EEG channels were visually inspected and the channels with fewer artefacts were selected.

A



B



C

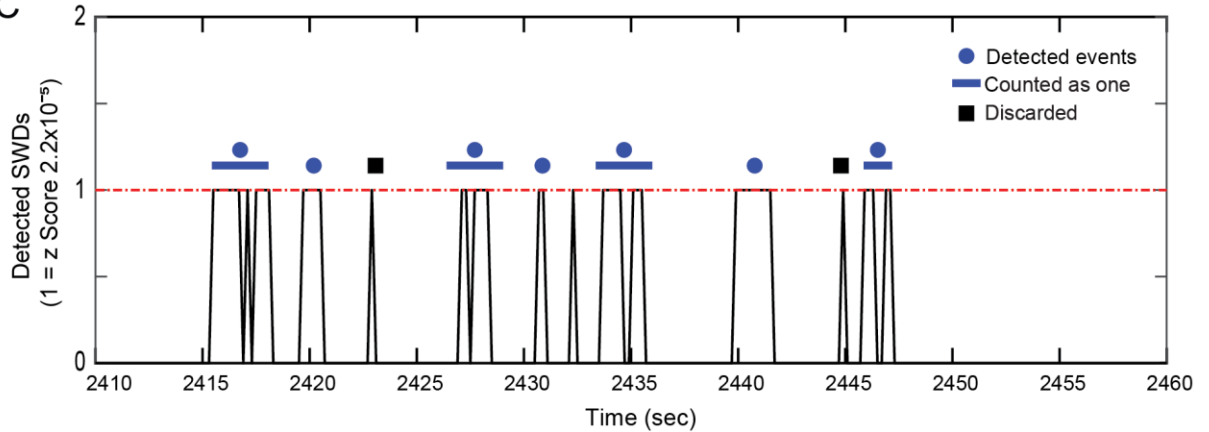


**Figure 2.2. Method for automatic detection of SWD.** (A) Raw EEG trace of a representative SWD (bottom), with two selected SWD epochs (0.2 seconds each) marked with blue and red vertical lines. Inserts (top) show respective cepstral power analysis showing the fundamental frequency ( $f_0$ ) peak on

*the pseudo-time domain (top inset) and pseudo-frequency domain (bottom inset). (B) Peak cepstral power in the theta band range (5-10 Hz) calculated in 0.2 second epochs, normalized to its absolute maximum value, and transformed into z-scores. The threshold for detecting SWD events is marked with dashed red line. (C) Detected SWDs transformed into zeros (below threshold) or ones (threshold or over) on the time interval shown in panels A and B. Events counted as one (< 1 second between events) are marked by blue lines, whereas black squares designate discarded events (length < 0.8 seconds). Figure adapted from Katsanevaki et al. (Cell Reports, 2024).*

## 2.7. Automatic sleep-wake scoring

A custom-made automated sleep scoring algorithm (Hristova et al., 2024) was developed, based on a previously published method (Madrid-López et al., 2017). It clusters EEG and EMG recording epochs assigning each to either wake, NREM or REM sleep according to the spectral power at specific frequency bands associated with each brain state. Traces from EEG channels overlying the somatosensory cortex on either brain hemisphere and the two EMG channels were visually inspected for excessive movement artefacts. The EEG and EMG channels with fewer artefacts were chosen for analysis and if both EEG or EMG traces had similar artefact levels, one channel for each modality was randomly chosen for analysis. Spectral logarithmic EEG and EMG power was calculated for non-overlapping 5 second epochs in the 0.2-125 Hz frequency range using the multitaper package (Rahim et al., 2014) for R (Posit team, 2022), using non-overlapping window-size of 0.2 Hz half-time and bandwidth product of three. Each epoch was plotted in a three-dimensional metric space with values representing theta EEG power (maximum of the power in the 6.0 – 8.2 Hz range), delta and sigma power (1 - 20 Hz mean power, excluding the theta range), and neck muscle activity power (EMG power in the 60–90 Hz range). For clustering into brain states, centroids were estimated using an iterative algorithm that maximizes the local density by segregating the epochs based on the following characteristics: NREM sleep displayed high delta and sigma power with low EMG power, REM sleep displayed high theta power and low EMG power, and wake displayed high EMG power and low delta and sigma power. The distribution of clusters around the estimated centroid were approximated by Gaussian models assuming ellipsoidal shapes. The covariance matrix of the Gaussian models was used to calculate the Mahalanobis distance from a given epoch to each cluster. The epochs are assigned to a sleep stage according to a minimum Mahalanobis distance to the respective cluster (Figure 2.3A and B). The automated sleep scoring code is available at <https://github.com/Gonzalez-Sulser-Team/AUTOMATIC-SLEEP-SCORER>.

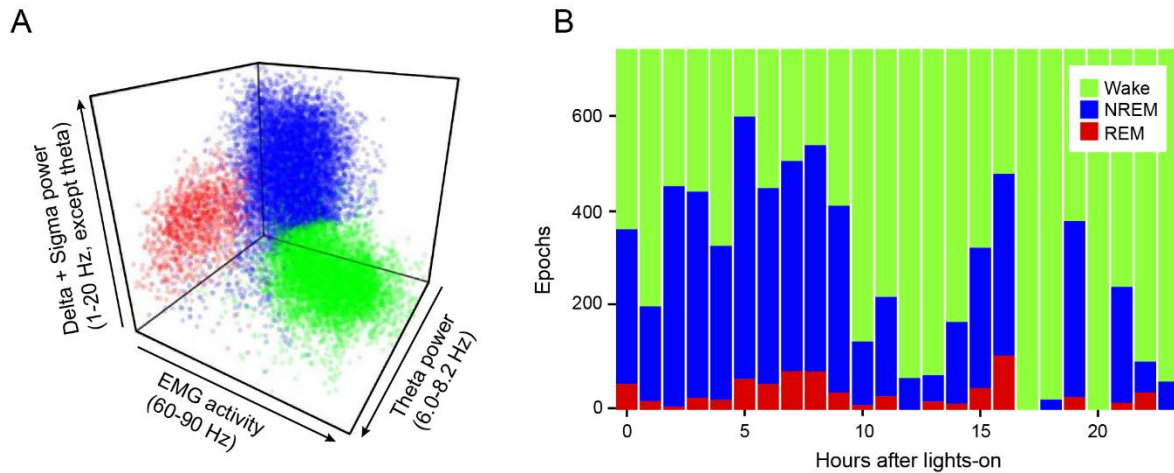


**Figure 2.3. Automated sleep scoring output from a representative rat. (A)** Clustering by state of 5 second epochs into 3D space from an example baseline recoding day. Epochs are segregated based on the following characteristics: high delta and sigma power with low EMG power – NREM, high theta power and low EMG power – REM, and high EMG power and low delta and sigma power – wake. Dots correspond to individual epochs assigned to one of the three states. Arrowheads indicate direction of increasing power within the specific frequency dimension. **(B)** Distribution of sleep and wake state epochs by hour for the full 24-hour period following automated scoring of the example day shown in panel A. Figure partially adapted from Madrid-López et al., (2017).

Automated sleep scoring performance was validated by comparing the automatically generated vigilance state output with visual EEG state scoring of identical 5 second epochs. Researchers visually scoring were blind to automated output. 4 hours of EEG were manually scored in seventeen animals, between 10:00 – 14:00 o'clock, when approximately equal distributions of wake, NREM and REM epochs were observed. Percent agreement and Cohen's kappa were calculated to assess interrater reliability between visual and automatic scoring of brain states (McHugh, 2012). Agreement between visual and automatic scoring was 88.1%, 87.5% and 95% for REM, NREM and wake states respectively. Overall agreement between the scoring methods was 90.8%. The kappa coefficient was 0.83 ( $\pm 0.002$  SE) (Figure. 2.4A and Table 2.1).

SWD and sleep-wake state data was analysed over 48 hours starting at zeitgeber time 07:00 am of the day after connection, under a 12-hour/12-hour light–dark schedule. Values per hour over the two days were then averaged over the 24-hour period.

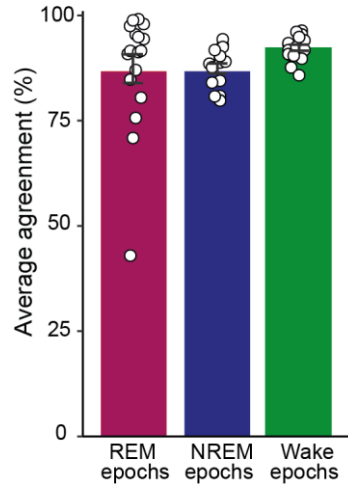


**Figure 2.4. Comparison between sleep-wake automated scoring algorithm and visual scoring.** Agreement between visually and automatically scored epochs of REM, NREM and wake states is 88.1%, 87.5% and 95% respectively. Overall agreement between the scoring methods is 90.8%. The kappa coefficient was  $0.83 (\pm 0.002 \text{ SE})$ . Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

|                                                  | Visually scored epochs |       |       |
|--------------------------------------------------|------------------------|-------|-------|
|                                                  | REM                    | NREM  | Wake  |
| Automatically scored epochs                      |                        |       |       |
| REM                                              | 2378                   | 107   | 233   |
| NREM                                             | 119                    | 20637 | 781   |
| Wake                                             | 201                    | 285   | 19254 |
| Agreement (%)                                    | 88.1                   | 87.5  | 95    |
| Global agreement (%)                             | 90.8                   |       |       |
| Cohen's kappa ( $\kappa$ ) ( $\pm Se_{\kappa}$ ) | $0.83 \pm 0.002$       |       |       |

**Table 2.1. Comparison of the performance of sleep-wake automated scoring algorithm and visual scoring with results from statistical comparison.** Numbers represent epoch counts. The diagonal elements (blue) represent instances of agreement in epoch classification for REM, NREM and wake states between automated scoring and visual scoring. Elements off-diagonal (orange) represent instances of disagreement in state scoring.

## 2.8. Analysis of spike and wave discharges by sleep state

In the *Grin2b* haploinsufficiency line an additional peak detection algorithm (van Brakel, 2014) was required to identify SWDs during wake epochs as harmonics reminiscent of SWDs were detected in the average power spectra suggesting inconsistent labelling. This algorithm utilises z-scores to identify peaks by calculating a moving mean and flagging datapoints that deviate from the moving mean by a given threshold. If peaks were identified in both 5 - 9 Hz and 10 -18 Hz range, this was indicative of the presence of harmonics, which are evident in SWD epochs. After automated scoring of all additional SWD epochs the sleep quantification was adjusted accordingly.

All epochs identified to contain SWDs were classed separately from the other three brain states. SWD distribution across wake and REM was determined by evaluating the first 5 second epoch prior to a seizure occurring, while the first 30 seconds (six epochs) prior were used to assess SWD initiation in NREM and wake-NREM transitions.

## 2.9. EEG spectral power analysis

After initial brain state scoring, further processing was performed on the data to remove epochs with excessive noise. A third-order bandpass Butterworth filter (0.2 – 100 Hz) was applied to the full-length raw data. 5 second epochs were extracted from the filtered recording and discarded if any data point exceeded 3000mV, which was beyond the range of physiological activity.

To calculate the power spectral density, brain state epoch averages were Hanning-tapered and Fourier transformed using the SciPy welch method (with 50% overlapping windows). The spectral slope, a regression-based fitting measurement which describes the rate of change of the EEG power spectra, was calculated between 1 - 48 Hz using the NumPy polyfit function (Fasol et al., 2023). Epochs that were outside of the visually selected threshold of  $-5 \log_{10}(\text{mV}^2)/\text{Hz}$  for the spectral slope and 500 mV for the spectral offset were removed from further analyses.

## 2.10. Manual scoring of spike and wave discharge propagation

SWDs are reported to initiate in the somatosensory cortex prior to generalising (Crunelli et al., 2020; Meeren et al., 2002). Therefore, SWD onset across all depth LFP channels (two per location) bilaterally targeting somatosensory cortex, nRT and substantia nigra were visually scored. Time matched 2-hour windows between 11:00 – 13:00 o'clock were used, based on 24-hour sleep data

acquired, which identified the highest likelihood of all three brain states (wake, NREM and REM) occurring was within this timeframe. The duration of individual SWDs was recorded, along with the SWD onset time for each channel. Seizures were labelled as either originating in somatosensory cortex or as generalised (when onset time was shared across all channels).

## 2.11. Histology

### 2.11.1. Perfusion fixation

Rats were anaesthetized with isoflurane followed by a lethal dose of sodium pentobarbital (between 0.7 and 1ml based on weight) and transcardially perfused with ~ 50 ml phosphate-buffered saline (PBS; Invitrogen) followed by ~ 50 ml 4% paraformaldehyde (PFA; Sigma Aldrich) in 0.1 M phosphate buffer (Sigma Aldrich). Brains were removed and post-fixed overnight in 4% PFA, then rinsed in PBS and incubated overnight in 30% sucrose in PBS. Tissue was then placed in Optimum Cutting Temperature (OCT) embedding matrix and sliced coronally in 60 µm thick sections using a cryostat. Free floating sections of interest containing and in proximity to cannula track marks were collected and stored in PBS with sodium azide 0.05% (Sigma Aldrich) at 4°C until used for immunohistochemical labelling.

### 2.11.2. Immunohistochemistry

Sections were rinsed in PBS three times and incubated overnight at 4°C with Neurotrace 640/660 (1:500, Life Technologies) in 0.3% Triton™ X-100 (Sigma-Aldrich) in PBS. Slices were triple rinsed in PBS and mounted on glass slides with Mowiol (Sigma Aldrich) mounting medium. Slides were kept in the dark at 4°C until imaged.

### 2.11.3. Imaging

To evaluate the anatomical location of infusion cannulas, tiled fluorescent images were acquired across all immunohistochemically labelled tissue sections using a Zeiss Axio Scan.Z1 microscope and a Plan-Apochromat 10×/0.45 M27 objective. Cannula placement coordinates were then validated against Paxinos and Watson's Rat Brain Atlas (Paxinos & Watson, 2018).

## 2.12. Statistical analysis

Sample size estimates were based off prior polysomnography and pharmacology studies in rodents (Buller-Peralta et al., 2022; F. Cao et al., 2020; Katsanevaki et al., 2024; Seok et al., 2018; Thomas et al., 2017). Normality and homoscedasticity for all data were estimated by Shapiro–Wilk and Levene’s tests (rejection value set at  $< 0.05$ ), and based on these, comparisons between genotypes were made using two-sample unpaired t-test, Welch’s two-sample t-test or Wilcoxon rank sum test (Mann-Whitney U Test equivalent). Two-way ANOVA tests were performed where more than two groups were compared (genotype and frequency). A Linear Mixed Model (LMM) approach was used for time series analysis, where multiple measures were taken from the same animal. The method is robust against unbalanced data and allowed for comparisons to be made across groups using genotype, sex and treatment as fixed effect and individual animals as random effects. This model avoids pseudoreplication and is appropriate for the analysis of longitudinal data with within-subject design, whereby subject specific response trajectory is accounted for (Baayen et al., 2008; Fitzmaurice & Ravichandran, 2008). Wherever appropriate, these were followed by *post hoc* Tukey’s multiple comparisons, set at a 95% confidence level (alpha level of 0.05) to control the family-wise error rate. Pearson correlation was used to test the relationship of SWDs with brain state abnormalities. Analyses were conducted in R (R Core Team, 2022), RStudio (Posit team, 2022), using the ggplot2, car and lmerTest packages (Fox & Weisberg, 2019; Kuznetsova et al., 2017; Wickham, 2016). All data in figures are presented as mean  $\pm$  SEM, and  $P < 0.05$  was considered as significant and indicated with \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .



# Chapter 3

## Altered Sleep Physiology in *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-y</sup> and *Grin2b*<sup>+/-</sup> Rats Modelling Neurodevelopmental Disorders

### 3.1. Introduction

Sleep disturbances negatively impact on quality of life, daytime behaviour, and seizure thresholds, and are extremely common in individuals with NDDs with prevalence rates reported to be as high as 80% (Barone et al., 2019; Dosier et al., 2017). The predominant sleep complaints reported in children and adults with NDDs include difficulty in settling down at night and initiating sleep, frequent nocturnal awakenings and sleep maintenance, as well as daytime somnolence. Sleep fragmentation throughout the day and night determines daytime sleepiness, and can itself contribute to irregular sleep schedules, disruption of circadian rhythms, and complete reversal of the night–day cycle (Angriman et al., 2015). Sleep disturbances in individuals with NDDs have consistently been associated with a range of functional impairments, encompassing cognitive deficits (reduced IQ performance), adaptive behaviour, and dysregulation in emotional processing and behavioural control mechanisms (Kamara & Beauchaine, 2020). Where epilepsy is comorbid with NDDs, sleep is usually further impaired by seizures and ictal activity, while sleep disruptions can lower seizure thresholds (Bazil et al., 2000; Moore et al., 2021; Stirling et al., 2023). Findings of the sort underscore the pervasive impact of sleep dysfunction on multiple domains of functioning in those with NDDs and bring emphasis to the need for a better understanding of the nature of sleep in NDDs.

The high rate of sleep disorders in individuals with NDDs is somewhat intuitive, given that sleep is controlled by the central nervous system, the function of which is commonly perturbed in NDDs. Sleep impairment in NDDs is thought to arise from intrinsic neurologic pathophysiology, while comorbid psychiatric disorders, behavioural disorders, epileptic seizures, sleep disordered breathing, and medication side effects (e.g. from antiseizure and antipsychotic drugs) further negatively impact on

sleep processes (Dosier et al., 2017). From a pathophysiology perspective, many of the genes associated with genetic NDDs are also mechanistically involved in specific sleep related biological pathways. For instance, a screen for molecular correlates of sleep need recently identified many synaptic proteins, including *SYNGAP1*, *SHANK3*, and *GRIN2B*, responsible for *SYNGAP1* haploinsufficiency, Phelan-McDermid syndrome (*SHANK3* deficiency) and *GRIN2B* related NDD respectively. Phosphorylation of these proteins accumulates in relation to sleep need, to serve as a molecular signature of sleep pressure that modulates subsequent sleep cycles (Z. Wang et al., 2018). Furthermore, in humans and mammalian species, the highest level of sleep happens during the neonatal period, when the brain develops rapidly and is highly plastic, and it is speculated that NDD causing *de novo* mutations in developmentally critical genes could be responsible for the perturbation of sleep and sleep related networks in early developmental ages (Barone et al., 2019). Additional evidence suggest that circadian system desynchronization may also be implicated in NDDs, and research has identified alterations in melatonin synthesis and key circadian genes resulting in sleep fragmentation and sleep-wake rhythm dysregulation (Carmassi et al., 2019; Kamara & Beauchaine, 2020; Laposky et al., 2005; Walker et al., 2020; Z. Yang et al., 2016). What remains unknown is whether sleep and circadian dysfunctions in NDDs are resultant from genetic mutation alone, from mutations indirectly affecting downstream sleep and circadian pathways, or as a consequence of abnormally developed brain networks.

Cognitive deficits, sleep disturbances, difficulty falling asleep, as well as hyperactivity and impulsivity at time of sleep are reported in individuals with *16p11.2* deletions (B. A. Fernandez et al., 2010; Hanson et al., 2010; Kamara et al., 2023; Tabet et al., 2012). Melatonin and risperidone, a drug used for the management of psychiatric and behavioural issues in ASD patients, have been trialled for the treatment of sleep impairment in some individuals with the CNV, however, long-term efficacy data on sleep management is limited. In probands with severe sleep impairment polysomnography has shown non-epileptic abnormalities consisting of temporo-occipital and parietal hypersynchrony and rapid rhythmic waves in the EEG (Tabet et al., 2012). Such features are likely to disrupt normal sleep architecture, interfere with progression through sleep stages, and potentially lead to fragmented or non-restorative sleep.

Several mouse models have recapitulated the sleep disorder phenotypes observed in patients with *16p11.2* deletion CNVs, though with varied results. Male heterozygous *16p11.2* deletion mice exhibit home cage hyperactivity throughout both light and dark phases, independent of stress/ anxiety, while maintaining normal circadian rhythm dynamics (Angelakos et al., 2017). During the light phase, male *16p11.2* deletion mice spend significantly more time awake, experience reduced NREM sleep, particularly in the light phase, and have longer wake bouts. Additionally, they display increased alpha power during wakefulness (Angelakos et al., 2017). In contrast to males, female heterozygous *16p11.2* deletion mice exhibit physiologically normal sleep patterns (Angelakos et al., 2017). In a different study male mice from the same heterozygous *16p11.2* deletion line, similarly, presented with increased time

awake and reduced time in both REM and NREM sleep. In these mice the number and duration of REM bouts was decreased, suggesting deficits in REM-NREM transitions and impairment of REM maintenance (H.-C. Lu et al., 2019). The authors demonstrate that in male *16p11.2* heterozygous deletion mice PAG-projecting GABAergic paraventricular nucleus neurons in the ventral medulla, have hyperpolarized resting membrane potential and increased membrane resistance, which contributes to observed deficits in NREM-REM transition and REM maintenance (H.-C. Lu et al., 2019). In addition, heterozygous male *16p11.2* deletion mice were found to exhibit state and phase dependent spectral power alterations. During the light phase, these mice had increased theta during wake, decreased delta during NREM, and both decreased theta and increased beta during REM. In contrast, during the dark phase, they showed decreased theta during wake and decreased theta alongside increased delta during REM (H.-C. Lu et al., 2019). In a recent preprint from Choi and colleagues, altered sleep physiology consisting of frequent NREM to wake microarousals was observed in the same *16p11.2* deletion mouse line as above. These microarousals, defined as short ( $\leq 20$  seconds) wake-like episodes during sleep, resulted in decreased NREM duration and increased NREM bout frequency. Furthermore, the strength of the infraslow sigma rhythm, an oscillation related to the amplitude modulation of sigma power over time, was significantly reduced in male *16p11.2* deletion mice. The increased frequency of microarousals was linked to heightened LC-NE activity during NREM sleep, with inhibition of LC-NE neurons or noradrenergic transmission improving sleep continuity in male heterozygous mice. Anatomically, heterozygous mice exhibited more wake-promoting inputs from the lateral hypothalamus and ventral medulla, suspected to contribute to sleep fragmentation and increased microarousals, along with fewer inhibitory inputs from the cortex and hippocampus (Choi et al., 2024).

Sleep dysfunction is commonly present in ASD and ID and has been specifically reported in three related individuals with *NLGN3* variants contributing to both NDDs (Bian et al., 2023; Cohen et al., 2014; Quartier et al., 2019). In a recent study, *NLGN3* knockout rats exhibited significant sleep and behavioural abnormalities, including decreased NREM sleep, increased REM sleep, and reductions in beta and gamma spectral power (Thomas et al., 2017). These animals also displayed reduced social interactions, hyperactivity in open field tests, an increased tendency for chewing, and an exaggerated startle response. Activity levels were significantly elevated during the dark phase of the 24-hour cycle. In terms of sleep architecture, *Nlgn3* knockout rats had prolonged wake bouts, particularly in the final hours of the dark phase, and spent overall less time in NREM sleep. In contrast, they showed increased REM sleep during the light phase, with significantly longer REM bout durations. Spectral beta and gamma power were reduced in knockout animals across wake and sleep states, while elevated alpha power during wake and REM, and delta power during NREM, were also observed, suggesting altered sleep depth and cortical excitability (Thomas et al., 2017). Altered spectral power profiles during sleep and wake have also been reported in mice carrying the *Nlgn3*-R451C mutation first identified by Jamain et al. (Bian et al., 2023; Jamain et al., 2003; J. J. Liu et al., 2017). These animals exhibit significantly

reduced delta, theta, and alpha spectral power during NREM sleep, reduced alpha power and increased beta power during REM sleep, and displayed increased sigma and beta power during wake. Spectral power for all frequency bands was found to be higher during the dark period for wake and higher during the light period for NREM and REM sleep, suggesting that EEG power follows expected circadian patterns (J. J. Liu et al., 2017). Interestingly, *Nlgn3* knockin mice did not show differences in time spent in wake, NREM, or REM sleep, nor in the number and duration of wake, NREM, and REM sleep bouts (J. J. Liu et al., 2017).

Finally, sleep dysfunction is observed in over 60% of individuals with NDDs who have pathogenic *GRIN2B* variants, but this has not been rigorously investigated. Recently, severe early-onset insomnia at two weeks of age, characterized by difficulties falling asleep and frequent nighttime awakenings, was documented in a patient with a pathogenic *GRIN2B* missense variant. This individual also exhibited an electrical status epilepticus-like pattern during sleep, which is expected to impede the achievement of deep sleep stages and result to arousal (den Hollander, Veenvliet, et al., 2023). Additional data regarding sleep dysfunction in *GRIN2B* related NDDs comes from parental surveys and reported issues amongst patients consist of sleep initiation difficulty, breathing irregularities during sleep and daytime somnolence (Freunscht et al., 2013; Platzer et al., 2017; Simons Searchlight, 2021).

Clinical data and quantifiable sleep research of altered sleep and circadian architecture in patients with NDDs is sparse and hindered by variable sleep assessment methods, small sample sizes, and significant heterogeneity in patient age, variant type and symptomatology, even in individuals with the same NDDs causing mutations. This variability poses substantial challenges in formulating conclusions regarding sleep-related issues in specific cohorts and in accurately evaluating the efficacy of sleep interventions. Considering this, the recent sleep phenotypes uncovered in preclinical rat and mouse models of *16p11.2* deletion and *Nlgn3* related NDDs have produced valuable, yet contrasting results, stressing the need for further sleep assessment in these rodent lines, as well as a more standardized and unbiased sleep scoring approach. In addition, sleep in many rare genetic NDDs including *GRIN2B* related NDDs has not been evaluated, representing a significant gap in the field of NDD and sleep research. Therefore, further controlled research in animal models could aid in identifying sleep and circadian related clinically translatable biomarkers specific to certain genetic NDDs, which could be useful for monitoring of disorder progression. Moreover, these models offer valuable insights into the mechanisms underlying dysfunction in NDDs.

In this thesis chapter, I address the need for quantifiable sleep data in three rat lines of NDDs using a custom-built sleep stage automatic scoring algorithm (for algorithm validation results see Chapter 2, section 2.7). I conducted long-term uninterrupted wireless polysomnography recordings in rats with *16p11.2* deletion, complete knockout of *Nlgn3*, and heterozygous knockout of *Grin2b*, and hypothesised that sleep distribution and circadian rhythmicity will be altered in these animals. To

determine this, measurable sleep parameters and spectral power properties of the three vigilance states – REM, NREM and wake were evaluated.

## 3.2. Methods

Detailed descriptions of animals and animal husbandry are described in Chapter 2, sections 2.1 – 2.4. Across all animal models sleep screening employed 14-channel EEG grids coupled with 2 EMG channels. Animals were recorded wirelessly for 48 hours, and sleep and wake states were quantified offline using a custom-made automatic sleep scoring algorithm. Chapter 2 contains relevant methods for surgical implantation (2.4.1 and 2.4.2), wireless EEG recording (2.4.6), automated sleep scoring and quantification (2.7), power spectral analysis (2.9), and statistical analysis details (2.12) used in this chapter. Of note, *Grin2b*<sup>+/-</sup> animals also presented with seizures which were quantified as an independent state and sleep quantification was adjusted accordingly (see Chapter 2, sections 2.6 and 2.8). In addition, *16p11.2*<sup>+/-</sup> male rats had an unexpected REM sleep phenotype, therefore all identified REM sleep epochs in these animals were also visually inspected and proofed.

## 3.3. Results

To investigate the sleep-wake patterns across the circadian cycle in each line, animals were implanted with 14-channel surface electrode grids and EMG electrodes in neck muscles (Figure 3.1A). Wireless freely moving 24-hour recordings were performed in the rats' home cages to acquire EEG and EMG during REM, NREM and wake states (Figure 3.1B-D). I quantified for each brain state, the number of bouts, average bout duration and total time spent in REM, NREM and wake to determine if *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> animals exhibited abnormal sleep physiology, as is reported in patients with NDDS of corresponding genetic aetiologies. I tested for differences in sleep-wake patterns for the full 24-hours, and in the light-dark phase distribution of each state. Hourly time course analysis across the 24-hour cycle was also conducted, as this could reveal more subtle trends and abnormalities in sleep-wake rhythms and circadian activity that are lost in net 24-hour and 12-hour analyses. For example, delayed sleep onset, which can be indicative of dysregulation in circadian signalling or sleep-wake homeostatic processes (Chang et al., 2009; Nesbitt, 2018).

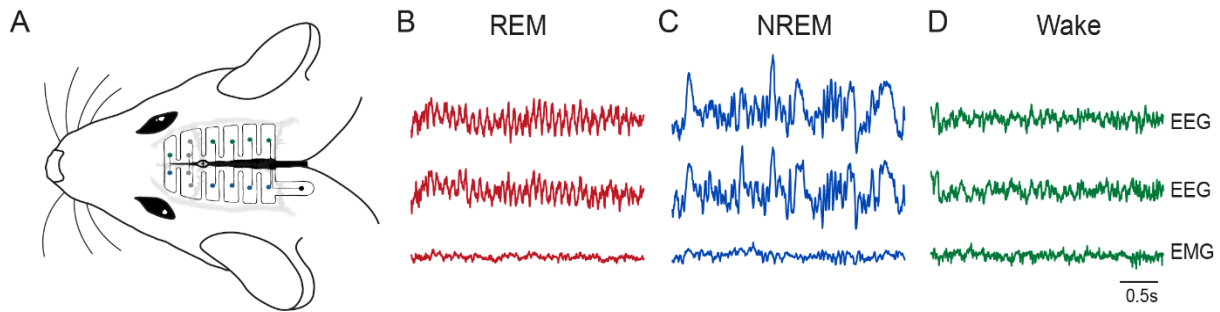


**Figure 3.1. Recordings of rat EEG and EMG used to identify vigilance states.** (A) Schematic of a 16-channel skull-surface EEG implant illustrating approximate placement of electrodes relative to the skull. Representative EEG and EMG voltage traces for REM (B), NREM (C) and wake (D). REM is characterized by high theta activity and low EMG, and NREM by high delta and sigma power with low EMG tone. Wake is characterised by high EEG theta and high EMG activity; EEG is dyssynchronous during this state. Bottom scale bar = 0.5 seconds.

Spectral power analysis of sleep EEG is a powerful tool that provides rich information about sleep architecture, quality, and underlying neurophysiology. Historically, spectral abnormalities during sleep, such as decreased delta, and increased beta and gamma power have been reported in patients with ASD, ID and ADHD, who struggle with dysfunctional sleep (Lanigar & Bandyopadhyay, 2017; Ostrowski et al., 2021; Robinson-Shelton & Malow, 2016; Y. Shi et al., 2022; Y. Zhang et al., 2022). Therefore, I hypothesized that deleterious mutations of *16p11.2*, *Nlgn3* and *Grin2b* in rat models affects the physiological dynamics of individual brain states. I tested whether spectral properties across REM, NREM and wake in *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> rats differed from those of wild-type littermate animals. For each rat line, the average EEG spectral power from one representative channel across all animals and epochs of REM, NREM and wake states was calculated. Statistical comparisons between genotypes were made for delta (1-5 Hz), theta (5-10 Hz), sigma (10-16 Hz), beta (16-30 Hz) and gamma (30-48 Hz) frequency bands of interest.

Sleep-wake distribution and spectral power results for the *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> models investigated in this thesis are described below. Additionally, I report on gene expression validation results in *Grin2b*<sup>+/-</sup> rats modelling a *Grin2b* haploinsufficiency phenotype, a novel NDD rat line, which unlike *16p11.2*<sup>+/-</sup> and *Nlgn3*<sup>-/-</sup> lines, has not previously been described.

### 3.3.1. Sleep-wake abnormalities in *16p11.2*<sup>+/-</sup> rats

Sleep disturbances are observed in both human patients (Kamara et al., 2023) and rodent models (Angelakos et al., 2017; H.-C. Lu et al., 2019) with deleterious mutations in the *16p11.2* loci. To explore this further, I first considered the relationship between REM sleep and genotype. Surprisingly,

*16p11.2<sup>+/-</sup>* rats in this study displayed a significant increase in REM sleep minutes in comparison to wild-type littermates, when analysed on an hour-by hour basis over 24 hours, during individual hours 0-2, 4, 6-9 and 11 of the light phase, and hours 12 and 13 at the very beginning of the dark phase (LMM, effect of hour  $F = 22.85$ ,  $DF = 23$ ,  $P < 0.00001$ ; genotype  $F = 9.021$ ,  $DF = 1$ ,  $P = 0.0062$ ; hour x genotype  $F = 3.31$ ,  $DF = 23$ ,  $P < 0.00001$ ; Tukey *post hoc* test, hour 0  $P = 0.0009$ , hour 1  $P = 0.018$ , hour 2  $P = 0.022$ , hour 4  $P = 0.0008$ , hour 6  $P < 0.0001$ , hour 7  $P = 0.0062$ , hour 8  $P = 0.0082$ , hour 9  $P = 0.0021$ , hour 11  $P < 0.0001$ , hour 12  $P = 0.018$ , hour 13  $P = 0.024$ ; Figure 3.2A). Correspondingly, when REM was quantified by light and dark phases, the observed increase in this state was only present during the 12-hour light period and no differences were found between the two genotypes during the dark period (LMM, effect of phase  $F = 53.29$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 9.021$ ,  $DF = 1$ ,  $P = 0.0063$ ; phase x genotype  $F = 8.47$ ,  $DF = 1$ ,  $P = 0.0079$ ; Tukey *post hoc* test, light  $P = 0.0002$ , dark  $P = 0.52$ ; Figure 3.3A). This resulted in an overall net increase of REM sleep during the full 24-hours quantified in *16p11.2<sup>+/-</sup>* animals relative to *16p11.2<sup>+/+</sup>* rats (Two-sample unpaired t-test,  $DF = 23$ ,  $T = 3.0035$ ,  $P = 0.0063$ ; Figure 3.2D). For the full 24-hours, the increase in REM minutes was due to a higher number of REM sleep bouts in *16p11.2<sup>+/-</sup>* animals relative to *16p11.2<sup>+/+</sup>* littermates (Two-sample unpaired t-test,  $DF = 23$ ,  $T = 2.41$ ,  $P = 0.025$ ; Figure 3.2D), and this increase was again restricted to the 12-hour light phase, as no differences in REM bout number were observed between the two groups during the dark phase (LMM, effect of phase  $F = 61.054$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 5.79$ ,  $DF = 1$ ,  $P = 0.025$ ; phase x genotype  $F = 8.76$ ,  $DF = 1$ ,  $P = 0.00703$ ; Tukey *post hoc* test, light  $P = 0.0007$ , dark  $P = 0.77$ ; Figure 3.3A). Finally, no differences were observed between in *16p11.2<sup>+/-</sup>* and *16p11.2<sup>+/+</sup>* rats in their average REM bout durations during the full 24-hour timeframe (Two-sample unpaired t-test,  $DF = 23$ ,  $T = 1.1$ ,  $P = 0.28$ ; Figure 3.2D), and specifically during the light and dark periods (LMM, effect of phase  $F = 0.52$ ,  $DF = 1$ ,  $P = 0.48$ ; genotype  $F = 1.39$ ,  $DF = 1$ ,  $P = 0.25$ ; phase x genotype  $F = 3.007$ ,  $DF = 1$ ,  $P = 0.59$ ; Figure 3.3A).

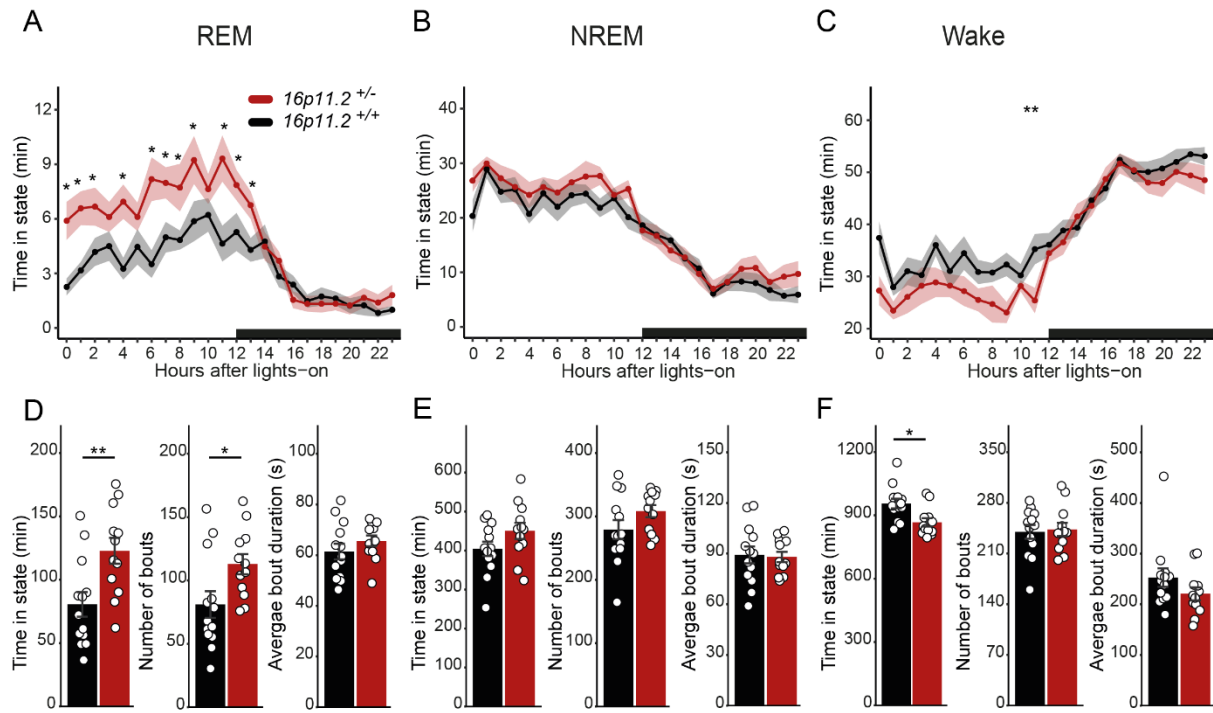


**Figure 3.2.  $16p11.2^{+/-}$  animals display sleep-wake abnormalities.** Quantification showing the time spent in REM (A), NREM (B) and wake (C) by hour, across the 24-hour light-dark cycle with black bar on x-axis indicating lights off in the animal facility.  $16p11.2^{+/-}$  rats had overall significantly more REM sleep compared to wild-type littermates, and this increase was specific to hours 0-2, 4, 6-9 and 11 of the lights phase, and hours 12 and 13 at the very beginning of the dark phase (REM hour  $P < 0.00001$ , genotype  $P = 0.0062$ , hour  $\times$  genotype  $P < 0.00001$ ; LMM with Tukey post hoc tests;  $P$  values for individual hours can be found in results section). \* = effect from genotype. No net or individual hour differences were observed between genotypes for NREM sleep (NREM hour  $P < 0.00001$ , genotype  $P = 0.109$ , hour  $\times$  genotype  $P = 0.82$ ; LMM), while an overall decrease was found in the wake state, although this reduction was not specific to any hour of the day (wake hour  $P < 0.00001$ , genotype  $P = 0.0081$ , hour  $\times$  genotype  $P = 0.27$ ; LMM). \*\* = effect from genotype. Points indicate group mean values (mean  $\pm$  SEM). Bar plots showing total time (left), number of bouts (middle) and average bout duration (right) for REM (D), NREM (E) and wake (F) during the full 24 hours.  $16p11.2^{+/-}$  animals spent more time in REM sleep due to increased amount of REM sleep bouts and had reduced minutes of wake (\* =  $P < 0.05$ , \*\* =  $P < 0.01$ , unpaired Two-sample  $t$ -tests and Wilcoxon rank sum tests specified in results text). Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

Following this, the effect of genotype on NREM sleep activity was examined. There were no differences in the minutes of NREM between  $16p11.2^{+/-}$  and  $16p11.2^{+/+}$  animals on an hour-by-hour basis (LMM, effect of hour  $F = 37.76$ ,  $DF = 23$ ,  $P < 0.00001$ ; genotype  $F = 2.77$ ,  $DF = 1$ ,  $P = 0.109$ ; hour  $\times$  genotype  $F = 0.72$ ,  $DF = 23$ ,  $P = 0.82$ ; Figure 3.2B), or over the full 24-hour cycle (Two-sample unpaired  $t$ -test,  $DF = 23$ ,  $T = 1.66$ ,  $P = 0.1097$ ; Figure 3.2E).  $16p11.2^{+/-}$  rats had a comparable amount of NREM bouts to  $16p11.2^{+/+}$  animals (Two-sample unpaired  $t$ -test,  $DF = 23$ ,  $T = 1.59$ ,  $P = 0.12$ ; Figure 3.2E), and the average NREM bout duration of  $16p11.2^{+/-}$  animals was also not different to that of wild-type littermates (Two-sample unpaired  $t$ -test,  $DF = 23$ ,  $T = -0.204$ ,  $P = 0.84$ ; Figure 3.2E). Similarly,

during the light and dark phases, *16p11.2*<sup>+/-</sup> animals did not differ from *16p11.2*<sup>+/+</sup> rats in the total amount of NREM sleep (LMM, effect of phase  $F = 152.23$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 2.78$ ,  $DF = 1$ ,  $P = 0.11$ ; phase x genotype  $F = 0.75$ ,  $DF = 1$ ,  $P = 0.39$ ; Figure 3.3B), the amount of NREM bouts (LMM, effect of phase  $F = 102.54$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 2.56$ ,  $DF = 1$ ,  $P = 0.13$ ; phase x genotype  $F = 1.15$ ,  $DF = 1$ ,  $P = 0.29$ ; Figure 3.3B) and the average NREM bout duration (LMM, effect of phase  $F = 58.93$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 0.0079$ ,  $DF = 1$ ,  $P = 0.93$ ; phase x genotype  $F = 0.59$ ,  $DF = 1$ ,  $P = 0.45$ ; Figure 3.3B).

Finally, the association between wake and genotype was considered. In relation to the increase in REM sleep observed in *16p11.2*<sup>+/-</sup> rats, when analysed hour by hour, heterozygous animals spent less time in the wake state. However, this reduction was not confined to specific hours of the day, rather it was observed more broadly across the 24-hour period (LMM, effect of hour  $F = 39.19$ ,  $DF = 23$ ,  $P < 0.00001$ ; genotype  $F = 8.39$ ,  $DF = 1$ ,  $P = 0.0081$ ; hour x genotype  $F = 1.16$ ,  $DF = 23$ ,  $P = 0.27$ ; Figure 3.2C). Consistent with this, analysis of total wake minutes over the 24-hour period confirmed a slight but statistically significant overall decrease in *16p11.2*<sup>+/-</sup> animals (Wilcoxon rank sum test,  $W = 33$ ,  $P = 0.016$ ; Figure 3.2F). This subtle reduction in the time *16p11.2*<sup>+/-</sup> rats spent awake was lost when separate light and dark 12-hour cycles were analysed. Instead, the decrease in wake time reflected a more general reduction across both phases combined (LMM, effect of phase  $F = 159.26$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 7.21$ ,  $DF = 1$ ,  $P = 0.01$ ; phase x genotype  $F = 2.75$ ,  $DF = 1$ ,  $P = 0.104$ ; Figure 3.3C). The subtlety in decreased minutes of wake is further emphasized by the lack of differences between *16p11.2*<sup>+/-</sup> rats and *16p11.2*<sup>+/+</sup> littermates in the number of wake bouts (Two-sample unpaired t-test,  $DF = 23$ ,  $T = 0.25$ ,  $P = 0.806$ ; Figure 3.2F) and the average wake bout durations (Wilcoxon rank sum test,  $W = 49$ ,  $P = 0.12$ ; Figure 3.2F). The same observation is true for when 12-hour light and dark periods were analysed, where there were no distinguishable changes in the number of wake bouts during the light or dark cycle (LMM, effect of phase  $F = 96.84$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 0.062$ ,  $DF = 1$ ,  $P = 0.81$ ; phase x genotype  $F = 0.036$ ,  $DF = 1$ ,  $P = 0.85$ ; Figure 3.3C), or the average wake bout durations during either of the two 12-hour phases (LMM, effect of phase  $F = 77.54$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 1.45$ ,  $DF = 1$ ,  $P = 0.24$ ; phase x genotype  $F = 0.082$ ,  $DF = 1$ ,  $P = 0.78$ ; Figure 3.3C). The increased minutes of REM are at the expense of time spent in wake and do not appear to directly influence NREM physiology.

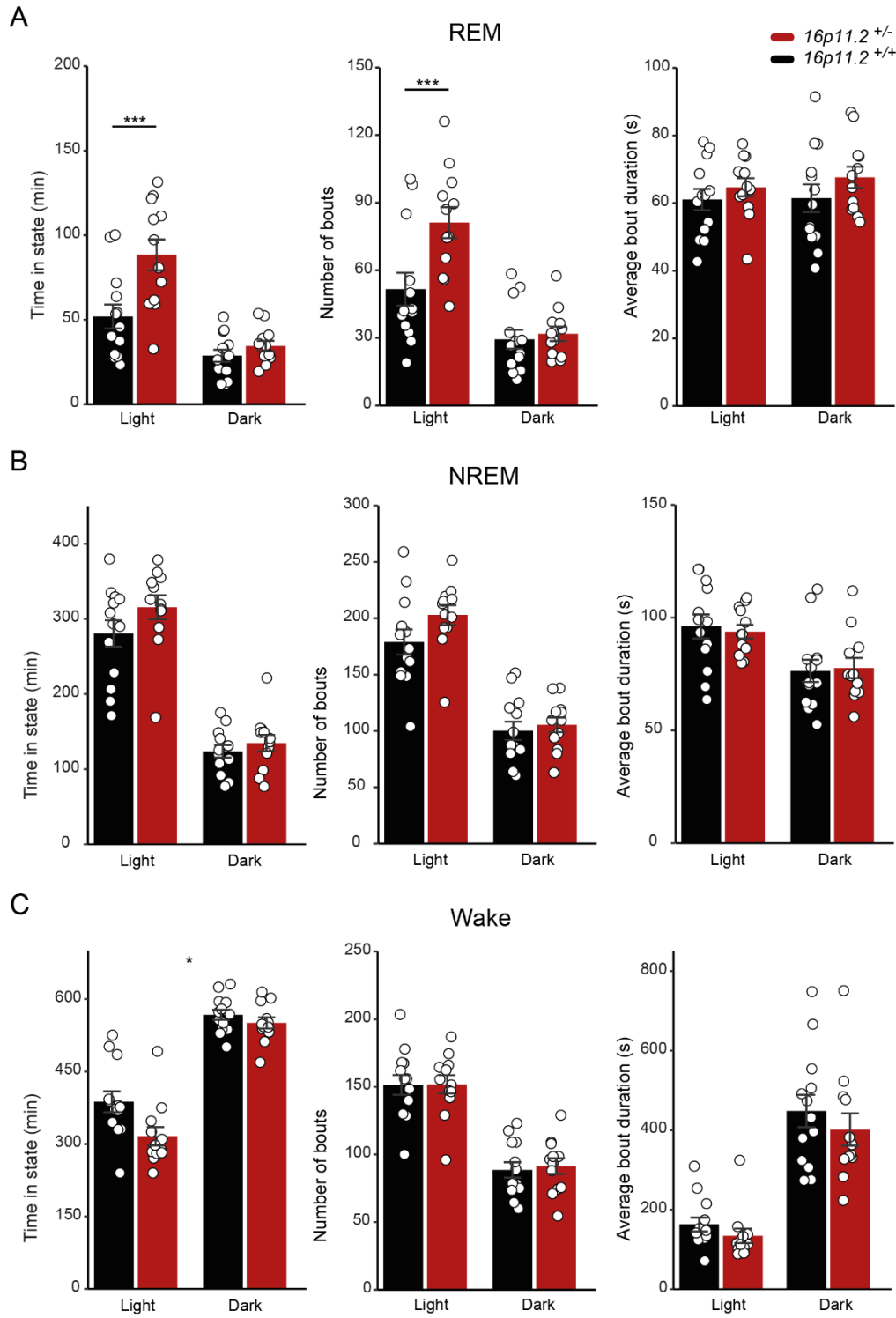


**Figure 3.3. Increased REM in 16p11.2<sup>+/-</sup> rats is specific to the light period.** Quantification showing total time (left), number of bouts (middle) and average bout duration (right) for REM (A), NREM (B) and wake (C) during the 12-hour light and dark periods. Bars indicate mean values (mean  $\pm$  SEM) and points correspond to values from individual rats. 16p11.2<sup>+/-</sup> rats had significantly more REM sleep compared to wild-type littermates during the light phase but not the dark (phase  $P < 0.00001$ , genotype  $P = 0.0063$ , phase  $\times$  genotype  $P = 0.0079$  (light  $P = 0.0002$ , dark  $P = 0.52$ ), LMM with Tukey post hoc tests). Number of REM bouts were similarly increased in 16p11.2<sup>+/-</sup> animals during the light cycle only (phase  $P < 0.00001$ , genotype  $P = 0.025$ , phase  $\times$  genotype  $P = 0.00703$  (light  $P = 0.0007$ , dark  $P =$

0.77), LMM with Tukey post hoc tests), while average REM bout duration did not differ between the two genotypes during both light and dark period (phase  $P = 0.48$ , genotype  $P = 0.25$ , phase x genotype  $P = 0.59$ , LMM) (\*\*\*) = post hoc test for genotype x phase interaction) (A). No differences were found between  $16p11.2^{+/-}$  and wild-type rats in NREM sleep when quantified for the light and dark periods (Time in NREM phase  $P < 0.00001$ , genotype  $P = 0.11$ , phase x genotype  $P = 0.39$ ; NREM bouts phase  $P < 0.00001$ , genotype  $P = 0.13$ , phase x genotype  $P = 0.29$ ; NREM bout duration phase  $P < 0.00001$ , genotype  $P = 0.93$ , phase x genotype  $P = 0.45$ ; LMMs) (B). Minutes of wake were subtly reduced in  $16p11.2^{+/-}$  animals, but the reduction was due to a net deficit and not specific to either light or dark period (phase  $P < 0.00001$ , genotype  $P = 0.01$ , phase x genotype  $P = 0.104$ , LMM) (\* = effect of phase). There were not differences in the number of wake bouts and average wake bout durations between the two genotypes during the light and dark cycles (wake bouts phase  $P < 0.00001$ , genotype  $P = 0.81$ , phase x genotype  $P = 0.85$ ; wake bout duration phase  $P < 0.00001$ , genotype  $P = 0.24$ , phase x genotype  $P = 0.78$ ; LMMs) (C). Detailed statistic can be found in results text.

#### *Sex-specific differences in REM and NREM sleep in $16p11.2^{+/-}$ rats*

$16p11.2$  deletion syndrome affects both males and females. However, several lines of evidence in rodent model of  $16p11.2$  deletion syndrome suggest the CNV contributes to male-specific impairments in gene expression patterns, dopaminergic signalling, social behaviour and sleep, as well as morpho-structural brain alteration. I therefore tested whether the increase in REM minutes and bouts, and reduction in time spent awake was sex, or sex and genotype specific (e.g. specific to male or female  $16p11.2^{+/-}$  animals, or both), as well as whether there were additional subtle sleep-wake related differences between  $16p11.2^{+/-}$  female,  $16p11.2^{+/-}$  male and wild-type littermates of both sexes. Here the same group of  $16p11.2^{+/-}$  and  $16p11.2^{+/+}$  animals were analysed, this time looking for an effect from sex.

Indeed, hour-by-hour analysis revealed the increase in REM minutes was observed in  $16p11.2^{+/-}$  male rats relative to wild-types of both sexes and  $16p11.2^{+/+}$  females. This was observed in hours 0-1, 4, 6-9, 11 and 12 predominantly restricted to the light phase. In female  $16p11.2^{+/-}$  animals REM minutes were no different from those in male and female wild-type littermates (for statistical results see Table 3.1, Figure 3.4A). Accordingly, minutes of REM in male  $16p11.2^{+/-}$  rats were also increased for the full 24-hour day relative to  $16p11.2^{+/+}$  controls and female  $16p11.2^{+/-}$  animals (Table 3.2, Figure 3.4B). The observed increase in number of REM bout was specific to sex but not to genotype of  $16p11.2$  rats, as male animals displayed more REM bouts overall. There were no differences in the average REM bout duration between male and female rats of both genotypes (Table 3.2, Figure 3.4B).

| Linear mixed model | Effect of genotype |    |               | Effect of sex |    |              | Effect of genotype x sex x hour |    |              |
|--------------------|--------------------|----|---------------|---------------|----|--------------|---------------------------------|----|--------------|
|                    | F                  | DF | P             | F             | DF | P            | F                               | DF | P            |
| REM hour-by-hour   | 6.5                | 1  | <b>0.019</b>  | 5.56          | 1  | <b>0.028</b> | 1.67                            | 1  | <b>0.028</b> |
| NREM hour-by-hour  | 6.28               | 1  | <b>0.021</b>  | 7.15          | 1  | <b>0.014</b> | 1.13                            | 1  | 0.31         |
| Wake hour-by-hour  | 10.37              | 1  | <b>0.0041</b> | 1.64          | 1  | 0.21         | 1.27                            | 1  | 0.19         |

| Tuckey post hoc tests for REM hour-by-hour interaction |                     |                     |                     |                     |                     |                     |
|--------------------------------------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                                                        | $F^{+/-} - M^{+/-}$ | $F^{+/+} - M^{+/+}$ | $F^{+/-} - F^{+/-}$ | $M^{+/+} - M^{+/-}$ | $F^{+/-} - M^{+/+}$ | $F^{+/+} - M^{+/-}$ |
| Hour 0                                                 | 0.11                | 0.89                | 0.94                | <b>0.0013</b>       | 0.67                | <b>0.0035</b>       |
| Hour 1                                                 | 0.23                | 0.99                | 0.74                | <b>0.022</b>        | 0.9                 | <b>0.0023</b>       |
| Hour 2                                                 | <b>0.05</b>         | 0.99                | 1.00                | 0.065               | 0.99                | <b>0.011</b>        |
| Hour 3                                                 | 0.53                | 0.65                | 0.99                | 0.099               | 0.89                | 0.56                |
| Hour 4                                                 | 0.38                | 0.7                 | 0.8                 | <b>0.0011</b>       | 0.3                 | <b>0.013</b>        |
| Hour 5                                                 | 0.7                 | 0.99                | 0.99                | 0.36                | 0.98                | 0.35                |
| Hour 6                                                 | <b>0.0011</b>       | 0.87                | 0.99                | <b>&lt; 0.0001</b>  | 0.84                | <b>&lt;0.0001</b>   |
| Hour 7                                                 | 0.067               | 0.99                | 0.99                | <b>0.027</b>        | 0.99                | <b>0.0037</b>       |
| Hour 8                                                 | <b>0.0052</b>       | 0.96                | 0.99                | <b>0.022</b>        | 0.93                | <b>0.0009</b>       |
| Hour 9                                                 | <b>0.001</b>        | 0.29                | 0.99                | <b>0.049</b>        | 0.58                | <b>&lt;0.0001</b>   |
| Hour 10                                                | 0.27                | 0.43                | 0.99                | 0.9                 | 0.71                | 0.059               |
| Hour 11                                                | <b>&lt;0.0001</b>   | 0.91                | 0.99                | <b>&lt; 0.0001</b>  | 0.99                | <b>&lt;0.0001</b>   |
| Hour 12                                                | 0.19                | 0.83                | 0.92                | 0.25                | 0.99                | <b>0.008</b>        |
| Hour 13                                                | 0.7                 | 0.99                | 0.88                | 0.11                | 0.79                | 0.12                |
| Hour 14                                                | 0.96                | 0.32                | 0.87                | 0.48                | 0.88                | 0.99                |
| Hour 15                                                | 0.98                | 0.99                | 0.99                | 0.86                | 0.99                | 0.86                |
| Hour 16                                                | 0.99                | 0.99                | 0.91                | 0.93                | 0.86                | 0.97                |
| Hour 17                                                | 0.93                | 0.97                | 0.98                | 0.99                | 0.9                 | 0.99                |
| Hour 18                                                | 0.85                | 0.99                | 0.81                | 0.99                | 0.3                 | 0.99                |
| Hour 19                                                | 0.98                | 0.84                | 0.88                | 0.97                | 1.0                 | 0.97                |
| Hour 20                                                | 0.7                 | 0.99                | 0.84                | 0.94                | 0.96                | 0.99                |
| Hour 21                                                | 0.7                 | 0.92                | 0.99                | 0.98                | 0.87                | 0.72                |
| Hour 22                                                | 0.97                | 0.87                | 0.99                | 0.73                | 0.97                | 0.99                |
| Hour 23                                                | 0.79                | 0.99                | 0.7                 | 0.99                | 0.68                | 0.99                |

**Table 3.1. Values for statistical analysis of time course sleep-wake distribution across brain states, genotypes and sex in 16p11.2<sup>+/-</sup> rats.** Left most column contains description of test statistic and measurements analysed. Columns 2-4 contain relevant F, DF and P values for the effects of genotype and sex, and interaction effect between them. Values in rows 3-5 correspond to results from LMMs used in hour-by-hour time course analysis of sleep-wake distribution. Values in rows 8-31 correspond to post hoc Tuckey multiple comparisons for the genotype x sex x hour interaction effect observed in REM sleep (row 3). F = female 16p11.2, M = male 16p11.2. P values are given for each relevant hour and comparison combination. Statistically significant post hoc results are shown in bold blue text. Statistical results relate to section 3.3.1 ‘Sex-specific differences in REM and NREM sleep in 16p11.2<sup>+/-</sup> rats’ and Figure 3.4.

Considering the effect of sex on NREM sleep across genotypes, no male-specific NREM alterations in *16p11.2*<sup>+/-</sup> rats were found. However, overall sex differences were observed in this sleep state. Female rats from this line spent more time in NREM sleep relative to male rats, although this was not due to any specific hour of the day (Table 3.1, Figure 3.4C). This observation was reflected in the net 24-hour quantification that similarly showed female animals had more minutes of NREM than male animals (Table 3.2, Figure 3.4D). There were no differences in the amount of NREM bouts between the two sexes, and the increase time in NREM in female rats was due to increased average NREM bout duration in these animals (Table 3.2, Figure 3.4D).

| Two-way ANOVA                                                   | Effect of genotype |    |               | Effect of sex |    |               | Effect of genotype x sex |    |              |
|-----------------------------------------------------------------|--------------------|----|---------------|---------------|----|---------------|--------------------------|----|--------------|
|                                                                 | F                  | DF | P             | F             | DF | P             | F                        | DF | P            |
| <b>Total REM duration</b>                                       | 6.49               | 1  | <b>0.016</b>  | 5.91          | 1  | <b>0.034</b>  | 5.0                      | 1  | <b>0.036</b> |
| <b>Number of REM bouts</b>                                      | 3.29               | 1  | 0.084         | 4.54          | 1  | <b>0.045</b>  | 1.46                     | 1  | 0.24         |
| <b>Average REM duration</b>                                     | 0.84               | 1  | 0.37          | 0.066         | 1  | 0.8           | 3.48                     | 1  | 0.076        |
| <b>Total NREM duration</b>                                      | 5.56               | 1  | <b>0.034</b>  | 7.28          | 1  | <b>0.028</b>  | 0.86                     | 1  | 0.37         |
| <b>Number of NREM bouts</b>                                     | 1.75               | 1  | 0.2           | 0.26          | 1  | 0.62          | 0.023                    | 1  | 0.88         |
| <b>Average NREM duration</b>                                    | 0.35               | 1  | 0.59          | 9.45          | 1  | <b>0.0058</b> | 0.8                      | 1  | 0.38         |
| <b>Total wake duration</b>                                      | 9.78               | 1  | <b>0.0043</b> | 1.67          | 1  | 0.17          | 3.019                    | 1  | 0.097        |
| <b>Number of wake bouts</b>                                     | 0.23               | 1  | 0.64          | 0.57          | 1  | 0.46          | 0.43                     | 1  | 0.52         |
| <b>Average wake duration</b>                                    | 2.33               | 1  | 0.14          | 0.703         | 1  | 0.41          | 0.34                     | 1  | 0.57         |
| <b>Tuckey post hoc tests for total REM duration interaction</b> |                    |    |               |               |    |               |                          |    |              |
| <b>F<sup>+/-</sup> – M<sup>+/-</sup></b>                        | <b>0.024</b>       |    |               |               |    |               |                          |    |              |
| <b>F<sup>+/+</sup> – M<sup>+/+</sup></b>                        | 0.99               |    |               |               |    |               |                          |    |              |
| <b>F<sup>+/+</sup> – F<sup>+/-</sup></b>                        | 0.99               |    |               |               |    |               |                          |    |              |
| <b>M<sup>+/+</sup> – M<sup>+/-</sup></b>                        | <b>0.01</b>        |    |               |               |    |               |                          |    |              |
| <b>F<sup>+/-</sup> – M<sup>+/+</sup></b>                        | 0.99               |    |               |               |    |               |                          |    |              |
| <b>F<sup>+/+</sup> – M<sup>+/-</sup></b>                        | <b>0.0026</b>      |    |               |               |    |               |                          |    |              |

**Table 3.2. Values for statistical analysis for 24-hour sleep-wake distribution across brain states, genotypes and sex in *16p11.2*<sup>+/-</sup> rats.** Left most column contains description of test statistic and measurements analysed. Columns 2-4 contain relevant F, DF and P values for the effects of genotype and sex, and interaction effect between them. Values in rows 3-11 correspond to results from Two-way ANOVA test statistics used in net 24-hour analysis of sleep-wake distribution. Values in rows 12-18 correspond to post hoc Tuckey multiple comparisons for the genotype x sex interaction effect observed for total REM sleep duration (row 3). F = female *16p11.2*, M = male *16p11.2*. P values are given for

each relevant comparison combination. Statistically significant post hoc results are shown in bold blue text. Statistical results relate to section 3.3.1 ‘Sex-specific differences in REM and NREM sleep in 16p11.2<sup>+/-</sup> rats’ and Figure 3.4.

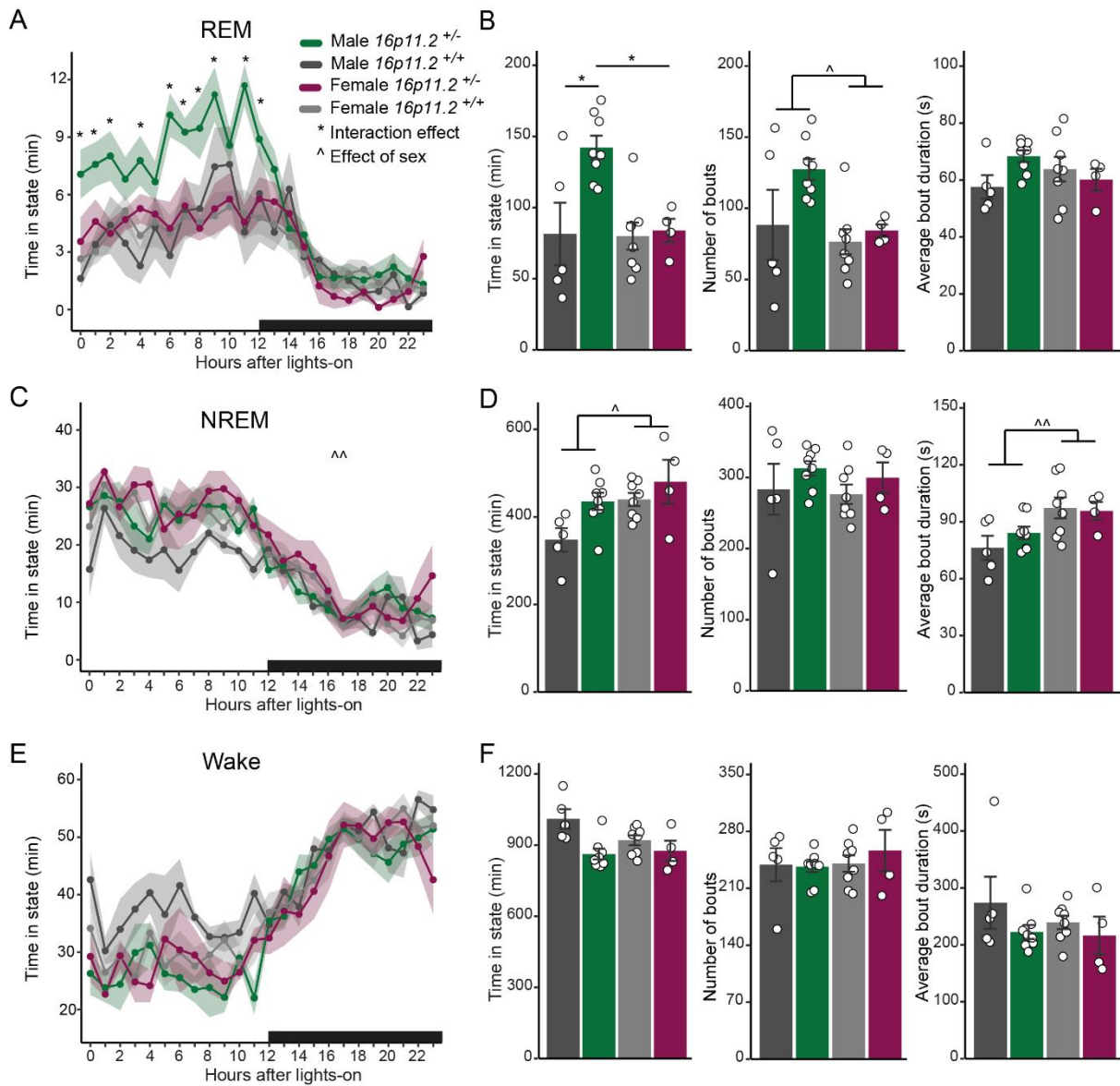


**Figure 3.4. REM sleep increases in 16p11.2<sup>+/-</sup> rats are male-specific.** Quantification showing the time spent in REM (A), NREM (C) and wake (E) by hour across the 24-hour light-dark cycle; points indicate group mean values (mean  $\pm$  SEM). Bar plots showing total time (left), number of bouts (middle) and average bout duration (right) for REM (B), NREM (D) and wake (F) during the full 24 hours; bars indicate mean values (mean  $\pm$  SEM) and points correspond to values from individual rats. Note: significance (\*) where effect from genotype is found is not indicated. Time in REM is increased in male 16p11.2<sup>+/-</sup> rats relative to female 16p11.2<sup>+/-</sup> rats and wild-type littermates, and this difference is restricted to specific hours 0-1, 4, 6-9 and 11 during the light period; \* = post hoc tests for genotype  $\times$  sex  $\times$  hour interaction showing differences between male 16p11.2<sup>+/-</sup> rats compared to female 16p11.2<sup>+/-</sup> rats and male/female wild-type animals (A). For the full 24 hours, male 16p11.2<sup>+/-</sup> animals spent

significantly more total minutes in REM relative to female  $16p11.2^{+/-}$  rats and littermate controls (\* = effect of sex x genotype interaction) (**B**, left panel). However, the increased number of REM bout was specific to male animals overall, with the genotype of rats having little effect on observed sleep increase (^ = effect of sex) (**B**, middle panel). No differences were found in the average REM bout duration between male and female rats of both genotypes (**B**, right panel). Overall minutes of NREM were increased in female animals relative to male rats, and this difference was not due to any particular hour (^ = effect of sex) (**C**). 24-hour quantification similarly showed female animals had more minutes of NREM than male animals (^ = effect of sex) (**D**, left panel). Female rats had longer NREM sleep bouts, resulting in more total NREM sleep time, but both sexes had the same number of NREM episodes (^ and ^^ = effect of sex) (**D**, middle and right pane). Time in wake is similar between  $16p11.2^{+/-}$  and wild-type animals of both sexes throughout each hour of the day (**E**). Over the course of 24 hours, sex did not influence total wake, wake bout counts, or average wake bout duration, and these measures were similar between male and female  $16p11.2^{+/-}$  and  $16p11.2^{+/+}$  animals (**F**). For detailed statistics see Tables 3.1 and 3.2).

Lastly, I looked at whether wake was influenced by sex between the two genotypes. No sex or male  $16p11.2^{+/-}$  specific differences were found in the wake state on an hour-by-hour basis, however the analysis revealed an overall genotype difference as before (Table 3.1, Figure 3.4E). This was also true for the full 24-hour period (Table 3.2, Figure 3.4F). There were no differences between males and females of either genotype in the number of wake bouts or the average wake bout duration (Table 3.2, Figure 3.4F). These data indicate male-specific sleep abnormalities in  $16p11.2^{+/-}$  rats, consisting of considerable increases in REM sleep total minutes and number of bouts. These observations could be suggestive of impairment in NREM or REM to wake transitional mechanisms, or alternatively may reflect a greater homeostatic sleep drive for REM sleep in these animals. The apparent loss of wake differences when stratifying by both sex and genotype likely stems from the heterogeneous distribution of time in wake across the four groups. Although statistically non-significant, time in wake appears greater in male wild-type animals, in comparison to wild-type females and  $16p11.2^{+/-}$  male and female rats, which may mask the interaction effect. Furthermore, the significantly greater overall proportion of wake in comparison to REM might also reflect the observed results, as based on the relevant amounts of the two states, and their distribution between the four groups, the differences in REM sleep are likely to be much more pronounced than those in wake.

#### *Reduced spectral power during wake and sleep in $16p11.2^{+/-}$ rats*

Spectral power analyses of REM, NREM and wake revealed that in comparison to  $16p11.2^{+/+}$  controls,  $16p11.2^{+/-}$  animals had notable decreases in power, in all states across all five frequency bands measured. Statistically, the largest cross-frequency power decrease observed for  $16p11.2^{+/-}$  rats relative to wild-type controls was found in NREM sleep (Two-way ANOVA, effect of genotype  $F = 22.25$ ,  $DF = 1$ ,  $P < 0.00001$ ; frequency  $F = 30.44$ ,  $DF = 4$ ,  $P < 0.00001$ ; genotype x frequency  $F = 1.55$ ,  $DF = 4$ ,  $P = 0.18$ ; Figure 3.5B ), followed by wake (Two-way ANOVA, effect of genotype  $F = 14.45$ ,  $DF = 1$ ,

$P = 0.00023$ ; frequency  $F = 87.38$ ,  $DF = 4$ ,  $P < 0.00001$ ; genotype x frequency  $F = 0.41$ ,  $DF = 4$ ,  $P = 0.8$ ; Figure 3.5C ), and finally REM sleep (Two-way ANOVA, effect of genotype  $F = 8.69$ ,  $DF = 1$ ,  $P = 0.0039$ ; frequency  $F = 8.42$ ,  $DF = 4$ ,  $P < 0.00001$ ; genotype x frequency  $F = 0.24$ ,  $DF = 4$ ,  $P = 0.92$ ; Figure 3.5A ). In summary, the decrease spectral power in *16p11.2*<sup>+/-</sup> animals for each sleep-wake state was not statistically reduced in specific frequency bands, but did span across multiple frequency ranges of brain activity. This could indicate a broad impact of the *16p11.2* deletion on brain oscillatory activity during sleep and wake periods and potentially reflects alterations in underlying neural circuits mediating all three vigilance states.

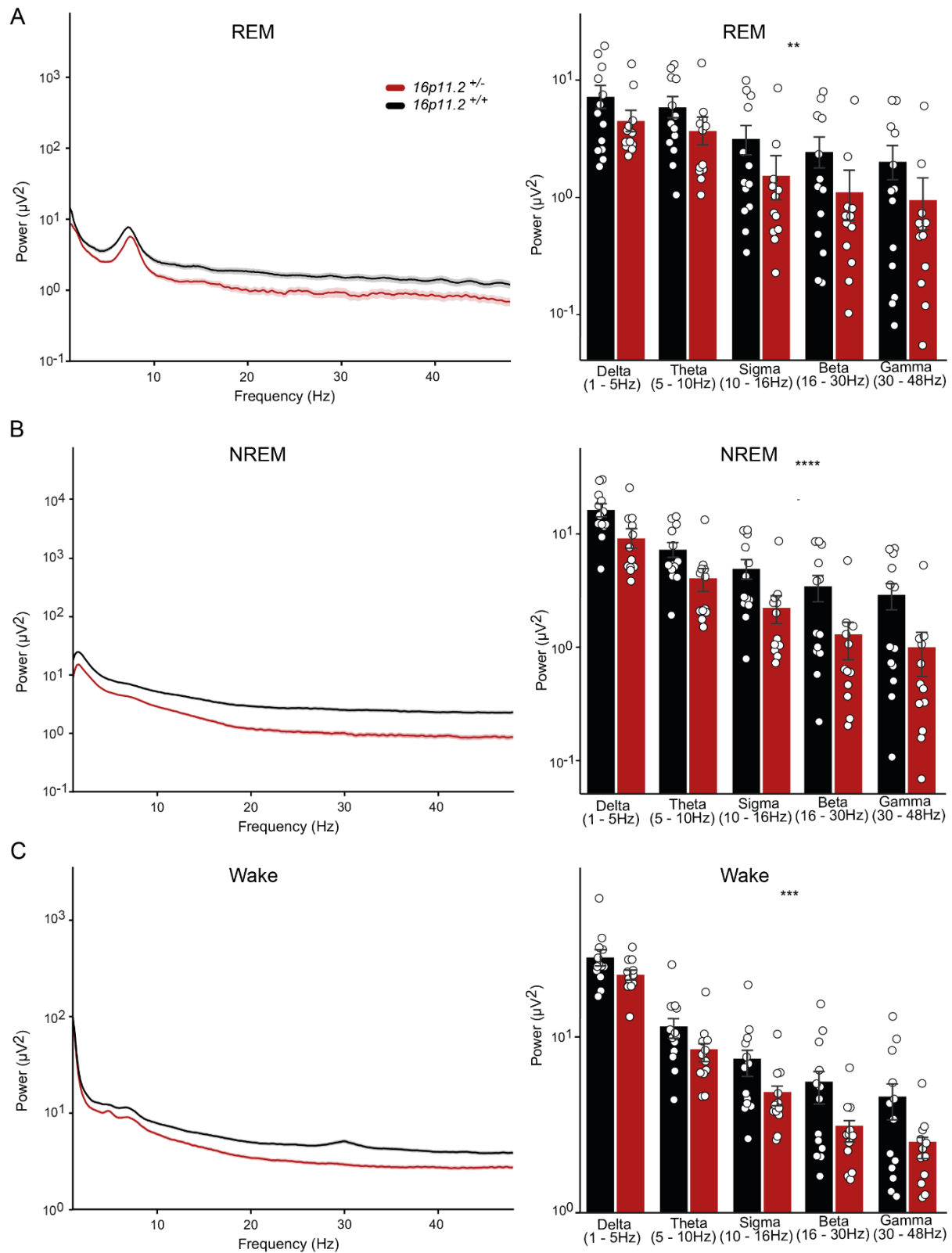


**Figure 3.5. Spectral power is overall decreased in 16p11.2<sup>+/-</sup> rats.** Average EEG power spectra across wild-type and 16p11.2<sup>+/-</sup> rats (left; traces represent group averages (mean  $\pm$  SEM)) and quantification by individual frequency bands (right) during REM (A), NREM (B) and wake (C) epochs. Quantification of average power in specific frequency bands during REM, NREM and wake epochs, showed significantly reduced power in 16p11.2<sup>+/-</sup> rats relative to wild-type animals although no significant differences were found in individual frequency bands (REM genotype  $P = 0.0039$ , frequency  $P <$

0.00001, genotype  $\times$  frequency  $P = 0.92$ ; NREM genotype  $P < 0.00001$ , frequency  $P < 0.00001$ , genotype  $\times$  frequency  $P = 0.18$ ; wake genotype  $P = 0.00023$ , frequency  $P < 0.00001$ , genotype  $\times$  frequency  $P = 0.8$ ; Two-way ANOVA tests). \*\*, \*\*\* and \*\*\*\* = effect from genotype. Detailed statistics in main text. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

### 3.3.2. Sleep-wake abnormalities in *Nlgn3*<sup>-/-</sup> rats

Deleterious *NLGN3* variants contribute to sleep impairment in male individuals with ASD and ID (Cohen et al., 2014; Quartier et al., 2019). Sleep deficits were recently recapitulated in the knockout *Nlgn3* rat line by Thomas and colleagues (Thomas et al., 2017), who investigated sleep in the same model as used in this thesis. In contrast to the work by Thomas et al., I did not find specific deficits in either REM or NREM sleep, however, I did observe altered sleep patterns.

Beginning with the effect of *Nlgn3* knockout on REM physiology, hour-by-hour analysis revealed no differences in the amount of time spent in REM sleep between *Nlgn3*<sup>-/-</sup> and *Nlgn3*<sup>+/+</sup> rats (LMM, effect of hour  $F = 14.73$ ,  $DF = 23$ ,  $P < 0.00001$ ; genotype  $F = 0.6$ ,  $DF = 1$ ,  $P = 0.45$ ; hour  $\times$  genotype  $F = 1.0203$ ,  $DF = 23$ ,  $P = 0.44$ ; Figure 3.6A). Minutes of REM across the full 24-hours were not different between the two genotypes (Two-sample unpaired t-test,  $DF = 26$ ,  $T = 0.77$ ,  $P = 0.45$ ; Figure 3.6D), nor were there any differences in the number of REM bouts (Two-sample unpaired t-test,  $DF = 26$ ,  $T = 0.84$ ,  $P = 0.408$ ; Figure 3.6D) and average REM bout duration (Two-sample unpaired t-test,  $DF = 26$ ,  $T = -0.69$ ,  $P = 0.5$ ; Figure 3.6D) between *Nlgn3*<sup>-/-</sup> and *Nlgn3*<sup>+/+</sup> animals. Similarly, during the light and dark phases the total amount of REM sleep (LMM, effect of phase  $F = 32.91$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 0.59$ ,  $DF = 1$ ,  $P = 0.45$ ; phase  $\times$  genotype  $F = 0.36$ ,  $DF = 1$ ,  $P = 0.56$ ; Figure 3.7A), the amount of REM bouts (LMM, effect of phase  $F = 51.97$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 0.71$ ,  $DF = 1$ ,  $P = 0.41$ ; phase  $\times$  genotype  $F = 0.34$ ,  $DF = 1$ ,  $P = 0.58$ ; Figure 3.7A) and the average REM bout duration (LMM, effect of phase  $F = 32.29$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 0.97$ ,  $DF = 1$ ,  $P = 0.33$ ; phase  $\times$  genotype  $F = 0.62$ ,  $DF = 1$ ,  $P = 0.26$ ; Figure 3.7A) did not differ in *Nlgn3*<sup>-/-</sup> animals relative to wild-type littermates.

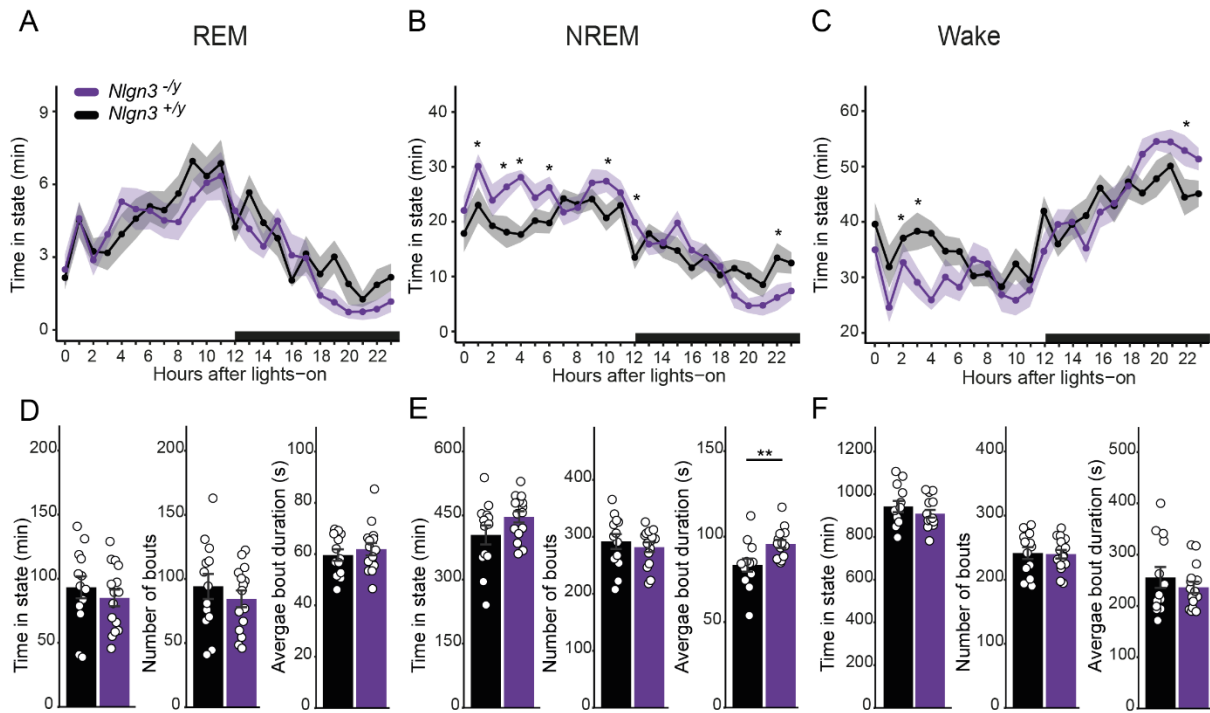


**Figure 3.6. *Nlgn3*<sup>-/-</sup> animals have altered NREM sleep and wake distribution.** Quantification showing the time spent in REM (A), NREM (B) and wake (C) by hour, across the 24-hour light-dark cycle with black bar on x-axis indicating lights off in the animal facility. On an hourly basis, REM sleep in *Nlgn3*<sup>-/-</sup> rats was not different to that in wild-type littermates (REM hour  $P < 0.00001$ , genotype  $P = 0.45$ , hour  $\times$  genotype  $P = 0.44$ ; LMM). Altered time course dynamics during NREM sleep in *Nlgn3*<sup>-/-</sup> rats, which have more minutes of NREM sleep during hours 1, 3, 4, 6, 10 and 12, and less minutes of NREM at hour 22 compared to *Nlgn3*<sup>+/-</sup> animals (NREM hour  $P < 0.00001$ , genotype  $P = 0.103$ , hour  $\times$  genotype  $P < 0.00001$ ; LMM with Tukey post hoc tests;  $P$  values for individual hours can be found in results section). In hour-by-hour analysis *Nlgn3*<sup>-/-</sup> rats spent less time in wake in hours 2-3, and more time in wake during hour 22 (NREM hour  $P < 0.00001$ , genotype  $P = 0.28$ , hour  $\times$  genotype  $P = 0.00063$ ; LMM with Tukey post hoc tests;  $P$  values for individual hours can be found in results section). \* = effect from genotype. Points indicate group mean values (mean  $\pm$  SEM). Bar plots showing total time (left), number of bouts (middle) and average bout duration (right) for REM (D), NREM (E) and wake (F) during the full 24 hours. *Nlgn3*<sup>+/-</sup> animals had longer NREM sleep bout duration compared to wild-types (\* =  $P < 0.05$ , \*\* =  $P < 0.01$ , unpaired Two-sample  $t$ -tests and Wilcoxon rank sum tests specified in results text). Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

Analysis of the relationship between genotype and NREM sleep revealed that *Nlgn3*<sup>-/-</sup> rats displayed altered time course and physiology dynamics in the NREM state. Knockout animals were found to have significantly more minutes of NREM sleep during the first half of the 24 light-dark period, during individual hours 1, 3, 4, 6, 10 and 1. NREM sleep was generally lower during the final quarter of the 24-hour cycle, with a significant reduction observed at hour 22, when compared to *Nlgn3*<sup>+/-</sup> animals (LMM, effect of hour  $F = 17.34$ ,  $DF = 23$ ,  $P < 0.00001$ ; genotype  $F = 2.85$ ,  $DF = 1$ ,  $P = 0.103$ ; hour  $\times$  genotype  $F = 2.61$ ,  $DF = 23$ ,  $P < 0.00001$ ; Tukey post hoc test, hour 1  $P = 0.024$ , hour 3  $P =$

0.0086, hour 4  $P = 0.0009$ , hour 6  $P = 0.039$ , hour 10  $P = 0.033$ , hour 12  $P = 0.042$ , hour 22  $P = 0.021$ ; Figure 3.6B). Indeed, light and dark phase analysis reflected the above observations, as significant increases in NREM sleep minutes were found in the 12-hour light period, but not during the dark cycle of  $Nlgn3^{-/-}$  animals compared to  $Nlgn3^{+/+}$  rats (LMM, effect of phase  $F = 144.81$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 2.87$ ,  $DF = 1$ ,  $P = 0.103$ ; phase x genotype  $F = 9.26$ ,  $DF = 1$ ,  $P = 0.0053$ ; Tukey *post hoc* test, light  $P = 0.0019$ , dark  $P = 0.48$ ; Figure 3.7B). The increased amount of NREM sleep in the light phase and the less pronounced reduction of NREM sleep during the dark phase in  $Nlgn3^{-/-}$  rats, was due to changes in both the number of bouts and average bout duration of this sleep state, which may reflect compensatory sleep homeostatic processes. During the light phase NREM bout amount was not different between  $Nlgn3^{-/-}$  and  $Nlgn3^{+/+}$  animals, however, during the dark period  $Nlgn3^{-/-}$  rats had statistically fewer NREM sleep bouts than wild-type littermate controls (LMM, effect of phase  $F = 112.57$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 0.44$ ,  $DF = 1$ ,  $P = 0.52$ ; phase x genotype  $F = 4.501$ ,  $DF = 1$ ,  $P = 0.044$ ; Tukey *post hoc* test, light  $P = 0.33$ , dark  $P = 0.05$ ; Figure 3.7B). In contrast,  $Nlgn3^{-/-}$  rats displayed significantly longer average NREM bout durations, during both light and dark 12-hour cycles, relative to  $Nlgn3^{+/+}$  rats (LMM, effect of phase  $F = 16.43$ ,  $DF = 1$ ,  $P = 0.00041$ ; genotype  $F = 9.012$ ,  $DF = 1$ ,  $P = 0.0059$ ; phase x genotype  $F = 0.24$ ,  $DF = 1$ ,  $P = 0.63$ ; Figure 3.7B). Ultimately this resulted in a trend towards more time spent in NREM sleep in  $Nlgn3^{-/-}$  rats compared to wild-type littermates, which was not statistically significant, during the full 24-hours (Two-sample unpaired t-test,  $DF = 26$ ,  $T = -1.69$ ,  $P = 0.103$ ; Figure 3.6E). Average NREM bout durations, however, remained slightly increased in  $Nlgn3^{-/-}$  relative to  $Nlgn3^{+/+}$  animals (Two-sample unpaired t-test,  $DF = 26$ ,  $T = -0.85$ ,  $P = 0.0085$ ; Figure 3.6E). Finally, during the full 24-hour cycle, the number of NREM bouts was not any different between  $Nlgn3^{-/-}$  and  $Nlgn3^{+/+}$  rats (Two-sample unpaired t-test,  $DF = 26$ ,  $T = 0.66$ ,  $P = 0.52$ ; Figure 3.6E).

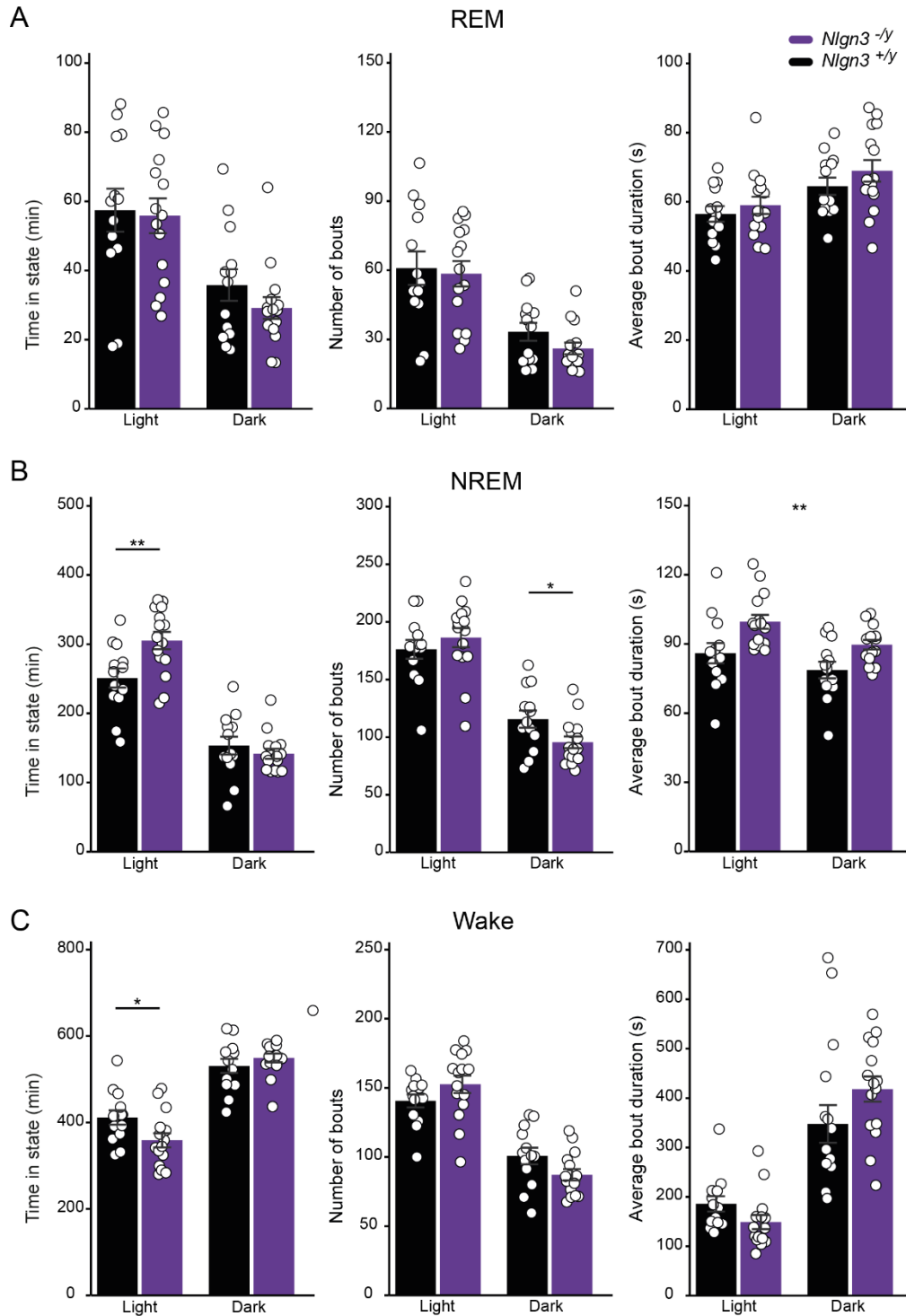


**Figure 3.7. Altered NREM and wake physiology in *Nlgn3*<sup>-/-</sup> rats during the light period.** Quantification showing total time (left), number of bouts (middle) and average bout duration (right) for REM (A), NREM (B) and wake (C) during the 12-hour light and dark periods. Bars indicate mean values (mean  $\pm$  SEM) and points correspond to values from individual rats. Regardless of phase, the total minutes of REM, REM bouts and average REM bout durations were unaltered in *Nlgn3*<sup>-/-</sup> rats relative to littermate controls (Time in REM phase  $P < 0.00001$ , genotype  $P = 0.45$ , phase  $\times$  genotype  $P = 0.56$ ; REM bouts phase  $P < 0.00001$ , genotype  $P = 0.41$ , phase  $\times$  genotype  $P = 0.58$ ; REM bout duration phase  $P < 0.00001$ , genotype  $P = 0.33$ , phase  $\times$  genotype  $P = 0.26$ ; LMMs) (A). In comparison to wild-type rats, *Nlgn3*<sup>-/-</sup> animals spent more time in NREM sleep during the light phase, had fewer

NREM bouts in the dark period, and have overall increased NREM bout durations, irrespective of whether NREM occurred in the light and dark phase (Time in NREM phase  $P < 0.00001$ , genotype  $P = 0.103$ , phase x genotype  $P = 0.0053$  (light  $P = 0.0019$ , dark  $P = 0.48$ ); NREM bouts phase  $P < 0.00001$ , genotype  $P = 0.52$ , phase x genotype  $P = 0.044$  (light  $P = 0.33$ , dark  $P = 0.05$ ); NREM bout duration phase  $P = 0.00041$ , genotype  $P = 0.0059$ , phase x genotype  $P = 0.63$ ; LMMs with Tukey post hoc tests)(\*\* and \* = effect from genotype x phase interaction; \*\* = effect from genotype) (**B**). Total minutes of wake were fewer in *Nlgn3<sup>-/-</sup>* animals during the light period when compared to *Nlgn3<sup>+/-</sup>* rats (phase  $P < 0.00001$ , genotype  $P = 0.28$ , phase x genotype  $P = 0.019$  (light  $P = 0.016$ , dark  $P = 0.38$ , LMM with Tukey post hoc tests). The number of wake bouts and average wake bout durations in *Nlgn3<sup>-/-</sup>* animals were not different to those in wild-type littermates (wake bouts phase  $P < 0.00001$ , genotype  $P = 0.89$ , phase x genotype  $P = 0.018$  (light  $P = 0.12$ , dark  $P = 0.079$ ); wake bout duration phase  $P < 0.00001$ , genotype  $P = 0.18$ , phase x genotype  $P = 0.61$ ; LMMs with Tukey post hoc tests) (\* = effect from genotype x phase interaction) (**C**). Detailed statistic can be found in results text.

In addition to the alterations observed in NREM sleep, the time course of wake in *Nlgn3<sup>-/-</sup>* rats also exhibited genotype-dependent deviations, which were proportionally inverse to those found for the NREM state. Namely, hour-by-hour analysis showed that *Nlgn3<sup>-/-</sup>* animals had less minutes of wake during individual hours 2 and 3 at the start of the 24-hour cycle, and more minutes of wake during hour 22 at the end of the 24-hour day (LMM, effect of hour  $F = 17.83$ ,  $DF = 23$ ,  $P < 0.00001$ ; genotype  $F = 1.19$ ,  $DF = 1$ ,  $P = 0.28$ ; hour x genotype  $F = 2.29$ ,  $DF = 23$ ,  $P = 0.00063$ ; Tukey post hoc test, hour 2  $P = 0.018$ , hour 3  $P = 0.0022$ , hour 22  $P = 0.032$ ; Figure 3.6C). Accordingly, when all hours of the day were combined, the time spent in wake was not different between *Nlgn3<sup>-/-</sup>* and *Nlgn3<sup>+/-</sup>* animals (Two-sample unpaired t-test,  $DF = 26$ ,  $T = 1.093$ ,  $P = 0.28$ ; Figure 3.6F), although quantification by light and dark phases showed a subtle yet statistically significant decrease in minutes of wake in *Nlgn3<sup>-/-</sup>* rats during the light period, while no differences were found in time spent awake between the two groups in the dark phase (LMM, effect of phase  $F = 116.62$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 1.19$ ,  $DF = 1$ ,  $P = 0.28$ ; phase x genotype  $F = 6.62$ ,  $DF = 1$ ,  $P = 0.019$ ; Tukey post hoc test, light  $P = 0.016$ , dark  $P = 0.38$ ; Figure 3.7C). There were no differences in the number of wake bouts during the full 24-hour day (Two-sample unpaired t-test,  $DF = 26$ ,  $T = 0.14$ ,  $P = 0.89$ ; Figure 3.6F), and the 12-hour light or dark phases between *Nlgn3<sup>-/-</sup>* and *Nlgn3<sup>+/-</sup>* littermates (LMM, effect of phase  $F = 104.47$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 0.0201$ ,  $DF = 1$ ,  $P = 0.89$ ; phase x genotype  $F = 6.16$ ,  $DF = 23$ ,  $P = 0.018$ ; Tukey post hoc test, light  $P = 0.12$ , dark  $P = 0.079$ ; Figure 3.7C). Similarly, *Nlgn3<sup>-/-</sup>* average wake bout durations were not different from those observed in littermate controls during the 24-hour period (Two-sample unpaired t-test,  $DF = 26$ ,  $T = 0.87$ ,  $P = 0.39$ ; Figure 3.6F), as well as the 12-hour light and dark phases (LMM, effect of phase  $F = 42.47$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 0.18$ ,  $DF = 1$ ,  $P = 0.68$ ; phase x genotype  $F = 0.27$ ,  $DF = 1$ ,  $P = 0.61$ ; Figure 3.7C). These data indicate that male rats lacking *Nlgn3* have subtle time dependent abnormalities in sleep-wake patterns and an enhanced ability to sustain NREM sleep during the light phase of the 24-hour cycle, when rodents naturally sleep more. This could reflect irregularities in sleep and wake promoting circuits, which are pathways critical for

circadian regulation of sleep, as well as alteration in homeostatic sleep pressure accumulation or dissipation mechanisms.

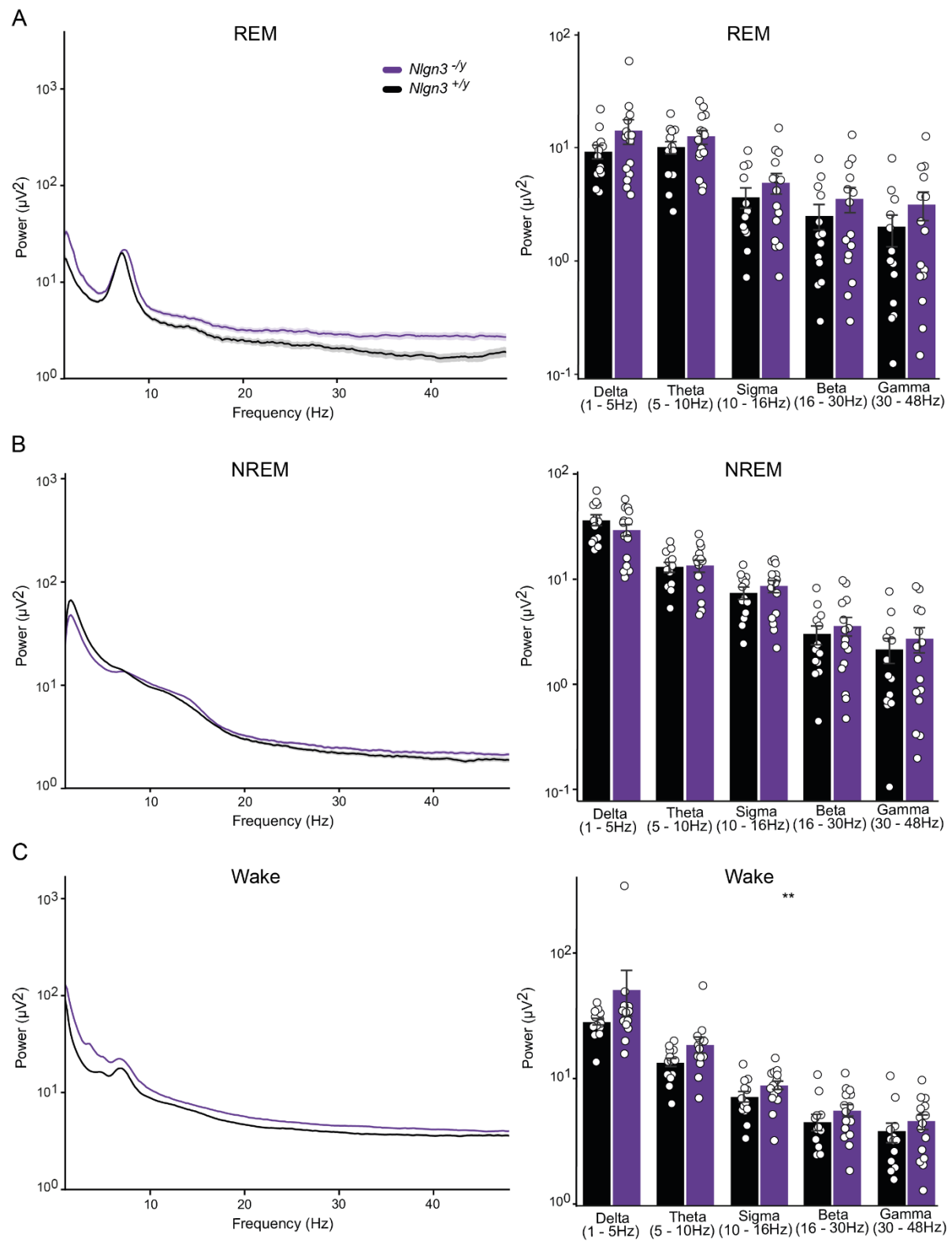


**Figure 3.8. Spectral power during wake is increased in *Nlgn3*<sup>-/-</sup> rats.** Average EEG power spectra across wild-type and *Nlgn3*<sup>-/-</sup> rats (left; traces represent group averages (mean  $\pm$  SEM)) and quantification by individual frequency bands (right) during REM (A), NREM (B) and wake (C) epochs. Quantification of average power in specific frequency bands during REM, NREM epochs, showed no spectral power differences in *Nlgn3*<sup>-/-</sup> animals relative to wild-type littermates (REM genotype  $P = 0.078$ , frequency  $P < 0.00001$ , genotype  $\times$  frequency  $P = 0.99$ ; NREM genotype  $P = 0.49$ , frequency  $P < 0.00001$ , genotype  $\times$  frequency  $P = 0.25$ ; Two-way ANOVA tests). *Nlgn3*<sup>-/-</sup> rats showed significantly increased power in comparison to *Nlgn3*<sup>+/+</sup> animals although no significant differences were found in individual frequency bands (genotype  $P = 0.0043$ , frequency  $P < 0.00001$ , genotype  $\times$  frequency  $P = 0.78$ ; Two-way ANOVA). \*\* = effect from genotype. Detailed statistics in main text. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

#### *Increased spectral power during wake in *Nlgn3*<sup>-/-</sup> rats*

In terms of spectral composition properties, no differences in power were observed in any of the five frequency bands analysed between *Nlgn3*<sup>-/-</sup> and *Nlgn3*<sup>+/+</sup> animals, during REM sleep (Two-way ANOVA, effect of genotype  $F = 3.15$ ,  $DF = 1$ ,  $P = 0.078$ ; frequency  $F = 29.8$ ,  $DF = 4$ ,  $P < 0.00001$ ; genotype  $\times$  frequency  $F = 0.047$ ,  $DF = 4$ ,  $P = 0.99$ ; Figure 3.8A) and NREM sleep (Two-way ANOVA, effect of genotype  $F = 0.46$ ,  $DF = 1$ ,  $P = 0.49$ ; frequency  $F = 72.64$ ,  $DF = 4$ ,  $P < 0.00001$ ; genotype  $\times$  frequency  $F = 1.38$ ,  $DF = 4$ ,  $P = 0.25$ ; Figure 3.8B). However, during wake epochs *Nlgn3*<sup>-/-</sup> rats had significantly increased cross-frequency power when compared to *Nlgn3*<sup>+/+</sup> littermates (Two-way ANOVA, effect of genotype  $F = 8.47$ ,  $DF = 1$ ,  $P = 0.0043$ ; frequency  $F = 98.71$ ,  $DF = 4$ ,  $P < 0.00001$ ; genotype  $\times$  frequency  $F = 0.032$ ,  $DF = 4$ ,  $P = 0.78$ ; Figure 3.8C). This finding complements the sleep-wake distribution patterns observed in this rat line and, suggests that NLGN3 may have important role in modulating brain oscillations specifically during the wake state.

#### 3.3.3. Gene expression in a novel rat model of *GRIN2B* neurodevelopmental disorder

This thesis utilized a novel model of *GRIN2B* related NDD generated by the Medical College of Wisconsin Gene Editing Rat Resource Centre, in which CRISPR/Cas9 was used to introduce a mutation in the *Grin2b* gene of Long-Evans rats. This model has not been previously described or characterised. Therefore, I first tested whether expression levels of GluN2B, encoded by *Grin2b*, are reduced in *Grin2b*<sup>+/-</sup> animals. As decreased GluN2B expression may be compensated by mechanisms that contribute to the normal development, properties and function of neurons (Gambrill and Barria et al., 2011; Gray et al., 2011; Sepulveda et al., 2011), I evaluated for changes in the expression of GluN2A, GluR1 and PSD95 postsynaptic proteins. These proteins were selected as they broadly inform on potential compensatory modifications at synapses, including NMDAR composition (Wyllie et al., 2013), NMDAR/AMPA ratio, trafficking and localisation, as well as the volume, strength and density

of dendritic spines (Cane et al., 2014; Huganir and Nicoll, 2013). Such adaptations would be expected to influence synaptic function and network signalling, and while compensatory, could also support pathological mechanisms in *Grin2b*<sup>+/-</sup> rats.

Protein expression was confirmed by immunoblotting somatosensory cortex (Figure 3.9A and Figure 3.11A) and hippocampus (Figure 3.10A- and Figure 3.11C) homogenates. This was done in collaboration with Dr Sarfaraz Nawaz and Prof Peter Kind (Hristova et al., 2024). Immunoblotting of somatosensory cortex and hippocampal synaptosome fractions showed that protein expression was located in synapses (Figure 3.9B and Figure 3.10B, Figure 3.11B and D). GluN2B protein levels in homogenates and synaptosome fractions were reduced by approximately 50% in adult *Grin2b*<sup>+/-</sup> rats compared to *Grin2b*<sup>+/+</sup> wild-types in somatosensory cortex and hippocampus (somatosensory cortex homogenate, Two-sample unpaired t-test,  $DF = 10$ ,  $T = 5.107$ ,  $P = 0.00046$ ; synaptosome, Wilcoxon rank sum test,  $W = 36$ ,  $P = 0.0051$ ; hippocampal homogenate, Two-sample unpaired t-test,  $DF = 10$ ,  $T = 6.86$ ,  $P = 0.000044$ ; synaptosome, Two-sample unpaired t-test,  $DF = 10$ ,  $T = 3.061$ ,  $P = 0.012$ ; Figure 3.9C and Figure 3.10C). The expression of GluN2A, GluR1 and PSD95 synaptic proteins in *Grin2b*<sup>+/-</sup> rats was unaffected in the somatosensory cortex (GluN2A homogenate, Two-sample unpaired t-test,  $DF = 10$ ,  $T = 0.3$ ,  $P = 0.77$ ; synaptosome, Two-sample unpaired t-test,  $DF = 10$ ,  $T = 0.46$ ,  $P = 0.66$ ; GluR1 homogenate, Two-sample unpaired t-test,  $DF = 10$ ,  $T = 0.85$ ,  $P = 0.42$ ; synaptosome, Welch's two-sample unpaired t-test,  $DF = 5.46$ ,  $T = 1.27$ ,  $P = 0.26$ ; PSD95 homogenate, Two-sample unpaired t-test,  $DF = 10$ ,  $T = 2.11$ ,  $P = 0.061$ ; synaptosome, Two-sample unpaired t-test,  $DF = 10$ ,  $T = 0.16$ ,  $P = 0.88$ ; Figure 3.9D-F) and the hippocampus (GluN2A homogenate, Two-sample unpaired t-test,  $DF = 10$ ,  $T = -0.38$ ,  $P = 0.71$ ; synaptosome, Two-sample unpaired t-test,  $DF = 10$ ,  $T = -0.025$ ,  $P = 0.98$ ; GluR1 homogenate, Two-sample unpaired t-test,  $DF = 10$ ,  $T = 0.65$ ,  $P = 0.53$ ; synaptosome, Wilcoxon rank sum test,  $W = 10$ ,  $P = 0.23$ ; PSD95 homogenate, Two-sample unpaired t-test,  $DF = 10$ ,  $T = -0.14$ ,  $P = 0.89$ ; synaptosome, Two-sample unpaired t-test,  $DF = 10$ ,  $T = -0.34$ ,  $P = 0.74$ ; Figure 3.10D-F). Despite the reduction in GluN2B expression, *Grin2b*<sup>+/-</sup> animals were fertile and appeared physically indistinguishable from wild-type littermates.

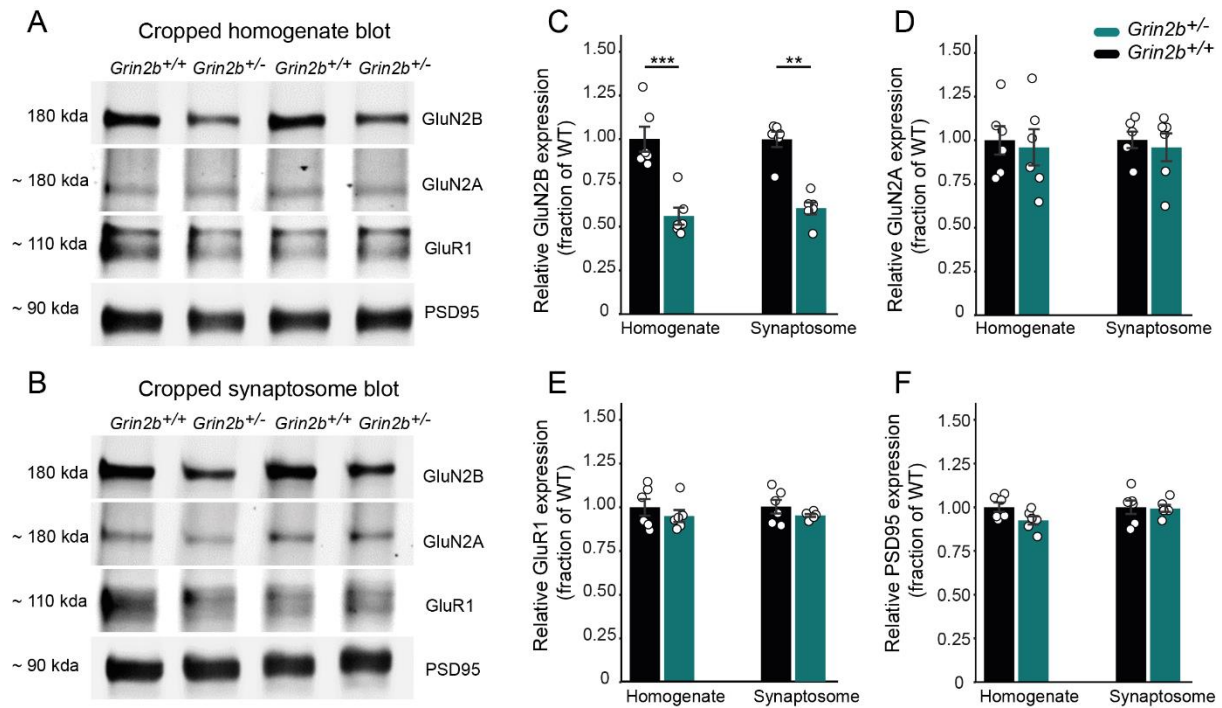


**Figure 3.9. Heterozygous *Grin2b* deletion in rats results in reduction of endogenous *GluN2B* expression in somatosensory cortex.** Representative cropped Western blots of extracts from rat somatosensory brain homogenates (**A**) and synaptosomes (**B**), full length blots in Supplementary Fig 3. Bands in the molecular weight range expected for full length *GluN2B*, *GluN2A*, *GluR1* and *PSD95* were detected in homogenates and synaptosomes from wild-type and *Grin2b*<sup>+/-</sup> animals. (**C**) Quantification of *GluN2B* protein from homogenates and synaptosomes reveals a significant decrease in *Grin2b*<sup>+/-</sup> rats ( $P = 0.00046$ ,  $P = 0.0051$ , Two-sample unpaired *t*-test and Wilcoxon rank sum test). There was no change in *Grin2b*<sup>+/-</sup> rats in expression levels of *GluN2A* ( $P = 0.77$ ,  $P = 0.66$ , Two-sample unpaired *t*-tests) (**D**), *GluR1* ( $P = 0.42$ ,  $P = 0.26$ , Two-sample unpaired *t*-tests) (**E**), or *PSD95* ( $P = 0.061$ ,  $P = 0.88$ , Two-sample unpaired *t*-tests) (**F**). Detailed statistics in text. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

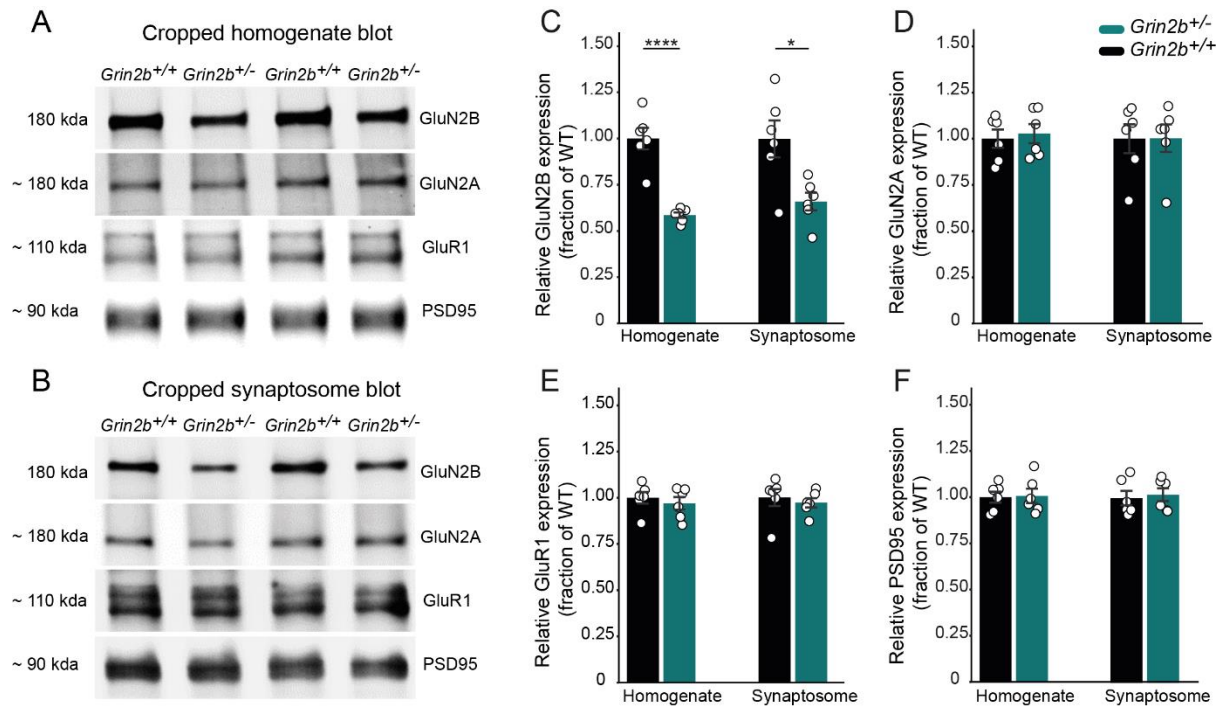


**Figure 3.10. Heterozygous *Grin2b* deletion in rats results in reduction of endogenous *GluN2B* expression in hippocampus.** Representative Western blots of extracts from rat somatosensory brain homogenates (**A**) and synaptosomes (**B**), full length blots in Supplementary Fig 3. Bands in the molecular weight range expected for full length *GluN2B*, *GluN2A*, *GluR1* and *PSD95* were detected in homogenates and synaptosomes from wild-type and *Grin2b*<sup>+/-</sup> animals. (**C**) Quantification of *GluN2B* protein from homogenates and synaptosomes reveals a significant decrease in *Grin2b*<sup>+/-</sup> rats ( $P = 0.000044$ ,  $P = 0.012$ , Two-sample unpaired *t*-tests). There was no change in *Grin2b*<sup>+/-</sup> rats in expression levels of *GluN2A* ( $P = 0.71$ ,  $P = 0.98$ , Two-sample unpaired *t*-tests) (**D**), *GluR1* ( $P = 0.53$ ,  $P = 0.23$ , Two-sample unpaired *t*-test and Wilcoxon rank sum test) (**E**), and *PSD95* ( $P = 0.89$ ,  $P = 0.74$ , Two-sample unpaired *t*-tests) (**F**). Detailed statistics in text. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

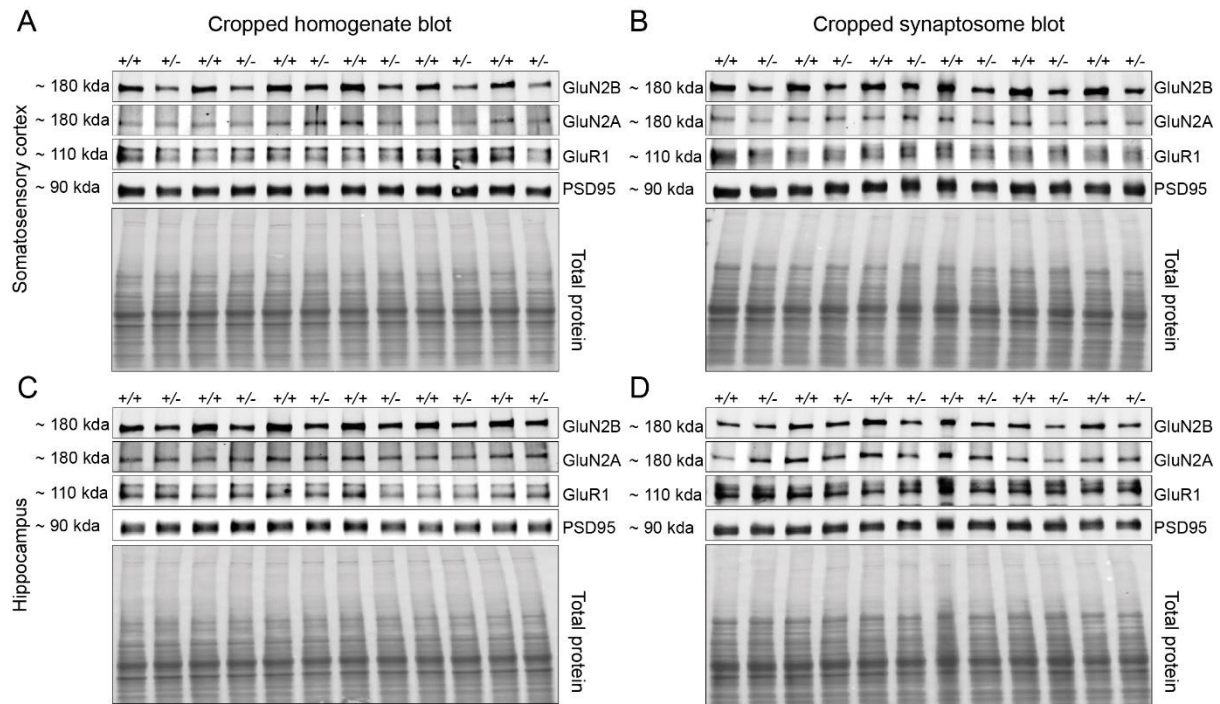


**Figure 3.11. Heterozygous *Grin2b* deletion in rats results in reduction of endogenous *GluN2B* expression in somatosensory cortex and hippocampus.** Entire set of Western blots of extracts from rat somatosensory brain homogenates (A) and synaptosomes (B), and extracts from rat hippocampal brain homogenates (C) and synaptosomes (D). Bands in the molecular weight range expected for full length *GluN2B*, *GluN2A*, *GluR1* and *PSD95* were detected in homogenates and synaptosomes from wild-type and *Grin2b*<sup>+/-</sup> animals.

### 3.3.4. Sleep-wake abnormalities in *Grin2b*<sup>+/-</sup> rats

Sleep disturbances are prevalent in individuals with *GRIN2B* mutations. Proband case studies and parental reports indicate that at least 60% of these patients experience difficulties in initiating and maintaining sleep, exhibit breathing irregularities during sleep, and suffer from daytime somnolence (Freunscht et al., 2013; Platzner et al., 2017; Simons Searchlight, 2021). However, sleep impairment related to *GRIN2B* NDDs has not previously been quantified or studied, representing a significant knowledge gap, which this thesis aimed to address in an animal model.

The relationship between *Grin2b* haploinsufficiency and REM sleep in rats was investigated first. I found that *Grin2b*<sup>+/-</sup> rats displayed a statistically significant net decrease in REM sleep minutes over the entire 24 hours when analysed hour by hour relative to wild-type animals, however this reduction was not specific to any hours of the day (LMM, effect of hour  $F = 16.13$ ,  $DF = 23$ ,  $P < 0.00001$ ; genotype  $F = 9.98$ ,  $DF = 1$ ,  $P = 0.0045$ ; hour x genotype  $F = 0.95$ ,  $DF = 23$ ,  $P = 0.53$ ; Figure 3.12A). Similarly, *Grin2b*<sup>+/-</sup> rats spent less total time in REM sleep than wild-type littermate controls when total minutes per animal were analysed (Two-sample unpaired t-test,  $DF = 22$ ,  $T = 3.16$ ,  $P =$

0.0045; Figure 3.12D). This reduction was due to fewer REM sleep bouts in *Grin2b*<sup>+/-</sup> animals relative to *Grin2b*<sup>+/+</sup> rats (Two-sample unpaired t-test,  $DF = 22$ ,  $T = 3.94$ ,  $P = 0.00069$ ; Figure 3.12D). Interestingly, the average duration of REM sleep bouts was longer for *Grin2b*<sup>+/-</sup> animals than wild-type littermates (Wilcoxon rank sum test,  $W = 33$ ,  $P = 0.043$ ; Figure 3.12D), however, this was insufficient to compensate for the net REM deficit observed. Reduced REM sleep in *Grin2b*<sup>+/-</sup> mutants was not specific to light or dark periods, instead it was a consequence of overall loss of time spent in this state across the 24 hour period (LMM, effect of phase  $F = 119.42$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 9.99$ ,  $DF = 1$ ,  $P = 0.0046$ ; phase x genotype  $F = 0.85$ ,  $DF = 1$ ,  $P = 0.37$ ; Figure 3.13A). I did, however, observe significantly fewer REM sleep bouts during both the light and dark periods in *Grin2b*<sup>+/-</sup> rats when compared to *Grin2b*<sup>+/+</sup> animals (LMM, effect of phase  $F = 105.93$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 15.52$ ,  $DF = 1$ ,  $P = 0.00067$ ; phase x genotype  $F = 4.53$ ,  $DF = 1$ ,  $P = 0.045$ ; Tukey *post hoc* test, light  $P = 0.0001$ , dark  $P = 0.032$ ; Figure 3.13A). The average duration of REM sleep bouts did not statistically differ between the light and dark phase between the two genotypes (LMM, effect of phase  $F = 2.26$ ,  $DF = 1$ ,  $P = 0.13$ ; genotype  $F = 1.88$ ,  $DF = 1$ ,  $P = 0.18$ ; phase x genotype  $F = 2.15$ ,  $DF = 1$ ,  $P = 0.16$ ; Figure 3.13A).

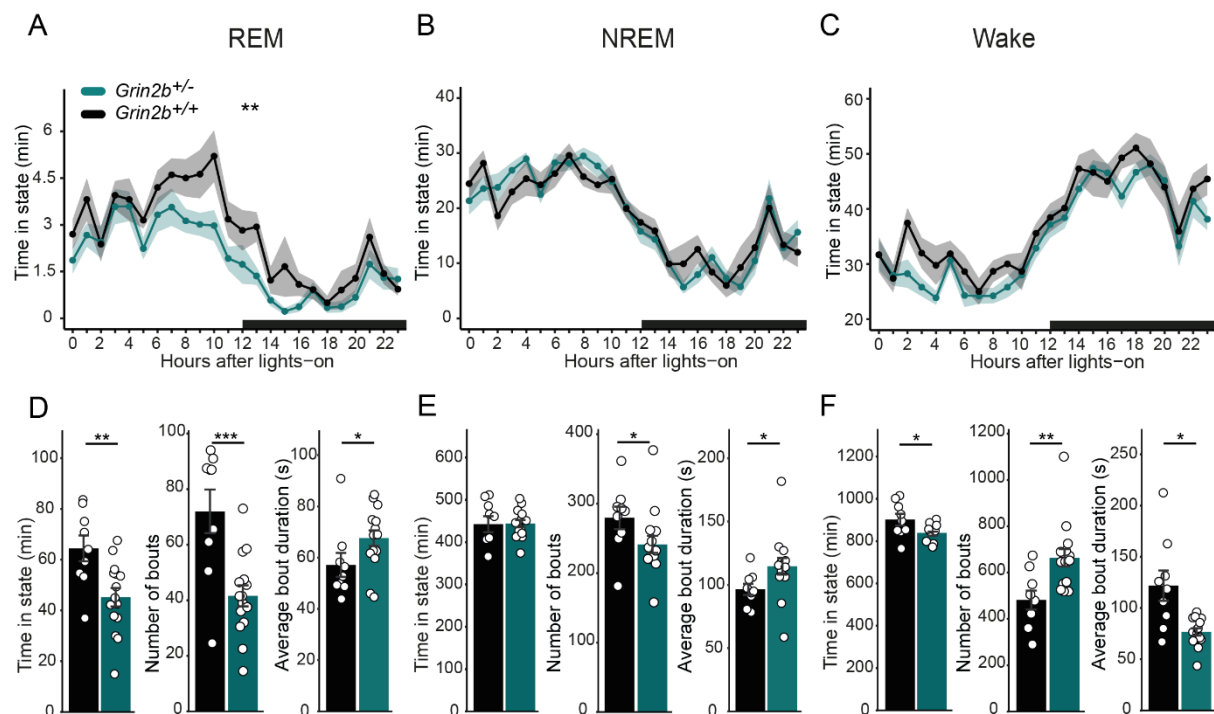


**Figure 3.12. *Grin2b*<sup>+/-</sup> animals display sleep-wake abnormalities.** Quantification showing the time spent in REM (A), NREM (B) and wake (C) by hour, across the 24-hour light-dark cycle with black bar on x-axis indicating lights off in the animal facility. *Grin2b*<sup>+/-</sup> rats had overall significantly less REM sleep compared to wild-type littermates, and this reduction was no specific to hours of the day (hour  $P < 0.00001$ , genotype  $P = 0.0045$ , hour x genotype  $P = 0.53$ ; LMM). No net or individual hour differences were observed between genotypes for NREM and wake states (NREM hour  $P < 0.00001$ , genotype  $P = 0.93$ , hour x genotype  $P = 0.66$ ; wake hour  $P < 0.00001$ , genotype  $P = 0.014$ , hour x

genotype  $P = 0.84$ ; LMMs). \*\* = effect from genotype. Points indicate group mean values (mean  $\pm$  SEM). Bar plots showing total time (left), number of bouts (middle) and average bout duration (right) for REM (D), NREM (E) and wake (F) during the full 24 hours (\* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ , unpaired Two-sample t-tests and Wilcoxon rank sum tests specified in results text). Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

Next, I looked at the impact *Grin2b* heterozygous deletion had on NREM physiology. On an hour-by-hour basis the distribution of NREM sleep was not different between *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> rats (LMM, effect of hour  $F = 21.52$ ,  $DF = 23$ ,  $P < 0.00001$ ; genotype  $F = 0.0081$ ,  $DF = 1$ ,  $P = 0.93$ ; hour x genotype  $F = 0.86$ ,  $DF = 23$ ,  $P = 0.66$ ; Figure 3.12B). Similarly, there was no difference between *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> rats in the total amount of time spent in NREM over the full 24 hours (Two-sample unpaired t-test,  $DF = 22$ ,  $T = -0.09$ ,  $P = 0.93$ ; Figure 3.12E). Nonetheless, *Grin2b*<sup>+/-</sup> rats demonstrated abnormal NREM distributions when compared to *Grin2b*<sup>+/+</sup> littermates, as they had fewer NREM bouts (Wilcoxon rank sum test,  $W = 29.5$ ,  $P = 0.025$ ; Figure 3.12E), which were longer in average duration (Wilcoxon rank sum test,  $W = 26$ ,  $P = 0.015$ ; Figure 3.12E). During the light and dark phases, however, *Grin2b*<sup>+/-</sup> animals did not differ from their wild-type littermates in the total amount of NREM sleep (LMM, effect of phase  $F = 237.34$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 0.0065$ ,  $DF = 1$ ,  $P = 0.94$ ; phase x genotype  $F = 0.94$ ,  $DF = 1$ ,  $P = 0.34$ ; Figure 3.13B), the amount of NREM bouts (LMM, effect of phase  $F = 198.28$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 3.62$ ,  $DF = 1$ ,  $P = 0.07$ ; phase x genotype  $F = 0.041$ ,  $DF = 1$ ,  $P = 0.84$ ; Figure 3.13B) and the average NREM bout duration (LMM, effect of phase  $F = 16.34$ ,  $DF = 1$ ,  $P = 0.00055$ ; genotype  $F = 4.091$ ,  $DF = 1$ ,  $P = 0.055$ ; phase x genotype  $F = 0.0023$ ,  $DF = 1$ ,  $P = 0.99$ ; Figure 3.13B), suggesting net full day differences observed are subtle.

Lastly, wake was evaluated in both *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> animals to assess for any differences between the two genotypes. I looked at the distribution of wake across each hour of the light-dark cycle and found that while overall *Grin2b*<sup>+/-</sup> animals had significantly reduced time in wake relative to *Grin2b*<sup>+/+</sup> controls, this was not specific to any given hour (LMM, effect of hour  $F = 22.55$ ,  $DF = 23$ ,  $P < 0.00001$ ; genotype  $F = 7.14$ ,  $DF = 1$ ,  $P = 0.014$ ; hour x genotype  $F = 0.71$ ,  $DF = 23$ ,  $P = 0.84$ ; Figure 3.12C). When compared to wild-type littermates, the total minutes awake were significantly reduced in *Grin2b*<sup>+/-</sup> rats (Welch's two-sample unpaired t-test,  $DF = 10.25$ ,  $T = 2.26$ ,  $P = 0.047$ ; Figure 3.12F) with mutants having more wake bouts (Wilcoxon rank sum test,  $W = 18$ ,  $P = 0.0035$ ; Figure 3.12F) of shorter duration (Welch's two-sample unpaired t-test,  $DF = 8.91$ ,  $T = 2.97$ ,  $P = 0.016$ ; Figure 3.12F). Wake state differences were due to the overall changes across the full 24 hours, as the differences between *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> animals were irrespective of the light or dark period for the time spent awake (LMM, effect of phase  $F = 185.12$ ,  $DF = 1$ ,  $P < 0.00001$ ; genotype  $F = 6.64$ ,  $DF = 1$ ,  $P = 0.013$ ; phase x genotype  $F = 0.23$ ,  $DF = 1$ ,  $P = 0.64$ ; Figure 3.13C), number of wake bouts (LMM, effect of phase  $F = 1.39$ ,  $DF = 1$ ,  $P = 0.25$ ; genotype  $F = 9.76$ ,  $DF = 1$ ,  $P = 0.0049$ ; phase x genotype  $F = 1.27$ ,  $DF = 1$ ,  $P = 0.27$ ; Figure 3.13C) and average wake bout duration (LMM, effect of phase  $F = 26.25$ ,  $DF = 1$ ,  $P$

$< 0.00001$ ; genotype  $F = 13.14$ ,  $DF = 1$ ,  $P = 0.0015$ ; phase  $\times$  genotype  $F = 2.34$ ,  $DF = 1$ ,  $P = 0.14$ ; Figure 3.13C).

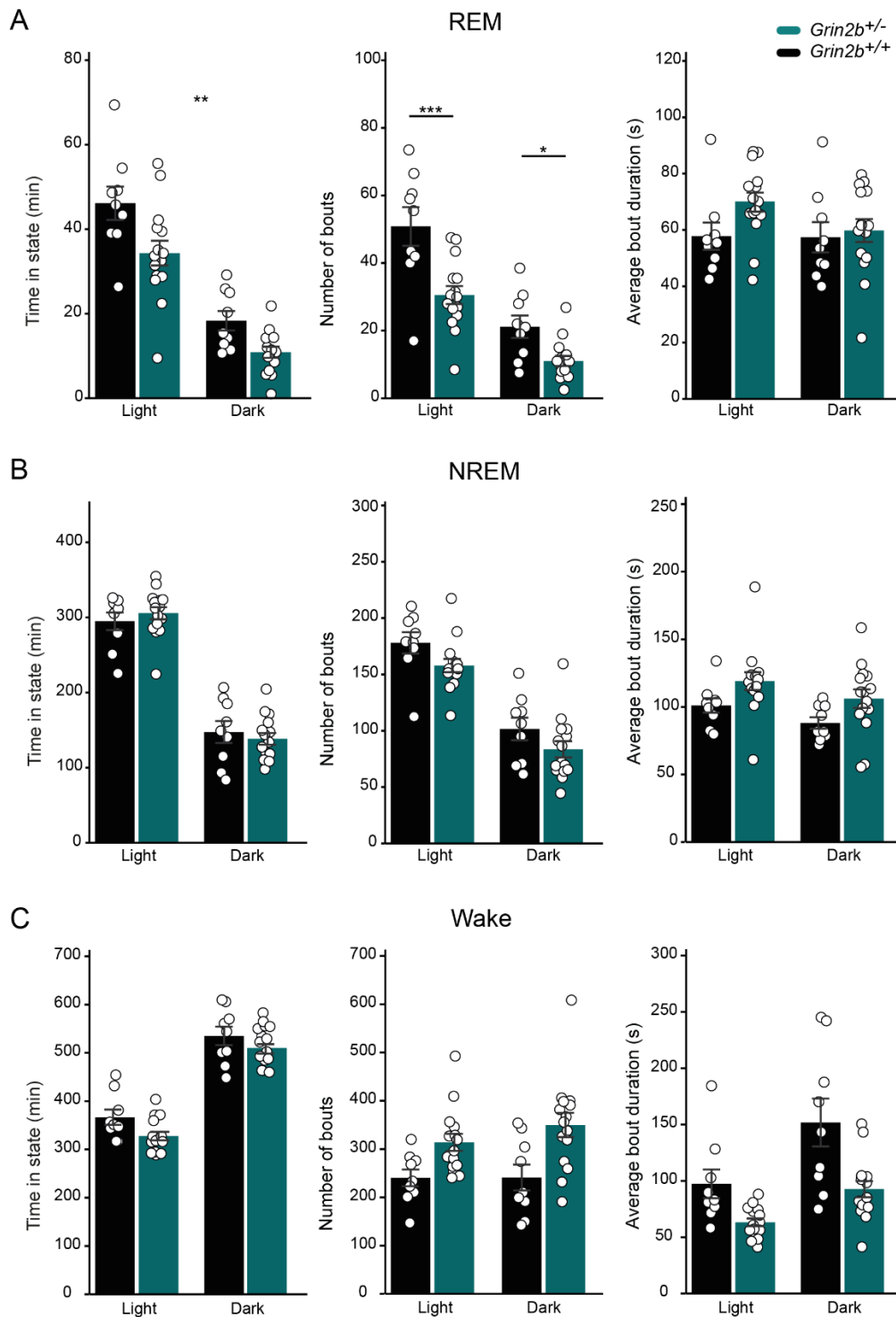


**Figure 3.13. Reduced REM sleep in *Grin2b*<sup>+/-</sup> rats during the light and dark periods.** Quantification showing total time (left), number of bouts (middle) and average bout duration (right) for REM (A), NREM (B) and wake (C) during the 12-hour light and dark periods. Bars indicate mean values (mean  $\pm$  SEM) and points correspond to values from individual rats. Total time in REM sleep was overall

reduced in *Grin2b*<sup>+/-</sup> rats compared to wild-types, as REM sleep reductions were irrespective of light and dark phases (phase  $P < 0.00001$ , genotype  $P = 0.0046$ , phase x genotype  $P = 0.37$ , LMM) (\*\* = effect from genotype). In comparison to *Grin2b*<sup>+/+</sup> animals, the number of REM bouts in *Grin2b*<sup>+/-</sup> rats was reduced, both during the light and the dark periods, while no differences were found between the two groups in the average length of REM bouts (REM bouts phase  $P < 0.00001$ , genotype  $P = 0.00067$ , phase x genotype  $P = 0.45$  (light  $P = 0.0001$ , dark  $P = 0.032$ ); REM bout duration REM phase  $P = 0.13$ , genotype  $P = 0.18$ , phase x genotype  $P = 0.16$ ; LMMs with Tukey post hoc tests) (\*\*\*) and \* = post hoc test for genotype x phase interaction) (A). Analysis by light-dark phase revealed no difference between the two genotypes for total time spent in NREM sleep, the number of NREM bouts and the average NREM bout durations (Time in NREM phase  $P < 0.00001$ , genotype  $P = 0.94$ , phase x genotype  $P = 0.34$ ; NREM bouts phase  $P < 0.00001$ , genotype  $P = 0.07$ , phase x genotype  $P = 0.84$ ; NREM bout duration phase  $P = 0.00055$ , genotype  $P = 0.055$ , phase x genotype  $P = 0.99$ ; LMMs) (B). Relative to wild-type littermates, *Grin2b*<sup>+/-</sup> rats had fewer total minutes of wake, and more wake bouts of increased duration, however, differences were irrespective of the light or dark periods (Time in wake phase  $P < 0.00001$ , genotype  $P = 0.013$ , phase x genotype  $P = 0.64$ ; wake bouts phase  $P = 0.25$ , genotype  $P = 0.0049$ , phase x genotype  $P = 0.27$ ; wake bout duration phase  $P < 0.00001$ , genotype  $P = 0.0015$ , phase x genotype  $P = 0.14$ ; LMMs) (C). Detailed statistic can be found in results text.

#### *Sex does not influence sleep-wake physiology in Grin2b<sup>+/-</sup> rats*

*GRIN2B* related NDDs are observed in both male and female individuals (Freunscht et al., 2013; Platzer et al., 2017; Simons Searchlight, 2021). I found that sleep states are altered in *Grin2b*<sup>+/-</sup> rats, and next tested for the effect of sex on REM, NREM and wake states across genotypes in the same set of animals used above. Analysis revealed no statistically significant differences between male and female *Grin2b*<sup>+/-</sup> animals and wild-type littermate controls (for statistical results see Table 3.3, Figure 3.14A-F), indicating that heterozygous deletion of *Grin2b* affects sleep related mechanisms similarly in both sexes.

| Two-way ANOVA         | Effect of genotype |    |               | Effect of sex |    |              | Effect of genotype x sex        |    |       |
|-----------------------|--------------------|----|---------------|---------------|----|--------------|---------------------------------|----|-------|
|                       | F                  | DF | P             | F             | DF | P            | F                               | DF | P     |
| Total REM duration    | 8.23               | 1  | <b>0.0095</b> | 0.0094        | 1  | 0.92         | 0.83                            | 1  | 0.37  |
| Number of REM bouts   | 12.46              | 1  | <b>0.0021</b> | 0.033         | 1  | 0.86         | 0.39                            | 1  | 0.54  |
| Average REM duration  | 2.49               | 1  | 0.13          | 1.76          | 1  | 0.41         | 0.036                           | 1  | 0.85  |
| Total NREM duration   | 0.0074             | 1  | 0.93          | 0.46          | 1  | 0.5          | 0.064                           | 1  | 0.8   |
| Number of NREM bouts  | 2.96               | 1  | 0.101         | 0.0066        | 1  | 0.94         | 0.0045                          | 1  | 0.95  |
| Average NREM duration | 0.28               | 1  | 0.11          | 0.83          | 1  | 0.37         | 0.0025                          | 1  | 0.96  |
| Total wake duration   | 5.054              | 1  | <b>0.036</b>  | 1.66          | 1  | 0.21         | 0.12                            | 1  | 0.73  |
| Number of wake bouts  | 7.13               | 1  | <b>0.015</b>  | 3.77          | 1  | 0.066        | 0.025                           | 1  | 0.62  |
| Average wake duration | 10.38              | 1  | <b>0.0043</b> | 5.69          | 1  | <b>0.027</b> | 1.9                             | 1  | 0.18  |
| Linear mixed model    |                    |    |               |               |    |              | Effect of genotype x sex x hour |    |       |
|                       | F                  | DF | P             | F             | DF | P            | F                               | DF | P     |
| REM hour-by-hour      | 8.84               | 1  | <b>0.0075</b> | 0.85          | 1  | <b>0.037</b> | 1.24                            | 1  | 0.21  |
| NREM hour-by-hour     | 0.043              | 1  | 0.52          | 0.45          | 1  | 0.5          | 1.46                            | 1  | 0.078 |
| Wake hour-by-hour     | 5.16               | 1  | <b>0.038</b>  | 1.76          | 1  | 0.41         | 1.31                            | 1  | 0.16  |

**Table 3.3. Values for statistical analysis across brain states, genotypes and sex in *Grin2b*<sup>+/-</sup> rats.** Left most column contains description of test statistic and measurements analysed. Columns 2-4 contain relevant F, DF and P values for the effects of genotype and sex, and interaction effect between them. Values in rows 3-11 correspond to results from Two-way ANOVA test statistics and values in rows 14-16 correspond to results from LMMs used in hour-by-hour time course analysis of sleep-wake distribution. Statistically significant post hoc results are shown in bold blue text. Statistical results relate to section 3.3.4 ‘Sex does not influence sleep-wake physiology in *Grin2b*<sup>+/-</sup> rats’ and Figure 3.14.

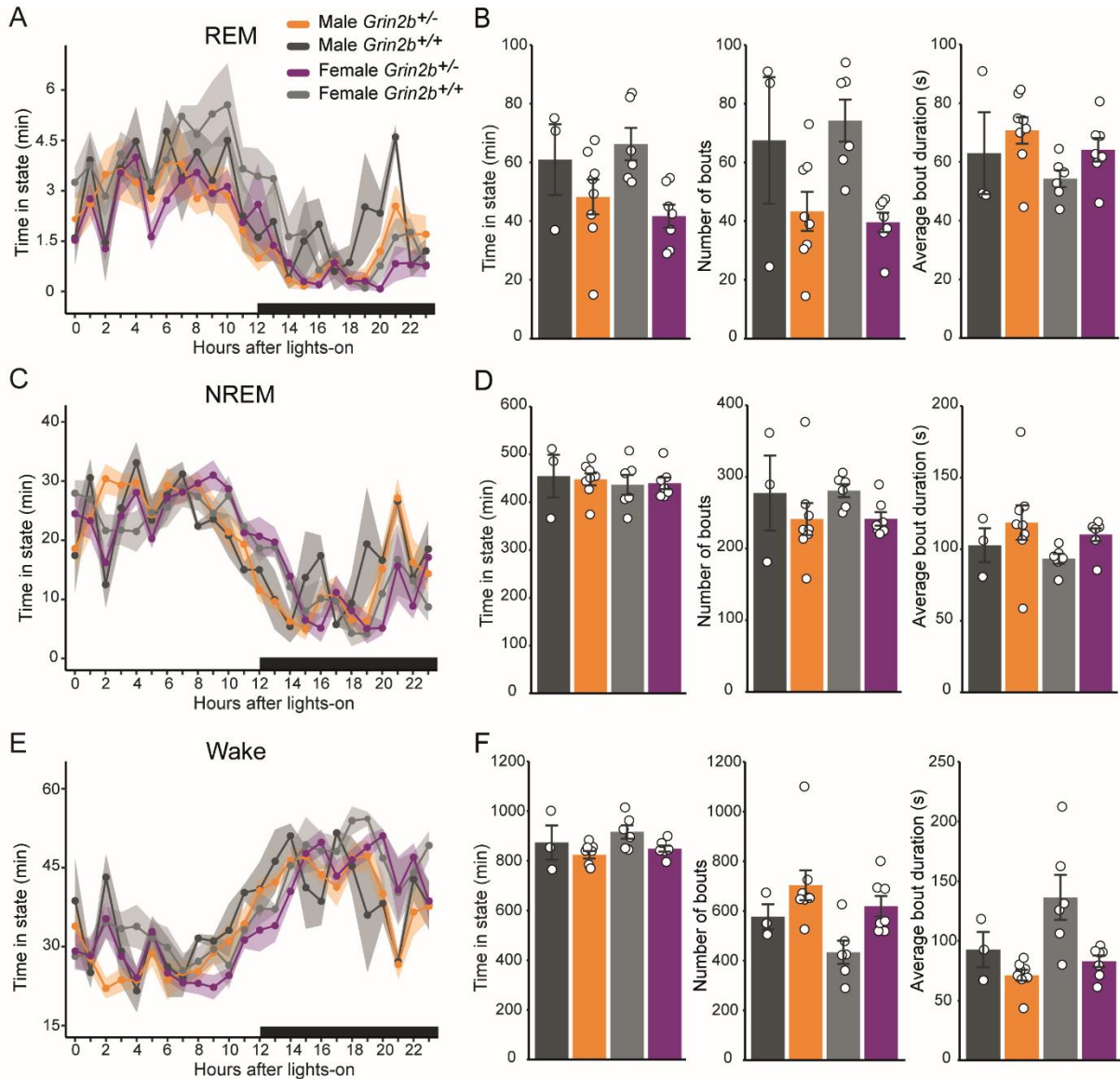


**Figure 3.14. Sleep-wake physiology in *Grin2b*<sup>+/-</sup> animals does not differ between male and female animals.** Quantification showing the time spent in REM (A), NREM (C) and wake (E) by hour across the 24-hour light-dark cycle; points indicate group mean values (mean ± SEM). Bar plots showing total time (left), number of bouts (middle) and average bout duration (right) for REM (B), NREM (D) and wake (F) during the full 24 hours; bars indicate mean values (mean ± SEM) and points correspond to values from individual rats. Note: significance (\*) where effect from genotype is found is not indicated. Time in REM is reduced in both female and male *Grin2b*<sup>+/-</sup> rats relative to female and male wild-type littermates, however, this difference is not due to animals' sex or to specific hours of the day (A). For the full 24 hours, sex did not impact on total REM sleep or REM bouts, as each was reduced in both male and female *Grin2b*<sup>+/-</sup> mutants relative to their wild-type sex-matched littermates (B). Average REM bout duration was not different between both sexes and genotypes (B). Time in NREM is similar between *Grin2b*<sup>+/-</sup> and wild-type animals of both sexes throughout each hour of the day (C). For the 24-hour day, sex did not impact on total NREM sleep, NREM bouts and average NREM bout duration, and these metrics did not differ between male and female *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> animals (D). Time in wake is reduced in both female and male *Grin2b*<sup>+/-</sup> rats relative to female and male *Grin2b*<sup>+/+</sup> littermates, and this difference is not due to animals' sex or to specific hours of the day (E). There we no sex dependent differences for the total wake time, number of wake bouts and average wake bout duration, when

quantified for the full 24 hours (**F**). Total time in wake and average wake bout duration were decreased, while number of bouts was increased in male and female *Grin2b*<sup>+/-</sup> rats compared to male and female *Grin2b*<sup>+/+</sup> rats (**F**). For detailed statistics see Table 3.3).

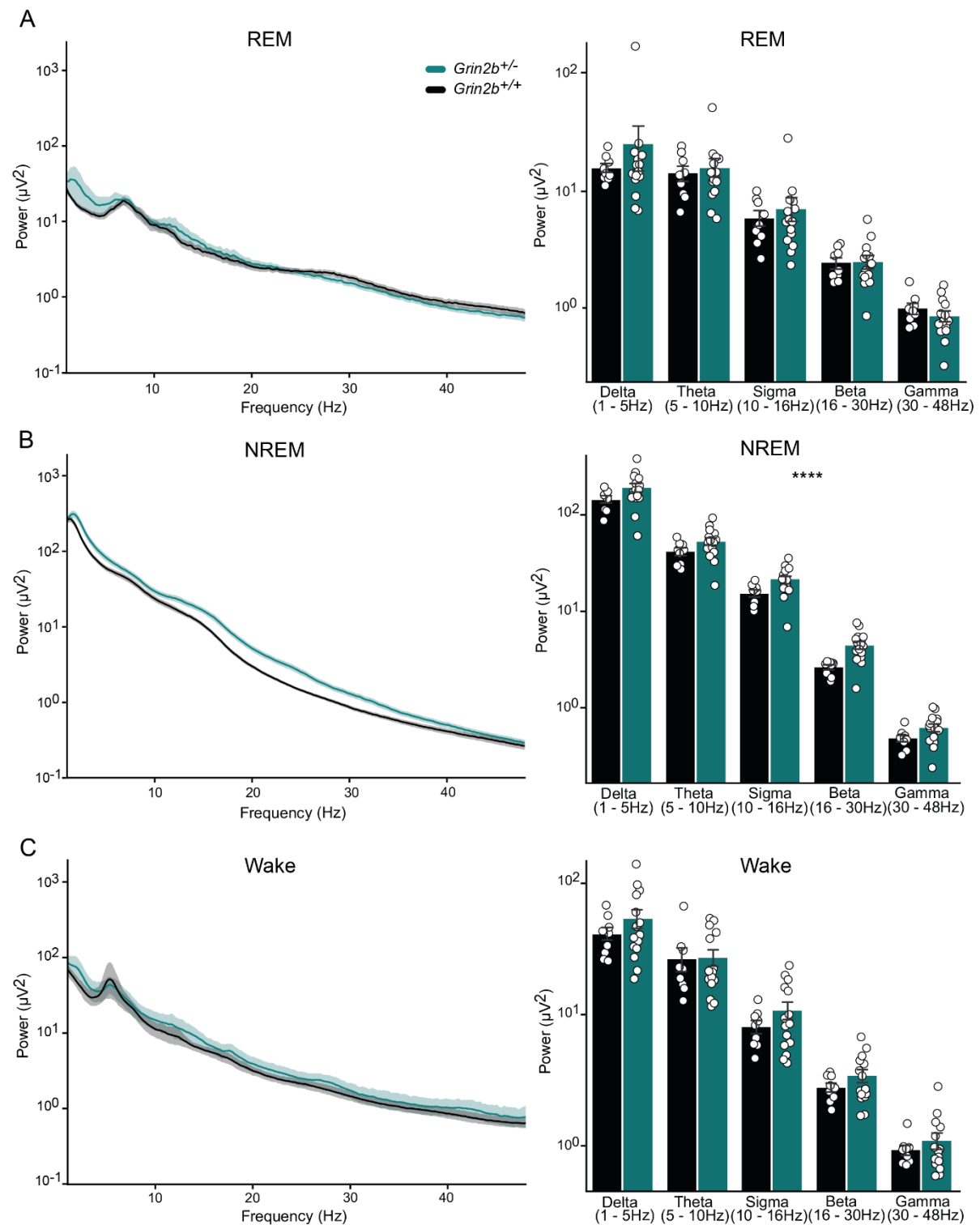


**Figure 3.15. Spectral power is increased in NREM sleep in *Grin2b*<sup>+/-</sup> rats.** Average EEG power spectra across wild-type and *Grin2b*<sup>+/-</sup> rats (left; traces represent group averages (mean  $\pm$  SEM) and quantification by individual frequency bands (right) during REM (A), NREM (B) and wake (C) epochs. Quantification of average power in specific frequency bands during REM epochs and wake, showed no spectral differences between *Grin2b*<sup>+/-</sup> and wild-type animals (REM genotype  $P = 0.98$ , frequency  $P < 0.0001$ , genotype  $\times$  frequency  $P = 0.89$ ; wake genotype  $P = 0.17$ , frequency  $P < 0.00001$ , genotype  $\times$  frequency  $P = 0.98$ ; Two-way ANOVA tests). Spectral power was significantly higher during NREM epochs in *Grin2b*<sup>+/-</sup> rats although no significant differences were found in individual frequency bands (genotype  $P = 0.000023$ , frequency  $P < 0.0001$ , genotype  $\times$  frequency  $P = 0.66$ , Two-way ANOVA). \*\*\*\* = effect from genotype. Detailed statistics in main text. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

#### *Increased spectral power during NREM in *Grin2b*<sup>+/-</sup> rats*

Finally, no statistical spectral power differences were found between *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> rats during REM sleep (Two-way ANOVA, effect of genotype  $F = 0.0003$ ,  $DF = 1$ ,  $P = 0.98$ ; frequency  $F = 139.83$ ,  $DF = 4$ ,  $P < 0.00001$ ; genotype  $\times$  frequency  $F = 0.28$ ,  $DF = 4$ ,  $P = 0.89$ ; Figure 3.15A) and wake (Two-way ANOVA, effect of genotype  $F = 1.89$ ,  $DF = 1$ ,  $P = 0.17$ ; frequency  $F = 258.1$ ,  $DF = 4$ ,  $P < 0.00001$ ; genotype  $\times$  frequency  $F = 0.13$ ,  $DF = 4$ ,  $P = 0.98$ ; Figure 3.15C). However, power was significantly increased across frequency bands in NREM sleep, although there were no statistical differences in specific bands (Two-way ANOVA, effect of genotype  $F = 19.61$ ,  $DF = 1$ ,  $P = 0.000023$ ; frequency  $F = 932.18$ ,  $DF = 4$ ,  $P < 0.00001$ ; genotype  $\times$  frequency  $F = 0.61$ ,  $DF = 4$ ,  $P = 0.66$ ; Figure 3.15B). Grouped together, the above results show that *Grin2b*<sup>+/-</sup> rats have altered sleep-wake brain state distributions and spectral properties.

### 3.4. Discussion

Using home-cage, long-term wireless polysomnography coupled with offline automated sleep-wake scoring, altered sleep-wake distribution phenotypes and EEG spectral properties were identified in rat models of *16p11.2* deletion syndrome, *Nlgn3* insufficiency (knockout) and *Grin2b* haploinsufficiency. The findings, implications and potential mechanisms leading to abnormal sleep phenotypes are discussed below, separately for each line. I outline potential neuroanatomical, circuit and regulatory pathway signalling alterations that have previously been reported in *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> rodent models, and that could reflect the sleep abnormalities observed in this thesis.

### 3.4.1. Male-specific REM sleep augmentation in *16p11.2*<sup>+/-</sup> rats

In this work male *16p11.2*<sup>+/-</sup> rats displayed a significant increase in time spent in REM sleep, which was due to animals having more bouts of REM during the light phase of the 24-hour day. Time in NREM sleep, NREM bout distribution and durations were unaltered in male *16p11.2*<sup>+/-</sup> rats relative to wild-types. Physiologically similar distributions of REM, NREM and wake states were seen in *16p11.2*<sup>+/-</sup> female rats and wild-type littermates of both sexes. *16p11.2*<sup>+/-</sup> rats displayed reduced spectral power across REM, NREM and wake, which was not specific to any given frequency range and could be suggestive of generalized dysfunction in neural circuits regulating both sleep and wake states.

The sex-specific effect presented here complements prior research in mice with heterozygous *16p11.2* deletions that present with male-specific alterations in GABAergic gene expression (Openshaw et al., 2023), striatal dopaminergic signalling (Grissom et al., 2018), striatal structural changes (V. J. Kumar et al., 2018), impairment of memory and social interaction (Martin Lorenzo et al., 2023) , and importantly, hyperactivity and decreases in both REM and NREM sleep (Angelakos et al., 2017; H.-C. Lu et al., 2019).

The precise nature of male vulnerability to *16p11.2* deletions is still under investigation, but it's theorised to be a consequence of male and female differences in the function and baseline expression patterns of some of the 28 genes contained in the chromosomal region. For example, there is evidence for sex differences in the function of ERK1 (encoded by *MAPK3* in the *16p11.2* locus) and ERK2 kinases, which are critical for neuronal function, synaptic plasticity and memory formation via their recruitment in the ERK/MAPK pathway (Mizuno & Giese, 2010). ERK/MAPK regulates plasticity-associated genes by mediating the activation of the transcription factor cAMP response element-binding protein (CREB), which is involved in memory formation and various cognitive processes (Mizuno & Giese, 2010). It has been shown that activation of ERK/MAPK is higher in the ventral hippocampus of male, but not female, rats following contextual fear conditioning (Kudo et al., 2004), and that this contributes to male specific CREB activation (Gresack et al., 2009; Mizuno & Giese, 2010). In line with this, Grissom and colleagues recently demonstrated paradoxically elevated ERK1 phosphorylation in male *16p11.2* deletion mice both at baseline and in response to sucrose consumption in striatum, a region heavily involved in reward driven signalling and behaviour. Hyperphosphorylation of ERK1 in the striatum of these animals was concordant with distinctly impaired reward-directed learning and maintenance of motivation. Notably, augmented ERK1 activation and impaired learning were not observed in female mice carrying the same mutation (Grissom et al., 2018). In a separate study increased ERK1 signalling in male *16p11.2* deletion mice resulted in reduced brain size and cortical cytoarchitecture perturbations of animals (Pucilowska et al., 2015). Therefore, genetic lesion of *16p11.2* impacts ERK1-mediated signalling, and cortico-striatal development and circuitry, in a sex-dependent

manner to at least in part contribute to male-specific vulnerability to *16p11.2* CNVs. This could be a relevant mechanism in the abnormal sleep phenotype observed in male *16p11.2*<sup>+/-</sup> rats in this thesis, as ERK/MAPK plays a crucial role in entrainment of the circadian rhythm, particularly in response to light stimuli (X.-L. Wang et al., 2020).

The ERK/MAPK pathway interacts with and phosphorylates *CLOCK*, *BMAL1*, *CRY1*, and *CRY2* in the circadian system with ERK1/2 activation and subsequent CREB phosphorylation in the SCN being crucial for light-induced resetting of the circadian clock (X.-L. Wang et al., 2020). ERK hyperactivation observed in male *16p11.2* mice (Grissom et al., 2018; Pucilowska et al., 2015) has also been shown in *Syngap1* haploinsufficient and *Fmr1* knockout mice (Krueger et al., 2011; Rumbaugh et al., 2006). Mice and rats with these mutations present with NDD and sleep impairment phenotypes (Boone et al., 2018; Buller-Peralta et al., 2022; Katsanevaki et al., 2024; Krueger et al., 2011; Saré et al., 2017). In the context of sleep, genetic knockout of *Erk1* or *Erk2* and pharmacological suppression of ERK phosphorylation in mice, leads to reduced sleep duration and extended periods of wakefulness, which highlights the critical role for ERK signalling in regulating sleep-wake cycles and maintaining normal sleep patterns (Mikhail et al., 2017). It then follows that increased ERK activity could induce higher rates of sleep, however, the predicted functional consequences of dysregulated ERK1/2 signalling are insomnia, abnormal sleep patterns and delayed sleep phase onset, characterized by a relative delay in circadian phase (Kalkman, 2012). Such sleep disturbances are found in individuals with *16p11.2* CNVs and in mouse models of *16p11.2* deletion (Angelakos et al., 2017; Choi et al., 2024; B. A. Fernandez et al., 2010; Hanson et al., 2010; Kamara et al., 2023; H.-C. Lu et al., 2019; Tabet et al., 2012). Interestingly, I found no strong indication of perturbations to circadian rhythm timing or insomnia phenotypes in *16p11.2*<sup>+/-</sup> rats, rather male *16p11.2*<sup>+/-</sup> animals in this study presented with significantly more REM sleep bouts, which increased the total amount of REM sleep in these animals. It is plausible that increased ERK signalling, such as that observed in striatum (Grissom et al., 2018; Portmann et al., 2014) and cortex (Pucilowska et al., 2015), is also present elsewhere in the brain, and leads to increases in sleep and reduced wake time. Accordingly, the data presented in this thesis are suggestive of inappropriate ERK activation in REM promoting regions (e.g. LC, DRN, PPT, LTD and PRF nuclei) or of disruption in ERK-dependent circadian cues related to REM initiation. However, the mechanisms resulting in augmented REM, but not NREM sleep, are not fully understood. Additionally, REM sleep triggers ERK/MAPK cascade activation independently of the noradrenergic system, which is active during wake. During REM sleep, the ERK signalling cascade is primarily stimulated through cholinergic mechanisms and is therefore distinct from the ERK activation patterns observed in the wake state (Soulé et al., 2012). The effects of *16p11.2* heterozygous deletion on ERK signalling in various brain regions remain to be investigated. It will also be interesting to study whether and how potential alterations in ERK signalling might specifically impact REM sleep dynamics. Finally, it is likely that the functions of other genes in the *16p11.2* locus, such as *MVP*, the product of which forms a

structural scaffold for ERK (Kolli et al., 2004), also converge on the ERK/MAPK pathway to regulate its activity. This highlights the need for further examination of the collective impact of *16p11.2* deletion on molecular signalling and sleep.

Striatal dopaminergic pathways have a strong sleep-wake regulating role (Dong et al., 2019) and structural and dopaminergic signalling abnormalities in this area have been demonstrated in male mice with *16p11.2* deletions (Grissom et al., 2018; V. J. Kumar et al., 2018; Portmann et al., 2014; Pucilowska et al., 2015). Specifically, striatal GABAergic medium spiny neurons that are critically for the wake state, overexpress dopamine D2 receptors in *16p11.2* deletion male mice (Grissom et al., 2018; Portmann et al., 2014; Vetrivelan et al., 2010). Activation of D2 receptors during development decreases inhibitory cell migration from the basal forebrain to the cortex (Crandall et al., 2007) and importantly, some evidence suggests that basal ganglion D2 receptor activation, including in the striatum, may promote sleep (Vetrivelan et al., 2010). Furthermore, dopamine signalling in the basolateral amygdala during NREM sleep triggers the transition to REM sleep by hyperpolarising dopamine D2 receptor expressing neurons (Hasegawa et al., 2022). Interestingly, oestrogen in females has been shown to downregulate D2 receptor function (Bazzett & Becker, 1994), which could further explain the male-specific sleep abnormalities found in this and other studies mentioned. Taken together, these findings indicate that abnormalities in dopaminergic signalling, particularly in cortico-striatal and amygdala circuitry, could be responsible for the observed changes in sleep patterns in male *16p11.2*<sup>+/-</sup> rats. It will be interesting to determine whether similar observations can be made in *16p11.2*<sup>+/-</sup> animals used in this study, if they indeed correlate to the observed REM sleep phenotypes, and whether striatal or basolateral amygdala circuits and dopaminergic signalling can be treated as therapeutic targets for sleep dysfunction related to *16p11.2* CNVs.

Besides dopaminergic signalling irregularities, there is evidence of compromised noradrenergic and serotonergic system activity in *16p11.2* deletion models (Brown et al., 2012; Choi et al., 2024; Mitchell et al., 2020; Panzini et al., 2017; Walsh et al., 2018). Noradrenergic signalling during sleep is predominantly mediated by LC norepinephrine neurons (NE), which excite ARAS regions and inhibit sleep promoting VLPO and MnPO. The LC-NE population is continuously active during wake, displays slower firing rate in NREM sleep and is silent during REM sleep (Brown et al., 2012). Aberrant LC-NE connectivity with wake promoting regions was recently shown in *16p11.2*<sup>+/-</sup> male mice, which presented with a higher rate of NREM microarousals ( $\leq 20$  seconds) but physiologically normal REM (Choi et al., 2024). It is therefore unlikely that the REM sleep increases observed here are resultant from altered noradrenergic activity, although this cannot be ruled out, as LC-NE connectivity and signalling could indirectly impact on REM sleep dynamics. Additionally, alternate mechanisms affecting this neuronal population may be impacted by the CNV mutation in *16p11.2*<sup>+/-</sup> rats used here. For instance, LC-NE neurons selectively express melatonin MT<sub>1</sub> receptors, which specifically enhance REM sleep and associated theta power, but not NREM sleep, by decreasing the firing activity of LC-NE cells

(López-Canul et al., 2024). Therefore, maladaptive melatonin activity could in part result in augmented REM sleep in *16p11.2*<sup>+/-</sup> male rats. Abnormal increases and decreases in melatonin levels throughout the day are observed in individuals with ASD (Maurer et al., 2023), although notably, there is currently no direct evidence supporting irregular melatonin production and receptor function specific to *16p11.2* deletion variants. Sleep microarousals were not investigated in the present work. Given the sleep state scoring paradigm I employed uses shorter 5 second windows akin to those used by Choi et al. (Choi et al., 2024; H.-C. Lu et al., 2019), it will be interesting to determine if a similar NREM phenotype can be observed in *16p11.2*<sup>+/-</sup> animals in this research. Importantly, this would indicate whether similar LC-NE activity mechanisms are perturbed in the *16p11.2* heterozygous deletion rat model.

Serotonin signalling promotes wake and inhibits REM sleep (Arnaldi et al., 2015; Brown et al., 2012). REM generating GABAergic PRF and SLD neurons are activated by cholinergic and glutamatergic inputs from PPT and LDT. The DRN provides the principal inhibitory serotonergic innervation to PPT and LDT, with wake promoting serotonergic brainstem and motor neurons inhibited by the glutamatergic SLD pathway during REM sleep (Brown et al., 2012; S.-H. Lee & Dan, 2012; Scammell et al., 2017). Thus, reduction in serotonin release reinforces REM sleep and REM sleep atonia (Arnaldi et al., 2015). Indeed, mice lacking serotonin receptors 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> exhibit greater amounts of REM sleep, while NREM sleep remains normal (Boutrel et al., 1999, 2002). Prior work using two different *16p11.2* deletion mouse lines indicates impairment in endogenous serotonergic transmission of male and female animals (Mitchell et al., 2020; Panzini et al., 2017; Walsh et al., 2018), which present with stress induced hyperlocomotion, reduced sociability, and a strong anxiolytic phenotype (Mitchell et al., 2020). Furthermore, *16p11.2* deletion restricted to serotonergic neurones in mice is sufficient to impair normal social behaviour and decreases DRN serotonin neuronal activity (Walsh et al., 2018). Given the above findings and relevance of serotonergic DRN neurons in indirectly modulating wake, it is reasonable to suggest that reduced or compromised serotonergic signalling in brainstem regions, particularly the DRN, might result in the increased REM sleep phenotype observed in male *16p11.2*<sup>+/-</sup> rats in this study. The increased number of REM bouts in male *16p11.2*<sup>+/-</sup> animals further supports this, as in the absence of sufficient wake-promoting serotonergic transmission in the brainstem, NREM sleep transitional circuitry may favour REM sleep over wake, leading to the male-specific phenotype I observed here. It will be important to first address whether the abnormal serotonergic signalling found in *16p11.2* deletion mouse models is phenocopied in rat lines with deleterious *16p11.2* CNVs, such as the model used in this thesis, whether activity of serotonergic neurons is impaired during sleep, and subsequently in which REM or wake promoting regions, for example the DRN and SLD, cellular serotonin properties and signalling could be disrupted.

From a circuitry perspective, REM sleep dynamics involve the recruitment of multiple structures and neuronal populations. They include lateral hypothalamic neurons that produce melanin-concentrating hormone and, GABAergic neurons in the lateral paraventricular nucleus (LPGi) of

the ventral medulla (Brown et al., 2012; J. Lu, Sherman, et al., 2006; Scammell et al., 2017; Weber et al., 2015). The increased REM found in male *16p11.2*<sup>+/-</sup> animals could reflect altered cellular properties or connectivity between REM-promoting and suppressing neurons. In the hypothalamus, melanin-concentrating hormone expressing neurons promote REM sleep and their acute activation maintains REM sleep (Jego et al., 2013). Interestingly, *16p11.2* deletion mice display sensorimotor behavioural phenotypes consistent with those observed in rats with hypothalamic lesions (Golani et al., 1979; Horev et al., 2011; Schallert et al., 1978). The increased REM sleep observed in male *16p11.2*<sup>+/-</sup> animals might therefore reflect altered activity or connectivity of REM-promoting neurons in the hypothalamus, particularly those expressing melanin-concentrating hormone. GABAergic LPGi neurons, which project to the REM suppressing ventrolateral PAG, have strong modulatory influence over REM sleep. Upon activation, these cells increase both the probability of NREM to REM transitions and REM duration (Weber et al., 2015), and their firing activity correlates with REM sleep initiation and maintenance (Weber et al., 2018). Recently, Lu and colleagues showed reduced excitability in these neurons in male *16p11.2* deletion mice with NREM and REM sleep deficits (H.-C. Lu et al., 2019). Given the intrinsic properties of GABAergic LPGi neurons are impacted by *16p11.2* deletion, hypothetically, this cellular population could be paradoxically hyperexcitable in *16p11.2*<sup>+/-</sup> rats used in this study. Alternatively, activity abnormalities in glutamatergic SLD populations, which project to and activate LPGi inhibitory cells (Luppi et al., 2011) may produce similar abnormal REM sleep initiation phenotypes such as those observed in *16p11.2*<sup>+/-</sup> rats here. With the above in mind, it is worth noting that the mechanisms governing transitions between different states of vigilance and sleep remain poorly understood and observed REM sleep increases in *16p11.2*<sup>+/-</sup> animals are likely a consequence of irregularities spanning multiple regions and circuits.

Imaging studies have uncovered significant structural and connectivity changes in patients with *16p11.2* deletion CNVs (Chung et al., 2021; Owen et al., 2014; Qureshi et al., 2014). Children with *16p11.2* deletions display extensive white matter abnormalities, particularly in the corpus callosum and the external and internal capsules. The internal capsule contains long range fibres, which are crucial for motor control and connectivity between the cortex, striatum and spinal cord (Owen et al., 2014). Analogous male-specific abnormalities in white matter projection tracts are also observed near the striatum in *16p11.2* deletion mice (V. J. Kumar et al., 2018) and such structural abnormalities could underly the unusual sleep phenotype in male *16p11.2*<sup>+/-</sup> rats used here. There is abundant evidence for brainstem and cortico-striatal circuit involvement in REM sleep behaviour disorder (RBD), which is characterized by REM muscle tone increases, abnormal dreams and altered limb movements (H.-J. Zhang et al., 2021). RBD is frequently associated with neurodegenerative diseases (H.-J. Zhang et al., 2021), however, dedicated polysomnography studies have shown that RBD is also diagnosed in a proportion of individuals with ASD, ADHD and epilepsy (Shukla et al., 2020; Thirumalai et al., 2002). Cortico-striatal circuits appear to be heavily impacted by *16p11.2* mutations (V. J. Kumar et al., 2018;

Owen et al., 2014), which compromise up to 1% of ASD cases (Chung et al., 2021) and a quarter of these also have epilepsy (Steinman et al., 2016; Zufferey et al., 2012). Considering the above, cortico-striatal network activity may be a contributing factor to the increased REM sleep found in *16p11.2*<sup>+/-</sup> rats. Furthermore, this raises the question whether in addition to increased REM sleep, REM sleep muscle atonia and REM limbic abnormalities might be present in male and female *16p11.2*<sup>+/-</sup> animals used in this study. This warrants further research and could be a means to elucidate whether cortical and striatal circuits are compromised in this rat model.

It is possible that the increased REM sleep observed here is due to an increased homeostatic need for REM in response to potential behavioural or cognitive abnormalities that were not investigated. Alternatively, increased REM sleep could be indicative of poor-quality REM sleep, as *16p11.2*<sup>+/-</sup> rats presented with overall decreased spectral power during this state. Prior studies showed decrements in both REM and NREM sleep alongside reduced theta power in REM sleep and wake (H.-C. Lu et al., 2019), and increased alpha and low delta power during wake (Angelakos et al., 2017; Bian et al., 2023), indicating dysfunction in theta, alpha and delta generating neural circuits in mutant mice. Power analysis in this work, however, identified overall power deficits across multiple frequency ranges in all three vigilance states suggesting broad alterations in multiple brain networks. Finally, I found no circadian phase abnormalities in *16p11.2*<sup>+/-</sup> animals, and increased REM in male *16p11.2*<sup>+/-</sup> rats occurred during the expected light phase of the 24-hour cycle. The lack of REM increases during dark periods implies that the observed REM phenotype may be indirectly tied to specific circadian processes involving photic stimulation pathways and light-dependent dysregulation in related clock gene expression. The SCN receives direct environmental light signals through the retinohypothalamic pathway to synchronises physiological and behavioural responses to the light-dark cycle by interacting with various sleep and wake promoting nuclei (Brown et al., 2012). The timing and intensity of REM sleep is closely linked to the expression of clock genes, including *Per1*, within the dorsomedial SCN (Fisk et al., 2018; M. L. Lee et al., 2009) and data from this study could be indicative of irregular REM related clock gene expression in SCN and abnormalities within the retinohypothalamic tract. Alternatively, the increased REM sleep during the light period might reflect biochemical processes that contribute an abnormal molecular signal for REM sleep need.

In summary, I demonstrate a robust male-specific increase in REM sleep resultant from a higher number of REM sleep bouts in male *16p11.2*<sup>+/-</sup> rats, which aligns with the male-specific effect of *16p11.2* mutations in multiple reports (Angelakos et al., 2017; Grissom et al., 2018; V. J. Kumar et al., 2018; H.-C. Lu et al., 2019; Martin Lorenzo et al., 2023; Openshaw et al., 2023). Interestingly, the data presented here contrast previous work in *16p11.2* deletion male mice that showed decrements in both REM and NREM sleep (Angelakos et al., 2017; H.-C. Lu et al., 2019) and increased frequency of NREM microarousals (Choi et al., 2024), leaving an open-ended question as to whether the latter phenotype might be observed in the rats used in this study. Based off prior evidence, aberrant

monoaminergic signalling, as well as cortico-striatal, hypothalamic, amygdala and brainstem circuit abnormalities likely influence over REM sleep dynamics in *16p11.2*<sup>+/-</sup> animals. While sleep-related network and cellular abnormalities are probable, the finding that REM sleep abnormalities occurred only during the light phase of the 24-hours is highly suggestive of potential irregularities in either molecular signals governing REM sleep need, circadian clock signalling, particularly of *Per1*, as well as the integration of light-dependent cues. The results presented here do not directly phenocopy observed sleep disturbances and difficulty falling asleep in patients with *16p11.2* CNVs (B. A. Fernandez et al., 2010; Hanson et al., 2010; Kamara et al., 2023; Tabet et al., 2012). With the above in mind, sleep is different in *16p11.2*<sup>+/-</sup> rats relative to *16p11.2*<sup>+/+</sup> littermates. Therefore, assessing for abnormal network connectivity and activity, neuronal function and gene expression may enable the discovery of the mechanisms contributing to abnormal sleep and behaviour in NDDs associated with *16p11.2* CNVs. Furthermore, investigating NREM microarousals and the possibility for RBD phenotypes in *16p11.2*<sup>+/-</sup> rats could produce clinically relevant findings. Ultimately, underlying signalling and structural abnormalities discussed above could be shared between the model used here, *16p11.2* deletion mouse models and human patients with these CNVs, which make the *16p11.2*<sup>+/-</sup> rat line a useful model for investigating neurobiological mechanisms mediating sleep abnormalities and sex differences in NDDs.

### 3.4.2. Sleep-wake abnormalities in *Nlgn3*<sup>-/-</sup> rats

ASD and NDDs associated with *NLGN3* mutation are typically observed in males, as the gene is X-linked. Therefore, this thesis assessed sleep physiology in male *Nlgn3*<sup>-/-</sup> rats only. *Nlgn3*<sup>-/-</sup> male rats did not differ in the time spent in REM, NREM and wake compared to wild-type littermates. However, they were found to have more NREM sleep during the light phase of the 24-hour cycle, which was due to an increased average duration of NREM bouts. Longer NREM bouts were also observed during the dark phase in *Nlgn3*<sup>-/-</sup> mutants, however, animals presented with fewer bouts during this period. The increase in NREM sleep during the light period was at the expense of reduced time *Nlgn3*<sup>-/-</sup> rats spent awake. No abnormal REM sleep phenotypes were observed in *Nlgn3*<sup>-/-</sup> animals relative to wild-type littermates, suggesting REM related processes are minimally impacted by knockout of the *Nlgn3* gene.

Sleep dysfunction is commonly observed in individuals with ASD (Cohen et al., 2014), however, it has only been reported in three related male patients with the *NLGN3* R597W variant (Quartier et al., 2019). Studies assessing sleep in *Nlgn3*-R451C knock-in mice conclude this variant contributes minor changes to sleep architecture, as mice present with normal amounts of REM, NREM and wake (Bian et al., 2023; J. J. Liu et al., 2017). However, research in rodents and drosophila

demonstrates that neuroligins have a putative role in sleep regulation (O’Callaghan et al., 2017). Subtly altered REM sleep physiology was demonstrated in mice with the R451C variant and consisted of fewer REM bouts of slightly longer duration, and a normal amount of REM overall (Bian et al., 2023). In a separate study these mice presented with decreased power of low frequency (< 10 Hz) activity during NREM sleep but had normal sleep architecture (J. J. Liu et al., 2017). Both R451C and R597W mutations impair *NLGN3* migration and localisation to the plasma membrane, with protein retained in the endoplasmic reticulum triggering the cellular UPR pathway (Quartier et al., 2019). As in the mouse models discussed above, *Nlgn3*<sup>-/-</sup> rats did not present with abnormalities in sleep, although slight differences were noted in NREM dynamics, particularly during the light phase. This suggests that the *NLGN3* adhesion protein contributes to the mediation and expression of sleep-wake activity and circadian processes (El Helou et al., 2013; Seok et al., 2018). This notion is further supported by decreased time in NREM and increase time spent in REM sleep observed by Thomas et al., in the same *Nlgn3* knockout rat model used here (Thomas et al., 2017), as well as the findings showing reduced sleep in mice with conditional *Nlgn3* knockout restricted to the medial septum (MS) (Sun et al., 2024).

Glutamate modifies synaptic transmission and network connectivity through NMDAR and AMPAR activation (Hansen et al., 2021). The balance between these receptors influences neuronal firing synchrony, with high NMDAR activity correlating to desynchrony in active wake, while lower NMDAR activity is associated with NREM sleep-like synchrony (Vyazovskiy et al., 2009). Sleep deprivation decreases NMDAR function and plasticity, leading to intense sleep (Aton et al., 2009; C. Chen et al., 2006; Kopp et al., 2006). It's believed that prolonged wakefulness desensitizes NMDARs, shifting signalling towards AMPAR-mediated transmission. During NREM sleep, reduced glutamate transmission may restore NMDAR function, gradually decreasing sleep intensity. Thus, elements modulating NMDA and AMPA receptor function are highly relevant for sleep and circadian regulating processes (El Helou et al., 2013). ASD has repeatedly been linked to mutations in the NRXN-NLGN system (Südhof, 2008), and research has found that proper AMPAR and NMDAR function requires both the presence of NRXNs and NLGNs (Barrow et al., 2009; Chubykin et al., 2007). Recently, El Helou and colleagues showed that *Nlgn1* critically modulates waking duration and waking quality, with abnormalities in sleep initiation and homeostasis found in mice with complete *Nlgn1* knockout. They observed that these animals had decreased wake and increased NREM sleep during the 12-hour dark period, due to an inability to maintain wakefulness. The authors further noted that *Nlgn1* knockout mice spent more time awake towards the end of the dark period, when wild-type mice usually slept (El Helou et al., 2013). This hourly time course finding during the latter portion of the dark phase is consistent with the observation I made in *Nlgn3*<sup>-/-</sup> rats, which also had less sleep during the final third of the dark cycle. Furthermore, *Nlgn3*<sup>-/-</sup> animals had significantly more NREM sleep during the light phase of the 24-hour cycle suggesting the regulatory roles of *NLGN3* on sleep and circadian dynamics are alike those of *NLGN1*, although to a lesser extent as evidenced by the lack of NREM differences across the

full 24-hour day. Mechanistically, NLGN1, which is predominantly found at excitatory synapses, tunes NMDAR function and is required for the maintenance of NMDAR-mediated synaptic transmission (Barrow et al., 2009). Similarly, NLGN3 modulates NMDAR- dependent activity in hippocampal PV interneurons (W. Cao et al., 2018; Polepalli et al., 2017), but is also heavily involved in the regulation of AMPAR transmission via controlling AMPAR organisation at postsynaptic sites (Chanda et al., 2017; Etherton et al., 2011; Han et al., 2022; Hosie et al., 2018). Therefore, irregular, and especially decreased NMDAR and/ or AMPAR activity, which likely differs by region and cell type, could represent an underlying mechanism for the longer NREM bout duration in *Nlgn3*<sup>-/-</sup> animals. Notably, the sleep phenotype in *Nlgn3*<sup>-/-</sup> rats used here is mild and could be due to compensatory expression of NLGN1 and NLGN2, which may reduce potentially distorted AMPAR or NMDAR transmission, as shown in mouse hippocampal and cortical neuronal cultures (Chanda et al., 2017), thus positively impacting on sleep impairment severity.

*Nlgn1* and *Nlgn2* contain several E-boxes, which are cis-regulatory elements that allow for transcriptional control of the gene by transcription factors CLOCK and BMAL1 (El Helou et al., 2013; Hannou et al., 2018). Indeed, CLOCK and BMAL1 regulate the expression of both *Nlgns* (El Helou et al., 2013; Hannou et al., 2018). This demonstrates that *Nlgn1* and *Nlgn2* are under strong circadian control, which through their expression, modulates daily fluctuations in synaptic plasticity and neuronal excitability that are essential for normal sleep-wake physiology. Expression of *Nlgn2* is elevated under high sleep need conditions (Hannou et al., 2018), and likely serves to reinforce the strong GABAergic tone, particularly in sleep-promoting VLPO and MnPO nuclei that are fundamental to sleep induction and maintenance. In contrast, increased sleep pressure modulates synaptic *Nlgn1* levels in a brain area specific manner (El Helou et al., 2013). The presence of E-box sequences was only recently shown in *Nlgns1* and 2, and given the association of ASD and cooccurring sleep dysfunction related to other *NLGN* mutations, it is possible that core clock genes may also regulate the expression patterns of *Nlgn3*. However, circadian modulation of *Nlgn3* has not yet been confirmed. If indeed this is the case, it offers insights into the molecular regulation of neuronal signalling critical for sleep processes, which is relevant to the understanding of sleep-wake abnormalities in *Nlgn3*<sup>-/-</sup> animals in this research. This is especially probable given the importance of NLGN3 in regulating NMDAR and AMPAR activity, meaning that sleep reduction of NMDAR and AMPAR- dependent transmission could be achieved via CLOCK and BMAL1 transcriptional control of *Nlgn3*. Circadian signalling mechanisms could therefore present a viable therapeutic target for the treatment of sleep impairment in associated NDDs. It will be important for future research to address if *Nlgn3* is under circadian modulation and in which brain areas this control is most necessary with respect to sleep physiology.

NLGN3 is expressed at inhibitory and excitatory cells, and thus is a modulator of both forms of signalling. Furthermore, preferential cell-type and region-specific expression of the gene is demonstrated by several studies (Földy et al., 2013; Rothwell et al., 2014). For example, NLGN3

regulates inhibitory synaptic transmission in hippocampal CCK basket cells, but not in PV interneurons (Földy et al., 2013). GABAergic transmission is fundamental to sleep regulation, and increased time in wake and significantly reduced REM was observed in mice with complete knockdown of *Nlgn2*, which is preferentially localises at inhibitory synapses (Seok et al., 2018). *Nlgn2* knockout mice also presented with reduced NREM sleep restricted to the 12-hour dark period (Seok et al., 2018), and I likewise found a reduction of nighttime NREM bouts in *Nlgn3<sup>-/-</sup>* animals, suggesting that NLGN3 and NLGN2 may contribute in a similar manner to the control of NREM-related signalling. Notably, epileptic activity consistent with SWDs was also observed in *Nlgn2* knockout mice (F. Cao et al., 2020; Seok et al., 2018), presenting a confounding element in bridging findings between the two studies. Excitatory and inhibitory signalling, in various brain regions and circuits is likely impacted on differentially in *Nlgn3<sup>-/-</sup>* animals, leading to the sleep phenotypes observed. Reduced sleep and social memory deficits were observed in mice with conditional *Nlgn3* knockout in the MS. Specifically, an increase in the firing rate of MS GABAergic neurons, which project to the preoptic area and hippocampal CA2 region, which regulate sleep and memory respectively, was observed in *Nlgn3* MS-specific knockout animals. Authors were able to rescue sleep and memory impairment by inactivating this neuronal population, thus showing that NLGN3 regulates the synaptic partnering and transmission of MS inhibitory cells, which can modulate sleep via their divergent downstream targets (Sun et al., 2024). Similar circuit mechanisms could be associated with the sleep abnormalities observed in *Nlgn3<sup>-/-</sup>* rats in this study. The PAG is involved in arousal regulation, with a small PAG dopaminergic population observed to be wake promoting, particularly in response to salient environmental stimuli. Endogenous and optogenetic activation of these cells leads to waking from sleep in mice, while lesioning results in profound hypersomnia (J. R. Cho et al., 2017; J. Lu, Jhou, et al., 2006). PAG neurons were recently shown to be intrinsically hyperexcitable in *Nlgn3* knockout rats (Anstey et al., 2022), and this may underpin the increased amount of wakefulness found in *Nlgn3<sup>-/-</sup>* animals during the final hours of the 12-hour dark cycle. *Nlgn3* knockout mice exhibited imbalance between synaptic inhibition and excitation in nucleus accumbens and the ventral striatum region (Rothwell et al., 2014), which upon activation promote NREM sleep by increasing the number and duration of NREM episodes (Oishi et al., 2017). Recently, the loss of NLGN3 was shown to result in cell-type-specific reduction of synaptic inhibition (via reduced frequency of mIPSC) onto dopamine D1- expressing medial spiny neurons of the nucleus accumbens (Rothwell et al., 2014). It may be that decreased inhibitory tone onto these neurons increases their excitatory output, and thus leads to augmented NREM sleep, as observed in *Nlgn3<sup>-/-</sup>* rats in the daytime hours.

The increase in NREM sleep during the light phase in *Nlgn3<sup>-/-</sup>* rats, particularly the first half of which, could alternatively be due to homeostatic sleep drive. This is plausible because *Nlgn3<sup>-/-</sup>* males also spent less time in NREM sleep, and more time awake towards the end of the 12-hour dark period, which further indicates that increased wake activity during the active dark cycle may contribute to

increased sleep need and result in the sleep-wake distribution found in *Nlgn3*<sup>-/-</sup> animals here. *Nlgn3* knockout rats were previously shown to be hyperactive in an open field activity paradigm, which measures exploratory activity in novel environments. Importantly, this hyperactivity phenotype was predominantly expressed during the dark phase of the 24-hours (Thomas et al., 2017), supporting the likelihood that increased NREM sleep during the light phase emerges from a homeostatic sleep need in response to excessive nighttime activity in *Nlgn3*<sup>-/-</sup> rats. Furthermore, the authors also describe increased theta, beta and gamma power during wake in *Nlgn3* knockout rats, while I observed overall higher power across frequencies in *Nlgn3*<sup>-/-</sup> animals. An EEG hallmark found in children with ADHD is relatively greater spectral power, particularly in the theta range (Robertson et al., 2019), and findings from this and prior research (Thomas et al., 2017) support the idea that *Nlgn3*<sup>-/-</sup> rats are hyperactive, especially at night. Finally, sleep deprivation experiments have demonstrated that augmented NREM delta power is characteristic of increased NREM sleep need (B. Kim et al., 2020), however, power analysis during NREM found no differences in *Nlgn3*<sup>-/-</sup> animals during this sleep state. The observed increase in NREM sleep duration without concomitant changes in spectral power may indicate that the adaptive alteration in sleep regulation mechanisms in *Nlgn3*<sup>-/-</sup> rats potentially favour temporal extension of sleep over augmentation of sleep intensity.

*Nlgn3*<sup>-/-</sup> rats presented with increased NREM sleep, particularly during the light hours of the day due to an increased duration of NREM bouts. The mechanisms underlying this sleep phenotype are likely multifaceted. First, prior work demonstrates *Nlgn3* knockout leads to signalling abnormalities in striatal, forebrain and midbrain structures associated with sleep-wake dynamics (Anstey et al., 2022; J. R. Cho et al., 2017; Földy et al., 2013; Oishi et al., 2017; Rothwell et al., 2014; Sun et al., 2024). Increased sleep during nighttime hours and increased wake in daytime hours could be a consequence of altered network activity of one or multiple such regions. Alternatively, circuit irregularities increase wakefulness in *Nlgn3*<sup>-/-</sup> animals and potentially contribute hyperactivity, which is observed in this rat model (Thomas et al., 2017), but was not specifically assessed in this thesis. Under these circumstances the increased NREM sleep in *Nlgn3*<sup>-/-</sup> rats, during the light phase could constitute a homeostatic response to elevated sleep need. Second, normal NMDAR and AMPAR expression in response to wake activity may be impaired in the absence NLGN3, thus impacting on sleep-wake activity (W. Cao et al., 2018; Chanda et al., 2017; Etherton et al., 2011; Han et al., 2022; Hosie et al., 2018; Polepalli et al., 2017). And third, like with other *Nlgn* genes, *Nlgn3*-mediated excitatory and inhibitory transmission may be under circadian CLOCK and BMAL1 transcriptional regulation (El Helou et al., 2013; Hannou et al., 2018). Therefore, time of day signalling changes, buildup of sleep pressure and associated homeostatic sleep responses may be altered in *Nlgn3*<sup>-/-</sup> rats, which do not express this protein. Finally, NLGN3 localises to glutamatergic and GABAergic synapses and the lack of severe sleep decrements or circadian abnormalities suggests compensatory *Nlgn1* and *Nlgn2* mechanisms may be active.

Taken together, findings from this study add to the existing literature demonstrating sleep dysfunction associated with *Nlgn3* mutations (Sun et al., 2024; Thomas et al., 2017). Several key aspects relating to the role of *NLGN3* in sleep regulation remain to be investigated. Namely, whether *NLGN3* contains E-box sequences and is under circadian control, and if *NLGN3* expression is altered in response to sleep pressure, whether cellular activity and circuit mechanisms mediating NREM sleep initiation and maintenance (e.g. GABAergic neurons VLPO and MnPO) are affected by *NLGN3* mutations, and finally, if upregulation of other *NLGNs* can be observed and compensates when there is lack of *NLGN3* expression. Such studies will provide deeper insights into the complex interplay between neuroligins, sleep regulation, and the broader implications of *NLGN3* mutations on sleep-wake cycles.

### 3.4.3. REM sleep deficits and altered sleep-wake physiology in *Grin2b*<sup>+/-</sup> rats

I identified clinically relevant sleep-wake phenotypic abnormalities in a novel model of *GRIN2B* haploinsufficiency. *Grin2b*<sup>+/-</sup> animals had a 50 % reduction in *Grin2b* expression compared to wild-type levels both in the somatosensory cortex and the hippocampus in adult rats. Dysfunctional sleep is commonly observed in children and adults with neurodevelopmental disorders and severe sleep fragmentation and difficulties falling asleep have been reported in individuals with pathological *GRIN2B* variants (Freunscht et al., 2013; Platzer et al., 2017; Simons Searchlight, 2021). REM, NREM and wake brain states were all significantly altered in *Grin2b*<sup>+/-</sup> rats. Despite a slight increase in the average duration of individual REM epochs, there was a striking reduction in total REM minutes over the 24-hour cycle. The reduced net REM was underpinned by a lower occurrence of REM bouts in *Grin2b* heterozygous knockouts and may indicate altered REM initiation mechanisms. Total NREM minutes were unchanged over 24 hours but *Grin2b*<sup>+/-</sup> rats had fewer NREM bouts that were longer in duration. Wake total minutes over 24 hours were also slightly decreased in mutants, which had an increased number of wake bouts, that were shorter in their average duration. Spectral power was increased in *Grin2b*<sup>+/-</sup> animals during NREM sleep and was comparable to that of wild-type littermates during wake and REM sleep. Finally, as *GRIN2B* mutations affected both sexes, analyses for any sex-specific REM deficit and sleep physiology difference between male and female *Grin2b*<sup>+/-</sup> animals and wild-type littermate were conducted. No differences were found between male and female mutant rats, which presented with consistent sleep-wake abnormalities and reduced REM sleep.

Sleep modulation and diurnal rhythmicity are in part driven by NMDAR-dependent processes. In rats, pharmacological block of NMDAR transmission results in sleep deficits, with REM sleep being particularly sensitive to receptor antagonism (Stone et al., 1992). Pharmacological NMDAR activation on the other hand has been shown to strongly modulate light-dark locomotor and social behaviour transitions in relation to sleep-wake physiology (Burgdorf et al., 2019). Furthermore, NMDAR subunit

composition confers functional differences on network activity in a vigilance state-dependent manner. Antagonism of GluN2A-NMDARs suppresses sleep and produces high-power gamma oscillations (Campbell & Feinberg, 1999; Kocsis, 2012), while blocking GluN2B containing receptors only produces region specific alterations to REM sleep gamma rhythms (Kocsis, 2012). Thus, REM sleep is particularly reliant on NMDAR signalling and GluN2B subunits have a role in modulating the expression of REM sleep oscillatory activity. Although REM sleep physiology was not further studied in the above literature, it is possible that GluN2B-NMDAR antagonism could elicit altered REM sleep dynamics, such as the deficits observed in *Grin2b* haploinsufficient rats in this study. In rats, NMDAR antagonists reduce sleep while agonists enhance sleep (Burgdorf et al., 2019). Therefore, given the widespread distribution of GluN2B-NMDARs across various forebrain structures, the observed sleep disturbances in *Grin2b*<sup>+/-</sup> rats may result from disruptions in multiple neural circuits.

This is conceivable as the GluN2B subunit is the most abundantly expressed NMDAR subunit throughout the adult forebrain (Wyllie et al., 2013a). GluN2B containing NMDARs are critically expressed in the SCN and receive inputs from the retinohypothalamic tract neurons in response to light. These GluN2B-NMDARs, through eliciting long calcium transients when activated, have a crucial role in bridging membrane events to changes in gene expression relevant to light-induced phase shifts of the circadian system (L. M. Wang et al., 2008). Calcium influx via NMDARs is hypothesized to be a component of the sleep homeostatic mechanism that monitors waking duration. This process is thought to contribute to the accumulation of sleep pressure during periods of wakefulness, potentially serving as a molecular correlate of time spent awake (S. Liu et al., 2016; Z. Wang et al., 2018). The exact contribution of GluN2B-NMDARs to REM and NREM sleep distribution is unknown. However, given that the subunit is involved in facilitating specific sleep oscillations and is an important component of NMDAR-mediated responses within SCN neurons contributing to circadian regulation of sleep, it is possible that molecular signalling in SCN may be altered in *Grin2b*<sup>+/-</sup> animals. More specifically, NREM sleep onset and maintenance, depend on circadian cues (Easton et al., 2004), and dysregulated activity of GluN2B-NMDAR transmission and receptor mediated molecular pathways within the SCN may in part contribute to the alteration of NREM sleep dynamics in *Grin2b*<sup>+/-</sup> rats. Indeed, increased SCN neuronal excitability in response to light was shown in mice with ASD associated *Shank3* heterozygous deletion. These animals exhibited increased light sensitivity at the cellular level resulting in disorganised circadian phase shifts (Alamilla et al., 2021). In addition, abnormal SCN signalling was shown in mice modelling Alzheimer's disease, which also presented with disrupted circadian rhythms (Paul et al., 2018). Similar mechanisms in SCN could also reflect the deficits in REM sleep observed in mutant rats, as the timing and intensity of REM sleep is correlated to clock gene expression (M. L. Lee et al., 2009).

Beside the extra-thalamic circuits regulating NREM sleep discussed above, NREM sleep oscillations and transitions between wake and NREM sleep are modulated by the firing activity of

neurons of thalamocortical circuits and local thalamic networks integrating the nRT and thalamocortical neurons (Herrera et al., 2016; Mátyás et al., 2018; McCormick & Bal, 1997). The thalamocortical circuits also contribute to the appearance of NREM sleep spindles (L. M. J. Fernandez & Lüthi, 2020; Huguenard & Prince, 1994; McCormick & Bal, 1997). During sleep this network involves complex interactions between several key areas and cell types. In the thalamus the nRT, which is primarily comprised of GABAergic neurons, acts as a pacemaker for sleep spindles and modulates thalamic relay neuron activity to generate and propagate slow oscillations across the cortex. At NREM sleep transitions, reduced neuromodulator release in the thalamus leads to hyperpolarization of both nRT and thalamocortical neurons. This hyperpolarization selectively deinactivates low-threshold T-type calcium channels, which facilitate the characteristic bursting activity of thalamic neurons observed in NREM sleep (McCormick & Bal, 1997). Furthermore, highly collateralized thalamic neurons, particularly in the medial thalamus, serve as key hubs for orchestrating graded state transitions between arousal levels by modulating widespread forebrain activity. These thalamic neurons transmit arousal-predicting activity patterns through extensive axonal projections, allowing them to coordinate the initiation and maintenance of NREM sleep by influencing both cortical and subcortical structures involved in sleep-wake regulation (Mátyás et al., 2018). A recently published preprint investigating the input specific localisation of GluN2 subunits observed that GluN2B was the most abundant postsynaptic NMDAR subunit localised to a large proportion of thalamocortical synapses receiving inputs from layer VI cortical neurons (Topolski et al., 2024). In addition, NMDARs containing GluN2B were previously found to be the dominant postsynaptic receptor subtype at thalamoreticular synapses facilitating local thalamic transmission (Astori & Lüthi, 2013). Therefore, thalamocortical network function is reliant on GluN2B-NMDAR activity, which modulates circuit signalling in an input and cell-type specific manner. As such, disruption to thalamocortical signalling due to insufficient GluN2B expression within relevant synapse could be a relevant mechanism underlying the irregular NREM sleep phenotype observed in *Grin2b*<sup>+/-</sup> animals in this thesis. This is an interesting hypothesis to further investigate and is further supported by findings from other studies, which have shown that abnormal thalamocortical circuit transmission impairs sleep processes. For example, reduced nRT firing contributes to sleep fragmentation, impaired sleep homeostasis and deficits in spindle activity in a mouse model of schizophrenia (Czekus et al., 2022), while hyper and abnormal thalamocortical connectivity is observed in patients with ASD (Ayub et al., 2021; Iidaka et al., 2019; Nair et al., 2013), which often have dysfunctional sleep (Cohen et al., 2014).

Recently, NMDARs in the VLPO of hypothalamus were shown to be essential for NREM and REM sleep, demonstrating the highest amount of activity during REM (J. Lu et al., 2000, 2002; Miracca et al., 2022). Pan-neuronal knockout of NMDARs in these cells significantly reduced both NREM and REM sleep, while reduced NMDAR expression in GABAergic populations produced severe NREM fragmentation (Kroeger et al., 2018; Miracca et al., 2022). These observations are consistent with the

decreased time in REM sleep in *Grin2b*<sup>+/-</sup> rats. As such, it is possible that NMDAR transmission in the VLPO may be disrupted in these animals. Indeed, it has been suggested that tonic neuronal excitation reliant on extrasynaptic NMDARs (Sah et al., 1989), which are predominantly composed of GluN2B subunits (Tovar & Westbrook, 1999), serves to stabilize hypothalamic sleep-promoting cells in their firing activity (Miracca et al., 2022). Thus theoretically, signalling mediated by GluN2B containing NMDARs could be disrupted in VLPO of *Grin2b*<sup>+/-</sup> rats, although this idea assumes GluN2B expression in this sleep promoting structure. GluN2B subunits are expressed in hypothalamic regions (L. M. Wang et al., 2008), however, subunit expression patterns specific to VLPO have not yet been examined. The VLPO is a crucial region governing sleep-wake physiology and is highly dependent on NMDAR signalling, making it an important region for further investigation in *Grin2b*<sup>+/-</sup> rats. Assessing NMDAR subunit contributions to signalling of inhibitory and excitatory cellular populations in VLPO, as well as studying the intrinsic properties of neurons in this structure will be important for future sleep research in the animal model.

The calcium- dependent hyperpolarization pathway is a proposed mechanism that links neuronal activity, calcium signalling, and sleep regulation. The model states that neuronal activation during wake leads to accumulated influx of calcium in cells, which initiates hyperpolarizing mechanisms including calcium and potassium-activated channels that rapidly hyperpolarizes neurons. Inhibiting calcium entry into the cell therefore results in induced neuronal excitability through the inhibition of the hyperpolarization pathway. This pathway is an important mediator of NREM slow wave oscillations (S. Shi & Ueda, 2018; Tatsuki et al., 2016; Yamada & Ueda, 2020; K. Yoshida et al., 2018). A recent computational model, validated in mice, demonstrated this pathway is involved in sleep induction and maintenance during the light phase, and notably, is dependent on cortical layer IV to VI GluN2B containing NMDARs. Specifically, NMDAR antagonism increased cortical excitation, particularly of excitatory neurons, with all active cells expressing high levels of the GluN2B subunit (Tatsuki et al., 2016). These findings suggests that the NREM sleep phenotypes observed in *Grin2b*<sup>+/-</sup> rats could be a consequence of altered cortical excitability and deficits in the calcium-dependent hyperpolarization mechanisms. Indeed, the spatial and cell-type specific expression of postsynaptic NMDARs containing GluN2B subunits is an important factor for normal network activities responsible for various processes including sleep (Krone et al., 2021). For example, GluN2B-NMDARs are selectively found in layer V local intracortical synapses and facilitate intracortical integration of synaptic inputs from thalamocortical and subcortical connections. Neurons in layer V are essential for the initiation and propagation of NREM cortical slow waves (Krone et al., 2021) and are the primary means by which the neocortex communicates with the rest of the brain (S. S. Kumar & Huguenard, 2003). Furthermore, cellular hyperpolarizing calcium influx is also associated with activation of extrasynaptic NMDARs (Isaacson & Murphy, 2001), which are predominantly GluN2B expressing (Tovar & Westbrook, 1999). Taken together, the above findings imply that cortical GluN2B-NMDARs

are functionally important for sleep initiation and maintenance, as well as the regulation of sleep homeostasis. This could be a relevant mechanism to study in relation to the NREM sleep distribution observed in *Grin2b*<sup>+/-</sup> animals here.

Impairment in molecular signalling pathways leads to sleep dysfunction. GluN2B is the dominant NMDAR subunit mediating ERK1/2 activity, which is known to regulate sleep-wake distributions and NREM sleep duration (Mikhail et al., 2017). As previously mentioned ERK/MAPK plays an important role in the entrainment of circadian rhythms and phosphorylates core clock genes and clock gene regulators (Coogan & Piggins, 2003; El Gaamouch et al., 2012; Hirayama et al., 2005; Karin, 1995; Tao et al., 2015; X.-L. Wang et al., 2020; Yap & Greenberg, 2018). These pathways are affected in *Syngap1*, *Nlgn3*, *Fmr1* and *Scn2a* deficient mice and rats (Gkogkas et al., 2014; Hörnberg et al., 2020; Komiyama et al., 2002; Z. Ma et al., 2022), which also present with sleep impairments (Boone et al., 2018; Buller-Peralta et al., 2022; Z. Ma et al., 2022; Thomas et al., 2017). Furthermore, it was recently shown that NMDAR mediated sleep drive in part requires the phosphorylation of GluN2B subunits (Z. Wang et al., 2018). Thus, inadequate GluN2B-NMDAR-dependent molecular signalling could in part contribute to the sleep alterations observed in *Grin2b*<sup>+/-</sup> mutants, although future research is required to evidence this.

REM and NREM sleep are dependent on homeostatic sleep drive, with the expression of REM sleep also being modulated by NREM sleep dynamics (Franken & Dijk, 2024). I found no differences in the overall amount of time *Grin2b*<sup>+/-</sup> rats spent in NREM sleep across the 24-hour day, indicating that although NREM sleep dynamics differed between the two genotypes, the homeostatic system responses governing the amount of NREM sleep are likely functionally intact or compensated for in these animals. In contrast, REM sleep was decreased in *Grin2b*<sup>+/-</sup> rats. An interesting finding is that the probability of NREM to REM sleep transitions is reduced particularly after long REM sleep periods (>1 min) in rats (Bassi et al., 2009). REM bout duration was statistically longer in *Grin2b*<sup>+/-</sup> rats, with an average length of approximately 65-70 seconds. Therefore, the increased length of REM sleep bouts, which may be attributed to a homeostatic response to reduced REM sleep bouts, could paradoxically also impair the propensity and sleep drive for future REM initiation. Sleep homeostatic mechanisms were not the focus of the present study, however, it would be interesting to further investigate them in the *Grin2b*<sup>+/-</sup> model. Specifically, experiments utilising sleep deprivation and phase shift paradigms would allow for determination of the role of *Grin2b* in regulating sleep and circadian processes in response to accumulating sleep pressure.

Inadequate GluN2B-NMDAR cellular and subcellular signalling likely contributes to the altered NREM sleep physiology and decrements to REM sleep in *Grin2b*<sup>+/-</sup> mutants. In addition, consistent sleep abnormalities were observed in both male and female *Grin2b*<sup>+/-</sup> animals, therefore, both sexes are likely similarly impacted by the loss of one *Grin2b* allele. The processes by which sleep is

disturbed likely includes compromised network activity in cortical, thalamocortical and subcortical structures, as well as potential dysregulation in GluN2B-mediated ERK1/2 activation and, abnormal molecular sleep homeostasis signalling cues dependent on GluN2B phosphorylation and increased intracellular calcium levels. REM and NREM sleep initiation and maintenance are dependent on VLPO activity, which is heavily reliant on NMDAR function, thus presenting an interesting structure for future investigation into sleep impairment mechanisms in *Grin2b*<sup>+/-</sup> rats. Furthermore, GluN2B-NMDARs regulate thalamocortical signalling at least to a certain extent, while abnormalities in this network are associated with disrupted sleep. Notably, *Grin2b*<sup>+/-</sup> animals also presented with SWDs, consistent with a seizure phenotype, which is discussed in detail in the following Chapter 4, along with the relationship between SWD and sleep phenotypes observed in *Grin2b*<sup>+/-</sup> mutants. However, the fact that SWDs emerge as a consequence of compromised thalamocortical circuitry further implies that pathological activity in thalamus, cortex and structures projecting to the thalamocortical circuit (e.g. the VLPO) could also underpin the abnormal sleep found in *Grin2b*<sup>+/-</sup> rats. Moreover, SWDs could also account for the reduction in REM sleep through downstream seizure-related processes, such as disruption of regular circadian patterns or neuromodulator release. Future work will need to investigate whether dysregulation in diurnal behavioural patterns is present, and whether and to what extent ERK/MAPK activity and clock gene modulation is affected by GluN2B haploinsufficiency. As GluN2B subunits are highly expressed at cortical thalamic inputs from layer VI to the ventrobasal thalamus and are the dominant NMDAR subunit at thalamoreticular synapses, it will be interesting to determine whether targeted thalamic cells receive sufficient input and if this could mechanistically disrupt sleep and its associated network oscillatory activity. Finally, GluN2B expression is developmentally regulated, and is the dominant subunit expressed throughout the brain during early postnatal development contributing to circuit development (Paoletti et al., 2013; Vieira et al., 2020). Thus, sleep circuitry may be perturbed from the earliest developmental period, which could in turn disrupt developing connectivity, which is reliant on neonatal sleep (Del Rio-Bermudez et al., 2020; Del Rio-Bermudez & Blumberg, 2018). Although circadian regulation of sleep and sleep homeostasis does not begin to emerge until P17 (Frank et al., 2017), after possible NMDAR functional compensation by GluN2A, cellular and network connectivity mediating sleep processes may be functionally compromised due to reduction in *Grin2b* expression during development. Considering the time-dependent expression patterns of GluN2B-NMDARs it will be important to evaluate sleep phenotypes and related circuit formation in neonatal and juvenile *Grin2b*<sup>+/-</sup> rats. Collectively these data suggest a compromised sleep architecture in this model, which is consistent with clinical reports, and to my knowledge, offers the first pre-clinical quantitative evidence of dysfunctional sleep patterns relating to *GRIN2B* haploinsufficiency.

#### 3.4.4. Conclusions and considerations for future work

Sleep problems are among the most common complaints reported amongst individuals with NDDs and often contribute to the worsening of learning, memory and behavioural issues. The data presented here provides evidence for sleep dysfunction in rat models of *16p11.2* deletion syndrome, *Nlgn3* and *Grin2b* related NDDs. Findings from the latter two models align with prior literature documenting sleep impairment in mice and rats with *16p11.2* and *Nlgn3* deletions (Angelakos et al., 2017; Choi et al., 2024; H.-C. Lu et al., 2019; Thomas et al., 2017), while this study offers the first description for altered sleep physiology and deficits specific to heterozygous deletions of *Grin2b*. With this in mind, the specific sleep-wake distributions and spectral power alterations I identified differ from those reported in similar studies, which also demonstrate contrasting finding with each other. As prior sleep studies suggest, factors including species differences, experimental techniques, animal husbandry and housing environments could have contributed to the variability in sleep phenotype results (H.-C. Lu et al., 2019; Thomas et al., 2017). Nevertheless, I observed distinctly different sleep phenotypes in all three rat models used in this research, which were subjected to the same husbandry, experimental protocols and analyses. This implies that *16p11.2*, *Nlgn3* and *Grin2b* deletions differentially impact on brain development and network activity leading to variable sleep physiology in rats. Overall, my findings demonstrate that the *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> rat models display specific sleep-wake alterations and thus will be useful for future investigation into brain dysfunction underlying sleep impairment.

To robustly characterize these sleep phenotypes, this study employed 24-hour polysomnography, the gold standard for sleep assessment, using skull surface EEG. This approach provides continuous, functional data on sleep-wake states in freely behaving animals. The study design allowed for distinguishing undisturbed home-cage sleep from novelty-induced activity, minimizing potential anxiogenic effects, such as those observed in short open-field tests (Dahlhaus & El-Husseini, 2010; Ding et al., 2014; Mineur et al., 2002; Pietropaolo et al., 2011). Moreover, sleep architecture was evaluated using an automated sleep-scoring algorithm, which classified states based on spectral power features, ensuring consistency while reducing human bias and inter-rater variability. Despite these advantages, several limitations should be considered. The automated sleep-scoring algorithm, which segments EEG and EMG data into 5 second epochs, improves reliability but may overlook atypical sleep microstructures or brief arousals relevant to NDDs. Additionally, all sleep and circadian measurements were conducted in adult rats, despite the models representing neurodevelopmental disorders. Evaluating sleep across developmental stages would provide a clearer understanding of when disruptions emerge and how they evolve. Lastly, quantitative EEG band-specific power spectral analysis, performed in this work using the Welch method, provides insight into frequency-dependent changes in sleep regulation. This method is frequently used in rodent and human sleep research

(Angelakos et al., 2017; Choi et al., 2024; J. J. Liu et al., 2017; H.-C. Lu et al., 2019; Tabet et al., 2012; Thomas et al., 2017), and has yielded translatable biomarkers for Angelman syndrome (Ostrowski et al., 2021). A significant caveat with this approach, however, is the loss of information from the continuous signal. Furthermore, segmentation of power into discrete frequency bands is not standardized and band definitions are highly variable across the literature (Newson and Thiagarajan, 2019), which complicates cross-study comparisons. This study would have therefore benefited from complementary methods, such as spectral slope analysis, which captures the aperiodic (1/f) component of the power spectrum, a marker of neural excitability and inhibition. A steeper 1/f slope indicates greater inhibition, while a shallower slope suggests increased excitability. This approach would have provided a more fundamental measure of brain function, reducing dependence on arbitrary band definitions and offering novel insights into sleep and cognition (Donoghue et al., 2020).

The aetiology of dysfunctional sleep in NDDs remains unknown, although it is believed to arise as consequence of pleiotropic effects of genetic mutations on brain development, circuit organisation and cellular and subcellular signalling (Charrier et al., 2017). Therefore, it will be important to investigate the underlying circuit and intracellular mechanisms contributing to altered sleep processes in genetic NDDs. Furthermore, such studies may aid in the identification of convergent signalling abnormalities that contribute to sleep dysfunction in NDDs of varying genetic origins.

The MAPK/ERK cascade is of particular interest as abnormalities in pathway signalling are observed across various NDD rodent models (Gkogkas et al., 2014; Grissom et al., 2018; Hörnberg et al., 2020; Komiyama et al., 2002; Z. Ma et al., 2022), many of which also present with sleep impairments (Angelakos et al., 2017; Boone et al., 2018; Buller-Peralta et al., 2022; H.-C. Lu et al., 2019; Z. Ma et al., 2022; Thomas et al., 2017). The presence of both altered MAPK/ERK signalling and sleep disturbances in these genetically distinct rodent models suggests that dysregulation of this pathway may represent a convergent mechanism underlying various NDDs with sleep abnormalities. Dysregulation in MAPK/ERK activity is observed in *Nlgn3* and *16p11.2* knockout mice (Grissom et al., 2018; Hörnberg et al., 2020; Pucilowska et al., 2012), while GluN2B mediates long lasting phosphorylation of ERK1/2 (El Gaamouch et al., 2012). It will be interesting to determine to what extent MAPK/ERK signalling in *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> rats used in this study may be affected and whether abnormal sleep phenotypes can be rescued via pharmacological modulation of this kinase cascade. If so the MAPK/ERK pathway could represent a unifying target for therapeutic interventions across different genetic NDDs.

Previous studies investigating sleep have employed a range of techniques, including *in vitro* electrophysiology, optogenetic manipulation of sleep-promoting circuits and neuronal subtypes, viral tagging and molecular sequencing (Choi et al., 2024; C. Ma et al., 2019; Z. Ma et al., 2022). For example, combining monosynaptic rabies viral tracing with fluorescence in situ hybridization and

immunohistochemistry, was used to identify a novel thalamo-amygdalar circuit involved in NREM sleep initiation and maintenance (C. Ma et al., 2019). Similar approaches could be used to determine which neuronal populations and regions in the brain related to sleep-wake physiology are most affected, as well as whether connectivity between different regions is altered by *16p11.2*, *Nlgn3* and *Grin2b* deleterious mutations.

For instance, I suggest that higher levels of REM sleep in male *16p11.2*<sup>+/-</sup> rats could be due to increased D2 expression in medium spiny neurons in the striatum. D2 receptor reporter Sprague Dawley rats, which express the fluorescence protein tdTomato under the control of the endogenous D2 receptor promoter, have successfully been used to map out the cellular expression pattern of D2 receptors in adult and postnatal rat forebrain (Q. Yu et al., 2019). Crossing this reporter line with *16p11.2*<sup>+/-</sup> rats, would allow for histological quantification of D2 receptor expression in striatal MSNs. In addition, combining this approach with immunostaining and imaging for D1 receptor expressing medium spiny neurons could establish changes in D1/ D2 expression ratio of MSNs, which is critical for goal directed behaviours and sleep-wake regulation (Dong et al., 2022; Grissom et al., 2018; Kato et al. 2023). As connectivity in sleep associated nuclei is altered in *16p11.2*<sup>+/-</sup> rat and mouse models (Choi et al., 2024; Portland et al., 2014), and D2 receptor activation in the striatum, may promote sleep (Vetrivelan et al., 2010), a further experiment could employ mono-synaptically restricted rabies virus tracing to map and compare presynaptic inputs to striatum. Here, *16p11.2*<sup>+/-</sup> animals could be crossed with a D2-Cre transgenic line (e.g. LE-Tg(Drd2-iCre)10Ttc rats; NIDA transgenic rat project; Rat Resource and Research Center (RRRC), Columbia, MO) that would allow for viral tracing restricted to D2 expressing medium spiny neurons, and therefore the identification and quantification of synaptic inputs to medium spiny neurons from various sleep or wake related structures (Choi et al., 2024). This combined approach would provide crucial insights into whether dopaminergic signalling and striatal connectivity may be altered in *16p11.2*<sup>+/-</sup> rats to contribute the sleep-wake disturbances observed. It would also offer the foundations for further investigating striatal dopaminergic activity in relation the increased REM sleep phenotype in these animals. Finally, given the abundant variety of molecular tools and transgenic lines, these experiments could potentially be tailored investigate alternate or parallel mechanisms contributing to abnormal sleep, such as serotonergic transmission or aberrant REM sleep related connectivity patterns.

Homeostatic sleep need and sleep recovery processes are reliant on the expression of various genes, amongst which are those encoding Nlgn adhesion proteins and NMDAR subunits (El Helou et al., 2013; Tatsuki et al., 2016). For instance, *Nlgn1* knockout mice subjected to sleep deprivation display exacerbated delta activity rebound, indicative of accelerated accumulation of sleep pressure in the absence of NLGN1 protein (El Helou et al., 2013). Sleep deprivation polysomnography experiments coupled with RNA sequencing analyses could be useful in determining the role of *Nlgn3*, *Grin2b* and genes found on the *16p11.2* locus in sleep regulation (Z. Wang et al., 2018). These experiments could

reveal alterations in markers of sleep need and the expression of genes critically involved in circadian entrainment pathways. In this chapter, I propose that *Nlgn3*, similarly to *Nlgn1* and *Nlgn2* (El Helou et al., 2013; Hannou et al., 2018), might contain E-box motifs, allowing circadian clock proteins to regulate *Nlgn3* transcription and, consequently, synaptic dynamics relevant to sleep-wake physiology. Chromatin immunoprecipitation, a technique used to investigate protein-DNA interactions, could be employed to confirm CLOCK/ BMAL1 binding at E-box sequences in the *Nlgn3* promoter (Mongrain et al., 2011). This would establish a molecular link between circadian regulation of *Nlgn3* expression and the sleep phenotype observed in *Nlgn3*<sup>-/-</sup> rats, potentially driven by altered transcriptional control over synaptic AMPAR and NMDAR expression. The above coupled with sleep deprivation polysomnography experiments and spectral analysis would further inform on the role of *Nlgn3* in the homeostatic regulation of sleep need (El Helou et al., 2013).

Taken together, such studies may produce valuable insights relating to the pathophysiology underlying sleep dysfunction in patients with NDDs and allow for personalised therapeutic approaches in their treatment.



# Chapter 4

## Absence Seizures in a Novel Rat Model of GRIN2B Related Neurodevelopmental Disorder and their Association with the Sleep-Wake Cycle

### 4.1. Introduction

*GRIN2B* pathogenic variants cause multiple neurodevelopmental clinical phenotypes such as epilepsy, ASD, ID, sleep impairments and movement abnormalities (Endele et al., 2010; Epi4K Consortium., 2013; Freunscht et al., 2013; O’Roak et al., 2011; Platzer et al., 2017). An estimated 52% of patients with *GRIN2B* related NDD present with severe epilepsy, which is refractory to therapy in half of diagnosed cases (Platzer et al., 2017). A variety of seizure types occur in patients including infantile spasms and generalized tonic-clonic, focal, and absence seizures (Epi4K Consortium., 2013; Platzer et al., 2017). *De novo GRIN2B* variants are one of the primary monogenic causes of epileptic encephalopathies, in which epilepsy is thought to worsen neurodevelopmental comorbidities (Epi4K Consortium., 2013; Lemke et al., 2014; Platzer et al., 2017; Scheffer et al., 2017; Smigiel et al., 2016). The predicted incidence of *GRIN2B* pathogenic mutations is 5.91 per 100.000 births (Lemke, 2020), making it the most prevalent disorder associated with NMDAR coding *GRIN* genes.

NMDARs are sodium, potassium and calcium permeable tetrameric ligand-gated ion channels, which play a critical role in glutamatergic transmission, synaptic plasticity and the development of the nervous system (Paoletti et al., 2013; Traynelis et al., 2010b). *GRIN2B* encodes GluN2B, which is highly expressed in the prenatal and early postnatal periods (Paoletti et al., 2013; Traynelis et al., 2010b). Later in development the expression of GluN2A, which is encoded by *GRIN2A*, is increased, while GluN2B-NMDARs become restricted to forebrain regions. GluN2B containing receptors have a lower open probability, slower deactivation kinetics and an increased sensitivity to glutamate and glycine compared to GluN2A containing receptors, which are important properties mediating circuit

formation, plasticity and the regulation of intracellular calcium dependent signalling (Paoletti et al., 2013; Wyllie et al., 2013b). In the context of neurodevelopmental disorders, rodent *in vitro* data demonstrates that pathogenic *GRIN2B* variants alter NMDAR function (Kellner et al., 2021), neuronal differentiation and migration (Bell et al., 2018; H. Jiang et al., 2015), dendrite morphogenesis (Bahry et al., 2021; Sceniak et al., 2019b) and synaptic density (S. Liu et al., 2017b; Sceniak et al., 2019b). Given the spatiotemporal expression profile of the gene, such abnormalities are expected to disrupt circuit connectivity and alter excitatory and inhibitory transmission in the brain to variable extents across regions, ultimately contributing to aberrant network properties that manifest in *GRIN2B* related NDD.

Epileptic activity in *GRIN2B* related NDDs has been challenging to model effectively, especially as complete loss of GluN2B-NMDARs is perinatally lethal. Adult rats carrying the *Grin2b*-Trp373 loss of function variant have a reduced threshold to pentylenetetrazol induced seizures, however, do not present with spontaneous epileptic phenotypes (X. Wang et al., 2022). Severe seizures were reported in P14-21 mice lacking GluN2B specifically in inhibitory neurons, yet these seizures were lethal (Kelsch et al., 2014). Therefore, the lack of a rodent model presenting with a robust epileptic phenotype has made researching how mutations in *GRIN2B* alter brain connectivity and signalling difficult. Given that approximately half of individuals with *GRIN2B* variants have epilepsy that is often severe (Platzer et al., 2017), a model which replicates spontaneous epileptic activity, as a consequence of abnormal *GRIN2B* function and expression, is crucial for elucidating the underlying mechanisms and potentially informing the development of targeted therapeutic strategies to improve patient outcomes.

Here, I test the role of *Grin2b* on brain network function, specifically looking for the presence of epileptic activity, in a novel rat model of *GRIN2B* haploinsufficiency. I identify spontaneously occurring absence seizures in these animals by performing *in vivo* chronic wireless EEG recordings. As sleep is frequently reported to be dysfunction in patients with *GRIN2B* mutations (Freunsch et al., 2013; Platzer et al., 2017; Simons Searchlight, 2021), and rats with heterozygous *Grin2b* deletion were demonstrated to have altered sleep-wake distributions and REM sleep deficits in Chapter 3, I additionally correlate seizure activity to the sleep-wake phenotypes observed (see Chapter 3, section 3.3.4).

## 4.2. Methods

Descriptions of animals and animal husbandry are detailed in Chapter 2, sections 2.1.1, 2.1.4 and 2.2. Across all animals, seizure screening employed 14-channel EEG grids coupled with 2 EMG channels. Animals were recorded wirelessly for 48 hours, and seizures were quantified offline using a custom-made automatic seizure scoring algorithm. Chapter 2 contains information on relevant methods for surgical implantation (2.4.1, 2.4.2 and 2.4.5), wireless EEG recording (2.4.6), seizure scoring and

quantification (2.6, 2.8 and 2.10), power spectral analysis (2.9), and statistical analysis details (2.12) used in this chapter.

## 4.3. Results

### 4.3.1. Absence seizures in *Grin2b*<sup>+/-</sup> rats

To assess whether heterozygous knockout of *Grin2b* leads to the development of epileptic seizures, animals were fitted with 14-channel surface electrode grids and 2 EMG electrodes in neck muscles (Figure 4.1A). The exact same experimental setup was previously used in Chapter 3 to evaluate sleep patterns. In fact, the data presented here and in Chapter 3 were obtained from the same 24-hour continuous recordings, from the same animals, which allowed for comprehensive analysis on how identified seizure activity is related to the abnormal sleep physiology observed in *Grin2b*<sup>+/-</sup> rats.

*Grin2b*<sup>+/-</sup> rats displayed spontaneous behavioural arrests reminiscent of absence seizures which correlated with electrographic SWDs (Figure 4.1A). Notably, wild-type animals also presented with SWDs, which had the same distinct electrographic pattern seen in *Grin2b*<sup>+/-</sup> rats (Figure 4.1A), however, these events occurred sparingly and were infrequent and brief as detailed below. Time-of-day factors including sleep, circadian rhythm, and light-dark phase dynamics frequently influence seizure expression (C.-H. Cho, 2012; Matos et al., 2010). I therefore first looked at the distribution of SWDs across each hour of the 24-hour cycle to evaluate whether the occurrence of SWDs in *Grin2b*<sup>+/-</sup> animals was higher than in wild-type littermates at specific hours of the day. I then tested whether there were any differences in the number of SWDs during the 12-hour light and dark phases, as collectively the above analyses could inform on relevant seizure activity patterns in *Grin2b*<sup>+/-</sup> animals. I found that there was a statistically higher number of SWDs in *Grin2b*<sup>+/-</sup> animals compared to controls across the entire day, and that hour of the day did not impact on this difference, indicating that SWD occurrence in both *Grin2b*<sup>+/-</sup> and wild-type animals fluctuates in a similar way over the course of the day (LMM, effect of genotype  $F = 55.36$ ,  $DF = 1$ ,  $P < 0.00001$ ; effect of hour  $F = 1.29$ ,  $DF = 23$ ,  $P = 0.16$ ; hour x genotype  $F = 0.97$ ,  $DF = 23$   $P = 0.49$ ; Figure 4.1B). The number of SWD events in *Grin2b*<sup>+/-</sup> animals was similar between light and dark phases, and significantly higher than that observed in wild-type rats (LMM, effect of genotype  $F = 55.36$ ,  $DF = 1$ ,  $P < 0.00001$ ; effect of phase  $F = 1.23$ ,  $DF = 1$ ,  $P = 0.28$ ; phase x genotype  $F = 0.41$ ,  $DF = 1$   $P = 0.53$ ; Figure 4.2). The above reflected a significant increase in the total number of SWDs over the full 24 hours in *Grin2b*<sup>+/-</sup> rats when compared to wild-type *Grin2b*<sup>+/+</sup> littermates (Wilcoxon rank sum test,  $W = 4.5$ ,  $P = 0.0002$ ; Figure 4.1C). Furthermore, *Grin2b*<sup>+/-</sup> rats had a longer average SWD duration than *Grin2b*<sup>+/+</sup> rats (Two-sample unpaired t-test,  $DF = 22$ ,  $T = -5.26$ ,  $P = 0.00003$ ; Figure 4.1C) and the total duration of SWDs was also significantly longer in *Grin2b*<sup>+/-</sup>

animals when compared to *Grin2b*<sup>+/+</sup> rats (Wilcoxon rank sum test,  $W = 5.5$ ,  $P = 0.0002$ ; Figure 4.1C). These results suggest that *Grin2b* haploinsufficiency drives the network to a state of increased absence seizure activity.

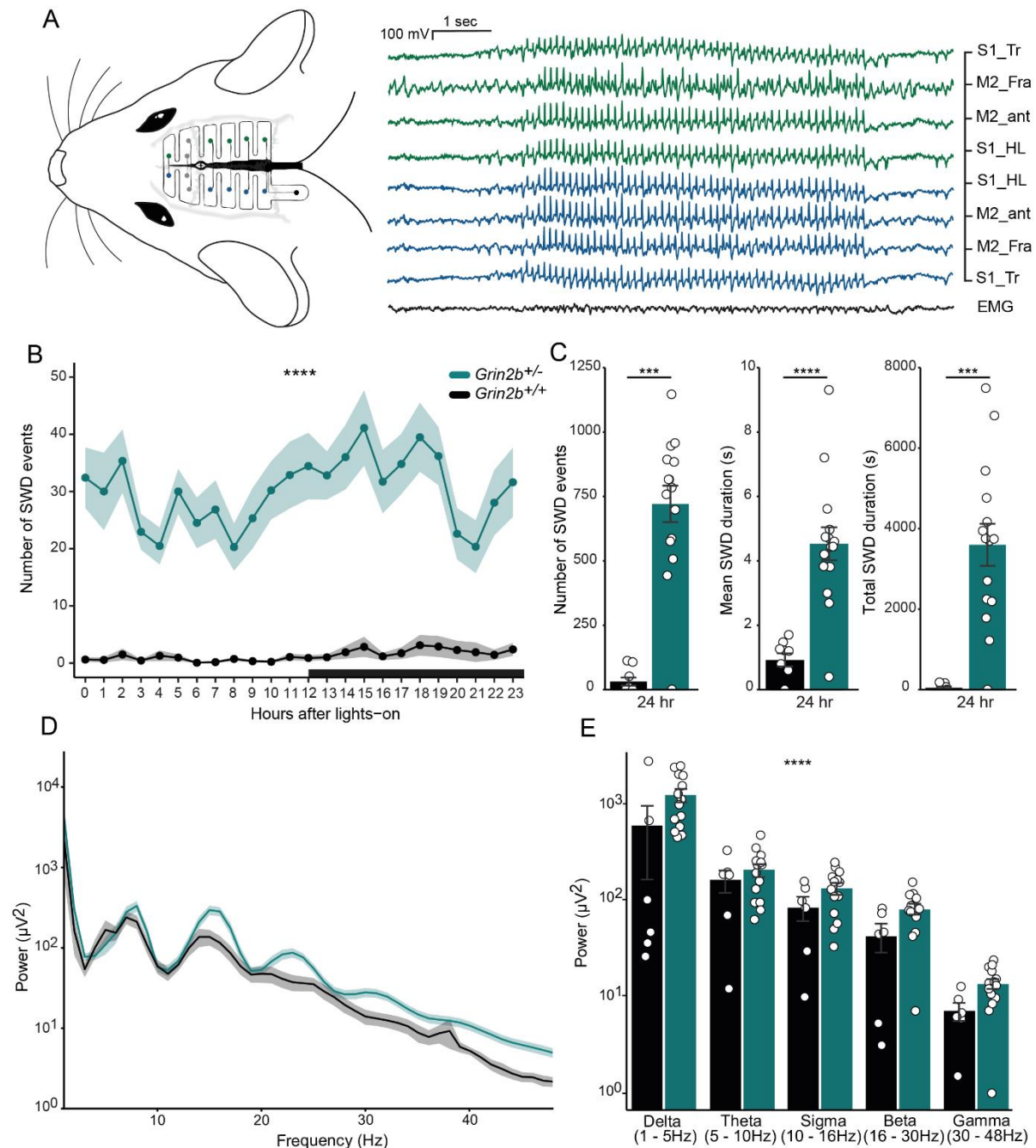


**Figure 4.1. Prevalence and spectral properties of absence seizures in *Grin2b*<sup>+/-</sup> animals.** (A) Schematic of a 16-channel skull-surface EEG implant illustrating approximate placement of electrodes relative to the skull (left) and representative EEG and EMG voltage traces from bilateral electrode pairs in diagram showing a SWD (right). Left hemisphere EEG (blue), right hemisphere EEG (green); S1 = primary somatosensory cortex, M2 = secondary motor cortex, Tr = trunk area, Fra = frontal association cortex, ant = anterior, HL = hindlimb region, EMG = electromyogram. (B) Number of SWD

events plotted by hour over the 24-hour light-dark cycle with black bar on x-axis indicating lights off in the animal facility. Hour of the day had no effect on the number of seizures between the two genotypes and *Grin2b*<sup>+/-</sup> rats had significantly more seizures overall when compared to wild-type littermates (genotype  $P < 0.00001$ , hour  $P = 0.16$ , hour  $\times$  genotype  $P = 0.49$ , LMM), (\*\*\*\* = effect from genotype). Points indicate group mean values (mean  $\pm$  SEM). (C) Bar plots of total number of SWD events (left), SWD average duration (middle) and total SWD durations (right) across the entire 24 hours with *Grin2b*<sup>+/-</sup> rats having significantly higher values ( $P = 0.0002$ ,  $P = 0.00003$ ,  $P = 0.0002$ , Two-sample unpaired *t*-test and Wilcoxon rank sum tests). (D) Average EEG power spectra for *Grin2b*<sup>+/-</sup> rats and wild-type littermates during all SWD epochs across all animals in each group. Traces represent group averages (mean  $\pm$  SEM). (E) Quantification of average power in commonly used frequency bands during SWDs, showing an overall increased spectral power (genotype  $P < 0.00001$ , frequency  $P < 0.0001$ , genotype  $\times$  frequency  $P = 0.12$ , Two-way ANOVA). Detailed statistics in text. \*\*\*\* = effect from genotype. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

I performed spectral analysis on SWDs to determine if these differed between genotypes. In SWD epoch averages we found a clear peak in the theta frequency range with a secondary harmonic in both genotypes (Figure 4.1D). Spectral power during SWDs in *Grin2b*<sup>+/-</sup> rats was higher when compared to wild-type littermates and individual frequency bands did not contribute to power increases (Two-way ANOVA, effect of genotype  $F = 23.99$ ,  $DF = 1$ ,  $P < 0.00001$ ; frequency  $F = 43.36$ ,  $DF = 4$ ,  $P < 0.0001$ ; genotype  $\times$  frequency  $F = 1.97$ ,  $DF = 4$ ,  $P = 0.12$ ; Figure 4.1E). The overall increased power in *Grin2b*<sup>+/-</sup> rats may indicate differences in the underlying mechanisms of SWD generation.

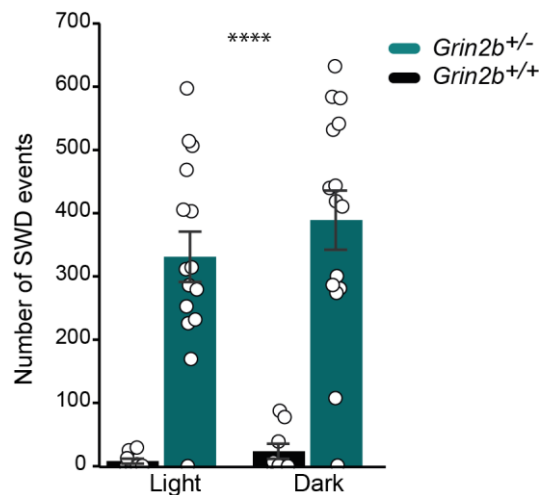


**Figure 4.2. *Grin2b*<sup>+/-</sup> animals present with increased amount of SWDs during light and dark phases.** *Grin2b*<sup>+/-</sup> rats showed similar SWD event numbers in both light and dark periods, which significantly exceeded SWD amounts in wild-type littermates during both phases (effect of genotype  $P < 0.00001$ ; effect of phase  $P = 0.28$ ; phase  $\times$  genotype  $P = 0.53$ , LMM). Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

To determine whether male and female *Grin2b*<sup>+/-</sup> rats have different susceptibility to the occurrence of SWDs, I compared both sexes from each genotype from our animal cohort discussed above. I found that sex had no significant effect on the number of SWDs across all hours of the day, and that differences were specific to genotype (LMM, effect of hour  $F = 1.24$ ,  $DF = 23$ ,  $P = 0.021$ ; genotype  $F = 47.83$ ,  $DF = 1$ ,  $P < 0.00001$ ; sex  $F = 0.12$ ,  $DF = 1$ ,  $P = 0.73$ , hour x genotype x sex  $F = 0.85$ ,  $DF = 23$ ,  $P = 0.66$ ; Figure 4.3A). Similarly, while there were still statistically significant differences between genotypes, there were no differences between male and female *Grin2b*<sup>+/-</sup> rats in the total number of SWD events (Two-way ANOVA, effect of sex  $F = 0.12$ ,  $DF = 1$ ,  $P = 0.74$ ; genotype  $F = 47.663$ ,  $DF = 1$ ,  $P < 0.0001$ ; sex x genotype  $F = 0.2$ ,  $DF = 1$ ,  $P = 0.66$ ; Figure 4.3B), the mean duration of SWDs (Two-way ANOVA, effect of sex  $F = 0.0002$ ,  $DF = 1$ ,  $P = 0.99$ ; genotype  $F = 23.17$ ,  $DF = 1$ ,  $P = 0.0001$ ; sex x genotype  $F = 0.017$ ,  $DF = 1$ ,  $P = 0.89$ ; Figure 4.3B) or the total time spent in a state of SWDs (Two-way ANOVA, effect of sex  $F = 0.0001$ ,  $DF = 1$ ,  $P = 0.99$ ; genotype  $F = 22.9$ ,  $DF = 1$ ,  $P = 0.0001$ ; sex x genotype  $F = 0.002$ ,  $DF = 1$ ,  $P = 0.96$ ; Figure 4.3B).

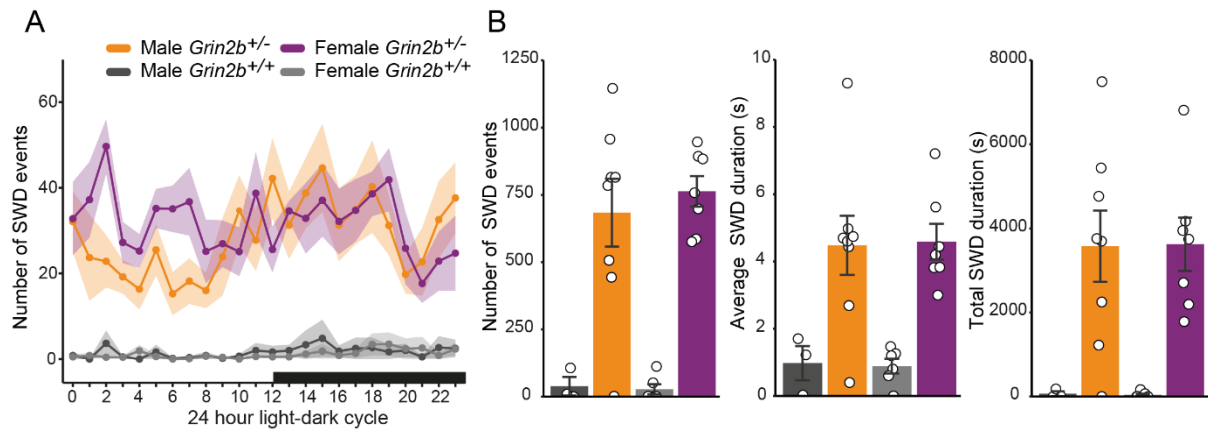


**Figure 4.3. No differences in SWD properties between male and female *Grin2b*<sup>+/-</sup> rats.** (A) Number of SWD events plotted by hour over the 24-hour light-dark cycle with black bar on x-axis indicating lights off in the animal facility. Hour of the day and sex do not impact on the amount of SWDs in *Grin2b*<sup>+/-</sup> male and female rats, which consistently had significantly more seizures than wild-type sex-matched littermates (hour  $P = 0.021$ , genotype  $P = 0.00001$ , sex  $P = 0.73$ , hour x genotype x sex  $P = 0.66$ , LMM). Points indicate group mean values (mean  $\pm$  SEM). (B) Bar plots of total number of SWDs events (left), SWD average duration (middle) and total SWD duration (right). *Grin2b*<sup>+/-</sup> rats (SWD events sex  $P = 0.74$ , genotype  $P < 0.0001$ , sex x genotype  $P = 0.66$ ; SWD average duration sex  $P = 0.99$ , genotype  $P = 0.0001$ , sex x genotype  $P = 0.89$ ; SWD total duration sex  $P = 0.99$ , genotype  $P = 0.0001$ , sex x genotype  $P = 0.96$ , Two-way ANOVA tests). Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

The thalamocortical circuit plays a crucial role in the generation and propagation of SWDs associated with absence seizures. Studies in genetic Wistar Albino Glaxo from Rijswijk (WAG/Rij) and

Genetic Absence Epilepsy Rats from Strasbourg (GAERS) rat models of absence epilepsy, which are strains of rats that spontaneously display SWDs, established that SWDs initiate in the primary somatosensory cortex and are driven particularly by layer V/VI excitatory pyramidal neurons in the barrel field (Crunelli et al., 2020; Meeren et al., 2002; Polack et al., 2007; Studer et al., 2019). Thus, the next aim of this study was to investigate whether this cortical initiation pattern was replicated in *Grin2b*<sup>+/-</sup> rats. This was indeed the case, as 63.67% of SWDs in *Grin2b*<sup>+/-</sup> rats originated (had spiking activity at least 0.5s before other channels) in the somatosensory cortex before propagating subcortically (Figure 4.4A-C). This implies that *Grin2b* is likely to be critically involved in regulating corticothalamic signalling. There was no difference between the average duration of seizures that initiated in the somatosensory cortex and those that appeared simultaneously in the cortex and subcortical structures from the start (Two-sample unpaired t-test,  $DF = 8$ ,  $T = 1.17$ ,  $P = 0.28$ ; Figure 4.4C). Notably, it is possible that the latter group were also of cortical origin but were of non-somatosensory cortex initiation. In this scenario the temporal pattern of SWD initiation and generalisation may be difficult to accurately observe.

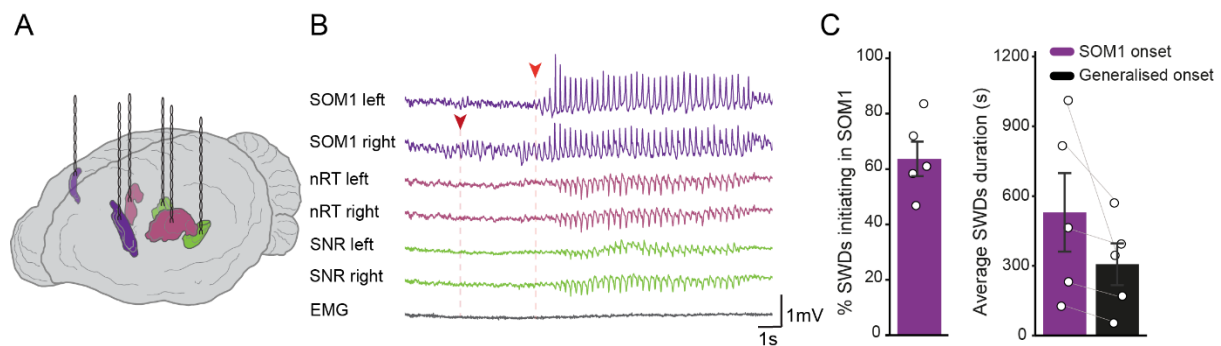


**Figure 4.4. SWDs initiate in the somatosensory cortex in *Grin2b*<sup>+/-</sup> rats.** (A) Schematic of bilateral electrode implantation targeting S1, nRT and SNR. (B) Representative EEG and EMG voltage traces from bilateral electrode pairs from diagram in (A) showing SWD onset in S1. Red arrowheads indicate seizure onset in right and then left S1 prior to seizure generalising. (C) Quantification showing 63.67% of SWDs initiate in S1 in *Grin2b*<sup>+/-</sup> rats (left) and no differences in the average SWD duration between SWDs of S1 origin and SWDs of generalised onset ( $P = 0.28$ , Two-sample unpaired t-test) (right). Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats. S1 = somatosensory cortex, nRT = reticular thalamic nucleus, SNR = substantia nigra.

#### 4.3.2. Absence seizures in *Grin2b*<sup>+/-</sup> rats preferentially occur during wake

I hypothesized that the increased SWD activity in *Grin2b*<sup>+/-</sup> rats may contribute to the altered sleep-wake brain states (see Chapter 3, section 3.3.4). I therefore first tested whether SWDs preferentially occurred during wake, NREM or REM sleep and whether there were any differences

between *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> rats. Collectively for both genotypes, I observed that seizures predominantly occurred during wake (94.8%), followed by NREM (4.4%) and scarcely during REM sleep (0.5%) (Figure 4.5A). Rats have polyphasic sleep patterns, with wake bouts occurring throughout the light and dark phase periods of the 24-hour cycle (Yasnikov & Deboer, 2012), and these data further highlight SWD occurrence is likely more state dependent than light-dark phase dependent. Interestingly, a higher percentage of SWDs initiated during NREM in *Grin2b*<sup>+/-</sup> rats than in wild-type littermates, while a higher percentage of seizures in wild-type animal occurred during the wake state (Two-way ANOVA, effect of genotype  $F = 1$ ,  $DF = 1$ ,  $P = 0.98$ ; state  $F = 594.056$ ,  $DF = 2$ ,  $P < 0.00001$ ; genotype x state  $F = 10.94$ ,  $DF = 2$ ,  $P = 0.00008$ ; Tukey *post hoc* test, wake  $P = 0.0022$ , NREM  $P = 0.0011$ ; Figure 4.5A). The increased frequency of *Grin2b*<sup>+/-</sup> SWDs in NREM sleep prompted me to examine whether the wake-NREM transitional state (Figure 4.5B and C), when synchronous subcortical output is increased (Halassa et al., 2014), is linked to SWD occurrence and if this differed between groups. I tested all SWD epochs identified to originate in NREM and found that all *Grin2b*<sup>+/-</sup> rats had SWDs initiating at wake-NREM transitions, while this was the case for only a small fraction of *Grin2b*<sup>+/+</sup> littermates (33.33 %, 3/9 rats) (Fishers exact test,  $P = 0.00062$ ; Figure 4.5C). I subsequently evaluated for differences in the proportion of wake-NREM transitional SWDs between *Grin2b*<sup>+/-</sup> animals and wild-type rats that presented with transitional state seizures. However, I did not observe any variation in the percent of wake-NREM transitional SWDs between the two groups, 24.2% of SWDS in *Grin2b*<sup>+/-</sup> rats occurred during the wake-NREM transitional period, which was similar the 22.39% observed in *Grin2b*<sup>+/+</sup> littermates (Two-sample unpaired t-test,  $DF = 16$ ,  $T = -0.34$ ,  $P = 0.74$ ; Figure 4.5D).

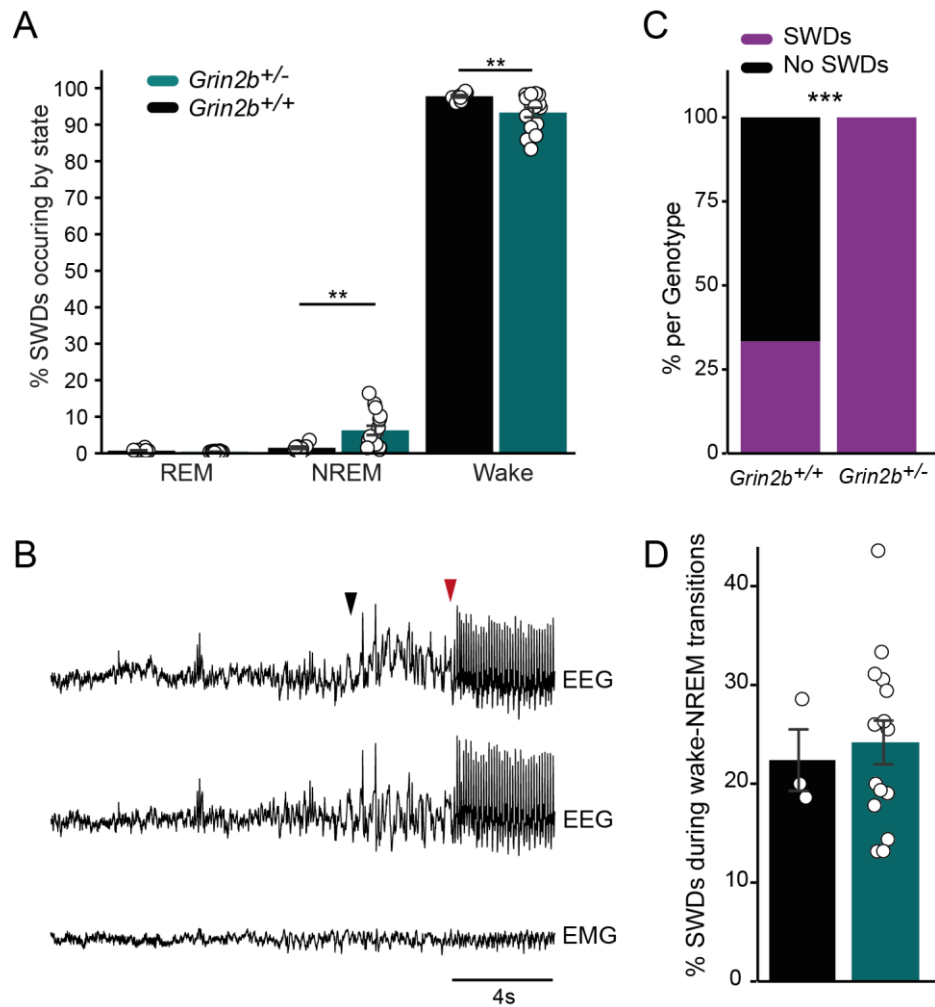


**Figure 4.5. SWDs preferentially occur during wake and SWDs initiate at wake-NREM transitions in all *Grin2b*<sup>+/-</sup> animals.** (A) Plot of average percentage of SWDs occurring during REM, NREM and wake brain states. Compared to wild-type littermates, *Grin2b*<sup>+/-</sup> rats have less SWDs in wake and more SWDs in NREM (genotype  $P = 0.98$ , state  $P < 0.00001$ , genotype  $\times$  state  $P = 0.00008$  (wake  $P = 0.0022$ , NREM  $P = 0.0011$ ), Two-way ANOVA with Tukey post hoc tests). Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats. (B) A higher proportion of SWDs in NREM initiate during wake-NREM transitions in *Grin2b*<sup>+/-</sup> when compared to wild-type animals ( $P = 0.00062$ , Fishers exact test). Bars indicate all animals per each genotype and fill colouring indicates the relevant proportion of animals with SWDs. Points correspond to values from individual rats. (C) Representative EEG and EMG voltage traces during a wake-NREM transition with a SWD. Black arrowhead indicates start of NREM and red arrowhead indicates start of SWD. (D) Amount of SWDs initiating at wake-NREM transitions were not different between *Grin2b*<sup>+/-</sup> rats and *Grin2b*<sup>+/+</sup> wild-types that did have SWDs at transitional periods (3/9 animals) ( $P = 0.74$ , Two-sample unpaired t-test). Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats.

#### 4.3.3. Brain state abnormalities in *Grin2b*<sup>+/-</sup> rats are not correlated with absence seizure activity

I next hypothesized that there might be a mechanistic link between sleep-wake abnormalities (see Chapter 3, section 3.3.4) and SWD occurrence, and therefore next determined whether there was a correlation between sleep-wake properties and total SWD duration in *Grin2b*<sup>+/-</sup> rats. Total seizure duration in *Grin2b*<sup>+/-</sup> animals did not correlate with total time in REM (Pearson correlation,  $T = -0.055$ ,  $DF = 13$ ,  $R = -0.015$ ,  $P = 0.96$ ; Figure 4.6A) and NREM sleep (Pearson correlation,  $T = -1.47$ ,  $DF = 13$ ,  $R = -0.13$ ,  $P = 0.65$ ; Figure 4.6B). There was also no correlation between total SWD duration and bouts of REM (Pearson correlation,  $T = 0.704$ ,  $DF = 13$ ,  $R = -0.19$ ,  $P = 0.49$ ; Figure 4.6A) and NREM (Pearson correlation,  $T = 2.006$ ,  $DF = 13$ ,  $R = 0.49$ ,  $P = 0.066$ ; Figure 4.6B), as well as average bout duration of REM (Pearson correlation,  $T = 0.61$ ,  $DF = 13$ ,  $R = 0.17$ ,  $P = 0.55$ ; Figure 4.6A), NREM (Pearson correlation,  $T = -1.44$ ,  $DF = 13$ ,  $R = -0.37$ ,  $P = 0.17$ ; Figure 4.6B). Finally, while I found a relationship between *Grin2b*<sup>+/-</sup> total SWD durations and total time in wake (Pearson correlation,  $T = -2.16$ ,  $DF = 13$ ,  $R = -0.51$ ,  $P = 0.048$ ; Figure 4.6C), there was no correlation between total duration of seizures with wake bouts (Pearson correlation,  $T = 0.61$ ,  $DF = 13$ ,  $R = 0.17$ ,  $P = 0.55$ ; Figure 4.6C) or average wake bout duration (Pearson correlation,  $T = -0.93$ ,  $DF = 13$ ,  $R = -0.25$ ,  $P = 0.37$ ; Figure 4.6C) in *Grin2b*<sup>+/-</sup> mutants. The general lack of correlation between SWDs and sleep properties in *Grin2b*<sup>+/-</sup> animals suggests that the two phenotypes may have independent underlying mechanisms.

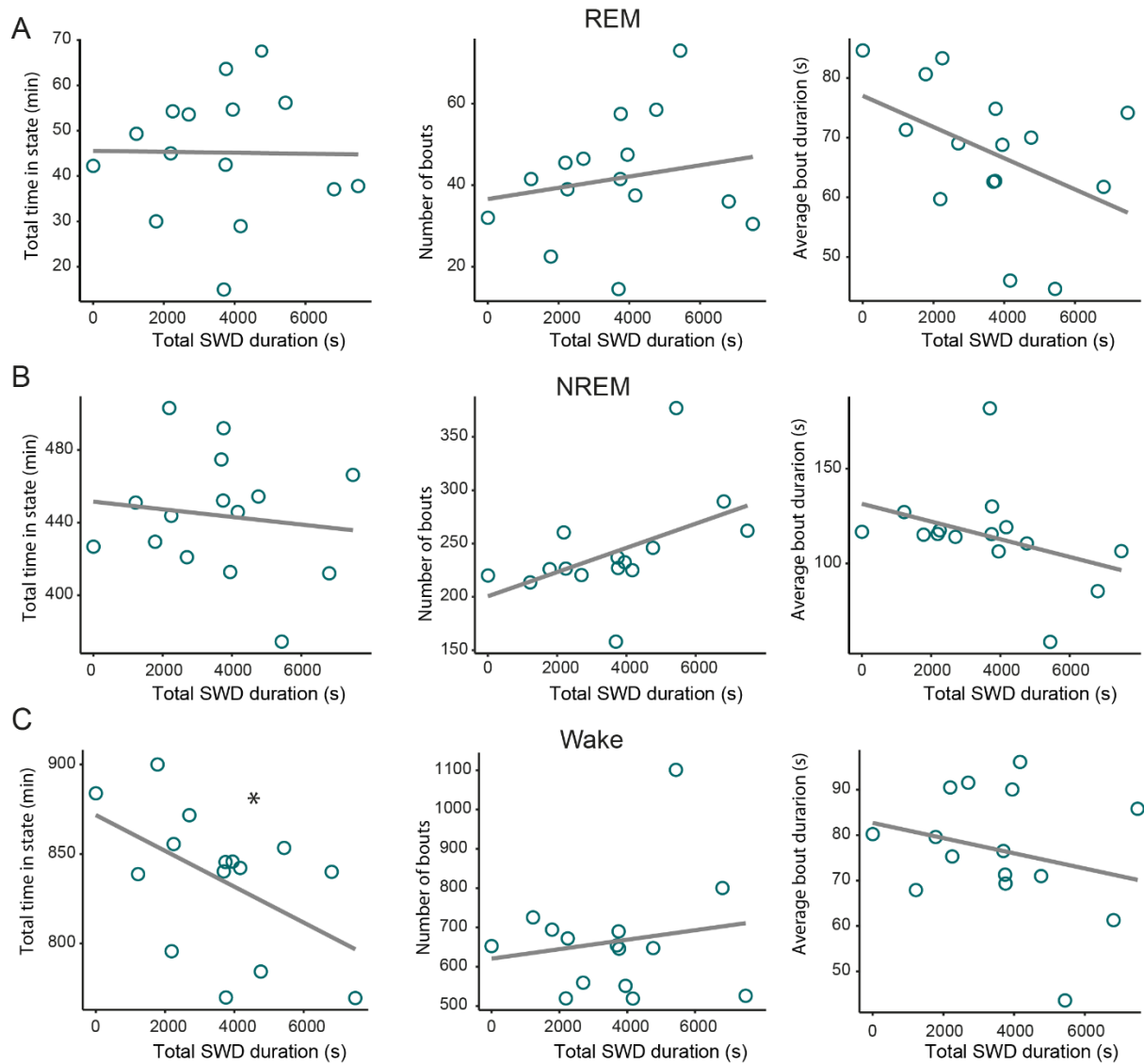


**Figure 4.6. SWDs do not correlate with sleep abnormalities in *Grin2b*<sup>+/-</sup> animals.** Correlation plots of total time in state (left), number of bouts (middle) and average bout duration (right) with SWD duration for REM (A), NREM (B) and wake (C), plotted against total SWD duration. Quantification revealed total SWD duration is negatively correlated to total time awake ( $P = 0.048$ , Pearson correlation) and is uncorrelated to all other sleep measurements (see results section for detailed statistics). Points correspond to values from individual rats.

## 4.4. Discussion

I identify a clinically relevant epileptic phenotype in a novel model of GRIN2B haploinsufficiency. *Grin2b*<sup>+/-</sup> rats displayed a higher prevalence of absence seizure-associated SWDs, which were significantly longer than those observed in wild-type littermates, throughout both light and dark phases of the 24-hour cycle. Furthermore, spectral power of SWDs in *Grin2b*<sup>+/-</sup> animals was greater. In Chapter 3, I described abnormal sleep-wake patterns in *Grin2b*<sup>+/-</sup> rats. Here, I found that the

occurrence of SWDs was largely uncorrelated to the severity of sleep-wake abnormalities in these animals. SWDs predominantly occurred during wake and interestingly, also initiated at wake-NREM transitions in *Grin2b*<sup>+/-</sup> rats, which was seldom observed in *Grin2b*<sup>+/+</sup> animals.

Although absence seizures are not the most prominent seizure type amongst patients with *GRIN2B* mutations, they have been reported in some individuals with loss of function variants (Epi4K Consortium., 2013). I showed that *Grin2b*<sup>+/-</sup> rats had a higher number of absence seizures, which were significantly longer in duration and had higher spectral power when compared to wild-type littermates. Over 24 hours, *Grin2b*<sup>+/-</sup> mutants had a total of 3598 seconds of SWDs, almost an entire hour of the day in an absence seizure brain state. In contrast, wild-type littermates had day average totals of less than a minute. Mice with heterozygous *Grin2b* knockout or a single copy of the *Grin2b*-C456Y ABD variant exhibit behaviours reminiscent of ASD, including repetitive self-grooming, hypoactivity with brief anxiolytic-like behaviours, but notably lack epileptic activity (O’Roak et al., 2011; Shin et al., 2020). Hence, the spontaneously occurring SWDs observed in *Grin2b*<sup>+/-</sup> mutants here represents an important finding, validating the model’s utility for future research into the contribution of GluN2B to the development and pathophysiology of seizures.

In the context of *GRIN2B*, a recent publication showed that in utero electroporation of the loss of function truncating variant *Grin2b*-Trp373 in rats, resulted in reduced GluN2B surface expression and NMDAR hypofunction in hippocampal tissue, which in adult animals manifested as core ASD behavioural phenotypes and an enhanced risk of pentylenetetrazol-induced seizures (X. Wang et al., 2022). NMDAR hypofunction is also observed in hippocampal tissue from *Shank2* knockout mice and in transfected rat cortical neurons null for *Shank3*. In both cases excitation-inhibition network dynamics are altered and *Shank2* knockout mice also exhibit ASD-like impairments in social interactions, as well as repetitive behaviours (Duffney et al., 2013; Lim et al., 2017; Won et al., 2012). Such pathological shifts in network excitation-inhibition ratios are hallmark of ASD, epilepsy and NDDs (Bozzi et al., 2018; Rubenstein & Merzenich, 2003), and *GRIN2A* variants that decrease NMDAR activation are reported in individuals with developmental delay and severe epilepsy (Gao et al., 2017). This implies that similarly to the above, *Grin2b* haploinsufficiency could contribute to NMDAR hypofunction and seizure pathogenesis in *Grin2b*<sup>+/-</sup> animals.

The short (~ 1s) SWD events observed in *Grin2b*<sup>+/-</sup> rats may not represent true absence seizures but rather proto-SWDs, which are brief 5–10 Hz oscillations that have been described in the literature as physiological rather than pathological (Nicolelis and Fanselow, 2002; Shaw et al., 2004; Taylor et al., 2017, 2019). These proto-SWDs are proposed to be alpha mu rhythms, which relate to attentiveness and tactile sensory processing (Nicolelis & Fanselow, 2002; Shaw et al., 2004), and as suggested by Taylor and colleagues, are voluntarily controlled in response to reward stimulus (Taylor et al., 2019). A key feature of absence seizures is impaired consciousness, and the ability to respond to sensory

stimulation during SWDs does not demonstrate that these events are not seizures, since human patients have been reported to show responsiveness of sensory cortex during seizures (Chipaux et al., 2013). Consciousness can be partially maintained during mild human absences, and the extent of impairment can vary between seizures and subjects (Blumenfeld, 2005, 2012). Moreover, consciousness is impaired in rodent absence seizures, as evidenced in female GAERS that have diminished response rates following auditory stimulus (McCafferty et al, 2023). Therefore, while the extremely low occurrence of these events and their short durations in wild-type animals (~ 2 per hour) raises questions about their functional significance and whether they warrant direct comparison to the more frequent and prolonged SWDs seen in *Grin2b*<sup>+/-</sup> rats, further work is needed to establish if SWDs in *Grin2b*<sup>+/+</sup> animals represent pathological or physiological oscillations. Indeed, the spectral characteristics of SWDs in wild-types differed from those of *Grin2b*<sup>+/-</sup> littermates, as evidenced by a lack of resonant harmonic above 15 Hz in wild-types. Future work could investigate the morphological properties of SWDs, such as the presence or absence of spikes in each cycle and Root Mean Square amplitude computation that is useful for quantifying the strength or magnitude of oscillatory events. These analyses would help further differentiate SWDs in *Grin2b*<sup>+/+</sup> rats and could provide support for the physiological nature of these oscillations in this genotype. Ultimately, as this was not the aim of the present study, SWDs in wild-type rats were treated as short pathological absence seizures.

Increased cortical excitation in layer V/VI is thought to trigger SWDs (Meeren et al., 2002; Polack et al., 2007; Pumain et al., 1992; Studer et al., 2019). Activity is thought to spread to thalamic structures and generalises bilaterally through thalamocortical circuits. Within the thalamus, excitatory thalamocortical relay neurons in the ventrobasal region and inhibitory cells in the nRT play crucial roles in modulating circuit oscillations, including SWD rhythms. These neurons shift between tonic firing (during wakefulness) and burst firing (during sleep and absence seizures), with the latter activity mode being dependent on T-type voltage-gated calcium channels, which activate at near-hyperpolarizing potentials between -60 and -70mV (Crunelli et al., 2020; Lindquist et al., 2023). Notably, burst firing in the nRT is critical for SWDs, and inhibiting this nucleus has been shown to attenuate seizures (Cain et al., 2018; McCafferty et al., 2018). The two-fold decrease in Glu2B expression observed in *Grin2b*<sup>+/-</sup> rats likely alters thalamocortical synaptic dynamics and connectivity, similarly to other mutations that have been shown to change physiological synaptic transmission, and thus modulate the brain circuitry mediating SWDs. For example, decreased AMPAR-mediated excitatory transmission in stargazer and *Gria4* knockout mice at corticothalamic synapses projecting to nRT, which is critical for pacing corticothalamic rhythmic oscillations, results in diminished feedforward reticular thalamic inhibition and SWDs (Barad et al., 2012; Menuz & Nicoll, 2008; Paz et al., 2011). Deficits in inhibitory synaptic transmission, specifically at nRT-thalamic GABAergic terminals underlies SWDs in a *Nlgn2* knockout mouse model (F. Cao et al., 2020), while mice with selective loss of the *Scn8a* gene in inhibitory

neurons, display deficits in intrinsic intra-reticular excitatory output, leading to imbalanced circuit properties resulting in excessive burst firing and the emergence of SWDs (Makinson et al., 2017).

GluN2B containing NMDARs are expressed throughout the thalamocortical circuit. In the cortex, they are the dominant postsynaptic receptor subtypes facilitating intra-cortical signalling between layer V excitatory cells (S. S. Kumar & Huguenard, 2003), which also project to thalamus and nRT (Deschênes et al., 1994; Hádinger et al., 2023; Paz et al., 2011; Pinault et al., 1995). SWDs initiate as a consequence of irregular signalling properties of layer V/VI pyramidal neurons, which could be a result of insufficient GluN2B mediated NMDAR function at short range cortico-cortical synapses in *Grin2b*<sup>+/-</sup> animals. This possibility is further supported by the observation that over half of seizures in these animals originated in the somatosensory cortex prior to generalising. In the thalamus, NMDAR signalling plays a major role in the control of genetically determined absence seizures, thus dysregulation of NMDAR activity contributes to the development of SWDs (Koerner et al., 1996). GluN2 expression in the thalamus is highly diverse and subunit localisation is thought to be organised in an input and cellular subtype manner to allow processing of an abundant input range. Previously, GluN2B containing NMDARs were found to mediate thalamoreticular signalling (Astori & Lüthi, 2013), and therefore regulate communication between relay thalamus and nRT. In addition, the nRT contains tonically active GluN2B-NMDARs that are located on reticulo-reticular GABAergic terminals and generate a persistent facilitation of vesicular GABA release in nRT. When blocked, mIPSC frequency in nRT neurons is significantly decreased (Crabtree et al., 2013). The recurrent intra-reticular inhibitory connections are thought to have a desynchronizing effect on network activity because they provide inhibition that is out of phase with the overall network oscillation. Importantly, reductions to IPSCs arising from nRT-nRT synapses was demonstrated to dysregulate intra-reticular transmission and contributed to the emergence of SWDs in *Scn8a* knockout mice (Makinson et al., 2017). Deficits in GluN2B expression could contribute similarly to nRT impairments in *Grin2b*<sup>+/-</sup> rats.

Lastly, a recently published preprint found that postsynaptic GluN2B-NMDARs are dominantly expressed at posteromedial thalamocortical inputs from layer VI cortical neurons (Topolski et al., 2024). In terms of *Grin2b* haploinsufficiency, this is interesting, as in rodents the posterior ventrobasal thalamic region is hypothesized to be a critical node for absence seizure generalization, given its pivotal role in pre- and post-ictal activity and its involvement in both the initiation and termination of SWDs (Crunelli et al., 2020). These recent findings, strongly suggest that heterozygous loss of GluN2B may lead to aberrant NMDAR-dependent neurotransmission in the ventral cortex, and posterior thalamus. Such dysregulation is likely to affect layer V pyramidal neurons, nRT and thalamocortical relay neurons. This would potentially alter the excitability and synchronization properties of these cellular populations in the thalamocortical circuit, and consequently lead to the emergence and propagation of SWDs. Further research is necessary to explore the precise mechanisms underlying the hypothesised relationship between GluN2B haploinsufficiency and absence seizures. Furthermore, GluN2B-NMDARs are

enriched at extrasynaptic sites, functioning to regulate neuronal excitability and thus firing output (Isaacson & Murphy, 2001). It is plausible that deficits in extrasynaptic receptor expression may serve as an alternative or complementary mechanism contributing to seizure generation in *Grin2b* haploinsufficient rats.

In *Grin2b*<sup>+/-</sup> rats the spectral slope of SWD epochs showed a peak frequency in the theta range with strong resonant harmonics in higher frequency bands. This could explain the amplified power across multiple frequencies in *Grin2b*<sup>+/-</sup> animals and may indicate enhanced neuronal synchronization or heightened excitability within the thalamocortical circuit during seizure events (Hu et al., 2024). In addition, the increased power could also be related to the prolonged SWD durations observed in *Grin2b*<sup>+/-</sup> rats, as longer seizures may allow for greater accumulation of synchronized neuronal activity, thereby contributing to higher overall spectral power. Ultimately, the spectral power results presented here suggest that GluN2B haploinsufficiency could lead to altered connectivity strength and a more robust or widespread engagement of neural networks during SWDs, potentially reflecting altered oscillatory properties of the epileptic network.

I found no correlation between REM and NREM sleep abnormalities (see Chapter 3, section 3.3.4) and total SWD activity. Indeed, REM sleep is not critically dependent on thalamocortical circuitry, and while this network is recruited to generate NREM spindles and pace slow oscillatory activity, NREM sleep is also dependent on multiple other hypothalamic and midbrain structures that operate through distinct neurophysiological mechanisms. It is therefore plausible that the lack of correlation between sleep abnormalities and SWDs stems from loss of GluN2B contributing similar effects to thalamocortical and other sleep circuits that function independently of one another. Alternatively, the impact of *Grin2b* haploinsufficiency may vary depending on the spatial expression of GluN2B, potentially affecting brain networks that mediate absence seizures differently from those that underlie sleep-wake physiology. Both hypotheses are probable and present interesting avenues for future research into the role of *Grin2b* in epilepsy and sleep mechanisms.

SWDs primarily occurred when *Grin2b*<sup>+/-</sup> rats were awake and interestingly, absence seizures were more likely to initiate during wake-NREM transitions in *Grin2b*<sup>+/-</sup> rats compared to wild-type controls. This could explain the reduced amount of NREM bouts, which were longer in mutant animals (see Chapter 3, section 3.3.4), as the increased bout duration could represent a compensatory response to NREM initiation difficulties. Quiet-restful wake and NREM are states in which absence seizures occur more frequently in Long Evans rats, with a high probability of SWDs occurring around wake to NREM transitions (Kozák et al., 2020), when thalamic synchrony is increased to gate sensory input (Halassa et al., 2014). This data suggests that the mechanisms mediating wake-sleep transition in *Grin2b*<sup>+/-</sup> animals are functioning abnormally and may be a result of hypersynchronous activation in NREM initiating circuits, particularly of the thalamus.

Mechanistically, absence epilepsy is thought to also be associated with insufficiency of sleep-promoting mechanisms, as reciprocally interconnected arousal and sleep-promoting neuronal groups control thalamocortical excitability (Danober et al., 1998; Snead, 1995). Reduced activity of sleep promoting GABAergic VLPO and MnPN were previously shown in epileptic WAG/Rij rats, while inappropriately timed activation of MnPN during waking, although insufficient to fully suppress the arousal system, induces SWDs in non-epileptic rats (Suntsova et al., 2009). Indeed, absence epilepsy is associated with deficits in REM sleep, increased latencies to REM sleep onset, sleep fragmentation and decreased sleep efficiency (Baldy-Moulinier, 1992; Maganti et al., 2005; Saad et al., 2019). Similarly, WAG/Rij rats present with SWDs and have altered sleep patterns consisting of prolonged transitional periods from wake to sleep, lengthened intermediate stages of sleep, more frequently followed by arousals, and decreased REM sleep percentage (Coenen & van Luijtelaar, 2003; Gandolfo et al., 1990; van Luijtelaar & Bikbaev, 2007). These findings are in alignment with the sleep and SWD phenotypes I observed in *Grin2b*<sup>+/-</sup> rats. Based off the above observations and the findings that NMDARs in the VLPO are essential for NREM and REM sleep (J. Lu et al., 2000, 2002; Miracca et al., 2022), it is possible that loss of sufficient GluN2B expression impacts on and dysregulates the circuit activities of basal hypothalamic sleep-promoting structures. This scenario will be interesting to explore in *Grin2b*<sup>+/-</sup> rats, especially in light of the reduced REM, altered NREM dynamics (see Chapter 3, section 3.3.4), and presence of SWDs in these animals.

The identification of SWDs in rats with heterozygous *Grin2b* deletion opens the potential for in-depth research on their emergence, circuit mechanisms, and functional consequences, such as cognitive and behavioural effects, and neuronal network alterations. Groundwork investigations could examine cellular properties that underlie SWDs in *Grin2b*<sup>+/-</sup> animals. These could include a detailed characterization of intrinsic excitability in layer V and VI cortical pyramidal neurons, which are implicated in the initiation of SWD activity, as well as in nRT and thalamocortical relay neurons, which are essential for the propagation and maintenance of oscillatory thalamocortical rhythms. For example, current-clamp recordings in acute brain slices taken from *Grin2b*<sup>+/-</sup> rats could be used to assess resting membrane potential, input resistance, rheobase, and the threshold for action potential generation in these neuronal populations (Gray et al., 2011; B. J. Hall et al., 2007; Kelsch et al., 2014; Polack et al., 2007). These measures would help determine whether *Grin2b* haploinsufficiency alters baseline excitability, potentially contributing to aberrant burst-firing patterns associated with SWDs. In parallel, voltage-clamp experiments, could assess the NMDAR/AMPA ratio at excitatory synapses in these cellular populations. When paired with selective antagonism of GluN2A or GluN2B-containing NMDARs using Zn<sup>2+</sup> or ifenprodil, respectively, voltage-clamp experiments could allow for the dissection of NMDAR subunit contributions to synaptic transmission and plasticity in *Grin2b*<sup>+/-</sup> rats (Astori & Lüthi, 2013). Specifically, this approach could reveal whether a shift in NMDAR subunit composition occurs following heterozygous *Grin2b* knockout, potentially leading to altered synaptic integration and

excitatory drive. Given the developmental role of GluN2B-containing NMDARs, these investigations, done at various stages of development, would give insight as to when network activity becomes abnormal *Grin2b*<sup>+/-</sup> rats and whether and how it is compensated for at a cellular signalling level. Such evaluation of cellular properties in *Grin2b*<sup>+/-</sup> animals would lay the foundation for more targeted circuit level interrogation techniques, utilizing acute slice preparations from dedicated *Grin2b* Cre-driver lines, viral-mediated genetic manipulations, and optogenetic approaches to precisely dissect cell-type-specific contributions to network dynamics and their role in thalamocortical dysfunction and SWD generation.

The methods used throughout this chapter enabled a detailed characterization of seizures in *Grin2b*<sup>+/-</sup> rats, each with distinct advantages and limitations. Skull surface EEG allowed for seizure identification and quantification in freely behaving animals without disrupting their habitual environment or requiring handling, which could otherwise induce stress and influence seizure susceptibility (Deszi et al., 2023; Jones et al., 2023). The 24-hour recording setup, also used for sleep characterization in Chapter 3, enabled seizure analysis across both light and dark periods, facilitating correlations with circadian and sleep-wake dynamics, which have previously been shown in human patients and in rodent models of absence epilepsy (Kozák et al., 2020; Malow, 2004; Roliz & Kothare, 2023). Furthermore, the automated SWD quantification algorithm, which incorporates cepstral analysis, provided efficient and unbiased seizure detection over multiple days while reducing manual scoring variability. Although EEG recordings were visually inspected, a potential limitation of automated scoring is that any seizure activity falling outside predefined threshold values would go undetected. Intracerebral EEG, a well-validated and widely used technique in rodent electrophysiology, enabled precise recording of neural activity from deep brain structures, offering high temporal resolution within targeted brain regions and allowed for detailed characterization of network activity and region-specific oscillatory patterns. In this study, depth electrodes implanted in the somatosensory cortex and subcortical structures confirmed seizure onset in the somatosensory cortex (Crunelli et al., 2020; Meeren et al., 2002; Polack et al., 2007; Studer et al., 2019), providing insight into seizure propagation and subcortical involvement. One consideration, however, is that stereotaxic depth electrodes may induce localized tissue damage, potentially altering seizure dynamics compared to pre-implantation activity. Therefore, addressing these factors in future studies will further refine seizure characterization in this model. Finally, while this thesis provided an initial characterisation of seizure activity in *Grin2b*<sup>+/-</sup> rats, limiting analysis to adult animals precluded investigation of seizure onset and progression across development, a critical factor in NDDs. Expanding investigations to include developmental timepoints will be crucial for a more comprehensive understanding of seizure evolution in this model.

In conclusion, I report on absence seizure in a novel rat model of *GRIN2B* haploinsufficiency. *Grin2b*<sup>+/-</sup> rats exhibited a significantly higher prevalence of absence seizures, which were longer in duration and had greater spectral power compared to wild-type littermates. A significant proportion of

individuals with GRIN2B mutations present with a range of seizure types, including absence seizures (Epi4K Consortium., 2013; Platzer et al., 2017), and these findings validate the model's utility for investigating the role of GluN2B in seizure pathophysiology. The observed SWDs in *Grin2b*<sup>+/-</sup> animals likely result from altered thalamocortical circuit dynamics due to reduced GluN2B expression, potentially leading to NMDAR hypofunction and imbalance in network transmission. However, the precise mechanisms relating to such alterations required future circuit and cellular level studies. Interestingly, I found that SWDs predominantly occurred during wakefulness and at wake-NREM transitions in *Grin2b*<sup>+/-</sup> rats, suggesting sleep and arousal mechanisms may be dysregulated, as discussed in Chapter 3. Overall, these data are consistent with clinical observations, and as such, provide evidence for a translationally relevant tool for future research that should aim at defining the underlying pathological mechanisms and developing targeted therapeutics for *GRIN2B* related disorders.

# Chapter 5

## Efficacy of Pharmacological Interventions on Absence Seizures in a Novel Rat Model of *GRIN2B* Related Neurodevelopmental Disorder

### 5.1. Introduction

Approximately 52% of individuals with *GRIN2B* related NDDs experience severe epilepsy, which presents a substantial challenge for medical management, as seizures in roughly half of diagnosed cases of *GRIN2B* NDDs and epilepsy are resistant to conventional treatments. Patients exhibit a diverse range of seizures, including infantile spasms, generalized tonic-clonic seizures, focal seizures, and absence seizures (Endele et al., 2010; Epi4K Consortium., 2013; Freunscht et al., 2013; O’Roak et al., 2011; Platzer et al., 2017). Furthermore, *GRIN2B* variants have been identified in patient cohorts with West Syndrome and Lennox-Gastaut Syndrome (Lemke et al., 2014), where in the latter absence seizures represent one of the predominant seizure types (Camfield, 2011).

Current epilepsy treatment in patients with *GRIN2B* NDD includes standard mono and poly therapy with anti-seizure medications, such as valproic acid, levetiracetam, lamotrigine and sulthiame amongst others (Krey et al., 2022; Lemke et al., 2014; Platzer et al., 2017). Many of these drugs are effective, but to date none have been demonstrated to be specifically effective for this disorder. *In vitro* studies using *Xenopus laevis* oocytes indicate potential therapeutic efficacy of memantine and radiprodil, in treating pathogenic *GRIN2B* gain of function missense variants (Platzer et al., 2017). The former is a non-specific NMDAR antagonist, and the latter specifically blocks GluN2B-NMDARs. However, despite promising preclinical findings, significant clinical benefits from NMDAR antagonist treatment have yet to be conclusively demonstrated in patients with *GRIN2B* related disorders. L-serine is a natural nonessential amino acid and the precursor of D-serine, an endogenous NMDAR coagonist that, like glycine, binds the GluN1 subunit increasing the propensity for channel opening (Mothet et al., 2000). Recently, dietary supplementation with L-serine, which is found in various foods was shown to

increase D-serine levels in both plasma and cerebrospinal fluid, which markedly improved neurocognitive, behavioural, sleep, and seizure frequency outcomes in individuals with loss of function and null *GRIN2B* mutations (den Hollander, Rothuizen-Lindenschot, et al., 2023; Krey et al., 2022; Soto et al., 2019). The observed beneficial outcomes of memantine, radiprodil and L-serine suggest that modulating NMDAR activity is a viable therapeutic approach in treating disorders characterized by abnormal NMDAR function.

In Chapter 4, I discussed the presence of an absence seizure phenotype in *Grin2b*<sup>+/-</sup> rats, which have significantly more SWDs of longer durations when compared to their wild-type littermates (see Chapter 4, section 4.3.1). As mentioned above, a proportion of patients with *GRIN2B* variants present with absence seizures. These seizures result from aberrant oscillatory activity within the thalamocortical circuit, characterized by 2.5-4 Hz SWDs (5-11 Hz in rodents) that rapidly generalize across the brain (Crunelli et al., 2020; Lindquist et al., 2023). SWDs are thought to initiate in the cortex with activity propagating to the thalamus (Meeren et al., 2002; Polack et al., 2007; Pumain et al., 1992; Studer et al., 2019). The intrinsic properties of thalamic neurons and the synaptic interconnections between the inhibitory nRT, thalamocortical relay nuclei and cortex are crucial in generating the diverse firing patterns that modulate brain states. Under physiological condition, rhythmic burst firing in thalamocortical and nRT neurons is associated with sleep spindles and NREM sleep delta waves. In pathological circumstances the shift from tonic to highly synchronized bursting of these populations precipitates seizures that rapidly spread through the brain, resulting in altered consciousness. Burst firing within the thalamocortical network is dependent on T-type voltage-gated calcium channels (Crunelli et al., 2020; Lindquist et al., 2023), and notably, the bursting activity of these channels in nRT cells is essential for the generalisation and maintenance of SWDs (Cain et al., 2018; McCafferty et al., 2018). Multiple lines of evidence show that mutations altering thalamocortical synaptic dynamics and connectivity lead to the emergence of SWDs (Barad et al., 2012; F. Cao et al., 2020; Makinson et al., 2017; Menuz & Nicoll, 2008; Paz et al., 2011), therefore, thalamocortical circuit dysfunction, driven by maladaptive network connectivity and aberrant burst firing underlies the characteristic rhythms and loss of awareness in absence seizures.

NMDAR are essential to the flow of information through the thalamocortical circuit, and in the thalamus, dysregulation of NMDAR-dependent signalling contributes to the development of SWDs (Koerner et al., 1996; Lacey et al., 2012). For instance, enhanced activation of NMDARs generates nRT overexcitation, and therefore the hyperpolarisation of nRT and thalamocortical neuron, which promotes thalamocortical hypersynchronous bursting contributing to seizures (Lacey et al., 2012). Paradoxically, NMDAR antagonism in the nRT produces comparable effects *in vitro* (Vaisanen et al., 2004; Y. Zhang et al., 2009), although *in vivo* studies remain limited. These findings highlight that NMDAR activity within the thalamus is finely tuned and that irregular NMDAR transmission in either direction can contribute to pathological thalamocortical dynamics. This is further evidenced in individuals with

*GRIN2A* variants that decrease NMDAR activation, who present with developmental delay and absence seizures (Gao et al., 2017). Interestingly, both NMDAR agonists and antagonists infused intrathalamically attenuate SWDs in GAERS, by presumably altering firing type and output frequency (Koerner et al., 1996), thus thalamic NMDARs present a putative therapeutic target in the treatment of absence epilepsy.

Given the critical role of NMDAR signalling in modulating thalamocortical oscillations and the observation that *Grin2b*<sup>+/-</sup> rats exhibit SWDs reminiscent of absence seizures in individuals with de novo *GRIN2B* related NDDs, this chapter aimed to investigate the potential for pharmacologically blocking seizures in *Grin2b*<sup>+/-</sup> animals. I conducted acute pharmacology experiments by either treating animals with systemic drug administration or infused drugs directly to SWD-mediating brain structures. These were namely the somatosensory cortex, where SWDs initiate in rats, and the inhibitory nRT, which is crucial for pacing SWD oscillatory tone and propagating seizure activity (Crunelli et al., 2020; Lindquist et al., 2023). In both systemic and site-specific experiments, the efficacy of ethosuximide and memantine were tested. Ethosuximide is a well-established anti-epileptic drug primarily used for the treatment of absence seizures (Zimmerman & Burgemeister, 1958). The drug suppresses absence seizure activity by inhibiting T-type calcium channels, thereby reducing pathological burst firing and hypersynchronous oscillations that contribute to SWD generation in various mouse and rat models of epilepsy (Terzioglu et al., 2006). Considering the role of T-type calcium channels in modulating thalamocortical rhythms, ethosuximide represents a targeted intervention for SWDs in *Grin2b*<sup>+/-</sup> rats. Furthermore, given its proven efficacy in diminishing SWDs, the drug serves as a benchmark for other tested compounds, while also confirming the necessity of T-type calcium channel-mediated burst firing for SWD generation in *Grin2b*<sup>+/-</sup> animals. Memantine is a low-affinity, non-competitive NMDAR antagonist that preferentially blocks excessive receptor activation while preserving physiological neurotransmission (Bormann, 1989; H. S. Chen & Lipton, 1997; Parsons et al., 1999). Therefore, by selectively inhibiting overaction of NMDARs, memantine may potentially mitigate hyperexcitability and network instability within the thalamocortical circuit, potentially reducing seizure occurrence in *Grin2b*<sup>+/-</sup> rats. Trialling both drugs in *Grin2b* heterozygous deletion rats could provide insights into their potential therapeutic efficacy for absence seizure activity and further our understanding of the underlying mechanisms driving thalamocortical dysfunction in *GRIN2B* related NDDs.

## 5.2. Methods

Detailed descriptions of animals and animal husbandry are described in Chapter 2, sections 2.1.1, 2.1.4 and 2.2. Across all *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> animals, 2-channel EEG recordings were used for systemic and intracerebral pharmacology experiments. 2-hour (systemic experiments) and 1-hour (site-specific experiments) wireless EEG baseline and drug response recordings were made, and seizures were quantified offline using a custom-made automatic seizure scoring algorithm. Chapter 2 contains relevant methods for surgical implantation (2.4.1, 2.4.3 and 2.4.4), wireless EEG recording (2.4.6), systemic and site-specific drug treatment (2.5.1 and 2.5.2), seizure scoring and quantification (2.6, 2.8 and 2.10), histology and imaging (2.11.1, 2.11.2 and 2.11.3), and statistical analysis details (2.12) used in this chapter.

## 5.3. Results

### 5.3.1. Spike and wave discharges in *Grin2b*<sup>+/-</sup> rats are blocked by systemic pharmacological treatment

This work aimed to evaluate whether SWDs in *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> rats could be suppressed pharmacologically. I tested the efficacy of ethosuximide, a T-type calcium channel blocker used in the treatment of absence seizures in humans (Zimmerman & Burgemeister, 1958) and known to block SWDs in other absence seizure rodent models (Terzioglu et al., 2006). In addition, I evaluated seizure responses to memantine, a noncompetitive NMDAR antagonist currently being tested as a mono or adjunctive treatment option in NMDAR related epilepsies (Bormann, 1989; H. S. Chen & Lipton, 1997; Parsons et al., 1999; Platzer et al., 2017). The experimental timeline is shown in Figure 5.1A. As *Grin2b*<sup>+/-</sup> rats had a more severe seizure phenotype compared to wild-type littermates (see Chapter 4, section 4.3.1), I hypothesized that altered NMDAR-mediated signalling may contribute to aberrant brain circuits function underlying SWDs and that blocking NMDARs would therefore reduce SWD activity (Koerner et al., 1996).

I found that in both *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> rats there was a statistically significant reduction in SWDs with pharmacological treatment, as systemic ethosuximide resulted in significantly fewer SWDs than memantine and saline (LMM, effect of treatment  $F = 6.48$ ,  $DF = 2$ ,  $P = 0.0049$ ; genotype  $F = 0.42$ ,  $DF = 1$ ,  $P = 0.53$ ; treatment x genotype  $F = 0.34$ ,  $DF = 2$ ,  $P = 0.71$ ; Tukey *post hoc* test,

ethosuximide-saline  $P = 0.0081$ , ethosuximide-memantine  $P = 0.015$ , memantine-saline  $P = 0.96$ , Figure 5.1B). Both ethosuximide and memantine, however, were effective in significantly reducing the average duration of SWDs in *Grin2b*<sup>+/-</sup> but not *Grin2b*<sup>+/+</sup> animals (LMM, effect of treatment  $F = 9.17$ ,  $DF = 2$ ,  $P = 0.00048$ ; genotype  $F = 0.71$ ,  $DF = 1$ ,  $P = 0.41$ ; treatment x genotype  $F = 3.34$ ,  $DF = 2$ ,  $P = 0.044$ ; Tukey *post hoc* test for interaction effect by genotype, *Grin2b*<sup>+/-</sup> ethosuximide-saline  $P < 0.0001$ , *Grin2b*<sup>+/-</sup> memantine-saline  $P = 0.0009$ , *Grin2b*<sup>+/-</sup> memantine-ethosuximide  $P = 0.76$ , *Grin2b*<sup>+/+</sup> ethosuximide-saline  $P = 0.88$ , *Grin2b*<sup>+/+</sup> memantine-saline  $P = 0.99$ , *Grin2b*<sup>+/+</sup> memantine-ethosuximide  $P = 0.99$ , Figure 5.1C). The specific effect on *Grin2b*<sup>+/-</sup> rats, but not wild-types, may stem from the significantly fewer and shorter seizures observed in *Grin2b*<sup>+/-</sup> animals (see Chapter 4, section 4.3.1). Consequently, the potential for reduction in average SWD duration of wild-type rats in response to drug treatment is much lower overall. While this is highly likely, it does not preclude the possibility of distinct seizure mechanisms between genotypes. Furthermore, memantine's effect on reducing SWD duration without affecting SWD occurrence may be attributed to its use-dependent mechanism of action. This mechanism requires the opening of NMDARs to allow access to the channel pore, which occurs abundantly after seizure initiation. As a result, memantine's impact may primarily be observed in modulating ongoing seizure activity rather than preventing seizure onset (Parsons et al., 1993). The combined effect of less SWDs with lower duration likely resulted in ethosuximide, but not memantine, reducing total seizure durations in both rat groups (LMM, effect of treatment  $F = 9.44$ ,  $DF = 2$ ,  $P = 0.0011$ ; genotype  $F = 0.67$ ,  $DF = 1$ ,  $P = 0.43$ ; treatment x genotype  $F = 1.15$ ,  $DF = 2$ ,  $P = 0.34$ ; Tukey *post hoc* test, ethosuximide-saline  $P = 0.0005$ , ethosuximide-memantine  $P = 0.083$ , memantine-saline  $P = 0.98$ ; Figure 5.1D). Statistical analysis with absolute values showed similarly reduced SWD activity following ethosuximide and memantine treatment and can be found in Appendix A.1. Both ethosuximide and memantine could therefore potentially be tested as treatments for SWDs in *GRIN2B* patients.

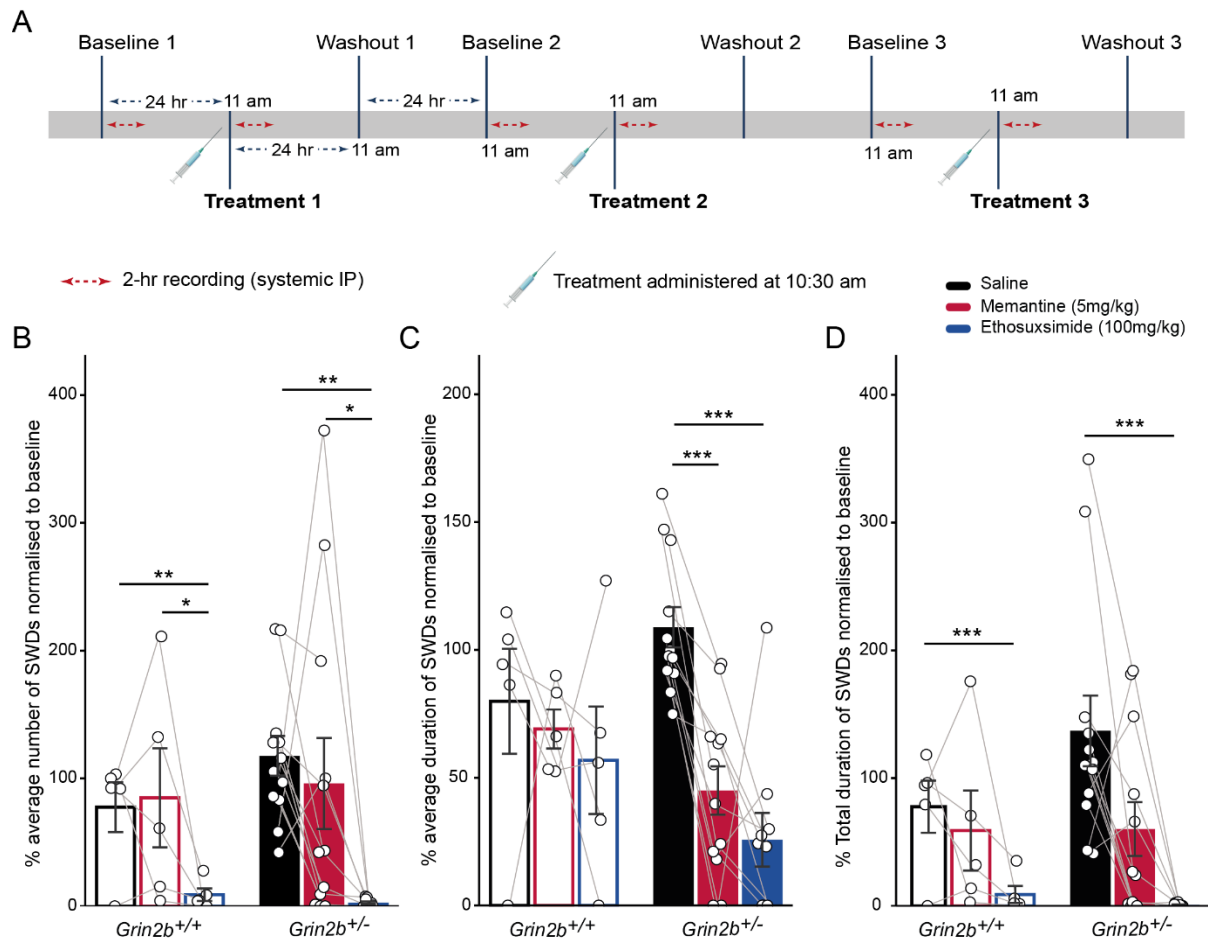


**Figure 5.1 Acute administration of anti-seizure drugs attenuates SWDs in *Grin2b*<sup>+/-</sup> and wild-type animals.** (A) Diagram of pharmacology experiment timeline. Panels showing effects of acute drug treatment on SWD average number (B), average duration (C) and total duration (D) of SWDs in *Grin2b*<sup>+/-</sup> and wild-type animals. Treatment with ethosuximide successfully blocked SWD events in both groups (treatment  $P = 0.0049$  (ethosuximide-saline  $P = 0.0081$ , ethosuximide-memantine  $P = 0.015$ ), genotype  $P = 0.53$ , treatment  $\times$  genotype  $P = 0.71$ , LMM with Tukey post hoc tests) (B). Memantine and ethosuximide reduced average SWD duration in *Grin2b*<sup>+/-</sup> rats but not in wild-type littermates (treatment  $P = 0.00048$ , genotype  $P = 0.71$ , treatment  $\times$  genotype  $P = 0.044$  (*Grin2b*<sup>+/-</sup> ethosuximide-saline  $P < 0.0001$ , *Grin2b*<sup>+/-</sup> memantine-saline  $P = 0.0002$ ), LMM with Tukey post hoc tests) (C). Total seizure duration was also significantly reduced following ethosuximide treatment in both *Grin2b*<sup>+/-</sup> and wild-type rats (treatment  $P = 0.0011$  (ethosuximide-saline  $P = 0.0005$ ), genotype  $P = 0.43$ , treatment  $\times$  genotype  $P = 0.34$ , LMM with Tukey post hoc tests) (D). For detailed statistics see results section. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats and grey lines follow treatment response of each individual rat.

### 5.3.2. Site-specific drug infusion to the reticular thalamic nucleus attenuates spike and wave discharges in *Grin2b*<sup>+/-</sup> rats

Previous research demonstrates that inhibiting T-type calcium channels in nRT and the perioral region of the somatosensory cortex effectively reduces SWDs in GAERS (Manning et al., 2004; McCafferty et al., 2018; Polack et al., 2007). Additionally, in GAERS thalamic NMDAR signalling exerts significant influence over the regulation of absence seizures of genetic origin, as application of both NMDAR agonists and antagonists to the thalamus blocks SWD activity (Koerner et al., 1996). Therefore, I next investigated the effect of ethosuximide and memantine applied directly to either the somatosensory cortex or the nRT of *Grin2b*<sup>+/-</sup> animals (Figure 5.2B and C). The experimental timeline is illustrated in Figure 5.2A. This experimental protocol was also applied to a small group of *Grin2b*<sup>+/+</sup> rats (four animals per infusion site), which overall have significantly fewer SWDs than *Grin2b*<sup>+/-</sup> animals (see Chapter 4, section 4.3.1). The short recording and analysis period of 1-hour meant that seizures expressed by *Grin2b*<sup>+/+</sup> rats were too few for appropriate data normalisation and statistical comparisons. Consequently, the data from wild-type littermates were excluded from further analysis.

I found infusion of ethosuximide and memantine into the nRT of *Grin2b*<sup>+/-</sup> animals lead to a significant decrease in the amount of SWDs compared to saline (LMM, effect of treatment  $F = 31.35$ ,  $DF = 2$ ,  $P = 0.00067$ ; Tukey *post hoc* test, ethosuximide-saline  $P = 0.0016$ , ethosuximide-memantine  $P = 0.75$ , memantine-saline  $P = 0.0009$ ; Figure 5.2B and C). Relative to treatment with saline however, ethosuximide and memantine had no effect on reducing average SWD durations of seizures that did occur (LMM, effect of treatment  $F = 1.41$ ,  $DF = 2$ ,  $P = 0.32$ ; Figure 5.2B and C). Ultimately, the strong reduction in SWD events following ethosuximide and memantine administration to the nRT, resulted in decreased total seizure durations in *Grin2b*<sup>+/-</sup> rats, when treated with either drug compared to saline control (LMM, effect of treatment  $F = 31.87$ ,  $DF = 2$ ,  $P = 0.00064$ ; Tukey *post hoc* test, ethosuximide-saline  $P = 0.0016$ , ethosuximide-memantine  $P = 0.68$ , memantine-saline  $P = 0.0008$ ; Figure 5.2B and C). In contrast, ethosuximide and memantine application to the somatosensory cortex of *Grin2b*<sup>+/-</sup> rats had no effect on the number of SWDs (LMM, effect of treatment  $F = 0.15$ ,  $DF = 2$ ,  $P = 0.87$ ; Figure 5.2D and E), the average duration of seizures (LMM, effect of treatment  $F = 0.51$ ,  $DF = 2$ ,  $P = 0.62$ ; Figure 5.2D and E), and total SWD durations (LMM, effect of treatment  $F = 0.7$ ,  $DF = 2$ ,  $P = 0.53$ ; Figure 5.2D and E). The lack of effect in the somatosensory cortex is intriguing, as previous studies demonstrate SWD attenuation following block of T-type calcium channel bursting. The opposing observation here could be due to the differences in experimental protocols and pharmacological agents used between this and prior studies (Manning et al., 2004; McCafferty et al., 2018). Moreover, the somatosensory cortex is structurally large, encompassing a significant portion of the rostro-caudal and dorso-ventral cortex. It's therefore possible that the 1  $\mu$ l infusion volume, administered bilaterally (0.5  $\mu$ l in each hemisphere), did not sufficiently diffuse across the somatosensory cortex, or that too low a

concentration was used to elicit a detectable response. Overall, these preliminary data suggest that although both ethosuximide and memantine were able to modulate nRT activity to reduce SWD, likely by inhibiting nRT burst firing (McCafferty et al., 2018), once seizures initiated within the thalamocortical circuit, the network dynamics sustaining SWD activity remain largely unaffected. Notably, result found in Appendix A.2, which contains statistical analyses of site-specific infusion performed on absolute values, contrast the above finding adding emphasis on the need for replication of these experiments with sufficiently large sample sizes.

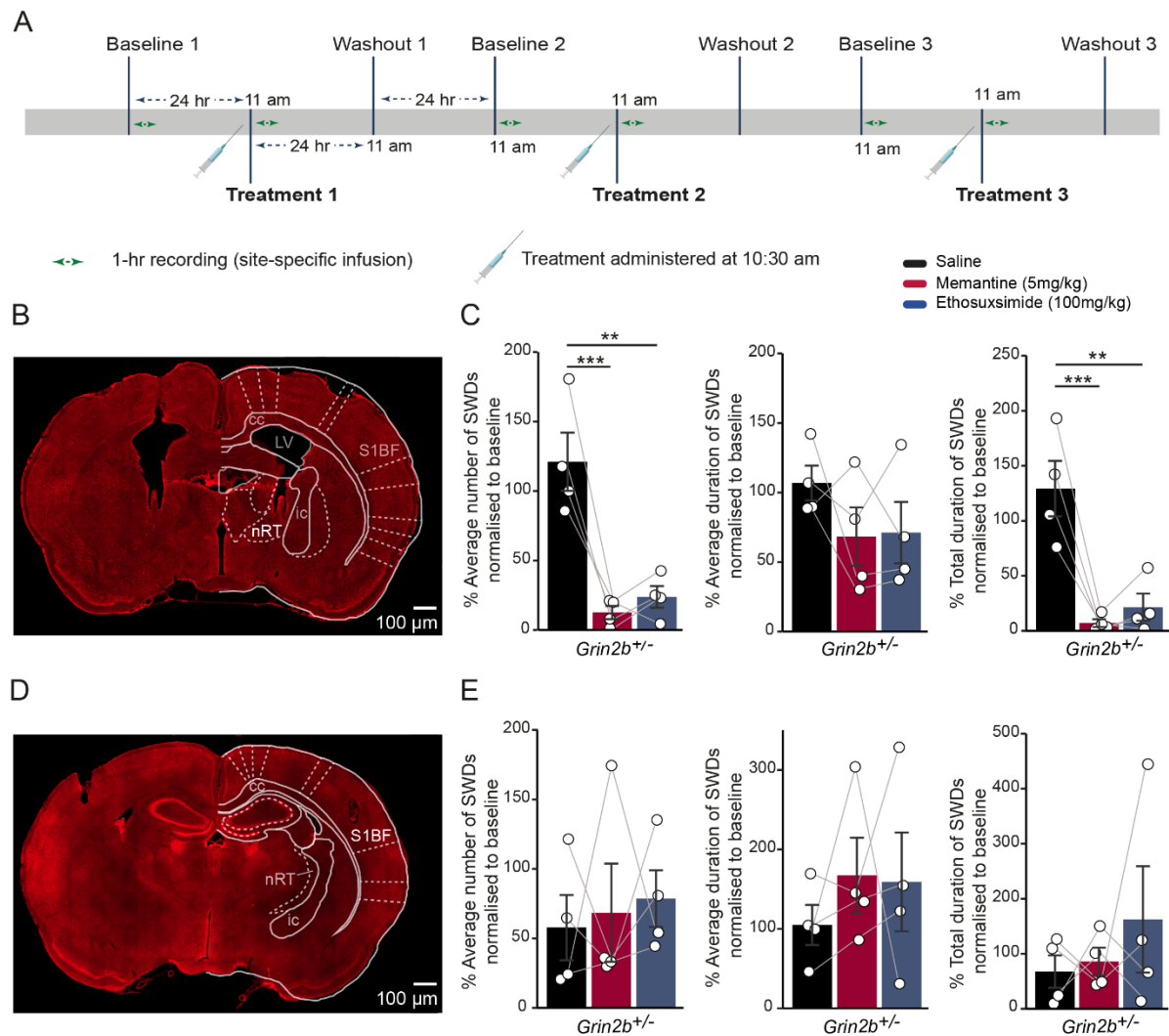


**Figure 5.2 Site-specific infusion of anti-seizure drugs in nRT attenuates SWDs in *Grin2b*<sup>+/-</sup> rats.** (A) Diagram of pharmacology experiment timeline. (B) Representative histology image showing bilaterally implanted cannulas in the rostral nRT, overlaid with approximate coordinates from Paxinos and Watson's Rat Brain Atlas (Paxinos & Watson, 2018). (C) Panels showing effects of acute nRT drug infusion on SWD average number (left), average duration (middle) and total duration (right) of SWDs in *Grin2b*<sup>+/-</sup> rats. Treatment with ethosuximide and memantine successfully blocked SWD events in *Grin2b*<sup>+/-</sup> animals (treatment  $P = 0.00067$  (ethosuximide-saline  $P = 0.0016$ , ethosuximide-memantine  $P = 0.75$ , memantine-saline  $P = 0.0009$ , LMM with Tukey post hoc tests), however, neither drug reduced average SWD durations in *Grin2b*<sup>+/-</sup> rats (treatment  $P = 0.32$ , LMM). Total seizure duration

was also significantly reduced following ethosuximide and memantine infusion to the nRT (treatment  $P = 0.00064$  (ethosuximide-saline  $P = 0.0016$ , ethosuximide-memantine  $P = 0.68$ , memantine-saline  $P = 0.0008$ ), LMM with Tukey post hoc tests). (D) Representative histology image showing bilaterally implanted cannulas in the somatosensory cortex, overlaid with approximate coordinates from Paxinos and Watson's Rat Brain Atlas (Paxinos & Watson, 2018). (E) Panels showing effects of acute somatosensory cortex drug infusion on SWD average number (left), average duration (middle) and total duration (right) of SWDs in *Grin2b*<sup>+/-</sup> rats. Infusion of ethosuximide and memantine had no effect on the number of SWDs (treatment  $P = 0.87$ , LMM), the average duration of seizures (treatment  $P = 0.62$ , LMM), and the total SWD durations (treatment  $P = 0.53$ , LMM). SIBF = primary somatosensory cortex - barrel field, nRT = reticular thalamic nucleus, LV = lateral ventricle, cc = corpus callosum, ic = internal capsule. For detailed statistics see results section. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats and grey lines follow treatment response of each individual rat.

## 5.4. Discussion

In this chapter, I demonstrate that SWDs in *Grin2b*<sup>+/-</sup> rats are attenuated by treatment with both ethosuximide and memantine. Systemic pharmacological treatment with ethosuximide was able to both reduce the number and average duration of absence seizures while memantine only reduced average SWD duration. However, both drugs decreased absence seizure durations only in *Grin2b*<sup>+/-</sup> animals and not wild-types, suggesting that memantine is effective in suppressing longer SWDs, such as those observed in *Grin2b*<sup>+/-</sup> rats. Alternatively, seizure generation and maintenance mechanisms are potentially different between the two genotypes. Preliminary data showed that infusion of either drug to the nRT produced a strong reduction in number of SWDs, however neither drug affected the duration of seizures once they initiated. Therefore, modulating T-type calcium channel and NMDAR activity in the nRT may serve as an effective treatment strategy by specifically preventing the initiation of SWDs. Both drugs are approved for clinical use and could prove to be translationally relevant options. Finally, I was unable to replicate SWD attenuation following drug infusion of either ethosuximide or memantine to the somatosensory cortex, which is at odds with previous studies (Manning et al., 2004; McCafferty et al., 2018; Polack et al., 2007).

I found SWDs in *Grin2b*<sup>+/-</sup> rats were sensitive to systemic treatment with ethosuximide and memantine. Ethosuximide is one of most widely used drugs for the treatment of absence seizures, and as in this study, successfully diminishes SWDs in rodent models and patients (Glauser et al., 2013; Penovich & James Willmore, 2009; Richards et al., 2003; Taylor et al., 2019), mainly through its antagonistic effect on T-type calcium channels. In contrast, I found memantine had no effect on the frequency of occurrence of SWDs, but did however, decrease the average duration of seizures. This effect could be due to memantine's action at the NMDAR magnesium binding site, which is only exposed during periods of membrane depolarization (H. Chen et al., 1992; H. S. Chen & Lipton, 1997).

Consequently, absence seizure initiation may remain unaffected but, after successive bouts of membrane depolarization during a SWD, the events may shorten when memantine blocks the receptor at the magnesium site. Generally, the effectiveness of memantine on drug-resistant early-onset epilepsy in individuals with gain of function *GRIN2* variants (*GRIN2A*, *GRIN2B* and *GRIN2D*) is mixed (Li et al., 2016; Pierson et al., 2014; Platzer et al., 2017; XiangWei et al., 2018) and previously, memantine treatment did not improve seizure frequency in four patients with *GRIN2B* gain of function mutations (Platzer et al., 2017). Moreover, there is an absence of documented memantine use in patients with loss of function mutations, although this is consistent with the current understanding of NMDAR pharmacology and the differential effects of gain versus loss of function mutations on glutamatergic signalling. With the above in mind, my data suggest that memantine could have favourable outcomes with respect to seizure manifestations in *Grin2b* haploinsufficiency, which contributes either loss of function or null NMDAR properties. However, the potential effects of memantine on seizures associated with *GRIN* mutations resulting in NMDAR hypofunction, or its use as an adjunctive therapy require further research and clinical evaluation before any firm conclusions can be drawn.

T-type calcium channels in thalamic nRT neurons are essential for absence seizures and blocking them with ethosuximide or selective channel antagonist TTA-P2 successfully diminishes SWDs (McCafferty et al., 2018; Richards et al., 2003). In *Grin2b*<sup>+/-</sup> rats, direct infusion of ethosuximide to the nRT acutely reduced the occurrence of SWDs. This aligns with prior studies in the GAERS model (McCafferty et al., 2018; Richards et al., 2003) and in mice with *Nlgn2* knockout (F. Cao et al., 2020), and supports the view that the thalamus is critically engaged, through generation of low-threshold calcium bursting, in the generalisation and maintenance of SWDs in *Grin2b*<sup>+/-</sup> rats.

While the exact pathophysiological mechanism leading to aberrant T-type channel activation in *Grin2b*<sup>+/-</sup> animals remains unknown, it is well established that the rhythmic output of thalamocortical circuitry is dependent on glutamatergic signalling, maintained within a narrow range. Altering excitatory input to and within the network leads to SWDs, as shown in stargazer, *Gria4* and *Scn8a* knockout mice (Barad et al., 2012; Makinson et al., 2017; Menuz & Nicoll, 2008; Paz et al., 2011). Considering the widespread expression of GluN2B-NMDARs throughout the thalamocortical circuit, and their role in facilitating intracortical, intrathalamic, and corticothalamic communication (discussed in Chapter 4, section 4.4) (Astori & Lüthi, 2013; Crabtree et al., 2013; S. S. Kumar & Huguenard, 2003; Topolski et al., 2024), it is reasonable to infer that thalamocortical glutamatergic transmission is likely compromised in *Grin2b*<sup>+/-</sup> rats. Shifting thalamic NMDAR activity to both extremities suppresses seizures (Koerner et al., 1996), therefore directly modulating NMDARs could potentially rescue the seizure phenotype in these animals.

Koerner et al., showed that intrathalamic NMDAR antagonism in the relay thalamus reduced SWDs in GAERS, by partially inhibiting the glutamatergic component of the thalamocortical network

in the thalamus (Koerner et al., 1996). My findings are complementary to this, as memantine infusion directly to the nRT produced a strong reduction in the amount of SWDs in *Grin2b*<sup>+/-</sup> rats. One potential mechanism for this effect is that blocking nRT NMDARs reduces firing of GABAergic neurons. Indeed, postsynaptic NMDARs excite inhibitory cells, thus NMDAR antagonism might reduce inhibitory firing in the nRT. A reduction in nRT inhibitory tone would then be expected to decrease the likelihood of membrane hyperpolarization, subsequently diminishing rhythmic low-threshold calcium currents in both the nRT and reciprocally connected thalamocortical relay neurons. This would ultimately lead to fewer SWDs. However, one contradiction to the above explanation is that prior work demonstrates increased nRT activation in response to NMDAR blockade. Specifically, Vaisanen and colleagues showed that expression of *c-fos*, an immediate early gene used as a marker of cell activation, was significantly higher in nRT neurons following treatment with a non-specific NMDAR antagonist (Vaisanen et al., 2004). Similarly, Zhang and colleagues found that blocking NMDARs induced hyperpolarisation of nRT cells *in vitro* (Y. Zhang et al., 2009). The authors further demonstrated that in nRT, NMDARs composed of GluN2C and GluN2B subunits, crucially contribute to inward currents at resting membrane potential in response to ambient glutamate (Crabtree et al., 2013; Y. Zhang et al., 2009). This implies that a subset of NMDARs within the nRT, particularly those containing GluN2C and GluN2B subunits, contribute to the maintenance of tonic cation influx, which potentially regulates inhibitory conductance and transmission of nRT GABAergic neurons. As mentioned in Chapter 4, intra-reticular GABAergic connections are thought to have a desynchronizing effect on thalamocortical network activity and decreasing inhibitory tone within the nRT does result in SWDs (Makinson et al., 2017). Thus, blocking NMDARs could paradoxically diminish inhibitory nRT output and consequently lead to increased SWDs. This does not appear to be the case in the present study, although the decrease in SWDs of *Grin2b*<sup>+/-</sup> animals does not categorically rule out the possibility of nRT activation following memantine infusion. The impact of heterozygous *Grin2b* knockout on thalamocortical structures and circuit activity is not yet known. Given the spatiotemporal *Grin2b* expression patterns, partial loss of GluN2B-NMDAR signalling presumably affects neuronal populations differentially based on their specific GluN2B-dependent signalling properties, resulting in seizures. Complete NMDAR blockade following memantine infusion, however, may lead to overall reduced excitatory neurotransmission, and decreased SWD activity. Further electrophysiological and molecular work is necessary to definitively characterize the effects of memantine on nRT neuronal activity and how this reflects SWD dynamics in *Grin2b*<sup>+/-</sup> rats.

*Grin2b*<sup>+/-</sup> animals exhibited SWDs in both the somatosensory cortex and thalamus that were temporally correlated (see Chapter 4, section 4.3.1). Although in *Grin2b*<sup>+/-</sup> rats, seizures were found to initiate in the somatosensory cortex, here, they appear to critically depend on the thalamus, as local infusion of either ethosuximide or memantine to the somatosensory cortex did not attenuate SWDs. The lack of SWD suppression by localized drug infusions was unexpected, considering that blocking activity

in the cortex diminishes SWDs in rats (Manning et al., 2004; McCafferty et al., 2018; Polack et al., 2007). The reasons for the lack of effect in *Grin2b*<sup>+/-</sup> rats are unclear, but could be due to limited drug diffusion, suboptimal drug concentrations and seizure network complexities that differ from those described in other rodent models of absence epilepsy. In *Grin2b*<sup>+/-</sup> rats, the circuit activities generating SWDs may be more extensive, involving broader cortical and subcortical regions beyond the drug application site. This hypothesis is supported by similar observations in *Nlgn2* knockout mice, where cortical application of ethosuximide also failed to reduce SWDs (F. Cao et al., 2020). Thus, while experimental factors may have contributed to the lack of SWD suppression, it is plausible that SWDs in *Grin2b*<sup>+/-</sup> animals could arise from alternate circuit mechanisms. Consequently, further investigation into the pathophysiology underlying absence seizures in this genetic model is needed to disentangle both cortical and thalamic contributions to SWDs in *Grin2b*<sup>+/-</sup> rats. A final yet substantial consideration is that the lack of drug-specific effects on SWDs when applied to the cortex may be due to a general reduction in SWDs following infusions altogether. Specifically, similar decreases in SWD numbers were observed after ethosuximide, memantine, and saline (control treatment) infusions. Therefore, my results do not fully clarify whether genuine drug-specific responses leading to the observed reductions were simply a result of the infusion process itself. Ultimately, replication of this dataset is necessary before conclusions regarding the efficacy of ethosuximide and memantine infused to the somatosensory cortex in *Grin2b*<sup>+/-</sup> rats can be made.

The data regarding the effects of memantine and ethosuximide infusion to the nRT and somatosensory cortex, as currently presented, are preliminary and require further refinement of investigation techniques before validity can be established. Drug infusion experiments, as employed here, represent a cost-effective and well-established approach for assessing the region-specific contributions of the brain to seizure generation and propagation. However, this method is not without its disadvantages, including low cell-type specificity, potential off-target effects, and limited temporal resolution in modulating neural activity. These constraints could be mitigated in future by employing more advanced techniques such as optogenetics and chemogenetics (Eelkman et al., 2021; Lindquist et al., 2023; Panthi and Leitch, 2021; Sorokin et al., 2017; Teng et al., 2022). Nevertheless, site-specific drug infusions via cannulation have proven useful in dissecting fundamental seizure mechanisms across multiple epilepsy models (F. Cao et al., 2020; Koerner et al., 1996; Manning et al., 2004; McCafferty et al., 2018; Polack et al., 2007; Richards et al., 2003), and as such were deemed appropriate for the investigation of SWDs in *Grin2b*<sup>+/-</sup> rats. One notable limitation of this study is the brief use of isoflurane anaesthetic during infusions. While this was necessary for achieving technical precision, it represents a significant confound in interpreting drug infusion results. Contemporary haloether volatile agents, including isoflurane, significantly inhibit T-type calcium channels, modestly block NMDARs and enhance GABA<sub>A</sub>-mediated inhibitory currents (Brosnan, 2011; Feseha et al., 2020). Although SWDs in *Grin2b*<sup>+/-</sup> animals were quantified 30 minutes post infusions and exposure to isoflurane, when most of

the isoflurane is expected to be eliminated from the rat brain (see Chapter 2 for reasoning, section 2.5.2) (Subcommittee on Laboratory Animal Nutrition et al., 1995; Wyrwicz et al., 1987), the observed pharmacological effects of memantine and ethosuximide are likely to be influenced by trace amounts of residual isoflurane. An additional caveat is that while the majority of infused ethosuximide and memantine likely blocked T-type calcium channels and NMDARs within the nRT, it is possible that the same occurred in adjacent thalamocortical relay neurons as a result of diffusion dynamics. Regarding memantine, given that SWD attenuation was observed following NMDAR blockade in the relay thalamus (Koerner et al., 1996), and that evidence indicates nRT is active and hyperpolarized in response to NMDAR inactivation (Vaisanen et al., 2004; Zhang et al., 2009), it seems probable that the observed results in *Grin2b*<sup>+/-</sup> rats are at least partly due to NMDAR antagonism of thalamocortical cells in addition to blockade in nRT. This consideration likely extends to the effects of ethosuximide.

To address these limitations and provide a clearer understanding of the mechanisms underlying SWD reduction in *Grin2b*<sup>+/-</sup> animals, it will be important to replicate site-specific drug infusion experiments using more precise drug localization techniques, while also eliminating the need for anaesthetics. For instance, habituating animals to head restriction during infusions, or alternatively, delivering drugs through reverse microdialysis probes (McCafferty et al., 2018), would significantly improve on experimental technique, data quality, and animal welfare. To achieve this, the exact concentration and dialysis time period needed to block activity in the nRT but not ventrobasal thalamus would first need to be established. Dose and time response calculations for thalamic reverse microdialysis have previously been conducted using silicone neural probes in conjunction with microdialysis implants (David et al., 2013). For instance, dose and time dependent analyses for the T-type calcium channel blocker TTA-P2 have demonstrated that administering a 300  $\mu$ M concentration via a probe placed at the core of the relay thalamus effectively suppresses T-channel bursts within a region encompassing the entire nucleus. This effect occurs within approximately 20 minutes and remains specific to relay thalamic neurons, leaving T-type channel activity in the nRT unaffected (David et al., 2013; McCafferty et al., 2018). By employing the above methods and establishing dose and time response variables for ethosuximide and memantine, a microdialysis application similar to that of McCafferty and colleagues (2018) would ensure localized and sustained drug delivery, minimizing off-target diffusion and allowing for precise pharmacological interrogation of thalamic network contributions to SWD generation in *Grin2b*<sup>+/-</sup> animals. A further step would then be to dissect nRT NMDAR subunit specific contributions to SWDs by replicating these experiments using ifenprodil, Zn<sup>2+</sup> and QNZ46, which block GluN2B, GluN2A and GluN2C/D expressing NMDARs respectively (Mosley et al., 2010; Paoletti et al., 2013). Ultimately, refined experimental protocols, such as the above, would enable a more precise investigation of the distinct contributions of different thalamic and cortical regions to SWD generation and suppression in *Grin2b*<sup>+/-</sup> rats, providing clearer insights into the mechanisms underlying absence seizures in this model.

In summary, I demonstrate seizures in *Grin2b*<sup>+/-</sup> animals are sensitive to ethosuximide and memantine and propose both drugs could favourably impact on absence seizure severity. I found *Grin2b*<sup>+/-</sup> rats showed a strong reduction in SWDs following systemic and nRT-specific drug administration, however, drug infusion into the somatosensory cortex failed to decrease seizure occurrences. This differs from previous research that shows pharmacologically targeting the cortex attenuates SWDs in multiple rodent models of absence epilepsy (Manning et al., 2004; McCafferty et al., 2018; Polack et al., 2007) and suggests there may be an alternative seizure generating mechanisms in the *Grin2b*<sup>+/-</sup> rats. Whilst this may be the case, I highlight possible experimental confounds that should be addressed prior to drawing definitive conclusions.



# Chapter 6

## Concluding Remarks and Future Directions

A significant proportion of individuals with NDDs exhibit sleep dysregulation, characterized by impaired sleep onset latency, reduced sleep efficiency, compromised sleep architecture, and circadian rhythm perturbations (Angriman et al., 2015; Barone et al., 2019). Such sleep abnormalities are frequently accompanied by comorbid neurological conditions, and notably epilepsy, which is prevalent in up to 26% of NDD cases (American Psychiatric Association, 2013). The interplay between genetic factors, neurological pathophysiology, and comorbid conditions poses significant challenges in understanding and treating sleep dysfunction and seizures in rare NDDs. Furthermore, clinical evidence on sleep architecture and circadian rhythm alterations relating to specific genetic NDDs is scarce, hampered by methodological inconsistencies, insufficient statistical power, and substantial phenotypic heterogeneity. To date, preclinical genetic NDD models that demonstrate face validity have proven to be useful tools for investigating neurobiological mechanisms underlying seizures, sleep and circadian disturbances, as well as testing potential therapeutic strategies (Gonzalez-Sulser, 2020; Lázaro & Golshani, 2015; Silverman et al., 2022). As such, the possibility of providing quantitative data on seizure symptomatology and sleep physiology impairments in genetic rodent lines that accurately recapitulate clinical disorder features motivated the present work. Using continuous wireless polysomnography and pharmacological intervention, this thesis explored sleep and seizure characteristics in rat models of NDDs associated with deletions in the *Nlgn3* and *Grin2b* genes and the *16p11.2* chromosomal locus.

In Chapter 3, I presented distinct abnormal sleep phenotypes in *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> rat models of NDDs. I show REM sleep increases in male *16p11.2*<sup>+/-</sup> animals, which aligns with the male-specific effect of *16p11.2* mutations in multiple previous reports (Angelakos et al., 2017; Grissom et al., 2018; V. J. Kumar et al., 2018; H.-C. Lu et al., 2019; Martin Lorenzo et al., 2023; Openshaw et al., 2023). I describe altered sleep-wake physiology in *Nlgn3*<sup>-/-</sup> rats, consistent with the role of *Nlgns* in regulating sleep and circadian processes (El Helou et al., 2013; Hannou et al., 2018; Seok et al., 2018; Thomas et al., 2017). Finally, I demonstrate REM sleep deficits and a compromised sleep architecture in the novel *Grin2b* haploinsufficiency rat line, therefore providing initial quantitative assessment of aberrant sleep patterns associated with *GRIN2B* haploinsufficiency. In Chapter 4, I reveal the presence of absence seizures in *Grin2b*<sup>+/-</sup> rats, and correlate seizure events to sleep-wake physiology

abnormalities in these animals. Notably, my data suggest this is the first preclinical rodent model of *Grin2b* haploinsufficiency to exhibit a robust and reliable seizure phenotype. This will be useful for studying underlying pathophysiological processes associated with the mutation and the effects of therapeutic interventions. Finally, in Chapter 5, I successfully blocked seizures in *Grin2b*<sup>+/-</sup> animals using ethosuximide and memantine and demonstrate that the thalamocortical circuit is critically involved in mediating absence seizures in this rat line.

The neurobiological mechanisms underlying cognitive impairment, sleep dysfunction, and seizures in genetic NDDs likely overlap or significantly influence each other (Malow, 2004), although the exact mechanisms are still unknown. The long-term objective of this thesis was to therefore lay the groundwork for future research focused on identifying brain region, cell type-specific, and molecular mechanisms that underly the various sleep and circadian disruptions and mediate seizures in these disorders, thereby enabling the development of more targeted and personalized treatment strategies.

## 6.1. Genetic mutations lead to variable sleep and seizure phenotypes

NDDs arise from the alteration of normal brain developmental trajectories, impacting the function of specific neuronal networks by disrupting broad regulatory pathways, synaptic function and normal circuit assembly processes. Furthermore, numerous lines of evidence indicate that genetic mutations associated with NDDs exert heterogeneous effects on specific brain regions and neural circuits that manifest in a range of phenotypes, including in sleep impairment, epilepsy, and alterations in learning, memory, and social behaviour. This is because the developmental expression patterns and functional significance of genes are generally distinct, and contribute to different molecular, synaptic and signalling mechanisms (Barone et al., 2019; Exposito-Alonso & Rico, 2022). Findings from this thesis demonstrate that deletions in *16p11.2*, *Nlgn3*, and *Grin2b* variably impair sleep and circadian rhythmicity and lead to the emergence of seizures, therefore their impacts on brain development and network activity are specific, affecting different brain structures and cellular subtypes.

A large proportion of genes that confer risk of NDDs encode cell-surface proteins involved in the structural and functional assembly of synapses, receptor subunits crucial to synaptic transmission, and molecules that mediate intracellular regulatory cascades. Neuroligins are key synaptic molecules that play vital roles in synaptic organization and are frequently implicated in ASD and ID. *Nlgn3* mutations disrupt these mechanisms in cortical, striatal, and midbrain circuits amongst others, affecting circuit development and connectivity between specific inhibitory and excitatory synapses (Exposito-Alonso & Rico, 2022; Uchigashima et al., 2021). Chapter 3 revealed that *Nlgn3* knockout in male rats induces mild, time-dependent alterations in NREM sleep and wake without affecting overall sleep-wake

distribution. These findings align with previous research showing *Nlgn3* loss leads to signalling abnormalities in sleep-wake associated structures, including the striatum and PAG (Anstey et al., 2022; J. R. Cho et al., 2017; Földy et al., 2013; Oishi et al., 2017; Rothwell et al., 2014; Sun et al., 2024). Moreover, *Nlgn3* likely undergoes circadian transcriptional regulation (El Helou et al., 2013; Hannou et al., 2018), and its absence may impair circadian modulation of synaptic excitatory and inhibitory transmission leading to imbalanced sleep-wake modulation signalling, reflected in the observed altered sleep phenotypes.

Strong genetic evidence supports impaired glutamate receptor signalling in NDDs, and synaptic strength changes in response to neuronal activity rely on NMDAR activation. Mutations affecting NMDARs are believed to render developing circuits more susceptible to dysfunction by disrupting glutamate receptor-dependent synaptic homeostatic scaling, crucial for network stability during critical developmental periods (Exposito-Alonso & Rico, 2022). Indeed, *Grin2b* variants impair neuronal differentiation, migration and prenatal circuit activity during early development (Bell et al., 2018; H. Jiang et al., 2015; Munz et al., 2023) and in adults differentially impacts intrinsic cellular excitability and morphology of hippocampal, cortical and olfactory bulb neurons (Akashi et al., 2009; Bahry et al., 2021; Brigman et al., 2010; Espinosa et al., 2009; Gray et al., 2011; B. J. Hall et al., 2007; Kelsch et al., 2012, 2014; M. J. Kim et al., 2005; S. Liu et al., 2017a; Sceniak et al., 2019a), leading to variable NDD outcomes and epilepsy. Here, *Grin2b*<sup>+/-</sup> male and female rats, were observed to have REM sleep deficits, altered sleep-wake physiology (Chapter 3), and pharmacologically sensitive absence seizure SWDs (Chapters 4 and 5). Absence seizure and NREM sleep oscillations are dependent on thalamocortical interactions (Crunelli et al., 2015; Crunelli & Hughes, 2010; David et al., 2013; Lindquist et al., 2023), making it likely that the altered NREM sleep phenotype and the presence of SWDs in this line arise from shared network abnormalities, including those in cortical, thalamic and hypothalamic circuits. Conversely, REM deficits presumably stem from distinct mechanisms and regions impacted by loss of GluN2B-NMDAR activity, for instance the VLPO of hypothalamus, which mediates NMDAR-dependent REM and NREM expression (Miracca et al., 2022). Alternatively, downstream seizure-related processes, such as imbalance of neuromodulators or disruption of regular circadian patterns may account for reduced REM sleep (Bazil et al., 2000).

NDD and epilepsy related CNVs encompass a range of genes that impact on multiple domains of intracellular and circuit level signalling (Exposito-Alonso & Rico, 2022; Parenti et al., 2020). Many chromosome *16p11.2* genes, converge on the ERK/MAPK cascade, involved in brain development, synapse formation, as well as sleep regulation (Leone et al., 2024). ERK1 expression is abnormal in *16p11.2*<sup>+/-</sup> male mice and thought to contribute to the pathology profiles associated with *16p11.2* deletions by impacting on ERK/MAPK activity (Grissom et al., 2018; Pucilowska et al., 2015). Additionally, heterozygous *16p11.2* deletion in mice results in irregular dopaminergic and serotonergic transmission, both crucial for sleep-wake physiology, influencing sleep timing, induction, and duration

in a region and cell type-specific manner (Arnaldi et al., 2015; Brown et al., 2012; Grissom et al., 2018; Hasegawa et al., 2022; Mitchell et al., 2020; Panzini et al., 2017; Portmann et al., 2014; Vetrivelan et al., 2010; Walsh et al., 2018). From a network perspective, cortico-striatal, hypothalamic, and brainstem circuit signalling, and connectivity abnormalities are found in patients with *16p11.2* deletion CNVs (Chung et al., 2021; Owen et al., 2014; Qureshi et al., 2014) and in preclinical mouse models of *16p11.2* deletion syndrome (Choi et al., 2024; V. J. Kumar et al., 2018; H.-C. Lu et al., 2019). Importantly, transmission between the hypothalamus and the brainstem mediates REM and NREM sleep expression (Brown et al., 2012; Scammell et al., 2017). I propose that similarly dysregulated signalling properties and aberrant connectivity could mechanistically account for the increased REM sleep phenotype observed in *16p11.2*<sup>+/-</sup> male rats in Chapter 3 of this study.

Perturbations to the described processes are linked to epilepsy, sleep dysfunction, ID, and stereotyped behaviours associated with ASD. The sleep and seizure phenotypes observed in rat models with mutations in *16p11.2*, *Nlgn3* and *Grin2b* (Chapters 3 to 5) provide valuable insights into potential molecular and network mechanisms contributing to these disorders. These findings highlight the complex relationship between genetic alterations and their effects on neuronal and network activity, demonstrating that various genetic mutations can lead to NDDs through diverse biological pathways. The variability in sleep and seizure phenotypes across the three genetic models used here reflects this heterogeneity and emphasizes the importance of understanding mutation-specific effects on brain function.

## 6.2. Implications for sleep, epilepsy and neurodevelopmental disorder research

Findings from this thesis advance the understanding of sleep dysfunction and seizure pathology in rare genetic NDDs by capturing distinct, mutation-specific alterations in sleep architecture and seizure manifestations. Prior research has predominantly relied on mouse models, which, while informative, have yielded conflicting findings, specifically, regarding the impact of *16p11.2* deletions and *Nlgn3* mutations on sleep. The use of rat models in this study has revealed sleep phenotypes that diverge from those previously reported, such as the unexpected increase in REM sleep in male *16p11.2*<sup>+/-</sup> rats. These findings highlight the necessity of cross-species validation in preclinical research and raise important questions about species-specific differences in sleep-wake regulation. Moving forward, comparative studies across multiple rodent models, alongside unified sleep analyses protocols, could refine our understanding of how genetic mutations differentially affect sleep processes and

provide insight into the translational relevance of specific animal models for human NDDs and epilepsies.

This study provides the first quantitative characterization of sleep abnormalities in *Grin2b*<sup>+/-</sup> rats, revealing REM sleep deficits and pharmacologically modifiable absence seizures. The presence of absence seizures in this model aligns with evidence implicating the thalamocortical circuit in both seizure activity and sleep regulation, suggesting a potentially shared pathophysiological basis. This overlap raises important questions about how disrupted NMDAR function in this circuit contributes to both epileptiform activity and sleep impairments. Future studies could leverage these findings to dissect the molecular and network-level mechanisms underlying sleep-seizure interactions, potentially identifying therapeutic targets that simultaneously address both impairments.

More broadly, this work supports the view that sleep dysfunction in NDDs is a fundamental neurobiological feature rather than a secondary consequence of cognitive or neurological deficits. By demonstrating distinct, mutation-specific sleep and seizure phenotypes, this study reinforces the need for personalised investigative and therapeutic strategies to address both sleep disturbances and seizure susceptibility in NDDs. Given the high comorbidity of epilepsy and sleep disorders in individuals with NDDs, understanding their bidirectional relationship will be crucial for developing targeted treatments. Determining when and how sleep disruption shapes neurodevelopmental trajectories, cognitive function, and seizure susceptibility will be key to designing interventions that improve both sleep quality and overall neurological outcomes. The findings from this thesis lay the foundation for these future investigations.

### 6.3. Sleep and brain network dysfunction

Sleep plays a crucial role in brain development and function, modulating synaptic plasticity, pruning, and connectivity. It is essential for synaptic homeostasis, with upscaling and downscaling of synaptic strength occurring during wake and sleep respectively (Kaida et al., 2023; Klinzing et al., 2019). The frequency of sleep impairments observed in NDDs and epilepsy are suggestive of a complex interplay between these conditions. The onset of sleep problems often correlates with the progression of comorbid conditions including ASD, ID and epilepsy, whilst further evidence indicates reciprocal relationships between these disorders (Doldur-Balli et al., 2022). This relationship creates a cycle where the worsening of one condition contributes to the deterioration of others and is particularly evident in the co-occurrence of sleep disorders and epilepsy (Moore et al., 2021). Furthermore, sleep disturbances may serve as a marker of circuit pathology in NDDs and epilepsy. Changes in sleep architecture, such as reduced sleep spindles or altered REM sleep patterns reflect underlying neural network dysfunctions.

These sleep-related markers provide valuable insights into the extent of network alteration and may serve as early indicators of disease progression or treatment efficacy (Buckley et al., 2010; Farmer et al., 2018; Ostrowski et al., 2021).

Sleep impairments and epilepsy, alongside other NDDs are believed to arise from shared network mechanisms, although the precise aetiology remains unclear. Presumably these disorders stem from synaptic dysfunction and atypical brain network development, spanning circuits involved in cognitive function, sensory processing and sleep (Barone et al., 2019; Doldur-Balli et al., 2022; Moore et al., 2021). Moreover, sleep impairment likely exacerbates synaptic dysfunction in related disorders, creating a feedback loop where disturbed sleep further compromises synaptic connectivity and neural circuitry, accelerating the progression and severity of NDDs and epilepsy (Doldur-Balli et al., 2022). Sleep disturbances thus are both a symptom and a contributing factor to the progression of neurodevelopmental and epileptic disorders, and therefore present an important avenue for therapeutic interventions in these interrelated disorders that could mitigate the progression of associated symptoms and enhance overall brain function.

## 6.4. Future directions

The work presented in this thesis highlights novel sleep dysfunction and epilepsy phenotypes in three genetic rat models of human NDDs and provides a foundation for exploring the pathophysiology underlying sleep problems and seizures reported in individuals diagnosed with these disorders. The different sleep patterns and seizure events found between *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>+/-</sup> and *Grin2b*<sup>+/-</sup> rats point to alternate network signalling abnormalities in distinct circuits and structures. As such the next step forward will be to investigate these in depth. *In vivo* unit recordings, *ex vivo* electrophysiology, molecular techniques (e.g. immunoblotting, fluorescence in situ hybridization), and genetic manipulations including optogenetics and viral tracing will provide further insight into the neuronal and circuit level changes mediating the phenotypes reported in this dissertation (Choi et al., 2024; C. Ma et al., 2019; Z. Ma et al., 2022; Topolski et al., 2024). In addition, transcriptomic analysis could be useful in determining the influence of *Nlgn3*, *Grin2b* and genes found on the *16p11.2* locus on sleep regulation and circadian entrainment pathways (Z. Ma et al., 2022; Z. Wang et al., 2018).

A critical feature of NDDs is their emergence during the developmental periods. The experimental insights from this work are limited to adult animals and do not provide information on when sleep impairment and seizure activity might emerge in *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> rats. Therefore, future studies employing innovative technologies, such as those mentioned above, to characterize sleep and seizures across development would be beneficial and could elucidate on when

specific network alterations arise and would be best targeted. Considering the importance of neonatal sleep on developing brain circuits and connectivity (Del Rio-Bermudez et al., 2020; Del Rio-Bermudez & Blumberg, 2018), characterising the sleep profile of *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> neonatal rats could inform on whether sleep perturbations are present during the developmental periods, and therefore potentially contribute to the abnormal circuit formation, seizures and overall dysregulation of sleep and circadian rhythms in adult rats. Additionally, advanced genetic techniques, such as viral-mediated FLEX Cre and tamoxifen-inducible Cre systems, combined with cell-type and time specific promoters, could inform on the critical regional dependencies and developmental stages of certain genes (Atasoy et al., 2008; Donocoff et al., 2020; Schnütgen et al., 2003). Such approaches may also allow for adult-specific restoration of NDD-associated genes or chromosomal regions and thus elucidate whether observed phenotypes in *16p11.2*<sup>+/-</sup>, *Nlgn3*<sup>-/-</sup> and *Grin2b*<sup>+/-</sup> rat models persist following genetic restoration in adulthood. Persistence of these phenotypes would suggest a limited window for intervention, indicating that certain alterations may become irreversible beyond specific developmental stages. Such findings would significantly impact therapeutic strategies for NDDs, emphasizing the importance of early intervention or necessitating alternative approaches for adult patients.

A benefit of the findings from this thesis is the possibility to exploit these rat models to test pharmacologic interventions and potential treatment strategies for the amelioration of sleep impairments and seizures (Riva et al., 2021; Yuge et al., 2020). I was able to demonstrate this in Chapter 5 by pharmacologically attenuating SWDs in *Grin2b*<sup>+/-</sup> animals. Looking towards the future, it will be interesting to replicate these experiments with a focus on restoring sleep to physiologically normal levels. Additionally, other therapeutic modalities including brain stimulation, targeting of neuroinflammation, and gene therapy could be investigated to further enhance treatment outcomes and provide comprehensive strategies for managing these disorders in the clinic (Riva et al., 2021). Ultimately, understanding the mechanisms underlying NDDs and epilepsy will allow for the development of more targeted, efficacious therapeutic interventions that could significantly improve the well-being of individuals affected by these disorders.



# Appendix A

## A.1 Absolute value analysis of systemic pharmacological block on SWDs in *Grin2b*<sup>+/-</sup> rats

To assess the robustness of the findings presented in Chapter 5 section 5.3.1 (Figure 5.1), additional analyses using absolute values rather than normalised data were conducted. Pharmacological treatment decreased SWDs in both *Grin2b*<sup>+/-</sup> and *Grin2b*<sup>+/+</sup> rats, with *post hoc* analyses showing that ethosuximide effectively reduced seizures compared to saline (LMM, effect of treatment  $F = 5.69$ ,  $DF = 2$ ,  $P = 0.0091$ ; genotype  $F = 2.08$ ,  $DF = 1$ ,  $P = 0.18$ ; treatment x genotype  $F = 0.84$ ,  $DF = 2$ ,  $P = 0.44$ ; Tukey *post hoc* test, ethosuximide-saline  $P = 0.0074$ , ethosuximide-memantine  $P = 0.066$ , memantine-saline  $P = 0.61$ ; Figure A.1B). Similarly to the normalised dataset in Chapter 5, both ethosuximide and memantine were effective in significantly reducing the average duration of SWDs in *Grin2b*<sup>+/-</sup> but not *Grin2b*<sup>+/+</sup> animals (LMM, effect of treatment  $F = 15.16$ ,  $DF = 2$ ,  $P < 0.0001$ ; genotype  $F = 2.19$ ,  $DF = 1$ ,  $P = 0.16$ ; treatment x genotype  $F = 5.65$ ,  $DF = 2$ ,  $P = 0.0089$ ; Tukey *post hoc* test, *Grin2b*<sup>+/-</sup> ethosuximide-saline  $P < 0.0001$ , *Grin2b*<sup>+/-</sup> ethosuximide-memantine  $P = 0.2$ , *Grin2b*<sup>+/-</sup> memantine-saline  $P < 0.0001$ , *Grin2b*<sup>+/+</sup> ethosuximide-saline  $P = 0.5$ , *Grin2b*<sup>+/+</sup> ethosuximide-memantine  $P = 0.99$ , *Grin2b*<sup>+/+</sup> memantine-saline  $P = 0.48$ , Figure A.1C). The reduction in both the number and duration of SWDs likely contributed to ethosuximide, but not memantine, significantly decreasing total seizure duration in both rat genotypes (LMM, effect of treatment  $F = 3.85$ ,  $DF = 2$ ,  $P = 0.034$ ; genotype  $F = 2.28$ ,  $DF = 1$ ,  $P = 0.16$ ; treatment x genotype  $F = 1.28$ ,  $DF = 2$ ,  $P = 0.29$ ; Tukey *post hoc* test, ethosuximide-saline  $P = 0.025$ , ethosuximide-memantine  $P = 0.28$ , memantine-saline  $P = 0.45$ ; Figure A.1D).

The results shown in Figure A.1 are alike those obtained from the normalised data, indicating that the observed effects are not artifacts of data normalisation but instead reflect genuine differences between conditions. Given the reasonable 2-hour duration of the analysis, inter-animal variability remained relatively low, allowing for meaningful interpretation of absolute values. These findings support the reliability of the reported effects and reinforce the conclusions drawn in Chapter 5 section 5.3.1.

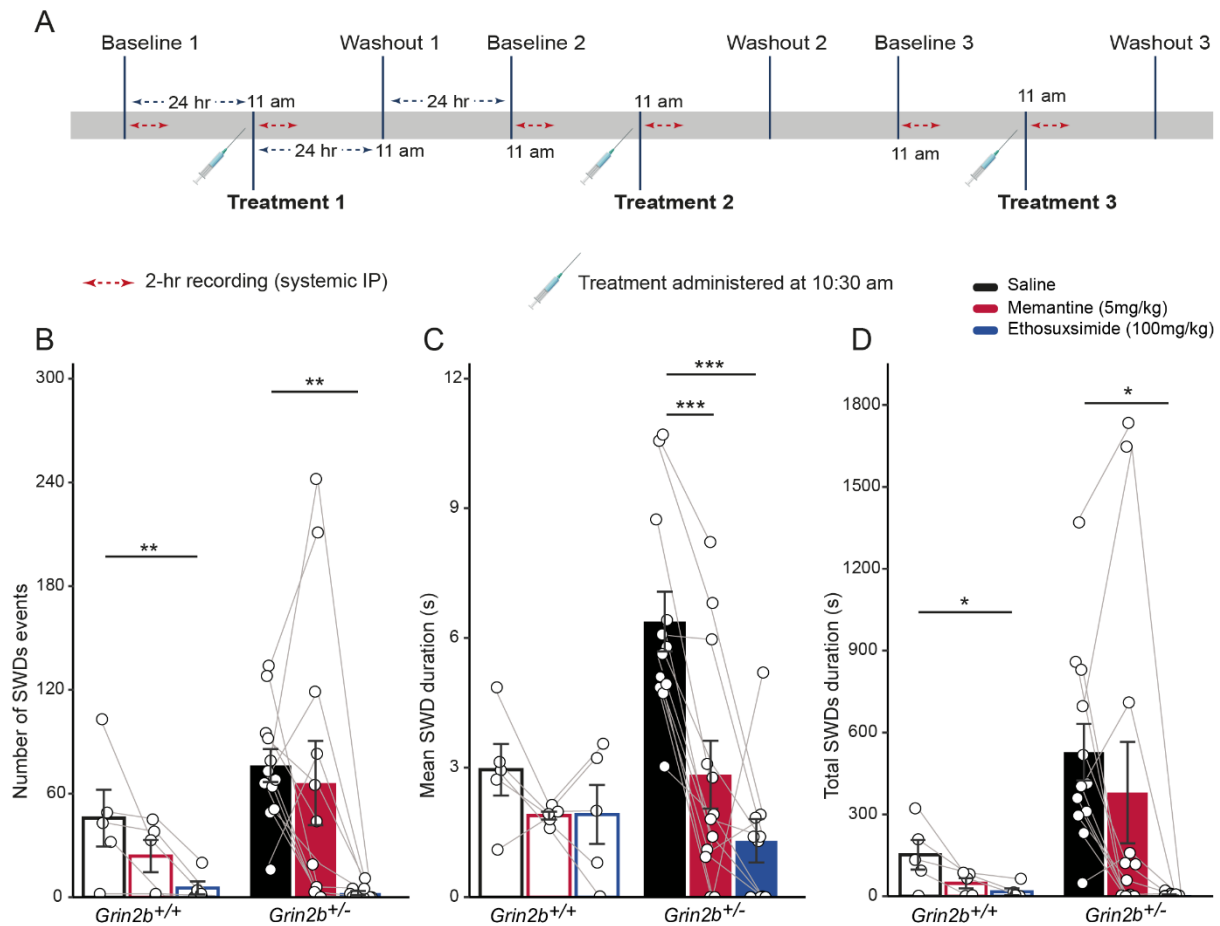


**Figure A.1 Acute administration of anti-seizure drugs attenuates SWDs in *Grin2b*<sup>+/-</sup> and wild-type animals - absolute value analysis.** (A) Diagram of pharmacology experiment timeline. Panels showing effects of acute drug treatment on SWD number (B), average duration (C) and total duration (D) of SWDs in *Grin2b*<sup>+/-</sup> and wild-type animals. Treatment with ethosuximide successfully blocked SWD events in both groups (treatment  $P = 0.0091$  (ethosuximide-saline  $P = 0.0074$ ), genotype  $P = 0.18$ , treatment  $\times$  genotype  $P = 0.44$ , LMM with Tukey post hoc tests) (B). Memantine and ethosuximide reduced average SWD duration in *Grin2b*<sup>+/-</sup> but not wild-type rats (treatment  $P < 0.0001$ , genotype  $P = 0.16$ , treatment  $\times$  genotype  $P = 0.0089$  (*Grin2b*<sup>+/-</sup> ethosuximide-saline  $P < 0.0001$ , memantine-saline  $P < 0.0001$ ), LMM with Tukey post hoc tests) (C). Total seizure duration was also significantly reduced following ethosuximide treatment in both *Grin2b*<sup>+/-</sup> and wild-type rats (treatment  $P = 0.034$  (ethosuximide-saline  $P = 0.025$ ), genotype  $P = 0.16$ , treatment  $\times$  genotype  $P = 0.29$ , LMM with Tukey post hoc tests) (D). For detailed statistics see Appendix A.1. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats and grey lines follow treatment response of each individual rat.

## A.2 Absolute value analysis of site-specific pharmacological block on SWDs in *Grin2b*<sup>+/-</sup> rats

In Chapter 5 section 5.3.2 (Figure 5.2), direct infusion of ethosuximide and memantine to the nRT reduced the number of SWDs in *Grin2b*<sup>+/-</sup> animals, while infusion of either drug to the somatosensory cortex had no effect SWD numbers or durations. To evaluate the reliability of these findings, supplementary statistical analyses were performed using absolute values instead of normalized data.

Analyses using absolute values showed infusion of drugs to the nRT had no effect on the number of SWDs (LMM, effect of treatment  $F = 3.33$ ,  $DF = 2$ ,  $P = 0.11$ ; Figure A.2B and C), average SWD duration (LMM, effect of treatment  $F = 1.67$ ,  $DF = 2$ ,  $P = 0.27$ ; Figure A.2B and C), and total SWD duration (LMM, effect of treatment  $F = 2.38$ ,  $DF = 2$ ,  $P = 0.17$ ; Figure A.2B and C). These data contrast finding in section 5.3.2, where normalisation was applied. Likewise, when absolute values were considered in statistical analyses of treatment infusions in the somatosensory cortex, no differences were found between treatments for the number of SWDs (LMM, effect of treatment  $F = 0.27$ ,  $DF = 2$ ,  $P = 0.77$ ; Figure A.2D and E), average SWD duration (LMM, effect of treatment  $F = 3.29$ ,  $DF = 2$ ,  $P = 0.11$ ; Figure A.2D and E), and total SWD duration (LMM, effect of treatment  $F = 1.39$ ,  $DF = 2$ ,  $P = 0.32$ ; Figure A.2D and E), which is consistent with the observations in Chapter 5 section 5.3.2.

Although not statistically significant, the data on drug infusions in the nRT (Figure A.2) followed a pattern similar to that observed in the normalized dataset (Chapter 5, Figure 5.2), suggesting that the effects of memantine and ethosuximide may be genuine, particularly with larger sample sizes. The discrepancies in findings here and in Chapter 5 section 5.3.2 are likely due to high variability, which normalization helps mitigate. In contrast, analyses of drug infusions into the somatosensory cortex using absolute values showed no significant treatment effects, which is consistent with the findings from the normalized dataset. As discussed in this thesis, site-specific infusion data remain preliminary and require replication with sufficient sample sizes and statistical power to ensure reliability. The absence of a significant effect in the absolute value analysis adds further emphasis to this.

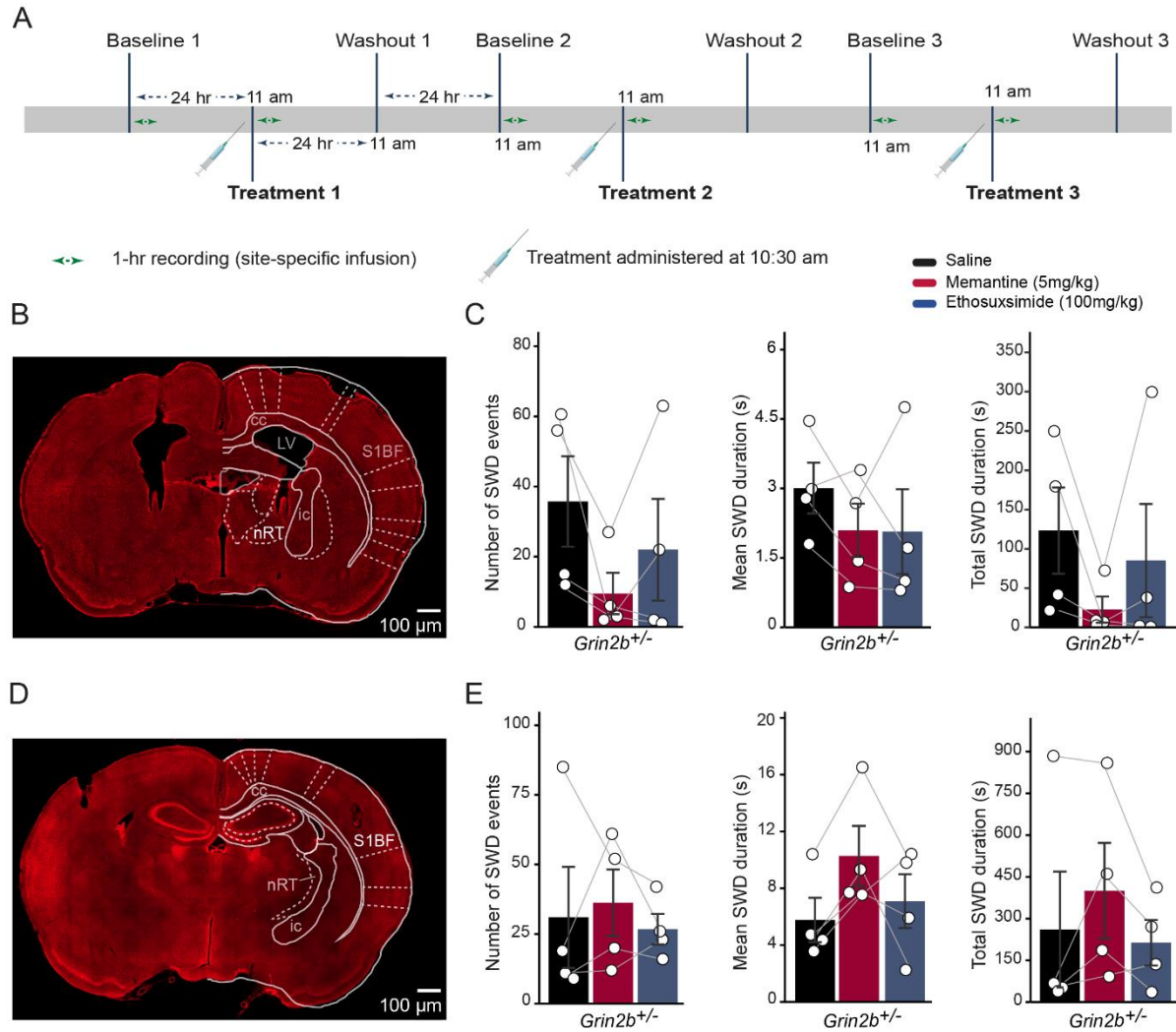


**Figure A.2 Site-specific infusion of anti-seizure drugs in nRT attenuates SWDs in  $Grin2b^{+/-}$  rats - absolute value analysis.** (A) Diagram of pharmacology experiment timeline. (B) Representative histology image showing bilaterally implanted cannulas in the rostral nRT, overlaid with approximate coordinates from Paxinos and Watson's Rat Brain Atlas (Paxinos & Watson, 2018). (C) Panels showing effects of acute nRT drug infusion on SWD average number (left), average duration (middle) and total duration (right) of SWDs in  $Grin2b^{+/-}$  rats. Treatment with ethosuximide and memantine does not block SWD events in  $Grin2b^{+/-}$  animals relative to saline control treatment (treatment  $P = 0.11$ , LMM). Neither drug reduces average SWD durations (treatment  $P = 0.27$ , LMM) or total seizure durations (treatment  $P = 0.17$ , LMM) compared to saline in  $Grin2b^{+/-}$  rats. (D) Representative histology image showing bilaterally implanted cannulas in the somatosensory cortex, overlaid with approximate coordinates from Paxinos and Watson's Rat Brain Atlas (Paxinos & Watson, 2018). (E) Panels showing effects of acute somatosensory cortex drug infusion on SWD average number (left), average duration (middle) and total duration (right) of SWDs in  $Grin2b^{+/-}$  rats. Infusion of ethosuximide and memantine had no effect on the number of SWDs (treatment  $P = 0.77$ , LMM), the average duration of seizures (treatment  $P = 0.11$ , LMM), and the total SWD durations (treatment  $P = 0.32$ , LMM). S1BF = primary somatosensory cortex - barrel field, nRT = reticular thalamic nucleus, LV = lateral ventricle, cc = corpus callosum, ic = internal capsule. For detailed statistics see Appendix A.2. Bars indicate mean values (mean  $\pm$  SEM). Points correspond to values from individual rats and grey lines follow treatment response of each individual rat.



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