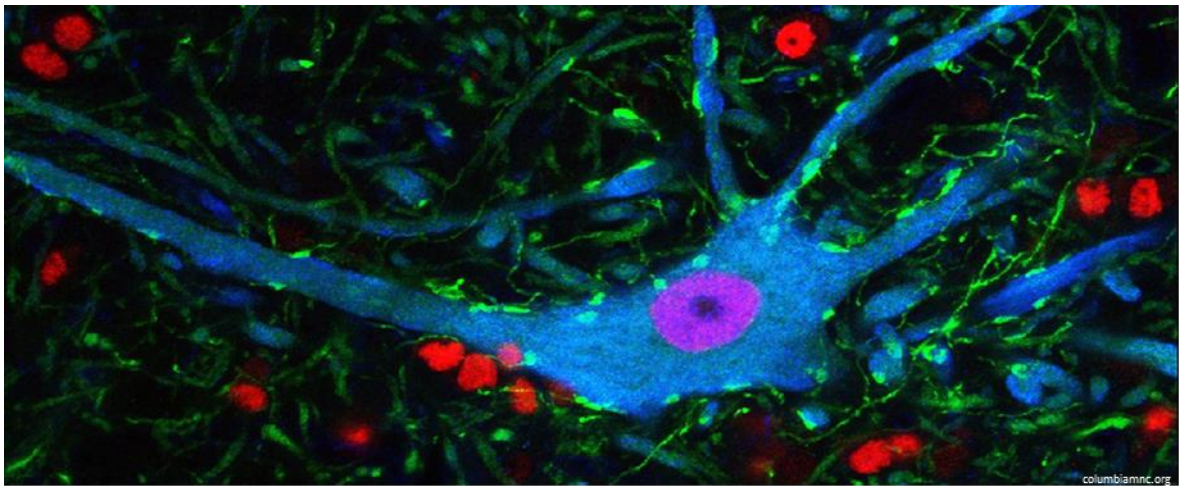


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# Investigation on the role of $Cl^-$ homeostasis and GABAergic transmission in sleep disorders of Down syndrome and in Prader Willi syndrome: a possible contributor to cognitive impairment.

**Ph.D Thesis**

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# Index

## Abstract

GABA is the main inhibitory neurotransmitter of the central nervous system (CNS). Recently, GABAergic transmission has been reported to be depolarizing and possibly excitatory rather than inhibitory in a number of neurodevelopmental disorders both in patients and mouse models.

In particular, the Ts65Dn mouse model of Down syndrome (DS) exhibits depolarizing GABA due to upregulation of the $Cl^-$ importer NKCC1 both in the hippocampus and in the cortex. Moreover, NKCC1 inhibition by the FDA-approved diuretic bumetanide is able to rescue inhibitory GABAergic transmission, synaptic plasticity and cognitive functions in Ts65Dn mice.

Beside cognitive impairment, DS mice and people with DS show sleep disturbaces. Since sleep pattern is regulated by GABAergc transmission, we reasoned that the alteration of GABAergic transmission due to upregulation of NKCC1 might be underlying at least some of the sleep disturbances in DS mice. So, we characterized sleep in Ts65Dn mice and investigated the effects of a chronic treatment with bumetanide. We found that bumetanide ameliorates the quality of sleep in NREM and REM sleep phases before and after sleep deprivation and decreases abnormal wakefulness during light phase at baseline in Ts65Dn mice. Moreover, we also found abnormalities in other parameters, which could contribute to sleep abnormalities of Ts65Dn mice: an increase of food intake and activity (partially rescued by bumetanide) with a reduction of body temperature during dark phase.

Because of the association of altered GABAergic signaling by dysregulation of the expression of NKCC1 (but also of the Cl- exporter KCC2) in many neurodevelopmental disorders characterized by cognitive or social impairment, and sleep disorders, we extended our studies to Prader Willi syndrome (PWS). PWS is a neurodevelopmental disorder, caused by defects of genomic imprinting and characterized by cognitive, social and sleep abnormalities. Here, we observed that the *Snord116*

mutant mouse model of PWS, PWScr$^{m+/p-}$ show an increased expression of NKCC1, specifically in the hippocampus in comparison to their wild-type mice. Moreover, we report that PWS mice have altered cognition and the circadian period in free-running conditions. In particular, mutant mice present defects of long-term memory and a reduced shortening of their circadian period together with an increase of alpha activity in dark-dark (DD). Moreover, they also show alteration of pain sensitivity, that could be linked to defects in the thermoregulation. Interestingly, in constrast with PWS people, *Snord116* mutant mice showed no alterations of anxiety, repetivive, obsessive and social behaviors.

In an effort to rescue cognition and the circadian phenotype by rescuing NKCC1 inhibition, we treated *Snord116* mutant mice with bumetanide. Remarkably, bumetanide treatment resulted in a complete rescue of the cognitive defects and circadian alteration in DD, with no effects in controls. Our results suggest an important link between GABA transmission and the regulation of cognition and the circadian clock in PWS. In addition, the current study extends the repertoire of disorders in which NKCC1 inhibition attenuates behavioural deficits and proposes a new potential mechanism for the investigation of PWS.

# Introduction

## 1. Neurodevelopmental Diseases

Development and maturation of the nervous system are the result of a biological process known as "neurodevelopment". In humans, this process arises during the third week of embryonic growth, characterized by the formation of the neural tube (Bishop KM et al, 2002; Sur M et al 2005; Stiles J et al, 2010; Tau GZ and Peterson BS, 2010; Stiles J, 2011). Afterwards, starting from the ninth week onward, a gradual process of maturation takes place in the brain, allowing its typical structure acquisition. Several events are involved under a tight organization, such as a large cell proliferation, migration, and differentiation (Stiles J et al, 2010; Tau GZ et al, 2010; Stiles J, 2011) (Fig1).

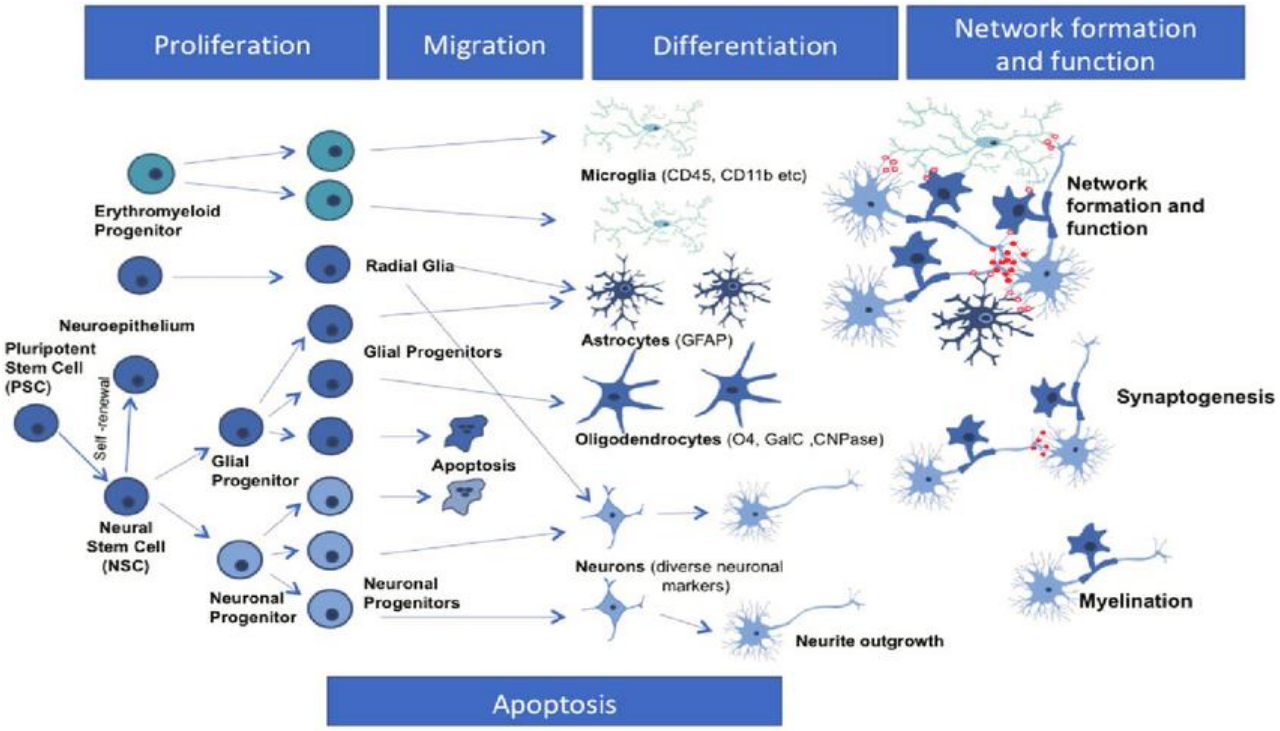



**Fig.1.** Mechanisms of neuronal formation during brain development (Bal-Price et al, 2018).

Any alteration or interference on these ordered and complex processes may result in aberrant brain development and give rise to the onset of a neurodevelopmental phenotype, where falls the definition of neurodevelopmental disorders (NDDs) (Moreno-De-Luca A, et al 2013; Mullin AP et al, 2013; Platzer K, et al, 2019).

Neurodevelopmental disorders are alterations that spring during childhood. These disorders comprise a wide variety of diseases (e.g., Down syndrome, autism spectrum disorders, Prader Willi syndrome). Nevertheless, there are a number of common symptoms that these diverse diseases share. These include abnormal brain development, cognitive defects, language disorders, sleep disturbances, circadian rhythms alterations, psychopathologies (e.g. anxiety), and increased susceptibility to seizures (Soni S et al, 2008; Mullin AP et al, 2013; Viggiano et al, 2015; Robinson-Shelton A and Malow BA, 2016; Thomas S et al, 2017; Diamandopoulos and Green 2018; Niemi MEK et al, 2018; Carmassi et al, 2019).

Currently, the high incidence rate of these diseases is a serious issue for the national health systems of various countries, affected individuals and their families. Recent reviews in various populations showed an average global estimate of 62/10,000 for autism (Elsabbagh M et al, 2012) , 10.37/1000 for intellectual disability (Maulik PK et al, 2011), 1/1000 for DS (Weijerman, ME and de Winter JP, 2010) 1/15,000–1/20,000 for PWS (Soni S et al, 2008), and a prevalence of median lifetime around 4/1000 for schizophrenia (Welham J et al, 2008). Manifold possible causes have been correlated with NDDs; those include genetic factors, environmental conditions, infectious and traumatic events. Interestingly, these factors show a significant interaction between each other, highlighting the impact of their reciprocal influence (Moreno-De-Luca A, et al 2013). As matter of fact, the co-occurrence of different NDDs has been often demonstrated in the literature (e.g. Singh T et al, 2017), possibly indicating the presence of common biological/cellular mechanisms (Gilman SR et al, 2011; Kilinc M et al, 2018).

The genetic mechanisms underlying NDDs are far from being understood, although much progress has been made in this filed in recent years. Mutations in several hundred genes, known to be associated with neurodevelopmental pathways, such as chromatin remodeling, synaptic function and transcriptional regulation genes have been associated to a wide spectrum of NDDs (Darnell Jennifer C, et al 2011; De Rubeis S et al, 2014; Iossifov I et al, 2014). Interestingly there is a marked overlap of these genes results across the diverse NDDs, further strengthening the idea of a possible presence of common biological/cellular mechanisms. Among studies on genetic mechanisms implicated in the NDDs, investigation on the epigenetic mechanisms are also gaining a significant importance (Kubota T et al, 2013). Epigenetic mechanisms are fundamental to determine physiological development during embryogenesis and a proper differentiation of neural cells (Li E et al, 1993; Takizawa T et al, 2011). These epigenetic mechanisms comprise DNA methylation and histone modification and have been implicated in different congenital disorders affected by problems of genomic imprinting. (Payer et al, 2011; Kubota T et al, 2013). In particular, genomic imprinting refers to a type of epigenetic mechanism, where transcriptional mechanisms of regulation are strictly regulated in a parent of origin manner. During gametogenesis imprinted regions of DNA are specifically marked through methylation, determining a parent-specific expression (Mac Donald, 2012). Interestingly, mutations or alterations of these regions may be implicated in the onset of NDDs, as happens in PWS or AS (Kubota T et al, 1997). Moreover, large evidence has shown how different environmental factors such as a poor nutrition, use of drugs, and mental stress, may cause alterations on the epigenetic gene regulation in the brain, thus impacting brain function (Jirtle Rl, et al, 2007; Alegria-Torres et al, 2011). Interestingly, epigenetic mechanisms are reversible mechanisms, which involves modifications of factors binding on and detachment from DNA and histone proteins. Hence, the exploitation of epigenetic reversibility based on the

rearrangement of abnormal epigenomic patterns may be potentially helpful for therapies for NDDs (Handy D E et al, 2011).

In recent years, a lot of NDDs have been also associated to dysfunction of inhibitory GABAergic transmission (Ben-Ari 2017). In particular, experiments carried out on both animal models and postmortem human samples have demonstrated an excitation/inhibition imbalance in neuronal circuits linked to neurodevelopmental defects in GABAergic transmission common to many neurodevelopmental disorders including DS (Deidda et al, 2015) and PWS (Lucignani G et al, 2004; Ebert MH et al, 1997; Rice L J et al, 2016). Interestingly, some of the symptoms common to all NDDs (i.e., increased susceptibility to seizures, cognitive impairment, sleep disorders) have been previously associated to impairment in GABAergic transmission (Plante David T et al, 2012; Ben-Ari et al, 2017). In this thesis, I investigated the contribution of impaired GABAergic transmission to sleep disorders in DS mice and cognitive impairment of PWS mice.

## 2. GABA and $Cl^-$ homeostasis

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. GABA plays an important role as regulator of neuronal networks during mammalian ontogenesis. In particular, neuronal development is orchestrated by GABAergic signaling in terms of neural proliferation, migration, differentiation, and neuronal-network wiring. In the adulthood, the neurotransmitter GABA regulates the activity of different neuronal cell-types widely interconnected, by strongly modulating synaptic activity. GABA exerts these functions by binding to chloride-permeable ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors (Deidda et al, 2014). Synaptic currents induced when GABA binds to $GABA_A$ receptors are carried by chloride ($Cl^-$) and, to a lesser degree, by bicarbonate ($HCO3^-$). $GABA_A$ receptor-mediated conductance either

hyperpolarize or depolarize a neuron depending on its internal $Cl^-$ and $HCO3^-$ concentrations and membrane potential. Two cation-chloride cotransporters are fundamental in controlling neuronal $Cl^-$ (Payne JA et al, 2003; Sipila ST et al, 2006): the Na-K-2Cl cotransporter NKCC1, which imports $Cl^-$ in the neurons and promotes depolarizing responses to GABA, and the K-Cl cotransporter KCC2, which releases $Cl^-$ outside of the cell, supporting hyperpolarizing responses. Expression and function of both transporters are controlled at multiple levels.

During development, KCC2 expression is upregulated, while NKCC1 is downregulated (Yin D et al, 1996; Plotkin MD et al, 1997; Rivera C et al, 2004; Dzhala VI et al, 2005). These changes are associated with a hyperpolarizing shift in the reversal potential of GABAergic responses (Ben-Ari et al, 1989; Payne JA et al, 2003; Farrant M et al, 2007). Thus, under physiological conditions, adult neurons present reduced intracellular $Cl^-$ concentration [(Cl-)i] underlying the GABAergic inhibitory drive (Fig.2).

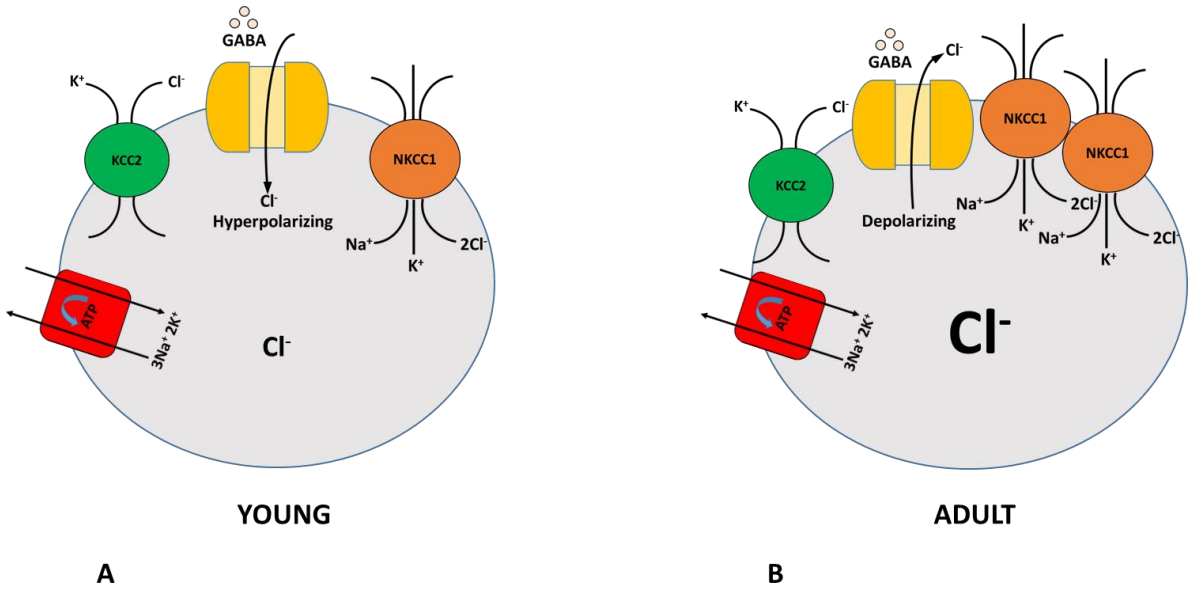



**Fig.2 $Cl^-$ levels of concentrations in young (A) and adult (B).**

In contrast, neurons have high [($Cl^-$)i] levels with depolarizing and possibly excitatory GABA actions in a large range of pathological conditions such as Down, Autism and Rett syndromes, spinal cord lesions, chronic pain, brain trauma, cerebrovascular infarcts, and, different types of epilepsies, and other genetic or environmentally-related conditions. Interestingly, of the NKCC1-inibitor bumetanide efficiently restores reduced [($Cl^-$)i] levels, GABAergic transmission in mouse models of these disorders. Most notably, bumetanide can also rescue core behavioral symptoms in mice and some clinical trials patient case studies (Ben-Ari, 2017).

## 3. Chloride transporters in NDDs

### 3.1 Epilepsy

Epilepsy is a neurological disorder characterized by epileptic seizures. These are caused by altered, excessive or hypersynchronous neuronal activity in the brain (Chang & Lowenstein, 2003). Several pieces of evidence suggest that neuronal hyperexcitability and hypersynchronization are the result of an alteration of the delicate balance between excitatory and inhibitory synaptic activity. Interestingly, an imbalance in NKCC1 and KCC2 activity together with depolarizing GABAergic action have been observed in several animal models of epilepsy (Ben-Ari, 2017; Di Cristo et al., 2018). The first lines of evidence about the involvement of the two cation chloride cotransporters (CCCs) in epileptogenic activity came from the late 90s, when four independent studies found that the antagonization of NKCC1 with furosemide or bumetanide caused a block of epileptic activity both in vitro and in vivo in rats (Hochman et al., 1995; Hochman et al., 1999; Hochman & Schwartzkroin, 2000; Schwartzkroin et al., 1998). In 2002, four other works demonstrated the direct relationship between NKCC1, KCC2 and epileptic pathogenesis. In particular, high NKCC1 expression was

indicated as a factor influencing the increased susceptibility to seizures in the developing brain. In this study, bumetanide administration was able to rescue epileptiform activity both in vitro and in vivo during development in rodents (Dzhala et al., 2005). Moreover, increased expression of NKCC1 was found in the amygdala-kindling model of seizures in rats (Okabe et al., 2002). Furthermore, a decrease in KCC2 expression was found in the mouse hippocampus after kindling-induced seizures (Rivera et al., 2002). Finally, mice deficient in KCC2 showed frequent seizures. This may be possibly due to a shift in the $E_{Cl}$, which leads to impaired efficacy of $GABA_AR$-mediated inhibition and/or to less reuptake of potassium and chloride during high-frequency spikes (Woo et al., 2002). Stemming from these first works, several other studies in rodent models confirmed the involvement of an altered NKCC1/KCC2 ratio in the pathogenesis of epilepsy. A number of these studies also confirmed positive outcomes upon bumetanide treatment (Almeida et al., 2011; Amadeo et al., 2018; Baek et al., 2016; Cleary et al., 2013; Dzhala et al., 2008; Dzhala et al., 2010; D. A. Edwards et al., 2010; Eftekhari, Mehrabi, et al., 2014; Hu et al., 2017; Kelley et al., 2018; Koyama et al., 2012; X. Li et al., 2008; Loscher et al., 2013; MacKenzie & Maguire, 2015; MacKenzie et al., 2016; Marguet et al., 2015; Mazarati et al., 2009; Nardou et al., 2009; Reid et al., 2013; Robel et al., 2015; Santos et al., 2017; Sivakumaran & Maguire, 2016; Tao et al., 2016; Tollner, F. Wang et al., 2017; J. Zhang et al., 2016).

Interestingly, an imbalance in the NKCC1/KCC2 ratio is also present in human patients. First, upregulation of NKCC1 and/or downregulation of KCC2 was found in the hippocampal subiculum and hippocampi obtained from patients affected by temporal lobe epilepsy (Huberfeld et al., 2015; Huberfeld et al., 2007; Munoz et al., 2007; Palma et al., 2006; Sen et al., 2007). Then, other studies found altered expression of NKCC1 and/or KCC2 in the cortical malformation of patients affected by medically intractable epilepsy (Aronica et al., 2007; Sen et al., 2007; Shimizu-Okabe et al., 2011) in hypothalamic hamartoma, a rare epileptogenic lesion associated with gelastic seizures (D. Y. Kim et

al., 2008), in cortical samples from epileptic children (Jansen et al., 2010), and in peritumoral tissues with high seizure susceptibility (Conti et al., 2011; Pallud et al., 2014). Notably, an increased expression of NKCC1 and a decreased expression of KCC2 were observed also in brain samples obtained from patients affected by Dravet syndrome, an infantile encephalopathy characterized by severe epilepsy and cognitive impairment (Ruffolo et al., 2018). Interestingly, bumetanide treatment ameliorated seizure frequency in temporal lobe epilepsy (Eftekhari, Mehrabi, et al., 2014). Moreover, bumetanide was able to reduce seizure duration and frequency in a child affected by intractable multifocal seizures (Kahle et al., 2009). Nevertheless, the NEMO trial, assessing the efficacy and safety of the use of bumetanide for the treatment of acute neonatal encephalopathy seizures (Pressler et al., 2015), was recently interrupted due to poor bumetanide antiepileptic action and ototoxicity (Ben-Ari et al., 2016). Moreover, the involvement of KCC2 in the pathogenesis of epilepsy has been recently questioned based on conflicting results showing increased KCC2 expression in epileptic brain tissue from both human (Jansen et al., 2010; Karlocai et al., 2016) and rodent models (Awad et al., 2016; Galanopoulou, 2008; Khirug et al., 2010). Nevertheless, the conflicting results showed both decreased and increased KCC2 expression in epilepsy; this discrepancy could depend on brain region, stage of disease, gender, or the influence of seizures themselves. Indeed, KCC2 activity causes potassium elevation in the extracellular compartment, which could contribute to the lowering of the threshold for the generation of the seizures and to the synchronization of the epileptiform discharges (Di Cristo et al., 2018). Thus, although the involvement of alterations of NKCC1 and KCC2 expression/activation in epilepsy is clearly demonstrated, deeper studies to better investigate their delicate modulation and assess the possibility of targeting them with pharmacological approaches are still required.

### 3.2 Autism spectrum disorder (ASD)

Autism spectrum disorder (ASD) is a group of syndromes characterized by different etiologies, but common core symptoms (e.g., repetitive behaviors, deficits in social interaction and language impairment; (Pizzarelli & Cherubini, 2011), suggesting that possibly there are common mechanisms underlying ASD pathology. Moreover, ASD can be comorbid with other neurodevelopmental syndromes such as epilepsy (M.L. Lewis et al, 2018), Rett syndrome (Percy, 2011), Fragile X syndrome (Kaufmann et al, 2017), or Down syndrome (J. Moss et al, 2013). Several pieces of evidence, both from rodent models and humans, indicate that commonly altered GABAergic transmission could underlie ASD pathology (Cellot & Cherubini, 2014). In particular, the pioneering observation of a paradoxical effect upon the administration of $GABA_A$ signaling enhancing benzodiazepine diazepam in autistic children (e.g., anxiety and aggression (Marrosu et al, 1987), suggested the possibility of depolarizing GABA action in ASD. This idea prompted researchers to test whether the inhibition of NKCC1 by bumetanide could be a valid therapeutic strategy in five autistic children (Lemonnier & Ben-Ari, 2010). The amelioration of some behavioral aspect related to ASD upon bumetanide treatment opened the way for a larger clinical trial designed for 54 autistic patients (Lemonnier et al., 2012) and consequently a phase II clinical study (Lemonnier et al., 2017). These studies confirmed that bumetanide is able to ameliorate the core symptoms of ASD measured by the Childhood Autism Rating Scale (CARS). Moreover, bumetanide resulted efficient in the treatment of a young girl with Asperger syndrome, a neurodevelopmental disorder belonging to ASD (Grandgeorge et al., 2014). In parallel to the clinical studies, KCC2 expression was downregulated in the VPA rat model of ASD. Interestingly, bumetanide administration in VPA-treated pregnant rats resulted in the rescue of core behaviors related to ASD in their offspring (Eftekhari, Shahrokhi, et al., 2014; Tyzio et al., 2014). Nevertheless, the lack of preclinical studies

addressing bumetanide treatment at the developmental stages comparable to those of patients enrolled in clinical trials and the paucity of mouse models of autism tested among the many that exist, highlights the need for further investigation.

### 3.3 Fragile X syndrome (FRX)

Fragile X syndrome (FRX) is a genetic disorder caused by mutations in the X-linked *FMR1* gene encoding for Fragile X mental retardation protein (FMRP). FMRP is a regulator of the translation of several mRNAs. FXS individuals show cognitive deficits, autistic behavior, hypersensitivity to sensory stimuli and comorbidity with epilepsy (Morel et al., 2018). These symptoms led researchers to hypothesize an excitatory/inhibitory imbalance, as previously observed in ASD and epilepsy. In particular, driven by the positive outcome of the earlier pilot study on autistic patients (Lemonnier et al., 2012), the same authors treated a FXS child with bumetanide. Interestingly, bumetanide administration resulted in the amelioration of the score of each of the 5 clinical tests performed to probe autistic core symptoms (Lemonnier et al., 2013), opening the route to larger clinical trials. In agreement with the clinical study, FXS mice showed a delay in the developmental switch of GABA polarity from depolarizing to hyperpolarizing, due to increased expression of NKCC1 (He et al., 2014). The same year, Tyzio and colleagues found increased $Cl^-$ concentrations in hippocampal slices from FXS mice at P15 and P30 due to a decreased level of KCC2. Fetal treatment with bumetanide right before birth was able to recover the intracellular $Cl^-$ concentration, GABAergic transmission and the behavioral features related to autism later in life (Eftekhari, Shahrokhi, et al., 2014; Tyzio et al., 2014). Recently, treatment of FXS mice with bumetanide during the critical period of somatosensory cortex plasticity rectified GABA polarity and synaptic plasticity and allowed long-lasting restoration of proper somatosensory-circuit formation (He et al., 2018). Moreover, a recent

study found that bumetanide treatment by itself was insufficient to completely rescue social impairment in the automated tube test in FXS mice, suggesting the need for a combination therapy. Nevertheless, in the same study, the combination of the genetic reduction of mGluR5 expression together with bumetanide treatment worsened social impairment, indicating that the combination therapy needs to be better investigated in terms of drug type, targeting pathway and time window of administration (Zeidler et al, 2017). Although there are only a few studies in animal models and in humans, the abovementioned evidence confirms the involvement of the NKCC1/KCC2 imbalance in the pathogenesis of FXS syndrome and their modulation as a possible therapeutic strategy.

### 3.4 Rett Syndrome (RTT)

Rett syndrome (RTT) is a neurodevelopmental disorder caused by mutations in the X-linked Methyl-CpG-binding protein (*MECP2*) gene. Mecp2 binds to methylated DNA and regulates the transcription of a large number of genes. Individuals affected by RTT grow normally until the age of 6-18 months, but then develop various symptoms (e.g., cognitive impairment, seizures, altered motor function and stereotyped behaviors; (Ehinger et al., 2018). As for autism, several pieces of evidence indicate a possible alteration in the GABAergic signaling in RTT rodent models and humans (Cellot & Cherubini, 2014). The first evidence regarding alterations in the NKCC1/KCC2 balance came from a study of corticospinal fluid (CSF) obtained from RTT patients, where a decreased level of KCC2 expression was found (Duarte et al., 2013). More recently, deficits in KCC2 expression have been found in human RTT patient stem cell-derived neurons (Tang et al., 2016) and in a mouse model of RTT (Banerjee et al., 2016). Interestingly, Insulin-like Growth Factor-1 (IGF1) treatment ameliorated the severity of the syndrome both in RTT mouse models (Castro et al., 2014; Tropea et al., 2009) and in RTT patients (Khwaja et al., 2014; Pini et al., 2016), thus suggesting its implementation in the

treatment of NDs (Bou Khalil, 2017). Moreover, the pharmacological enhancement of KCC2 gene expression is able to exert therapeutic effects on human Rett syndrome neurons. Indeed, it rescues the electrophysiological and morphological abnormalities of these neurons. In addition, enhancement of KCC2 expression is able to ameliorate disease-related breathing pauses and locomotion deficits in *Mecp2* mutant mouse model of RTT (*Mecp2* −/y) (Tang X at al, 2019). Nevertheless, further investigation of the NKCC1/KCC2 ratio and its consequence on GABA signaling are needed to better clarify the role of CCCs in the pathogenesis of RTT and their possible involvement as therapeutic targets.

### 3.5 Schizophrenia

Schizophrenia is a neurodevelopmental disorder characterized by psychosis and cognitive impairments, leading to disability and premature mortality. In particular, the clinical manifestations can be divided into three categories: positive symptoms (e.g., hallucinations), negative symptoms (e.g., depression and apathy) and cognitive symptoms (D.A. Lewis, 2012). The etiology of schizophrenia is still under investigation, but a large body of literature agrees on the contribution of both genetic and environmental factors. GABAergic transmission seems again to play an important role in the pathogenesis of this ND (Balu & Coyle, 2011). In particular, the first pieces of evidence of impaired $Cl^-$ homeostasis in schizophrenia came from a study on the prefrontal cortex (and later the hippocampus) of schizophrenic patients, where NKCC1 expression was increased (Dean et al., 2007; Hyde et al., 2011). A few years later, alterations in *SLC12A2* and *SLC12A5* genes, encoding for NKCC1 and KCC2, respectively, were indicated as susceptibility genes for schizophrenia development in patients (J.Y. Kim et al., 2012; Merner et al., 2015; Merner et al., 2016; Potkin et al., 2009). Furthermore, increased expression of two kinases regulating NKCC1 and KCC2 activity, OXSR1 and

WNK3, was found in the prefrontal cortex of schizophrenic subjects, indicating a possible increase in NKCC1 activity and a decrease in KCC2 function in schizophrenic patients (Arion & Lewis, 2011). In addition, an altered NKCC1/KCC2 ratio was described in two different mouse models of schizophrenia (Larimore et al., 2017; Yang et al., 2015). Finally, an interplay between NKCC1 and the protein Disrupted in schizophrenia 1 (DISC1, an intrinsic regulator of neurogenesis implicated in schizophrenia) has been demonstrated to be fundamental for the regulation of the dendritic development of newborn neurons during adult neurogenesis in the mouse hippocampus (J.Y. Kim et al, 2012), suggesting possible involvement of NKCC1 in the pathogenic mechanisms underlying schizophrenia.

Of note, in vitro evidence from Amin and coworkers revealed an imbalance in NKCC1 and KCC2 expression also in DiGeorge Syndrome (a condition conferring high risk of schizophrenia), which caused hyperexcitability of the network recovered by bumetanide application to the neuronal culture (Amin et al, 2017).

Bumetanide treatment in schizophrenic patients reduced the severity of the symptoms and hallucinations (Lemonnier et al., 2016; Rahmanzadeh, Eftekhari, et al., 2017), without ameliorating the total score of the general positive and negative syndrome scale (PANSS) and the brief psychiatric rating scale (BPRS) (Rahmanzadeh, Shahbazi, et al, 2017). Interestingly, intranasal administration of oxytocin reduced the severity of symptoms in schizophrenic patients in several studies (Brambilla et al., 2016; Davis et al., 2014; Davis et al., 2013; Feifel et al., 2012; Feifel et al., 2010; Fischer-Shofty et al., 2013; Gibson et al., 2014; Goldman et al., 2011; Lee et al., 2013; Modabbernia et al., 2013; Ota et al., 2018; Pedersen et al., 2011; Shin et al., 2015; Woolley et al., 2017; Woolley et al., 2014), but see (Cacciotti-Saija et al., 2015; Caravaggio et al., 2017; Dagani et al., 2016; Horta de Macedo et al., 2014; Jarskog et al., 2017). In light of the ability of oxytocin to regulate GABA signaling in fetal and newborn rodents (Ben-Ari, 2018; Eftekhari, Shahrokhi, et al., 2014; Khazipov et al., 2008;

Leonzino et al., 2016; Tyzio et al., 2006; Tyzio et al., 2014), it is tempting to hypothesize that oxytocin exerts its therapeutic effect on schizophrenic patients also by regulating CCCs. A deeper investigation of the molecular mechanisms underlying the possible relation between the oxytocin system and CCCs could open new avenues for the treatment of schizophrenia and other NDs.

### 3.6 Tuberous Sclerosis Complex

Tuberous sclerosis complex (TSC) is a multiorgan genetic disorder caused by loss of function mutations of the *TSC1* or *TSC2* genes (van Slegtenhorst et al., 1997). This pathology is characterized by the presence of cortical tubers (i.e., dysplastic lesions), source of focal epilepsy, autistic behaviors and intellectual disability. Given the imbalance of the NKCC1/KCC2 ratio in epilepsy (Schulte et al., 2018), the investigation of CCCs in tuberous sclerosis has gained interest. TSC patients present an increased NKCC1/KCC2 ratio in extracts from cortical tubers (Ruffolo et al., 2016; Talos et al., 2012). An altered GABA reversal potential was also described in Xenopus oocytes injected with membranes from TSC patient cortical tissues (Ruffolo et al., 2016). Altogether, these studies suggest a possible involvement of NKCC1/KCC2 imbalance in the pathogenesis of TSC in patients. Nevertheless, a better investigation of both the pathogenic mechanisms and possible therapies needs to be performed in rodent models.

### 3.7 Neurodevelopmental Abnormalities Caused by Traumatic brain injury

Traumatic brain injury (TBI) is caused by an injury to the brain due to external objects or forces. When TBI occurs in early childhood, the cortical and subcortical lesions lead to altered neurodevelopmental processes and consequent cognitive defects persisting for the lifetime of the

individual (Bonnier et al., 2007; Jonsson et al., 2013; Keenan et al., 2007). The neurodevelopmental damages occurring after a TBI are the result of a cascade of events, called secondary brain injury, including damage of the blood-brain barrier, inflammation, excitotoxicity, edema, ischemia and neuronal damage (e.g., excitotoxicity, aberrant ionic homeostasis, axonal disconnection and death; (Ghajar, 2000; Park et al., 2008). One of the mechanisms underlying this cascade of events is possibly an imbalance of NKCC1 and KCC2 expression and function. Indeed, three independent studies found that NKCC1 was upregulated in the hippocampus and choroid plexus of traumatic brain injury rat models and that bumetanide administration decreased the inflammatory response and neuronal damage (Lu et al., 2008; Lu et al., 2006; Lu et al., 2007). Then, other studies confirmed the fundamental role of NKCC1 in TBI-induced rodent hippocampal aberrant neurogenesis (Lu et al., 2015), neuronal and astrocytic apoptosis (Hui et al., 2016; M. Zhang et al., 2017), cerebral edema (Lu et al., 2017; M. Zhang et al., 2016), seizures (Liang & Huang, 2017; F. Wang et al., 2017) BBB disruption (J. Zhang et al., 2017) and microvascular failure (Simard et al., 2010). Finally, a recent work described decreased KCC2 expression in the rat parietal cortex after TBI, which was rescued by melatonin administration leading to amelioration of neural apoptosis and brain edema (Wu, Shao, et al., 2016). The deep understanding of the involvement of both NKCC1 and KCC2 in the secondary brain injury upon TBI suggests timely pharmacological interventions to prevent the consequent neurodevelopmental alterations observed in TBI children.

### 3.8 Glioma

Gliomas are brain tumors, which generates from glial cells and they represent the most common solid tumor of childhood. On the base of their unique properties compared to those affect the adults, pediatric gliomas can be considered to be neurodevelopmental disorders (Baker et al., 2016).

Studies on humans evidences that the intracellular volume regulation promoted by NKCC1 as well as aquaporin 4 support glia cell invasion (Haas, B.R. and Sontheimer H, 2010; Garzon-Muvdi T et al, 2012; Turner K.L. and Sontheimer H, 2014). NKCC1 appears to be upregulated in different kind of glioblastoma and anaplastic astrocytoma tissues. Bumetanide pharmacological treatment or NKCC1 knock down cause a decrease of cell migration (Garzon-Muvdi T et al, 2012; Turner K.L. and Sontheimer H, 2014).

In particular, bumetanide treatment (25–50 mM) allows a dose-dependent reduction of cell migration (Garzon-Muvdi T et al, 2012). Cortical GABAergic excitation around human gliomas concur to epileptic activities (Pallud J et al, 2014) and NKCC1 activity is increased (Garzon-Muvdi T et al, 2012). So, high $(Cl^-)i$ levels may be reached through the action of NKCC1, determining changes in cell volume and a damaged cell migration. Nevertheless, clinical trials have not been evaluated in humans.

## 4. Down syndrome

Down syndrome (DS), also called trisomy 21, is a genetic disorder caused by the presence of all or part of a third copy of chromosome 21. The most important clinical hallmarks of individuals with DS are congenital birth defects and the intellectual disability, which gives rise to a reduction of the intelligence quotient, learning problems and deficits of memory abilities, particularly related to hippocampal functions (Desai, 1997; Nadel, 2003; Antonarakis & Epstein, 2006; Parker et al., 2010 Dierssen, 2012).

Most of the studies investigated possible mechanisms involved in cognitive impairment, taking advantage of diverse murine genetic models of DS (Dierssen, 2012). The Ts65Dn mouse (Reeves et al., 1995) is the most characterized and widely used. These mice are characterized by the presence

of an extra chromosome derived from mouse chromosome 16, representing the long arm of human chromosome 21, fused to the centromere of the murine chromosome 17 (Antonarakis et al, 2004). Interestingly, Ts65Dn mice recapitulate many features of DS. In particular, these mice show impairment in neuronal development (Belichenko et al, 2004; Chakrabarti et al., 2010; Chakrabarti et al., 2007; Contestabile et al., 2010; Contestabile et al., 2007), defects of synaptic plasticity (Contestabile et al., 2013; Costa & Grybko, 2005; Kleschevnikov et al., 2004; Siarey et al., 1999; Siarey et al., 1997), impaired hippocampus-dependent memory functions (Contestabile et al., 2013; Costa et al., 2008; Fernandez et al., 2007; Reeves et al., 1995), hyperactivity (Escorihuela et al., 1995; Reeves et al., 1995; Sago et al., 2000) and sleep disorders (Colas et al., 2008). In 2015, Deidda and colleagues proposed a new perspective about $GABA_AR$ transmission in DS (Deidda, et al, 2015). In their work, the efficacy and polarity of $GABA_AR$ signaling were investigated in adult Ts65Dn mice. Surprisingly, they found that GABAergic transmission was depolarizing and mostly excitatory rather than hyperpolarizing and inhibitory in adult DS mice. In particular, they described an increase in spike frequency in Ts65Dn hippocampal and neocortex acute slices in comparison to WT, both in baseline conditions and upon application of GABA. Accordingly, blockade of endogenous $GABA_A$ signaling by the application of the $GABA_AR$ antagonist bicuculline resulted in a reduction in the spike frequency in neurons from Ts65Dn brain slices. Interestingly, the same study found that the defective GABAergic signaling was due to an increased expression of NKCC1 protein, which they found in the entire hippocampus, the CA3-CA1 subregion and cortices of adult Ts65Dn mice compared to WT littermates. Interestingly, Deidda and coworkers found increased NKCC1 expression also in hippocampi from DS individuals, providing a parallel between the animal model and humans. Conversely, no changes in KCC2 protein expression both in Ts65Dn mice and DS individuals were detected. Considering that the increased expression of NKCC1 is the possible cause of the aberrant GABAergic transmission in Ts65Dn mice, Deidda and colleagues evaluated NKCC1

inhibition by bumetanide as a potential therapeutic strategy. Bath application of bumetanide was able to rescue $E_{Cl}$, with a reduction of spontaneous spiking activity and a decrease in the GABA-induced spike frequency in acute hippocampal slices of adult Ts65Dn mice. Moreover, bumetanide bath application to acute brain slices was able to recover the hippocampal CA1-CA3 LTP to WT levels, with no effect on the LTP in WT mice. Finally, Deidda and colleagues tested Ts65Dn mice and their WT littermates in three independent behavioral tasks to assess hippocampus-dependent long-term explicit memory after either an acute (1 time only), subchronic (1 week) or a chronic (4 weeks) systemic (intraperitoneal) treatment with bumetanide. Interestingly, they proved that all three treatments with bumetanide were able to fully recover the poor associative memory of Ts65Dn mice in the contextual fear conditioning test. Moreover, bumetanide was able to rescue the performance of Ts65Dn mice to the level of WT mice in the object-location test, showing a full recovery of spatial-memory performance. Finally, bumetanide administration was also able to rescue the novel-discrimination memory of Ts65Dn mice in the novel object recognition test.

Notably, bumetanide exerted acute activity on NKCC1, and it did not provide long-lasting effects. Indeed, a drug withdrawal experimental protocol (i.e., a week of bumetanide washout after a four-week treatment) completely abolished the rescue observed in both LTP and behavioral tasks, indicating the requirement for chronic treatment (Deidda et al, 2015).

Beside cognitive impairment, individuals with DS present also with many other health issues (Asim et al, 2015). Among them are sleep disorders, which are perceived as one of the problems mostly affecting the quality of life of individuals with DS and their family members, possibly also affecting cognitive performance (Andreou G et al, 2002). Considering the aberrant GABAergic transmission reported in DS (Deidda et al, 2015) and the influence of GABA signaling in sleep, we decided to

realize an accurate characterization of sleep and its correlation with mechanisms of $Cl^-$ homeostasis in Ts65Dn mice. Below is a short introduction to sleep physiology and its regulation by GABAergic transmission under physiological condition.

## 5. Sleep and GABAergic transmission

### 5.1 Sleep-wake cycle

The sleep-wake cycle is regulated by two main processes: a homeostatic process (S) and a circadian process (C). The homeostatic process indicates the propensity of sleep to increase or decrease, that depends on the previous wakefulness (Borbely, 2009; Cirelli, 2009). The circadian process is involved in dictating the time of sleep and it is self-sustained (C.B Saper et al, 2005; Saper,C.B et al, 2013; Waterhouse et al, 2012) (Fig3).

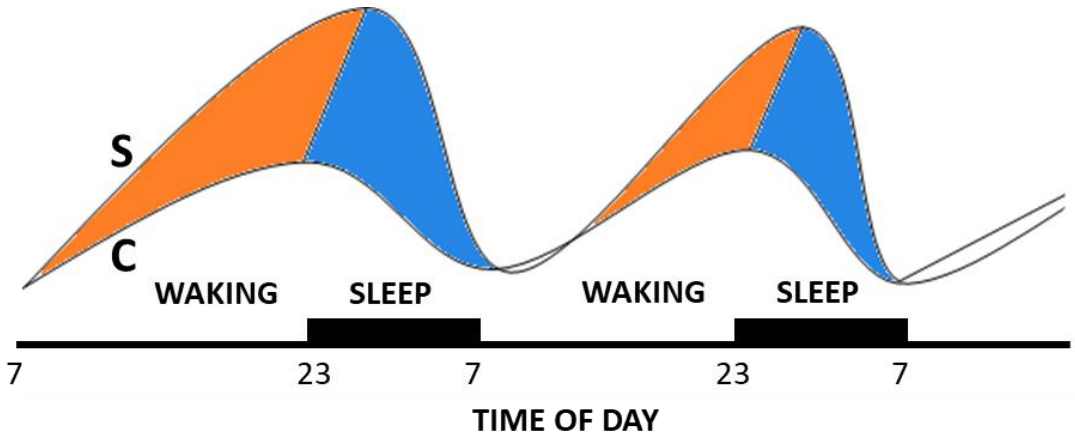



**Fig3.** The sleep-wake cycle regulation by a homeostatic process (S) and a circadian process (C). Orange and blue indicate waking and sleep respectively. Waking and sleep periods are related to humans. (rearranged by Borbély & Achermann, 1999)

The sleep-wake cycle alternates wakefulness and sleep in a circadian way, where in diurnal species, like humans, the wakefulness takes place during daylight hours, while sleep at night. Differently, nocturnal animals, such as mice and rats, have a period with greater activity in the dark phase, showing during the light phase a much smaller activity (Tobler,1995).

One of the main regulators of sleep that presents with a circadian rhythm is body temperature. A decrease in temperature is associated to sleep period, whereas an increase of temperature coincides with the awakening (P.L Parmeggiani, et al, 1971; P.L Parmeggiani, 1980; P.L Parmeggiani, 1987). Changes in body temperature have been associated with insomnia (Leon.C. Lack et al, 2008) and occur in individuals following sleep deprivation. Sleep deprivation makes subjects more vulnerable to heat loss, with less ability to warm themselves to a comfortable temperature (Landic CA et al,1998; Masanobu.H et al, 2017).

The sleep-wake cycle is composed by different arousal states: the wakefulness and an important ultradian cycle (less than 24 hours) that includes 90 - 110 minutes cycle of non-rapid eye movement sleep (NREM) and rapid-eye movement (REM) in humans. The overall architecture of the sleep-wake cycle is well represented by a hypnogram, a graphic representation, showing the hours of recording and the different phases of sleep-wake cycle (Fig4).

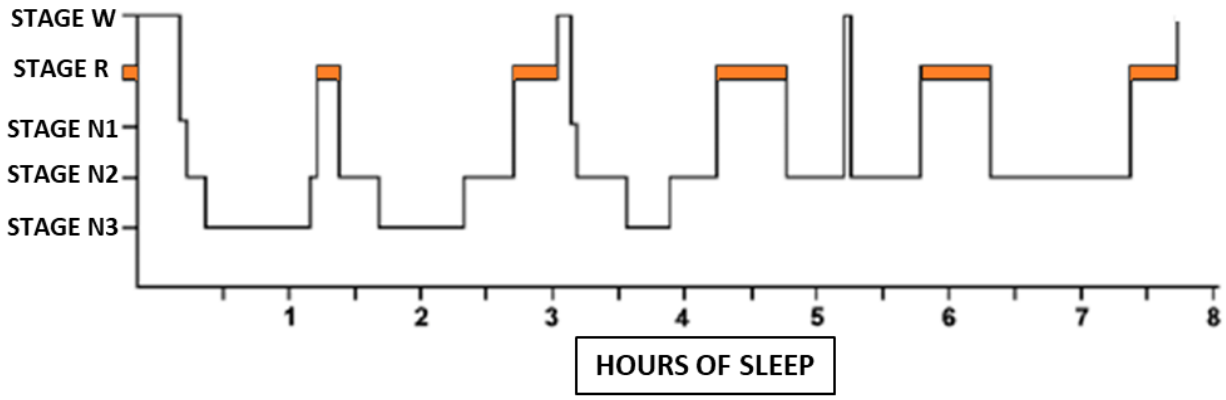


**Fig.4.** Different phases of the sleep-wake cycle in humans. In horizontal are showed the recording hours (hours of the night); in vertical are represented (wake (W), REM sleep (stage R, indicated by orange) and the three sub-stages of sleep NREM (stage N1, N2 and N3) (rearranged by Bahammam et al, 2016).

However, rodents have an ultradian cycle with a much shorter duration (10-12 minutes) and a polyphasic sleep, rather than monophasic as humans, with an alternation of waking and sleeping phases (Tobler,1995; D'Olimpio F and Renzi P, 1998).

Each phase of sleep-wake cycle is characterized by a particular range of EEG activity and an involvement of different brain circuities, which lead to a wide range of behaviors, both during wakefulness and sleep.

From the wakefulness state, we observe usually a transition to NREM sleep phase, which in turn is characterized in humans by three different substates (N1, N2 and N3), correlated to increasing depth of sleep. The waking state passes to N1, which is a superficial state of sleep, then goes to N2 and N3, which represent deep stages. In particular, at the beginning of sleep, the transition from the waking phase to the N3 occurs very quickly. In this phase, waves of high voltage and low frequency predominate in the EEG trace of the substate N3, also called "Slow Wave Sleep" (SWS). Then, after a time around 70 minutes in humans sleep enters a REM stage, and then the cycle starts again from the N1 phase of NREM sleep. Usually, five NREM and REM sleep cycles alternate, which can be interspersed with very brief awakenings. (Carskadon and Dement, 2011).

However, both NREM and REM phases have shown an importance in synaptic plasticity and cognition. Indeed, experiments of REM or non-REM sleep deprivation in rodents have been correlated to a decrease in brain plasticity and cognitive performance (Tononi and Cirelli, 2006; Fogel.S et al, 2012; Wei.Li et al, 2017).

## 5.2 The phases of the sleep-wake cycle

In the following paragraphs, I will enter in detail on the different type of phases of the sleep-wake cycle.

## 5.3 Wakefulness

The wakefulness state is distinguished by the presence of beta waves (frequency: 15-30 Hz) in the EEG recordings during an active waking state, while alpha waves (frequency: 8-12 Hz) are predominant in a state of relaxed wakefulness. The cortical activation is involved in the wakefulness, and it generates a desynchronized (high amplitude and low frequency) EEG tracing (De Gennaro,L and Casagrande,M, 1998) (Fig.5).


**Fig.5** EEG wakefulness state. Adapted from Lydic et al 2002.

The desynchronization is caused by the neuronal activity originating from the projections departed from the reticular formation (RF), a fundamental structure for the state of wakefulness (Lindsley et al., 1950; Starzl et al., 1951). Probably, the FC drives its action through the release of the glutamate neurotransmitter (Jones, 1995). Notably, the function-mediated by several anesthetics inhibits the glutamatergic transmission (Rudolph and Antkowiak, 2004).

The waking state is also controlled by the locus coeruleus (LC), where noradrenergic transmission promotes the excitation of neuronal networks implicated in the waking state, and the inhibition of

neuronal networks which supports the sleep (Aston-Jones and Bloom, 1981; McCarley and Hobson, 1975). Moreover, dopaminergic neurons originating from the substantia nigra, tegmental ventral area (VTA) and the grey periaqueductal ventral (vPAG) project in the striatum, cortex and basal forebrain (Lu et al., 2006). These projections are characterized by high level of activity during the waking state and a very low activity during slow waves sleep (Lena et al, 2005; Maloney et al, 2002). The hypothalamus is another important structure for the wakefulness state. In the posterior hypothalamic tuberomammillary nucleus (TMN), the neurotransmitter histamine is released to promote cortical activation to keep the state of wakefulness, exerting an excitatory effect on the ascending reticular activating system (ARAS) and inhibiting the neurons of the ventrolateral preoptic area (VLPO) implicated in sleep promotion, through the action of excitatory synapses on inhibitory interneurons (Brown et al., 2001b; Saper et al., 2001; Liu et al., 2010). In addition, also orexin/hypocretin neurons are projected from lateral hypothalamus to aminergic systems, in particular the locus coeruleus (LC) and maintain wakefulness. These neurons inhibit the REM sleep and their absence was associated with the insurgence of narcolepsy (Chemelli et al, 1999; Lin et al, 1999; Mignot et al, 2002; Peyron et al., 2000).

On the other hand, the mesencephalic structures laterodorsal tegmental nucleus (LDT) and tegmental peduncular pontin nucleus (PPT), are implicated in the desynchronization during the waking state through the release of acetylcholine. Notably, these structures are also important in REM sleep (el Mansari M. et al., 1989; Steriade et al., 1990; Jones, 1995; McCormick, 1992). Finally, also serotoninergic neurons located in the raphe dorsal nucleus (DR) and the raphe medial nucleus (MR) of the encephalic trunk, project to the neocortex, limbic system and diencephalon. DR and MR neurons have the highest firing during the wakefulness, reducing the activity in NREM sleep phase and become silent during REM sleep phase (Portas CM et al, 2000; Commons KG, 2015).

## 5.4 NREM sleep phase

During NREM sleep the features are: a disappearance of muscle tone, a synchronized (low amplitude and high frequency) EEG and the absence of rapid ocular movements. NREM sleep phase is mainly characterized by delta waves (frequency: 0,5-4 Hz) linked to a deeper sleep, that is defined as “the slow wave sleep” (Fig.6). The three stages N1, N2 and N3 of NREM sleep have a greater grade of deep sleep and a different brain activity. N1 is linked to the presence of alpha waves, accompanied by the first theta waves, N2 shows K complex (bi-triphasic waves, with a high voltage, over 75 µV) and sleep spindles, which are waves with a progressive increase and a gradual decrease in the amplitude (frequency around 12 - 14 Hz) (Gennaro and Casagrande, 1998; Rechtschaffen and Kales, 1968; Iber et al., 2007). The last stage, N3, is determined by the onset of delta waves, which reveal thalamus-cortical and cortico-thalamic neuronal firings (McCormick and Bal, 1997; McCormick e Westbrook, 2001; Steriade et al., 1993; Steriade, 1999).

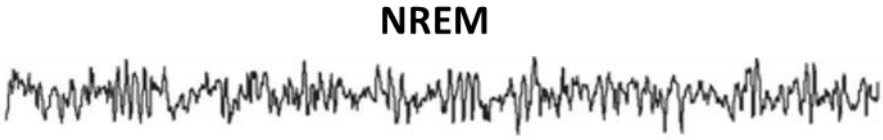



**Fig.6** EEG NREM state. Adapted from Lydic et al 2002.

The brain area implicated in the generation of NREM sleep phase is the Preoptic Area (POA) of the hypothalamus, where the ventrolateral preoptic nucleus (VLPO) and the Median preoptic area (MnPO) are the fundamental regulators of the NREM sleep. Injury of the POA causes the loss of NREM sleep, while stimulation increases the percentage of time spent in this phase (McGinty and Szymusiak, 2001). VLPO and MnPO neurons make use of inhibitor neurotransmitters as GABA and

galanin (gal), which have projections directed to brain area of the ARAS, implicated in the regulation of wakefulness (Sherin et al., 1998; Steininger et al., 2001; Uschakov et al., 2007).

During the sleep VLPO and MnPO neurons show a greater activity during sleep deprivation, highlighting their importance in the regulation of sleep homeostasis (Suntsova et al., 2002).

### 5.5 REM sleep phase

The REM sleep phase, also called paradoxical sleep, show a desynchronized rhythm characterized by rapid eyes movements and muscle atony. REM sleep phase is very similar to wakefulness in terms of EEG tracing (Fig.7). Nevertheless, the theta waves (frequency: 4-7 Hz), are predominant in the EEG recording during REM sleep in the temporal lobal structures of the brain, mostly the projections from the hippocampus (McCormick e Westbrook, 2001).


**Fig.7** EEG REM state. Adapted from Lydic et al 2002.

The most relevant feature that is linked to REM sleep is the experience of dreams. Indeed, individuals waked up during this phase mostly remember to have dreamed. However, dreaming is also reported in NREM sleep, but less realistic and thought-similar (Dement and Kleitman, 1957; Hobson, 2009). Moreover, REM sleep is distinguished by the presence of an irregular respiratory and cardiovascular activity (Aserinsky and Kleitman, 2003; Duron, 1972; Coccagna et al., 1971; Dufour and Court, 1977; Lacombe et al., 1988; Meunier et al., 1988). Notably, the homeostatic mechanisms of body thermoregulation that works normally in the other phase appear to be absent

during REM sleep (Dickerson et al., 1993; Parmeggiani et al., 1971). The main structure involved in the REM sleep onset is the pontine reticular formation (PRF) (Jones, 1991). Studies have indicated an increase of glutamatergic neurons excitability, particularly in the sublaterodorsal nucleus (SLD, sub-lateral dorsal) and the pericoeruleus (PC) of PRF during REM sleep. SLD and PC neurons receive projections from mesencephalic areas, which are implicated in the generation of the EEG pattern typical of REM sleep (Sakai et al., 1979; Sakai, 1986). Moreover, other two neuronal systems are responsible of REM sleep regulation: the cholinergic system involved to activate the REM phase (Mitani et al., 1988; McCarley, 2007) and the monoaminergic system, which promotes the inhibition of REM sleep (Hobson et al., 1975; Aston-Jones and Bloom, 1981; Hobson et al., 1983; Lydic et al., 1987a; Lydic et al., 1987b; Trulson and Jacobs, 1979). Finally, also the GABAergic system regulates the REM sleep especially in PRF. In particular, different kind of GABAergic neurons are involved in the REM sleep: GABAergic neurons REM-on of the sub-later dorsal nucleus (SLD), which inhibit neurons of the monoaminergic system and GABAergic neurons REM-off of the lateral nucleus of the Pontine tegmentum (LPT) and of the periaqueductal ventral-lateral gray (vlPAG), which avoid the activation of REM-on system of PRF (Boissard et al, 2002; Boissard et al., 2003; Lu et al., 2006). Notably, the Ach regulates GABAergic neurons REM-on and REM-off during wakefulness and NREM sleep phase (Brown et al., 2012).

## 5.6 Circadian rhythms

The daily solar cycle determines several and regular changes in terms of light, temperature, and food availability in the environment. So, the presence of an internal biologic timer, common to almost all organisms including humans, allows the regulation of physiology and behavior in order to anticipate daily changes of the day/night cycle.

Some processes such as absorption, metabolization and production of fatty proteins, hormones and other biological components must take place at a precise time. Optimizing these physiological processes means ensuring proper cell development, reproduction and repair.

The circadian rhythms give rise to circadian oscillations in gene expression regulated by the core clock genes, protein modification, and behavior. It is important that these circadian rhythms are synchronized and adapted to the external environment, in particular to factors such as light, temperature, predation and food availability. These environmental signals, or time-givers are called "zeitgebers". The particularity of circadian rhythms is that they have their own internal regulation, but that it can be influenced by external signals in order to synchronize with the environment. The circadian clock plays a fundamental role in the physiology of living organisms by controlling the sleep/wake cycle, reproduction, thermoregulation and metabolic control. Human physiology is based on the daily rhythm of activity and sleep. In particular, the activity requires the internal organization of the organs belonged to the digestive system during the day, while the sleep suspends most of these processes promoting other activities such as cellular repair, toxin removal and memory consolidation. Therefore, circadian rhythms have a great impact on life and their impairment could strongly alter our health and well-being. (Pittendrigh C.S, 1993; Foster R.G and Kreitzman L, 2004; Dunlap J.C et al, 2011).

Underlying the control of circadian rhythm is a molecular mechanism based on a positive and negative transcriptional feedback loop that involves different genes. These include *Clock* and *Bmal1* that encode for proteins which give rise to a heterodimer involved in a positive feedback circuit which determines the activation of many genes. Among the genes that are activated *Per* (*Per1* and *Per2*) and *Cry* (*Cry1* and *Cry2*) are involved in the activation of a negative feedback circuit, where PER and CRY proteins dimerize by inhibiting the clock. Next, CLOCK-BMAL1 heterodimer promoting

the transcriptional activity, determining a cycle repetition starting from low transcription levels (Partch C.L et al, 2013; Aguilar-Arnal and Sassone-Corsi, S, 2014) (Fig.8).

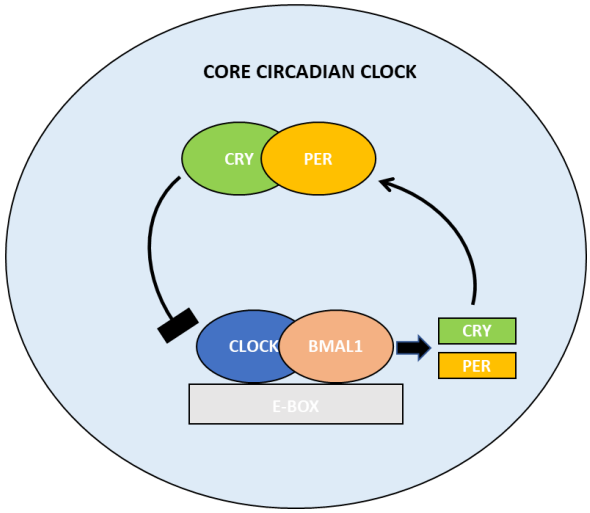



**Fig8.** Core circadian clock. Clock and Bmal1 feedback (+) circuit. CRY and PER feedback (-) circuit.

## 5.7 GABA in sleep and circadian rhythms

GABAergic transmission is a key regulator of sleep (Stenberg D, 2007). GABA is increasingly recognized as an important neurotransmitter for the initiation and maintenance of sleep (Morgan PT et al, 2012). Hypothalamic GABAergic neurons have projections able to inhibit the firing of cells that promote wakefulness. Inhibited neurons include neurons containing norepinephrine, serotonin, glutamate, hypocretin, histamine, and serotonin (Siegel Jerome M, 2004).

Notably, studies have suggested how GABAergic signaling alterations may play a role in the etiology of chronic insomnia (Plante et al, 2012). For example, reduced levels of GABA are presented in patients with primary insomnia (Morgan PT et al, 2012; Plante et al, 2012). In addition to this,

treatment with $GABA_B$ receptor antagonists leads to enhanced behavioral states linked to brain activation (wakefulness and the dreaming stage during sleep) (Gottesmann C, 2002).

Moreover, low levels of GABA have been associated with difficulty in falling asleep, whereas high levels of GABA are involved in promoting sleep (Bollu and Kaur, 2019). Nevertheless, high GABA levels also prevent the achievement of deep sleep stages, which are essential for rest and the recovery. Accordingly, the use of benzodiazepines and barbiturates, drugs that act on the $GABA_A$ receptor opening frequency by increasing the effect of GABA, leads to a greater speed in falling asleep, enhance the slow-wave sleep, promote fewer nocturnal awakenings, but generate problems in reaching the REM sleep phase (Gottesmann C, 2002).

Another way in which GABA can influence sleep is through the regulation of circadian rhythms, which are involved in the regulation of the sleep-wake cycle (Cirelli C, 2009). Indeed, GABA has a key role in the function of this master circadian pacemaker and represents the main neurotransmitter of a tiny hypothalamic structure involved in the control of circadian rhythms called “suprachiasmatic nucleus” (SCN) (Shlomo Wagner et al, 2001; Nygård M and Palomba M, 2006). In mice, GABA exerts a dual effect on neurons in the SCN, excitatory during the day and inhibitory at night. This is due to changes in $[(Cl^-)]i$ during the circadian cycle. Interestingly, SCN neurons show a huge number of somatic $GABA_A$ receptors, which contribute to a modifiable, tonic $Cl^-$ conductance. In fact, studies showed that two different mechanisms of $Cl^-$ transport operated by NKCC1 and KCC2 are in place in SCN neurons. NKCC1 action is implicated in the accumulation of $Cl^-$ inside the cells, and KCC2 action is implicated in removing $Cl^-$ from cells. The process of replenishment is less efficient during the subjective night in the light phase, that is the period when mice are common to sleep. This results in a lower $[(Cl^-)i]$ during the night phase of the circadian cycle (Shlomo Wagner et al, 2001).

## 6. DS and sleep disorders

One of the major causes of sleep disorders in DS is sleep apnea (OSA). This is mostly due to the particular craniofacial profile of DS people, the overweight and the alterations of muscle tone of upper airways (Andreu G et al, 2002; Donnelly LF et al, 2004). Nevertheless, there is a component of the sleep abnormalities of DS people which is independent of OSA. Indeed, individuals who underwent to upper airway surgery improving OSA, have continued to report important sleep disorders (Dudoignon et al, 2017).

Sleep disorders of DS people are characterized by an increase of wakefulness with a prolonged sleep latency (in particular for NREM phase) and an amount of sleep fragmentation during the night, followed by light sleepiness during the day. Other prominent features are the reduction of the REM sleep phase (with a decrease of alpha power) and alterations in the waking EEG (slower alpha band and/or a theta band power increment) (Grubar JC et al, 1986; Diomedi M et al 1999; Levanon A, et al, 1999). However, circadian rhythms result to be unaffected by trisomy (Fernandez et al, 2017), suggesting that sleep disorders are independent by a desynchronization of the circadian clock.

## 7. Animal models of DS and sleep disorders

While various studies on humans have shown the mentioned changes in sleep, the knowledge about the sleep of the different animal models of DS are really based on very few and limited experiments. So far, research works have displayed only the disruption in the rest activity patterns and hyperactive episodes and no change of wake, NREM, and REM sleep phase, with a moderate sleep rebound after sleep deprivation in Ts1Cje mice (Colas et al, 2008). Fragmented patterns of sleep-like behavior have been observed in Tc1 mice, during the light phase with a prolongation of

wakefulness at the beginning of the dark phase (Heise et al, 2015). Other studies on Dp(16)1Yey/+ mice have reported an increase of wakefulness during light and dark phases and an enhanced activity during light phase. In addition to this, the EEG of these mice indicated alterations, with a decrease of NREM phase linked to delta power reduction (Levenga J et al, 2018). Similar sleep abnormalities were found in Ts65Dn mice, which show an increase of wakefulness during dark period with a decrease of NREM sleep phase (low frequency range including delta) and a slight increase of REM sleep amounts in the light period. Moreover, T65Dn mice have a delayed but increased sleep rebound after sleep deprivation (Colas et al, 2008). No alterations were found for circadian rhythms (Stewart et al, 2007), confirming results linked to DS individuals.

Although sleep patterns have been addressed in numerous mouse models of DS, the results have included one only study per mouse model. Moreover, all studies lacked the evaluation of activity, body temperature and metabolism, which are other factors that could be decisive in influencing the course of sleep (C Leon Lack et al 2008; Owens J A, 2009; Sharma and Kavuru, 2010).

## 8. Current treatment and clinical trials for DS

There is no pharmacological treatment for cognitive deficits in DS, only few clinical trials under development. Bartesaghi's group has promoted fluoxetine (Prozac – drug approved for depression), an inhibitor of serotonin reuptake, where the first clinical trial for safety in DS soon recruiting. However, mechanism of actions of fluoxetine are not known for DS. Finally, fluoxetine is a psychotropic drug, which is causing doubts in parents and difficulty in recruiting for the trial. Another study supported by de la Torre's group support the use of epigallocatechin-3-gallate (EGCG, phytochemical derived from green tea), an inhibitor of DYRK1A kinase. Also, for this treatment there are drawbacks such as that the mechanism of action is not known for DS; green tea active substance

is unknown; and there is a poor efficacy in clinical trials in young adults with DS (NCT01394796 and NCT01699711). An additional trial (NCT03624556) in pediatric patients with DS or Fragile X syndrome is currently recruiting.

Finally, Costa's group has proposed memantine hydrochloride (Namenda - drug approved for Alzheimer-type dementia), an antagonist of NMDA-Receptor. Also, in this case the mechanism of action is not known for DS, and poor efficacy was reported in a clinical trial in adults (NCT01112683) with DS. Nevertheless, an additional trial (NCT02304302) in young adults with DS currently recruiting.

## 9. Prader-Willi syndrome

PWS is a rare genomic imprinting or epigenetic disorder, with an incidence estimated at the birth of approximately 1/15,000–1/20,000 individuals (Soni S et al, 2008).

PWS is caused by alterations linked to allele paternal chromosome 15 (15q11-q13) deletion. The loss of paternal genetic inheritance characteristic of PWS, can originate from the partial or total deletion of the q11-13 region (65-75 %) or a maternal uniparental disomy 15 (UPD) (20-30%) with the presence of two copy of maternal chromosome 15 or the absence of chromosomal paternal copy. Notably, other cases of PWS arise for imprinting defects (1-3%) (Cassidy SB et al, 2012) (Fig.9).

A recent scientific research has highlighted the existence of two different sites of the breakpoint involving the deletion: Type 1 (T1D) and Type (T2D). T1 comprises the breakpoint site 1 (BP1), which is found in the centromere zone of chromosome 15, T2 includes the breakpoint 2 (BP2), placed ~500 kb distal to BP1. Therefore, T1 involves an additional zone of genetic material in comparison to T2 (Nicholls RD et al, 2001; Mewborn SK et al, 2002) (Fig.10).

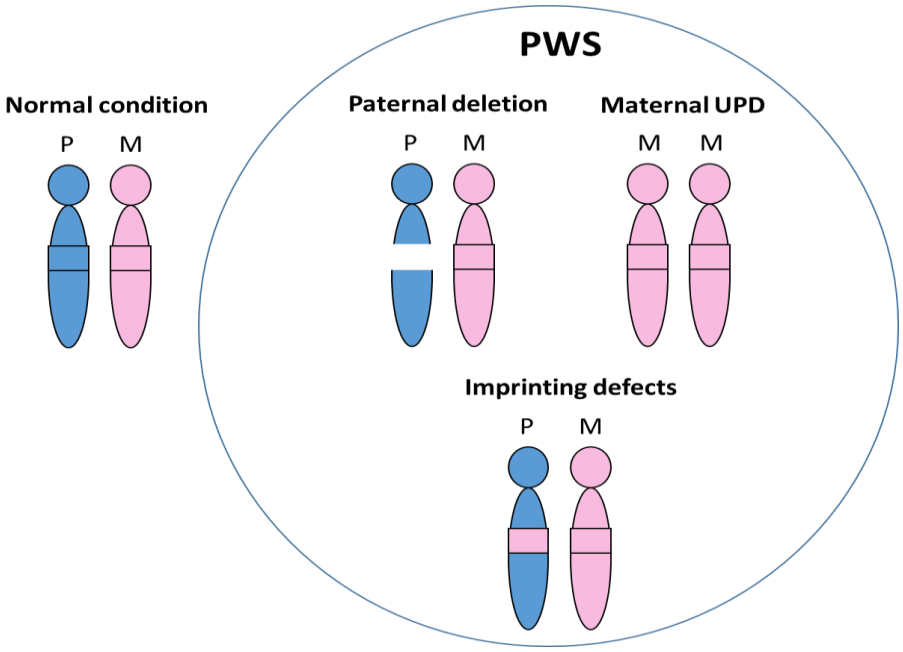



**Fig.9.** Mechanisms implicated in the insurgence of PWS paternal chromosome 15 (15q11-q13) deletion.

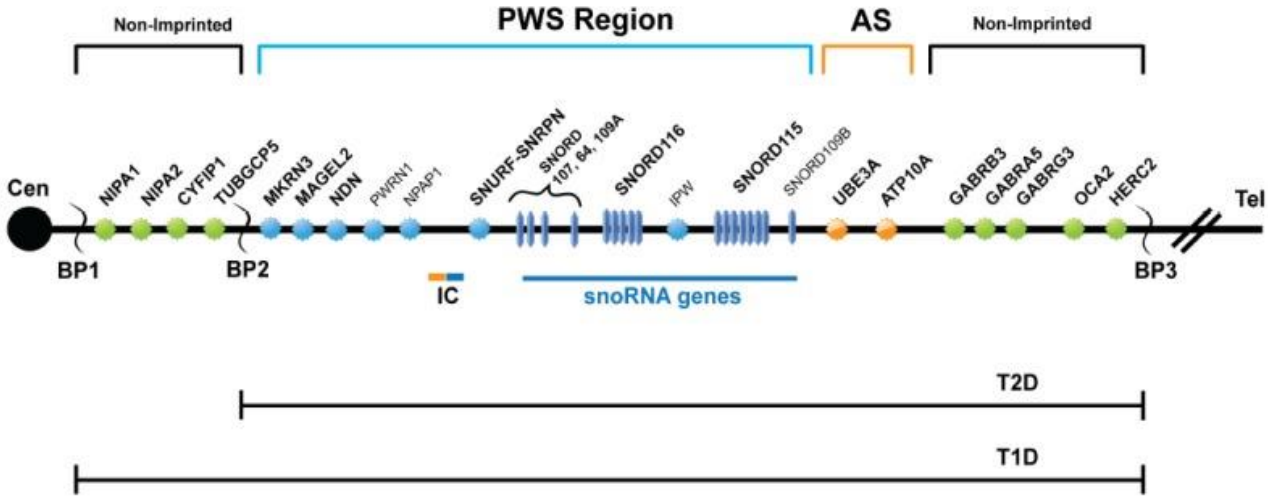



**Fig10.** Breakpoint 1 and 2 involving in the generation of T1 or T2 deletion of chromosome 15 (15q11-q13)

PWS is mainly characterized by developmental abnormalities, behavioral deficits, sleep disorders and metabolic dysfunctions. Developmental abnormalities comprise neonatal hypotonia, growth retardation, short stature, frequent hypogonadism and defects in brain region showing lower brainstem volume, and a trend towards a reduction of cortical surface area and white matter volume (Lan An Thuy, 2017; Whittington J and Holland A, 2017; Miscogiuri et al, 2019).

The most important PWS features are associated with an impulsive tendency to feeding getting into obesity, an intellectual disability from mild to moderate, a high presence of separation anxiety in childhood (Steinhausen et al, 2004), aggressive behavior, impulsivity and hyperactivity related to attention deficit hyperactivity disorder (ADHD), psychosis aspects, obsessive-compulsive-behaviors during the adulthood and sleep disorders (Gillet and Perez, 2016; Guinovart et al, 2019). Peculiarly, children with PWS show poor feeding and lack of appetite in infancy, developing an uncontrolled appetite with lack of satiety that get to weight gain, mostly after the age of 2–3 years (Crinò et al, 2018). Moreover, seizures are also reported in some patients (Verrotti et al, 2014). Thus, many of these hallmarks common to other NDDs maybe also related to altered intracellular $Cl^-$ concentration and depolarizing GABAergic signaling. In addition to this, PWS is also related to deficits of sociability, emotional states and joint attention (Dickens et al, 2019). In particular, a relationship between PWS and specific certified clinical features of autism spectrum disorder (ASD) is present in approximately 40% of people with PWS due to UPD, 15–18% of PWS cases due to deletions (Dyckens et al, 2011).

The imprinting defect leads to the loss of genes expression on allele paternal chromosome locus 15q11-q13, while the same region linked to maternal chromosome is involved to the onset of Angelman syndrome (AS), highlighting a key role in the expression of these genes (Nicholls, 2000). Among the lack of genes caused by the deletion on paternal chromosome 15 in PWS, *SNORD116* (also called H/MBII-85), plays a critical role on the clinical phenotypic manifestation of PWS. Indeed,

PWS individuals, presenting deletion of *SNORD116* gene only, present with the typical phenotypic features associated to PWS (Bieth E et al, 2015).

*Snord116* is a cluster of small nucleolar/noncoding RNAs (snoRNAs), mainly expressed in the brain, which are involved in the control of the expression of other small nucleolar RNAs (Sahoo et al, 2008). Lack of *Snord116* in different mouse models with deletion of diverse lengths (all including *Snord116*) lead to growth deficiency, hyperphagia, cognitive alterations and sleep disorders (Ding et al, 2008; Lassi et al, 2016; Lassi, Maggi, et al 2016; Adhikari et al, 2018). Nevertheless, how *Snord116* affects these functions remains still unclear.

Among the experimental models, the mouse mutant PWScr$^{m+/p-}$, carry a very restricted deletion just involving *Snord116* and *IPW* (imprinted gene in the Prader Willi syndrome region) exons A1/A2, B, and C, which is a maternally imprinted gene related to the PWS and AS imprinted domain on human chromosome 15 and mouse chromosome 7, probably implicated in mechanisms of regulation of imprinting (Wevrick R et al, 1994; Skryabin BV et al, 2007). Thus, PWScr$^{m+/p-}$ represents one of the best models to examine the specific phenotype associated with the absence of *Snord116*. PWScr$^{m+/p-}$ mutant mice are characterized by postnatal growth retardation, a major reduction in the size of the hippocampus and sleep disorders (Skryabin BV et al, 2007; Lassi et al, 2016). However, only few studies have been executed on PWScr$^{m+/p-}$ and these studies did not include cognition and other behaviors correlated to ASD and anxiety disorders.

## 10. Cognition and PWS

Cognitive deficits in PWS individuals are characterized by impairment in memory, a low intelligent quotient (IQ), impairment to elaborate an abstract idea, and difficulties of comprehension (Warren and Hunt, 1981; Bertella et al, 2005; Whittington and Holland, 2017). Interestingly, performances

on basic literacy and numeracy are very variable in PWS, but they shared a decline of the attainment with age (Whittington et al, 2004). Generally, language development is delayed and compromised, presenting problems in the articulation, which need speech therapy (Whittington et al, 2002). In particular, different studies, (Chen et al, 2010; Dimitropoulos et al, 2013 a,b), have shown that expressive language is more damaged than receptive language. Moreover, people with PWS also reported impairments of auditory modality in an event-related brain activity study (Stauder et al, 2002), and low scores relative to investigations of executive functions, with a severe impairment of working memory and a slight impairment in tasks evaluating the inhibition (Jauregi et al, 2007; Chevalere et al, 2015). However, smaller brain volumes and less cortical complexity, probably are responsible for low IQ, while specific disorders of executive functions and language may be related to specific brain areas (Manning and Holland, 2015; Whittington and Holland, 2017). Anyway, clinical diagnostic criteria for PWS enclose excellent abilities with jigsaw puzzles and a lot of time doing them (Verdine et al, 2008).

Finally, social cognition is commonly impaired in PW, with the presence of absent or very poor peer group relationship, similarly to features associated to ASD (Dykens EM et al, 2019). For example, some studies indicated that the observance of social norms are not well considered in individuals with PWS (Dimitropoulos et al, 2013 a,b), disturbing conversation and invading others' personal space (Lo et al, 2013).

## 11. PWS mouse models and cognition

Studies on mouse models of PWS started in 1992 aimed at looking at physical and behavioral characteristics, particularly feeding behavior, size, weight, and hypotonia. Cognition was investigated only later (Whittington and Holland, 2017).

In particular, an imprinting centre deletion mouse model of PWS (PWS-IC) has reported reduced attentional capabilities using a five-choice serial reaction time task (5-CSRTT) decreasing accuracy and presenting a greater number of omissions and longer correct reaction times in comparison to wild type mice. Interestingly motor function and motivation were not significantly impaired compared to controls in these same mice. Remarkably, these results are comparable to features of people with PWS (Relkovic et al, 2010). Of note, PWS-IC model has shown better performances, spending less time to reach criteria and few errors, during acquisition and reversal learning in a behavioral test based on the influence of palatable food reward on learning. Probably, this cognitive effort can be explained considering the importance of food acquisition for people with PWS (Relkovic et al, 2012).

Impairments in social recognition, social interaction and a reduced learning ability were found in adult male mice lacking the *Magel2* gene ($M2^{-}$), while no cognitive deficits were showed in female $M2^{-}$mice. However, working memory tested in the Y-maze remained unchanged independently of gender (Meziane et al, 2015). Social deficits were displayed for a *Maged1* knock-out mouse model (Dombret et al, 2012). Conversely, investigations on spatial learning and memory in *Necdin* deficient mice using the Morris water maze indicated that the mutants reported better memories for the platform location (Muscatelli et al, 2000).

Researches based on the lack of SnoRNA with a deletion of the 40+ copies of *Snord116*, have showed a normal short-term working memory measuring rates of spontaneous alternation in the Y maze, motor learning deficiency between WT mice and mutant mice (Ding et al, 2008). In addition, experiments on *Snord116*$^{+/-}$ mutant mice, carrying a ~150 kb deletion of *Snord116*, have reported deficits in the learning and memory abilities using the novel object recognition, location memory and tone cue fear conditioning assays compared to littermate control *Snord116*$^{+/+}$ mice (Adhikari et al, 2018). Anyway, in the model PWScr$^{m+/p-}$ with the restricted deletion involving *Snord11*6, has a

better performance and decision making in the working-for-food task have been confirmed (Lassi et al, 2016). These results highlight the strong cognitive effort to eat in PWS.

## 12. Psychopathologies and PWS

The various psychopathological traits associated with PWS are very varied and most patients report generalized anxiety. Interestingly, anxiety-related and depressive disorders have been associated with genetic modifications involving the region of chromosome 15 responsible for the PWS. Several cases of patients showing anxiety symptoms also reported outbursts of anger. However, the most overwhelming (or preponderant cases of mental illness) have been seen in UDP cases. This suggests the importance of the genetic component in PWS disorders (Guinovart et al, 2019).

PWS is one of several disorders associated with autism. This is probably because the region 15q11p-13 represents a place of significant impact on ASD susceptibility genes. In particular, PWS individuals exhibit repetitive behaviors, which may be different in type among the various subjects, and they are more expressed in males. Among the obsessive-compulsive disorders, self-injurious behavior is a very present feature, especially skin picking (Dykens et al, 2011).

Furthermore, PWS individuals also report communicative impairments and social interaction issues (Dimitropoulos A, 2013b). For example, during experiments of face processing, PWS people present eye-tracking alterations similar to ASD people (Dalton KM et al 2005; Klin A et al, 2002). Moreover, PWS individuals perform poorly in the social attribution task, again similarly to ASD individuals (Koenig K et al, 2004).

A hypothesis to explain the psychopathological traits that are often associated with PWS may be the implication of the GABAergic system. Indeed, the deletion region of PWS chromosome 15 contains the genes implicated in the coding of the alpha5 and beta3 subunit of the $GABA_A$ receptor. These

$GABA_A$ receptors present a reduction in the number and abnormalities in the subunit composition in the neocortex and in the insula of PWS individuals (Lucignani G et al, 2004).

In addition, low levels of GABA have been reported using single-voxel proton magnetic resonance spectroscopy (1H-MRS) in PWS people (Rice L.J et al 2016).

## 13. Current treatment and clinical trials for PWS

Obesity and hyperphagia are mostly involved to induce morbidity and mortality in PWS that needed an accurate diagnosis, appropriate medical management and treatment (Butler et al, 2016).

Management is actually operated by multidisciplinary teams including clinical geneticists, endocrinologists, dietitians, primary care physicians and mental health experts who aim to regulate weight gain, monitoring the diet and give a specific treatment to comorbid conditions associated with PWS (Butler MG, 2006; Soni.S et al, 2007; Goldstone AP et al, 2008; McCandless SE et al, 2011).

Hyperphagia and obesity complications can be managed by strict control of the diet and practicing regular exercise plans. Therapies with the goal to replace growth and sex hormones represent other significant strategies involved in the management (McCandless SE et al, 2011; Griggs.G et al, 2015).

However, problems related to cognition are also very important, preventing a normal course of life for these individuals inside society. Therefore, the necessity of a clinical treatment in order to allow an improvement of intellectual disabilities of PWS appears to be essential.

So far, only the growth hormone treatment has produced beneficial effects on cognition in children and adults with PWS (Grugni and Marzullo 2016). This was described in different case studies (Vogt KS and Emerick JE, 2015; Dykens et al, 2017, Lo ST et al, 2015) But this treatment commonly prescribed in PWS during infancy, childhood and adolescence may worsen scoliosis (Murakami N et

al, 2012; Deal CL et al, 2013) and the safety of treatment is not well clarified. Moreover, therapies or clinical trials to improve symptoms of PWS related to ASD have not been carried out yet.

Thus, new therapeutic approaches for the treatment of cognition and ASD in PWS is urgently needed. In this context, the study of $Cl^-$ homeostasis represents a still unexplored field of the research (Lucignani G et al, 2004; Rice L.J et al 2016) in animal models and individuals with PWS. From a growing literature that describes the involvement of an aberrant chloride homeostasis in a very high number of pathologies of neurodevelopment (Ben-ari, 2017) that share with PWS various symptoms, our desire is born to investigate the mechanisms underlying the action of the GABA neurotransmitter and the mechanisms of $Cl^-$ homeostasis in neuronal activity of PWS.

## Aim of Project

### 1. The role of $Cl^-$ homeostasis in sleep disorder of DS

In the recent past, Deidda et al. proposed that GABAergic transmission, which is normally hyperpolarizing and inhibitory in adult WT animals, is depolarizing and possibly excitatory in adult DS mice. Accordingly, they demonstrated that DS mice overexpress NKCC1, leading to higher $[Cl^-]i$ and depolarizing/excitatory $Cl^-$ efflux upon $GABA_A$-receptor activation. Surprisingly, NKCC1 inhibition by the FDA-approved diuretic bumetanide has shown to be able to rescue inhibitory GABAergic transmission, synaptic plasticity and cognitive functions in adult DS mice (Deidda et al, 2015).

The alteration of GABAergic transmission due to upregulation of NKCC1 in DS might be a reason of sleep disturbances. This is useful to shed light on whether defective $Cl^-$ homeostasis is involved in sleep alteration in adult DS mice, and possibly to indicate bumetanide as a therapeutic approach to ameliorate sleep disorders in DS patients. Thus, we propose an in vivo study of sleep and its relation to altered $Cl^-$ homeostasis in adult TS65Dn mice, the most used animal model of DS (Reeves et al., 1995; Escorihuela et al., 1995; Reeves et al., 1995; Siarey et al., 1997; Siarey et al., 1999; Sago et al., 2000; Belichenko et al, 2004; Kleschevnikov et al., 2004; Costa & Grybko, 2005; Chakrabarti et al., 2007; Contestabile et al., 2007; Fernandez et al., 2007; Colas et al., 2008; Costa et al., 2008; Contestabile et al., 2010; Chakrabarti et al., 2010; Contestabile et al, 2013).

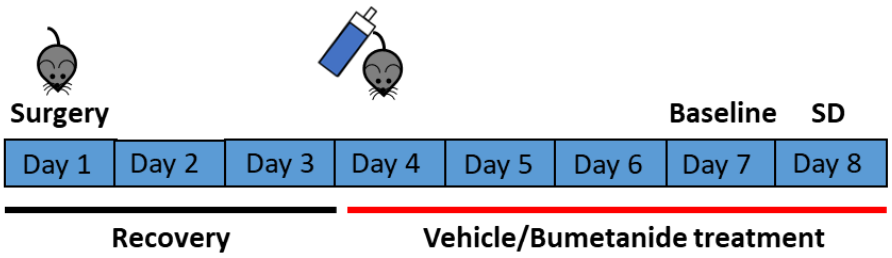
We used a unique setup that allows the simultaneous and synchronized monitoring of EEG, temperature and activity along the 24 hrs. So, we investigated about a possible implication of an aberrant GABAergic transmission on sleep and whether rescuing an impaired $Cl^-$ homeostasis by bumetanide could normalize the sleep-wake cycle in adult Ts65Dn mice.

## 2. The role of $Cl^-$ homeostasis in behaviors and circadian rhythms of PWS

PWS is a neurodevelopmental disorder characterized by anxiety, cognitive alterations, sleep disorders and ASD behaviors (Steinhausen et al, 2004; Dykens EM et al, 2019; Guinovart et al, 2019; Miscogiuri et al, 2019). As in a growing number of neurodevelopmental disorders these same health issues have been associated to impaired intracellular $Cl^-$ homeostasis (Ben-Ari, 2017), we decided to evaluate possible changes in the expression of the two chloride co-trasporters NKCC1 and KCC2 in a in a mouse model of PWS ($PWScr^{m+/p-}$ mouse model of PWS) in the diverse brain regions. We conducted our experiments in the $PWScr^{m+/p-}$ mice. This mouse model has the shortest deletion of the chromosome 7, just involving *Snord116* and IPW exons A1/A2, B, and C (Wevrick R et al, 1994; Skryabin BV et al, 2007). We chose this model because it was the best to specifically recapitulate PWS symptoms, considering that *SNORD116* deletion is mostly implicated in the phenotypic manifestations of PWS (Bieth E et al, 2015). Then, we investigated the impact of altered $Cl^-$ homeostasis on cognition, anxiety and ASD behaviors in $PWScr^{m+/p-}$ compared to WT. Finally, we also investigated possible abnormalities in the circadian rhythm of PWS mice and its $Cl^-$ dependence.

## Results: The role of $Cl^-$ homeostasis in sleep disorder of DS

All experiments were performed in collaboration with Dr. Tucci's lab (Neurobehavioural Genetics group). EEG recordings led to a detailed overview during the day-night cycle on wakefulness analysis (Fig.11), NREM (Fig.12,Fig.15, Fig.16), REM (Fig.13,Fig.17,Fig.18), and total sleep (Fig.14) of wild type (WT) and Ts65Dn (Ts) mice (3-4 month old) treated with vehicle (WT_VE and Ts_VE saline) and bumetanide 2mg/ml in drinking water (WT_BU and Ts_BU), following the experimental procedure represented below in the scheme 1.




**Scheme1.** Representation of experimental procedure

In total, 24 animals were treated (6 WT_VE, 6 Ts_VE, 6 WT_BU and 6 Ts_BU) before (Fig.11-15, 17) during and after sleep deprivation (SD) of 6 h (Fig.11-14, 16,18). Through the same wireless devices, we also performed the analysis of activity and temperature (Fig.19-22) during the 24 hours.

## 1. Bumetanide decreases abnormal wakefulness at the early stage of light phase and improves sleep quality in Ts65Dn mice

During the 24 hours of EEG recordings, the amount of wakefulness was greater in Ts_VE mice than WT_VE in the light period at baseline (Fig.11A). Interestingly bumetanide treatment decreases this abnormal wakefulness in the light period. Indeed, no differences emerged by bumetanide treatment in Ts_BU compared to WT_VE at baseline, but a significative increase of wakefulness amounts were reported in the recovery after SD (Fig.11B). However, the comparison between WT_VE and WT_BU (Fig.11C), did not shown any significative effect, as well as, Ts_VE and Ts_BU (Fig.11D).

Moreover, we reported an alteration of architecture of sleep with a reduction of amount of NREM sleep across light phase in Ts_VE compared to WT_VE at baseline and after SD (Fig.12A). Of note,

we displayed a decrease in NREM sleep amounts of Ts_BU respect to WT_VE though light phase at baseline and in light and dark phase during the recovery (Fig.12B). No statistically significant difference was found between WT_VE and WT_BU, as well as, Ts_VE and Ts_BU (Fig.12C, D).

As regards the REM sleep phase, we did not find any alteration difference between WT_VE and Ts_VE (Fig.13A), WT_VE and WT_BU (Fig.13C), and Ts_VE and Ts_BU (Fig.13D). Peculiarly, we showed a reduction of amount of REM sleep in Ts_BU compared to WT_VE across dark period after SD, (Fig.13B).

In addition to this, analysis on the total sleep have confirmed a statistically significant decrease of sleep in Ts_VE compared to WT_VE during light at baseline (Fig.14A) and a lowering sleep amounts in dark phase after SD in Ts_BU (Fig.14D).

Further analyses were performed on the spectral characteristics of the EEG. EEG power density was compared for delta (0, 5–4 Hz) (Ito J et al, 2014) and theta (5–10 Hz) (Tort A. BL. et al, 2018) in NREM and REM sleep phases, as an indication of the sleep quality across 24h at baseline and 18h after SD. We found a statistically significant reduction of delta power during NREM and an increase of theta power before and after SD in Ts_VE compared to WT_VE (Fig.15A and Fig.16A). Interestingly, we reported an improvement of power density of delta and theta power in Ts_BU compared to Ts_VE (Fig.15D and Fig.16D), towards the level of WT_VE at baseline and after SD (Fig.15B and Fig.16B). No differences have shown in the comparison between WT_VE and WT_BU (Fig.15C and Fig.16C). In addition, we showed a statistically significant increase of theta power density in Ts_VE compared to WT_VE before and after SD, and a decrease of delta power at baseline (Fig.17A and Fig.18A). Peculiarly, Ts_BU mice present a lower power density of theta compared to Ts_VE before and after SD (Fig.17D and Fig.18D), improving theta power towards the level of WT_VE (Fig.17B and Fig.18B). Moreover, Ts_BU also show an improvement of delta power at baseline towards the level of WT_VE

(Fig.17D and Fig.17B). No differences were found between WT_VE and WT_BU (Fig.17C and Fig.18C).

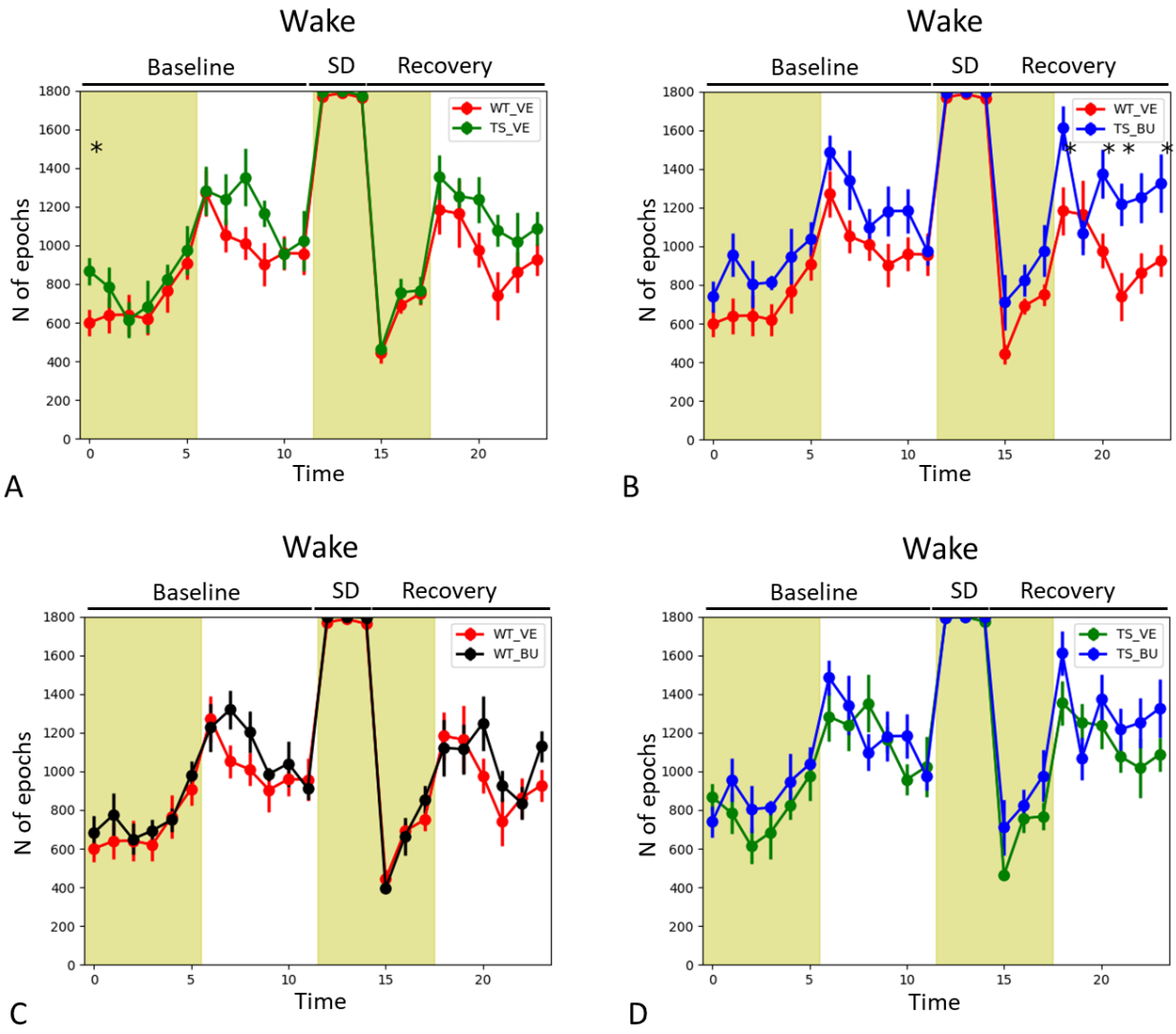



**Fig.11.** Amount of the epochs number of wake, per two hours, across the 24 h in A) 6 WT_VE versus 6 Ts_VE, B) 6 WT_VE versus 6 Ts_BU, C) 6 WT_VE versus 6 WT_BU D) 6 Ts_VE versus 6 Ts_BU at baseline, sleep deprivation (SD) and in the recovery after SD. An epoch is intended 4 seconds of EEG registration period. All graphs are presented as mean ± SEM across 24 h. Light and dark phases are indicated respectively by the yellow and white on each graph. Statistical significance is represented as follows: *P < 0.05, by t.test student.

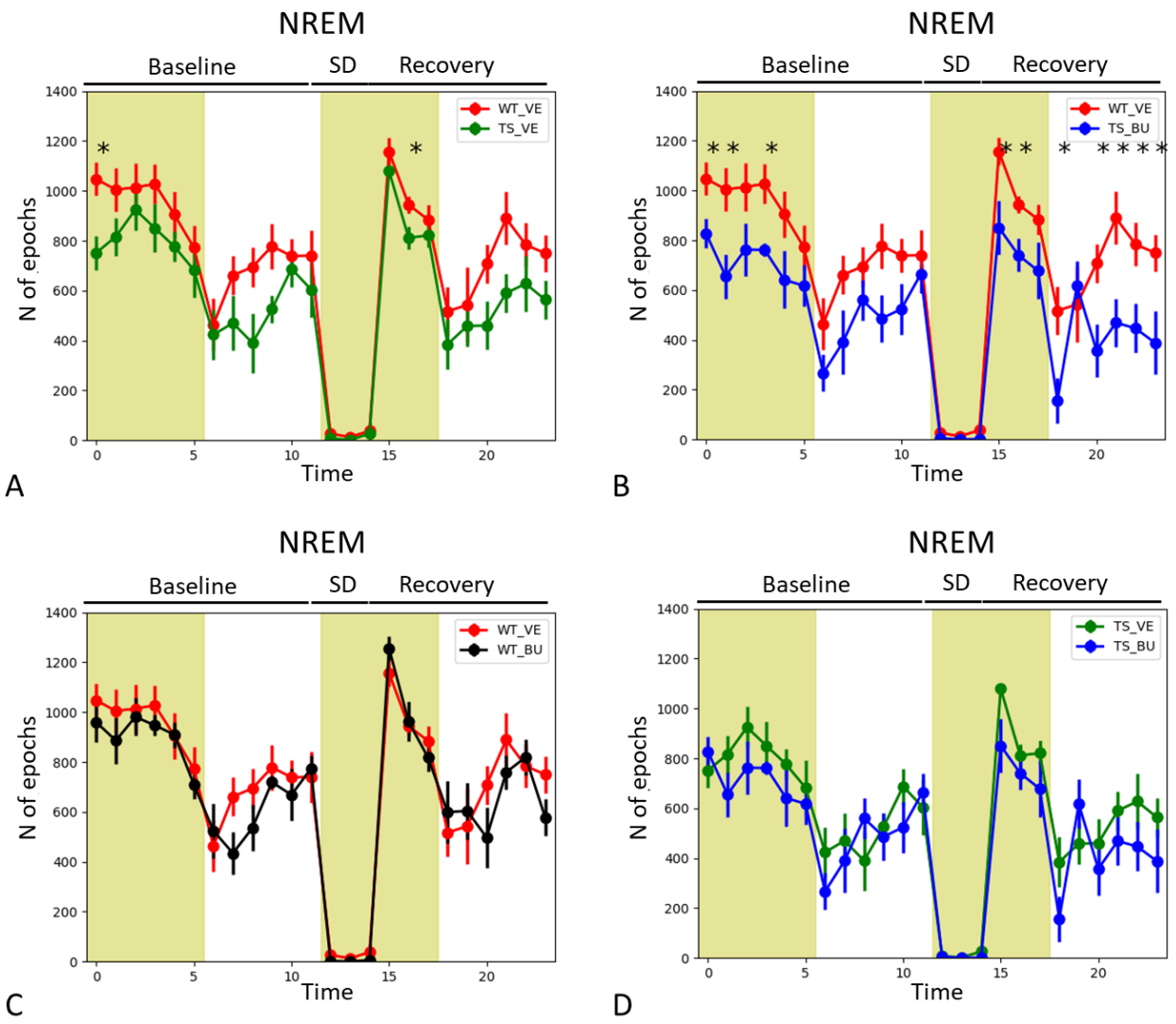



**Fig.12**. Amount of the epochs number of NREM, per two hours, across the 24 h in A) 6 WT_VE versus 6 Ts_VE, B) 6 WT_VE versus 6 Ts_BU, C) 6 WT_VE versus 6 WT_BU D) 6 Ts_VE versus 6 Ts_BU at baseline, sleep deprivation (SD) and in the recovery after SD . An epoch is intended 4 seconds of EEG registration period. All graphs are presented as mean ± SEM across 24 h. Light and dark phases are indicated respectively by the yellow and white on each graph. Statistical significance is represented as follows: *P < 0.05, by t.test student.

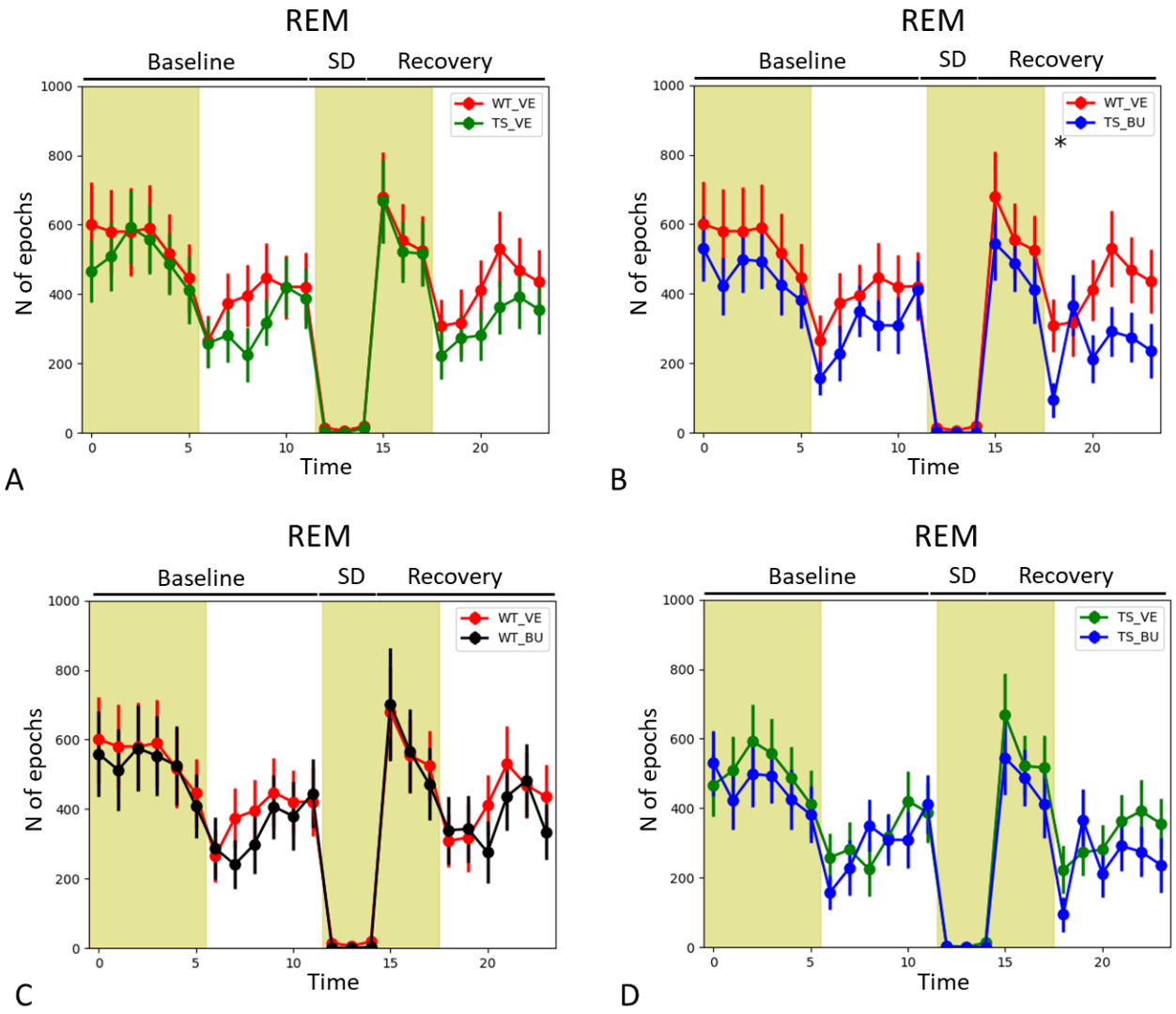



**Fig.13**. Amount of the epochs number of NREM, per two hours, across the 24 h in A) 6 WT_VE versus 6 Ts_VE, B) 6 WT_VE versus 6 Ts_BU, C) 6 WT_VE versus 6 WT_BU D) 6 Ts_VE versus 6 Ts_BU at baseline, sleep deprivation (SD) and in the recovery after SD . An epoch is intended 4 seconds of EEG registration period. All graphs are presented as mean ± SEM across 24 h. Light and dark phases are indicated respectively by the yellow and white on each graph. Statistical significance is represented as follows: *P < 0.05, by t.test student.

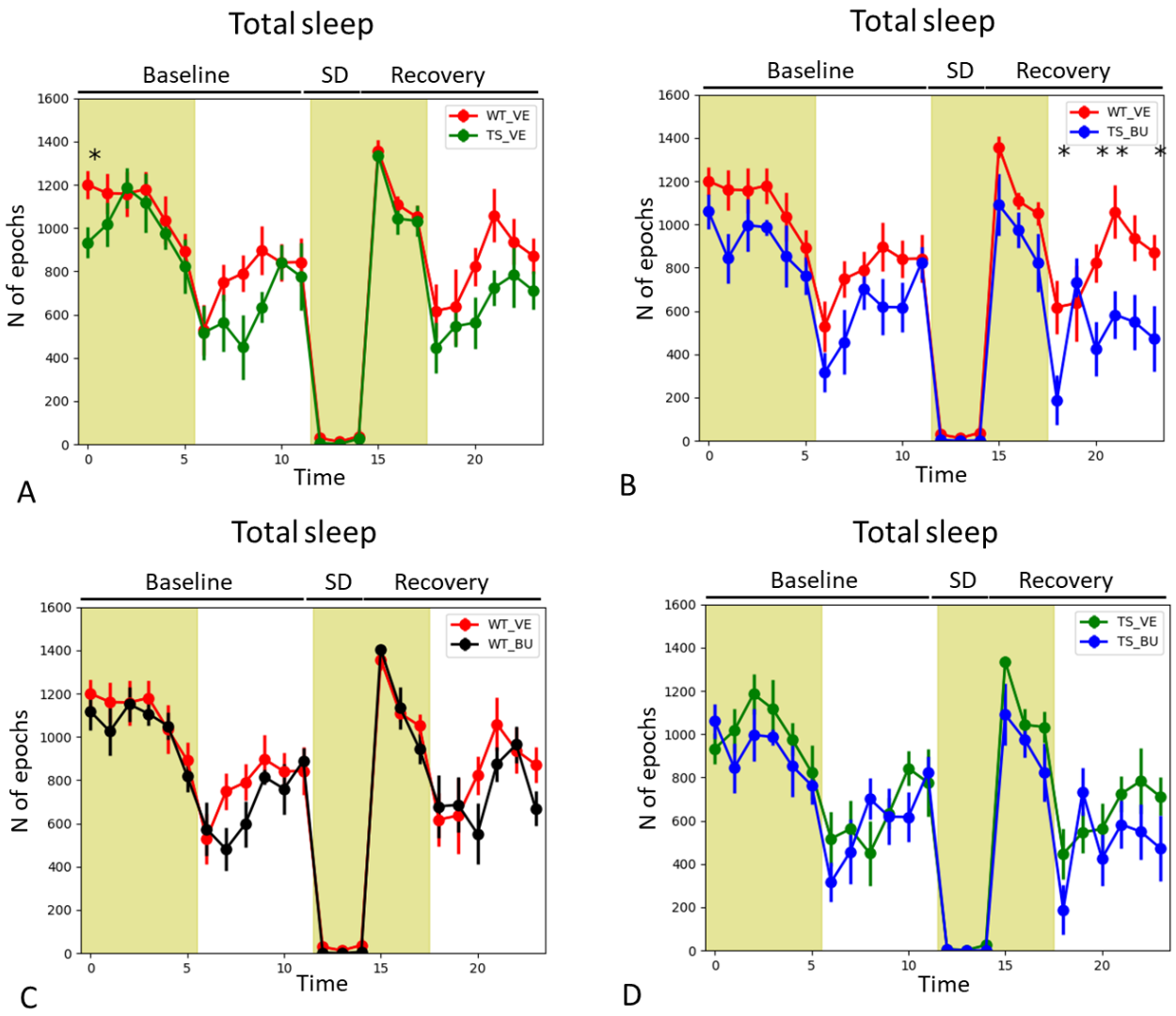



**Fig.14.** Amount of the epochs number of NREM, per two hours, across the 24 h in A) 6 WT_VE versus 6 Ts_VE, B) 6 WT_VE versus 6 Ts_BU, C) 6 WT_VE versus 6 WT_BU D) 6 Ts_VE versus 6 Ts_BU at baseline, sleep deprivation (SD) and in the recovery after SD. An epoch is intended 4 seconds of EEG registration period. All graphs are presented as mean ± SEM across 24 h.Light and dark phases are indicated respectively by the yellow and white on each graph. Statistical significance is represented as follows: *P < 0.05; **P < 0.01, by t.test student.

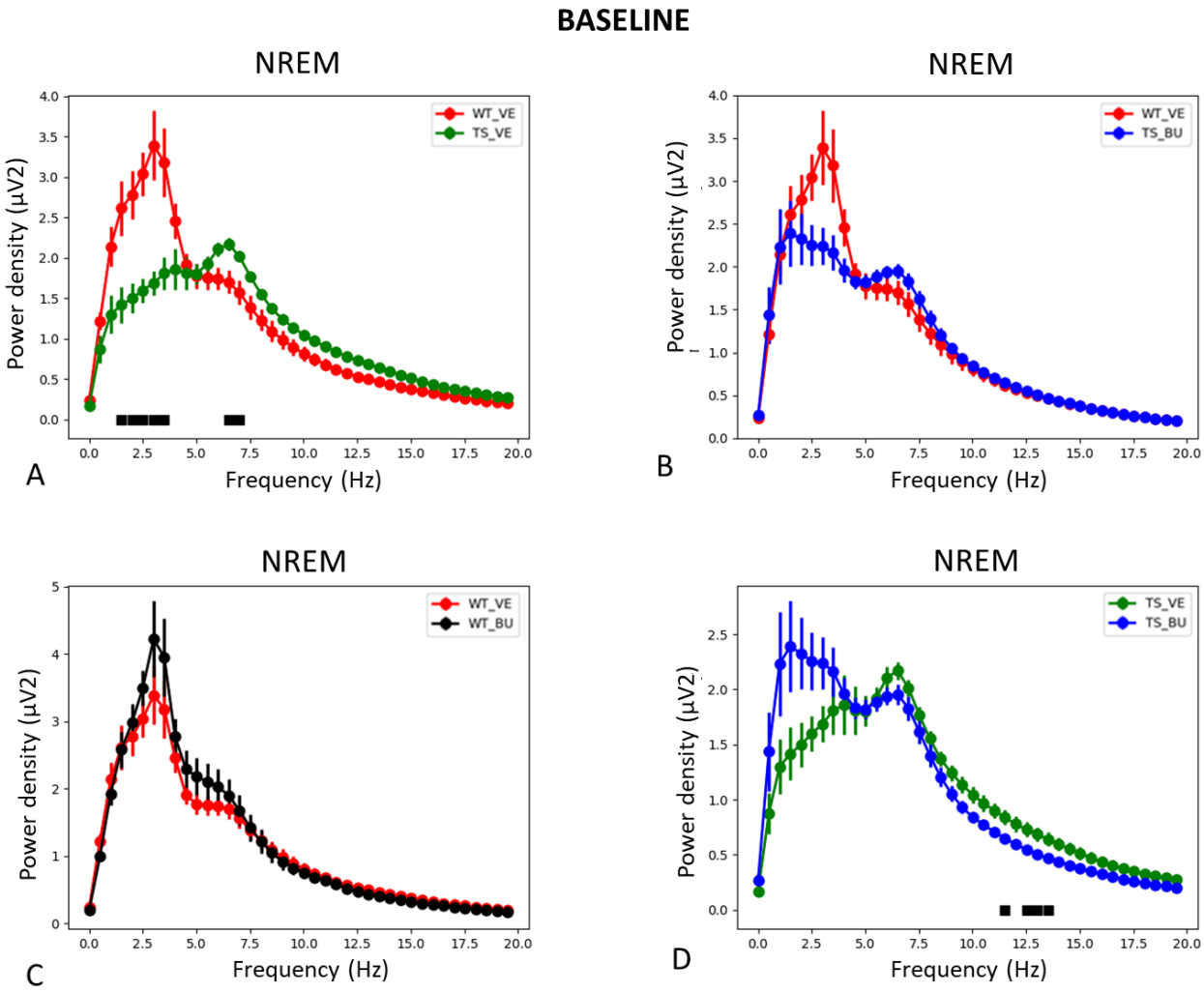



**Fig.15**. EEG power densities of the whole spectrum of frequencies (µV2 ) during NREM sleep at baseline in A) 6 WT_VE versus 6 Ts_VE B) 6 WT_VE versus 6 Ts_BU, C) 6 WT_VE versus 6 WT_BU D) 6 Ts_VE versus 6 Ts_BU. ■ =*P<0,05, ■■ = P<0,001, by t.student test.

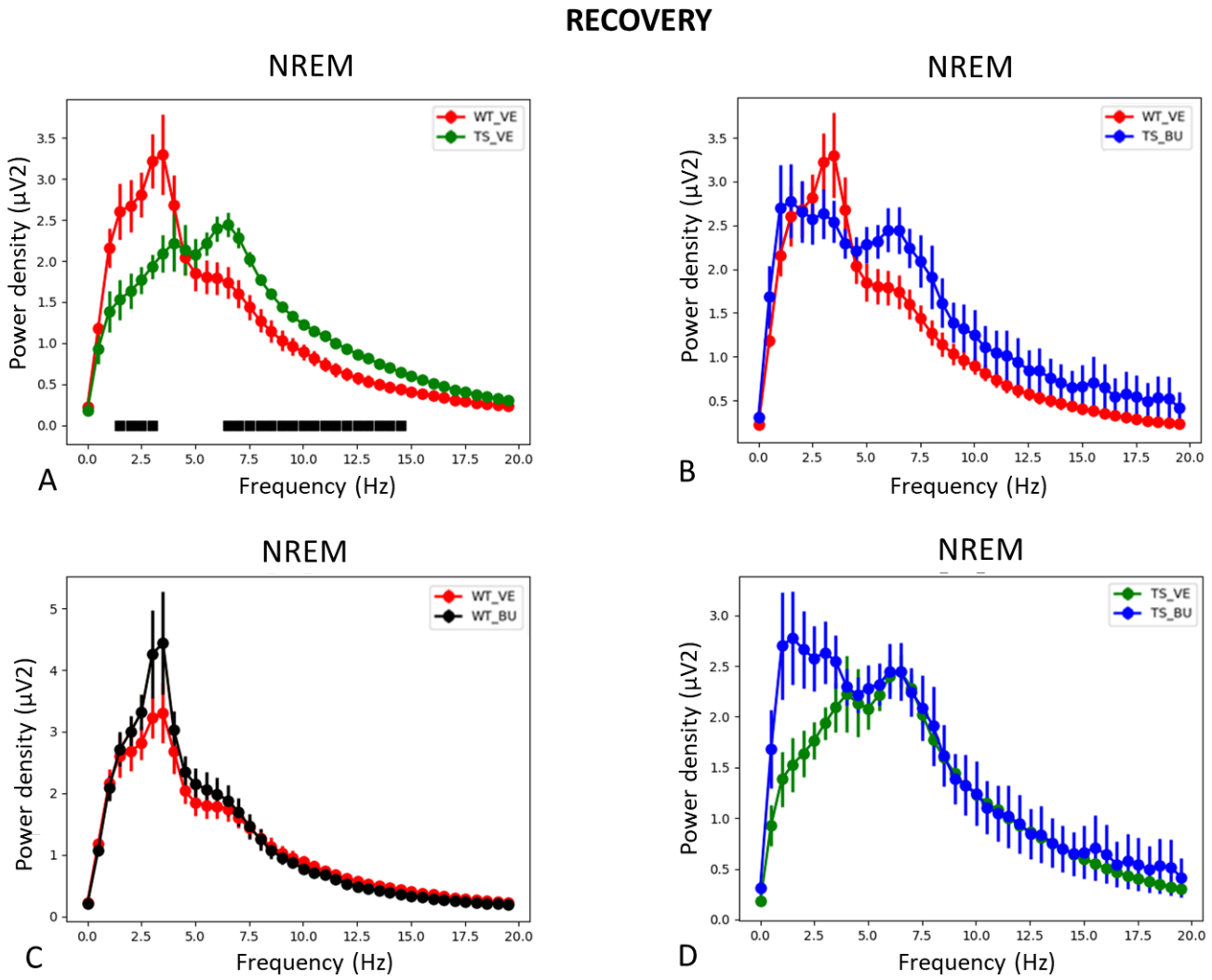



**Fig.16**. EEG power densities of the whole spectrum of frequencies (µV2) during NREM sleep after sleep deprivation in A) 6 WT_VE versus 6 Ts_VE B) 6 WT_VE versus 6 Ts_BU, C) 6 WT_VE versus 6 WT_BU D) 6 Ts_VE versus 6 Ts_BU. ■ =*P<0,05.

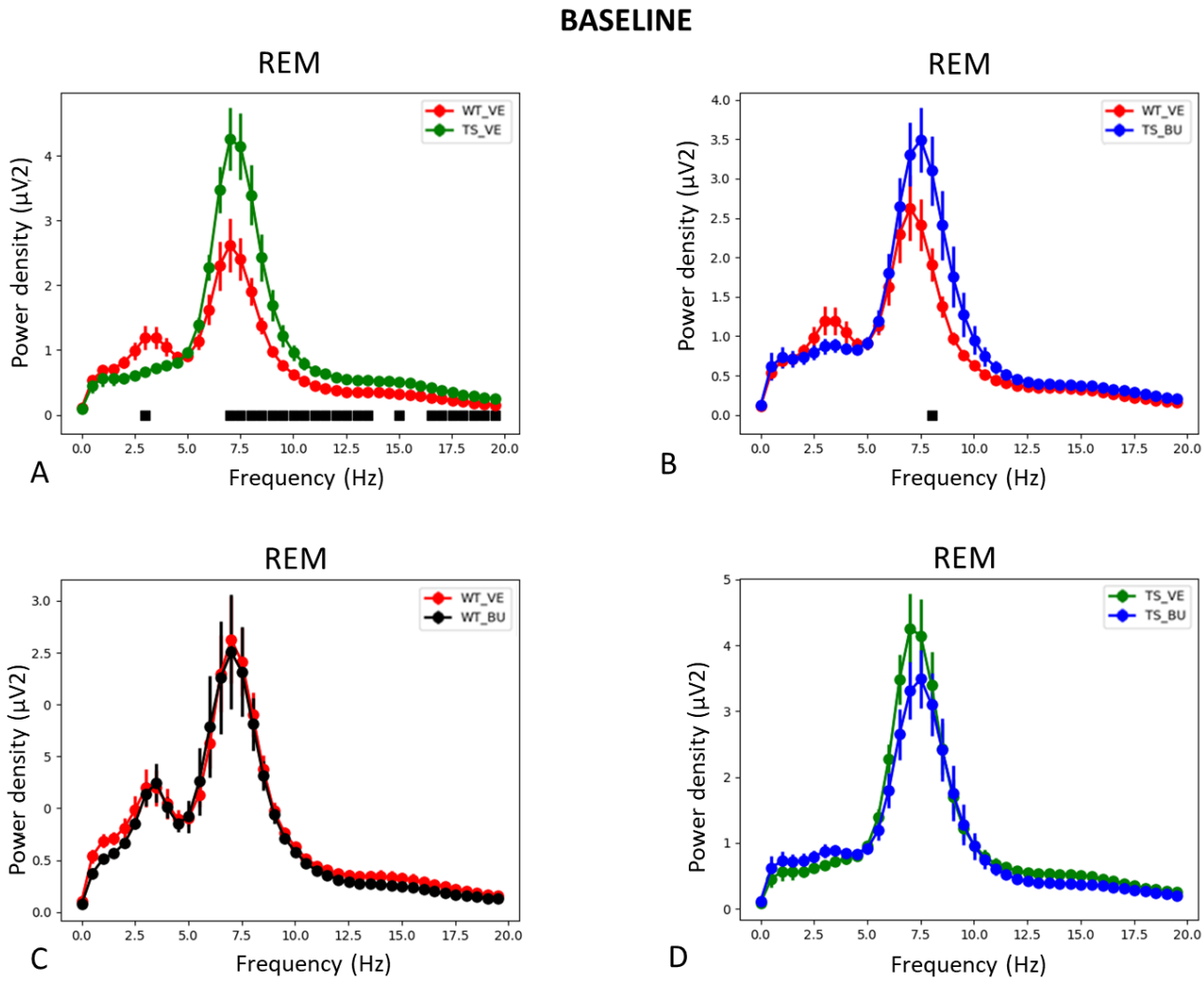



**Fig.17**. EEG power densities of the whole spectrum of frequencies (µV2) during REM sleep at baseline in A) 6 WT_VE versus 6 Ts_VE B) 6 WT_VE versus 6 Ts_BU, C) 6 WT_VE versus 6 WT_BU D) 6 Ts_VE versus 6 Ts_BU. ■ = P<0,05, by t.student test.

**RECOVERY**

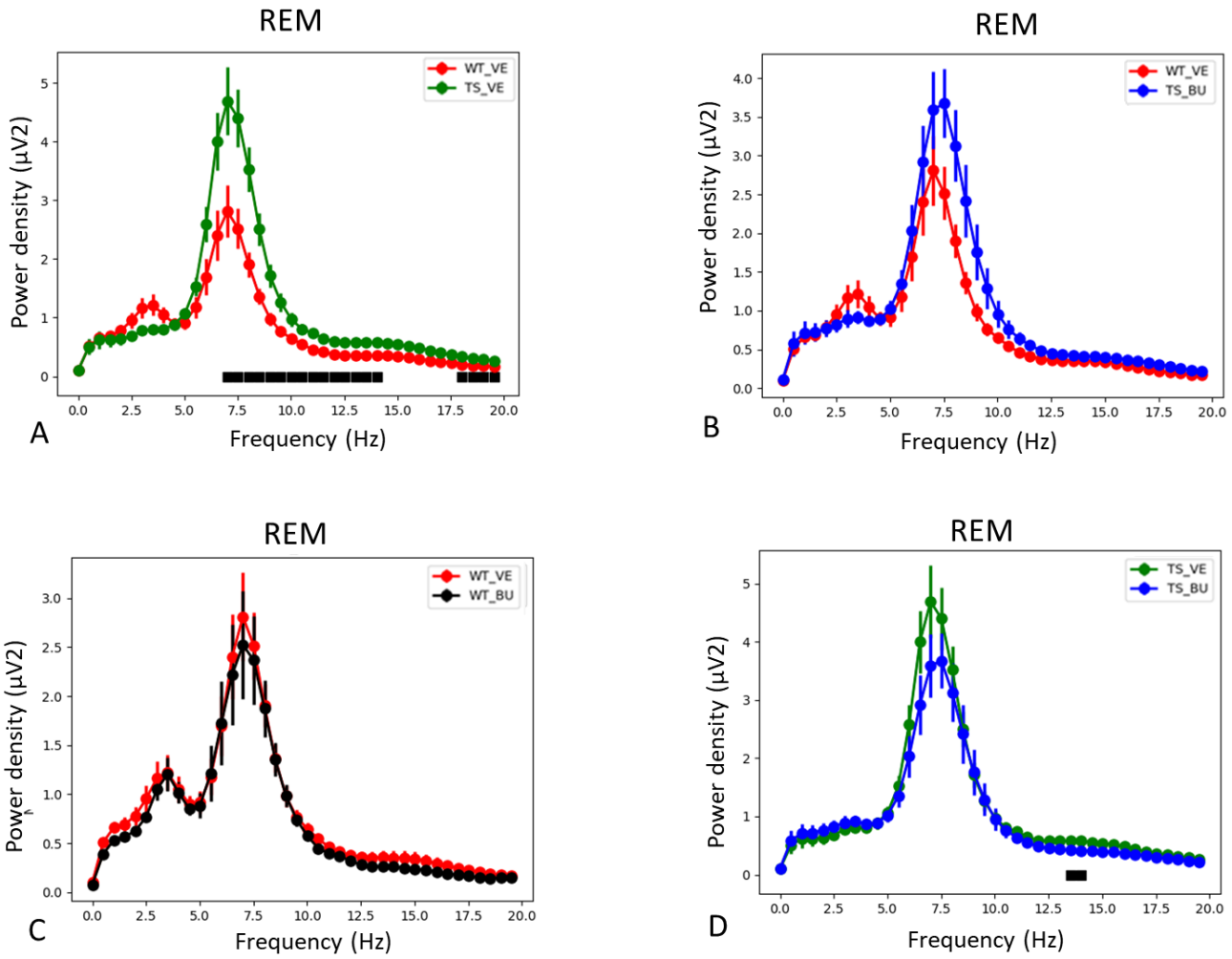



**Fig.18**. EEG power densities of the whole spectrum of frequencies (µV2) during REM sleep after sleep deprivation. A) 6 WT_VE versus 6 Ts_VE B) 6 WT_VE versus 6 Ts_BU, C) 6 WT_VE versus 6 WT_BU D) 6 Ts_VE versus 6 Ts_BU. ■ = P<0,05, by t.student test.

## 2. Body temperature is reduced in Ts65Dn mice during dark phases, and bumetanide does not have an effect on temperature.

To assess physiological parameters across long-term recording, we recorded the circadian profile of body temperature in mice using intraperitoneal implants. Although, Ts_VE mice have not showed notable alterations of their body temperature throughout 24 h in both phase light and dark before and after sleep deprivation, we found a statistically significant genotyping effect on Ts65Dn mice in terms of a lower body temperature in dark phase at baseline (Fig.19A,B and Fig.20A,B).
Peculiarly, bumetanide treatment has not shown any effect both in WT_BU and Ts_BU mice (Fig.19 A,B; Fig.20 A,B).

## 3. Ts65Dn mice display hyperactivity during 24 h hours, partially rescued by bumetanide treatment

Physiological activity of mice was assessed throughout 24 h. It resulted significantly increased in Ts_VE mice compared to WT_VE at baseline in light phase (Fig.21A) and presented an elevated trend during dark phase (Fig.21B). Interestingly, both conditions are partially rescued by bumetanide treatment in Ts_BU (Fig.21A). After SD, we confirmed the increasement trend of activity in Ts_VE, during light and dark phase, reduced by bumetanide (Fig.22,A,B). Conversely, no differences emerged during the light and dark phase in WT_BU for this parameter before and after SD (Fig.21A,B and Fig.22A,B).

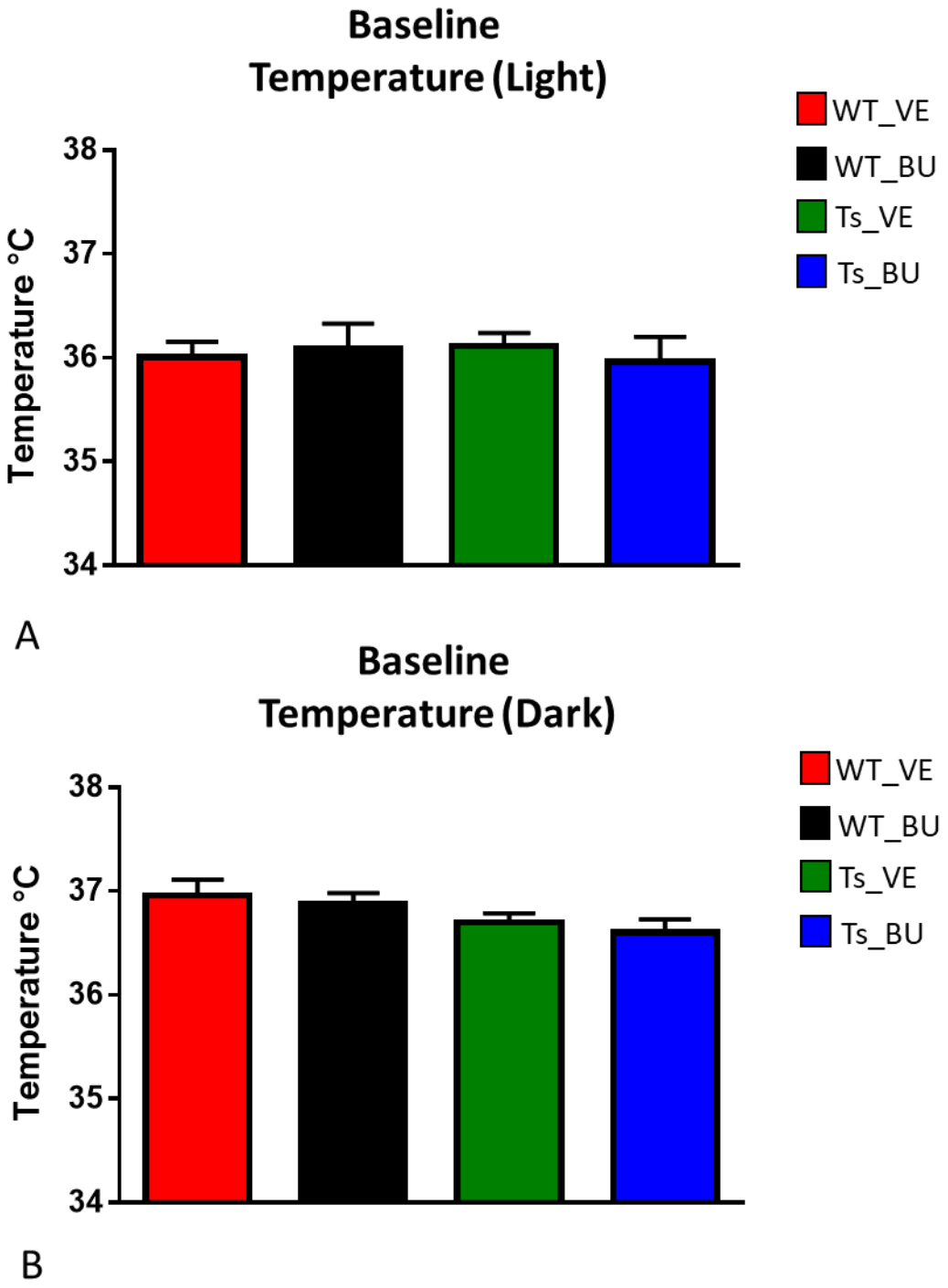



**Fig.19.** Body temperature across 24h at baseline in 6 WT_VE, 6 WT_BU, 6 Ts_VE, 6 Ts_BU during light (A) and dark (B) period. All graphs are presented as mean ± SEM across 12 h. Statistical significance is represented as follows: P > 0.05, two-way ANOVA followed by Tukey's post hoc test.

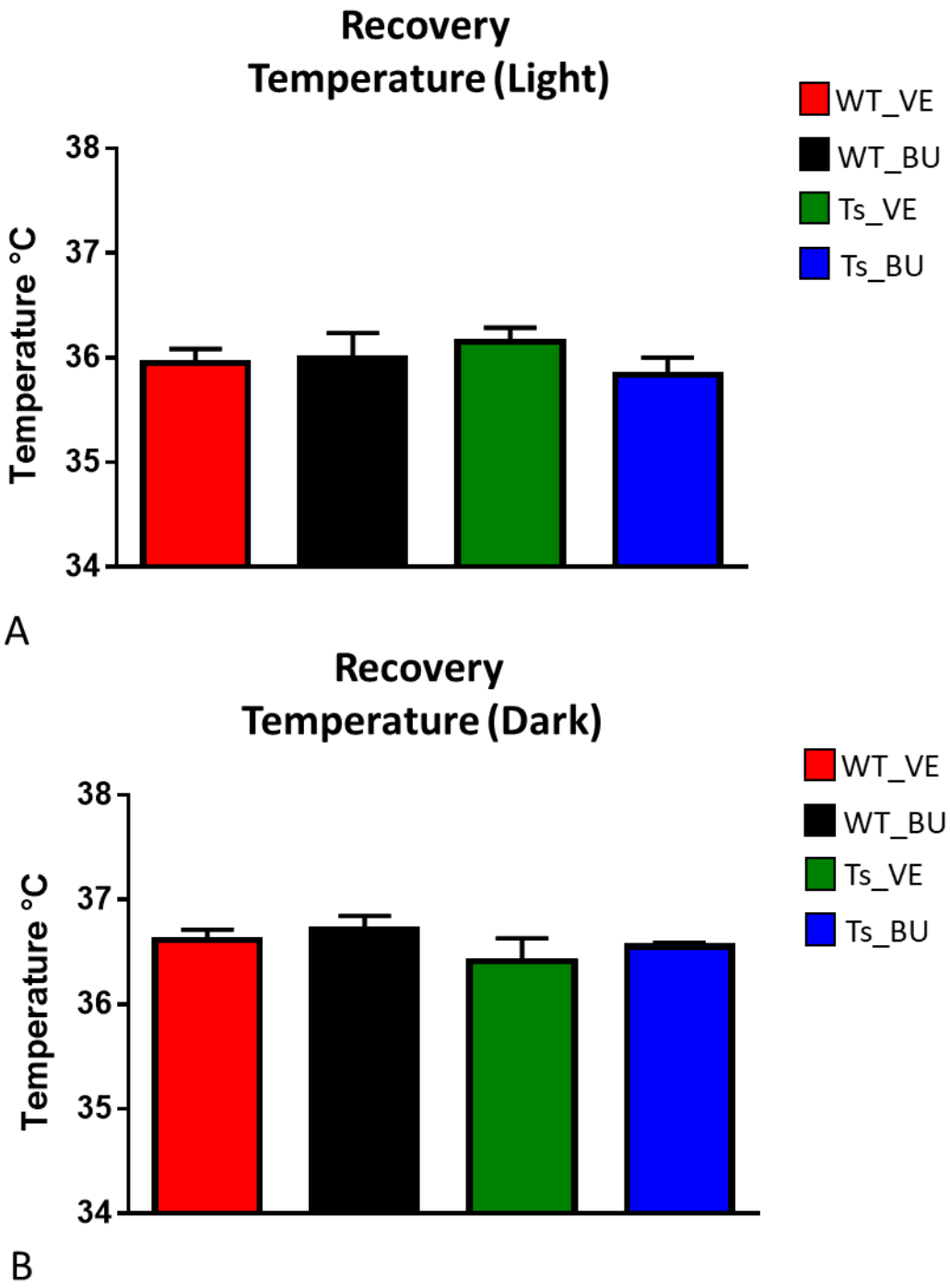



**Fig.20**. Body temperature across 24h after sleep deprivation in 6 WT_VE, 6 WT_BU, 6 Ts_VE, 6 Ts_BU, during light (A) and dark (B) period. All graphs are presented as mean ± SEM across 12 h. Statistical significance is represented as follows: P > 0.05, two-way ANOVA followed by Tukey's post hoc test.

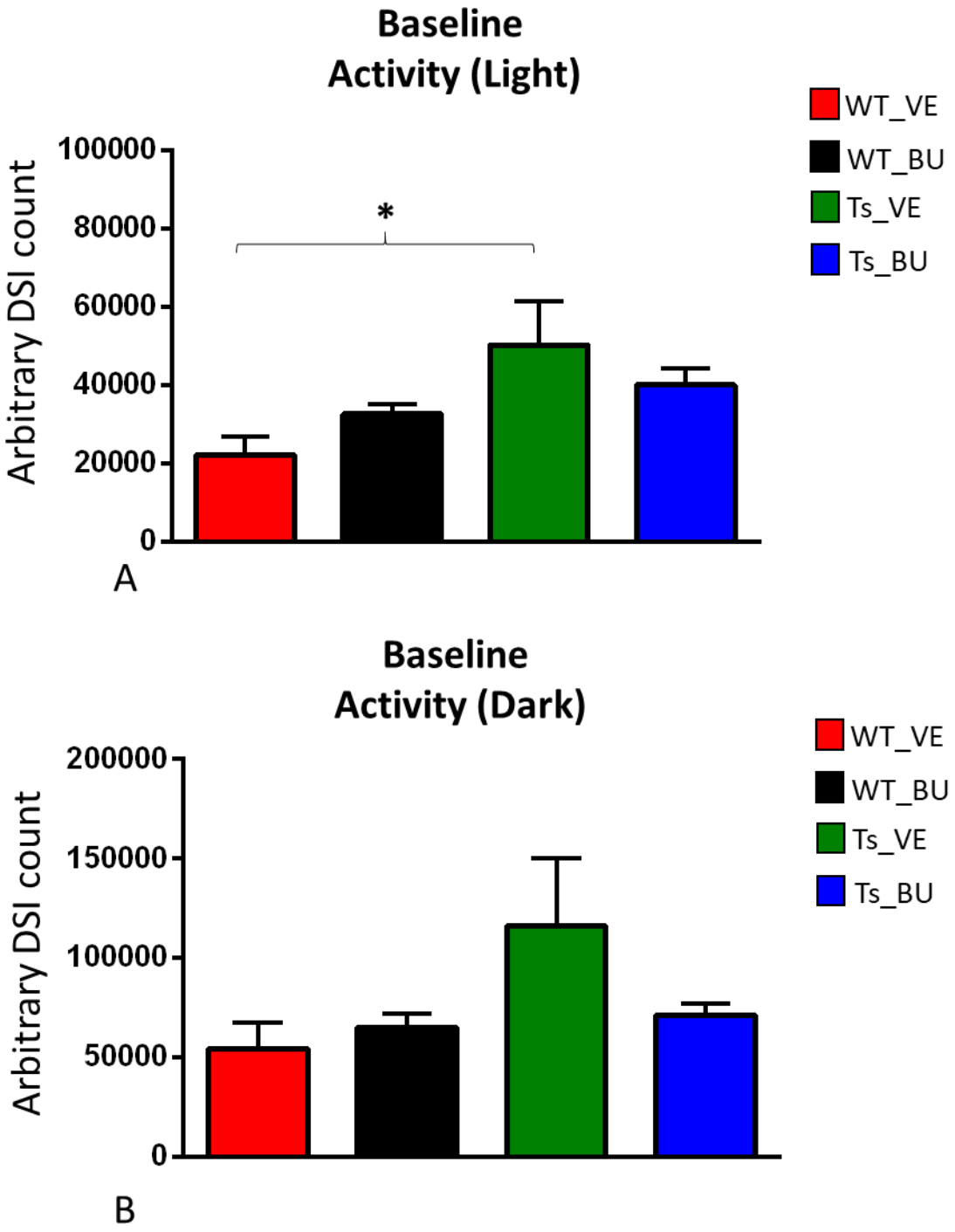



**Fig.21**. Activity across 24 h at baseline in 6 WT_VE, 6 WT_BU, 6 Ts_VE, 6 Ts_BU, during light (A) and dark (B) period. All graphs are presented as mean ± SEM across 12 h. Statistical significance is represented as follows: P > 0.05, two-way ANOVA followed by Tukey's post hoc test.

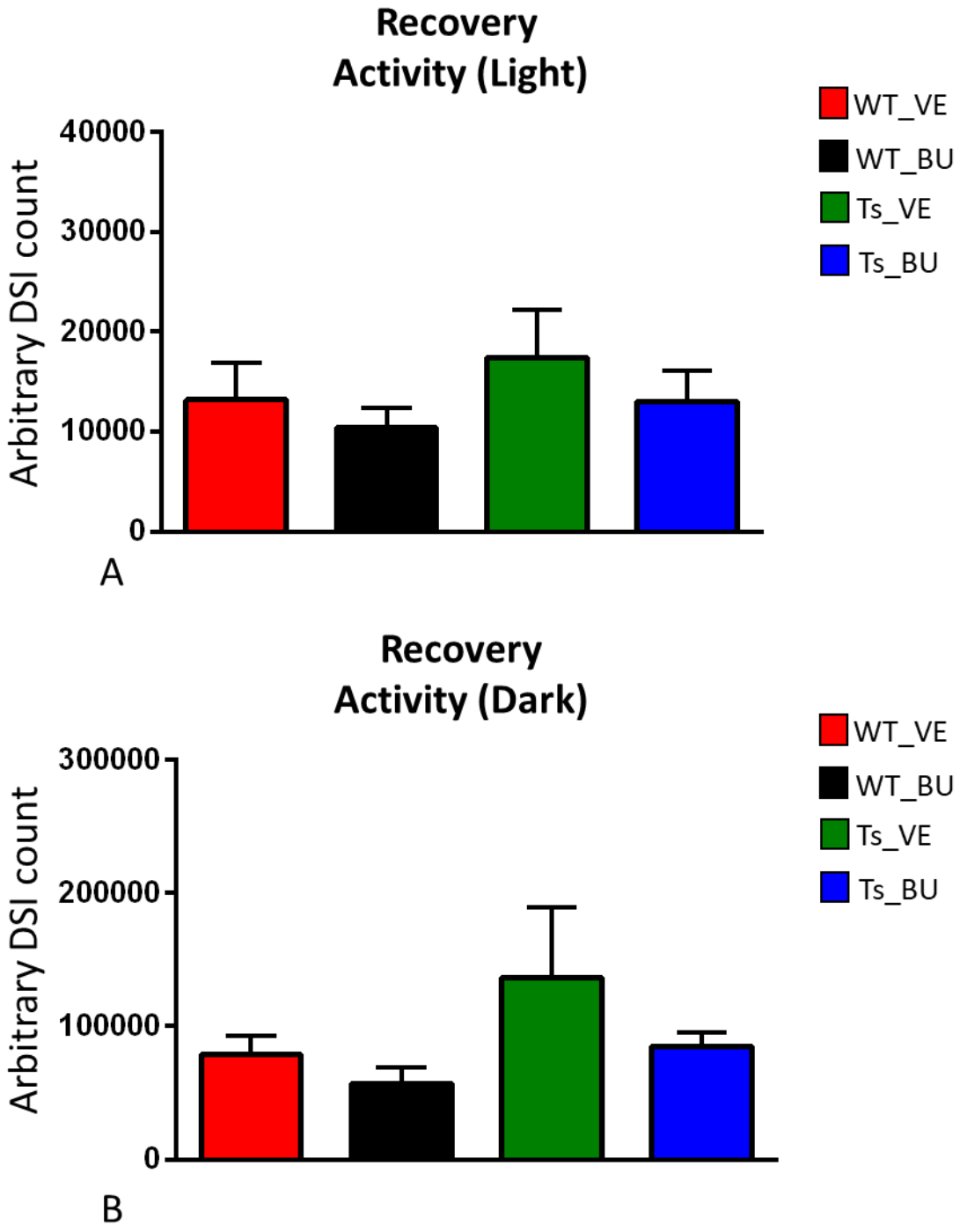



**Fig.22**. Activity across 24 h after sleep deprivation in 6 WT_VE, 6 WT_BU, 6 Ts_VE, 6 Ts_BU, during light (A) and dark (B) period. All graphs are presented as mean ± SEM across 12 h. Statistical significance is represented as follows: P > 0.05, two-way ANOVA followed by Tukey's post hoc test.

## 4. Ts65Dn mice show an increase in food intake, not reduced by bumetanide treatment

To evaluate different parameters linked to sleep, we also considered monitoring the food intake of mice throughout 24 h, considering the relationship between eating activity and sleep patterns reported in literature (Imaki M et al, 2002; Weiss et al, 2010). Notably, we found an increase of food intake in Ts_VE mice compared to WT_VE. (Fig23).

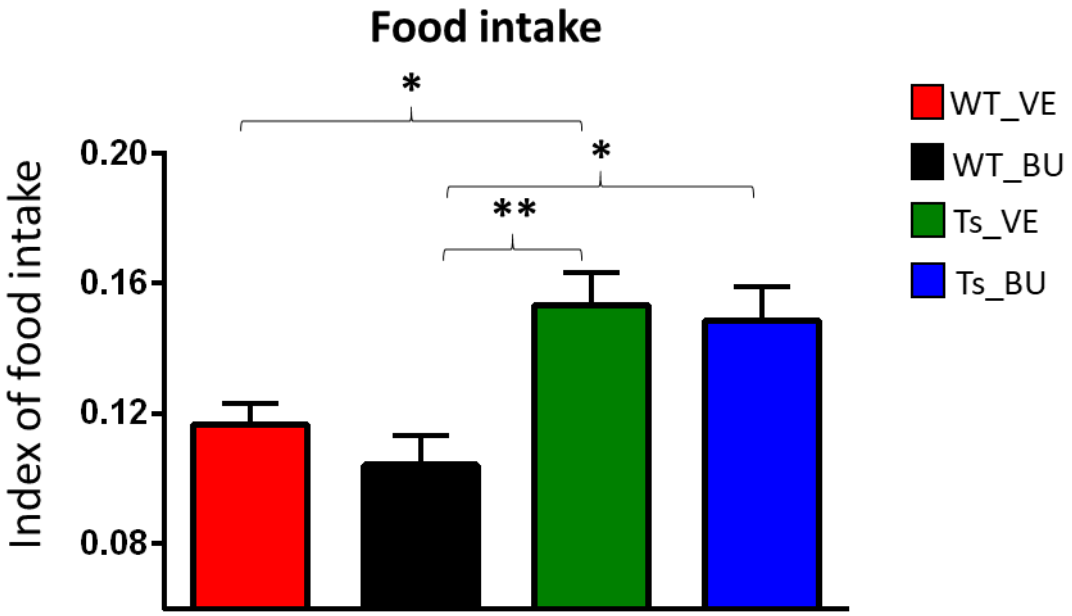



**Fig.23**. Food intake across 24 h in 6 WT_VE, 6 WT_BU, 6 Ts_VE, 6 Ts_BU. All graphs are presented as mean ± SEM across 24 h. Statistical significance is represented as follows: *P < 0.05, **P<0,01 two-way ANOVA followed by Tukey's post hoc test.

## Results: The role of $Cl^-$ homeostasis in behaviors and circadian rhythms of PWS

### 1. NKCC1 protein expression is specifically upregulated in the hippocampus of PWScr$^{m+/p-}$mice compared to controls

To investigate whether an alteration of the $Cl^-$ homeostasis could be linked to PWS, we first assessed the expression level of NKCC1 and KCC2 in the hippocampus from adult (3months) PWScr$^{m+/p-}$mice and their WT littermates by western blot technique. We found that NKCC1 expression was significantly increased in PWScr$^{m+/p-}$mice in comparison to controls (Fig.24a). Conversely, we found comparable levels of KCC2 expression in the two experimental groups. (Fig.24b).

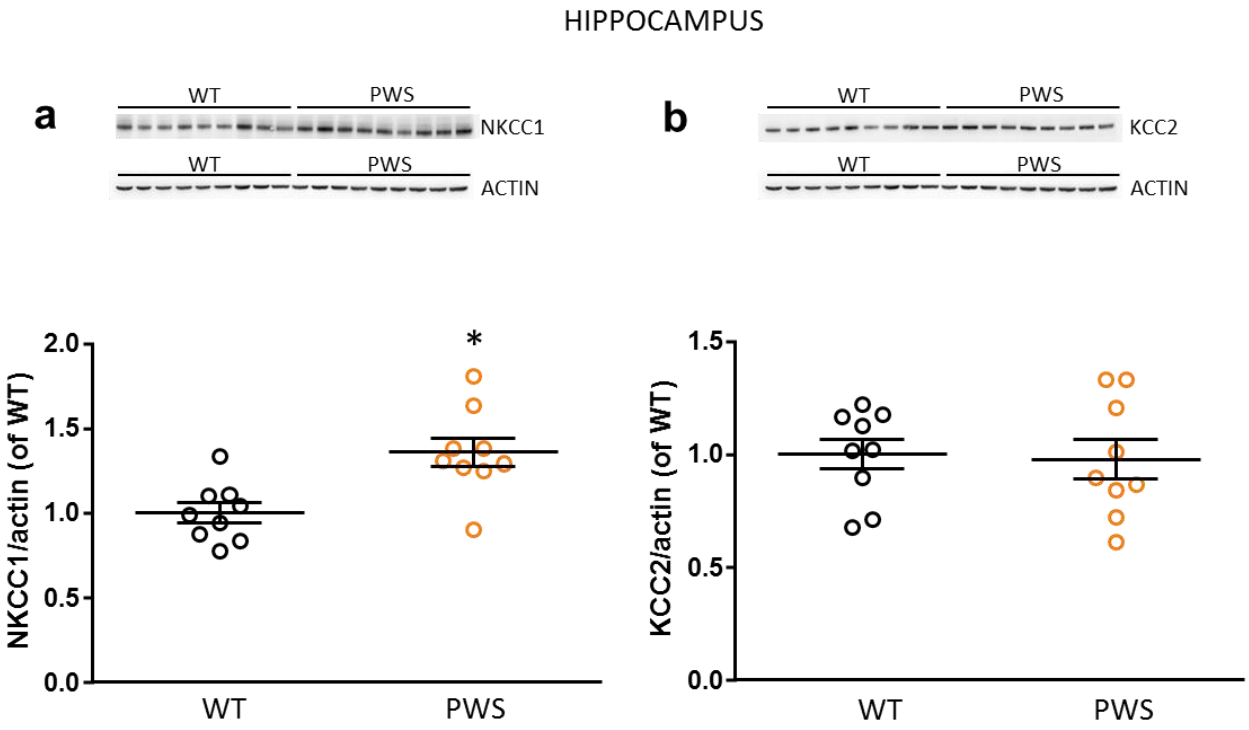



**Fig.24**. NKCC1 protein expression is increased in the hippocampi of PWScr$^{m+/p-}$mice (**a**) Top, representative immunoblots for NKCC1 in protein extracts from samples of mouse whole hippocampus in PWScr$^{m+/p+}$ (WT) =9 and PWScr$^{m+/p-}$ (PWS)

=9. (**b**) Top, representative immunoblots for KCC2 in protein extracts from samples of mouse whole hippocampus in PWScr$^{m+/p+}$ (WT) =9 and PWScr$^{m+/p-}$ (PWS)=9. Bottom, quantification of NKCC1 and KCC2 in samples from PWS in comparison to WT mice, respectively (*P<0,05 and P>0,05) in **a**, **b** respectively, by Student's *t*-test. Actin was used as an internal standard.

Next, we also evaluated NKCC1 and KCC2 protein levels in the prefrontal cortex (Fig.25), hypothalamus (Fig.26) and SCN (Fig.27). In none of these brain regions, we found any significative difference both for NKCC1 and KCC2 leves between PWScr$^{m+/p-}$mice and controls.

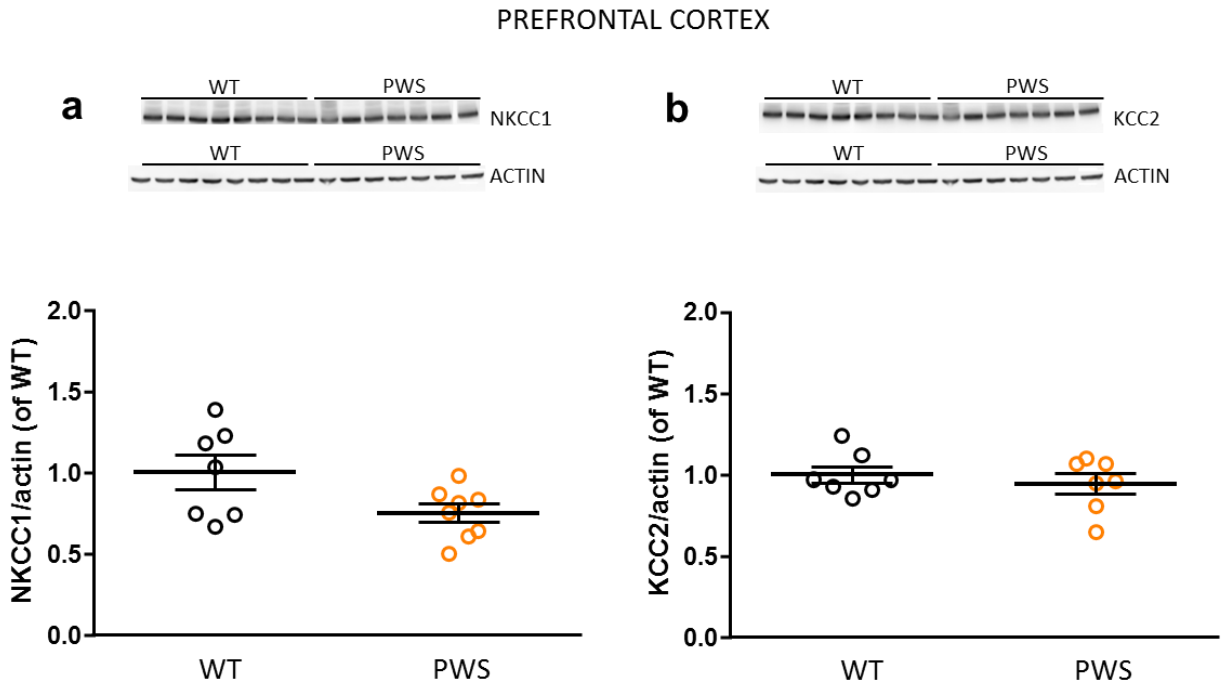



**Fig.25**. (**a**) Top, representative immunoblots for NKCC1 in protein extracts from samples of mouse prefrontal cortex in PWScr$^{m+/p+}$ (WT) =7 and PWScr$^{m+/p-}$(PWS) =8. (**b**) Top, representative immunoblots for KCC2 in protein extracts from samples of mouse whole hippocampus in PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$ =7. Bottom, quantification of NKCC1 and KCC2 in samples from PWS in comparison to WT mice, P>0,05 in **a**, **b**, by Student's *t*-test. Actin was used as an internal standard.

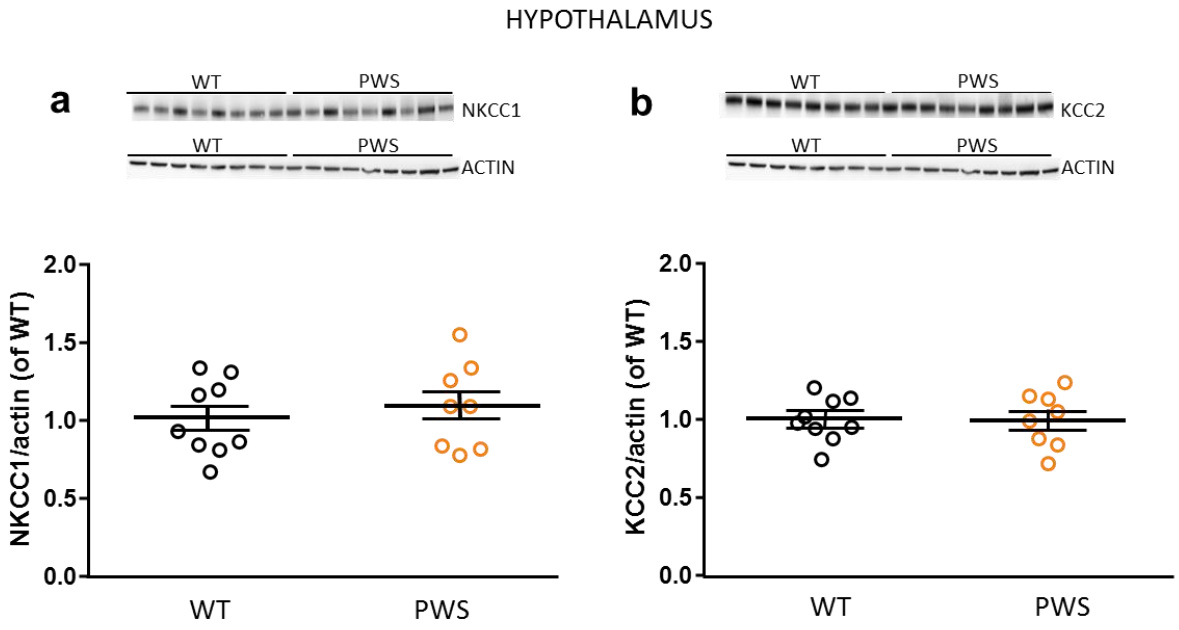



**Fig.26**. (**a**) Top, representative immunoblots for NKCC1 in protein extracts from samples of mouse whole hypothalamus in PWScr$^{m+/p+}$ (WT) =9 PWScr$^{m+/p-}$ (PWS)=8. (**b**) Top, representative immunoblots for KCC2 in protein extracts from samples of mouse whole hippocampus in PWScr$^{m+/p+}$ (WT) =9 and PWScr$^{m+/p-}$ (PWS)mice =8. Bottom, quantification of NKCC1 and KCC2 in samples from PWS in comparison to WT mice, P>0,05 in **a**, **b**, by Student's *t*-test. Actin was used as an internal standard.

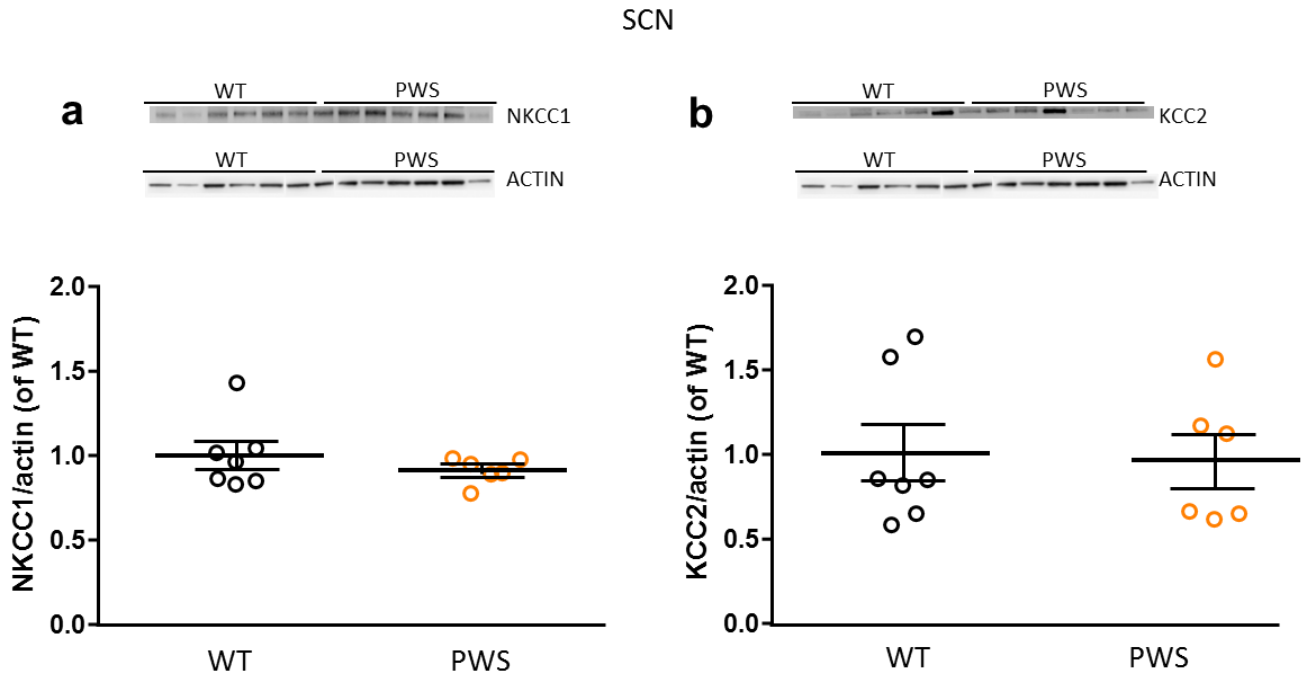


**Fig.27**. (**a**) Top, representative immunoblots for NKCC1 in protein extracts from samples of mouse whole SCN in PWScr$^{m+/p+}$ (WT) = 6 and PWScr$^{m+/p-}$(PWS). (**b**) Top, representative immunoblots for KCC2 in protein extracts from samples of mouse whole hippocampus in PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$ (PWS) =6. Bottom, quantification of NKCC1 and KCC2 in samples from PWS mice with PWS in comparison to WT mice, (P>0,05) in **a**, **b,** by Student's *t*-test. Actin was used as an internal standard.

## 2. A chronic treatment with bumetanide rescues cognitive deficits in PWScr$^{m+/p-}$ mice.

Upregulation of NKCC1 has been linked to cognitive impairment in Down syndrome mice (Deidda et al, 2015). We investigated the possible contribution of increased expression of NKCC1 in cognitive behavior of PWScr$^{m+/p-}$ mice by treating the animals with a NKCC1 inhibitor (bumetanide) and then assessing their cognitive performance. In particular, we evaluated the long-term hippocampus-dependent explicit memory after subchronic, systemic treatment with bumetanide (0.2 mg Kg$^{-1}$, IP, daily; Fig. 28), as described for DS in Deidda et al 2015.

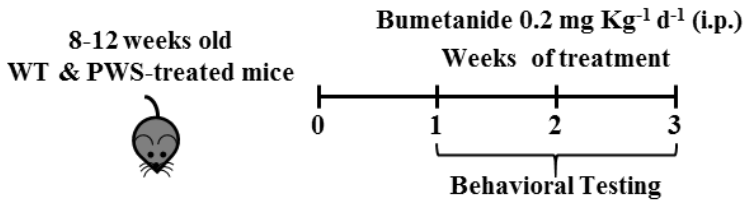



**Fig.28**. Schematic cartoon of the experimental protocol for the treatment of WT and PWScr$^{m+/p-}$mice with bumetanide and its efficacy assessment.

Thus, we assessed the long-term memory in three independent tasks (i.e., novel object recognition (NOR), object location recognition (OL), Contextual and Cue fear conditioning).

First, we performed the NOR test. This task measures the preference of mice for a novel object versus previously encountered familiar objects.

Interestingly, we found that a poor novelty-discrimination capability during the performance with the presence of the new object in PWScr$^{m+/p-}$ in comparison to WT. This deficit was completely rescued by bumetanide administration (Fig.29).

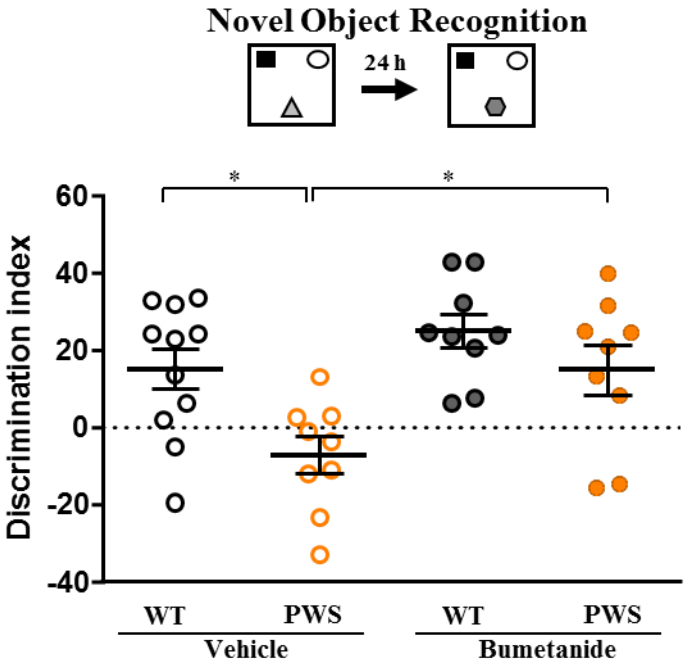



**Fig.29.** Schematic representation of NOR. Quantification of the discrimination index in vehicle-treated PWScr$^{m+/p+}$ (WT)=11 and PWScr$^{m+/p-}$ (PWS) =9 or bumetanide-treated PWScr$^{m+/p+}$ WT=9 and PWScr$^{m+/p-}$ (PWS) =9 in the NOR test (*P < 0.05, two-way ANOVA followed by Tukey's post hoc test).

Next, we also evaluated spatial memory in the OL. The test measures the ability of mice to recognize the new location of a familiar object with respect to spatial, external cues (Fig.30).

Here, we found an impairment of spatial memory in vehicle-treated PWScr$^{m+/p-}$ mice. Bumetanide treatment was able to restore the performance of PWScr$^{m+/p-}$ to the level of WT (Fig.30).

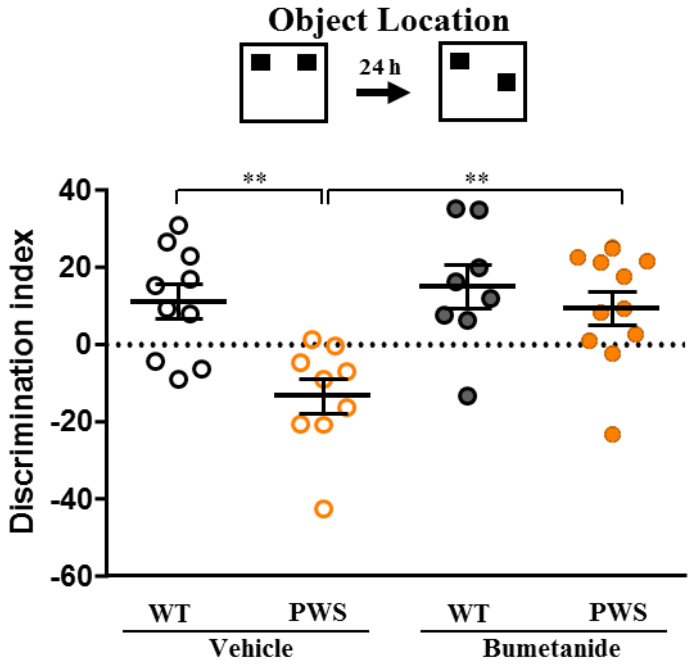



**Fig.30**. Schematic representation of the OL. Quantification of the discrimination index in vehicle-treated PWScr$^{m+/p+}$ (WT) =10 and PWScr$^{m+/p-}$ (PWS) =9 or bumetanide-treated PWScr$^{m+/p+}$ (WT)= 8 and PWScr$^{m+/p-}$ (PWS) =11 in the OL test. (*P < 0.01, two-way ANOVA followed by Tukey's post hoc test).

Finally, we assessed associative memory in the contextual and post cued fear conditioning tests. These tasks measure the freezing response that takes place after pairing of a foot shock (conditioning) with a particular context represented by a grid releasing an electric shock (Fig.31) or after a cue, represented by an acustic signal related to the releasing of the electric shock during the day of training. We did not find any difference among groups for any of the two tests However, bumetanide significantly increased associative memory in PWScr$^{m+/p-}$ vs vehicle treatment in the Post cued test (Fig.32).

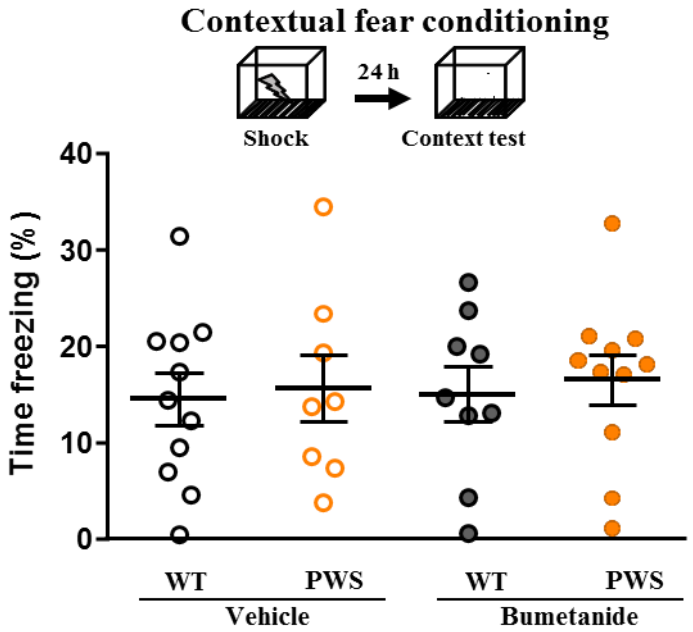



**Fig.31**. Schematic representation of the Contextual Fear Conditioning. Quantification of the freezing response in vehicle-treated PWScr$^{m+/p+}$ (WT)=10 and PWScr$^{m+/p-}$ (PWS) =10 or bumetanide-treated PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$(PWS) =11 in the Contexual fear conditioning test. (*P*> 0.05, two-way ANOVA followed by Tukey's *post hoc* test).

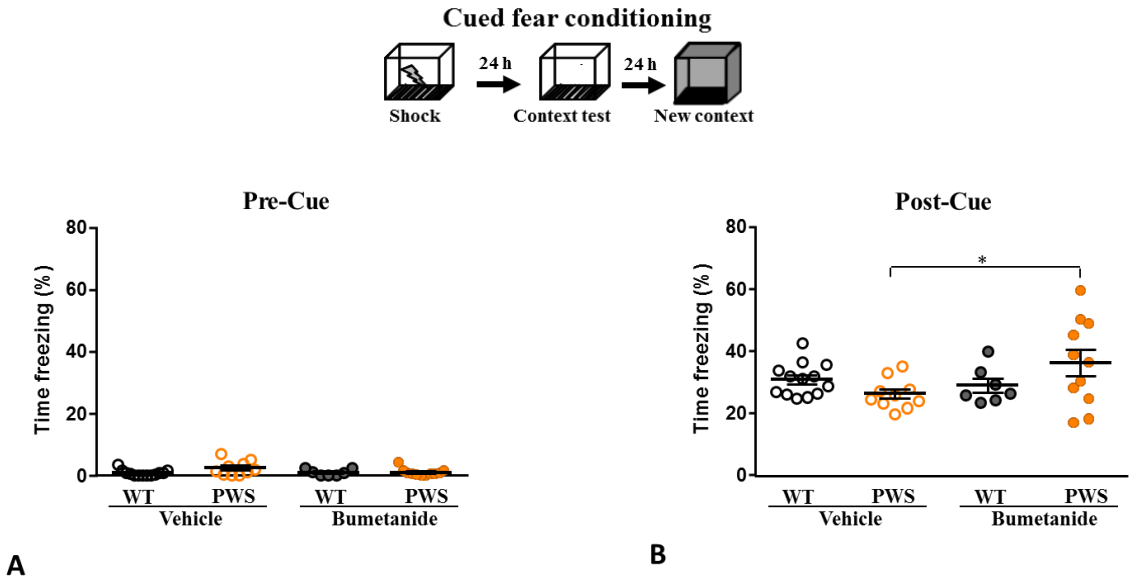


**Fig.32**. Schematic representation of the Cued Fear Conditioning. Quantification of the freezing response in vehicle-treated PWScr$^{m+/p+}$ (WT) =13 and PWScr$^{m+/p-}$mice (PWS) =10 or bumetanide-treated PWScr$^{m+/p+}$ (WT) =7 and PWScr$^{m+/p-}$mice (PWS) =11 in the Pre-cue (A) and Post-cue (B) fear conditioning test. (*$P$< 0.05, two-way ANOVA followed by Tukey's *post hoc* test).

## 3. PWScr$^{m+/p-}$mice do not show significant anxiety and stereotypic behavior in the Open Field

Next, we also assessed anxiety-like behavior in PWScr$^{m+/p-}$mice in the open field, from the habituation of NOR (see protocol), to investigate about a possible contribution of NKCC1 upregulation on anxiety disorders in PWS individuals. In particular, we evaluated the time spent in the center of the arena and the number of entries in the center of arena as an index of reduced anxiety levels showed by mice. We also analyzed general motor activity levels. No significant difference was found for number of entries and time spent in the center of arena between PWScr$^{m+/p-}$ and WT (Fig.33A and Fig.33B), independently of motor activity level measured as the travelled distance (Fig.33C).

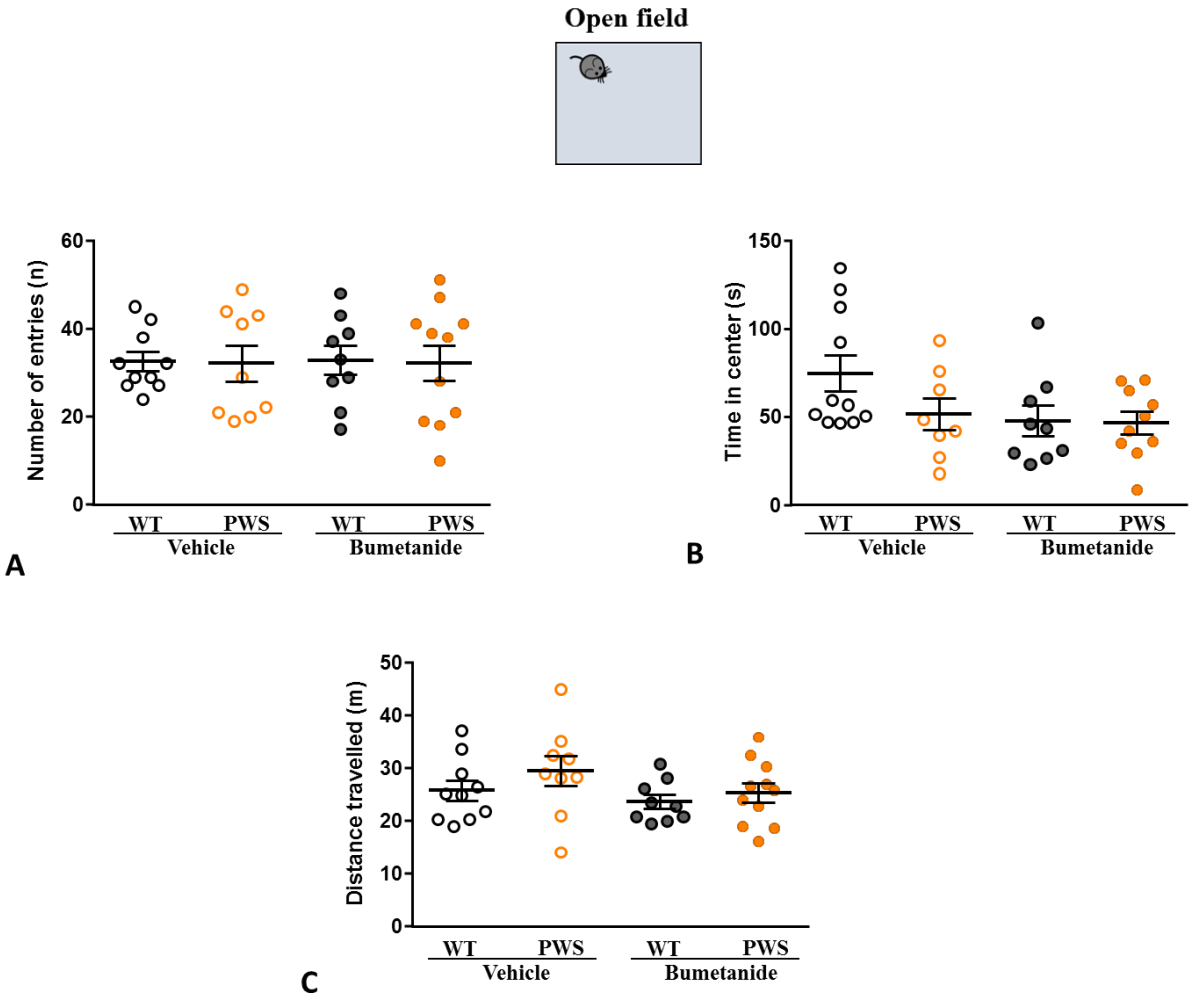



**Fig.33**. Top= Schematic representation of the Open field (a) Quantification of the number of entries in the center of field in vehicle-treated PWScr$^{m+/p+}$ (WT)=10 and PWScr$^{m+/p-}$ (PWS) =9 or bumetanide-treated PWScr$^{m+/p+}$ (WT)=9 and PWScr$^{m+/p-}$ (PWS) =11 in open field (P >0,05, two-way ANOVA followed by Tukey's post hoc test). (b) Quantification time in the center of field in vehicle-treated PWScr$^{m+/p+}$ (WT)=11 and PWScr$^{m+/p-}$ (PWS) =9 or bumetanide-treated PWScr$^{m+/p+}$ (WT)=9 and PWScr$^{m+/p-}$ (PWS) =8 in the open field. (P >0,05, two-way ANOVA followed by Tukey's post hoc test). (c) Quantification of the distance travelled in PWScr$^{m+/p+}$ (WT)=10 and PWScr$^{m+/p-}$ (PWS) =8 or bumetanide-treated PWScr$^{m+/p+}$ (WT)=9 and PWScr$^{m+/p-}$ (PWS) =11 in open field (P >0,05, two-way ANOVA followed by Tukey's post hoc test).

## 4. PWScr$^{m+/p-}$ mice do not present any significant feature related to obsessive-compulsive disorders

Next, we investigated whether defect of $Cl^-$ homeostasis of PWScr$^{m+/p-}$ are implicated in repetitive behaviors, important hallmarks linked to ASD, and obsessive-compulsive disorders showed in PWS individuals. To this aim, we performed diverse behavioral tests such as self-grooming, digging and marble burying.

Self-grooming is a very complex innate behavior, characterized by the presence of a sequence of evolutionary conserved patterns. Rodents show frequently behavioral activities involving self-grooming, which are important in hygiene maintenance and other physiologically important processes such as thermoregulation, social communication and de-arousal (JC Fentress, 1968 a,b; BM Spruijt et al 1988; BM Spruijt et al,1992; ST Leonard et al, 2005; A Kalueff et al, 2010). However, self-grooming attitude can change during stressful states or in certain neuropsychiatric disorders, such as autism (JN Crawley, 2007; SE Ahmari et al 2013; A Roth et al,2013). Indeed, it represents a relevant aspect related to the neurobiology complexity, repetitive and sequential pattern of behavior (AM Graybiel et al, 2002; A Kalueff et al,2007; A Kalueff et al ,2010).

Thus, we measured the total time and the number of episodes of self-grooming in PWScr$^{m+/p-}$ mice compared with controls (Fig.34A, B). We found no changes for self-grooming in presence of *Snord116* deletion (Fig34A, B). In addition, we also evaluated the digging behavior in a new closed field and the marble burying test. Digging and marble burying has been strongly used to assess obsessive/compulsive behaviors and ASD-like behaviors in rodent models (DA Amodeo et al 2012; SL Andersen et al 2010; RM Deacon, 2006; SS Moy et al, 2014; ML Scattoni et al, 2008; JL Silverman et al, 2015; H Takeuchi et al,2002). We found that the time spent and the number of episodes of digging were not statistically different between groups (Fig.35A, B). As well as, no differences we

found for the Marble burying test between groups (Fig.36). Finally, we also analyzed the number of wall rearing and twirling as index of stereotypic behavior. Again, we found no evidence of alterations of these behaviors in PWScr$^{m+/p-}$ compared to WT (Fig.37 and Fig.38).

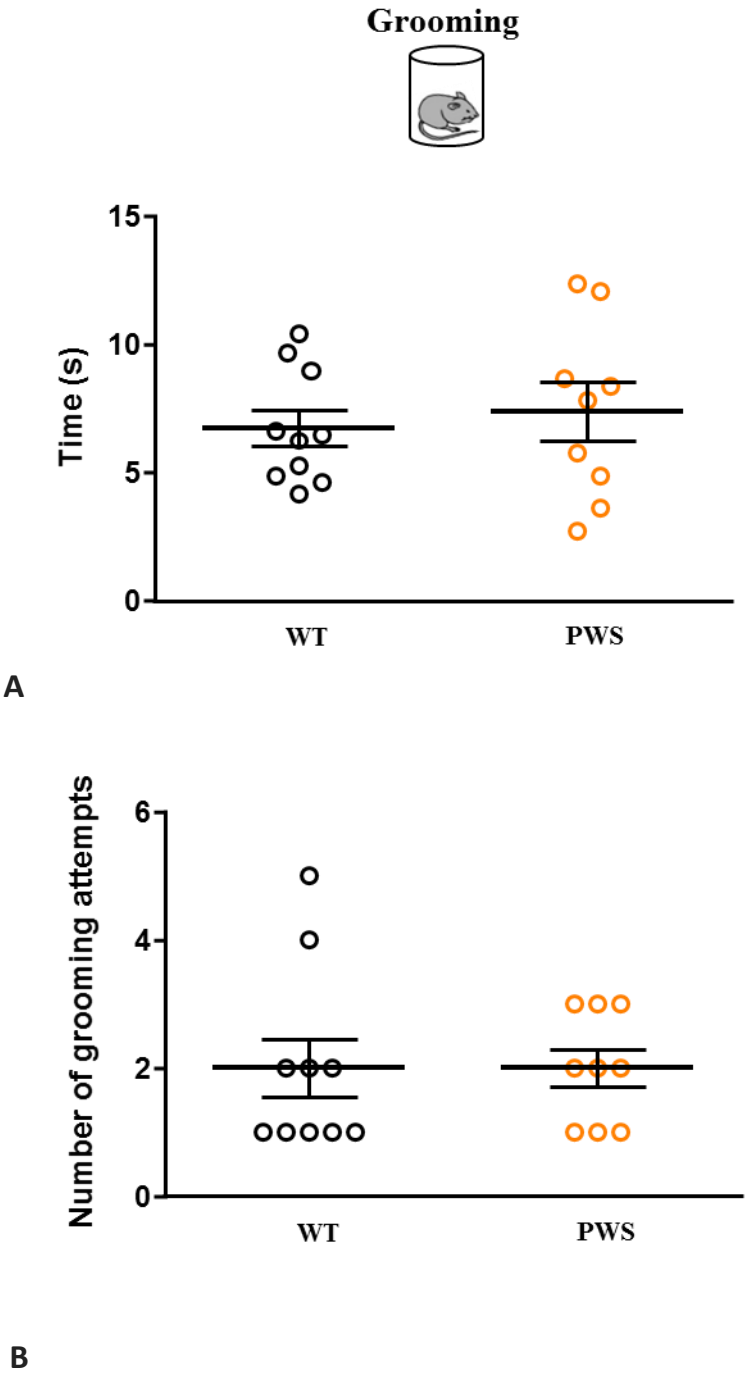
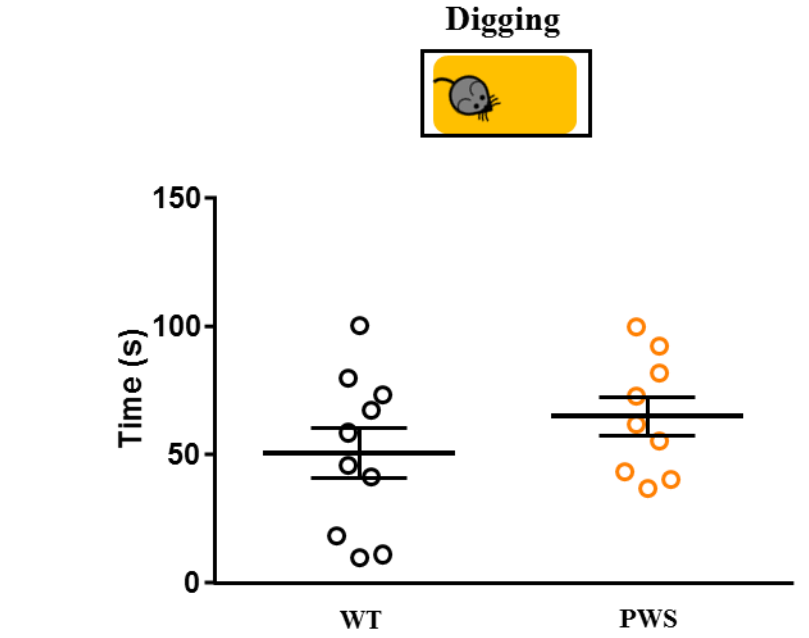
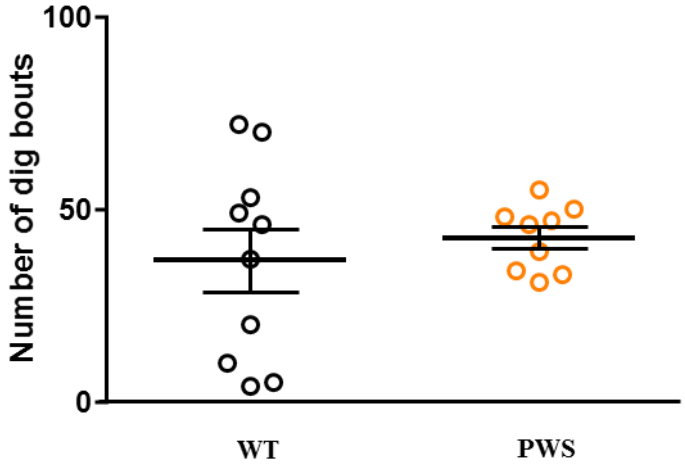



**Fig.34**. Top= Schematic representation of the mice self-grooming. (A) Time spent in self-grooming and (B) number of grooming attempts (episodes) in PWScr$^{m+/p+}$ (WT) =10 and PWScr$^{m+/p-}$ (PWS) =9. P>0,05, by t.test student.




A




B

**Fig.35**. Top= Schematic representation of the mice digging. (A) Time spent in digging and (B) number of dig bouts in PWScr$^{m+/p+}$ (WT) =10 and PWScr$^{m+/p-}$ (PWS) =9. P>0,05, by t.test student.

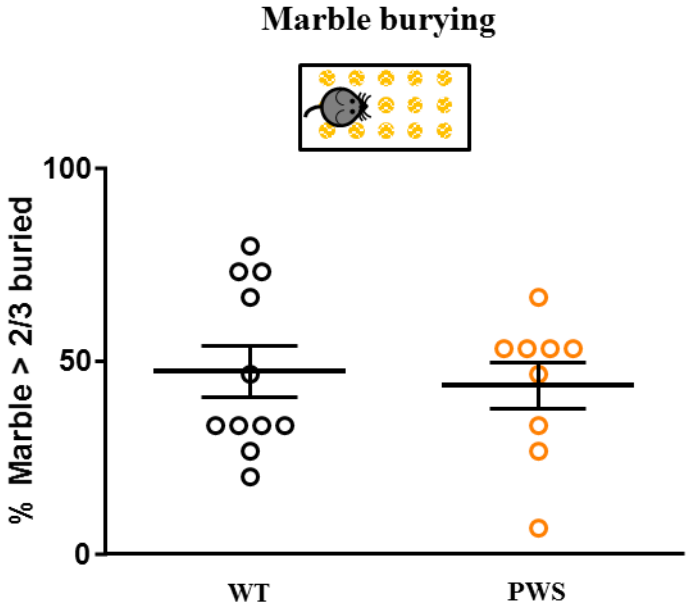



**Fig.36**. Top= Schematic representation of the Marble burying. Quantification of % of Marble >2/3 buried in PWScr$^{m+/p+}$ (WT) =10 and PWScr$^{m+/p-}$ (PWS) =9. P>0,05, by t.test student.

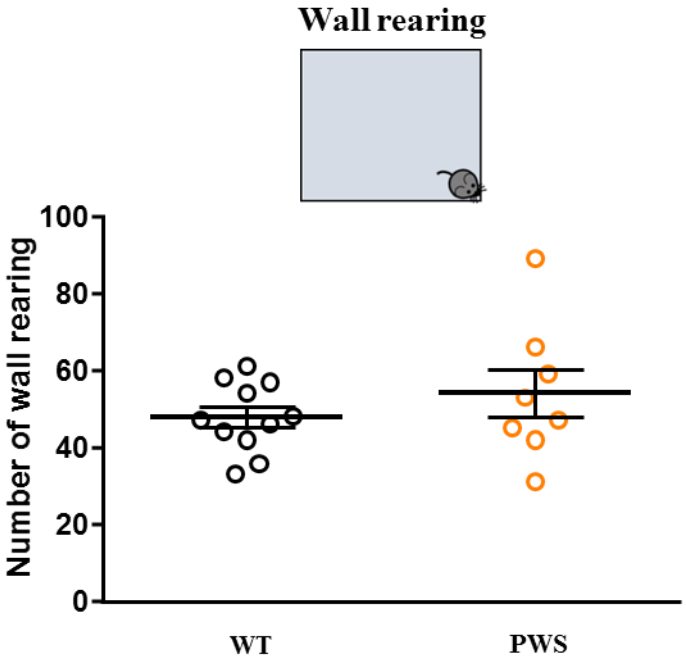



**Fig.37**. Top= Schematic representation of the Wall rearing. Quantification of number of Wall rearing in PWScr$^{m+/p+}$ (WT) =11 and PWScr$^{m+/p-}$ (PWS) =8. P>0,05, by t.test student.

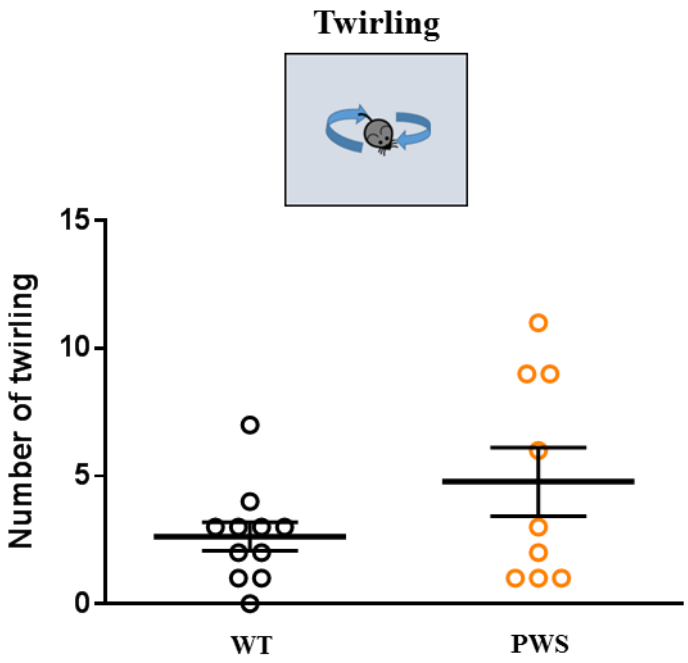



**Fig.38**. Top= Schematic representation of the Twirling. Quantification of number of Twirling in PWScr$^{m+/p+}$ (WT) =11 and PWScr$^{m+/p-}$ (PWS) =9. P>0,05, by t.test student.

## 5. Sociability is not significantly affected in mutant PWScr$^{m+/p-}$ mice

In order to investigate on the implication of aberrant $Cl^-$ homeostasis in ASD-like behaviors (Ben-Ari, 2017), which are present also in PWS individuals (Dykens EM et al, 2019), we focalized our attention on the sociability of PWScr$^{m+/p-}$ mice. In mutant PWScr$^{m+/p-}$ mice, we thus evaluated whether *Snord116* paternal deletion affects sociability. To this aim, we performed two tests strictly related to sociability, the Male-Female interaction test (Fig.39) and three-chamber test (Fig.40) (Kaidanovich-Beilin. O et al, 2011; Ricceri et al, 2016).

First, we performed a Male-Female interaction test (Fig.39). Mice are usually very active to interact with each other during social interaction, and they are commonly show sniffing of anogenital region, head, and of the rest of their bodies, and dedicate time in touching each other with a reciprocal following (mostly observed in male versus female mice).

In particular, we measured the time spent in sniffing as index of the social approach in tested male mice vs a never-met-female mouse. Results have confirmed no significant differences in sociability in PWScrm+/p- compared to controls (Fig.39).

Next, in the three-chamber test, we assessed the sociability index (Fig.40A,B), in terms of the social approach of the tested mice with a never-met-intruder in comparison to an object; and the social novelty (Fig.40C,D), as a social approach of tested mice with a novel never-met-intruder in comparison to the already met intruder. PWScr$^{m+/p-}$ mice did not show any significant alteration in sociability index respect to WT, for time spent in compartment and sniffing of a never-met-intruder compared to an object (Fig.40A,B). No differences between groups were present also in the social novelty for time spent in compartment and sniffing of a novel never-met-intruder compared to an already met intruder (Fig.40C,D).

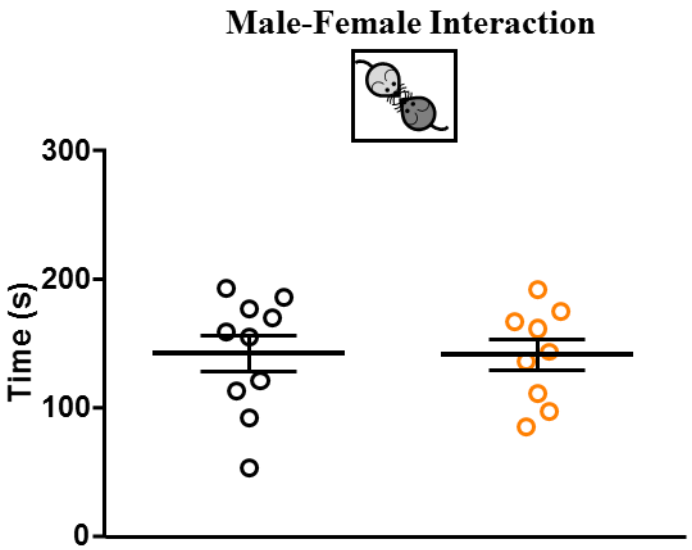



**Fig.39**. Top= Schematic representation of the male-female interaction. Time spent of a male mouse versus a never met female mouse, in PWScr$^{m+/p+}$ (WT) =10 and PWScr$^{m+/p-}$ (PWS)=9. P>0,05, by t.test student.

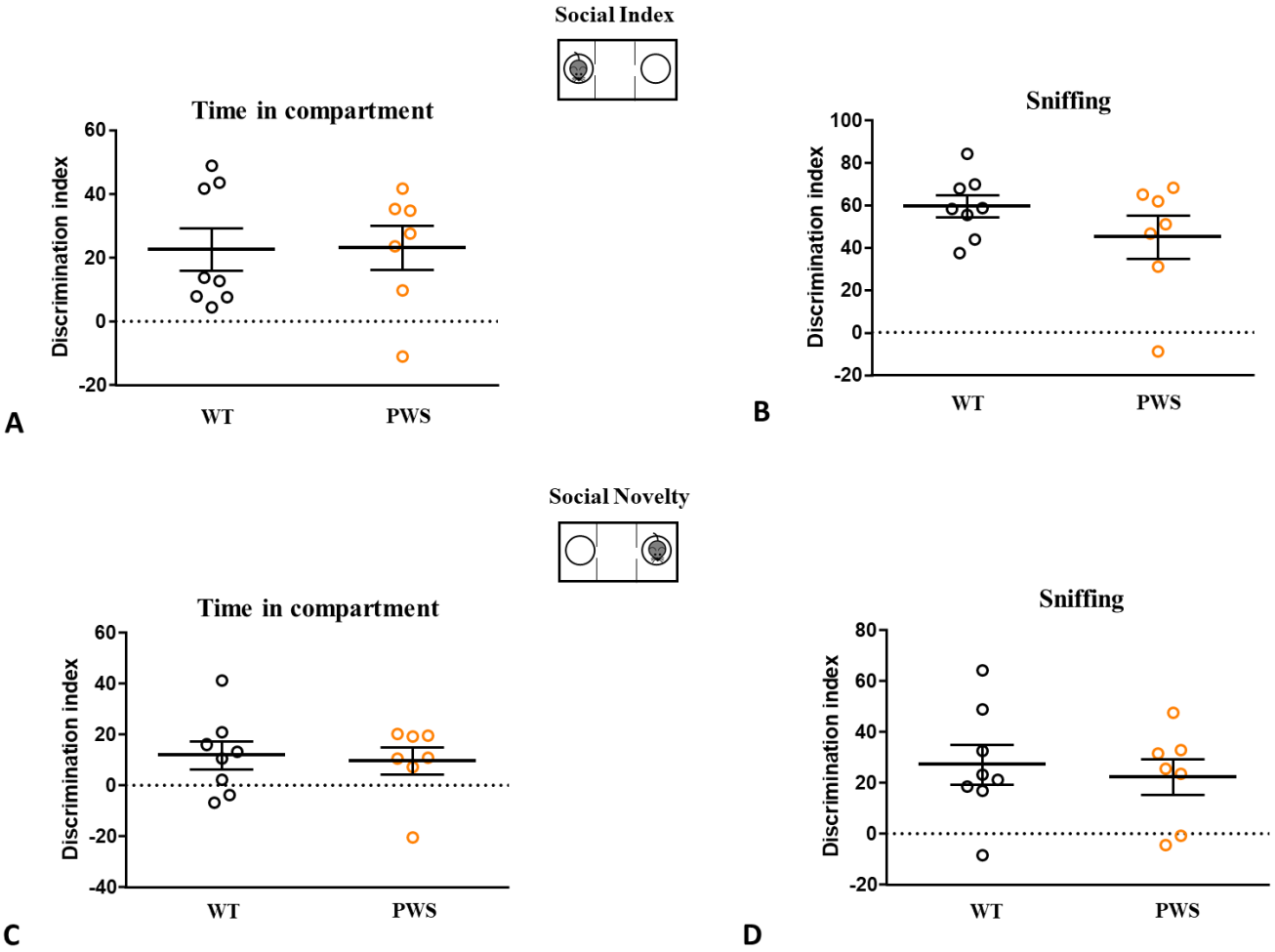



**Fig.40**. Top= Schematic representation of the Three chamber test for social index. (A) Discrimination index of time spent in compartment of never met mouse versus an object, in PWScr$^{m+/p+}$ (WT) =8 and PWScr$^{m+/p-}$ (PWS)=7. P>0,05, by t.test student. (B) Discrimination index of time spent in sniffing of never met mouse versus an object, in PWScr$^{m+/p+}$ (WT) =8 and PWScr$^{m+/p-}$ (PWS)=7. P>0,05, by t.test student. Top= Schematic representation of the Three chamber test for social novelty. (C) Discrimination index of time spent in compartment of never met mouse versus a familiar mouse, in PWScr$^{m+/p+}$ (WT) =8 and PWScr$^{m+/p-}$ (PWS)=7. P>0,05, by t.test student. (D) Discrimination index of time spent in sniffing of never met mouse versus a familiar mouse, in PWScr$^{m+/p+}$ (WT) =8 and PWScr$^{m+/p-}$ (PWS)=7. P>0,05, by t.test student.

## 6. PWScr$^{m+/p-}$ mice show an increase of pain sensitivity in presence of a thermal stimulus

Finally, we assessed the pain sensitivity to an acute thermal stimulus in PWScr$^{m+/p-}$ using a hot plate platform kept at constant temperature of 52°C. We measured the response in terms of latency to lick the paws from the hot plate, as sign of pain sensitivity, applying a cut-off latency of 30 seconds. Interestingly we found that PWScr$^{m+/p-}$ present significantly a greater sensitivity to pain compared to controls (Fig.41).

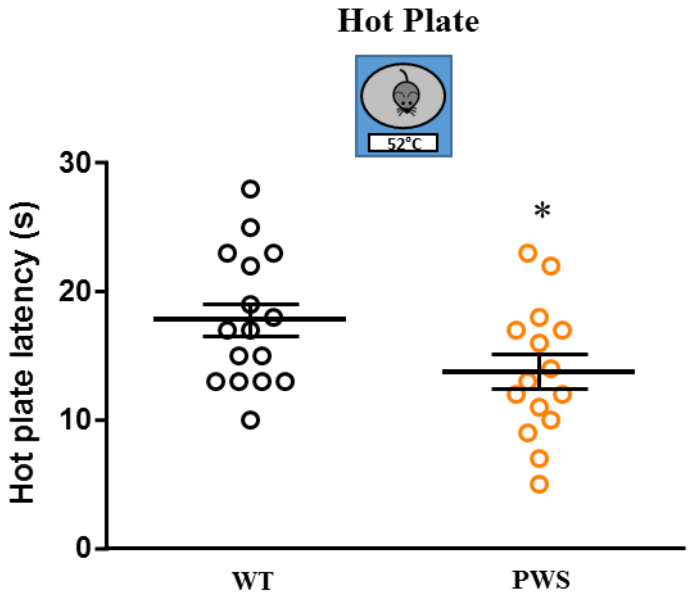



**Fig.41**. Top= Schematic representation of the Hot plate test. Quantification of pain sensitivity in PWScr$^{m+/p+}$ (WT) =16 and PWScr$^{m+/p-}$ (PWS)= 15. *P<0,05, by t.test student.

## 7. PWScr$^{m+/p-}$ show a reduced shortening of the circadian period and an increase of activity in DD, rescued by bumetanide chronic treatment.

Sleep disorders have been well characterized in both PWS individuals and PWScr$^{m+/p-}$ mutated mice (Lassi et al, 2016), while no evidences are reported on circadian rhythms. *Snord116* gene deletion has been associated to different clock gene expression of *Clock*, *Cry1*, *Cry2*, *Per1* and *Per2*, enhancing circadian zeitgebers (Powell et al, 2013). Therefore, we decided to assess the circadian clock of PWScr$^{m+/p-}$mice using running wheels in Light-Dark (LD) and Dark-Dark (DD) cycle, used to evaluate the endogenous circadian period also called as the free running period.

Thus, to characterize the circadian period and the impact of $Cl^-$ homeostasis in the presence of *Snord116* deletion, we tested PWScr$^{m+/p-}$ and control mice treated with vehicle or bumetanide (2mg/kg body weight in drinking water) using running wheels in their home-cages during 24 hr for 20 total days (10 days of LD cycle followed by 10 days of constant darkness), without any interruptions.

The circadian period of each mouse was derived from wheel running activity. We observed that PWScr$^{m+/p-}$ mice showed a significant lengthening of their internal clock in DD condition compared to the WT mice, completely rescued by bumetanide treatment (Fig.42C). Conversely, we did not find any changes during LD cycle among groups (Fig.42B).

In addition, we also evaluated the time devoted to the behavioral activity, expressed as the length of the segment of the daily activity (alpha) during LD and DD conditions.

Interestingly, we reported a significant increase of alpha activity in PWScr$^{m+/p-}$ compared to WT, reduced by bumetanide treatment.

However, bumetanide in WT significantly strengthened the difference with PWScr$^{m+/p-}$ treated with vehicle or bumetanide during constant darkness (Fig.42E). Conversely, we did not observe any difference of alpha activity among groups during LD condition (Fig.42D).

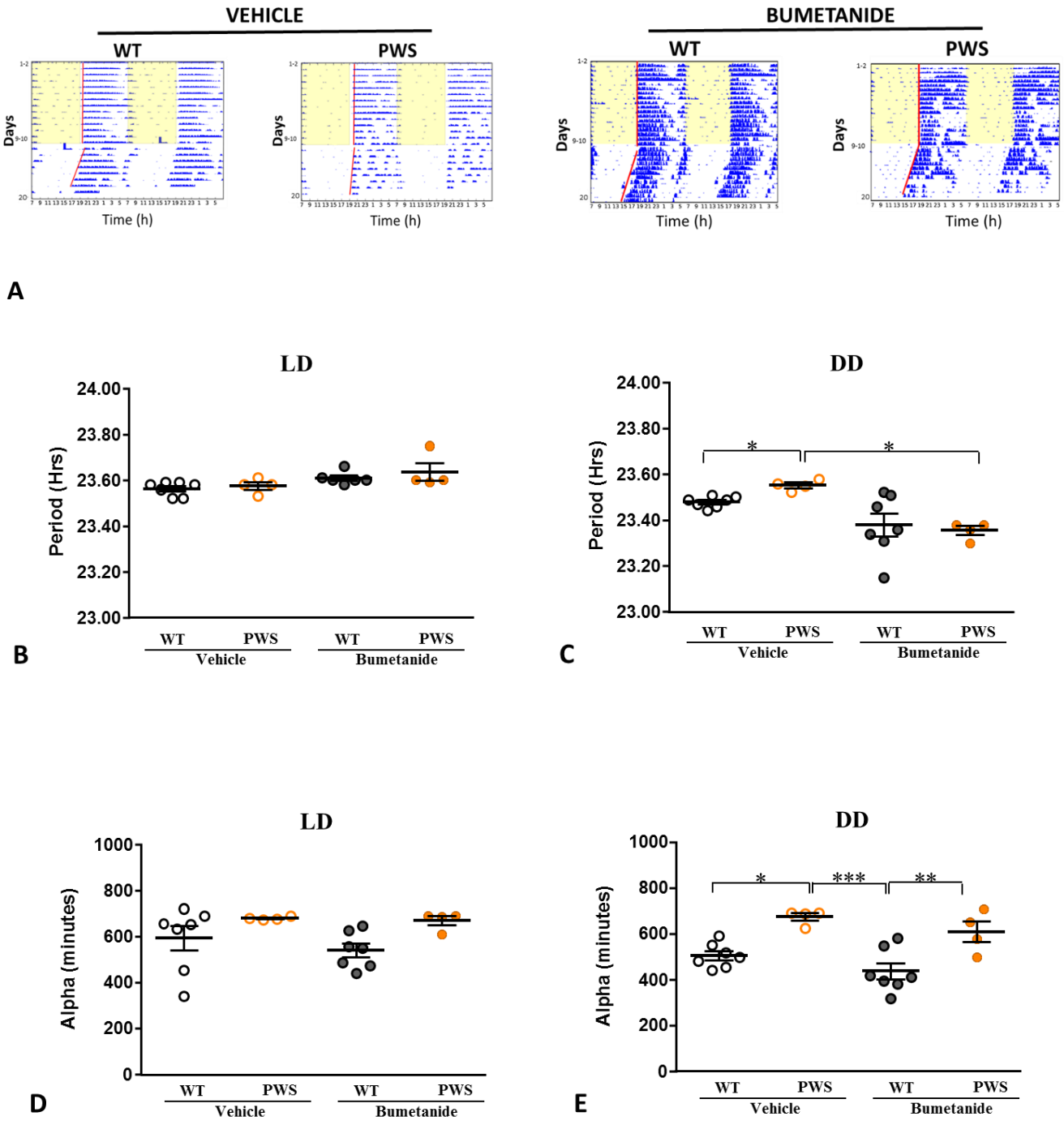


**Fig.42. (**A) Representative actograms of wheel-running activity under light-dark (LD) conditions, followed by constant darkness (DD). Periods of light and dark are respectively indicated by yellow and white panels. (B) Quantification of circadian period in LD vehicle-treated PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$ (PWS) =4 or bumetanide-treated PWScr$^{m+/p+}$ (WT)=6 and PWScr$^{m+/p-}$ (PWS) =4. P>0,05, two-way ANOVA followed by Tukey's post hoc test. (C) Quantification of circadian period in DD vehicle-treated PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$ (PWS) =4 or bumetanide-treated PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$ (PWS) =4.*P<0,05, two-way ANOVA followed by Tukey's post hoc test. (D) Quantification of alpha activity in LD vehicle-treated PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$ (PWS) =4 or bumetanide-treated PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$ (PWS) =4. P>0,05, two-way ANOVA followed by Tukey's post hoc test. (E) Quantification of alpha activity in DD vehicle-treated PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$ (PWS) =4 or bumetanide-treated PWScr$^{m+/p+}$ (WT)=7 and PWScr$^{m+/p-}$ (PWS) =4 . *P<0,05, **P<0,01, ***P<0,001, two-way ANOVA followed by Tukey's post hoc test.

# Discussion

## 1. The role of $Cl^-$ homeostasis in sleep disorder of DS

In this study, we have explored the sleep and EEG abnormalities, and the implication of $Cl^-$ mechanisms in a mouse model of DS, which could be translated to the human pathology. To this aim, we selected the strain of Ts65Dn mice, which are the most studied and widely used model, characterized by NKCC1 upregulation in hippocampus and cortex. This has been linked to depolarizing and possibly excitatory *vs* hyperpolarizing and inhibitory GABAergic transmission in Ts65Dn animals (Deidda et al, 2015).

Ts65Dn mice recapitulate many features of DS showing impairment in neuronal development (Belichenko et al., 2004; Chakrabarti et al., 2010; Chakrabarti et al., 2007; Contestabile et al., 2010; Contestabile et al., 2007), defects of synaptic plasticity (Contestabile et al., 2013; Costa & Grybko, 2005; Kleschevnikov et al, 2004; Siarey et al., 1999; Siarey et al., 1997), impaired hippocampus-dependent memory functions (Contestabile et al., 2013; Costa et al., 2008; Fernandez et al., 2007; Reeves et al., 1995), hyperactivity (Escorihuela et al., 1995; Reeves et al., 1995; Sago et al., 2000) and sleep disorders (Colas et al., 2008). Previously, sleep pattern in Ts65Dn mice was poorly characterized by just one study that had been conducted by Colas et al. Nevertheless, this study highlighted major sleep abnormalities, including increased wakefulness, decreased NREM sleep, increased theta power during NREM and REM sleep and a delayed, but increased sleep rebound after sleep deprivation (Colas et, al 2008).

Our study extends the understanding of sleep physiology in Ts65Dn mice, including more detailed investigation of the architecture of sleep-wake cycles, with the addition of temperature and motor activity recordings.

In particular, we found an increase of wakefulness, and a reduction of NREM sleep before and after SD, but no abnormalities in REM phase in Ts65Dn mice. However, these mice present with a reduction of delta waves in REM at baseline and NREM before and after SD together with an increase of theta power density before and after SD. Interestingly, bumetanide treatment exerted on Ts65Dn mice an effect on power density of delta and theta waves, improving the quality of sleep in both NREM and REM sleep phases before and after SD, without any changes in WT. Moreover, bumetanide does not give rise to significant variations on epochs amounts of NREM and REM suggesting that the major architecture of sleep is not compromised by the GABAergic transmission. However, important alterations regarding the quality of sleep were evident, and bumetanide has shown to be able to decrease the abnormal wakefulness in Ts65Dn at baseline. Accordingly, our results displayed that bumetanide could have a potential effect to improve sleep quality in DS individuals.

Spectral power of theta was also found increased and correlated with a decline of cognitive function in the Parkinson Disease (Cozac et al, 2016), so it may represent a possible implication in cognitive alterations and the age-related neurodegeneration associated with DS (Lockrow at al, 2012).

Moreover, the balance of inhibitory and excitatory septohippocampal inputs affects theta oscillations, which are mostly expressed in hippocampal networks (Buzsaki, 2002). In this context, the increased EEG theta power that we describe in Ts65Dn mice may be the result of increased synaptic connectivity/activity at hippocampus-network level or could be the result of the engagement of further oscillating networks, either directly or *via* septohippocampal networks (Colas et al, 2008). So, our results showing the increase of theta waves in Ts65Dn mice may be linked to a dysregulation of $Cl^-$ homeostasis and consequent GABAergic neurotransmission within the hippocampus, as found in (Deidda et al, 2015). In support of this, GABAergic transmission has been implicated of in theta oscillations (Buzsaki, 2002) by experiments reporting elimination of theta in

terms of rhythm generation and current generation through, blocked or potentiation of $GABA_A$ receptor signaling during picrotoxin-induced epilepsy or pentobarbital anesthesia, (Vanderwolf, 1988). On the other hand, the effect that we described on theta power could also be linked to App gene triplication, which is present in Ts65Dn mice (and individuals with DS). (Colas et al, 2008). Indeed, DS studies on humans, mouse models and induced human pluripotent stem cells have shown the presence of the triplication for the Amyloid Precursor Protein (APP) gene, which determines an increase of Aβ brain levels (Wiseman FK et al 2015). In particular, App plays a role in regulating synaptic function and vesicular axonal transport (Kamal et al, 2001, Kamenetz et al, 2003) and its overexpression is involved in disruption of the retrograde transport of NGF, at level of septal cholinergic neurons in the Ts65Dn model. This alteration is related to an augmentation of theta power in Ts65Dn mice at 3 months of age (Salehi et al, 2006).

Another behavioral characteristic already described in Ts65Dn mice (and in individuals with DS) is an augmentation of motor activity. This is characterized by a greater exploratory behavior compared to controls, mainly during the dark period (Coussons-Read and Crnic, 1996, Martinez-Cue et al., 2002, Deidda et al 2015). Our data agree with the last studies showing an increase of motor activity and wakefulness. Interestingly, bumetanide treatment caused a reduction of activity, suggesting a regulation of motor behavior promoted by GABAergic transmission. A possible hypothesis to conciliate these data may be that increased wake and activity could be a compensatory mechanism to defective information processing, as well as, the increase of theta power (Colas et al, 2008). We also characterized body temperature, which represents a main regulator of sleep that follow the circadian rhythms. In particular, a decrease of temperature is present during the sleep period, whereas an increase coincides with the awakening (Parmeggiani et al, 1971; Parmeggiani, 1980; Parmeggiani, 1987). Interestingly, changes in body temperature have been associated with insomnia and occur in individuals following sleep deprivation, when subjects become more vulnerable to heat

loss, with less ability to warm themselves to a comfortable temperature (Landic CA et al,1998; Masanobu.H et al, 2017). Interestingly, we showed a significantly lower body temperature in Ts65Dn mice compared to controls. More investigation will be needed to investigate whether this difference in body temperature between Ts65Dn mice and WT has some influence on their sleep pattern.

Considering the relationship between eating activity and sleep patterns reported in literature, we also evaluated food intake in Ts65Dn mice. In particular, independent studies on humans have reported that short sleepers have higher energy intakes compared to normal sleepers (Imaki M et al, 2002; Weiss et al, 2010). Further studies will also be required to investigate whether the increase of food intake that we describe here in Ts65Dn mice is correlated to sleep abnormalities in these mice intake.

Although sleep disturbances are among the health issues mostly affecting the quality of life of people with DS not many studies have been conducted in DS individuals so far. This is mainly due to technical difficulties of people with DS in cooperating during EEG recordings and the presence of sleep apnea. For this reason, correlation with our results reported in Ts65Dn mice on the human condition is somehow incomplete. Nevertheless, there is evidence of an increase of wakefulness and hyperactivity in people with DS, which parallels our results. On the other hand, an augmentation of theta power seems to be not reported in DS humans, even if specific investigations have never been performed. Moreover, human studies, using appropriate age-matched controls, and characterized by careful spectral analysis across different brain regions, are needed. This is a crucial issue also when considering that sleep disturbances may be involved in the poor cognitive performance in DS people and the clinical evolution of DS, characterized by early dementia in the

elderly people (Colas et al 2008). In this perspective, the use of bumetanide as a therapy to improve sleep quality could also benefit the lives of DS people.

## 2. The role of $Cl^-$ homeostasis in behaviors and circadian rhythms of PWS

Several studies reported in the literature indicates that an aberrant $Cl^-$ homeostasis, caused by imbalance in NKCC1 vs KCC2 activity and/or expression, is involved in a large number of neurological disorders. In addition, NKCC1 inhibition promoted by bumetanide treatment appears to be an effective therapeutic strategy to improve behavioral alterations in many animal models of these neurological disorders (Ben-Ari, 2017; Schulte et al, 2018). Notably, benefits and efficacy of bumetanide treatment have been also showed in several clinical trials and case studies performed on patients (Ben-Ari, 2017).

Cognitive impairment, anxiety, obsessive-compulsive and ASD symptoms reported in PWS have never found explanation in a specific alteration of some molecular mechanisms. However, abnormal GABA metabolism and $GABA_A$ receptors in PWS were reported in literature (Lucignani G et al, 2004; Rice L.J et al 2016). These deficits were related to abnormal expression of $GABA_A$ receptor subunit genes (GABRB3, GABRA5, and GABRG3), present on the PWS/AS chromosomal region (15q11–13) (Lucignani G et al, 2004). These supportive evidences for a GABA dysfunction have encouraged the hypothesis of a possible involvement of GABA in behavioral alterations of PWS. Here, we completed these investigations by addressing the role $Cl^-$ homeostasis in PWS, prompted by its in several other neurodevelopmental disorders (Ben-Ari, 2017). We conducted our experiments on mutant $PWSCr^{m+p-}$ characterized by a very short paternal deletion involving *Snord116* gene, mostly implicated in the phenotypic manifestations of PWS (Bieth E et al, 2015).

Interestingly, we reported the imbalance of NKCC1 levels specifically for the hippocampus of PWSCr$^{m+p-}$ mice compared to controls, without any changes in the hypothalamus, SCN and the prefrontal cortex. Conversely, KCC2 expression did not significantly modify in the hippocampus of mutant PWSCr$^{m+p-}$ mice.

As demonstrated for other neurodevelopmental disorders (e.g., Down syndrome, autism spectrum disorders) (Ben-Ari, 2017) this is suggestive of the presence of a depolarizing and possibly excitatory GABAergic transmission (rather than hyperpolarizing and inhibitory). Nevertheless, further electrophysiological recordings are needed in the future to directly prove this hypothesis.

Moreover, Snord116 is a cluster of small nucleolar/noncoding RNAs (snoRNAs), mainly expressed in the brain, which are involved in the control of the expression of other small nucleolar RNAs (Sahoo et al, 2008), and may influence the transcription of other genes. So, our results also suggest that probably Snord116 could also regulate the expression of NKCC1 through mechanisms that need to be explored. Whether these expression changes are a primary event due to the imprinting mechanisms of *Snord116* related to PWS or a secondary response to other modifications is yet to be established.

Next, we characterized behavior that had been previously shown to be affected by impaired GABAergic transmission and/or $Cl^-$ homeostasis imbalance (Ben-Ari, 2017). In particular, we focused on long-term memory, anxiety, pain sensitivity, and other behaviors linked to obsessive/compulsive disorders and ASD in PWScr$^{m+/p-}$ mice.

The absence of anxiety, obsessive/compulsive and ASD behaviors that we found in PWSCr$^{m+p-}$ mice has evidenced that *Snord116* deletion and GABAergic transmission may not be correlated with these symptoms showed in PWS people.

On the other hand, we did report the presence of cognitive impairments in the long-term memory of PWSCr$^{m+p-}$ mice. Notably, the cognitive impairments, completely rescued by bumetanide chronic

treatment, highlighting the implication of abnormal regulation of $Cl^-$ homeostasis and GABAergic transmission in cognitive deficits of PWS. Moreover, we found an increase of pain sensitivity in PWSCr$^{m+p-}$ mice, which could be associated with defects in the thermoregulatory system. Indeed, these mice are characterized by a higher peripheral temperature compared to controls (Lassi et al, 2016), and variations of skin temperature are reported to have an important effect on the latency of behavioral responses during studies of nociception. In support of our results, higher temperatures have been correlated into a decrease in withdrawal latency (Bitar Ne et al, 2014).

Experiments to evaluate circadian clock in the PWS have never been conducted until now. However, individuals with PWS and PWSCr$^{m+p-}$ mice model have reported defect in the thermoregulation and abnormalities of sleep/wake cycles (Richdale et al, 1999; Ince E et al, 2005; Gillett E and Iris A. Perez, 2016; Lassi et al,2016), which could be related to a circadian rhythm dysregulation. Moreover, GABAergic signaling and mechanisms of $Cl^-$ homeostasis have been recently reported to be involved in the regulation of state plasticity in SCN network for maintaining behavioral responses to seasonal change (Rohr KE, 2019). For these reasons, we also executed circadian rhythm studies in mutant PWSCr$^{m+p-}$ and investigated on a possible contribution of an aberrant GABAergic transmission. Peculiarly, we observed that PWScr$^{m+/p-}$ mice have an elongation of their internal clock in DD condition compared to controls, completely rescued by bumetanide treatment. In contrast, we did not find any changes during LD cycle. In addition, we also evaluated the time devoted to behavioral activity alpha during LD and DD conditions, showing an increase of alpha activity in mutant PWScr$^{m+/p-}$ mice, reduced by bumetanide treatment. Altogether, these results indicate that cation $Cl^-$ cotransporters, NKCC1, but also KCC2, could represent promising targets for therapeutic interventions aimed at ameliorating the cognitive dysfunctions or disorders related to circadian clock of individuals with PWS though the modulation of $GABA_AR$ signaling. Interestingly, beside the

NKCC1 inhibitor bumetanide, compounds able to increase KCC2 activity/expression have been recently described (Tillman L and Zhang J, 2019).

Here, we proposed a pharmacological treatment using bumetanide, an FDA-approved drug, for a disease linked to imprinting defects. We chose to use bumetanide because it has a strong potential to be rapidly translated as a safe therapeutic approach for clinical trials in PWS individuals. Conversely, new developed molecules (Braudeau, J. et al. 2011) need to have further evaluations for factors as safety, tolerability and pharmacokinetics (e.g., trials NCT01436955, NCT01684891 and NCT01667367 listed at http://clinicaltrials.gov/). In fact, bumetanide has a long clinical history as a diuretic in both acute and chronic (lifelong) treatments (Ward, O.C. & Lam, L.K 1977, Maa et al, 2011). Moreover, bumetanide has just minor side effects such as the manifestation of a mild hypokalemia. Nevertheless, this can be addressed by a specific dietary supplementation. Furthermore, bumetanide did not show aversive effects during clinical trials in children with autism (trial NCT01078714, http://clinicaltrials.gov/) (Lemonnier, E. et al, 2012) (Lemmonier et al, 2017; James BJ.et al, 2019).

Different studies have reported that bumetanide is able to reach the brain (Li, Y. et al 2011, Cleary, R.T. et al, 2013 Deidda et al ,2015), even if its brain penetration may not be optimal (Puskarjov M et al 2014). Our results, together with results of the systemic effects of bumetanide to treat other disorders related to the brain (Dzhala et al, 2005 Sipilä et al, 2006; Kahle et al, 2009; Mazarati et al, 2009; Edwards et al, 2010; Lemonnier & Ben-Ari et al 2010; Lemonnier, et al, 2012; Hadjikhani et al, 2013; Lemonnier et al, 2013; Tyzio et al 2014) strengthen these evidences. The dose of 0.2 mg $kg^{-1}$ used during chronic injections here was chosen on the basis of previous studies on rodents (Dzhala et al. 2005; Mares, 2009; Mazarati et al, 2009; Brandt et al 2010; Wang & Kriegstein, 2011; Deidda et al, 2015). While, the dose 2mg $kg^{-1}$ for drinking water was conducted based on a previous

study showed in Tyzio et al, 2014. However, the dose that is used in mice is higher than the normal therapeutic range for humans (total daily dose, 0.5–2 mg; maximum daily dose, 10 mg) (http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/018225s024lbl.pdf; 2008). This was justified by the fast-metabolic rate of bumetanide in rodents (Pentikäinen, et al 1977; Ostergaard et al, 1972, Löscher, et al, 2013). Moreover, the use of this dosage is also supported by the encouraging results of other studies for the treatment of autism conducted with dosages similar to those described before for patients and animal models (Lemonnier et al, 2012; Lemonnier & Ben-Ari, 2010).

To conclude, our data describe the first and innovative pharmacological approach for PWS, with a safe, FDA-approved drug that can be readily translatable directly on people with PWS. We adopted a strategy of 'drug repurposing' (Strittmatter, 2014) in order to reduce the high costs and long time periods, considering the long period between testing new therapeutic approaches in animal models and the commercialization of newly developed drugs.

Finally, our data on bumetanide treatment of adult animals encourage the possibility to be able to ameliorate cognitive symptoms and circadian alterations that result from imprinting defects originating prenatally still in adulthood.

# Methods

## Animals and treatment (DS)

All animal procedures were approved by IIT licensing in compliance with the Italian Ministry of Health (D.Lgs 26/2014) and EU guidelines (Directive 2010/63/EU). A veterinarian was employed to maintain the health and comfort of the animals. Mice were housed in filtered cages in a temperature-controlled room with a 12:12 hour dark/light cycle and with ad libitum access to water and food. All efforts were made to minimize animal suffering and use the lowest possible number of animals required to produce statistical relevant results, according to the "3Rs concept". In this study, we used Ts65Dn mice (R.H Reeves et al, 1995) generated by repeated backcross of Ts65Dn female to C57BL/6JEi x C3SnHeSnJ (B6EiC3) F1 males (Jackson Laboratories). Ts65Dn mice were genotyped by PCR as previously described (A Duchon et al, 2011). Animals aged between 3-4 months were used for experiments. Only males were used for sleep experiments. Ts65Dn and WT littermates were randomly assigned to bumetanide (Sigma; 2 mg/kg body weight, as described in Tyzio et al, 2014) or vehicle groups (2% DMSO in saline) in drinking water and treated chronically after 4 days from surgery for 4 days. We used bumetanide treatment in drinking water to allow a constant daily assumption of bumetanide to monitor its effect on the sleep parameters.

## Telemetry system

Sleep was monitored by using a telemetry system. Telemetry is a wireless monitoring system that allows monitoring sleep. This procedure is feasible in mice using a radio-telemetry system for recording heart rate, electromagnetic phenomena (EEG) and electromyogram (EMG) (Data Sciences Int, St Paul, Minnesota 55126- 6164, USA - see internet website: http://www.datasci.com/products/implantable_telemetry/mouse.asp). The system includes a

miniature telemetric instrument (the transmitter) that contains a battery to which two pairs of electrodes (DSI physiotel TL11M-F20-EET) are connected. The battery allows the monitoring of the activity of two biopotentials for 6 weeks without interruption. This miniature plant is capable of measuring 2.5 mV peak to peak, but also overall mouse activity and body temperature. Digitized signals are transmitted from this system through radio frequencies to the receiver located under the cage. The receiver then transmits the data to the computer where a Dataquest Data Acquisition System allows you to view and then analyze the data. This system will allow the measurement of EEG, EMG, motor activity and temperature for long periods without the need of any animal restriction. For each mouse, a daily food intake was calculated. Food intake was adjusted for body weight and expressed as “food intake index”.

**Surgery**

We used 3-4 months adult mice of at least 20 grams weight. Before the implant, all the mice were anesthetized and therefore all surgical procedures were performed in animals subjected to total anesthesia. Isofluorane by means of a vaporizer (Dose: - Induction (chamber): 4.0 - 5.0 vol. % Isofluorane - Maintenance (mask): 1.5 - 2.0 vol. % Isofluorane). The level of anesthesia was constantly monitored by the nosocomial reflection at the tail and / or the paw level and the corneal reflection. Body temperature was maintained through the use of a thermal blanket. After trichotomy and disinfection, with the animal in general anesthesia, animals were carved and subcutaneously cut along the median line of approximately 2 cm for the introduction of the miniature transmitter into the peritoneal cavity. The transmitter wireless containing the electrodes was placed cranially. A sterile catheter was passed under the skin to reach the back of the neck. The cables passed through the hose and hence the tube was removed leaving the cables at subcutaneous level. In some cases of surgery, we put the transmitter under the skin and therefore

no abdominal wall incision was made. At this point, through a central incision on the head, the electrodes were positioned over the right hemisphere (approximately 2 mm from the central line and about 1 mm from the bramble) and left hemisphere (about 3 mm from the central line and about 1 mm from the lambda). The electrodes were fixed using miniaturized screws or placed in a special hole and then covered with acrylic of the type used in dental procedures. After positioning the pair of electrodes for the EEG, the electrodes were positioned for the detection of muscle activity. In this case, the electrodes were inserted into the neck muscle through the use of a needle. The same electrodes were fixed to the muscle through 1-2 suture points. Then, the incision on the scalp and neck were closed and sutured with absorbable material (eg Dexon II, 3-0, Vicryl, 2-0). For post-operative recovery, some analgesics (eg ketorolac or carprofen) were used.

## Deprivation of sleep

For short Sleep Deprivation (6hrs), mice were kept awake by the experimenter's intervention. In particular, an air jet or a brush was used by the experimenter any time that the animal had a sleeping posture. Various objects were introduced in the mouse cages to stimulate their curiosity, without inducing stress. The goal of this method is to get a sleep deprivation with the least stress since stress modifies the fundamental sleep properties.

## Statistical analysis

The statistical tests were performed with MATLAB and GraphPad Prism 8.3. Two-way mixed analyses of variance (ANOVAs) (Genotype × Treatment) were run temperature, activity and food intake in order to compare WT with Ts65Dn mice across light and dark phases. For all sleep parameters and the power spectral analysis of NREM and REM the effects of two parameters (genotype and treatment) were decomposed using two-tailed t-tests as described in Lassi et al, 2016

and Diessler et al 2017. Outliers were excluded only from the final pool of data by a Grubb's test run iteratively until no outliers were found.

## Animals and treatment (PWS)

All animal procedures were approved by IIT licensing in compliance with the Italian Ministry of Health (D. Lgs 26/2014) and EU guidelines (Directive 2010/63/EU). A veterinarian was employed to maintain the health and comfort of the animals. Mice were housed in filtered cages in a temperature-controlled room with a 12:12 hour dark/light cycle and with ad libitum access to water and food. All efforts were made to minimize animal suffering and use the lowest possible number of animals required to produce statistical relevant results, according to the "3Rs concept". In this study, we investigated mice carrying a deletion of the PWS critical region ($PWScr^{m+/p-}$) including *Snord116* and IPW exons compared to WT controls ($PWScr^{m+/p+}$). In the Istituto Italiano di Tecnologia (IIT), mice were bred and maintained through paternal inheritance on a C57BL/6J background. Male mice of 3-4 months old were tested for all behavioral tests. WT controls female mice of 3-4 months old were used just for male-female interaction test.

$PWScr^{m+/p-}$ and WT littermates were untreated or randomly assigned to bumetanide (Sigma; 0.2 mg/kg body weight for injection or administration in water solution 2mg/ kg body) or vehicle groups (2% DMSO in saline) for cognitive and circadian rhythms investigations.

## Protein extraction

For total protein extraction, hippocampal and cortical samples were homogenized in RIPA buffer (1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.4) containing 1 mM PMSF, 10 mM NaF, 2 mM sodium orthovanadate and 1% (v/v) protease and phosphatase inhibitor cocktail (Sigma). The samples were clarified by centrifugation at 20,000g, and

the protein concentration was determined using a Bicinchoninic Acid Assay (BCA) kit (Pierce). Western blotting For immunoblot analysis, equal amounts of protein were run on 4–12% Bis-Tris NuPAGE (Invitrogen) or Criterion-XT (Bio-Rad) gels and transferred onto nitrocellulose membranes (GE Healthcare). Membranes were probed with mouse anti-NKCC1 (clone T4, Developmental Studies Hybridoma Bank; 1:4,000), rabbit anti-KCC2 (Millipore, catalog no. 07-432; 1:4,000), rabbit anti β-actin (Sigma, catalog no. A2066; 1:10,000), mouse anti-Na+/K+-ATPase (clone C464.6, Millipore, catalog no. 05-369; 1:2,000), mouse anti-APP (clone 22C11, Millipore, catalog no. MAB248; 1:2,000), followed by HRP-conjugated secondary antibodies goat antirabbit and goat anti-mouse (Thermo Scientific, catalog nos. 31460 and 31430, respectively; 1:5,000). Chemiluminescence signals were digitally acquired on an LAS 4000 Mini Imaging System (GE Healthcare), and the band intensities were quantified using ImageQuant software (GE Healthcare). In some experiments, membranes were stripped and reprobed with a second antibody. The specificity of antibodies to NKCC1 and KCC2 was verified on brain samples from NKCC1- and KCC2-deficient mice, respectively (kind gift of Dr. K. Kaila, University of Helsinki;).

## Behavioral testing

Mice were tested after 1 week of treatment with vehicle, DMSO or bumetanide (0.2 $mg*kg^{-1}$ i.p.). The battery of tests was run over a total period of 3 weeks (behavioral tests for $PWScr^{m+/p-}$ and WT littermates in the following order NOR, OL, Fear conditioning). During the days of behavioral testing, animals were treated daily with the drug, with tests beginning 1 h after injection. The tasks were video-recorded and then analyzed manually by a blind operator. After each trial or experiment, the diverse apparatus and objects were cleaned with 70% ethanol.

Next another cohort of mice, not subjected to any treatment, was used to investigate on ASD, pain sensitivity and obsessive/compulsive disorders in $PWScr^{m+/p-}$ mice.

## Novel object recognition test (NOR)

The NOR test was conducted in a gray acrylic arena (44 × 44 cm). On the day before the NOR test, mice were allowed to become habituated to the apparatus by freely exploring the open arena for 10 min. NOR is based on the preference of mice for a novel object *versus* a familiar object when they are allowed to explore freely. The objects used were different in shape, color, size and material. During the acquisition sessions, three different objects were placed into the arena, and the mice were allowed to explore for 10 min. Object preference was evaluated during these sessions. Testing occurred 24 h later in the same arena. In the test, one of the objects used in the acquisition session was replaced by a novel object, and mice were allowed to explore freely for 10 min. The objects were counterbalanced between the sessions and were cleaned with 70% ethanol after each trial. Exploratory behavior toward an object was defined as direct contact with the object by the animal's mouth, nose or paws or as an instance when the animal approached the object so that its nose was within 1 cm of the object. Any indirect or accidental contact with the objects was not included in the scoring. The time spent exploring each object, expressed as a percentage of the total exploration time, was measured for each trial. The discrimination index was calculated as the difference between the percentage of time spent investigating the novel object and that of time spent investigating the familiar objects: Discrimination Index = ((Novel Object Exploration Time/Total Exploration Time) × 100) − ((Familiar Object Exploration Time/Total Exploration Time) × 100).

Experiments of Open field, Twirling and Wall rearing were derived from the videos recorded during the habituation phase of NOR.

## Object location test (OL)

The OL test evaluates spatial memory by measuring the ability of mice to recognize the new location of a familiar object on the basis of the available extra-maze cues. The test was conducted in a gray

acrylic arena (44 × 44 cm). Mice were habituated to the chamber for 15 min on the day before testing. The next day, mice were exposed to two identical objects for 15 min during the acquisition phase. Object preference was evaluated during this session. Testing occurred 24 h later in the same arena. During the trial session, one of the objects was moved to a novel location, and mice were allowed to explore the objects for 15 min. The objects and the arena were cleaned with 70% ethanol after each trial. The time mice spent exploring each object was measured. 'Exploration' was defined as any investigative behavior toward (i.e., head orientation, sniffing occurring within <1.0 cm) or deliberate contact with an object. A discrimination index was calculated as the percentage of time spent investigating the object in the new location minus the percentage of time spent investigating the object in the old location: Discrimination Index = ((New Object Location Exploration Time/Total Exploration Time) × 100) − ((Old Object Location Exploration Time/Total Exploration Time) × 100).

**Fear conditioning (FC)**

Delay contextual and cued fear conditioning was conducted using an automated fear-conditioning chamber (Med Associates, St Albans, VT, USA). The conditioning chamber was interfaced to a PC installed with VideoFreeze software (version 1.12.0.0, Med Associates) and enclosed in a sound-attenuating cubicle. Training consisted of a 2-min acclimation period followed by three tone-shock (CS–US) pairings tone-shock (CS–US) pairings (80 dB tone, duration 30-s; 0.5 mA footshock, duration 1 s; intershock interval 90-s) and a 2.5-min period, during which no stimuli were presented. The environment was well lit (~100 lx), with a stainless steel grid floor. A 5-min test of contextual fear conditioning was performed 24-hr after training, in the absence of the tone and footshock, but in the presence of 100 lx overhead lighting, and an identical context to that used on the training day. Cued fear conditioning, conducted 48-hr after training, mice were moved to a new environmental context (black chamber with plastic gray floor and vanilla odor). Overhead lighting was turned off.

The cued test consisted of a 3-min acclimation period followed by a 3-min presentation of the tone CS and a 90-s exploration period. The freezing behavior was scored by a trained operator blinded to the experimental groups.

**Three-chamber test**

The test evaluates the social approach of the tested mouse vs a never-met-intruder in comparison to an object (sociability) or vs a novel never-met-intruder in comparison to the already met intruder (social novelty), and it was performed similarly to what previously described in mouse models of autism. The three-chamber apparatus consists in a rectangle, three-chambered box of grey acrylic. The chambers are accessible by rectangle openings with sliding doors. In the first 10 minutes (habituation) the tested mouse was free to explore the apparatus containing two inverted stainless-steel wire pencil cups (one in each of the two side chambers), with a weighted plastic cup on the top of them, to prevent the mouse climbing on the top. Then, the tested mouse was briefly confined in the center chamber while a never-met-intruder (previously habituated to the apparatus) was placed in one of the side chambers, under the pencil cup. For the following 10 minutes (sociability test), the tested mouse was allowed to explore all the three chambers. Then, the tested mouse was again briefly confined in the center chamber while a novel never-met-intruder (previously habituated to the apparatus) was placed in the other side chamber under the pencil cup. Thus, for the following 10 minutes (social novelty test) the tested mouse was allowed to explore all the three chambers. The time spent exploring the object or the intruder was calculated by measuring the second when the mice showed investigative behavior (i.e., head orientation, sniffing occurring within < 1.0 cm) or time spent in compartment. The sociability index was calculated as the difference between the time spent investigating the never-met-intruder and the time spent investigating the familiar object divided by the total exploration time: sociability index = (never met intruder

exploration time - object exploration time)/( never met intruder exploration time + object exploration time). The social novelty index was calculated as the difference between the time spent investigating the novel never-met-intruder and the time spent investigating the already met intruder divided by the total exploration time: social novelty index = (novel never met intruder exploration time - already met intruder exploration time) /(novel never met intruder exploration time + already met intruder exploration time).

### Male-Female interaction

The test evaluates the social approach, based on male-female interaction of the tested male mouse *vs* a never-met-female mouse. For the habituation, the tested mouse was placed singularly in a cage (26 cm × 48 cm × 20 cm). It was performed in a similar way to that previously described for mouse models of ASD (E Drapeau et al, 2018). After 5 minutes, an unfamiliar C57BL/6J female mouse was placed into the home-cage of the isolated male mouse, and behavior was recorded for a 5-min test session. The time spent interacting with the female intruder was calculated by measuring the second when the mice showed any of the following interacting behavior: anogenital sniffing, body sniffing, head sniffing, following and mounting. Digging and grooming analysis were executed during the habituation.

Experiments of grooming and digging were derived from the videos of the habituation of Male-Female interaction protocol.

### Hot Plate test

The response to an acute thermal stimulus was assessed. In particular, the experimenter held the mouse and gently placed the paws of the animal on the surface of the hot plate kept at constant

temperature of 52°C. The latency to lick the paws from the hot plate was measured. To prevent any heat injury to pups, a cut-off latency of 30 seconds was applied.

**Marble burying**

Mice are placed for thirty minutes in a standard cage empty just filled with 4 cm depth of wood chip bedding. After thirty minutes of habituation, 15 marbles are put evenly spaced in the cage. The amount of marbles buried is measured after thirty minutes of testing. In this procedure a marble is considered buried if 2/3 of the marble is covered with bedding.

**Running Wheels**

The cages were equipped with a wheel connected to a system that measures in automatic the number of wheel circumvolutions in their home-cage during the 24 hours a day for 20 days. In a variant of this test was used a plate placed under the cage and allowing to monitor the motor activity of mice. Both of these techniques are totally non-invasive and mice they have access to libitum for food and water. Mice were kept under normal conditions of lighting (12:12 LD) for 10 days and in constant darkness (DD) for other 10 days to study the rhythms circadian in a free-running condition, without any interruption. The animals were kept in conventional cages. Mice were treated with vehicle or bumetanide in drinking water (2mg/kg) for the whole period of experiments. We used bumetanide treatment in drinking water to allow a constant daily assumption of bumetanide to monitor its effect on the circadian rhythms. The circadian period of each mouse was calculated by wheel running activity.

The analyses were performed using the MATLAB (www.mathworks.com). The time devoted to ehavioral activity (alpha) during LD and DD conditions, where alpha represents the length of the

segment of the daily activity was analyzed and computed with the algorithm described in Leise, 2013 and Leise et al, 2013.

## Statistical analysis

The statistical analysis was performed using GraphPad Prism 8.3. Where appropriate, the statistical significance was assessed using the following parametric test: Student's t test, two-way ANOVA followed by all pairwise Tukey post hoc test. Outliers were excluded only from the final pool of data by a Grubb's test run iteratively until no outliers were found.

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