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**The chloride/potassium co-transporter KCC2 in synaptic
plasticity, hippocampal rhythmogenesis and memory**

*Le co-transporteur de chlore et potassium KCC2 dans la plasticité synaptique,
rythmogénèse hippocampique et mémoire*

Par Clémence Simonnet

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Dirigée par Jean-Christophe Poncer

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Devant un jury composé de :

Dr Alberto Bacci

Dr Lisa Genzel

Dr Peter Blaesse

Dr Pascale Quilichini

Dr Stéphanie Daumas

Dr Jean-Christophe Poncer

Président du Jury

Rapporteur

Rapporteur

Examineur

Membre invitée

Directeur de thèse

Abstract

Information transfer, storage and retrieval in the brain rely on an exquisite balance between excitation and inhibition. At the cellular level, memory encoding involves long-term potentiation of excitatory synapses, while at the network level, cortical rhythmogenesis underlies memory encoding as well as consolidation and requires inhibitory, GABAergic signaling to synchronize neuronal ensembles. Therefore, alterations of this balance are observed in most disorders associated with cognitive deficits. In the mature central nervous system, GABA is the main neurotransmitter mediating fast inhibitory transmission and activation of GABAARs usually leads to an influx of chloride ions. Therefore, a tight control of transmembrane chloride gradients is crucial to maintain the efficacy and polarity of GABA transmission and primarily involves the chloride/potassium co-transporter KCC2. Down-regulation of KCC2 expression and subsequent alteration of GABA signaling is therefore thought to be involved in many neurological and psychiatric disorders such as epilepsy, autism or Rett syndrome. However, recent evidence suggests the role of KCC2 extends beyond the mere control of GABAergic signaling in neurons. Thus, through interactions with a variety of molecular partners, KCC2 influences neuronal membrane excitability as well as the function and plasticity of glutamatergic synapses. Altogether, KCC2 therefore appears at the crossroads of excitatory and inhibitory transmission.

During my PhD, I explored the consequences of KCC2 down-regulation in the dorsal hippocampus on learning and memory, and the underlying mechanisms both at the cellular and network levels. My results demonstrate that KCC2 knockdown in principal neurons of the dorsal hippocampus affects both spatial and contextual memory. This effect is associated with deficits in long-term potentiation of hippocampal synapses as well as neuronal hyperexcitability and hippocampal rhythmopathy, including abnormal sharp-wave ripple generation and gamma-band activity during sleep. These alterations likely contribute to impair both memory encoding and consolidation. Importantly, however, KCC2 knockdown in dorsal hippocampus is not sufficient to generate spontaneous, epileptiform activity. Since KCC2 is down-regulated in many disorders associated with cognitive impairment, my results suggest that strategies aiming to restore KCC2 expression may hold therapeutic potential in these disorders. I therefore started testing this hypothesis in experimental models of Rett syndrome.

Résumé

Dans le cerveau, le transfert d'information nécessite un parfait équilibre entre signalisations excitatrice et inhibitrice. Au niveau cellulaire, la formation d'un souvenir dépend de la potentiation à long-terme des synapses excitatrices alors qu'au niveau du réseau neuronal, la rythmogenèse hippocampique contrôle la formation et la consolidation de ce même souvenir et nécessite une transmission GABAergique inhibitrice afin de synchroniser les ensembles neuronaux. Ainsi, une altération de cet équilibre existe dans la plupart des pathologies associées à des troubles cognitifs. Dans le système nerveux central mature, GABA est le principal neurotransmetteur responsable de la transmission inhibitrice et l'activation de récepteurs GABAA entraîne un influx d'ions chlore. l'efficacité et la polarité de la transmission GABAergique dépendent du maintien de la concentration intra-neuronale de chlore, et ce rôle revient principalement au co-transporteur de chlore et potassium KCC2. La diminution d'expression de KCC2 et l'altération de la transmission GABAergique qui en découle sont souvent invoqués pour expliquer les troubles présentés par les patients épileptiques, ou atteints du Syndrome de Rett. Cependant, de récents résultats suggèrent que le rôle de KCC2 ne se limite pas seulement à un contrôle de la signalisation GABAergique. A travers des interactions avec des partenaires protéiques, KCC2 influence l'excitabilité neuronale ainsi que le fonctionnement et la plasticité des synapses glutamatergiques. KCC2 participe ainsi au maintien des transmissions excitatrices et inhibitrices.

Au cours de ma thèse, j'ai donc étudié le rôle d'une diminution d'expression de KCC2 dans l'hippocampe dorsal, sur l'apprentissage et la mémoire, ainsi que les mécanismes sous-jacents au niveau cellulaire et du réseau. Mes résultats démontrent une altération de la mémoire spatiale et contextuelle lorsque l'expression de KCC2 est diminuée dans les neurones principaux. Cet effet est associé à une diminution de la potentiation à long terme des synapses hippocampiques ainsi qu'une hyperexcitabilité neuronale et des déficits de rythmogenèse, plus spécifiquement des sharp-wave ripples et des oscillations gamma anormales lors du sommeil. Ces altérations contribuent probablement à des déficits d'apprentissages et de consolidation. De plus, un point important est l'absence d'activité spontanée épileptique chez ces animaux. Puisque l'expression de KCC2 est diminuée dans des pathologies associées à des troubles cognitifs, mes résultats suggèrent que des stratégies permettant de stabiliser l'expression de KCC2 pourraient être considérées comme de potentielles options thérapeutiques. J'ai donc commencé à tester cette hypothèse dans un modèle murin pour le syndrome de Rett.

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List of abbreviations

4-AP	4 Amino-pyridine
AIS	Axon initial segment
AMPA	α -Amino-3-hydroxy-5methyl-4-isoxazolepropionic acid
ASD	Autism spectrum disorder
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CA	<i>cornu Ammonis</i>
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CCC	Cation-Chloride Co-transporter
CNS	Central nervous system
CREB	cAMP response element-binding protein
CTD	Carboxy-terminal domain
DBS	Deep-brain stimulation
DG	Dentate gyrus
dHPC	Dorsal hippocampus
DLPFC	Dorsolateral prefrontal cortex
Dlx	Distal-less homeobox
DMSO	Dimethyl sulfoxide
EC	Entorhinal cortex
EEG	Electroencephalogram
E _{GABA}	Reversal potential of GABA _A R mediated currents
Egr4	Early growth response transcription factor 4
EPSC	Excitatory postsynaptic current
Erk1/2	Extracellular signal-regulated kinases 1 and 2
F/G-actin	Microfilament/globular-actin
FERM	Four.one, Ezrin, Radixin, Moesin
GABA	γ -Amino Butyric Acid
GABA _A R	Receptor subtype A for GABA
GDP	Giant depolarizing potentials
HEK	Human embryonic kidney
HFS	high frequency stimulation
HPCD	Hydroxypropyl- β -cyclodextrin
IGF1	Insulin-Growth Factor 1
iPSC	Induced-pluripotent stem cell
IPSC	Inhibitory postsynaptic current
KCC2	K ⁺ /Cl ⁻ cotransporter type 2
LIMK	Lin-11, Isl-1 and Mec-3 kinase
LTD	Long-Term Depression
LTP	Long-Term Potentiation

mAChR	Muscarinic acetylcholine receptor
MBD	Methyl-binding domain
MecP2	Methyl CpG binding protein 2
MF	Mossy fiber
MS-DBB	Medial septum – diagonal band of broca
MUA	Multi-unit activity
NEM	N-Ethylmaleimide
NKCC1	Na ⁺ /K ⁺ /2Cl ⁻ cotransporter type 1
NLS	Nuclear localization signal
NMDAR	N-methyl-D-Aspartate receptor
NOR	Novel object recognition
NREM	Non Rapid Eye Movement
NTD	Animo-Terminal Domain
OR	Object recognition
OSR	Osmotic stress resistant protein
PAK	Serine/threonine-protein kinase
PKC	Protein kinase C
PLC γ	Shc/FRS-2 and phospholipase C γ
PP	Perforant path
PPI	Protein phosphatase 1
PR	Place recognition
PSD	Post-synaptic density
PV	Parvalbumin
REM	Rapid eye movement
REST	RE1-silencing transcription factor
RTT	Rett syndrome
SC	Schaffer collateral
SCI	Spinal cord injury
shRNA	Short hairpin RNA
SOM	Somatostatin
SPAK	Ste20p-related proline/alanine-rich kinase
SPW-R	Sharp-wave ripple
TLE	Temporal lobe epilepsy
TRD	Transcriptional repression domain
TrkB	Tropomyosin receptor kinase B
USF	Upstream stimulating factor
V _{REST}	Resting membrane potential
WNK	With no lysine kinase
β -PIX	β isoform of Rac/Cdc42 guanine nucleotide exchange factor

INTRODUCTION

Introduction

In the central nervous system, information processing relies mostly on a transfer of information through chemical synapses. Following the emission of an action potential, a presynaptic neuron releases its neurotransmitter into the synaptic cleft. The postsynaptic neuron can be either activated or inhibited, depending on the nature of the neurotransmitter and transmembrane ion gradients. In the mature CNS, excitation relies mainly on the release of glutamate while γ -Amino butyric acid (GABA) signaling usually produces neuronal inhibition.

Learning and memory rely on the coordinated action of these synaptic signals. At the cellular level, long-term potentiation of glutamatergic transmission results in changes in synaptic function and morphology. At the network level, network oscillations (such as theta and gamma-band oscillations) in brain regions such as the hippocampus allow information encoding during exploration while other oscillatory activities such as sharp-wave ripples and theta during rapid eye movement (REM) sleep participate in memory consolidation. These rhythms rely on the coordinated activity of excitatory and inhibitory signaling as well as neuronal excitability. Therefore, mechanisms acting to perturb either glutamatergic or GABAergic signaling or neuronal excitability are likely to result in alterations in the ability of an animal to learn and store new information.

Both excitatory and inhibitory transmission require ions flux through the neuronal plasma membrane. At rest, the neuronal membrane is electrically polarized with a net negative charge on the inside of the membrane. An action potential therefore corresponds to an abrupt depolarization of the membrane potential. Indeed, fast excitatory transmission results from glutamate receptor activation, leading to an entrance of cations (Na^+ , Ca^{2+}) underlying excitatory postsynaptic currents (EPSCs). On the other hand activation of ionotropic GABAA receptors (GABAARs) mediate hyperpolarizing inhibitory postsynaptic currents (IPSCs) through the influx of anions (Cl^- , HCO_3^-) and prevent action potential firing.

Electrochemical gradients are thus essential to synaptic transmission and the control of neuronal excitability. The Na/K ATPase maintains intraneuronal concentration of Na^+ and K^+ while Cation-Chloride Cotransporters (CCCs) regulate transmembrane Cl^- gradients. Among these proteins, two are mainly expressed in the mature CNS: KCC2 usually extrudes chloride ions under isotonic conditions, typically maintaining a low intraneuronal chloride concentration, while NKCC1 participates in the influx of Cl^- ions. The subsequent transmembrane Cl^- gradient largely determines the polarity of GABAARs (Figure 1). Hence, changes in the activity of these transporters directly impact GABAergic signaling and have been involved in various disorders.

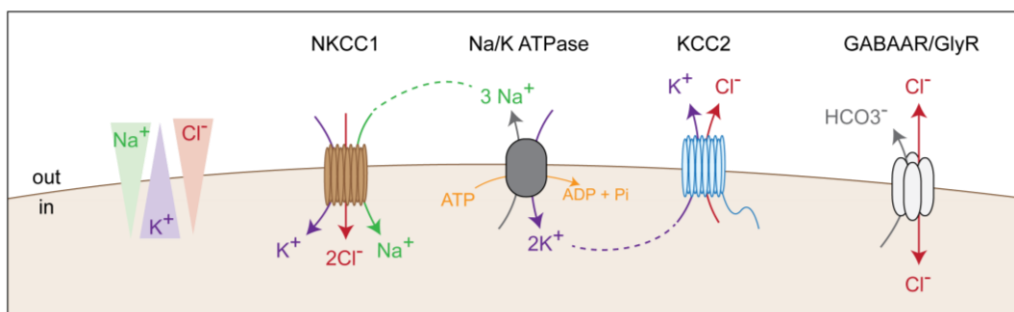


Figure 1. CCCs expression control the chloride ion flux through GABAARs

KCC2 extrude chloride ions using the K^+ gradient created by the Na/K ATPase while NKCC1 intrudes Cl^- . The resulting chloride gradient determines the direction of Cl^- flux through GABAAR.

Nevertheless, modulation of synaptic transmission is also observed under physiological conditions. Indeed, in all brain regions and neuronal subtypes, synaptic efficacy is modulated in response to neuronal activity. This synaptic plasticity allows neurons to strengthen or weaken their connections through long-term potentiation (LTP) and depression (LTD), respectively. LTP mechanism requires the influx of Ca^{2+} via the N-methyl-D-Aspartate receptor (NMDAR) resulting in the addition of α -Amino-3-hydroxy-5methyl-4-isoxazolepropionic acid receptor (AMPA) in the postsynaptic density (PSD). Somewhat surprisingly, KCC2 has been shown to influence these forms of plasticity through an ion-transport independent mechanism.

My PhD thesis focus on the role of KCC2 in learning and memory and the impact of KCC2 down-regulation on cellular and network mechanisms underlying memory encoding and consolidation. Before presenting my experimental results, I will introduce the function

and regulation of KCC2 in physiological and pathological conditions and explore the different mechanisms involved in learning and memory. My experimental results will then be described and discussed in the second and third parts of the manuscript.

I. KCC2, a neuronal cation-chloride cotransporter influencing both GABAergic and glutamatergic signaling

1. Expression of KCC2 in the CNS

a. The Cation-Chloride Co-transporters (CCC) family

Fast excitatory and inhibitory signaling rely on transmembrane ion fluxes through primarily AMPA and GABA_A receptors, respectively. While AMPA receptors are mainly permeable to sodium and potassium ions (Dingledine et al., 1999), GABA_AR are predominantly permeable to chloride and, to a lesser extent, bicarbonate ions (Hübner and Holthoff, 2013). Transmembrane electrochemical gradients of these ions are maintained through the function of various ionic pumps and transporters. In particular, the Na/K ATPase serves as an active ion pump allowing the influx of K⁺ and efflux of Na⁺ ions and thereby contributes to maintain the transmembrane gradients of these ions (Schmidt and Dubach, 1971). Intraneuronal chloride concentration on the other hand is under the control of cation-chloride cotransporters (CCCs) that are non-electrogenic, secondary active transporters using Na⁺ and K⁺ gradients to transport chloride ions (Blaesse et al., 2009; Gamba, 2005).

All genes encoding CCCs belong to the *SLC12* gene family (*SLC12A1-9*). CCCs are expressed widely in the body and can be classified in 4 families, depending on their stoichiometry and the cations that are co-transported (Gamba, 2005):

- Na⁺/Cl⁻ co-transporters (NCC): expressed selectively in kidney
- Na⁺/K⁺/2Cl⁻ cotransporters (NKCC1-2): NKCC1 is the only one expressed in the CNS
- K⁺/Cl⁻ (KCC1-4): KCC2 is almost exclusively expressed in the brain
- CCC-interacting protein CIP1 and the polyamine transporter CCC9, identified on the basis of their homology of structure and mRNA sequence with other CCCs. However, their role as co-transporters is not defined yet. CIP1 regulates the transport activity of NKCC1 (Caron et al., 2000) and KCC2 (Wenz et al., 2009).

CCCs have multiple physiological roles. They are mainly acting to control intracellular chloride concentration. Another major function is their role in osmotic regulation that I will address later in this introduction.

b. KCC2 structure and regulatory sequences

KCC2 was only identified in the late 90s, by screening a DNA library to identify a gene with homology to the NKCC1 and 2 transporters (Payne et al., 1996). So far, only NKCC1 topology has been characterized (Gerelsaikhan and Turner, 2000) and the secondary structure of other CCCs is expected to be similar. KCC2 three-dimensional structure was predicted using hydropathy profile alignment, i.e. by analyzing the hydrophobic and hydrophilic domains of the protein based on its amino acids composition (Payne et al., 1996). KCC2 is 140 kDa glycoprotein with 12 putative transmembrane domains, flanked with intracellular amino- (NTD, amino acids 1-103) and carboxy-terminal domains (CTD, last 500 amino acids), and a large extracellular loop containing glycosylation sites between the 5th and 6th transmembrane domains. More recently, mass spectrometry experiment confirmed the presence of 6 glycosylation sites and some of the regulatory sites in the CTD (Agez et al., 2017).

The ternary structure of KCC2 is unknown, as the protein has not been crystallized yet. This is probably due to the double difficulty of purifying a transmembrane protein and obtaining a crystal. However, a plausible ternary structure of CCCs is based on the crystallographic model of the CTD of MaCCC, a prokaryotic CCC transporter from the archaea *Methanosarcina acetivorans* (Warmuth et al., 2009). MaCCC CTD is shorter than that of eukaryotic CCCs, but the difference is mainly due to insertion of loops, which are not predicted to significantly impact the 3D structure.

Finally, the quaternary structure of KCC2 and its role are still a matter of debate. It is known that KCC2 can form multimeric structures with itself as well as other CCCs as bi-, tri- and tetrameric KCC2 complexes can be detected by biochemical assay (Blaesse et al., 2006; Simard et al., 2007; Uvarov et al., 2009). The proteins are interacting together mainly through disulfide bridges, as a majority of the monomeric form can be observed in western blot after treatments with DTT or β -mercaptoethanol (Agez et al., 2017; Blaesse et al., 2006). Moreover, recent experiments suggested CTD is important for the dimerization (Agez et al.,

2017), as also demonstrated for KCC1 (Casula et al., 2001) and MaCCC (Warmuth et al., 2009).

KCC2 oligomerization might be necessary for its transport function. Indeed, throughout development, the proportion of oligomer forms increase in all brain regions. In the rat lateral superior olive, KCC2 is mainly in its monomeric form at post-natal day 3 (P3), which correlates with low chloride extrusion capacity. But as neurons mature, KCC2 forms oligomers and by P12, the chloride extrusion capacity increases, reflecting functional KCC2 (Blaesse et al., 2006).

Two groups have also suggested that lipid rafts could influence KCC2 oligomerization and function. However, they disagree on the role of these lipid rafts on KCC2 function. Watanabe and colleagues showed KCC2 lipid raft insertion and oligomerization were facilitated by the phosphorylation of Y1089 residue (Watanabe et al., 2009). In neurons expressing a constitutively dephosphorylated Y1089D residue, KCC2 activity was decreased and its localization within lipid rafts disrupted. As the function of numerous transporters depends on their localization in lipid rafts (Martens et al., 2004), they proposed that KCC2 function may be increased when localized within the lipid rafts. On the other hand, Hartmann and colleagues suggested lipid rafts might decrease KCC2 function, as chemical alteration of lipid rafts increased KCC2 transport function and affected KCC2 clustering. This apparent discrepancy may be explained by the fact that the first study did not investigate the effect of disrupting lipid rafts on KCC2 function but directly acted on its phosphorylation and its redistribution at the membrane. Therefore, the conclusion of this work arise from mostly correlative observations.

KCC2 membrane stability and function rely on phosphorylation and dephosphorylation of KCC2 key tyrosine, threonine or serine, most of them localized in the CTD (Figure 2). I will only concentrate on a few of them that are important in mature neurons but more information can be found in recent reviews (Alessi et al., 2014; Côme et al., 2019).

In our group, GABAAR activation was shown to stabilize KCC2 at the plasma membrane. Indeed, a transient rise in $[Cl^-]_i$ leads to inhibition of WNK1 kinase. In the absence of GABAAR activation, WNK1-SPAK-OSR1 pathway phosphorylates KCC2 T906 and T1007 residues and increases KCC2 membrane diffusion and reduces its membrane clustering and function (Heubl et al., 2017; de Los Heros et al., 2018; Rinehart et al., 2009).

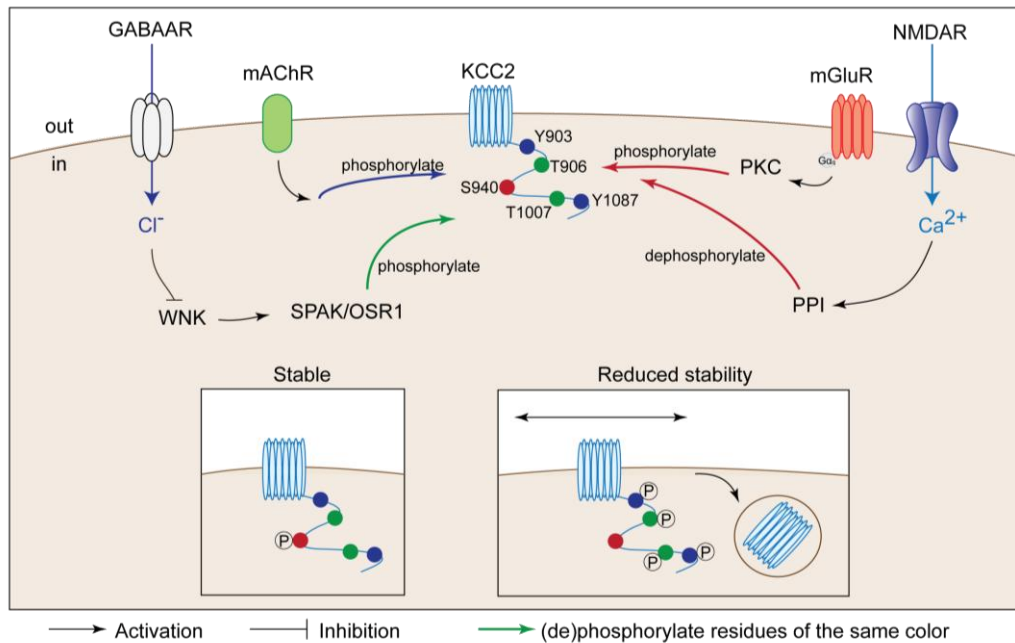


Figure 2. KCC2 regulation through phosphorylation of specific residues

KCC2 membrane expression is modulated through a variety of intracellular signaling pathways. Its C-terminal domain hosts different residues important for its stability. Indeed, phosphorylation of S940 increases KCC2 membrane stability and function while phosphorylation of T1007 or T906 is associated with opposite effects. Phosphorylation of Y903 and Y1087 may on the other hand lead to increased membrane diffusion/recycling.

Excitatory transmission has also been shown to control KCC2 transport function and stability (Kaila et al., 1997; Wang et al., 2006; Woodin et al., 2003). Indeed, postsynaptic Ca^{2+} influx through NMDARs induces protein phosphatase 1 (PP1) – dependent dephosphorylation of KCC2 S940 residue (Lee et al., 2011) which in turn reduces the stability of KCC2 and increases its membrane diffusion near excitatory but not inhibitory synapses (Chamma et al., 2013). On the opposite, phosphorylation of S940 by the protein kinase C (PKC) increases KCC2 activity and membrane stability (Lee et al., 2007). Finally, in HEK-293 cells, phosphorylation of Y903 and Y1087 decreases the cell surface stability of KCC2. Moreover, activation of the muscarinic acetylcholine receptors (mAChRs) in cultures of hippocampal neurons increases KCC2 tyrosine phosphorylation (Lee et al., 2010).

Interestingly, among the various rodent models of epilepsy, one of them consists in using pilocarpine, a mAChR agonist. Moreover, mouse models with constitutively dephosphorylated S940 residue are associated with increased seizure susceptibility, as I will discuss later (see Introduction II.2.b, Silayeva et al., 2015).

c. KCC2 isoforms

The human KCC2 gene (*SLC12A5*) is predicted to encode different transcripts variants, possibly with different roles. Two full-length KCC2, referred to as KCC2a and KCC2b have been identified (Uvarov et al., 2007), and can interact together (Uvarov et al., 2009). KCC2a has a longer NTD (40 more amino acids) with an extra regulatory site for SPAK. KCC2a and KCC2b form functional transporters, but their expression patterns during development and in the brain differ. While KCC2b is largely expressed throughout the whole adult brain, KCC2a expression is really low in cortex, hippocampus, thalamus and cerebellum. Both isoforms are expressed in the hypothalamus, brain stem and spinal cord (Markkanen et al., 2014).

In mouse neonatal brain, both isoforms are expressed but only KCC2b is up-regulated during postnatal development (Markkanen et al., 2014; Rivera et al., 1999). Importantly, knock-out mice for both isoforms die at birth from severe motor and respiratory deficits, whereas mice expressing KCC2a only survive for almost 2 weeks, then die of spontaneous seizures (Uvarov et al., 2007). This suggests a role of KCC2a in spinal cord and brain stem development. Thus, KCC2a expression in these structures might be sufficient to control for intraneuronal chloride concentration and neuronal function required for normal breathing (Dubois et al., 2018), whereas its low expression in the cortex might rapidly promote hyperactivity and seizures.

Shorter transcripts have also been identified and lack some of the exons of the *SLC12A5* gene (AK294059, AK295096, AK098371, EXON6B) (Tao et al., 2012; Wang et al., 2008). These transcripts may not encode for functional transporter proteins but may have a regulatory effect still to be characterized. Moreover, these transcripts may be species-specific (Gagnon and Di Fulvio, 2013) or even tissue-specific. For instance, one such transcript was recently identified in the pancreas but not in the brain (Kursan et al., 2017). I will address later how some of these transcripts may be associated to the pathology (see Introduction II.1).

d. Spatial and temporal expression of KCC2

- **KCC2 expression profile in the brain**

Although KCC2 was thought to be expressed only in the CNS, recent evidence revealed KCC2 expression in other tissues such as chicken cardiomyocytes (Antrobus et al., 2012), human osteoblasts (Bräuer et al., 2003) or pancreatic islets β -cells where it regulates insulin secretion (Kursan et al., 2017).

In the CNS, KCC2 is expressed only in neurons, due to the presence of two upstream and intronic RE-1 binding sites for the neuron-restrictive silencing factor (NRSF), also called RE1-silencing transcription factor (REST) (Karadsheh and Delpire, 2001; Yeo et al., 2009). Other binding sites have been described for early growth response transcription factor 4 (Egr4), mainly expressed in postnatal neurons (Uvarov et al., 2006) and upstream stimulating factor (USF1 and 2) via an E-box controlling element (Markkanen et al., 2008).

As described before, KCC2 (hereby referring to both isoforms) is expressed throughout the brain (Markkanen et al., 2014), including spinal cord (Hübner et al., 2001), cerebellum (Williams et al., 1999), thalamus (Barthó et al., 2004), auditory brainstem (Blaesse et al., 2006) and cortical structures (Gulyás et al., 2001; Rivera et al., 1999) (Figure 3). KCC2 is expressed in excitatory neurons (Rivera et al., 1999), Purkinje cells (Mikawa et al., 2002) and in, at least, parvalbumin-containing and some calbindin-containing interneurons (Gulyás et al., 2001). Although KCC2 seems ubiquitously expressed, the reversal potential of GABA_AR mediated currents (E_{GABA}), which depends in part on transmembrane chloride gradients, shows differences depending on the structure and neuronal type (Klein et al., 2018; Schmidt et al., 2018). This could be due to differential expression or function of the protein, co-expression with other CCCs such as NKCC1 and/or differences in bicarbonate metabolism.

At the subcellular level, KCC2 shows diffuse expression throughout the somatodendritic membrane of neurons but also forms clusters, in particular in the vicinity of both GABAergic and glutamatergic synapses (Chamma et al., 2013; Gauvain et al., 2011; Gulyás et al., 2001). However, its expression is strictly excluded from the axon and axon initial segment (AIS) (Williams et al., 1999), except in bipolar retinal cells (Vardi et al., 2000). Notably, the sodium/potassium/chloride cotransporter NKCC1 is, on the contrary, expressed in both the somatodendritic and axonal membrane (Jang et al., 2001; Khirug et al., 2008).

The AIS preserves the axonal identity by acting as a gatekeeper through two main mechanisms. Sub-membrane ankyrin-spectrin scaffolding complex forms a surface diffusion barrier impeding lipids and membrane proteins trafficking. The AIS also acts as an intracellular traffic filter. Indeed, specific motor proteins, such as myosin VI, are transporting the vesicles to the axon (Letierrier and Dargent, 2014; Lewis et al., 2011). Recent work in epithelial cells suggests that the subcellular localization of NKCC1 depends on alternative splicing (Carmosino et al., 2008). A similar mechanism can be hypothesized for KCC2. It is also possible that vesicles containing KCC2 are not expressing the cargo proteins for axonal targeting. As a consequence of KCC2 and NKCC1 differential expression in the axon, chloride concentration in axon terminals is higher than in the somatodendritic compartment (Price and Trussell, 2006).

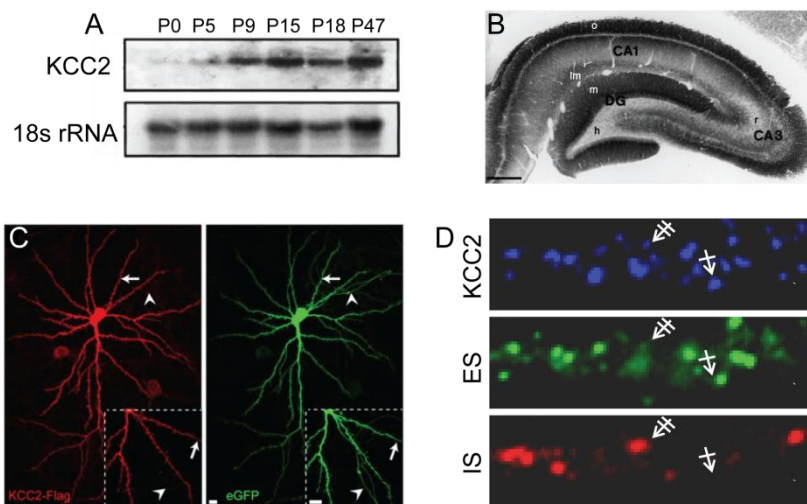


Figure 3. Spatio-temporal expression of KCC2

A. KCC2 expression increases in the rodent brain during post-natal development. B. KCC2 expression in the hippocampus. Scale bar 600 μ m. B. Immunostaining of a neuron transfected with recombinant KCC2 (red) and GFP (green). Higher magnification of the region of interest: KCC2 is expressed in the soma and dendrites (arrow) of neurons but is excluded from the axon (white arrowhead). D. KCC2 is localized close to both excitatory synapses (ES) and inhibitory synapses (IS).

(Adapted from Chamma et al., 2013; Gulyás et al., 2001; Rivera et al., 1999)

- **KCC2 developmental expression**

KCC2 expression in the forebrain increases during development (Clayton et al., 1998; Jansen et al., 2010; Rivera et al., 1999). In humans, KCC2 expression increases *in utero*

around 25 weeks post-conception with its maximum after birth (Sedmak et al., 2016), in contrast with mice and rats where KCC2 expression mainly increases after birth (Watanabe and Fukuda, 2015) (Figure 3). However, these results cannot be generalized to all rodents as guinea pig neonates already exhibit a high amount of KCC2 mRNA by embryonic day 42 (E42) without any up-regulation after birth (Rivera et al., 1999).

Since over-expression of brain-derived neurotrophic factor (BDNF) in embryos was shown to increase KCC2 expression at E18 (Aguado et al., 2003), its effect on developmental up-regulation of KCC2 was studied. BDNF binding to tropomyosin receptor kinase B (TrkB) activates the extracellular signal-regulated kinases 1 and 2 (Erk1/2) pathway. This in turn increases KCC2 expression through Egr4-dependent KCC2 transcription (Ludwig et al., 2011a). Another *in vitro* study also reported the importance of both RE-1 sites to drive the effect of BDNF (Yeo et al., 2009). As KCC2a mRNA expression is relatively constant during development and restricted to specific brain regions (Markkanen et al., 2014), these regulations might only affect KCC2b transcription (Ludwig et al., 2011a; Yeo et al., 2009). However, a recent *in vivo* experiment reported a normal increase in KCC2 expression in BDNF^{-/-} mice at P5-6 and P13-14 (Puskarjov et al., 2015). This suggests that other mechanisms exist to allow for KCC2 up-regulation during development (Ludwig et al., 2011b) and BDNF expression is not strictly necessary.

Nevertheless, this effect of BDNF on KCC2 expression differs in adult neurons. BDNF incubation for 2 to 3 hours is sufficient to reduce KCC2 expression in acute hippocampal slices and decrease Cl⁻ extrusion capacity of the cell membrane (Rivera et al., 2002). This regulation depends on the activation of both Shc/FRS-2 and phospholipase C γ (PLC γ) – cAMP response element-binding protein (CREB) signaling (Rivera et al., 2004).

KCC2 expression is stable during aging in both rodents and humans (Ferando et al., 2016; Sedmak et al., 2016). However, KCC2 down-regulation was observed following LTP-induction protocol in aged mice (21 to 28 weeks old) (Ferando et al., 2016). fEPSP amplitude was reduced upon bicuculline application in old but not young mice, suggesting a possible contribution of a depolarizing GABAergic component. Moreover, in these old mice, unstimulated synapses were also potentiated, which did not occur in younger mice. Interestingly, application of the KCC2 enhancer CLP-257 (Gagnon et al., 2013) restored the synapse specificity of LTP in old mice, suggesting an age-dependent role of KCC2 membrane expression in LTP, with a possible impact on learning and memory that, however, was not

explored in this study. I will further address this point later in this introduction (see Introduction I.3.b).

2. KCC2 controls neuronal chloride homeostasis

a. KCC2 co-transport potassium and chloride : thermodynamic considerations

Under physiological conditions, KCC2 extrude chloride ions using the potassium gradient maintained by the Na/K ATPase. It is the only KCC working in isotonic conditions, thanks to its ISO domain (Acton et al., 2012). Since the reversal potential of both Cl^- and K^+ ions are equal, KCC2 operates close to its thermodynamic equilibrium (Payne, 1997). The reversal potential of an ion is calculated according to the Nernst equation:

$$E_{ion} = \frac{RT}{zF} * \ln \left(\frac{[ion]_i}{[ion]_o} \right)$$

where R represent the ideal gaz constant, T the temperature in Kelvin, F the Faraday constant and z the valence of the studied ion.

Hence, the equation $E_{\text{Cl}^-} = E_{\text{K}^+}$ is equivalent, after simplification, to $[\text{Cl}^-]_i * [\text{K}^+]_i = [\text{Cl}^-]_o * [\text{K}^+]_o$

Both extracellular chloride concentration and intracellular potassium concentration can be considered as fixed and close to 140 mM. Such approximations suggests that for $[\text{Cl}^-]_i$ higher than $[\text{K}^+]_o$, KCC2 extrudes both choride and potassium ions. Conversely, KCC2 will intrude ions when $[\text{K}^+]_o$ is higher than $[\text{Cl}^-]_i$. Physiological $[\text{Cl}^-]_i$ has been estimated to vary between 7 and 13 mM in a computational model (Doyon et al., 2011) while physiological $[\text{K}^+]_o$ ranges between 2 and 4 mM. However, intense neuronal activation induces a rise in $[\text{K}^+]_o$ up to 10 mM in cortical neurons (Avoli et al., 1996; Thompson et al., 1988). This would result in a change of ion transport directionality through KCC2.

Hence, KCC2 is in an interesting position to reduce excitability. In basal conditions, KCC2 will extrude chloride and potassium ions, restoring a low intraneuronal chloride concentration and preserving the inhibitory GABAergic signaling. However, following an

intense neuronal activation, reverse transport through KCC2 will cope with the increased potassium concentration in the extracellular compartment.

b. KCC2 controls the reversal potential of GABAAR mediated currents

- **Developmental switch in the polarity of GABA transmission**

A few years before KCC2 was identified (Payne et al., 1996), Ben-Ari and collaborators recorded spontaneous giant depolarizing potentials (GDPs) in rat immature CA3 neurons (Ben-Ari et al., 1989). These GDPs were observed in 85% pyramidal neurons during the first postnatal week, then their occurrence decreased and disappeared by post-natal day 12 (P12). These GDPs were dependent on GABA signaling as application of the GABAAR blocker bicuculline suppressed them, while GABA application increased their frequency and depolarized the membrane. Around P5, a switch in the polarity of GABA responses from depolarizing to hyperpolarizing occurs in hippocampal neurons. The precise timing of the switch was later refined and placed around P13 in the hippocampus (Khazipov et al., 2004).

As described previously, KCC2 up-regulation parallels the shift in the polarity of GABAAR mediated currents in rat forebrain (Rivera et al., 1999). Moreover, inhibition of KCC2 expression by incubation of slices with antisense oligonucleotides against KCC2 mRNA was sufficient to prevent this shift. Thus, GABA transmission depends on KCC2 expression which dynamically controls intraneuronal concentration. Several studies later revealed that the precise timing of KCC2 up-regulation differs depending on the brain region but is highly correlated with the timing of the shift in the polarity of GABAAR-mediated currents in each brain region (see Watanabe and Fukuda, 2015 for review).

As I will discuss later in this introduction, KCC2/NKCC1 expression ratio is altered in different disorders (see Introduction II) and delayed shift in the polarity of GABA transmission has been observed in two rodent models of autism (Tyzio et al., 2014) and more recently in a mouse model of Rett syndrome (Lozovaya et al., 2019). Hence, alterations of GABA signaling around birth might affect brain function and be responsible for at least some of the symptoms associated with these disorders.

- **Impact of KCC2 knockdown on GABAergic signaling in adulthood**

As observed during development, KCC2 expression controls the polarity of GABA signaling (Rivera et al., 1999). This suggests that in disorders associated with KCC2 down-regulation, GABA might become paradoxically excitatory. In line with this hypothesis, several studies reported a depolarized value of E_{GABA} upon KCC2 knockdown (e.g., Huberfeld et al., 2007; review in Côme et al., 2019) and computational models revealed that decreasing KCC2 activity may reduce synaptic inhibition (Doyon et al., 2011).

However, KCC2 interacts with protein partners, including some involved in the trafficking or recycling of various transmembrane proteins and receptors (Mahadevan et al., 2017). This suggests that KCC2 down-regulation might also affect other neuronal properties, including intrinsic neuronal properties. Indeed, our team recorded both E_{GABA} and the resting membrane potential (V_{rest}) in rat dentate granule cells and CA1 pyramidal neurons and reported that both values were similarly depolarized in neurons lacking KCC2, leaving the driving force of ion flux through GABAARs almost intact upon KCC2 knockdown (Goutierre et al., 2019). The driving force reflects the difference between V_{rest} and E_{GABA} and therefore determines the amplitude and polarity of GABAergic currents. Therefore, KCC2 knockdown has little effect on GABAergic signaling at rest but instead increases neuronal excitability through reduced membrane trafficking of the leak potassium channel Task-3 which normally interacts with KCC2.

Despite the importance of V_{rest} in setting the efficacy and polarity of GABA signaling, such a value is rarely reported in studies looking at the effect of KCC2 down-regulation or is not commented. Upon conditional ablation of KCC2, another team reported a depolarized value of V_{rest} compensating the shift in E_{GABA} in cerebellar granule cells but not Purkinje cells (Seja et al., 2012). This observation coincides with the fact that Purkinje cells express mainly Task-1 and Task-5 (Karschin et al., 2001) while cerebellar granule cells, like dentate granule cells and pyramidal CA1 neurons, express mainly Task-3 (Marinc et al., 2014). Altogether, these data suggest that KCC2 down-regulation might affect neuronal excitability and GABAergic signaling efficacy differently depending on the neuron types and brain regions.

Activation of GABAAR has been shown to lead to intra-neuronal accumulation of Cl^- associated with an increase in KCC2 activity in order to restore chloride homeostasis (Heubl et al., 2017; Viitanen et al., 2010). Hamidi and collaborators therefore used a protocol inducing epileptiform activities in the hippocampus to study the impact of KCC2 blocker

VU0240551 (Hamidi et al., 2015). Spontaneous interictal discharges can be induced in hippocampal slices by 4-AP bath application (Voskuyl and Albus, 1985) and two types of epileptiform activities can be recorded: short- and long-lasting events (Perreault and Avoli, 1991). Bath application of VU0240551 reduced the occurrence of short-lasting events and increased the interval of occurrence of long-lasting events (Hamidi et al., 2015), suggesting the mechanisms maintaining these two types of activity might be different. Indeed, blocking KCC2 might prevent the increase in $[K^+]_o$ resulting from intense GABAAR activation (Viitanen et al., 2010), thereby reducing cell excitability and long-lasting event frequency. Blocking KCC2 also increased the interval of occurrence of pharmacologically isolated GABAergic events and decreased their duration (Hamidi et al., 2015). This is presumably due to the fact that blocking KCC2 affects the intraneuronal concentration of Cl^- and therefore the efficacy of GABAergic signaling.

Altogether, these experiments suggest that even though chronic KCC2 down-regulation might not predominantly affect GABAergic signaling at rest, upon intense neuronal activation (for example during LTP induction or during a seizure) the lack of KCC2 might affect the efficacy and polarity of GABA transmission. Understanding whether KCC2 knockdown affects GABAergic signaling *in vivo*, and to what extent, is necessary to develop and validate therapeutic options for the pathology. Indeed, restoring chloride homeostasis with bumetanide might be of therapeutic interest (see Introduction II.4.b).

c. Osmotic regulation by KCC2

In addition to their critical role in controlling transmembrane ion gradients, CCCs also play an essential role in osmotic regulation. Thus, CCCs possess an important ion efflux or influx capacity and are used by many cells to adjust their internal osmolarity. Notably, KCCs were first described in epithelial and red blood cells as swelling-activated transporters, the activity of which was critical to face osmotic challenges (Dunham and Ellory, 1981; Lauf and Theg, 1980; Zeuthen and MacAulay, 2002). When cells are exposed to increased extracellular osmolarity, water molecules are moving outside of the cell, thus reducing cell volume and conversely. To compensate for osmolarity gradients, mechanisms are activated to reduce the movement of water molecules. As CCCs co-transport K^+ and Cl^- , ionic movements across the

cell membrane are indirectly or directly coupled to water transport (MacAulay and Zeuthen, 2010; Zeuthen and MacAulay, 2002). It has been estimated that up to 500 molecules of water may cross the membrane for each chloride ion transported by NKCC1 (MacAulay and Zeuthen, 2010).

In the CNS, postsynaptic ionotropic receptors lead to net ion influx into the postsynaptic neuron and water influx. Thus, intense neuronal activity directly impacts cell volume and may lead to cell swelling and cell death in extreme cases (Pasantes-Morales and Tuz, 2006). Since neurons lack aquaporins (Amiry-Moghaddam and Ottersen, 2003), other mechanisms may be necessary for coping with synaptic activity-induced swelling. Hence, digital holographic microscopy revealed an important water influx through NKCC1 and NMDARs following glutamate application. Furosemide, but not bumetanide application strongly reduced cell swelling, suggesting a role of KCC2 in water extrusion (Jourdain et al., 2011). In one study, KCC2 function was proven essential to neuronal survival following NMDA-induced excitotoxicity (Pellegrino et al., 2011). Although this latter study did not fully explore the mechanisms underlying such neuroprotective effect, it may involve the role of KCC2 in coping with activity-induced cell swelling. Moreover, other KCCs are activated during osmotic challenges and KCC3 and KCC4 participate to the water extrusion and recovery of cell volume (Boettger et al., 2003; Frenette- Cotton et al., 2018; Race et al., 1999).

In order to restore the neuronal osmolarity, the swelling-induced activity of KCC2 is most likely dependent on the inhibition of the WNK/SPAK/OSR1 pathway, which results in dephosphorylation of T906 and T1007 residues (Gagnon et al., 2006; de Los Heros et al., 2018; Rinehart et al., 2009). Conversely, extracellular hyperosmotic challenges activate the WNK/SPAK/OSR1 pathway, thus inhibiting KCC2 membrane expression. Notably, NKCC1 is also regulated by this pathway in the opposite direction (Kahle et al., 2010).

3. Emerging role of KCC2 in dendritic spines

In 2001, Gulyas et al reported for the first time KCC2 expression in dendritic spines, close to glutamatergic synapses in the hippocampus (Gulyás et al., 2001). Interestingly, they observed high level of KCC2 in dendritic spines and lower level in the shafts, hosting

GABAergic synapses. This observation suggested that KCC2 aggregates in dendritic spines and was later confirmed by our team (Gauvain et al., 2011b). This protein seems to be excluded from the post-synaptic density (PSD) as KCC2 does not colocalize with PSD-95 (Figure 4). However, the limitations of optical fluorescence imaging currently preclude further analysis of the relation between KCC2 and the PSD.

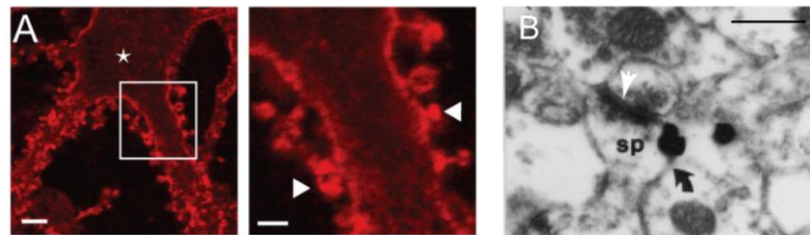


Figure 4. KCC2 is expressed in the vicinity of glutamatergic synapses

A. Maximum intensity projection of confocal optical sections showing KCC2 expression (red) is localized at the membrane in dendrites (left) and spines (right). Scale bar left 5 μm , and right 2 μm . **B.** Silver-intensified gold particle (black arrow) staining KCC2 are localized closed to the post-synaptic density (white arrow) in dendritic spines (sp) of pyramidal cells. Scale 0.6 μm .

(Adapted from Gauvain et al., 2011 and Gulyás et al., 2001)

Since KCC2 controls chloride homeostasis, its role in GABAergic signaling has been extensively studied in both normal and pathological conditions (Hübner et al., 2001; Tyzio et al., 2014). However, what could be the functional role of KCC2 at glutamatergic synapses?

a. KCC2 is necessary in spinogenesis

As we have seen previously, KCC2 expression increases after birth. To understand how this affects brain development, Li et al. took advantage of a KCC2 knockout mouse (Li et al., 2007). As these mice die shortly after birth, they used primary hippocampal neuronal cultures from embryos to study the dendritic morphology and synaptic function of these neurons. They observed long, abnormal dendritic protrusions and a decrease in the number of functional glutamatergic synapses in KCC2^{-/-} neurons. The abnormal dendritic morphology was confirmed in brain slices from P16 KCC2^{hypo/null} mice, a mouse model that still retains around 20% of KCC2 expression compared to WT mice (Li et al., 2007). These effects were independent of GABA signaling as blocking GABAARs had no impact on spine morphology.

However, over-expression of a transport-deficient KCC2 in KCC2^{-/-} neurons rescued normal spine morphology, suggesting a transport-independent mechanism. Moreover, transfecting WT neurons with KCC2-CTD, likely acting to disrupt the interaction of endogenous KCC2 with its protein partners, also altered dendritic morphology.

The authors then screened for proteins interacting with KCC2 in immunoprecipitation assays, and identified the actin-related protein 4.1N (Denker and Barber, 2002). KCC2 interacts with the FERM domain of 4.1N through its CTD (Li et al., 2007). Hence, the over-expression of FERM-4.1N to disrupt its interaction with KCC2 in WT neurons was sufficient to mimic the altered morphology observed upon KCC2 knockout. This study was the first to observe a transport-independent function of KCC2, involving an interaction with actin-related protein 4.1N. In this context, it is also striking that KCC2 expression increases concomitantly with the development of dendritic spines (Miller and Peters, 1981).

While KCC2 expression seems to be required for normal spinogenesis, precocious KCC2 over-expression, however, also perturbs dendritic development in a brain-region specific manner. Thus, in the hippocampus, premature KCC2 overexpression induces abnormal dendritic arborization with decreased dendritic length and branching (Cancedda et al., 2007). Instead, neurons from the somatosensory cortex show an increase in dendritic spine density (Fiumelli et al., 2013). In addition, these effects on dendritic spine development are dependent on its chloride transport function in the hippocampus, but not in the somatosensory cortex (Awad et al., 2018). Since KCC2 expression at the membrane is higher in hippocampal neurons compared to somatosensory neurons at P7, we might hypothesize different impact of KCC2 expression and function during development. This suggests caution is necessary before generalizing molecular mechanisms to different neurons and brain regions.

Interestingly, no change in synapse density was observed when KCC2 was suppressed in mature neurons, after the period of synaptogenesis. In these conditions, instead, dendritic spines exhibited larger heads, likely owing to altered water export (Gauvain et al., 2011). Indeed, this effect was due to KCC2 transport function and was mimicked by chronic exposure to the KCC2 antagonist VU0240551. Altogether, these data suggest that KCC2 plays a crucial role in spinogenesis during development but is not required for the structural maintenance of dendritic spines in mature neurons.

b. KCC2 affects LTP expression, independent of its ion transport function

The function of KCC2 in dendritic spines of mature neurons is not, however, limited to osmotic regulation of spine head volume. Although spine head volume usually positively correlates with quantal size (Ashby et al., 2006; Harris et al., 1992), KCC2 knockdown in mature neurons leads to both spine head enlargement and reduced mEPSC amplitude (Gauvain et al., 2011). This effect on quantal size was shown to be independent of KCC2 function but instead involve interaction of its CTD with intracellular partners, as it was mimicked by overexpressing KCC2-CTD. Single particle tracking experiments further revealed that KCC2-actin interaction, likely via 4.1N (Li et al., 2007) forms a molecular barriers that hinders the lateral diffusion of AMPA receptors within dendritic spines. Upon KCC2 extinction, perisynaptic receptors diffuse faster and escape dendritic spines, resulting in reduced postsynaptic clustering and reduced quantal size of glutamatergic currents (Gauvain et al., 2011).

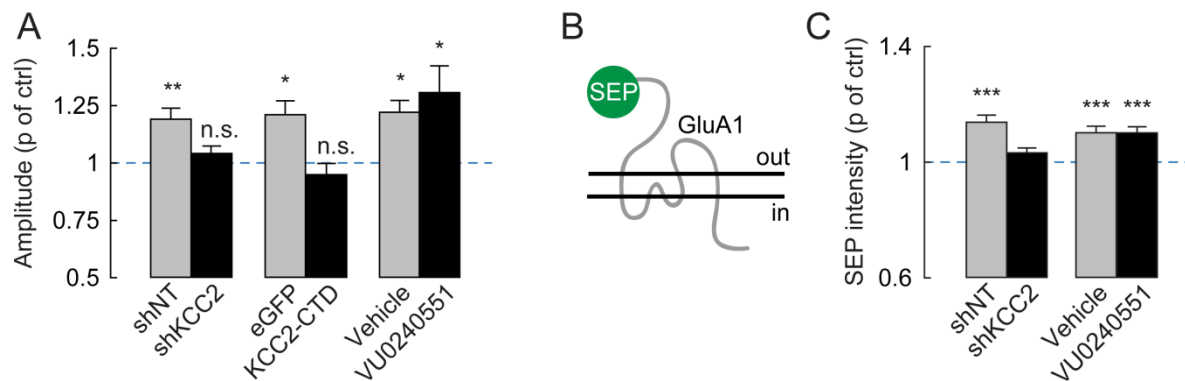


Figure 5. KCC2 is necessary for LTP and GluA1 activity-driven insertion at the membrane

A. Change of mEPSP amplitude upon chemical LTP (cLTP) protocol: neurons lacking KCC2 (shKCC2) or over-expressing its CTD (KCC2CTD) do not express LTP while blocking KCC2 transport function with VU0240551 has no effect. B. Neurons were transfected with GluA1-SEP, SEP fluorescence is only visible when the receptor subunit is expressed at the membrane. C. Upon cLTP induction, GluA1 is not transported to the membrane in the absence of KCC2. Treatment with VU0240551 has no effect on GluA1 trafficking.

(Adapted from Chevy et al., 2015)

Since lateral diffusion of AMPARs is also key to the expression of long term potentiation, promoting their diffusion by suppressing KCC2 expression may be expected to influence LTP expression. This hypothesis was then tested in our lab using RNA interference-based KCC2 knockdown both *in vivo* and *in vitro* (Figure 5). Chronic extinction of KCC2

expression in the rat dentate gyrus compromised LTP at perforant path synapses (Chevy et al., 2015). To test whether this effect was dependent on KCC2 transport function, chemical LTP (cLTP) was induced in the presence of the KCC2 antagonist VU0240551 in hippocampal neuronal cultures. In this condition, cLTP was not altered. However, KCC2-CTD over-expression again mimicked the effect of KCC2 knockdown, suggesting this effect on LTP was ion transport independent.

How may then KCC2 influence LTP at glutamatergic synapses? In the hippocampus, at least two different forms of LTP can be observed, as I will discuss later (see Introduction III.3.a). Here, I will focus on NMDAR-dependent LTP, as expressed at perforant path to granule cell and Schaffer collateral to CA1 synapses. Briefly, this form of LTP requires the exocytosis and trafficking of GluA1-containing AMPARs to the synapse upon NMDAR activation (Shi et al., 1999). Spine actin cytoskeleton is critically involved in AMPARs exocytosis (Gu et al., 2010; Kopec et al., 2007) and clustering (Allison et al., 1998). Actin microfilaments (F-actin) are highly dynamic, with an equilibrium between F-actin and G (globular)-actin, the monomeric form of actin. Following LTP induction, cofilin activity transiently increases, thereby inducing transient actin depolymerization acting to promote AMPAR exocytosis (Gu et al., 2010). Its subsequent inactivation by phosphorylation promotes actin polymerization which is necessary for spine growth, receptor anchoring and clustering (Borovac et al., 2018). Actin depolymerization/repolymerization therefore represents a critical step in the expression of LTP.

Upon KCC2 knockdown in hippocampal neurons, actin turnover was reduced (Llano et al., 2015) and F-actin content in dendritic spines was very significantly increased (Chevy et al., 2015). In the two studies, this effect was shown to i) reflect reduced cofilin activity due to its enhanced phosphorylation and ii) involve a previously unsuspected interaction between KCC2-CTD and the β isoform of Rac/Cdc42 guanine nucleotide exchange factor (β -PIX) (Chevy et al., 2015; Llano et al., 2015). β -PIX specifically activates the Rac1-PAK pathway (but not the RhoA pathway) (ten Klooster et al., 2006), converging onto the LIM kinase involved in cofilin phosphorylation (Llano et al., 2015; Van Troys et al., 2008). KCC2 knockdown may then promote β -PIX activity, leading to the over-activation of the Rac1-PAK-LIMK pathway. Thus, in neurons lacking KCC2, treatments to block this pathway restored activity-driven AMPAR insertion following cLTP (Chevy et al., 2015). These experiments suggest that KCC2 might serve as an anchor to keep β -PIX away from the PSD.

This would hinder the ability of β -PIX to form its signaling complex, by preventing its interaction with GIT1 and/or Shank in the PSD (Park et al., 2003; Zhang et al., 2003), whereas relieving β -PIX interaction with KCC2 may then promote its relocation to the PSD and activation of its downstream effectors (Figure 6). Alternatively, KCC2 might somehow alter β -PIX phosphorylation state (Saneyoshi et al., 2008).

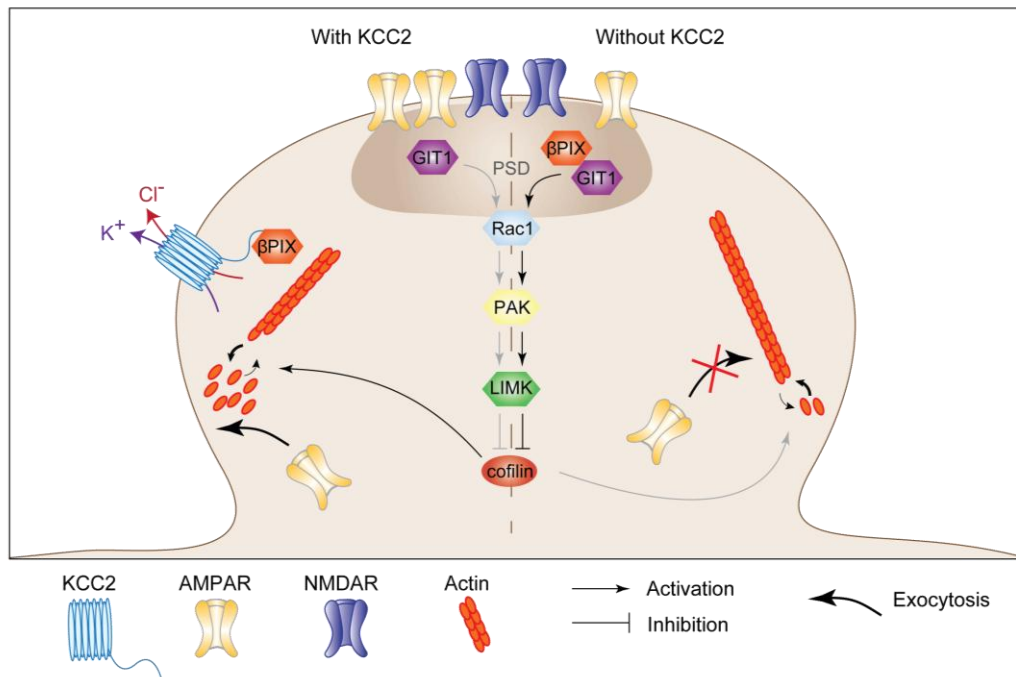


Figure 6. KCC2 expression controls cofilin activation

Our hypothesis is that KCC2 traps β PIX outside the PSD, thereby preventing activation of the GIT1-Rac1-PAK-LIMK pathway and subsequent cofilin inactivation. Upon high-frequency stimulation, actin transiently depolymerizes allowing GluR1-containing AMPARs trafficking. Without KCC2, cofilin activity is reduced and AMPARs exocytosis is prevented.

(Adapted from Chevy et al., 2015)

Since long-term potentiation is thought to represent the cellular substrate for learning and memory (Bliss and Collingridge, 1993; Nicoll, 2017; Teyler and Discenna, 1984), these observations predict KCC2 down-regulation, as observed in the pathology, may then result in cognitive deficits, independently of (or in addition to) alterations in GABA signaling. In support of this hypothesis, several studies have shown that blocking either the LIMK pathway (Lunardi et al., 2018), Rac1 expression (Haditsch et al., 2009) or GluA1-containing AMPARs insertion (Rumpel et al., 2005) alters hippocampal-dependent learning and memory. As we will see later, this was the rationale and central hypothesis of my PhD thesis.

II. KCC2 is down-regulated in neurological and psychiatric disorders

KCC2 membrane expression and function control both GABAergic and glutamatergic signaling. Thus, down-regulation of KCC2 expression or function affects intraneuronal chloride concentration and subsequently influences GABAergic signaling (Huberfeld et al., 2007; Pellegrino et al., 2011; Rivera et al., 1999). On the other hand, loss of KCC2 interaction with several protein partners also influence the efficacy and long term plasticity of glutamatergic synapses in particular through actin remodeling (Chevy et al., 2015).

KCC2 down-regulation has been observed in various neurological and psychiatric disorders such as epilepsy (Huberfeld et al., 2007; Palma et al., 2006), neuropathic pain (Coull et al., 2003), spinal cord injury (Boulenguez et al., 2010), brain trauma (Bonislawski et al., 2007), schizophrenia (Hyde et al., 2011a), autism (Tyzio et al., 2014) or Rett syndrome (Tang et al., 2016). Recent identification of KCC2 mutations, epigenetic dysregulation, abnormal mRNA or protein expression may help better understand how KCC2 dysfunction underlies the pathology and affects neuronal function thereby offering options for personalized therapeutic intervention.

In this section, I will explore abnormal KCC2 expression in humans and mouse models of neurological and psychiatric disorders. Most data from human brain come from post-mortem tissue, the integrity of which is necessary to study mRNA and protein expression. Indeed, some proteins are known to be degraded quickly after patients' death and it is argued that mRNAs start degrading very early, probably in the first hour post-mortem (Sidova et al., 2015). Brain banks collect information about pH, RNA integrity and post-mortem interval. However, none of these values correlate with the amount of synaptic proteins extracted from post-mortem samples (Bayés et al., 2014). Data from post-mortem human brains should therefore be taken with caution, as information provided in publications usually does not clearly indicate brain integrity. Comparing data across multiple studies both in human and mouse models may be most appropriate and informative.

1. KCC2 mutations in human pathology

Until five years ago, no mutation in the *SLC12A5* gene encoding KCC2 gene had been identified. But the rapid development of large-scale DNA sequencing methods have allowed scientists to explore genetic variation more rapidly and efficiently (Levy and Boone, 2019).

In 2014, two teams identified an Histidine-to-Arginine mutation (R952H) affecting the carboxyl-terminal domain of KCC2 in an Australian family with a history of febrile seizures (Puskarjov et al., 2014) and a Canadian family with idiopathic generalized epilepsy (Kahle et al., 2014). Then, in 2016, this mutation was also identified in schizophrenic and autism spectrum disorder (ASD) patients (Merner et al., 2015). The first study showed that HEK-293 cells over-expressing KCC2-R952H exhibit impaired neuronal Cl⁻ extrusion, reduced membrane expression and present altered dendritic spine formation/shape in hippocampal neurons (Puskarjov et al., 2014). These observations were confirmed by Kahle and collaborators, showing that reduced KCC2 membrane expression reflected impaired phosphorylation of serine 940, a key regulatory site for KCC2 stability (Kahle et al., 2014). S940 phosphorylation status was subsequently associated with epilepsy, as mice with a constitutively dephosphorylated S940 KCC2 (S940A mutant) exhibit more severe progression towards *status epilepticus* and death (Silayeva et al., 2015).

KCC2 mutations associated with the pathology are mainly located either (Figure 7):

- within the carboxyl-terminal domain (Kahle et al., 2014; Merner et al., 2015; Puskarjov et al., 2014), containing some of the major phosphorylation sites (S940, T906, T1007, Y903, Y1087) controlling KCC2 stability and function (Introduction I.1.b ;Côme et al., 2019), and the ISO domain (amino acid 1022-1037) required for KCC2 function under isotonic conditions (Acton et al., 2012),

- in the loop between the 5th and the 6th transmembrane segments (Saito et al., 2017; Saitsu et al., 2016; Stöberg et al., 2015), containing six identified glycosylation sites (Agez et al., 2017). Mutations in the vicinity of these regulatory sites could affect KCC2 trafficking, membrane stability and/or function through conformational changes, possibly affecting KCC2 interaction with other proteins. Indeed, HEK-293 cells over-expressing some of the mutations identified in patients with epilepsy of infancy with migrating focal seizures (KCC2-L311H, L426P or G551D) showed impaired glycosylation of KCC2 and reduced membrane expression (Stöberg et al., 2015). Since impaired glycosylation of KCC4 is known to affect its membrane trafficking (Weng et al., 2013), the same mechanism might underlie this

reduced membrane expression. However, no study so far has shown a direct link between KCC2 glycosylation and its trafficking to the membrane.

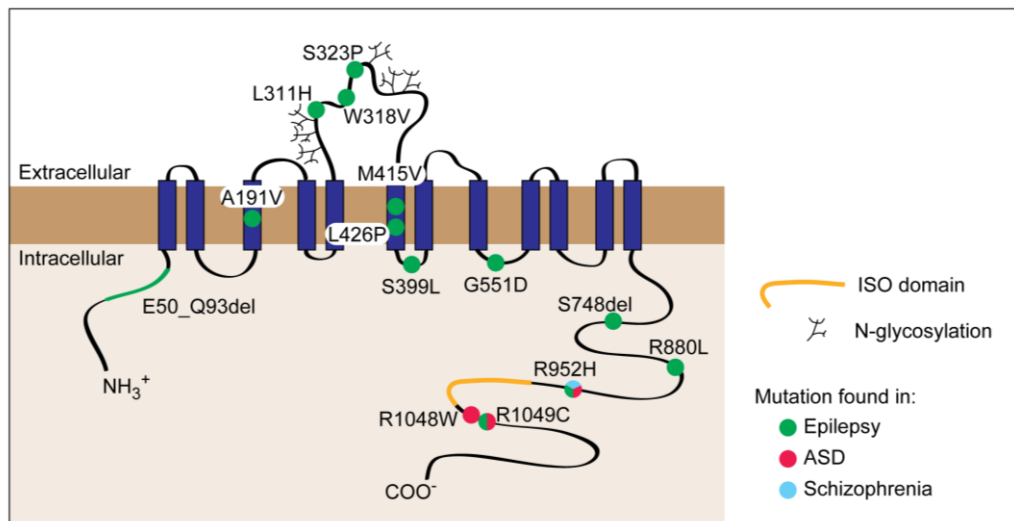


Figure 7. KCC2 mutations associated with epilepsy, autism or schizophrenia

Mutations in KCC2 have been associated with neurological and psychiatric disorders such as epilepsy, ASD or schizophrenia.

Just as the aforementioned KCC2-R952H mutation, other mutations such as R1049C were shown to have a similar impact on S940 phosphorylation (Kahle et al., 2014). This mutation, identified in patients with idiopathic generalized epilepsy was also found in patients with autism spectrum disorders (ASD; Merner et al., 2015). Merner et al. explored exome data from 2517 families in the ASD Simon Simplex Collection. They concluded that mutations in the CTD of KCC2 are more likely in ASD patients compared to controls. Moreover, these mutations tend to introduce or disrupt a CpG site. This could lead to epigenetic regulation of KCC2 expression that has not been fully studied yet.

Finally, *SLC12A5* has 26 exons and, due to alternative splicing, can generate different KCC2 mRNAs (Tao et al., 2012; Wang et al., 2008). Interestingly, two of these transcripts, EXON6B and AK098371, are respectively more expressed in the dorsolateral prefrontal cortex (DLPFC) of schizophrenic patients or reduced. AK098371 expression is also associated with one of the risk SNPs for schizophrenia (Tao et al., 2012). Both transcripts lack the PEST (proline, glutamic acid, serine, threonine) domains in the C-terminus, which is critical for KCC2 function in isotonic conditions (Mercado et al., 2006). This suggests these transcripts may play another role in neurons, rather than serving as a transporter.

2. KCC2 down-regulation in the pathology

For obvious reasons, the impact of KCC2 down-regulation in the pathology has mainly be considered with respect to its role in controlling chloride gradients and GABAergic signaling (Blaesse et al., 2009). However, as our group and others have shown, KCC2 down-regulation also alters the function and long term plasticity of glutamatergic synapses (Introduction I.3.b) (Chevy et al., 2015; Gauvain et al., 2011a) as well as intrinsic neuronal excitability (Goutierre et al., 2019) and it may be expected that these alterations also contribute to the pathology.

As alterations of glutamatergic or GABAergic signaling have been associated with LTP and rhythmogenesis deficits (Menendez de la Prida and Trevelyan, 2011; Rumpel et al., 2005; Viereckel et al., 2013), we hypothesize that KCC2 knockdown might affect cognitive function. Indeed, memory deficits are reported in several disorders associated with KCC2 down-regulation such as epilepsy (Gröticke et al., 2008; Thompson and Corcoran, 1992), autism (Bennetto et al., 1996), schizophrenia (Gur and Gur, 2013) or Rett Syndrome (Chahrour and Zoghbi, 2007). In addition, hypomorphic KCC2-deficient mice, which express KCC2 at levels only 15-20% of that in wild-type littermates, show spatial learning deficits in the Morris water maze (Tornberg et al., 2005).

a. Psychiatric and neurological disorders

Psychiatric disorders such as schizophrenia or autism spectrum disorders are considered as developmental disorders and synaptopathies (Grant, 2012). Indeed, most of the mutations associated with higher risk of developing these disorders affect genes encoding synaptic proteins (Bayés et al., 2011; Chen et al., 2014; Osimo et al., 2019). Moreover, even though symptoms may only appear later in the patient's life, neuronal and synaptic deficits may already be present in the first weeks of life (Courchesne et al., 2007; DeLisi, 1997).

In schizophrenia, studies in human patients looked for deficits in KCC2 or NKCC1 mRNA expression. In the hippocampus and prefrontal cortex, NKCC1/KCC2 mRNA ratio is increased compared to controls (Dean et al., 2016; Hyde et al., 2011b) while two distinct studies observed no such change in the dorsolateral prefrontal cortex (DLPFC) (Arion and

Lewis, 2011; Hyde et al., 2011b). However, several elements complicate the interpretation of these post-mortem data. First, the etiology of schizophrenia involves a constellation of cognitive, behavioral and emotional symptoms. Therefore, not all subjects in these studies may display the same symptoms. In addition, patients might have been on or off medication at the time of death, and this parameter was not accounted for in these early studies. This was, however, addressed in a more recent study showing stronger down-regulation of KCC2 protein in the DLPFC of off-medication schizophrenic patients (Sullivan et al., 2015).

Moreover, recent evidence suggests KCC2 function might be altered in schizophrenia. Indeed, in humans, OSR1 and WNK3 mRNA expression levels are increased in the DLPFC (Arion and Lewis, 2011). This is probably a downstream consequence of the disease, as monkeys treated with antipsychotics did not exhibit higher mRNA expression levels of OSR1 and WNK3. As discussed earlier (Introduction I.1.b), WNK3 and OSR1 kinases regulate neuronal chloride homeostasis through phosphorylation of both KCC2 and NKCC1 with opposing impact on their function (Kahle et al., 2005). Enhanced WNK3/OSR1 activity in the DLPFC of schizophrenic patients is then predicted to perturb chloride transport and, subsequently, affect GABAAR-mediated transmission.

KCC2 function depends on post-translational modifications (Côme et al., 2019, for review). Therefore, affecting its phosphorylation state and transport activity might be responsible for some cognitive deficits. Yang and collaborators took advantage of the SPAK^{-/-} mice, as phosphorylation by SPAK inhibits KCC2 function while activating NKCC1 (de Los Heros et al., 2014). In a post-weaning social isolation paradigm - a purely environmental model of schizophrenia (Jones et al., 2011) - wild-type mice failed to learn the novel object recognition task and showed KCC2 over-expression in the prefrontal cortex while the opposite was observed in the hippocampus (Yang et al., 2015). Interestingly, KCC2 expression can be rescued in the prefrontal cortex of SPAK^{-/-} mice following post-weaning social isolation (Yang et al., 2015) while novel object recognition was improved. This study therefore suggests that exploring only protein expression levels might not be sufficient to fully predict the underlying mechanisms of these disorders, and information about phosphorylation state may be critical in this respect.

However, it is unclear how long after death KCC2 phosphorylation remains stable. Indeed, phosphorylation of some protein may be stable only 10 minutes while that of others persists for hours (Oka et al., 2011; Wang et al., 2015). In this respect, studying the phosphorylation status of KCC2 in human brain might be proven difficult and animal models of disorders associated with KCC2 down-regulation might yield more information on the

exact mechanisms – whether KCC2 function is altered or its interaction with protein partners – and potential therapeutic targets.

In ASD, the perinatal and early postnatal shifts of GABAergic signaling polarity is thought to be altered (Tyzio et al., 2014). Indeed, in the hippocampus of two different rodent models of autism (fragile X syndrome mice and rats exposed to valproate *in utero*), KCC2 is down-regulated during the first month of life compared to control animals and bath application of the GABAAR agonist isoguvacine leads to a paradoxical increase in neuronal firing. In these models, blocking NKCC1 function using bumetanide in pregnant dams was able to rescue neuronal excitability but also rhythmogenesis and behavior in the offspring (Tyzio et al., 2014).

However, another study on fragile X syndrome mice failed to observe a decrease in KCC2 expression at P15 but showed enhanced NKCC1 expression in the somatosensory cortex (He et al., 2014). This result again highlights the fact that KCC2 and NKCC1 expression may vary in opposite directions in distinct brain subregions, potentially contributing to promote intracellular chloride accumulation and dampening GABA signaling.

Other neurological disorders are associated with KCC2 down-regulation. Indeed, **Huntington disease** mouse models R6/2 and YAC128 show increased NKCC1/KCC2 ratio associated with excitatory GABA in the hippocampus (Dargaei et al., 2018). **Rett syndrome** patients and mouse models are also presenting KCC2 down-regulation. I will develop these results later in this introduction (Introduction II.3.b-c).

Finally, **stress** can affect KCC2 expression. In a model of early maternal separation, KCC2 up-regulation after birth is delayed (Furukawa et al., 2017). However another study on maternal separation reported on the contrary an increase in KCC2 expression in adult mice (Hu et al., 2017). In adult mice, repeated stress can lead to KCC2 down-regulation (Tsukahara et al., 2015, 2016). However, this later study used a stress protocol (forced water administration for 3 weeks with a gastric tube too big for the mouse) that was also probably painful and did not explore the impact of KCC2 down-regulation on GABAergic signaling. With a chronic restraint stress (30 min/day for 14 consecutive days) but not an acute stress, MacKenzie and Maguire observed a dephosphorylation of KCC2 on S940 residue that might explain KCC2 down-regulation (MacKenzie and Maguire, 2015). Moreover, a depolarizing shift of E_{GABA} was observed and intrinsic activity of CA1 pyramidal neurons was increased.

Chronic stress affects KCC2 expression and evidence suggests this might underlie increased seizure susceptibility in stressed patients and animals (MacKenzie and Maguire, 2015; Maguire, 2014; Matsumoto et al., 2003; Neugebauer et al., 1994). Therefore, more work is needed to fully understand the impact of stress on CCCs expression and its subsequent effect on behavior.

b. Epilepsy

Epilepsy is among the most frequent neurological disorders and is characterized by the occurrence of unusually unpredictable and unprovoked seizures that may remain focal or generalize to large cerebral regions. It is usually assumed that epilepsy reflects an imbalance between excitation and inhibition at the expense of the latter (Engel, 1996; Kaila et al., 2014). As KCC2 and NKCC1 expression at the neuronal membrane regulates intraneuronal chloride concentration, and therefore the polarity and efficacy of GABAAR-mediated transmission, their expression and role has been extensively studied in relation to epilepsy.

Temporal lobe epilepsy (TLE) is a form of epilepsy that often originates in the hippocampus proper or the subiculum and can be associated with hippocampal sclerosis (Engel, 2001; Lewis, 1999). As of today, around 30% of epileptic patients and up to 75% of mesial TLE patients are resistant to drug-treatment (Cockerell et al., 1995; Spencer, 2002). Therefore, understanding the etiology and underlying mechanisms of TLE is critical to help identify novel pharmacological targets. A large body of literature has addressed the expression of KCC2 and NKCC1 in the context of temporal lobe epilepsy.

Well before KCC2 mutations associated with epilepsy were discovered, a series of experiments on human hippocampal tissue resected from TLE patients shed new light on a possible role of KCC2 transport function in the context of epilepsy. Cohen and collaborators recorded synchronous discharges *in vitro* resembling the interictal events observed in the EEG of patients (Cohen et al., 2002). These synchronous events were suppressed by blockers of glutamatergic transmission, and more surprisingly, by GABAAR antagonists. Interestingly, some of the principal cells of the subiculum were excited during interictal events and displayed depolarizing responses to GABA. This led the authors to suggest that paradoxical excitatory GABA might participate to the interictal activity. In a follow-up study, Huberfeld and colleagues reported that around 30% of subicular pyramidal neurons were depolarized

during interictal events and this correlated with reduced or undetectable KCC2 expression (Huberfeld et al., 2007). To restore normal chloride concentration, NKCC1 antagonist bumetanide was applied in the bath while recording neuronal activity and this was sufficient to suppress interictal activity.

There is no clear consensus on KCC2 and NKCC1 protein expression in epileptic patients. In the subiculum, a fraction (20-30%) of pyramidal neurons have no detectable KCC2 expression at the mRNA or protein level (Huberfeld et al., 2007; Muñoz et al., 2007; Palma et al., 2006) and the NKCC1/ KCC2 ratio increases up to 40 times (Palma et al., 2006). However, in the hippocampus *proper*, KCC2 protein expression was reported constant (Muñoz et al., 2007) or slightly down-regulated, with NKCC1/KCC2 mRNA ratio increased only by a factor 2 or 3 (Palma et al., 2006). In contrast, a recent study reported an increase in KCC2 protein expression in non-sclerotic hippocampus and no change in sclerotic hippocampus compared to control (Karlócai et al., 2016). As other studies investigated KCC2 expression in heterogenous group of patients with and without sclerosis, it is possible that they might have missed this observation. Altogether, these data suggests KCC2 expression level in human TLE differs between subiculum and sclerotic or non-sclerotic hippocampus *proper*.

Are these changes in KCC2 expression a consequence or a cause of epilepsy? Some data clearly support the notion that KCC2 down-regulation occurs because of epileptic activity. Indeed, its expression is tightly regulated by neuronal activity (Introduction I.1.b). *In vitro* treatments aiming to increase synaptic activity in neuronal cultures reported reduced KCC2 clustering and membrane expression through dephosphorylation of S940 residue (Chamma et al., 2013; Lee et al., 2011). Moreover, KCC2 down-regulation has been observed in several experimental seizure models in mice (Karlócai et al., 2016; Li et al., 2008; Pathak et al., 2007; Rivera et al., 2002).

Other data support that KCC2 down-regulation or partial loss of function may promote seizures. Indeed, early KCC2 down-regulation might set the ground for development of epilepsy by weakening inhibitory transmission. As developed previously, KCC2 mutations are associated with epilepsy in human (Fukuda and Watanabe, 2019). Moreover, increased susceptibility to seizures is observed in a mouse model of hypomorphic KCC2 (with 80% reduction in expression) (Tornberg et al., 2005) or in mice expressing constitutively a

dephosphorylated S940 residue (Silayeva et al., 2015). Finally, in a recent study, the Moss lab induced full neuronal KCC2 genetic ablation by expressing the Cre-recombinase in the hippocampus of adult KCC2^{flox/flox} mice and reported the emergence of spontaneous seizures (Kelley et al., 2018). Another study using KCC2 knockdown by lentivirus-based RNA interference in juvenile rats also reported a facilitation of seizures (Chen et al., 2017). However, the latter conclusions need to be considered with caution, as they were based on only 3 KCC2 knockdown rats vs 2 controls. Moreover, the authors did not provide any video in support of spontaneous, Racine stage III seizures (defined by unilateral forelimb clonus). Instead, they reported a short (< 1 second) ‘abnormal’ activity in EEG recordings, whereas stage III seizures usually last several seconds.

Since KCC2 is down-regulated in the mouse model we used, we might expect the emergence of spontaneous seizures. However, as I will show you in the results, we never observed such phenotype or recorded abnormal neuronal activity.

c. KCC2 down-regulation in brain insults

KCC2 down-regulation is observed in pathologies following insults such as **traumatic brain injury** (Bonislowski et al., 2007; Shulga et al., 2008), **cerebral ischemia** (Jaenisch Nadine et al., 2010), **spinal cord injury**, and **in cerebral tissue surrounding glioma** (Pallud et al., 2014). It is interesting to note that most of these affections are also often associated with development of epilepsy (Kruitbosch et al., 2006; Lucke-Wold et al., 2015).

Spinal cord injury (SCI) results in chronic pain and spasticity affecting the sensory-motor system. In SCI mouse models, KCC2 down-regulation has been observed in motoneurons in the case of spasticity (Boulenguez et al., 2010) and in neurons of the lamina I of the superficial dorsal horn in the case of chronic pain (Coull et al., 2003), and appears to depend on activation of the BDNF/TrkB pathway (Boulenguez et al., 2010). A working hypothesis in neuropathic pain is the “gate control theory” (Price et al., 2005; Vinay and Jean-Xavier, 2008). Briefly, C-fibers are contacting neurons within the dorsal horn to conduct the pain signal. However, C-fibers terminals are also contacted by GABAergic interneurons relaying the tactile information from A-beta fibers, thus inhibiting C-fibers and preventing

transmission of the painful input. Upon KCC2 down-regulation following SCI, the inhibitory signalization is altered, leading to the activation of pain-conducting fibers and hyperalgesia.

3. Rett syndrome and KCC2

Rett syndrome (RTT) is a developmental disorder affecting primarily females with an incidence of 1/10000. Patients develop normally up to 6-18 months old, then start to exhibit motor deficits, loss of language, impaired social interaction, stereotypies, mental deterioration and seizures (Chahrour and Zoghbi, 2007).

a. MecP2 gene expression in RTT

In 1999, Huda Zoghbi's team identified the gene responsible for over 95% of RTT cases: the methyl CpG binding protein 2 (MecP2) (Amir et al., 1999). This protein is a transcription factor expressing a methyl-binding domain site (MBD), a transcriptional repression domain (TRD), two nuclear localization signals (NLS) and its C-terminal facilitates MecP2 interaction with DNA and nucleosomal proteins. Mutation spectrum is broad, which might explain the variety and severity of the symptoms (Allemang-Grand et al., 2017; Lyst and Bird, 2015). It is important to note that most of the mutations affect the NLS, MBD and TRD domains, while truncations may lead to loss of the C-terminal domain. In line with this observation, a mouse model retaining only the MBD and TRD domains (Δ NIC-MecP2) exhibit only a mild RTT-like phenotype (Tillotson et al., 2017). In some cases, a stop codon is introduced early in the gene, with a complete loss of MecP2 function (Chahrour and Zoghbi, 2007). Among these hundreds of mutations (Rettdbase, <http://mecp2.chw.edu.au/index.shtml>), eight 'hotspots' have been identified (encoding the amino acid substitution R106W, R133C, T158M, R168X, R255X, R270X, R294X and R306C) and represent more than 60% of documented cases (Figure 8. ; Neul et al., 2008).

MecP2 gene is expressed on the X chromosome, explaining why mainly females are affected by RTT. However, in rare cases, males can also exhibit RTT (Schönewolf- Greulich et al., 2019; Villard, 2007). They may have two X chromosomes and then exhibit classical RTT. If they also only have one X chromosome, then their life expectancy is reduced and

symptoms are usually more severe. For this reason, most of the research on RTT has been done on hemizygous males as they exhibit a stronger behavioral deficit. However, the best mouse model should be heterozygous females.

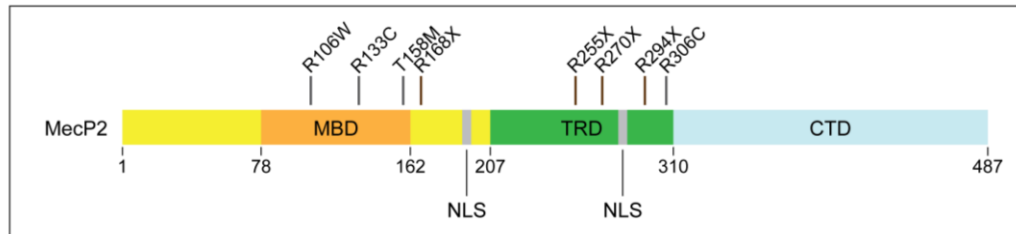


Figure 8. Schema of MecP2 protein and major mutations associated with RTT

Eight hotspots are associated with over 60% of documented RTT cases (Neul et al., 2008) and localized mainly in the MBD (methyl-binding domain) and TRD (transcriptional repression domain).

NLS: nuclear localization signals; CTD: C-terminal domain

b. Circuit dysfunction in RTT

Neurological symptoms in RTT involve circuit alterations and E/I balance deficits, due to MecP2 down-regulation or mutations. Different mouse models have been developed to study the impact of MecP2 suppression/mutations on motor and cognitive function. Interestingly, while suppressing MecP2 in all brain cells or just a neuronal subtype is associated with altered behavioral phenotype, all mouse models do not mimic RTT to the same extent (see Table 1). Indeed, suppressing MecP2 in interneurons (Vgat-MecP2^{y/-}) or in all neurons (MecP2^{y/-}) has similar impact, with animals exhibiting stereotypies, premature death, motor and cognitive deficits (Chao et al., 2010; Guy et al., 2001; Hao et al., 2015; Lu et al., 2016). In contrast, mice with MecP2 suppression in glutamatergic neurons only (VGlut2-MecP2^{y/-}) or in subsets of interneurons (Dlx5/6-MecP2^{y/-}, PV-MecP2^{y/-}, SOM-MecP2^{y/-}) only recapitulate some of the behavioral traits (Chao et al., 2010; Ito-Ishida et al., 2015; Meng et al., 2016).

Electrophysiological studies of RTT models remain partly contradictory, possibly due to the heterogeneity of brain regions that were recorded and the timing of the recordings with respect to the ontology of the disease (Shepherd and Katz, 2011). I have summarized data from these studies in the Table 1 but will focus on results obtained in the hippocampus of symptomatic mice in this thesis.

Table 1. RTT mouse models and associated behavioral and electrophysiological deficits

RTT mouse model	Behavior	Electrophysiology	Reference
MecP2 ^{y/-} (male)	- Locomotor deficits - Increased weight - Premature death	<u>Recordings in L5 pyramidal neurons in somatosensory cortex</u> - Reduced firing rate - Reduced spontaneous action potential firing - Normal intrinsic excitability - Reduced mEPSCs amplitude	(Dani et al., 2005; Guy et al., 2001)
		<u>Recordings in hippocampus</u> - Increased EPSC amplitude - Smaller paired-pulse facilitation - Increased AMPAR-NMDAR ratio - Reduced LTP	(Asaka et al., 2006; Li et al., 2016)
		<u>Recordings in L2/3 pyramidal neurons in visual cortex</u> - Reduced excitatory and inhibitory conductances - Depolarized E _{Cl} ⁻	(Banerjee et al., 2016)
	- Motor deficits - Hypoactive - Stereotypies - Reduced anxiety - Cognitive deficits (cued memory in fear conditioning, object and place recognition)		(Stearns et al., 2007)
<i>Mecp2</i> ^{flax/y} ; CreER ^{+/-} (suppression of MecP2 in adult mice)	- Similar phenotype to MecP2 ^{y/-}		(McGraw et al., 2011)
MecP2 ^{+/-} (female)	Deficits appear later than in males MecP2 ^{y/-} - Motor deficits - Hypoactive - Reduced anxiety - Cognitive deficits (fear conditioning, inhibitory avoidance, object and place recognition) - Increased weight	<u>Recordings in hippocampus</u> - Reduced LTP	(Guy et al., 2001, 2007; Samaco et al., 2013; Stearns et al., 2007)
	- Impaired contextual (fear conditioning) and spatial (water maze) memory	<u>Recordings in hippocampus</u> - Increased synchrony between CA1 neurons	(Hao et al., 2015; Lu et al., 2016)
MecP2 ^{+/-} + DBS in the fornix (female rescue model)	- Rescue all cognitive deficits observed before (contextual + spatial memory)	- Rescue CA1 hypersynchrony	(Hao et al., 2015; Lu et al., 2016)

VGlut2-MecP2 ^{y/-} (MecP2 KO in glutamatergic neurons)	<ul style="list-style-type: none"> - Tremors - Anxiety-like behavior (decreased in open field but increased in light/dark box) - Normal social interactions - Increased weight - Premature death 	<u>Recordings in L5 pyramidal neurons in somatosensory cortex</u> <ul style="list-style-type: none"> - Reduced spontaneous action potential firing - Normal intrinsic excitability <u>EEG:</u> <ul style="list-style-type: none"> - seizure-like discharges 	(Meng et al., 2016)
Viaat-MecP2 ^{y/-} (MecP2 KO in interneurons)	<ul style="list-style-type: none"> - Motor deficits - Stereotypies - Increased social interaction - Cognitive deficits (novel object recognition, water maze) - Premature death 	<u>Recordings in L2/3 pyramidal neurons in somatosensory cortex</u> <ul style="list-style-type: none"> - Reduced mIPSC amplitude - No change in mEPSC <u>EEG:</u> <ul style="list-style-type: none"> - Electrographic seizures <u>Recordings in hippocampus</u> <ul style="list-style-type: none"> - Reduced LTP 	(Chao et al., 2010)
Dlx5/6-MecP2 ^{y/-} (MecP2 KO in interneurons of forebrain)	<ul style="list-style-type: none"> - Motor deficits - Stereotypies - Increased social interaction 		(Chao et al., 2010)
PV-MecP2 ^{y/-}	<ul style="list-style-type: none"> - Motor deficits - Increased social interaction - Cognitive impairment (cued memory deficits) - Premature death 		(Ito-Ishida et al., 2015)
SOM-MecP2 ^{y/-}	<ul style="list-style-type: none"> - No motor deficits - Stereotypies - Spontaneous seizures - Premature death 		(Ito-Ishida et al., 2015)
ΔNIC-MecP2 (male, retaining only MBD and NID sites)	<p>Mild neurological phenotype from 1 year old</p> <ul style="list-style-type: none"> - Motor deficits - Stereotypies - Increased weight 		(Tillotson et al., 2017)
MecP2 ^{y/-} + ΔNIC (Rescue model)	<ul style="list-style-type: none"> - Reduced symptom severity - Increased life expectancy 		(Tillotson et al., 2017)
MecP2 ^{308/Y} (from 5 months old)	<ul style="list-style-type: none"> - Motor deficits - Decreased locomotor activity (dark phase) - Stereotypies - Increased anxiety - Cognitive deficits (fear conditioning, water maze) - Decreased social interaction 	<u>Recordings in CA1</u> <ul style="list-style-type: none"> - Reduced LTP and LTD - Decreased paired-pulse facilitation <u>Recordings in layer 2/3 of primary motor and sensory cortex</u> <ul style="list-style-type: none"> - Reduced LTP 	(De Filippis et al., 2010; Moretti et al., 2005, 2006)

Overall, neuronal activity increases in the hippocampus of RTT mice, with impaired synaptic inhibition (Calfa et al., 2011; Moretti et al., 2006; Zhang et al., 2008) and naive excitatory synapses onto pyramidal neurons showing features of potentiated connections: enhanced AMPAR content, higher surface levels of GluA1 at synapses, and larger dendritic spines (Li et al., 2016). Moreover, *MecP2^{y/-}* mice exhibit reduced hippocampal LTP (Asaka et al., 2006; Li et al., 2016) associated with a deficit in GluA1-containing AMPARs exocytosis (Li et al., 2016). A possible interpretation is that synapses are already saturated and cannot undergo further AMPAR enrichment.

Among the different RTT mouse models, one of them, *MecP2³⁰⁸* seemed of particular interest in my thesis. Indeed, these mice are expressing a truncated version of the MecP2 protein and symptomatic mice have a reduced LTP associated with cognitive deficits (Moretti et al., 2006). Moreover, they start to exhibit motor and cognitive alterations later than *MecP2^{y/-}* mice (5 months old), which is an advantage. Indeed *MecP2^{y/-}* die young (2 months old) and have a strong motor phenotype (Guy et al., 2001) that complicates exploratory behavioral experiments involving locomotion and exploration, such as place recognition task (see Introduction III.2.b).

c. KCC2 imbalance in RTT

Obviously, MecP2 has a wealth of potential downstream targets and regulates the expression of many genes that may in turn influence neuronal and synaptic functions (Chahrour et al., 2008). KCC2 down-regulation, however, was recently reported, first in human cerebrospinal fluid of RTT patients (Duarte et al., 2013) and later in patient-derived human induced pluripotent stem cells (iPSCs; Tang et al., 2016), mouse cortical neurons transfected with a shRNA against MecP2 or in male *MecP2^{y/-}* mice (Banerjee et al., 2016).

Pyramidal neurons from *MecP2^{-y}* mice (P20-P25) show reduced KCC2 expression and exhibit a more depolarized value of E_{GABA} compared to WT littermates, while their resting membrane potential is unaffected suggesting an excitatory GABAergic signaling (Banerjee et al., 2016). In order to understand when this alteration of KCC2 expression takes place, another study used iPSCs to mimic neuronal development. Indeed, in WT iPSCs, KCC2 expression increases while neurons mature *in vitro* (Tang et al., 2016). This correlates with a

progressive hyperpolarization of E_{GABA} (roughly, from -50 mV to -70 mV). However, in iPSCs from RTT patients, neither KCC2 expression nor E_{GABA} changed over time. These experiments suggest that MecP2 down-regulation affects KCC2 developmental up-regulation and, subsequently, the functional shift of GABA signaling.

Following this hypothesis, one would expect GABAergic transmission to be altered in RTT models. In the visual cortex of MecP2^{-y} mice, both excitatory and inhibitory synaptic conductance in pyramidal neurons were reduced (Banerjee et al., 2016). Neurons derived from RTT iPSCs show reduced frequency and amplitude of spontaneous excitatory and inhibitory synaptic currents compared to WT neurons (Marchetto et al., 2010). This reduced frequency could reflect a reduction in the density of release sites or decreased release probability. Indeed, these neurons exhibit fewer glutamatergic synapses. This effect is a consequence of MecP2 dysfunction as down-regulating MecP2 by RNA interference in WT neurons also affects the number of excitatory synapses (Marchetto et al., 2010). Unfortunately, no study so far has explored the impact on synapse stability of KCC2 over-expression in RTT iPSCs or mouse model. Since KCC2 was shown to be required for synapse maturation and early spinogenesis in cortical neurons (Li et al., 2007; Introduction I.3.a), one could hypothesize that synaptic deficits observed in RTT be due, at least in part, to KCC2 extinction.

d. MecP2, a regulator of KCC2 expression?

When MecP2 was first described in 1992 as a nuclear protein binding to a single methyl-CpG pair, the authors hypothesized a role in chromatin compaction (Lewis et al., 1992). However, a few years later, they observed that the effect of MecP2 on transcription was not a consequence of changes in chromatin structure *per se*, and identified a transcriptional repressor domain (TRD) able to interfere with other components of the transcription machinery. Interestingly, they also observed an up-regulation of gene expression when the unmethylated CpG motifs were present, even though they did not comment this result much (Nan et al., 1997). Then, several studies explored its role as a transcriptional repressor and identified different protein complexes associated to MecP2 (Lunyak et al., 2002; Nan et al., 1998).

However, MecP2 function is even more complex. Indeed, while studying gene expression in the hypothalamus of two mouse models - one lacking MecP2 and the other one over-expressing the protein - Charhour and colleagues identified that over 80% of the genes controlled by MecP2 were activated. Among them, transcription of *SLC12A5*, which encodes KCC2, showed a 30% increase when MecP2 was over-expressed compared to WT and a 12% decrease when MecP2 was suppressed. Today, MecP2 is considered to have different transcriptional effects and its exact function may depend on molecular complexes it is engaged in. Multiple roles have been reported, including that as a transcriptional repressor, activator or its involvement in micro-RNA processing (Ip et al., 2018).

Then, how is MecP2 regulating KCC2 expression? As discussed earlier (Introduction II.d), KCC2 transcription can be regulated through four sites: two upstream and intronic RE-1 binding sites for REST, one binding site for Egr4 and an E-box controlling element (Markkanen et al., 2008; Uvarov et al., 2006; Yeo et al., 2009). Interestingly, in the brain of RTT patients and MecP2-deficient mice (heterozygous females or hemizygous males), REST and CoREST mRNA and protein levels are up-regulated (Abuhatzira et al., 2007), while KCC2 is down-regulated. REST is known to act as a repressor of KCC2 expression (Yeo et al., 2009). Indeed, its over-expression in cortical neurons further represses KCC2 transcription and increases intraneuronal chloride concentration while a REST dominant-negative peptide rescues KCC2 transcription (Yeo et al., 2009). Since KCC2 promoter exhibits CpG motifs (Uvarov et al., 2007), REST can recruit CoREST and MecP2 (Lunyak et al., 2002) and this REST-CoREST-MecP2 complex interacts with RE-1 sites of KCC2 (Yeo et al., 2009). Interestingly, MecP2 also controls REST expression, by binding its promoter either directly or through the interaction with other, not yet identified proteins to control their expression (Abuhatzira et al., 2007).

In conclusion (Figure 9), MecP2 could regulate KCC2 expression in adulthood by controlling REST and Co-REST expression. As over-expressing or suppressing MecP2 respectively increases or decreases *SLC12A5* gene expression (Chahrour et al., 2008), it is also probable that MecP2 either acts as an activator of KCC2 transcription or competes with REST by blocking its interaction with RE-1 sites. We will see later in my work that KCC2 is not down-regulated in all RTT mouse models, suggesting that distinct mutations of MecP2 might not affect its activator/repressor activity in the same way. This may explain the diversity and differential severity of RTT in patients bearing distinct MecP2 mutations.

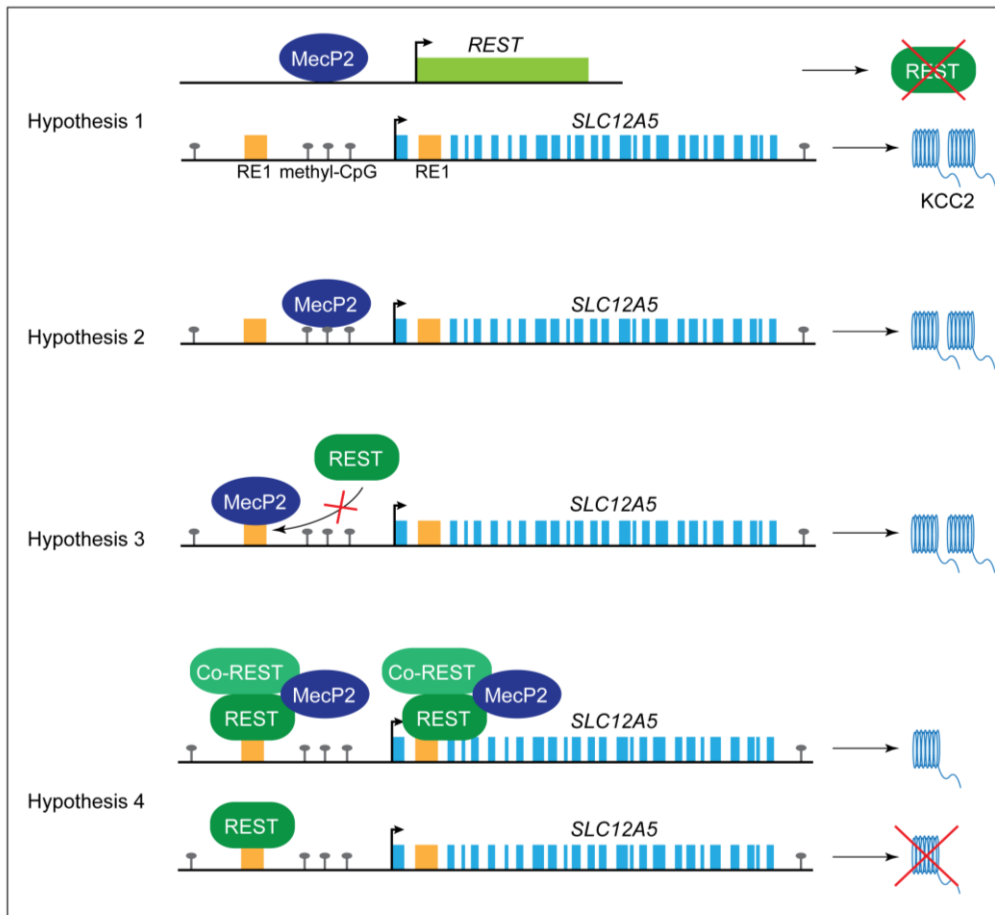


Figure 9. Hypotheses on MecP2 role in KCC2 transcription regulation

Different hypotheses might explain how MecP2 act as an activator of KCC2. Note that these hypotheses are not mutually exclusive. Hypothesis 1: MecP2 suppresses REST transcription allowing *SLC12A5* transcription. Hypothesis 2: MecP2 recognizes methyl-CpG domains and activate *SLC12A5* transcription. Hypothesis 3: MecP2 is in competition with REST to interact on RE1 sites. Hypothesis 4: the transcriptional complex REST-CoREST-MecP2 allows moderate *SLC12A5* transcription while REST alone completely abolishes it.

4. Therapeutic strategies

Two main strategies have so far been considered in order to rescue altered neuronal chloride homeostasis in the pathology. One first approach consists in using the NKCC1 antagonist bumetanide to try and prevent chloride import and compensate for the loss of KCC2 function. However, this strategy may only restore GABAergic signaling. As discussed in section 1 of this introduction, KCC2 down-regulation affects not only GABA signaling through impaired chloride transport but also the formation, function and plasticity of

glutamatergic synapses through interactions with spine actin cytoskeleton (Chevy et al., 2015; Gauvain et al., 2011a; Li et al., 2007) as well as membrane excitability through regulation of leak potassium conductance (Goutierre et al., 2019), respectively. Therefore, another strategy aiming to restore KCC2 function in the pathology may then rescue a variety of synaptic and intrinsic neuronal membrane properties beyond the mere control of chloride transport.

In this thesis, as I was exploring the consequences of KCC2 down-regulation on learning and memory in wild-type animals as well as in a model of RTT, I considered which strategy would be most appropriate to rescue memory deficits in conditions associated with reduced KCC2 expression. I will now discuss and compare these different approaches.

a. KCC2 over-expression

Whereas many studies explored the consequences of KCC2 down-regulation in the pathology, what is the impact of its over-expression and could it represent a therapeutic option?

Over-expression of KCC2 in the mouse adult brain in CaMKII-expressing neurons in cortex, amygdala and hippocampus does not seem to affect health, locomotor activity or anxiety (Goulton et al., 2018; Nakamura et al., 2019). In the motor cortex, however, this caused an increase in spine density and mice performed better in an accelerating rotarod, suggesting a role of KCC2 expression level in motor learning (Nakamura et al., 2019). In addition, over-expressing KCC2 may have a neuroprotective effect against seizures *in vitro* and upon kainate or pilocarpine-induced seizures *in vivo*, but not after PTZ injection (Goulton et al., 2018; Magloire et al., 2019). These studies suggest that KCC2 over-expression in principal cells may protect against epilepsy and improve motor learning. Whether such approach could be translated into the clinic using gene therapy after the diagnosis of seizures or psychiatric disorders remains unclear.

As discussed above, following spinal cord injury, KCC2 is down-regulated, which results in interneuron hyper-excitability. Using an AAV-PHP.B virus that crosses the blood-brain barrier, Chen and colleagues recently re-expressed KCC2 in neurons of a partially lesioned spinal cord and observed motor improvement of the mice. Moreover, re-expressing KCC2 or the inhibitory DREADD hM4Di only in interneurons was sufficient to mimic this

effect. These treatments helped rescuing the functional state of the circuit, facilitating the motor command and coordination (Chen et al., 2018).

This last study suggests the possibility of at least partially rescuing a pathological phenotype using KCC2 re-expression by gene therapy. However, more work is needed to improve gene therapy safety and to develop viruses crossing the blood-brain barrier (BBB; Joshi et al., 2017). Finding drugs that could affect KCC2 expression, function or restore chloride homeostasis would probably be a more suitable and readily applicable option.

b. Restoring chloride concentration with bumetanide

Many studies explored the benefits of the NKCC1 antagonist bumetanide in disorders associated with KCC2 down-regulation with the aim to restore intraneuronal chloride concentration (Kharod et al., 2019; Figure 10). Indeed, bumetanide is an FDA-approved loop diuretic, already available for human use, and shows a high affinity for NKCC1 as compared with other CCCs.

In vitro, bumetanide application rescues E_{GABA} in mouse models of autism (Tyzio et al., 2014), Down syndrome (Deidda et al., 2015) and Rett syndrome (Banerjee et al., 2016) and also decreases the interictal activity on human epileptogenic tissue (Huberfeld et al., 2007; Pallud et al., 2014; Palma et al., 2006).

In vivo, combined bumetanide and phenobarbital i.p injections in rodent epilepsy models reduced the frequency and duration of seizures (Brandt et al., 2010; Rahmanzadeh et al., 2018). In female rats treated with valproate and Fmr1 KO mice, bumetanide treatment before birth reduced the behavioral deficits of the autistic pups and the spontaneous activity in *in vitro* electrophysiological recordings (Tyzio et al., 2014). However, maternal care was not assessed in this study and it is well known that WT pups of Fmr1^{+/-} females exhibit social deficits (Zupan et al., 2016). Therefore, it is possible that the behavioral alterations might be due partly to better maternal care and/or less anxious mother.

Interestingly, bumetanide increases LTP and behavioral performances in a mouse model of Down syndrome in which NKCC1 is up-regulated while KCC2 expression remains unchanged (Deidda et al., 2015). Moreover, mice performed in the same way whether they

received the drug chronically or acutely. Therefore, experiments aiming to test the effect of bumetanide on a mouse model of pathology might only require acute treatment.

Bumetanide is currently envisaged as a treatment for schizophrenia (Lemonnier et al., 2016; Rahmanzadeh et al., 2017), autism (Hadjikhani et al., 2018; Lemonnier et al., 2012, 2017) or epilepsy (Eftekhari et al., 2013; Pressler et al., 2015) and a phase-III clinical trial is currently underway to evaluate the potential benefits of a liquid form of bumetanide in young children with ASD (<https://clinicaltrials.gov/ct2/show/NCT03715153>).

However, clinical use of bumetanide in brain disorders raises several problems, in particular regarding its bio-availability. First, bumetanide is >98% bound to plasma proteins and its ability to cross the BBB has been questioned (Römermann et al., 2017). Indeed, even though bumetanide could partially enter the brain, its concentration would likely not be high enough to act on NKCC1. To overcome this issue, prodrugs have been developed (Töllner et al., 2014) and more work is underway to facilitate the BBB-crossing of drugs. In addition, NKCC1 is widely expressed in many organs, therefore, a treatment with bumetanide could have multiple side effects. Indeed, the NEMO trial aiming to treat seizures in newborns with hypoxic ischaemic encephalopathy was stopped following hearing loss in 3/11 infants (Pressler et al., 2015). In other clinical trials for autism or schizophrenia with children of 3 years old minimum, hearing loss was not reported while an occasional hypokalaemia was observed (Lemonnier et al., 2017) and treated with oral potassium supplements.

c. Acting on KCC2 stability and/or expression

As discussed above, KCC2 dysfunction may be due to genetic mutation or activity-dependent alterations of its function, trafficking and/or recycling. Since KCC2 is involved in a variety of molecular interactions and affect both GABAergic and glutamatergic signaling as well as neuronal excitability (Introduction I.2-3), restoring chloride homeostasis exclusively might not be sufficient to fully compensate for KCC2 down-regulation. Hence, new compounds acting to promote KCC2 function, stability and/or expression represent a particularly promising direction.

Through a currently unknown mechanism, **Insulin-Growth Factor 1 (IGF1)** treatment *in vitro* and *in vivo* facilitate the developmental GABAergic switch from excitatory to inhibitory (Baroncelli et al., 2017; Kelsch et al., 2001). Interestingly, IGF1 treatment has a strong impact on RTT iPSCs and MecP2^{-y} mice brain. Thus, MecP2^{-y} mice treated with IGF1 exhibit behavioral ameliorations with increased lifespan and respiratory function as well as improved locomotor activity. IGF1 also restores the density of glutamatergic synapses and dendritic spine (Marchetto et al., 2010). Moreover, in RTT iPSCs (Tang et al., 2016) as in MecP2^{-y} mice (Banerjee et al., 2016), treatment with IGF1 restores KCC2 developmental up-regulation and normal E_{GABA}. Whether IGF1 may have similar effects in other disorders associated with KCC2-down-regulation remains to be explored.

As discussed earlier (Introduction I.1.b, Figure 2), KCC2 membrane stability and function rely on phosphorylation of key residues, mostly in its carboxy-terminal domain. Thus, phosphorylation of S940 enhances while that of T906 and T1007 reduces KCC2 membrane stability and function (Chamma et al., 2013; Heubl et al., 2017; Lee et al., 2007). Treatments could therefore be developed to target specifically the pathways acting on KCC2 phosphorylation status. A mouse model with T906/T1007 mutated to alanine in order to prevent their phosphorylation are more resistant to kainate-induced seizures *in vivo* (Moore et al., 2018). In addition, these mutations lead to a hyperpolarized E_{GABA}, reflecting decreased intraneuronal chloride concentration. Similarly, **N-Ethylmaleimide (NEM)** has been shown to act on WNK/SPAK pathway and enhance KCC2 membrane stability in neurons by promoting S940 phosphorylation and T1007 dephosphorylation (Conway et al., 2017).

Other compounds have recently been identified by screening of molecular libraries and suggested to act as KCC2 enhancers, even though their mode of action remains unknown. For instance, **CLP-257** was shown to specifically enhance KCC2 function (as compared with other CCCs) and membrane expression and to restore Cl⁻ transport both in spinal cord neurons treated with BDNF and in rats with neuropathic pain upon peripheral nerve injury, two models associated with reduced KCC2 activity and expression (Gagnon et al., 2013). *In vivo*, however, CLP-257 has a poor half-life and a pro-drug, **CLP-290**, was therefore developed and increased analgesia in rats with peripheral nerve injury (Gagnon et al., 2013; Figure 10). In a rat model of neuropathic pain, morphine treatment induces side effects such as morphine-induced hyperalgesia, associated with KCC2 down-regulation and Cl⁻ extrusion deficits in spinal cord neurons. *In vivo*, treatment with CLP-290 reduced these specific side effects

(Ferrini et al., 2017). In another study on spinal cord injury, treatment with CLP-290 improved motor phenotype, but only after 7 weeks and the effect was abolished when treatment was interrupted (Chen et al., 2018). This suggests the necessity of a long treatment with CLP-290 in order to observe an improved phenotype and should be taken into account while designing experiments.

While the mechanism of action of CLP drugs on KCC2 remains unknown, their target itself was recently questioned by another team suggesting that CLP-257 may not even act directly on KCC2. This team failed to reproduce part of the results of Gagnon and colleagues and instead suggested that CLP-257 may act to potentiate GABA_ARs (Cardarelli et al., 2017). However, as discussed by Gagnon and colleagues, it is here important to note that most of the experiments were done using different techniques, models and experimental protocols (Gagnon et al., 2017). This issue therefore remains controversial.

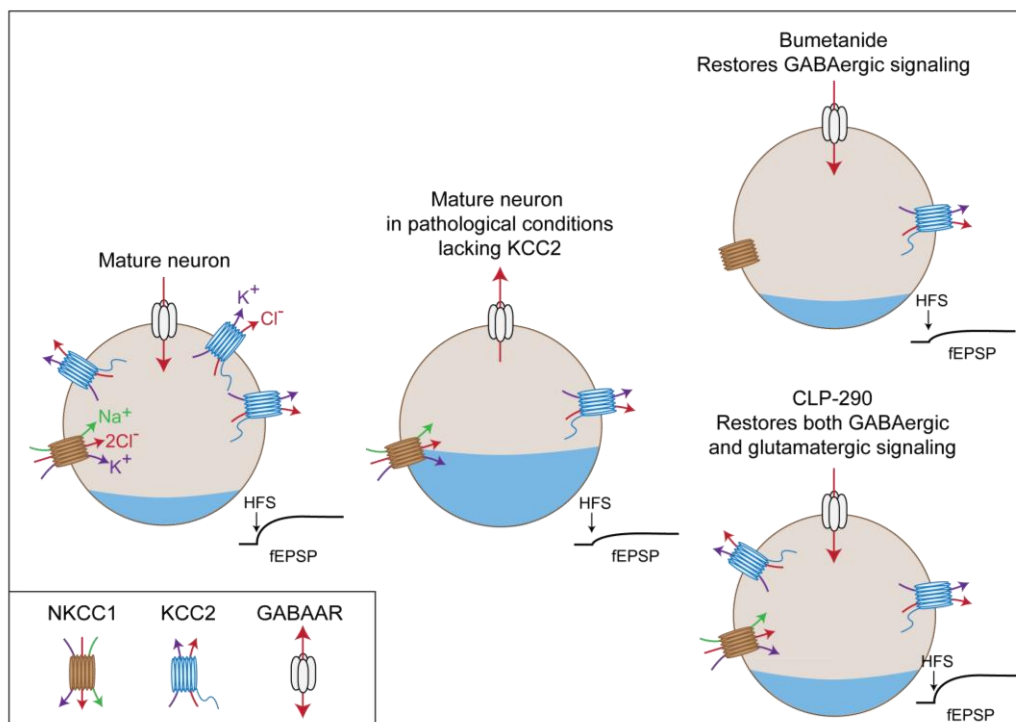


Figure 10. Different therapeutic options to rescue deficits following KCC2 down-regulation

Mature neurons exhibit a low intraneuronal concentration (in blue). In neurons lacking KCC2, high intracellular chloride concentration might affect GABAergic signaling. In addition, the efficacy and plasticity of glutamatergic synapses is also impaired in neurons with reduced KCC2 expression. Whereas bumetanide may only rescue GABA signaling, CLP-290 on the contrary may rescue both GABAergic and/or glutamatergic signaling.

It should be noted that most KCC2 enhancers discussed so far have their flaws. Thus, CLP-290 is extremely difficult to prepare for systemic injection and is unstable. It therefore needs to be prepared fresh daily. From my own experience, the drug may be altered if the temperature of the bath used to sonicate and dissolve the drug increases or if the pH exceeds 5-6. Its mechanism of action is still unknown and possible side effects have not yet been fully addressed. On the other hand, NEM is not specific of KCC2 and acts on different CCCs (Gamba, 2005) and a variety of other targets. Similarly, IGF1 acts on signaling pathways involved in numerous cells functions, such as MAPK and PI3K/pAKT pathways (Laviola et al., 2007), somewhat complicating its clinical use. However, one of the advantages of IGF1 is that it can cross the blood brain barrier (Baker et al., 2005) and is FDA approved. A clinical trial is currently underway to study the effect of trofinetide (an IGF1 analog) in RTT patients. Phase II results show an improvement of repetitive behaviors, respiratory deficits, some motor deficits, social interactions and seizures (Glaze et al., 2019).

As discussed previously, evidence suggests that KCC2 knockdown might affect learning and memory, which is the central hypothesis of my work. KCC2 down-regulation is a common alteration in many disorders associated with cognitive deficits and its effect on the excitation/inhibition balance has been extensively studied. However, KCC2 expression in neurons varies depending on the brain region and a pathology, such as RTT, does not seem to affect all neurons in the same extent. Therefore, I choose to focus on the hippocampus and explored the impact of KCC2 down-regulation in different neuronal subtypes. Moreover, treatments aiming at restoring KCC2 membrane stability or chloride homeostasis are two options that are currently explored and clinical trials are currently ongoing.

III. The hippocampus, a key structure in learning and memory

During the 16th-century Renaissance, cities in the north of Italy were leading the advances in medicine and surgery. In particular, Julius Caesar Arantius (1530-1589), a surgeon at Bologna University, focused on human anatomy. His dedication to the anatomy of the brain led him to distinguish different structures, such as the hippocampus. At the time, names were usually related to zoology or mythology and this might have influenced Arantius. Indeed, *hippocampus* in Latin, corresponds to seahorses and was first used in 1587 in *De Humano Foetu Liber*. However, if Arantius is considered the discoverer of the hippocampus, the structure he described was not yet the full hippocampus we know today (Bir et al., 2015). Other scholars suggested different names for the hippocampus. For instance, French anatomist de Garengeot, suggested *cornu Ammonis* (1742), related to the Egyptian god Amun, represented with ram's horn (Iniesta, 2014; Pearce, 2001). In 1750, French anatomist Pierre Tarin distinguished the *dentate gyrus* from the rest of the hippocampus (Bir et al., 2015). Finally, Camillo Golgi introduced a technique allowing the observation of the brain structure that was later modified by Ramon y Cajal (Swanson, 1999). This great advancement led to a better understanding of the hippocampal structure that was described by Lorente de No, Cajal's student, in 1934 (Lorente De Nó, 1934).

Patients with amnesia following brain trauma were the first to provide insights into the cerebral structures involved in learning and memory. When talking about the hippocampus, Henry Molaison, better known as H.M patient, is famous (Scoville and Milner, 1957). In 1953, his surgeon removed both temporal lobes to treat his drug-resistant epilepsy. Following this surgery, he suffered from severe anterograde amnesia. By comparing his amnesia with that of other patients, Scoville and Milner concluded on the importance of the hippocampus in declarative long-term memory, including semantic and episodic memory (for a review on H.M, see Corkin, 2002).

Since then, decades of research on the role of the hippocampus in learning and memory and the advancement in experimental technology has allowed dissecting the hippocampal circuits to understand their involvement in different memory tasks.

1. Overview of the hippocampal structure and circuits

a. Anatomy of the hippocampus

The hippocampus is a bilateral cortical structure present in humans in the temporal lobes. Despite the difference of localization in rodents and humans, studies have shown a similar connectivity and anatomy of this structure (Bergmann et al., 2016) even though it is still important to keep in mind that some differences can exist between species (Clark and Squire, 2013; van Groen et al., 2002).

It is composed of two interlocked folds of cortex, the *cornu Ammonis* (CA) subdivided into areas CA1, CA2 and CA3, and the dentate gyrus (DG). Although the terminology varies among scientists, the hippocampus proper usually refers to these four sub-regions. In a broader version, the hippocampus also includes the subiculum, the pre- and para-subiculum and the entorhinal cortex (EC) (Figure 11). Contrary to the cortex, composed of 6 layers, the hippocampus exhibits an alignment of the cell bodies of principal cells in one thin layer. Since most of my PhD work was performed on the hippocampus proper, I will focus on the anatomy and connectivity of this region, specifically on the dorsal hippocampus.

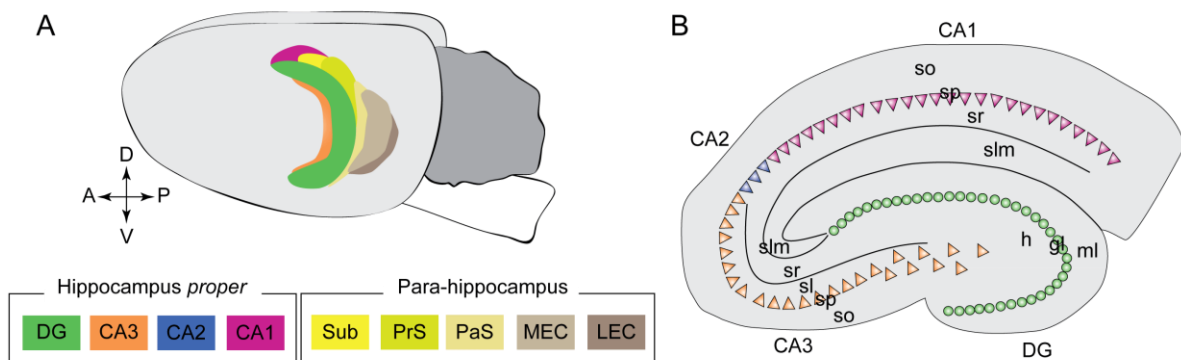


Figure 11. Anatomy of the hippocampus

A. Lateral view of the hippocampus anatomy in the rodent brain consisting of the hippocampus proper and the para-hippocampus.

(Sub: subiculum, PrS: pre-subiculum, PaS: para-subiculum, MEC-LEC: medial and lateral enthorinal cortex)

B. Schema of the hippocampus with the different layers and fields.

(so: *stratum oriens*, sp: *stratum pyramidale*, sl: *stratum lucidum*, sr: *stratum radiatum*, slm: *stratum lacunosum moleculare*, ml: *molecular layer*, gl: *granular layer*, h: hilus)

A cross section of the **CA areas** highlights the different strata (Figure 11). Principal cells are called pyramidal neurons due to the shape of their somata and are localized in the *stratum pyramidale*. They are extending their basal dendrites and axons in the deep external *stratum oriens* and their apical dendrites through *stratum radiatum* then *stratum lacunosum moleculare* on the other side. The lamination of the hippocampus is roughly the same along the CA3 to CA1 axis, except for an additional *stratum lucidum* above the pyramidal layer in CA3.

In rats, a single pyramidal cell in CA1 receive ~30,000 excitatory and ~1700 inhibitory inputs, mainly localized in the *stratum pyramidale* (40%) (Megías et al., 2001). Interestingly, in C57Bl/6J mice, the length of dendrites and approximative density of synapses is similar to those in the rat. As the hippocampus is smaller in size, this could be due to fewer neurons rather than smaller neurons (Routh et al., 2009).

One might assume hippocampal excitatory neurons from one sub-region are a uniform population. However, recent evidences challenge this view. Indeed, pyramidal neurons in CA1 can be subdivided into deep and superficial neurons based on anatomical and functional differences, with deep neurons more likely to be place cells (Cembrowski et al., 2016; Mizuseki et al., 2011).

The **dentate gyrus** has a U-shape, with granule cells somata densely packed in the *granular layer* while their spiny dendrites are dispersed in the superficial *molecular layer* and their axons run through the hilus (Figure 11). Other excitatory neurons are located in the hilus: the mossy cells (Fujise and Kosaka, 1999). Their role is unclear, as they can activate both granule cells and interneurons (Hsu et al., 2016; Scharfman, 1995). They discharge at theta frequency, which suggests a role in phase-locking granule cells activity and some studies reported their involvement in different behaviors (Bui et al., 2018; Scharfman, 2016; Soltesz et al., 1993).

In rodents, the DG is one of the three regions with the subventricular zone and the olfactory bulb to undergo neurogenesis (Altman and Das, 1965). These past two years, the presence of neurogenesis in humans has been debated as Sorrells et al. failed to detect hippocampal neurogenesis in adult brains in 2018 (Sorrells et al., 2018). However, another group identified newborn cells in adults this year and argued the failed attempt to identify these neurons can be explained with a poor human brain preservation or prolonged/uncontrolled fixation (Moreno-Jiménez et al., 2019; Steiner et al., 2019).

The newborn granule cells are localized at the limit of the hilus and migrate into the *granular layer* as they mature (Toni and Schinder, 2015). These neurons exhibit an enhanced synaptic plasticity during the first 4-6 weeks of their life: (1) it is easier to induce LTP in these neurons, (2) their membrane properties are different, allowing them to be more excitable than mature neurons and respond to sparse glutamatergic synaptic inputs (Ge et al., 2007; Schmidt-Hieber et al., 2004). Young neurons are thought to be necessary in memory processing (Deng et al., 2010) as they are more likely to be recruited into spatial memory networks by the age of 4 weeks than mature neurons (Kee et al., 2007).

While 80-90% of hippocampal neurons are principal cells, the remaining 10-20% are interneurons. GABAergic interneurons are localized throughout the different strata of the hippocampus. They are extremely diverse with respect to their morphology, connectivity, gene expression or physiology, which complicates their classification (for a review, see Booker and Vida, 2018). CA1 itself hosts over 20 different interneurons subtypes (Klausberger and Somogyi, 2008) and this number keeps increasing as experimental techniques are evolving.

Interneurons can be divided in three main groups, depending on their post-synaptic targets. Principal cells receive perisomatic inhibition from parvalbumin-containing (PV) and cholecystokin-containing (CCK) basket cells but also from axo-axonic interneurons contacting the axonal intermediate segment (AIS). While perisomatic interneurons make up approximately 50% of all interneurons, dendritic-contacting interneurons, such as somatostatin-containing interneurons (SOM) are the most diverse group (Klausberger and Somogyi, 2008). Finally, some interneurons, such as vasoactive intestinal peptide (VIP) positive interneurons specifically contact other GABAergic cells.

b. The hippocampal tri-synaptic loop

The hippocampal circuit has first been described as a unidirectional tri-synaptic glutamatergic circuit (Andersen et al., 1971). As some specific species differences in connectivity have been observed (van Groen et al., 2002), I will try to focus on circuits that have been observed in mice (Figure 12).

The main hippocampal input arises from neurons of layer II, and to a lesser extent from the deep layers IV-VI, of the enthorinal cortex (EC). They send their projections to the granule cells of the dentate gyrus (DG) through the perforant path (PP) (Witter, 2007). The axons of the granule cells, called the mossy fibers (MF), are contacting pyramidal cells of CA3 in the *stratum lucidum* to form the second excitatory synapse (Henze et al., 2000; Ishizuka et al., 1990). Then, CA3 neurons project onto proximal dendrites in *stratum radiatum* of CA1 neurons through their Schaffer collaterals (SC). Finally, the main output pathway of the hippocampus corresponds to CA1 pyramidal cells contacting the layer V of the EC, either directly or through the subiculum (Amaral et al., 1991; Naber et al., 2001). Layer V neurons are in turn the major output connecting other cortical structures (Insausti et al., 1997). Moreover, within the EC, a loop controls the information processed in the hippocampus as layer V neurons also contact layers II-III (Ohara et al., 2018).

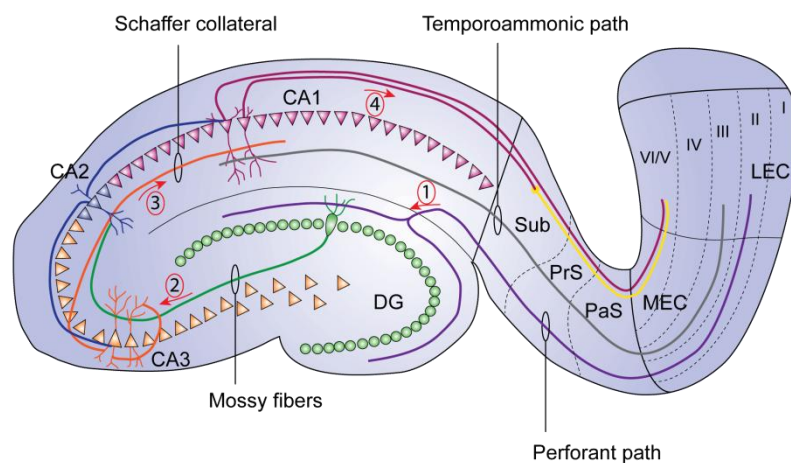


Figure 12. Intra-hippocampic connectivity

The main hippocampic connectivity corresponds to the tri-synaptic pathway: 1. Granule cells receive an afferent from the EC through the perforant path, 2. Mossy fibers contact CA3 neurons 3. CA3 neurons send their Schaffer collaterals to synapse onto CA1 neurons. 4. CA1 neurons are the main output of the hippocampus, contacting the subiculum and EC.

CA3 neurons are also establishing recurrent collaterals, CA2 neurons contact CA3 and CA1 neurons and receive afferents from the EC (establishing a di-synaptic pathway : EC-CA2-CA1). CA1 neurons also receives a direct input from the EC through the temporoammonic path.

c. Hippocampal connectivity, a more complete view

However, as research evolved, with new tracing methods, it quickly became apparent that hippocampal connectivity was more complex (Amaral and Witter, 1989; Sloviter and Lømo, 2012). Thus, why is this tri-synaptic loop simplistic?

First of all, the intra-hippocampal circuit is more complex than a single tri-synaptic loop. Indeed, CA2, described by Lorente de No in 1934 (Lorente De Nó, 1934) is not included. CA2 neurons receive a direct input in the *stratum lacunosum moleculare* from the EC layer II and project onto CA1 pyramidal neurons, forming a di-synaptic hippocampal circuit (Chevalyere and Siegelbaum, 2010; Cui et al., 2013). CA2 pyramidal neurons also receive direct inputs from DG granule cells, CA3 and CA2 and send projections to areas CA1, CA3 and EC layer II (Cui et al., 2013; Kohara et al., 2014; Rowland et al., 2013). Interestingly, CA2 acts very strongly onto CA1 neurons, probably influencing the hippocampal output, while the CA3 to CA2 input is dominated by a strong feed-forward inhibition (Chevalyere and Siegelbaum, 2010; Cui et al., 2013). This suggests CA2 is part of two supplementary excitatory loops: DG-CA2-CA1 and EC-CA2-CA1.

Among other intra-hippocampic circuits, CA3 has a specific connectivity. First of all, MF axons are innervating more interneurons whose dendrites are located in the *stratum lucidum* than excitatory neurons (Acsády et al., 1998). Then, CA3 pyramidal neurons can form reciprocal connections with other CA3 pyramidal neurons (Le Duigou et al., 2014; MacVicar and Dudek, 1980). This forms an associative network with some single cells able to recruit large ensembles of neurons and involved in the generation of sharp-wave ripples (Csicsvari et al., 2000).

As seen previously, 10-20% of hippocampal cells are interneurons and can act on principal cells through different circuits. Two main types of inhibition can be observed based on connectivity (Kullmann, 2011). On one hand, excitatory afferents recruit interneurons, acting on downstream targets. This feed-forward inhibition is considered essential to prevent runaway propagation of excitatory information and to precisely control spike timing. On the other hand, feedback inhibition refers to local recurrent network between principal cells and interneurons. This represents one mechanism to synchronize cells and control rhythmic activity involved in cognitive function (see Introduction III.3.b).

Second, considering the EC as a single entity is also a mis-representation of the circuit. In 1909, Brodmann sub-divided the cerebral cortex into 52 distinct regions based on their cytoarchitecture (Brodmann, 1909). The entorhinal cortex was defined as areas 28a and 28b. Indeed, more research confirmed this structure can be divided in two parts: the medial (MEC) and the lateral entorhinal cortex (LEC) (Witter et al., 2017). Entorhinal cortex does not only contact the DG and its projections can be species specific (van Groen et al., 2002). Projections from the layer III in C57Bl6/J mice also contact CA3 and CA1 neurons in *stratum lacunosum moleculare* through the temporoammonic path (van Groen et al., 2003; Witter et al., 1988). However, in rats and monkeys, CA3 inputs come from the EC layer II, and in some extent from the layer III in rats at least, while CA1 inputs originates from layer III (Tamamaki and Nojyo, 1993; Witter, 2007). These projections are spatially organized, as neurons originating from the medial part of LEM and MEC innervate the dorsal hippocampus, whereas the ventral hippocampus receives inputs from the lateral part of the EC (van Groen et al., 2003; Naber et al., 2001).

Third, because all neurons from a single area are not identical, their connectivity can change. In CA1, deep neurons receive inputs from the MEC and CA2, while LEC projects to superficial neurons in CA1 (Kohara et al., 2014; Masurkar et al., 2017). Newborn neurons in DG are sending their axons to CA3 (Sun et al., 2013; Toni et al., 2008) and receive afferents from interneurons, mossy cells, CA3, LEC (Vivar et al., 2012).

Furthermore, the hippocampus is a large structure that can be subdivided in dorsal and ventral regions. These regions do not receive the same inputs and have been involved in different cognitive function that will be developed later (see Introduction III.2) (Fanselow and Dong, 2010).

Finally, the hippocampus is not a brain region isolated with only one single input and output through the EC (Figure 13). Indeed, the hippocampus receives cholinergic inputs from the diagonal band of Broca (DBB) and the septum (Frotscher and Léránth, 1985; Teles-Grilo Ruivo and Mellor, 2013). These two structures are also sending long range projecting GABAergic neurons on hippocampal interneurons (Freund and Antal, 1988), and glutamatergic neurons onto pyramidal neurons (Colom et al., 2005; Hajszan et al., 2004; Huh et al., 2010; Müller and Remy, 2018). Two hypothalamic nucleus also contact the hippocampus: the supramammillary nucleus (SuM) can co-release glutamate and GABA onto DG cells (Haglund et al., 1984; Hashimoto et al., 2018) and vasopressinergic neurons of

the paraventricular nucleus (PVN) specifically contact CA2 neurons (Cui et al., 2013). Other afferences include a direct glutamatergic connection from the amygdala to the ventral CA1 (McDonald and Mott, 2017), noradrenergic inputs from locus coeruleus (Jones and Moore, 1977), serotonergic inputs from dorsal and median raphe (Conrad et al., 1974; Tanaka et al., 2012), dopaminergic inputs from the VTA (Scatton et al., 1980) and the nucleus accumbens (Verney et al., 1985) towards the ventral hippocampus and from the locus coeruleus towards the dorsal hippocampus (Smith and Greene, 2012).

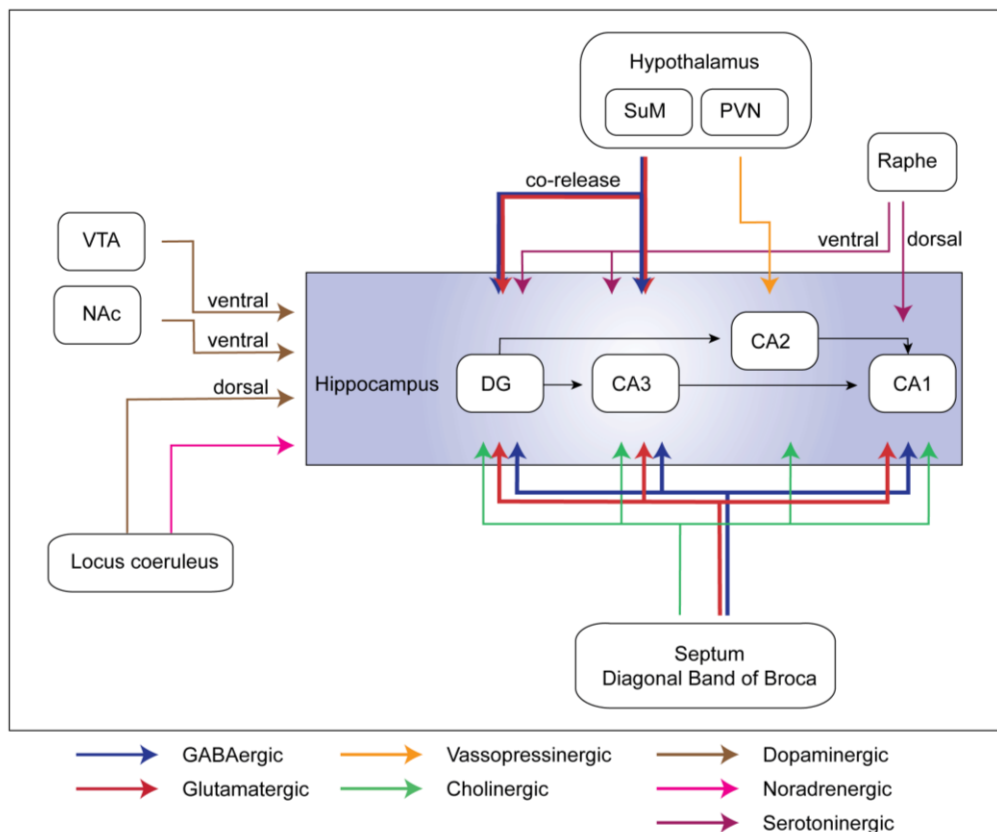


Figure 13. Extra-hippocampic inputs

The hippocampus receives inputs from different brain regions. I highlighted the neurotransmitter released by these different projections. Note that some projections are known to contact specifically one hippocampal subregion and/or ventral vs dorsal hippocampus.

SuM: supramammillary nucleus, PVN: paraventricular nucleus, VTA: ventral tegmental area, NAc: nucleus accumbens

2. Hippocampus-dependent learning and memory

The hippocampus is a key structure for spatial and non-spatial forms of declarative memory, which corresponds to the ability to remember places, people and events (Eichenbaum, 2004; Tulving and Donaldson, 1972). It is considered as a cognitive map (see discussion in Lisman et al., 2017) with neurons encoding spatial information (O'Keefe, 1976) and the timing of events (Agster et al., 2002; Eichenbaum, 2017).

During my PhD, I explored the role of KCC2 down-regulation in the dorsal hippocampus and will therefore focus on this subregion here. Indeed, the hippocampus is not a uniform structure and can be subdivided into dorsal, intermediate and ventral zone (Fanselow and Dong, 2010; Moser and Moser, 1998). Neurons within these regions differ in gene expression (Dong et al., 2009; Thompson et al., 2008) and extra-, intra-hippocampic connectivity (Swanson and Cowan, 1977). The dorsal hippocampus has mainly been associated with cognitive function while the ventral hippocampus relates to emotion and stress. Indeed, lesions of the dorsal hippocampus impair spatial learning and fear contextual memory (Broadbent et al., 2004; Moser et al., 1995; Phillips and LeDoux, 1992). The importance of dorsal hippocampus in spatial memory correlates with a higher number of place cells with better spatial selectivity than in the ventral hippocampus (Jung et al., 1994). On the other hand, lesions of the ventral hippocampus tend to reduce fear cued memory in the fear conditioning paradigm (Maren and Holt, 2004). As the ventral hippocampus interacts with the amygdala (McDonald and Mott, 2017), it is possible that the transfer of information between both structures is necessary for cued memory.

a. Hippocampal networks underlying spatial and contextual memories

Contextual and spatial memories rely on different hippocampal processing. Both require the formation of a spatial map allowing self-representation within the environment. Moreover, specific mechanisms allow pattern separation - in order to discriminate between different environments - and pattern completion - in order to navigate with only a set of partial cues (Kesner et al., 2016; Leutgeb and Leutgeb, 2007).

In 1971, John O'Keefe and Jonathan Dostrovsky identified neurons within the hippocampus, the 'place cells', that fire when the animal occupies a restricted location in the environment called 'place field' (O'Keefe and Dostrovsky, 1971). When the animal is moving, it crosses different place fields and the corresponding place cells firing are coding the ongoing trajectory. Place cells are activated when the animal entered a new environment and their specificity stabilizes with time (Frank et al., 2004; Hill, 1978; Wilson and McNaughton, 1993). Some place fields can be stable for days (Muller et al., 1987) while others are not maintained for more than a day (Ziv et al., 2013). As blocking NMDARs impairs place cell stabilization (Kentros et al., 1998), it is possible that long term plasticity mechanisms, such as LTP (Cobar et al., 2017), are necessary to form a spatial memory. Moreover, as I will describe later, when an animal explores its environment, a theta rhythm is generated and place cells firing is compressed to fit within theta oscillations (see Introduction III.3.b) (O'Keefe and Recce, 1993). This participates in the encoding and then to consolidation during sleep (Buzsáki, 2002a).

The ability to detect changes in space or time, which is necessary to discriminate between two similar contexts, may rely on pattern separation process performed by the DG (Kassab and Alexandre, 2018; Senzai, 2019). There are a few physiological properties that might explain why the DG can act as a pattern separator. Granule cells are less excitable as their resting membrane potential is lower compared to neurons from other brain regions (Fricke and Prince, 1984). Moreover, there are no recurrent collateral between granule cells. Altogether, granule cells display sparse activity (Jung and McNaughton, 1993) with just a few neurons recruited to form a memory (Liu et al., 2012).

Lesions of the DG have demonstrated the importance of this structure to discriminate between two similar objects (Gilbert et al., 2001) or detect novelty in an environment (Hunsaker et al., 2008). More recently, optogenetic tools have been used to manipulate precisely the neurons forming the memory for a context and showed that DG is necessary for contextual memory acquisition (Bernier et al., 2017; Kheirbek et al., 2013). During learning, a subset of neurons are recruited to form the engram in the DG and reactivation of this trace is sufficient to generate a fear response (Liu et al., 2012). However, inhibition of the DG during memory retrieval does not affect the fear response, except when the animal needs to identify the right context, for example when a similar context was presented to the animal before learning (Bernier et al., 2017). Altogether, these results suggest that DG is necessary during retrieval for its pattern separation ability and that other neuronal regions also encode the

contextual memory (Bernier et al., 2017; Madroñal et al., 2016). Indeed, it is known that memory trace in the DG is transferred to – at least – the medial prefrontal cortex (mPFC) (Kitamura et al., 2017) after a few days post-conditioning, which might explain why some studies were not able to observe memory retrieval deficits following DG inhibition (Madroñal et al., 2016).

The DG is the main first input to the hippocampus, which might explain its crucial role in contextual memory acquisition. However, experiments aiming to block CA3 and CA1 also affect memory acquisition and retrieval (Daumas et al., 2005), probably by blocking the transfer of information outside of the hippocampus. Moreover, CA3 and CA1 are necessary for the generation of different rhythms such as theta and sharp-wave ripples, involved in encoding and consolidation (Buzsáki, 2002a, 2015) of spatial and contextual information (Boyce et al., 2016; Girardeau et al., 2009; Nakashiba et al., 2009). The mechanisms will be developed later in this introduction (see Introduction III.3.b).

If you have been walking in a city during winter when it is snowing, and then in the summer, you know you can find your way even though visual cues are not exactly the same. This ability to retrieve memory with an incomplete set of information is called pattern completion. In the Morris water maze, suppression of NMDARs in CA3 does not affect the acquisition and memory retrieval of the mice (Nakazawa et al., 2002). However, the animals cannot find the platform upon partial cue removal. It has been hypothesized that CA3, through its recurrent collaterals, reiteratively processes highly convergent information from DG and EC to stabilize it (Leutgeb and Leutgeb, 2007).

b. Spatial and contextual behavioral paradigms

The purpose of memory is to guide our future behavior and we rely on contextual information such as time, space or internal state to help discriminate between similar situations (for a review, see: Stark et al., 2018). Over the years, bilateral lesions in humans and rodents hippocampus have been associated with spatial and episodic memory deficits (Morris et al., 1982; Scoville and Milner, 1957). Thus, different tasks have been developed in rodents to study these memories.

- **Spatial memory paradigms**

1. Spatial memory has been tested in mazes. In ‘dry’ mazes, rodents have to learn a route allowing them to receive a reward (radial arm maze: Olton et al., 1979) or find a refuge to avoid an aversive situation (Barnes maze: Barnes, 1979). Alternatively, in the Morris water maze, rodents are using extra-maze cues to navigate into a pool and find the hidden platform (Morris, 1984). On the test day, the platform is removed and the time spent in each quadrant is recorded. This task allows the distinction between learning deficits (how long does the animal need to learn to find the platform) and memory deficits (how long is the animal trying to find the platform in the quadrant it was localized previously). This task has been extensively used in the literature. Indeed, it avoids problem with ‘dry’ mazes such as olfactory cues and does not require for the animals to be food deprived. However, mice, contrary to rats, do not like swimming which can induce some unexpected behavior such as passive strategy (extensive floating) or thigmotaxis (time spent near the wall) (Sousa et al., 2006; Wolfer et al., 1998).

2. Place recognition task (Vogel-Ciernia and Wood, 2014) is probably more ethologically relevant than water maze as it is based on the natural ability of the animals to explore their environment and is less stressful (Sousa et al., 2006). Rodents are allowed to explore two identical objects in an arena with visual cues. Then, either a few minutes (short-term memory) or hours/day later (long-term memory) the animal is placed in the same arena with one of the objects moved to a new location that he will explore more than the unmoved object. Indeed rodents have a preference for novelty.

- **Contextual memory paradigms**

1. One very common experimental design is the contextual fear memory paradigm (Curzon et al., 2009; Izquierdo et al., 2016). The animal is introduced in a chamber and receives a foot shock associated with a cue (tone, flash of light). On the next day, when reintroduced in this chamber, the animal exhibits a *freezing* behavior, i.e. a complete absence of movement, considered as the behavioral response showing the animal remembers the association between the chamber and the stimulus. Then, the animal is placed in a new chamber, which he can explore before the cue is presented. However, cued memory has been

associated with the amygdala while dorsal hippocampal is associated with contextual memory (Phillips and LeDoux, 1992).

2. Fear memory has also been used in the inhibitory avoidance paradigm (Izquierdo et al., 2016). The animal learns that one context is associated with an aversive stimulus and also that this experience is contingent on the subject's choice to move towards this context. Indeed, the animal can explore two chambers, one brightly lit and the other devoid of light. The animal will have a tendency to move towards the dark room, however, it will then receive a foot shock. On the next day, the animals tends to avoid the chamber associated with the stimulus (Jarvik and Kopp, 1967). Experiments aiming to lesion the dorsal hippocampus or to block LTP have been shown to impair the memory retention (Izquierdo et al., 2006; Mitsushima et al., 2011).

3. Some tasks are relying on 'what-when-where' information (Ergorul and Eichenbaum, 2004; Kart-Teke et al., 2006). The context therefore requires the spatiotemporal integration of information. In this paradigm, the animal has to remember the order of appearance ('when') of an odor or an object ('what') as well as the location in the arena ('where'). Different tasks exist and need to be modified depending on the rodents used. Indeed, rats are able to learn faster a more complex task than mice. Moreover, such experiments may require the use of a reward.

4. The object-in-context paradigm uses the association between an object and a specific context (Dix and Aggleton, 1999; Langston and Wood, 2010). The animal must discriminate between two familiar objects, one that was presented in the same context while the other was presented in a different context.

Among these different paradigms, I decided to use the fear conditioning paradigm in my experiments. Indeed, this task requires the involvement of two brain regions: the hippocampus and the amygdala. As I down-regulated KCC2 only in the dorsal hippocampus while the amygdala was left intact, I was expecting to observe contextual but not cued memory deficits. On the contrary, the inhibitory avoidance paradigm does not allow the formation of two distinct memories that can easily be tested. Moreover, the fear conditioning experiment is passive, allowing animals with motor deficits to learn, such as mice exhibiting RTT-like deficits. Indeed, animals with locomotor deficits might show difficulties learning tasks such as the object-in-context or 'what-when-where'. They also require at least a week of training as the animals first need to get habituated to the arena before the learning. In this

respect, the fear conditioning can be done faster (three days with my paradigm, see Materials and Methods IV).

c. Other behaviors related to the hippocampus

The role of the hippocampus in novel object recognition (NOR) is still a matter of debate. Indeed, some lesion studies reported an impairment of NOR (Clark et al., 2000) while other did not (Barker and Warburton, 2011; Cohen et al., 2013; Duva et al., 1997). However, the size of the lesion was different between studies, which may account for the difference. In line with this idea, Broadbent and collaborators studies the relationship between memory impairment and lesion size and observed that NOR was altered following a lesion of more than 75% of the hippocampus (Ainge et al., 2006; Broadbent et al., 2004). Moreover, rats failed in the NOR when the lesion was done after the acquisition phase while they performed as well as controls when the lesion was done prior to learning (Gaskin et al., 2003), suggesting that another brain structure can support the object recognition, but only if the hippocampus did not participate in the encoding (Bird, 2017; Gaskin et al., 2003). Altogether, these experiments suggest that i) only large hippocampal lesions affect object recognition ii) if the hippocampus is lesioned after learning, then the recognition is altered, iii) most of the time, the perirhinal cortex may support object recognition (Winters and Bussey, 2005; Winters et al., 2004).

The hippocampus, and especially its ventral part, has also been associated with anxiety (Fanselow and Dong, 2010). Indeed, optogenetic inhibition of ventral DG neurons decreases the innate anxiety response of mice in the open field and/or elevated-plus-maze (Kheirbek et al., 2013). In this study, the authors argue that locomotor activity increases upon inhibition of dorsal DG and therefore the increased time spent in the open arms of the elevated-plus-maze is only a consequence of increased exploratory behavior. However, this hypothesis does not fully explain why the animals are suddenly spending nearly 80% of their time in open arms. In general, most of the research on anxiety focused on the ventral hippocampus as it exhibits direct connection with the amygdala (McDonald and Mott, 2017), and it is possible that a role of the dorsal hippocampus may have been overlooked. Indeed, a recent study reported that neurons from the amygdala and dorsal hippocampus, coding respectively for the emotional

and spatial content of a memory, are reactivated concomitantly during sleep (Girardeau et al., 2017).

3. Cellular and network substrates for memory encoding and consolidation

What are the mechanisms underlying learning and memory? What is the substrate of memory? Memory is the ability to encode, store and retrieve information. Encoding is the first step to create a new memory and happens during learning. Then, neurons are reactivated during sleep and participate in the consolidation and storage of the memory by stabilizing neuronal ensembles. Finally, retrieval corresponds to the ability of the brain to re-activate these ensembles.

In this section, I will describe different mechanisms involved in encoding and consolidation. Synaptic plasticity and long-term potentiation (LTP) are thought to represent the cellular mechanism of memory encoding (Bliss and Collingridge, 1993). Then, I will describe how neuronal networks participate in the generation of theta and gamma-band oscillations as well as sharp-wave ripples to support encoding and consolidation (Colgin, 2016).

a. Long-term plasticity mechanism and relevance to memory encoding

In 1949, Donald Hebb postulated the existence of a synaptic modification, either pre- and/or post-synaptically, that may underlie learning and memory. However, the first experimental evidence in support for long-term potentiation (LTP) was obtained over 20 years later. In 1966, Terje Lømo presented at the XII Scandinavian Congress of Physiology, the first observation of long-lasting field spike and EPSP potentiation occurring after afferent tetanization (Lømo, 1966). He then started his PhD on another topic. When Tim Bliss joined the team in 1968, they started studying this phenomenon in anaesthetized rabbits (Bliss and Lømo, 1973). In 1969, Tim Bliss left to London and observed LTP in un-anaesthetized rabbits with Tony Gardner-Medwin (Bliss and Gardner-Medwin, 1973). Both papers were published only in 1973 as the authors tried reproducing their results in other labs and failed for years.

Terje Lømo speculates these difficulties may have been due to stress in experimental animals. Indeed, stress can alter LTP and rabbits might have had different susceptibility to stress in different labs (Lømo, 2018).

As of today, LTP is established beyond doubt and its mechanisms have been extensively studied. After years of intense debate regarding the locus of LTP expression (pre- vs. post-synaptic) it appears this may primarily depend on synapses. Here, I will mainly focus on synapses with post-synaptic expression dependent on NMDARs, such as perforant path to DG (PP-DG) or Schaffer collateral to CA1 synapses (SC-CA1).

- **Glutamatergic receptors composition**

As discussed earlier in this introduction, fast excitatory synaptic transmission is mediated by glutamate release from the presynaptic element onto the postsynaptic neuron expressing glutamate gated-channels (iGluRs). iGluRs have been identified based on their specific ligand (kainate, AMPA, NMDA). Glutamate release can also bind to metabotropic receptors (mGluRs) and activate G-protein mediated signaling cascades. For the purpose of this thesis, I will focus on AMPAR and NMDAR but readers could refer to (Carta et al., 2014; Riedel and Reymann, 1996) for complementary information.

NMDARs are associated with a cation conductance with high Ca^{2+} permeability (Ascher and Nowak, 1988; MacDermott et al., 1986) and are critical to induction of most forms of LTP as I will discuss later. The binding of both glutamate and a co-agonist such as glycine or glia-released D-serine is necessary to trigger Ca^{2+} influx (Johnson and Ascher, 1987; Panatier et al., 2006). Moreover, NMDARs exhibit a voltage-dependent blockade by extracellular Mg^{2+} (Mayer et al., 1984; Nowak et al., 1984). NMDARs are therefore qualified as *coincidence detectors* since Ca^{2+} influx only occurs when pre-synaptic glutamate release coincides with post-synaptic depolarization.

NMDA receptors function as heterotetrameric assemblies usually composed of two GluN1 and two GluN2 subunits. There are eight isoforms of the GluN1 subunit encoded by a single gene (GluN1-1a to 4a and GluN1-1b to 4b) and four members of the GluN2 family encoded by four paralogous genes (GluN2A to D). Additional subunits (GluN3A and GluN3B) may also contribute to heterotetrameric receptor complexes at some specific synapses (for more information, see Kehoe et al., 2013). The diversity in subunit composition

of NMDA receptors may also vary according to their subcellular localization, the type of neuron or the age (Magnusson et al., 2002; Paoletti and Neyton, 2007). In the hippocampus, receptors containing GluN2A and GluN2B are predominant and the latter exhibit a higher affinity for glutamate, slower deactivation and desensitization kinetics and a higher affinity for CaMKII (Dingledine et al., 1999; Paoletti, 2011). Therefore, the ratio of these two subunits has functional implications. Moreover, in aging rodents, GluN2A expression increases while GluN2B is highly expressed at birth and declines at the onset of adulthood (Monyer et al., 1994). Currently, it is not well known how NMDARs composition might change in advanced age, but different studies are suggesting an involvement in cognitive decline (Liu et al., 2008; Magnusson et al., 2007; Zhao et al., 2009).

AMPARs are also tetrameric ion channels. They are formed by the combination of two identical dimers expressing GluA1, GluA2, GluA3 or GluA4 (Dingledine et al., 1999). Messenger RNAs undergo important post-transcriptional modifications, conferring specific biophysical properties and constraining the subunit assembly. Notably, GluA2 containing AMPARs are Ca²⁺ impermeable due to RNA editing of a glutamine into a positively-charged arginine within the pore (Arg586) and cannot form homo-tetrameric structures (Cull-Candy et al., 2006). Moreover, GluA2 and GluA4 mRNAs are alternatively spliced leading to two supplementary variants: GluA2L display a longer carboxy terminal domain (CTD) while GluA4S has a shorter CTD.

It is commonly admitted that long-tailed subunits-containing AMPARs - such as GluA1, GluA4 and GluA2L - allow for activity-dependent regulation of AMPAR trafficking (Anggono and Huganir, 2012), whereas short-tailed subunits-containing AMPARs - GluA2, GluA3 and GluA4S - are constitutively expressed at the membrane. Hence, it is accepted that GluA2/GluA3 containing AMPARs recycle in and out of the synapse while GluA1-containing AMPARs are delivered in an activity-dependent manner (Hayashi et al., 2000; Kopec et al., 2006; Plant et al., 2006; Shi et al., 1999). The importance of this last mechanism will be addressed in Introduction III.3.b. However, a study suggested neurons lacking GluA1 could still undergo LTP as long as the pool of AMPARs at the membrane was sufficient, independent of its subunit composition (Granger et al., 2013).

- **NMDAR-dependent LTP expression mechanism**

LTP is initiated by a brief tetanus or high-frequency stimulation (~1 s), called induction (Figure 14), that activates postsynaptic NMDARs (Harris et al., 1984; Kauer et al., 1988). In 1979, two independent teams observed the importance of both Ca^{2+} and Mg^{2+} in LTP induction. Indeed, lowering extracellular Ca^{2+} or increasing Mg^{2+} antagonizes LTP (Dunwiddie and Lynch, 1979; Wigström et al., 1979).

Following a high-frequency stimulation, glutamate is released into the synaptic cleft (Dolphin et al., 1982) and activates postsynaptic AMPARs. The subsequent depolarization (Kelso et al., 1986; Malinow and Miller, 1986) relieves NMDARs from their blockade by the Mg^{2+} ions leading to Ca^{2+} influx (Baimbridge and Miller, 1981) which in turn binds to calmodulin and activates CaMKII (Lee et al., 2009; Shen and Meyer, 1999). The kinase dissociates from actin (Kim et al., 2015) and translocates to the post-synaptic density (PSD) of activated synapses to interact with the intracellular tail of NR2B and NR1 subunits (Bayer et al., 2001; Foster et al., 2010; Leonard et al., 1999). Lee et al. recorded the diffusion and activation of CaMKII following LTP induced by 2-photon glutamate uncaging onto a single dendritic spine (Lee et al., 2009). They found CaMKII activation was restricted to the activated synapse and explained this specificity with the rapid inactivation of the kinase (5 s in spines and 1 min in dendrites) while its diffusion in spine and dendrites is slow (1 and 20 minutes, respectively). Therefore, CaMKII activation might be important for the synapse specificity of LTP.

In the late 1990's, a series of studies from Roberto Malinow's group at the Cold Spring Harbor Laboratory demonstrated that GluA1-containing AMPARs insertion underlies LTP expression. Using overexpression of a recombinant, GFP-tagged GluA1, they showed that tetanic stimulation (Shi et al., 1999) or overexpression of the constitutively active CaMKII (Hayashi et al., 2000) lead to the synaptic aggregation of GluA1-containing AMPARs. The importance of GluA1 in LTP was further confirmed using mice carrying a complete genetic ablation of *GRIA1* (Zamanillo et al., 1999), which exhibit an altered LTP. Moreover, affecting only the activity-dependent insertion of AMPAR either by overexpressing either a truncated CTD-lacking GluA1 (Shi et al., 2001) or the GluA1-CTD (Rumpel et al., 2005) or in mice carrying a trafficking-compromised GluA1 (Bannerman et al., 2018), was sufficient to preclude LTP expression.

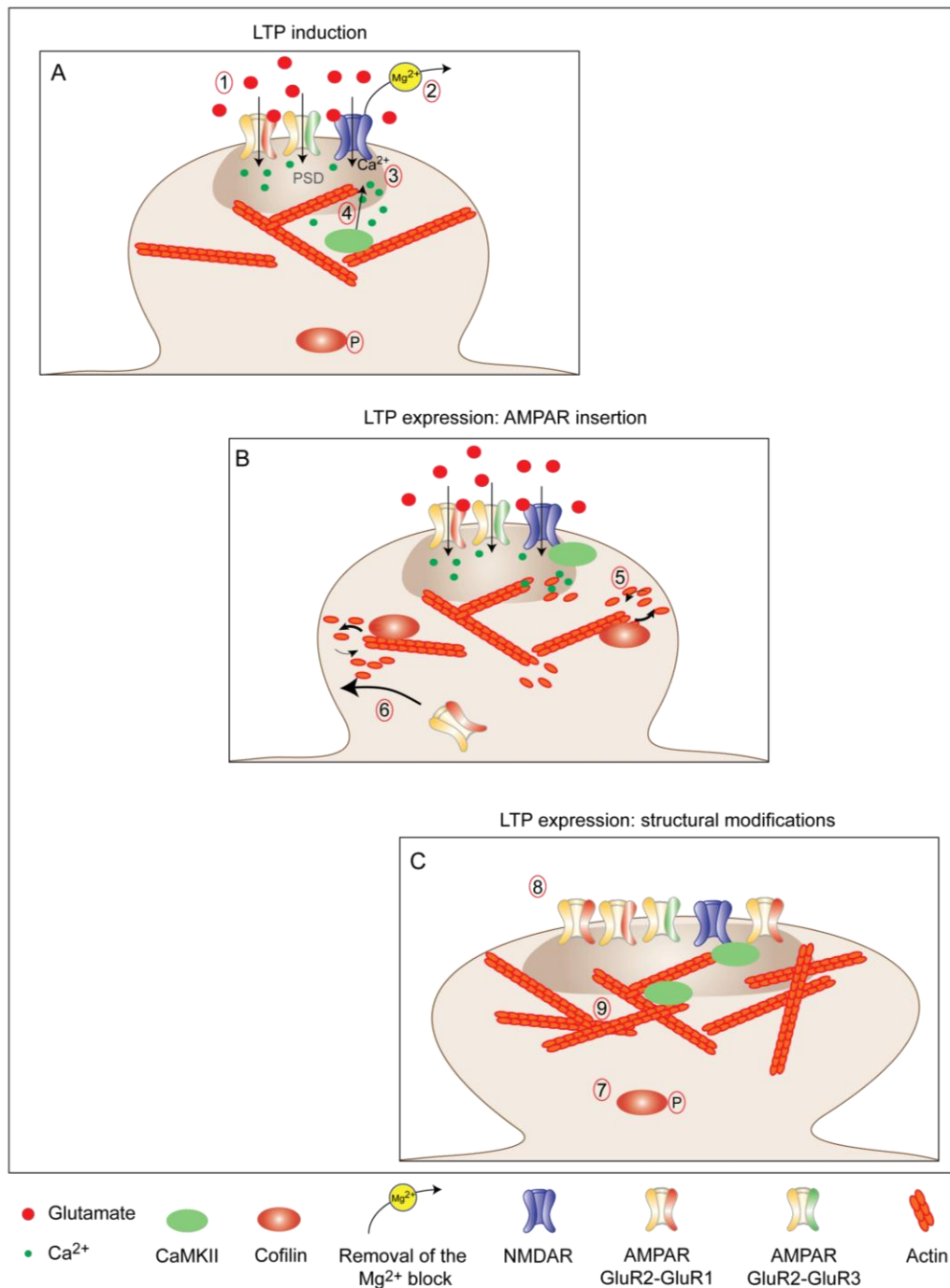


Figure 14. LTP induction and expression mechanisms

A. Following glutamate release into the synaptic cleft (1), postsynaptic depolarization relieves NMDARs from Mg²⁺ block (2) allowing postsynaptic Ca²⁺ influx (3) and CaMKII activation and recruitment at the PSD (4). **B.** Then, transient activation of cofilin allows for spine actin depolymerization (5) and the exocytosis of GluA1-containing AMPARs (6). **C.** Finally, cofilin is phosphorylated and therefore inactivated (7) while the AMPARs are recruited into the PSD (8) and actin polymerization contributes to spine head enlargement (9).

Synaptic plasticity also requires actin remodeling (Bosch et al., 2014) and the equilibrium between F- and G-actin involves different proteins. Profilin controls the

polymerization at the barbed end (plus end) while cofilin is involved in the depolymerization at the pointed end (minus end). Finally, nucleation and branching of F-actin are controlled by the cortactin promoting Arp2/3 complex recruitment while capping proteins block F-actin polymerization at the barbed end (Mizuno, 2013).

GluA1-containing AMPARs are rapidly inserted at the synapse following LTP induction (Shi et al., 2001) and this process requires a transient cofilin activation (Chen et al., 2007; Gu et al., 2010). By severing a cortex of submembrane actin, cofilin might allow the insertion of proteins at the synapse (Gu et al., 2010). On the other hand, cofilin activation might also participate in the delayed actin ramification. In presence of a large concentration of actin monomers, cofilin can depolymerize F-actin to create new barbed end, allowing Arp2/3 branching activity and promoting F-actin ramification (Ichetovkin et al., 2002; Van Troys et al., 2008). So far, this mechanism has only been observed in cell motility but could be hypothesized to act similarly in the potentiated synapse. Indeed, actin monomer content increases shortly after LTP induction while cofilin is transiently activated (Bosch et al., 2014). Once cofilin is phosphorylated and therefore inactive, actin can repolymerize leading to spine head enlargement. At this point, inactivated CaMKII β interacts with F-actin and stabilizes the new dendritic spine structure (Kim et al., 2015).

Finally, whereas the initial phase of LTP (i-LTP) expression relies primarily on post-translational protein modifications, glutamate receptors trafficking and actin remodeling, a later, more persistent phase involves *de novo* protein synthesis (Abraham and Williams, 2003). Thus, various inhibitors of protein synthesis affect LTP persistence (Fifková et al., 1982). NMDARs activation and the subsequent Ca²⁺ increase recruit multiple signaling cascades involving protein kinases (e.g. CaMKII, PKC), transcription factors (e.g. CREB), growth factors (e.g. BDNF) and translation initiation factors (e.g. eIF4E) (for a review, see Abraham and Williams, 2008). In this conventional view, the newly synthesized proteins contribute to the activity-dependent structural changes. However, evidence suggest LTP can be maintained up to 8 hours without protein synthesis (Abbas, 2013; Abbas et al., 2009), likely owing to the pool of proteins already present at the synapse.

- **LTP can be pre-synaptic in the hippocampus**

As I mostly explored LTP at PP-DG and CA3-CA1 synapses during my PhD and both synapses undergo predominantly postsynaptic LTP, I decided to focus particularly on this form of of NMDAR-dependent, postsynaptic LTP. However, other LTP mechanisms have been described in the hippocampus. In particular, CA3 pyramidal neurons exhibit two different mechanisms to induce LTP. In 1984, Harris and Cotman observed LTP at mossy fiber to CA3 (MF-CA3) synapse was independent on NMDARs activation while NMDARs antagonists were blocking LTP at the associational-commissural fibers to CA3 synapse (Harris and Cotman, 1986).

Synaptic plasticity at MF-CA3 synapse involves presynaptic modulation (for a review, see Castillo, 2012). Indeed, paired-pulse facilitation, an index of neurotransmitter released, is reduced upon LTP induction (Zalutsky and Nicoll, 1990), suggestive of presynaptic modifications. Moreover, in the same study, manipulation of postsynaptic intracellular Ca^{2+} concentration failed to preclude LTP induction. Although the underlying mechanisms remain partially unclear, some evidence indicate a presynaptic locus of induction and expression for this form of LTP: presynaptic Ca^{2+} increase may recruit a cAMP/PKA pathway leading to the increased glutamate release (Weisskopf et al., 1994).

- **LTP, a substrate for memory?**

LTP is considered a key molecular mechanism underpinning learning and memory (Bliss and Collingridge, 1993). Indeed, manipulations of the cascades of events involved in LTP mechanism have an effect on the ability of animals to learn. One of the first evidence came from alteration of both hippocampal LTP and spatial memory, following *in vivo* chronic intraventricular infusion of AP5, a NMDAR antagonist (Morris et al., 1986). However, one has to keep in mind that manipulating different proteins also means manipulating different phases of the learning and memory process. In a behavioral paradigm, animals first have to learn the task and form a memory. Then, a test phase – recall – is presented to the animal, in which it needs to remember what was learned previously. Designing precise behavioral experiments is therefore important to conclude on the role of a protein.

Delivering an inhibitor prior to learning and removing it afterwards allows one to study the role of a protein in memory formation. Indeed, in this case, the recall process (i.e.

the ability to remember) should be unaltered. Comparing the effect on the memory recall of an antagonist infusion starting before or after learning would be another way to distinguish between learning and recall. In this case, if the protein is only important in learning, infusing the antagonist after task learning would have no effect on memory retrieval. Some behavioral tests (e.g. Morris water maze) are designed to distinguish the involvement of a protein in either learning or recall. Indeed, when animals need to learn over a few trials/days, the ability to perform faster can be recorded. By using different experimental designs, the importance of NMDARs (Day et al., 2003; Morris et al., 1986), GluA1-containing AMPARs insertion (Rumpel et al., 2005) and CaMKII activation (Giese et al., 1998; Silva et al., 1992) in the formation of long-term memory have been demonstrated, as well as the role of AMPARs in memory retrieval (Bast et al., 2005; Day et al., 2003).

To find out whether previously stored information is lost or if recall is altered, learning of a behavioral task has to occur before any inhibitor is delivered and then the recall of the memory can be tested. If an alteration is observed, this cannot be due to the learning as the molecule was not present at the time of learning. However, a positive result can indicate a role either in recall or in storage. To decipher between recall and storage, the inhibitor can be applied after learning and removed before memory retrieval. This type of experiment highlighted the role of CaMKII (Rossetti et al., 2017) in memory storage.

However, all these experiments do not establish a causal link between synaptic plasticity and information storage. To tackle this question, Nabavi et al. used optogenetics to elicit LTD or LTP in the lateral amygdala (Nabavi et al., 2014). They injected an AAV expressing a variant of the light-activated channel ChR2, oChIEF, in the medial geniculate nucleus and auditory cortex and installed the optic fiber above the lateral amygdala. Then, they trained rats to associate the activation of this pathway with blue light with a foot-shock. This associative memory could be inactivated and then reactivated by optogenetically-induced LTD and LTP protocols, respectively.

In a similar experiment where mice were trained to associate a tone with a foot shock, LTP was expressed post-synaptically only at synapses activated to form the associative memory, as detected by an increased AMPA/NMDA ratio. Moreover, depotentiation of the auditory to LA pathway using optogenetics suppressed the conditioned fear response to the tone (Kim and Cho, 2017). Other studies have shown how neuronal assemblies (i.e. engrams) can support memory (Liu et al., 2012; Ramirez et al., 2013). Altogether, these data support a causal role of LTP to recruit neuronal assemblies contributing to memory specificity.

b. The role of hippocampal rhythmogenesis in memory encoding and consolidation

Population activities in the brain can be detected using electrical recordings of extracellular signals. Those can be performed either at the skull or brain surface, as in electrocorticographic recordings. When they are directly recorded inside the brain, these signals are called local field potentials (LFP) (Buzsáki et al., 2012). They reflect synchronized activity in large neuronal ensembles. Indeed, unsynchronized action potentials from individual neurons cannot sum and thus remain undetected. In the adult hippocampus, LFP recordings detect three main rhythmic activities (Colgin, 2016; Mizuseki and Miyawaki, 2017): theta (~4-12 Hz), sharp wave–ripples (~150–200 Hz ripples superimposed on ~0.01–3 Hz sharp waves) and gamma (~25–100 Hz).

Memory relies on the formation of neuronal ensembles activated concomitantly. Brain rhythms are then thought to play a role in memory by synchronizing the activity of these ensembles. In this part, I will describe how these rhythms are generated and how their expression sustains memory encoding and/or consolidation in the rodent brain.

- **Theta rhythm and memory encoding**

Theta-band activity (~4-12 Hz) arises during exploratory behaviors and rapid eye movement (REM) sleep. These oscillations are of largest amplitude in the *stratum lacunosum moleculare* of CA1 and their phase reverses in the pyramidal layer. Even though I will focus specifically on hippocampal theta, it should be noted that this rhythm can be observed in other cortical and subcortical structures (Jones and Wilson, 2005; van der Meer and Redish, 2011; Vertes et al., 2004).

Theta oscillations in CA1 arise from extra- and intra-hippocampic pathways (Figure 15). In unanesthetized animals, they depend on pacing from the medial septum – diagonal band of Broca (MS-DBB) and excitatory inputs from the EC and CA3 (Buzsáki, 2002a). As lesions of the MS-DBB were shown to consistently abolish theta oscillations in cortical structures (Petsche et al., 1962), the MS-DBB is considered the main pacemaker of theta rhythm in the brain through its cholinergic and GABAergic afferents. Blockade of cholinergic transmission in urethane-anesthetized and unanesthetized animals do not have the same effect

on theta (Kramis et al., 1975). This suggests two types of theta-generating mechanisms may co-exist: one visible during anesthesia relying on cholinergic transmission ('atropine-sensitive theta'), while the other requires GABAergic transmission but no cholinergic inputs ('atropine-resistant theta'). Since atropine-sensitive theta is mainly observed under anesthesia, I will rather focus on the mechanisms underlying the generation of atropine-resistant theta.

Long range projecting GABAergic neurons from MS-DBB are contacting hippocampal interneurons in DG, CA3 and CA1 (Freund and Antal, 1988; Müller and Remy, 2018). In the classical model of theta generation, MS-DBB rhythmically disinhibit CA1 pyramidal cells, promoting theta oscillations through perisomatic inhibition (Buzsáki, 2002a). Indeed, when GABAergic projections from MS-DBB cannot inhibit PV interneurons, theta oscillations are disrupted (Wulff et al., 2009). Concomitantly, the entorhinal cortex, which is known to also oscillate at theta frequency (Mitchell and Ranck, 1980), excites apical dendrites of the principal cells (Buzsáki, 2002a). However, the reality is more complex as different interneurons subtypes can be contacted by MS-DBB GABAergic neurons (Freund and Antal, 1988; Unal et al., 2015). Finally, the idea that theta oscillations are only controlled through extra-hippocampic inputs has been challenged as they were observed also in intact hippocampal preparations *in vitro* (Goutagny et al., 2009). This preparation allowed the identification of CA3 as an additional generator for theta.

Parvalbumin- (PV) and somatostatin-expressing (SOM) interneurons control theta oscillation in CA1 and are activated in the trough of the theta wave and in the ascending phase, respectively (Royer et al., 2012). This result is surprising as a previous study in urethane-anesthetized rats showed that PV interneurons fire in the descending phase while SOM interneurons are active in the trough of the theta wave (Klausberger and Somogyi, 2008; Klausberger et al., 2005). Royer et al. recognized the datasets were limited which could account for the difference. One could also argue for interspecies specificity or a difference due to the anesthetized versus behaving animal.

Royer et al showed that, in head-fixed mice running on a treadmill with visual cues, optogenetic inhibition of SOM interneurons increased the probability of pyramidal cell burst firing with the discharge of longer bursts, while PV interneurons inhibition only mildly affected this burst firing but induced a phase shift in the timing of spikes (Royer et al., 2012). These findings suggest that dendritic inhibition is critical for selecting the CA1 inputs and outputs to be strengthened as burst firing promotes synaptic plasticity (Lisman, 1997; Takahashi and Magee, 2009) while somatodendritic inhibition controls the timing of spikes within theta-phase.

A recent study evaluated the importance of PV interneurons in theta generation using the intact hippocampus preparation *in vitro*. By optogenetically silencing PV interneurons, they disrupted theta rhythm (Amilhon et al., 2015), which is not observed in behaving animals (Royer et al., 2012). This suggests that in rodents, excitatory inputs from the EC might sustain theta waves in the absence of perisomatic inhibition.

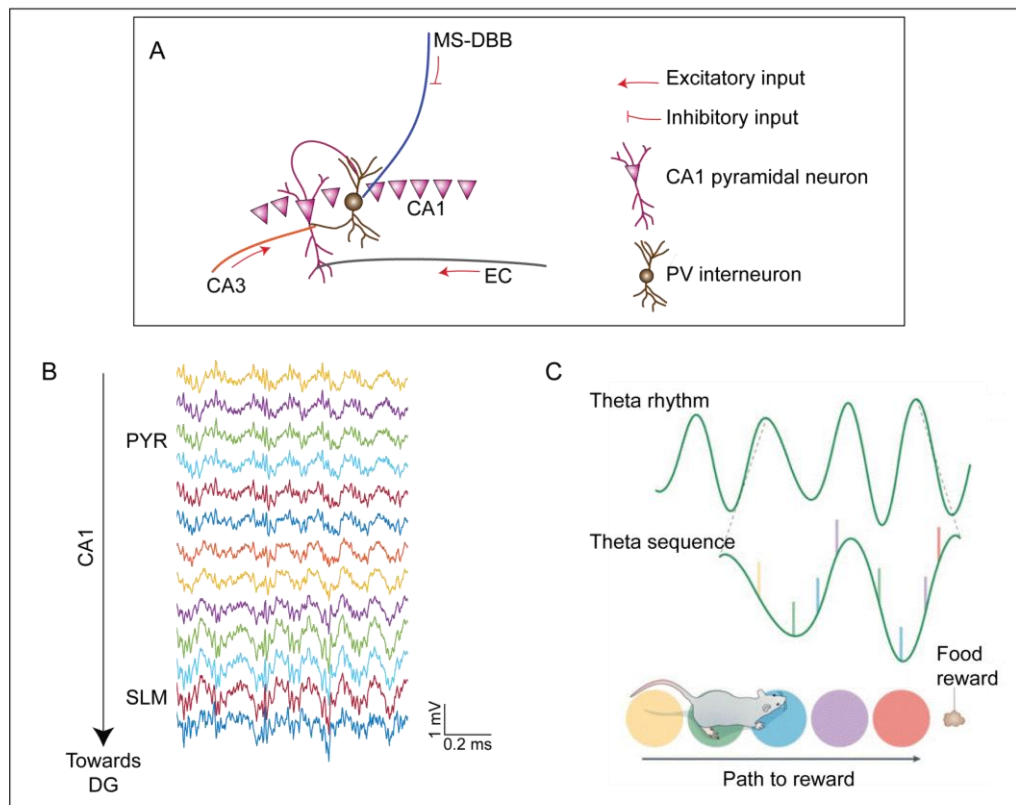


Figure 15. Theta oscillations allow spatial memory encoding and consolidation

A. CA1 PV interneurons receive inhibitory inputs from GABAergic projection of MS-DBB, the main theta generator in the brain, while pyramidal neurons receive excitatory inputs from CA3 and the EC. **B.** Trace example of theta oscillations during REM sleep. Notice the increase in theta power in the SLM (*st. lacunosum moleculare*) compared to the PYR (pyramidal layer). **C.** Place cells are firing while the animal crosses different fields while walking towards a reward. These place cells are compressed into theta oscillations, participating in the encoding of spatial information.

(Adapted from Colgin, 2016)

The relevance of theta oscillations in the formation of memory has been suggested for a long time. Indeed, burst stimulation at theta frequency induces LTP (Larson and Munkácsy, 2015) and deep brain stimulation at theta frequency increases human ability to recognize previously presented pictures (Titiz et al., 2017). Accordingly, studies aiming to disrupt theta oscillations in behaving animals revealed memory deficits (Boyce et al., 2016; Winson,

1978), while EEG recordings in humans showed a correlation between high theta power and contextual memory retrieval (Staudigl and Hanslmayr, 2013).

Theta rhythm is observed in awake animals and during REM sleep, suggesting two distinct roles of these oscillations. Awake theta oscillations are critical for the formation of neuronal ensembles in moving animals. Indeed, when the animal is exploring its environment, place cells fire sequentially as the animal enters their firing field (O'Keefe, 1976), thereby creating a behavioral sequence of firing which is contained in the theta oscillation (O'Keefe and Recce, 1993). Thus, awake theta is participating to the encoding of a memory (Figure 15).

On the other hand, REM sleep has been associated with memory consolidation (Boyce et al., 2017). Indeed, selective REM sleep deprivation in which the animal or human subjects are awakened when they transition from non-REM to REM sleep result in memory impairment (Fishbein, 1971; Karni et al., 1994). However, rapid hormonal response to stress and subsequent metabolic changes (Knutson et al., 2007; McEwen, 2006) are a potential caveat of such experiments. In order to overcome such challenges, Boyce and colleagues used optogenetic manipulations of the MS GABAergic neurons projecting to the hippocampus (Boyce et al., 2016). Following learning of a place recognition task or fear conditioning, they specifically inhibited these neurons during REM sleep, thus reducing the power of theta oscillations. On the next day, mice exhibited altered spatial and contextual memories but not cued memory recall, thus suggesting theta oscillations are supporting hippocampo-dependent but not amygdalo-dependent memories.

- **Gamma oscillations**

Gamma oscillations points to activity in a large frequency band ranging from 25 to 100 Hz. Activities in the whole range of gamma frequencies are observed in the hippocampus during both exploration and sleep (Bragin et al., 1995).

For a long time, gamma was considered as a unique rhythm in the hippocampus. However, in a groundbreaking study, Laura Colgin and colleagues demonstrated for the first time a very clear distinction between slow (25-55 Hz) and fast (60-100 Hz) gamma (Colgin et al., 2009). Both rhythms are observed during theta oscillations in CA1 (Belluscio et al., 2012) but rarely appear simultaneously. Moreover, they emerge from different mechanisms: slow gamma is directly entrained by CA3 afferents while fast gamma is generated by inputs from the medial EC (Colgin et al., 2009).

Since the identification of distinct gamma oscillations, attempts have been made to assign each rhythm a specific role in memory. Advances toward this goal have been made through the study of CA1 pyramidal cells firing during theta oscillations associated with fast or slow gamma. Indeed, slow gamma is associated with neurons firing in the first half of the theta oscillations while neurons are activated in the second half during fast gamma (Bieri et al., 2014). Hence, this suggests a role of fast gamma in memory encoding while slow gamma predicts the next location. Consistent with this hypothesis, theta-slow gamma coupling in CA3 increases during an associative task learning and remains high during retrieval (Tort et al., 2009) and disruption of encoding memory affects fast gamma oscillations (Newman et al., 2013).

However, recent evidence suggested other roles for these rhythms. Indeed, a transient burst of fast gamma synchrony can be recorded before correct choice on a spatial working memory task (Yamamoto et al., 2014) suggesting a role in working rather than encoding memory. Furthermore, pyramidal cells phase locking to slow gamma increases during novelty but not during memory retrieval (Kitanishi et al., 2015). Hence, these discrepancies between studies still need further investigations to understand the relationship between slow and fast gamma coupling to theta.

- **Sharp-wave ripples and memory encoding**

Sharp-wave ripples (Figure 16) are generated during waking immobility, slow-wave sleep and consummatory behavior in the hippocampus (Buzsáki, 2015). The sharp wave is a large oscillation (~0.01–3 Hz) with maximal amplitude in the *stratum radiatum* while the ripple arises in the pyramidal layer of CA1 with a high frequency oscillation usually comprised between 150-200 Hz. Unlike theta, SPW-R expression does not require extra-hippocampic inputs as they can spontaneously be generated in the isolated hippocampus (Kubota et al., 2003; Maier et al., 2003).

Sharp-waves arise from the transient burst of collective firing in CA3 permitted by the high recurrent connectivity of this region (Buzsáki, 1986) and lead to the massive depolarization of CA1 pyramidal cells. This activation is required to allow the generation of ripples (Stark et al., 2014). Then, ripples are generated within CA1 by the recruitment of fast-

spiking parvalbumin basket cells interneurons firing at ripple frequency due to both their intrinsic properties and their reciprocal connections (English et al., 2014; Schlingloff et al., 2014). These interneurons provide a time window in which a subset of pyramidal cells can fire in a compressed manner (Buzsáki, 2015; Ylinen et al., 1995). Indeed, shunting inhibition allows only neurons receiving a strong excitation to be activated, selecting probably neurons that were already potentiated during learning. Moreover, this CA1 spiking activity during ripple is unlikely entrained by CA3 as CA1 spiking activity correlates with the ripples while CA3 spiking activity does not (Csicsvari et al., 1999; Sullivan et al., 2011).

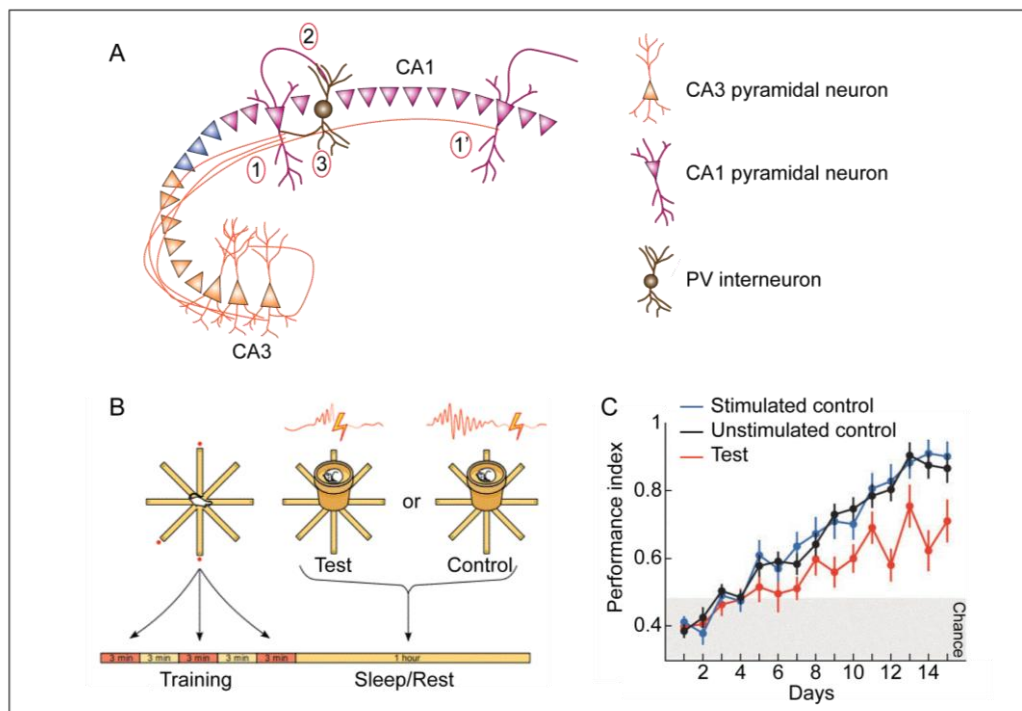


Figure 16. Hippocampal ripples are necessary for consolidation

A. SPW-R generation mechanism. CA1 neurons receive strong excitation from CA3 pyramidal neurons (1). CA1 pyramidal neurons then activate the PV interneurons (2). They are synchronizing their activity and fire at high frequency, generating a ripple and allowing CA1 neurons to be activated in a compressed manner (3). If the input from CA3 is weak, there will be no ripples (1'). **B.** Girardeau and collaborators trained rats to learn a task, allowing them to rest while suppressing ripples (Test) or not (Control). **C.** Upon suppression of sleep ripples, rats showed altered performance in the memory test.

(Adapted from Girardeau et al., 2009)

SPW-R are observed in the awake immobile animal and during sleep, corresponding to online and offline replay of events, respectively (Joo and Frank, 2018; Roumis and Frank, 2015). Sleep ripples are considered to carry-out offline mnemonic function such as memory

consolidation and transfer of memories to the neocortex (Ji and Wilson, 2007; Kudrimoti et al., 1999; Lee and Wilson, 2002). Indeed, during ripples, re-activation of spike sequences relevant to previous waking experiences can be detected and hippocampal ripples precede neocortical oscillations, such as spindles, suggesting a synchrony in information transfer between these two structures (Maingret et al., 2016; Siapas and Wilson, 1998). Moreover, a causal relationship between ripples and memory consolidation was demonstrated by Girardeau and collaborators (Figure 16). They trained rats to learn a complex spatial memory task over multiple days. Following each training session, rats were allowed to rest and ripples were detected online and subsequently suppressed by closed-loop electrical stimulations. Ripple disruption resulted in impaired learning, an effect that could not be attributed to the electrical stimulation since delayed stimulations did not affect the animal's performance (Girardeau et al., 2009).

If offline replay is necessary for consolidation, online replay may participate in the guided decision-making of the animal. Indeed, suppressing the awake ripples alters the ability of rats to learn how to navigate in the W maze spatial alternation task, which requires the ability to integrate immediate past experience to decide on the next step (Jadhav et al., 2012). Moreover, a recent study was able to enhance the performance of rats in a similar behavioral paradigm, by increasing the awake ripple duration (Fernández-Ruiz et al., 2019). This allowed more CA1 neurons among the low-firing population of pyramidal cells to be recruited to participate in the memory and probably contribute to stabilize the place field.

IV. Rationale and objective of the project

Learning and memory require an exquisite balance between excitation and inhibition. Different mechanisms have been suggested over the years for encoding, consolidating and storing memories. At the cellular level, LTP allows the strengthening of synaptic connections (Bliss and Collingridge, 1993; Nicoll, 2017). In LTP, synaptic AMPAR content increase together with structural and morphological modifications. At the network level, groups of neurons are activated and organized within theta-gamma oscillations for encoding (Buzsáki, 2002b; Colgin, 2016). Then during sleep, SPW-R in NREM and theta oscillations in REM participate to memory consolidation and storage (Boyce et al., 2017; Buzsáki, 2015). Rhythmogenesis relies on intact GABAergic transmission in order to synchronize neuronal ensembles. Therefore, any modification at the molecular level disrupting the excitation/inhibition balance might subsequently impair learning and memory.

In the adult brain, fast inhibitory transmission activates chloride ions-permeable GABAARs. The flux of Cl^- depends on the electrochemical gradient of this ion and mechanisms controlling chloride homeostasis directly influence GABAergic transmission. In mature cortical neurons, intracellular chloride concentration is controlled by the activity of the chloride/potassium co-transporter KCC2. Over the last 20 years, KCC2 has been extensively studied in the context of epilepsy. Indeed, in slices resected from epileptic patients, pathological interictal activities can be suppressed by inhibition of GABAergic transmission, suggesting GABA might have a paradoxical excitatory effect (Cohen et al., 2002). Furthermore, this observation correlates with a depolarizing effect of GABA and the loss of KCC2 expression in a subset of principal cells (Huberfeld et al., 2007). Since then, KCC2 expression has been found to be down-regulated in many neurological and psychiatric conditions. Moreover, hippocampal neurons of ASD, RTT or Huntington mouse models display a depolarized E_{GABA} (Banerjee et al., 2016; Dargaei et al., 2018; Tyzio et al., 2014). Altogether, these data suggest KCC2 knockdown in the pathology alters GABAergic transmission.

However, recent evidence from our lab have shown that dentate granule cells lacking KCC2 present a more depolarized E_{GABA} associated with a depolarized resting membrane

potential, thus unexpectedly preserving steady-state GABAergic transmission (Goutierre et al., 2019). KCC2 knockdown therefore modifies the intrinsic properties of the neurons. In line with this observation, Marie Goutierre and collaborators found that neurons display an increased intrinsic excitability due to a deficit in membrane trafficking of the leak potassium channel Task-3, associated with deficits in rhythmogenesis, specifically an increase in dentate spikes amplitude and frequency. These data suggest KCC2 knockdown might alter hippocampal rhythms beyond the mere control of chloride homeostasis and GABA signaling.

Finally, KCC2 expression is not restricted to GABAergic synapses and has been observed near the PSD. In the past 10 years, KCC2 down-regulation during development has been shown to impair dendritic spine maturation (Li et al., 2007) while in mature neurons, its suppression decreases the efficacy of glutamatergic transmission (Gauvain et al., 2011a) and precludes long-term potentiation at glutamatergic synapses (Chevy et al., 2015). All of these alterations are independent of KCC2 transport function but rely on its interaction with proteins such as 4.1N or β PIX (Chevy et al., 2015; Gauvain et al., 2011a; Li et al., 2007; Llano et al., 2015). KCC2 knockdown in adult mice might therefore results in memory deficits through altered glutamatergic signaling as well.

KCC2 appears to be a protein at the crossroads of excitatory and inhibitory transmission while also influencing neuronal excitability and the ability of cortical neurons to respond to stimuli in the appropriate manner. Since KCC2 is down-regulated in many disorders associated with cognitive impairment, I asked whether KCC2 knockdown on its own might affect learning and memory and explored how mechanisms of memory encoding and consolidating were affected. To address these questions, I used a viral-based, chronic extinction of KCC2 in the dorsal hippocampus to investigate the impact of KCC2 down-regulation on synaptic plasticity, network activity and memory. Finally, I tried to find an appropriate mouse model of pathology to examine whether KCC2 over-expression might rescue memory deficits.

MATERIALS AND METHODS

Materials and methods

1. How to knockdown or rescue KCC2?

- **Plasmid cloning**

In order to suppress KCC2, I used a short hairpin RNA (shRNA) approach to knockdown KCC2 expression (shKCC2) in mice. This shKCC2 sequence AGCGTGTGACAATGAGGAGAA has been previously reported and validated (Bortone and Polleux, 2009). As KCC2 is only expressed in neurons (Karadsheh and Delpire, 2001), the constitutive polymerase III promoter U6 was chosen to drive expression of the small hairpin RNAs (shRNAs), while the polymerase II promoter CMV drove GFP expression (U6-shRNA-CMV-GFP).

Next, we aimed at suppressing KCC2 specifically in principal neurons or interneurons. I then used the cell-type specific promoters CaMKII (for principal cells) (White et al., 2011, Addgene #32578) and mDlx (for GABAergic interneurons) (Dimidschstein et al., 2016, Addgene #83900). As those are polymerase II promoters, I embedded shRNA sequences in a micro-RNA sequence (miR-30 backbone) (Fellmann et al., 2013). The resulting shmirRNA can be processed by the cell using the micro-RNA biogenesis pathway and has several advantages. Indeed, unlike shRNAs, shmirRNAs can be transcribed by polymerase II, allowing for use of cell-type specific promoters. In addition, shmirRNA sequence can be fused to that of a reporter protein, allowing for expression under a single promoter region (see mDlx-GFP-shmirRNA construct developed based on Yu et al., 2015). ShmirRNAs are also less toxic than shRNAs (Boudreau et al., 2009).

Finally, in order to over-express KCC2-CTD, we used the gene sequence already used in our team (Gauvain et al., 2011a). Indeed, even though the gene sequence corresponds to the rat, the encoded protein is identical to that of the mouse.

In the following table 2, I summarize information of the different constructs used in this study, such as the vector of origin, the inserted sequences and the enzymes used for cloning. Only the plasmids in bold were used for viral production.

Table 2. Information about plasmid constructs

Final plasmid	Vector of origin	Inserted sequence(s)	Cloning enzymes
pAAV-U6-shRNA-CMV-GFP	pAAV-U6-shLuc-CMV-GFP	U6-shRNA	EcoRI ; BglII
pAAV-CaMKII-shmirRNA-CMV-GFP	pAAV-U6-shLuc-CMV-GFP	CaMKII-shmirRNA	EcoRI ; BglII
pAAV-CaMKII-shmirRNA-CMV-GFP-SV40	pAAV-CaMKII-shmirRNA-CMV-GFP	SV40	EcoRI
pAAV-mDlx-GFP-shmirRNA-WPRE-SV40	pAAV-mDlx-GFP-WPRE-SV40 (Addgene #83900)	shmirRNA	BsrGI ; AscI
pAAV-CaMKII-KCC2CTD-WPRE-pA-CMV-GFP-SV40	pAAV-CaMKII-shmirRNA-CMV-GFP-SV40	KCC2-CTD (from a lab construct) WPRE-pA (from plasmid Addgene #61463)	XhoI ; EcoRI
pAAV-CaMKII-KCC2CTD-WPRE-CaMKII-GFP	pAAV-CaMKII-KCC2CTD-WPRE-CMV-GFP	CaMKII	HindIII ; NheI

Finally, once the plasmids were cloned, viruses were produced by the translational vector core in Nantes, France (CPV, UMR-1089). In order to ensure infection of dorsal hippocampal neurons with minimal retrograde transport, we chose AAV serotype 2.1 as a vector (Aschauer et al., 2013).

- **Rescue of KCC2 expression**

In order to rescue KCC2 expression following its down-regulation with U6-shKCC2 expressing virus, we need a KCC2 gene sequence shRNA proof. In our team, we already had such a viral construct expressing KCC2-flag with the rat sequence, under CaMKII promoter. We confirmed the mouse shRNA against KCC2 does not recognize the rat KCC2 sequence and decided to use both virus together.

2. Animals and surgical procedures

- **Animals**

Mice were housed in standard laboratory cages on a 12-hours light/dark cycle, in a temperature-controlled room (21°C) with free access to food and water. C57Bl/6J mice were purchased from Janvier Labs and were delivered to our animal facility at least a week before surgery or behavioral testing. B6.129S-*Mecp2*^{tm1Hzo}/J (MecP2³⁰⁸) and B6;129P2-*Mecp2*^{tm1Bird}/J (MecP2^{flox}) mice were purchased from Jackson laboratory and bred in our animal facility with C57Bl/6Jr mice to generate hemizygous males and heterozygous females.

Chronic treatments with bumetanide (0.2 mg/kg in 2% DMSO (dimethyl sulfoxide)) or CLP-290 (100 mg/kg in 10% HPCD (hydroxypropyl-β-cyclodextrin)) were given through daily intraperitoneal (i.p.) injection (volume of injection was 100 μL per 10 g of body weight). s

- **Stereotaxic surgery**

To inject a virus in the dorsal hippocampus (dHPC), mice were anesthetized with ketamine/xylazine (100/15 mg/kg) and placed on a heating pad at 36-37°C for the entire surgery. 10 minutes before opening the skin, lidocaine (2%) was applied locally on the skin. After surgery, body temperature was maintained using a heating pad under the cage until the animal recovered from anesthesia. Behavioral experiments or electrophysiological recordings were then conducted 10 to 14 days after surgery.

In a first experimental group, AAV1-U6-shKCC2-CMV-GFP or AAV1-U6-shNT-CMV-GFP (*see Plasmid cloning for more information*) were bilaterally injected in dHPC (500 nl in both the dentate gyrus and CA1 for each hemisphere) at the following stereotaxic coordinates from Bregma: -1.8 mm anteroposterior (AP), +/- 1.2 mm mediolateral (ML) and -2.1/-2.0/-1.9/-1.3/-1.25 mm dorsoventral (DV).

For the next experimental groups, different viruses were used to knockdown KCC2 in principal cells, AAV1-CaMKII-shmirKCC2(NT)-CaMKII-GFP or in interneurons, AAV1-mDlx-GFP-shmirKCC2(NT). Other viruses were used to over-express KCC2 in principal cells, AAV1-CaMKII-KCC2flag.

In the second project on Rett syndrome, the same protocol and coordinates were used to inject bilaterally the AAV1-hSyn-GFP-Cre (Addgene, #105540-AAV1) or AAV1-hSyn-GFP (Addgene, #105539-AAV1) in MecP2^{flox} mice.

- **Silicon probe implantation**

One week after viral injection, some mice were implanted with a 16 or 32-channel linear silicon probe (Neuronexus). Mice were anesthetized with isoflurane (4% for induction, 1.0–2.0% afterwards) in a stereotaxic frame for the entire surgery and their body temperature was maintained with a heating pad. To reduce pain during the surgery, mice were injected with buprenorphine before to start the surgery (0.1 mg/kg).

First, the skull was cleaned and the craniotomy at the probe location was re-opened. Two screws were implanted on the frontal bone (1 per hemisphere), 1 on the parietal bone (contralateral to the probe) and 1 reference screw above the cerebellum.

Then, the probe was lowered into the brain at the following coordinates from Bregma: -1.8 mm (AP), -1.2 mm (ML), -2.4 mm (DV, for the 32-channels silicon probes) or - 2.0 mm (DV, for the 16-channels silicon probes). Once the probe was positioned, the craniotomy was covered with Vaseline to protect the probe.

The headstage was then built using a thin layer of SuperBond dental cement applied onto the skull, followed with Unifast Trad dental cement on the probe and the screws. Pieces of copper mesh were then arranged around the probe to create a Faraday cage and cemented onto the skull after soldering the ground and reference of the probe with the reference electrode above the cerebellum to the copper mesh.

After surgery, body temperature was maintained using a heating pad under the cage until the animal recovered from anesthesia. Mice were then housed separately to avoid any fight that could damage the probe. On the next 3 days following surgery, mice were monitored twice a day and buprenorphine (0.1 mg/kg) was administered each time. To ensure mice would not lose weight, they were fed with liquid high-calorie chocolate. Behavioral experiments and recordings were conducted a week after the surgery, once the animals recovered.

3. Electrophysiology

- **Slice preparation**

Methods of slicing and tissue preservation are critical for neuron survival. When I arrived in the lab, the classical preparation was the following. Mice were deeply anesthetized with ketamine/xylazine (100/10 mg/kg) and transcardially perfused with an ice-cold choline-based solution containing (in mM): 110 Choline Cl, 25 Glucose, 25 NaHCO₃, 11.6 Ascorbic acid, 3.1 pyruvic acid, 1.25 NaH₂PO₄, 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂ saturated with 95% O₂ / 5% CO₂. The brain was then extracted and 350 µm slices were prepared using a vibratome (Microm, Thermo Fisher). Slices were then transferred and allowed to recover for 1 hour in a humidified interface chamber filled with bicarbonate-buffered ACSF pre-heated at 37°C and oxygenated with 5% CO₂ in O₂, containing (in mM): 126 NaCl, 26 NaHCO₃, 10 Glucose, 3.5 KCl, 1.25 NaH₂PO₄, 1.6 CaCl₂ and 1.2 MgCl₂.

However, when I started LTP experiments using fEPSP recordings, we noticed that the potentiation was small compared to that reported in previous publications. We then reasoned that choline is a precursor of acetylcholine, and acts as a weak agonist on cholinergic receptors, in particular the alpha7 nicotinic acetylcholine receptors (nAChRs) (Alkondon et al., 1997), a subunit expressed in the hippocampus (Fabian-Fine et al., 2001). Ondrejcek et al (Ondrejcek et al., 2012), showed that the application of an alpha7 nAChRs agonist enhances hippocampal synaptic transmission, with long lasting effects even after washout of the agonist. In addition, choline may also activate muscarinic receptors in the mM range (Costa and Murphy, 1984) and may therefore perturb LTP expression upon prolonged activation (Dennis et al., 2016).

In order to circumvent this putative problem, we decided to slice brains using a N-Methyl-D-Glucamine (NMDG)-based solution (based on Ting et al., 2014) containing (in mM): 93 NMDG, 25 Glucose, 30 NaHCO₃, 20 HEPES, 5 Ascorbic acid, 3 pyruvic acid, 1.2 NaH₂PO₄, 2.5 KCl, 0.5 CaCl₂, 10 MgCl₂, adjusted to pH 7.4 adding HCl and saturated with 95% O₂ / 5% CO₂. Using this new paradigm, LTP of fEPSP evoked at Schaffer collateral was significantly increased (Figure 17). Therefore, I decided to use this slicing protocol for all my experiments.

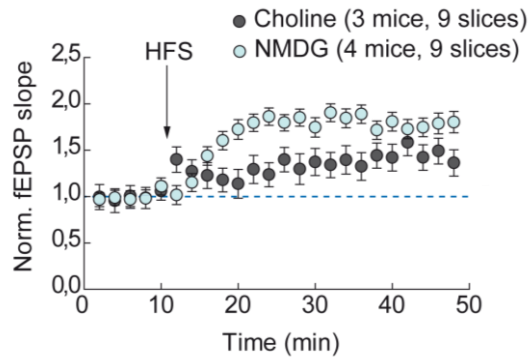


Figure 17. LTP expression depends on the cutting solution used

Choline- or NMDG-based choline solution were used. Following high frequency stimulation (HFS), LTP response is increased in slices cut in NMDG.

- **LTP recordings**

For *ex vivo* field EPSPs (fEPSPs) recordings, slices were transferred in a submerged recording chamber and super-perfused with bicarbonate-buffered ACSF as above, after a cut was made between the CA3 and CA1 areas in order to prevent propagation of epileptiform activities. A recording borosilicate glass pipette (2–4 mΩ) filled with ACSF was inserted in the molecular layer of a densely infected CA1 area, and a tungsten bipolar electrode (0.5 mΩ) was positioned 100-150 μm apart in the *st. radiatum*, and used to stimulate the Schaffer collateral pathway. fEPSPs were isolated in the presence of the GABAA receptor antagonist bicuculline methochloride (20 μM), using a Multiclamp 700B amplifier (Molecular Devices), amplified 20X, low-pass filtered at 5 kHz, and digitized at 20 kHz.

fEPSPs were evoked at 0.1 Hz for 10 minutes before a high frequency stimulation (HFS: 5 x 1 second at 100 Hz) was delivered to induce LTP. fEPSPs were then recorded for another 40-50 minutes after LTP induction. fEPSP slope were analyzed offline using Clampfit software (Molecular Devices). Briefly, baseline potential was set to zero, and recordings were low-pass filtered at 1 kHz using a Bessel filter. The initial slope of the fEPSP was then automatically measured using a 1 ms time-window manually positioned at the onset of the fEPSP. Data were acquired and analyzed blind to the experimental condition.

- ***In vivo* recordings**

Recordings of awake behavior were performed in a white 50 x 50 x 40 cm white arena with a black cue card for the spatial orientation while sleep recordings took place when the mouse was in its home cage. We recorded 30 minutes of sleep before the introduction to the

white arena to perform the place recognition task (see Materials and Methods IV) and up to one hour of sleep after. However, we only analyzed the 30 first minutes of sleep.

Data were acquired at 20 kHz using an Intan recording controller (Intan Technologies, Los Angeles, USA) and Intan Recording Controller software (version 2.05).

All analyses were performed offline using Matlab built-in functions, Chronux (Bokil et al., 2010), the FMAToolbox (<http://fmatoolbox.sourceforge.net/>), as well as custom-written scripts.

Power spectra and spectrograms were computed using multi-tapers estimates on the raw LFP. Gamma band was defined at 25-90 Hz. Slow and fast gamma ranges (respectively 25-55 and 60-90 Hz) were determined according to Colgin et al. 2009. Theta-gamma coupling was estimated using the measure described in Tort et al., 2008. Briefly, theta-gamma coupling allows to estimate the modulation of the amplitude of an oscillation (here in the gamma band) by the phase of another oscillation (here theta). A value of 0 indicates lack of phase to amplitude modulation while an increased value of the modulation index reveal stronger amplitude-phase coupling.

Theta power was determined in the 5-10 Hz band. Spike detection was performed by high-pass filtering and setting a threshold (which was determined manually). Bursts of spikes were defined as a minimum of 3 spikes with inter-spike interval below 20 ms. We selected this 20 ms value by looking at the distribution of all inter-spikes intervals (ISI): between 40 and 50% of neurons have an ISI smaller than 20 ms in our data set. Ripple detection was performed by band-pass filtering (100-600 Hz), squaring and normalizing, followed by thresholding of LFP recorded in CA1 pyramidal layer. Thresholds were different between each animal and were determined manually, in order to select only SPW-R events.

4. Behavioral experiments

- **Open field**

The open field test can be used to test both locomotion and anxiety. Mice have a tendency to stay along the sides of an open field when the light is bright and avoid the center of the arena. The arena was cleaned with 10% ethanol between each trial.

We first used a 40 x 40 x 20 cm white rectangular arena with a light intensity in the center of 90-100 lux. By the end of my PhD, we started using a larger arena (50 x 50 x 40

cm). As this new environment was larger, we were expecting to see more clearly the differences of anxiety between our groups. Mice were gently placed facing a wall for a 10 minutes recording period. Their activity was recorded and analyzed using the Ethovision software. Mice were considered in the center of the arena (1/9 of the arena) when their body center was in the central zone.

- **Elevated-O-maze**

This task allows to test anxiety and confirm results obtained in the open field test. The apparatus consists in a white circular maze (35 cm of diameter, lane is Z cm wide), elevated 50 cm above the floor, and divided in 4 quadrants. Two opposite “closed” quadrants surrounded by 12 cm high opaque walls, the safe environment, and two “open” quadrants which represent the anxiogenic environment as mice can fall. The luminosity was low (8-10 lux in the open arms). The elevated-O-maze was cleaned with 10% ethanol between each trial. Mice were placed randomly in one of the closed arms, facing towards the open arm. Their activity was recorded for 10 minutes and analyzed using the Ethovision software. Mice were considered in the open arms when the four paws were placed in the arm.

- **Rotarod**

Motor activity and coordination was tested with the rotarod. Mice were pre-trained for 3 days on an automated 5-lane rotarod unit. Each day, mice were placed on the rotarod for 5 minutes at constant low speed. 30 minutes later, they were placed on the accelerating rotarod, going from 4 rpm to 45 rpm in 5 minutes.

On the training day, mice were placed on a rod that accelerated smoothly from 4 to 45 rpm over a period of 5 minutes. This training was repeated three times, with 30 minutes of rest between two consecutive sessions. The latency to fall from the rod was recorded. If mice stayed up to 5 minutes, they were removed and scored at 300 seconds.

- **Grip strength test**

To test the grip strength, the mouse was placed on a grid that was flipped at 30 cm above an empty cage. We controlled the 4 paws of the mouse were in contact with the grid after flipping it. The latency to fall (i.e none of the paws in contact with the grid) was recorded.

- **Object and place recognition**

The arena and the objects were cleaned with 10% ethanol between each trial and the luminosity in the center of the arena was set to 10 lux in order to reduce anxiety while mice are learning these tasks. A cue card was placed on one wall to allow for orientation in the arena.

On the three first days, mice were habituated to the arena, with two habituations per day, at least 3 hours apart. On the first habituation (H1), all mice from a single cage are placed in the arena for 30 minutes, allowing them to explore together the environment to help reduce the stress. For the four next habituations (H2 to H5), mice are placed alone in the arena for 10 minutes. On the last habituation (H6), two identical objects are introduced to help reduce the stress associated with novelty.

The same arena was used for both object and place recognition (OR and PR). In order to avoid any bias due to the habituation to the arena and a loss of interest, half of the mice first learned the OR then the PR, and the other half learned the PR then the OR. In the course of my PhD, I tried different protocols and different arenas. The last, optimized version of the task is explained here.

The OR task is based on the rodent's natural preference to novel objects. During the first exposure, two identical objects were presented to the mouse for 10 minutes. Then, 10 minutes (short-term memory) or 24 hours later (long-term memory), mice were placed back in the arena, facing the wall opposite to the objects with a new object and an old object for 5 minutes.

In the PR task, mice are placed back in the arena with one object moved and the other in the same position.

Mice behavior was video-recorded and analyzed manually during the exposure session, in order to ensure that mice were exploring enough and did not have an initial preference for one of the objects. The data recorded during the memory test were manually scored. A discrimination index (DI) was used as a measure of discrimination between familiar and novel object or between novel and old location:

$$DI(OR) = \frac{\textit{Time spent on novel object} - \textit{Time spent on old object}}{\textit{Total time of exploration}}$$

$$DI(PR) = \frac{\textit{Time on object in new location} - \textit{Time on object in old location}}{\textit{Total time of exploration}}$$

- **Morris water maze**

The Morris water maze task (Morris, 1984) is a spatial memory task where mice learn to find a platform, hidden under the water surface. We used a large circular pool (1.5 meter diameter, depth 25 cm) filled with 21°C water colored in white with a non-toxic paint to prevent animals from seeing the platform. The room temperature was at 25°C. Mice had at least 30 minutes of rest between trials in a cage placed under a heating lamp.

Mice first learned to find a visible platform with no cues on the wall. They were trained 4 times per day for 5 days. All mice learned correctly this task. Then the platform was hidden in this same location. Mice started each trial from a different position to ensure they will use the external cues to look for the platform. They were trained 4 times per day for 5 days. If a mouse did not find the platform after 90 s, it was placed on the platform for 15s and then removed. 30 minutes (short-term memory) or 72 hours (long-term memory) later, we recorded 90 s of swimming without any platform to control for spatial memory.

Task performance was evaluated by calculating the distance and time to reach the platform during the training phase. To control for spatial memory, we compared the time spent in the target quadrant, where the platform was previously located, versus the three other quadrants.

- **Fear conditioning**

In order to form a strong contextual memory, mice underwent a first day of exposition to the chamber without receiving a foot shock (Brown et al., 2011). Each mouse was placed four minutes in the box 1 (27x27 cm): black environment, white light (0 lux), square box, cleaned with 70% ethanol.

On the second day, they were allowed to explore box 1 for 4 minutes before to be exposed to a tone of 30 seconds (4 Hz, 85 dB) that co-terminated with a two-seconds foot shock (0,25 mA) delivered through the floor (Fanselow, 1986). After 30 seconds without any tone, the tone - foot shock was introduced again. In total, mice received 4 foot shocks.

On the third day, mice were placed back in box 1 for 4 minutes to evaluate contextual memory, without receiving any foot-shock. Two hours later, they were placed in a box 2 to test cued memory. Box 2 was white, with a different shape than box 1, a new grid on the floor, red light (1 lux), and was cleaned with 1% acetic acid. After 4 minutes of exploration, a tone (4 Hz, 85 dB) was played for 30 seconds, followed by 30 seconds of silence. This sequence was repeated 6 times.

The freezing behavior was recorded initially with Packwin v2.0.05 software. However, over the course of my PhD, this software stopped working and I started analyzing data manually: every 5 seconds, I was looking at the video for 2 seconds and noted whether the mouse was freezing or not and calculate the proportion of freezing time for each animal.

5. Biochemistry and Immunohistochemistry

- **Postsynaptic density extraction**

This protocol was adapted from an existing protocol I helped improving (Bayés et al., 2014). Dissected tissues (hippocampus or cortex) were homogenized in Buffer A (0.32 M sucrose, 2 mM HEPES, pH 7.4, 1 tablet of complete EDTA-free protease inhibitor (Roche), 30 mM NaF and 5 mM sodium-o-vanadate using a 2 ml Dounce homogenizer. The resulting homogenates were centrifuged at 1000 g for 10 min at 4°C. The pellets were then re-homogenized in the same Buffer A and centrifuged at 1000 g for 10 min at 4°C. The combined supernatants (S) were centrifuged at 18600 g for 15 min at 4°C. The pellets were re-suspended in Buffer B (1.5 M sucrose, 50 mM Tris-HCl, pH 7.4) and layered at the bottom of the sucrose gradient containing 0.85 M sucrose (middle) and 0.3 M sucrose (top) in 50 mM Tris-HCl, pH 7.4. Sucrose gradients were centrifuged at 60000 g for 90 min using the SW60 Ti rotor (Optima L90K ultracentrifuge, Beckman-Coulter). The synaptosomes (SYN) purified at this step were collected from the interface between 1.5 M and 0.85 M sucrose. They were then diluted in 2 volumes of 50 mM Tris-HCl, pH 7.4 and centrifuged at 48,000 x g for 20 min at 4°C using the MLA-80 rotor (Optima Max Ultracentrifuge). The pellets were re-suspended in 1.5 % Triton X-100, 25 mM Tris-HCl, pH 7.4, incubated on ice for 30 min, layered on top of a 0.85 M sucrose solution in 50 mM Tris-HCl, pH 7.4 and centrifuged at 104000 g for 40 min at 4°C using the SW60 Ti rotor (Optima L90K ultracentrifuge). The pellets (PSD) were collected as PSD preparations and re-suspended in a re-suspension buffer containing 50 mM Tris-HCl, pH 7.4.

- **Western blot analysis**

The protein concentration in all samples was determined using the Pierce assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher). Equal amounts of proteins were then separated on a 4%–12% SDS polyacrylamide gradient gel (Invitrogen) and transferred onto a

nitrocellulose membrane (GEHealthcare) at 100 V for 1 hour in order to ensure efficient transfer of high molecular weight proteins using a liquid transfer system (Bio-Rad).

The membrane was blocked for one hour at room temperature in 5% milk (diluted in 1X TBS – 0,1% Tween 20) then washed three times for 5 min in 1X TBS – 0,1% Tween 20. These washing conditions were used after incubation with primary and incubation with secondary steps. The membrane was incubated overnight at 4°C with the primary antibody (diluted in 5% milk, 1X TBS – 0,1% Tween 20). Then the secondary antibody (diluted in 5% milk, 1X TBS – 0,1% Tween 20) was added for 1 hour at room temperature. The membrane was then probed using the Odyssey blot imaging system (LI-COR).

The primary antibodies used were: rabbit anti-KCC2 (1:1000, Millipore), mouse anti-Tuj1 (1:10000, R&D systems). The secondary antibodies used were: goat anti-rabbit 800 (1:5000, Tebu-Bio or 1:15000, LiCor), goat anti-mouse 700 (1:5000, Tebu-Bio or 1:15000, LiCor).

- **Histology**

Mice were deeply anesthetized using sodium pentobarbital (200 mg/kg i.p.) and then transcardially perfused with ice-cold PBS solution, followed by ice-cold 4% paraformaldehyde (PFA) in PBS pH 7.4. Extracted brains were fixed in 4% PFA overnight and then equilibrated in 30% sucrose in phosphate buffer saline (PBS). A sliding cryotome was used to section the brains into 40 µm-thick coronal sections. Immunohistochemistry experiment was performed when necessary.

Table 3. Infection score and related virus spread in the hippocampus

Infection score	Size of the infection
0	No infection in either one hippocampus or in one subregion
1	Minimum 320 µm in all hippocampus, usually one in one subregion
2	Minimum 600 µm of infection in one subregion then over 800 µm in the rest of the hippocampus
3	Over 800 µm of infection in both hippocampi (DG, CA1, CA3)

The size of the infection spread was quantified, with a score ranging from 0 to 3 (see table 3). In some experiments using mice infected with CaMKII-shmirKCC2 expressing viruses, I noticed that the freezing responses in contextual retention test was correlated with the infection score (Figure 18, CamKII-shmirKCC2: linear regression $p=0.168$, but mice with an infection score of 3 have a tendency to freeze less than mice with an infection score of 1, t-

test one-tailed $p=0.056$). Based on this observation, in all behavioral tasks and for all virus used, only mice with an infection score of 2 or 3 were kept for further analysis.

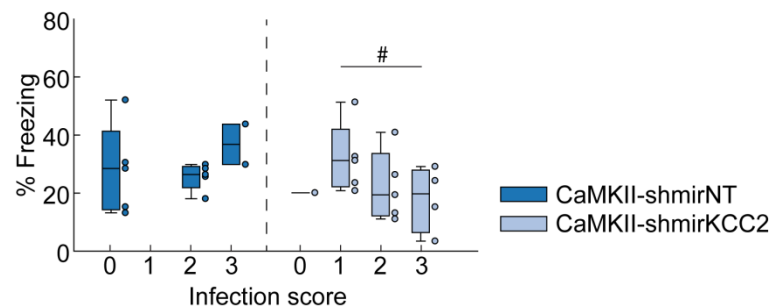


Figure 18. Infection size with CaMKII-shKCC2 seems to correlate with ability to remember

Even though a linear regression test did not show any effect, mice infected with the vector expressing CaMKII-shmirKCC2 with an infection score of 1 tend to freeze more than mice with an infection score of 3.

t-test, one-tail $p = 0.056$

- **Immunohistochemistry**

Slices were incubated for 1 hour at room temperature in a blocking solution containing 10% goat serum, 0.1% Triton X-100 in PBS. Incubation with primary antibody (rabbit anti-KCC2 (1:500, Millipore), chicken anti-GFP (1:1000, Millipore), mouse anti-Flag (1:1000, Sigma-Aldrich), mouse anti-GAD67 (1:1000, Abcam)) was then performed in blocking buffer overnight at 4°C. After 3 x 10 min washes in PBS, slices were incubated with secondary antibodies with a dilution of 1:1000 in blocking buffer for 1 hour (goat anti-rabbit-cy3, donkey anti-mouse-cy5, donkey anti-chicken-488 (Jackson Labs)). After another 3 x 10 min washes, slices were mounted on a coverslip in Mowiol/Dabco (25 mg/mL) solution. Immunofluorescence images were acquired using an upright confocal microscope (Leica TCS SP5), using a 40X 1.30-N.A. objective and Ar/KR laser set at at 491 and 561 nm for excitation of Cy3 and FITC, respectively, for images of the entire hippocampus. Stacks of 25-30 μm optical sections were acquired at 512x512 pixel resolution with a z-step of 1 μm .

6. Statistics

All statistical tests were performed using SigmaPlot software. Two tables with all information related to the statistical tests used are provided in the Results section below (see

Table 4 and 5). When necessary, proportions were arcsin-transformed prior to applying the appropriate statistical test. Comparison of means was performed using Student's t-test for normally distributed variables (as tested with Shapiro-Wilk test) of equal variances (Brown-Forsythe test). Otherwise, comparison of mean was performed using the non-parametric Mann-Whitney test. Two-way ANOVA was used for comparison of distributions when data were normally distributed with equal variances.

RESULTS

Results

I. Part I : The effect of chronic KCC2 down-regulation on learning and memory

KCC2 is down-regulated in numerous neurological disorders such as epilepsy (Huberfeld et al., 2007), autism (Tyzio et al., 2014), schizophrenia (Arion and Lewis, 2011) or Rett syndrome (Duarte et al., 2013). Most of these disorders are associated with mild to severe forms of cognitive impairment, including episodic and working memory deficits (Bennetto et al., 1996; Gur and Gur, 2013; Tramoni-Negre et al., 2017).

As discussed in the introduction, the potassium/chloride cotransporter KCC2 controls the intraneuronal concentration of chloride and thereby influences the efficacy and sometimes even the polarity of GABA signaling (Pellegrino et al., 2011; Rivera et al., 1999). Besides its role in ion transport, KCC2 also interacts with a variety of molecular partners and thereby influences neuronal properties beyond the mere control of chloride homeostasis. Thus, KCC2 influences the function and plasticity of glutamatergic synapses through interactions with actin-related proteins (Chevy et al., 2015; Gauvain et al., 2011a; Llano et al., 2015) and therefore acts as a master regulator of both GABAergic and glutamatergic signaling (Chamma et al., 2012). In addition, KCC2 also interacts with the leak potassium channel Task-3 and controls its membrane expression, thereby regulating neuronal excitability (Goutierre et al., 2019).

Down-regulation of KCC2 expression in the pathology may then be expected to affect various cellular and synaptic mechanisms concurring to perturb neuronal and network activity and compromise memory encoding and consolidation. Thus, in the hippocampus, KCC2 down-regulation was shown to compromise LTP expression (Chevy et al., 2015), a key molecular mechanism underpinning learning and memory (Bliss and Collingridge, 1993; Whitlock et al., 2006). On the other hand, GABAergic signaling and neuronal excitability both critically shape hippocampal rhythmogenesis, including the generation of theta-band

activity (Royer et al., 2012) and sharp wave ripples (SPW-R, (English et al., 2014; Schlingloff et al., 2014)), which are required for memory encoding and consolidation (Buzsáki, 2002b, 2015).

During my PhD, I therefore wished to test the hypothesis that chronic KCC2 down-regulation might affect learning and memory and explore the underlying mechanisms. Understanding these mechanisms could then be key to establish the best strategies to try and rescue memory deficits in disorders associated with KCC2 down-regulation. This aspect will be further explored in the second part of this work.

1. KCC2 down-regulation in dorsal hippocampal neurons impairs spatial and contextual memory in mice

Genetic KCC2 ablation is associated with severe developmental defects and yields to premature death around birth, due to respiratory deficits (Hübner et al., 2001) and seizures (Uvarov et al., 2007). Such animal models were therefore not appropriate to evaluate the impact of KCC2 down-regulation on learning and memory. Instead, I then decided to use a viral-based, chronic extinction by RNA interference in the dorsal hippocampus. Adult mice (P56) were infected bilaterally in the dorsal hippocampus with vectors expressing either non-target (shNT) or KCC2-specific (shKCC2) shRNAs. We first verified the effectiveness of KCC2 knockdown by western blot analysis of microdissected hippocampal extracts (Figure 19A) and by immunostaining (Figure 19B). KCC2 expression was reduced in hippocampal extracts from mice infected with virus expressing shKCC2 sequences as compared to shNT (t-test, one-tailed $p < 0.001$). Viral infection itself yielded no change in KCC2 expression, as no significant difference was detected in samples from mice infected with the control virus and non-infected animals (t-test, one-tailed $p = 0.388$).

Since behavioral analysis of hippocampal-dependent memory relies on object exploration and locomotion, we first tested whether KCC2 knockdown in the dorsal hippocampus had any effect on locomotor activity (Figure S1). Whereas speed was unchanged between KCC2-knockdown and control mice (Mann-Whitney, $p = 0.249$), KCC2-knockdown mice showed a tendency - yet not significant - to walk shorter distances (Mann-Whitney, $p = 0.093$) and spend more time immobile (t-test, one-tailed $p = 0.079$). We next tested

mice behavior in two anxiety paradigms. In the open field (Figure S2A-C), KCC2-knockdown mice spent more time in the center of the arena (Mann-Whitney, $p=0.006$) and entered more frequently the central zone than control mice (Mann-Whitney, $p=0.013$), suggestive of reduced anxiety. In contrast, in the elevated-O-maze (Figure S2D-E), no difference was observed in the time spent in the open arms between KCC2-knockdown and control mice (Mann-Whitney, $p=0.176$). These results suggest KCC2 down-regulation in dorsal hippocampus might only marginally reduce anxiety in mice.

We next tested whether down-regulating KCC2 might affect different forms of hippocampus-dependent memory. To evaluate spatial memory, we used the Morris water maze and a place recognition paradigm. In the Morris water maze, mice first had to find a visible platform without visual cue (Figure S3A). KCC2-knockdown mice showed increased latency to reach the platform on the two first days of training but then learned the task as control mice. The next week, mice were trained to find a hidden platform by using visual cues in the room (Figure S3B-C). All mice learned this task similarly, as assessed by the time to reach the platform. Moreover, the swimming speed was identical between both groups. In order to further test for spatial memory retention (Figure S3D), mice underwent short delay (30 minutes) and long delay (72 hours) probe trials. In both trials, KCC2-knockdown and control mice explored the target quadrant similarly, suggesting neither short nor long-term spatial memory were altered upon KCC2 down-regulation. In the second spatial memory task (Figure 19C-D), however, mice explored two identical objects for 10 minutes in an arena, then 24 hours later, one object was displaced. Whereas control mice showed a significant preference for the moved object, this preference was significantly reduced (and nearly abolished) in KCC2-knockdown mice (t-test, one-tailed $p=0.017$). These results show that KCC2 extinction in dorsal hippocampus compromises spatial memory in some but not other experimental paradigms.

Then, we asked whether KCC2 suppression might also affect contextual memory, another form of learning relying on hippocampal function (Phillips and LeDoux, 1992). Mice received 4 foot shocks (FS) associated with 4 sounds on the training day (Figure 19E). On the next day, contextual and cued memories were tested. In the contextual retention memory test, over 4 minutes of exploration (Figure 19F), freezing increased in all mice with a peak at 2 minutes and then decreased. However, freezing in control mice was significantly higher than for KCC2-knockdown mice both during the 4-minutes exploration (two-way ANOVA,

p=0.006) or during the 3 first minutes only (t-test, one-tailed p=0.050; Figure 19G). However, no difference in freezing behavior was observed between the two groups in the cued memory test (two-way ANOVA, p=0.208; Figure 19H), which was expected as cued memory is primarily dependent on amygdala, not hippocampus (Phillips and LeDoux, 1992). Our results therefore suggest that chronic KCC2 suppression in the dorsal hippocampus impairs both spatial and contextual memory.

2. KCC2 down-regulation in principal cells is sufficient to affect contextual memory

We next aimed to explore the cellular and network mechanisms underlying memory deficits upon KCC2 knockdown in dorsal hippocampus. Since KCC2 is expressed in hippocampal principal cells and at least some interneurons subtypes (Gulyás et al., 2001), we first asked whether KCC2 down-regulation in a subset of neurons might be sufficient to recapitulate these memory deficits. In order to specifically suppress KCC2 expression in principal cells or interneurons, respectively, we designed two new vectors using a principal cell promoter (CaMKII) and a forebrain interneuron-specific promoter (mDlx) (Dimidschstein et al., 2016). As those are polymerase II promoters, we embedded shRNA sequences in a micro-RNA sequence (shmirRNA, see Methods). These constructs efficiently suppressed KCC2 expression in mice injected in the dorsal hippocampus, as illustrated by immunofluorescence detection (Figures 20A-B). Five to six weeks following viral injection, mice were then exposed to a fear-conditioning paradigm. During the retention test of contextual memory, mice infected with a virus expressing CaMKII-shmirKCC2 showed significantly less freezing than control mice during the 4 minute exploration (two-way ANOVA, p=0.02; Figure 20D) or during the 3 first minutes only (t-test, one-tailed p=0.019; Figure 20E), as in mice with non-specific neuronal knockdown. In contrast, mice infected with a virus expressing mDlx-shmirKCC2 displayed a freezing response comparable to that of the control group during the 4 minute exploration (two-way ANOVA, p=0.094) or during the 3 first minutes only (t-test, one-tailed p=0.215). Again, mice expressing either CaMKII-shmirKCC2 or mDlx-shmirKCC2 in the dorsal hippocampus showed no deficit in cued memory (see Table 4 for statistics; Figure 20F).

These results suggest KCC2 down-regulation only in dorsal hippocampus principal cells, but not GABAergic interneurons, is sufficient to impair contextual memory. In order to further test the specificity of our knockdown approach, we then performed a rescue experiment in which KCC2 down-regulation was induced in dorsal hippocampus using the AAV1-U6-shKCC2 vector and then specifically rescued in principal cells using a AAV1-CaMKII-KCC2flag vector, expressing recombinant, Flag-tagged and shRNA-proof KCC2 (see Materials and Methods 2.). Co-infection was confirmed by immunohistochemistry using anti-GFP and anti-Flag antibodies (Figure 20C). Recombinant KCC2 expression only in principal cells fully restored contextual memory in KCC2 knockdown mice (two-way repeated measures ANOVA, $p=0.424$; see Table 4 for statistics; Figure 20F) while cued memory was unaffected. None of these manipulations led to significant change in locomotor activity (Figure S1) or anxiety (Figure S2), as evaluated in open field and elevated-O-maze tests (see Table 4 for statistics).

3. KCC2 down-regulation impairs hippocampal LTP

As discussed in the introduction, deficits in spatial and contextual memory may reflect alterations in memory encoding, consolidation, or both. Long term potentiation is thought to represent the cellular substrate for memory encoding (Bliss and Collingridge, 1993). Previous data from our lab reported altered LTP at perforant path inputs onto dentate granule cells in the rat upon KCC2 knockdown (Chevy et al., 2015). This reflected deficits in activity-dependent AMPAR membrane delivery, as discussed in the introduction. We asked whether this effect was also observed at Schaffer collateral synapses onto CA1 cells, where LTP has been associated with hippocampal-dependent memory (Whitlock et al., 2006). A fEPSP was evoked in CA1 by stimulation of Schaffer collaterals in the *st. radiatum* of hippocampal slices from mice infected with AAV1-U6-shRNA, in the presence of the GABAAR antagonist bicuculline (Figure 21A). High-frequency stimulation (5 x 1 s at 100 Hz) elicited a long-lasting increase in the initial slope of the fEPSP in these slices (Figure 21B-C). However, this form of LTP was reduced in amplitude in slices from mice infected with AAV1-U6-shKCC2 (Figure 21C, t-test, one-tailed $p=0.067$). More recordings are required to confirm this observation.

This effect could be rescued by re-expressing KCC2 specifically in principal cells, using co-injection of AAV1-U6-shRNA and AAV1-CaMKII-KCC2flag vectors as above. Thus, upon overexpression of shRNA-proof recombinant KCC2 in principal cells, no difference was observed on the decay or amplitude of LTP in slices from mice infected with U6-shNT vs U6-shKCC2-expressing vectors (Figure 21D; t-test, one-tailed $p=0.370$). Our results demonstrate that memory deficits induced by KCC2 knockdown in the dorsal hippocampus are associated with impaired hippocampal LTP. Both defects are specific to KCC2 as they can be rescued by KCC2 re-expression in principal cells.

4. KCC2 knockdown impairs hippocampal rhythmogenesis

Hippocampal rhythms underlying memory consolidation, such as sharp wave ripples (SPW-R) and theta-band activity in particular during rapid eye movement (REM) sleep, rely on temporally precise interactions between glutamatergic and GABAergic signaling (Buzsáki, 2002b, 2015). Since KCC2 knockdown is known to affect both glutamatergic (Gauvain et al 2011; Chevy et al 2015) and GABAergic (Pellegrino et al., 2011; Rivera et al., 1999) synapses as well as neuronal membrane excitability (Goutierre et al 2019; see Introduction I.2.b), we hypothesized these effects may perturb hippocampal rhythmogenesis. To test this hypothesis, we bilaterally injected mice with AAV1-U6-shNT or AAV1-U6-shKCC2 vectors in the dorsal hippocampus and then implanted 16- or 32-channel silicon probes in the right hippocampus (Figures 22A-B, see Materials and Methods 2.). The silicon probe was positioned to record LFP signal throughout the CA1 to DG axis.

Theta-band activity (5-10 Hz) was recorded throughout hippocampal layers during REM sleep (Figure 22C). Its power peaked within *st. lacunosum moleculare*, as previously reported (Buzsáki, 2002; Figure 22D). No difference was observed in the power profile of theta-band activity between KCC2 knockdown and control mice (Kruskal-Wallis, $p=0.774$). Gamma-band activity (25-100 Hz) is also detected during various behavioral states including REM sleep and is known to be modulated by theta, with gamma phase and amplitude both coupled to the phase of the theta rhythm (Colgin, 2016). We therefore compared theta-gamma coupling during REM sleep and again found no difference between KCC2 knockdown and control mice (Kruskal-Wallis, $p=0.558$, Figure 22E). However, the power gamma-band

activity was reduced upon KCC2 knockdown (Kruskal-Wallis, $p=0.012$, Figure 22F). Gamma oscillations can be divided in slow (25-55 Hz) and fast (60-90 Hz) gamma components, with distinct underlying mechanisms and functional impact on memory encoding and consolidation (Colgin et al., 2009; 2016). We therefore distinguished the two components and observed a specific decrease in slow gamma oscillations with no significant effect on fast gamma in KCC2 knockdown animals as compared with control (Kruskal-Wallis, $p=0.003$ and 0.269 , respectively, Figures 22G-H).

Although two studies reported spontaneous seizures upon KCC2 knockdown (Chen et al., 2017) or conditional genetic ablation (Kelley et al., 2018) in the hippocampus, we did not record any interictal or ictal activity in 6 mice with hippocampal KCC2 knockdown. We also did not detect high frequency oscillations (HFOs), such as fast ripples that may represent the hallmark of an epileptic hippocampus (Lévesque and Avoli, 2019; Menendez de la Prida and Trevelyan, 2011; Figure 23A). This lack of epileptiform activity was in agreement with the lack of spontaneous seizures in KCC2 knockdown animals observed during weeks of behavioral evaluation.

Sharp wave-ripples (SPW-Rs) are large amplitude, hippocampal LFP events that occur during slow-wave sleep and waking immobility (Buzsáki, 2015). As discussed earlier (see Introduction III.3.b), sharp waves are large amplitude negative polarity deflections generated from CA3 and transmitted to the *st. radiatum* in CA1. Ripples on the other hand are fast network oscillations detected in the *st. pyramidale*, generated within both CA3 and CA1 and involve local GABAergic inputs from perisomatic interneurons (Schlingloff et al., 2014). SPW-Rs were shown to be critically involved in memory consolidation during sleep, as their chronic disruption by electrical stimulation compromises memory consolidation in a spatial memory task (Girardeau et al., 2009). We first compared the properties of ripples in the CA1 *st. pyramidale* during slow wave sleep either prior to or following a spatial memory task (Figure S4). Unlike a previous reports (Eschenko et al., 2018), we found no evidence for a change in ripple duration or frequency before *vs.* after the task (t-test, $p=1$ for both tests; Figure S4C-D). As described previously (Figure 19D), whereas control mice learned the task and showed preference for the moved object (one-sample t-test $p=0.015$; Figure S4B), mice infected with U6-shKCC2 expressing vector showed no preference (one-sample t-test $p=0.371$; Figure S4B).

We then tested whether KCC2 down-regulation might affect ripple occurrence or intrinsic properties during slow-wave sleep after the spatial memory task (see Materials and Methods). Ripple frequency profiles were similar in control and KCC2 knockdown mice, with no sign of fast-ripple component (250-500 Hz), as discussed above (Figure 23A). However, ripple frequency and duration were increased in KCC2 knockdown as compared to control mice (Figures 23B-E; Kolmogorov–Smirnov, $p < 0.001$ and < 0.001 , respectively). In addition, multi-unit activity (MUA) in CA1 *st. pyramidale*, also increased in KCC2 knockdown mice (Figure 23D). Thus, spike frequency, burst frequency and burst duration were increased as compared to control animals (Figure 23E-G ; Kolmogorov–Smirnov, $p < 0.001$ for all conditions). The burst duration was not just a mere reflection of the increased MUA as the burst event fraction (number of bursts divided by the number of spike trains, see Materials and Methods) was significantly increased in KCC2 knockdown mice (Figure 23H, Mann-Whitney, $p = 0.010$). Moreover, spike bursts were more often associated with ripples than in control mice (Figure 23I, Mann-Whitney, $p = 0.010$). Altogether, these results show that KCC2 down-regulation in dorsal hippocampus but profoundly affects hippocampal rhythmogenesis by i) reducing slow gamma rhythm, ii) increasing ripple frequency and duration and iii) increasing MUA and bursting in CA1 as well as promoting spike bursts during SPW-Rs. These alterations may act to deteriorate memory consolidation and thereby contribute to impair hippocampus-dependent memory.

5. Conclusion

In conclusion, in this part of my work, I showed that KCC2 knockdown in dorsal hippocampus (either in all neurons or only in principal cells) alters spatial and contextual memory in mice. This behavioral effect is associated with deficits in LTP expression and alterations in hippocampal rhythmogenesis. Thus, whereas KCC2 knockdown was not sufficient to generate epileptiform activity (as in Goutierre et al., 2019; but see Chen et al., 2017; Kelley et al., 2018), slow gamma power was decreased in CA1, suggestive of an alteration in CA3 inputs, and the frequency and duration of ripples were increased. More strikingly, recordings in CA1 pyramidal layer showed that neurons were hyperexcitable with an increased frequency of occurrence of spike bursts that often interfered with ripples. We hypothesize this might create a *background noise* deteriorating the information content of

ripples, thereby impairing memory consolidation. These results will be discussed in detail in the last part of this thesis (see Discussion).

Interestingly, restoring *KCC2* expression in principal cells only in *KCC2* knockdown mice was sufficient to rescue contextual memory and LTP. This suggests this paradigm might be efficient to rescue the phenotype of mouse models of pathology associated with *KCC2* down-regulation. This hypothesis led to the second part of my experimental work.

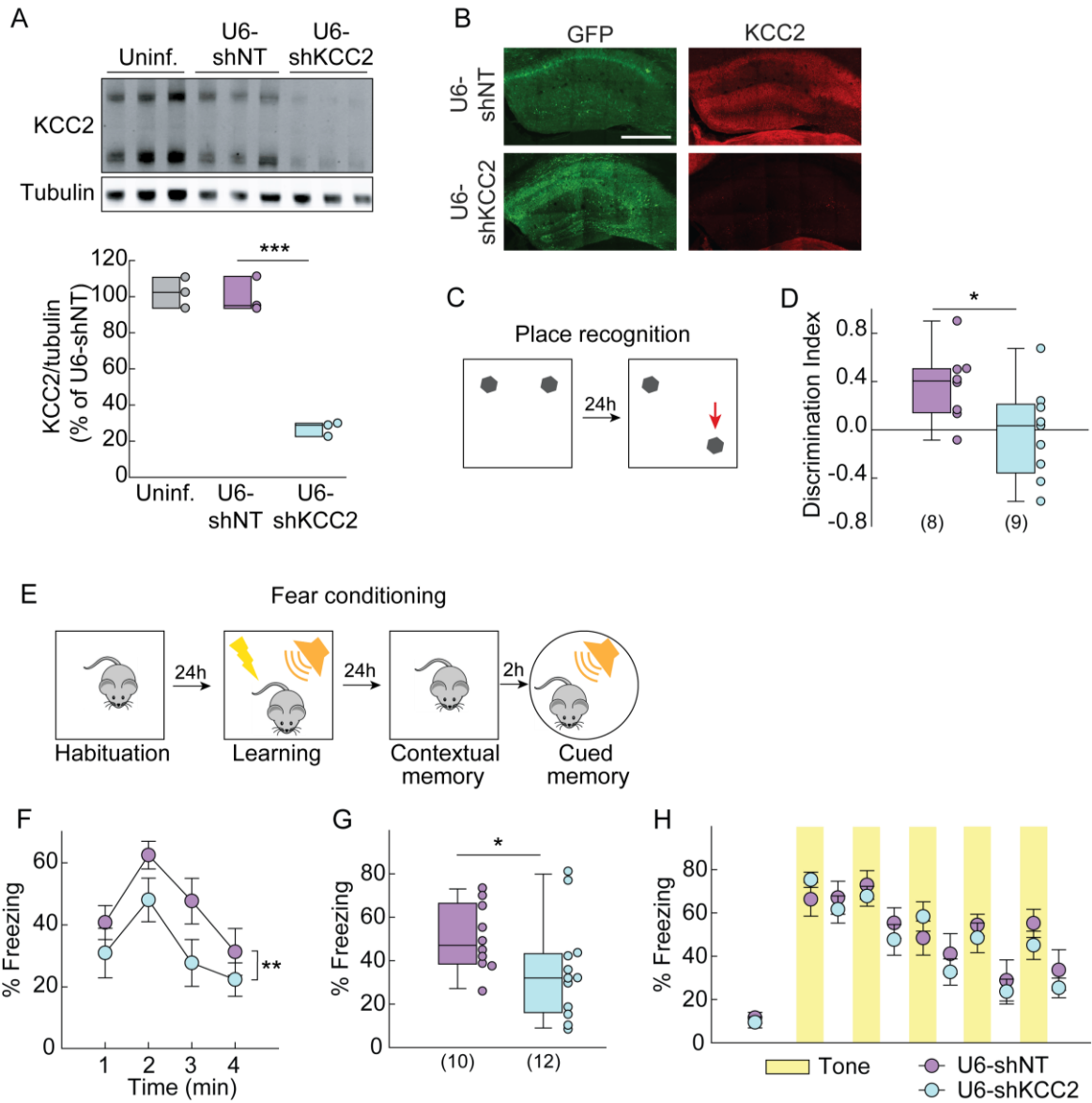


Figure 19. KCC2 down-regulation in dorsal hippocampal neurons impairs spatial and contextual memory

A. Top, representative immunoblot of KCC2 in hippocampal protein extracts from non-infected mice (n=3) and mice infected either with U6-shNT (n=3) or U6-shKCC2 (n=3) expressing viruses. Bottom, quantification of KCC2 expression, relative to that from mice infected with U6-shNT vector (t-test, one-tailed $p < 0.001$). Tubulin was used as an internal standard. **B.** Confocal micrographs of hippocampal coronal sections immunostained for GFP and KCC2 from mice infected with U6-shNT or U6-shKCC2-expressing vectors, showing massive KCC2 knockdown in the later. Scale: 500 μ m. **C.** Protocol of the place recognition task. **D.** Discrimination index showing control mice learned to identify the moved object better than KCC2-knockdown mice (t-test, one-tailed $p = 0.017$). **E.** Protocol of the fear conditioning task. **F.** Time course of freezing during the first 4 minutes of exploration of the foot-shock associated cage. Mice infected with U6-shKCC2 vector display reduced freezing time compared to mice infected with U6-shNT vector (two-way ANOVA, Bonferonni post-hoc test, $p = 0.006$), **G.** Summary data for the 3 first minutes of exploration of the cage (t-test, one-tailed $p = 0.050$) **H.** Time course of freezing upon exposure to foot-shock associated sound. Cued memory retention show no difference between both groups.

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

All statistical tests and data are in Table 4.

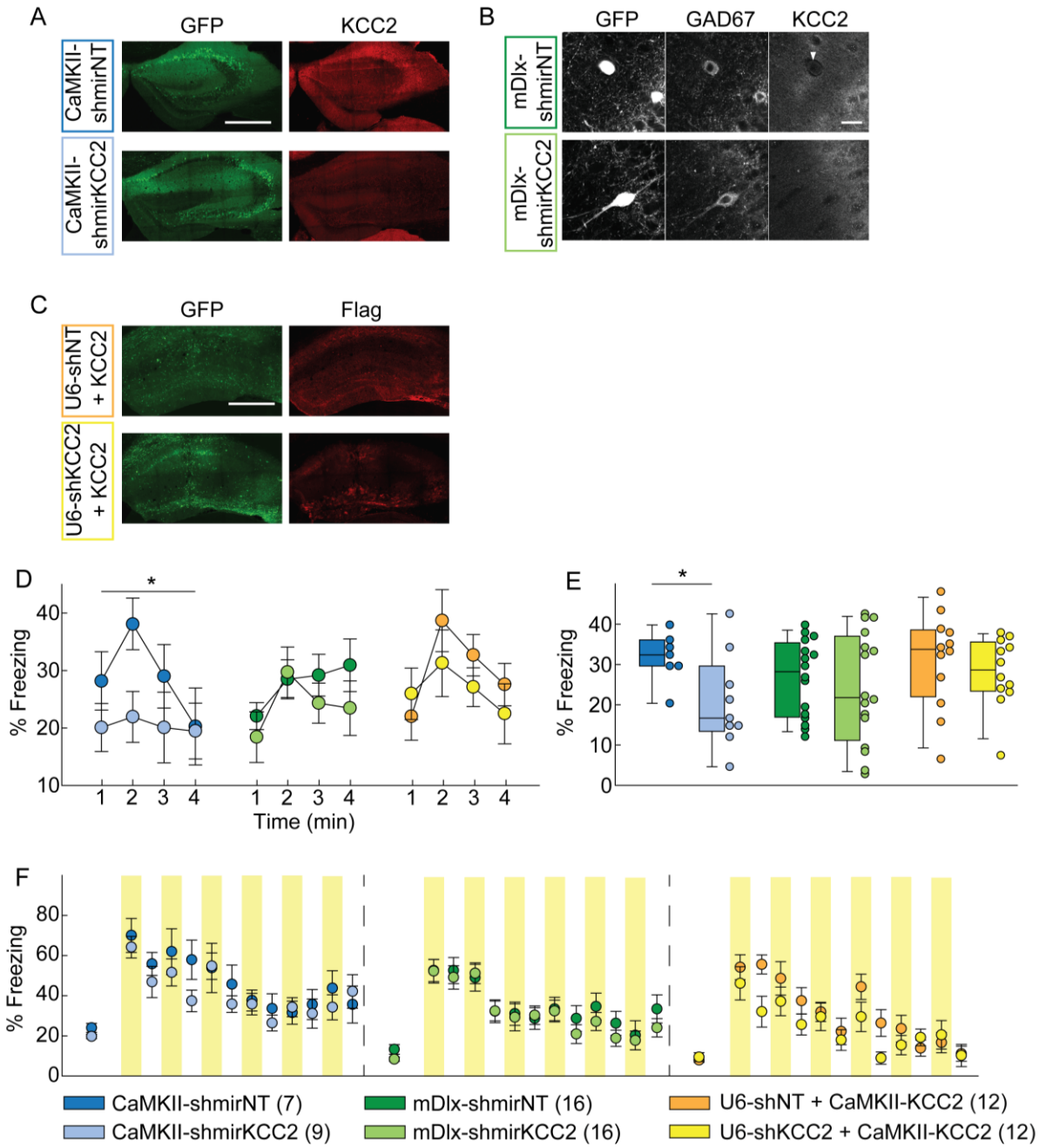


Figure 20. KCC2 down-regulation in principal cells is sufficient to alter contextual memory

A. Confocal micrographs of hippocampal coronal sections immunostained for GFP and KCC2 from mice infected with CaMKII-shNT or CaMKII-shKCC2-expressing vectors, showing massive KCC2 knockdown in the later. Scale: 500 μ m. **B.** Confocal micrographs of CA1 area in hippocampal coronal sections immunostained for GFP, GAD67 and KCC2 from mice infected with vectors expressing mDlx-shmirNT or mDlx-shmirKCC2. Arrowheads show that all infected cells are GAD67+ interneurons and that mDlx-shmirKCC2 efficiently suppressed KCC2 expression in these cells. Scale: 20 μ m. **C.** Confocal micrographs as in A immunostained for GFP and Flag from mice infected with U6-shNT or U6-shKCC2 and CamKII-KCC2flag expressing vectors showing both vectors are expressed together. Scale : 500 μ m. **D.** Time course of freezing during the first 4 minutes of exploration of the foot-shock associated context, showing reduced freezing in mice infected with CaMKII-shmirKCC2 expressing vector as compared with mice infected with CaMKII-shmirNT vector (two-way ANOVA, Bonferonni post-hoc test, $p=0.020$). Mice infected with mDlx-shmirKCC2 vector or with U6-shKCC2 and CamKII-KCC2 show no alteration of freezing behavior as compared to control mice. **E.** Summary data for the 3 first minutes of exploration (t-test, one-tailed $p=0.019$ for CaMKII-shRNA) **F.** Cued memory retention with no difference between groups. * $p < 0.05$; n.s. non significant.

All statistical tests and data are in Table 4.

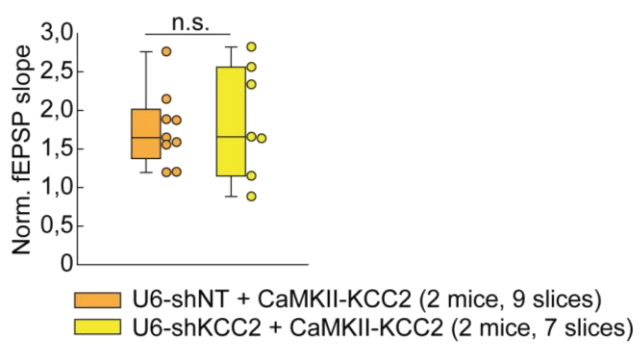
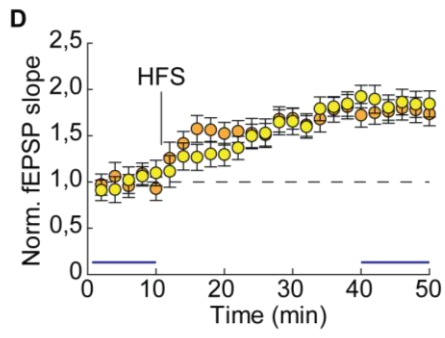
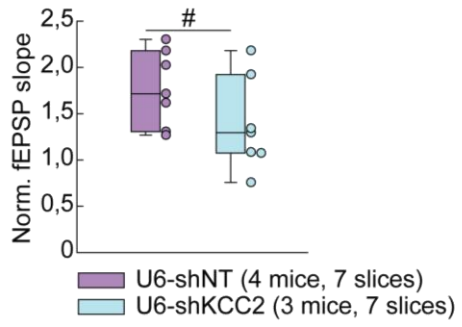
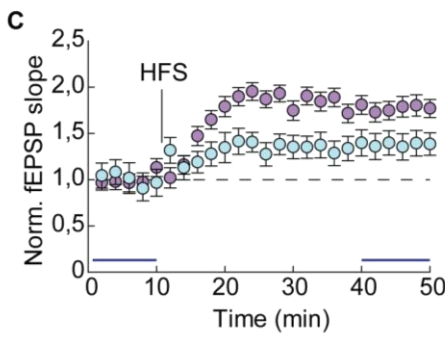
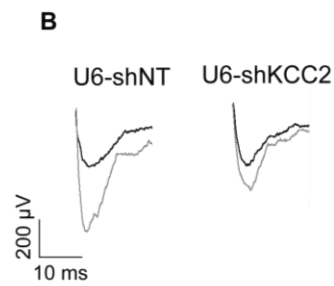
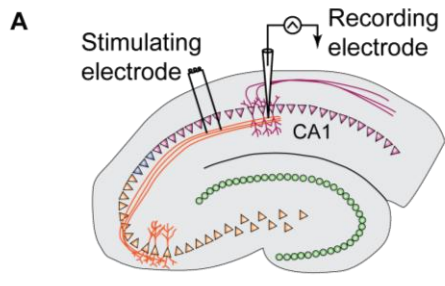


Figure 21. KCC2 knockdown impairs hippocampal LTP

A. Hippocampal schema with the position of the stimulating and recording electrodes. **B.** Average of 60 consecutive fEPSPs evoked in CA1 upon stimulation of Schaffer collaterals before (black) and 40 min after (grey) high-frequency train (HFS, 5×1 s at 100 Hz) . **C.** Left, Time course of changes in fEPSP slope upon HFS in slices from mice infected with U6-shNT or U6-shKCC2 vectors. Blue bars represent the time windows used for averages. Right, Summary data of fEPSP slope 40 min after HFS normalized to the mean of 10 min baseline. LTP expression in slices from mice infected with U6-shKCC2 vector is reduced compared to control slices from mice infected with U6-shNT vector (t-test, one-tailed $p = 0.067$). **D.** Same as in B for recordings performed from mice infected with U6-shKCC2 vs U6-shNT together with CaMKII-KCC2flag vectors. Restoring KCC2 expression in principal cells only in KCC2 knockdown mice fully rescued LTP expression.

$p < 0.1$; n.s. non significant

All statistical tests and data are in Table 4.

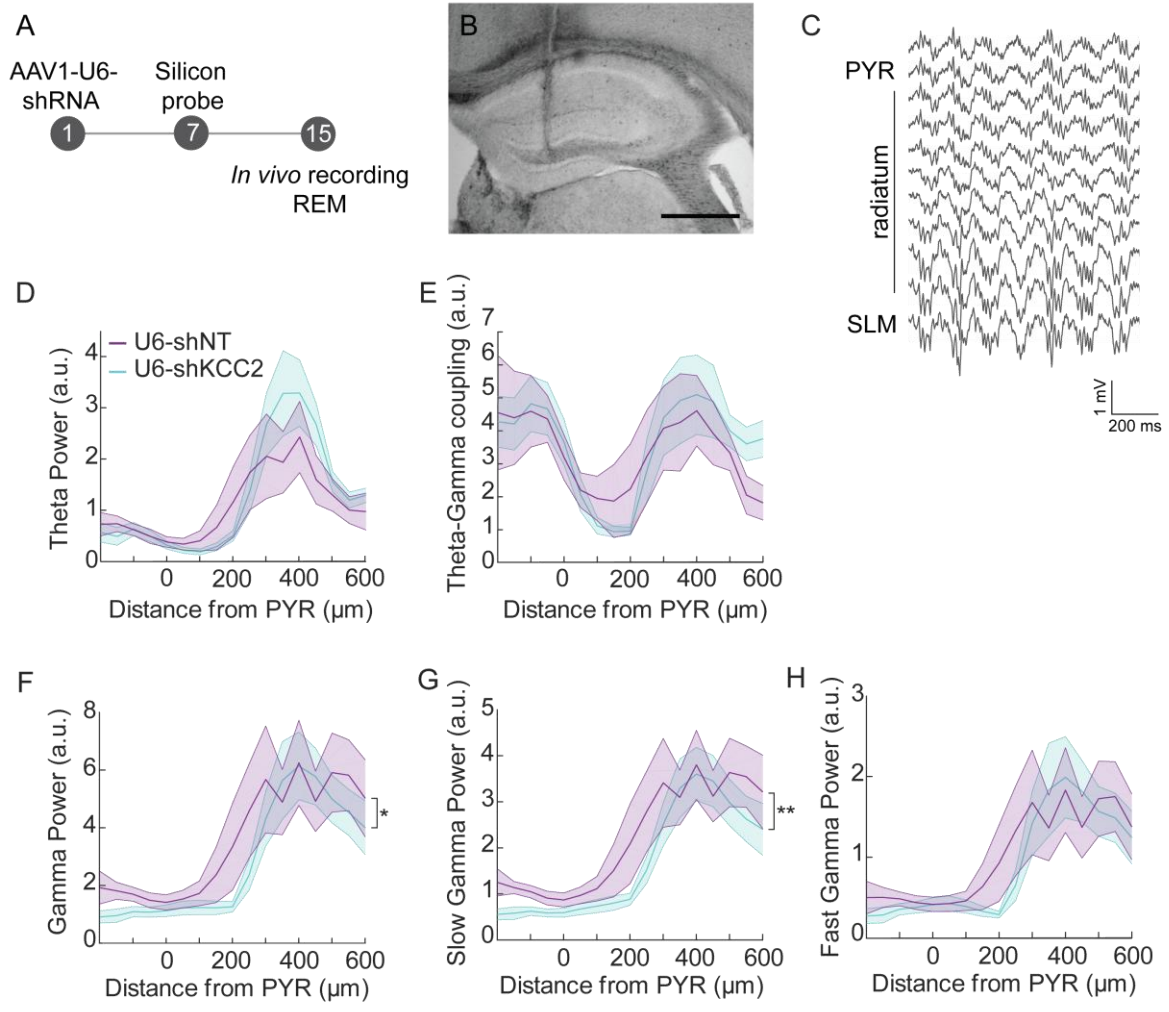


Figure 22. Effect of KCC2 knockdown on hippocampal rhythmogenesis during REM sleep

A. Timeline of the experiment. **B.** Wide field microscope image of dorsal hippocampus near implantation site showing the track of the implanted 32-channel linear probe. Scale: 500 μm . **C.** Example of REM sleep recordings in mice infected with U6-shNT expressing vectors. **D.** Theta power in the 5-10 Hz frequency band measured during REM sleep using multi-tapers estimates. Power (in arbitrary units, a.u.) is plotted as a function of the electrode localization with respect to *st. pyramidale* (PYR). Theta power profile was not affected upon KCC2 knockdown (Kruskal-Wallis, $p=0.774$). **E.** Theta-gamma coupling was not significantly affected following KCC2 knockdown (Kruskal-Wallis, $p=0.558$). **F-H.** Gamma (25-90 Hz, **F**), slow gamma (25-55 Hz, **G**) and fast gamma (60-90 Hz, **H**) power plotted as a function of the electrode localization with respect to *st. pyramidale* (PYR). Gamma power was reduced upon KCC2 knockdown (Kruskal-Wallis, $p=0.012$), mostly as a result of reduced slow gamma (Kruskal-Wallis, $p=0.003$) but not fast gamma power (Kruskal-Wallis, $p=0.269$).

* $p<0.05$; ** $p<0.01$

All statistical tests and data are in Table 4.

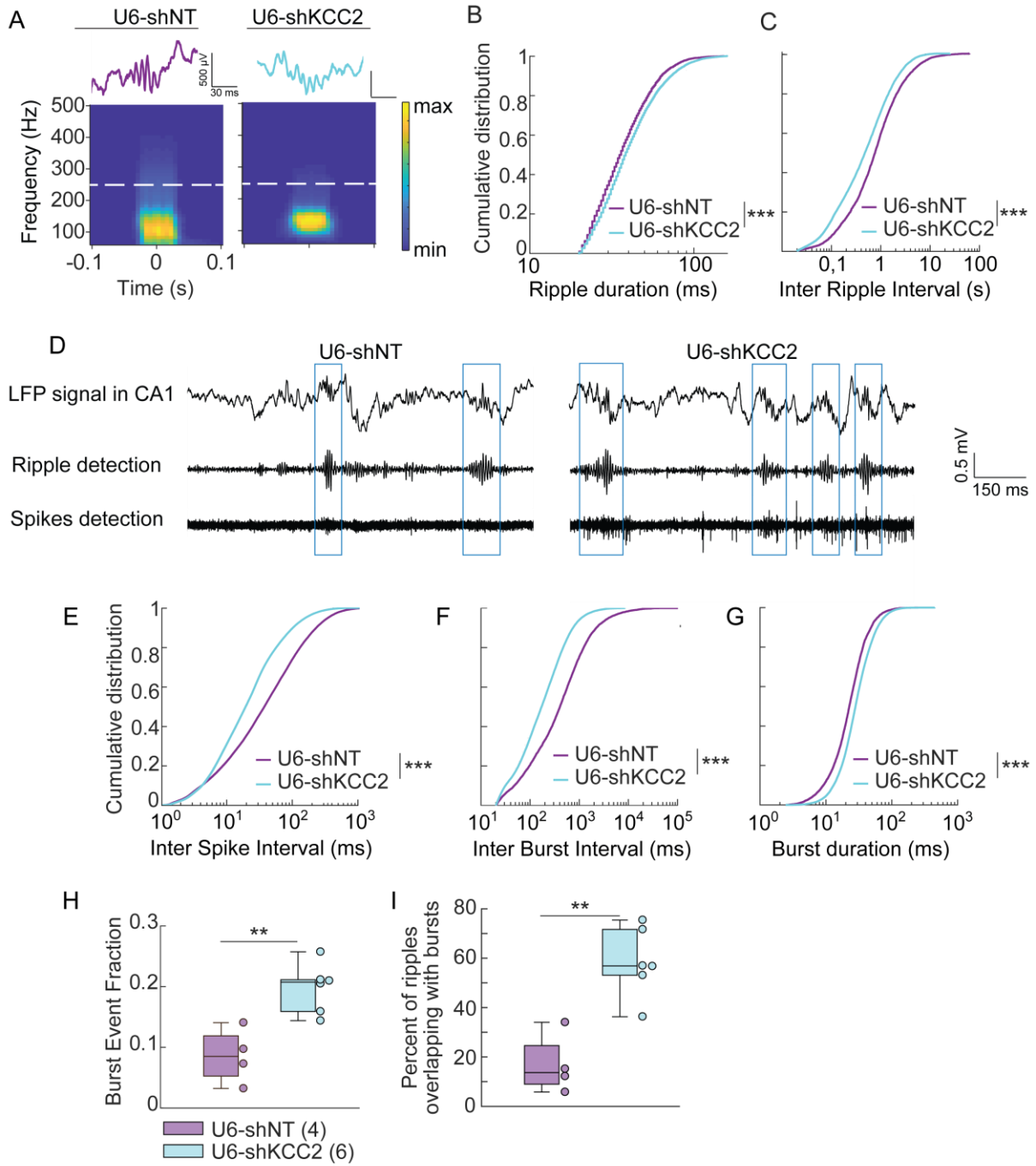
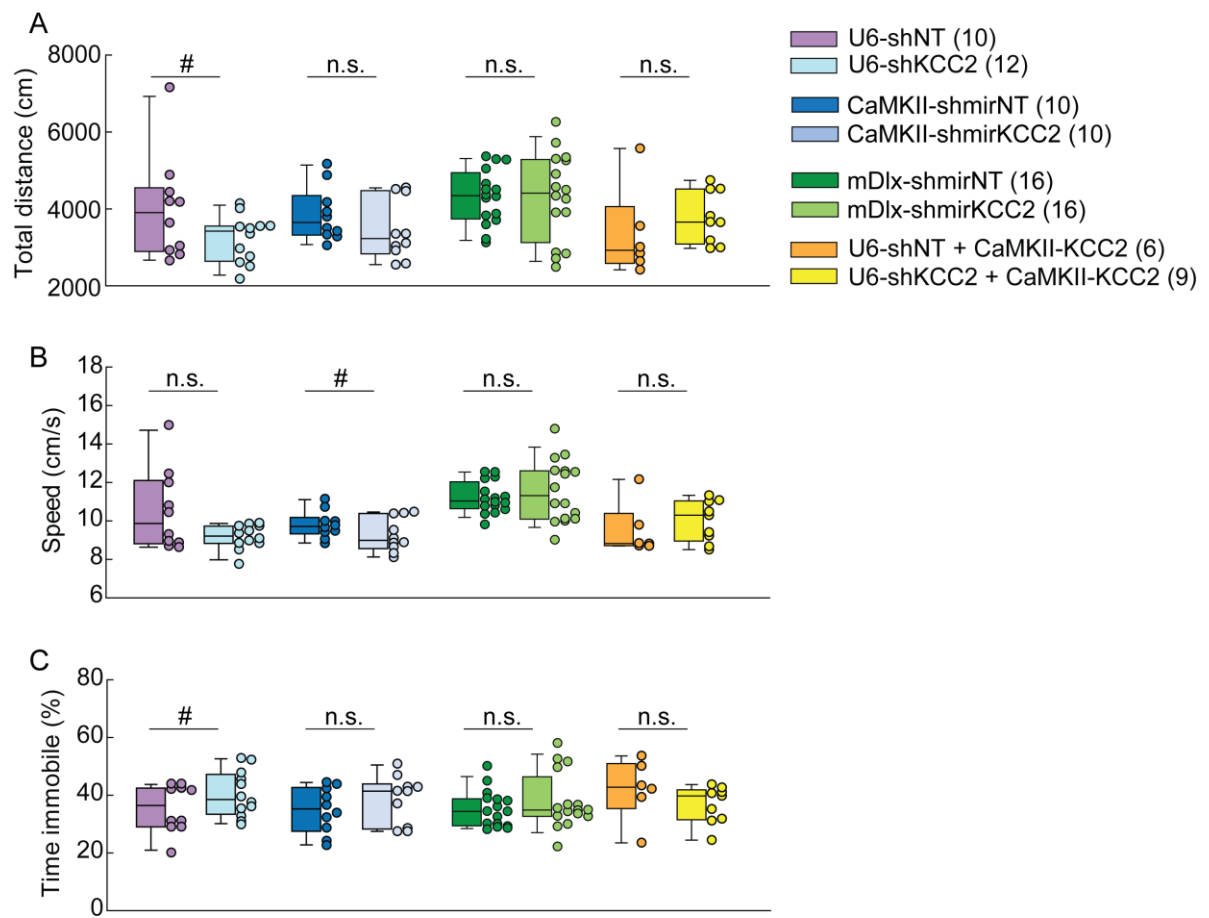


Figure 23. KCC2 knockdown induces hyperexcitability in CA1 and altered SPW-Rs

A. Top, representative recordings of individual ripples from mice infected with U6-shNT or U6-shKCC2 expressing vectors. Bottom, spectrograms showing the average frequency over time of all ripples in recordings from 4 control and 5 KCC2 knockdown mice. **B-C,** Cumulative histograms of ripple duration (**B**) and inter event interval (**C**). Upon KCC2 knockdown, ripple duration and frequency significantly increased (Kolmogorov–Smirnov test, $p < 0.001$ for both conditions) **D.** Traces examples of LFP signal recorded in CA1 *st.pyramidale* mice infected with U6-shNT or U6-shKCC2 expressing vectors. The signal was filtered to detect ripples (boxed in blue, 100-300 Hz) or multiunit activity (> 500 Hz). **E-G.** Cumulative histograms of inter-spike (**E**) and inter-burst (**F**) intervals as well as burst duration (**G**). MUA frequency as well as burst frequency and duration were increased in KCC2 mice (Kolmogorov–Smirnov test, $p < 0.001$ for all three conditions). **H-I.** Boxplots showing the distribution of burst event fraction (number of bursts relative to the number of spike trains (**H**) and proportion of ripples associated with bursts of MUA activity (**I**). Both were significantly increased upon KCC2 knockdown (Mann-Whitney, $p = 0.010$ and $p = 0.010$, respectively).

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

All statistical tests and data are in Table 4.

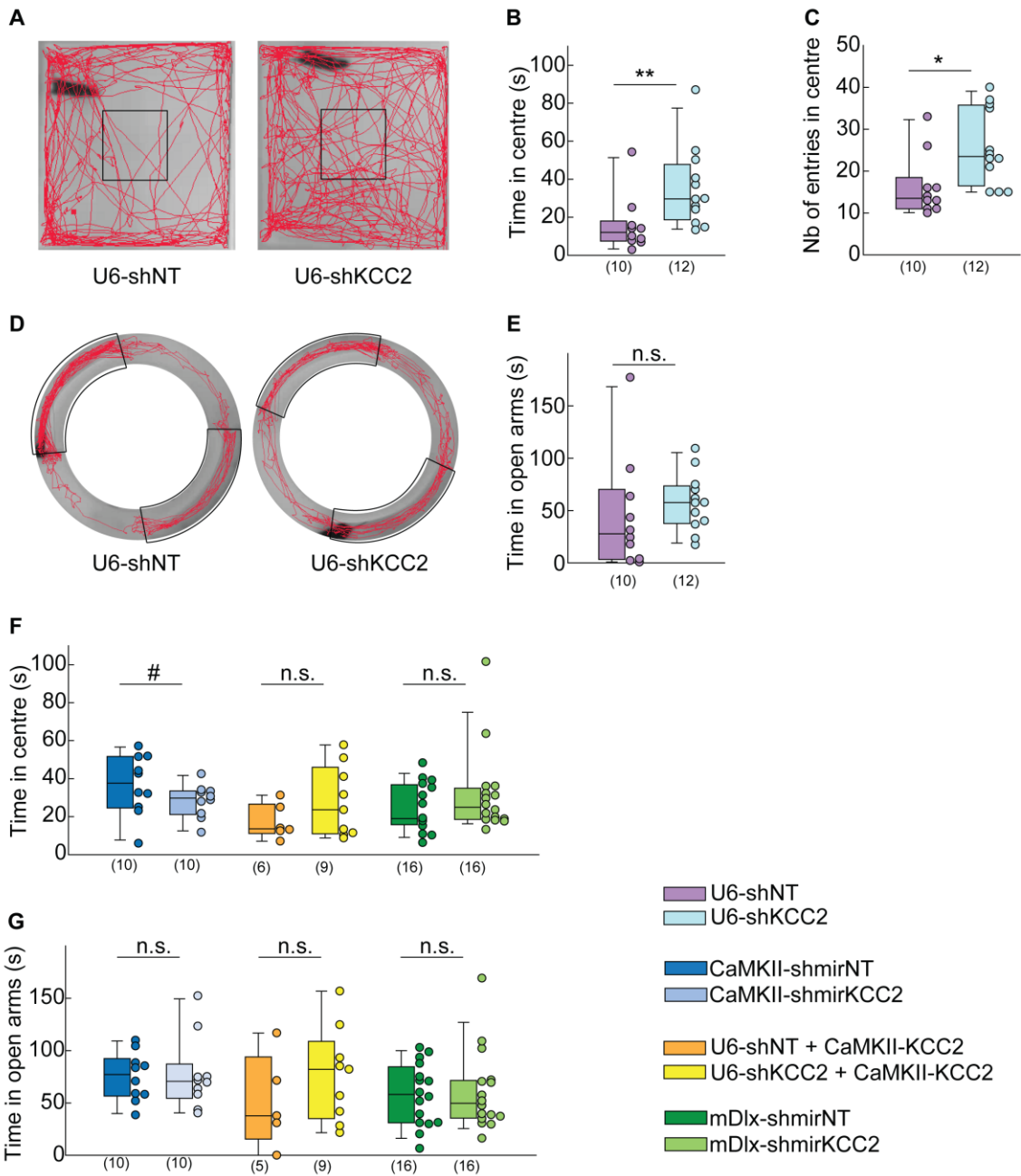


Supplementary Figure 1. Locomotor activity is not altered upon KCC2 down-regulation

A. Distribution of total distance traveled during 10 minutes exploration of an open field by mice infected with various vectors shown on the right. Number of animals are shown in brackets. No significant difference was observed between test and control groups. **B.** Distribution of running speed (total distance/time in movement) for the same groups as in A. Again, no significant difference was observed between test and control groups. **C.** Distribution of immobility time for the same groups as in A and B, again showing no significant difference between test and control mice.

$p < 0,1$; n.s. non significant.

All statistical tests and data are in Table 4.

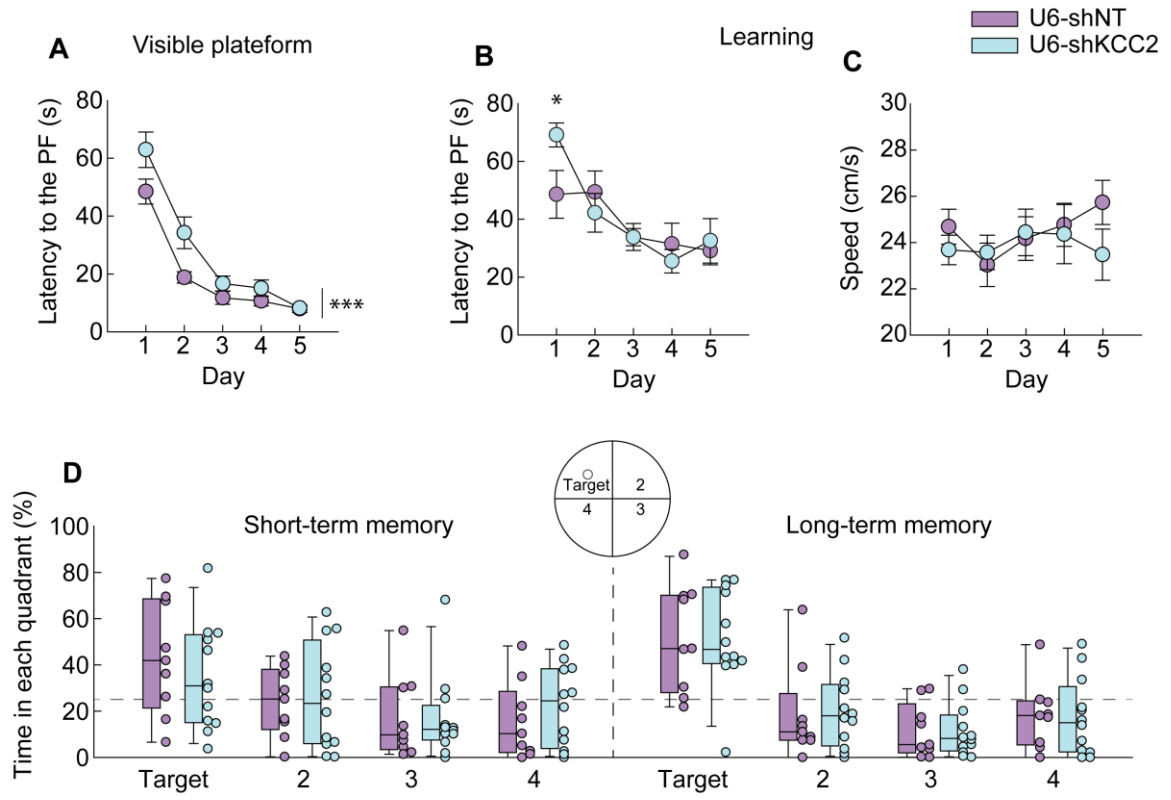


Supplementary Figure 2. Evaluation of anxiety upon KCC2 down-regulation

A. Representative trajectories of individual mice in the open field. The center square corresponds to 1/9 of the total surface of the arena. **B-C.** Distributions of time spent in the center (B) and number of entries into the center square (C). Mice infected with U6-shKCC2 expressing vector (cyan) spent more time in the center than control mice (purple, Mann-Whitney, $p=0.006$) and showed an increased number of entries in the center square (Mann-Whitney, $p=0.013$). **D.** Representative trajectories of individual mice in the elevated O-maze. Boxed areas correspond to closed arms. **E.** Distribution of time spent in open arms showing no difference between KCC2 knockdown and control mice (Mann-Whitney, $p=0.176$). **F-G.** same as in B and E, respectively, for mice infected with various vectors shown on the right. No significant difference was observed between test and control mice for any of these vectors.

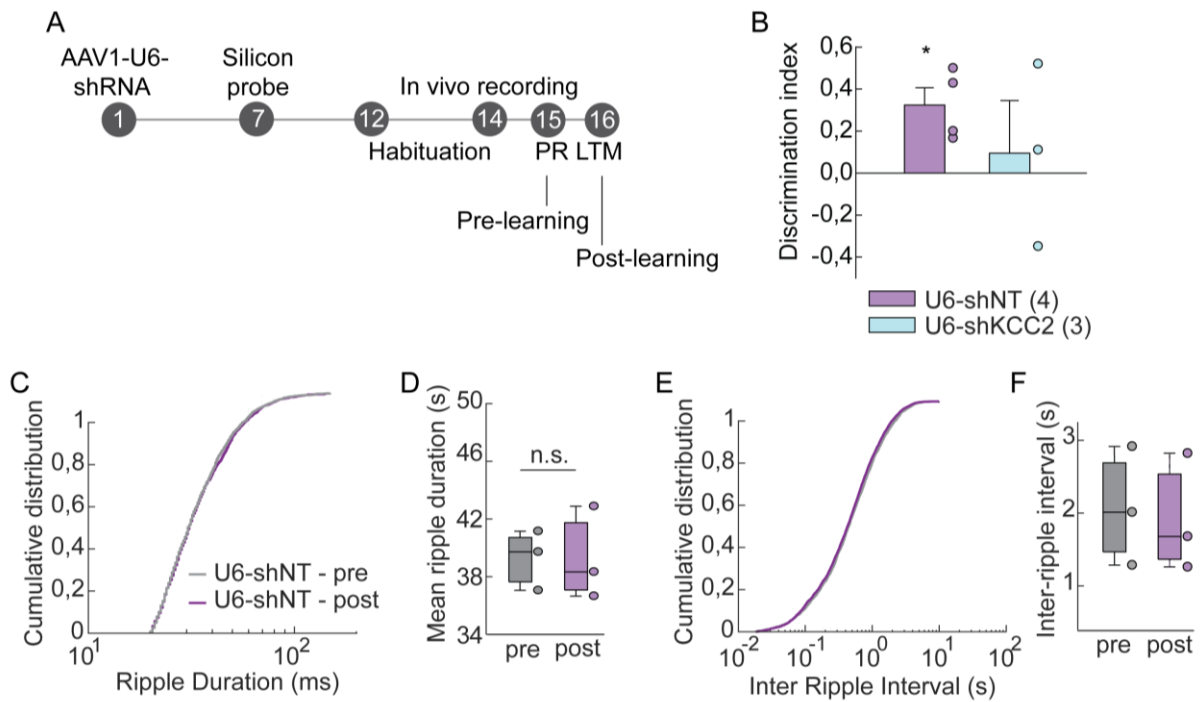
** $p < 0.01$; * $p < 0.05$; # $p < 0.1$; n.s. non significant

All statistical tests and data are in Table 4.



Supplementary Figure 3. KCC2 down-regulation in neurons does not affect spatial memory in Morris water maze

A. Latency to reach to the visible platform plotted against session number. Mice infected with U6-shKCC2 expressing vector show delayed learning as compared to control mice (two-way ANOVA, $p < 0.001$). **B.** Same as in A for hidden platform. On day 1, mice infected with U6-shKCC2 expressing vector show increased latency to reach the platform as compared to control mice (t-test, one-tailed $p = 0.039$). However, overall, there is no difference in the learning process between both groups (two-way ANOVA, $p = 0.851$). **C.** Swimming speed plotted against session number. No difference was observed between the two groups (two-way ANOVA, $p = 0.104$). **D.** Distribution of time spent in each quadrant in probe trials performed 30 minutes (short term) or 72 hours (long term) after training, for mice infected with U6-shKCC2 (cyan) or U6-shNT (purple) expressing vectors. All mice spent more time exploring the target quadrant (t-test one-tailed, short term : $p = 0.216$; long term : $p = 0.441$). All statistical tests and data are in Table 4.



Supplementary Figure 4. Compared SPW-R properties before and after learning

A. Timeline of this experiment. Intra-hippocampal recordings were performed just before and right after spatial memory task (place recognition). **B.** Distributions of discrimination index showing KCC2 knockdown mice show no preference for the moved object as compared to control mice (one-sample t-test, $p=0.015$). **C-D,** Cumulative distribution (C) and mean (D) ripple duration before and after learning in control mice, showing no significant difference. **E-F.** Cumulative distribution (E) and mean (F) inter-ripple intervals for the same experiments as in C-D, showing again no significant difference before and after learning.

* $p<0.05$; n.s. non significant

All statistical tests and data are in Table 4.

Table 4. Statistical data of project 1

		Number of mice	Type of data	Transformation of data	Statistical test	p value		Power	F, t, z	Post-hoc test or supplementary information
Figure 19A		3 (uninf.) vs 3 (shNT)	Ratio	no	t-test, one tailed p value	0.388		0.083	-0.305	
	U6-shRNA	3 (shNT) vs 3 (shKCC2)	Ratio	no	t-test, one tailed p value	<0.001	***	1	11.931	
Figure 19D	U6-shRNA	8 (shNT) vs 9 (shKCC2)	Ratio	no	t-test, one tailed p value	0.017	*			
		8 (shNT)	Ratio	no	one-sample t-test, one tailed p	0.005		0.929	3.471	
		9 (shKCC2)	Ratio	no	one-sample t-test, one tailed p	0.423		0.072	-0.201	
	U6-shRNA	10 (shNT) vs 12 (shKCC2)	Percent	arcsin	Two-way ANOVA	0.021			5.441	Bonferonni
Figure 19F	U6-shRNA	10 (shNT) vs 12 (shKCC2)	Percent	arcsin	Two-way ANOVA	0,006	**		2,819	Bonferonni
Figure 19G	U6-shRNA	10 (shNT) vs 12 (shKCC2)	Percent	arcsin	t-test, one tailed p value	0,050	*	0,507	1,721	
Figure 19H	U6-shRNA	9 (shNT) vs 12 (shKCC2)	Percent	arcsin	Two-way ANOVA	0,208			1,592	
Figure 20D	CaMKII-shRNA	7 (shNT) vs 9 (shKCC2)	Percent	arcsin	Two-way ANOVA	0,020	*		5,718	Bonferonni
	mDlx-shRNA	16 (shNT) vs 16 (shKCC2)	Percent	arcsin	Two-way RM ANOVA	0.267			1.277	
	U6-shRNA + KCC2	12 (shNT) vs 12 (shKCC2)	Percent	arcsin	Two-way RM ANOVA	0.424			0.665	
Figure 20E	CaMKII-shRNA	7 (shNT) vs 9 (shKCC2)	Percent	arcsin	t-test, one tailed p value	0,019	*	0,702	0,139	
	mDlx-shRNA	16 (shNT) vs 16 (shKCC2)	Percent	arcsin	t-test, one tailed p value	0,215		0,194	0,799	
	U6-shRNA + KCC2	12 (shNT) vs 12 (shKCC2)	Percent	arcsin	Mann-Whitney	0,402				
Figure 20F	CaMKII-shRNA	7 (shNT) vs 9 (shKCC2)	Percent	arcsin	Two-way ANOVA	0,072	#			Equal Variance Test failed
	mDlx-shRNA	14 (shNT) vs 16 (shKCC2)	Percent	arcsin	Two-way ANOVA	0,235				Normality test failed
	U6-shRNA + KCC2	12 (shNT) vs 12 (shKCC2)	Percent	arcsin	Two-way RM ANOVA	0.259			1.342	
Figure	U6-shRNA	7 sl. (4 shNT) vs 7 sl. (3	ratio	no	t-test, one-tailed p value	0,067	#	0,449	1,607	

21C		shKCC2)								
Figure 21D	U6-shRNA + KCC2	9 sl. (2 shNT) vs 7 sl. (2 shKCC2)	ratio	no	t-test, one-tailed p value	0,37		0,093	-0,34	
Figure 22D	U6-shRNA	5 (shNT) vs 6 (shKCC2)	arbitrary units	no	Kruskal-Wallis	0,774				
Figure 22F	U6-shRNA	5 (shNT) vs 6 (shKCC2)	arbitrary units	no	Kruskal-Wallis	0,558				
Figure 22G	U6-shRNA	5 (shNT) vs 6 (shKCC2)	arbitrary units	no	Kruskal-Wallis	0,012	*			
Figure 22H	U6-shRNA	5 (shNT) vs 6 (shKCC2)	arbitrary units	no	Kruskal-Wallis	0,003	**			
Figure 22I	U6-shRNA	5 (shNT) vs 6 (shKCC2)	arbitrary units	no	Kruskal-Wallis	0,269				
Figure 23B	U6-shRNA	4 (shNT) vs 6 (shKCC2)	Measure (ms)	no	Kolmogorov–Smirnov	<0,001	***			
Figure 23C	U6-shRNA	4 (shNT) vs 6 (shKCC2)	Measure (s)	no	Kolmogorov–Smirnov	<0,001	***			
Figure 23E	U6-shRNA	4 (shNT) vs 6 (shKCC2)	Measure (ms)	no	Kolmogorov–Smirnov	<0,001	***			
Figure 23F	U6-shRNA	4 (shNT) vs 6 (shKCC2)	Measure (ms)	no	Kolmogorov–Smirnov	<0,001	***			
Figure 23G	U6-shRNA	4 (shNT) vs 6 (shKCC2)	Measure (ms)	no	Kolmogorov–Smirnov	<0,001	***			
Figure 23H	U6-shRNA	4 (shNT) vs 6 (shKCC2)	Ratio	no	Mann-Whitney	0,010	**			
Figure 23I	U6-shRNA	4 (shNT) vs 6 (shKCC2)	Percent		Mann-Whitney	0,010	**			

Figure S1A	U6-shRNA	10 (shNT) vs 12 (shKCC2)	Measure (cm)	no	Mann-Whitney	0,093	#			
	CaMKII-shRNA	10 (shNT) vs 10 (shKCC2)	Measure (cm)	no	Mann-Whitney	0,162				
	mDlx-shRNA	16 (shNT) vs 16 (shKCC2)	Measure	no	t-test, one tailed p value	0,483		0,054	-0,043	

			(cm)							
	U6-shRNA + KCC2	6 (shNT) vs 9 (shKCC2)	Measure (cm)	no	Mann-Whitney	0,141				
Figure S1B	U6-shRNA	10 (shNT) vs 12 (shKCC2)	Measure (cm/s)	no	Mann-Whitney	0,249				
	CaMKII-shRNA	10 (shNT) vs 10 (shKCC2)	Measure (cm/s)	no	t-test, one tailed p value	0,075	#	0,422	1,504	
	mDlx-shRNA	16 (shNT) vs 16 (shKCC2)	Measure (cm/s)	no	Mann-Whitney	0,720				
	U6-shRNA + KCC2	6 (shNT) vs 9 (shKCC2)	Measure (cm/s)	no	t-test, one tailed p value	0,221		0,186	-0,791	
Figure S1C	U6-shRNA	10 (shNT) vs 12 (shKCC2)	Percent	arcsin	t-test, one tailed p value	0,079	#	0,411	-1,47	
	CaMKII-shRNA	10 (shNT) vs 10 (shKCC2)	Percent	arcsin	t-test, one tailed p value	0,151		0,267	-1,062	
	mDlx-shRNA	16 (shNT) vs 16 (shKCC2)	Percent	arcsin	Mann-Whitney	0,534				
	U6-shRNA + KCC2	6 (shNT) vs 9 (shKCC2)	Percent	arcsin	t-test, one tailed p value	0,132		0,295	1,165	
Figure S2B	U6-shRNA	10 (shNT) vs 12 (shKCC2)	Measure (s)	no	Mann-Whitney	0,006	**			
Figure S2C	U6-shRNA	10 (shNT) vs 12 (shKCC2)	Number	no	Mann-Whitney	0,013	*			
Figure S2E	U6-shRNA	10 (shNT) vs 12 (shKCC2)	Measure (s)	no	Mann-Whitney	0,176				
Figure S2F	CaMKII-shRNA	10 (shNT) vs 10 (shKCC2)	Measure (s)	no	t-test, one tailed p value	0,081	#	0,404	1,456	
	mDlx-shRNA	16 (shNT) vs 16 (shKCC2)	Measure (s)	no	Mann-Whitney	0,386				
	U6-shRNA + KCC2	12 (shNT) vs 12 (shKCC2)	Measure (s)	no	Mann-Whitney	0,444				
Figure S2G	CaMKII-shRNA	6 (shNT) vs 9 (shKCC2)	Measure (s)	no	t-test, one tailed p value	0,434		0,069	-0,169	
	mDlx-shRNA	16 (shNT) vs 16 (shKCC2)	Measure (s)	no	Mann-Whitney	0,895				
	U6-shRNA + KCC2	5 (shNT) vs 9 (shKCC2)	Measure (s)	no	t-test, one tailed p value	0,165		0,247	-1,017	
Figure S3A	U6-shRNA	9 (shNT) vs 12 (shKCC2)	Measure (s)	no	Two-Way ANOVA	<0,001				normality and equal variance failed
Figure S3B	U6-shRNA	9 (shNT) vs 12 (shKCC2)	Measure (s)	no	Two-way ANOVA	0,851				Normality test failed
Figure S3C	U6-shRNA	9 (shNT) vs 12 (shKCC2)	Measure (cm/s)	no	Two-way ANOVA	0,104			2,699	
Figure S3D	STM - target	9 (shNT) vs 12 (shKCC2)	Percent	arcsin	t-test, one-tailed p value	0,216		0,192	0,802	

	STM	9 (shNT) vs 12 (shKCC2)	Percent	arcsin	Two-way ANOVA	0,996		3E-05		
	LTM - target	9 (shNT) vs 12 (shKCC2)	Percent	arcsin	t-test, one-tailed p value	0,441		0,067	0,152	
	LTM	9 (shNT) vs 12 (shKCC2)	Percent	arcsin	Two-way ANOVA	0,956		0,003		
Figure S4B	U6-shRNA	4 (shNT)	ratio	no	one-sample t-test, one tailed p	0,015	*		3,916	
	U6-shRNA	3 (shKCC2)	ratio	no	one-sample t-test, one tailed p	0,371			0,377	
	U6-shRNA	4 (shNT) vs 3 (shKCC2)	ratio	no	Mann-Whitney	0,629				
Figure S4D	U6-shRNA	3 (shNT) vs 3 (shKCC2)	Measure (s)	no	t-test	1				
Figure S4E	U6-shRNA	3 (shNT) vs 3 (shKCC2)	Measure (s)	no	t-test	1				

II. Part 2: Rescuing KCC2 function/expression in a mouse model of Rett syndrome

In my first project, I showed that KCC2 knockdown in dorsal hippocampal neurons was sufficient to impair spatial and contextual memory in mice. In addition, I was able to rescue contextual memory by restoring KCC2 expression in principal cells only in KCC2 knockdown mice. In line with this experiment, we then hypothesized that restoring KCC2 expression in a mouse model of pathology involving KCC2 suppression might rescue some of the memory deficits associated with the pathology. To this end, we decided to use a mouse model of Rett syndrome (RTT), a genetic pathology involving motor and cognitive impairment (Chahrour and Zoghbi, 2007) and associated with KCC2 down-regulation both in patients and patient-derived iPSCs (Duarte et al., 2013; Tang et al., 2016).

1. KCC2 over-expression in MecP2³⁰⁸ males rescues LTP

Different mouse models of RTT have been generated, with various mutations in the *MECP2* gene located on the X chromosome (see Introduction II.3.a). We first decided to work with the MecP2^{308/Y} mouse model, expressing a truncated MecP2 protein, and displaying a milder phenotype than the full knockout animals (Guy et al., 2001; Stearns et al., 2007). Thus, while breeding normally, MecP2^{308/Y} males are known to exhibit motor and cognitive deficits from 5 months of age (De Filippis et al., 2010; Moretti et al., 2006). In particular, MecP2^{308/Y} mice were shown to display spatial and contextual memory deficits as well as impaired LTP and synaptic function (Moretti et al., 2006).

As discussed above (see Introduction II.3 and Table 1), several arguments suggested KCC2 may be down-regulated in RTT patients, but also in the neocortex of MecP2 KO mice (Banerjee et al., 2016). We therefore first tested whether KCC2 expression was also down-regulated in the hippocampus of MecP2^{308/Y} mice. Western blot analysis performed on hippocampal extracts from 4 animals from 2 different litters indeed revealed reduced KCC2 expression in mutant mice compared to WT controls (Figure 24A).

Next, we explored LTP expression at Schaffer collateral inputs onto CA1 pyramidal neurons, in the presence of the GABAAR antagonist bicuculline, as above. As previously described (Moretti et al., 2006), we found reduced LTP expression in slices from MecP2^{308/Y} mice as compared to WT littermates, even though the difference did not reach statistical significance (Figure 24B-C; t-test, one-tailed $p=0.072$). We then tested whether over-expressing KCC2 in principal cells of MecP2^{308/Y} mice might rescue LTP expression. In slices from MecP2^{308/Y} mice infected with AAV1-CaMKII-KCC2flag vector, the amplitude of LTP was indistinguishable from that of WT, uninfected littermates (Figure 24B-C, t-test, one-tailed $p=0.462$). These results suggest that over-expressing KCC2 in principal neurons of MecP2^{308/Y} mice might be sufficient to rescue hippocampal LTP.

2. Treatments to restore chloride homeostasis or KCC2 stability in MecP2^{308/Y} mice

Based on these initial observations and the results presented in the previous section of this thesis, we hypothesized that restoring KCC2 expression or function in MecP2^{308/Y} mice may rescue some cognitive deficits associated with this RTT mouse model. Two strategies were then considered: either restoring neuronal chloride homeostasis only, using the loop diuretic and NKCC1 antagonist bumetanide (as in Banerjee et al., 2016), or promoting KCC2 membrane expression using the recently described KCC2 enhancer CLP-290 (Gagnon et al 2013). Since KCC2 down-regulation impairs LTP independent of its transport-function (Chevy et al., 2015), our hypothesis was that CLP-290 would more likely compensate for the cognitive deficits in MecP2^{308/Y}.

Mice were treated daily with one intraperitoneal (i.p) injection and tested for the motor and cognitive deficits previously reported in MecP2^{308/Y} mice (De Filippis et al., 2010; Moretti et al., 2006). The following data are preliminary and were collected in order to test the validity of our hypothesis. As I was establishing the MecP2³⁰⁸ strain in the lab and the number of mice available was initially limited, the same mice were used to assess the effect of both bumetanide and CLP-290, at least one month apart. Thus, 5-6 month old mice first received an injection of bumetanide in DMSO (0.2 mg/kg) or DMSO alone as a control, and their behavior was tested 1 to 4 hours later (as in Deidda et al., 2015). At least one month after the

last experiment, mice received a daily injection of CLP-290 in HPCD (100 mg/kg) or HPCD alone as a control, and behavior was tested from the 6th day, within 2 to 6 hours of the last injection (as in Chen et al., 2018; Gagnon et al., 2013). In order to control for early effect of CLP-290 treatment, we recorded the locomotor activity and grip strength over the five first days of treatment (Figure S5). However, we did not observe any improvement in MecP2^{308/Y} mice.

5 month-old MecP2^{308/Y} mice initially showed increased locomotion and reduced immobility time as compared to WT littermates (Figure 25A-C, see Table 5 for statistical data). This difference, however, was still observed, yet to a lesser extent, upon bumetanide injection and was not observed in the same animals tested one month later for CLP-290 or with the control HPCD. MecP2^{308/Y} showed no difference in motor coordination, as evaluated in rotarod test (Figure 25E). More consistent, however, were strength deficits in the grip test (Figure 25D). In this test, MecP2^{308/Y} mice showed significantly reduced latency to fall from the grid as compared to WT littermates (Mann-Whitney, $p=0.004$ and 0.016 , for mice treated with DMSO or HPCD, respectively). However, again, these deficits were not improved upon bumetanide injection or chronic CLP-290 treatment (Figure 25D, t-test, one tailed $p<0.001$ and $p=0.002$ for mice treated with bumetanide or CLP-290, respectively).

MecP2^{308/Y} mice have been previously reported to display anxiety-like deficits and contextual memory alteration in the fear-conditioning paradigm (Moretti et al., 2006). We therefore wished to test whether bumetanide may alleviate these deficits. We first tested MecP2^{308/Y} mice for anxiety in the open field and elevated O-maze (Figure 26A-B). MecP2^{308/Y} showed slightly reduced anxiety in the open field, as estimated by increased time spent in the center (t-test, one-tailed $p=0.017$), and this difference was abolished in bumetanide-injected animals. However, it was not observed in the elevated O-maze (Figure 26B, t-test, one-tailed $p=0.482$). Surprisingly, our result in the open field is the opposite of the one previously reported (Moretti et al., 2006). Animals were then tested for contextual retention memory (Figure 26C-D). Over 4 minutes of exploration of the foot-shock associated cage, freezing increased in all mice. However, unlike previous reports (Moretti et al., 2006) freezing in MecP2^{308/Y} mice was significantly increased, not decreased, as compared to that of WT littermates both during the first 4-minutes exploration (two-way RM ANOVA, $p=0.024$) or during only the first 3 minutes (t-test, one-tailed $p=0.021$). Moreover, in the cued memory retention test, MecP2^{308/Y} mice exhibited increased freezing response as compared to WT littermates (Figure 26E, two-way ANOVA, $p<0.001$). Overall, these results suggest

MecP2^{308/Y} mice do not show major deficits in contextual or cued memory retention, in contrast to previously reported observations (Moretti et al., 2006).

In summary, my results show that MecP2^{308/Y} mice may have a much milder phenotype than previously reported. In addition, in contrast to recombinant human IGF1 which rescued both KCC2 expression and sensory information processing in full MecP2 mice (Banerjee et al., 2016), neither bumetanide or CLP-290 rescued the most prominent deficits (grip strength) observed in MecP2^{308/Y} mice. This discrepancy led us to re-examine KCC2 expression more thoroughly in cortex and hippocampus in these mice. In contrast to our initial observations, in a larger sample, we no longer detected significant reduction in KCC2 expression in MecP2^{308/Y} mice as compared to WT littermates (Figure 27). In fact, KCC2 expression was slightly increased in the cortex of MecP2^{308/Y} mice (t-test, one-tailed $p=0.065$). This is in striking contrast with results obtained in full MecP2 KO mice (Banerjee et al., 2016) and suggests truncation of MecP2 protein at residue 308 may not suffice to significantly affect KCC2 expression. The molecular substrate for such differential regulation of KCC2 expression by various MecP2 mutant proteins will be further discussed later in this manuscript (see Discussion III).

3. Development of a new model of MecP2 deficiency

MecP2 full KO mice are reputedly very difficult to breed and display a very complex phenotype associating motor, sensory and cognitive deficits (Guy et al., 2001; Stearns et al., 2007). Exploring spatial and/or contextual memory deficits in these mice is then complicated by interference with both sensory and motor deficits. On the other hand, I showed that, contrary to previous reports, the milder phenotype of MecP2^{308/Y} mice did not include detectable memory deficits that could be used to assess the efficiency of KCC2 rescue strategies. I therefore sought to develop a model of conditional MecP2 invalidation restricted to dorsal hippocampus. I took advantage of the MecP2^{fl^{ox}} mouse strain and used local infection with an AAV1-hSyn-Cre-GFP vector in dorsal hippocampus in order to induce MecP2 gene ablation selectively from dorsal hippocampal neurons. Since RTT is a pathology affecting primarily females carrying a mutation on one X chromosome (Amir et al., 1999; Chahrour and Zoghbi, 2007), I tested this approach on both heterozygous females and

hemizygous males. The female mouse model was expected to better mimic the human pathology, while the male mouse model may yield a more pronounced phenotype.

One week after AAV1-hSyn-Cre-GFP injection in the right hippocampus of heterozygous *MecP2*^{fl^{ox}} females, *KCC2* expression was reduced compared to the non-infected side in one out of three mice, as detected by western blot (Figure 28). In order to allow more time to the Cre-recombinase to act, we decided to reproduce this experiment and wait one month. Then we will test the effect of a local, hippocampus-specific *MecP2* deletion, on anxiety and fear conditioning memory but will not test for motor deficits as we are not expecting any. This work is currently ongoing.

4. Conclusion

Overall, our results demonstrate that *MecP2*^{308/Y} mice, in contrast to full *MecP2* KO mice, do not exhibit a down-regulation of *KCC2* expression. This suggests *KCC2* down-regulation may depend on the position of the mutation on the *MECP2* gene. Remarkably, however, LTP was impaired in these mice and could nevertheless be rescued by *KCC2* overexpression, supporting the notion that *KCC2* overexpression may be beneficial in rescuing synaptic deficits associated with RTT. However, preliminary experiments aiming to restore chloride homeostasis using bumetanide or *KCC2* stability using CLP-290 failed to rescue the behavioral phenotype of *MecP2*^{308/Y} mice. Therefore, I am currently developing another mouse model lacking *MecP2* specifically in the dorsal hippocampus.

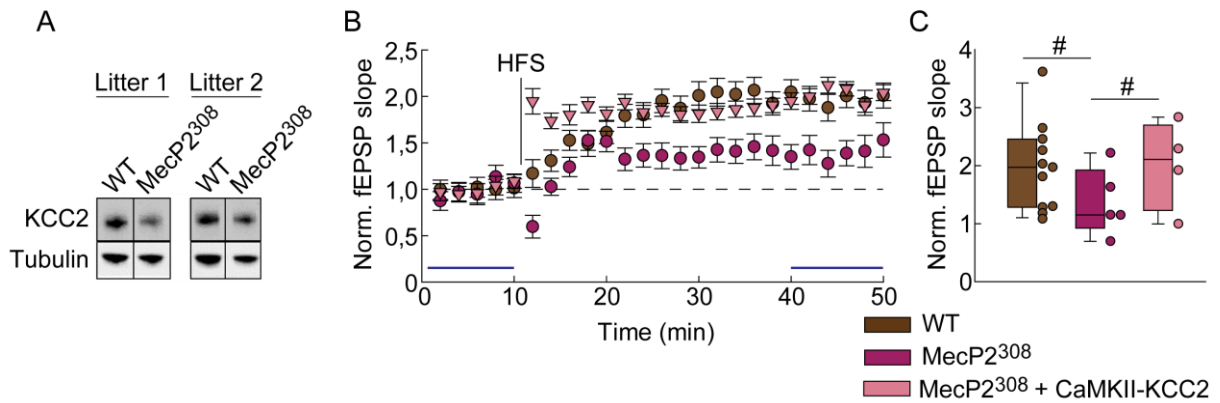


Figure 24. Over-expressing KCC2 in MecP2³⁰⁸ mice rescues LTP

A. Immunoblots of hippocampal protein extracts from 2 WT and 2 MecP2³⁰⁸ mice from 2 different litters probed with KCC2 and tubulin antibodies. **B.** Time course of changes in fEPSP slope upon HFS of Schaffer collaterals in slices from MecP2^{308/Y}, WT littermates and MecP2^{308/Y} mice overexpressing recombinant KCC2. Blue bars represent the time windows used for averages. **C.** Distributions of fEPSP slope 40 min after HFS normalized to the mean of 10 min baseline. Each point represents one recording from one slice. LTP expression in MecP2^{308/Y} mice was reduced compared to that in WT littermates (t-test, one-tailed p=0.072), while overexpressing KCC2 in MecP2^{308/Y} mice rescued LTP expression (t-test, one-tailed p=0.098).

p<0.1

All statistical tests and data are in Table 5.

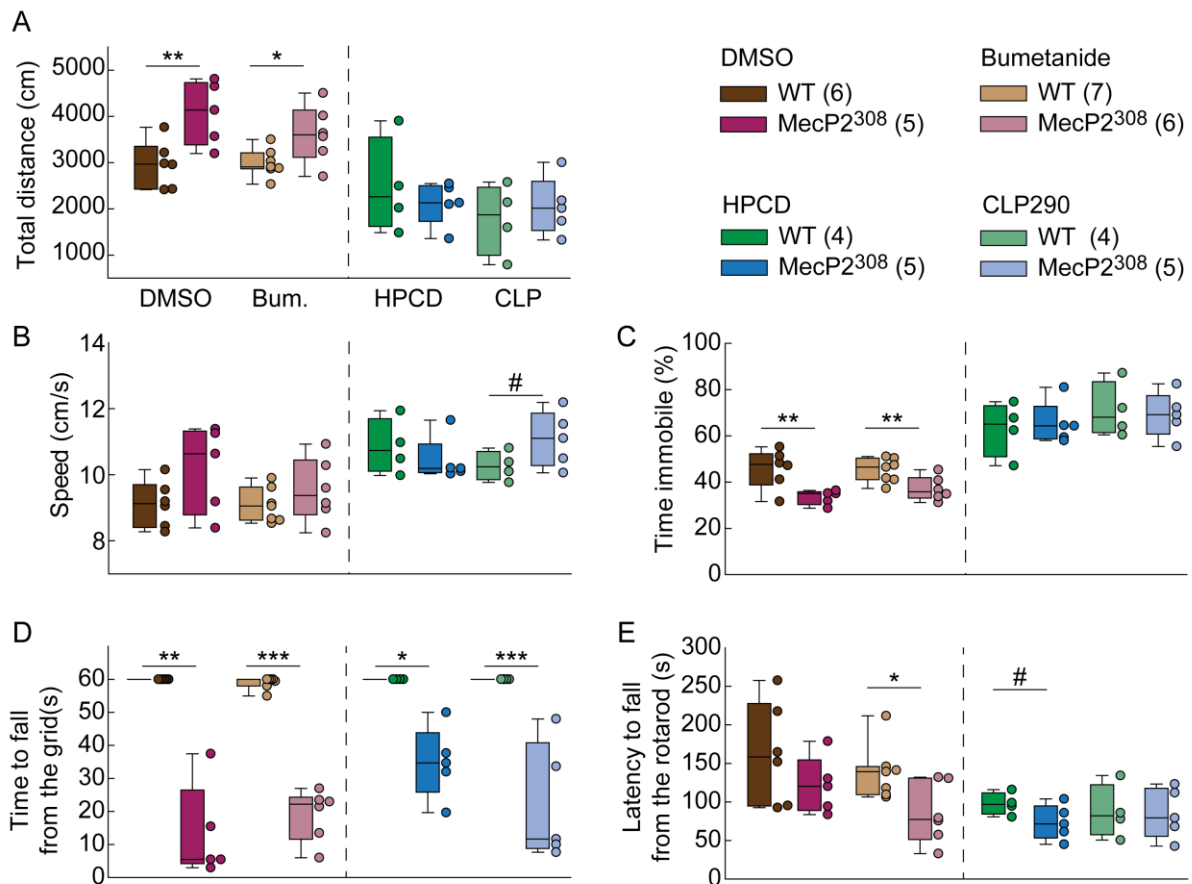


Figure 25. Bumetanide or CLP-290 do not rescue locomotor activity and grip strength in *MecP2*^{308/Y} mice

A. Distributions of total distance traveled during 10-minute exploration of an open field by WT and *MecP2*^{308/Y} mice treated with either bumetanide (Bum.), CLP-290, or DMSO or HPCD as control, respectively. **B.** Distributions of running speed (total distance/time in movement) for the same groups as in A. **C.** Distributions of immobility time for the same groups as in A and B. **D-E.** Motor deficits assessed by latency to fall from a grid (D) or from an accelerating rotarod (E) for the same groups as in A.

p<0.1 ; * p<0.05 ; ** p<0.01 ; *** p<0.001

All statistical tests and data are in Table 5.

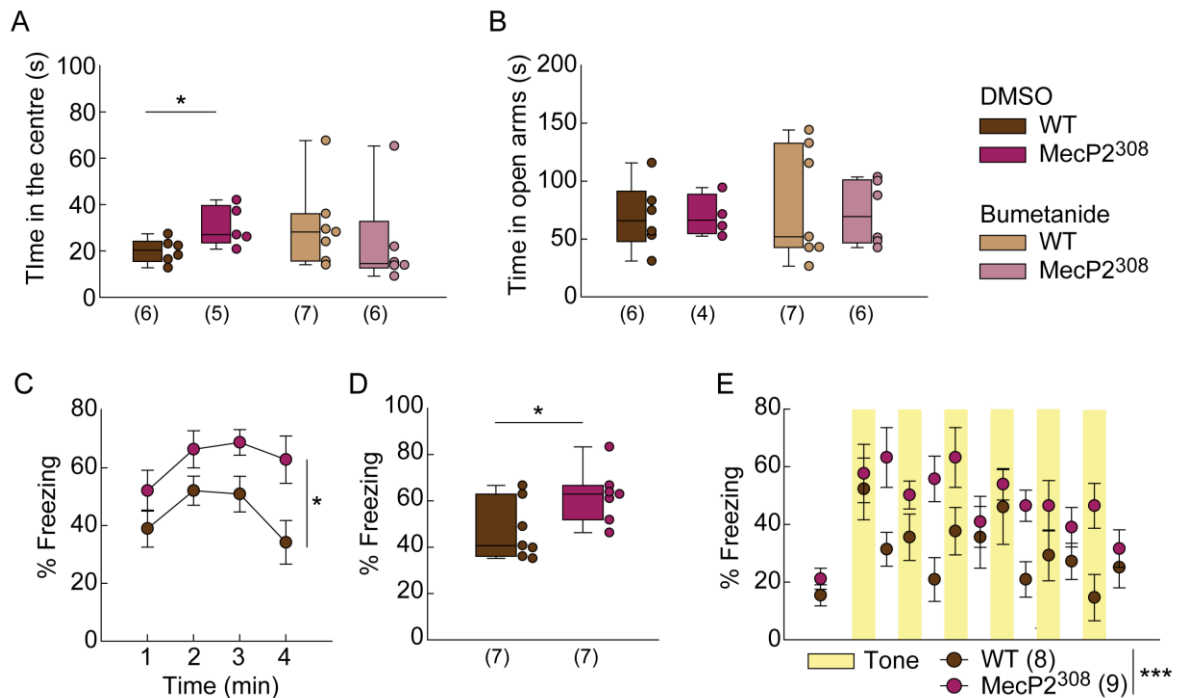


Figure 26. MecP2^{308/Y} mice in anxiety and fear-conditioning memory tasks

A. Distributions of time spent in the center of an open field for WT and MecP2^{308/Y} mice treated with bumetanide or DMSO alone as control. MecP2^{308/Y} mice are spending more time in the center (t-test, one-tailed $p=0.017$). **B.** Distributions of time spent in open arms showing no difference between the different groups. Number of animals are indicated in brackets for A-B. **C.** Time course of freezing during the first 4 minutes of exploration of the foot-shock associated cage in a fear conditioning paradigm. MecP2^{308/Y} mice ($n=7$) display an increased freezing response compared to WT littermates ($n=7$) (two-way RM ANOVA, $p=0.024$). **D.** Summary data for the 3 first minutes of exploration (t-test, one-tailed $p=0.021$). **E.** Time course of freezing upon exposure to foot-shock associated sound for the same group as in C-D. MecP2^{308/Y} mice ($n=8$) are freezing more than WT littermates ($n=9$) in the cued memory retention test (two-way ANOVA, $p<0.001$).

* $p<0.05$

All statistical tests and data are in Table 5.

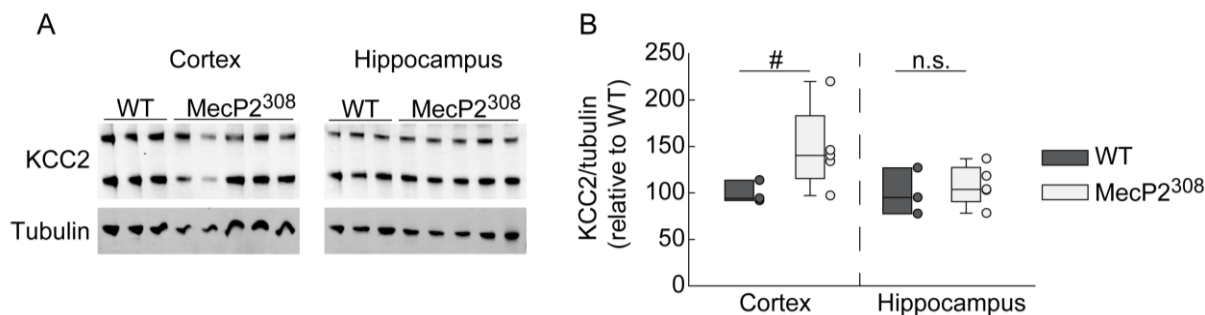


Figure 27. KCC2 is not down-regulated in MecP2³⁰⁸ mice

A. Immunoblots of protein extracts from cortex or hippocampus of WT mice (n=3) and MecP2³⁰⁸ mice (n=5), probed for KCC2 and tubulin. **B.** Quantification of KCC2 expression relative to tubulin, normalized to WT mice.

See table X for the statistical tests.

p<0.01 ; n.s. non significant

All statistical tests and data are in Table 5.

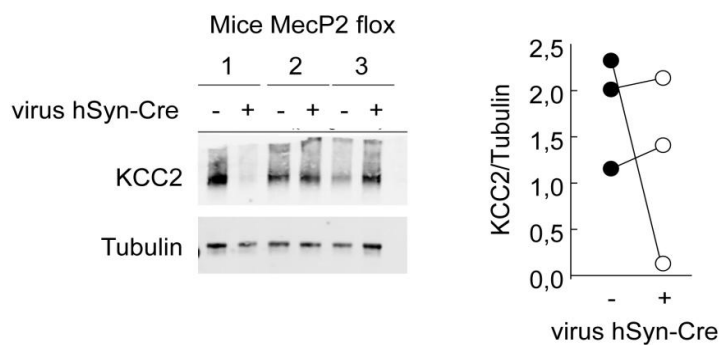
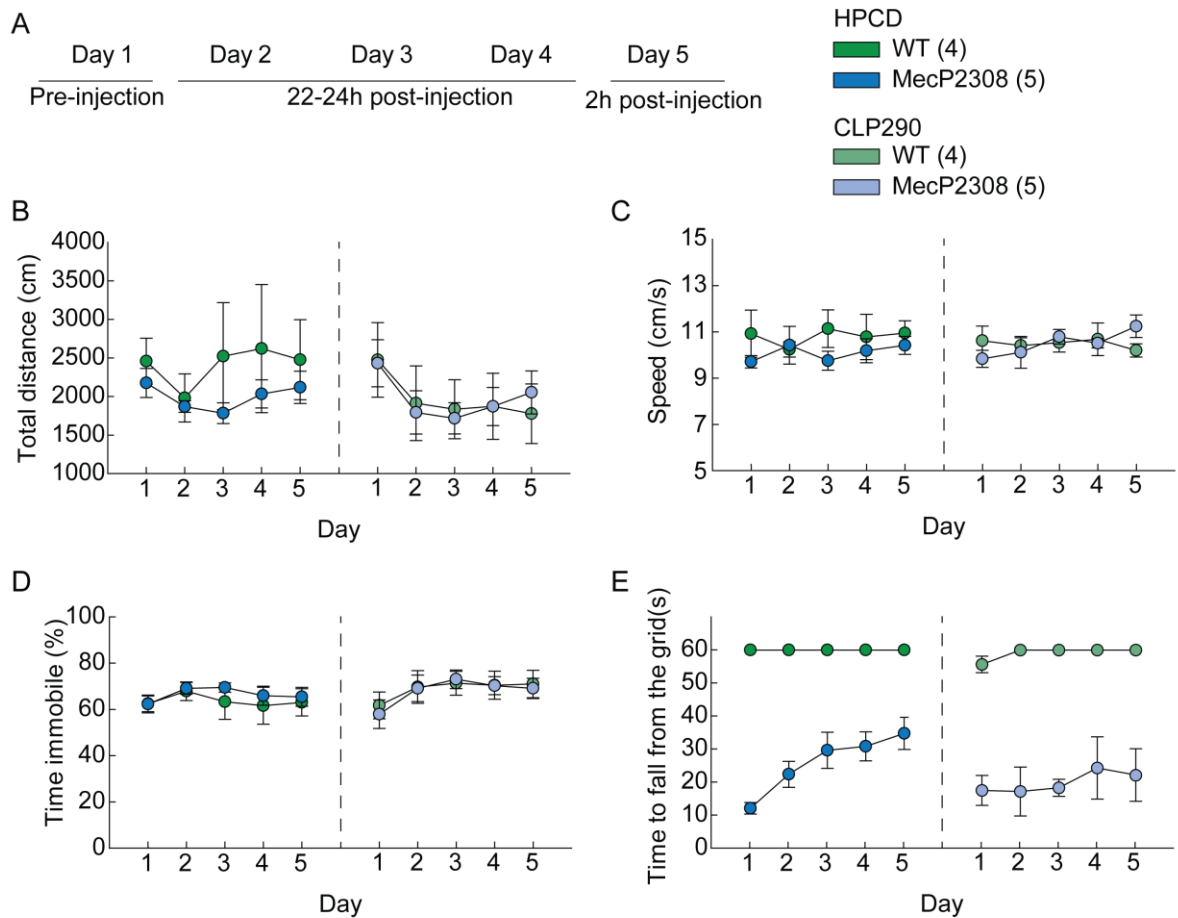


Figure 28. KCC2 expression following MecP2 suppression in dorsal hippocampus

Left, Immunoblots of protein extracts from left and right hippocampus of MecP2^{flox} heterozygous female mice infected (+) with a vector carrying the Cre-recombinase, or not (-) and probed for KCC2 and tubulin. Right, Quantification of KCC2 expression relative to tubulin.



Supplementary figure 5. Effect of chronic treatment with CLP-290 on locomotor activity and grip strength

A. Timeline of the behavioral tests following CLP-290 or HPCD i.p. injection. **B.** Day by day distributions of total distance traveled (B), running speed (C) and immobility time (D) during 10 minutes exploration of an open field in MecP2³⁰⁸ mice and WT littermates treated with CLP-290 or HPCD alone as a control. **E.** Day by day plot of latency to fall from a grid for the same mice than in A-C.

All statistical tests and data are in Table 5.

Figure 5. Statistical data of project 2

		Number of mice	Type of data	Transformation of data	Statistical test	p value		Power	F, t, z	Post-hoc test or supplementary information
Figure 24C	Mecp2 ^{308/Y}	11 sl (4 WT) vs 5 sl (2 MecP2 ^{308/Y})	ratio	no	t-test, one-tailed p value	0,072	#	0,432		
	Mecp2 ^{308/Y}	11 sl (4 WT) vs 4 sl (2 MecP2 ^{308/Y} /KCC2)	ratio	no	t-test, one-tailed p value	0,462		0,06		
	Mecp2 ^{308/Y}	5 sl (2 MecP2 ^{308/Y}) vs 4 sl (2 MecP2 ^{308/Y} /KCC2)	ratio	no	t-test, one-tailed p value	0,0979	#	0,362		
Figure 25A	DMSO	6 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm)	no	t-test, one-tailed p value	0,0066	**	0,884	-3,082	
	Bumetanide	7 (WT) vs 6 (MecP2 ^{308/Y})	Measure (cm)	no	t-test, one-tailed p value	0,0191	*	0,713	-2,355	
	HPCD	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm)	no	t-test, one-tailed p value	0,254		0,156	0,698	
	CLP-290	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm)	no	t-test, one-tailed p value	0,285		0,135	-0,597	
Figure 25B	DMSO	6 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm/s)	no	t-test, one-tailed p value	0,0611		0,473	-1,706	
	Bumetanide	7 (WT) vs 6 (MecP2 ^{308/Y})	Measure (cm/s)	no	t-test, one-tailed p value	0,153		0,262	-1,074	
	HPCD	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm/s)	no	t-test, one-tailed p value	0,222		0,182	0,812	
	CLP-290	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm/s)	no	t-test, one-tailed p value	0,0635	#	0,467	-1,731	
Figure 25C	DMSO	6 (WT) vs 5 (MecP2 ^{308/Y})	Percent	arcsin	t-test, one-tailed p value	0,0064	**	0,887	3,099	
	Bumetanide	7 (WT) vs 6 (MecP2 ^{308/Y})	Percent	arcsin	t-test, one-tailed p value	0,0091	**	0,829	2,772	
	HPCD	4 (WT) vs 5 (MecP2 ^{308/Y})	Percent	arcsin	t-test, one-tailed p value	0,369		0,092	-0,348	
	CLP-290	4 (WT) vs 5 (MecP2 ^{308/Y})	Percent	arcsin	t-test, one-tailed p value	0,389		0,084	0,293	
Figure	DMSO	6 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	Mann-Whitney	0,004	**			

25D	Bumetanide	7 (WT) vs 6 (MecP2 ^{308/Y})	Measure (s)	no	t-test, one-tailed p value	<0,001	***	1	13,137	
	HPCD	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	Mann-Whitney	0,016	*			
	CLP-290	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	t-test, one tailed p value	0,002	**	0,982	4,187	
Figure 25E	DMSO	6 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	t-test, one-tailed p value	0,12		0,316	1,260	
	Bumetanide	7 (WT) vs 6 (MecP2 ^{308/Y})	Measure (s)	no	t-test, one-tailed p value	0,0125	*	0,783	2,592	
	HPCD	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	t-test, one-tailed p value	0,0546	#	0,504	1,835	
Figure 26A	CLP-290	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	t-test, one-tailed p value	0,464		0,059	0,093	
	DMSO	6 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	t-test, one-tailed p value	0,017	*	0,747	-2,503	
	Bumetanide	7 (WT) vs 6 (MecP2 ^{308/Y})	Measure (s)	no	Mann-Whitney	0,101				
Figure 26B	DMSO	6 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	t-test, one-tailed p value	0,482		0,054	-0,046	
	Bumetanide	7 (WT) vs 6 (MecP2 ^{308/Y})	Measure (s)	no	t-test, one-tailed p value	0,378		0,089	0,32	
Figure 26C	DMSO	7 (WT) vs 7 (MecP2 ^{308/Y})	Percent	arcsin	Two-way RM ANOVA	0,024	*		6,698	Bonferonni
Figure 26D	DMSO	7 (WT) vs 7 (MecP2 ^{308/Y})	Percent	arcsin	t-test, one-tailed p value	0,0213	*	0,689	-2,268	
Figure 26E	DMSO	8 (WT) vs 9 (MecP2 ^{308/Y})	Percent	arcsin	Two-way ANOVA	<0,001	***		34,68	Bonferonni
Figure 27B	Cortex	3 (WT) vs 5 (MecP2 ^{308/Y})	Ratio	no	t-test, ont-tailed p value	0,065	#			
	Hippocampus	4 (WT) vs 5 (MecP2 ^{308/Y})	Ratio	no	t-test, ont-tailed p value	0,321				

Figure S5B	HPCD	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm)	no	Two-way ANOVA	0,091				Normality test failed
	CLP-290	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm)	no	Two-way ANOVA	0,996			3E-05	
Figure S5C	HPCD	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm/s)	no	Two-way ANOVA	0,088				Normality test failed

	CLP-290	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (cm/s)	no	Two-way ANOVA	0,969			0,002	
Figure S5D	HPCD	4 (WT) vs 5 (MecP2 ^{308/Y})	Percent	arcsin	Two-way ANOVA	0,362			0,854	
	CLP-290	4 (WT) vs 5 (MecP2 ^{308/Y})	Percent	arcsin	Two-way ANOVA	0,762			0,093	
Figure S5E	HPCD	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	Two-way ANOVA	<0,001				Normality test failed
	CLP-290	4 (WT) vs 5 (MecP2 ^{308/Y})	Measure (s)	no	Two-way ANOVA	<0,001				Normality test failed

DISCUSSION

Discussion

During my PhD, I evaluated the consequences of chronic KCC2 down-regulation on learning and memory. I showed KCC2 knockdown in neurons of the dorsal hippocampus alters contextual memory in a fear-conditioning paradigm as well as spatial memory in a place recognition task. Moreover, I extended previous observations reporting reduced hippocampal long-term potentiation *in vitro*. I also revealed hippocampal rhythmopathy induced upon KCC2 knockdown. Specifically, I observed that the power of slow gamma-band activity was decreased during REM sleep in KCC2 knockdown mice. Perhaps more important, ripples occurring during NREM sleep, which have been shown to be important for consolidation of spatial memory (Buzsáki, 2015; Girardeau et al., 2009), were increased in duration and frequency and were often contaminated with bursts of multiunit activity (MUA), reflecting hyperexcitability within the CA1 network. I further attempted to decipher the role of neuronal subtypes involved in memory deficits upon KCC2 knockdown and found that KCC2 down-regulation in principal cells only, but not in GABAergic interneurons only, was sufficient to impair contextual memory. Finally, I showed that restoring KCC2 expression in principal neurons upon KCC2 knockdown in the entire dorsal hippocampus rescues both LTP and contextual memory.

Although these observations strongly support an impact of KCC2 down-regulation, as observed in a variety of neurological and psychiatric disorders, on cognitive performances, it raises several important questions yet to be addressed. In particular, it remains unclear whether memory deficits observed upon KCC2 knockdown primarily reflect encoding, consolidation or retrieval defects. Thus, we observed deficits in both LTP - that may compromise encoding - and in altered rhythmogenesis - that may impair memory consolidation during sleep. What is the relative contribution of these different defects in memory performance remains to be determined. Finally, whether memory deficits in conditions associated with down-regulation of KCC2 expression may be rescued by targeting its membrane expression and/or function also remains to be fully evaluated.

I. KCC2 knockdown induces hyperexcitability and hippocampal rhythmopathy

1. Mechanisms for abnormal ripple generation

Ripples are generated locally in CA1 and are associated with cell firing replay. In rodents, this phenomenon is associated with reactivation of place cell firing patterns that occurred during exploration (Buzsáki, 2015). Mechanism of ripple generation in CA1 requires a buildup of excitation, arising from a population burst generated through the recurrent network of CA3 (Schlingloff et al., 2014). The sharp waves generated in CA3 propagate to CA1 by depolarizing CA1 pyramidal cells, bringing them closer to their firing threshold (English et al., 2014). However, the strong shunting inhibition from PV-interneurons prevents most cells from firing during the ripple by increasing their firing threshold, only allowing neurons with the largest intracellular depolarization to fire (Hulse et al., 2016). This mechanism might allow only neurons receiving sufficient excitation - such as neurons previously potentiated - to overcome this inhibition (Behrens et al., 2005). Since LTP is reduced following KCC2 knockdown, it is unlikely that neurons might be able to fire because of prior potentiation. However, *in vivo* hippocampal recordings of LFP allowed us to observe a hyperexcitability of the network in the CA1 region, with an increase in bursts of spikes associated with ripples. Two hypotheses may explain this hyperexcitability.

First, KCC2 down-regulation might affect chloride homeostasis and GABAergic signaling, therefore reducing the inhibition drive. Thus, many studies have reported a >10 mV depolarizing shift in the reversal potential of GABAAR-mediated currents (E_{GABA}) upon KCC2 knockdown or knockout (Chen et al., 2017; Dargaei et al., 2018; Kelley et al., 2018; Pellegrino et al., 2011). In principle, such a shift may reduce the efficacy or even reverse the polarity of GABA signaling in neurons. A recent study from our group, however, recently showed that most of the depolarization in E_{GABA} upon KCC2 knockdown is actually compensated by an equivalent shift in resting membrane potential, leading to negligible alteration of GABAergic signaling under steady-state conditions (Goutierre et al. 2019). Nevertheless, it is conceivable that during regimes of intense activation of PV interneurons - which induce a massive charge flux through GABAARs in postsynaptic pyramidal cells (Magloire et al., 2019) - KCC2 down-regulation may accelerate the buildup of intracellular

chloride homeostasis. This in turn would be expected to alter the efficacy and/or polarity of GABA signaling and would act to shorten ripples and even lead to the generation of fast ripples (250-500 Hz) (Stark et al., 2014). Instead, we observed longer ripples and an absence of fast ripples upon KCC2 knockdown. It is therefore possible that the short activation of interneurons during the course of individual ripples is not sufficient to alter GABAergic signaling even in the absence of KCC2 function. Moreover, *in vitro* recordings of ripples in hippocampal slices showed no change in ripple duration or frequency upon application of the KCC2 antagonist VU0463271 (unpublished data from our lab). These observations argue against a major role of KCC2 ion transport function in ripple generation.

Second, it was recently shown that KCC2 down-regulation is associated with reduced leak potassium currents through Task-3 channels, leading to increased input resistance, EPSP/spike coupling and membrane excitability both in dentate gyrus granule cells and CA1 pyramidal cells (Goutierre et al., 2019). This reflects a direct interaction between KCC2 and Task-3 channels that controls the membrane expression of the latter, and more likely underlies ripple alterations upon KCC2 knockdown. Thus, following KCC2 knockdown, CA1 pyramidal neurons are expected to fire more upon CA3 excitatory input, with more spikes generated than in control animals. This buildup of activity would then lead to enhanced recruitment of PV interneurons, leading to their prolonged firing and generation of longer-lasting ripples. This effect may be even reinforced if PV interneurons, which also express KCC2 (Gulyás et al., 2001, unpublished data from our team), are also more excitable upon KCC2 knockdown. This hyperexcitability would also explain the increase in ripple frequency, as pyramidal cells and interneurons might be more easily engaged into ripple generation due to increased EPSP/spike coupling (Goutierre et al., 2019). It also likely contributes to enhanced MUA and bursts generation, as detected in hippocampal recordings from KCC2 knockdown mice.

2. Relevance to pathology and memory deficits

SPW-Rs are necessary for memory consolidation, by replaying neuronal ensembles and sustaining memories during sleep (Girardeau et al., 2009). Moreover, several papers reported abnormal SPW-Rs in pathologies associated with cognitive deficits such as

schizophrenia (Altimus et al., 2015; Suh et al., 2013), dementia (Witton et al., 2016) or epilepsy (Valero et al., 2017).

Following chronic KCC2 knockdown using RNA interference *in vivo*, we observed an increased frequency and duration of SPW-Rs, as well as a hyperactivity of the neuronal network in the form of increased MUA and bursting behavior. Similar alterations were observed in some, but not all, animals models of disorders associated memory impairments. Thus, adult Frm1-KO mice - a model for autism - and rats with pilocarpine-induced epilepsy both exhibit down-regulation of KCC2 (Li et al., 2008; Pathak et al., 2007; Tyzio et al., 2014) and display CA1 pyramidal cell hyperactivity (Boone et al., 2018; Valero et al., 2017). Moreover, Frm1-KO mice also exhibit prolonged ripples compared to WT mice (Boone et al., 2018). However, to my knowledge, such alteration was not explored in rodent epilepsy models, for which most studies focused primarily on high frequency oscillations such as fast ripples (250-500 Hz), a hallmark of epileptic hippocampus (Buzsáki et al., 1989).

In mouse models of RTT, ripples alterations have not been reported yet, but this work is ongoing in our team. However CA1 principal cells hypersynchrony has been observed (Kee et al., 2018; Lu et al., 2016), probably altering the precision of neuronal ensembles for memory encoding. Following exploration of a novel environment, place cells do not increase their firing synchrony, both during the next exploration or during awake ripples, suggesting encoding may be affected (Kee et al., 2018). It would be interesting to test whether the CA1 hyperactivity observed upon KCC2 knockdown might also reflect a hypersynchrony of neuronal activity.

Although my recordings demonstrate increased MUA in the CA1 area upon KCC2 knockdown, it should be noted, however, that our recordings do not allow for neuronal identification (principal cells *vs.* interneurons). Tetrode recordings would allow spike sorting analysis and discrimination of spike features corresponding to neuronal subtypes (Jog et al., 2002; Rey et al., 2015). Instead, linear silicon probes as those used in our recordings, with a 50 μm spacing between electrodes, only let us record MUA with no indication regarding cell identity, even though a recent study showed accurate estimations of neural population dynamics can be achieved without spike sorting (Trautmann et al., 2019).

How may changes in ripple generation and network excitability affect learning and memory? A recent study reported that prolonged awake ripples (over 100 ms) are actually associated with better performance in a working spatial memory task (Fernández-Ruiz et al., 2019). However, online replay is hypothesized to underlie guided-behavior (Jadhav et al., 2012) while offline replay is necessary for memory consolidation (Girardeau et al., 2009; Joo and Frank, 2018). Since we examined only offline (NREM sleep) ripples, it is difficult to establish how these observations relate to ours.

In exploratory behavior, elements within an experience might be encoded in the form of high frequency oscillations such as gamma, embedded into larger theta oscillations that organize these sequences (Heusser et al., 2016). Slow-gamma oscillations are associated with the first half of the theta oscillation (Bieri et al., 2014), suggesting a role in predicting next location and might therefore be necessary for memory retrieval (Tort et al., 2009). During REM sleep, we observed a decreased power of slow-gamma activity in KCC2 knockdown mice. If slow-gamma power is also decreased during exploration, which remains to be determined, this effect might contribute to the spatial and contextual memory deficits observed in KCC2 knockdown mice.

Finally, hyperexcitability during learning is undesirable, as only the strongest-driven assemblies should be activated to encode memory. Any other neuronal activation would likely increase noise and reduce specificity. For example, the ability of the DG to perform pattern separation relies on its sparse firing (Aimone et al., 2011; Jung and McNaughton, 1993). As neurons are more excitable following KCC2 knockdown, it is possible that too many neurons are activated, hiding some specific neuronal engrams. This might explain the contextual memory deficits observed upon KCC2 knockdown. Moreover, the formation of neuronal ensembles during theta might be altered as neuronal hyperactivity might increase the number of neurons spiking at all time, hiding the specific place cells firing and/or disrupting the ability to encode new memories. Indeed, aging rodents and humans display increased hippocampal activity correlating with cognitive decline that can be rescued using low doses of antiepileptic drugs acting to decrease neuronal excitability (Bakker et al., 2012; Koh et al., 2010; Wilson et al., 2005). Finally, hyperexcitable neurons might be activated during SPW-Rs even though they are not part of a neuronal ensembles encoding for a memory. CA1 pyramidal neurons fire only sparsely during SPW-Rs (English et al., 2014). Again, increased

and non-specific firing and bursting during SPW-Rs, as observed in KCC2 knockdown mice, may contribute to a background noise that may compromise their information content.

3. KCC2 knockdown in dorsal hippocampus is not sufficient to trigger epileptiform activities

KCC2 down-regulation has been reported both in brain tissue from epileptic patients and in experimental epilepsy models (review in Moore et al., 2017; but see Karlócai et al., 2016). Whether KCC2 down-regulation is causal to the pathology or a mere consequence of anomalous, hypersynchronous activity however remains debated. Thus, a modeling study predicted that KCC2 suppression in about 30 % principal neurons, as observed in the human epileptic subiculum (Cohen et al., 2002; Huberfeld et al., 2007), may be sufficient to initiate synchronous, epileptiform activities (Buchin et al., 2016). Interestingly, however, those were only observed upon raising extracellular potassium to non-physiological levels, suggesting KCC2 down-regulation on its own might not be sufficient to generate such activities under resting conditions. Experimental data addressing this question remain so far controversial. Thus, whereas two recent studies reported epileptiform activities upon conditional KCC2 ablation in the dorsal hippocampus (Kelley et al., 2018) or focal KCC2 knockdown in dorsal dentate gyrus (Chen et al., 2017), no sign of such activities were detected under similar conditions (Goutierre et al., 2019 and the present study). Remarkably, the two studies from our lab both reported increased neuronal excitability without spontaneous, epileptiform activities or high frequency oscillations (HFOs) reminiscent of an epileptic network, even though using distinct interfering RNA sequences, making them unlikely to be due to off-target, non-specific effects.

Thus, in my recordings, I was not able to detect any electrophysiological marker of an epileptic network, such as fast ripples or interictal events (Buzsáki et al., 1989). Fast ripples are HFOs comprised between 250 and 500 Hz, arising through synchronous burst discharge of pyramidal cells (Menendez de la Prida and Trevelyan, 2011). Loss of inhibition may be responsible for these abnormal events as picrotoxin, a GABAAR blocker, increases the occurrence of fast ripples originating in CA3/CA2 both *in vitro* (Wong and Traub, 1983) and *in vivo* (Stark et al., 2014). Since KCC2 participates in the control of chloride homeostasis,

thereby influencing GABAergic signaling, one might have expected to record fast ripples upon KCC2 knockdown. Indeed, tissue around glioma cells or epileptic rodent models show reduced KCC2 expression and a high incidence of fast ripples (Pallud et al., 2014; Valero et al., 2017).

How may I then reconcile these results with this set of observations? First, in pilocarpine model (Kourdougli et al., 2017), temporal lobe epilepsy patients (Palma et al., 2006) and in peritumoral tissue (Pallud et al., 2014), down-regulation of KCC2 was paralleled by NKCC1 up-regulation. This is expected to produce an even greater shift in intraneuronal chloride concentration and loading during repetitive interneuron activation. Instead, in our model, we have been using a shRNA against KCC2 which is not expected to alter NKCC1 expression, even though this was not specifically tested due to the lack of a specific NKCC1 antibody. Second, in the pilocarpine model of temporal lobe epilepsy, KCC2 suppression is massive with 60% down-regulation in the entire hippocampus (Kourdougli et al., 2017). It is therefore possible that the spread of the virus and subsequent KCC2 down-regulation in my model (70% down-regulation compared to control, only in dorsal hippocampus) was not sufficient to allow the generation of fast ripples.

Finally, and perhaps more importantly, there is one major difference between animal models of epilepsy or human epileptic tissue and our model of KCC2 knockdown. In epileptic tissue, an initial seizure or *status epilepticus* leads to epileptogenesis (the process leading to the emergence of epilepsy) before ictogenesis (the mechanisms contributing to the generation of a seizure in an epileptic network) occurs. In our model, however, there is no initial insult leading to epileptogenesis. KCC2 down-regulation might therefore only promote anomalous activities in an already epileptic network. In line with this hypothesis, hypomorphic KCC2 null mice retaining only 15-20% of KCC2 expression do not exhibit spontaneous epileptic seizures. However, their susceptibility to pentylenetetrazole (PTZ)-induced seizures is increased (Tornberg et al., 2005), while KCC2 over-expression protects against pilocarpine-induced seizures (Magloire et al., 2019). Alternatively, KCC2 down-regulation may participate, along with other mechanisms (such as inflammation, cell death, reactive gliosis... (Pitkänen and Lukasiuk, 2011)) in initial epileptogenesis (Kourdougli et al., 2017; Pathak et al., 2007) but not in ictogenesis. In support of this hypothesis, KCC2 down-regulation was observed during epileptogenesis but not during the chronic phase of mouse epilepsy models

(Karlócai et al., 2016; Pathak et al., 2007), further suggesting that KCC2 down-regulation might play a role to destabilize the neuronal network following an insult.

II. What mechanism(s) underlies memory impairment upon KCC2 knockdown?

I have shown that KCC2 knockdown in dorsal hippocampus neurons of adult mice leads to network hyperexcitability, hippocampal rhythmopathy as well as LTP impairment, correlated with memory deficits. Moreover, KCC2 down-regulation only in principal cells is sufficient to induce contextual memory deficits. However, these observations are primarily correlative and the main mechanism(s) involved in memory deficits remain elusive. Indeed, KCC2 is a potassium/chloride co-transporter involved in neuronal chloride homeostasis and its function might be crucial to control GABAergic signaling (Viitanen et al., 2010). On the other hand, KCC2 interacts with actin-related proteins and its down-regulation has been shown to impact glutamatergic signaling (Chevy et al., 2015; Gauvain et al., 2011a). Finally, a recent study revealed KCC2 promotes membrane traffic of the potassium channel Task-3 and thereby controls neuronal intrinsic excitability (Goutierre et al. 2019). Here I will discuss 2 non-mutually exclusive hypotheses and how they could be tested experimentally.

1. Hypothesis 1: KCC2 ion transport-function is required for memory

Theta-band activity and SPW-Rs are necessary in encoding and consolidating memories (Colgin, 2016; Mizuseki and Miyawaki, 2017). As these rhythms rely on GABAergic signaling to synchronize ensembles of pyramidal cells, we could expect them to be disrupted upon KCC2 knockdown. However, as previously discussed (see Discussion I.1) impairment of GABAergic signaling would be expected to result in shorter, not longer, ripples (Stark et al., 2014). This result, in line with previous data from our team (Goutierre et al.,

2019), suggests KCC2 down-regulation on its own may not result in major changes in inhibitory signaling.

In order to test whether memory deficits following KCC2 knockdown are due to altered GABA signaling, one might knockdown KCC2 as in the present study, and then express a transport-deficient KCC2. Several point mutants with normal membrane expression but impaired ion transport function have been described, in particular the C568A mutant (Reynolds et al., 2008). Such recombinant KCC2 is expected to traffic normally to the cell membrane and interact with intracellular partners, such as actin-related proteins and Task-3 and therefore would rescue LTP (Chevy et al., 2015) and neuronal excitability (Chevy et al., 2015) but not transmembrane chloride gradients. Another possibility would be to infuse the KCC2 antagonist VU0463271 (Delpire et al., 2012) in the dorsal hippocampus (as in Sivakumaran et al., 2015). However, in the latter study, mice experienced behavioral arrest and epileptiform activities. Therefore, combining such paradigm with a spatial or contextual memory task and possibly intrahippocampal recordings during sleep would be experimentally challenging.

2. Hypothesis 2: KCC2 interaction with protein partners is necessary for learning and memory

During my PhD, I showed KCC2 knockdown impairs LTP expression at the Schaffer collateral to CA1 synapses, as previously observed in the rat dentate gyrus (Chevy et al., 2015). This experiment was performed in the presence of the GABA_A antagonist bicuculline, allowing us to isolate the impact of KCC2 down-regulation on glutamatergic signaling, independent of GABA signaling. Moreover, rescuing KCC2 expression only in principal cells allowed us to restore LTP upon KCC2 knockdown in the dorsal hippocampus.

LTP is considered the synaptic substrate to form long-term memory (Bliss and Collingridge, 1993). The mechanisms underlying LTP expression at several hippocampal synapses, as described in Introduction (see Introduction III.3.a), relies on actin dynamics in dendritic spines, with a transient activation of cofilin (Gu et al., 2010) and insertion of synaptic, GluA1-containing AMPARs (Shi et al., 1999). These mechanisms were shown to be

impaired upon KCC2 knockdown (Chevy et al., 2015; Gauvain et al., 2011a), due to changes in actin polymerization and loss of interaction of spine membrane proteins with KCC2. Interestingly, KCC2 down-regulation in dorsal hippocampus affects contextual memory as well as spatial memory in the place recognition paradigm, but not in the Morris water maze. This result is consistent with some reports showing that altering GluA1-containing AMPAR exocytosis, although impairing LTP and some forms of memory (Mitsushima et al., 2011; Rumpel et al., 2005) does not compromise spatial memory in the Morris water maze (Bannerman et al., 2018; Zamanillo et al., 1999).

This lack of alteration in the Morris water maze test upon KCC2 knockdown may have another explanation. Indeed, studies on spatial memory have shown that hippocampal lesions particularly affect single-trial learning tasks, such as the delay matching-to-place (DLM) paradigm. In a series of experiments, Richard Morris and collaborators observed a strong deficit in rats with ibotenate-induced hippocampal lesion in the DLM paradigm, in which the platform position in the water maze changes every day (Steele and Morris, 1999) while rats were still learning with overtraining in the classic water maze paradigm (Morris et al., 1990). This might explain the discrepancy we observed upon KCC2 knockdown, with altered memory in the single-trial place recognition task but not following one-week training in the water maze. Moreover, other brain regions are known to be activated in the water maze, such as the cerebellum (Petrosini et al., 1996; Rochefort et al., 2013) and might participate to learning upon KCC2 knockdown in the hippocampus.

KCC2 down-regulation affects LTP in a transport-independent manner and relies on the loss of interactions with actin-related proteins through its C terminal domain (CTD) (Chevy et al., 2015). Therefore, over-expressing KCC2-CTD might be expected to affect learning and memory similarly as KCC2 knockdown. Such a paradigm would let us preserve KCC2 transport function, which is unaffected upon KCC2-CTD over-expression (Chamma et al., 2013), while specifically disrupting KCC2 interactions with some intracellular partners. It is currently underway. I injected an AAV1-CaMKII-KCC2CTD vector in the dorsal hippocampus of mice and tested them for contextual memory retention in the fear conditioning paradigm. If memory deficits are observed, these will be attributable to mechanisms independent of altered GABAergic signaling. However, it should be noted that the interaction domain between KCC2 and Task-3 channels has not been identified yet (Goutierre et al., 2019). Therefore, such experiment will not let me disentangle the role of

altered hippocampal LTP vs. increased neuronal excitability in memory upon KCC2 knockdown.

In order to specifically test the impact of altered glutamatergic signaling on memory upon KCC2 suppression, one could act on the Rac1-PAK-LIMK-cofilin pathway following KCC2 knockdown. Indeed, this pathway is over-activated in the absence of KCC2 (Chevy et al., 2015; Llano et al., 2015), likely impairing GluA1-containing AMPARs exocytosis (Gu et al., 2010) and altering LTP (Chevy et al., 2015; Shi et al., 1999). Treatment with a LIMK inhibitor or the PAK inhibitor IPA-3 rescues activity-driven GluA1 synaptic insertion and LTP in KCC2 knockdown neurons (Chevy et al., 2015). Interestingly, BMS-5, a potent LIMK inhibitor has already been used *in vivo* (Lunardi et al., 2018). As this pathway is necessary for LTP expression, acute treatment of KCC2 knockdown mice with BMS-5 on the day of learning should be sufficient to rescue LTP and memory in these mice.

Finally, the contribution of increased intrinsic excitability in memory deficits upon KCC2 knockdown could be tested using a viral approach to specifically shut down Task-3 expression. This tool is already available in our team. Such manipulation is expected to induce neuronal hyperactivity and ripples abnormal structure without affecting glutamatergic and GABAergic signaling. However, one should keep in mind that KCC2 interacts with a variety of proteins (Mahadevan et al., 2017; Al Awabdh et al., in preparation). Its down-regulation might then affect other, yet unidentified pathways and mechanisms.

III. Rescuing KCC2 expression in a mouse model of RTT

KCC2 is down-regulated in many neurological and psychiatric disorders associated with memory impairment such as epilepsy (Huberfeld et al., 2007), Huntington's disease (Dargaie et al., 2018), autism (Tyzio et al., 2014) or Rett syndrome (Duarte et al., 2013; Tang et al., 2016). Therefore, understanding whether and how KCC2 down-regulation contributes

to memory deficits associated with these disorders might unravel novel target for therapeutic intervention.

During my PhD, I have demonstrated that KCC2 down-regulation in the hippocampus impairs some form of spatial and contextual memory. Next, I therefore wanted to test whether restoring KCC2 expression in a mouse model of pathology associated with KCC2 down-regulation and memory impairment might help rescue the memory deficits. To address this question, I chose a mouse model for RTT. Indeed, KCC2 expression is down-regulated in the CSF and in iPSCs of patients (Duarte et al., 2013; Tang et al., 2016). RTT is a genetic disorder with >95% cases associated with a mutation in the *MECP2* gene. Therefore, different mouse models for this pathology have been developed, recapitulating most of the behavioral phenotype such as motor deficits, memory alterations and/or epilepsy (Chahrour and Zoghbi, 2007).

In order to test my hypothesis, I needed a mouse model of RTT with spatial and/or contextual memory deficits (as well as KCC2 down-regulation in the hippocampus). We did not use the **MecP2 KO mice**, as these animal breed very poorly, have a very short lifespan (7-8 weeks) and exhibit prominent motor deficits which may complicate investigation of their behavior in the various behavioral tasks used in spatial memory tests (Bird et al., 2001).

Instead, we selected a mouse model expressing a truncated MecP2, the **MecP2³⁰⁸ mouse model**. MecP2^{308/Y} mice were reported to exhibit deficits in both spatial and contextual memory from 20 weeks old (De Filippis et al., 2010; Moretti et al., 2006). However, in contrast to these studies, we did not observe such deficits. In fact, Mecp2^{308/Y} mice from 22 to 26 weeks old showed increased - not reduced - freezing response in a contextual fear memory task. Since our mice were bred on a C57Bl6/JRj background while previous behavioral experiments were performed on mice with a mixed background, this difference might account for the different phenotype in contextual memory. In addition, Moretti and colleagues used a different fear conditioning protocol (Moretti et al., 2006).

Moreover, this mouse model turned out to express normal KCC2 levels of KCC2 compared to WT, in contrast to our initial observations. As discussed in the Introduction section of this thesis (see Introduction II.3), MecP2 is a transcription factor controlling - among many other target genes - KCC2 expression either by interacting with mCPG binding sites and/or other transcription factors, such as REST and CoREST to form protein complexes

controlling gene expression (Chahrour et al., 2008; Lunyak et al., 2002; Yeo et al., 2009). Truncation of MecP2 at residue 308 amino acid has been shown to disrupt MecP2 interaction with SMRT complex (Lyst et al., 2013). However, it is unknown whether its interaction with other protein complexes is also impaired. Moreover, this shorter MecP2 still contains DNA binding site (Hite et al., 2009). Altogether, our data suggests MecP2 protein truncated after amino acid 308 may still control KCC2 expression through a mechanism yet to be fully determined, but that does not require its carboxy-terminal domain, suggesting not all MecP2 mutations may result in altered KCC2 expression. Interestingly, in the study conducted by Duarte and colleagues, 2 patients carrying a stop mutation at residue 294 (R294X) display higher KCC2 expression than other patients and KCC2/NKCC1 ratio is then either only slightly reduced or even increased in these patients (Duarte et al., 2013). On the contrary, patients expressing a shorter MecP2 with a stop insertion either after amino acid 255 or 270 show prominent decrease in KCC2 expression and KCC2/NKCC1 ratio. It is therefore plausible that MecP2 sequence necessary in KCC2 transcription regulation involves residues comprised between amino acid 270 and 294. Since this sequence corresponds to the TRD site (transcriptional repression domain, see Figure 8 in Introduction II.3.a), MecP2 may control KCC2 transcription through interactions with other transcription factors rather just the methyl CpG site in KCC2 promoter.

To circumvent this problem, I decided to use the **MecP2^{flox} mouse strain**. These floxed mutant mice possess loxP sites flanking exons 3-4 of the *MECP2* gene. Following Cre-mediated recombination, only the first 8aa will be transcribed. We then used an AAV vector expressing the Cre-recombinase under a neuronal promoter in the dorsal hippocampus in heterozygous females, which represent a better model for human pathology (Samaco et al., 2013), and hemizygous males, which are likely to exhibit a stronger phenotype. This mouse model has three advantages. First, we can ablate almost the full MecP2 sequence, therefore allowing complete suppression of MecP2 functions. Second, genetic ablation is local and conditional and can be restricted to the dorsal hippocampus, thereby avoiding motor deficits associated with the full KO. As suppressing MecP2 expression in adult recapitulates RTT phenotype in particular with respect to fear conditioning memory (McGraw et al., 2011), we are still expecting some cognitive deficits in this model and will be able to test the impact of KCC2 overexpression.

We observed a KCC2 down-regulation in one heterozygous $MecP2^{fllox}$ females infected with the Cre-recombinase in one hippocampus but not the other, suggesting this mouse model to be a best fit to test our hypothesis. However, the experiment to confirm this result are ongoing. Remarkably, RTT mouse models with cognitive deficits are usually $MecP2^{fllox}$ mice crossed with mice expressing the Cre-recombinase in specific neuronal subsets (Chao et al., 2010; Ito-Ishida et al., 2015; Meng et al., 2016), resulting in more widespread MecP2 deletion than with viral injection. An alternative to this approach may then be to cross $MecP2^{fllox}$ mice with T29-1 mice which express the Cre-recombinase mainly in CA1 pyramidal neurons (Tsien et al., 1996).

IV. General conclusion

During my PhD, I aimed to characterize the impact of a chronic KCC2 knockdown on learning and memory in adult mice and the underlying mechanisms. My results reveal that KCC2 down-regulation in dorsal hippocampus impairs spatial and contextual memory. This effect is associated with reduced hippocampal LTP expression and increased neuronal excitability, as previously reported *in vitro*, but also with alterations of hippocampal rhythmic activities involved in memory encoding and consolidation. These defects do not only rely on altered chloride transport and GABA signaling but also involve chloride transport-independent alterations in synaptic plasticity and neuronal excitability. Therefore, I propose that strategies aiming solely to restore chloride homeostasis (and GABAergic transmission) in the pathology using loop diuretics such as bumetanide might not fully compensate for KCC2 suppression and associated cognitive deficits. In contrast, rescuing KCC2 expression - using recently developed KCC2 enhancers such as CLP-290 (Gagnon et al., 2013) - or neuronal excitability - using K2P channel openers such as the dihydroacridine analogue (ML67-33; Bagriantsev et al., 2013) or the Trek-1 specific opener Gi-530159 (Loucif et al., 2018) - either alone or in combination, may prove a more effective strategy.

ANNEXES

Annexes

In the Annexe 1, I present the abstracts of posters and oral presentations I did during my PhD.

- My doctoral school organize a seminar in Roscoff once during our PhD. I did an oral presentation of 10 minutes in front of my colleagues.
- I also went to international conferences (SfN 2017 and FENS 2018) in the course of my PhD and participated to a local conference held in Paris (ENP days). For these three events, I presented a poster with the my work's progress.

In the Annexe 2, I present the abstracts of different publications I participated in.

- During my master's degree, I decided to perform two long-term internships in international labs with very different scientific approaches. In Pr Seth Grant's lab at *Edinburgh's University*, I used molecular approaches and improved a protocol to extract post-synaptic density proteins from human brains. I then joined Dr Kay Tye's lab at *MIT* where I combined chemogenetics, behavioral approaches and immunohistochemistry in a project aiming to study the involvement of different subcategories of neurons from the basolateral amygdala in valence coding. These internships led to three publications (Bayés et al., 2014; Beyeler et al., 2016; Roy et al., 2018)
- Finally, the 4th publication was a collaboration with Albert Giralt when I started my PhD (Giralt et al., 2017). We took advantage of one technique I learned while doing my intership with Pr Seth Grant. I therefore extracted post-synaptic proteins from mouse hippocampi to look for glutamate receptors subunits enrichment differences between a Pyk2 deficient mouse model and WT mice.

Annexe 1: Posters and oral presentations

2018: Poster presentation at the FENS Forum 2018, Berlin, Germany

Chronic KCC2 extinction in mouse dorsal hippocampus compromises contextual and spatial memory

Simonnet C, Goutierre M., Moutkine I, Daumas S, Poncer JC

Intraneuronal chloride concentration is controlled by the potassium/chloride cotransporter KCC2 which thereby influences the efficacy and polarity of GABA signaling. In addition to ion transport, KCC2 also controls the function and plasticity of glutamatergic synapses through interactions with actin-related proteins. Thus, KCC2 acts as a master regulator of both synaptic inhibition and excitation. Since KCC2 expression is altered in a variety of neurological and psychiatric conditions associated with cognitive impairment, we asked whether chronic down-regulation of KCC2 may impact cognitive performances in mice.

Chronic KCC2 suppression using viral-based RNA interference, impaired both contextual memory in a fear conditioning paradigm and spatial memory in a place recognition task. Our data show these deficits are associated with impairment of both hippocampal LTP and rhythmogenesis. In order to further dissect the underlying mechanisms, we compared suppression of KCC2 expression in principal neurons vs. GABAergic interneurons. Somewhat unexpectedly, we observed that KCC2 extinction only in principal neurons fails to impact contextual and spatial memory, suggesting KCC2 expression in GABAergic interneurons may be of particular importance in this phenotype.

We conclude that KCC2 down-regulation in dorsal hippocampus compromises contextual and spatial memory, through mechanisms that remain to be fully explored. Strategies aiming to stabilize KCC2 membrane expression or restore chloride homeostasis may then prove beneficial in rescuing cognitive impairments in conditions associated with KCC2 down-regulation.

2018: Oral presentation at Roscoff seminar for PhD students, Roscoff, France

**Synaptic and cognitive deficits induced by down-regulation of the neuronal transporter
KCC2**

Intraneuronal chloride concentration is controlled by the potassium/chloride cotransporter KCC2 which thereby influences the efficacy and polarity of GABA signaling. In the pathology, KCC2 suppression may compromise network activity through alterations of GABA signaling and rhythmogenesis that underlie memory encoding and consolidation. In addition, recent studies revealed ion transport-independent functions of KCC2 at excitatory synapses. Thus, chronic KCC2 down-regulation in hippocampal neurons impairs long-term potentiation (LTP) of glutamatergic synapses through modifications of actin dynamics in dendritic spines. Since KCC2 expression is altered in a variety of neurological and psychiatric conditions associated with cognitive impairment, we asked whether chronic down-regulation of KCC2 may impact cognitive performances in mice.

Chronic KCC2 suppression using viral-based RNA interference, impaired both contextual memory in a fear conditioning paradigm and spatial memory in a place recognition task. Our data show these deficits are associated with impairment of both hippocampal LTP and rhythmogenesis. I specifically focused on sharp-wave-ripples (SWRs) that are necessary for memory consolidation and theta-band activity associated with REM sleep and exploration, which is involved in both memory acquisition and consolidation. My data reveal a specific impairment of SWR dynamics upon chronic KCC2 down-regulation.

In order to further dissect the underlying mechanisms, we suppressed KCC2 expression in principal neurons. Somewhat unexpectedly, we observed this extinction failed to impact contextual and spatial memory, suggesting KCC2 expression in GABAergic interneurons may be of particular importance in this phenotype.

We conclude that KCC2 down-regulation in dorsal hippocampus compromises contextual and spatial memory, through mechanisms that remain to be fully explored. Strategies aiming to stabilize KCC2 membrane expression or restore chloride homeostasis may then prove beneficial in rescuing cognitive impairments in conditions associated with KCC2 down-regulation.

2017: Poster presentation at the Society for Neuroscience conference, Washington DC, USA

Chronic KCC2 extinction in mouse dorsal hippocampus compromises contextual memory

Simonnet C, Goutierre M., Kouidri Y. , Moutkine I, Daumas S, Poncer JC

GABA is the main inhibitory neurotransmitter in the adult brain, and provides fast inhibitory neurotransmission mostly through activation of GABAA receptors. As these receptors are mainly permeable to chloride ions, mechanisms controlling chloride homeostasis directly influence GABAergic transmission. In mature cortical neurons, intracellular chloride concentration is controlled by the activity of neuronal chloride/potassium co-transporter KCC2, the expression of which is down-regulated in several neurological and psychiatric conditions associated with cognitive impairment. In the pathology, KCC2 suppression may compromise network activity through alterations of GABA signaling and rhythmogenesis that underlie memory encoding and consolidation. In addition, recent studies revealed ion transport-independent functions of KCC2 at excitatory synapses. Thus, chronic KCC2 down-regulation in hippocampal neurons impairs both the efficacy and long-term potentiation of glutamatergic synapses through modifications of actin dynamics in dendritic spines. We therefore asked whether chronic down-regulation of KCC2 may impact cognitive performances in mice and explored the underlying mechanisms.

Using a viral-based, chronic extinction by RNA interference, we suppressed KCC2 expression in the dorsal hippocampus of adult mice and tested hippocampal LTP, learning and memory 2-4 weeks post-infection. Chronic KCC2 suppression impaired LTP both at perforant path synapses onto granule cells and Schaffer collateral synapses onto CA1 neurons. Next, we tested the behavioral impact of chronic KCC2 suppression in several hippocampus-dependent and independent memory tasks. In a fear conditioning paradigm, we found that contextual memory was specifically compromised upon KCC2 suppression in dorsal hippocampus while cued memory was intact. Next we asked whether these deficits primarily depend on alteration of GABAergic vs. glutamatergic signaling using overexpression of a dominant-negative peptide of KCC2. This approach allows us to specifically disrupt KCC2 interaction with protein partners without affecting its ion transport function. However this did not lead to

detectable deficits in contextual memory, suggesting these did not primarily depend on KCC2 transport-independent functions.

We conclude that KCC2 down-regulation in the dorsal hippocampus compromises contextual memory, through mechanisms that remain to be fully explored. Strategies aiming to stabilize KCC2 membrane expression or restore chloride homeostasis may then prove beneficial in rescuing cognitive impairments in conditions associated with KCC2 down-regulation.

2017: Poster presentation at the ENP (Neuroscience school of Paris) days, Paris, France.

**Chronic extinction of the chloride transporter KCC2 compromises contextual
memory**

Simonnet C, Chevy Q, Moutkine I, Daumas S, Poncer JC

Many neuropsychiatric disorders with cognitive impairment such as schizophrenia, autism and Rett syndrome are associated with reduced expression of the neuronal chloride/potassium co-transporter KCC2. Since KCC2 controls intracellular chloride, its activity directly influences GABAergic transmission efficacy. However, recent studies also revealed ion transport-independent functions of KCC2 at excitatory synapses (Gauvain et al PNAS 2011, Chevy et al J Neurosci 2015). Thus, chronic KCC2 down-regulation in hippocampal neurons impairs both the efficacy and long-term potentiation (LTP) of glutamatergic synapses through modifications of actin dynamics in dendritic spines. We are therefore exploring whether KCC2 down-regulation in the dorsal hippocampus may impact learning and memory. Our data reveal a specific alteration of contextual memory but not cued memory upon KCC2 suppression in dorsal hippocampal neurons. We will next explore whether these deficits depend on alterations of GABAergic *vs.* glutamatergic signaling. These results will help predict the best approach to rescue cognitive deficits in pathologies associated with KCC2 down-regulation.

Annexe 2: Publications

Proteomic analysis of postsynaptic proteins in regions of the human neocortex.

Roy M, Sorokina O, Skene N, **Simonnet C**, Mazzo F, Zwart R, Sher E, Smith C, Armstrong JD, Grant SGN.

Nature Neuroscience. 2017

The postsynaptic proteome of excitatory synapses comprises ~1,000 highly conserved proteins that control the behavioral repertoire, and mutations disrupting their function cause >130 brain diseases. Here, we document the composition of postsynaptic proteomes in human neocortical regions and integrate it with genetic, functional and structural magnetic resonance imaging, positron emission tomography imaging, and behavioral data. Neocortical regions show signatures of expression of individual proteins, protein complexes, biochemical and metabolic pathways. We characterized the compositional signatures in brain regions involved with language, emotion and memory functions. Integrating large-scale GWAS with regional proteome data identifies the same cortical region for smoking behavior as found with fMRI data. The neocortical postsynaptic proteome data resource can be used to link genetics to brain imaging and behavior, and to study the role of postsynaptic proteins in localization of brain functions.

Pyk2 modulates hippocampal excitatory synapses and contributes to cognitive deficits in a Huntington's disease model.

Giralt A, Brito V, Chevy Q, **Simonnet C**, Otsu Y, Cifuentes-Diaz C, de Pins B, Coura R, Alberch J, Ginés S, Poncer JC, Girault JA.
Nature Communication. 2017

The structure and function of spines and excitatory synapses are under the dynamic control of multiple signalling networks. Although tyrosine phosphorylation is involved, its regulation and importance are not well understood. Here we study the role of Pyk2, a non-receptor calcium-dependent protein-tyrosine kinase highly expressed in the hippocampus. Hippocampal-related learning and CA1 long-term potentiation are severely impaired in Pyk2-deficient mice and are associated with alterations in NMDA receptors, PSD-95 and dendritic spines. In cultured hippocampal neurons, Pyk2 has autophosphorylation-dependent and -independent roles in determining PSD-95 enrichment and spines density. Pyk2 levels are decreased in the hippocampus of individuals with Huntington and in the R6/1 mouse model of the disease. Normalizing Pyk2 levels in the hippocampus of R6/1 mice rescues memory deficits, spines pathology and PSD-95 localization. Our results reveal a role for Pyk2 in spine structure and synaptic function, and suggest that its deficit contributes to Huntington's disease cognitive impairments.

Divergent routing of positive and negative information from the amygdala during memory retrieval.

Beyeler A, Namburi P, Glober GF, **Simonnet C**, Calhoun GG, Concers GF, Luck R, Wildes CP, Tye KM.
Neuron. 2016

Although the basolateral amygdala (BLA) is known to play a critical role in the formation of memories of both positive and negative valence, the coding and routing of valence-related information is poorly understood. Here, we recorded BLA neurons during the retrieval of associative memories and used optogenetic-mediated phototagging to identify populations of neurons that synapse in the nucleus accumbens (NAc), the central amygdala (CeA), or ventral hippocampus (vHPC). We found that despite heterogeneous neural responses within each population, the proportions of BLA-NAc neurons excited by reward predictive cues and of BLA-CeA neurons excited by aversion predictive cues were higher than within the entire BLA. Although the BLA-vHPC projection is known to drive behaviors of innate negative valence, these neurons did not preferentially code for learned negative valence. Together, these findings suggest that valence encoding in the BLA is at least partially mediated via divergent activity of anatomically defined neural populations.

Human post-mortem synapse proteome integrity screening for proteomic studies of postsynaptic complexes.

Bayés A, Collins MO, Galtrey CM, **Simonnet C**, Roy M, Croning MD, Gou G, van de Lagemaat LN, Milward D, Whittle IR, Smith C, Choudhary JS, Grant SG.
Molecular Brain. 2014

BACKGROUND:

Synapses are fundamental components of brain circuits and are disrupted in over 100 neurological and psychiatric diseases. The synapse proteome is physically organized into multiprotein complexes and polygenic mutations converge on postsynaptic complexes in schizophrenia, autism and intellectual disability. Directly characterising human synapses and their multiprotein complexes from post-mortem tissue is essential to understanding disease mechanisms. However, multiprotein complexes have not been directly isolated from human synapses and the feasibility of their isolation from post-mortem tissue is unknown.

RESULTS:

Here we establish a screening assay and criteria to identify post-mortem brain samples containing well-preserved synapse proteomes, revealing that neocortex samples are best preserved. We also develop a rapid method for the isolation of synapse proteomes from human brain, allowing large numbers of post-mortem samples to be processed in a short time frame. We perform the first purification and proteomic mass spectrometry analysis of MAGUK Associated Signalling Complexes (MASC) from neurosurgical and post-mortem tissue and find genetic evidence for their involvement in over seventy human brain diseases.

CONCLUSIONS:

We have demonstrated that synaptic proteome integrity can be rapidly assessed from human post-mortem brain samples prior to its analysis with sophisticated proteomic methods. We have also shown that proteomics of synapse multiprotein complexes from well preserved post-mortem tissue is possible, obtaining structures highly similar to those isolated from biopsy tissue. Finally we have shown that MASC from human synapses are involved with over seventy brain disorders. These findings should have wide application in understanding the synaptic basis of psychiatric and other mental disorders.

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