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Role of the cotransporter KCC2 in cortical excitatory synapse development and febrile seizure susceptibility

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Résumé

Le co-transporteur KCC2 spécifique au potassium et chlore a pour rôle principal de réduire la concentration intracellulaire de chlore, entraînant l'hyperpolarisation des courants GABAergic l'autorisant ainsi à devenir inhibiteur dans le cerveau mature. De plus, il est aussi impliqué dans le développement des synapses excitatrices, nommées aussi les épines dendritiques. Le but de notre projet est d'étudier l'effet des modifications concernant l'expression et la fonction de KCC2 dans le cortex du cerveau en développement dans un contexte de convulsions précoces.

Les convulsions fébriles affectent environ 5% des enfants, et ce dès la première année de vie. Les enfants atteints de convulsions fébriles prolongées et atypiques sont plus susceptibles à développer l'épilepsie. De plus, la présence d'une malformation cérébrale prédispose au développement de convulsions fébriles atypiques, et d'épilepsie du lobe temporal. Ceci suggère que ces pathologies néonatales peuvent altérer le développement des circuits neuronaux irréversiblement. Cependant, les mécanismes qui sous-tendent ces effets ne sont pas encore compris. Nous avons pour but de comprendre l'impact des altérations de KCC2 sur la survenue des convulsions et dans la formation des épines dendritiques.

Nous avons étudié KCC2 dans un modèle animal de convulsions précédemment validé, qui combine une lésion corticale à P1 (premier jour de vie postnatale), suivie d'une convulsion induite par hyperthermie à P10 (nommés rats LHS). À la suite de ces insultes, 86% des rats mâles LHS développent l'épilepsie à l'âge adulte, au même titre que des troubles d'apprentissage. À P20, ces animaux presentent une augmentation de l'expression de KCC2 associée à une hyperpolarisation du potentiel de réversion de GABA. De plus, nous avons observé des réductions dans la taille des épines dendritiques et l'amplitude des courants post-synaptiques excitateurs miniatures, ainsi qu'un déficit de mémoire spatial, et ce avant le développement des convulsions spontanées. Dans le but de rétablir les déficits observés chez les rats LHS, nous avons alors réalisé un knock-down de KCC2 par shARN spécifique par électroporation *in utero.* Nos résultats ont montré une diminution de la susceptibilité aux convulsions due à la lésion corticale, ainsi qu'une restauration de la taille des épines. Ainsi, l'augmentation de KCC2 à la suite d'une convulsion précoce, augmente la susceptibilité aux

convulsions modifiant la morphologie des épines dendritiques, probable facteur contribuant à l'atrophie de l'hippocampe et l'occurrence des déficits cognitifs.

Le deuxième objectif a été d'inspecter l'effet de la surexpression précoce de KCC2 dans le développement des épines dendritiques de l'hippocampe. Nous avons ainsi surexprimé KCC2 aussi bien *in vitro* dans des cultures organotypiques d'hippocampe, qu' *in vivo* par électroporation *in utero*. À l'inverse des résultats publiés dans le cortex, nous avons observé une diminution de la densité d'épines dendritiques et une augmentation de la taille des épines. Afin de confirmer la spécificité du rôle de KCC2 face à la région néocorticale étudiée, nous avons surexprimé KCC2 dans le cortex par électroporation *in utero*. Cette manipulation a eu pour conséquences d'augmenter la densité et la longueur des épines synaptiques de l'arbre dendritique des cellules glutamatergiques. En conséquent, ces résultats ont démontré pour la première fois, que les modifications de l'expression de KCC2 sont spécifiques à la région affectée. Ceci souligne les obstacles auxquels nous faisons face dans le développement de thérapie adéquat pour l'épilepsie ayant pour but de moduler l'expression de KCC2 de façon spécifique.

Mots-clés : co-transporteur KCC2, cortex, convulsions fébriles atypiques, dysplasie corticale, modèle animal de TLE, susceptibilité aux convulsions, développement des synapses excitatrices.

Abstract

The potassium-chloride cotransporter KCC2 decreases intracellular Cl^- levels and renders GABA responses inhibitory. In addition, it has also been shown to modulate excitatory synapse development. In this project, we investigated how alterations of KCC2 expression levels affect these two key processes in cortical structures of a normal and/or epileptic developing brain.

First, we demonstrate that KCC2 expression is altered by early-life febrile status epilepticus. Febrile seizures affect about 5% of children during the first year of life. Atypical febrile seizures, particularly febrile status epilepticus, correlate with a higher risk of developing cognitive deficits and temporal lobe epilepsy as adults, suggesting that they may permanently change the developmental trajectory of neuronal circuits. In fact, the presence of a cerebral malformation predisposes to the development of atypical febrile seizures and temporal lobe epilepsy. The mechanisms underlying these effects are not clear. Here, we investigated the functional impact of this alteration on subsequent synapse formation and seizure susceptibility.

We analyzed KCC2 expression and spine density in the hippocampus of a well-established rodent model of atypical febrile seizures, combining a cortical freeze lesion at post-natal day 1 (P1) and hyperthermia-induced seizure at P10 (LHS rats). 86% of these LHS males develop epilepsy and learning and memory deficits in adulthood. At P20, we found a precocious increase in KCC2 protein levels, accompanied by a negative shift of the reversal potential of GABA (E_{GABA}) by gramicidin-perforated patch. In parallel, we observed a reduction in dendritic spine size by DiI labelling and a reduction of miniature excitatory postsynaptic current (mEPSC) amplitude in CA1 pyramidal neurons, as well as impaired spatial memory. To investigate whether the premature expression of KCC2 played a role in these alterations in the LHS model, and on seizure susceptibility, we reduced KCC2 expression in CA1 pyramidal neurons by *in utero* electroporation of shRNA using a triple-probe electrode. This approach lead to reduced febrile seizure susceptibility, and rescued spine size shrinkage in LHS rats. Our results show that an increase of KCC2 levels induced by

early-life insults affect seizure susceptibility and spine development and may be a contributing factor to the occurrence of hippocampal atrophy and associated cognitive deficits in LHS rats.

Second, we investigated whether KCC2 premature overexpression plays a role in spine alterations in the hippocampus. We overexpressed KCC2 in hippocampal organotypic cultures by biolistic transfection and *in vivo* by *in utero* electroporation. In contrast to what was previously published, we observed that both manipulations lead to a decrease in spine density in the hippocampus, as well as an increase in spine head size *in vivo*. In fact, it has been previously shown that overexpressing KCC2 leads to an increase of spine density in the cortex *in vivo*. To prove that this discrepancy is due to brain regional differences, we overexpressed KCC2 in the cortex by *in utero* electroporation, and similarly found an increase in spine density and length. Altogether, our results demonstrate for the first time, that alterations of KCC2 expression are brain circuit-specific. These findings highlights the obstacles we will face to find adequate pharmacological treatment to specifically modulate KCC2 in a region-specific and time-sensitive manner in epilepsy.

Keywords : KCC2 cotransporter, cortex, atypical febrile seizures, cortical dysplasia, animal model of TLE, seizure susceptibility, excitatory synapse development.

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List of abreviations

$[Cl^-]_i$: Intracellular chloride concentration

5HT3aR: ionotropic serotonin receptor 5HT3a

aa: Amino acid

AIS: Axon Initial Segment

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR: AMPA receptor

Bax: Bcl-2-associated X

BDNF: Brain-derived neurotrophic factor

CA (1 and 3): Cornus ammonis

CC: Corpus callosum

CCC: Cation chloride cotransporters

CCK: Cholecystokinin peptide

CD: Cortical Dysplasia

ChC: Chandelier cells

CKB: Brain-type creatine kinase

Cof1: Cofilin

CP: Cortical Plate

CP1: CCC interacting protein 1

CR: Calretinin

CRN: Cajal-Retzius neurons

DG: Dentate gyrus

DS: Down syndrome

E: Embryonic day

ECM: Extracellular matrix

EEG: Electroencephalogram

eEPSP: evoked Excitatory PostSynaptic Potentials

E_{GABA}: Reversal potential of GABA

Egr4: Early growth response 4

EM: Electron microscopy

ERK 1/2: Extracellular signal-regulated kinase 1/2

FS-BC: Fast-spiking basket cells

FSs: Febrile seizures

GABA: Gamma-aminobutyric acid

$GABA_AR$: $GABA_A$ receptor

$GABA_BR$: $GABA_B$ receptor

GAD: Glutamic acid decarboxylases

GC: Granule cells

GlyR: Glycine receptor

GPR 39: Gq-coupled receptor 39

HEK293: Human embryonic kidney 293

HS: Hyperthermic seizures

IGF-1: Insulin-like growth factor 1

INs: Interneurons

IPC: Intermediate progenitor cells

IZ: Intermediate zone

KAR: Kainate-type glutamate receptor

KCC2-ΔNTD: KCC2 construct with N-terminal deleted (transporter deficient)

mEPSC: miniature Excitatory Postsynaptic Current

MRI: Magnetic Resonance Imaging

mTLE: mesial Temporal Lobe Epilepsy

MZ: Marginal zone

NE: Neuroepithelium

Neto2: Neuropilin and toloid like-2

NMDA: N- methyl-d-aspartate

NMDAR: NMDA receptor

NRSE: Neuron-restrictive silencing elements

NRSF: Neuron-restrictive silencing factors

OPT: Optomotor task

OSR1: Oxidative stress-responsive kinase 1

P: Postnatal day

P2X4R: purinergic receptor

Pak: p21-activated serine/threonine-protein kinase

PAM: Protein Associated with Myc

PCs: Pyramidal cells

PKC: Protein kinase C

PLC: Phospholipase C

PNN: Perineuronal net

PP: Preplate

PP1: Protein phosphatase 1

PV: Parvalbumin

PV+ cells: ChC and FS-BC

Rac: Small GTPase of the Rho family

RCC1: Regulator of chromatin condensation

RGCs: Radial glial cells

sEPSC: spontaneous Excitatory PostSynaptic Currents

Ser: Serine

SCI: Spinal cord injury

SL: Stratum lacunosum

SLM: Stratum lacunosum-moleculare

SM: Stratum moleculare

SO: Stratum oriens

SOM: Somatostatin

SP: Subplate

SPAK: Ste20p-related proline/alanine-rich kinase

SPN: subplate neurons

SPN: Subplate neurons

sPSC: spontaneous Postsynaptic current

sPSP: spontaneous Postsynaptic potential

SR: Stratum radiatum

SRS: Spontaneous recurrent seizures

SVZ: Subventricular zone

Thr: Threonine

TTX: Tetrodotoxin

Tyr: Tyrosine

USF1/2: Upstream stimulating factors 1 and 2

VEP: Visual evoked potentials

VGAT: vesiculat $GABA_A$ transporter

VIAAT: Vesicular inhibitory amino acid transporter

VZ: Ventricular zone

WM: White matter

WNK: With-no-lysine kinase

βPix: Beta isoform of guanine nucleotide exchange factor

To my mother whom I miss deeply

And to my father, sister and brother who inspired my hard work and dedication

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I would like to take a few lines to personally thank everyone who has been part of my journey. First, I would like to acknowledge and thank my supervisors Graziella and Lionel for giving me this great opportunity, as well as for teaching me so much. This thesis would not be what it is today without the both of you, and I am extremely grateful. I would like to add a special note for Graziella who has taken so much time on a daily basis, to help and guide me through the many challenges I faced. Your dedication to your lab and passion for neuroscience is remarkable and inspiring; and if I were to ever have my own lab, I would aspire to be as great as you!

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Chapter I

General Introduction

This thesis' project focuses on understanding the role of the cotransporter KCC2 in seizure susceptibility and hippocampal glutamatergic synapse alterations in a rat model of atypical febrile seizures. I will first review the normal cortical circuit development from embryonic to postnatal stages. Secondly, I will review KCC2 expression pattern, function and activation in the developing brain. Thirdly, I will review the key mechanisms implicated in epileptogenesis of mesial Temporal Lobe epilepsy (MTLE), febrile seizures and cortical dysplasia; and lastly, I will describe the chosen rodent model of atypical febrile seizure, which is a well-accepted preclinical model for MTLE.

1. Cortical circuit development

Proper brain function relies on the establishment of a precise and selective pattern of synaptic connectivity and an appropriate balance between excitatory and inhibitory synapses. Glutamatergic and GABAergic neurons are two major types of neurons that form excitatory and inhibitory outputs to their target neurons, respectively. The equilibrium between excitatory and inhibitory inputs is critical for the proper performance of the brain, and their imbalance may lead to various neurological disorders such as autism, schizophrenia and epilepsy[1]. My project focuses on cortical structures (neocortex and hippocampus), therefore I will emphasize on GABAergic and glutamatergic circuit development in these regions.

In the cortex, the main source of inhibition comes from a very heterogeneous GABAergic interneuron population that balances the predominant excitatory pyramidal cell population. Although interneurons (INs) represent a smaller percentage of all cortical neurons (~20%), and GABAergic synapses only represent approximately 5% of all synapses onto PCs

mostly concentrated in perisomatic regions, their function is extremely crucial in shaping the spatial and temporal profile of principal cell firing, thereby controlling network activity and plasticity[2-5]. GABAergic innervation also controls the temporal synchrony of activity among large numbers of pyramidal neurons within a population, because one single GABAergic interneuron can innervate hundreds of pyramidal cells[2,6,7]. Futhermore, GABAergic INs differ greatly in dendritic and axonal morphology, gene expression, electrophysiological properties, input and output connectivity, and subcellular domain distribution and properties of their synapses. Recent reports suggest that there may be at least 20 different subtypes of INs in the neocortex or hippocampus[8-17]. This astounding variety of GABAergic INs indicates that they may serve distinct functions in cortical networks[18-20]. Further, INs expressing similar molecular markers but located in different brain regions may show differences in their functional or/and connectivity properties, making their classification overall controversial. For example, fast-spiking perisomatic-targeting GABAergic INs (basket cells), one of the most easily identifiable and accepted GABAergic cell type, primarily target either excitatory (in the neocortex and hippocampus) or inhibitory projection neurons (in the striatum and central and medial amygdala) depending on which structure they reside in. For all these reasons, classifying INs across different telencephalic regions is both a daunting task and a hotly debated issue, which no doubt will see a strong evolution in the next years with the application of recently developed techniques to identify neuron connectivity and single-cell genetic profiles[18].

The pyramidal cell population, representing the majority of neurons in the cortex, was for a long time thought to be homogenous. However, it is now well accepted that pyramidal neurons differ between layers and even within the same layer. Tyler W.A. et al. (2015) demonstrates that several neural precursor populations actually generate distinct pyramidal neurons in cortical layers II/III, which in turn exhibit different electrophysiological and structural properties depending on the precursor cell type from which they originated[21]. However, independent of their differences, all these populations are still grouped under the same general term of pyramidal cells (PCs). Altogether, these various types of excitatory and inhibitory neurons form the complex circuitry of the neocortex and hippocampus. To decipher through this intricate structure, I will first discuss more in details the embryonic development,

when these neurons are generated, and how they make their way to their final destinations. Secondly, I will explain how mature circuits are established through synaptogenesis, apoptosis and cortical plasticity.

1.1. Embryonic development

The cortex starts to develop into the neocortex and the hippocampus *in utero*. These structures gain their complexity when the neurons that compose them reach their final location. To establish such precise synaptic patterns in these regions, neurons pass through multiple checkpoints during development, such as cell fate determination, cell migration and localization. I will first describe the development of the neocortex and hippocampus. I will then review the diversity of neurons in these structures and when and where they are generated. Lastly, I will explain how they migrate toward and within the cortex.

1.1.1. Cortex anatomy and structure

The telencephalon consists of the pallium and subpallium. It is the pallium that gives rise to the neocortex and hippocampus. As described above, there are two broad classes of cortical neurons: INs that make local connections (mostly inhibitory GABAergic neurons), and projection neurons, which extent axons to distant intracortical, subcortical and subcerebral targets (mostly glutamatergic PCs[22]; and GABAergic neurons[23]). Until well after birth, these structures continue to develop into their mature state by circuits refinement[2]. This section will briefly describe how the hippocampus and the neocortex develop into their mature form.

Hippocampus

At the preplate stage (embryonic day 14; E14) the hippocampus neuroepithelium lobule of the lateral ventricale is composed of three morphogenetic components (see figure 1). The first one is the ammonic neuroepithelium, which generates PCs and INs of stratum oriens (SO)

and radiatum (SR). The second component is the dentate neuroepithelium, which generates granule cells and large neurons of stratum moleculare and hilus. Finally, the glioepithelium, the third component, produces glial cells. These components represent the ventricular zone where neurons emerge from to form the hippocampal plate stage (from E15-E19) to finally become the hippocampus [2,24].

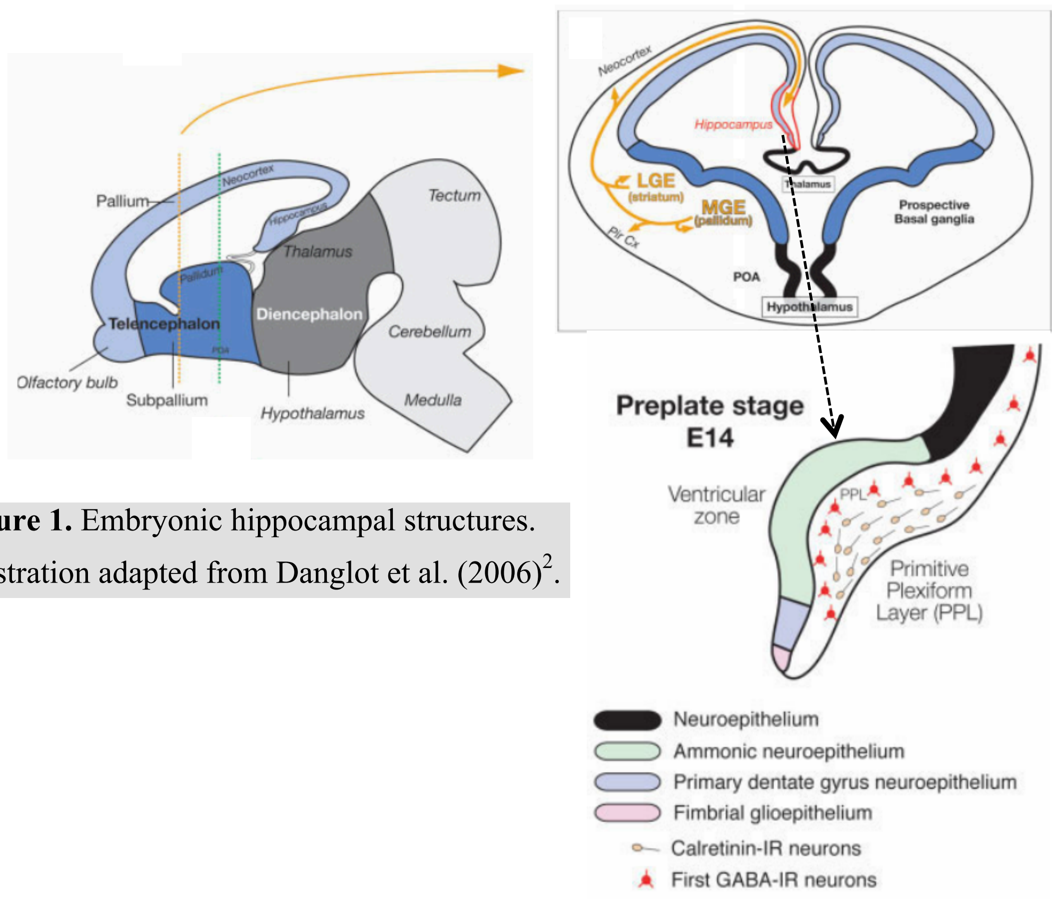


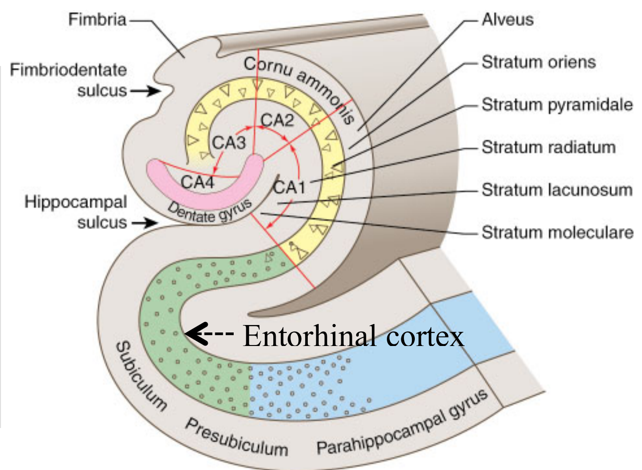
Figure 1. Embryonic hippocampal structures. Illustration adapted from Danglot et al. (2006)[2].

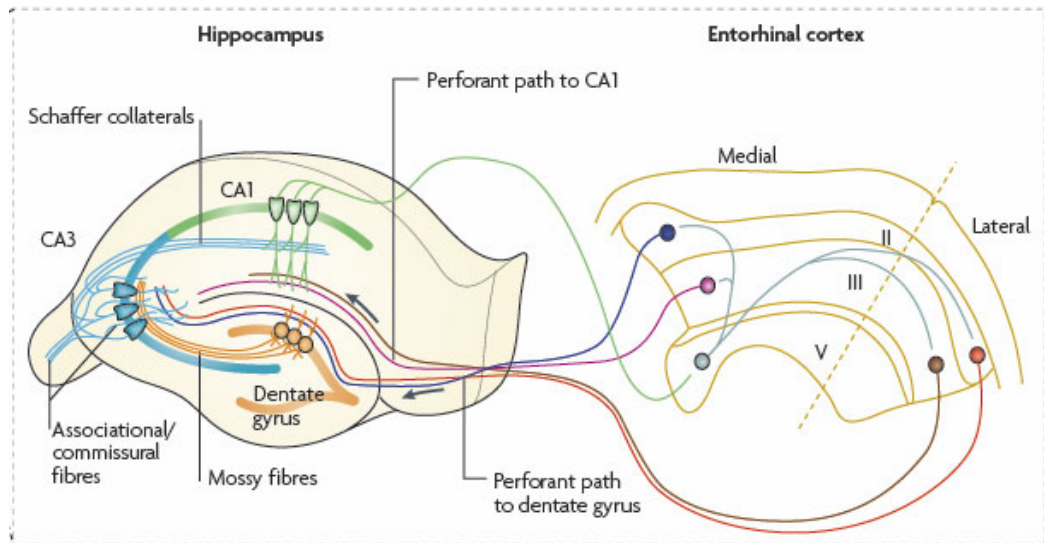
The mature hippocampus resembles a sea horse in shape, where PCs are densely packed together, creating a very tightly organized circuit. This circuit is also termed the trisynaptic excitatory pathway, because is formed by the connections between principal excitatory cells from the dentate gyrus (DG) and the *cornus ammonis* 3 and 1 (CA3, CA1; see Figure 2). The perforant path lead axons originating from the entorhinal cortex, which is located in the parahippocampal gyrus, to granule cells in the DG in the stratum moleculare (SM) layer in majority, and to the distal-most part of the apical dendrites of PCs in the stratum lacunosum-moleculare (SLM) layer. This path is the main site of interaction between the hippocampus

and other brain regions. Further, granule cell axons, termed mossy fibers, innervate PCs in the CA3. These particular fibers are highly affected in many neurological disorders such as epilepsy, which leads to network reorganization, mossy fiber sprouting and the emergence of ectopic granule cells, suggesting that a tight regulation of the circuitry is pivotal for normal circuit development and connectivity. Finally, CA3 PCs axons contact the apical dendrite of CA1 pyramidal neurons, in a path termed Shaffer collaterals. CA1 pyramidal neurons then input back to the entorhinal cortex and subiculum[2].

Figure 2. Hippocampal anatomy and connectivity pattern.

Image adapted from Neves et al. (2008)[25], where the top figure illustrates the hippocampus as it is located in the mouse brain, and the bottom image illustrates the excitatory synaptic connectivity between the hippocampus and the entorhinal cortex.





The CA1 represents the output of the hippocampus, and is the focus of my project. CA1 PCs as well as parvalbumin-expressing GABAergic INs are located in the SM. From this layer, basal dendrites and the axon of pyramidal cells extend upwards to the stratum oriens

(SO) and alveus layer, while the apical dendrites extend downwards to the stratum radiatum (SR) and stratum lacunosum-moleculare (SLM) layer. The laminar segregation of afferent fibers and the compartmentalized structure of PCs in the hippocampus allow GABAergic INs to perform spatially segregated information processing at the same time. As they usually innervate distinct domains of their target cells, the strategic placement of inhibitory synapses indicates that they serve distinct functions in the hippocampal network[4]. Additionally, INs in the DG inhibit granule cell activity and allow to control and gate the incoming signals to the hippocampus. As such, blocking $GABA_A$ receptor in the hippocampus, *in vivo* and *in vitro*, induces epileptiform activity[26,27], while potentiating GABAergic inhibition increases the amplitude of spontaneous gamma oscillations, impacting working memory[28] and hippocampus-dependent memory tasks[2,29]. Altogether, the hippocampus formation is developed from very early on, and gains complexity through its diverse populations of neurons, and its precise organization of synaptic input. At the mature state, the hippocampus is critical for navigation, context-dependent learning and episodic memory[30].

Neocortex

The mammalian cerebral cortex is comprised of six layers, which contains similar neuron subtypes as the hippocampus characterized by distinct projection patterns and gene expression profiles[31]. During early development, the cortical neuroepithelium located on the dorsolateral wall of the rostral neural tube expands, and give rise to neocortical PCs. From E10.5 the first layer adjacent to the ventricle is born and termed the ventricular zone (VZ) (see figure 3). As neurogenesis proceeds, an additional proliferative layer, which is termed the subventricular zone (SVZ), forms above the VZ, and an intermediate zone (IZ) forms above the SVZ. The VZ and SVZ contain the progenitor cells that will produce glutamatergic neurons of all neocortical layers. The first-born neurons form the preplate layer (PP) above the VZ/SVZ/IZ, which is then split into the superficial marginal zone (MZ) and subplate (SP) by around E12.5. In between these two layers begins to develop the cortical plate (CP), which will eventually grow to become the multilayered cortex. This laminar organization occurs in an ‘inside-out’ manner, meaning that newly generated neurons bypass those previously

generated (older) from the PP to reach the superficial CP[32]. Moreover, the superficial marginal zone will grow to become layer 1, while the IZ will be replaced by white matter. As development advances, and new neurons are generated and migrate towards their final destination, each cortical layer gets more clearly defined. Eventually, in the adult, there are no ventricular zones, just the white matter, the subplate and the six cortical layers.

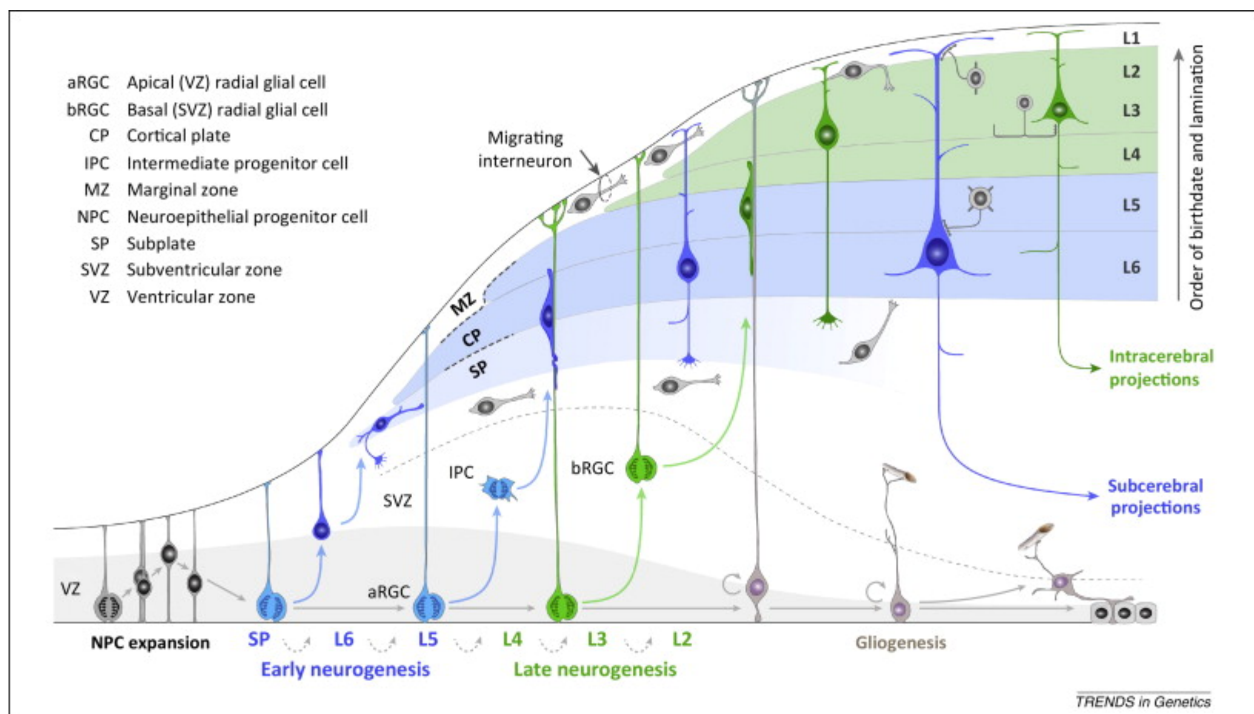


Figure 3. Cortical layers development.
Illustration adapted from Shibata et al, (2015)[33].

The neocortex is quite larger and thus, a more complex structure than the hippocampus. It extends to the whole brain, and controls most cognitive, executive, emotional and autonomic functions[33]. Cortical neurons form a variety of long-rage connections, from one region of the neocortex to the other, but also from the neocortex to other structures in the brain. As such, there are three major subtypes of cortical projection neurons: associative, commissural and corticofugal (see figure 4). Layers I to III are the primary origin and termination of intracortical afferents, which are either associative or commissural. Associative projection neurons project their axons within the ipsilateral cerebral hemisphere. Commissural, or callosal, projection neurons are located in layers II/III, V and VI, extend their axons across the

corpus callosum (CC) to the opposite hemisphere. Within this group, there are different PCs projections: one that extends a single projection to the contralateral cortex (PCs from layer II/II, V/VI); one that extends to the contralateral cortex as well as the ipsilateral and contralateral striatum (PCs from layer V); finally, one that extends to the contralateral cortex as well as the ipsilateral frontal cortex (PCs from superficial layer V). Furthermore, corticofugal projection neurons project out of the cortex towards subcerebral regions (subcerebral projection neurons) and the thalamus (corticothalamic neurons). Subcerebral projection neurons, termed type I layer V projection neurons, originate from the sensorimotor area of the cortex or from the visual cortex and extend towards the basal ganglia, brainstem and spinal cord. Corticothalamic neurons mostly from layer VI project subcortically to different nuclei in the thalamus[22]. The pathway from cortical neurons of layer VI to the thalamus is a glutamatergic excitatory pathway. They interconnect in a reciprocal manner since most thalamic neurons receive feedback from layer VI of the same cortical column they innervate. Additionally, the thalamic reticular nucleus is composed of GABAergic neurons that are mostly located dorsal and lateral to the thalamic relay nuclei. Cortical neurons from layer VI actually project to these thalamic reticular cells, on their way to innervate thalamic relay cells. Thus, while exciting these relay cells directly, they also inhibit them indirectly by activating the GABAergic reticular cells[34]. Consequently, corticothalamic modulation depends on the balance and communication of its monosynaptic excitation and its disynaptic feedforward inhibition, which is dynamic and activity-dependent. During low-frequency corticothalamic activity, there is a strong and long-lasting inhibition that blocks spiking of thalamic relay neurons. Contrastingly, high-frequency corticothalamic activity leads to an overall excitation and increased spiking probability of thalamic relay neurons[35]. Contrary to what was previously thought, there are ten times more cortical synapses than thalamic synapses onto layer IV stellate neurons. Using high-throughput light microscopy, validated by electron microscopy, Schoonover et al. (2014) demonstrated that thalamocortical synapses are slightly more proximal to the soma than cortical synapses. Despite the subtle anatomical bias, the fact that they only control one of ten excitatory synaptic inputs, thalamocortical and corticocortical inputs equally influence the membrane potential of cortical neurons from layer IV barrel cortex. Both classes of synapses are equivalent in strength; therefore, strength is likely not a predictor of thalamic efficacy and influence on cortical neurons[36].

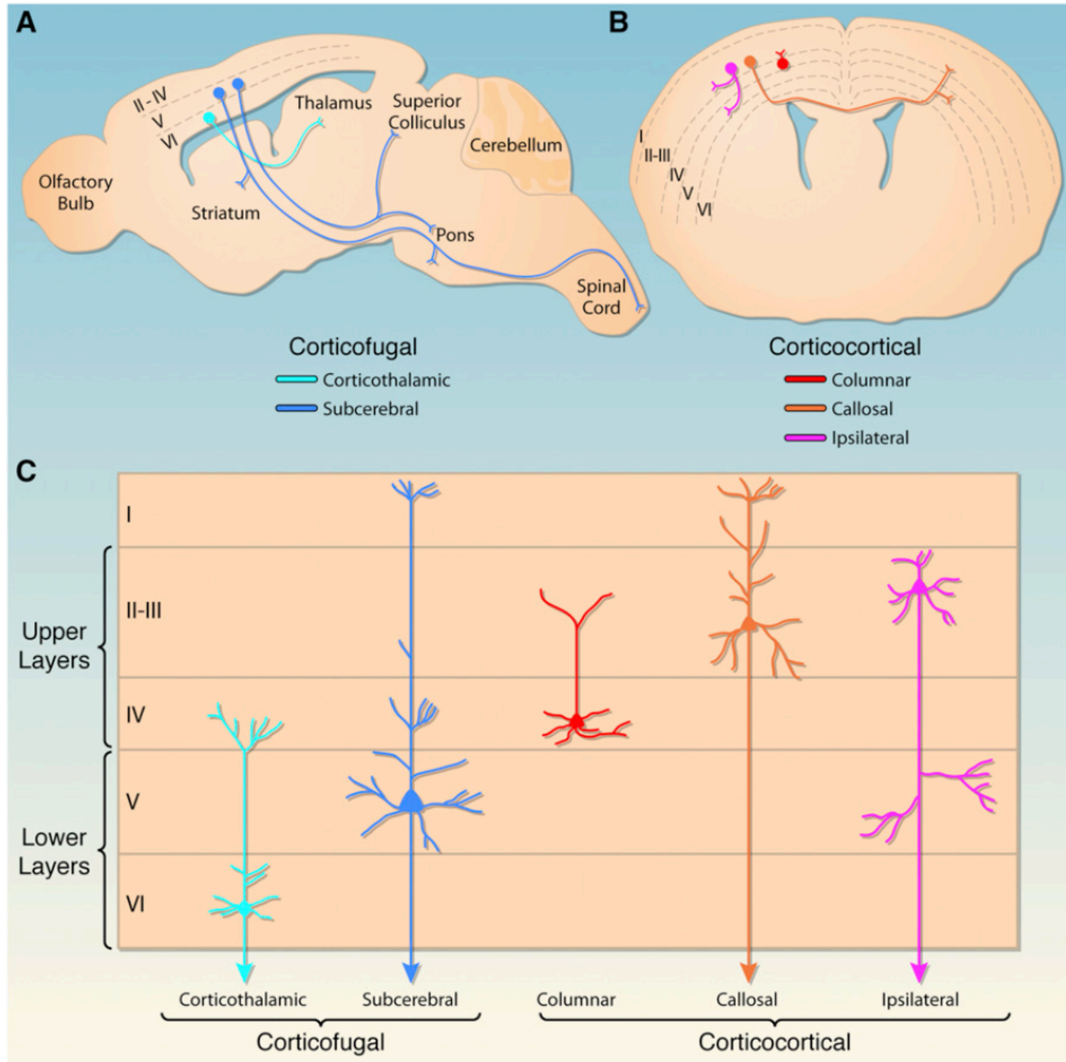


Figure 4. Major subtypes of neocortical projection neurons.
Illustration adapted from Franco and Müller (2013)[37].

Cortical neurons connect vertically to form small microcircuits, termed cortical columns, which span all six layers. Axons and apical dendrites of layer IV stellate cells (spiny glutamatergic neurons), or layer II/III and V/VI PCs all project vertically. Thalamocortical input is therefore transmitted to a narrow vertical column of PCs whose apical dendrites are contacted by stellate cell axons. This means that the same information is relayed up and down through the thickness of the cortex in columnar fashion. Neurons within a particular column tend to have very similar response properties. In the somatosensory cortex for example, all neurons from a particular column receive inputs from the same local area of the skin and

respond to a single class of receptors[38]. Finally, GABAergic neurons are spread out into all cortical layers, which I will discuss more in detail in the following section, and allow intracolumnar and intercolumnar activity synchronization.

Primary cortical areas process sensory information or deliver motor commands directly to the spinal cord. The primary sensory areas receive most of their information directly from the thalamus; and a few synaptic relays are interposed between the thalamus and the peripheral receptors[39]. It has long been thought that layer IV excitatory and inhibitory cells were the principal targets of sensory information from the thalamus, which then relayed the information to layer II/III that then relayed it to layer V/VI PCs. However, accumulating evidence suggests that direct thalamocortical input onto layer VI, V and onto layer I may have been underestimated. Electrophysiological evidence suggests that many layer V/VI neurons respond and process sensory information just as quickly as layer IV neurons, in fact, even in parallel, which suggests that they receive direct input from the thalamus. As such, it seems that there are two processing systems that can possibly serve different functions: an upper strata from layer IV to layers II/III, and a lower strata in layer V/VI[40]. In addition, a recent study demonstrates that thalamocortical neurons project equally to layer IV and layer VI, and the input onto layer VI neurons are just as strong at P3-P5. However, after the first postnatal week, synapses in layer IV, and not layer VI, display experience-dependent strengthening through long-term potentiation (LTP). This process allows specific layer IV synapses to stabilize over layer VI synapses, most likely relying on axon branching increase and connectivity divergence, and not necessarily on individual strengthening of connections[41]. Finally, layer I is the molecular layer where most apical dendrites of PCs terminate, and has been shown to be essential for the feedback interactions in the cerebral cortex that underlie cognitive processes such as associative learning and attention[42,43]. It was thought that the excitatory input onto these apical dendrites were mostly cortical. However, Rubio-Garrido and others (2009) demonstrated, using various pathway tracers, that a large number of thalamocortical neurons, originating from most thalamic nuclei, converge onto layer I apical dendrites directly[42]. However, it is still unknown whether this thalamocortical input is different from the thalamocortical input that innervates layer IV.

The relative size of each layer differs across all cortical regions. For example, the primary motor cortex has essentially no layer IV, though they have PCs that exhibit the prototypical properties of layer IV excitatory neurons in layer Va[44]. The motor cortex is primarily an output region and thus receives little sensory information directly from the thalamus. On the other hand, the primary visual cortex has a very prominent layer IV. Visual input onto the lateral geniculate nucleus creates a large and highly organized input onto layer IV; which can even be divided into three sublayers in humans and monkeys[45,46]. Furthermore, sensory information is processed in the neocortex through different pathways that extend towards secondary and tertiary sensory cortices, which produces progressively more complex information contributing to neural circuitry underlying complex behaviors.

1.1.2. Neuron and interneuron generation and diversity

GABAergic diversity

Here, we will follow the GABAergic INs classification suggested by two recent reviews[47,48]; keeping in mind that other classifications may evolve based on future functional/genetic studies. Briefly, three groups of GABAergic INs account for nearly if not all cortical GABAergic neurons, at least in the somatosensory cortex. The first group comprises of GABAergic INs expressing the calcium-binding protein parvalbumin (PV), which traditionally include chandelier cells (ChC, or axo-axonic cells) and fast-spiking basket cells (FS-BC). Of note, it has been recently shown that only part of chandelier cells tested PV+ by immunostaining, ranging from 15% in the medial prefrontal cortex to around 50% in the barrel cortex[49], showing current classifications are not perfect. The second group includes the somatostatin (SOM)-positive cells, which comprises Martinotti cells and X-94-cells[50] in the cortex and the O-LM and bistratified cells in the hippocampus. PV+ and SOM+ cells represent the two largest population of INs in rodent neocortex (about 70% all of cortical INs). The third group includes GABAergic cells that express the ionotropic serotonin receptor 5HT3a (5HT3aR), which primarily populates neocortical layers I–III. In primary somatosensory

cortex, ~40% of 5HT3a receptors-positive INs expresses vasointestinal peptide (VIP), while the large majority of the rest expresses reelin (See figure5).

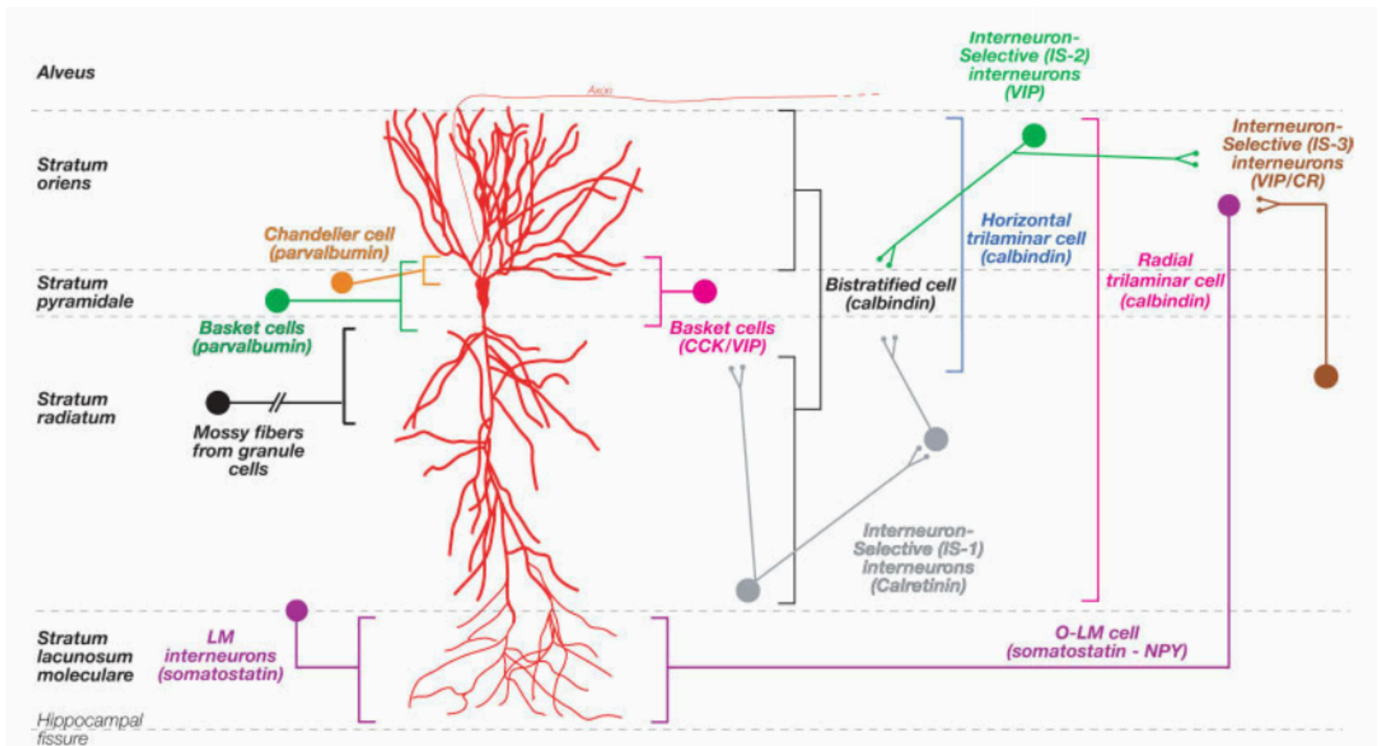


Figure 5. GABAergic interneuron diversity in the hippocampus. Illustration adapted from Danglot et al, (2006)[2].

PV-positive FS-BCs represent around 40% of all INs, and include different subtypes, differing in size of innervation field (large, small or nested BC), location of axonal projection (intra or inter-columnar), gene expression and electrophysiological properties. As unifying characteristics, all BCs can produce action potentials at high frequency, hence the name fast-spiking, and innervate the soma and proximal dendrites of both inhibitory and excitatory cells. This specific localization of synapses allows BCs to control action potential generation, timing, and synchrony in pyramidal cell populations, and make them very crucial for regulating their output. ChC or axo-axonic cells have a few similar electrophysiological properties as BCs such as high firing frequency with minimally adapting trains of action potentials, especially in the cortex[49,51-54]. However, their properties differ in their amplitude of afterhyperpolarization and their firing frequency[55]. ChCs also show a higher degree of spike frequency adaptation than BCs in the hippocampus[53]. Nevertheless, the main difference

between BCs and ChCs is the subcellular domain where they innervate PCs. ChCs specifically target the axon initial segment (AIS) of pyramidal cells, thus may strongly control the output of PCs[49,54]. In addition, it has been recently suggested that the synapses at the AIS made from ChC can actually be either excitatory or inhibitory[52,56], but this remains controversial. Furthermore, a single FS-BC can innervate more than 1500 PCs and contact 60 other PV^+ INs in the CA1, while it has been estimated that one ChC innervates from ~100 PCs in somatosensory cortex[57] up to ~1000 PCs in the hippocampus [2,4,6,7]. This large innervation field allows PV+ cells to exert a widespread influence on network activity[58]. In the hippocampus, FS-BCs and ChC are localized in the stratum pyramidale layer (SP) where PCs are concentrated, which permits their specific subcellular innervation pattern. In the cortex, the proportion of PV-expressing INs is biggest in layer IV, though they are also localized in all layers except layer I[48]. This spatial organization allows for an important synchronization of glutamatergic neurons[59]. In particular, FS-BCs play a critical role for the rhythmic synchronization on neuronal populations in the gamma band (30-80HZ)[28].

INs expressing the neuropeptide SOM usually show either a bursting firing pattern or an adapting regular-spiking, and target PC distal dendrites, thereby allowing them to control the efficacy and plasticity of synaptic inputs that terminate in the same dendritic domain[3,4,60,61]. In general, SOM+ cells can be very diverse and many recent studies have been focusing on identifying potential subtypes[9,50,62,63]. The most cited subclasses in the hippocampus are the O-LM (also weakly positive for PV) or bistratified cells in the SR[64-66]. Altough O-LM INs innervate the distal apical dendrite of PCs in the SLM, their somata are located in the stratum oriens (SO); while bistratified cells, which also express the calcium-binding protein calbindin, innervate all PC dendrites. In the cortex, the major subclasses are Martinotti cells and X-94-cells. Martinotti cells comprise 2 types that project their axon in layer I but differ in the position of the cell body (layer V vs layer II/III). X-94-like cell bodies are located in layer IV primarily and V, and specifically target other cells in layer IV[50,67]. Furthermore, around 30% of SOM+ cells also express the molecular marker calretinin (CR)[68], and are mainly located in layer II/III and have similar properties as Martinotti cells but differ in dendritic field morphology[68].

Finally, 5HT3aR+ INs are arguably the less characterized IN populations. Within this group, Reelin+ INs show late-spiking properties, and include neurogliaform cells; while VIP+ INs includes CR+ cells with irregular spiking, and CR- cells with fast-adapting spiking. In the hippocampus, these INs selectively inhibit other INs located in different layers[2]; while in the cortex, they are mainly concentrated in layer I and represent approximately 50% of INs in layer II/III. Additionally, another subtype that expresses 5HT3aR, also expresses the peptide cholecystokinin (CCK). Similarly to FS-BC, CCK-positive cells innervate soma and proximal dendrite of target cells (hence they are also called basket cells), however they have different firing patterns (spike trains at moderate frequencies), higher input resistance and slower membrane time constant. Finally, CCK+ cells express the type 1 cannabinoid receptors (Cb1R) presynaptically in the hippocampus[69-72]. These receptors mediate short-term depression of GABA release following depolarization of postsynaptic cells[73], referred as depolarization-induced suppression of inhibition, which is activity-dependent[69,70]. A recent study shows that CCK binds to CCK_B receptors on PCs, leading to synthesis and postsynaptic release of endocannabinoids. Activation of presynaptic CB1 receptors located on the axon terminals of CCK+ BCs results then in suppression of GABA release[74]. Likely, FS-BC and CCK-BC differentially impact information processing and rhythmic activity patterns[75]. CCK immunostaining within the cortex show sparse cellular labeling, raising the question of whether CCK-BC are much more abundant in hippocampus (in SP) than in cortex.

This short description of different INs types does not intend to be exhaustive but to convey the complexity underlying GABAergic circuits. Indeed, even within each group, there are many different types of cells, which emphasize the diversity of INs. Another important aspect is that the percentage of different INs may change depending from the brain region, and the species. For example, the total proportion of GABAergic neurons in the cortex might be much higher in primates (24–30%) compared with rodents (15%)[76-78]. Further, several differences have been reported in the phenotypic properties of cortical INs between rodent and primate brains[77,79]. Another example comes from the ChC, which are more numerous in the prefrontal cortex than in sensory cortices[49,80].

Even when INs share common characteristics, such as subcellular targets or molecular markers, their individual function can change depending on the circuit they are

incorporated in, which adds another level of complexity. The advantage of INs diversity is that they can control neuronal networks in a very specific and finely tuned manner, releasing GABA at different time points and at different subcellular compartments. Further, recent studies show that different INs types act in concert to modulate and synchronize relevant signals depending on the behavioral state[20,81,82]. It is therefore plausible that any disruption of GABAergic interneuron development strongly affects neuronal networks dynamics, signal processing and ultimately brain cognition. In fact, GABAergic dysfunctions, or the more largely used "alterations of excitatory/inhibitory balance" have been implicated in numerous neurodevelopmental disorders like epilepsy, autism and schizophrenia[83-88].

GABAergic interneuron generation

Neocortical GABAergic INs are generated embryonically in the ventral telencephalon (subpallium), in the medial, lateral and caudal ganglionic eminence (MGE, LGE and CGE, respectively) and in small part in the preoptic area (POA), from where they then migrate to their final destination[2,89,90]. The most generally accepted hypothesis is that the location and the timing of IN birth determine its identity. The generation of GABAergic INs occurs prior to principle cells generation, between embryonic day 13 (E13) and E18 in rat, and between E9 and E17 in mouse[91,92]. In the hippocampus, CA1 and CA3 INs are generated before INs of the dentate gyrus [2,93,94,95] (E12-13 vs. E13-14). By using inducible genetic fate mapping, Miyoshi and colleagues (2007) showed that, similarly to PCs, INs that are generated earlier (E9.5/E10.5) migrate to deeper layers in the cortex, whereas INs that are generated later (around E15.5) migrate to more superficial layers[8,9,96]. PV+ and SOM+ INs derive from the MGE, but while FS-BCs are continuously generated throughout E9.5 to E16.5, SOM+ INs can be generated as early as E9.5 but are absent when progenitors are labeled at E15.5. CR+ and VIP+ INs are generated later (around E15.5) in the CGE and migrate preferentially to superficial layers of the cortex, where they represent the most numerous population of INs[8,9,96]. A recent study performing genetic fate mapping of Nkx2.1-expressing progenitors using Nkx2.1-CreER mice has demonstrated that ChCs generated from the ventral germinal

zone of the lateral ventricle, an Nkx2.1-expressing remnant of the MGE, starting from late gestation until close to birth[7,97].

Time and location specific determination of INs fate suggests that each IN subtype may be defined by a unique temporal combination of transcription factor expression. For example, SOM+ and PV+ cells that will migrate to the cortex and hippocampus preferentially derive from the dorsal and ventral zones of the MGE, respectively, and requires the expression of the transcription factor Nkx2.1. Nkx2.1 knockdown at E10.5 results in a switch of IN subtypes observed at more mature ages, with more VIP/CR+ neurons, which normally originate from the CGE, in place of PV+ and SOM+ INs[98]. The downstream cascade of Nkx2.1 includes the sequential activation of Lhx6 and Sox6. Lhx6 is essential for specification and migration of PV+ and SOM+ INs[99]. $Lhx6^{-/-}$ MGE cells may also acquire a CGE-like fate[100]. On the other hand, Sox6 genetic manipulation showed that this transcription factor is necessary for proper laminar migration and maturation but not specification of the MGE-derived INs[10]. The complete chain of transcriptional events that differentiate PV+ from SOM+ INs specification is still not fully understood. One of the suggested mechanisms is the preferential postnatal expression of distinct members of the Dlx gene family. Indeed, loss of Dlx5 or Dlx5/6 preferentially reduces the number of mature PV+ INs[101], while loss of Dlx1 affects the maturation and survival of SOM+ and CR+ INs[102]. Interestingly, early GABAergic neurogenesis, however, does not necessarily imply that they reach their final destination and are functional before glutamatergic PCs.

Glutamatergic neuron diversity

Pyramidal cells represent 80% of neurons in the cortex, and are less diverse than GABAergic INs. In the neocortex, excitatory neurons located in layer II/III and V/VI are projection PCs, while spiny stellate cells in layer IV are excitatory INs. They all use glutamate as their primary neurotransmitter. Layer II/III PCs have smaller cell bodies than layer V/VI PCs. Stellate cells have dendrites extending in all direction, and form synapses with neurons near the cell body. These excitatory INs are the primary recipients of sensory information from the thalamus. PCs from layer II/III and layer V also have different electrophysiological

features. They differently express persistent sodium channels, which are important for maintaining recurring firing, and generating depolarizing afterpotentials and action potential bursts, therby generating different firing properties and different functional implications as they innervate different cortical and subcortical regions[103]. As such, a recent study published in Cerebral Cortex (2015) reveals that there are 10 different subtypes of PCs between layer II and VI in the prefrontal cortex of rats, depending on morphological and electrophysiological parameters. Briefly, layer II PCs have a unique morphology with a narrow basal field, but a wide apical dendritic field. Layer III PCs specifically displayed bursts of action potential upon current injection; whereas, layer V PCs have the largest voltage sags. Finally, layer VI PCs have the most diverse morphological features, where approximately 40% of them have long apical dendrites that extend all the way to layer I[104].

In the hippocampus, there are at least three types of excitatory cells: PCs from CA1, PCs from CA3 and granule cells (GCs) in the dentate gyrus. DG is the primary gate that filters and processes sensory input entering the hippocampus. Consequently, GCs have low intrinsic excitability and fire sparsely[105]; which protects hippocampal circuits from runaway excitation[106]. As for CA regions, different layers of entorhinal cortex actually transmit distinct types of information to the CA3 vs. CA1 regions, suggesting specific regional differences[107]. CA3 place cells, which are hippocampal cells that discharge in a particular location in the environment, are important for the quick representation of spatiotemporal sequences, while CA1 place cells are important for comparing these new sequences to stored sequence information, suggesting that there is a heterogeneity between the CA3 and CA1[108]. Additionally, place cells are less frequently formed by CA3 than by CA1 cells, yet they were more stable and transmit more spatial information per spike than CA1 PCs[109]. On the other hand, there are differences in individual CA1 or CA3 pyramidal cells[30]. As such, it has been shown that CA1 and CA3 pyramidal cells have different firing rates, spike bursts, as well as other features of spike dynamics the are brain state-dependent. Another level of heterogeneity involves laminar differences, comparing deep and superficial layers of the CA1 region. Deep PCs have higher firing rate, more frequent bursts, and are differently influenced by oscillations during sleep-dependent brain states[110], and project to different brain regions (amygdala vs

prefrontal cortex)[111]. Overall, these data suggest that hippocampal PCs differ in their activity dynamics, which likely contributes to distinctive computational roles.

Glutamatergic neuron generation

Pyramidal neurons and GCs derive from different neuroepithelium matrices from the hippocampal plate, as seen in figure 1. GCs are mostly generated postnatally (85%), starting by E20, until reaching a peak within the first postnatal week. Prenatally generated GCs rise from the dentate NE and migrate towards the future DG. However, postnatally generated GCs derive from the subgranular zone of the dentate gyrus itself, and more specifically, from the precursors cells born in the dentate NE that retained their proliferative capacity[2,93]. Of these postnatally born cells, around 10% are generated after P18[24,93].

The different populations of PCs on the other hand, originate from the periventricular NE, and rise from a variety of neural stem and progenitor cells[22,38,112]. Neocortical precursors are grouped into four major classes. The major neural precursors of the neocortex are radial glial cells (RGCs), which undergo self-renewing asymmetrical cell divisions in the VZ and SVZ to generate neurons and astrocytes[21,22,113] (see figure 3). It is well established that RGCs are progressively fine-tuned to produce molecularly and morphologically distinct excitatory neurons between the six neocortical layers and hippocampus[114]. RGCs also divide to generate three types of intermediate progenitors (IPCs), to help generate PCs diversity, who divide themselves symmetrically in the VZ and SVZ. The different types of IPC classes are apical (aIPSC) in the VZ, basal (bIPC) that form the SVZ and basal radial glia (bRG)[113,115].

Recently, the classical model of corticoneurogenesis has been challenged (see figure 6). The classical model suggests that there is a homogeneous population of multipotent progenitor cells that produce different classes of PCs in sequential order, from deeper layers, to superficial layers from E11.5 to E17.5 in the mouse cortex and between E14-15 for CA3 PCs and E15-16 for CA1 PCs[2,91,92]. This model suggests that while progenitors can generate all PCs in early development, they become progressively restricted to generating only superficial neurons at later embryonic stages. Franco and others (2012)[31], proposes another model, where there are two classes of progenitor cells with a defined fate of giving rise either to PCs deep in

layers V and VI, or to superficial PCs in layers II to IV. In fact, SVZ-expressed transcripts (*Svet1*) and cut-like 2 (*Cux2*) are expressed in a population of dividing neurons in the SVZ (IPCs) during the generation of superficial-layer PCs, and specifically in postnatal PCs from layer II-IV. These results suggest that *Svet1* and *Cux2* are markers for upper-layer progenitor cells from the SVZ, and that laminar fate may be established even before differentiation[31]. Therefore, progenitors that are destined to form PCs in superficial layers may proliferate during early development, before they start to produce neurons, and then the SVZ may contribute to neurogenesis of superficial layers. A unifying model suggested by Marín (2012) hypothesizes that several progenitors lineages exist, which are restricted to producing either deep or superficial neurons. However, within each lineage there are intrinsic and extrinsic factors that control the production of different classes of PCs[116]. In accordance to this hypothesis, Tyler and colleagues (2015) demonstrates that even within the same superficial layer II/III, there are several neural precursor populations that give rise to electrophysiologically and structurally distinct PCs[21], suggesting that electrophysiological and morphological properties of PCs are intrinsically specified at the precursor stage and that divergent routes of neurogenesis generate PC diversity[21]. Time-lapse microscopy revealed that cortical progenitors that are cultured *in vitro*, can continue to divide and produce neurons that express laminar markers, suggesting that generation of PC diversity seems, at least in part, to be due to intrinsic factors in the progenitor cells[117].

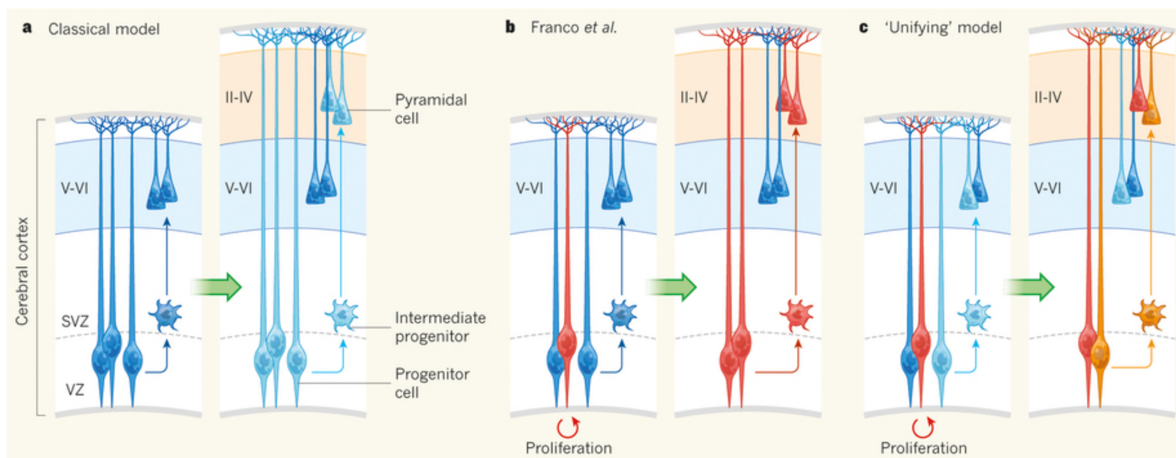


Figure 6. Representation of the different models of corticoneurogenesis proposed. Illustration adapted from Marin (2012)[118].

There are a number of genes that control cortical neurogenesis such as LIM homeobox 2 (*Lhx2*), forkhead box G1 (*Foxg1*), empty spiracles homologue 2 (*Emx2*) and paired box 6 (*Pax6*). They each have an essential role in cortical projection neuron generation, by repressing dorsal midline (Lhx2 and Foxg1) and ventral (Emx2 and Pax6) fates and specification of neocortical progenitors[22,119]. It has also been shown that Pax6 and Nr2e1 control the VZ proliferation of progenitors, by controlling the kinetics of cell division and the choice of a progenitor to divide symmetrically or asymmetrically. Pax6 mutants have more progenitors undergoing asymmetrical division, as well as a decrease of number of Cux2-expressing cells in the SVZ, suggesting they control the expansion of the SVZ and the generation of PCs of superficial layers[22,120]. In situ hybridization and transgenic mice studies reveal that there are a number of genes that determine layer- and subtype-specificity in the mouse neocortex (see[22] for full list;[121]). For example, the orthodenticle homeobox 1 (*Otx1*) is expressed in 40 to 50% of subcerebral projection and layer VI neurons[122]; and Fez family zinc finger 2 (*Fezf2*) is expressed in all subcerebral projection neurons in the cortex and PCs in the hippocampus[123]. Both *Otx1* and *Fezf2* are expressed in VZ prior to the generation of layer V and VI neurons[124]. The list of genes involved in this process is quite extensive, but altogether, it seems that there are sequential steps of progressive differentiation, which are guided by transcription factor expression in subsets of progenitor cells, which thus differently generate lower-layers or upper-layers projection neurons.

1.1.3. Migration

After neurogenesis, postmitotic GABAergic and glutamatergic neurons position themselves in the developing neocortex through defined modes of migration, starting from soma translocation in early corticogenesis, to glia-guided and tangential migration later on[22]. I will first explain the modes of migration employed by GABAergic INs, and then those taken by PCs.

GABAergic interneuron

Using different labeling techniques such as DiI or electroporation of fluorophores *in vivo* or *in vitro* and time-lapse imaging, it has been widely shown that INs migrate tangentially from the MGE to the cortex[125,126] and the hippocampus[94,127,128]. INs born before E14.5 from the LGE reach the olfactory bulb, the nucleus accumbens and the ones born after E14.5 reach the neocortex[129,130]. Finally, INs born in the CGE reach the cerebral cortex, the striatum, the amygdala, the nucleus accumbens and the hippocampus (both CA regions and dentate gyrus)[131]. INs derived from all these regions enter the neocortex through two different pathways, one that is superficial and reaches the marginal zone (MZ) and cortical plate (CP), and the other most prominent pathway goes through the SVZ and IZ[94]. There is also some crosstalk between these two paths, as INs that originate from the MGE reach the SVZ and then migrate radially or obliquely towards the CP and MZ[132]. Interestingly, most INs reach the hippocampus through a superficial pathway, below the MZ, where they can then change their mode of migration to radial to reach the different layers of the hippocampus[94].

A large bulk of evidence suggest that the migratory fate of INs is specified by transcription factors, which modulate the expression of signaling receptors and adhesion molecules, which in turn allow migrating cells to respond selectively to route-specific guidance molecules and growth factors. Further, it has been shown that $GABA_B$, $GABA_A$ and AMPA receptor-mediate signaling stimulate INs migration as well, likely through membrane depolarization and increase of intracellular calcium levels[94,128,133,134]. In summary, GABAergic INs specification, migration and final positioning is determined by a strictly regulated sequence of combinatorial gene expression. Deficits in INs specification and migration, induced by either specific mutations or deletions of genes implicated in these developmental processes, are often associated with seizure in animal models. For example, mouse mutants for Dlx1/2[135], Dlx5/6[101], Sox 6[10], Arx[136] (which are implicated in INs migration, final positioning and phenotypical maturation) to cite a few, show both deficits in specific INs population and seizure of varying severity. Interestingly, migration disorder may be associated to deficits in structures that arise later in development, such as synapses. For example, Sox6 mutants mice show both a reduction of PV+ INs in all cortical layer and an immature synaptic phenotype of the PV INs that successfully reached the cortex[10]. A recent study found an association of

mutations in ARX and *CXCR4,* both essential for proper migration and laminar positioning of cortical GABAergic INs, with infantile spasms, a form of early-onset epileptic encephalopathy[137]. The astonishing speed of development of sequencing techniques has made whole exon sequencing cost and time effective in clinical settings. Therefore, I believe it is likely that in the next years research will identify mutations that, by altering the temporal/spatial regulation and expression levels of INs development regulators, may constitute a risk factor for the development of brain diseases.

Glutamatergic neurons

One day after PCs are generated, they emerge from the NE in VZ up to the IZ forming an agglomeration of cells[2,24]. The following day, they leave the IZ and migrate radially toward the cortical or hippocampal plate, depending on their final destination. Initially, somal translocation from the VZ towards the plia surface is predominant. At this stage, cells typically have a long (radially-oriented) leading process and a short transient trailing process, and they quickly and continuously migrate[138]. At later stages, it has been suggested that there are four different stages of migration of cortically derived neurons. First, bipolar neurons rapidly move to the SVZ; second, they remain in the IZ-SVZ for 24h, but develop a multipolar morphology, where they have the option of migrating tangentially, while extending and retracting its multiple processes, termed 'multipolar migration'. Third, some neurons, but not all, can then return toward the ventricule through a reversal of polarity of its processes; while the neurons that remained in the IZ transform into a bipolar morphology, then slowly migrate to the CP through radial migration, guided by the RGCs[2], and this migration mode is termed 'locomotion'. Lastly, when neurons reach the MZ, they anchor the tip of the leading process and detach from the radial glial fiber, this mode is refered to as 'terminal translocation'. From generation to final destinations in the CP, this process can take about 4-5 days.

Migration to the hippocampal plate (future CA regions) can take up to 4 days for CA1 neurons, and longer for the CA3 neurons as they migrate further and through the CA1. Migration in the hippocampus and neocortex are similar in the beginning, however, when neocortical neurons begin the locomotion migration mode, CA1 PCs adopt the 'climbing

mode'. Essentially, bipolar-like neurons (future CA1 PCs) migrate through the IZ to the hippocampal plate by using several scaffold RGCs, and dynamically extend and retract their branched leading processes in search of another RGC, and thus migrate in a zig-zag manner and causing migration speed to be lower than in the cortex. Despite CA1 and CA3 pyramidal layers being identifiable as early as E20-22, migration continues at birth[139]. In fact, postnatal local migration has been observed in the hippocampus. It has been postulated that GABAergic interneurons somata are redistributed from the dendritic to the cell body layers, and to the SR-SLM border in the hippocampus from P5 to P15[2].

Several extracellular matrix components, like fibronectin, chondroitin sulfate proteoglycans, reelin, neurocan and others are distributed along radial glial fibers, and are closely associated to migrating neurons and are believed to serve as a scaffold for CP formation, and guidance signal. Moreover, specific surface molecules are required for the recognition and adhesion during neuronal cell migration, but also to stop migration[32]. These surface molecules are specifically and transiently expressed in the leading process of migrating neurons, as well as the surface of neighboring radial glial fibers. For example, NJPA1 is a glial membrane protein localized at the junction of migrating neurons and radial glial fibers; blocking it by antibody causes the leading process to retract, microtubules to reorganize and the precocious detachment of migrating neuron from the radial glial shafts[140]. However, NJPA1 is only an example among hundreds of molecules that guide migrating neurons towards its final destination[32]. Indeed, most migration-regulating factors are either membrane bound, or secreted diffusible molecules that attract or repel neurons to a particular structure[32]. For example, brain-derived neurotrophic factors (BDNF), that influence the laminar-fate of PCs[141]. The phosphoprotein synapsin III was also shown to be important for radial migration and orientation of PCs *in vivo*; and the mechanism involves the upstream activation of Semaphorin-3A and phosphorylation of cyclin-dependent kinase-5 (CDK5) site of synapsin III[142]. Another signaling pathway involved in proper cell migration is the Wnt signal transduction. Mutations of this pathway have been found in models of neural tube defects[143], which underlie their importance during neocortical and hippocampal development. In addition, a recent study evaluated that both Wnt canonical and non-canonical signaling are activated in pyramidal precursors during radial migration, and regulate the transition of

migrating neurons from multipolar to bipolar morphology. They found that the canonical pathway is triggered by Wnt5A expression, while the activation of the non-canonical pathway through ephrin-B1 controls the polarization and orientation of migrating neuron [144]. Another example of molecular mechanism governing radial migration is through the activation of non-hyperpolarizing $GABA_B$ receptors. Knocking down this receptor impaired neuronal migration and the polarized neurite morphology through alterations of the cAMP signaling and downstream phosphorylation of the kinase LKB1[145].

Furthermore, Cajal-Retzius neurons (CRN) are transient subpopulations of neurons that are present during gestation and are essential for migration. CRN are born by E10-12, and disappear by the second and third postnatal week respectively. They secrete reelin to help guide radial migration of projection neurons, and laminar specificity. CRNs also express the immunoglobulin-like adhesion molecule nectin1, and neocortical projection neurons express its binding partner nectin3, and their interaction is critical for radial migration[146,147].

Proper migration is the key to a structurally organized cortical circuit, and disrupting this process can lead to detrimental interference of proper cortical wiring. In fact, dysfuntion in migration have been known to cause cortical malformations, like cortical dysplasia, and are a risk factor for epilepsy.

1.2. Postnatal development

GABAergic and glutamatergic neurons are generated embryonically, nevertheless, their morphological maturation largely extends during the postnatal period. Many different processes characterize early postnatal development that lead to mature cortical circuits; such as local migration, axon guidance, synaptogenesis, apoptosis, and network formation and refinement. In fact, when do neurons stop migrating? When do they start to make synapses and form a mature stable and refined network? We will try to understand these processes in this next section.

During migration, ambient GABA and glutamate are part of the cues that stimulate interneuron motility. However, after reaching the neocortex and hippocampus, migrating neurons start to express the cotransporter KCC2. It has been established that KCC2 lowers the intracellular concentration of chloride, thereby controlling the reversal potential of Cl^- and rendering GABAergic transmission hyperpolarization, through $GABA_A$ receptor activation. In fact, Bortone and Polleux (2009) have shown that the upregulation of KCC2 is necessary and sufficient to make migrating interneurons respond to GABA as a stop signal by negatively regulating the frequency of spontaneous intracellular calcium transients. Therefore, migrating cortical interneurons are able to sense the ambient levels of GABA and glutamate as a stop signal[133]. Once these neurons have stoped migrating, and reached the final destinations, their axons begin to explore their new environement to find potential suiters and start to make new synapses to allow network communication. I will briefly present an overview of these processes.

1.2.2. Axon guidance and synaptogenesis

The establishment of precise neuronal connectivity requires neurons to extend axons and dendrites, which are guided to their target region and extended into specific layers. Axonal guidance and target recognition guides laminar and subcellular specificity, to finally produce the pre- and post-synaptic specialization components to form synapses. Within that region and those layers, the growth cone is the "exploratory" tip of extending axons that guides them to finer target fields to form synapses[148,149].

Growth cones are a highly motile structures at the tip of growing neurites which allow axons to advance, retract, turn and branch, and these behaviors are regulated by the reorganization and dynamics of the actin and the microtubule cytoskeleton. They are guided by the aid of molecular cues in the environment[150,151], and can even respond to multiple attractive and/or repulsive molecular cues at the same time, with distinct signaling pathways[148]. They also respond to cell-surface adhesion molecules to regulate spatial specificity[152], involving multiple structural protein families, such as the immunologlobin (Ig) superfamily, semaphorins, netrins, ephrins, neuropilins, plexins, Eph kinases and numerous

extracellular matrix glycoproteins. Some of these proteins can be associated to the plasma membrane, and thus act as receptors for secreted ligands, or extracellular matrix (ECM) components, or they may interact with proteins expressed on neighboring cells[148].

For example, neurofascin is an L1 family immunoglobulin cell adhesion molecule (L1CAM), which has been shown to promote IN axon pathfinding. The neuronal NF186 isoform is associated to the ankyrinG-based cytoskeleton at the axon initial segment (AIS) of purkinje cells in the cerebellum. In fact, it is expressed in a subcellular gradient along the soma-AIS axis, which guides FS-BC axon terminals to the AIS of purkinje cells. AnkyrinG-associated NF186 is also necessary for 'pinceau' synapse formation (FS-BC synapses ressembing a paint brush), and/or stabilization[153]. On the other hand, NF166, which interacts with NrCAM and axonin-1, is implicated in the induction of neurite outgrowth via FGFR1 signaling. It is also expressed at the AIS and soma of dissociated hippocampal neurons and has been suggested to control gephyrin clusters formation at the axon hillock[154,155]. Another example involving the semaphorins signaling family, where knockouts of its signaling components lead to extended infrapyramidal mossy fiber axonal pathways and spontaneous seizures[156-158]. Furthermore, mechanical force has also been shown to regulate axon guidance and growth, as well as synapse formation and plasticity by regulating receptor-ligand interactions, protein conformational changes and cytoskeleton dynamics[159]. Altogether, axons are guided to their target by multiple mechanisms, involving the extracellular matrix, secreted and membrane-bound proteins and even mechanical force, displaying its complexity.

Once an exploring axon recognizes a potential target, the mechanisms of synaptogenesis occur. In fact, this process can start very early on during development. As such, neurons that are present during gestation, such as CRN and subplate neurons (SPN) can already make synapses. SPNs constitutes a heterogeneous glutamatergic and GABAergic neuronal cell population that is curcial for axonal projection development and neocortical column formation[147]. Both CRN and SPN have glutamatergic and GABAergic synapses[147,160]. However, recording of evoked and spontaneous post-synaptic currents (sPSC) revealed that GABAergic synapses represent the major input to CRN from E14[147,161], although they mediate excitatory responses in CRN and SPN[147,162]. Martinotti cells have been shown to form direct GABAergic synapses to CRN[147], though they are not necessarily the only IN subtype

contacting CRN. Futhermore, there is evidence of direct synaptic contact between CRN and SPN[163], thus proving that synaptogenesis occurs very early on.

Nevertheless, mature and stable synapses are formed during postnatal development. PCs synapses are formed prior to GABAergic synapses, which are present from P5, however these newly formed synapses are immature, and are characterized by a small size and few synaptic vesicles[2]. GABAergic terminals and axon growth strongly increase in number between P7 and P21, where they finally reach their mature perisomatic innervation patterns[6]. FS-BC synapses, in particular, are detected only after P4 in layer V/VI of the visual cortex[147,164]; while in the barrel cortex, layer IV FS-BC only receive reliable excitatory thalamic input by P7[147,165]. Maturation of inhibitory innervation in the cortex is regulated by sensory experience, and thus activity-dependent.

There are two modes of synapse formation (Figure 7), where a spine can either grow towards an 'empty' axon to form a new bouton, or can form a synapse towards an already existing boutons and create multiple synapse boutons. In this case, as they mature, the previously exisiting synapses can be eliminated. As single synapses start to form, the pre and postsynaptic components start to accumulate at the future site of the synapses. Initial immature GABAergic synapses function by multivesicular release, and synaptic transmission starts with GABA, which in early development can serve as a depolarizing transmitter. Immature synapses have a higher probability than mature synapses to release vesicles. Finally, maturation leads to an increase of synchronous over asynchronous release, characterized by the increase in release sites and the expression of the necessary vesicular release machinery components, thus increasing the probability of release at a particular synaptic contact in response to presynaptic action potential.

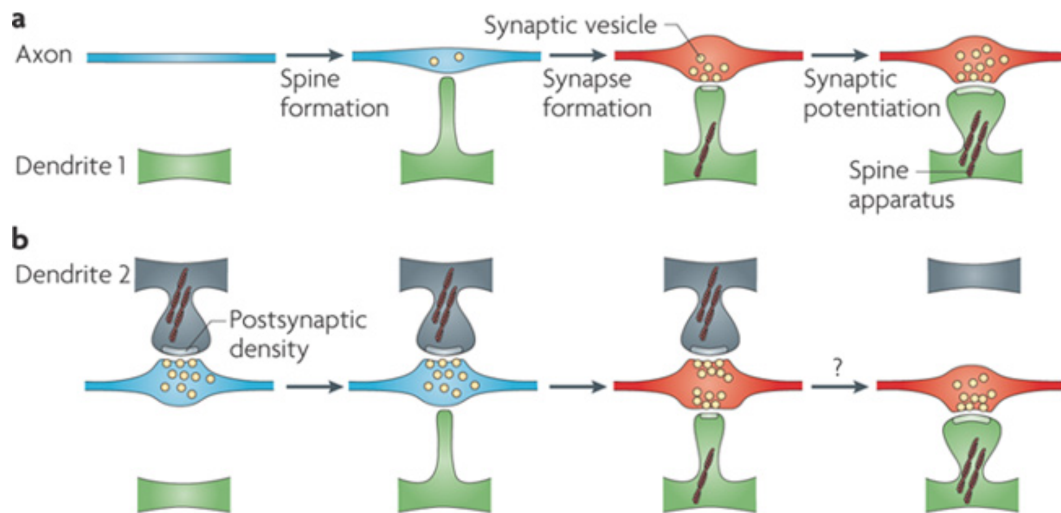


Figure 7. Two possible mechanisms of synapse formation.
Illustration adapted from Holtmaat and Svoboda (2009)[166].

1.2.2. Network formation & Refinement

During synaptogenesis, a lot of synapses are formed and axonal and dendritic arbors are even more widely distributed than in the adult. Then as a process of refinement, maturation eliminates inappropriate or weak synapses and axon collaterals. Another process of refinement is programmed cell death that occurs during early development, thereby reducing the number of neurons, also in the effort to produce strong and stable neuronal networks and connectivity. In this section, I will describe the process of apoptosis and critical period plasticity during which major changes occur.

Apoptosis

Refinement at the circuit level consists of a portion of neurons that undergo programmed cell death after interneuron migration. A recent paper published by Southwell et al., in Nature letters (2012) has proven that interneuron cell death is intrinsically determined. As evaluated

by the expression of an apoptosis marker, caspase 3, they assessed that most cell death occur from P7 to P11. Around 40% of interneurons were eliminated by Bax-dependent (the Bcl-2-associated X) programmed cell death. Three important experiments analyze whether the process of apoptosis is dependent on neurotropic competition, or is intrinsically predetermined. Firstly, the process of programmed cell death in interneurons is maintained when cells are cultured *in vitro*. Moreover, this process is also maintained when embryonic interneuron precursor were transplanted *in vivo*, and were 6 to 10 days younger then endogenous interneurons. Indeed, programmed cell death occurred in a timely-manner for the endogenous neurons (at P7), whereas the transplanted cells only underwent apoptosis when they had reached their equivalent cellular age. Finally, the rate of apoptosis was independent of the amount of interneurons that were transplanted, or the lack of the neurotrophic TrkB receptor. These pivotal experiments strongly suggest that programmed cell death is intrinsic to the developing cell, and is the first *in vivo* and *in vitro* evidence that cell survival is not, at least solely, dependent on BDNF and TrkB as it was previously believed[167-169]. However, these experiments do not rule out that there may be other neurotrophic factors involved in cell survival. BDNF also binds to p75NTR to a lesser affinity, a receptor involved in apoptosis, thus should be studied. In fact, one hypothesis is that cell-cell contact and synaptic transmission could regulate cell survival. In fact, it has been shown that synapse maturation is activity-dependent. Could cell survival be activity-dependent? The first synapses appear by P2 and progressively develop, while the peak of apoptosis occurs at P7. Could it be that the cells that cannot form synapses because there was not enough activity be the ones that die? In fact, Southwell et al. also show that transplanted interneurons get integrated to the circuit, and the frequency, but not the amplitude, of sIPSC recorded from endogenous pyramidal cells increased, regardless of the amount of cells that were transplanted. One hypothesis that arises is that inhibition is independent of population size, but is controlled by homeostatic regulation of synaptic strength and number[169]. These findings could explain why apoptosis takes place, because the control of inhibition doesn't require as many interneurons. Early electrical activity can influence cell death. Studies in culture have shown that silencing spontaneous activity increases apoptosis[170,171]. Interestingly however, Southewell and colleagues reported that the percentage of cell death was independent of the number of transplated cells. Altogether,

though it seems clear that apoptosis is a normal occurrence in early development, the mechanism that regulate their programmed cell death is not yet fully understood.

Critical period of plasticity

Network formation and refinement can take place at the circuit level, cellular level and molecular level. Indeed, once migration is completed, and synapses are forming and contributing to GABAergic maturation, the circuit is increasingly more complexe. Part of the normal refining of neuronal circuit requires activity and experience, and there is a specific timing where they are most important, to establish their mature wiring patterns. This is called the critical period, which has been defined as 'a strict window during which experience provides information that is essential for normal development and permanently alters performance.' by Hensch in 2005[172]. This process applies to the different regions of the cortex, but the clearest example is in the visual cortex, after eye opening. The closure of one eye, referred as monocular deprivation, during this critical period can cause a permanent loss of visual acuity from the closed eye, and a reorientation of binocular neurons to respond to visual input from the open eye[172,173]. It has been already established that GABAergic inhibition maturation takes part in the onset and time course of the critical period. In GAD65 knockout mice, where GAD65 is one of two enzymes necessary for GABA synthesis, normal ocular dominance plasticity is absent, and preserve the potential for plasticity throughout their life; however, delivery of diazepam to increase inhibition reverses the effects[174,175]. A few mechanisms involved in GABAergic maturation have been elucidated. For example, when BDNF expression is accelerated, GABAergic maturation and inhibition is also accelerated in the visual cortex, which prematurely closes the critical period of plasticity, and precociously increases visual acuity[176] (see figure 8). BDNF-overexpressing transgenic mice also lead to a premature shift in the critical period opening, causing it to open and close sooner than it should[177]. Along with BDNF, it has been shown that activity-dependent downregulation of PSA, attached to NCAM cell adhesion molecule, regulates the timing of GABAergic maturation. As such, removing PSA prematurely promotes a precipitated maturation of functional FS-BC synapses, leading to enhanced inhibitory synaptic transmission, as well as a

premature onset of the critical period for ocular dominance plasticity[173]. In addition, the retina-derived homeoprotein Otx2 was found to transfer to the visual cortex in an experience-dependent manner, and promote critical period plasticity[178]. The developmental shift of NMDA receptor, from NR2B to NR2A, has also been involved in GABAergic maturation during critical period in the barrel cortex[179]. Moreover, reducing intracortical inhibition in adult wild type rats can partially reactivate ocular dominance (OD) plasticity in the visual cortex, and therefore was sufficient to reopen plasiticty after critical period is normally closed[180]. Conversely, interfering with the normally 'excitatory' GABAergic transmission during early development (from P3 to P8) prolonged the critical period plasticity in the visual cortex, without altering the structural or functional development of the region[181]. This suggests that inhibitory maturation, as well as early excitatory GABAergic transmission is crucial for the normal development of ocular dominance plasticity, and the adult level of inhibition actively restricts cortical plasticity; but it also suggest that the balance between excitation and inhibition is an important component of this plasticity.

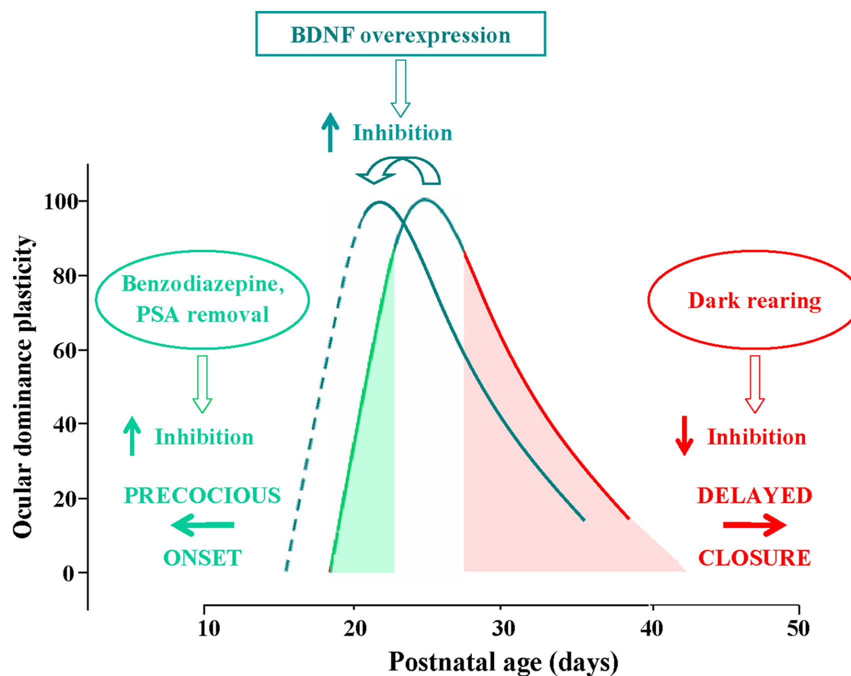


Figure 8. Representation of the time window where critical period for ocular dominance plasticity can occur in response to monocular deprivation.

Illustration adapter from Sale et al (2010)[182].

In the absence of sensory experience, how do neuronal circuit develop? Adult cats that were dark reared from birth had immature visual cortical functions; as well as monocularly deprived cats during the critical period, which dramatically shifted neuronal spiking response in favor of the open eye[183]. These functional changes are accompanied by synapses pruning and axonal rearrangements[184]. However, a recent paper from Kang E et al. (2013) showed that sensory deprivation, by dark rearing mice from birth, doesn't stop visual cortical development as was suggested previously, instead it delays it[185]. In fact, analyzing spatial frequency threshold of the behavioral optomotor task (OPT; reports mainly sub-cortical function) and visual acuity by visual evoked potentials (VEP; reflects mostly thalamocortical function) show an immature spatial vision until P34-35, however there is a slow recovery until P55-65. On the other hand, dark-reared mice re-exposed to light exhibit adult OPT threshold faster then control mice, and experience a rapid recovery of visual acuity, and plasticity of cortical circuitry[185,186]. Therefore, sensory deprivation delays the development of spatial vision but spontaneous activity is sufficient for potential visual circuitry and function. In addition, it also shows that depending when the pathology is present, and the length of it can determine how fatal the damages are. What happens if the timing of cortical plasticity is affected? In fact, these studies suggest that the timing of critical period is plastic itself depending on the timing of sensory experience. There are factors involved in GABAergic maturation that are expressed later in life to limit excessive circuit rewiring. For example, perineuronal nets (PNNs) that enwrap around FS-BCs in an activity-dependent fashion, and restrict plasticity in the adult by reaching its full maturation around the end of the critical period in the visual cortex. However, after dark rearing from birth PNN expression was overall reduced, as well as the density of FS-BCs, preferentially in layers II/III and V/VI[187]. Consequently, any alteration of one of these factors can affect the length of critical period. If critical period is open too long, the circuit may remain immature. The potential of making new connections could enhance learning, but it could also affect long-term memory if these connections do not remain. As such, the presence of the extracellular matrix (ECM) ends developmental plasticity by allowing structural stability. ECM removal in the auditory cortex of adult Mongolian gerbils during a specific phase of cortex-dependent auditory relearning, promotes an increase in relearning performance without erasing already established capacities. Promoting synapse plasticity via a reduction of ECM also enhanced the cognitive flexibility of reversal of

learning[188]. This study demonstrates that indeed we can possibly increase the learning process, as well as reversal of learning, due to circuit flexibility. However, certain deficits can be rescued by homeostatic mechanisms. For example, GAD67 is the other enzyme necessary for GABA synthesis. Selectively removing one allele of GAD67 in FS-BCs causes substantial inhibitory neurotransmission deficits, and increases PC excitability. However, these deficits were rescued in adult mice, suggesting that adult mice have the potential to compensate for early development alterations[189].

There has been recent evidence of cellular refinement, by pruning/elimination of inappropriate synapses and axon collaterals (see figure 9). In fact, this process can be activity-dependent and/or guided by molecular signals. There is evidence of both. Firstly, the pattern of subcellular targeting of basket cells is maintained in the absence of sensory and thalamic input, suggesting that this process requires mainly genetically defined cell surface labels[190]. On the other hand, there is also evidence showing that the maturation of these synapses, defined in terms of perisomatic density, is strongly dependent on sensory inputs[6], in addition to molecular cues[191,192]. As for pruning of inappropriate axon collaterals, there has been recent *in vitro* evidence that this process occurs in FS-BC[193].

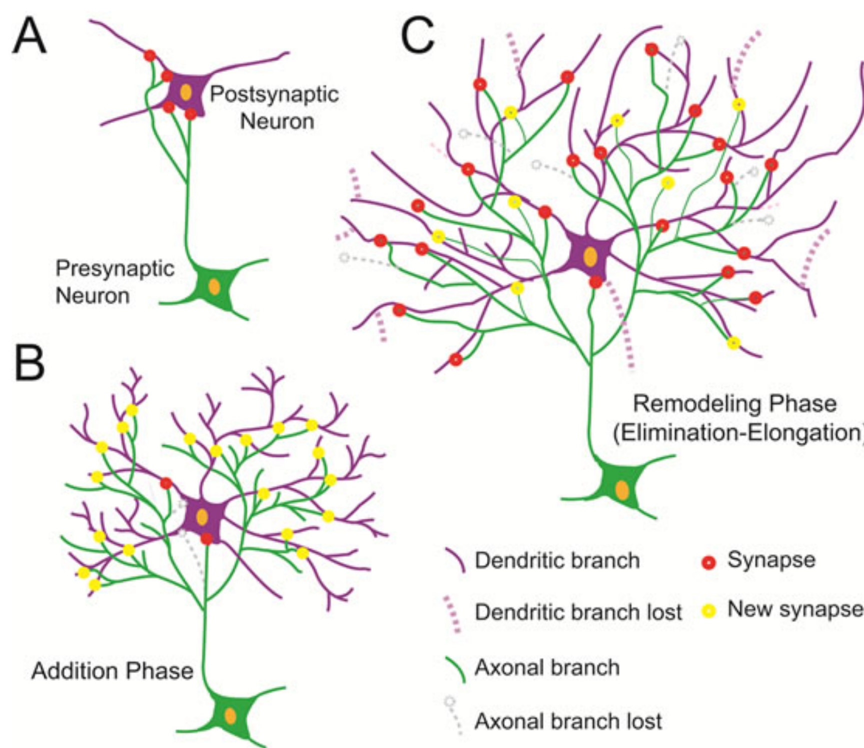


Figure 9. Representation of morphology development, synaptogenesis and pruning during cortical circuit maturation and refinement

Illustration adapted from Uribe-Querol et al (2013)[194].

Synaptic plasticity in the hippocampus has been extensively studied, for its capacity to store information, as this region is associated with episodic and spatial memory. The model of synaptic plasticity is long-term potentiation (LTP)[195], which is enhanced following learning. LTP is produced when high frequency electrical stimulation of afferent fibers enhances synaptic transmission that can last a very long time (months). This process can be reversed with low-frequency stimulation in a timely manner, and this process is termed depotentiation, and only depotentiates recently activated synapses. Along with LTP, there are other activity-dependent plasticity mechanisms, such as long-term depression (LTD), EPSP-spike potentiation, as well as spike-timing-dependent plasticity (STDP), and de-depression. LTD is the opposite process of LTP, where low-frequency stimulation weakens synaptic transmission. This form of plasticity is more present during development and less in the adult. It has been suggested to be a homestatic mechanisms to prevent overactivation of networks, or also serves as a learning mechanisms. This form of plasticity can also be revesed by high-frequency stimulation, causing de-depression. EPSP-spike potentiation refers to the potentiation of action potential probability of a given synaptic input. STDP is when pre- and postsynaptic cells are independently stimulated, and the timing at which the spikes are evoked determines the direction of plasticity. Although many molecular components involved in these forms of plasticity have been elucidated, how synaptic plasticity in overall circuits leads to the storage and recall of information is still an open question[25].

In summary, hippocampal and neocortical development are specifically and temporally organized. *In utero* neuron generation by mutitple precursor cells produces the several subtypes of glutamatergic and GABAergic cells; which then use different locomotive processes to migrate to their final laminar positions. Once located in the right region, their neurites explore their environment for potential connections, thus initiates synapse formation. Finally, activity-dependent mechanisms of refinement allow to solidify strong connections and weed out innapropriate ones, thus forming stable and mature neuronal circuits. Each of these processes require numerous key factors to guide them, and to allow proper circuit activity for normal brain development. One important factor in normal circuit development is KCC2, a cation chloride cotransporter that I will discuss in the following section.

2. The Cation Chloride Cotransporter KCC2

Cation Chloride Cotransporter (CCC) family consists of the solute carrier 12 (SLC12) gene family which encodes four types of K^+-Cl^- cotransporters (KCC1-4), two types of Na^+-K^+-$2Cl^-$ cotransporters (NKCC1-2) and an Na^+-Cl^- cotransporter (NCC). The uptake of Cl^- by NKCC1, NKCC2 and NCC is driven by the inwardly directed Na^+ gradient, which is maintained by the Na^+/K^+-ATPase. Conversely, all KCCs (KCC1-4) mediate net Cl^- efflux, which is driven by the respective outwardly directed K^+ gradient, also through Na^+/K^+-ATPase. Nevertheless, all CCC members are electroneutral, meaning they do not contribute to current changes, but rather they are implicated in the flux of ions, which is dependent on the intracellular-extracellular ionic gradient. Their principal role is the cotransporter activity, however, they are also involved in a number of biological processes, such as cell volume and blood pressure regulation, and either neuronal or neuroendocrine signalling, depending on the specific CCC member and posttranslational activation[196-201]. Some CCC members are expressed in all organ systems ubiquitously like NKCC2, and some are solely expressed in the CNS, like KCC2 and KCC4. NKCC2 and NCC are both primarily expressed in the kidney[202]. Finally, NKCC1 as well as KCC3 are also expressed in the peripheral nervous system (PNS), and even more specifically in neurons and glial cells for NKCC1[196]. Their C- and N-terminal domains are important for regulation of membrane expression[203-206], basolateral and apical sorting in polarized epithelial cells[207] and oligomerization[208-211].

Less is known about the NCC, NKCC2 and KCC1, KCC3 and KCC4, although they share similar structure, function and regulatory domains. First, KCC3 is widely expressed mainly in neurons in the rodent CNS and PNS, and undergoes a developmental upregulation, similar to KCC2[212]. Although KCC3 also contributes to neuronal Cl^- regulation, its physiological function is still poorly understood[213]. Nevertheless, its absence or genetic impairment causes neurodegeneration with a reduced threshold for seizures[214], as well as peripheral neuropathy associated with agenesis of the corpus callosum, referred to as the Andermann syndrome[215]. Furthermore, KCC1 mRNA is expressed in neurons and non-neuronal cells in the rodent CNS at low levels[216,217], whereas in the embryonic brain, it is only detected in the choroid plexus[218]. Finally, KCC4 mRNA is highly expressed in the VZ in the

embryonic brain, but its expression decreases to low levels in most of the adult rodent CNS, apart from the suprachiasmatic nucleus, brainstem and spinal cord[218]. Knockout mice of either KCC1 or KCC4 do not exhibit any obvious CNS phenotype[219,220]. In the human cortex, the expression of both KCC1 and KCC4 is very low, while KCC3 is consistently highly expressed throughout life[196].

KCC2 and NKCC1 are the two-most characterized CCC members, and are essential in determining the driving force of GABAergic currents. NKCC1 is almost ubiquitously expressed[221] in the adult rodent CNS and PNS, in neurons and in glial cells [222]. It was initially thought that NKCC1 underwent a downregulation during development in the rat and human brains[223,224]; however, several other studies reported a developmental upregulation of NKCC1 mRNA[225-227]. This discrepancy is most likely due to the differential expression of the two slice variants, NKCC1a and NKCC1b, produced by the *Slc12A2* gene[221]. In fact, the use of probes and antibodies that target exon 21, only detect NKCC1a and not NKCC1b[226,228]. Additionally, NKCC1b undergoes developmental upregulation, and its expression is higher than that of NKCC1a in the adult human brain[229].

Moreover, KCC2 shares a high homology with other KCCs (KCC1, KCC3 and KCC4); nevertheless, KCC2's role is unique in the CNS development and function. It is preferentially and progressively expressed in neurons during development, and establishes the neuronal chloride concentration under isotonic conditions. It fact, its expression is essential for the switch to hyperpolarizing GABA current, and thus alterations of its expression and/or function has been implicated in many neurological disorders, such as epilepsy[223,230-234], neuropathic pain[235], spasticity following spinal cord injury[236], autism[237], and schizophrenia[225,238,239]. My thesis focuses on the effects of KCC2 alterations on seizure susceptibility and synaptic formation. In this section, I will first review KCC2 expression profile in the cortex and hippocampus, followed by a detailed review of its functions in the CNS and the mechanisms involved in its activation.

2.1. KCC2 structure and expression

2.1.1. Structure and intrinsic KCC2 activity

KCC2 is a glycosylated protein with twelve transmembrane domains and an intracellular N- and C-terminal that weighs 140KDa[240,241]. The electroneutral transporter carries ions at a stoichiometric ratio of $1K^{+}$: $1Cl^{-}$; however the coupled translocation mechanism is still unknown[197]. There is a twofold symmetry between the transmembrane 1 to 5 and transmembrane 6 to 10, in an inverted orientation (see figure 10). This pseudo-symmetry could be the basis for the transporting activity[242] and there is evidence that the intracellular release may be regulated through the intracellular loop 1 (ICL1) that produces a flexible intracellular gate[243]. In addition, to the short loop connecting the transmembrane segments, there is a long extracellular loop (LEL 3) of approximately 100 amino acid (aa) residues[244,245] where multiple N-linked glycosylation sites were found[246]. However, their importance has not yet been identified[197]. Beside the glycosylated sites, there are four cysteines in the LEL of KCC2, which are likely substrates of intra- or intermolecular disulfide bonds. To evaluate their role, Hartmann et al. (2010), mutated these cysteines in HEK293 cells. These mutations rendered KCC2 fully inactive, likely due to the formation of incorrect disufide bridges; however, they did not affect the cotransporters' expression and surface targeting[197,247]. KCC4 is the closest CCC member to KCC2, and interestingly, mutations of the corresponding cysteines in KCC4 did not affect its transporter activity. Furthermore, a mutation of a specific amino acid, C568A, located in the 10th transmembrane domain, inactivates KCC2, and affects its interaction to the cytoskeleton (through protein 4.1N; see below for more details). However, it is unclear whether this mutation inhibits its intrinsic activity or affects its membrane trafficking, and whether it causes a conformational change[248,249].

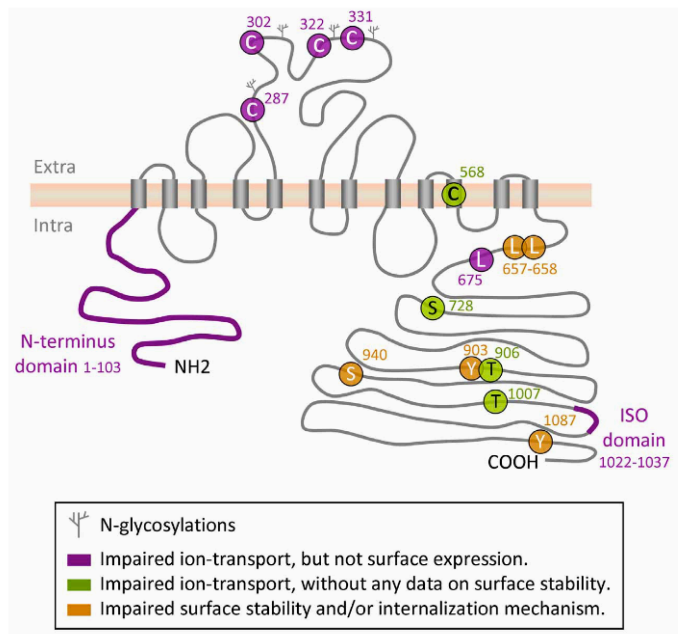


Figure 10. Schematic illustration of KCC2 structure.

Image adapted from Medina et al, 2014 that features the aa residues implicated in the transporter function, its cell surface stability and membrane internalization.

Several studies demonstrate that both the N- and C-terminal domain are important for the intrinsic transporter activity of KCC2. First, truncating the N-terminal region inactivates the transporter activity, but did not affect its plasma membrane expression[250]. In the C-terminal there is a leucine (L675), which has also been shown to be critical for KCC2's transporter activity, but not its surface expression in heterologous expression systems[251]. The C-terminal domain is also packed with multiple tyrosine, serine and threonine phosphorylation sites that play a key role in the activation of the transporter; and they will be reviewed in detail in the following sections. This structure model of KCC2 (as seen in figure 10) is the only putative known structure to date; the three dimensional structure of KCC2, and other KCCs for that matter, are still not known[196,252,253]. In addition, the ion-transporting elements, the rate of transport, the mechanisms regulating the transport, but also the intracellular signalling cascades regulating the cotransporter have yet to be determined. Further studies regarding these issues will be important in order to develop potential (and clean) drugs targeting KCC2.

A defining feature of KCC2 is its high constitutive transport activity under isotonic conditions, where the osmotic pressure across the plasma membrane is equivalent and water can move freely across the membrane without changing ionic concentrations[254]. This feature is crucial to maintain a low intracellular chloride concentration ($[Cl^-]_i$) in neurons. KCC2 contains a structural motif of 15 amino acids (aa) long (1022–1037aa in exon 23) referred to as

the ISO domain, which is important for its isotonic activity[255-257]. This feature constitutes a pivotal difference between KCC2 and other KCCs, since the latter have low transporting activity under isotonic conditions, and would require cell swelling, or osmotic stress, to have a high transporting activity[198]. KCC4 could be strongly activated by hypotonic swelling, but not in isotonic conditions[258,259]. In fact, transferring the ISO domain to KCC4 of cultured hippocampal neurons rendered this transporter constitutively active in isotonic conditions, while deleting it from KCC2 reduces its cotransporter activity. Additionally, replacing this sequence in KCC2 by the corresponding KCC4 sequence abolished its constitutive activity, and instead allowed activation under hypotonic conditions like KCC4. These data suggest that KCC2 probably has two functionally different domains, the first being the specific ISO domain, and the second may be shared with the other KCCs that actually transport ions under hypotonic conditions (lower osmotic pressure, with more water and less solutes;[197,252,256,257]). Taken together, it is evident that both the N- and C-terminal region of KCC2 are involved in its intrinsic cotransporter activity, however their regulatory mechanisms are not yet completely understood.

2.1.2. Expression profile

KCC2 is abundantly expressed in neuronal cells of the CNS, as evaluated by western blot, electrophysiology, immunofluorescence and ribonuclease protection analysis[216,226,240,244,260,261]. KCC2 is the only KCC member that is not expressed in glia cells[240,262]. There are, however, certain subpopulations of adult CNS neurons that do not express KCC2, such as dopaminergic neurons of the substantia nigra[263]. Moreover, the number of KCC2-expressing neurons is developmentally upregulated in parallel with neuronal maturation in a caudal-rostral pattern[218,227,264]. As such, KCC2 protein is first expressed in the spinal cord and subcortical neurons during embryogenesis, and gradually increases in higher brain structures. At birth, mature KCC2 expression levels are already established in the spinal cord and brainstem; however, in the more rostral parts such as cortex, upregulation begins perinatally and mature pattern is reached by the second postnatal week[227,264]. This section will describe more specifically the expression pattern of KCC2 during development.

KCC2 transcript and protein expression starts at E12.5 in the immature brainstem and in developing spinal cord motoneurons as they first start to differentiate in the medulla and ventral horn[222,264], and gradually increases across all the spinal cord and medulla at E15.5. The cerebellum starts to develop and Purkinje and granular cells start to be generated by E11. When Purkinje cells start to differentiate at E15, KCC2 is already visible, and increases until E18.5. Conversely, neurons destined for the granular and molecular layers differentiate later and migrate inward from the external cerebellum. As they reach their final destination beginning at P3, they start to express KCC2. Finally, all neurons of the cerebellum continue to gradually increase KCC2 expression postnatally. In parallel, the thalamus starts to develop from E12, and neurons in the dorsolateral nuclei begin to differentiate and start to express KCC2 by E15.5. However, dorsomedial thalamic nuclei differentiate later, and express KCC2 at E18.5; while in the ventral thalamus and ventral lateral geniculate, KCC2 is expressed at E14.5. In addition, in the olfactory bulb originating from the telencephalon, KCC2 starts to be expressed by mitral cells (oldest olfactory neurons) at E15, and appear in all other neurons (for ex. tufted cells) by E18.5. The striatum differentiates after the olfactory bulb, yet KCC2 follows the same transcriptional pattern in medium-sized spiny neurons. Neurons from the 'roof' of the telencephalon begin to migrate to form the neopallial cortex, which becomes the outer gray layer of the cortex at E12. Finally, the basal part, which includes the piriform cortex finishes its differentiation before the cortical plate differentiation process begins, and thus, starts to express KCC2 before the rest of the neocortex, at E15.5 [218,264].

In the cortex, KCC2 has a particular area-specific distribution and age-related increase. It is first identifiable in the basal part of the neocortex (piriform and entorhinal cortices, referred to as the paleocortex). KCC2 (mRNA and protein) is already present in the piriform cortex at E15.5 in differentiated neurons. At birth, the labelling is already strong in dendrites and somas in superficial layers and also diffused in the neuropil; it then gradually increases and reaches its mature pattern after the first postnatal week. In contrast, in isocortical areas (neocortex and hippocampus), KCC2 is absent from the VZ or IZ during neurogenesis and migration. And, in the somatosensory or motor cortices, KCC2 labelling is very faint at birth in all layers but layer 1, where it is expressed in the neuropil. It is then progressively increased

in the entire neocortex during development. In fact, neocortical neurons differentiate later than the paleocortex suggesting that KCC2 expression correlates with the telencephalon development, as well as cortical neuron differentiation and maturation[218,227,264,265]. In fact, as soon as an immature neuron reaches their final position, the mRNA of KCC2 can be detected.

To further understand KCC2 expression patterns, a few studies have evaluated layer-specific differences in the sensory neocortex and hippocampus (see Figure 11). Firstly, as previously explained, KCC2 is already expessed in cortical layer 1 at birth, and this pattern continues throughout postnatal development. Other layers have a more complex pattern since neurons are still migrating to superficial layers in the cortical plate at birth. In layer V/VI, KCC2 is first localized in dendrites and cell bodies of non-pyramidal neurons, as seen by weak immunolabeling in small multipolar neurons, present until P3. Nonetheless, at P3, KCC2 is also weakly labeled in the cell body and dendrites of PCs. At P5, KCC2 levels increase in dendrites and on the surface of PCs somata and in the neuropil of layer V and in granule cells in layer IV. The staining intensity in the neuropil increases in layers IV/V/IV at P7, and appears homogeneous by P10. At this time, cell bodies as well as apical dendrites no longer have any clear KCC2 signal, as they enter the membrane. KCC2 immunolabelling increases until P14. However, in layer II/III KCC2 immunolabelling is negative at P5, and moderately increases starting from P7 in the bottom half of these layers. At P10, most neurons express KCC2 but they maintained a gradient pattern, where the intensity decreases from deep to superficial neurons; whereas after P12, KCC2 levels increase and are consistently homogeneous in the neuropil. In summary, KCC2 is localized in deeper layers after P7, and all layers after P10, and reaches its mature expression pattern (somata membrane and in the neuropil) across all cortical layers by P12[266]. Altogether, it seems KCC2 in expressed in a timely manner as neurons settle into their final destination in the neocortical layers. Interestingly, the timing of KCC2 expression in PCs corresponds to the development of GABAergic synapses, which appear at P3 and significantly increases after P5. Furthermore, both electrophysiological analysis of GABAergic driving force coupled with immunolabelling of GABA and VGAT (vesicular GABA transporter) suggest that GABA is 'excitatory'/shunting in superficial layers, while it is inhibitory in deeper layers of the cortex, during the first postnatal week[266,267]. After P10, GABA staining extends to superficial layers

and plays an inhibitory action in all neocortical neurons[266-268]. These expressional changes suggest that the developmental timing of GABAergic synapse formation in each cortical layer correlates to the timing of KCC2 expression[266].

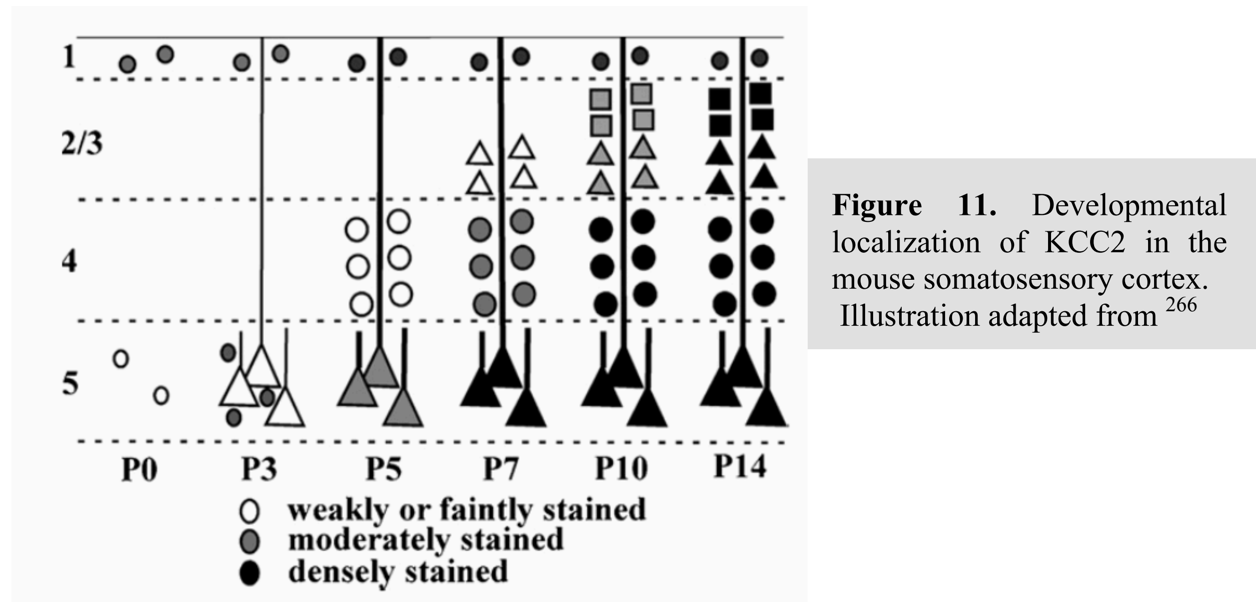


Figure 11. Developmental localization of KCC2 in the mouse somatosensory cortex. Illustration adapted from [266]

In the hippocampus, KCC2 transcript expression starts in the CA3 region at around E15.5, where the first pyramidal neurons settle in; and spreads to the CA1 region by E18.5. In the DG, where granule neurons are mostly generated postnatally, KCC2 expression increases during the first postnatal week. Finally, KCC2 mature levels are reached by P15[218,227,264,266,269].

Interestingly, the subcellular localization of KCC2 differs in the paleocortex, neocortex and hippocampus. From P0 to P12 in the paleocortex and neocortex, KCC2 is localized in transport vesicles and mostly in dendritic plasma membrane as observed by electron microscopy (EM) imaging. In superficial layers of the neocortex and paleocortex, KCC2-positive transport vesicles increase with age; while in the hippocampus, they gradually decrease with age[265,269]. This difference may be due to either an increase in KCC2 synthesis and transport, or to an increase in the recycling of KCC2 at the membrane, in the cortex. Furthermore, KCC2 is highly expressed in the vicinity of excitatory synapses in the hippocampus close to extra-synaptic $GABA_A$ receptors ($GABA_AR$)[269,270] as well as in thalamic relay neurons, that receive cortical afferents[271]. The expression of KCC2 in dendritic spine heads in the cortex is still controversial, as a recent report has shown by EM that at P6 and

P12, KCC2 is located at the spine neck and not spine heads[265]. Finally, distribution of KCC2 mRNA or protein levels discussed in this section are similar in the mouse and rat developing brains, with the only difference that the developmental timing is shifted about two days later in the rat compared to the mouse[264].

KCC2 expression is not only age- but also gender-specific. For example, at P1, KCC2 protein levels are significantly higher in females than in males in the rat entorhinal cortex and hippocampus[272]. Consistently, CA1 pyramidal neurons show more hyperpolarized GABAergic postsynaptic currents in females from P4 to P14[273]. Interestingly, seizure susceptibility is strongly influenced by sexual hormonal difference between genders in the developing brain; which can regulate the excitation threshold and thus regulate seizure generalization[274-276]. The gender difference in KCC2 expression, and consequently in the onset of inhibitory GABAergic neurotransmission during brain development could partly explain why males are more susceptible to seizure than females. Other studies are necessary to understand whether these gender-based differences are due to differences in KCC2 protein levels only, or to the number of cells expressing this cotransporter[272].

Finally, the developmental upregulation of KCC2 is not only brain region- and gender-specific, but also species-specific. Rodents are born with low cortical KCC2 expression and depolarizing $GABA_A$R-mediated responses in cortical neurons. However, in the human neocortex, KCC2 mRNA is strongly upregulated during the second half of gestation[225,228,277]. In addition, immunohistochemical analysis demonstrates that most cortical neurons express KCC2 from the 25th postconceptional week onwards[278]. While an earlier study suggested that KCC2 is predominantly expressed postnatally in humans[224], recent evidences support the hypothesis that KCC2 expression begins prenatally in the human neocortex[196].

2.1.3. Isoforms

There are two different isoforms of KCC2: KCC2a and KCC2b. These isoforms are encoded by alternative exon one, and have different promoters regulating them, though both have similar transporter properties when expressed in HEK293 cells. The major structural

difference between the two isoforms is in the N-terminal section of the protein[279]. In mature cortex, hippocampus and cerebellum, the major isoform is KCC2b, which represents 80% of total KCC2. In the embryonic and neonatal brainstem, however, both isoforms have similar distribution (colocalization) and expression pattern[280], suggesting they are initially regulated by common regulatory mechanisms. Moreover, KCC2b expression is increased during the first postnatal week in the cortex and cerebellum, while KCC2a does not increase postnatally in these regions, and even disappears in the adult thalamus and cerebellar Purkinje cells. In several noncortical regions including the basal forebrain, hypothalamus, brainstem and spinal cord, postnatal KCC2a expression is high. Concerning the subcellular localisation, both isoforms target dendrites, but they colocalize only partly. In the mature CNS, KCC2b is primarily located at the plasma membrane of neuronal somata and proximal dendrites in the hippocampus, entorhinal cortex, Purkinje neurons[269,281], brainstem[282], and spinal motoneurons[222,236]. Endogenous KCC2a, however, is not found at the plasma membrane of neuronal somata and proximal dendrites in any area of the postnatal CNS. On the other hand, KCC2a is present in neuronal dendrites, and colocalizes with dendritic MAP2, but only partly with KCC2b; thus, it may be that these isoforms are either separately localised in the dendrite (intracellular vs membrane) or they are located in different areas of the dendrite (proximal vs distal). The different N-terminal region of these isoforms can target different signals or interacting proteins, which could lead to different transporting mechanisms, or possibly intracellular retention mechanisms[281], however further studies are needed to address this discrepancy. Its important to bear in mind that most studies on KCC2 expression have used mRNA probes and antibodies that detect both KCC2a and KCC2b, thus, unless stated otherwise, KCC2 refers collectively to both splice variants.

2.2. Function

Although low levels of KCC2 are expressed in the cortex at birth, its expression in the spinal cord and brainstem is already mature. As such, $KCC2^{-/-}$ mice (knockout of both isoforms) die at birth. They exhibit an increase in depolarizing GABA response, severe

deficits in motoneuron control, abnormal muscle tone and respiratory failure[222,250]. These phenotypes underline KCC2 importance in spinal cord and medulla development around birth. Similarly, overexpressing KCC2 precociously impairs neural development[249]. On the other hand, KCC2b-specific knockout mice maintain a small amount of KCC2 (~5%) and survive up to three weeks postnatally; however, they develop spasticity and recurrent generalized seizures associated with a substantial reduction of inhibitory interneurons in the cortex and hippocampus, until their eventual demise[283]. Finally, a partial knockout of both KCC2 isoforms, which maintains 20-30% of functional KCC2, survive but suffer motor deterioration and present a reduced sensitivity to diazepam[284]. Even a 20–50% change of KCC2 expression in neurons alters neuronal network activity and circuit development[285] (and our data). Silencing other CCC members expressed in the brain (NKCC1, KCC1, KCC3, and KCC4) causes markedly less dramatic changes phenotypes[199,252].

Overexpression of KCC2 produces a rapid and evident ion flux (within a few seconds; [254,257,286-289]), which can be reversed by furosemide, a non-selective blocker of KCC2[254,289] or by VU0463271, a KCC2-specific inhibitor[290]. Dependent or independently of its cotransporter activity, KCC2 is implicated in several neuronal processes such as mature inhibitory GABAergic responses, neuron migration, dendritic outgrowth, synapse formation and maturation [252]. In the following section, I will discuss these different functions in detail.

2.2.1. GABA switch

NKCC1 is the main functional cotransporter present during early embryonic development, starting its expression from E12.5-15.5[291]. In immature neurons, Na^+/K^+ATPase generates the Na^+ gradient, and drives NKCC1 to increase the intracellular chloride concentration by importing Na^+, K^+ and $2Cl^-$ inside the cell (see figure 12). In these circumstances, opening of the ionotropic $GABA_AR$ allows chloride to go outside the cell, to maintain normal gradient homeostasis, creating a depolarizing chloride current. As such, GABAergic neurotransmission in early development is shunting or, more controversially, ‘excitatory’, by essentially reducing the threshold for action potential generation and

increasing intracellular concentration of Ca^{2+} [292]. The depolarizing response of GABA does not necessarily imply that GABA excites neurons, alternatively, it could entail that it still has an inhibitory action on neurons due to the increased conductance than can shunt excitatory synaptic currents[264,293]. These characteristics allow GABA to serve as a trophic factor, and to tightly regulate cell proliferation, migration, axonal growth, synapse formation and cell death[294-296].

In the mature CNS on the other hand, GABA is predominantly inhibitory in function. This developmental shift in GABA action is due to a shift in the Cl^- reversal potential. It has been widely shown that the developmental upregulation of KCC2 is responsible for the change in Cl^- influx through $GABA_AR$ and GlyR (glycine receptors), and the shift of GABA function from excitatory/shunting to inhibitory[222,287,292,297]. The extrusion of Cl^- by KCC2 is driven by the K^+ gradient generated by Na^+/K^+ATPase. This gradual shift was observed towards the end of the first postnatal week in the neocortex and a week later in the hippocampus[216,265,298]. As proof of concept, blocking KCC2 in hippocampal culture by a KCC2-specific antisense oligonucleotide prevented the switch to inhibitory GABA action[216]. Additionally, all knockout models of KCC2, whether it is fully or partially inactivated, exhibit an increase in the intracellular chloride concentration and thus a reduction of GABAergic and glycinergic inhibitory strength[222,283,299]. In parallel, a precocious overexpression of KCC2 in immature cortical neurons produced a significant negative shift in the reversal potential of GABA (E_{GABA}), demonstrating that the presence of KCC2 is sufficient to end the depolarizing period of GABA in developing cortical neurons[248,287]. Impaired KCC2 in neurological disorders such as epilepsy underlie an imbalance in chloride reversal potential and in GABA function, which has been shown in some cases to be one of the major causes of hyperexcitability in these paradigms[198,231,232,300].

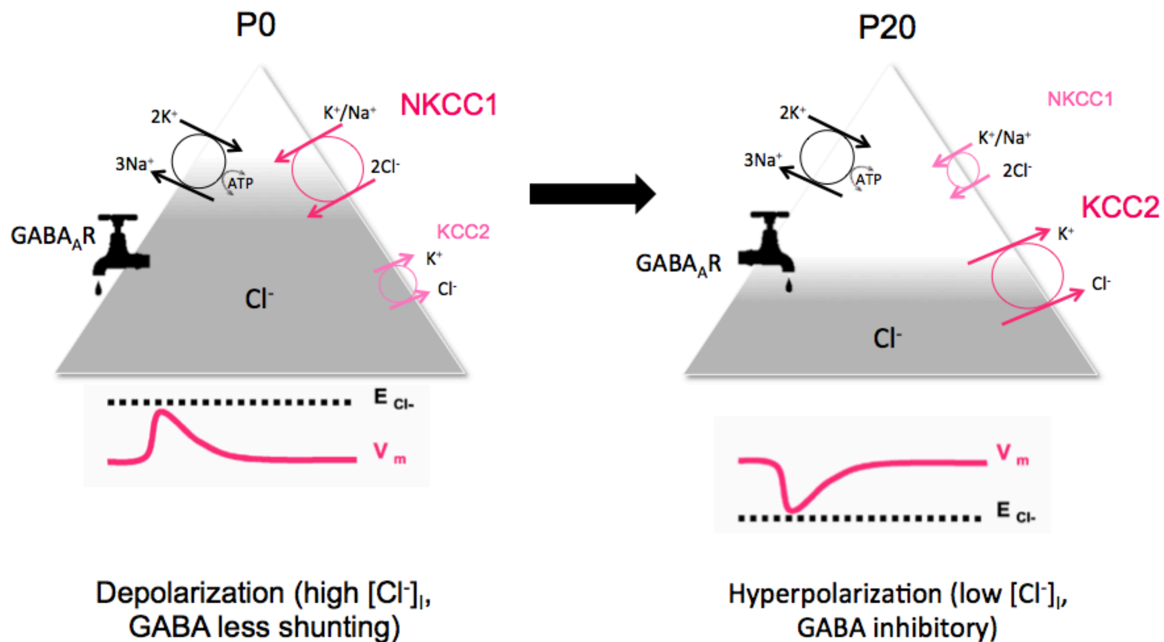


Figure 12. KCC2 and NKCC1 cotransporters are key for the developmental switch of GABAergic driving force.

At birth (left), NKCC1 expression is prominent and loads the cell with Cl-. In this case, the reversal potential of GABA is more depolarized than the resting membrane potential, thus GABA is depolarizing. In the mature brain however (right), KCC2 is prominently expressed, and extrudes Cl- outside the cell. Adult neuron resting membrane potentials are more depolarized than the reversal potential of GABA, and thus hyperpolarize and inhibit.

2.2.2. Role in neocortical development

KCC2 is implicated in several neuronal processes such as migration and dendritic outgrowth. As such, overexpressing KCC2 in mouse embryos at E9.5-11.5 impairs the development of the neural tube (which remains thinner), and neural crest-related structures. It also leads to an abnormal body curvature and smaller brain structures. These results were mimicked when overexpressing a KCC2 mutant which cannot bind the cytoskeleton, suggesting that KCC2 structural interaction with cytoskeleton plays an important role in neuronal differentiation and migration[249].

During migration into the cortex, ambient GABA and glutamate initially stimulate the motility of interneurons through both $GABA_A$ and AMPA/NMDA receptor activation. Once in

the cortex, upregulation of KCC2 is necessary and sufficient to reduce interneuron migration by hyperpolarizing GABAergic action. Initially, $GABA_AR$ activation in migrating interneurons, with low expression of KCC2, induces calcium transients. These calcium transients disappear upon KCC2 upregulation. As such, blocking L-type voltage-sensitive calcium channel (VSCC) successfully stopped interneuron migration. These results suggest that the upregulation of KCC2 and hyperpolarizing $GABA_AR$ decreases the frequency of spontaneous intracellular Ca^{2+} transients initiated by VSCC activation which sends a stop signal to migrating interneurons[133].

Depolarizing GABA and glycine activity is believed to be important for the ontogeny of developing neuronal circuits before the maturation of sensory inputs[147,295]. As mentioned, GABA in early development act in concert with glutamatergic mechanisms and intrinsic excitatory circuits[301] to produce intracellular Ca^{2+} transients, which activates downstream cascades with trophic actions. For example, depolarizing GABA was specifically proven to be important for the morphological maturation of cortical neurons. Cancedda et al. (2007) demonstrated that overexpressing KCC2 embryonically by *in utero* electroporation leads to a decrease in branch number and in total dendritic length, while there was no difference in pyramidal neuron migration. Comparably, they obtained the same results when they overexpressed an inward-rectifying K^+ channel, which lowers the membrane resting potential like an overexpression of KCC2 does. Finally, the KCC2 transporter-deficient mutant did not affect neocortical morphology, suggesting that the early GABA 'excitatory' function is important for the morphological maturation of neonatal cortical neurons *in vivo*[302].

2.2.3. Role in glutamatergic development

Recent reports reveal another important role for KCC2 postnatally: its role in dendritic spine development. The developmental upregulation of KCC2 is associated with synaptogenesis and in fact, a significant amount of KCC2 in cortical neurons is localized either at, or in the vicinity of dendritic spines[269,270,303]. The first evidence was provided by Li et al, (2007), that analyzed spine morphology in primary cultures of immature cortical neurons

from KCC2$^{-/-}$ mice, after two weeks in culture[250]. The lack of KCC2 in cortical neurons prevented spine maturation altogether leading to an increase of filopodia-like dendritic protrusions (≅5µm) that were branched and highly motile. They also observed less active excitatory synapses, by a reduction of VGLUT1 and PSD95 clusters (pre and postsynaptic structures), as well as a reduction in the frequency of miniature excitatory postsynaptic current (mEPSC). In KCC2$^{+/-}$ mice, where ~20% of KCC2 remains, there was no difference in spine density, but protrusions were longer at P16, similar to what reported in their previous *in vitro* analysis. This effect was shown to be independent of KCC2 cotransporter activity since transfecting KCC2$^{-/-}$ neurons with either wild-type or the N-terminal deleted KCC2 mutant construct (KCC2-ΔNTD), which is transport-deficient, rescued spine development. Furthermore, they found that the structural interaction of KCC2 to spine cytoskeleton is through the C-terminal domain and the protein 4.1N. They also observed aberrant dendritic protrusions in KCC2$^{-/-}$ neurons when they were continuously cultured with TTX, demonstrating that this effect is not due to a decrease of inhibitory function and subsequent hyperexcitability, and is in fact due to transporter- and activity-independent mechanism. On the other hand, removing KCC2 by shRNA in mature cultured hippocampal neurons, after spine formation and when KCC2 expression is higher, did not affect spine density or spine length. However, the authors found a reduced efficacy of excitatory synapses, due to an alteration of aggregation of AMPA receptors (AMPAR) in dendritic spines. This effect is independent of KCC2 cotransporter function; instead KCC2 seems to constraint the lateral movement of AMPAR, directly or indirectly at the spine head. Interestingly, the 4.1N protein that binds KCC2 and actin, also binds to GluR1 subunit of AMPAR[304]. Moreover, the actin cytoskeleton is important for synaptic AMPAR stabilization[305], thus, direct or indirect interaction between KCC2, actin and AMPAR may affect the maintenance of AMPAR in dendritic spines. Therefore, KCC2 knockdown in early development reduces spine formation, while in mature neurons, after spine formation, does not compromise spine maintenance but reduces the efficacy of excitatory synapses[303].

Finally, a premature expression of KCC2 in the somatosensory cortex *in vivo* by *in utero* electroporation (E17.5) increases dendritic spine density of layer II/III pyramidal neurons at P10, P15 and P90, as well as increases frequency of mEPSC. The authors showed that

overexpressing both KCC2 mutant with N-terminal deletion (transporter deficient), or the isolated C-terminal domain (involved in transporter function and binding to actin) induces a similar increase in spine density; whereas overexpressing KCC2 C568A mutant, which affects both the transporter and structural function, did not affect spine density. These results imply KCC2 effect on spine density is transporter-independent, and instead mediated by the structural binding of its C-terminal domain to the actin-associated protein 4.1N[306,307]. Interestingly, blocking the expression of protein 4.1N in mature cultured hippocampal neurons increased the lateral diffusion of KCC2 away from excitatory synapses[308]. Consequently, modifications of KCC2 localization between the dendritic spines and the dendritic shaft could control the efficacy of excitatory synapses by constraining AMPA receptors at spine heads[196,303,308]. Altogether, these results demonstrate that KCC2 has an important function in dendritic spine development, and thus potentially in cognitive functions.

Recent structural data suggests that KCC2 at the plasma membrane is responsible for Cl^- homeostasis, while KCC2 in the cytoplasm is associated to its role in synaptogenesis and spinogenesis, which is independent of its cotransporters activity[265], however further studies are required to confirm this hypothesis. In addition, KCC2 expression levels were thought to be negligible in the embryonic cortex. However, in embryonic hippocampi of $KCC2^{-/-}$ mice at E18.5, Khalilov and collegues (2011)[309] observed an increase in spontaneous neural network activity, increased glutamatergic and GABAergic synapse density and increased frequency of spontaneous excitatory and inhibitory postsynaptic current (sEPSC, sIPSC). Interestingly, E_{GABA} was unaffected, suggesting that KCC2 was not functionally active, but demonstrating that KCC2 already serves a function in embryonic hippocampus[309], which warrants further exploration.

The molecular signaling pathways that trigger the developmental upregulation of KCC2 in spines remain inadequately understood[196]. BDNF embryonic overexpression increases synaptogenesis and precociously upregulates KCC2[310]. On the other hand, in $BDNF^{-/-}$ mice, KCC2 is normally upregulated during development[234], suggesting that BDNF is not necessary to KCC2 expression.

Furthermore, another important cell adhesion molecule termed neuroligin 2 also appears to be important in regulating KCC2 and the GABAergic functional switch, as well as

maintaining dendritic spines by increasing KCC2 expression[311]. Further experiments are essential to clarify the molecular mechanisms controlling KCC2 developmental upregulation and their interplay with synaptogenesis.

2.2.4. Role as a neuroprotective agent

Recently, it has been suggested that endogenous KCC2 may play a novel role as a neuroprotective agent[288]. KCC2-specific shRNA-mediated knockdown in primary and organotypic hippocampal cultures leads to an increase in $[Cl^-]_i$ and prolongs the recovery time of intracellular chloride after imposing a chloride increase. Most importantly, these transfected neurons show a higher susceptibility to lipofectamine-dependent oxidative stress, as well as NMDA receptor-dependent excitotoxicity, and this effect is dependent on KCC2 cotransporter activity. Finally, coexpressing rat shRNA-KCC2 with mouse KCC2 restored the resistance of neurons to toxicity. Therefore, silencing KCC2 reduces neuronal resistance to toxicity and should be taken into account when performing these expression modifications. In fact, a minimal KCC2 protein domain in the N-terminal was recently identified as sufficient for KCC2-dependent neuroprotection[312]. Altogether, these results reveal new roles of KCC2 and open new neuroprotective strategies for a wide range of neurodegenerative diseases.

2.3. Activation

While the cotransporter activity of KCC2 is dependent of both the N-terminal and C-terminal domain, the latter is also involved in KCC2 activity regulation through membrane expression, oligomerization and phosphorylation[196,197]. In fact, it has been shown that the presence of KCC2 in the plasma membrane does not necessarily imply that its cotransporter activity is activated[313]. It is only once it is activated that the number of active cotransporters in the membrane can determine its capacity and the ion-turnover.

NKCC1 and KCC2 are reciprocally regulated by the intracellular chloride concentration ($[Cl^-]_i$). When $[Cl^-]_i$ falls below the physiological threshold, NKCC1 is directly phosphorylated and leads to its functional activation, and thus restores the $[Cl^-]_i$[196,314]. Conversely, an increase in $[Cl^-]_i$ promotes the activation of KCC2, to extrude Cl^- [289]. In the following section, we will see more precisely how KCC2 is activated.

2.3.1. Membrane activation

Membrane insertion and internalization

The following section will focus on the surface expression of KCC2, from its insertion in the membrane and stabilization, to its turnover and internalization. Interestingly, in response to seizures, KCC2 undertakes quick withdrawal from the plasma membrane, thus reducing its cotransporter activity, and resulting in the reduction of hyperpolarizing GABA responses[203,307,308,315-318]. Therefore, it is important to understand the mechanisms involving this type of activation of KCC2. By visualizing the internalization process of KCC2, thanks to an extracellular tag, in HEK293 cells, Zhao and colleagues (2008) found a novel non-canonical di-leucine motif (657LLXXEE662) in the C-terminal domain of KCC2 that is important for its constitutive internalization and for binding to the clathrin-binding adaptor protein-2 complex (See figure 14). The clathrin-mediated endocytosis pathway regulates the internalization of KCC2 in HEK cells[205] and in cultured neuronal cells[318]. There are also a number of phosphorylation sites in the C-terminal region that are also implicated in the turnover regulation of KCC2, and they will be discussed later. The specific recycling mechanisms and the signaling pathways that regulate the membrane insertion of KCC2 are however, not completely elucidated.

Several studies have evaluated the turnover rate of KCC2 in mature neurons. First, Rivera and colleagues (2005), used a biotinylation methodology and prepared hippocampal slice cultures with a slice chopper and found that KCC2 is reintroduced in the neuronal plasma membrane within 30 to 40 minutes[297]. However, Puskarjov and colleagues (2012) prepared slice cultures with a vibratome, which usually causes less neuronal damage, and reported that

the half time of turnover is much longer, ranging from several hours to days[319]. On the other hand, treating cultured hippocampal neurons with an inhibitor of endocytosis (dynasore) for 45 minutes doubled the surface expression of KCC2[204]. Lastly, in heterologous expression systems, the entire functional pool of membrane KCC2 is recycled every 10 minutes[203,205], demonstrating that it is possible for KCC2 to undergo rapid endocytosis, which can perhaps respond quickly to more stringent conditions. Longer processes can also be due to genomic regulation of KCC2, creating more indirect surface expression mechanisms. Few known molecules important for KCC2 membrane trafficking, such as TrkB receptor, protein kinase C (PKC) and 5-HT2A serotonin receptors[320,321], will be touched on later.

Lipid rafts

There are two important studies that suggest that lipid rafts control KCC2 activity[322,323]. Lipid rafts represent a microdomain of specialized membranes that are enriched in glycosphingolipids and glycoproteins. They operate as organization centers where signaling molecules assemble and cluster and where they can regulate their activity. In neurons, approximately 50% of KCC2 molecules are associated with lipid raft markers, while the others are bound to non-raft markers[322,323]. Hartmann et al. (2009) stated that KCC2 is inactive when it is located in lipid rafts[322]. Conversely, Watanabe et al. (2009) suggested that KCC2 is actually active when they are located in rafts-like clusters[323]. These opposing statements can be explained by the use of different experimental models, and distinct wide-range acting pharmacological agents. After detailed critical analysis, Medina et al, (2014) proposed that KCC2 might be active as a membrane raft-associated protein, but concurs that more studies will be required to understand the role of KCC2 in lipid rafts[252].

Oligomerization

An important, but complex feature of KCC2 activity is its aptitude to oligomerize. Multiple biochemical and functional interaction have proven that KCC2, as well as other CCC members, form homo-oligomeric structures[208-211,282,324]. KCC2 has been reported to form molecular complexes consistent to monomers, dimers, trimers and tetramers[282,325], either with

other KCC2 or with other CCC family members[324,326]. In addition, KCC2a and KCC2b isoforms form homo- and heterodimers *in vivo*, as well as in heterologous system (HEK293)[280,281]. It has been suggested that in the immature brain, there are more KCC2 monomers, whereas in the adult, KCC2 is mostly in oligomeric forms[282], and thus proposing that only the oligomeric form of KCC2 is cotransporter active. First, in the auditory brainstem nuclei, KCC2 complexes of higher molecular weight parallel transport activation[282]. KCC2 tends to form aggregates at high concentrations in heterologous expression systems[280], suggesting that the developmental upregulation of KCC2 could potentially push towards an oligomer state. However, it is also possible that KCC2 complexes are formed by association with other interacting proteins, like Neto2 (neuropilin and tolloid like-2) and the kainate receptors forming hetero-oligomers[197]. For example, Neto2, which is necessary for KCC2 cotransporter function, preferentially associates with oligomeric KCC2[327]. Further, it was recently shown that kainate receptors form hetero-oligomeric complexes with KCC2. Interestingly, in GluK1/2 knockout mice, Mahadevan and colleagues (2014) reported an increase in monomeric KCC2 and a decrease in oligomeric KCC2[325], thus suggesting that the kainate receptor may affect the KCC2 monomer/oligomer ratio. Interestingly, in conditions where the cotransporter activity of KCC2 was reduced, oligomeric KCC2 levels are reduced too[323,325], suggesting that KCC2 activity promotes oligomerization.

It is important to mention that although different groups have consistently found KCC2 oligomerization, there are technical issues that limit the interpretation of their presence and functionality. KCC2 has been found to be resistant to SDS dissociation of protein complexes and denaturation[328]. The ability to form oligomers also changes depending on the experimental approach, whether we are looking at endogenous levels of KCC2 or whether it is overexpressed in heterologous systems. Depending on the length of the protein extraction protocol, there may be more complexes that are dissociated. Therefore, it can be difficult to interpret western blot analysis[252].

The mechanisms involved in oligomerization and how they affect KCC2 transporter activity also remains elusive. For example, we do not know whether oligomerization is critical for translocation to the surface, and/or internalization; or how many subunits are required to form an active transporter; and whether homo-oligomerization is necessary for transport

activity, or more precisely if each subunit mediates transport, but requires oligomerization to assume a transport active conformation. Most likely, when the crystal structure of KCC2 will be available, it will be easier to understand how oligomerization is required for cotransporter activity. Despite the lack of answers to these questions, I will describe the advancements we have seen so far in the field. Firstly, a yeast two-hybrid system analysis demonstrated that the full length C-terminal of KCC2 (or) and KCC4 form a dimer, but not truncated C-terminal fragments[324]. Another study using the rat hypothalamic cell line GT1-7 show that removing the last 28 aa of KCC2 caused an increase of monomers and a decrease of oligomers[323]. However, its not known whether this change in oligomerization is due to the absence of an important sequence for dimerization, or it is due to a change in the overall conformation of the protein; but taken together with the yeast two-hybrid experiments, these studies suggests that the C-terminal region may be important for oligomerization[197]. Furthermore, the tyrosine kinase inhibitor genistein triggered a substantial shift towards monomeric KCC2 in GFT1-7 cells and a more depolarized reversal potential of GABA (E_{GABA}), however, a phosphomimetic substitution of Tyr1087 in KCC2 in hippocampal neurons gave similar results[323]. Another study recently indicated that tyrosine phosphorylation of KCC2 causes an increase in the cotransporter degradation in hippocampal neurons and HEK293 cells[204]. However, the level of KCC2 parallels its tyrosine phosphorylation during development[264]. Taken together, these data show that the role of tyrosine phosphorylation in KCC2 oligomerization is still poorly understood.

2.3.2. Signaling pathways regulating KCC2 function

There are multiple signaling pathways involved in KCC2 activity (see Figure 14). In this section, I will provide an overview of the most characterized pathways involved in regulatory function, such as the phosphorylation of KCC2, its Zinc-mediated regulation, its protein-protein interaction, its transcriptional regulation, and the role of trophic factors, like BDNF.

Phosphorylation

One of KCC2 activation mechanisms relies on posttranslational modifications through phosphorylation[198,329-332]. KCC2 has multiple phosphorylation sites in its C-terminal region, which allow its specific functional and surface expression regulation. Initially, it was suggested that KCC2 is activated by endogenous protein tyrosine kinases[333], and the amount of phosphorylated KCC2 increases during development[264]. However, it became evident that the regulation of KCC2 through phosphorylation is more complex than initially anticipated. Moreover, not only are there multiple phosphorylation sites in the KCC2 sequence, but there is also cross talk between these different sites[197,252,329,334], only further complicating the issue.

The first evidence came from the coexpression of WNK3 (with-no-lysine kinase 3, serine-threonine kinase) with CCCs in Xenopus oocytes, which resulted in the activation of NKCC1 and inactivation of KCC1 and KCC2[335]. The WNK family together with SPAK (a Ste20p-related proline/alanine-rich kinase) and OSR1 (an oxidative stress-responsive kinase -1), also expressed in the CNS, form a signaling pathway implicated in the control of swelling-induced regulation of CCC members, and is regulated by $[Cl^-]_i$ and extracellular osmolarity[336]. All four WNK family members effectively block the cotransporter activity in KCC2-overexpressed neurons[337]. The regulation of KCC2 by the WNK-SPAK/OSR1 signaling pathway involves the N-terminal Thr6 in KCC2a, and the C-terminal Thr906 and Thr1007 in KCC2b[329,331,334,337]. Phosphorylation of both Thr906 and Thr1007 significantly hinders the cotransporter activity, whereas their dephosphorylation leads to constitutively active cotransporter activity[329,331,334]. As such, Thr906 is partially phosphorylated in the neonatal mouse brain and dephosphorylated in the adult brain, suggesting that dephosphorylation of these aa residues contributes to the developmental upregulation of functional KCC2[252]. Precisely, Inoue et al. (2012) demonstrated that the phosphorylation of Thr906 and Thr1007 in the immature brain is supported by taurine, which acts through the WNK-SPAK/OSR1 phosphorylation cascade. However, these studies were obtained by exogenous addition of taurine, KCC2 and WNK1 kinase, and it will be necessary to prove this model *in vivo*[252]. Finally, it was recently shown that WNK-regulated SPAK/OSR1 kinases directly phosphorylate Thr1007, but not Thr906[334], suggesting a more indirect activation of Thr906 (see summary table I below).

On the other hand, there are three residues in the C-terminal region of KCC2, Thr934, Ser937 and Ser 940, which are involved in phosphorylation-mediated activation. Interestingly, these residues are located in an exon that is only present in vertebrates, suggesting that the increased activation of KCC2 may have been an adapting mechanism to cope with a more complex CNS[197,332]. Phosphorylation of Ser940 by protein kinase C (PKC) increases surface expression of KCC2 as well as its transporter activity[203] and clustering at the membrane[338], and thus, decreased the internalization of KCC2[203]. More specifically, phosphorylation of S940 limits adaptor-protein 2 (AP2)-mediated endocytosis of KCC2, resulting in a stable population of membrane-localized KCC2. Consequently, dephosphorylating Ser940 inactivated KCC2 and increased its endocytosis. In fact, there is a high level of phosphorylated Ser940 in resting conditions in cultured hippocampal neurons[318]; suggesting that the phosphorylation of this site is necessary for maintaining KCC2 activity under physiological conditions. Treating cultured hippocampal neurons with glutamate rapidly dephosphorylates Ser940 in a NMDA receptor and protein phosphatase-1 (PP1) dependent manner[318]. Furthermore, phosphorylation of Ser937 or Thr934 increases intrinsic KCC2 transport activity, without affecting its total abundance or surface expression, suggesting these residues are implicated in kinetic regulation of the transporter activity[332]. However, stausporine and NEM (N-ethylmaleimide), two broadband serine/threonine kinase inhibitors, have opposite effects on transporter activity depending on the phosphorylation state of Thr934 and Ser937[332]. Both agents activated transporter activity when Thr934/Ser937 were dephosphorylated, but inhibited it when Thr934/Ser937 were phosphorylated, which suggests that these agents may target other phosphorylation sites. Taken together, serine/threonine kinases can have a dual modulation on KCC2 activity, in which the intrinsic transport activity of KCC2 is increased by phosphorylation of Thr934, Ser937 or Ser940[318,332] and decreased by phosphorylation of Thr906 and Thr1007[331].

Furthermore, a mutation in the residue Ser728 of KCC2 to alanine displayed an increase in the constitutive transporter activity, when PKC is activated; suggesting that dephosphorylation of this residue is required for KCC2 activation[203]. Although, this is likely an indirect effect since PKC does not directly phosphorylate Ser728 in HEK293 cells. Intriguingly, the constitutive activity associated with the phosphorylation of all these residues

(Ser940, 937, 728 and Thr934) requires a lot of metabolic energy and can affect the pH of the cell[196], demonstrating that posttranslational modifications like phosphorylation can be an approach to reduce energetic costs.

Moreover, the endogenous effect of tyrosine phosphorylation of KCC2 seems controversial. It is also unclear whether tyrosine phosphorylation occurs under physiological conditions. Basal activity was seen in cortex[264] and in primary hippocampal cultures[315,323], however, it was negligible in basal conditions in cultured hippocampal neurons in a study by Lee and colleagues (2010)[204]. In addition, Wake et al. (2007) demonstrated that neuronal activity, oxidative stress and BDNF treatment decreased tyrosine phosphorylation of KCC2, while Lee et al. (2010) shows an activation of tyrosine phosphorylation after pilocarpine-induced status epilepticus, or muscarinic receptor agonist application[315]. In addition, there are two residues that are phosphorylated by tyrosine kinases: Tyr1087 and the less characterized Tyr903[204]. Lee and colleagues (2010) observed that phosphorylation of Tyr903/1087 decreases the cell surface stability and total amount of KCC2 by increasing lysozomal degradation, while their dephosphorylation increases cell surface stability. This process was mediated specifically by Src-family tyrosine kinases through activation of the G-protein coupled muscarinic acetylcholine receptors (mAChRs), and this study suggested that tyrosine phosphorylation targets internalized KCC2 for lysozomal degradation. Conversely, other studies had previously found that phosphorylation of Tyr1087 abolishes KCC2 transporter activity, without affecting its cell surface expression, while dephosphorylation does not affect KCC2 transport in either oocytes, mammalian cell lines or neurons[286,288,323]. Watanabe et al. (2009) also found an implication of tyrosine phosphorylation of KCC2 in its recruitment into lipid rafts; which might recruit active or inactive KCC2 as explained above[323]. These conflicting results were explained as a difference of systems (oocytes vs. HEK293 cells), or the possibility that there are other targets of tyrosine phosphorylation in KCC2 *in vivo*, or they may activate different signaling cascades[203]. Additionally, both phosphorylation and dephosphorylation can increase in response to oxidative stress[323], and thus, turnover of KCC2, which reduces cell surface expression, can also affect other kinases and phosphatases involved in cell surface stability of KCC2[203]. Taken together, these results suggest however, that tyrosine phosphorylation is associated with reduced KCC2 activity, while its

dephosphorylation potentiates the transporter. The mechanisms controlling its activity, through a change in surface expression or through changes in intrinsic activity, are still unclear and require more analysis. What is clear however, is that there are two different signalling pathways that are activated by tyrosine phosphorylation: cytosolic c-Scr kinase[204,333] and BDNF-dependent TrkB receptor tyrosine kinase[230,236,316,339]. These kinases can themselves regulate multiple signalling cascades, and thus, it will be necessary to discover the exact regulatory pathways controlling tyrosine phosphorylation-mediated KCC2 activity[252].

In summary, these studies demonstrate that phosphorylation can activate or inactivate KCC2 transporter activity. Phosphorylation of tyrosine 903 & 1087 and threonine 906 & 1007 are all associated with the inactivation of KCC2 transport activity, while phosphorylation of Ser940 activates KCC2. Table I summarizes what is currently known regarding the effect of phosphorylation on KCC2 activity. Moreover, according to public databases, there are a number of other phosphorylation sites in KCC2, implying that further studies will be necessary to better understand the role of phosphorylation on KCC2 function, the interaction between phosphorylated residues under fluctuating conditions, and to establish their role *in vivo*[197]. This will be especially important for designing pharmacological therapy aimed at regulating KCC2 function by modulating KCC2 phosphorylation.

Table I. The effect of phosphorylation of key amino acids in KCC2

	Amino acid residue	Region in KCC2	Cotransporter activity	Membrane surface level	Signalling pathway involved
Serine	Ser940	C-term	+	Increased	PKC
	Ser937	C-term	+	No difference	?
	Ser728	C-term	-	?	?
Threonine	Thr6	N-Term	-?	?	?
	Thr934	C-term	+	No difference	?
	Thr906	C-term	-	?	WNK – SPAK/OSR1
	Thr1007	C-term	-	?	WNK – SPAK/OSR1
Tyrosine	Tyr 903	C-term	-	Controversial	c-Scr kinases &/or
	Tyr1087	C-term	-	Controversial	BDNF-TrkB tyrosine kinases

Protein-Protein interactions

Another mechanism of KCC2 activation is dependent on its interaction to other proteins. There are multiple key molecules involved in KCC2 activity and the most important ones will be listed in this section. The first protein found to interact with KCC2 is the brain-type creatine kinase (CKB), which activates its cotransporter activity in HEK293 cells[340] (see Figure 13). Furthermore, α2 subunit of the Na^{+}-K^{+}-ATPase pump interacts with KCC2. Na-K-ATPase α2 subunit knockout mice ($Atp1a2^{-/-}$) have decreased functional KCC2, and display respiratory deficits similar to those observed in $KCC2^{-/-}$ mice, suggesting that this interaction may be linked to normal breathing function[341]. Another interacting candidate is the protein associated with Myc (PAM), through which its regulatory chromatin condensation (RCC1) domain activates KCC2 function in HEK293 cells[342]. The CCC interacting protein 1 (CP1) was shown to physically and functionally interact with KCC2, and activate its cotransporter activity; however, CIP1 is downregulated during maturation, thus, its precise role is still under investigation[326]. Moreover, it was recently discovered that KCC2 interacts with the auxiliary subunit Neto2 of the kainate-type glutamate receptor (KAR), or directly to KAR [325,327]. As described briefly before, Neto2 preferably binds to the active oligomeric form of KCC2, and losing this interaction leads to reduced KCC2-dependent Cl^{-} extrusion and depolarizing GABAergic responses [327]. Neto2 is part of a neuronal scaffolding platform that regulates the synaptic abundance of kainate receptors[343], and may therefore have multiple regulatory functions on KCC2 activity, dependent on changing conditions and neuronal circuits activity, which can affect the organization of scaffolding proteins [252]. Furthermore, the C-terminal domain of KCC2 binds to the cytoskeleton-associated protein 4.1N and regulates dendritic spine formation by stabilizing KCC2 at the plasma membrane of spines [250,306] and the aggregation of AMPA receptors at spine heads [303]. Blocking this interaction leads to lateral diffusion of KCC2 away from glutamatergic synapses, and enhancing NMDAR activity also resulted in lateral diffusion [308]. Finally, KCC2 also directly interacts with the beta isoform of Rac/Cdc42, the guanine nucleotide exchange factor (βPix), which itself binds to Rac (small GTPase of the Rho family) and Pak (p21-activated serine/threonine-protein kinase). The KCC2: βPix interaction activation results in a reduction of the phosphorylation of cofilin (Cof1) and a change of glutamatergic synapse properties[252].

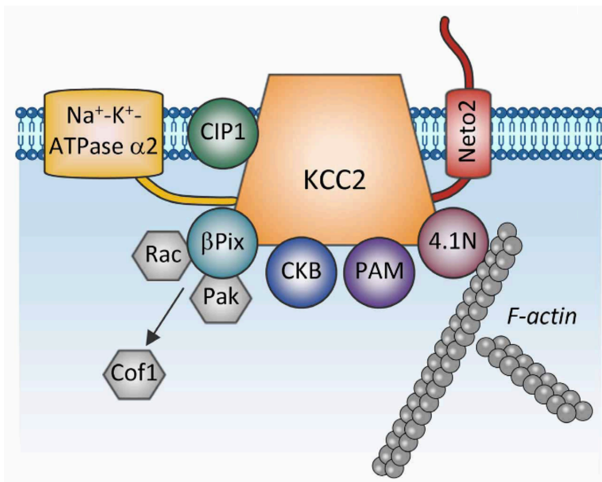


Figure 13. Illustration depicting both membrane-bound and cytosolic proteins that directly interacts with KCC2.

Adapted from Medina et al (2014)[252]. It is not yet known whether they form a single complex, or whether they interact individually with different KCC2 pools.

Transcriptional regulation

In the *Slc12A5* gene (encoding KCC2) promoter and proximal intron-1 regions, 10 putative transcription factors binding sites have been found[344], however only a few have been characterized. First, the early growth response 4 (Egr4) is a transcription factor that regulates KCC2 expression through BDNF and its receptor TrkB in immature neurons. Extracellular signal-regulated kinase 1/2 (ERK1/2) activates Egr4 [344,345], however, blocking Egr4 signalling only decreases total KCC2 expression by less than ~50%[344], suggesting that KCC2 transcription is regulated by additional mechanisms. Another regulatory element implicated in the developmental upregulation of KCC2 is the E-box that binds to ubiquitously expressed USF1/2 (upstream stimulating factors 1 and 2[346]). Finally, there are two neuron-restrictive silencing elements (NRSE) that were also found in the *Slc12A5* gene, however their regulation of KCC2 seems controversial[347-349]. Nevertheless, it seems that pathways regulating neuron-restrictive silencing factor (NRSF), which binds to both NRSE, may contribute to the downregulation of KCC2 during epileptogenesis[196,350]. The other transcription factor binding elements have not been characterized yet.

Role of trophic factors

So far, we know that three trophic factors can modulate KCC2 functional expression, the brain-derived neurotrophic factor (BDNF), the insulin-like growth factor 1 (IGF-1) and

neurturin[252,333,345]. Since the regulatory action of BDNF on KCC2 has been the most characterized, I will focus on its function in this section. First evidence came from a transgenic overexpression of BDNF *in vivo*, which strongly promoted KCC2 mRNA expression[310]. Accordingly, deleting BDNF's main receptor TrkB greatly reduced KCC2 mRNA expression[351]. Moreover, Ludwig et al. (2011) demonstrated that applying BDNF to immature hippocampal slice culture increased KCC2 protein levels, through Erg4-dependent transcription[345]. In contrast, BDNF application to mature neurons, *in vivo* and *in vitro*, had the opposite effect, and reduced KCC2 expression (mRNA and protein) and function (internalization and tyrosine phosphorylation), suggesting that BDNF action may be age- and maturation-dependent[230,236,315,316,352]. Consistently, overexpressing BNDF *in vivo* leads to a depolarizing shift in E_{GABA} in mature dorsal horn neurons in the spinal cord[235,339]. Furthermore, scavenging endogenous BDNF or inhibiting the downstream cascade of BDNF-TrkB, by introduction of a point mutation that uncouples TrkB from PLCγ1, prevents the activity-dependent reduction of KCC2 mRNA and protein expression[230,316]. The difference between the effect in immature vs. mature brain may be due to the age-related difference of TrkB phosphorylation and activation of its downstream cascades, such as PLCγ1[353].

Interestingly, in mature but damaged neurons, BDNF can revert to its ability to promote KCC2 expression after an acute insult[236,352]. It is possible that injured neurons acquire properties of immature ones as an adaptive response to stimulate neuronal survival and rewiring[300,352], which is the case when KCC2 is downregulated by seizures or ischemia[354].

Recent evidence suggests that KCC2 is regulated by calpain, the Ca^{2+} and BDNF-activated protease. Calpain cleaves KCC2 in its C-terminal region and causes the loss of a ~20-40kDa fragment[308,319], which contains multiple critical sites for KCC2 function and regulation[255,329], and thus causes a downregulation of both total and membrane-bound KCC2 and compromises its cotransporter activity[308,319]. Additionally, calpain activation, as well as the dephosphorylation of Ser940, has been suggested to regulate the lateral mobility of KCC2 within the plasma membrane[308]. Since the C-terminal region also contains the residue mediating KCC2 structural interaction to the cytoskeleton, calpain activation and cleavage of KCC2 may alter dendritic spine formation, and could potentially affect AMPA clustering in dendritic spines[303]; however, this remains to be substantiated.

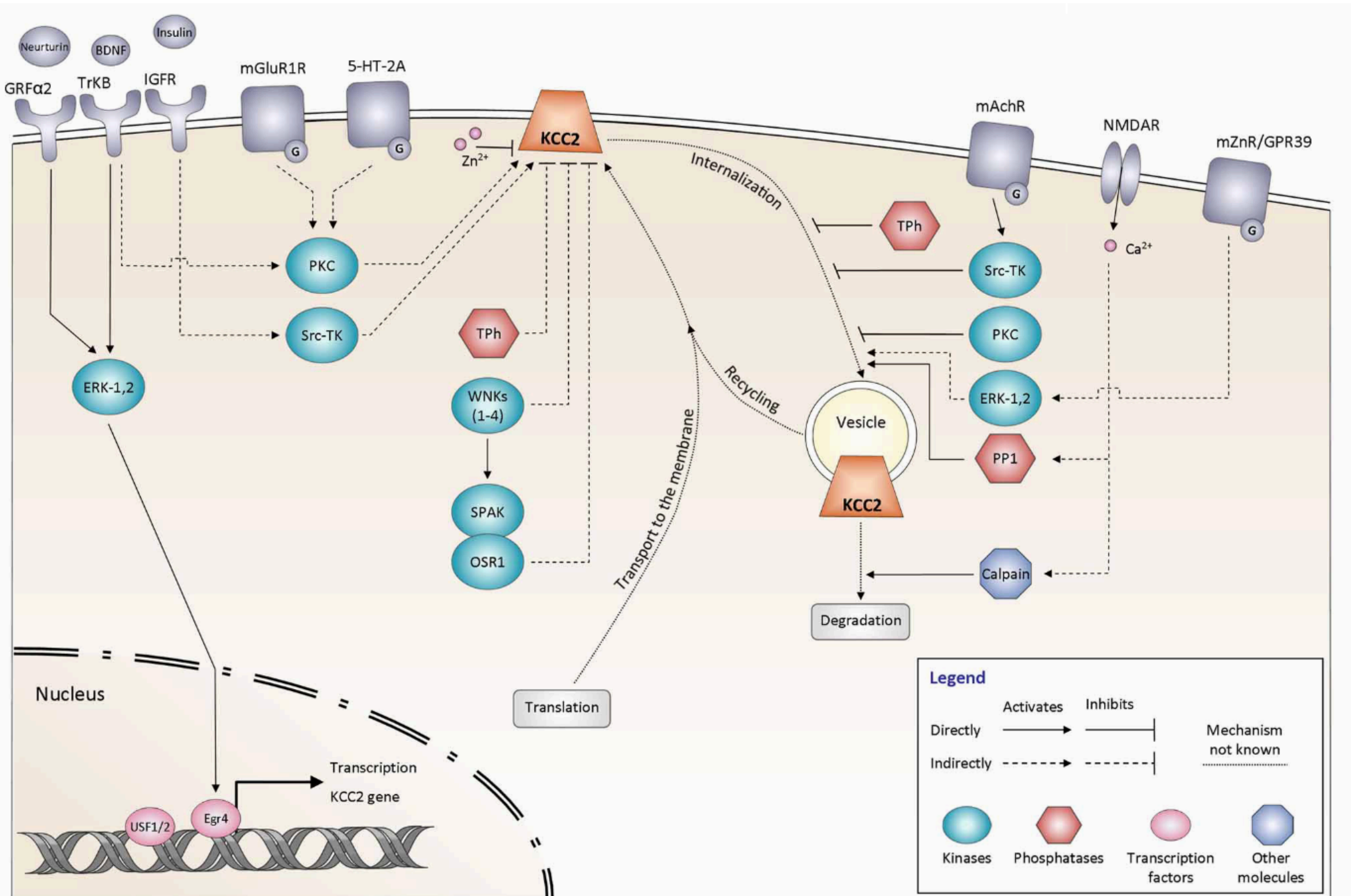
The regulatory action of BDNF is tightly related to the thyroid hormone metabolism during development[355]. Interestingly, thyroxine regulates KCC2 expression in a similar manner than BDNF, by either activating or blocking it[356,357]. However, this effect was insensitive to inhibiting BDNF signaling, suggesting that these two molecules regulate KCC2 expression through distinct pathways[252]. There is also evidence that steroid hormones regulate KCC2 expression, which has important implications for gender differences in the susceptibility to early-life seizures[273,358]. However, the specific mechanisms implicated in hormone regulation of KCC2 function require more detailed analysis.

Zinc-mediated control

Zinc has a bidirectional role in the regulation of KCC2. Firstly, intracellular Zn^{2+} rapidly blocks KCC2 activity[359], however, the mechanisms of its action is not yet identified. In contrast, extracellular Zn^{2+} released from mossy fibres terminals significantly activates KCC2 by increasing its insertion into the plasma membrane. Specifically, metabotropic zinc-sensing receptor (mZnR) is encoded by orphan Gq-coupled receptor (GPR39) and is coupled to PLC/ERK pathway (phospholipase C/extracellular-signal-regulated kinases). Zn^{2+}-mediated activation of KCC2 is abolished by silencing GPR39 or inhibiting PLC or ERK [360], suggesting that Zinc may be one of the factors involved in regulating KCC2 surface insertion. In addition, it was shown that SNARE proteins are essential for the increased activity of KCC2 after Zn^{2+} stimulation of mZnR/GPR39[361]. More detailed analysis of the regulation of Zinc and its control of KCC2 intrinsic activity is necessary. In the following page, figure 14 illustrates the known signaling mechanisms involved in KCC2 regulation.

Figure 14. Signaling pathways involved in KCC2 regulation, surface expression, endocytosis and membrane trafficking.

(Next page) Illustration adapted from Medina et al. (2014).



Neurturin
BDNF
Insulin
GRFα2
TrKB
IGFR
mGluR1R
5-HT-2A
G
Zn^{2+}
KCC2
PKC
Src-TK
ERK-1,2
TPh
WNKs (1-4)
SPAK
OSR1
Internalization
Recycling
Transport to the membrane
Translation
mAchR
NMDAR
mZnR/GPR39
Ca^{2+}
Vesicle
PP1
Calpain
Degradation
Nucleus
Transcription
KCC2 gene
USF1/2
Egr4
Legend
Activates
Inhibits
Directly
Indirectly
Mechanism not known
Kinases
Phosphatases
Transcription factors
Other molecules

2.3.3. Activity-dependent regulation

Whether physiological patterns of neuronal activity control developmental upregulation of KCC2 expression and its cotransporter activity remains controversial. There are a few studies that demonstrate that a prolong blockade of GABAergic[362] or glycinergic[363] inputs in developing neuronal networks blocks the developmental upregulation of functional KCC2. In contrast, other studies have not found any changes in KCC2 functional expression after chronic inhibition of neuronal spiking (application of TTX which blocks sodium channels), or GABAergic neurotransmission (application of $GABA_A$ blocker picrotoxin) or glutamatergic neurotransmission (NMDAR and AMPAR inhibitors)[311,364]. Similarly, upregulation of KCC2 is unaffected in vesicular inhibitory amino acid transporter (VIAAT)-knockout mice, despite the widespread absence of GABAergic synaptic transmission[365]. It is possible, however, that activity-dependent changes contribute to fine adjustments of post-translational regulation, such as phosphorylation, which activates KCC2 function[252]. In fact, neuronal activity controls BDNF release from both pre- and postsynaptic terminals[366], and as seen previously, BDNF controls the developmental expression of KCC2, which suggests that BDNF can contribute to activity-dependent modulation of KCC2 in a context-dependent manner[252].

2.4. KCC2 modulation in disease

Regulation of GABA driving force in different pathological conditions has recently come under scrutiny. More specifically, recent studies have looked at alterations of KCC2 expression and function in epilepsy, neuropathic pain, autism, Down syndrome and motor spasticity. My project focuses on alterations of KCC2 in epilepsy, however, I will first briefly discuss the main hypothesis on the role of KCC2 alterations on these neurological disorders.

2.4.1 KCC2 alterations in different neurological disorders

Neuropathic pain

KCC2 has been greatly associated with neuropathic pain. In these conditions, nociceptive transmission drives through spinal nociceptive pathways, where peripheral neurons in the dorsal root ganglia contact neurons in the spinal dorsal horn, which then projects sensory information to the thalamus. In the spinal dorsal horn, the pain pathway is regulated by a network of local inhibitory INs, which discriminate nociceptive sensory pathways from non-nociceptive sensory pathways by releasing GABA or glycine (Gly). Tactile allodynia is a clinical condition, and a classical symptom of neuropathic pain, where a harmless stimulus is perceived as painful, which underlines a dysfunction of nociceptive channel threshold[196,367]. In fact, blocking GABAergic input (through spinal administration of $GABA_AR$ and GlyR antagonists) can cause this sensitivity to sensorial stimuli[368]. One of the mechanisms promoting spinal disinhibition, and underlying neuropathic pain symptoms, is an alteration of the Cl^- homeostasis in the superficial spinal dorsal horn mediated by microglia-released BDNF. Microglia-dependent synthesis and release of BDNF is dependent on the upregulation of the purinergic receptors P2X4Rs, which are typically expressed at negligible levels in resting microglia[369]. Chloride homeostasis alterations is brought upon microglial activation through multiple extracellular signals (which are still controversial[367]) associated with neuropathic pain, and a subsequent upregulation of P2X4Rs in microglia, release of BDNF, and activation of TrkB receptors in neurons of the superficial dorsal horn[370] (see figure 15). BDNF-TrkB activation then alters KCC2 function and thus chloride homeostasis[339]. As such, blocking this mechanism at any level rescues spinal inhibitory transmission, and the allodynia in neuropathic animals[339,371]. This mechanism of spinal disinhibition mediated by microglia to neuron signals, through P2X4Rs-BDNF-TrkB-KCC2 cascade, was also found following spinal cord injury[372], and in morphine-induced hyperalgesia (pain hypersensitivity) where spinal microglia activation is dependent of opioid receptors in microglia[371]. Recently, the use of adenosine agonists (A_3AR) has been shown to reverse neuropathic pain, through modulation of GABAergic neurotransmission by enhancing KCC2 activity[373]. Interestingly, anticonvulsant drugs also offer successful treatment for neuropathic pain, and suggests that

KCC2 regulation through BDNF-TrkB, and calpain are common causes of epilepsy and neuropathic pain[367,374-376].

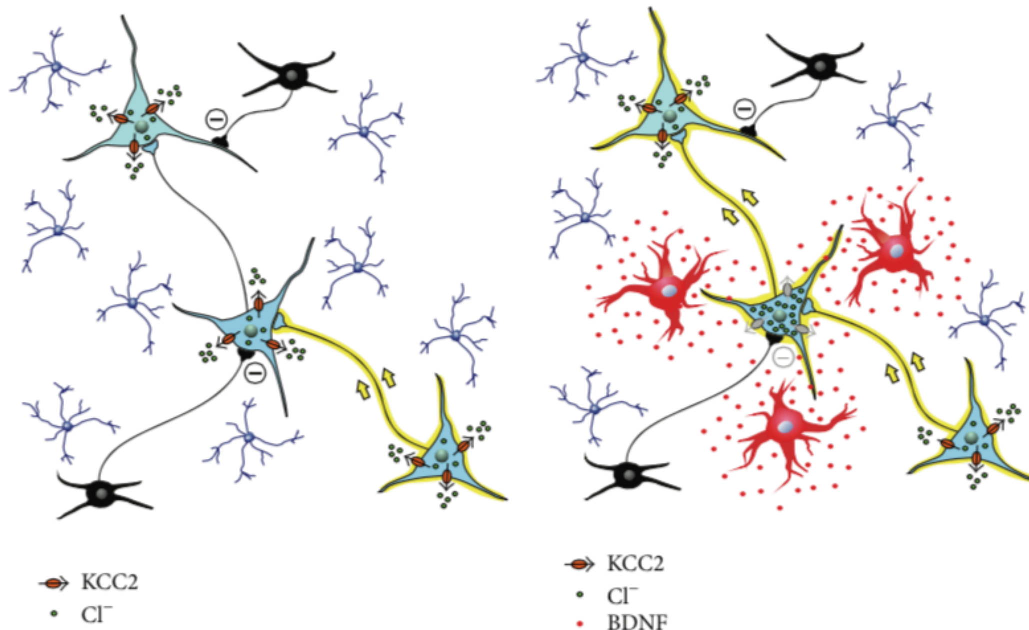


Figure 15. Microglia can regulate neuronal network excitability through BDNF-dependent release.

Illustration adapted from Ferrini and De Koninck (2013)[367] demonstrating a mature CNS network in normal conditions (left), where microglia is typically in the resting state (blue cells), and INs release GABA or Gly and KCC2 is actively extruding chloride. The illustration on the right represents the same network after an external incident activates microglia (red cells), which then activates the BDNF-TrkB-KCC2 cascade that results in spinal disinhibition.

Autism

Autism is a neurodevelopmental disorder characterized by impairment in communication and social interactions. There are multiple factors causing autism and they regroup both genetic and environmental vulnerabilities. Oxytocin is a hormone that is important for communication, and modulation of this hormone can induce labor[377]. Tyzio and colleagues (2014) focused on two animal models of autism, the first being mice that carry the fragile X mutation, and the second a valproate treatment *in utero*, to specifically understand the cellular and network alterations of GABAergic signaling that occur during delivery[237]. As

explained previously, $GABA_AR$ driving force is high in the fetal brain (E20-21), and reduced to adult levels at P15-20, due to the upregulation of KCC2. However during labor in rodents, there is an abrupt oxytocin-mediated decrease of the intracellular chloride levels, wherein $GABA_AR$ driving force is reduced, establishing inhibitory signaling, which exerts a neuroprotective and analgesic effect on newborns[237,378,379]. They reveal that this labor-specific switch is abolished in hippocampal CA3 PCs in both autism models, with GABAergic 'excitatory' signaling even at P30, a downregulation of KCC2 expression and a shift from plasma membrane to cytoplasmic KCC2, and finally an enhancement of glutamatergic activity. Alternatively, bumetanide (NKCC1 antagonist) treatment to the mother restores the GABAergic developmental switch to inhibition at P0 in both conditions, and rescues the autistic-like phenotypes in the offsprings. In addition, blocking oxytocin signaling in naïve mothers causes the offsprings to display autistic-like phenotypes and reproduced the electrophysiological alterations observed in the autistic mouse models. Taken together, these results identify a deficiency in chloride regulation and GABAergic-mediated inhibition in both animal models of autism, and highlight the importance of oxytocin-mediated GABAergic inhibition during labor.

Down Syndrome

Down syndrome (DS) is the most frequent genetic cause of intellectual disability. Patients with DS have hippocampus-related learning and memory deficits and a low IQ. The best-characterized animal of trisomic DS is the Ts65Dn mouse, which carries an extra copy of the distal segment of mouse chromosome 16 (syntenic to the long arm of human chromosome 21)[380,381]. This model replicates the hippocampus-dependent learning and memory impairments[381,382], with impaired synaptic plasticity, such as LTP[383]. In some studies, an increase in GABAergic INs in the forebrain is observed[384,385], and this increase is believed to increase in inhibition in DS mice, which could in turn affect synaptic plasticity and cognition. In fact, treatment with $GABA_AR$ antagonists rescues the LTP and cognitive impairments. However, other studies have not reported an increase in $GABA_AR$-mediated inhibition in DS mice[386,387]; although, neither of these studies measured the functional activity of GABAergic

signaling. Deidda and colleagues (2015) recently reported that $GABA_AR$ signaling is excitatory in adult Ts65Dn mice, which induced a depolarizing shift of the reversal potential of $GABA_AR$-driven Cl^- currents (E_{Cl}), and an increase of NKCC1 surface expression in the hippocampus of DS mice, and of specimen from DS patients. Treating Ts65Dn mice with bumetanide, an NKCC1 inhibitor, rescued the shift of E_{Cl}, the LTP impairment as well as hippocampus-mediated memory deficits. In this case, how is inhibition sustained in DS mice? They observed that $GABA_BR$ inhibition was increased, suggesting this could compensate the $GABA_AR$-mediated excitation. Additionally, it is possible that specific cell compartments allow local shunting inhibition[388]. Altogether, this is another instance where the chloride gradient can have a major influence on neuronal network activity.

Motor spasticity after spinal cord injury

Spasticity is a severe complication following spinal cord injury. It is characterized by velocity-dependent increase in muscle tone, which causes hyperexcitable stretch reflexes, spasms and hypersensitivity to sensory stimulations. Approximately 75% of patients will develop muscle spasticity one year after the spinal cord injury, but only half receive medication for these symptoms[389]. Furthermore, the drugs commercially available only partially relieve the symptoms.

It is believed that these symptoms are due to increased motoneuron excitability[390]. Additionally, inhibition is reduced below the lesion in spinal reflex pathways, which is observed as a decrease of presynaptic and recurrent inhibition[391]. This reduction of inhibition increases synaptic inputs in response to muscle stretch. A recent study showed that KCC2 is downregulated particularly in the membrane of motoneurons in the ventral horn following spinal cord injury in rats, thereby depolarizing E_{GABA}, and reducing the strength of postsynaptic inhibition. This mechanism underlies the disinhibition and electrophysiological correlates of spasticity. The downregulation of KCC2 after spinal cord injury is prevented by BDNF sequestering at the time of the injury[236].

2.4.2. KCC2 in epilepsy

Epilepsy is the second most common neurological disorder, with a prevalence of approximately 1% worldwide[392]. Altering GABAergic neurotransmission in experimental conditions can lead to seizure generation *in vivo* and epileptiform activity *in vitro*. In fact, ictal discharges (during seizures) are associated with intense interneuron firing and $GABA_A$ receptor ($GABA_AR$) activation[393]. However, the hypothesis that epilepsy derives from an imbalance between excitation and inhibition has recently been seriously questioned, as it does not highlight the complexity and variety of circuit alterations that occur in the epileptic brain. This notion will be described more in detail in the following section. Nevertheless, GABAergic transmission has been shown to be greatly modified in multiple models of epilepsy, and considering the key role of KCC2 and NKCC1 in controlling the efficacy of inhibition, understanding alterations of their expression and function is an important aspect in understanding the subsequent circuit based alterations. In the following section, I will discuss modifications of KCC2 in adult epilepsy and neonatal seizures.

Epilepsy in the adult brain

Human studies

Pathological activity has been studied in brain tissue obtained from surgical removal of epileptogenic zones from patients with phamarcoresistant epilepsy, and KCC2 deficits were observed. In fact, Huberfeld and colleagues analyzed tissue slices from patients with mesial temporal lobe epilepsy (MTLE) associated with hippocampal sclerosis, and found that human tissue slices generated spontaneous interictal-like discharges (between ‘ictal’ or between seizures) that were initiated in the subiculum. PCs from the subiculum displayed depolarized $GABA_AR$-mediated postsynaptic events, while ~30% of PCs were devoid of KCC2 mRNA. More specifically, the cells that were devoid of KCC2 were depolarized, while the other KCC2-positive cells were hyperpolarized during interictal events. Finally, they observed that bumetanide treatment to block NKCC1 produced a hyperpolarizing shift of the E_{GABA} and supressed interictal discharges[232,394]. Furthermore, in human resected tissue, they also evaluated that KCC2 was downregulated in non-dysplatic neurons (non-giant neurons) that did

not migrate properly in patients with focal cortical dysplasia[395]. However, in one particular study, it was shown that membranes taken from human peritumoral epileptic cortex that were injected in Xenopus oocytes saw a stricking increase in NKCC1 expression, and a more modest upregulation of KCC2, however E_{GABA} was depolarized[396], suggesting that KCC2 alterations may be context-dependent.

Animal studies

Numerous studies in rodents have also demonstrated that in mature cortical neurons (and spinal cord neurons) KCC2 is downregulated in response to seizure. It was observed in multiple models of epilepsy, such as models of temporal lobe epilepsy[231,397], focal cortical dysplasia[395,398], hypoxia-ischemia[399]. In particular, both *in vivo* and *in vitro* experiments display an aberrant shift towards increased NKCC1 and decreased KCC2 expression and the re-emergence of depolarizing $GABA_A$ergic signaling, which may underlie certain epileptic discharges in the adult brain[231,232,400]. Therefore, it is likely that activity-dependent mechanisms lead to KCC2 downregulation in pathological conditions.

In fact, genetic impairment targeting KCC2 in rodents caused an increase in seizure susceptibility[283,284], and typically affected the cotransporter activity and interaction with the cytoskeleton. A particular study demonstrated that status epilepticus induced by the administration of pilocarpine led to a progressive downregulation of KCC2, which decreased the efficacy of inhibition and thus abolished the function of the dentate gyrus as a hippocampal barrier against seizure originated from the entorhinal cortex[401].

The exact mechanisms leading to a downregulation of KCC2 are not completely understood, however there is evidence that they involve the activation of NMDARs and TrkB, calpain and potentially PP1 (protein phosphatase 1;[230,319]), as explained in the previous section. The TrkB has been shown to specifically activate PLCγ1 during epileptogenesis[402]. Additionally, there is evidence that KCC2 is specifically downregulated in models of seizures where there is the highest increase in BDNF and TrkB expression[230].

Furthermore, Silayeva and colleagues (2015), demonstrate directly that status epilepticus (SE; state of continuous seizures, lethal and leads to long-term neurological deficits in

survivors) inactivates KCC2. In particular, kainate-induced SE rapidly dephosphorylates KCC2 at the Ser940 site, and genetically ablating Ser940 phosphorylation accelerated the development and lethality of SE. This study suggests that deficits in KCC2 activity directly contribute to the pathophysiology of SE[403]. Furthermore, in a pilocarpine model of temporal lobe epilepsy, it was found that muscarinic acetylcholine receptors mAChRs activation was prolonged, and induced an increase of tyrosine phosphorylation of KCC2 and its subsequent degradation, thus reducing KCC2 activity[204]. However, some animals did not develop SE nor exhibited tyrosine phorsphorylation and internalization of KCC2 following pilocarpine injection, suggesting that there are other cellular mechanisms present to regulate tyrosine phosphorylation and subsequent degradation of KCC2 upon muscarinic activation[203,204].

In a clinical perspective, agents that control chloride gradient and reinstate inhibitory actions of GABA may thus open novel therapeutic approaches in these adult neurological conditions. Interestingly, a KCC2-selective analog (CLP257) was recently found, and this compound restores impaired Cl^- transport in neurons with dysfunctional KCC2 activity and rescued KCC2 surface expression, thus offering chloride enhancer as a novel therapy for neurological diseases[404]. However, in the neonatal brain the situation is different, and we will see how in the next section.

Epilepsy in the neonatal brain

Depolarizing GABA during early development was found accountable for the reduced seizure threshold and increased seizure propensity in the neonatal brain, and thus explaining why the use of established GABAergic anticonvulsants (such as phenobarbital and benzodiazepine) were not very successful. In addition, inhibiting NKCC1 with bumetanide treatment *in vitro* and *in vivo* in animal models of neonatal seizures was shown to inhibit epileptic activity, and made it a candidate for anticonvulsant therapy[224,300]. The scientific and medical communities were hoping to treat refractory neonatal seizures by blocking NKCC1 (with bumetanide) and thus restore the inhibitory balance and improve seizure susceptibility. In fact, this was the basis of two clinical trials (NCT01434225; NCT00380531). Unfortunately, one of the clinical trials (NCT01434225) was recently terminated, and they

concluded that treatment of NKCC1 antagonist to complement with phenobarbital was not a viable option to treat neonatal seizures. These clinical studies have not produced positive results, suggesting that the situation may be more complex[405].

Indeed, epilepsy in the developing brain is different than in adult epilepsy. The developing immature brain possesses different physiological properties, including neuronal ionic currents that typically last longer and are less selective. This allows immature neurons a higher probability of spiking activity and allows them to connect and fire together at the same time, and readily synchronize, thus making the developing brain more susceptible to seizures[406,407]. Interestingly, KCC2 is an important factor that underlies this susceptibility to seizure generation and facilitation.

Human studies

Until last year, no mutations of the *Slc12A5* gene in patients had been reported, even though it was cloned two decades ago[240]. The first SLC12A5 point variant (KCC2-R952H) reported is a missence mutation identified in an Australian family with early onset febrile seizures. When introduced in neural stem cells or cortical $KCC2^{-/-}$ neurons *in vitro*, and by in utero electroporation *in vivo*, this variant caused cotransporter and spinogenesis deficits caused by an internalisation of KCC2 in rodent neurons, and strongly suggests that it is a susceptibility variant for febrile seizures[307]. The second study identified a new point variant, KCC2-R1049C, as well as KCC2-R952H, in a French-Canadian cohort with idiopathic generalized epilepsy. They found that these mutations alter its transporter function, trafficking and S940 phosphorylation regulation, and their predicted pathogenicity suggested that they are disease causing. Altogether, the authors reported that these mutations were a risk factor or contributed to the pathogenesis of human idiopathic generalized epilepsy. These data strengthen the genetic link between KCC2 and human epilepsy, and offer two alternative hypotheses where either the impairment of $GABA_AR$-mediated inhibition enhances seizure susceptibility and/or the reduction of functional spines leads to a desynchronization of neuronal circuits that are seizure-promoting[196,392,408]. Finally, these two independent studies provide evidence that KCC2 dysfunction can predispose to epilepsy in humans.

A study published in 2010 was the first evidence that pediatric epilepsy differentially affects KCC2 expression. Analyzing human neocortex specimen of pediatric patients with focal cortical dysplasia (FCD), a form of intractable focal epilepsy, and age-matched controls demonstrated that KCC2 expression increases markedly with age in normal conditions, but in FCD tissue from patients older than 1 year of age had a reduction of KCC2 expression, whereas FCD patients younger than 1 year old had an increase in KCC2[409]. These results suggest that there is a time-sensitive modulation of KCC2 in epilepsy.

Animal studies

The first evidence of the dual effect on KCC2 expression and function comes from two studies by Galanopoulou, where three episodes of status epilepticus were induced by kainic acid treatment at P4-P6 rats. She observed that KCC2 mRNA expression was upregulated in the CA3 region of the neonatal hippocampus. In addition, CA1 pyramidal neurons displayed a hyperpolarizing shift of E_{GABA}, associated with an increased or decreased immunoreactivity for KCC2 and NKCC1, respectively[410,411]. Furthermore, Khirug and colleagues (2010) provided another evidence of the differential expression of KCC2 due to a neonatal seizure[412]. The authors found that a single neonatale seizure induced by kainite injections between P5 and P7 leads to a precocious hyperpolarization of E_{GABA}. While, they did not see a difference in the total amount of KCC2, they found an increase in the membrane surface expression of KCC2 and Na^+-K^+-ATPase α2, 1h after seizure onset. They found similar results when exposing hippocampal slices to kainite, and interestingly, effects on KCC2 surface expression and E_{GABA} were blocked by the presence of the kinase inhibitor K252a. In cases of early-life seizure, it was suggested that KCC2 upregulation could be the brain trying to counteract the seizure-induced increase of intracellular Cl-, which leads to an increase in hippocampal hyperexcitability. However, this hypothesis remains to be proven.

A recent study found that a single episode of kainate-induced neonatal seizure triggers a TrkB-dependent upregulation of neuronal Cl- extrusion by an increase in surface expression of KCC2, and can be temporally limited by calpain. However, this effect is transient and calpain-mediated downregulation of KCC2 alters its function to extrude Cl^-. Additionally, in $BDNF^{-/-}$

mice, this KCC2 upregulation is impaired, through a significant reduction of surface expression, suggesting that the fast and functional activation of KCC2 following neonatal SE is BDNF-dependent. These results suggest that the seizure-induced release of BDNF in the early postnatal brain enhances $GABA_AR$-mediated inhibition and hyperpolarizing E_{GABA}. Interestingly however, in these BDNF-deficient mice (with no seizures), the normal developmental increase of KCC2 in not altered, suggesting that although BDNF can induce KCC2 upregulation, this mechanism is not BDNF-dependent[234]. In fact, it was shown that other trophic factors act in parallel, and even in a redundant manner to activate transcriptional pathways downstream of BDNF that regulate KCC2 expression[413]; suggesting that in $BDNF^{-/-}$ mice, other trophic factors may compensate this deficit, and induce the developmental increase of KCC2 expression.

Conversely, it was shown that in an animal model of ischemia in the immature brain there was a downregulation of KCC2 acutely post-ischemia at P7, P10 or P12, and recovers from ischemic insult within a few days. These results suggest that ischemic injury significantly modulates the developmental profile of KCC2 and dictates the efficacy of anticonvulsant medications that follow[414]. In addition, it was reported that KCC2 was downregulated in the intact neonatal hippocampal preparation when seizure was induced with a sustained treatment of kainate[415]. Lastly, a recent study demonstrated a calpain-mediated cleavage of hippocampal KCC2 in a rat model of late gestation hypoxia-ischemia[416]. The fact that KCC2 upregulation is transient following a single neonatal seizure could potentially explain these differences[234].

In summary, it is evident that KCC2 is crucial in normal brain development, and the effects of its dysfunction in pathological conditions seem to be age- and context-dependent. The time at which an initial insult occurs, differentially affects KCC2 expression and function. In addition, numerous signalling pathways could rapidly modulate the activity of this transporter. However, the mechanism distinguishing the effects of KCC2 in adult brain versus developing brain are still under investigation. I am more specifically interested in the modulation of KCC2 in a model of temporal lobe epilepsy, where neonatal rats are subjected to cortical dysplasia and atypical febrile seizures. In the following section, I will discuss these subtypes of epilepsy and the main mechanisms that have been elucidated.

3. Epilepsy

3.1. Definition

Epilepsy is the second most common neurological disorder characterized by spontaneous recurrent seizures and affects 65 million people worldwide. Seizure incidence is highest in the first month of life[417], and can trigger epilepsy and cognitive disorders in later life[418]. Epileptogenesis can occur due to genetic mutations or following an initial insult, which causes aberrant molecular, cellular and neuronal network activity modifications. The pharmacological treatments currently available are only seizure suppressing or antiepileptic, but they do not prevent the development of epilepsy. These drugs also lack a temporal, regional and cell-type specificity, therefore often causing side effects such as nausea, tremor, fatigue, low blood counts, abnormal liver function, cognitive impairment, bone loss, mood changes and teratogenic effects[419]. Additionally, not all patients respond to the available drugs; in fact a third of epilepsy patients develop drug-refractory epilepsy, meaning they are resistant to treatments[420]. In some cases, patients can undergo surgical removal of the epileptic region in the brain, which however, can also lead to negative side effects[421]. Further, this option does not always offer a long-term recovery from generalized seizures[422]. In addition, there are patients where even a surgical approach is not possible because the location of the seizure focus is such that removing it would lead to devastating neurological and cognitive consequences[423]. It is therefore very important to elucidate the mechanisms involved in epileptogenesis to develop more targeted treatments, which will hopefully benefit more patients.

Studies of animal models of epilepsy and human tissue aimed at understanding the process of epileptogenesis following an initial insult demonstrate that there are multiple alterations that span from acute changes within minutes and days to chronic changes over weeks and months (see figure 16). The first changes include alterations of membrane depolarization and consequently ion channel kinetics, post-translational modifications and activation of immediate early genes. These initial changes then lead to neuronal death, activation of inflammatory cascades to finally, mossy fiber sprouting, network reorganization,

neurogenesis and gliosis. These processes are developmentally regulated, which could potentially explain the differences in epileptogenesis between adult and developing brains[424].

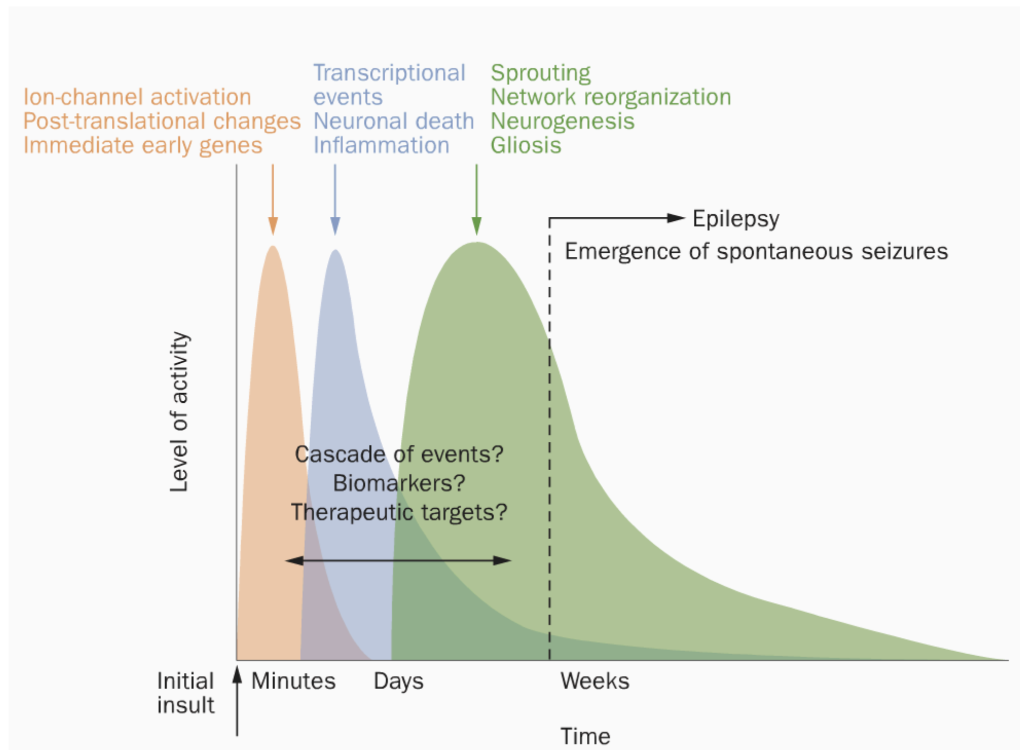


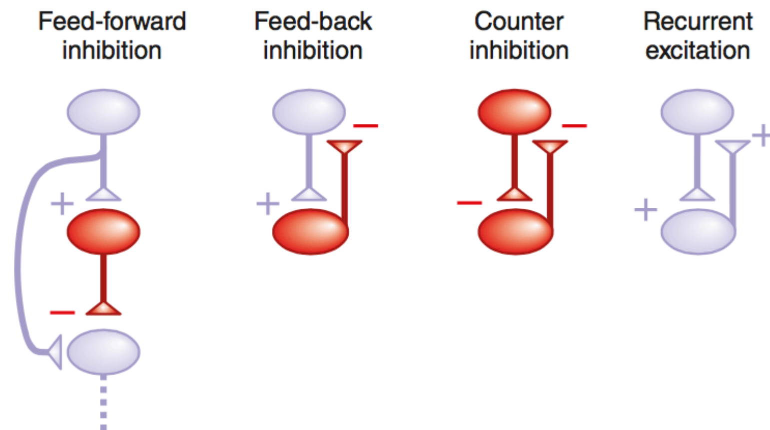
Figure 16. Illustration depicting the timing of key alterations during epileptogenesis following an initial insult.
Illustration adapted from Rakhade and Jensen (2009) [424].

3.2. Mechanisms of ictogenesis and epileptogenesis

Epileptic convulsions are due to dysfunctional neuronal circuits subject to excessive and/or hyper-synchronous activity. In recent years, technological advances such as multi-site extracellular arrays, optogenetics and paired intracellular recordings are starting to enlighten how micro-scale networks are organized and their role in generating and modulating seizure activity. These recent discoveries are also challenging the well-established belief that epilepsy is an outcome of an excitation/inhibition imbalance[425]. In this section, I will describe the concepts regarding mechanisms of seizure generation, specifically termed ictogenesis, and mechanisms leading to epilepsy (epileptogenesis). First, all seizures seem to originate in local

microcircuits and then spread from that initial ictogenic zone. Second, seizures propagate through cerebral networks and engage critical microcircuits in distal nodes. This process can be diminished or blocked by suppressing activity in these nodes. The microcircuit motifs include feed-forward inhibition, feed-back inhibition, counter-inhibition and local recurrent excitatory circuits (see figure 17). Another important aspect is the outside influence to these local microcircuits, which include long-range excitatory or inhibitory connections. In fact, these microcircuit motifs are not isolated, but they are part of larger networks. However, dysfunction in these microcircuits (through epilepsy for example), which engages other local microcircuits, can then alter the overall dynamic of large-scale networks. I will next explain the concepts of these microcircuits more in details and how they are affected in epilepsy.

Figure 17. Representation of the different microcircuit motifs involved in epilepsy. Schematic adapted from Paz and Huguenard (2015) [425].



Recurrent excitation

Local recurrent excitatory circuits are the simplest and most clearly affected in epilepsy. Most experimental models of epilepsy demonstrate that there is an increase in excitatory activity leading to hyperexcitability. For example, in the DG of the hippocampus, intricate changes in connectivity were observed as inputs onto GCs were increased from other GCs but also from hilar excitatory neurons and CA3 PCs after kainate treatment[426], leading to hyperconnected neurons. Hyperconnected PCs were also observed in the neocortex following cortical malformations, such as focal cortical dysplasia[427].

Feed-forward inhibition

Feed-forward inhibition is a type of microcircuit motif that has also been broadly implicated in several epilepsies. Feed-forward inhibition occurs when excitatory inputs from remote brain regions recruit local inhibitory networks that tune/control the strength and form of the efferent signals (see figure 17). It commonly occurs in neocortical and hippocampal networks, where I will focus on, but it can also occur in basal ganglia and thalamic networks. More specifically, sensory signal from the thalamus projects onto sensory receptive zone in the cortex in layer IV mostly, in the form of glutamatergic excitation. In fact, a single thalamic fiber can contact and excite both excitatory (PCs) and inhibitory (FS-BCs) cortical neurons in layer IV in a di-synaptic circuit. Therefore, while the PCs amplify and process the signals and propagate it to other PCs in other layers in their cortical column, FS-BCs also fire and release GABA onto excitatory cells in layer IV. This causes feed-forward inhibition, which sets a brief window for temporal synaptic integration, which in turn determines temporal precision in response to stimulation [428] and limits the overexcitation of the neocortex[429]. Feed-forward inhibition was found to rely on FS-BCs primarily, and more so than SOM-expressing INs [430]. In addition, FS-BCs receive convergent excitatory inputs from multiple thalamocortical cells. Furthermore, considering individual FS-BCs can output to numerous PCs and spiny stellate neurons in layer IV, they can powerfully suppress their output and shunt the thalamocortical-mediated excitation[431]. The same mechanism of feed-forward inhibition was observed in the dentate gyrus in the hippocampus, where entorhinal cortical excitatory project (perforant path) onto both FS-BC and granule cells, but their interaction is more dynamic. In fact, the perforant path preferentially targets FS-BC, and maybe other GABAergic interneurons in the DG, thus driving a large feed-forward inhibitory conductance in GCs[432]. However, it was also shown that FS-BCs specifically targets PCs with specific output projections in the CA1[111]. Altogether, this illustrates the underlying complexity of cortical large-scale networks, considering each of these particular interactions and microcircuits can have significant effects.

Consequently, disruption of feed-forward inhibition can lead to overexcitation of cortical networks and seizures. Incidentally, this was observed in multiple models of epilepsy, including those generated by cortical malformation like focal cortical dysplasia (which will be explained in details later)[433], generalized-absence epilepsy models (absence seizures cause the

patient to zone-out or stare into space)[434], and in *in vitro* models with chemoconvulsant-induced epileptiform activity [430,435]. A reduction of feed-forward inhibition is also consistent with the controversial hypothesis that BCs are 'dormant' in epilepsy. This hypothesis suggests that connectivity onto these cells would be reduced to a point, where they would fail to fulfill their role and regulate PCs in a timely-manner[436]. Consistently, loss of function of $Ca_V2.1$ calcium channels selectively in neocortical PV INs altered GABA release, impaired their ability to restrain cortical pyramidal cell excitability, and thus altering feed-forward inhibition, and was sufficient to cause generalized seizures [437]. Mutations of Scn1a gene, coding $Na_v1.1$ voltage-gated sodium channels essential for action potential generation and propagation of primarily GABAergic INs, underlie 80% of cases of Dravet Syndrome, a severe myoclonic epilepsy in infancy[438]; and 10% of generalized epilepsy with febrile seizure plus (GEFS+)[439]. In animal models of these disorders, PV- and SOM-expressing INs have reduced excitability, resulting in dis-inhibition of cortical networks [440]. Further, Scn1a knockout in PV-expressing INs increased seizure susceptibility, while seizure threshold was unaltered when Scn1a was specifically inactivated in excitatory cells[441]. It was also found that feed-forward inhibition is involved in the propagation of epileptiform activity in mouse neocortical slices[442-444]. Considering the importance of PV-expressing INs in feed-forward inhibition on functional local excitatory neurons, the loss of this process can cause strong dysfunction of circuits, and can bridge epileptic activity between microcircuits. It is proposed that rescuing functional alterations of inhibition by reinstating proper feed-forward inhibition could prevent seizures[425].

Feed-back inhibition

Feed-back inhibition occurs when locally activated inhibitory neurons shape recurrent excitatory activity. Inhibitory cells can mediate both feed-back and feedforward inhibition, but it seems that PV-INs play a significant role in feed-forward inhibition, while SOM INs appear to be more important for feed-back inhibition. Cortical Martinotti neurons (expressing somatostatin) target distal dendrites of PCs, and provide a relative weaker inhibitory strength at baseline compared to PV INs. However, they are recruited by simultaneous repetitive activity of multiple presynaptic PCs, through a di-synaptic inhibitory feed-back pathway

between PCs. More specifically, Martinotti cells receive facilitating excitatory connections from PCs (from layer II/II and layer V) and provide inhibitory GABAergic input onto the apical and basal dendrites of the same PCs in layer I (self-inhibition) and neighboring PCs, thus influencing their dendritic integration. In the hippocampus, CA1 PCs activated by high-frequency stimulation of neighboring PCs cause feed-back inhibition through O-LM interneuron activation[445]. Therefore, when there is an intense activation of local microcircuits during seizure, SOM INs can be recruited to dampen this activity to locally suppress the hyperactivity. Consistently, as mentioned previously, in the Dravet Syndrome, SOM INs are also affected and contribute to hyperexcitability of cortical circuits[440]. DLX1 deficient mice (Dlx1 is a transcription factor essential for GABAergic INs generation) display loss of SOM INs and calretinin-positive INs, which leads to reduction of inhibitory drive in the neocortex and hippocampus and generalized seizures[135]. Furthermore, ChCs can potentially also respond to hyperexcitability related to seizures, as a recent study suggests that ChC are not recruited by sensory stimulation (of whiskers), but by disinhibition following bicuculine (GABAR antagonist) application[446]. These results suggest that ChCs can be recruited by a significant increase in activity, which in the context of epilepsy can potentially prevent PCs action potential generation, and could act as a microcircuit 'emergency brake'[425].

Counter-inhibition

Lastly, counter-inhibition, which is essentially inhibition of inhibition, occurs as local connections between activated inhibitory neurons can decrease output of other inhibitory cells and induce disinhibition or altered oscillatory coupling. First, there are INs that can make synapses onto themselves, such as PV INs[447]. Counter-inhibition of PV INs can potentially suppress its feed-forward inhibition of local microcircuits, and promote seizure propagation between regions. Furthermore, there are other interneurons that only contact, chemically and/or electrically, other INs of the same or different class, as discussed in the first section. For example, VIP-expressing INs specifically suppress SOM- (preferentially) and PV-expressing INs, and thus mediate disinhibition [81]. In the context of epilepsy, activation of these INs can promote overexcitation through disinhibition. For example, if SOM INs are suppressed by VIP INs, it would prevent SOM INs-mediated feed-back inhibition. However, if

these INs are suppressed, this could prevent hyperexcitation.

It is important to bear in mind that these individual microcircuits are not isolated, and epilepsy results from propagation of ictal activity through all microcircuits. Paz and Huguenard (2015) [425] suggest that an imbalance between different microcircuits can be ictogenic. However, it is also possible that there is an accumulative effect, such that small disruptions of microcircuits can occur spontaneously, but not have a harmful effect; whereas after reaching a certain threshold, disruption of multiple microcircuits at the same time could lead to seizure development.

Finally, although a lot of studies on epilepsy started to reveal some key molecular mechanisms of ictogenesis and epileptogenesis, new studies have uncovered over 400 genes that were closely associated with epilepsy. Hopefully in the future, we will have a better idea of the molecular mechanisms inducing spontaneous recurrent seizures, as they are multi-faceted and likely combine genetic and acquired alterations. We are interested in a rodent model of mesial temporal lobe epilepsy, with two predisposing early-life insults, and thus I will focus on few key molecular mechanisms implicated specifically in mTLE, and these neonatal insults.

3.3. MTLE

3.3.1. Definition and Incidence

Mesial temporal lobe epilepsy (MTLE) is the most prevalent form of epilepsy, which accounts for a third of all cases, and is usually refractory to treatment[448]. Seizures originate from limbic structures such as the hippocampus, the parahippocampal gyrus and the amygdala. The hippocampus, located in the mesial temporal lobe, is particularly prone to generate seizures. This disease results in severe hippocampal sclerosis in approximately 50% of cases[449]. MTLE also leads to pyramidal cell loss in both CA regions, granule cell loss in dentate gyrus and gliosis as observed from epileptic tissues from surgical resections[450-455]. In addition, numerous GABAergic INs populations are affected, such as SOM- INs and neuropeptide Y-positive INs [456]. MTLE typically begins in teenagers and even adults, however

it is believed that the initial insult occurs in early life during neural development. In fact, numerous studies, including one with a cohort of ~4000 patients, demonstrate that complex febrile seizures is the most common pathology associated with MTLE [449,457-460]. The mean latency to develop MTLE after febrile seizures is between 8 to 11 years[459,461,462]. One possibility is that hippocampal sclerosis predisposes to prolonged febrile seizures and MTLE. A second possibility is that prolonged febrile seizures develop following an anatomical and/or genetic insult, and these seizures lead to hippocampal sclerosis and MTLE later in life [463]. In fact, a retrospective study from Dr. Carmant's group revealed that 66% of MTLE patients had a history of febrile seizures associated with focal cortical dysplasia, a cortical malformation [464]. Other studies provided evidence of dual pathology leading to MTLE. In fact, Magnetic resonance imaging (MRI) analysis found the presence of focal cortical dysplasia with febrile seizures[465,466]. Also, an MRI study of MTLE patients with or without a history of febrile seizures demonstrated that those with the history had a smaller hippocampus ipsilateral to the seizure focus, suggesting that there are at least two types of mesial temporal sclerosis originating from different pathogenetic pathways depending on the initial insult[466,467]. Indeed, other early childhood lesions observed in MTLE are birth trauma, head injury or meningitis. Moreover, both genders seem to be equally affected, and several cases of familial MTLE have been described[468,469]; however the prevalence of familial MTLE is uncertain.

3.3.2. Mechanisms

Alterations of inhibition have been found in patients with MTLE and animal models of MTLE. First, loss of GABAergic INs is reported in human tissue[461,470,471], as well as in experimental animal models[472,473]. In addition, $GABA_AR$-mediated inhibitory responses is increased in GCs[474], but decreased in CA1 PCs[475], revealing regional reorganization differences and selective alterations of $GABA_AR$ subtypes. Vesicular neurotransmission of GABA is also affected in MTLE[475]. In fact, despite dysfunction of GABAergic INs in the DG following TLE, the homeostatis activity of FS-BCs is maintained and limits overexcitation of the DG[476]. However, apart for the feed-forward inhibition circuit in the DG, there are direct projections from the entorhinal cortex to the CA1, through the temporoammonic pathway. In models of temporal lobe epilepsy (TLE) induced by pilocarpine or kainic acid injections, there

is neuronal loss in layer III of the entorhinal cortex, which is prevented by diazepam treatment[477,478]. Coincidently, neurons from layer III project to the CA1[479], and in animal models of TLE, there is a loss of this particular feed-forward inhibition[477]. In the pilocarpine model of TLE, CA1 O-LM interneurons[480], important for feed-back inhibition and feed-forward inhibition in the extra-hippocampal inputs from the entorhinal cortex[481], are lost. Consistent with the observation that mainly O-LM interneurons are reduced in CA1[481], dendritic inhibition on PCs is specifically decreased[482]. Taken together, these changes would lead to a reduction of feed-forward inhibition from the entorhinal cortex to CA1. In fact, it was observed in a model of MTLE that the cortical input onto the DG of the hippocampus was not affected, and the input onto CA3, via Schaffer collaterals, is decreased (despite reduction of Schaffer-evoked inhibition), while the normally weak direct excitatory cortical input onto the CA1, via the temporoammonic pathway, is increased ten-fold[483]. These alterations of the temporoammonic pathway are critically situated to facilitate seizure generation and/or propagation in the hippocampus. In parallel to the O-LM-mediated reduction of dendritic CA1-PC inhibition, there is an increase in spontaneous GABAergic inhibition in the soma of PCs, resulting from an increase of activity of somatic projecting INs, namely FS-BC, in MTLE[482]. Furthermore, recurrent excitation in the hippocampus between the GD, CA3 and CA1 is increased following seizures. Altogether, these data show that the hippocampus contains different pathways, whose alterations can be important for ictogenesis.

In summary, a better understanding of the pathological alterations of MTLE in the developing brain will help develop more targeted treatments, depending on the initial insult. As such, the Carmant laboratory has developed a dual-pathology rodent model of MTLE. This model consists of the association of a focal cortical dysplasia with atypical febrile seizures, since patients with these pathologies have a higher risk of developing MTLE as adults. To understand the importance of studying MTLE, and the impact that cortical dysplasia (CD) and febrile seizures (FS) have on the brain, I will focus on these topics in the following sections.

3.4. Febrile seizures

3.4.1. Definition and Incidence

Febrile seizures (FSs) are by definition an epileptic seizure that occurs in infancy or childhood associated with fever, but without evidence of a CNS infection or other afebrile seizures (NIH & ILAE definition, 1993). FSs are the most common convulsive events in children between 6 months and 5 years of age, with a peak incidence at approximately 18 months, and a prevalence of 2–14% depending on the population affected[484]. Febrile seizures can be divided into two subtypes: simple and complex FSs. Simple FSs are brief (<15min) and generalised convulsions, and occurs in isolation (only one seizure event within 24h). Simple FSs have not been associated with subsequent epilepsy or cognitive deficits in prospective and retrospective studies, and are considered benign[485,486]. On the other hand, complex FSs exhibit seizures that are atypical, prolonged (>15min) and focal or multiple (more than one convulsion in each episode of fever; or recurrent within 24h). These complex FSs account for 30-40% of FSs cases [487], and are a risk factor for MTLE [459,488,489]. Behavioral manifestations of FSs and electroencephalogram (EEG) recordings demonstrate that seizures originate from limbic structures. MRI signals were also altered in these structures, mainly in the hippocampus and the piriform cortex, and to a lesser extend in the amygdala, without cell death[490-492]. Finally, the gender differences in FSs are controversial. Most studies suggest that boys have a higher risk of developing FSs than girls[493,494], while the opposite has also been reported[495]. Other longitudinal studies concluded that there are no gender-based differences[485,496].

Generalized epilepsy with febrile seizures plus (GEFS+) is a clinical subset of familial FS. Patients with GEFS+ have FS beyond the age of 6, and can also have afebrile seizures, such as absences or myoclonic (brief muscle jerks) seizures. Numerous mutations have been associated with this disorder, and a classification system is starting to take form depending on the chromosomal location of these mutations. GEFS+ type 1 is associated with mutations of SCN1B, encoding the β1 subunit of the voltage-gated sodium channel NaV; GEFS+ Type 2 is associated with SCN1A encoding NaV1.1; and GEFS+ Type 7 has mutations in SCN9A encoding NaV1.7[497].

3.4.2. Potential causes and risks

FSs can be familial in some cases and sporadic in others, demonstrating that both genetic and environmental components contribute to their generation. I will discuss these two factors in this section.

It is commonly believed that the temperature rise of the fever per se is the main risk factor for FS development, and is more critical than the actual temperature reached; however there is no evidence available to date to support this hypothesis[498,499]. Seizures typically develop within the first day of fever; however, they do not necessarily occur at the peak temperature during the fever. Nevertheless, the average temperature during fever of children with FS (average of 39.8^{o}C) is usually higher than children with similar fever-illnesses[500]. However, measurements of fever reported in the literature differ due to the divergence of methodology used (axillary or rectal). In addition, any virus or bacteria that causes fever can provoke FSs. In particular, viral infections are regularly associated with FS[501,502], while the prevalence of bacterial infections is low, but can be severe[493,499].

A family history of FS increases the risk for developing FS, in fact 25-45% of cases have a positive family history. Siblings or monozygotic twins of children experiencing FSs are reported to have a higher risk of developing FSs[495,500,503]. However, whether family history is a predictor of the severity of FSs is controversial[504].

A clear factor for severity and recurrence of FSs is the age of onset. When children had their first convulsions between one and two years of age, 33% of them would go on to have recurrent seizures, suggesting that the earlier the age of onset, the more the likelihood of recurrence[485]. Although the severity of the FSs has a negative correlation with behaviour outcome, FSs alone is not associated with severe cognitive deficits [486,504].

In addition, FSs are more likely to develop in patients with a predisposition of genetic or perinatal insult nature, compared to the general population[505]. As such, high incidences of patients with initially focal or prolonged FSs reveal pre-existing brain abnormalities by MRI[465]. Additionally, since the family history of FS or epilepsy increases the likelihood of children developing FSs, a genetic influence on the development of FS has identified genetic loci by linkage analysis in families with FS[506,507]. I will discuss this in detail in the next

section. Furthermore, neuronal migration disorders have shown to increase susceptibility to FSs[508]. In fact, cortical dysplasia (CD) is a typical pre-existing structural lesion that affects the susceptibility to FSs [509]. In addition to predisposing to febrile seizures, there is a strong correlation (~80%) with CD and MTLE patients with hippocampal sclerosis as shown by MRI analysis[510], suggesting CD may be a potential predisposing factor for the development of both FSs and MTLE [510,511].

3.4.3. Long-term effects

Children with complex FSs have an eighfold-increased risk of developing epilepsy compared to children with simple FSs, as well as control children [487]. A 25-year follow-up study showed that while ~2% of children with simple FSs will develop spontaneous recurrent seizures (SRS), the overall risk rises to 7-49% when children with atypical FSs are included [512]. More specifically, it was reported that children with FSs with a single complex feature (focal, recurrent or prolonged) had a 6-8% chance to develop MTLE, while this risk increases to 17-22% for those with two complex features, and finally, the risk increase to 32-49% (depending on the cohorts) for children with FSs that have all three atypical features [512-514]. This risk is also associated with the number of generalized seizures the children suffered from, and their family history, as explained previously[512].

Febrile status epilepticus (FSE; seizures lasting >30min) occurs in approximately 10% of children with FSs [465,515], and these children have a greater risk of developing epilepsy. It has become increasingly proven that a significant proportion of these children develop acute hippocampal injury on MRI that progress over the years toward hippocampal sclerosis, the pathological/radiological hallmark of MTLE, and even mesial temporal sclerosis in some cases[457,458,492,516]. Another recent study indicates that 20-30% of children with complex FSs - of either type - have definite hippocampal volume loss in the year following FSs, and authors suggest that the number of recurrent FSs has a role in the pathogenesis of long-term hippocampal injury[517]. Another prospective study suggests that the duration of FSs is a key factor leading to hippocampal injury[466]. However, a study with a larger population, and a long-term evaluation of hippocampal injury is still necessary. In fact, a long-term and large

prospective study is currently in place to specifically understand the relationship between FSs, hippocampal/mesial temporal sclerosis and the development of MTLE. This study is named FEBSTAT and plans to understand the consequences of prolonged FSs in childhood. Preliminary results indicate that 25% of children experiencing prolonged FSs have definite MRI abnormalities[518] but only 12% show neuroradiological evidence of hippocampal injury, and a subgroup of children with FSE also appear to have subtle pre-existing hippocampal abnormalities[519]. While the relationship between FSE, subsequent hippocampal sclerosis and MTLE is still not fully understood, all these studies show a causal relationship between FSE and TLE, but not other forms of epilepsy. Unfortunately, it has been proven that no treatment, acute or continuous, can prevent the development of MTLE[520].

The long-term effects of FSs on cognitive development remain controversial. Several studies have demonstrated that FSs alone are not associated with severe cognitive deficits[485,486,504,521]. Furthermore, it was specifically shown that semantic memory was not impaired following FSs[522]. However, recent studies looking at particular memory processing and hippocampal-dependent recognition tests illustrate that there may be more subtle abnormalities than previously recognized[523-525]. Chang et al (2001) demonstrated that 6-year-old children with a history of FSs actually outperformed control children on several working memory tasks, but underperformed on a task of delayed recognition, another working memory measure. In addition, experiencing FSs before the age of 1 was the only significant risk factor for deficits in mnemonic function[525]. Children with complex FSs had more severe impairments on episodic memory[526] and delayed language development[527]. In addition, Roy et al (2011) showed that even a single FSE impacts visuomotor development, eye-hand coordination, and general cognitive development in infants from 3 to 21 months[528]. Moreover, Kipp and colleagues (2010) observed no difference of hippocampal volume on their group of 17 children with both simple and complex FSs, however they had functional changes in MTL memory tasks and processing. They conducted a recognition memory experiment, which can help dissociate between familiarity (a quick matching process that is hippocampus-independent) and recollection (slow recall-like process where detailed memories of a prior episode is retrieved, which is hippocampus-dependent). These tests revealed that recollection was deficient, and they hypothesised that children with FSs use familiarity processing to compensate for their degraded recollection[524]. Another study focused on children shortly after

prolonged FSs (~38 days), since they are the ones showing strong association with a hippocampal injury. They specifically investigated hippocampal-dependent memory, by visual paired comparison tasks, which is essentially like the novel-object recognition paradigm used to study rodent behaviour. This task was chosen because of the young age of the subjects (about 3 years old). Children were familiarised with two objects, and then with one familiar and one new object, immediately after or with a 5 min delay (same paradigm with faces). Children with prolonged FSs had recognition memory impairment following the 5min delay, and bigger deficits correlated with smaller hippocampal volumes. These impairments persisted a year later. The authors suggested that these children have a problem either in retaining the memory for 5 min or with retrieving the memory following the delay[523]. Finally, a recently published report from Tsai and colleagues (2015) used other tests that are more sensitive in detecting cognitive abnormalities and demonstrated that children with all complex aspects of FSs (prolonged, multiple or focal) have a lower full-scale intelligence quotient, and several cognitive impairments including perceptual reasoning and working memory deficits[529]. Altogether, these studies reveal persistent deficits in specific hippocampus-dependent memory tasks. Hopefully, the FEBSTAT study will address and conclude on whether FSE causes memory deficits in children with hippocampal injury[519].

3.4.4. Molecular alterations from human and animal studies

Genetic mutations

Recent genetic evidence suggests that FSs are genetically complex. There are reports of six susceptibility FS loci on several chromosomes, in particular 2q, with linkage to genes encoding sodium channel receptors, termed FEB1 to 6. In particular, mutations have been found in voltage-gated sodium channel α-1, α2 and β-1 subunit (*SCN1A, SCN2A, SCN1B;*[439]). However, the risk varies between families with similar conditions[530]. It has also been reported that FSs may be a hyperpolarization-activated cyclic nucleotide gated channels (HCN) channelopathy[514,531]. In addition, a mutation in the Slc12A5 gene, which encodes KCC2, has recently been reported in a particular Australian family with early childhood onset of FSs.

This mutation causes a missense variant in KCC2b (Arg952His;[307]). Mutations in the gene encoding $GABA_AR$ γ2 subunit have also been found in association with FSs[532]. Altogether, these findings suggest that the genetic aspect of FS is complex and likely multifactorial.

Temperature increase

The most accepted and stable experimental paradigm to induce FSs and increase the core body temperature is by exposure to heated dry air, which reproducibly generates generalized seizures in rodents[463,533,534]. The long-term effects of hyperthermia-induced seizures in rats are similar to those observed following FSs in humans, with a similar age-dependent manner. In fact, short FSs do not lead to the development of spontaneous recurrent seizures (SRS), whereas a third of rats with prolonged FSs have SRS in adulthood[533]. Although, there is no neuronal loss or mossy fiber sprouting, which are typically observed in models of MTLE, there is evidence of hippocampal excitability, with altered inhibitory drive and altered hippocampal circuits[535,536].

Essentially, the increase in temperature by hyperthermia induces seizures in all rodents, suggesting that genetic susceptibility is not necessarily required for induction of FSs [532]. However, it was reported that different mouse strains have different threshold of temperatures required to generate seizures [537], suggesting that genetic background may influence the susceptibility of developing FSs. Several genes have been implicated in the susceptibility to FSs in rodent models, such as genes encoding for sodium channels[439], $GABA_AR$[538] and interneukins[537], without discarding the possibility of interactions between these genes. The change in brain temperature can, in itself, influence neuronal functions, through temperature-sensitive ion channels for example[539], and consequently increasing the probability of generating general synchronized neuronal activity. Interestingly, a medication overdose that provokes hyperthermia, or hot baths, can induce seizures in young children[540], suggesting that the increase in brain temperature is sufficient to generate seizures. It was also recently shown that during hyperthermia-induced seizures, there is an over-regulation of body temperature, through dysfunction of the thermoregulation process in the preoptic area and anterior hypothalamus (PO/AH), which appears to contribute to seizure generation[541].

Inflammation

Nevertheless, fever also induces an inflammatory response, in addition to the rise in temperature, which leads to cytokine secretion in the brain as well as in periphery in humans and rodents[532,542]. Interleukin-1b contributes to fever generation and conversely, fever induces cytokine synthesis in the hippocampus[542]. In rodents, following hyperthermia, there is an increase in inflammatory response, such as a release of cytokines, including interleukin-1β (IL-1β;[533,537]). In fact, in interleukin-1 receptor type I knockout mice, the temperature threshold for FSs is higher [532,537]; conversely, when it was directly introduced in the immature brain, it triggered seizures. Overall, these results suggest that IL-1β may be implicated in the development of FSs. The signaling pathway downstream of interleukin-1 activates mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) signaling, which could lead to proconvulsive epileptic circuits[463,514,532]. Ultimately, it is believed that IL-1β can lead to FSs by reducing $GABA_A$R currents, or through enhancing calcium conductance through the NMDAR[543] thereby increasing neuronal excitability. In humans, there was an increased frequency of IL-1β in children with FS[544]. However, studies measuring IL-1β expression in children with FSs have given contradicting results[545], and this issue remains debatable. However, hyperthermia and fever activate several other inflammatory cytokines, such as interleukin-6, Interleukin 1-RA and the alpha tumor necrosis factor (TNF α) but results are conflicting.

Respiratory alkalosis

Another mechanism implicated in the development of FSs is the hyperventilation and respiratory alkalosis induced by hyperthermia. In rodent models of FS, it has been shown that this factor can influence seizures by increasing intracortical pH[546], and that alkalosis in the brain can lead to neuronal excitability. However, the clinical relevance of these findings remains controversial and lacks sustainable evidence [532,547]. In summary, there are a multitude of alterations in response to FSs, but the causative events remain unidentified.

3.5. Cortical dysplasia

3.5.1. Definition and subtypes

As seen in the first section, brain development undergoes very timely and organized stages to generate a functional laminar cortex. Disruptions during these processes can lead to malformations of cortical development (MCD), commonly associated with intractable epilepsy, cognitive impairment and motor and sensory deficits[548]. Focal cortical dysplasias (FCDs) were first reported in phamaco-resistant patients who underwent resection surgery. Examination of the resected brain tissue lead to the discovery of histopathologies, such as cortical disorganization and large 'bizarre' neurons, eventually termed dysmorphic neurons, and balloon cells (malformed cells with potentially multiple nuclei) in half of these initial cases[549]. FCDs represent a subgroup of MCD that are characterized by a spectrum of pathological changes of cortical development and are thought to underlie pharmacoresistant focal epilepsy. FCDs are specifically characterized by abnormal cortical lamination (dyslamination), defects of neuronal migration, growth and differentiation leading to cytoarchitectural lesions and abnormalities of white matter, which can either be restrained to one discrete cortical area, or can involve several lobes and even the entire hemisphere[464,511,550]. In fact, FCD has been found to be the most common etiology in pediatric epilepsy surgery patients [551]. In 2011, the International League Against Epilepsy (ILAE) classified FCDs into three types based on their histopathological and neuroradiological features[550]. FCD type I refers to isolated lesions, where the microscopic dyslamination of the neocortex (misaligned or disorganized layers) is either radial (Type Ia) or tangential (Type Ib), and is present in one or multiple lobes. FCD type II also refers to isolated lesions categorized by cortical dyslamination and abnormal cell development and maturation (dysmorphic neurons), without balloon cells (Type IIa) or with balloon cells (Type IIb). Finally, FCD type III occurs in association with hippocampal sclerosis (Type IIIa), or with epilepsy-associated tumours (EATs; Type IIIb), next to vascular malformations (Type IIIc), or in association with epileptogenic lesions acquired in early life (i.e. traumatic brain injury, encephalitis, etc.; Type IIId). This classification is still under discussion, in particular regarding the addition of a subtype that was previously thought more rare, where there is a combination of FCD type II

and hippocampal sclerosis or with EATs [552]. Future studies are required to better understand the etiology and pathogenesis of each of these FCD subtypes.

3.5.2. Risks and long-term effects

Technical advances in neuroimaging can now detect more subtle cortical abnormalities as potential epileptogenic foci before the resection surgery, and will hopefully allow more successful presurgical screening [550,552]. These abnormalities have been associated with both genetic and acquired factors, however the association between these factors and the mechanisms leading to epilepsy are still under investigation [548]. These factors may directly affect hippocampal development and its connections, which could increase the susceptibility to seizures. In fact, retrospective studies as well as a study of two families afflicted with FSs indicate that pre-existing malformations of the hippocampus could predispose to FSs and epileptogenesis[464,465]. Alternatively, these genetic and environmental factors may affect the susceptibility to seizures indirectly, by enhancing the susceptibility of hippocampal neurons to seizure-induced neuronal loss. As such, hyperthermia caused significant cell loss in rats with a predisposed, but experimentally acquired, neocortical migration disorders[508].

Individuals affected with FCD display a wide range of symptoms that is dependent on the extent and region of the brain affected [553]. However, epilepsy is the main symptom, it usually begins in childhood and is typically refractory to phamarcological treatment [554]. Other symptoms include maladaptive behaviour and disorders, psychomotor retardation and learning disability[555]. Lastly, FCD patients can also suffer of homonymous hemianopsia (loss of half the field of view on the same side in both eyes;[556,557]) and have increased risk of developing sleep-related epilepsy.

Incidence reports are sparse and since clear subtypes and terminology were only recently modified, the prevalence of each subtype of FCD remains to be evaluated. However, there are studies illustrating the differences between groups. Analysis of postsurgical resected tissue from patients with intractable epilepsy revealed that isolated FCD type I occurred approximately in 30% of patients. These patients suffered from more frequent seizures, with

either frontal or multilobar lesions, and had the worse postsurgical outcome compared to FCD type III [558,559]. Patients with FCD type I associated with hippocampal sclerosis (which would now be considered type IIIa) had surgery in the temporal lobe (99% of cases) and displayed the longest duration of epilepsy. FCD with hippocampal sclerosis was also the most common pathological lesion association (35% of cases) compared to either isolated FCD (type I) or with other lesions (type III). Interestingly, FSs significantly correlated with the presence of FCD type IIIa (focal cortical lesion + hippocampal sclerosis) [558]. In addition, timing of the developmental insult during gestation affects the severity of CD; thus, the earlier the insult, the greater the damage[560]. Accordingly, patients with larger lesions appear to present symptoms earlier than patients with smaller lesions. In addition, patients with central and parietal lesions displayed motor and sensory limb deficits and facial nerve palsy (swelling of facial nerves) and poor bilateral hand coordination. When the seizures are medically refractory, the motor defects become permanent. Cognitive impairment varies from mild impairment to severe cognitive and autism spectrum disorders depending on the severity of the FCD and the age of onset of epilepsy. As such, earlier onset and severe lesions were associated with psychiatric symptoms[556]. Evidence suggests that the surgical outcome of patients with FCD type II has a better seizure-free outcome, although they have an earlier age of seizure onset and more frequent seizures than FCD type I. Whereas, FCD type I has a worse surgical outcome, behavioural deficits and lower intelligence than FCD type II patients[553].

Importantly, studies in animal models as well as in patients with MTLE support a causal link between the presence of cortical dysplasia, the incidence of atypical FSs, and the subsequent development of hippocampal sclerosis and MTLE[463,509,534,558,561,562]. Subtle malformations of cortical development have been increasingly identified in patients with MTLE, and their combination is often referred to as dual pathology [464,561]. As such, in children with refractory TLE, the most frequent pathological association was cortical dysplasia (64%), with or without mesial temporal sclerosis[464] in association with FSs as well[558,561]. This strong association between FCD and hippocampal sclerosis in children with refractory TLE suggests that they may be common causal factors for FCD and hippocampal injury. Altogether, these results lead the group of Dr. Lionel Carmant to the hypothesis that during development the presence of a genetic or acquired subtle FCD causes the brain to be more susceptible to

prolonged FSs and seizure-induced limbic injury, therefore increasing the risk of developing MTLE.

3.5.3. Mechanisms involved in ictogenesis and epileptogenesis

Concerning the epileptogenesis mechanisms in FCD, the challenge has been to identify whether seizures originate from the dysplastic cortex and/or the surrounding area, as a proportion of patients continue to have seizures after surgical resection of the abnormal tissue. Another challenge is to identify the effects and mechanisms of dysmorphic cells in these regions, and the effect on the surrounding tissue on neuronal hyperexcitability and the development of epilepsy. The cellular mechanisms underlying FCDs in the development of intractable epilepsy are largely unknown, and believed to be multifactorial. In general, FCDs have been hypothesized to ensue from somatic mutations in progenitor cells during brain development [563].

Cells types in FCDs

FCD causes alterations of cell proliferation and differentiation, among other things, which causes the development of abnormal cells, in particular dysmorphic neurons, balloon cells and immature pyramidal neurons, which could contribute to seizure generation in FCD. Electrophysiological recordings of freshly resected dysplastic tissue from pediatric patients demonstrate that there are four types of abnormal-appearing cells. The first being the dysmorphic pyramidal neurons, which have an altered shape, are enlarged with abnormal orientation, a cytoskeletal structure with enriched neurofilaments, but do not have altered electrophysiological properties. The second type, intermediate cells (or cytomegalic or dysplastic cells), share both glial and neuronal features. These cells generated large and increased Ca^{2+} currents when depolarized, and displayed atypical hyperexcitable intrinsic membrane properties. Balloon cells have a thin membrane, with a pale and glassy cytoplasm with eccentric nucleus (sometimes two nucleus), and they express both neuronal and glial markers, although they present mostly glial-like features. Balloon cells do not display active

voltage- or ligand-gated currents, do not appear to receive synaptic input, and are thought to be electrically silent cells that could however still potentially promote synaptic reorganization in the surrounding cortex resulting in net excitability. Finally, the immature pyramidal neurons which derive from neuroblasts have a small soma, do not accumulate neurofilaments, and do not display any aberrant physiological features[550,553]. As described above, these 4 cell types are not expressed in all cases of FCD. Patients who do express the cytomegatic dysmorphic neurons and balloon cells usually have a higher seizure frequency than patients with FCDIIa, yet they have the best outcome after surgery[564], suggesting that these cells clearly have an epileptogenic effect, and when they are removed, patients have a better outcome.

Glutamate and GABAergic receptors

Based on the observation that all subunits of NMDA and AMPA receptors are enhanced in FCD, although to a variable extent depending on experimental conditions in animals models, glutamatergic receptors are believed to contribute to the mechanism leading to hyperexcitability[565]. Furthermore, resection tissue analysis revealed alterations of synaptic density and of the proportion of excitatory and inhibitory synapses, compared to the surrounding tissue[566], demonstrating that neuronal circuits are likely affected in the dysplastic tissue, which communicates and affects the surrounding tissue and could potential promote epileptogenesis. In fact, intracranial EEG recording of FCD patients revealed that the epileptogenic zone may encompass the lesion identified by MRI[567], and may reveal complex propagation patterns to neighbouring or distant microcircuits, as explained previously.

In addition, in human neocortical resected tissue, they found a functional increase of NMDA and GABA responses. In brain slice models of ictal activity, treatment with 4-aminopyridine (4-AP), a K^+ channel blocker that increases neurotransmitter release usually does not generate ictal activity; however, in CD tissue it leads to NMDAR-mediated ictal discharges and GABAR-mediated interictal discharges. Importantly, ictal discharges can then blocked by NMDAR antagonists[568].

In tissue with FCD, GABAergic INs density and the expression of GABA transporters are reduced, leading to altered inhibition. In addition, parvalbumin and calbindin expression is reduced in CD, and these IN populations are abnormally localised in the cortex[569]. In FCD

type II, there is a greater decrease of GAD-labelled INs as compared to FCD type I[557]. In addition, neurons in dysplastic cortex have immature-like features, and GABA neurotransmission retains the immature feature of shunting/excitatory neurotransmitter[570]. Further, in FCD type II, GABAAR subunit composition recruits more of the immature γ2 subunit, compared to the mature α1 subunit[571]. These results can explain the lack of effect of benzodiazepine treatment.

Interleukin receptors

Few molecules have been shown to play an important role in hyperexcitability development leading to seizures. IL-1β (interleukin-1β) and its receptor IL-1R are particularly interesting. In fact, IL-1β and IL-1R expression levels are increased in FCD surgical resections, and the number of neurons expressing both proteins positively correlated with the frequency of seizures[572,573]. The significance of these alterations, however, is still unknown.

mTOR pathway

A relationship between the mammalian target of rapamycin (mTOR) signalling pathway activation in dysmorphic neurons in FCDs and in several other cortical malformations has been recently identified. mTOR is a serine/threonine kinase protein involved in multiple stages of brain development, such as cell growth and proliferation, energy metabolism, inflammation and regulation of autophagy[574] (see figure 18 below). Thus, this protein integrates multiple inputs and serves as a 'focal node' to nutrients, cytokines, hormones, cellular stressors and oxygen sensors. mTOR is formed by mTORC1 and mTORC2, which are two protein complexes. mTORC1 is the complex that is sensitive to rapamycin and integrates several inputs through the PI3K-AKT pathway (phosphoinositide -3-kinase/protein kinase B, both serine/threonine kinases;[575]). One of its role is to activate downstream signalling cascades involved in protein synthesis activation, through two different pathways, the first being the ribosomal protein S6/ribosomal protein S6 kinase beta-1 (S6-S6K1) pathway and the second being the eukaryotic initiation factor 4E binding protein-1/eukaryotic translation initiation factor 4E (4E-BP1/eIF4E) pathway. Conversely, mTORC2 is insensitive to rapamycin and

regulates cytoskeletal organization through different kinases. mTOR is regulated by different proteins, such as tuberous sclerosis complex 1 (TSC1) and 2 (TSC2), as well as PTEN (phosphatase and tensin homolog), neurofibromin 1 (NF1) and STE20-related kinase adaptor alpha (STRADA). An increase in mTOR signalling, through an increase of phosphorylated S6 protein and kinase 1 (pS6, pS6K1) was observed in patients with FCD type IIb, but not FCD type I; it was also observed in other forms of cortical malformations that incidentally, can be associated with FCD such as gangliogliomas (AET)[223,575]. In fact, FCD type IIb and tubers, where >80% of balloon cells and giant cells, respectively, show increased phosphorylated S6K1 and S6. Additionally, some FCD type IIb also exhibit an activation of upstream P13K and AKT, and downstream substrates vascular endothelial growth factor (VEGF) and signal transducer and activator of transcription 3 (STAT3). Furthermore, PTEN kockout mouse model exhibited similar histopathological features to FCD, which were mostly rescued by rapamycin. Finally, mTOR signalling was also increased in TLE tissue resection, and their epileptic phenotype were rescued by rapamycin; suggesting that mTOR may be implicated in epileptogenesis. Balloon cells express immature markers, such as microtubule-associated protein 1B (MAP1B) or alpha-internexin. They also express stem cell markers, such as Sox2, Oct4, c-Myc, which is a feature linked with mTOR activation. Moreover, enhanced mTOR signaling can also modulate the expression of neurotransmitter receptors and ion channels. As such, these alterations, which have been shown to change membrane properties and synaptic organization, can result in neuronal hyperexcitability[548]. Overall, these observations suggest that cells in FCDs undergo impaired cell differentiation and retain immature features, and that morphological and functional changes at the cellular and circuit levels may lead to epilepsy, as well as focal deficits and cognitive impairment.

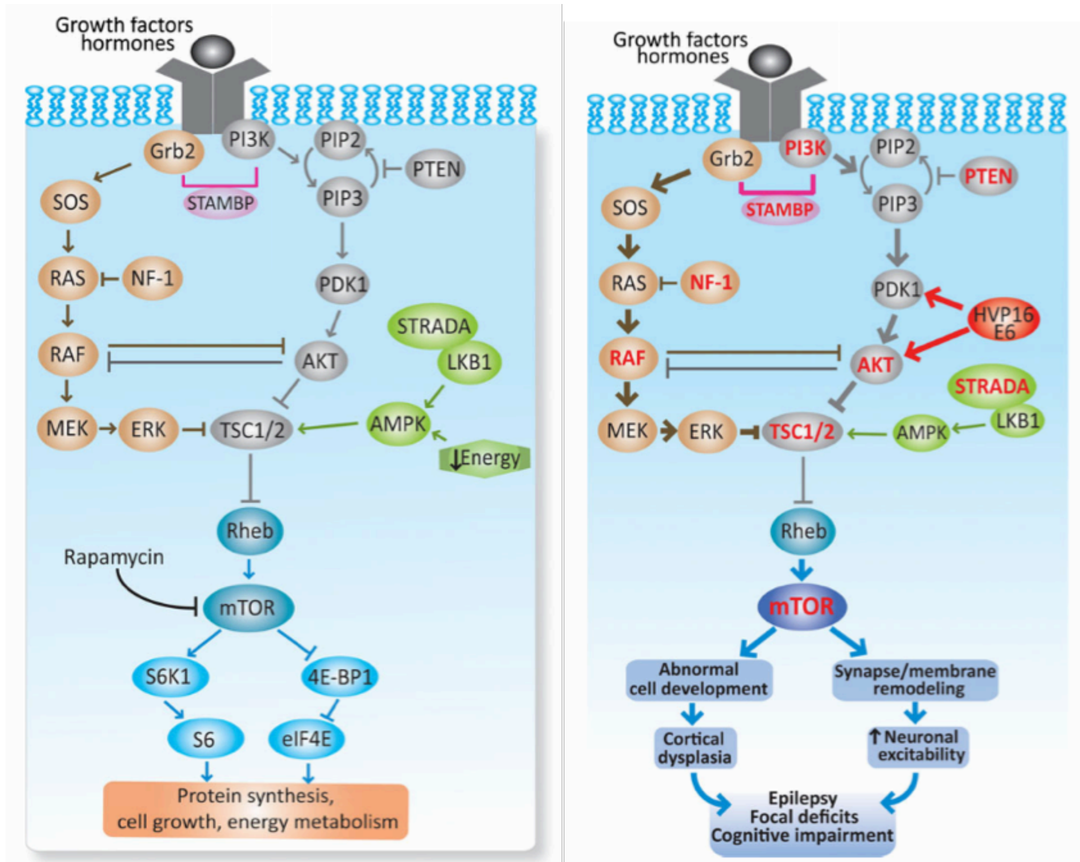


Figure 18. Schematic illustrating the mTOR signaling pathway in normal (left) and pathological in FCD (right) conditions.
Several pathways converge on the TSC1/TSC2 complex, such as the RAS-MAPK, PI3K-AKT-mTOR and AMPK. TSC1/TSC2 activation negatively regulates mTOR through the Ras homolog enriched in brain (Rheb). mTOR, in turn, regulates downstream substrates (S6K1/S6, 4E-BP1/eIF4E) that subsequently control protein synthesis, cell growth, and energy metabolism. In cortical dysplasias, the mTOR pathway becomes hyperactive (thicker lines) by mutations of genes encoding upstream regulators, or through hyperactive components of the pathways that converge on TCS1/2 and mTOR itself following FCD (highlighted in red). This hyperactivity leads to increased cell growth and proliferation (for ex. balloon cells), which could result in lesion formation. Image adapted from [548].

Human papilloma virus

Recently, it was suggested that a virus may cause FCD, more particularly the human papilloma virus (HPV) 16 oncoprotein E6, which was found in some FCD type IIb patients[576,577]. Studying postsurgical human tissue demonstrated that E6 expression colocalized with pS6 protein in balloon cells. In fact, transfecting HPV16 in mice during

embryonic development lead to focal cortical malformations and an increase of pS6; suggesting that HPV16 may alter cortical development through mTOR activation (see figure 18). More specifically, HPV16 E6 oncoprotein activates mTORC1 through two different paths. First, it binds TSC2 and thus promotes its ubiquitin-mediated degradation. Second, it activates phosphoinositide-3-dependent kinase 1 (PDK1) and AKT, thus inhibiting TSC1/TSC2[578]. The P13K-AKT-mTOR pathway promotes protein translation and thus favours the replication of double-stranded DNA virus (ex. CMV), which could thus lead to an easier spread of the infection to other cells[579]. Further studies will be necessary to confirm whether these viral infections cause FCD features directly, or indirectly via an increased inflammatory response.

The etiologic relationship between focal cortical dysplasia, complex/atypical febrile seizures and mesial temportal lobe epilepsy remains to be elucidated. In the lab, we focus on FCD associated with FSs to gain insight into the mechanisms underlying epileptogenesis of TLE and the neuronal circuit alterations that occur in the hippocampus and cortex before the onset of spontaneous recurrent seizures.

3.6. Dual pathology: LHS model

Based on these observations, the Carmant laboratory developed a rodent model combining both cortical focal dysplasia and FSs (LHS rats). Pups carrying a cortical dysplasia induced by unilateral and localized freeze lesion at P1, show shorter seizure onset time and more prolonged seizures when exposed to hyperthermia at P10 compared to naïve rats [534], a condition resembling febrile status epilepticus in children. In addition, the temperature necessary to induce generalized tonic-clonic convulsions during hyperthermia is also reduced, while the posthyperthermia depression is longer in rats carrying a cortical dysplasia, compared to naïve rats [534]. The LHS model therefore reproduces the vulnerability, or increased susceptibility of a predisposed brain to FS. In addition, by adulthood, more than 90% of LHS rats develop spontaneous recurrent seizures and hippocampal sclerosis accompanied by synaptic alterations and spatial memory deficits [580,581]. Thus, this model is clinically relevant,

as it reproduces well the association between the presence of a predisposing brain malformation, atypical FSs and the development of MTLE [463,582]. However, the mechanisms underlying increased atypical FSs susceptibility and synaptic deficits are currently poorly understood.

Only two days after the hyperthermia-induced seizures (HSs), pups with a cortical lesion showed a decrease in the total volume of the brain, and in the ipsilateral hemisphere, cortex, subcortex and hippocampus volumes, which persisted at P22. The ipsilateral hippocampal atrophy was present despite the lack of cell death[583]. Moreover, limiting the duration of the seizures by diazepam treatment prevented the hippocampal atrophy. Finally, at P80, when rats develop spontaneous recurrent seizures, the hippocampal atrophy was more severe, and both the total hippocampus as well as the individual pyramidal layers were affected. This volume loss was accompanied by cell death, which was predominant in the CA1 region, and spine density reduction in CA1 pyramidal neurons. Low field potentials recordings confirmed that spontaneous recurrent seizures originate from the atrophied hippocampus[581]. Further, LHS rats suffered from learning and memory deficits in adulthood[580]. However, it was not clear before the start of my studies whether these deficits were a consequence of spontaneous recurrent seizures or whether they preceded them.

Electrophysiological recordings demonstrated that, already by P20, well before the onset of spontaneous recurrent seizures, CA1 pyramidal neurons were hyperexcitable, with an increase in evoked excitatory postsynaptic potentials (eEPSP) and an increase in the frequency of spontaneous excitatory postsynaptic current (sEPSC) onto CA1 interneurons[584,585]. The inhibitory activity in LHS rats was also affected. As such, the amplitude of both $GABA_A$ and $GABA_B$ inhibitory postsynaptic portentials (IPSP), and the evoked inhibitory postsynaptic currents (eIPSC) were increased[584]. In parallel, western blot analysis accompanied with electrophysiological recordings indicated that the ionotropic glutamate receptor NMDA expression was altered in the LHS rats. At P20, NR2B subunit was upregulated; while at P80, NR2A, but not NR2B, subunit was markedly overexpressed[534,581]. These results are in agreement with two other dual hit models, wherein the cortical dysplasia is induced either by treatment with the neurotoxin MAM (Methylazoxymethanol acetate) followed by pilocaprine-induced seizures[586], or by a prenatal freeze lesion followed by electrical kindling[587]. Therefore, network reorganization occurs relatively rapidly in the dual pathology model and likely leads

to the development of spontaneous recurrent seizure in adulthood.

In summary, both individual pathologies (FCD and FSs) make the brain more susceptible to epilepsy, however the association of both strongly promotes epileptogenesis. Whether and how changes in inhibition promote seizures or protect the brain from seizures in the LHS model was yet to be established and it is the focus of my graduate work.

Aims & Hypothesis

The neuron-specific KCC2 cotransporter is crucial for regulating the inhibitory GABAergic driving force and for glutamatergic spine development. This thesis project focuses on the effect of alterating KCC2 on both these aspects in both normal and seizure-related conditions.

Epilepsy typically causes severe GABAergic and glutamatergic dysfunction; however, the underlying molecular mechanisms remain to be elucidated. Recent efforts have focused on targeting molecules that establish the chloride gradient as a tool to modulate more efficiently GABAergic inhibition in epilepsy. The first project focuses on a dual-hit model, which reproduces the clinical situation where children with prolonged and atypical FSs are at higher risk of developing MTLE and cognitive problems. In fact, early-life seizures in a predisposed individual interfere with the proper development of hippocampal circuits and cause long-term neurological and psychiatric deficits. Ultimately, a better understanding of the consequences of atypical febrile seizures could help reduce the incidence of MTLE. Our hypothesis is that KCC2 alterations in the LHS model have detrimental effects on seizure vulnerability and spine development. Therefore, my project aims to evaluate KCC2 expression and function in our dual-hit model, and its effect on seizure susceptibility and excitatory synapse development.

A few studies have demonstrated that reducing KCC2 expression can directly affect both GABAergic drive and glutamatergic synaptic signaling, by distinct mechanisms. On the hand, the effect of premature KCC2 expression in circuit formation and spine development are less understood. Understanding how precocious KCC2 expression in the developing brain may affect specific long-term synapse formation is important, since in the neonatal brain, opposite to what occurs in adult, traumatic events such as seizures can lead to an increase in KCC2 expression and activation. The second project aims to investigate the effects of premature KCC2 expression on dendritic spine formation in CA1 hippocampal pyramidal neurons, since this region is particularly affected by early-life seizures.

Chapter II

Reducing premature KCC2 expression rescues seizure susceptibility and spine morphology in atypical febrile seizures

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Abstract

Atypical febrile seizures are considered a risk factor for epilepsy onset and cognitive impairments later in life. Patients with temporal lobe epilepsy and a history of atypical febrile seizures often carry a cortical malformation. This association has led to the hypothesis that the presence of a cortical dysplasia exacerbates febrile seizures in infancy, in turn increasing the risk for neurological sequelae. The mechanisms linking these events are currently poorly understood. Potassium-chloride cotransporter KCC2 affects several aspects of neuronal circuit development and function, by modulating GABAergic transmission and excitatory synapse formation. Recent data suggest that KCC2 downregulation contributes to seizure generation in the epileptic adult brain, but its role in the developing brain is still controversial.

In a rodent model of atypical febrile seizures, combining a cortical dysplasia and hyperthermia-induced seizures (LHS rats), we found premature and sustained increase in KCC2 protein levels, accompanied by a negative shift of the reversal potential of GABA. In parallel, we observed a significant reduction in dendritic spine size and mEPSC amplitude in CA1 pyramidal neurons, accompanied by spatial memory deficits. To investigate whether KCC2 premature overexpression plays a role in seizure susceptibility and synaptic alterations, we reduced KCC2 expression selectively in hippocampal pyramidal neurons by *in-utero* electroporation of shRNA. Remarkably, KCC2 shRNA-electroporated LHS rats show reduced hyperthermia-induced seizure susceptibility, while dendritic spine size deficits were rescued. Our findings demonstrate that KCC2 overexpression in a compromised developing brain increases febrile seizure susceptibility and contribute to dendritic spine alterations.

Key words: atypical febrile seizures, temporal lobe epilepsy, cortical dysplasia, KCC2, seizure susceptibility, dendritic spines, *in utero* electroporation, shRNA, hippocampus.

Introduction

Febrile seizures (FSs) are the most common convulsive events in children between 6 months and 5 years of age, with a prevalence of 2–14% in this population. Simple FSs are considered benign, whereas atypical febrile seizures, which account for 30-40% of FSs cases (Nelson and Ellenberg, 1976), are a risk factor for Mesial Temporal Lobe Epilepsy (MTLE) (Finegersh et al., 2011; French et al., 1993; Hamati-Haddad and Abou-Khalil, 1998). In fact, while only 2% of all children with FSs will develop epilepsy, the long-term risk of developing MTLE is much higher (32-49%, depending on the cohorts) for children with FSs that have all three atypical features: lateralized, prolonged and multiple FSs in a day (Annegers et al., 1987; Birca et al., 2004). Notably, individuals carrying a developmental defect in cortical architecture, termed focal cortical dysplasia, are more likely to develop atypical febrile seizures than the general population (Bocti et al., 2003; Hesdorffer et al., 2008). Studies in animal models as well as in patients with MTLE support a causal link between the presence of cortical dysplasia, the incidence of atypical FSs, and the subsequent development of hippocampal sclerosis and MTLE (Park et al., 2010; Scantlebury et al., 2004; Tassi et al., 2010; Tassi et al., 2009). Based on these observations, we developed a rodent model combining both cortical focal dysplasia and FSs (LHS rats). Pups carrying a cortical dysplasia show shorter seizure onset time and more prolonged seizures when exposed to hyperthermia at P10 compared to naïve rats (Scantlebury et al., 2004), a condition resembling febrile status epilepticus in children. In addition, by adulthood, most LHS rats develop spontaneous recurrent seizures and hippocampal sclerosis accompanied by synaptic alterations and spatial memory deficits (Gibbs et al., 2011; Scantlebury et al., 2005). Thus, this model is clinically relevant, as it reproduces well the association between the presence of a predisposing brain malformation, atypical FSs and the development of neurological sequelae. The mechanisms underlying increased atypical FSs susceptibility in brains carrying a focal dysplasia are poorly understood.

KCC2 and NKCC1 are cation-chloride cotransporters, which are responsible for establishing the neuronal Chloride (Cl^-) gradient that governs GABAergic inhibition. During development, the increase in KCC2 expression is responsible for the shift of GABA function from excitatory to shunting/inhibitory (Kaila et al., 2014). In addition to regulating the Cl- gradient, KCC2 modulates several aspects of neuronal development, including synapse formation (Fiumelli et al., 2013; Gauvain et al., 2011; Li et al., 2007). The immaturity of the GABAergic system and the presence of higher levels of intracellular Cl^- in the developing neurons may in part account for the higher incidence of seizures during early postnatal development (Dzhala et al., 2005). This hypothesis led to the prediction that KCC2-activating or NKCC1-inibiting compounds would improve seizure controls in neonates (Dzhala et al., 2005); however clinical trials testing this hypothesis have been so far disappointing (Pressler et al., 2015). Recent data suggest that different traumatic events in the developing brain, including seizures, induce an increase in KCC2 expression levels and/or activation (Galanopoulou, 2008; Khirug et al., 2010; Puskarjov et al., 2014). Thus, KCC2/NKCC1 balance may be altered in a brain with pre-existing conditions, making it harder to predict the outcome of targeting their activity on seizure susceptibility. Further, whether and to what extent pathology-induced premature KCC2 expression contributes to long-term synaptic alterations remains to be investigated.

Here, we test the hypothesis that KCC2 expression and function is prematurely increased in the LHS model, and that this event causes increased seizure susceptibility and altered synapse formation in the developing brain.

Materials and Methods

Animals

Sprague–Dawley pups were obtained from Charles River Laboratories (St. Constant, Quebec, Canada) at postnatal day 1 (P1). Pups were culled to 12 per dam, matched by gender, weighed and kept with their mother in a 12 h light/dark cycle with food and water *ad libitum*. Animal care and use conformed to institutional policies and guidelines (CIBPAR, Sainte-Justine Hospital Research Centre, Université de Montréal, Montreal, QC, Canada). This study complies with the ARRIVE guidelines.

Freeze lesions and Induction of hyperthermic seizure

A focal microgyrus and hyperthermic seizures were induced as described in (Scantlebury et al., 2004). Briefly, in anesthetized P1 rats, a cylindrical 2-mm diameter copper probe, cooled in liquid nitrogen, was brought into contact with the skull overlying the right fronto-parietal cortex, 2 mm caudal to bregma and 2 mm lateral to the sagittal suture, for 10s after skin incision. At P10 pups were placed individually at the bottom of a Plexiglas box through which warm dry air (45–52°C) was circulated by a standard hairdryer fitted on the uppermost part of a side panel. Each pup remained in the box until a generalized convulsion occurred. Pups were then moved to an ambient temperature surface and remained untouched for 30 min of observation.

Western Blot

Western blots were performed as described in (Ouardouz et al., 2010). Membranes were probed with the following primary antibodies: anti-KCC2 1:1000 (rabbit polyclonal IgG; Cat. no. 07-432, Millipore) and 1: 200 anti-NKCC1 (kindly gifted by Dr. Jim Turner, NIH/NIDCR) and anti-glyceraldehyde-3-phosphate dehydrogenase 1:4000 (GAPDH, mouse monoclonal IgG; Cat. no. AM4300; Applied Biosystems, Streetsville, Ontario, Canada). Each experimental group included 3 to 5 animals. All samples were run simultaneously. Bands were quantified using Image J software. The intensity of each KCC2 and NKCC1 band was first normalized over the intensity of the GAPDH band in the same lane (internal loading control). For each experimental group, KCC2 intensity levels were then normalized over the

control group represented by naïve animals of the same sex.

Electrophysiology

Electrophysiological recordings were essentially performed as in (Ouardouz et al., 2010). Hippocampal slices were prepared from male rats at P18-P22 (mEPSC experiment) or P11-P15 (E_{GABA} experiment). Male rats were anesthetized with isoflurane and decapitated. Brain tissue was quickly removed and placed in cold artificial cerebrospinal fluid (ACSF) containing in mM: 126 NaCl, 3 KCl, 2 $MgSO_4$-H_2O, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 D-Glucose, bubbled with 95% O_2 ⁄-5% CO_2. The pH and osmolarity were adjusted to 7.3-7.4 and 300-310mOsm, respectively. Hippocampal slices (300µm thick) were cut with a vibratome (VT1000S, Leica Microsystems) and transferred to a container filled with oxygenated ACSF at room temperature. After an hour of incubation, individual slices were placed into a recording chamber and continuously superfused with oxygenated ACSF heated at ±32°C with a temperature controller (TC-324B, Warner Instruments).

Hippocampal CA1 pyramidal cells were visualized with an upright microscope (Olympus) fitted with a near-infrared CCD camera (XC-EI50, Sony). Patch pipettes were pulled from borosilicate glass tubing with a resistance ranging from 4 to 7MΩ. Intracellular solutions used were K^+-based or Cs^+-based according to the experiment and contained in mM: 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 Hepes buffer, 0.5 EGTA, 10 phosphocreatine, 2 ATP Tris, 0.4 GTP Li. For CsCl-based solution all K^+ ions were replaced by Cs^+, and the solution included the Na^+ inhibitor N-(2,6-dimethyl-phenylcarbamoylmethyl) triethylammonium bromide QX-314 (2mM; Sigma). The pH was adjusted to 7.2–7.3 with KOH or CsOH, respectively, and biocytine was added to the intracellular solutions for post-hoc confirmation of cell identity. Signals were digitized with a Digidata 1440A analog-digital converter (Molecular Devices), acquired at a sampling rate of 2kHz, low-pass filtered at 1kHz using an Axopatch 200B amplifier (Axon Instruments) and visualized using pClamp software 10 (Molecular Devices). Miniature excitatory postsynaptic currents (mEPSCs) were recorded in whole-cell configuration at a holding potential of -60mV and in presence of $GABA_A$ receptor antagonist Bicuculline methiodide (5µM) and TTX (1µM). All events detected over 10min were analyzed. A total of 11 animals (11 cells from 6 male LHS, 10 cells from 5 male Ctrl) were

used for this experiments. To measure the reversal potential of GABA (E_{GABA}), eIPSCs were evoked by a puff of 10uM GABA delivered in the vicinity of the CA1 pyramidal cell soma (30psi, 3ms, 20sec interval). In this protocol, 7,5ug/ml of gramicidin diluted in DMSO, and Alexa Fluor 488 were added to the intracellular solution containing in mM: 135 KCl, 4 NaCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES. E_{GABA} current amplitudes were measured at the peak current evoked at 10mV incrementing holding potentials from -120 to -30mV, E_{GABA} value representing the intercept of the current-voltage curve with the abscissa. All drugs were purchased from Sigma-Aldrich (Canada). A total of 13 male rats (7 cells from 6 LHS, 8 cells from 7 Ctrl) were used for this experiments.

DiI labeling

DiI labeling was performed essentially as previously described in (Gibbs et al., 2011). Labeled pyramidal cells were imaged 40-60 minutes after diolistic transfection. Only pyramidal cells with a complete soma and clearly labeled primary basal dendrite branches were imaged and traced. Because of the variability of DiI labeling, we chose to reconstruct only the first 120µm of the basal dendrites from the soma. We did not analyze apical dendrites because in the first 120µm from the soma, the density of spine was low and very variable. A total of 12 pyramidal neurons from 4 male rats per group were imaged using a Leica SP8 confocal microscope and a 63x glycerol immersion objective (NA 1.3). Three labeled typical pyramidal neurons were randomly selected from the CA1 region of each animal. Image stacks of basal dendrites were acquired at 0.5 µm intervals and then analyzed with Neurolucida (MicroBrightField) software.

Immunolabeling

Brains were perfused in 4% paraformaldehyde/phosphate buffer, pH 7.4, submerged in 30% sucrose/PBS, then frozen in Tissue Tek. Slices were cut 80µm thick by cryostat, blocked in 10% NGS and 1% Triton for 2h at room temperature, and incubated overnight at 4°C in 10% NGS, 0.1% Triton and different primary antibodies- NeuN, 1:400 (mouse monoclonal, Cat. no. MAB377 Millipore); KCC2, 1:200 (rabbit polyclonal, Cat. no. 07-432 Millipore); GFP, 1:500 (chicken polyclonal, Cat. no. Ab13970 Abcam). The following secondary antibodies were used: Alexa (488, 555 or 633)-conjugated goat IgG (1: 400; Molecular Probes,

Invitrogen) or Alexa 488 goat anti-chicken IgY H&L (1:500, Cat. no. Ab150169 Abcam). NeuN staining was used to unequivocally identify the CA1 region in hippocampal slices. GFP staining was used to enhance GFP signal from transfected and electroporated cells. To quantify KCC2 expression levels, pyramidal cell membranes were outlined and the intensity levels were measured (ImageJ software), after background subtraction, for both the electroporated (GFP-positive) pyramidal cell and three neighboring cells in the same confocal plane. Ratios of KCC2 intensity levels in the transfected versus untransfected neighboring cells were then calculated and compared across the different experimental groups. For each construct, pyramidal cells from three different sections in the CA1 hippocampus were imaged from three different electroporated pups and quantified.

In utero electroporation

In utero electroporation was performed as in (Dal Maschio et al., 2012) at E17. KCC2 shRNA and Scramble shRNA were as described and characterized in (Succol et al., 2012). Both scramble and KCC2 shRNA were co-transfected with pCAG-ires-GFP. Scramble shRNA (co-electroporated with pCAG-ires-GFP) and pCAG-IRES-GFP alone were used as controls, and pooled, as they did not show statistical difference in any of the analyzed parameters. Electroporated pyramidal neurons were imaged using Leica confocal microscopes (SPE and a SP8; 63x glycerol immersion objective; NA 1.3). An average of 2 labeled pyramidal neurons were randomly selected from CA1 area of each animal. Image stacks of basal dendrites were acquired at 0.5 µm intervals and then analyzed with Neurolucida (MicroBrightField) software. To avoid problems in identifying dendrite provenance in highly transfected areas, we analyzed only the basal dendrites included in four identically sized stacks centered around the soma, which in our imaging conditions represent the first 120µm of basal dendrites originating from the soma. Between 10 and 20 hippocampal CA1 pyramidal cells from at least four males were used for each experimental condition. Tiled image in Fig. 5B was acquired with a Zeiss confocal microscope (LSM880).

Imaging and spine analysis

Hippocampal CA1 pyramidal neurons were imaged only from male experimental animals. Spine density, spine length, morphology and head diameter were quantified in 3-D using Neurolucida software, as described in (Fiumelli et al, 2013). We classified spines as: mushroom spines, identified by a clearly distinguishable enlargement of the head of the spine (compared to the neck); stubby spines, identified as structures with equal thickness between head and neck (minimum 0.3µm thick); thin spines as long and thin protrusions lacking a clearly defined head (maximum of 0.3µm thick). All quantification was done blind to the treatment. To measure spine head diameter in the shRNA-mediated knockdown experiments, we first deconvolved the acquired stacks using Volocity software. Images were then converted from green to rainbow lookup table, allowing a better delineation of the spine head area.

Morris Water Maze Test

Spatial learning memory was assessed as in (Scantlebury et al., 2004). Each group comprised of 11 to 14 animals.

Statistical analysis

All data analysis was performed in blind of treatment and construct use for electroporation. Differences between two experimental groups were assessed using Students t-test for normally distributed data and Mann-Whitney test for not normally distributed data. E_{GABA} differences between groups were determined using an unpaired Student's t-test. Differences between 3 or more experimental groups were assessed with one-way ANOVA and *post hoc* comparison. For non-normally distributed data, nonparametric Kruskal–Wallis one-way ANOVA test was used. In the shRNA experiment, two-way ANOVA with Holm-Sidak's multiple comparison *post hoc* analysis was used to determine the relative contribution of the electroporated constructs (Factor 1, Ctrl constructs vs. KCC2shRNA) and of the presence of the dual pathology (Factor 2, LHS (cortical dysplasia at P1+hyperthermia induced seizure at P10) vs. naïve rats). Cumulative distributions were analyzed using the Kolmogorov-Smirnov test. All bar graph represent mean ± SEM.

Results

Expression levels of functional KCC2 are prematurely increased specifically in male LHS rats.

Alterations of KCC2 protein levels, and consequently, shifts in the reversal potential of GABA (E_{GABA}), have been reported in a number of studies on seizure and trauma, with the direction of the change depending on the seizure model and on the age at which seizures occur (Galanopoulou, 2008; Huberfeld et al., 2007; Khirug et al., 2010). In this study, we used a "double-hit" model of atypical febrile seizures, where rats were subjected to a cortical dysplasia induced by freeze lesion (L) at post-natal day 1 (P1) and to hyperthermia-induced seizures (HS) at P10 (Fig.1A; LHS rats). We focused our analysis on the hippocampus because we have previously shown that in adult LHS animals, seizures start in this region (Lévesque et al., 2009). To evaluate KCC2 expression, we performed western blot analysis in LHS hippocampus 10 days after hyperthermia-induced seizures, since we have previously shown that neuronal circuit alterations are already present at P20 (Ouardouz et al., 2010), even though spontaneous recurrent seizures occur only after the third postnatal month (Scantlebury et al., 2005). KCC2 expression levels were specifically increased in male LHS rats compared to male control animals (Ctrl), while they were not significantly different in the hippocampus of male rats subjected either to the cortical dysplasia (L) or the hyperthermia-induced seizure (HS) alone (Fig.1B; Ctrl: 100±5%, L=104±16%, HS=119±11%, LHS (ipsilateral LHS male): 148±11%; 1-way Anova, $p<0.05$) or in male rats that received a sham surgery (125±46%; one-way Anova, $p>0.05$). Interestingly, this increase was specific to the hippocampus ipsilateral to the cortical focal dysplasia in LHS rats (Ctrl: 100±5%; contralateral LHS 99±3%, Student's t-test $p>0.05$). Further, KCC2 levels were not significantly different in P20 LHS female rats as compared to female controls (Fig. 1B2, Ctrl female: 100±17%; LHS female: 80±15%, Student's t-test: $p>0.05$), supporting the hypothesis that the effects of early-life insults on KCC2 expression are gender-specific (Galanopoulou, 2008).

Next, we investigated whether the presence of the cortical dysplasia was sufficient to alter the time course of KCC2 expression before the hyperthermia-induced seizures at P10.

We found that KCC2 expression levels were significantly increased in the hippocampus of lesioned male pups already at P10 compared to age-matched littermates (Fig.1C; Ctrl: 100±22%, L: 346±87%, Student's t-test $p<0.05$); while NKCC1 levels were not changed (Suppl. Fig. 1; Ctrl: 100±15%, L: 84±25%, Student's t-test $p>0.05$). Thus, the presence of the cortical malformation induced a premature overexpression of KCC2, but not NKCC1, which was maintained up to P20 only in male rats that experienced the second hit, the hyperthermia-induced seizures. The smaller difference in KCC2 expression levels between LHS and controls at P20 compared to P10 was likely due to the developmental increase in KCC2 expression occurring physiologically in untreated animals. Interestingly, we have previously reported that KCC2 expression levels did not differ significantly in male LHS rats compared to controls at P80 (Gibbs et al., 2011), when the first spontaneous seizures occur, suggesting that the overexpression of KCC2 levels is confined to a critical period following the double insults.

To confirm that elevated KCC2 protein levels have a functional impact, we measured E_{GABA} by gramicidin-perforated patch of pyramidal CA1 neurons in brain slices from P13-P15 male LHS rats. Summary of all the recordings showed a hyperpolarizing shift of E_{GABA} in LHS compared to control rats (Fig.1D, E; E_{GABA} for Ctrl rats: -39.4±5.4mV vs. LHS rats: -55.7±4.3mV, Student's t-test $p<0.05$). Altogether, these data suggest that E_{GABA} is more hyperpolarized in CA1 neurons in LHS pups, consistent with what was previously reported in rats, which experienced kainate-induced status epilepticus during the first postnatal week (Galanopoulou, 2008; Khirug et al., 2010).

Excitatory synapses onto CA1 pyramidal cell basal dendrites are reduced in LHS rats.

Glutamatergic inputs impinging on pyramidal cells are formed almost exclusively on specialized dendritic protrusion called dendritic spines. Dendritic spine alterations have been observed both in experimental animal models of epilepsy and in human epileptic patients (Freiman et al., 2011; Jiang et al., 1998). To investigate whether spine density and morphology were affected in male LHS rats, we used diolistic transfection to label relatively sparse pyramidal neurons in the CA1 region of the hippocampus in P20 rats (Fig.2A). Spine density on CA1 pyramidal neuron basal dendrites in the hippocampus ipsilateral to the cortical dysplasia was not significantly different in LHS and control animals (Fig.2B-D; ctrl: 1.05±0.05spine/μm vs. LHS: 0.93±0.07μm, Student's t-test $p>0.05$), while spine head size was significantly reduced in LHS rats (Fig.2E; K-S test $p<0.001$).

To examine whether morphological spine alterations had a functional impact, we recorded miniature excitatory postsynaptic current (mEPSC) from CA1 pyramidal neurons. Consistent with the observed smaller spine sizes in CA1 pyramidal neurons, we found that the mean mEPSC amplitude was significantly smaller (Fig.3A,B; -15.1±1.5pA for Ctrl vs. -10.3±1.5pA for LHS, Student's t-test $p<0.05$), whereas interevent intervals (IEI) were not significantly different (Fig.3C; Student's t-test $p>0.05$) in male LHS pups compared to control littermates. In addition, the mean decay constant (τ) was significantly shorter in LHS pups as compared to controls (LHS=30±3ms vs. Ctrl=84±26ms; t-test $p=0.005$), suggesting differences in glutamate receptor type or/and subunits composition between the two groups. Taken together, these results demonstrate that excitatory synapses impinging onto CA1 neuron dendritic spines are weaker in LHS rats. These alterations may contribute to cognitive deficits; indeed, LHS male rats showed spatial memory deficits in the Morris water maze probe test already by P40 (Suppl. Fig. 2), long before the onset of spontaneous seizures, which occurs after P90 (Scantlebury et al., 2005).

Reducing KCC2 expression in hippocampal pyramidal neurons decreases hyperthermia-induced seizure susceptibility and rescues spine size in LHS rats.

VO0463271, the only available KCC2-specific antagonist, is rapidly metabolized when administered systematically (Delpire et al., 2012; Sivakumaran et al., 2015). Further, pharmacological treatments could affect KCC2 function in the whole brain, while seizure-induced KCC2 upregulation is likely circuit and/or region specific. Therefore, to evaluate whether a premature increase in KCC2 expression in LHS rats contributed to seizure susceptibility and dendritic spine size reduction, we turned to a targeted genetic approach that allowed cell-type and brain-region specific manipulation of KCC2 levels, namely *in utero* electroporation of shRNAs at E17 using a triple-electrode probe (Dal Maschio et al., 2012) (Fig.4A). In particular, we specifically targeted pyramidal cells in the hippocampus underlying the cortical dysplasia, since KCC2 expression levels were selectively increased in this region (Fig.1). To label neurons at high resolution, we co-electroporated the shRNA constructs with a construct encoding for GFP. Pups electroporated with scramble shRNA+GFP or GFP alone were used as controls and pooled together as they did not show any significant difference on the specific parameters analyzed (hereon referred as the Ctrl group). Labeled pyramidal neurons were mostly confined to the dorsal region of the electroporated hippocampus (Fig. 4A). KCC2shRNA electroporation significantly decreased KCC2 intensity in electroporated pyramidal cells compared to neighboring, GFP-negative ones in both control and LHS rats (Fig.4B-C, Ctrl: 0.98±0.03a.u., KCC2 shRNA: 0.52±0.03a.u.; Ctrl+LHS: 0.84±0.03a.u.; KCC2 shRNA+LHS: 0.61±0.03a.u.; two-way ANOVA, $p<0.001$ for Ctrl vs shRNA). As previously shown, the occurrence of cortical dysplasia at P1 increased hippocampal KCC2 expression (Fig.1C). However, since KCC2 expression is hardly detectable in the CA1 region of P1 naïve pups, electroporation of KCC2shRNA at E17 and the subsequent embryonic expression of KCC2shRNA are unlikely to affect CA1 neuron development prior to birth.

Interestingly, we found that male pups electroporated with KCC2-shRNA and subjected to a cortical dysplasia at P1 (KCC2shRNA+LHS) showed significantly longer seizure onset

time when exposed to hyperthermia at P10 compared to male lesioned rats that were not electroporated (LHS) or male lesioned pups that were electroporated with control constructs (Ctrl+LHS) (Fig.5A,B). Conversely, the mean seizure onset time in KCC2-shRNA pups did not significantly differ from the one recorded in naïve rats (Ctrl) (Fig.5B, Ctrl: 12:56±0:33min/sec; LHS: 10:24±0.24min/sec; Ctrl shRNA+LHS: 11:33±0.23min/sec; KCC2shRNA+LHS: 13:44±0.37min/sec, one-way ANOVA $p<0.05$). Most remarkably, the difference in seizure onset time was already evident in single litters electroporated with KCC2-shRNA when comparing littermates that were successfully transfected (identified by the presence of GFP+ pyramidal neurons) with littermates that were not (seizure onset time was in average 2.51±0.04min longer, in successfully transfected pups; n=3 litters). On the other hand, the difference in seizure onset time between pups showing GFP+ neurons *vs.* those that did not was negligible when control constructs were electroporated (mean difference 0.310±0.004min, n=4 litters). We performed this intra-litter analysis using litters where about 50% of male rats were successfully electroporated. Figure 6C shows the difference in seizure onset time in transfected *vs.* untransfected LHS animals in all electroporated litters combined (Ctrl (Scramble + GFP): transfected: 11:32±0:22min/sec *vs.* non-transfected: 11:33±0:19min/sec; KCC2 shRNA: transfected: 13:44±0:37min/sec, non-transfected: 10:59±0:24min/sec; n=8 litters electroporated with KCC2-shRNA and n=11 litters electroporated with control constructs; two-way ANOVA, $p<0.001$). Altogether, these data suggest that reducing KCC2 expression levels in hippocampal pyramidal neurons may actually have anticonvulsive effects in a young brain compromised by a pre-existing brain malformation. Another significant implication of these findings is that the premature increase in hippocampal KCC2 expression induced by early-life insults may actually contribute to increased seizure susceptibility.

As a next step, we asked whether reducing KCC2 upregulation in male LHS rats affected the spine size alterations observed in CA1 pyramidal neurons at P20 (Fig.6). To avoid problems in identifying dendrite provenance in highly transfected areas, we analyzed only the basal dendrites included in four identically sized stacks centered around the soma, which in our imaging conditions represent the first 120µm of basal dendrites originating from the soma. When comparing LHS (Ctrl+LHS) and naïve (Ctrl) rats electroporated with control

constructs, we found no significant difference in spine density (Fig.6B,D, Ctrl: 0.99±0.04 spine/µm vs. Ctrl + LHS: 1.15±0.05 spine/µm; two-way ANOVA $p>0.05$) between the two groups. On the other hand, spine diameter was significantly reduced in LHS rats compared to age-matched controls (Fig. 6E; K-S test $p<0.001$), consistent with what we observed using DiI labeling approach in post-fixed hippocampal slices (compare Fig.6 with Fig. 2).

shRNA-mediated KCC2 reduction induced a small but significant decrease of overall spine density (Fig.6D; Ctrl: 0.99±0.04 spine/µm, KCC2shRNA: 0.82±0.06 spine/µm, Ctrl+LHS: 1.15±0.05 spine/µm KCC2shRNA+LHS: 0.92±0.06 spine/µm; two-way ANOVA $p<0.05$) and an increase in spine length (Fig.6F, K-S test, $p<0.001$). All the above changes were independent of the occurrence of the dual pathology (LHS factor within KCC2 shRNA, two-way ANOVA $p>0.05$). These data are in accordance with the reported critical role of KCC2 expression levels in spine formation (Fiumelli et al., 2013; Li et al., 2007). On the other hand, while spine head size was significantly reduced in LHS rats, electroporated with control constructs (Fig.6E; K-S test $p<0.001$), it was drastically increased following KCC2 shRNA transfection exclusively in LHS rats (Fig.6E, K-S test $p<0.001$). Scatter plots of spine length *vs.* diameter (Fig.6G, H) clearly show that dendritic spines were markedly larger and longer specifically in KCC2shRNA+LHS rats. All together, these data suggest that overall KCC2 expression levels may determine the direction of the change in dendritic spine size following early-life insults.

In summary, our data showed that premature KCC2 expression induced by the cortical dysplasia may be a significant factor contributing to the increased hyperthermia-induced seizure susceptibility and permanent alterations in excitatory circuit connectivity later in life. Conversely, reducing KCC2 expression in a region-specific subset of pyramidal cells is sufficient to delay hyperthermia-induced seizure onset and rescue long-term dendritic spine morphological deficits.

Discussion

$GABA_A$ receptor function changes during development, becoming increasingly more inhibitory as neurons mature. This functional change is due to age-related differences in the relative abundance of KCC2 and NKCC1, which regulates Cl^- homeostasis (Kaila et al., 2014). Both *in vivo* and *in vitro* experiments have shown that a variety of insults, including trauma, nerve transections and seizures lead to the reappearance of less hyperpolarizing and sometimes depolarizing GABA responses in adult brain (Coull et al., 2003; Huberfeld et al., 2007; Palma et al., 2006; van den Pol et al., 1996) (but see also (Karlocai et al., 2015)). In particular, in human epileptic temporal lobe tissue and epileptic adult rats, an aberrant shift towards increased NKCC1/KCC2 ratio and re-emergence of depolarizing GABAergic signaling may underlie epileptic discharges (Huberfeld et al., 2007; Palma et al., 2006). In a clinical perspective, agents that control Cl^- gradient and reinstate inhibitory actions of GABA may thus open novel therapeutic approaches in these adult neurological conditions.

Contrary to what occurs in the adults, traumatic events in the developing brain induce an increase in KCC2 expression and/or activation (Galanopoulou, 2008; Khirug et al., 2010; Puskarjov et al., 2014) (and our data). It has been previously hypothesized that upregulation of KCC2 function may be a mechanism to protect the brain from further damage by counteracting the massive increases in intracellular Cl^- loads induced by trauma and seizures, which could in turn contribute to increased neural network activity (Galanopoulou, 2008). By using a novel approach to specifically direct the expression of KCC2 shRNA in hippocampal pyramidal neurons *in vivo*, we showed that premature KCC2 expression in the postnatal brain increases susceptibility to hyperthermia-induced seizures. Therefore our data suggest that premature KCC2 expression in the young brain is not protective but may instead contribute to long-term pathological outcomes.

We found that KCC2 expression levels were already strongly elevated in P10 pups with the cortical dysplasia, as compared to controls. Cortical dysplasia, caused either by genetic or environmental factors, promotes circuit hyperexcitability (Chevassus-au-Louis et al., 1999), which may in turn promote KCC2 expression. In fact, sustained neural activity is a major modulator of KCC2 expression and function (Fiumelli et al., 2005; Fiumelli and Woodin,

2007). On the other hand, KCC2 expression levels remained consistently higher at P20 only in animals subjected to hyperthermic seizures too, suggesting a cumulative effect of the double insult on KCC2 long-term expression. Interestingly, this effect was specific to males, consistently with previous findings showing that KCC2 expression time course in rat hippocampal pyramidal neurons are gender-specific in normal development and following multiple status epilepticus episodes in the neonate (Galanopoulou, 2008). Intriguingly, we have recently showed a sexual dimorphism in long-term vulnerability to develop epilepsy in the LHS model (Desgent S, 2012). Sexual dimorphic outcomes have been reported both in children (Donders and Hoffman, 2002; Lauterbach et al., 2001) and in rodent model of neonatal hypoxic-ischemic encephalopathy (Mirza et al., 2015); thus it will be interesting to investigate whether gender-specific stress or/and inflammatory signaling pathways are responsible for preferential KCC2 upregulation, which in turn may increase the risk for epilepsy and cognitive impairments in males.

In our model, we observed consistently more negative E_{GABA} in LHS CA1 pyramidal neurons compared to controls. Further, we have previously reported that the excitatory drive onto CA1 GABAergic interneurons is increased in LHS rats, which correlates with an increase in sIPSC onto CA1 pyramidal cells (Ouardouz and Carmant, 2012). At first glance, these changes aiming at enhancing the efficacy of GABAergic neurotransmission might act as an intrinsic mechanism to limit network hyperexcitability caused by the dual pathology (Ouardouz et al., 2010). On the other hand, shRNA-mediated reduction of KCC2 in hippocampal neurons was sufficient to lower seizure susceptibility in LHS rats. The most likely explanation is that KCC2 has a seizure-promoting action in specific conditions (Hamidi and Avoli, 2015; Kaila et al., 1997; Ruusuvuori et al., 2013; Vitanen et al., 2010). In hippocampal CA1 neurons, functionally excitatory GABAergic responses can be induced by high-frequency stimulation (Kaila et al., 1997). Such stimulation causes intense GABAergic interneuron firing, a consequent HCO_3^--dependent increase in $[Cl^-]_i$ and a large depolarizing shift of E_{GABA} in the target pyramidal neurons. Because CO_2 readily permeates neuronal membranes, intraneuronal HCO_3^-, which is lost owing to net efflux through $GABA_A$Rs is replenished by the activity of cytosolic carbonic anhydrases (Ruusuvuori et al., 2013). Under these conditions, the increase in $[Cl^-]_i$ leads to enhanced K^+ and Cl^- extrusion by KCC2, and

to a consequent increase in $[K^+]_o$, which further depolarizes both neurons and glia (Vitanen et al., 2010). In this scenario, increased functional levels of KCC2 may counter-intuitively contribute to the emergence of highly synchronized spontaneous network events, including seizures (Ruusuvuori et al., 2013). Our data demonstrate that limiting the increase of KCC2 levels in a relatively small number of hippocampal pyramidal neurons is sufficient to rescue heightened febrile seizure susceptibility caused by cortical dysplasia, therefore supporting the hypothesis that premature KCC2 expression may have pro-convulsive effects in a predisposed brain.

Recently, a point mutation in SLC12A5, the gene coding for KCC2 in humans, was reported in an Australian family with early childhood onset of FSs and two other point mutations were reported in French-Canadian cohort with idiopathic generalized epilepsy (Kahle et al., 2014). All these variants have been shown to exhibit significantly impaired Cl^- extrusion capacities (Kahle et al., 2014). In these cases a large part of the KCC2 molecular pool exhibit altered function since very early embryonic development, while KCC2 expression is altered only after birth in the LHS model. In addition, KCC2 shRNA electroporation was limited to pyramidal neurons in the dorsal hippocampus and did not abolish completely KCC2 expression (Fig. 4). Therefore, whether reduction of KCC2 expression/function plays a pro- or anticonvulsive role may depend on the cellular and developmental context and on the extent of the impairment of KCC2 function.

Several studies show that KCC2 plays a role in different aspects of neuronal development, including synapse formation and AMPA receptor trafficking, by both ion-transporter dependent and independent mechanisms (Chevy et al., 2015; Fiumelli et al., 2013; Gauvain et al., 2011; Li et al., 2007). It is thus possible that pathology-induced premature KCC2 expression may directly change the developmental trajectory of dendritic spine development in the hippocampus of LHS rats. In fact, we observed that in LHS rats, dendritic spine size is reduced well before the onset of spontaneous recurrent seizures, which can exacerbate spine loss (Gibbs et al., 2011), and this loss is rescued by KCC2shRNA electroporation. In addition, it also possible that reduced seizure threshold in KCC2shRNA electroporated rats may indirectly contribute to rescue spine size. Interestingly, LHS rats

electroporated with KCC2 shRNA showed a significant increase in spine size compared to both control LHS and no-LHS groups. One possible explanation is that activity-dependent molecular signaling induced by seizures may affect spine development differently depending on KCC2 expression levels. For example, BDNF plays dual and opposite effects on GABAergic synaptic transmission in Purkinje neurons depending on KCC2 activity levels (Huang et al., 2012). In addition, it will be important to study whether and how alterations in spine size induced by KCC2 shRNA expression correlate with changes in synaptic transmission *in vivo*.

Our results show that pyramidal cells electroporated with KCC2 shRNA have reduced spine density independently of the presence of the dual pathology. It is possible that the observed effects of KCC2 reduction on febrile seizure susceptibility may partly depend on these alterations. On the other hand, LHS rats electroporated with KCC2 shRNA show seizure onset time comparable to naïve rats, while control LHS rats seize faster, even if they do not show any significant difference in spine density compared to control rats. Nevertheless, future studies are required to understand whether the effects of reducing KCC2 expression in hippocampal pyramidal neurons on febrile seizure susceptibility are mainly transporter-dependent or if they rely on long-term changes in pyramidal cell inputs.

Recently, it has been suggested that modulating chloride homeostasis might be a viable clinical tool to treat seizures in the developing brain. As the developing brain is more prone to seizure because of the immaturity of GABAergic inhibition and of higher level of intracellular chloride, a pharmaceutical approach designed to reduce intracellular chloride, by treatment with the NKCC1-inhibitor bumetanide, seemed a rational strategy (Dzhala et al., 2005). However, clinical studies have failed so far to show reproducible results (Pressler et al., 2015). In addition to the different abundance of NKCC1, KCC2 and carbonic anhydrase in full term babies compared to neonatal rats (Kaila et al., 2014), it is possible that, depending on the underlying seizure cause, KCC2 upregulation may in fact contribute to network hyperexcitability. Interestingly, a recent study found an overall increase in KCC2 expression in surgically removed hippocampi of patients with pharmacoresistent TLE, that developed seizures mostly in their teens or later, compared to control human tissue (Karlocai et al.,

2015). Further, depending on the cortical focal dysplasia type, both increased and decreased levels of KCC2 have been reported in patients (Talos et al., 2012). Therefore, understanding how NKCC1 and KCC2 are affected by pre-existing conditions is a critical prerequisite for establishing whether pharmaco-modulation of chloride co-transporters may be therapeutically effective.

Finally, our data suggest caution in interfering with chloride gradient for long-periods in the developing brain. Supporting this notion, bumetanide treatment *in vivo* during early postnatal period leads to altered timing of the critical period for experience-dependent plasticity (Deidda et al., 2015). These effects are dependent on the age of bumetanide-treatment, underscoring the importance of studying the role of KCC2/NKCC1 in specific developmental and pathological conditions, by using animal models, which reproduce as closely as possible the human pathology.

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Potential Conflict of interest

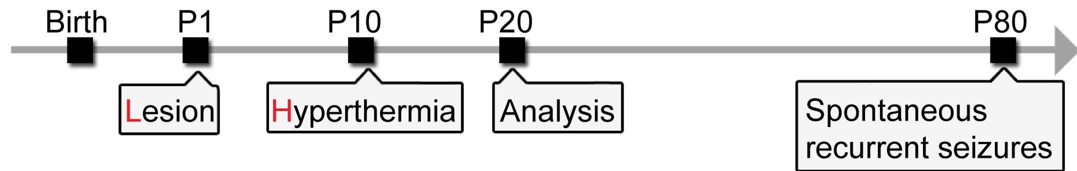
The authors have declared that no competing financial interests.

Authors Contributions

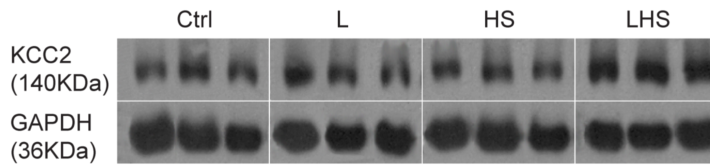
P.N. Awad, L. Carmant and G. DiCristo designed the study, and wrote the manuscript.
Patricia performed western blot, whole-cell recordings (with help from Nathalie and Mohamed), and in utero electroporation experiments. Analyzed all spine density and morphology, seizure threshold and RNAi downregulation.
Nathalie performed the gramicidin-perforated patch experiment and analysis.
Bidisha performed the DiI labeling experiment.
Jonathan performed the Morris Water Maze experiments and analysis.
Josianne offered technical assistance with the *in utero* electroporation experiments.
Laura Cancedda gave crucial feedback on the project and manuscript.

Figures

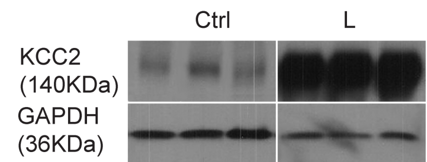
A.



B1. P20



C1. P10



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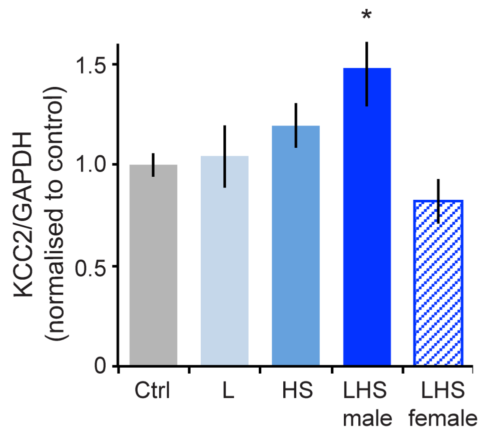

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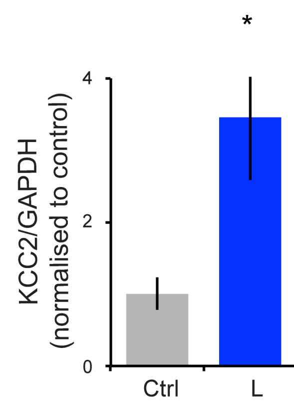

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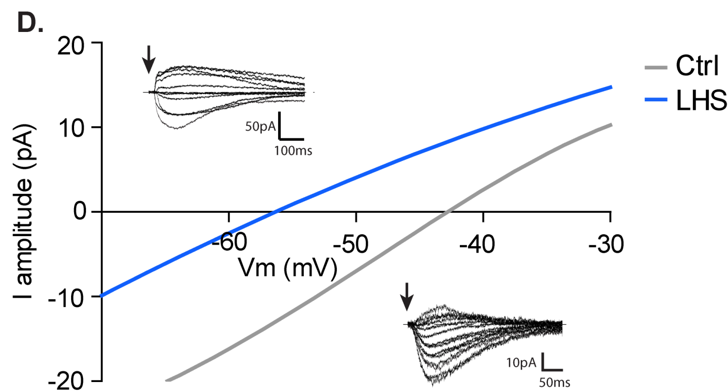

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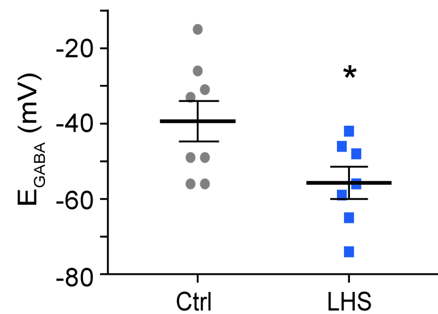

Figure 1. KCC2 expression levels are specifically increased in male LHS rats.

Figure 1. KCC2 expression levels are specifically increased in male LHS rats.

A. Schematic representation of the LHS animal model. A freeze lesion is induced at P1, to mimic a cortical dysplasia and hyperthermic seizures are induced at P10. **B1**. Western blot analysis of KCC2 expression levels (band at 140KDa) in the hippocampus of different experimental groups at P20. Each lane represents a different animal. **B2.** Quantification shows that KCC2 is significantly increased only in male LHS (one-way Anova, *$p<0.05$). n=5 Control rats (Ctrl); n=3 rats subjected only to cortical dysplasia (L); n=5 rats subjected only to Hyperthermia-induced Seizure (HS); n=3 male LHS; n=3 female LHS. **C1.** Western blot analysis of KCC2 expression levels in the hippocampus of P10 control rats (Ctrl) and of rats subjected to a cortical dysplasia (L). Each lane represents a different animal. **C2.** Quantification shows a threefold increase of KCC2 expression levels in L compared to Ctrl male pups (Student's t-test, *$p<0.05$; Ctrl: n=8 males; L: n=5 L males). **D**. IV curve measured in CA1 pyramidal neurons in the gramicidin-perforated patch configuration. Example traces of current amplitudes in response to a GABA puff (arrow) at varying holding membrane potential of control (bottom insert) and of LHS (top insert). **E**. Mean E_{GABA} values calculated from all CA1 pyramidal neurons recorded from Ctrl (black) and LHS (blue) rats. Each data point represents the result from one neuron, and the average value is depicted by the line (±SEM). E_{GABA} is more hyperpolarized in LHS compared to Ctrl rats (Unpaired Student's t-test $p<0.05$). Ctrl: n=8 cells from 7 male rats, LHS: n=7 cells from 6 male rats.

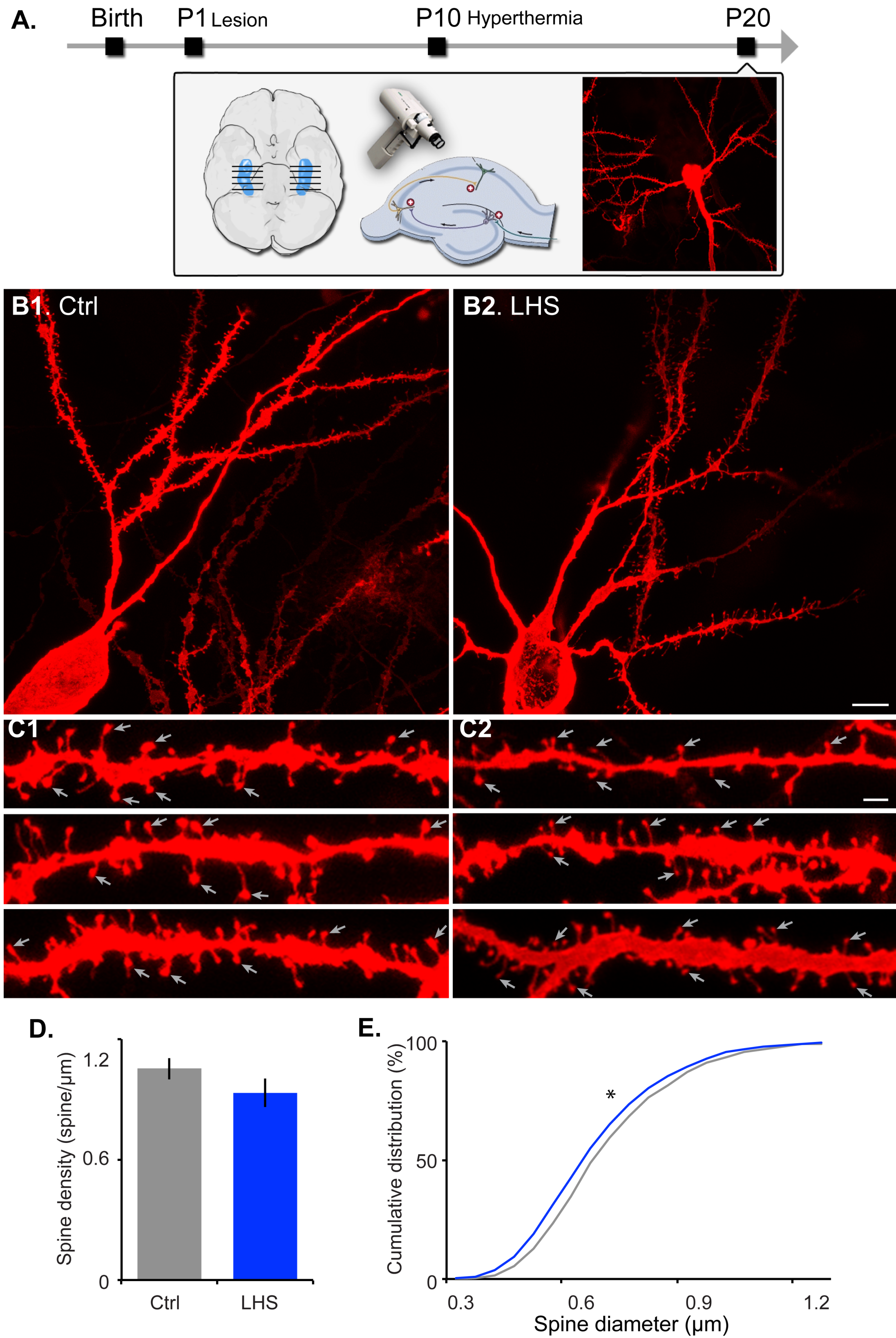

Figure 2. Spine size of CA1 pyramidal neuron basal dendrites is reduced in LHS rats.

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A. Schematic representation of DiI labeling approach. **B**. DiI labeled CA1 pyramidal neurons from control (Ctrl, **B1**) and LHS (**B2**) rats. Scale bar, 10µm **C**. High magnification of basal dendrites of CA1 pyramidal neuron in the lesioned hemisphere of a P20 LHS male, showing smaller spines (**C2**, arrows), compared to those observed in control pyramidal neurons (**C1**); Scale bar, 1µm. **D**. Mean spine density is not significantly different between LHS and Ctrl groups (Student t-test, $p>0.05$). **E**. The cumulative distribution of spine diameter is shifted toward smaller value for CA1 pyramidal neurons from LHS (blue) compared to Ctrl (grey) rats (K-S test, $p<0.001$). n= 12 CA1 pyramidal neurons from 4 Ctrl rats; n= 12 CA1 pyramidal neurons from 4 LHS rats.

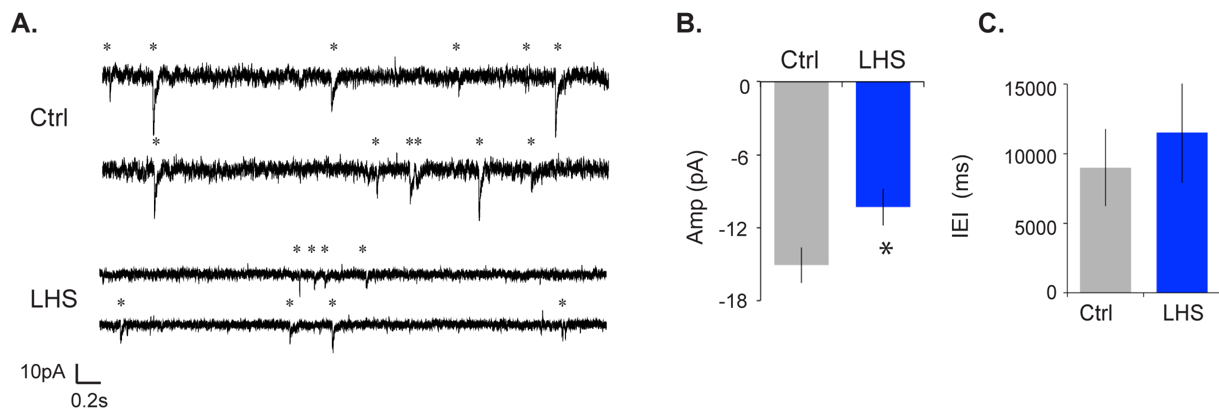


Figure 3. Functional maturation of glutamatergic synapses is impaired in CA1 pyramidal neurons from LHS rats.

A. Example traces of mEPSC recorded on CA1 pyramidal cells from a control (Ctrl) and a LHS rat at P20. Asterisks indicate single mEPSC events. **B-C.** Mean mEPSCs peak amplitude is significantly smaller in LHS compared to Ctrl rats (**B,** Student's t-test, *p<0.05), while mean mEPSC interevent intervals (IEI) is not affected (**C,** Student's t-test, p>0.05). N = 11 cells from 6 male LHS and n=10 cells from 5 Ctrl male rats.

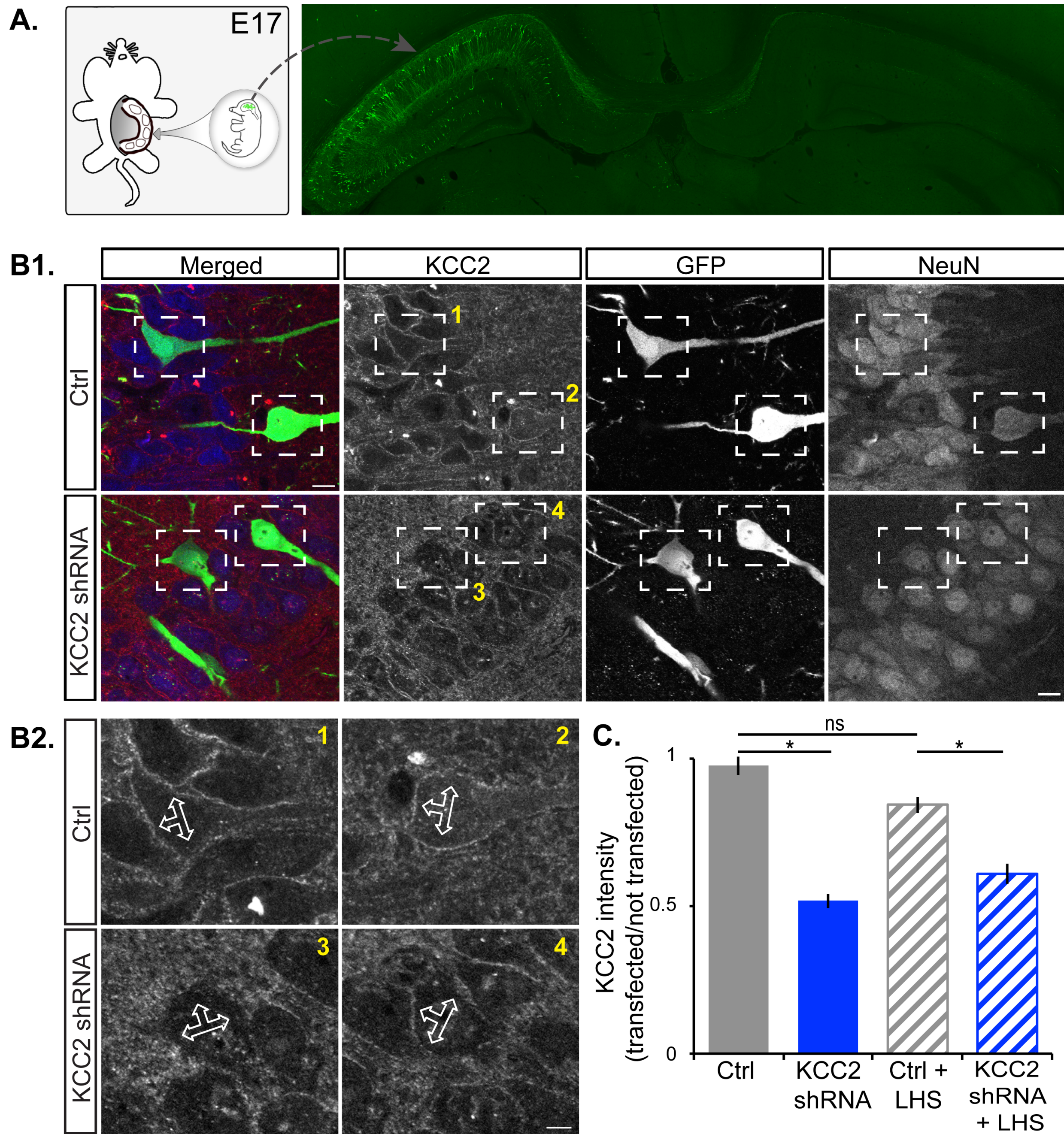


Figure 4. In utero electroporation of KCC2 shRNA significantly reduces membrane KCC2 expression in CA1 pyramidal neurons.

Figure 4. In utero electroporation of KCC2 shRNA significantly reduces membrane KCC2 expression in CA1 pyramidal neurons.

A. Schematics of experimental procedure (left) and coronal brain section of electroporated P20 rat (right) showing that transfection is limited to one hippocampus. **B.** Image representing the reduction of KCC2 intensity. (**B1**) Membrane KCC2 expression (red) is clearly visible in electroporated Control CA1 pyramidal cells (top, green, boxed region), while it is reduced in KCC2shRNA-electroporated cells (bottom, green, boxed region). CA1 region is identified using NeuN immunostaining (blue). Scale bar, 10µm. (**B2)** High magnification images of boxed regions in **B1**. Arrows indicate KCC2 expression at the membrane. Scale bar, 5µm. **C.** KCC2 intensity is significantly reduced in pyramidal cells electroporated with KCC2 shRNA, compared to neighbouring, untransfected cells in both naive and LHS rats (two-way ANOVA, $p<0.001$). N = 40 cells from 3 Ctrl; n=65 cells from 3 KCC2 shRNA electroporated rats; n=70 cells from 3 Ctrl + LHS rats; n=48 cells from 3 KCC2 shRNA + LHS rats.

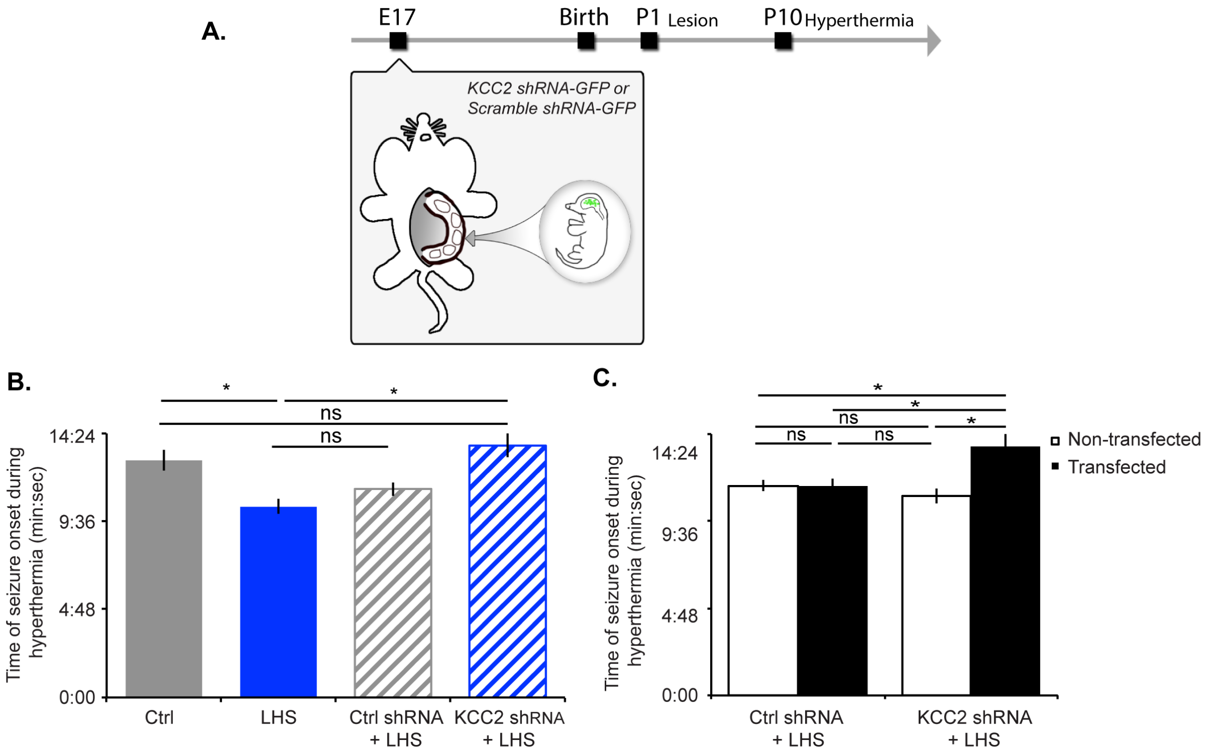


Figure 5. shRNA-mediated KCC2 reduction in CA1 pyramidal neurons decreases hyperthermia-induced seizure susceptibility in LHS rats.

A. Schematics of experimental procedure. **B.** Seizure onset time during hyperthermia is significantly lower in non-electroporated LHS pups (LHS) or LHS pups electroporated in the hippocampus *in utero* with control constructs (Ctrl shRNA+LHS) compared to naïve pups (Ctrl) (one-way Anova, *p<0.05). On the other hand, mean seizure onset time in LHS pups electroporated with KCC2 shRNA (KCC2 shRNA+LHS) is not significantly different from the one recorded in Ctrl pups (one-way Anova, n.s. p>0.05). Ctrl: n=12, LHS: n= 32, Ctrl RNAi+LHS: n=17, KCC2 shRNA+LHS: n=7. **C.** Difference in mean seizure onset time between pups showing GFP-positive pyramidal neurons in CA1 region (transfected) versus pups lacking GFP-positive cells (untransfected), in litters electroporated with either KCC2shRNA (two-way Anova, *p<0.001) or Control constructs (two-way Anova, n.s. p>0.05). Ctrl shRNA+LHS: transfected pups n=17, untransfected pups n=26; KCC2 shRNA: transfected pups n=7, untransfected pups n=18.

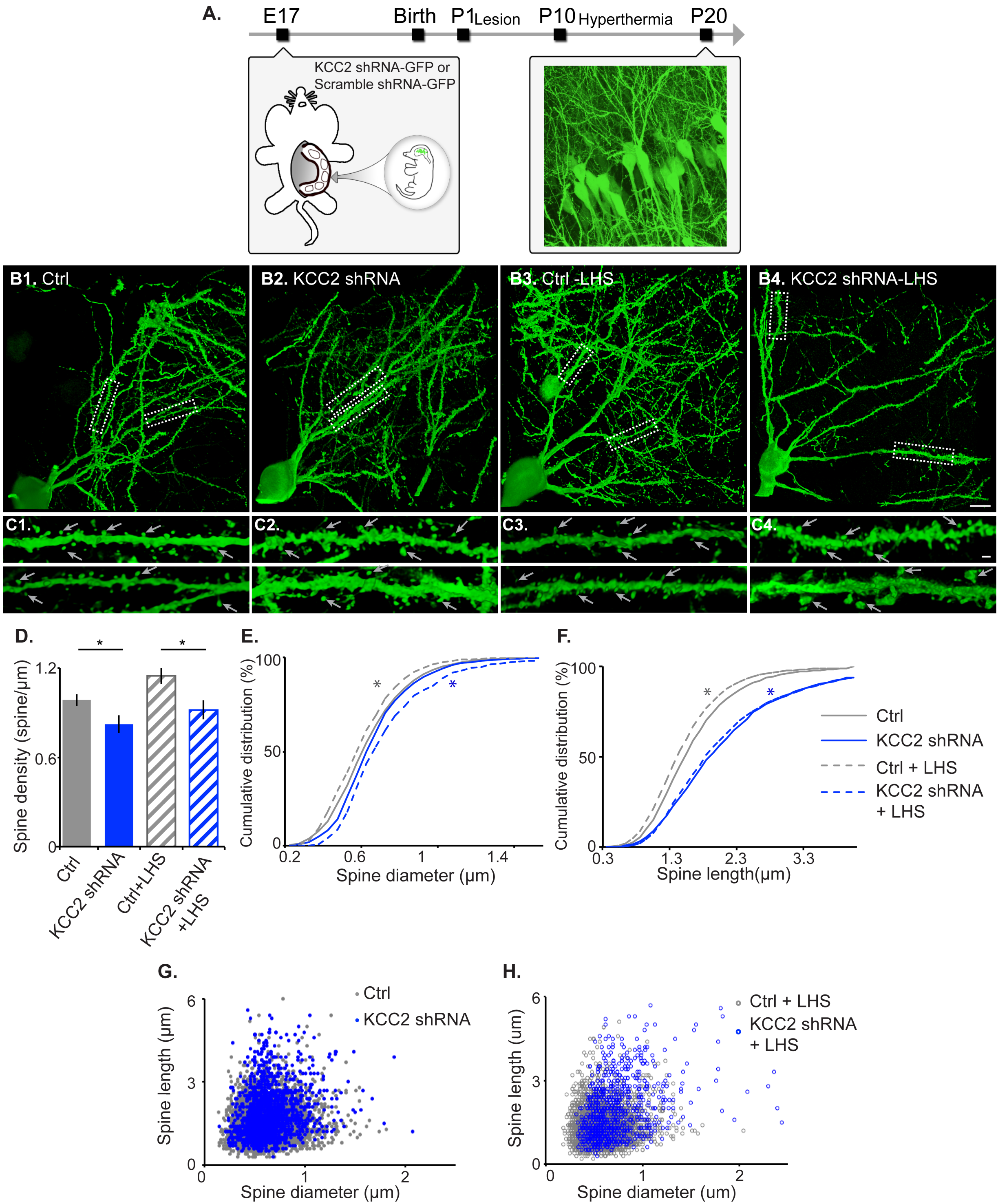
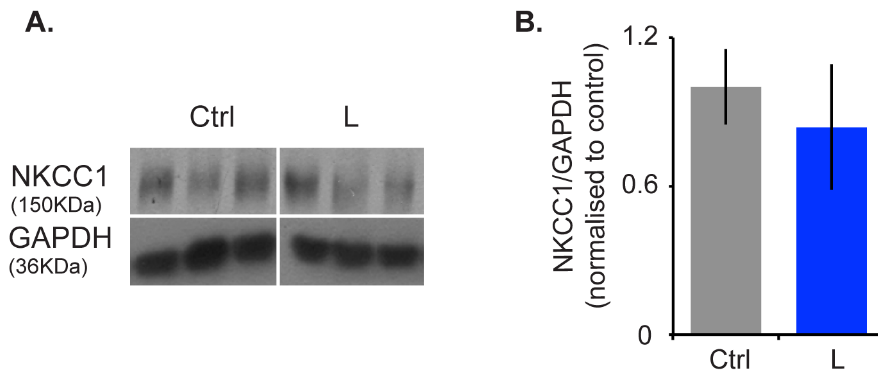


Figure 6. shRNA-mediated KCC2 reduction in CA1 pyramidal neurons rescues spine size in LHS rats.

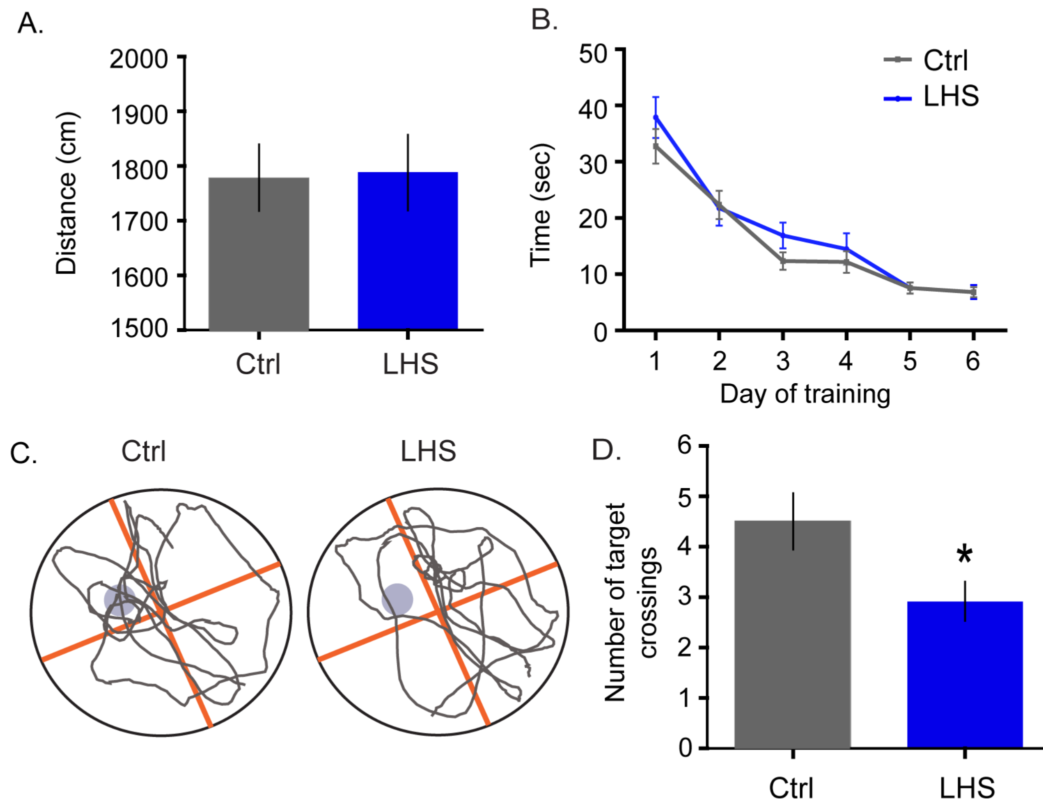
Figure 6. shRNA-mediated KCC2 reduction in CA1 pyramidal neurons rescues spine size in LHS rats.

A. Schematics of experimental procedure. Image in right panel is representative of the level of transfection obtained with this technique. **B.** Examples of CA1 pyramidal neurons from naive (**b1**, **b2**) or LHS (**b3**, **b4**) pups electroporated with Control (Ctrl, **b1**, **b3**) or KCC2shRNA (**b2**, **b4**) constructs. Scale bar, 10µm; **C**. High magnifications images of basal dendrites from boxed region in the respective neurons showed in **B**. Arrows indicate mushroom spines. Scale bar, 1µm. **D.** Spine density is reduced in rats electroporated with KCC2 shRNA, independently on the presence of the dual pathology (two-way Anova, $^{*}p<0.05$). **E**. Cumulative distribution of spine head size is shifted toward smaller values in Ctrl-LHS compared to naïve rats electroporated either with Ctrl or KCC2shRNA constructs (K-S test, $p<0.001$). On the other hand, LHS rats electroporated with KCC2shRNA show significantly larger spine diameters (K-S test, $p<0.001$; Ctrl: n= 18 pyramidal cells from 8 animals, KCC2 shRNA: n=9 pyramidal cells from 4 animals, Ctrl+LHS: n=15 pyramidal cells from 8 animals, KCC2shRNA+LHS: n=7 from 6 animals). **F.** Spine length is increased in rats electroporated with KCC2 shRNA, independently on the presence of the dual pathology (K-S test, $p<0.001$). **G-H**. Scatter plots where each symbol represents a spine (diameter x spine length) in naïve (**G**) or LHS (**H**) rats electroprotaed with Ctrl or KCC2-shRNA constructs. Data point distribution in LHS rats electroporated with KCC2-shRNA is shifted to the right compared to the one in LHS rats electroporated with control constructs (**H**), which is indicative of the presence of larger and longer spines in the former group. Ctrl: n= 19 pyramidal cells from 8 animals, KCC2 shRNA: n=9 pyramidal cells from 4 animals, Ctrl+LHS: n=14 pyramidal cells from 12 animals, KCC2shRNA+LHS: n=10 from 6 animals.



Supplementary Figure 1. NKCC1 expression is not affected in P10 lesioned pups.

A. Western blot analysis of NKCC1 expression levels (band at 150KDa) in the hippocampus of P10 control rats (Ctrl) and of rats subjected to a cortical dysplasia (L). Each lane represents a different animal. **B**. Quantification shows no difference in NKCC1 expression levels between L and Ctrl male pups (Student's t-test, $p>0.05$). Ctrl: n=8 males; L: n=4 males.



Supplementary Figure 2. LHS rats show spatial memory deficits.

A. Distance travelled by P40 control (Ctrl) and LHS male rats in the Open Field, the day prior to Morris Water Maze testing. There is no statistical difference between control and LHS rats, demonstrating the lack of locomotive deficits in the latter group (Unpaired Student t-test, $p>0.05$). **B**. Mean time to reach the platform during training (Days 1-6) is no statistically different between control and LHS rats, suggesting that LHS rats have no major learning deficits. **C.** Examples of typical swimming pattern trajectories in the Morris Water Maze of a control and a LHS rat during the probe test (Day 7). **D**. The mean number of target crossings during the probe test is significantly lower for LHS than for Ctrl rats (Unpaired Student T test, $*p<0.05$), suggesting memory retention deficits in LHS rats. Ctrl: n= 14, LHS: n= 11 male rats.

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Chapter III

Opposite effects of KCC2 on synapse formation in hippocampus and cortex

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Abstract

The electroneutral KCC2 co-transporter is the major chloride (Cl^-) extruder in mature neurons. The spatio-temporal regulation of KCC2 transcription and translation orchestrates the developmental shift of synaptic GABAergic transmission from depolarizing to hyperpolarizing or shunting. Recent studies also indicate a critical role for KCC2 in both glutamatergic synapse formation and functional maintenance. In fact, it has been shown, by either global or cell-and temporal-restricted downregulation of KCC2 expression, that KCC2 reduction leads to immature dendritic spines in young cortical neurons, while it reduces AMPA receptor clustering in mature hippocampal neurons. Conversely, premature KCC2 overexpression leads to an increased spine density in pyramidal cortical neurons. Whether the effects of alterations of KCC2 overexpression on dendritic spines are circuit-specific is unknown. Here, we performed site-specific *in utero* electroporation of KCC2 cDNA using a novel triple-electrode approach, to target either hippocamapal CA1 or somatosensory cortical pyramidal neurons. We found that KCC2 premature expression significantly decreased spine density in CA1 pyramidal neurons, while it had the opposite effect in cortical principal neurons. These effects were cell autonomous, because single-cell biolistic overexpression of KCC2 in hippocampal organotypic cultures also induced a reduction of dendritic spine density. Altogether, these results demonstrate that premature increase in KCC2 expression affects dendritic spine development in a brain region specific manner.

Introduction

KCC2 is a potassium-chloride cotransporter, the only member of the Cation Chloride Cotransporter family that is almost exclusively expressed in neurons. It has been widely shown that the increase in KCC2 expression during development is responsible for the shift of GABA function from excitatory to shunting/inhibitory (Chudotvorova et al., 2005; Fiumelli et al., 2005; Lee et al., 2005; Blaesse et al., 2006). KCC2 has gained a lot of attention recently because its downregulation, either as a putative cause or as a consequence of a pathological condition, can occur in diverse neurological conditions, including epilepsy, neuropathic pain and ischemia (Ferrini and De Koninck, 2013; Loscher et al., 2013; Kaila et al., 2014). By increasing intracellular Cl^- concentration, downregulation of KCC2 reduces GABA transmission efficacy, thereby increasing network hyperexcitability.

On the other hand, alterations of KCC2 expression likely have more complex consequences on circuit activity. In fact, in addition to regulating the Cl^- gradient, recent data suggest that KCC2 may also modulate several aspects of neuronal development, including dendritic spine formation (Li et al., 2007; Gauvain et al., 2011; Fiumelli et al., 2013). Removing KCC2 in immature cortical neurons prevented spine maturation altogether, leading to an increase of filopodia protrusions (Li et al., 2007). Conversely, removing KCC2 in mature hippocampal neurons, after spine formation and when KCC2 expression is higher, did not affect spine density but reduced the efficacy of excitatory synapses, through an alteration of aggregation of AMPA receptors in dendritic spines (Gauvain et al., 2011). Interestingly, these effects were not due to reduction of transporter activity, but to altered interactions of KCC2 with the cytoskeleton (Li et al., 2007; Gauvain et al., 2011). All together these studies show that reduction of KCC2 expression can directly affects both GABA drive and glutamatergic synaptic signaling, by distinct mechanisms.

Conversely, the effects of premature KCC2 expression in circuit formation are less understood. One study showed that *in utero* electroporation of KCC2 in cortical pyramidal cells induced a premature negative shift in E_{GABA} that was in turn responsible for a reduction

of dendrite complexity (Cancedda et al., 2007). Using an identical strategy, a second study did not find any effects on dendritic arborisations but showed that premature expression of KCC2 in cortical pyramidal neurons *in vivo* induced a long-lasting increase in dendritic spine density and spontaneous excitatory activity, through a mechanism that was independent of its ion transport function (Fiumelli et al., 2013). Understanding how precocious KCC2 expression in the developing brain may affect specific long-term synapse formation is important, since, opposite to what occurs in adult, in the neonatal brain traumatic events, including seizures (Galanopoulou, 2008, Awad et al, 2016), injury (Bos et al., 2013) and the presence of cortical malformation (Awad et al., 2016) actually lead to increased in KCC2 expression and activation. Here, we investigate the effects of premature KCC2 expression on dendritic spine formation in CA1 pyramidal neurons in the hippocampus, because this region is particularly affected by early-life seizures or the presence of cortical malformations (Galanopoulou, 2008, Awad et al, 2016).

Materials and methods

Animals

Sprague–Dawley pups were obtained from Charles River Laboratories (St. Constant, Quebec, Canada) at postnatal day 1 (P1). Pups were culled to 12 per dam, matched by gender, weighed and kept with their mother in a 12 h light/dark cycle with food and water *ad libitum*. Animal care and use conformed to institutional policies and guidelines (CIBPAR, Sainte-Justine Hospital Research Centre, Université de Montréal, Montreal, QC, Canada).

Hippocampal organotypic culture

Slice culture preparation was essentially as described in (Chattopadhyaya et al., 2004). Postnatal day 4 (P4) mouse pups were decapitated, and brains were rapidly removed and immersed in ice-cold culture medium (containing MEM, 20% horse serum, 1 mM glutamine, 13 mM glucose, 1 mM CaCl2, 2 mM MgSO4, 0.5 µm/ml insulin, 30 mM HEPES, 5 mM NaHCO3, and 0.001% ascorbic acid). Coronal brain slices of hippocampus, 400 µm thick, were cut with a Chopper (Stoelting, Wood Dale, IL) into ice-cold culture medium. Slices were then placed on transparent Millicell membrane inserts (Millipore, Bedford, MA), usually 3-5 slices/insert, in 30 mm Petri dishes containing 0.75 ml of culture medium. Finally, they were incubated in a humidified incubator at 34°C with a 5% CO2-enriched atmosphere, and the medium was changed three times per week. All procedures were performed under sterile conditions. Constructs to be transfected were incorporated into “bullets” or “co-bullets” made using 1.6 µm gold particles coated with 30 µg of the DNA of interest. These bullets were used to biolistically transfect slices by gene gun (Bio-Rad, Hercules, CA) at high pressure (180 Pa). Cultures were biolistically tranfected with either pCI-Tomato-pCI GFP alone, pCI-wtKCC2-IRES-eGFP or pCI-mutKCC2-IRES-eGPF together with pCI-tdTomato, from equivalent post-natal day (EP) 6 to EP20. Transfected slices were incubated under the same conditions as described above, before fixation and imaging.

In utero electroporation

The day of mating (limited to 4 h in the morning) was defined as embryonic day zero (E0), and the day of birth was defined as postnatal day zero (P0). E17 timed-pregnant Sprague Dawley rats were anesthetized with isoflurane (induction, 4%; surgery, 2%), and the uterine horns were exposed by laparotomy. The DNA (2 µg/µl in water) together with the dye Fast Green (0.3 mg ml−1; Sigma, St Louis, MO, USA) was injected (5 µl) through the uterine wall into one of the lateral ventricles of each embryo by a sterile 30-gauge needle. After soaking the uterine horn with a phosphate-buffered saline (PBS) solution, the embryo's head was carefully held between tweezer-type circular electrodes (10 mm diameter; Nepa Gene, Bulldog Bio, Canada) to transfect the cortex. To target the hippocampus, we used the tweezer-type circular electrodes as well as a third electrode (7×6×1 mm, platinum-plated copper) which was accurately positioned for electroporation (Dal Maschio et al., 2012). For the electroporation, five electrical pulses (50 V, 50 ms, 150 ms intervals) were delivered with a square-wave electroporation generator (ECM 830, BTX, Harvard Apparatus). Following electroporation, the uterine horns were returned into the abdominal cavity, and embryos continued their normal development. At P20, rats were fixed by transcardial perfusion with 4% PFA in PBS (0.1M, pH 7.4). Perfused brains were then dissected, sectioned coronally in 80 µm thick-slices with a vibratome (Leica VT1000S), imaged and analyzed. Hippocampal CA1 and layer II/III pyramidal cells from at least four animals were used for each experimental condition.

The cDNA encoding KCC2 full-length WT and the loss-of-function mutant KCC2-C568A were previously described and cloned into pCAG-IRES-EGFP (Fiumelli et al., 2005; Cancedda et al., 2007). The pCAG vector bears a modified chicken b-actin promoter with a cytomegalovirus immediate-early enhancer that directs high and persistent expression levels in neurons *in vivo* of both KCC2 and Tomato (Niwa et al., 1991). The expressing vectors pCAG-KCC2-IRES-EGFP and pCAG-KCC2-C568A-IRES-EGFP were co-transfected with pCAG-IRES-tdTomato (Cancedda et al., 2007), and pCAG-IRES-tdTomato alone was used as a control. They will thereafter be termed ‘KCC2wt’, ‘KCC2mut’ and ‘Control’ respectively.

Imaging and spine analysis

Pyramidal neurons were imaged using a Leica confocal microscope SPE or SP8 (63x oil immersion objective; NA 1.3). Two-three labeled typical pyramidal neurons were randomly selected from CA1 area or the somatosensory cortex of each analyzed animal. Pyramidal cells from at least four animals were used for each experimental condition. Four image stacks of basal dendrites were acquired (0.5 μm intervals) all around the pyramidal cell soma. To avoid bias due to variability in labeling between different cells and problems in identifying dendrite provenance in highly transfected areas, we analyzed only the basal dendrites included in four identically sized stacks centered around the soma, which in our imaging conditions represent the first 120μm of basal dendrites originating from the soma.

Total spine density, spine morphology, spine length and dendritic length were analyzed and quantified using Neurolucida software (MicroBrightField). Mushroom spines were defined as spine with a neck and bearing a head, which was at least twice as large as the neck. Values for animals of the same experimental group were not statistically different and were pooled. All quantification were done blind to the treatment. To measure spine head diameter we first deconvolved the stacks using Volocity software, and the images were converted from GFP or tomato to rainbow coloring, allowing a better delineation of the spine area, by becoming white where fluorescence is brightest, all the way to black where there is no signal.

Statistical analysis

Differences between two experimental groups were assessed with t-test for normally distributed data and Mann-Whitney test for not normally distributed data. Differences between 3 or more experimental groups were assessed with one-way ANOVA and post hoc comparison. For non-normally distributed data, nonparametric Kruskal–Wallis one-way ANOVA test was used. Cumulative distributions were analysed using the Kolmogorov-Smirnov test. All results are expressed as mean ± SEM.

Results

Premature KCC2 expression induces a long-term increase in dendritic spine density in cortical neurons (Fiumelli et al., 2012). However, whether KCC2 premature expression affects spinogenesis in hippocampal pyramidal neurons in the same fashion is unknown. To address this question, we overexpressed KCC2 by *in utero* electroporation at embryonic day 17 (E17) specifically in the CA1 region of the hippocampus, by using a triple-electrode probe (Dal Maschio et al., 2012). We expressed two different forms of KCC2, the wild type form (KCC2wt) as well as KCC2 carrying the C568A mutation (KCC2mut), which lacks both the cotransporter activity and its binding activity to the protein 4.1N, which mediates the interaction with the cytoskeleton. KCC2-GFP plasmids were co-electroporated with Td-Tomato, to label neuronal morphology. Control neurons were transfected with Td-Tomato alone. Transfected CA1 pyramidal cells were imaged and analyzed at P20 (Fig. 1A).

Overexpression of KCC2wt, but not KCC2mut, significantly decreased spine density in basal dendrites of pyramidal cells (Fig 1B and C; Ctrl: 0.78±0.06 spine/μm; KCC2mut: 0.82±0.08 spine/μm; KCC2wt: 0.62±0.04 spine/μm; one way ANOVA *:$p<0.05$). On the other hand, KCC2mut expressing cells showed significantly longer spines (Fig 1D; Ctrl: 1.42±0.05 μm, KCC2mut: 1.63±0.02 μm, KCC2wt: 1.42±0.05 μm; one way ANOVA *:$p<0.05$). Longer spines have been suggested to represent immature thin or filopodia spine (Bosch and Hayashi, 2011), however KCC2mut expressing cells showed long spines with a distinctive mushroom head, which indicated that they were not immature. Although mushroom spine density was not significantly different in KCC2mut cells (Suppl. Fig. 1A; Ctrl: 0.30±0.03 spine/μm, KCC2wt: 0.23±0.03 spine/μm; KCC2mut: 0.35±0.05 spine/μm; one-way ANOVA $p>0.05$), spine diameter analysis revealed that KCC2mut cells had significantly larger spines compared to Ctrl and KCC2wt cells (Fig 1E; average Ctrl: 0.650±0.009 μm, KCC2wt: 0.675±0.003 μm; KCC2 mut: 0.86±0.01 μm; one way ANOVA, *$p<0.05$; K-S test, **$p<0.001$) The scatter plot representation clearly showed the rightward shift to longer and bigger spines induced by KCC2mut overexpression (Fig.1F, red; Suppl. Fig. 1E). Additionally, overexpression of KCC2wt lead to larger spine heads compared to

controls, but smaller diameter than KCC2mut (Fig. 1E, KS test, **$p<0.001$). KCC2 precocious overexpression in cortical neurons has been reported to affect dendrite development (Cancedda et al., 2007). As such, we reconstructed basal dendrites for up to 120 µm from cell bodies, where we could reliably separate dendrites belonging to different pyramidal cells, and found that dendritic branch number and length were not majorly affected by KCC2wt or KCC2mut overexpression (Suppl. Fig 1C and D). The lack of effect of KCC2 premature expression on CA1 basal dendritic length and complexity may be due to the different developmental stage of CA1 and cortical pyramidal neurons at the time of transfection.

Next, we asked whether the effects of KCC2 overexpression in CA1 pyramidal neurons were due to cell autonomous or circuit-based effects. To address this question, we overexpressed KCC2wt in isolated pyramidal cells in hippocampal organotypic cultures, by biolistic transfection, from equivalent post-natal day (EP) 6 to EP20 (Fig. 2A). Similarly to what was observed following in utero-electroporation *in vivo*, KCC2 wt-overexpressing cells displayed a significant decrease in spine density compared to control pyramidal neurons (Fig. 2B to D, Ctrl: 0.99 ± 0.12 spine/µm; KCC2wt: 0.56 ± 0.05 spine/µm; Mann-Whitney Rank Sum Test $p<0.05$). Spine density reduction was widespread along the dendritic arbor (Fig. 2E) and affected different spine types, including mushroom (Fig.2F, Ctrl: 0.61 ± 0.07 spine/µm; KCC2wt: 0.36 ± 0.05 spine/µm; t-test $p<0.05$), thin (Ctrl: 0.15 ± 0.02 spine/µm; KCC2wt: 0.08 ± 0.01 spine/µm; t-test $p=0.001$) and stubby spines (Ctrl: 0.23 ± 0.04 spine/µm; KCC2wt: 0.12 ± 0.01 spine/µm; trend but not significantly different, $p>0.05$). In contrast, average spine length was unaltered in KCC2wt-expressing cells compared to controls (Fig. 2G, Ctrl: 1.59 ± 0.04 µm; KCC2wt: 1.75 ± 0.12 µm; t-test $p>0.05$). Overexpression of KCC2 from EP6-20 did not significantly affect the overall morphology of pyramidal cells, in terms of total dendritic length (Suppl. Fig. 2A, t-test $p>0.05$), and Scholl analysis of dendritic arbor complexity (Suppl. Fig 2B, t-test $p>0.05$ per radius, apart for first radius closest to soma). Thus, the reduction in spine density we observed was not due to a general delay in the maturation of pyramidal cell dendritic arbor.

All together, these results confirm that premature overexpression of KCC2 leads to a

decrease of spine density onto CA1 pyramidal neurons, both *in vivo* and *in vitro*. Further this effect is cell autonomous, as low-density overexpression of KCC2 in organotypic cultures showed similar results as high-density KCC2 electroporation *in vivo*.

It was previously reported that KCC2 premature expression following in utero electroporation in the somatosensory cortex caused a long-term increase in pyramidal cell spine density, while we observed the opposite effect following in utero electroporation of the hippocampus. To verify that our results were not due to technical or analysis differences, we electroporated the same constructs described above in somatosensory cortex, as described by (Fiumelli et al., 2013) (Fig. 3A-B). Consistently with this previous study, we found that KCC2wt precocious expression caused spine density increase (Fig. 3D, Ctrl: 1.41 ± 0.12 µm; KCC2wt: 2.17 ± 0.17 µm; one-way ANOVA, *$p<0.05$), while it did not affect spine head size (Fig. 3E, 3G, Ctrl: 0.61 ± 0.02 µm; KCC2wt: 0.61 ± 0.02 µm, one-way ANOVA $p>0.05$, K-S test, $p>0.001$) or spine length (Fig. 3F, Ctrl: 1.85 ± 0.04 µm; KCC2wt: 2.14 ± 0.12 µm, one-way ANOVA $p>0.05$). KCC2mut expression, on the other hand, did not affect spine density, length or head size (Fig. 3D, one way ANOVA, $p>0.05$).

All together our data demonstrate that the effects of KCC2 premature expression are circuit- and brain-region specific.

Discussion

Proper function of neural circuits requires the orchestrated formation of trillion of synapses. Studying how synapses form is necessary to understand both how the brain functions normally and how this process goes awry in disease. These studies often focus on specific neuron cell types from specific brain region. However, one important question is whether molecular mechanisms regulating synapse formation in a specific neuronal cell type (for example, glutamatergic neurons) can be generalized to similar neurons localized in different brain regions. Our findings suggest caution is necessary before generalizing; in fact, we demonstrated that during the same developmental time window, KCC2 plays two distinct and opposing roles on synapse formation in CA1 region of the hippocampus and in the overlying somatosensory cortex, two structures that are often considered similar when synapse formation mechanisms are tackled.

Because of KCC2 dual actions as mediator of inhibitory electrical signals, which is based on its regulation of Cl^- currents, and as regulator of glutamatergic synapse formation, which is based on its structural interaction with the cytoskeleton, it has been suggested that KCC2 is perfectly poised to be a key molecular player in the synchronization of excitatory and inhibitory activities. Following this working hypothesis, an increase in KCC2 would both increase the inhibitory drive, by decreasing intracellular Cl^- concentration, and the strength of excitatory synapses, by increasing both synapse density (Fiumelli et al., 2013) and GluR1-containing AMPA receptor clustering (Gauvain et al., 2011). However, our results show that, while KCC2 overexpression indeed increases spine density in cortex, it actually has the opposite effect in hippocampus. Therefore, the role of KCC2 in matching glutamatergic and GABAergic activities is likely more complex than hypothesized so far and may be circuit and likely age-dependent.

During neural circuit formation, synapse number is regulated both by hard-wired and activity-dependent mechanisms. In this context, we could envision the battling of two actions induced by KCC2 overexpression, the spinogenesis promoting effects, mediated by cytoskeleton interactions, and the transporter-mediated alteration of GABA driving force. In

fact, premature overexpression of KCC2 leads to more negative E_{GABA} (Rivera et al., 2004; Cancedda et al., 2007; Galanopoulou, 2008; Khirug et al., 2010; Pellegrino et al., 2011), which may render spike generation less likely. Reduced neuronal activity may in turn impair synapse potentiation, therefore leading to synaptic loss. It is possible that the effects of KCC2 premature expression on spinogenesis may be mediated by global alterations of neuronal activity, which may occur following high-density *in utero* electroporation. On the other hand, single-cell KCC2 overexpression in CA1 pyramidal neurons in hippocampal organotypic cultures, which most likely did not perturb network activity, induced dendritic spine loss similarly to what observed following in utero electroporation *in vivo*. Therefore, it seems more likely that it is the specific cellular context in which the transfected neurons develop that determine how KCC2 overexpression affects spine formation.

What are the molecular mechanisms underlying the different effects of precocious KCC2 expression in cortical vs. CA1 pyramidal neurons? The Brain Derived Neurotrophic Factor (BDNF) is one the strongest modulator of KCC2 activity. Most importantly, this modulation is age-dependent as BDNF accelerates KCC2 expression, and, possibly, its phosphorylation-mediated activation in developing neurons in normal condition or following seizures (Fiumelli et al., 2005; Fiumelli and Woodin, 2007; Khirug et al., 2010), while it has the opposite effects in adult neurons following injury, trauma or seizures (Kaila et al., 2014). BDNF expression levels increase after birth with a different developmental time course depending on the brain region. In particular, BDNF levels increase sharply between P10 and P15 in the visual cortex (Bozzi et al, 1995), but at least few days earlier in the dorsal hippocampus (data not shown). BDNF itself can either facilitate or inhibit GABAergic transmission depending on which specific age and neural circuits are investigated (Mizoguchi et al., 2003; Huang et al., 2012). Therefore, the different expression of BDNF, its receptor(s) and, possibly, downstream signaling components may contribute to the opposite effects of KCC2 on spine formation in the cortex and hippocampus. Another possibility is that KCC2 localization may be regulated differently in CA1 vs. cortical pyramidal neurons during the first postnatal week. In fact, we observed KCC2 signal localized at the membrane in sparse CA1 pyramidal neurons at P1, while in the overlying cortex, KCC2 immunosignal, when it was detectable, was localized exclusively in pyramidal cell cytoplasm (data not shown).

However, whether KCC2 needs to be localized at the membrane to interact with the cytoskeleton, and thus influence synapse formation, is still a matter of controversy.

We recently reported that electroporation of shRNA against KCC2 at E17.5 induced a reduction of spine density in CA1 pyramidal neurons quantified at P20 (Awad et al, 2016). At first glance, this observation may appear discordant with the data presented here, showing that KCC2 overexpression by the same technique during the same developmental period also causes spine loss. Using shRNA-mediated manipulation and KCC2-deficient mice, a recent study showed that KCC2 interacts with and inhibits β-PIXb, a GEF for small GTPases Rac1 and Cdc42, in hippocampal dissociated cultures (Llano et al., 2015). β-PIX, through activation of Rac1, forms part of the signaling cascade controlling cofilin-1 phosphorylation (Saneyoshi et al., 2008; Mizuno, 2013). Rac1 and cofilin-1 are known to play a pivotal role in spine morphogenesis. Interestingly, the activity of these two proteins must be tightly regulated to obtain normal spine formation. For example, knockdown of cofilin-1 by shRNA and overexpression of constitutively active cofilin-1 induces similar phenotype in developing neurons: long filopodia-like structures (Hotulainen et al., 2009; Shi et al., 2009). Similarly, both long-term increase and decrease of Rac1 activity leads to reduced number of spines (Nakayama et al., 2000; Zhang et al., 2003). In other words, the shift in actin turnover balance in either direction results in disrupted spine development. Therefore, either reducing or increasing KCC2 expression may alter the ratio between active and inactive forms of β-PIX, which in turn would alter both Rac1 and cofilin-1.

Recently, several studies, including ours, showed that insults in the developing brain, such as seizures or cortical dysplasia (Galanopoulou, 2008; Khirug et al., 2010; Awad et al, 2016), induced a premature expression of KCC2. Overall, our results suggest that these pathology-induced alterations of KCC2 expression may differentially affect the development of distinct neural circuits. It will be important to understand whether and how these specific circuit alterations affect diverse cognitive functions.

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Author Contributions

G. Di Cristo, L. Carmant and P.N. Awad designed the study and wrote the manuscript.

Patricia performed the organotypic culture and *in utero* electroporation in the cortex experiments, and analyzed spine density and morphology.

Joanna performed the overexpression of KCC2 in the hippocampus by *in utero* electroporation experiment.

Elie performed a spine morphology analysis.

Laura Cancedda has given crucial feedback on the project and manuscript.

Potential Conflict of interest. The authors have declared that no conflict of interest exists.

Figures

Figure 1

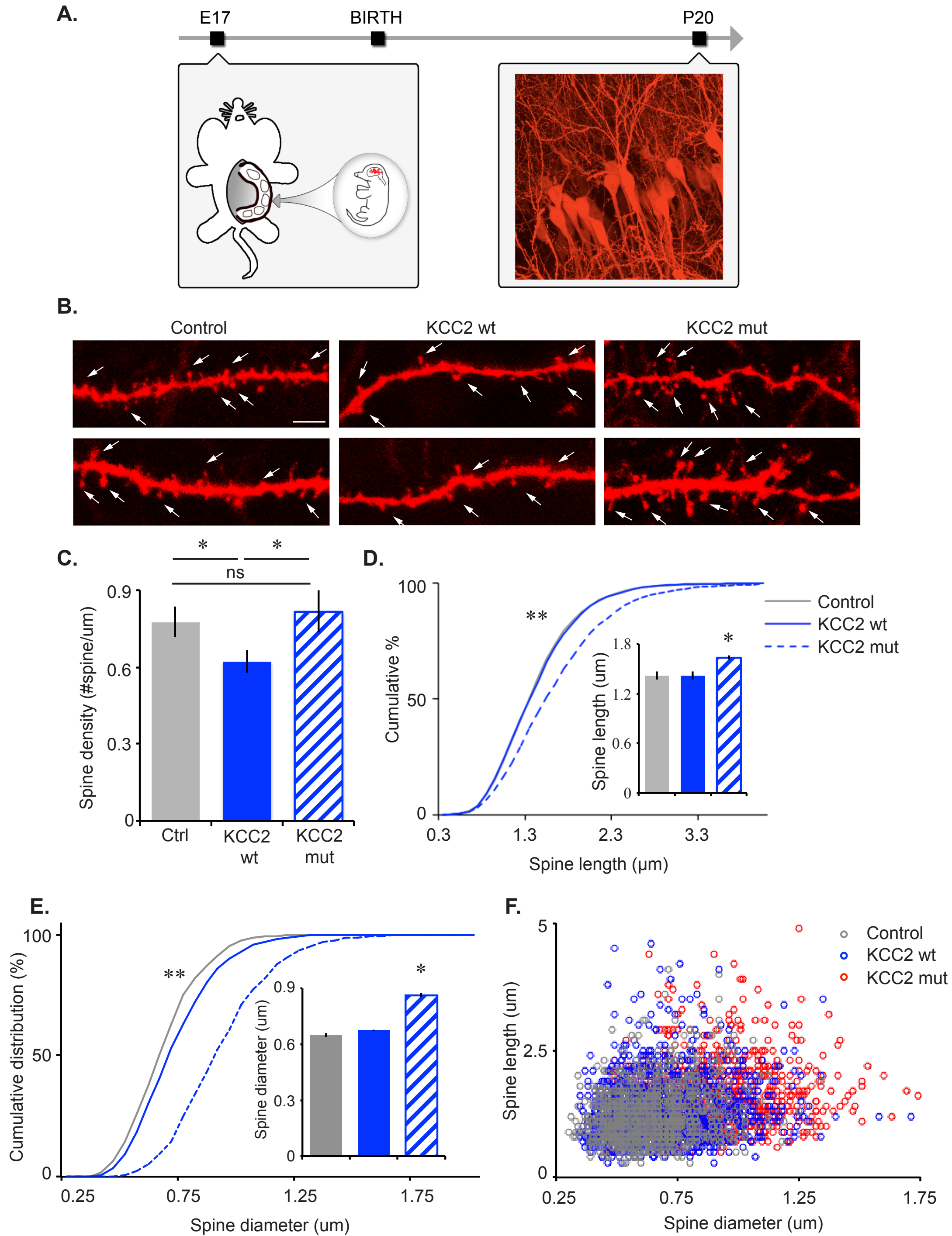


Figure 1. Precocious expression of KCC2wt from E17.5 to P20 *in vivo* decreases spine density in hippocampal CA1 pyramidal cells.

A. Schematics of experimental procedure. Hippocampal pyramidal cells were electroporated *in utero* at E17.5 with Td-Tomato (Ctrl) or KCC2wt-GFP+Td-Tomato (KCC2wt) or KCC2mut-GFP+Td-Tomato (KCC2mut), then fixed and imaged at P20. Image at P20 is representative of the level of transfection obtained with this technique. Only tdTomato signal is shown. **B.** Representative basal dendrite segments from two different CA1 pyramidal cells showing that KCC2wt-expressing cells have fewer spines, while KCC2mut have longer spines (arrowheads). Scale bar 5 μm. **C.** Spine density is significantly reduced in KCC2wt-expressing cells compared to control and KCC2mut-expressing ones. (1-way Anova, $*p<0.05$). **D.** Cumulative distribution and average (insert) spine length (1-way Anova $*p<0.05$; Kolmogorov-Smirnov test, $**p < 0.001$). n= 8 for controls from 5 animals, n= 9 for KCC2wt from 5 animals, n=5 for KCC2mut from 3 animals. Values in ***C*** and ***D*** (insert) represent mean ± SEM.

Figure 2

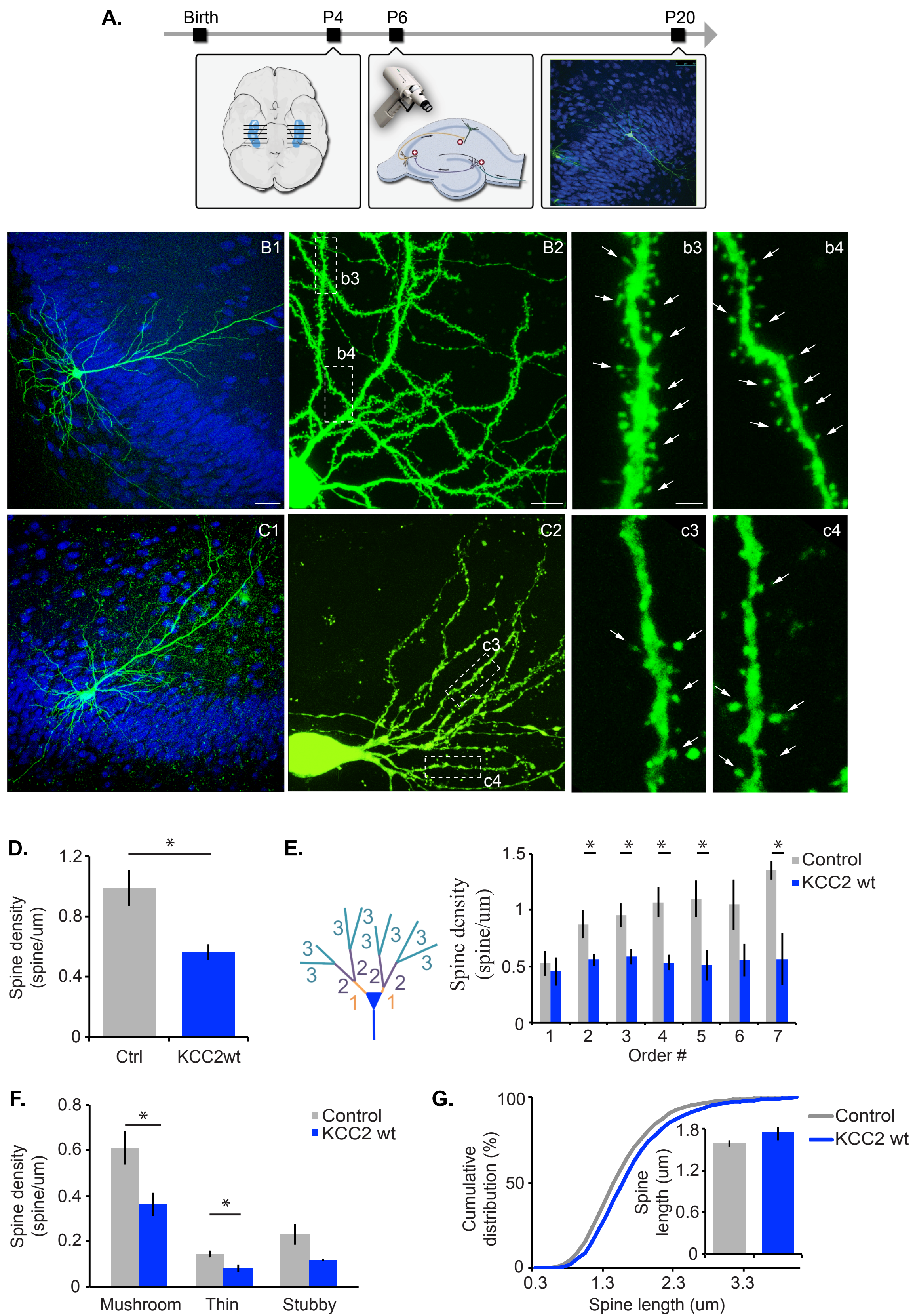
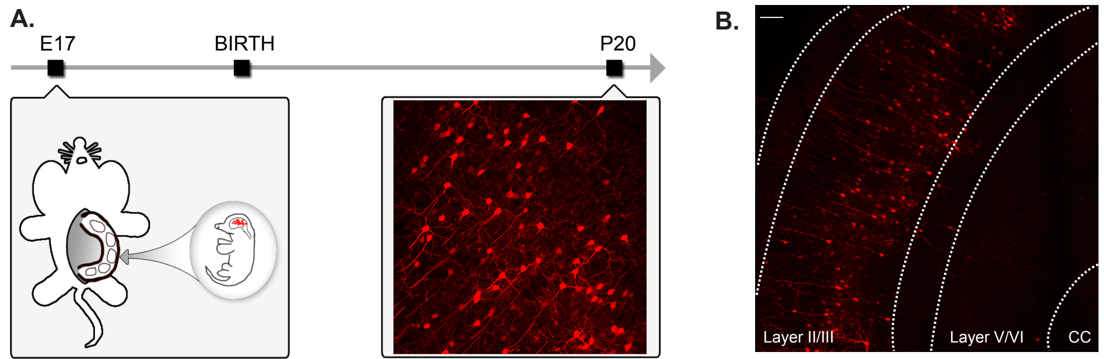
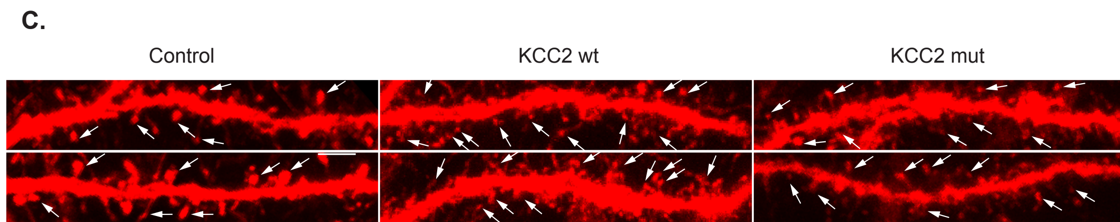
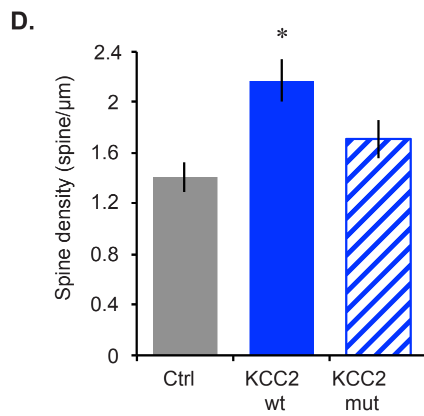


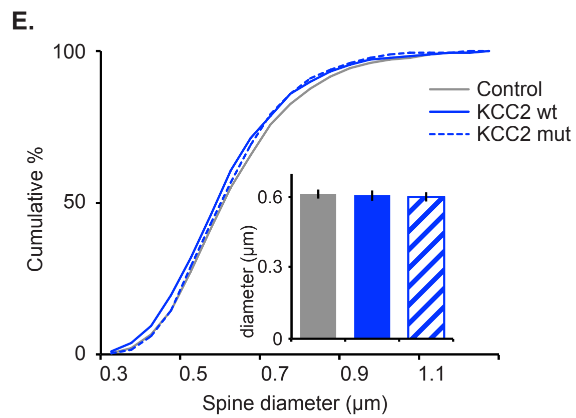
Figure 2. KCC2wt precocious expression decreases spine density in hippocampal CA1 pyramidal cells in organotypic cultures.

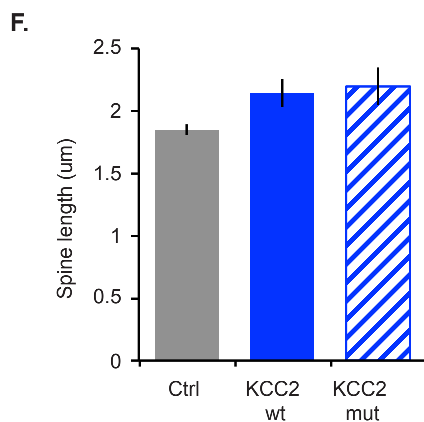
A. Schematics of experimental procedure. Hippocampal organotypic culture slices were biolistically transfected with pCI-GFP (Ctrl) and pCI-KCC2wt-IRES-GFP (KCC2wt) at P6, then fixed and imaged at P20. **B-C.** Low (B1,C1) and high (B2, C2) magnification of CA1 transfected pyramidal cells show no gross difference in dendritic arbor morphology (B1-C1; NeuN immunostaining, blue). However, KCC2wt-expressing cells have fewer spines (c3, c4, arrowheads) compared to control cells (b3, b4, arrowheads). b3, b4 and c3, c4 are from boxed regions in B2, C2. Scale bars B1-C1, 50 μm; B2-C2, 10 μm; b3-b4-c3-c4, 2 μm. **D.** Spine density is strongly reduced in KCC2wt-overexpressing cells (Mann-Whitney Test, $*p<0.05$). **E.** *Left*, schematic of branch order definition. *Right*, Spine density in KCC2wt-expressing cells is overall reduced independently from the dendritic branch order (Mann-Whitney Test, $*p<0.05$). **F.** Both mushroom (Student's t-test $*p<0.05$) and thin (Student's t-test $*p=0.001$) spines are significantly reduced in KCC2wt-expressing cells. **G.** Cumulative distribution and average (insert) spine length show no statistical difference between KCC2wt-expressing and control cells (Student's t-test $p>0.05$, ns). n= 6 pyramidal cells from controls, n= 6 pyramidal cells from KCC2wt. Values in ***D-G*** represent mean ± SEM.


A.
E17
BIRTH
P20
B.
Layer II/III
Layer V/VI
CC


C.
Control
KCC2 wt
KCC2 mut


D.
Spine density (spine/µm)
2.4
2
1.6
1.2
0.8
0.4
0
*
Ctrl
KCC2 wt
KCC2 mut


E.
Cumulative %
100
50
0
0.3
0.5
0.7
0.9
1.1
Spine diameter (µm)
Control
KCC2 wt
KCC2 mut
diameter (µm)
0.6
0.3
0


F.
Spine length (um)
2.5
2
1.5
1
0.5
0
Ctrl
KCC2 wt
KCC2 mut

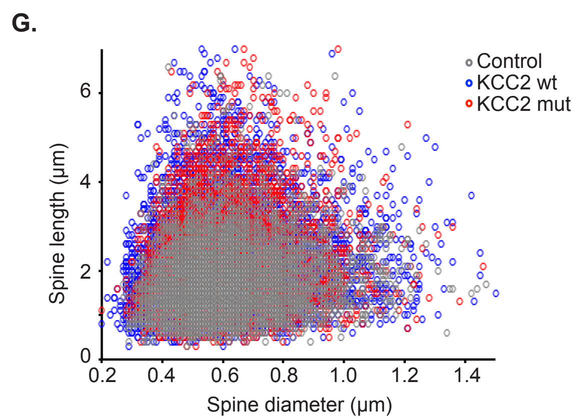
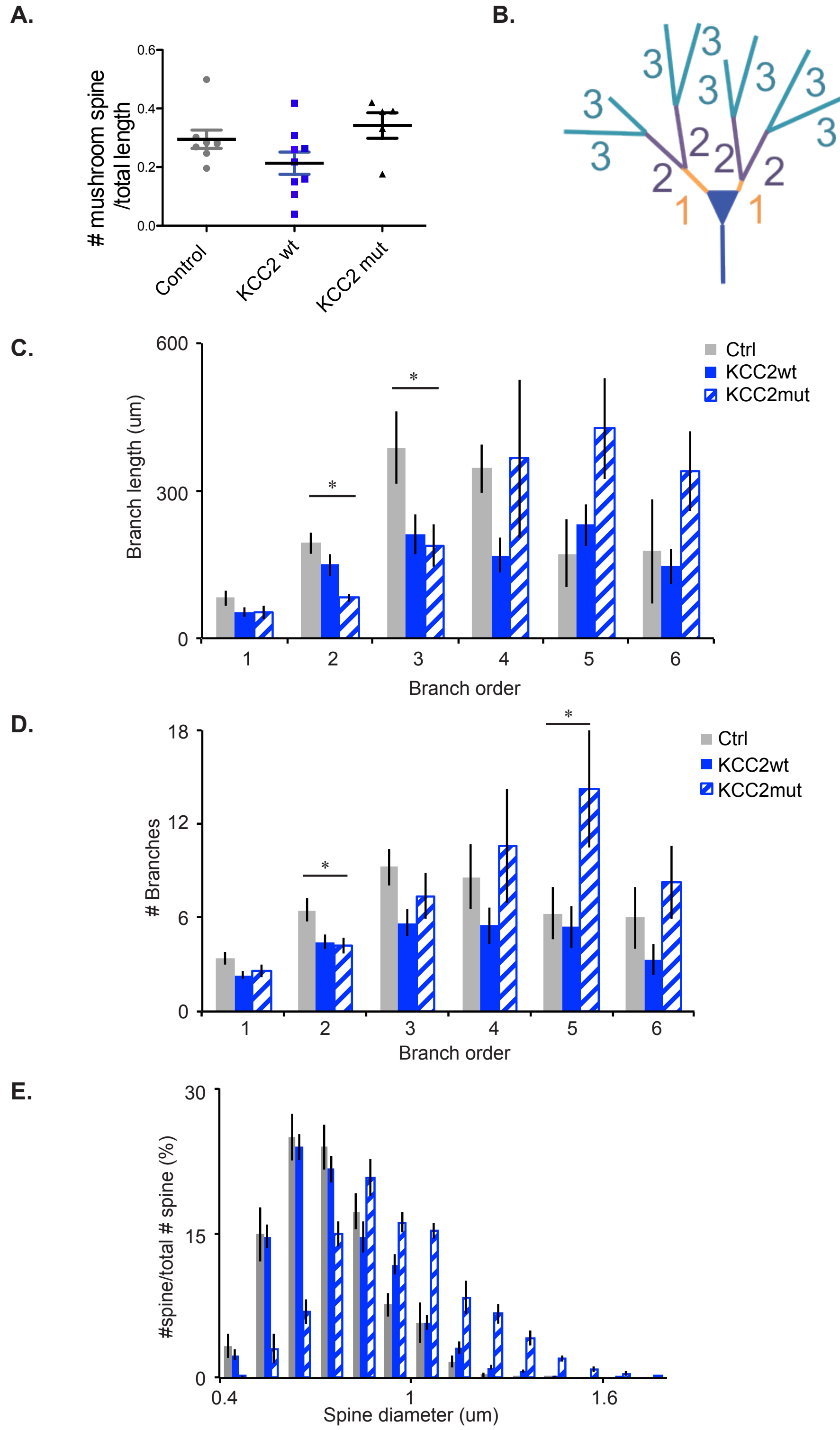

G.
Spine length (µm)
6
4
2
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Spine diameter (µm)
Control
KCC2 wt
KCC2 mut

Figure 3. Precocious expression of KCC2wt in layer II/III pyramidal cells from the somatosensory cortex from E17 to P20 *in vivo* increases spine density.

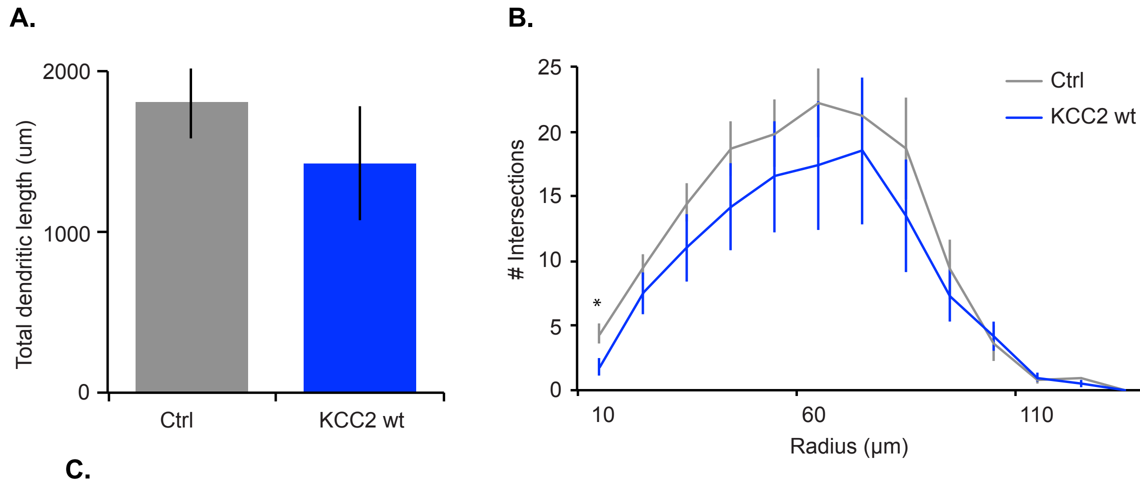
A. Schematics of experimental procedure. Cortical pyramidal cells were electroporated *in utero* at E17.5 with Td-Tomato (Ctrl) or KCC2wt-GFP+Td-Tomato (KCC2wt) or KCC2mut-GFP+Td-Tomato (KCC2mut), then fixed and imaged at P20. Image at P20 is representative of the level of transfection obtained with this technique. Only tdTomato signal is shown. B. Image including all layers of the somatosensory cortex at P20, illustrating the specific transfection of layer II/II pyramidal cells. C. Representative basal dendrite segments from two different layer II/III pyramidal cells showing that KCC2wt-expressing cells have more spines, while KCC2mut have shorter spines (arrowheads). Scale bar 1 µm. D. Spine density is significantly increased in KCC2wt-expressing cells compared to control and KCC2mut-expressing ones (1-way Anova, $*p<0.05$). E. Cumulative distribution and average (insert) spine head diameter illustrating that KCC2mutant have smaller spine heads (Kolmogorov-Smirnov test, $**p < 0.001$). F. Spine length average demonstrates that both KCC2wt and mutant overexpression in layer II/III pyramidal neurons leads to longer spines (two-way Anova, $*p<0.05$). G. Scatter plots where each symbol represents a spine (diameter x spine length) in naïve (grey open circle), KCC2 wt (blue) and KCC2 mut (red). Data point distribution shows that while spines of control cells are very condensed, both electroporated KCC2 constructs is more spread out, which is indicative of the presence of larger and longer spines. Ctrl: n= 7 cells from 4 animals; KCC2wt: n=8 cells from 5 animals, n=6 for KCC2mut from 3 animals. Values in *D* and *F* represent mean ± SEM.


A.
B.
C.
D.
E.
mushroom spine /total length
0.0
0.2
0.4
0.6
Control
KCC2 wt
KCC2 mut
1
2
3
Ctrl
KCC2wt
KCC2mut
Branch length (um)
Branch order
Branches
#spine/total # spine (%)
Spine diameter (um)
0.4
1
1.6

Supplementary figure 1. Dendritic arbor analysis of electroporated CA1 pyramidal cells.

A. Mushroom spine density was not different in KCC2mut cells, while it showed a non-significant trend towards reduction in KCC2wt compared to controls. **B-D**. Dendritic arbor analysis of CA1 cells electroporated with tdTomato (ctrl) or KCC2wt-GFP+Td-Tomato (KCC2wt) or KCC2mut-GFP+Td-Tomato (KCC2mut), then fixed and imaged at P20. Only basal dendrites for up to 120 µm from cell bodies, where we could reliably separate dendrites belonging to different pyramidal cells, were reconstructed. **B**. *S*chematic of branch order definition. Branch length (**C**) and number (**D**) per order. Dendritic branch number and branch length were not majorly affected by KCC2 wt or KCC2 mut overexpression (* 1-way Anova, $p<0.05$). **E**. Graph representing the amount of spine of a specific diameter, compared to the total number of spines. This graph shows that drastic shift of spine enlargement in KCC2mut-expressing cells n= 8 for controls from 5 animals, n= 9 for KCC2wt from 5 animals, n=5 for KCC2mut from 3 animals. Bar graphs *A, C, D* and *E* represent mean ± SEM.

Supplemental figure 2



Supplementary figure 2. Dendritic arbor analysis of KCC2wt-expressing and control CA1 pyramidal cells in organotypic cultures.

A. Total dendritic length is not significantly different. **B.** Scholl analysis representing the complexity of dendritic arbor. Overall, dendritic arbors were not singificantly affected by KCC2 wt overexpression. n= 6 pyramidal cells from controls, n= 6 pyramidal cells from KCC2wt. Bar graphs represent mean ± SEM.

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Chapter IV

General Discussion

KCC2 alterations in epilepsy: pro- or anti-convulsive?

Large evidence suggests that seizures may leave their imprint on the developing brain by altering the way that neurons differentiate, connect, and communicate to each other. This in turn may have functional and behavioral long-lasting consequences[589]. The cellular and molecular mechanisms underlying early-life seizure-induced alterations of neuronal circuit development are still not well understood. Contrary to what occurs in the adult brain, different traumatic events in the nervous system, including seizures and neuronal injury, induce an increase in KCC2 expression levels and function in the young brain[411,412]. In line with these findings, we showed that KCC2 expression is precociously increased in a rodent model of mesial temporal lobe epilepsy (MTLE) induced by two early-life insults. This observation likely explains the more negative E_{GABA} we consistently recorded in CA1 pyramidal neurons from LHS rats compared to controls. Further, we have previously reported that the excitatory drive onto CA1 GABAergic interneurons is increased in LHS rats, which correlates with an increase in sIPSC onto CA1 pyramidal cells[584]. One possibility is that these functional changes enhance the efficacy of GABAergic inhibition, which might act as an intrinsic mechanism to protect the developing brain from hyperexcitability-induced damage caused by early-life insults (see summary figure 19).

Interestingly, shRNA-mediated reduction of KCC2 in a small population of CA1 pyramidal cells was sufficient to rescue the heightened febrile seizure susceptibility caused by the cortical dysplasia, suggesting that precocious KCC2 overexpression may have pro-convulsive effects in a predisposed brain. As recently reported by the Kaila group, it is possible that the seizure-promoting effects of KCC2 occur in specific conditions. As such,

seizure-induced hyperactivity can lead to a deregulation of ionic homeostasis, as intracellular Cl^- starts to accumulate through HCO_3^- mediated-efflux, which increases Cl^- and K^+ extrusion through KCC2. Seizure activity can then cause an increase in extracellular K^+ concentrations, making it increasingly difficult for Na^+K^+-ATPase to maintain homeostasis, causing depolarization of the potassium reversal potential. This ionic homeostasis deregulation compromises the resting membrane potential of neurons and subsequently reduces the threshold for seizure susceptibility[590-592]. Consequently, increased functional levels of KCC2 may counter-intuitively contribute to the emergence of highly synchronized spontaneous network events, including seizures[234,354,592].

An alternative possibility, that cannot be excluded, is that the reduction of KCC2 during a critical period for circuit development may alter neuronal connectivity rendering the network somewhat less excitable. Reduction of KCC2 expression either in constitutive knockout mice or by shRNA transfection suggests that KCC2 plays a role in several aspects of neuronal development, including neuron migration[133], synapse formation[250,284,286,306], transcriptional switch of neurotransmitter receptor subunits[593]. While we did not observe any notable effects of shRNA-KCC2 on electroporated pyramidal cells localization, which ruled out neuron migration alterations, we found that CA1 neurons showed significantly reduced basal dendritic complexity and spine density in P20 electroporated rats. However, these alterations show no direct correlation with seizure onset time following exposure to hyperthermia, as LHS rats electroporated with KCC2-shRNA show seizure onset time comparable to naïve rats, while control LHS rats seize faster, even if they do not show alteration in basal dendrite complexity or spine density. Nevertheless, future studies are required to understand whether and how targeted alterations of KCC2 expression in CA1 neurons after neuronal differentiation alters network activity and, possibly, cognitive functions.

Potential circuit-based mechanisms underlying KCC2 effects on seizure susceptibility

Although only a limited percentage of PCs were electroporated, they were sufficient to rescue seizure susceptibility. A possibility is that since we are electroporating on a specific embryonic day, we may be targeting a subset of CA1 PCs with specific projections. A study recently published in Neuron[111] demonstrated that PV INs specifically target a subset of pyramidal cells according to their specific projections, and depth in the CA1 pyramidal layer. More specifically, Lee and collaborators found that PV INs evoked greater inhibition in deep CA1 PCs; and further segregated among deep PCs to preferentially innervate those that project to the amygdala. In parallel, reciprocal connectivity analysis showed more frequent excitatory inputs of superficial CA1 PCs onto PV INs; and PV INs preferentially received excitation from PCs that project to the prefrontal cortex. These results revealed bias in target selection and innervation by both glutamatergic and GABAergic local CA1 circuitry and further demonstrated the presence of heterogeneous IN-PC microcircuits. Consequently, an attractive hypothesis is that when we are electroporating at E17.5, we might be targeting a subpopulation of PCs, preferentially projecting to the amygdala. A recent study from our group demonstrated a tight relationship between the amygdala and hippocampus during kainic acid-induced seizures. In particular, this study suggested that gamma oscillations in the amygdalo-hippocampal network could facilitate long-range synchrony and contribute to seizure propagation, while synchrony of this network correlated with seizure severity[594]. Thus, downregulating KCC2 in PCs specifically projecting to the amygdala may potentially have a bigger impact on seizure propagation and severity, compared to downregulating KCC2 in CA1 PCs projecting to the prefrontal cortex. On the other hand, KCC2 reduction in prefrontal cortex-projecting PCs may be directly affecting the local feedback and/or feed-forward microcircuits of the CA1. As we discussed in the introduction, alterations of these microcircuits are closely related to seizure-induced alterations[425]. Perhaps, reestablishing normal levels of KCC2 in the hippocampus of LHS rats allows a rebalancing of altered hippocampal microcircuits. Nevertheless, it is possible that all types of CA1 PCs are targeted

by *in utero* electroporation; in this case the resulting outcome may be due to a cumulative effect of all these microcircuit changes.

Mechanisms underlying differential KCC2 regulation following insults in young and adult brain

As discussed previously, the effects of seizures on KCC2 expression and function are age-dependent, and in line with several pieces of evidence suggesting that neuronal signaling mechanisms are radically different in developing and mature brain. Whether alterations of KCC2 expression/function may play a pro- or anticonvulsive role may, therefore, depend from the cellular and developmental context and from the extent of the impairment of KCC2 function. Supporting this notion, treatment with the NKCC1 inhibitor bumetanide *in vivo* during late embryonic development leads to permanent decrease in excitatory synaptic transmission and sensorimotor gating deficits[595], while similar treatment during early postnatal period altered timing of the critical period for experience-dependent plasticity[388]. These effects are dependent on the age of bumetanide-treatment, underscoring the importance of studying the role of KCC2/NKCC1 balance in specific developmental and pathological conditions, by using animal models which reproduce as closely as possible the human pathology.

Age-related differences in KCC2 alterations following neurological insults only came to light very recently, and thus, the causes leading to these differences remain to be interpreted. A potential age-dependent mechanism involves the BDNF-mediated TrkB activation. In immature neurons, activation of BDNF-TrkB signaling increases KCC2 expression, whereas it downregulates KCC2 in the adult brain[310,345,351,413]. In addition, in neurons damaged by acute oxidative stress or excitotoxicity, BDNF promotes KCC2 expression[352], which could be an adaptive response to promote neuronal survival and potential rewiring[288]. However, microglia-released BDNF decreases KCC2 expression in spinal cord neurons following nerve transection, a mechanism that is thought to contribute to the onset of neuropathic pain. On the

other hand, in mature hippocampal neurons, BDNF-TrkB-mediated downregulation of KCC2 seems to require the activation of intracellular signalling cascades initiated by the binding of PLCγ1 and Shc/FRS-2 (Src homology 2 domain containing transforming protein (Shc) and FGF receptor substrate 2) to the intracellular domain of the TrkB receptor. These signaling pathways activate second messengers and other downstream effectors, including cAMP CREB, which activates transcriptional machinery and thus regulates several genes involved in neuronal plasticity[596]. When the TrkB-PLCγ1 signaling cascade is blocked, Shc/FRS-2-mediated pathway leads to an upregulation of KCC2[316]. These results suggest that there are distinct TrKB-mediated signaling pathways leading to KCC2 downregulation in mature neurons. Furthermore, BDNF also exerts an age-dependent effect on $GABA_AR$ function. In CA1 immature pyramidal neurons, BDNF quickly potentiates $GABA_AR$-mediated currents whereas, in mature neurons, BDNF suppresses $GABA_AR$ function[597]. Furthermore, TrkB phosphorylation at its PLC binding site was found to be age-dependent[353]. The age- and insult-dependent effects of BDNF on KCC2 expression, and $GABA_ARs$ may be due to differential activation of TrkB and subsequent activation of distinct downstream signaling pathways in mature vs immature cortical neurons. Based on these observations, I hypothesize that LHS rats have an activity-dependent BDNF-TrkB activation, which in turn leads to upregulation of KCC2.

Another possible mechanism implicated in age-related differences involves the mTOR signaling pathway. A study treating young rats (3-4 weeks old) with rapamycin, to block the mTOR pathway, demonstrated an increase in severity to pilocarpine-induced seizures, as well as a reduction of the onset of seizure, but an increase of the total duration. This treatment also reduces the dosage necessary to induce seizures, and reduces KCC2 expression in the thalamus and hippocampus, without altering NKCC1 expression. In contrast, treating mature rats with rapamycin did not affect seizure sensitivity to pilocarpine, nor altered KCC2 expression. Interestingly, blocking KCC2 with furosemide globally, or specifically in the thalamus also increased pilocarpine-induced seizure severity in young rats[598]. Previous reports have suggested that rapamycin may suppress epileptogenesis and seizures[599,600], suggesting a role of the mTOR pathway in age-dependent seizure susceptibility. Considering that cortical dysplasia greatly alters the mTOR pathway, and pharmacological inhibition suppresses

seizures and neuronal hypertrophy in a mouse model of cortical dysplasia[601], it is possible that KCC2 upregulation after P1 lesion in our model is associated to alterations in the mTOR pathway. The fact that mTOR signaling in epileptogenesis is also age-dependent makes it an attractive mechanism.

KCC2 function before birth

One potential caveat of our study is that we introduced shRNA into neurons before birth, thus, before the cortical lesion-induced increase in KCC2 expression. As discussed in the introduction, KCC2 mRNA is expressed in differentiated neurons when they reach their final position, and this process starts from E18.5 for CA1 PCs. We electroporated KCC2 shRNA in migrating pyramidal neurons from the lateral ventricle of the right hemisphere at E17.5. We, therefore, transfected neurons before they expressed KCC2 mRNA, and the shRNA should not have any effects until the migrating PCs reach their final positions. Another important point is that KCC2 protein expression is not clearly detectable before birth; instead it is faintly expressed and then gradually increases postnatally, within the first week. Furthermore, a study of $KCC2^{-/-}$ mouse embryo demonstrated that at E18.5, CA3 pyramidal cells did not have altered chloride gradient or E_{GABA}. In fact, immunolabelling revealed that KCC2 was cytoplasmic in the majority of CA3 neurons at that age[309]. Consequently, although we are introducing shRNA before birth, the effects on hippocampal circuit formation should be limited. In addition, although KCC2 has been shown to affect the migration of interneurons[133], we did not observe any alterations in layer formation or displaced neurons. In the extremely rare case that an animal presented with an abnormal-looking hippocampus, it was discarded and not analyzed (maybe 1-2 in hundreds).

A recent study by Inoue et al, 2012 provides another evidence suggesting that KCC2 functions mainly postnatally. The authors found that the ectopic expression of KCC2 is functional in postnatal, but not embryonic brain. They suggest that this difference may be related to the embryonic abundance of endogenous taurine, which acts through WNK1

signaling pathway to block KCC2 function[337,602]. In addition, in hippocampal neurons *in vitro,* although KCC2 was present at early stages, it was not functional, and needed to be activated (by phosphorylation, leading to increase in membrane surface expression, or/and oligomerization, etc.)[299,333]. As such, we reason that KCC2-shRNA affects KCC2 expression mainly in pups lesioned at P1, which show a dramatic increase in KCC2 expression levels over the next 10 days than in control brains, where there is a limited amount of KCC2.

Long-term effects of the dual pathology

Our group has previously reported that LHS rats exhibited spontaneous recurrent seizures from P80 onwards, spatial memory deficits both before and after the appearance of these spontaneous seizures, and finally dendritic spine loss. Glutamatergic synaptic alterations may contribute to the deficits in spatial memory. Moreover, the latent phase between the two early-life insults and the development of epilepsy demonstrates the occurrence of several neuronal circuit alterations well before seizure onset, as was also demonstrated by my work. In fact, the expression levels of KCC2 return to baseline in LHS at P80[581], suggesting that the effects on KCC2 expression and function are crucial during epileptogenesis, but not in the recurrent generation of seizures. This is another evidence that KCC2 alterations are time-sensitive.

Rescuing KCC2 overexpression in LHS pups during a critical postnatal window may also ameliorate the cognitive deficits observed in adult LHS rats. Preliminary experiments suggest that P40 LHS pups electroporated with KCC2-shRNA show improved learning compared to age-matched LHS pups electroporated with scramble-shRNA. On the other hand, KCC2shRNA electroporation in naïve rats does not appear to affect learning or memory as tested with the Morris water maze (preliminary observations). Another important question to explore is whether KCC2shRNA electroporation blocks epileptogenesis, which can be analyzed by EEG recording and seizure analysis in >P100 LHS rats.

KCC2 function in spine development: time and region-specificity

Several studies suggested that KCC2 accumulates at glutamatergic synapses in spine heads of hippocampal pyramidal cells[269,303]. Further, the developmental increase of KCC2 expression coincides with the most intense phase of synaptogenesis, increasing the likelihood that KCC2 modulates synapse formation, in normal conditions. In fact, it has yet to be established whether KCC2 proteins located in spines, which structurally promote spinogenesis, also actively transport K^+/Cl^- [196].

Here, we report that spine head size and mEPSC amplitude in CA1 pyramidal cells is reduced in P20 LHS rats, well before the onset of spontaneous recurrent seizures, which eventually results in spine loss[581] (See figure 19 below). In accordance with these data, we found that electroporation of KCC2-shRNA decreased spine density but did not affect spine head size, in CA1 pyramidal neurons in naive animals. In contrast, Khalilov et al (2011), reported a significant increase in the frequency of spontaneous IPSCs and EPSCs as well as enhanced network activity in CA3 hippocampal neurons from $KCC2^{-/-}$ E18.5 mouse embryos[309]. Further, suppressing KCC2 expression in mature neurons, after spine formation, did not affect spine density but reduced the efficacy of excitatory synapses through alteration of AMPA receptor aggregation in dendritic spines, an effect independent of KCC2 transporter function[303]. Finally, Purkinje-cell specific KCC2 knockout neither altered spine density nor their morphology[213]. Overall, these data suggest once again that the age and localization of the genetic manipulations need to be taken into account when studying KCC2 functional effects on circuit development.

Surprisingly, LHS rats electroporated with KCC2-shRNA showed a significant increase in spine head size both compared to control LHS and control no-LHS groups (see figure 19). Multiple molecular and activity-dependent mechanisms likely underlie these alterations, as both GABAergic and glutamatergic receptor expression and function are altered in LHS compared to controls[585] and inhibitory/excitatory balance regulates synapse formation and plasticity. As reported previously, BDNF is required for seizure-induced upregulation of

KCC2 in neonatal hippocampus[234]. One possible explanation as described in our first paper is that spine morphology may depend on the expression and interaction of activity-dependent factors, such as KCC2 and BDNF, both in normal and hyperactive networks.

Evidence that timing of KCC2 modulation is crucial on its effects on spine development first came from Li et al. (Neuron 2007), who showed that disrupting KCC2 expression in early-life leads to a lack of spine development and an increase in dendritic protrusions in the cortex, suggesting that KCC2 is important in spine formation [250]. On the other hand, Gauvain et al. (2011) showed that reducing KCC2 expression by shRNA in mature hippocampal neurons (14DIV) no longer affected the density and formation of spines, but affected spine morphology through a loss of clustering of AMPAR at the membrane [303]. This study suggests that KCC2 is required for proper spine maintenance in mature hippocampal neurons. Our data from shRNA-mediated downregulation of KCC2 in the hippocampus are consistent with these findings.

Effects of KCC2 alterations are also sensitive to the brain region implicated. Overexpression of KCC2 in the cortex *in vivo* leads to long-term spine density increase[306]. In contrast to the cortex, overexpressing KCC2 in the hippocampus *in vivo* leads to spine density reduction (our data). These results propose that KCC2 is differently regulated in the hippocampus compared to the cortex, and/or the timing of KCC2 expression is different in these regions. Interestingly, preliminary observations suggest that KCC2 is already localized at the membrane of some CA1 PCs in the hippocampus at P1, while KCC2 immunoreactive signal is completely localized in PC cytoplasm in the cortex. These preliminary observations require further quantitative analysis; nevertheless they support the hypothesis of regional specificity of KCC2 expression and, possibly, function during early postnatal development. Additionally, the most probable mechanism underlying the regional differences of KCC2 effects on spines may involve distinct BDNF-TrkB signaling activity. It will be interesting to investigate if the expression levels of BDNF and of activated TrkB increase earlier in the hippocampus than in cortex. Additionally, calpain activation (which cleaves a C-tern region of KCC2), as well as the dephosphorylation of Ser940, has been suggested to regulate the lateral mobility of KCC2 within the plasma membrane[308], as previously discussed. KCC2's

cytoskeleton binding domain is in the C-terminal region, suggesting that calpain activation and cleavage of KCC2 may alter dendritic spine formation, and could potentially affect AMPA clustering in dendritic spines[303]. It will be interesting to examine whether calpain expression is region specific, and how it is affected in an epileptic brain.

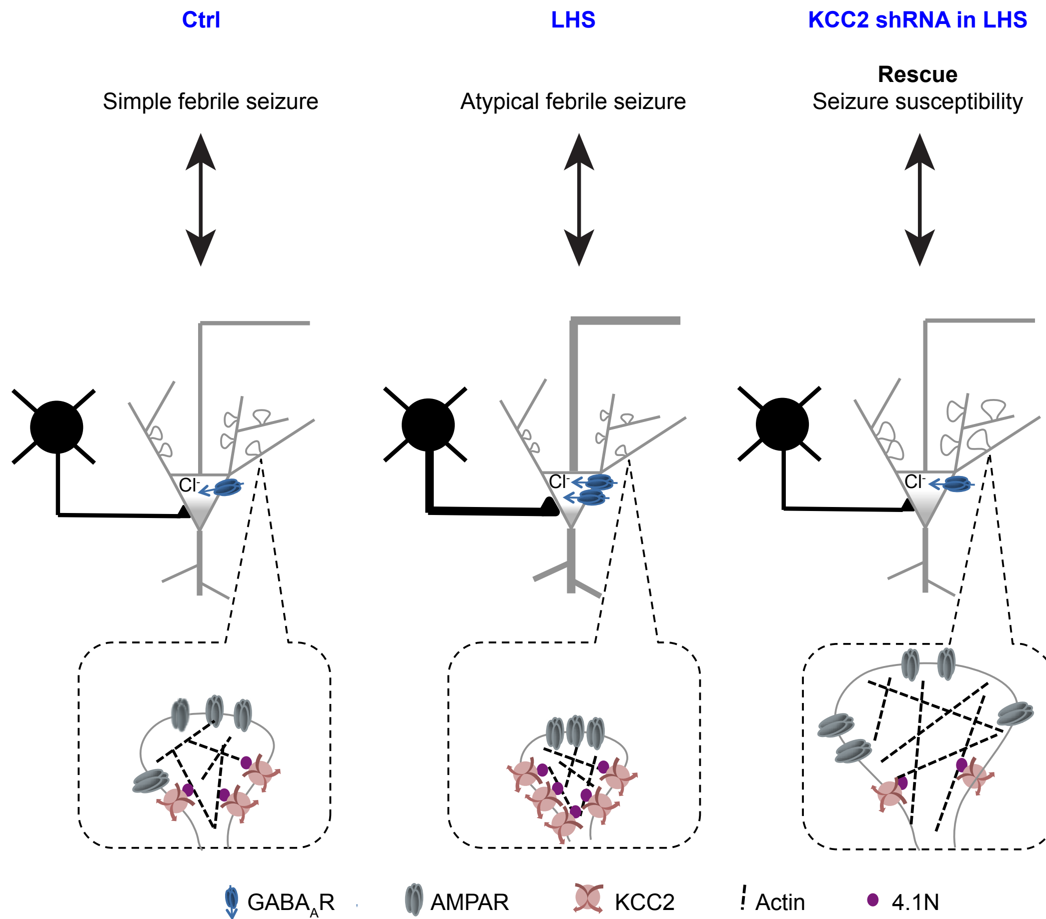

Figure 19. Summary illustration depicting the alterations of KCC2 in the LHS model.

The gray cells represent pyramidal cells, and the black cells represent PV-INs. In control conditions (left), a simple febrile seizure will not have any pathological effects. In the LHS rats (middle), there is a lesion that predisposes to febrile seizure susceptibility, causing atypical febrile seizures. The LHS rats show precocious overexpression of KCC2 and an increase of inhibitory drive onto CA1 PCs (thicker lines from basket cell). Finally, dendritic spines heads are smaller. Conversely, knockdown of KCC2 by shRNA in LHS rats (right) rescues febrile seizure susceptibility, KCC2 levels, and spine shrinkage.

Overall, it is clear that the timing of KCC2 modulation can differently affect different circuits, and perhaps the earlier expression of KCC2 in the hippocampus is necessary for its proper wiring and spine development, which reaches plateau before similar events in cortex. This is particularly important when we are looking for NKCC1 and KCC2 as therapeutic targets for pediatrics epilepsy[224,405] or other developmental diseases, such as autism[237,603] as we discussed previously. As such, in a pathological context, the hippocampus is particularly sensitive in epilepsy. Therefore, an early-life insult resulting in an increase of KCC2 particularly in the hippocampus, where there already is initially more KCC2, may differently affect the overall network activity, and potentially cause a pro-convulsive effect. Therefore, treating children and adults with different types of epilepsy, implicating different brain areas, will most likely not produce the same effect. Altogether, changes of KCC2 expression or/and function in the brain may be harder to understand than we previously thought. An important question that remains to be resolved is whether and to what extent cognitive abilities are affected by alterations of KCC2 expression levels in specific circuits following pathological insults during development.

Gender-based differences

Consistent to what previously reported following status epilepticus or trauma in neonate rodents *in vivo*[410,412], we found that KCC2 expression was increased specifically in male LHS rats. Interestingly, KCC2 expression in females, while more variable, was not significantly different compared to controls. This is consistent with previous findings showing that the expression time course of KCC2 and the efficacy of $GABA_A$ receptor signaling in rat CA1 pyramidal neurons are gender-specific in normal development and following multiple status epilepticus episodes in the neonate[410]. Our lab has recently demonstrated that there are gender-based differences in the long-term vulnerability to developing epilepsy in the LHS rats. First, the occurrence of spontaneous recurrent seizure occurred only in the adult male, and not female LHS rats[534,580,604]. While the mechanisms underlying this dimorphism are still not clearly elucidated, it is likely the stress response to the cortical dysplasia induced a P1 that plays a role[604]. While the histological damage induced by the cortical lesion was the same in

both sexes, corticosterone blood levels increased at P1 following the lesion in males but not female. On the other hand, androgenized females which received testosterone treatment showed a similar rise in corticosterone at P1 as their males littermates and also developed MTLE in adulthood[604]. These results suggest that the hormonal response to the freeze lesion may predispose the male brain to more severe hyperthermic seizures and the development of epilepsy. Finally, KCC2 expression may be preferentially increased in males in response to stress-induced $GABA_A$ receptor-mediated depolarization of CA1 pyramidal neurons[605] or other gender-specific stress or/and inflammatory signaling pathways.

Limitations of our study – Alternative strategies

Studying the role of seizure-induced KCC2 variations *in vivo* has been so far hindered by the lack of specific KCC2 pharmacological inhibitors, which can be administrated via intraperitoneal or intravenous injections. We used shRNA-mediated knockdown, which is the best option to moderately reduce KCC2, and allow region-, time- and cell-specific alterations of KCC2 expression. The ideal condition would have been to use a tamoxifen-inducible plasmid, to selectively knockdown KCC2 starting from P1. The laboratory of Dr. Cancedda (our collaborators) has tried to produce this plasmid, however, it was never successful, as they could not reduce expression leakage. A limitation of our strategy is that shRNA-mediated KCC2 reduction may affect circuit development prior to the initial insult at P1 although as we suggested above, this effect is unlikely. However, pyramidal cell spine density reduction induced by KCC2shRNA expression prior to the hyperthermia could affect the overall circuit activity. On the other hand, we only transfected a small subpopulation of CA1 pyramidal neurons, thus it is unlikely that the entire hippocampal network activity is altered, an effect that would instead be induced by the use of pharmacological KCC2 inhibitors.

As for KCC2 inhibitors, furosemide was the only available drug when we started this study. Furosemide, however, is not specific to KCC2 as it also inhibits NKCC1; it is also poorly permeant across the blood-brain barrier, and thus, not adequate for our *in vivo* experiments either. Recently, two new specific KCC2 inhibitors were produced. VU0240551

has been used in electrophysiology recordings *in vitro*, whereas VU046327 (generated by Dr. Delpire) effect following *in vivo* administration has only recently been demonstrated[290]. Administration of VU046327 actually increased seizure susceptibility, which is the opposite effect compared to what we found. The most likely explanation is that most if not all KCC2 activity is blocked by the concentration used in this study, while our approach reduces but does not block KCC2. Importantly, this compound is unstable, thus the authors had to infuse it directly into the brain during EEG recordings. Therefore, long-term treatment with this drug is simply not feasible during the first postnatal weeks, unless we cause further lesion by introducing stable cannula implant in the brain. Therefore, a 'drug-free' approach was and still is the best option. Additionally, it's important to bear in mind that we did not want to fully eliminate KCC2 from the LHS brains, instead we wanted to reduce the overexpression observed in the hippocampus underneath the cortical dysplasia in these animals and see whether this would reverse the observed alterations. Therefore, the most appropriate experiment we could have done to limit KCC2 expression was shRNA-mediated knockdown in individual neurons without affecting the overall activity of cortical neuronal networks.

A concern with using shRNAs is the possibility of having off-target effects or immune-reactive side effects. Therefore, we used an established scramble control and KCC2 shRNA previously published. The reversal potential of GABA (E_{GABA}) was successfully depolarized in the presence of KCC2 shRNA[302]. We also measured the level of KCC2 in electroporated animals and saw a ~50% decrease in membrane surface expression at P20. Transfected cells were still GFP+ at P50, which allowed us to perform learning and memory test in electroporated rats. We are currently investigating whether KCC2shRNA affects KCC2 expression levels at P50 or later.

An alternative experiment to prove that KCC2 upregulation is pro-convulsive in LHS animals is to pharmacologically enhance GABAergic signaling with a very low dose of diazepam, and see whether the seizure time onset is decreased[590-592]. This experiment would more specifically link KCC2 modifications to seizure susceptibility.

Finally, an important experiment would have been to examine the synaptic changes following KCC2 modulation by electrophysiology. In fact, both KCC2shRNA-mediated

knockdown and KCC2 overexpression *in vivo* affected spine density and morphology. Measuring miniature excitatory postsynaptic currents would have indicated the effect of spine alterations on estimated transmitter release, and thus, synaptic strength.

Significance of this project

Basic and clinical studies indicate that seizures in neonates have long-term neurological and psychiatric consequences. Current therapy for neonatal seizures is usually focused on early treatment with benzodiazepines and barbiturates, which exert their effects via modulation of GABA action at the $GABA_A$ receptor. Although these drugs are effective in adults, they do not control neonatal seizures well and may have adverse long-term effects on neurodevelopment, highlighting the need for novel approaches for treatment of seizures in the developing brain.

Recent efforts have focused on targeting molecules that establish the chloride gradient $[Cl^-]_i$ as a tool to modulate more efficiently GABAergic inhibition. It has been hypothesized that pharmacologically decreasing NKCC1 or promoting KCC2 function may promote the anticonvulsant effects of benzodiazepines and barbiturates in young brains. Unfortunately, two clinical trials evaluating bumetanide as a treatment for neonatal seizures (NCT00830531 and NCT01434225) have been so far largely disappointing [606], indicating the need for a better understanding on how KCC2/NKCC1 regulates network excitability in the developing brain, particularly in a brain compromised by pre-existing conditions.

Our dual-hit model has been well accepted by the medical community as it reproduces the clinical situation where children with prolonged, atypical FSs are at higher risk of developing medial temporal lobe epilepsy and cognitive problems than those experiencing simple FSs [580,581]. This project constitutes an important advancement to the field since the role of pathology-induced, premature KCC2 upregulation in the developing brain is still highly controversial. The strength of this study resides in the innovative, targeted genetic approach to modulate KCC2 expression selectively in the dorsal hippocampus, using a triple-electrode

probe in our *in utero* electroporation experiments. This strategy allowed us to show for the first time that decreasing KCC2 expression in a limited number of hippocampal pyramidal neurons is sufficient to lower seizure susceptibility and rescue synaptic deficits in a clinically relevant model of atypical febrile seizures in the developing brain. It also brought to light regional differences of KCC2 modulation, further emphasizing the complexity of treating neonatal seizures.

Finally, we believe these findings are of interest for its potential translational applications. Our data show that understanding the injury-specific alterations of KCC2 in clinical-relevant models is a critical prerequisite for investigating whether pharmaco-modulation of chloride cotransporters may be therapeutically effective.

Conclusion

Recent efforts have focused on targeting molecules that establish the chloride gradient as a tool to modulate GABAergic inhibition. KCC2 cotransporter progressively decreases the intracellular chloride concentration, and in turn is responsible for the shift in polarity of GABAergic transmission from mostly excitatory during early development to inhibitory in the adult. In addition, KCC2 has an important function in spine development and functional maintenance. It seems that the timing of the cotransporter's developmental upregulation is ever so slightly different between the neocortex and the hippocampus. We showed that this region-specific difference leads to opposite effects on spine development, when KCC2 is prematurely increased. These results suggest that KCC2 in the developing brain is particularly sensitive to region-specific alterations, a phenomenon that could be heightened in a brain compromised by pre-existing conditions. As such, we evaluated the effect of two neonatal insults on febrile seizure susceptibility and spine development.

Febrile seizures (FSs) are the most common convulsive events in humans between 6 months and 5 years of age, with a prevalence of 2–14% in this population. Simple FSs are considered benign, whereas children experiencing atypical FSs, which account for 30-40% of FS cases, run a higher risk (7%-49% depending on FS characteristics) of developing cognitive deficits and temporal lobe epilepsy as adults. Interestingly, patients with temporal lobe epilepsy that have experienced atypical FSs often carry a cortical malformation. This association has led to the hypothesis that a double-hit (cortical dysplasia and atypical febrile seizures) in the developing brain may precipitate the onset of neurological problems later in life. Our dual-hit model has been well accepted by the medical community as it reproduces the clinical situation where children with prolonged, atypical FSs are at higher risk of developing medial temporal lobe epilepsy and cognitive problems than those experiencing simple FSs [580,581]. In this project, we report that LHS rats have a precocious and sustained increase of hippocampal KCC2 protein levels, resulting in a negative shift of the reversal potential of GABA (E_{GABA}). In addition, excitatory synapses are significantly altered in CA1 pyramidal neurons, which was accompanied by spatial memory deficits occurring before the

onset of spontaneous recurrent seizures. By using a novel approach to specifically direct the expression of KCC2 shRNA in CA1 pyramidal neurons, namely a triple-electrode probe during *in utero* electroporation experiments. We demonstrated that the precocious increase of KCC2 expression contributes to the occurrence of long-term alterations in dendritic spine size in CA1 pyramidal neurons and, surprisingly, to lowering the threshold for febrile induced seizures *in vivo*. Altogether, our results demonstrate for the first time, that alterations of KCC2 expression are region-specific and time-sensitive. Further, future studies will help us to better design targeted manipulation of KCC2 to alleviate neurological and cognitive problems caused by a variety of early-life insults.

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