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GABAergic Signaling and Neuronal Chloride Regulation in the Control of Network Events in the Immature Hippocampus

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ACADEMIC DISSERTATION

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To Albert

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Abstract

Spontaneously arising network events are a characteristic feature of all developing neural networks. This activity is crucial for normal neuronal development and the establishment of appropriate synaptic connectivity. In the developing hippocampus, depolarizing GABAergic drive is essential in generation of early network events, known as giant depolarizing potentials (GDPs). Blockade of GABAergic signaling leads to hypersynchronization of the network and emergence of ictal-like events, pointing to dual, both excitatory and inhibitory roles for GABA, in regulation of these events.

In Studies I-III of this thesis, we examined the role of $GABA_A$ receptor ($GABA_AR$) - mediated neurotransmission with some parallel work on glycinergic signaling as well as neuronal Cl^- regulation in modulation of GDPs in the developing rodent hippocampus.
In Study I, we demonstrate that low levels of GABA and glycine suppress GDPs by activating extrasynaptic receptors. This implies that regardless of the depolarizing drive for Cl^- currents at this developmental stage, a low conductance via Cl^- - permeable $GABA_ARs$ and glycine receptors (GlyRs) can cause efficient shunting and inhibition of the network events.
In Study II, we discovered that sustained activation of a subset of hippocampal interneurons, caused by the neuropeptide arginine vasopressin (AVP), silences the network events in the perinatal hippocampus, regardless of the maturational level of the GABAergic system as compared across species. This is attributed to decreased synchronous interneuronal input that is essential for the GDP generation.
In Study III, we demonstrate that transport-functional K-Cl cotransporter 2 (KCC2) is present in the CA3 pyramidal neurons already in the perinatal stages in mice and rats. Cl^- extrusion by KCC2 counteracts the dominant Na-K-2Cl cotransporter 1 (NKCC1) - mediated Cl^- uptake and restrains the depolarizing GABAergic drive onto the CA3 pyramidal cells. Thereby, function of KCC2 limits pyramidal neuron spiking and synchronization during GDPs and participates in the modulation of GDPs from their developmental onset.

This work describes novel physiological GABAergic mechanisms that control GDPs in the perinatal rodents and establishes a role for KCC2 in regulation of pyramidal neuron excitability and synchronization during GDPs starting from their developmental onset.

Contents

List of original publications

This thesis is based on the following studies:

I. Sipilä ST, Spoljaric A, Virtanen MA, **Hiironniemi I**, Kaila K (2014) Glycine transporter-1 controls nonsynaptic inhibitory actions of glycine receptors in the neonatal rat hippocampus *J Neurosci* 34(30), 10003-9

II. Spoljaric A*, Seja P*, **Spoljaric I**, Virtanen MA, Lindfors J, Uvarov P, Summanen M, Crow AK, Hsueh B, Puskarjov M, Ruusuvuori E, Voipio J, Deisseroth K, Kaila K (2017) Vasopressin excites interneurons to suppress hippocampal network activity across a broad span of brain maturity at birth *PNAS* 114(50), E10819-E10828

III. **Spoljaric I**, Spoljaric A*, Mavrovic M*, Seja P, Puskarjov M, Kaila K (2019) KCC2-mediated Cl^- extrusion modulates spontaneous hippocampal network events in perinatal rats and mice *Cell Reports* 26(5), 1073-1081

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The studies are referred to in the text by their roman numerals.

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I. The candidate performed and analyzed the majority of local field potential experiments.

II. The candidate performed and analyzed the majority of local field potential experiments and a set of single-cell voltage-clamp experiments, as well as participated in writing and revising the manuscript.

III. The candidate performed and analyzed the majority of local field potential experiments in the manuscript, participated in planning of the experiments, prepared most of the figures and wrote the manuscript together with KK and MP.

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Brandt C, Seja P, Töllner K, Römermann K, Hampel P, Kalesse M, Kipper A, Feit P W, Lykke K, Toft-Bertelsen TL, Paavilainen P, **Spoljaric I**, Puskarjov M, MacAulay N, Kaila K, Löscher W (2018) Bumepamine, a brain-permeant benzylamine derivative of bumetanide, does not inhibit NKCC1 but is more potent to enhance phenobarbital's anti-seizure efficacy. *Neuropharmacology* 143, 186-204

Abbreviations

AHP	afterhyperpolarization
ASD	autism spectrum disorders
AVP	arginine vasopressin
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
CA	*cornu ammonis* area of hippocampus
CCC	cation-chloride cotransporter
CGE	caudal ganglionic eminence
$[Cl]_i$	intracellular Cl^- concentration
CNS	central nervous system
DF_{GABA}	driving force of $GABA_AR$-mediated currents
DLX2	distal-less homeobox gene 2
DMSO	dimethyl sulfoxide
DOHaD	developmental origin of health and disease
E	embryonic day
E_{GABA}	equilibrium potential of $GABA_AR$-mediated currents
GABA	gamma-aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
$GABA_BR$	$GABA_B$ receptor
GAD67	glutamate decarboxylase 67
GAT1	GABA transporter 1
GDP	giant depolarizing potential
GlyR	glycine receptor
GlyT1	glycine transporter 1
iGluR	ionotropic glutamate receptor
$I_{K(Ca2+)}$	Ca^{2+} activated K^+ current
I_{Nap}	Persistent Na^+ current
KCC2	K-Cl cotransporter 2
LFP	local field potential
MGE	medial ganglionic eminence
mIPSC	miniature inhibitory postsynaptic current
MUA	multiunit activity
NKCC1	Na-K-2Cl cotransporter 1
NMDAR	N-methyl-D-aspartate receptor
OT	oxytocin
OTR	oxytocin receptor

P	postnatal day
sEPSC	spontaneous excitatory postsynaptic current
sIPSC	spontaneous inhibitory postsynaptic current
SLR	*stratum lucidum* and *stratum radiatum*
(e)SPW	(early) sharp wave
TrkB	tropomyosin-related kinase receptor B
V1aR	vasopressin 1a receptor
V1bR	vasopressin 1b receptor
WNK1	lysine deficient protein kinase 1
5-HT	5-hydroxytryptamine (serotonin)

1 Introduction

The adult human brain has been estimated to contain approximately 86 billion neurons that are connected to each other with trillions of precisely formed synaptic connections (Pakkenberg *et al.*, 2003; Azevedo *et al.*, 2009; Herculano-Houzel, 2009; Kirkby *et al.*, 2013). The construction of this complex system, from the proliferation of the progenitor cells to the formation of synaptic connectivity requires seamless cooperation of multiple systems. The building of the brain is guided by predetermined genetic programs, inductive tissue interactions and molecular guidance factors that assist in the migration of neurons and organization of the neuronal processes (Goodman and Shatz, 1993; Kirkby *et al.*, 2013; Muller and Marin, 2014; Lim, Llorca and Marin, 2018), but it also critically relies on the neuronal activity and activity dependent signaling cascades (Katz and Shatz, 1996; Blankenship and Feller, 2010; Kirkby *et al.*, 2013; Lim, Llorca and Marin, 2018).
Developing cortical networks intrinsically generate synchronous network events prior to the maturation of most sensory systems. These spontaneous events are thought to guide the initial organization of the circuits and set the foundations for the more specialized network functions in the adult brain (Katz and Shatz, 1996; Blankenship and Feller, 2010; Kirkby *et al.*, 2013; Colonnese and Phillips, 2018).

In the developing hippocampus, these events are known as giant depolarizing potentials (GDPs) (Ben-Ari *et al.*, 1989; Sipilä and Kaila, 2007; Griguoli and Cherubini, 2017). GDPs are paced by intrinsic currents generated in the hippocampal CA3 pyramidal neurons, and triggered by tonic depolarizing $GABA_A$ receptor ($GABA_AR$) - mediated drive from local interneurons (Ben-Ari *et al.*, 1989, 2007; Sipilä *et al.*, 2005; Sipilä, Huttu, *et al.*, 2006; Spoljaric *et al.*, 2017). The events synchronize within the concurrently connected neuronal network and propagate throughout the hippocampus. Other neurotransmitter systems, like glycine acting via glycine receptors (GlyRs) and NMDA receptors (NMDARs), participate in modulation of GDPs (Gaiarsa *et al.*, 1990; Sipilä *et al.*, 2014). In mice and rats, GDPs disappear by the end of the second postnatal week due to gradually increasing functional expression of the K-Cl cotransporter 2 (KCC2) and consequent maturation of the hyperpolarizing $GABA_AR$-mediated inhibition (Rivera *et al.*, 1999; Khazipov *et al.*, 2004; Tyzio *et al.*, 2007; Kaila, Price, *et al.*, 2014).

The high plasticity of the developing brain makes it vulnerable to pathophysiological insults and, indeed, various nervous system disorders are suggested to originate already during the early stages of brain development (Andersen, 2003; Schaefers and

Teuchert-Noodt, 2013; Hanson and Gluckman, 2014; Faa *et al.*, 2016). Certain mouse models of central nervous system (CNS) disorders, such as epilepsy and autism, exhibit abnormal GDP activity (Pizzarelli and Cherubini, 2013; Vargas, Petrou and Reid, 2013; Cellot *et al.*, 2016) which could, at least partly, account for the wider functional deficits that occur in the later stages of the disease.
A thorough understanding of the normal regulation of intrinsic network events in the developing brain will help us to better understand abnormalities of this activity, and may lead to the discovery of novel pharmacological targets for the treatment of brain disorders.

In this thesis we examine the mechanisms of GABAergic control of GDPs in the perinatal rodent hippocampus. Furthermore, using pharmacological tools, we address the role of KCC2-mediated Cl^- extrusion in regulation of the neuronal excitability and hippocampal network events prior to the maturation of hyperpolarizing $GABA_AR$-mediated inhibition.

2 Review of the literature

2.1 Spontaneous network events in the developing hippocampus

2.1.1 Role of spontaneous network events in the CNS development

Developing neural networks exhibit intrinsically generated synchronous events prior to the maturation of most sensory systems. These events are thought to be crucial in driving the initial organization of the networks to set the foundation for more specialized functions in the mature brain (Goodman and Shatz, 1993; Katz and Shatz, 1996; Blankenship and Feller, 2010; Kirkby *et al.*, 2013).
Spontaneous events in the developing networks typically occur as slow synchronous bursts of neuronal activity ("events"), separated by long periods of quiescence and often propagating within the neural network (Zhang and Poo, 2001; Blankenship and Feller, 2010). Similar network activity patterns are found in various areas of the developing CNS and in different species (Hamburger and Balaban, 1963; Ben-Ari *et al.*, 1989; Menendez de la Prida, Bolea and Sanchez-Andres, 1998; Garaschuk *et al.*, 2000; Khazipov *et al.*, 2001; Vanhatalo and Kaila, 2006; Triplett *et al.*, 2009; Watt *et al.*, 2009; Rockhill, Kirkman and Bosma, 2009; Blankenship and Feller, 2010; Ackman, Burbridge and Crair, 2012; Ackman and Crair, 2014; Colonnese and Phillips, 2018). Despite the differences in the mechanisms promoting the activity at different areas of the CNS, spontaneous network events appear to be a fundamental feature of all developing neural networks (Blankenship and Feller, 2010).

The significance of spontaneous events in the construction of neuronal networks is most thoroughly studied in relation to the topographical organization of the visual system, where even subtle manipulations of the spontaneous retinal waves have been shown to induce permanent changes in the organization and function of the visual pathways (Katz and Shatz, 1996; Grubb *et al.*, 2003; McLaughlin *et al.*, 2003; Huberman, Feller and Chapman, 2008; Xu *et al.*, 2011; Ackman and Crair, 2014; Colonnese and Phillips, 2018). Diverse studies in the visual system suggest that the spontaneous network events guide the initial arrangement of the network which later, after eye-opening, is refined by sensory evoked activity (Katz and Shatz, 1996; Huberman, Feller and Chapman, 2008; Colonnese and Phillips, 2018).

Spontaneous events in the spinal cord participate in development of pattern-generating circuits and create the first movements of the fetus in the womb (Hamburger and Balaban, 1963; O'Donovan and Landmesser, 1987; Blankenship and Feller, 2010; Momose-Sato and Sato, 2013). At different levels of the developing auditory system, cells that later respond to similar frequencies create synchronous bursts of activity (Tritsch *et al.*, 2007; Babola *et al.*, 2018), potentially guiding the stabilization of connections within different domains of the system. Somatosensory cortex produces bursts of activity in response to spontaneous myoclonic jerks during sleep as well as tonic spiking during goal-oriented movements in awake rats (Khazipov *et al.*, 2004; Tiriac, Del Rio-Bermudez and Blumberg, 2014; Colonnese and Phillips, 2018). In neonatal rats, cortical somatosensory responses to goal-oriented movements are blocked at an inhibitory gate in the external cuneate nucleus that relays sensory information (Tiriac, Del Rio-Bermudez and Blumberg, 2014; Tiriac and Blumberg, 2016; Colonnese and Phillips, 2018). Presumably, this allows the synchronous bursts to guide the development of topographic maps without perturbation from the persistent activity patterns.

Studies suggest that the circuits maintaining synchronous network events during early development differ from the ones generating more continuous patterns of network activity in the mature brain (Khazipov and Luhmann, 2006; Blankenship and Feller, 2010; Colonnese and Phillips, 2018). This highlights the fact that early spontaneous oscillations are not immature versions of the mature activity, and should not be studied as such. Instead, these events are a manifestation of a unique phase of the development with a specific relevance and function.

2.1.2 Network events in the developing hippocampus *in vitro* and *in vivo*

GDPs, first characterized in the late 80s, are the most pronounced network activity pattern found in the developing hippocampus *in vitro* (Ben-Ari *et al.*, 1989, 2007; Khazipov *et al.*, 2004; Griguoli and Cherubini, 2017). GDPs are generated by coordinated action of intrinsic conditional pacemaker currents in CA3 pyramidal neurons and the GABAergic drive from interneurons which at this developmental stage is depolarizing (Bolea *et al.*, 1999; Sipilä *et al.*, 2005; Sipilä, Huttu, *et al.*, 2006; Ben-Ari *et al.*, 2007; Valeeva *et al.*, 2010). Both GABAergic and glutamatergic neurons are recruited to these events that occur approximately at a frequency of 0.1 - 0.3 Hz. In intact hippocampal preparations, GDPs are preferably generated at the septal end of the hippocampus and propagate from there to the medial and temporal regions (Leinekugel *et al.*, 1998; Sipilä, Huttu, *et al.*, 2006). Synchronous network events activate voltage-gated channels and glutamatergic NMDARs leading to a rise in intracellular Ca^{2+} (Leinekugel *et al.*, 1997). The rise in intracellular Ca^{2+} triggers

downstream cascades that can lead, for example, to changes in gene expression, secretion of neurotrophic factors or changes in synaptic efficacy, thereby affecting various developmental processes (Ben-ari *et al.*, 1997; Kasyanov *et al.*, 2004; Spitzer, 2006; Mohajerani *et al.*, 2007; Kuczewski *et al.*, 2008; Winnubst *et al.*, 2015).
As the GABAergic system matures, GDPs are replaced by more specialized types of activity. Network events known as sharp waves (SPWs) are the predominant type of network events in more mature hippocampus *in vitro* (Kubota *et al.*, 2003; Maier, Nimmrich and Draguhn, 2003; Behrens *et al.*, 2005).

Hippocampal network events, known as early sharp waves (eSPWs) can be recorded from neonatal, awake or anesthesized, rats and mice *in vivo* (Leinekugel *et al.*, 2002). Like GDPs, the eSPWs depend on both GABAergic and glutamatergic signaling and they occur at a similar frequency and in a similar developmental time window as GDPs, making them a putative *in vivo* counterpart of GDPs (Leinekugel *et al.*, 2002; Buzsáki, 2015; Griguoli and Cherubini, 2017). However, eSPWs often follow spontaneous muscle twitches in immature rats (Karlsson *et al.*, 2006) and network events in the medial entorhinal cortex (Valeeva *et al.*, 2019) indicating that in addition to the local hippocampal circuit, extrahippocampal inputs participate in the generation of eSPWs.

In the mature hippocampus, SPWs occur during feeding, awake immobility and slow wave sleep, but they are somewhat different from the eSPWs. eSPWs often contain a slow tail of unit activity, that is never associated with the adult SPWs and have a different current-source density profile. In addition, the mature SPWs contain fast frequency ripple oscillations and are never triggered by movement (Buzsáki, 2015; Valeeva *et al.*, 2019), suggesting that the mechanisms driving this activity differ, at least in part, from those in the developing hippocampus. Mature SPWs and associated ripples are believed to be important in memory consolidation and planning. Like GDPs, these events are highly dependent on the synchronous activity of the local interneurons (Klausberger and Somogyi, 2008). As mentioned above, SPWs can be recorded *in vitro*, but there is considerable variation between different *in vitro* models in their characteristics, such as pharmacological responses and coupling with the ripple oscillations (Buzsáki, 2015). These differences may reflect the degree of damage to the local axon collaterals essential in generation of these population bursts, which calls for careful consideration when making comparisons between the *in vivo* and *in vitro* events (Traub *et al.*, 2004; Buzsáki, 2015).

2.2 Cell-autonomous mechanisms in the generation of GDPs

2.2.1 Intrinsic pacemaker currents in the CA3 pyramidal neurons

GDPs are initiated in the CA3 pyramidal cells that fire action potentials in regular spontaneous bursts when depolarized, thus working as conditional pacemakers (Sipilä *et al.*, 2005). In the absence of blockers, the intact CA3 pyramidal neurons fire at a preferred frequency of 0.3 Hz which is characteristic for GDPs (Sipilä *et al.*, 2005). The intrinsic bursting of CA3 neurons is driven by the persistent Na^+ current I_{Nap} that depolarizes the neuron towards the firing threshold. The Ca^{2+} activated K^+ current $I_{K(Ca2+)}$ is responsible for terminating the bursts and for the refractory period that follows them (Sipilä, Huttu, *et al.*, 2006). Low expression of $Kv_{7/M}$ channels that mediate the muscarinic K^+ current in mature neurons, has been suggested to facilitate bursting in immature CA3 neurons (Safiulina *et al.*, 2008).

Although the CA3 region carries the highest propensity for generation of GDPs, the other subfields of hippocampus are also able to intrinsically produce these events. GDPs in the CA1 region typically follow GDPs in CA3 with an approximately 200 ms delay (Menendez de la Prida, Bolea and Sanchez-Andres, 1998). When the connections between these two areas are cut, CA1 still generates GDP-like events but they occur at lower frequency (Menendez de la Prida, Bolea and Sanchez-Andres, 1998). This suggests that some conditional pacemaker cells are also present in the CA1 region.

2.2.2 Regulation of intraneuronal chloride

2.2.2.1 Chloride regulation and neuronal development

The conditional pacemaker cells in the CA3 area receive depolarizing drive from the GABAergic interneurons to generate GDPs (Sipilä *et al.*, 2005; Sipilä, Huttu, *et al.*, 2006; Sipilä, Schuchmann, *et al.*, 2006; Cherubini *et al.*, 2011). Both the qualitative and quantitative features of GABAergic signaling in the CNS are directly coupled to the regulation of intracellular Cl^- concentration ($[Cl]_i$) in central neurons (Payne *et al.*, 2003; Kaila, Price, *et al.*, 2014; Schulte, Wierenga and Bruining, 2018). While $GABA_A$Rs pass both Cl^- and HCO_3^-, they are approximately four times more permeable to Cl^- making $[Cl]_i$ the main determinant of the equilibrium potential of the $GABA_A$R-mediated currents (E_{GABA}) (Kaila, 1994). The deflection of E_{GABA} from the membrane voltage (V_m) creates the driving force for $GABA_A$R-mediated currents (DF_{GABA}) (DF_{GABA}

= V_m - E_{GABA}), which defines the direction and the magnitude of the $GABA_AR$-mediated currents (Kaila, 1994; Kaila, Price, *et al.*, 2014).

The level of $[Cl]_i$ is a dynamic equilibrium set by the passive Cl^- flux via open $GABA_ARs$ and other Cl^- channels as well as the active transport of Cl^- through the cell membrane (Kaila, 1994). Active regulation of $[Cl]_i$ in adult neurons is mainly dealt with by two electroneutral cation-chloride cotransporters (CCCs), Na-K-2Cl cotransporter 1 (NKCC1) and KCC2, that mediate the uptake and extrusion of Cl^-. NKCC1 and KCC2 utilize the energy of Na^+ and K^+ gradients across the cell membrane, respectively, both created by the Na-K ATPase (Payne *et al.*, 2003; Kaila, Price, *et al.*, 2014). In mature cortical neurons, the function of KCC2 typically predominates Cl^- transport, maintaining low $[Cl]_i$ (approximately 5-7 mM in quiescent neurons *in vitro*) (Kaila *et al.*, 1993; Farrant and Kaila, 2007; Paredes *et al.*, 2016) and setting E_{GABA} to around -70 mV (Kaila *et al.*, 1993; Stein *et al.*, 2004; Banke and McBain, 2006; MacKenzie and Maguire, 2015).

Depolarizing responses to GABA in immature neurons arise from their relatively high $[Cl]_i$. Under these conditions, $GABA_AR$ activation typically leads to an efflux of Cl^-. The high $[Cl]_i$ results from Cl^- uptake by NKCC1 and negligible Cl^- extrusion due to low expression of KCC2 in the immature neurons (Rivera *et al.*, 1999; Payne *et al.*, 2003; Stein *et al.*, 2004; Kaila, Price, *et al.*, 2014; Sulis Sato *et al.*, 2017, but see also Valeeva *et al.*, 2016). Because of the critical role of NKCC1 in maintaining depolarizing GABAergic responses, GDPs are completely abolished by the NKCC1 blocker bumetanide (Dzhala *et al.*, 2005; Sipilä, Schuchmann, *et al.*, 2006; Spoljaric *et al.*, 2017; Brandt *et al.*, 2018).

The functional expression of KCC2 shows a steep developmental upregulation. The increase in Cl^- extrusion capacity leads to a gradual decrease in $[Cl]_i$ and consequently to the maturation of hyperpolarizing $GABA_AR$-mediated responses typical for most mature cortical neurons (Figure 1, Rivera *et al.*, 1999; Stein *et al.*, 2004; Spoljaric *et al.*, 2017). This developmental change in $[Cl]_i$ has recently been demonstrated for the first time *in vivo* in mouse cortical neurons using a genetically encoded fluorescent sensor (Sulis Sato *et al.*, 2017). The depolarizing-to-hyperpolarizing shift in GABAergic function is, indeed, a key part of neuronal differentiation in the CNS and the timing of it varies between brain areas and animal species (Rivera *et al.*, 1999; Li *et al.*, 2002; Kaila, Price, *et al.*, 2014; Sedmak *et al.*, 2016). In mouse and rat cortical structures, including hippocampus, this shift occurs by the end of the second postnatal week. Consequently, GDPs typically disappear around the 12^{th} postnatal day (P) (Khazipov *et al.*, 2004; Stein *et al.*, 2004; Ben-Ari *et al.*, 2007).

It is important to keep in mind that, in different species, the rate of brain development and the timing to reach various developmental milestones shows

enormous variation, hence interspecies age comparisons are often hard to make (Clancy, Darlington and Finlay, 2001; Erecinska, Cherian and Silver, 2004). Rats and mice are examples of altricial species that give birth to relatively immature young, in contrast to precocial species, like guinea pigs, that are born at a relatively well-developed stage. In guinea pigs, KCC2 upregulation takes place *in utero* and the hyperpolarizing $GABA_AR$-mediated responses are fully mature at birth (Rivera *et al.*, 1999; Spoljaric *et al.*, 2017). With respect to brain maturation, the neonate human is an intermediate between these two extremes: the GABAergic system matures around birth and in prematurely born infants, the depolarizing-to-hyperpolarizing shift may still be incomplete (Kaila, Price, *et al.*, 2014; Puskarjov, Kahle, *et al.*, 2014).

2.2.2.2 *Expression and functional regulation of NKCC1 and KCC2*

NKCC1 and KCC2 are both members of the solute carrier 12 family of electroneutral CCCs (Blaesse *et al.*, 2009). Both KCC2 and NKCC1 are expressed in two splice variants, a and b, with similar ion transport characteristics (Uvarov et al. 2007, Morita et al. 2014, Kaila et al. 2014). KCC2b constitutes over 90 % of KCC2 in the mature

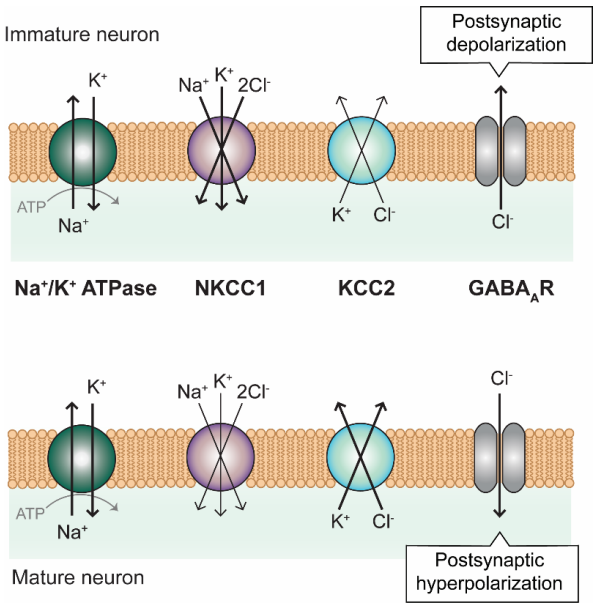

Figure 1. Regulation of intraneuronal chloride

The polarity and magnitude of $GABA_AR$-mediated currents depend on the intraneuronal chloride concentration $[Cl]_i$. The active regulation of $[Cl]_i$ in central neurons is managed by two secondary active cation-chloride cotransporters, Na-K-2Cl cotransporter 1 (NKCC1) and K-Cl cotransporter 2 (KCC2). NKCC1 and KCC2 utilize the energy of Na^+ and K^+ transmembrane gradients to move Cl^- into and out of the cell, respectively. Na^+ and K^+ gradients are maintained by Na-K ATPase. In immature neurons, KCC2 expression is low whereas NKCC1-mediated Cl^- uptake is highly active, resulting in a relatively high $[Cl]_i$. In such conditions activation of $GABA_ARs$ leads to the outflow of Cl^- and depolarization of the postsynaptic cell. KCC2 expression increases with development and, in mature neurons, KCC2-mediated Cl^- extrusion dominates the transport. In most mature neurons $[Cl]_i$ is low and $GABA_AR$ activation hyperpolarizes the cell.

mouse cortical areas and it is responsible for the developmental upregulation of KCC2 (Uvarov *et al.*, 2007; Kaila, Price, *et al.*, 2014). While KCC2 is almost exclusively expressed in the central neurons, NKCC1 exhibits a rather ubiquitous expression pattern across all organ systems of the body (Vibat *et al.*, 2001; Kaila, Price, *et al.*, 2014). In the human brain, the NKCC1b isoform is predominant over NKCC1a (Vibat *et al.*, 2001). Most mature cortical neurons co-express NKCC1 and KCC2, but the distributions of the two differ, creating subcellular gradients in E_{GABA} and DF_{GABA} and allowing region specific GABAergic responses within one cell (Khirug *et al.*, 2008; Báldi, Varga and Tamás, 2010; Kaila, Price, *et al.*, 2014).

The mere presence of NKCC1 and KCC2 protein in a cell is not a direct indication of functional Cl^- transport due to diverse post-translational mechanisms regulating their activity (Kahle *et al.*, 2013; Medina *et al.*, 2014). Both KCC2 and NKCC1 carry several regulatory phosphorylation sites that strongly affect their transport activity and/or membrane trafficking (Lee *et al.*, 2007; Rinehart *et al.*, 2009; Kahle *et al.*, 2013; Kaila, Price, *et al.*, 2014; Medina *et al.*, 2014). In the developing cortical areas, KCC2 has been suggested to be predominantly transport-inactive due to the inhibitory phosphorylation by lysine deficient protein kinase 1 (WNK1) on its threonine residues Thr^{906} and Thr^{1007} (Rinehart *et al.*, 2009; de los Heros *et al.*, 2014; Friedel *et al.*, 2015). The phosphorylation of these sites declines with brain maturation, suggesting that de-phosphorylation of these threonines may contribute to the depolarizing-to-hyperpolarizing shift in $GABA_A$R-mediated responses (Rinehart *et al.*, 2009; Friedel *et al.*, 2015). An activity-induced increase in intracellular Ca^{2+} and activation of group I metabotropic glutamate receptors or 5-hydroxytryptamine (5-HT) type 2A receptors, have been implicated in phosphorylation of the KCC2 serine residue 940 (Ser^{940}) (Fiumelli, Cancedda and Poo, 2005; Banke and Gegelashvili, 2008; Lee *et al.*, 2011; Bos *et al.*, 2013). Phosphorylation of Ser^{940} enhances KCC2 activity and stability on the cell membrane (Lee *et al.*, 2007).
Finally, diverse cellular protein-protein interactions have been implicated in the regulation of KCC2 function (Medina *et al.*, 2014). In interaction with specific cytoskeletal proteins, KCC2 serves a structural role in the dendritic spines that is independent of the ion-transport function (Li *et al.*, 2007; Llano *et al.*, 2015). As KCC2 is critically involved in spinogenesis, it forms a link and a possible synchronizing factor in the development of the excitatory glutamatergic and inhibitory GABAergic neurotransmission (Li *et al.*, 2007; Fiumelli *et al.*, 2013).

Cl^- uptake by NKCC1 can be selectively inhibited with low concentrations of the loop-diuretic bumetanide, whereas the functional studies on KCC2 have long been limited by lack of specific pharmacological compounds to modulate its function. Furosemide can be used inhibit Cl^- extrusion, but it also affects NKCC1 with a similar potency

(Payne *et al.*, 2003). The first putatively selective antagonist for KCC2 was described 2012 (Delpire *et al.*, 2012). In the original study, VU0463271 exhibited more than 100-fold selectivity to KCC2 over NKCC1 and no additional targets besides KCC2 were detected in a wide screen of other transporters, receptors and ion channels (Delpire *et al.*, 2012), making it an attractive novel tool in a study of KCC2 function.

2.3 Network factors contributing to GDPs

2.3.1 GABAergic signaling and interneurons in regulation of GDPs

2.3.1.1 Role of $GABA_AR$- and $GABA_BR$-mediated signaling in generation and modulation of GDPs

In the hippocampus, the GABAergic interneurons constitute approximately 10-15 % of the total neuronal population and practically all neurons are sensitive to GABA (Kaila, 1994; Isaacson and Scanziani, 2011; Pelkey *et al.*, 2017). GABA activates two types of receptors. $GABA_ARs$ are ligand-gated ion channels permeable to Cl^- and HCO_3^- and they are the receptor type responsible for the fast synaptic responses of GABA. In addition, certain types of $GABA_ARs$ are found in the extrasynaptic locations. Unlike synaptic $GABA_ARs$, the extrasynaptic $GABA_ARs$ typically have a very high affinity for GABA and they typically mediate persistent, tonic GABAergic currents (Farrant and Kaila, 2007; Belelli *et al.*, 2009). $GABA_AR$-mediated inhibition can result from hyperpolarization of the membrane potential or an increase in membrane conductance that causes shunting of the excitatory inputs (Farrant and Kaila, 2007; Kaila, Price, *et al.*, 2014). $GABA_B$ receptors ($GABA_BRs$) are G-protein coupled receptors that are functionally linked to K^+ or Ca^{2+} channels. They mediate slow inhibitory responses, resulting from inhibition of presynaptic Ca^{2+} currents or increase in the postsynaptic K^+ conductance (Kaila, 1994).

Under physiological conditions, the depolarization that primes the conditional pacemaker cells to trigger is provided by tonic GABAergic input (Sipilä *et al.*, 2005), and synchronous activity of the GABAergic interneurons during GDPs forms a major component driving these events (Khazipov *et al.*, 1997; Bolea *et al.*, 1999). Interestingly, both *in vivo* and *in vitro* studies indicate dual effects of $GABA_AR$ activation in neonates. Kirmse and colleagues (2015) demonstrated that a puff application of GABA on neonatal cortical plate neurons *in vivo*, depolarizes the majority of immature neurons, but inhibits the spontaneous network oscillations in this area. In P0-5 hippocampal slices, application of $GABA_AR$ agonists was shown first to increase the frequency of GDPs, followed by complete blockade of the activity

(Khalilov *et al.*, 1999). Correspondingly, an increase in endogenous GABA levels by blockade of GABA transporter 1 (GAT1) that is responsible for the uptake of GABA in the presynaptic terminal, reduces the frequency of GDPs (Sipilä *et al.*, 2004). These results suggest that GABAergic signalling can suppress neuronal activity already in the immature brain. In agreement with this, various studies demonstrate that blockers of $GABA_AR$-mediated signalling produce hypersynchronization and ictal-like activity in immature networks (Khalilov *et al.*, 1999; Lamsa *et al.*, 2000; Wells *et al.*, 2000).

All of the above studies suggest that the inhibitory actions arise from the $GABA_AR$-mediated shunting of the glutamatergic inputs that can also take place at slightly depolarizing levels of E_{GABA}. It has remained elusive, however, how such inhibitory effects can be maintained during intense neuronal activity, as in the absence of Cl^- extrusion E_{GABA} should rapidly be drawn to excitatory values (Buzsáki, Kaila and Raichle, 2007; Raimondo, Richards and Woodin, 2017). In Study III of the present work, we demonstrate the presence of transport-functional KCC2 in hippocampal CA3 pyramidal neurons of perinatal mice and rats. The KCC2-mediated Cl^- extrusion restricts the depolarizing GABAergic drive onto CA3 pyramidal neurons, and likely also provides a mechanism to maintain shunting inhibition during pronounced neuronal activity.

In addition to $GABA_AR$-mediated effects, activation of $GABA_BRs$ has been suggested to be involved in the regulation of GDPs. In a recent study, Khalilov and colleagues (2017) showed that $GABA_BR$ activation and the associated K^+ current, contribute to the afterhyperpolarization (AHP) that follows GDPs. During AHP, $GABA_BR$ activation acts in concert with $I_{K(Ca2+)}$ (see above), but unlike the latter, the $GABA_BR$-mediated component is not dependent on the spiking of the cell during GDPs. Blockade of $GABA_BRs$ robustly reduced the GDP-AHP and prolonged GDPs.

A summary of the key mechanisms in GDP generation are illustrated in Figure 2.

2.3.1.2 Contribution of distinct interneuron types to GDP generation

In mice and rats, cortical interneurons are generated starting at embryonic day (E) 9 from progenitor cells in the embryonic subpallium. From subpallium, interneurons migrate tangentially to their final destinations in the neocortex and hippocampus. There are two main pools or progenitors giving rise to the diverse cortical interneuron subtypes. Most parvalbumin and somatostatin expressing interneurons originate from the medial ganglionic eminence (MGE), whereas the caudal ganglionic eminence (CGE) gives rice to the cholecystokinin, reelin, calretinin, and vasointestinal

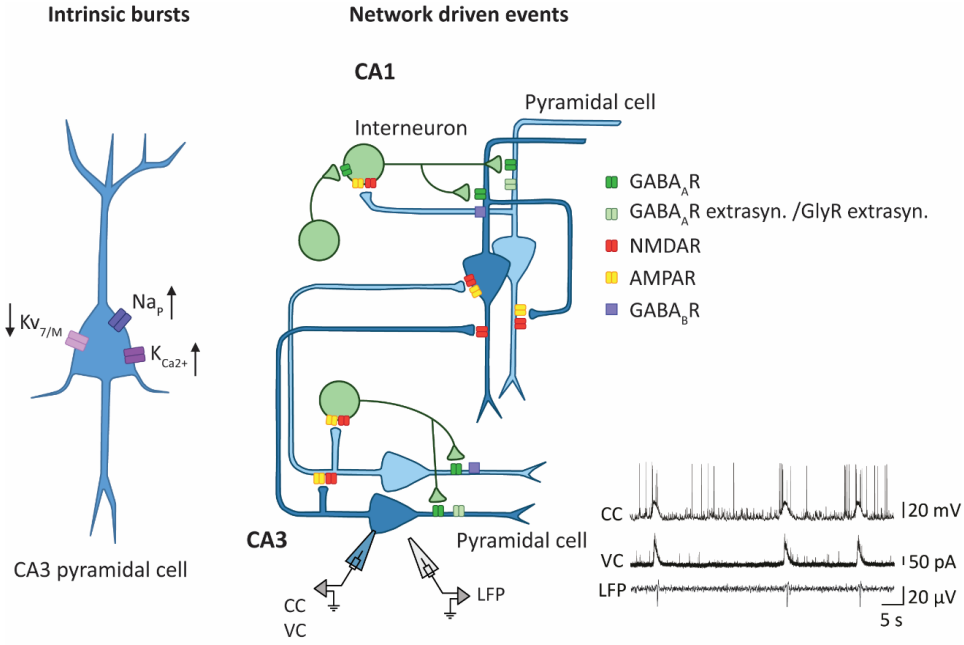

Figure 2. Cellular and network mechanisms in GDP generation

CA3 pyramidal neurons function as conditional pacemakers of GDPs, firing at a regular 0.1-0.3 Hz bursts when depolarized. The bursts in the pacemaker cells are initiated by persistent Na+ current (I_{Nap}) and terminated by the Ca^{2+} activated K^+ current ($I_{K(Ca2+)}$). Low expression of $Kv_{7/M}$ channels that mediate muscarinic K^+ current facilitate bursting of immature neurons. Under physiological conditions, the depolarization facilitating the GDP initiation in the pacemaker cells is provided by tonic $GABA_AR$-mediated input, and the synchronous activity of the GABAergic interneurons during GDPs forms a major component driving these events. Furthermore, $GABA_BR$ activation participates in termination of GDPs. Thus, both the glutamatergic pyramidal neurons and the GABAergic interneurons are involved in GDPs and their cooperative function is necessary for GDP generation. GDPs can be electrophysiologically monitored both with local field potential (LFP) and single cell recordings. Current clamp tracks activity of a single cell, while recordings of spontaneous synaptic currents (sIPSCs or sEPSCs) in voltage clamp mode allow monitoring activity of a population of neurons targeting the recorded cell. Examples of GDP recordings from a CA3 pyramidal neuron in whole-cell current-clamp (CC) and voltage clamp (VC, sIPSCs) mode as well as an LFP trace are presented in the right-most panel.

peptide expressing interneurons (Danglot, Triller and Marty, 2006; Cossart, 2011; Pelkey *et al.*, 2017). The first interneurons can be detected in the hippocampus as early as E15.5 by immunostaining using glutamate decarboxylase 67 (GAD67) or distal-less homeobox gene 2 (DLX2) as a marker (Pleasure *et al.*, 2000; Danglot, Triller and Marty, 2006; Manent *et al.*, 2006). Interneurons are characterized by their axonal arborization, specific chemical markers and electrophysiological features. In the mature brain, multiple interneuron subtypes can be indentified in all layers of the hippocampus (Klausberger and Somogyi, 2008; Pelkey *et al.*, 2017). Due to the late maturation of the anatomical and physiological characteristics used for subtype identification, classification of interneurons is more complicated in the developing brain (Tyzio *et al.*, 1999; Danglot, Triller and Marty, 2006; Cossart, 2011; Magueresse and Monyer, 2013).

The MGE-derived interneurons (MGE-INs) have been suggested to be more important in the generation of GDPs compared to the CGE-INs. A recent study (Wester and McBain, 2016) demonstrated that optogenetic inhibition of MGE-INs in P6 mouse brain slices strongly suppressed GDPs, whereas only a moderate reduction in GDP frequency was seen upon CGE-IN inhibition. The study suggests that the P6 MGE-INs are highly recurrently connected, often target perisomatic regions and have a high initial release probability during bursts, as opposed to more sparsely connected CGE-INs that typically target dendritic regions and exhibit slower asynchronous GABA release. The authors tentatively suggest that these features make the MGE-INs and the CGE-INs well-suited to contribute to the initiation and cessation of GDPs, respectively.

Astonishingly, even individual, widely connected GABAergic interneurons can potently influence network synchrony in the hippocampus (Bonifazi *et al.*, 2009; Picardo *et al.*, 2011). These hub neurons develop, at least in part, from the first populations of GABAergic precursors leaving the ganglionic eminences (Picardo *et al.*, 2011) and when stimulated, can either increase or decrease the frequency of GDPs.

2.3.2 Glycinergic modulation of GDPs

In addition to GABA, glycinergic signaling has been implicated in the regulation of GDPs (Cherubini *et al.*, 1990; Gaiarsa *et al.*, 1990). In the caudal structures of CNS, the brainstem and the spinal cord, glycine is the major neurotransmitter mediating fast synaptic inhibition, but in cortical areas it predominantly operates as a modulatory neurotransmitter (Lynch, 2004; Avila, Nguyen and Rigo, 2013). Glycine acts via strychnine sensitive GlyRs, which are ligand-gated Cl^- and HCO_3^- - permeable ion channels closely related to $GABA_A$Rs and similarly to them, GlyR-mediated currents are directly influenced by $[Cl]_i$. In mature CNS, GlyRs are abundantly

expressed in the spinal cord but are also commonly detected in higher brain areas like cerebral cortex, hippocampus, cerebellum, thalamus and olfactory bulb (Malosio *et al.*, 1991). In addition to activating GlyRs, glycine serves as a co-agonist for the glutamatergic NMDARs via strychnine-insensitive binding site (Johnson and Ascher, 1987; Zhang *et al.*, 2008).

Glycinergic synapses have not been identified in the hippocampus but extrasynaptic GlyRs are present in this area already perinatally (Ito and Cherubini, 1991; Malosio *et al.*, 1991; Fatima-Shad and Barry, 1992; Aroeira *et al.*, 2011). In adult rodents, glycine is co-released from glutamatergic (Muller *et al.*, 2013) and GABAergic synapses (Danglot *et al.*, 2004; Song, Chattipakorn and McMahon, 2006). In addition, it has been suggested that astrocytes would release glycine in the hippocampus (Zhang *et al.*, 2008). Similarly to GABA, high (300 µM- 1 mM) concentrations of glycine depolarize CA3 pyramidal neurons in P2-4 rats in a strychnine-sensitive manner, and hyperpolarize them in the later stages of the development (Cherubini *et al.*, 1990; Ito and Cherubini, 1991).

Glycine has been shown to increase the frequency of GDPs when bath-applied at 10-50 µM. This excitatory effect has been attributed to NMDAR activation as it was insensitive to strychnine and was mimicked by D-serine, an agonist of the NMDAR glycine-binding site (Cherubini *et al.*, 1990; Gaiarsa *et al.*, 1990; Ito and Cherubini, 1991; Avila, Nguyen and Rigo, 2013). It should be noted, however, that the concentrations of glycine used in most studies are higher than the reported endogenous concentrations of glycine in the CNS (Baker *et al.*, 1991; Globus *et al.*, 1991; Whitehead *et al.*, 2001). In Study I of the present work, we demonstrate that, unlike the high glycine levels, application of low physiological concentrations of glycine *suppress* GDPs by activating extrasynaptic GlyRs. This effect could be mimicked by an increase in endogenous glycine levels induced by inhibiting glycine uptake via glycine transporter 1 (GlyT1).

2.3.3 Role of arginine vasopressin in the developing hippocampus

Oxytocin (OT) and arginine vasopressin (AVP) are peptide hormones primarily produced in the hypothalamic supraoptic and paraventricular nuclei. In addition to targeting the posterior pituitary from where the hormones are released into the bloodstream, the processes from OT- and AVP-releasing neurons innervate various areas in the brain, including the hippocampus (Ludwig and Leng, 2006; Stoop, 2012; Cui, Gerfen and Young, 2013; Zhang and Hernández, 2013). OT and AVP act via G-protein coupled receptors that regulate either K^+ or non-selective cation conductances (Koshimizu *et al.*, 2012; Cilz, Cymerblit-Sabba and Young, 2018). In the mature rodent hippocampus, activation of vasopressin receptors 1a (V1aR) and 1b

(V1bR), and oxytocin receptor (OTR) modulate the activity of both the principal cells and interneurons (Ramanathan *et al.*, 2012; Cilz, Cymerblit-Sabba and Young, 2018; Tirko *et al.*, 2018). Activation of OTRs has also been implicated in suppression of SPWs in juvenile mice (Maier *et al.*, 2016), but next to nothing is known about the actions of OT and AVP in the developing hippocampus.

In rats, the OT system matures postnatally (Altstein and Gainer, 1988). It has been suggested that around birth, maternal OT, passing via the placenta and the fetal blood-brain barrier (BBB), causes a transient shift (in rats E19-P1) in the polarity of the GABAergic responses in the fetal brain by OTR-mediated downregulation NKCC1 (Tyzio *et al.*, 2006, 2014). However, considering the maternal plasma OT levels that remain at subnanomolar range during birth (Higuchi *et al.*, 1986; Chard, 1989) and the poor permeability of BBB to OT (Mens, Witter and van Wimersma Greidanus, 1983; Leng and Ludwig, 2016), it seems unlikely OT in the fetal brain could reach a level high enough to activate the OTRs (K_i in rats 1 nM) (Manning *et al.*, 2012).

In contrast to OT, a fully processed form of AVP can be detected in the rat hypothalamus already at E16 (Altstein and Gainer, 1988). AVP is an important stress hormone in mammals starting from the fetal stages (Benzing *et al.*, 2011; Burkhardt *et al.*, 2012; Summanen *et al.*, 2017). Mammalian birth is known to be associated with a strong activation of the fetal HPA axis resulting a massive surge of fetal stress hormones into the circulation (Stark *et al.*, 1979; Lagercrantz and Slotkin, 1986; Wellmann *et al.*, 2010; Schlapbach *et al.*, 2011; Hillman, Kallapur and Jobe, 2012; Summanen *et al.*, 2017, 2018). Stress hormones play a crucial role in assisting the transition of the infant to its life outside the uterus (Pohjavuori and Fyhrquist, 1980; Lagercrantz and Slotkin, 1986; Hillman, Kallapur and Jobe, 2012; Evers and Wellmann, 2016). During birth, fetal AVP orchestrates adaptive peripheral processes such as analgesia, maintainance of the blood-pressure and initiation of lung-based breathing (Pohjavuori and Fyhrquist, 1980; Wallace, Hooper and Harding, 1990; Evers and Wellmann, 2016).
In mammals, birth is always associated with a period of perinatal asphyxia. This would be expected to be a particularly great risk for the fetal brain that relies on oxidative metabolism, yet the physiological processes that have evolved to protect the brain during parturition are relatively unknown. Hypoxia is a strong stimulant of AVP release during birth (Evers and Wellmann, 2016), but while the importance of AVP in mediating peripheral adaptations is well known, the neuroprotective potential of this peptide in association to birth has not been previously studied.

In Study II of the present work we show that fibers from AVPergic cells innervate the rat hippocampus as early as P0. Furthermore, we demonstrate that AVP activates a

subset of interneurons and robustly suppresses hippocampal network events in perinatal rats and guinea pigs. This suggests an evolutionarily conserved role for AVP in reducing the energy demand of the brain during birth in mammals.

2.4 GDPs in circuit formation

Spontaneous network events have been implicated in developmental processes such as synapse maturation and pathfinding (Blankenship and Feller, 2010; Kirkby *et al.*, 2013). GDP-associated Ca^{2+} oscillations have been suggested to act as Hebbian type coincidence detectors, allowing long-term potentiation of synapses which are active during GDPs. Pairing of the mossy fiber or Schaffer collateral stimulation with GDPs, has been shown to selectively strengthen the active synapses (Kasyanov *et al.*, 2004; Mohajerani *et al.*, 2007). This potentiation of the paired connections was dependent on the postsynaptic Ca^{2+} influx and the voltage-gated Ca^{2+} channels (Kasyanov *et al.*, 2004) as well as on the endogenous brain-derived neurotrophic factor (BDNF) and activation of presynaptic tropomyosin-related kinase receptor B (TrkB) (Mohajerani *et al.*, 2007). By strengthening the concurrently active synapses, GDPs would promote the development of the local circuits.

Impairments in $[Cl^-]_i$ and $GABA_AR$ signaling have been associated with many neurodevelopmental disorders like the autism spectrum disorders (ASD) (Ben-Ari, 2017; Moore *et al.*, 2017). Changes in the GABAergic signaling would be expected to directly affect GDPs. Indeed, changes in GDP frequency and developmental timing have been reported in mouse models of Down-Syndrome and autism (Pizzarelli and Cherubini, 2013; Cellot *et al.*, 2016; Lysenko *et al.*, 2018). Maladaptive changes in network events may also be involved in fetal alcohol syndrome, as ethanol strongly interferes with network events in the developing hippocampus (Galindo, Zamudio and Valenzuela, 2005; Chernova *et al.*, 2017). Such changes in GDPs could, at least in part, account for the subsequent structural and functional impairments of hippocampal network, associated with these disorders (Galindo, Zamudio and Valenzuela, 2005; Meyza *et al.*, 2012; Chernova *et al.*, 2017).

Increasing knowledge on early brain plasticity and epigenetics has emphasized the impact of early life phases in long-term health outcomes, creating a whole new research field known as the developmental origins of health and disease (DOHaD)(Gluckman and Hanson, 2004). It is becoming more evident that developmental factors can contribute to the risk of many late-onset brain disorders, such as Alzheimer's and Parkinson's disease (Schaefers and Teuchert-Noodt, 2013; Hanson and Gluckman, 2014; Faa *et al.*, 2016). During development various environmental cues can affect the developmental processes and lead to the

establishment of various phenotypes from a single genotype. This plasticity allows flexible adaptation to the prevailing conditions but also makes the brain more vulnerable to pathological insults, which may partly explain why the most sensitive periods are restricted to a narrow developmental time window (Michel and Tyler, 2005; Schaefers and Teuchert-Noodt, 2013).

GDPs define a critical window of hippocampal development, during which the construction of the circuit is driven by mechanisms which are no longer functional in the mature brain. Thus, they have a potential to permanently affect neuronal structure and function. Since the circuits driving the activity patterns in the early phases of development are essentially different than the mature ones, traces of early insults may be hidden by the maturing network and some of the consequences may only emerge later on in life (Khazipov and Luhmann, 2006; Colonnese and Phillips, 2018). A thorough understanding of the circuits that drive and regulate the early network events during the brain development will help us to better understand disorders of the developing brain and may provide new strategies to treat them.

3 Aims

The general aim of this thesis is to study the role of $GABA_AR$-mediated signaling and Cl^- regulation in restraining GDPs in the perinatal rodent hippocampus:

i) examine how low levels of GABA and glycine modulate GDPs.
ii) investigate central effects of the stress hormone AVP around birth, focusing on hippocampal network events and GABAergic neurotransmission.
iii) study the functional expression and physiological role of KCC2 in controlling GDPs in the perinatal hippocampus.

4 Experimental procedures

For a detailed description of the experimental procedures used, the reader is referred to the original Studies I-III. The methods used by the candidate are described in brief below.

All the electrophysiologal experiments in Studies I-III were done using acute hippocampal slices or intact hippocampal preparations (*in toto* hippocampi, Khalilov *et al.*, 1997). Such preparations allow detailed examination of local circuit function as they conserve the main structure and connectivity in the tissue, and enable direct pharmacological manipulations.
Local field potential (LFP) recordings provide a technique to study network function without perturbation of the intracellular milieu by measuring the electrical fields in the extracellular space. LFP signal is created by the current sinks and sources resulting from the activity of the nearby neurons. In this thesis, LFP recordings were used to monitor GDPs and multiunit activity (MUA).
In whole-cell voltage clamp recordings, the membrane voltage of the recorded neuron is controlled to monitor the transmembrane currents. In this thesis, whole-cell voltage clamp recordings were used to monitor spontaneous synaptic and extrasynaptic currents as well as to estimate E_{GABA} level using GABA uncaging. Here, recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) were used to monitor the activity of interneurons, while miniature inhibitory postsynaptic currents (mIPSCs) recorded in the presence of TTX served in examining activity-independent changes in the function of inhibitory synapses.

4.1 Acute brain slices and *in toto* hippocampi

Perinatal (E18.5-P5) Wistar rat pups and E17.5-18.5 $KCC2^{+/+}$ and $KCC2^{-/-}$ mice (Vilen *et al.*, 2001) of either sex were used in the experiments. The work was approved by the Local animal ethics committee of the University of Helsinki.
Embryos collected from timed-pregnant dams and neonatal pups were decapitated and brains were quickly dissected into ice cold (< 4 ˚C) cutting solution. For the preparation of *in toto* hippocampi, cerebellum was removed and the hemispheres were separated with a scalpel blade. The deeper parts of the brain (brainstem, midbrain and striatum) were removed and the hippocampi were gently separated from the neocortex using two spatulas (see also Khalilov *et al.*, 1997). Horizontal brain slices (400 µm) were cut using a vibratome (Campden istruments 7000 smz-2).

After preparation, the slices and *in toto* hippocampi were moved to standard solution and let to recover for 1 hour in + 34˚C. After the recovery, the slices and *in toto* hippocampi were stored in room temperature for up to 6 hours. All extracellular solutions used in the preparation, for the recovery and during the recordings, were equilibrated with carbogen (95 % O_2, 5 % CO_2).

4.2 Electrophysiological recordings

LFP recordings were performed in a submerged recording chamber (32±1 ˚C) with constant double-sided perfusion (3.5 ml/min for the slices and 5 ml/min for the *in toto* hippocampi). Both preparations were anchored into the chamber with silver wires and were let to adjust for minimum of 10 minutes before the start of the recordings. LFP recordings were obtained with a custom-made amplifier and collected to the disk with WinEDR (Strathclyde electrophysiology) using 20 kHz sampling frequency, 1000 x amplification and 5 kHz low pass filter. Thin filament class-capillary pipettes (tip diameter 4-8 µm) filled with 150 mM NaCl were placed in CA3 pyramidal layer. Slices with stable GDP activity were included in the analysis.

For the whole-cell voltage clamp recordings of mIPSCs the recording solution was supplemented with TTX (0.5 µM), CNQX (10 µM) and D-AP5 (20 µM). The recordings were obtained from visually identified CA3 pyramidal neurons using EPC-10 patch amplifier and Patch Master software (HEKA) with a sampling rate of 50 kHz. Borosilicate patch pipettes (resistance 3-5 MΩ) were filled with intracellular solution containing (in mM): 140 Cs-methanesulfonate, 2 MgCl2, and 10 Hepes (280 ± 5 mOsm) pH adjusted to 7.2 with CsOH, calculated liquid junction potential 13 mV. Neurons were held at a holding potential of 0 mV and series resistance compensation was done online. Neurons with stable holding current and series resistance below 25 MΩ (change < 30 % during the recording) were included in the analysis.

4.3 Analysis

GDPs were detected manually in Clampfit (Molecular Devices) after band-pass filtering (1-10 Hz) and with a threshold individually chosen for each recording. GDP area (mV × ms) was quantified using numerical integration for each event in Clampfit (Spoljaric *et al.*, 2017). The event areas were analyzed in consecutive 10-s bins to obtain total area that takes into account both frequency and amplitude of the events. mIPSCs were manually detected in MiniAnalysis (Synaptosoft) after 1000 Hz low pass filtering and using a threshold of 4 x RMS noise.

5 Results

5.1 Glycine transporter-1 controls nonsynaptic inhibitory actions of glycine receptors in the neonatal rat hippocampus (I)

High, non-physiological levels of exogenous glycine are known to facilitate GDPs via NMDAR activation (Cherubini *et al.*, 1990; Gaiarsa *et al.*, 1990) but besides this, not much is known about the actions of glycine in the neonatal hippocampus. In this study, we demonstrated single-channel currents in P0-6 rat CA3 pyramidal neurons that were mediated by GlyRs, judged by their sensitivity to the GlyR blocker strychnine and to the GlyR and $GABA_AR$ blocker picrotoxin. In the presence of blockers for NMDARs and $GABA_ARs$, the currents were strongly enhanced by both bath applied glycine (10-30 μM) and the GlyT1 blocker NFPS. When the perfusion was further supplemented with TTX, glycine induced a dose-dependent increase in the holding current. In the presence of blockers for ionotropic glutamatergic receptors (iGluR block), glycine (30 μM) induced an increase in the baseline current, which was further augmented in the presence of NFPS. Altogether, these results strongly suggest that glycine acts as an endogenous agonist for nonsynaptic GlyRs in the neonatal rat hippocampus and demonstrate a powerful uptake of glycine by GlyT1 in control conditions and during experimental glycine load. NFPS-induced GlyR-mediated currents were not dependent on neuronal activity, as shown by their insensitivity to TTX and depletion of Ca^{2+} from the perfusion medium.

Surprisingly, GlyT1 blockade by NFPS reduced the frequency of GDPs in LFP recordings in the CA3 area. This effect was mimicked by bath-application of a low (2 μM) concentration of glycine, suggesting that the inhibitory effect was due to a slight increase in GlyR conductance. Indeed, in the presence of strychnine, glycine did not affect GDP frequency, confirming the pivotal role of GlyRs in mediating this effect.

In order to determine whether the effect is unique for GlyR activation, we next examined if similar effect could be produced by activating $GABA_ARs$. In a manner similar to glycine, bath-application of a low concentration (100 nM) of $GABA_AR$ agonist isoguvacine reduced the frequency of GDPs.

Finally, in the presence of synaptic $GABA_AR$ and iGluR blockers, NFPS and 2 μM glycine also inhibited MUA recorded in the CA3 pyramidal layer, suggesting that the suppression of GDPs by GlyR activation was due to a direct effect on pyramidal neuron excitability rather than network-mediated actions.

5.2 Vasopressin excites interneurons to suppress hippocampal network activity across a broad span of brain maturity at birth (II)

During birth, fetal AVP is known to mediate a variety of peripheral adaptive processes that assist the transition of the infant for life outside of the womb (Pohjavuori and Fyhrquist, 1980; Wallace, Hooper and Harding, 1990; Evers and Wellmann, 2016). While AVPergic neurons also form central projections in the adult (Stoop, 2012), a role of this stress hormone in the fetal brain during and around birth remains unknown. In this study, we examined the AVP-mediated responses in the perinatal rodent brain.

AVPergic fibres originating from hypothalamic paraventricular and supraoptic nuclei are known to innervate adult hippocampus (Cui, Gerfen and Young, 2013; Zhang and Hernández, 2013) where AVP regulates the excitability of interneurons and principal cells (Ramanathan *et al.*, 2012). Here, by using the whole-tissue clearing method CLARITY (Chung and Deisseroth, 2013) and immunohistochemistry to provide a three-dimensional visualization of the connections, we confirmed that AVPergic fibers innervate the rat hippocampus already at P0. To address the functional effects of AVP in the perinatal hippocampus, we wanted to examine the effect of AVP on GDPs that strongly rely on depolarizing drive provided by GABA. However, as mentioned above, some studies suggest that GABA transiently switches polarity due to downregulation of NKCC1 around birth (Tyzio *et al.*, 2006, 2014). Therefore, we first studied whether GDPs are present in the time window of our study (E21.5 – P2). Using LFP recordings, we detected bumetanide-sensitive GDPs in hippocampal slices and *in toto* hippocampi throughout the perinatal period starting from E21.5. Furthermore, pharmacological $GABA_AR$ activation evoked spiking in 95 % and intracellular Ca^{2+} transients in 98 % of pyramidal neurons, monitored by loose-cell attached recordings and Ca^{2+} imaging, respectively. Altogether, these data indicate that the DF_{GABA} remains depolarizing throughout the perinatal period in the rat hippocampus.

Bath applied AVP (10 nM) caused a robust transient suppression of GDPs at all age points studied. The transient nature of AVP effect was likely caused by receptor desensitization and after a 10-minute washout of the drug, the effect was fully reproducible. Suppression of GDPs by AVP was strongly attenuated by the V1aR blocker SR49059, suggesting that this receptor mediates the effect. Since AVP binds to the OTRs with similar affinity as to the V1aR, we also examined the possible involvement of OTR in the observed AVP effect. However, the selective activation of OTRs by low concentration of OT (10 nM), had no effect on GDPs.

Next, we wanted to study whether the activity of interneurons and/or pyramidal neurons is affected by AVP. Interestingly, AVP failed to change CA3 pyramidal neuron activity monitored by extracellular MUA activity in the presence of picrotoxin as well as by whole-cell spontaneous excitatory postsynaptic currents (sEPSCs) recorded from the CA3 pyramidal cells. Furthermore, neither the holding current nor the input resistance in the pyramidal neurons were affected by AVP, indicating that they were insensitive to AVP. In contrast to this, AVP caused a robust increase in the frequency of sIPSCs recorded in whole-cell voltage-clamp from pyramidal neurons in iGluR block, and the effect was strongly suppressed by SR49059. Conversely, AVP had no effect on the whole-cell mIPSCs, recorded in the presence of iGluR block and TTX, nor on DF_{GABA} in gramicidin-perforated patch recordings from CA3 pyramidal neurons. Overall, these experiments suggest that AVP enhances the activity of hippocampal interneurons without affecting the pyramidal cells.

Whole-cell current clamp recordings from visually identified interneurons in *stratum lucidum* and *stratum radiatum* denoted in the original paper and below as "SLR interneurons" in P0-2 VGAT-Venus rat brain slices (Uematsu *et al.*, 2008), confirmed that AVP induced spiking of pharmacologically isolated (picrotoxin, iGluR block) interneurons in a SR49059-sensitive manner. Interestingly, only the SLR interneurons responded to AVP whereas the interneurons located in *stratum oriens* and in *stratum pyramidale* were not affected by AVP. In line with the electrophysiological data, a highly sensitive fluorescent *in situ* assay RNAscope (Wang *et al.*, 2012) revealed that the V1aR staining in P0 rat hippocampal CA3 area localized mainly in the SLR interneurons.

To assess the AVP induced changes in the dynamics of GABAergic drive and pyramidal neuron firing during GDPs, simultaneous three-electrode recordings were performed from the CA3 pyramidal layer, with one voltage-clamped and one current-clamped pyramidal neuron, combined with an LFP electrode. The voltage clamp recordings demonstrated a steep decrease in the synchronous sIPSC bursts associated with field GDPs after the application of AVP, whereas the sIPSC activity between the bursts was robustly increased. The decrease in synchronous GABAergic bursts was paralleled by decreased depolarization and spiking of the current-clamped pyramidal neuron and the abolishment of field GDPs in the LFP recording. These results suggest that the suppression of GDPs by AVP is caused by sustained activation of interneurons that prevents the synchronous GABAergic drive crucial for the generation of GDPs.

Finally, we wanted to examine whether the AVP-mediated suppression of synchronous network events is dependent on the level of maturation of the hippocampal network. In contrast to rats, the guinea pigs are born at a more mature state of cortical development and they have high levels of KCC2 and hyperpolarizing GABAergic responses already at birth (Rivera et al 1999). In agreement with the

previous studies, we demonstrated that upregulation of KCC2 protein expression in guinea pigs takes place *in utero*. Furthermore, strong KCC2-mediated Cl^- extrusion was already detected in the P0 guinea pig hippocampal CA3 pyramidal neurons, demonstrated by the presence of a somato-dendritic E_{GABA} gradient under a sustained somatic Cl^- load. In the neonatal guinea pig brain slices, GDPs have already been replaced by SPWs akin to those found in slices from juvenile rats and mice. SPWs are not dependent on depolarizing GABA, like GDPs are, but they do rely on synchronous interneuronal input.

Astonishingly, bath application of AVP suppressed SPWs in LFP recordings from P0-2 guinea pig hippocampus and, like in rats, this effect was sensitive to SR49059. Furthermore, AVP induced an SR49059-sensitive increase in sIPSC frequency, indicating that suppression of the network events also in more mature hippocampus resulted from desynchronization. These data suggest that the AVP-mediated suppression of hippocampal network events is independent on the maturational level of the network.

5.3 KCC2-mediated Cl^- extrusion modulates spontaneous hippocampal network events in perinatal rats and mice (III)

The existing literature suggests negligible KCC2 expression in the cortical neurons of perinatal mice and rats (Rivera *et al.*, 1999; Li *et al.*, 2002; Stein *et al.*, 2004). In the present study, we show that blocking KCC2 with a novel KCC2 inhibitor VU0463271 (10 µM) (Delpire *et al.*, 2012; Sivakumaran *et al.*, 2015), induces a robust increase in GDP frequency and amplitude in the neonatal (P0-5) rats. This suggests a contribution of KCC2 in the regulation of GDPs already in perinatal stages. In line with this, GDPs detected in *in toto* hippocampi from E17.5-18.5 $KCC2^{-/-}$ mice (Vilen *et al.*, 2001) were bigger in amplitude compared to $KCC2^{+/+}$ littermates. In a manner similar to its effect in neonatal rats, VU0463271 enhanced GDP frequency and amplitude in embryonic $KCC2^{+/+}$ mice but not in the $KCC2^{-/-}$ mice. Accordingly, immunohistochemical stainings revealed KCC2 expression at E18.5 $KCC2^{+/+}$ mouse CA3 pyramidal layer and *stratum radiatum*, which was not seen in the $KCC2^{-/-}$ mice. KCC2 immunohistochemistry in perinatal GAD67-GFP mice (Tamamaki *et al.*, 2003) suggested that the majority of cells with distinct KCC2 expression were non-GABAergic. Indeed, a detailed analysis of somatic KCC2 staining in E18 - P1 mice confirmed, that KCC2 staining in CA3 pyramidal neurons was higher (89/290 neurons) than in interneurons (48/300 neurons). Since such data does not take into account the KCC2 expressed in the dendritic region nor does it denote the functional state of the transporter, we next moved on to study the functional responses of interneurons and pyramidal neurons to VU0463271. In loose-cell attached recordings from CA3

pyramidal cells, VU0463271 robustly increased the GABA-driven spiking of the pyramidal neurons, whereas no change was observed in the sIPSCs recorded in whole-cell patch clamp from the pyramidal neurons, both performed in the presence of iGluR blockers. We have shown earlier that the Cl^- extrusion capacity in the rat perinatal pyramidal neurons is extremely weak and goes through gradual upregulation during the first two postnatal weeks (Khirug *et al.*, 2005, 2010). In agreement with the previous results, in the presence of bumetanide and under a somatically imposed Cl^- load, E_{GABA} did not differ significantly from the levels predicted by passive Cl^- distribution either in the soma or in the dendrite at a 50 µm distance from the soma. However, when the resolution of the technique was improved by moving along the dendrite to a distance of 200 µm from the soma, E_{GABA} values revealed a negative deflection from the calculated E_{GABA} value. VU0463271 caused a positive shift in E_{GABA} towards the passive values indicating that the negative deflection of E_{GABA} in the distal dendrites resulted from KCC2-mediated Cl^- extrusion. This method shows that KCC2 is functional in the distal dendrites, but it does not exclude possible somatic KCC2 function.

Next, we performed gramicidin-perforated voltage clamp recordings of mIPSCs from the CA3 pyramidal neurons (in the presence of iGluR blockers and TTX). In these experiments, VU0463271 induced a significant increase in mIPSC amplitude, indicating an increase in the driving force of GABAergic postsynaptic currents.
Having shown that the KCC2-mediated Cl^- extrusion restrains the depolarizing GABAergic drive in pharmacologically isolated CA3 pyramidal neurons, we next wanted to study how KCC2 inhibition affects the spiking of the pyramidal neurons during GDPs. To achieve this, we performed simultaneous loose cell-attached recordings from CA3 pyramidal neurons and LFP recordings from the CA3 pyramidal layer. Application of VU0463271 had no effect on the total number of GDP-nested spikes in either MUA or loose cell-attached recordings. Instead, it increased the spiking probability during the GDP rising phase. This shows that inhibition of KCC2 enhances the excitability of the pyramidal neurons and leads to increased synchronization of pyramidal cell firing during the rising phase of GDP.

6 Discussion and Conclusions

In this thesis, the main goal was to investigate perinatal Cl^- extrusion in mouse and rat hippocampus and the $GABA_AR$-mediated control of GDPs. Our results demonstrate that NKCC1-mediated depolarizing GABAergic drive is maintained in the hippocampal CA3 neurons throughout the perinatal period in rats, but it is modulated by KCC2-mediated Cl^- extrusion that opposes the predominant Cl^- intake. KCC2 function limits the excitability and synchronization of the pyramidal neurons during GDPs restraining the frequency and amplitude of the events. Our data support the dual role of GABA (Khalilov *et al.*, 1999; Lamsa *et al.*, 2000) in the perinatal brain showing that low activation of $GABA_ARs$ or GlyRs reduces the excitability of the hippocampal pyramidal neurons and inhibits GDPs by shunting inhibition. Furthermore, we demonstrate that sustained activation of a subset of interneurons can suppress GDPs by desynchronizing the network.

6.1 Study I

The main finding in Study I is that modest activation of extrasynaptic GlyRs and $GABA_ARs$ by low levels of agonists can be functionally inhibitory in the neonatal hippocampus despite the depolarizing driving force of the Cl^- currents in this developmental stage.

In addition to glycine, β-alanine and taurine can activate GlyRs and have been suggested to act as endogenous agonists for this receptor in hippocampus (Mori, Gähwiler and Gerber, 2002). In this study, we demonstrated that the increase of endogenous glycine levels by inhibition of glycine transporter GlyT1 robustly enhanced the frequency of spontaneous strychnine sensitive currents in CA3 neurons. Furthermore, the blockade of GlyT1 mimicked the inhibitory effect of exogenous glycine on GDPs and spontaneous unit activity in LFP recordings. Hence, our results strongly support the idea that glycine is an endogenous agonist for GlyRs in the neonatal hippocampus, but do not exclude possible effects of β-alanine and taurine (Saransaari and Oja, 1997, 1999; Mori, Gähwiler and Gerber, 2002).

Previous studies in the neonatal hippocampus, have reported biphasic, dual effects of $GABA_AR$ activation on GDPs. Application of relatively high concentrations of $GABA_AR$ agonists muscimol or isoguvacine induce an increase in GDP frequency followed by suppression of the events (Khalilov *et al.*, 1999; Lamsa *et al.*, 2000). A

similar dual action was also recently reported for glycine in regulating seizures in a neonatal cortico-hippocampal preparation (Chen *et al.*, 2014). Chen and colleagues demonstrated that a moderate concentration (10 μM) of glycine augmented whereas a high concentration (100 μM) inhibited the spontaneous seizure activity. The applied concentrations of GABA and glycine, used in our study closely resemble the physiological extracellular values of these neurotransmitters in the CNS (Baker *et al.*, 1991; Globus *et al.*, 1991; Whitehead *et al.*, 2001). The observation that such concentrations of these transmitters suppress activity are in line with studies reporting emergence of seizure-like activities after the blockade of $GABA_AR$- or GlyR-mediated signaling (Khalilov *et al.*, 1999; Lamsa *et al.*, 2000; Chen *et al.*, 2014). Our data also readily explain the suppression of GDP frequency following pharmacological inhibition of GAT1 (Sipilä *et al.*, 2004).

Together with previous reports, our data imply that the effects of $GABA_AR$ and GlyR activation on the hippocampal network activity are highly sensitive to the concentration of the agonist (Khalilov *et al.*, 1999; Lamsa *et al.*, 2000; Sipilä *et al.*, 2005; Chen *et al.*, 2014). Overall, the data suggest a triphasic response of GDPs to an increasing concentration of bath applied GABA and glycine with the lowest concentrations inhibiting, intermediate exciting and highest concentrations again suppressing the events (Khalilov *et al.*, 1999; Lamsa *et al.*, 2000; Sipilä *et al.*, 2004, 2005, 2014). Considering the complexity of GABAergic signaling mechanisms, such variation in GABA-induced effects is not that surprising. The $GABA_AR$-mediated effects in hippocampus involve tonic and phasic mechanisms mediated by separate types of receptors with distinct subunit compositions that differ in their agonist affinities, desensitization and other functional characteristics (Farrant and Kaila, 2007). Furthermore, differences in intrinsic characteristics and distribution of $GABA_AR$ subtypes among neuronal populations may cause distinct cell populations to respond differently to changes in agonist concentration (Banke and McBain, 2006; Marchionni, Omrani and Cherubini, 2007; Cossart, 2011; Pelkey *et al.*, 2017; Lombardi *et al.*, 2018). Finally, during intense neural activity like GDPs, ionic plasticity in neurons produces changes in DF_{GABA} in different subcellular compartments dynamically affecting $GABA_AR$ function (Blaesse *et al.*, 2009; Kaila, Price, *et al.*, 2014; Kaila, Ruusuvuori, *et al.*, 2014; Lombardi *et al.*, 2018). All the variables considered, predicting the network effects of exogenously applied GABA is not an easy task.

Compared to GABA, studies focusing on glycinergic signaling in the perinatal hippocampus are scarce. Nevertheless, extrasynaptically located GlyRs are known to be expressed in the perinatal hippocampus (Malosio *et al.*, 1991; Aroeira *et al.*, 2011) and GlyR-mediated currents induced by exogenous glycine application have been reported in cultured hippocampal neurons (Fatima-Shad and Barry, 1992) and in

hippocampal slices from neonatal rats (Ito and Cherubini, 1991). In slices, GlyR activation was shown to depolarize CA3 pyramidal neurons, but this was only seen when applying high (300 µM – 1 mM) concentrations of glycine (Ito and Cherubini, 1991). In the same study, application of 30-50 µM glycine evoked a strychnine-insensitive increase in the frequency of sIPSC and GDPs recorded in the CA3 pyramidal neurons. In contrast, our data show that the excitability of CA3 pyramidal neurons is directly regulated by GlyR activation. A minor increase in extracellular glycine leads to a suppression of GDPs and also reduces MUA recorded in the pyramidal layer in pharmacological isolation. This suppressing effect appears to be independent of NMDAR activation as the effect on GDPs was completely abolished by strychnine and MUA was recorded in the presence of the NMDAR blocker D-AP5. To my best knowledge, our study is the first one to address the role of GlyRs in the regulation of GDPs. Our data readily demonstrate the significance of glycine as an endogenous modulatory neurotransmitter in the early stages of hippocampal development.

6.2 Study II

In Study II we demonstrate AVP-induced sustained activation of SLR interneurons at the CA3 area of the perinatal rat hippocampus, which leads to complete suppression of GDPs. Importantly, we show that since the described effect results from the desynchronization of the network rather than changes in polarity of the GABAergic responses, AVP attenuates network events regardless of the brain maturity.

Suppression of the energetically costly synchronous events during birth is expected to significantly reduce the energy consumption of the fetal brain and prevent neuronal damage during the hypoxic period (Volpe, 1976; Inder and Volpe, 2000; Buzsáki, Kaila and Raichle, 2007). In addition, birth associated intense but unique sensory stimuli could potentially cause permanent changes in the developing networks. Inhibition of the synchronized activity during birth would prevent such irrelevant plastic changes.

The transient OT-induced hyperpolarizing shift in the polarity $GABA_AR$-mediated responses, that has been suggested to take place in the perinatal rat and mouse brain (Tyzio *et al.*, 2006, 2014), would reduce the NKCC1-dependent activity like GDPs during birth, and could therefore in principle be protective in these species. However, some points in the proposed hypothesis require reconsideration.

i) As mentioned previously, the subnanomolar OT levels in the maternal circulation (Higuchi *et al.*, 1986) combined with the poor permeability of BBB to pituitary hormones (Mens, Witter and van Wimersma Greidanus, 1983; Leng and Ludwig, 2016) and high oxytocinase activity in the placenta (Naruki *et al.*, 1996; Matsumoto and Mori, 1998) challenge the role of maternal OT in activating OTRs in the fetal brain. Moreover, as the fetal OT system is not mature at birth (Altstein and Gainer, 1988), it seems unlikely that OT could account for the hyperpolarizing shift. ii) Whereas the proposed hyperpolarizing shift could protect the brain during birth in the altricial mammals, it would not reduce the energy costs of the brain in the precocial species that already exhibit hyperpolarizing GABAergic responses at birth. iii) There are numerous papers reporting GDPs in rats and mice at P0, at the time of peak of the suggested hyperpolarizing shift (Ben-Ari *et al.*, 1989; Khazipov *et al.*, 2004; Sipilä, Voipio and Kaila, 2007; Sipilä *et al.*, 2009). Also our present data strongly challenge the presence of the transient hyperpolarizing shift in E_{GABA} by demonstrating depolarizing GABAergic responses in CA3 pyramidal neurons as well as presence of bumetanide-sensitive GDPs between E21.5 and P2.

The crucial role of the fetal HPA axis in mediating birth-related physiological adaptations in the periphery is well documented (Stark *et al.*, 1979; Pohjavuori and Fyhrquist, 1980; Lagercrantz and Slotkin, 1986; Chard, 1989; Wallace, Hooper and Harding, 1990; Evers and Wellmann, 2016; Wood and Walker, 2016). The release of AVP from the pituitary is stimulated by a drop pO_2 in fetal blood, as a result of the first contractions (Stark *et al.*, 1979; Pohjavuori and Fyhrquist, 1980; Evers and Wellmann, 2016). Levels of AVP in the fetal blood during birth readily outweigh the values recorded under any other circumstances later in life (Wellmann *et al.*, 2010; Evers and Wellmann, 2016), emphasizing the significance of AVP in the process. Complications during delivery further augment the fetal AVP surge (Schlapbach *et al.*, 2011; Summanen *et al.*, 2017). While AVP obviously is a strong candidate in mediating birth-related adaptations also in the fetal brain, our study is the first one to examine the central actions of this neurohormone around birth

Recently, our laboratory has shown that the birth-related AVP surge takes place also in fetal rats (Summanen *et al.*, 2018), indicating an evolutionarily conserved role for AVP during delivery. In the present study, we demonstrated AVPergic fibers in the rat hippocampus at the time of birth. This enables the neuroendocrine actions in this brain area in the fetus and thus confirms the relevance of our model. Throughout the perinatal period, AVP induced a robust suppression of GDPs in *in toto* hippocampi and acute brain slices. The effect of bath-applied AVP was transient which probably reflects receptor desensitization and *in vivo* would likely be prevented by pulsatile release distinctive for AVPergic signaling (Wakerley, Poulain and Brown, 1978).

The interspecies differences in the brain development are often overlooked in the neurodevelopmental research, although the degree of brain development at specific age points varies widely between different mammals. The human cortical structures at birth are far more developed than those of a perinatal rat or a mouse, corresponding roughly a two-week old rat (Clancy, Darlington and Finlay, 2001; Erecinska, Cherian and Silver, 2004). Nonetheless, conclusions based on work on neonate rats or mice are often directly applied to a newborn human. In order to obtain a wider perspective to mammalian birth in our study, we tested precocial guinea pigs alongside with altricial rats, to compare the effect of AVP in two species born at completely different stage of the brain development. In guinea pigs, the hyperpolarizing GABAergic responses mature *in utero* and hippocampal slices from neonatal guinea pigs display SPWs already at birth. While the mechanisms driving the generation of GDPs and mature SPWs are different, they both strongly rely on synchronous firing of GABAergic interneurons during the events (Khazipov *et al.*, 1997; Bolea *et al.*, 1999; Klausberger and Somogyi, 2008). Our data demonstrate that sustained activation of interneurons induced by AVP interferes the synchronous drive regardless of the polarity of the GABAergic drive, leading to suppression of the network events. Such mechanism would function in analogous manner across a wide range of species, including humans. This considered, we propose that centrally-released AVP provides an evolutionarily conserved pan-mammalian mechanism to protect the fetal brain during birth.

In summary, Study II introduces a novel GABAergic mechanism to suppress synchronous network events in the perinatal hippocampus. Generation of GDPs requires seamless cooperation of neurons within the densely interconnected hippocampal network. Here, we demonstrate that an extrahippocampal input affecting a specific subpopulation of neurons can crucially interfere with this synchrony. Whether the SLR interneurons in particular, play a cardinal role in driving GDPs, remains to be studied.

6.3 Study III

In Study III we demonstrate for the first time that perinatal hippocampal CA3 pyramidal neurons in mice and rats are equipped with transport-functional KCC2. By using the KCC2 inhibitor VU0463271 (Delpire *et al.*, 2012; Sivakumaran *et al.*, 2015), we show that KCC2-mediated Cl^- extrusion opposes the dominant Cl^- uptake by NKCC1, and limits the GABAergic depolarizing drive onto these cells. Pharmacological silencing of KCC2 enhances the spiking and synchronization of the CA3 pyramidal neurons during the initial rising phase of GPDs, resulting in a robust increase in the

frequency and amplitude of these events. It is worth noting that this was the first study to use $KCC2^{-/-}$ mice to validate VU0463271 as a selective inhibitor of KCC2.

Based on *in vitro* work in cortical explants, it has been suggested that KCC2 is upregulated in the GABAergic interneurons prior to principal cells (Bortone and Polleux, 2009). Our *ex vivo* data demonstrate that the opposite is true for hippocampus. In perinatal hippocampal slices, expression of KCC2 was more often found in pyramidal cells than interneurons in the CA3. Furthermore, pharmacological inhibition of KCC2 in the presence of iGluR blockers markedly increased the GABA-driven spiking of CA3 pyramidal cells whereas no change was observed in the activity of interneurons targeting these cells.

In toto preparations from embryonic $KCC2^{-/-}$ mice have been reported to exhibit increased level of network activity and seizure-like events (Khalilov *et al.*, 2011), but the cause of the enhanced excitability has remained unclear. Whereas no seizure-like events were observed in the $KCC2^{-/-}$ hippocampi in our study, the amplitude of GDPs was significantly higher in $KCC2^{-/-}$ compared to their wild-type littermates, which is in line with an increased network excitability in the absence of KCC2. In their work, Khalilov and colleagues did not detect a difference in the somatic E_{GABA} between the $KCC2^{-/-}$ and $KCC2^{+/+}$ hippocampi, but the E_{GABA} values in both groups were highly variable, making definite conclusions infeasible. It is also possible that at this age point, Cl^- extrusion is more pronounced in the dendritic region. Developing synaptic connections are known to first innervate the dendritic regions in hippocampal pyramidal neurons (Tyzio *et al.*, 1999; Marty *et al.*, 2002). While our recordings of E_{GABA} under a fixed somatic Cl^- load do not exclude somatic function of KCC2, they readily demonstrate KCC2-mediated Cl^- extrusion in the CA3 pyramidal neuron distal dendrites. Importantly, our gramicidin-perforated patch recordings of synaptic GABAergic currents demonstrate that, regardless of the location of the inputs, the synaptic depolarizing GABAergic drive is restrained by KCC2 function in the intact CA3 pyramidal neurons.

Recently, it was shown that during GDPs, the DF_{GABA} switches polarity from depolarizing to hyperpolarizing at the peak of the field event (Khalilov *et al.*, 2015). In the complete absence of Cl^- extrusion, the pronounced glutamatergic activity during GDPs would lead to progressive depolarization of E_{GABA} (Kaila, Price, *et al.*, 2014) preventing such a switch. Our work now reveals a mechanism by which the CA3 pyramidal neurons are able to maintain the E_{GABA} at a level that allows hyperpolarizing GABAergic effects during GDPs. Furthermore, we show that KCC2 controls GDPs by constraining the pyramidal neuron synchronization and firing to the

rising phase of GDPs. Our work thus demonstrates a pivotal role for KCC2 in regulating GDPs, starting from their developmental onset.

Whereas the full KCC2 knockouts die soon after birth due to respiratory failure (Hubner *et al.*, 2001), the mice deficient in KCC2b isoform survive slightly longer, but exhibit frequent seizures and cell loss that is most pronounced in the hippocampus, entorhinal and temporal cortices (Woo *et al.*, 2002). Such epileptic phenotype may be a direct consequence of KCC2 disruption on network excitability, but might also reflect impaired wiring of the underlying circuits in the absence of KCC2-mediated control of the network events. In human, deficits in KCC2 function have been implicated in various developmental disorders such as ASD, fragile X, Down syndrome, epilepsy and schizophrenia (Puskarjov, Seja, *et al.*, 2014; Deidda *et al.*, 2015; Moore *et al.*, 2017; Schulte, Wierenga and Bruining, 2018). Our present results extend the impact of KCC2 malfunction to earlier developmental stages providing new insights in the study of these disorders.

7 References

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